ISSN 1022-7954, Russian Journal of Genetics, 2018, Vol. 54, No. 6, pp. 643–651. © Pleiades Publishing, Inc., 2018.
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ANIMAL GENETICS

Genetic Variability in the Yellow-Necked Field Mouse (*Sylvaemus flavicollis* Melch., 1834, Muridae, Rodentia) at the Eastern Border of the Range

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Received April 3, 2017; in final form, August 3, 2017

Abstract—The first characterization of the genetic variability in populations of *S. flavicollis* on the eastern border of the range is presented. Seven individuals from the most northeastern habitat of the species (Middle Urals) were karotyped. No deviations from the standard chromosome set, either by the chromosome number or morphology, were revealed. Analysis of the mtDNA cytochrome *b* gene sequences (1133 bp) in 44 individuals from five populations on the eastern border of the species range (Middle and Southern Urals) resulted in identification of 17 haplotypes. All haplotypes were new and not found earlier in other parts of the species range. The genetic diversity indices and analysis of the demographic and genetic structure indicate a relatively recent origin of the populations under study as a result of rapid expansion. Phylogenetic analysis (97 haplotypes, including the GenBank data) showed that all haplotypes described at the eastern border of the range belonged to the same phylogroup distributed in the Balkan region, Northern and Eastern Europe, and Russia (Samara oblast). Close relationships between the examined populations and the populations from the northern part of the species range in Eastern Europe was demonstrated.

Keywords: genetic differentiation, B chromosomes, mitochondrial genome, cytochrome *b*, phylogeography, peripheral populations, intraspecific variability

DOI: 10.1134/S1022795418060157

INTRODUCTION

The yellow-necked field mouse (*Sylvaemus flavicollis* Melch., 1834) is a typical representative of the European fauna, whose distribution range is confined to the broad-leaved forest zone and occupies a considerable territory of Eurasia from Great Britain and northern Spain to the Urals [1–3]. Genetic analysis of this species was performed in a large number of studies. In particular, the existence of chromosomal polymorphism in *S. flavicollis* caused by the appearance in the karyotype of additional chromosomes (B chromosomes) was demonstrated. The presence of B chromosomes was observed in the majority of the studied populations of the yellow-necked field mouse, and the correlation of their frequency with a number of biochemical, physiological, and environmental factors was analyzed [4–14]. On the basis of the analysis of chromosomal polymorphism distribution patterns within the species range, suppositions on both the increase in the number of individuals with B chromosomes at the border of the range [5, 8, 9] and the decrease in the frequency of additional chromosomes from the center to the periphery of the distribution area [7, 10, 13] were made. A number of genetic studies of the yellow-necked field mouse focused on taxonomy, phylogeny, and phylogeography were performed using the sequence of the mtDNA cytochrome *b* gene as a marker [15–21]. The differentiation of *S. flavicollis* into three relatively isolated clades was demonstrated. Clade A included the specimens from the Balkan region and Northern Europe; clade B, from the Balkan region and southern Russia; and clade C, from Western, Northern, and Eastern Europe. Moreover, the possible pathways of the formation of species phylogeographic structure were considered [20, 21].

However, at present, the data on the genetic variability of *S. flavicollis* mostly concern the western and central parts of the range. For a considerable territory in the eastern part of the species distribution range (East European Plain), karyological and molecular genetic studies are few [4–6, 20, 21], and for the eastern border of the range, data are completely absent. At the same time, the ideas on intraspecific genetic variability, phylogeographic structure, and the history of the range formation of widespread species, like the yellow-necked field mouse, cannot be considered complete without analysis of representative data from all parts of the distribution range, including peripheral populations. In addition, the study of marginal populations is important in assessing the stability and adap-

