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GENOMICS. TRANSCRIPTOMICS. PROTEOMICS

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If a gene tree conflicts with an accepted tree,
one should stop and ponder why.
C. B. Stewart [1].

The Use of Nuclear DNA Molecular Markers for Studying Speciation and Systematics as Exemplified by the "*Lacerta agilis* complex" (Sauria: Lacertidae)

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Abstract—Four types of nuclear DNA markers identified by the taxonprint, RAPD, and IMP (Inter-MIR-PCR) methods, and the nucleotide sequences of satellite DNA monomers have been used to analyze the molecular genetic similarity between some populations, subspecies, and species of lizards combined into the group *Lacerta* s. str., as well as representatives of some other genera. The notions on the systematics and phylogeny of this group based on morphological and zoogeographic criteria have been compared to the conclusions based on molecular genetic data. The genus and species subdivisions of populations based on nuclear molecular markers and morphological characters generally agree with each other, the degree of genetic differences being correlated with the taxonomy suggested by zoomorphologists. The degree of differences between the subspecies of one of the species studied, *Lacerta agilis*, varies depending on the molecular markers used: according to the results of RAPD analysis, all subspecies substantially differ from one another, the variation within populations being small; with respect to other markers, the differences are smaller and not equivalent. The existence of the so-called eastern and western clades of this species earlier assumed by other researchers on the basis of mtDNA and morphological data has been confirmed. There are no distinct gradations exceeding individual variation in 14 populations of *L. agilis exigua* (the eastern clade) with respect to IMP markers, although these populations inhabit a vast area from the Ural Mountains to the Kabardino-Balkar Republic (the Caucasus). These data suggest that the subspecies has been rapidly spreading northwards since the Pleistocene glaciation (about 15,000 years ago).

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Key words: molecular evolution, molecular systematics, nuclear DNA markers, reptilian genome, lizard, Lacertidae

INTRODUCTION

The correspondence between the morphological similarity of organisms and their molecular genetic relationship is the key problem when studying evolution and speciation. Numerous examples of contradictions between the generally accepted systematics, which, in principle, must be based on the hierarchical genetic relationships and reflect evolution, and the results of the genetic analysis of various DNA markers often discourage biologists. Some authors believe that these two approaches are incompatible and that systematics does not "have to" (or may not) use data on the degree of molecular homology (the results of molecular genetic analysis are reviewed in [2]). On the other hand, there is also a notion that modern systematics should be based only on molecular biological data, without regard to morphological data (see review [3]). It seems likely that both extremes are unfounded. As shown in [3], a synthesis of the two is necessary—or at least an understanding of how much morphological and molecular data combined together will help to form scientific systematics based on DNA structure.

This entails many difficulties, both theoretical (e.g., the absence of a self-consistent concept of species and problems related to the analysis of complex sets of molecular data to obtain unambiguous phylogenetic constructions [4–7]) and practical, because the most popular cladistic theory cannot as yet offer a

convenient and testable method for constructing biological systematics (see review [8]).

The cladistic phylogenetic approaches may continue developing successfully and become the basis for a system of the animate world based on its molecular phylogeny. However, we believe that genetic similarity and genetic distances, at least at the level of lower taxa, also seem informative for testing and revising the systematic positions of taxa. First, the morphological and molecular genetic characters of taxa are often coincident or congruent (see reviews [9, 10]), which indicates that there is no homoplasy in these cases, and that the two types of characters are orthologous. Second, it is obvious that the degree of genetic differences as such indirectly reflects the phylogenetic relationship between the taxa compared and the monophyletic origin of the most similar ones.

In the very beginning of the molecular-evolution research boom, D. Hillis, one of the most prominent researchers in this field, formulated the following fundamental consideration. Since an organism studied has only one history, "…systematic study of any set of genetically determined characters should be congruent with other such studies based on different sets at the characters of the same organism. Congruence between studies is strong evidence that the underlying historical pattern has been discovered; conflict may indicate theoretical or procedural problems in one or both analyses, or it may indicate that additional data are needed to resolve the phylogenetic relationship in question" [11].

Proceeding from this principle, we may assume that, if the use of one type (or a limited number) of external characters or molecular markers has lead to contradiction between morphological and molecular systematics, this should not be considered a catastrophe until data on other markers are obtained. Taxonomy should be tested by several methods, and systematics is valid if the biological relationship derived from morphological and zoogeographic characters is correlated with the molecular genetic relationship even based on only one type of markers. If there is no such correlation, this fact should be tested using other molecular markers, and, if these results agree with one another, it should be admitted that the morphological criteria have been chosen incorrectly in the given case, and the system should be changed taking into account direct data on relationships at the DNA level. Unfortunately, this logic is not always held to; most researchers study only one type of molecular markers (usually, mitochondrial genes), which often leads to ambiguous, or at least intermediary, conclusions.

The contradiction between morphological systematics and conclusions based on a single molecular method may result from homoplasy (convergence in the broad sense), which may occur at both the morphological and molecular levels [4]. In the latter case, homoplasy is less harmful, because its probability decreases with an increase in the number of characters (number of nucleotides) and types of molecular markers characterizing different DNA regions; therefore, molecular markers of different types should underlie phylogenetic and genetic conclusions in different cases [4]. Nevertheless, morphological systematics often proves valid and agrees with molecular systematics even if one type of markers is used (see review [12]); therefore, we think that the risk of these contradictions is overestimated (see also review [13]).

We proceeded from these premises to compare the data on genetic relationships within one group of reptiles, namely, some species and subspecies of lizards from the genus *Lacerta* s. str. (the "*Lacerta agilis* complex") obtained with the use of four types of molecular markers. The species from the *L. agilis* complex have a wide geographic range, from Gibraltar and Sweden to Lake Baikal and Kazakhstan. The spread of these lizards over most of their current range was secondary: it occurred after the end of the last Pleistocene glaciation of Eurasia. It is now generally believed that the glacier and permafrost began to recede about 15000 years ago (see review [14]).

