

Phylogeography and morphological variation in a narrowly distributed Caucasian rock lizard, *Darevskia mixta*

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Abstract. The Caucasian rock lizard *Darevskia mixta* was sampled and studied from throughout its range, using mitochondrial cytochrome *b* sequences and scalation. The populations of the Greater and the Lesser Caucasus are reciprocally monophyletic matrilineally, and the respective lineages have been separated since the mid-Pleistocene. The lizards from the Greater Caucasus commonly have an unpaired preanal scale, whereas the lizards from the Lesser Caucasus have an additional scale behind the central temporal and subdivided interparietal scale more commonly than those from the Greater Caucasus. The Lesser Caucasus populations are further subdivided into two geographically distinct matrilineages, and are more diverse genetically and morphologically than the Greater Caucasus populations. The central part of the Lesser Caucasus is suggested to be the ancestral area for the entire *D. mixta* lineage. Successive Pleistocene periods of glaciation appear to be responsible both for the isolation of *D. mixta* from its common relatives, and for subdivision within *D. mixta*. Presence of *D. mixta* in NE Turkey is challenged.

Keywords: Caucasus, endemics, glacial refugia, Ice Age, Lacertidae, mitochondrial phylogeny, scalation.

Introduction

Darevskia (Caucasian rock lizards; Arribas, 1997) is a monophyletic species group mainly from the Caucasus Ecoregion (Zazanashvili et al., 2004). The group is highly speciose, with between 20 and 30 bisexually breeding species, most of them with very limited geographic range (Tarkhnishvili, 2012; Ahmadzadeh et al., 2013). One narrow-ranged rock lizard, *Darevskia mixta*, is probably the sole vertebrate fully endemic to the country of Georgia. Although Darevsky (1967) mentioned a single specimen from Giresun province in Turkey, and this information was later circulated (e.g. Sindaco et al., 2000), we hypothesize that this specimen was misidentified, and this will be addressed in the discussion. *D. mixta* differs from other closely related lizards, exhibiting a nearly diagnostic scalation trait: the presence of a sin-

gle large scale (post-centraltemporal, PCT) located between similarly large central temporal and tympanal scales. Other characteristic traits of this species include its small size (snout-vent length between 49 and 63 cm), paired preanal scales (PA), dark flanks, and emerald green dorsum in reproductive males (Darevsky, 1967; Tarkhnishvili, 2012). The closest relatives of *D. mixta*, which simultaneously have neighboring geographic ranges, are *D. clarkorum* (and possibly *D. dryada*) from SW Georgia and NE Turkey, and *D. caucasica* from the Central and Eastern Greater Caucasus (Murphy et al., 2000). These species do not have the scalation of the temporal area typical for *D. mixta*.

Mehely (1909) first described *D. mixta*, suggesting its hybrid origin between rock-dwelling *Lacerta saxicola* sensu Lato (= *D. saxicola*) and ground-dwelling *Lacerta* (= *Darevskia*) *derjugini*. Darevsky (1967) also hypothesized a hybrid origin of *D. mixta*, with *D. derjugini* as one of the parental species. *D. mixta* itself is a maternal parent species to two unisexual forms, *D. "dahli"* and *D. "armeniaca"* (Murphy et al., 2000).

The current distribution range of *D. mixta* is fully associated with a Colchic humid for-

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est refugium east of the Black Sea (Zeist and Bottema, 1991; Tarkhnishvili, Gavashelishvili and Mumladze, 2012), specifically with its easternmost part. Phylogeographic studies of small-bodied animals with limited dispersal ability (Tarkhnishvili et al., 2000; Mumladze et al., 2013) suggest that this refugium might be, in fact, subdivided into even smaller refugia and populations of salamanders, snails, and perhaps lizards of those refugia could be totally or partly isolated.

Summarized field findings of *D. mixta*, available from the literature and documented during field studies in Georgia and Turkey since 2006, are shown in fig. 1. The general pattern is that the species is sporadically found in river gorges from both the Lesser and the Greater Caucasus, although its presence in NE Turkey, in SW Georgia (Ajara) and in Likhi Range joining the Greater and the Lesser Caucasus mountains remains unconfirmed.

The aims of the present study were to describe variations in scalation characters of *D. mixta* within its range, and to infer underlining phylogeography. Specifically, we questioned if

there are (1) fixed matrilineal differences among the populations of *D. mixta* from throughout the range, in particular between the Greater and the Lesser Caucasus populations; (2) identify where maternal lineages of *D. mixta* expanded from, i.e., where the ancestral area of the species is located; (3) whether there are morphological differences among populations of *D. mixta*, and how they correlate with the haplotype distribution.

