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Species diversity and DNA barcode library of freshwater Molluscs of South Caucasus

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Abstract

This study provides the first attempt to investigate the molecular diversity of South Caucasian freshwater molluscs (Mollusca, Gastropoda) and lay down the first bricks to build up a DNA-barcode library. In total 289 CO1 barcode sequences were obtained from 34 morpho-species belonging to 24 molluscan genera and 10 families that represent nearly 30% of known freshwater molluscan diversity of the South Caucasus region. DNA barcodes were analyzed by means of the Barcode Index Number (BIN) and the other tools available in BOLD system. Results showed that the knowledge of freshwater miolluscs diversity in the South Caucasus is far to be comprehensive. For the studied 34 morpho-species, 298 barcodes were clustered into 42 BINs from which unique BINs were defined for 14 species, and 6 species were characterized with more than a single BIN. From the studied taxa, 60% were characterized larger than 2.2% sequence divergence indicating the possibly high genetic variation or cryptic diversity. Within our limited taxonomic coverage, we found one new species for the republic of Georgia (*Galba schirazensis*), and at least three undescribed species taxa belonging to genera *Stagnicola, Segmentina* and *Anisus*. Uniqueness and high molecular diversity of the studied species emphasizing the need for further intensive morphological and molecular investigation of the South Caucasian freshwater molluscan taxa.

Keywords

South Caucasus, DNA barcode library

Introduction

Under increasing anthropogenic pressure the conservation of freshwater biodiversity and maintaining freshwater ecosystem functioning remains one of the most critical challenges for the 21st century's world (Butchart et al. 2010, Hoffmann et al. 2010). A sufficient knowledge of the species diversity and distribution of freshwater taxa is crucial for understanding the needs and implementation of conservations measures to save species

and maintain ecosystem integrity (Collier et al. 2016). Freshwater molluscs constitute a diverse and functionally important component of freshwater communities (Runck 2007, Strong et al. 2007) inhabiting a full range of freshwater habitats (Dillon 2000, Strong et al. 2007) and the same time, are the most vulnerable taxa among other freshwater inhabitants (Cuttelod et al. 2011). The accurate biodiversity information on freshwater molluscs is often missing, especially in the species-reach and economically poorly devolved parts of the world, hindering the management and conservation activities. A good example is the Caucasus biodiversity hot-spot where in spite of the recent advancements (e.g.Bikashvili et al. 2021, Chertoprud et al. 2020, Chertoprud et al. 2021, Grego et al. 2020, Neiber et al. 2021, Vinarski et al. 2014) the diversity and distribution of freshwater molluscs are still far to be comprehensive (Mumladze et al. 2020, Mumladze et al. 2019). Most probably this is due to the absence of local taxonomic expertise during the last 50 years in the region.

A recent developments of DNA barcoding technology helped a lot to revive and advance biodiversity inventory and monitoring at an unprecedented rate (Waugh 2007, Trivedi et al. 2016). DNA barcoding proved to be an effective tool in helping taxonomists to distinguish taxa and even confidently solve the taxonomic problems especially when the other traditional (morphology - based) methods alone are failing (Hebert et al. 2003, Hajibabaei et al. 2006, Sheth and Thaker 2017, Goldstein and DeSalle 2011). Perhaps more importantly, DNA barcoding trigger even non-taxonomists and the young generation to put the effort in biodiversity investigation (Packer et al. 2009, Ellis et al. 2010, Ebach 2011). For instance, in Georgia, a number of research projects have been conducted very recently investigating the freshwater biodiversity including or exclusively based on DNA barcoding approaches done by an experienced and amateur scientists (Bikashvili et al. 2021, Grego et al. 2020, Epitashvili et al. 2020, Japoshvili et al. 2020). In addition, DNA barcoding (and in particular environmental DNA, or eDNA meta-barcoding) is a promising tool in fast, non-invasive and cost-effective means for biodiversity inventory/monitoring (Thomsen et al. 2012, Carew et al. 2013, Thomsen and Willerslev 2015). However, in order to make DNA barcoding approaches an useful tools, it is essential to build barcode reference libraries against to which newly obtained barcodes can be compare (Leese et al. 2018, Weigand et al. 2019). Barcode reference library is basically a data infrastructure that requires a routine input from the taxonomy and molecular-genetics. Currently the largest reference library is available in BOLD systems (http://www.boldsystems.org) which is on the other hand less effective when dealing with taxa and barcodes from the poorly investigated areas (Weigand et al. 2019). For instance, for the Caucasus region, barcode information is lacking for a great deal of taxa including the freshwater molluscs. Here in the present publication, we provide a first stage of an ongoing project that aims to build the DNA barcode reference library for South Caucasian freshwater molluscs within the framework of the Caucasus Barcode of Life incentive (https://ggbc.eu). In particular, the aim of the given study was to (1) generate CO1 barcode sequences for part of freshwater molluscan taxa, (2) investigate within vs. between species sequence variation, (3) identify gaps in species level taxonomic knowledge of freshwater molluscs and (4) develop subsequent research agenda.

