Mammalian Biology 81 (2016) 255–265

Contents lists available at ScienceDirect

Mammalian Biology

journal homepage: www.elsevier.com/locate/mambio

Original Investigation

Genetic and alarm call diversity across scattered populations of speckled ground squirrels (*Spermophilus suslicus*)

Vera A. Matrosova[a,*], Mikhail Yu. Rusin[b], Elena V. Volodina[c], Svetlana V. Proyavka[d], Ludmila E. Savinetskaya[e], Olga N. Shekarova[e], Hanna V. Rashevska[b], Ilya A. Volodin[c,f]

[a] *Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow 119991, Russia*
[b] *Schmalhausen Institute of Zoology, National Academy of Sciences of Ukraine, Kiev 01601, Ukraine*
[c] *Moscow Zoo, Moscow 123242, Russia*
[d] *Lipetsk State Pedagogical University, Lipetsk 398020, Russia*
[e] *Severtsov Institute of Ecology and Evolution, Russian Academy of Sciences, Moscow 119071, Russia*
[f] *Lomonosov Moscow State University, Moscow 119991, Russia*

ARTICLE INFO

Article history:
Received 16 September 2015
Accepted 15 January 2016
Handled by Frank E. Zachos
Available online 1 February 2016

Keywords:
Alarm call
Control region
Mitochondrial DNA
Spermophilus suslicus
Vocal communication

ABSTRACT

Alarm calls of ground squirrels are innate signals, showing substantial geographical variation across populations without the masking effects of sex and age- related variation. This makes them a convenient model for studying population genetic effects on the evolution of alarm communication. We compared data on the alarm call structure and the mitochondrial DNA (mtDNA) complete control region (C-region) (998–1002 bp) polymorphism in the same 90 individual speckled ground squirrels (*Spermophilus suslicus*) across 6 populations (15 individuals per population), separated by distances from 12 to 1274 km. We calculated acoustic distances between each pair of populations based on acoustic variables of alarm calls, averaged for each individual using Euclidean distances of population centroids, in the space of canonical axes of discriminant function analysis. Genetic distances ranged of 0–1.1% within populations and of 0.5–4.9% between populations. Prominent differences were found between eastern and western populations separated by the Dnieper River. Both genetic and acoustic distances showed a significant positive correlation with geographical among populations. Positive correlation between acoustic and genetic distances did not reach significance. These results support effects of ecological selection on the alarm call variables rather than the genetic drift hypothesis. In addition, these results support the current taxonomic separation between subspecies of speckled ground squirrels differing in diploid chromosome sets $2n = 34$ (*Spermophilus suslicus guttatus*) and $2n = 36$ (*Spermophilus suslicus odessanus*).

Introduction

Relations between geographical variation in vocal and genetic data attract a wide research interest focused on the evolution of acoustic communication in rodents (mole-rats: Nevo et al., 1987; singing mice: Campbell et al., 2010; grounds squirrels: Eiler and Banack, 2004; Schneiderová and Policht, 2012a,b; domestic mice: von Merten et al., 2014). In general, acoustic divergence may be predicted by different selection regimes: ecological selection, sexual selection, a combination of both and by neutral evolution, i.e., genetic drift (Wilkins et al., 2013). Particular selection regimes do not specifically relate to particular call functions, e.g., acoustic divergence of male courtship calls are not necessarily related to sexual selection and may be explained by genetic drift (Campbell et al., 2010).

Alarm calls of ground squirrels are strongly subject to geographical variations within and between species, which makes them excellent vocal indicators of species (Matrosova et al., 2012b; Schneiderová and Policht, 2012a,b), subspecies (Eiler and Banack, 2004; Nikol'skii et al., 2007), and even populations (Eiler and Banack, 2004; Nikol'skii et al., 2007; Matrosova et al., 2012a). These differences are expected to be genetically predetermined, as interspecies hybridization in ground squirrels results in intermediate acoustic characteristics between parental patterns of alarm calls (Koeppl et al., 1978; Nikol'skii et al., 1984; Titov et al., 2005). In addition, the vocal learning in ground squirrels could not be proved even by direct cross-foster interspecies parenting experiments (Matocha, 1975). The speckled ground squirrel has been

---

[*] Corresponding author.
E-mail address: v.matrosova@gmail.com (V.A. Matrosova).

http://dx.doi.org/10.1016/j.mambio.2016.01.001
1616-5047/

Table 1
Characteristics of 6 study populations.

Population	Location	Habitat	Year
Zaraysk (ZAR)	54°47′68″N 38°42′23″E	An open dry floodable meadow near the village Velikoe pole, Zaraysk district, Moscow region, Russia	2005–2009
Lipetsk (LIP)	52°36′28″N 39°26′38″E	A municipal cemetery established in 1980 in place of farmlands near the village Kosyrevka, Lipetsk region, Russia	2010
Michurinsk (MIC)	52°51′44″N 40°47′01″E	Remains of a formerly successful colony along the M-6 federal highway near the village Dmitrievka, Michurinsk district, Tambov region, Russia	2013
Novosel'skoe (NOV)	45°20′44″N 28°36′33″E	A pasture with moderate grazing pressure at the low bank of Yalpug Lake near the village Novosel'skoe, Reni district, Odessa region, Ukraine	2013
Ozernoe (OZE)	45°25′92″N 28°40′04″E	A pasture with overgrazed grass stand at the high bank of the Yalpug Lake near the village Ozernoe, Izmail district, Odessa region, Ukraine	2013
Odessa (SOV)	46°20′66″N 30°40′31″E	A waste ground along tram ways in Sovinyon suburb of the city Odessa, Odessa region, Ukraine	2014

previously studied separately for genetic variation among five populations (Matrosova et al., 2014) and for acoustic variation in two populations (Matrosova et al., 2012a). However, the relationships between acoustic and genetic characteristics have not been studied to date in any ground squirrel.

The speckled ground squirrel *Spermophilus suslicus* (Güldenstädt, 1770) is a small-sized (body mass 180–220 g, body length without tail 190–220 mm), diurnal, herbivorous, obligate hibernating sciurid (Lobkov, 1999), which was formerly continuously distributed over the steppes and forest-steppes of Russia, Ukraine, Moldova, Belarus and locally in Poland (Wilson and Reeder, 2005; Helgen et al., 2009). However, the current distribution area is highly fragmented into separate colonies due to human activities (Nedosekin, 2007; Shekarova et al., 2008; Matrosova et al., 2012a). Three to five subspecies have been previously recognized in this species (Wilson and Reeder, 2005; Helgen et al., 2009). The species range is subdivided by the insurmountable geographical barrier of the Dnieper River into the eastern and western parts. Eastern speckled ground squirrels *Spermophilus suslicus guttatus* (Pallas, 1770) have a diploid chromosome set $2n = 34$, NF = 68, whereas the western speckled ground squirrels *Spermophilus suslicus odessanus* (Nordmann, 1840) have $2n = 36$, NF = 72 (Denisov et al., 1969; Lyapunova and Vorontsov, 1970). Recent mtDNA barcoding study confirmed the noticeable level of genetic differences between the eastern and western ground squirrels (Ermakov et al., 2015).

Alarm calls of speckled ground squirrels consist of weakly modulated tonal notes about 200 ms in duration, with fundamental frequencies ranging from 8 to 10 kHz (Fig. 1), that are typically produced in series with intervals substantially longer than the duration of the notes themselves (Nikol'skii, 1979; Nikol'skii et al., 1984; Volodin, 2005). Very rarely can the alarm calls be complicated with an ultrasonic component (Matrosova et al., 2012b). All previous studies of the acoustic variation of alarm calls in speckled ground squirrels have been conducted on colonies from the eastern part of the distribution area (Nikol'skii, 1979; Nikol'skii et al., 1984; Volodin, 2005; Matrosova et al., 2007, 2009, 2011, 2012a,b). At the same time, the acoustic variation of alarm calls in the western ground squirrels has not been described to date.

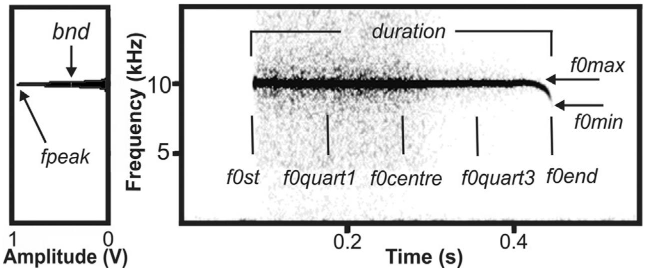

Fig. 1. Measured variables of the alarm call. Left: the mean power spectrum; right: spectrogram. *f0max* – the maximum fundamental frequency; *f0min* – the minimum fundamental frequency; *f0st* – the fundamental frequency at the beginning; *f0quart1* – the fundamental frequency at the 1st quarter; *f0centre* – the fundamental frequency in the middle; *f0quart3* – the fundamental frequency at the 3rd quarter; *f0end* – the fundamental frequency at the end; *fpeak* – the maximum amplitude frequency; *bnd* – the bandwidth of maximum amplitude frequency.

In the lack of differences across sex and age-classes in the alarm call structure in the eastern speckled ground squirrels (Matrosova et al., 2011, 2012a), the geographic-related variation affects all the age- and sex-classes uniformly. Significant 15% differences (about 1 kHz) in the alarm call fundamental frequency were found between two eastern populations of speckled ground squirrels (Matrosova et al., 2012a). These differences were observed in both pups and adults and in both sexes. Similarly noticeable genetic differences (5 nucleotide substitutions) in the full-size control region of mitochondrial DNA (C-region) were found between these populations, although within populations, the C-region was conservative (Matrosova et al., 2014). Comparative studies of nucleotide sequences of partial C-region (310 b.p.) of 20 colonies (Ermakov et al., 2011) or full-size C-region (999–1001 b.p.) (Matrosova et al., 2014) revealed higher genetic diversity within western populations of the distribution area of the species compared to the eastern ones.