Material and methods

Sampling

During 2009-2014 our team members studied several hundred rock lizard locations in Georgia and NE Turkey (Tarkhnishvili et al., 2010; Tarkhnishvili, 2012; Tarkhnishvili, Murtskhvaladze and Gavashelishvili, 2013; Tarkhnishvili et al., unpublished). All previously recorded locations for *D. mixta* (Darevsky, 1967; Murphy et al., 2000; Tarkhnishvili et al., 2010) were included, and augmented with additional populations. We identified 26 locations of *D. mixta*, and collected tissue samples from thirteen locations (fig. 1, table 1). These locations can be grouped into two mountain systems (the Greater and the Lesser Caucasus Mountains) (fig. 1). Only the individuals with a diagnostic trait – one scale between the central temporal and tympanal scales – were considered. Surveys done in several gorges in

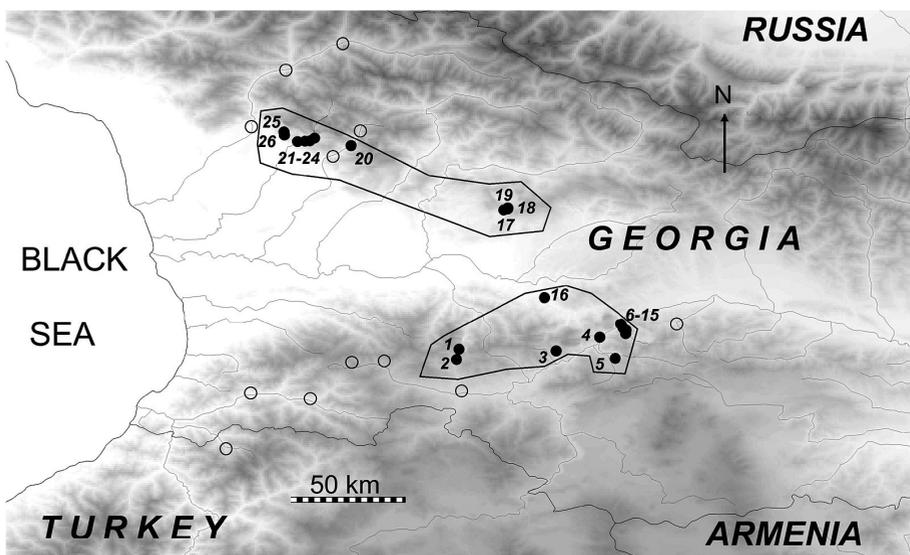


Figure 1. Sampling locations of *D. mixta*. Solid black circles 1-26: sampling locations of *D. mixta* (see table 1 for details). Open circles: the surveyed rock lizard locations close to the range of *D. mixta*, where the presence of this species was not established. The solid outline shows the approximate range of *D. mixta*.

Table 1. Specimens of *D. mixta* used in the analysis (see fig. 1 for the locations).

Mountain system	Region	Location	Morphology	Sequencing
Lesser Caucasus	Zekari Pass	1	10	2
		2	1	1
	Borjomi Gorge	3	2	3
		4	1	1
		5	3	3
		7	3	3
		15	2	2
Greater Caucasus	Rioni Valley	17	1	1
	Samegrelo Range	19	5	2
		20	5	3
		21	2	2
		24	2	2
		26	5	3

Turkey, close to Georgian border, did not confirm presence of lizards with morphological traits of *D. mixta*, nor did the survey in the Lesser Caucasus west of Abastumani Gorge (locations 1-2, fig. 1), or in the Greater Caucasus west of the Khobi River Gorge (locations 25-26, fig. 1). Each individual was photographed as described in Tarkhishvili et al. (2010); Tarkhishvili, Murtskhaladze and Gavashelishvili (2013), tail-tips were collected in the study were stored in 95% ethanol to preserve the DNA for later extraction and analysis.

Scalation analysis

42 individuals from 13 locations were described morphologically (table 1). The scalation characters scored from each individual lizard image are shown in fig. 2. In total, 18 characters were included in the analysis. These morphological characters were analyzed using Categorical Principal Component Analysis (Gifi, 1990; see Tarkhishvili, Murtskhaladze and Gavashelishvili, 2013 for details) and multivariate hierarchical ANOVA (type I sum of squares), with mountain systems (Greater and Lesser Caucasus) and "areas" as predictors. SPSS 21 (2012) was used for these analyses.

