Acta Herpetologica 18(2): 147-158, 2023
DOI: 10.36253/a_h-14756

Molecular genetic characteristics of *Darevskia portschinskii* lizard populations based on microsatellite markers analysis

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Submitted on: 2023, 26th May; revised on: 2023, 24th August, 2023; accepted on: 2023, 21st October
Editor: Andrea Villa

Abstract. The Caucasian rock lizard species *Darevskia portschinskii* is one of the bisexual species participating in interspecific hybridisation as the paternal ancestor with the maternal ancestors *D. mixta* and *D. raddei* resulting in the successful formation of the parthenogenetic *D. dahli* and *D. rostombekowi*, respectively. Populations of *D. portschinskii* have been previously divided into two subspecies, *D. p. portschinskii* and *D. p. nigrita* according to their geographical distribution and the morphological data, but they have not been characterised genetically. Here, we used ten microsatellite markers to determine the genetic structure of the *D. portschinskii* populations. The utility of the developed microsatellite markers for investigating the genetic variability within and among populations with a heterogeneous spatial distribution was demonstrated. Our results showed that the intra- and interspecific differentiation of the studied populations were consistent with the morphological data on the subspecies status of the *D. p. portschinskii* and *D. p. nigrita* populations. A potential applicability of the developed microsatellite markers to study genetic diversity of *Darevskia* species and subspecies complexes is suggested.

Keywords. *Darevskia* lizards, *Darevskia portschinskii*, taxonomy and population genetics, microsatellite loci, genetic polymorphism, genetic differentiation.

INTRODUCTION

The Caucasian rock lizard *Darevskia portschinskii* was described for the first time by K.F. Kessler in 1878 based on samples from the Tbilisi region (Kessler, 1878). The main range of this species is concentrated along the right bank of the Kura River valley and the ravines of its right tributaries within Georgia, northern Armenia, and northwestern Azerbaijan at altitudes of 300-1700 meters above sea level. There is a large, isolated population in the valley of the middle part of the Iori River (left tributary of the Kura River) on the southern slopes of the Tsivi-Gombori mountain range, as well as in the ravine of the Akera River (part of the Araks River basin). The species inhabits relatively arid zones along the banks of rivers and mountain slopes with xerophytic shrubs and herbaceous vegetation (Darevsky, 1967).

The increased interest in the study of *D. portschinskii* populations is due to the fact that this species acts as a paternal taxon in interspecific hybridisation with *D. mixta* (maternal species), resulting in the formation of unisexual (parthenogenetic) *D. dahli* (Uzzel and Darevsky, 1975), and is one of the examples of sympatric speciation in rock lizards of the genus *Darevskia* within the framework of the theory of reticulate or reticular evolution (Dobzhansky, 1937; Borkin and Darevskiy, 1980). Furthermore, in part of its range, *D. portschinskii* is sympatric with the parthenogenetic *D. dahli*, and in such

ISSN 1827-9635 (print)
ISSN 1827-9643 (online)

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zones there is the possibility of interspecific hybridisation and the appearance of triploid hybrids (Darevsky, 1967), whose evolutionary potential as participants in new stages of reticular evolution has not yet been established. However, unlike other studies on triploid hybrids between parthenogenetic and sexual species (Danielyan et al., 2008; Freitas et al., 2019; Freitas et al., 2022), the ongoing processes of hybridisation and triploid hybrids from sympatry sites between *D. dahli* and *D. portschinskii* are less known and only few information about hybrid zone from previous years are available (Darevsky, 1967; Petrosyan et al., 2020a).

Darevskia lizards, including *D. portschinskii*, show strong heterogeneity in their geographical distribution with many isolated populations due to the high topographic and climatic variability of the Caucasus region (Petrosyan et al., 2020b). According to morphological analysis and geographical distribution, *D. portschinskii* lizards were divided by Bakradze (1976) into two subspecies, *D. p. portschinskii* and *D. p. nigrita*. *D. p. nigrita* (originally designated *Lacerta portschinskii nigrita*) was found in the gorge of the river Mashavera (Georgia), as well as in the gorge of the middle reaches of the river Dzoraget (Armenia, outskirts of the city of Stepanavan). Populations of *D. p. nigrita* subspecies live at an altitude of 1400 - 1500 m, whereas other populations have been found at lower altitudes. Lizards of the *D. p. nigrita* subspecies have a much darker colorations than those of the *D. p. portschinskii* subspecies, with two distinct longitudinal rows of pale ocelli running along the upper edge of the temporal stripes. In addition, they are characterised by a brighter orange-yellow in vivo colouration of the underside of the body of males. *D. p. portschinskii* lizards are characterised by gray, grayish-beige or brownish colouration above, and longitudinal rows of light ocelli along the edges of the temporal stripes are absent or weakly expressed.

