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ANIMAL GENETICS

Polymorphism and Genetic Structure of *Microtus maximowiczii* (Schrenck, 1858) (Rodentia, Cricetidae) from the Middle Amur River Region as Inferred from Sequencing of the mtDNA Control Region

I. N. Sheremetyeva[a], I. V. Kartavtseva[a], L. V. Frisman[a, b], T. V. Vasil'eva[a], and A. V. Adnagulova[c]

[a] *Institute of Biology and Soil Science, Far Eastern Branch of the Russian Academy of Sciences, Vladivostok, 960022 Russia*
e-mail: sheremet76@yandex.ru
[b] *Institute for Complex Analysis of Regional Problems, Far Eastern Branch of the Russian Academy of Sciences, Birobidzhan, 679016 Russia*
[c] *Khabarovsk Antiplaque Station, Rospotrebnadzor, Khabarovsk, 680031 Russia*

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Abstract—The genetic variability of the mitochondrial DNA control region sequences was estimated for the Maximowicz's vole *Microtus maximowiczii* from the Middle Amur River region located between the confluence of Amur River with Ussuri River and Zeya River. The species as a whole was characterized by a high level of genetic variability. For each individual sample, low nucleotide diversity was observed, except for two samples in which a more than twofold increase in this index was revealed. The presence of the contact zone of two genetically distinct populations in the area between Bira and Bidzhan rivers is suggested.

Keywords: phylogeography, genetic diversity, mtDNA control region, Microtus maximowiczii, Middle Priamurye
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INTRODUCTION

The Maximowicz's vole *Microtus maximowiczii* (Schrenck, 1858) is a species with a wide range that is found in humid biotopes of the forest and steppe forest zones of eastern Asia [1]. The range of this species has a complex mosaic structure and extends from the eastern shore of Lake Baikal to the western slope of the Sikhote-Alin Mountain Range. According to some authors, the main part of the species range is located in northern Mongolia and northeast China, and there are peripheral populations on the territory of Trans-Baikal and the Amur Region of Russia that penetrate like tongues from the main part of the range [2, 3]. In the Pleistocene, the range of this species was wider, since its fossils are known from the caves of the southern slopes of the Sikhote-Alin in Primorye [4].

By the end of the last century, three subspecies were recognized in the species [1, 5]: *M. m. maximowiczii* Schrenck, 1858, found in the upper reaches of the Amur River in the mouth of Omutnaya River and widespread in the Amur region and Northeast China; *M. m. ungurensis* Kastchenko 1913, found in the Chita oblast, Ungur River, and living in Trans-Baikal and northeastern Mongolia; *M. m. gromovi* Vorontsov, 1988, found in the southeast of Yakutia (eastern shore of Lake Bol'shoe Toko). However, studies in recent years have made it possible raise the taxonomic rank of the last subspecies to species [6, 7].

The Maximowicz's vole is characterized by extremely complex chromosomal polymorphism [8–14]. The diploid chromosome number can vary from 36 to 44, and the number of arms varies from 52 to 62. The variability of the chromosome number in this species is determined by the high number of structural mutations, while karyotype variability is observed both within the local populations, as well as between them. Most authors are inclined to consider the geographical confinement of chromosomal differences and distinguish from three to five chromosomal forms [10, 14]. Four chromosomal forms (A, B, V, and D) were described for the voles of Buryatia and Trans-Baikal krai, and one form (C) was described for the voles of the Middle Amur River region [14–16].

In previous molecular genetic studies of Maximowicz's vole [17, 18] from the territory of Middle Amur River region, only ten specimens from the five samples were examined [18], which is clearly insufficient for such a large and geographically challenging territory.

