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Coincidence of genotypes at two loci in two parthenogenetic rock lizards: how backcrosses might trigger adaptive speciation

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The parthenogenetic lizards *Darevskia armeniaca* and *Darevskia dahli* are widespread throughout the Lesser Caucasus, although they occupy different habitats. While these forms differ in size, colour and scalation, both *D. armeniaca* and *D. dahli* have a hybrid origin and a common maternal progenitor species. Current evidence about the patrilineal origin is inconclusive, and the exact number of ancestral lineages remains unknown. This study aimed to investigate the distribution of mitochondrial haplotypes and alleles at five microsatellite loci in both the parthenogenetic forms and their presumed ancestors, in order to infer the number of ancestral hybridization events. Mitochondrial DNA analysis confirmed that both forms descend matrilineally from *D. mixta* from a limited geographic area in Central Georgia. Simultaneously, the majority of both *D. armeniaca* and *D. dahli* shared the same genotypes at two microsatellite loci, but differed at the other three. This observation and overall study of the distribution of the microsatellite genotypes suggests that (1) both *D. armeniaca* and *D. dahli* descend from very few, possibly single original hybridization event, most likely *D. mixta* and *D. portschinskii*, hence the hypothesis of different paternal species for these two forms is rejected; (2) expansion of the original parthenogenetic hybrid form into the range of *D. valentini*, followed by backcrosses of *D. valentini* males with the parthenogens, triggered development of a new parthenogenetic form, *D. armeniaca*; (3) backcrosses and mutation are more likely reasons of genetic diversity both between and within the parthenogenetic forms than their multiclonal origin.

ADDITIONAL KEYWORDS: *Darevskia* – evolution of parthenogens – geographic parthenogenesis – microsatellites – mitochondrial DNA – parthenogenesis – probability of shared ancestry.

INTRODUCTION

Obligatory parthenogesis exists in eight squamate families (Kearney, Fijita & Ridenour, 2009; Jančúchová-Lásková, Landová & Frynta, 2015a), with the best-known examples found in teiids (genus *Aspidoscelis*, syn. *Cnemidophorus*; Parker & Selander, 1976), geckos (genus *Lepidodactylus*; Bolger & Case, 1994; genus *Nactus*; Eckstut, Hamilton & Austin, 2013), agamids (genus *Leiopelis*; Grismer & Grismer, 2010) and lacertids (*Darevskia*; Darevskii, 1967). Generally, lizard parthenogens descend from interspecific hybridization events occurring between congeneric species (Dawley, 1989; Moritz *et al.*, 1992). Among these, Caucasian rock lizards in the genus *Darevskia* are the only reptile group from temperate climate that exhibits obligate parthenogenesis, containing seven morphologically and ecologically distinct asexual forms (Darevsky, 1992; Murphy *et al.*, 2000; Tarkhnishvili, 2012; Freitas *et al.*, 2016a). Although Busack *et al.* (2016) suggest that *Darevskia* is a junior synonym of *Caucasilacerta* proposed earlier for the Caucasian rock lizards, here and below we keep using the synonym *Darevskia* because this is a name widely used in recent papers studying parthenogenesis in these reptiles, and the present paper does not have taxonomic focus.

These parthenogenetic *Darevskia* taxa are diploids that reproduce without gynogenesis (Darevsky, 1992). Diploid, triploid and tetraploid hybrids between parthenogenetic *D. armeniaca* and sexually reproducing *D.*

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valentini are found in some locations (Darevskii, 1967; Darevsky & Danielyan, 1968; Danielyan, Arakelyan & Stepanyan, 2008), as well as triploid hybrids between parthenogenetic *D. dahli* and sexual *D. portschinskii* (Darevsky & Kulikova, 1961; Davoyan, 2007). Darevskii (1967) suggested that such polyploid hybrids are sterile, although Danielyan *et al.* (2008) showed that some sexually reproducing males and females of hybrid origin, with various ploidy, can occasionally be fertile. Fertile triploid forms are known to occur in another parthenogenetic lizard genus, *Aspidoscelis* (Reeder, Dessauer & Cole, 2002; Cole *et al.*, 2010; Wynn *et al.*, 1987) and in blind snakes of genus *Ramphotyphlops* (Wynn *et al.*, 1987). In general, it is not clear whether parthenogenetic lizards can enrich their gene pool by hybridizing with the related sexual forms.

D. dahli and *D. armeniaca*, the two parthenogenetic forms, are widespread in southern Georgia and northern Armenia (Darevskii, 1967; Tarkhnishvili, 2012). *D. armeniaca* lives in uplands of the Southern Caucasus, at elevations of 1500–2500 m, whereas *D. dahli* inhabits at elevations between 800 and 2000 m, mostly in mountain forest (Darevsky, 1967; Tarkhnishvili *et al.*, 2010) (Fig. 1). Morphological differences between them are comparable to the morphological characters that distinguish sexually reproducing species of rock lizards. *D. armeniaca* has a larger body size (maximum snout-vent length 74 mm vs. 63 mm in *D. dahli*) and exhibits a different dorsal colour pattern than *D. dahli*. In addition, *D. armeniaca* has larger and fewer scales in the temporal region, and a single very broad preanal scale, in contrast to paired preanal scales in *D. dahli* (Fig. 2; Darevskii, 1967). While common mtDNA haplotypes found in *dahli and armeniaca* parthenogens provide strong evidence that *Darevskia mixta* is the maternal progenitor of both *D. dahli* and *D. armeniaca* (Moritz *et al.*, 1992; Murphy *et al.*, 2000), exactly which species is the paternal progenitor is less obvious. Previous authors (Uzzell & Darevsky, 1975; Darevsky, Kupriyanova & Uzzell, 1985; Kupriyanova, 1989, 1992) hypothesized that *D. dahli* and *D. armeniaca* derived through their paternal lineage from *Darevskia portschinski* and *D. valentini*, respectively, which have ranges that broadly overlap with the ranges of the respective parthenogenetic forms. Murphy *et al.* (2000) suggested that the distribution of alleles at 24 polymorphic allozyme loci in the parthenogens and their presumptive paternal progenitors supports this view. Moreover, the exact number of individual hybridization events triggering parthenogenesis in the ancestors of *D. dahli* and *D. armeniaca* remains unknown. Parker *et al.* (1989) suggest that parthenogenetic lizard forms descend from a single hybrid individual, although sometimes accompanied by a few rare clones descending from other hybridization occurrences. However, analysis of the extent of variation in nuclear markers (allozymes and microsatellite DNA genotypes) has identified genetic polymorphisms in all parthenogenetic forms of *Darevskia* (McCulloch et al., 1995; Murphy *et al.*, 1997; Kan *et al.*, 1998; Fu, Murphy & Darevsky, 1999; Fu *et al.*, 2000; Tokarskaya *et al.*, 2003; Davoyan *et al.*, 2007; Korchagin et al., 2007, 2013; Vergun *et al.*, 2014). Some authors attribute these polymorphisms to multiple origins of individual forms (Fu et al., 1999, 2000; Vergun *et al.*, 2014), whereas others ascribe this variation to the accumulation of mutations and recombination within the asexual lineages (Murphy *et al.*, 1997; Tokarskaya *et al.*, 2003) subsequent to their becoming

