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Harvest mouse *Micromys minutus* of the Urals and Western Siberia: range boundaries and genetic diversity

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ABSTRACT. As a result of the analysis of the *cyt b* complete sequences of 26 harvest mice *Micromys minutus* from 13 localities of the Urals and Western Siberia, the distribution boundaries and genetic diversity of the Russia phylogeographic lineage have been clarified. The distribution of the lineage in the south of the central part of Northern Eurasia from the Tyva Depression to the Southern Trans-Urals coincides with the southern boundary of the species range. The northern area of species distribution was previously considered to be limited to the taiga zone. However, our study showed that the northern boundary of the range passes through the subpolar regions of the West Siberian Plain and Cis-Urals in the zone of modern forest-tundra. Phylogeographic analysis with the inclusion of new data has confirmed the existence of four previously described phylogenetic lineages (Europe, Russia, Taiwan, Korea-Japan), the genetic diversity and demographic analysis of which have confirmed the suggestion that the modern species genetic structure and range has formed during the Late Pleistocene–Holocene period.

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KEY WORDS: *Micromys*, central part Northern Eurasia, cytochrome *b* gene, genetic structure, phylogeography, Quaternary.

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Мышь-малютка *Micromys minutus* Урала и Западной Сибири: границы ареала и генетическое разнообразие

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РЕЗЮМЕ. В результате анализа полных последовательностей гена цитохрома *b* 26 особей мыши-малютки (*Micromys minutus*) из 13 локалитетов Урала и Западной Сибири уточнены границы распространения и генетическое разнообразие «российской» клады. Ее распространение на юге центральной части Северной Евразии от Тувинской котловины до Южного Зауралья совпадает с общепринятой южной границей видового ареала. Северная граница проходит по приполярным районам Западно-Сибирской равнины и Предуралья в зоне современных лесотундр за пределами таежной зоны, которой, как считалось ранее, ограничивается северная область распространения вида. Филогеографический анализ мыши-малютки с включением новых данных показал существование четырех описанных ранее филогенетических линий («европейской», «российской», «тайваньской» и «корейско-японской»), показатели генетического разнообразия и демографии которых подтверждают предположение о формировании современной генетической структуры и ареала вида на протяжении позднего плейстоцена–голоцена.

КЛЮЧЕВЫЕ СЛОВА: *Micromys*, центральная часть Северной Евразии, ген цитохром *b*, генетическая структура, филогеография, четвертичный период.

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Introduction

The harvest mouse *Micromys minutus* (Pallas, 1771) is the smallest rodent of the family Muridae of Eurasia, whose range occupies a significant part of the forest and forest-steppe zones of the Palearctic from northern Spain and Great Britain to the Korean Peninsula, as well as the islands of Japan and Taiwan (Gromov & Erbaeva, 1995; Kryštufek *et al*., 2019). Despite the species inhabiting a wide variety of open habitats (floodplains, forest edges, fields, fallows, uprooting, thickets of shrubs and weeds in wastelands, as well as variety of anthropogenic habitats, including gardens, and arable land drainage ditches, and grain or rice paddies), one of the main conditions for successful existence is the presence of high and dense herbage, on the stems of which the harvest mouse spends most of the time during reproductive period.

For the island (Great Britain, Japan) and Central European populations of the harvest mouse, a decrease in number and a fragmentation of the range has been noted, caused by anthropogenic impact (Haberl & Kryštufek, 2003; Kuroe *et al*., 2007; Hata *et al*., 2010; Sawabea & Natuhara, 2016). At the same time, a number of captures of *M. minutus* (namely in the territory of Magadan Region, the north of Khabarovsk Territory, Amur Region) beyond the eastern boundary of the distribution area of the species have been associated with the broadening of the range as a result of climate change (Tiunov, 2003; Dokuchaev, 2004; Cheriomkin *et al*., 2018). It is generally accepted that the distribution of the harvest mouse in the north is limited to the taiga zone, but in Western Siberia this species has been repeatedly recorded in the forest-tundra zone, where, settling intrazonal biotopes, it reached the Arctic Circle (Boikov & Bolshakov, 1972; Gromov & Erbaeva, 1995). Nevertheless, it remains unclear whether the penetration of *M. minutus* so far to the north territories is random or whether the species inhabits the forest-tundra permanently, and its rare findings are associated with the difficulty of trapping it by standard methods (Kettell *et al*., 2016).

