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MOLECULAR GENETICS

Molecular Nature of Allelic Polymorphism of Highly Variable Microsatellite Locus *Du161(arm)* in Unisexual Lizard *Darevskia armeniaca* (Lacertidae)

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Abstract—In the genome of unisexual (parthenogenetic) lizard *Darevskia armeniaca*, highly variable locus *Du161* (*arm*) was discovered. Analysis of allelic polymorphism was carried out using locus-specific PCR of the lizard DNA specimens from 13 isolated Armenian populations ($N = 138$). In the sample examined, a total of 12 *Du161(arm)* alleles were identified, and their differences at the level of primary DNA structure were determined. Sequence analysis of the *Du161(arm)* alleles showed that their microsatellite clusters contained repeats of one type (GATA repeats). Allelic *Du161(arm)* variants differed in the number of GATA monomers in microsatellite, point mutations of transition and transversion types, located at fixed distances from microsatellite cluster, and by single nucleotide insertions, as well as by longer insertions located within and outside of the microsatellite cluster. Moreover, point mutations formed different combinations (haplotypes), typical of certain alleles. These combinations can be used for the analysis of the origin and inheritance of these alleles in *D. armeniaca*, as well as for investigation of their interspecific variation in the representatives of the genus *Darevskia*.

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INTRODUCTION

Hypervariable mini- and microsatellites represent a universal system of molecular markers, widely used in population and evolutionary studies, genome marking, assessment of paternity, and medical genetic studies [1–3]. These repeats belong to highly unstable regions of the eukaryotic genome, displaying maximum mutation rates known for the genetic loci [4]. Investigations of individual mini- and microsatellite loci showed that changes occurring in these sequences seemed to be highly variable with distinct differences between the species, type of repeats, alleles, as well as age and sex [5]. Despite of the intense studies, performed in human and some other bisexual species, genesis of mini- and microsatellite loci remains poorly studied in the species with clonal type of reproduction. Investigation of these processes in unisexual lizards, used as model systems, can make a considerable contribution to the understanding of the mechanisms of variation and evolution of hypervariable DNA repeats. It will also provide the new information on the nature of genetic and clonal diversity of unisexual species themselves.

Earlier we cloned and characterized a number of microsatellite loci in unisexual diploid lizards *Darevskia unisexualis*, *D. armeniaca*, and *D. dahli* [6–8]. It was found out that most of these loci were monomorphic, containing two alleles identical in all individuals from the samples examined. Polymorphic loci characterized by rather low variability were also identified. However, exactly characterization of the polymorphic loci provided new information on molecular nature of their variation [9, 10]. Identification of new highly variable loci in the genomes of unisexual vertebrate species is very important for gaining insight into mechanisms of the appearance of genetic and clonal diversity in these species and evaluating the level of this diversity.

Darevskia armenica is one of seven obligate parthenocarpic rock lizard species of the genus *Darevskia*. The species appeared as a result of interspecific hybridization between *D. valentini* ("paternal species") and *D. mixta* ("maternal species") [11]. *D. armeniaca* is characterized by diploid chromosome set ($2n = 38$), as well as chromosome morphology (acrocentrics, A), typical of lizards of the genus *Darevskia*. In addition to acrocentric chromosomes, the genome of the species of interest was found to contain microchromosomes, which is typical of all species of the genus *Darevskia* [12].

Allozyme analysis of 35 loci in *D. armeniaca* from seven populations demonstrated wide distribution of one clone along with the presence of two rare clones [13]. However, unlike other parthenogenetic rock lizards, in *D. armeniaca* one of rare clones was prevalent in one of the populations examined (Papanino, 19 out of 27 individuals), and in the second populations, 16 out of 22 lizards examined belonged to the rare clone.

Table 1. Characteristic of the primers designed for the *Du161(arm)* locus

Locus *Du161*	Primer	Sequence	Number of nucleotides	*T* annealing, °C
	F–direct	5' GTGAGACTTTCATGTATAACTGAC 3'	24	50
	R–reverse	5' ATAATGGGCTGTACCTAATCC 3'	21	

More recent studies showed the existence of four forms, two of which were predominant, and other two, minor forms [14, 15]. At the same time, the use of multilocus DNA fingerprinting with different microsatellite probes revealed considerably higher level of genetic diversity in the populations of *D. armeniaca* [16–19]. The nature of locus variability identified with the help of this approach still remains obscure. More complete information can be obtained through the analysis of cloned microsatellite loci characterized by high variability.

