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ESTIMATION OF THE SUBSPECIFIC LEVEL OF DIFFERENTIATION IN CAUCASIAN LIZARDS OF THE GENUS *Darevskia* (SYN. "*Lacerta saxicola* complex," LACERTIDAE, SAURIA) USING GENOME DNA MARKERS

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The taxonomic categories such as population and subspecies were studied on the example of three Caucasian lizard species of genus *Darevskia — D. praticola*, *D. derjugini*, and *D. rudis* by comparing the morphological data and results inferred from nuclear DNA markers. RAPD and new inter-MIR-PCR (IM-PCR) methods were used. The IM-PCR was used to characterize the lacertid DNA fragments located between dispersed SINE type repeats which occurred to be ortologous to mammalian repeats of the same type. It was shown that separation of the Northern population of *D. derjugini* (subspecies *silvatica*) is supported by the comparison with two Southern populations (*derjugini* and *barani*). The latter ones, in their turn, are very similar and hardly can be considered as good subspecies by the genetic distance. The subspecific division of *D. praticola* (*praticola* and *pontica*) also requires more specification. For example, several populations from North Caucasus (ssp. *praticola*) occurred to be heterogeneous. The level of differences between ssp. *praticola* and *pontica* is of the same order as in some of the *praticola* populations. Low level of molecular differences between two subspecies of *D. rudis* (*obscura* and *bischoffi*) does not confirm their validity as a full subspecies.

Key words: lizard, Lacertidae, *Darevskia*, systematics, RAPD, inter-MIR-PCR method, population, subspecies, species.

One of the most intriguing and serious systematics problem is the determination of subspecies and species criteria and the difference between subspecies and population. Morphological criteria appear to be often inconsistent and incomplete. Moreover, it is not easy to obtain enough specimens for statistically significant inferences. In many cases it is impossible to evaluate the evolutionary importance of chosen morphological features. It is worth mentioning, that morphological status of some of the species populations may depend, for example, on the habitation and feeding of the population. Molecular markers are supposed to establish the genetic relationships of populations and to choose the objective criteria for developing of taxon systematics on the basis of their phylogeny (Hillis, 1987). At the lowest level — population and species — the variable mitochondrial DNA regions are usually used, but these markers have some limitations (Grechko, 2002). Another approach involves markers of the RAPD method, which is more convenient to elucidate the population (intra and inter) genetic relationships (see Grechko, 2002).

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In this work we used RAPD technique to solve the question whether there is some genetic basis for extant systematics of Caucasian rock lizards. Earlier we used this method to establish the population and subspecific status in the lacertid group *Darevskia saxicola*, *Lacerta agilis*, and, partly, *D. raddei* (Ryabinina et al., 1998). In this paper the results of examination of *D. praticola*, *D. derjugini* (as a forest), and *D. rudis* (as a rock lizards representatives) are presented.

Our earlier results showed that according to sequence analysis of the DNA satellite repeats specific for the genus *Darevskia* these two forest species are more distant of the closely related rock lizards group of *Darevskia*. By sequence and contents in DNA of the CLsat (Caucasian Lacerta Satellite) subfamilies *D. derjugini* appeares to be more closely related to *D. saxicola* relatives (*D. saxicola*, *D. valentini*, *D. portschinskii*, *D. raddei*, and *D. alpina*), whereas *D. praticola* was closer to the group of *D. mixta* (*D. dryada*, *D. caucasica*, and *D. dagestanica*) (Ciobanu et al., 2002). The same relationships were found by the taxonoprint method (Grechko et al., 1998), which is confirmed by us here (data not presented).

Besides RAPD, we used so-called inter-MIR-PCR method, or IM-PCR (Buntjer, 1997). It is based on PCR with one or two specific primers which are complementary to the conserved fragment sequences of one of the SINE type dispersed DNA repeats. These primers ensure the amplification of regions situated between MIR element copies. As a result a highly informative set of fragments is obtained. It can be electrophoretically fractionated and analyzed. Earlier the MIR SINE repeats were shown as mammal characters (Yurka et al., 1995). Later the orthologous MIR repeats containing highly conserved CORE-fragment were found in other taxa — from plants to vertebrates (Gilbert and Labuda, 2000). In preliminary experiments we have shown that reptilian (lizards) genome seems also to possess the same type of SINE as mammalian genome. When MIR-specific primers were applied to reptilian DNA, a very conserved and specific for lacertid genera and species set of amplified fragments was obtained.

In our work the data of genetic comparisons based on RAPD and IM-PCR markers were obtained on the same set of lacertid specimens as in morphological examination of species *D. derjugini*, *D. praticola*, and *D. rudis*.