Figure 1. Geographic distribution of *D. armeniaca* (circles) and *D. dahli* (squares). All locations from Georgia shown on the map are sampled and used in the analysis. Star indicates location of the mitochondrial haplotype of *D. mixta*, ancestral to both *D. dahli* and *D. armeniaca*.

Figure 2. Temporal and anal scalation in *D. armeniaca* (left) and *D. dahli* (right). Diagnostic characters indicated by arrows.

parthenogenetic. Recent combined study of both mitochondrial and nuclear genes suggest multiclonal origin of three parthenogenetic *Darevskia* from Armenia and Turkey (Freitas *et al.*, 2016a). Vergun *et al.* (2014), based on the analysis of genotypes at three microsatellite loci in *D. dahli* from Northern Armenia, suggested coexistence of clones descending from three independent hybridization events. Two individuals had microsatellite alleles with specific substitutions in the flanking regions of allele Du215; the authors considered them to represent clones descending from hybrid individuals other than one ancestral to the most of *D. dahli*. Hence, there is no conclusive evidence on the origin of genetic polymorphisms in the studied parthenogens.

Remarkably, the previous studies did not include parthenogenetic samples collected in proximity to the maternal species' range, nor have they compared parthenogen microsatellites to the microsatellite genotypes in the putative ancestral species. Here we describe comparative analysis of *D. dahli* and *D. armeniaca* sampled throughout their Georgian range, and that of their presumed parental species, *D. mixta*, *D. portschinskii* and *D. valentini*. We analysed the distribution of both mitochondrial haplotypes and microsatellite genotypes. The goal of this study was to clarify the origin of genetic diversity within the parthenogens and to compare the results with what is known for other parthenogenetic reptiles. Our results explored genotype distribution, and suggested that both parthenogenetic forms descend from a single or few hybrids from a limited geographic area.

MATERIAL AND METHODS

Sampling

Tissue samples (tail-tips) were collected and stored in 95 % ETOH, taken from 40 locations of *D. dahli*, 25 of *D. armeniaca*, 46 of *D. portschinskii*, 22 of *D. valentini* and 15 of *D. mixta*, across the entire Central Lesser Caucasus, where these taxa are found in sympatry (Table S1, Fig. 1). One to thirteen individuals from each site were sampled and analysed. Digital images were taken for every animal, for confirmation of species identifications made in the field. For identification, diagnostic morphological traits described in Darevskii (1967) were used. In total, 78 *D. dahli*, 37 *D. armeniaca*, 81 *D. portschinskii*, 42 *D. valentini* and 29 *D. mixta* individuals were sampled.

Mitochondrial DNA sequencing

DNA extraction was performed using a Qiagen tissue kit following the manufacturer's instructions (QIAamp DNA Mini and Blood Kit Handbook), using ~20 mg of muscle tissue for each individual. To check for contamination, a negative control (5 µL of reagents only) was included for each set of extractions.

A partial, 320 bp fragment of the mitochondrial Cytochrome *b* (Cyt *b*) gene was amplified and sequenced from 46 *D. dahli* and 20 *D. armeniaca* individuals throughout the sampling area, and from 14 samples of *D. mixta* throughout its range (Gabelaia *et al.*, 2015; Table S1). The primers used were L14841 5′-CCA TCCAACATCTCAGCATGATGAAA-3′ and H15149 5′-GCCCCTCAGAATGATATTTGTCCTCA-3′ (Kocher *et al.*, 1989), as described for these taxa in Murphy *et al.* (2000). PCR was carried out in a 20 µL total volume, with 2–4 µL template DNA (~20–50 ng/µL), and final concentrations: 1U of Taq polymerase (Promega), 1× PCR buffer (Promega) 1.25 mM of $MgCl_2$, 0.1 mM of each dNTP and 0.1 µM of each primer. The PCR profile included denaturing at 95 °C for 2 min, followed by 30 cycles of 94 °C for 45 s, 46 °C for 1 min and 72°C for 2 min, with final extension 72 °C for 10 min. An aliquot of 3–5 µL from each PCR was run on a 1% agarose gel to visualize the DNA fragments. Sequencing reactions were performed with the primers used for PCR, using Big Dye Terminator v.3.1 (Applied Biosystems, Foster City, CA, USA). The amplicons were sequenced on an automated sequencer (ABI 3130). Sequence alignment was performed with BioEdit v7.0 (Hall, 1999). In the alignment, mtDNA sequences of three *D. mixta*, two *D. dahli* and one *D. armeniaca* individuals available from GenBank were added to our aligned sequences (accession numbers KY349165-KY349224).

The sequences were edited using SEQSCAPE v2.5 (Applied Biosystems Inc., Foster City, CA, USA), and unique sequences were deposited in GenBank (accession numbers JN546146–JN546194). Because putative numt is found in one of *Darevskia* species (Freitas *et al.*, 2016b), prior to further analysis, we tested whether our sequenced fragments or GenBank sequences represent pseudogenes following the procedures described in Tarkhnishvili *et al.* (2016); 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 NJ tree constructed using our sequences. No pseudogenes were found in this procedure (results not shown).

