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ANIMAL GENETICS

In memoriam of I.S. Darevskii

Molecular Genetic Relationships and Some Issues of Systematics of Rock Lizards of the Genus *Darevskia* (Squamata: Lacertidae) Based on Locus Analysis of SINE-Type Repeats (Squam1)

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Abstract—To study the molecular genetic relationships and correlate them with the taxonomy within the complex of lacertid lizards of the genus *Darevskia*, the locus analysis of the copies of the SINE-type repeat (Squam1) specific for the order Squamata was used. It was demonstrated that one of the loci (No. 34) contained the Squam1 copy insert in all species and subspecies of the examined genus. SINE allelic copies in some of the loci contained large indels and specific sets of mutations. The allelic variant M (medium, about 340 bp) was found most frequently; it was detected in all subspecies of *D. saxicola* (*saxicola*, *darevskii*, *szczerbaki*, *lindholmi*) and in most of the other species of the genus. Two species, *D. derjugini* and *D. praticola*, differed from the other species in the presence of long (L) and short (S) alleles. The longest allele was characteristic of the *D. derjugini* population from the Northern Caucasus (L, 379 bp, ssp. *silvatica*), while the shortest allele (97 bp) united the *derjugini* and *barani* subspecies. The second allele S (279 bp) characterizes the subspecies *D. praticola praticola*, some individuals of which also carry allele M. The second subspecies, *D. p. pontica*, contains allele L2, which differs from all other medium alleles in the presence of strictly specific short indel. In addition to apomorphic indels, the specificity and mutation distribution patterns among the Squam1 alleles were also examined. An analysis of the NJ tree indicated the concordance between morphological and molecular genetic characters of the species *derjugini, praticola,* and *saxicola.* Furthermore, four subspecies of *D. saxicola* were much closer to each other than the subspecies within the first two species; *D. d. silvatica* and the group of *D. d. derjugini* + *barani* were clearly separated. It cannot be excluded that populations from Azerbaijan and Serbia can be treated as the independent subspecies of *D. praticola*.

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INTRODUCTION

Up to now, the molecular phylogenetics of lizards was based mainly on the mitochondrial DNA markers. However, in recent years, the use of these markers has been criticized (see review [1]). At present, it has been suggested that, in the era of mass whole genome sequencing, the future of phylogenomics is associated with the use of sets of nuclear DNA sequences (e.g., the sums of conservative noncoding regions [2, 3], protein gene clusters [4, 5], or anonymous loci [6]). Attempts are being made to find common primers for far-distant taxa based on known full-size DNA sequence of one of them. These searches, however, are still limited by the quite insufficient number of whole genome sequences for a great number of taxa, and mostly concern the intron–exon genome structures. It should also be noted that these studies are mostly focused on phylogenetics of higher taxa [7]. The use of these data is complicated by the differences in the rates of evolution/mutation of the protein-coding genes with different functional loads in different taxa (see [8]). For these reasons, searches for DNA regions suitable for universal practical use in the analyses of taxa evolution, especially at the early stages of speciation, are still topical and being continued.

At present, due to their specific features and patterns of evolution, short, interspersed SINE-type repeats are thought to be the most promising phylogenetic markers [9–11]. One of the advantages of these markers is that, in the course of the taxon formation, they seem to have been repeatedly amplified and, in different evolutionary periods, their copies are integrated in different genomic loci. Furthermore, almost no removal of the copy from the loci ever occurs; their evolution is unidirectional. This makes it possible to find the copies that characterize either the most ancient events of the taxon formation (e.g., upon the description of large mammal taxa [12, 13]), or those that characterize the stages of speciation at the population level (e.g., upon the analysis of species and population structure of cichlid fish in the lakes of Africa [14]). The search for and selection of copy markers of different evolutionary ages usually presents no experi-

mental difficulties. The use of these markers at the generic and species levels was validated in the studies of Okada et al. [15, 16] and some other authors [11, 17].

In the present study, using this type of molecular markers of chromosomal DNA, we investigated the genetic relationships within the *Darevskia* lizard complex. Specific interspersed repeats in the order of squamate reptiles (Squamata) were independently and simultaneously (2006) described by us [18] and Piskurek et al. [19]. These repeats were called Squam (or Sauria-SINE [19]).

The systematics and taxonomy of the large species complex of rock and terrestrial lizards from the Caucasus, Balkan Peninsula, and Greece (previously included into the *Lacerta saxicola* Linn, 1758 complex [20]) are based on morphological characteristics and mitochondrial markers; they remain controversial and are subjected to continuous revisions. According to the suggestion of Arribas [21], this group was separated from the broad and heterogeneous *Lacerta* group into an independent genus *Darevskia* with the retention of specific and subspecific categories, suggested in the aforementioned monograph by Darevsky [20]. In this study, the true rock lizards of the *saxicola* group were united with *D. mixta, D. dryada, D. clarcorum, D. derjugini*, and *D. praticola*.

More recently, Arnold et al. [22] renamed some lizard groups with the inclusion of geographic comments into the generic names. Correspondingly, Caucasian lizards (analogously to *Iberolacerta, Dalmatolacerta, Iranolacerta*, etc.) were named as *Caucasilacerta*. However, in this case, it remains unclear, why other species of this genus, inhabiting the Balkans, Turkey, and Turkmenistan, should be included into this group. In addition, the description of the *Darevskia* genus presented in the summary report of Arnold et al. does not take into account the revision of Darevsky [20], which is based on his extensive morphometric analysis of the lizards of this region. According to this analysis, the taxa *saxicola, brauneri*, as well as *darevskii* and *szczerbaki* (not mentioned in the summary report), are treated as the subspecies of *D. saxicola*, rather than the independent species, similar to the populations of *valentini, portschinskii, alpina, daghestanica, raddei, parvula*, and *rudis. D. mixta, D. caucasica, D. derjugini*, and *D. praticola* were recognized by Darevsky as independent species. The subspecies of the latter two species are not mentioned in the summary report of Arnold et al. [22]. However, following the logic of these authors, these subspecies should be given species status and mentioned in their revisions. In the study on the Balkan meadow lizards [23], these lizards are treated as the species of *Darevskia pontica*, despite the initial subspecies category of the name *pontica*. For these reasons, prior to the clarification of this issue, in the present study, we mostly use the taxonomy of Darevsky.