The *L. agilis* complex comprises, according to different authors, seven or eight species (*L. agilis, L. strigata, L. viridis, L. bilineata?, L. media, L. schreiberi, L. trilineata*, and *L. pamphilica*) [15–17]; four of them are subdivided into numerous subspecies, although there is some doubt as to whether they occupy the same systematic level (see review [17]). For a long time, as numerous European, Caucasian, and Asian lizards were being described, they were all combined under the common name *Lacerta* suggested by C. Linnaeus about 250 years ago. After a recent revision, a group of species related to *L. agilis* L., 1758 from the genus *Lacerta* s. lato (sensu lato, i.e., in a broad sense) was combined into the genus *Lacerta* s. str. (sensu stricto, i.e., in a strict sense) [18, 19]. In addition, the genera *Podarcis*, *Archaeolacerta, Iberolacerta, Darevskia*, and some others are now distinguished [19–21]. In the genus *Lacerta* s. str., the morphology of *L. agilis* has been studied in more detail than that of other species [15, 17]. Initially, this induced taxonomists to subdivide it into about 20 subspecies, but their number and names remained unstable. Some subspecies were afterwards regarded as synonymic [15] and others (e.g., *altaica* and *kurtuana* [22]) were devalued. There are other uncertainties. Some authors regard the subspecies *bilineata* of the species *L. viridis* as a separate species; the significance of the separation of the species *media* and *trilineata* is likewise unclear [17].

These and other examples reflect the instability and incompleteness of the system of the *Lacerta* s. str. complex; obviously, the time has come to use molecular marker to evaluate the genetic relationships

between geographical populations of this genus. Such studies were not conducted until recent time (see Discussion).

We used markers determined by the taxonprint [23, 24], Inter-MIR-PCR (IMP) [25, 26], and RAPD [27] methods, as well as satellite DNAs that we found when studying the *L. agilis* complex [28–30]. We earlier used this approach for studying *Darevskia*, another genus from the same family of lizards, and it proved to be sufficiently informative [28].

EXPERIMENTAL

The species and subspecies studied are listed in the table. Figure 1 shows a schematic map of the sites where representatives of these species and subspecies were collected.

The isolation and analysis of DNA were described earlier (see references in the Introduction). These articles describe experimental details and the methods for the analysis of genetic similarity.

The distance NJ trees [31, 32] were constructed as described in the cited studies.

RESULTS

Analysis of Genetic Similarity Based on Taxonprint Data

This method permits obtaining the electrophoretic distribution patterns of labeled DNA repeats (the taxonprint) after exhaustive hydrolysis of DNA with a restriction endonuclease. Each taxonprint contains a set of all repetitive DNA regions (not only satellite and dispersed ones) after DNA digestion by a given endonuclease. We demonstrated that the distribution patterns and intensities of taxonprint bands were identical in all animals from the same population and were species- and genus-specific [23, 24]. Obviously, the greater the similarity between populations (with respect to as many taxonprints as possible), the closer the genetic relationship. In the cases when we could collect animals from different populations of the same species (as was the case, e.g., with several populations of *Darevskia raddei*), we found that geographically close populations were almost identical with respect to taxonprint markers, whereas some of the more remote populations had characters that were outapomorphic for them, which reflected the start of speciation [33]. Conversely, the DNAs of all lacertid genera studied (*Darevskia, Lacerta, Podarcis, Eremias, Gallotia, Zootoca*, and *Ophisops*) substantially differed from one another in all taxonprints. However, they had several fractions that were synapomorphic for all genera, i.e., specific for the family Lacertidae [23]. It is especially important that species of the *L. agilis* complex and the Caucasian lizards from the "*L. saxicola* complex," which were earlier included in the same genus *Lacerta* s. lato, substantially differed (with respect to most taxonprints). These data, together with other, purely morphological ones, served as the basis for classifying the *L. saxicola* complex as a separate genus *Darevskia* [19].

Species and subspecies of *Lacerta* s. str. analyzed in this study

Species and subspecies	Number of specimens	Place of collection
L. agilis agilis	1	Darmstadt, Germany
L. a. exigua	2	Tula Region, Russia
″	3	Voronezh Region, Russia
″	1	Lipetsk Region, Russia
″	2	Orenburg Region, Russia
″	3	Astrakhan Region, Russia
″	2	Kabardino-Balkar Republic, Russia
″	1	Adygei Republic, Russia
″	2	Udmurt Republic, Russia
″	1	Kalmyk Republic, Russia
″	2	Krasnodar Region, Russia
″	4	Kharkiv Region, Ukraine
″	1	Dnepropetrovsk Region, Ukraine
″	1	Crimea, Ukraine
L. agilis boemica	1	Kabardino-Balkar Republic, Russia
L. agilis brevicaudata	3	Kuchak, Armenia
L. agilis chersonensis	3	Tula Region, Russia
″	1	Pskov Region, Russia
″	1	Kaliningrad Region, Russia
″	1	Leningrad Region, Russia
″	1	Minsk Region, Belarus
L. strigata	1	Dagestan, Russia
″	2	Lake Sevan, Armenia
L. viridis	2	Dnepropetrovsk Region, Ukraine
L. media	1	Aranler, Armenia

Figure 2 shows, as an example, three of the nine taxonprints obtained. These taxonprints illustrate the intergeneric difference (between the genera *Darevskia* and *Zootoca*) as compared to the species of the *agilis* complex, between species of this complex (*agilis, strigata*, and *viridis*), and between three *L. agilis* proper (*chersonensis, boemica*, and *agilis*). Analysis of all these and other taxonprints involving the construction of matrices of pairwise similarity makes it possible to construct the NJ tree (Fig. 3). All genera of

