Phylogeny and morphological variation in the rock lizards of the genus *Darevskia*

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Statement

As an author of this doctoral thesis, I confirm that this compilation thesis is my original work and does not include the materials of other authors that have already been published, accepted for publication or submitted for a degree, which have not been cited in an accepted manner.

Mariam Gabelaia --

აბსტრაქტი

Darevskia გვარის კავკასიური კლდის ხვლიკები მცირე ზომის ხვლიკების მრავალფეროვანი ჯგუფია, რომლებიც ძირითადად დიდსა და მცირე კავკასიონზე ცხოვრობენ. *Darevskia*-ს სახეობების საზღვრების დადგენა რთულია, მათი გარეგნული მსგავსებისა და სახეობებს შორის გადაფარვადი მორფოლოგიური ნიშნების გამო. ამასთან ერთად, ისინი ხშირად ჰიბრიდიზირებენ და შედეგად ხშირია შუალედური მორფოლოგიისა და გენეტიკის მქონე ინდივიდები, რომელთა რომელიმე ცალკეული სახეობისთვის მიკუთვნება ძალიან რთულია. ეს განსაკუთრებით ვლინდება თვისობრივი მორფომეტრიული მეთოდებით შეფასებისას, რომელიც სხეულისა და ქერცლების სხვადასხვა განაზომებს ეფუძნება.

ამ კვლევაში, ჩვენი მიზანია ვიპოვოთ თანხვედრა ფილოგენეტიკურ კავშირებსა და მორფოლოგიურ მსგავსებებს შორის *Darevskia* სახეობებში და გამოვიყენოთ გენეტიკური და მორფოლოგიური მეთოდები, ან მათი კომბინაცია სახეობების საზღვრების დასადგენად. კვლევისას ძირითადად ვიყენებთ მიტოქონდრიული დნმ-ის ხაზსა და გეომეტრიულ მორფომეტრიულ მეთოდებს (ფორმის ორ-განზომილებიანი ფურიეს კონტურული ანალიზი და სამ-განზომილებიან ლენდმარკებზე დაფუძნებული ანალიზი, ‘ტრადიციული’ მორფომეტრიის საპირისპიროდ) და ვცდილობთ გამოვავლინოთ ის მორფოლოგიური ნიშნები, რომლებიც ცალკეული სახეობებისთვისაა დამახასიათებელი და კარგად აღწერს სახეობათა დივერგენციას.

კვლევის შედეგად დადგინდა, რომ *Darevskia* სახეობების თავისა და ზოგიერთი ქერცლების ფორმაზე გეომეტრიულ მორფომეტრიული ანალიზის გამოყენებით შესაძლებელია მათი ფილოგენეტიკური კავშირების მსგავსი სურათის მიღება, ან სახეობათა საზღვრების დადგენა, თუმცა უფრო ეფექტურია როდესაც გენეტიკურ (მიტოქონდრიული დნმ) ანალიზთან ვაერთიანებთ.

ძირითადი საძიებო სიტყვები: *Darevskia* , ფილოგენია, გეომეტრიული მორფომეტრია.

Abstract

Caucasian rock lizards of the genus *Darevskia* are a diverse group of small lizards mainly living in the Greater and the Lesser Caucasus. It is difficult to delimit *Darevskia* species due to a high degree of morphological similarity and overlapping identification characters. Additionally, they also strongly hybridize and thus individuals with intermediate morphology and genetic characters are found. As a result, there are extreme difficulties in assigning found individuals to a distinct species. This is particularly true for the traditional morphological methods such as qualitative morphometrics, where multiple measurements of the body and scales are compared with each other.

In the present project, we aimed to find the links between the phylogenetic relationships and morphological similarities in *Darevskia,* and apply genetic and morphological methods, or their combination, for delimiting the species boundaries. We mainly focus on the matrilineal phylogeny and geometric morphometric techniques (two-dimensional Fourier outline shape analysis and three-dimensional landmark-based analysis in comparison with 'traditional' morphometrics). More specifically we aim to reveal the morphological features which are species-specific and follow the species evolutionary divergence pattern.

In conclusion to our study, the head shape and even some scalation features of *Darevskia* species can be suitable markers for depicting the species phylogenetic relationships or delimiting the species boundaries when assessed with geometric morphometric techniques, but perform the best if integrated with genetic (mtDNA) analysis.

Keywoords: *Darevskia,* phylogeny, geometric morphometrics.

Overzicht

Kaukasische rotshagedissen van het geslacht *Darevskia* vormen een diverse groep van kleine hagedissen die hoofdzakelijk voorkomen in de Grote en de Kleine Kaukasus. *Darevskia* soorten zijn echter zeer moeilijk van elkaar te onderscheiden als gevolg van hun grote mate van morfologische gelijkenissen en de overlap in kenmerken die gebruikt worden om de soorten te identificeren. Bovendien vertonen ze intensieve hybridisatie, waardoor individuen met een intermediaire morfologie en genetische merkers worden teruggevonden. Dit maakt het extreem moeilijk om de gevonden specimens toe te wijzen aan gekende soorten. Dit is vooral zo indien gebruik wordt gemaakt van traditionele morfologische onderzoeksmethodes, zoals kwalitatieve morfometrie, waarbij meerdere lengtemetingen worden uitgevoerd op het lichaam en de schubben, om dan onderling te vergelijken.

In dit project trachtten we de verbanden te achterhalen tussen de fylogenetische relaties en de morfologische gelijkenissen in *Darevskia,* waarbij op basis van genetische en morfologische methods (afzonderlijk en gecombineerd) gebruikt worden om soorten af te bakenen. We richten ons hier vooral op de matrilineaire fylogenie en geometrisch morfometrische technieken (twee-dimensionale Fourier analyse om de vorm op basis van contouren te analyseren, en drie-dimensionale vorm analyses op basis van landmarks, en dit vergeleken met meer 'traditionele' morphometrie). Meer specifiek is het doel om de morfologische eigenschappen te achterhalen die soort-specifiek zijn, en zodoende de evolutionaire divergentiepatronen van de soorten te achterhalen.

Een conclusie van dit onderzoek is dat zowel de kopvorm, maar ook bepaalde eigenschappen in het schubpatroon bij de *Darevskia* species bruikbare indicatoren zijn om fylogenetische verwantschappen te achterhalen, of voor het identificeren en afbakenen van soorten. Betrouwbare resultaten worden echter enkel verkregen indien de data geïntegreerd werd met de genetische (mtDNA) analyses.

Keywoorden: *Darevskia,* fylogenie, geometrisch morfometrie.

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Abbreviations

2D - two-dimensional

3D - three-dimensional

3DPCA - principal component describing the shape of the three-dimensional head shape

5thUL - fifth upper labial scale

AN – anal

ANOVA - analysis of variance

ASPCA - principal component describing the shape of the anal scale

BG-PC - between-group principal component

BG-PCA - between-group principal component analysis

BIC – Bayesian information criterion

Bp - base pair

BV – bootstrap support value

CT – central temporal

CV – canonical variate

CVA – canonical variate analysis

Cytb – cytochrome *b*

Db – *Darevskia brauneri*

DFA - discriminant function analysis

Dl – *Darevskia lindholmi*

Ds – *Darevskia saxicola*

EFDs - elliptic Fourier descriptors

ESS – effective sample size

EV – Enguri valley

FN - frontal-nasal

FP – frontoparietal

Fp (in chapter 5) – number of femoral pores

Fr – frontal

FSPCA - principal component describing the shape of the frontal scale

GM - geometric morphometrics

GPSA - generalized Procrustes surface analysis

HKY - Hasegawa-Kishino-Yano

HKY+G - Hasegawa-Kishino-Yano plus Gamma

HL/HW – length of pileus divided by the maximum width of pileus

ICP - iterative closest point

IM – intermaxillary

IN – internasal

IP - interparietal

Kya – thousand years ago

LM_CVA - canonical variate analysis based on the Procrustes coordinates

LO – loreal

M – mandibular

M.a.s.l – metres above sea level

MCMC – Markov chain Monte-Carlo

MJ – median-joining

ML – maximum likelihood

Mp – mutated positions

MtDNA - mitochondrial DNA

Mya – million years ago

N – nuchal

N1/2 – nasal

NE – northeast

NP – nonparmetric

NP (in chapter 5) – the number of large preanal scales

NP-MANOVA – nonparametric multivariate analysis of variance

Nsc – the number of small scales between the inner and outer rows of supraoculars

P – parietal

PA – preanal scales

PC - principal component

PC_CVA – canonical variate analysis based on the principal components

PCA - principal component analysis

PCR - polymerase chain reaction

PCT – post-central temporal

PF – prefrontal

PN - post-nasal

PO – postorbital

PP – posterior probabilities

PPO – post-postorbital

R – rostral

S. D. – standard deviation

SD - standard deviation

SDs - small dorsal scales

SMs - small mandibular scales

SO - supraorbital

SVL - snout vent length

TM – tympanal

TV – Tekhuri valley

UCs - upper ciliated scales

ULPCA - principal component describing the shape of the fifth upper labial scale

UPGMA - unweighted pair group method with arithmetic mean

UT – upper temporal

Chapter 1. Phylogenetic relationships and species' boundaries in *Darevskia*

1.1 General Introduction

Rock lizards of the genus *Darevskia* (Arribas, 1999) are a diverse group of small lizards from the Lacertidae family, mainly distributed on the Greater and the Lesser Caucasus. *Darevskia* lizards are small; their body length (snout-vent length) is 4.5 – 8.5 cm in adult individuals. The genus comprises of up to 30 species including seven unisexual and three terrestrial species. Terrestrial *Darevskia* have deeper heads and more round trunk compared to rock-dwellers, which have a flat head and body suitable for hiding in the rock splits. The scalation pattern in *Darevskia* is variable, which strongly overlaps among the species; thus the specific morphological characters differentiating species significantly are unknown (Tarkhnishvili, 2012). *Darevskia* mainly occupy similar habitats, but with different preferences in humidity, temperature, and elevation. The closely related species tend to have allopatric or parapatric distributions, while distant species are more often found to live sympatrically. Parapatric habitats lead to intensive interspecific hybridization between the majority of *Darevskia,* and are responsible for the large number of intermediate individuals, which are difficult to attribute to any particular species (Tarkhnishvili, 2012).

The phylogeny of the Caucasian rock lizards (genus *Darevskia*) has been unsuccessfully linked with the morphological similarities and dissimilarities among its species for a while (Darevsky, 1967; Fu et al., 1997; MacCulloch et al., 2000; Murphy et al., 2000; Tarkhnishvili, 2012; Tarkhnishvili et al., 2013), due to their variable but strongly overlapping morphological characters and genetic diversity (Tarkhnishvili et al., 2013). The first major study about the taxonomic and phylogenetic relationships of *Darevskia* (Fig. 1) was conducted by Darevsky using 'traditional' morphometrics, which relied on the multiple measurements and qualitative scalation traits of the organisms (Darevsky 1967). The taxonomy of *Darevskia* comprised six nominal, species - *Darevskia praticola* Eversmann, 1834; *Darevskia derjugini* Nikolsky, 1898; *Darevskia saxicola* Eversmann, 1834; *Darevskia* *caucasica* Méhely, 1909; *Darevskia mixta* Méhely, 1909 and *Darevskia rudis* von Bedriaga, 1886 and their subspecies (chapter 2 - Gabelaia et al., 2017).

The phylogenetic relationships were first revised by Murphy and Moritz, based on allozyme electrophoresis (Murphy et al., 1996; Moritz et al., 1992) and finally by Murphy et al. (2000), who used mitochondrial DNA (mtDNA) to attribute 15 species (only bisexual) to three monophyletic groups (clades) – '*caucasica*', '*rudis*' and '*saxicola*' (Fig. 1). This work led not only to the phylogenetic but also taxonomic revision of the *Darevskia* species group and has not changed till now, with an exception of *Darevskia parvula* which was included in clade '*rudis*' in Murphy's study but now is placed closer to '*saxicola*' clade instead (Tarkhnishvili, 2012).

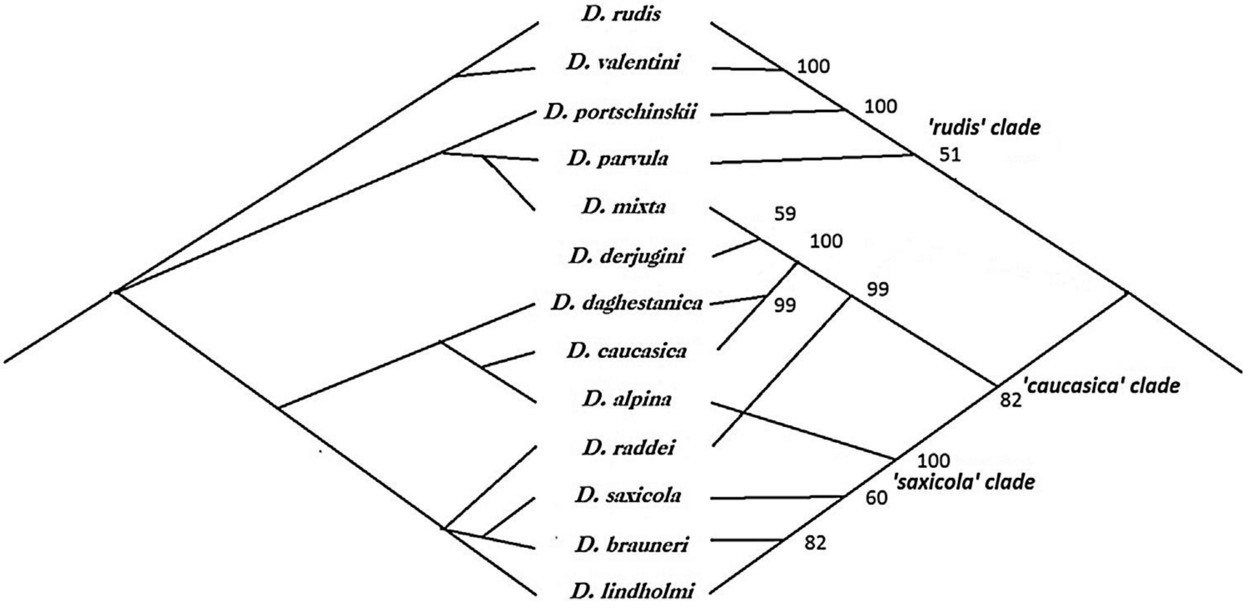

Figure 1. Topology based on qualitative traits of scalation pattern, according to Darevsky (1967) (on the left) and topology based on the parsimony analysis of molecular data (mtDNA), according to Murphy et al. (2000) (on the right). Numbers indicate bootstrap values. (Figure from chapter 2 - Gabelaia et al., 2017).

In general, mitochondrial DNA is used very commonly for delimiting the species and resolving their phylogenetic relationships (Fu et al., 1997; Murphy et al., 2000; Tarkhnishvili et al., 2013; Camargo et al., 2010; Wiens and Penkrot, 2002). According to Wiens and Penkrot (2002) mtDNA is more advantageous compared to the nuclear-based markers when studying species delimitation, as mtDNA haplotypes are able to diverge fully in much less time than nuclear-based haplotypes, because of the smaller effective population size of the mitochondrion.

The discrepancies between the phylogeny based on mtDNA analysis and the qualitative 'traditional' morphometrics have led us to the idea of exploring the methodological approaches for *Darevskia* species' morphological delimitation based on quantitative rather than qualitative analysis, and in this way reconstruct the phylogenetic relationships that are congruent with the mtDNA phylogeny.

The techniques of geometric morphometrics (GM) for studying biological organisms' shape variation started to mature in the late 80s (Bookstein, 1986; Rohlf, 1986). Two-dimensional (2D) outline shape analysis was the first technique of geometric morphometrics to be developed, but was soon substituted by landmark-based GM (Kaliontzopoulou, 2011). Unlike 'traditional' (qualitative) morphometrics, which considers only linear measurements and meristic characters of the animals, GM takes into account the geometric relationship of these characters and the shape of the organism itself (Zelditch et al. 2004) and thus is able to capture shape variation better than 'traditional' morphometrics (Kaliontzopoulou, 2011; Rohlf and Marcus, 1993; Zelditch et al., 2004). Visualization of the shape variation is another advantage of GM, which is very convenient when studying organisms' morphology (Klingenberg, 2013; Zelditch et al., 2004).

The advantages of the GM over the 'traditional' morphometrics have been described in multiple works. For example Breno together with coauthors (2011) showed that GM is more sensitive in quantifying the variation of the skull in an African rat species (*Mastomys natalensis*) than 'traditional' morphometrics (Breno et al., 2011). Other examples of the superiority of GM over 'traditional' morphometrics are described by Maderbacher et al. (2008), Abdel-Rahman et al. (2009), Bernal (2007), Blanco and Godfrey (2006). Geometric morphometrics has been shown to be more effective in reconstructing phylogenetic relationships than 'traditional' morphometrics, too (Smith and Hendricks, 2013).

In this PhD study, we use molecular (mtDNA), morphological ('traditional' morphometrics, geometric morphometrics) approaches and the combination of both, in order to delimit the species' boundaries and reveal the morphological features and the appropriate methodology for reconstructing the phylogenetic relationships congruent to the genetic (mtDNA) one.

1.2 Methodology

1.2.1 Sample collection

The part of the samples used for the genetic and morphological analysis was already collected by the researchers of Institute of Ecology (Ilia State University) starting from 2008. During my research period, the additional samples have been collected for genetic and morphometric analysis.

The studied species were: *D. mixta, D. clarkorum, D. derjugini, D. caucasica, D. daghestanica, D. raddei, D. rudis, D. obscura, D. valentini, D. portschinskii, D. parvula, D. brauneri,* and *D. praticola* (Fig. 2). The first six species belong to the clade '*caucasica'* (Murphy et al., 2000). *Darevskia mixta* is found in the western part of both the Lesser and the Greater Caucasus; *Darevskia clarkorum* in the Western Lesser Caucasus; both are found in mesophylic mountain forest belt. *Darevskia caucasica* – throughout the central Greater Caucasus, and *D. daghestanica* in the Eastern Greater Caucasus; both live in subalpine and alpine belts. Finally, *D. raddei* occurs sporadically between the Lesser Caucasus Mountains and Lake Van area and is only representative of the clade that lives in reasonably dry landscape. All these species but *D. derjugini,* which is a ground-dweller with a broad West Caucasus range, are rock dwelling forms.

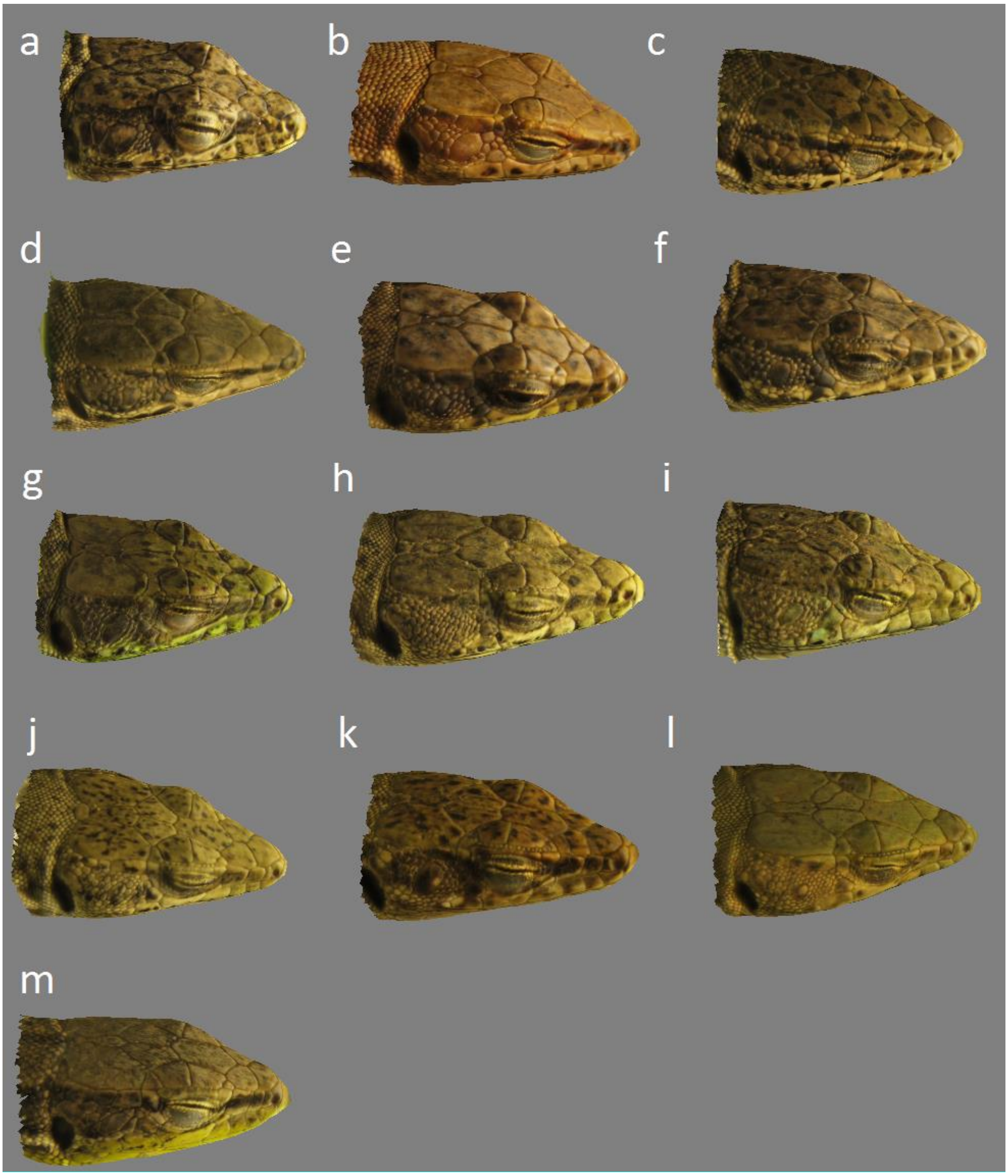

Figure 2. Species of *Darevskia* used in the study. a - *D. mixta,* b – *D. derjugini,* c – *D. caucasica,* d – *D. daghestanica,* e – *D. clarkorum,* f – *D. raddei,* g – *D. rudis,* h – *D. obscura,* i – *D. valentini,* j – *D. portschniskii,* k – *D. parvula,* l – *D. brauneri,* m – *D. praticola.* (Figure from chapter 3 – Tarkhnishvili et al., submitted).

Darevskia rudis, D. obscura, D. valentini, and *D. portschinskii* belong to the clade '*rudis*'. All of them are rock-dwellers. *Darevskia rudis* has a broad range between the Eastern Greater Caucasus and the northwestern Anatolia. *Darevskia valentini* lives between the Lesser Caucasus Mountains and Lake Van. *Darevskia portschinskii* is a dwarf form found in the eastern part of the Lesser Caucasus. The range of *D. obscura* (formerly *D. rudis obscura*) lays at the crossing of the ranges of the other three species of the group (chapter 6 - Gabelaia et al., 2018). All these species are genetically very close to each other and gene introgression between them takes place (Tarkhnishvili et al., 2013).

Darevskia brauneri and *D. praticola* belong to the clade '*saxicola*'. The former species is a rock-dweller from the Western Greater Caucasus, and the latter is a ground-dweller found between the Northern Iran and Balkan Peninsula.

Finally, Murphy et al. (2000) attributed a small rock-dweller from the Western Lesser Caucasus, *D. parvula*, to the clade '*rudis*', although later findings (Tarkhnishvili, 2012; Murtskhvaladze et al., in press) suggest that it belongs to a basal clade within *Darevskia*.

In total, 415 individuals of 13 species of the genus *Darevskia* from 16 locations have been used in the study (Fig. 3, Table 1). The amount of samples per each location and per species used in this PhD study can be found in Table 1.

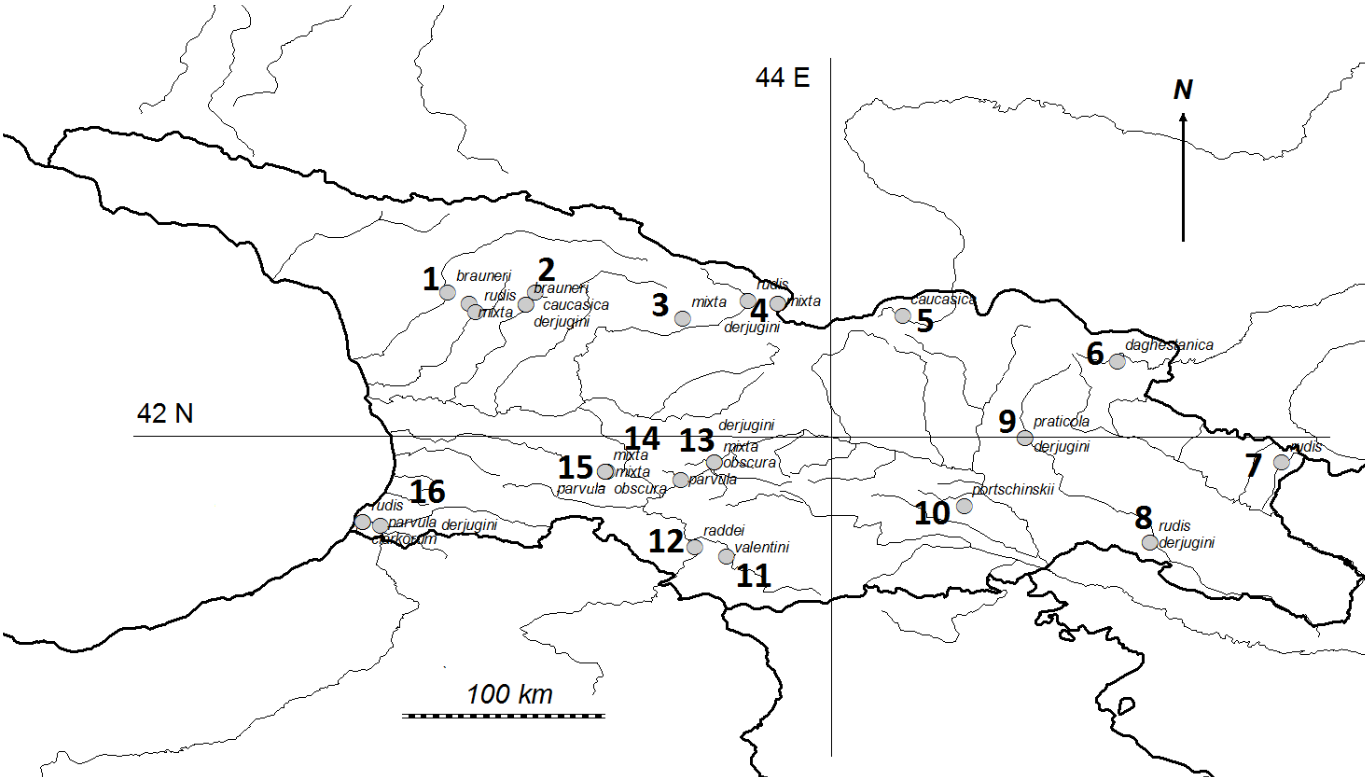

Figure 3. Sampling locations for each species. Numbers represent the locations in the Table 1.

Location	*mixta*	*brauneri*	*derjugini*	*parvula*	*rudis*	*valentini*	*portschinskii*	*obscura*	*clarkorum*	*caucasica*	*praticola*	*daghestanica*	*raddei*
1 - Enguri		8											
2 - Samegrelo	19	17	4		2					3			
3 - Rioni	6												
4 - Racha	9		3		16								
5 - Truso										3			
6 - Tusheti												6	
7 - Lagodekhi					9								
8 - Iori			4		2								
9 - Sioni			3								6		
10 - Kojori							63						
11 - Javakheti						44							
12 - Vardzia													6
13 - Borjomi	20		3	9				37					
14 - Zekari	11												
15 - Abastumani	13			9				11					
16 - Adjara			9	32	22				6				

Table 1. Locations and the number of samples per sampling location.

The samples of *D. mixta* (see also chapter 4 - Gabelaia et al., 2015; chapter 3 - Tarkhnishvili et al., submitted) have been collected from Samegrelo (northwestern Greater Caucasus) and Borjomi-Abastumani area (central Lesser Caucasus) in 2014 and 2017. The samples of *D. brauneri* were collected from Tekhuri (Samegrelo) and Enguri valleys (northwestern Greater Caucasus) in 2014 and 2017 (chapter 5 - Tarkhnishvili et al., 2016; chapter 3 - Tarkhnishvili et al., submitted). The photographs of *D. derjugini, D. mixta, D. rudis, D. valentini, D. portschinskii* and *D. parvula* used for the 2D geometric morphometric analysis have been collected by the researchers from the Institute of Ecology in 2008-2013 years (see also chapter 2 - Gabelaia et al., 2017). The samples for three dimensional (3D) reconstructions of *D. rudis* (from Charnali, Adjaria – southwest of Georgia and Lagodekhi – Eastern Georgia), *D. obscura* (from Borjomi Gorge area), *D. valentini* (from Javakheti – Southern Georgia), *D. portschinskii* (from Kojori – central Georgia) and *D. parvula* (from Borjomi Gorge area) were collected during my studies in 2017. The samples for the 3D reconstructions of *D. praticola* and *D. derjugini* originate from the Sioni area (central Eastern Georgia), *D. raddei* from Vardzia (Southern Georgia), *D. clarkorum* from the Charnali Gorge, *D. caucasica* from the Truso Gorge (central Northern Georgia) and Samegrelo area and *D. daghestanica* from the Tusheti area (eastnorthern Georgia) (chapter 6 - Gabelaia et al., 2018; chapter 3 – Tarkhnishvili et al., submitted).

For the genetic analyses, lizards' tail tissues were collected in field and stored in 95% ethanol until the DNA extraction procedure. For 'traditional' morphometric and 2-dimensional GM analyses, we took 2D digital photographs of the lizards' dorsal, lateral and anal areas.

1.2.2 DNA analysis

We extracted DNA from tissue samples using the QIAGEN tissue kit. The extraction protocol followed the recommendations by the manufacturer (QIAamp DNA, 2007).

For the genetic analysis we used mtDNA cytochrome *b* fragment (˜700 bp) with primer pairs: H15915-L15369 and H15488-L15153 (Fu, 2000; Murphy et al., 2000). The total volume of the PCR reaction was 21µl including DNA - 2-4µ, Promega Taq polymerase - 1 U, 5x Promega buffer, MgCl2 - 1 µM, dNTP - 0.1 µM each, and primer concentrations at 0.1 µM. In order to check for the errors during the preparing of the PCR (contamination, pippeting, etc.) we used a negative and positive controls with each reaction. The thermo cycler profile was as follows: 93°C for 3 min, 30 cycles at 93 °C for 1 min, 53 °C for 1 min, 69°C for 2 min and 70°C for 10 min. We ran the sequences on ABI 3130 sequencer in both directions. For editing the sequences we used SEQSCAPE 2.5 (Applied Biosystems Inc., Foster City, CA, USA) (chapter 4 - Gabelaia et al., 2015; chapter 5 - Tarkhnishvili et al., 2016).

For sequence alignment we used BioEdit 7.1.3.0 software (Hall, 1999). For data analysis we used various software packages, depending on the research question: MEGA v.6.06 (Tamura et al., 2013), BEAST v.1.5.1 and v.1.8.0 (Drummond and Rambaut, 2007), NETWORK v.4.6 and 4.6.1.1 (Fluxus Technology Ltd., 2004).

1.2.3 'Traditional' morphometric analysis

For the 'traditional' morphometric analysis we used 2D photographs of lizards' dorsal, lateral and anal areas, taken during field works. The number and type of scalation characters (Fig. 4) were chosen according to the research question (see chapter 4 - Gabelaia et al., 2015 and chapter 5 - Tarkhnishvili et al., 2016).

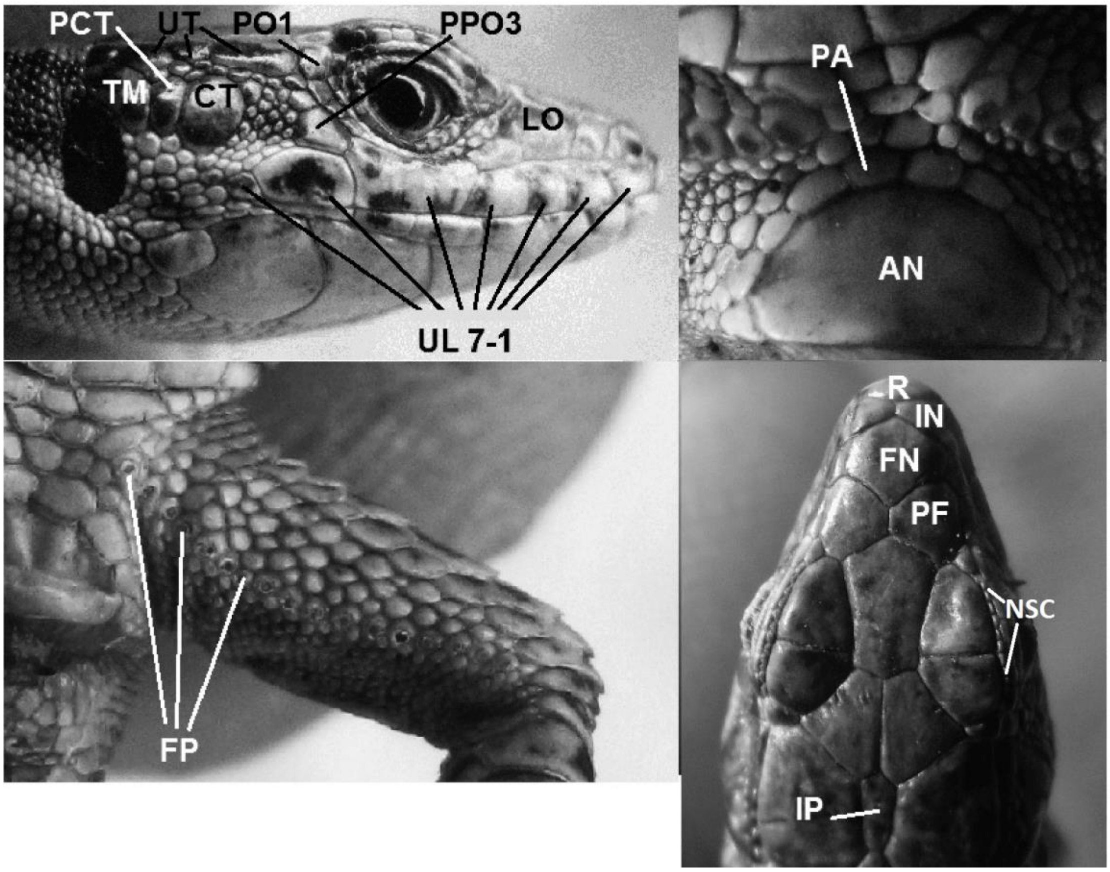

Figure 4. Scalation characters used for 'traditional' morphometric analyses: CT – central temporal scale; PCT – post central temporal; TM – tympanal; PO1 – postorbital 1; PPO3 – post postorbital 3; UL – upper labial; LO – loreal; AN – anal scale; PA – preanal scales; PF – prefrontal scales; IN – internasals; FN – frontonasals; R – rostral; IP – interparietal; FP – femoral pores; HL/HW – length of pileus divided by maximum width of pileus; NSC – small scales between the inner and outer rows of supraoculars (modified from chapter 4 - Gabelaia et al., 2015).

