A DNA barcode library for the water mites of Montenegro

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Abstract

Water mites (Acari, Hydrachnidia) are a significant component of freshwater ecosystems inhabiting a wide range of aquatic habitats. This study provides a first comprehensive DNA barcode library for the water mites of Montenegro. DNA barcodes were analysed from 233 specimens of water mites morphologically assigned to 86 species from 28 genera and 15 families. In the course of the study, four species, i.e. *Lebertia reticulata* (Koenike, 1919), *Atractides inflatipalpis* K.Viets, 1950, *A. latipes* (Szalay, 1935) and *Parabrachypoda montii* (Maglio, 1924) were molecularly confirmed as new for Montenegro and three species, i.e. *Protzia octopora* Lundblad, 1954, *Piona laminata* (Thor, 1901) and *Unionicola ypsilophora* (Bonz, 1783) are new for the Balkan Peninsula. Results are analysed using the Barcode Index Number system (BIN) and the Refined Single Linkage (RESL) of BOLD. The BIN assigned sequences to 98 clusters, while the RESL reveal 103 operational taxonomic units (OTUs). Unique BINs were revealed for 72 species (83.7%), whereas twelve species (14%) were characterised by two BINs and two species (2.3%) with three BINs. Amongst the studied taxa, 14 species were found with a high intraspecific sequence divergences (> 2.2%), emphasising the need for additional comprehensive morphological and molecular analysis of these species.
Keywords
DNA barcoding, COI, water mites, Montenegro, species delimitation

Introduction

Hydrachnidia, also known as water mites, is a most diverse and abundant group of arachnids in freshwater habitats (Davids et al. 2007). With nearly 7,500 species grouped into 550 genera (Smit 2020), they inhabit a wide range of aquatic habitats, including lotic, lentic, interstitial and temporary waters. Water mites have a complex life cycle that includes two pupa-like resting stages, i.e. protonymph and tritonymph and three active stages: larva is almost always parasitic, deutonymphs and adults that are predators of minute invertebrates (Davids et al. 2007). Some recent studies have shown that water mites can be good indicators of ecosystem health, especially of groundwater-dependent ecosystems (Pešić et al. 2019b). However, their time-consuming taxonomic identification has been identified as a major constraint for more significant involvement in rapid assessment programmes (Weigand et al. 2019).

Traditional morphology often underestimates the true diversity of water mites and, in recent years, it has been successfully replaced by an integrative approach that combines both morphological characteristics and molecular data (Martin et al. 2010, Pešić et al. 2017, Fisher et al. 2017, Pešić et al. 2019a, Pešić et al. 2020d, Pešić and Smit 2020). This process has been enhanced by the formation of the comprehensive DNA barcode reference libraries, such as the BOLD System (https://www.boldsystems.org/) and GenBank (https://www.ncbi.nlm.nih.gov/). DNA barcodes have been proposed and successfully adopted for water mites as an efficient method for detecting previously overlooked and/or misidentified species (Martin et al. 2010, Pešić et al. 2017, Pešić et al. 2019). The significant increase in the number of studies using DNA barcodes in recent years, especially in some regions, has laid the foundations for building a comprehensive library of DNA barcodes at the national and/or regional level (e.g. Blattner et al. 2019).

Of the Balkan countries, Montenegro is one of the best studied from the taxonomic and faunistic point of view (Pešić et al. 2018). Water mite research began in 1903 when the Czech zoologist Karl Thon published the first list of 13 species (Thon 1903). For more than one century, a large number of papers on the Montenegrin water mites have been published (Musselius 1912, Viets 1936, Pešić 2001, Pešić 2002b, Pešić 2002d, Pešić 2002a, Pešić 2002c, Pešić 2003a, Pešić 2003c, Pešić and Gerecke 2003, Di Sabatino et al. 2003, Pešić 2003b, Pešić 2004b, Pešić 2004a, Smit and Pešić 2004, Baker et al. 2008, Pešić et al. 2010, Pešić et al. 2012, Pešić et al. 2017, Bańkowska et al. 2016, Pešić et al. 2018, Zawal and Pešić 2018, Pešić et al. 2019a, Pešić et al. 2019c, Pešić et al. 2020a, Pešić et al. 2020b, Pešić et al. 2020c, Pešić et al. 2020d, Zawal et al. 2020, Pešić and Smit 2020, Pešić et al. 2021b)

Currently, 201 species of water mites have been reported for Montenegro (Pešić et al. 2018, Pešić et al. 2019c, Pešić et al. 2020c, Pešić et al. 2020d, Pešić and Smit 2020). This
number makes up about 50% of the species known from the Balkans, which is estimated at about 400 species (Pešić et al. 2018). This is still a small number for the area of such hydrogeological characteristics and the turbulent geological history as the Balkans. Therefore, there is no reason not to believe that the expected number of water mites in the Balkans is at least at the level of Central Europe which is home to approximately 745 species (Gerecke et al. 2016).

The aim of the study is to develop and evaluate the first library of barcodes for water mites from Montenegro, targeting a COI fragment of ≈ 658 bp. Taking advantage of publicly available DNA barcode reference libraries, such as the BOLD and the use of the universal Barcode Index Number (BIN), allows us to assess the molecular diversity of water mite species inhabiting the territory of Montenegro, as well as to explore their distribution patterns in Europe. Moreover, this approach will allow us to also identify problematic species groups both for traditional taxonomy and for DNA barcoding.

**Material and methods**

Water mites were collected by hand netting, sorted live in the field and immediately preserved in 96% ethanol (EtOH) for the molecular analysis. Water mites were collected from 54 sampling sites in Montenegro (Fig. 1) during several sampling campaigns from 2018-2020. Photos from each studied specimen were taken before molecular work started. The photographs were made using a camera on a Samsung Galaxy smartphone.

Molecular analysis were conducted in the Canadian Centre for DNA Barcoding (Guelph, Ontario, Canada; (CCDB; [http://ccdb.ca/](http://ccdb.ca/)) and in the Department of Invertebrate Zoology and Hydrobiology (DIZH), University of Łódź, Poland. For the methods used for cytochrome c oxidase subunit I (COI) gene amplification in DIZH, see Pešić et al. (2017). In CCDB, the specimens were sequenced for the barcode region of COI using standard invertebrate DNA extraction (Ivanova et al. 2007), amplification (Ivanova and Grainger 2007a) and sequencing protocols (Ivanova and Grainger 2007b). The DNA extracts were archived in −80°C freezers at the Centre for Biodiversity Genomics (CBG; biodiversitygenomics.net) and the specimen vouchers were stored in 95% EtOH and returned to the first author for morphological examination. Some of these vouchers were dissected as described elsewhere (Davids et al. 2007) and slide-mounted in Faure’s medium, while the rest were transferred to Koeneke’s medium and stored in the collection of the first author at the Department of Biology in Podgorica.

**DNA barcode analysis**

In CCDB, the chromatograms were assembled into consensus sequences for each specimen and uploaded to BOLD. The taxonomic account, voucher specimen ID, collecting locality and voucher depositor were incorporated into the system for further analysis. Water mite sequences, obtained during this study, were grouped in the “MNHYD” (DNA barcode reference library of Montenegrin water mites) dataset. Detailed voucher information, taxonomic classifications, photos, DNA barcode sequences, primer pairs used and trace
files (including their quality) were uploaded to the dataset “MNHYD” on the Barcode of Life Data Systems (BOLD; www.boldsystems.org).

The translation of the COI sequences into amino acids did not contain any stop codon positions and blasting the sequences confirmed the absence of contaminations. In cases of the four *Unionicola ypsylophora* mites, we amplified *Anodonta exulcerata* DNA instead of water mite DNA. These specimens were excluded from further analysis.

The reference library for the molecular identification of water mites sequenced in this study was analysed using the Barcode Index Number system (BIN) (Felsenstein 1985). The distribution of BINs was performed by the Barcode of Life Data System v.4 (accessed 15 November 2021). The two-phase BIN analysis system in the first phase applies a first threshold of 2.2% (that allows a rough differentiation between intraspecific and interspecific distances), followed by refinements through Markov clustering into the final BINs (Ratnasingham and Hebert 2013). BOLD ID and accession numbers for all specimens included in final dataset are given in Table 1.
Table 1. Details on barcoded specimens from Montenegro.

