FINAL REPORT

**Taxonomic revision of the southern birch mice, superspecies *Sicista subtilis***
**(PD 105116; NKFI-6)**

**Tamás Cserkész**

**EÖTVÖS LORÁND UNIVERSITY, Faculty of Science, Institute of Biology, Department of Systematic Zoology and Ecology**

## INTRODUCTION

The Palaearctic birch mice (genus *Sicista* Gray, 1827) represent one of the longest-living extant genera of rodents (Kimura 2010). The earliest records are 17 million years old, and hint at a central Asian centre of origin, from where the genus conquered wide variety of habitats in Eurasia and North America (Kimura 2013). They represent an early diverged group of the superfamily Dipodoidea showing unspecialised morphological adaptations; these characteristics can validate the separate classification of the genus into family Sminthidae, which was considered as separate family within superfamily Dipodoidea by Shenbrot *et al*. (1995) and this position was supported by genetic analysis (Lebedev et al. 2013).

Seven taxa are currently recognised in the *subtilis* group, most of these have an allopatric distribution in the western Eurasian steppe zone:

- *S. subtilis subtilis* (Pallas, 1773) has the easternmost and largest range extending from the River Ob and Lake Baikal to the river Volga (Shenbrot et al. 1995, Kovalskaya et al. 2011).
- The least known members of the group are *S. subtilis vaga* (Pallas, 1778) and,
- *S. subtilis sibirica* Ognev, 1935, as both have a limited and ambiguous distribution in southern Russia and northern Kazakhstan.
- *Sicista severtzovi* (Ognev, 1935) is a separate species since 1986 (Sokolov *et al.* 1986) and occupies the eastern-central part of European distribution of the genus in E Ukraine and W Russia (Shenbrot *et al.* 1995).
- A geographically restricted subspecies is *S. severtzovi cimlanica* Kovalskaya *et al*., 2000, only known from the Tsimlyansk Sands near river Don (Kovalskaya et al. 2000).
- *Sicista subtilis nordmanni* (Keyserling & Blasius, 1840) can be found on most of the territory of S Ukraine (Zagorodnyuk & Kondratenko 2000), in small part of Russia (Kovalskaya et al. 2011) and the area extending to SE and E Romania (Ausländer et al. 1959, Cserkész et al. 2015).
- Only two populations of the westernmost taxa, *S. subtilis trizona* (Frivaldszky, 1865) are currently known: one in Hungary and the other one in Transylvania, central Romania (Cserkész et al. 2015).

The above classification is currently the most widely accepted and widespread (Holden & Musser 2005) although some authors suggested modifications (Zagorodnyuk & Kondratenko 2000, Zagorodniuk 2009, Kovalskaya et al. 2011). Moreover, based on karyological evidences, Kovalskaya et al. (2011) supposed the existence of undescribed species of *Sicista* in the middle Don basin (named '*Sicista* sp.n.1' and '*Sicista* sp.n.2'). The strict chromosomal differences between *S. subtilis subtilis* and *S. subtilis nordmanni* may indicate reproductive isolation and already warrant for separate specific status (Zagorodnyuk & Kondratenko 2000, Kovalskaya et al. 2011). While most taxonomic studies of the group have used standard chromosome analysis and comparative chromosome banding analysis to understand relationships, molecular phylogenetic analysis of the genus was long awaited.

The lack of a comprehensive survey utilizing molecular approaches may contribute to taxonomic instability and incompletely resolved phylogeny of the *S. subtilis* group. The main goal of this study is to provide a comprehensive analysis of the group by using an integrative approach of genetic and morphological methods. To confirm this, we use the whole mitochondrial cytochrome *b* (CytB) to show the levels of genetic divergence according to the genetic species concept of (Bradley & Baker 2001); we use the CytB and a nuclear genetic marker (IRBP) to build a phylogenetic hypothesis.

