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Phenotypic divergence, convergence and evolution of Caucasian rock lizards (*Darevskia*)

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Phenotypic evolution can cause either divergent or convergent phenotypic patterns. Even adaptation to the same environment may result in divergence of some elements of phenotype, whereas for other morphological traits it could cause phenotypic convergence. We hypothesize that at least some phenotypic characters diverge monotonically, hence they evolve irreversibly even in very closely related species, and this happens in spite of multiple convergent adaptive patterns. We studied the evolution of phenotype in 13 closely related Caucasian rock lizards (*Darevskia*), whose phylogenetic relationships are well known. We used head shape and the outlines of three important scales, using geometric morphometrics. We studied the association of the overall head shape, individual principal components of head shape and scale outlines with four predictors: phylogeny, habitat, sex and size. The overall head shape was not correlated with any of these predictors, whereas some principal components were correlated with habitat or phylogeny. Habitat type explained the highest fraction of variation in head shape and anal scale area. The relatedness inferred from the components of phenotype not correlated with habitat was congruent with the phylogenetic tree inferred from molecular data. Although adaptation to local environments may obscure the phylogenetic signal present in phenotype, there are components of phenotype whose evolution is irreversible.

ADDITIONAL KEYWORDS: adaptation – *Darevskia* – Dollo's law – geometric morphometrics – lizards – phenotypic vs. genotypic evolution – phylogeny – rock-dwelling – three-dimensional head shape.

INTRODUCTION

After Mullis *et al.* (1986) invented polymerase chain reaction, it became clear that molecular genetic data perform better for reconstruction of phylogenies than traditional morphological analyses (Patterson *et al.*, 1993; Hillis & Moritz, 1996; Felsenstein, 2004; Kelly *et al.*, 2014). The reason for this is that adaptive evolution can often result in convergent phenotypes (Losos, 2011), which confounds morphologically derived phylogenetic analysis. The repeated appearance of similar traits as a result of convergent evolution has occurred across the tree of life, including examples in lizards (Vitt *et al.*, 1997; Harmon *et al.*, 2005; Barros et al., 2011; Edwards *et al.*, 2012). As a result, molecular phylogenies often contradict previous phylogenies that are based on the analysis of phenotypic characters. On the contrary, adaptation to similar environments can also result in divergent evolution, if there are species-specific evolutionary constraints (Arnold, 1992). Hence, phenotypic evolution within a group of species may manifest a complex system of both divergent and convergent evolution.

Dollo (1893; Gould, 1970) posited a principle of irreversibility in evolution. It declares that an 'organism never returns exactly to a former state, even if it finds itself placed in conditions of existence identical to those in which it has previously lived'. An important consequence of Dollo's law is that absolute phenotypic convergence of once separated species is impossible, and at least some phenotypic structures diverge over time. However, some reviews suggest that Dollo's law has limited application, because repeated evolution of similarity of phenotype is common (Pagel, 2004; Pagel *et al.*, 2004; Domes *et al.*, 2007).

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Goldberg & Igić (2008), however, concluded that most of the described instances of reversible evolution are not real, but instead attributable to methodological flaws in the analysis of phenotypes. The lack of full phenotypic convergence owing to reversible evolution is most easily seen when comparing higher taxonomic categories; professional paleontologists will not, by way of example, confuse the skeletons of a dolphin and an ichthyosaur, in spite of their very similar body shapes. Likewise, one would not confuse a true lizard from family Lacertidae, an agama and a gecko, irrespective of any ecological equivalency that might exist (Losos, 2011). This is, however, not so obvious in lower taxonomic categories; congeneric species adapted to similar environments may be difficult to distinguish (Smith *et al.*, 2011). Considering multiple examples of convergent evolution, it is apparent that some components of shape can achieve substantial similarity of structure as a consequence of convergent adaptation in similar environments (e.g. body shape in dolphins and ichthyosaurs), whereas other characters or body parts never do (e.g. skull structure in mammals and reptiles).

With respect to reptiles, Openshaw & Keogh (2014) have suggested that evolution of head shape depends primarily on body size and habitat-related factors, which could trigger convergent changes of phenotype and obscure phylogenetic relationships. We would add to this list reinforcement, understood as selection acting against the production of hybrids (Sawyer & Hartl, 1981). Reinforcement may cause divergence in mating behaviour, including mating bites (Vincent & Herrel, 2007), and enhanced sexual dimorphism (Johnson *et al.*, 2005), hence divergence in head shape. Phrased another way, some traits will track phylogeny and be phylogenetically informative, whereas others will respond to selection in a fashion that leads to spurious or false conclusions owing to convergence. Convergent patterns may draw us to a false conclusion about reversibility of phenotypic evolution, if only few selected phenotypic traits are analysed. But is it possible to separate convergent and irreversibly divergent components of phenotype effectively while studying species in the same genus? Here, we examine a variety of traits in lizards of the genus *Darevskia*, in order to determine which traits are similar owing to convergent evolution and to distinguish this similarity from that attributable to relatedness.

We used Caucasian rock lizards (*Darevskia*). Systematics and species boundaries in this group have been revised many times during the 20th century, as knowledge of the genus has expanded (Nikolski, 1913; Darevsky, 1967; Böhme, 1984; Tarkhnishvili, 2012; Ahmadzadeh *et al.*, 2013). Reconstruction of molecular phylogenies showed multiple incongruencies between phylogenies of *Darevskia* inferred from phenotypic vs. genotypic data (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). A strict consensus mitochondrial phylogeny of *Darevskia*, with the positions of the studied species indicated, is shown in Figure 1 (Murphy, 2000; Tarkhnishvili, 2012; Ahmadzadeh *et al.*, 2013; Murtskhvaladze *et al.*, 2020). Currently, this monophyletic genus is composed of 26 sexually reproducing species (Uetz & Hošek, 2020) that belong to three (Murphy *et al.*, 2000) or four (Tarkhnishvili, 2012) clades. Although phylogeny based on traditional phenotypic analysis was shown to be irrelevant, Gabelaia *et al.* (2017) demonstrated that morphometric analysis of the anal scale helped to separate *Darevskia* that belong to two different clades, the *caucasica* and *rudis* clades, irrespective of their habitat preferences. This preliminary finding indicated phylogenetic signal in shape dimensions, and furthermore, that geometric morphometrics might be more powerful for such analysis than traditional scalation analysis. However, that analysis was not able to distinguish species within the same clade. Further analysis of three-dimensional (3D) head shapes effectively discriminated among closely related species of the *rudis* clade (Gabelaia *et al.*, 2018), but that study did not identify characters that track phylogeny.