The study of the harvest mouse genetic diversity using the cytochrome *b* gene and the mtDNA control region revealed that the formation of the modern genetic structure and the species range were relatively recent events that have occurred during the Late Pleistocene–Holocene period (Yasuda *et al*., 2005). At the same time, the significant genetic differences between Russian and European *M. minutus* on the one hand and closeness of the European with Korean and Japanese populations on the other hand are perplexing. Since genetic data from a significant part of the species range is absence, the suggestion "that European and East Asian populations were derived from a refugium, perhaps via a corridor situated to the north of a simultaneously expanding central Siberian population" (Yasuda *et al*., 2005) needs to be confirmed. The central part of Northern Eurasia remains a "blank spot", although it has been shown that the physiographic conditions of such regions as the Urals and Western Siberia played an important role in the evolutionary history of a number of widespread small mammals (Bilton *et al*., 1998; Brunhoff *et al*., 2003; Haynes *et al*., 2003; Sibiryakov *et al*., 2018).

The aim of this study was to analyze the genetic diversity and intraspecific phylogenetic relationships of the harvest mouse of the Urals and Western Siberia, including the forest-tundra zone, using the sequence data of the mtDNA cytochrome *b* gene (*cyt b*).

Material and methods

In this study we used samples from 26 individuals from 13 previously unstudied localities on the Urals and Western Siberia. Seven localities are near the northern border of the species range; localities pts. 1–3 are from the forest-tundra zone and pts. 4–7 from the northern taiga zone (Fig. 1, Table 1). The own data and museum collections (Museum of the Institute of Plant and Animal Ecology, Ural Branch of Russian Academy of Sciences, Yekaterinburg and Museum of the Ural Federal University (Appendix 1)) were used. All work has been carried out in accordance with the European Convention for the protection of vertebrate animals used for experiments or other scientific purposes.

Total genomic DNA has been extracted using method of salt extraction (Aljanabi & Martinez, 1997) from small pieces of muscle tissue preserved in 96% ethanol or skulls and skins. PCR of the mtDNA fragments containing the *cyt b* has been performed in two ways. The first variant was performed for muscle tissue: two fragments of the *cyt b* about 550 bp and 700 bp in length have been amplified using two pairs of primers: L7 (5'-ACCAATGACATGAAAAATCATCGTT-3') and H2 (5′-TAGTTGTCTGGGTCTCC-3′), and L8 (5′-CTGCCATGAGGACAAATATCATT-3′) and H6 (5'-TCTCCATTTCTGGTTTACAAGAC-3') (Tougard *et al*., 2001; 2008). For museum samples, containing degraded DNA, a two-stage nested PCR was conducted. At the first stage, primer pairs L7–H2 and L8–H6 have been used to amplify the main DNA fragment. At the second stage, to amplify short fragments lying inside the main amplicon, we used an additional set of species-specific primers developed by the authors (Pilevich & Kroholeva, 2021), which made it possible to obtain sequences of ≈170–320 bp (Appendix 2).

The chromatograms have been analyzed using the BioEdit v.7.2.0 (04.30.2013) software program (Hall, 1999). The results of the automatic analysis of the obtained capillary electrophoresis data have been manually cross-checked. The sequence alignment (MUSCLE algorithm), calculation of genetic distances, and construction of phylogenetic trees with the neighbor joining (NJ) and maximum likelihood (ML) methods have been carried out in the MEGA v6 software program (Tamura *et al*., 2013). The Bayesian inference (BI) implemented in the MrBayes v.3.2.2 software program (Ronquist *et al*., 2012) has been used. The search for optimum models of nucleotide sequence evolution has been performed in the MrModeltest 2.3 software program (Nylander, 2004). For constructing the BI phylo-

