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Genetic variation in the *Sicista subtilis* (Pallas, 1773) species group (Rodentia, Sminthidae), as compared to karyotype differentiation

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Abstract: Genetic variation in chromosomally polymorphic *Sicista subtilis* complex and related *Sicista betulina* species group was analysed using two mitochondrial markers (*COI* and *Cytb*). The *S. subtilis* group is shown to include six lineages, five of which correspond to species currently recognized based on cytogenetic and genetic data: *Sicista nordmanni*, *Sicista trizona*, *S. subtilis sensu stricto*, *Sicista severtzovi* and *Sicista cimlanica*. A previously unknown genetic lineage of *S. subtilis* was found in the North Caucasus. The existence of two divergent lineages within *Sicista strandi* is supported. It is suggested that the speciation rate in *Sicista* was strongly affected by rapid chromosomal evolution.

Keywords: birch mice; DNA barcoding; Palearctic; phylogeography; steppe fauna.

Introduction

While most birch mice (*Sicista*) inhabit forests and meadows of the temperate Palearctic, the members of the *Sicista subtilis* species group represent a unique case of adaptation to arid/semiarid environments as they occur in the steppe and semidesert zones of Eastern Europe, Kazakhstan and Siberia. Morphological taxonomies of the genus, which were based on external morphology and glans penis anatomy (Méhely 1913, Vinogradov 1925, Ognev 1948), regarded steppe birch mice as a single species with several subspecies. However, subsequent cytogenetic studies revealed a plethora of cryptic species and karyomorphs (e.g. Sokolov et al. 1986, Shenbrot et al. 1995), the status of which is debatable. According to the most comprehensive review (Kovalskaya et al. 2000, 2011), there are six main karyomorphs, which can be regarded as separate species:

Sicista subtilis sensu stricto (Pallas, 1773); 2n = 24; NFa = 38–44. Its range extends from the Volga River far eastwards to Khakassia, Tuva and the west Baikal region (Shenbrot et al. 1995, Kovalskaya and Fedorovich 1997).

Sicista nordmanni (Keyserling *et* Blasius, 1840); 2n = 26; NFa = 46. It can be found in most of the territory of southern Ukraine (Zagorodniuk and Kondratenko 2000), in an adjacent small part of Russia (Kovalskaya et al. 2011) and in the area extending to south-eastern and eastern Romania (Ausländer et al. 1959, Cserkész et al. 2015).

Sicista severtzovi Ognev, 1935; 2n = 26; NFa = 46. This taxon has a complicated history. It was originally described as a subspecies that was supposed to include dark-coloured birch mice from the northern part of the Russian steppes. Later, it was elevated to full species rank based on a specific karyotype (2n = 18–20, NFa = 26–28) described from the western part of its presumed range (Sokolov et al. 1986). However, a subsequent study showed that the chromosome complement of birch mice from the vicinity of terra typica (northern Voronezh region) is different and, hence, that the previous cytogenetic data should be attributed to another taxon. According to Kovalskaya et al. (2011), the distribution of *S. severtzovi sensu stricto* is limited by the Don and Hoper rivers (in the west, south and east), whereas the northern limits remain to be determined.

Sicista cimlanica Kovalskaya et al. 2000; 2n = 22; NFa = 33–34. This birch mouse is known only from the Tsimla Sands in the lower Don basin. It was originally described as a subspecies of *Sicista severtzovi* and its status was not discussed in Kovalskaya et al. (2011).

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Nevertheless, taking into account the high level of karyotypic differentiation between *S. severtzovi* and *S. cimlanica*, it appears appropriate to treat the latter as a full species.

Sicista sp. 1 (Kovalskaya et al. 2011); 2n = 22–26; NFa = 39–44. Based on available chromosomal data, the range of *Sicista* sp. 1 is bordered by the Don and Hoper rivers in the west and by the Volga River in the east. It is likely that its southernmost extension coincides with the narrowest part of the divide of the Volga and Don rivers, a region that provides contact with *Sicista subtilis s. str*. The northern distributional limit of *Sicista* sp. 1 is uncertain.

Sicista sp. 2 (Kovalskaya et al. 2011); 2n = 16–22; NFa = 26–29. Animals that were tentatively grouped in this taxon were collected at the confluence of the Don and Severskii Donetz rivers. The karyotype of this taxon was at first erroneously attributed to *Sicista severtzovi*.

It should be emphasized that considerable chromosomal variation is also found within three chromosomal species (*Sicista subtilis s. str.*, *Sicista* sp. 1, *Sicista* sp. 2), with the highest level of polymorphism observed in *Sicista* sp. 1. At the same time, some parts of the range are still studied insufficiently; thus, there are no cytogenetic data for most of the North Caucasus.