The character descriptions included the number, absence/presence, displacement and/or shape of the specific character or their intersections (the character placements on the lizards and exact descriptors for each of them can be found in the chapters 4 – Gabelaia et al., 2015 and 5 – Tarkhnishvili et al., 2016).

For statistical analysis we used IBM SPSS v21.0 (IBM Corp., 2012).

1.2.4 Geometric morphometric analysis

The approaches of GM used in the research are two-dimensional Fourier outline shape analysis (Lestrel, 1997), as well as three-dimensional landmark-based GM analysis (Dean, 1996).

For the 2D outline shape analysis we used 2D photographs of dorsal, lateral and anal areas. We traced the outlines of the study areas in Corel-draw Graphics Suite x7 (Corel Corporation). Outline digitization error was quantified according to the protocol by Adriaens (2007) – http://tiny.cc/htuz4y. We then applied an elliptic Fourier analysis using the software Shape (Iwata and Ukai, 2002). The process of the shape evaluation in Shape software is the following: the outline shape is extracted as a chain-code and transformed in shape harmonics described by elliptic Fourier descriptors (four elliptic Fourier descriptors for each harmonic). The software then normalizes for size, orientation and a starting point of the outlines according to the first harmonic's (shape descriptor) major axis. The software then conducts a principal component analysis (PCA) on the elliptic Fourier descriptors (Lestrel, 1997) and quantifies the principal component (PC) scores for each sample.

For the 3D GM analysis we used 3D models (constructed from multiple 2D photographs). The process for 3D model construction is as follows: we collect live individuals at the field, anesthetize them temporarily using chloroform and place them in small tube in the centre of a cardboard circle, so that the head of the lizard is pointed upwards. After, by moving the camera around the lizard, we photograph the head 36 times from a perspective of 90° to the midline and 36 times from a 45° perspective. The 2D digital images are uploaded in AgiSoft PhotoScan Pro v.1.2.6 (Agisoft, 2016) in order to generate 3D models of the lizards' head.

After creating the 3D external head models, we digitize homologous landmarks on their scalation pattern using AgiSoft PhotoScan Pro v.1.2.6, extract the 3D coordinates of the landmarks and arrange them in Microsoft Excel (Microsoft Corporation, 2007). For aligning and scaling 3D coordinates we apply Procrustes superimposition and conduct PCA analysis using the software PAST (Hammer et al., 2001).

For the analysis of 3D shape data, we use a number of statistical packages – PAST (Hammer et al., 2001), MorphoJ (Klingenberg, 2011), R (R Development Core Team, 2016), and IBM SPSS v21.0 (IBM Corp., 2012).

In order to see how the phenotypes vary along the species, we integrate the genetic and morphological approaches in each chapter.

1.3 General Discussion

Since the beginning of the organisms' speciation study, morphological data has been widely used for delimiting the boundaries of the species and reconstructing their evolutionary relations, and has later been paired with genetic (mostly mtDNA) studies for the better perspective on taxonomy and phylogenetic relationships (Wiens and Penkrot, 2002; Kaliontzopoulou, 2011). In this doctoral study we tried to find the links of the morphological variation among *Darevskia* with their phylogenetic relationships and species' boundaries.

1.3.1 Phylogeny versus morphology

The congruence of the morphological variation with the species' phylogeny has been discussed in many vertebrate studies (Ivanović et al., 2008; Gentilli et al., 2009; Henderson et al., 2013), but not always confirmed (Wiens and Penkrot, 2002; López-Fernández et al., 2005;

Steppan, 1998; Serb et al., 2001), especially when dealing with morphologically and genetically diverse species groups. The methodology of assessing the morphology is important: as described in the introduction, the 'traditional' qualitative morphological analysis has been unable to associate the morphological characteristics of the Caucasian rock lizards with their phylogenetic relationships (Fig. 1), while multiple studies (including ones described in chapters 2 – Gabelaia et al., 2017 and 3 – Tarkhnishvili et al., submitted) have shown that geometric morphometric techniques assess the shape variation better than 'traditional' morphometrics (see chapters 4 – Gabelaia et al., 2015 and 5 – Tarkhnishvili et al., 2016) and perform better in reconstructing phylogenetic relationships. Indeed, according to the 2-dimensional Fourier outline shape analysis, the phylogenetic signal was detected in the anal scale shape of the six species (the divergence seems to be among the major clades rather than individual species – the same pattern was observed once 13 species from all four clades were included in the analysis) and head's frontal scale shape of 13 species of *Darevskia* lizards, and the dendrogram of the 'species mean shape' (UPGMA clustering of anal scale shapes based on Euclidean distances between the species medians - separately for males and females) perfectly complemented the mtDNA phylogenetic tree. Moreover, 3D GM was able to extract a phylogenetic signal from the external head shape for most of the species of *Darevskia*, despite the strong convergent pattern in it. Once we excluded the shape components associated with habitat, size and sex (the effect of the size and sex on the head shape variation were minimal compared to the habitat effect) from the morphological data, we were able to build an unrooted neighbor-joining tree that was congruent to the mtDNA one (chapter 3 - Tarkhnishvili et al., Submitted). Consequently, according to our study, GM shape analysis shows to be more effective in reflecting the species phylogenetic relationships than 'traditional' morphometric analysis.

Besides the advantageous and disadvantageous methodological approaches discussed here, the concordance level of morphological and genetic variation strongly depends on the morphological feature being studied. Our analysis (in chapter 3 – Tarkhnishvili et al., submitted) showed that in spite of the convergent patterns in *Darevskia* lizards' head shape there were still components of the head shape carrying clear phylogenetic signal. Indeed multiple studies have successfully reconstructed phylogenetic relationships using head structure (Gentilli et al., 2009; Ivanović et al., 2008; Ivanović et al., 2009).

According to Thorpe et al. (1995) different morphological characters (which substantially vary among different populations) can either reflect the influence of only phylogeny, environmental conditions or both at once. In their study Thorpe with coauthors (1995) showed that different populations of the Canary island lizards *Gallotia galloti* showed different associations of morphological variation (with phylogeny, environmental conditions or both) when examining their scalation, color pattern and body dimensions. In *Darevskia* lizards, some features of the head shape (associated with flatness of the rostral part of the head, width of the lower jaw with concave vs straight edges, and relative length of the interparietal scale) and frontal scale (associated with a more angled anterior edge of the frontal scale) seem to be less affected by environmental conditions and are more phylogenetically conservative than other shape components. On contra, the variation of the shape component associated with head height (the largest variation in the head shape) completely depended on the habitat, irrespective of phylogeny. This is also in line with the observed fact, that lacertid's head shapes often show a significant influence of the habitat type (Kaliontzopoulou, 2011).

The presence of convergence doesn't necessarily cover up the phylogenetic signal in the shape. The study by Claude et al. (2004) also showed that Testudinoidea turtles' skull shape variation was mostly explained by the habitat type and diet, but still remained phylogenetically informative. Indeed, according to our study (see chapter 3 - Tarkhnishvili et al., submitted), the high level of divergence among the 3D head structure of *Darevskia* lizards is reflecting both the ecological preferences of the species as well as phylogenetic.

1.3.2 Species boundaries versus morphology

The chapters 4, 5, 6 (Gabelaia et al., 2015; Tarkhnishvili et al., 2016; Gabelaia et al., 2018 respectively) are associated with finding the congruence between genetically and morphologically defined species boundaries. The application of 'traditional' morphometrics based on qualitative scalation traits provided the congruent pattern with *D. mixta* phylogeographic pattern, but not for *D. brauneri* (chapters 4 – Gabelaia et al., 2015 and 5 – Tarkhnishvili et al., 2016). In *D. mixta* the morphological differences between the Greater and the Lesser Caucasus populations, which belong to isolated, long separated mitochondrial lineages, were still not fixed and strong morphological overlap occurred (chapter 4 - Gabelaia et al., 2015).

Unlike, 'traditional' (qualitative) morphometrics, the application of 3D geometric morphometrics was more powerful for species morphological delimitation and was able to separate (with minor overlaps) the closely related species within a single major clade '*rudis*' (2D GM failed to do so), and even separated the two subspecies of a single species, supporting earlier genetic studies on the same taxa (chapter 6 - Gabelaia et al., 2018).

In conclusion, according to this study reconstructing phylogenetic relationships from geometric morphometric data that match with the species' molecular phylogeny is possible, once the convergent components are removed from it. Yet, revealing the sole feature or method for species' morphological delimitation remains ambiguous. Three-dimensional geometric morphometrics has been most precise as a sole method for defining the species boundaries, though has been most effective when integrated with mitochondrial DNA analysis, which (the integrative approach) has often been used successfully for delimiting the species boundaries (Leache et al., 2009; Siriwut et al., 2015; Davis et al., 2016).

1.3.3 Future perspectives

The 3D GM analysis of the lizards head shape was able to separate the close related species of *Darevskia* and even helped to reconstruct the species phylogeny congruent to that based on mtDNA only after detailed assessment of habitat-related traits and their elimination from the analysis.

The morphological analysis in this study was based mainly on scalation features, which are highly variable and homoplastic morphological traits in *Darevskia*; even though the 3-dimensional GM analysis reflected the shape of the head structure as a whole, the shape data was largely dependent on scalation, as we digitized landmarks on the homologous intersections of the scalation characters. There have been multiple studies describing the homoplasy in the external characters of the reptiles. Harmon et al. (2005) revealed ecomorphic convergences in the external head shape of 21 Caribbean *Anolis* lizard species. de Broin et al. (2006) has also described multiple homoplastic carapace characters among the *Testudo, Agrionemys* and *Eurotestudo* lineages.

I hypothesize that the convergent patterns in head shape and other peculiarities of external phenotype may depend on different structural changes and developmental patterns, which one can infer only in the course of deeper analysis of anatomical structures of the animals.

On the contrary, the skull shape is less prone to homoplasy (Ivanović et al., 2008) and consequently, the application of the 3D GM on the skull surface could be more species-specific and conservative for delimiting the species' morphological boundaries than the external head shape analysis.

Even though our study didn't highlight the notable methodological limitations associated with GM approaches we've used, we plan to use the relatively new GM approach: landmark-free method - Generalized Procrustes Surface Analysis (GPSA), in comparison to the 3D landmark-based analysis. This method is different from the landmark-based one, because it uses surface scans itself for the superimposition of the shapes. Superimposition is achieved through iterative closest point (ICP) algorithm. ICP chooses the closest points from each surface, creates the mean 'reference' surface and then superimposes the surfaces such, that the distances between theirs and 'reference' surface points is reduced. By each iteration: the mean 'reference' surface is recalculated, the distances between the 'reference' and individual points are recalculated and surfaces are superimposed until the changes in the 'reference' surface cannot be significantly reduced. After the surfaces are finally superimposed, distance metrics (similar to Procrustes distance used for landmark-based data) are calculated for the further analysis (Pomidor et al., 2016).

The advantage of the GPSA over landmark-based analysis is the better visualization and the larger amount of the shape information obtained when studying smooth objects like skull, whilst landmark-based method is limited in potential homologous landmark points (Pomidor et al., 2016).

Finally, the comparison of these two methods will allow us to see which method performs better in assessing phylogenetic signal and delimiting *Darevskia* species' boundaries based on skull shape.

1.3.4 Summary

Caucasian rock lizards of the genus *Darevskia* are a diverse group of small lizards mainly living in the Greater and the Lesser Caucasus. It is difficult to delimit *Darevskia* species due to a high degree of morphological similarity and overlapping identification characters. Additionally, they also strongly hybridize and thus individuals with intermediate morphology and genetic characters are found. As a result, there are extreme difficulties in assigning found individuals to a distinct species. This is particularly true for the traditional morphological methods such as qualitative morphometrics, where multiple measurements of the body and scales are compared with each other.

In the present project, we aimed to find the links between the phylogenetic relationships and morphological similarities in *Darevskia,* and apply genetic and morphological methods, or their combination, for delimiting the species boundaries. We mainly focus on the matrilineal phylogeny and geometric morphometric techniques (two-dimensional Fourier outline shape analysis and three-dimensional landmark-based analysis in comparison with 'traditional' morphometrics). More specifically we aim to reveal the morphological features which are species-specific and follow the species evolutionary divergence pattern.

In the Chapter 1, we briefly summarize the aim of this PhD study, the used methodological approaches, the major results and findings and future perspectives. Geometric morphometrics is indeed able to capture the shape variation with clear phylogenetic signal in it and is even able to reconstruct the tree based on the scalation features of *Darevskia* lizards. This is congruent to the mtDNA tree, once the environmental adaptive morphological features are detected and removed from the data. None of the sole morphological methods are powerful enough for delimiting the species boundaries (especially the closely related ones), but in general, 3D geometric morphometrics is the most effective approach, especially when integrated with mtDNA.

In chapter 2, we aim to resolve phylogenetic relationships using 2D outline Fourier shape analysis based on anal and pileus shapes and compare it with the trees based on mtDNA and 'traditional' morphometrics. UPGMA analysis grouped ~200 individuals representing six species according to their basal matrilineal clades ('*rudis*' and '*caucasica*'). Consequently, 2D GM shape analysis showed to be more effective in reflecting the species phylogenetic relationships than 'traditional' morphometric analysis.

In chapter 3, we apply 3D geometric morphometrics on the heads of 13 species of *Darevskia* lizards to see if the phylogenetic relationships can be reflected in some features of head shape. We also analyze the 2D anal, 5th upper labial and head's frontal scales' shapes of the same species using Fourier outline shape analysis. The largest variation associated with the 3D head shape resembled ecological preferences (such as higher vs flat head), clearly separating the terrestrial species of *Darevskia* from the rock-dwellers, though other shape components did resemble the significant phylogenetic signal in the head shape, as well as in the frontal scale shape. Centroids of taxa obtained from the discriminant function analysis even helped to reconstruct a tree that was congruent to the mtDNA tree of the species, once the habitat, sex and size-related (only habitat-related shape variation was substantial, others were minor) shape components were initially excluded from the analysis. Anal scale shape, on the other hand helped to distinguish among the major mtDNA clades and sex, rather than individual species.

Chapter 4 describes the two distinct populations of *D. mixta* species distributed in Colchic humid forest refugium (east of the black sea on the Greater and the Lesser Caucasus). According to mtDNA analysis the populations of the Greater and the Lesser Caucasus are monophyletic and their evolutionary lineages diverged in the middle Pleistocene. Indeed, according to the studies by Tarkhnishvili et al. (2000) and Mumladze et al. (2013) this area often serves as two separate refugia of the Greater and the Lesser Caucasus populations for the small animals. The scalation pattern in these two populations is different too: the lizards from the Greater Caucasus don't have a double preanal scale, and the individuals from the Lesser Caucasus have an additional scale in temporal area. Also the individuals from the Lesser Caucasus represent two geographically distinct populations and are genetically and morphologically more diverse than the Greater Caucasus population. The central part of the Lesser Caucasus is most probably where the *D. mixta* evolutionary lineage initiated.

Chapter 5 describes another example of the intraspecific genetic and morphological divergence due to the geographic isolation caused by Pleistocene-time fragmentation in the Western Greater Caucasus. Even though the '*saxicola*' clade species have been studied extensively, none of the studies included *D. brauneri* samples from the Georgian populations. Thus we decided to study the genetic variation of *D. brauneri* in order to assess its taxonomic status and look into the matrilineal relationships of the Georgian *D. brauneri* populations.

We studied the mtDNA fragments of *D. saxicola* clade lizards – *D. brauneri* from the two easternmost populations in Western Georgia - Tekhuri and Enguri valleys. We found that these two populations are genetically very distinct, both from each other and from the earlier described *D. brauneri*. More specifically, Tekhuri Gorge lizards are placed basally within the '*saxicola*' clade, while Enguri Gorge lizards are closer to the Northern Caucasus *D. saxicola,* than to the other *D. brauneri.* Morphologically, Tekhuri Gorge lizards have wider heads compared to the other *D. brauneri.*

In chapter 6, we aimed to differentiate the closely related species within the single major matrilineal clade by using 3-dimensional geometric morphometric techniques, which was not possible with the application of 2-dimensional outline shape analysis. We quantified the head shape in three closely related species of the '*rudis*' clade: *D. rudis, D. portschinskii* and *D. valentini.* The 3D GM analysis was able to differentiate the closely related species and could even separate the subspecies *D. rudis obscura* from the rest of *D. rudis* nominal species. This pattern was in line with the previous mtDNA observations, thus we suggested the elevation of *D. rudis obscura* to species level – *Darevskia obscura.*

In conclusion to our study, the head shape and even some scalation features of *Darevskia* species can be suitable markers for depicting the species phylogenetic relationships or delimiting the species boundaries when assessed with geometric morphometric techniques, but perform the best if integrated with genetic (mtDNA) analysis.

1.3.4 Samenvatting

Kaukasische rotshagedissen van het geslacht *Darevskia* vormen een diverse groep van kleine hagedissen die hoofdzakelijk voorkomen in de Grote en de Kleine Kaukasus. *Darevskia* soorten zijn echter zeer moeilijk van elkaar te onderscheiden als gevolg van hun grote mate van morfologische gelijkenissen en de overlap in kenmerken die gebruikt worden om de soorten te identificeren. Bovendien vertonen ze intensieve hybridisatie, waardoor individuen met een intermediaire morfologie en genetische merkers worden teruggevonden. Dit maakt het extreem moeilijk om de gevonden specimens toe te wijzen aan gekende soorten. Dit is vooral zo indien gebruik wordt gemaakt van traditionele morfologische onderzoeksmethodes, zoals kwalitatieve morfometrie, waarbij meerdere lengtemetingen worden uitgevoerd op het lichaam en de schubben, om dan onderling te vergelijken.

In dit project trachtten we de verbanden te achterhalen tussen de fylogenetische relaties en de morfologische gelijkenissen in *Darevskia,* waarbij op basis van genetische en morfologische methods (afzonderlijk en gecombineerd) gebruikt worden om soorten af te bakenen. We richten ons hier vooral op de matrilineaire fylogenie en geometrisch morfometrische technieken (twee-dimensionale Fourier analyse om de vorm op basis van contouren te analyseren, en drie-dimensionale vorm analyses op basis van landmarks, en dit vergeleken met meer 'traditionele' morphometrie). Meer specifiek is het doel om de morfologische eigenschappen te achterhalen die soort-specifiek zijn, en zodoende de evolutionaire divergentiepatronen van de soorten te achterhalen.

In Hoofdstuk 1 worden de doelstellingen van de thesis kort toegelicht, evenals de toegepaste methodologieën, de belangrijkste resultaten en bevindingen, evenals enkele toekomstperspectieven. De geometrisch morfometrische benadering slaagt er wel degelijk in om een fylogenetisch signaal te capteren in de vormvariatie, en laat zelfs toe om een verwantschapsboom te reconstrueren op basis van eigenschappen van de schubben bij *Darevskia*-hagedissen. Deze boom is congruent met de boom verkregen op basis van mtDNA, van zodra omgevingsgebonden, adaptieve kenmerken worden gedetecteerd en verwijderd uit de dataset. Geen enkele van de strikt morfologische methodes was krachtig genoeg om soorten volledig ondubbelzinnig af te bakenen, maar de 3D geometrisch-morfometrische benadering gaf wel het beste resultaat, vooral wanneer deze met mtDNA data werd geïntegreerd.

In Hoofdstuk 2 hebben we getracht om de fylogenetische relaties van de soorten te achterhalen op basis van 2D contour-data op de vorm van de anale en pileus-schub, welke werden geanalyseerd via een elliptische Fourier analyse. Deze resultaten werden vergeleken met de cladogrammen op basis van de mtDNA en de resultaten van de 'traditionele' morphometrie. Een UPGMA analyse groepeerde de ~200 individuen, die zes soorten vertegenwoordigen, volgens hun basale, matrilineaire clades ('*rudis*' and '*caucasica*'). Er kon dus worden besloten dat de 2D GM vormanalyse het meest accuraat de fylogenie van de soorten weerspiegelt.

In hoofdstuk 3 worden de resultaten beschreven van de 3D geometrisch morfometrische analyse op de kop van 13 *Darevskia* species, om na te gaan of de fylogenetische verwantschappen weerspiegeld worden in de kopvorm. We hebben ook de 2D vorm geanalyseerd van de anale, 5^{e} bovenste labiale and de frontale (op de kop) schubben met behulp van op contouren gebaseerde elliptische Fourier analyse. De grootste variatie in de 3D kopvorm vertoonde gelijkenissen die eerder ecologisch gerelateerd zijn (zoals een hogere of plattere kop), waarbij terrestrische *Darevskia* soorten duidelijk gescheiden worden van de rotsbewoners. Ook andere aspecten van de vorm bleken een fylogenetisch signaal weer te geven, zoals de vorm van de frontale schub. Op basis van de centroid-data van de taxa, verkregen uit de discriminant functie-analyse, kon een verwantschapsboom worden gegenereerd die congruent was met de boom verkregen uit mtDNA data, van zodra data over habitat, seks en grootte-gebonden vormcomponenten uit de analyse werden gelaten. Hiervan was vooral habitat-gerelateerde data belangrijk. De vorm van de anale schub liet dan beter toe om de belangrijkste mtDNA-clades te onderscheiden, evenals verschillen tussen geslachten, eerder dan verschillen tussen soorten.

In Hoofdstuk 4 worden de twee verschillende populaties van *D. mixta* besproken, welke voorkomen in de vochtige Colchic wouden (ten oosten van de Zwarte Zee, in de Grote en Kleine Kaukasus). De mtDNA analyse leert dat de populaties van de Grote en Kleine Kaukasus monofyletisch zijn, en dat hun evolutionaire lijnen van elkaar gesplitst zijn gedurende het Midden Pleistoceen. Zoals aangetoond door Tarkhnishvili et al. (2000) en Mumladze et al. (2013) vormen beide gebieden twee gescheiden refugia voor de Grote en Kleine Kaukasus populaties. Beide populaties vertonen ook een verschil in schubpatroon: de hagedissen van de Grote Kaukasus hebben géén dubbele preanale schub, en deze van de Kleine Kaukasus hebben een additionele schub in de temporale regio. Deze laatste groep wordt ook vertegenwoordigd door twee verschillende geografische populaties die ook genetisch en morfologisch diverser zijn dan die van de Grote Kaukasus. De oorsprong van de *D. mixta* evolutielijn situeert zich vermoedelijk in het centraal gedeelte van de Kleine Kaukasus.

In Hoofdstuk 5 wordt een ander voorbeeld van genetische en morfologische divergentie op intraspecifiek niveau besproken. Deze divergentie is het gevolg van een geografische isolatie als gevolg van een fragmentatie in het westelijk deel van de Grote Kaukasus, gedurende het Pleistoceen. Desondanks dat de soorten van de '*saxicola*' clade reeds intensief warden bestudeerd, toch werden in deze studies nooit de *D. brauneri* specimens van de Georgische populaties mee opgenomen. Vandaar dat hier de genetische variatie van *D. brauneri* werd bestudeerd, zodat de taxonomische status kon worden bepaald, en er meer inzicht werd verworven in hun positie binnen de matrilineaire verbanden.

mtDNA fragmenten van de hagedissen van de *D. saxicola* clade werden bestudeerd, meer bepaald *D. brauneri* uit de twee meest oostelijke populaties in West-Georgië - Tekhuri en Enguri valleien. We konden aantonen dat deze beide populaties genetisch zeer verschillend zijn, zowel van elkaar als van de eerder beschreven *D. brauneri*. Meer specifiek, de Tekhuri-vallei hagedissen vormen een basale clade in de '*saxicola*' clade, terwijl deze uit de Enguri vallei nauwer verwant zijn aan de *D. saxicola* uit de noordelijke Kaukaus dan aan de andere *D. brauneri.* De hagedissen van de Tekhuri-vallei hebben ook bredere koppen dan die van *D. brauneri.*

In Hoofdstuk 6 hebben we getracht om de nauw verwante soorten binnen één matrilineaire clade te onderscheiden door gebruik te maken van 3-dimensionale, geometrisch morfometrische technieken, iets wat niet gelukt was op basis van de 2D contour data. We kwantificeerden de kopvorm in drie nauwverwante soorten uit de '*rudis*' clade: *D. rudis, D. portschinskii* en *D. valentini* . De 3D GM analyse liet toe om de nauwverwante soorten van elkaar te onderscheiden, en zelfs de subspecies *D. rudis obscura* e onderscheiden van de rest van de *D. rudis* nominale species. Dit patroon komt overeen met de eerdere mtDNA observaties, op basis van welke het advies wordt geformuleerd om *D. rudis obscura* op te waarderen tot soortsniveau: *Darevskia obscura.*

Een conclusie van dit onderzoek is dat zowel de kopvorm, maar ook bepaalde eigenschappen in het schubpatroon bij de *Darevskia* species bruikbare indicatoren zijn om fylogenetische verwantschappen te achterhalen, of voor het identificeren en afbakenen van soorten. Betrouwbare resultaten worden echter enkel verkregen indien de data geïntegreerd werd met de genetische (mtDNA) analyses.

1.3.4 შეჯამება

Darevskia გვარის კავკასიური კლდის ხვლიკები მცირე ზომის ხვლიკების მრავალფეროვანი ჯგუფია, რომლებიც ძირითადად დიდსა და მცირე კავკასიონზე ცხოვრობენ. *Darevskia*-ს სახეობების საზღვრების დადგენა რთულია, მათი გარეგნული მსგავსებისა და სახეობებს შორის გადაფარვადი მორფოლოგიური ნიშნების გამო. ამას დამატებით ისინი ხშირად ჰიბრიდირებენ და შუალედური მორფოლოგიისა და გენეტიკის მქონე ინდივიდები ხშირია. შედეგად, ამ ინდივიდების რომელიმე ცალკეული სახეობისთვის მიკუთვნება ძალიან რთულია. ეს განსაკუთრებით ვლინდება თვისობრივი მორფომეტრიული მეთოდებით შეფასებისას, რომელიც სხეულისა და ქერცლების სხვადასხვა განაზომებს ეფუძნება.

ამ კვლევაში, ჩვენი მიზანია ვიპოვოთ თანხვედრა ფილოგენეტიკურ კავშირებსა და მორფოლოგიურ მსგავსებებს შორის *Darevskia* სახეობებში და გამოვიყენოთ გენეტიკური და მორფოლოგიური მეთოდები, ან მათი კომბინაცია სახეობების საზღვრების დასადგენად. კვლევისას ძირითადად ვიყენებთ მიტოქონდრიული დნმ-ის ხაზსა და გეომეტრიულ მორფომეტრიულ მეთოდებს (ფორმის ორ-განზომილებიანი ფურიეს კონტურული ანალიზი და სამ-განზომილებიან ლენდმარკებზე დაფუძნებული ანალიზი, 'ტრადიციული' მორფომეტრიის საპირისპიროდ) და ვცდილობთ გამოვავლინოთ ის მორფოლოგიური ნიშნები, რომლებიც ცალკეული სახეობებისთვისაა დამახასიათებელი და კარგად აღწერს სახეობათა დივერგენციას.

პირველ თავში, ჩვენ მოკლედ ვაჯამებთ ამ სადოქტორო კვლევის მიზანს, გამოყენებულ მეთოდოლოგიას, მთავარ შედეგებს და კვლევის სამომავლო მიმართულებას. კვლევის შედეგად აღმოჩნდა რომ გეომეტრიული მორფომეტრიის გამოყენებით შესაძლებელია *Darevskia*-ს სახეობებში არსებულ მორფოლოგიურ ცვალებადობაში ფილოგენეტიკური სიგნალის დაჭერა და მიტოქონდრიულ დნმზე დაფუძნებული ხის შესაბამისი ხის აგება, იმ შემთხვევაში თუ ანალიზიდან გამოვრიცხავთ ფორმის ისეთ კომპონენტებს რომლებიც გარემო პირობებთან არის დაკავშირებული. არცერთი ცალკეული მორფოლოგიური მეთოდი არ იძლევა სახეობათა საზღვრების ზუსტად დადგენის საშუალებას (განსაკუთრებით ახლო

მონათესავე სახეობების), თუმცა სამ-განზომილებიანი გეომეტრიული მორფომეტრია ყველაზე ეფექტურია, განსაკუთრებით როდესაც თან მიტოქონდრიული დნმ-ის ანალიზს ვურთავთ.

მეორე თავში, ჩვენი მიზანია ხვლიკების ფილოგენეტიკური კავშირების დადგენა მათ ანალურ ქერცლსა და პილეუსის ფორმაზე ორ-განზომილებიანი ფურიეს კონტურული ანალიზის გამოყენებით. შედეგებს ჩვენ ვადარებთ მიტოქონდრიული დნმ-ის ანალიზზე დაფუძნებულ ხესთან და 'ტრადიციული' მორფომეტრიის შედეგებთან. UPGMA ანალიზმა ჩვენს მიერ შერჩეული ექვსი სახეობის 200-მდე ინდივიდი დააჯგუფა მათი შესაბამისი მიტოქონდრიული ხაზის კლადაში ('*rudis*' და '*caucasica*'). შესაბამისად, ორ-განზომილებიანი გეომეტრიულ მორფომეტრიული ანალიზი უფრო ეფექტური გამოდგა სახეობების ფილოგენეტიკური კავშირების დასადგენად ვიდრე 'ტრადიციული' მორფომეტრიული ანალიზი.

მესამე თავში, ჩვენ ვიყენებთ სამ-განზომილებიან გეომეტრიულ მორფომეტრიას *Darevskia*-ს 13 სახეობის თავის ფორმაზე, რათა ვნახოთ მოიცავს თუ არა თავის ფორმა ფილოგენეტიკურად ინფორმატიულ ნიშნებს. ასევე, ჩვენ ვაანალიზებთ იგივე 13 სახეობის ანალური, ზემო ყბის მე5 ქერცლის და თავის ფრონტალური ქერცლების ორ-განზომილებიან ფორმას ფურიეს კონტურული ანალიზის საშუალებით. თავის სამ-განზომილებიანი ფორმის მთავარი კომპონენტი ეკოლოგიურ მახასიათებლებს ავლენს (მაღალი თავის ფორმა ბრტყელ თავის ფორმასთან შედარებით), რაც მკაფიოდ განასხვავებს *Darevskia* გვარის მიწაზე მცხოვრებ სახეობებს კლდეზე მცოცავი სახეობებისგან, თუმცა თავის სამ-განზომილებიანი ფორმის სხვა კომპონენტებმა, ისევე როგორც თავის ფრონტალური ქერცლის ორ-განზომილებიანმა ფორმამ გამოავლინეს ფილოგენეტიკური სიგნალი. მას შემდეგ რაც ისეთი კომპონენტები გამოვრიცხეთ თავის ფორმიდან, რომლებიც ჰაბიტატზე, სქესსა და ზომაზე იყვნენ დამოკიდებული (ჰაბიტატი ყველაზე მნიშვნელოვანი ფაქტორი იყო თავის სამ-განზომილებიანი ფორმის შეფასებისას, დანარჩენების წილი საკმაოდ მცირეა), დისკრიმინანტული ანალიზის შედეგად მიღებულ სახეობათა ცენტროიდებზე ავაგეთ მიტოქონდრიულ დნმზე დაფუძნებული ხის იდენტური ხე. რაც შეეხება

ანალური ქერცლის ფორმას, ის აღწერს მთავარ კლადებს შორის (და სქესთა შორის) ვარიაციას და არა ცალკეულ სახეობებს შორის.

მეოთხე თავში აღწერილია *D. mixta*-ს ორი განსხვავებული პოპულაცია, რომლებიც კოლხური ტყის რეფუგიუმში მდებარეობენ (შავი ზღვის აღმოსავლეთით დიდსა და მცირე კავკასიონზე). მიტოქონდრიული დნმის ანალიზის საფუძველზე დიდი და მცირე კავკასიონის პოპულაციები მონოფილეტურებია და მათი ევოლუციური ხაზები შუა პლეისტოცენის დროს გაიყო. ამასთან შესაბამისობაშია თარხნიშვილის და თანაავტორების (2000) და მუმლაძის და თანაავტორების (2013) კვლევები, რომელთა თანახმადაც ეს ტერიტორია პატარა ზომის ცხოველებისთვის ორ განსხვავებულ რეფუგიუმს წარმოადგენს - დიდი კავკასიონის და მცირე კავკასიონის პოპულაციები. ქერცლების განლაგება *D. mixta*-ს ამ ორ პოპულაციაში ასევე განსხვავებულია ერთმანეთისგან: დიდი კავკასიონის ინდივიდებს არ აქვთ ორმაგი პრეანალური ქერცლი, ხოლო მცირე კავკასიონის ინდივიდებს აქვთ დამატებითი ქერცლი საფეთქლის არეში. მცირე კავკასიონის ინდივიდები თავის მხრივ წარმოადგენენ ორ გეოგრაფიულად განსხვავებულ პოპულაციას და გენეტიკურადაც და მორფოლოგიურადაც უფრო მრავალფეროვანნი არიან ვიდრე დიდი კავკასიონის პოპულაცია. სწორედ მცირე კავკასიონის ცენტრალურ ნაწილს მოვიაზრებთ *D. mixta*-ს ევოლუციური ხაზის საწყისად.

