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BIOCHEMISTRY, BIOPHYSICS, AND MOLECULAR BIOLOGY

Genetic Differentiation of Parthenogenetic Lizards *Darevskia rostombekowi* (Family Lacertidae) As Determined Using Nuclear and Mitochondrial DNA Markers

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In this study, using analysis of nucleotide sequences of mitochondrial genome (cytochrome *b* gene) of parthenogenetic Rostombekov's lizards, we demonstrated that three northwestern populations of the parthenogenetic species *Darevskia rostombekowi* and an isolated high-mountain population dwelling the southeastern coast of Lake Sevan, which has not been studied earlier, have the same origin. They originated from the same population of the maternal bisexual species *D. raddei* from Egegnadzor, one of southern populations of this species in Armenia. At the same time, using RAPD markers of nuclear genome, we showed that the high-mountain population of *D. rostombekowi* at Lake Sevan significantly differs from the northwestern populations of this species ($p < 0.05$). These data are indicative of the same origin of the populations of this species and initial stages of intraspecific differentiation of *D. rostombekowi*.

One of the central problems in studying unisexual vertebrate species is the assessment of their intraspecific variability and clonal diversity. Possible sources of genetic diversity of populations of these species may include the emergence of the original clones from different founders, mutations occurring in the course of their evolutionary history, as well as the process of genetic recombination and rare cases of repeated crossings, whose contribution to variability is negligible [1, 2]. Apparently, the range size, the age of species, and some other factors may also determine the degree of clonal diversity of a parthenogenetic species.

The Rostombekov's rock lizard *D. rostombekowi* occupies a relatively small range consisting of isolated populations of various size inhabiting the northern foothills of the Small Caucasus situated in North Armenia, contiguous territories of northwestern Azerbaijan, and a small high-mountain (2000 m) population inhabiting the southeastern coast of Lake Sevan. The isolated population at Lake Sevan occupies small outcrops of basalt rocks located approximately 2 km from the lake, in the vicinity of village Zagalu. It is separated from the nearest populations inhabiting the foothills near the Kura River (environs of Lake Gai-Gel' in Azerbaijan) by the Sevan and Vardenis mountain ranges rising to 3000 m and more, which are an insurmountable geographical barrier for *D. rostombekowi*.

Similarly to other parthenogenetic species of the genus *Darevskia*, Rostombekov's rock lizards have a hybrid origin from the maternal bisexual species *D. raddei* and the parental species *D. portchinskii* [1]. They have a double chromosome set ($2n = 38$) [3], are characterized by fixed heterozygocity of allozyme loci [4] and a low variability of restriction sites of mitochondrial DNA [5]. Earlier studies of the allozyme spectra of 35 loci of *D. rostombekowi* from the northwestern and central Armenian populations revealed no allozyme variability in this species. In view of this fact, it was assumed that this is a monoclonal species [6], unlike the parathenogenic species *D. dahlia, D. armeniaca*, and *D. unisexualis*, in which two to five allozyme clones were found [7].

Studies of the restriction polymorphism of mitochondrial DNA [5] and nucleotide analysis of the cytochrome *b* (1044 bp) gene of three northwestern populations of the parthenogenetic species *D. rostombekowi* and related bisexual species of this genus unambiguously demonstrated that the northwestern populations dwelling near the town of Spitak and villages Gosh and

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Papanino have the same origin from one of southern Armenian populations of *D. raddei*—the population of Egegnadzor (39°45' N, 45°08' E) [4].

Earlier, we showed with the use of DNA fingerprinting that lizards of the parthenogenetic species *D. rostombekowi* from the population at Lake Sevan significantly differ from the lizards of this species from the populations of northwestern Armenia ($p < 0.001$) [8]. For this reason, the goal of this study was to investigate the intraspecific structure and relationship of all the four populations of *D. rostombekowi* using nuclear (RAPD) and mitochondrial (cytochrome *b* gene) DNA markers.

Parthenogenetic *D. rostombekowi* females were caught in June and July of 1999–2001 in isolated populations of Northern Armenia in the vicinity of villages Gosh (4 animals) and Papanino (the environ of Dilizhan, 12 animals), in the environs of the town of Spitak (9 animals), as well as in the isolated population on the southeastern coast of Lake Sevan (Zagalu, 5 animals). Blood obtained from adult females was conserved with 0.5 M EDTA (pH 8.0) and used to isolate DNA as described earlier [8].