| Taxa                      | Voucher Code | BOLD Process ID | BIN               | Locality                                  | Coordinates       |
|---------------------------|--------------|-----------------|-------------------|--------------------------------------------|-------------------|
| **Limnocharidae**         |              |                 |                   |                                            |                   |
| *Limnocharaes aquatica*   | 31. CG2020_6_C10 | DNAEC032-20   | BOLD:ACS0438      | Podgorica, Zeta River at Pričelje          | 42.5022N, 19.2225E |
| **Hydryphantidae**        |              |                 |                   |                                            |                   |
| *Panisus michaeli*        | CCDB 38361 A04 | DCDDJ004-21    | BOLD:ADT7504      | Kolašin, Lalevića Dolovi, spring #1       | 42.899N, 19.631E  |
|                           | CCDB 38361 A05 | DCDDJ005-21    |                   | Kolašin, Lalevića Dolovi, spring #1       | 42.899N, 19.631E  |
|                           | CCDB 38361 A06 | DCDDJ006-21    |                   | Kolašin, Lalevića Dolovi, spring #1       | 42.899N, 19.631E  |
| *Trichothys jadrankae*    | CCDB-38679-A08 | DNCBD008-20    | BOLD:AEF1286      | Bar, Poseljanski stream at Poseljani       | 42.3095N, 19.0518E |
| *Partnunia naprintua*     | CCDB 38361 A08 | DCDDJ008-21    | BOLD:AEI6734      | Andrijevica, spring at Trešnjevik          | 42.7392N, 19.6933E |
| *Protzia invalvaris*      | CCDB 38361 C11 | DCDDJ035-21    | BOLD:AEI2833      | Kolašin, Bistrica stream                   |                   |
|                           | CCDB38233 A08 | DCCDB008-21    |                   | Kolašin, Kolašinska rijeka stream          | 42.8391N, 19.5749E |
|                           | CCDB38233 A09 | DCCDB009-21    |                   | Kolašin, Kolašinska rijeka stream          | 42.8391N, 19.5749E |
|                           | CCDB38233 A10 | DCCDB010-21    |                   | Kolašin, Kolašinska rijeka stream          | 42.8391N, 19.5749E |
| *Protzia squamosa paucipora* | CCDB 38361 A09 | DCDDJ009-21    | BOLD:AEL1015      | Kolašin, spring on road to Trešnjevik      | 42.7405N, 19.6801E |
|                           | CCDB 38361 A10 | DCDDJ010-21    |                   | Kolašin, spring on road to Trešnjevik      | 42.7405N, 19.6801E |
|                           | CCDB 38361 A11 | DCDDJ011-21    |                   | Kolašin, spring on road to Trešnjevik      | 42.7405N, 19.6801E |
| *Protzia octopora*        | CCDB38233 D09 | DCCDB045-21    | BOLD:AEI5747      | Kolašin, Bistrica stream                   | 42.9871N, 19.4338E |
| *Protzia halberti*        | Hyd_MN_VP7   | DNAEC081-20    | BOLD:AED9646      | Bijelo Polje, Lještanica stream            | 43.0631N, 19.5808E |
|                           | 3. CG2020_8_2 | DNAEC002-20    |                   | Bijelo Polje, Lještanica stream            | 43.0631N, 19.5808E |
|                           | 4. CG2020_1   | DNAEC003-20    |                   | Bijelo Polje, Lještanica stream            | 43.0631N, 19.5808E |
|                           | 5. CG2020_1_3 | DNAEC004-20    |                   | Bijelo Polje, Lještanica stream            | 43.0631N, 19.5808E |
| Taxa                        | Voucher Code | BOLD Process ID | BIN         | Locality                                      | Coordinates         |
|-----------------------------|--------------|-----------------|-------------|-----------------------------------------------|---------------------|
| **Protzia rotunda**        | 6. M18_01_1_D10 | DNAEC045-20     | BOLD-AED8976 | Žabljak, Sedlo, spring Studenac | 43.0973N, 19.0702E  |
|                            | CCDB-3867-E04 | DNCBD052-20     |             | Bar, Međurječka rijeka stream              | 42.0363N, 19.2179E  |
|                            | CCDB-3867-E05 | DNCBD053-20     |             | Bar, Međurječka rijeka stream              | 42.0363N, 19.2179E  |
| **Protzia rugosa**         | 6. CG2020_1_4 | DNAEC005-20     | BOLD-AEE010  | Bijelo Polje, Lještanica stream            | 43.0631N, 19.5808E  |
|                            | 7. CG2020_8_B6 | DNAEC017-20     |             | Berane, spring nr Mon. Djurdjevi Stupovi   | 42.8527N, 19.862E   |
|                            | CCDB38233 D05 | DCCDB041-21     |             | Mojkovac, Bistrica stream                   | 42.9871N, 19.4338E  |
| **Hydrodromidae**          |              |                 |             |                                               |                     |
| **Hydrodroma reinhardi**   | CCDB-3867-G04 | DNCBD076-20     | BOLD-AEF0798 | Podgorica, Cijevna River at Dinoša          | 42.4057N, 19.3569E  |
| **Hydrodroma torrenticola**| CCDB-3867-E06 | DNCBD054-20     | BOLD-AEF3799 | Bar, Međurječka rijeka stream               | 42.0363N, 19.2179E  |
| **Lebertiidae**            |              |                 |             |                                               |                     |
| **Lebertia jadensis**      | CCDB 38361 C09 | DCDDJ033-21     | BOLD-ADK0383 | Kolašin, Bistrica stream at Crkvine         | 42.8054N, 19.4456E  |
|                            | CCDB-3867-G08 | DNCBD080-20     |             | Podgorica, Cijevna River at Dinoša          | 42.4057N, 19.3569E  |
|                            | CCDB 38361 C08 | DCDDJ032-21     |             | Kolašin, Bistrica stream at Crkvine         | 42.8054N, 19.4456E  |
|                            | CCDB-3867-F10 | DNCBD070-20     |             | Danilovgrad, spring below the bridge        | 42.5542N, 19.1059E  |
| **Lebertia cuneifera**     | CCDB 38363 A01 | SEPTA001-21     | BOLD-ADV4392 | Nikšić, spring “Babino sicelo”              | 42.8043N, 19.2152E  |
| **Lebertia varolata**      | CCDB-3867-B05 | DNCBD017-20     | BOLD-ADK0996 | Bar, stream in Godinje Village               | 42.2206N, 19.1118E  |
|                            | CCDB-3867-B07 | DNCBD019-20     |             | Bar, stream in Godinje Village               | 42.2206N, 19.1118E  |
|                            | CCDB-3867-D03 | DNCBD039-20     |             | Bar, Rikavac stream above Old Bar           | 42.1001N, 19.1432E  |
|                            | CCDB-3867-D04 | DNCBD040-20     |             | Bar, Rikavac stream above Old Bar           | 42.1001N, 19.1432E  |
|                            | CCDB-3867-D05 | DNCBD041-20     |             | Bar, Rikavac stream above Old Bar           | 42.1001N, 19.1432E  |
|                            | CCDB-3867-D06 | DNCBD042-20     |             | Bar, Rikavac stream above Old Bar           | 42.1001N, 19.1432E  |
| Taxa                  | Voucher Code     | BOLD Process ID | BIN                        | Locality                        | Coordinates       |
|----------------------|------------------|----------------|-----------------------------|----------------------------------|--------------------|
| **Lebertia natans**  |                  |                |                             |                                  |                    |
| 16. M19_24_3_E7      | DNAEC054-20      | Bar, Medjurječka rijeka stream | 42.0226N, 19.22E               |                    |
| 17. M19_24_3_E8      | DNAEC055-20      | Bar, Medjurječka rijeka stream | 42.0226N, 19.22E               |                    |
| **Lebertia glabra**  |                  |                |                             |                                  |                    |
| CCDB38233 C04        | DCCDB028-21      | Kolašin, Kolašinska rijeka stream | 42.8391N, 19.5749E               |                    |
| CCDB38233 D03        | DCCDB039-21      | Kolašin, Bistrica stream at Crkvine | 42.9871N, 19.4338E               |                    |
| CCDB38233 D04        | DCCDB040-21      | Kolašin, Bistrica stream at Crkvine | 42.9871N, 19.4338E               |                    |
| CCDB38233 D01        | DCCDB037-21      | Mojkovac, Bistrica stream | 42.9871N, 19.4338E               |                    |
| CCDB38233 D02        | DCCDB038-21      | Mojkovac, Bistrica stream | 42.9871N, 19.4338E               |                    |
| CCDB38233 C05        | DCCDB029-21      | Kolašin, Kolašinska rijeka stream | 42.8391N, 19.5749E               |                    |
| **Lebertia inaequalis** |              |                |                             |                                  |                    |
| CCDB-3867-C03        | DNCBD027-20      | Tuzi, Vitoja, pool | 42.324N, 19.3637E               |                    |
| CCDB-3867-B11        | DNCBD023-20      | Tuzi, Vitoja, pool | 42.324N, 19.3637E               |                    |
| CCDB-3867-C02        | DNCBD026-20      | Tuzi, Vitoja, pool | 42.324N, 19.3637E               |                    |
| CCDB 38363 B04       | SEPTA016-21      | Bar, Skadar Lake at Murići | 42.1637N, 19.2214E               |                    |
| CCDB 38363 B06       | SEPTA018-21      | Bar, Skadar Lake at Murići | 42.1637N, 19.2214E               |                    |
| CCDB 38363 B10       | SEPTA022-21      | Podgorica, Skadar Lake at Donja Plavnica | 42.2724N, 19.2007E               |                    |
| CCDB 38363 B11       | SEPTA023-21      | Podgorica, Gornja Plavnica, river | 42.2889N, 19.2108E               |                    |
| CCDB-3867-E12        | DNCBD060-20      | Bar, Medjurjecka rijeka stream | 42.0363N, 19.2179E               |                    |
| **Lebertia insignis**|                  |                |                             |                                  |                    |
| CCDB38233 B12        | DCCDB024-21      | Danilovgrad, River Zeta near Slap | 42.6001N, 19.0656E               |                    |
