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GENERAL BIOLOGY

Within- and Between-Population Polymorphism of the mtDNA Control Region of the Speckled Ground Squirrel (*Spermophilus suslicus*)

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Fragmentation of distribution ranges and their reduction frequently influence the genetic structure of wild animal populations interfering with gene exchange between different parts of the distribution area, decreasing effective population size, and elevating the level of inbreeding [1]. In small isolated populations, gene drift can decrease genetic diversity, thereby limiting the adaptation to environmental changes and increasing the risk for elimination of local populations [2–4].

Ground squirrels are a convenient model for studying how the fragmentation of distribution range influences the genetic structure of populations. It has been shown for different species and regions that transformation of habitats and their fragmentation caused by human activities limit the gene flow between isolated populations and reduce genetic diversity within populations, which elevates the risk of their elimination and demands special efforts for their preservation [2, 3, 5]. Reintroduction and exchange of individuals between isolated populations may help to maintain the genetic diversity of populations and their stability; however, this requires knowledge of their genetic structure, origin, and the degree of within- and between-population polymorphisms [2, 3, 5].

The speckled ground squirrel (*Spermophilus suslicus*) is a vulnerable species living in the steppe and forest–steppe zones of Russia, Ukraine, Moldova, and partially in Poland and Belarus. The ground squirrels from the northeastern part of the distribution range have a diploid chromosome set with $2n = 34$ and NF = 68 versus $2n = 36$ and NF = 72 in the southwestern part [6]. Although several researchers regard the 36-chromosome form as a separate species [7], we adhere in this study to the traditional systematics [8, 9]. In the past decades, the ground squirrel colonies have reduced in size, and the species has disappeared in some localities; only highly isolated small colonies are preserved [10]. This species is included into the list of endangered species in the Moscow, Nizhni Novgorod, Bryansk, Penza, and some other regions and the Republic of Tatarstan in Russia, as well as in Moldova. In Ukraine, both forms of the speckled ground squirrel were added to the third edition of endangered species list (2009), the 34-chromosome form being regarded as a Endangered species and the 36-chromosome form, as Not Evaluated. In Belarus and Poland, this species has also been protected since 2006 and 1984, respectively. In 1996–2008, this species had the status of a Vulnerable species in the IUCN Red List; however, its status was later unjustifiably decreased to Near Threatened.

Our earlier bioacoustic analysis of the populations living in Moscow and Lipetsk regions revealed significant between-population differences in the alarm calls, which suggested the presence of considerable genetic differences between these populations [11]. This assumption has been confirmed by the data on polymorphism in the left domain of mtDNA control region (310 bp) of the speckled ground squirrel, namely, a considerable level of differences between 20 populations living in different parts of the species distribution range [12]. It has been found that the phylogeographic structure in the western part of the distribution range is more pronounced as compared with

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Table 1. Characteristics of speckled ground squirrel populations (N, the number of assayed animals in population; N_{hapl}, number of found haplotypes; N_{uniq}, number of unique haplotypes; π, nucleotide diversity; and *S.E.*, standard error)

Population	N	N_{hapl}	N_{uniq}	π	(*S.E.*)
Zaraisk	10	1	0	0.000	(0.000)
Lipetsk	10	1	0	0.000	(0.000)
Michurinsk	10	3	2	0.001	(0.000)
Novosel'skoe	10	5	2	0.005	(0.001)
Ozernoe	10	4	2	0.009	(0.002)

the eastern part. All the examined individuals had unique haplotypes; however, only a single individual from each colony (except for four colonies, where two–three individuals were assayed) was analyzed, preventing estimation of within-population variation and its comparison with between-population polymorphism. In addition, only a fragment of the mtDNA control region was analyzed.

The goal of this study was to assess the within- and between-population polymorphisms of the complete mtDNA control region for five speckled ground squirrel populations of Central Russia and Ukraine.

MATERIALS AND METHODS

Sampling was performed in the April–August of 2005–2013 during aboveground activity of ground squirrels. The objects were 50 individually marked adult speckled ground squirrels, ten individuals from five isolated wild populations. The individuals were selected from the amount of available data so that the chance of including close relatives would be minimal.

(1) Zaraisk (54°47′68″ N, 38°42′23″ E) population: a community on an open dry meadow with a high grass stand near the village Velikoe pole, Zaraisk district, Moscow region.