Today, there are only a few molecular studies focused on birch mice (Cserkész et al. 2015, 2016, Baskevich et al. 2016, Rusin et al. 2018), and many of the aspects of their phylogeography and phylogenetic relationships still remain unclear. The available data (Pisano et al. 2015, Lebedev et al. 2019) demonstrated that the *Sicista subtilis* group is relatively close to the *Sicista betulina* group. The taxonomy of the latter was also revised based on chromosomal data (Sokolov et al. 1989), which allowed the recognition of *Sicista strandi* Formosov, 1931, with 2n = 44 occurring in the South-East Europe as a distinct species from *S. betulina* (Pallas, 1779) that is characterized by 2n = 32.

The only molecular study on variation in the *Sicista subtilis* group (Cserkész et al. 2015) has revealed several important facts. *Sicista trizona* (Petenyi, 1882) was found as a separate lineage placed as sister to *Sicista nordmanni*, which is also substantially differentiated from all other species. Other representatives of the *S. subtilis* group under study were found to be very close. Based on these results, Cserkész et al. (2015) considered *S. nordmanni* and *S. trizona* as separate species and lumped the rest under *S. subtilis*. This taxonomic decision is in sharp contradiction with the cytogenetic data, which suggest that chromosomal differentiation provides an effective barrier to gene flow among *Sicista severtzovi*, *Sicista* sp. 1 and *Sicista* sp. 2. It should be noted that Cserkész et al. (2015) did not study true *S. severtzovi sensu* Kovalskaya et al. (2011) and did not examine any samples from the Asian part of the range.

The current study is based on extended samples covering most of the range of the *Sicista subtilis* group and including animals that were karyotyped in previous studies. Therefore, the main focus is placed on the correlation between molecular variation and chromosomal differentiation in the *S. subtilis* and *Sicista betulina* groups.

Materials and methods

We analysed the alignment consisting of 65 sequences of cytochrome oxidase I (*COI*, 657 bp), most of which were taken from online projects housed by the Barcode of Life Data System (BOLD; www.boldsystem.org). Detailed information and process IDs of all sequences are presented in Table 1. We also sequenced the complete mitochondrial cytochrome b (*cytb*) gene for 10 specimens using the protocol described by Rusin et al. (2018). Twenty-eight sequences of *cytb* and seven sequences of *COI* were added from the GenBank (Cserkész et al. 2015, 2016, Baskevich et al. 2016, Schaffer et al. 2017). We did not include *cytb* sequences of *Sicista betulina* and *Sicista strandi* from Cserkézs et al. (2015) because they likely belong to a pseudogene lineage as shown by Rusin et al. (2018). Sequences obtained in this study were deposited in the GenBank with accession numbers MK758092–MK758103. *Sicista concolor* (Büchner, 1892) NC027579 (Yue et al. 2015) was used as the distant outgroup for *Sicista subtilis* and *S. betulina* species groups.

To assess the relationships among mitochondrial lineages, neighbour-joining trees were reconstructed in MEGA X (Kumar et al. 2018) for *cytb* and *COI* alignments separately. Clade support was estimated based on 1000 bootstrap pseudoreplicates. Formal species delimitation analysis was performed in ABGD (Puillandre et al. 2012) based on the matrix of uncorrected p-distances among *COI* sequences. The routine was conducted both with the alignment of the *Sicista subtilis* species group and the complete data matrix, also including the *Sicista betulina* species group. Estimates of node ages were obtained with the use of BEAST 1.8.4 (Drummond et al. 2012). The details of the molecular clock analysis are provided in Supplementary material 1.

Results and discussion

The results of the analysis of the two mitochondrial genes (Figure 1) demonstrated significant structures within

Table 1: Detailed information on tissue samples of *Sicista* used in the molecular analysis.

