

Article

Taxonomic Reassessment of Freshwater Mussels from the Western Balkans Reveals an Overlooked but Critical Refugium and Defines Conservation Priorities

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Abstract: Freshwater mussels are an endangered group of animals, especially in the water bodies of Central and Western Europe. Conservation priorities were established for many endangered freshwater mussel species in the last decade. Here, we present new data on a cryptic refugium of freshwater mussels in the Western Balkans. Two species, *Anodonta exulcerata* Porro, 1838 and *Unio elongatulus* Pfeiffer, 1825, were discovered in Montenegro for the first time. The phylogenetic position and taxonomic status of freshwater mussel species from Montenegro were studied by means of an integrative approach, combining morphological data and *COI* gene sequences. *Unio carneus* Küster, 1854 **stat. rev.** represents a species with a restricted range, which is endemic to the Western Balkans. A viable population of *Microcondylaea bonellii* (Férussac, 1827) was discovered in the Zeta River. This species was not mentioned in Montenegro since the 1900s. A significant level of genetic diversity was revealed for the studied species. In the Skadar Lake basin, freshwater mussels from diverse ecological groups were discovered. Rheophilic species of freshwater mussels may represent ancient lineages, which need special conservation planning. Conservation priorities should be aimed at the protection of freshwater mussel habitats on the Balkan Peninsula.

Keywords: freshwater mussels; the Balkan Peninsula; endemic species; phylogeography; taxonomy; conservation; phylogenetics; species diversity



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1. Introduction

Freshwater mussels are an ecologically and economically important group of aquatic animals [1] that are sensitive to water pollution, habitat loss, climate change, and other stressful anthropogenic and natural factors [2–4]. Some species of this taxonomic group (e.g., *Unio crassus*) are distributed in numerous river basins across Europe and they are endangered in certain regions with a high level of anthropogenic pressure. Endemic species or threatened populations of freshwater mussels, which are distributed in several large river basins in this region, were discussed recently in a few papers [5–7]. Endemic lineages of freshwater mussels were determined using the DNA sequence data that allow for solving the taxonomic uncertainty of local endemics, which were described on the basis of morphological data alone. These local endemic taxa may represent divergent intraspecific lineages of widely distributed species [8] or local endangered endemic species [7,9,10]. Therefore, the detection of endangered populations and the assessment of the current status of regional freshwater mussel assemblages should be considered important goals for biological studies. In some cases, a high level of genetic differentiation among freshwater

mussel populations with a narrow environmental niche could be linked to the influence of geographic barriers [11].

The Balkan Peninsula has served as a glacial refugium for multiple species of freshwater animals, including amphipods, gastropods and isopods [12–17]. For example, a cold-adapted amphipod species (*Homoeogammarus scutarensis*) is characterized by a high level of genetic divergence and was probably isolated there since the end of the Early Pliocene (4 Ma) [12,14]. Several endemic species of freshwater gastropods [15,16] and isopods [17] are also presented in the Lake Skadar basin, which confirms its importance for the conservation of freshwater biodiversity in the Balkans.

The distribution and conservation status of freshwater mussels are assessed in many regions of Southern Europe, including those of endemic and endangered species [18,19]. At the same time, freshwater mussels endemic to basins of the Western Balkans, particularly the Skadar Lake basin, are not sufficiently studied (see [20] for an overview of the freshwater mollusk diversity in this lake).

This study aims to provide new data on the species richness and phylogeography of freshwater mussels in Montenegro to clarify the taxonomic position and conservation status of keystone species from the Western Balkans.

2. Materials and Methods

2.1. Data Sampling

Freshwater mussel specimens were collected from seven sites across the Skadar Lake basin at the beginning of August 2021 (Figures 1 and 2). Totally, 71 specimens (i.e., 35 *Anodonta*, 30 *Unio*, and 6 *Microcondylaea*) were sampled (Table 1). A tissue snip from each specimen was preserved in 96% ethanol immediately after collection. The tissue snips and dry shell vouchers are deposited in the Russian Museum of Biodiversity Hotspots (RMBH thereafter), N. Laverov Federal Center for Integrated Arctic Research, the Ural Branch of the Russian Academy of Sciences, Arkhangelsk, Russia.

Table 1. List of studied localities in Montenegro.

No	Description of Sampling Sites	Coordinates		Number of Collected Specimens
		Latitude	Longitude	
1	Lower part of the Zeta River at Vranjske Njive (Podgorica)	42°28'6" N	19°15'29" E	4
2	Zeta River near Spuž town (Danilovgrad)	42°30'41" N	19°11'55" E	12
3	Crnojevića River near River Crnojevića village	42°21'16" N	19°1'40" E	23
4	Lower part of the Plavnica River near its mouth into Skadar Lake	42°16'20" N	19°12'3" E	13
5	Skadar Lake near Donji Murići village	42°9'48" N	19°13'18" E	3
6	Skadar Lake near sublacustrine spring at Podhum	42°18'48" N	19°21'13" E	6
7	Podgorica, Sitnica River, 800 m northeast of Čafa village	42°27'39" N	19°10'37" E	10

