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ORIGINAL ARTICLE

Phylogenetic relationship and variation of alarm call traits of populations of red-cheeked ground squirrels (*Spermophilus erythrogenys* sensu lato) suggest taxonomic delineation

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Abstract

Distribution area and taxonomic borders within the species complex *Spermophilus erythrogenys* sensu lato remain questionable. Early evidence suggests that red-cheeked ground squirrels of Southeast Kazakhstan are remarkably different in terms of the acoustic structure of their alarm calls from the red-cheeked ground squirrels of the Kurgan region in Russia. In this study, we analyzed the differences in the acoustic structure of the alarm call and mitochondrial DNA (complete control region, 1005–1006 bp and complete cytochrome *b* gene, 1140 bp) in 3 populations of red-cheeked ground squirrels (Tara, Altyn-Emel and Balkhash), all located within areas isolated by geographical barriers in Southeast Kazakhstan. We found that the alarm call variables were similar between the 3 study populations and differed by the maximum fundamental frequency (8.46 ± 0.75 kHz) from the values (5.62 ± 0.06 kHz) reported for the red-cheeked ground squirrels from the Kurgan region of Russia. Variation in mtDNA control region was only 3% and variation in cytochrome *b* gene was only 2.5%. Phylogenetic trees based on cytochrome *b* gene polymorphism of 44 individuals from the study area and adjacent territories indicated 3 clades with high (98–100%) bootstrap support: "*intermedius*," "*brevicauda*" and "*iliensis*"). We conclude that the 3 study populations in Southeast Kazakhstan belong to the clade *intermedius* and suggest a taxonomical revision of the species complex *Spermophilus erythrogenys* sensu lato, including analyses of nuclear DNA and alarm calls for populations of the *brevicauda* and *iliensis* clades.

Key words: alarm call, control region, cytochrome *b*, mitochondrial DNA, vocal communication

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INTRODUCTION

Red-cheeked ground squirrels of Southeast Kazakhstan, commonly considered within taxon *Spermophilus erythrogenys* sensu lato, represent an excellent model for studying biogeographic speciation (Nikol'skii & Rumyantsev 2004). Southeast Kazakhstan is a geo-mor-

phologically heterogeneous region with deep rivers, mountains and lowlands providing numerous eco-geographical barriers for ground squirrels, including the areas that are unfit for habitation although seem surmountable (Fig. 1). Studying populations of red-cheeked ground squirrels from this region might cast new light on their geographical divergence (Harrison *et al.* 2003; Herron *et al.* 2004; Thorington & Hoffmann 2005; Helgen *et al.* 2009) and raise new questions about the mechanisms by which populations diverged (Zachos 2018).

Geographical boundaries between ranges of different species and forms of ground squirrels from Southeast Kazakhstan are presently unknown. Approaches for identifying taxonomical ranks of different populations of ground squirrels from this area include both bioacoustical analyses (Nikol'skii & Rumyantsev 2004) and genetic analyses (Ermakov *et al.* 2015).

Figure 1 Map showing the study area (a) and data collection localities (b). Sites of matched alarm call and DNA sampling are marked in white; sites of DNA only sampling are marked in black (see Table 1 for details). Stars designate type localities: "*bre*" – *S. brevicauda* Brandt, 1843; "*int*" – *S. intermedius* Brandt, 1843; "*car*" – *S. carruthersi* (Thomas, 1912); "*ili*" – *S. e. iliensis* (Belyaev, 1945). The dotted line delineates the study area by Nikol`skii & Rumyantsev (2004).

Considering the acoustic structure of the alarm call (primarily based on frequency modulation pattern of the alarm call notes), Nikol'skii and Rumyantsev (2004) subdivided the alarm calls recorded in 13 populations of ground squirrels (distributed over the large area shown by the dotted line in Fig. 1) into 4 clusters, potentially corresponding to the 4 species (*S. erythrogenys*, *Spermophilus brevicauda*, *Spermophilus carruthersi* and *Spermophilus major*) with non-overlapping distribution areas; the boundaries between the clusters corresponded to large zoogeographic barriers (Fig. 1). Among them, most distinctive were 4 individual red-cheeked ground squirrels from the Altyn-Emel area: their alarm calls were unusually high-frequency, with the maximum fundamental frequency of over 8 kHz compared to values below 7 kHz in ground squirrels in other studied populations. The authors hypothesized that animals from the Altyn-Emel area belong to the form "*carruthersi*" within *S. erythrogenys* sensu lato; however, unequal samples of animals from different populations (from 1 to 9) and small numbers of alarm calls available for analyses (1 per individual) along with their unknown genetic status prevented more decisive conclusions about their taxonomical position (Nikol'skii & Rumyantsev 2004). To date, the alarm call acoustics of ground squirrels from the Altyn-Emel area have not been studied in detail.

A recent barcoding study (Ermakov *et al.* 2015), based on polymorphism of cytochrome *c* oxidase subunit 1 (COI) of all Eurasian species of ground squirrels, showed a paraphyly of *S. erythrogenys*, *S. brevicauda* and *Spermophilus pallidicauda*. Moreover, on the basis of mtDNA polymorphism, 5 individuals of supposed *S. erythrogenys* turned out to be 3 genetically distant forms, for which a putative species level rank was proposed: (i) *S. erythrogenys* (right bank of the Irtysh River); (ii) another, previously unknown, form from the right bank of the Ob' River (Kuznetsk Depression); and (iii) *S. carruthersi* (Zaysan Depression, the Dzungarian Alatau). Thus, although it has been suggested that ground squirrels inhabiting the area of the Dzungarian Alatau including the Altyn-Emel area should be considered as *S. carruthersi*, the available genetic and acoustic data are not sufficient to support this suggestion.