Materials and methods

Sample Collection

Sample collection campaign were carried out from 2015 to 2021 across the various regions of Georgia and also in Armenia and Azerbaijan during 2019 (Fig. 1). Samples were collected from various types of habitats including river banks, springs, channels, lake littorals, mires and temporal water bodies. Specimens were collected using sieving the substrates from different types of microhabitats and also directly from the surfaces of water plants and fallen leaves, stones and sink logs. In addition, where possible, bottoms of lotic/ lentic habitats were inspected with glass bottom viewing boxes for specimens of mussels (family *Unionidae*). Collected samples were immediately preserved in 96% ethanol. Sorting and taxonomic identification of individuals was conducted using the keys of Glöer (2002), Glöer (2019), Soldatenko and Starobogatov (2004), Welter-Schultes (2012), Piechocki and Wawrzyniak-Wydrowska (2016), Jackiewicz (1998) and Vinarski et al. (2020).

One to then specimens per morphologically defined species were selected for barcoding. In cases of genera - *Radix* and *Ancylus* for which the systematics of Caucasian taxa is not yet well understood, we took a larger number of specimens for each morpho-species. All selected specimens were first photographed according to BOLD standards (Milton et al. 2013) and in the case of larger specimens, only a part of tissue was separated for DNA extraction, while for small-bodied species (such as for instance *Ancylus* and most of Sphaeriidae), soft body of the complete individuals was submitted for DNA extraction. Collected materials/vouchers are deposited in the collection of the Institute of Zoology of Ilia State University, Tbilisi under the respective CaBOL identification numbers given in Suppl. material 1.

DNA processing

Genomic DNA was extracted from tissue samples using the Quick-DNA™ Miniprep Plus Kit (Zymo Research) (for 25 mg tissue), Quick-DNA™ Miniprep Plus Kit (Zymo Research) (for 5 mg tissue), DNeasy Blood & Tissue Kits (Qiagen, Germany) according to of manufacturer's instructions and the protocol proposed by Sokolov (2000) with slight modifications (Sauer and Hausdorf 2009). Partial sequences of cytochrome oxidase c subunits I (COI) were amplified by polymerase chain reaction (PCR) using the primer pair LCO1490-JJ and HCO2198-JJ (Astrin and Stüben 2008). Thermal conditions included denaturation at 95°C for 1 min, followed by first cycle set (15 cycles): 94°C for 30 sec, annealing at 55°C for 1 min (−1°C per cycle) and extension at 72°C for 1:30 min. Second cycles set (25 cycles): 94°C for 35 sec, 45°C for 1 min, 72°C for 1:30 min, followed by 1 cycle at 72°C for 3 min and final extension step at 72°C for 5 min. In addition, shorter CO1 sequences were amplified using the Folmer et al. (1994) forward (LCO1490) and Kuhn's reverse (LCO1491) primers (cited in Cordellier and Pfenninger (2008)). PCR cycling conditions were adopted from Wethington and Lydeard (2007) and was comprised of an initial denaturation step: 94°C for 3 min, followed by 30 cycles at 94°C for 40 sec, annealing temperature at 48°C for 1 min, 72°C for 1 min and final extension step at 72° for 10 min. Resultant amplicons were visualized on 1% agarose gels using 3 µl of PCR

product. The remaining PCR products were then performed using Big Dye Terminator v.3.1 (Applied Biosystems, Foster City, CA, USA) and run on an automated sequencer. Some of the PCR products were sequenced at Macrogen Europe Laboratory (Amsterdam, Netherlands). Both DNA strands of the PCR product were sequenced.

Data analyses

Sequences were edited in Geneious Pro v.7 (Drummond et al. 2011) to ensure the absence of indels and stop codons. Quality sequences (i.e. less than 1% base-pair ambiguity) were submitted to BOLD Systems (http://www.boldsystems.org) under the project acronym “GEOFM” including the specimen images, trace files, and the rest of the metadata (Suppl. material 1). In addition, we run a BOLD search for molluscan barcodes originating from the South Caucasus region and were supplemented to the “GEOFM” project under a dataset named “DS-FMOL” for part of the analyses.

Barcode Index Numbers (BIN) were then automatically assigned to each newly derived sequence by BOLD Systems v.4. That is a two-stage analysis where at the first stage an initial assignment of sequence to an Operational Taxonomic Unit (OUT) takes place based on Refined Single Linkage Clustering (RESL) with a threshold of 2.2% sequence differences. At the second stage, graphical analyses (Markov clustering) are applied to OTUs which in case of the existence of a clearly defined internal structure within OTU, can result in its split of two or more OTUs in spite of smaller than 2.2% sequence variation (Ratnasingham and Hebert 2013). RESL algorithm and ABGD (Automatic Barcode Gap Discovery - Puillandre et al. 2011) were further employed to generate OTUs and cluster histograms via BOLD Systems. Finally, a neighbor-joining (NJ) tree was reconstructed for the selected part of the dataset, based on the Kimura 2-parameter (K2P) model with 1000 bootstrap replicates using MEGA11 (Tamura et al. 2021).

Results

In total, 289 CO1 barcode sequences were obtained and uploaded in “GEOFM” BOLD project representing 34 species from 24 molluscan genera from 10 families. Prior to the present study, there were 47 freshwater molluscs CO1 barcode sequences available in the BOLD Systems (from the study area) including 11 sequences form unpublished “DNAqua-Net” project (*Viviparus costae* (2), *Theodoxus fluviatilis* (2), *Bithynia tentaculata* (1), *Corbicula fluminalis* (2), *Anisus* sp. (1), *Planorbis planorbis* (1), *Musculium lacustre* (1), *Pisidium* sp. (1)) and 36 sequences mined from GenBank (11 sequences of *Ancylus* spp. (Bikashvili et al. 2021), 23 sequences of Hydrobiidae spp. (Grego et al. 2017, Grego et al. 2020) and a single sequences of *Melanopsis mingrelica* (Neiber and Glaubrecht 2019) and *Radix euphratica* (Aksenova et al. 2019).