Taxa (Kovalskaya et al. 2011)	Taxa (Cserkész et al. 2016)	Mitochondrial lineage (this study)	Locality (*, coordinates are given for the nearest landmark)	Probe code	Voucher	*Col* BOLD/GenBank	*Cytb* GenBank
S. betulina group							
Sicista betulina	*Sicista betulina*	"BET"	1. Austria, Salzburg, 47°07′N, 12°56′E	Bet01		KY754549 (Schaffer et al. 2017)	
			2. Russia, Moscow reg.,	Bet02	ZMMU/tc-VSL-Sicista_YuMK_1757	SKMZM167_07	KY967412 (Rusin et al. 2018)
			Chernogolovka; 56°2′N, 38°24′E*	Bet03	ZMMU S-181480	SKMZM406_08	
			3. Russia, Kostroma reg.,	Bet04	ZMMU S-176700	SKMZM413_08	
			Manturovo; 58°19′N, 44°45′E*				
			4. Russia, Republic of Komi,	Bet05	ZMMU S-183236	SKMZM412_08	
			Pechoro-Ilychskiy reserve;	Bet06	ZMMU S-183257	SKMZM411_08	
			62°24′N, 57°11′E*				
			5. Russia, Krasnoyarsk territory,	Bet07	ZMMU S-183774	SKMZM407_08	
			Mirnoe; 62°17′N, 89°2′E*	Bet08	ZMMU S-185277	SKMZM821_09	
				Bet09	ZMMU S-185275	SKMZM820_09	
				Bet10	ZMMU S-185262	SKMZM819_09	
				Bet11	ZMMU S-185260	SKMZM818_09	
			6. Russia, Altai Republic,	Bet12	ZMMU/tc-VSL-Sicista_15.1		KY967411 (Rusin et al. 2018)
			Artybash; 51°47″N, 87°15′E*				
Sicista strandi	*Sicista strandi*	"STR N"	7. Russia, Belgorod reg.,	Bet13	ZMMU S-181441	SKMZM402_08	KM397209 (Pisano et al. 2015)
			"Yamskaya steppe" reserve;				
			51°11′N, 37°37′E*	Bet14	ZMMU S-181442	SKMZM403_08	
				Bet15	ZMMU S-181443	SKMZM404_08	
				Bet16	ZMMU S-181444	SKMZM405_08	MK758092
		"STR S"	8. Russia, Rostov reg., "Tsimla sands" reserve; 47°55′N, 42°30′E	Bet17	ZMMU S-178460	SKMZM027_07	KY967414 (Rusin et al. 2018)
			9. Russia, Saratov reg.,	Bet18	ZMMU S-190224	SKMZM121_07	
			Slavyanka; 51°50′N, 46°15′E*	Bet19	ZMMU S-190226	SKMZM123_07	KY967413 (Rusin et al. 2018)
				Bet20	ZMMU S-190227	SKMZM124_07	
S. subtilis group							
Unstudied	*S. trizona*	"TRI"	10. Hungary, Mezőcsát; 47°45′N, 20°47′E	Sub01			KP715871 (Cserkész et al. 2016)
				Sub02		KF854246 (Cserkész et al. 2015)	KP715873 (Cserkész et al. 2015)
				Sub03		KF854245 (Cserkész et al. 2015)	KP715872 (Cserkész et al. 2015)
			11. Romania, Juc-Herghelie; 46°52′N, 23°45′E	Sub04		KF854248 (Cserkész et al. 2015)	KP715875 (Cserkész et al. 2015)
				Sub05		KF854247 (Cserkész et al. 2015)	KP715874 (Cserkész et al. 2015)

Table 1 (continued)

