ISSN 1022-7954, Russian Journal of Genetics, 2023, Vol. 59, No. 3, pp. 297–306. © Pleiades Publishing, Inc., 2023.
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ANIMAL GENETICS

# Application of DNA Barcoding to the Study of Green Lizards (Sauria: Lacertidae: *Lacerta*)

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Received May 2, 2022; revised May 28, 2022; accepted June 3, 2022

**Abstract**—DNA barcoding remains a recognized and widely used method for taxon identification in biodiversity inventory and monitoring. In this publication, we present information about the results of using this method in the study of green lizards of the genus *Lacerta*. A total of 67 sequences of the *COI* gene fragment of three species (*L. agilis*, *L. media*, and *L. strigata*) were analyzed. For the first time, all subspecies of *L. agilis* known in the Caucasus were studied using DNA barcoding, and a phylogeographic hypothesis for *L. strigata* in the Caucasian part of the range was proposed. Genetic identification has been successful at the species level. In the composition of *L. agilis* within the Caucasus, we can diagnose only the subspecies *L. a. boemica* and *L. a. exigua*, while *L. a. brevicaudata*, *L. a. grusinica*, and *L. a. mzymtensis* do not differ in this marker from *L. a. exigua* and *L. a. ioriensis* from *L. a. boemica*.

**Keywords**: *Lacerta agilis*, *Lacerta media*, *Lacerta strigata*, mitochondrial DNA, *COI*

**DOI:** 10.1134/S1022795423030031

## INTRODUCTION

Systematics and phylogeny of green lizards of the genus *Lacerta* Linnaeus, 1758, which according to modern ideas includes ten species [1], is currently undergoing significant changes. In our opinion, the Caucasus is a promising area for the study of these issues [2, 3]. Three species of the genus live within this region—the Sand lizard *L. agilis* Linnaeus, 1758, represented here by six subspecies (*L. a. boemica*, Suchow, 1929, *L. a. brevicaudata* Peters, 1958, *L. a. exigua* Eichwald, 1831, *L. a. grusinica* Peters, 1960; *L. a. ioriensis* Peters et Muskhelischwili, 1968, and *L. a. mzymtensis* Tuniyev et Tuniyev, 2008); the nominative subspecies of the Three-lined lizard *L. m. media* Lantz et Cyrén, 1920; and the monotypic Caspian green lizard *L. strigata*, Eichwald, 1831 ([4], www.lacerta.de). Phylogenetic studies of *L. agilis* in the Caucasus region did not receive development after the work of S.A. Kalyabina-Hauf [5] and the release of her joint monograph with N.B. Ananjeva [6]. It has been proposed to upgrade the current status of *L. a. boemica* to species (as in the original publication of G.F. Sukhov [7]), while reducing a number of other Caucasian subspecies into junior synonyms *L. a. exigua* [6, 8]. Molecular genetic research of *L. a. ioriensis* and *L. a. mzymtensis* have not been carried out at all. Going beyond the Caucasus, we note that the question of the taxonomic position of the Transcarpathian populations of the Sand lizard remains unresolved [6, 9–11]. Phylogeography and phylogenetic relationships of populations are poorly studied *L. strigata* [12] in the Caucasus region. This also applies to *L. media* [13], despite the fact that this species is included in the superspecies complex *Lacerta* (*trilineata*), which is the focus of research on the phylogeny of the genus *Lacerta* [1, 14].

Previously published works have suggested that there are pathways of invasion and colonization of green lizards in the Transcaucasus region [15], as well as the relic nature of populations of the Caspian green lizard in the Terek-Kuma Lowland, Kumo-Manych Depression [16], and the Black Sea coast of the Caucasus in Abkhazia [17]. One of the objectives of this study was to check these hypotheses. It is also important that a number of designated taxa of green lizards are included in the regional and national lists of protected animal taxa (Red Data Books); this emphasizes the relevance of this study from the standpoint of studying and biodiversity conservation. Molecular genetic data make it possible to identify truly unique populations (beyond the currently accepted subspe-

(a)

