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Revealing Diversity in *Gammarus* (Amphipoda: Gammaridae) in the Freshwater Ecosystems of Armenia Using DNA Barcoding

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Abstract: *Gammarus* plays a central role in the detritus cycle and constitutes an important component in food webs. At the same time, taxonomy and morphological identification to species level is highly challenging in this genus. Thus, the freshwater gammarid diversity in the Caucasus biodiversity hotspot remains largely unstudied to date. We use DNA barcoding for the first time in assessing the biodiversity and taxonomy of gammarids within the amphipod genus *Gammarus* in the limnic ecosystems of Armenia. The results expand the knowledge on possible diversity and evolutionary lineages of *Gammarus* in the region. DNA barcodes obtained from our Armenian specimens consistently indicate four to six well-defined molecular operational taxonomic units (MOTUs) within three distinct morphospecies clusters. One to three MOTUs correspond to the *Gammarus balcanicus* species complex, two MOTUs to the *G. komareki* complex, and one MOTU to the *G. lacustris* complex. Five BINs out of six were unique and new to BOLD.

Keywords: Lake Sevan; Crustacea; Vorotan River; hidden diversity; DNA barcodes

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1. Introduction

Biodiversity provides humanity with essential ecosystem services that ensure the sustainability of life on Earth [1]. However, the ever-increasing anthropogenic impact and ever-increasing rate of invasions pose critical threats to biodiversity [2]. Thus, various environmental legislation and management approaches have been implemented at both the national and international level for the proper conservation of biodiversity. The key factor in determining the success of such measures consists of a quantification of biodiversity, inter alia by a proper identification of species. Morphology-based methods for biodiversity identification are widely accepted, but fail, e.g., when it comes to identifying cryptic species, or evaluating the loss of genetic variation [3]. These issues have partly been solved by the establishment of DNA barcoding [4]: a fast and efficient tool to monitor biological diversity and its ongoing loss.

Freshwater ecosystems cover only 0.001% of the surface area on Earth, but they are host to an over-proportionally large number of species [5]. Amphipod crustaceans are among the most important animals in temperate freshwater ecosystems. In many freshwater lotic ecosystems, they are dominant within macroinvertebrate species by biomass [6]. One of the most diverse epigean amphipod genus, *Gammarus* Fabricius, 1775 (Gammaridae), contains about 230 described species/taxa [7]. *Gammarus* plays an important role in the litter breakdown processes and by providing prey for secondary consumers. Furthermore, *Gammarus* is sensitive to many chemical stressors and can thus be used for assessing their

impact in freshwater [8]. Gammarids have also been deployed in cages in the field (i.e., in situ bioassays) and used to assess effluents, surface waters, and sediments [9].

The taxonomy of gammarids is complex and morphological identification to the species level requires immense expertise. However, in most of the cases the use of molecular tools turns the morphospecies into complexes of highly divergent lineages, e.g., *Gammarus fossarum* [10], *G. balcanicus* [11,12], *G. kischineffensis* [13], *G. komareki*/*G. lacustris* [3], *G. leopoliensis* [14], *G. nekkensis* [15], *G. pulex* [16], and *G. roeselii* [17]. *Gammarus* species are also well-known for sexual dimorphism and ontogenetic variations. Considering identification difficulties, the specimens from this genus are frequently being reported as *Gammarus* spp. in ecological studies [7]. Such an approach has been common in Armenia too within the last three decades, when the most attention has been paid to the estimation of the health status of surface water bodies and the rapid biological assessment methods used do not require species level identification for gammarids [18].

The Caucasus region is considered one of our planet's biodiversity hotspots [19] with unique ecosystems and many cryptic and endemic species, the conservation of which is highly important [20]. Crustaceans are common for almost all riverine ecosystems in the region, however, their diversity is far from being fully described. Moreover, phylogeographic studies are almost completely lacking for most of the groups of invertebrates. Recently, research on amphipods in the Caucasus has been revived, but these studies mainly focused on the subterranean [21,22], marine/brackish fauna [23], or south-western mountainous Russian Caucasus [24,25]. However, the recent state of the fauna of surface freshwater gammarids in the Caucasus is poorly studied [26]. A review of the literature shows that since the collapse of the Soviet Union, species level identification in Armenia was either biased or not conducted at all. As a result, only *Gammarus lacustris* has been documented in Armenia during the last 30 years. All the species records originate in the biggest freshwater lake in the Caucasus, Lake Sevan, and its tributaries [27–29]. However, up to date, three species have altogether been recorded for Lake Sevan. Besides *G. lacustris*, there were records of *G. sibiricus* and *G. pulex* [30,31]. Birstein [32] documented the latter species also in other water reservoirs in Armenia. Unfortunately, no reference material has survived to today for morphological comparisons with our specimens. Markosyan [33] documented *G. araxenus* from the major tributaries of Lake Sevan. Bening and Popova [34] documented *G. balcanicus zangesis*, *G. lacustris erevanensis*, and *G. komareki armeniacus* from the Hrazdan River ecosystem. It is difficult to assess the validity of these findings as there is no other information about these species/subspecies in the international databases and furthermore because their morphological descriptions are too vague.