MATERIAL AND METHODS

The methods of DNA isolation and RAPD procedure, adapted to our objects were published earlier (Ryabinina et al., 1998). The 10-mer oligonucleotide primers with different sequences were used (see Ryabinina et al., 1998). RAPD products were electrophoresed in agarose gel and photographed. Negatives were scanned and printed.

Inter-MIR method was used mainly as described in Bannikova et al. (2002). PCR was performed using primers complementary to the most conservative region of the CORE sequence of mammals' MIR element (Jurka et al., 1995). These are MIR17 – 5′-AGTGACTTGCTCAAGGT-3′ and MIL17 – 5′-GCCTCAGTTTCCTCATC-3′, labeled with ^{32}P from [γ-^{32}P]dATP by means of polynucleotide kinase (Sambrook et al., 1989). Polymerase chain reaction (PCR) was performed in 20 μliter of mixture, containing 10 mM Tris-HCl buffer, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.0001% gelatine, 0.2 mM dNTP, 4 pmole of each primers, 1 ng of Taq-polimerase ("Seleks," Russia), and about 25 ng of DNA. The PCR conditions were as described in Jurka et al. (1995): denaturation at 94°C, 30 sec; annealing at 56°C, 45 sec; elongation at 72°C, 2 min; last reaction — at 72°C, 5 min; altogether 27 cycles. DNA was subjected to preliminary denaturation at 94°C for 3 min. A "MJ Research" termocycler was used. The PCR products were denatured by heating and electrophoresed in 6% PAAG (Tris-borate buffer, pH 8.3, containing 8M urea). Autoradiography was done after gel drying on a "Retina" x-ray film (Germany). The pairwaise comparison of numbers of shared and unique bands were performed.

$$D = 2n_{A,B}/(n_A + n_B),$$

where n_A and n_B are numbers of fragments in A and B specimens, and $n_{A,B}$ is the number of shared electrophoretically coincided bands in both specimens of each pair studied, was used as a measure of genetic similarity.

RESULTS

RAPD Markers

The species studied and localities of studied specimens are listed in Table 1.

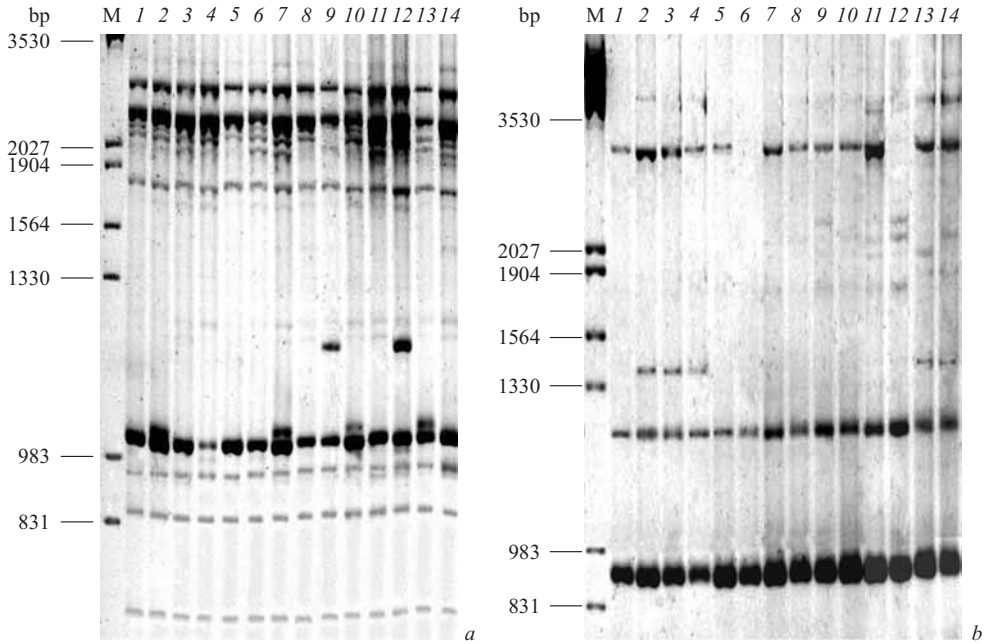

Fig. 1. Intrapopulational similarity of DNA RAPD products of 14 *Darevskia derjugini silvatica* specimens with primer 29 (*a*) and 45 (*b*). The specimens were gathered near Guseriple (see Table 1). There is no correlation of patterns with sex. M, Oligonucleotide length markers.

Figure 1 represents the amplified RAPD DNA fragments of 14 specimens of Northern population of *Darevskia derjugini* (ssp. *silvatica*) with two primers. It is shown that the level of individual (intrapopulational) polymorphisms is rather low; there is some reproducible set of main bands in all the specimens, but a few weak unique bands are also seen. Some specimens possess extra bands, not reffered to sex (Fig. 1*a*). Their presence should be taken into account in comparative experiments. It is worth mentioning that the number of fragments amplified in the IM-PCR method is much higher than obtained by RAPD (see later on).