(2) Lipetsk (52°36′28″ N, 39°26′38″ E) population: a community on a municipal cemetery near the village Kosyrevka. The cemetery occupies former farmlands (since 1980), currently being a complex anthropogenic landscape with prevalence of introduced plant species.

(3) Michurinsk (52°51′44″ N, 40°47′01″ E) population: this is the last preserved fragment of an earlier successful colony in the periphery of the village Dmitrievka, Michurinsk district, Tambov region, adjacent to federal highway M-6.

(4) Novosel'skoe (45°20′44″ N, 28°36′33″ E) population: a community near the village Novosel'skoe, Reni district, Odessa region, on a low bank of Lake Yalpug with prevalence of overgrazed pasture–ruderal plants at a moderate grazing pressure.

(5) Ozernoe (45°25′92″ N, 28°40′04″ E) population: a colony near the village Ozernoe, Reni district, Odessa region, on a high dry bank of the Yalpug Lake with overgrazed grass stand. The two last colonies were located at a distance of 12 km from one another and separated by two large meridionally stretched lakes, which may be regarded as a geographic barrier for ground squirrels.

The first three populations belong to the eastern part of the speckled ground squirrel distribution range and the last two, to its western part.

Animals were captured with live traps, labeled with permanent marks, assayed for sex and age, and released. The trapping point was determined with a GPS navigator. The geographic distances between populations were calculated as linear distances (km) based on GPS coordinates using MapSource.

DNA was isolated from soft tissue specimens (finger pad sections) fixed with 96% ethanol by phenol–chloroform extraction or a Kingfisher® Flex (Thermo Scientific, USA) robot for DNA extraction and Magna DNA Prep (Izogen, Russia) kit according to the manufacturers' protocols.

The complete control region flanked by a fragment of the tRNA–Pro gene and the tRNA–Phe gene (total length, 1148 bp) was amplified using the primers MDL1 and H00651 [13]. Polymerase chain reaction (PCR) was conducted in a volume of 25 mL using the reagents from Dialat (Russia); the reaction mixture contained 5 pM of each primer, 0.1–0.2 μg of DNA, and ddH_2O to the final volume. The PCR comprised 30 cycles of 1 min at 94°C, 1 min at 62°C, and 3 min at 72°C. The PCR products were separated in 1.5% agarose gel stained with ethidium bromide, visualized in UV light, cut off, and purified using a MinElute Gel Extraction kit (Qiagen, Germany). Sequencing was performed in an ABI 3730 automated genetic analyzer using a BidDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and each of the pair of internal primers (designed by V.L. Surin), namely, MDL2D (5'-CCAAATGACTATCCCCTACC-3') and MDL3R (5'-GACTAATAAGTCCAGCTACA-3').

The resulting nucleotide sequences were aligned with the help of SeqMan (Lasergene, USA) and BioEdit [14], as well as manually. The MEGA 5 software [15] was used for statistical data processing and construction of phylogenetic tree. The percentage of between-population differences was determined according to the number of nucleotide substitutions in the aligned sequences. The within- and between-group genetic differences (K_2P distances) were estimated according to the Kimura two-parameter model. The phylogenetic tree was constructed using unweighted pair group method average (UPGMA). Node support values in phylogenetic tree was estimated according to bootstrap support (1000 replicates). The nucleotide sequence of

	12	15	19	29	58	59	61	88	92	107	109	154	157	158	160	161	219	221	222	223	231	235	236	237	238	243	247	255	264	265	266	268	272
Z1	T	C	A	T	G	T	T	T	A	T	C	G	C	G	C	T	G	T	T	G	G	C	C	T	G	T	G	C	G	C	T	A	C
L1	.	.	.	.	.	.	.	C	.	.	.	.	T	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	T	.	.	.
M1	.	.	.	C	.	.	.	C	.	.	.	.	T	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.
M2	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.
M3	.	T	.	C	.	.	.	C	.	.	.	.	T	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.
N1	.	.	T	.	.	C	C	C	C	.	T	.	T	A	.	.	.	.	C	.	A	T	T	C	C	.	.	.	A	.	C	T	.
N2	.	.	T	.	A	C	C	C	C	C	T	.	T	A	.	C	A	.	.	A	A	T	T	C	T	C	A	.	A	.	C	T	.
N3	.	.	T	.	A	C	C	C	C	C	T	.	T	A	.	C	A	.	.	A	A	T	T	C	T	C	A	.	A	T	C	T	.
N4	.	.	T	.	A	C	C	C	C	C	T	.	T	A	.	C	A	.	.	A	A	T	T	C	T	C	A	.	A	.	C	T	.
N5	C	.	T	.	A	C	C	C	C	C	T	.	T	A	.	C	A	.	.	A	A	T	T	C	T	C	A	.	A	.	C	T	.
O1	.	.	T	.	.	C	C	C	C	.	T	.	T	A	G	.	.	.	C	A	A	T	T	C	C	.	.	A	A	.	C	T	.
O2	.	.	T	.	.	C	C	C	C	.	.	A	T	A	.	.	A	.	C	.	A	T	T	C	C	.	.	.	A	.	C	T	T
O3	.	.	T	.	.	C	C	C	C	.	T	A	T	A	.	.	A	.	C	.	A	T	T	C	C	.	.	.	A	.	C	T	T
O4	.	.	T	.	A	C	C	C	C	C	T	.	T	A	.	C	.	C	.	.	A	T	T	C	T	C	A	.	A	T	C	T	.