Thus, the aim of this work is to revise the diversity of gammarids in the freshwater ecosystems of Armenia using DNA barcoding. This work also strives to fill the gap in the knowledge regarding the phylogenetic diversity of some complexes of gammarids that can be encountered in the Minor Caucasus.

2. Materials and Methods

Sampling sites. A series of field works were conducted during June and July of 2019, May 2020, and September 2021. In total, we collected gammarids from 11 riverine sampling stretches of 100 m length each; four are located in the drainage basin of the Vorotan River (Southern part of Armenia) and seven are located in the drainage basin of Lake Sevan (Eastern part of Central Armenia). The only lacustrine station was in Lake Sevan (Figure 1).

Sampling stretches vary with regard to altitudes, substratum, and anthropogenic pressures (Table 1). Thus, the network of sampling sites was selected so as to comprise a wide range of habitats and abiotic factors to have adequate data on the diversity of gammarids in central and southern Armenia. However, one of the main limitations of this manuscript is in the space limit for the studies which does not allow for a description of the spatial features of species distribution in detail.

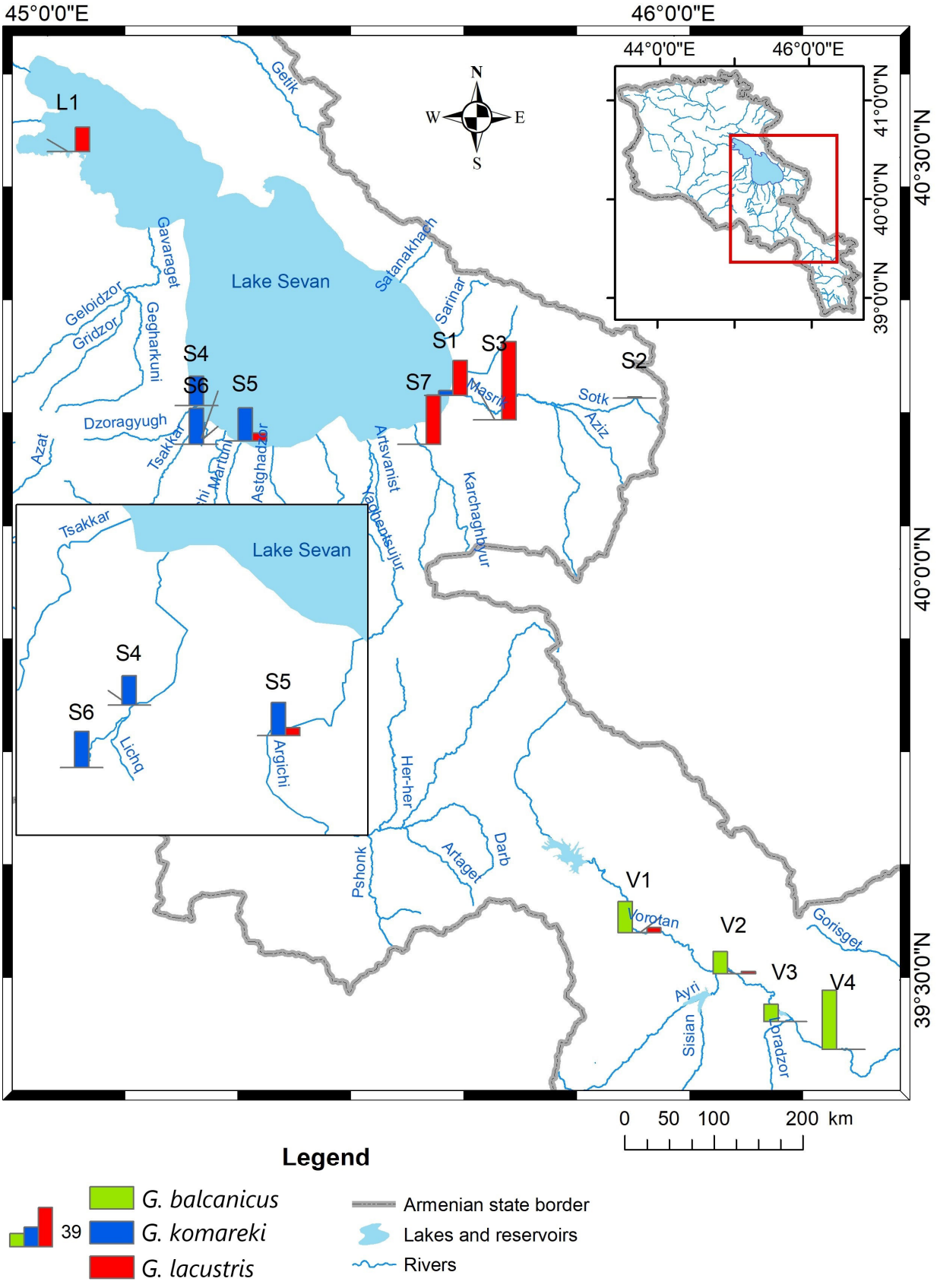

Figure 1. Sampling sites and *Gammarus* morphospecies distribution. S1–S7 sites are in the drainage basin of Lake Sevan, L1 is the only lacustrine site and V1–V4 are in the Vorotan basin.