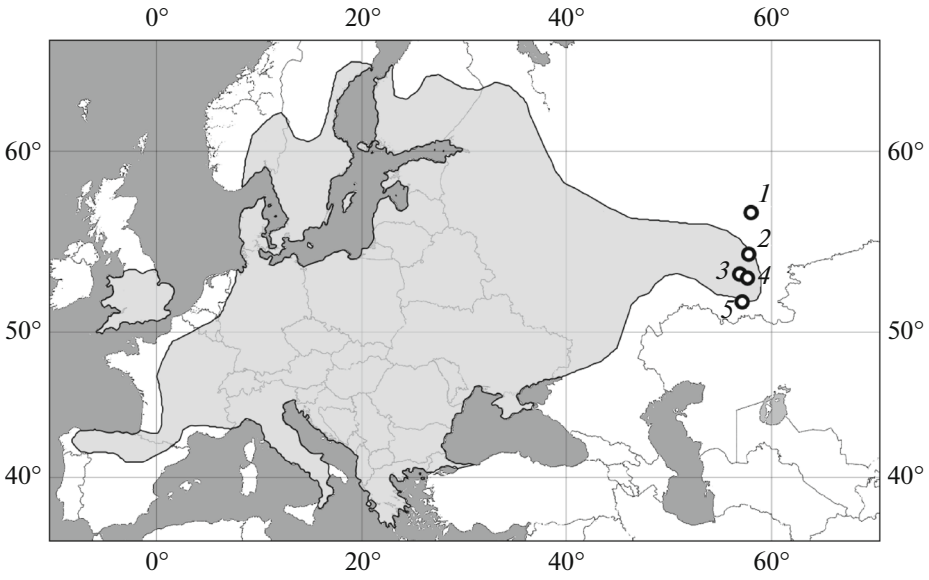

Fig. 1. Schematic map showing sampling sites for the analysis of genetic variability of *S. flavicollis* at the eastern border of the range. Middle Urals: *1*, Oak forest (Nizhneirginskaya oak forest, 56°56′ N, 57°26′ E); Southern Urals: *2*, Ignatievskaya (vicinity of the Ignatievskaya cave, 54°53′ N, 57°46′ E); *3*, Kinzebulatovo (vicinity of the settlement of Kinzebulatovo, 53°25′ N, 56°11′ E); *4*, Nugush (vicinity of the Nugush Reservoir, 53°05′ N, 56°26′ E); *5*, Verblyuzhka (vicinity of the Verblyuzhka Mountain, 51°22′ N, 56°48′ E). The black line is the border of the range of *S. flavicollis*.

tive potential of the species in the context of global climate change and increasing anthropogenic impact, as well as addressing the problem of biological invasions [22–24]. From this perspective, the objective of the present study was to analyze the genetic variability in populations of the yellow-necked field mouse across the eastern border of the species range on the basis of the analysis of chromosome sets and sequences of the mtDNA cytochrome *b* gene.

MATERIALS AND METHODS

Analyses of the genetic variability of the yellow-necked field mouse across the eastern border of the species range were carried out using specimens from five trapping sites (Fig. 1). One of the sites is located in the Middle Urals and is the most northeastern one known to date [25]; the remaining four localities are situated in the Southern Urals. The study was carried out using personal collections and the specimens from the collection of the Museum of the Institute of Plant and Animal Ecology, Ural Branch, Russian Academy of Sciences, Yekaterinburg.

Analysis of chromosome preparations made from bone marrow according to the standard method and stained according to Romanovsky–Giemsa [26] was performed for seven individuals from the most northeastern population (Middle Urals, Nizhneirginskaya oak forest (Fig. 1)). In each animal, 50 cells were analyzed, estimating the chromosome number and their morphology, as well as the presence of B chromosomes in the karyotype.

