Mitochondrial phylogeny of the *Darevskia saxicola* complex: two highly deviant evolutionary lineages from the easternmost part of the range

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The rock lizards of the *Darevskia saxicola* complex are found exclusively in the west of the Greater Caucasus and in southern Crimea. The earliest split within this group occurred between *D. saxicola* from the northern and *D. brauneri* from the southern slopes of the Greater Caucasus, followed by the split between *D. brauneri* and the Crimean *D. lindholmi*, and the expansion of *D. saxicola* to the westernmost slopes of the Greater Caucasus. We collected nominal *D. brauneri* from the two easternmost populations of the species range: the valleys of the Tekhuri and Enguri rivers in Georgia. Analysis of mitochondrial DNA showed that the lizards from both valleys are deeply differentiated from each other and from previously characterised *D. brauneri*. Lizards from Tekhuri keep a basal position within the clade *D. saxicola* (excluding *D. praticola*), and lizards from Enguri are closer matrilineally to the northern Caucasian *D. saxicola* than to either of the populations of nominal *D. brauneri*. Tekhuri lizards have broader heads and more small scales between the inner and outer rows of supraoculars than the other populations of the group. We suggest that the taxonomy of the group requires revision, considering the multiple deeply divergent mitochondrial lineages and introgressive gene flow between the continental populations of nominal *D. saxicola* and *D. brauneri*. The *D. saxicola* complex in the Caucasus resembles a “ring species” arrangement as described for other taxa and mountain regions.

**Key words:** Caucasus, cytochrome b, *Darevskia*, glacial refugia, mitochondrial DNA, scalation, Species Nova

**INTRODUCTION**

Caucasian rock lizards (*Darevskia*) have been assumed to consist of three genetically distinct clades (Murphy et al. 2000). The clades “rudis” and “caucasica” are widespread throughout the Caucasus except its northwestern part as well as parts of Anatolia, and the clade “saxicola” is limited to the western part of the Greater Caucasus and southern Crimea (Murphy et al., 2000; Tarkhnishvili, 2012). This Western Caucasian clade most likely split from the reminder of the genus *Darevskia* approximately 6 million years ago, and is perhaps associated with the ancient Caucasian Island, which was isolated from the Asia Minor until early Pliocene (Popov et al., 2004; Tarkhnishvili, 2012, 2014). The clade has five to six nominal species, including the polytypic *D. brauneri* and *D. saxicola* (MacCulloch et al., 2000; Murphy et al., 2000; Ciobanu et al., 2003; Milto et al. 2010; Doronin, 2011; Tuniyev & Tuniyev, 2012; Doronin et al., 2013). *Darevskia praticola*, the most distant from the other species of the clade (Murphy et al., 2000), has a range between the southern Caspian Sea and the Balkans (Agasyan et al., 2009). *Darevskia alpina* occurs at altitudes above 1,650 m a.s.l. in the Greater Caucasus west of the valley of the river Baksan (Darevsky, 1967; Tuniyev et al., 2009a). *Darevskia lindholmi* is found in southern Crimea (Darevsky, 1967), *D. saxicola* occurs west of the valley of river Chegem north of the Greater Caucasus Range, below 1,500 m a.s.l. (Darevsky, 1967; Tuniyev et al., 2009b), and *D. brauneri* occurs at the southern slopes of the Greater Caucasus between the river Tekhuri valley in Georgia and Anapa in Russia, and at the northern slopes west of the river Kuban valley (Darevsky, 1967; MacCulloch et al., 2000). The genetically distinct *D. [brauneri] szczerbaki* (a separate species according to Doronin et al., 2013) is found at the westernmost foothills of the Greater Caucasus. The subspecies *D. brauneri darevskii* from the north and the west of the river Psou valley was recently synonymised with the nominal form, and *D. b. miusserica* has been recently described from a small area in the north-westernmost Georgia (Doronin et al., 2013) (Fig. 1).