Similarity among haplotypes was visualized using a Median-Joining network (Network 5.0 software) (Bandelt, Forster & Rohl, 1999). Identifying the most basal haplotype of the parthenogenetic form (hence, identical with the haplotype of the maternal species) and linking those to particular haplogroup of *D. mixta* helped to infer the most likely area of matrilineal origin of the parthenogens.

Scoring microsatellite genotypes

Microsatellite genotypes at five tetranucleotide repeat loci were scored for all sampled individuals (Tables S1, S2). We used five primer pairs developed by Korchagin *et al.* (2007), here designated as Du215, Du281, Du418, Du47 and Du323. Some polymorphisms at loci Du215, Du281 and Du323 were described for *D. dahli* collected in Armenia (Davoyan *et al.*, 2007; Vergun *et al.*, 2014). PCR was performed using QIAGEN Multiplex PCR Kits with two sets of multiplex reactions, following manufacturer's recommended protocol. Primer concentrations varied as follows: in the first multiplex reaction Du215 0.11 μM, Du418 0.14 μM and Du47 0.12 μM; in the second multiplex reaction Du281 0.1 μM and Du323 0.13 μM. Thermal cycling was performed with the following steps: (1) initial denaturing at 95 °C for 15 min; (2) a touchdown program of 15 cycles of 94 °C for 30 s, 56 °C for 90 s, with a stepwise decrease of 0.5 °C at each cycle; 72 °C for 1 min; (3) 20 cycles of 94 °C for 1 min, 48 °C for 90 s and 72 °C for 1 min; (4) final extension at 60 °C for 30 min. Fragments were separated on an ABI 3130 Gene Analyzer, using the size standard LIZ 500 (Applied Biosystems Inc., Foster City, CA, USA). Genotypes were scored using Genemapper v3.5 software (Perkin-Elmer, Waltham, MA, USA). Every locus of each individual was repeated two to four times to control for allelic dropout and false allele amplification. If the amplified products showed unclear profiles due to overloading, they were diluted with dH_2O 1:60 and the fragment analysis subjected to repeated electrophoresis run, in order to obtain clear data. The numbers of size variants at four out of five studied loci were in correspondence with previously reported alleles (Korchagin *et al.*, 2007; Vergun *et al.*, 2014;Tarkhnishvili *et al.*, 2013). However, in both studied parthenogens, primers designed for Du281 amplified an almost monomorphic locus. This locus had alleles nearly diagnostic for both *D. dahli* and *D. armeniaca*, and simultaneously different from ones described by Vergun *et al.* (2014). For these reasons, we describe this locus as 'Du281-like'.

Population genetic analysis of microsatellite genotypes

We used Arlequin 3.5.1 (Excoffier & Lischer, 2010) to evaluate genetic diversity of the *D. dahli*, *D. armeniaca*, *D. portschinskii*, *D. valentini* and *D. mixta* samples. Specifically, we identified the mean number of alleles per locus for each studied taxon, expected and observed heterozygozity levels, theta *H*-value (Ohta and Kimura, 1973), and simple and modified Garza–Williamson indices, reflecting the ratio of allele number to allele range (Garza and Williamson, 2001; Excoffier, Laval & Schneider, 2005). STRUCTURE v2.2 (Pritchard, Stephens & Donnelly, 2000) was used to separate the entire dataset into groups with the least within-locus and between-locus disequilibria. We assigned POPFLAG=1 to sexual species and POPFLAG=0 to asexuals, therewith assigning parthenogens as 'migrants' and without population number prior (USERPOPINFO model). The number of K was set as 3, the same as the number of the anticipated parental sexual species included in the analysis. This was done in order to evaluate the proportion of loci gained by each parthenogenetic individual from each anticipated parental sexual species (the algorithm following the procedure of Murgia *et al.*, 2006). MCMC parameters were set with a burn-in period of 10 000 and 100 000 post-burn-in replicates.

We repeated the procedure ten times for each of a priori delimited number of clusters (K) ranging from 1 to 10, in order to calculate ΔK statistics (the rate of change of the log probability of the data between successive K-values). The number of clusters corresponding to the highest posterior probability of K (Pritchard, Wen & Falush, 2010) was assumed to reflect the most likely number of clusters within the dataset.

Inferring relations among the parthenogenetic genotypes

We developed a haplotype network of the parthenogenetic forms, using Network 5.0 software (Bandelt *et al.*, 1999), encoding microsatellite alleles as Y-STR data (the alleles for each individual stored in a single row,

assuming little or no recombination in parthenogens). Earlier, Vergun *et al.* (2014) used a similar approach for linking genotypes of *D. dahli* from Armenia, albeit with different software. The rationale is that point mutations in a parthenogenetic genome would cause divergence among the parthenogenetic individuals, but not recombination among the diverged genotypes, hence the divergence process is more similar to that in mitochondrial or Y-chromosome haplotypes than in autosomal genotypes. This procedure helped us to link individual parthenogenetic genotypes with a specific number of mutations [changes of number of repeats at individual short tandem repeat (STR) loci]. Genotypes sharing a sole mutation at a single locus were treated as deriving from one ancestral hybrid genotype as a result of point mutations. If differences were found among parthenogenetic individuals at two or more loci, we considered this to be indicative of multiclonal origins or backcrossing as a more likely reason for the differences among the individuals.

RESULTS

Mitochondrial DNA sequences

Based on digital images taken in the field, the majority of the studied individuals were easily assigned to one of the three sexually reproducing species or two parthenogenetic forms. Six individuals showed scalation characters intermediate between *D. valentini* and *D. armeniaca*, but their mitochondrial DNA was identical to *D. valentini* and not to *D. armeniaca* or *D. mixta*, suggesting that they were not parthenogens; these individuals were excluded from the analyses.