Table 1. Description of sampling sites. In each drainage basin, the sites are distributed from North to South. The first letter in the name identifies the drainage basin (V-Vorotan; S-Sevan). Lacustrine station labelled by L.

Name	River/Lake	Latitude	Longitude	Altitude	Sampled Substrate	Main Source of Pressure
L1	Lake Sevan	40.5155	45.0331	1900	Submerged macrophytes	Domestic and agricultural wastewater
S1	Masrik River	40.217	45.657	1915	Sand, gravel	Domestic and agricultural wastewater, Hydromorphological alteration
S2	Sotq River	40.212	45.931	2136	Pebble	Livestock grazing, Mining
S3	Akunq River	40.187	45.709	1926	Submerged macrophytes	Domestic and agricultural wastewater, Hydromorphological alteration
S4	Lichq River	40.167	45.244	1919	Submerged macrophytes	Domestic and agricultural wastewater
S5	Argichi River	40.163	45.269	1912	Pebble, sand	Agricultural wastewater
S6	Lichq River	40.159	45.236	1927	Pebble, gravel	Domestic wastewater
S7	Karchaghbyur River	40.158	45.589	1975	Cobble, Pebble	Livestock grazing
V1	Vorotan River	39.562	45.93	1680	Sand, gravel	Domestic and agricultural wastewater; hydropeaking
V2	Vorotan River	39.511	46.078	1506	Cobble, Pebble	Domestic wastewater, hydropeaking
V3	Loradzor River	39.452	46.157	1367	Pebble	Hydropeaking
V4	Vorotan River	39.417	46.248	1110	Cobble, pebble	Livestock grazing

Sampling procedure. Collection of material was realized using different techniques following the requirements of EN ISO 10870:2012 and EN ISO 16150:2012 [35,36]. Particularly, D frame and Surber samplers with a mesh size of 500 µm were used in the riverine sampling sites and Petersen snapper in the lacustrine. Some specimens were collected manually from the submerged macrophytes. All major habitats were sampled at each station, then animals sorted out in situ and gammarids were immediately preserved in 96% ethanol, alive. Coordinates and altitude of sampling stretches were registered using a Garmin eTrex20 (Garmin LTD, Kansas, USA) GPS receiver (Table 1). Processing of spatial data and further mapping of results were conducted in the ArcMap 10.5 software.

Morphological identification and data processing. Preserved material was transported to the Museum Koenig laboratory of the Leibniz Institute for the Analysis of Biodiversity Change (LIB/ZFMK; Bonn, Germany) in 2019 and to the laboratory of the Scientific Center of Zoology and Hydroecology of the National Academy of Sciences of Armenia in 2020 and 2021. Specimens were kept in the freezers and processed within the first month after arrival. In 2019 we analyzed 328 gammarid specimens morphologically and grouped these into three morphospecies using the available identification keys [37,38]. Then, we processed 74 specimens in 2020 and 32 in 2021 identically. From these three morphospecies groups, 22 representative specimens were selected randomly for molecular analysis. As a result, the distribution within morphospecies is the following: *Gammarus lacustris* (7 specimens, 4 localities), *Gammarus komareki* (7 specimens, 4 localities), and *Gammarus balcanicus* (8 specimens, 4 localities). All morphological specimen vouchers and extracted DNA were deposited at and are available from the LIB Biobank, Bonn.

DNA extraction, amplification and sequencing. Following the recommendations of Evans et al. [39], total genomic DNA was isolated from pereopods of the 22 analyzed specimens. After lysing the tissue overnight at 56 °C, DNA extraction was performed using the automated BioSprint96 magnetic bead extractor (Qiagen; Hilden, Germany), following the specifications of the BioSprint DNA Blood kit (Qiagen, Hilden, Germany).

Polymerase chain reaction targeted 658 bp of the 5′ part of the mitochondrial cytochrome c oxidase subunit 1 (COI) gene. In total, the reaction mixture contained a volume of 20 µL, where the undiluted DNA template was 2 µL, each primer was 0.8 µL, and the remaining volume contained standard amounts of the 'Multiplex PCR' kit reagents provided by Qiagen. Applied Biosystems 2720 Thermal Cyclers (Life Technologies; Carlsbad, CA, USA) were used to perform thermal cycling for all reactions. Two cycle sets of a PCR program based on a combination of a 'touchdown' and a 'step-up' routine was used starting with 15 min hot start Taq activation at 95 °C. First cycle set of 15 repeats was performed as follows: 35 s denaturation at 94 °C, 90 s annealing at 55 °C (−1 °C per cycle), and 90 s extension at 72 °C. Second cycle set of 25 repeats was performed as follows: 35 s denaturation at 94 °C, 90 s annealing at 40 °C, and 90 s extension at 72 °C; final elongation 10 min at 72 °C.