In our earlier studies, we examined the molecular genetic relationships within the lizards of *Darevskia* complex, using anonymous nuclear DNA markers for restriction endonuclease DNA mapping (taxonprint technique) [24], RAPD markers [25], and Inter-MIR-PCR [26]. Moreover, we compared the structures of the satellite DNA CLsat subfamilies, which were first described in our studies in lacertid lizards and specific to this order [27].

In the present study, the most attention was paid to the two most widely distributed species of the genus *Darevskia, D. derjugini*, and *D. praticola*. Up to now, both species were subdivided into a number of subspecies (six and three, respectively), the validity of which based on morphometric data is currently being questioned [28, 29]. For comparison, four (out of five accepted) subspecies of *D. saxicola* were used, each of which was represented by two individuals.

MATERIALS AND METHODS

DNA was extracted from blood using standard techniques after the cell lysis with proteinase K, deproteinization with the phenol–chloroform mixture (1 : 1), and subsequent precipitation with isoamyl alcohol [30]. To obtain loci containing Squam1 copies, DNA was digested and cloned, the clones carrying Squam1 copies were selected, and primers 17–23 bp in size complementary to the DNA regions flanking each of the copies from 5' and 3' ends were constructed at a distance of 70–100 bp. The structures of most promising primers are demonstrated in Table 1. Primers were synthesized by the Syntol Company (Russia).

PCR was carried out for the DNA samples of all examined species using flanking primers according to a protocol that included initial denaturation at 94°C for 4 min, followed by 30 cycles consisting of incubation at 95°C, 55–58°C, and 72°C for 1 min each stage. The reactions were performed using *Taq* polymerase produced by the Silex Company (Russia). PCR products were separated by electrophoresis in 1.0–1.5% agarose gels (Helicon, Russia). DNA fragments from the gel bands were purified using the DNA Purification Kit (Promega Wizard, United States) and sequenced using the ABI PRISM® BigDye™ Terminator kit v. 3.1 with subsequent analysis of the reaction products on the Applied Biosystems 3730 DNA Analyzer at the Genome Center for Collective Use (Moscow).

Sequences were aligned by hand using the GenDoc software program [31]. Distance tree was constructed using the neighbor-joining algorithm as implemented in the Mega4 software program [32]. The clade robustness was tested using bootstrap analysis (1000 replicates).

RESULTS

In the present study, populations of some Caucasian lacertids (family Lacertidae, subfamily Lacertinae) were screened at a number of orthologous loci

Table 1. Primer sequences used in the study

Primer	Forward (5'-3')	Reverse (5'-3')
34	AGTTGCTTGGAAGCACGTTG	AATCCGTGCAATCACGTACA
27	GAACATGGAAGCCTCTTAACATC	TGTGCTAGAGAAGTGGAATTAGGA
56	GCTGAACGATAGGGATGGAG	TGTGCAATGAAGGACTTTAGG
2	GTCAGGGCGCAATCCCA	ATTACTACTTGAGACTT
4	TTTGGGATAATCCCATT	GAGATTGGGGTGGCATG
6	TCAGCTTCAGAAGAAGC	GATATGACTGGGCATCC
11	CGTGCAGTGGATGGAAC	AACACTTCGGAAGTCGA
16	TAGACCGTAGTACCATA	TTCAGGATCTCATGGGT

containing copies of Squam1 interspersed SINE-type repeat, specific to squamate reptiles [18, 19]. First, to develop primers to the loci identified in the course of cloning and sequencing of DNA from one of the species of *saxicola* complex, *D. s. raddei*, a great number of clones containing Squam1 copy inserts at different loci were produced. Then, eight of these clones, which contain the most full-sized copies were selected. Then, as was described in the methodological section, primers located in the copy flanking regions at a distance of 70–100 bp from its 5' and 3' ends were constructed (Table 1). These primers were tested under the conditions of PCR to elucidate whether DNA sequences of the lizards that belong to all other species contained the corresponding loci. In most of the experiments, the sizes of amplification products generated corresponded to the expected summarized size of Squam1 and flanking regions (except No. 34, see below). These results were obtained using the DNA templates from all populations of *Darevskia*, and those from some other lacertid genera (data not shown).

Here, we present the data for locus 34, which, in the preliminary experiments, was characterized as the most informative, variable, and suitable for making estimates on molecular genetic relationships within the *Darevskia* genus. A set of populations of the species examined, the number of individuals tested in each of the species, and their geographic locations are demonstrated in Table 2.

Locus 34 is most specific to the *Darevskia* genus; the DNA samples from some other lacertid genera, including *Lacerta* s. str. (*L. agilis* complex), *Gallotia*, and *Zootoca*, contain no Squam1 at this locus. In most cases, the locus is homozygous, and rare individuals have two amplificate fractions (electrophoretic bands), which indicates the presence of at least two alleles, each of which was also sequenced.

The use of the primer pair described in the PCR reaction conducted for analyzing DNA samples from the lizards, which represent different populations of *D. derjugini*, resulted in the generation of a single amplification product, which in most of the individuals was found in the homozygous state. However, in the population corresponding to the subspecies *D. d. derjugini*, this product was of much smaller size (Fig. 1). At first, it was suggested that this band characterized the locus lacking the Squam1 copy. However, after isolating and purifying the material from bands of different sizes from the gel and sequencing them, it was found that the smaller band also contained an incomplete variant of the same repeat copy.

The systematic analysis of the amplification products of orthologous locus 34 in all populations of the genus examined and a comparison of their structures has been demonstrated in the following figures: the alignment of all sequences is shown in Fig. 2, and the schematic representation of their structure (on a scale) is shown in Fig. 3. All alleles can be subdivided into three groups based on size. Allele L1 (Large, 379 bp) was typical of all 11 lizards examined in the subspecies *sivlatica* of the species *D. derjugini*. This allele includes large indel (47 bp), not detected in any of the loci from all other populations (Figs. 2, 3). Allele L2 (344 bp) was found in nine Caucasian lizards of the subspecies *D. p. pontica*. Allele S1 (Small, S1, 97 bp) was detected in all individuals belonging to the subspecies *D. d. derjugini* (seven individuals) and *barani* (four individuals). Allele S2 (279 bp) was detected in the populations of *D. p. praticola* (12 individuals) from Talysh, Nalchik, and Zelenokumsk. Medium allele M (about 340 bp) was typical of all subspecies of *D. saxicola* mentioned (17 individuals), and was also detected in some lizards belonging to the first two species (see Discussion). Comparison of all alleles showed that all of them contained core sequence, typical of allele M, which