მეხუთე თავი აღწერს კიდევ ერთ მაგალითს გეოგრაფიული იზოლაციის შედეგად გამოწვეული შიდასახეობრივი გენეტიკური და მორფოლოგიური დივერგენციისა, რომელიც პლეისტოცენის დროს არსებული ფრაგმენტაციის შედეგად ჩამოყალიბდა დასავლეთ დიდ კავკასიონზე. მიუხედავად იმისა, რომ '*saxicola*' კლადის სახეობები დიდი ხნის განმავლობაში შეისწავლებოდნენ, არც ერთი კვლევა არ მოიცავდა *D. brauneri*-ის სინჯებს ქართული პოპულაციებიდან. ამ მიზეზის გამო ჩვენ გადავწყვიტეთ შეგვესწავლა *D. brauneri*-ის გენეტიკური ცვალებადობა, რათა გადაგვემოწმებინა მისი ტაქსონომიური სტატუსი და გავრკვეულიყავით *D. brauneri*-ის ქართული პოპულაციების მიტოქონდრიული ევოლუციური ხაზების ურთიერთობებში.

ჩვენ შევისწავლეთ *D. saxicola* კლადის სახეობის *D. brauneri*-ის მიტოქონდრიული დნმ ფრაგმენტები დასავლეთ საქართველოს ორი უკიდურესი აღმოსავლეთი პოპულაციებიდან - ტეხურის და ენგურის ხეობებიდან. ჩვენი კვლევის შედეგად აღმოჩნდა, რომ ეს ორი პოპულაცია გენეტიკურად ძალიან განსხვავდება ერთმანეთისგან და ადრე აღწერილი *D. brauneri*-გან. უფრო კონკრეტულად, ტეხურის ხეობის ხვლიკები ბაზალურ პოზიციას იკავებენ '*saxicola*' კლადაში, ხოლო ენგურის ხეობის ხვლიკები უფრო ახლოს არიან ჩრდილოეთ კავკასიის *D. saxicola*-თან, ვიდრე დანარჩენ *D. brauneri*-ის წარმომადგენლებთან. მორფოლოგიურად, ტეხურის ხეობის ხვლიკებს უფრო განიერი თავი აქვთ ვიდრე სხვა *D. brauneri*-ს.

მეექვსე თავში, ჩვენი მიზანია განვასხვავოთ ახლო მონათესავე სახეობები (ერთსა და იმავე კლადის შიგნით) სამ-განზომილებიანი გეომეტრიულ მორფომეტრიული მეთოდის გამოყენებით, რაც შეუძლებელი იყო ორ განზომილებიანი კონტურული ანალიზის შედეგად. ჩვენ გამოვიყენეთ '*rudis*' კლადაში შემავალი სამი ახლო მონათესავე სახეობის: *D. rudis, D. portschinskii* და *D. valentini* თავის ფორმები. სამ-განზომილებიანი გეომეტრიულ მორფომეტრიული ანალიზის შედეგად შევძელით გაგვესხვავებინა ახლო მონათესავე სახეობები; ასევე გამოიყო ქვესახეობა *D. rudis obscura* დანარჩენი *D. rudis*-გან. ეს შედეგი თანხვედრაშია მიტოქონდრიულ დნმზე დაფუძნებულ კვლევასთან, ამიტომ ჩვენ რეკომენდაცია გავუწიეთ ქვესახეობის *D. rudis obscura* ტაქსონომიური სტატუსის ამაღლებას სახეობის დონეზე - *Darevskia obscura.*

საბოლოოდ, *Darevskia* სახეობების თავისა და ზოგიერთი ქერცლების ფორმაზე გეომეტრიულ მორფომეტრიული ანალიზის გამოყენებით შესაძლებელია მათი ფილოგენეტიკური კავშირების მსგავსი სურათის მიღება, ან სახეობათა საზღვრების დადგენა, თუმცა უფრო ეფექტურია როდესაც გენეტიკურ (მიტოქონდრიული დნმ) ანალიზთან ვაერთიანებთ.

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Chapter 2. Phylogenetic signals in scale shape in Caucasian rock lizards (*Darevskia* species)

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Phylogenetic signals in scale shape in Caucasian rock lizards (*Darevskia* species)

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ABSTRACT

The genus *Darevskia* comprises over 20 species of small-bodied lizards, mainly occurring in the Caucasus Mountain Region. They show differences in body size, scalation and coloration, however, fully diagnostic characters that could separate the species with a high confidence level are still lacking. The early phylogenetic hypothesis of this group was based on 'traditional' analysis of morphology, based on multiple body and head measurements and scalation traits. Later, a molecular phylogeny of the genus rejected some of the proposed topology based on morphological traits. In this paper, we used quantitative morphological data (outline-based shape data) to test phylogenetic similarities, as proposed by these earlier hypotheses. We analyzed the pileus shape and the anal area of more than 200 individuals, representing six species of *Darevskia,* using outline based elliptic Fourier analysis. The analysis did confirm a clustering of the individuals and species (using UPGMA) from the same mitochondrial DNA clade. Hence, the phylogenetic affinity of the major clades in *Darevskia* could be identified using both molecular methods and outline analysis, whereas a traditional quantitative morphological analysis could not.

1. Introduction

Caucasian rock lizards of the genus *Darevskia* Arribas, 1999 are a highly diverse, monophyletic clade, mostly found in the Greater and the Lesser Caucasus (Tarkhnishvili, 2012). There are recent debates on the validity of the name *Caucasilacerta* Harris et al., 1998, a senior synonym of the same group according to Busack et al. (2016). However, considering the disputable taxonomic arguments (Arribas, 2016), we follow the widely used application of the name *Darevskia*.

Darevskia lizards occupy mostly the same type of habitats: rocks along rivers and roads, stony or break constructions, and pebbled river banks. The exceptions are *D. derjugini* Nikolsky, 1898 and *D. praticola* Eversmann, 1834; which prefer open soil or forest litter (Tarkhnishvili, 2012).

The ranges and habitats of a number of species are overlapping. This overlap has been shown in some cases to lead to interspecific hybridization and gene introgression (Darevsky, 1967; Murphy et al., 1996, 2000; Tarkhnishvili, 2012). Most of them are small lizards (SVL 50 mm–88 mm), their scalation patterns are variable and strongly overlap across the species.

The phylogenetic relationships among *Darevskia* species using molecular data was first studied by Murphy and Moritz (Murphy et al., 1996; Moritz et al., 1992). Later, Murphy et al. (2000) attributed bisexual species of *Darevskia* to three monophyletic clades using mtDNA analysis and allozyme electrophoresis: *'caucasica'*, *'saxicola'* and *'rudis'* (Murphy et al., 2000). These studies lead to a taxonomic revision of *Darevskia*, as well as a re-evaluation of their phylogenetic relationships, so far only based on morphological traits (qualitative traits of scalation pattern) in Darevsky's study (1967) (Fig. 1).

Darevsky (1967) identified only six bisexual forms as nominal species: *D. praticola, D. derjugini, D. saxicola* Eversmann, 1834; *D. caucasica* Méhely, 1909; *D. mixta* Méhely, 1909 and *D. rudis* Bedriaga, 1886 (some of them with several subspecies). He also reconstructed their phylogenetic relationships using 'traditional' morphometrics. Murphy and Moritz, on the other hand, identified 15 bisexual nominal species based on molecular data (Murphy et al., 1996; Moritz et al., 1992; Murphy et al., 2000) leading to an important taxonomic regroupings and revision of their phylogenetic relationships.

According to Darevsky (1967), *D. rudis* and *D. valentini* Boettger, 1892 were attributed to a clade different from the one including *D. portschinskii* Kessler, 1878 and *D. parvula* Lantz and Cyren, 1936;

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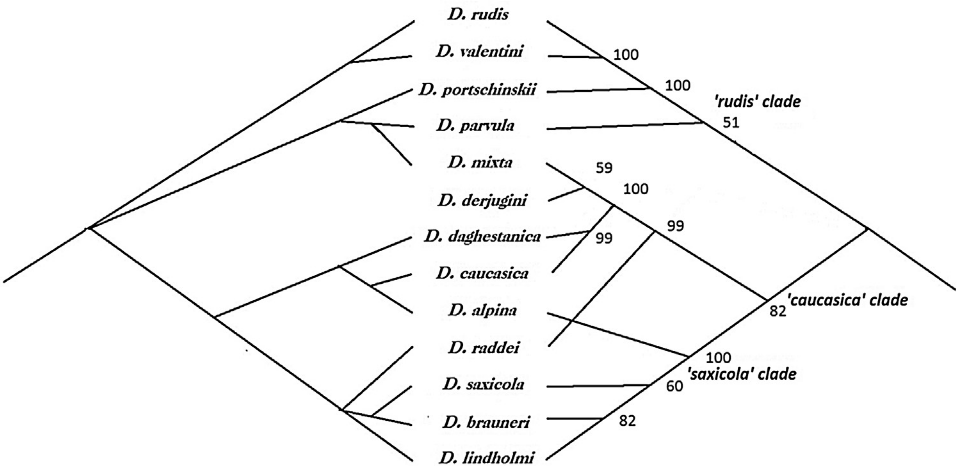

Fig. 1. Phylogenetic hypotheses of *Darevskia*: topology based on qualitative traits of scalation pattern, according to Darevsky (1967) (on the left) and topology based on the parsimony analysis of molecular data (mtDNA), according to Murphy et al. (2000) (on the right). Numbers indicate bootstrap values.

while the mtDNA analysis grouped those four species into a single clade. Also *D. mixta,* which was grouped with *D. parvula* in Darevsky's analysis, was re-grouped into the '*caucasica*' clade based on mtDNA. As a result, it was considered that phylogenetic relationships cannot be reconstructed based on qualitative, morphological traits of *Darevskia* lizards (Fu et al., 1997; MacCulloch et al., 2000; Murphy et al., 2000; Tarkhnishvili, 2012; Tarkhnishvili et al., 2013).

Considering that geometric morphometric approaches are more powerful in capturing shape variation than traditional morphometrics that rely on more qualitative traits (Zelditch et al., 2004; Breno et al., 2011; Maderbacher et al., 2008; Abdel-Rahman et al., 2009; Bernal, 2007; Blanco and Godfrey, 2006), we aimed to study whether interspecific phenotypic patterns (quantified using outline based geometric morphometrics) correspond with the molecular phylogenetic divergence pattern suggested by Murphy et al. (2000). Six species of *Darevskia* lizards, belonging to two clades – '*caucasica*' and '*rudis*', have been chosen for the study. These two clades were selected for this study because of their conterminous distribution across the Caucasus and the northeastern Turkey. This is important as it is known that the phenotypic variation may depend on the environmental conditions, such as altitude and climate (Darevsky, 1967). Comparing individuals of different species from the same geographic areas minimizes the influence of the environment. In contrast, the third clade '*saxicola*' is found exclusively in the western Greater Caucasus, with its range almost not overlapping with that of the other two clades. (Fig. 1): (1) '*caucasica*' clade: *D. mixta* and *D. derjugini* and (2) '*rudis*' clade: *D. valentini, D. rudis, D. portschinskii* and *D. parvula*. Although *D. parvula* is associated with the clade *'rudis'*, it is genetically quite distant from the rest of the species within this clade (Murphy et al., 2000). We analyzed to what degree shape variation in scalation patterns in the studied species reflect phylogenetic signals. We chose two areas: anal scale and pileus (dorsal head scales structure), as both were used by Darevsky for reconstructing the phylogenetic relationships of *Darevskia* species and both are highly suited for outline analysis. We tested whether scale shape can be linked to sex, and the most important, to what degree it reflects phylogenetic relationships between species and clades.

2. Material and methods

2.1. Selected species

All species selected for this study are rock dwelling, except for *D. derjugini* (Fig. 2), which is a ground dwelling species. It has a rounder head and body, different from rock dwelling lizards, which have a flattened head and body shape (Murphy et al., 2000; Tarkhnishvili, 2012) (Fig. 2).

Table 1
Sample sizes in the study.

Species	Anal scale	Pileus
D. porschinskii	27♀25♂	20♀21♂
D. valentini	21♀12♂	20♀14♂
D. rudis	40♀32♂	23♀21♂
D. derjugini	11♀11♂	10♀10♂
D. mixta	14♀16♂	14♀16♂
D. parvula	20♀24♂	17♀19♂

2.2. Sampling

The distribution range for the species used in this study varies per species. Two species have a wide range: *D. rudis* is found throughout most of the Caucasus and Turkey, and *D. derjugini* inhabits forests throughout the western part of the Caucasus Region. The other species have much narrower ranges: Georgian endemic *D. mixta* is found in the Central Greater Caucasus and Central lesser Caucasus, *D. parvula* occurs in the Central and Western Lesser Caucasus and black sea mountains in Turkey, *D. valentini* in the Southern Lesser Caucasus and *D. portschinskii* in the Eastern Lesser Caucasus (Tarkhnishvili, 2012). The sampling locations are shown in Fig. 3.

During 2009–2014, the research team of Ilia State University photographed the lizards with capture-release method during the field studies throughout Georgia, using a Canon PowerShot SX510 HS with 30× zoom lens. Digital images of anal and pileus (dorsal side of the head) areas were used in our study. In total, 253 anal scale and 205 pileus images of adult individuals throughout Georgia were analyzed (Table 1, Fig. 3).

2.3. Outline analysis

The outlines of the study areas (digitization error, which amounted 5% for both analysis, was checked according to the protocol by Adriaens, 2007 – http://www.fun-morph.ugent.be/Miscel/Methodology/Morphometrics.pdf) were traced in Coreldraw Graphics Suite x7 (Corel corporation) (Fig. 4), after which they were subjected to an elliptic Fourier analysis using the Shape Software (Iwata and Ukai, 2002). The software extracts the contour from the outline images as a chain-code, then normalizes size,

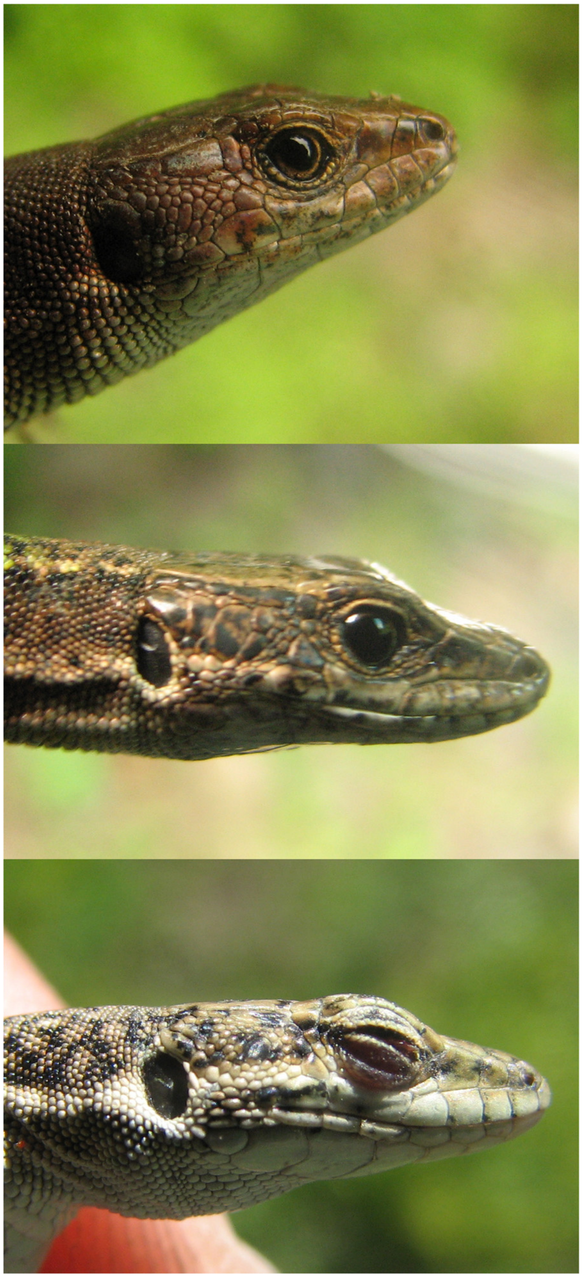

Fig. 2. Head shape of ground-dwelling *Darevskia derjugini* (top image), rock-dwelling *D. mixta* (middle image) and rock-dwelling *D. portschinskii* (bottom image). Rock-dwelling lizards have a more flattened head (and body) than the ground-dwelling one.

orientation and starting point for tracing the contour of the outlines according to the major axis of the first harmonic (first Fourier approximation to the shape). A PCA was done on the obtained elliptic Fourier descriptors (EFDs), being four EFDs for each of the 20 harmonics (goniometric shape descriptors) (Lestrel, 1997). The principal component scores were used as shape data for further statistical analyses.

2.4. Statistical analysis

Statistical analyses were performed in PAST and IBM SPSS software (Hammer et al., 2001; SPSS 21, 2012). In order to detect the principal shape axes that represent the maximal variation between the predefined groups, we applied a between-group PCA (Jolliffe, 1986). In order to test whether anal and pileus shape significantly differed across the species, clades, and sexes, a non-parametric MANOVA (Cooley and Lohnes, 1971) (parametric test assumptions were not met) was applied on the outline shape data.

Because there are indications of intermediate forms as a result of hybridization between the nominal species of *Darevskia* (Darevsky, 1967; Tarkhnishvili, 2012), we considered that higher order clades can more adequately reflect the morphological differences associated with phylogeny. In order to do so, we hierarchically defined groups in four different ways, reflecting boundaries between the groups at an increasing level of genetic differentiation: (1) individual species; (2) "1st level clustering", with most closely related species (*D. rudis*, *D. portschinskii* and *D. valentini*) grouped but other species taken separately (Fig. 5); (3) "2nd level clustering", with *D. derjugini* and *D. mixta* grouped, and (4) "3rd level clustering" according to the mtDNA clades as defined by Murphy et al. (2000) (two clades: 1st includes *D. rudis, D. valentini, D. portschinskii* and *D. parvula,* and 2nd includes *D. mixta* and *D. derjugini*). Post hoc tests were done by pairwise Hotelling's tests. We used both Hotelling's P values (not corrected for multiple testing) and Bonferroni corrected P values (multiplied by the number of pairwise comparisons; Rice, 1989).

2.5. Individual-based cluster analysis

We conducted a cluster analysis to test to what degree lizards of the same species/species groups/clades show morphological similarity at the individual level. We clustered shape data according to a UPGMA method, based on Euclidean distances between individuals, using PAST.

2.6. Phylogenetic signal

To test whether scale shape variation is correlated with genetic variations across the species, we quantified the K statistics for female and male shape data separately. This provides a statistical measure of the phylogenetic signal, where a test is done whether the similarity in the observed trait between two individuals is greater than that expected under Brownian motion ($K > 1$), less than expected ($K < 1$) or meets the model of Brownian motion (Adams, 2014). For quantifying the K-statistics, we used a neighbor joining phylogenetic tree derived from mean genetic distances between the species, using the software PAST. For calculating mean group genetic distances we used 73 sequences of Cytochrome *b* 302 bp fragments (4 – *D. derjugini*, 3 – *D. mixta*, 30 – *D. portschinskii*, 13 – *D. valentini*, 22 – *D. rudis*, 1 – *D. parvula*) downloaded from Genbank (see Genbank IDs in Appendix 1 at Supplementary data). The sequences were aligned using Bioedit version 7.2.5 (Hall, 2013). The genetic distances were computed using MEGA version 6 (Tamura et al., 2013). For the morphometric data, we used the median for each effective PC scores for each species per sex as a trait. All computations were done using package 'phytools' (Revell, 2012) in R Ver. 3.2.5 Software (R Development Core Team, 2016). The number of permutations was 10,000.

3. Results

3.1. Overall shape variation

Of the 77 principal components describing anal scale variation, 6 were effective (explained more variation than any of the individual variables used in the analysis) and explained 91% of the total variation. For the pileus data, 10 components out of 77 were effective and also explained 91% of the total variation.

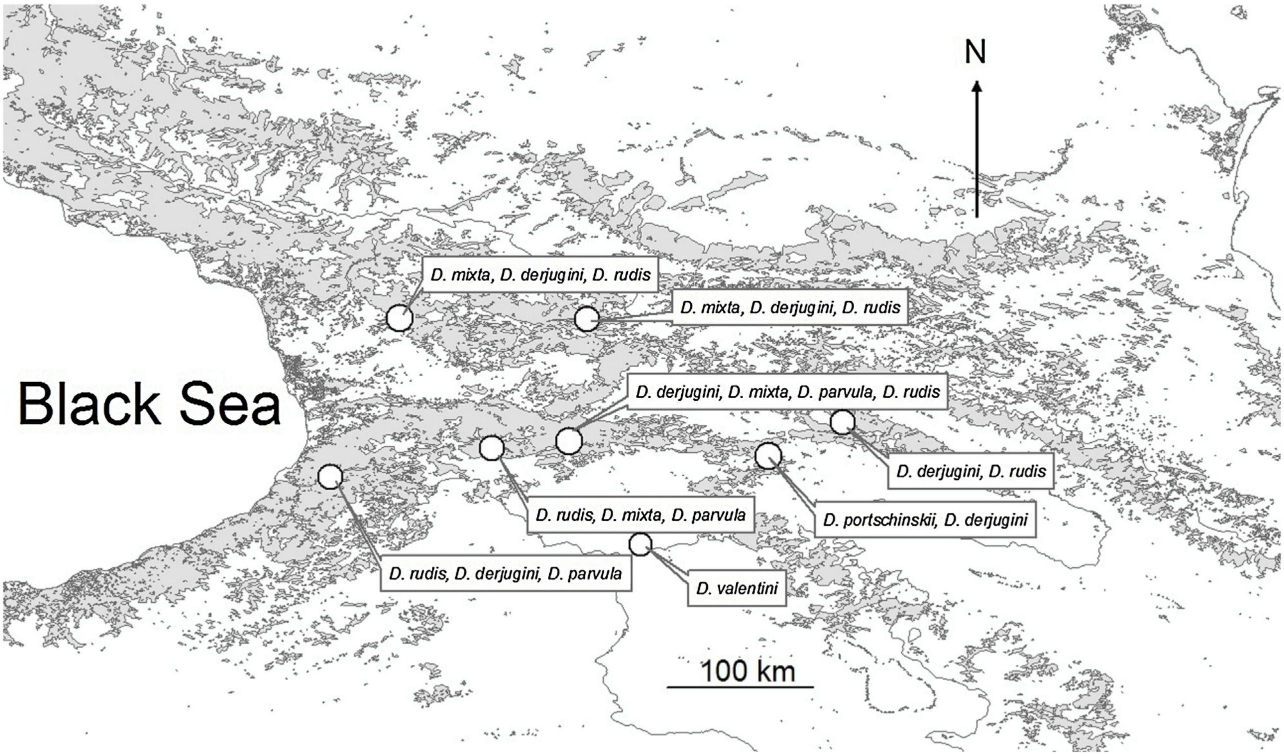

Fig. 3. Sampling locations of the species used in the present study. Grey area indicates presence of forested landscape; the range of most of *Darevskia* species coincides with forest distribution or is located above the forest belt.

Fig. 4. Anal scale outline of *Darevskia mixta* (top) and pileus outline of *D. valentini* (bottom).

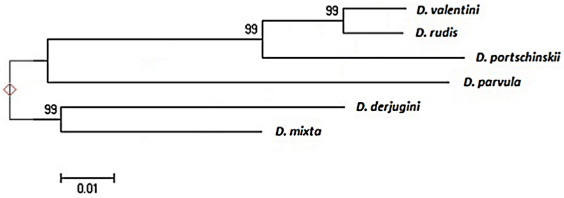

Fig. 5. MEGA-generated neighbor-joining tree computed using genbank sequences (Genbank IDs: AF206172, AF147798, U88611, U88614, U88615, U88609). Numbers indicate bootstrap values. Branch lengths show genetic distance between the species.

The visualization of the mean shape expressed by the first three effective principal components with its 2*standard deviation range is shown in Fig. 6.

According to the between-group PCA (BG-PCA), most of the variance between the nominal species was accounted for by the first principal component. Considering that for the anal scale, this BG-PC1 was largely correlated (0.75) with PC1 of the original analysis, BG-PC1 reflects variation between concave and convex anal scale shapes. For the pileus data, species-differences were accounted for by BG-PC2, which was largely correlated with original PC3 (0.54) and thus reflects variation in the concavity of the gap between the parietal and supraorbital scales (BG-PC1 is most probably related to size and sex).

On average, '*rudis*' clade lizards have more concave anal scales and '*caucasica*' clade lizards have more convex scales (Fig. 7A), while *D. parvula* keeps an intermediate position. Simultaneously, '*rudis*' clade lizards have a more concaved gap between the parietal and supraorbital scales than '*caucasica*' clade lizards (Fig. 7B).

The non-parametric MANOVA confirmed significant differences in anal scale and pileus shape for all four levels of groupings irrespective of sex. The differences increase with increasing levels of clustering for anal shape. For pileus shape, differences are high for both individual species and for 3rd level clustering (major clades '*rudis*' and '*caucasica*') but lower for intermediate level clustering (1st and 2nd level clustering) (Table 2). This may be related to the sexual dimorphism (significant for both traits): the F value for pileus variation related to sex is higher than the value related to species: 7.98 vs 7.09. The F value for anal scale variation related to

Table 2
Results from the NPMANOVA for the different genetic levels for the anal scale shape and pileus shape data (significance p-value).

Taxons	F value for anal scale data	F value for pileus data	F value for anal data (interaction species/clusters*sex)	F value for pileus data (interaction species/clusters*sex)
Individual species	22.87 (0.0001)	7.085 (0.0001)	15.01 (0.0001)	4.693 (0.0001)
1st level clustering	33.36 (0.0001)	6.211 (0.0001)	21.55 (0.0001)	4.699 (0.0001)
2nd level clustering	46.95 (0.0001)	5.609 (0.0001)	28.53 (0.0001)	4.469 (0.0001)
3rd level clustering (clades)	73.61 (0.0001)	8.129 (0.0001)	36.13 (0.0001)	6.201 (0.0001)

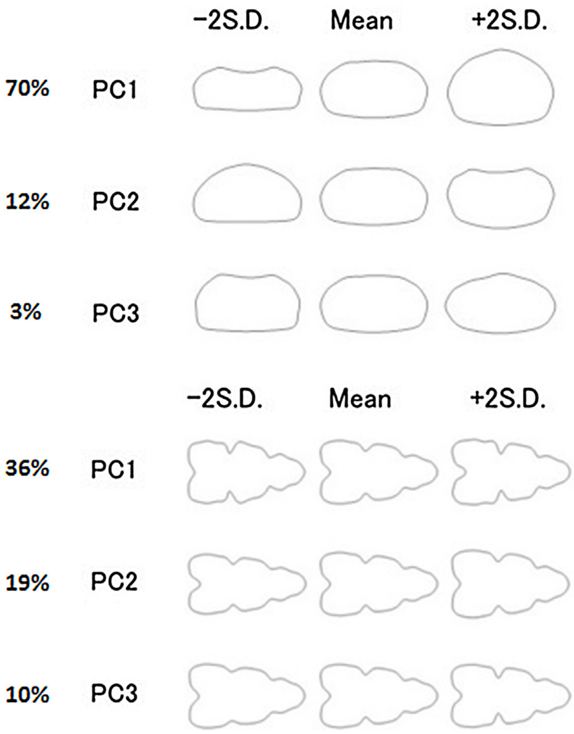

Fig. 6. PC contours representing shape variation for the first three effective principal components for anal scale (three upper rows) and pileus (three lower rows). 2S.D. indicates 2*standard deviation range.

sex is lower than the value related to species: 15.89 vs 22.87. The influence of the interaction between phylogeny and sex was also significant for both traits (Table 2).

The Hotelling's test with uncorrected p-values revealed significant differences for all the species/clusters pairs (both sexes combined) for both anal scale and pileus shape data, except for *D. valentini* and *D. rudis* in the individual species level for anal scale data. Between sexes, the anal scale was dimorphic in all studied species, except for *D. derjugini*. As for the pileus data, only *D. rudis, D. parvula* and *D. portschinskii* were sexually dimorphic.

After Bonferroni correction for pairwise comparisons, the differences in anal shape between some species (*D. parvula* and *D. valentini*, *D. rudis* and *D. portschinskii*, *D. valentini* and *D. rudis*) are no longer significant, although differences between the clusters remained significant. For the pileus data, differences between *D. mixta* and *D. portschinskii*, and between *D. parvula* and *D. rudis* were no longer significant. Moreover, *D. parvula* did not differ anymore from its matrilineal '*rudis*' group. Sexual dimorphism was only remained in *D. rudis* for both anal scale and pileus shape.

3.2. *Individual-based cluster analysis*

The cluster analysis based on anal scale shape grouped all studied individuals into two clusters, each one dominated by individuals from the '*rudis*' and '*caucasica*' clade, respectively (Fig. 8A). As for the pileus data, it did not cluster individuals according to their corresponding clades (Fig. 8B).

The misclassification rates were strongly asymmetric. Only 9% of '*rudis*' clade were misclassified into '*caucasica*' clade based on the anal scale shape and none based on the pileus shape. Simultaneously, one-third of the individuals of '*caucasica*' clade were misclassified into the '*rudis*' clade based on the anal scale shape, and the majority of the individuals were misclassified as '*rudis*' clade based on the pileus shape.

3.3. *Phylogenetic signal*

For the anal shape data, PC1 scores showed a strong phylogenetic signal for both females and males (females: $K = 1.477929$, P-value = 0.03; males: $K = 1.50718$, P-value = 0.03), thus showing that closely related species were more similar to one another than expected under Brownian motion. For the pileus shape data, all the traits showed a low but insignificant phylogenetic signal ($K < 1$; $p > 0.05$).

4. Discussion

Although the outline analysis on anal scale and pileus shape didn't show consistent differences between the species of the studied *Darevskia* lizards, it does indicate that morphological patterns do reflect phylogenetic ones to some degree. This could not be achieved with the more 'traditional' approach of Darevsky (1967). He used multiple measurements and scalation traits to reconstruct species relationships, but clustered together *D. mixta* and *D. parvula* and both with *D. portschinskii*, which does not correspond to phylogenetic pattern based on molecular data and outcome of the present study.

Geometric morphometrics being more successful to reflect phylogenetic relationships than 'traditional' morphometrics based on linear measurements has been shown in the past. For example, Smith and Hendricks (2013) reconstructed phylogenetic relationships using shape data of gastropod shells by applying outline-based geometric morphometrics. The advantage of geometric morphometrics is most probably associated with the fact that, besides the linear measurements, it also contains the information about the interrelations among the dimensions of the studied structure (Zelditch et al., 2004).