We amplified 658 bp from the 5′-end of the COI gene using HCO2198-JJ and LCO1490-JJ primers (Metabion, Planegg, Germany) [40]. Then, the PCR products were sent for bidirectional Sanger sequencing to BGI in Hong Kong (China).

Sequence alignment and data set assembly. The sequences were edited and aligned using MEGA 10 [41] and deposited in BOLD under accession numbers GAMAR001-20–GAMAR003-20, GAMAR005-20–GAMAR012-20, GAMAR014-20–GAMAR019-20, GAMAR022-20–GAMAR025-20, and CaBOL-1016044. DNA barcodes from Armenia were compared with other COI sequences of *Gammarus* species (Supplement S1) available in the online database of the Barcode of Life DataSystems (BOLD; www.boldsystems.org (Accessed on 3 May 2023) [42].

All 673 barcodes available from BOLD (downloaded on [1 January 2023]) with reliable geographical information for our species groups were added to the BOLD dataset named DS-GAMARM. In compiling this dataset, we considered only those specimens with information on the country of origin and included the broadest geographic range possible. Information on the location of sampling sites for specimens with BOLD IDs KT778323–KT778506 were added from Katouzian et al. [3]. Information on all sequences used in this manuscript is available in Table S1.

Phylogenetic analysis. By utilizing the maximum likelihood method and the general time reversible model, the evolutionary history was inferred [43]. To obtain the initial tree(s) for the heuristic search automatically we applied the neighbor-joining and BioNJ algorithms to a matrix of pairwise distances. The latter was estimated using the maximum composite likelihood (MCL) approach, and then the topology with superior log likelihood value was selected. To model evolutionary rate differences among sites a discrete Gamma distribution was used (5 categories (+G, parameter = 0.5750)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 20.54% sites). We drew the tree to scale and determined the branch lengths by the number of substitutions per site. This analysis involved 494 nucleotide sequences (more accurately they are haplotypes of 673 sequences). There were a total of 465 positions in the final dataset. Statistical support was assessed using 1000 bootstrap replicates. Evolutionary analyses were conducted in MEGA X [41].

Molecular species delimitation. Two molecular species delimitation methods were used. The first distance-based method involved the software Assemble Species by Automatic Partitioning (ASAP) [44] implemented on the web-interface (https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html (Accessed on 2 May 2023)). ASAP was conducted using a recursive split probability of 0.01. Then, we report (a) the partition with the best ASAP score and (b) the partition that is closest to the "correct" one of the two best partitions, according to their ASAP scores.

The BIN method is applied as part of BOLD where our sequences are compared with readily available sequences in BOLD and clustered based on their molecular divergence. A unique Barcode Index Number (BIN) was assigned to clusters if available in the database or automatically created ad hoc if the submitted sequences did not cluster with previously known BINs [45].

3. Results

Species diversity documented by morphological identification. Using the typical morpho-anatomical diagnostic features within *Gammarus*, the following species were identified morphologically: *G. balcanicus* (129 specimens, 4 localities), *G. lacustris* (201 specimens, 7 localities), and *G. komareki* (104 specimens, 5 localities). While *G. lacustris* specimens were found in both central and southern Armenia simultaneously, *G. balcanicus* specimens were found only in southern Armenia and *G. komareki* specimens only in central Armenia (Figure 1). However, this does not necessarily mean that there are no *G. balcanicus* specimens in central Armenia or *G. komareki* specimens in southern Armenia.

Local diversity analysis by molecular methods. All sequences from Armenia were clustered with BOLD sequences of the three known *Gammarus* species: *G. balcanicus*, *G. lacustris*, and *G. komareki* (Figure 2) which is similar to the results of the clusterization based on morphological identification. Moreover, all the morphospecies identified by morphological observation have corresponded to the results of molecular identification.

Eight sequences (GAMAR001–GAMAR003, GAMAR005–GAMAR009) belong to the *G. balcanicus* group that was grouped into five haplotypes and clustered with sequences from the southern European region. Seven sequences (CaBOL-1016044, GAMAR011, GAMAR012, GAMAR014–GAMAR017) belong to the *G. lacustris* group that was grouped into three haplotypes and clustered with sequences from Finland, Norway, and Iran. Seven sequences (GAMAR010, GAMAR018, GAMAR019, GAMAR022–GAMAR025) belong to the *G. komareki* group that was grouped into five haplotypes and clustered with sequences from Iran.

We delineate the freshwater amphipod species from Armenian freshwater ecosystems and reveal the potential Molecular Operational Taxonomic Units (MOTUs) that could represent putative cryptic species within the studied species. Two different molecular species delimitation methods (ASAP and BIN) showed four/six and six MOTUs, respectively (Supplement Table S1, Figure 2). Of the four/six MOTUs, one/three MOTUs were morphologically identified as belonging to the *Gammarus balcanicus* complex, two MOTUs to the *G. komareki* complex, and one MOTU to the *G. lacustris* complex (Figure 2).