The average fragment length of CO1 barcodes in a “DS-FMOL” dataset (combining “GEOFM” project plus pre-existing barcodes) were 534 bp (min: 409bp and max: 658bp). Nucleotide base frequencies were: A-25.4%, G-18.4, C-14.4%, T-41.8%) - a similar to the reported frequencies for molluscs (e.g. Weigand et al. 2011), while GC content equal to

32.8% was lowest compared to results from other molluscan studies (35.8% and 36.9% from Kumar et al. (2015) and Layton et al. (2014), respectively.

The families Planorbidae and Lymnaeidae are represented by the highest number of barcodes (116 and 99, respectively). The two families Unionidae and Neritidae are represented each with 19, 12 barcodes, respectively. The two families Cyrenidae and Sphaeriidae are represented by an equal number of barcodes (each with 11 barcodes). The two families Physidae and Viviparidae are represented each with 10, and 5 barcodes, respectively and the family Melanopsidae and Acroloxidae by the lowest number of barcodes (3 barcodes). The most common genus was *Ancylus*, for which 93 barcodes (3 species) were generated, followed by *Radix* and *Unio* (73 and 16 barcodes, respectively and 3 species each of them). The 18 genera were represented by a single species, 2 genera with 2 species and a single genus by the 4 species. Of all species obtained, three species *Ancylus capuloides*, *Radix auricularia* and *Ancylus benoitianus* were represented the highest number of barcodes (each with 58, 52 and 31, respectively), followed by *Radix euphratica*, *Lymnaea stagnalis*, *Theodoxus fluviatilis*, *Corbicula fluminalis*, *Unio crassuss* and *Physella acuta* (each with 21, 14, 12, 11, 13 and 10 barcodes, respectively). Most of the species are represented with less than 10 barcodes, including 6 species, with a single barcode (Fig. 2).

The BIN and RESL analyses resulted in 43 BINs united into 40 OTUs. In addition, 13 OTUs were also formed for 23 sequences for which no BINs had been defined due to small barcode size (less than 500 bp (Ratnasingham and Hebert 2013)). However, all of these sequences were mined from GenBank. Also, the BIN had not been defined to the three sequences of *Ancilus benoitianus*. From the 43 BINs, 34 (79%) were concordant and 9 (21%) were represented with singletons. Sequences (107) of 18 BINs (42%) are only known from the study area for the time of publishing.

Average within species divergence were 0.69±0.0% (ranged from 0% to 4.1%) followed with divergence of 6.4±0.0% within genera (ranged from 0 to 16.7%) and 17.8±0.0% divergence within families (ranged from 10.42% to 21.9%).

In most cases, morphologically determined specimens (comprising 28 species) were matched with a single OTU/BIN cluster with intraspecific (or within BIN) sequence divergence of less than 2.2%. More than one BINs were found in 6 species-level taxa - *Ancylus capuloides* (2 BINs), *Planorbis planorbis* (2 BINs), *Physella acuta* (2 BINs), *Lymnaea stagnalis* (2 BINs), *Radix auricularia* (2 BINs) and *Radix euphratica* (4 BINs) (Table 1).

Tree-based reconstruction using the NJ algorithm on K2P distance produced highly congruent results with the morphological and BIN assignment of specimens (Fig. 3). With more than 98% bootstrap support values, 33 morpho-species out of 34 were produced well-defined clusters. Only *Ancylus benoitianus/capuloides* species complex were not complementary with morphological identification, rather reflecting the entangled BIN structure.

Discussion

South Caucasian freshwater molluscs (and all invertebrates in general) are still poorly known (Japoshvili et al. 2016, Mumladze et al. 2020). The check-list of freshwater molluscs species for the South Caucasus or any separate country within it is more than 50 years old and completely outdated (Zhadin 1952, Javelidze 1973, Akramowski 1976). While a number of papers have appeared during the last three decades providing information on the taxonomy and systematics of separate taxa (given below), only three articles were published reporting the field research-based inventory results of all freshwater molluscs of a particular area: for Sevan Lake in Armenia Mashkova et al. (2018), Javakheti region of Georgia - Bikashvili et al. (2021) and Kazbegi municipality in Georgia - Neiber et al. (2021). Thus, it is clear that the current knowledge of freshwater mollsucs species diversity and distribution in the South Caucasus region remains far to be comprehensive.

Within the current project, we were able to generate 298 new barcodes resembling 34 freshwater mollusc species-level taxa. Roughly, this is no more than 30% of the expected species number in the South Caucasus (based on Vinarski and Kantor (2016), Glöer (2019), Mumladze et al. (2019), Grego et al. (2020)). Nearly all morphologically identified species were further validated with barcode data while several species were turned out to be unmatched with BOLD taxonomy. This latter category includes species of the pond snails of the family Lymnaeidae, ramshorn snails (family Planorbidae) freshwater calms (family Sphaeriidae). While the aim of this article is not to deal with the systematics and taxonomy of species, in the following we will revise each of the studied taxa and outline gaps in the knowledge.