Formerly, taxon *S. erythrogenys* sensu lato was interpreted as a single wide-range polymorphic species complex involving all ground squirrels from Kazakhstan to Central Mongolia from west to east and all ground

squirrels from Southwest Siberia to Tien Shan from north to south (Belyaev 1955; Gromov *et al.* 1965; Vasilyeva 1968; Kryštufek & Vohralík 2013). From another point of view, *S. erythrogenys* sensu lato includes a few morphologically different forms considered as separate species (Afanas'ev *et al.* 1953; Ognev 1963; Sludskiy *et al.* 1969). Recently, these forms with unclear taxonomic status were separated into 3 species based on cytochrome *b* gene (cyt *b*) polymorphism: *S. erythrogenys*, inhabiting the northern part of the area; *S. brevicauda*, inhabiting the southern part of the area and *S. pallidicauda*, inhabiting the eastern part of the area (Harrison *et al.* 2003; Herron *et al.* 2004; Thorington & Hoffmann 2005; Helgen *et al.* 2009).

In rodents, vocalizations are not learned but inherited genetically (Kikusui *et al.* 2011). Ground squirrels have species-specific alarm calls (Nikol'skii 1979, 1984; Schneiderová & Policht 2011; Matrosova *et al.* 2012). Bioacoustic analysis has proved to be a useful taxonomic tool for identifying ground squirrel species (Titov *et al.* 2005; Nikol'skii *et al.* 2007; Schneiderová & Policht 2012a,b), subspecies (Matrosova *et al.* 2016) and interspecies hybrids (Nikol'skii *et al.* 1984; Formozov & Nikol'skii 1986; Nikol'ski & Starikov 1997; Titov *et al.* 2005).

Within species, alarm calls of ground squirrels are similar in the acoustics across ages and sexes (Matrosova *et al.* 2007, 2011; Swan & Hare 2008; Volodina *et al.* 2010), including the red-cheeked ground squirrels *S. erythrogenys* (Zhilin 2002). Alarm calls of red-cheeked ground squirrels are either single notes (hereinafter "single-note alarm calls") or represent clusters of several similar notes ("multi-note alarm calls"). In both the single-note and multi-note alarm calls, the maximum fundamental frequency of the notes ranges from 2.91 to 5.52 kHz, whereas note duration ranges from 22 to 179 ms (Nikol'skii 1979; Nikol'skii & Starikov 1997; Zhilin 2002). Both the single-note and multi-note alarm call types have similar frequency modulation patterns of the notes.

In this study, we used representative genetic and acoustic samples to obtain new data that enables testing of a potentially separated taxonomical position of ground squirrels from Southeast Kazakhstan (on the territory of the Dzungarian Alatau mountains), taking into account the existing natural eco-geographical barriers (Fig. 1). The aims of this study were to compare intrapopulation and interpopulation variation of the alarm call structure and to provide, by using 2 mtDNA markers (control region and cytochrome *b* gene), insights into the phylogeography of the ground squirrels inhabiting this area.

MATERIALS AND METHODS

Study sites, animals and dates

Alarm call and DNA samples of red-cheeked ground squirrels were collected in Southeast Kazakhstan from 10 to 24 June 2015 in the 3 natural colonies which are representative of the 3 study populations, "Tara," "Altyn-Emel" and "Balkhash" (Table 1). All the 3 habitats represented a dry grazing steppe. We examined 38 living subjects: 30 live-trapped and 8 free-ranging; 10 were from the Tara, 10 from the Altyn-Emel and 18 from the Balkhash population. Of the 38 living subjects, 22 provided matched samples of the alarm calls and DNA, whereas 16 individuals from the Balkhash population provided unmatched samples of alarm calls and DNA (8 individuals provided only alarm calls whereas the other 8 individuals provided only DNA samples). In addition, we sampled DNA from 8 museum specimens: "*brevicauda*" (2), "*intermedius*" (4), "*carruthersi*" (1) and "*iliensis*" (1) forms obtained from their type locality or adjacent areas (Table 1, Fig. 1). We also included in the analyses 2 sequences of the form "*iliensis*" from Genbank (AF157856 and AF157857). Nucleotide sequences of the cyt *b* gene of *S. relictus* (AF157876), *S. pallidicauda* (AF157866, AF157869) and *S. erythrogenys* (AF157875) were used as outgroups (Table 1, Fig. 1). The latter sequence was obtained near the terra typica (Vasilyeva 1968; Thorington & Hoffmann 2005) and is, therefore, considered a nominative subspecies *S. e. erythrogenys*.

Alarm call, DNA and GPS data collection

For acoustic recordings (48 kHz, 16 bit), we used 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). Twenty-two individuals (10 individuals from the Tara population, 10 individuals from the Altyn-Emel population and 2 individuals from the Balkhash population) were live-captured and recorded for calls emitted toward a researcher when sitting singly in wire-mesh live-traps (10 × 10 × 35 cm). The alarm calls were either produced spontaneously or in response to additional stimulation (walking the researcher near the traps or in response to movements of a hand-held hat). The distance from the microphone to a caller was approximately 1 m. After recording, the animals were