Except for *D. praticola*, neither of these species has diagnostic morphological characters that could easily distinguish them from their closest relatives. *Darevskia alpina* is morphologically intermediate between *D. brauneri* and *D. caucasica*, a representative of another clade of *Darevskia*. The differences among the remaining...
species of the *D. saxicola* complex are fragile. On average, *D. saxicola* has larger scales in temporal areas than *D. brauneri* or *D. lindholmi*, but this character is not diagnostic. All other characters described as characteristic for the listed forms (Darevsky, 1967, Fig. 2) overlap among *D. brauneri*, *D. lindholmi*, and *D. saxicola*, and only the non-overlapping ranges can safely distinguish among these forms. Adults of the subspecies *D. [brauneri] szczerbaki* and *D. b. darevskii* are larger than those of *D. b. brauneri* (Darevsky, 1967).

Genetic analyses showed controversial results. Murphy et al. (2000) suggested *D. praticola* and *D. alpina* to be the most basal species in the clade, although a discrepancy between the mitochondrial and allozyme phylogenies may suggest hybrid origin of the latter. The resolution of genetic relations among the reminder of the nominal taxa (*D. saxicola*, *D. lindholmi* and *D. brauneri*) is low. According to mitochondrial data, *D. lindholmi* and *D. brauneri* are matrilineally closer to each other than to *D. saxicola* (Murphy et al., 2000; Doronin et al., 2013). This suggests that the first split within this group occurred between the northern and the southern slopes of the Greater Caucasus, and only later between the Crimean and the Caucasian populations. However, mitochondrial sequences of *D. [brauneri] szczerbaki* from the south westernmost Greater Caucasus are closest to the northern Caucasian *D. saxicola*. Because allozyme alleles (MacCulloch et al., 2000) and morphology (Darevsky, 1967) of *D. [brauneri] szczerbaki* are close to those of the other populations of *D. brauneri*, a hybrid origin of *D. [b.] szczerbaki* with *D. saxicola* as the matrilineal and *D. brauneri* as the patrilineal ancestor is possible.

The mitochondrial lineage of Crimean *D. lindholmi* is effectively isolated by the Kerch Strait, although it has split from the reminder of the group later than the Caucasian lineages from each other. In contrary, gene flow between nominal *D. saxicola* and *D. brauneri* may exist. Hybridisation events may happen millions of years after the split of the lineages and does not necessarily cause assimilation (e.g., Frost & Hillis, 1990; Mallet, 2010). It was recently shown that a similar pattern is observed in another clade of *Darevskia* (*D. "rudis"*), where *D. valentini* became recently separated from the closely related *D. rudis* and *D. portschinskii*, whereas the more ancient *D.

**Table 1.** The comparative variation scale of several traits is given for the individuals from Tekhuri and Enguri valleys (our data), *D. brauneri*, *D. saxicola* and *D. lindholmi* (Darevsky, 1967; our data). fp – femoral pores; np – the number of large preanal scales; HL/HW – length of pileus divided by maximum width of pileus; nsc – the number of small scales between the inner and outer rows of supraoculars.

<table>
<thead>
<tr>
<th>Character</th>
<th>Tekhuri Valley</th>
<th>Enguri Valley</th>
<th>D. saxicola</th>
<th>D. brauneri</th>
<th>D. lindholmi</th>
</tr>
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<tbody>
<tr>
<td>fp</td>
<td>18–21 (19.4)</td>
<td>17–21 (18.1)</td>
<td>13–20 (17.1)</td>
<td>15–22 (18.5)</td>
<td>16–23 (19.1)</td>
</tr>
<tr>
<td>np</td>
<td>2–3 (2.40)</td>
<td>1–2 (1.75)</td>
<td>1–3 (1.9)</td>
<td>2–3 (2.40)</td>
<td>1–2</td>
</tr>
<tr>
<td>HL/HW</td>
<td>1.52–1.91 (1.7)</td>
<td>1.82–2.00 (1.89)</td>
<td>1.82–2.0 (1.89)</td>
<td>1.73–1.98 (1.90)</td>
<td>2.0–2.08 (2.05)</td>
</tr>
<tr>
<td>nsc</td>
<td>12–14 (12.63)</td>
<td>13 (13)</td>
<td>3–15 (9.4)</td>
<td>8–13 (10.62)</td>
<td>7–21 (12.4)</td>
</tr>
</tbody>
</table>
portschinskii lineage is intensively hybridising with *D. rudis* (Tarkhnishvili et al., 2013).