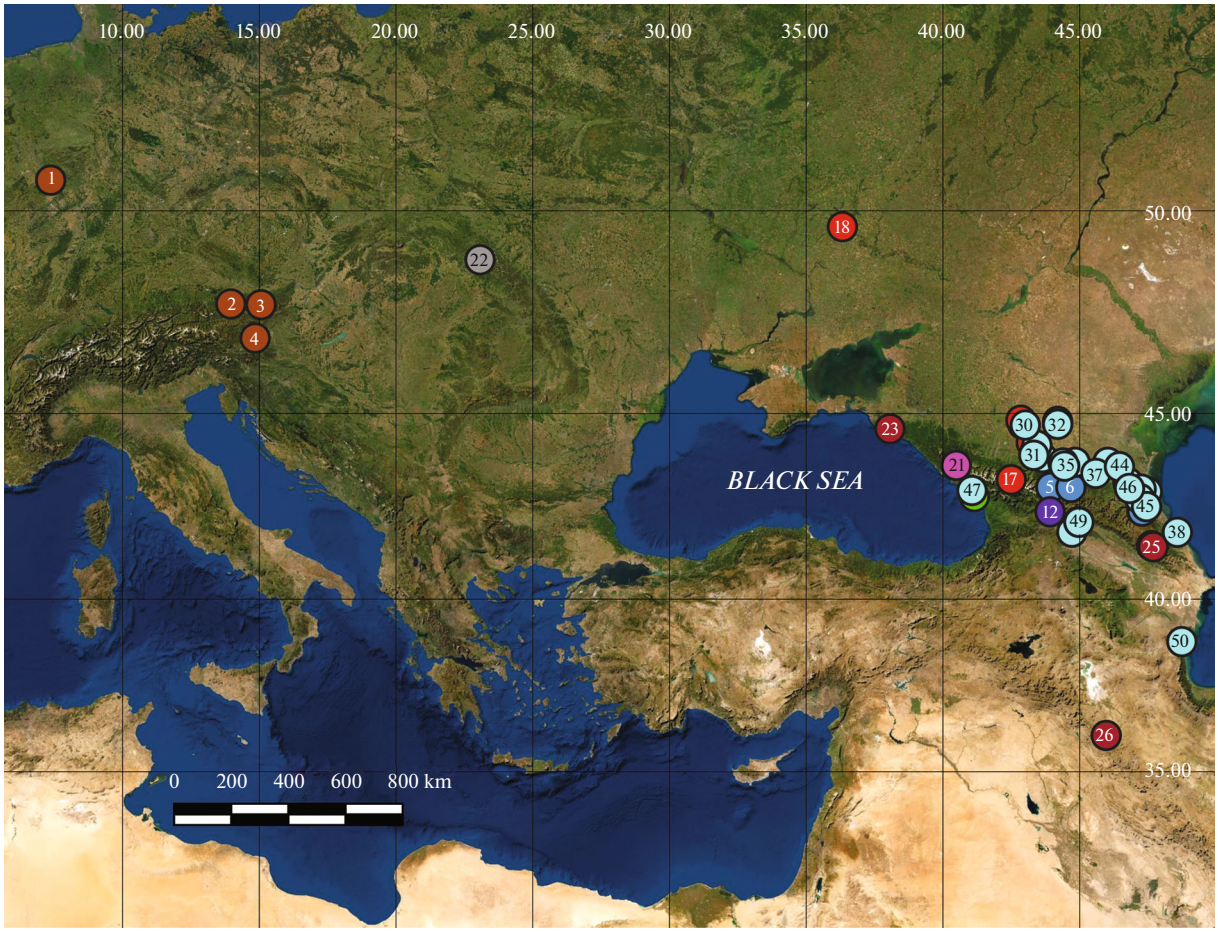


**Fig. 1.** Locality records of *Lacerta* used for molecular genetic analysis: (a) all species, (b) *L. agilis*, (c) *L. media*, and (d) *L. strigata*. The numbering corresponds to that in the appendix. The color of the circle corresponds to the taxon in Figs. 2 and 3.

cies taxonomy, which is largely outdated in the case of *L. agilis*) and justify the need for their protection. The diagnostics of species also remains relevant, since we noted errors in many published works and databases on their distribution [18].

The foregoing indicates the need for a more detailed analysis of the phylogeography, taxonomy and distribution of lizards of the genus *Lacerta* in the Caucasus and adjacent territories. DNA barcoding (biological identifications through DNA barcodes) continues to be an effective method in this area. In herpetological studies, its effectiveness has been shown for studies at the global [19] and regional [20–22] levels. This tool is also successfully used in studies of systematically complex groups of lizards [23].

## MATERIALS AND METHODS

To isolate the genomic DNA, parts of regenerated tails, phalanges of forelimbs, or skin of lizards fixed in 96% ethanol were used. Voucher specimens are stored in the collection of the Zoological Institute of the Russian Academy of Sciences (ZISP; the collection was maintained within the framework of project of the Ministry of Science and Higher Education of the Russian Federation no. 075-15-2021-1069) and the Zoological Museum of Moscow State University (ZMMU). Phylogenetic analysis included 67 sequences of the fragment of the mitochondrial gene of the first subunit of cytochrome oxidase (*COI*) *L. agilis*, *L. media*, and *L. strigata* collected in 50 localities (Appendix) (Fig. 1). Of these, 62 were obtained in the course of this study, five were taken from GenBank (http://www.ncbi.nlm.nih.gov/gene/) [20–22].

In the course of studying the external morphometric characters, on which the taxonomy of the Sand lizard is based [24], we encountered the absence of reliable markers for diagnosing a number of its subspecies in the Caucasus. For this reason, the identification of subspecies was carried out on the basis of information about their ranges, according to the current ideas [6, 24–26]. To compare the Caucasian subspecies belonging to the eastern group [6], we used *COI* sequences of representatives of the western group of

(b)