Since there is no unambiguous definition of the Middle Amur River region, in this study, we included in this region the territory from the left bank of Zeya River in the west to the Ussuri River basin in the east,

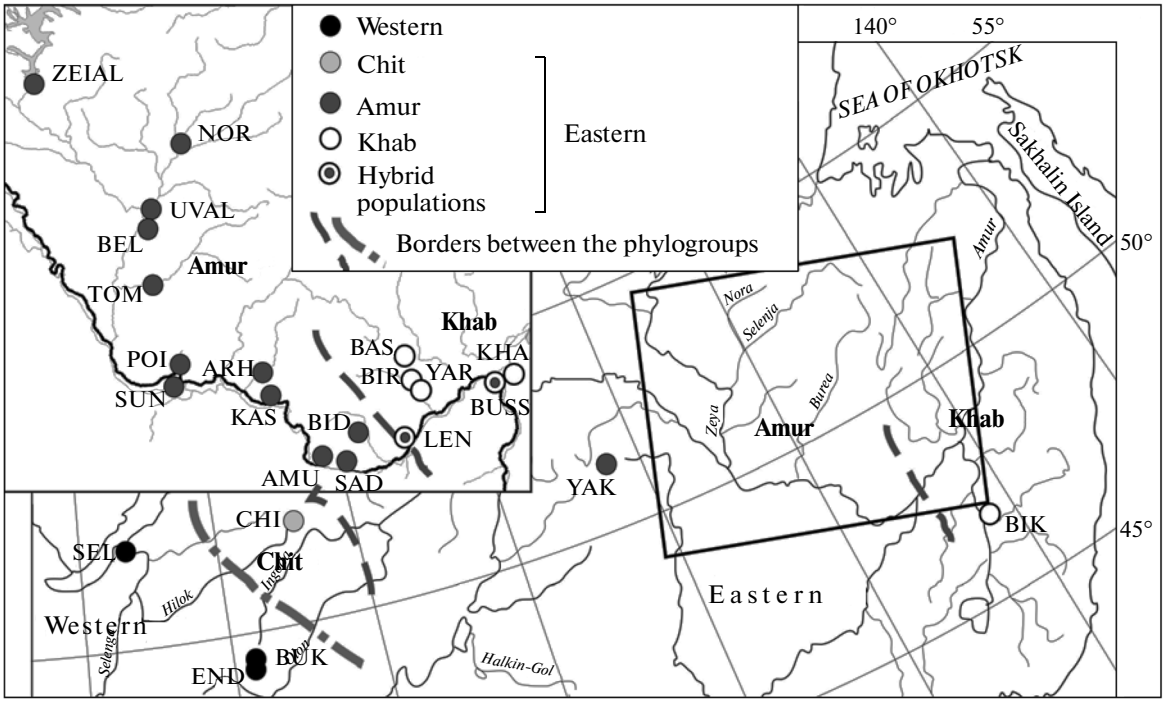

Fig. 1. Sampling localities and phylogenetic structure of Maximowicz's vole *Microtus maximowiczii*; western and eastern phylogenetic groups. Chit, Khab, and Amur are the geographical subgroups in eastern phylogenetic group. The borders between phylogenetic groups and subgroups are shown by the dashed line. See the text for designations of sampling localities.

and we limited it in the north to the southern foothills of the Stanovoy Ridge and Dzhagdy Ridge. This territory is characterized by its location in different geographical regions with different natural conditions. Most of the territory is in a monsoon region of temperate climate, but there are areas with a strong degree of continentality [19]. The mixing of natural environment contributes to the interpenetration of representatives of the various groups of animals and plants into geographical regions uncharacteristic for them. In this regard, the biogeographical value of this region, within which lie the boundaries between the East Siberian, Dauro-Mongolian, Beringian, and Manchurian fauna, is great [20, 21]. It is also a geographically complex region, because its relief is characterized by the presence of a large number of ridges and rivers, which may have an insulating effect on animals. For example, some species have there the edges of their distribution ranges. The range-edge animal populations in this region lead to high species diversity [21, 22]; for example, some related species living in sympatry can be found in this region [23].

The objective of this study was to assess the level of genetic diversity Maximowicz's voles in the Middle Amur River region, as well as to elucidate the peculiarities of the phylogenetic structure of the species in the examined region and to compare the obtained data with the results of karyological studies. For this purpose, the mtDNA control region was analyzed.

