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ANIMAL GENETICS

Mitochondrial Evidence of Refugial Distribution of the Pygmy Field Mouse *Sylvaemus uralensis* Pall. (Rodentia, Muridae) in the Northwestern Caucasus

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Abstract—Variation of the 838-bp fragment of the mitochondrial *cytb* gene was analyzed in *Sylvaemus uralensis* from the northern macroslope of the Western Caucasus. On the basis of two fixed nonsynonymous substitutions, *cytb* sequences of the population sample studied can be considered as a distinct Lago-Naki haplogroup, which is clustered in the European *cytb* lineage. As estimated on the basis of the known rate of substitutions per third codon position in *S. sylvaticus,* the population must have been isolated for all or a part of the last glaciation period (10000 to 100000 years ago). The observed differentiation of *cytb* haplotypes is indicative of the refugial distribution of *S. uralensis* in the northern macroslope of the Western Caucasus, as well as of a secondary contact between the Caucasian and the Russian Plain populations during the Holocene.

Keywords: pygmy field mouse, *Sylvaemus uralensis*, phylogeography, *cytb*, refugium
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INTRODUCTION

The Caucasus is traditionally considered as a center of species endemism with faunistic connections to the Russian Plain, the Balkan Peninsula, and Asia Minor. In the recent decades, a novel aspect of strong endemism of Caucasian mammals has been revealed by cytogenetic and molecular genetic studies that identified geographic vicarious species-level taxa in the Russian Plain and the Caucasus. It was found that the faunistic break in the South of the Russian Plain is comparable to the Beringian break. Most geographic vicarious species of the Russian Plain and the Caucasus are mesophilic forest and grassland inhabitants, and prolonged refugial distribution of forest vegetation during glaciation periods was the principal factor that affected the divergence of mammalian species of the Russian Plain and the Caucasus.

The area of the pygmy field mouse, *Sylvaemus uralensis* Pall., is continuous across the Kuban–Azov Lowland (between the Lower Don and the Kuban streams); this species is widely present in small river valleys and in field-protecting tree belts. Molecular genetic analysis based on the mitochondrial genes *cytb* and *COI* did not detect any differences between *S. uralensis* populations of the Russian Plain and the Caucasus [1–4]; therefore, it remained unclear whether this species, much like other forest species, was preserved in the Caucasus during the last glaciation, or it spread there from the Russian Plain after the glaciation.

Previously, two chromosomal forms of *S. uralensis* that differ in the number of chromosomes featuring large pericentromeric heterochromatin blocks (C-blocks) have been described: the Russian Plain form and the Caucasian form [5–8]. This led to a hypothesis that the modern continuous area of this species developed by secondary distribution of refugial populations that survived during the glaciation periods in the Russian Plain and the Caucasus. However, the rate of C-block evolution has not been established so far, and the reported significant variation in the number of chromosomes featuring pericentromeric heterochromatin [7] may also be associated with the choice of techniques used for chromosome staining.

In the present work, a statistical analysis of haplotype differentiation in the phylogenetic tree of the mitochondrial cytochrome *b* gene (*cytb*) was complemented with a study of haplotype similarity based on fixed nucleotide and amino acid substitutions. The goal of this work was to determine the structure of intraspecies variation in *S. uralensis* by fixed *cytb* mutations and to assess the probability that its Pleistocene refugia may have existed in the Caucasus.

MATERIALS AND METHODS

The study was performed with 16 specimens of *Sylvaemus uralensis* from three sites located in the upper stream of the Belaya River in the Caucasian State Nature Biosphere Reserve (CBR), situated on the northern macroslope of the Western Caucasus. Specimens were collected in a fir and broad-leaved forest of Partizanskaya Polyana 44°00′40.80″ N, 40°02′04.70″ E, 1500 m above sea level ($n = 8$; CBR-1); in a subalpine forest near Yavorova Polyana on the Lago-Naki Plateau, 44°00′39.40″ N, 39°59′01.56″ E, 1820 m above sea level ($n = 5$; CBR-2); and in the Lago-Naki cordon, 44°02′55.60″ N, 40°01′03.78″ E, 1800 m above sea level ($n = 3$; CBR-3).