	275	276	290	337	361	411	460	501	502	626	689	703	712	755	784	816	823	844	840	848	845	857	881	895	896	900	908	911	912	920	978	986	998	999
Z1	T	C	C	G	C	A	A	A	T	A	A	T	C	C	A	C	T	T	C	T	A	C	G	–	C	A	T	T	T	T	C	–	–
L1	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	–	.	.	.	.	.	.	.	–	–
M1	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	–	.	.	.	.	.	.	.	–	–
M2	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	–	.	.	.	.	.	.	.	–	–
M3	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	–	.	.	.	.	.	.	.	–	–
N1	A	A	T	A	T	T	G	G	C	.	G	C	A	T	C	T	.	–	A	C	T	T	.	A	.	T	C	C	C	C	T	T	G
N2	A	A	T	A	T	C	G	G	.	.	G	C	A	T	C	T	.	.	A	C	T	T	.	A	T	T	C	C	C	C	T	T	G
N3	A	A	T	A	T	C	G	G	.	.	G	C	A	T	C	T	.	.	A	C	T	T	.	A	.	T	C	C	C	C	T	T	G
N4	A	A	T	A	T	C	G	G	.	.	G	C	A	T	C	T	.	–	A	C	T	T	A	A	T	T	C	C	C	C	T	T	G
N5	A	A	T	A	T	C	G	G	.	.	G	C	A	T	C	T	.	–	A	C	T	T	.	A	T	T	C	C	C	C	T	T	G
O1	A	A	T	A	T	T	G	G	C	.	.	C	A	T	C	T	.	–	A	C	T	T	.	A	.	T	C	C	C	C	T	T	G
O2	A	A	T	A	T	T	.	G	.	.	G	C	A	T	C	T	–	–	A	C	T	T	.	A	.	T	C	C	C	C	T	T	G
O3	A	A	T	A	T	T	.	G	C	.	G	C	A	T	C	T	.	–	A	C	T	T	.	A	.	T	C	C	C	C	T	T	G
O4	A	A	T	A	T	T	G	G	.	G	G	C	A	T	C	T	.	–	A	C	T	T	.	A	.	T	C	C	C	C	T	T	G

Fig. 1. Variation in the nucleotide sequence of mtDNA control region (999–1001 bp) of the speckled ground squirrel. Only variable positions are shown; numbering corresponds to the complete structures aligned relative to haplotype Z1; dots denote identical nucleotides and dashes, deletions.

homologous mtDNA fragment of the Perote ground squirrel (*Xerospermophilus perotensis*) (NCBI acc. no. JQ326958.1 [5]) was used as an outgroup.

RESULTS AND DISCUSSION

We have determined the nucleotide sequence of the complete mtDNA control region (999–1001 bp) in 50 speckled ground squirrel individuals belonging to five populations (Table 1, Fig. 1). Totally, 61 sites (6% of the total fragment length) have proved to be variable and 56 of them, phylogenetically significant. Nucleotide diversity (π) varied from zero to 0.009 in different populations, amounting to 0.024 (*S.E.* = 0.003) in the pooled sample set (Table 1). The mean nucleotide contents were 31% for A, 34% for T, 12% for G, and 23% for C. The ratio of transitions to transversions was 3 : 1; 14 haplotypes were identified among the examined individuals (Table 2, Fig. 1). The same haplotype was never met beyond the population where it was observed.

