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MOLECULAR GENETICS

RAPD–PCR Analysis of Ground Squirrels from the Tobol–Ishim Interfluve: Evidence for Interspecific Hybridization between Ground Squirrel Species *Spermophilus major* and *S. erythrogenys*

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Abstract—Populations of two ground squirrel species, *Spermophilus major* and *S. erythrogenys,* from the interfluvial area of the Tobol and Ishim rivers, where their ranges overlap, have been examined using RAPD–PCR. We have identified 253 loci, which included taxon-specific markers for *S. major* and *S. erythrogenys* as well as markers for geographic populations. Estimation of genetic diversity and construction of phylogenetic relationships were performed using software programs POPGENE, TEPGA, and TREECON. In all, based on morphological traits, animals from the Tobol–Ishim interfluve were assigned to the two parental morphotypes and showed similar levels of genetic variability (H, n_a, n_e). However, the total polymorphism level proved to be higher in ground squirrels with the *major* morphotype (P = 40.32%, P_{95} = 27.27%) than in animals with the *erythrogenys* morphotype (P = 32%, P_{95} = 22.13%). Nevertheless, the number of rare alleles was high in both cases, constituting about 70% of the total number. Interpopulation differentiation was considerably higher in *S. major* (δ = 0.50) than in *S. erythrogenys* (δ = 0.41). The genetic differentiation between local samples from the Tobol–Ishim interfluvial area was lower than that between the parental species. A significant part of the genetic diversity of the species examined and animals from the zone of overlapping ranges was accounted for by intrapopulation variability. Animals from the northern and southern parts of the Tobol–Ishim interfluve were characterized by the core traits of *S. major* and *S. erythrogenys*, respectively, falling into two distinct clusters in the UPGMA and NJ reconstructions. In addition to three hybrid individuals, identified by the bioacoustic method, three hybrid animals were distinguished using RAPD analysis. These animals earlier were thought to be "pure" species and formed their own clusters in phylogenetic reconstructions. Thus, the RAPD–PCR results directly showed the existence of stable hybridization (20% genetic hybrids) between *S. major* and *S. erythrogenys* in the Tobol–Ishim interfluvial area, which is more extensive than inferred previously from morphological and bioacoustic data.

INTRODUCTION

Two species of the genus of ground squirrels *Spermophilus* (*S. major* Pallas, 1778 and *S. erythrogenys* Brandt, 1843) attract the attention of researchers because of their debatable taxonomic attribution [1–3], unclear range boundaries, and the existence of hybridization between them in contact zones [4, 5]. In the present work, we have accepted the view of Gromov on the systematic position of these ground squirrel species [2, 3] and the boundaries of their ranges, taking into account the results of bioacoustic and craniometric studies [4–6], as well as cytological and biochemical analyses [7]. According to these data, ground squirrels from the left bank of the Irtysh River (the *heptneri* = *ungae* form) are assigned to the species *S. erythrogenys*. To date, the species status of *S. major* and *S. erythrogenys* is quite clear. In particular, these species are well differentiated on the basis of a number of cytogenetic [7–9] and genetic biochemical traits [10]. Some light was shed on these issues by the results of bioacoustic diagnostics of ground squirrels, based on the species-specificity of danger-warning signals in all species of the genus *Spermophilus* from Eurasia [4, 5, 11–13]. These studies did not only verify important features of both species, but also revealed the intergradation zone (Kurgan oblast, the Tobol–Ishim interfluve), where stable settlements of interspecific hybrids were found. At present, two contact zones harboring settlements shared by the species, North Vargashinskaya and Romanovskaya, have been established in the area of overlapping of the *S. major* and *S. erythrogenys* ranges in Kurgan oblast [11]. An unusual for both species signal with predominant (in frequency) elements similar to *S. major* was recorded in the great majority of animals from this area [5]. Since the bioacoustic diagnostics,

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Table 1. Specimens examined in the study

Species	Locality	Number of specimens	Locality no.
S. major	Ul'yanovsk oblast, vicinity of Malovka village	3	1
S. e. erythrogenys	Novosibirsk oblast, Karasukskii district, settlement of Karasuk	3	2
S. e. brevicauda	Kazakhstan, Chilikskii district, settlement of Tolkin	1	4
	Kazakhstan, Chilikskii district, settlement of Prudkhoz	1	5
	Kazakhstan, 149 km along Almaty–Narynkol highway	3	6
	Kokchetav oblast, Ruzaevskii district, village of Andreevka	6	15
S. e. heptneri	Pavlodar oblast, Aksukskii district, settlement of Kyzyl-Zhar	1	3
Animals from Tobol–Ishim interfluve			
Morphotype "*major*"	Kurgan oblast, Pritobol'nyi district, village of Zaborskoe	1	9
	Kurgan oblast, Pritobol'nyi district, village of Obukhovo	4	10
	Kurgan oblast, Ketovskii district, village of Temlyakovo	4	8
	Kurgan oblast, Pritobol'nyi district, village of Utyatskoe	5	7
Hybrid with traits of "*major*"	Kurgan oblast, Vargashinskii district, village of Shastovo	2	11
Hybrid with traits of "*erythrogenys*"	Kurgan oblast, Polovinskii district, village of Romanovo	1	12
Morphotype "*erythrogenys*"	Kurgan oblast, Polovinskii district, village of Privol'noe	9	13
	Kurgan oblast, Polovinskii district, village of Sukhmen'	5	14

like most traditional methods, can only indirectly confirm a hybrid origin of animals, classic approaches and modern molecular genetic methods should be combined to resolve the issue on interspecific hybridization in ground squirrels.

Using RAPD analysis, many independent loci dispersed throughout the genome can be examined. Recently, it has been shown that amplification products include all types of DNA sequences [14]. In closely related species that are poorly differentiated by other characters, RAPD markers in most cases can detect cryptic genetic variation [14]. Species-specific RAPD markers can also be used to identify hybrids and study hybrid speciation, for which the information in individual variability of the parental populations is required [15].