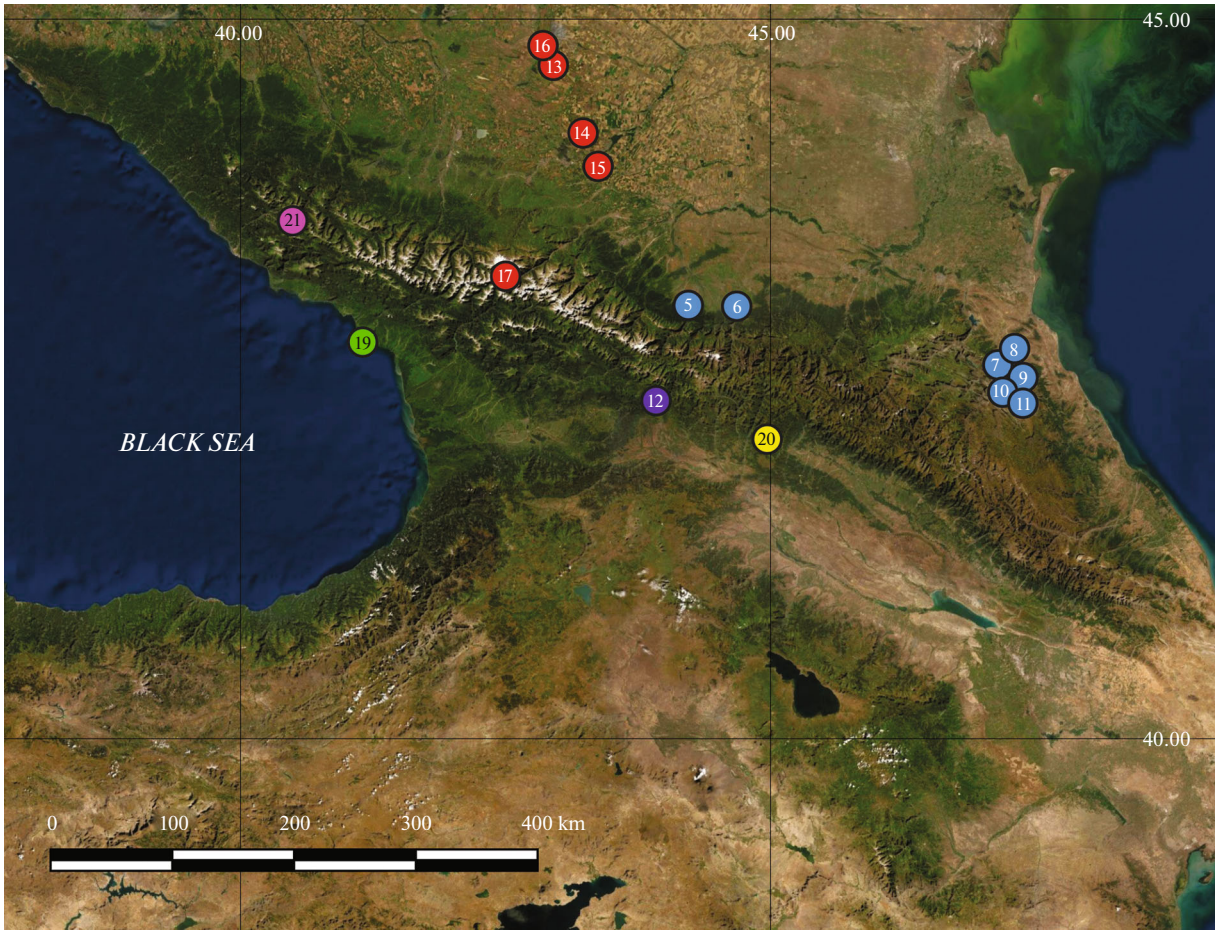


**Fig. 1.** (Contd.)

subspecies *L. agilis argus* (Laurenti, 1768) and Transcarpathian *L. agilis* ssp. It is important to emphasize that the analysis used type specimens (holotype *L. a. mzymtensis*, ZISP24648) and topotypes (*L. a. boemica*, ZISP31113, *L. a. ioriensis*, ZISP29878), which makes our conclusions about the taxonomy reliable and justified.

Isolation of total DNA was carried out by the standard salt method with lysis of proteinase K [27]. Amplification of a section of gene *COI* (643 bp) was carried out using universal primers UTF 5'-TGT AAA ACG GCC AGT TCT CAA CCA AYC AYA ARG AYA TYG G-3' and UTR 5'-CAG GAA ACA GCT ATG ACT ARA CTT CTG GRT GKC CRA ARA AYC A-3' [28] under the following conditions: initial denaturation 95°C for 3 min and, then, 30 cycles at 95°C for 30 s, 50°C for 30 s, and 72°C for 50 s, with final synthesis being at 72°C for 5 min.

The reaction mixture for PCR (25 μL) contained 50–100 ng of DNA, 0.5 μM of each primer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 2.5 μL of 10× PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), and 2 units. Taq polymerase (Thermo Scientific). Sequencing was performed on an ABI PRIZM 3500xL genetic analyzer (Applied Biosystems). The resulting sequences were deposited at GenBank NCBI (OM267788–OM267849).

Sequence alignment was carried out using the Geneious Prime 2021.0.1 (https://www.geneious.com) and AliView 1.6 programs [29]. Phylogenetic relationships were reconstructed using Bayesian analysis (BA) in MrBayes 3.1.2 [30–32] and the maximum likelihood (ML) method in the MEGA X software package [33]. For BA, 1.5 million generations were used; the statistical reliability of ML tree nodes was assessed by bootstrap analysis (2000 pseudoreplicas). The stability of dendrogram nodes of phylogenetic relationships in the case of BA was assessed by calculating posterior probabilities. The node support levels were 0.95, high; 0.90–0.95, medium; and less than 0.90, unsupported; the bootstrap support values in ML analysis were more than 75%, significant; 50–75%; trend; and less than 50%, no support [34]. Sequences are chosen as the outer group *Zootoca vivipara* (Lichtenstein, 1823) (MN015068) and *Timon*

(c)