Thus, the classification of subspecies of *D. portschinskii* populations was based solely on morphological and geographic data. At the same time, genetic studies on this problem have not yet been carried out. Therefore, the main objective of this study is the molecular genetic characterisation of *D. p. portschinskii* and *D. p. nigrita* populations based on the analysis of the variability of microsatellite markers and obtaining interrelated estimates of the phenetic and genetic classification of these populations. To solve this problem, ten di- and trinucleotide microsatellite loci were searched in and selected from the genome assembly of *D. valentini* (Ochkalova et al., 2022), and their orthologs were also found in the draft genomic assemblies of *D. unisexualis* and *D. raddei*. Based on these loci, a PCR analysis system was developed for the molecular genetic characterisation of *Darevskia* species, including *D. portschinskii*. As an outgroup for comparison, *D. valentini* lizards with a firmly established species status, belonging to the same 'rudis' clade as *D. portschinskii* (Arribas, 1999; Rato et al., 2021), were taken.

MATERIALS AND METHODS

Sample collection

Tail fragments of 43 *D. portschinskii* lizards were collected in three main areas: in the vicinity of the villages of Zuar and Marts, and in the Dzoraget region (Fig. 1 and Table 1). After sampling the biomaterial, the lizards were released into their habitats. Total genomic DNA were isolated by the standard phenol-chloroform method using proteinase K. As an outer group we used eight previously obtained DNA samples of *D. valentini* lizards from the populations of Lchashen, Tezh, and Adis (Armenia, 40°51'N, 44°90'E; 40°70'N, 44°61'E; 40°30'N, 44°73'E, respectively).

Developing of the microsatellite markers

The search for microsatellite loci in the *D. valentini* genome (Ochkalova et al., 2022) was carried out using a pipeline that included several programs and Python scripts. First, we searched for microsatellite repeats with a given monomer length of two and three nucleotides and with a number of at least ten repeats in the genome of *D.*

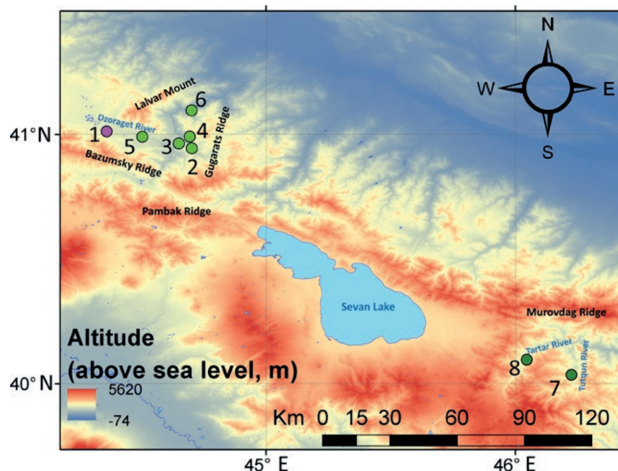

Fig. 1. Sampling localities of *D. portschinskii* used in the study, as reported in Table 1. The licensed ArcGIS Desktop 10.4.1 program (http://desktop.arcgis.com) was used to create the map.

Table 1. Samples of *D. portschinskii* used in the present study. The geographic localities are shown in Fig. 1.

Species/ Subspecies	Population (n)	Locality	Map locality	Sample size	Coordinates	
D. p. nigrita	"Dzoraget" (12)	Dzoraget	1	12	40°95'N	44°70'E
D. p. portschinskii	"Marts" (14)	Marts	2	10	40°95'N	44°70'E
		Dzekh	3	1	40°96'N	44°65'E
		Karinj	4	1	40°97'N	44°69'E
		Karmir Ageg	5	1	40°98'N	44°55'E
		Zarni Monastery (Haghpat)	6	1	41°09'N	44°71'E
	"Zuar" (17)	Zuar	7	16	40°04'N	46°23'E
		Karvachar	8	1	40°11'N	46°04'E

valentini using the MISA perl script (Thiel et al., 2003). Then, using BedTools v2.30.0 (Quinlan and Hall, 2010), all sequences that met the following criteria were selected: 1) the size of the sequences adjacent to the 5' and 3' ends of the microsatellite is at least 100 bp, 2) the microsatellite and flanking regions do not contain N, and 3) the flanking regions do not contain repeats and sequences that hinder primer hybridisation. A similar search found orthologous loci in the draft genome assemblies of *D. unisexualis* and *D. raddei*. For the selection of PCR primers, the Primer3 program was used (Untergasser et al., 2012; Koressaar and Remm, 2007) at a given length of the desired product, including a microsatellite repeat of at least 100 bp, a distance from the primers to the microsatellite of at least 10 bp, a primer length of 20–25 bp, and a melting point of 58-62 °C. The most suitable primer pairs were tested for the uniqueness of the amplified product by local *in silico* search in the *D. valentini* genome using BLAST+ 2.12.0 (Camacho et al., 2009). Ten microsatellite loci were selected for molecular genetic analysis (supplementary Table S1).

Amplification procedure.