Up to now we do not have a sufficiently big set of *D. derjugini* specimens from any other populations different from *silvatica* population. But assuming that individual polymorphism of other related subspecies have to be of the level of ssp. *silvatica*, we compared RAPD markers of three populations, one of which seems to belong to a morphological subspe-

TABLE 1. Populations of *Darevskia praticola*, *D. derjugini*, and *D. rudis* Studied

	Population localities	Species	
		specified as species	specified as subspecies
1	Guseriple, Krasnodar Territory, Russia	*derjugini*	*silvatica*
2	Akhaldaba, Georgia	*derjugini*	*derjugini*
3	Batumi, Georgia	*derjugini*	*barani*
4	Golubye Ozera, Kabardino-Balkaria, Russia	*praticola*	*praticola*
5	Nalchik, Kabardino-Balkaria, Russia	*praticola*	*praticola*
6	Zelenokoumsk, Kouma river, Stavropol Territory, Russia	*praticola*	*praticola*
7	Sochi, Red Valley, Russia	*praticola*	*pontica*
8	Tuapse, Black Sea, Russia	*praticola*	*pontica*
9	Akhaldaba, Georgia	*rudis*	*obscura*
10	Gonio, Georgia	*rudis*	*bischoffi*

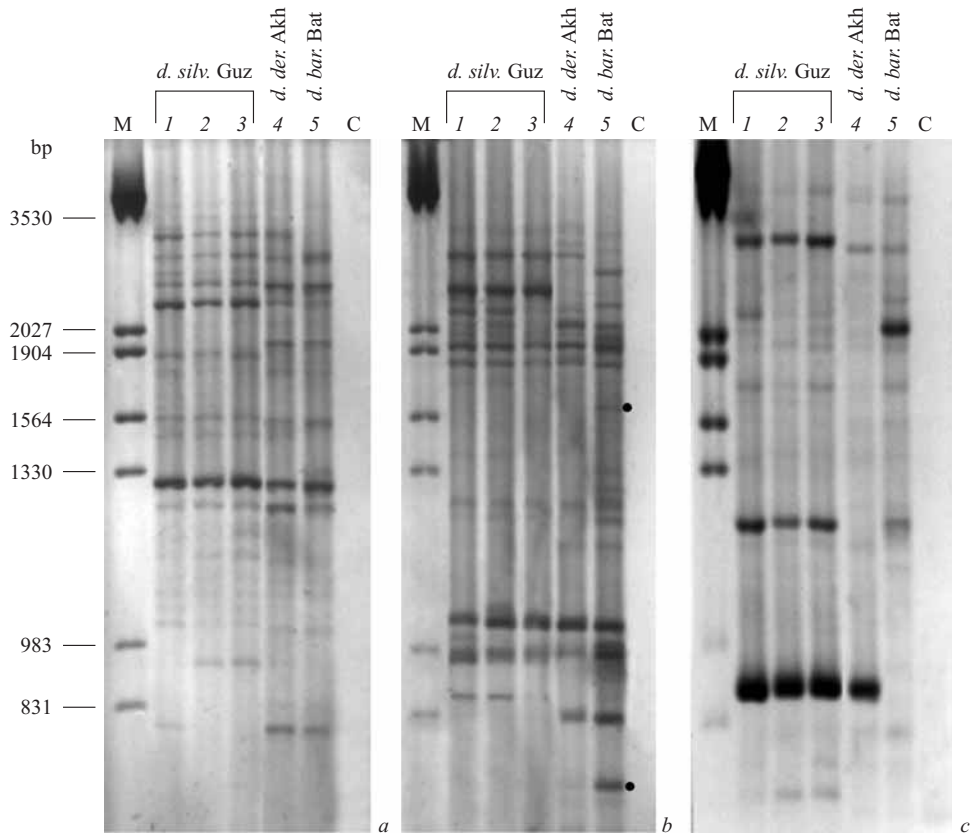

Fig. 2. The comparison of DNA RAPD marker patterns of *D. derjugini* populations treated as subspecies *silvatica* (Gus, Guseriple), *derjugini* (Akh, Akhaldaba) and *barani* (Bat, Batumi, see Table 1) with primers 1001 (*a*), 29 (*b*), and 45 (*c*). M, Fragment length markers; C, control probe without DNA.

cies *derjugini* (Akhaldaba, Akh), the second one — to ssp. *barani* (Batumi, Bat), and the third one is obviously ssp. *silvatica* (Guseriple, Gus) (Fig. 2).