The present study was the first to identify and characterize highly variable *Du161(arm)* locus in the genome of *D. armeniaca*. The molecular structure of the locus allelic variants was established.

MATERIALS AND METHODS

Locus-specific PCR was carried out using DNA specimens from 138 *D. armeniaca individuals* from 13 populations of Armenia. Blood samples taken from adult female lizards were preserved in 0.05 M EDTA solution, pH 8.0. DNA was extracted with the help of standard phenol–chloroform technique with the use of proteinase K [20].

PCR amplification was carried out using a pair of primers earlier designed for the *Du161* locus of closely related species *D. unisexualis*. The reaction was run in a total volume of 20 μL reaction mixture containing 1× buffer for *Taq* polymerase (Dialat); 250 μM of each dNTP; 2 mM $MgCl_2$; 10 μM of each primer; and 0.8 units of *Taq* polymerase (Dialat). The reaction mixture was supplemented with 20 ng of genomic DNA. Amplification was performed using the four-channel TP4-PCR-01 DNA amplifier (Tertsik). Primer sequences for PCR reactions and annealing temperatures are shown in Table 1. Amplification products were separated by electrophoresis in 8% polyacrylamide gel (PAAG) and visualized by staining with ethidium bromide. Sequencing of amplification products was carried out using the method of Sanger and the ABI PRISM BigDye Terminator V.3.1 reagent kit (GE Healthcare, United States). Further analysis of the reaction products was carried out using the ABI PRISM 3100-Avant automated DNA sequencer (Applied Biosystems, United States). Sequencing of chosen fragment was performed in two directions. Forward and reverse sequences were compared to one another to avoid possible sequencing errors. The sequences belonging to one sample were completely identical. DNA sequences of different individuals were aligned using the Clustal W procedure of the MegAlign 4.05 software program.

RESULTS

An example of monolocus PCR analysis of *D. armeniaca* lizards (selective samples from different populations) is shown in Fig. 1. It was demonstrated that in a sample of 138 individuals, the *Du161*(*arm*) locus was represented by at least 12 allelic variants, differing in electrophoretic mobility of amplification products within the interval from 400 to 300 bp. To examine molecular nature of the *Du161*(*arm*) allelic polymorphism, sequencing of the PCR products of the corresponding allelic variants was performed. Comparison of 12 *Du161*(*arm*) allelic variants is presented in Fig. 2. Sequencing of the *Du161*(*arm*) alleles showed that their microsatellite clusters contained repeats of one type, the GATA repeats (Table 2). Allelic variants differed in the number of GATA monomers in microsatellites, point mutations of transition and transversion types, located at fixed distances from microsatellite cluster, and by single nucleotide insertions, as well as by more long insertions, located inside and outside of microsatellite cluster. Moreover,

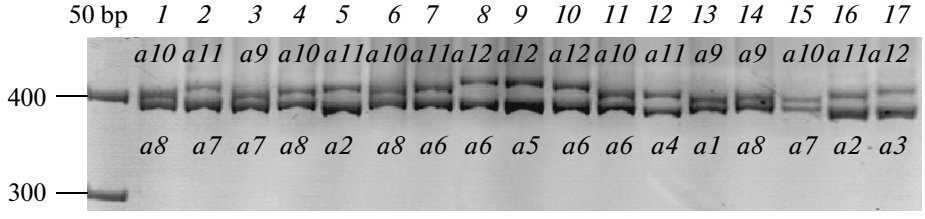

Fig. 1. PAAG electrophoresis of the *Du161(arm)* amplification products from selective DNA samples of *D. armeniaca*. *a1* to *a12*, allelic variants of the *Du161(arm)* locus. Molecular size marker, 50-bp Ladder (Fermentas) with the step of 50 bp.