## METHODS

### Field samples

All European members of the *Sicista subtilis* group were sampled in field by us during six subsequent East-European expeditions in 2012-2015. This sampling covers all but two currently recognized subspecific taxa in the group (Table 1). In all cases, except *S. subtilis subtilis*, our sampling represents the entire ranges of the taxa. The animals sampled were trapped with live-catching method using pitfalls (details given in Cserkész et al. 2015). Animals were released at the capture site after external measurements, samples and photographs were taken.

### Molecular methods

For the molecular work, whole genomic DNA was extracted from tissue samples (hair with the bulb attached) collected in the field using manual lysis and extraction method as detailed in (Cserkész et al. 2015). The amplification and sequencing of the IRBP gene also followed the above protocol. As for the mitochondrial CytB, we devised new primers based on the publicly available complete mitochondrion of *Mus musculus* (NC_005089) and *Rattus rattus* (NC_012374). Successfully amplified products were submitted to be sequenced from both directions by commercially available service provider (Macrogen Inc., South-Korea) using the original primers for sequencing. All newly generated sequences were submitted to GenBank (IRBP accession numbers: KP715879–KP715887; CytB: KP715861–KP715878). Besides own sequences, we downloaded publicly available IRBP sequences from GenBank.

## RESULTS

### Phylogenetic tree reconstruction

1100 bp of the first exon of the IRBP gene were obtained, and the sequences were aligned without the need of introducing gaps. Altogether, there were 250 variable positions of which 151 were parsimony-informative. If we focus only on the in-group (*Sicista* spp.), the number of variable/parsimony-informative sites decreases to 57 and 28, respectively. The 1000-times repeated heuristic search based on the above DNA-matrix found a single most parsimonious tree (Fig. 1) at 286 steps with negligible signs of homoplasy [Consistency Index(CI)=0.96, Homoplasy Index(HI)=0.042, Retention Index(RI)=0.94]. The ML search on the RaxML cluster found the same maximally plausible tree, therefore we only display bootstrap support values of that analysis on the corresponding branch of the MP-tree (Fig. 1).

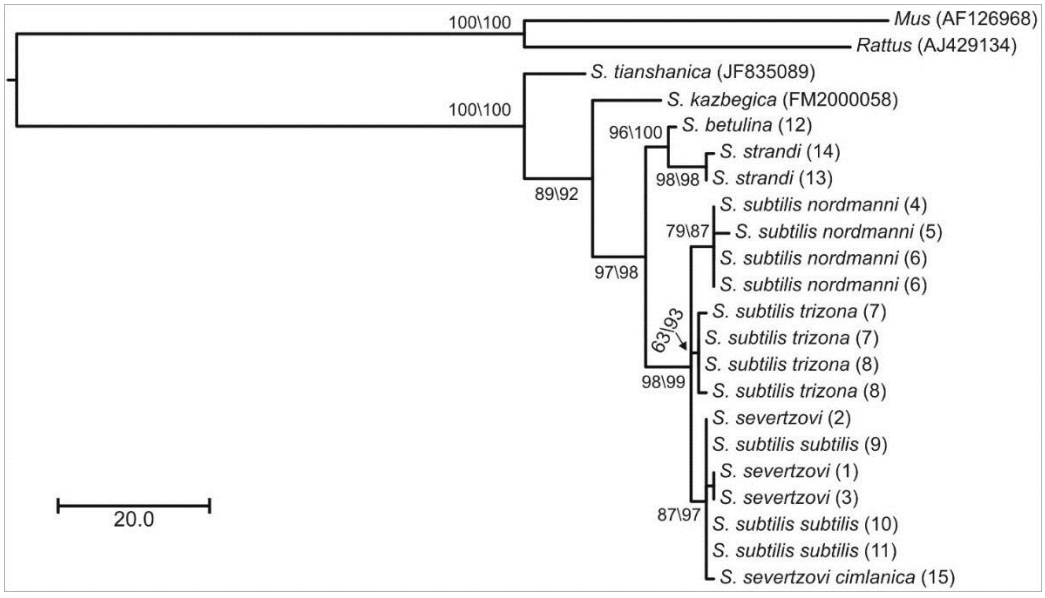


Figure 1. Phylogenetic tree of the genus *Sicista* with a focus on the subtilis group based on maximum parsimony analysis of IRBP-sequences. The tree is displayed as a phylogram, the scale bar stands for 20 mutational changes. Bootstrap percentages above 50% of 1000/100 pseudo-replicate resulting from MP/ML searches are displayed at the corresponding branches.