Total DNA was isolated from muscle tissue specimens fixed in 96% ethanol using the method of salt extraction [27]. PCR of the mtDNA fragment containing the cytochrome *b* gene was performed with a pair of primers L7 (5'-ACCAATGACATGAAAAATCATCGTT-3') and H6 (5'-TCTCCATTTCTGGTTTACAAGAC-3') [28] in 25 μL of reaction mixture (3 mM of each of the dNTPs (SibEnzyme), *Taq* Buffer 10× + KCl – $MgCl_2$ (Fermentas), 6.25 mM $MgCl_2$ (Fermentas), 7.5 pM of each primer, 2.5 U *Taq* DNA Polymerase (SibEnzyme), and 50–100 ng DNA template) according to the protocol (94°C for 3 min; [94°C for 20 s, 58°C for 15 s, 72°C for 1 min 20 s], 35 cycles; 72°C for 10 min) on a MyCycler thermal cycler (BioRad). The PCR product was purified by electrophoresis on a 1% agarose gel, after which the gel segment containing the desired fragment was excised, and the PCR product was reprecipitated using a Silica (Sigma-Aldrich S-S5631). The sequencing reaction was performed using the Big Dye Terminator Cycle Sequencing Kit v. 3.1 (Applied Biosystems, United States) according to the manufacturer's recommendations in two directions with primers L7 and H6, respectively, followed by sequence determination on an ABI Prism 3130 automated analyzer (Applied Biosystems, United States).

Primary treatment of the obtained sequences was carried out using the BioEdit v. 7.2.0 software program [29]. All results of automated analysis were manually double-checked. In the case of controversial regions and/or nucleotides in the specimen, repeated sequencing

was performed to eliminate the inconsistencies that occurred.

Sequence alignment, calculation of genetic distances, and construction of phylogenetic trees by the neighbor joining (NJ) and maximum likelihood (ML) methods were carried out in the MEGA v. 6 software program [30]; the search for the best-fit models of nucleotide sequence evolution was performed in the MrModeltest 2.3 software program [31]. Construction of phylogenetic trees using Bayesian analysis was performed using the MrBayes v. 3.2.2 software program [32]; construction of median-joining networks was accomplished using the Network v. 5.0.0.0 software program [33]. Data on the genetic diversity of nucleotide sequences were obtained using the Arlequin v. 3.1 [34] and DnaSP [35] software programs.

The study of the *S. flavicollis* population genetic diversity on the eastern border of the range was carried out on the basis of the data on 44 cytochrome *b* gene sequences (1133 bp) determined in the specimens from five localities of the Middle and Southern Urals (Fig. 1). For the comparative analysis and phylogenetic reconstructions, the 838-bp fragment was used (17 haplotypes were our own data from five peripheral populations; 80 haplotypes were data represented in the GenBank database (Table 1)). Six haplotypes of *S. ponticus* (AJ605667, AJ605668, AJ6056676, AJ605677, AJ605688, AJ605690 [20]) were used as the outgroup.

To exclude erroneous sequences or sequences of nuclear pseudogenes that could lead to incorrect results in phylogenetic reconstructions, sequences of maximum length were selected in the GenBank database that did not contain ambiguously read nucleotides and insertions/deletions leading to a reading-frame shift.

Analysis of mismatch distribution among partial cytochrome *b* gene sequences (838 bp) of *S. flavicollis* in a phylogroup containing specimens from the eastern border of the range was performed on the basis of the data on 61 sequences. Forty-four sequences were determined in our study and 17 sequences were taken from the GenBank database (one sequence for each of Mich05–Mich08, Mich10, Mich11, Mich21, F2, F8, F10, F12, TK67707, and TK96207 haplotypes and two sequences for each of Mich25 and Mich48 haplotypes (Table 1)).

RESULTS

Cytogenetic analysis showed that chromosome characteristics of seven yellow-necked field mice from the territory of the Middle Urals (Nizhneirginskaya oak forest, Fig. 1) corresponded to the standard chromosome set of *S. flavicollis* ($2n = 48$, $NF = 48$). The karyotypes of all examined individuals (350 metaphase cells analyzed) consisted of 48 chromosomes, including 23 pairs of autosomes represented by acrocentric chromosomes gradually diminishing in size and sex chromosomes that were also acrocentric. B-chromosomes were not detected in any of the seven animals studied.

Sequences of the 1133-bp *S. flavicollis* mtDNA cytochrome *b* gene were obtained for 44 individuals from five populations of the Middle and Southern Urals. Twenty polymorphic sites (5 transitions and 15 transversions) were revealed, of which 11 sites were parsimoniously informative. The cytosine and guanine content in the sequence was 0.393.