mtDNA was isolated from liver specimens fixed with 96% ethanol. Total DNA was isolated using the standard technique that involves tissue lysis with proteinase K in the presence of SDS and DNA extraction with phenol–chloroform with subsequent precipitation. Amplification was performed with universal *cytb* primers as described in [9]. The resulting *cytb* fragment was 838 bp long (nucleotides 16–853 of the complete *cytb* sequence). Phylogenetic analysis was performed on the basis of the maximum likelihood (ML) and Bayesian inference (BI) approaches. Data were analyzed using the program packages MEGA, PHYML, JMODELTEST (with HKY+I model; *P*-inv = 0.668), MRBAYES (ngen = 100000; nruns = 4), and FIGTREE. The *p*-distances were calculated with MEGA using the distance estimation method (the number of bootstrap replicates was 1000). The median network of *cytb* haplotypes was constructed with POPART v. 1.7 using the MJ, TCS, and ancestral MP algorithms. A phylogenetic tree based on amino acid sequences was constructed using the ML approach; for the sake of convenience, the radial presentation was selected. To reveal the effects of selection, changes in the physicochemical properties of amino acids were evaluated using the MM01 model [10] and the TreeSAAP software [11]. In this test, 31 properties of amino acids are evaluated with a score of 1 to 8 (*mc*). Nonconservative substitutions ($mc = 6–8$, $P < 0.001$) imply the presence of directed selection pressure and adaptation, since such substitutions alter the molecule's shape and functions. In contrast, conservative substitutions ($mc = 1–5$, $P < 0.001$) are an evidence of stabilizing selection [11]. The molecular clock hypothesis was tested with MEGA using the RelTime method [12] and the T3P model [13]. For the purposes of phylogenetic analysis, the sample collected in the original study was supplemented with sequences from the GenBank database. The newly obtained sequences were also deposited in GenBank (Table 1). As an outgroup, the phylogenetic tree included GenBank *cytb* sequences from *Sylvaemus flavicollis* (AJ298603) and *Apodemus agrarius* (AB303226). Nucleotide and amino acid positions are indicated relative to the full-size gene and protein sequence, respectively.

RESULTS

The original sequences obtained in this work were not pseudogenes, since they did not contain aberrations typical of nuclear copies of mitochondrial genes and could be completely aligned to *cytb* sequences available in the GenBank. In the phylogenetic tree constructed based on *cytb* nucleotide sequences, the previously described *S. uralensis* clades, the European and the Asian (Kazakhstan, Turkmenistan, and Uzbekistan) [2, 3], formed distinct branches with high bootstrap support levels and post hoc probabilities; they were separated by *P*-dist = 6.7 ± 0.8% (Fig. 1). In this tree, specimens of *S. uralensis* collected in the western part of the northern macroslope of the Greater Caucasus belonged to the European lineage of *cytb* sequences and did not form a separate branch. The mean intraspecies haplotype distance within the European clade was calculated as *P*-dist = 0.9 ± 0.2% (the novel sample included) and was similar to the distance between *COI* haplotypes of the Caucasus and the Russian Plain (0.7 ± 0.2% [4]).

The new population sample of *S. uralensis* was characterized by two fixed mutations that lead to amino acid substitutions in positions 136 (Val → Gly) and 156 (Met → Ile) (Table 1), as well as by fixed synonymous substitution in the third position of codon TCC (A → C) corresponding to amino acid position 139. It can also be mentioned that one animal in this sample had an Ile → Val amino acid substitution in position 236 (it has not been detected in other samples of the European clade, but in the Asian clade this substitution is fixed), and position 42 was polymorphic (Thr/Ala), in contrast to the Met monomorphism observed in the Asian lineage.

The median networks constructed using different techniques were identical: median joining (MJ), TCS, and ancestral MP. Figure 2 shows the MJ median network. Although all specimens of the Lago-Naki featured two nonsynonymous substitutions, they did not form a separate group in the median network. Moreover, this group also included specimens Krasnodar31, Krasnodar31-1, and KB14, which were characterized by a different combination: 136Val–156Met. Probably, this was because the number of informative sites was large even within the European lineage ($n = 22$). Thus, it was impossible to strictly define the Lago-Naki haplogroup using only these phylogenetic markers. However, in a phylogenetic tree constructed on the basis of amino acid sequences, the sample in question could be recognized as a separate cluster (Fig. 3).