Hence, phylogenetic signal is present in the phenotypes of *Darevskia*, and there are some characters that differ more strongly in phylogenetically more distant species irrespective of their specific habitats and life mode; this might suggest irreversibility of phenotypic evolution within this group. Phylogenetic inference is, however, not simple, probably because convergent adaptations mask phylogenetic signal. In traditional taxonomy, researchers separate the characters into more and less conservative types, considering the former more informative for phylogenetic reconstructions (Farris, 1966). We assumed that, in order to detect phylogenetic signal in a phenotype, one should identify those characters that diverge monotonically, rather than relying only on the more conservative characters. We extracted and analysed the principal components (PCs) of phenotype of 13 species of *Darevskia* and estimated the phylogenetic and adaptive correlates of these components of phenotype. Our hypotheses were as follows: (1) phenotypic differences between different species of *Darevskia*, even those adapted to similar environments and with similar body size, increase with time of divergence; and (2) those components of phenotype that are less influenced by environment, body size or sexual dimorphism might better reflect phylogeny.

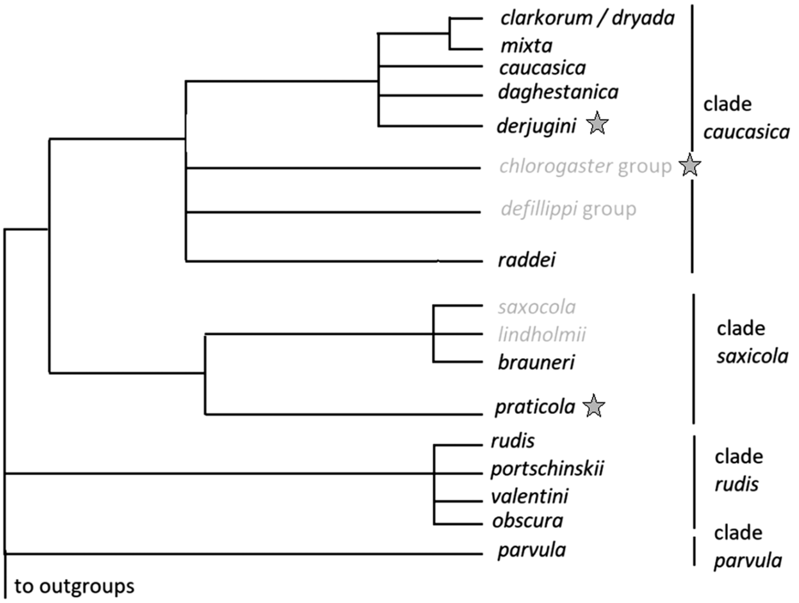

Figure 1. Strict consensus tree of *Darevskia*, based on the publications of Murphy *et al.* (2000), Tarkhnishvili (2013), Ahmadzadeh *et al.* (2013) and Murtskhvaladze *et al.* (in press). The four major clades discussed in the present study are shown. Grey letters indicate species not included in the present study. Ground-dwelling taxa are marked with a star.

MATERIAL AND METHODS

Taxa included in this analysis

We selected 13 species representing four major clades within *Darevskia*: *Darevskia parvula* (clade '*parvula*'), *Darevskia portschinskii*, *Darevskia valentini*, *Darevskia rudis*, *Darevskia obscura* (clade '*rudis*'), *Darevskia praticola*, *Darevskia brauneri* (clade '*saxicola*'), *Darevskia raddei*, *Darevskia daghestanica*, *Darevskia derjugini*, *Darevskia caucasica*, *Darevskia mixta* and *Darevskia dryada* (= *Darevskia clarkorum*; see Schmidtler *et al.*, 2002) (clade '*caucasica*'). These species differ by geographical range (e.g. *D. brauneri* and *D. caucasica* are found only in the Greater Caucasus mountains, whereas *D. parvula* and *D. portschinskii* are found 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 maximal body length is 55–56 mm, to *D. rudis*, whose body length in some populations reaches 85 mm; Tarkhnishvili, 2012). The molecular phylogeny shown in Figure 1 was used as the standard to assess the phylogenetic signal in morphometric analyses. Some nominal species are not completely separated genetically (Murphy *et al.*, 2000); for instance, broad genetic introgression is evident for the species of the clade '*rudis*' (Tarkhnishvili *et al.*, 2013).

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 are not diagnostic. Ground-dwelling species (*D. praticola* and *D. derjugini*) have taller heads and differently positioned preanal scales compared with the rock dwellers (Bannikov *et al.*, 1977; Tarkhnishvili, 2012). It is not clear how these characters differ among the rock-dwelling species (Fig. 2).

Sampling

Seventy-eight individuals of the 13 listed species were collected by hand from 12 locations within Georgia (Fig. 3) and used for the morphometric analysis: three males and three females of each species. The number of males and females from each location is presented in the Supporting Information (Table S1). The Ethical Commission for Research Projects of Ilia State University reviewed the methodology and study protocols and approved this research (permit #1018).

