ISSN 0026-8933, Molecular Biology, 2007, Vol. 41, No. 5, pp. 764–775. © Pleiades Publishing, Inc., 2007.
Original Russian Text © V.V. Grechko, A.A. Bannikova, S.A. Kosushkin, N.L. Ryabinina, K.D. Milto, I.S. Darevsky, D.A. Kramerov, 2007, published in Molekulyarnaya Biologiya, 2007, Vol. 41, No. 5, pp. 839–851.

GENOMICS. TRANSCRIPTOMICS. PROTEOMICS

UDC 575.174:575.858:553.2

Molecular Genetic Diversification of the Lizard Complex *Darevskia raddei* (Sauria: Lacertidae): Early Stages of Speciation

V. V. Grechko[a], A. A. Bannikova[b], S. A. Kosushkin[a], N. L. Ryabinina[a], K. D. Milto[c], I. S. Darevsky[c], and D. A. Kramerov[a]

[a] *Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow, 119991 Russia; e-mail: grechko@eimb.ru*

[b] *Moscow State University, Moscow, 119992 Russia*

[c] *Zoological Institute, Russian Academy of Sciences, St. Petersburg, 199034 Russia*

Received December 26, 2006

Accepted for publication February 19, 2007

Abstract—To characterize the molecular genetic diversity of the genus *Darevskia*, several populations were examined by the inter-SINE-PCR method, reporting the number and sizes of the spacers between individual copies of SINE-like interspersed repeats. Examination of 17 *D. raddei* geographical populations and several reference species revealed unequal genetic differences, measured as Nei and Li's genetic distances (D_{NL}), for different groups of samples. The highest homogeneity was observed for the apparently panmictic *D. raddei nairensis* population from the basin of the Hrazdan River: genetic differences within each of the five samples and between them were similarly low (less than 0.1). The difference between ten samples of *D. raddei raddei* from Armenia and Karabakh (0.2–0.3) was somewhat higher than the interindividual difference within each sample (0.1–0.2), indicating that the samples belonged to different populations. The assumption was supported by the phylogenetic tree topology and multidimensional scaling. The differences between samples from the morphological subspecies *D. raddei raddei* and *D. raddei nairensis* ranged 0.3–0.4. The difference of two *D. raddei raddei* samples of Talysh (Azerbaijan) from other samples of the same subspecies corresponded to the subspecific level. The genetic distances between the good species *D. raddei* and *D. rudis* was 0.6–0.7. In terms of D_{NL}, a questionable population from northwestern Turkey ("*D. tristis*") was closer to *D. rudis* ($D_{NL} = 0.45$), probably representing its subspecies. The phylogeography of *D. raddei* is discussed.

DOI: 10.1134/S0026893307050093

Key words: DNA markers, inter-SINE spacers, molecular evolution, reptiles, speciation

INTRODUCTION

The problem of speciation is among the most important problems of biology and has many aspects. Two particular questions are which molecular genetic criteria allow related populations to be assigned to one taxon of a certain rank and how significant these criteria are for the taxonomy of life.

These questions have not received unequivocal answers as of yet. It is clear that the molecular genetic approach holds the greatest promise for testing the phylogenetic hypotheses underlying the new taxonomy, but some problems arise in this field as well. The results vary in significance even with common markers such as genes of the mitochondrial and chloroplast genomes or the ribosomal genes; for instance, this is the case when phylogenetic trees are constructed and interpreted [1]. Hence, comparative evaluation of particular molecular markers is of immense importance for evolutionary genetic studies [2, 3]. It seems necessary to expand the range of molecular markers characterizing various DNA regions (or the complete genome sequence in the ideal case) at least until congruent results are obtained [4].

In this work, we studied the molecular genetic diversity in several populations of Azerbaijani lizard *Darevskia raddei*, belonging to the group of rock lizards of the Caucasus and Asia Minor. Populations of this species complex are found in a vast area including Armenia, southern Azerbaijan, the adjacent regions of Iran, northern Turkey, and the Lake Van region. The species is highly polymorphic in morphology. It is of interest to estimate its intra- and interpopulation genetic polymorphism, to identify the ancestral form, and to reconstruct the distribution of the species through the given area.

The taxonomy of the genus *Darevskia* and, in particular, the species complex *D. raddei* is rather intricate, as evident from the history of their analysis and classification. In early studies, all rock lizard populations of the Caucasus and Asia Minor were assigned to

a single species, *Lacerta saxicola*, with many subspecies, including *L. saxicola raddei* [5]. As data and collection material accumulated, many subspecies were isolated as separate species (with their own subspecies), still with some discrepancies. Upon subsequent reviews, some populations were renamed or assigned to another species or subspecies. Many such examples have been described in [5]. It became clear that morphological and zoogeographical criteria are incapable of resolving all discrepancies and molecular genetic methods should be employed.

We have previously studied the association between the molecular evolution of nuclear DNA and the genesis of taxa in lizards of the Caucasus and several other regions [6–12]. Here, we report the results of studying the *D. raddei* complex with the use of the inter-SINE-PCR method (IS-PCR), which has earlier been used to analyze the intra- and interpopulation structure of *D. praticola, D. derjugini* [11], and the *L. agilis* complex [12]. Apart from reptiles, IS-PCR has been employed in estimating the molecular genetic relationships of ungulates [13], insectivores, and chiropters [14]. In works [12–14], length polymorphism has been studied for genomic fragments flanked by copies of SINE-family MIR [15], which is of an extremely ancient origin and is found in the genomes of many higher eukaryotes. MIR has a conserved core and, therefore, it is possible to construct primers suitable for examining the reptile group of interest. Specific oligonucleotide primers (Fig. 1) are used to obtain amplification patterns. Since the positions of individual SINE copies is fixed in the genome from the moment of their integration [16], the heterogeneity of inter-SINE spacers is specific and reflects the history of genome divergence of a population and, indirectly, the phylogeny of the taxon. Owing to this specificity, IS-PCR is superior to RAPD analysis: RAPD markers, amplified with short arbitrary primers, are less specific and the results may be distorted by occasional fluctuations. Moreover, RAPD markers amplified in the presence of one arbitrary primer are far fewer than in the case of IS-PCR [8, 11]. The amplified fragments are better resolved in IS-PCR compared with RAPD analysis because high-resolution polyacrylamide gel is used in place of agarose gel.

As observed in studies of *D. praticola* and *D. derjugini* [11], the number and sizes of the amplified spacers only slightly vary among individuals of one population and the extent of this variation can be used to estimate the genetic distance between populations. Some good species of the genus *Darevskia* significantly differ in these characters, while individual genera of the family Lacertidae have almost no synapomorphy (or share only a few fragments) [11, 12]. Thus, IS-PCR addresses the variation at the intrageneric and intraspecific levels, at least in Lacertidae. In this work, we used IS-PCR to examine 17 populations

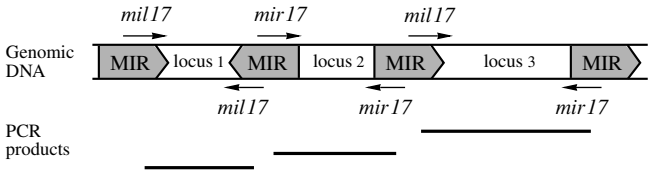

Fig. 1. Scheme of DNA amplification in IS-PCR.

of the *D. raddei* species complex (table). The results of RAPD analysis were used for comparisons in some cases.