PCR was carried out in a volume of 20 μl per 50 ng of DNA using the GenPakPCRCore kit (Isogene Laboratory) according to the manufacturer's protocol under the following temperature conditions: denaturation at 94 °C - 3 minutes, amplification for 30 cycles (denaturation: 94 °C - 1 minute, annealing: 60 °C - 40 seconds, elongation: 72 °C - 30 seconds), last cycle - 5 minutes at 72 °C. The concentration of each primer was 0.2 μM. One of the primers for amplifying individual loci was labelled with a fluorescent dye at the 5' end. Table S1 shows the characteristics of the loci and primers used in the work. The PCR amplification products were fractionated in 0.8% agarose gel followed by DNA fragment isolation using the GeneJET Gel Extraction Kit (ThermoScientific).

Fragment analysis.

Fragment analysis was performed at the Synthol company using the NANOFOR-05 genetic analyser. The size of the amplified fragments was determined using the Peak Scanner v1.0 software (Applied Biosystems). Each amplification product was considered as a biallelic locus, heterozygous in the presence of two fragments of varied sizes and homozygous if a single fragment was detected.

Genetic analysis.

Number of alleles/genotypes observed, Simpson's index (1-D), and alleles distribution (Evenness) for all microsatellite loci were estimated using *poppr* (Kamvar et al., 2014) package. For analysis of allelic richness (A_R), observed and expected heterozygosity (H_{obs}, H_{exp}), we used the *hierfstat* (Goudet, 2005). The deviation from Hardy-Weinberg equilibrium for each locus was tested with allele randomizations (1000 permutations per test) with the package *pegas* (Paradis, 2010). The degree of genetic differentiation between samples was determined by calculating F_{ST} (Nei, 1987) and a modified version of Hedrick's G_{ST} (Hedrick, 2005; Meirmans and Hedrick, 2011) using *mmod* package (Winter, 2012), taking into account the tendency of the standard G_{ST} test to underestimate the degree of differentiation between a small number of populations. An UPGMA tree based on the matrix of Nei (1972) pairwise genetic distances (implemented in *poppr*) was used to visualise the relationships among all individuals and populations studied. Support for each node was tested by 1000 bootstrap replicates. The association index used to assess multilocus linkage disequilibrium was determined using the *poppr* package (Kamvar et al., 2014).

Two approaches have been used to infer clusters or subpopulations from a sample by genetic mixture analysis. Firstly, to determine the population structure of *D. portschinskii*, we used discriminant principal component

analysis (DAPC) (Jombart et al, 2010) implemented in the *adegenet* package (Jombart 2008; Jombart 2011). A sample of *D. valentini* was added as an outgroup, while the analysis was carried out without taking into account the a priori population structure of *D. portschinskii* known by geographic origin. Before the DAPC analysis, we checked all loci and found 214 missing values in the total sample (Fig. S1). The sc7287 and sc1872 loci contained more than 5% missing values and were corrected. To avoid bias, the missing data were replaced by mean allele frequencies. The optimal number of genetic clusters in populations obtained by the k-means algorithm was determined using the Bayesian information criterion. The probability of the relationship of specific individuals to genetic groups was calculated.

An additional analysis of the genetic structure of the species *D. portschinskii* was carried out using the Bayesian approach implemented in the STRUCTURE 2.3.4 program (Pritchard et al., 2000). Taking into account the probability of the origin of populations from a common ancestor, we used the admixture model (allele frequencies correlated among populations), which does not exclude the possibility of crossing. The posterior probabilities were estimated using a Markov chain Monte Carlo method (MCMC) based on 1,000,000 iterations of this chain, following a burn-in period of 200,000 iterations. Since the sample of *D. portschinskii* comes from eight collection points (Fig. 1, Table 1) combined into three known populations ("Marts", "Zuar", and "Dzoraget"), we conditioned our data on different values of K ranging from 1 to 8 initially containing the optimal value, but the calculations did not include information on the population structure (option LOCPRIOR=0). Ten runs were performed for each K to test the stability of the results. The best value of K that captured the main structure in the data was determined by the ΔK value estimation method according to Evanno et al. (2005) using the web-based program Structure Harvester (version 0.6.8) (Earl and vonHoldt, 2012). CLUMPAK (Kopelman et al., 2015) with defaults parameters (LargeKGreedy algorithm, random input order and 2000 replicates) was used for merging the replicate runs, and graphical representation of the results obtained by STRUCTURE.

RESULTS

Molecular genetic characteristics of microsatellite loci

Comparative data on the analysis of allelic polymorphism on 10 microsatellite loci, expected heterozygosity (H_{exp}), distribution of alleles (Evenness), and the Simpson diversity index (1–D) for the loci used, obtained on the samples of *D. portschinskii* and *D. valentini*, are presented in Table 2. It can be seen that the loci differ in a wide range in the number of alleles and genotypes in these samples. The average values of the above indicators allow us to use these loci to assess the intraspecific polymorphism of these species. The smallest number of alleles, as well as the smallest values of the Simpson index (1–D) and expected heterozygosity (H_{exp}) were obtained at the sc10877 and sc12962 loci. The alleles of the microsatellite loci form distinct genotypes, the number of which varies depending on the locus and the subspecies (Table 2).