The 320 bp alignment of Cyt *b* sequence for *D. dahli*, *D. armeniaca* and the maternal *D. mixta* was variable at 21 positions. Eleven unique haplotypes of *D. dahli*, eight unique haplotypes of *D. mixta* and eight haplotypes of *D. armeniaca* were identified. The Median-joining network (Fig. 3) indicated that the most common haplotype (central circle) in the parthenogens was also found in *D. mixta* from Borjomi Gorge of Central Georgia but not in other parts of the range of *D. mixta*. Ten out of twenty-one individuals of *D. armeniaca*, irrespective of the geographic location, had haplotypes substantially different from the other individuals of this taxon; however, these haplotypes were not linked to other extant haplotypes of *D. mixta* from throughout the range and hence cannot be used as an evidence of polyclonal maternal origin of this form. The output is consistent with the hypothesis of a single maternal lineage of *D. mixta* ancestral to both *D. dahli* and *D. armeniaca*. Overall, 88% of the studied *D. dahli* and 33% of the *D. armeniaca* from the entire study area shared this ancestral haplotype.

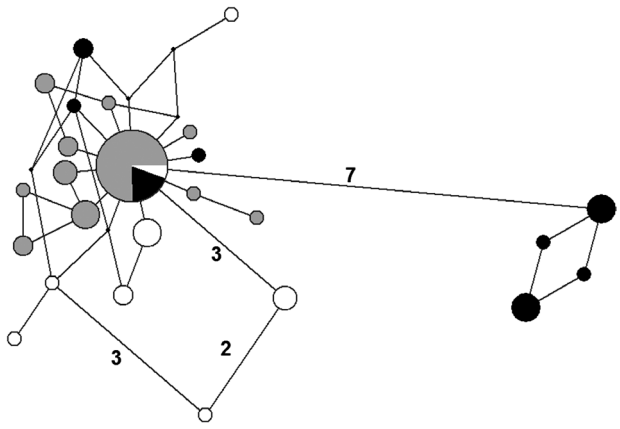

Figure 3. Median-joining network of mitochondrial DNA haplotypes identified in the studied individuals. Node size is proportional to the number of sequenced specimens, the numbers indicate number of point mutations between two linked haplotypes. White area – *D. mixta*, grey area – *D. dahli* and black area – *D. armeniaca*. The most common haplotype (centre of the network) is found in some of the studied *D. mixta*, the majority of *D. dahli* and some *D. armeniaca* individuals; other haplotypes found in the parthenogens are linked to this haplotype with one or two substitutions. The numbers at the links between the haplotypes or haplotypes and median vectors show the number of substitutions between them if more than one.

The distribution of microsatellite genotypes

Table S1 shows the distribution of genotypes at five studied loci. The genotypic diversity was much higher in sexual than in parthenogenetic species, as expected. However, parthenogenetic individuals also exhibited high level of variation at individual loci, both within and between the parthenogenetic forms. At Du232, 93% of *D. dahli* and 77% of *D. armeniaca* shared genotype 184/215 (numbers indicate allele size in bp). At Du47, 84% of *D. dahli* and 86% of *D. armeniaca* shared genotype 272/280. Simultaneously, at three other loci the most common genotypes were different for *D. dahli* and *D. armeniaca* (Table 1). Table 2 shows frequencies of common alleles found in parthenogens from three candidate paternal species of *Darevskia*. The most common alleles found in both *D. dahli* and *D. armeniaca* at loci Du47 and Du232 are also present in *D. mixta* and *D. portschinskii*, and less commonly in *D. valentini* (Table 2).

The commonest alleles identified at Du215 and Du323 in *D. dahli* were the same as the common alleles described by Vergun *et al.* (2014). About 62.7% of our *D. dahli* exhibit the most common genotype (228/244, 184/215), compared with 65.8% of Armenian samples showing this genotype (Vergun *et al.* 2014).

GENETIC DIVERSITY OF THE STUDIED SAMPLES

Analysis of microsatellite data revealed the presence of 3–8 alleles (5.8 on average) at each locus in the parthenogenetic individuals irrespective of species, and 6–14 alleles (10.3 on average) in sexual species (Table 3). The highest number of alleles (22 in total) was detected for Du215 and the lowest (13 in total) for locus Du47. Overall, the parthenogens showed lower allele numbers than sexuals, lower theta (*H*) values and relatively lower modified Garza–Williamson index values (Table 3). However, the differences were not significant ($P > 0.05$) for most of the parameters compared with *D. portschinskii*, *D. valentini* and *D. mixta*. The observed heterozygozity exceeded expected heterozygozity in *D. dahli* and (not significantly) in *D. armeniaca*, and was below expectation in *D. portschinskii* and *D. mixta* (Table 3).

STRUCTURE simulations are shown in Fig. 4. Of sexual species, *D. portschinskii* showed high assignment probabilities to cluster 3 (0.53 on average, Table S3), but also high assignment probabilities to cluster 1 (0.24 on average). *D. valentini* had high assignment probabilities to cluster 2 (0.84 on average), and *D. mixta* to cluster 1 (0.74 on average). Both parthenogenetic forms showed the highest assignment probabilities to the cluster associated with *D. mixta* (0.75 on average for *D. dahli* and 0.73 for *D. armeniaca*, the differences were insignificant). The assignment probabilities to cluster 3 (dominating in *D. portschinskii*) were 0.14 for *D. dahli* and 0.1 for *D. armeniaca* (differences significant, $P = 0.0005$). The assignment probabilities to cluster 2 (dominating in *D. valentini*) were 0.10 for *D. dahli* and 0.17 for *D. portschinskii* (differences significant, $P \sim 0.05$; see Fig. 4, Table S3).

