COXI based phylogenetic analysis of Caucasian clade of European Troglocaris (Crustacea: Decapoda: Atyidae) with the suggestion of a new taxonomic group structure

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New genetic data on Caucasian troglobiotic shrimps collected from the territory of Russia, Abkhazia and Western Georgia are presented. Based on new genetic data on the marker region of subunit I of cytochrome oxidase of mitochondrial DNA (COXI mtDNA) of Caucasian species and other taxa of European cave shrimps (Troglocaris s.l.) from GenBank (NCBI) database, a new generic structure is presented. Based on a significant genetic divergence of COXI mtDNA subgenera of Troglocaris s.l., namely Troglocaris s.s., Xiphocaridinella and Speleocaridella, should be considered as separate genera while Troglocaris (Troglocaridella) hercegovinensis (Babić, 1922) is suggested to be transferred within Dinaric genus Speleocaridella as Speleocaridella hercegovinensis (Babić, 1922) comb. nov. Besides, Troglocaris bosnica shows a significant genetic difference (at the level of the separate genus) from the remaining representatives of the genus Troglocaris s.s. Moreover, p-distances (COXI) of about 17% are suggested for generic separation within European Troglocaris-related atyid shrimps and 5% for separation of cryptic species within Caucasian Xiphocaridinella. A list of all known taxa of Troglocaris-related atyid shrimps of the European part and a discussion of the general distribution of troglobdrid atyid shrimps in the Balkans and the Caucasus are presented. A new version of divergence events between Dinaric–Caucasian Troglocaris lineages (genera) based on new genetic data is suggested.

Keywords: phylogeny; COXI mtDNA; Xiphocaridinella; stygobiotic; stygobionts; shrimps; Caucasus

Introduction

Karst biocenoses due to their isolated location are especially rich in endemic species. It is very likely that each karst system has its own unique set of inhabiting species. One of the richest troglobiotic faunas in the world exists on the territory of the Southern Caucasus, followed by the cave and underground water fauna of the Balkan Peninsula. At the same time, the degree of study of the European hypogean communities obviously considerably exceeds that of the Caucasian ones. Troglobiotic higher crustaceans (Decapoda, Isopoda and Amphipoda) are the most important component of the hypogean cave ecosystems, sometimes forming them entirely. As a rule, in cave associations, crustaceans lead in number and diversity, forming the basis of the food pyramid of these communities. The relatively sparse data from the karst regions of the Caucasus indicate the extreme richness of the fauna of 37 troglobiotic crustaceans (about 50 endemic species in the Caucasus are known) (see review in Turbanov and Marin, 2015; Turbanov et al., 2016). At the moment, both the habitation of a larger number of species, and the wider distribution of this group in the Caucasus are assumed (e.g. Sadowsky, 1930; Birštein, 1939, 1948; Juzbaš'jan, 1940; Sendra and Reboleira, 2012; Marin and Sokolova, 2014; Marin, 2017; Turbanov et al., 2016). At the same time, subterranean fauna is characterized by a large number of endemics (local endemism), due to special ecological conditions and isolated location and low potential for dispersal (see Sket and Zakšek, 2009).