MATERIALS AND METHODS

The 69 tissue specimens used in the analysis were obtained from Maximowicz's voles trapped in the region of Middle Amur River basin (Fig. 1):

NOR, Amur oblast, Norsky Nature Reserve, left bank of Nora River ($n = 10$);

ARH, Amur oblast, outskirts of the settlement of Arkha ($n = 3$);

POI, Amur oblast, outskirts of the settlement of Poyarkovo ($n = 2$);

BEL, Amur oblast, outskirts of the settlement of Beloyarovo, left bank of Selemdzha River ($n = 4$);

UVAL, Amur oblast, outskirts of the settlement of Novokievsky Uval, left bank of Selemdzha River ($n = 6$);

TOM, Amur oblast, outskirts of the city of Belogorsk, Tom River ($n = 1$);

ZEIAL, Amur oblast, outskirts of the city of Zeya, left bank of Zeya River ($n = 5$);

SAD, Jewish Autonomous oblast, outskirts of the settlement of Sadovoe ($n = 9$);

AMU, Jewish Autonomous oblast, outskirts of the settlement of Amurzet ($n = 3$);

BAS, Jewish Autonomous oblast, Bastak Nature Reserve ($n = 4$);

LEN, Jewish Autonomous oblast, outskirts of the settlement of Leninskoe ($n = 4$);

BIR, Jewish Autonomous oblast, near the city of Birobidzhan ($n = 4$);

KHA, Khabarovsk krai, outskirts of the settlement of Galkino, right bank of Amur River ($n = 10$);

BIK, Khabarovsk krai, outskirts of the settlement of Orenburgskoe, near the city of Bikin, right bank of Ussuri River ($n = 2$);

SUN, Northeast China, outskirts of the settlement of Hunke ($n = 2$).

The analysis also included 28 sequences of the mtDNA control region of Maximowicz's voles obtained earlier [18] and deposited in the GenBank/NCBI database with the accession numbers HM135863 to HM135873 and HM135875 to HM135890. Of these, ten sequences were obtained from the voles caught in the Middle Amur River basin: NOR—Amur oblast, Norsky Nature Reserve ($n = 5$), HM135863 to HM135867; BIR—Jewish Autonomous oblast, outskirts of the city of Birobidzhan ($n = 1$), HM135869; KAS—Amur oblast, outskirts of the settlement of Kasatkino ($n = 2$), HM135870 and HM135871; YAR—Jewish Autonomous oblast, outskirts of the settlement of Zhelty Yar ($n = 1$), HM135869. The remaining 18 sequences were included in the analysis for comparison and calculation of the genetic characteristics for the species as a whole: SEL—Buryatia, outskirts of the settlement of Istomono, delta of Selenga River ($n = 1$); END—Trans-Baikal krai, Sokhondinsky Nature Reserve, Enda River ($n = 9$); BUK—Trans-Baikal krai, Sokhondinsky Nature Reserve, Bukukun River ($n = 4$); CHI—Trans-Baikal krai, bank of Maly Undugun Lake ($n = 4$). In addition, Maksimowicz's vole mtDNA control region sequences obtained from the GenBank/NCBI database (BUSS—the Chinese part of Bolshoy Ussuriysky Island, formed at the confluence of Ussuri River and Amur River ($n = 11$), accession numbers KJ857292 to KJ857294 and KJ857303 to KJ857310; YAK—outskirts of the settlement of Yakeshi, China ($n = 2$), accession numbers KJ857295 to KJ857296) were examined [24]. A total of 90 specimens of Maximowicz's vole were examined for the Middle Amur River region.

Phylogenetic trees were constructed with sequences of a homologous mtDNA fragment of the reed vole *Microtus fortis* Buchner, 1889 retrieved from the GenBank/NCBI database (accession number, HM135828) as outgroup.