To perform RAPD analysis, in preliminary experiments we tested seven primers differing in the nucleotide composition, size, and GC content. In further experiments, the following five primers were used: no. 45 (5'GCCGTCCGAG3'), no. 29 (5'CCGGCCTTAC3'), no. 343 (5'AGGTCACTGA3'), no. SB2 (5'GACGGCCAGTATT3'), and no. 92 (5'CATTCCGGCC3'). Polymerase chain reaction (PCR) was performed in a reaction mixture (25 μl) containing 10 mM Tris–HCl (pH 8.0), 50 mM KCl, 0.1% Tween 20, 0.8 mM of each deoxynucleoside trisphosphate (dGTP, dTTP, dCTP, and dATP), 3.0 mM $MgCl_2$, genomic DNA (0.1 μg per sample), and Taq polymerase (0.5 U). The concentration of primers varied from 0.5 to 1.5 mM. Amplification was performed in a TP4-PTsR-01 amplifier (Tertsik) under the following conditions: denaturation (94°C, 5 min), 35 cycles of amplification (94°C, 1 min; annealing at 32–40°C (depending on primer); and 72°C, 2 min), and elongation (72°C, 5 min). Amplification products were separated by electrophoresis in 2.0% agarose gel in 1 × TRE buffer for 1 day at $U = 50$ V. Gels were stained with an aqueous solution of ethidium bromide (50 μg/l) and photographed under UV light. Data obtained using RAPD markers were summarized in a binary matrix table of an object–trait type, which was then analyzed using the Biosystem 1.0 information system [9].

To study the 1044-bp fragment of mitochondrial DNA containing the cyt *b* gene, three pairs of primers were used, as described in [4]. For sequencing, the amplificants were isolated from agarose gel by elution on a DEAE paper. The nucleotide sequences of the amplification products were determined by the method of Sanger using an ABI PRISM®BigDye™ Terminator 3.1 kit, with subsequent analysis of the reaction products in an ABI PRISM 3100 Avant sequencer. The sequences of the studied fragment of mitochondrial DNA of different animals were aligned using the MegAlign 4.05 software. Genetic distances between nucleotide sequences were determined using the special software MEGA 3 and represented in the form of an NJ dendrogram (neighbor-joining tree; bootstrap, 1000 replicas).

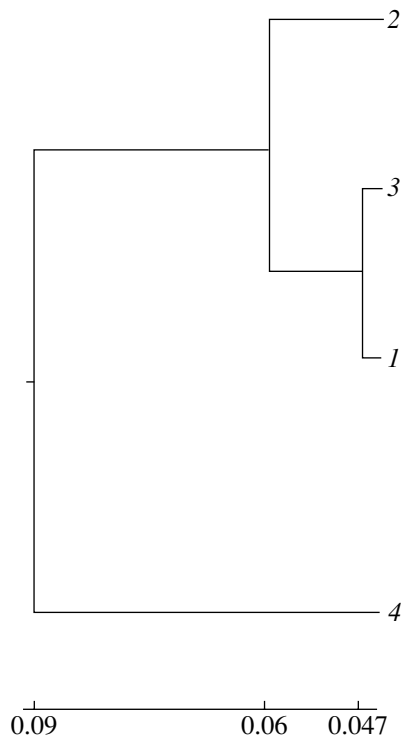

Fig. 1. Genetic differences between the *D. rostombekowi* populations. The dendrogram was constructed on the basis of the difference indices between the lizards of different populations by the UPGMA method using the cumulative matrix table ($p < 0.05$). Designations: *1–3*, North Armenian populations; *4*, Lake Sevan population.

The dendrogram shown in Fig. 1 illustrates genetic differences between the populations of *D. rostombekowi*, determined with the use of RAPD DNA markers. The RAPD spectra of *D. rostombekowi* are characterized by species specificity, low polymorphism of the marker DNA fragments compared to the bisexual species of the genus *Darevskia* [10], and prevalence of invariant zones for primers nos. 45, 343, and SB2 in the region of 2.0 to 0.5 kb. The most polymorphous spectra of amplified DNA were obtained using primers nos. 29 and 92. The genetic differences between the populations of *D. rostombekowi* were estimated using multiple comparative analysis based on the Bonferroni test modified by Holmes [9]. Multiple pairwise comparisons of the mean intrapopulation similarity indices revealed significant differences ($p < 0.05$) among the populations analyzed. According to the results of RAPD analysis, all studied lizards belonging to the spe-