Also in our study on *Darevskia* lizards, the use of outline data proved to show phylogenetic signals up to specific levels of genetic differentiation. Although shape differences between individual species were not always statistically significant, the analysis of larger inclusive phylogenetic groups (matrilineal clades) did show significant differences for both anal scale and pileus shape. The K-statistics also confirmed a significant phylogenetic signal in the anal scale shape, indicating that the variance increases with the genetic distance between the evolutionary lineages. This was, however, not the case for the pileus shape. Here shape variation more reflects sexual dimorphism than differences between the nominal species, as inferred by NPMANOVA. This is in line with previous observations, since males usually have wider and larger head than females (Darevsky, 1967). Sexual dimorphism is well observed in

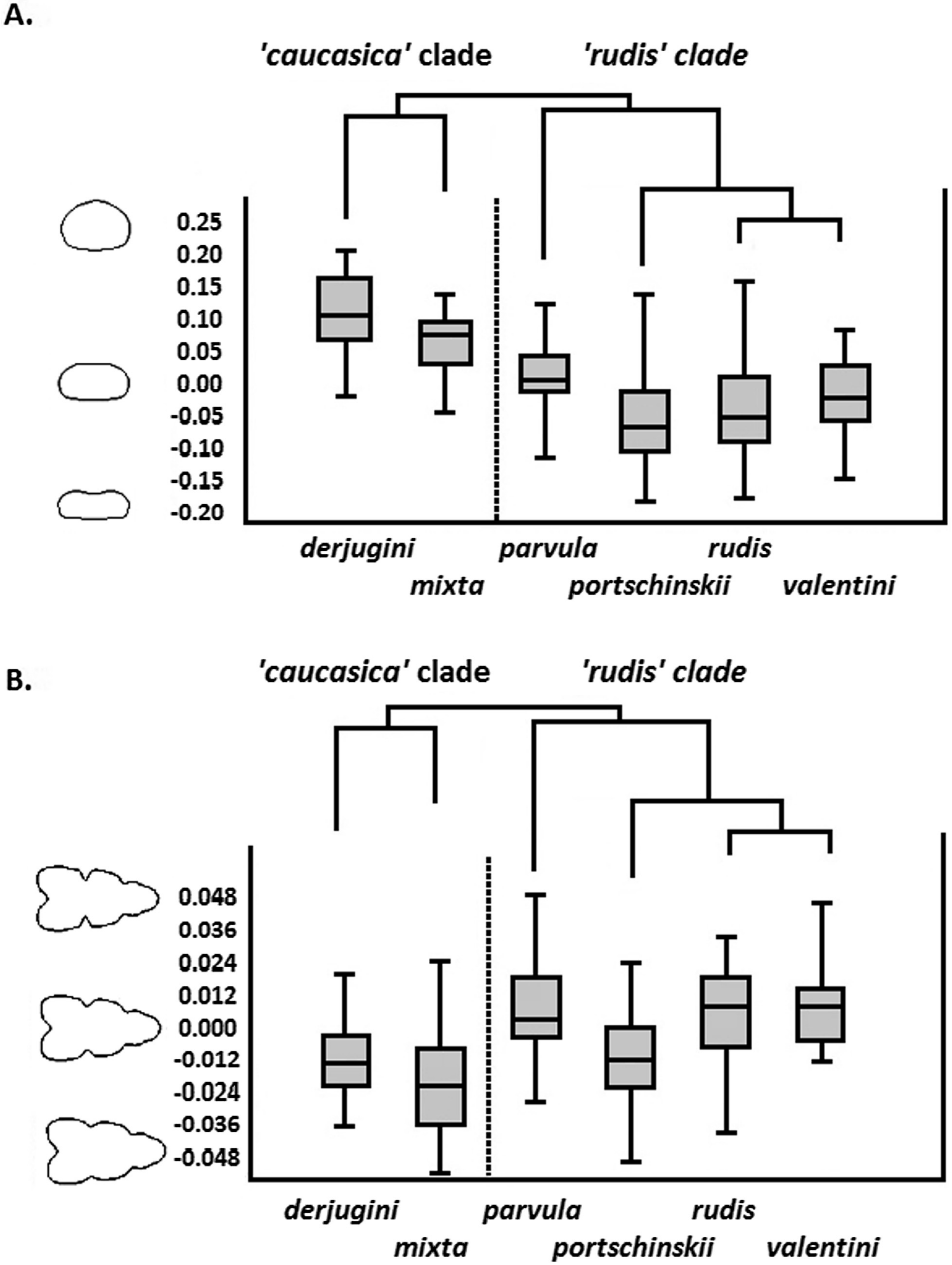

Fig. 7. A – Box plot of between-group PC1 scores for each species for anal scale shape (Outlines on the left based on original PC1 scores; tree above – phylogenetic tree based on mtDNA cyt-b fragment). B – Box plot of between-group PC1 scores for each species for pileus shape. (Outlines on the left based on original PC3 scores; tree above – phylogenetic tree based on mtDNA cyt-b fragment).

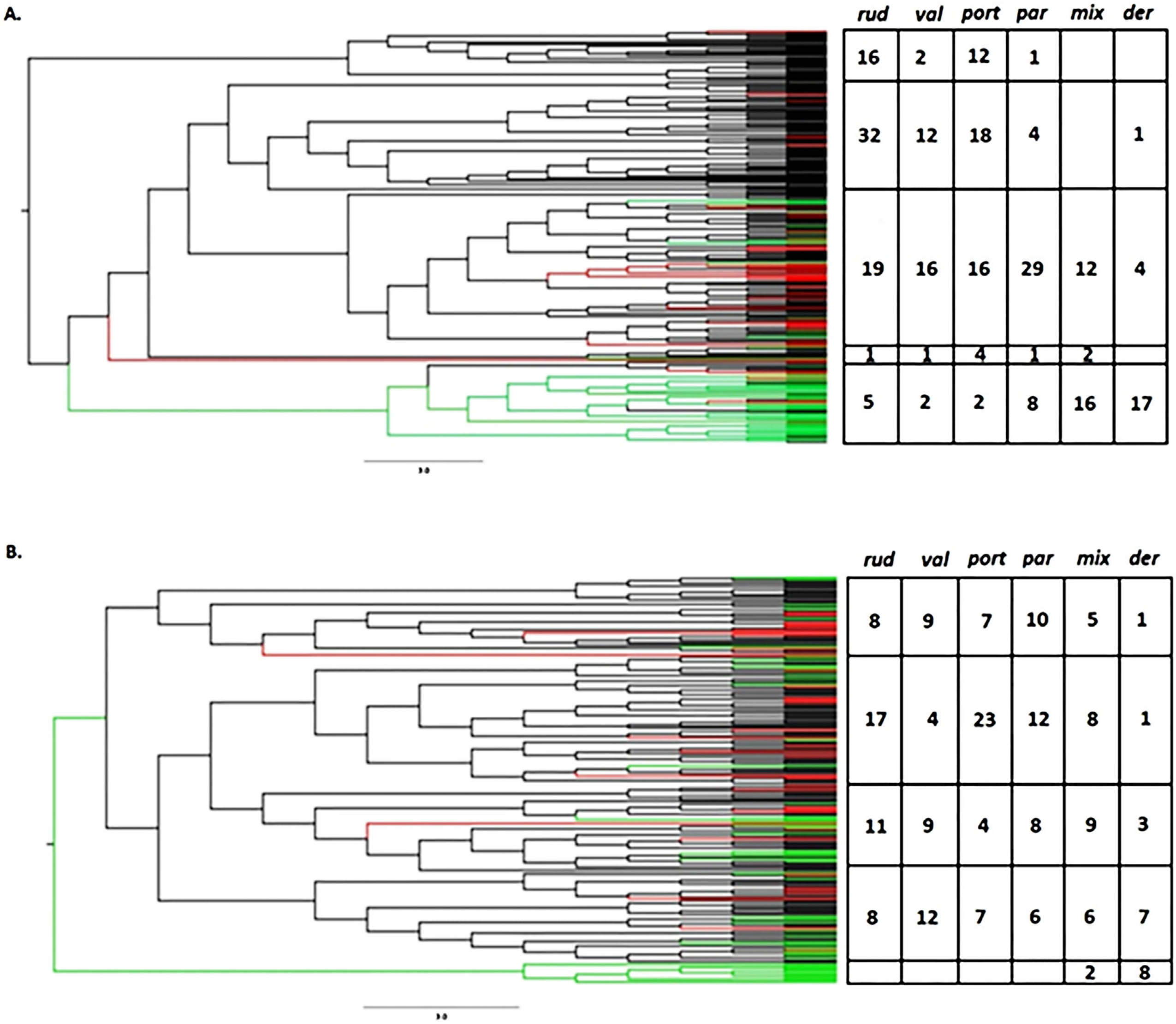

Fig. 8. Cluster analysis generated UPGMA trees based on anal scale data (A) and pileus data (B) ('*caucasica*' clade lizards marked with green, '*rudis*' clade lizards – black, *D. parvula* – red. Numbers in matrix number of individuals in the respective area of the tree). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

most of the lizard species (Herrel et al., 1996). There are two main functional explanations for such a dimorphism: (1) sexual selection (intrasexual/intersexual interactions); (2) natural selection due to food competition. Herrel et al. (1996) showed that sexual dimorphism resulting in divergent bite force performances in males and females of *Podacris hispanica atrata* Steindachner, 1870 has an ecological relevance, such as reducing food competition between the sexes. On the other hand, Herrel et al. (1999) showed in their work that lizards (*Gallotia galloti* Duméril and Bibron, 1839) with larger heads bite harder and the maintenance of dimorphism in head sizes of males and females is most likely associated with sexual selection (intrasexual as well as intersexual interactions). Male *Darevskia*, similar to other lacertids, bite other males and females during the mating period, although there are no specific studies linking head size and sexual selection in this group (Galoyan, 2011). It is highly likely that the situation does not substantially differ from those in other lizards that show selective importance of the head size and dimensions.

In *Darevskia* species, both anal scale and pileus shape, the analyses confirmed that the largest difference was between the basal clades. This can potentially be associated with an increase in morphological divergence with increasing genetic distance, possibly being correlated with an increase in the isolation level between the lineages overtime. Basal clades are genetically more isolated from each other than species within the same clade, which can have intermediate forms as a result of hybridization. Indeed, according to Murphy et al. (2000), within-clade hybridization occurs among the most of sympatric species. *D. derjugini* and *D. mixta* can produce hybrids, which may mate with their parental species afterwards. Also, according to Tarkhnishvili et al. (2013), there is an introgression of both mitochondrial and autosomal alleles between *D. rudis, D. portschinskii* and *D. valentini*.

However, it appears that the level of introgressive hybridization reduces with time. Time calibrated Bayesian phylogenetic tree of *Darevskia* (Tarkhnishvili, 2012) suggests the split between the two clades studied in this paper occurred 4.9–7.3 mya with 95% significance interval; the split between *D. parvula* and its closest relatives – 4.7–7.1 mya; between *D. derjugini* and *D. mixta* lineages – 2.4–3.8 mya; and, between *D. portschinskii* and *D. rudis-D. valentini* – 1.3–2.3 mya. Correspondingly, the mismatches are the fewest among the representatives of different clades, but very high for individual species or 1st level species clusters (Table 2, Fig. 8).

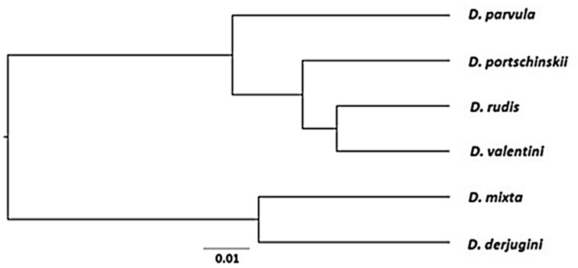

Fig. 9. UPGMA tree on the species per sex medians based on Euclidean distances (identical result for both sexes).

The UPGMA clustering analysis on anal scale shape outline, based on the Euclidean distances between the species medians (separately taken for different sex) (Fig. 9), shows that the dendrogram corresponds with the mtDNA tree (Fig. 5), unlike the one based on 'traditional' morphometrics (Fig. 1). It supports both the two main clades and the species relationships within the clades in the mtDNA tree. In contrast, 'traditional' morphometrics harvests some important discrepancies from the DNA-based phylogeny. For instance, *D. portschinskii* is placed in the same cluster with *D. parvula* and *D. mixta* and not with *D. valentini* and *D. rudis*, whose closest relative it is (Murphy et al., 2000; Tarkhnishvili et al., 2013). Further, *D. parvula* is clustered together with *D. mixta* and not with the other species of the '*rudis*' clade, as it should be if molecular data are considered. These discrepancies are non-existing when the outline approach is used.

Outline approach didn't show substantial differences between a ground-dwelling *D. derjugini* and its rock-dwelling matrilineal relative, *D. mixta,* supporting predominant importance of phylogeny on this trait.

Scale shape reflecting the observed phylogenetic signal does not seem to be linked to the general differences in overall body size. Body length (SVL) of *D. rudis* and *D. valentini* males is 70–88 mm and that of females is 69–83 mm, making them the largest of the species studied (SVL below 70 mm for both genders, even below 60 mm for *D. parvula*) (Darevsky, 1967). Being the smallest of the studied species, *D. parvula* does group with its larger sized, matrilineal relatives (*D. valentini* and *D. rudis*) rather than with the smaller species (*D. derjugini* and *D. mixta*). Darevsky (1967) indicated that a larger body in rock lizards is associated with the increased number of chest and abdominal cross rows, however, dependence of other scalation traits on body size were not observed. Pileus and especially anal scale shape disparities thus seem to reflect evolutionary divergences in *Darevskia*. Whereas the differences in the shape of anal scale and pileus cannot be distinguished between some closely related species of *Darevskia*, the distinction is consistent when more inclusive clades are compared.

The outline analysis used in this paper helped us to identify those shape characters that prove to be more informative from a phylogenetic viewpoint. A next step could be to try to disentangle this shape variation from that resulting from local adaptations or traits that show patterns of random variation. In the future, this approach could markedly improve our understanding of morphological evolution in rock lizards and possibly other groups of reptiles.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jcz.2017.04.004.

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Chapter 3: Phenotypic divergence, adaptation, and cladogenesis in Caucasian rock lizards (*Darevskia*)

Phenotypic divergence, adaptation, and cladogenesis in Caucasian rock lizards (*Darevskia*)

(Submitted)

David Tarkhnishvili, Mariam Gabelaia & Dominique Adriaens

Abstract

Three-dimensional geometric morphometrics has shown to be an effective methodology for multiple purposes, including discrimination among the closely related species, analyzing morphological adaptations, or mapping patterns of divergent and convergent evolution. The applicability of this methodology for searching a phylogenetic signal in morphological structures is contradictory. The theory is not very optimistic, suggesting that complexes of intercorrelated morphological characters will phylogenetically hardly be informative; on the other hand, some empirical studies reveal the presence of phylogenetic signal in variables derived from GM analysis. We studied 13 species of Caucasian rock lizards (*Darevskia*), whose phylogenetic relationships are well-known, thanks to molecular methods, using GM approach: those included 3D head shapes as well as outlines of three important scales in the head and anal area. We compared the derived variables with sex, adult body size, and habitat type of a species (ground-dwellers, rock dwellers, and intermediate). 3D head shapes were shown to be more informative characters than the scale outlines; individual principal components correlated with habitat type and body size. When the ecological correlates were excluded from the analysis, the resulting information was congruent with the phylogenetic pattern of the studied taxon; this is taken as an illustration of Dollo's law on irreversibility of phenotypic evolution of species.

Keywords: *Geometric morphometrics, 3D head shape, adaptation, rock-dwelling, phylogeny, phenotypic vs genotypic evolution, lizards, Darevskia.*

Introduction

After Mullis et al. (1986) discovered the PCR reaction, it became clear that molecular genetic data perform much better for reconstructing phylogenies than "traditional" phenotypic analyses (Hillis & Moritz, 1996; Felsenstein, 2004). The reason is adaptive evolution resulting in convergent phenotypic patterns (Losos, 2011). This is shown on multiple organisms, including lizards (Vitt et al., 1997; Harmon et al., 2005; Barros et al., 2011; Edwards et al., 2012). Losos (2011) suggested however that "head shape of lizards primarily reflects phylogeny; members of the same family cluster together in a multivariate morphological space regardless of what they eat." Although, this statement hardly applies to the congeneric species. On the contrary, phylogenetic hypotheses based on the analysis of phenotypes are commonly falsified by molecular phylogenetic.

One example of this is about Caucasian rock lizards (*Darevskia*), which systematics and species boundaries have changed many times during the 20th century (Nikolski, 1913; Darevsky, 1967). Reconstructing molecular phylogenies showed multiple incongruences between the phenotypic and genotypic based grouping of *Darevskia* species (Fu et al., 1997; MacCulloch et al., 2000; Murphy et al., 2000; Tarkhnishvili, 2012; Tarkhnishvili et al., 2013; Ahmadzadeh et al., 2013; Gabelaia et al., 2017). This monophyletic genus aggregates 26 sexually reproducing species and seven parthenogenetic forms (Uetz & Hošek, 2019). Mitochondrial phylogeny revealed presence of three monophyletic clades within the genus: *"rudis"*, *"saxicola"*, and "*caucasica*" (Murphy et al., 2000). Later molecular genetic studies suggested that (1) a species from the western Lesser Caucasus, *D. parvula*, belongs to a separate clade and is not associated with the "*rudis*" clade (Tarkhnishvili, 2012; Ahmadzadeh et al., 2013); (2) there is broad introgression of mitochondrial DNA between closely related taxa *D. portschinskii, D. rudis,* and *D. valentini* (Tarkhnishvili et al., 2013); (3) *D. daghestanica* is not a sister species for *D. caucasica* but a basal taxon for the subclade *daghestanica- derjugini - caucasica - mixta - clarkorum* (Murtskhvaladze et al., in press) (Fig. 1). Finally, (4) *D. dryada* may be a synonym of *D. clarkorum* rather than a separate species, a conclusion based on the analysis of morphology (Schmidtler et al., 2002) and unpublished mitochondrial DNA analysis.

Gabelaia et al. (2017) showed that geometric morphometrics, in particular an outline analysis of the anal scale, helps to separate *Darevskia* from two different clades *sensu* Murphy et al. (2000): clade "*caucasica*" and clade "*rudis*", irrespective of their habitat preferences. Later, Gabelaia et al. (2018) showed that geometric morphometrics based on the 3-dimensional head shape data can effectively discriminate between very closely related species of the "*rudis*" clade and, moreover, suggest phenotypic distinctness of a matrilineally monophyletic subspecies *D. rudis obscura*; the authors recommended to elevate its taxonomic status to full species.

In this paper, we analyzed 3-dimensional head shapes and outlines of three important scales of 13 species of *Darevskia* from the western Caucasus, representing all major clades within the genus. The purpose of this analysis was to test if the convergent patterns in *Darevskia* shades phylogenetic signal that otherwise may present in the phenotypic variation. For this goal, we identified those components of phenotypic variation, which significantly correlated with specific environmental conditions, and removed them from the analysis for inferring whether the residual phenotypic variation reflects phylogeny better. As the reference data, we used full consensus mitochondrial phylogeny of *Darevskia,* based on several recent publications (Murphy et al., 2000; Tarkhnishvili, 2012; Ahmadzadeh et al., 2013; Tarkhnishvili et al., 2013; Murtskhvaladze et al., in press) (Fig. 1).

The analysis showed strong convergent patterns in the evolution of *Darevskia*, but also helped to extract phylogenetic correlates based on the geometric morphometrics.

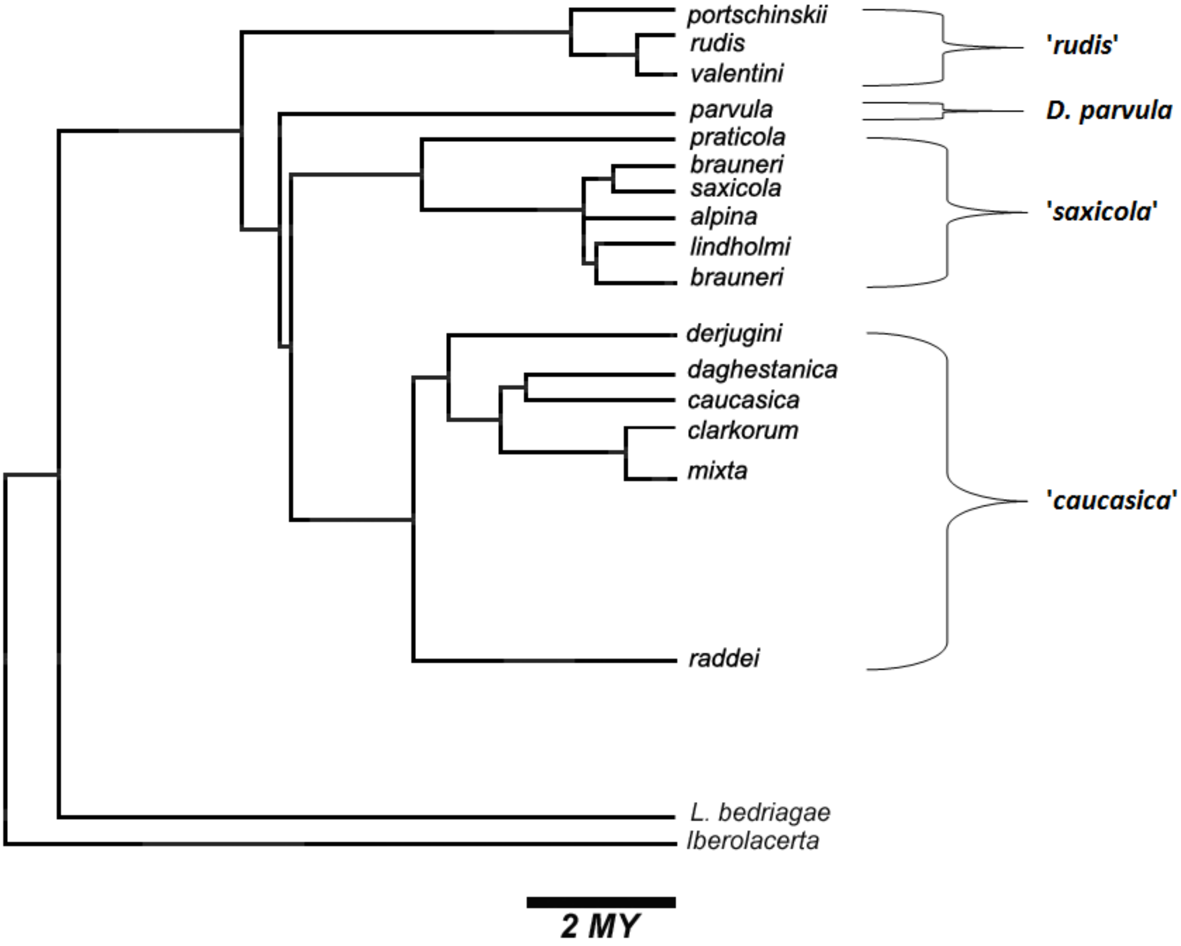


Figure 1. Currently accepted topology based on Bayesian phylogenetic analysis of cytochrome *b* sequence fragments (1051 bp) of *Darevskia* lizards and two outgroup species (*Archaeolacerta bedriagae* and *Iberolacerta spp.*), after Tarkhnishvlili (2012). On the right: '*rudis*', '*saxicola*' and '*caucasica*' represent distinct monophyletic clades, while *D. parvula* keeps separate position on the tree. Topology is similar to the one by Murphy et al. (2000), except for the position of *D. parvula* which was included in the clade '*rudis*' in Murphy's study.

Material and Methods

Studied system

We selected 13 species representing four major clades within *Darevskia*: *D. parvula* (clade "*parvula*"), *D. portschinskii, D. valentini, D. rudis, D. obscura* (clade "*rudis*"), *D. praticola, D.*

brauneri (clade "*saxicola*"), *D. raddei, D. daghestanica, D. derjugini, D. caucasica, D. mixta, D. dryada* (= *D. clarkorum*) (clade "*caucasica*"). These species differ by geographic range (e.g. *D. brauneri* and *D. caucasica* are found only in the Greater Caucasus mountains, whereas *D. parvula* and *D. portschinskii* only in the Lesser Caucasus); by habitat (*D. praticola* and *D. derjugini* are ground-dwellers, others are rock-dwellers, or use habitats opportunistically); by adult body size (from *D. parvula* whose maximum body length is 55-56 mm, to *D. rudis*, whose body length in some populations reaches 85 mm (Tarkhnishvili, 2012).

Previous studies showed that the anal scale in lizards from the clades *"rudis"* and *"parvula"* is broader than in those from the clade "*caucasica"* (Gabelaia et al., 2017), although the differences cannot help to identify separate species. Visual observations suggest that ground-dwelling species (*D. praticola, D. derjugini*) have taller heads, and differently positioned preanal scales than the rock-dwellers (Tarkhnishvili, 2012). It is not clear how these characters differ among the rock-dwelling species, although some differences one may detect (Fig. 2). In general, head shape is an informative phenotypic system sometimes used for the analysis of phylogeny (Gentilli et al., 2009; Ivanović et al., 2013), in spite of its association with habitat type (Kaliontzopoulou et al., 2008; Openshaw and Keogh, 2014). Our previous study showed a very high sensitivity of head shape to species boundaries in *Darevskia* (Gabelaia et al., 2018).

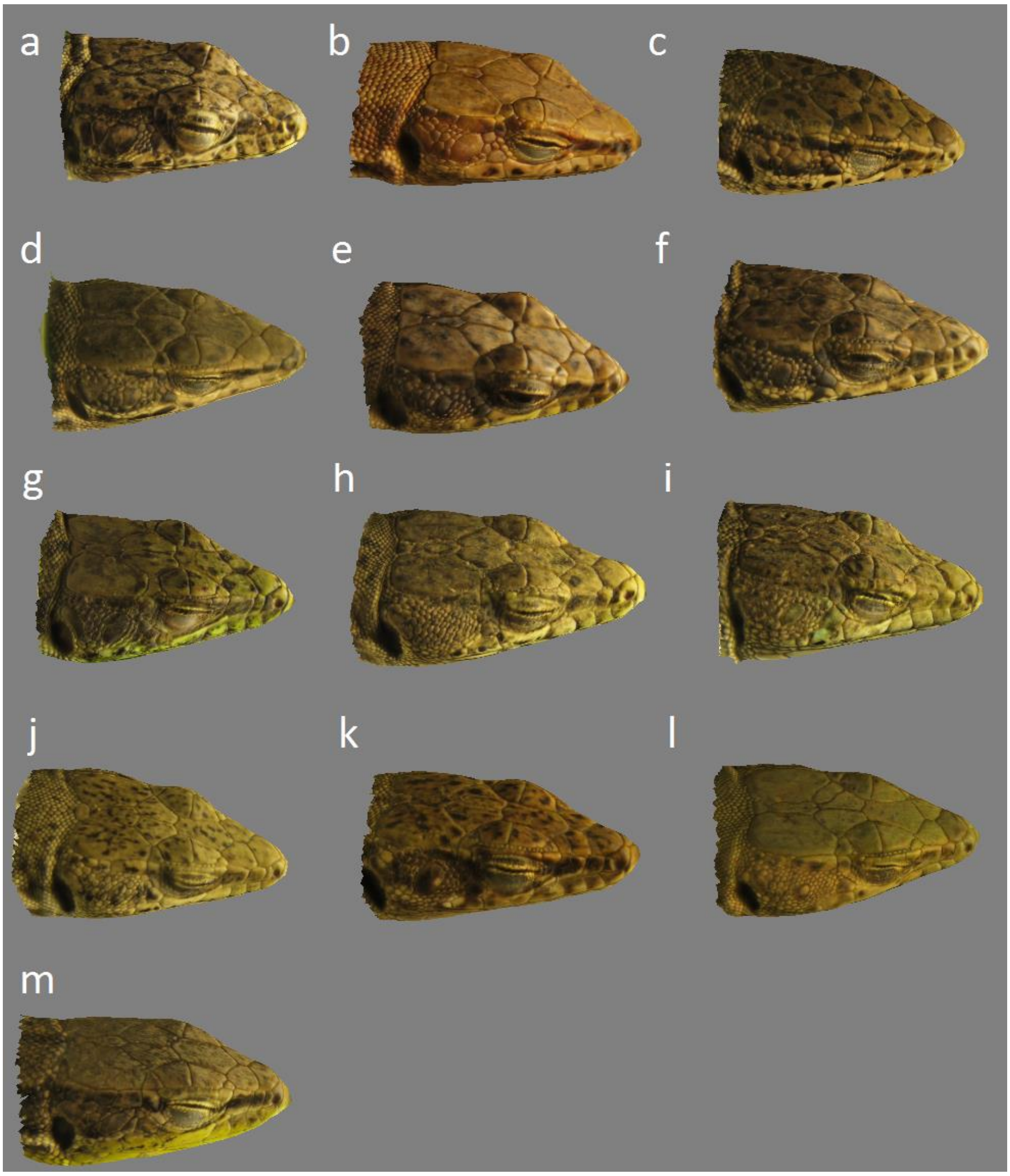


Figure 2. Heads of *Darevskia* species used in the study. a - *D. mixta,* b – *D. derjugini,* c – *D. caucasica,* d – *D. daghestanica,* e – *D. clarkorum,* f – *D. raddei,* g – *D. rudis,* h – *D. obscura,* i – *D. valentini,* j – *D. portschniskii,* k – *D. parvula,* l – *D. brauneri,* m – *D. praticola.*

Sampling

78 individuals of the 13 listed species were collected from 12 locations within Georgia and used for the morphometric analysis: three males and three females of each species (Fig. 3).

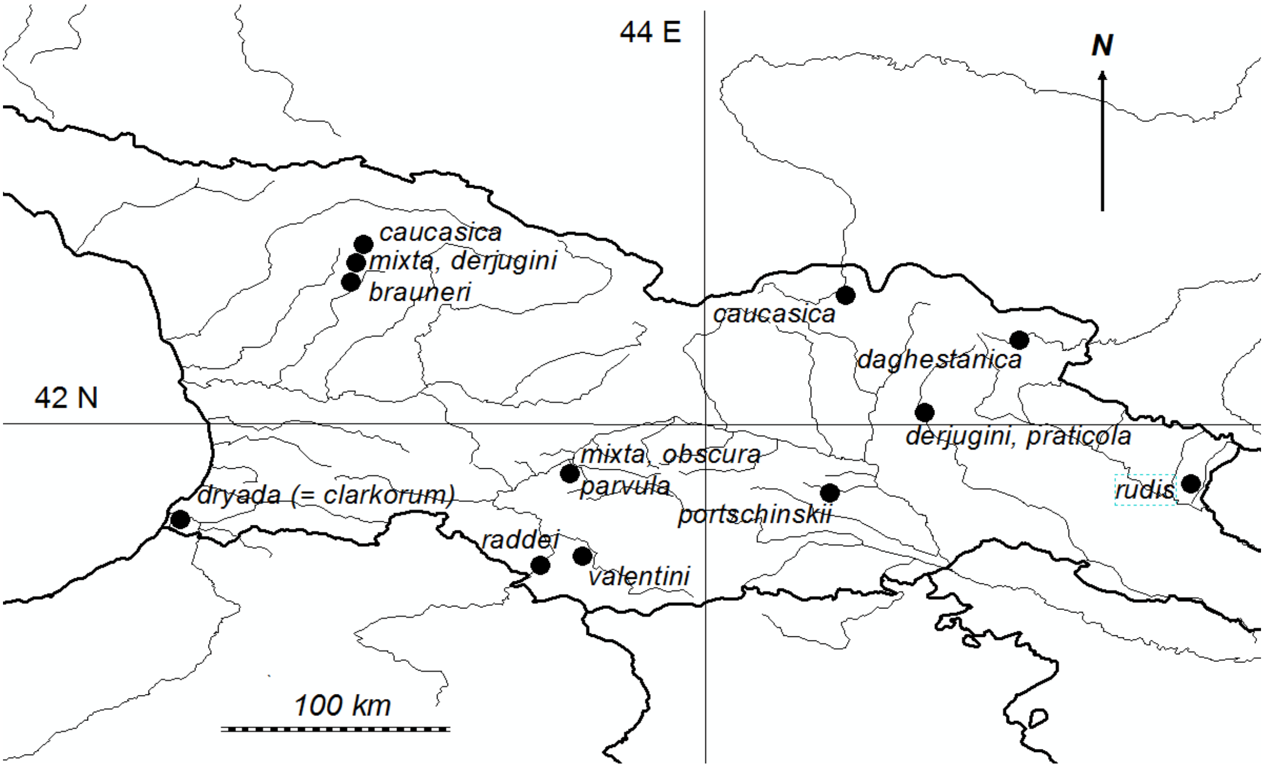


Figure 3. Sampling locations for each species.

The data used for geometric morphometrics

We used 3D head models constructed from multiple 2D photographs for further analysis following the procedure described in (Gabelaia et al., 2018). The individuals were anesthetized and placed in a tube in the centre of a cardboard circle, so that the head of the lizard was pointed upwards. By moving the camera around the animal, we photographed the head 36 times from a perspective of 90° to the midline and 36 times from a 45° perspective. The 2D digital images were uploaded in AgiSoft PhotoScan Pro v.1.2.6 (Agisoft, 2016) in order to generate 3D models of the lizards' head.

After creating the 3D head models, we digitized 54 homologous landmarks on their scalation pattern (Fig. 4) using AgiSoft PhotoScan Pro v.1.2.6, extracted the 3D coordinates of the landmarks and arranged them in a spreadsheet. For aligning and scaling 3D coordinates we applied Procrustes superimposition (Rohlf & Slice, 1990; Dryden and Mardia, 1998). Finally, in order to extract the 'meaningful' components from the shape data, we ran principal component analysis (PCA) using the software PAST (Hammer et al., 2001). ‘Meaningful’ components (from here onwards 3DPCA1 - 3DPCAn) were defined after a broken stick analysis (Jackson, 1993) in software PAST.

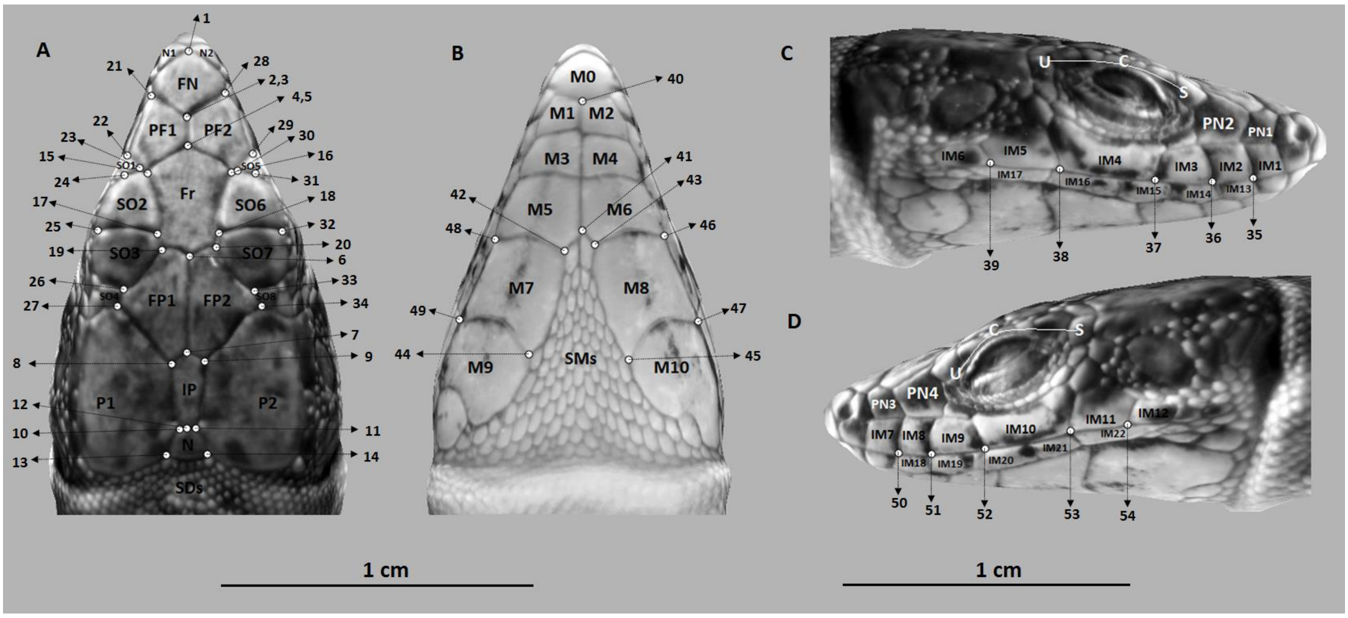


Figure 4. Fifty four three-dimensional landmarks on *Darevskia rudis rudis* individual. Names of the scales are indicated with text, and landmarks are identified with arrows and numbers (see description of the scales and landmarks in Supporting Information, Appendix S1). A, dorsal view of the head. B, ventral view of the head. C, view of the right profile of the head. D, view of the left profile of the head.