| **Lebertia maculosa**|                  |                |                             |                                  |                    |
| 32. CG2020_1_C11     | DNAEC033-20      | Bijelo Polje, Lještanica stream | 43.0631N, 19.5809E               |                    |
| Taxa                  | Voucher Code       | BOLD ID        | BIN       | Locality                                      | Coordinates       |
|-----------------------|--------------------|----------------|-----------|-----------------------------------------------|-------------------|
| **33. CG2020_1_C12**  | DNAEC034-2         |                |           | Bijelo Polje, Lještanica stream              | 43.0631N, 19.5809E |
| **1. CG2020_8**       | DNAEC001-20        | BOLD-AED9718   |           | Berane, spring nr. Mon. Đurđevi Stupovi       | 42.8527N, 19.862E  |
| CCDB 38361 H01        | DCDDJ085-21        |                |           | Kolašin, spring at Monastir Morača           | 42.7668N, 19.3906E |
| **Lebertia porosa**   | CCDB-3867-G09      | DNBCBD081-20   | BOLD-ACS0974 | Podgorica, Cijevna River at Dinoša           | 42.4057N, 19.3569E |
| CCDB 38363 C10        | SEPTA034-21        |                |           | Cetinje, River Crnojevića                    | 42.3557N, 19.0228E |
| CCDB38233 A01         | DCCDB001-21        | BOLD-AED4662   |           | Podgorica, spring Mareza                     | 42.4801N, 19.1822E |
| **7. CG2020_10**      | DNAEC006-20        |                |           | Tuzi, Vitoja spring                          | 42.3254N, 19.3628E |
| **Lebertia reticulata** | **Hyd_MN_VP13**   | DNAEC086-20    | BOLD-ADT9218 | Šavnik, spring of Bukovica stream           | 43.0589N, 19.1103E |
| **Hyd_MN_VP14**       | DNAEC087-20        |                |           | Šavnik, spring of Bukovica stream           | 43.0589N, 19.1103E |
| CCDB 38363 A11        | SEPTA011-21        |                |           | Nikšić, spring Vukovo Vrelo                 | 42.8574N, 18.9426E |
| **Lebertia schecheltii** | **9. CG2020**    | DNAEC008-20    | BOLD-AED9612 | Žabljak, Sedlo, spring Studenac              | 43.0973N, 19.0702E |
| **10. CG2020_2_3**    | DNAEC009-20        |                |           | Žabljak, Sedlo, spring Studenac              | 43.0973N, 19.0702E |
| **Oxidae**            | **Oxus angustipositus** | **CCDB 38361 C03** | **DCDDJ027-21** | **BOLD-AEB9099** | Ulcinj, Šasko Lake | 41.9768N, 19.3388E |
| **CCDB-38679-A11**    | DNBCBD011-20       |                |           | Cetinje, Poseljanski stream, lower part       | 42.3057N, 19.0557E |
| **CCDB 38363 B05**    | SEPTA017-21        |                |           | Bar, Skadar Lake at Murići                   | 42.1637N, 19.2214E |
| **CCDB 38363 B07**    | SEPTA019-21        |                |           | Bar, Skadar Lake at Murići                   | 42.1637N, 19.2214E |
| **Teutoniidae**       | **Teutonia cometes** | **33. M19_20_3_F11** | **DNAEC068-20** | **BOLD-ACH7884** | Podgorica, Mareza canal | 42.479N, 19.1813E |
| **Hyd_MN_VP5**        | DNAEC079-20        |                |           | Danilovgrad, spring Svinjiška vrela          | 42.6384N, 19.0074E |
| **Sperchontidae**     | **Sperchon brevirostris** | **CCDB38233 D07** | **DCCDB043-21** | **BOLD-ACP6107** | Mojkovac, Bistrica stream | 42.9871N, 19.4338E |
| Taxa                        | Voucher Code | BOLD Process ID | BIN              | Locality                                      | Coordinates        |
|-----------------------------|--------------|-----------------|------------------|-----------------------------------------------|--------------------|
|                            | CCDB38233 D08| DCCDB044-21     | BOLD:AED3857     | Mojkovac, Bistrica stream                     | 42.9871N, 19.4338E |
|                            | CCDB38233 A11| DCCDB011-21     |                  | Kolašin, Kolašinska rijeka stream             | 42.8391N, 19.5749E |
| Sperchon clupeifer         | Hyd_MN_VP11  | DNAEC084-20     | BOLD:AEE4061     | Žabljak, Ljutica stream                       | 43.1378N, 19.3023E |
|                            | CCDB-3867-B04| DNCBD016-20     | BOLD:ACS1100     | Bar, stream in Godinje Village                | 42.2206N, 19.1118E |
| Sperchon hibernicus        | CCDB-3867-D02| DNCBD038-20     | BOLD:AEF3824     | Bar, Rikavac stream above Old Bar             | 42.1001N, 19.1432E |
| Sperchon hispidus          | M19 29A 8 E3 | DNAEC050-20     | BOLD:AED3202     | Danilovgrad, Zeta River at Spuž               | 42.5113N, 19.1982E |
|                            | 29. CG2020_7_C8C7 | DNAEC030-20 | BOLD:AED3202     | Danilovgrad, Zeta River at Spuž               | 42.5113N, 19.1982E |
| Spechon denticulatus       | 10. CG2020_8 B8 | DNAEC019-20 | BOLD:AEF4077     | Berane, spring nr. Mon. Djurdjevi Stupovi    | 42.8527N, 19.862E  |
| Sperchon papillosus        | M19 12B 1 D7 | DNAEC043-20     | BOLD:AED2134     | Budva, Lastva Grbaljska, stream               | 42.3103N, 18.8138E |
| Sperchon thienemanni       | Hyd_MN_VP4   | DNAEC078-20     | BOLD:AED2134     | Šavnik, spring Kikov izvor near Boan          | 42.9465N, 19.1893E |
|                            | Hyd_MN_VP10  | DNAEC083-20     |                  | Žabljak, Sedlo, Studenac spring               | 43.0972N, 19.0702E |
|                            | CCDB 38361 A03| DCDD003-21     |                  | Kolašin, Lalevića Dolovi, spring #1          | 42.899N, 19.631E   |
|                            | CCDB 38363 A02| SEPTA002-21    |                  | Nikšić, Lukavica Mt., spring Babino Sicelo   | 42.8043N, 19.2152E |
|                            | CCDB 38363 A04| SEPTA004-21    |                  | Nikšić, Lukavica Mt., spring Babino Sicelo   | 42.8043N, 19.2152E |
|                            | CCDB 38363 A05| SEPTA005-21    |                  | Nikšić, Lukavica Mt., spring Babino Sicelo   | 42.8043N, 19.2152E |
| Sperchon violaceus         | Hyd_MN_VP8   | DNAEC088-20     | BOLD:AAN0076     | Žabljak, Mlinski potok stream                | 43.1494N, 19.0898E |
|                            | 27. M19 16A 3 F5 | DNAEC062-20   |                  | Kolašin, Biogradska River                    | 42.8968N, 19.6047E |
|                            | 56. CG2020_1 | DNAEC010-20     |                  | Bijelo Polje, Lještanica stream              | 43.0631N, 19.5809E |
|                            | 57. CG2020_8 | DNAEC011-20     |                  | Bijelo Polje, Lještanica stream              | 43.0631N, 19.5809E |
| Taxa                          | Voucher Code | BOLD Process ID | BIN             | Locality                          | Coordinates       |
|------------------------------|--------------|-----------------|-----------------|------------------------------------|-------------------|
| **58.** CG2020               | DNAEC012-20  |                 |                 | Bijelo Polje, Lještanica stream    | 43.0631N, 19.5809E|
| **26.** M19_16A_3_F4         | DNAEC061-20  |                 |                 | Kolašin, Biogradska River          | 42.8968N, 19.6047E|
| **28.** M19_16A_3_F6         | DNAEC063-20  |                 |                 | Kolašin, Biogradska River          | 42.8968N, 19.6047E|
| CCDB38233 D06                | DCCDB042-21  |                 |                 | Mojkovac, Bistrica stream          | 42.9871N, 19.4338E|
| CCDB38233 H10                | DCCDB094-21  |                 |                 | Mojkovac, spring in Bistrica Village| 42.9862N, 19.4349E|
| CCDB38233 H11                | DCCDB095-21  |                 |                 | Mojkovac, spring in Bistrica Village| 42.9862N, 19.4349E|
| **Sperchonopsis verrucosa**  | CCDB 38361 B11| DCCJJ023-21     | BOLD:AEK8297    | Cetinje, spring “Smokov Vijenac”   | 42.254N, 18.9902E  |
| **46.** M19_16B_1_G10        | DNAEC040-20  |                 | BOLD:ACS9705    | Kolašin, Biogradska River          | 42.8968N, 19.6047E|
| **Monattractides madritensis**| CCDB-3867-G11| DNBC083-20      | BOLD:AED3803    | Podgorica, Cijevna River at Dinoša  | 42.4057N, 19.3569E|
| **44.** M19_12B_3_G8         | DNAEC075-20  |                 |                 | Budva, Lastva Grbaljska, first order stream | 42.3103N, 18.8138E|
| CCDB-3867-B01                | DNBC013-20   |                 | BOLD:AEL3852    | Bar, stream in Godinje Village     | 42.2206N, 19.1118E|
| **Monattractides stadleri**  | CCDB38233 C03| DCCDB027-21     | BOLD:AED3802    | Bar, Rikavack stream above Old Bar  | 42.1001N, 19.1432E|
| **45.** M19_129_3_G9         | DNAEC076-20  |                 |                 | Budva, Lastva Grbaljska, first order stream | 42.3103N, 18.8138E|
| **Torrenticola amplexa**      | CCDB-3867-F08| DNBC068-20      | BOLD:ACR0665    | Danilovgrad, spring below the bridge | 42.5542N, 19.1059E|
| CCDB-3867-F09                | DNBC069-20   |                 |                 | Danilovgrad, spring below the bridge | 42.5542N, 19.1059E|
| CCDB38233 G04                | DCCDB076-21  |                 |                 | Danilovgrad, spring below the bridge | 42.5542N, 19.1059E|
| **Torrenticola brevirostris** | 42. M19_29A_5_G6 | DNAEC073-20     | BOLD:AED9586    | Danilovgrad, Zeta River at Spuž     | 42.5113N, 19.1982E|
| CCDB 38363 C12               | SEPTA036-21  |                 |                 | Podgorica, Morača River in Podgorica| 42.4368N, 19.2559E|
| **Torrenticola dudichi**      | CCDB38233 D11| DCCDB047-21     | BOLD:AED7520    | Mojkovac, Bistrica stream          | 42.9871N, 19.4338E|
| **43.** M19_16A_4_G7         | DNAEC074-20  |                 |                 | Kolašin, Biogradska rijeka stream   | 42.8968N, 19.6047E|
| Taxa                        | Voucher Code | BOLD Process ID | BIN             | Locality                                | Coordinates          |
|-----------------------------|--------------|-----------------|------------------|------------------------------------------|----------------------|