DNA extraction, PCR, and sequencing of mitochondrial DNA

DNA was extracted from tissue samples (tail tips) of 25 *Darevskia mixta* using a Qiagen tissue kit, according to the manufacturer's instructions (QIAamp DNA, 2007).

Partial DNA sequence from the mitochondrial locus cytochrome *b* (cytb) was used in the analysis – cytb has been shown to be sufficiently variable among closely related species of *Darevskia* to allow resolution of species relationships (Fu, Murphy and Darevsky, 1997; Murphy et al., 2000; Tarkhishvili, Murtskhaladze and Gavashelishvili, 2013). A cytb fragment (714 bp) was amplified using primer pairs H15915-L15369 and H15488-L15153 (Fu, 2000; Murphy et al., 2000). PCR was carried out in 21- μ l total volume,

with 2-4 μ l template DNA, 1 U of Go Taq DNA polymerase and 5X buffer (Promega), 1 μ M of MgCl₂, 0.1 μ M of each dNTP, and primer concentrations at 0.1 μ M. The thermocycling profile included starting 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. An aliquot of 3-5 μ l from each PCR was electrophoresed on a 1% agarose gel with SyberSafe DNA dye. The amplicons were sequenced on the automatic sequencer ABI 3130.

Single-stranded sequencing was performed with PCR primers using Big-Dye Terminator 3.1. PCR fragments were sequenced in both directions to assure sequence accuracy. mtDNA sequences were edited using SEQSCAPE 2.5 (Applied Biosystems Inc., Foster City, CA, USA) and the unique sequences were deposited in GenBank.

Phylogenetic analysis

714 bp sequences of 25 individuals were aligned using BioEdit7.1.3.0 software (Hall, 1999), along with four sequences downloaded from Genbank (one sample of *D. clarkorum* used as an outgroup, and three samples of *D. mixta*. Genbank accession numbers of downloaded sequences are: *D. clarkorum* – U88605.2; *D. mixta* – AF147796.1, AF147797.1, AF147798.1. Accession numbers for then novel sequences generated in this study: KM496573-KM496582).

We inferred the best-fit substitution model using MEGA 6.0 (Tamura et al., 2013). The inferred model was HKY (Hasegawa et al., 1985). We reconstructed a maximum likelihood phylogenetic tree of the inferred haplotypes with this model applied. We also used MEGA 6.0 for inferring molecular diversity of the lizards from the Lesser and the Greater Caucasus, in order to infer the most likely area of origin of *D. mixta* evolutionary lineage. For this purpose, mean Jukes-Cantor (1969) genetic distance between the individuals was calculated separately for the individuals from the Greater and from the Lesser Caucasus. We reconstructed a Bayesian tree topology for the same haplotypes using the software BEAST v. 1.8.0 (Drummond and Rambaut, 2007). The analysis was initiated from a random starting tree, employing the best-fit substitution model, assuming the coalescent model with constant population size. Posterior distributions of the parameters were approximated using Markov chain Monte-Carlo with chain settings as suggested by BEAST manual. Finally, a Median-Joining (MJ) algorithm (Bandelt et al., 1999) was applied to reconstruct all possible evolutionary pathways among the inferred haplotypes. Software NETWORK 4.6.1.1 (Fluxus Technology Ltd.) was used for the network construction, with the default settings applied.

In order to estimate the time of separation between the inferred clades within the studied dataset, we tested a molecular clock hypothesis. To test equality of substitution in different clades, we used Tajima's (1993) relative substitution rate test. After Crochet et al. (2004), we used minimum and maximum substitution rates for cytochrome *b* 1.5 and 2.5% per Mya (millions of years ago), respectively, with 2% as an average value.