Genetic polymorphism in populations of D. portschinskii

Darevskia portschinskii portschinskii is characterised by a larger number of alleles and genotypes compared

Table 2. Genetic variability of the loci used in a mixed sample of *D. portschinskii* and *D. valentini* individuals. 1-D - Simpson index, H_{exp} - expected heterozygosity (Nei's, 1978, gene diversity), Evenness - distribution, A_R - allelic richness.

Locus	Number of alleles	Number of genotypes	1-D	H_{exp}	H_{obs}	Evenness	A_R
sc12962	4	5	0.2609	0.3034	0.30	0.53	3.9785
sc4525	14	15	0.3830	0.8060	0.79	0.68	13.2006
sc138	9	14	0.5417	0.7737	0.76	0.72	8.8333
sc149	12	18	0.4348	0.8408	0.83	0.72	11.7690
sc12560	14	24	0.7234	0.8802	0.87	0.80	13.4590
sc7287	13	22	0.5854	0.8277	0.81	0.69	13.0000
sc1872	10	10	0.3095	0.4640	0.46	0.43	9.8804
sc4045	11	16	0.3750	0.7535	0.74	0.62	10.6804
sc10877	3	3	0.0638	0.2969	0.29	0.58	2.9984
sc6476	24	31	0.7083	0.9102	0.90	0.67	22.5750
Overall	11.40 ± 5.85	15.50 ± 8.56	0.44 ± 0.21	0.69 ± 0.24	0.68 ± 0.23	0.64 ± 0.11	11.04 ± 5.46

Table 3. Genetic characteristics of the loci used in the three analysed samples. 1-D - Simpson index; H_{exp} and H_{obs} - expected and observed heterozygosity (Nei's, 1978, gene diversity); Evenness - distribution, A_R - allelic diversity.

Samples	Locus	Number of alleles	Number of genotypes	H_{obs}	H_{exp}	1-D	Evenness	A_R
D. valentini	sc12962	2	2	0.2500	0.2321	0.22	0.61	1.9917
	sc4525	10	7	1.0000	0.9286	0.88	0.88	9.2333
	sc138	4	4	0.0000	0.8214	0.72	0.93	3.9917
	sc149	7	6	0.6250	0.8839	0.81	0.87	6.6167
	sc12560	1	1	0.0000	0.0000	0.00	0.00	1.0000
	sc7287	6	5	1.0000	0.7679	0.73	0.78	5.6167
	sc1872	6	6	0.8570	0.6667	0.63	0.60	6.0000
	sc4045	7	8	0.7500	0.8661	0.80	0.84	6.6167
	sc10877	2	2	0.3750	0.3214	0.30	0.71	2.0000
	sc6476	12	8	1.0000	0.9554	0.90	0.89	10.8583
	Overall	5.7±3.56	4.9±2.56	0.58±0.40	0.64±0.34	0.60±0.31	0.79±0.12	5.39±3.2
D. portschinskii	sc12962	3	4	0.2778	0.2117	0.31	0.60	2.2669
	sc4525	4	8	0.2083	0.2222	0.70	0.92	3.7402
	sc138	8	12	0.6319	0.6349	0.76	0.72	5.3348
	sc149	9	13	0.3889	0.5082	0.78	0.72	5.7252
	sc12560	13	23	0.8699	0.8344	0.85	0.77	7.0208
	sc7287	10	17	0.4806	0.7845	0.77	0.67	5.7787
	sc1872	4	4	0.2235	0.2520	0.23	0.47	2.3147
	sc4045	7	9	0.2917	0.4236	0.65	0.69	4.9570
	sc10877	1	1	0.0000	0.0000	0.00	0.00	1.0000
	sc6476	16	23	0.6528	0.7896	0.86	0.73	7.4568
	Overall	7.5±4.7	11.4±7.7	0.4±0.26	0.47±0.29	0.59±0.30	0.7±0.12	4.56±2.15
D. p. nigrita	sc12962	2	3	0,8333	0.5076	0.50	1.00	2.0000
	sc4525	1	1	0.0000	0.0000	0.00	0.00	1.0000
	sc138	3	3	0.7500	0.5076	0.50	0.82	2.833
	sc149	1	1	0.0000	0.0000	0.00	0.00	1.0000
	sc12560	7	9	0.9167	0.7992	0.77	0.78	6.6230
	sc7287	6	9	0.6000	0.8556	0.80	0.92	6.0000
	sc1872	2	2	0.5455	0.4091	0.40	0.83	2.0000
	sc4045	1	1	0.0000	0.0000	0.00	0.00	1.0000
	sc10877	1	1	0.0000	0.0000	0.00	0.00	1.0000
	sc6476	6	9	0.5833	0.7538	0.72	0.73	5.7880
	overall	3±2.4	3.9±3.6	0.42±0.38	0.38±0.36	0.37±0.34	0.85±0.1	2,92±2.31
D. p. portschinskii	sc12962	2	2	0.0000	0.0638	0.07	0.45	1.9887
	sc4525	4	7	0.3125	0.3329	0.65	0.82	4.0000
	sc138	8	11	0.5729	0.6975	0.81	0.82	7.8159
	sc149	9	12	0.5833	0.7612	0.83	0.81	8.8619
	sc12560	11	17	0.8466	0.8518	0.85	0.82	10.4047
	sc7287	9	12	0.4208	0.7490	0.71	0.60	9.0000
	sc1872	3	3	0.0625	0.1735	0.12	0.43	2.9574
	sc4045	7	9	0.4375	0.6339	0.72	0.75	6.7587
	sc10877	1	1	0.0000	0.0000	0.00	0.00	1.0000
	sc6476	14	16	0.6875	0.8075	0.86	0.72	12.8615
	Overall	6.8±4.2	9±5.7	0.39±0.30	0.51±0.33	0.56±0.35	0.69±0.16	6.56±3.92
D. p. portschinskii ("Marts")	sc12962	1	1	0.0000	0.0000	0.00	0.00	1.0000
	sc4525	1	1	0.0000	0.0000	0.00	0.00	1.0000
	sc138	7	7	0.3333	0.7424	0.72	0.64	5.8643

