Chapter 1

The New Approach for Genotyping of Parthenogenetic Lizard Populations of the Genus Darevskia Based on Microsatellite-Containing Loci

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Abstract

Microsatellites, comprising tandemly repeated short nucleotide sequences, are ubiquitous in eukaryotic genomes. Mutations within microsatellites are frequent, altering their overall length by insertion or deletion of a small number of repeat units. Mutation rates at microsatellite sites can be several orders higher in comparison with low rates for genomic nucleotide substitutions. Despite their high mutability, stable allele frequency distributions are typically observed for microsatellites in humans as well as other primates, although the mechanism maintaining these stable distributions remains unclear. Genetic variation at many microsatellite loci is characterized by

high heterozygosity and the presence of multiple alleles. Therefore, microsatellites have been the most widely used markers in genetic studies. Here we briefly provide general information concerning microsatellites, – their definition, genomic distribution, genetic variation, some applications in genetic analysis. New approach for microsatellite genotyping of unisexual lizard species is described in more detail. The approach reveals parent-specific markers consisting of microsatellites and single nucleotides located out of microsatellite cluster for each allele of each locus. The single nucleotide variable markers yield direct information about interspecific hybridization founder events, and microsatellite variability provides additional information concerning possible mutations in the initial hybrid clones that give rise to new genotypes.

Introduction

Hundreds of thousands of microsatellites or short tandem repeats (STRs) are interspersed throughout mammalian genomes. They are of special interest because the numbers of repeats within specific STRs tend to be highly variable, and because these short tandem repeat polymorphisms can be rapidly analyzed using PCR. Thousands of STRs have been developed and mapped for the human, mouse, rat, and other genomes, and they have been used to map dozens of genes responsible for heritable disorders [1-3]. The bulk of simple repeats are embedded in noncoding DNA, either in the intergenic sequence or in the

introns. They are so abound in eukaryotic genomes that many genes, include variable STRs within regulatory and/or coding domains. The distribution of particular motif classes within a genome can vary substantially among different species. In humans, STRs can originate in coding regions, leading to the appearance of repetitive patterns in protein sequences. It was reported that tandem repeats are common in many proteins, and the mechanisms involved in their genesis may contribute to the rapid evolution of proteins [4,5]. Mono-, di-, tri and tetranucleotide repeats are the main types of microsatellite, but repeats of five or six nucleotides are also classified as microsatellites. According to their composition, STRs can be classified as: *i)* perfect if composed of a single motif; *ii)* imperfect if other base pair occurs between repeats; *iii)* interrupted if a sequence of a few base pairs is inserted into the motif; or *iv)* composite if formed by several, repetitive motifs [6]. Trinucleotide repeats associated with human disease comprise a special class of microsatellites in coding DNA. These loci undergo extensive repeat expansions, the mutational mechanism of which is thought to differ from that of most microsatellites in the genome. For instance, the establishment of hairpin structures with a relatively high amount of base-pair complementarities might stabilize loops that are generated during replication slippage [7].

From the beginning tandem repeats have been designated as nonfunctional DNA. With the exception of

tandem repeats involved in human neurodegenerative diseases, repeat variation was often believed to be neutral with no phenotypic consequences [8]. The detection of microsatellites in transcripts and regulatory regions of the genome encouraged scientific interest in discovering their possible biological functions. Some studies have presented data that microsatellites play a role in such processes as the regulation of transcription and translation, organization of chromatin [9,10]. Probably the biological function of a microsatellite is related to its position in the genome. It was suggested that STRs may play an important role in contributing to the different expression profiles of housekeeping and tissue-specific genes [11]. Microsatellites located in introns can play a role in the transport and alternative splicing of mRNA. In the promotor, STRs can influence gene expression to possible alterations caused by expansion or contraction of repeat sequences. These alterations can result in an increase or reduction in the level of gene expression caused by changes in transcription factor linkage sites. Tandem repeats in intergenic regions can cause changes in the secondary structure of the DNA altering the chromatin structure, which can results in alterations in the expression of nearby genes.

An attractive model of microsatellite evolution holds that a genome-wide distribution of microsatellite repeat length that is at equilibrium results from a balance between length and point mutations [12]. According to

this model, two opposing mutational forces operate on microsatellite sequences. Length mutations, the rate of which increases with increasing repeat count, favour loci to attain arbitrarily high values, whereas point mutations break long repeat arrays into smaller units. The high diversity mutation rates of STRs support a hypothesis that their mutability can be evolutionarily beneficial. Another explanation is that natural selection has limited ability to eliminate mutation.