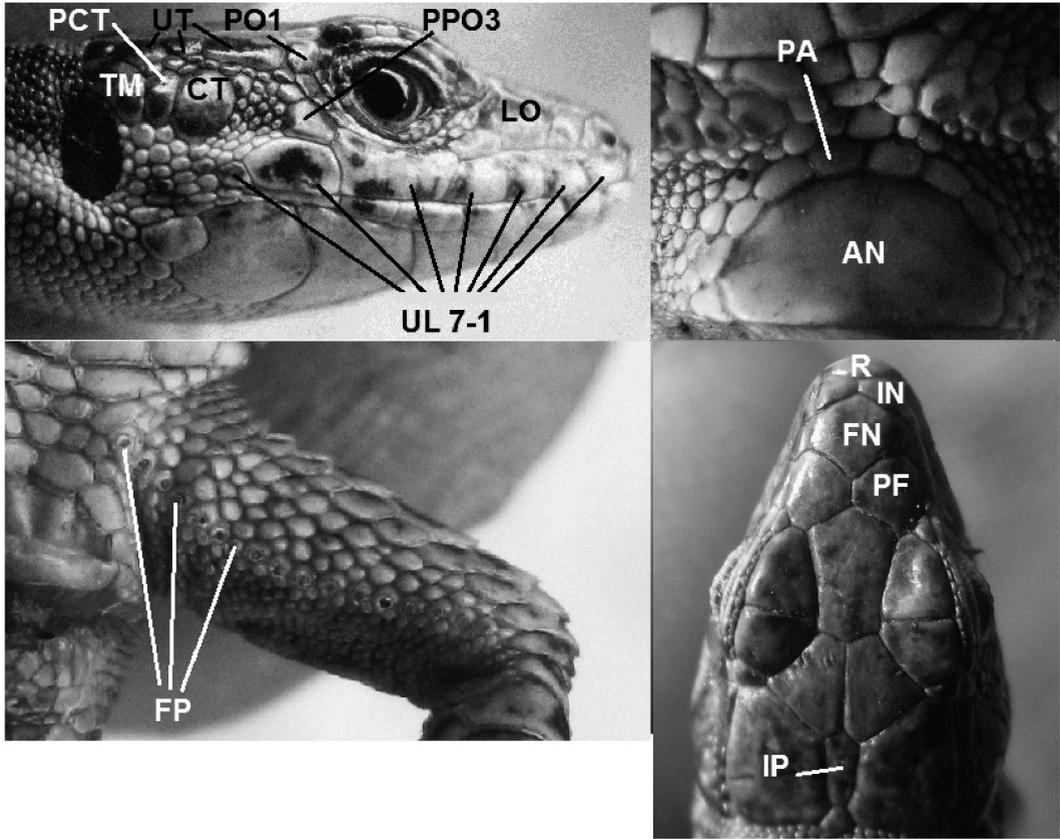


Figure 2. Scalation characters described for the studied individuals. CT – central temporal scale, PCT – post-central-temporal, TM – tympanal, PO1 – postorbital 1, PPO3 – post-postorbital 3, UL – upper labials, LO – loreal, AN – anal scale, PA – preanals, PF – prefrontals, IN – internasals, FN – fronto-nasal, R – rostral, IP – interparietal. Character description (see also table 2): (1) PCT subdivided vs. not subdivided vertically, (2) absence, presence of small or large additional scale between CT and PCT, (3) CT and UT not in contact vs. in contact, (4) PCT and UT not in contact vs. in contact, (5) TM and UT not in contact vs. in contact, (6) least number of scales between CT and PO1, (7) between CT and PPO3, (8) between CT and UL6, (9) between TM and UL7, (10) LO and UL4 separated, contacting, or overlapping, (11) number of FP, (12) number of scales contacting AN, (13) PA – symmetric pair, asymmetric pair, or single, (14) small scale between two PA absence or presence, (15) small scale inclined between PF absence vs. presence, (16) FN and R contacting, separated, or small scale inclined between IN, (17) IP with a sharp vs. obtuse end, or subdivided (18) nostril scales contacting vs. separated.

Results

Matrilineal phylogeny and mt-DNA diversity in the two geographic populations

We identified 10 novel *D. mixta* haplotypes. The studied fragment of 714 bp mitochondrial cytochrome *b* gene had 24 variable positions. Seven to nine positions were different in the lizards from the Greater vs. the Lesser Caucasus. Six positions were different between the lizards from the Central and the Eastern parts of Meskheta Range, the Lesser Caucasus (locations

1-2 vs. 3-15, fig. 1). Other variations marked in individual animals.