Preliminary estimation of genetic similarity using the *D* value in experiments with primers 1001 and 29 shows that the distance between the southern populations (Akh and Bat) is smaller ($D \sim 0.80 - 0.95$) than distances of both of them together with Northern population from Guseriple ($D = 0.5 - 0.7$). This means that regarded as subspecies *D. derjugini* and *D. barani* are more similar than each of them to *D. silvatica* and the levels of genetic similarity between all three subspecies are not equivalent. It should be noticed that the Southern morphological subspecies are not valid enough for the subspecies status from genetic point of view.

Figure 3 shows RAPD patterns of two populations of *Darevskia praticola* referred as subspecies *praticola* and *pontica*. Individual polymorphism among four specimens of *p. praticola* (from Zelenokoumsk, Zel) (Fig. 3, lines *3 – 6*) is quite low (*D* is close to 1). On this ground one can see that there are obvious differences between two populations of *p. praticola* (from Nalchik and Golubye Ozera) (Fig. 3, lines *1* and *2*), and less differences between two populations of *D. p. pontica* (Sochi and Tuapse) (Fig. 3, lines *7* and *8*).

In the latter case, *pontica* populations practically do not differ in the experiments with primers 1001 and 45 (Fig. 3*a*, *c*). Preliminary calculations of *D* ratio on the basis of the primers 1001 and 29 show that in this case (*D* is about 0.1) genetic distance of the Nalchik population from two others (from Golubye Ozera and Zelenokoumsk) is much larger whereas the latter two occur to be closer to each other (*D* is about 0.6). Thus on the basis of genetic similarity these two populations and populations referred as ssp. *pontica* (Sochi and Tuapse) (*D* is about 0.3 – 0.5)

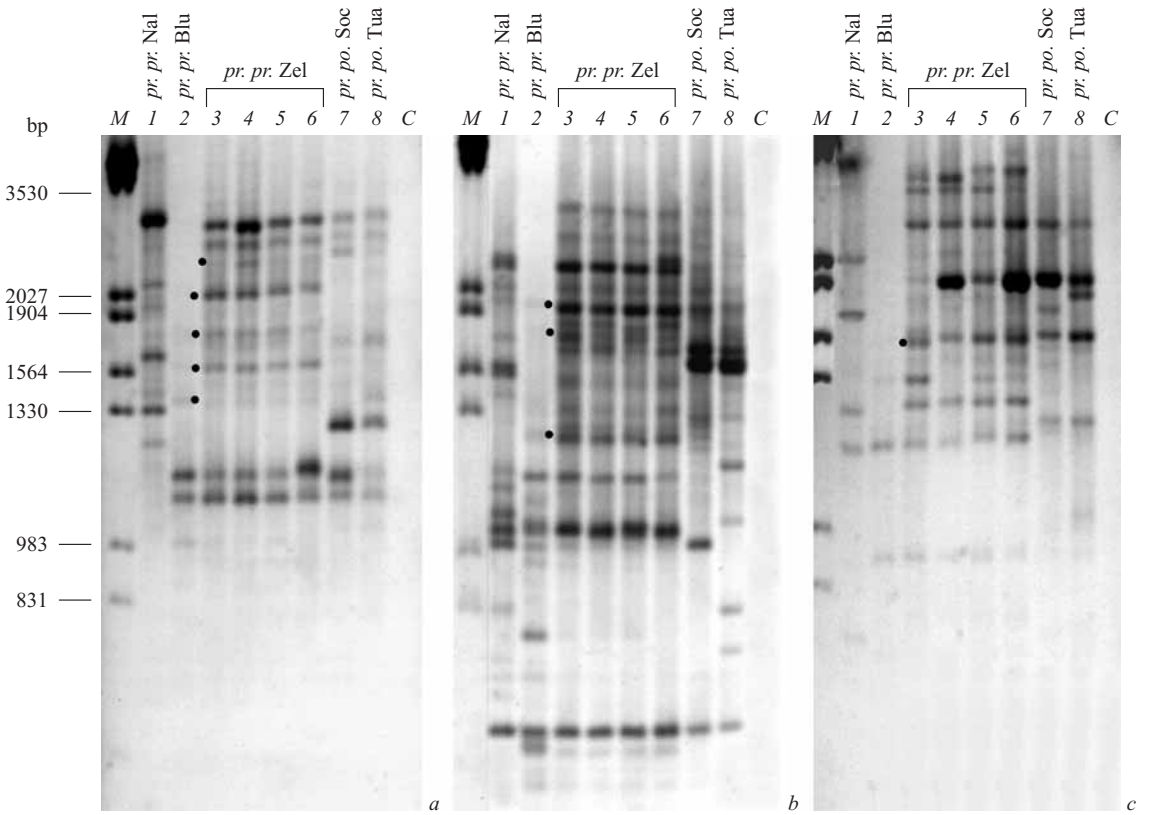

Fig. 3. The comparison of DNA RAPD marker patterns of *D. praticola* populations treated as subspecies *praticola* (Nal, Nalchik; Blu, Blue Lakes; and Zel, Zelenokoumsk) and ss*p. pontica* (Soc, Sochi; Tua, Tuapse, see Table 1) with primers 1001 (*a*), 29 (*b*), and 45 (*c*). M, Fragment length markers. Well reproducible weak bands are marked by points.