The aim of the present work was finding molecular genetic evidence for extensive hybridization between *S. major* and *S. erythrogenys* in the zone where their ranges overlap (the Tobol–Ishim interfluve) and establishing the *S. erythrogenys* form hybridized by *S. major.* The tasks of the study were as follows: (1) detecting taxon-specific and population molecular markers; (2) quantifying genetic variability of the local populations and their differentiation; (3) estimating the total level of genetic diversity in the pooled sample; (4) reconstructing phenetic and phylogenetic relationships between the ground squirrels; and (5) assessing correlation between acoustic signals and genetic characters.

MATERIALS AND METHODS

We examined 49 ground squirrels from 15 localities, which were represented by animals from the zone of overlapping ranges of *S. major* and *S. erythrogenys*, as well as by "pure" parental species outside of this zone (Table 1, Fig. 1). Samples represented only by one animal were analyzed in the total sample, but excluded from population analysis. A member of a related genus, marmot *Marmota bobak*, was taken as an outgroup. DNA was isolated using a standard procedure [16].

For RAPD–PCR analysis, we used nine primers (OPC-02, OPC-05, OPC-08, OPC-09, OPC-12, OPC-16, OPC-20, OPD-05, and OPE-20, Operon Technologies, United States), which produced clearly read and reproducible amplification products. PCR was run in 25 μl of a reaction mixture, containing 60 ng of total DNA, 1× buffer (67 mM Tris-HCl, pH 8.8; 2 mM $MgCl_2$, 0.01% Tween-20, 0.01 M 2-mercaptoethanol), 0.2 mM each dNTP, 0.5 μl of the primer, 1 unit of *Taq* polymerase, in the following regime: denaturing for 2 min at 94°C; 41 cycles (denaturing for 1 min at 94°C, annealing for 30 s at 37°C, 15 s at 45°C, and elongation for 2 min at 72°C). The final elongation was conducted

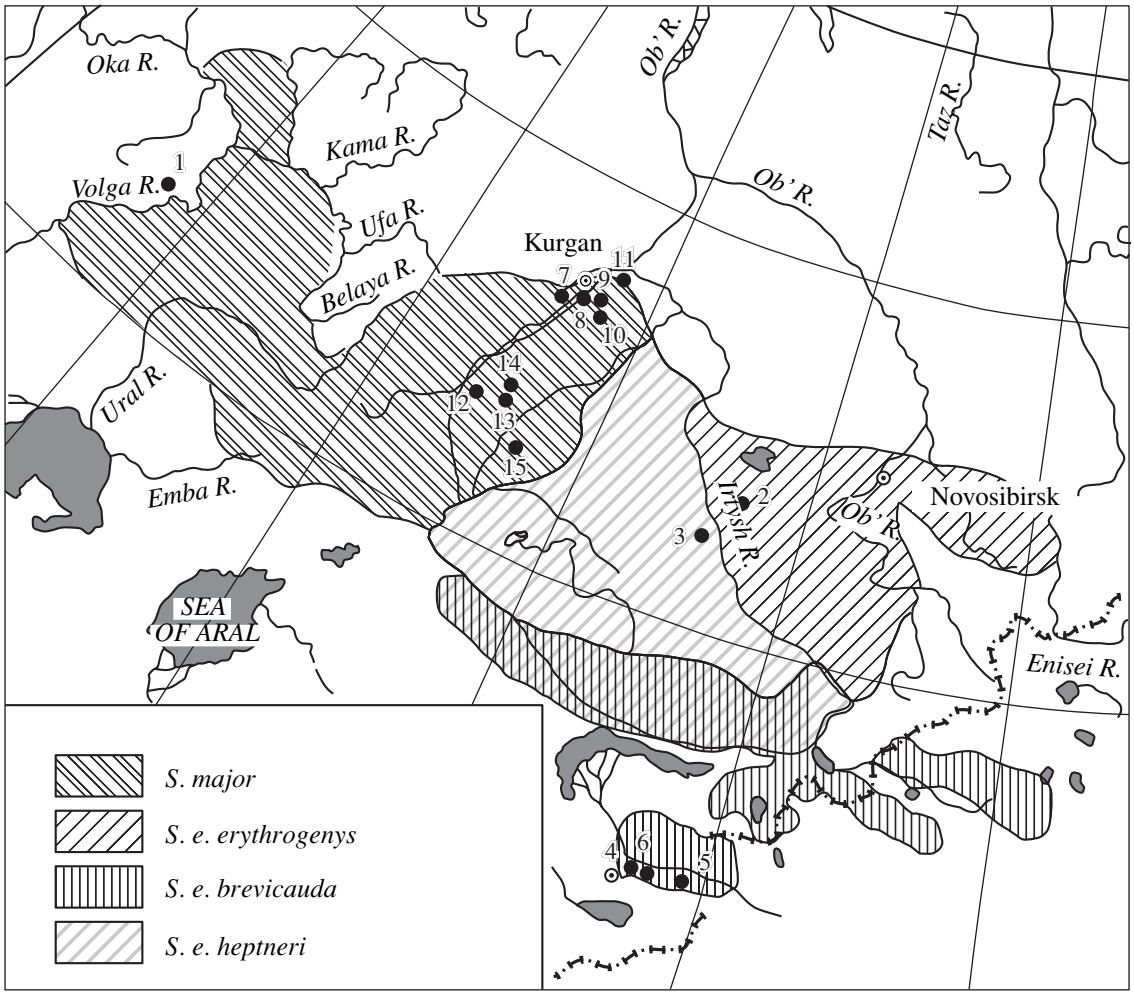

Fig. 1. Ground squirrel ranges and sampling localities (designated by numerals as in Table 1).

for 6 min at 72°C. The RAPD–PCR products were fractionated by electrophoresis in 2% agarose gel, containing 0.5 μg/ml of ethidium bromide in 1× TBE buffer, and photographed in UV light. Lambda phage DNA digested with *Pst*I was used as a molecular mass marker. The electrophoregrams were used for constructing binary matrices, where the presence or absence of a band was indicated as 1 or 0, respectively. In counting the fragments, all visually detected bands were scored.