Table 1. One to three most common genotypes (length of microsatellite alleles) at five studied microsatellite loci in *D. armeniaca* and *D. dahli*. *CA*, *CD* – the commonest genotypes of *D. armeniaca* and *D. dahli*, respectively

Locus	*CD*		*arm*	*dahli*	*CA*		*arm*	*dahli*
Du215	*228*	244	0	0.760	*206*	232	0.667	0.027
	232	244	0	0.146	*192*	236	0.233	0
Du418	*150*	158	0	0.280	*138*	138	0.600	0
	150	150	0.100	0.213	*150*	150	0.100	0.213
	146	158	0	0.120	*130*	138	0.100	0
Du47	***272***	**280**	**0.857**	**0.840**	***272***	**280**	**0.857**	**0.840**
	272	272	0	0.053	*272*	276	0.067	0.027
Du281-	*199*	199	0.033	0.827	*187*	187	0.867	0
Like	*203*	203		0.093	*195*	195	0.067	0
Du323	***184***	**215**	**0.767**	**0.933**	***184***	**215**	**0.767**	**0.933**

Note: Numbers indicate frequencies of the respective genotypes. Genotypes which dominate at individual loci in both unisexual forms are shown in bold. *D. armeniaca* (arm), *D. dahli* (dahli).

NETWORK OF PARTHENOGENETIC MICROSATELLITE GENOTYPES

The genotype networks for *D. dahli* and *D. armeniaca* are shown in Fig. 5. The total number of microsatellite genotypes, which differed from others at least at one mutation, was 37 in *D. dahli* and 18 in *D. armeniaca*. The most common genotype of *D. dahli* was present in 20% of the 70 individuals with fully scored genotypes, and the most common genotype of *D. armeniaca* was present in 30% of the individuals. In addition, 33% of *D. dahli* had genotypes differing from the most common one at a single locus/one mutation, and another 12.9% differed from the most common genotype at one out of five loci. Together with the most common genotype, they represented 66% of the individuals studied.

Table 2. The most common alleles (length of microsatellite alleles) at five studied microsatellite loci in *D. armeniaca* and *D. dahli*, and the frequencies of the respective alleles in these forms and their presumed sexual progenitors. *CA*, *CD* – the commonest alleles of *D. armeniaca* and *D. dahli*, respectively (length of alleles in bp)

Locus	*CA*	*arm*	*dahli*	*mixta*	*port*	*val*	*CD*	*arm*	*dahli*	*Mixta*	*port*	*val*
Du215	*206*	0.333	0.013	0.000	0.000	0.000	*228*	0.000	0.380	0.060	0.125	0.036
	232	0.350	0.087	0.160	0.125	0.000	*244*	0.000	0.487	0.220	0.224	0.000
Du418	*140*	0.700	0.053	0.160	0.072	0.063	*152*	0.100	0.429	0.080	0.217	0.125
	140	0.700	0.053	0.160	0.072	0.063	*160*	0.000	0.257	0.160	0.092	0.083
Du47	***272***	**0.417**	**0.500**	**0.120**	**0.128**	**0.019**	***272***	**0.417**	**0.500**	**0.120**	**0.128**	**0.019**
	280	**0.433**	**0.459**	**0.120**	**0.108**	**0.250**	***280***	**0.433**	**0.459**	**0.120**	**0.108**	**0.250**
Du281-like	*187*	0.867	0.040	0.333	0.129	0.036	*199*	0.033	0.827	0.104	0.106	0.232
	187	0.867	0.040	0.333	0.129	0.036	*199*	0.033	0.827	0.104	0.106	0.232
Du323	***184***	**0.400**	**0.480**	**0.500**	**0.013**	**0.000**	***184***	**0.400**	**0.480**	**0.500**	**0.013**	**0.000**
	215	**0.517**	**0.473**	**0.115**	**0.270**	**0.148**	***215***	**0.517**	**0.473**	**0.115**	**0.270**	**0.148**

Note: Numbers indicate frequencies of the respective alleles. Genotypes which dominate at individual loci in both unisexual forms are shown in bold. *D. armeniaca* (arm), *D. dahli* (dahli), *D. mixta* (mixta), *D. portschinskii* (port) and *D. valentini* (val).

Table 3. Measures of genetic diversity (mean and standard deviation) for five polymorphic microsatellite loci for two parthenogenetic forms (*D. dahli*, *D. armeniaca*) and three sexual species (*portschinskii*, *valentini*, *mixta*) of lizards

Variable	*D. dahli*	*D.armeniaca*	*D. portschinskii*	*D. valentini*	*D. mixta*
Ho					
Mean ± SD	0.71 ± 0.41	0.59 ± 0.46	0.74 ± 0.16	0.78 ± 0.13	0.66 ± 0.20
He					
Mean ± SD	0.55 ± 0.15	0.54 ± 0.19	0.85 ± 0.03	0.78 ± 0.08	0.82 ± 0.09
Theta (H)					
Mean	1.52	1.51	3.46	2.41	2.87
AN					
Mean	6.0	5.6	12.4	8.4	10.0
GW					
Mean ± SD	0.26 ± 0.07	0.18 ± 0.09	0.28 ± 0.07	0.21 ± 0.04	0.20 ± 0.04
GWM					
Mean ± SD	0.11 ± 0.06	0.10 ± 0.05	0.20 ± 0.04	0.15 ± 0.05	0.16 ± 0.04

Note: Ho – observed heterozygozity; He – expected heterozygozity; Theta (H) – theta for microsatellite data, assuming stepwise mutation model (Ohta & Kimura, 1971); AN – allele number; GW – Garza–Williamson Index; GWM – modified Garza–Williamson Index.

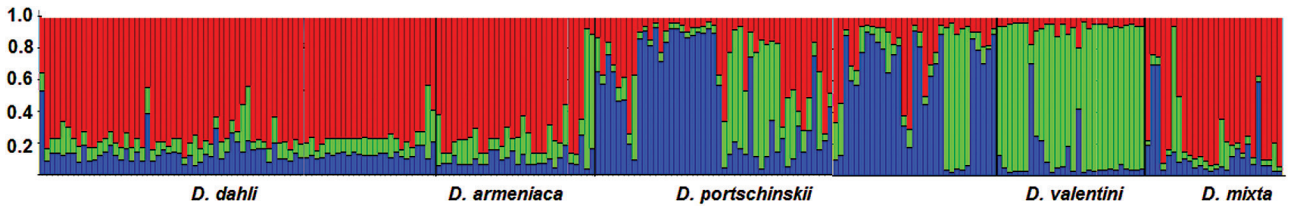

Figure 4. The output of STRUCTURE simulations. Different colours indicate assignment probabilities for each individual to one of seven inferred clusters. Allele frequencies analysed using sexually breeding species only. The analysis failed to cluster all individuals of *D. portschinskii*, but cluster 3 (shown in blue) is more typical for this species.