The IRBP-based tree provides basic insights into the phylogeny of the analysed species; the monophyly of the genus *Sicista* is highly supported, and within the genus four main clades are identified by IRBP. Within the highly supported (bs: 98%/99%) *S. subtilis* group, three well-defined clades can be found: the clade of the *S. subtilis nordmanni* samples (bs: 79%/87%); the clade of the samples of *S. subtilis trizona* (bs: 63%/93%); the clade of the samples of *S. subtilis subtilis* and *S. severtzovi* (incl. subspecies *cimlanica*). Although the phylogenetic relationship within the studied species remains unresolved with this nuclear regions, it helps to identify the main lineages within the *S. subtilis* group and informs us about possible hybridisation events between these lineages.

The mitochondrial CytB region provides much more resolution (see topology on Fig. 2), which is clearly attributable to the higher number of phylogenetically informative DNA-positions: there were 289 variable position, 250 of which are informative for parsimony in the 1132 bp-long alignment that did not require the introduction of gaps. The MP heuristic search identified the same six equally most parsimonious trees at length of 434 steps in 1000 repetitions. These trees had slightly higher levels of homoplasy (CI=0.73; HI=0.26, RI=0.88), but these figures are still normal for such an alignment. The topological difference between these six trees concerned finally unsupported branches (see Fig. 4). Similar to the IRBP-based tree, the ML analysis found a fully compatible tree with these MP-trees, therefore again just the support values are displayed on the corresponding branches. As all of the trees were compatible with the dendrogram, we constructed in the genetic distance analysis, we only show the statistical supports on the corresponding branches. The overall topology of the mitochondrial tree is consistent with that of the nuclear marker (Fig. 2) implying the lack of hybridization between the identified lineages. Within the CytB-based tree, more resolution is found both between the main clades and within two of the three main lineages also identified by the IRBP-tree. The phylogenetic relationship of the main clades is resolved here with high (MP analysis) and moderate (ML analysis) support (bs: 93%/76%) for the *Sicista subtilis trizona* and *S. subtilis nordmanni* lineages resolved as sister. Furthermore, the *S. subtilis trizona* clade is split into two main, highly supported (bs: 99%/92%) branches, one representing the Transylvanian, another the Pannonian lineage of samples; whereas the *S. subtilis subtilis* clade also splits into two highly (MP analysis) and moderately (ML analysis) supported lineages (bs: 100%/75%) one containing only samples of the nomenclatural type (bs: 100%/94%) and another including all *S. severtzovi* samples (bs: 92%/94%) including subspecies *cimlanica*.