An analysis of potential selective pressure effects identified two nonconservative substitutions: Met156Ile (equilibrium constant (ionization of COOH), $mc = 8$, $P < 0.001$) and Val136Gly (mean r.m.s. fluctuation displacement, $mc = 8$, $P < 0.001$). With respect to the three other properties (solvent accessible reduction ratio, $mc = 4$, $P < 0.001$; thermodynamic transfer, $mc = 4$, $P < 0.001$; hydrophobicity, buriedness, $mc = 3$,

Table 1. Characterization of material studied and variation of cytochrome *b* amino acid sequences in *Sylvaemus uralensis*

No.	Location	Haplotype	GenBank acc. no.	Amino acid position in cytochrome *b*			
				42	136	156	236
European lineage							
Western part of the northern macroslope of the Greater Caucasus							
1	CBR 1	13	KY001666	Ala	Gly	Ile	Ile
	″	14	KY001667	.	.	.	.
	″	15	KY001668	Thr	.	.	.
	″	31	KY001669	Thr	.	.	.
	″	32	KY001670	.	.	.	.
	″	38	KY001671	Thr	.	.	.
	″	39	KY001672	.	.	.	.
	″	40	KY001673	Thr	.	.	.
2	CBR 2	46	KY001674	Thr	.	.	Val
	″	47	KY001675	.	.	.	.
	″	55	KY001676	Thr	.	.	.
	″	56	KY001677	Thr	.	.	.
	″	57	KY001678	Thr	.	.	.
3	CBR 3	88	KY001679	Thr	.	.	.
	″	89	KY001680	Thr	.	.	.
	″	90	KY001681	Thr	.	.	.
Western part of the southern macroslope of the Greater Caucasus							
4	Khosta*	Krasnodar31	FN430758	.	Val	Met	.
	″	Krasnodar31-1	FN430761	.	Val	Met	.
5	Krasnaya	Krasnodar88	FN430759	Thr	Val	Met	.
	Polyana*	Krasnodar95	FN430760	Thr	Val	Met	.
Central Caucasus							
5a	Kabardino-	KB10	FN430754	Thr	Val	Met	.
	Balkaria*,	KB14	FN430755	Thr	Val	Met	.
	Nalchik	KB19	FN430756	Thr	Val	Met	.
	″	KB19-1	FN430757	Thr	Val	Met	.
Russian Plain							
6	Kursk oblast*	Kursk30	FN430769	Thr	Val	Met	.
7	Samara oblast*	Samara27	FN430765	Thr	Val	Met	.
8	Ryazan oblast*	Ryazan46	FN430767	Thr	Val	Met	.
9	Tambov oblast*	Tambov06	FN430766	Thr	Val	Met	.
10	Saratov oblast*	Saratov63	FN430768	Thr	Val	Met	.
Asian lineage							
11	Turkmenistan*	Turkmenistan51	FN430745	Met	Val	Met	Val
12	Uzbekistan*	Uzbekistan45	FN430744	Met	Val	Met	Val
13	Kazakhstan*	Kazakhstan55	FN430743	Met	Val	.	Val
	″	Kazakhstan27	FN430742	Met	Val	Met	Val
	″	Kazakhstan18	FN430741	Met	Val	.	Val
	″	Kazakhstan14	FN430740	Met	Val	.	Val

CBR, Caucasian State Nature Biosphere Reserve.
* Sequences representing these locations were obtained from GenBank; specimens from the Caucasus were collected by A.S. Bogdanov [3].