Among the 44 determined sequences, 17 haplotypes (Ur1–Ur17) were described, all of which were new (deposited in the GenBank (NCBI) database under accession numbers MF621854–MF621870). Most of the haplotypes (13 of 17) were found only in one of the investigated localities (Table 2). Moreover, sequences unique for a sample were not found in only one locality (vicinity of the Nugush Reservoir, Nugush; Fig. 1).

Comparison of the genetic diversity indices in the samples of *S. flavicollis* from the studied marginal populations showed the tendency of their increase in the north–south gradient (Table 2, Fig. 1). Analysis of the molecular variance (AMOVA) showed a rather high level of interpopulation differences ($\Phi_{ST} = 0.173$, $P < 0.001$), accounting for 17.26% of variability, while intrapopulation differences accounted for 82.74% of variability. Comparison of population pairs with respect to the Φ_{ST} index showed that the most differentiated was the sample of *S. flavicollis* from the Nizhneirginskaya oak forest, the most northeastern point in the species range (Fig. 1). Comparison of this sample with other samples showed the presence of statistically significant interpopulation differences, while interpopulation differentiation of the remaining four samples was not statistically significant (Table 3).

In general, for *S. flavicollis* from the eastern border of the range (Middle and Southern Urals), the genetic diversity indices values (Table 2) are lower than those found earlier in the western and central parts of the species distribution range [21]: Balkan–Italian region, $h = 0.988$, $\pi^{\times 100} = 1.5$; Iberian Peninsula and Southern France, $h = 0.986$, $\pi^{\times 100} = 0.54$; Western, Northern, and Central Europe, $h = 0.992$, $\pi^{\times 100} = 0.75$; Ukraine and Russia (Volgograd, Voronezh, Samara oblasts), $h = 0.952$, $\pi^{\times 100} = 0.92$. In addition, considerably lower nucleotide diversity was observed along with relatively small differences in haplotype diversity.

Phylogenetic reconstruction was performed using both our own and published data for the 838-bp cytochrome *b* gene fragment. Figure 2b shows a phylogenetic tree obtained with the help of Bayesian analysis; the tree topologies obtained using other methods, ML and NJ, were not critically different. All analyzed sequences of *S. flavicollis* clustered into three phylogroups that had relatively clear geographic localization (Fig. 2). For instance, the Southeastern phylogroup includes haplotypes from Southern (Serbia,

Table 1. Geographic origin, names, and GenBank accession numbers of *S. flavicollis* cytochrome *b* gene (838 bp) haplotypes used in the study

Geographic location	Haplotype name (GenBank accession number)
Austria	Mich61 (AJ605600)
Belarus	Mich08 (AJ605601), Mich09 (AJ605603)
Belgium	Mich58 (AJ605604)
Bosnia and Herzegovina	F14 (JF819968), F15 (JF819969), F16 (JF819970), F11 (JF819965), F12 (JF819966)
England	AF5 (JX457731)
Hungary	Mich41 (AJ605634)
Germany	Mich39 (AJ605616)
Greece	Mich24 (AJ605624), Mich66 (AJ605628), Mich71 (AJ605626), Mich72 (AJ605627), Mich77 (AJ605625), Mich21 (AJ605629), Mich29 (AJ605631), Mich35 (AJ605633), Mich20 (AJ605619), Mich26 (AJ605617), Mich34 (AJ605618), Mich75 (AJ605622), Mich79 (AJ605620), JRM-706 (AJ605621)
Spain	Mich53 (AJ605661), Mich62 (AJ605660)
Italy	Mich47 (AJ605637), Mich49 (AJ605640), JRM-19 (AJ311150)
Lithuania	Mich10 (AJ605641)
Macedonia	Mich32 (AJ605644), Mich55 (AJ605643), Mich68 (AJ605642), F1 (JF819955), F2 (JF81956), F4 (JF819958), F5 (JF819959),F6 (JF819960), F7 (JF819961), F8 (JF819962), F9 (JF819963), F10 (JF819964)
Netherlands	Mich52 (AB032853)
Poland	AF4 (JX457730)
Russia	Mich12 (AJ605652), Mich15 (AJ605653), Mich05 (AJ605650), Mich06 (AJ605651)
Romania	Mich13 (AJ605646), Mich17 (AJ605647)
Serbia	Mich19 (AJ605691)
Slovenia	Mich70 (AJ605657), Mich22 (AJ605656)
Turkey	Mich48 (AJ605672), Mich69 (AJ605673)
Ukraine	TK67530 (AY158443), TK67707 (AY158444), TK67715 (AY158445), TK67970 (AY158450), TK84424 (AY158451), TK96182 (AY158452), TK96183 (AY158453), TK96207 (AY158454)
France	T666 (AJ311151), Mich25 (AJ605614), AF6 (JX457732)
Czech Republic	Mich33 (AJ605605), Mich40 (AJ605608), Mich51 (AJ605606), Mich63 (AJ605609)
Switzerland	Mich78 (AF159392)
Sweden	Mich04 (AJ605663), Mich43 (AJ605664)
Estonia	Mich07 (AJ605611), Mich11 (AJ605610)
England, Spain, Poland, Finland	AF1 (JX457727)
Spain, Poland	AF2 (JX457728)
Poland, France	AF3 (JX457729)