The ML tree of the studied haplotypes is shown in fig. 3a. The rooted tree is separated into two well-supported clades, from the Greater and from the Lesser Caucasus, respectively. The Lesser Caucasian clade, in turn, is separated into the populations of the Eastern and Central Meskheta Range (locations 1-2 vs. 3-15, fig. 1). Hence, there are three areas that have monophyletic lineages inferred from haplotypes, those from the locations (1-2, 3-15,

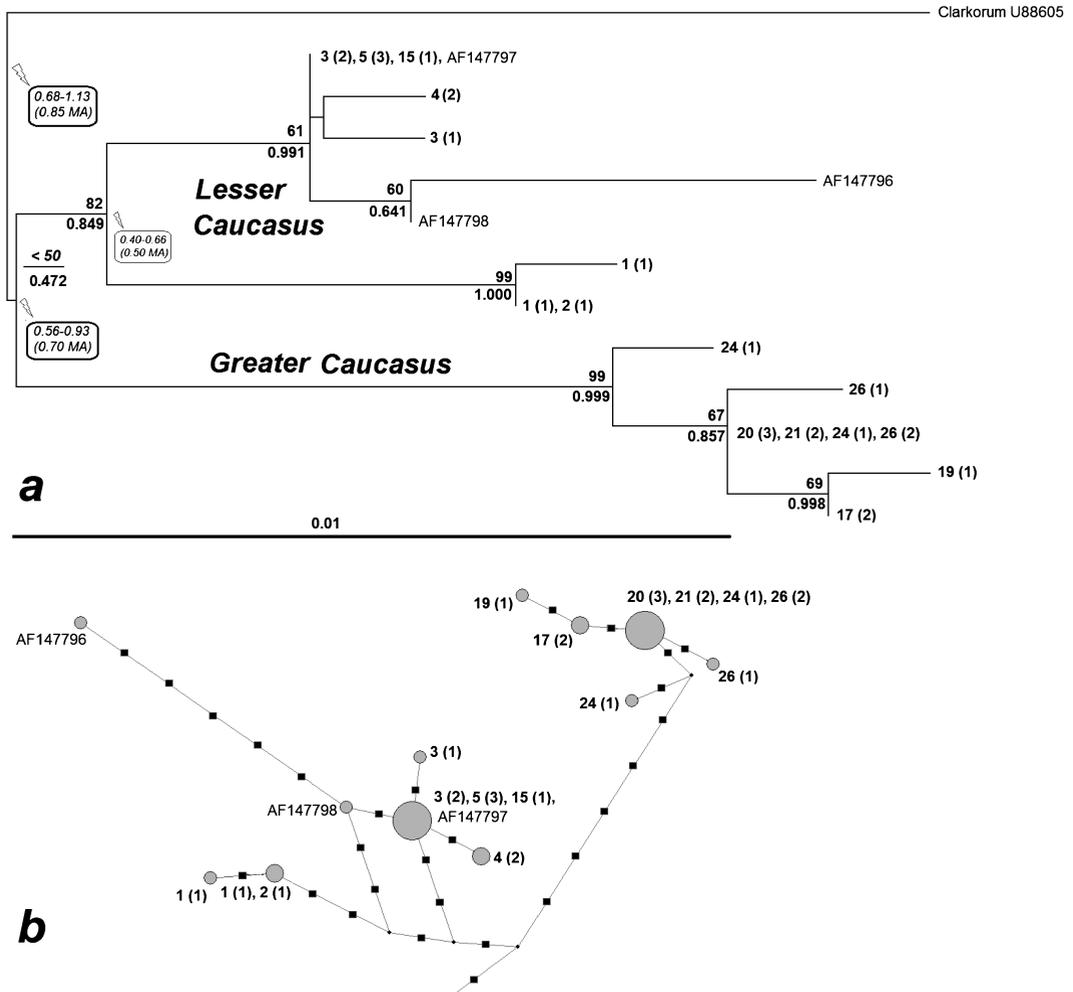


Figure 3. The topology of mitochondrial haplotypes of *Darevskia mixta*. (a) Maximum likelihood tree of the mitochondrial haplotypes (cytochrome *b*) of *D. mixta*. Tip labels indicate locations (fig. 1), with the number of individuals in parenthesis. Node labels indicate bootstrap values (1000 bootstrap replications) above the line, and Bayesian posterior probabilities below the line; figures in frames – estimated minimum, maximum, and average time of split. (b) Median-Joining network of the same haplotypes. Small squares – individual substitutions. Size of nodes showing individual haplotypes correspond to the respective sample size.

and 17-26, fig. 1). Bayesian inference was in full concordance with the ML tree; the posterior probabilities showed even stronger support of the individual clades than the bootstrap values for the ML tree (fig. 3a). Median-Joining network of the haplotypes is shown in fig. 3b. Mean Jukes-Cantor genetic distance between the individuals in the Lesser Caucasus was significantly higher than that in the Greater Caucasus: 0.00484 ± 0.00013 vs. 0.00143 ± 0.00072 .