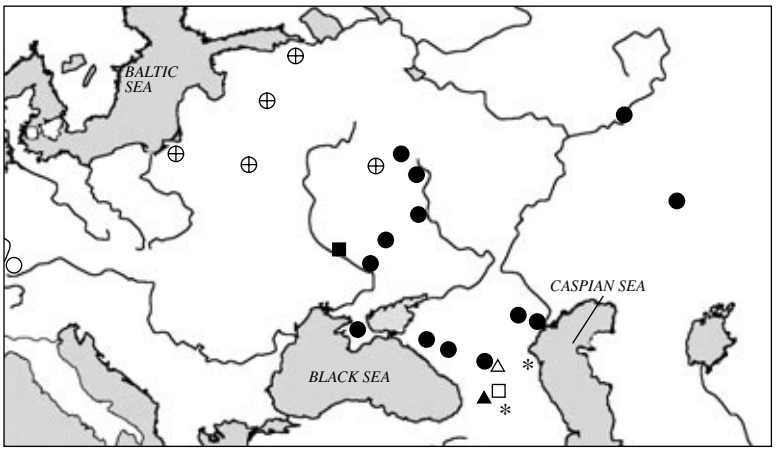

Fig. 1. Geographic distribution of populations of the species and subspecies of *Lacerta* s. str. studied: ● *L. agilis exigua*; △ *L. a. boemica*; □ *L. a. brevicaudata*; ⊕ *L. a. chersonensis*; ○ *L. a. agilis*; * *L. a. strigata*; ■ *L. viridis*; ▲ and *L. media.*

the family Lacertidae form clades, which confirms their morphological systematics. Zoologists believe that the genera *Zootoca, Darevskia*, and *Podarcis* were genetically close to *Lacerta* s. str., and the genera *Eremias, Gallotia*, and *Ophisops* were more remote from it [18]. Harris *et al.* [20, 21] and Fu [34] obtained similar relationships in experiments with fragments of three mitochondrial genes (the 12S and 16S rRNA genes and *cyt b*).

Thus, three species from the *L. agilis* complex (*agilis, strigata*, and *viridis*) differed in these molecular markers from the other genera, whereas the differences between species of this complex were insignificant. This suggests that either the repeats remained conserved during the genus evolution or the three species diverged only recently.

The close similarity of populations of the same genera that are regarded as separate species in systematics was confirmed later when studying the nucleotide sequences in monomers of one of the repeats isolated from *Taq*I and *Hind*III taxonprints.

Analysis of Genetic Similarity Based on Data on Satellite DNA

The nucleotide sequences of satellite DNA monomers and the pattern of their organization into tandem arrays are taxon-specific markers; they are widely used for studying speciation and molecular genetic relationships (see reviews [9, 10]). Earlier, we found and characterized satellite DNA families of lizards from the genus *Darevskia* [26] and the aforementioned three species from the *L. agilis* complex (Agi160 [29, 30]). The two satellite DNA families, in which monomer lengths are 150 and 160 bp, respectively, have a common ancestral form, because they have long shared conservative sequences [29]. However, they also have distinct differences exceeding the differences within each genus.

Figure 4 shows the alignment of the Agi160 monomers of three species from the *L. agilis* complex (after multiple alignment), which served as the basis for constructing the NJ tree of genetic relationship (Fig. 5). We also studied the DNA of three subspecies of *L. agilis* (Fig. 2). As evident from Fig. 4, monomer sequences could be subdivided into three clades generally corresponding to three morphological species. However, the positions of subspecies *L. a. boemica* and *L. a. exigua* in the species clade were not resolved significantly, whereas the third subspecies, *L. a. agilis*, was segregated from them.

In addition to three main species of this complex, we used a sample of DNA of another species, *L. media* from Transcaucasia (subspecies *isaurica*), in which the nucleotide sequences of satellite DNA have not been determined thus far. According to the results of dot and Southern hybridization, Agi160 repeats are also found in the DNA of *L. media*; however, either they are exceptionally degenerate or the number of their copies is substantially smaller than that of the repeats in other species from this complex [30], because the intensity of hybridization with a probe isolated from *L. agilis* is small and is expressed at lower temperatures of washing [29, 30]. In both cases, this species can be assumed to be more distantly related to the first three species; however, it belongs to the clade *Lacerta* s. str.

Analysis of Genetic Similarity Based on RAPD Data

It is known that markers of this method are insufficiently informative when studying taxa of a higher rank than species; they are mainly used in population analysis [4]. For a number of reasons, the variation of electrophoretic band patterns cannot serve as a mea-