EXPERIMENTAL

DNA was isolated from the blood or organs (frozen or fixed with ethanol) of lizards as described previously [8].

IS-PCR employed two systems of primers complementary to the consensus sequences of the MIR core: MIR17 (5'AGTGACTTGCTCAAGGT-3') with MIL17 (5'-GCCTCAGTTTCCTCATC-3') and OMIR17 (5'-ACCTTGAGCAAGTCACT-3') with OMIL17 (5'-GATGAGGAAACTGAGGC-3') (Fig. 1). The amplification conditions and PAGE of the products labeled with radioactive phosphorus were described previously [14]. The electrophoretic patterns were tested for the presence or absence of particular bands (fragments) and were used to construct binary matrices. The matrices obtained with the two primer systems were combined in one matrix, which was used for phylogenetic analysis by the neighbor-joining (NJ) [17] and maximum parsimony (MP) [18] algorithms and multidimensional scaling [19]. A tree including all *D. r. raddei* populations but not *D. r. nairensis* and other *Darevskia* species was constructed for 53 operational taxonomic units (OTUs) with 372 characters, of which 8 were constant in all OTUs and 258 were informative. A tree with a reduced set of *D. r. raddei* populations and the total set of *D. r. nairensis* populations included 59 OTUs and was constructed with 367 characters, of which 61 were constant and 275 were informative.

Genetic distances (D_{NL}) were computed according to Nei and Li [20], using the PAUP* v. 4.0b10 [18] and TREECONw [21] software programs. The genetic distances obtained with the two programs slightly differed because of the difference in their algorithms, but the relationships of all values and the main trends were much the same for all data sets.

RAPD analysis was performed with three arbitrary decanucleotide primers as described previously [11, 12]. The amplification products were electrophoretically resolved in 1% agarose gel and visualized with ethidium bromide.

Lizard populations examined and the extent of molecular genetic differences between forms of various taxonomic ranks

Species, subspecies	No.	Collection locality	Sample size (total 91)	D_{HL} range			
				individual variation in pairwise comparisons	variation of geographical samples in pairwise comparisons	difference between the two *D. raddei* subspecies	difference between *Darevskia* species
		ARMENIA					
	1	Lchap	4	0.02–0.04 (mean 0.03)			*D. r. nairensis* vs. "*D. tristis*": 0.60–0.65
	2	Lchashen	11	0.03–0.05 (mean 0.04)	Samples 1–5 vs. samples 1–5: 0.02–0.10		
D. r. nairensis	3	Bjnee	1				*D. r. nairensis* vs. *D. rudis obscura*: 0.72–0.78
	4	Arzni	6	0.02–0.04 (mean 0.03)			
	5	Yerevan	6	0.04–0.10 (mean 0.07)			
	6	Yeranos	6	0.08–0.13 (mean 0.10)			
	7	Geghard	2	0.07			
	8	Khosrov	3	0.11–0.15 (mean 0.13)	Samples 6–15 vs. samples 6–15: 0.10–0.20		*D. r. raddei* vs. "*D. tristis*": 0.65–0.70
	9	Yeghegnadzor	8	0.06–0.30 (mean 0.20)			
	10	Goris	4	0.11–0.18 (mean 0.15)		*D. r. raddei* 6–15 vs. *D. r. nairensis*: 0.30–0.40	
	11	Galidzor	3	0.15–0.20 (mean 0.18)	Samples 16 and 17 vs. samples 16 and 17: 0.12–0.25		*D. r. raddei* vs. *D. rudis obscura*: 0.65–0.72
D. r. raddei	12	Tatev	3	0.12–0.16 (mean 0.14)			
	13	Gosh	11	0.03–0.12 (mean 0.07)		*D. r. raddei* 6 and 17 vs. *D. r. nairensis*: 0.40–0.45	
		AZERBAIJAN					
	14	Kelbajar	3	0.07–0.11 (mean 0.09)	Samples 6–15 vs. samples 16, 17: 0.30–0.40		*D. r. raddei* vs. *D. rudis* sp.: 0.63–0.73
	15	Shusha	5	0.07–0.15 (mean 0.11)			
	16	Komijorkoy	4	0.08–0.12 (mean 0.10)			
	17	Divagach	2	0.16			
"*D. tristis*"		TURKEY					
	18	Adapazari	4	0.04–0.12 (mean 0.08)			
D. rudis obscura		GEORGIA					*D. rudis obscura* vs. "*D. tristis*" 0.43–0.51
	19	Borjomi	3	0.20–0.30 (mean 0.25)			
D. chlorogaster		AZERBAIJAN					
	20	Dasdatuk	1				

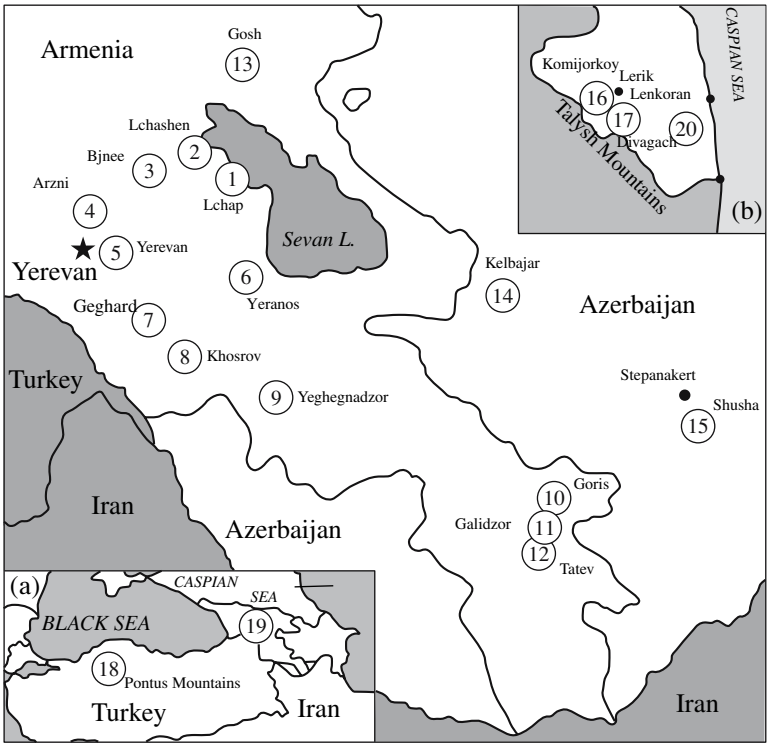

Fig. 2. Distribution of populations belonging to *D. raddei* (1–17) and other species (18–20) in Armenia and Azerbaijan. Inset (a) shows the capture localities for the Turkish population conventionally termed "*D. tristis*" (Pontus Mountains, Adapazari, 18) and for *D. rudis obscura* (Georgia, Borjomi, 19). The approximate distances are 1000 km between the Armenian and Turkish populations, 200 km between the Armenian and Azerbaijani populations, and 100 km between the Armenian and Georgian species. The samples are designated according to the capture localities. Inset (b) shows the localities of two *D. r. raddei* samples from Talysh (Komijorkoy and Divagach, 16 and 17) and one *D. chlorogaster* sample (Dasdatuk, 20) from southwestern Azerbajian.