Parameters of genetic variation and differentiations were estimated using software programs POPGENE [17] and TFPGA version 1.3 [18]. Genetic variability of populations was estimated as the proportion of polymorphic loci at the 95% significance criterion (P_{95}) and without it (P), the average (n_a) and the effective (n_e) number of alleles per locus, mean heterozygosity (H), gene diversity (h), and intrapopulation differentiation (δ_S), and the similarity/dissimilarity parameter (S/D) [17]. To assess genetic differences among the populations and the level of population differentiation, we used the index of interpopulation differentiation (G_{ST}) and the number of migrants per generation among local populations (N_m), as well as total genetic diversity among the samples (D_{ST}), which was computed from the total genetic diversity (H_T) and the mean sample genetic diversity (H_S), as described by Gregorius [19]. In addition, we computed interpopulation genetic distances D_N [17] and performed exact test for population differentiation [18]. To generate phylogenetic reconstructions (NJ, MST) and UPGMA phenograms, software packages TREECON version 1.3 [20] and NTSYS pc version 1.7 [21] were used.

RESULTS

Variability of the RAPD Patterns

The amplification products showed a distribution of amplified fragment unique for each primer. Apart from rare exceptions, primers OPC-09, OPC-20, and OPE-20 produced monomorphic profiles for the total ground squirrel sample. Six out of nine primers (OPC-02, OPC-05, OPC-08, OPC-12, OPC-20, OPD-05) proved

to be specific for polymorphic DNA sequences (Fig. 2). The band number in the RAPD patterns varied from two to twelve, and molecular weight of the fragments, from 0.279 to 2.431 kb, constituting on average 1.36 kb. In all, we have identified 253 characters. In most cases, the RAPD profiles of all animals from the zone of overlapping ranges of *S. major* and *S. erythrogenys* (populations 7–15, see Table 1) were very similar, but also had unique fragments occurring in some specimens or populations.

Molecular markers of different taxonomic levels were found. For instance, fragments OPC-02_{2331}, OPC-20_{737}, and OPD-05_{1672} were common for the genera *Spermophilus* and *Marmota* that are highly differentiated in all other characters. Primers OPD-05, OPC-02, and OPC-05 proved best for differentiating among the *Spermophilus* species and their populations (see Fig. 2); the RAPD profiles of *S. major* and *S. e. erythrogenys* showed closer similarity to one another than to *S. e. brevicauda*. The OPD-05_{398} fragment marked the species *S. major* and *S. erythrogenys*, occurring in all animals from localities Temlyakovo, Obukhovo, Utyatskoe, Shastovo, Zaborskoe, and Romanovo. Moreover, all animals from localities Malovka, Privol'noe, and Sukhmen' differed from the other individuals by the presence of the OPD-05_{483} fragment, which seems to be a molecular marker for the species *S. major*. The OPC-05_{718} fragment discriminated the *S. major* sample from the other species. This fragment was polymorphic and found only in populations Temlyakovo (in two of four animals), Obukhovo (in three of four animals), and Romanovo. The OPC-05_{1373} character, characteristic of *S. e. erythrogenys, S. e. brevicauda*, and *S. e. heptneri*, was absent in all *S. major* individuals. This fragment was also found in the genome of each animals from Sukhmen', Privol'noe, and Andreevka, while in the local samples from Obukhovo, Shastovo, and Romanovo (where OPC-05_{718}, characteristic of *S. major*, occurs), this fragment was found only in some individuals.

In addition to the qualitative differences among the ground squirrel samples, some fragments manifested clear quantitative differences in fragment majority. For example, all *S. major* animals from the vicinity of the village of Malakhovka, as well as from the local samples from Temlyakovo, Utyatskoe, and Shastovo, had a prominent fragment OPC-20_{1093}, which was underrepresented in the other populations (Fig. 2c). The OPD-05_{295} character was observed in all ground squirrels examined, but had different frequencies in different localities.

By the RAPD profiles, the animals from the northern part of the Tobol–Ishim interfluve (populations 7–11, see Table 1) are visually very similar to *S. major*, and the animals from its southern part (populations 14–15), to *S. e. brevicauda*.

Hybrid animals from Shastovo, which are morphologically very similar to *S. major*, and a ground squirrel from Romanovo, more similar to *S. erythrogenys*, in some cases exhibited specific fragment distributions, distinguishing them from the other animals.

Population Genetic Variability

The average (n_a) and effective (n_e) numbers of alleles per locus in the samples from different populations were similar and low (Table 2). Animals with the *major* and *erythrogenys* morphotypes showed the same values of genetic diversity parameters, except intrapopulation differentiation, which varied in a wide range, being maximum in *S. major*. In animals from all interfluvial populations (the total sample), n_a was as high as 1.72, while n_e remained low (1.18).

Heterozygosity of ground squirrels from different samples also proved to be rather low. On average, each individual had few heterozygous loci ($H = 0.11$). The minimum H (0.06) was found in animals from the village of Privol'noe, the maximum (0.12), in animals from the settlement of Temlyakovo (see Table 2). However, heterozygosity was somewhat higher in ground squirrels with the *major* morphotype than in those with the *erythrogenys* morphotype.

Intrapopulation differentiation δ_S of ground squirrels from different samples was high, varying from 0.4 in *S. e. brevicauda* in the combined sample from Andreevka and Southeastern Kazakhstan, to 0.86 in *S. major* from Malovka. Comparing the δ_S values in animals of different morphotypes from the interfluve, we have shown that differentiation among the animals of the *major* morphotype was higher than that in animals with the *erythrogenys* morphotype.

Genetic polymorphism was estimated as the total proportion of the variable RAPD characters in their total number. This parameter was 40.32% in the *major* morphotype and 32.02% in the *erythrogenys* morphotype. At the 95% significance level, the polymorphism value decreased to respectively 27.27 and 22.13%. This means that the number of rare alleles was high in both morphotypes, constituting 68% from the total number of polymorphic loci. Individuals of *S. e. brevicauda* from Andreevka and Southeastern Kazakhstan exhibited the highest polymorphism (see Table 2).