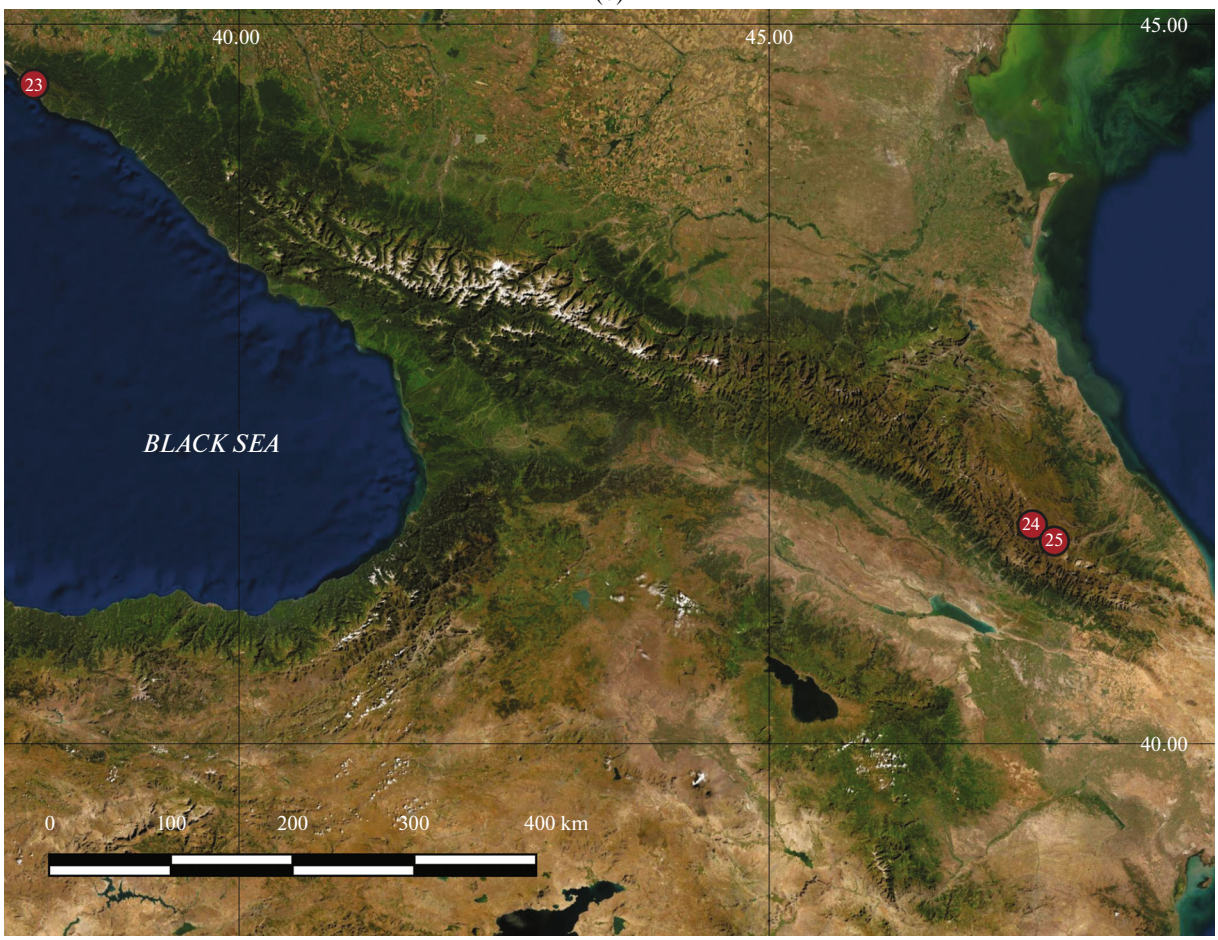


**Fig. 1.** (Contd.)

*lepidus* (Daudin, 1802) (MN015075). In addition, the analysis involved *L. trilineata* Bedriaga, 1886 (MN015090).

The selection of an evolutionary model for nucleotide sequences was carried out in the MrModeltest 2.4 program [35] using the Akaike (AIC)–GTR (General Time Reversible) information criterion with parameters $I = 0.3691$ and $G = 0.1844$. Graphic images of trees were obtained using the FigTree 1.4.4 software (http://tree.bio.ed.ac.uk/software/figtree). The haplotype network was constructed using the TCS method in the PopART program [36]. When analyzing genetic variability using the DnaSP v.5.10.01 program [37], the following parameters were calculated: total number of polymorphic positions $S$, total number of substitutions $\eta$, number of haplotypes $H$, haplotype diversity $h$, diversity of nucleotides per site $\pi$, average number of nucleotide substitutions $K$, Tajima's test value (Tajima's $D$), and Fu's neutrality test (Fu's $Fs$). Genetic distances ($p$ distances) were calculated using the MEGA X program.

The automated simultaneous analysis phylogenetics (ASAP) [38] and automatic barcode gap discovery (ABGD) algorithms [39] were used to analyze taxonomic boundaries based on molecular genetic characters. They make it possible to identify molecular phylogenetic taxonomic units (MOTU), which are monophyletic clades of indeterminate rank. The analysis was performed using the following parameters: $P_{min}$ (minimum a priori distance) = 0.01, $P_{max}$ (maximum a priori distance) = 0.1, and X (relative interval width) = 0.5; a matrix of pairwise K80 distances was used.