Data used for geometric morphometrics

Head shape is an informative character sometimes used for phylogenetic inference (Gentilli *et al.*, 2009; Ivanović *et al.*, 2013), in spite of its association with habitat type (Kaliontzopoulou *et al.*, 2008; Openshaw & Keogh, 2014). Our previous study showed that head shape is species specific in *Darevskia* (Gabelaia *et al.*, 2018). In the present study, we used 3D head models constructed using a photogrammetry approach from multiple two-dimensional (2D) photographs, following

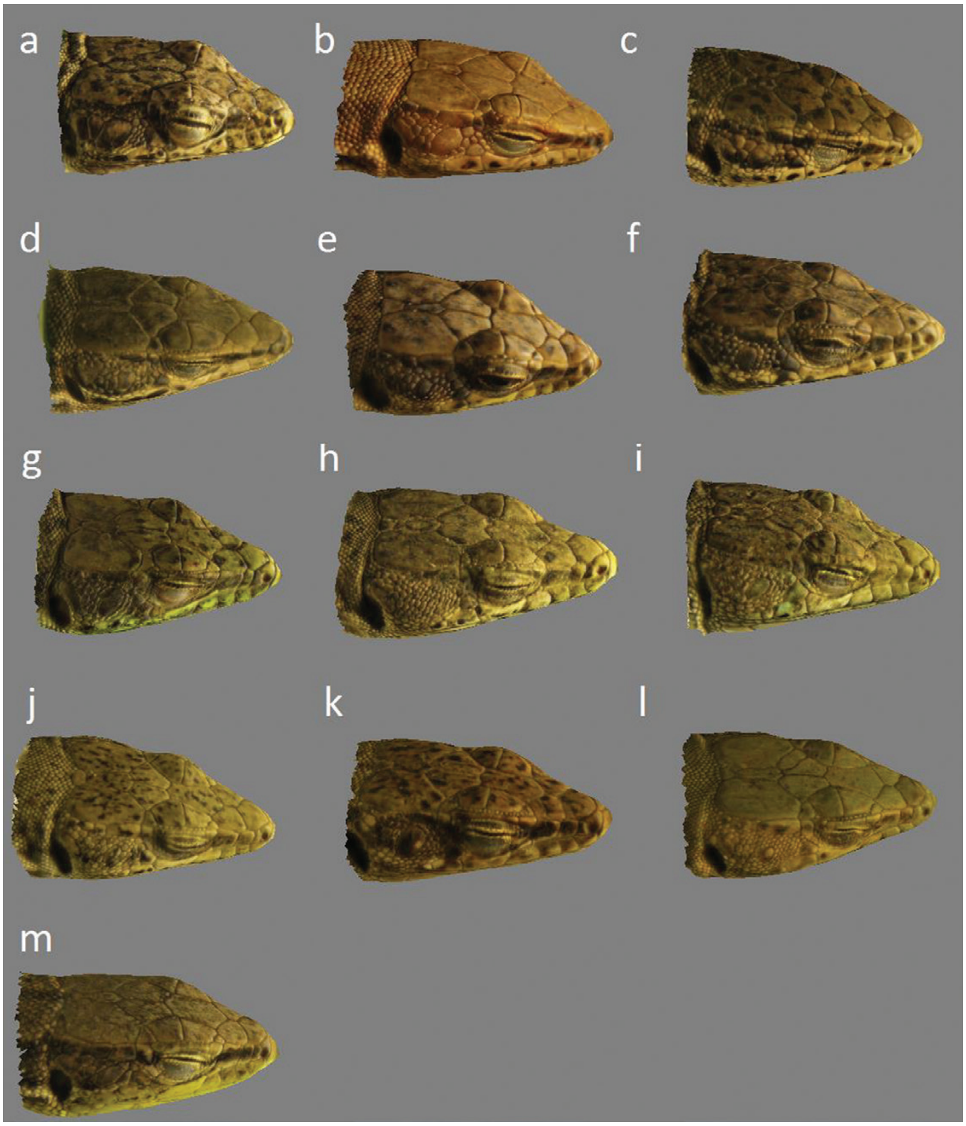

Figure 2. Heads of *Darevskia* species used in the study. A, *Darevskia mixta*. B, *Darevskia derjugini*. C, *Darevskia caucasica*. D, *Darevskia daghestanica*. E, *Darevskia clarkorum*. F, *Darevskia raddei*. G, *Darevskia rudis*. H, *Darevskia obscura*. I, *Darevskia valentini*. J, *Darevskia portschniskii*. K, *Darevskia parvula*. L, *Darevskia brauneri*. M, *Darevskia praticola*.

the procedure described by Gabelaia *et al.* (2018). The individuals were anaesthetized with chloroform 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 (2016) in order to generate 3D models of the heads.

After creating the 3D head models, we digitized 54 landmarks on their scalation pattern (Supporting Information, Fig. S1; Table S2) 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 & Mardia, 1998), using the software PAST (Hammer *et al.*, 2001).

For assessing asymmetry in the landmark configurations, we followed the procedure described by Klingenberg *et al.* (2002). The analysis showed that there was no asymmetry in our data worthy of consideration during further analysis. Finally, in order to extract the 'meaningful' components from the shape data, we ran principal components analysis (PCA) in the software PAST. Meaningful components (3DPCA1–3DPCAn) were defined via broken stick

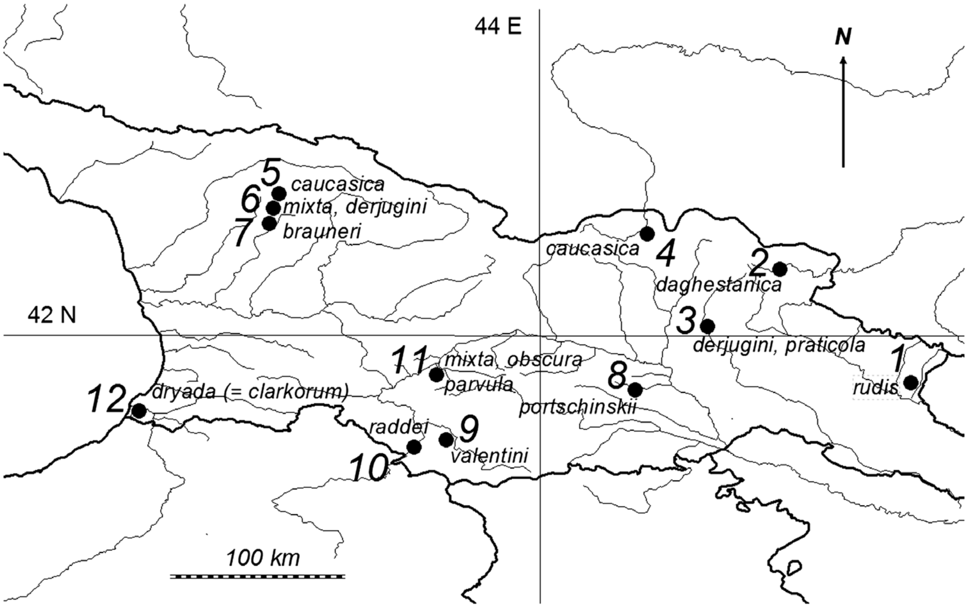

Figure 3. Sampling locations for each species.