RESULTS

The number of lizards of the *D. raddei* complex is given in the table; the localities of their capture are shown in Fig. 2.

The primary result of our experiments was an electrophoretic pattern of amplified inter-MIR spacers (Fig. 3). Each band corresponds to a particular spacer between two MIR copies. The absence of a band indicates that the corresponding DNA region and, possibly, one or both of the flanking MIR copies diverged to change the size and/or nucleotide sequence of the spacer and, consequently, its position in gel [11]. Spacers were PCR-amplified with two pairs of primers complementary to the MIR core (Fig. 1) [14]. The primer pairs allow spacer amplification between both similarly and oppositely oriented MIR copies. The resulting patterns were analyzed as described in Experimental.

The patterns were compared for the two morphological subspecies (*D. r. raddei* and *D. r. nairensis*), *D. chlorogaster*, and the questionable species "*D. tristis*" (table, Fig. 2). The *D. raddei* and "*D. tristis*" patterns significantly differed even at the qualitative level.

An NJ tree (Fig. 4a) revealed two sister monophyletic groups with a high bootstrap support (90–100%). The most compact and monomorphic cluster included lizards from the five sites located along the Hrazdan River from Lake Sevan to Yerevan (50–60 km, Fig. 2). In addition, the cluster included the sample from the basin of a Hrazdan tributary located near former Akhundovo (now Pyunik; shown in Fig. 3). The genetic distances (D_{NL}) between individuals in these samples and between the samples ranged from 0.01 to 0.10 (table). Morphologically, the five samples are assigned to one subspecies, *D. r. nairensis*, which is often regarded as a separate species. It is probable that the samples represent one population of the basin of the Hrazdan River and are not divided by geographical barriers.

The second monophyletic group combined lizards belonging to *D. r. raddei* and captured in the other 12 localities (Fig. 2). This group was more heterogeneous, consisting of three subgroups with a high bootstrap support. One included lizards from two Azerbaijani localities, Komijorkoy and Divagach (Talysh region). Another subgroup included lizards captured south and southeast of Lake Sevan (Yeranos, Geghard, Goris, Tatev, and Galidzor) and in two localities of

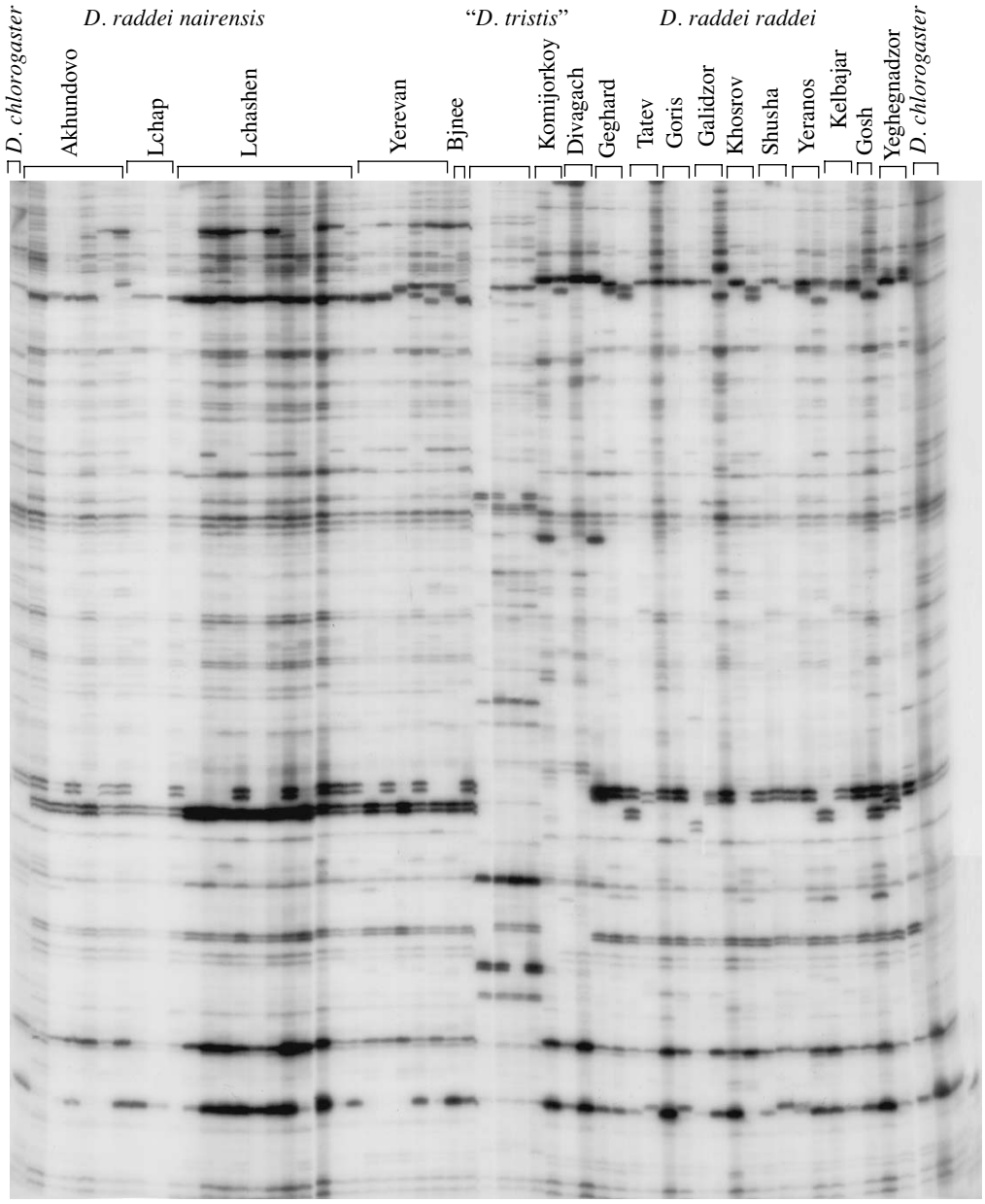

Fig. 3. Fragment of an electrophoretic pattern with DNA fragments amplified with the primers specific to the conserved regions of SINE-family MIR (see Fig. 1).

western Azerbaijan (Shusha and Kelbajar, Karabakh). Two clusters were detectable in this subgroup but had a low bootstrap support (<50%). The third subgroup included one sample from Gosh (north of Lake Sevan, Fig. 2).

In the above experiment, DNAs of only two lizards were examined for each *D. r. raddei* geographical sample because of the gel capacity. Hence, another experiment was carried out to compare the patterns of all lizards of this subspecies in our sample (Fig. 4, table). The separation of lizards from Talysh (Komijorkoy and Divagach) was supported again. The structure of the other clusters had only a weak support. The most distinct separation was observed for all lizards from Gosh and Yeghegnadzor. The Gosh sample was the most homogeneous and the Yeghegnadzor sample was the most divergent.