The GenBank haplotypes having in the accession number the AB abbreviation [18]; AF [15]; AJ [19, 20]; JF [16]; JX [36]; AY, unpublished data.

Romania) and Eastern (Ukraine) Europe and Russia (Volgograd oblast). The Northeastern group is represented by haplotypes from the territory of the Balkan Peninsula (Greece, Bosnia and Herzegovina, Eastern Turkey, Macedonia), Northern (Estonia, Lithuania) and Eastern (Belarus, Ukraine) Europe, and Russia (Samara oblast). All haplotypes of *S. flavicollis*, described in the present study for five localities on the eastern border of the species range belong to this group. The Western phylogroup consists of haplotypes

Table 2. Distribution of the cytochrome *b* gene haplotypes and the genetic diversity indices in populations of *S. flavicollis* at the eastern border of the range in the Urals

Haplotype	Oak forest	Ignatievskaya	Kinzebulatovo	Nugush	Verblyuzhka	Middle and Southern Urals
Ur1	6					6
Ur2	4		1	2		7
Ur3	1					1
Ur4		2	4	2	1	9
Ur5					3	3
Ur6					1	1
Ur7					1	1
Ur8			2			2
Ur9			1			1
Ur10			1			1
Ur11			2			2
Ur12		1	2	1		4
Ur13			1			1
Ur14			1	1		2
Ur15		1				1
Ur16		1				1
Ur17		1				1
Genetic diversity indices						
N	11	6	15	6	6	44
Nh	3	5	9	4	4	17
$h \pm SD$	0.618 ± 0.104	0.933 ± 0.122	0.914 ± 0.052	0.867 ± 0.129	0.800 ± 0.172	0.911 ± 0.023
$\pi^{\times 100} \pm SD^{\times 100}$	0.106 ± 0.02	0.206 ± 0.057	0.207 ± 0.03	0.135 ± 0.038	0.306 ± 0.085	0.214 ± 0.023
k	1.20	2.33	2.34	1.53	3.47	2.43

N, the number of individuals; *Nh*, the number of haplotypes; *h*, haplotype diversity; π, nucleotide diversity; *k*, the mean number of pairwise differences between haplotypes; SD, standard deviation.

Table 3. The Φ_{ST} values (below the diagonal) and the level of statistical significance of the observed differences (above the diagonal) in interpopulation analysis of the genetic variability of *S. flavicollis* near the eastern border of the range

Sample	Oak forest	Ignatievskaya	Kinzebulatovo	Nugush	Verblyuzhka
Oak forest		<0.002	<0.001	>0.05	<0.001
Ignatievskaya	0.254		>0.05	>0.05	>0.05
Kinzebulatovo	0.260	0.003		>0.05	*<0.05*
Nugush	0.200	0.000	0.000		>0.05
Verblyuzhka	0.371	0.144	0.168	0.182	

from Western (France, Germany, Austria, Switzerland, Netherlands), Northern (Sweden), Eastern (Czech Republic, Hungary, Romania, Belarus, Ukraine), and Southern (Spain, Italy, Greece, Macedonia, Slovenia, Turkey) Europe.