For the two-dimensional Fourier shape analysis, we used the outlines of three scales: anal scale, fifth upper labial scale (5thUL) and frontal scale (Fig. 5) and analyzed using Shape software (Iwata and Ukai, 2002). The procedure is described in Gabelaia et al., 2017. Meaningful PCs (explaining more variation than any single variable in the analysis) were extracted by the Shape software, hence producing variables describing shape of the anal scale (ASPCA), 5thUL (ULPCA), and frontal scale (FSPCA).

Figure 5. An anal, fifth upper labial and frontal scale outlines on *D. clarkorum* individual (contoured in red).

Statistical procedures

The further analysis was applied to the meaningful PCA scores of each of the four studied structures (3DPCA, ASPCA, ULPCA, FSPCA) in order to infer their importance in the distinction between the studied species and clades, their association with sex, body size, and habitat type, and correlation with phylogenetic pattern based on the DNA analysis.

We separated the lizards into categories arranged in increasing order, according to their habitat preferences and body size in the following way. Habitat preferences: (1) ground dwellers (*D. praticola, D. derjugini*); (2) species that are preliminarily rock-dwellers but also commonly found away from the rocks (*D. rudis, D. valentini, D. daghestanica, D. caucasica, D. mixta, D. clarkorum*); and (3) strict rock-dwellers (*D. parvula, D. portschinskii, D. obscura, D. brauneri, D. raddei*). Body size: (1) species with body (snout-vent) length of adults up to 55-56 mm (*D. raddei, D. parvula*); (2) species with body length of adults up to 58-65 mm (*D. praticola, D. derjugini, D. caucasica, D. mixta, D. clarkorum, D. portschinskii*); (3) species with the body length of adults between 66-73 (*D. brauneri, D. obscura, D. valentini*); and (4) species with the body length of adults between 75-85 mm (*D. rudis*) (Darevsky, 1967; Böhme, 1984; Tarkhnishvili, 2012; in some species, e.g. in *D. raddei*, body size markedly differs among the populations, and the numbers above apply to the populations used in our analysis).

We analyzed, for each meaningful PCA axis, the significance of their association with (1) species, (2) sex, (3) clade (as shown in Fig. 1) coded as nominal variables, (4) body size category, (5) habitat preference, and (6) interaction between species and sex, using univariate

General Linear Model scheme (SPSS 21, IBM corp. 2012). We then applied phylogenetic ANOVA using 'phylANOVA' function from package 'phytools' in R software (Revell, 2012) for inferring the significant influence of habitat and body size controlled for phylogenetic signal on the same PCA axes.

Finally, we used Blomberg's K statistics and Pagel's lambda using package 'phytools' (Revell et al., 2007) in R (R Development Core Team, 2008) for estimating strength and significance of association of each meaningful PCA axis with phylogeny. This was done for inferring and visualizing those phenotypic dimensions which are stronger than the others associated with phylogeny. The tree used for these calculations was that of the same species, based on the full mitochondrial genome (Murtskhvaladze et al. in press).

After visualizing the dimensions associated with ecological adaptations and phylogeny, we inferred multivariate phenotypic pattern and tested its association with the phylogenetic tree of *Darevskia*. For this reason, we ran stepwise discriminant function analysis (DFA) designed for equal samples (Huberty and Olejnik, 2006) (1) for the entire set of meaningful PCA scores based on the 3D head shape and the scale outlines, (2) for the set of the meaningful PCA scores, with the exception of those which were significantly correlated with habitat, body size, or sex. We used the Euclidean distances between DFA centroids (considering absence of correlation between the axes) for constructing unrooted Neighbor-Joining tree (Saitou and Nei, 1987) and compared this tree visually with the Neighbor-Joining tree based on the mitochondrial DNA analysis (Murtskhvaladze et al., in press). The software used for the tree building was MEGA 10.1 (Tamura et al., 2013).

Results

Selecting important variables

PCA based on the three-dimensional head shapes extracted eight significant PC axes explaining 67% of the total shape variation. The analysis of the outline of the anal, 5th UL, and frontal scale extracted six, eight, and nine PC axes respectively; altogether 31 significant components of variation of head shape and three large scales presenting in all studied species.

Univariate ANOVA, after stepwise Bonferroni correction (Rice, 1989) applied across the rows, showed that (1) two out of 31 meaningful PCAs were significantly associated with sex (and one was significantly associated with sex dependent on species); (2) seven were significantly associated with habitat type (rock-dwelling vs ground-dwelling or intermediate life mode); (3) four were significantly associated with body size, (4) ten were significantly associated with "major" clades within *Darevskia* (Table 1). The latter suggests the influence of phylogeny on the head shape and outline of the important scales; indeed, after applying phylogenetic ANOVA, only one meaningful PCA out of 31 (3DPCA1; 28% of the total variation in head shape) remained significantly associated with habitat type if controlled for phylogeny (Table 1, Fig. 6), and none of the dimensions - with body size.

Blomberg's *k* - and Pagel's *lambda* coefficient analysis inferring the association of each of the 31 significant components of variation with mitochondrial phylogenetic tree suggested presence of such association in three characters: 3rd PC based on the analysis of 3D head shape (7.7% of the variation in head shape; $k = 1.09$; $p = 0.011$; lambda = 1.00; $p = 0.051$); 4th PC based on the 3D head shape (6.7% of the variation in head shape; $k = 0.89$; $p = 0.026$; lambda = 0.77; $p = 0.030$); and 4th PC based on the outline of the frontal scale (4.9% of the variation in shape of frontal scale; $k = 1.00$; $p = 0.017$; lambda = 1.00; $p = 0.082$). In fact, only the 4th PC based on the 3D head shape showed a significant ($P<0.05$) association with phylogeny for both *k* and lambda coefficients, and simultaneously significantly differentiated the studied species.

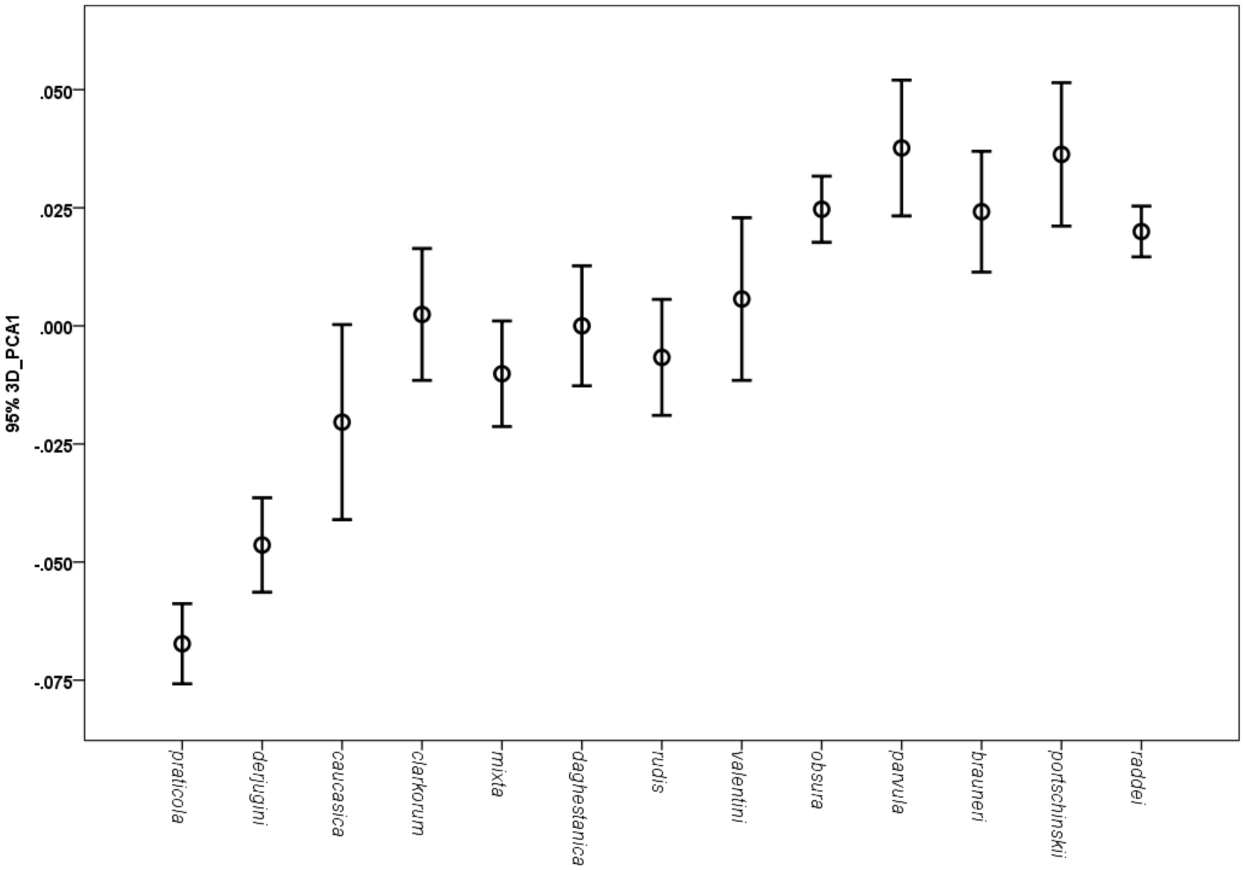
Hence, the differences in head shape and scale outlines may be related to the adaptation to habitat type and, to a lesser extent, to body size; however, only the first PCA describing 3D head shape varies depending on the habitat, irrespective of phylogeny. One-third of meaningful PCA axes differed between the clades within *Darevskia* that means the separation of four basal clades within this taxon aggregated irreversible phenotypic characters. 4th PCA describing head shape holds especially strong phylogenetic signal, together with the 4th dimension of frontal scale; these variables are associated neither with habitat or body size, nor with sex.

Table 1. Significance (P values) of individual PCA axes with the preferred habitat, body size, sex, attribution to a particular phylogenetic clade, and species of *Darevskia*. The values that remain significant considering P<0.05 after Bonferroni correction applies across columns, shown in cursive*.

	sex	clade	hb	hb-ph	size	sz-ph	spec	sex*sp	K	Lambd
3DPCA1	0.169	*0.000*	*0.000*	*0.001*	0.438	0.063	*0.002*	0.976	0.254	0.990
3DPCA2	0.830	0.025	*0.000*	0.170	0.060	0.460	0.016	0.982	0.809	1.000
3DPCA3	0.136	*0.000*	0.907	0.699	0.035	0.743	0.082	0.068	*0.011*	0.051
3DPCA4	0.039	*0.000*	0.448	0.856	0.061	0.229	0.000	0.351	*0.026*	*0.030*
3DPCA5	0.039	*0.000*	0.060	0.738	*0.000*	0.443	*0.000*	0.275	0.275	1.000
3DPCA6	0.880	0.104	*0.000*	0.477	0.810	0.909	0.574	*0.003*	0.078	1.000
3DPCA7	0.086	*0.005*	0.531	0.860	0.020	0.905	0.204	0.318	0.434	1.000
3DPCA8	0.029	0.358	0.753	0.925	*0.000*	0.127	0.271	0.712	0.863	1.000
ASPCA1	*0.000*	*0.000*	0.010	0.094	*0.002*	0.198	0.126	0.878	0.412	0.510
ASPCA2	*0.001*	*0.000*	0.031	0.866	0.730	0.367	*0.001*	0.176	0.350	0.226
ASPCA4	0.389	0.069	0.708	0.041	0.762	0.367	0.685	0.425	0.212	1.000
ULPCA1	0.014	*0.004*	*0.000*	0.022	0.052	0.368	*0.003*	0.609	0.068	0.418
ULPCA2	0.416	*0.004*	*0.002*	0.881	0.029	0.657	0.047	0.336	0.522	1.000
FSPCA1	0.540	0.259	*0.000*	0.054	0.247	0.334	0.075	0.265	0.589	1.000
FSPCA2	0.285	0.526	*0.000*	0.029	0.352	0.874	0.085	0.791	0.240	1.000

FSPCA4	0.838	*0.000*	0.093	0.188	*0.003*	0.993	0.043	0.685	*0.017*	0.083

* clade - the differences between the four clades of *Darevskia* (Fig. 1). Hb - habitat type (rock-dwellers, ground-dwellers, intermediate); hb-ph - habitat type controlled for phylogeny; size - maximum body size of adults; sz-ph - influence of size controlled for phylogeny; spec - species; sex*sp - influence of sex dependent on a species; K and lambda - significance of phylogenetic signal at species level.


95% 3D_PCA1
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praticola
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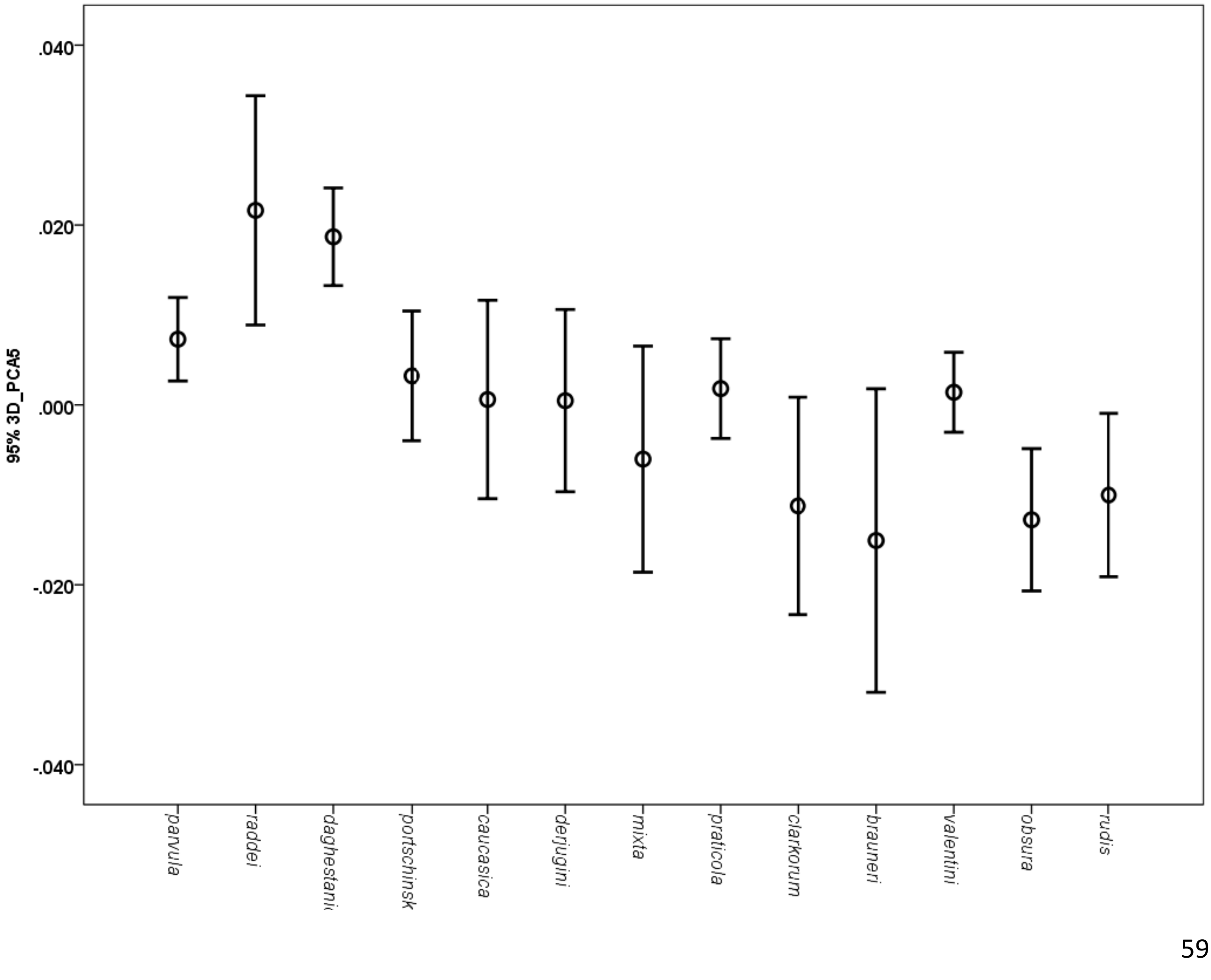
brauneri
portschinskii
raddei


95% 3D_PCA5
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raddei
daghestani
portschinsk
caucasica
derjugini
mixta
praticola
clarkorum
brauneri
valentini
obsura
rudis

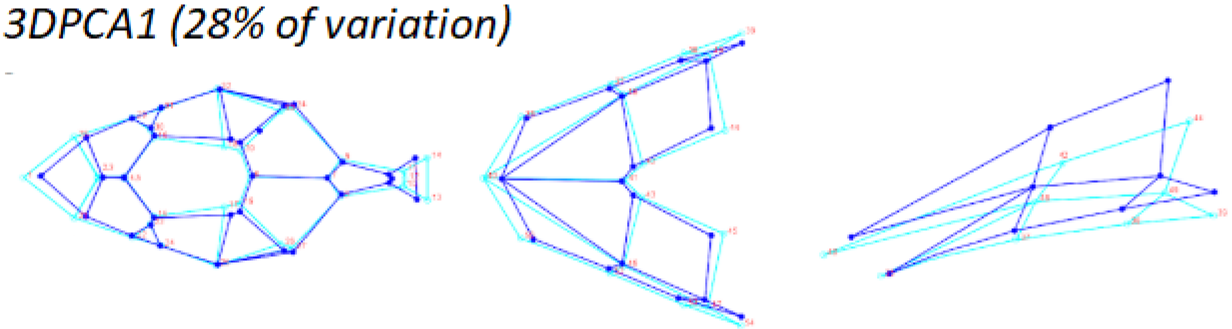
Figure 6. ‘Mean and Whisker’ plot of the 3DPCA1 axis (associated with the preferred habitat type: the first two species are ground dwellers, 3-8 - preliminary rock-dwellers, 9-13 - strict rock-dwellers) and 3DPCA5 axis (associated with body size of adults: in the first 3 species snout-vent length less than 60 mm, 4-8 - 60-65 mm, 9-13 - more than 65 mm). The dots represent species’ mean; whiskers represent 95% confidence interval.

Explaining the important principal components

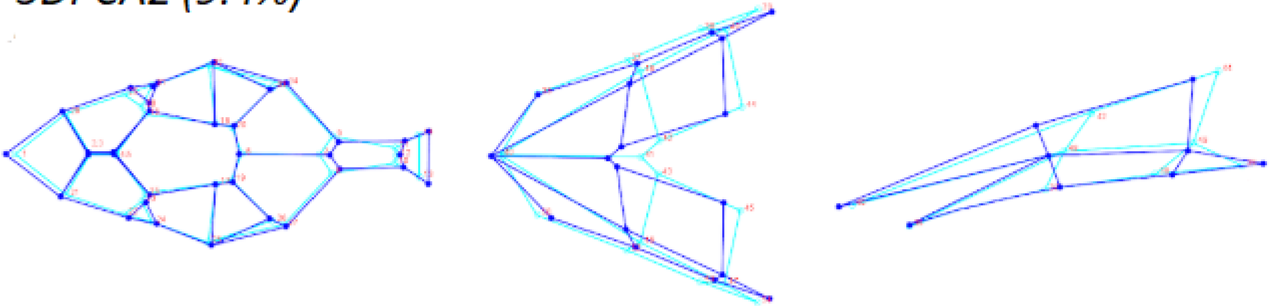
The most important components explaining phenotypic evolution of the lizards are associated with head shape: 1st, 2nd, and 6th PC of the 3D head shape helps to discriminate among the species with more and less dependence on the rocky habitats; PC5 is correlated with body size; PC3 and 4 are significantly associated with phylogeny but not with the habitat or size. PC6 is associated with sex. PC1 and 2 of anal scale shape variations are associated with sex (and helps to distinguish between the clades), and PC6 of the 3D head shape showed significant variation among the sexes dependent on the species (for the interaction sex*species, $P = 0.003$); besides, PC4 of the frontal scale shape significantly correlates with body size, and PC1 of the 5thUL correlates with habitat type.

PC1 of the 3DPCA (28% of the head shape variation) is associated with head height. The lizards with high scores along this axis (ground dwellers) have taller and shorter heads, and frontal scale with parallel edges, whereas the lizards with low scores (rock dwellers) have flat elongated heads with angled edges of the frontal scale. PC2, also associated with habitat type, varies with relative length of the rostral part (Fig. 7, a, b). PC4 (6.7% of the head shape variation), i.e. the most phylogenetically informative dimension of the head shape, is associated with flatness of a rostral part of the head, breadth of the lower jaw with concave vs straight edges, and relative length of the interparietal scale (Fig. 7, d). PC5 (5.3% of the head shape variation) is associated with the breadth of the lower jaw. Specifically, large-bodied lizards (e.g. *D. rudis*) have wider lower jaw in its proximal part, whereas small-bodied ones (e.g. *D. parvula*) have narrower lower jaw (Fig. 7, e).

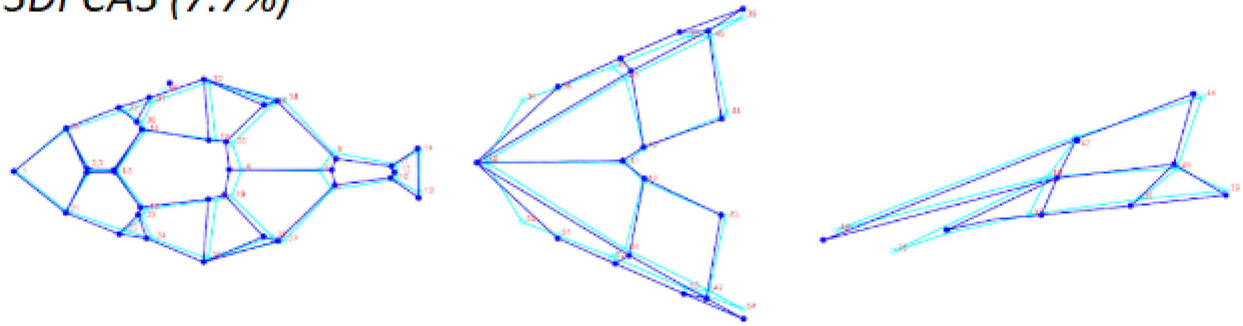
3DPCA1 (28% of variation)



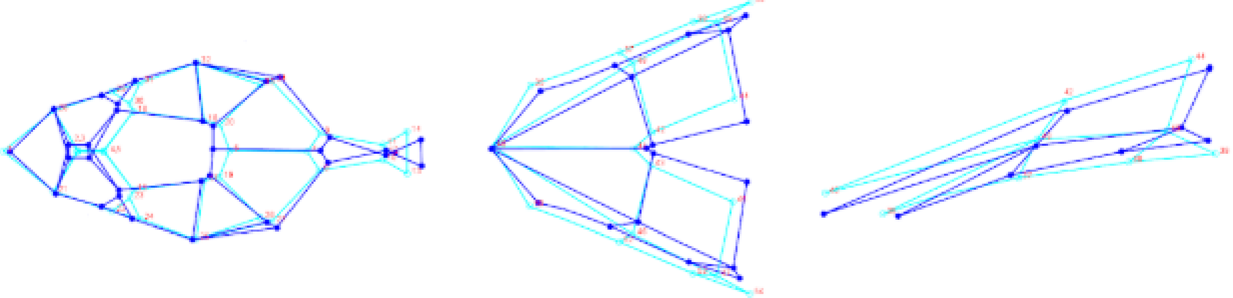
3DPCA2 (9.4%)



3DPCA3 (7.7%)



3DPCA4 (6.7%)



3DPCA5 (5.3%)

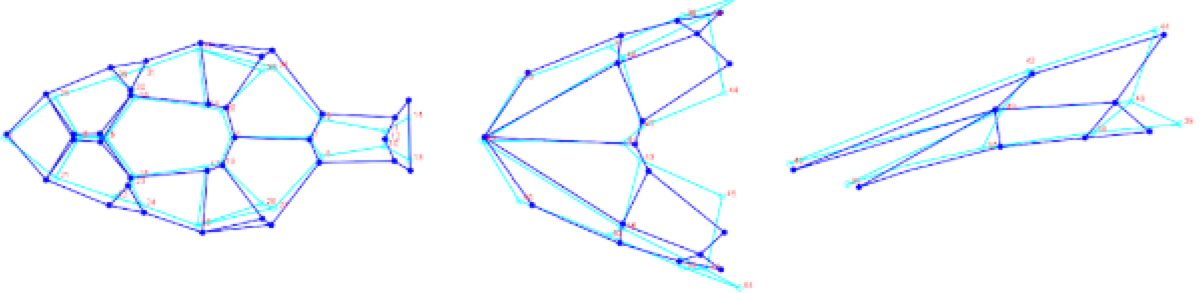


Figure 7. Dorsal, ventral and jaw wireframes (from left to right) of 3DPCA1-5. Mean values along the respective PCA axes are marked with light blue dots and lines; maximal values are marked with dark blue dots and lines.

Lower PC1 values for anal scale shape (females and lizards of the clades "*rudis*" and "*parvula*") differ from the higher values (males and lizards of the clades "*caucasica*") by wider and shorter, less round shape. Lower values of PCA2 differ from the higher values by the pointed hind part of the anal scale (Fig. 8, a, b). Higher values of ULPCA1 (ground-dwelling forms) is associated with relatively taller 5th UL (Fig. 8, b). Higher values of PCA4 for the frontal scale shape (larger species) are associated with a more angled anterior edge of the frontal scale (Fig. 8, c).

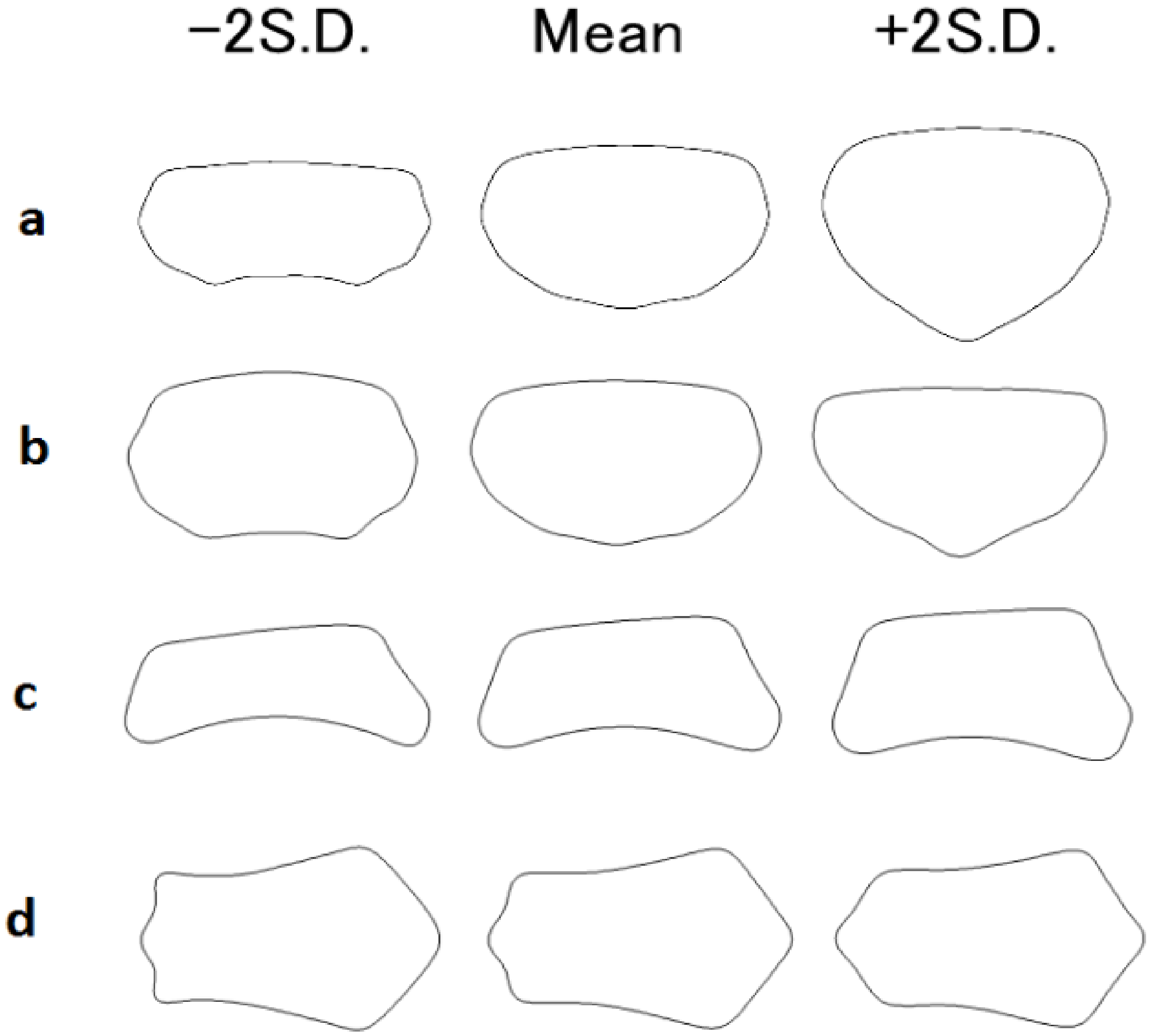


Figure 8. PC contours explaining the shape variation for ASPCA1 (a), ASPCA2 (b), ULPCA1 (c), FSPCA4 (d). 2S.D. is 2* standard deviation.

Discriminant analysis and centroid-based tree

The stepwise DFA analysis run for 31 meaningful PC axes retained four variables that significantly discriminate among the species: 1st, 2nd, 4th and 5th PC for the 3D head shape. The distance trees based on the centroids along the four significant discriminant functions was not congruent with the phylogenetic tree of *Darevskia* (results not shown).

The stepwise DFA analysis run for 19 meaningful PC axes not associated with habitat, size, or sex (Table 1) retained two variables significantly discriminating among the species: 2nd and 4th PC for the 3D head shape (3DPCA2 and 3DPCA4). The discriminant function centroids retained based on these variables produced distance matrix, strongly correlating with the molecular distances. Unrooted Neighbor-Joining tree based on this distance matrix was congruent with the neighbor-joining tree based on the mitochondrial DNA analysis. The coincidence of the mitochondrial and phenotypic tree topologies were full in part describing the relations of the species within the clade "*caucasica*" (Fig. 9)

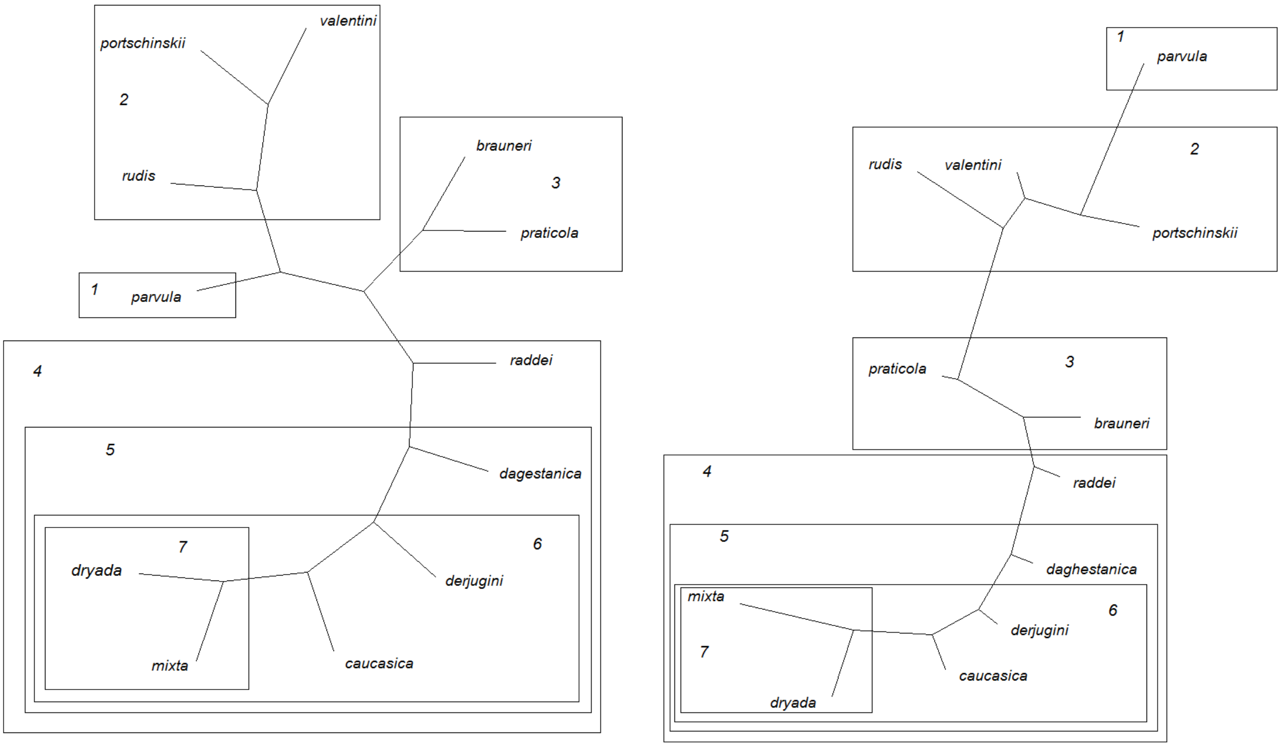


Figure 9. Left: Unrooted Neighbor-Joining mitochondrial DNA tree of the studied *Darevskia* species based on the full mitochondrial sequences of these species (Murtskhvaladze et al., in press). Right: the unrooted distance-based Neighbor-Joining tree (the distances between the DFA centroids; the analysis conducted for meaningful PCAs not associated with sex, habitat, or size retained only 3DPCA2 and 3DPCA4 as significantly discriminating among the species).