Figure 4. Anal, fifth upper labial and frontal scale outlines (in white) on a *Darevskia clarkorum* individual.

analysis (Jackson, 1993) in the software PAST. This method is based on comparison of the observed distribution of eigenvalues of the individual PCs with the random distribution of numbers with the same cumulative value. Those PCs whose eigenvalue exceeds the corresponding random value of the same order are considered to be meaningful.

For the two-dimensional Fourier shape analysis (Kennedy *et al.*, 1990), we used images of the same animals collected using a camera. The analysis was based on the outlines of three scales: the anal scale, fifth upper labial scale (5thUL) and frontal scale (Fig. 4). The outlines were traced in Corel-draw Graphics Suite x7 (Corel Corporation) and analysed using SHAPE software (Iwata & Ukai, 2002). This software extracts the contour from the outline images, then normalizes the size, orientation and starting point for tracing the contours of the outlines according to the major axis of the first harmonic (first Fourier approximation to the shape). Principal components analysis was done on the obtained elliptic Fourier descriptors (EFDs), with four EFDs for each of the 20 harmonics (goniometric shape descriptors; Lestrel, 1997). The procedure was described by Gabelaia *et al.* (2017). Meaningful PCs were extracted using SHAPE software, producing 2D coordinates describing the shapes of the anal scales (ASPCA), 5thUL (ULPCA) and frontal scale (FSPCA).

Finally, the body length of each studied individual, from the tip of the snout to the hip fold, was recorded with precision to 1 mm.

STATISTICAL PROCEDURES

We separated the lizards into three habitat preference categories, as follows: (1) ground dwellers (*D. praticola* and *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* and *D. clarkorum*); and (3) strict rock dwellers (*D. parvula*, *D. portschinskii*, *D. obscura*, *D. brauneri* and *D. raddei*). This division is based on published descriptions (Darevsky, 1967; Bannikov *et al.*, 1977; Tarkhnishvili, 2012) and personal observations (by D.T.).

We conducted an overall test of correlation of 3D head shape (based on the 54 original landmark coordinates) with habitat type and body size, controlled for phylogeny (phylogenetic ANOVA), conducted separately for the males and the females, using the procD.pgls function from the package *geomorph* (Adams & Otárola-Castillo, 2013) in R (R Development Core Team, 2008), and estimated the correlation between the overall shape and phylogeny using the physignal function from the same package. This was done in order to infer whether there was a phylogenetic signal in overall head shape of lizards and whether the overall shape was correlated with habitat type, size or sex even if controlled for phylogeny. The tree used for these calculations was that of the 13 species studied in this paper, based on the full mitochondrial genome (Murtskhvaladze *et al.*, in press).

We then applied phylogenetic ANOVA using the phylANOVA function from the *phytools* software package (Revell, 2012) in R to assess the influence of habitat and body size, again controlled for phylogenetic signal, on individual components of phenotype (meaningful PC axes based on 3D head shape and scale outlines). In addition, we used a univariate general linear model scheme (SPSS v.21; IBM Corp., 2012) for almost the same purpose, specifically for estimating the significance of the association of each meaningful PC axis with the following: (1) species; (2) sex; (3) habitat preference; and (4) clade (as shown in Fig. 1), all coded as nominal variables; (5) body length coded as a covariate; and (6) the interaction between species and sex. This was done to identify those individual components of phenotype that are linked with adaptation to specific environments and those that are not linked with the environment but diverge with time.

To estimate the strength and significance of association of each meaningful PC axis with phylogeny, we calculated Blomberg's K statistics and Pagel's λ using the phylosig function from the *phytools* package (Revell *et al.*, 2007) and the physignal function from the R package *geomorph* (for Blomberg's K only).

The sequential Bonferroni correction procedure (dividing P-values by the number of tests; Rice, 1989) was applied in correlation tables across columns to adjust for the testing of multiple hypotheses.

Lastly, we inferred multivariate phenotypic patterns for each taxon and tested their associations with the phylogenetic tree of *Darevskia*. For this reason, we ran a stepwise discriminant function analysis (DFA) designed for equal samples (Huberty & Olejnik, 2006): (1) for the entire set of meaningful PCA scores based on the 3D head shape and the outlines of 2D images of the scales; and (2) for the set of the meaningful PCA scores, with the exception of those that were significantly correlated with habitat, body size or sex. We used the Euclidean distances between DFA centroids (considering absence of correlation between the axes) to construct an unrooted neighbor-joining tree (Saitou & Nei, 1987) and compared this tree visually with the unrooted neighbor-joining tree based on the mitochondrial DNA analysis (Murtskhvaladze *et al.*, in press) by visual analysis of coinciding and non-coinciding nodes in the species-level trees based on the phenotype and mitochondrial DNA analysis. The software used for the tree building was MEGA v.10.1 (Tamura *et al.*, 2013).

RESULTS

SELECTION OF IMPORTANT VARIABLES

The analysis did not show a significant association of overall head shape with either habitat type or body size, if controlled for phylogeny (phylogenetic ANOVA, $P > 0.30$). Head shape was not correlated with phylogeny (physignal function, $P = 0.405$ for females and 0.149 for males).

The PCA based on the three-dimensional head shapes (3DPCA) extracted eight meaningful PC axes, explaining 67% of the total shape variation. The analysis of the outline of the anal, 5th UL and frontal scales (ASPCA, ULPCA and FSPCA) extracted six, eight and nine PC axes, respectively; altogether, 31 meaningful components of variation of head shape and three large scales were present in all studied species (Supporting Information, Table S3).