| *Torrenticola laskai*       | CCDB-3867-G06 | DNBCD078-20     | BOLD-AEF5471     | Podgorica, Cijevna River at Dinoša       | 42.4057N, 19.3569E   |
|                             | CCDB-3867-B10 | DNBCD022-20     |                  | Kolašin, Tara River near Mateševo        | 42.7898N, 19.5374E   |
|                             | CCDB-3867-E11 | DNBCD059-20     | BOLD-AED2306     | Bar, Međurječka rijeka stream            | 42.0363N, 19.2179E   |
| *Torrenticola lukai*        | CCDB 38361 C12 | DCCDJ036-21     | BOLD-ACH9685     | Kolašin, Bistrica stream at Crkvine      | 42.8054N, 19.4456E   |
| *Torrenticola meridionalis* | CCDB 38361 D02 | DCCDJ038-21     | BOLD-AED7519     | Kolašin, Bistrica stream at Crkvine      | 42.8054N, 19.4456E   |
|                             | CCDB-3867-G02 | DNBCD074-20     |                  | Bar, Orahovštica River                   | 42.2476N, 19.0798E   |
|                             | CCDB-3867-G01 | DNBCD073-20     |                  | Bar, Orahovštica River                   | 42.2476N, 19.0798E   |
|                             | CCDB-3867-B09 | DNBCD021-20     |                  | Kolašin, River Drčka near Mateševo       | 42.7619N, 19.5549E   |
|                             | CCDB-3867-E01 | DNBCD049-20     |                  | Bar, Rikavac stream above Old Bar        | 42.1001N, 19.1432E   |
|                             | CCDB-3867-E03 | DNBCD051-20     |                  | Bar, Rikavac stream above Old Bar        | 42.1001N, 19.1432E   |
|                             | CCDB 38361 D01 | DCCDJ037-21     | BOLD-AEI3402     | Kolašin, Bistrica stream at Crkvine      | 42.8054N, 19.4456E   |
|                             | CCDB 38361 B08 | DCCDJ020-21     |                  | Kolašin, Bistrica stream at Crkvine      | 42.8054N, 19.4456E   |
|                             | CCDB38233 B10 | DCCDB022-21     |                  | Kolašin, Kolašinska rijeka stream        | 42.8391N, 19.5749E   |
|                             | CCDB38233 D12 | DCCDB048-21     |                  | Mojkovac, Bistrica stream                | 42.9871N, 19.4338E   |
| *Torrenticola similis*      | CCDB 38361 B09 | DCCDJ021-21     | BOLD-AEK9661     | Kolašin, Bistrica stream at Crkvine      | 42.8054N, 19.4456E   |
| *Torrenticola barsica*      | CCDB-3867-E09 | DNBCD057-20     | BOLD-AEF1219     | Bar, Međurječka rijeka stream            | 42.0363N, 19.2179E   |
|                             | CCDB-3867-F04 | DNBCD064-20     |                  | Bar, Međurječka rijeka stream            | 42.0363N, 19.2179E   |
| *Torrenticola elliptica*    | CDB38233 B11  | DCCDB023-21     | BOLD-AEI9183     | Kolašin, Kolašinska rijeka stream        | 42.8391N, 19.5749E   |
| *Torrenticola ungeri*       | 19. M19_24_6_E10 | DNAEC057-20      | BOLD-AED2307     | Bar, Međurječka rijeka stream            | 42.0226N, 19.22E     |
|                             | 20. M19_24_6_E11 | DNAEC058-20      |                  | Bar, Međurječka rijeka stream            | 42.0226N, 19.22E     |
|                             | CCDB-3867-D08 | DNBCD044-20     |                  | Bar, Rikavac stream above Old Bar        | 42.1001N, 19.1432E   |
| Taxa                                | Voucher Code | BOLD Process ID | BIN                | Locality                                | Coordinates        |
|-------------------------------------|--------------|-----------------|--------------------|------------------------------------------|--------------------|
| Pseudotorrenticola rynchota         | CCDB-3867-G07 | DNCBD079-20     |                    | Podgorica, Cijevna River at Dinoša       | 42.4057N, 19.3569E |
|                                     | CCDB-3867-B02 | DNCBD014-20     | BOLD:AEF1632      | Bar, stream in Godinje Village           | 42.2206N, 19.1118E |
|                                     | CCDB-3867-B03 | DNCBD015-20     |                    | Bar, stream in Godinje Village           | 42.2206N, 19.1118E |
| Limnesiidae                         |              |                 |                    |                                          |                    |
| Limnesia undulata                   | CCDB-3867-C05 | DNCBD029-20     | BOLD:AAX5286       | Tuzi, Vitoja, pools                      | 42.324N, 19.3637E  |
|                                     | C38363 C03   | SEPTA027-21     |                    | Tuzi, Skadar Lake at Podhum              | 42.3139N, 19.3534E |
| Hygrobatidae                        |              |                 |                    |                                          |                    |
| Atractides fluviatilis              | CCDB-3867-G10 | DNCBD082-20     | BOLD:AEF1143       | Podgorica, Cijevna River at Dinoša       | 42.4057N, 19.3569E |
| Atractides fissus                   | CCDB38233 B03 | DCCDB015-21     | BOLD:AEI1811       | Kolašin, Kolašinska rijeka stream        | 42.8391N, 19.5749E |
|                                     | CCDB38233 D10 | DCCDB046-21     |                    | Mojkovac, Bistrica stream                | 42.9871N, 19.4338E |
| Atractides anae                     | 1 CG2020 8 B3 | DNAEC014-20     | BOLD:AED1201       | Berane, spring nr. Mon. Djurdjevi Stupovi| 42.8527N, 19.862E  |
| Atractides inflatipalpis            | 29 M19 24 4 F7 | DNAEC064-20     | BOLD:AED3549       | Bar, Međurječka rijeka stream            | 42.0226N, 19.22E   |
| Atractides inflatipes               | CCDB-3867-G03 | DNCBD075-20     | BOLD:AEF1144       | Bar, Orahovštica stream                  | 42.2476N, 19.0798E |
| Atractides fonticolus               | CCDB38233 B09 | DCCDB021-21     | BOLD:AEI8720       | Podgorica, Pričelje, spring Studenac     | 42.4835N, 19.2429E |
|                                     | CCDB38233 B08 | DCCDB020-21     |                    | Podgorica, Pričelje, spring Studenac     | 42.4835N, 19.2429E |
| Atractides gibberipalpis            | CCDB 38361 C07 | DCDDJ031-21     | BOLD:AEK7766       | Mojkovac, Bistrica stream                | 42.8054N, 19.4456E |
|                                     | CCDB38233 B02 | DCCDB014-21     | BOLD:AEI3946       | Kolašin, Kolašinska rijeka stream        | 42.8391N, 19.5749E |
| Atractides inflatus                 | 14 M19 12 4 E5 | DNAEC052-20     | BOLD:ACB4677       | Budva, Lastva Grbaljska, first order stream | 42.3103N, 18.8138E |
| Atractides nodipalpis               | CCDB-3867-F07 | DNCBD067-20     | BOLD:ACR0209       | Danilovgrad, spring below the bridge     | 42.5542N, 19.1059E |
|                                     | 41 M19 29A 1 G5 | DNAEC072-20     |                    | Danilovgrad, Zeta River at Spuž           | 42.5113N, 19.1982E |
|                                     | CCDB-3867-F05 | DNCBD065-20     | BOLD:AED3547       | Danilovgrad, spring below the bridge     | 42.5542N, 19.1059E |
| Taxa                        | Voucher Code | BOLD Process ID | BIN         | Locality                        | Coordinates     |
|-----------------------------|--------------|-----------------|-------------|---------------------------------|-----------------|
| *Atractides pennatus*       | 40. M19_29A_1_G4 | DNAEC071-20    | DNBCD071-20 | Danilovgrad, Zeta River at Spuž | 42.5113N, 19.1982E |
|                            | CCDB-3867-F11 | DNBCD009-20    | BOLD-ADF7007 | Bar, Orahovštica stream         | 42.2476N, 19.0798E |
|                            | CCDB-38679-A09 | DNBCD009-20    |             | Bar, Poseljani, Poseljanski stream | 42.3057N, 19.0575E |
|                            | 25. CG2020_9_C6 | DNAEC028-20    |             | Podgorica, Mareza spring         | 42.4801N, 19.1821E |
|                            | 23. CG2020_9_C5 | DNAEC027-20    |             | Podgorica, Mareza spring         | 42.4801N, 19.1821E |
|                            | 3. CG2020_2_B4 | DNAEC015-20    |             | Žabljak, Sedlo, Studenac spring   | 43.0973N, 19.0702E |
|                            | 32. M19_23_1_F10 | DNAEC067-20    |             | Nikšić, Vidrovan, Vukovo Vrelo spring | 42.8575N, 18.9414E |
|                            | 31. M19_23_1_F9 | DNAEC066-20    |             | Nikšić, Vidrovan, Vukovo Vrelo spring | 42.8575N, 18.9414E |
|                            | 4. M19_22_1_D8 | DNAEC042-20    |             | Nikšić, spring in Miločani Village | 42.8265N, 18.9018E |
|                            | CCDB 38363 C01 | SEPTA025-21    |             | Budva, spring Smokov Vjenac      | 42.2346N, 18.907E |
|                            | CCDB 38363 B12 | SEPTA024-21    |             | Budva, spring Smokov Vjenac      | 42.2346N, 18.907E |
|                            |              |                 |             |                                 |                 |
| *Atractides robustus*       | CCDB-3867-D12 | DNBCD048-20    | BOLD-ADZ9348 | Bar, Rikavac stream above Old Bar | 42.1001N, 19.1432E |
|                            | CCDB-3867-D11 | DNBCD047-20    |             | Bar, Rikavac stream above Old Bar | 42.1001N, 19.1432E |
|                            | CCDB-3867-D10 | DNBCD046-20    |             | Bar, Rikavac stream above Old Bar | 42.1001N, 19.1432E |
|                            | CCDB 38361 H02 | DCDDJ086-21    |             | Kolašin, spring nr. Monastir Morača | 42.7668N, 19.3906E |
|                            | CCDB38233 B01 | DCCDB013-21    |             | Kolašin, Kolašinska Rijeka stream | 42.8391N, 19.5749E |
| *Atractides latipes*        | 18. M19_08B_7_E9 | DNAEC056-20    | BOLD-AED4000 | Podgorica, River Cijevna at Trgaja | 42.3964N, 19.3798E |
| *Atractides stankovici*     | CCDB38233 C08 | DCCDB032-21    | BOLD-AED3550 | Dnilovgrad, River Zeta near Slap | 42.6001N, 19.0656E |
|                            | CCDB38233 C07 | DCCDB031-21    |             | Danilovgrad, River Zeta near Slap | 42.6001N, 19.0656E |