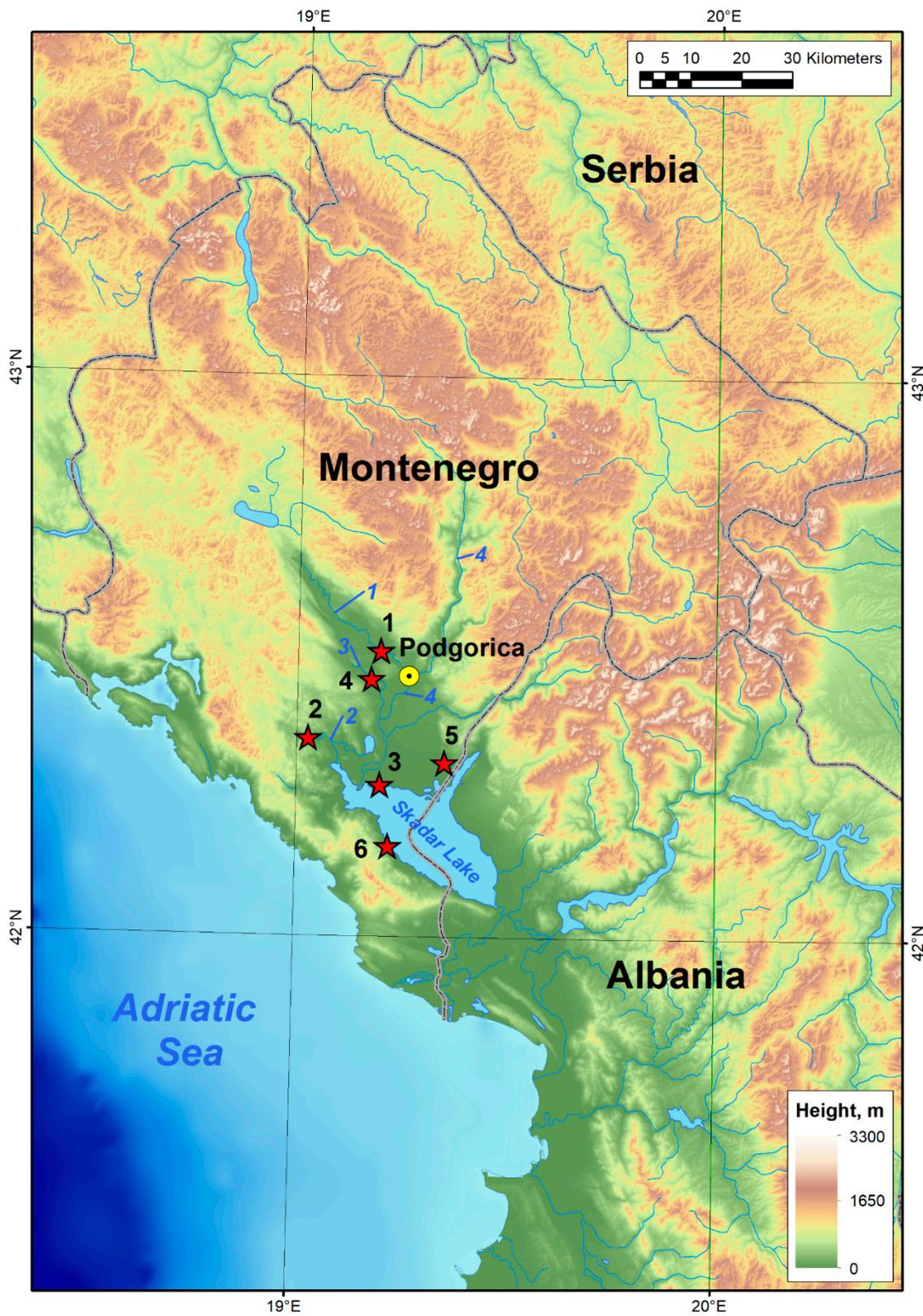


Figure 1. Map of Montenegro with our sampling localities. Red stars indicate studied sites: Zeta River (1); Crnojevića River (2); Plavnica River (3); Sitnica River (4); Skadar Lake (Podhum), site 1 (5); and Skadar Lake (Donji Murići village), site 2 (6). Violet numbers indicate rivers: Zeta River (1); Crnojevića River (2); Sitnica River (3); and Morača River (4).