The European stygobiotic atyid shrimp genus Troglocaris Dormitzer, 1853 (Crustacea: Decapoda: Atyidae) is currently divided into 4 subgenera: Speleocaris Matjašič, 1956 (4 species), Troglocaridella Babić, 1922 (1 species), Xiphocaridinella Sadowsky, 1930 (6 species) and Troglocaris s. str. (4 valid species and 6 subspecies) (Zakšek et al., 2006, 2009; Sket and Zakšek, 2009; Marin and Sokolova, 2014; Marin and Turbanov, 2015; Marin 2017; Marin and Sinelnikov, 2017). The main morphological peculiarity which distinguishes Troglocaris from other European atyid shrimps, such as Atyaphrya de Brito Capello, 1867, Dugastella Bouvier, 1912, Gallocaris Sket et Zakšek, 2009 and Typhlatya Creaser, 1936 (Sket and Zakšek, 2009) is the length of the subapical and apical spines on the appendix masculina in males, which are significantly smaller than the diameter of the appendix masculina in all representatives of the genus Troglocaris (see key in Sket and Zakšek, 2009). At present, the division into subgenera does not satisfy modern morphological requirements, since most of the considered morphological features vary greatly even within the species of the same subgenus (see, for example, the length of the rostrum within the subgenus Xiphocaridinella (see Marin, 2017)) and such division is mainly supported by geographical distribution.
Troglocaris (Troglocaris) anopthalmus peridiastica Jugovic, Jažić, Prevorčnik and Sket, 2012
Troglocaris (Troglocaris) anopthalmus sonica Jugovic, Jažić, Prevorčnik and Sket, 2012
Troglocaris (Troglocaris) boxnica Sket et Zakšek, 2009
Troglocaris (Troglocaris) planinensis Birstein, 1948
Troglocaris (Troglocaris) schmidtii Dormitzer, 1853
Subgenus Spelaearcis Maťašič, 1956 – distributed exclusively in the Balkans
Troglocaris (Spelaearcis) kapelana Sket et Zakšek, 2009
Troglocaris (Spelaearcis) neglecta Sket et Zakšek, 2009
Troglocaris (Spelaearcis) prænescet Sket et Zakšek, 2009
Troglocaris (Spelaearcis) prenteri (Maťašič, 1956)
Subgenus Troglocaridella Břách, 1922 – distributed exclusively in the Balkans
Troglocaris (Troglocaridella) hervegovinensis (Břách, 1922)
Subgenus Xiphocaridinella Sadowsky, 1930 – exclusively Caucasian group
Troglocaris (Xiphocaridinella) ablasaki Birstein, 1939
Troglocaris (Xiphocaridinella) fagei Birstein, 1939
Troglocaris (Xiphocaridinella) jasbachjani Birstein, 1948
Troglocaris (Xiphocaridinella) kutasissianus (Sadowsky, 1930)
Troglocaris (Xiphocaridinella) osterloffi Juzbaš’jan, 1940
Troglocaris (Xiphocaridinella) kumistavi Marin, 2017

The active study of cave shrimps in the Caucasus began in the 1930s–1950s. At the moment, 6 species of the genus Troglocaris (subgenus Xiphocaridinella) are described from the caves from the territory of the southwestern part of Russia, Abkhazia and Western Georgia, which are relics of the freshwater fauna of the Sarmatian territory of the southwestern part of Russia, Abkhazia and Western Georgia, which are relics of the freshwater fauna of the Sarmatian or Pontine seas (basins) (Sadowsky, 1930; Birstein, 1939, 1948; Juzbaš’jan, 1940; Sendra and Reboleira, 2012; Marin and Sokolina, 2014; Marin, 2017). This group is assumed to be much more diverse in the Caucasus than is presently known and many species will be discovered in the nearest future. Shrimps of the genus Troglocaris have been recorded at a depth of more than 2,000 meters in the lower siphon of Knubera Cave in Abkhazia, which at the moment is considered the deepest in the world (Sendra and Reboleira, 2012). To mark species within crustacean genera, including the Troglocaris (Xiphocaridinella) species complex, it is useful to use the mitochondrial cytochrome c oxidase subunit I gene marker (COI), as one of the most informative markers for population and species-level genetic studies (Avise, 1993). At the same time, data on the Caucasian clade are not numerous and special criteria are needed for recognition of species and genera within this group of atyid cave shrimps.