## RESULTS

During the study, 62 *COI* gene sequences of green lizards were obtained (OM267788–OM267849); five previously published sequences (KP697820, MN993135, MN993136, MN993138, and MG815780) ranged in length from 630 to 657 bp. Thirty of the studied sequences of *L. agilis* form 17 haplotypes (Hap 5–8, 10–15, 20, 22, 24–28); 54 (8.4% of the total length of the fragment) variable positions were identified, of which 43 (6.7%) were parsimony informative,

(d)

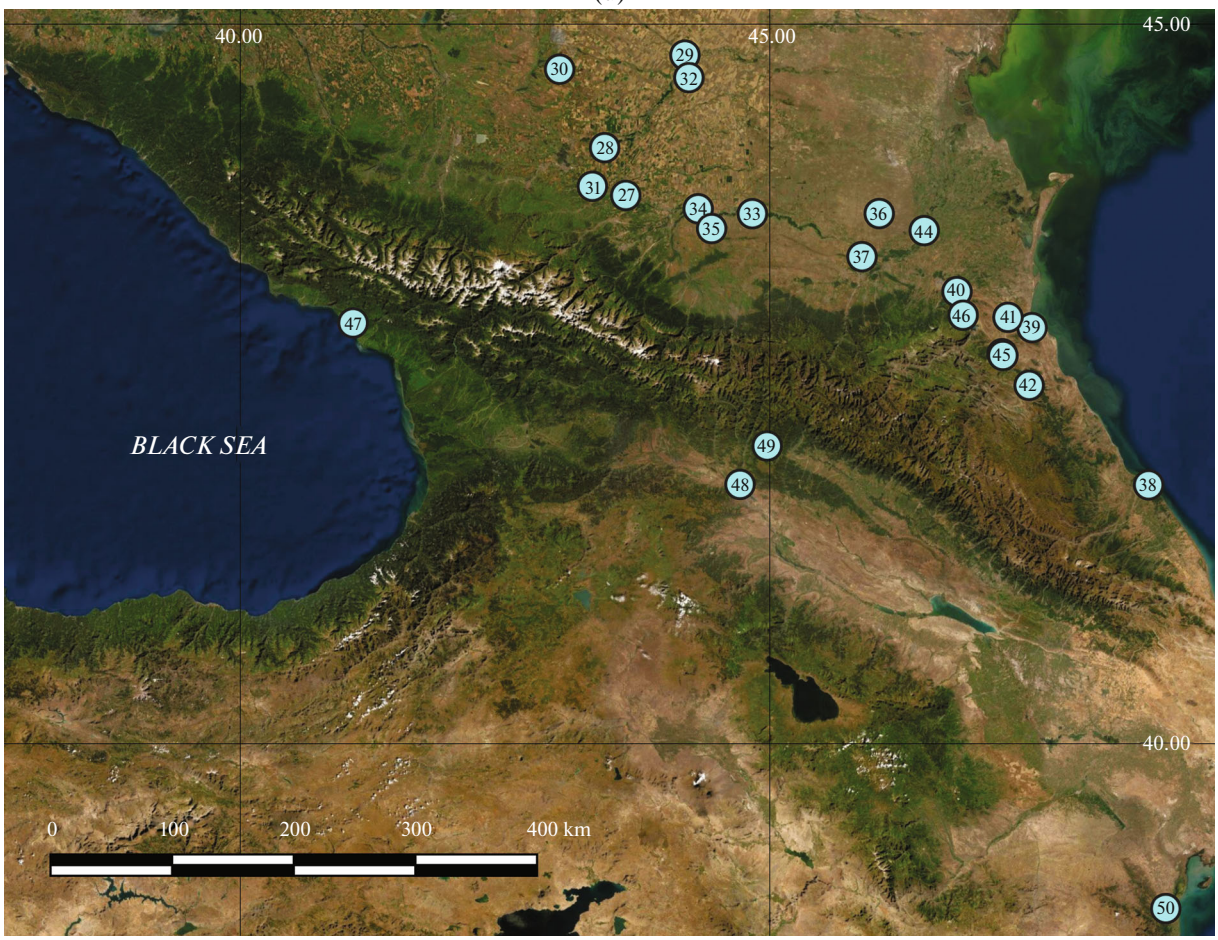


**Fig. 1.** (Contd.)

and the ratio of transitions to transversions was 7.6. Four *COI* gene sequences of *L. media* form three haplotypes (Hap 16–18); 14 (2.2% of the total length of the fragment) variable positions were identified, of which one (0.2%) was parsimony informative; the ratio of transitions to transversions was 2.1. Thirty-three sequences of *L. strigata* form seven haplotypes (Hap 1–4, 9, 20, 22); 13 (2% of the total length of the fragment) variable positions were identified, of which four (0.6%) were parsimony informative; the ratio of transitions to transversions was 7.7.

Indicators of genetic diversity in the sample are presented in Table 1. Relatively high values of haplotype and nucleotide diversity in the Sand lizard indicate a stable population with a high abundance or a possible secondary contact of historically separated populations [40]. A possible recent expansion of the Caspian green lizard from the refugium may be indicated by low values of genetic diversity indicators, and negative significant indicators *D* may suggest the presence in the past of a sudden population growth (expansion) and/or positive selection [41].

**Table 1.** Genetic diversity in the studied samples of green lizards of the genus *Lacerta*

| Species | *n* | *S*/η | *h* | π | *K* | Tajima's *D* | Fu's *Fs* |
|---|---|---|---|---|---|---|---|
| *L. agilis* | 30 | 54/54 | 0.94 ± 0.02 | 0.029 ± 0.002 | 17.72 | 1.12 ($p > 0.1$) | 0.89 ($p > 0.1$) |
| *L. media* | 4 | 14/14 | 0.83 ± 0.02 | 0.011 ± 0.005 | 7.16 | 0.62 ($p > 0.1$) | 2.08 ($p > 0.1$) |
| *L. strigata* | 33 | 13/13 | 0.38 ± 0.11 | 0.002 ± 0.001 | 1.32 | −1.90 ($p < 0.05$) | −1.62 ($p < 0.05$) |

*n* is sample size, *S* is total number of polymorphic positions, η is total number of substitutions, *h* is diversity of haplotypes, π is diversity of nucleotides per site, *K* is average number of nucleotide substitutions, Tajima's value *D* is Tajima test value, and Fu's *Fs* is the value of Fu's test for neutrality.