are closer to *pontica* rather than to the Nalchik *praticola* population. These estimations should be supported by a larger set of specimens, but the tendency is obvious. This means that subspecies *praticola* is heterogeneous and should be studied in more detail to clarify the status of different populations. These tendencies are also revealed when the inter-MIR markers are applied.

Inter-MIR-PCR Markers

In order to obtain more informative marker pictures (patterns) we used the IM-PCR procedure on the same DNA samples. An example of electrophoretic separation of the IM-PCR ampliphication products is presented in Fig. 4. DNAs of 12 specimens of *Darevskia derjugini* (Guseriple population, regarded as ssp. *silvatica*) are monomorphous enough by these markers: for 3 – 4 individuals only a few additional bands are detected among approximately 30 bands (Fig. 4*a*). Three populations of *D. derjugini* treated as subspecies (lines *1 – 14*) share a large number of bands (only 6 – 7 out of 36 – 37 bands occured to be dissimilar). Specimens from Southern populations, disposed in localities of subspecies *derjugini* and *barani*, are very similar by the band patterns and make up a separate (from Northern ssp. *silvatica*) group of similarity. The band pattern of the Northern population (Guseriple, ssp. *silvatica*) contains 5 – 6 more saturated bands (marked by points) than Southern populations.

The preliminary evaluation of genetic similarity using *D* ratio between three populations of *D. derjugini* shows that the difference between two Georgian populations referred to ssp. *barani* (Bat) and ssp. *derjugini* (Akh) is very small in terms of their intrapopulational index of *D* ratio and genetic similarity of 12 individuals of Guseriple population ($D \sim 0.85$).