Material and methods

This paper presents an attempt to evaluate the genetic diversity on the marker region of subunit I of cytochrome oxidase of mitochondrial DNA (COXI mtDNA) of troglobiotic shrimps of the genus Troglocaris and representatives of the genus from the Caucasian basins on available collections and new collected material. For the Caucasian clade freshly collected topotypic material was used, which allowed us to obtain genetic data that had not previously been reported for this genus – Troglocaris (Xiphocaridinella) kutasissian Sadowsky, 1930 (Takašitsae Cave, Western Georgia), Troglocaris (Xiphocaridinella) fagei Birstein, 1939 (New Athon Cave, Abkhazia), Troglocaris (Xiphocaridinella) jasbachjani Birstein, 1948 (Agura River, Sochi, Russia), Troglocaris (Xiphocaridinella) ablasaki Birstein, 1939 (Abrskila Cave, Abkhazia) and Troglocaris (Xiphocaridinella) osterloffi Juzbaš’jan, 1940 (the Lower Shakuran Cave, Abkhazia) and Troglocaris (Xiphocaridinella) kumistavi Marin, 2017 (Prometheus (Kumistavi) Cave, Western Georia). The name “Troglocaris birsteinii Muge, Zueva et Ershov, 2001”, proposed for the species reported from Otapa Cave, should be considered as nomen nudum (Franjevic et al., 2010). All collected material is deposited in the collection of the Laboratory of Ecology and Morphology of Marine Invertebrates of the Institute of Ecology and Evolution. A. N. Severtsov RAS (Moscow). Species names and a modern taxonomic position are given according to the international database of the World Register of Marine Species (WoRMS) and the Marine Species Identification Portal.

To resolve the taxonomy of cryptic diversity of species complex fragments of the mitochondrial gene coding for cytochrome oxidase c subunit I (COI) as one of the most popular markers for population genetic studies (Avise, 1994, 2000) were amplified and compared. Total genomic DNA was extracted from pleopods or abdominal muscle tissue using the innuPREP DNA Micro Kit (AnalytikJena, Germany) following the manufacturer’s protocol. The mitochondrial marker COI was amplified with the help of «PCR Core» (Iisona Lab., Moscow) using the universal primers LCO1490 (5'-ggtaaccaaatcaataagatgtagt-3') and HC02198 (5'-taaactctagggcaataataaatac-3') (Felmer et al., 1994). PCR products were performed on amplification «Tereck DNA Technology» under the following conditions: initial denaturation at 94 °C for 2 min followed by 40 cycles of 95 °C for 2 min, 59°C for 45 s, and 72 °C for 1.5 min, followed by chain extension at 72 °C for 7 min. The volume of 20 μl of reaction mixture contained 5 μl of total DNA, 10 μl of PCR Dilaent and 2.5 μl of each primer. Synthesis of the full-length fragment was performed at an annealing primer temperature 59 °C during 45 seconds. The amplification products were separated by using gel electrophoresis of nucleic acids on a 2% agarose gel in 1xTBE, and then stained and visualized with 0.003% EtBr using imaging UV software. The resulting PCR products were sequenced in both forward and reverse direction on the basis of "EuroGen" (Moscow, Russia). Uniformity of sequences obtained was performed using the program BioEdit v. 5.0.9. The resulting markers of COXI gene of mtDNA with 598 bp long sequence were used for further phylogenetic analysis. The received nucleotide alignments of COI gene were used to construct the phylogenetic relations (tree) in MEGA 7.0 using k-nearest neighbor’s algorithm (k-NN, Neighbor-Joining method) and Maximum-likelihood algorithm. Uncorrected pairwise genetic distances (p-distance) were calculated based on COI sequences using MEGA 7.0. Data on “molecular clocks” are used according to Zakšek et al. (2006) calculated for Troglocaris s.s. as well as Knowlton et al. (1993) and Knowlton & Weigt (1998) suggested for shrimps of the genus Alpheus (Crustacea: Decapoda: Alphidae) as sequence divergence rate of about 1.4–2.4% per MYA for COXI gene marker.

Results

All known Caucasian Xiphocaridinella species are strictly restricted to certain karst cave ecosystems showing significant genetic divergence between known species (Table 1), which is usually not less than 5%. At the same time, the interspecies genetic variability within populations is very low (Table 2). Relatively high variability within Troglocaris (Xiphocaridinella) fagei is explained by mixture of three separate populations from the closely spaced New Athon, Habu and Mchishita caves, while variability within population from each of caves is similar to other species, being about 0.003 substitutions per 100 nucleotides.