The focus of this study was to examine whether the acoustic variation in the alarm call was connected to genetic differences across populations of speckled ground squirrels. The goals were: (1) to describe within and between-population genetic polymorphism and acoustic variation; (2) to make a comparison of genetic and acoustic data between eastern ($2n = 34$) and western ($2n = 36$) chromosomal races of speckled ground squirrels; (3) to evaluate the relationship between genetic and acoustic variation in 6 scattered populations of ground squirrels.

Material and methods

Study sites, animals and dates

Data were collected in April–August of 2005–2014 during above ground activity from 6 natural colonies of speckled ground squirrels, representing 6 populations (Table 1). Three eastern populations (ZAR, LIP, MIC) were located in Russia, to the east of the Dnieper River; and 3 western populations (NOV, OZE, SOV), were located in Ukraine, to the west of the Dnieper River. The NOV and OZE, although closely located (12 km) to each other, were considered as separate populations, as they were separated by two large meridianally stretched lakes, representing an insurmountable geographic barrier for ground squirrels. The two populations are not continual around these lakes for at least last a few dozen years, because of the impassable swamps around their shorelines. The subjects were 90 adult (one year and older) speckled ground squirrels, 15 individuals per population. Animals were captured with live-traps, recorded for calls, sampled for DNA, assayed for sex and age and released back at the place of capture (for details see Matrosova et al., 2007, 2009). The trapping point was determined with a GPS navigator (Garmin Ltd., Schaffhausen, Switzerland). The geographical distances between populations were calculated as linear distances (in km) based on GPS coordinates using the MapSource Internet resource.

Call and DNA collection

Matched alarm call and DNA samples have been collected from the same individuals. Acoustic recording always preceded animal handling. Alarm calls were recorded from animals captured singly in wire-mesh live-traps and calling toward a researcher either spontaneously or in response to additional stimulation (walking near the traps or the movements of a hand-held hat). Normally recording session lasted 3–5 min providing 30–40 calls per animal. The distance to the microphone was kept of about 1 m. Pattern of calling toward humans and structure of alarm calls in live-traps are similar to those that occurred under natural conditions toward predators or humans (Nikol'skii, 1979; Volodin, 2005; Matrosova et al., 2007, 2010a,b). Alarm calls were recorded (48 kHz, 16 bit) using a solid state recorder Marantz PMD-660 (D&M Professional, Kanagawa, Japan) with an AKG-C1000S cardioid electret condenser microphone (AKG-Acoustics GmbH, Vienna, Austria).

As DNA samples, we sampled thin cuts of a middle finger pad from a hind leg for DNA analyses. The place of the cut was treated with anticeptic solution of hydrogen peroxide. In ZAR population, the genetic relationships among individuals have been documented in a long-term ecological study. All individuals were permanently marked with passive integrated transponders – microchips (Bayer AG, Leverhusen, Germany), to avoid inclusion into the genetic and acoustic study close relatives (mothers/offspring, siblings). In other populations, where data have been collected within a short time space, traps were spread evenly throughout the area separated with the maximum possible distance ranged commonly within a few dozen meters, to reduce non-random sampling of related individuals.

Molecular analyses

We used the full-size C-region of mtDNA as the genomic marker. The C-region is highly variable because it is not involved in encoding proteins, therefore the changes in sequences of its nucleotides are selectively neutral. Whole genomic DNA was extracted from tissue samples (finger pads) using 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 C-region flanked by a fragment of the tRNA–Pro and the tRNA–Phe genes was amplified using the primers MDL1 and H00651 (Syntol, Moscow, Russia) following Ermakov et al. (2002), total length of amplified fragment was 1148 b.p. Polymerase chain reaction (PCR) was conducted in a volume of 25 ml using the reagents from Dialat Ltd., (Moscow, 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. For most gene samples, we have been obtaining two PCR products of approximate similar size (differing by not more than 20–40 b.p.), probably because of the presence of nuclear copies of mtDNA (Ermakov et al., 2015). The brightest target PCR product band was shorter by the length, whereas the satellite PCR product band was longer and colored less intensively or missing. We obtained sequences of satellite PCR product which could represent putative pseudogenes in a small number of samples. We are confident that the new sequences represent the C-region rather than nuclear pseudogenes since they closely match the previously reported sequences obtained for the genus *Spermophilus* (Gündüz et al., 2007; Ermakov et al., 2011; Ochoa et al., 2012) and because they did not display any anomalym e.g., ambiguous bases, typical for the nuclear copies (Bensasson et al., 2001). We separated the target PCR product in 1.5% agarose gel, stained it 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 v3.1Cycle 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′) as well as external primers if needed. The resulting nucleotide sequences were aligned with the SeqMan (Lasergene, USA) and BioEdit (Hall, 1999) software, as well as manually. In total, we analyzed the nucleotide sequence of the complete C-region in 90 speckled ground squirrel individuals, belonging to six populations and included into the acoustic analysis (Supplementary Table S1). Fifty sequences were published previously in Matrosova et al. (2014; GenBank Acc. no. KF934335-KF934384) and the 40 sequences generated in this study have been deposited in GenBank (GenBank Acc. no. KT597935-KT597974). We used the MEGA 5 software (Tamura et al., 2011) for statistical data processing. Nucleotide indels were treated as missing data. 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 were estimated according to the Kimura two-parameter model (K2P) calculated in MEGA 5. Haplotype diversity (h) and nucleotide diversity (π) within each population were calculated in Arlequin v. 3.5 (Excoffier and Lischer, 2010). We estimated genetic differentiation levels between population group (fixation index F_{ST}) after 1000 permutations with analysis of molecular variance (AMOVA) in the same program. We tested the assump-

Table 2
Genetic characteristics of 6 populations of speckled ground squirrels.

Population	N	N_{hapl}	N_{uniq}	π (SD)	h (SD)	Tajima's D, P	Fu's Fs, P
ZAR	15	1	0	0.000 (0.000)	0.000 (0.000)	0.000, P = 1.000	0.000, N.A.
LIP	15	1	0	0.000 (0.000)	0.000 (0.000)	0.000, P = 1.000	0.000, N.A.
MIC	15	3	2	0.001 (0.001)	0.257 (0.142)	−1.911, P = 0.011	0.106, P = 0.411
NOV	15	8	5	0.004 (0.003)	0.895 (0.053)	−0.514, P = 0.326	−0.484, P = 0.400
OZE	15	4	1	0.009 (0.005)	0.619 (0.120)	1.409, P = 0.942	7.362, P = 0.994
SOV	15	10	4	0.010 (0.006)	0.943 (0.040)	−0.435, P = 0.365	0.109, P = 0.514
Eastern group	45	5	2	0.003 (0.002)	0.709 (0.024)	2.021, P = 0.981	3.366, P = 0.922
Western group	45	22	11	0.011 (0.006)	0.942 (0.021)	0.055, P = 0.598	−1.637, P = 0.315
Total	90	27	13	0.028 (0.014)	0.914 (0.015)	2.364, P = 0.992	8.019, P = 0.953

N – the number of assayed animals in population; N_{hapl} – number of found haplotypes; N_{uniq} – number of unique haplotypes; π – nucleotide diversity (averaged over loci); h – haplotype diversity; SD – standard deviation; tests of selective neutrality: Tajima's D and Fu's Fs (no. of simulations = 10,000).

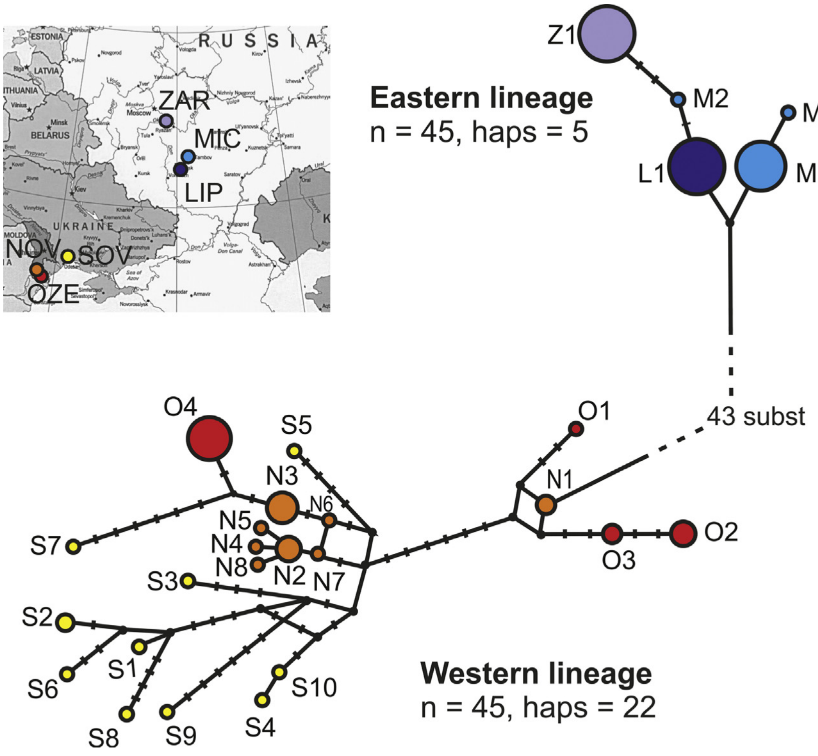

Fig. 2. Unrooted haplotype network of absolute distances between mtDNA haplotypes of C-region. Each circle represents a unique haplotype, its size proportional to the haplotype frequency. Small black circles represent hypothetical haplotypes. Colors represent the 6 colonies within the species range. Lines connecting each haplotype represent a single nucleotide substitution, and the hatch marks along those branches represent additional substitutions. Haplotypes with more than one branch connecting them to other haplotypes represent alternative pathways of equal likelihood. Population haplotypes: Z – Zaraysk; L – Lipetsk; M – Michurinsk; N – Novosel'skoe; O – Ozernoe; S – Odessa. Sample numbers correspond to those listed in Supplementary Table S1.

tion of neutral evolution for C-region sequences with Tajima's *D*, and Fu's *Fs* neutrality tests implemented in Arlequin software after 10,000 permutations.