|                            | 13. CG2020_4_B10 | DNAEC020-20    |             | Podgorica, Mareza canal          | 42.479N, 19.1813E |
|                            | 14. CG2020_4_B11 | DNAEC021-20    |             | Podgorica, Mareza canal          | 42.479N, 19.1813E |
| Taxa                        | Voucher Code | BOLD Process ID | BIN          | Locality                                      | Coordinates       |
|-----------------------------|--------------|-----------------|--------------|-----------------------------------------------|-------------------|
| *Hygrobates calliger*       | CCDB 38361 C06 | DCDDJ030-21     | BOLD-AEL5782 | Kolašin, Crkvine, Bistrica stream             | 42.8054N, 19.4456E |
|                            | CCDB-38679-A04 | DNCD004-20      | BOLD-AEF4261 | Bar, Poseljanski stream at Poseljani          | 42.3095N, 19.0518E |
|                            | CCDB-38679-A03 | DNCD003-20      |              | Bar, Poseljanski stream at Poseljani          | 42.3095N, 19.0518E |
| *Hygrobates foreli*         | Hyd_MN_VP6   | DNAEC080-20     | BOLD-AEE3281 | Žabljak, Mlinski potok stream                 | 43.1494N, 19.0898E |
| *Hygrobates lacrima*        | 27, CG2020_3_C7 | DNAEC029-20     | BOLD-AED2490 | Kolašin, Tara River near Mateševo             | 42.7897N, 19.5383E |
| *Hygrobates limnocrenicus*  | 13, M19_20_5_E4 | DNAEC051-20     | BOLD-AED2489 | Podgorica, Mareza canal                       | 42.479N, 19.1813E  |
| *Hygrobates longipalpis*    | CCDB-3867-C07 | DNCD031-20      | BOLD-ACR9783 | Tuzi, Vitoja, pool                            | 42.324N, 19.3637E  |
|                            | CCDB-3867-C09 | DNCD033-20      |              | Tuzi, Vitoja, pool                            | 42.324N, 19.3637E  |
|                            | CCDB-38679-A10 | DNCD010-20      |              | Bar, Poseljani, Poseljanski stream            | 42.3057N, 19.0557E |
|                            | CCDB 38363 C04 | SEPTA028-21     |              | Tuzi, Skadar Lake at Podhum                   | 42.3139N, 19.3534E |
| *Hygrobates mediterraneus*  | 7, M19_24_2_D11 | DNAEC046-20     | BOLD-AED2190 | Bar, Medurječka rijeka stream                 | 42.0226N, 19.22E   |
|                            | 8, M19_24_2_D12 | DNAEC047-20     |              | Bar, Medurječka rijeka stream                 | 42.0226N, 19.22E   |
|                            | 36, M19_24_1_G1 | DNAEC070-20     |              | Bar, Medurječka rijeka stream                 | 42.0226N, 19.22E   |
|                            | CCDB-3867-F01 | DNCD061-20      |              | Bar, Medurječka rijeka stream                 | 42.0363N, 19.2179E |
| *Hygrobates norvegicus*     | Hyd_MN_VP3   | DNAEC077-20     | BOLD-ACH7323 | Šavnik, spring Kikov izvor near Boan          | 42.9465N, 19.1893E |
|                            | CCDB 38361 A01 | DCDDJ001-21     |              | Kolašin, Laleviča Dolovi, spring #1          | 42.899N, 19.631E   |
|                            | CCDB 38361 A02 | DCDDJ002-21     |              | Kolašin, Laleviča Dolovi, spring #1          | 42.899N, 19.631E   |
|                            | CCDB 38361 A07 | DCDDJ007-21     |              | Kolašin, Laleviča Dolovi, spring #1          | 42.899N, 19.631E   |
| **Unionicolidae**           |              |                 |              |                                               |                   |
| *Neumania imitata*          | 15, M19_29C_2_E6 | DNAEC053-20     | BOLD-AED4073 | Danilovgrad, River Zeta at Spuž               | 42.5113N, 19.1982E |
| *Neumania limosa*           | CCDB-3867-C10 | DNCD034-20      | BOLD-AEF5902 | Tuzi, Vitoja, pool                            | 42.324N, 19.3637E  |
| Taxa                          | Voucher Code | BOLD Process ID | BIN          | Locality          | Coordinates          |
|-------------------------------|--------------|-----------------|--------------|-------------------|----------------------|
|                              | CCDB-3867-C01| DNCBD025-20     |              | Tuzi, Vitoja, pool| 42.324N, 19.3637E    |
| Unionicola minor              | CCDB38233 G06| DCCDB078-21     |              | Tuzi, Vitoja, pool| 42.324N, 19.3637E    |
|                              | CCDB-3867-G12| DNCBD084-20     | BOLD:AEF4865| Ulcinj, Šasko Lake| 41.9768N, 19.3389E   |
|                              | CCDB 38361 C02| DCDDJ026-21    |              | Ulcinj, Šasko Lake| 41.9768N, 19.3389E   |
|                              | CCDB 38361 C05| DCDDJ029-21    |              | Ulcinj, Šasko Lake| 41.9768N, 19.3389E   |
|                              | CCDB 38363 B09| SEPTA021-21    |              | Tuzi, Vitoja, pool| 42.324N, 19.3637E    |
|                              | CCDB 38361 C04| DCDDJ028-21    | BOLD:AAU0335| Ulcinj, Šasko Lake| 41.9768N, 19.3389E   |
| Unionicola ypsilophora       | CCDB 38363 D04| SEPTA040-21    |              | Cetinje, River Cnojevica (Anodonta exulcerata)| 42.3546N, 19.0286E   |
| Piona damkoehleri            | CCDB 38361 B03| DCDDJ015-21    | BOLD:AEK5107| Danilovgrad, Moromiš pond| 42.5322N, 19.1993E  |
|                              | CCDB 38361 B04| DCDDJ016-21    |              | Danilovgrad, Moromiš pond| 42.5322N, 19.1993E  |
|                              | CCDB 38361 B05| DCDDJ017-21    |              | Danilovgrad, Moromiš pond| 42.5322N, 19.1993E  |
|                              | CCDB 38361 B06| DCDDJ018-21    |              | Danilovgrad, Moromiš pond| 42.5322N, 19.1993E  |
|                              | CCDB 38361 B07| DCDDJ019-21    |              | Danilovgrad, Moromiš pond| 42.5322N, 19.1993E  |
| Piona laminata               | CCDB 38361 A12| DCDDJ012-21    | BOLD:AEL3248| Danilovgrad, Moromiš pond| 42.5322N, 19.1993E  |
| Piona disparilis             | Hyd_MN_VP12 | DNAEC085-20    | BOLD:AEE3977| Šavnik, spring of Bukovica stream, pool| 43.0589N, 19.1103E  |
|                              | CCDB 38363 A08| SEPTA008-21    |              | Nikšić, Vukovo Vrelo spring, pool| 42.8577N, 18.9416E  |
|                              | CCDB 38363 A09| SEPTA009-21    |              | Nikšić, Vukovo Vrelo spring, pool| 42.8577N, 18.9416E  |
| Typhis torris                | CCDB-3867-C08| DNCBD032-20    | BOLD:AEF2208| Tuzi, Vitoja, pool| 42.324N, 19.3637E    |
| Typhis ornatus               | CCDB 38361 B01| DCDDJ013-21    | BOLD:ACS0401| Danilovgrad, Moromiš pond| 42.5322N, 19.1993E  |
|                              | CCDB 38361 B02| DCDDJ014-21    |              | Danilovgrad, Moromiš pond| 42.5322N, 19.1993E  |
| Taxa                  | Voucher Code  | BOLD Process ID | BIN            | Locality                      | Coordinates       |
|----------------------|---------------|-----------------|----------------|-------------------------------|-------------------|
| **Wettinidae**       |               |                 |                |                               |                   |
| *Wettina lacustris*  | 30. M19_20_4_F8 | DNAEC065-20     | DNA-ADL2726    | Podgorica, Mareza canal       | 42.479N, 19.1813E |
| **Mideopsidae**      |               |                 |                |                               |                   |
| *Mideopsis milankovici* | 22, M19_24_2_E12 | DNAEC059-20    | BOLD-AED2191   | Bar, Medjuječka rijeka stream | 42.0226N, 19.22E  |
| *Mideopsis roztoczensis* | CCDB-3867-A02 | DNBC002-20     | BOLD-AC1492    | Podgorica, Cijevna stream     | 42.3095N, 19.0518E |
|                      | CCDB-3867-G05 | DNBC077-20     |                |                               | 42.4057N, 19.3569E |
|                      | CCDB38233 C12 | DCCDB036-21    |                | Danilovgrad, Zeta River at Spuž | 42.5112N, 19.1991E |
|                      | CCDB38233 C11 | DCCDB035-21    |                | Danilovgrad, Zeta River at Spuž | 42.5112N, 19.1991E |
|                      | CCDB 38363 D07 | SEPTA043-21    |                | Danilovgrad, Zeta River at Vranjske Njive | 42.4683N, 19.2579E |
| **Athienemanniidae** |               |                 |                |                               |                   |
| *Mundamella germanica* | 1. KIA_20B_D6 | DNAEC041-20    | BOLD-AED6269   | Danilovgrad, Spuž, spring near Zeta River | 42.5113N, 19.1982E |
| **Aturidae**         |               |                 |                |                               |                   |
| *Hexaxonopsis serrata* | CCDB 38363 B01 | SEPTA013-21    |                | Bar, Skadar Lake at Murići    | 42.1637N, 19.2214E |
|                      | CCDB 38363 B02 | SEPTA014-21    |                | Bar, Skadar Lake at Murići    | 42.1637N, 19.2214E |
|                      | CCDB 38363 B03 | SEPTA015-21    |                | Bar, Skadar Lake at Murići    | 42.1637N, 19.2214E |
| **Parabrachypoda montii** | 5. M19_20_6_D9 | DNAEC044-20    | BOLD-AED5455   | Podgorica, Mareza canal       | 42.479N, 19.1813E |
| **Woolastokia rotundifrons** | 10. M19_27_2_E1 | DNAEC048-20    | BOLD-AEE0289   | Šavnik, Tušina River at Boan   | 42.9432N, 19.205E  |
|                      | 11. M19_27_2_E2 | DNAEC049-20    |                | Šavnik, Tušina River at Boan   | 42.9432N, 19.205E  |
| **Arrenuridae**      |               |                 |                |                               |                   |
| *Arrenurus cylindratus* | 34, M19_20_1_F12 | DNAEC069-20    | BOLD-AED6864   | Podgorica, Mareza canal       | 42.479N, 19.1813E |
| *Arrenurus refractarioulus* | CCDB 38363 A07 | SEPTA007-21    |                | Nikšić, Lukavica Mt., pools   | 42.8118N, 19.1872E |
All obtained BINs were inspected for concordance using BOLD Workbench. The Refined Single Linkage (RESL) algorithm was used to assign water mite barcodes to Operational Taxonomic Units (OTUs).