Genetic Differentiation

The estimates of genetic differentiation between all animals from the Tobol–Ishim interfluve, where hybridization presumably occurs (arbitrarily referred to as "hybrids"), and pure parental species are presented in Table 3. Pairwise comparisons yielded low values of mean within-sample gene diversity (H_S = 0.04–0.11). By contrast, the values of total gene diversity H_T = 0.12–0.16) in some cases were several times higher than the latter parameter, which suggests significant differentiation of the pairs (see Table 3). Total genetic diversityD_{ST} among the pairs varied from 0.02 to 0.08, i.e., most genetic diversity is within populations. The total genetic diversity was maximum in *S. major* and

Table 2. Intrapopulation genetic diversity of *S. major*, *S. erythrogenys*, and individuals from the Tobol–Ishim interfluvial area

Species, morphotype, locality		n_a	n_e	h	H	δ_S	S/D	P, %	P_{95}, %
Morphotype *major* from interfluve	Temlyakovo (n = 4)	1.29 ± 0.5	1.16 ± 0.61	0.10 ± 0.17	0.12	0.45 ± 0.04	0.96 ± 0.04/0.04	20.55	–
	Obukhovo (n = 4)	1.19 ± 0.4	1.1 ± 0.25	0.06 ± 0.14	0.09	0.46 ± 0.05	0.95 ± 0.05/0.04	18.58	.
.	Utyatskoe (n = 5)	1.14 ± 0.4	1.1 ± 0.29	0.06 ± 0.15	0.1	0.54 ± 0.06	0.96 ± 0.05/0.03	14.23	–
Total for morphotype "*major*" (n = 13)		**1.43 ± 0.5**	**1.15 ± 0.27**	**0.10 ± 0.15**	**0.10**	**0.50 ± 0.05**	**0.98 ± 0.04/0.02**	**40.32**	**27.27**
S. major, Malovka (n = 3)		1.14 ± 0.4	1.09 ± 0.30	0.05 ± 0.1	0.08	0.86 ± 0.03	0.96 ± 0.03/0.03	13.83	–
Morphotype "*erythrogenys*" from interfluve	Sukhmen' (n = 5)	1.19 ± 0.4	1.1 ± 0.24	0.06 ± 0.13	0.07	0.66 ± 0.05	0.97 ± 0.02/0.03	18.58	–
	Privol'noe (n = 9)	1.21 ± 0.4	1.09 ± 0.20	0.05 ± 0.13	0.06	0.69 ± 0.04	0.93 ± 0.02/0.07	20.95	–
Total for morphotype "*erythrogenys*" (n = 14)		**1.32 ± 0.47**	**1.13 ± 0.26**	**0.08 ± 0.15**	**0.08**	**0.56 ± 0.05**	**0.97 ± 0.05/0.03**	**32.02**	**22.13**
Total for animals from interfluve (n = 27)		**1.72 ± 0.5**	**1.18 ± 0.28**	**0.12 ± 0.15**	**0.11**	**0.63 ± 0.04**	**0.96 ± 0.04/0.04**	**58.10**	**31.62**
S. e. erythrogenys, Karasuk (n = 3)		1.26 ± 0.5	1.17 ± 0.30	0.09 ± 0.17	0.09	0.53 ± 0.03	0.93 ± 0.04/0.06	25.85	–
S. e. brevicauda (n = 11)		1.49 ± 0.5	1.19 ± 0.27	0.12 ± 0.16	0.13	0.40 ± 0.05	0.98 ± 0.04/0.03	45.45	28.46
.									
Phenotypic hybrids, Shastovo and Romanovo (n = 3)		1.23 ± 0.5	1.13 ± 0.27	0.08 ± 0.15	0.11	0.58 ± 0.05	0.85 ± 0.01/0.15	22.53	–

Note: H, heterozygosity with correction for the sample size.

S. erythrogenys (combined sample), and the lowest estimate characterized the morphotype *major–S. e. brevicauda* pair.

The population genetic subdivision, measured by fixation index, varies in a considerable range but is generally high (G_{ST} = 0.15–0.65). The number of migrants per generation (N_m = 0.26–12.9) shows, on the one hand, a different gene flow between different localities and taxonomic forms, and on the other hand, reveals its selective character. For instance, animals from the Tobol–Ishim interfluve, belonging to both *major* and *erythrogenys* morphotypes display twofold higher gene exchange with *S. e. brevicauda* from Southeastern Kazakhstan (N_m = 2.87 and 2.52, respectively) than with *S. major* from Malovka (N_m = 1.66). Comparison of individuals from the southern and the northern parts of the Tobol–Ishim interfluve also showed a considerable gene flow between them (G_{ST} = 0.18, N_m = 2.18). Although these values proved to be lower than the corresponding estimates for each of these groups with *S. e. brevicauda*, they indicate hybridization between ground squirrels from the Tobol–Ishim interfluve. By contrast, gene exchange between "pure" *S. major* and *S. e. brevicauda* is virtually absent (G_{ST} = 0.65, N_m = 0.26).

Pairwise analysis of the local samples by exact test for differentiation did not detect any differences in each particular case. However, combined analysis of all localities demonstrated that the samples did not belong to the same population (χ^2 = 915.33, *d.f.* = 504, p = 0.000).