The phylogenetic relationships among haplotypes were reconstructed using the five different algorithms. The unweighted pair group method average (UPGMA) and neighbor-joining (NJ) trees constructed in MEGA 5 under the assumption of the K2P model. The maximum parsimony (MP) tree constructed in PAUP v. 4.0. An initial heuristic search of 20 random taxon addition replicates was conducted with tree-bisection-reconnection branch swapping (TBR), a bootstrap analysis (1000 replicates) was done to determine the bootstrap support of the heuristic search. The Maximum likelihood (ML) tree constructed in Treefinder v. 2011 and the Bayesian inference of phylogeny (BI) tree constructed in Mr. Bayes v. 3.2.2 software used the best-fit DNA substitution model for our sequence dataset selected by jModeltest v. 2.1.6 (Darriba et al., 2012) under BIC criterion (HKY + G). Node support values in phylogenetic trees were estimated according to bootstrap support (1000 replicates). The nucleotide sequence of homologous mtDNA fragment of the Perote ground squirrel *Xerospermophilus perotensis* (NCBI acc. no. JQ326958.1, Ochoa et al., 2012) was used as an outgroup because suitable sequences from closely related species were not available from the GenBank. A Haplotype Network was constructed using the Network v. 4.612 software (Fluxus Technology Ltd, UK, www.fluxus-engineering.com).

Acoustic analyses

All calls were analyzed spectrographically using Avisoft SASLab Pro software (Avisoft Bioacoustics, Berlin, Germany). Before analysis, the calls were downsampled to 24 kHz and high-pass filtered at 1 kHz to remove background noise. Spectrograms were created with Hamming window, FFT 1024 points, frame 50% and overlap 96.87%.

We took measurements from up to 10 calls per individual, 15 individuals per population, 856 alarm calls in total, because 11 individuals provided less than 10 measurable alarm calls (from 4 to 9 alarm calls). The number of calls included per individual did not noticeably affect the results, so we used the average parameter values of calls per individual (averaged calls). We selected calls randomly among calls of good quality, i.e., not superimposed with wind, noise, or sounds animals make by hitting the live-trap. All measurements were taken from the fundamental frequency band (*f0*), because it was the highest in amplitude in a call spectrum (Matrosova et al., 2007). We used the "automatic parameter measurements" option of Avisoft SASLab Pro to extract the fundamental frequency values. We measured *f0* for each of 5 single power spectra, taken with equal intervals from beginning to end of a call: *f0st*, *f0quart1*, *f0centre*, *f0quart3* and *f0end* of a call, and the *f0max*, *f0min*, were automatically extracted (Fig. 1). With this option, we measured the *duration* of each alarm call. From the mean power spectrum, we measured automatically the maximum amplitude frequency (*fpeak*) and the bandwidth of maximum amplitude frequency (*bnd*) at 20 dB below maximum. All measurements were exported automatically to Microsoft Excel (Microsoft, Redmond, Washington). Overall, for each alarm call we measured 1 temporal, 7 frequency and 2 power variables. For each animal, we calculated mean values for each call variable, thus obtaining one averaged call per individual.

Statistic analysis

We quantified the population distinctiveness of the alarm calls by performing a principal component analysis (PCA) followed by a discriminant function analysis (DFA). We used PCA to eliminate redundancy due to the high intercorrelation of the acoustic variables in our data set and to examine clustering among variables. PCA transforms a large number of correlated variables into a few orthogonal variables (principal components) that explain the maximum amount of variation in the original data set with a minimum loss of information. Principal components are weighted linear combinations of the original variables. We retained three components with highest eigenvalues, which describe 98% of variation. The scores of the three extracted principal components were confirmed for normality (Kolmogorov–Smirnov test) and used as input variables in the DFA.

To calculate acoustic distances across populations, we calculated Euclidean distances between populations according to the scores of three PCs (Briefer and McElligott, 2012; Volodin et al., 2014). First, we calculated the position of each individual's mean call in the space of canonical axes of DFA. Then we obtained population centroids and calculated Euclidean distances between the centroids for each pair of populations. Euclidean distances were used as a measure of similarity in the structure of calls for each pair of populations. Shorter Euclidean distances indicate greater similarity in alarm call structure.

We used a nested design of ANOVA with a population nested within population groups (eastern or western) with a Tukey honestly significant difference (HSD) test to assess whether acoustic values differed between populations. Inter-population relationships between acoustic, genetic and geographical distances were evaluated with one-tailed permutation Mantel test (5000 permutations, ten times per each pairwise comparison). Statistical analyses were made with STATISTICA v. 8.0 (StatSoft, Tulsa, OK, USA) and PAST v. 2.17c (Hammer et al., 2001); all means are given as mean ± SD. Significance levels were set at 0.05, and two-tailed probability values (for the exlusion of Mantel test) are reported.

Results

Genetic structure and diversity

We analyzed the nucleotide sequence of the full-size mtDNA C-region (998–1002 b.p.) in 90 speckled ground squirrels, belonging to 6 populations and included into the acoustic analysis of alarm call variation (Supplementary Table S1). Totally, 83 sites (about 8% of the full fragment length) were variable and 67 of them, parsimony-informative. The mean nucleotide composition was 30.96% (A), 33.81% (T), 11.97% (G) and 23.26% (C) and the mean transition/transversion ratio (over all sequence pairs) was $R = 3.277$.

We summarized the genetic characteristics in Table 2. Nucleotide diversity (π) varied from 0 to 0.01 in different populations, amounting to 0.028 ± 0.014 in the pooled sample set (Table 2). Haplotype diversity (h) varied substantially higher, from 0 to 0.943 in different populations, amounting to 0.914 ± 0.015 in the pooled sample set. Both nucleotide and haplotype diversities were higher for western populations than for eastern populations (Table 2). The genetic distance between western and eastern population groups comprised 0.046 ± 0.008.

Twenty seven haplotypes were identified among the 90 examined individuals (Fig. 2, Supplementary Table S1; Supplementary Fig. S2). There were no shared haplotypes among any two studied populations. Two haplogroups (eastern and western) were clearly distinguished. The western haplogroup contained much more prominent genetic diversity compared to the eastern one in terms of the number of haplotypes (22 vs 5) (Fig. 2).

The C-region sequence data showed clear phylogeographic structure. Five different phylogenetic methods (UPGMA, NJ, ML, MP and BI) identified the same two lineages with all the C-region haplotypes: one lineage contained the all western populations NOV, OZE and SOV, the second lineage contained all eastern populations ZAR, LIP, and MIC (Supplementary Fig. 1). Bootstrap support was very high (87–100%) for the two lineages irrespective of the applied algorithm for creating the tree except BI tree with bootstrap support of the western lineage was less than 70%.

The differences between eastern and western speckled ground squirrels included 33 single nucleotide substitutions, mostly in the right domain of the C-region: transversions A/T at 5 positions (19, 268, 275, 854, 900 b.p.), transitions C/T at 15 positions (59, 61, 236, 266, 290, 703, 775, 816, 848, 875, 908, 911, 922, 970, 986 b.p.), transitions A/G at 3 positions (158, 264, 501 b.p.) and deletions at 3 positions (895, 998, 999 b.p.) (Supplementary Fig. S2). In addition, in all specimens, living to the west of the Dnieper River, two loci had complex substitutions: at 238 position all eastern animals had G, whereas the western animals had either T or C; at 411 position all eastern animals had A, whereas the western animals had either T or C (Supplementary Fig. S2). The raw and net pairwise distances between mtDNA C-region sequences of the 6 study populations are given in Supplementary Table 3. The lowest divergences are between LIP and MIC populations, the highest divergences are between ZAR and NOV populations.

The fixation index indicated significant differences between the eastern and western population groups ($F_{ST} = 0.857$, $P < 0.001$). An AMOVA showed that variation between the eastern and western population groups account for 82.83% of genetic variability ($df = 1$), whereas variation among populations within groups account for 8.99% ($df = 4$), and variation within populations account for only 8.18% of genetic variability ($df = 85$).

Western populations lineage has a nonsignificant positive value for Tajima's D (0.055, $P = 0.598$) and nonsignificant negative value for Fu's Fs (−1.637, $P = 0.315$), while eastern populations lineage has nonsignificant positive values for Tajima's D (2.021, $P = 0.981$) and for Fu's Fs (3.366, $P = 0.922$) (Table 2).

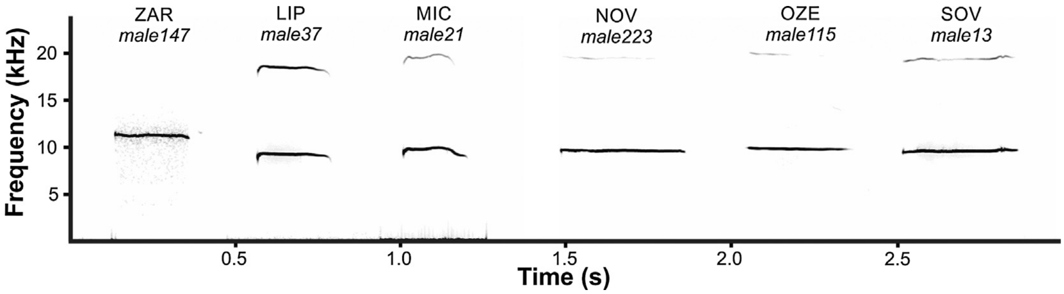

Fig. 3. Representative spectrograms of adult male alarm calls from speckled ground squirrels originating from 6 study populations. Spectrograms were created with sampling rate 48 kHz, FFT 1024, frame 50%, overlap 93.75%. Audio file is available in Supplementary.