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, version 10.1 (Kumar et al. 2018). The Neighbour-Joining (NJ) tree (edited in MEGA7, Kumar et al. 2016), based on K2P distances and pairwise deletion of missing data, was used to visualise similarity. The support for tree branches was calculated by the non-parametric bootstrap method (Felsenstein 1985) with 1000 replicates and shown next to the branches.

Results

DNA barcodes of 233 specimens morphologically assigned to 86 species from 28 genera and 15 families of water mites from Montenegro were newly generated for this study. The specimens were collected through the “DNA-Eco” (DNA barcode reference library as a tool for sustainable management of freshwater ecosystems in the highly threatened Lake Skadar Basin) project. The current study develops the first COI barcode reference library of water mites for Montenegro with the focus on Skadar/Shkodra Lake catchment area.

Fragment lengths of the analysed DNA barcode fragments ranged from 201 to 658 (mean: 636.2) base pairs, including no stop codons, insertions or deletions. The DNA barcode region was characterised by a high AT-content: the mean sequence compositions were A = 30.82 ± 0.1252%, C = 20.39 ± 0.1222%, G = 14.91 ± 0.0709% and T = 33.88 ± 0.1253%. The obtained results are similar to those found in other arthropod studies (e.g. Raupach et al. 2015).

The families Hygrobatidae Koch, 1842 and Lebertiidae Thor, 1900 are represented by the highest number of sequences (53 and 44, respectively). The opposite, the three families Arrenuridae Thor, 1900, Teutoniidae Koenike, 1910 and Limnesiidae Thor, 1900 are represented each with two sequences and the two families Athienemanniidae K. Viets, 1922 and Wettinidae Cook, 1956 by the lowest number of sequences (each with one sequence). The most common genus was Lebertia Neuman, 1880, for which 44 barcode sequences (11 species) were generated, followed by Atractides Koch, 1837 (35 barcodes; 13 species), Torrenticola Piersig, 1896 and Sperchon Kramer, 1877 (29 and 26 barcodes, 10 and 8 species, respectively). Six genera were represented by a single specimen. The highest number of barcodes per species was reached for Atractides pennatus (K. Viets, 1922), Sperchon violaceus Walter, 1944 and Torrenticola meridionalis Di Sabatino and Cicolani, 1990 (each with 10 barcodes), followed by Lebertia inaequalis (Koch, 1837) and L. variolata Gerecke, 2009 (each with 8 barcodes) and Sperchon thienemanni Koenike, 1907 (6 barcodes). On the other hand, most species are represented by less than 5 DNA barcodes. Thirty-three species are represented by a single DNA barcode not allowing us to estimate the intraspecific distances. BOLD ID and accession numbers for all specimens included in final dataset are given in Table 1.
The mean intrageneric K2P distance was 20.2 ± 0.0% (range 6.09-42.37%). The mean intraspecific nucleotide K2P distances were 2.43 ± 0.01% (ranging from 0% to 24.16%). The summary statistics showing significant changes of average K2P distances within the different taxonomic levels are given in Table 2.

Table 2.
Summary table of K2P genetic distances within the different taxonomic levels derived from 233 analysed water mite specimens from Montenegro. The list of studied species is provided in Table 1. Deletion Method: Pairwise Deletion. Alignment: BOLD Aligner (Amino Acid based HMM).

| Label          | n   | Taxa | Comparisons | Min Dist. (%) | Mean Dist. (%) | Max Dist. (%) | SE Dist. (%) |
|----------------|-----|------|-------------|---------------|----------------|---------------|--------------|
| Within Species | 200 | 53   | 391         | 0.00          | 2.43           | 24.16         | 0.01         |
| Within Genus   | 207 | 14   | 2291        | 6.09          | 20.20          | 42.37         | 0.00         |
| Within Family  | 168 | 7    | 1054        | 16.17         | 37.14          | 63.16         | 0.01         |

The BIIN and RESL (OTU) analyses assigned sequences to 98 BIINs and 103 OTUs, respectively. Fifty BIINs (159 records) were concordant (51%) and 48 BIINs were represented by a single sequence (49%). At the time of publication of the dataset, fifty-five (56.1%) of these BIINs (with 102 sequences) included sequences only from Montenegro, while the remaining BIINs included sequences also from other countries.