Since the local interfluvial samples showed a trend for division into the southern part (samples 12–15 with morphotype *erythrogenys*) and northern part (samples 7–11 with morphotype *major*), we estimated genetic distances both within and between them. The genetic distances between the animals from the southern and northern parts of the Tobol–Ishim interfluve proved to be higher (D_N = 0.04) than the intrapopulation distances in each of them (D_N = 0.02 for the northern and D_N = 0.03 for the southern part, respectively). Interestingly, the genetic differences between all interfluve animals and both *S. major* and *S. e. brevicauda* are in the same range as those of the morphotypes. Conversely, the genetic distances between the animals from the range overlapping zone and *S. erythrogenys* from the settlement of Karasuk (which is outside of this zone) are twofold higher, approaching the between-species values (D_N = 0.11). Note that genetic differences are greater between animals with morphotypes *erythrogenys* and "pure" *S. erythrogenys* than between subspecies *S. e. erythrogenys* and *S. e. brevicauda* (see Table 2). Thus, our results clearly indicate hybridization of *S. major* with subspecies *S. e. brevicauda*, rather than *S. e. erythrogenys*. It may well be that the local samples of ground squirrels from the northern and southern parts of the interfluve mainly belong to *S. major* or *S. e. brevicauda,* respectively.

Table 3. Genetic differentiation of *S. major*, *S. e. erythrogenys*, and individuals from the Tobol–Ishim interfluvial area

Species	H_T	H_S	D_{ST}	G_{ST}	N_m	S/D_N
Morphotype "*major*"/*S. e. brevicauda*	0.13 ± 0.02	0.11 ± 0.02	0.02 ± 0.02	0.15	2.87	0.96/0.04
Morphotype "*major*"/*S. major*	0.10 ± 0.03	0.07 ± 0.01	0.03 ± 0.02	0.23	1.66	0.94/0.06
Morphotype "*major*"/*S. e. erythrogenys*	0.16 ± 0.03	0.10 ± 0.02	0.06 ± 0.03	0.35	0.92	0.88/0.13
S. major*/*S. e. brevicauda	**0.12 ± 0.02**	**0.04 ± 0.003**	**0.08 ± 0.02**	**0.65**	**0.26**	**0.87/0.14**
S. major*/*S. e. erythrogenys	**0.15 ± 0.03**	**0.08 ± 0.01**	**0.07 ± 0.02**	**0.46**	**0.60**	**0.85/0.16**
S. e. brevicauda*/*S. e. erythrogenys	**0.15 ± 0.03**	**0.11 ± 0.02**	**0.04 ± 0.02**	**0.25**	**1.47**	**0.91/0.09**
Morphotype "*erythrogenys*"/*S. e. brevicauda*	0.12 ± 0.03	0.10 ± 0.02	0.02 ± 0.03	0.17	2.52	0.95/0.05
Morphotype "*erythrogenys*"/*S. major*	0.10 ± 0.03	0.07 ± 0.01	0.03 ± 0.03	0.37	0.85	0.92/0.09
Morphotype "*erythrogenys*"/*S. e. erythrogenys*	0.15 ± 0.03	0.10 ± 0.01	0.05 ± 0.03	0.36	0.88	0.88/0.13
Morphotype "*major*"/morphotype "*erythrogenys*"	**0.12 ± 0.02**	**0.11 ± 0.02**	**0.01 ± 0.02**	**0.18**	**2.18**	**0.96/0.04**
All animals from interfluve/*S. e. erythrogenys*	0.16 ± 0.03	0.11 ± 0.01	0.05 ± 0.03	0.30	1.16	0.89/0.11
All animals from interfluve/*S. e. brevicauda*	0.13 ± 0.03	0.11 ± 0.01	0.02 ± 0.02	0.15	2.90	0.96/0.04
All animals from interfluve/*S. major*	0.11 ± 0.02	0.08 ± 0.01	0.03 ± 0.01	0.24	1.58	0.95/0.06

Phenogenetic and Phylogenetic Reconstructions

To examine phylogenetic relationships among ground squirrels, we have constructed an NJ tree using the TREECON software and an MST tree using NTSYS-pc version 1.7. These trees have fairly similar topologies. All individuals in them group into two distinct clusters according to their morphotypes, *major* or *erythrogenys*. In the NJ tree (Fig. 3), the first cluster includes *S. e. brevicauda*, *S. e. erythrogenys*, and *S. e. heptneri*, as well as animals from the southern part of the Tobol–Ishim interfluve. The second cluster is formed by *S. major* and animals from the northern part of the interfluve. Note that hybrid individuals show some phenotypic and genetic isolation, forming their own subcluster within the parental cluster of *S. major.*

The MST tree of phylogenetic relationships among ground squirrels is presented in Fig. 4. As the previous reconstruction, this tree differentiated the local samples of the southern and northern parts of the Tobol–Ishim interfluve, but distinct geographical subdivision was established only in four cases: Sukhmen', Privol'noe, Obukhovo, and Malovka. Interestingly, one animal from the northern part of the interfluve (the village of Utyatskoe) was assigned to the southern group of animals. Phenotypically hybrid animals from Shastovo and Romanovo, identified by bioacoustic analysis, formed a link with the genetically "pure" parental species through phenotypically "pure", but genetically hybrid animals identified by RAPD–PCR (see Fig. 4). Phenotypically hybrid individuals from Shastovo and Romanovo were associated to the genetically pure parental species *S. major* through genetic hybrids (identified in RAPD–PCR), which had the *major* phenotype.

The UPGMA similarity tree (Fig. 5), apart from to two major clusters, has also two additional clusters, one of which, according to bioacoustic analysis, includes both hybrid and "pure" animals. The other additional cluster consists of two *S. e. brevicauda* and one *S. e. heptneri* individual. The topology of each cluster more clearly correlates with the geographic origin of the animals than that of the previous reconstructions. It is remarkable that in the UPGMA tree, the animals fell in two groups, though, judging by their phylogenetic relationships (shown in the NJ tree), they group in one cluster, while the animal from Romanovo, assigned to hybrids by vocal signal, occupies a separate branch near the tree base in both reconstructions.

DISCUSSION

We have carried out a molecular genetic study of the region of overlapping ranges of *S. major* and *S. erythrogenys* in the Tobol–Ishim interfluve, where a zone of hybridization of these species had been hypothesized. The results of the study revealed the presence of *S. major–S. e. brevicauda* hybrids among the ground squirrels from the Tobol–Ishim interfluve.