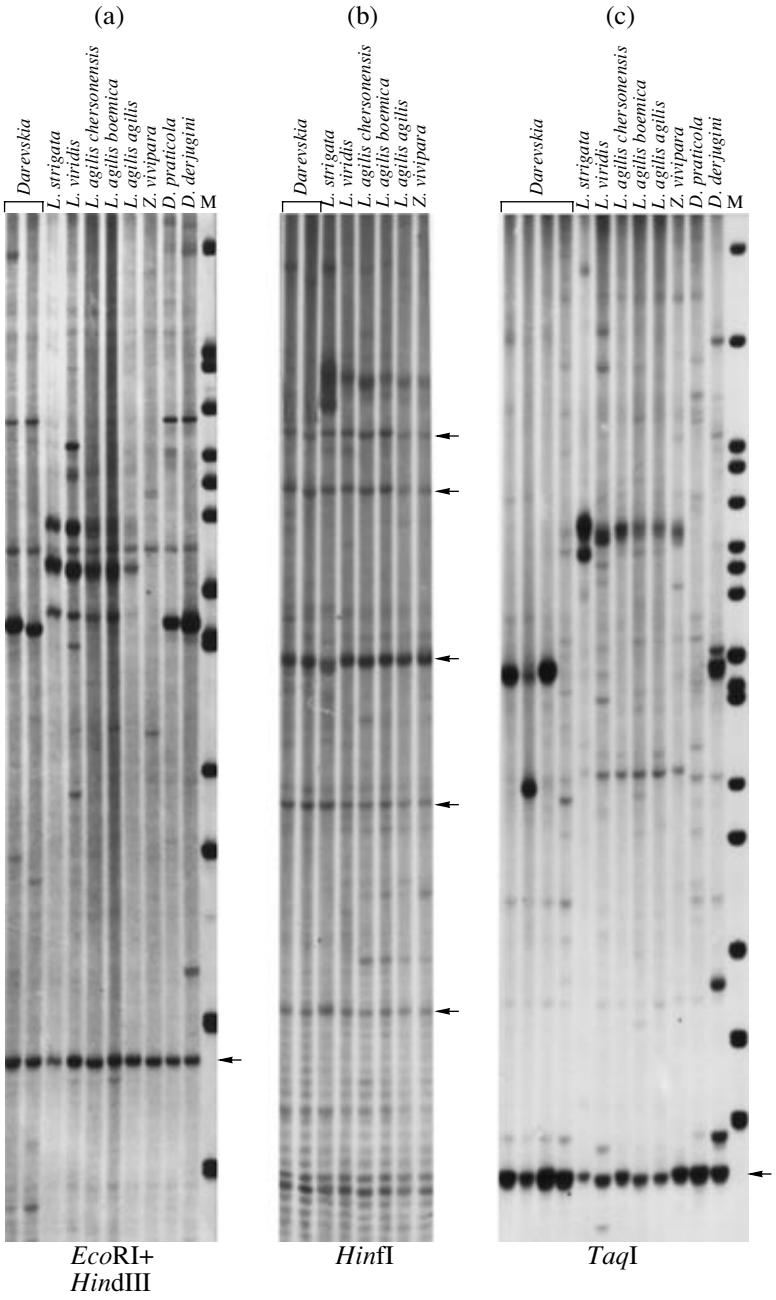

Fig. 2. Comparison of DNA taxonprints of some species and subspecies of *Lacerta* s. str. and the genera *Darevskia* and *Zootoca* after DNA cleavage by restriction endonucleases (a) *Eco*RI + *Hin*dIII, (b) *Hinf*I, and (c) *Taq*I. The fractions of the repeats common for different genera of the family Lacertidae are indicated with arrows. M, DNA fragment length (bp) markers.

sure of genetic relationship when comparing taxa; however, a complete identity of the patterns is conclusive evidence for the identity of genetic material for the primers studied, which indicates that the populations are closely related or identical to one another. Therefore, similarity or pronounced differences are indirect evidence that the animals belong to the same taxon or different taxa, respectively.

As can be seen from Fig. 6a, neither *L. strigata* (two populations from Dagestan and Azerbaijan) nor *L. viridis* (one population from Ukraine) exhibited individual DNA heterogeneity with respect to these markers. Earlier, we obtained similar data on five populations of *D. saxicola* formally distinguished as subspecies in the system (*saxicola, darevskii, brauneri, szczerbaki*, and *lindholmi*), four of which (living in the Northern Caucasus) were practically identical to one another with respect to RAPD markers, whereas the fifth subspecies, *lindholmi* (living in Crimea) substantially differed from them with respect to both RAPD

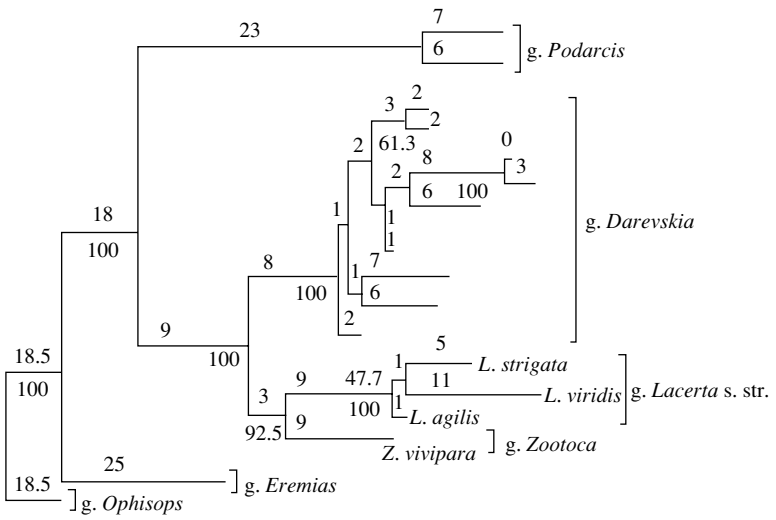

Fig. 3. An unrooted NJ tree of some genera of lizards from the family Lacertidae based on the comparison of all taxonprints studied. Numbers above and below the lines show Fitch's distances and bootstrap indices (100 iterations), respectively. Bootstrap values lower than 50% are not shown.

markers and the results of taxonprint analysis [23, 25]. These data were confirmed by the results of comparison between subfamilies of the CLsat satellite (specific for the genus *Darevskia*), which also demonstrated that the Crimean subspecies differed from the Caucasian ones in both the set of subfamilies of this satellite and the presence of a unique satellite variant (autapomorphic for this species) that was not found in other species of this genus [36].

Regarding RAPD markers in the DNAs of different species and subspecies of the *L. agilis* complex, all of them proved to be entirely different (Fig. 6b). We consider the characterization of populations based on RAPD markers to be a good species character if these patterns are either entirely identical or entirely different. Earlier, we showed that the RAPD marker patterns of *D. valentini* and *D. portschinskii* (which were very similar to each other with respect to other molecular characteristics) were practically the same, which allowed us to question the validity of their separation into two species, although they differ in the morphological criteria generally accepted in systematics [23]. These two species are also practically indistinguishable from each other in taxonprints [33] and the nucleotide sequences of the monomers of the CLsat satellite [37]. Conversely, in the case of *Lacerta* s. str. (Fig. 6), the three species substantially differed from one another with respect to the RAPD patterns; they had no common bands at all. Five subspecies of *L. agilis* also differed, but they had synapomorphies for the pair *a. agilis* and *a. chersonensis* (Fig. 6, lanes *3* and *4*) and the triplet *a. boemica, a. brevicaudata*, and *a. exigua* (Fig. 6, lanes *1, 2, 5*, and *6–8*). These data are sufficient to support the subspecies subdivision of *L. agilis*, although the number of characters detected by this method was small, and it became necessary to use another more informative method. Therefore, we subsequently used IMP markers, which proved to be more sensitive in the intraspecific range in studies on the genus *Darevskia* [25] and which yielded more characters.

