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Riverine barriers and geographic variation in little ground squirrel *Spermophilus pygmaeus* (Sciuridae, Rodentia) based on mitochondrial cytochrome *b* gene sequences

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ABSTRACT. Little ground squirrel (*Spermophilus pygmaeus*) typically inhabit semi-deserts and dry steppes of the European plains and Kazakhstan. Range-wide latitudinal distribution along with major river separation zones makes this species a proper model for testing the riverine barrier hypothesis. For the first time, we have evaluated genetic variation in little ground squirrel populations throughout its range based on the analysis of cytochrome *b* gene sequences. A high level of genetic diversity (4.9%) among "western" and "eastern" populations of the little ground squirrel has evidenced the Volga River to be an effective biogeographic barrier. A barrier role of the Ural River is expressed to a lesser extent, and the Don River has neither effect on the restriction to gene flow and phylogeographic structure. The genetic diversity data has confirmed the hypothesis on *S. pygmaeus* speciation in the long-term Ciscaucasia refugium, and the appearance of the mountain ground squirrel (*S. p. musicus*) in the Middle Pleistocene.

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Речные барьеры и географическая изменчивость малого суслика *Spermophilus pygmaeus* (Sciuridae, Rodentia) по данным секвенирования митохондриального гена цитохрома *b*

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РЕЗЮМЕ. Малый суслик (*Spermophilus pygmaeus*) типичный обитатель полупустынь и сухих степей европейских равнин и Казахстана. Широтное распространение на обширном ареале, разделенном крупными реками, делает этот вид удобным модельным объектом тестирования гипотезы речных барьеров. Впервые на основе анализа последовательностей гена цитохрома *b* мы оценили генетическую изменчивость среди популяций малого суслика во всех частях его ареала. Высокий уровень генетических различий (4.9%) между «западными» и «восточными» популяциями малого суслика подтвердили роль Волги как эффективного биогеографического барьера. Барьерная роль реки Урал выражена в меньшей

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степени, влияния реки Дон на ограничение потока генов и филогеографическую структуру не выявлено. Данные генетической изменчивости поддерживают гипотезу формирования *S. pygmaeus* в долговременном Предкавказском рефугиуме и среднеплейстоценовый возраст горного малого суслика *S. p. musicus*.

КЛЮЧЕВЫЕ СЛОВА: суслики, генетическое разнообразие, изоляция, филогеография.

Introduction

The riverine barrier hypothesis by Alfred Russel Wallace (1825) suggests that large rivers represent geographical barriers to gene flow for terrestrial organisms, leading to population differentiation and ultimately allopatric speciation. Little ground squirrel *Spermophilus pygmaeus* (Pallas, 1778) are best suited for testing this hypothesis, since this species is characterized by the widest range in the Palearctic (west-east extent is about 3000 km), separated by major rivers (the Don, the Volga, and the Ural) of a meridional-type flow. A high level of genetic diversity among "western" and "eastern" lineages of *S. pygmaeus* from the Volga River drainage (7% and 3.5%, respectively) was revealed in our previous papers (Ermakov *et al.*, 2006, 2015, 2018). Those results were conducted using on the mitochondrial DNA (mtDNA) sequence analysis of the control region (C-region, 311 bp) fragments, and the cytochrome oxidase subunit I (COI, 657 bp) gene. Taking into account the level of differentiation of "western" and "eastern" little ground squirrels, some authors consider them as semispecies (allospecies) within the superspecies "*pygmaeus*" (Pavlinov & Khlyap, 2012).

Besides, the indicated higher genetic diversity of the western ground squirrels confirmed the hypothesis on the European origin of the species, and the subspecific status of the Caucasian mountain ground squirrel *S. p. musicus* was suggested (Ermakov *et al.*, 2006, 2018). However, we found neither major effect of the Don and the Ural River, unlike the Volga, on the phylogeographic structure of little ground squirrels.