Table 1 Geographic origin of all *Spermophilus* specimens analyzed

Population/ Museum specimen	Location	Region	Date of collection/Collector(s)
Tara, *n* = 10	44.9166N 78.0833E	Alma-Ata region, Koksus district, village Jarlyosek (former village Oktyabr).	10-24.06.2015/ Ivanova A.D., Alexandrov D.Yu., Sibiryakova O.V.
Altyn-Emel, *n* = 10	44.1923N 78.5833E	Alma-Ata region, Kerbulak district, National Park Altyn-Emel, the pass «Stariy».	
Balkhash, *n* = 18	46.2530N 78.9200E	Alma-Ata region, Sarkand district, village Lepsy.	
126* (1)	46.1951N 80.9165E	Alma-Ata region, Alakol district, town Usharal.	30.05.1953/ Strautman E.I.
142* (1)	46.4277N 80.9363E	Alma-Ata region, Alakol district, lake Baybol.	20.06.1948/ Sludsky A.A.
59552* (2)	45.3807N 80.1397E	Alma-Ata region, Sarkand district, aul (village) Ekiasha (former village Pokatilovka).	07.06.2007/ Lopatina N.V.
59553* (2)	46.7275N 79.2150E	Alma-Ata region, Sarkand district, lake Balkhash, estuary of Ayaguz River.	08.06.2007/ Lopatina N.V.
S-64538* (3)	45.5835N 82.0498E	Dzungarian gates, Alma-Ata region, Alakol district, lake Zhalanashkol.	28.05.1959/ Kuzmina M.A.
S-151799* (3)	47.6121N 85.1692E	Zaysan depression, East-Kazakhstan region, Zaysan district, village Karatal.	11.06.1954/ Ivanov O.A.
S-131114* (3)	47.9751N 85.2406E	Zaysan depression, East-Kazakhstan region, Kurchum district, aul (village) Boran (former Buran).	06.05.1967/ Prokopov K.P.
S-143254* (3)	48.3547N 80.4635E	Kazakh Upland, East-Kazakhstan region, Ayagoz district, station Enkerey.	22.06.1987/ Shenbrot G.I.

Museum specimens are labeled with asterisks (*): (1) the Zoological Museum of Institute of Zoology of the Republic of Kazakhstan; (2) the Zoological Museum of Institute of Systematics and Ecology of Animals, Siberian Branch of Russian Academy of Sciences; and (3) the Zoological Museum of Moscow University. *n*, sample size

sampled for the genomic samples (claws of fingers from a hind leg). The place of the cut was treated with antiseptic solution of hydrogen peroxide; the presence of the cut was checked to prevent a repeated inclusion of animals in the analyses. After sampling, the animals were released back at the place of capture. In addition, in the Balkhash population, unmatched call and genomic samples were collected, as 8 live-trapped individuals provided only the genomic samples and 8 free-ranging individuals provided only the alarm calls. Inclusion in the same analyses of calls from live-trapped and free-ranging animals should not affect the results because pattern of calling toward humans and structure of alarm calls are similar in ground squirrels in live-traps and in natural conditions (Nikol'skii 1979; Matrosova *et al*. 2010). Age and sex of the animals were not taken into account, as alarm calls of red-cheeked ground squirrels do not differ between ages and sexes (Zhilin 2002).

To reduce the likelihood of collecting individuals from the same family, we distributed the traps as far as possible based on the size of the colony. The largest distances between traps, up to several hundred meters, were in the Balkhash population, which covered the long coastline of the lake. The smallest distances between traps, up to a few dozens meters, were in the Altyn-Emel population, represented by a small isolated valley. In mid-June, the body weight of the juveniles was already close to those of adults and the natal dispersion had occurred, so we did not observe any mothers associated with the young, which minimized the possibility of individuals belonging to the same families. The trapping points from which the alarm calls and/or gene

samples were labeled by GPS coordinates using Garmin GPSmap 60CS (Garmin, Olathe, KS, USA) at a point close to the point of recording/sampling. Geographical distances between populations were calculated as linear distances (in km) based on GPS coordinates using the Google Earth Internet resource (https://earth.google.com/web).

Molecular analyses

We used a full-size mitochondrial control region and cytochrome *b* gene (cyt *b*) as genomic markers. The control region is highly variable, so it was previously used for both estimating the within-species geographical differences (Ochoa *et al.* 2012; Matrosova *et al.* 2016; Ivanova *et al.* 2017) and for identification of species (Gündüz *et al.* 2007). The cyt *b* is the most commonly used marker for comparison of taxa, and is widely applied in studies of different species of ground squirrels (e.g. Harrison *et al.* 2003; Herron *et al*. 2004; Helgen *et al.* 2009).

Whole genomic DNA was extracted from tissue using the phenol–chloroform method (Sambrook *et al.* 1989). The complete control region flanked by fragments of the tRNA–Pro and the tRNA–Phe genes was amplified using the primers MDL1 and H00651 following Ermakov *et al.* (2002). Polymerase chain reaction (PCR) comprised 35 cycles of 1 min at 94 °C, 1 min at 62 °C and 1 min at 72 °C.

The cyt *b* gene was amplified using 2 primer pairs: L14723 (L7)/GlCbR5a and 397-Sp (L397)/GlCbendR (Ducroz *et al.* 2001; Faerman *et al.* 2017). The PCR comprised 35 cycles of 1 min at 94 °C, 1 min at 56 °C, and 1 or 2 min at 72 °C for 2 pairs of primers, respectively.

Sequencing was performed in an ABI 3730 automated genetic analyzer using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) using PCR primers. The nucleotide sequences were aligned both with BioEdit (Hall 1999) software and manually. We used the MEGA v. 7.0. software (Kumar *et al.* 2016) for data processing. Nucleotide indels were treated as missing data. DNA raw and net divergences were estimated under the Kimura 2-parameter (K2P) model using MEGA v. 7.0. SE were estimated from 1000 bootstrap replicates. For constructing the phylogenetic tree, the maximum likelihood (ML) method was used. The most appropriate DNA substitution model for the dataset was established using jModelTest 2.1.10 (Posada 2008). The ML tree was created with the Hasegawa–Kishino–Yano model, gamma distributed (HKY+G) (–lnL = 2592.53, BIC = 5831.58, AICc = 5389.03). The minimum evolution (ME) and the neighbor-joining (NJ) methods were used to check the stability of the branch topology. Node support values in phylogenetic trees were estimated according to bootstrap support (1000 replicates). The *S. relictus*, *S. pallidicauda* and *S. erythrogenys* were selected as outgroups for creating the phylogenetic tree. Haplotype diversity (h) and nucleotide diversity (π) within each population were calculated in Arlequin v. 3.5 (Excoffier & Lischer 2010) and DnaSP v. 5.10.01 (Librado *et al.* 2009). Haplotype networks were constructed using the median joining method in the PopART software (Leigh & Bryant 2015).

