AN INTEGRATIVE TAXONOMIC STUDY OF MOLLUSCS FROM BELSHI KARSTIC LAKES USING MOLECULAR GENETIC ANALYSIS

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ABSTRACT
High species diversity and the large number of endemic species make the mollusc fauna of the Balkan Peninsula one of the most important hotspots of the Holarctic region. Belshi lakes, situated in central Albania, represent a typical and interesting area of karstic lakes. As their mollusc fauna has been randomly investigated, information remains very limited. Although molecular genetic methods, especially DNA barcoding techniques have significantly become important in biodiversity research, they have been rarely applied in studies of aquatic fauna of Albania, and even lesser in molluscan studies. This paper represents a survey of the malacofauna of the Belshi lakes and first steps into DNA barcoding analysis of selected molluscs collected there. Molluscs samples were collected from 34 lakes in the spring and autumn of 2018. The DNA barcoding analyses included sequencing of a short fragment of the mitochondrial cytochrome c oxidase subunit I (COI) gene and comparisons with a library of DNA barcodes of known species (Barcode of Life Data Base BOLD or GenBank). Standard DNA extraction and PCR of the COI marker sequence were carried out via standard barcoding primers and Sanger sequencing of the PCR products. DNA analysis confirmed the presence of six molluscs species, two of which did not match well with sequences available for comparison. The species thought to be *Gyraulus albus* was quite distantly related to that taxon. Also, the species that was initially identified as *Dreissena polymorpha* turned out to be very closely related to the *Dreissena carinata* clade. The results here reported show also the importance of integrative taxonomy, which along with morphological and genetical analysis help to better understand the relationships among freshwater mollsucs in the Balkans and their taxonomic issues.
***Keywords*:** barcoding, freshwater malacofauna, Balkan Peninsula, Albania

1. INTRODUCTION

Molluscs, like many other groups of macroinvertebrates have an essential role in the functional processes of aquatic ecosystems such as marine, freshwater and brackish. They have a key position within food networks, being at the same time decomposers and / or filters even for higher trophic levels (Griffiths, 1991).

Integrated taxonomy is the study of taxonomy, genetics, origin, phylogenetic relationships, biogeography, development, behaviour, ecology etc. of species, combining (integrating) morphological and anatomical characteristics with those of molecular genetics (Araujo, 2007).

In Albania, DNA barcoding techniques have been scarcely used, mainly for some species of medicinal plants and domesticated animal species in agriculture. The application of these techniques is almost absent in studies of wild fauna, aquatic fauna and especially molluscs in Albania.

Belshi lakes are of karstic origins and located in Central Albania. They represent the group with the largest number of lakes (88 lakes in total) in the hydrogeographical network of Albania.

Currently, there is no information about the malacofauna of Belshi Lakes. The summary lists of continental molluscs of Albania, referred to Dhora and Welter-Schultes (1999), Fehér and Eröss (2009) and Dhora (2010) do not mention mollusks from Belshi lakes.

The present paper aims to: i) identify the mollusks' species based on the integrated taxonomy, meaning the combination of morphological and anatomical data with those of molecular genetics to increase the accuracy and confidence in species identification and, ii) to compare the mollusks' species from Belshi lakes with the same species from the Balkan region and larger via molecular genetic analysis.

This investigation is the first attempt to obtain information about the malacofauna of these lakes and to use molecular genetic analyses and integrative taxonomy for a better understanding of relationships among freshwater mollsucs in the region.

2. MATERIAL AND METHODS

Sampled lakes and sampling periods

Mollusk sampling was carried out in 34 lakes in Belshi. The selection of lakes has generally been random, but also taking into account the possibility of easy access.

Fig. 1. Map of Belshi area, showing the sampled lakes.

Table 1. List of sampled lakes and their respective geographical coordinates

	Lake	COORDINATES
1	Liqeni i Katundit	40°57'54.1"N 19°58'23.3"E
2	Liqen pa emër	40°57'45.1"N 19°58'37.2"E
3	Liqeni i Miloshit	40°57'49.9"N 19°58'45.3"E
4	Liqeni i Bicit	40°57'39.0"N 19°58'42.1"E
5	Liqeni i Ulzës	40°58'07.0"N 19°58'12.0"E
6	Liqeni Kashaj	40°57'55.3"N 19°58'01.4"E
7	Liqen pa emer	40°58'03.2"N 19°57'56.1"E
8	Liqeni i Komnecit	40°58'00.4"N 19°57'24.3"E
9	Liqeni i Godës	40°58'37.1"N 19°57'47.9"E
10	Liqeni i Kashtës	40°59'05.7"N 19°56'52.0"E