Tajima's relative substitution rate test inferred $P = 0.13$, therefore the hypothesis of equal substitution rates was not rejected. Given the calibration of Crochet et al. (2004), the inferred time of separation of *D. mixta* evolutionary lineage is 0.68-1.13 (0.85) Mya (fig. 3). The timing of separation between the Greater and the Lesser Caucasus lineages was 0.56-0.93 (0.75) Mya, and the timing of separation of the matrilineally monophyletic population from the locations 1 and 2 is 0.40-0.66 (0.50) Mya.

Scalation

Categorical Principal Component Analysis was unable to separate the individuals from different geographic areas (results not shown). Hierarchical ANOVA (Type I sum of squares) showed significant effect of mountain systems for four out of 18 studied characters (table 2).

Over 25% of the lizards from the Lesser Caucasus (but none from the Greater Caucasus) had a large additional scale inserted between CT and PCT. Lizards from the Greater Caucasus have significantly more scales (usually 3-4) between CT and PPT3 compared to those from the Lesser Caucasus (usually 2-3), especially those from the locations 1-2 (fig. 1). Over 20% of lizards from the Lesser Caucasus (but none from the Greater Caucasus) have a separated interparietal scale. Lastly, more than half of the lizards from the Greater Caucasus exhibited an unpaired preanal scale, but none of those from the Lesser Caucasus (table 2, fig. 4A-D). In addition, there were significant ($P = 0.04$) dif-

ferences between the locations 1-2 and the rest of the locations, with respect to the number of scales between CT-PPO3 (fig. 2).

For 12 out of 18 characters, the lizards from the Lesser Caucasus had Greater coefficients of variation (SD divided by average value) than those from the Greater Caucasus, and only for two characters was the pattern opposite (table 2).

Discussion

Populations of *D. mixta* from the Greater and the Lesser Caucasus are likely completely isolated, this isolation most likely occurred shortly after separation of the *D. mixta* evolutionary lineage, ca. 0.8 Mya. The central part of the Lesser Caucasus was the most likely area of origin for *D. mixta*. Both genetic and morphological variations among the populations of *D. mixta* suggest a Lesser Caucasian origin of the *D. mixta* lineage.

Table 2. Variation of scalation characters and multivariate hierarchical ANOVA output.*

#	Character	CVAR (Greater Caucasus)	CVAR (Lesser Caucasus)	F (Greater vs. Lesser Caucasus)	P (Greater vs. Lesser Caucasus)
1	PCT shape	36	36	0.011	0.92
2	Two PCT	22	57	5.612	0.02
3	CT-UT contact	29	36	1.588	0.22
4	PCT-UT contact	36	36	0.001	0.97
5	TM-UT contact	31	26	0.575	0.45
6	Scales between CT-POC1	26	31	0.253	0.62
7	Scales between CT-PPO3	14	27	8.717	0.01
8	Scales between CT-UL6	18	27	1.152	0.29
9	Scales between TM-UL7	18	27	0.924	0.34
10	Contact between LO-UL4	36	43	0.675	0.42
11	Number of FP	9	6	0.030	0.86
12	Scales around AN	16	16	1.392	0.25
13	Unpaired PA	51	31	13.802	0.00
14	Smaller scale between PA	0	20	0.810	0.37
15	Incline between PAR	0	20	0.810	0.37
16	Incline between NS	53	54	0.215	0.65
17	PAR vs. PPAR	0	57	4.494	0.04
18	Nostril scales	51	55	0.265	0.61
	Multivariate			2.202	0.781
	N	20	22		

* F calculated with Wilk's Lambda. Boldface in columns CVAR show characters that vary Greater in the Lesser than in the Greater Caucasus. Boldface in column P indicates significant ($P < 0.05$) values. For detailed descriptions of the scalation traits see fig. 2.