Genomic DNA was isolated from ethanol tissues (liver and muscles) that were either fresh or fixed in 95% by phenol–chloroform extraction [25]. The control region fragment was amplified by polymerase chain reaction (PCR) with forward Pro+ (5'-ACC ATC AGC ACC CAA AGC TG-3') and reverse Phe– (5'-AAG CAT TTT CAG TGC TTT GCT T-3') primers. Amplification was performed on the UNOII–Thermoblock (Biometra, Germany) in 25 μL of the reaction mixture, containing 1 to 2 μg of total DNA; 2.5 μL 10× buffer (SibEnzim, Novosibirsk, Russia); 1 μL of 20 mM dNTP mixture; 0.5 μL of each primer; 3 units of *Taq* polymerase (SibEnzim, Novosibirsk, Russia); and deionized water. The PCR conditions included initial DNA denaturation (94°C for 120 s), followed by 40 cycles of amplification (94°C for 10 s; 52°C for 10 s; 72°C for 60 s) and final extension (72°C for 420 s). Amplification products were subjected to cyclic sequencing with a Big Dye terminator kit v. 3.1 (Applied Biosystems, United States) and forward and reverse primers. The reaction conditions included initial DNA denaturation (96°C for 60 s), followed by 25 cycles of amplification (96°C for 30 s; 50°C for 10 s; 60°C for 240 s). Nucleotide sequences were determined on the automated ABI Prizm 3130 sequencer (Applied Biosystems, United States) at the Institute of Biology and Soil Science, Far Eastern Branch of the Russian Academy of Sciences, (Vladivostok).

The editing and alignment of the obtained sequences were performed with the use of the BioEdit 7.0.9.0 software program [26].

The choice of the model for phylogenetic tree reconstruction and calculation of the genetic distances was performed in the MEGA 5.1 software program [27]. Phylogenetic reconstructions were made with the neighbor-joining (NJ) and maximum likelihood (ML) approaches, and the robustness of the clustering pattern was assessed by bootstrap analysis (1000 replicates). The haplotype network was built with the MP approach as implemented in the Network 4.5.0.0 software program, in which the calculations were made with the use of the median-joining algorithm [28]. The haplotype diversity (h) and nucleotide diversity (π) were calculated in the DnaSP 5.10.01 software program [29].

RESULTS AND DISCUSSION

The complete (940-bp) sequences of the mtDNA control region were obtained for all 69 new Maximowicz's vole specimens. These sequences were deposited in the GenBank/NCBI database with the accession numbers KM403496 to KM403564. The mtDNA control region sequences from 90 Maximowicz's vole specimens from Middle Amur River region contained 148 variable sites, among which 77 were parsimony-informative. The mean nucleotide composition for the voles from Middle Amur River region was T, 32.8%; C, 25.4%; A, 29.2%; G, 12.6%. Altogether, 85 haplotypes were described for the individuals from Middle Amur River region. Five of these haplotypes were found in two individuals, while 80 haplotypes were found to be unique. The haplotype and nucleotide diversity for the species as a whole was high and constituted 99 and 1.55%, respectively. The mean degree of haplotype genetic divergence (p distance) between pairs of individuals for the species constituted 0.0177 ± 0.0022. For Maximowicz's voles from Middle Amur River region, the haplotype and nucleotide diversity was high in all populations. In general, these values for the region were only slightly different from those for the species (99.9 and 1.41%, respectively). The mean level of hap-

Haplotype (h) and nucleotide (π) diversity and the values of selective neutrality tests in Maximowicz's vole samples