Table 3
Values (mean ± SD) of alarm call variables and nested ANOVA results for their comparison between eastern (ZAR, LIP, MIC) and western (NOV, OZE, SOV) population groups and among 6 populations.

Acoustic variable	Population						ANOVA results for	
	ZAR	LIP	MIC	NOV	OZE	SOV	Population groups	Six populations
duration (ms)	248 ± 44^{x}	228 ± 34^{x}	207 ± 42^{x}	360 ± 81^{y}	328 ± 71^{y}	332 ± 76^{y}	$F_{1,84} = 76.58$, $P < 0.001$	$F_{4,84} = 1.49$, $P = 0.21$
f0min (kHz)	9.47 ± 0.49^{x}	7.76 ± 0.74^{y}	8.84 ± 0.73^{x}	9.39 ± 0.95^{x}	9.17 ± 0.77^{x}	9.07 ± 0.92^{x}	$F_{1,84} = 9.93$, $P = 0.002$	$F_{4,84} = 9.44$, $P < 0.001$
f0max (kHz)	9.86 ± 0.54^{x}	8.38 ± 0.66^{y}	9.38 ± 0.68^{x}	9.77 ± 0.86^{x}	9.72 ± 0.74^{x}	9.49 ± 0.73^{x}	$F_{1,84} = 9.28$, $P = 0.003$	$F_{4,84} = 8.83$, $P < 0.001$
f0start (kHz)	9.69 ± 0.54^{x}	8.08 ± 0.65^{y}	9.13 ± 0.72^{x}	9.56 ± 0.91^{x}	9.52 ± 0.77^{x}	9.40 ± 0.70^{x}	$F_{1,84} = 12.01$, $P < 0.001$	$F_{4,84} = 9.59$, $P < 0.001$
f0quart1 (kHz)	9.67 ± 0.49^{x}	8.27 ± 0.68^{y}	9.21 ± 0.72^{x}	9.70 ± 0.86^{x}	9.58 ± 0.74^{x}	9.34 ± 0.74^{x}	$F_{1,84} = 10.55$, $P = 0.002$	$F_{4,84} = 8.03$, $P < 0.001$
f0centre (kHz)	9.63 ± 0.49^{x}	8.28 ± 0.65^{y}	9.26 ± 0.72^{x}	9.66 ± 0.85^{x}	9.54 ± 0.72^{x}	9.32 ± 0.75^{x}	$F_{1,84} = 9.17$, $P = 0.003$	$F_{4,84} = 7.74$, $P < 0.001$
f0quart3 (kHz)	9.62 ± 0.50^{x}	8.27 ± 0.65^{y}	9.24 ± 0.67^{x}	9.57 ± 0.86^{x}	9.49 ± 0.72^{x}	9.31 ± 0.77^{x}	$F_{1,84} = 7.77$, $P = 0.007$	$F_{4,84} = 7.58$, $P < 0.001$
f0end (kHz)	9.65 ± 0.58^{x}	7.83 ± 0.74^{y}	8.94 ± 0.73^{x}	9.48 ± 0.94^{x}	9.23 ± 0.80^{x}	9.26 ± 0.81^{x}	$F_{1,84} = 11.13$, $P = 0.001$	$F_{4,84} = 10.83$, $P<0.001$
fpeak (kHz)	9.65 ± 0.50^{x}	8.27 ± 0.67^{y}	9.21 ± 0.72^{x}	9.67 ± 0.85^{x}	9.55 ± 0.73^{x}	9.33 ± 0.75^{x}	$F_{1,84} = 9.77$, $P = 0.002$	$F_{4,84} = 7.84$, $P<0.001$
bnd (kHz)	0.41 ± 0.15^{x}	0.31 ± 0.97^{y}	0.36 ± 0.10^{xy}	0.31 ± 0.07^{y}	0.33 ± 0.08^{xy}	0.30 ± 0.06^{y}	$F_{1,84} = 5.42$, $P = 0.02$	$F_{5,84} = 2.34$, $P = 0.06$

$N = 15$ individuals for each population, one averaged call per individual. The same superscripts indicate which populations did not differ significantly ($P > 0.05$, Tukey HSD post-hoc test).

Table 4
Factor loadings of the measured alarm call variables on the first three principal components.

Acoustic variable	Principal component		
	PC1	PC2	PC3
Duration	−0.222	**0.681**	**0.698**
f0min	**−0.972**	0.020	−0.064
f0max	**−0.995**	−0.048	0.009
f0start	**−0.989**	−0.004	−0.026
f0quart1	**−0.994**	0.004	−0.005
f0centre	**−0.992**	−0.016	−0.015
f0quart3	**−0.992**	−0.021	−0.043
f0end	**−0.968**	0.008	−0.077
Fpeak	**−0.994**	−0.002	−0.009
Bnd	−0.115	**−0.793**	**0.598**
Eigenvalue	7.857	1.096	0.857
Cum %variance	78.57	89.52	98.10

Bold types indicate the heaviest factor loadings ($|r| > 0.60$). Eigenvalues and cumulative explained variances are given at the bottom of the table.

Acoustic differences between populations

Alarm calls of speckled ground squirrels from all 6 studied populations represented tonal high-frequency vocalizations, with either weakly modulated or flat contour, with the maximum energy concentrated on the fundamental frequency band (Fig. 3). Among populations the mean maximum fundamental frequency of the alarm calls ranged from 8.38 to 9.86 kHz, the mean minimum fundamental frequency ranged from 7.76 to 9.47 kHz, and the duration ranged from 207 to 360 ms (Table 3).

ANOVA revealed a strong effect of population group on all the 10 alarm call variables (Table 3). Call duration, the peak frequency and all fundamental frequency variables were higher, whereas the bandwidth was lower in the western than in the eastern population group. The effect of population was found on the peak frequency and all fundamental frequency variables, but not on call duration or bandwidth (Table 3). A comparison of call durations showed that calls in any of the three eastern populations were significantly shorter than calls in any of the three western populations ($P < 0.01$ for all comparisons, Tukey HSD test). Neither within eastern nor within western populations, alarm calls did not differ by duration (Table 3, Fig. 4A). The differences between populations by all fundamental frequency and peak frequency variables were similar, the values did not differ between all populations for the exception of LIP, where it was significantly lower compared to all other populations ($P < 0.01$ for all comparisons, Tukey HSD test) (Table 3, Fig. 4B). The bandwidth in ZAR was significantly wider compared to those in LIP, NOV and SOV ($P < 0.05$ for all comparisons, Tukey HSD test), the differences were non-significant between all other pairs of populations (Table 3).

The PCA performed on alarm calls revealed three components (PC1–PC3) which described 98.1% of the variation in the original data set (Table 4). All fundamental frequency and peak frequency variables were grouped in the first principal component. Two other components (PC2 and PC3) were related to call duration and bandwidth. Scatterplot based on two first principal components (PC1 and PC2) displays high similarity of the western populations. Meanwhile ground squirrels from the eastern populations show much wider diversity by the alarm call acoustics (Fig. 5). The LIP population is found in the most isolated position among all populations because of a distinctive PC1 related to frequency variables (Supplementary Table S2).

Correlations between acoustic, genetic and geographical distances

We compared inter-population genetic distances (estimated according to the Kimura two-parameter model), acoustic distances (based on Euclidean distances of DFA) and geographical distances (calculated as linear distances between populations in km)

Table 5
Acoustic, genetic and geographical distances among 6 study populations.

Populations	Geographical distance (km)	Genetic distance (K2P) (%)	Acoustic distance (DFA Euclidean distances)
ZAR-LIP	249	0.503	2.441
ZAR-MIC	255	0.496	1.117
ZAR-NOV	1274	4.928	2.266
ZAR-OZE	1262	4.793	1.688
ZAR-SOV	1096	4.739	1.947
LIP-MIC	95	0.207	1.595
LIP-NOV	1130	4.515	3.143
LIP-OZE	1119	4.336	2.630
LIP-SOV	940	4.398	2.501
MIC-NOV	1217	4.618	2.823
MIC-OZE	1206	4.479	2.206
MIC-SOV	1026	4.447	2.298
NOV-OZE	12	0.933	0.625
NOV-SOV	196	1.208	0.648
SOV-OZE	185	1.478	0.376

K2P – Kimura two-parameter model, used for genetic differences estimated; DFA – Euclidean distances – calculated with DFA using PCA scores of alarm call variables.

(Table 5). The shortest geographical distance was found between NOV and OZE populations, whereas the minimum genetic distance was found between LIP and MIC populations, and the shortest acoustic distance was found between OZE and SOV populations. However, the maximum geographical distance coincided with the maximum genetic distance between ZAR and NOV populations, whereas the maximum acoustic distance was observed between LIP and NOV populations (Table 5).

A comparison of correlations between acoustic, genetic and geographical distances with permutation Mantel test showed a significant positive correlation between genetic and geographical distances ($r = 0.97$, $P = 0.023 \pm 0.002$) and between acoustic and geographical distances among populations ($r = 0.74$, $P = 0.043 \pm 0.003$). Positive correlation between acoustic and genetic distances did not reach significance ($r = 0.66$, $P = 0.089 \pm 0.004$).

