SHORT COMMUNICATIONS

Genetic Differentiation among Natural Populations of the Lizard Complex *Darevskia raddei* as Inferred from Genome Microsatellite Marking

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Abstract—The article presents the genetic parameters of the populations of lizards of the *Darevskia raddei* complex (*D. raddei nairensis* and *D. raddei raddei*) and the populations of *D. valentini* calculated on the basis of the analysis of variability of 50 allelic variants of the three nuclear genome microsatellite-containing loci of 83 individuals. It was demonstrated that the F_{st} genetic distances between the populations of *D. raddei nairensis* and *D. raddei raddei* were not statistically significantly different from the F_{st} genetic distances between the populations of different species, *D. raddei* and *D. valentini*. At the same time, these distances were statistically significantly higher than the F_{st} distances between the populations belonging to one species within the genus *Darevskia*. These data suggest deep divergence between the populations of *D. raddei raddei* and *D. raddei nairensis* of the *D. raddei* complex and there arises the question on considering them as separate species.

Keywords: species, subspecies, populations, lizards, *Darevskia*, microsatellites, genetic distances, Wright's *F*-statistics, F_{st}

Caucasian rock lizards of the genus *Darevskia* (family Lacertidae) include 27 bisexual and seven unisexual (parthenogenetic) species that are evolutionarily related to each other. Molecular genetic study of this lizard group is of particular interest because they were the subject of intensive ecological and biogeographic studies and, in addition, parthenogenesis in this group, probably, arose repeatedly [1]. All seven parthenogenetic species of the genus *Darevskia* were formed as a result of different combination patterns of interspecific hybridization with the participation of the four bisexual species, *D. raddei*, *D. mixta*, *D. valentini*, and *D. portschinskii*. However, the mechanisms that determine the specificity of these hybridizations remain unclear. According to Murphy et al. [2], the formation of parthenospecies in this group has phylogenetic limitations and is associated be associated with the sex chromosome genes of bisexual parental species. For example, the populations of *D. raddei* complex (*D. r. raddei* and *D. r. nairensis*) are always maternal in interspecific hybridizations with *D. valentini* and *D. portschinskii* upon the formation of five out of seven parthenospecies of the genus *Darevskia*. At the same time, the taxonomic status of these populations has

remained a subject of scientific studies for many years, during which *D. raddei* and *D. nairensis* were assigned to different species and subspecies or to a single species, depending on the nature of the study and the type of genetic markers. In this study, to get new information on the taxonomic status of the populations of the *D. raddei* complex, we first used microsatellite DNAs as genetic markers. This is because the mutation rate of microsatellites makes it possible to detect microevolutionary events within and between the populations and in the species. The populations of bisexual lizards *D. valentini* with well-established species status were used as control group.

The DNA specimens used in the study were from the lizard DNA collection of *D. r. raddei* (seven populations, including Yeghegnadzor, 39°47′48.4″ N, 45°19′52.4″ E; Geghard, 40°08′49.4″ N, 44°48′26.9″ E; Gosh, 40°42′20.3″ N, 45°00′57.7″ E; Kelbajar, 40°06′03.1″ N, 45°59′27.1″ E; Tatev, 39°23′13.2″ N, 46°15′11.2″ E; Doroga, 39°22′53.98″ N, 46°21′6.63″ E; and Goris, 39°33′09.5″ N, 46°21′19.7″ E, 19 individuals), *D. r. nairensis* (six populations, including Pyunik, 40°36′49.9″ N, 44°35′06.4″ E; Lchap, 40°28′02.4″ N, 45°03′43.5″ E; Lchashen, 40°30′45.92″ N,

44°54′3.22″ E; Yerevan, 40°10′37.0″ N, 44°36′09.3″ E; Bjni, 40°27′42.6″ N, 44°39′07.3″ E; and Hayrivank, 40°26′02.3″ N, 45°06′27.2″ E, 45 individuals), and *D. valentini* (four populations, including Lchashen, 40°30′45.92″ N, 44°54′3.22″ E; Kuchak, 40°31′49.81″ N, 44°17′3.43″ E; Tezh, 40°42′8.08″ N, 44°36′30.80″; and Hatis, 40°18′14.91″ N, 44°43′40.71″ E, 19 individuals) from Armenia. The molecular genetic methods used in the study (DNA isolation, selection of primers and PCR conditions) were described earlier [3]. Genotyping analysis was carried out using the Du215 (21 allelic variants), Du281 (21 allelic variants), and Du323 (eight allelic variants) microsatellite loci. All identified alleles were sequenced on an ABI PRISM 3100-Avant automated sequencer. The allele sequences obtained were deposited in the Gen Bank database under accession numbers from GU972551 to GU972552; from KM573717 to KM573727 (*D. valentini*); from HM014002 to HM014003; from KM573728 to KM573762 (*D. raddei* complex).