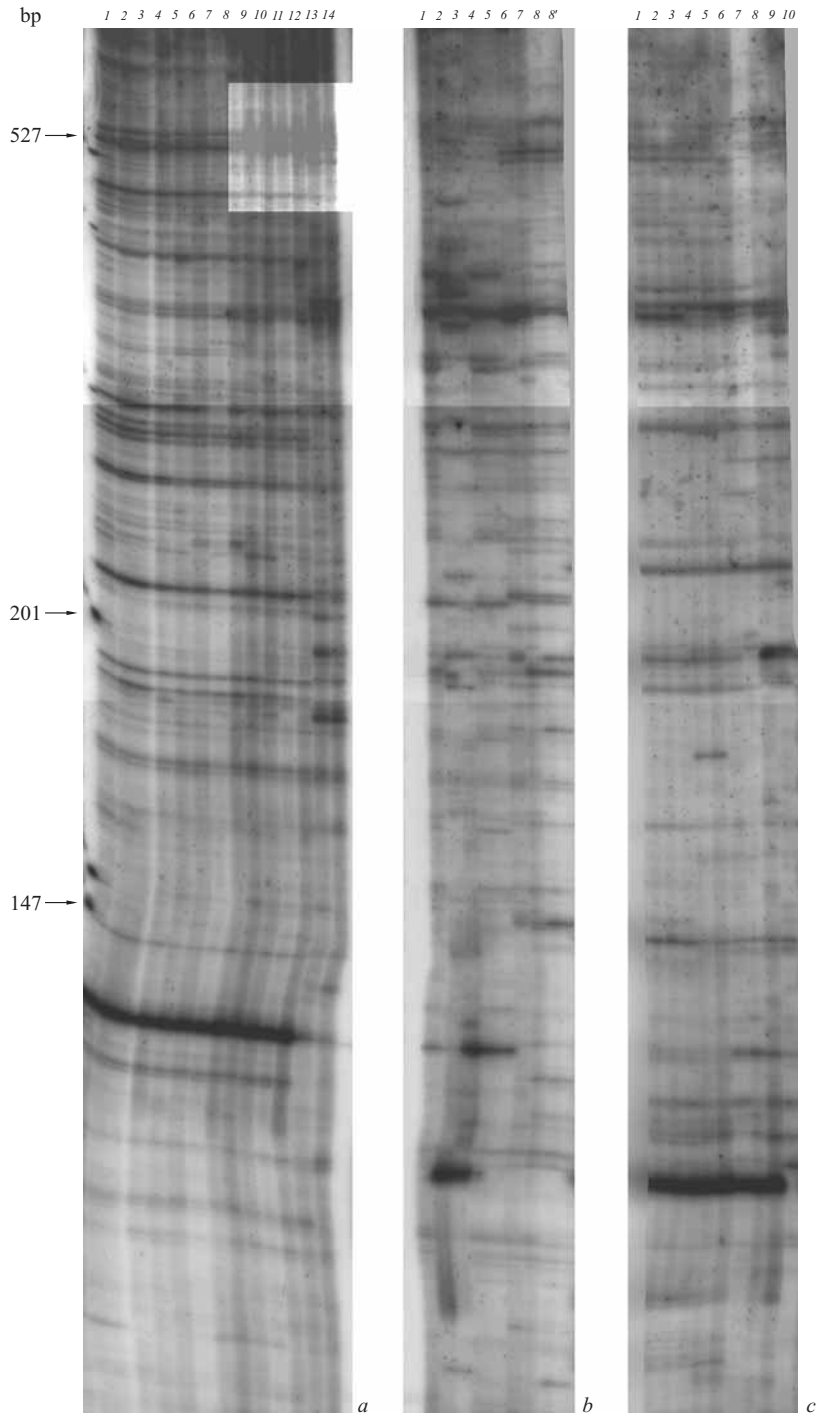

Fig. 4. The comparison of DNA inter-MIR-PCR products patterns of species studied. *a*, Populations of *D. derjugini*: *1 – 12*, Guseriple (ssp. *silvatica*); *13*, Akhaldaba (ssp. *derjugini*); *14*, Batumi (ssp. *barani*). *b*, Populations of *D. praticola*: *1*, Golubye Ozera (ssp. *praticola*); *2*, *3*, Nalchik (ssp. *praticola*); *4*, *5*, Zelenokoumsk (ssp. *praticola*); *6*, Tuapse (ssp. *pontica*); *7*, *8*, Sochi (ssp. *pontica*, *8'*, *8''*, aliquots of one specimen). *c*, Populations of *D. rudis* and species *D. valentini* and *D. portschinskii*: *1 – 4*, Gonio (ssp. *bischoffi*, aliquots of first specimen); *5*, *6*, Gonio (aliquots of the second specimen); *7*, *8*, Akhaldaba (ssp. *obscura*); *9*, *D. valentini*; *10*, *D. portschinskii*. Markers of nucleotide length are shown by arrows. *a*, *b*, *c*, Parts of one electrophoregram divided for convenience. The upper part is enlightened.

This may mean that either the specification of specimen was incorrect or the subspecies nomination is not valid. Further investigations will clarify this problem. At the same time the difference between the Northern population of Guseriple and both above mentioned Southern populations is somewhat larger ($D \sim 0.7 - 0.8$).

As for *D. praticola* (Fig. 4*b*) it was shown that, like in the case of RAPD markers, Nalchik population of ss*p. praticola* is distant from both Zelenokoumsk and Golubye Ozera populations of the same subpecies *praticola*. The latter both are more similar — *D* value is 0.6 vs. 0.35 when compared with the Nalchik population. These values are of the same order of magnitude as the value of genetic similarity between ss*p. praticola* and ssp. *pontica* (0.3 – 0.7). At the same time intrapopulational polymorphisms of IM-PCR products are about 1.0 — both in the Sochi and Zelenokoumsk *praticola* populations and about 0.9 between the near Sochi and Tuapse populations of *pontica*. This means that subspecies *praticola* is not homogeneous and Nalchik population may pretend at least to separate subspecies status.

Data on *D. rudis* (Fig. 4*c*) show that four aliquots DNA products of a specimens of the same population (Gonio, ssp. *bischoffi*) (lines *1 – 4*) and two aliquots of the second specimens (lines *5* and *6*) are the same ($D \sim 0.95$). This indicates a good reproducibility and low intrapopulational variability of inter-MIR-PCR products in this subspecies. The same concerns two specimens of ssp. *obscura* (lines *7* and *8*). The genetic distance between both subspecies is not very prominent — *D* does not exceed 0.8. In other words, the *D* value for two *rudis* subspecies is not as high as the *D* value for subspecies *p. praticola* and *p. pontica* (~0.3) and for subspecies, for example, *d. silvatica* and *d. derjugini* (~0.3 – 0.5).