Taxa (Kovalskaya et al. 2011)	Taxa (Cserkész et al. 2016)	Mitochondrial lineage (this study)	Locality (*, coordinates are given for the nearest landmark)	Probe code	Voucher	*Col* BOLD/GenBank	*Cytb* GenBank
S. nordmanni	*S. nordmanni*	"NORD"	12. Romania, Iași; 47°11′N, 27°27′E	Sub06		KF854244 (Cserkész et al. 2015)	KP715876 (Cserkész et al. 2015)
			13. Ukraine, Odessa prov., Tarutino; 46°16′N, 29°23′E	Sub07	ZMMU/tc-VSL-Sicista_YUMK_09_21	SKMZM848_09	
				Sub08	ZMMU/tc-VSL-Sicista_YUMK_09_55	SKMZM847_09	
			14. Ukraine, Kherson prov., Black Sea Reserve; 46°27′N, 32°0′E	Sub09		KF854243 (Cserkész et al. 2015)	KP715878 (Cserkész et al. 2015)
			15. Russia, Belgorod reg., Borisovka; 50°33′N, 36°03′E	Sub10	ZMMU/tc-VSL-Sicista_YUMK_05_1	SKMZM034_07	KY967418 (Rusin et al)
				Sub11	ZMMU/tc-VSL-Sicista_YUMK_05_9	SKMZM033_07	
				Sub12	ZMMU/tc-VSL-Sicista_YUMK_1688	SKMZM029_07	MK758093
				Sub13	ZMMU/tc-VSL-Sicista_YUMK_1694	SKMZM032_07	MK758094
				Sub14			KP715877 (Cserkész et al. 2016)
Sicista sp. 2	*S. subtilis*	"CIM"	16. Russia, Belgorod reg., "Yamskaya steppe" reserve; 51°11′N, 37°37′E*	Sub15	ZMMU S-178461	SKMZM037_07	MK758095
				Sub16	ZMMU/tc-VSL-Sicista_YUMK_1562	SKMZM028_07	
				Sub17	ZMMU/tc-VSL-Sicista_YUMK_1563	SKMZM038_07	
				Sub18	ZMMU/tc-VSL-Sicista_YUMK_1580	SKMZM046_07	
				Sub19	ZMMU/tc-VSL-Sicista_YUMK_1582	SKMZM048_07	
				Sub20	ZMMU/tc-VSL-Sicista_YUMK_1592	SKMZM043_07	MK758096
				Sub21			KP715869 (Cserkész et al. 2016)
			17. Russia, Belgorod reg., Makeshkino; 50°38′N, 37°48′E	Sub22	ZMMU/tc-VSL-Sicista_YUMK_1589	SKMZM044_07	
				Sub23	ZMMU/tc-VSL-Sicista_YUMK_1594	SKMZM039_07	
				Sub24	ZMMU/tc-VSL-Sicista_YUMK_1595	SKMZM047_07	
S. severtzovi		"SEV"	18. Russia, Voronezh reg., Krasnoe; 51°09′N, 41°28′E	Sub25	ZMMU S-182605	SKMZM089_07	
				Sub26	ZMMU S-182606	SKMZM090_07	MK758097
				Sub27	ZMMU S-182607	SKMZM093_07	MK758098
				Sub28	ZMMU S-182608	SKMZM094_07	
				Sub29	ZMMU S-182609	SKMZM166_07	
Sicista sp. 1		"CIM"	19. Russia, Volgograd reg., Serafimovich; 49°39′N, 42°43′E	Sub30	ZMMU/tc-VSL-Sicista_YUMK_1556	SKMZM035_07	MK758099
				Sub31			KP715865 (Cserkész et al. 2016)
S. (severtzovi) cimlanica			20. Russia, Volgograd reg., "Tsimla sands" reserve; 48°09′N, 42°51′E	Sub32	ZMMU S-173549	SKMZM025_07	MK758100
				Sub33	ZMMU S-173550	SKMZM026_07	
				Sub34	ZMMU S-178462	SKMZM040_07	
			21. Russia, Rostov reg., "Tsimla sands" reserve; 47°49′N, 42°39′E	Sub35			KP715870 (Cserkész et al. 2016)
Unstudied			22. Ukraine, Lugansk prov., Trokhizbenka; 48°48′N, 38°57′E	Sub36			KP715864 (Cserkész et al. 2016)

Table 1 (continued)

Taxa (Kovalskaya et al. 2011)	Taxa (Cserkész et al. 2016)	Mitochondrial lineage (this study)	Locality (*, coordinates are given for the nearest landmark)	Probe code	Voucher	*Col* BOLD/GenBank	*Cytb* GenBank
Unstudied	Unstudied	"NC"	23. Russia, Rostov reg., Remontnoe; 46°29′N, 43°1′E	Sub37	ZMMU/tc-VSL-Sicista_Rem_2		MK758101
				Sub38	ZMMU/tc-VSL-Sicista_Rem_3		MK758102
subtilis	Unstudied		24. Russia, Republic of Kalmykia, Achinery; 45°24′N, 45°20′E	Sub39	ZMMU/tc-VSL-Sicista_YUMK_1687	SKMZM031_07	MK758103
S. subtilis	*S. subtilis*	"SUB"	25. Russia, Astrakhan reg., Grachi; 47°49′N, 46°14′E	Sub40	ZMMU S-197172		KY967417 (Rusin et al. 2018)
			26. Russia, Volgograd reg., Ilovlya; 49°14′N, 44°07′E	Sub41			KP715866 (Cserkész et al. 2016)
			27. Russia, Volgograd reg., Kamyshin; 49°55′N, 45°14′E	Sub42			KP715867 (Cserkész et al. 2016)
				Sub43	ZMMU/tc-VSL-Sicista_YUMK_1559	SKMZM036_07	
			28. Russia, Saratov reg., Slavyanka*; 51°50′N, 46°15′E	Sub44			KR107032 (Baskevich et al. 2016)
				Sub45			KR107031 (Baskevich et al. 2016)
				Sub46	ZMMU S-190229	SKMZM125_07	
				Sub47	ZMMU/tc-VSL-Sicista_IT_2005_188	SKMZM126_07	
			29. Russia, Saratov reg., Novokamenka; 50°45′N, 46°27′E	Sub48			KP715868 (Cserkész et al. 2016)
			30. Russia, Saratov reg., Diakovka; 50°44′N, 46°46′E	Sub49	ZMMU/tc-VSL-Sicista_YUMK_1772	SKMZM849_09	
			31. Russia, Saratov reg., Oktyabrskiy; 51°38′N, 49°7′E	Sub50	ZMMU S-190230	SKMZM127_07	
			32. Russia, Orenburg reg., Kuvandyk; 51°22′N, 57°25′E	Sub51	ZMMU S-178642	SKMZM030_07	
			33. Kazakhstan, Pavlodar prov., Kudaykol; 51°49′N, 75°55′E	Sub52	ZMMU S-182802		KY967416 (Rusin et al. 2018)
				Sub53	ZMMU/tc-VSL-Sicista_53_07_21	SKMZM390_08	
				Sub54	ZMMU S-182286	SKMZM391_08	
				Sub55	ZMMU S-182287	SKMZM392_08	
			34. Kazakhstan, Pavlodar prov., Sharbakty; 51°23′N, 78°20′E	Sub56	ZMMU S-182289	SKMZM393_08	
				Sub57	ZMMU S-182290	SKMZM394_08	
				Sub58	ZMMU S-182292	SKMZM396_08	
			35. Kazakhstan, East Kazakhstan prov., Zaisan depression; 48°02′N, 83°22′E	Sub59	ZMMU S-182804	SKMZM401_08	