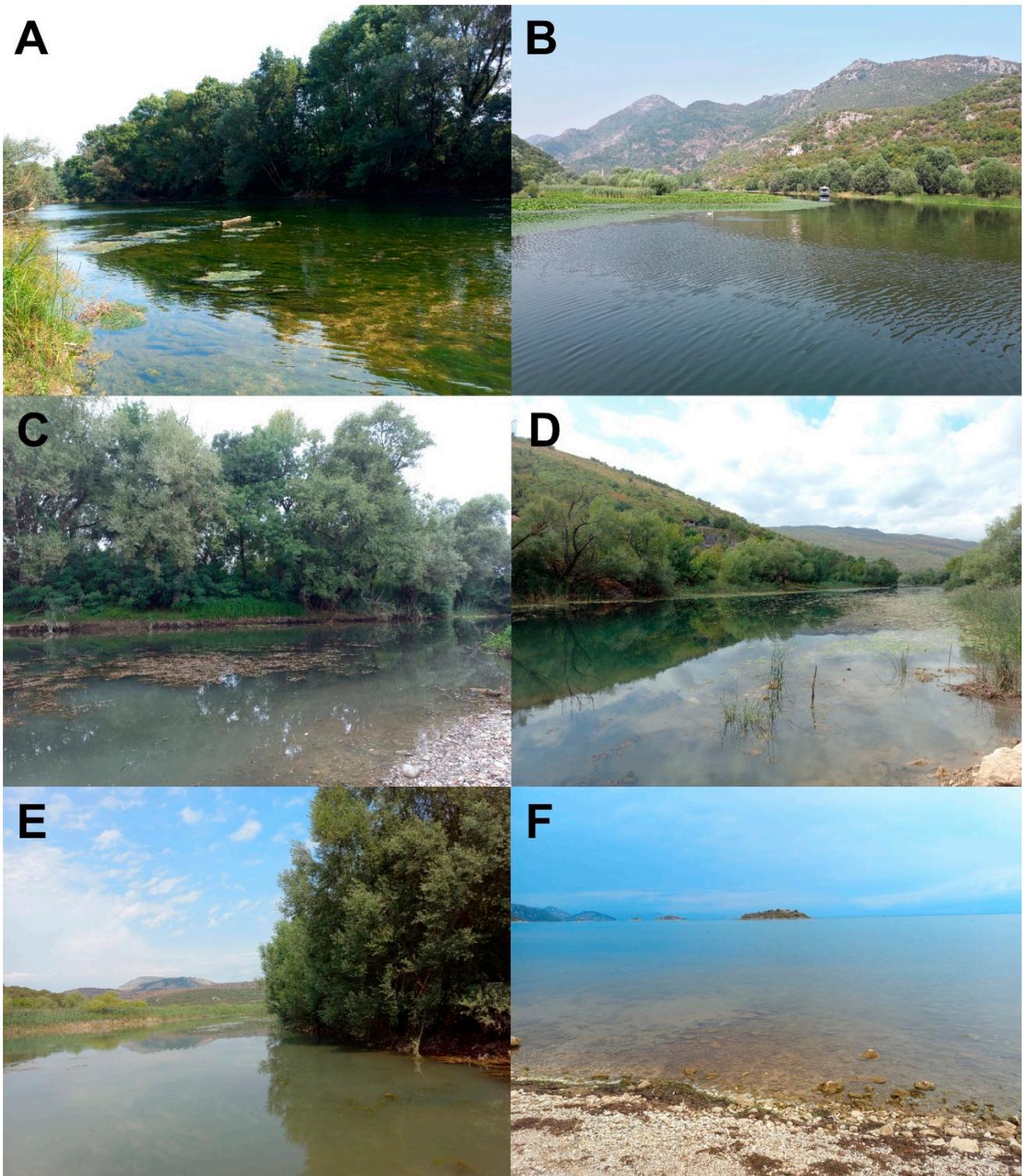


Figure 2. Habitats of freshwater mussels (Unionidae) in Montenegro. (A) Zeta River (*Microcondylaea bonellii* and *Unio carneus* **stat. rev.**). (B) Crnojevica River (*Anodonta exulcerata*, *Unio carneus* **stat. rev.**, and *U. elongatulus*). (C) Plavnica River (*A. exulcerata* and *U. elongatulus*). (D) Sitnica River (*A. exulcerata*). (E) Skadar Lake (Podhum), site 1 (*A. exulcerata*). (F) Skadar Lake (Donji Murići village), site 2 (*U. elongatulus*).

For comparative morphological studies, we analyzed the shell shape, structure of pseudocardinal and lateral teeth, and the sculpture and position of umbo. The type series of target nominal taxa and other freshwater mussels from museum collections, original

descriptions and figures from appropriate scientific literature, and available images from the MUSSELP database (<https://mussel-project.uwsp.edu>, accessed on 10 June 2022) were used for morphological investigations.

2.2. DNA Extraction and Sequencing

Total genomic DNA was extracted from available tissue snips using the NucleoSpin Tissue Kit (Macherey-Nagel GmbH and Co., KG, Düren, Germany), following the manufacturer's protocol. For molecular analyses, we obtained sequences of the mitochondrial *cytochrome c oxidase subunit I (COI)* gene fragment. The sequences were amplified and sequenced using primers LCO1490 and HCO2198 [21]. The PCR mix contained approximately 200 ng of total cellular DNA, 10 pmol of each primer, 200 μ mol of each dNTP, 2.5 μ L of PCR buffer (with 10×2 mmol $MgCl_2$), 0.8 units of Taq DNA polymerase (SibEnzyme Ltd., Novosibirsk, Russia), and H_2O , which was added up to a final volume of 25 μ L. Thermocycling included one cycle at 95 °C (4 min), followed by 28–32 cycles of 95 °C (50 s), 46–48 °C (50 s), and 72 °C (50 s), and by a final extension at 72 °C (5 min). Forward and reverse sequencing were performed on an automatic sequencer (ABI PRISM3730, Applied Biosystems) using the ABI PRISM BigDye Terminator v.3.1 reagent kit. The resulting *COI* sequences were checked manually using BioEdit software version 7.2.5 [22]. In addition, 176 *COI* sequences of *Unio*, *Anodonta*, and *Pseudanodonta* specimens were obtained from NCBI's GenBank. The *COI* sequences of *Parreysia* spp. were used as outgroup for the phylogenetic analyses (Table S3).

2.3. Phylogeographic and Phylogenetic Analyses

The alignment of the *COI* sequences was performed directly using the ClustalW algorithm [23]. The dataset (total length of 660 bp) was collapsed from 103 available haplotypes to a set of 91 unique haplotypes (Tables S1 and S3) using an online FASTA sequence toolbox (FaBox v1.61) [24]. Three partitions (3 codons of *COI*) were used for phylogenetic analysis.

The phylogenetic analyses were carried out using maximum likelihood and Bayesian inference approaches. Maximum likelihood phylogenetic searches were performed through IQ-TREE (W-IQ-TREE) web-server with automatic identification of the best-fit substitution model for each partition [25]. Best-fit models were estimated automatically according to Bayesian informational criteria (BIC) as F81+F for the 1st codon, TIM3+F+G4 for the 2nd codon, and TN+F+G4 for 3rd codon using ModelFinder software [26]. An ultrafast bootstrap (UFBoot) algorithm with 1000 replicates was implemented for estimation of the internal branches' probability [27].

The Bayesian analyses were performed in MrBayes v. 3.2.6 [28]. We assigned the same evolutionary model for each codon of the *COI* fragment as those used in our maximum likelihood phylogenetic analysis. We completed four runs, each with three heated (temperature = 0.1) and one cold Markov chain, using 15,000,000 generations with sampling every 1000th generation. All calculations were carried out at the San Diego Supercomputer Center through the CIPRES Science Gateway [29]. The first 25% of trees were discarded as burn-in. A convergence of the MCMC chains to a stationary distribution was checked through Tracer v. 1.7.1 [30].

The phylogeographic analysis was performed on the basis of a median-joining network approach using Network v. 5.0.0.1 software with default settings [31]. We used the *COI* datasets with 55 sequences of *Anodonta exulcerata*, 36 sequences of *Microcondylaea bonellii*, and 122 sequences of *Unio elongatulus* (Tables S1 and S2). Missing sites were removed from these datasets, and all sequences in each dataset were shortened in accordance with the minimum sequence length (567 bp for *Anodonta exulcerata*, 587 bp for *Microcondylaea bonellii*, and 531 bp for *Unio elongatulus*).

2.4. Distribution Mapping

The distribution maps for each species were created using ESRI ArcGIS v. 10 software, Redlands, CA, USA (www.esri.com/arcgis, accessed on 10 June 2022). Additional occurrence data were obtained from published sources [7,9,10,32–42], as well as from the following museum collections: NCSM Mollusk Collection (North Carolina State Museum of Natural Sciences, Raleigh, NC, USA) [43], SMF Mollusca Collection (Senckenberg Museum, Frankfurt, Germany) [44], Musée d'histoire naturelle (MHNN Collection, Neuchâtel, Switzerland) [45] and Biologiezentrum Linz (Linz, Austria) [46].

3. Results

3.1. Species Richness of Freshwater Mussels in Montenegro

Four species of freshwater mussels (Unionidae) were found in the studied sites (Figures 3–8, Table 2). Two species belong to the genus *Unio* (*Unio carneus* and *Unio elongatulus*), while two others belong to the genera *Anodonta* (*Anodonta exulcerata*) and *Microcondylaea* (*Microcondylaea bonellii*). The greatest number of freshwater mussel species was observed at the natural sampling locality in the Crnojevića River (Table 2). In contrast, each of the three sampling localities in urban and suburban areas impacted by anthropogenic loads houses only one species of freshwater mussels.