Analysis of Genetic Similarity Based on Inter-MIR-PCR (IMP) Data

This method permits the comparison of the sizes of spacer DNA regions dividing copies of dispersed SINE repeats. Primers specific for conserved regions of these repeats are used, which makes it possible to amplify spacers of copies several tens to several hundreds of nucleotides in size that are located relatively close to one another. Since MIR homologs of mammalian SINE repeats have been found in all taxa of eukaryotes [38], we used primers for conserved regions of this repeat [25, 26]. It has been found that the individual specificity of the electrophoretic patterns of amplification products in lizards is usually low and can be revealed in the analysis. This was originally demonstrated in experiments with DNA from 12 lizards of the same subspecies of *D. derjugini* [25]. Our experiments with DNA from several lizards of the same species (Fig. 7) confirm this conclusion. In the case of pairwise comparison, the bands that occupied the same position in the gel were considered homologous (irrespective of their intensities), whereas the absence of the band was regarded as the absence of the character (a 1/0 matrix). The resultant matrices were presented in the form of trees with the use of the TREECON software [32] using the neighbor-joining (NJ) algorithm.

Figure 7 shows the electrophoretic pattern obtained in one experiment on IMP markers. Differences between species of the complex were expressed in the presence of either synapomorphic fractions or those outapomorphic for each species (indicated by arrows), which were sufficiently distinct. The differences between subspecies of *L. agilis* were less manifest and more difficult to analyze because of pronounced individual variations (the subspecies *agilis, chersonensis, exigua, boemica*, and *brevicaudata*). This heterogeneity especially strongly masked the population structure of the subspecies *L. a. exigua.*

The results of this and other experiments formed the basis for an NJ tree (Fig. 8), in which none of the subspecies *exigua, boemica*, and *brevicaudata* clustered separately. This group lives in eastern and southeastern Europe; *chersonensis* and *agilis* from western Russia and Europe are located separately from each other (Fig. 1).

In general, these data agree with the data on RAPD markers (see above) indicating that the subspecies *exigua, boemica*, and *brevicaudata* are close to one another, as are *chersonensis* and *agilis*. Since we studied different numbers of lizards from different subspecies (from 1 to 25), it was difficult to take into account the individual heterogeneity of each subspecies with small number of specimens. In addition, some subspecies were unavailable (e.g., *bosnica, argus*, and eastern populations of *exigua*). The question remains open until we obtain results on a sample of all subspecies.

Nevertheless, we have already obtained enough data to suppose that the current subspecies categories are far from equivalent, and their systematics requires further analysis.

DISCUSSION

Comparing the data obtained with the use of different molecular methods, we can draw the following conclusions.

First, the group of lizards collectively termed the *L. agilis* complex indeed forms a separate clade of closely related species, judging by the taxonprint and satellite DNA markers; this fact supports the status of this group as a genus, along with other genera of the lacertid family (*Darevskia, Podarcis, Eremias,*

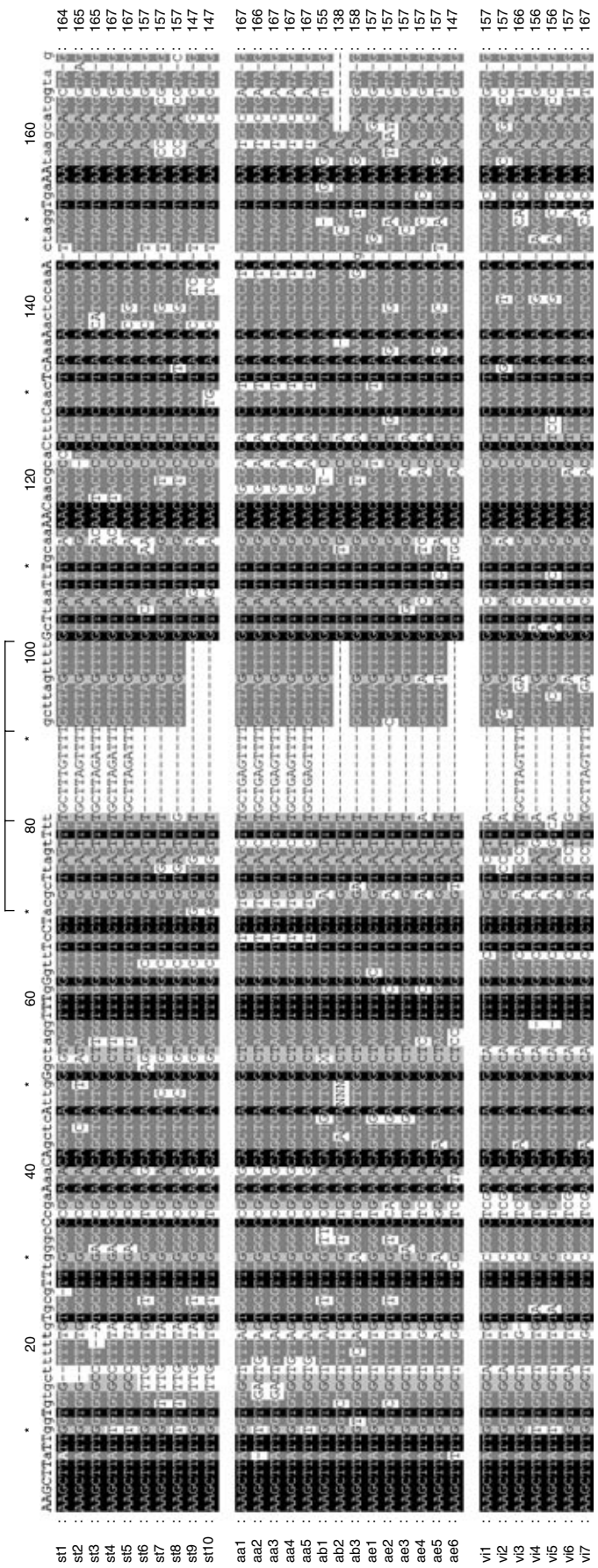

Fig. 4. Alignment of the nucleotide sequences of monomers of the Agi160 satellite family based on published data [26, 27] and the results of this study. The regions of complete identity are shown in black; the regions carrying single substitutions (individual variation of monomers) are shown in gray. The positions of three decanucleotides are shown above; some of them are deleted. Heterogeneity with respect to the number of decanucleotides is characteristic of different monomers of the same organism. Designations: aa, *L. agilis agilis*; ab, *L. a. boemica*; ae, *L. a. exigua*; st, *L. strigata*; vi, *L. viridis.*