Table 1
Uncorrected pairwise genetic (COXI) distances between known Caucasian species of subgenus Xiphocaridinella (asterisk indicates minimum values of p-distances)

<table>
<thead>
<tr>
<th>Species</th>
<th>Kutasissian</th>
<th>Kumistavi</th>
<th>Ablasaki</th>
<th>Osterloffi</th>
<th>Fagei</th>
</tr>
</thead>
<tbody>
<tr>
<td>X. kumasnavi</td>
<td>0.058 ± 0.010*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X. ablasaki</td>
<td>0.112 ± 0.014</td>
<td>0.099 ± 0.013</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X. osterloffi</td>
<td>0.120 ± 0.014</td>
<td>0.108 ± 0.013</td>
<td>0.103 ± 0.014</td>
<td></td>
<td></td>
</tr>
<tr>
<td>X. fagei</td>
<td>0.120 ± 0.014</td>
<td>0.107 ± 0.014</td>
<td>0.106 ± 0.014</td>
<td>0.100 ± 0.013</td>
<td></td>
</tr>
<tr>
<td>X. jasbachjani</td>
<td>0.109 ± 0.014</td>
<td>0.104 ± 0.015</td>
<td>0.104 ± 0.015</td>
<td>0.108 ± 0.014</td>
<td>0.064 ± 0.010*</td>
</tr>
</tbody>
</table>
Uncorrected pairwise genetic (COXI) distances within studied populations of known Caucasian species of subgenus Xiphocaridinella (asterisk indicates minimum values of p-distances)

<table>
<thead>
<tr>
<th>Species</th>
<th>Troglocaris</th>
<th>Troglocaris bosnica</th>
<th>Speleocaris</th>
<th>Troglocaridella</th>
<th>Xiphocaridella</th>
<th>Atyaephyra</th>
<th>Dogatella</th>
<th>Galocaris</th>
</tr>
</thead>
<tbody>
<tr>
<td>Troglocaris bosnica</td>
<td>0.153 ± 0.024*</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Speleocaris</td>
<td>0.217 ± 0.024</td>
<td>0.222 ± 0.024</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Troglocaridella</td>
<td>0.243 ± 0.024</td>
<td>0.241 ± 0.025</td>
<td>0.169 ± 0.024*</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Xiphocaridella</td>
<td>0.214 ± 0.024</td>
<td>0.225 ± 0.024</td>
<td>0.189 ± 0.025*</td>
<td>0.207 ± 0.024</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Atyaephyra</td>
<td>0.265 ± 0.027</td>
<td>0.261 ± 0.029</td>
<td>0.295 ± 0.027</td>
<td>0.322 ± 0.031</td>
<td>0.275 ± 0.020</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Dogatella</td>
<td>0.264 ± 0.022</td>
<td>0.279 ± 0.026</td>
<td>0.287 ± 0.024</td>
<td>0.308 ± 0.026</td>
<td>0.296 ± 0.017</td>
<td>0.275 ± 0.018</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Galocaris</td>
<td>0.200 ± 0.025*</td>
<td>0.213 ± 0.023</td>
<td>0.276 ± 0.025</td>
<td>0.295 ± 0.026</td>
<td>0.255 ± 0.020</td>
<td>0.257 ± 0.025</td>
<td>0.260 ± 0.021</td>
<td>–</td>
</tr>
<tr>
<td>Typhlatya</td>
<td>0.275 ± 0.023</td>
<td>0.264 ± 0.020</td>
<td>0.260 ± 0.023</td>
<td>0.290 ± 0.023</td>
<td>0.289 ± 0.018</td>
<td>0.255 ± 0.023</td>
<td>0.288 ± 0.019</td>
<td>0.235 ± 0.016</td>
</tr>
</tbody>
</table>

Uncorrected pairwise genetic (COXI) distances (p-distances) between subgenera of Troglocaris s.l. (Table 3; Fig. 1) show well supported divergences with p-distances not less than 17–20%. The division of the subgenus Speleocaris and Troglocaridella for the Dinaric group is not justified on the basis of available molecular data. The genetic subdivision between subgenera of Troglocaris s.l. is similar to generic level divergence between Galocaris and Troglocaris s.s. (Table 3). The data obtained clearly support the monophyly of Xiphocaridinella and Troglocaris s.s. while Speleocaris represents a paraphyletic taxon (Fig. 1).