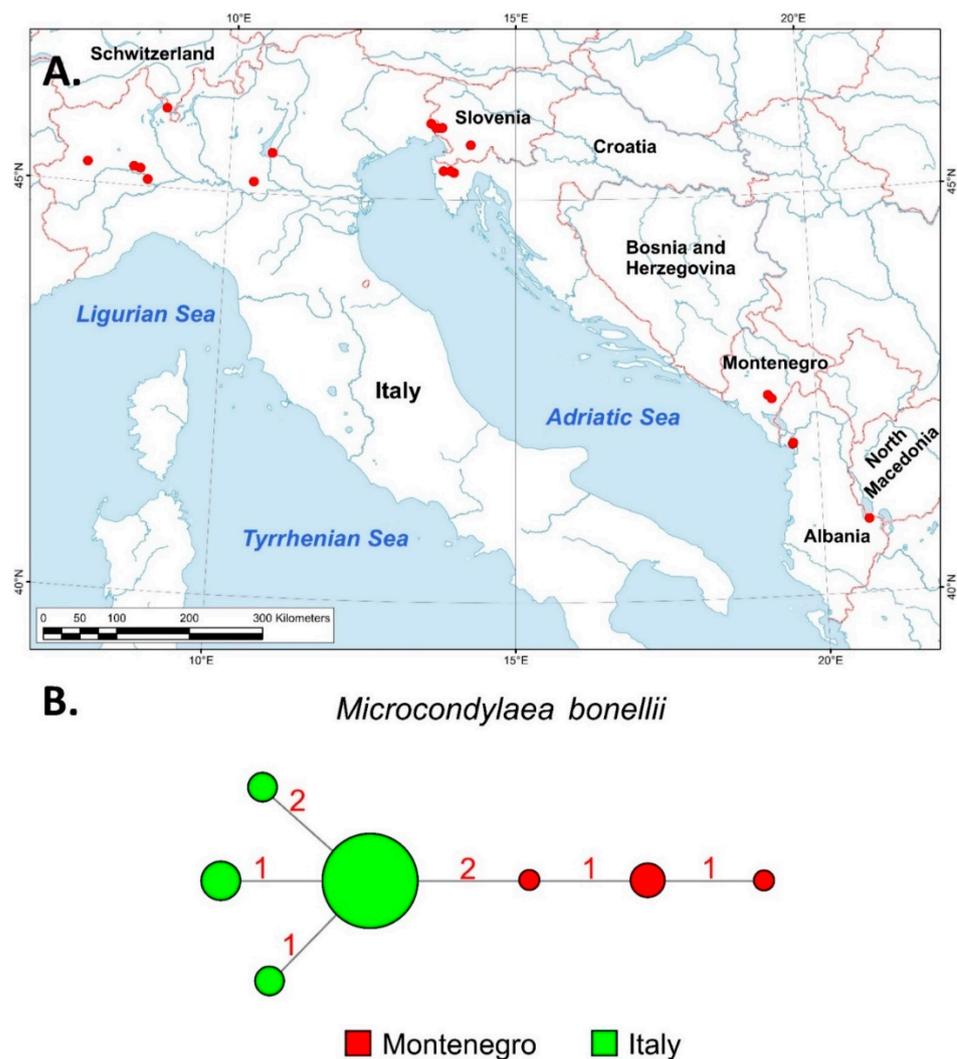


Figure 3. Distribution and phylogeography of *Microcondylaea bonellii*: range map (A) and median-joining network (B).



B. *Anodonta exulcerata*

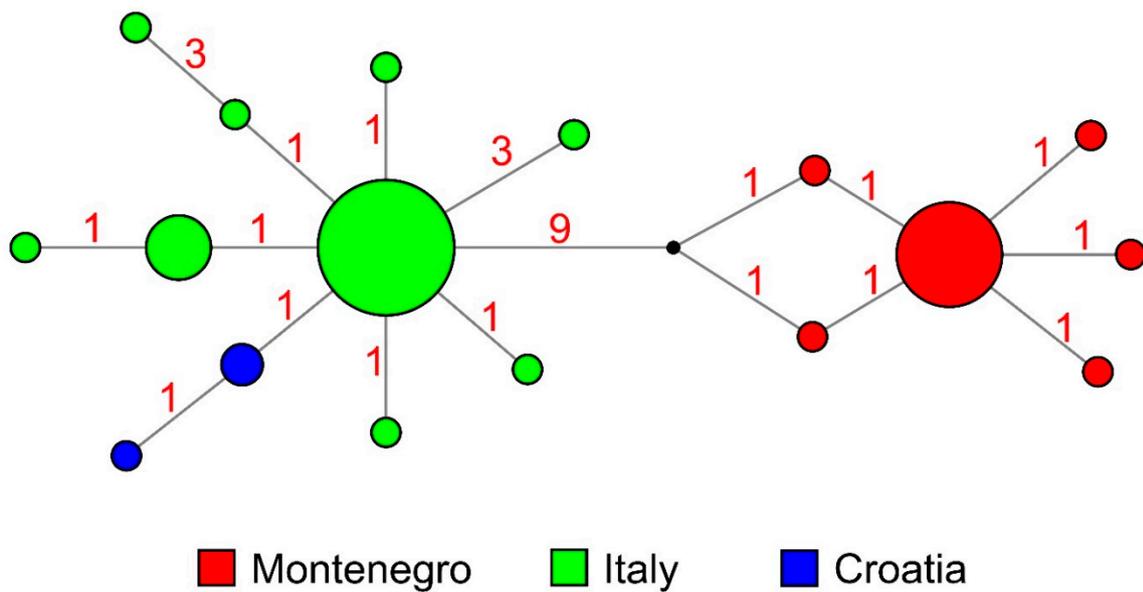


Figure 4. Distribution and phylogeography of *Anodonta exulcerata*: range map (A) and median-joining network (B).