11	Liqeni i Thatë	40°59'15.5"N 19°56'20.7"E
12	Liqen pa emër	40°59'00.1"N 19°55'43.4"E
13	Liqen pa emër	40°58'58.3"N 19°55'39.7"E
14	Liqeni i Strehës	40°58'41.1"N 19°55'38.0"E
15	Liqen pa emër	40°58'44.2"N 19°55'23.3"E
16	Liqeni i Belshit	40°58'42.4"N 19°53'32.1"E
17	Liqen pa emër	40°58'57.1"N 19°54'15.7"E
18	Liqeni i Gjatë	40°59'04.2"N 19°54'25.5"E
19	Liqen pa emër	40°59'16.6"N 19°54'27.6"E
20	Liqeni i Trojsit	40°59'18.6"N 19°53'50.4"E
21	Liqen pa emër	40°59'02.5"N 19°53'48.6"E
22	Liqeni i Dorbnit	40°57'06.3"N 19°52'29.2"E
23	Liqeni i Merhojës	40°56'36.6"N 19°52'25.1"E
24	Liqeni i Cestijes	40°55'09.1"N 19°52'01.7"E
25	Liqeni i Seferanit	40°57'18.8"N 19°53'55.0"E
26	Liqeni i Civiles	40°57'21.6"N 19°54'28.8"E
27	Liqen pa emër	40°57'33.5"N 19°55'30.9"E
28	Liqeni i Paçit	40°57'33.1"N 19°55'38.3"E
29	Liqeni i Këlvorës	40°57'16.2"N 19°55'33.9"E
30	Liqen pa emër	40°53'34.9"N 19°53'48.9"E
31	Gjoli i Turbullt	40°53'49.7"N 19°53'29.8"E
32	Liqeni Gropa e Selamit	40°53'40.8"N 19°53'09.1"E
33	Liqen pa emër	40°53'47.3"N 19°52'16.2"E
34	Liqeni i Gropës së Madhe	40°53'47.9"N 19°52'01.5"E

Sampling was carried out in spring (April - May) 2018 and autumn (September - October) 2018. In the lakes of the Mediterranean climatic zone (which includes the lakes of Belshi, and all the other lakes in Albania), these periods generally coincide with the highest values of dissolved oxygen in the water and their better environmental condition (in spring), and the decrease of the values of dissolved oxygen and increase of eutrophication and environmental stress (in late summer and early autumn) as stated in (Wetzel 2001).

Mollusks' sampling

The molluscs sampling is based on (Hájek *et al*., 2006; Horsak *et al*., 2015). Each lake was sampled at three equidistant points that were selected based on the diversity of micro-habitats in the lake, type of substrate, the presence or absence of macrovegetation, and the presence of stones, wood, or artificial materials on substrate. Sampling of aquatic mollusks was done in transects at 1 m distance from the shore, along a 10 m linear stripe on lake substrata. Sampling time interval was 20 minutes at each point. Samples on soft bottom and macrovegetation were taken with a hand net of 0.5 mm mesh size and a diameter of 20

cm. Hand picking with forceps was applied on hard substrata and woods. The samples were stored in plastic bottles with 90% alcohol and transported to the laboratory.

Taxonomic identification and molecular genetic analyses of mollusks

Taxonomic identification of mollusks was initially done at the Laboratory of Environmental Biology, Faculty of Natural Sciences, University of Tirana, Albania. Later on, the samples were transferred for molecular genetic analysis at the Laboratory of Molecular Systematics of the Museum of Natural History, Vienna, Austria. Small tissue fragments were taken from each animal's body for DNA extraction and they were allowed to dry from the remaining ethanol. Tissues were treated for extraction using the Qiagen 2011 method (QIAamp®DNA Mini Kit). After extraction, the next step in DNA sequencing was the PCR. The results were processed through applications for processing DNA sequences such as: BioEdit, referred to Hall T. A (1999), GenBank (https://www.ncbi.nlm.nih.gov/), Molecular Evolutionary Genetics Analysis (MEGA) (https://www.megasoftware.net) and The Barcode of Life Database (BOLD) (http://www.barcodinglife.org).

3. RESULTS AND DISCUSSIONS

There are eight mollusks species found in Belshi lakes as listed below:

GASTROPODA
Radix auricularia (Linnaeus, 1758)
Ampullaceana balthica (Linnaeus, 1758)
Galba truncatula (O. F. Müller, 1774)
Gyraulus chinensis (Dunker, 1848)
Physella acuta (Draparnaud, 1805)
Valvata piscinalis (O. F. Müller, 1774)

BIVALVIA
Musculium lacustre (O. F. Müller, 1774)
Dreissena carinata (Dunker, 1853)

Six mollusks' taxa underwent molecular genetic analyses. Two species, namely *Ampullaceana balthica* and *Valvata piscinalis*, did not undergo such analysis as they were found only as empty shells. Consequently, molecular genetic analyses appeared impossible.

Genus *Radix*

Based on data from the Laboratory of Environmental Biology in Tirana and the Department of Invertebrate Zoology, Museum of Natural History, Vienna, Austria, it was initially thought that from the genus Radix in our samples, in addition to *(Radix) Ampullaceana balthica* (Linnaeus, 1758), we had two other species: *Radix auricularia* (Linnaeus, 1758) and *Radix balthica* (Linnaeus, 1758). DNA analysis reported that both supposed taxa belong to the species *Radix auricularia* (Linnaeus, 1758). This is could be clearly noted in the phylogenetic tree (Figure 3), where the sequences of individuals found in Belshi lakes stand in the same lineage with *Radix auricularia* sequences found in Greece, Montenegro, Croatia, and Germany. These sequences appear to be far from *Radix balthica* species (Linnaeus, 1758).