Table 2. Species and subspecies of *Darevskia* genus with indication of geographic location

Species	Subspecies	Abbrevations	Number of individuals	Total	Geographic location	Abbrevations
D. derjugini	*silvatica*	dsi	11		Guzeripl, Russia	Guz
	barani	dba	4	22	Batumi, Georgia	Bat
	derjugini	dde	7		Akhaldaba, Serbia	Akha
D. praticola	*pontica*	ppo	3		Belgrade, Serbia	Bel
			2		Tuapse, Russia	Tua
			1		Armavir, Russia	Arm
			1		Salme, Abkhazia	Sal
			3	24	Sochi, Russia	Soch
			2		Petropavlovskoe, Russia	Pet
	praticola	ppr	1		Nalchik, Russia	Nal
			4		Zelenokumsk, Russia	Zel
			7		Talysh, Azerbaijan	Tal
D. saxicola	*darevskii*	sda	2		Sochi, Russia	Soch
	saxicola	ssa	1		Kruglolesskaya, Russia	Kru
			5	17	Kislovodsk, Russia	Kis
	szczerbaki	ssz	4		Anapa, Russia	Ana
	lindholmi	ssl	5		Crimea, Ukraine	Cri

was modified into large and small alleles at the expense of different indels (Fig. 3).

It is evident that the copy flanking regions of locus 34 also contain phylogenetic signals in their structure (Fig. 2). The 5' flanking fragment of the locus is more homogenous in all taxa examined. It contains no indels, which indicates strict orthology between the loci compared. The 3' flanking regions of the locus are more diverged and contain indels 6–20 bp in size. Moreover, each of the indel groups corresponds to the signal, which can be identified based on a structure comparison of the same copy. Thus, it can be concluded that different populations of the three genera examined are monophyletic (similarity of about 80%

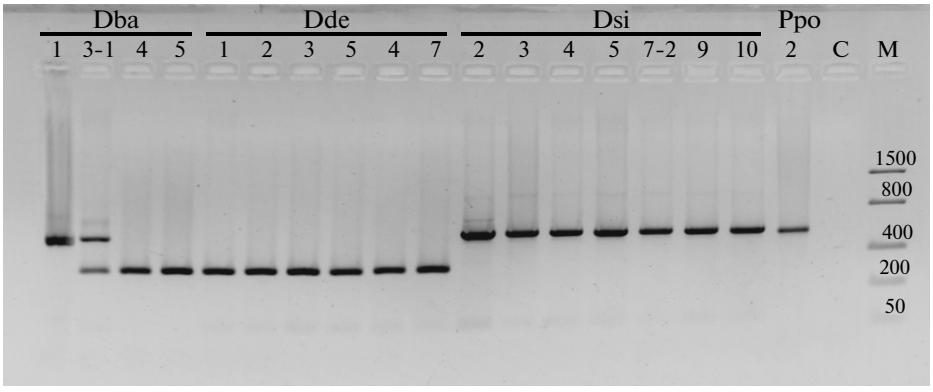

Fig. 1. Electrophoretic separation of PCR amplification product obtained with the use of primer pair to locus 34, containing the Squam1 copy, on the DNA templates of the lizards from the populations of *D. derjugini*. M, molecular size marker, bp. C, control without DNA template. For designations of the subspecies names, see Table 2.