Acoustic analyses

All calls were analyzed spectrographically using Avisoft SASLab Pro software (Avisoft Bioacoustics, Berlin, Germany). Before analysis, the calls were high-pass filtered at 1 kHz to reduce the low-frequency background noise, as preliminary visual analysis of the spectrograms showed that call fundamental frequency always exceeded 1 kHz. Spectrograms were created with Hamming window, FFT 1024 points, frame 50% and overlap 93.75%. For the acoustic analysis, we randomly selected up to 10 alarm calls of good quality per individual, 10 individuals per population, with 282 alarm calls in total, because 6 individuals provided fewer than 10 (from 5 to 8) measurable alarm calls.

Because the alarm call of red-cheeked ground squirrels may contain a different number of notes, we measured the acoustic parameters of each note sequentially and uniformly. In total, we analyzed 344 notes (245 notes from the 245 single-note alarm calls and 99 notes from the 37 multi-note alarm calls). Alarm calls of 12 individuals who provided both the single-note and multi-note alarm calls were used for comparison of the acoustic structure between the notes of the single-note alarm calls and the first notes of the multi-note alarm calls. For this analysis, we averaged within individuals all available alarm calls of each alarm call type (single-note or multi-note).

In the alarm calls, the fundamental frequency band had the highest energy relative to the harmonics (Fig. 2a). As alarm calls of red-cheeked ground squirrels represent tonal notes deeply modulated in frequency (Zhilin 2002), the maximum fundamental frequency (*f0 max*) was clearly visible on the spectrogram (Fig. 2a). We measured the *f0 max*, the start fundamental frequency (*f0 st*) and the end fundamental frequency (*f0 end*) of a note manually from the screen with the reticule cursor. We

measured *duration* of each note within alarm calls manually with the standard marker cursor. From the values of *f0 st* and *f0 end*, we automatically selected the minimum fundamental frequency (*f0 min*). The depth of frequency modulation (*df*) was calculated as the difference between the *f0 max* and the *f0 min*. In addition, the between-note *interval* (Fig. 2a) was calculated where presented. All measurements were exported automatically to Microsoft Excel (Microsoft, Redmond, WA, USA).

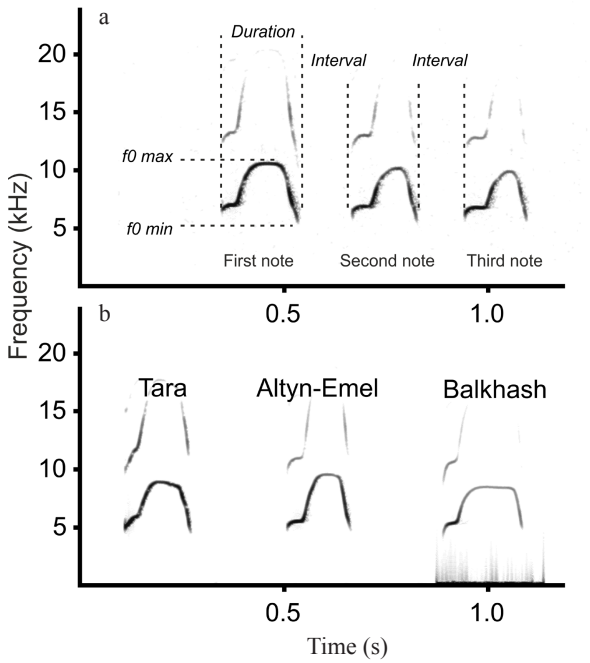

Figure 2 (a) Measured alarm call variables: *f0 max* – the maximum fundamental frequency; *f0 min* – the minimum fundamental frequency; *duration* – duration of a note; *interval* – between-note interval. (b) Examples of three single-note alarm calls of three red-cheeked ground squirrels from study populations (Tara, Altyn-Emel and Balkhash).

Statistical analysis

Statistical analyses were made with STATISTICA v. 6.0 (StatSoft, USA); all means are given as mean ± SD. Significance levels were set at 0.05 and 2-tailed probability values are reported. We used repeated measures analysis of variance (RM ANOVA) to compare the acoustics between the averaged per individual notes of the single-note alarm calls and the first notes of the multi-note alarm calls. To estimate the acoustic differences between populations, we performed a standard procedure of discriminant function analysis (DFA) using the 3 least correlated acoustic variables that mainly contributed to discrimination (*duration*, *f0 min*, *f0 max*). We used ANOVA with a Tukey honestly significant difference (HSD) test with the averaged per individual values of the acoustic parameters of the single-note alarm calls and/or the first note of the multi-note alarm calls to assess whether the acoustic variables differed between populations.

