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MOLECULAR GENETICS

Nucleotide Sequences of the Microsatellite Locus *Du215* (*arm*) Allelic Variants in the Parthenospecies *Darevskia armeniaca* (Lacertidae)

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Abstract—Using monolocus PCR analysis with the pairs of primers designed for the *Du215* locus of *Darevskia unisexualis*, allelic polymorphism at the orthologous locus in the populations of the related parthenospecies *D. armeniaca* was investigated. It was demonstrated that *Du215* (*arm*) locus was polymorphic and in the populations of parthenospecies *D. armeniaca* ($n = 127$) represented by at least three allelic variants, differing from each other by the size and composition of microsatellite cluster, and by single nucleotide substitutions in flanking DNA. Unlike the *Du215* locus, *Du215* (*arm*) was shown contain not only GATA, but also (GACA) repeats, which were absent in *D. unisexualis*. Thus, in this study, the data on the molecular nature of allelic polymorphism at one of the microsatellite loci of the parthenospecies *D. armeniaca* were reported.

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INTRODUCTION

Tandemly organized repetitive DNA sequences of different types are a characteristic feature of the eukaryotic genome and can be found both in coding and noncoding regions. Tandem repeats vary in length of the cluster and size of the repetitive unit. Owing to structural similarities with satellite DNA, these sequences were named mini- and microsatellites. Due to high mutation rate, microsatellites play an important role in the genome evolution, producing and maintaining population genetic diversity [1]. The family of GATA/GACA microsatellites [2] is thoroughly studied. It was first discovered in the W chromosome of the snake *Bungarus fasciatus* [3]. The total number and organization of these repeats and their derivatives significantly vary among species. The members of this repeat family are found in all eukaryotic genomes studied so far (plants, different invertebrate species, and mammals, including primates and human). This feature makes possible their use as the genetic markers in different fields of population and evolutionary biology [2, 3].

Studies of individual microsatellite loci showed that changes taking place within these sequences are very diverse, depending on the species of living organism, repeat type, alleles, age, and sex. However, genesis of microsatellite loci, intensively studied in human [4] and some other bisexual species [5], appears to be poorly investigated in the species with the clonal type of reproduction.

Parthenogenetic lizards *Darevskia armeniaca* are widely distributed in Central Armenia and southern regions of Georgia. Like other unisexual species of the genus *Darevskia*, *D. armeniaca* is the species of hybrid origin (parental species are *D. valentini* and *D. mixta*) and is characterized by diploid chromosome number, high fixed heterozygosity of allozyme loci [6], and low mitochondrial DNA restriction site polymorphism [7]. Studies of the allozyme patterns in *D. armeniaca* revealed variability of four out of thirty-five loci investigated, which was manifested as the distribution of two major and two minor allozyme variants in the populations of this species [6]. In addition to allozyme clones, *D. armeniaca* was shown to have two morphological forms with typical variation of dorsal body coloration [8].

Earlier studies [9, 10] showed that all *D. armeniaca* individuals were characterized by species-specific DNA fingerprint profile, differing from DNA fingerprint patterns of other parthenospecies of the genus *Darevskia*. In addition, at the background of species-specific profiles, genetic heterogeneity in some microsatellite loci was demonstrated [10]. Moreover, family fingerprint analysis revealed rather high mutation rate at the $(GATA)_n$- and $(GACA)_n$-containing loci among the first generation progeny [11]. Mutant fingerprint phenotypes revealed in the progeny differed from the maternal ones by electrophoretic mobility of one or several fragments, and were found also in the population sample. Thus, mutations detected in the families of

D. armeniaca positively correlated with high population variability of these markers. At the same time, the nature of the fingerprint markers variability still remains obscure. It can be caused by different reasons, including mutations in the restriction sites and variations in the DNA regions, containing microsatellite cluster [12–15]. In our earlier studies, some loci of *D. unisexualis* containing the $(GATA)_n$ microsatellite were cloned and sequenced [16]. Among these, the *Du215* locus was shown to be polymorphic, and in the populations of *D. unisexualis* ($n = 65$) it was represented by at least three allelic variants, differing by the length of microsatellite cluster.

In the present study, using locus-specific PCR, allelic variants of the *Du215* locus were revealed in another parthenospecies of the genus *Darevskia*, *D. armeniaca* (orthologous locus was designated as *Du215* (*arm*)), and their differences at the level of DNA primary structure were determined.

MATERIALS AND METHODS

Locus-specific PCR analysis was conducted using the sample of 127 DNA specimens of *D. armeniaca* from 15 populations inhabiting Central and Northeastern Armenia. Blood samples were conserved in 0.5 M EDTA solution pH 8.0, and DNA was isolated by standard phenol–chloroform extraction with the use of proteinase K.