Discussion

This study is the second (after Ochoa et al., 2012) examining the full-size mtDNA C-region in ground squirrels. We found that the three eastern populations ($2n = 34$) did differ strongly from the 3 western populations ($2n = 36$) by the C-region. By contrast, a detailed acoustic analysis did not reveal principal differences in the alarm call pattern among the 6 study populations, scattered over the species distribution area. Although based on principal components analysis of the acoustics there are some slight tendencies in separation of eastern and western groups, all populations have a large overlap with all others (Fig. 5). At the same time, one eastern population was distinctive in its alarm call frequency values from all other populations, either eastern or western. Genetic and geographical distances and acoustic and geographical distances showed a significant positive correlation among populations. At the same time, a positive correlation between acoustic and genetic distances was not statistically significant.

We found the high degree of genetic variation in the C-region (about 8% of the length), as was expected for the non-coding DNA fragment. However, this variation was non-uniformly distributed among study populations. Ground squirrels from the eastern populations displayed considerably lower genetic diversity compared to those in the western populations, either in terms of the number of unique haplotypes or in terms of nucleotide diversity. Consistently, a low genetic variability was found within eastern populations, for instance, in two of them (ZAR and LIP), all specimens within populations shared the same haplotype. At the same time, a high amount of genetic variability was found within all western populations. Unequal levels of genetic variation between different parts of the speckled ground squirrel distribution area were reported. The speckled ground squirrel populations from the southern Ukraine had the higher diversity of microsatellite loci compared to Polish or western Ukrainian populations (Biedrzycka and Konopiński, 2008). In European ground squirrels *Spermophilus citellus* in their northwestern part of distribution area in the Czech Republic, the microsatellite- and MHC-based genetic diversity were lower than in central parts of the area in Hungary or Slovakia (Říčanová et al., 2011). In Idaho ground squirrels *Urocitellus brunneus*, three northern populations had significantly lower genetic diversity by either mtDNA or microsatellite markers than 8 southern populations (Garner et al., 2005). In antelope ground squirrels *Ammospermophilus leucurus*, 5 northern populations had significantly lower haplotype diversity by mtDNA markers: partial cytochrome *b* gene (555 b.p.) and C-region (510 b.p.), than eight southern populations (Whorley et al., 2004).

The detected genetic differences might result from the enhanced fragmentation and isolation together with suboptimal ecological conditions for the eastern, as compared to the western populations. The eastern populations and especially ZAR, that is located at the northernmost border of the species distribution area, have shorter periods of above ground activity and hibernates at relatively lower ambient temperatures than the western populations (Shekarova et al., 2003; Tchabovsky et al., 2005). All age-classes have less time for fattening that is critically important for successful hibernation (Huber et al., 2001; Vasilieva and Tchabovsky, 2014). The dwindling genetic diversity in fragmented habitats was reported for three ground squirrel species: European (Hulová and Sedláček, 2008; Kryštufek et al., 2009; Říčanová et al., 2011; Slimen et al., 2012), Perote (Ochoa et al., 2012) and speckled (Biedrzycka and Konopiński, 2008; Biedrzycka and Radwan, 2008). At the same time, for any pair of populations, even for the most close-distant (only 12 km) NOV and OZE, we did not detect any shared haplotype. This suggests a high degree of geographical isolation and a low degree of migration between populations in either eastern or western part of the species distribution area.

Preliminary demographic analyses, carried out with Tajima's *D*, Fu's *Fs* neutrality tests, gave non-significant results so we can only say about some trends. Neither of the two haplogroups meets the criteria of the sudden expansion model (Rogers, 1995). Probably, both eastern and western populations (i.e., haplogroups) are characterized by a stable population numbers; probably, the eastern populations have trends toward a decrease in the population numbers. Thus, our data do not allow us to examine historical changes in population size. Evidently, that demographic history of ground squirrels is complex and deserves a separate detailed study with inclusion of many populations and molecular markers.

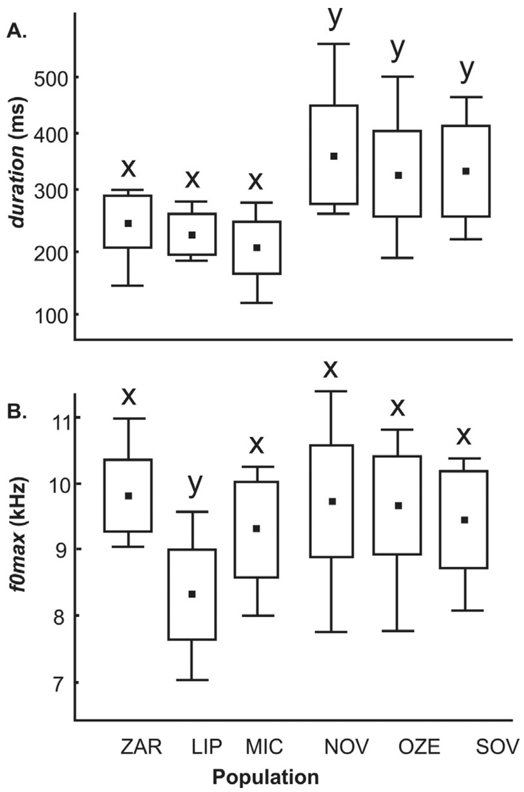

Fig. 4. Inter-population differences of the alarm call duration (A) and maximum fundamental frequency (*f0max*) (B). Central points show means, boxes show ± SD, whiskers show min–max values. Results for comparison of acoustics between populations (ANOVA with Tukey HSD test) are given with letters (x or y); means sharing the same letter are not significantly different.

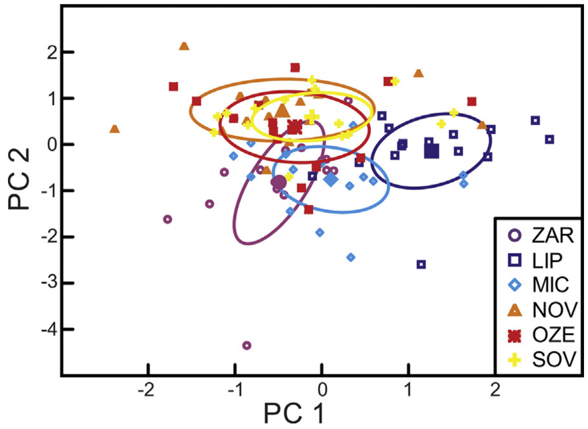

Fig. 5. Scatterplot showing separation produced by the first two principal components (PC1 and PC2) of alarm calls from 6 populations: ZAR – Zaraysk; LIP – Lipetsk; MIC – Michurinsk; NOV – Novosel'skoe; OZE – Ozernoe; SOV – Odessa. Large solid figures indicate centroids, ovals indicate 50% range intervals.

Based on the full-size C-region (998–1002 b.p.) genetic distance of 4.6%, obtained in this study between the eastern and the western populations of speckled ground squirrels did not reach the species-level degree of differentiation. For comparison, the species-level raw K2P genetic distances between three closely related *Spermophilus* species (*S. citellus*, *Spermophilus taurensis*, *Spermophilus xanthoprymnus*), based on a partial (572 b.p.) C-region, were substantially larger and ranged of 7.1–10.9% (Gündüz et al., 2007). Similar genetic distance ranges of 4.8–8.7% based on left domain C-region (311 b.p.) have been reported within species with a wide area, the little ground squirrel *Spermophilus pygmaeus*, between populations distributed to the east or to the west of Volga River (Ermakov et al., 2006).

The reported by Ermakov et al. (2011, 2015) values of the genetic distance between eastern and western populations of speckled ground squirrels based on the left domain of the C-region (311 b.p.), comprised 8% (6.5–10.4%). The lower values of genetic distances obtained in the current study, might be accounted for by the larger analyzed fragment of mtDNA which also contained more conservative central domain of the C-region.

Alarm calls of ground squirrels are highly species-specific (Eiler and Banack, 2004; Matrosova et al., 2012b; Schneiderová and Policht, 2012b), although all of them are given in response to a potential threat (Woodland et al., 1980; Sherman, 1985; Eiler and Banack, 2004) to decrease the success rate of predators hunting alerted victims (Woodland et al., 1980; Krebs and Davies, 1987; Eiler and Banack, 2004). According to Nikol'skii (1979), distinctive alarm call could serve as reliable indicators of the full species status.

This study considers the alarm calls of ground squirrels as innate signals. Formerly, direct interspecies cross-foresting experiments failed to confirm the vocal learning (Matocha, 1975). At the same time, a strong support of innate structure of alarms comes from intermediate acoustic characteristics between parental species of interspecies hybrids (Koeppl et al., 1978; Nikol'skii et al., 1984; Titov et al., 2005), suggesting strongly that these calls are started at birth.

An earlier study of geographical variation of the alarm call in *Spermophilus lateralis* and *Spermophilus saturatus* ground squirrels (Eiler and Banack, 2004) suggests that changes of vocalization parameters within local sites and between years suggests that alarm calling is at least partially a learned behavior. Unless that can be solidly substantiated, this approach might be confusing for ground squirrels. Alarm calls of speckled ground squirrels and yellow ground squirrels *Spermophilus fulvus* display a very high momentary individuality (Matrosova et al., 2009, 2010a,b), but these individual characteristics are very instable already at terms as short as three days (Matrosova et al., 2009). So, neither individuality nor stability may serve as arguments for the experiencing vocal learning by ground squirrels. Mammals experiencing vocal learning from conspecific tutors are bats (e.g., Jones and Ransome, 1993; Boughman, 1997, 1998; Knörnschild et al., 2010, 2012), cetaceans (Janik and Slater, 1997; Tyack, 1997, 2008; Weiß et al., 2006), pinnipeds (Sanvito et al., 2007; Schusterman, 2008) and humans. In all other mammalian groups, the vocal modification under social effects is very subtle (nonhuman primates: Snowdon and Elowson, 1999; Lemasson et al., 2003, 2011; Rukstalis et al., 2003; Crockford et al., 2004; Tanaka et al., 2006; carnivores: Townsend et al., 2010; ungulates: Briefer and McElligott, 2012; Volodin et al., 2014; rodents: Arriaga et al., 2012; Arriaga and Jarvis, 2013). For ground squirrels, the presence of at least subtle social effects on vocalizations (vocal learning or plasticity) has not yet been proved.