RESULTS

General acoustic structure of alarm calls

The subject red-cheeked ground squirrels mostly produced the single-note alarm calls (87% of the total number). Much more rarely (13%) they produced the multi-note alarm calls, consisting of 2–6 notes separated with internote intervals ranging from 33 to 312 ms (on average, 132 ± 74 ms). To account for the potential differences between the first and other alarm calls within clusters (Randall & Rogovin 2002), we compared the acoustic structure between notes of the single-note

Table 2 Values (mean ± SD) of variables of the notes differing in position within the alarm call (single, first, other position) and RM ANOVA results for their comparison

Acoustic variable	Single-note alarm calls, $n = 245$	Multi-note alarm calls, $n = 99$		ANOVA	
		First notes, $n = 37$	Other notes, $n = 62$	Single notes *vs* First notes	First notes *vs* Other notes
duration (ms)	168 ± 25	153 ± 17	139 ± 17	$F_{1,11} = 0.11$, $P = 0.75$	$F_{1,11} = 29.37$, $P < 0.001$
f0 min (kHz)	4.48 ± 0.46	4.66 ± 0.47	4.26 ± 0.41	$F_{1,11} = 0.52$, $P = 0.49$	$F_{1,11} = 8.15$, $P = 0.02$
f0 max (kHz)	8.45 ± 0.72	8.56 ± 0.63	8.07 ± 0.68	$F_{1,11} = 0.004$, $P = 0.95$	$F_{1,11} = 28.02$, $P < 0.001$
df (kHz)	3.98 ± 0.60	3.90 ± 0.61	3.81 ± 0.52	$F_{1,11} = 0.21$, $P = 0.66$	$F_{1,11} = 2.16$, $P = 0.17$

n, number of averaged per individual notes (one note per alarm call).

alarm calls, the first notes of the multi-note alarm calls and other notes of the multi-note alarm calls. In the multi-note alarm calls, *duration*, *f0 max* and *f0 min* were significantly higher in the first notes compared to other notes (2nd, 3rd, etc.), whereas *df* did not differ significantly between the first and other notes (RM ANOVA, Table 2). At the same time, the first notes of the multi-note alarm calls and the notes of the single-note alarm calls did not differ in any acoustic variable (RM ANOVA, Table 2). Thus, for each individual, we used for further analysis only the averaged per individual first notes of the alarm calls regardless of the presence or absence of other notes.

The *duration* of the first alarm call note (n = 282) ranged from 125 to 237 ms (on average, 167 ± 25 ms); *f0 max* ranged from 7.19 to 9.90 kHz (on average, 8.46 ± 0.70 kHz), *f0 min* ranged from 3.63 to 5.33 kHz (on average, 4.49 ± 0.42 kHz) and *df* ranged from 3.01 to 5.05 kHz (on average, 3.96 ± 0.54 kHz).

Interpopulation acoustic differences

Alarm call variables were very similar between the 3 study populations (Fig. 2b). Minor, although significant, differences were only revealed in *duration* (Table 3). DFA, based on the averaged per individual values of 3 acoustic variables of the first notes of alarm calls, showed 56.7% correct assignment of the alarm calls to population.

Genetic comparison with closely related taxa

We found 15 unique cyt *b* haplotypes: 7 haplotypes among the examined 8 museum specimens obtained near the type localities of different closely related forms and 8 haplotypes among the 30 examined live-trapped individuals (Table S1). The cyt *b* sequence data displayed a clear phylogeographic structure (Fig. 3a). On the phylogenetic tree, ground squirrels from Southeast Kazakhstan display 3 clades with very high (98–100%) bootstrap support irrespective of the applied algorithm for creating the tree (NJ, ML or ME). The first clade comprises ground squirrels from the Balkhash, Tara and Altyn-Emel (form "*intermedius*" MH518104-MH518107); among them, the Altyn-Emel ground squirrels are most separated. The second clade comprises specimens from the Zaysan depression (form "*brevicauda*" MH518109-MH518110) and from surroundings of the Dzungarian Gate (form "*carruthersi*" MH518108). The third clade comprises specimens from the left bank of the Ily River (form "*iliensis*" MH518111) and from the southeast part of the Kazakh Upland (Fig 3b; see also Fig 1).

Raw genetic distances between the 3 clades inhabiting Southeast Kazakhstan ("*brevicauda*," "*iliensis*" and "*intermedius*") varied from 2.9% to 3.9% (Table 4). The raw distances between these clades and *S. erythrogenys* varied from 6.2% to 7.0%. Similarly, net genetic distances between clades "*brevicauda*," "*iliensis*" and "*intermedius*" varied from 2.3% to 2.9% (Table 4) and from 6.1% to 6.5% between these clades and *S. erythrogenys*.