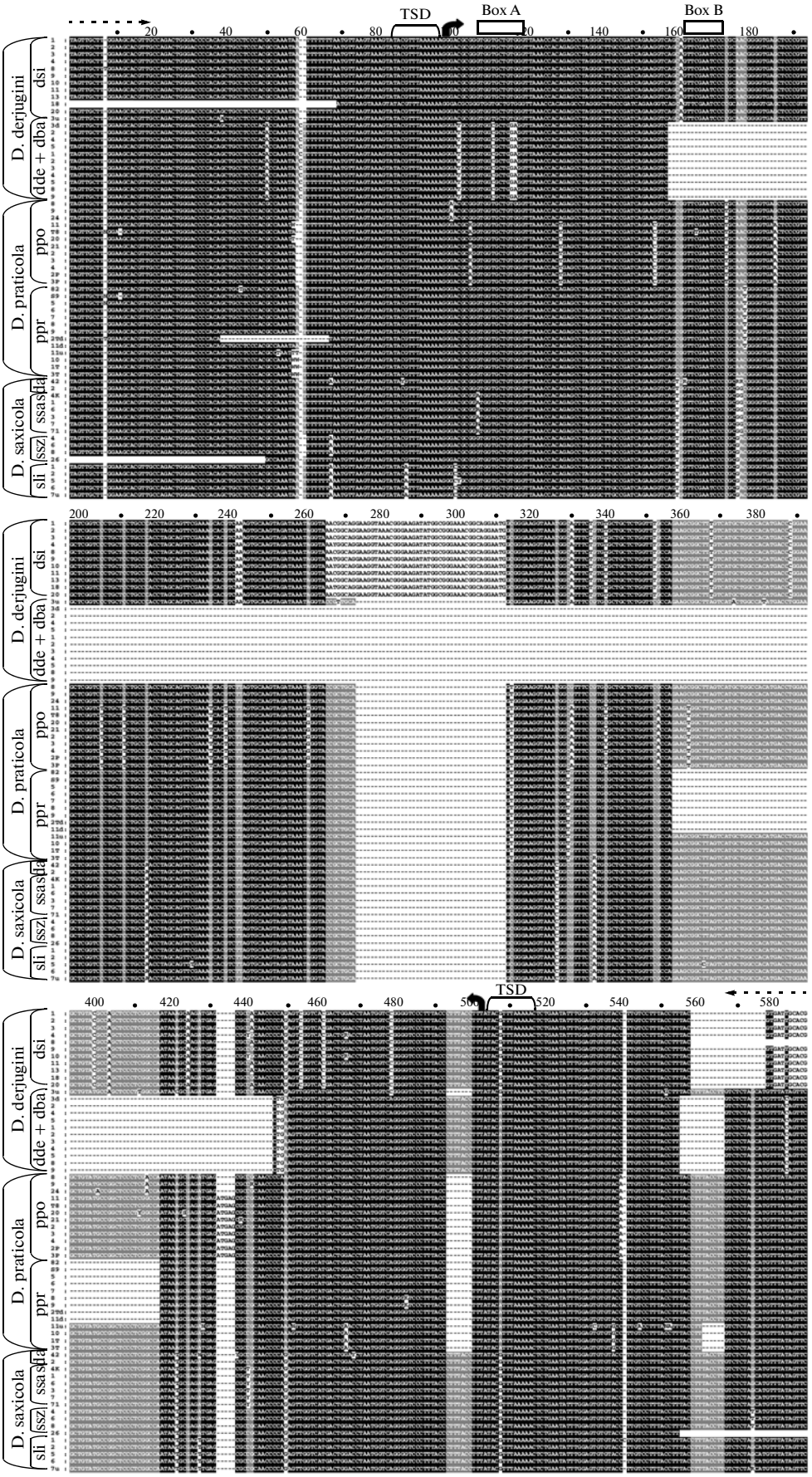

Fig. 2. Alignment of 65 sequences of locus 34 from all representatives of the genus *Darevskia* examined (shown on the left). Sequences are represented in the form of three vertical blocks with continuous nucleotide numbering. (Scaled-up version of the figure is available on request). A and B are boxes of RNA polymerase III (PolIII) promoter; TSD (Target Site Duplication) is typical region of target-site duplication at insertion point of interspersed SINE-type repeats; bent arrows show start and end of Squam1 copy; dashed arrows show positions of locus primes; black field shows identical nucleotides; gray field shows the regions of weak heterogeneity (single mutations); dashes indicates indels, empty spaces—missing data. For designations of subspecies names, see Table 2.

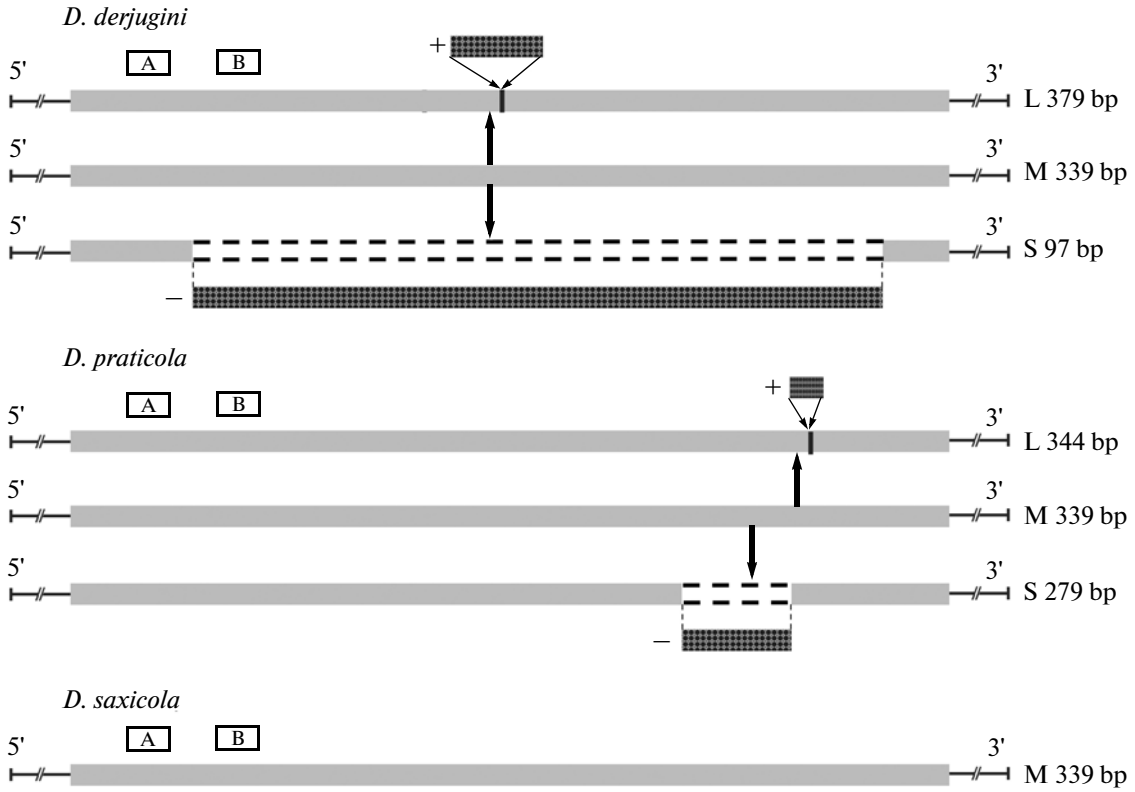

Fig. 3. Scheme of consensus structures (with retention of alignment scale) of Squam1 copy alleles based on the initial data demonstrated in Fig. 2 and with the indication of possible pathway of the formation of L and S alleles from the locus 34 allele M. Conventional size ratio of three alleles is presented: L is Large; M is Medium; and S is Small. The primer positions, as well as the start and end positions of the locus, copy, and two boxes (A and B) of RNA polymerase III promoter, are indicated. The parts characteristic of the consensus of the M allele are shown in solid gray; apomorphic indels are hatched. Plus and minus designate the most possible pathway of the indel formation through insertion (+) or deletion (–). Straight lines with double skew lines show conditional 5' and 3' flanking regions of the locus (abridged).

of the locus), but possess phylogenetically informative differences in the structure of the locus itself, which carries shorter and longer indels.

Since the method of recording the indels of different sizes and localization has no unambiguous resolution in sequence alignment, the similarity of the sequences homologous to allele M in all populations carrying this allele (similarity of single mutation patterns in this allele) was examined individually. Alleles L and S, as apomorphies for the populations carrying these alleles, were also recorded individually.

A sequence comparison of the locus copy by specific single mutations was carried out as it was suggested earlier [33], by means of their tabulating in the positions common for one or another group of individuals. In Fig. 4, all single mutations of alleles M (relative to consensus), which in our case make up about 20% of the full-size sequence, are summarized and compared to the subdivision of the species based on morphological characters. Positions of the mutations in the full-size locus sequence are shown at the top of the figure. It can be seen that mutations are specifically grouped in some respect corresponding to the population and species assignment. In this context, the consensus nucleotides (shown at the bottom) are treated as the most similar to the ancestral sequence in this position and are designated by dots. Apomorphies are designated by letters or dashes (in the case of deletions).