Sample identifier (number of specimens)	N	V_s	$h \pm$ S.E.	$\pi \pm$ S.E.	Tajima's D	Fu's F	$D \pm$ S.E.
Amur							
NOR ($n = 15$)	14	17	0.99 ± 0.028	0.0048 ± 0.0008	0.291	**−6.085**	0.0049 ± 0.0013
UVAL + BEL ($n = 10$)	9	22	0.98 ± 0.054	0.0084 ± 0.0011	−0.148	−2.350	0.0084 ± 0.0019
SAD + AMU ($n = 12$)	12	29	1.00 ± 0.032	0.0089 ± 0.0011	−0.792	**−5.909**	0.0089 ± 0.0018
Khab							
LEN ($n = 4$)	4	38	1.00 ± 0.177	0.0212 ± 0.0069	−0.467	1.158	0.0213 ± 0.0034
BUSS ($n = 11$)	10	52	0.98 ± 0.046	0.0134 ± 0.0026	−1.537	−1.760	0.0134 ± 0.0018
BIR + BAS + YAR ($n = 10$)	9	30	0.98 ± 0.055	0.0091 ± 0.0022	−1.119	−2.145	0.0091 ± 0.0018
KHA ($n = 10$)	9	12	0.98 ± 0.054	0.0037 ± 0.0012	−1.266	**−6.259**	0.0037 ± 0.0011
Middle Amur River region as a whole ($n = 90$)	85	148	0.99 ± 0.002	0.0155 ± 0.0007	**−1.773**	**−33.691**	0.0153 ± 0.0019

N, number of haplotypes; V_s, number of variable sites; S.E., standard error. Statistically significant test values are in semibold type.

lotype genetic divergence between pairs of individuals for the voles from Middle Amur River region was slightly lower than for the species as a whole: 0.0141 ± 0.002 (table). In individual samples from the Middle Amur River region, the nucleotide diversity value ranged from 0.0037 to 0.0212. The highest values of nucleotide diversity were found in the samples from the outskirts of the settlement of Leninskoe (LEN) and from Bolshoy Ussuriysky Island (BUSS), 0.0212 ± 0.0069 and 0.0134 ± 0.0026 respectively. In the other samples from this region, the values were more than 1.5 times lower (table).

The ML phylogenetic reconstructions were made with the Hasegawa, Kishino, and Yano model with G distribution (BIC = 8847.319). All haplotypes were divided into two phylogroups. The western phylogroup consisted of voles from the outskirts of the settlement of Istomino (Buryatia) and individuals from Sokhondinsky Nature Reserve. The eastern phylogroup contained individuals from Trans-Baikal krai, the bank of Malyi Undugun Lake, and from the Middle Amur River region (Figs. 1 and 2). The distance between these phylogroups constituted 0.0282 ± 0.0043. In the eastern phylogroup, three geographical subgroups can be distinguished. The first subgroup is represented by individuals from the outskirts of the city of Chita (Chit), and the second and third subgroups can be represented by voles from the Middle Amur River region (Figs. 1 and 2). The second subgroup (Khab) contains voles from the samples from the Middle Amur River region below the confluence of the Amur River and Bira River (BAS, YAR, BIR, KHA, BIK); three of the four voles from the outskirts of the settlement of Leninskoe (LEN), which is located on Amur River, between Bidzhan and Bira rivers, as well as ten of the 11 voles from Bolshoy Ussuriysky Island (BUSS), are also present in this subgroup. The third subgroup (Amur) contains the voles caught in the Middle Amur River region above the confluence of Amur and Bidzhan rivers, as well as one individual from Bolshoy Ussuriysky Island and one vole caught near the settlement of Leninskoe.

The haplotype network (Fig. 3) also shows the presence of two well-differentiated phylogroups, western and eastern, that are spaced at 17 mutational steps. The eastern phylogroup is also divided into three subgroups. In the network, subgroup Chit has the intermediate position between the Amur and Khab subgroups. Specimens from the outskirts of the settlement of Leninskoe and Bolshoy Ussuriysky Island, similarly to their position in phylogenetic trees, do not cluster together and are located in different subclusters. The distances between the subgroups were Chit/Amur, 0.0151 ± 0.0028; Chit/Khab, 0.0149 ± 0.0032; Amur/Khab, 0.0186 ± 0.0031.