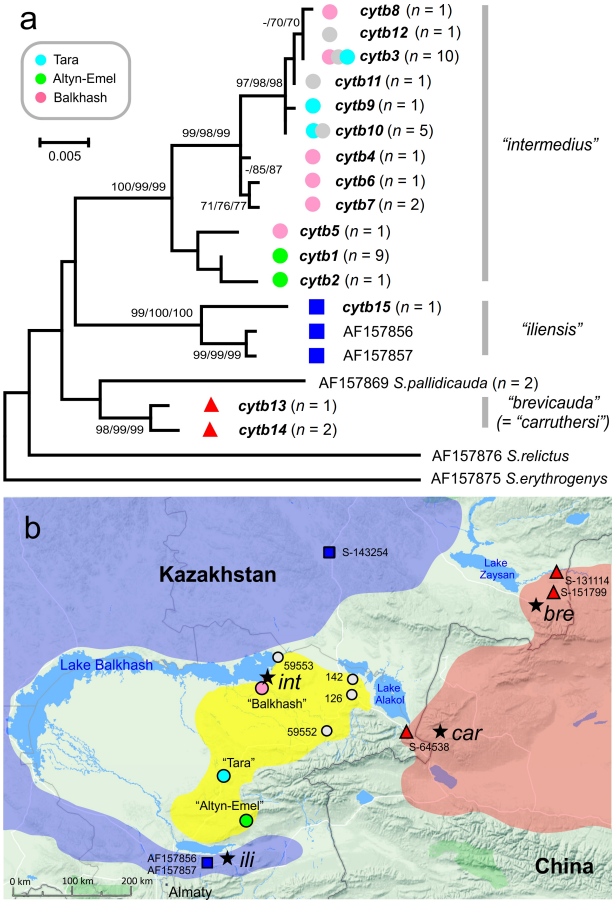

Figure 3 Suggested phylogenetic relationships of red-cheecked ground squirrels and the closely related taxa. (a) ML phylogenetic tree (HKY+G model) based on analyses of 15 unique cyt *b* haplotypes found among study specimens from Southeast Kazakhstan. Numbers near the branches denote percentage bootstrap resampling support from 1000 replications (ML/ME/NJ). Bootstrap support is only shown for the values exceeding 70%. *n* – number of sequences representing each haplotype. (b) Suggested distribution areas for the given forms. Figures near a sample name designate clustering of the sample with a particular taxon accordingly to the cyt *b* polymorphism (triangles – "*brevicauda*", squares – "*iliensis*", circles – "*intermedius*"). Stars designate type locality ("*brevicauda*", "*carruthersi*", "*iliensis*", "*intermedius*"). For details, see Table S1.

Table 3 Values (mean ± SD) of the alarm call first notes and ANOVA results for their comparison between 3 populations

Acoustic variable	Population			ANOVA results for 3 populations
	Tara, $n = 10$	Altyn-Emel, $n = 10$	Balkhash, $n = 10$	
duration (ms)	158 ± 15[a]	158 ± 16[a]	185 ± 32[b]	$F_{2,27} = 4.57$, $P < 0.05$
f0 min (kHz)	4.48 ± 0.42	4.39 ± 0.35	4.62 ± 0.50	$F_{2,27} = 0.73$, $P = 0.49$
f0 max (kHz)	8.36 ± 0.80	8.56 ± 0.67	8.45 ± 0.67	$F_{2,27} = 0.21$, $P = 0.81$
df (kHz)	3.87 ± 0.58	4.18 ± 0.60	3.84 ± 0.39	$F_{2,27} = 1.21$, $P = 0.31$

The same superscripts indicate which populations did not differ significantly ($P > 0.05$, Tukey HSD post-hoc test). *n*, number of averaged alarm calls (1 per individual).

Interpopulation genetic differences

Overall genetic diversity

We analyzed in detail the nucleotide sequences of the full-size mtDNA control region (1005-1006 bp, GenBank Acc. No.: MH518112-MH518141) and full cyt *b* (1140 bp, MH518074-MC518103) in 30 red-cheeked ground squirrels from Tara, Altyn-Emel and Balkhash populations (10 individuals per population). For the control region, 33 sites (approximately 3% of the full fragment length) were variable, of which 21 were parsimony informative. Similarly, for the cyt *b*, 29 sites (approximately 2.5% of the full fragment length) were variable, of which 21 were parsimony informative.

We summarize the genetic characteristics in Table 5. Both mtDNA markers yielded similar results. Nucleotide diversity (π) was very low; in the pooled sample set of the 30 individuals it was 0.0097 ± 0.0051 for the control region and 0.0088 ± 0.0046 for cyt *b*. Haplotype diversity (*h*) was substantially higher; in the pooled sample set of the 30 individuals it was 0.8575 ± 0.0423 for control region and 0.8184 ± 0.0457 for cyt *b*.

Table 4 Raw (below the diagonal) and net (above the diagonal) K2P-distances between the 3 main genetic (cyt *b*) linages of ground squirrels inhabiting the Southeast Kazakhstan, with associated standard errors (SE) based on 1000 bootstrap replicates in parentheses

	intermedius $N = 34$	*brevicauda* $N = 3$	*iliensis* $N = 3$
intermedius	—	0.023(0.004)	0.029(0.006)
brevicauda	0.029(0.005)	—	0.025(0.005)
iliensis	0.039(0.006)	0.032(0.005)	—

N, number of sequences.

Table 5 Genetic characteristics of 3 study populations of red-cheeked ground squirrels

Population	C-region			Cytochrome *b*		
	N_{hapl}	π (SD)	*h* (SD)	N_{hapl}	π (SD)	*h* (SD)
Tara, $n = 10$	3[†]	0.0046 (0.0028)	0.6444 (0.1012)	3[†]	0.0012 (0.0009)	0.6444 (0.1012)
Altyn-Emel $n = 10$	3	0.0006 (0.0006)	0.3778 (0.1813)	2	0.0007 (0.0006)	0.2000 (0.1541)
Balkhash $n = 10$	8[†]	0.0087 (0.0049)	0.9333 (0.0773)	6[†]	0.0069 (0.0040)	0.8444 (0.1029)
Total $n = 30$	13	0.0097 (0.0051)	0.8575 (0.0423)	10	0.0088 (0.0046)	0.8184 (0.0457)
Mean nucleotide composition	33.5% (T), 30.5% (A), 23.7% (C), 12.3% (G)			34.1% (T), 28.3% (A), 24.8% (C), 12.7% (G)		

[†]One haplotype was shared between Tara and Balkhash populations. Designations: *h*, haplotype diversity; *n*, the number of assayed animals in population; N_{hapl}, number of found haplotypes; π, nucleotide diversity (averaged over loci); SD, standard deviation.