Univariate ANOVA, after stepwise Bonferroni correction applied across the columns, showed that: (1) three out of 31 meaningful PCA axes were significantly associated with sex (none was significantly associated with sex dependent on species); (2) nine were significantly associated with habitat type (rock-dwelling vs. ground-dwelling or intermediate life mode); (3) four were significantly associated with body size; and (4) ten were significantly associated with 'major' clades within *Darevskia* (Table 1). The last of these findings suggests the influence of phylogeny on the head and scale shape; indeed, after application of phylogenetic ANOVA, only one meaningful PCA axis out of 31 (3DPCA1; 28% of the total variation in head shape) remained significantly associated with habitat type if controlled for phylogeny ($P < 0.001$; Fig. 5), and none of the PCA axes was correlated with body size.

Table 1. Significance (*P*-values) of individual principal components analysis axes with the preferred habitat, body size, sex, attribution to a particular phylogenetic clade, and species of *Darevskia* (based on a single univariate general linear model analysis; columns 3–8); the two last columns show the significance of phylogenetic signals estimated with Blomberg's *K* statistics and Pagel's λ (Bonferroni correction not applied)

Analysis	% var	sex	clade	hb	size	spec	sex*sp	*K*	λ
3DPCA1	28.06	0.124	**0.000**	**0.000**	0.174	0.010	0.881	0.254	0.990
3DPCA2	9.35	0.926	**0.003**	**0.002**	0.014	0.028	0.919	0.809	1.000
3DPCA3	7.74	0.176	**0.000**	0.352	0.071	0.038	0.241	**0.011**	0.051
3DPCA4	6.67	0.153	**0.000**	0.323	**0.000**	**0.000**	0.365	**0.026**	**0.030**
3DPCA5	5.29	**0.004**	0.061	0.811	**0.000**	**0.000**	0.795	0.275	1.000
3DPCA6	3.88	0.874	0.036	**0.002**	0.014	0.869	0.019	0.078	1.000
3DPCA8	3.27	0.035	0.382	0.759	0.545	**0.000**	0.712	0.863	1.000
ASPCA1	69.01	**0.006**	**0.000**	**0.002**	0.790	**0.001**	0.935	0.412	0.510
ASPCA2	12.21	**0.001**	**0.000**	**0.030**	**0.000**	0.022	0.103	0.350	0.226
ULPCA1	42.05	0.012	**0.003**	**0.000**	0.940	**0.002**	0.544	0.068	0.418
ULPCA2	21.88	0.488	**0.005**	**0.002**	0.327	0.012	0.279	0.522	1.000
ULPCA6	2.81	0.095	**0.000**	0.072	**0.000**	0.032	0.343	0.445	0.507
FSPCA1	46.39	0.592	0.135	**0.000**	0.508	0.107	0.186	0.589	1.000
FSPCA2	17.64	0.368	0.779	**0.000**	0.158	0.098	0.742	0.240	1.000
FSPCA4	4.87	0.697	**0.000**	0.496	0.139	0.012	0.950	**0.017**	0.083
FSPCA7	2.53	0.993	0.513	0.512	**0.007**	**0.007**	0.944	0.843	0.934
FSPCA8	2.18	0.479	0.014	0.244	0.566	**0.000**	0.083	0.935	1.000

The values that remain significant ($P < 0.05$) are shown in boldface. For columns 2–8, Bonferroni correction applied.
Abbreviations: 3DPCA, principal component analysis (PCA) scores based on three-dimensional head shape; ASPCA-PCA, scores based on the outlines of the anal scale; clade, the differences between the four clades of *Darevskia* (Fig. 1); FSPCA-PCA, scores based on the outlines of the frontal scale; hb, habitat type (rock dwellers, ground dwellers or intermediate); hb-ph, habitat type controlled for phylogeny; *K* and λ, significance of phylogenetic signal at species level; sex*sp, influence of sex dependent on the species; size, maximal body size of adults; spec, species; sz-ph, influence of size controlled for phylogeny; ULPCA-PCA, scores based on the outlines of the fifth upper labial scale.

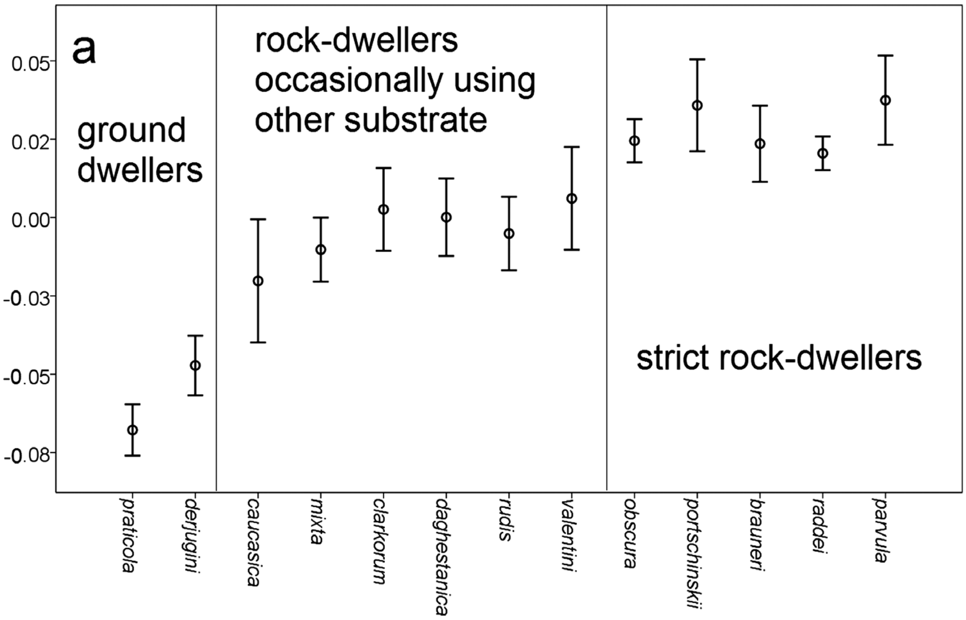

Figure 5. 'Mean and whisker' (95% confidence interval) plot of the 3DPCA1 axis scores (that associated with the preferred habitat type: the first two species are ground dwellers; species 3–8, preliminary rock dwellers; and species 9–13, strict rock dwellers).