PCR amplification of *D. armeniaca* DNA samples was conducted using the pair of primers selected earlier for the *Du215* locus of the parthenospecies *D. unisexualis* [16, 17]. The reaction was carried out in a final volume of 20 μl, containing 1× buffer for *Taq* polymerase (Dialat); 250 μM of each dNTP; 2 mM $MgCl_2$; 10 μM of each primer (5'-CAACTAGCAGTAGCTCTCCAGA-3'; 5'-CCAGACAGGCCCCAACTT-3'); 0.8 units of *Taq* polymerase (Dialat). The reaction mixture was supplemented with 20 ng of genomic DNA. Amplification was performed in the Tertsik four-channel thermal cycler (TP4-PCR-01). The reaction conditions included denaturing for 3 min (94°C), followed by 30 cycles of 94°C for 1 min; 58°C for 40 s; 72°C for 40 s; and the 5-min final extension at 72°C.

Amplification products were fractionated in 8% polyacryamide gel (PAAG) and visualized by staining with ethidium bromide. To establish the nature of the amplified DNA fragments, the latter were extracted from polyacrylamide gels and cloned into *Escherichia coli* in the pMos Blue vector (Amersham), according to the standard protocol of the manufacturer. Sequencing of amplification products was performed according to the method of Sanger with the ABI PRISM(R)BigDye™ Terminator v. 3.1 reagent kit and subsequent analysis of the reaction products on the automated sequencer DNA ABI PRISM 3100-Avant. Nucleotide sequences were aligned using the MegAlign 4.05 software program.

RESULTS AND DISCUSSION

The results of monolocus PCR analysis of *D. armeniaca* individuals from 15 isolated populations from Armenia are shown in Fig. 1. It can be seen that the *Du215* (*arm*) locus is heterozygous, and in the population of the parthenospecies *D. armeniaca* it is represented by at least three allelic variants, differing in electrophoretic mobility of amplification products in the interval of 200 to 250 nucleotide pairs (designated by arrows). Note that the populations examined differed in frequencies of these allelic variants (Table 1).

Interestingly, all individuals from the Takyarlu population represented by two morphological forms, distinguished by body coloration, appeared to be electrophoretically invariant and heterozygous at the locus examined (Fig. 1, lanes 1 to 21). The animals from the so-called Ukrainian population (animals from local, isolated population Medved–Gora from Armenia, introduced to the Ukraine in 1964 [18]) were also heterozygous. It should be noted that in this population, three *Du215* (*arm*) allelic variants were observed (lanes 22 to 38). Three allelic variants of this locus, differing in electrophoretic mobility, were also detected in the population of Alaverdy (lanes 40 to 42). In the remaining populations examined, the *Du215* (*arm*) produced two amplificants with clearly different electrophoretic mobilities, and seemed to be represented by only two allelic variants.

To examine the molecular nature of the *Du215* (*arm*) allelic polymorphism in populations of *D. armeniaca*, sequencing of the PCR products, corresponding to its three allelic variants was performed. As shown in the Fig. 2, sequences of the *Du215* (*arm*) allelic variants in *D. armeniaca* differ from the sequence of the *Du215* clone in parthenospecies *D. unisexualis* (GenBank accession no. AY574978) by the size and composition of microsatellite clausters, as well as by some single nucleotide substitutions and indels in the flanking DNA regions. Analysis of the *Du215* (*arm*) allele sequences showed that their microsatellite clusters (Table 2) have complex structure. The *Du215* (*arm*) alleles 1 and 2 contain $(GATA)_n$ and $(GACA)_n$ clusters and differ from one another by one (GATA) monomeric unit. The *Du215* (*arm*) allele 3 contains no $(GACA)_n$ repeats and has shorter $(GATA)_n$ cluster. Variability of terminal microsatellite regions in the *Du215* (*arm*) and orthologous *Du215* alleles should be also mentioned. This variability is manifested as either the presence or absence of the 5'-terminal GACA monomer along with different copy number of the GCAA unit at the 3' end of the corresponding variant of the given locus. In addition, the *Du215* (*arm*) alleles 1 to 3 differ from one another in point mutations of transition and transversion types, which are located at fixed distances (–19, –38, and –58 bp from the beginning of microsatellite cluster). Furthermore, point mutations form two haplotypes, T–G–C (alleles 1 and 2) and A–C–T (allele3). Interestingly, alleles of the *Du215* orthologous locus in *D. uni-*