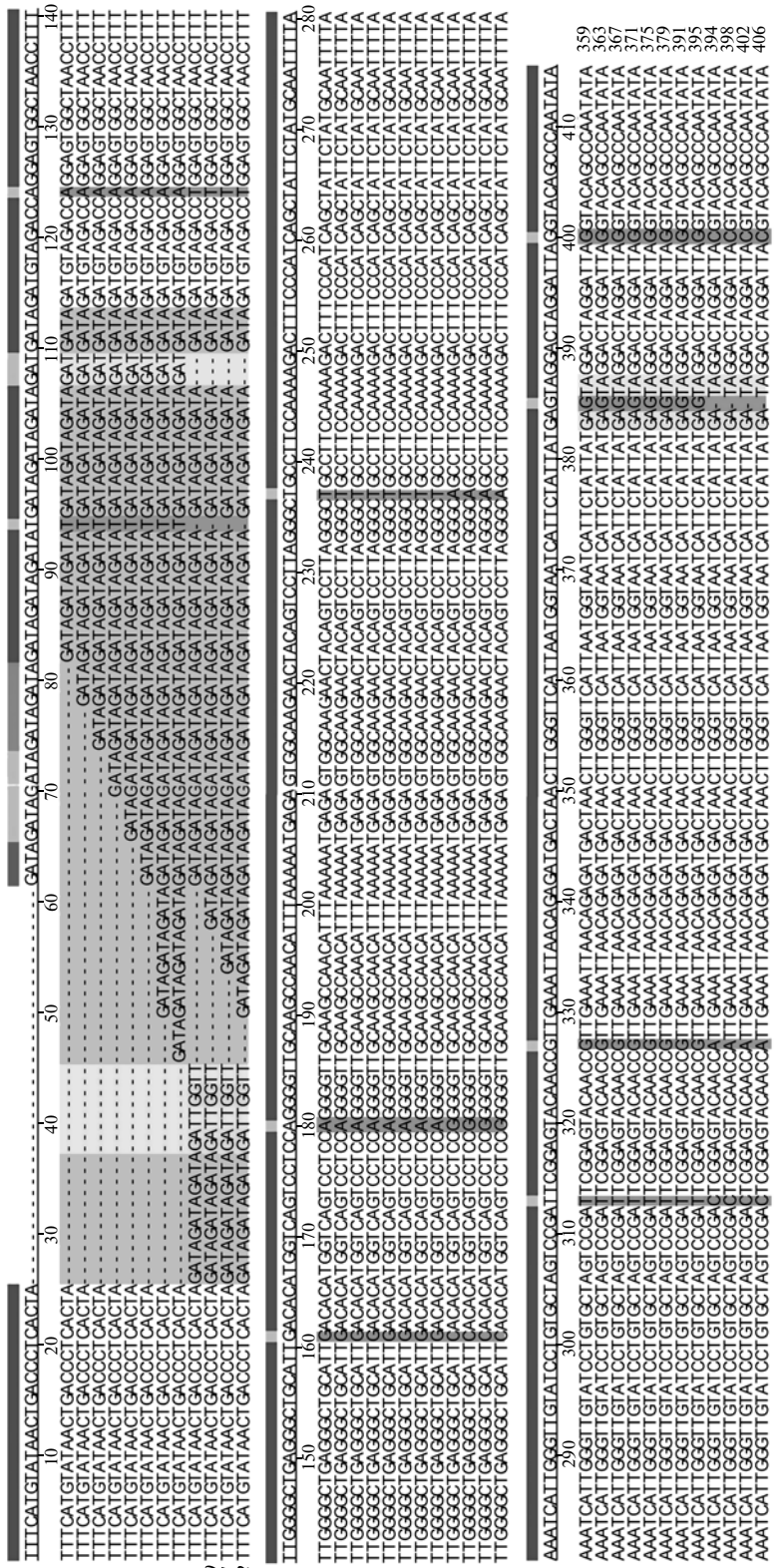

Fig. 2. Sequence comparison of 12 *Du161(arm)* allelic variants from *D. armeniaca*. Sequence matches are indicated by gray color on the band above. Single nucleotide substitutions are indicated by dark gray color. Microsatellite cluster units are indicated by pale gray color.

Table 2. Specific structure of allelic variants of the *Du161(arm)* microsatellite locus in parthenogenetic species *D. armeniaca*