Blomberg's *K* and Pagel's λ coefficient analysis inferring the association of each of the 31 meaningful components of variation with the mitochondrial phylogenetic tree suggested the presence of an association for three characters: 3DPCA3 (7.7% of the variation in head shape; $K = 1.09$; $P = 0.011$; $\lambda = 1.00$;

P = 0.051); 3DPCA4 (6.7% of the variation in head shape; K = 0.89; P = 0.026; λ = 0.77; P = 0.030); and FSPCA4 (4.9% of the variation in shape of the frontal scale; K = 1.00; P = 0.017; λ = 1.00; P = 0.082). In fact, only 3DPCA4 showed a significant ($P < 0.05$) association with phylogeny for both K and λ coefficients.

One-third of meaningful PCA axes differed between the clades within *Darevskia*. Blomberg's K showed significant association of 3DPCA3 and 3DPCA4, together with FSPCA4 (Fig. 6), with phylogeny. However, the nominally more sensitive Pagel's λ suggested a significant phylogenetic signal only for 3DPCA4. This variable was not associated with habitat or sex, although it was correlated with body size.

Explaining the important principal components

Nine out of 31 analysed PCs, including the first and the second PCs describing 3D head shape (Table 1), helped to discriminate among the species with more and less dependence on the rocky habitats. Five PCs, including the second PC describing the outline of the anal scale, diverged in species with different body length. Three PCs, including both the first and the second PC describing the outline of the anal scale, were correlated with sex (Table 1). Not all of these PCs significantly differentiated individual species, however. The following variables simultaneously discriminated among individual species and showed significant differences between the species occupying different habitats, between those with different body size and between males and females: 3DPCA1, ASPCA1 and ULPCA5 (habitat type); 3DPCA4 and 3DPCA5 (body length); and 3DPCA5 and ASPCA1 (sex). Finally, 3DPCA4 was correlated with phylogeny and simultaneously discriminated individual species.

For 3DPCA1 (28% of the head shape variation), dorsoventral head height showed the highest loading. The lizards with high scores along this axis (ground dwellers) had deeper and shorter heads and a frontal scale with parallel edges, whereas the lizards with low scores (rock dwellers) had flat, elongated heads, with angled edges of the frontal scale (Fig. 6). 3DPCA4 (6.7% of the head shape variation), i.e. the most phylogenetically informative dimension of the head shape, showed high loadings of dorsoventral height of a rostral part of the head, lateral width of the lower jaw with concave vs. straight edges, and relative length of the interparietal scale. 3DPCA5 (5.3% of the head shape variation) was associated with the lateral width of the lower jaw. Specifically, larger lizards had a wider lower jaw in its proximal part, whereas small-bodied ones (e.g. *D. parvula*) had a narrower lower jaw (Fig. 6).

Lower ASPCA1 values (females and lizards of the clades *rudis* and *parvula*) differed from the higher values (males and lizards of the clades *caucasica*) in having a wider and shorter, less round shape (Fig. 7A). Higher values of ULPCA1 (ground-dwelling forms) were associated with a relatively taller 5th UL (Fig. 7B).

Discriminant analysis and centroid-based tree

The stepwise DFA run for 31 meaningful PC axes identified four significantly discriminant functions; however, the neighbor-joining tree, based on the distances among the centroids, was not congruent with the phylogenetic tree of *Darevskia* (results not shown).

The second run of the stepwise DFA was for only those meaningful PC axes that were not associated with habitat (Table 1). The unrooted neighbor-joining tree based on this distance matrix was partly congruent with the neighbor-joining tree based on the mitochondrial DNA analysis. Congruence of the mitochondrial and phenotypic tree topologies was complete for taxa in the *caucasica* clade (Fig. 8, nodes 1–5), but not for the *rudis* clade. The phenotypic tree, unlike the DNA tree, positioned *D. brauneri* and *D. saxicola* as a paraphyletic group; it displaced *D. parvula* into the *D. rudis* clade, and suggested a closer relationship between *D. valentini* and *D. rudis*, not *D. portschinskii* (remarkably, in accordance with early mitochondrial tree published by Murphy *et al.*, 2000).

DISCUSSION

If at least some individual phenotypic characters are considered, the differences between rock lizard species increase with the time of divergence, even if these species are adapted to similar environments. That entails monotonic divergence, which supports Dollo's law regarding the irreversibility of phenotypic evolution. This divergence is obvious for the fourth component of 3D head shape of the lizards, which contains information on the shape of the rostral part. This PC is significantly correlated with phylogeny. It is not clear whether it has adaptive importance. Potentially, dimensions of the rostral part might be related to bite strength, hence to the diet or mating behaviour (Vincent & Herrel, 2007; Galoyan, 2013), although it can also be non-adaptive in that it is not correlated with habitat type. Meanwhile, this PC explains < 7% of the total variability in head shape, which means that most of the variation in head shape is not correlated with phylogeny.

Our results also suggest that removal of the characters heavily influenced by habitat type can improve the phylogenetic signal in the phenotype