According to BOLD, of all the sequences from Armenia grouped under six BINs (Table 2), five of them were unique (BOLD:AED1628, BOLD:AED1629, BOLD:AED2057, BOLD:AED2058, BOLD:AED2059), and one BIN (BOLD:AAA2702) contains sequences from *Gammarus lacustris* collected in Armenia and 17 other countries such as Norway, Finland, Russia, Canada, Iran, Tajikistan, etc. Of the unique BINs three belong to the *G. balcanicus* complex and two belong to the *G. komareki* complex. Thus, no unique BIN was obtained only for the *G. lacustris* complex. All six BINs are the same as the MOTUs obtained via the ASAPb approach that is closest to the "correct" one among the two best partitions, while the ASAPa partition with the best ASAP score showed only four MOTUs with additional lineage for the *G. komareki* group.

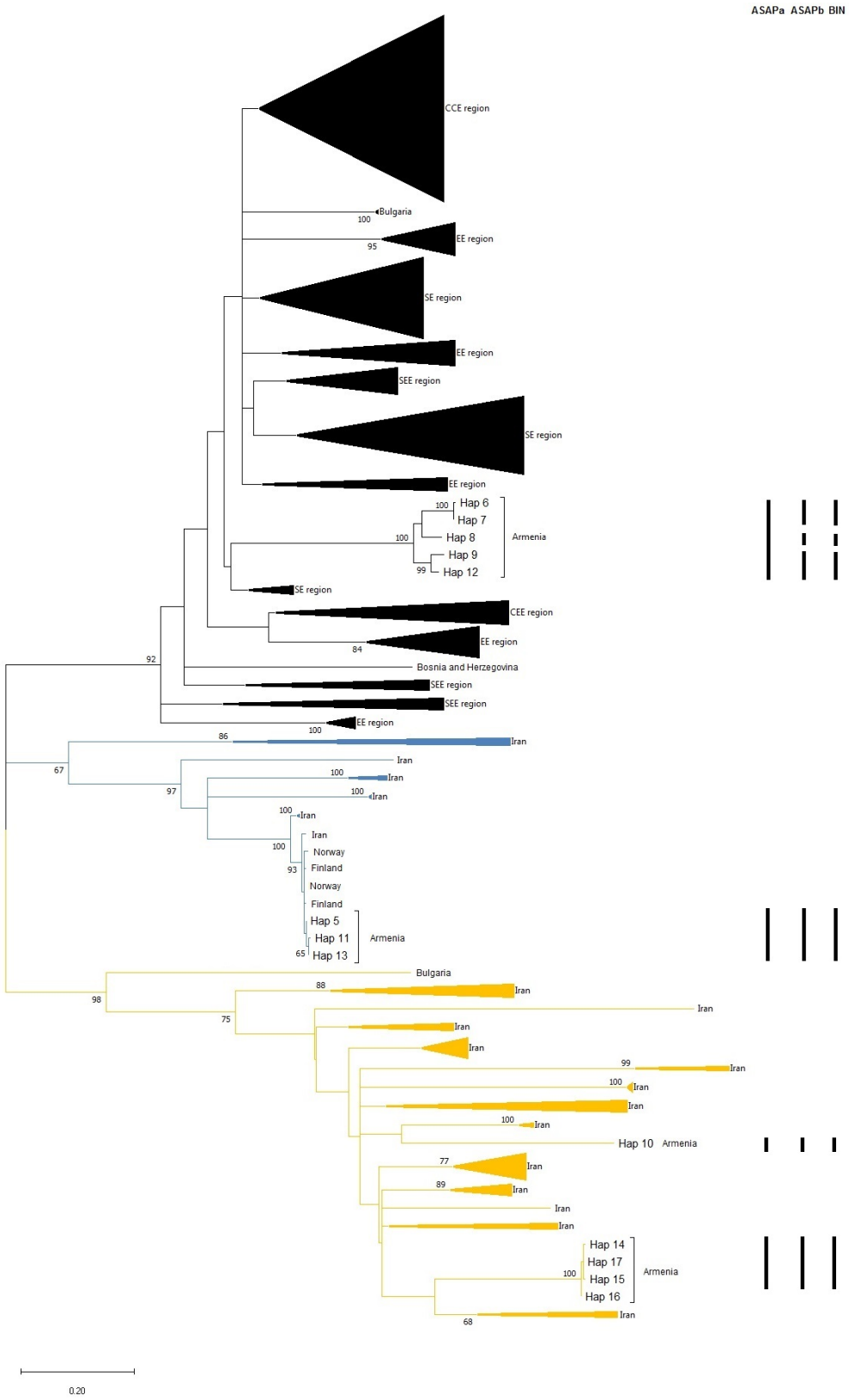

Figure 2. Phylogenetic tree of *Gammarus* species and the results of molecular species delimitation. Black color used for *G. balcanicus*, blue color is used for *G. lacustris* and orange color is used for *G. komareki*.