This comparison showed distinct differences between *D. d. silvatica* and *D. d. barani* + *D. d. derjugini* (Fig. 4). The northern population was characterized by the presence of nine population-specific mutations of allele L1. At the same time, allele S1, found in the lizards from the populations of ssp. *barani* + ssp. *derjugini*, was distinguished for six mutations in quite different positions. It should be noted that, with respect to this character, allele S1 of one *D. d. barani* lizard completely corresponded to that of *D. d. derjugini*, while its allele M contained eight mutations in common with allele L1 of *D. d. silvatica*. The tree structure is consistent with a visual examination of the scheme in Fig. 4. Specifically, the subspecies *silvatica* clusters together with allele M of *D. d. barani*. The absence of synapomorphies at this locus in L1 (ssp. *silvatica* and ssp. *barani*) and S1 (ssp. *barani* + ssp. *derjugini*) is explained in terms of the presence of a large SINE deletion in the latter alleles.

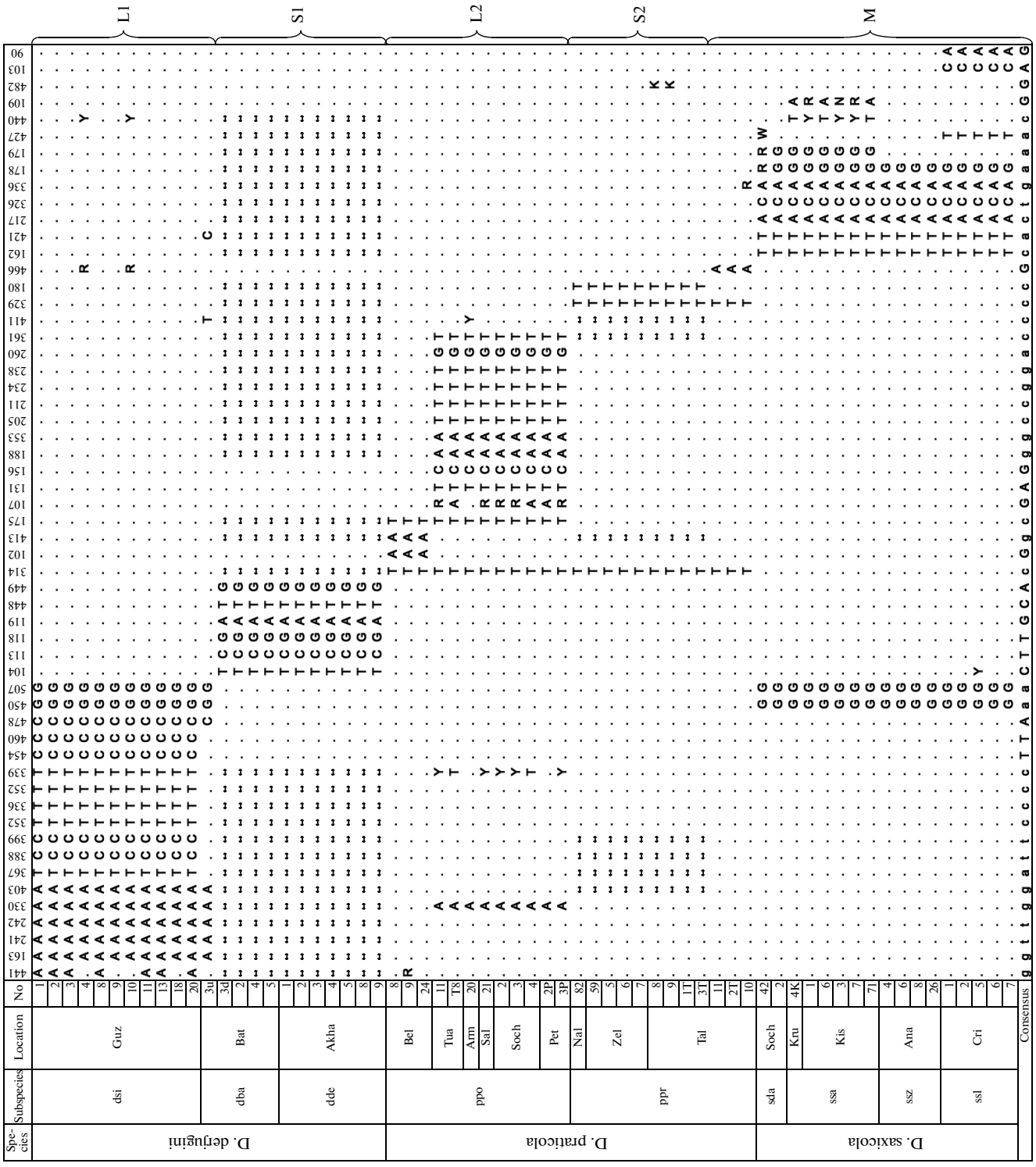

Fig. 4. Distribution of specific single mutations in the Squam1 copies of locus 34 in the species examined (relative to consensus, shown at the bottom). Mutations are grouped based on similarity to the full-size sequence at this position (numbers are shown at the top). Dots show identical nucleotides; dashes show absence of nucleotide. For designations of subspecies names and geographic localities, see Table 2.

All populations of *D. praticola* were united by a single synapomorphy, a mutation in the alignment position. In addition, these populations have many characters similar to the consensus. The set of populations from the Black Sea part of the range and belonging to ssp. *pontica* seems to be most different from the other populations. This is evidenced by at least 11 apomorphies. The lizards from Serbia, formally attributed to