(Continued)

Table 3. (Continued).

Samples	Locus	Number of alleles	Number of genotypes	H_{obs}	H_{exp}	1-D	Evenness	A_R
	sc149	7	8	0.6667	0.8750	0.87	0.92	6.5059
	sc12560	9	10	0.8182	0.8864	0.88	0.84	7.8177
	sc7287	7	7	0.3750	0.9018	0.87	0.85	7.0000
	sc1872	3	3	0.1250	0.3571	0.34	0.57	3.0000
	sc4045	5	4	0.5000	0.6402	0.63	0.66	4.5310
	sc10877	1	1	0.0000	0.0000	0.00	0.00	1.0000
	sc6476	13	10	0.7500	0.9167	0.91	0.75	9.8641
	Overall	5.4±3.98	5.2±3.65	0.36±0.32	0.53±0.40	0.52±0.40	0.75±0.13	4.76±3.17
D. p. portschinskii ("Zuar")	sc12962	2	2	0.0000	0.1250	0.12	0.50	1.9879
	sc4525	4	6	0.6250	0.6604	0.64	0.77	4.0000
	sc138	5	5	0.8125	0.6562	0.64	0.74	4.9637
	sc149	5	6	0.5000	0.6484	0.62	0.71	5.0000
	sc12560	8	11	0.8750	0.8188	0.79	0.80	7.6129
	sc7287	5	6	0.4667	0.6071	0.58	0.71	4.8644
	sc1872	1	1	0.0000	0.0000	0.00	0.00	1.0000
	sc4045	6	8	0.3750	0.6271	0.60	0.60	5.8387
	sc10877	1	1	0.0000	0.0000	0.00	0.00	1.0000
	sc6476	6	7	0.6250	0.7000	0.68	0.74	5.7371
	Overall	4.3±2.3	5.3±3.2	0.43±0.33	0.48±0.31	0.47±0.30	0.70±0.10	4.20±2.21

to *D. p. nigrita* at eight of the 10 loci analysed ($P<0.05$). At the sc12962 locus, the number of alleles in both subspecies was the same; however, in the *D. p. nigrita* alleles form three genotypes, and two in the subspecies *D. p. portschinskii*.

In the analysed sample of each species and subspecies, the sc12962 locus is represented by an equally small number of alleles, two alleles each. The sc10877 locus, represented by only three alleles, turned out to be the least polymorphic: in individuals of *D. portschinskii*, this locus was represented by one allele, and in the sample of *D. valentini*, by two alleles. The sc1872 locus is represented by 10 alleles; however, the Simpson index and expected heterozygosity were less than 0.5 (Table 2). This is because the alleles of this locus are unevenly distributed between species (Table 3): six alleles were found in the sample of *D. valentini* individuals, and four alleles were found in the sample of *D. portschinskii*. The expected and observed heterozygosity (H_{exp}) values in the total analysed sample range from 0.3 to 0.9, and the average value of this parameter is 0.68 (Table 2). However, each of the studied species differs in the main characteristics of the loci (Table 3). The sc10877 locus turned out to be species-specific: in all studied individuals of *D. portschinskii*, the locus is monomorphic, and all individuals are homozygotes (Table 3). According to these data, the sc10877 locus cannot be used to analyse the intraspecific polymorphism of *D. portschinskii*, but it can be used for interspecific comparisons. The number of alleles at the studied loci varies significantly in distinct groups. Some loci, which have one or two alleles, are characterised by low diversity, low expected heterozygosity (H_{exp}), and uneven distribution of alleles. For example, loci sc4525, sc149, and sc4045 in the subspecies *D. p. nigrita* are represented by one allele. Characteristics of the loci also vary between populations of *D. p. portschinskii*. In the "Marts" population, the mean number of alleles is higher than in "Zuar" population, but the mean number of genotypes in both populations is nearly the same (Table 3). Observed heterozygosity in "Marts" population is lower than in "Zuar". This could be explained by formation of individuum-specific homozygous genotypes. In total, "Marts" is more polymorphic than the "Zuar" population, which may be related with higher sampling localities (Table 1).