The genetic distances computed according to Nei and Li [20] are summarized in the table. The range of individual heterogeneity was 0.02–0.10 in the *D. r. nairensis* cluster and 0.10–0.20 in the cluster including the other *D. raddei* lizards. The differences among samples 6–15 from Armenia and Karabakh and between samples 16 and 17 from Azerbaijan were much the same (0.10–0.25). However, the difference between these two groups (6–15/16 and 17) reached

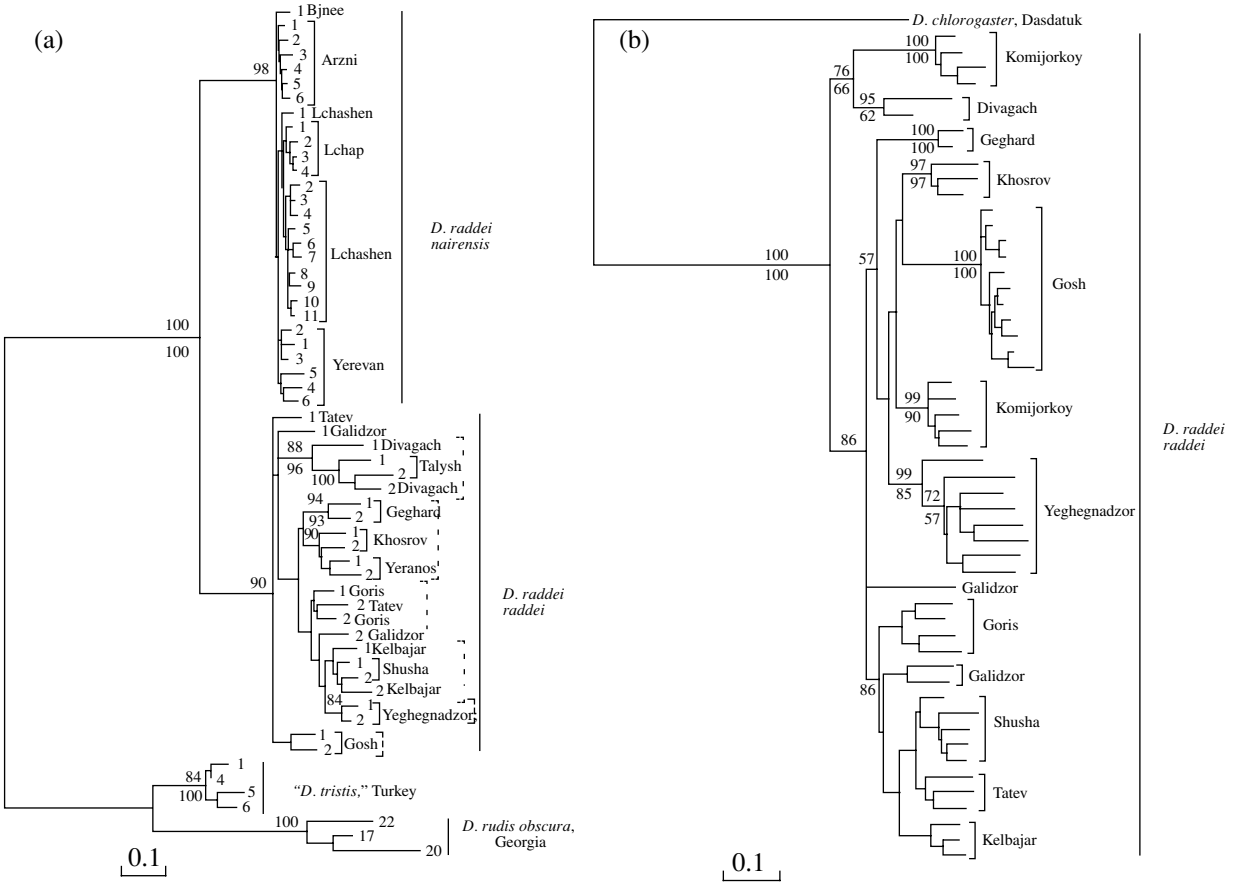

Fig. 4. (a) NJ tree constructed for the *D. r. raddei, D. r. nairensis, D. rudis obscura*, and "*D. tristis*" populations on the basis of IS-PCR markers. The first clade includes 28 lizards from five samples belonging to the subspecies *D. r. nairensis* by morphology. The other clade (24 lizards from 12 populations) includes samples belonging to *D. r. raddei* by morphology. Bootstrap indices (percent of 1000 replicates) are shown at the top of branches for clusters having at least 50% significance. The tree was rooted via midpoint rooting. (b) NJ tree constructed for *D. r. raddei* populations with the total sample available. *Darevskia chlorogaster* was used as an outgroup. Bootstrap indices of NJ and MP analyses are shown, respectively, at the top of and below branches for clusters having at least 50% significance.

0.30–0.40, which agrees with their position on the trees (Fig. 4).

The distance D_{NL} between the *D. r. nairensis* and *D. r. raddei* clusters ranged from 0.30 to 0.45. The distance between *D. raddei* and *D. rudis* and between *D. raddei* and "*D. tristis*" was 1.5–2 times higher (0.60–0.75), while the distance between *D. rudis* and "*D. tristis*" was about 0.45.

The results of multidimensional scaling (Fig. 5) of the *D. r. raddei* samples fully agreed with the NJ and MP phylogenetic trees, clearly illustrating the molecular genetic relationships among the populations under study. Multidimensional scaling revealed five nonoverlapping groups, supporting the separation of the Gosh, Komijorkoy + Divagach, and Yeghegnadzor populations from the other samples. The Goris and Galidzor samples grouped variously with the Shusha and Kelbajar samples or with Yeranos, Geghard, and Khosrov samples, suggesting that all of these samples belong to one cluster (Fig. 5, dashed line).

When the phylogenetic relationships among the populations were studied by MP and UPGMA (data not shown), the topology of the trees did not significantly differ from that of the NJ tree (bootstrap indices of the MP tree are shown in Fig. 4b).

The IS-PCR findings agree with the results of earlier RAPD analysis of some of the populations represented in our sample. For instance, analysis with three primers showed that the *D. r. raddei* samples from Geghard, Gosh, Yeghegnadzor (Figs. 6a, 6c), Yeranos, and Khosrov (data not shown) differ from each other to a greater or lesser (Fig. 6b) extent depending on the primer but still have many synapomorphic fragments. The Gosh population was most homogeneous for these markers (Fig. 6a) while the Yeghegnadzor population was most heterogeneous (Fig. 6b), which agrees with a greater heterogeneity revealed for the latter by IS-PCR (D_{NL} 0.07 and 0.20, respectively).