Allele	Structure of microsatellite cluster	Fixed nucleotide substitutions outside of microsatellite*								Haplotype
		+11	+48	+67	+124	+200	+214	+272	+286	
Du161 (arm)1	5' $(GATA)_3$ T $(GATA)_3$ GAT (GATA) 3'	A	G	A	T	T	G	G	G	T-A-G-A-T-T-G-G-G
Du161 (arm)2	5' $(GATA)_4$ T $(GATA)_3$ GAT (GATA) 3'	A	G	A	T	T	G	G	G	T-A-G-A-T-T-G-G-G
Du161 (arm)3	5' $(GATA)_5$ T $(GATA)_3$ GAT (GATA) 3'	A	G	A	T	T	G	G	G	T-A-G-A-T-T-G-G-G
Du161 (arm)4	5' $(GATA)_6$ T $(GATA)_3$ GAT (GATA) 3'	A	G	A	T	T	G	G	G	T-A-G-A-T-T-G-G-G
Du161 (arm)5	5' $(GATA)_7$ T $(GATA)_3$ GAT (GATA) 3'	A	G	A	T	T	G	G	G	T-A-G-A-T-T-G-G-G
Du161 (arm)6	5' $(GATA)_8$ T $(GATA)_3$ GAT (GATA) 3'	A	G	A	T	T	G	G	G	T-A-G-A-T-T-G-G-G
Du161 (arm)7	5' $(GATA)_{11}$ T $(GATA)_3$ GAT (GATA) 3'	A	G	A	T	T	G	G	G	T-A-G-A-T-T-G-G-G
Du161 (arm)8	5' $(GATA)_{12}$ T $(GATA)_3$ GAT (GATA) 3'	A	G	A	T	T	G	G	G	T-A-G-A-T-T-G-G-G
Du161 (arm)9	5' $(GATA)_3$ – GATTG-GTT $(GATA)_{12}$ ... GATA 3'	T	C	G	A	C	A	–	C	T-C-G-A-C-A-C
Du161 (arm)10	5' $(GATA)_3$ – GATTG-GTT $(GATA)_{13}$ ... GATA 3'	T	C	G	A	C	A	–	C	T-C-G-A-C-A-C
Du161 (arm)11	5' $(GATA)_3$ – GATTG-GTT $(GATA)_{14}$ ... GATA 3'	T	C	G	A	C	A	–	C	T-C-G-A-C-A-C
Du161 (arm)12	5' $(GATA)_3$ – GATTG-GTT $(GATA)_{15}$ ... GATA 3'	T	C	G	A	C	A	–	C	T-C-G-A-C-A-C

* The distances in bp after (+) microsatellite cluster are presented.

point mutations formed different combinations (haplotypes), typical of certain alleles. It can be seen that alleles formed two groups in accordance to marking haplotypes, which probably, reflected their origin from bisexual parental species. Interestingly, alleles belonging to different haplotype groups differ in the structure of microsatellite clusters. This finding also points to the independent origin of these alleles. Thus, we can say that combination of specific microsatellite structure and the structure of flanking DNA can be treated as an integrated marking character for each group of the alleles.

The group I includes alleles with haplotype T-A-G-A-T-T-G-G-G (from *Du161(arm)1* to *Du161(arm)8*), and group II includes alleles with haplotype T-C-G-A-C-A-C (from *Du161(arm)9* to *Du161(arm)12*). In alleles of group I, two single nucleotide insertions absent from alleles of group II were discovered, including insertion of T nucleotide inside of microsatellite cluster and insertion of G nucleotide in position +272 outside of microsatellite (Fig. 2, Table 2). In addition to GATA repeats, alleles of group I contain GAT insertion inside of microsatellite cluster. Unlike alleles of group I, alleles of group II contain the GATTGGTT insert in microsatellite cluster.

According to the data obtained, all lizard individuals tested were heterozygotes. In addition, the populations examined differed from one another in the frequencies of allelic variants (Table 3) and in their combinations (Table 4). Most of the populations examined contained from four to six allelic variants differing in frequencies. Three populations (Ukrainian, Megradzor, and Takyarlu) were characterized by a great number of allelic combinations. The most abundant (present in many populations) were alleles *a9*, *a11*, and *a10*. The least common were alleles *a1*, *a2*, *a4*, *a6*, and *a8*. At the same time, rare alleles could be the prevalent in certain populations, like allele *a4*, in the Artik population, and allele *a8*, in the Ukrainian population.

DISCUSSION

Investigation of individual mini- and microsatellite loci in bisexual species showed that changes occurring in them are greatly variable [5]. It is known that the mutation rate in hypervariable microsatellite loci is three to four orders of magnitude higher than the mean values for the structural genes [4]. Although the mechanisms of microsatellite instability are not com-

Table 3. Frequency of the *Du161(arm)* allelic variants in the populations of parthenopgenetic species *D. armeniaca*

Population	Number of individuals	Allelic variants *Du161*											
		a1	*a2*	*a3*	*a4*	*a5*	*a6*	*a7*	*a8*	*a9*	*a10*	*a11*	*a12*
Alaverdy	3		2	1								2	1
Ukrainian	35							9	26	3	8	16	8
Artik	18				15	3				14	4		
Gosh	1							1				1	
Kuchak	8			3	4	1				6	2		
Lchap	1							1		1			
Lchashen	1							1				1	
Megradzor	16	2		8		8				11	3	4	
Papanino	7						3	2	2			5	2
Semenovsky pass	8				2		6				3	3	2
Stok	3		2	1						1		2	
Stepanavan	9					5	4			3	6		
Tkyarlu	15		9	5						6	4	5	1
Tkyarlu (mutants)*	13							1	12	12		1	

* Morphological forms with changed body coloration.