The results of RAPD analysis and data on bioacoustic signals [11] are in a good agreement, recording fairly high differentiation of *S. major* and *S. erythrogenys*. According to our results, such gene diversity indices as n_a and n_e, do not significantly differ between the two ground squirrel species and the hybrids from the overlapping range zones, being somewhat higher in the latter, which is probably accounted for by a larger sample size in this case. The range of the number of heterozygous loci in animals from different localities (H = 0.06–0.132) may be explained, on the one hand, by the

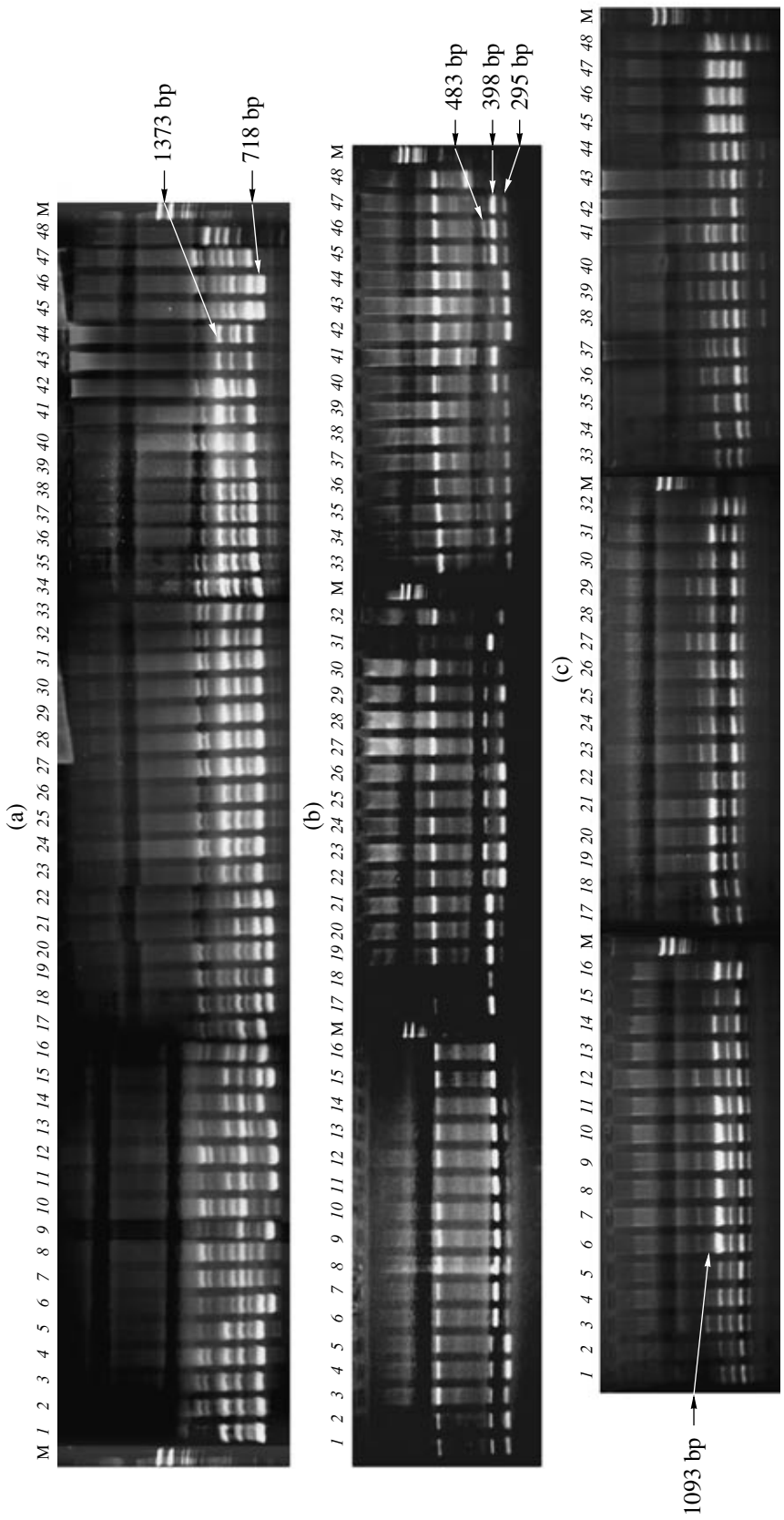

Fig. 2. Electrophoregrams of ground squirrels obtained using primers OPC-05 (a), OPD-05 (b), and OPC-20 (c). *1–5*, Sukhmen'; *6–9*, Temlyakovo; *10*, Zaborskoe; *11–14*, Obukhovo; *15*, Romanovo; *16, 17*, Shastovo; *18–22*, Utyatskoe; *23–31*, Privol'noe; *32–37*, Andreevka; *38–40*, *S. e. brevicauda; 41*, *S. e. erythrogenys; 42*, *S. e. heptneri; 43, 44*, *S. e. brevicauda; 45–47*, *S. major; 48*, *Marmota.* M, phage λ DNA digested with *Pst*I. Marker loci are indicated by arrows.