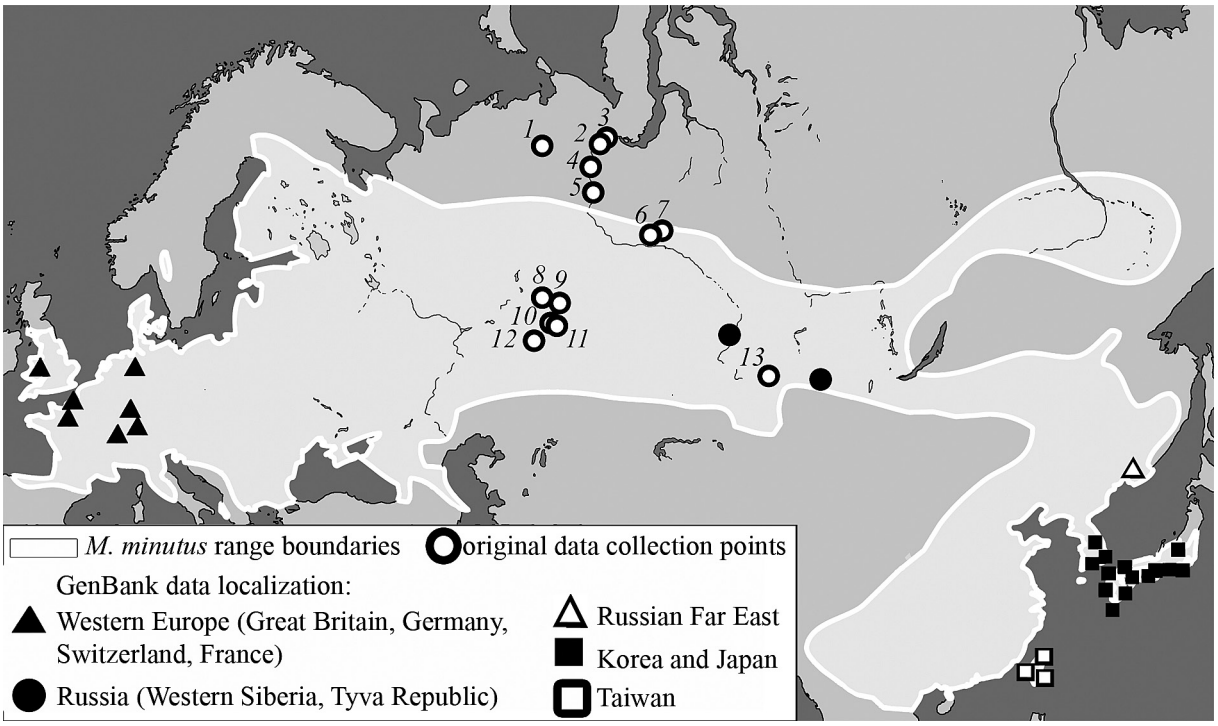

Fig. 1. *Micromys minutus* range (Kryštufek *et al*., 2019) and geographical localization of samples used in the study: 1–13 — original data collection point; geometric shapes — GenBank data.

genetic tree, a complex approach with a separate choice of the model for each of the three codon positions has been used. For all three positions the optimal model has been proven to be the general time reversible model (GTR+G). In order to construct trees using the NJ and ML methods, respectively, Tamura's three-parameter model (T3P) and GTR have been chosen.

The construction of median-joining networks (MJ network) has been carried out in the Network v5.0.0.0 software program (Bandelt *et al*., 1999). The assessment of genetic diversity indexes and the tests for selective neutrality have been performed in the Arlequin v.3.1 (Excoffier *et al*., 2005) and DnaSP v.5.10 (Librado & Rozas, 2009) software programs.

In addition to the original data, the phylogeographical analysis included 39 haplotypes presented in GenBank (Fig. 1, Table 1).The complete *cyt b* sequences (1140 bp) were used in the study, since the inclusion of partial sequences of about 1100 bp from the Scandinavian peninsula (JX531446–450, JX457740 (Råberg *et al*., 2013; Barbosa *et al*., 2013)) did not affect the results of the analysis (Appendix 3). *Micromys erythrotis* (AB201996, FJ827491, FJ827492 (Yasuda *et al*., 2005; Abramov *et al*., 2009) has been used as outgroup.

Results

A total of ten haplotypes were obtained among the complete sequences of *cyt b* (1140 bp) from 26 harvest mice (Table 1). One haplotype (WS1) has been previously found in the vicinity of Novosibirsk and Kyzyl (Tyva Republic) cities (Yasuda *et al*., 2005). Our study has shown this haplotype has a wide distribution in the central part of Northern Eurasia from Altai and the Southern Trans-Urals to the forest-tundra zone of the Lower Ob' and subpolar Cis-Urals. Nine haplotypes are new (SU1–SU4 and WS9, WS11–WS13; GenBank IDs OP859026–34). Haplotypes WS11 was found in the taiga zone of the Surgut Ob' region, WS9 in the northern taiga and forest-tundra of the Lower Ob' region, while WS12 and WS13 were restricted to the forest-tundra zone of the Lower Ob' region. Haplotypes SU1 through SU4 have been identified in populations on the border of the southern taiga and forest-steppe zones of the Trans-Urals (Table 1).