Table 1 (continued)

Taxa (Kovalskaya et al. 2011)	Taxa (Cserkész et al. 2016)	Mitochondrial lineage (this study)	Locality (*, coordinates are given for the nearest landmark)	Probe code	Voucher	*Col* BOLD/GenBank	*Cytb* GenBank
			36. Russia, Republic of Khakassia, Novonikolaevka; 53°12′N, 91°16′E	Sub60	IEE_100	SKMZM041_07	
				Sub61	IEE_120	SKMZM042_07	
			37. Russia, Tuva Republic, 90km north from Kyzyl *; 52°32′N, 94°18′E	Sub62	ZMMU/tc-VSL-Sicista_Tuva_32		KY967415 (Rusin et al. 2018)

ZMMU, Zoological Museum of Moscow University, main collection; ZMMU/tc, Zoological Museum of Moscow University, tissue collection; IEE, Institute of Ecology and Evolution, tissue collection.

both *Sicista subtilis* and *Sicista betulina* species groups. Species delimitation (ABGD analysis) produced different numbers of clusters (ranging from six to 11, depending on the value of the prior and the alignment type); however, in most cases, the *S. subtilis* group was divided into six lineages, while the *S. betulina* group was divided into three (Figure 1). Lineages within species groups are distributed allopatrically (Figure 2). As follows from the comparison with the cytogenetic data presented in Kovalskaya et al. (2011), there is fairly good correspondence between the distribution of the mitochondrial DNA (mtDNA) lineages and karyotypes throughout most of the range (Figure 2, Table 1).

The "NORD" lineage is distributed from the Belgorod region to south-western Ukraine and eastern Romania and corresponds to *Sicista nordmanni*, while the "TRI" lineage from Hungary and Transylvania correspond to *Sicista trizona*, as shown by Cherkész et al. (2015). In agreement with the latter study, our data demonstrate the isolated position of *S. nordmanni* and *S. trizona*, which are placed as sister branches. The Kimura-2-parameter (K2P) distance between these two western lineages is 6.0% (*COI*) and 7.8% (*cytb*).

Four other lineages cluster together, with the K2P distances between them being 1.9–4.2% (*COI*) and 3.0–6.3% (*cytb*). The most distant lineage from the others (2.9–4.2% *COI*; 5.8–6.3% *cytb*) is the "NC" one, which was found in two points in the North Caucasus (Figure 2, Table 1, localities 23 and 24). The specimen from Kalmykia (locality 24) was examined cytogenetically using routine staining and G-banding (Kovalskaya et al. 2011). Its karyotype (2n = 24, NFa = 44) was found to be very close to that of the *Sicista subtilis s. str*. This is the only case when the same karyotype is shared by animals belonging to two different genetic lineages. The status of the "NC" lineage deserves a separate study.

The "SUB" lineage is distributed from the Volga River, where it is found on both banks, eastwards to eastern Kazakhstan, Khakassia and Tuva and corresponds to *Sicista subtilis s. str*. There is no clear structure within this lineage. Despite large geographic distance between the westernmost and easternmost localities, the difference among specimens is low (<1.0% *COI*). This result suggests the lack of differentiation between typical *Sicista subtilis subtilis*, *Sicista subtilis vaga* (Pallas, 1779) (Kazakhstan) and *Sicista subtilis sibirica* Ognev, 1935 (forest steppes of South Siberia), which were described based on external morphology (details of coloration).

The "SEV" lineage is found only in the Voronezh region and corresponds to *Sicista severtzovi s. str*. as defined by Kovalskaya et al. 2011.