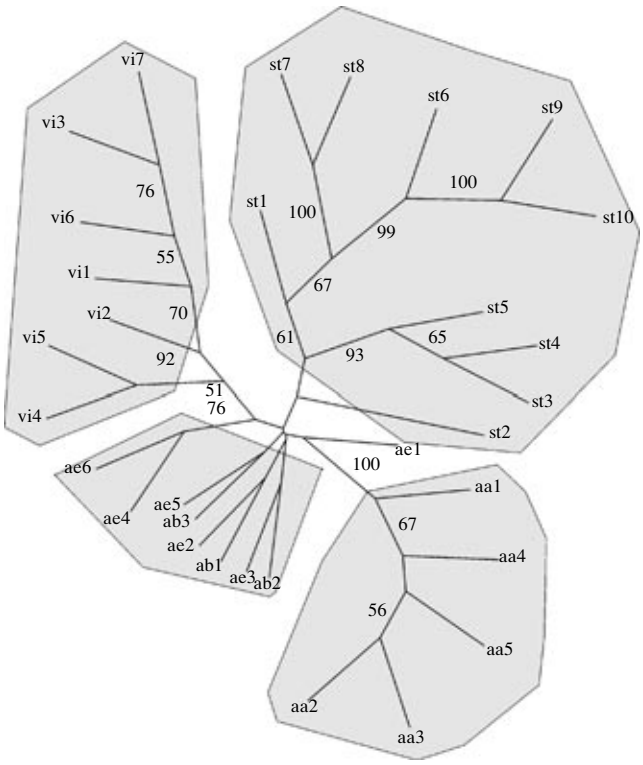

Fig. 5. An unrooted NJ tree based on the comparison between the nucleotide sequences of the monomers of the Agi160 satellite DNA shown in Fig. 4. Designations are the same as in Fig. 4. Monomer clusters are outlined. Bootstrap values lower than 50% are not shown.

Ophisops, and *Gallotia*) (Fig. 3) [29]. This conclusion is based on the data obtained on four species. We have included one of them, *L. media*, in the *L. agilis* complex on the basis of several synapomorphic markers revealed by the IMP method (Fig. 7) and the presence of the Agi160 satellite, which does not hybridize with DNA of any other genus studied, except for the aforementioned four species from the genus *Lacerta* s. str. [30]. Other authors combine the Iberian species *L. schreiberi* [39], which we have not studied, with *L. agilis* and *L. media* on the basis of their similarity with respect to mitochondrial genes [20]. This species may also be regarded as a member of the clade *Lacerta* s. str., as was suggested earlier, on the basis of zoological characters alone.

Second, our data indicate that the subdivision of the complex into species is, in general, correlated with species clustering with respect to all types of markers studied (at least regarding the species dealt with in this study) (Figs. 3, 5, 8).

Third, it was difficult to determine the small degree of genetic differences between morphological subspecies of the species *L. agilis*, because we had representative samples only for subspecies *exigua* and *chersonensis* (Fig. 8). In the subspecies *L. a. exigua*, we studied, with the use of the IMP method, many populations from a vast area stretching from the Udmurt Republic and the Orenburg Region southwards, through the central region of Russia and the Kalmyk

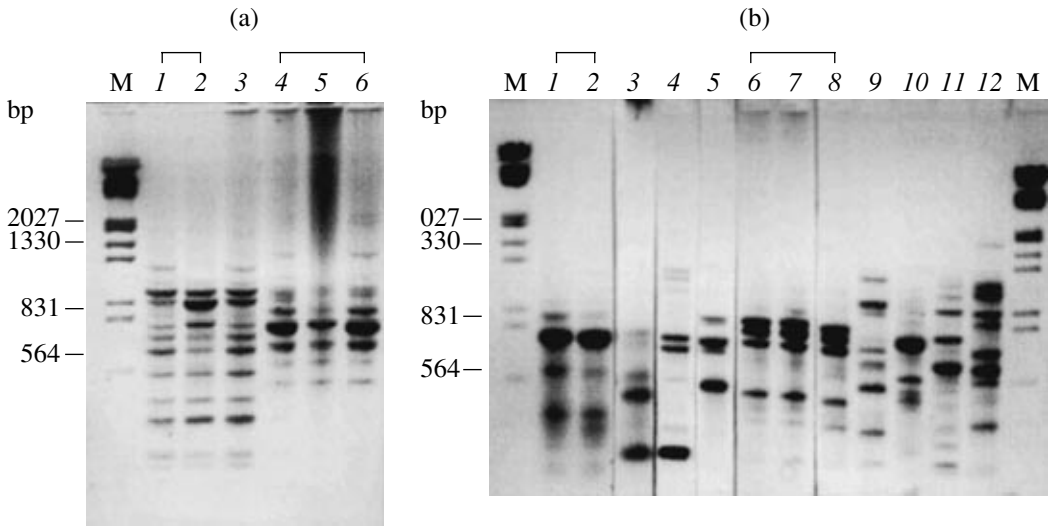

Fig. 6. Comparison of the electrophoretic patterns of RAPD markers of DNA from different species of *Lacerta* s. str. Each lane contains the amplification product of DNA fragments from one animal. (a) DNA of (*1–3*) *L. strigata* and (*4–6*) *L. viridis*, primer A: *1*, the Dagestan population; *2, 3*, the Dilizhan (Armenia) population; *4–6, L. viridis*, the Dnepropetrovsk (Ukraine) population. (b) DNA of *L. agilis* subspecies amplified with the use of primer B: *1, 2, a. exigua*; *3, a. agilis*; *4, a. chersonensis*; *5, a. brevicaudata*; *6–8, a. boemica*; *9, L. strigata*; *10, L. viridis*; *11, Zootoca (Lacerta) vivipara*; *12, Podarcis taurica.* The brackets above show the number of animals of the same (a) species or (b) subspecies. M, markers (bp)