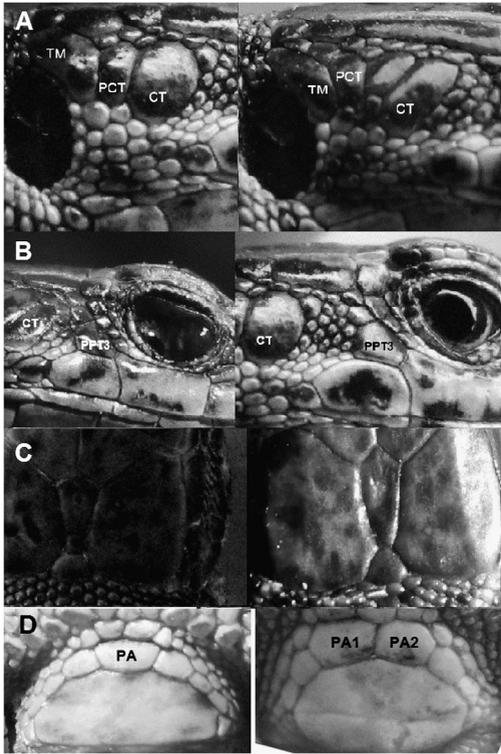


Figure 4. Most typical differences between *D. mixta* from the Lesser and the Greater Caucasus. (A) additional PCT (right image; more common in the Lesser Caucasus); (B) low vs. high number of scales between eye and CT (left – low number; more common in the Lesser Caucasus); (C) separated interparietal scale (left image; more common in the Lesser Caucasus); (D) singular (unpaired) preanal scale (left image; more common in the Greater Caucasus).

D. mixta are small, territorial lizards. They strongly depend on humidity and temperature in microhabitats, avoiding dry or too warm locations (Tarkhnishvili, 2012). They are never found in lowland areas and at elevations below 250–280 meters above sea level (our data). Hence, their dispersal ability is limited. Our molecular genetic data are in line with this fact. The studied, albeit limited, samples suggest that the lizards from three parts of the range: region (1) (Middle part of Meskheti Range), region (2) (Borjomi Gorge), and the Greater Caucasus Mountains (locations 17–26, fig. 1) are, each matrilineally monophyletic. In contrast, the individual locations within these three parts of the range have admixtures of the haplo-

types found in different locations of the same area. If the calibration of the molecular clock earlier suggested by Crochet et al. (2004) for small lizards of genus *Iberolacerta*, closely related to *Darevskia* (Tarkhnishvili, 2012) is accepted, the differences between the Greater and Lesser Caucasus populations date back to “Mid-Pleistocene Revolution” ca. 800 KY ago, and almost coincide in time with the separation of *D. mixta* and its closest relative, *D. clarkorum*. This climatic transition was associated with increasing of glacial cycles to ca. 100 Kya (thousands of years ago) and deepening of temperature fall during the glacial maxima (Imbrie et al., 1993). Multiple studies on different small animals suggest that the Mid-Pleistocene Revolution was an important event that triggered multiple splits of evolutionary lineages in the Caucasus (Tarkhnishvili, 2014). Most likely, these climatic transitions built impenetrable barriers between the Lesser and the Greater Caucasian populations of sporadically distributed *D. mixta*. The lizards from the Greater and the Lesser Caucasus have apparently never exchanged maternal lineages since then.

Genetic studies of other small, less mobile animals, the Caucasian salamander (*Mertensiella caucasica*), for example, or the large endemic Caucasian snails (*Helix buchi*, *H. goderdziana*) (Tarkhnishvili et al., 2000; Mumladze et al., 2013), also suggest that humid forest refugia of the Western Caucasus (Zeist and Bottema, 1991; Tarkhnishvili et al., 2012; Tarkhnishvili, 2014) were historically fragmented, rather than continuous. According to these studies, the most prominent division was between current Central Georgia (where the entire range of *D. mixta* is located) and the south-eastern Black Sea Coast (where the range of *D. clarkorum*, the sister species of *D. mixta* is located). Our current study suggests that the “Central Georgian” refugium has also been separated into Greater and Lesser Caucasian parts, although lizard populations from these two areas experienced a lesser degree of isolation than did the populations of *D. mixta* and *D.*

clarkorum from the Central and Southwestern Georgia. How did this differentiation into distinct Greater and Lesser Caucasus populations happen? The Lesser and the Greater Caucasus are currently connected via the forested Likhi Range. Meanwhile, the upper reaches of the largest river of the Eastern Black Sea Basin, the Rioni River, split in foothills of these mountains, with tributary streams coming from both the Greater and the Lesser Caucasus. Our repeated surveys of the Likhi Range habitats did not discover the presence of *D. mixta*, although this habitat is populated by another rock lizard, *D. rudis*, a species that is more tolerant of dry habitats (Tarkhnishvili, 2012). It is likely that *D. mixta* can survive only in middle and upper reaches of smaller rivers and, hence, the lower part of Rioni Valley is a barrier separating the Greater and the Lesser Caucasus populations. It is possible that this split became more pronounced after the Mid-Pleistocene, because the decline of rainfall during glacial cycles would likely have caused further fragmentation of suitably humid habitat.