There was a single individual with a highly deviant genotype, which differed at five loci from the most common genotype. STRUCTURE clustered this individual with *D. valentini* rather than with other *D. dahli*. For *D. armeniaca*, 30% differed from the most common genotype at a single locus, hence together with the individuals with the commonest genotype they represented 60% of the individuals. There were two other individuals with highly deviant genotypes, which differed at five loci from the most common genotype. STRUCTURE clustered these individuals with *D. mixta* rather than with other *D. armeniaca* or *D. dahli* (Fig. 4).

DISCUSSION

Our microsatellite data suggest relatively high genotypic diversity in both parthenogenetic forms, although less than in sexually breeding *Darevskia*. This can in part be attributed to their hybrid origin, as has been proposed by other authors (McCullogh et al., 1995; Fu et al., 1999, 2000; Vergun *et al.*, 2014), and/or mutation or recombination as suggested by Murphy *et al.* (1997). However, the most common genotypes at two loci, which coincide in *D. dahli* and *D. armeniaca*, might also indicate that occasional interbreeding of a single initial parthenogenetic lineage with sexual species has been an important force of genetic diversification within parthenogens, and even a mechanism of 'speciation', to an extent to which this term can be applied to parthenogenetic forms.

Three different biological processes could cause the observed genetic diversity within *D. dahli* and *D. armeniaca*: (1) independent hybridization events between *D. mixta* and *D. portschinskii*, or *D. mixta* and *D. valentini* (i.e. multiclonality); (2) new mutations or recombination as a part of an automictic process and (3) backcrosses with males of parental species or their close relatives.

If we reduce the number of studied loci to those three described in Vergun *et al.* (2014), the number of genotypes in *D. dahli* would reduce from 37 to 18. This is still a higher diversity than in the Armenian populations. However, the majority of genotypes are separated from each other by one or a few mutations and hence a large part of the measured genetic variation

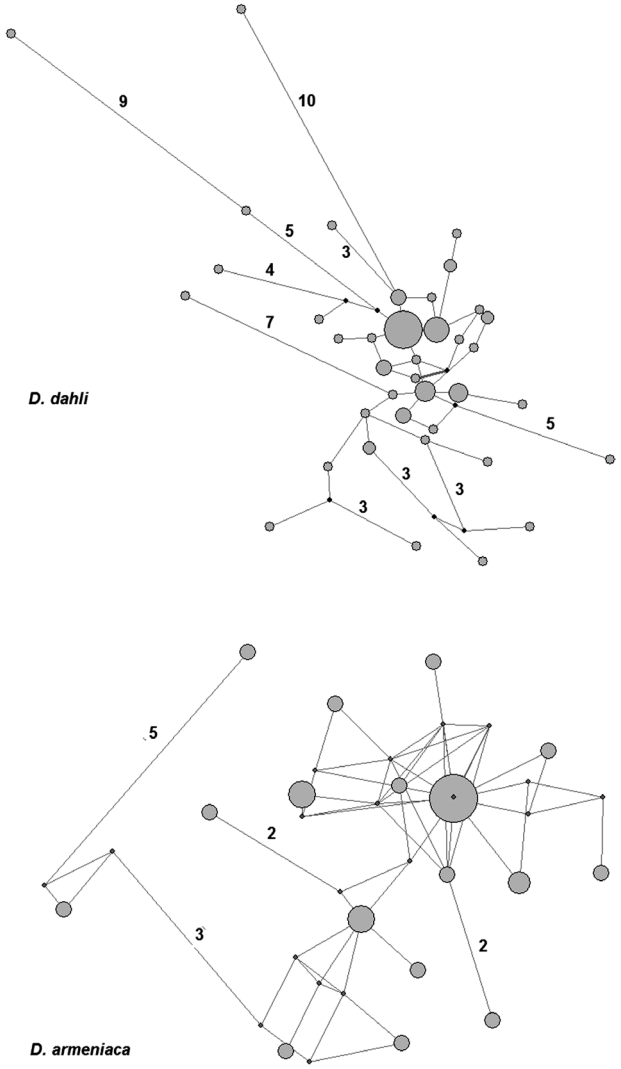

Figure 5. Network of microsatellite genotypes of *D. dahli* and *D. armeniaca*. Numbers indicate the number of microsatellite repeats distinguishing two genotypes (based on all four studied loci; no numbers are shown if two genotypes are differing by a single mutation). Size of circle is proportional to a genotype frequency.

can easily be attributed to mutation processes within a clone. Korchagin et al. (2007, 2013) suggest that the mutation rates (change of the number of repeats) at the described microsatellite loci are high. Vergun *et al.* (2014) attributed most of the observed diversity within *D. dahli* from five Armenian locations (9 out of 11 identified genotypes) to mutations within the most common parthenogenetic clone, and only two individuals out of 111 studied ones were suspected to represent different clones. Higher genetic diversity was described for *D. armeniaca*, and a multiclonal origin for this species was suggested (McCullogh et al., 1995).

On the other hand, identical genotypes of *D. dahli* and *D. armeniaca* at loci Du47 and Du323 cannot be explained by multiple origins, or mutation processes within the clones. These loci are highly polymorphic in *D. mixta*, *D. portschinskii* and *D. valentini*. Consequently, there is a minute probability that two parthenogenetic hybrids with coincident, heterozygotic genotypes at two unlinked loci emerged as a result of two or more hybridization events, or in hybridization events in more than one geographic area.