Microsatellite mutations can be studied using a number of approaches. The most straightforward way is the direct detection of mutation events in pedigree genotyping. An alternative strategy is to use inbred lines that have been propagated for several generations. More indirect approaches rely on sequence analyses of different allele types within a species or of orthologous sequences in related species [13]. The high rate of mutation at microsatellite loci that ranged from 10^{-4} to nearly 10^{-2} per locus per gamete per generation in humans makes it possible to observe mutation events directly; data on *de novo* mutations have now been reported for a range of loci and organisms [14,15]. Length changes in microsatellite DNA are generally thought to arise from replication slippage – that is, transient dissociation of the replicating DNA strands followed by misaligned reassociation. When the nascent strand realigns out of register, renewed replication will lead to the insertion or deletion of repeat units relative

to the template strand. Most of these primary mutations are corrected by the mismatch repair system, and only the small fraction that was not repaired ends up as microsatellite mutation events [16].

Microsatellite markers are enormously useful in studies of population structure, genetic mapping, and evolutionary processes. Microsatellites with core repeats 3 to 5 nucleotides long are preferred in forensics and parentage analysis. It is worth noting that a number of STR search algorithms have been developed [17-19]. After identifying the sequences containing STRs, specific primers must be synthesized, complementary to the flanking regions, followed by amplification and polymorphism testing [20-22]. Microsatellite genomic distribution, biological function and practical utility have been reviewed in a number of articles [6-9,23-28].

Microsatellites in Multilocus DNA Fingerprinting of Parthenogenetic Lizard Species

To study clonal and genome diversity in unisexual vertebrate populations, several approaches including histocompatibility analysis, extensive surveys of mtDNA and allozyme variations, and in more rare cases, DNA fingerprinting have been used. Of these techniques, histocompatibility analysis and DNA fingerprinting were clearly

more sensitive. At the same time, in its present form, the histocompatibility technique is difficult to use on a survey scale.

Jeffreys et al. [29] were first to use the minisatellite probes 33.6 and 33.15 to detect numerous homologous loci in human DNA, and have demonstrated the individual-specific pictures of blot hybridizations that were termed DNA fingerprints. Later, the M13 family of minisatellites was characterized [30,31], as well as a number of microsatellites [32]. At present, DNA fingerprinting is considered potentially very powerful tool for population studies of species whose genomes are poorly investigated. The features and possible applications of DNA fingerprinting were considered in earlier reviews [33,34]. To date, only limited number of unisexual fish and lizard species were investigated with the use of DNA fingerprinting.

Unisexual vertebrates recognized first in 1932 [35] represent exciting cases of wild populations consisting of predominantly or exclusively females. According to [36], they share four features: (1) they are of interspecific hybrid origin, (2) they are exclusively female or nearly so, (3) they reproduce via an aberrant gametogenetic mechanism (meiotic or premeiotic) that inhibits genetic recombination and causes clonal inheritance, and (4) they often include polyploids. At present, there are more than 80 known unisexual vertebrate species and forms of fishes, amphibians, and reptiles, distributed on five continents

[37]. Three modes of reproduction can be distinguished within the unisexual vertebrates whose reproductive mechanisms are understood: (1) parthenogenesis, in which the hybrid genome is transmitted intact to the eggs, which develop into genetically identical offspring in the absence of sperm, (2) gynogenesis, in which sperm from a related bisexual ancestor activates development of the eggs but syngamy of egg and sperm does not occur and resulting offspring are genetically identical to their mother and to each other, and (3) hybridogenesis, in which the part of the hybrid genome derived from one parental species is inherited clonally, while the genome from the other parental species is lost and replaced in each generation through fertilization.

Among vertebrates, true parthenogenesis, where reproduction proceeds without male participation, has been detected only in squamate reptiles, especially in lizards. The Lacertidae was the first family in which this phenomenon was discovered [38]. In most known instances, parthenogenetic species originated from interspecific hybridization between closely related bisexual species [39,40]. Because of their hybrid origin, parthenogenetic species combine much of the genetic diversity of two parental sexual species. The more divergent hybridizing parental species are, the greater ability to persist in nature over many generation because distinct parental alleles form heterozygous loci in the hybrid genome. This provides evolutionary stability for parthenogenetic species. Each

parthenogenetic species usually consists of several clonal lineages, possibly caused by either mutations (especially in hypervariable microsatellite loci), multiple origins from different pairs of founders, rare new hybridization events, or some level of genetic recombination [41-43]. Clonal diversity also appears to correlate with the size of the area of origin, distinct ecological conditions of the habitat, and age of parthenogenetic species [44-46].

The lacertid genus *Darevskia* is of particular significance because its species have been the subjects of extensive ecological and biogeographical studies, and because parthenogenesis has arisen several times within the group [39]. *Darevskia* includes 17 bisexual species and seven parthenogenetic, diploid species of hybrid origin [40]. Previous studies on these parthenogenetic species revealed some degree of allozyme variation and low variability of mitochondrial DNA [46-50]. The basic biology including comparative anatomy, chromosome cytology, biochemical genetics, ecology, and biogeography have been studied in details in some of these species [51,52]. However, only four diploid parthenogenetic lizard species of the genus *Darevskia* (*D. dahli, D. armeniaca, D. unisexualis,* and *D. rostombekowi*) were investigated by DNA fingerprinting technique.