Combinations of alleles at ten analysed loci for each individual of *D. portschinskii* form multilocus genotypes (MLG). Supplementary Fig. S1 shows the accumulation curve of MLG. For 48 analysed individuals (eight individuals of *D. valentini* and 40 individuals of *D. portschinskii*) the curve "reaches" a plateau starting from the genotype consisting of seven loci. Thus, this curve makes it possible to identify the minimum number of loci required to separate population samples. Based on the results obtained, a Nei's UPGMA tree (bootstrap 1000)

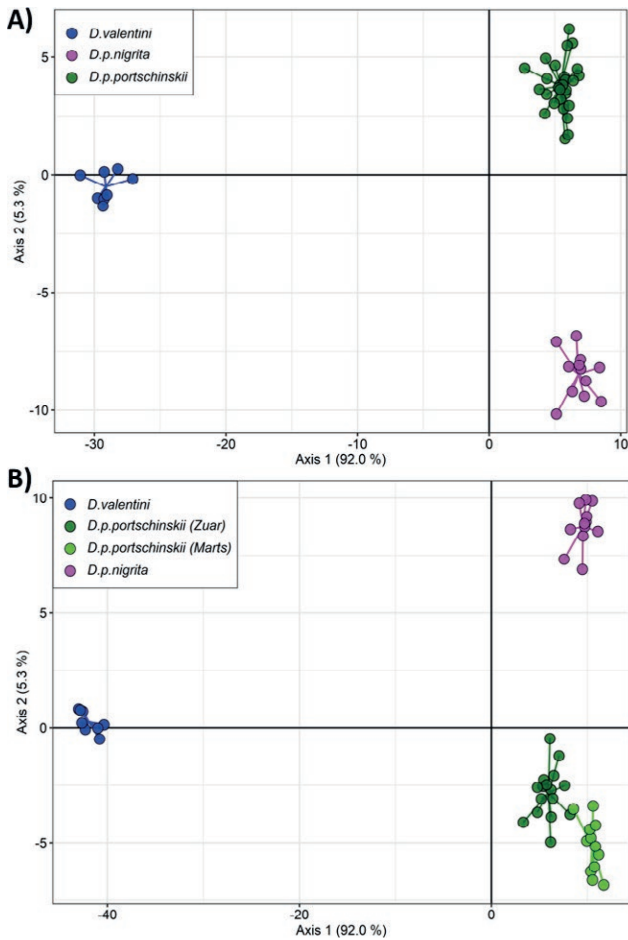

Fig. 2. Clustering of *D. portschinskii* and *D. valentini* individuals based on discriminant principal component analysis (DAPC). A) K=3; B) K=4.

Table 4. Values of the parameters of intraspecific and interspecific differentiation of *D. portschinskii* and *D. valentini*.

	F_{ST}	G_{ST}
D. portschinskii – *D. valentini*	0.298	0.797
D. p. portschinskii – *D. valentini*	0.303	0.782
D. p. nigrita – *D. valentini*	0.443	0.908
D. p. portschinskii – *D. p.nigrita*	0.286	0.556
D. p. portschinskii ("Zuar") – *D. valentini*	0.357	0.819
D. p. portschinskii ("Marts") – *D. valentini*	0.328	0.798
D. p. portschinskii ("Zuar") – *D. p. nigrita* ("Dzoraget")	0.353	0.624
D. p. portschinskii ("Marts") – *D. p. nigrita* ("Dzoraget")	0.332	0.617
D. p. portschinskii ("Marts") – *D. p. portschinskii* ("Zuar")	0.222	0.461

was constructed, demonstrating a significant division of the *Darevskia* lizards sample into two groups corresponding to *D. valentini* and *D. portschinskii* and supposed but non-significant subdivision of *D. portschinskii* into two clusters according of subspecies status, *D. p. portschinskii* and *D. p. nigrita* (Fig. S2).