The presence of the three phylogroups described above in the phylogenetic structure of *S. flavicollis*, as well as the inclusion of haplotypes from the analyzed marginal populations from the territory of the Urals to the Northeastern phylogroup, is supported both by phylogenetic reconstructions using different methods and by the median-joining network analysis (Fig. 2c). The differences between the three phylogroups range from four (Western and Northeastern) to five (Northeastern and Southeastern) mutational steps. In the Western and Northeastern groups, star-like structures were revealed.

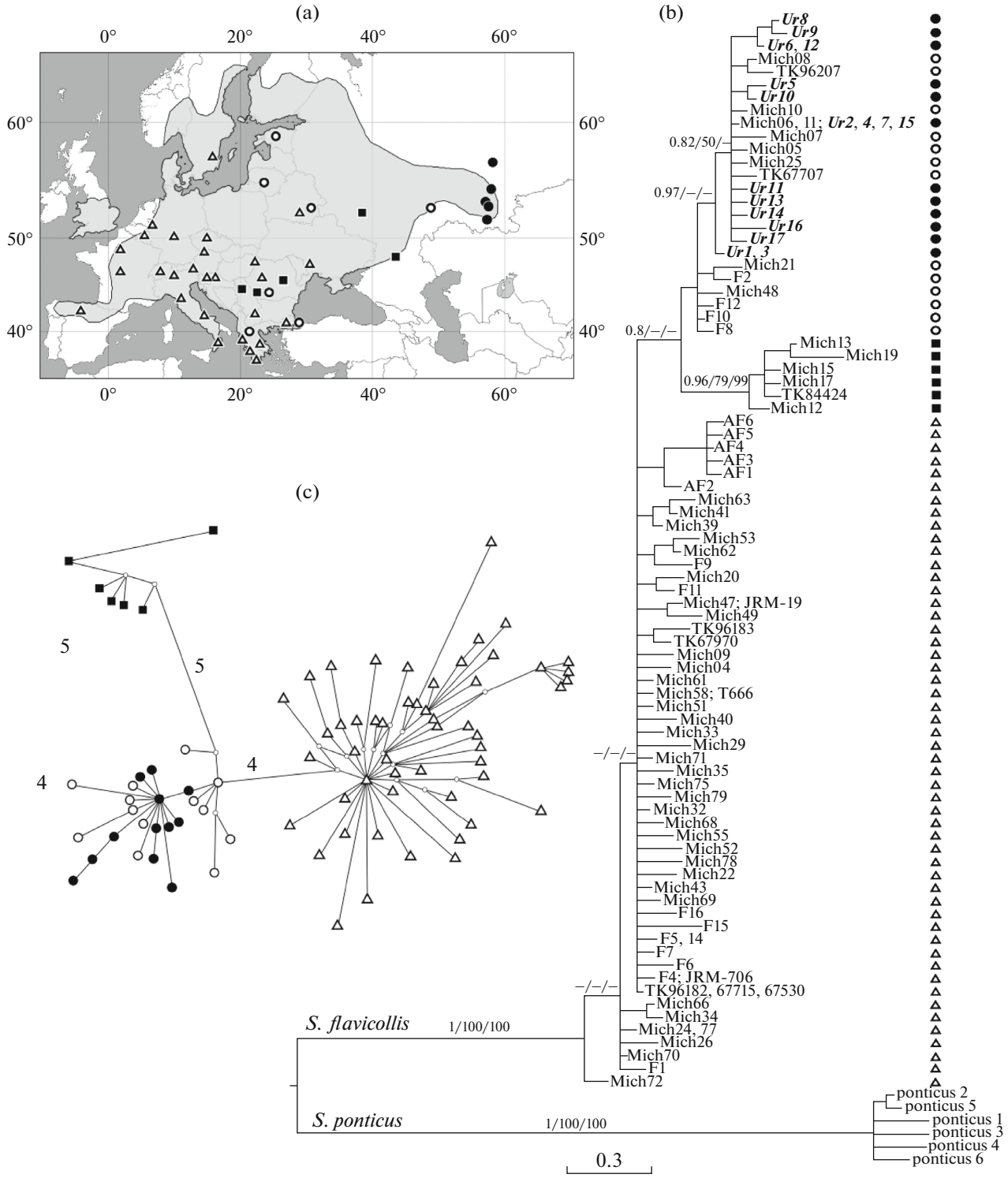

Fig. 2. The distribution of *S. flavicollis* phylogenetic lineages across the species range (a); phylogenetic tree (b) reconstructed using the Bayesian analysis on the basis of 97 haplotypes of the cytochrome *b* gene (838 bp); over the branches are the BI > 75/ML > 50/NJ > 50 probabilities; median-joining network of these haplotypes (c); figures above the branches designate the numbers of substitutions. Geometrical figures designate the haplotype distribution over phylogroups: circle, Northeast phylogroup; square, Southeastern phylogroup; triangle, Western phylogroup. Black circles indicate the haplotypes described by us for the eastern border of the range.