Parthenogenetic lizards *D. dahli* from two populations of Armenia were first fingerprinted using hybridization probes M13 DNA, $(TCC)_{50}$, $(GACA)_4$, and $(CAC)_5$

[53,54]. DNA fingerprints produced by minisatellite probe M13 DNA were practically identical for all specimens in each population, while some interpopulation differences were observed. Microsatellite probes also produced species specific fingerprints but a number of variable fragments were clearly detected in both populations. Out of 25 individuals of *D. dahli* analyzed, 17 (68%) yielded 5 fingerprint phenotypes (clones), represented by 6 (24%), 4 (16%), 3 (12%), and 2 (8%) individuals. Similar analysis was performed with *D. armeniaca* [54,55] and *D. unisexualis* [54,56-58] lizards. Out of 36 individuals of *D. armeniaca* from three populations of Armenia, 19 (52.7%) were classified into 6 clones represented by 5 (13.8%), 4 (11%), 3 (8%), 2 (5.5%), and 2 (5.5%) lizards. Out of 40 lizards of *D. unisexualis* from three Armenian populations analyzed with M13 DNA and $(GATA)_4$ probes, 28 (70%) were assigned to 4 clones consisting of 16 (40%), 7 (17.5%), 3 (7.5%), and 2 (5%) individuals, respectively. The most polymorphic, practically individual-specific fingerprints were observed when *D. unisexualis* populations were analyzed with the $(TCC)_{50}$ and $(TCT)_5$ probes [56,57]. Typical picture of fingerprint analysis of parthenogenetic population is shown in Figure 1. Variation of mini- and microsatellites-containing loci in populations of parthenogenetic lizards *D. rostombekowi* was studied in [59]. Previously, the allozyme data for 35 loci of *D. rostombekowi* (sample of 65 animals from three Armenian populations) did not show any variation [60]. Therefore, *D. rostombekowi* was

considered a monoclonal species. In contrast, DNA fingerprinting with M13 minisatellite, $(GATA)_4$ and $(TCC)_{50}$ microsatellite probes in samples of 21 animals from 3 isolated populations of North Armenia and in a sample of 5 animals from relict population of Sevan Lake indicated that relict population of Sevan Lake was significantly different from others [56]. It should be mentioned that the biological significance of genetic heterogeneity detected by DNA fingerprinting of natural populations of parthenogenetic lizards with respect to clonal differentiation remains uncertain.

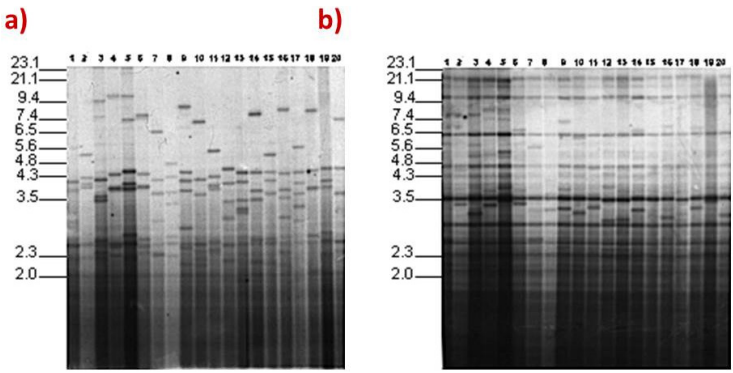

Figure 1: DNA fingerprinting of parthenogenetic lizard individuals of *Darevskia unisexualis* using microsatellite probes $(TCC)_{50}$ (a) and $(TCT)_{10}$ (b).

To date, only parthenogenetic families of *D. armeniaca* and *D. unisexualis* consisting of mother and their progeny of first generation were investigated by DNA fingerprinting. Malysheva et al. [61] have fingerprinted 43 *D. armeniaca* families (131 siblings) using $(GACA)_4$,

$(GGCA)_4$, $(GATA)_4$, and $(CAC)_n$ probes, and revealed mutant fingerprints in 4 families (16 siblings) that differed from their mothers in several restriction DNA fragments. Mutation rate for new restriction fragment length estimated by these probes varied from $0.8x10^{-2}$ to $4.9x10^{-2}$ per fragment/sibling. As a rule, identical mutant fingerprints were observed in all siblings of one family. It means that the most variations, detected as RFLPs, had germ line origin, but somatic changes of $(CAC)_n$ fingerprints were also found. In similar experiments, rather high intrafamily variation of $(GATA)_n$, $(TCT)_n$, and $(TCC)_n$ containing DNA fragments was shown for cogeneric parthenogenetic species *D. unisexualis* [55,57,62]. For instance, mutation rate for new $(GATA)_n$-containing fragments, revealed in 25 families (overall 84 siblings), was as high as $0.9x10^{-2}$ per fragment/sibling. The case of somatic variation of $(GATA)_n$ detectable loci in adult *D. unisexualis* lizards was also described [62]. Typical pictures of intrafamily variability of DNA fingerprints are shown in Figure 2. It is clear that differences in fragment size detected as RFLP appear to be too large to reflect microsatellite repeat number variation and seem likely to reflect nucleotide variation in restriction sites of hybrid parthenogenetic genomes due to its high heterozyosity.