Genetic differentiation in D. portschinskii *populations*

The standardised association index for the subspecies *D. p. nigrita* (-0.0247, P = 0.816) reflects the free recombination of alleles within this subspecies (Fig. S3A). For the subspecies *D. p. portschinskii*, the value of this parameter is 0.0677 (P = 0.001), which indicates the absence of allele recombination between the Zuar and Marts populations (Fig. S3B) and reflects the geographical remoteness of these groups. The value of the standardised association index shows the free recombination of alleles within each group: 0.000293 (P = 0.624) and -0.00396 (P = 0.655) for the Zuar and Marts groups, respectively (supplementary Fig. S4). The level of statistical significance P > 0.05 does not allow us to reject the hypothesis of the absence of linkage of alleles between loci and their independent recombination within each subspecies. A value of 0.0953 (P = 0.001) for *D. portschinskii* as a whole (i.e. for both subspecies) indicates no allele recombination between subspecies (Fig. S3C), i.e. these subspecies are isolated and crosses between their individuals are absent.

The main values of the parameters of intra- and interspecific differentiation, obtained by pairwise comparison of individuals of *D. portschinskii*, its subspecies, and the species *D. valentini*, are shown in Table 4. The F_{ST} parameter changes insignificantly during interspecific and intraspecific comparison. The G_{ST} value between *D. portschinskii* and *D. valentini* is 0.797, while between the subspecies of *D. portschinskii* it is 0.556. *Darevskia portschinskii portschinskii* in this study is represented by two geographically distant populations, "Zuar" and "Marts", although individuals of this subspecies from the population "Marts" are geographically closer to the population of the subspecies *D. p. nigrita* "Dzoraget" (Fig. 1). The values of inter- and intraspecific G_{ST} and F_{ST} indices are shown in Table 4. According to the data obtained, the values of these parameters and the level of differentiation between the "Marts" and "Zuar" populations are lower than that between the "Dzoraget" - "Marts" and "Dzoraget" - "Zuar" populations.

The use of the DAPC method on the entire sample of *D. portschinskii* and the outgroup *D. valentini* made it possible to determine the presence of three genetic groups (K = 3) with a clear division into subspecies according to the taxonomic status, which form a three non-overlapping graphs array (Fig. 2A). The same analysis with K =

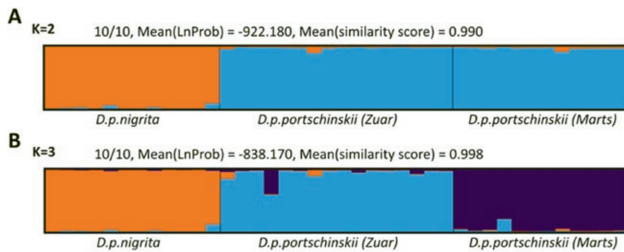

Fig. 3. Genetic structure of the species *D. portschinskii* according to the results of clustering in the STRUCTURE program. K=2 allows differentiating *D. portschinskii* into the subspecies *D. p. nigrita* and *D. p. portschinskii* (A), while K=3 allows the exact definition of two distinct populations within *D. p. portschinskii* (B).

4 shows differentiation on the groups, corresponding to *D. valentini*, *D. p. nigrita*, and *D. p. portschinskii* "Marts" and "Zuar" (Fig 2B.) The populations "Mars" and "Zuar" are geographically isolated, individuals can't mate, so there is no exchange of alleles between individuals. The partial overlapping between these two groups can be explained by gene flow before their isolation from each other (Cota et al., 2017), and can reflect their belonging to the same subspecies. The DAPC on the samples of *D. portschinskii* species only determines the presence of three genetic groups (K = 3) with a clear division into populations "Marts", "Zuar", and "Dzoraget" (Fig. S6).

At the same time, the probability of attracting individuals from *D. valentini* species to the corresponding cluster was 100% [95% confidence interval 67.6-100%]. The same accuracy of classification is observed for the subspecies *D. p. nigrita*. Evanno's method was used to determine the optimal number of clusters *K* (supplementary Fig. S7A) for the analysis of interpopulation subdivision of the species *D. portschinskii* in the STRUCTURE program. Already at the value of K=2, two large clusters are distinguished, corresponding to the subspecies *D. p. nigrita* and *D. p. portschinskii* (Fig. 3A). The optimal number of clusters is K=3, at which the separation of *D. p. portschinskii* into two populations "Marts" and "Zuar" (Fig. 3B) is observed. However, an increase in the number of clusters, and performing the same analysis with adding *D. valentini* (K = 4), does not affect the reliable division of *D. portschinskii* into two groups with subspecies status (Fig. S8). The slight admixture between subspecies can be explained by gene flow before their isolation from each other (Cota et al., 2017).