The pairwise differences between the haplotypes within different samples from the Middle Amur River region ranged from 2.64 to 20.8 positions. Minimal differences were found in the sample from Norsky Nature Reserve. The between-haplotype differences in the samples from the outskirts of the settlement of Sadovoe (JAO) and of the settlement of Galkino (Khabarovsk krai) constituted 9.06 and 3.49 positions, respectively. The differences between the haplotypes in the sample from Bolshoy Ussuriysky Island constituted 11.98, and those in the sample from the outskirts

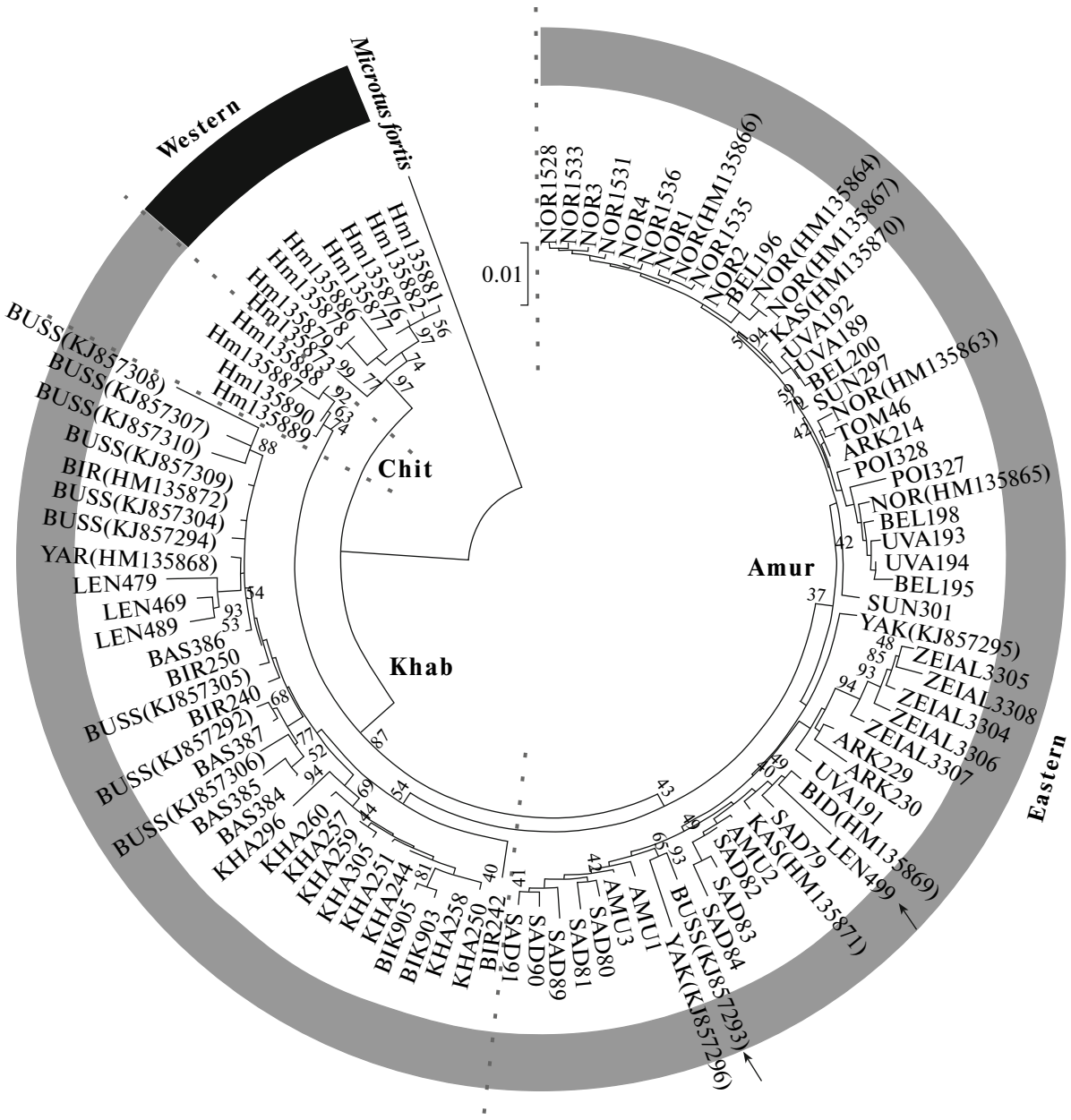

Fig. 2. Dendrogram of phylogenetic relationships of the mtDNA control region haplotypes of Maximowicz's vole *Microtus maximowiczii*: western and eastern phylogenetic groups. Chit, Khab, and Amur are the geographical subgroups in eastern phylogenetic group.