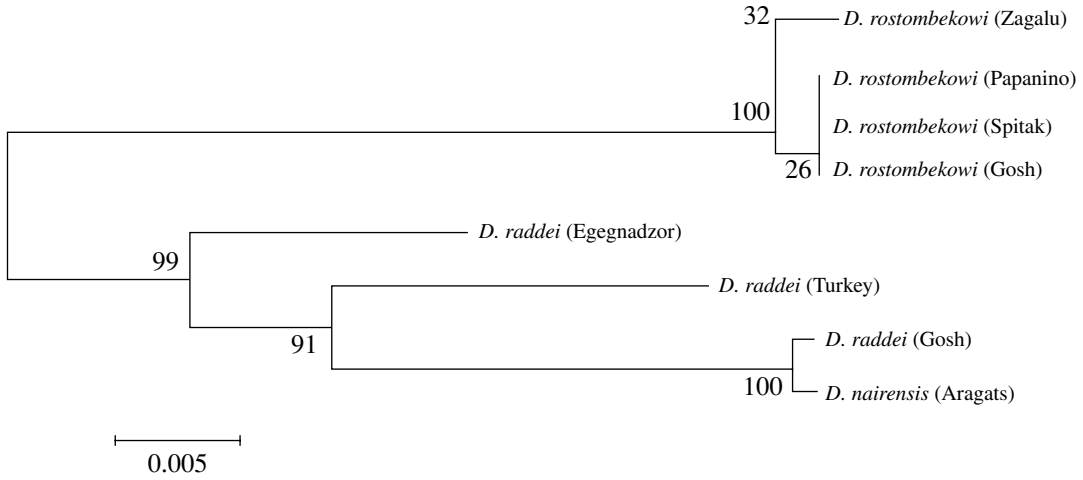

Fig. 2. NJ dendrogram (neighbor-joining tree) based on the results of analysis of the nucleotide sequences of the cyt *b* gene (1044 bp) of unisexual and bisexual lizard species of the genus *Darevskia.* A bootstrap support (1000 replicas) is given for each branching point. The corresponding regions of nucleotide sequences of mitochondrial DNA of *D. raddei* and *D. nairensis* were taken from [4].

cies *D. rostombekowi* can be divided in to two groups, the first of which combines the animals from the Sevan population, and the second one combines all other animals from the North Armenian populations.

RAPD analysis can be sufficiently effectively used for determination of intraspecific and interpopulation variability of both bisexual and unisexual species. However, the variability of RAPD markers in the populations of parthenogenetic Caucasian rock lizards is not high, which agrees well with the data of Grechko et al. [11] and is considerably lower than in some species of gynogenetic fish [12]. Despite this fact, the results obtained using RAPD analysis, are consistent with the data on the interpopulation variability of fingerprinting markers in *D. rostombekowi* [8] and testify to a significant divergence in the Sevan population.

To study the phylogenetic relationships, origin, and clonal diversity of *D. rostombekowi* populations inhabiting northwestern Armenia and the coast of Lake Sevan, we sequenced the amplified regions of mitochondrial cytochrome *b* gene. A comparative analysis of the nucleotide sequences of these regions of 18 *D. rostombekowi* lizards from four Armenian populations revealed only a single nucleotide substitution (transition C $\rightarrow$ T at position 535 bp) in the animals from the Sevan population (Fig. 2) versus the animals form the other populations studied.

Figure 3 shows the dendrogram constructed on the basis of the results of analysis of polymorphism of DNA nucleotide sequences obtained in this work and the data reported by Fu et al. [4]. The results of analysis of this dendrogram showed that the Sevan population forms a single cluster with the other *D. rostombekowi* populations and that it clusterizes most closely with the population of the maternal species *D. raddei* from Egegnadzor. At the same time, our data indicate the existence of a second mitotype in *D. rostombekowi*, which was discovered in the Sevan population and distinguishes from the other populations of this species in only one nucleotide substitution.

According to modern ideas, all parthenogenic species of the genus *Darevskia* appeared on the Caucasus in the postglacial period and are sufficiently young species that have equal chances for occurrence of new clones [1]. According to the results of electrophoretic studies of proteins and mitochondrial DNA, parthenogenic reptile species are multiclonal to various extent. A high level of variability of allozyme loci was discovered, for example, in *Heteronotia bionoei* (Gekkonidae) [13]. Genetic heterogeneity was also demonstrated for the American parthenogenic lizards of the genus *Cnemidophorus* (*C. tessalis* and *C. neomexicanus*; family Teiidae) [14] and the parthenogenic species of Caucasian rock lizards of the genus *Darevskia* (*D. dahli, D. armeniaca*, and *D. unisexualis*; family Lacertidae) [7].