The genetic population parameters (heterozygosity, Hardy–Weinberg equilibrium, F_{st}) were determined with the help of the GenePop v. 4.2 and Web version of POPTREEW (http://www.med.kagawa-u.ac.jp/~genome1b/takezaki/poptreew/index.html) software programs. These genetic parameters were compared between the populations of one species (only for *D. r. raddei*, only for *D. r. nairensis*, and only for *D. valentini*), as well as between the populations of knowingly different species (*D. r. raddei*–*D. valentini* and *D. r. nairensis*–*D. valentini*) and among the studied (target) populations of *D. r. raddei*–*D. r. nairensis*. Calculation of descriptive statistics (arithmetic mean, variance, range, standard deviation and errors) and analysis of variance (ANOVA) was performed using the STATISTICA 7 software program. Phylogenetic trees were constructed using the MEGA 6.06 software program [4].

Genetic diversity in the populations (heterozygosity), *p*-values for evaluating the Hardy–Weinberg equilibrium, and F_{st} statistics are presented in the table (for small samples (from one to two individuals), these data are not shown). The mean value of heterozygosity for the populations of *D. r. raddei* was 0.434 (±0.219); for the populations of *D. r. nairensis*, 0.507 (±0.127); and for the populations of *D. valentinii*, 0.525 (±0.052). These differences in the results of the analysis of variance were not statistically significant ($F(2, 14) = 2.3336$; $p = 0.13316$).

As follows from the table, the population allele frequencies are mostly in the Hardy–Weinberg equilibrium. However, in some populations (Yeghegnadzor and Tatev), deviation from this relationship toward the reduction of heterozygote frequencies was observed. In some other populations (Pyunik, Lchashen (in *D. valentini*), and Yerevan), the deviation was associated with the increased number of heterozygous individuals. In the studied populations, there can be several reasons for the deviation of the genetic parameters from the Hardy–Weinberg expectations. In the present study, the effect of the mutational process on the shift of Hardy–Weinberg equilibrium was assessed taking into account the rate of variation of the loci for which the Hardy–Weinberg equilibrium was calculated. Furthermore, in this study, the individuals were genotyped at hypervariable microsatellite loci, and therefore, it is obvious that the Hardy–Weinberg equilibrium indices will statistically significantly depend on the mutations in these regions of the genomes and that the rates of allele variability at these loci are quite sufficient to use this type of genetic markers to assess the Hardy–Weinberg equilibrium indices. However, even highly variable microsatellite loci differently (sometimes unequally and multidirectionally) affect the shift of the Hardy–Weinberg equilibrium. For instance, the Du215 Du323 loci demonstrate the shift of the Hardy–Weinberg equilibrium toward increasing number of heterozygotes in populations (for H1, the number of heterozygotes increased ($p = 0.0063$ and $p \ll 0.0001$, respectively)). On the other hand, the Du281 locus demonstrates the shift of the Hardy–Weinberg equilibrium toward decreasing number of heterozygotes (for H1, the number of heterozygotes decreased ($p = 0.0075$)). In small populations, the increase in the proportion of heterozygotes occurs when in the population there is a difference in the frequencies of different alleles between the sexes. Thus, it can be suggested that, in the populations of Pyunik, Lchashen (in *D. valentini*), and Yerevan, males and females show a statistically significant imbalance of the frequencies of different alleles, while in the populations of *D. r. raddei*, the frequencies of different alleles are the same regardless of the sex of the individual. It should be noted that nonuniform action of selection on males and females leads to the increase in heterozygote frequency in subsequent generations. It seems likely that this observation can serve as an explanation for the increase in heterozygote frequency in the populations of Pyunik, Yerevan (in *D. r. nairensis*), and Lchashen (in *D. valentini*).

Moreover, by example of the populations of Yeghegnadzor and Tatev, in *D. r. raddei*, the action of the Wahlund effect can be observed. This means that the studied populations are subdivided into subpopulations where hybridization occurs preferably. This generally leads to the reduction in the number of heterozygotes in the population.