It is worth to mention that control rock lizard species *D. valentini* and *D. portschinskii* (lines *9* and *10*), taken for the comparison with rock *D. rudis*, are also very similar by IM-PCR markers ($D \sim 0.8$). The similarity of the same level of significance was revealed between these two species and *D. rudis*. At the same time the distance between this species and *D. praticola* and *D. derjugini* is much higher ($D \sim 0.1 - 0.2$)

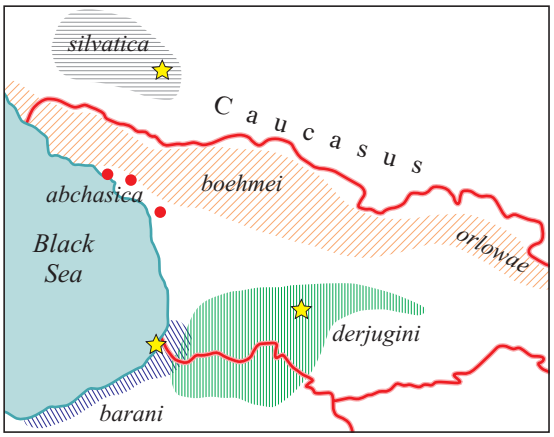

Fig. 5. The scheme of *Darevskia derjugini* morphological subspecies area in Caucasus [modified from (Orlova, 1978a)], based on data accumulated to 1981. The localities of specimens studied are designated by asterisk.

DISCUSSION

The Caucasian lizards of genus *Darevskia* are clearly subdivided into two main groups by their morphology and ecology. One of them consists of the true rock lizards, another, so called forest, inhabits in the wood bedding. *D. derjugini* and *D. praticola*, studied in this work, are among the latter.

D. derjugini is a Caucasian endemic species and it seems to be more phylogenetically younger than *D. praticola*. Now it is subdivided into three geographically separated groups with 6 morphologically discriminated subspecies (Bischoff, 1984). Ssp. *silvatica*, Bartenef and Resnikova, 1931, is located in piedmont regions of Krasnodar Area and separated from the rest of populations by the Main Caucasian Ridge. The Southern populations of ssp. *boehmei*, Bischoff, 1982, inhabits the South slope of Caucasian region in Georgia and North-Eastern Azerbaidjan border with ssp. *abchasica*, Bischoff, 1982, on the West, and ssp. *orlowae*, Bischoff, 1984, — on the East (Bischoff, 1982). In the South-East Transcaucasia and in the neighboring Turkey regions semisympatric subspecies *derjugini*, Nikolskij, 1898, and *barani*, Bischoff, 1984, are distributed. Figure 5 shows a scheme of *D. derjugini* area where the localities of the populations studied marked by asterisks. One of the populations was gathered in Northern

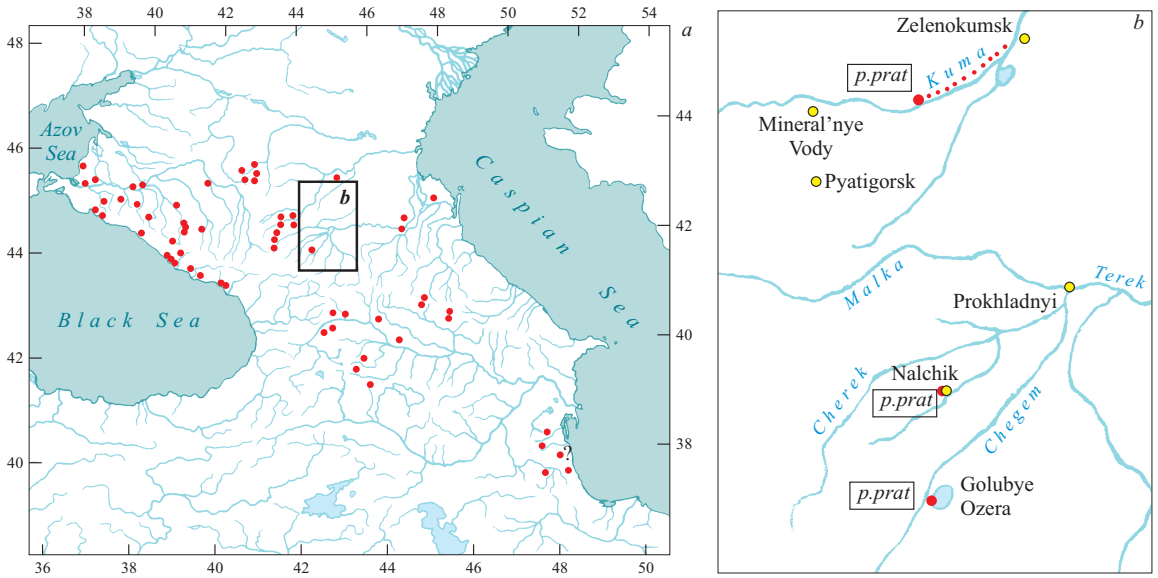

Fig. 6. *a*, The scheme of *Darevskia praticola* morphological subspecies area in Caucasus [modified from (Orlova, 1978b)]; *b*, the scheme of localities of *D. praticola praticola* populations studied (Kouma river valley, designated as Zelenokoumsk, Nalchik, and Golubye Ozera).