DISCUSSION

The study of intraspecific diversity of *D. portschinskii* is of particular interest in connection with the participation of its populations in interspecific hybridisations of various types, with the formation of unisexual (parthenogenetic) forms, *D. dahli* and *D. rostombekowii* (Uzzel and Darevsliy, 1980), and possible hybridisation of *D. portschinskii* with the related parthenogenetic species. Until now, the population classification of this species has been based only on morphological and geographical data that suggested the subspecies taxonomic status of *D. p. portschinskii* and *D. p. nigrita* populations (Bakradze, 1976). The aim of our work was to perform molecular genetic characterization of *D. portschinskii* populations based on microsatellite markers and to state the value of their genetic differentiation. For this, 40 lizards with wide morphological variation from different geographic locations were genotyped using 10 microsatellite loci, each present in the *D. valentini*, *D. raddei*, and *D. unisexualis* genomes. As follows from the data obtained, the value of one of the main indicators of intraspecific differentiation F_{ST} for *D. portschinskii* is 0.298 (Table 4), which is higher than shown for a number of vertebrate species. For example, the value of F_{ST} parameter between some subspecies of *Darevskia bithynica* lizards is 0.048 (Koç et al., 2017), for the introduced American mink is 0.144 (Korablev et al., 2018), and for the toothed smelt is 0.0701 (Semenova et al., 2019). This suggests that the populations studied in this work are not only geographically isolated, but also diverged to the subspecies level (Mikulícek et al., 2007). The values of the F_{ST} parameter when comparing the species *D. valentini* and *D. portschinskii* differ insignificantly and are comparable with those obtained when comparing the subspecies *D. p. portschinskii* and *D. p. nigrita* (Table 4). However, it seems more correct to use the G_{ST} criterion according to Hedrick (2005) (Table 4), as it more accurately reflects intraspecific and interspecific relationships in the *Darevskia* group. The use of F_{ST} and G_{ST} criteria to assess intraspecific diversity and differentiation of *D. portschinskii* shows similar results (Table 4). Populations belonging to the same subspecies *D. p. portschinskii*, despite significant geographical remoteness, are characterised by greater genetic similarity than populations of different subspecies *D. p. portschinskii* and *D. p. nigrita* living at a relatively close distance. According to the data obtained, *D. p. nigrita* is characterised by a lower level of intrapopulation polymorphism than *D. p. portschinskii*, which is probably due to the small range of this subspecies. This is consistent with the earlier results of the analysis of the genetic diversity of allozyme loci in the species *D. portschinskii*, which showed that small disjunct populations isolated from the main range of the species are characterised by a low level of genetic polymorphism compared to non-isolated contiguous populations (MacCalloch et al., 1997). In general, our genetic data on the

population differentiation of *D. portschinskii* confirms the morphological data on the subspecies status of the *D. p. portschinskii* and *D. p. nigrita* populations. It is evident that 10 developed microsatellite loci were effective for studying intraspecific diversity of investigated *Darevskia* species: we also suggested a potential applicability of the developed microsatellite markers for study other *Darevskia* lizard species and subspecies. In general, they are more polymorphic than tetranucleotide microsatellite markers developed by us previously (Korchagin 2007), and which are widely used for genetic study of *Darevskia* lizards (Badaeva et al., 2008; Girnyk et al., 2018; Vergun et al., 2014, 2020; Ryskov et al., 2017; Tarkhnishvili et al, 2017; Freitas et al., 2019; Koç, et al., 2017; Kropachev et al., 2023). We suppose that they can be useful to study different *Darevskia* lizard species.

The study of evolutionary relationships at the population, subspecies, and species levels requires the analysis of rapidly evolving markers. Microsatellites are characterised by a high mutation rate and genetic polymorphism (Jarne and Lagoda, 1996; Estoup and Corneut, 2000; Ellegren, 2004; Badaeva et al., 2008), and a number of studies confirms the successful use of microsatellites for the reconstruction of phylogenetic relationships of such taxa at the level of species/subspecies/population (Estoup et al., 1995; Harr et al., 1998; Petren et al., 2005; Richard and Thorpe, 2001; Peréz et al., 2002; Orsini et al., 2005; Chirhart et al., 2005; Hughes et al., 2005; Kankare et al., 2005; Knaden et al., 2005). Here, we first present phylogenetic relationships within *D. portschinskii* based on the polymorphism of developed trinucleotide (sc12962, sc138, sc149, sc4045) and dinucleotide (sc12560, sc6476, sc4525, sc10877, sc7287, sc1872) microsatellite loci. The main genetic characteristics of these loci were determined, and their suitability for molecular genetic study of *Darevskia* species was demonstrated. In particular, it has been shown that they are effective for studying the intraspecific diversity of *Darevskia* lizards: the number of alleles is sufficient to reliably separate morphologically different populations; multilocus genotypes formed by alleles of only seven microsatellite loci make it possible to construct various types of phylogenetic trees.

ACKNOWLEDGEMENTS

The authors are grateful to F.A. Osipov, Ph.D., for assistance in compiling a map of the collection sites of population samples of the lizards *D. portschinskii*. All applicable international, national and/or institutional guidelines for the care and use of animals have been followed. Animal work was carried out in strict accordance with the rules of the Ministry of Nature Protection of Armenia (permit number 5 / 22.1 / 51043) and the ethics committee of Moscow State University (permit number 24-01). The authors declare that they have no conflict of interest. This work was performed using the equipment of IBG RAS core facility. The study was supported by the Russian Science Foundation Grant No. 19-14-00083.

SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found at <http://www-262 9.unipv.it/webshi/appendix/index.html> manuscript number 14756.

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