We assumed that a weak phylogeographic signal is due to the inconsistency of the used divergence markers of little ground squirrel levels. Being the non-coding mtDNA region with a high mutation rate, the C-region is widely used in population genetic studies. Yet, a less variable marker is needed for species-level analysis with a wide geographic coverage (Bannikova, 2004; Abramson, 2007). On the contrary, the COI gene is the most slowly evolving gene of the mitochondrial protein coding genes (Simon *et al.*, 1994), being a popular marker in species delimitation and above-species rank phylogenies (Hebert *et al.*, 2003).

Therefore, in the present study, we use the mitochondrial cytochrome *b* (*cyt b*) gene sequences as a proper phylogeographic marker, being widely used in the research on long-tailed ground squirrels (genus *Urocitellus*) (Eddingsaas *et al.*, 2004; Cook *et al.*, 2010; Galbreath *et al.*, 2011; Faerman *et al.*, 2017; McLean *et al.*, 2018), and short-tailed ground squirrels (genus *Spermophilus*) (Kryštufek *et al.*, 2009; Říčanová *et al.*, 2013; Gür *et al.*, 2018; Asgharzadeh *et al.*, 2019; Matrosova *et al.*, 2019).

Here, the *cyt b* sequences are used to consider the impact of riverine barriers on the level of genetic diversity for little ground squirrels. In the future, the geographic context and sampling design of this study will allow us testing the hypothesis on a European origin for the species and the isolation of its mountain populations.

Material and methods

This study included 59 *S. pygmaeus* specimens from 26 locations covering almost complete natural range of little ground squirrel. Moreover, two *cyt b* sequences were downloaded from GenBank. The total sample set included 61 sequences: 33 specimens were collected from 15 localities on the west of the Volga River and 28 specimens were collected from 11 localities on the east of the Volga River (Table 1, Fig. 1A). Tissue samples from the examined specimens were deposited in the Collection of the Department of Zoology and Ecology of Penza State University (Penza, Russia) and the Collection of Wild Animal Tissues for Genetic Research of Koltzov Institute of Developmental Biology of Russian Academy of Sciences (Moscow, Russia).

Whole genomic DNA was extracted from the tissue samples using the phenol-chloroform method (Sambrook *et al.*, 1989). The complete *cyt b* gene (1140 bp) was amplified using primer pairs GlCbSTD — 5'-AAT GAC ATG AAA AAT CAT CGT TGT-3' and GlCbendR — 5'-CTT CAT TTT TGG TTT ACA AGA CCA-3' (Faerman *et al.*, 2017). The reaction conditions were as follows: initial denaturation at 95°C for 3 min, followed by 32 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min. The PCR products were analyzed using 6% polyacrylamide gel electrophoresis. The gel was stained with ethidium bromide, and the DNA bands were visualized under ultraviolet light. The sequencing was done on the ABI 3500 automated capillary sequencer (Applied Biosystems) with the BigDye Terminator v.3.1 Kit (Applied Biosystems), using the same primers. The sequences were aligned manually using the BioEdit v.7.0 sequence alignment editor (Hall, 1999).

DnaSP v.5.10.01 (Librado & Rozas, 2009) was used to analyze the number of haplotypes (*H*), haplotype diversity (*Hd*), nucleotide diversity per site (π), the number of polymorphic sites (*S*), fixation index (F_{ST}), gene flow (N_m), and Fu's F-statistics (*Fs*). We assessed