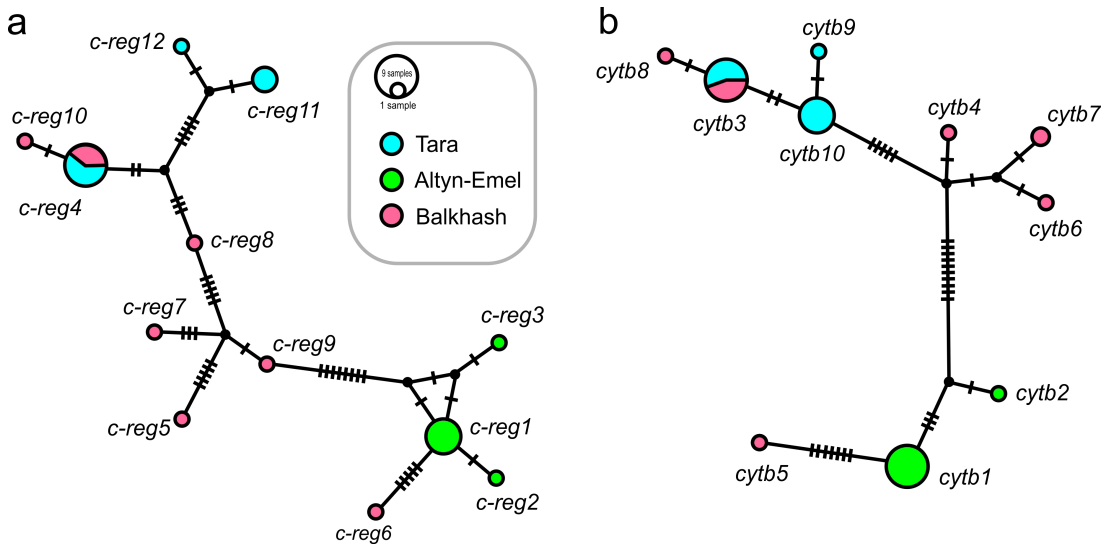

Figure 4 Unrooted haplotype network of absolute distances between mitochondrial DNA haplotypes of (a) control region and (b) cytochrome *b* gene. Each circle represents a unique haplotype; its size proportional to the haplotype frequency. Small black circles represent hypothetical haplotypes. Colors represent the three study populations. The hatches on the lines connecting haplotypes represent nucleotide substitutions. For details, see Table S1.

Interpopulation genetic diversity

Among the 30 individuals examined, 13 control region haplotypes and 10 cyt *b* haplotypes were identified (Table 4 and Table S1). Both markers demonstrated 1 shared haplotype between individuals from Tara and Balkhash populations. Most unique haplotypes were found in the Balkhash population. In contrast, individuals from the Altyn-Emel population were practically identical genetically; only 2 individuals had single substitutions in their haplotypes. Both the control region and the cyt *b* sequence data showed a weak phylogeographic structure, without any subdivision into phylogroups (Fig. 4). Clusters of close haplotypes do not correspond to certain geographical areas, with the exception of the Altyn-Emel population, which is the most remote and isolated within the Dzungarian Alatau southwest ridges. Within-group pairwise distances (K2P) were 0.0046 ± 0.0016 (Tara), 0.0006 ± 0.0004 (Altyn-Emel) and 0.008 ± 0.0020 (Balkhash) for the control region and 0.0012 ± 0.0007 (Tara), 0.0007 ± 0.0004 (Altyn-Emel) and 0.007 ± 0.0016 (Balkhash) for the cyt *b*.

Between-group distances (K2P) comprised 0.014 ± 0.003 (Altyn-Emel–Tara), 0.012 ± 0.003 (Altyn-Emel–Balkash) and 0.008 ± 0.002 (Tara–Balkhash) for the control region and 0.016 ± 0.003 (Altyn-Emel–Tara), 0.014 ± 0.003 (Altyn-Emel–Balkash) and 0.005 ± 0.001 (Tara–Balkhash) for the cyt *b*.

DISCUSSION

Alarm call differences

Based on the acoustics of alarm calls, 3 study populations were very similar, in agreement with findings for *Spermophilus suslicus* (Matrosova *et al.* 2016), indicating that the bioacoustical changes of alarm calls are more conservative compared to the genetic changes in speciation of ground squirrels. At the same time, our results confirmed the early evidence of noticeable differences in the acoustics of the alarm calls between the red-cheeked ground squirrels of Southeast Kazakhstan and the red-cheeked ground squirrels of the Southern Trans-Ural (Kurgan region) of Russia (Nikol'skii 1979; Nikol'skii & Starikov 1997; Zhilin 2002; Nikol'skii & Rumyantsev 2004).

In the 3 studied "*intermedius*" populations from Southeast Kazakhstan, the alarm call note duration was 167 ± 25 ms compared to much shorter (119.8 ± 73.98 ms) duration reported for the "*iliensis*" red-cheeked ground squirrels from Russia (Zhilin 2002). In addition, an average maximum fundamental frequency was substantially higher in "*intermedius*" populations from

Southeast Kazakhstan (8.46 ± 0.75 kHz) than in "*iliensis*" red-cheeked ground squirrels from Russia (5.62 ± 0.06 kHz) (Zhilin 2002). Further differences were observed in ratios of single-note/multi-note alarm calls: whereas the ground squirrels from Southeast Kazakhstan mostly produced the single-note alarm calls (this study), the ground squirrels from Russia mostly produced the multi-note alarm calls (Zhilin 2002; Nikol'skii & Rumyantsev 2004). The degree of acoustic differences between the "*intermedius*" and "*iliensis*" ground squirrels was comparable with those between the Taurus (*Spermophilus taurensis*), Anatolian (*Spermophilus xanthoprymnus*) and European ground squirrels (*Spermophilus citellus*) (Gündüz *et al.* 2007). The Taurus and Anatolian ground squirrels were recently separated to different species based on their alarm call traits (Schneiderová & Policht 2011), as they differed from each other by the alarm call structure as strongly as from the closely related species, the European ground squirrel (Schneiderová & Policht 2012). Our results provide similar arguments in favor of species autonomy of potential *S. e. intermedius* from Southeast Kazakhstan and potential *S. e. iliensis* from Russia. Information about the acoustics of the third line of red-cheeked ground squirrels ("*brevicauda*") is scarce; the frequency pattern of the alarm call is probably different (Nikol'skii & Rumyantsev 2004). Further research is necessary to investigate the acoustic differences between the potential species *S. e. intermedius* and *S. brevicauda* etc.