Table 2. BINs from Armenian rivers and their characteristics.

BIN (Species)	Average K2P Distance (%) *	Maximal K2P Distance *	Notes **
BOLD:AED1629 (*G. balcanicus*)	-	-	1 sequence (1 COI haplotype)
BOLD:AED2058 (*G. balcanicus*)	2.09	2.09	2 sequences (2 COI haplotypes)
BOLD:AED2059 (*G. balcanicus*)	0.06	0.16	5 sequences (2 COI haplotypes)
BOLD:AAA2702 (*G. lacustris*)	2.07	5.68	498 sequences (7 of them from Armenia and are belonging to 3 COI haplotypes)
BOLD:AED1628 (*G. komareki*)	1.25	2.57	9 sequences (6 of them are belonging to 4 COI haplotypes)
BOLD:AED2057 (*G. komareki*)	-	-	1 sequence (1 COI haplotype)

4. Discussion

Past research on* Gammarus *in Armenia. The first note on the *Gammarus* in Armenia was made by Kessler [30] and then by Brand [31] back in the late 19th century, which were the only mentions of the genus in the pre-Soviet era of Armenian history. All further mentions were made since the establishment of the Soviet regime and we noticed significant changes in the species composition of gammarids when comparing species documented in pre-Soviet, Soviet, and post-Soviet periods in Armenia which is due partially to the difficulties of morphological identification of *Gammarus* species. However, the species registered for Lake Sevan before the 1940s were later never recorded in the region, which leads us to conclude that those records were flawed due to taxonomic misidentification. The same conclusion was made by Kasimov [46], as since the 1940s [33] only *G. lacustris* has been documented in Lake Sevan. Current data [28,29] underpin our conclusion. Moreover, neither the morphological nor molecular analysis of samples collected from the only lacustrine station on Lake Sevan allow us to assume that *G. komareki* have penetrated the lake recently or that the only *Gammarus* species there is still *G. lacustris.*

Diversity in Armenian* Gammarus *species. State hydrobiological monitoring of surface water bodies in Armenia was launched only about one decade ago, however, hydrobiological studies have been common for some water bodies in Armenia since the establishment of the Sevan hydrobiological station back in 1923. As a result, the *Gammarus* species was regularly reported in the central regions of Armenia [27–29,32–34]. This study offers the first DNA-based insights into the diversity of *Gammarus* species in Armenia and can be used as a starting point for further work. The results consistently show that Armenia harbors three species/groups of the genus *Gammarus*. However, the spatial extent of our studies does not allow us to suppose that there are no other species groups in the country. Based on the evidence from the molecular studies we can only assume that there are several other lineages in the *G. balcanicus* and *G. komareki* groups in the Caucasus, but to conclusively prove this, a larger number of samples from Armenia and neighboring countries needs to be sequenced, along with additional genetic markers (including nuclear ones).

In general, Yousefi et al. [47] note the cryptic diversity of the *G. komareki* group in the Caucasus based on a very limited number of sequences from Northern Iran. Mamos et al. [11] conclude that there is a high cryptic diversity of the *G. balcanicus* group in Europe based on geographically broader sampling. About 70% of our sequences belong to cryptic species and cluster within the two species complexes of *G. komareki* and *G. balcanicus*. Both these MOTUs have unique BINs in BOLD. Only one freshwater species could be unambiguously linked with morphology, i.e., a single morphospecies comprising a single genetically identified species *G. lacustris*. Such an issue is also well-described by Mamos et al. [11] and Behrens-Chapuis et al. [48]. Thus, five *Gammarus* MOTUs appear to be cryptic and with some likelihood of also being endemic species for our region or Armenia, all new

to BOLD. Additionally, one specimen (haplotype 10 in Figure 2) arouses interest because it is highly distinct from the rest of the haplotypes within the *G. komareki* group, and because the specimen was collected in the upper course of the Sotk river, with 2136 m representing the highest sampling site in this study. Copilaș-Ciocianu [49] has already noticed high sequence divergence within the *G. komareki* complex when comparing the available data from GenBank for the Iran–Bulgaria geographic boundary at the beginning of the 2010s. He theorized that more divergent genetic lineages still can be found, taking into consideration the lack of research in our region and recent data as well just proves that assumption. The lesser Caucasus along with the adjacent mountain ranges of Elburz, Zagros, and Pontos can be home to endemic, insular distributions of not only *G. komareki*, but also *G. balcanicus* species.

Although additional molecular data on *Gammarus* would help in enlightening the colonization history of the genus in the Caucasus, we nevertheless consider the evidence sufficient to assume the potentially new *Gammarus* species are regional or local endemics of Armenia.