The complete sample set of ground squirrels fell into two large groups, comprising three eastern (Zaraisk, Lipetsk, and Michurinsk) and two western (Novosel'skoe and Ozernoe) populations, respectively (Fig. 2). The eastern populations were weakly structured: the genetic distances between individuals within the Zaraisk and Lipetsk populations were zero, while only single nucleotide substitutions were observed in the Michurinsk population (Table 1, Fig. 2). The within-population variation of the western Novosel'skoe and Ozernoe populations was considerably higher compared with the eastern ones; different phyletic lineages were present there. The between-population distances were larger than the within-population ones, including the distances for the adjacent

Table 2. Examined speckled ground squirrel individuals and the found haplotypes of mtDNA control region (Z, Zaraisk population; L, Lipetsk; M, Michurinsk; N, Novosel'skoe; O, Ozernoe; m, male; and f, female; unique haplotypes are boldfaced)

Specimen	Sex	Haplotype	NCBI acc. no.	Specimen	Sex	Haplotype	NCBI acc. no.
Z147	m	Z1	KF934335	M18	m	M1	KF934360
Z153	m	Z1	KF934336	M19	m	M1	KF934361
Z381	m	Z1	KF934337	M21	m	M1	KF934362
Z412	f	Z1	KF934338	M22	f	M1	KF934363
Z4057	f	Z1	KF934339	M23	f	M1	KF934364
Z4064	m	Z1	KF934340	N14	m	N1	KF934365
Z4066	m	Z1	KF934341	N16	f	N2	KF934366
Z4070	f	Z1	KF934342	N27	f	N1	KF934367
Z4143	m	Z1	KF934343	N48	f	N3	KF934368
Z4144	f	Z1	KF934344	N52	m	N3	KF934369
L34	f	L1	KF934345	N56	m	N2	KF934370
L35	f	L1	KF934346	N211	f	N2	KF934371
L36	m	L1	KF934347	N213	m	N3	KF934372
L37	m	L1	KF934348	N222	m	**N4**	KF934373
L38	m	L1	KF934349	N226	m	**N5**	KF934374
L39	f	L1	KF934350	O101	f	**O1**	KF934375
L46	m	L1	KF934351	O103	f	O2	KF934376
L47	m	L1	KF934352	O105	m	**O3**	KF934377
L48	f	L1	KF934353	O108	m	O4	KF934378
L49	m	L1	KF934354	O109	f	O4	KF934379
M11	m	M1	KF934355	O111	f	O4	KF934380
M13	f	M1	KF934356	O112	f	O4	KF934381
M15	m	**M2**	KF934357	O113	f	O2	KF934382
M16	f	**M3**	KF934358	O114	f	O4	KF934383
M17	f	M1	KF934359	O201	m	O4	KF934384

Table 3. Matrix of the genetic and geographic distances between the examined speckled ground squirrel populations

Population	Zaraisk	Lipetsk	Michurinsk	Novosel'skoe	Ozernoe
Zaraisk		0.005	0.005	0.049	0.048
Lipetsk	249		0.002	0.045	0.043
Michurinsk	255	95		0.046	0.045
Novosel'skoe	1274	1130	1217		0.010
Ozernoe	1262	1119	1206	12	

Nucleotide distances between populations (K_2P distance) are shown above diagonal and geographic distances between populations (km) are shown below.

highly variable Novosel'skoe and Ozernoe populations (Tables 2 and 3). The genetic distance between all eastern and all western populations was 4.4%.

The genetic distances between geographically remote populations have proved to be considerably larger compared with the isolated populations of the same or adjacent regions (Table 3). The nucleotide differences accumulate gradually with an increase in the distance between populations due to gene drift. This effect is enhanced by fragmentation of the distribution range and the presence of ecological or geographical barriers, as suggested by considerable between-population differences of adjacent but isolated populations.

Analysis of the variation in the complete mtDNA control region suggests that the ground squirrels from the east of the distribution range display considerably