In addition, we cannot exclude the effect of small sample sizes, for which the calculated values of the Hardy–Weinberg equilibrium may show bias. However, in our study, the population samples where the Hardy–Weinberg equilibrium parameter values are biased are not the smallest.

Next, the table shows the F_{st} values for the pairs of populations of definitely different species and definitely one species and the populations of *D. r. raddei*–*D. r. nairensis*. The presented data show that the pop-

P-values for evaluation of the hypotheses of Hardy—Weinberg equilibrium, heterozygosity (averaged over all loci), and the F_{st} genetic distances between the populations of *D. r. raddei*, *D. r. nairensis*, and *D. valentini*

| Species | Population | Number of individuals | Heterozygosity (standard error) | Hardy—Weinberg equilibrium (*P*-value) | | Average F_{st} genetic distance from the populations | | |
				H1: number of heterozygotes decreased	H1: number of heterozygote increased	*D. raddei*	*D. nairensis*	*D. valentini*
D. r. raddei	Yeghegnadzor	8	0.539 (0.271)	**0.000**	1.000	0.284	0.346	0.468
	Geghard	3	0.500 (0.251)	0.464	0.869	0.316	0.381	0.488
	Gosh	1	–	–	–	0.432	0.486	0.571
	Kelbajar	1	–	–	–	0.675	0.688	0.738
	Tatev	3	0.481 (0.241)	**0.005**	1.000	0.301	0.375	0.497
	Doroga	2	–	–	–	0.315	0.320	0.509
	Goris	2	–	–	–	0.422	0.427	0.592
D. r. nairensis	Pyunik	17	0.437 (0.236)	1.000	**0.000**	0.488	0.154	0.519
	Lchap	5	0.527 (0.128)	0.956	0.098	0.427	0.102	0.474
	Lchashen	14	0.611 (0.111)	0.898	0.111	0.345	0.123	0.432
	Yerevan*	6	0.593 (0.093)	0.998	**0.003**	0.366	0.108	0.441
	Bjni	1		–	–	0.432	0.185	0.488
	Hayrivank	2		–	–	0.533	0.175	0.550
D. valentini	Lchashen	4	0.542 (0.028)	1.000	**0.030**	0.543	0.476	0.083
	Kuchak	2	0.500 (0.072)	–	–	0.564	0.497	0.207
	Tezh	6	0.505 (0.005)	0.996	**0.004**	0.562	0.494	0.114
	Hatis	7	0.554 (0.104)	0.525	0.462	0.537	0.469	0.082

P- values at which the alternative hypothesis is accepted (H1), are highlighted in bold.

* Currently, the issue of belonging of the Yerevan population to *D. r. nairensis* is debatable. Initially, I.S. Darevsky assigned this population to *D. r. nairensis*, although some researchers (including F.D. Danielyan) consider it belonging to *D. r. raddei*.

ulations of lizards belonging to the same species are characterized by the F_{st} value of 0.239 (±0.014); the populations of lizards from different species, by the F_{st} value of 0.519 (±0.014); and the populations of *D. r. raddei*—*D. r. nairensis*, by the value of 0.432 (±0.027). To eliminate the influence of random fluctuations of the F_{st} values, we conducted the φ transform for these values and conducted their analysis of variance. The analysis showed that all three groups of populations were statistically significantly different from each other relative to this parameter: $F(2, 48) = 29.344$ ($p \ll 0.0001$). Specifying the differences with the help of Tukey's a posteriori test showed that these differences were mainly due to the differences in the F_{st} values between the lizard populations of one species and the lizard populations from different species ($p = 0.000126$). Moreover, the F_{st} values of the populations of *D. r. raddei*—*D. r. nairensis* were statistically significantly different from those for the lizard populations

of definitely one species ($p = 0.000169$) and did not differ from the F_{st} values for the populations of different species ($p = 0.122981$). It should be noted that the F_{st} values obtained for the populations of one lizard species of the genus *Darevskia* are consistent with the F_{st} values for the populations of lizards of one species from other genera. For instance, analysis of the population genetic structure in green lizard *Lacerta viridis* [5] showed that the F_{st} values for sympatric populations ranged from 0.076 to 0.167, and for allopatric populations, they varied from 0.134 to 0.345.