Formal taxonomy relies on expert opinion, and the information on gene flow between nominal species rarely leads to clumping of once separated taxa. Given the current state of knowledge one can suggest that there are sufficient grounds to keep different species names for *D. saxicola*, *D. brauneri*, and *D. lindholmi*, although *D. [b. ] szczerbaki* is a hybrid population between *D. saxicola* and *D. brauneri* rather than a separate monophyletic lineage. An additional challenge remains for final conclusion on the group's taxonomy. None of the Georgian populations of *D. brauneri* occurring between the rivers Gumista and Tekhuri (south-eastern part of the species range), were ever studied by means of molecular genetics, and the analysis of these populations is important for validating monophyletic matrilineal origin of *D. brauneri*. We therefore aimed to investigate molecular variation as a proxy for assessing the real taxonomic status of nominal *D. brauneri* from this area by studying their mitochondrial DNA variation. Another issue was the analysis of matrilineal relationships of these easternmost populations, in a broader context of *Darevskia saxicola* group evolution.

**METHODS**

We surveyed four valleys in Western Georgia (east to west): Abasha, Tekhuri, Khobi and Enguri. All these valleys originate from rivers either of southern slope of the Greater Caucasus (Enguri), or Samegrelo Range, which is a southern branch of the Greater Caucasus (Fig. 1). The Tekhuri river is reported as the easternmost point of the distribution for *D. brauneri*, and the Enguri Valley is also reported for *D. brauneri* locations (Darevsky, 1967). The neighbouring valleys were surveyed for specifying the easternmost distribution of *D. brauneri*. Five species of *Darevskia* were recorded within the study area: *D. brauneri*, *D. mixta*, *D. caucasica*, *D. rudis* and *D. derjugini*. The specimens of *D. brauneri* were distinguished from the coexist *D. mixta*, *D. caucasica*, and *D. derjugini* by (i) the presence of multiple scales between the central temporal and tympanal scale (Fig. 2); (ii) a higher number of femoral pores (18-21), compared with *D. caucasica*, and more scales across the back; (iii) a more robust body and head than *D. caucasica* or *D. mixta*, but less robust than *D. rudis* from the same geographic area, and a dorsal pattern (salad-green background, usually with contrasting black spots) different from the other species. Comparison of obtained sequences with already available mitochondrial sequences of coexisting species did not suggest misidentification (results not shown).

We photographed each of 6 males and 5 females collected from the valley of the river Tekhuri, ca. 400–550 m a.s.l., and 2 males and 6 females collected from the valley of the river Enguri (427–1363 m a.s.l.) from five different perspectives, as described in Tarkhnishvili et al. (2013). We scored four morphological characters (fp – the number of femoral pores; np – the number of large preanal scales; HL/HW – length of pileus divided by maximum width of pileus; nsc – the number of small scales between the inner and outer rows of supraoculars) for each of these individuals, and compared the obtained figures with those described by Darevsky (1967) for *D. brauneri*, *D. saxicola* and *D. lindholmi*.

We collected and stored tail-tips in 95% ethyl alcohol for DNA extraction and molecular analysis for 7 individuals from the valley of the river Tekhuri and 6 individuals from the valley of the river Enguri. DNA was extracted from tissue samples using a Qiagen tissue kit (QiAamp DNA, 2007, Qiagen, Hilden, Germany) according to the manufacturer's instructions. To check for contamination and pipetting error, a negative control (reagents only) was used for each extraction procedure and PCR to avoid cross-contamination problems. A 714 bp fragment of the mitochondrial gene cytochrome b was selected for the analysis (Murphy et al., 2000; Tarkhnishvili et al., 2013) and PCR-amplified using two different primer pairs in order to increase the total length of the sequenced fragment (H15915 – L15369 and H15488 – L15153, Fu, 2000; Murphy et al., 2000). PCRs were carried out in a 21 µl total volume, with 2–4 µl template DNA, 1 U of Promega Taq polymerase, 5x Promega buffer, 1 µM of MgCl2, 0.1 µM of each dNTP, and primer concentrations at 0.1 µM. The PCR profile included initial denaturation at 93°C for 3 min, followed by 30 cycles at 93 °C for 1 min, 53 °C for 1 min, and 69 °C for 2 min and 70°C for 10 min for final extension. The amplicons were sequenced on an ABI 3130 sequencer in both directions to ensure sequence accuracy. Sequences were edited using SEQUACE 2.5 (Applied Biosystems Inc. Foster City, CA, USA) and unique sequences (haplotypes) were deposited in GenBank (accession no KR265093, KR265094, KR265102-KR265106 for Enguri Valley, KR265095-KR265101 for Tekhuri Valley samples).