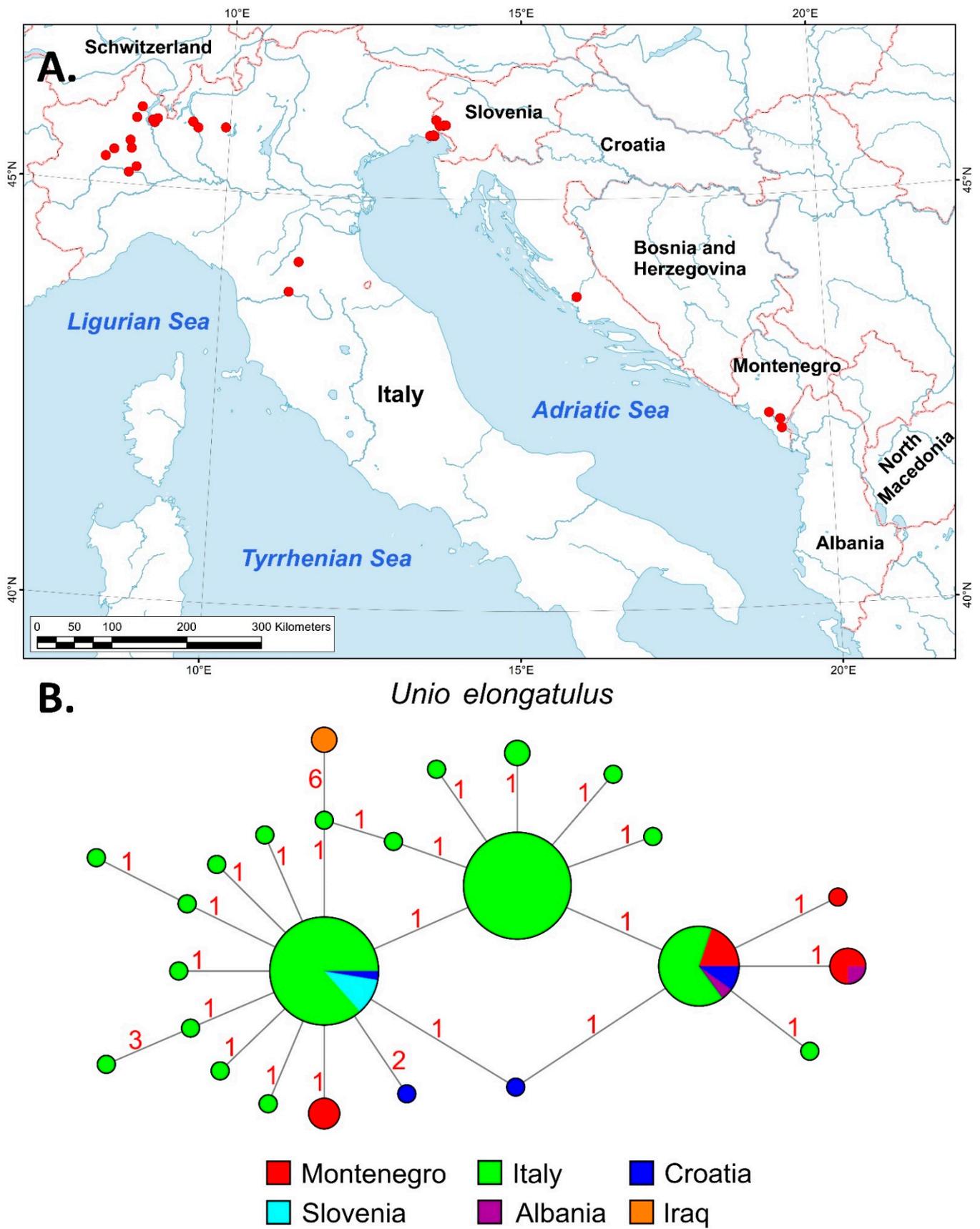


Figure 5. Distribution and phylogeography of *Unio elongatulus*: range map (A) and median-joining network (B).



Figure 6. Distribution of *Unio carneus* stat. rev.

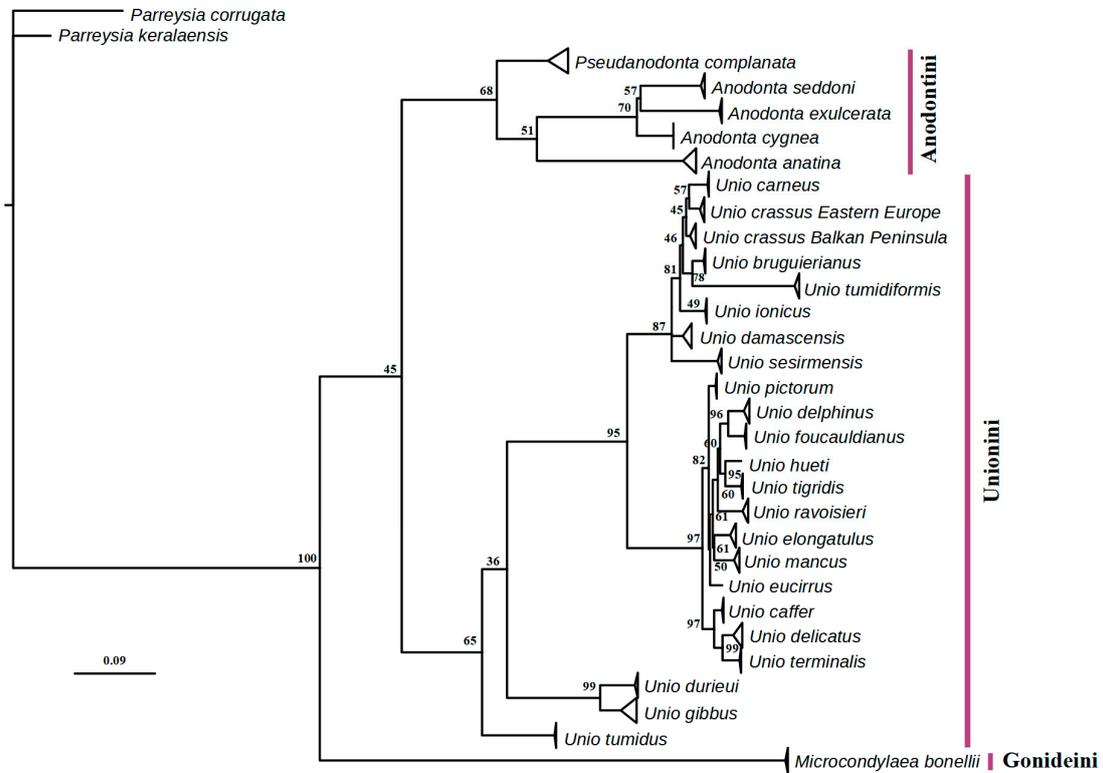


Figure 7. Maximum likelihood phylogeny of freshwater mussels (Unionidae) from Montenegro and adjacent countries with supplement of related taxa based on the COI barcode fragment. Black numbers near nodes are BS values.