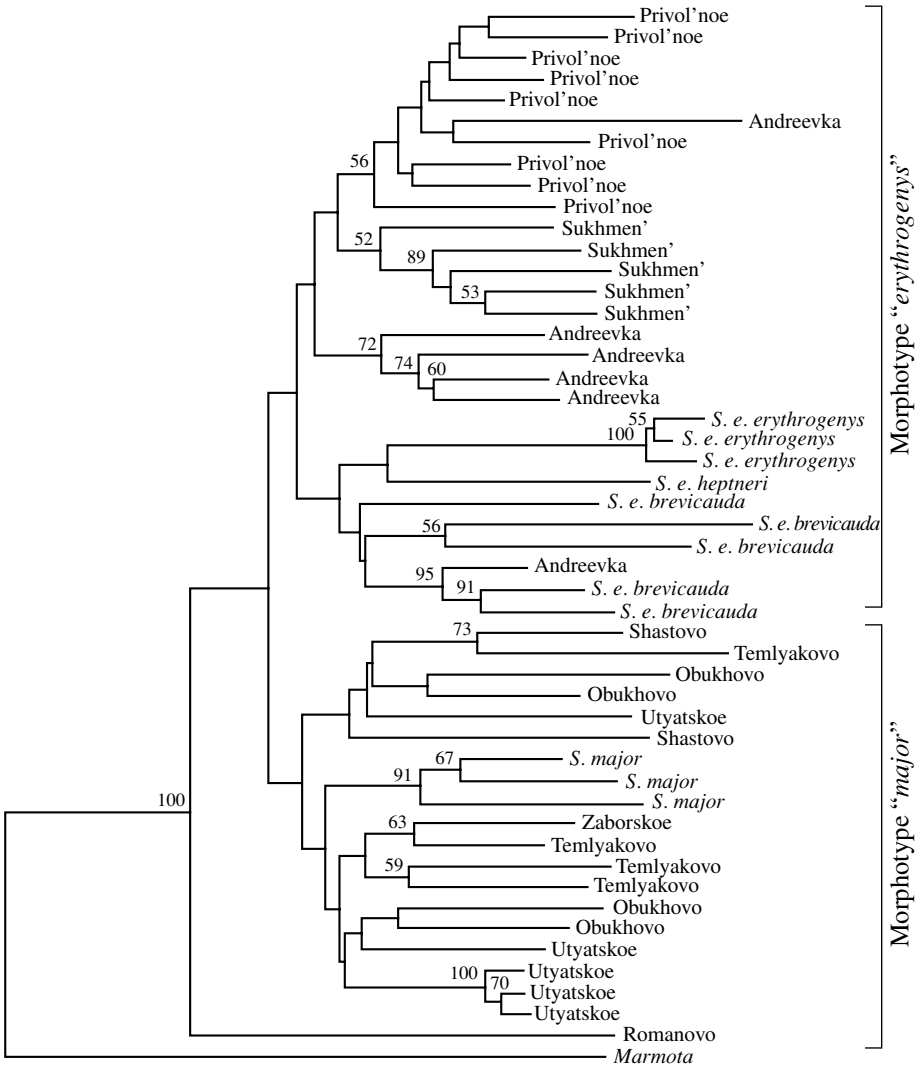

Fig. 3. Phylogenetic relationships among ground squirrels from the Tobol–Ishim interfluve constructed by the NJ method using the TREECON program.

presence of closely related individuals in the samples (Privol'noe), and on the other, by a small number of specimens used in the study. Notwithstanding, these estimates are well in the limits established for other ground squirrel species using biochemical genetic methods. For instance, heterozygosity for protein polymorphisms varies from 0 in *S. erythrogenys* to 0.035 in *S. major* [22]. Similar heterozygosity estimates were obtained for other ground squirrel species [23–25]. The maximum values were reported for *S. mexicanus* (H = 0.137) [23], and the minimum values, for *S. musicus* and *S. pygmaeus* (H = 0.000 and H = 0.015) [22].

The index of genetic differentiation δ_S varied in a very wide range (0.40–0.86), with the highest values observed in the lowest polymorphic animals (see Table 2).

The greatest differences in genetic distances were detected in hybrid animals from Shastovo and Romanovo (see Table 2). This result may be related to the hybridization of *S. major* with other sympatric ground squirrel species (inhabiting other parts of the range). Evidence in favor of occasional interspecific hybridization was recorded in other parts of the range of several ground squirrel species. Thus, a molecular genetic study conducted in a sympatric zone of four ground squirrel species, detected sporadic hybridization in pairs *S. major*/*S. pygmaeus* and *S. major*/*S. fulvus* [26]. In this study, analysis of intraspecific variability of the mtDNA D-loop region in *S. major* showed, in addition of the haplotype of this species, the presence of haplotypes of *S. fulvus* and *S. pygmaeus*. It is interesting that, in spite of high heterozygosity, the nuclear genomes of the phenotypic hybrids were closer to the parental species than to one another (see Tables 2, 3). Moreover, five animals (including three phenotypic hybrids) differed from the remaining sample by the spectrum of the amplification products (see Fig. 2), thereby forming a distinct cluster in the genetic similarity tree. On the one hand, this result reveals cryptic