The distance based on the first discriminant function centroids significantly correlated with the mitochondrial phylogeny (lambda = 0.70, P = 0.047).

Discussion

Our results suggest that it is possible to extract a phylogenetic signal, using 3D geometric morphometrics, even for a group of closely related species with clear patterns of adaptive divergence, in spite of the convergent evolution complicating this task.

Openshaw and Keogh (2014) suggest that morphological shape variation can be affected by the three most important factors: body size, phylogenetic background and habitat-related factors. Sex differences should be obviously added to this list. It makes sense to separately discuss these four effects in respect to the study species group. Sexual dimorphism in lizards is expected to be associated with bite strength, since bite is an important element of mating behavior (Vincent & Herrel, 2007). In *Darevskia*, this is a potentially important behavioral element because type of mating bite may be a mechanism of reinforcement (Darevsky, 1967; Galoyan, 2013). However, our analyses suggests that only a small subset of head shape variation (PC6, explaining 3.9% of the variation) is associated with sex, along with the outline of the anal shape.

Habitat type is another factor obviously affecting phenotypic evolution in lizards. Losos et al. (1997) showed that the body shape of anoles undergo significant change just a few generations after they were introduced to new island habitats, and these changes depend on the difference in vegetation between the original and new habitat. Barros et al. (2011) showed that skull evolution in lizards of the family Gymnophthalmidae is driven by their mode of living (burrowing vs ground-dwelling). Vanhooydonck & Van Damme (1999) and

Herrel et al. (2001) demonstrated a decrease in head depth in rock dwelling lizards, related to the ground-dwellers. Revell et al. (2007) showed, for four different lizard clades representing two families, that adaptation to the rock-dwelling life mode is usually correlated with longer limbs and flatter heads. Urošević et al. (2012) showed this rule to also apply for European, small-bodied lacertids of the genus *Podarcis*, close relatives of *Darevskia* (Zheng & Wiens, 2015; Murtskhvaladze et al., in press).

On the other hand, Slayton (2006) showed that herbivorous lizards from different taxonomic groups, in spite of similarity in the amount of force produced during jaw closure, do not converge in jaw shape. Our research suggests that habitat preference is an important agent of phenotypic evolution in small-bodied lizards, which significantly affected head shape and anal area. The position of scales around the anal scale in rock-dwelling *Darevskia* is different than in the ground-dwelling congeneric species: the latter ones have preanals overlaying the fore part of the anal scale similar to most of other lacertids, whereas in rock-dwellers edges of the scales in the anal area contact each other (Tarkhnishvili, 2012); but without affecting their outlines (Gabelaia et al., 2017; this paper). Geometric morphometrics associates the main axis of 3D head shape variation (PC1, explaining 28% of the total head shape variation) with habitat preferences. Ground dwellers have taller heads with shorter and more obtuse snout than rock dwellers; moreover, similar but less prominent differences exist between rock-dwelling *Darevskia* that depend on rock habitats to different extent (e.g. heads are taller and shorter in *D. mixta* or *D. caucasica* than in *D. parvula* and *D. raddei*). It is likely that the flat heads help lizards to increase the number of rock clefts suitable for hiding and egg laying. This analysis supports the predictions suggesting higher effectiveness of 3-dimensional geometric morphometrics over other methods of phenotypic analysis (Sztencel-Jabłonka et al., 2009; Adams et al., 2013; Ivanović et al., 2013; Gabelaia et al., 2018). Some other traits, including outline of upper labial and frontal scales, correlate with habitat type only if they are not controlled for phylogeny. Hence, they are phylogenetically constrained and, different from the head shape, do not produce convergent patterns.

Variability in body size can be an important adaptive feature that can be driven by a change in the available food (Meiri, 2008), primary productivity of the ecosystem (Aragon & Fitze,

2014) or niche shift as a result of coexisting with closely related species (Meiri, 2008; Moritz et al., 2018). Body shape changes as a result of allometric growth (Johnson et al., 2005; Kaliontzopoulou, 2011; Openshaw & Keogh, 2014). Small size promotes fast diversification in body shape in lizards (Meiri, 2008). *Darevskia* comprise small-bodied lizard species; in different species maximum body length varies between 55 - 85 mm. *Darevskia* species occupying the same location and the same habitat (i.e. coexisting rock dwellers) usually differ in body size, such as *D. parvula* and *D. rudis* in southwestern Georgia, or *D. rudis* and *D. mixta* in the central part of the country. This may be related to either a niche shift for avoiding competition (Tarkhnishvili, 2012), or reinforcement understood as selection acting against the production of hybrid individuals (Sawyer & Hartl, 1981). Body size is not associated with substantial differences in shape. Only PC5 of the 3D head shape shows some differences among small-bodied and large-bodied rock lizards, specifically the former ones having concave edges of the lower jaw and a pointed parietal scale in the hind part (Fig. 7, b). This character is phylogenetically constrained and does not produce convergent patterns.

Multiple studies have discussed the congruence of the species' morphological variation with their phylogeny (Gentilli et al., 2009; Henderson et al., 2013), but not always confirmed (Wiens and Penkrot, 2002; López-Fernández et al., 2005; Steppan, 1998; Serb et al., 2001). Bookstein (1991) suggested that biological shapes comprised by several intercorrelated morphological characters hardly will be phylogenetically informative, because they are prone to morphological integration ('coordinated variation among traits that are closely related in development and/or function' – Singh et al., 2012). This would reduce the exposure of features that are only phylogenetically informative (Ivanović et al., 2013). Smith et al. (2011) showed that adaptive changes during lizard evolution are constrained, which explains multiple examples of convergent or parallel evolution in different lizard families, shading the phylogenetic pattern (Thorpe et al., 1994; Harmon et al., 2005; Köhler et al., 2010). A statement of Losos (2011) on the stable differences in head shape among lizards of different families, despite their divergent adaptations, is very much true; however, this hardly applies to different species from the same genus: congeneric but non-sister species, adapted to similar environments, may be very difficult to recognize (Smith et al., 2011).

Discordance between the phenotypic (Darevsky, 1967) and genotypic (Murphy et al., 2000; Ahmadzadeh et al., 2013) systems of *Darevskia* is a clear example of this (Gabelaia, 2017). Body size and head shape brodly vary among the closely related species of this genus and may be more similar in species that belong to different clades, such as *D. portschinskii, D. parvula,* and *D. raddei*.

However, our data suggest that, in spite of these convergent patterns, there are dimensions of head shape and scale outline that hold clear phylogenetic signals, which changes are irreversible, following Dollo's law (Gould, 1970). Gabelaia et al. (2017) showed this for the outline of the anal scale that indicates the differences between the clades "*rudis*" and "*parvula*" on one hand, and clade "*caucasica*" on the other. Table 1 of this paper suggests that there are multiple shape dimensions (principal components) that significantly differ between the four major clades within *Darevskia*. Those include adaptive ones, such as head depth associated with habitat type (different between the basal clades "*parvula*" and "*rudis*" on one hand and terminal clades "*saxicola*" and "*caucasica*" on the other) but also the dimensions which adaptive importance is less clear, such as the relative depth of the rostral and cranial parts of the head (3DPCA4), breadth of the hind part of the lower jaw, the outline of the anal scale, more or less elongated upper labials, and frontal scale with concaved vs parallel edges.

The congruence of the phenotypic tree (based on the head dimensions unrelated to habitat type) and genotypic tree suggests that the convergent evolutionary patterns cannot completely annihilate phenotypic divergence even between the most closely related species of rock lizards. Geometric morphometrics is shown to be an effective methodology that is able to infer the evolutionary signal even in cases where traditional multivariate morphometry is powerless (Bernal, 2007; Maderbacher et al., 2008; Abdel-Rahman et al., 2009; Breno et al., 2011); it is an effective instrument for studying correlations between the molecular and phenotypic evolution. Simultaneously, it is important to understand that a substantial part of shape variation in close relatives is due to adaptation that may show convergent patterns. One needs to carefully analyze the entire picture and remove these convergent patterns for identifying phylogenetically informative traits.

Obviously, the phenotypic evolution of *Darevskia* requires further detailed studies exploring adaptive importance of individual phenotypic dimensions and interaction between convergent and divergent evolutionary patterns in the course of speciation.

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Chapter 4: Phylogeography and morphological variation in a narrowly distributed Caucasian rock lizard, *Darevskia mixta*

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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 single 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

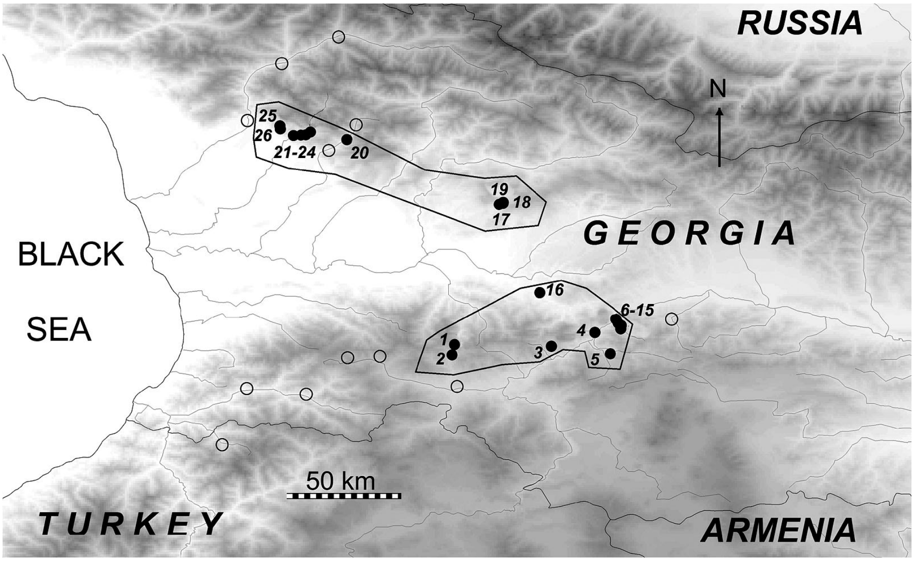

Figure 1. Sampling locations of *D. mixta*. Solid black circles 1-26: sampling locations of *D. mixta* (see table 1 for details). Open circles: the surveyed rock lizard locations close to the range of *D. mixta*, where the presence of this species was not established. The solid outline shows the approximate 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
		19	5	2
	Samegrelo Range	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 Tarkhnishvili et al. (2010); Tarkhnishvili, Murtskhvaladze 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 Tarkhnishvili, Murtskhvaladze 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; Tarkhnishvili, Murtskhvaladze 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-μl total volume, with 2-4 μl template DNA, 1 U of Go Taq DNA polymerase and 5X buffer (Promega), 1 μM of $MgCl_2$, 0.1 μM of each dNTP, and primer concentrations at 0.1 μM. 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 μl 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 Rambault, 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.

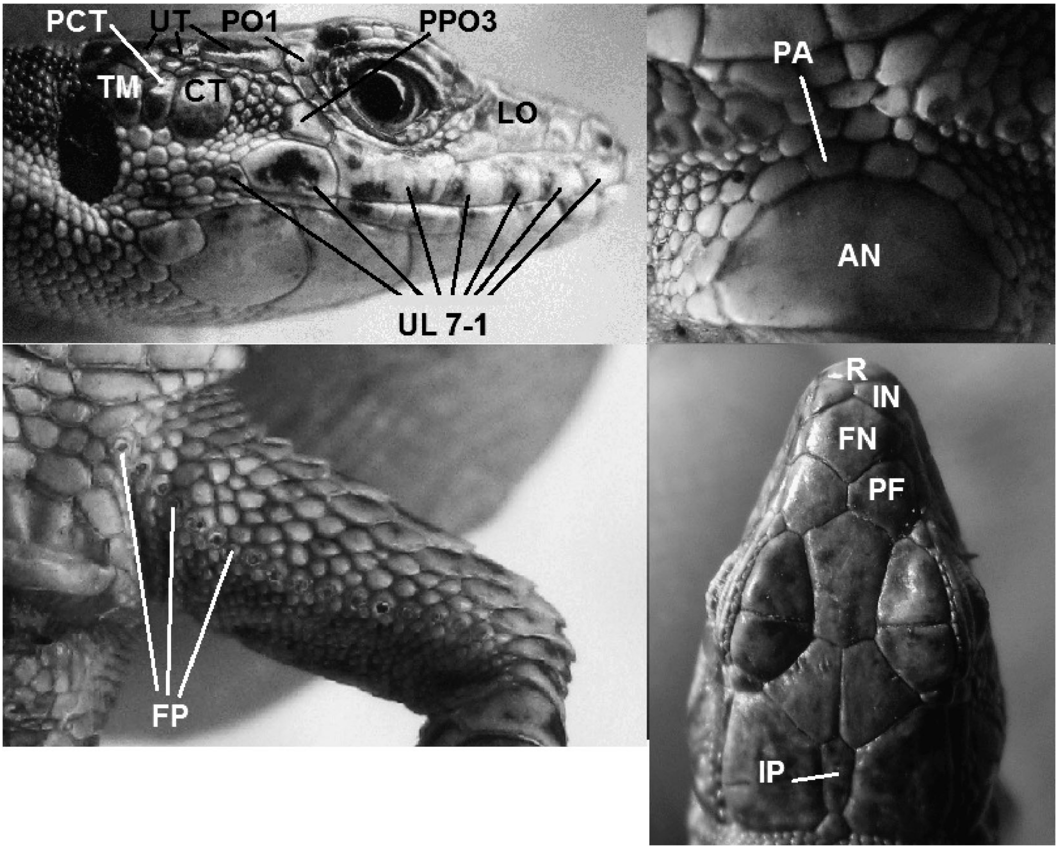

Figure 2. Scalation characters described for the studied individuals. CT – central temporal scale, PCT – post-central-temporal, TM – tympanal, PO1 – postorbital 1, PPO3 – post-postorbital 3, UL – upper labials, LO – loreal, AN – anal scale, PA – preanals, PF – prefrontals, IN – internasals, FN – fronto-nasal, 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 Meskheti Range, the Lesser Caucasus (locations 1-2 vs. 3-15, fig. 1). Other variations marked 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 Meskheti 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,

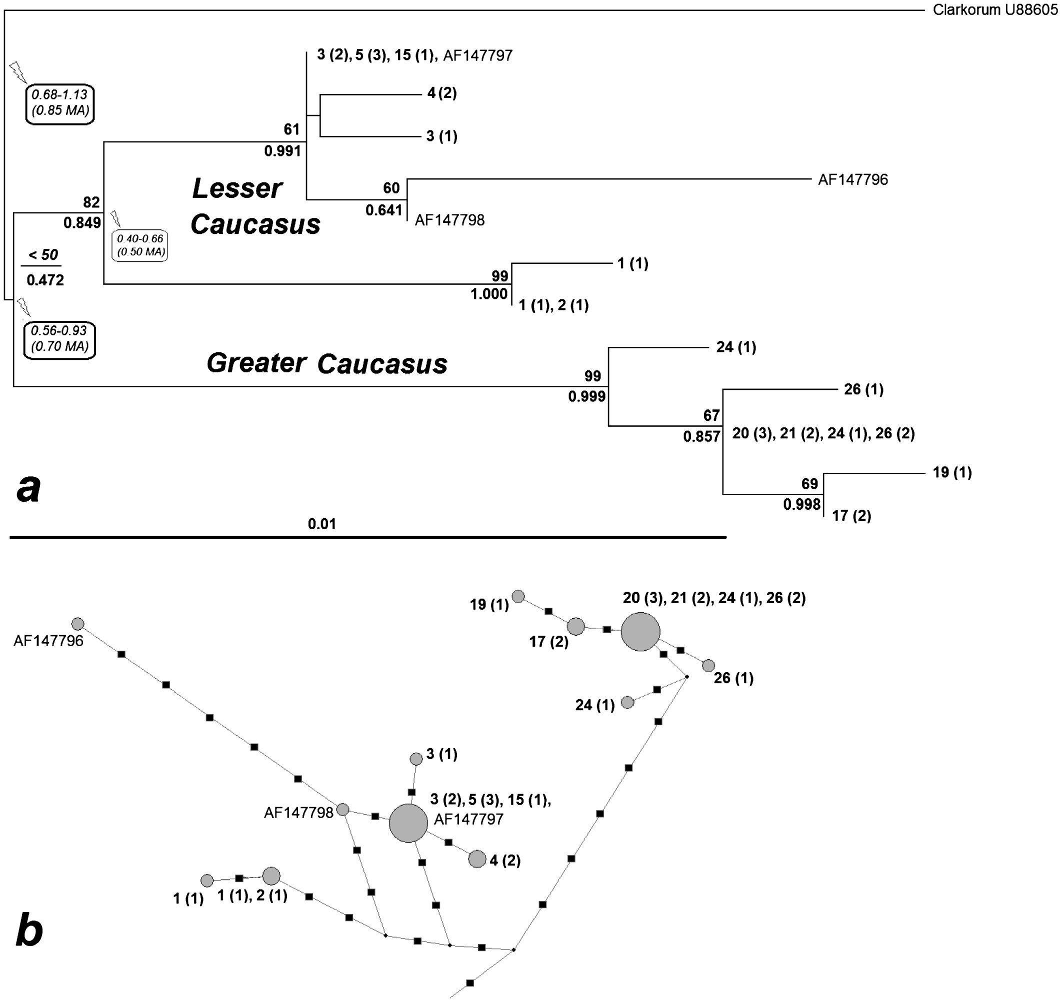

Figure 3. The topology of mitochondrial haplotypes of *Darevskia mixta*. (a) Maximum likelihood tree of the mitochondrial haplotypes (cytochrome *b*) of *D. mixta*. Tip labels indicate locations (fig. 1), with the number of individuals in parenthesis. Node labels indicate bootstrap values (1000 bootstrap 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 ($P = 0.04$) differences 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.

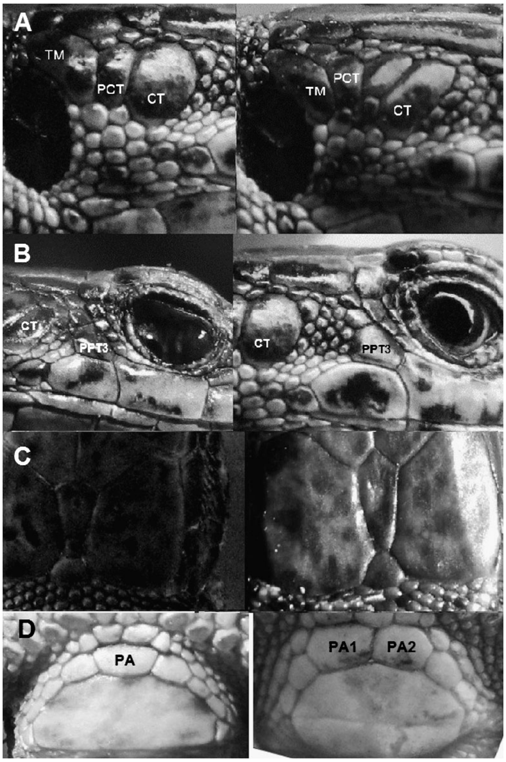

Figure 4. Most typical differences between *D. mixta* from the Lesser and the Greater Caucasus. (A) additional PCT (right image; more common in the Lesser Caucasus); (B) low vs. high number of scales between eye and CT (left – low number; more common in the Lesser Caucasus); (C) separated interparietal 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 haplotypes 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 Meskheti 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 Yavuzkemal, 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 Yavuzkemal, and discovered only *D. clarkorum* at these locations. Our field studies since 2006, including those conducted throughout NE Turkey close to Yavuzkemal (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.

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Chapter 5: Mitochondrial phylogeny of the *Darevskia saxicola* complex: two highly deviant evolutionary lineages from the easternmost part of the range

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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 Caucasus 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* (MacCullogh 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; MacCullogh 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

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Fig. 1. Sketched ranges (After Darevsky, 1967; MacCullogh et al., 2000; Doronin et al., 2013) of the nominal species and subspecies of *D. saxicola* clade and locations used for sampling for the genetic studies by Murphy et al. (2000), Doronin et al. (2013) and in this paper. *D. praticola* and *D. alpina* not included. *Ds – D. saxicola, Db – D. brauneri, Dl – D. lindholmi.*

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.

Character	Tekhuri Valley	Enguri Valley	*D. saxicola*	*D. brauneri*	*D. lindholmi*
fp	18–21 (19.4)	17–21 (18.1)	13–20 (17.1)	15–22 (18.5)	16–23 (19.1)
np	2–3 (2.40)	1–2 (1.75)	1–3 (1.9)	2–3 (2.40)	1–2
HL/HW	1.52–1.91 (1.7)	1.82–2.00 (1.89)	1.82–2.0 (1.89)	1.73–1.98 (1.90)	2.0–2.08 (2.05)
nsc	12–14 (12.63)	13 (13)	3–15 (9.4)	8–13 (10.62)	7–21 (12.4)

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 coexistent *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 SEQSCAPE 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. mjusserica*: 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 &

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

	D. b. b.	*D. b. d.*	*D. b. m.*	*D. l.*	*D. a.*	*D. s.*	*D. [b] s.*	Enguri
D. b. brauneri								
D. b. darevskii	0.026							
D. b. myusserica	0.028	0.036						
D. lindholmi	0.041	0.039	0.046					
D. alpina	0.050	0.048	0.048	0.050				
D. saxicola	0.042	0.040	0.040	0.037	0.044			
D. [b.] szczerbaki	0.043	0.040	0.035	0.034	0.043	0.021		
Enguri Valley	0.049	0.046	0.048	0.046	0.049	0.026	0.036	
Tekhuri Valley	0.051	0.046	0.045	0.046	0.047	0.030	0.034	0.028

Kumar, 2000). 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 & Rambault, 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.*

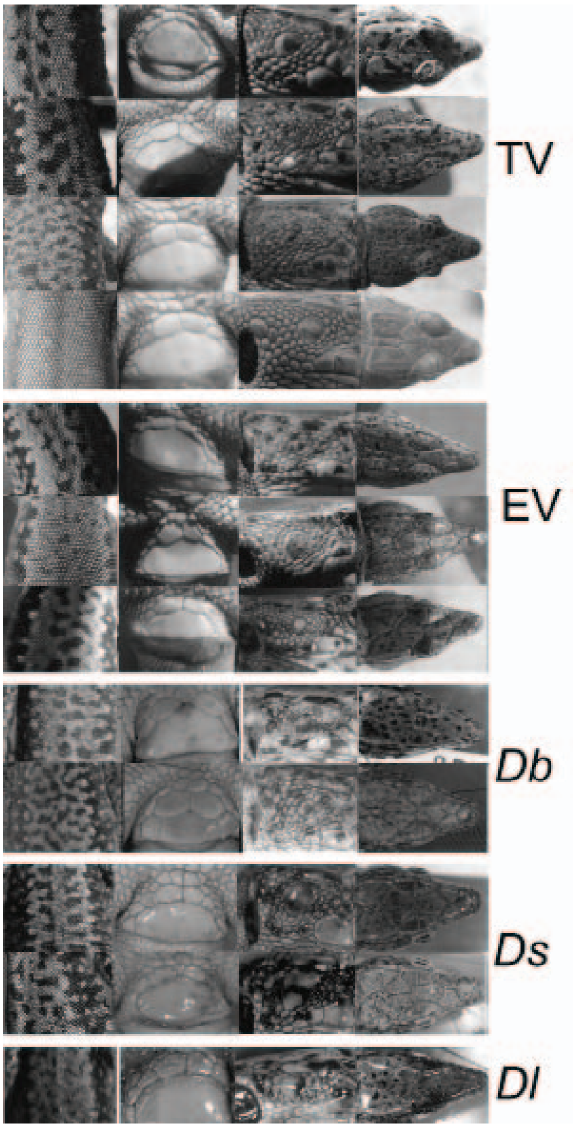

Fig. 2. Dorsal side (left), anal area (middle left), temporal area (middle right) and pileus (right) of males of the population from the river Tekhuri Valley (TV), river Enguri Valley (EV), Sochi (*D. brauneri, Db*), upper currents of river Kuban' (*D. saxicola, Ds*), Yalta, Crimea (*D. lindholmi, Dl*).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

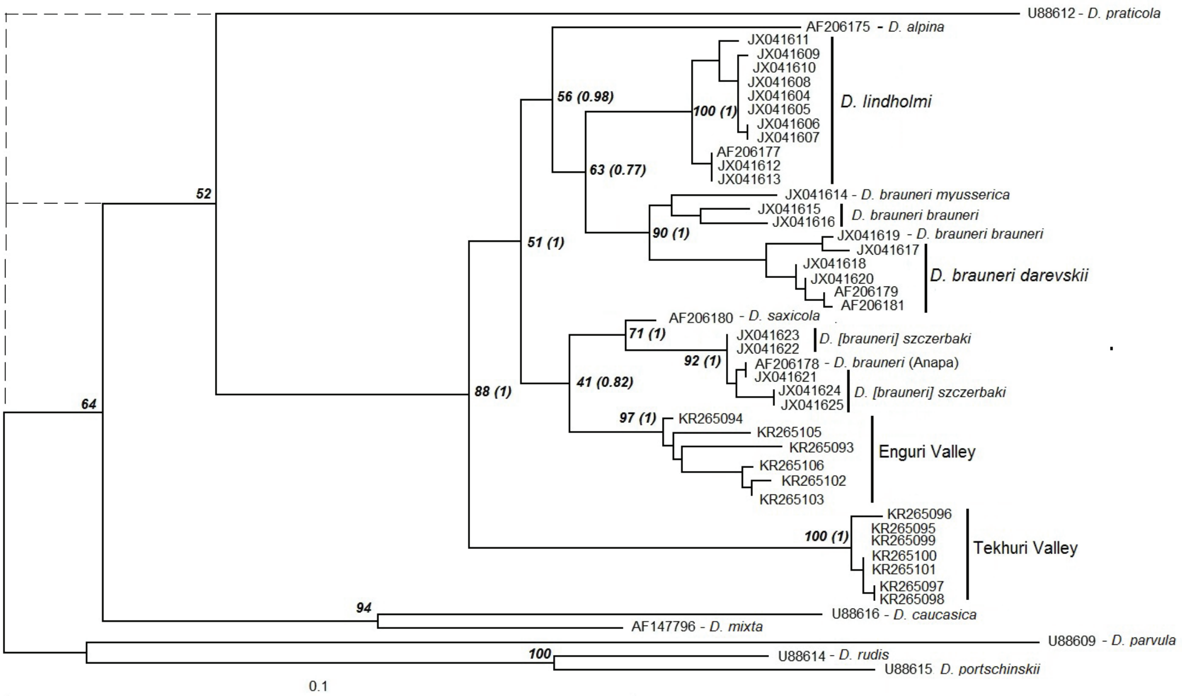

Fig. 3. Tree topology for the studied sequences (Fu et al., 1996; Doronin et al., 2013; our data), inferred with ML method (model HKY + G, equal branch length hypothesis rejected). Dashed lines indicate conflicting topologies inferred from Bayesian algorithm. Bootstrap support values are shown near the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

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

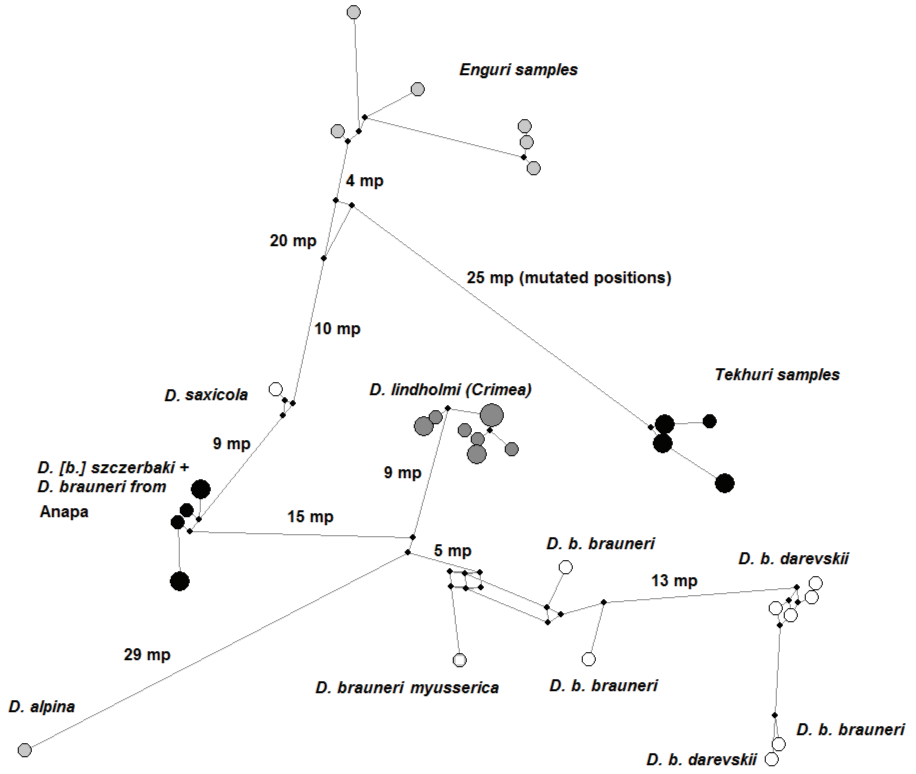

Fig. 4. Median-Joining network of haplotypes of clade *D. saxicola* (Fu et al., 1996; Doronin et al., 2013; our data). Branch length proportional to the number of substitutions.

1.00) + *D. " brauneri"* from Anapa (geographic location of *D. [b] szczerbaki*); (4) lizards from the valley of the river Tekhuri (BV 100, PP 1.00); (5) lizards from the valley of the river Enguri (BV 97, PP 1.00); (6) a clade, albeit with a low bootstrap support, comprised of the latter one *and D. saxicola* (BV 41, PP 0.82). It appears that the mitochondrial haplogroup of lizards from the valley of the river Tekhuri keep a basal position in the tree of *D. saxicola* group, excluding *D. praticola* (Fig. 3). Molecular distances between the obtained and downloaded haplotypes of *D. saxicola, D. lindholmii, D. brauneri* and their subspecies, and the specimens from Enguri and Tekhuri valleys are shown in Table 2. The network of the haplogroups (Fig. 4) suggests comparable distances between the nominal species of the group (*D. saxicola, D. brauneri, D. lindholmii*) and the samples described in this paper.

Although the null hypothesis of the equal evolutionary rate throughout the maximum likelihood tree was rejected, we can speculate that the range of differences (percent substitutions) between individual sequences of Tekhuri Valley lizards, Enguri Valley lizards, and the reminder of specimens of *D. brauneri, D. saxicola, D. lindholmi*, and *D. alpina* correlate with variations of the divergence time between these lineages. Considering a 95% confidence interval of the differences among the branches of different length and the rates varying between 1.5% and 2.5% of sequences per mya (Crochet et al., 2004), the time of separation among these branches should vary between 1.22-1.70 mya. Hence, even if a very broad range of the molecular evolution rates is considered, the split between the *D. saxicola* clade from Tekhuri Valley, Enguri Valley, the northern slope of the Greater Caucasus, and from the southern slope should have been completed before the Mid-Pleistocene revolution 800 kya, when the glacial cycles became deeper and longer (Imbrie et al., 1993). As a hypothesis, the expansion of ice cover over large parts of the Western Greater Caucasus fragmented existing habitats and caused mtDNA divergence. A similar matrilineal pattern of Pleistocene-time fragmentation in the central southern Caucasus was recently described for another taxon, *Darevskia mixta sensu lato* (Gabelaia et al., 2015).

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 *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 *D. saxicola, D. lidholmi,* and the subspecies of *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 *D. saxicola*, and never have large central temporal scales. Different from *D. brauneri* from the Russia's Black Sea Coast and studied specimens of *D. saxicola* and *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 *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).

Evolutionary lineages and taxonomic implications

Excluding the basal *D. praticola*, there are three almost equidistant mitochondrial lineages within the *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 *D. brauneri brauneri, D. b. darevskii, D. b. miusserica, D. lindholmii,* and in the hybrid form *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 *D. saxicola* and lizards usually indicated as *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 existent taxonomy of the group. There are two possible taxonomic solutions. It is possible to consider the nominal *D. brauneri, D. saxicola, D. lindholmi* and specimens described here as subspecies within the polytypic *D. saxicola* (Eversmann, 1834). However, taxa differentiated to the extent as we observe it for *D. saxicola* and *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 *D. lindholmi* is a separate species. The populations from the rivers Enguri and Tekhuri should not be considered to be part of *D. brauneri*, because matrilineally they are more distant from this species than *D. lindholmi*.