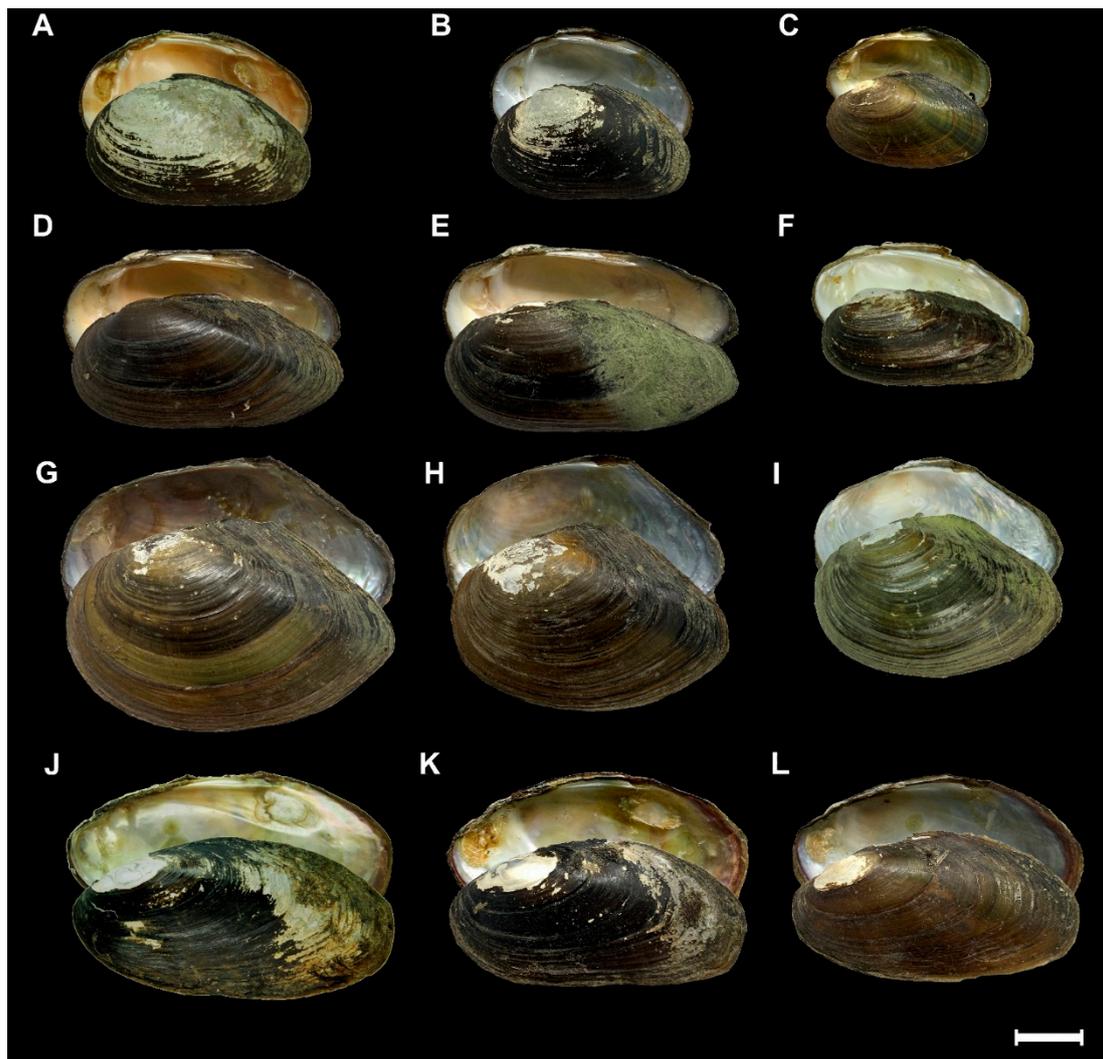


Figure 8. Shells of freshwater mussels (Unionidae) from Montenegro (RMBH collection): *Unio carneus*: RMBH biv1201/2 (A), RMBH biv1204/1 (B), and RMBH biv1204/2 (C); *U. elongatulus*: RMBH biv1206/8 (D), RMBH biv1209/2 (E), and RMBH biv1210/5 (F); *Anodonta exulcerata*: RMBH biv1208/1 (G), RMBH biv1207/2 (H), and RMBH biv1205/5 (I); *Microcondylaea bonellii*: RMBH biv1203/6 (J), RMBH biv1203/4 (K), and RMBH biv1203/5 (L).

Table 2. Species richness and number of collected specimens of freshwater mussels in Montenegro.

No	Species	Sampling Sites						
		Zeta River Near Vranjske Njive	Zeta River at Spuž	Crnojevića River	Skadar Lake (Donji Murići Village)	Plavnica River	Skadar Lake (Podhum)	Sitnica River
1	<i>Unio carneus</i>	4	6	4	-	-	-	-
2	<i>Unio elongatulus</i>	-	-	10	3	3	-	-
3	<i>Microcondylaea bonellii</i>	-	6	-	-	-	-	-
4	<i>Anodonta exulcerata</i>	-	-	9	-	10	6	10
Number of species		1	2	3	1	2	1	1

3.2. Phylogeographic and Distributional Patterns

We obtained 44 *COI* sequences for the four species of freshwater mussels collected from Montenegro (Table S1). Totally, 28 unique *COI* haplotypes were recorded for these species. The maximum likelihood tree and the Bayesian phylogeny of the Unionidae based on the

COI haplotypes revealed several separate lineages of the *Unio crassus* group, including a distant lineage from the Skadar Lake basin (Figure 7 and Figure S1). Taxonomically, the latter lineage belongs to *Unio carneus* stat. rev. (Figure 6). The mean uncorrected COI p-distance between *Unio carneus* stat. rev. from Montenegro and *U. crassus* from water bodies of the Russian Plain and Baltic Sea basin is $3.7 \pm 0.7\%$.