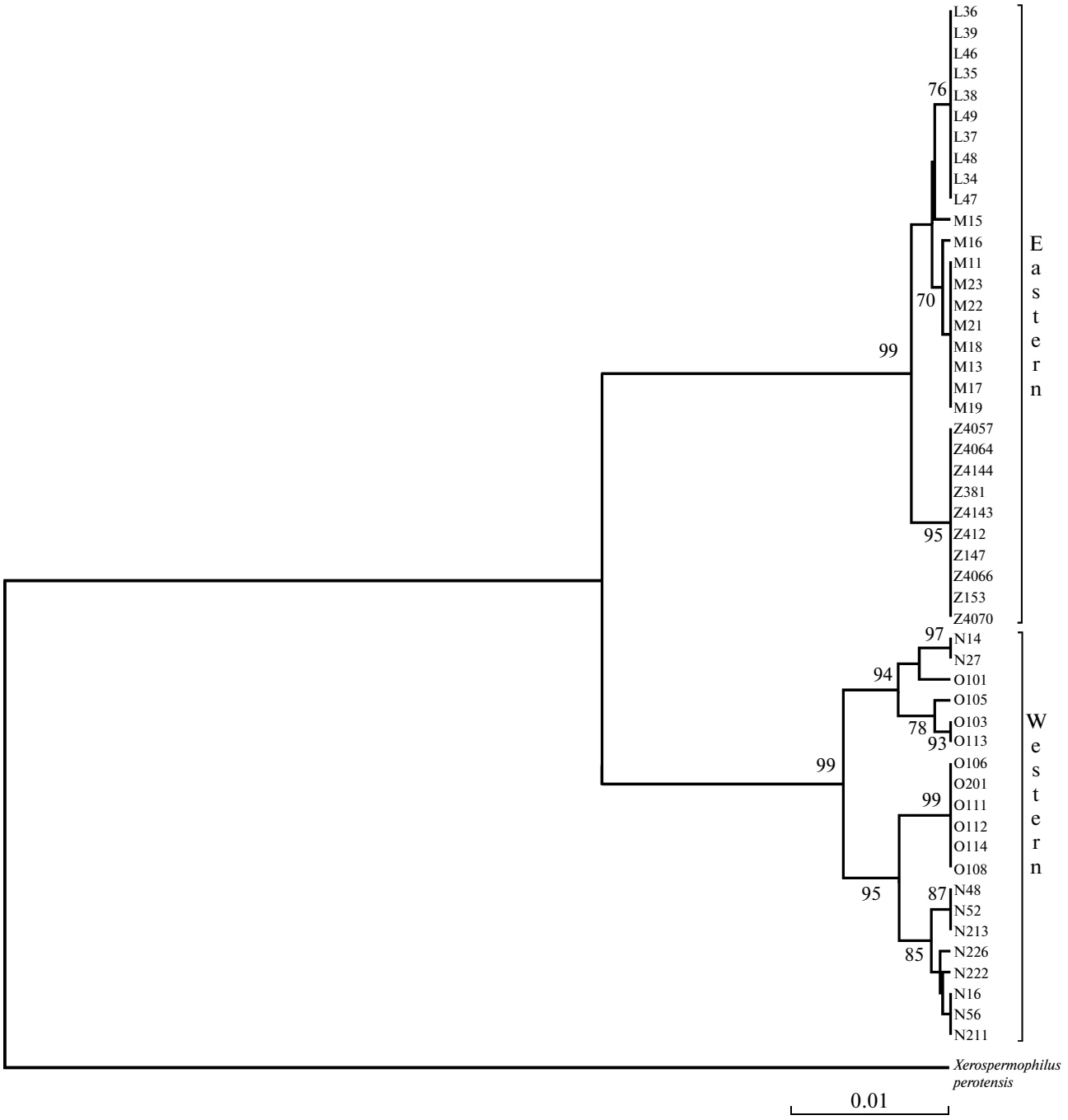

Fig. 2. Phylogenetic tree (UPGMA) for the haplotypes of mtDNA control region of the speckled ground squirrel. Bootstrap support for 1000 replicates is shown near branches only for the values exceeding 70%; sample numbers correspond to those listed in Table 2.

lower genetic diversity as compared with the western populations of this species. Thus, our data agree with a recent estimation of the polymorphism in the left domain of speckled ground squirrel mtDNA control region from different parts of the distribution range [12].

The pooled genetic diversity of the eastern populations studied suggests that they are less successful as compared with the western populations. The former are considerably fragmented and intensely stressed by anthropogenic factors. The examined western populations from Ukrainian Bessarabia are more successful, on the one hand, in genetic diversity and, on the other, in the population size and area of colonies.

Molecular genetic methods are widely used for solving various problems in nature conservation, including the search for the populations suitable for

animal reintroduction. A donor population should be maximally close genetically to the restored one.

It is evident that only integrated analysis of both genomic (nuclear and mitochondrial DNAs) and phenotypic (including acoustic) markers can provide more comprehensive information. It is necessary to increase the number of examined populations and to use more variable genomic markers, such as microsatellites from the nuclear DNA.

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