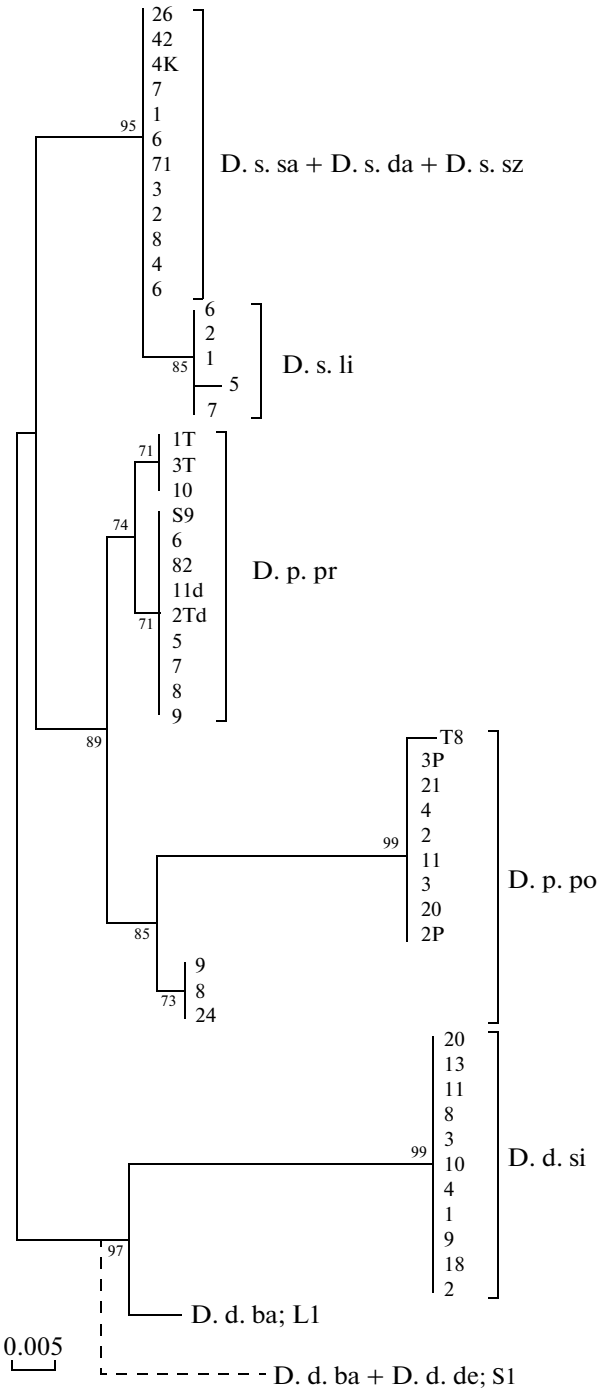

Fig. 5. NJ tree inferred from data in Fig. 4 for locus 34 alleles L and M from the populations that carry them. Possible position of allele S is designated by dotted line (see Discussion).

this subspecies have none of these mutations. At the same time, they have two other synapomorhies and one more mutation, which brings them closer to ssp. *pontica*. Thus, compared to the consensus, the degree of sequence conservatism observed in Serbian lizards is much higher than in the Caucasian individuals of ssp. *pontica*.

With regard to single mutations (as in the case of the indel described above), the lizards from the main part of the range of nominative subspecies ssp. *praticola* (Nalchik and Zelenokumsk) are almost indistinguishable from the four Azerbaijan populations. Three other sequences of Azerbaijan lizards form a close similarity group, and they have only one mutation in common with the representatives of ssp. *praticola*. These lizards have only one synapomorphic substitution, while the remaining part of the sequence is indistinguishable from the consensus.

D. saxicola is the least different from the others in the set of substitutions at the subspecies level. The subspecies within this species are grouped at the six synapomorphies (as well as at two more mutations similar to L1 mutations (*D. d. silvatica* and one *D. d. barani* specimen). Within the species, the similarity groups generally coincide with subspecies subdivision. For instance, ssp. *lindoholmi* has three complete apomorphies; ssp. *saxicola* has two apomorphies and one position, which groups this subspecies together with ssp. *darevskii*. The *szczerbaki* subspecies has no its own apomorphies, and differs from conspecific individuals in the similarity with the consensus positions.

Distant NJ analysis of these data (Fig. 5) produces a tree on which almost all populations cluster in accordance with the subspecific (morphology-based) composition of the three species examined. The probable position of the populations that carry allele S is shown tentatively; it is consistent with the results of superposition of specific mutations of short and long alleles in the parts that were able to be compared (Fig. 4, indels are designated by dashes). In other words, mutations in the common parts (excluding indels) of all allele types also reflect their species-specificity, and enables them to be compared with a given morphological species and subspecies.

Figure 6 demonstrates geographic distribution pattern of the populations and species with different copy variants of locus 34. The number of individuals in each population is given in Table 2.

DISCUSSION

Thus, in our study, it was demonstrated that locus analysis described in the reviews [9, 10] and based on the SINE copy presence/absence in the loci of different taxa has one more aspect that was not taken into consideration in the earlier phylogenetic studies performed at lower phylogenetic levels. Namely, the repeat copy can be incompletely excised from the locus with the retention of some of its part and the appearance of another allele of the same DNA locus. In general, the possibility of removal of the already inserted into the genome SINE copy has been discussed in the literature. In other words, it is discussed whether the principle of unidirectional evolution is maintained along the pathway of only the multiplication of the number of copies and the increase in the

degree of their divergence, which in turn makes it possible to assess the relative taxon age (see review [10] and [34]). Using the example of the primate and some plant repeats, it was demonstrated that, in principle, deletions with the precise removal of the whole copy can occur, although their frequency is negligible (0.01–0.5% from the number of copies). Other events, which can affect the phylogenetic analysis, are also discussed. However, the frequency of these events is also very low (≤0.03%) [11]). However, as far as we know, no cases of the removal of the considerable part of the copy (of about tens or hundreds of nucleotides) with the retention of its start and end sequences within single species, were recorded. Irrespectively of the mechanisms and reasons of the appearance of incomplete loci, they can be considered as molecular apomorphy and valuable morphological character, especially in the homozygous state. The interpretation of the cases of the incomplete lineage sorting (fixation) of alleles in the populations is more difficult and requires the investigation of a greater number of loci.

The group of *D. saxicola* is the most homogeneous with respect to the structure of the locus examined. First, it should be noted that *D. saxicola* (Caucasian rock lizards), which are very morphologically similar [20], appeared to be similar relative to the locus 34. Namely, all representatives of this group examined so far contained the allele of M type and size. The latter allele is also typical to the three (out of seven) *D. p. praticola* individuals from Talysh and three lizards from the Balkans. However, the allele of *D. saxicola* differs from the latter in the pattern of single mutations (Fig. 2). It was demonstrated that DNA samples of single representatives from some other populations of the *D. saxicola* complex (*valentini, portschinskii, rudis, chlorogaster, alpina, nairensis, raddei, clarcorum, dryada, mixta*, and *caucasica*), which are recognized by some authors as independent species, contained only allele M (data not shown).

In our case, allelic variants of *D. derjugini* and *D. praticola* appeared as a result of the modification of allele M through either the integration of an alien fragment (as in the case of L alleles) or the disappearance of large (as in the case of S1 and S2 alleles) and small (as in the case of some variants of M alleles) fragments in different populations. Thus, allele M can be considered to be basal for all three species of the genus *Darevskia* examined. These considerations are schematically represented in Fig. 3. As was mentioned above, within the group of M alleles, there are specific mutational and deletion differences, expressed to a considerably lower degree, compared to other variants. However, these differences provide certain conclusions on the genetic relationships in the *D. saxicola* group (the results will be published separately).