We did not find pronounced differences in the general acoustic pattern of the alarm call between eastern and western populations of speckled ground squirrels for the exception of call duration. Nevertheless, the LIP population was substantially distinctive from all other populations due to the fundamental frequency that was 14–18% lower than the respective frequency variables found in alarm calls of other populations. In other species of ground squirrels, the alarm calls similarity did not correlate with between-species relatedness in Anatolian *S. xanthoprymnus* and Taurus *S. taurensis* relative to European ground squirrels (Schneiderová and Policht, 2012a,b), although molecular data indicate that the Taurus ground squirrel is the most closely related to the European ground squirrel (Gündüz et al., 2007). Inconsistently, the acoustic differences in alarm calls could be used as a tool for verifying the subspecies-level taxonomy of Caucasian mountain *Spermophilus musicus* and the little ground squirrels (Nikol'skii et al., 2007). The acoustics of alarm calls were closer between the little ground squirrel populations located to the west of Volga River and Caucasian mountain ground squirrel populations in comparison with the little ground squirrel populations located to the east of Volga River, consistent with molecular (Ermakov et al., 2006) and cariotypical (Vorontsov and Lyapunova, 1970) data.

Although genetic and acoustic differences between populations increased with the increase of geographical distance, the acoustic and genetic distances did not correlate significantly to each other. Therefore, the hypothesis of genetic drift is inapplicable to our data, although earlier it was applied to explain the concordant variation of male advertisement songs and mtDNA C-region (495 b.p.) in singing mice (*Scotinomys teguina* and *Scotinomys xerampelinus)* from 7 localities in Costa Rica and Panama (Campbell et al., 2010). Mainly, the distinctive alarm call frequency values in the LIP population could be responsible for the lack of correlation between the acoustic and genetic distances. More appropriately, changes in alarm call variables of the LIP population can be explained by the hypothesis of ecological selection (Campbell et al., 2010; Wilkins et al., 2013). Unlike other five open-habitat populations with good visibility allowing the detection of predators at a great distance, the LIP population inhabits the 60-ha cemetery, representing a relatively isolated colony because of the ploughing of the surrounding fields (Nedosekin, 2007; Matrosova et al., 2012a). Many prominent memorials, trees and bushes in the environment provide multiple hides, but strongly limit visibility, enhancing the probability of close encounter with predators. At the same time, the frequency of appearance of human visitors decreases the alertness and fleeing distance of the animals compared to other populations. Nevertheless, these singular ecological conditions do not directly explain the observed decrease of e.g., the maximum fundamental frequency of alarm calls from 9.4 to 9.8 kHz in 5 other populations to 8.4 kHz at the LIP population, as this decrease does not noticeably affect the alarm call propagation ability. Probably, the ecological selection for these environmental conditions could affect the coding regions of genes. Further research of the LIP population genetics (primarily nuclear DNA), is necessary to reveal the reasons for the unusual acoustic characteristics of the alarm call.

Our data confirm the genetic differences between the western ($2n = 36$) and the eastern ($2n = 34$) speckled ground squirrels that probably evolved during a prolonged period of their separate evolution (Ermakov et al., 2015). At the same time, the inconsistent acoustic differences between populations, representing these two chromosomal races, suggest that the processes of divergence of alarm call structure between the eastern and western population groups are not yet completed. Consistent data were obtained in the study of the acoustic and chromosomal diversity for 11 populations of blind mole rats *Spalax ehrenbergi*, belonging to 4 morphologically indistinguishable chromosomal races with $2n = 52$, 54, 58, and 60 (Nevo et al., 1987). The chromosomal races differed by the acoustics in their courtship calls. During experimental playbacks, females of the three phylogenetically older races ($2n = 52$, 54, and 58) preferred males from their own race; whereas, females of the phylogenetically young ($2n = 60$) chromosomal race did not recognize calls of different race males. Results of the current study of acoustic and genetic diversity in rodent alarm calls are consistent with earlier findings with rodent advertisement calls, indicating the gradual acoustic differentiation during speciation (Nevo et al., 1987; Campbell et al., 2010).

The speckled ground squirrel is a typical representative of steppe and even forest-steppe vegetation. It is obvious that the modern species distribution corresponds with the distribution of the forest-steppe vegetation ecosystems during the Last Glaciation Maximum (LGM) (Markova et al., 2009) (Supplementary Fig. 3). Our studied populations from south-western Ukraine represent at the same time the south-western limit of the modern species distribution and the area that were covered with suitable habitats during LGM. Areas, where our studied eastern populations are located, were covered by tundra-forest-steppe periglacial (LIP and MIC) or even tundra-steppe periglacial (ZAR) ecosystems during LGM. Normally, ground squirrels were not present in these ecosystems (Markova et al., 2009). Although during Mikulinian (also known as Eemian) Interglacial representatives of the genus *Spermophilus* (possibly suslicus) were found all across the modern distribution of the speckled ground squirrel, from south-western Ukraine and even to the Yaroslavl region of Russia (Markova, 2000), it is obvious that most of the steppe species including speckled ground squirrel were wiped out from northern regions during LGM. We suggest that our data can prove that the Odessa region contained one of the refugia of the species. This is based on the high level of genetic variability in the western phylogroup. The same results were obtained for the European ground squirrel where Pannonian populations had much higher variability of cytochrome b (960 b.p.) compared to those from the Central Europe (Říčanová et al., 2013). Low genetic diversity in northern populations of both speckled and European ground squirrels could be explained as a founding effect during recolonization of periglacial areas. Populations from the southern genetically rich parts of the distribution area are especially important for the survival of the species as the ground squirrels are one of the big losers of European wildlife.

Acknowledgements

This study was supported by the Russian Foundation for Basic Research (grant 15-34-20589). We thank the students engaged in work experience from various universities for their help with data collection. We are grateful to staff of the DNA sequencing centre “Genome” (Engelhardt Institute of Molecular Biology, Russian Academy of Sciences). We thank A. Ovcharov, A. Gaidash, D. Achramenko, Yu. Milobog, A.V. Tchabovsky, S.A. Schilova and N.S. Vasiliev for collaboration in the field and valuable discussion. We thank V.S. Lebedev, A.A. Bannikova, O.A. Ermakov, S.V. Titov, M.V. Kholodova, P.A. Sorokin, N.A. Poyarkov, E.N. Solovjeva, V.S. Artamonova, M.A. Ghazali and I.V. Pal’ko for their methodical advices. We are sincerely grateful to Stephen Pollard for his courteous corrections of style and language and are sincerely grateful to two anonymous referee, whose comments were useful and inspiring. We adhered to the “Guidelines for the treatment of animals in behavioural research and teaching” (Anim. Behav., 2006, 71, 245–253) and to the laws for scientific research of Ukraine and the Russian Federation, the countries where the study was conducted. Data collection protocol #2011-36 was approved by the Committee of Bio-ethics of Lomonosov Moscow State University.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mambio.2016.01.001.

References

Arriaga, G., Jarvis, E.D., 2013. Mouse vocal communication system: are ultrasounds learned or innate? Brain Lang. 124, 96–116, http://dx.doi.org/10.1016/j.bandl.2012.10.002.

Arriaga, G., Zhou, E.P., Jarvis, E.D., 2012. Of mice, birds, and men: the mouse ultrasonic song system has some features similar to humans and song-learning birds. PLoS One 7 (10), e46610, http://dx.doi.org/10.1371/journal.pone.0046610.

Bensasson, D., Zhang, D.-X., Hartl, D.L., Hewitt, G.M., 2001. Mitochondrial pseudogenes: evolution’s misplaced witnesses. Trends Ecol. Evol. 16, 314–321, http://dx.doi.org/10.1016/S0169-5347(01)02151-6.

Biedrzycka, A., Konopiński, M.K., 2008. Genetic variability and the effect of habitat fragmentation in spotted suslik *Spermophilus suslicus* populations from two different regions. Conserv. Genet. 9, 1211–1221, http://dx.doi.org/10.1007/s10592-007-9442-8.

Biedrzycka, A., Radwan, J., 2008. Population fragmentation and major histocompatibility complex variation in the spotted suslik, *Spermophilus suslicus*. Mol. Ecol. 17, 4801–4811, http://dx.doi.org/10.1111/j.1365-294X.2008.03955.x.

Boughman, J., 1997. Greater spear-nosed bats give group-distinctive calls. Behav. Ecol. Sociobiol. 40, 61–70, http://dx.doi.org/10.1007/s002650050316.

Boughman, J., 1998. Vocal learning by greater spear-nosed bats. Proc. R. Soc. Lond. B 265, 227–233, http://dx.doi.org/10.1098/rspb.1998.0286.

Briefer, E.F., McElligott, A.G., 2012. Social effects on vocal ontogeny in an ungulate, the goat, *Capra hircus*. Anim. Behav. 83, 991–1000, http://dx.doi.org/10.1016/j.applanim.2011.05.012.

Campbell, P., Pasch, B., Pino, J.L., Crino, O.L., Phillips, M., Phelps, S.M., 2010. Geographic variation in the songs of neotropical singing mice: testing the relative importance of drift and local adaptation. Evolution 64, 1955–1972, http://dx.doi.org/10.1111/j.1558-5646.2010.00962.x.

Crockford, C., Herbinger, I., Vigiland, L., Boesch, C., 2004. Wild chimpanzees produce group-specific calls: a case for vocal learning? Ethology 110, 221–243, http://dx.doi.org/10.1111/j.1439-0310.2004.00968.x.