Pond snails of the family Limnaeidae are distributed worldwide (Correa et al. 2011). They are of major medical and veterinary importance since they act as vectors of parasites (Bargues et al. 2006, Medeiros et al. 2014). The morphological and anatomical plasticity among and within lymnnaeid representatives remains challenging (Bargues and Mas-Coma 1997, Jackiewicz 1998, Pfenninger et al. 2006, Aksenova et al. 2018) however a recent large scale multi-marker molecular genetics and morpho-anatomical investigations refined species-level taxonomy at least for a part of taxa within this family (Aksenova et al. 2018, Vinarski et al. 2020). Unfortunately, only 4 sequences of a single species (*Radix auricularia*) were available for the whole south Caucasus (in particular from Armenia) at the time of the studies cited above. According to literature, there are at least 6 genera of two subfamilies distributed in the South Caucasus including *Ampullaceana, Peregriana, Radix* (all three from the subfamily Amphipepleinae)*, Galba, Stagnicola* and *Lymnaea* (all three form the subfamily Lymnaeinae).

Amphipepleinae represents one of the most spacious and taxonomically challenging groups. Morphologically identified species - *Ampullaceana lagotis* formed the unique BIN BOLD:AEN6567 with the divergence of 4.97% to The nearest neighbor (NN) BIN BOLD:ACI0501 that includes specimens of yet unresolved "*Radix zazurnensis*" from Russia (3) and China (32) (Aksenova et al. 2016). Thus this species is represented in our

database as *Ampulaceana* sp. (Fig. 3) awaiting further taxonomic clarification. In contrast, specimens identified as *Peregriana peregra* (widely referred to as *Radix labiata*) perfectly matched with the BIN BOLD:AAD0368 (with a maximum intra-BIN distance 4.92%) representing the same species from western Palearctic.

The genus *Radix* turned out to be the most complex within the family. Based on morphology alone we were able to confidently identify only *R. auricularia*, barcodes of which formed two separate BINs: 12 specimen were allocated under BIN BOLD:ACI2007 (with 2.88% divergence to NN, BOLD:AAD6712) and 40 specimens were formed under the BIN BOLD: AAD6712 (2.88% divergence to NN BOLD: ACI2007). Both BINs seem to characterize geographically variable *R. auricularia* populations . Other unidentified specimens of *Radix* (22 in total) formed four unique BINs which were clustered together as a single putative species given the divergence threshold 2.2% (Fig. 3). Within this cluster, 17 Georgian specimens were classified under the BIN BOLD:ADJ8863. With our specimens, this BIN includes specimens from Iraq, Iran, Uzbekistan and Russia and represents species *R. euphratica* (with NN BIN BOLD:AEI7975 (2.82%divergence) representing a single specimen of *R. euphratica* from Iran). Five other specimens of *Radix* sp. formed 3 different BINs, BOLD:ADK5204 (with 3.37% divergence to NN BIN, BOLD:ADJ8863), BOLD:ADK6106 (with 1.92% divergence to NN BIN BOLD:ADR3052), BOLD:ADR3052 (with 1.92% divergence to NN, BIN BOLD:ADK6106). Due to its small within BIN distances, this clade can be named as *R. euphratica* (Fig. 3) which first mentioned from the Tbilisi Reservoir (voucher number Mlym68) (Aksenova et al. 2019). Our research has shown that *Radix euphratica* much widespread in Georgia (13 sampling points for this study).

Subfamily Lymnaeinae includes three representative genera in South Caucasus each with single species. *Galba* is characterized by high phenotypic plasticity and extremely uniform anatomical traits, which is often the reason for species misidentification (Samadi et al. 2000, Standley et al. 2013). Three of our specimens of *Galba truncatula* formed the BIN BOLD:ABA2623 which represents the cluster of *G. truncatula* specimens from all over its distribution area. Distance to its NN BIN (BOLD:AAI7214) is 4.03% and is named as *G. truncatula* as well. The single specimen (Samegrelo region, western Georgia) in our dataset (also morphologically identified as *G. truncatula*) clustered under BIN BOLD:AAY4012 comprising specimens of *G. schirazensis*. The NN (with 7.84% divergence) BIN is BOLD:ADR2784 including the specimens of *Galba truncatula* form Japan. According to Kruglov (2005) *G. schirazensis* is distributed in Azerbaijan. For Georgia, it is a new country record. From the genus *Lymnea* a single species – *L. stagnalis* is known. Our specimens of *L. stagnalis* formed two BINs. Eight specimens were matched with BIN BOLD:AEN6037, for which only single barcode was available From Ukraine. The NN BIN is BOLD:ACQ0092 with 2.43% divergence, includes specimens also belonging to *L. stagnalis*. The remaining 6 specimens were formed the unique BIN (BOLD:AEM9638) with the NN BIN - BOLD:ACQ2679 (with 2.12% divergence) comprising specimens of *L. stagnalis*. Thus in South Caucasus at least two haplotype of *L. stagnalis* occurs both in a mountainous Javakheti region (southern Georgia). The last genera in this subfamily is *Stagnicola* which is also represented with a single species (*S. palustris*) in South

Caucasus. Only two specimens *Stagnicola* were represented in our dataset forming the unique BIN - BOLD:AEN6388 which were diverged by 4.83% from the NN BIN BOLD:ACV7473 representing the specimens of *S. turricula* from Poland. Most probably the genus *Stagnicola* in Georgia (and in South Caucasus) is not a *S. palustris* , or the genus is represented with more than one species in the region. Thus additional sampling and taxonomic investigation are required.