Summarizing, DNA fingerprinting studies provide some indirect evidence for existing variations in parthenogenetic genomes. To obtain more direct information about polymorphism and molecular structure of micro-

satellite-containing loci in clonal species, new molecular approaches should be applied. It is evident that DNA cloning and sequencing of allelic variants of microsatellite-containing loci may give detailed information on the nature of their variability. At present, very limited information is available concerning structural organization and allelic polymorphism of individual microsatellite loci in unisexual vertebrates.

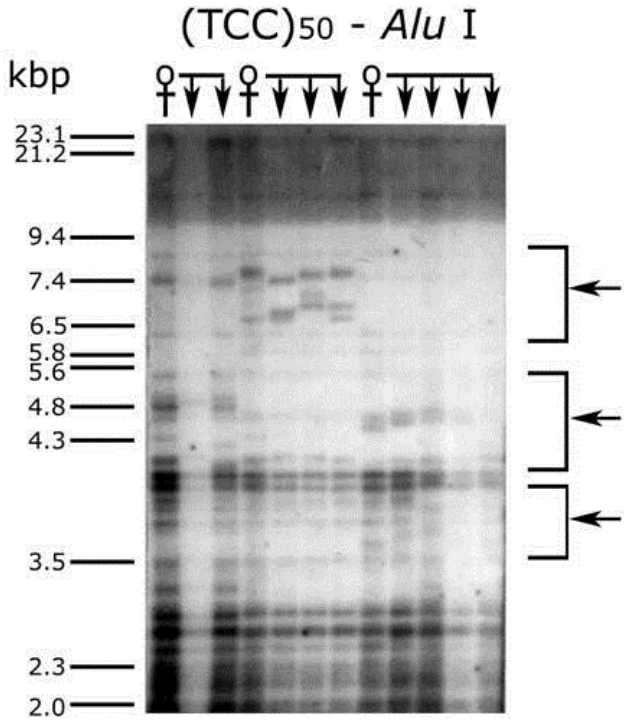

Figure 2: DNA fingerprinting of parthenogenetic lizard families of *Darevskia unisexualis* using microsatellite probe $(TCC)_{50}$.

Polymorphism of Microsatellite-Containing Loci in Hybrid Genomes of Parthenogenetic Lizard Species

Parthenogenetic species of hybrid origin combine the genetic diversity of two parental sexual species. The more divergent hybridizing parental species are, the higher heterozygosity of genome of hybrid species at codominant loci exists. Therefore, to further study genetic and clonal diversity in parthenogenetic species, more information on organization and polymorphism of individual microsatellite-containing loci as well as the data on the rate of microsatellite mutations in the genomes should be obtained. The most extensive information about origin allelic variation of microsatellite-containing loci in parthenogenetic species was followed from our studies of *Darevskia* lizards.

Korchagin et al. [63] have cloned and sequenced a number of $(GATA)_n$ microsatellite loci of *D. unisexualis*. Among several loci analyzed in detail, two (Du 215 and Du 281) were polymorphic. Three and six allelic variants were detected among 65 lizards investigated for Du 215 and Du 281, respectively. Sequencing the PCR products amplified from these variants showed that allelic differences at the polymorphic loci were caused by variation in a number of tandem repeats and point mutations in microsatellite cluster as well as single nucleotide variation (SNV) in the flanking regions. Comparison of polymor-

phic and monomorphic $(GATA)_n$-containing loci showed that the monomorphic ones contained more point mutations in microsatellite clusters as well as degenerative $(GATA)_n$-like sequences in the vicinity of these clusters or large insertions of $(GACA)_n$ in the middle of $(GATA)_n$ cluster. These data are consistent with the view that loci containing less perfect microsatellites are more stable and vice versa. Authors carried out cross-species analysis of polymorphic Du 215 and Du 281 and monomorphic Du 323 indicating that the PCR priming sites at the *D. unisexualis* were conserved in other parthenogenetic and bisexual species of genus *Darevskia*. Using these locus-specific primers, similar analysis have shown that alleles of all microsatellite-containing loci studied in parthenogenetic species *D. dahli*, *D. armeniaca* and *D. rostombekowi* differ from each other by the structure of microsatellite clusters and by single nucleotides at fixed positions out of microsatellites [61,64,65]. Typical picture that shows interallelic differences at microsatellite loci in parthenogen is presented in Figure 3.