In RAPD analysis, lizards from Lchashen and Lchap—two out of the five samples characterized in

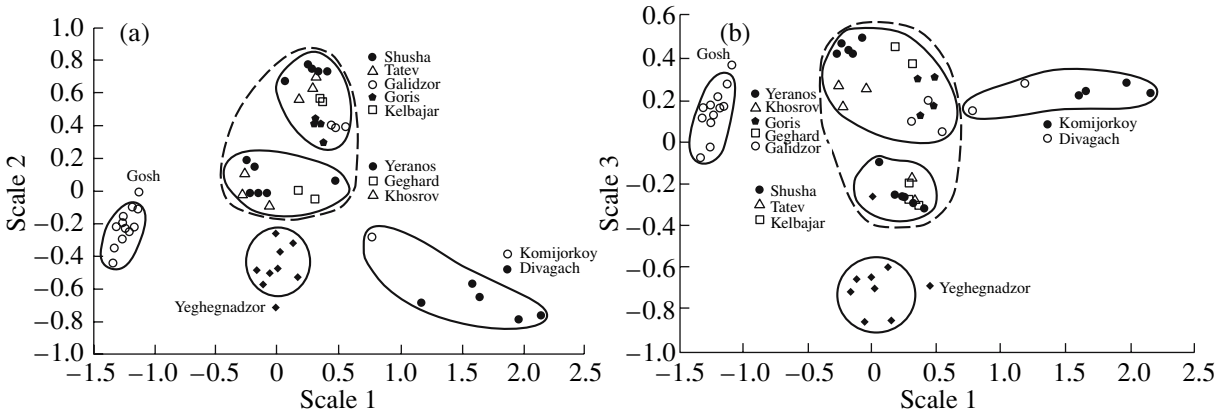

Fig. 5. Two-dimensional scaling of the *D. r. raddei* samples shown in Fig. 4b in two different coordinate systems (a, b). Groups with unstable positions of the Goris and Galidzor samples (see Fig. 2) are shown with dashed lines.

Fig. 4 and morphologically assigned to *D. r. nairensis*—had several synapomorphic characters and a few minor differences, which were lower than the differences between the *D. r. raddei* populations (Fig. 7). The *D. r. nairensis* group was far more homogeneous than any *D. r. raddei* population, which agreed with the IS-PCR data (Fig. 4) and testified again that the samples from the Hrazdan River basin belong to one population. A comparison of the *D. r. raddei* and *D. r. nairensis* patterns (Figs. 6, 7) revealed synapomorphic characters and a few apomorphic characters for each subspecies. It is rather difficult to estimate the significance of the results obtained with these markers for our small samples, and the difference between the two subspecies is nonsignificant. A similar situation has been observed with mitochondrial markers (the cytochrome *b* gene (*cytb*)): the two subspecies seemed conspecific [22].

DISCUSSION

The definition of species, which is essential for well-grounded taxonomy and systematics, is a long-standing and widely discussed problem. At least four theoretical concepts of the species (morphological, biological, phylogenetic, and genetic) are possible, each isolating and considering only one aspect of the problem. Such a consideration is important but practically unproductive because particular ideas of each concept are mingled with the others, sometimes in an unobvious manner. Practical biology utilizes both morphological and molecular genetic methods to identify species and to study their phylogeny [4, 23, 24]. We prefer the genetic concept, which associates speciation with the accumulation of genetic changes, leading to subsequent genetic isolation and separate evolution of the given line. The notion of reproductive isolation from other concepts is involved, though implicitly, in the notion of genetic isolation. We think that the former is unnecessary now, as the possibility of speciation via hybridization of two genetically distinct lines is actively discussed [24, 25]. Baker and Bradley [26] have comprehensively considered the problem of genetic species in mammals, using the original Bateson–Dobzhansky–Mayr (BDM) model. The model combines several ideas common for all concepts of species. It is recommended that the experimental definition of species employ the estimation of genetic distances, phylogenetic analysis of monophyly and sister groups, and purely biological methods of studying a taxon.

This approach was used in this work as well as in our previous studies, performed independently and simultaneously with [26]. The approach reveals the genetic distance even between poorly distinguishable forms, including subspecies, whose taxonomic status is often determined by the geographical location of a population rather than by objective and reliable morphological differences. Shubina et al. [27] have also considered this issue when using the genetic distances to establish the actual phylogenetic relationships in the intricate group of salmonids of the genus *Salvelinus.*

To infer the subdivision of a taxon at the levels of population $\longrightarrow$ subspecies $\longrightarrow$ species $\longrightarrow$ genus in mammals from the differences in the nucleotide sequence of mitochondrial *cytb* (and other genes), differences in simple percent substitution frequencies have been estimated on the basis of the individual variation in a sample (see Table 3 in [26]). However, mitochondrial (or chloroplast) DNA markers often yield questionable results, leading to discrepancies (for a review, see [2]), as is the case in Lacertidae [28], and should be considered in combination with nuclear DNA markers. It is rather difficult to choose a particular nuclear DNA region for studying the phylogeny of a taxon, since individual genes greatly differ in the extent of evolutionary variation and their significance for speciation is usually obscure. Hence, genetic distances estimated by nucleotide sequence analysis of a