On the BI phylogenetic tree (NJ and ML trees demonstrated same topologies) all *cyt b* haplotypes clustered, with varying support levels, into four main clades (Fig. 2A). The differentiation of *M. minutus* revealed by this study supports the results obtained by Yasuda *et al*. (2005), who named these lineages according to their geographical distribution: Europe, Russia, Taiwan, and Korea-Japan clades. Two haplotypes from the Russian Far East (FE1, FE2) are significantly differentiated from all the others, and significantly different one from another in the same time. All sequences sequenced by us belong to the Russia clade, which has been previously identified based on the analysis of single samples from the vicinity of the cities of Novosibirsk and Kyzyl (Yasuda *et al*., 2005).

Table 1. Geographical information, sample size (*n*), abbreviation and GenBank accession number of *cyt b* haplotypes of *Micromys minutus* analyzed in the study.

Locality	*n*	Abbreviation (accession ID)
Original data*		
1. Vicinities of Vorkuta City, Komi Republic, Polar Cis-Urals, 67.03ºN 64.12ºE	1	WS1 (AB201975)
2. Varna-Pugor Island, lower Ob' River, YaNAO, Western Siberia, 65.58ºN 65.23ºE	3	WS9 (OP859026), WS12 (OP859030)
3. Vylposl village, Priuralsky District, YaNAO, Western Siberia, 66.68ºN 66.50ºE	1	WS13 (OP859034)
4. Khashgort village, Shuryshkarsky District, YaNAO, Western Siberia, 65.52ºN 65.67ºE	2	WS1 (AB201975), WS9 (OP859026)
5. Polnovat village, Beloyarsky District, KhMAO, Western Siberia, 63.78ºN 65.93ºE	1	WS1 (AB201975)
6. Surgut City, KhMAO, Western Siberia, 61.16ºN 73.21ºE	1	WS11 (OP859029)
7. Ult Yagun railway station, Surgut District, KhMAO, Western Siberia, 61.5ºN 74.22ºE	1	WS1 (AB201975)
8. Sabik railway station, Sverdlovsk Region, Middle Urals, 57.12ºN 59.18ºE	2	WS1 (AB201975)
9. Dvurechensk village, Sverdlovsk Region, Middle Urals, 56.6ºN 61.07ºE	7	SU1 (OP859028), SU3 (OP859032), MU1 (OP859033), WS1 (AB201975)
10. Metlino village, Chelyabinsk Region, Southern Urals, 55.8ºN 61ºE	2	SU4, SU1 (OP859027, OP859028)
11. Muslyumovo village, Chelyabinsk Region, Southern Urals, 55.6ºN 61.48ºE	3	SU2 (OP859031), SU3 (OP859032)
12. Mt. Iremel, Republic of Baskortostan, Southern Urals, 54.52ºN 58.85ºE	1	SU4 (OP859027)
13. Yaylyu village, Turochaksky District, Altai republic, 51.77ºN 87.6ºE	1	WS1 (AB201975)
GenBank data**		
Great Britain	1	UK1 (AB201983)
Germany	5	G1 (AF159399), G2 (AB201981), G3 (AB201982), G4 (AB125099), G5 (AB125100)
Russia	10	FE1 (AB125091), FE2 (FJ827490), WS1 (AB201975), WS2 (AB201974), WS3 (AB201973), WS4 (AB201972), WS5 (AB201971), WS6 (AB201970), WS7 (AB201969), WS8 (AB201976)
Taiwan	12	T1 (AB201985, 91, 94), T2 (AB201986–89, AB201992, 95), T3 (AB201990, 93), T4 (AB201984)
France	1	F1 (AB125098)
Switzerland	4	SW1 (AB125094–96), SW2 (AB125097)
South Korea	6	K1 (AB201980), K2 (AB201978–79, AB201993), K3 (AB201977), K4 (AB125092)
Japan	34	J1 (AB033697), J2 (AB201966–68, AB125085–87), J3 (AB201965, AB125080–81), J4 (AB201960–64, AB125070–71, 73), J5 (AB201959), J6 (AB201958), J7 (AB125088–90), J8 (AB125084), J9 (AB125069, 72, 74–76, 82–83), J10 (AB125079), J11 (AB125078), J12 (AB125077)

* the numbers of localities correspond to those given in Fig. 1; YaNAO — Yamalo-Nenets Autonomous Okrug; KhMAO — Khanty-Mansi Autonomous Okrug.