It is possible that the evolutionary pattern within the *D. saxicola* clade (excluding *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 *D. brauneri* and *D. lindholmi*. The northern lineage was split between the northern (*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 *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 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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Chapter 6: Use of three-dimensional geometric morphometrics for the identification of closely related species of Caucasian rock lizards (Lacertidae: *Darevskia*)

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Use of three-dimensional geometric morphometrics for the identification of closely related species of Caucasian rock lizards (Lacertidae: *Darevskia*)

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Geometric morphometrics is a better tool to evaluate the variation of shape than 'traditional' morphometrics. In reptiles, it outperforms morphometrics based on linear measurements and scalation. In an earlier study, two-dimensional outline-based geometric morphometrics in six species of rock lizards (*Darevskia*) showed that their shapes reflected the species divergence pattern. This allowed us to separate species from different clades, but we could not distinguish closely related species within a clade. We hypothesized that three-dimensional (3D) head shape data would be sufficiently discriminative to identify closely related species. To test this hypothesis, we compared head shapes of three closely related species of the '*rudis*' clade, *Darevskia portschinskii*, *Darevskia valentini* and *Darevskia rudis*, with the last species treated as two distinct groups (*D. r. obscura* vs. *D. r. rudis*), using 3D landmark data. The 3D analysis isolated the species of the '*rudis*' clade from each other and even separated individuals from populations that showed a genetic introgression pattern. The analysis showed that *D. r. obscura* is morphologically as distinct from *D. r. rudis* as from the other nominal species. For this reason, we suggest elevating the status of *D. r. obscura* to species level, i.e. *Darevskia obscura* Lantz & Cyrén, 1936.

ADDITIONAL KEYWORDS: head shape – species boundaries – three-dimensional photogrammetry.

INTRODUCTION

Caucasian rock lizards (genus *Darevskia* Arribas, 1999) are a small-bodied, speciose group of rock lizards mostly found in the Caucasus (Darevsky, 1967; Tarkhnishvili, 2012). This group is composed of three ('*rudis*', '*caucasica*' and '*saxicola*') matrilineal clades, each consisting of several species, according to Murphy *et al.* (2000). The scalation pattern in *Darevskia* is highly variable at the individual level, with individual scalation traits strongly overlapping among the species. As such, there are no fully diagnostic scalation differences for most of the *Darevskia* species (Darevsky, 1967; Tarkhnishvili, 2012).

Geometric morphometrics (GM) has been proved to assess shape variation better than 'traditional' (linear measure based) morphometrics (Rohlf & Marcus, 1993; Zelditch *et al.*, 2004; Blanco & Godfrey, 2006; Bernal, 2007; Maderbacher *et al.*, 2008; Abdel-Rahman *et al.*, 2009; Breno *et al.*, 2011; Schwarzfeld & Sperling, 2014). In a previous study, Gabelaia *et al.* (2017) used GM techniques, more specifically outline-based elliptic Fourier analysis, for comparing and identifying rock lizards from different clades based on the anal and pileus scales. Gabelaia *et al.* (2017) focused on six species from the '*rudis*' and '*caucasica*' clades. The analysis separated individuals from different clades and produced a dendrogram congruent with a species-level molecular phylogeny (Murphy *et al.*, 2000; Tarkhnishvili, 2012) but was unable to distinguish between closely related species within the same clade.

In the present work, we hypothesized that the application of GM on lizard head shape, which contributes to more evolutionarily informative data (Kaliontzopoulou *et al.*, 2007), would provide a more powerful dataset on species-specific variation, especially when quantified in three dimensions (3D), and this would allow discrimination between closely related species. Starting from the 2000s, the use of 3D GM has increased in studies that examine patterns of organismal morphological variation (Goricki & Trontelj, 2006; Sztencel-Jabłonka *et al.*, 2009;

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Adams *et al.*, 2013; Ivanovic *et al.*, 2013; Mangiacotti *et al.*, 2014; Gray *et al.*, 2017). The advantage of 3D GM compared with 'traditional' morphometrics and/or two-dimensional (2D) GM is that not only does it capture subtle shape variation in more detail, but also it allows a better visualization of that variation (Zelditch *et al.*, 2004; Klingenberg, 2013). For this study, we analysed head shape variation in three closely related species of the '*rudis*' clade: *Darevskia rudis* Bedriaga, 1886 (including the subspecies *Darevskia rudis obscura* and *Darevskia rudis rudis*); *Darevskia portschinskii* Kessler, 1878 and *Darevskia valentini* Boettger, 1892 (Murphy *et al.*, 2000; Tarkhnishvili, 2012). The respective evolutionary lineages of the '*rudis*' clade species separated from a common ancestor supposedly during the late Pleistocene and are still in a stage of incomplete lineage sorting (Tarkhnishvili *et al.*, 2013). *Darevskia rudis* is found in most of the Caucasus and Asia Minor, whereas *D. valentini* replaces *D. rudis* in the south of the Lesser Caucasus (Fig. 1). In the central part of the Lesser Caucasus, only *D. portschinskii* is found. Hence, the species are parapatrically distributed and form contact zones (Tarkhnishvili, 2012; Tarkhnishvili *et al.*, 2013). This parapatrical distribution explains the broad gene introgression zone between *D. portschinskii* and *D. r. obscura*. Another form, *Darevskia rudis macromaculata*, is found in some locations geographically close to the range of *D. r. obscura* (Darevsky, 1967) and belongs to the same monophyletic mitochondrial clade as *D. r. obscura* (Tarkhnishvili *et al.*, 2013). This clade is equidistant from *D. portschinskii*, *D. valentini* and *D. r. rudis* (Tarkhnishvili *et al.*, 2013). Arribas *et al.* (2013) suggested a conspecific status for all forms within *D. rudis* and synonymized *D. r. macromaculata* with *D. r. obscura*.

The three species investigated in this study (*D. rudis*, *D. valentini* and *D. portschinskii*) differ in body size, colour pattern and some scalation traits. Adult *D. r. rudis* have a larger body than adult *D. portschinskii*, with *D. valentini* (together with the subspecies *D. r. obscura*) being larger than *D. portschinskii* but smaller than most of the *D. r. rudis* populations (Darevsky, 1967). *Darevskia valentini* has a brighter coloration compared with the other species, with large contrasting dark spots on the back. All subspecies of *D. rudis* have shin scales markedly larger than the dorsal scales, different from the other species of the clade, whose shin scales are not larger than the dorsal scales (Darevsky, 1967; Tarkhnishvili, 2012).

In this work, we aimed to compare 3D head shape variation between *D. portschinskii*, *D. valentini* and *D. rudis* by collecting 2D images from lizards that were then used to generate 3D meshes through photogrammetry. The last of these species was treated as two groups: *D. r. obscura* and *D. r. rudis.* Using 3D landmark data, we aimed to determine whether 3D morphometrics provides more reliable diagnostic information that could separate the studied taxa than the 2D outline data on scalation patterns used before (Gabelaia *et al.*, 2017) or 'traditional' analysis of scalation applied to the same taxonomic groups (Darevsky, 1967; Tarkhnishvili *et al.*, 2013).

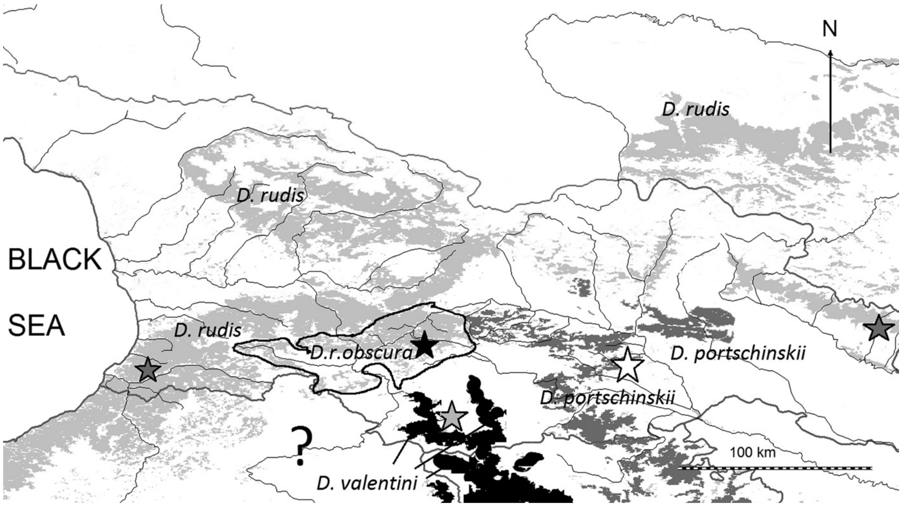

Figure 1. The distribution (according to Darevsky, 1967; Tarkhnishvili *et al.*, 2013) of the studied taxa in the Central and Western Caucasus. The ranges of *Darevskia rudis* (light grey areas) and *Darevskia portschinskii* (dark grey areas) coincide with the distribution of mountain forests. The distribution of *Darevskia valentini* (black area) is along the valleys of the major rivers and in the Abul-Samsari mountain range in southern Georgia. The range of *D. r. obscura* is delimited with a thick line. The question mark shows the area in NE Turkey where we refrain from naming the exact taxon of the *D.* '*rudis*' clade (most probably *D. r. obscura* or *D. valentini*). The stars indicate sampling locations (Table 1).

Table 1. Sample size and sampling locations of the *Darevskia* specimens studied

Taxon	Samples	Location	Geographical coordinates
D. portschinskii	3♂ 8♀	Kojori	41.649N 44.683E
D. rudis obscura	8♂ 3♀	Borjomi	41.873N 43.411E
D. rudis rudis	6♂ 10♀	Charnali 2♂ 5♀ Lagodekhi 4♂ 5♀	41.554N 41.607E 41.855N 46.300E
D. valentini	7♂ 4♀	Akhalkalaki	41.301N 43.389E

MATERIAL AND METHODS

We collected 49 adult (male and female) individuals from five locations in Georgia. Single sites were sampled for *D. portschinskii*, *D. valentini* and *D. r. obscura*; and two sites were sampled for *D. r. rudis*, including the south-west and the north-east of Georgia (Fig. 1; Table 1). All five locations/populations were studied earlier using mitochondrial DNA sequencing and microsatellite genotyping (Tarkhnishvili *et al.*, 2013); hence, their attribution to one of the four studied taxa had been genetically validated. Each individual was anaesthetized using chloroform. The individual was positioned in the centre of a cardboard circle in a tube with its head pointed upwards; the head was then photographed 36 times from a perspective of 90° to the midline and 36 times from a perspective of 45° to the midline, by moving the camera around the lizard (Fig. 2). Images were uploaded in AgiSoft PhotoScan Pro v.1.2.6 (Agisoft, 2016), which first aligned the images in 3D spaces and then generated 3D models of the head surface, onto which 66 homological landmarks were subsequently digitized (Fig. 3; Supporting Information, Appendix S1).

After digitizing the landmarks in AgiSoft PhotoScan Pro v.1.2.6, we exported the 3D coordinates in a DXF (drawing exchange format) and arranged them in Microsoft Excel (Microsoft Corporation, 2007) to retain landmark names and coordinates. We aligned and scaled 3D coordinates by performing a Procrustes superimposition using the software PAST (Hammer *et al.*, 2001). Aligned and scaled coordinates were then used for principal components analysis (PCA) in PAST (Hammer *et al.*, 2001). The individual scores along the meaningful principal component (PC) axes (obtained through a broken-stick analysis; Jackson, 1993) were used for the ordination of the individuals, in order to explore overall shape variation and to infer whether the axes already differentiated between the studied taxa.

To test for group differences in head shape, given that parametric test assumptions were not met, we applied a nonparametric (NP) MANOVA (Cooley & Lohnes, 1971) to seven meaningful PC scores. This method did not reveal significant differences in the head shape 3D data between the males and the females ($P > 0.05$); therefore, the sexes were pooled for the further analyses. We then applied the NP-MANOVA on two levels of grouping: the three nominal species (*D. portschinskii*, *D. valentini* and *D. rudis*), and these nominal species with *D. r. obscura* treated as a separate taxon (*D. portschinskii*, *D. valentini*, *D. r. rudis* and *D. r. obscura*). We included *D. r. obscura* as a separate taxon for two reasons: its monophyletic matrilineal origin, and the preliminary general PCA results showing that individuals of *D. r. obscura* grouped separately from those of *D. r. rudis* (see the Results section). Post-hoc tests (Hotelling's *P*-values and Bonferonni-corrected *P*-values) were performed to determine whether the differences were significant for each level of grouping.

As an ordination to visualize the levels of between-group differences in head shape between the four taxa, both a canonical variate analysis (CVA) and a between-group PCA (BG-PCA) were performed. To avoid ordination bias in the CVA owing to the low sample size per group (compared with the high number of variables), the CVA was done on the scores of the seven meaningful PCs (the robustness of the CVA was verified through a classifier analysis, combined with confusion matrix analysis, showing that the a priori grouping was well supported by the shape data). The BG-PCA was performed on the total landmark coordinates dataset. All these analyses were done in PAST (Hammer *et al.*, 2001).

To visualize which shape patterns were reflected in these ordinations, and thus which were the most discriminating shape differences between groups, we generated landmark wireframes that reflect canonical variate (CV) axis variation from a CVA on the total dataset of the 66 original landmarks in MorphoJ

(Klingenberg, 2011). Given that the orientations of the CV axes (with respect to the taxa group means) in this CVA were similar to those of the CVA on the meaningful PCs, the wireframes could be used to represent group differences as obtained through the latter CVA.

To check whether 3D GM analysis is more powerful in differentiating closely related lizard species than 2D GM study using outlines, we repeated the analysis described by Gabelaia *et al.* (2017) for all specimens described in the present paper. We conducted a Fourier outline shape analysis on the anal scale, which was shown to be the most effective approach for distinguishing evolutionary lineages of rock lizards, compared with other methods, including the analysis of the dorsal view of the head (Gabelaia *et al.*, 2017). We applied a NP-MANOVA and CVA on the 'meaningful' PCs to compare the results with 3D GM results.

All procedures with live animals were ethically approved by the Ilia State University Commission for Ethical Issues and were in accordance with Article 259 of Georgian Criminal Law. General anaesthesia of the lizards was used to avoid killing the animals collected in the wild. After photographing, the lizards were released to their natural habitats.

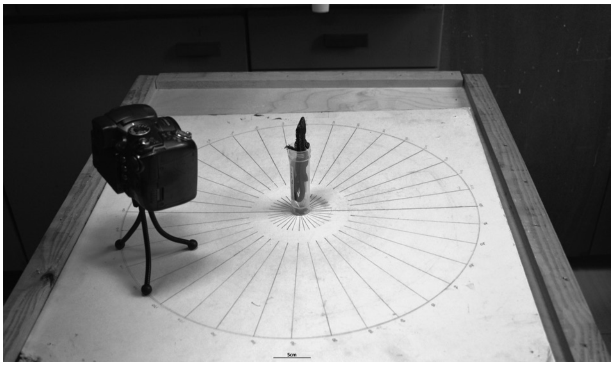

Figure 2. Set-up used for photographing the head of an immobilized lizard. The circle indicates the different positions at which a picture was taken.

RESULTS

The first seven PCs explained 62% of the overall shape variation (for eigenvalues and character loadings, see Supporting Information, Appendix S2). The first PC axis clearly discriminated between *D. r. obscura* and *D. r. rudis*. This axis also separated *D. portschinskii* and *D. r. rudis*. The first and the second axes showed different average scores between the studied taxa (Fig. 4). The NP-MANOVA confirmed significant differences between the taxa for both levels of groupings. Higher *F*-values were obtained for the analysis where *D. r. obscura* was included as a separate taxon ($F_{3,48}$ = 12 vs. $F_{2,48}$ = 8.8; $P < 0.05$ in both cases), which means higher overall differentiation among the taxa. The post-hoc tests, both uncorrected and Bonferonni-corrected Hotelling's *P*-values, confirmed significant

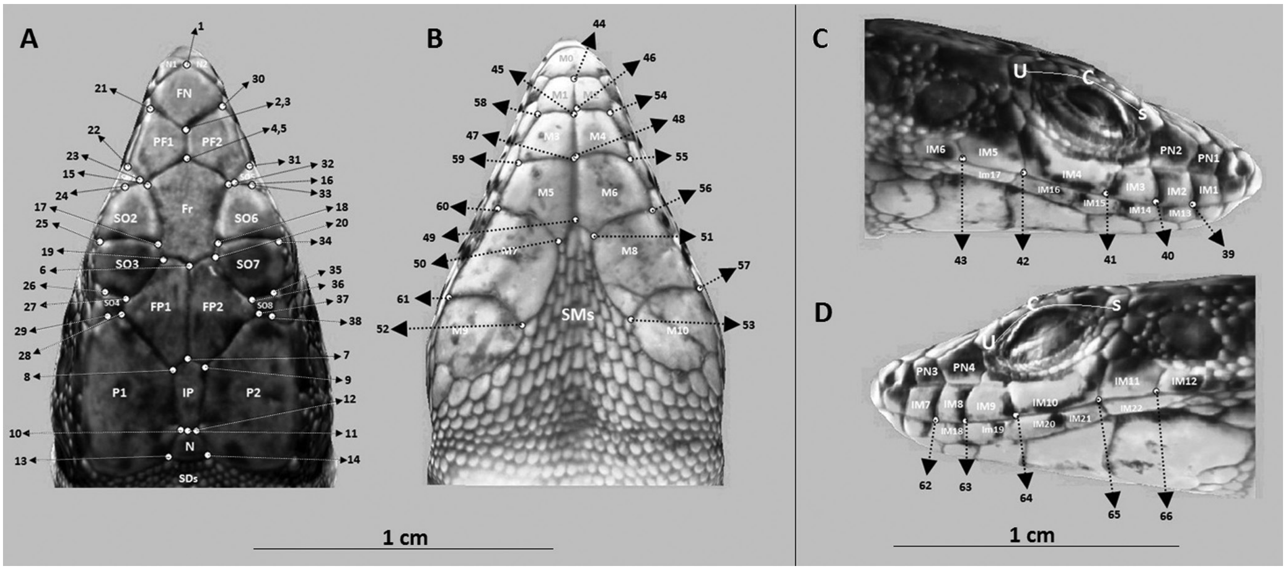

Figure 3. Digitized three-dimensional landmarks. Names of the scales are indicated with white text, and landmarks are identified with black numbers (see description of the scales and landmarks in Supporting Information, Appendix S1). A, dorsal view of the head. B, ventral view of the head. C, view of the right profile of the head. D, view of the left profile of the head. The specimen in the photograph is *Darevskia rudis rudis*.

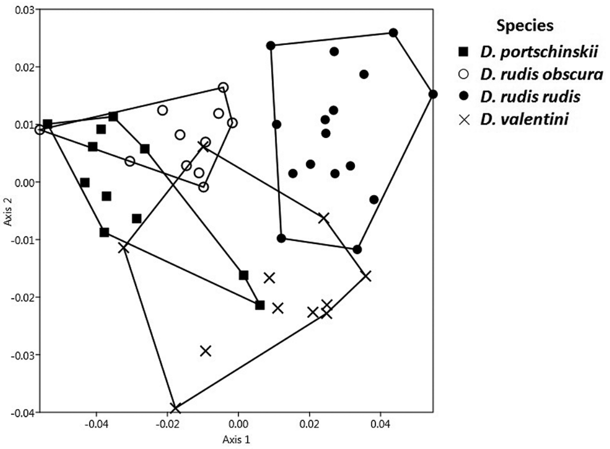

Figure 4. Plot of the first axis vs. the second axis from the principal components analysis on the three-dimensional head shape of the *Darevskia* lizards, analysing all 66 landmarks.

differences ($P < 0.05$) for each pairwise comparison across the four included taxa.

The BG-PCA axes completely separated all four studied taxa (Fig. 5A, B). The first axis (explaining 67% of the variation) fully separated *D. r. rudis* from *D. r. obscura* and from *D. portschinskii*. The second axis (19% of the variation) fully separated *D. valentini* from *D. r. rudis* and from *D. r. obscura*. The third axis (14% of the variation) fully separated *D. r. obscura* from *D. portschinskii*. Plotting the first axis vs. the third axis fully separated *D. portschinskii* from *D. valentini*.

The CVA based on seven meaningful PCs (PC_CVA) and CVA based on the Procrustes coordinates of all 66 landmarks (LM_CVA) discriminated the four taxa. The orientations of the first axis (CV1) and the third axis (CV3) from both the PC_CVA and LM_CVA coincided, whereas that of the second axis (CV2) showed opposite directions (see plots in Supporting Information, Appendix S3). For the PC_CVA, CV1 fully separated *D. r. rudis* from *D. portschinskii* and *D. valentini* (explaining 58.5% of the variation), CV2 (28.5% of the variation) separated *D. valentini* from *D. portschinskii*, and CV3 (13% of the variation) partly separated *D. valentini* from *D. r. obscura*. Combining CV1 with CV2 showed a complete separation of *D. r. obscura* and *D. portschinskii*, whereas *D. r. obscura* was separated from *D. r. rudis* when plotting CV1 vs. CV3. The confusion matrix classified 96% of the individuals correctly; after jackknifing, the preciseness of classification went down to 86% of the individuals (see Supporting Information, Appendix S4). In summary, discrimination in the PC_CVA was incomplete only for *D. r. obscura* and *D. valentini*, but completely separated all other taxa (Supporting Information, Appendix S3).

Group differences explained by CV1 included differences in snout length, head height, width at the level of the jaw joint and the size of interparietal (IP) scale (Fig. 6A). Individuals with lower CV1 scores have a taller and narrower head in the jaw joint area and a smaller IP scale (especially *D. r. rudis*), whereas individuals with high CV1 scores have a flatter and wider head in the jaw joint area and a larger IP scale (*D. portschinskii*) (Fig. 6A; Supporting Information, Appendix S3A, B). The second axis (CV2) reflects differences in the shape of the frontal (Fr) and IP scales (Fig. 6B). Individuals with lower CV2 scores (*D. valentini*) have wider and shorter Fr and narrower IP scales (here, we consider the opposite directions of the CV axes produced by PC_CVA and LM_CVA), whereas individuals with higher scores have narrower and more elongated Fr and wider IP scales (*D. portschinskii*) (Fig. 6B; Supporting Information, Appendix S3A, B). The third CV axis mainly reflects differences in the configuration of the scales on the ventral head, in which *D. r. obscura* (lower scores) was partly differentiated from the rest (Fig. 6C; Supporting Information, Appendix S3C, D).

In 2D GM outline shape analysis, the NP-MANOVA also showed significant differences when *D. r. obscura* was treated as a separate taxon ($F_{3,48} = 4.4$; $P < 0.05$). However, post-hoc tests failed to differentiate *D. portschinskii* from *D. r. rudis* and *D. valentini*, or *D. valentini* from *D. r. obscura*. After Bonferroni correction, only *D. r. rudis* and *D. r. obscura* remained significantly different from each other. The CVA based on the seven 'meaningful' PCs generated three components explaining 51.7, 34.2 and 14.1% of the total variation; however, all taxa still partly overlapped along these axes (results not shown). The confusion matrix classified only 65% of the individuals correctly; after jackknifing, the precision of classification went down to 49% of the individuals (see Supporting Information, Appendix S5).

DISCUSSION

This paper suggests that a 3D analysis of head shape might provide important information not accessible using 'traditional' morphometrics or 2D GM, enabling separation of even very closely related species of lizards that are otherwise difficult to differentiate. Our study also suggests that the taxon *D. r. obscura* is morphometrically distinct from *D. r. rudis*, and there are sufficient reasons to qualify it as a separate species, *Darevskia obscura*.

The example considered in this paper contributes both to the methodology of morphometric comparisons of existing species and to the general understanding of species boundaries. De Queiroz (2007) defined a species

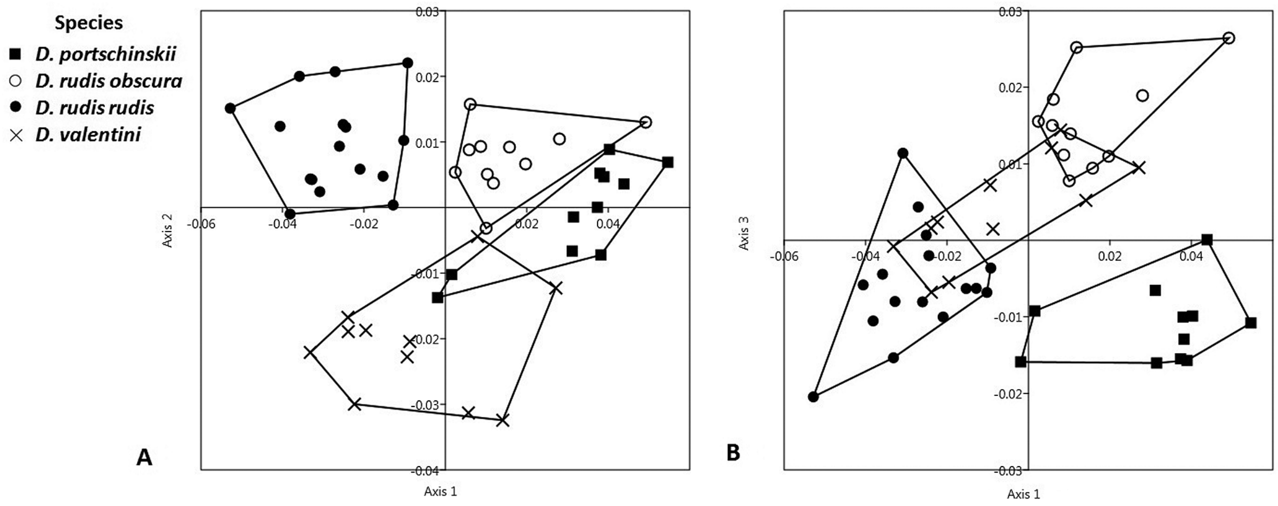

Figure 5. Plots of the between-group principal components analysis (BG-PCA) on the three-dimensional head shape of the *Darevskia* lizards, analysing all 66 landmarks. A, first vs. second axis. B, first vs. third axis.

as an evolutionary lineage with 'its own evolutionary pathway'. This definition acknowledges the fact that incipient species may hybridize and exchange alleles for a long period before achieving full reproductive isolation, which does not always prevent their divergence (Mallet, 2005). The studied nominal species of rock lizards did not achieve the stage of complete lineage sorting ('genealogical concordance' in terms of Avise & Ball, 1990) and, most probably, they continue to hybridize and show gene introgression patterns (Tarkhnishvili *et al.*, 2013). In the border areas, there are multiple individuals that cannot be allocated easily to either of the taxa, based solely on superficial examination (D. Tarkhnishvili, unpublished observations) or even study of the scalation pattern (Darevsky, 1967; Tarkhnishvili *et al.*, 2013).

Additionally, the nominal species have some characteristic features that apply to most of the populations or individuals. Adult specimens of *D. portschinskii* are almost always smaller than adult *D. r. rudis* or *D. r. obscura* from neighbouring locations. *Darevskia rudis obscura* adults are usually smaller than those of *D. r. rudis* from habitats with similar environmental conditions and reach higher elevations. *Darevskia valentini* has brighter dorsal coloration and smoother scales than most of the *D. rudis* populations, although some populations geographically intermediate between *D. valentini* and *D. r. obscura* (which Darevsky referred to as *D. r. macromaculata*) have individuals that are difficult to attribute to either nominal species [Arribas *et al.* (2013) even synonymized *D. r. macromaculata* with *D. r. obscura*]. Some specimens of the nominal species of the '*rudis*' clade show individual traits that are more similar to those of other species of the clade than to those of the species to which they belong. In this case, the geographical context should be considered before attributing these specimens to one or another taxon.

Our morphometric study showed that the vast majority of conspecific individuals are identifiable if the entire head shape is taken into account. Head shape helps to distinguish not only between nominal species (*D. rudis*, *D. portschinskii* and *D. valentini*) but also between them and a taxon previously considered to be a subspecies, *D. r. obscura*. Remarkably, the 2D GM outline analysis performed in the present study was unable to discriminate closely related species of the '*rudis*' clade, whereas 3D GM analysis could do so.

Several studies have shown 3D GM to be a powerful tool for differentiating reptilian taxa based on their head shape or head elements. Andjelković *et al.* (2016) differentiated closely related ring and dice snakes (*Natrix natrix* Linnaeus, 1758 and *Natrix tessellata* Laurenti, 1768) using 3D GM on the cranial elements. Three-dimensional GM was also able to differentiate significantly three Montpellier snakes: *Malpolon insignitus insignitus*, *Malpolon insignitus fuscus* (subspecies of *M. insignitus* Geoffroy Saint-Hilaire, 1827) and *Malpolon monspessulanus monspessulanus* (subspecies of *M. monspessulanus* Hermann, 1804) based on their head shape (Mangiacotti *et al.*, 2014). Three-dimensional GM has also been used for identifying fossil lizards (Gray *et al.*, 2017).

The head is a solid structure covered with easily recognizable large scales in Lacertids and some other lizards, making placement of homologous landmarks convenient. Consequently, 3D analysis of head shape is a good tool for taxonomic analysis based on morphology, much more reliable than 'traditional' analyses including a qualitative comparison of scalation, traditional morphometrics or GM based on the 2D images. Most

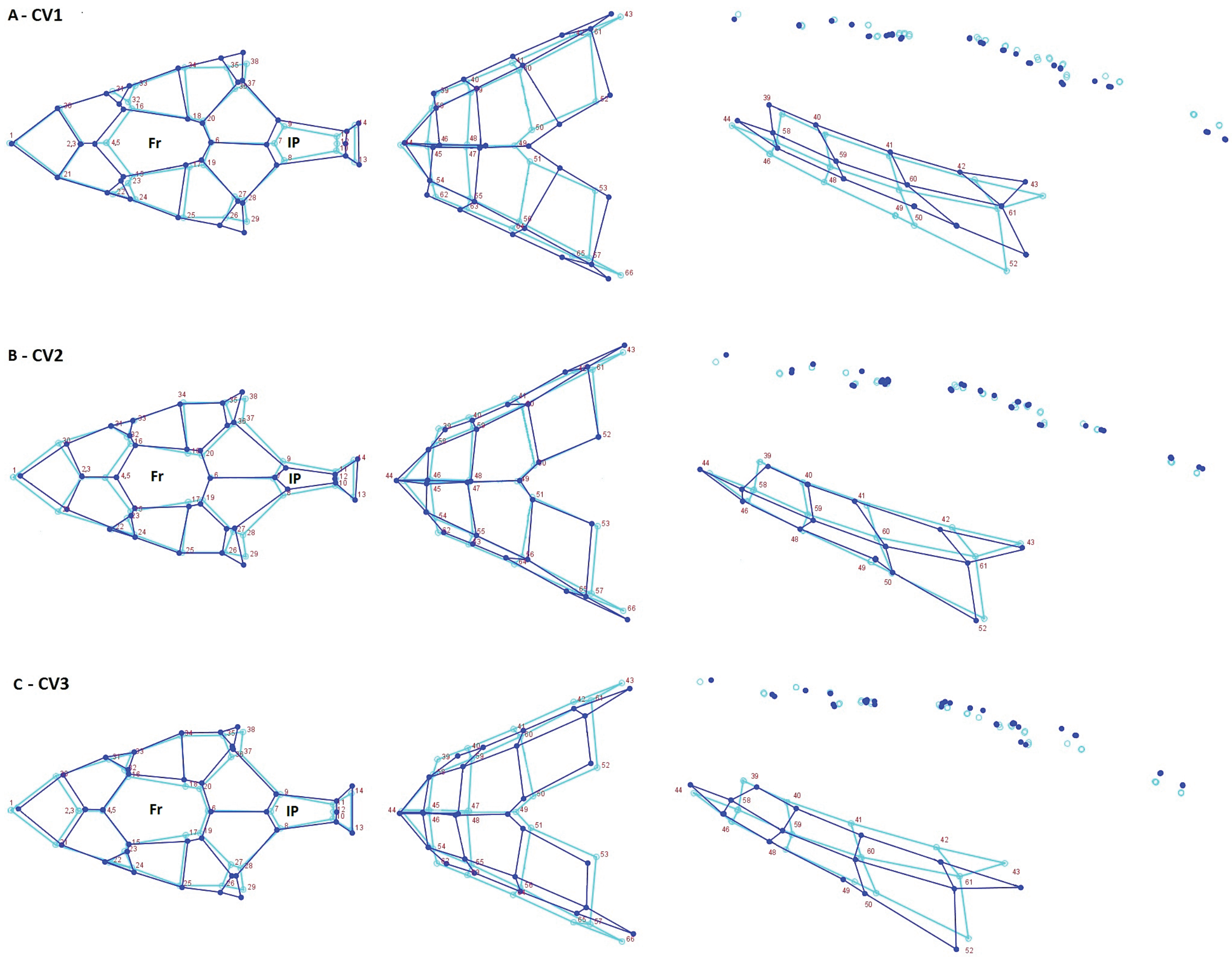

Figure 6. Wireframes for three canonical variate (CV) axes for visualizing shape changes. Dorsal, ventral and jaw wireframes (dots above the jaw wireframe are dorsal landmarks) from left to right. A–C, shape changes along the first (A), second (B) and third (C) CV axis. Light blue dots and lines represent the shape corresponding to the mean values along the respective CV axis. Dark blue dots and lines represent the shape corresponding to the maximal values along a respective CV axis, extrapolated up to 10.0 units to emphasize the subtle changes in shape.

importantly, it helps to discriminate even between species that did not achieve the stage of complete lineage sorting and individuals collected from the populations where molecular genetic analysis suggests the presence of introgressive gene flow (Tarkhnishvili *et al.*, 2013). Such individuals are commonly impossible to attribute to one or another species based on a limited number of genetic characters. However, 3D GM is able to cluster even these individuals with the other members of the same population.