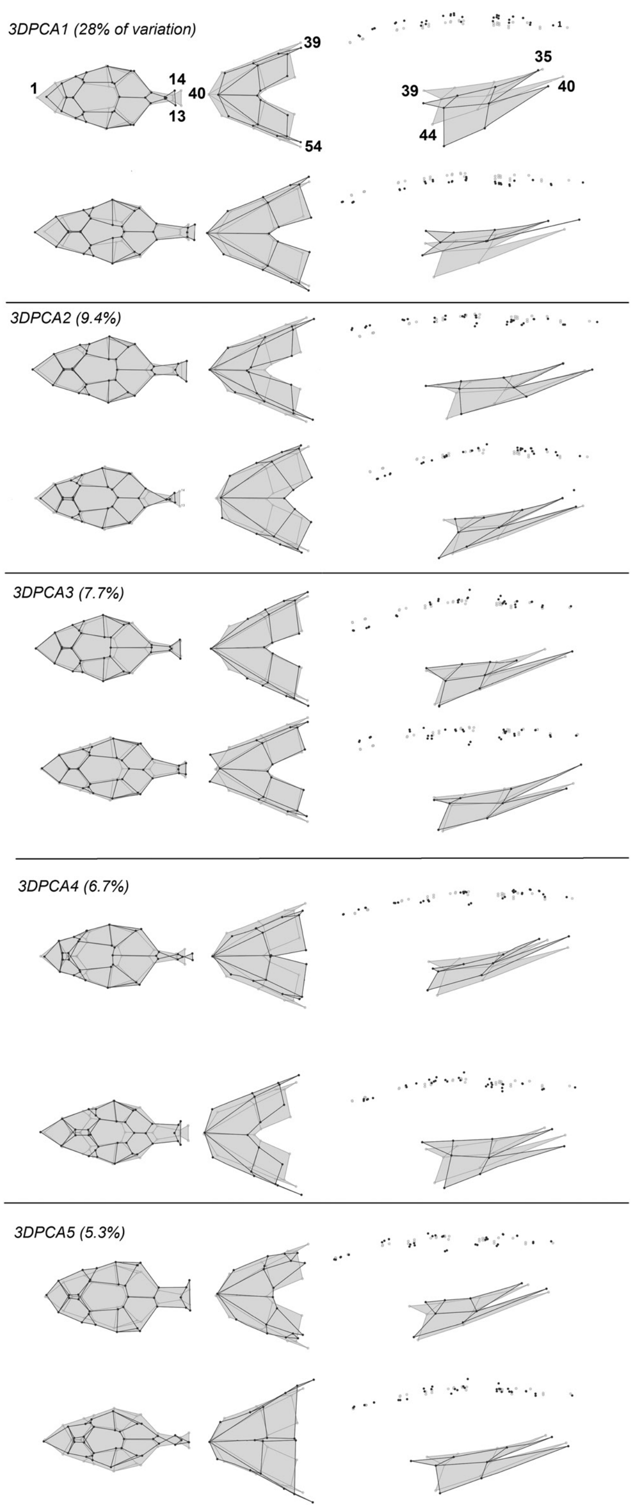

Figure 6. Dorsal, ventral and lateral wireframes (from left to right) of 3DPCA1–3DPCA5. Mean values along the respective principal components analysis axis are marked with grey dots and lines; maximal and minimal values (upper and lower diagram in each panel) are marked with black dots and lines. On the lateral view, wireframes for jaws are shown, and the position of landmarks on the dorsal part of the head. Numbers on the wireframes of 3DPCA1 refer to the landmark numbers from the Supporting Information (Fig. S1).

of *Darevskia*. This addresses the second hypothesis formulated in the Introduction. The congruence between the phenotypic distance-based tree and the phylogenetic tree based on the molecular data increased when the phenotypic variables that separate ground-dwelling lizards from the rock-dwelling species were excluded from the analysis. Our results also support the greater efficacy of 3D geometric morphometrics over other methods of phenotypic analysis (see also Sztencel-Jabłonka *et al.*, 2009; Adams *et al.*, 2013; Ivanović *et al.*, 2013; Gabelaia *et al.*, 2018).

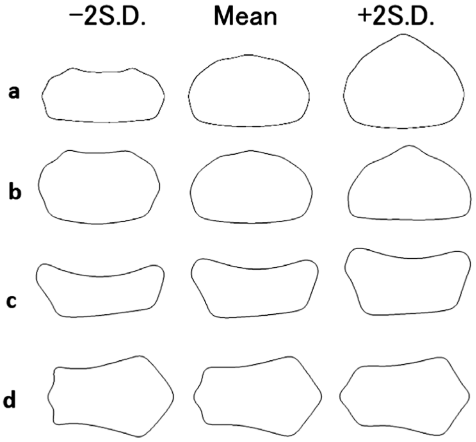

Figure 7. Principal component contours explaining the shape variation for ASPCA1 (A), ASPCA2 (B), ULPCA1 (C) and FSPCA4 (D). Abbreviation: 2S.D. is 2 × standard deviation.

How do these findings relate to the existent views on the phenotypic evolution? Bookstein (1991) suggested that biological shapes composed of several integrated morphological characters will seldom be phylogenetically informative, because their integrated function resists easy or rapid evolutionary change ('coordinated variation among traits that are closely related in development and/or function'; Singh *et al.*, 2012). This would reduce the detectability of features that are phylogenetically informative (Ivanović *et al.*, 2013). Indeed, phylogenetic signal in the phenotype of various vertebrates is not always detected (Steppan, 1998; Serb *et al.*, 2001; Wiens & Penkrot, 2002; López-Fernández *et al.*, 2005), although some other studies suggest its presence (Gentilli *et al.*, 2009; Henderson *et al.*, 2013). Smith *et al.* (2011) showed that adaptive changes during lizard evolution provide multiple examples of convergence, obscuring the phylogenetic pattern (Thorpe *et al.*, 1994; Harmon *et al.*, 2005; Köhler *et al.*, 2010). The discordance between the phenotypic (Darevsky, 1967) and genotypic (Murphy *et al.*, 2000; Ahmadzadeh *et al.*, 2013) systems of *Darevskia* are in agreement with this statement. Body size and head shape vary widely among closely related species of this genus and may be more similar in species from different clades, such as *D. portschinskii*, *D. parvula* and *D. raddei*, than in the closest relatives.