Spatial features of* Gammarus *species distribution in Armenia. Analyzing the results spatially (Figure 3), allows for some additional observations. Although Bening and Popova [34] registered the presence of *G. balcanicus* along the course of the Hrazdan River, which drains Lake Sevan, we have not been able to find it in the tributaries of the lake yet. One of the reasons for this could be the many disruptions in the Hrazdan River continuity due to a hydropower capacity development in the Soviet period [50], however, considering the period of time that has passed since the first registration and the number of invasions in the region, *G. balcanicus* had all the opportunities to colonize the drainage basin of Lake Sevan too. Thus, the abiotic factors in this catchment of one of the biggest high-altitude lakes in the world are probably not so favorable for this species. To shed more light on this issue, thorough phylogeographic studies are still necessary.

It is noticeable that all three cryptic species of *G. balcanicus* are "isolated" from each other in the Vorotan River system by either hydro-engineering structures or natural phenomena. In general, the establishment of hydroenergetics in the drainage basin of the Vorotan River has led to interruptions of the river continuity in several sites since the late 1970s [51]. The *G. balcanicus* genospecies comprising haplotypes 9 and 12 were recorded in the Loradzor tributary which is isolated from the Vorotan River through underground pressure channels that transfer the water from the river into the Shamb reservoir. The species represented by haplotype 8 was only recorded upstream from the Angeghakot reservoir which has no outlet to release water to the river downstream. And the third genospecies with haplotypes 6 and 7 was recorded in the Harjis gorge where the Vorotan River disappears underground to re-emerge as a strong source a few hundred meters upstream from a sampling site. There is evidence that *G. balcanicus* rarely co-occurs with other gammarids [52], so possibly the existence of such artificial obstacles does not play a major role in their spatial isolation. However, there is a possibility that the further application of molecular methods to material from the Vorotan River system may further expand the number of known *G. balcanicus* MOTUs/species—possibly more or less evenly arranged along the course of the river. Overall, the three new MOTUs/candidate species add to the picture of a megadiverse *G. balcanicus* which in Eurasia already exceeds 50 MOTUs [12]. Noteworthy about the *G. balcanicus* species complex in Armenia is the high genetic diversity also outside of the wider Balkans area, for which this phenomenon was described [17]. Thus, our findings add new geographic evidence to the existing data pool on *G. balcanicus*. Considering such facts, and the limited number of specimens in our study, we restrain ourselves from delving deeper into the morphological descriptions of *G. balcanicus* morphospecies in Armenia at this stage.