Table 4. Combination of the *Du161(arm)* allelic variants in the populations of parthenopgenetic species *D. armeniaca*

Population	Number of individuals	Combination of allelic variants (*a1–a12*)*							
Alaverdy	3	*a2–a11*	*a3–a12*						
Ukrainian	35	*a7–a9*	*a7–a10*	*a7–a11*	*a7–a12*	*a8–a9*	*a8–a10*	*a8–a11*	*a8–a12*
Artik	18	*a4–a9*	*a4–a10*	*a5–a9*	*a5–a10*				
Gosh	1	*a7–a11*							
Kuchak	8	*a3–a9*	*a3–a10*	*a4–a9*	*a4–a10*	*a5–a9*			
Lchap	1	*a7–a9*							
Lchashen	1	*a7–a11*							
Megradzor	16	*a1–a9*	*a2–a10*	*a2–a11*	*a3–a9*	*a3–a10*	*a3–a11*	*a4–a10*	
Papanino	7	*a6–a11*	*a7–a11*	*a8–a12*					
Semenovsky pass	8	*a4–a11*	*a6–a10*	*a6–a12*					
Stok	3	*a2–a9*	*a2–a11*	*a3–a11*					
Stepanavan	9	*a5–a10*	*a6–a9*	*a6–a10*					
Tkyarlu	15	*a2–a9*	*a2–a10*	*a2–a11*	*a3–a9*	*a3–a10*	*a3–a11*	*a4–a10*	
Tkyarlu (mutants)**	13	*a7–a9*	*a8–a9*	*a8–a11*					

Notes: * *a1–a1 – Du161(arm)1–Du161(arm)12.*

** Morphological forms with changed body coloration.

pletely clear, there are a number of hypothesis on the issue [21, 22]. It is suggested that microsatellite instability depends on a number of factors, among which the key role is played by nucleotide composition and characteristics of the genome context [23, 24]. According to the modern concepts, mutational changes in microsatellite loci can serve as the equilibrium between point mutations and the DNA replication and repair errors [5, 21, 22, 25].

The main result of the present study is the discovery of highly variable locus in the genome of parthenogenetic species *D. armeniaca* and elucidation of the nature of its allelic polymorphism. At present, the data on cloning and characterization of microsatellite loci

in unisexual vertebrates are scarce. Wilmhoff et al. [26] cloned and sequenced 16 variable dinucleotide microsatellite loci of parthenogenetic mourning gecko *Lepidodactylus lugubris*. Genetic polymorphism of these loci was evaluated with the help of PCR analysis. Considerable genetic differences were observed between different subgroups of the individuals examined, pointing to the existence of clonal lineages. Gardner et al. [27] isolated and sequenced six polymorphic loci, containing $(AAC)_n$ and $(AAAG)_n$, in triploid parthenogenbetci form of Australian lizard *Menetia greyii*. The authors suggested using these loci for analysis of bisexual species of the *M. greyii* complex. However, in the studies cited molecular characterization of allelic variants of the polymorphic loci was not performed. Analysis of 14 microsatellite loci in two parthenogenetic species of the genus *Lepidophyma* (*L. reticulatum* and *L. flavimaculatum*) was the first, providing sufficiently strong evidence on nonhybrid origin of parthenogenesis in natural populations of vertebrates [28]. The authors showed that heterozygosity (high in case of the hybrid origin of parthenogenetic species) was very small in unisexual lizards *L. reticulatum*, and was almost absent from *L. flavimaculatum* individuals. Spontaneous origin of both parthenogenetic species from bisexual ancestral species is suggested.

Up to now, the most comprehensive information on microsatellite loci in unisexual vertebrates was obtained in our studies of four parthenogenetic species of the genus *Darevskia* [19]. Using a number of examples it was demonstrated that allelic variants of these loci differed in the structure of microsatellite clusters and in point mutations in the flanking DNA regions. The contribution of mutational variation to the genetic diversity of parthenogenetic species was shown only for one locus (*Du281*), characterized by rather high variability (six allelic variants identified in the sample of 69 specimens) [9]. We showed that microsatellite mutations of deletion/insertion type in the first generation progeny took place in early embryogenesis and could involve either one or both alleles. Moreover, these mutations were not detected upon the analysis of lowly polymorphic loci, which probably, was associated with their low mutability.