**Table 2.** Average genetic *p* distances (%) (under the diagonal) and their standard deviations (above the diagonal) between species of the genus *Lacerta* according to the analysis of *COI* gene fragment sequences (643 bp)

| Species | *L. strigata* | *L. agilis* | *L. media* |
|---|---|---|---|
| *L. strigata* | | 1.1 | 1.3 |
| *L. agilis* | 10.1 | | 1.2 |
| *L. media* | 11.8 | 11.1 | |

The minimum genetic distance value (*p* distance) was found in the pair of species *L. agilis* and *L. strigata* (10.1%), maximum in *L. media* and *L. strigata* (11.8%) (Table 2). According to the results of the reconstruction of the phylogenetic relationships of representatives of the genus *Lacerta*, dendrograms with identical topology were obtained, in which five clades with high bootstrap supports can be distinguished (Fig. 2). On the resulting median network of haplotypes (Fig. 3), three haplogroups can be identified: (I) *L. agilis* with three subgroups (Ia, *argus*; Ib, *boemica*; Ie, *exigua*), (II) *L. media*; and (III) *L. strigata*. The most distant from all others is haplogroup III. It is 25 nucleotide substitutions distant from haplogroup I and 22 from haplogroup II. The ASAP/ABGD analysis showed a clear gap between the values of subspecies and species variability (threshold distance is 2.9%; barcoding gap is 3.0%). The application of these algorithms made it possible to identify five monophyletic MOTUs in the volume of the studied sample.

## DISCUSSION

The studied species of green lizards demonstrate a high level of differentiation by gene *COI*; i.e., its species specificity was confirmed on our material. On the resulting dendrogram (Fig. 2), the first to be separated are *L. triliniata* and *L. media*, which are traditionally included in *Lacerta* (*triliniata*) [14]. This is inconsistent with published data obtained using fragments as mitochondrial (*cytb*, *12S*, *16S*) and nuclear (*C-mos*, β-*fib*) genes [12, 14]. Thus, it was shown that *L. strigata* appeared at earlier stages of the radiation of the genus *Lacerta* [12]. This indicates a low efficiency of the DNA barcode for studying the phylogenetic relationships of species of the genus *Lacerta*. The next to be isolated on the dendrogram is *L. strigata*, and then, finally, *L. agilis*.

Sand lizard sequences, in turn, formed three clades. Inside *L. agilis*, the first clade to be isolated is *L. a. boemica*, which also included *L. a. ioriensis* (*p* distance 0.8%). This confirms the hypothesis that the Iora lizard is genetically close to the *L. a. boemica* [15, 24, 25, 42]. It is noteworthy that the most distant