** the GenBank haplotypes having the AB abbreviation in the accession number (Yasuda *et al*., 2005) exclude AB033697 (Suzuki *et al*., 2000); AF (Martin *et al*., 2000); FJ (Abramov *et al*., 2009).

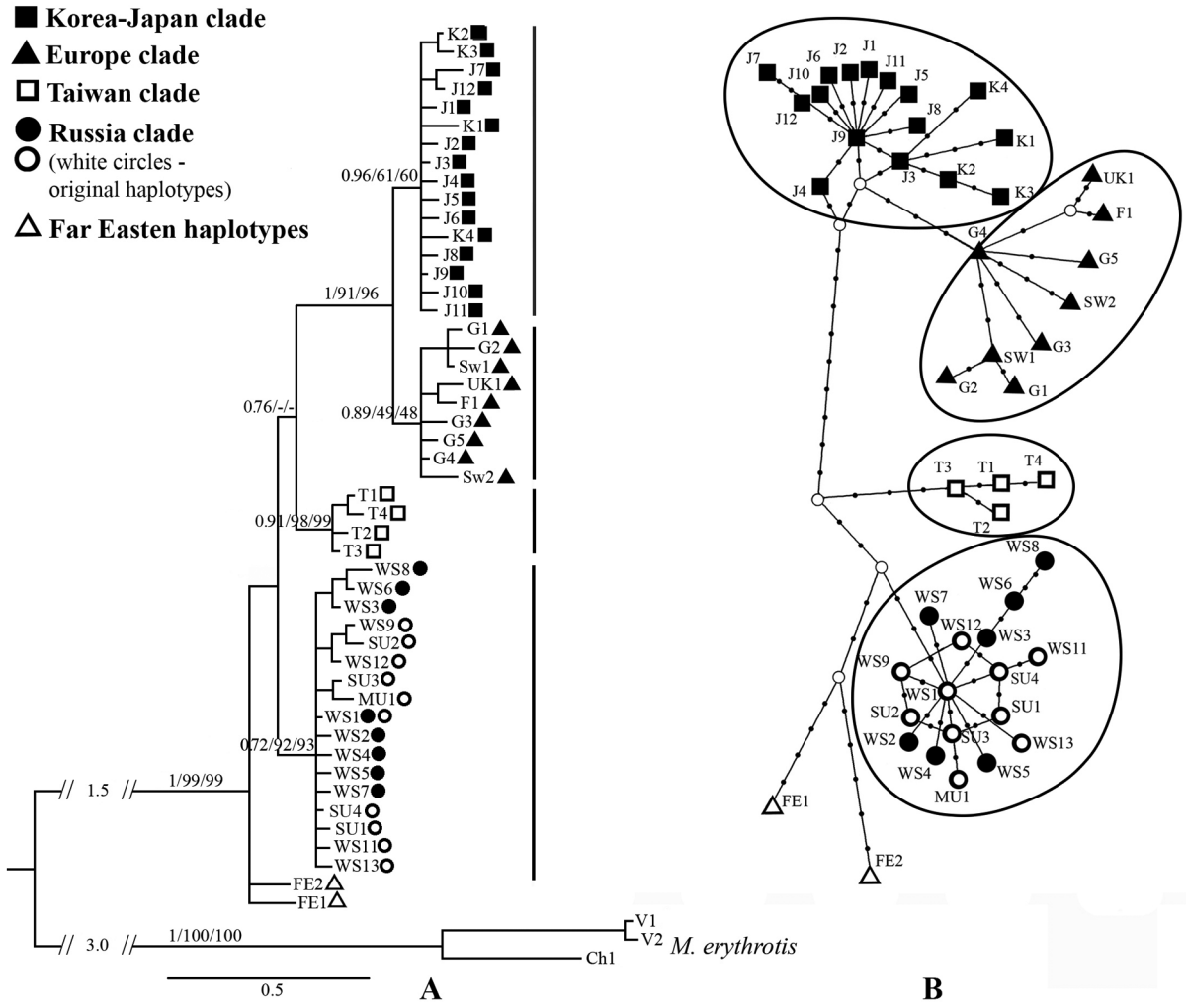

Fig. 2. *Micromys minutus* phylogenetic tree constructed using the Bayesian analysis on the basis of *cyt b* haplotypes (A). Near the branches are the BI > 0.70/ML ≥ 49/NJ ≥ 48 probabilities. Median-joining network of *cyt b* haplotypes (B).