Haplotype diversity of the Lesser Caucasian populations of *D. mixta* significantly exceeds haplotype diversity of the Greater Caucasus populations. Scalation analysis also suggests higher individual variation of the most of the studied characters in the Lesser Caucasus populations, compared to those in the Greater Caucasus. If selection were driving the divergence, then the sharper environmental gradients in the Greater Caucasus would have engendered higher diversity there. This is opposite to our findings, we therefore conclude that 1) either the Lesser Caucasus population was ancestral to the Greater Caucasus population of the species, or 2) the Greater Caucasian population passed through a bottleneck during one of the consecutive glacial maxima, e.g. during the Last Glacial Maximum over 20 Kya (Frenzel, 1968).

The central part of the Lesser Caucasus was probably a more important refuge for this species, and other animals and plants with similar ecological requirements, compared to the

Greater Caucasus. It seems likely that, because its less extreme terrain, the Lesser Caucasus will experience fewer avalanches, landslides, or other catastrophic changes that cause local extinctions compared to the Greater Caucasus. The Greater Caucasus therefore, especially its central, the highest and the steepest part, has limited importance as a glacial refugium. Our study supports the existence of two distinct refugia in the Lesser Caucasus, based on the deep genetic differences between *D. mixta* lineages from the south-eastern Black Sea Coast, and central Georgia (Tarkhnishvili, Thorpe and Arntzen, 2000; Tarkhnishvili, Gavashelishvili and Mumladze, 2012). It suggests that the “Central Georgian” refugium was located in the eastern part of Meskheta Mountains (locations 1-2, fig. 1).

In light of these findings, we believe that the reported Turkish specimen of *D. mixta* (Darevsky, 1967) was probably misidentified. This specimen is described from Yavuzkema, near Giresun, i.e. over 400 km west from the closest *D. mixta* population in Georgia. Subsequently, not a single verified finding of *D. mixta* has been reported from Turkey, and meantime the information on this lone specimen has been recycled. The closest relative of *D. mixta*, *D. clarkorum*, has been studied morphometrically by Ilgaz (2007). This author explored ten locations of rock lizards, including Yavuzkema, and discovered only *D. clarkorum* at these locations. Our field studies since 2006, including those conducted throughout NE Turkey close to Yavuzkema (Tarkhnishvili et al., 2008), and the Georgian mountains adjacent to the *D. mixta* range, also did not confirm the presence of the species outside the range described here. Occasionally, individuals of closely related lizards of *D. “caucasica”* clade (Murphy et al., 2000) have a large PCT scale, making them externally indistinguishable from *D. mixta* (e.g. specimen of *D. caucasica* from Gudauri, number ZFMK 76418 stored at the Zoological Forschungsmuseum A. Koenig, Bonn). For this reason we suggest that the specimen mentioned by Darevsky

(1967) was in all likelihood *D. clarkorum*, with a PCT scale similar to that of *D. mixta*. The range of *D. mixta*, therefore, covers the eastern part of Meskheti Range of the Lesser Caucasus Mountains and southern slopes of the Greater Caucasus between the valleys of rivers Rioni and Khobi, and is fully endemic to Georgia.

Acknowledgements. The authors wish to thank L. Mumladze, L. Ninua, D. Dekanoidze, and A. Gavashelishvili for assistance with fieldwork, A. Avaliani, T. Beridze, and G. Golubiani for assistance in the laboratory, and to W. Boehme for assisting with the morphological study of the lizards from the ZFMK (Zoologisches Forschungsmuseum Alexander Koenig) collection, and two anonymous reviewers for valuable comments on the first draft of the paper. Our special thanks are to Cort Anderson, who corrected the English of the manuscript and provided additional helpful comments. The study was financed by the Georgian National Science Foundation (award no. GNSF/ST07/6-217) and internal budget of the Institute of Ecology, Ilia State University.

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Submitted: September 10, 2014. Final revision received: January 29, 2015. Accepted: January 29, 2015.
Associate Editor: Uwe Fritz.