It was assumed earlier that the instability of the karyotype of hybrid genomes may also lead to the genetic diversity of parthenogenic species and appearance in them of chromosomal mutations [3]. It is believed that the latter govern the formation of new clones and/or geographically isolated chromosomal races. Such clones was discovered, for example, in the parthenogenic species of the genus *Hemidactylus.* In the light of available data, it cannot be ruled out that the lizards from the isolated population inhabiting the coast of Lake Sevan, which differ genetically from the *D. rostombekowi* lizards from the northwestern Armenian populations, represent such chromosomal race and/or a clone that emerged as a result of chromosomal mutations occurred in the course of karyological evolution of this species.

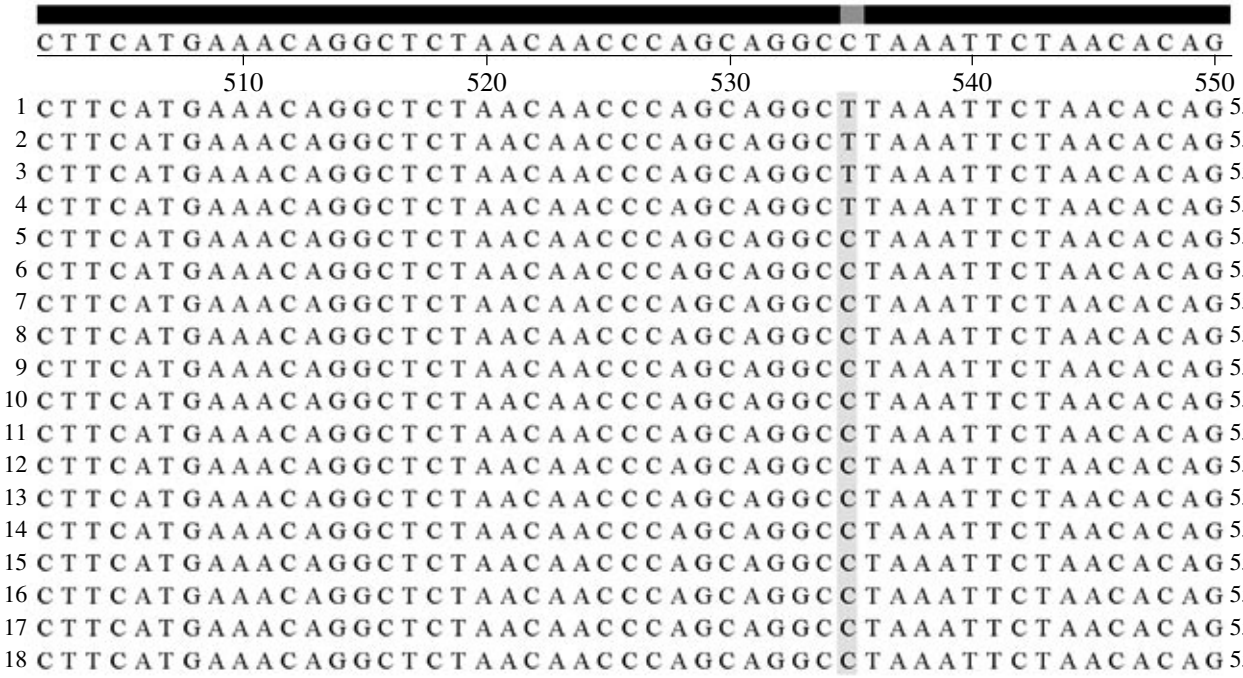

Fig. 3. Nucleotide sequence of the region of the cyt *b* gene (positions 500 to 550 bp from the 5' end of the amplification product of mitochondrial DNA). The only nucleotide substitution C → T (position 535 bp) is marked. Designations: *1–4*, lizards from the population dwelling at Lake Sevan (Zagalu); *5–18*, lizards from the populations of northwestern Armenia (Papanino, Spitak, Gosh).

Thus, the results of analysis of nuclear and mitochondrial DNA markers provide another view on the populational structure, origin, and evolutionary history of this species.

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