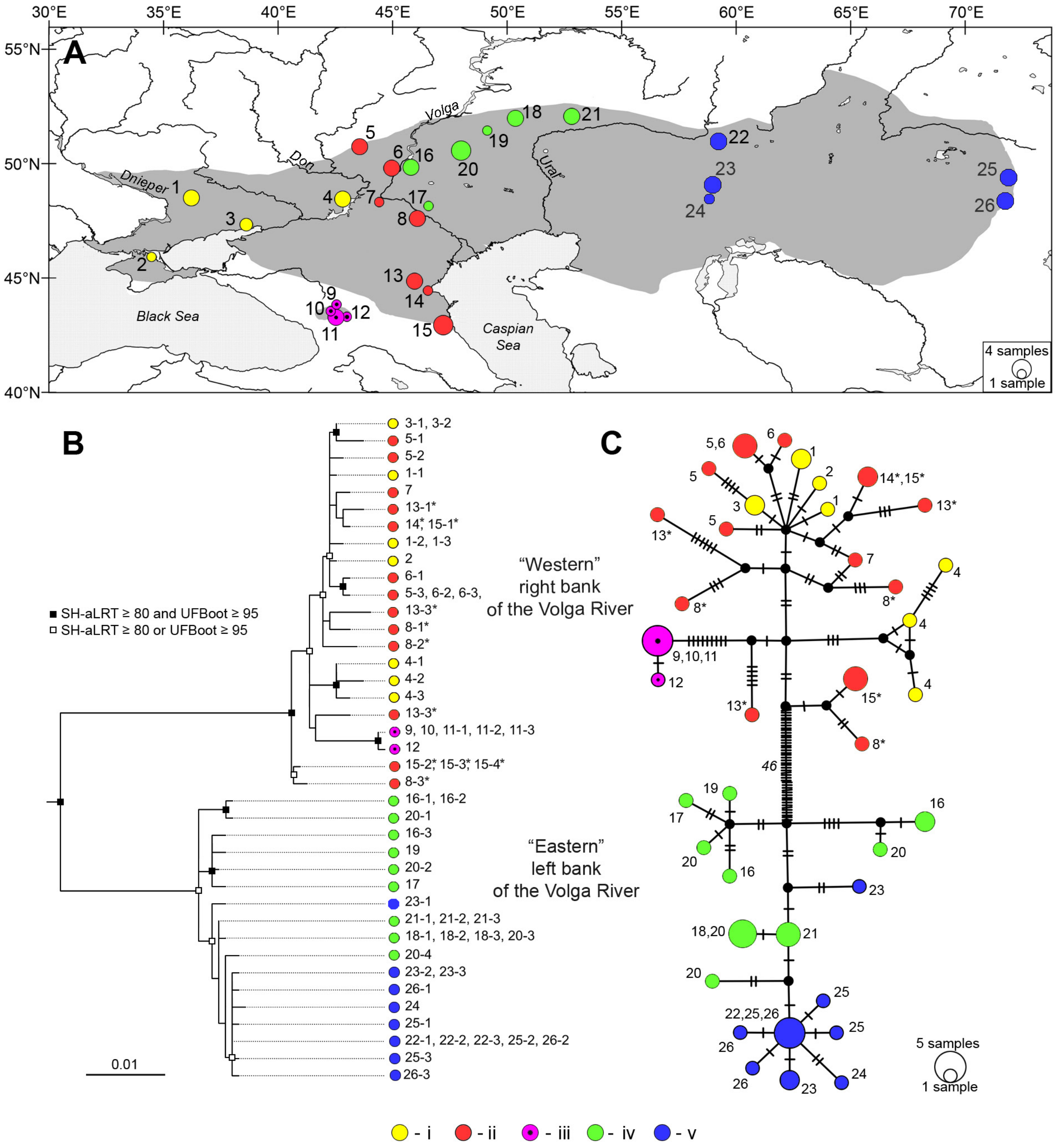

Fig. 1. Distribution (A), ML phylogenetic tree (B) and haplotype network (C) of "western" and "eastern" *Spermophilus pygmaeus* haplogroups. Collection localities: (i), the Dnieper–Don interfluve; (ii), the Don–Volga interfluve; (iii), vicinity of Mt. Elbrus; (iv), the Volga–Ural interfluve; (v), east of the Ural River. The numbers of points on the map, the phylogenetic tree and the network correspond to the locality numbers in Table 1 (designations 3-1, 3-2, etc., refer to different individuals of the same population); * indicates populations of the Lower Volga and Ciscaucasia. Outgroup is not shown.

the significance via 1000 permutations. The genetic distance matrices (*p*-distances) were calculated in the MEGA7 software (Kumar *et al.*, 2016).