Darriba, D., Taboada, G.L., Doallo, R., Posada, D., 2012. jModelTest 2: more models, new heuristics and parallel computing. Nat. Methods 9 (8), 772, http://dx.doi.org/10.1038/nmeth.2109.

Denisov, V., Bielianin, A., Jordan, M., Rudek, Z., 1969. Karyological investigations of two species *Citellus* (*Citellus pygmaeus* Pall. and *Citellus suslicus* Guld.). Folia Biol. 17, 169–174.

Eiler, K.C., Banack, S.A., 2004. Variability in the alarm call of golden-mantled ground squirrels (*Spermophilus lateralis* and *S. saturatus*). J. Mammal. 85, 43–50, http://dx.doi.org/10.1644/1545-1542(2004)085<0043:VITACO>2.0.CO;2.

Ermakov, O.A., Surin, V.L., Titov, S.V., Tagiev, A.F., Luk'yanenko, A.V., Formozov, N.A., 2002. A molecular genetic study of hybridization in four species of ground squirrels (*Spermophilus*: Rodentia, Sciuridae). Russ. J. Genet. 38 (7), 796–809, http://dx.doi.org/10.1023/A:1016395722664.

Ermakov, O.A., Titov, S.V., Savinetsky, A.B., Surin, V.L., Zborovsky, S.S., Lyapunova, E.A., Brandler, O.V., Formozov, N.A., 2006. Molecular genetic and palaeoecological arguments for conspecificity of little (*Spermophilus pygmaeus*) and Caucasian mountain (*S. musicus*) ground squirrels. Zool. Zh. 85 (12), 1474–1483.

Ermakov, O.A., Surin, V.L., Titov, S.V., 2011. Genetic diversity and differentiation of the speckled ground squirrel inferred from sequencing of mtDNA control region. Izv. Penz. Gos. Pedagog. Univ. im. V.G. Belinskogo 25, 176–180.

Ermakov, O.A., Simonov, E., Surin, V.L., Titov, S.V., Brandler, O.V., Ivanova, N.V., Borisenko, A.V., 2015. Implications of hybridizatio. NUMTs, and overlooked diversity for DNA barcoding of Eurasian ground squirrels. PLoS One 10 (1), e0117201, http://dx.doi.org/10.1371/journal.pone.0117201.

Excoffier, L., Lischer, H.E.L., 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Mol. Ecol. Res. 10, 564–567.

Garner, A., Rachlow, J.L., Waits, L.P., 2005. Genetic diversity and population divergence in fragmented habitats: conservation of Idaho ground squirrels. Conserv. Genet. 6, 759–774, http://dx.doi.org/10.1007/s10592-005-9035-3.

Gündüz, I., Jaarola, M., Tez, C., Yeniyurt, C., Polly, P.D., Searle, J.B., 2007. Multigenic and morphometric differentiation of ground squirrels (*Spermophilus*, Sciurida, Rodentia) in Turkey, with a description of a new species. Mol. Phylogenet. Evol. 43, 916–935, http://dx.doi.org/10.1016/j.ympev.2007.02.021.

Hall, T.A., 1999. BioEdit: a user friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95–98.

Hammer, Ø., Harper, D.A.T., Ryan, P.D., 2001. PAST: paleontological statistics software package for education and data analysis. Palaeontol. Electron. 4, 1–9.

Helgen, K.M., Cole, F.R., Helgen, L.E., Wilson, D.E., 2009. Generic revision in the holarctic ground squirrel genus *Spermophilus*. J. Mammal. 90, 270–305, http://dx.doi.org/10.1644/07-MAMM-A-309.1.

Huber, S., Hoffmann, I.E., Millesi, E., Dittami, J., Arnold, W., 2001. Explaining the seasonal decline in litter size in European ground squirrels. Ecography 24, 205–211.

Hulová, Š., Sedláček, F., 2008. Population genetic structure of the European ground squirrel in the Czech Republic. Conserv. Genet. 9, 615–625, http://dx.doi.org/10.1007/s10592-007-9378-z.

Janik, V.M., Slater, P.J.B., 1997. Vocal learning in mammals. Adv. Stud. Behav. 26, 59–99.

Jones, G., Ransome, R.D., 1993. Echolocation calls of bats are influenced by maternal effects and change over a lifetime. Proc. R. Soc. Lond. B 252, 125–128.

Knörnschild, M., Nagy, M., Metz, M., Mayer, F., von Helversen, O., 2010. Complex vocal imitation during ontogeny in a bat. Biol. Lett. 6, 156–159, http://dx.doi.org/10.1098/rsbl.2009.0685.

Knörnschild, M., Nagy, M., Metz, M., Mayer, F., von Helversen, O., 2012. Learned vocal group signatures in the polygynous bat *Saccopteryx bilineata*. Anim. Behav. 84, 761–769, http://dx.doi.org/10.1016/j.anbehav.2012.06.029.

Koeppl, J.W., Hoffman, R.S., Nadler, C.F., 1978. Pattern analysis of acoustical behavior in four species of ground squirrels. J. Mammal. 59, 677–696, http://dx.doi.org/10.2307/1380133.

Krebs, J.R., Davies, N.B., 1987. An Introduction to Behavioural Ecology. Blackwell Scientific Press, Oxford.

Kryštufek, B., Bryja, J., Bužan, E.V., 2009. Mitochondrial phylogeography of the European ground squirrel, *Spermophilus citellus*, yields evidence on refugia for steppic taxa in the southern Balkans. Heredity 103, 129–135, http://dx.doi.org/10.1038/hdy.2009.41.

Lemasson, A., Gautier, J.-P., Hausberger, M., 2003. Vocal similarities and social bonds in Campbell's monkey (*Cercopithecus campbelli*). C. R. Biol. 326, 1185–1193.

Lemasson, A., Ouattara, K., Petit, E.J., Zuberbühler, K., 2011. Social learning of vocal structure in a nonhuman primate? BMC Evol. Biol. 11, 362, http://dx.doi.org/10.1186/1471-2148-11-362.

Lobkov, V.A., 1999. The Speckled Ground Squirrel of North-western Shores of the Black Sea: Biology and Population Dynamics. Astroprint, Odessa.

Lyapunova, E.A., Vorontsov, N.N., 1970. Chromosomes and some issues of the evolution of the ground squirrel genus *Citellus* (Rodentia: Sciuridae). Experientia 26, 1033–1038.

Markova, A.K., 2000. The Mikulino (=Eemian) mammal faunas of the Russian Plain and Crimea. Neth. J. Geosci. 79, 293–301.

Markova, A.K., Simakova, A.N., Puzachenko, A.Y., 2009. Ecosystems of Eastern Europe at the time of maximum cooling of the Valdai glaciation (24–18 kyr BP) inferred from data on plant communities and mammal assemblages. Quat. Int. 201, 53–59, http://dx.doi.org/10.1016/j.quaint.2008.05.020.

Matocha, K.G., 1975. Vocal Communication in Ground Squirrels, Genus *Spermophilus*. PhD Thesis. Graduate Faculty of Texas Tech University, pp. 67 http://repositories.tdl.org/ttu-ir/handle/2346/16471.

Matrosova, V.A., Volodin, I.A., Volodina, E.V., Babitsky, A.F., 2007. Pups crying bass: vocal adaptation for avoidance of age-dependent predation risk in ground squirrels? Behav. Ecol. Sociobiol. 62, 181–191, http://dx.doi.org/10.1007/s00265-007-0452-9.

Matrosova, V.A., Volodin, I.A., Volodina, E.V., 2009. The short-term and long-term individuality in speckled ground squirrel alarm calls. J. Mammal. 90, 158–166, http://dx.doi.org/10.1644/08-MAMM-A-032.1.

Matrosova, V.A., Volodin, I.A., Volodina, E.V., Vasilieva, N.A., 2010a. Stability of acoustic individuality in the alarm calls of wild yellow ground squirrels *Spermophilus fulvus* and contrasting calls from trapped and free-ranging callers. Naturwissenschaften 97, 707–715, http://dx.doi.org/10.1007/s00114-010-0686-7.

Matrosova, V.A., Volodin, I.A., Volodina, E.V., Vasilieva, N.A., Kochetkova, A.A., 2010b. Between-year stability of individual alarm calls in the yellow ground squirrel *Spermophilus fulvus*. J. Mammal. 91, 620–627, http://dx.doi.org/10.1644/09-MAMM-A-143.1.

Matrosova, V.A., Blumstein, D.T., Volodin, I.A., Volodina, E.V., 2011. The potential to encode sex, age and individual identity in the alarm calls of three species of Marmotinae. Naturwissenschaften 98, 181–192, http://dx.doi.org/10.1007/s00114-010-0757-9.

Matrosova, V.A., Pivanova, S.V., Savinetskaya, L.E., Volodin, I.A., Volodina, E.V., Shekarova, O.N., 2012a. The between-population variation of the alarm call in the speckled ground squirrel (*Spermophilus suslicus*, Rodentia, Sciuridae): effects of sex, age and body mass. Zool. Zh. 91, 453–463.

Matrosova, V.A., Schneiderová, I., Volodin, I.A., Volodina, E.V., 2012b. Species-specific and shared features in vocal repertoires of three Eurasian ground squirrels (genus *Spermophilus*). Acta Theriol. 57, 65–78, http://dx.doi.org/10.1007/s13364-011-0046-9.

Matrosova, V.A., Savinetskaya, L.E., Shekarova, O.N., Pivanova, S.V., Rusin, M.Yu., Volodin, I.A., Volodina, E.V., Tchabovsky, A.V., 2014. Within- and between-population polymorphism of the mtDNA control region of the speckled ground squirrel (*Spermophilus suslicus*). Doklady Biol. Sci. 455, 143–148, http://dx.doi.org/10.1134/S0012496614020197.