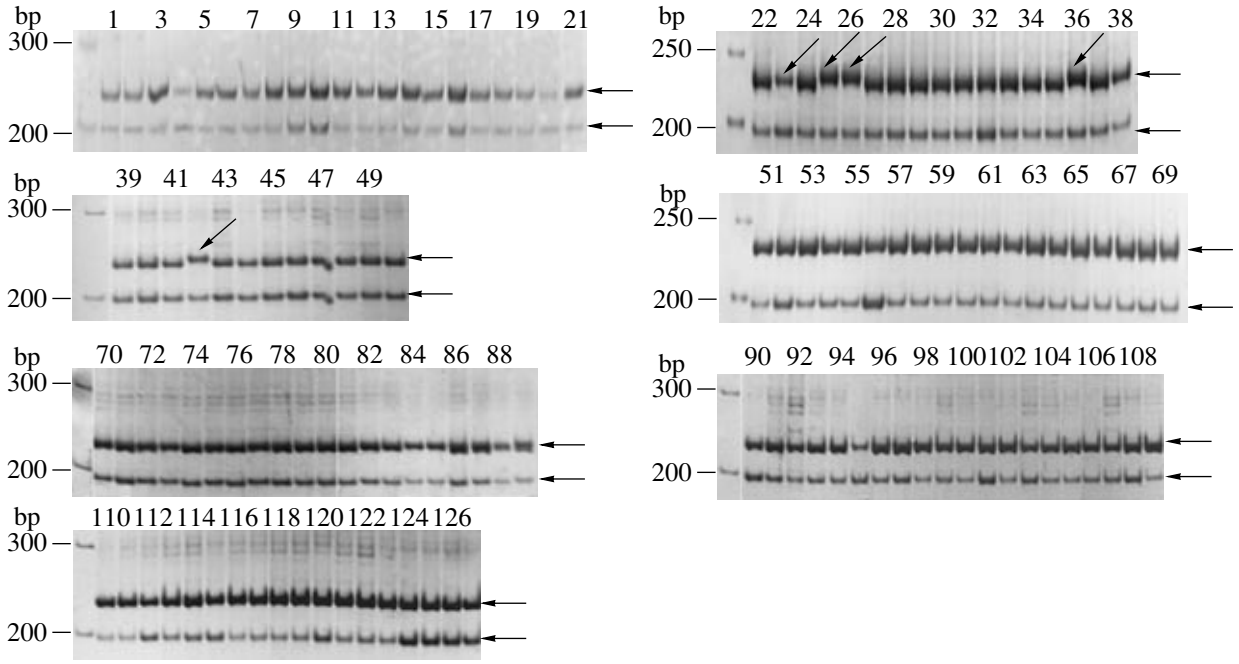

Fig. 1. PAAG electrophoresis of the *Du215* (*arm*) PCR products in *D. armeniaca* from different populations: Takyarlu (1–21), Ukrainian population (22–38), Lchashen (39), Alaverdy (40–42), Megradzor (43–50), Medved'-Gora (51–62), Pushkin Pass (63–69), Semenovskii Pass (70–77), Papanino (78–81), Tezh (82–89), Stepanovan (90–98), Kutchak (99–105), Gosh (106), Sotk (107–109), and Artik (110–127). Marker fragment, 50 bp (Amersham). Allelic variants are designated by the arrows.

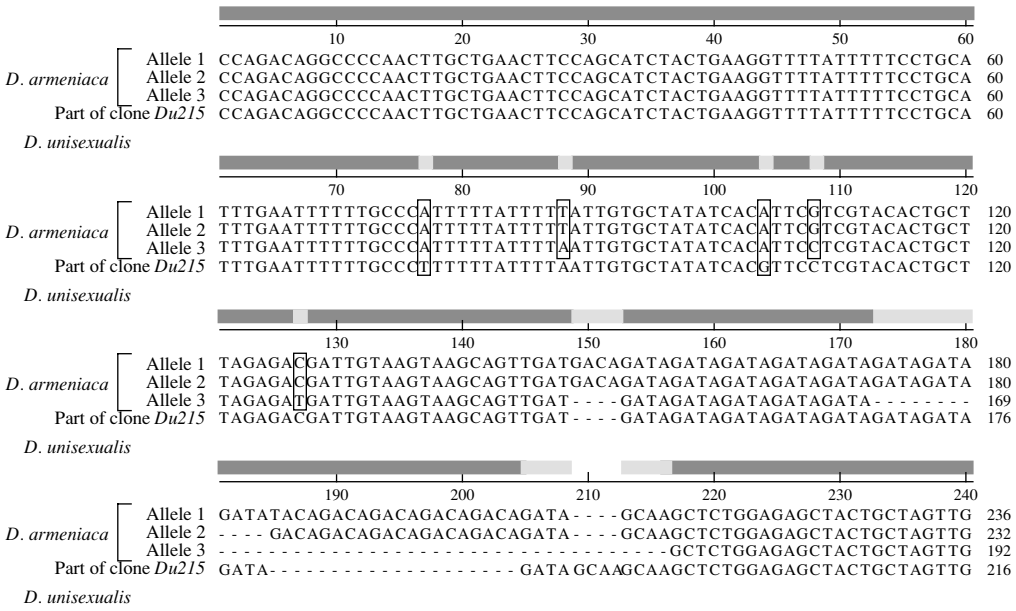

Fig. 2. Sequence comparison of *D. armeniaca* three *Du215* (*arm*) allelic variants with one another and with the orthologous locus *Du215* (GenBank, ac. no. AY574978) from the relative parthenospecies *D. unisexualis*. Coinciding regions are marked by darker color on the upper lane. Single nucleotide substitutions are marked by the frames.