We performed a maximum likelihood (ML) phylogenetic analysis of the unpartitioned *cyt b* sequences in the IQ-TREE software (Nguyen *et al.*, 2015) using its online web interface W-IQ-TREE (Trifinopoulos *et al.*, 2016). The best-fit model of sequence evolution according to the Bayesian information criterion (BIC) scores was HKY+F+I (Kalyaanamoorthy *et al.*, 2017). Branch support was assessed using the ultrafast bootstrap (UFBoot) approximation algorithm (Minh *et al.*, 2013; Hoang *et al.*, 2018) and approximate likelihood-ratio test

Table 1. Characteristics of the studied material.

No.	Collection locality	Coordinates	*n*	GenBank IDs
	Ukraine:			
1	Kharkiv Region, Konnoe	49.014°N 36.561°E	3	OP588847–849
	Russia:			
2	Crimea, Dzhankoi	45.971°N 34.488°E	1	OP588846
3	Rostov Region, Lakademonovka	47.185°N 38.521°E	2	OP588850–851
4	Volgograd Region, Popov-2	48.724°N 42.881°E	3	OP588858–860
5	Volgograd Region, Rodinskoe	50.905°N 43.391°E	3	OP588852–854
6	Volgograd Region, Gorniy Balykley	49.600°N 45.060°E	3	OP588855–857
7	Volgograd Region, Dubovyj Ovrag	48.352°N 44.608°E	1	OP588861
8	Astrakhan Region, Nikolskoe	47.741°N 46.395°E	3	OP588862–864
9	Kabardino-Balkaria, Shidzhatmaz	43.702°N 42.675°E	1	OP588903
10	Kabardino-Balkaria, Dzhylysu	43.436°N 42.541°E	1	OP588904
11	Kabardino-Balkaria, Elbrus	43.291°N 42.596°E	3	OP588866–868
12	Kabardino-Balkaria, Baksan	43.326°N 42.793°E	1	OP588865
13	Dagestan, Sukhokumsk	44.873°N 45.833°E	3	OP588895–897
14	Dagestan, Kochubey	44.364°N 46.682°E	1	OP588902
15	Dagestan, Paraul	42.750°N 47.354°E	4	OP588898–901
16	Volgograd Region, Krasnoselec	49.663°N 45.718°E	3	OP588875–877
17	Astrakhan Region, Baskunchak	48.218°N 46.777°E	1	OP588878
18	Saratov Region, Rakhmanovka	51.937°N 49.663°E	3	OP588869–871
19	Saratov Region, Dergachi	51.299°N 48.999°E	1	AF157910*
20	Saratov Region, Novotulka	50.914°N 47.557°E	4	OP588872–874, AF157907*
21	Orenburg Region, Shestakovka	52.081°N 52.809°E	3	OP588879–881
22	Orenburg Region, Ashchebutak	51.043°N 59.145°E	3	OP588882–884
	Kazakhstan:			
23	Aktobe Region, Emba	49.057°N 58.536°E	3	OP588885–887
24	Aktobe Region, Alabas	48.454°N 58.555°E	1	OP588888
25	Karaganda Region, Karazhal	49.394°N 71.643°E	3	OP588889, 893, 894
26	Karaganda Region, Arzabaj	48.454°N 71.047°E	3	OP588890–892

*Harrison *et al*., 2003.

based on the Shimodaira-Hasegawa-like procedure (SH-aLRT) with 10000 replicates each (Guindon *et al*., 2010). In the ML analyses, clades with support values of both UFBoot ≥ 95 and SH-aLRT ≥ 80 were considered strongly supported, while clades with only one of UFBoot ≥ 95 or SH-aLRT ≥ 80 were weakly supported; nodes with both UFBoot < 95 or SH-aLRT < 80 were unsupported. Haplotype networks were constructed using the median-joining method in the PopART software (Leigh & Bryant, 2015).

The obtained sequences were deposited in the GenBank database (accession numbers: OP588846–588904). The sequences of speckled ground squirrel (*S. suslicus* — AF157895) (Harrison *et al*., 2003), the European ground squirrel (*S. citellus* — AF157858) (Harrison *et al*., 2003), and the Taurus ground squirrel (*S. taurensis* — AM691696) (Gündüz *et al*., 2007) were used as outgroups.