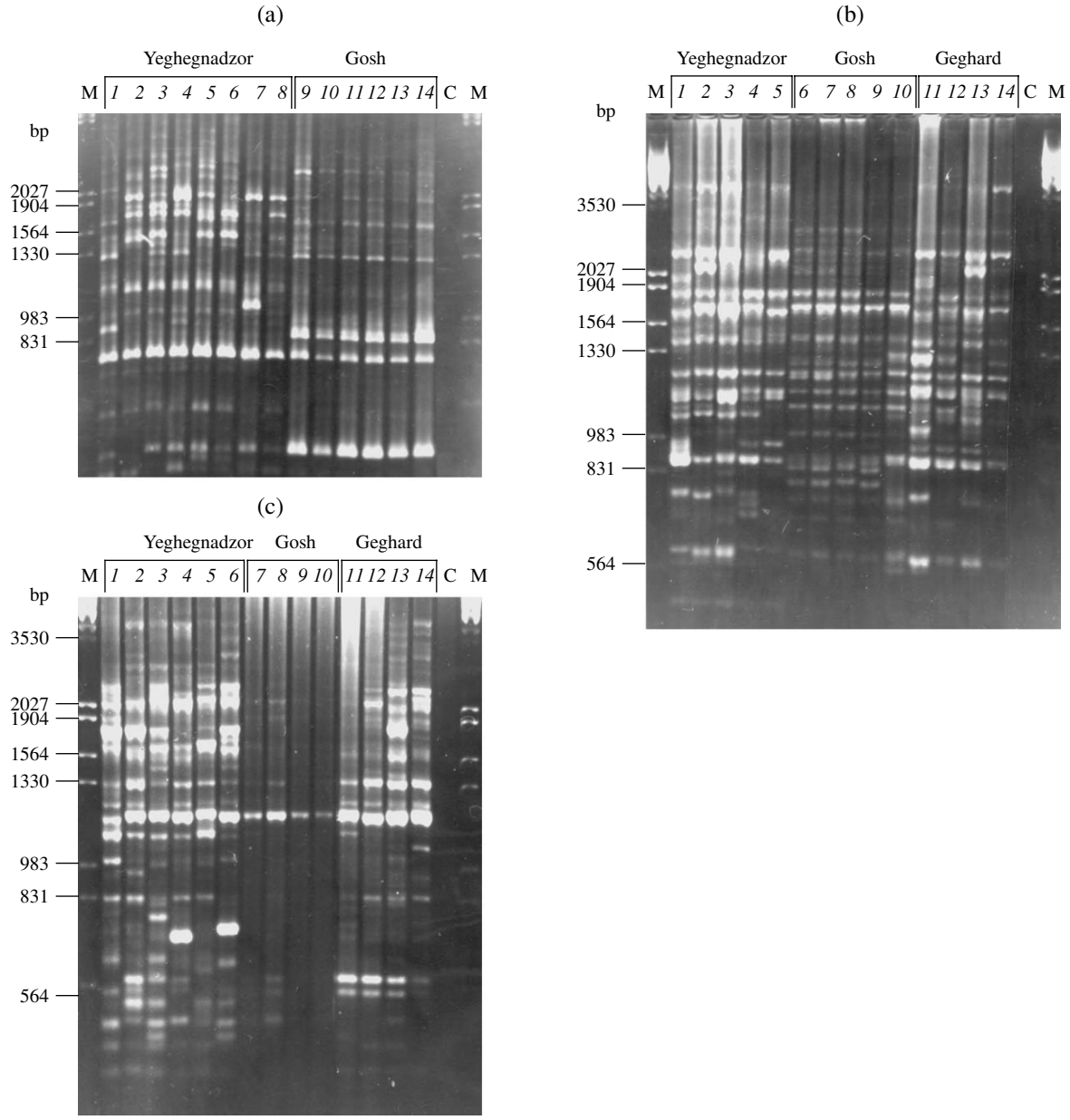

Fig. 6. Intra- and interpopulation differences estimated for some *D. raddei* populations by comparing RAPD patterns obtained with three arbitrary primers (a–c). The patterns did not vary with sex (data not shown). Each track shows the product amplified from DNA of one lizard. Here and in Fig. 7: *M*, molecular weight markers (phage λDNA/*Hin*dIII); *C*, control (without DNA).

particular gene may be poorly informative or even misleading.

A more promising approach is to estimate the genetic distances with as many as possible DNA fragments varying in length and having a greater total length as compared with any individual marker; in particular, multiple fragments whose positions in the genome are determined by the fixed positions of a nonautonomous SINE-family repeat are preferable [16]. The fragments are defined by the primers specific to a particular repeat (MIR in our case). SINE copies are multiple, allowing amplification of many spacers between them in a certain length range, which depends on the polymerase properties and the resolution range of electrophoresis. The method reports the mutations that change the lengths and, consequently, the electrophoretic mobility of fragments. A limitation is that some nonorthologous fragments may acquire similar electrophoretic mobilities as a result of mutations. In addition, some fragments may have a non-monoallelic inheritance so that two alleles differing in length would be regarded as different genes. These drawbacks are compensated for to a certain extent by the facts that the detectable fragments are multiple

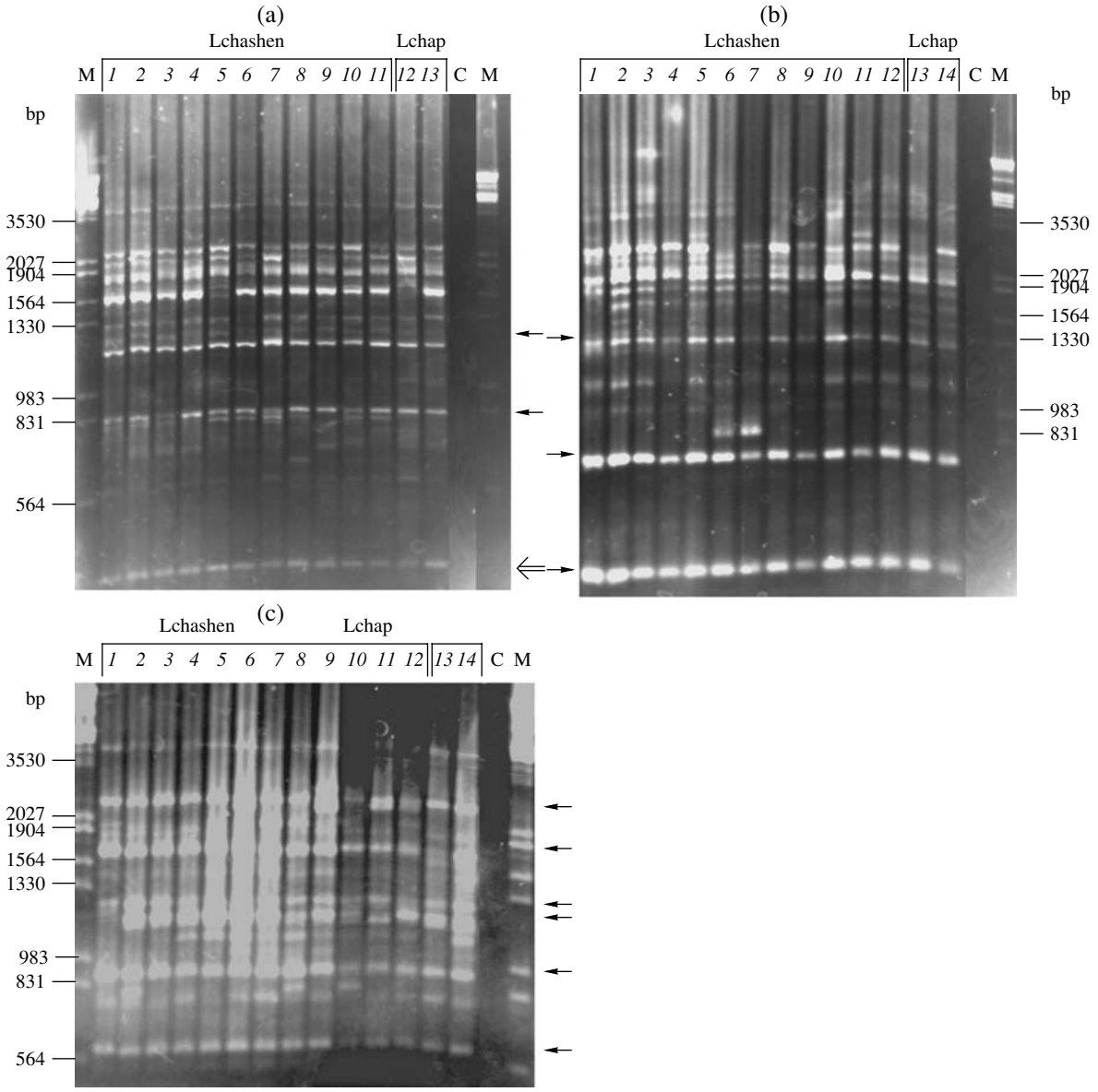

Fig. 7. RAPD analysis of differences among individual lizards and between two samples morphologically assigned to *D. r. nairensis*. Three arbitrary primers were used (a–c). Synapomorphic bands common for all *D. raddei* populations are indicated with filled arrows. The bands without arrows are found in the patterns of some *D. r. raddei* populations. The only autapomorphic fragment of *D. r. nairensis* is indicated with an open arrow.

and the above cases are rare, if the results do not contradict biological concepts or lead to absurd conclusions.

Our results demonstrate that the genetic distance strongly correlates with the relationships in the genus *Darevskia*, ranging from a high similarity of individuals within a sample to a substantial difference between good species. Analysis makes it possible to identify the D_{NL} ranges corresponding to different geographical samples of one population or to different populations, as well as to correlate the differences with the morphological subdivision of the species into subspecies. Our results support the morphological division of *D. raddei* into at least two subspecies, the difference between which approximates $D_{NL} = 0.5$.