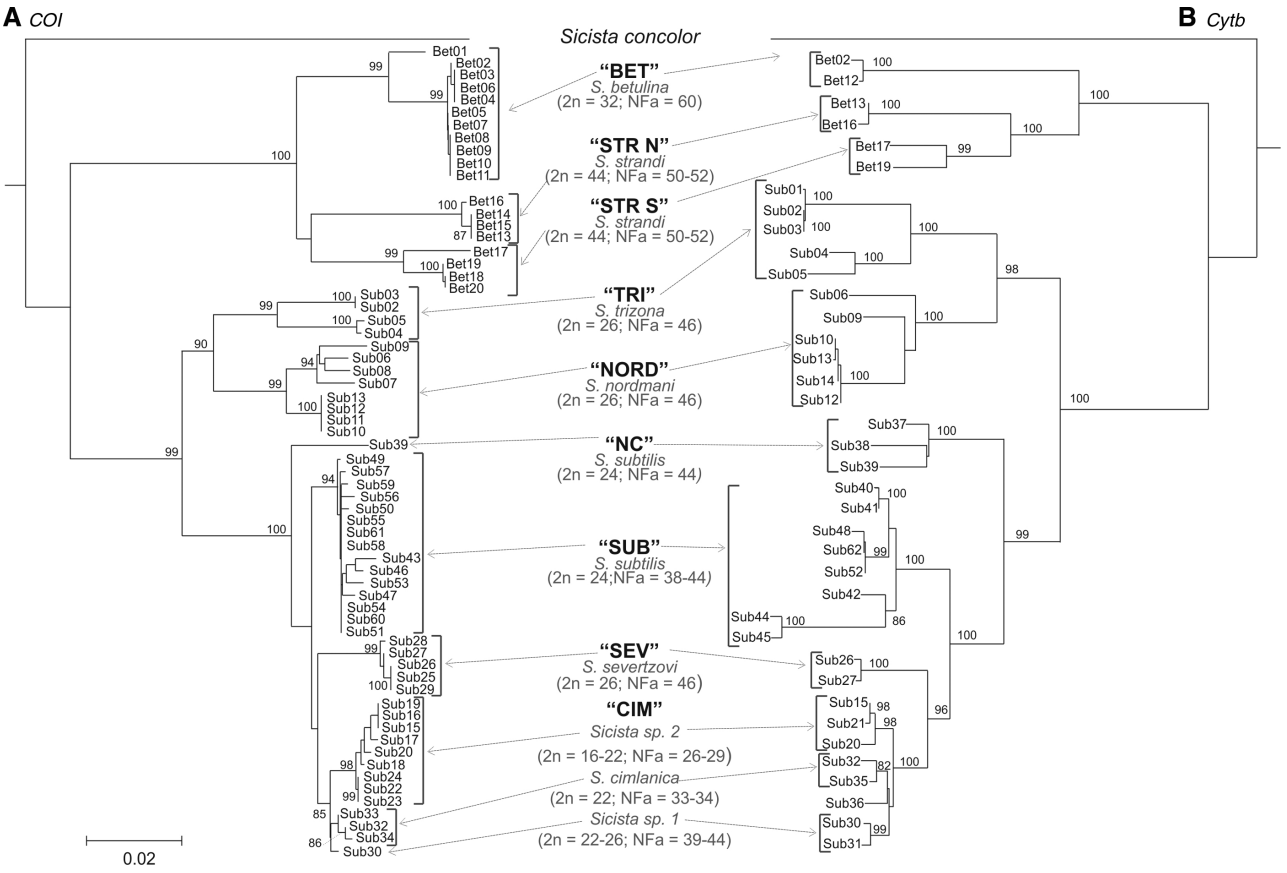

Figure 1: Phylogenetic relationships among mitochondrial lineages of *Sicista subtilis* and *Sicista betulina* species groups. The neighbour-joining trees reconstructed based on the alignments of two mitochondrial genes: (A) *COI* and (B) *Cytb*. The numbers above and below branches correspond to bootstrap support values and are not shown for the intrapopulation clusters.