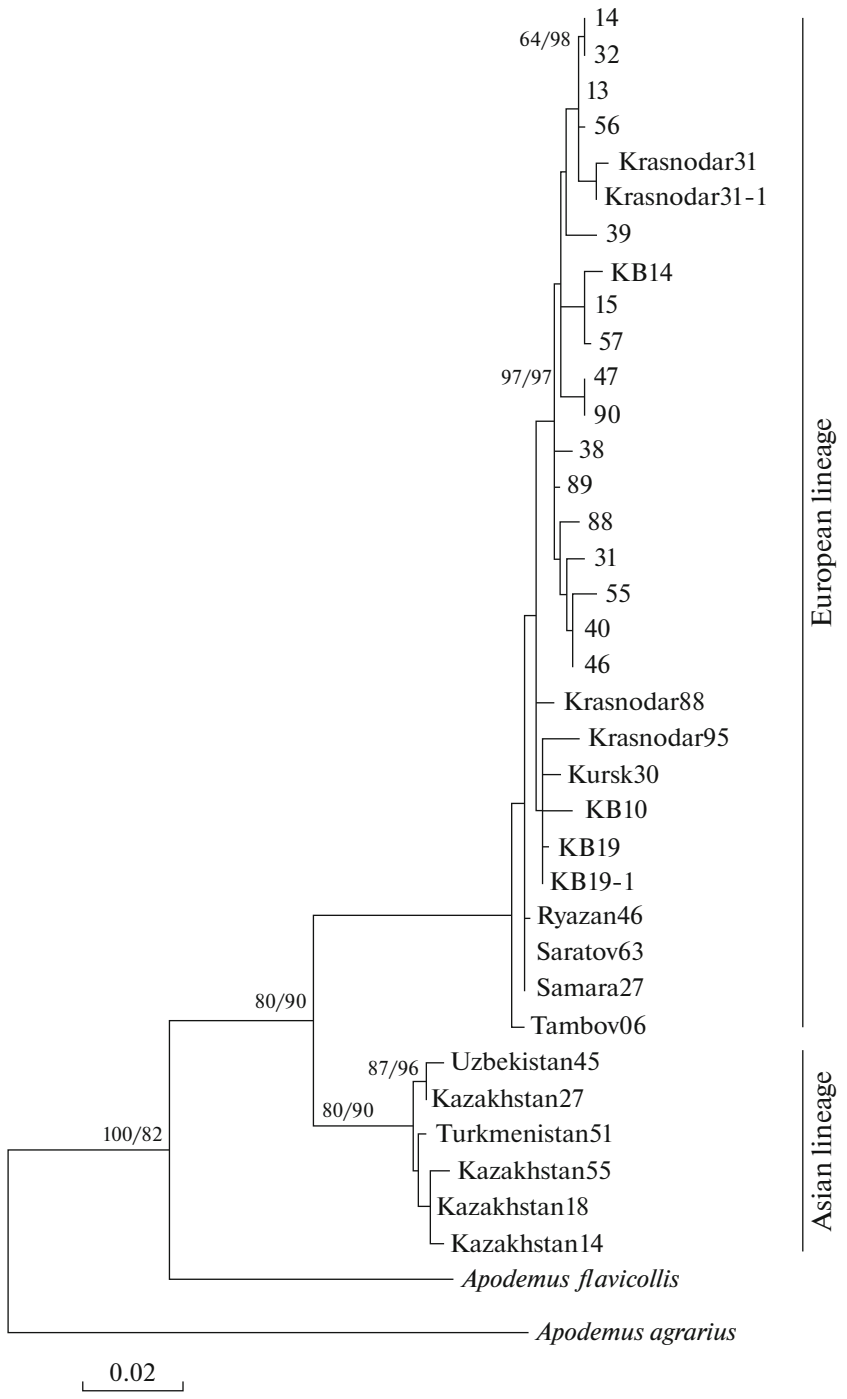

Fig. 1. Phylogenetic tree of *Sylvaemus uralensis* constructed using the maximum likelihood and the Bayesian inference approaches. Both trees had identical topology; the figure shows the ML tree. Bootstrap support values (ML) and Bayesian post hoc probabilities (BI) exceeding the threshold of 50 are shown above the corresponding nodes as (ML/BI). Haplotype characteristics are given in Table 1.

$P < 0.001$), Met156Ile was a conservative substitution. It should be noted that substitutions in positions 136 and 156 involve a functionally important fragment of the cytochrome b molecule: the cd loop of the Qo redox center.

If we assume the paleontological estimate for the reference point of divergence between yellow-necked mouse, *S. flavicollis*, and other mouse species of the genus *Sylvaemus* as 2.3 ± 0.2 Ma (Upper Pliocene), and the rate of mtDNA mutation in the field mouse as