**Discussion**

The genetic subdivision between subgenera of Troglocaris s.l. is similar to generic level divergence between Galocaris and Troglocaris s.s. (Table 3). Such phylogenetic relations within Troglocaris are also supported by Sket and Zakšek (2009) based on sequences of cytochrome oxidase subunit 1 (COI) + 16S rDNA. Thus, subgenera of the genus Troglocaris s.l. should be considered as separate genera. It is possible to use p-distances calculated for COXI gene marker larger than 17% for generic separation within European Troglocaris-related atyid shrimps and 5% for separation of cryptic species within Caucasian Xiphocaridinella, which is rather similar to criteria suggested in other groups of decapod crustaceans and invertebrates (see Knowlton et al., 1993; Knowlton and Weigt, 1998; Hebert et al. 2003; Sites and Marshall 2004; Zakšek et al., 2006, 2009; Lefèbure et al. 2006; Lushai et al., 2003). Besides, Troglocaris bosnica shows a significant genetic difference (at the level of the separate genus) from the remaining representatives of the genus Troglocaris s.s. (Table 3).

At the same time, the monotypic genus Troglocaridella is separated from the representatives of the genus Speleocaris mostly based on the presence of supraorbital spines on the carapace in T. hercegovinensis vs. their absence in Speleocaris (after Sket and Zakšek, 2009). Such morphological features are variable within genera (Skel and Zakšek, 2009; Marin and Sokolova, 2014) while generic data (Fig. 1; Table 3) do not allow clear separation of T. hercegovinensis from other representatives of the genus Speleocaris and it is presently suggested to synonymize genera Speleocaris and Troglocaridella of the Dinaric group and further consider the species as Speleocaris hercegovinensis (Babić, 1922) comb. nov.

**Table 2**

Uncorrected pairwise genetic (COXI) distances within studied populations of known Caucasian species of subgenus Xiphocaridinella (asterisk indicates minimum values of p-distances)

<table>
<thead>
<tr>
<th>Species</th>
<th>Uncorrected pairwise genetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xiphocaridinella fagei</td>
<td>0.014 ± 0.003*</td>
</tr>
<tr>
<td>X. kautaisiana</td>
<td>0.0006 ± 0.0006*</td>
</tr>
<tr>
<td>X. abakiri</td>
<td>0.003 ± 0.001</td>
</tr>
<tr>
<td>X. jasbachiani</td>
<td>0.002 ± 0.001</td>
</tr>
<tr>
<td>X. osterloffi</td>
<td>0.003 ± 0.0015</td>
</tr>
<tr>
<td>X. kumistavi</td>
<td>0.0009 ± 0.0008*</td>
</tr>
</tbody>
</table>

**Fig. 1.** The evolutionary tree of Troglocaris s.1. based on COXI gene marker obtained using the Maximum-Likelihood method based on the Kimura 2-parameter model.
Similar morphological variability in the armature of the rostrum in the carapace is known from the Caucasian Xiphocaridinella currently including 6 valid species known from the Russian part of the Caucasus (Krasnodar Territory), Abkhazia and Western Georgia. Xiphocaridinella jusebachjiani known exclusively from the Agura River (Sochi, Russia) shows an extremely short (reduced) unarmed rostrum and the absence of supraorbital or suborbital teeth. The Agura River (Sochi, Russia) shows an extremely short (reduced) rostrum of Troglocaris s. str. (Dormitzer, 1853), taxonomic revision and description of new taxa after phylogenetic and morphometric studies. Zootaxa, 3421, 1–31. The hypothesis of the recent split (about 6–11 million years ago) between the Caucasian and Dinaric cave shrimps of the Parathethis relict (Zakšek et al., 2006) supports the idea of a closer relationship between the subgenus Spelaecaris and Xiphocaridinella than with the Dinaric group, which we also believe is correct on the basis of our research. Besides, it is possible to suggest two separate divergence events between within Dinaric–Caucasian Troglocaris lineages (genera), which occurred about 9–13MYA (ancestors of Troglocaris separated from Spelaecaris–Xiphocaridinella) and about 8–13MYA when ancestors of Xiphocaridinella separated from Troglocaris vs single split about 6–11MYA suggested by Zakšek et al. (2006).

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References