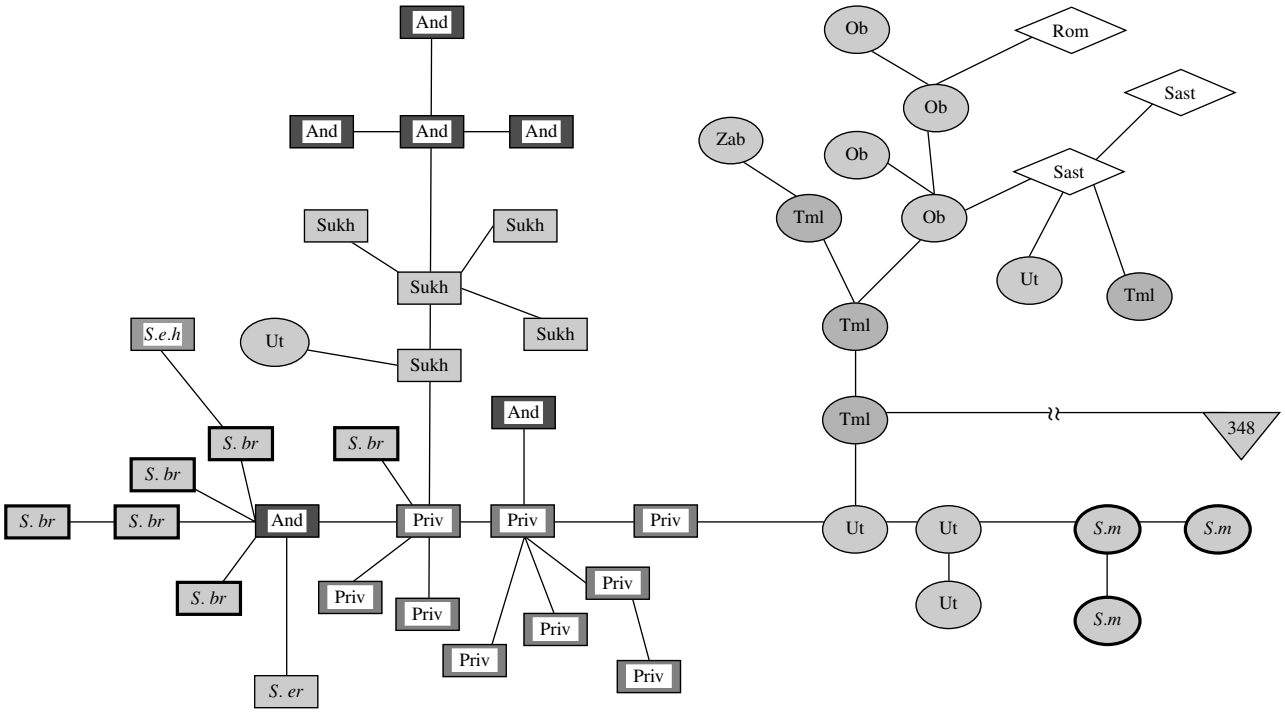

Fig. 4. MST tree of phylogenetic relationships among ground squirrels from the Tobol–Ishim interfluve constructed using the NTSYS program package. The parental forms are boxed in bold. Rectangles indicate morphotype *erythrogenys*; ovals, morphotype *major*; diamonds, phenotypic hybrids. Ground squirrels: *S. er.*, *S. erythrogenys*; *S. m.*, *S. major; S. br., S. e. brevicauda; S. e. h., S. heptneri.* Geographic localities: And, Andreevka; Sukh, Sukhmen'; Priv, Privol'noe; Ut, Utyatskoe; Tml, Temlyakovo; Zab, Zaborskoe; Shast, Shastovo; Rom, Romanovo; 348, *Marmota bobak.*

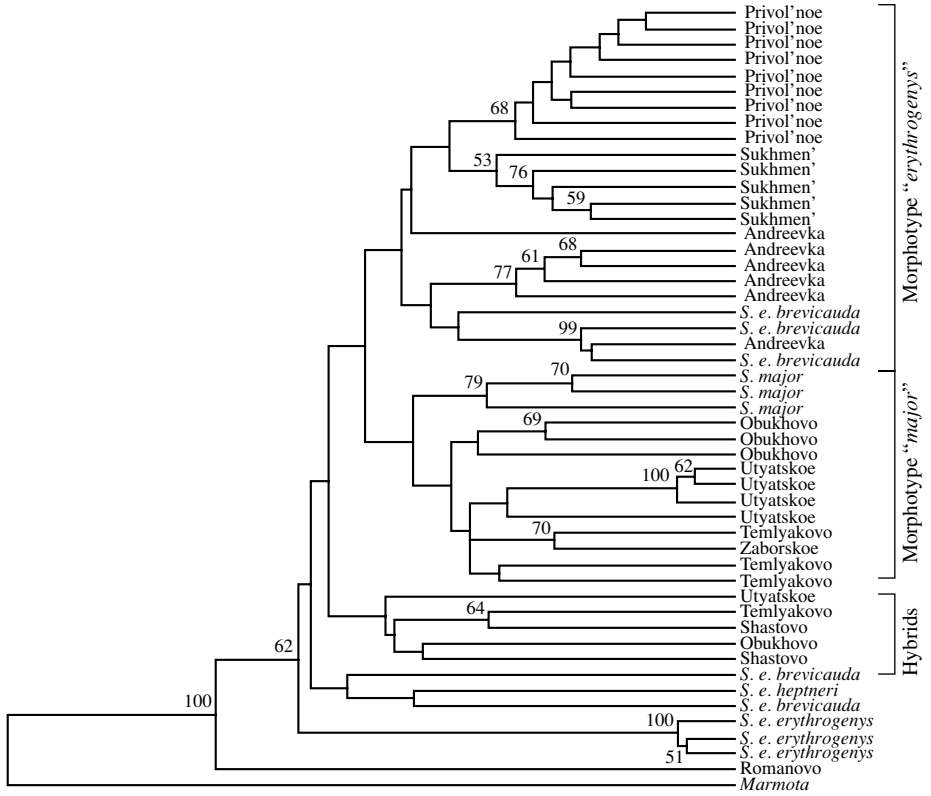

Fig. 5. UPGMA dendrogram of ground squirrels from the Tobol–Ishim interfluve constructed using the TREECON program.

genotypic hybrids; on the other, it shows their higher genetic differentiation with the remaining ground squirrels. The results of RAPD analysis revealed hybridization in ground squirrels in the Tobol–Ishim interfluvial area, confirming the earlier conclusions inferred from studies of bioacoustic signals [4, 5]. The number of hybrid animals detected by the bioacoustic (3 of 31) and genetic (6 of 31, i.e., about 20%) methods suggests considerable hybridization on the area. Apparently, *S. major* hybridizes not with *S. erythrogenys* in general, but likely with form *brevicauda*, whose area, according to the current zoological information, virtually does not overlap with that of *S. major.*

In spite of the hybridization processes, we have observed consistently preserved morphotypes and the prevalence of the nuclear genotype *major* in the north, and *brevicauda*, in the south, which may indicate selection against hybrids. In contrast to the proposals, inferred from zoological observations, genetic similarity of the phenotypic hybrids with *S. e. brevicauda* (D = 0.04) proved to be higher than with *S. major* (D = 0.06), which may imply introgressive hybridization, when some species are accepted by others. However, these differences may be partly explained by high genetic variability of *S. major* itself, and partially by the small sample size in the case of this species.

Thus, our results suggest the existence of interspecific hybridization of ground squirrels in the Tobol–Ishim interfluve, which occurs at a larger scale than was believed on the basis of morphological and bioacoustic data. Furthermore, *S. major* is more likely to hybridize with *S. e. brevicauda*, rather than with *S. e. erythrogenys*. Although the RAPD analysis results are fairly informative, they are not complete without mtDNA analysis, which will be reported in the forthcoming paper.

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