Results

A total of 61 *S. pygmaeus* samples yielded 39 different *cyt b* haplotypes. There were 121 polymorphic sites found out of 1140 bp sequenced, 86 of which were parsimony-informative (7.5% of the total length). The average value of uncorrected genetic distances (*p*-distance) within the sample set was 2.8 ± 0.3%, with the maximum value of 5.5%.

The ML-tree evidences separation of the little ground squirrel haplotypes into “western” (right bank of the Volga River) and “eastern” (left bank of the Volga River) supported groups (Fig. 1B). The “western” group shows the basal dichotomy between the Lower Volga and Ciscaucasia populations (localities 8 and 15), and the rest of the *S. p. musicus* clade from the Elbrus region (localities 9–12). Ground squirrels from the west and east banks of the Don River do not form separate groups within the western clade. Ground squirrel populations from the Lower Volga and Ciscaucasia (localities 8, 13–15) are characterized by a basal position in the western clade with a high genetic diversity. There were seven haplotypes found in the Ciscaucasia and the Lower Volga regions out of 22 described in the west of the Volga River. The eastern clade contains a polytomy of *S. pygmaeus* sequences from the entire Volga–Ural interfluve. Ground squirrels from the eastern bank of the Ural River form a distinct subclade (localities 22–26). In five cases, we recorded identical haplotypes in various populations (localities 5 and 6; 9–11; 14 and 15 in the western clade; 18 and 20; 22, 25, 26 in the eastern clade).

The median-joining network of 39 haplotypes (Fig. 1C) showed results similar to the ML-tree and confirmed clear separation (46 mutational steps) of the "western" group from the "eastern" one. No geographic substructure was found within remarkably diverse "western" group. The haplotypes clustered together on the median-joining network occurred in populations located in different interfluves.

Spermophilus p. musicus haplotypes (localities 9–12), separated by nine mutational steps, were the most divergent within the "western" group. The "eastern" group was less diverse but more structured. Haplotypes of ground squirrels living in the interfluve of the Volga and the Urals (localities 16–21) are located closer to the center of the network. The star-like pattern (localities 22–26) in the "eastern" group implies population expansion to the east of the Ural River. Only one specimen from the left bank of the Ural River does not fall into this group (locality 23).

The average genetic distance (*p*-distance) between the "western" and "eastern" groups of haplotypes was 4.9 ± 0.6%. The genetic diversity parameters of particular groups (Table 2) were higher in the "western" group than those in the "eastern" group, with the exception of *S. p. musicus* (Elbrus group) having minimal values. The values of genetic diversity in populations living on both banks of the Volga (Don–Volga and Volga–Ural groups) were higher than those in the western (Dnieper–Don group) and eastern (East of Ural group) parts of the range.

The estimates of genetic diversity and gene flow showed moderate genetic differences (F_{ST} = 0.07) and sufficient gene flow (N_m = 6.28) between the two "western" groups (Dnieper–Don and Don–Volga) (Tab. 3). The populations of the Volga–Ural interfluve are more strongly differentiated in the eastern part of *S. pygmaeus* range (F_{ST}=0.38), and the gene flow being limited (N_m = 0.83). A high level of differentiation is observed between populations of ground squirrels from both banks of the Volga River (F_{ST} of 0.87–0.92, N_m of 0.04–0.08), and the maximum values of isolation are characteristic of *S. p. musicus* (Elbrus group) (F_{ST} of 0.72–0.78, N_m of 0.14–0.19 from "western" groups and F_{ST} of 0.95–0.98, N_m of 0.01–0.03 from "eastern" groups). This is consistent with the results of a pairwise comparison of the genetic distances (*p*-distance) between the groups studied (Table 3).

Discussion

In this study, we have analyzed the mitochondrial genetic structure of little ground squirrel across its range using *cyt b* sequences. The deep divergence between the "western" and "eastern" *S. pygmaeus* groups has confirmed the role of the Volga River as an effective biogeographical barrier. The level of differences between little ground squirrels on both banks of the Volga (*p*-distance — 4.9%; net distance — 4.2%) is comparable to that for the pair of sister species *S. citellus–S. taurensis* (5%) (Gündüz *et al.*, 2007), as well as between the taxa of the red-cheeked ground

Table 2. Genetic diversity in *Spermophilus pygmaeus* samples from different group populations.