We also discount the possibility of genetic divergence of a single founding parthenogenetic clone into two distinct lizard forms, *D. dahli* and *D. armeniaca*. Leaving aside major morphological and ecological differences between these forms, the differences at three microsatellite loci (Du215, Du418 and Du281-like) between the dominating genotypes of *D. dahli* and *D. armeniaca* cannot be attributed, with any countable probability, solely to spontaneous mutations or recombination (which would reduce heterozygosity levels and explain homozygotic genotypes at some loci in the parthenogens). Therefore, we suggest that the most plausible explanation of these coincident genotypes in *D. dahli* and *D. armeniaca* is the mating of the initial parthenogenetic form with males of *D. valentini*. *D. valentini* has a current distribution that is separated from *D. mixta* by approximately 30 km, while *D. portschinskii* is parapatric with *D. mixta*. If *D. valentini* and *D. mixta* are indeed the progenitor species, hybridization would require some historical range overlap between these species, which is unlikely because the intervening terrain is unsuitable habitat, followed by range contraction to their current, more restricted distributions. Here, we suggest a more plausible hypothesis that includes (1) an initial switch to parthenogenesis as a result of hybridization between *D. mixta* and *D. portschinskii* (whose populations have limited geographic contact); (2) expansion of the parthenogens (*D. dahli*) into the range of *D. valentini* and (3) formation of parthenogenetic *D. armeniaca* as a result of a series of crosses between *D. valentini* and *D. dahli*, rather than between *D. valentini* and *D. mixta*.

There are documented hybrids between *D. dahli* and *D. portschinskii* (Darevsky & Kulikova, 1961; Davoyan, 2007), which are triploid and suggested to be sterile (Davoyan, 2007). Hybrids between *D. valentini* and *D. armeniaca* are also commonly reported and their ploidy can vary (Darevsky & Danielyan, 1968, 2001; Darevsky, Kupriyanova & Uzell, 1985; Darevsky, 1995; Danielyan, 2003 Danielyan FD. 2003. Natural hybridization of rock lizards of genus *Darevskia*. In: Study and protection of animals of South Caucasus, p. 60–61. Gasparyan G, Ed., Yerevan (in Russian)). Although early authors suggested sterility of the hybrids, Danielyan *et al.* (2008) showed that the crosses between *D. valentini* and *D. armeniaca* can produce fertile individuals of both sexes, a fact supported by the presence of mature female and male gonads.

Diploid, triploid and tetraploid hybrids were also identified in the studied populations. Cole *et al.* (2010) demonstrated the presence of multiple triploid parthenogenetic clones in *Aspidoscelis* descending from hybridization between parthenogenetic diploids and sexual species; hence, the pattern may be common in systems including coexisting parthenogens and their sexually breeding ancestors. In conclusion, occasional development of fertile triploid hybrids, as a result of hybridization between parthenogenetic *Darevskia* and males of *D. valentini*, is highly probable. Figure 6 shows a hypothetical scenario of hybridization events that can transform diploid parthenogenetic *D. dahli*, through two consequential hybridizations with *D. valentini*, or crosses between first generation hybrids, into morphologically, ecologically and genetically distinct forms, sharing genotypes with *D. dahli* at individual loci. The scheme includes (1) hybridization of male *D. valentini* with *D. dahli*, causing the development of triploid offspring of one or both sexes and (2) meiotic reduction of ploidy by a triploid individual, producing diploid eggs that can develop without fertilization (Fig. 6). Our hypothesis suggests that parthenogenetic *D. dahli*, and not sexual *D. mixta*, is the most likely maternal progenitor of *D. armeniaca*. Similar genetic origins are hypothesized for *D. uzzelli* and *D. unisexualis* as an alternative to independent origins of these forms as a result of independent hybridization events (Freitas *et al.*, 2016a), although the authors retain both hypotheses as equally plausible.

Our hypothesis posits expansion of *D. dahli* southwards from the original hybrid zone between *D. portschinskii* and *D. mixta*, followed by a crossing with *D. valentini* at the southern range of the expansion. This was possible because *D. dahli* can survive in habitats which are inhospitable to the parental species. *D. dahli* is very successful ecologically, at least under post-glacial climatic conditions, and tends to outcompete its patrilineal ancestor *D. portschinskii* in areas of range overlap (Tarkhnishvili *et al.*, 2010). The parthenogen perhaps realizes advantages of heterosis, which is a likely explanation of the 'geographic parthenogenesis' phenomenon (Glesener & Tilman, 1978; Kearney, Wahl & Autumn, 2004; Vrijenhoek & Parker, 2009).

This backcrossing hypothesis also potentially explains the presence of some deviant genotypes within both *D. dahli* and *D. armeniaca*, which otherwise must invoke multiple hybridization events. The competing hypothesis of polyclonal ancestry is based on the presence of single individuals, which descend from the different hybrid parthenogens from the original hybridization area. If this hypothesis were true, one should expect that all or almost all microsatellite loci would have different genotypes in different clones – coincidence of genotypes, as seen in *D. dahli* and *D. armeniaca*, is unlikely. Supposed representatives of different clones in Virkun et al. (2014) coincide with the common clone at two out of three studied loci, and a similar pattern as presented in our data. Hence, we suggest that backcrosses/hybridization with sexual *Darevskia* rather than multiple hybridization events between *D. mixta* and patrilineal ancestors of the parthenogens best explains the presence of strongly diverged genotypes in *D. dahli* and *D. armeniaca* at some loci, but not in others.