In this context, the *Du161(arm)* locus of *D. armeniaca*, characterized by the highest variability, compared to other such loci examined in unisexual lizards of the genus *Darevskia*, deserves special interest. It can be suggested that this locus is highly mutable in the genome of *D. armeniaca*. The differences between 12 allelic variants of *Du161(arm)* were associated with the change of the repeat unit number in microsatellite cluster. It seems likely, that these differences can be explained by the effect of DNA polymerase slippage during replication [29, 30]. It should be noted that in most of the alleles microsatellite is changed by one GATA monomer. The presence of a great number of allele-specific fixed point mutations outside of the microsatellite provides easy allele identification, which can be useful for family and phylogenetic analysis. Interestingly, similar structural variations in the microsatellite cluster flanking regions were described in the genomes of different (unisexual and bisexual) species of the genus *Darevskia* [6–8]. These variations can correspond to the mutation hot points, arising near repeated elements of the genome [31].

According to classification of Weber and Wong [23], most of the alleles of microsatellite loci described in the present study, can be referred to as simple, since they contain only the GATA monomers. Our earlier comparative analysis of a number of loci in parthenogenetic species *D.unisexualis* showed that the loci containing the repeat units of one type in microsatellite cluster were more polymorphic, compared to the loci with complex microsatellites (with two or more types of the repeat units) [23]. This can be the reason for high variability of the *Du161(arm)* locus. At the same time, it seems likely, that microsatellite flanking regions can influence its polymorphism [32].

As a result of detailed structure analysis, the *Du161(arm)* allelic variants were divided into two groups, differing in structural organization of microsatellite clusters (the presence of deletions and insertions), as well as in fixed single nucleotide mutations (haplotypes) outside of the cluster. These differences are thought to be associated with the hybrid nature of parthenogenetic species *D. armeniaca*. The evidence can be obtained in further comparisons of the *Du161(arm)* alleles in parthenogenetic species and its parental bisexual species (*D. valentine* and *D. mixta*), as was already done for some other loci [10].

To explain mutational behavior of microsatellites, a number of basic models and their modifications were suggested. The basic models are represented by "stepwise mutation model" (SMM) [33] and its modified variant, "two-phase mutation model" [34]. Analysis of the individual microsatellite locus mutations in different organisms showed that the changes occurring in 5–75% of the cases followed the multi-phase mutation pattern [3, 35]. Sometimes, mutations in microsatellite loci are associated with the presence of mobile elements in the cluster flanking regions, for example, the *Alu* repeats [36]. Interestigly, our investigations showed that genome of parthenogenetic lizards of the genus *Darevskia* also contained mobile element (retroposon) *Boy-B* LINE [37].

Microsatellite differences observed among the *Du161(arm)* alleles were manifested as the changes of the size of the cluster by one GATA monomer, and corresponded to classical one-step model [33]. However, the changes observed in alleles 6 and 7 corresponded to the modified variant of this model [34], according to which some microsatellites can change the repeat length by more than one repeat unit (multistep variation pattern).

The discovery of haplotype markers and typical nucleotide variations in microsatellite of the *Du161(arm)* locus in parthenogenetic species *D. armeni-*

aca makes it possible to establish the origin of allelic variant of this locus from one or another parental species, as well as to examine phylogenetic relationships between the species of the genus *Darevskia* at the molecular level. As was mentioned above, DNA fingerprint analysis revealed high level of genetic diversity in the samples of *D. armeniaca* [7]. However, the biological significance of such diversity level from the point of view of clonal differentiation remains unclear. Analysis of the distribution patterns of *Du161(arm)* alleles and their combinations (genotypes) in the populations of *D. armeniaca* (Tables 3 and 4) pointed to their rather high genetic diversity. Comparative analysis is possible only for several populations with large sample sizes examined. The most polymorphic of them are the populations of Takyarlu, Megardzor, and Ukrainian, where the number of genotypes identifies constituted 10, 7, and 8, respectively. At the same time, the presence of certain subgroups of the individuals with identical genotypes in these populations seems to reflect clonal diversity of *D. armeniaca* at the *Du161(arm)* locus.

Taken together, the results of the present study provide new information on the origin of allelic diversity of the hybrid genomes of parthenogenetic species and evidence in favor of the existence genetically unstable loci in the genomes of parthenogenetic lizards. Mutations at these loci can make a contribution to the genomic and clonal diversity of unisexual populations.

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