We analysed our sequences along with the sequences of *D. brauneri* and closely related species of *Darevskia* deposited in GenBank (*D. lindholmi*: accession numbers JX041604-JX04613, AF206177; *D. saxicola*: AF206180; *D. brauneri brauneri*, *D. b. darevskii*, *D. b. mjuesserii*: JX041614-JX041620, AF206178, AF206179, AF206181; *D. b. szczerbaki*: JX041621-JX041625; *D. alpina*: AF206175; *D. praticola*: U88612; *D. caucasica*: U88616; *D. mixta*: AF147796; *D. parvula*: U88609; *D. portschinskii*: U88615; *D. rudis*: U88614; Fu et al., 1997; Murphy et al., 2000; Doronin et al., 2013). Prior to further analysis, we tested whether our sequenced fragments or GenBank sequences represent pseudogenes (Zhang & Hewitt, 1996; 2003; Dubey et al., 2009); this was done by investigating whether premature stop-codons occur in the obtained sequences, and whether synonymous are several times more common than non-synonymous substitutions in all branches of the tree and the branches comprised of our sequences (Dubey et al., 2009).

For phylogenetic analysis, 682 bp sequences from 47 individuals each (our 13 samples, 28 sequences of *D. saxicola* group except *D. praticola* derived from Genbank, and six outgroups) were aligned using BioEdit v.7.1.3.0 software (Hall, 1999). Following Hasegawa et al. (1985) and using the software MEGA v.6.06 (Tamura et al., 2013), we found the best model of nucleotide substitution using the Bayesian Information Criterion (BIC) as HKY+G (Nei &
Based on this model we reconstructed a maximum likelihood phylogenetic tree using the Nearest-Neighbour-Interchange ML Heuristic Method, and tested phylogeny using bootstrap method (1000 replicates). We then validated the topology, inferred from the maximum likelihood method, applying Bayesian phylogenetic analysis with software BEAST v.1.5.1 (Drummond & Rambaut, 2007). Posterior distributions of parameters of the Bayesian tree were approximated using Markov chain Monte-Carlo (MCMC) with chain length set at 100,000,000 to provide sufficient sample size for each parameter (i.e. effective sample size (ESS)>100), including an ESS for the posterior probability of 2047. The null hypothesis of the equal evolutionary rate throughout the maximum likelihood tree was rejected at a 5% significance level. Hence, we did not attempt to estimate the exact time of split between the branches and the confidence intervals. However, we inferred the approximate split time between some clades using minimum and maximum substitution rates for cytochrome b 1.5 and 2.5% per myr with 2% as an average value, based on estimations by Crochet et al. (2004) for Iberolacerta lizards which are close relatives of Darevskia (Tarkhnishvili, 2012).

In order to compare genetic distances between the ingroup haplotypes (D. saxicola group excluding D. praticola) we (i) calculated a distance matrix between the haplotypes based on numbers of base differences per site between sequences using MEGA v.6.06, and (ii) inferred the haplogroup network using a median-joining algorithm (Bandelt et al., 1999) with the software NETWORK v.4.6.

### RESULTS AND DISCUSSION

The tests conducted for our sequences and the GenBank sequences of the D. saxicola group showed no stop codon. The transition/transversion ratio was 6.24. The mean difference between the non-synonymous and synonymous substitutions was 0.054 for the entire dataset, 0.047 for the Tekhuri Valley specimens and 0.058 for Enguri Valley specimens, similar to that of other individual branches of the inferred phylogenetic tree. Consequently, there is no indication that any sequences used in the analysis are pseudogenes.