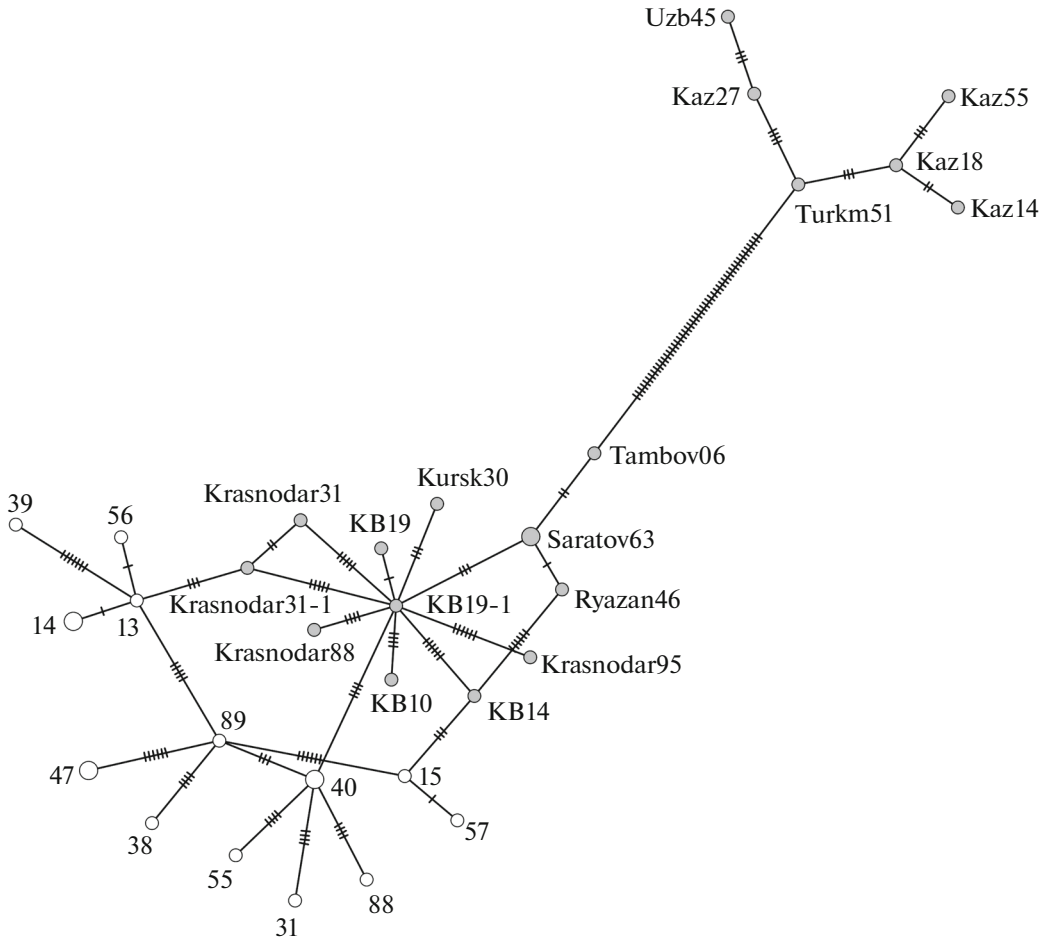

Fig. 2. Median-joining network of mitochondrial haplotypes of *Sylvaemus uralensis*. Specimens sequenced in this study are shown with white circles (all belong to the Lago-Naki haplogroup). Circle diameters correspond to the number of haplotypes. The number of mutations between haplotypes is shown with crosses.

2.85% substitutions per 1 Ma, the moment of divergence between the European and the Asian clades of *S. uralensis* can be determined as 1.5 Ma (Lower Eopleistocene) [3]. A similar calculation suggests that the Lago-Naki haplogroup diverged 130000 years ago. The age of divergence was also estimated using an alternative approach based on the rate of substitution fixation in the third codon position in *S. sylvaticus*, which constitutes 0.22 (0.082–0.5) per triplet per Ma [14, 15]. In the Lago-Naki haplogroup, there are two fixed nonsynonymous substitutions (in a second and a third position) and a fixed synonymous substitution in a third position. Since a substitution in a second position is a rarer evolutionary event, all three of them were included in the calculation. The *cytb* fragment analyzed comprises 279 triplets, and the number of substitutions per triplet is 3/279 = 0.0108. Thus, it can be roughly estimated that the Lago-Naki haplogroup diverged within the European lineage on average at 48.9 ka (21.5–131.1 ka); therefore, it remained isolated for a part or for the whole of last Valdai glaciation (10000 to 100000 years ago).

Small isolated groups accumulate fewer mutations, but these are fixed faster. The mean rates of substitution fixation calculated per 1 Ma may be considerably higher in small isolated populations than in large open ones. If mice of the current Lago-Naki haplogroup descend from a population that survived the glaciation period as a small isolate, the rate of substitution fixation could have been higher than the average level of 0.22 substitutions per triplet per 1 Ma; accordingly, the period of isolation could have been shorter, closer to the minimum estimate of 21500 years. Thus, isolation could have begun during the last glaciation maximum (24000 to 17000 years ago) [16]. In contrast, the age of divergence between the European and the Asian lineages of *S. uralensis* determined on the basis of substitution fixation is probably strongly underestimated.