of the settlement of Leninskoe were more than two times greater than those in other samples (20.8). The average number of nucleotide differences between the individual samples ranged from one (between the samples from Sadovoe and Leninskoe, the geographically closest samples) to seven (between samples from Norsky Nature Reserve and the settlement of Galkino in Khabarovsk krai, the geographically most remote samples).

Comparison of the data on the phylogenetic structure with the results of earlier karyological studies showed that the western phylogroup matched with chromosomal forms B and V and the eastern phylogroup matched with chromosomal forms A and C. Differences between the haplotypes of individuals belonging to chromosomal forms B and V were revealed by 12 substitution mutations, and differences between chromosomal forms A and C were revealed by eight substitutions. The differences between the haplotypes of the individuals from different phylogenetic subgroups, Amur and Khab, which belong to the same chromosomal form C, constituted 12 substitutions, which is 1.5 times higher than those between the individuals of chromosomal forms A and C.

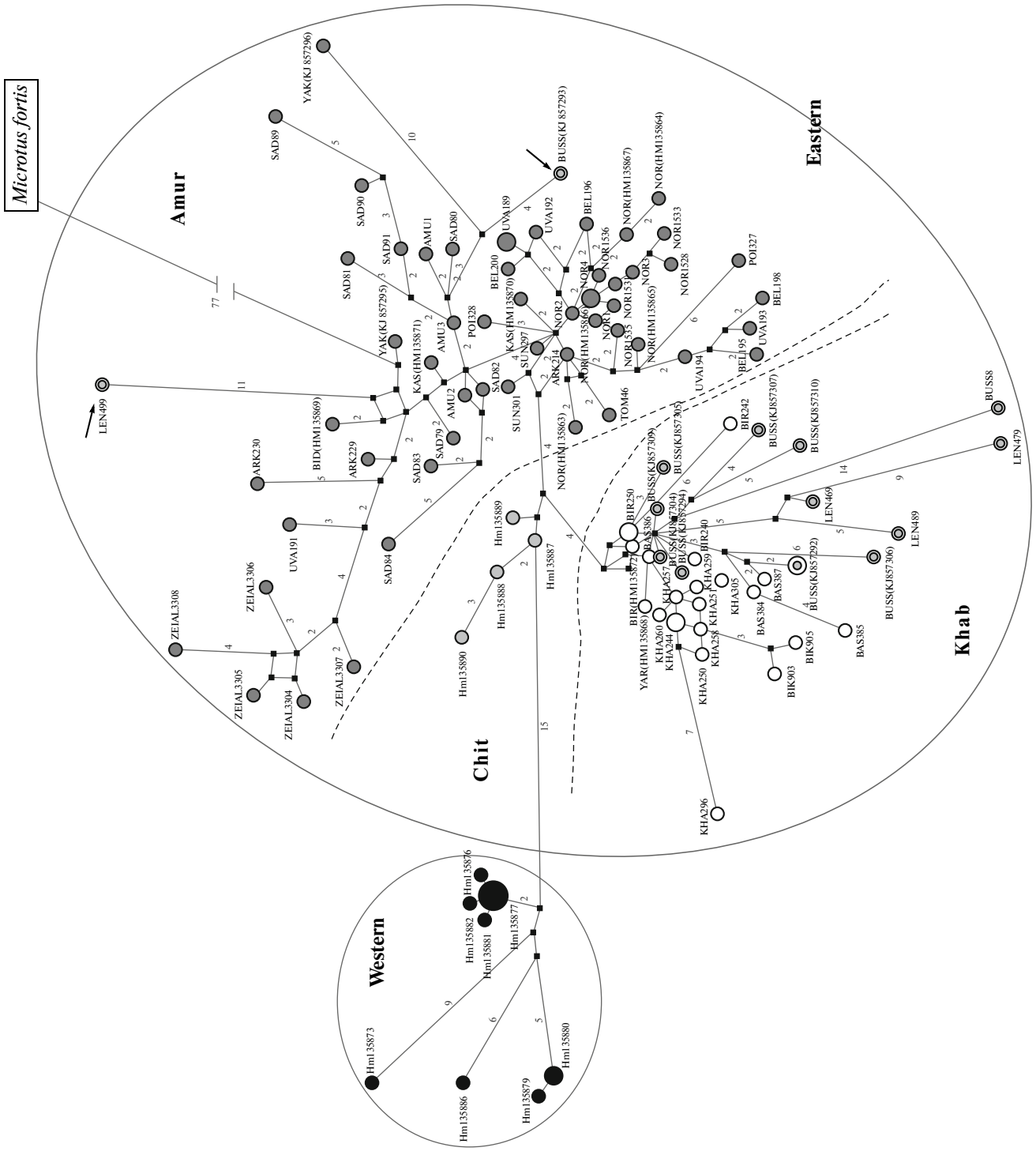

Fig. 3. Phylogenetic network of mtDNA haplotypes (390 bp) of *Microtus maximowiczii* built in the Network 4.5.0.0 software program. The circle sizes are proportional to the number of specimens with corresponding haplotype; figures show the number of nucleotide substitutions.

The species as a whole demonstrated a high level of differentiation ($F_{st} = 0.624$) with the higher proportion of variation falling on the interpopulation component. Pairwise comparison of the individual samples of Maximowicz's vole from Middle Amur River region produced $F_{st} = 0.499$ and $N_m = 0.25$. The minimum F_{st} value was observed between the sample from Norsky Nature Reserve and the samples from the outskirts of the settlements of Beloyarovo and Novokievsky Uval (0.141), which are located relatively close to each

other on the left bank of Selemzha River in Amur oblast, while the maximum value of this index was found between the most distant samples, in those from Norsky Nature Reserve in Amur oblast and the outskirts of the settlement of Galkino in Khabarovsk krai (0.748).