The *D. derjugini* complex contains a great number of interpopulation differences (Figs. 2 and 3). One of the populations (*silvatica*) that inhabit the northern slope of the Caucasus Range (in vicinity of the Guzeripl settlement) carries the largest allele L1 (379 bp) of the Squam1 locus. This allele appeared as a result of the indel (47 bp), which is alien in origin and specific exclusively to this population. The second population examined located in the type territory of this species (from the Artvin Mountains on the coast of the Black Sea to the southern foothills of the central part of the Caucasus Range [28]), carries only the S1 allele of the Squam1 copy (97 bp). In this allele, there was a deletion of the most part of the Squam1 body, excluding its 5' and 3' ends (Figs. 2 and 3). This copy residue contains only one (box A) out of two fragments of the RNA polymerase III promoter, part of tRNA sequence, and a fragment of the Squam1 3' end sequence, including specific terminal repeats. The allele lacks box B of the promoter, which means that it lost the ability to direct RNA polymerase transcription and, hence, to enter into the next cycle of amplification and integration of the new copies into the genome. This copy of the Squam1 locus 34 is found in completely inactive terminal state.

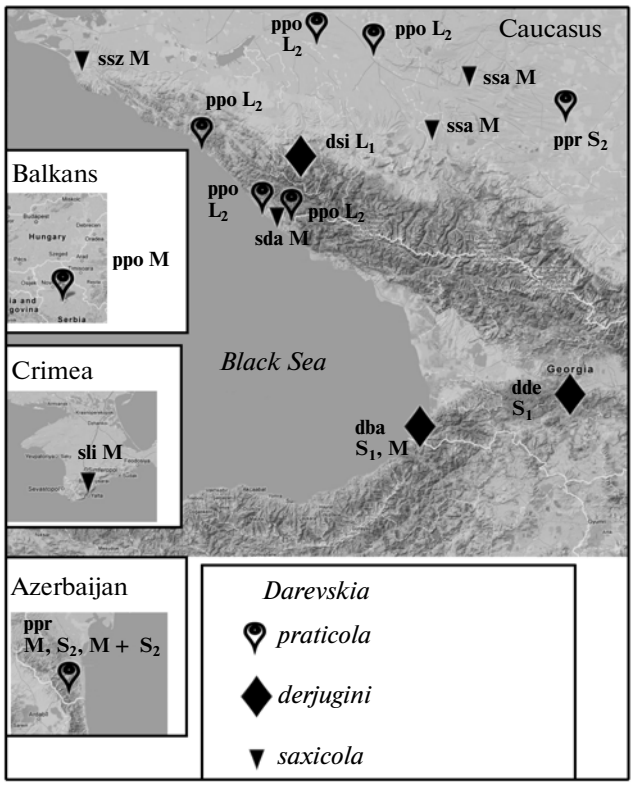

Fig. 6. A map of Western Caucasus with indication of the localization of the species and subspecies (populations) examined. The inserts demonstrate the localities in the Balkans (vicinity of Belgrade), in Crimea (vicinity of Yalta), and in Azerbaijan (Talysh). The types of the alleles, typical to each population (M, L, S) are indicated. Heterozygosity of *D. d. barani* (dba) was observed only in one out of four individuals examined, as well as in the only available individual of *D. d. boehmei* (not shown). For designations of subspecies names, see Table 2.

The allele distribution pattern described is partly consistent with the systematic subdivision of the genus *D. derjugini* into the subspecies based on morphological characteristics [28]. Based on this character, the subspecies *D. d. silvatica* is isolated because it contains allele L1 (Fig. 3). At the same time, allele S1 is characteristic of populations of the two other subspecies, *D. d. barani* and *D. d. derjugini*, which is consistent with the new concepts of the morphologists that these two subspecies are identical ([28], see below). However, the conclusion of the authors that all subspecies of this species are synonymous seems to be premature.

In this context, it should be noted that, in addition to a single lizard, which was at our disposal from the population of *D. d. boehmei* and was found to carry only allele M (not shown), this allele was detected in one out of four lizards of subspecies *barani* examined. The latter lizard also characterized by the S1 allele, which was the only allele found in other *D. d. barani* and *D. d. derjugini* individuals examined. Despite the fact that these two lizards from the *boehmei* and *barani* subspecies were caught in isolated localities (Table 2), it has been suggested that possible hybridization takes place between representatives of both subspecies, which are carriers of only the M and S1 alleles. This issue will remain open until a larger set of *D. d. boehmei* samples has been analyzed. The only thing that is obvious is that at least one of the populations of *D. derjugini* carries allele M. An analysis of other populations of this species deserves special interest.

The suggestion of hybridization is based on the fact that cases of interspecific hybridization in the group of the Caucasian lacertids have been recorded repeatedly and are currently not questioned. The most obvious of these cases are the parthenogenetic populations, of which the unisexual *D. armeniaca, D. dahli, D. rostombekovi, D. unisexualis*, and *D. uzzelli* originated from different combinations of crosses between bisexual species of *D. mixta*, *D. valentini*, *D. portschinskii* and *D. raddei*. Even the origin of bisexual *D. mixta* is thought to be associated with earlier interspecific hybridization between *D. saxicola* and *D. derjugini* [20]. These events (in the case of the formation of parthenogenetic species) were first approved at the morphological level, then supported by molecular genetic data obtained using allozyme and RAPD analysis [25], as well as a comparison of satellite DNA of the presumptive parents and parthenogenetic progeny [36]. In this context, an analysis of the DNA sequences from the populations of *D. d. abchasica* (not examined in our study) deserves special interest, especially because the range of this subspecies has no isolating barriers with that of *D. d. boehmei.* It should be noted that recent extensive zoogeographic studies by Tuniev et al. suggested the absence of distinct isolating barriers between Caucasian populations. Even the high-mountain passes between the northern regions and Transcaucasia do not represent an insurmountable obstacle for the reptiles [28].

A detailed analysis of the Squam1 sequence similarities between the species and populations relative to specific mutations is presented in the Results section. The molecular genetic characteristics of the populations related to these characters generally coincide with the morphological concepts on the subspecific structure of the species (excluding evident synonymy of *D. d. barani* and *D. d. derjugini*) and the subspecific features of Talysh population, as well as the Zelenokumsk and Nalchik populations of *D. praticola* (see below).