We differentiated between designations SNV and single nucleotide polymorphisms (SNPs). In hybrid genomes of parthenogenetic lizards, SNVs originated via combining the codominant loci in divergent parental genomes. SNVs were fixed in all individuals of a distinct clonal lineage and represented parent-specific markers inherited by parthenogenetic species from a distinct pair of parents. Thus, distinct clonal SNVs likely owed to independent hy-

bridization founder events. In bisexual species, SNPs designate inter-individual allelic differences in polymorphic loci that originated via mutation in a population.

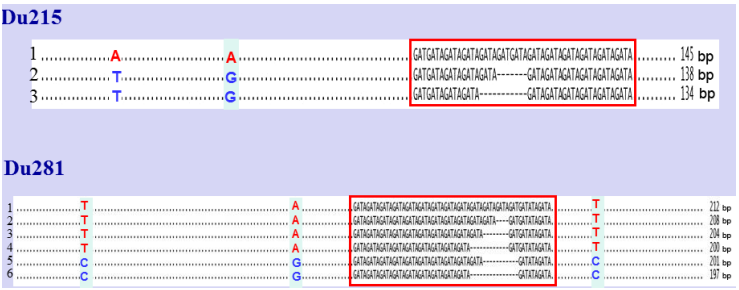

Figure 3: Interallelic microsatellite and single nucleotide Du215 and Du281 loci in parthenogen *Darevskia unisexualis.*

To study microsatellite mutations in parthenogenetic lizard progeny, DNA samples of 217 lizards (49 mothers and 168 offspring) of *D. unisexualis* and 197 lizards (50 mothers and 147 offspring) of *D. armeniaca* were screened by locus-specific PCR [66]. Mutant alleles were detected as changes in the electrophoretic mobility of PCR amplification products obtained from mother and their offspring. No mutant alleles were detected in *D. armeniaca* offspring at both Du281 and Du215 loci, and in *D. unisexualis* offspring at Du215 locus. At the same time, 15 mutant alleles among offspring of four *D. unisexualis* mothers were found at the Du281 locus. These data show that the Du281 locus of *D. unisexualis* is highly mutable, with an estimated mutation rate of 0.1428 events per germ line tissue. Comparison of nucleotide sequences of mothers and their

offspring revealed mutations only in $(GATA)_n$ microsatellite clusters, while no mutations were found in the flanking regions (Table 1). The haplotypes (T-A-T and C-G-C), formed by fixed point mutations in the flanking regions of microsatellite cluster, and specific for allelic variants of Du281 [63] were used to mark maternal and corresponding offspring alleles. In family 1, consisting of the mother and one offspring, the deletion of one GATA monomer in microsatellite cluster was found in both offspring alleles. In family 2, consisting of the mother and five offspring, only one offspring allele marked by haplotype C-G-C was mutant with the deletion of GATATA in the microsatellite cluster of all offspring. In family 3, consisting of the mother and two offspring, an insertion of one GATA monomer was found in the microsatellite cluster of both offspring alleles. Family 4, consisting of the mother and four offspring, represent a more complicated case. While no mutations were observed among offspring alleles marked by haplotype T-A-T, different pattern of mutation was found among offspring alleles marked by haplotype C-G-C. In three offspring the mutant alleles revealed a deletion of one GATA monomer, but in another offspring a GATATA sequence was lost in the microsatellite cluster. Thus, in this study authors characterized highly unstable $(GATA)_n$ containing locus in parthenogenetic lizard species *D. unisexualis*. Additionally, they characterized various types of mutant alleles of this locus found in the *D. unisexualis* offspring of the first generation. Comparison of maternal and offspring alleles of two polymorphic loci revealed de novo mutations only at the Du281 locus in *D. unisexualis*.

The obtained mutation rate for Du281 is comparable with the earlier results of DNA fingerprinting analysis.

Table 1: Allelic variants of microsatellite clusters of Du281 locus in parthenogenetic lizard families *D. unisexualis*.

Family 1.	
Maternal (M1) allele 1	•••T•••A•••(GATA)$_9$ (GATA) GAT(GATA)TA(GATA)•••T•••
Offspring (1) allele 1	•••T•••A•••(GATA)$_9$ - - - - GAT(GATA)TA(GATA)•••T•••
Maternal (M1) allele 2	•••C•••G•••(GATA)$_9$ (GATA) (GATA)TA(GATA)•••G•••
Offspring (1) allele 2	•••C•••G•••(GATA)$_9$ - - - - (GATA)TA(GATA)•••G•••
Family 2.	
Maternal (M2) allele 1	•••T•••A•••(GATA)$_9$ GAT(GATA)TA(GATA)•••T•••
Offspring (1 – 5) allele 1	•••T•••A•••(GATA)$_9$ GAT(GATA)TA(GATA)•••T•••
Maternal (M2) allele 2	•••C•••G•••(GATA)$_9$ (GATA)TA(GATA)•••G•••
Offspring (1 – 5) allele 2	•••C•••G•••(GATA)$_9$ - - - - - - (GATA)•••G•••
Family 3.	
Maternal (M3) allele 1	•••T•••A•••(GATA)$_9$ - - - - GAT(GATA)TA(GATA)•••T•••
Offspring (1, 2) allele 1	•••T•••A•••(GATA)$_9$ (GATA)GAT(GATA)TA(GATA)•••T•••
Maternal (M3) allele 2	•••C•••G•••(GATA)$_9$ - - - - (GATA)TA(GATA)•••G•••
Offspring (1, 2) allele 2	•••C•••G•••(GATA)$_9$ (GATA) (GATA)TA(GATA)•••G•••
Family 4.	
Maternal (M4) allele 1	•••T•••A•••(GATA)$_9$ GAT(GATA)TA(GATA)•••T•••
Offspring (1, 3, 4) allele 1	•••T•••A•••(GATA)$_9$ GAT(GATA)TA(GATA)•••T•••
Offspring (2) allele 1	•••T•••A•••(GATA)$_9$ GAT(GATA)TA(GATA)•••T•••
Maternal (M4) allele 2	•••C•••G•••(GATA)$_9$ (GATA) (GATA)TA(GATA)•••G•••
Offspring (1, 3, 4) allele 2	•••C•••G•••(GATA)$_9$ - - - - (GATA)TA(GATA)•••G•••