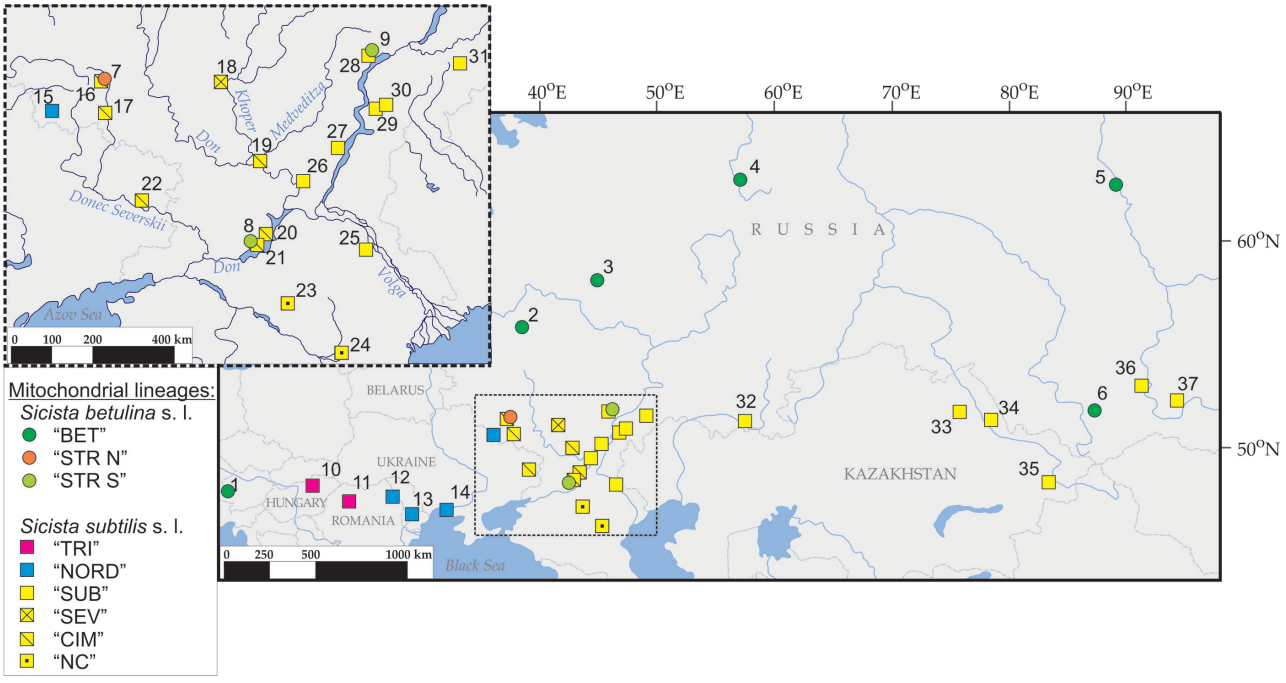

Figure 2: Geographical variation in mitochondrial DNA of *Sicista subtilis* and *Sicista betulina* groups. Designations of localities and mitochondrial lineages correspond to those in the Table 1.

The "CIM" lineage is distributed from the Tsimla sands in the south to the Belgorod region in the north and includes animals that are attributed to *Sicista* sp. 1, *Sicista* sp. 2 and *Sicista cimlanica* based on cytogenetic data (Kovalskaya et al. 2000, 2011). Within this lineage, all animals belonging to *Sicista* sp. 2 (points 16 and 17) form a subclade. Unfortunately, only one locality within the range of highly polymorphic *Sicista* sp. 1 was sampled. The "SEV" and the "CIM" lineages are consistently placed as sister branches, and the K2P distances between them are 2.5% (*COI*) and 3.0% (*cytb*).

The "BET" lineage corresponds to *Sicista betulina* and occurs from Central Europe to Central Siberia. The codon position 175 in the *COI* alignment contains a stop-codon (TAA) in most sequences, potentially suggesting that a pseudogene was amplified by the standard International Barcode of Life (IBOL) procedure for this species. However, these putative pseudogene sequences are rather close to the single sequence that does not contain stop-codons (KY754549, Schaffer et al. 2017) and demonstrate no unusual amino-acid replacements relative to the latter. Our *cytb* data suggest that mitochondrial variation in the "BET" lineage is low throughout the range; however, the isolates of this species from North Europe remain unstudied.

Lineages "STR N" and "STR S" both belong to *Sicista strandi*. "STR N" is found in the north-western part of the species range, while "STR S" occurs in the southern and eastern parts. The level of divergence of *cytb* and *COI* between these two lineages is 6% (K2P-distance), which may indicate species-level divergence according to the genetic species concept (Bradley and Baker 2001). The degree of nuclear differentiation between "STR N" and "STR S" (Lebedev et al. 2019) is consistent with the mitochondrial results. Chromosomal variation within *S. strandi* was examined by Baskevich et al. (2005), and minor differences in C-heterochromatin banding patterns between northern and southern populations were revealed. However, to determine the status of the two lineages, additional studies based on extensive geographic sampling are required.