Four haplotypes were recorded in *Unio elongatulus* samples from Montenegro (Figure 5). The mean uncorrected COI p-distance between the closest (Albanian, Italian, Croatian, and Slovenian) populations and our samples from Montenegro varies from 0.26 to 0.39%. The samples of *Microcondylaea bonellii* from Montenegro are a highly differentiated group in our phylogenetic tree, forming a separate clade with full bootstrap support. The mean uncorrected COI p-distance between its population in Zeta River and the Italian populations is $0.57 \pm 0.27\%$. *Anodonta exulcerata* from the Skadar Lake basin formed a separate group within the Anodontini clade. This species is more closely related to *Anodonta seddoni* (BS = 57) and *A. cygnea* (BS = 70). The mean uncorrected COI p-distance ranges from 2.04 to 2.18% between the samples from Montenegro and the Italian and Croatian populations.

Our network analyses revealed that there is a highly divergent lineage of *Anodonta exulcerata* in Montenegro, which differs from other populations of this species (Apennine Peninsula) by 10–12 nucleotide substitutions (Figure 4). *Unio carneus* stat. rev. seems to be a unique phylogenetic lineage endemic to Montenegro (Figure 6). Two other species, that is, *Unio elongatulus* and *Microcondylaea bonellii*, do not share such a strong geographic differentiation (Figures 3 and 5). In particular, Montenegro haplotypes of *Unio elongatulus* are mixed in the network among haplotypes from the Italian and Croatian rivers (Figure 5). The lineage of *Microcondylaea bonellii* from Montenegro differs from that of the Apennine Peninsula by two nucleotide substitutions (Figure 3).

4. Discussion

4.1. Taxonomic and Biogeographic Issues

Our integrative taxonomic analysis has returned a list of freshwater mussel species (Figure 4), which inhabit different types of water bodies in the Skadar Lake basin. Available molecular data allow for providing a critical reassessment of the taxonomic status of freshwater mussel taxa in the region.

In particular, *Unio carneus* should be considered a separate species based on a significant genetic distance from its sister species, *U. crassus* ($3.7 \pm 0.7\%$ for the COI sequences) [47]. Here, we show that the external conchological features of this species are variable (Figure 8). Other allopatric species in the *Unio crassus* group were uncovered by Lopes-Lima et al. [48] from Turkey (*U. bruguierianus*) and by Araujo et al. [19] from Greece and Albania (*U. ionicus*).

Anodonta exulcerata was previously defined based on samples from the Italian Peninsula to Croatia, west of the Dinaric Alps [7]. Here, we report on new records of *A. exulcerata* in water bodies of the Skadar Lake basin (e.g., Plavnica, Crnojevica, and Sitnica rivers) (Figure 6). Our results suggest that the population of this species in Montenegro was isolated for several millions of years according to a large number of nucleotide substitutions in the COI gene sequences. These data support the hypothesis on the presence of a glacial refugium for freshwater taxa in Montenegro [49]. The mean uncorrected COI p-distance between this population and those from the Apennine Peninsula and northern Dinaric Alps suggests a divergence event of 5.7 Ma, assessed by the mean COI substitution rate for the family Unionidae [50]. A star-shaped network probably reflects a recent bottleneck in populations of *Anodonta exulcerata* from water bodies of the Skadar Lake basin with a subsequent demographic expansion. Similar phylogeographic patterns were observed earlier for two lineages of the widespread species *Anodonta anatina* from South European freshwater basins [51]. It may be explained by the long-term isolation of mussel populations in the freshwater refugia of Southern Europe during cold climatic episodes of the Late Pliocene and Pleistocene.

Unio elongatulus was generally known from freshwater basins of the Italian Peninsula, Croatia, and Albania [7,52]. There are data on records of *Unio elongatulus* in Iraq (Figure 7). Here, we report on the discovery of this species in Montenegro for the first time. It was collected along the Skadar Lake shoreline and from inflowing rivers (Plavnica and Crnojevica rivers). There are several phylogenetic lineages of *Unio elongatulus* in the Mediterranean Region, each of which is separated by one or two nucleotide substitutions in the *COI* gene sequences. The haplotype network of *Unio elongatulus* has a star-like shape, indicating a recent population expansion scenario [53–55], and, probably, a recent population bottleneck [56].

A viable population of *Microcondylaea bonellii* was discovered in the Zeta River. This species was not mentioned in Montenegro since 1909 [57]. We observed only one living population of this species near Podgorica (Figures 1 and 2), which shares three *COI* haplotypes. *Microcondylaea bonellii* has a disjunctive range, which is interrupted by the Dinaric Alps (Figure 5). The populations on both sides of the Dinaric Alps have a relatively high level of genetic diversity. This species was assessed as vulnerable (VU) due to the narrow and disjunctive distribution of its viable populations, many of which are threatened by economic activity and tourism (Table S4). Hence, the development of conservation priorities for *M. bonellii* is urgently needed because suitable habitats for this species are decreasing rapidly.

Several mussel species with narrower environmental niches are recorded in small cold-water mountain rivers of Montenegro (e.g., *Microcondylaea bonellii* and *Unio carneus* in Zeta River). This distribution pattern may lead to local extinctions in isolated streams due to human-mediated impacts and the decreasing genetic diversity in populations [58]. Climate changes and anthropogenic impacts in riverine basins may also trigger the local extinction processes. Conservation studies on freshwater mussels are specifically important for water bodies of Montenegro due to the active development of tourism and other infrastructure on lake and river shores (e.g., around Lake Skadar) [59]. For instance, riverbed changes in Northeastern Italy (e.g., Torrente Versa) led to the decreasing area of suitable habitats for stenobiont species of freshwater mussels such as *Microcondylaea bonellii* [60]. Therefore, future conservation efforts should be led for the maintenance of suitable habitats for endangered species. Additionally, strict protection of these habitats from anthropogenic impacts may be recommended.

4.2. Taxonomy

Family Unionidae Rafinesque, 1820

Tribe Unionini Rafinesque, 1820

Genus *Unio* Retzius, 1788

Type species: *Mya pictorum* Linnaeus, 1758 (by original designation)

4.2.1. *Unio elongatulus* Pfeiffer, 1825

Type: Neotype MZUF BC/53148 (designated by [52]).

Type locality: in den Flüssen Illyriens; auch in Main und Rhein, jedoch nur selten (in the rivers of Illyria; also in the Main and Rhine, but only rarely).