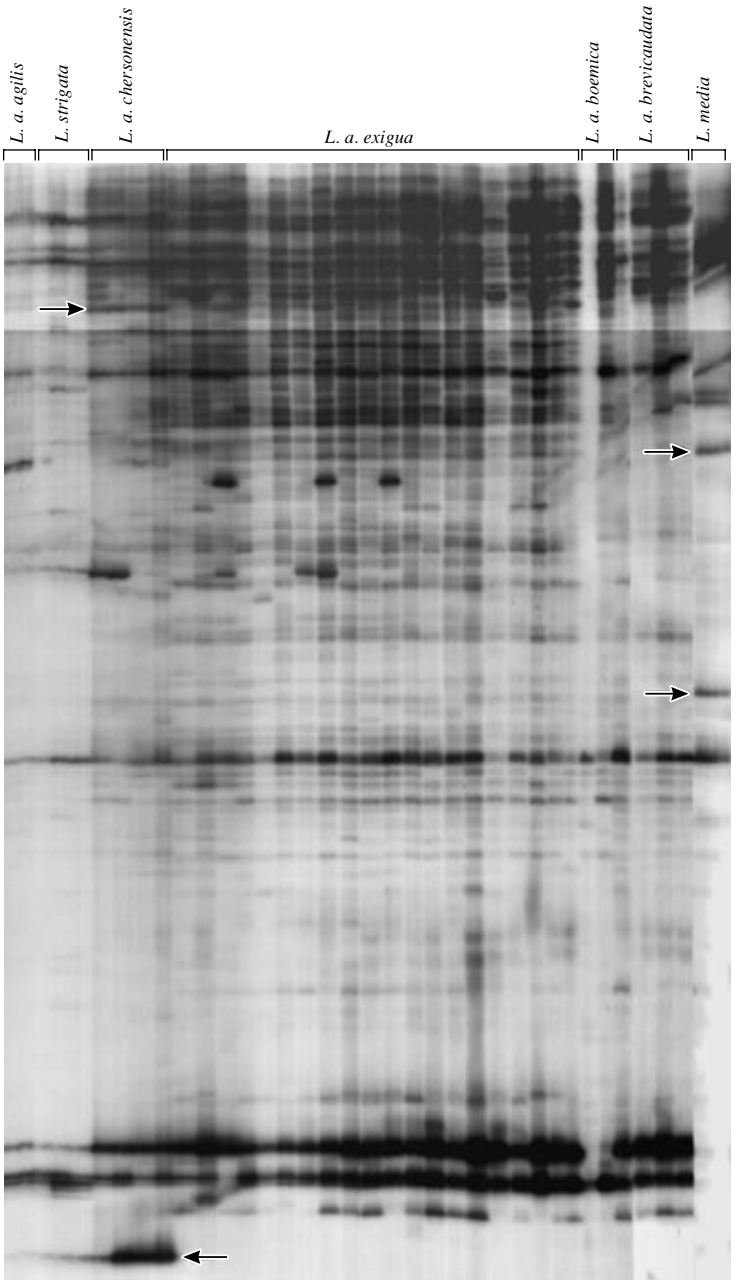

Fig. 7. The electrophoretic patterns of DNA markers amplified in the presence of primers complementary to the conserved regions of SINE repeats (the IMP method [22, 23]). Each lane contains the DNA product of one animal. Populations of *L. a. exigua* are shown in the table and Fig. 8. Autapomorphies are indicated by arrows.

Republic, to the Kabardino-Balkar Republic (Fig. 1). This analysis did not show any differences outside the limits of individual variation within populations studied. The subspecies *brevicaudata* and *boemica*, although they were represented by a few animals, were indistinguishable from *exigua*, falling within the same range of individual and interspecific variation (Fig. 8). These data agree with our data on RAPD markers (Fig. 6) and satellite DNAs, which also showed that these subspecies were more similar to one another within each combination (Fig. 5). Note, however, that there are doubts regarding the correct identification of a lizard from the subspecies *boemica* captured in the vicinity of the city of Nalchik, where the range of this subspecies bordered on that of the subspecies *exigua*; therefore, the conclusion on the clustering of *boemica* and *exigua* should be considered preliminary.

The correctness of the subdivision of *L. agilis* into at least two subspecies, *exigua* and *chersonensis*, is also evidenced by the fact that populations of *chersonensis* located almost sympatrically with the subspe-