As already mentioned, *D. r. nairensis* is highly homogeneous, while *D. r. raddei* tends towards a higher heterogeneity of lizards within each sample and of individual samples. The greatest difference was observed between the Talysh and the other ten samples. In terms of D_{NL}, the difference is similar to that between the morphological subspecies *D. r. raddei* and *D. r. nairensis* (0.30–0.40). The molecular genetic data make it possible to consider the Talysh population as a candidate subspecies. The other samples also

tend to a certain clustering pattern (Figs. 4, 5; table), suggesting intense current speciation.

The Yeghegnadzor population, which is the most divergent (Fig. 4, table), and the Gosh population, which is the most homogeneous (similarly to *D. r. nairensis*; see table, samples 1–5), attract the greatest interest in this context. The clustering of highly homogeneous populations allows certain phylogeographical inferences in some taxa (for a review, see [29]). The *D. raddei* complex seems to advance northwards, from the "phalanx," advancing slowly and continuously accumulating individual genetic differences (as the Yeghegnadzor population in our case), to pioneer populations (those of Gosh and the Hrazdan River basin), which colonize new areas and have a higher homozygosity, as characteristic of founder species. This assumption agrees with our data on satellite DNAs [30, 31] and the accepted zoogeographical views of migrations of the Caucasian fauna after the last Pleistocene glaciation episode [5].

It is clear that the analysis used in this work is indecisive and is commonly performed to preliminarily correlate the morphological and molecular genetic characters. Yet its findings can be of critical importance in the case of a taxonomically intricate genus such as *Darevskia.* This is evident from comparisons at the population, subspecific, and specific levels in the genus. The morphological criteria commonly used to distinguish between *D. raddei* and *D. rudis* (e.g., folidosis and other standard features [5]) do not seem statistically significant and convincing. However, as estimated with molecular genetic characters, the distance between these two species is far greater than the distances between populations within either of them. The same is true for the formal subspecies (this work and [11]). Thus, the results of molecular genetic analysis agree with the taxonomic ranks established morphologically and zoogeographically.

It should be noted, however, that such a correlation is not observed in some cases. For instance, "*D. saxicola tristis*" was described as a separate subspecies of rock lizards and was thought to be close to *D. saxicola* from the Caucasus [5]. More recently, Böhme and Bischoff [32] assigned this form to *L. rudis* as its subspecies *L. rudis tristis.* We (Darevsky) think that the Turkish species is morphologically more similar to Transcaucasian *D. raddei*, since the Turkish sample differs from *D. rudis* in several characters, as well as in body habitus.

The molecular data support our previous assumption [31]. The two reference species, *D. rudis obscura* (Georgia) and "*D. tristis*" (Turkey), each formed a sister group to the *D. raddei* complex (D_{NL} = 0.65–0.75) with different extents of divergence (Fig. 4, table). The distance between "*D. tristis*" and *D. raddei* is much the same as the distance between *D. rudis* and *D. raddei.* The molecular genetic difference between *D. rudis* and "*D. tristis*" (D_{NL} about 0.40, Fig. 4) falls in the range characteristic of the difference between *D. r. raddei* and *D. r. nairensis* (D_{NL} = 0.30–0.45). Thus, the putative species "*D. tristis*" actually belongs to *D. rudis*, rather than to *D. raddei*, and is possibly a *D. rudis* subspecies, as suggested in [32].

Several examples demonstrate that the D_{NL} criteria developed for *D. raddei* are applicable to other species of the genus and to other genera. We have previously used IS-PCR to study a group of forest *Darevskia* species and subspecies (*D. praticola* and *D. derjugini*) and the Georgian species *D. rudis* [11]. For each pair of these species, D_{NL} approached 0.8–0.9. For taxonomic subspecies, D_{NL} is 0.3–0.7 in *D. praticola* (*D. p. praticola* and *D. p. pontica*), 0.3–0.5 in *D. derjugini* (*D. d. derjugini* and *D. d. silvatica*), and only about 0.2 in *D. rudis* (*D. r. obscura* and *D. r. bischoffi*). The D_{NL} ranges of the first two species are comparable with the ranges observed for subspecies in this work (table). However, the difference observed in the two subspecies of *D. rudis* is too low for the subspecific level. Only an insignificant molecular genetic distance has also been found between *D. derjugini barani* and *D. d. derjuguni* [11]. Other methods are necessary to resolve this apparent discrepancy.

The same method has been used to study the subspecies and species composition of *Lacerta* s. str., another genus of the family Lacertidae [12]. Three *L. agilis* subspecies (*argus, chersonensis*, and *exigua*) and two *L. agilis* sister species (*L. strigata* and *L. media*) cluster with a similarly high D_{NL} range, 0.65–0.75. The genetic distances among 13 populations of the broad-area subspecies *L. agilis exigua* fall within the range 0.05–0.20, characteristic of the interpopulation level. Almost no difference has been observed between samples of *L. a. chersonensis.* Thus, the genetic difference between the taxonomic subspecies is extremely high.

Likewise, substantial distances between the above subspecies have been revealed with mitochondrial *cytb* [33], but the results of complex analysis suggests the integrity of *L. agilis* [33, 34]. It should be noted that this species has not been compared with other species of the same genus.

Thus, the above three morphological subspecies of *L. agilis* (*argus, chersonensis*, and *exigua*) reach the level characteristic of the accepted species *L. strigata* and *L. media* or, conversely, these species are actually subspecies of *L. agilis.* Since the interspecific difference in the genus *Darevskia* corresponds to D_{NL} = 0.7–0.9, the three Lacerta subspecies can also be regarded as individual species. Another morphological subspecies, *L. a. brevicaudata*, does not reach the subspecific rank in terms of D_{NL}. The subspecific rank of *L. a. brevicaudata* is also discredited by data on *cytb* [33] and satellite DNA [12, 30].

Thus, the genetic distances estimated using IS-PCR markers provide additional logical and unequivocal information for solving controversies in taxonomy of some lizards.

Based on our findings, D_{NL} ranges characteristic of various taxa can be used as a semiquantitative index to establish the sister groups on a phylogenetic tree at the specific and/or subspecific level. Absolute D_{NL} values may differ in other groups of organisms, depending on the rate and extent of their divergence, evolutionary stage, and other zoogeographical factors. A similar approach based on simple genetic distances (percent similarity of *cytb* nucleotide sequences) is accepted in phylogenetic and taxonomic studies of mammals [26]. Studying the intraspecific genetic diversity is of immense importance for preserving the diversity of species. It can be accepted in this case that populations of the subspecific level reach a D_{NL} of about 0.4–0.5 while species have a D_{NL} of about 1.0.

Our findings indicate that all *D. raddei* samples examined in this work belong to one monophyletic clade and that the clade includes at least two sister groups of populations, which correspond to the two main *D. raddei* subspecies of Armenia [34]. To conclusively establish the rank of the populations within the species complex, it is necessary to study samples from Transcaucasia, Turkey, and Iran.