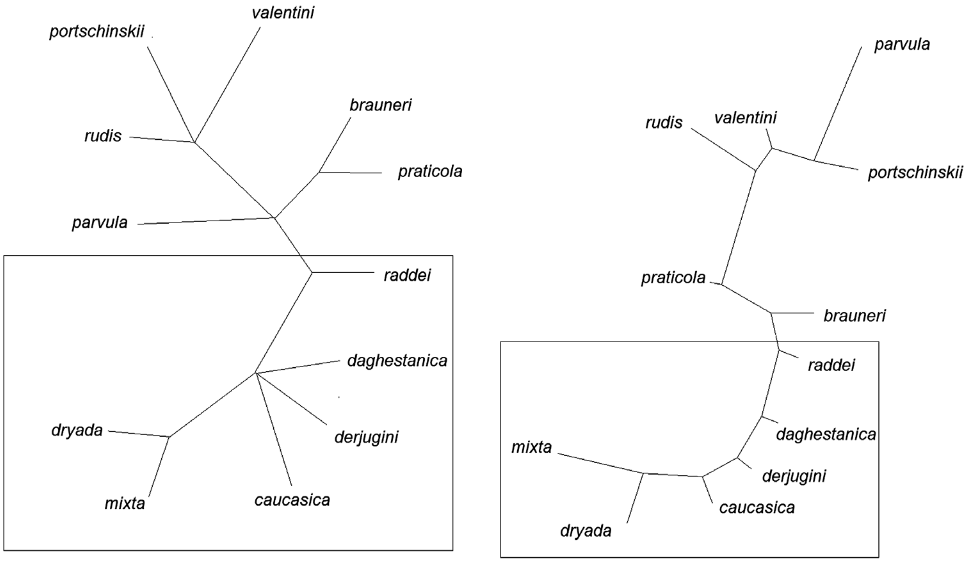

Figure 8. Left, the unrooted consensus tree of the studied *Darevskia* species (Fig. 1), based on the mitochondrial DNA sequences. Right, the unrooted distance-based neighbor-joining tree (the distances between the discriminant function analysis centroids; the analysis conducted for meaningful principal components analyses not associated with sex or habitat). Boxes delimit clade *caucasica*, according to Murphy *et al.* (2000).

Our research also suggests that convergent patterns, associated with habitat type, mask the phylogenetic signal. The position of scales around the anal scale in rock-dwelling and ground-dwelling *Darevskia* (but not between the species from different clades) is different, in that the ground dwellers have preanal scales overlying the anal scale, whereas in rock dwellers the edges of the scales in the anal area abut (Tarkhnishvili, 2012). The position of preanal scales in the ground-dwelling *Darevskia* is similar to that in ground dwellers from different genera, e.g. *Lacerta* and *Zootoka*, hence we hypothesize that these differences are adaptive; e.g. overlying preanal scales might complicate movement in rock clefts. Geometric morphometric analysis of 3D head shape also revealed convergent patterns related to habitat type. The first and the second PCA axes depend significantly on the habitat type. Ground dwellers have a deeper head, with shorter and more obtuse snout, than rock dwellers. Less prominent differences exist between rock-dwelling *Darevskia* that depend on rock habitats to different extents (e.g. heads are deeper and shorter in *D. mixta* or *D. caucasica* than in *D. parvula* and *D. raddei*). It is likely that flat heads help lizards to increase the number of suitable shelters.

The importance of habitat type in phenotypic evolution has been shown for different groups of lizards (Openshaw & Keogh, 2014). Losos *et al.* (1997) showed that the body shape of anoles underwent significant change within only a few generations after they were introduced to new island habitats, and these changes depended 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 was driven by their life mode (burrowing vs. ground dwelling). Vanhooydonck & Van Damme (1999) and Herrel *et al.* (2001) showed a decrease in head depth in rock-dwelling lizards, in comparison to the ground dwellers. In four different lizard clades, representing two families, adaptation to rock dwelling has been correlated with longer limbs and flatter heads (Revell *et al.*, 2007). Urošević *et al.* (2012) showed that this rule also applied to Lacertids of the genus *Podarcis*, close relatives of *Darevskia* (Zheng & Wiens, 2015; Murtskhvaladze *et al.*, in press).

In contrast, evolutionary constraints specific for individual taxa prevent convergence of some phenotypic traits. For instance, herbivorous lizards from different families, in spite of similarity in the amount of force produced during jaw closure, do not converge in jaw shape (Stayton, 2006). Outlines of the anal scales in *Darevskia* are not associated with habitat type, but differ between the representatives of distant clades of these lizards: *D. rudis*, *D. portschinskii* and *D. valentini* on the one hand, and *D. mixta* and *D. derjugini* on the other (Gabelaia *et al.*, 2017; present study). The same applies to the shape of the rostral part, which is significantly correlated with phylogeny. Hence, in spite of the convergent patterns, there are characters of head shape and scale outline that hold significant phylogenetic signals. The congruence of the phenotypic tree (based on the head dimensions unrelated to habitat type) and the genotypic tree suggests that the convergent patterns do not completely mask phenotypic divergence attributable to other causes, even between the closely related species of lizards, and some structures diverge even if different species adapt to similar environments.

Variability in body size is another important adaptive feature that can influence the overall phenotype. It 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 coexistence with closely related species (Meiri, 2008; Moritz *et al.*, 2018). *Darevskia* species occupying the same location and the same habitat (i.e. coexisting rock dwellers) commonly differ in body size, such as *D. parvula* and *D. rudis* in western Lesser Caucasus, or *D. rudis* and *D. mixta* in central Georgia. This might be related either to a niche shift for avoidance of competition or to reinforcement (Tarkhnishvili, 2012). Two PCs of head shape (3DPCA4 and 3DPCA5) are correlated with the body size. However, the influence of body size on head shape and anal outline is small, and this character does not complicate phylogenetic reconstruction.

The present study demonstrates that geometric morphology is an effective methodology, able to infer evolutionary signal where traditional multivariate morphometry is powerless (Bernal, 2007; Maderbacher *et al.*, 2008; Abdel-Rahman *et al.*, 2009; Breno *et al.*, 2011); it is especially effective for studying correlations between molecular and phenotypic evolution. Simultaneously, it is important to understand that convergent and divergent patterns may coexist in phenotypic evolution, and one needs to analyse the phenotypes of related species carefully, in order to separate and distinguish these patterns.

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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:

Figure S1. Fifty-four three-dimensional landmarks on a *Darevskia rudis rudis* individual.
Table S1. List of the locations (according to Fig. 3) and the number of lizards sampled from each location.
Table S2. Description of the landmarks. Abbreviations: FN, frontal–nasal; FP, frontoparietal; Fr, frontal; IM, intermaxillary; IP, interparietal; M, mandibular; N1, N2, nasals; N, nuchal; P, parietal; PF, prefrontal; PN, post-nasal; SDs, small dorsal scales; SMs, small mandibular scales; SO, supraorbital; UCs, upper ciliated scales. Landmark 'n' is the touching point of the scales of 'N'.
Table S3. Eigenvalues and percentage of explained variation of the first 20 principal components describing: three-dimensional head shape (3DPCA), outlines of the anal scales (ASPCA), fifth upper labial scale (ULPCA) and frontal scale (FSPCA).