The Ramshorn snails of the family Planorbidae is the most diverse group of freshwater pulmonates inhabiting a wide range of freshwater habitats (Jørgensen et al. 2004, Albrecht et al. 2007). Understanding of relationships within the Planrobidae remains confused due to the extreme variability of anatomical and shell morphological traits (Baker 1945, Hubendick 1978). In South Caucasus more than 15 species of Planorbidae are provisionally listed including the genera *Planorbis, Segmentina, Anisus, Hippeutis, Bathyomphalus, Gyraulus, Ancylus* and *Ferrissia* (Vinarski and Kantor 2016). For the current study, we obtained samples for 7 out of 8 genera including the following morpho-species: *P. planorbis, S. nitida, A. leucostoma, G. albus, B. contortus, F. californica, A. benoitianus, A. capuloides, A. major* and *Ancylus* sp.

Seven specimens of *Planorbis planorbis* formed two BINs - BOLD:AED0778 and BOLD:ADJ5964 diverged both from the same NN BIN (BOLD:ACS1294) with 3.4% and 2.1% respectively. All three BINs are considered as *P. planorbis* in BOLD systems comprising the specimens from different regions of Europe and Middle East.

The genus *Segemntina* is taxonomically understudied. Some authors are considering only a single *S. nitida* species within the genus (Falkner et al. 2001, Welter-Schultes 2012) while others (e.g. Kruglov and Soldatenko 1997) consider 14 separate species within the genus, including two species (*S. caucasica* and *S. malkae*) endemic to the north Caucasus. In this study, 3 specimens from South Caucasus (western Georgian lowlands) identified as *S. nitida* based on shell shape, formed the unique BIN BOLD:AEN3217 for which the NN BIN is BOLD:AAN3912 (with 11.89% divergence), comprising specimens of *Segmentina sp*. (52) and *S. nitida* (3) form Poland, Sweden and Germany. This specimens apparently does not belong to *S. nitida* and is instead either new species or belong to one of those species indicated by Kruglov and Soldatenko (1997) for which no DNA sequences are available. Further study is required to solve the taxonomy of South Caucasian *Segmentina*.

Two representatives of the genus *Anisus* is known for South Caucasus (*A. leucostoma* and *A. spirorbis*) (Glöer 2019, Vinarski and Kantor 2016). Six specimens of *Anisus* in our dataset formed an unique BIN BOLD:AEC8114 which were diverged from the NN BIN BOLD:AAR3430 (*A. spirorbis* from Germany) by 8.58%. Thus our specimens matched neither *A. spirorbis* nor *A. leucostoma* and most probably represents new, yet undescribed species.

The taxonomy of the genus of *Ancylus* is far to be resolved. For the Caucasus region, some 6 species are indicated (Akramowski 1976, Soldatenko and Starobogatov 2004). For the present study 104 specimens classified as 4 morpho-species (*A.* cf. *benoitianus, A.* cf.

capuloides, A. cf. *major* and *Ancylus* sp.) were collected from different areas of Georgia and Armenia. All specimens clustered together in a single taxon under 2.2% divergence assumption (Fig. 3). However, sequences were distributed among 4 different BINs including *A.* cf. *capuloides* subclade (2 bins: BOLD:AEN1069 (1.83% divergence to NN BOLD:AAD2028) and BOLD:AEN1070 (1.65% divergence to NN, BOLD:AEN1069)), *A.* cf. *benoitianus* subclade (BIN BOLD:AEN1066 with 1.1% divergence to NN BOLD:AAD2028) and *Ancylus sp.* - BOLD:AEN7656 with 4.58% divergence to NN BOLD:AAD2028). Thus, *Ancylus* in South Caucasus is characterized with a large number of lineages (resembling the taxonomy of Soldatenko and Starobogatov (2004)), although overall genetic (and morpho-anatomical) differentiation might not be enough to delimit the species. Nonetheless, the in-depth integrative taxonomic study is necessary to further progress in the systematics of Caucasian *Ancylus.*

The remaining Planorbiidae species – *Ferrisia californica, Gyraulis albus* and *Bathyomphalus contortus* all matched exactly within the conspecific representatives from the wide areas of western Palearctic. An exception is the *F. californica* which formed an unique BIN BOLD:AEJ3761 with 3.06% divergence from the NN BIN BOLD:AAE6642 (includes specimens under a name of *F. fragilis* (synonym of *F. califonica*)).

The freshwater clams (family Sphaeriidae) is a cosmopolitan group inhabiting all types of freshwater habitats (Korniushin 2002, Rassam et al. 2020). The taxonomy and distribution of freshwater clams still needs substantial clarification (Rassam et al. 2021). This is mainly because of limitations in diagnostically important morphological characters (Korniushin 2000, Voode 2017). From the South Caucasus region, a number of species are to be thought to belong to genus *Sphaerium*, *Musculium* and *Pisidium*. The former two genera are represented with single species (*M. lacustre* and *S. corneum*) while the latter genera is represented with at least 7 species (Akramowski 1976, Zhadin 1952). From these genera we were able to obtain DNA barcodes for several taxa identified as *S. corneum*, *M. lacustre* , *P. casertanum*, *P. subtruncatum*. Three specimens of *M. lacustre* were matched with a specimen from Spain (BIN BOLD:AEE5622) with maximum intra-BIN divergence of 0.36%. The NN BIN (with 1.6% divergence) is also represented with CO1 haplotype of *M. lacustre* specimens from Europe. In contrast, CO1 barcodes for morphologically identified specimens as *S. corneum* were matched with single specimens of *S. nucleus* from United Kingdom (BIN: BOLD:ACQ8004). Within this BIN only sequence was available before, which with our three additional sequences resulted within-BIN maximum p-distance 1.47%. The NN (with 3.85% divergence) BIN is BOLD:ABU6190 comprising *S. nucleus* specimens from central Europe, which are in their own closely related (2.87% divergence) to *S. corneum* (BOLD:ADF3777) from central and south-west Europe. *S. nucleus* was usually considered an intraspecific variety of *S. corneum* (Piechocki 1989). However, according to Korniushin (2001) and Petkevičiūtė et al. (2018) there are several stable morphological and anatomical characteristics, and even more importantly substantial genetic evidence that these two species are sister taxa. Due to observed genetic differences of our specimens to corneum/nucleus group, it is worthwhile to investigate the South Caucasian *Sphaerium* representatives in more details including multilocus phylogeny and morphology to solve its taxonomic affinities.