Group	*n*	*H*	*h* ± SD	π ± SD (%)	*S*	*K*	Fu's *Fs*
Dnieper–Don	9	7	0.944 ± 0.070	0.55 ± 0.11	16	6.22	–0.73
Don–Volga	15	13	0.954 ± 0.034	0.76 ± 0.06	44	8.66	–2.14
Elbrus	6	2	0.333 ± 0.215	0.03 ± 0.02	1	0.33	–0.003
"Western" in overall	33	22	0.964 ± 0.018	0.90 ± 0.06	65	10.22	–4.86
Volga–Ural	15	9	0.905 ± 0.054	0.51 ± 0.06	21	5.81	–0.66
East of Ural	13	8	0.859 ± 0.089	0.17 ± 0.05	12	1.97	–3.69*
"Eastern" in overall	28	17	0.944 ± 0.024	0.46 ± 0.06	29	5.24	–5.71
Total	61	39	0.978 ± 0.008	2.82 ± 0.06	121	32.05	–

n — sample size; *H* — number of haplotypes; *h* — haplotype diversity; π — nucleotide diversity (per site); *S* — total number of polymorphic positions; *K* — mean number of nucleotide substitutions; Fu's *Fs* — Fs-test value; $^{*}p < 0.05$.

Table 3. Pairwise genetic distance (*p*-distance ± SD (%) (below diagonal) and F_{ST}/ N_m (above diagonal) among various *Spermophilus pygmaeus* population groups based on sequence data.

Group	Dnieper–Don	Don–Volga	Elbrus	Volga–Ural	East of Ural
Dnieper–Don		0.07 / 6.28	0.78 / 0.14	0.89 / 0.06	0.92 / 0.04
Don–Volga	0.71 ± 0.12		0.72 / 0.19	0.87 / 0.08	0.90 / 0.05
Elbrus	1.31 ± 0.30	1.41 ± 0.30		0.95 / 0.03	0.98 / 0.01
Volga–Ural	4.82 ± 0.56	4.77 ± 0.55	5.18 ± 0.64		0.38 / 0.83
East of Ural	4.82 ± 0.58	4.80 ± 0.58	5.20 ± 0.66	0.55 ± 0.15	

squirrel species complex (*S. erythrogenys* sensu lato) (from 2.3% to 6.5%) (Matrosova *et al.*, 2019).

Ground squirrel ranges are generally located along the riverbeds. For example, the Volga is the eastern border of *S. suslicus* distribution and the western border of yellow ground squirrel (*S. fulvus*) and russet ground squirrel (*S. major*) ranges, except for anthropogenic dispersal and introduction cases (Ermakov & Titov, 2000; Aleksandrov *et al.*, 2019). The western border of *S. pygmaeus* passes along the Dnieper. In its turn, the Dnieper River divides speckled ground squirrels into two chromosome forms (*S. odessanus* 2n=36 and *S. suslicus* 2n=34) having significant differences in mtDNA markers (Matrosova *et al.*, 2014; Brandler *et al.*, 2015; Ermakov *et al.*, 2015).

Large rivers are a barrier for ground squirrels, including *S. pygmaeus*, due to their biological features (burrowing lifestyle, hibernation, coloniality, and poor ability to settle and overcome water barriers) (Popova *et al.*, 2019). Ground squirrels avoid settling within floodplains due to possible flooding of burrows, and hibernation does not allow them to cross rivers on ice. It was found that the majority of animals in the *S. pygmaeus* colony lead a sedentary life, and only a small part of individuals (3.5%) travel for a distance of more than 1 km (Kalabukhov & Raevskij, 1935). The cause of mass migrations is the lack of succulent fodder in dry years (Rall’ *et al.*, 1933), when little ground squirrels can swim across small steppe rivers singly or in groups of 3–5 individuals (Brudin, 1957). However, this species swims poorly, stays on the water for up to 12–18 minutes, and is able to cover a distance of about 100 meters during this time (Kalabukhov, 1978).