Most of the morphologically-identified species show an intraspecific variation of less than 2%. However, the 14 taxa listed in Table 3 showed a maximum interspecific divergence larger than 2%, resulting in these species in BOLD being spread over more than one BIIN. Two species, Lebertia glabra Thor, 1897 and L. inaequalis appeared each with 3 BIINs and twelve species, i.e. Lebertia maculosa Koenike, 1902, L. porosa Thor, 1900, Sperchon brevirostris Koenike, 1895, S. clupeifer Piersig, 1896, Sperchonopsis verrucosa (Protz, 1896), Monatracides madritensis (K. Viets, 1930), Torrenticola meridionalis, T. laskai Di Sabatino, 2009, Atractides gibberipalpis Piersig, 1898, A. nodipalpis Thor, 1899, Hygrobates calliger Piersig, 1896 and Unionicola minor (Soar, 1900), each with 2 BIINs (Table 3). In total, unique BIINs were revealed for 72 species (83.7%), two BIINs for 12 species (14.0%) and three BIINs for two species (2.3%).

The NJ analyses, based on K2P distances, revealed non-overlapping clusters with bootstrap support values > 95% for 50 species (58%) with more than one analysed specimen indicating a high congruence between BIINs affiliation and morphological species identification. Moreover, specimens showing high intraspecific distances are also clearly separated into different clades. A more detailed topology of all analysed specimens is presented in the supporting information (Suppl. material 2).

Discussion
This study provides COI barcodes for 233 specimens representing 86 morphologically identified species of water mites from Montenegro. These represent 42.8% of Montenegrin
water mite fauna, based on Pešić et al. (2018) and papers published thereafter (Pešić et al. 2019c, Pešić et al. 2020a, Pešić et al. 2020c, Pešić et al. 2020d, Pešić and Smit 2020). BOLD and RESL (OTU) analyses revealed 98 BINs and 103 OTUs, respectively, highlighting the high molecular diversity of the water mite fauna of Montenegro.

Table 3.
Species with intraspecific (ISD) maximum pairwise distances > 2.2% (p-dist.). Divergence values were calculated for all studied sequences, using the Nearest Neighbour Summary, implemented in the Barcode Gap Analysis tool provided by the Barcode of Life Data System (BOLD). BINs are based on the barcode analysis from 15 November 2021. Country codes (alpha-2 code): BG = Bulgaria, CH = Switzerland, DE = Germany, ES = Spain, FR = France, GB = United Kingdom, GL = Greenland, IT = Italy, NO = Norway, NL = Netherlands, ME = Montenegro, MK = North Macedonia, PL = Poland, RO = Romania, RS = Serbia, RU = Russia, SK = Slovakia. n = BIN member count.

| No. | Species          | BIN      | n  | MeanISD | MaxISD | Country          | Nearest BIN/Species | Distance to NN |
|-----|------------------|----------|----|---------|--------|------------------|---------------------|-----------------|
| 1.  | *Lebertia glabra* | BOLD:ACR9598 | 2  | 0.8     | 0.8    | ME, NL           | BOLD:ACS0595       | 12.52           |
|     | *Lebertia glabra* | BOLD:ACS0595 | 20 | 0.64    | 1.36   | NL, BG, ME, MK, IT, PL, SK | BOLD:AEJ3212 | 2.88 |
|     | *Lebertia glabra* | BOLD:AEI925 | 3  | 0.64    | 0.96   | ME               | BOLD:ACO2179       | 12.02           |
| 2.  | *Lebertia inaequalis* | BOLD:AEF5913 | 1  | N/A     | N/A    | ME               | BOLD:ADF6223       | 2.78 |
|     | *Lebertia inaequalis* | BOLD:ADF6223 | 18 | 0.18    | 0.34   | NL, PL, ME       | BOLD:AEF5913       | 2.78 |
|     | *Lebertia inaequalis* | BOLD:AEF2742 | 1  | N/A     | N/A    | ME               | BOLD:AEB4193       | 6.96 |
| 3.  | *Lebertia maculosa* | BOLD:AED9718 | 3  | 1.27    | 1.6    | ME, MK           | BOLD:AED9197       | 2.76 |
|     | *Lebertia maculosa* | BOLD:AED9197 | 2  | 0.16    | 0.16   | ME               | BOLD:AED9718       | 2.76 |
| 4.  | *Lebertia porosa*  | BOLD:ACS0974 | 133 | 0.81    | 2.37   | NL, FR, ME, DE, GB, BG, IT, PL, SK, ES, CH | BOLD:AED4662 | 3.89 |
|     | *Lebertia porosa*  | BOLD:AED4662 | 12 | 0.14    | 0.85   | ME               | BOLD:ACS0974       | 3.89 |
| 5.  | *Sperchon brevirostris* | BOLD:AED3857 | 3  | 0.32    | 0.48   | ME, MK           | BOLD:AEK3053       | 2.72 |
|     | *Sperchon brevirostris* | BOLD:ACP6107 | 28 | 0.55    | 3.12   | NO, DE, ME       | BOLD:AED3857       | 7.53 |
| 6.  | *Sperchon clupeifer* | BOLD:ACS1100 | 11 | 1.68    | 3.47   | NL, DE, NO, MK, ME, RU | BOLD:AAE4061 | 8.7 |
|     | *Sperchon clupeifer* | BOLD:AAE4061 | 1  | N/A     | N/A    | ME               | BOLD:ACS1100       | 8.7 |
| 7.  | *Sperchonopsis verrucosa* | BOLD:AEK8297 | 1  | N/A     | N/A    | ME, RO           | BOLD:ACS0908       | 4.83 |
| No. | Species                        | BIN          | n  | MeanISD | MaxISD | Country         | Nearest BIN/Species | Distance to NN |
|-----|-------------------------------|--------------|----|---------|--------|-----------------|---------------------|-----------------|
| 8.  | Sperchonopsis verrucosa       | BOLD:ACS9705 | 9  | 0.29    | 0.97   | NO, IT, ME      | BOLD:ADU8190       | 9.83            |
|     | Monatractides madritensis     | BOLD:AED3803 | 2  | 0.16    | 0.16   | ME              | BOLD:AEL3852       | 1.44            |
|     | Monatractides madritensis     | BOLD:AEL3852 | 2  | 0.64    | 0.64   | ME, SR          | BOLD:AED3803       | 1.44            |
| 9.  | Torrenticola meridionalis     | BOLD:AED7519 | 8  | 1.46    | 2.25   | ME, MK          | BOLD:AEI3402       | 6.57            |
|     | Torrenticola meridionalis     | BOLD:AEI3402 | 4  | 1.42    | 2.09   | ME              | BOLD:AEK9662       | 6.25            |
| 10. | Torrenticola laskai           | BOLD:AED2306 | 4  | 0.82    | 1.34   | RS, ME, RO      | BOLD:AED2306       | 6.57            |
|     | Torrenticola laskai           | BOLD:AED2306 | 4  | 0.82    | 1.34   | RS, ME, RO      | BOLD:AED2306       | 6.25            |
| 11. | Atractides gibberipalpis      | BOLD:AEK7766 | 1  | N/A     | N/A    | ME              | BOLD:AEI3946       | 4.81            |
|     | Atractides gibberipalpis      | BOLD:AEI3946 | 1  | N/A     | N/A    | ME              | BOLD:AEK7766       | 4.81            |
| 12. | Atractides nodipalpis         | BOLD:ACR0209 | 41 | 0.59    | 3.05   | NO, NL, GL, DE, ME, RS | BOLD:AED3548       | 13.3            |
|     | Atractides nodipalpis         | BOLD:AED3547 | 2  | 0       | 0      | ME              | BOLD:AAM4306       | 13.3            |
| 13. | Hygrobates calliger           | BOLD:AEF4261 | 2  | 1.2     | 1.2    | NO, ME          | BOLD:AEK4720       | 16.18           |
|     | Hygrobates calliger           | BOLD:AEL5782 | 2  | 1.03    | 1.03   | DE, ME          | BOLD:AEK4720       | 14.61           |
| 14. | Unionicola minor              | BOLD:AEF4865 | 3  | 0.59    | 0.7    | ME              | BOLD:ACI7165       | 17.02           |
|     | Unionicola minor              | BOLD:AAU0335 | 7  | 0.09    | 0.32   | NO, NL, ME      | BOLD:ACH3803       | 16.03           |

Of the 86 species recorded in this study, 79 species were previously reported for Montenegro. DNA barcoding confirmed the presence of four species new for Montenegro, i.e. *Lebertia reticulata* (Koenike, 1919), *Atractides inflatipalpis* K.Viets, 1950, *A. latipes* (Szalay, 1935) and *Parabrachypoda montii* (Maglio, 1924). Three species, i.e. *Protzia octopora* Lundblad, 154, *Piona laminata* (Thor, 1901) and *Unionicola ypsilophora* (Bonz, 1783) are recorded for the first time for the Balkan Peninsula. Specimens of the latter species were found between the gill blades of mussels *Anodonta exulcerata* Clesin, 1876, whose identification was confirmed by molecular data.

Moreover, species identification, based on molecular data conducted during this project, extended the list of Montenegrin water mites by description of several species new for science, i.e. *Atractides anae* Pešić, 2020, *Hygrobates lacrima* Pešić, 2020, *H. limnocrenicus* Pešić, 2020, *H mediterraneus* Pešić, 2020 and *Mideopsis milankovici* Pešić and Smit, 2020 (Pešić et al. 2020a, Pešić et al. 2020c, Pešić et al. 2020d, Pešić and Smit 2020).
2020). All of these studies highlighted the importance of an integrated approach that combines the morphology-based taxonomy and DNA barcodes.