Genus *Physella*

Although in the present in phylogenetic tree (Figure 3) and the existing databases, there are few species of *Physella acuta* from our region (the Balkans). Its position in the lineage of the species *Physella acuta* (Draparnaud, 1805) from samples originating from many countries like Greece, Italy and Austria is very much clear.

Genus *Galba*

Regarding this genus, there are no species from the Balkans in the presented phylogenetic tree and in the existing databases. Regarding the species *Galba truncatula* (O. F. Müller, 1774), there were considering similarities between the sequence of the species found in Belshi lakes and those from Germany and Spain, but also from countries far from our region such as Russia and Peru (Figure 3).

Genus *Gyraulus*

The individuals of genus *Gyraulus* were initially identified as *Gyraulus albus* (O.F. Muller, 1774), supported by existing reports of this species in Albania (Dhora and Welter-Schultes 1999; Fehér and

Erös 2009), but DNA sequences of it turned out to be similar by over 99.2% to those of *Gyraulus chinensis* (Dunker, 1848), (Figure 2), which is clearly distinguishable even in the phylogenetic tree (Figure 3). As aforementioned said, the species *Gyraulus albus*, stands far from the *Gyraulus chinensis* lineage and DNA sequences from our samples.

Fig. 2. Estimated similarity of DNA sequences between different *Gyraulus* sampled in the Belshi lakes and different *Gyraulus* according to the Barcode of Life Database (BOLD).

Genus *Musculium*

Regarding the genus *Musculium*, the species found in the Belshi lakes has been identified from the beginning as *Musculium lacustre* (O. F. Muller, 1774). Comparison of its DNA sequences has been made with the species found in Germany and Georgia. Although with few sequences to compare, from the analyzes performed there was no doubt about the position of the sequence of the species found in Belshi lakes as *Musculium lacustre* (Figure 3).

Genus *Dreissena*

Through identification keys, the species of this genus from Belshi lakes was initially identified as *Dreissena blanci*, referring to existing reports of this species in Albania (Dhora and Welter-Schultes, 1999; Dhora and Beqiraj, 2001; Dhora 2002; 2004; Beqiraj *et al*., 2006; Beqiraj *et al*., 2012; Beqiraj 2013; Peçulaj 2014).

Based on DNA analysis, the phylogenetic tree showed that this species found in Belshi lakes, is not only part of the clade *Dreissena carinata* (= *D. blanci, D. presbensis, D. stankovici*) from the *Dreissena* sequence analysed in other Balkan countries such as the Republic of

North Macedonia, Greece, Croatia, Montenegro, Hungary, and Mediterranean countries such as Italy but also in more distant countries such as Germany and the USA. Here, clarifying the name and the synonymy of this species would be necessary. There has been a recent revision with regard. Until a few years ago, the taxa *Dreissena stankovici* (Lvova and Starobogatov, 1982) and *Dreissena blanci* (Westerlund 1890) were considered as different species and as such they are still found in the database of Fauna Europaea (https://fauna-eu.org/). Following the recent revision, these two taxa, together with the *Dreissena blanci var. presbensis* (Kobelt 1915) are not accepted as species and have been replaced by *Dreissena carinata* (Dunker, 1853), as currently found in the MolluscaBase (https://www.molluscabase.org/) and WoRMS databases (http: //www.marinespecies .org).

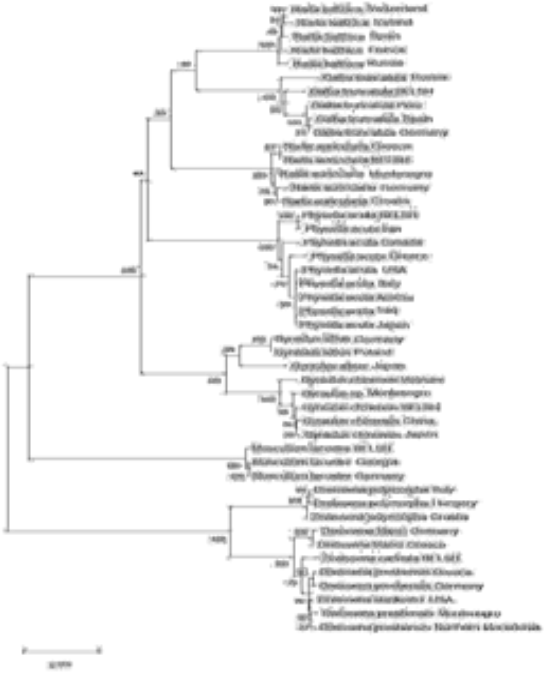

Fig. 3: Phylogenetic tree of live mollusk species found in Belshi lakes, compared to sequences of similar species from the region and larger.

DNA analyses carried out according to the methods of molecular genetics for the species *Gyraulus chinensis* and *Dreissena carinata*, and the revision of their taxonomy on a global scale, make the taxonomic review of the species from the genera *Gyraulus* and *Dreissena* previously reported in Albania from different habitats necessary.

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