Material examined: 16 specimens from Crnojevića River, Skadar Lake (Donji Murići village), and Plavnica River (RMBH biv1206, biv1209 and biv1210, including biv1206/1, biv1206/2, biv1206/7, biv1209/1, biv1209/2, biv1209/3, biv1210/1, biv1210/2, and biv1210/5 sequenced), Kondakov, Lyubas, and Pešić leg. (see Tables 1 and 2).

Distribution: All main tributaries of the Adriatic Sea from the Ofanto River to the Po River and other North Adriatic basins in Italy, and to Croatian and Albanian hydrographical basins flowing into the Adriatic Sea [7,18,52,61,62]. Here, we present its first record from Montenegro.

Comments: First confirmation of the presence of this species in the fauna of Montenegro. Our findings expand the known range of this species [7,52]. The list of its synonyms is given by Marrone et al. [52]. Several authors [7,18,61,62] described the distribution and

genetic structure of populations of this species in freshwater basins of the North Adriatic rivers and in riverine basins of the Italian Peninsula.

4.2.2. *Unio carneus* Küster, 1854 stat. rev.

=*Unio carneus* Küster [63]: 9(2): 103, pl. 28, Figures 1 and 2.

Type: Whereabouts unknown.

Type locality: Montenegro.

Topotypes examined: 14 specimens from Zeta and Crnojevića rivers (RMBH biv1201, biv1202, and biv1204, including biv1201/1, biv1201/2, biv1201/3, biv1202/2, biv1202/3, biv1202/4, biv1204/1, biv1204/2, biv1204/3 and biv1204/4 sequenced), Kondakov, Lyubas, and Pešić leg. (see Tables 1 and 2).

Distribution: Probably, endemic to Southwestern Balkans (Montenegro).

Comments: *Unio carneus* was described as a separate species based on conchological features, but later it was considered a synonym of *Unio crassus*. Recently, it was noted as a subspecies of *Unio crassus* (see Lopes-Lima et al. [18]; Araujo et al. [19]).

Tribe Anodontini Rafinesque, 1820

Genus *Anodonta* Lamarck, 1799

Type species: *Mytilus cygneus* Linnaeus (1758) (by original designation).

4.2.3. *Anodonta exulcerata* Porro, 1838

=*Anodonta subcircularis* [64]: 87, pl. 22, Figures 3 and 4; type locality: 'Europäische Turkei, in See von Skutari' (Lake Skadar, Montenegro).

Type: Lectotype NHMUK 1841.5.6.127 (designated by Riccardi et al. [32]).

Type locality: 'Nei piccoli laghi di Oggiono, Alserio, e più ancora di Pusiano in Brianza' (in the small lakes of Oggiono (=Lake Annone), Alserio, and even more in Pusiano, Brianza, Italy).

Material examined: Topotypes of the nominal taxon *Anodonta subcircularis* Clessin, 1873 were collected from Podhum (RMBH biv1205, biv1207, biv1208 and biv1211, including biv1205/1, biv1205/4, biv1205/5, biv1205/6, biv1205/7, biv1207/1, biv1207/2, biv1207/3, biv1208/3, biv1208/4, biv1208/5, biv1208/6, biv1208/7, biv1211/1, biv1211/2, biv1211/3, biv1211/4 and biv1211/5 sequenced), Kondakov, Lyubas, and Pešić leg. (see Tables 1 and 2).

Distribution: Earlier, it was stated that this species ranges from the Italian Peninsula to Croatia, west of the Dinaric Alps [7,32]. Here, we present its first records from Montenegro.

Comments: Riccardi et al. [32] compiled a comprehensive list of synonyms for this species. Here, we show that *Anodonta subcircularis* **syn. nov.** represents one more junior subjective synonym of *A. exulcerata* based on a series of newly collected topotypes of the first nominal taxon from the Skadar Lake.

Subfamily Gonideinae Ortmann, 1916

Tribe Gonideini Ortmann, 1916

Genus *Microcondylaea* Vest, 1866

Type species: *Unio (Alasmodonta) bonellii* Férussac, 1827 (by original designation).

4.2.4. *Microcondylaea bonellii* (Férussac, 1827)

Type: Lectotype SMF 4467.

Type locality: A.d. Lijak, Isonzo-Gebiet b. Gorz.

Material examined: six specimens from Zeta River (RMBH biv1203, including biv1203/2, biv1203/3, biv1203/4, biv1203/5 and biv1203/6 sequenced), Kondakov, Lyubas, and Pešić leg. (see Tables 1 and 2).

Distribution: Albania, Croatia, Italy, Montenegro, and Slovenia [7,9,10,60]. There is a doubtful historical record from Lake Trichonis, Greece [9]. In Montenegro, we re-discovered this species for the first time since 1909 [57].

Comments: It is assessed as a vulnerable species (VU) under criteria A2c [65]. Currently, this species has a disjunctive distribution due to losing its habitats under anthropogenic pressure [10].

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/d14110935/s1>, Table S1. Information on the new *COI* sequences of freshwater mussels from Montenegro generated in this study. Table S2. Information on additional *COI* sequences for *Microcondylaea bonellii*, *Anodonta exulcerata*, and *Unio elongatulus* obtained from GenBank and used in phylogeographic analyses (networks). Table S3. Information on additional *COI* sequences of freshwater mussels obtained from GenBank and used in phylogenetic analyses (one haplotype per species). Table S4. Species occurrence data for *Microcondylaea bonellii*, *Anodonta exulcerata*, *Unio elongatulus*, and *Unio carneus* stat. rev. Figure S1. Phylogenetic tree recovered from Bayesian inference analysis based on the *COI* barcode fragment of the Unionidae species from Montenegro and adjacent countries with supplement of related taxa. *Parreysia corrugata* spp. were used as outgroup. Scale bar indicates the branch lengths. Black numbers near nodes are Bayesian posterior probabilities values.

Author Contributions: I.N.B. developed the concept of this study; A.A.L., A.V.K. and V.P. collected the samples; A.V.K., A.A.T. and O.A.Y. designed and carried out the molecular analyses; A.A.L. and A.V.K. performed the phylogenetic modeling; M.Y.G. created the maps; T.A.E. took photos of the shells; I.V.V. and E.S.K. examined shell samples; A.A.L. and I.N.B. wrote the paper, with input from A.V.K. and V.P. All authors have read and agreed to the published version of the manuscript.

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