Another genus of clams with a complicated genetic structure is *Pisidium.* Specimens submitted to a barcoding pipeline were morphologically identified as either *P. casertanum* (5 specimens) or *P. Subtruncatum* (2 specimens). The only specimen of putative *P. subtruncatum* were validated under the BIN: BOLD:ACQ3092 while the rest of the specimens formed unique genetic clusters with no clear systematic position. As example, the BIN BOLD:ACQ7011 contains specimens from Greece, Albania, Germany and one specimen from Georgia with a maximum intra-specific divergence with 1.71%. The closest, NN BIN BOLD:AAG0350 (an unnamed clade) is diverged with 1.92%. The remaining 5 specimens were all turned out to belong to yet unknown species under the BINs BOLD:AEN6788 (5.13% divergence to NN) and BOLD:AEN0712 (3.8% divergence to NN BIN). Similar to *Sphaerium*, this genus is also difficult to classify based on shell morphology alone due to limitations if taxonomically meaningful characters (Korniushin and Glaubrecht 2006, Clewing et al. 2013, Voode 2017, Rassam et al. 2021). Accordingly, a more detailed study is necessary to solve species-level taxonomy and even to validate the taxonomic value of currently used identification (morphological) characters for the species level classification of *Phisidium*.

One more specious family in the study area is bivalve family Unionidae that includes at least 5 valid species occurring in South Caucasus including *Unio crassus, U. tumidus, U. pictorum, Anodonta cygnea* and *A. anatina* (Graf 2007). In the present study, we sequenced representative specimens for all 5 species that perfectly matched with the conspecific barcodes from the BOLD system (Table 1). Similarly, specimens of other 7 freshwater mollusc families represented with a single species in the South Caucasus including *Acroloxus lacustris* (Acroloxidae), *Physella acuta* (Physidae), *Bithynia tentaculata* (Bithyniidae), *Viviparus costae* (Viviparidae), *Melnaopsis mingrelica* (Melanopsidae), *Theodoxus fluviatilis* (Neritidae) and one bivalve species *Corbicula fluminalis* (Cyrenidae) also formed unambiguous barcode clusters matching the conspecific sequences originated outside the study area.

Conclusions

Our results clearly showed the insufficiency of the current knowledge of freshwater molluscs diversity in the South Caucasus region. In spite of the limited taxa coverage, nearly half of the studied taxa turned out to be in need of substantial taxonomic investigation/revision. South Caucasus region is Plio-Pleistocene refugia and occurrence of unique or endemic lineages are not a surprise, however, good understanding of its biodiversity is necessary to apply monitoring and conservation measures. A group of freshwater molluscs that were not investigated in the current project includes the representatives of the family Hydrobiidae – a minute prosobranch snails. Only recently, this group turned out to be very specious in the South Caucasus (particularly in Georgia) (Grego et al. 2020, Chertoprud et al. 2020, Chertoprud et al. 2021). Although the systematics of this family in South Caucasus is being studied by means of integrative approaches, still no quality barcodes are available for any of the species. Thus, diverse Hydrobiidae and some other freshwater mollusc families for which only a sample of

representatives have been studied until now, need to be further investigate in order to develop an useful barcode library. This particularly concerns the integrative taxonomic investigations to solve taxonomic ambiguities and clarify species level diversity in the region.

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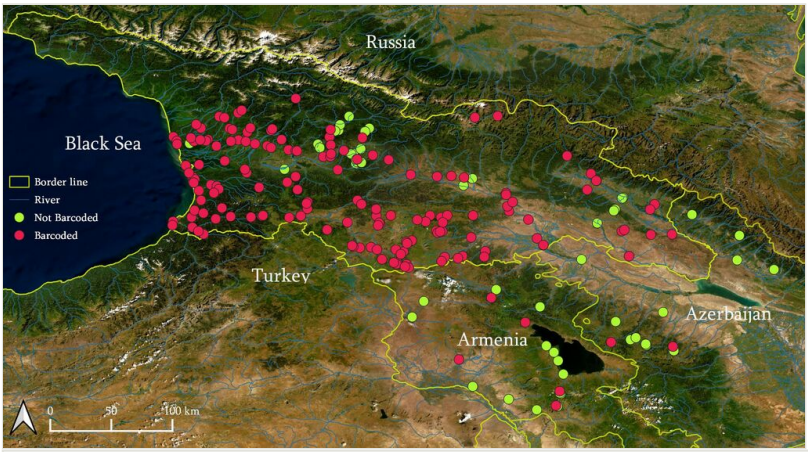

Figure 1.