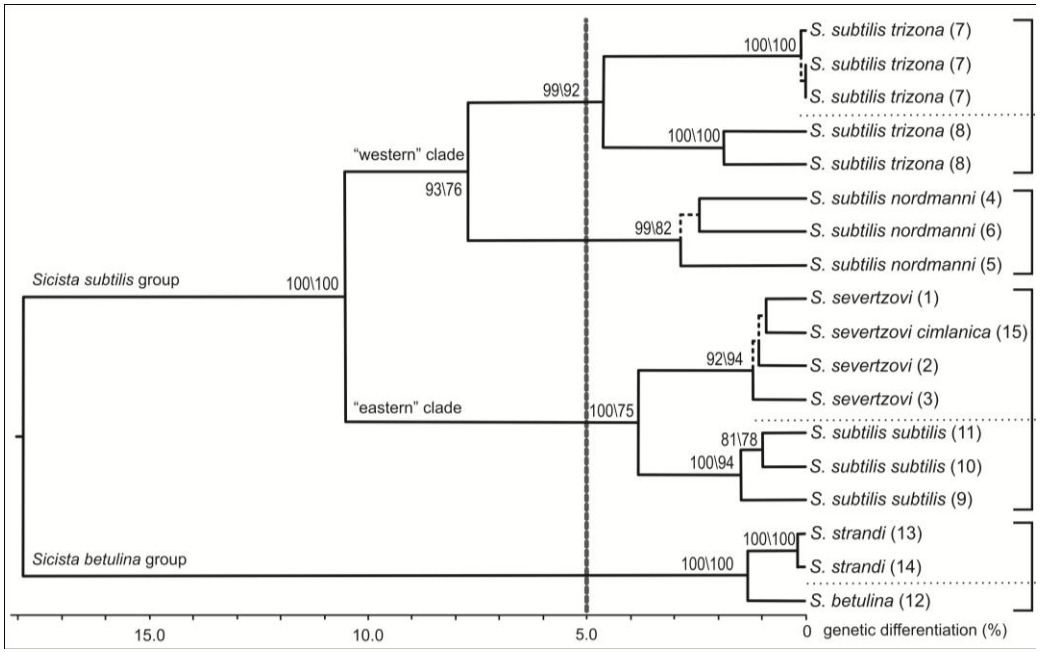


Figure 2. Kimura 2p genetic distance based dendrogram showing the *Sicista* species/populations analysed. The empirical 5% limit of sister species divergence in mammals, proposed by (Bradley & Baker 2001), is indicated on the dendrogram as dashed line. The bootstrap support from phylogenetic MP/ML analyses are shown next to the corresponding branches, whereas unsupported branches are indicated by dashing them.

**Genetic distances**

The dendrogram built upon the percentage differences of Kimura 2p genetic distance of the 1132 bp long CytB sequences (Fig. 2) mirrors the phylogenetic tree based on the same DNA region. The genetic difference between the two taxonomic groups of species shows a remarkable 18% difference, as do the main split between the two main lineages within the *subtilis* group, around 10%. There is again a substantial genetic difference between the *Sicista subtilis nordmanni* and *S. subtilis trizona* samples (7.25%), which is followed by the split (app. 4.5%) into two geographic clades in the latter subspecies. Interestingly, the *S. subtilis subtilis* and *S. severtzovi* lineages, although representing currently recognized separate species, only differentiated at a relatively low genetic level (app. 3.75%). The divergence between samples of *S. subtilis nordmanni* is similarly low (the highest is 2.9%) and can be explained by the geographic variation on a relatively large area. All other differences fall below 2%, which is defined as intraspecific variation in rodents (Bradley & Baker 2001), however, it is notable that the Transylvanian populations show 1.61% genetic difference in CytB, which is remarkable because it is displayed between specimens of two populations located approx. 10km away from each other.

## DISCUSSION

**Sequence divergence within the *S. subtilis* group**

The Genetic Species Concept (Bateson 1909, Dobzhansky 1937, Muller 1939) following Bradley and Baker (2001) and Baker and Bradley (2006) clearly defines a genetic species as "a group of genetically compatible interbreeding natural populations that is genetically isolated from other such groups" (Baker & Bradley 2006: 645). Our genetic distance based dendrogram (Fig. 2) readily shows the percentage divergence between the studied species. As expectable from taxonomy, the *S. betulina* and *S. subtilis* groups are well-diverged in their CytB region (18%). If looking at more shallow nodes, the western and eastern clade within the *subtilis* group shows a very significant, approximately 10% divergence. This is far above the 5% value assessed according to the Genetic Species Concept for allopatrically distributed phylogroups. Across several mammalian orders, species recognized by morphological distinction have CytB distance values >5%; notably, this is even true for sister species (Bradley & Baker 2001). This clearly hints at the specific-level separation of the two lineages. Moreover, the difference between *trizona* and *nordmanni* lineages is still well-above this threshold value (7.25%), implying again specific-level differentiation. Nevertheless, the difference between *severtzovi*, the alleged plesiomorphic member of the group according to other classifications (Sokolov et al. 1986), and *S. subtilis s.str.* is 3.75%, thus within the intraspecific divergence value of Bradley and Baker (2001), who reported values ranging from 0.0 to 4.7%. In accordance with this value, we found that intraspecific divergence is evident in most remaining cases in our dataset. The relatively high divergence between the two, geographically closely situated Transylvanian populations of *S. subtilis trizona* requires further evaluation, but we cannot exclude the possibility of high-levels of genetic diversity here.