Mitochondrial DNA differences

The within-species level of control region variation, studied in the 30 living specimens in this study, showed that 3% positions were variable. This degree of variation was comparable with that reported for the Perote ground squirrel *Xerospermophilus perotensis* (2%, Ochoa *et al.* 2012) but was substantially lower than that reported for the *S. suslicus* (8%, Matrosova *et al.* 2016), which diverged to 2 different geographically separated chromosome races with *p*-distance of 4.4–4.6% between them (Brandler *et al.* 2015; Matrosova *et al.* 2016). The within-species level of cyt *b* variation of 2.5% of variable positions was substantially lower than in the European ground squirrel *S. citellus* (6%, Kryštufek *et al.* 2009) but higher than in the Alashan ground squirrel *S. alashanicus* (0.11%, Kapustina *et al.* 2015).

Interspecies genetic distances based on cyt *b* sequences for "*intermedius*," "*brevicauda*" and "*iliensis*" lineages were 2.7–3.6% between these 3 forms. This degree of variation is comparable with the distances between such well-separated species as *Spermophilus fulvus*, *Spermophilus rallii* and *S. pallidicauda*, of 2.9–3.2% in all pairwise comparisons (Kapustina *et al.* 2015).

Net genetic distances between the *S. erythrogenys* from one side and the "*intermedius*," "*brevicauda*" or "*iliensis*" lineages from another side were highly significant and amounted of 6.1–6.5%, which indicates paraphilia of these taxa. Recently described as separated species, the Taurus ground squirrel *S. taurensis* differs from the most closely related *S. citellus* by cyt *b* genetic distance of 5.0%, whereas the difference between *S. taurensis* and *S. xanthoprymnus* comprised 9.6%, and the difference between *S. citellus* and *S. xanthoprymnus* comprised 9.8% (Gündüz *et al.* 2007). Our analyses of specimens from Southeast Kazakhstan suggest the presence of 3 major mtDNA lineages, displaying mtDNA distances of potential species-rank levels, "*intermedius*," "*brevicauda*" and "*iliensis*," and point to the potential role of geographical barriers in a substantial genetic differentiation of red-cheeked ground squirrels in Southeast Kazakhstan.

We conclude that the *Spermophilus erythrogenys* sensu lato complex needs further taxonomical revision. The 3 mtDNA lineages revealed in this study point, respectively, to the 3 putative species, which should be validated with inclusion of data on the alarm call acoustics from the "*brevicauda*" and "*iliensis*" lineages, with analyses of nuclear markers of specimens representative for all the 3 mtDNA lineages, "*intermedius*," "*brevicauda*" and "*iliensis*," and with a rigorous morphological examination. The inclusion of nuclear DNA markers, and acoustic and morphological data for validating potential species of ground squirrels is necessary because the recently speciated Eurasian *Spermophilus* species may be genetically distinct but not morphologically well-differentiated, as was revealed in the case of the geographically widespread Anatolian ground squirrel, *S. xanthoprymnus* (Gündüz *et al.* 2007).

"Intermedius" lineage

The 3 study populations (Tara, Altyn-Emel and Balkhash), comprising a common mtDNA clade, are all isolated by the Balkhash-Alakol depression from the north and east, by the Ili River and by the Dzungarian Alatau Mountains from the west and south (Fig. 1). Following Belyaev (1955), this clade can be considered as a potential new species *S. intermedius* Brandt, 1843, described earlier from this territory (southeast bank of the Balkhash Lake, village Lepsy) (Belyaev 1955).

"Brevicauda" lineage

Ground squirrels inhabiting territories from the Zaysan Lake and the Mongolian Altai Mountains to the Ebinur Lake and the Borohoro Mountains (from the

north to the south) (Fig. 1) are isolated from the "*intermedius*" lineage by the Balkhash-Alakol depression and probably also by the Tarbagatai, Saur, Urkashar and Birliktau Mountains. Grounds squirrels of this territory can be considered as a potential new species *S. brevicauda* Brandt, 1843 by a principle of priority, so *S. carruthersi* (Thomas, 1912) would be a synonym (Smith *et al.* 2008).

"Iliensis" lineage

Ground squirrels inhabiting a territory to the west from the Ily River barrier (including the right bank of the Ily River, the Chu-Ily Mountains, the western and northern bank of the Balkhash Lake (Fig. 1), probably belong to the same "*iliensis*" mtDNA lineage and can be considered as a potential new species *S. iliensis* (Belyaev, 1945), described from the right bank of the Ily River or *S. selevini* (Argyropulo, 1941) described from the Southeast Kazakh Uplands.

For further research, we suggest new molecular analyses of the *Spermophilus erythrogenys* sensu lato complex with more specimens included in analyses to identify potential "cryptic" species. For example, the studies by Harrison *et al.* (2003) and Herron *et al.* (2004) presented genealogies on the basis of the cyt *b* polymorphism that demonstrated that Eurasian *Spermophilus* form a monophyletic group within Marmotini. However, they studied too few specimens per species to confidently validate new cryptic species.

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SUPPLEMENTARY MATERIALS

Additional supporting information may be found in the online version of this article at the publisher's website.

Table S1 List of samples. In the names of the samples, "A" stands for the origin of the Altyn-Emel population, "T" for the Tara population and "B" for the Balkhash population

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