Nevertheless, large rivers are absolute barriers only in the lower flows with the maximum channel width to allow ground squirrels leaving hibernation during ice breakup (Vorontsov *et al.*, 1980). The rivers are less full-flowing in the upper reaches and the end of freezing in late March–early April may coincide with awakening from hibernation, when sexual active males can cross river upon spring ice. Probably, the westward dispersal of the little ground squirrel through the upper reaches of the Don Basin Rivers is the reason for the lack of a phylogeographic structure and differentiation between the settlements of the right and left banks of the Don (the Dnieper–Don and Don–Volga groups). An exception is the ground squirrels of the Lower Don (locality 4), which form a separate subgroup. In contrast to the Don, the Ural River is a certain barrier to ground squirrel settling, significantly limiting the flow of genes between the banks of the river. However, the barrier is not absolute, since a haplotype was found does not belong to the East of Ural group in the vicinity of Mugodzhar Hills (locality 23).

Mountain ranges are stable geographic barriers along with rivers, and mountain species being isolates of plains. According to the obtained data, the little mountain ground squirrel (Elbrus group) has practically no genetic variability. Only two haplotypes out of six individuals were found to differ by one nucleotide substitution. However, this subspecies being significantly differentiated and isolated from other little ground squirrels in the western part of the range confirms the penetration of *S. p. musicus* into the mountains in the Middle or early Late Pleistocene (Gromov *et al.*, 1965), rather than the recent (not earlier than 4000 years ago) expansion proposed by us (Ermakov *et al.*, 2006).

Finally, the data obtained support the hypothesis that the center of *S. pygmaeus* formation and postglacial expansion is the territory of Ciscaucasia (Popova & Zagorodniuk, 2016; Ermakov *et al.*, 2018; Popova *et al.*, 2019). The uniqueness and probable ancient origin of this group is evidenced by the high indices of haplotype and nucleotide diversity and the presence of specimens from Ciscaucasia and the Lower Volga in almost all subclades and phylogroups of the western part of the little ground squirrel range. The most ancient Pleistocene records of *S. pygmaeus* are known within Ciscaucasia (Terek-Kuma Lowland) since the Holsteinian interglacial (455–360000 years ago) (Bolikhovskaya *et al.*, 2016). Here, the little ground squirrel could have survived both during glacial and interglacial periods; therefore, this region can be viewed as constituting “long-term refugia” (Stewart *et al.*, 2010). According to our recent study, the separation of the “western“ and “eastern” genetic lineages of the little ground squirrel probably occurred on the eve of the Akchagylian transgression about 2.7 million years ago (Ermakov *et al.*, 2018).Colonization of the eastern part of the range took place in later eras. Low rates of genetic variability, the star-shaped structure of the haplotype network, and significant negative test values for the neutrality of little ground squirrels living east of the Ural River indicate a period of decline in numbers during one wave of colonization, while the rest of the groups remained in a state of stability. However, time-calibrated analyses are needed to uncover the origin and colonization roots of populations.

Recently, the colonization of the little ground squirrel has been in a state of deep depression in the European part of the range (Bystrakova *et al.*, 2005; Popov, 2016). On the one hand, global climatic changes have caused a decrease in the continentality of the climate, a shortage of winter and spring precipitation levels, and a shift in the maximum summer precipitation level. An increase in winter temperature could lead to early awakening with subsequent death of animals in conditions of a cold weather (Popov, 2016). On the other hand, climatic changes could provoke the replacement of primary semi-desert landscapes with anthropogenic steppe, in which the closeness and density of herbage creates unfavorable life conditions for the little ground squirrel (Shilova, 2011). The ongoing decrease in the number of animals and reduced northern and western boundaries of the range may result in lowering the level of biodiversity in this group and possible extinction of some little ground squirrel genetic lineages as a charismatic faunal element of short-grass steppe communities of the Palearctic, playing a vital role in maintaining these open habitats.

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