sexualis also posess fixed point substitutions, located at the distances of –42 and –69 bp from the beginning of microsatellite cluster, respectively. They form haplotypes A–A and T–G [19]. Figure 2 demonstrates the *Du215* allelic variant with haplotype T–G. In our earlier study, analysis of another polymorphic locus, *Du281*, of *D. unisexualis* showed the presence of point substitutions (and the corresponding haplotypes) within the microsatellite flanking DNA sequences [16]. Furthermore, according to our preliminary data, these

Table 1. Population genetic characteristics of the *Du215* (*arm*) locus in parthenospecies *D. armeniaca*

Population	No. of individuals	No. of allelic variants
Takyarlu	21	2
Ukrainian population	17	3
Alaverdy	3	3
Lchashen	1	2
Megradzor	8	2
Medved'-Gora	12	2
Pushkin Pass	7	2
Semenovskii Pass	8	2
Papanino	4	2
Tezh	8	2
Stepanovan	9	2
Kutchak	7	2
Gosh	1	2
Sotk	3	2
Artik	18	2

Note: Central populations: Takyarlu, Lchashen, Megradzor, Medved'-Gora, Pushkin Pass, Semenovskii Pass, and Tezh; northern populations: Alaverdy, and Stepanovan; northwestern population: Artik; northeastern populations: Papanino and Gosh; eastern populations: Kutchak and Sotk.

Table 2. Microsatellite clusters of the alleles of the *Du215* (*arm*) and orthologous *Du215* loci

Allele	Microsatellite cluster sequence
Allele 1	$GATGACA(GATA)_7(GACA)_5(GATA)_1GCAA$
Allele 2	$GATGACA(GATA)_8(GACA)_5(GATA)_1GCAA$
Allele 3	$GAT(GATA)_5$
Du215	$GAT(GATA)_9(GCAA)_2$

haplotypes were inherited by parthenospecies *D. unisexualis* from its two parental species, *D. raddei* and *D. valentini*. Thus, the presence of point substitutions in close vicinity of the polymorphic loci microsatellite clusters seems to be the characteristic feature of the parthenospecies examined, as well as of their bisexual parental species from the genus *Darevskia*.

The differences between the *Du215* (*arm*) alleles 1 and 2 observed were manifested as the change of the cluster size by one GATA monomeric unit, which corresponded to classic stepwise model [20, 21]. However, the changes in the structure of allele 3 corresponded to the modified variant of this model [22], according to which some microsatellites are capable of increasing the repeat length by more than one monomeric unit (multistep variability pattern). Analysis of the mutations in individual microsatellite loci from different organisms showed that the changes occurred within these sequences in 5 to 75% of the cases were multistep mutations [23]. For instance, in *Danio rerio* (zebrafish) 68% of mutations in microsatellite loci were multistep [24]. In addition, the data on the polarity of mutations in microsatellite DNA [25], as well as on the increased frequency of single nucleotide substitutions in the microsatellite flanking DNA sequences [26], were reported. Furthermore, the mutation frequency in the flanking regions can be higher than that in the microsatellite cluster, which was demonstrated for dinucleotide microsatellites of inbertebrates [27, 28]. It was demonstrated that in *D. unisexualis* and *D. armeniaca* allelic variants of the two loci, *Du215* and *Du281*, contained point substitutions within the microsatellite flanking regions ([17] and the present study). It is suggested that these nucleotide substitutions with certain periodicity can be found in the genomes of different species of the genus *Darevskia*. They may correspond to the mutation hot spots that arise near repetitive genome elements [29]. It is apparent that nucleotide substitutions should inevitably lead to restriction polymorphism, which was observed earlier in fingerprint profiles of the Caucasian rock lizards [9]. It should be noted that two single nucleotide substitution haplotypes were discovered in the human *DXS981* locus, containing the $(TATC)_n$ microsatellite sequence. It is suggested that the pattern of the microsatyellite allele distribution over the haplotypes point to the independent evolution of the alleles of this locus within the haplotype limits [30]. In case of parthenogenetic lizards characterized by the hybrid origin, the haplotypes revealed can be used for the analysis of phylogenetic relationships between the unisexual and bisexual species of the genus *Darevskia.*

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