All sequences of our samples build a monophyletic matrilineal clade together with the sequences of D. saxicola group excluding D. praticola.

#### Table 2. The average number of base differences per site between sequences of the ingroup taxa within D. saxicola clade excluding D. praticola.

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<tbody>
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<td>D. b. brauneri</td>
<td>0.026</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>D. b. darevskii</td>
<td>0.028</td>
<td>0.036</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>D. b. myussiarka</td>
<td>0.041</td>
<td>0.039</td>
<td>0.046</td>
<td></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>D. alpina</td>
<td>0.050</td>
<td>0.048</td>
<td>0.048</td>
<td>0.050</td>
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<tr>
<td>D. saxicola</td>
<td>0.042</td>
<td>0.040</td>
<td>0.040</td>
<td>0.037</td>
<td>0.044</td>
<td></td>
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<tr>
<td>D. [b.] szczerbaki</td>
<td>0.043</td>
<td>0.040</td>
<td>0.035</td>
<td>0.034</td>
<td>0.043</td>
<td>0.021</td>
<td></td>
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<tr>
<td>Enguri Valley</td>
<td>0.049</td>
<td>0.046</td>
<td>0.048</td>
<td>0.046</td>
<td>0.049</td>
<td>0.026</td>
<td>0.036</td>
<td></td>
</tr>
<tr>
<td>Tekhuri Valley</td>
<td>0.051</td>
<td>0.046</td>
<td>0.045</td>
<td>0.046</td>
<td>0.047</td>
<td>0.030</td>
<td>0.034</td>
<td>0.028</td>
</tr>
</tbody>
</table>

In order to compare genetic distances between the ingroup haplotypes (D. saxicola group excluding D. praticola) we (i) calculated a distance matrix between the haplotypes based on numbers of base differences per site between sequences using MEGA v.6.06, and (ii) inferred the haplogroup network using a median-joining algorithm (Bandelt et al., 1999) with the software NETWORK v.4.6.

**Fig. 2.** Dorsal side (left), anal area (middle left), temporal area (middle right) and pileus (right) of males of the population from the river Tekhuri Valley (TV), river Enguri Valley (EV), Sochi (D. brauneri, Db), upper currents of river Kuban’ (D. saxicola, Ds), Yalta, Crimea (D. lindholmi, Dl).

Interspecific differences are minor. D. saxicola has larger central temporal scales and, usually, larger scales beyond the preanal scales than the other forms. The lizards from the river Tekhuri Valley have broader heads than the others and smaller scales in temporal area.
Mitochondrial phylogeny of Darevskia saxicola brauneri, D. [b.] szczerbaki, D. saxicola, D. lindholmi and D. alpina downloaded from Genbank. Darevskia praticola was the closest outgroup species for this clade. The ingroup topology for the clade (D. saxicola clade without D. praticola) was slightly varying depending on the method applied (maximum likelihood vs Bayesian inference, Fig. 3). All analyses reveal the presence of the following matrilineal clades (Fig. 3): (1) D. lindholmi + D. brauneri (including D. b. brauneri, D. b. darevskii, and D. b. myusserica but not D. [b.] szczerbaki) (bootstrap support value, BV 63, PP 0.77); (2) all these forms + D. alpina (BV 56, PP 0.98); (3) D. [b.] szczerbaki + D. saxicola (BV 71, PP 0.98).

Fig. 3. Tree topology for the studied sequences (Fu et al., 1996; Doronin et al., 2013; our data), inferred with ML method (model HKY + G, equal branch length hypothesis rejected). Dashed lines indicate conflicting topologies inferred from Bayesian algorithm. Bootstrap support values are shown near the nodes; in parenthesis – posterior probabilities of Bayesian inference.