Variations in microsatellite clusters are denoted by bold letters. T-A-T and C-G-C are haplotypes specific for allelic variants of *D. unisexualis* [63]. In Family 2 the observed changes were the same in all offspring (1–5). In family 3 the observed changes were the same in all offspring (1, 2). In Family 4 the observed changes are the same in three offspring (1, 3 and 4).

Based on above presented information on allelic variation of microsatellite-containing loci the new genotyping to study clonal diversity and clone formation in parthenogenetic species was developed [64]. This analysis includes several stages:

I. study of genetic polymorphism of microsatellite-containing loci in lizard populations of parthenogen; revealing and sequencing of allelic variants of each locus;

II. determination of individual genotypes for all lizards, based on allelic combinations of all loci used;

III. identification of individuals with the same genotype as distinct clonal lineages;

IV. determination of markers specific for distinct clonal lineages, based on combinations of parent-specific SNV and microsatellite markers of both alleles of the loci.

Some data obtained in this way for parthenogenetic lizard species *D. dahli* are given below.

The New Approach For Locus-Specific Genotyping of Hybrid Genomes of Parthenogenetic Lizards Based on Microsatellite and Single Nucleotide Variations

D. dahli, which is one of the seven unisexual species, occurs in the southern Caucasus Mountains of Armenia and Georgia. Allozyme and mtDNA data analysis identified the sexual parents of *D. dahli* as *D. mixta* and *D. portschinskii* [39,67]. Examination of allozyme variation in *D. dahli* revealed the existence of five clones; one clone was widespread and abundant, and four other clones were rare and geographically restricted [47]. Based on such a pattern of allozyme clone distribution and the current allopatric occurrence of the parental bisexual species, these authors concluded that few bisexual individuals were involved in the formation of the *D. dahli* species, and that the initial successful clone was formed only once. In DNA fingerprinting studies of *D. dahli*, a rather high level of variation among individual fingerprint patterns was demonstrated when various microsatellite probes were used for blot hybridization [53,68]. However, the origin of this variation–whether it occurred as a result of point mutations or more complex genomic reorganization–remains obscure.

New information regarding genetic variation and clone formation in *D. dahli* was obtained via molecular

and genetic studies of individual genomic loci [64]. For this, 111 specimens of *D. dahli* from five populations of Armenia were analyzed using locus-specific PCR and DNA sequencing of PCR amplificants. It was shown that all *D. dahli* individuals but one were heterozygous at the three loci analyzed, and contained two alleles that differed from each other regarding the length and structure of microsatellite clusters, and regarding single nucleotide variations (SNVs) in fixed positions of the flanking regions (Table 2).

Table 2: Allelic variations of microsatellite containing loci Du215, Du281, and Du323 in parthenogenetic *D. dahli species.*