DISCUSSION

The area of the European and the Asian *S. uralensis* lineages by c*ytb* and *COI* encompasses the deserts of Central Asia and the Caspian Sea [2–4]. Mice of the

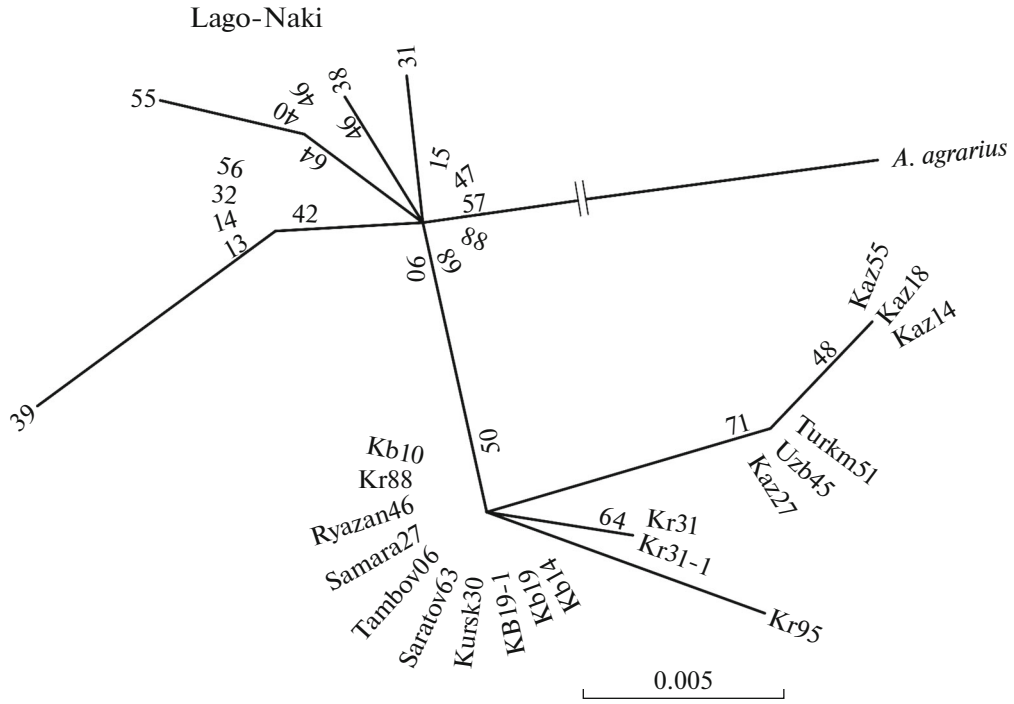

Fig. 3. ML phylogenetic tree of cytochrome *b* amino acid sequences of *Sylvaemus uralensis*, radial representation. Bootstrap support values exceeding the threshold of 40 are shown in italics next to the nodes.

European *COI* lineage inhabit territories from the Irtysh basin, where signs of their contact with the Asian lineage can be detected, to Eastern Europe, the Greater and the Lesser Caucasus, and the Lencoran region of Azerbaijan (Plateau of Iran), while mice of the Asian lineage are present in the southwest to the Kugitang Range in Turkmenistan. The distribution of mice that belong to the Asian and the European *cytb* clades is approximately the same, although less is known about the presence of the latter in the Caucasus. The GenBank database contains *cytb* sequences of *S. uralensis* from the western part of the southern macroslope and the Central Caucasus (Kabardino-Balkaria) [3]. There was a study on species identification of *S. uralensis* in the western Caucasus based on a *cytb* fragment, but the corresponding sequences were not described [14].

Haplotypes of the Russian Plain, the Central Caucasus, and the southern macroslope did not differ with respect to fixed amino acid substitutions in *cytb* sequences available in GenBank. The fact that *cytb* sequences of the sample studied encode two fixed amino acid substitutions makes it possible to describe it as a distinct Lago-Naki haplogroup. It should be mentioned that, in spite of considerable divergence, the European and the Asian *cytb* clades differ only in four fixed amino acid substitutions [3]. Mitochondrial distances by *cytb* and *COI* sequences among the populations of the Russian Plain and the Caucasus do not exceed 1% and are typical of geographic variation of widespread species.