An analysis of Central Caucasian populations of *D. d. orlowae*, the range of which probably overlaps with that of *D. d. derjugini,* deserves special interest [28]. This is even more necessary because the morphological systematics of the species of interest is quite ambiguous. The observed heterogeneity of the morphometric indices described for southern and northern slopes made it possible to make a conclusion on the premature description of six subspecific forms and the need for further detailed descriptions of the species (cited from [28]). However, the detailed and careful description of the above-mentioned subspecies of *derjugini* carried out in the study cited did not clarify the situation, and the authors of the study came to a conclusion that the subspecies of *silvatica*, *abchasica*, and *boehmei* are the synonyms of nominative subspecies *D. d. derjugini*.

Our data presented here offer the opposite view at least regarding the nominative subspecies *D. d. derjugini* (in combination with *D. d. barani*) and the northern subspecies *D. d. silvatica*, which are considerably and statistically significant different at molecular genetic characters, including the structure of orthologous locus 34, which contains a copy of interspersed Squam1 repeat, and the distribution and specificity of single mutations (Figs. 3, 4). As was demonstrated in our previous studies, the patterns of RAPD and Inter-MIR-PCR anonymous nuclear markers in *D. d. silvatica* differed considerably in this subspecies and in *D. d. barani* + *D. d. derjugini*. In turn, the latter two subspecies were almost indistinguishable from one another with regard to these markers [24, 25], which is consistent with the results of the present study. Thus, analyses of different nuclear markers produce congruent results, which favor the isolation of the northern population of *D. derjugini* and grouping of *D. d. barani* and *D. d. derjugini* into a single subspecies with the exclusion of ssp. *barani* from the taxonomy of the *Darevskia* genus.

An analysis of the allelic composition of locus 34, which contains the Squam1 copy, in the population of the second widely distributed in the Caucasus species, *D. praticola*, supports the radiation of at least two previously suggested Caucasian subspecies, *praticola* and *pontica*. The differences in the allele structures (Fig. 3) are less expressed than *D. derjugini*. However, these differences are statistically significant, since within the populations, all of the individuals examined (four to

eight individuals from each population) contain almost identical sequences. The carriers of the largest allele (L2), which contains a small insert clearly expressed in all individuals, inhabit the maritime regions of the northwestern slopes of the Caucasus, as well as more continental regions in vicinity of the city of Armavir and the settlement of Petropavlovskaya. These individuals correspond to the morphological subspecies *pontica*. The carriers of the smallest allele (S2) live in southeast of Azerbaijan and in the northernmost point of the range, they live to the north of the Central Caucasus in the region of Zelenokumsk and Nalchik.

The carriage of the intermediate in size allele M was detected in the three lizards from the examined Balkan population (near the city of Belgrade), as well as in three lizards from the Talysh region, which, until recently, was attributed to the *D. d. praticola* subspecies (although, see [37]). The structure of allele M in Balkan population, named after the species name, *D. pontica* [23], differs from that of allele M in Caucasian ssp. *pontica* in 13 specific mutations. In addition, allele M from Balkan population lacks Caucasian-specific indel ATGAG and the set of 13 mutations, characterizing allele L2 (Fig. 3).

The preliminary data, if only the Caucasian populations are considered, first point to the existence of molecular genetic differences between the populations attributed to the subspecies *pontica* and *praticola*. Second, within the *pontica*, the difference between the Central Caucasian population (Zelenokumsk–Nalchik) and southeastern population (Talysh) becomes visible. The first of these populations is characterized by the presence of only allele S2, which carries the large indel in the Squam1 copy. In heterozygous state, the lizards from the second population carry either the M (three individuals) or S2 (four individuals) alleles or both.

These data allow us to suggest that sorting of the M and S alleles, which appeared earlier, is either incomplete or this is the result of hybridization, e.g., in the case of Talysh population. It is also possible that the population of Zelenokumsk–Nalchik, which received only the S2 allele, is the daughter population relative to the Talysh population. This suggestion can be tested in the analysis of Caucasian meadow lizards conducted using a large number of populations and individuals in each population.

Based on the data obtained, it can be suggested that, in at least two subspecies, the subdivision of *D. practicola* does not contradict with the distribution of orthologous alleles, which contains the Squam1 copy, as well as with the single mutation patterns in these alleles. In addition to two subspecies suggested by morphological systematics, it can be suggested that the Talysh population, as well as the population from the vicinity of Belgrade (Balkans), can also be treated as independent subspecies. According to the proposal of Tuniev et al. [37], the Talysh population has already been recognized as the individual subspecies *D. praticola ghyrcanica* (based on morphometric data). On the other hand, the suggestion on the isolation of another population (or other populations) can be considered strictly preliminary and open for discussion and further investigation. At the same time, the systematics of Balkan meadow lizards distributed across the territory, including Rumania, Hungary, former Yugoslavia, and Greece, has only just begun to be investigated [23]. Furthermore, genetic relationships between these lizards and Caucasian populations were not examined so far. It should be also noted that the attribution of the species status to the Balkan population is not supported by a number of authors (for discussion, see analysis in [37]). The data of the present study seem to be useful for extensive phylogeographic analysis of a wide ly distributed *D. praticola* [37], since the use of the Squam1 locus could provide a resolution to contradictions.

Thus, detailed analysis of molecular genetic relationships of individual Squam1 copies is informative relative to their presence or absence in the genomic locus of interest, as well as in comparison of the locus copy properties at the sequences of the locus allelic variants. Similar to our case, or at least in the case of the lizards examined, the use of even singe locus produces valuable and logically relevant (correlated with morphological data) results and confirms the species status of the groups studied. These findings agree with the data obtained using other nuclear markers, e.g., anonymous markers of the taxoprint method [24, 25]. Considerable differences between the three species examined were observed in the content of specific satellite DNA subfamilies. Namely, *D. saxicola*—mostly contains the CLsatI subfamily; *D. derjugini*, CLsatIII; and *D. praticola*—mostly CLsatII [27].

In the present study, we do not discuss the actual phylogeny of the populations examined because it would be premature based on a single locus. The ongoing analysis of the other Squam1-containing loci in the same group of lacertids is necessary for phylogenetic generalizations. In a cross comparison of different markers, our model system of speciation, which is represented by the lizards of the *Darevskia* complex examined previously using different markers (mitochondrial, allozyme, and nuclear markers mentioned above), provides the possibility of obtaining congruent results.

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