slope of the Main Caucasian Ridge not far from Guseriple (ssp. *silvatica*), the second — at the Southern slope of the range near Akhaldaba (Georgia) on the territory designated for ssp. *derjugini*, and the third — near Batumi (Green Cape) on the territory designated for ssp. *barani*. This subdivision was suggested by Bischoff (Bischoff, 1982) who analyzed more than 300 specimens from different Caucasus localities by 16 meristic characters of sceleton, coloration and body proportions (traditionally used for the fam. Lacertidae lizard classification). The subspecies ssp. *silvatica* and ssp. *abchasica* were the most deviated (Bischoff, 1982). The first of them is studied in the present work.

In case of *D. derjugini* molecular IM-PCR markers argue in favour of separate status of Northern population (ssp. *silvatica*), which is correlated with morphological descriptions of Orlova (1978a) and Bischoff (1982). But the recognition of ssp. *derjugini* and ssp. *barani* as separate subspecies remains unclear.

The area of *D. praticola*, Eversmann, 1834, is widely expanded into the Balkan Peninsula and covers several countries and the Caucasus Mountains. In Caucasus this species is represented by several geographic populations, some of which are treated as ssp. *praticola*, while the others — ssp. *pontica*, Lantz et Cyren, 1919.

Figure 6 shows scheme of general distribution of these subspecies at Caucasus (*a*), with designations of localities of the populations studied here (*b*). One of them was taken from Kabardino-Balkaria near Nalchik, the second — 55 km to the South, at the Southern slope of Akhkaya range, the third — in the Kouma river valley between Zelenokoumsk and Mineral'nye Vody, all of them are considered as ssp. *praticola*. The populations of subspecies *pontica* were collected to the South-East from Sochi (Red Medows, about 70 km) and near Tuapse (125 km to the North-West from Sochi).

Morpho-systematical reinvestigation of the main *praticola* populations was done by Orlova (1978b) (Fig. 6), who studied 12 meristic and skeletal characters of 430 specimens from 9 localities in the North and South Caucasus. This analysis supports the view that there are some statistically reliable differences by some of characters between the populations examined. The results of this study show that specimens from Krasnodar and Abkhasia areas are the most different. Except of some meristic characters, which are generally very polymorphous, both groups differ by such alternative characters, important in lizard sys-

tematics, as the number and locations of mandibular shields. If the Northern population has 6 pairs touching in the middle of throat, then the rest of populations possess 5 pairs of mandibular shields and only two first of them touches each other (Orlova, 1978b). Thus the subdivision of at least two forms of *praticola*, suggested earlier as ss*p. pontica* and as ss*p. praticola*, was supported by molecular markers in our work. But there an opinion exists that *D. praticola* might have occured more complex as the number of populations studied is far from being complete up to now. Some evidence of this suggestion is demonstrated by the example of the Sochi population of *pontica*, which was earlier separated by Nikolsky (see Orlova, 1978b) as *Lacerta colchika*. Later this name was rejected. Our preliminary data based on the IM-PCR markers (not shown) support this decision. So this question must be reexamined.

The molecular data presented in this paper give the true molecular evidence of the separation of *D. praticola* into at least two subspecies (Figs. 3 and 4). However analysis of the data does not exclude the possibility of finding other systematic categories within species or subspecies of *D. praticola* (especially it concerns the *praticola* population from Nalchik). So the work will be continued later on using larger set of specimens from the other parts of the range.

As for two populations of *D. rudis*, Bedriaga, 1886, a high interpopulational similarity in *D. r. bischoffi*, Boehme et Budak, 1977 (Gonio) as well as in *D. r. obscura*, Lantz et Cyren, 1936 (Akhaldaba) was revealed. The level of their differentiation corresponds to the interpopulational level of differences between the other species. It means a weak support of *bischoffi* and *obscura* as a valid subspecies.

Elaboration of the Caucasian forest lizard systematics is far from being completed. The number of populations studied is comparatively small taking into account the enormous large area of each of them. These species have never been investigated in detail by molecular-genetic approaches, and their phylogeny still remains unclear.

The results obtained in this work show that in one case morphological and molecular inferences coincide, in the other molecular results can clarify some weakly supported systematical categories, support or reject the morphological meanings.

It worth to attract attention of taxonomists to the IM-PCR method (Buntjer, 1997; Bannikova et al., 2002). The distribution of interspersed repeats along the genomic DNA seems to be the specific character of different taxa. The IM-PCR marker patterns contain synapomorphic and apomorphic characters for populations and species. Our results show also, that reptilian genome may contain the orthologous to mammalian dispersed repeats family of the SINE type. The examination of this SINE family is the aim of our future work.

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