Nedosekin, V.Y., 2007. Current state of the speckled ground squirrel at the north of the Middle-Russian Hills. In: Sarychev, V.S. (Ed.), Ecological Research in Galichya Gora Reserve. Voronezh State University Press, Voronezh, pp. 133–135, Issue 1.

Nevo, E., Heth, G., Beiles, A., Frankenberg, E., 1987. Geographic dialects in blind mole rats: role of vocal communication in active speciation. Proc. Natl. Acad. Sci. U. S. A. 84, 3312–3315.

Nikol'skii, A.A., 1979. Species specificity of alarm call in sousliks (*Citellus*, Sciuridae) of Eurasia. Zool. Zh. 58, 1183–1194.

Nikol'skii, A.A., Denisov, V.P., Stoiko, T.G., Formosov, N.A., 1984. The alarm call in F1 hybrids *Citellus pygmaeus* X *C. suslicus* (Sciuridae, Rodentia). Zool. Zh. 63, 1216–1225.

Nikol'skii, A.A., Ermakov, O.A., Titov, S.V., 2007. Geographical variability of the little ground squirrel (*Spermophilus pygmaeus*): a bioacoustical analysis. Zool. Zh. 86, 1379–1388.

Ochoa, A., Gasca, J., Ceballos, G.J., Eguiarte, L.E., 2012. Spatiotemporal population genetics of the endangered Perote ground squirrel (*Xerospermophilus perotensis*) in a fragmented landscape. J. Mammal. 93, 1061–1074, http://dx.doi.org/10.1644/11-MAMM-A-371.1.

Říčanová, Š., Bryja, J., Cosson, J.F., Gedeon, C., Choleva, L., Ambros, M., Sedláček, F., 2011. Depleted genetic variation of the European ground squirrel in Central Europe in both microsatellites and the major histocompatibility complex gene: implications for conservation. Conserv. Genet. 12, 1115–1129, http://dx.doi.org/10.1007/s10592-011-0213-1.

Říčanová, S., Koshev, Y., Říčan, O., Ćosić, N., Ćirović, D., Sedláček, F., Bryja, J., 2013. Multilocus phylogeography of the European ground squirrel: cryptic interglacial refugia of continental climate in Europe. Mol. Ecol. 22, 4256–4369, http://dx.doi.org/10.1111/mec.12382.

Rogers, A.R., 1995. Genetic evidence for a Pleistocene population explosion. Evolution 49, 608–615, http://dx.doi.org/10.2307/2410314.

Rukstalis, M., Fite, J.E., French, J.A., 2003. Social change affects vocal structure in a callitrichid primate (*Callitrix kuhlii*). Ethology 109, 327–340, http://dx.doi.org/10.1046/j.1439-0310.2003.00875.x.

Sanvito, S., Galimberti, F., Miller, E.H., 2007. Observational evidences of vocal learning in southern elephant seals: a longitudinal study. Ethology 113, 137–146, http://dx.doi.org/10.1111/j.1439-0310.2006.01306.x.

Schneiderová, I., Policht, R., 2012a. Acoustic analysis of the alarm call of the Anatolian ground squirrel *Spermophilus xanthoprymnus*: a description and comparison with alarm calls of the Taurus *S. taurensis* and European *S. citellus* ground squirrels. Naturwissenschaften 99, 55–64, http://dx.doi.org/10.1007/s00114-011-0870-4.

Schneiderová, I., Policht, R., 2012b. Acoustic analysis of alarm calls of the European ground squirrel (*Spermophilus citellus*) and the Taurus ground squirrel (*S. taurensis*) (Mammalia: Sciuridae). Zool. Anz. 251, 139–146, http://dx.doi.org/10.1016/j.jcz.2011.07.005.

Schusterman, R.J., 2008. Vocal learning in mammals with special emphasis on pinnipeds. In: Oller, D.K., Gribel, U. (Eds.), The Evolution of Communicative Flexibility: Complexity, Creativity, and Adaptability in Human and Animal Communication. MIT Press, Cambridge, Massachusetts, pp. 41–70.

Shekarova, O.N., Krasnova, E.D., Shcherbakov, A.V., Savinetskaya, L.E., 2003. Settlements of the speckled ground squirrel *Spermophilus suslicus* (Guldenstaedt, 1770) in the south of the Moscow (Zaraysky region). Bull. Mosc. Soc. Nat. 108, 9–16.

Shekarova, O.N., Neronov, V.V., Savinetskaya, L.E., 2008. Speckled ground squirrel (*Spermophilus suslicus*): current distribution: population dynamics and conservation. Lynx (Praha) 39, 317–322.

Sherman, P.W., 1985. Alarm calls of Belding's ground squirrels to aerial predators: nepotism or selfpreservation? Behav. Ecol. Sociobiol. 17, 313–323, http://dx.doi.org/10.1007/BF00293209.

Slimen, H.B., Gedeon, C.I., Hoffmann, I.E., Suchentrunk, F., 2012. Dwindling genetic diversity in European ground squirrels? Mamm. Biol. 77, 13–21, http://dx.doi.org/10.1016/j.mambio.2011.10.001.

Snowdon, C.T., Elowson, A.M., 1999. Pygmy marmosets modify call structure when paired. Ethology 105, 893–908, http://dx.doi.org/10.1046/j.1439-0310.1999.00483.x.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731–2739, http://dx.doi.org/10.1093/molbev/msr121.

Tanaka, T., Sugiura, H., Masataka, N., 2006. Cross-sectional and longitudinal studies of the development of group differences in acoustic features of coo calls in two groups of Japanese macaques. Ethology 112, 7–21, http://dx.doi.org/10.1111/j.1439-0310.2006.01103.x.

Tchabovsky, A.V., Babitsky, A.F., Savinetskaya, L.E., 2005. Variation in annual cycle and mortality in spotted souslik in relation to population density in the northernmost part of its range. Doklady Biol. Sci. 405, 455–457, http://dx.doi.org/10.1007/s10630-005-0163-1.

Titov, S.V., Ermakov, O.A., Surin, V.L., Formozov, A.N., Kasatkin, M.V., Shilova, S.A., Shmyrov, A.A., 2005. Molecular genetic and bioacoustic diagnostics russet (*Spermophilus major* Pallas, 1778) and yellow (*S. fulvus* Lichtenstein, 1823) ground squirrels from mixed colony. Bull. Mosc. Soc. Nat. 110, 72–77.

Townsend, S.W., Hollen, L.I., Manser, M.B., 2010. Meerkat close calls encode group-specific signatures, but receivers fail to discriminate. Anim. Behav. 80, 133–138, http://dx.doi.org/10.1016/j.anbehav.2010.04.010.

Tyack, P.L., 1997. Development and social functions of signature whistles in bottlenose dolphins *Tursiops truncatus*. Bioacoustics 8, 21–46, http://dx.doi.org/10.1080/09524622.1997.9753352.

Tyack, P.L., 2008. Convergence of calls as animals form social bonds, active compensation for noisy communication channels, and the evolution of vocal learning in mammals. J. Comp. Psychol. 122, 319–331, http://dx.doi.org/10.1037/a0013087.

Vasilieva, N.A., Tchabovsky, A.V., 2014. Timing is the only thing: reproduction in female yellow ground squirrels (*Spermophilus fulvus*). Can. J. Zool. 92, 737–747, http://dx.doi.org/10.1139/cjz-2014-0084.

Volodin, I.A., 2005. Individuality of alarm calls in the spotted suslik (*Spermophilus suslicus*, Rodentia, Sciuridae). Zool. Zh. 84, 228–235.

Volodin, I.A., Volodina, E.V., Lapshina, E.N., Efremova, K.O., Soldatova, N.V., 2014. Vocal group signatures in the goitred gazelle Gazella subgutturosa. Anim. Cognit. 17, 235–349, http://dx.doi.org/10.1007/s10071-013-0666-3.

von Merten, S., Hoier, S., Pfeifle, C., Tautz, D., 2014. A role for ultrasonic vocalisation in social communication and divergence of natural populations of the house mouse (*Mus musculus* domesticus). PLoS One 9 (5), e97244, http://dx.doi.org/10.1371/journal.pone.0097244.

Vorontsov, N.N., Lyapunova, E.A., 1970. Chromosomal numbers and speciation in ground-dwelling sciurids (Sciurudae: Xerinoe et Marmotinae) of Holarctic. Bull. Mos. Soc. Nat. 75, 112–126.

Weiß, B.M., Ladich, F., Spong, P., Symonds, H., 2006. Vocal behavior of resident killer whale matrilines with newborn calves: the role of family signatures. J. Acoust. Soc. Am. 119, 627–635, http://dx.doi.org/10.1121/1.2130934.

Whorley, J.R., Alvarez-Castañeda, S.T., Kenagy, G.J., 2004. Genetic structure of desert ground squirrels over a 20-degree-latitude transect from Oregon through the Baja California peninsula. Mol. Ecol. 13, 2709–2720, http://dx.doi.org/10.1111/j.1365-294X.2004.02257.x.

Wilkins, M.R., Seddon, N., Safran, R.J., 2013. Evolutionary divergence in acoustic signals: causes and consequences. Trends Ecol. Evol. 28, 156–166, http://dx.doi.org/10.1016/j.tree.2012.10.002.

Wilson, D.E., Reeder, D.M. (Eds.), 2005. Mammal Species of the World. A Taxonomic and Geographic Reference. , 3rd ed. Johns Hopkins University Press, Baltimore.

Woodland, D.J., Jafaar, Z., Knight, M.L., 1980. The pursuit deterrent function of alarm signals. Am. Nat. 115, 748–753.