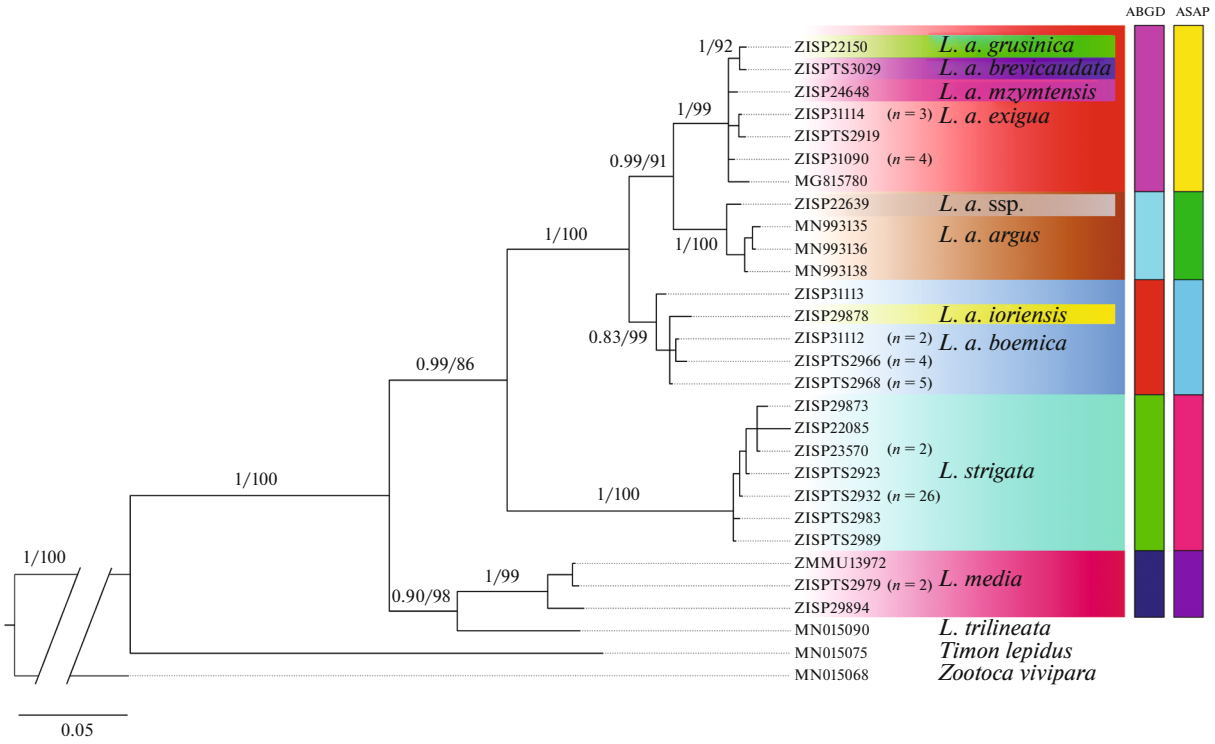


**Fig. 2.** Dendrogram of phylogenetic relationships of representatives of the genus *Lacerta* according to results of analysis of the *COI* gene fragment (643 bp). The nodes indicate Bayesian posteriors and bootstrap supports (BA/BS). The color designation of taxon corresponds to that in Fig. 1. The two columns indicate the highlighted molecular operational taxonomic units (MOTU) in different colors.

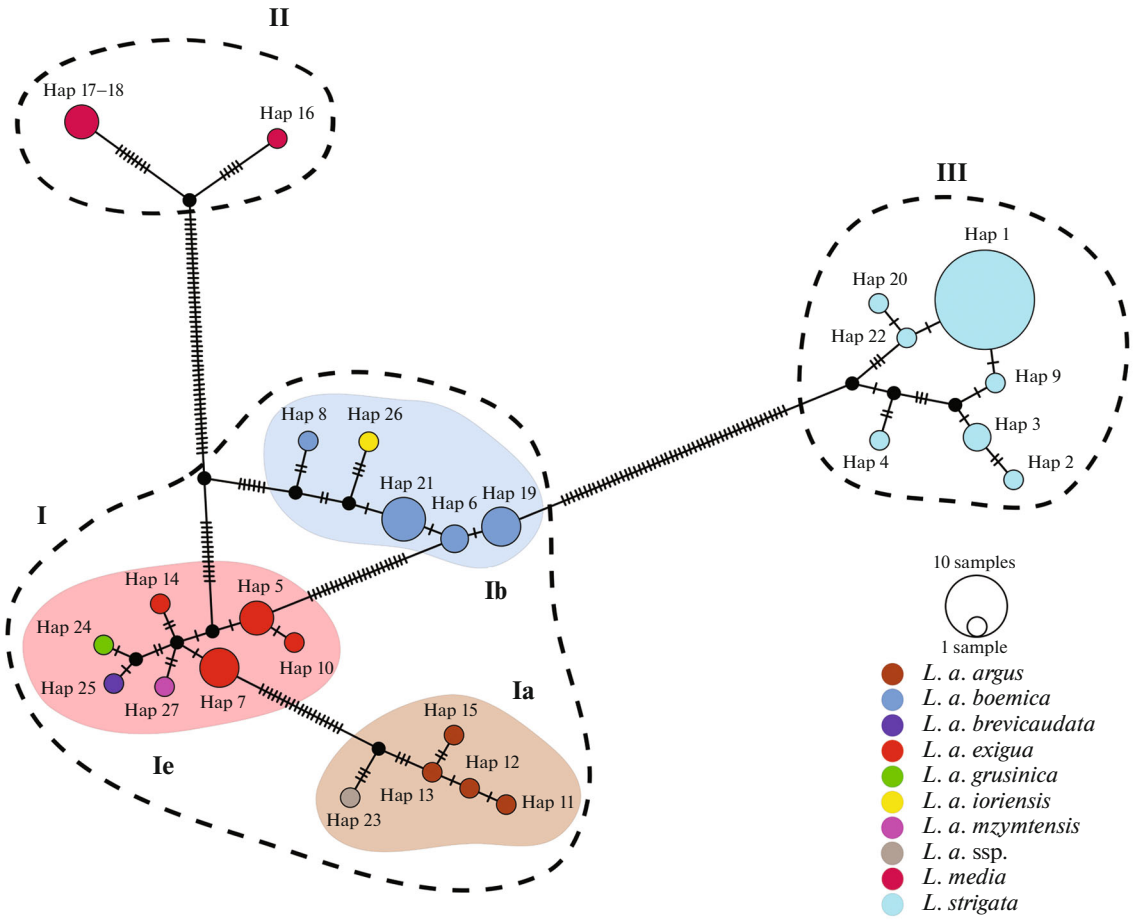


**Fig. 3.** Median haplotype network of *COI* (643 bp) of representatives of the genus *Lacerta* in the Caucasus and adjacent territory. Each colored circle is a unique haplotype the size of which is proportional to its occurrence in the sample. The color of the circle corresponds to the taxon in Figs. 1 and 2. Connecting lines are probable evolutionary relationships, serifs are nucleotide substitutions, and black circles at line nodes are predicted haplotypes. Haplotype groups (I–III) are bounded by a dotted line; subgroups of haplotypes are indicated by colored shading *L. agilis* (Ia, *argus*; Ib, *boemica*; Ie, *exigua*).

individual in this sample is from the Terek River valley in Vladikavkaz (Hap 8). In is likely that, in the past, in cold periglacial conditions on the Ossetian Sloping Plain, there were isolated microrefugia of this taxon confined to the valleys of large rivers. An individual from a nearby locality—the city of Alagir in the Ardon River valley, which also has a unique haplotype (Hap 6), may be indirect confirmation of this hypothesis. As in previous publications [6, 8] *L. a. boemica* demonstrated maximum differences with other *L. agilis* subspecies, this figure being 4.0–4.6% (Table 3). At the same time, the differences of *L. a. boemica* from other subspecies do not reach a level comparable to those between the recognized species of the genus.