The mismatch distribution between all haplotype pairs in the samples from Middle Amur River region was unimodal, which, along with the highly statistically significant negative value of the Tajima's test of selective neutrality, points to an increase in the population size of this group (table). The Fu's *F* values were also negative and statistically significant, indicating the quick colonization of the territory by the voles. The values of selective neutrality tests for individual samples were negative, except for the sample from Norsky Nature Reserve.

Thus, analysis of the mitochondrial DNA control region in Maximowicz's voles from Middle Amur River region revealed a high level of genetic variability. Each individual sample was characterized by a nucleotide diversity value lower than that for the species as a whole. The exception was the sample of voles from Bolshoy Ussuriysky Island, where the nucleotide diversity value was slightly lower than the species value. In addition, the sample of voles from the outskirts of the settlement of Leninskoe, despite its small size, was characterized by a nucleotide diversity value that was even higher than that for the species as a whole. It seems likely that the reduction of nucleotide diversity in some samples can be explained by the biology of the species, which is characterized by deep population size depressions during which the species is preserved in small individual colonies rather distant from each other. These depressions can/could result in a process leading to a decrease of nucleotide diversity.

The increased nucleotide diversity within the sample from the outskirts of the settlement of Leninskoe can be explained by two hypotheses. The first hypothesis suggests that this sample is the geographically closest to the center of the population with high diversity. The second hypothesis is that this sample consists of individuals from two or more differentiated populations. In our opinion, the second hypothesis is the most plausible, since the individuals from the sample from the outskirts of the settlement of Leninskoe never (even in haplotype network) clustered together. In addition, the detection of the vole with the Amur haplotype in the sample from Bolshoy Ussuriysky Island also supports the hypothesis that this area is a contact zone of two genetically different populations that correspond to the Amur and Khab phylogenetic subgroups. The dispersal of Maximowicz's vole individuals carrying haplotypes Amur subgroup probably occurs along the Amur River, with its flow.

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