TAXONOMIC INFERENCE

This morphometric study suggests that the populations of *D. rudis* from the upper part of the river Kura Valley, from the Borjomi Gorge southwards and west to the Goderdzi Pass, which Darevsky (1967) described as subspecies *D. r. obscura* and *D. r. macromaculata*, are morphologically not less distinct from *D. r. rudis* than the nominal species *D. valentini* and *D. portschinskii*. Different from *D. r. rudis*, the head of *D. r. obscura* is flatter but broader in the jaw joint area. It also has a relatively larger intraparietal scale than *D. r. rudis*. The earlier study of Tarkhnishvili *et al.* (2013) suggests that this form has a monophyletic matrilineal origin independent from the other *D. rudis* populations, and it is intermediate between *D. r. rudis*, *D. valentini* and *D. portschinskii* with respect to the distribution of microsatellite genotypes. This, however, does not apply to other populations of *D. rudis* (Tarkhnishvili *et al.*, 2013). Consequently, and following the original suggestion of Lantz & Cyrén (1936) who described this lizard as a subspecies of *D.* '*saxicola*' Eversmann, 1834 (that comprised most of the currently described species of *Darevskia*) and not of *D. rudis*, we suggest reinstating the status of this form to a species, *Darevskia obscura* Lantz & Cyrén, 1936.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Description of landmarks.
Appendix S2. Eigenvalues and character loadings for general principal components analysis (PCA) on three-dimensional shape data.
Appendix S3. Comparison of axes from canonical variate analysis based on seven meaningful principal components (PC_CVA) and canonical variate analysis based on the Procrustes coordinates of all 66 landmarks (LM_CVA). A, PC_CVA axis 1 vs. axis 2; B, LM_CVA axis 1 vs. axis 2; C, PC_CVA axis 1 vs. axis 3; D, LM_CVA axis 1 vs. axis 3.
Appendix S4. Upper panel, confusion matrix inferred from canonical variate analysis (CVA) assigning individuals to one of the four taxa based on the three-dimensional (3D) head shape. Lower panel, the jackknifed confusion matrix inferred from CVA assigning individuals to one of the four taxa based on the 3D head shape.
Appendix S5. Upper panel, confusion matrix inferred from canonical variate analysis (CVA) assigning individuals to one of the four taxa based on two-dimensional (2D) outline of anal scale. Lower panel, the jackknifed confusion matrix inferred from CVA assigning individuals to one of the four taxa based on 2D outline of the anal scale.

Appendices

Chapter 2

Appendix 1. Genbank accession numbers.

AF206172, AF164073, EF422420, U88608, AF147796, AF147798, AF147797, U88609, U88614, JN546167, JN546166, JN546165, JN546164, JN546163, JN546162, JN546161, JN546160, JN546159, JN546158, JN546157, JN546156, JN546155, JN546154, JN546153, JN546152, JN546151, JN546150, JN546149, JN546148 , JN546147 , GQ142123, U088611, JN546194, JN546193, JN546192, JN546191, JN546190, JN546189, JN546188, JN546187, JN546186, JN546185, JN546184, U88615, GU216649, GU216648, GU216647, GU216646, GU216645, GU216644, GU216643, GU216642, GU216641, GU216640, GU216633, GU216632, JN546183, JN546182, JN546181, JN546180, JN546179, JN546178, JN546177, JN546176, JN546175, JN546174, JN546173, JN546172, JN546171, JN546170, JN546169, JN546168, JN546146.

Chapter 3

Appendix S1. Description of landmarks.

Fr - frontal; P - parietal; IP - interparietal; N - nuchal; FP - frontoparietal; N - nasal; FN - frontal-nasal; PF - prefrontal; SO - supraorbital; M - mandibular; IM - intermaxillary, SMs - small mandibular scales; SDs - small dorsal scales; PN - post-nasal; UCs - upper ciliated scales.

Landmark ‘n’ - touching point of the scales of ‘N’:

1 - N1, N2, FN

2, 3 - FN, PF1, PF2

4, 5 - Fr, PF1, PF2

6 - Fr, FP1, FP2

7 - FP1, FP2, IP

8 - P1, FP1, IP

9 - P2, FP2, IP

10 - P1, IP, N

11 - P2, IP, N

12 - center between landmarks 10 and 11

13 - P1, N, SDs

14 - P2, N, SDs

15 - Fr, PF1, SO2

16 - Fr, PF2, SO5

17 - Fr, SO2, SO3

18 - FR, SO6, SO7

19 - Fr, SO3, FP1

20 - Fr, SO7, FP2

21 - PN3, FN, PF1

22 - PF1, SO1, PN4

23 - PF1, SO2, SO1

24 - SO1, SO2, UCs

25 - SO2, SO3, UCs

26 - SO3, FP1, SO4

27 - FP1, P1, SO4

28 - PN1, FN, PF2

29 - PF2, SO5, PN2

30 - PF2, SO5, SO6

31 - SO5, SO6, UCs

32 - SO6, SO7, UCs

33 - SO7, FP2, SO8

34 - FP2, P2, SO8

35 - IM1, IM2, IM13

36 - IM2, IM3, IM14

37 - Im3, IM4, IM15

38 - IM4, IM5, IM16

39 - IM5, IM6, IM17

40 - M0, M1, M2

41 - M5, M6, SMs

42 - M5, M7, SMs

43 - M6, M8, SMs

44 - M7, M9, SMs

45 - M8, M10, SMs

46 - M6, M8, M20

47 - M8, M10, M21

48 - M5, M7, IM15

49 - M7, M9, IM16

50 - IM7, IM8, IM18

51 - IM8, IM9, IM19

52 – IM9, IM10, IM20

53 - IM10, IM11, IM21

54 - IM11, IM12, IM22

Chapter 6

Appendix S1: Description of landmarks.

Fr - frontal; P - parietal; IP - interparietal; N1/2 - nuchal; FP - frontoparietal; N - nasal; FN - frontal-nasal; PF - prefrontal; SO - supraorbital; M - mandibular; IM - intermaxillary, SMs - small mandibular scales; SDs - small dorsal scales; PN - post-nasal; UCs - upper ciliated scales.

Landmark 'n' - touching point of the scales of 'N':

1 - N1, N2, FN

2,3 - FN, PF1, PF2

4,5 - Fr, PF1, PF2

6 - Fr, FP1, FP2

7 - FP1, FP2, IP

8 - P1, FP1, IP

9 - P2, FP2, IP

10 - P1, IP, N

11 - P2, IP, N

12 - center between landmarks 10 and 11

13 - P1, N, SDs

14 - P2, N, SDs

15 - Fr, PF1, SO2

16 - Fr, PF2, SO5

17 - Fr, SO2, SO3

18 - FR, SO6, SO7

19 - Fr, SO3, FP1

20 - Fr, SO7, FP2

21 - PN3, FN, PF1

22 - PF1, SO1, PN4

23 - PF1, SO2, SO1

24 - SO1, SO2, UCs

25 - SO2, SO3, UCs

26 - SO3, SO4, UCs

27 - SO3, FP1, SO4

28 - FP1, P1, SO4

29 - SO4, P1, UCs

30 - PN1, FN, PF2

31 - PF2, SO5, PN2

32 - PF2, SO5, SO6

33 - SO5, SO6, UCs

34 - SO6, SO7, UCs

35 - SO7, SO8, UCs

36 - SO7, FP2, SO8

37 - FP2, P2, SO8

38 - SO8, P2, UCs

39 - IM1, IM2, IM13

40 - IM2, IM3, IM14

41 - Im3, IM4, IM15

42 - IM4, IM5, IM16

43 - IM5, IM6, IM17

44 - M0, M1, M2

45 - M1, M3, M4

46 - M1, M2, M4

47 - M3, M5, M6

48 - M3, M4, M6

49 - M5, M6, SMs

50 - M5, M7, SMs

51 - M6, M8, SMs

52 - M7, M9, SMs

53 - M8, M10, SMs

54 - M2, M4,IM18

55 - M4, M6, M19

56 - M6, M8, M20

57 - M8, M10, M21

58 - M1, M3, IM13

59 - M3, M5, IM14

60 - M5, M7, IM15

61 - M7, M9, IM16

62 - IM7, IM8, IM18

63 - IM8, IM9, IM19

64 - IM9, IM10, IM20

65 - IM10, IM11, IM21

66 - IM11, IM12, IM22

Appendix S2. Eigenvalues and character loadings for general principal components analysis (PCA) on three-dimensional shape data.

PC	Eigenvalue	% variance
1	0.000798	29.006
2	0.000214	7.7874
3	0.000178	6.4686
4	0.00015	5.4635
5	0.000135	4.9083
6	0.000125	4.5473
7	0.000115	4.1725

Axis	Landmark	PC 1	PC 2	PC 3	PC 4	PC 5	PC 6	PC 7
x	1	0.050661	-0.10802	-0.06092	-0.06267	0.012879	-0.1061	0.030331
y	1	0.0204	-0.01873	0.00447	-0.04006	0.035199	0.009293	-0.02539
z	1	-0.02855	0.047198	0.029133	0.11173	-0.04877	-0.01641	-0.02108
x	2	0.045685	-0.06421	0.016917	0.027285	-0.04953	-0.07568	-0.00997
y	2	0.01556	-0.02853	-0.00452	-0.01402	0.038619	0.028731	-0.0007
z	2	0.002915	0.018893	0.027529	0.074966	-0.07752	-0.0741	0.002225
x	3	0.046196	-0.06169	0.014231	0.027925	-0.04604	-0.07384	-0.00761
y	3	0.015006	-0.02898	-0.00534	-0.01408	0.038629	0.028129	-0.00087
z	3	0.002806	0.019098	0.026661	0.075044	-0.07691	-0.074	0.002638
x	4	0.11767	0.05538	-0.01466	-0.12086	0.075779	-0.1524	0.075994
y	4	0.011932	-0.02117	-0.01275	-0.01476	0.013679	0.02422	0.004801
z	4	0.001429	0.029434	0.014996	0.058807	-0.04954	-0.08709	0.022549
x	5	0.11828	0.057673	-0.02134	-0.11963	0.074128	-0.1495	0.07967
y	5	0.011668	-0.02114	-0.01265	-0.01483	0.013852	0.022094	0.00259
z	5	0.001386	0.029444	0.014165	0.059034	-0.04957	-0.08714	0.022634
x	6	0.00987	-0.06241	-0.09716	0.047905	0.040881	-0.03479	0.0871

y	6	0.005538	0.018045	-0.0012	0.009145	0.001644	-0.00246	-0.03025
z	6	-0.03319	0.049112	0.022692	0.020167	-0.02537	-0.0116	0.076535
x	7	0.070776	0.14773	0.03344	-0.04199	0.061044	0.017569	-0.02585
y	7	0.015573	-0.02421	-0.0266	-0.01271	0.013159	0.004512	0.003057
z	7	-0.05109	0.086787	0.032184	-0.05162	0.057819	0.022989	0.024745
x	8	0.07244	0.11586	0.005865	-0.06079	0.11009	-0.0394	-0.06285
y	8	0.058769	0.021049	0.032298	-0.07864	0.041126	0.011389	0.053407
z	8	-0.04464	0.07686	0.012915	-0.05735	0.055088	0.020481	0.010904
x	9	0.055573	0.12032	0.007144	-0.02927	0.078736	-0.01711	-0.04223
y	9	-0.06449	-0.04505	-0.05553	0.092635	-0.05728	0.004246	-0.06021
z	9	-0.06732	0.068735	-0.00171	-0.01708	0.015734	0.022629	0.010494
x	10	-0.06667	-0.03633	0.13498	0.10899	0.001971	-0.18599	-0.05809
y	10	0.078505	0.054506	0.05711	-0.08413	-0.00691	0.025992	0.11371
z	10	-0.13072	0.017453	-0.01143	-0.00486	-0.06794	0.003662	-0.01787
x	11	-0.07412	-0.03247	0.13405	0.13096	-0.00282	-0.19119	-0.07795
y	11	-0.01928	-0.05603	-0.03091	0.090022	0.031633	-0.03711	-0.05038
z	11	-0.14894	0.00695	-0.02968	0.042106	-0.06241	-0.00618	-0.05924
x	12	-0.06699	-0.0335	0.12625	0.098523	-0.0273	-0.1486	-0.02652
y	12	0.029622	0.002993	0.0124	0.005052	0.011167	-0.0098	0.043342
z	12	-0.13945	0.013571	-0.02291	0.010105	-0.07761	0.008864	-0.01776
x	13	-0.02873	-0.00369	0.1376	0.059416	0.031173	-0.13842	-0.06744
y	13	0.046236	0.039791	0.006934	0.037137	-0.05212	3.71E-05	0.13857
z	13	-0.12172	0.019276	-0.04212	0.020536	-0.11244	0.012033	-0.04807
x	14	-0.02642	0.002686	0.11252	0.013133	0.043195	-0.17744	-0.072
y	14	0.011834	-0.092	0.037378	-0.01984	0.056547	0.042881	-0.04997
z	14	-0.12198	0.008855	-0.04587	-0.00747	-0.09187	0.016004	-0.07352
x	15	0.085794	0.044613	0.010839	-0.06045	0.056363	-0.09011	0.05531
y	15	-0.00017	-0.00979	-0.01561	-0.04207	-0.00492	-0.03466	0.040993
z	15	-0.00977	0.033328	0.019273	0.044161	-0.01871	-0.08288	-0.00935
x	16	0.089474	0.049653	-0.00785	-0.02434	0.072302	-0.1241	0.025415
y	16	0.015933	-0.0447	-0.03451	0.003192	0.001924	0.074074	-0.05195
z	16	-0.00349	0.023549	0.013344	0.055499	-0.02117	-0.05336	-0.01507

x	17	0.040064	-0.00725	0.013263	2.11E-05	0.008208	0.024847	0.031472
y	17	-0.03057	0.060007	-0.00026	0.031969	-0.05547	0.014454	0.026091
z	17	-0.02982	0.054382	0.045514	0.028637	0.002211	0.02082	0.011974
x	18	0.02196	0.021711	0.018321	0.027336	0.000405	0.013995	0.021785
y	18	0.02954	-0.08652	0.006292	-0.03812	0.029268	-0.03245	-0.02654
z	18	-0.01889	0.037542	0.032911	0.044871	0.026616	-0.00187	-0.00191
x	19	0.01547	-0.01392	-0.09088	0.034064	-0.03783	-0.04537	0.086201
y	19	-0.04019	0.056642	0.058666	0.048003	-0.03567	0.01676	0.002202
z	19	-0.0444	0.048556	0.036858	0.031211	-0.01798	-0.01169	0.045098
x	20	0.014084	-0.03204	-0.0364	0.041096	-0.03477	0.009312	0.082742
y	20	0.034948	-0.07465	-0.03725	-0.05723	0.011845	-0.03371	-0.05989
z	20	-0.02529	0.029299	0.018966	0.020419	-0.01041	-0.00908	0.03846
x	21	0.041359	-0.04903	0.066386	-0.08234	-0.00382	-0.11678	0.014752
y	21	-0.0004	0.007577	0.027143	-0.0559	0.008879	-0.02048	-0.01928
z	21	0.008896	0.034762	-0.00798	0.06668	-0.06583	-0.05061	0.005485
x	22	0.05169	0.10423	-0.04489	-0.00292	-0.02709	-0.0499	0.097941
y	22	-0.0188	0.014334	0.058423	-0.03543	-0.08205	0.007277	0.011219
z	22	0.002294	0.013201	0.023252	0.030843	0.000619	-0.07832	-0.03092
x	23	0.081263	0.028185	-0.00495	-0.03606	0.072346	-0.1011	0.008437
y	23	-0.02021	-0.00324	-0.00053	-0.03057	-0.04552	-0.06368	-0.01384
z	23	-0.01473	0.027127	0.02797	0.046477	-0.01423	-0.07873	-0.03213
x	24	0.061935	0.027996	0.039335	0.002059	0.041174	-0.08266	0.033547
y	24	-0.01822	0.086105	0.001134	-0.01018	-0.11375	0.027622	-0.02943
z	24	-0.02513	0.033065	0.057928	0.04045	0.050517	0.015786	-0.08747
x	25	0.057504	0.009818	-0.01516	0.024583	-0.10579	0.008837	0.06746
y	25	-0.01394	0.058148	0.047749	-0.03709	-0.13974	-0.04804	0.067099
z	25	-0.01104	0.008667	0.097426	-0.00557	0.24339	0.10565	-0.19294
x	26	0.04172	0.069797	-0.04805	0.026592	-0.08559	0.066439	0.046175
y	26	0.046807	0.17624	0.00723	-0.07337	-0.01859	0.029421	-0.05871
z	26	-0.02952	-0.00494	0.047431	0.006671	0.093217	0.074023	-0.07473
x	27	-0.03024	-0.00105	0.009054	0.11718	-0.10447	0.032506	0.087269
y	27	0.027905	0.18319	0.048372	-0.09685	-0.01718	0.019832	-0.03002

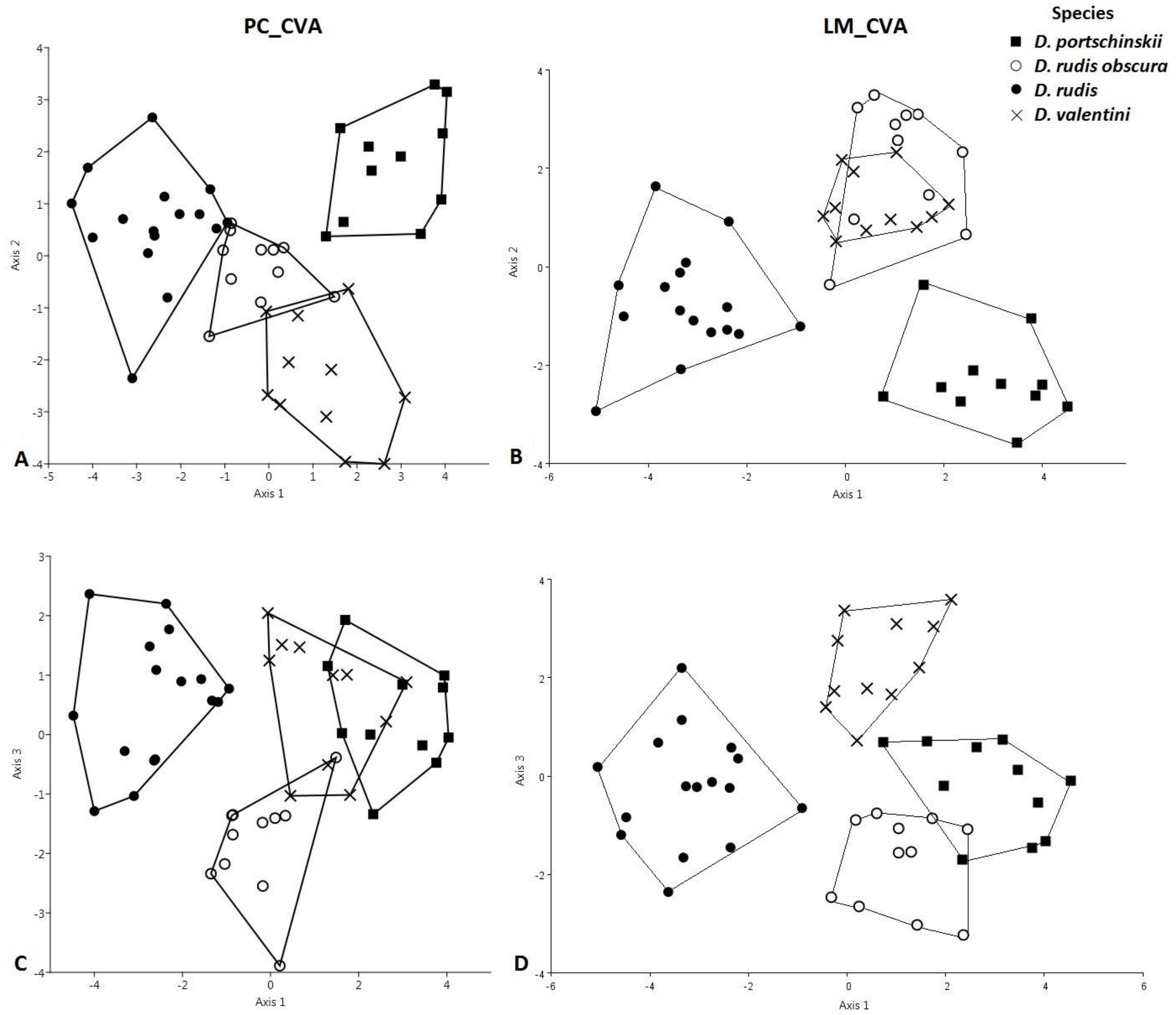
z	27	-0.04591	0.021954	0.055183	0.029735	-0.01058	0.032479	0.014315
x	28	-0.00687	0.084161	-0.02923	0.1043	-0.06581	0.10233	0.0332
y	28	0.02884	0.13908	0.10866	-0.12896	-0.03437	-0.055	-0.02279
z	28	-0.03776	0.034792	0.035096	0.037189	-0.0189	0.060029	0.016124
x	29	0.012375	0.038172	-0.00201	0.030063	-0.0413	0.049324	0.086759
y	29	0.090401	0.23215	-0.01608	0.026862	-0.04296	0.067	-0.05371
z	29	-0.04797	-0.05019	0.039441	0.005001	0.000266	0.067178	0.048855
x	30	0.045573	-0.06567	0.045099	-0.10061	-0.03335	-0.09649	-0.00236
y	30	-0.00167	-0.04008	-0.01906	-0.00024	0.037552	0.059901	0.021261
z	30	0.011075	0.017296	0.034052	0.060715	-0.0515	-0.0493	0.039223
x	31	0.063234	0.079113	-0.03107	0.034227	0.078252	-0.03307	0.042783
y	31	0.027744	-0.04236	-0.06449	-0.00554	0.049256	0.033384	0.013201
z	31	0.017041	-0.00086	0.017527	0.022044	0.003496	-0.06376	-0.00637
x	32	0.10076	0.027668	-0.02493	-0.00152	0.072577	-0.0923	-0.01531
y	32	0.024723	-0.03693	-0.03257	-0.02056	0.06482	0.076743	0.027104
z	32	-0.00264	0.018016	0.023247	0.04653	0.004091	-0.04531	-0.0038
x	33	0.070289	0.032733	0.018144	0.02954	0.049091	-0.01354	-0.01194
y	33	0.024166	-0.05882	-0.03918	0.021961	0.074669	0.055398	-0.01337
z	33	-0.00913	-0.00591	0.066595	0.044165	0.065055	0.008445	-0.07167
x	34	0.072305	0.009145	-0.00954	0.039185	-0.04674	0.060335	0.095778
y	34	0.008994	-0.05354	-0.0939	0.042648	0.004005	0.023215	0.002676
z	34	0.010336	-0.00567	0.095765	0.009524	0.26709	0.1321	-0.21418
x	35	0.042582	0.049541	-0.02544	0.03258	-0.0635	0.072804	0.050903
y	35	-0.06022	-0.12654	-0.04666	0.094727	-0.00956	-0.08108	0.071482
z	35	-0.03453	-0.03815	0.05048	0.046513	0.11584	0.067334	-0.04851
x	36	-0.01976	-0.03536	0.018313	0.12916	-0.08953	0.032445	0.10337
y	36	-0.02644	-0.19777	-0.07014	0.086358	0.045193	-0.03565	-0.08141
z	36	-0.04965	-0.03424	0.045775	0.06812	0.035022	0.040454	-0.00702
x	37	-0.01133	0.09783	-0.04998	0.11725	-0.07853	0.11274	0.037034
y	37	-0.03365	-0.12756	-0.11377	0.076642	0.044805	-0.06136	-0.05689
z	37	-0.04473	0.028084	-0.02008	0.069083	0.012364	0.054738	0.000777
x	38	0.014494	0.062515	-0.02	0.053276	-0.00657	0.095181	0.028728

y	38	-0.07429	-0.18339	-0.06571	0.043887	0.13099	-0.10334	-0.04461
z	38	-0.05068	-0.08312	-0.02096	0.062483	0.12076	0.059578	-0.00473
x	39	0.077669	-0.24332	0.008165	-0.10745	-0.11481	-0.02506	-0.08355
y	39	-0.01001	-0.02925	-0.03687	-0.04431	-0.05118	0.065648	-0.01621
z	39	0.034762	-0.13019	0.022961	-0.05561	-0.14302	-0.00094	-0.04917
x	40	0.05844	-0.16371	0.033394	-0.10233	-0.17059	0.037006	-0.08804
y	40	-0.04531	-0.01226	-0.03033	-0.03163	-0.07396	0.067192	-0.03351
z	40	0.032896	-0.09878	0.001341	-0.04404	-0.10914	0.015434	-0.04752
x	41	0.024318	-0.06195	0.05808	0.13673	-0.10228	-0.05027	-0.13847
y	41	-0.08435	-0.00404	-0.04665	0.023464	-0.05745	0.037988	-0.07079
z	41	0.02633	-0.06605	0.016237	0.045965	-0.02107	-0.00554	-0.0093
x	42	0.086	0.087408	-0.04946	0.09461	-0.0543	0.13541	-0.05398
y	42	-0.06652	0.06757	-0.10507	-0.0509	0.06052	0.013266	-0.06804
z	42	0.056048	-0.05341	0.004609	-0.01081	0.021539	0.016641	0.020263
x	43	0.12155	-0.04172	0.056047	0.000903	0.053903	0.17921	-0.11861
y	43	-0.08042	0.048203	-0.03535	-0.10362	0.12481	-0.12258	-0.09218
z	43	0.10084	-0.10373	0.002814	-0.0726	0.076411	0.082792	-0.00868
x	44	-0.04191	-0.02868	-0.18572	0.11669	0.041811	-0.01737	-0.00091
y	44	-0.02734	0.004958	0.037225	0.004643	0.011549	-0.01798	-0.03054
z	44	0.030962	0.078864	-0.06698	0.0841	0.013461	-0.06882	0.065808
x	45	-0.16307	-0.02209	-0.19284	-0.00597	0.082604	-0.01419	-0.04093
y	45	0.008023	0.027972	-0.00828	0.017436	-0.00317	0.01211	-0.03047
z	45	-0.00348	0.072189	-0.06668	-0.00579	0.015965	-0.0792	0.05341
x	46	-0.07698	-0.0371	-0.30353	0.093512	0.089341	0.013035	-0.05807
y	46	0.001246	-0.00428	0.028178	-0.00759	-0.03378	0.020752	0.000392
z	46	0.041736	0.057346	-0.1276	0.047987	0.016753	-0.04893	0.034966
x	47	-0.20519	0.066461	-0.08749	-0.07429	0.069706	-0.08842	-0.03983
y	47	0.00037	0.022003	0.008537	0.012561	0.000845	0.00913	-0.01579
z	47	0.021809	0.087441	-0.06021	-0.0251	-0.00298	-0.08344	0.071008
x	48	-0.18982	0.063659	0.006509	-0.06384	-0.00239	0.000813	-0.10851
y	48	0.010001	0.001981	0.007507	-0.01117	0.012987	0.035992	0.030366
z	48	0.026758	0.084876	-0.01612	-0.02517	-0.02686	-0.03873	0.049643

x	49	-0.14959	-0.12154	0.19987	-0.11354	0.21344	0.19385	0.3154
y	49	-0.00649	0.019931	0.005951	-0.0098	0.013564	-0.01274	0.006756
z	49	0.06145	-0.02625	-0.03123	-0.05696	-0.01206	0.11436	0.22504
x	50	-0.29703	-0.11654	0.056094	-0.13221	0.036804	-0.04023	0.10208
y	50	-0.06529	-0.00818	-0.03868	-0.07164	-0.05544	-0.01678	-0.03066
z	50	0.00743	-0.05874	-0.12116	-0.13367	-0.10795	0.037927	0.11648
x	51	-0.33919	-0.13672	0.13939	-0.14437	-0.07797	0.04074	0.078864
y	51	0.08507	0.045594	0.053297	0.093799	0.14786	0.043831	0.087222
z	51	0.013871	-0.04111	-0.04509	-0.0914	-0.125	0.060649	0.083635
x	52	-0.11823	-0.024	0.16952	0.021625	0.13127	0.13081	0.10055
y	52	-0.06575	-0.07007	-0.03094	-0.03761	-0.1508	0.00184	0.004679
z	52	0.12675	-0.06408	-0.08369	-0.17918	-0.12835	0.042048	0.14545
x	53	-0.13431	-0.02308	0.095574	0.054029	0.097786	0.16428	0.18186
y	53	0.035941	0.10339	0.084494	0.034748	0.17849	0.028542	0.066496
z	53	0.13516	-0.04347	-0.09411	-0.12146	-0.06643	0.048087	0.017904
x	54	0.00515	-0.03804	-0.30393	0.13545	0.12113	0.1497	0.050123
y	54	-0.02265	-0.00076	0.1744	-0.08995	-0.03259	-0.1117	-0.02203
z	54	0.046607	0.071238	-0.10201	0.007697	0.07048	-0.01342	0.025481
x	55	-0.07853	0.036356	0.011365	-0.14409	-0.00863	0.09486	-0.06672
y	55	0.061789	-0.0762	0.059803	0.1049	0.054809	-0.0286	0.0766
z	55	0.038167	0.014888	-0.0233	-0.06313	0.043869	0.006885	-0.00667
x	56	-0.07615	-0.03186	0.036648	-0.1691	-0.03126	0.086364	0.082876
y	56	0.10713	-0.05827	0.098838	0.13696	0.040554	-0.00803	0.000144
z	56	0.054716	-0.03723	0.045466	-0.09132	0.052307	0.001326	0.029881
x	57	-0.02457	0.095864	0.14622	0.031013	-0.14205	0.18517	-0.16078
y	57	0.10824	-0.13162	0.053812	0.12765	-0.03062	0.014106	0.10382
z	57	0.05557	-0.1301	0.02869	-0.03612	0.049977	0.012461	-0.01781
x	58	-0.07462	-0.00925	-0.1976	0.081547	0.09965	0.059063	-0.04588
y	58	-0.02832	0.007452	-0.09132	0.065753	0.000221	0.065666	-0.00524
z	58	0.014103	0.079777	-0.08319	-0.00767	0.038702	-0.02964	0.022282
x	59	-0.10819	0.056951	-0.14495	-0.10996	0.022607	-0.04089	-0.05759
y	59	-0.0842	0.077052	-0.10977	-0.03445	-0.06963	-0.04307	-0.04472

z	59	-0.00085	0.051528	-0.0689	-0.11795	0.035003	-0.04247	0.043219
x	60	-0.0548	0.001261	-0.05798	-0.16142	-0.0718	-0.00829	0.018607
y	60	-0.10263	0.078714	-0.11486	-0.09259	-0.08028	-0.04035	2.91E-05
z	60	0.024041	-0.0003	-0.03047	-0.15118	0.028396	-0.03542	0.026599
x	61	0.004689	0.1562	0.17755	0.010725	-0.11195	0.22654	-0.13554
y	61	-0.10196	0.18356	-0.01543	-0.10447	0.029828	-0.0202	-0.12709
z	61	0.020688	-0.06176	0.008839	-0.11476	0.060824	-0.00123	-0.02169
x	62	0.1204	-0.08859	-0.05307	-0.12523	-0.04778	0.026267	-0.21543
y	62	-0.03523	0.019814	0.065972	0.068817	0.038588	-0.06953	0.1056
z	62	0.065224	-0.02963	-0.03165	-0.02664	-0.02193	-0.02167	-0.08184
x	63	0.080054	-0.07518	0.00467	-0.11677	-0.0644	0.11082	-0.23793
y	63	0.00566	0.017162	0.053168	0.069613	0.043353	-0.09749	0.13446
z	63	0.066274	-0.04311	-0.01777	-0.02103	-0.02896	-9.33E-05	-0.08125
x	64	0.02271	-0.02824	0.062358	0.14692	-0.08306	-0.07615	-0.07832
y	64	0.064273	0.007545	0.054886	-0.06093	0.045663	-0.0125	0.032766
z	64	0.059349	-0.06926	0.013203	0.04011	0.029554	-0.03389	-0.02155
x	65	0.091833	0.040034	-0.04374	0.078186	-0.02181	0.13972	-0.04909
y	65	0.045134	-0.05278	0.11734	0.046719	-0.0941	-0.03405	0.0352
z	65	0.077598	-0.11039	0.046229	0.00296	0.007472	0.01405	0.000532
x	66	0.15939	-0.04248	0.031558	-0.04457	0.014789	0.024424	-0.08221
y	66	0.035462	-0.01333	0.045103	0.15854	-0.15301	0.13174	0.040156
z	66	0.1297	-0.16277	0.018215	-0.02284	0.033102	0.086921	-0.04085

Appendix S3: Comparison of axes from canonical variate analysis based on seven meaningful principal components (PC_CVA) and canonical variate analysis based on the Procrustes coordinates of all 66 landmarks (LM_CVA). A, PC_CVA axis 1 vs. axis 2; B, LM_CVA axis 1 vs. axis 2; C, PC_CVA axis 1 vs. axis 3; D, LM_CVA axis 1 vs. axis 3.



Appendix S4: Upper panel, confusion matrix inferred from canonical variate analysis (CVA) assigning individuals to one of the four taxa based on the three-dimensional (3D) head shape. Lower panel, the jackknifed confusion matrix inferred from CVA assigning individuals to one of the four taxa based on the 3D head shape.

Port	obs	Rud	Val	Total	
Port	11	0	0	0	11
obs	0	10	0	1	11
Rud	0	0	16	0	16

Val	0	1	0	10	11
Total	11	11	16	11	49

	Port	obs	Rud	Val	Total
Port	9	0	0	2	11
obs	0	10	0	1	11
Rud	0	2	14	0	16
Val	0	2	0	9	11
Total	9	14	14	12	49

Appendix S5: Upper panel, confusion matrix inferred from canonical variate analysis (CVA) assigning individuals to one of the four taxa based on two-dimensional (2D) outline of anal scale. Lower panel, the jackknifed confusion matrix inferred from CVA assigning individuals to one of the four taxa based on 2D outline of the anal scale.

obs	rud	por	val	Total	
obs	9	1	0	1	11
rud	2	11	2	1	16
por	3	1	5	2	11
val	2	0	2	7	11
Total	16	13	9	11	49

	obs	rud	por	val	Total

obs	8	1	1	1	11
rud	4	7	3	2	16
por	4	1	4	2	11
val	3	1	2	5	11
Total	19	10	10	10	49