Fig. 4. Median-Joining network of haplotypes of clade D. saxicola (Fu et al., 1996; Doronin et al., 2013; our data). Branch length proportional to the number of substitutions.
D. saxicola, D. lindholmi, D. brauneri

Evolutionary lineages and taxonomic implications

Excluding the basal \textit{D. praticola}, there are three almost equidistant mitochondrial lineages within the \textit{D. saxicola} clade (Murphy et al., 2000). One clade is likely derived from the southern slopes of the western part of the Greater Caucasus, and is currently found in the nominal \textit{D. brauneri brauneri}, \textit{D. b. darevskii}, \textit{D. b. miussserica}, \textit{D. lindholmi}, and in the hybrid form \textit{D. alpina}. The second lineage has probably originated at the northern slopes of the western Greater Caucasus and later expanded west-and southwards to the Greater Caucasus range, where the hybrid zone with the former lineage was formed. Nominal \textit{D. saxicola} and lizards usually indicated as \textit{D. [b.] szczerbaki} from the westernmost slopes of the Greater Caucasus share the same matrilineal haplogroup, probably related to a smaller lineage present in the lizards from the valley of the river Enguri. The third lineage was formed in the easternmost part of the group’s range, at the southern slopes of Samegrelo Range, a southern branch of the Greater Caucasus.

This pattern challenges the existing taxonomy of the group. There are two possible taxonomic solutions. It is possible to consider the nominal \textit{D. brauneri}, \textit{D. saxicola}, \textit{D. lindholmi} and specimens described here as subspecies within the polytypic \textit{D. saxicola} (Eversmann, 1834). However, taxa differentiated to the extent as we observe it for \textit{D. saxicola} and \textit{D. brauneri} are commonly considered as individual species, even if connected with a zone of introgression in the westernmost Greater Caucasus; we could further accept that \textit{D. lindholmi} is a separate species. The populations from the rivers Enguri and Tekhuri should not be considered to be part of \textit{D. brauneri}, because matrilineally they are more distant from this species than \textit{D. lindholmi}.

It is possible that the evolutionary pattern within the \textit{D. saxicola} clade (excluding \textit{D. praticola}) is close to what has been described as “ring species” (Irwin et al., 2001). The original differentiation of evolutionary lineages likely took place in the eastern part of their current ranges, where high mountains of the central-western Greater Caucasus ensured effective isolation between the populations, especially during glacial waves. This could have triggered the split among the northern, eastern and western mitochondrial lineages. The western lineage expanded to Crimea and, after isolation, developed into the geographically isolated species \textit{D. brauneri} and \textit{D. lindholmi}. The northern lineage was split between the northern (\textit{D. saxicola}) and southern (lizards from Enguri Valley) slopes of the Greater Caucasus, and the northern Caucasian branch later expanded westwards to the Taman Peninsula and Black Sea. The eastern lineage remained confined to the valleys of the Samegrelo Range. However, gene flow among the lineages appears likely, and neither of the nominal taxa except \textit{D. lindholmi}, is fully isolated from the others by geographic barriers. To estimate the degree of genetic isolation between populations, more data from recombinant loci would be available.

Morphological description of the specimens from the Tekhuri and Enguri river valleys

Morphologically, variations among the specimens from the Enguri river valley fall within the described variations of \textit{D. brauneri} (Darevsky, 1967; Table 1). The studied individuals from the Tekhuri river valley show some distinct morphological features. They have a broader head in the basal area than \textit{D. saxicola}, \textit{D. lindholmi}, and the subspecies of \textit{D. brauneri} described so far: 1.52–1.91 (1.7 in average, \(n=11\)) vs 1.73–2.08 (1.92, \(n=17\)). They have smaller scales in the temporal area than in \textit{D. saxicola}, and never have large central temporal scales. Different from \textit{D. brauneri} from the Russia’s Black Sea Coast and studied specimens of \textit{D. saxicola} and \textit{D. lindholmi}, males of lizards from the river Tekhuri valley have larger and contrasting black spots of irregular shape along the back, or the spotted pattern completely absent. Different from \textit{D. saxicola} from the river Enguri Valley, the belly of the males within reproductive season is yellow (as opposed to orange-yellow), and white outside the breeding season (see also Table 1).
desirable. Taken together, our findings of splits between the eastern, northern, and southern lineages contribute to our knowledge of cryptic mini-refugia in the western part of the Caucasus (Tarkhnishvili et al., 2000; 2014).

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REFERENCES


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