On the basis of the pairwise comparison of the populations with respect to the distances (F_{st} data) and using the neighbor joining algorithm with bootstrap support, the phylogenetic tree of the studied populations, represented in the figure, was constructed. On the tree, with rather high probability, the population group of *D. r. raddei* and that of *D. r. nairensis* are separated. The population of Kelbajar (*D. r. raddei*) was
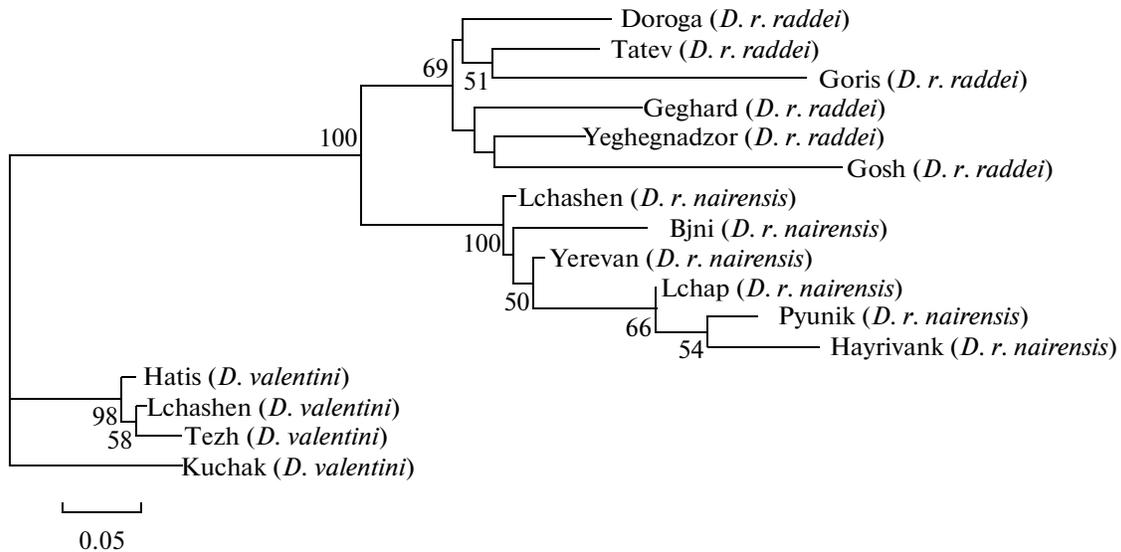
Phylogenetic tree of the populations of *D. r. raddei*, *D. r. nairensis*, and *D. valentini* constructed on the basis of the F_{st} distances using the neighbor joining algorithm with the bootstrap support of 1000 iterations.

excluded from the tree construction, as it was represented by a small sample and, in addition, it was strongly removed and isolated from the other populations. Moreover, one individual lizard from this population was characterized by unique mutations at microsatellite loci that led to the fact that, in the population of Kelbajar, the Du215 and Du323 alleles no. 1, as well as the Du281 allele no. 16, simultaneously transferred to the homozygous state (see GenBank KM573728; NM014002; KM573762).

Thus, the present study shows that the genetic distances between the populations of *D. r. raddei* and *D. r. nairensis* inferred from the F_{st} data did not differ from those between the populations of different species. It should be noted that, initially, all modern lizard species of the genus *Darevskia* were recognized as the subspecies of a single species, *Lacerta saxicola*. Then, with the accumulation of the data on the species biology, each of these subspecies was isolated into a separate species, including bisexual species *D. raddei*. Furthermore, *D. raddei nairensis* was considered the subspecies of *D. raddei*. In 1989, it was demonstrated that *D. raddei* and *D. nairensis* were reproductively isolated because they were characterized by different time of reproduction and different sexual behavior [6, 7]. These findings raise a serious reason to believe that *D. raddei* and *D. nairensis* are different species. Application of the methods of molecular genetic analysis led to the revision of their status. Examination of 36 allozyme loci revealed no differences between *D. r. raddei* and *D. r. nairensis* at fixed alleles [8]. Analysis of the cytochrome *b* gene sequence variation showed that these lizards did not form independent monophyletic groups [1]. These findings are consistent with data of allozyme analysis and suggest that the investigated populations belong to the same species. The use of RAPD and Inter-SINE-PCR [9] also showed no significant differences between the studied populations of the *D. raddei* complex. Therefore, these authors believe that there is no reason to isolate *D. r. nairensis* in a separate species and raise doubt on its subspecies status. At the same time, the use of more effective markers (such as microsatellite DNAs) and populations from a wider range, as follows from the combination of our data, provides the conclusion on deep divergence of the populations of *D. r. raddei* and *D. r. nairensis* of the *D. raddei* complex and on the possibility of assigning them to different species.

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