The median network analysis (Fig. 2B) confirmed the existence of four clades, three of which (Korea–Japan, Russia, and Europe) formed star-like structures. In the Russia clade, WS1 is the basal haplotype, widespread in the populations of the central part of Northern Eurasia from the Southern Urals and Altai to the lower reaches of the Ob' River and the Polar Cis-Urals, and it might be considered as ancestral for this genetic lineage. Haplotypes unique for the studied northern territories differ from the central one by one or two substitutions (Fig. 2B).

Analysis of *cyt b* polymorphism revealed high values of genetic diversity indices for the species as a whole (Table 2). Nucleotide diversity and the average number of pairwise differences between haplotypes are higher in the East Asian populations (Russian Far East, Korea, Taiwan, Japan) in comparison with Europe and the central part of Northern Eurasia, which combined with the nonsignificant values of the selective neutrality Fu's test indicates a longer evolutionary history of the species in this region. Low nucleotide diversity revealed for the harvest mouse from Europe and the central part of Northern Eurasia in combination with star-like structures on the median network suggest the recent increase in the population effective size, which is confirmed by the highly significant negative values of the Fu's test (the most sensitive for detecting recent population expansion).

Discussion

Differentiation of harvest mouse revealed in the course of phylogenetics reconstruction into four main clades Europe, Russia, Taiwan, and Korea–Japan clades (Fig. 2A) are consistent with the modern view on the harvest mouse genetic structure based on the analysis of *cyt b* and the mtDNA control region (Yasuda *et al*., 2005; Abramov *et al*., 2009). The median network structure, the presence of star-like structures in Korea-Japan, Russia, and Europe clades (Fig. 2B), and

Table 2. The genetic diversity indices and Fu's values of *M. minutus*.

Data	*n*	Nh	h ± SD	π ± SD(x100)	k	Fs*
Species in whole	99	49	0.970 ± 0.006	0.957 ± 0.025	10.91	–18.79
Europe	11	9	0.945 ± 0.066	0.316 ± 0.049	3.60	–3.93
central part of North Eurasia	34	18	0.906±0.035	0.166±0.021	1.90	–13.33
East Asian populations	54	22	0.936±0.015	0.665±0.077	7.59	–3.01

n — number of sequences; Nh — number of haplotypes; h — haplotype diversity; π — nucleotide diversity; SD — standard deviation; k — mean number of pairwise differences; Fs — values of Fu's test of selective neutrality; **p*<0.02

the data on *cyt b* polymorphism, the results of demographic analysis (Table 2) support the earlier suggestion that the formation of the modern genetic structure of *M. minutus* and expansion in the central and western Palearctic occurred during the Late Pleistocene-Holocene period (Yasuda *et al*., 2005).

All sequences of *M. minutus* of the central part of North Eurasia belong to the Russia clade, identified previously by few haplotypes from the Tyva Republic and the vicinity of the Novosibirsk city. Thus, the distribution area of this lineage includes the territories of the Urals and Western Siberia up to the northern borders of the range and reaches the European part of Russia, at least in the northeast (Komi Republic). Our data does not support the hypothesis about the northern route of the species expansion from East Asia to Europe (Yasuda *et al*., 2005), which has been suggested with regard to the significant genetic closeness of the European and Korean-Japanese clades compared to the Russian one. To make the evolutionary history of lineages clear, the data for significant still unstudied areas of the *M. minutus* range is required.

The presence of a widespread ancestral haplotype (WS1) and unique haplotypes (WS9, WS11–WS13) in *M. minutus* from the northern localities of the Urals and Western Siberia, including specimens from the forest-tundra zone, differing by one or two substitutions, indicates that the harvest mouse entering the northern territories has occurred simultaneously with the dispersal of the species within the modern distribution area of this lineage. It can be suggested that, in the presence of suitable biotopic conditions, the inhabitation of the harvest mouse in the forest-tundra zone of the central part of Northern Eurasia is permanent. However, to reach a definitive conclusion, further studies of northern populations with more genetic data are needed.