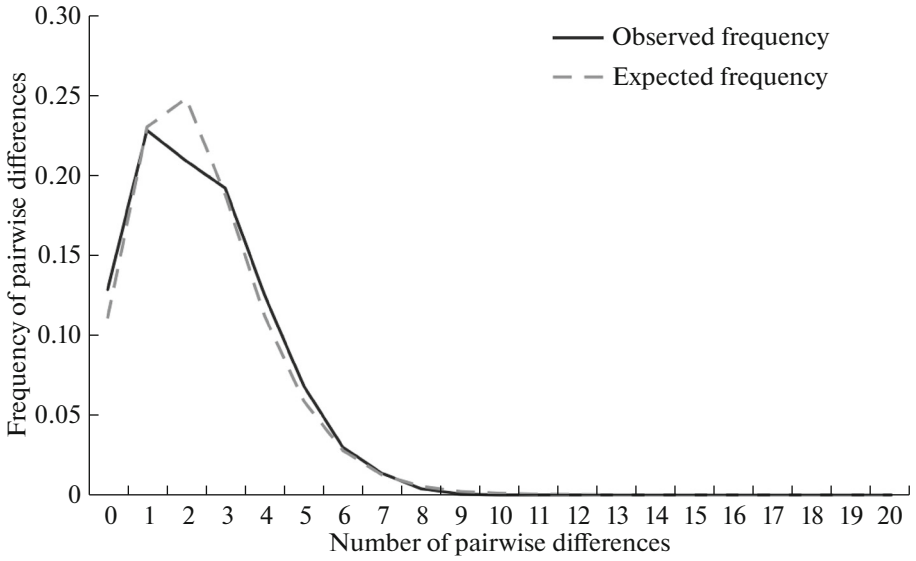

Fig. 3. The ratio of pairwise differences among the sequences of the cytochrome *b* gene fragment (838 bp) of *S. flavicollis* in the Northeastern phylogroup. Observed values, frequency distribution of pairwise differences among all sequences belonging to the Northeastern phylogroup. Expected values, theoretical frequency distribution of pairwise differences among the sequences using the model of the effective population size change.

The results of analysis of the mismatch distribution among partial cytochrome *b* gene (838 bp) sequences of *S. flavicollis* from the Northeastern group (Fig. 3) that includes haplotypes of the studied populations from the eastern border of the species range, as well as Tajima's ($D = -2.172$; $P < 0.05$) and Fu's ($Fs = -19.786$; $P < 0.01$) tests, indicate that this phylogroup passed through the stage of increase in the effective population size.

DISCUSSION

Karyotype and sequence analysis of the mtDNA cytochrome *b* gene in the yellow-necked field mouse from the Southern and Middle Urals resulted in the first description of the genetic variability in the populations of this species on the eastern border of the range.