Our data suggest two important evolutionary consequences. First, there is the frequency of hybridization between sexual lizards that causes parthenogenesis. Jančúchová-Lásková et al. (2015a, 2015b) showed that introgressive hybridization is common among distinct species of lizards; however, parthenogenesis is a rare result of hybridization, and occurs only if hybridizing species are genetically distant. Danielyan (1981

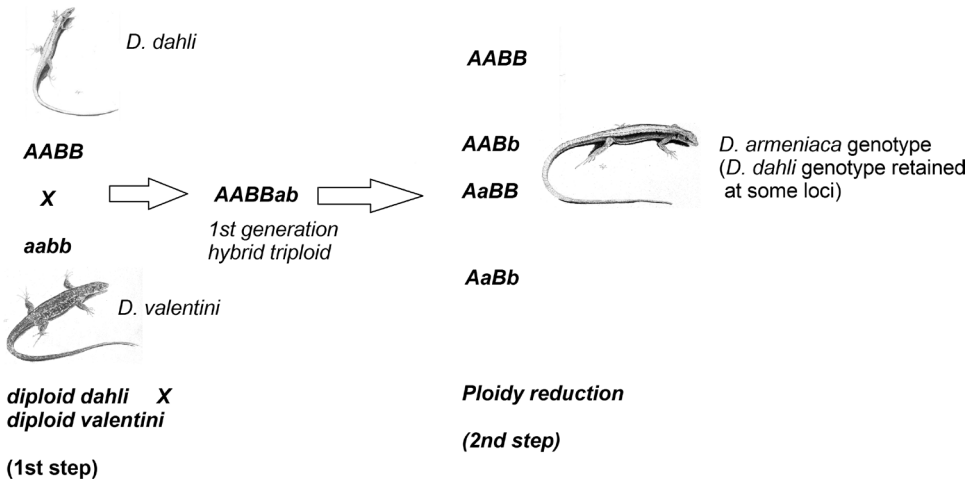

Figure 6. Pattern of hybridization between *D. dahli* with males of *D. valentini*, which results in the development of diploid parthenogen *D. armeniaca*. First step: triploid hybrids develop, with an additional chromosome inherited from the paternal individual; Second step: meiotic reduction of ploidy by a triploid individual, producing diploid eggs that can develop without fertilization.

Danielyan F. 1981. Study of relationships between bisexual and unisexual species of rock lizards using skin grafts and natural hybridization, pp. 46–47 in Abs. Fifth Herpetol. Conference: The Problems of Herpetology. 22–24 Sept., Ashgabat. Zoological Institute Academy of Sciences USSR, Leningrad [in Russian].) and Murphy *et al.* (1997) showed that even hybridization between the presumed ancestral species of the parthenogenetic lineages does not necessarily result in parthenogenesis. Consequently, substantial changes in genome structure should coincide with the hybridization event. We suggest that at least those parthenogenetic *Darevskia*, which descend matrilineally from *D. mixta*, may descend from a sole initial hybridization event, and further genetic diversification is caused by both post-hybridization mutations *and* gene introgression from paternal species. Since parthenogens can be ecologically successful (Vrijenhoek & Parker, 2009; Tarkhnishvili *et al.*, 2010; Freitas *et al.*, 2016a), their rarity in nature can be explained solely by the infrequency of novel parthenogenesis. This is in line with computer simulations of Janko *et al.* (2008), which showed that the relatively young age of parthenogenetic clones does not necessarily entail inferior viability in a long-term perspective, and may instead be a result of neutral turnover of successive clones.

This might appear to be at odds with a recent review (Lampert, 2008), suggesting that facultative parthenogenesis is relatively common in reptiles, and probably much more common than one might judge based on the recorded cases. Moreover, facultative parthenogenesis may be not just a rare occasion, but rather a reproductive strategy in some squamates, periodically switching between parthenogenetic and sexual reproduction. It was recently shown that two species of American pit vipers (cooperhead *Agkistrodon contotrix* and rattlesnake *Crotalus adamanteus*), whose females are able to store sperm for months or years, are in fact switching between parthenogenetic and sexual reproduction (Booth & Schuett, 2011). However, no evidence is available so far that facultative parthenogenesis, not including distant hybridization events, produces long-lasting unisexual forms. In contrast, parthenogenetic forms of hybrid origin are probably very rare (or rarely are viable), but if developed and fit to a habitat, they may proliferate for many generations without sex.

The second consequence of this study is that occasional backcrosses revise the point of view that hybrid parthenogenetic lizards represent an evolutionary dead end and are destined for extinction within a relatively short time (Ghiselin, 1974; Kondrashov, 1988; Stearns, 1988; Bell, 2008; but see Birky *et al.*, 2005). Hybrid parthenogens, if they happen to be viable and fertile, may rapidly increase population size (Bell, 1982, 2008; Meirmans et al., 2012), trigger fast range expansion (Tarkhnishvili *et al.*, 2010) and occupation of areas unsuitable for either of the parental species (Roughgarden, 1972; Glesener & Tilman, 1978; Kearney, Wahl & Autumn, 2004). However, they are thought to eventually lose in competition with sexually reproducing forms, because of lack of recombination and, hence, inferior genetic diversity essential for successful adaptation to changing conditions (Fisher, 1930; Ghiselin, 1974; Maynard Smith, 1978, 1998; Kondrashov, 1988; Cooper, Lenski & Elena, 2005; Song et al., 2011). The arguments for successful backcross pattern change the perspective of parthenogenetic lizards completely. Evidence of backcrosses producing new parthenogenetic forms suggests that this mechanism can be as successful as well-known examples of cyclical parthenogenesis, provided by invertebrate animals (Mittwoch, 1978; DeMeester, Gómez & Simon, 2004). In this case, the rarity of obligatory parthenogens in reptiles can be explained solely by the rarity of hybridization between sexual species of lizards resulting in viable unisexual individuals.

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SUPPORTING INFORMATION

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

Table S1. Sample locations (geographic coordinates indicated) and number of individuals of each species/location studied for microsatellite genotypes (STR) and mitochondrial haplotypes (mtDNA).
Table S2. The full description of the scored genotypes (alleles coded as the number of microsatellite repeats) for each of the individuals included. (1-30) *Darevskia armeniaca*; (31-75) *D. dahli*; (76-151) *D. portchinskii*; (152-180) *D. valentini*; (181-205) *D. mixta*. The most common parthenogenetic genotypes (both unisexual forms considered) are marked in green, the second most common genotype in blue.
Table S3. Assignment probabilities of five species to three clusters, as a result of STRUCTURE analysis. POPFLAG=1 is assigned to sexual species and POPFLAG=0 to asexuals, therewith assigning parthenogens as "migrants" and without population number prior (USERPOPINFO model). The number of K was set as 3, the same as the number of the anticipated parental sexual species included into the analysis.