Comparison to the previous data by Cserkész et al. (2016) indicates that the latter authors underestimated the true level of variation of *Sicista subtilis*, as they did not examine true *Sicista severtzovi* and populations from the North Caucasus. Following Bradley and Baker (2001), this level of differentiation in the *S. subtilis* group may correspond to both intraspecific and interspecific variation but is, however, more consistent with the former. Based on this consideration, Cserkezs et al. (2016) regarded the small distance between "SUB" and "CIM" (which they believed to represent *S. severtzovi*) as an indication of their conspecificity. However, high resolution G-banding shows that *S. subtilis s. str.* and *S. severtzovi* are separated by a substantial number of Robertsonian and non-Robertsonian transformations, including five tandem translocations (Kovalskaya et al. 2011), and the same is true for comparisons between other karyomorphs of *S. subtilis sensu lato* (2–7 tandem translocations). According to the latter work, "the high number and complex nature of at least some of the chromosomal rearrangements distinguishing the various taxa are likely to result in reproductive isolation between them". Therefore, it seems plausible that *S. subtilis s. l.* represents a rare case of extremely rapid speciation via fixation of aberrant karyotypes. The gene flow between the incipient species is blocked not through gene divergence but rather due to meiotic incompatibility. Similar cases of genetically close but chromosomally divergent species are known in other groups of mammals. Two species of voles *Microtus* (*Alexandromys*) *evoronensis* and *Microtus* (*Alexandromys*) *mujanensis* are both rather close genetically to a widespread and polymorphic *Microtus maximowiczii* (p-distance of 2.4 and 1.7%, respectively); however, their karyotypes are separated from that of the latter by numerous rearrangements and the hybridization data indicate sterility in F1 males (Meyer et al. 1996). Another example is presented by the *Sorex araneus-antinorii* species pair (*cytb* p-distance – 2.3%, Brünner et al. 2002, Bannikova and Lebedev 2010), which may have diverged as late as the Late/Middle Pleistocene boundary. Here, the examination of the hybrid zone demonstrated that the gene flow between these two chromosomally distinct species is limited (Brünner et al. 2002). We believe that the discrimination of species based on genetic divergence should be performed with more caution in groups with intensive chromosome evolution.

Molecular clock results (Supplementary Table S1) performed by two methods suggest that the divergence among the four closely related lineages of east Europe probably occurred in the second half of the Middle Pleistocene (400–250 kya). The steppe birch-mice are not sufficiently cold-tolerant to accommodate harsh conditions of glacial maxima throughout their contemporary range; hence, one should conclude that during the cold phases of the late Middle and Late Pleistocene, different lineages must have survived in different steppe refugia of southeastern Europe. The existence of multiple refugia in this region is also supported by the data on genetic variation in *Sicista strandi*, as well by the phylogeographic or vicariance patterns in other steppe rodents, including the grey hamster *Nothocricetulus* (*ex Cricetulus*) *migratorius* (Lebedev et al. 2018), mole vole *Ellobius talpinus* (Bogdanov et al. 2015) and mole rat *Spalax* spp. (Nemeth et al.

2013), although the details of the geographic structure vary among taxa. One may hypothesize that many of the refugial populations of steppe birch mice could be associated with isolated sand dune systems or "sand islands" *sensu* Sludski (1964) located in the steppes of the Don basin, which now harbours the largest diversity of chromosomal variants (*Sicista severtzovi, Sicista cimlanica, Sicista* sp. 1, *Sicista* sp. 2).

The lack of pronounced divergence in the eastern part of the range of the "SUB" lineage suggests its recent eastward expansion, presumably from the Volga region. This expansion can be hypothesized to take place in either postglacial or marine isotope stage 3 (MIS3) interglacial time. In contrast to other cases (*Nothocricetulus migratorius, Ellobius talpinus, Spermophilus pygmaeus*), the Volga is not the barrier between the lineages of *Sicista subtilis s. l.* as the "SUB" lineage is found on both banks. However, its distribution on the western bank is restricted to a rather narrow band along the river valley (points 26–28). The exact place of origin of the "SUB" lineage and the scenario of its dispersal remain to be elucidated.

Although our genetic data are generally concordant with chromosomal evidence, it only represents the genealogy of a single marker, so all results should be verified with nuclear DNA analysis. The most important point to be clarified by future studies is the nature of variation observed among populations belonging to the "CIM" lineage. The mechanisms responsible for the formation of the complex mosaic of cytotypes are unclear. The available data neither contradict nor firmly support the reciprocal monophyly of the sub-lineages corresponding to *Sicista cimlanica s. str.*, *Sicista* sp. 1 and *Sicista* sp. 2; however, it is evident that their divergence could be a recent (Late Pleistocene) event. If forthcoming nuclear studies corroborate the observed mitochondrial pattern and confirm the lack of gene flow among these taxa, it will validate the hypothesis that the rates of speciation in the *Sicista subtilis* group could be extremely high.

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