Map of collection localities for freshwater molluscs in the present study. The red dots correspond to the localities from where one or more specimens/species were submitted to barcoding, while the yellow dots correspond to localities from where the specimens are still waiting for genetic investigation.

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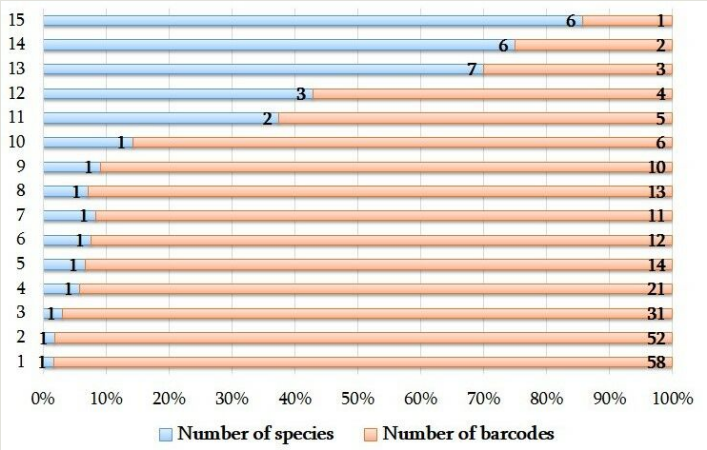

Figure 2.

Ranking of species according to the number of barcodes.

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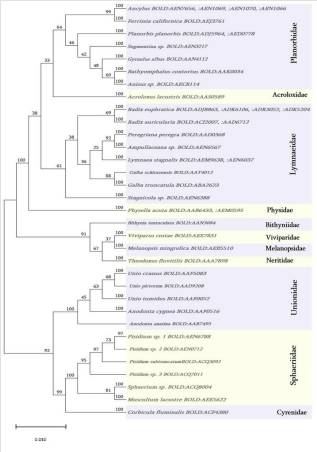

Figure 3.

NJ tree of freshwater molluscs based on the analyses of 313 cytochrome c oxidase (CO1) sequences using the Kimura 2- parameter (K2P) distance. Multiple BINs indicate species pairs with an intraspecific distance of 2.2%.

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Table 1.

BOLD summary data of barcoded Freshwater Molluscs with mean and maximum intraspecific and nearest neighbour (K2P) distances. Country Codes: AT = Austria, ALB = Albania, ARG = Argentina, AZR = Azerbaijan, ARM = Armenia, ALB = Albania, AU = Australia, BY = Belarus, BG = Bulgaria, BIH = Bosnia and Herzegovina, CH = Chaina, CO = Colombia, CU = Cuba, CA = Canada, HR = Croatia, CZ = Czech Republic, ECUA = Ecuador, FI = Finland, FR = France, DE = Germany, GE = Georgia, GR = Greece, HU = Hungary, IT = Italy, IR = Iran, IQ = Iraq, IN = India, JP = Japan, KZ = Kazakhstan, KE = Kenya, LT = Lithuania, MA = Morocco, MX = Mexico, MLO = Moldova, ME = Montenegro, MT = Malta, MY - Malaysia, MM = Myanmar, NZ = New Zeland, NP = Nepal, NL = Netherlands, MK = North Macedonia, PL = Poland, PT = Portugal, PE = Peru, RU = Russia, RO = Romania, RS = Serbia, SE = Sweden, SI = Slovenia, SK = Slovakia, ESP = Spain, CH = Switzerland, SG = Singapore, TH = Thailand, TR = Turkey, UKR = Ukraine, GB = United Kingdom, UZB = Uzbekistan, US = United States VE = Venezuela. n = BIN member count.

Species	BIN	n	MeanISD	MaxISD	Country	Nearest BIN/ species	Distance to NN
Ancylus sp.	BOLD:AEN7656	12	0.19	0.55	GE	BOLD:AAD2028	4.58
Ancylus benoitianus	BOLD:AEN1066	27	0.17	0.73	GE, ARM, AZR	BOLD:AAD2028	1.1
Ancylus capuloides	BOLD:AEN1069	57	0.41	1.47	GE	BOLD:AAD2028	1.83
Ancylus capuloides	BOLD:AEN1070	2	0.37	0.37	GE	BOLD:AEN1069	1.65
Bathyomphalus contortus	BOLD:AAK0034	20	0.75	1.61	DE, NL, AT, PL, GE	BOLD:ADR9065	9.45
Gyraulus albus	BOLD:AAN4112	19	1.16	3.02	DE, ME, AT, PL, RS, CZ, GE	BOLD:AEB5660	7.55
Segmentina sp.	BOLD:AEN3217	3	0.22	0.32	GE	BOLD:AAN3912	11.89
Anisus sp.	BOLD:AEC8114	6	0.43	0.81	GE	BOLD:AAR3430	8.58
Planorbis planorbis	BOLD:AED0778	5	0.39	0.97	GE	BOLD:ACS1294	3.4
Planorbis planorbis	BOLD:ADJ5964	4	0.28	0.5	IR, GE	BOLD:ACS1294	2.1
Ferrissia californica	BOLD:AEJ3761	3	0	0	GE	BOLD:AAE6642	3.06
Ampullaceana sp.	BOLD:AEN6567	2	0	0	GE	BOLD:ACI0501	4.97
Radix euphratica	BOLD:ADJ8863	53	1.34	2.96	IQ, IR, GE, USB, RU	BOLD:AEI7975	2.82
Radix euphratica	BOLD:ADK5204	5	0.96	1.7	IQ, GE	BOLD:ADJ8863	3.37