ACKNOWLEDGMENTS

This work was supported by the Russian Foundation for Basic Research (project nos. 06-04-48222 and 05-04-48147) and the program Dynamics of Gene Pools of the Russian Academy of Sciences.

REFERENCES

1. *Molecular Systematics*. 1996. Eds. Hillis D.M., Moritz C., Mable B.K. Sunderland, MA: Sinauer Assoc.
2. Grechko V.V. 2002. Using milanular DNA markars in phylogeny and systematics. *Russ. J. Genet*. **38**, 1013–1033.
3. Bannikova A.A. 2004. Molecular phylogeny and modern systematics of mammals. *Zh. Obshch. Biol*. **65**, 278–305.
4. Hillis D.M. 1987. Molecular versus morphological approaches in evolution. *Annu. Rev. Ecol. Syst*. **18**, 23–42.
5. Darevsky I.S. 1967. *Skal'nye yasheritsy Kavkasa* (Caucasian Rock Lizards). Leningrad: Nauka.
6. Grechko V.V., Fedorova L.V., Fedorov A.N., et al. 1997. Restriction endonuclease analysis of highly repetitive DNA as a phylogenetic tool. *J. Mol. Evol*. **45**, 332–336.
7. Fedorov A.N., Fedorova L.V., Grechko V.V., et al. 1999. Variable and invariable DNA repeat characters revealed by taxonprint approach are useful for molecular systematics. *J. Mol. Evol*. **48**, 69–76.
8. Ryabinina N.L., Grechko V.V., Darevsky I.S. 1998. Polymorphism of RAPD markers in lizard populations of the family Lacertidae. *Russ. J. Genet*. **34**, 1415–1420.
9. Grechko V.V., Ryabinin D.M., Fedorov A.N., et al. 1998. DNA taxonprints of some Lacertidae lizards: taxonomic and phylogenetic implications. *Mol. Biol.* **32**, 151–160.
10. Gobanu D.G., Grechko V.V., Darevsky I.S. 2003. Molecular evolution of satellite DNA CLsat in lizards from the genus *Darevskia* (Sauria: Lacertidae): correlation with species diversity. *Russ. J. Genet*. **39**, 1292–1305).
11. Ryabinina N.L., Bannikova A.A., Kosushkin S.A., et al. 2002. Estimation of the subspecies level of differentiation in Caucasian lizards of the genus *Darevskia* (syn. *Lacerta saxicola* complex, Lacertidae, Sauria) using genome DNA markers. *Russ. J. Herpetol*. **9**, 185–194.
12. Grecko V.V., Fedorov L.V., Ryabinin D.M., et al. 2006. The use of nuclear DNA molecular markers for studying speciation and systematics as exemplified by the "*Lacerta agilis* complex" (Sauria: Lacertidae). *Mol. Biol*. **40**, 51–62.
13. Buntjer J.B. 1997. *DNA Repeats in the Vertebrate Genome As Probes in Phylogeny and Species Identification*. Utrecht, Netherlands: Utrecht Univ. Press.
14. Bannikova A.A., Matveev V.A., Kramerov D.A. 2002. Experience in using inter-SINA-PCR in studies of mammalian phylogeny. *Genetika* (Moscow). **38**, 853–864.
15. Gilbert W., Labuda D. 2000. Evolutionary inventions and continuity of CORE-SINE in mammals. *J. Mol. Biol*. **298**, 365–377.
16. Kramerov D.A., Vassetzky N.S. 2005. Short retroposons in eukaryotic genome. *Int. Rev. Cytol*. **247**, 165–221.
17. Saitou N., Nei M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol*. **4**, 406–425.
18. Swofford D.L. 1998. PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4. Sunderland, MA: Sinauer Assoc. [software].
19. Kruskal J.B. 1986. Multidimensional scaling and other methods for discovering structure. In: *Statistical Methods for Digital Computers*, Eds. Einslein K, Ralston A., Wilf H.S. New York: Wiley. Translated under the title *Statisticheskie metody dlya EVM*, Moscow: Mir, pp. 301–347.
20. Nei M., Li W.-H. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci. USA*. **76**, 5269–5273.
21. van de Peer Y., de Wachter R. 1994. TREECON: A software package for the construction and drawing of evolutionary trees. *Cabios*. **9**, 177–182.
22. Fu J., Murphy R.W., Darevsky I.S. 2000. Divergence of the cytochrome *b* gene in the *Lacerta raddei* complex and in parthenogenetic daughter species: Evidence for recent multiple origins. *Copeia*. **2**, 432–440.
23. Brower A.V.Z., DeSalle R., Vogler A. 1996. Gene tree, species tree, and systematics. *Annu. Rev. Ecol. Syst*. **27**, 423–450.
24. Avise J. C. 2004. *Molecular Markers, Natural History, and Evolution*. 2nd ed., Sutherland, MA: Sinauer Assoc.
25. Arnold M.L. 1997. *Natural Hybridization and Evolution*. N.Y.: Oxford Univ. Press.

26. Baker R.J., Bradley R.D. 2006. Speciation in mammals and genetic species concept. *J. Mammal*. **87**, 643–662.

27. Shubina E.A., Ponomareva E.V., Gritsenko O.F. 2006. Population-genetic structure of chars in the northern Kuriles and the position of Dolly Varden char in the system of genus *Salvalinus* (Salminidaa: Oalaistai). *Zh. Obshch. Biol*. **67**, 280–297.

28. Fu J. 2000. Toward the phylogeny of the family Lacertidae: Why 4798 base pairs of mtDNA sequences cannot draw the picture. *Biol. J. Linn. Soc*. **71**, 203–217.

29. Hewitt G.M. 2004. Genetic consequence of climatic oscillations in the Quaternary. *Phil. Trans. Roy. Soc. London. Ser. B, Biol. Sci*. **359**, 183–185.

30. Ciobanu D.G., Grechko V.V., Darevsky I.S., Kramerov D.A. 2004. New satellite DNA in *Lacerta* s. str. lizards (Sauria: Lacertidae): Evolutionary pathways and phylogenetic impact. *J. Exp. Zool*. **302B**, 505–516.

31. Grechko V.V., Ciobanu D.G., Darevsky I.S., et al. 2006. Molecular evolution of satellite DNA repeats and speciation of lizards of the genus *Darevskia* (Sauria: Lacertidae). *Genome*. **49**, 1297–1307.

32. Böhme W., Bischoff W. 1984. *Lacerta rudis* Bedriaga, 1886 (Kielschwanz-Feldseidechse). In: *Handbuch der Reptilien und Amphibien Europas. Echsen-2 (Lacerta)*. Ed. Böhme W. Wisbaden: Aula-Verlag, vol. 2/1, pp. 332–344.

33. Kalyabina S.F., Milto K.D., Ananyeva N.B., et al. 2001. Phylogeography and systematics of *Lacerta agilis* based on mitochondrial cytochrome *b* gene sequences: First results. *Russ. J. Herpetol*. **8**, 149–158.

34. Arribas O.J. 1999. Phylogeny and relationships of the mountain lizards of Europe and Near East (*Archaeolacerta* Mertens, 1921, sensu lato) and their relationships among the Eurasian lacertid radiation. *Russ. J. Herpetol*. **l6**, 1–22.