Allelic variant	Size (bp)	Structure of microsatellite cluster	Fixed nucleotide variations[1]	EMBL/ Gene Bank ac. №
Du215(dahli)1	252	5' (GAT)(GACA)(GATA)$_{10}$(GACA)$_7$(GATA)(GCAA) 3'	T (–58), G (–38), C (–19)	FJ981592
Du215(dahli)2	248	5' (GAT)(GACA)(GATA)$_9$ (GACA)$_7$(GATA)(GCAA) 3'	T (–58), G (–38), C (–19)	FJ981593
Du215(dahli)3	244	5' (GAT)(GACA)(GATA)$_8$ (GACA)$_7$(GATA)(GCAA) 3'	T (–58), G (–38), C (–19)	FJ981594
Du215(dahli)4	232	5' (GAT)(GACA)(GATA)$_7$ (GACA)$_5$(GATA)(GCAA) 3'	T (–58), G (–38), C (–19)	FJ981595
Du215(dahli)5	232	5' (GAT)(GATA)$_{11}$(GCAA)$_4$ 3'	A (–58), C (–38), C (–19)	FJ981596
Du215(dahli)6	228	5' (GAT)(GATA)$_{10}$(GCAA)$_4$ 3'	A (–58) ,C (–38), C (–19)	FJ981597
Du215(dahli)7	192	5 '(GAT)(GATA)$_5$ 3'	A (–58), C (–38), T (–19)	FJ981598
Du281(dahli)1	225	5' (GGTA)(GATA)$_8$(GAT)(GATA)(GGTA)$_2$(GAT)(GATA)$_4$ 3'	T (+15)	FJ975076
Du281(dahli)2	221	5' (GGTA)(GATA)$_8$(GAT)(GATA)(GGTA)$_2$(GAT)(GATA)$_4$ 3'	T (+15)	FJ975077
Du281(dahli)3	199	5' (GATA)$_{12}$ 3'	C (+15)	FJ975078
Du281(dahli)4	195	5' (GATA)$_{11}$ 3'	C (+15)	FJ975079
Du281(dahli)5	191	5' (GATA)$_{10}$ 3'	C (+15)	FJ975080
Du281(dahli)6	183	5' (GATA)$_8$ 3'	C (+15)	FJ975081
Du323(dahli)1	215	5' (AC)$_6$...(GATA)$_{11}$(GAT)(GATA)(GATATAT)(GA)$_4$ 3'	C (–23), T (+39)	FJ981559
Du323(dahli)2	211	5' (AC)$_6$...(GATA)$_{10}$(GAT)(GATA)(GATATAT)(GA)$_4$ 3'	C (–23), T (+39)	FJ981560
Du323(dahli)3	184	5' (AC)$_5$...(GATA)(GGT)(GATA)$_5$(GAT)(GATATAT)(GA)$_4$ 3'	A (–23), C (+39)	FJ981561

[1]Distances before (–) and after (+) microsatellite cluster are given in bp.

The number of alleles varied from three to seven (depending on the locus). All allelic variants of every locus could be divided into distinct groups according to the fixed nucleotide variations in the microsatellite flanking regions. For example, fixed nucleotide variations in alleles 1–4 of Du215 formed the SNV set TGC, whereas those in alleles 5 and 6 of Du215 formed the SNV set ACC, and those in allele 7 of Du215 formed the SNV set ACT.

Table 3: Sample size, combined genotype structure, diversity and distribution in the *D. dahli* populations.

Genotype	Allelic combination	Populations					Number of individuals with definite genotype (genotype
		Pa	Ph	Va	Dz	De	
1	Du215(3+6)+Du281(1+4)+Du323(1+3)	61	9	2	0	0	72 (0,649)
2	Du215(3+6)+Du281(1+4)+Du323(2+3)	6	0	7	6	2	21 (0,189)
3	Du215(2+6)+Du281(1+4)+Du323(1+3)	0	0	0	0	6	6 (0,054)
4	Du215(3+5)+Du281(1+4)+Du323(2+3)	1	0	4	0	0	5 (0,045)
5	Du215(3+5)+Du281(2+4)+Du323(2+3)	0	0	1	0	0	1 (0,009)
6	Du215(3+6)+Du281(1+6)+Du323(1+3)	1	0	0	0	0	1 (0,009)
7	Du215(1+6)+Du281(1+4)+Du323(1+3)	0	0	0	0	1	1 (0,009)
8	Du215(2+6)+Du281(1+5)+Du323(1+3)	0	0	1	0	0	1 (0,009)
9	Du215(3+6)+Du281(1+3)+Du323(2+3)	0	0	1	0	0	1 (0,009)
10	Du215(4+7)+Du281(1+6)+Du323(2+3)	0	0	1	0	0	1 (0,009)
11	Du215(3)+Du281(1+4)+Du323(2+3)	0	0	1	0	0	1 (0,009)
Total number of individuals		69	9	18	6	9	111
Genotype diversity (%)		4 (5,8)	1 (0)	8 (44,4)	1 (0)	3 (33,4)	11 (9,9)

Pa – Papanino; Ph – Phioletovo; Va – Vaagni; Dz – Dzoraget; De – Dendropark

To identify genotypic diversity in *D. dahli* populations, allelic combinations of the three loci in each of the 111 individuals were constructed. In total, 11 genotypes that differed in population frequencies and geographical distribution were revealed (Table 3). The individuals with

identical genotypes formed distinct clonal lineages. One clone (genotype 1) was abundant (designated as major) and was represented by 72 individuals in three sampled populations; another (genotype 2) was less abundant (designated as intermediate) and was represented by 21 individuals in four populations; nine clones were rare, geographically restricted, and were represented by one (genotypes 5–11) or six and five (genotypes 3 and 4) individuals.