Amino acid substitutions in positions 136 and 156 typical of the Lago-Naki haplogroup are also present in the Asian lineage [3]. This lineage is additionally characterized by a fixed substitution in position 236 (Ile → Val), which was found in one haplotype of our sample.

Substitutions in the cd loop of the Qo redox center are thought to be associated with adaptive evolution [10, 17, 18]. Amino acid substitutions resulting from *cytb* mutations may facilitate adaptation to environmental conditions that require higher metabolic rates (oxygen deficiency, high altitudes, low-energy food) [10, 17–19]. Thus, the described amino acid substitutions in positions 136 and 156 may represent a result of selection pressure.

Forest species of the Greater Caucasus could have survived the last glaciation period in the known broad-leaved forest refugium in the west of Transcaucasia (Colchis) [20, 21]. Modern Colchis-like refugia of Tertiary flora have also been described on the northern macroslope [22, 23].

The pygmy field mouse might have survived the glaciation period not only in small forest refugia but also in refugia similar to modern subalpine habitats. In the latter case, the size of the refugial population must have been especially small, because the species abundance in modern subalpine environments is known to be lower than in forest habitats. The fact that no other haplotypes of the European lineage were found in the known area of this haplogroup (subalpine Lago-Naki

Plateau and upper part of the forest zone) may also indicate that the local population is better adapted to the harsh environments located 1500–1800 m above sea level. Most probably, other Caucasian haplotypes of the European lineage could be found in the habitats located lower along the northern macroslope, approximately 1000 m above sea level. The current distribution of the Lago-Naki haplogroup still remains to be determined. It is worth mentioning that the population of *Chionomys roberti* voles from the Lago-Naki plateau also represents a distinct monophyletic group, a haplogroup [24]. It is possible that evolution of both species partially occurred in the same small refugium of the last glacial period.

In early studies, it was observed that genomes of *S. uralensis* inhabiting the Russian Plain (*S. u. mosquensis* Ogn.) feature C-blocks in 20 pairs of autosomes, while in populations of the Caucasus (*S. u. ciscaucasicus* Ogn.) they are found only in seven pairs of the largest autosomes; the diploid chromosome number is the same for both groups ($2n = 48$) [5, 6]. Subsequently, these chromosome forms were termed East European (the Russian Plain, the Urals, and partially the basins of the Irtysh and the Tobol rivers) and South European (the Caucasus), respectively; the number of C-blocks was shown to vary from 14 to 18 autosome pairs in the populations of the Russian Plain [7] and from seven to nine autosome pairs in the populations of the Caucasus [8]. A FISH-based study of *S. uralensis* chromosomes revealed quantitative variation in the number of sequence repeats in C-positive regions [25]. In contrast, in other species of the genus *Sylvaemus*, e.g., *S. flavicollis* of the Russian Plain and *S. ponticus* from the Northern Caucasus, C-positive regions are composed of different sequences, i.e., the difference is qualitative [26]. Therefore, it can be assumed that chromosomal forms of *S. uralensis* began to diverge later and have the same age as the European and the Asian mtDNA lineages.

The observed differentiation of *cytb* haplotypes within the same South European chromosomal form speaks in favor of a secondary contact between *S. uralensis* populations of the Russian Plain and the Caucasus. At the same time, it becomes more probable that populations of the Central Caucasus, which do not differ from populations of the Russian Plain in fixed *cytb* substitutions, survived the glaciation period in some other refugium of the Caucasus rather than spreading from the Russian Plain during the postglacial time. Substitutions have not been fixed because of the large size of populations that inhabited the refugium (or refugia).

The hypothesis that forest species populations of the Russian Plain and the Caucasus established secondary contacts during the warmer period of Holocene agrees well with the data obtained by direct chorologic reconstruction and investigation of bone remains in locations of known age. The formation of forest mammalian species complexes of the Russian Plain and the Caucasus began simultaneously and independently in the Early Holocene (10200 to 8000 years ago), in particular, on the Russian Plain to the north of 50° N [27]. In the South of Rostov oblast, on the left bank of the Manych River, field mice possessing an intermediate number of C-blocks of pericentromeric heterochromatin (10–13 autosome pairs) have been described; presumably, they represent hybrids between the East European and the South European chromosomal forms [28]. The probability of a contact between these two chromosomal forms of *S. uralensis* occurring in this particular region is fairly high, since it is to the south of the Lower Don that areas of contacts between cryptic mammalian species of the Russian Plain and the Caucasus have been identified [29].

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