Our study confirmed efficiency of DNA barcoding as a tool for the identification of water mites. In particular, 72 of the 86 morphologically-identified species exactly matched the BINs defined from BOLD. This result coincides with high identification efficiency rates through the BOLD Best Close Match analysis. Nevertheless, our data revealed also 14 species listed in Table 3 that showed high intraspecific distances (> 2.2%) suggesting possible cryptic and/or pseudocryptic diversification. Most of these possible cryptic and/or pseudocryptic species, as seen in Table 3, appear to be hidden within common species.

Three species, i.e. *Lebertia maculosa*, *Monatractides madritensis* and *Torrenticola laskai* appeared each with 2 BINs in our dataset. The intraspecific maximum distances between BINs within each of these species were below 3% (Suppl. material 1). On the other hand, the intraspecific maximum distances between BINs within each of the other eleven species in the dataset were greater than 5% (Suppl. material 1).

*Lebertia glabra*, a species widely distributed in West Palaeartic (Di Sabatino et al. 2010) appeared in our dataset with 3 BINs. The first cluster (BIN:ACR9598) includes two specimens from Montenegro and The Netherlands; the second cluster (BIN:ACS0595) was more represented in BOLD and includes specimens from different parts of Europe - from The Netherlands and Poland to Montenegro, Italy and Macedonia. The third cluster (BOLD:AEI925) contained only specimens from Montenegro. The intraspecific K2P distances between all clusters ranged from 14.3 to 17.7% (Suppl. material 1).

*Lebertia inaequalis*, a species reported from the extended parts of the Palaeartic (Gerecke 2009, Di Sabatino et al. 2010), appeared in our dataset with 3 BINs, two of which each include only one specimen from Montenegro (BIN:AEF5913 and BIN:AEF2742, respectively). The third cluster (BIN:ADF6223), based on available records from BOLD, appears to be more widespread and contained specimens from The Netherlands, Poland and Montenegro. Intraspecific K2P distances between the latter cluster and BIN:AEF5913 was only 0.1%, while the distance from the second cluster (BIN:AEF2742) from Montenegro was rather large (17.3%; Suppl. material 1) highlighting the necessity of additional comprehensive morphological and molecular analysis.

*Lebertia porosa*, a eurytopic and eurythermous species, often reported from standing waters and pools of streams across the Holarctic (Gerecke 2009, Di Sabatino et al. 2010), is currently in the process of being revised (R. Gerecke, pers. communication) using DNA barcodes. Stur (2017) showed that 18 specimens of *L. porosa* from Norway comprise 7 BINs with a mean intraspecific p-distance of 11.7% and maximum up to 18.5%. In our dataset, specimens, morphologically assigned to *Lebertia porosa*, were presented with two clusters. Based on the available records from BOLD, the first cluster (BIN:ACS0974) appeared to be well represented in the BOLD database with 133 records from different parts of Europe; the second cluster (BIN:AED4662) contained specimens only from Montenegro. In our study, specimens of the latter BIN were collected in large limnocrenic springs, such as Mareza and Vitoja, while specimens from the first cluster (BIN:ACS0974)
were sampled in the lower reaches. The intraspecific K2P distance between these two *L. porosa* clusters in our dataset was estimated at 5.5% (Suppl. material 1).

*Sperchon brevirostris*, a species inhabiting low-and middle order streams in the study area (Pešić et al. 2010, Pešić et al. 2018), was represented in our material by two clusters. Based on the available records from BOLD, the first cluster (BIN:ACP6107) includes specimens from Norway, Germany and one specimen from Montenegro, while the second cluster (BOLD:AED3857) contained three specimens from Montenegro and North Macedonia. The K2P distance between these two clusters was 8.1% (Suppl. material 1). Similarly, *S. clupeifer*, a species frequently reported from Western Palaearctic (Di Sabatino et al. 2010), appeared with two clusters in our dataset. The first cluster (BIN:ACS1100) is well represented in BOLD and includes specimens from different part of Europe, while the second cluster (BIN:AEE4061) contained a single specimen from Montenegro. The intraspecific K2P distance between these two clusters in our dataset was estimated at 8.3% (Suppl. material 1).

*Sperchonopsis verrucosa*, a species often reported from the Holarctic Region (Gerecke et al. 2016), was represented in our study with two clusters. The first cluster (BIN:ACS9705) was more represented in BOLD and includes specimens from Norway, Italy and one specimen from Montenegro. The second cluster (BIN:AEK8297) includes two specimens from Montenegro and Romania. The intraspecific K2P distances between these two clusters was 11.2%, indicating the need for additional integrative analysis.

*Torrenticola meridionalis*, a species originally described from Italy, is widely distributed in Montenegro, inhabiting mainly low order streams (Pešić et al. 2018). It is morphologically closely related to *T. elliptica* which remains distinguishable in the male sex only, based on the stouter genital field. In our COI tree (Suppl. material 2), *T. elliptica* appeared as a sister clade to the clade that includes two clusters morphologically assigned to *T. meridionalis* (BIN:AEI3402 and BOLD:AED7519, respectively). The intraspecific K2P distances between *T. elliptica* and *T. meridionalis* clusters ranged from 8.6-9.0%. On the other hand, the K2P distance between *T. meridionalis* clusters in our dataset was estimated at 6.6%.

*Atractides nodipalpis*, a rhitrobiontic species, is the most frequently reported species of the genus in Europe (Gerecke et al. 2016). In our dataset, sequences of the specimens, morphologically assigned to the latter species, appeared as two clusters. Interestingly, specimens of both clusters were recorded syntopically. The first cluster (BIN:ACR0209) in the BOLD database was represented with 41 specimens from Norway (country of the type locality), The Netherlands, Montenegro and Russia, but also from Greenland. The second cluster (BIN:AED3547) includes two specimens from Montenegro. The intraspecific K2P distance between these two clusters was 18.8%, indicating the need for a comprehensive revision of this species complex.

*Hygrobates calliger*, a rhitrobiontic species widely distributed in the Palaearctic (Di Sabatino et al. 2010), was represented by two clusters in our dataset, each with two records in the BOLD database. The first cluster (BIN:AEF4261) includes specimens from Norway and Montenegro, while the second cluster (BIN:AEL5782) includes specimens
from Germany and Montenegro. The intraspecific K2P distance between these two clusters was 20.9% (Suppl. material 1), suggesting the existence of possible hidden cryptic and/or pseudocryptic species.

The sequences of *Atractides gibberipalpis*, a rhitrobiontic species often reported from the Palaeartctic (Pešić et al. 2021a), in our dataset were assigned to two different barcode clusters, each represented by a single specimen from Montenegro. The intraspecific K2P distance between these two clusters (BIN: BOLD:AEK7766 and BIN: BOLD:AEI3946, respectively) was estimated at 5% (Suppl. material 1).

*Unionicola minor*, a species widely distributed in Europe (Gerecke et al. 2016), was presented with two clusters in our dataset. Based on available data from BOLD, the first cluster (BIN:AAU0335) includes specimens from Norway and The Netherlands and one specimen from Lake Šasko in Montenegro. The second cluster (BIN:AAU0335) includes specimens only from Montenegro. The intraspecific K2P distances between these two clusters in our dataset was 23.8% (Suppl. material 1), suggesting the existence of cryptic (or pseudocryptic, see Pešić and Smit (2016) for a discussion about pseudocryptic speciation in water mites) species. Stålstedt et al. (2013) showed that the Swedish population of *Unionicola minor* consists of at least three cryptic species, emphasising the need for further research of the species in this complex.

Taxonomic studies of the above species were outside the scope of this paper. Further studies with material from a wider geographical area, were needed to clarify taxonomy and elucidate the delimitation of the species in the above complexes. This process should be accompanied by sufficient barcode coverage to allow the detection of phylogeographic patterns and/or even the existence of possible overlooked cryptic species. The build-up of DNA barcode library for water mites of Montenegro represents a long-term task, aimed at improving molecular identification and inclusion of this group in environmental assessment programmes and, on the other hand, to stimulate further biodiversity research of this limnofaunistic group in Montenegro and the Balkans.

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**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.

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Supplementary materials

Suppl. material 1: Molecular distances [doi]

Authors: Vladimir Pešić, Andrzej Zawal, Ana Manović, Aleksandra Bańkowska, Milica Jovanović
Data type: Molecular distances
Brief description: Molecular distances, based on the Kimura 2-parameter model of the analysed specimens of water mites from Montenegro. BINs are based on the barcode analysis from 15 November 2021.
Download file (79.41 kb)

Suppl. material 2: Compact Neighbour-Joining tree [doi]

Authors: Vladimir Pešić, Andrzej Zawal, Ana Manović, Aleksandra Bańkowska, Milica Jovanović
Data type: Neighbour-joining tree
Brief description: Compact Neighbour-Joining tree of all analysed water mite species based on Kimura 2-parameter distances. The tree was edited in MEGA7 (Kumar et al. 2016). Specimens are classified using ID numbers from BOLD and species name. BINs are based on the barcode analysis from 15 November 2021. Numbers next to nodes represent non-parametric bootstrap values (1,000 replicates, in %). The analyses involved all 233 COI nucleotide sequences.
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