To clarify whether the *D. dahli* species originated from single or multiple interspecies hybridization events, genotype-specific nucleotide markers formed by combinations of parent-specific SNVs of both alleles of three loci were established and compared. Figure 4 schematically shows the structural composition of 11 genotypes that included parent-specific microsatellite clusters and SNVs. It was clear that genotypes 1–9 had identical allelic combinations of parent-specific SNVs for all three loci: TGC/ACC (Du215), T/C (Du281), and CT/AC (Du323). This means that they have identical genotype-specific markers and, therefore, may have a common origin as the result of a single interspecies hybridization event. Genotypes 10 and 11 differ from each other and from genotypes 1–9 by parent-specific SNV combinations for locus Du215: TGC/ACT and TGC/TGC, respectively. Thus, it means that genotypes 10 and 11 have independent origins. Consequently, at least three independent interspecific hybridization events took place in the genesis of the 11 geno-

types. Several additional lines of evidence suggest that the microsatellite clones observed in *D. dahli* result from at least three independent interspecies hybridization events, rather than from a single origin. In addition to having the same allelic combination of parent-specific SNVs, genotypes 1–9 also exhibited a similar organization of allelic microsatellite clusters. Different situations occurred in individuals with genotypes 10 and 11. The microsatellite cluster of the ACT allele of genotype 10, unlike what was observed for all other alleles, had no $(GCAA)_4$ nucleotide group. The individual with genotype 11, unlike all other individuals, was homozygous at the Du215 locus.

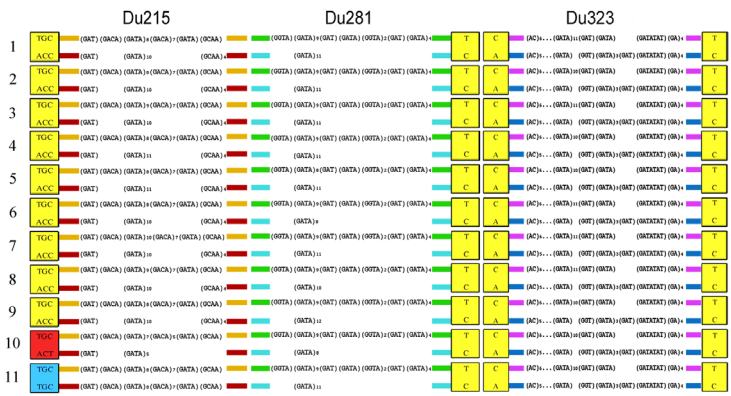

Figure 4: Schematic representation of 11 genotypes formed by allelic combinations of microsatellite loci Du215, Du281, and Du323 in 111 D. dahli individuals. Parental-specific SNV markers are shown in square. Variable microsatellite clusters are shown in each of two alleles.

An unresolved question remains regarding the relationships between genotypes 1–9. These genotypes differed from each other only by microsatellite sequences. These data do not allow the disclosure of the initial clone. One can predict that the major widespread clone with genotype 1 could be the initial clone, whether all others probably arose through postformation microsatellite mutations of the initial major clone. In summary, the data suggest that the clonal diversity observed for *D. dahli* derives from few interspecific hybridizations and postformation microsatellite mutations of the initial clones.

Conclusion

Microsatellites or short tandem repeats are abundant across genomes of most organisms. Some of them tend to be highly variable and represent a rich source of molecular markers useful in population studies, genetic mapping, evolutionary processes as well as in forensic and parentage analysis. Multilocus DNA fingerprinting with microsatellite probes for blot hybridization is very powerful tool for population studies of species whose genome structure is poorly investigated. In the cases, when molecular structure of microsatellite-containing loci is determined, their genetic polymorphism can be analyzed using PCR. Study of genetic diversity in unisexual species of hybrid origin is of particular interest because these species provide an example of clonal breeding system. Among vertebrates,

only parthenogenetic lizards reproduce clonally without male participation. In genetic studies of parthenogens, two main questions concerning clonal diversity and clone origin should be resolved. An analysis of allozyme patterns as well as an analysis of microsatellite variation using PCR, can provide information about clonal diversity in parthenogenetic species, while the latter method is more informative. However, they can not determine origin of detected clones. The new approach, described in our study, included lizard genotyping based on microsatellite and single nucleotide allelic variability of each locus used for analysis. This allowed to reveal genotype-specific markers formed by combinations of parent-specific single nucleotide markers located in the vicinity of microsatellite clusters of both alleles of all loci. Lizards with identical genotypes were assumed to represent distinct clonal lineages. The novelty of this approach consists of revealing parent-specific markers as variations of both, microsatellites and single nucleotides located outside of the microsatellite cluster for each allele of each locus. Based on single nucleotide variable markers direct information about interspecific hybridization founder events can be obtained, and microsatellite variability provides additional information concerning possible mutations in initial hybrid clones that provide arising new genotypes. The approach can produce new information about clonal variation and clone origin in other parthenogenetic species. Further-

more, this approach might be useful for clarification of these questions in any animal species of hybrid origin.

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