Conclusion

As a result of the analysis of the *cyt b* sequences of the harvest mouse from the Urals and Western Siberia, the distribution boundaries and genetic diversity of the Russia clade in the central part of Northern Eurasia have been clarified. According to our data, the distribution of the lineage in the south of the central part of Northern Eurasia from the Tyva Depression to the Southern Trans-Urals coincides with the southern boundary of the species range (Gromov & Erbaeva, 1995), while the northern boundary passes through the subpolar regions of the West Siberian Plain and Cis-Urals. The genetic diversity and phylogenetic relationships of the harvest mouse from the northern populations of the Urals and Western Siberia suggest a long-term presence of the species in these territories, including the modern forest-tundra zone. The *M. minutus* dispersion to the north of the forest-taiga zone is apparently due to the presence of intrazonal biotopes necessary for the habitation of the species outside the ecological optimum zone in these territories.

Phylogeographic analysis with the inclusion of new data has shown the existence of four previously described phylogenetic lineages, the genetic diversity and demographic analysis of which have confirmed the suggestion that the modern species range has formed during the Late Pleistocene–Holocene period (after LGM) (Yasuda *et al*., 2005). Nevertheless, a number of questions remain related to the low support on the phylogenetic tree of some nodes (Fig. 2A), which may be important to understanding the history of the species expansion within the modern distribution area, as well as the unresolved phylogeographic status of harvest mice from the Russian Far East. To answer these questions, new data for significant areas of the *M. minutus* range that remain practically unstudied, primarily the Eastern Siberia, Far East, as well as the east of Europe, is necessary.

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Appendix 1. The museum material of M. minutus used in the study. IPAE — Museum of the Institute of Plant and Animal Ecology UrB RAS, Yekaterinburg, Russia.

Map ID	2	7	8	12	13
Voucher ID	IPAE-25450, IPAE-24942, IPAE-17604	IPAE-17791	IPAE-268036	IPAE-268351	IPAE-331923

Appendix 2. Species-specific primers, which were designed based on the complete M. minutus sequences of cyt b (GenBank data (Table 1)), and PCR conditions.

Primers	5'–3' sequence	Approximate length of amplified fragment (bp)	Mix composition (V=25µl)	Temperature protocol and number of amplification cycles	
L7	TGACCAATGACATGAAAAATCATCG	253	H2O — 7.75µl, dNTP's 2.5мM each — 2.50µl, AS Buffer 10x — 2.50µl, MgCl2 50мM — 1.25µl, Primers 2pM — 3.75µl of each, Taq 5U/µl — 1.00µl, DNA 50ng/µl — 2.50µl	95°C — 3 min	x1
MM1-r	GTGAGTAACTGATGAGAATGCTG				
MM2-f	TCAGACACTATAACAGCATTCTCATC	170		95°C — 15 sec 60°C — 20 sec 72°C — 50 sec	x44
MM2-r	CATGTTTCTAGGAAGGCATAAGATCC				
MM3-f	ACGTAGGACGGGGAATCTACTA	287			
MM3-r	TAGGGCTGCGATGATGAAGG				
MM4-f	CCATGAGGACAAATATCCTTCTGAGG	266			
MM4-r	GGGTGGAATGGGATTTTATCTGC				
MM5-f	TCATCATCGCAGCCCTAGCA	326			
MM5-r	GATTAGGGCAATGACTCCTCCTAG				
MM6-f	CCTCCCCACATTAAACCAGAATGATA	253			
MM6-r	GATAAAGGGGTGTTCTACTGGTTG				
MM7-f	ATTCCGCCCAATCTCCCAAAC	246		72°C — 10 min	x1
MM7-r	TCTTCGTTTCTGGTTTACAAGACC				

Appendix 3. Results of phylogenetic reconstructions with the inclusion of partial *cyt b* sequences (1095 bp) from the Scandinavian Peninsula (JX531446–450, JX457740): median-joining network (A) and BI phylogenetic tree (B). Near the branches are the BI>0.70 probabilities. The haplotype's abbreviations correspond to those given in Table 1.