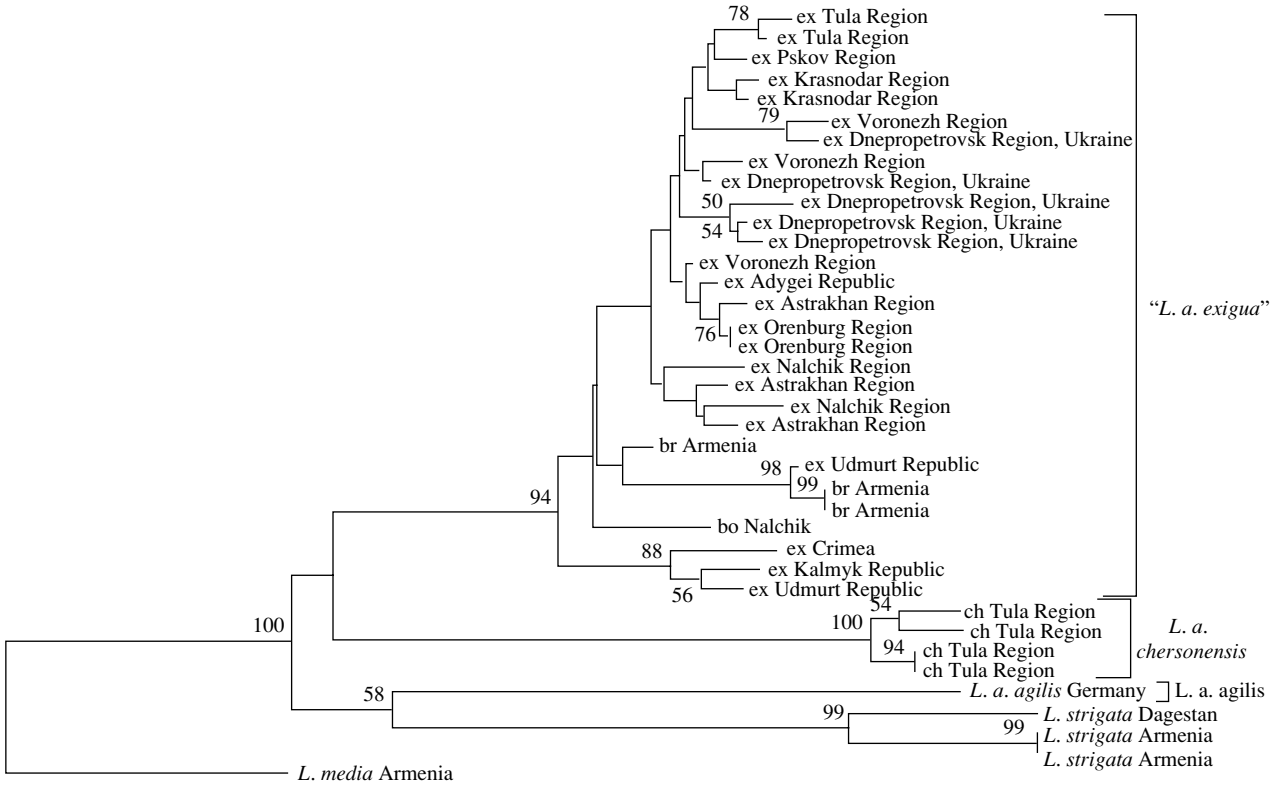

Fig. 8. An NJ tree based on the matrix of pairwise comparison between IMP markers of different subspecies of *L. agilis*, as well as *L. strigata* and *L. media.* See also Fig. 1 and the table for the positions and designations of the *L. agilis* populations studied. Abbreviations ex (*exigua*), bo (*boemica*), br (*brevicaudata*), and ch (*chersonensis*) apply to the morphological subspecies of *L. agilis*, representatives of which were captured in the places indicated in the tree.

cies *exigua* in the Tula and Pskov regions differ from it considerably more than any two of the 14 populations of *exigua* differ from each other (Fig. 1, solid circles).

Thus, we may only assume that subspecies of *L. agilis* may be grouped into eastern (including the Caucasian populations) and western branches; we arbitrarily call the former *L. exigua* and the latter *L. chersonensis.* Regarding the westernmost population, which is classified as a separate subspecies *L. agilis agilis*, we can only note that the only specimen that we had in our sample was located in the IMP tree separately from other subspecies, as could be expected taking into account its status and geographic location. In the NJ tree based on the comparison of monomers of Agi160 satellite DNA (Fig. 5), the subspecies *L. a. agilis* also formed a separate cluster. (We did not study the subspecies *argus*, which occupied the position intermediate between *agilis* and *chersonensis*.)

Kalyabina *et al.* [22] came to the same conclusion from their data on mtDNA markers in subspecies of *L. agilis.* In the sample used in [22], the subspecies *brevicaudata* and *boemica* were represented by large numbers of animals. Our data agree with the notion that the subspecies *brevicaudata* is not a separate taxon and should be regarded as a morph of the eastern clade that includes *exigua* [22].

The results of our study indicate that the results of molecular genetic analysis and morphological systematics agree well with each other. An accurate determination of the subspecies status in terms of molecular genetics is the most difficult and, probably, ultimately unsolvable problem, not only in the given case, but also in the case of any taxon. This reflects the actual situation with the transitional state in diverging and evolving populations, where the initial stages and rates of speciation undoubtedly differ, involving different DNA regions. The evolution of these regions is expected to vary depending on environmental conditions, and no equivalent and objective criteria (either morphological or molecular) of the subspecies status in any, even small, taxa are likely to appear. Apparently, this problem can be solved only at the qualitative level.

Finally, note that we did not find substantial differences between populations of the subspecies *L. agilis exigua* (beyond the range of individual differences within populations) with respect to IMP markers (Fig. 8). This indicates that the period after the last Pleistocene glaciation in Eurasia (about 15,000 years ago [14]),

when the species has been migrating over the area stretching over at least 1000 km from the south to north, is too short for interpopulation divergence and speciation. The results of our study confirm the data reported by Kalyabina-Hauf *et al.* [17], who demonstrated that the variation of the mtDNA marker (a fragment of gene *cyt b*) in *exigua* populations of the same areas was negligibly small. Moreover, they did not find differences with respect to this marker in the Kazakhstan population (near Lake Zaisan) located more than 1000 km farther to the east [17]. It seems likely that the colonization of such a vast area by the subspecies *L. agilis exigua* occurred in a burst, which may have been favored by the geographic situation: the subspecies spread across forest–steppes and steppes with relatively few water bodies and mountains that could hamper the spread. These considerations agree with Nichols and Hewitt's hypothesis [14, 39] that the migration to new habitats occurred very rapidly due to the "pioneer" populations, with maximum homozygosity being preserved and one species subsequently becoming the founder. This explains the low degree of genetic divergence in these populations compared to the slowly spreading populations of the main "phalanx" (according to the authors' terminology) [39] that we observed in our study.

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