Karyotypes of seven mice from the most northeastern of the known localities of *S. flavicollis* (Nizhneiriginskaya oak forest, Middle Urals) were represented by 48 acrocentric chromosomes gradually decreasing in size, including sex chromosomes. No additional chromosomes were found. At the same time, considering the considerable variability in the occurrence of B chromosomes in *S. flavicollis* [7, 10, 14], on the basis of a single analysis of a small sample, the possibility of their presence with a low frequency in the studied marginal population cannot be unambiguously excluded. Thus, in the yellow-necked field mouse on the eastern border of the species range, no deviations from the standard chromosome set, either in the number of chromosomes or in their morphology, were observed. The data of karyological analysis are consistent with the opinion of a number of authors on the decrease in the frequency of occurrence of additional chromosomes in peripheral populations of the yellow-necked field mouse [7, 10].

Sequence analysis of the 1133-bp mtDNA cytochrome *b* gene in 44 individuals from five populations of the yellow-necked field mouse from the Middle and Southern Urals resulted in the description of 17 haplotypes, none of which was previously found in other parts of the range. Comparison of the genetic diversity indices in the examined samples showed that, near the eastern border of the range, a trend toward the increase in nucleotide diversity in the direction from the north to the south, with the lowest diversity level observed in the northernmost population of the yellow-necked field mouse (Nizhneirginskaya oak forest, Middle Urals), could be tracked. Analysis of the population genetic structure (ANOVA, Φ_{ST}) also showed that this population was the most differentiated from the other four. It seems likely that the observed interpopulation differences of *S. flavicollis* in the eastern part of the species distribution range are associated with both relative isolation of the population in Middle Urals (the most northeastern one among presently known habitats [25]) and the history of the species dispersal over the territory of the Ural region and the eastern border of the range as a whole.

According to the ideas of intraspecific genetic structure formation [37], relatively high haplotype diversity at low values of nucleotide diversity observed in the yellow-necked field mouse at the eastern border of the range (Table 2) may be indicative of the relatively recent origin of the studied marginal populations

as a result of rapid dispersal. Analysis of the demographic history of Northeastern phylogroup, which includes haplotypes described by us in the eastern part of the *S. flavicollis* distribution range, also supports this supposition. Analysis of the mismatch distribution showed that this phylogroup passed through the stage of increase in the effective population size, which can be interpreted as active recent dispersal across the currently occupied territory.

Phylogenetic analysis of the cytochrome *b* gene sequences of the yellow-necked field mouse from the eastern border of the range determined in the present study and the sequences represented in the GenBank database demonstrates the belonging of all haplotypes described in the Urals to the Northeastern phylogroup. Analysis of the distribution and phylogenetic relationships within and between the mitochondrial lineages of the yellow-necked field mouse shows close relationships between the eastern and northern marginal populations (the Urals and the Baltic states).

The differentiation of *S. flavicollis* revealed in the course of comparative analysis does not conflict with the currently available concepts of the phylogeographic species structure. All analyzed sequences cluster into three phylogroups, Western, Southeastern, and Northeastern (Fig. 2), corresponding to the earlier described clades A, B, and C [21]. The inclusion of the data on *S. falvicollis* from the eastern border of the range in the analysis also supports the earlier proposal on the recent rapid dispersal of the Northeastern group (clade B [21]) across the currently occupied territory [20]. This is evidenced by the median network topology (Fig. 2) and analysis of the mismatch distribution among partial cytochrome *b* gene sequences (838 bp) within this group (Fig. 3), as well as by the results of Tajima's *D* and Fu's *Fs* tests.

Thus, our study of the genetic variability of the yellow-necked field mouse on the eastern periphery of the distribution range supplements the existing ideas on the intraspecific genetic structure of the species and facilitates verification of the suppositions made earlier without including data from this part of the species range in the analysis. The results indicate the need for further study of *S. flavicollis* on the East European Plain, in particular, for solving the problems of the chromosomal polymorphism distribution patterns and of the possible dispersal of other phylogroups to the east during the formation of the modern species range.

ACKNOWLEDGMENTS

This study was supported by the Russian Foundation for Basic Research (grant no. 16-04-01625).

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Translated by N. Maleeva