Radix euphratica	BOLD:ADK6106	3	0.32	0.48	IQ, GE	BOLD:ADR3052	1.92
Radix euphratica	BOLD:ADR3052	3	0.11	0.16	IQ, GE	BOLD:ADK6106	1.92
Radix auricularia	BOLD:ACI2007	14	0.46	0.84	ARM, GE	BOLD:AAD6712	2.88
Radix auricularia	BOLD:AAD6712	153	0.91	2.99	DE, PL, ME, HR, GR, MK, RU, ARM, CA, FR, ESP, CH, AT, US, GE	BOLD:ACI2007	2.88
Peregriana peregra	BOLD:AAD0368	74	2.03	4.92	ALB, FR, RS, GR, MK, ME, DE, SK, RU, AT, IR, GE	BOLD:AEN6567	10.14
Lymnaea stagnalis	BOLD:AEM9638	6	0	0	GE	BOLD:ACQ2679	2.12
Lymnaea stagnalis	BOLD:AEN6037	9	0.73	1.4	GE, DE	BOLD:ACQ0092	2.43
Galba truncatula	BOLD:ABA2623	50	0.99	2.74	FR, VE, IR, NP, SI, GR, RU, ME, ALB, GE	BOLD:AAI7214	4.03
Galba schirazensis	BOLD:AAY4012	64	0.42	0.02	CA, VE, PE, ECUA, MX, IR, FR, US, CO, JP, GE	BOLD:ADR2784	7.84
Stagnicola sp.	BOLD:AEN6388	2	0.16	0.16	GE	BOLD:ACV7473	4.83
Acroloxus lacustris	BOLD:AAS0589	29	1.44	2.92	DE, TR, MK,GR, RS, AT, ALB, UKR	BOLD:ADK8211	2.9
Physella acuta	BOLD:AAB6433	50	0.67	3.86	FR, US, GR, MK, IR, JP, MT, UKR, AZR, GE	BOLD:AEM0595	2.03
Physella acuta	BOLD:AEM0595	358	1.72	6.35	US, FR, NL, CU, AU, CA, IN, ARG, GR, MK, TH, SG, MY, NZ, MM, IR, CN, JP, AT, IQ, KE, ESP, MT, ME, DE, UKR, AZR, PE, GE	BOLD:AAB6433	2.03
Viviparus costae	BOLD:AEE7831	4	0.67	1.33	GE	BOLD:ADI2641	0.44
Bithynia tentaculata	BOLD:AAN3084	55	1.32	3.73	DE, US, AT, GE, RU, KZ, BY, UKR, RO	BOLD:AAF5645	7.77

Melanopsis mingrelica	BOLD:AEB5510	4	0.16	0.32	GE	BOLD:AEB0981	3.85
Theodoxus fluviatilis	BOLD:AAA7898	291	1.8	7.25	DE, FI, AT, HR, HU, BIH, UKR, ME, ALB, MK,GR, RU, TR, BG, GE, MLD, FR, RO, PT, ESP, LT, GB, MA, IT, SK	BOLD:ACF4500	5.08
Corbicula fluminalis	BOLD:ACF4380	64	0.15	3.07	FR, ARG, HU, IN, RU, GE, AZR	BOLD:ACF5867	1.92
Anodonta anatina	BOLD:AAB7495	897	1.93	5	PO, SE, PT, IT, ESP, FR, HR, RU, HU, CZ, UKR, AT, BG, MA, TR, DE, KZ, GE	BOLD:AAF6127	10.81
Anodonta cygnea	BOLD:AAF0516	110	0.28	2.1	SE, PT, DE, PL, FR, IT, CZ, GB, HU, AT, TR, RU, GE	BOLD:AEE8900	8.73
Unio crassus	BOLD:AAF5083	175	0.52	2.17	AT, UKR, TR, DE, GE	BOLD:ADR4461	2.28
Unio pictorum	BOLD:AAD9208	232	0.36	2.68	AT, PL, GB, UKR, RU, IR, GR, SK, FR, TR, DE, GE, MLD	BOLD:ADR3328	2.38
Unio tumidus	BOLD:AAF0052	78	0.22	1.28	SE, PL, UKR, AT, DE, GB, RU, SK, GE, MLD	BOLD:ADR6944	9.39
Sphaerium sp.	BOLD:ACQ8004	4	0.73	1.47	GE, GB	BOLD:ABU6190	3.85
Musculium lacustre	BOLD:AEE5622	4	0.18	0.36	ESP, GE	BOLD:ACQ4690	1.6
Pisidium sp. 1	BOLD:AEN6788	3	1.12	1.44	GE	BOLD:ACQ0055	5.13
Pisidium sp. 2	BOLD:AEN0712	1	N/A	N/A	GE	BOLD:ACQ0055	3.08
Pisidium sp. 3	BOLD:ACQ7011	4	1.03	1.71	ALB, GR, DE, GE	BOLD:AAG0350	1.92
Pisidium subtruncatum	BOLD:ACQ3092	7	0.77	1.61	GE, IT, MK, AT, US	BOLD:ACQ6136	3.09

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Supplementary material

Suppl. material 1: Supplementary Table S1

Authors: Ani Bikashvili
Data type: occurrences, sample data
Brief description: Details on barcoded freshwater molluscs from Georgia, Armenia and Azerbaijan
Download file (28.98 kb)