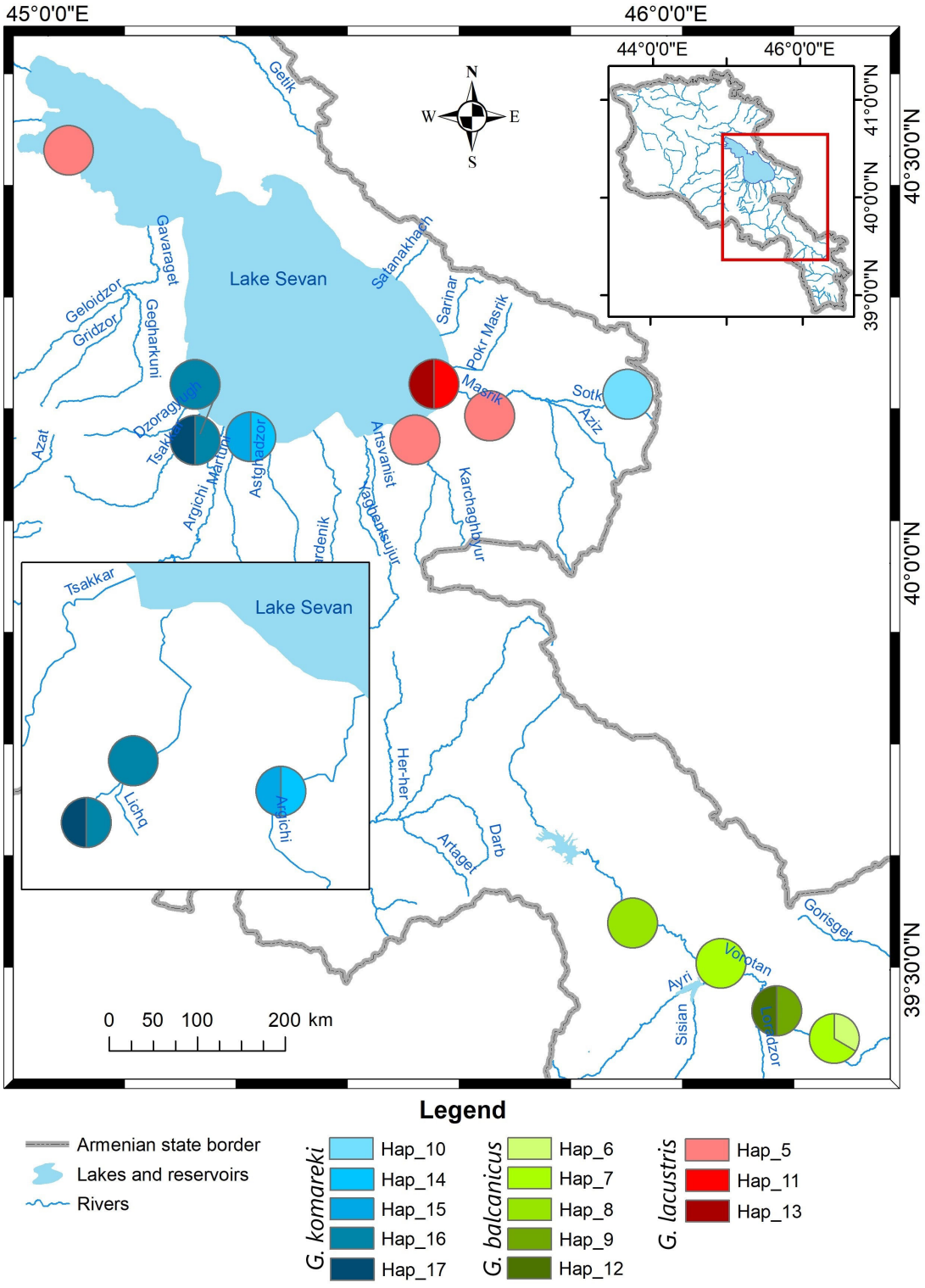

Figure 3. Spatial distribution of haplotypes. Red box shows the location of studied are in the territory of Armenia.

The common species for Lake Sevan—*G. lacustris*—was widely spread across Lake Sevan and recorded also in the Vorotan River system, covering the highest spatial and altitudinal range among all identified species complexes. This species was replaced by *G. komareki* in the upper course of another tributary of the Masrik River: the river Sotq. The specimen was found at a spring not impacted by the mining wastewater discharge that

regularly affects the Sotq River [53]. However, according to Karaman and Pinkster [54] *G. komareki* is also rather tolerant towards organic pollution, so it is expected that it could cover a wider geographic range within Armenia than was found recently. Also, it should be stressed that part of the Masrik River downstream from the confluence with the Sotq regularly dries out in the summer season due to water abstraction for irrigation [55]. Thus, the population of this species is also seasonally isolated. Considering that Lake Sevan was established only in the Pleistocene and Holocene [56], the history of the within-species divergence of *G. komareki* in this basin is relatively short. Thus, it is likely that this species also has a wider distribution in the region and could be found in the other headwaters of the area if it persisted there. Evidence from the Balkan peninsula also suggests that amphipods can create separate lineages in the same species and be isolated in different parts of the same river [17,57].

In both the studied rivers south-west of the Lake Sevan drainage basin—Argichi and Lichq—the same genospecies of the *G. komareki* complex was recorded. This is not surprising when considering that these tributaries have similar hydrology and biotopes as well as high spatial proximity [58]. However, the current worldwide lack of molecular data in *Gammarus* still mostly impedes broad analyses of their spatial distribution and endemism. Thus, the scrutiny of morphological differences among *Gammarus* specimens throughout the world remains a major open task and requires a copious material basis.

In general, our results were matched by data from the Irano-Anatolian and Caucasus biodiversity hotspots [3], where several lineages of widespread species complexes (*G. komareki* and *G. lacustris)* were found and *G. balcanicus* is supposed to be another candidate species complex comprising the cryptic lineages in the region.

However, our sampling is not appropriate to assess the hidden species diversity of this species in Armenia. The evidence from the studied rivers in Armenia and the results of molecular analysis allows us to assume that *Gammarus* species are mainly colonizing limited areas in the rivers and thus are very vulnerable to habitat degradations and hydromorphological alterations. Considering the recent developments in the Armenian economy and current challenges in water management due mainly to global climate change the pressure on river ecosystems will increase. Thus, the diversity of gammarids could be heavily threatened and further studies are necessary to protect them properly.

5. Conclusions

At this stage, a total of four/six MOTUs belonging to three species groups was revealed through our molecular analyses. Almost all these MOTUs occur spatially clearly isolated from each other in central and southern Armenia. *Gammarus balcanicus* is represented by three cryptic genospecies which were exclusively encountered in southern Armenia and *Gammarus komareki* is represented by two cryptic genospecies exclusively encountered in the rivers of the Lake Sevan basin. Only *G. lacustris* shows no unique MOTUs in the territory of Armenia and has wider geography in general. As for future research, we see the need for sampling at new sites in the different rivers to avoid geographic sampling bias and to add further molecular data points, as well as the need to use additional mitochondrial and nuclear markers. However, the gained results are already speaking to the distinct geographical features of three complexes of *Gammarus* in Armenia which opens up new perspectives for further research in the Caucasus biodiversity hotspot in regard to the evolutionary history of species and their phylogeography.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/w15193490/s1, Supplement S1: Geographical and molecular general description of 22 sequenced species; Supplement Table S1: Samples information with all BINs.

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