The Transcarpathian *L. agilis* ssp. is grouped with *L. a. argus* from Austria and Germany (*p* distance 1.2%). This is consistent with the conclusions of K.D. Milto [43] based on the study of external morphology. Publications the authors of which defend the taxonomic independence of the Transcarpathian populations on the basis of morphometric characters [9–11] did not include a comparison of it with the subspecies of the western group (with the exception of *L. a. chersonensis* Andrzejowski, 1832), including *L. a. argus*.

The endemic Caucasian subspecies *L. a. brevicaudata*, *L. a. grusinica*, and *L. a. mzymtensis* entered the same clade with *L. a. exigua* (*p* distance 0.3–0.8%). In it, in comparison with other clades, structuring is the least pronounced. It has been previously shown that populations of *L. a. exigua* are characterized by such low mitochondrial gene variability of *cytb* that geographically remote populations from the Caucasus to East Kazakhstan do not have significant differences in this marker [2, 6]. This may indicate a rapid colonization of the vast expanse of the steppe and forest–steppe zones of Eurasia by the Sand lizard at the end of the late Pleistocene–Holocene [2].

As in special work on the phylogeography of *L. media* based on gene analysis of *cytb* [13], the samples from the territory of the Greater Caucasus and the

**Table 3.** Average genetic *p* distances (%) (under the diagonal) and their standard deviations (above the diagonal) between subspecies of *Lacerta agilis* according to the analysis of *COI* gene fragment sequences (643 bp)

| Subspecies | *L. a. argus* | *L. a. boemica* | *L. a. brevicaudata* | *L. a. exigua* | *L. a. grusinica* | *L. a. ioriensis* | *L. a. mzymtensis* | *L. a.* ssp. |
|---|---|---|---|---|---|---|---|---|
| *L. a. argus* | | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 | 0.4 |
| *L. a. boemica* | 4.6 | | 0.8 | 0.8 | 0.8 | 0.3 | 0.8 | 0.8 |
| *L. a. brevicaudata* | 4.3 | 4.5 | | 0.3 | 0.2 | 0.8 | 0.4 | 0.8 |
| *L. a. exigua* | 4.1 | 4.0 | 0.8 | | 0.3 | 0.7 | 0.3 | 0.8 |
| *L. a. grusinica* | 4.3 | 4.5 | 0.3 | 0.8 | | 0.8 | 0.4 | 0.8 |
| *L. a. ioriensis* | 4.7 | 0.8 | 4.2 | 3.9 | 4.2 | | 0.8 | 0.8 |
| *L. a. mzymtensis* | 3.9 | 4.3 | 0.8 | 0.6 | 0.8 | 4.1 | | 0.8 |
| *L. a.* ssp. | 1.2 | 4.5 | 4.2 | 3.8 | 4.2 | 4.6 | 3.7 | |

Zagros attributed to the nominative subspecies demonstrated a fairly significant genetic distance from each other (*p* distance 2.1%) exceeding the interpopulation value in other studied taxa of green lizards.

It is worth noting the pronounced stellate structure of the haplotype network in *L. strigata* characterized by the predominance of the mass haplotype Hap 1, which is dominant in the North Caucasus (Appendix). Haplotypes from the territory of North Ossetia (Hap 9) and Dagestan (Hap 20 and 22) differ slightly from it—by one and three nucleotide substitutions, respectively. Three of them were found on the territory and in the vicinity of the Buglen village (Hap 1, 20, and 22). The sequence of an individual from the Sukhumi city is identical to that from the Mtskheta city (Hap 3); moreover, these localities are located at a distance of more than 320 km from each other (respectively, within the boundaries of the Colchis Lowland and on the southern slope of the Greater Caucasus) and are characterized by completely different physical and geographic conditions. This testifies to the dispersal of *L. strigata* to the Black Sea coast from the southeast, and not from the north, bypassing the Main Caucasian Range. In terms of haplotype diversity, Transcaucasia surpasses the North Caucasus, which was apparently inhabited only at the later stages of the formation of the species range. In general, despite the presence of isolated populations, no significantly distant haplotypes were found in the Caucasus, which can probably be considered evidence of the youth of the range of the Caspian green lizard in the region.

Molecular operational taxonomic units strictly correspond to the recognized species of the genus *Lacerta* and are not consistent with the subspecies structure of the Sand lizard. Identified contradictions between the currently accepted intraspecific taxonomy of *L. agilis* and differentiation of subspecies according to *COI* indicate that it needs to be revised with the use of more informative genetic markers. At the same time, the DNA barcode can be used for species diagnostics of green lizards in the Caucasus and adjacent territories.

## ACKNOWLEDGMENTS

The authors are grateful to K.D. Milto, V.F. Orlova, and B.S. Tuniyev for valuable advice and assistance in the work.

## FUNDING

Molecular genetic studies were carried out with the financial support of a grant from the Russian Science Foundation, no. 22-24-00079.

## COMPLIANCE WITH ETHICAL STANDARDS

*Conflict of interest*. The authors declare that they have no conflicts of interest.

*Statement on the welfare of animals*. All applicable international, national, and/or institutional guidelines for the care and use of animals have been followed.

## SUPPLEMENTARY INFORMATION

The online version contains supplementary material available at https://doi.org/10.1134/S1022795423030031

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