**Phylogenetic relationships within the *Sicista subtilis* group**

The phylogenetic trees based on differently inherited markers produced totally congruent topologies (Figs. 1, 2); unsurprisingly, the nuclear IRBP region produced less resolution than the mitochondrial CytB, which is reported to be highly variable in rodents (Spradling et al. 2001, Kryštufek et al. 2012). Within *Sicista subtilis* group, CytB unravels apparently surprising results: the steppic species are not only arranged in the east to west gradient, but also split into two main groups: the western clade contains *S. subtilis trizona* and *S. subtilis nordmanni*, whereas the eastern group consists of the nomenclatural type *S. subtilis subtilis* and *S. severtzovi*.

Sokolov *et al*. (1986) made the first attempt to reconstruct the phylogenetic relationship within the *subtilis*-group and placed *S. severtzovi* to the base of the tree, and to postulated the relationship of '(*S. subtilis nordmanni*, (*S. subtilis subtilis*, *S. subtilis vaga*, *S. subtilis sibirica*))'. Finally, along with a comparative chromosome banding analysis, Kovalskaya *et al*. (2011) provided an update of the latter tree based on karyotypic differences. In spite of the different approaches followed by the above workers, our phylogenetic trees do not correspond to the previous results. More importantly, we found that the stripe on the back of the animals is a synapomorphic character of the *S. betulina* and *S. subtilis* groups. This difference in phylogenetic hypothesis might be due to the sole utilisation of morphological characters that are more prone to be homoplastic than neutral genetic markers.

**Hybridization**

Kovalskaya and Fedorovich (1997) suggested that hybridization may occur between the 24 and 26 chromosome forms (*S. subtilis subtilis* and *S. subtilis nordmanni*) in their contact zone. Later, this hypothesis turned up again in connection to *S. severtzovi cimlanica* (2n=22), which was hypothesized to be a hybrid of *S. severtzovi* (2n=18–20) and *S. subtilis* (2n=24) by judging from mere diploid chromosomal numbers (Kovalskaya et al. 2000). In the light of the data presented in Kovalskaya *et al.* (2011) it is unlikely that hybridisation between S*. subtilis* (2n=24) and *S. nordmanni* (2n=26) or *S. severtzovi* (2n=26) as well as between the two latter took place. Our molecular results (Figs. 1, 2) neither support hybridisation hypothesis; the comparison of phylogenetic trees representing differently inherited molecular sequences (i.e. biparentally inherited nuclear regions *versus* uniparentally inherited mitochondrial regions) can reveal past hybridization if the same lineage is placed on different clades on the trees – there is a hard-incongruence between them (Wendel & Doyle 1998). As our nuclear (Fig. 1) and mitochondrial (Fig. 2) trees are fully compatible with each other, we cannot conclude from this on hybridization between the lineages. Nevertheless, Zagorodniuk (2011) argues for an ongoing hybridization in the contact zone of the two stable chromosomal forms, *S. subtilis subtilis* and *S. subtilis nordmanni*, and he interpreted the extensive karyotypic variability of *Sicista* spp. in the region as evidence for this process. Clearly, our data currently do not support this hypothesis, but we cannot fully exclude this explanation, as past hybridization between ancestral forms and subsequent speciation in the contact zone can admittedly blur present molecular signs.

**CONCLUSIONS**

Our new phylogenetic complemented with published cytogenetic, morphologic and craniometric results support the recognition of the existence of three discrete taxonomic entities within the *S. subtilis* group (see Fig. 2). These are (according to the nomenclature used throughout in the paper): *S. subtilis* (including *S. subtilis subtilis* and *S. severtzovi*), *Sicista subtilis nordmanni, Sicista subtilis trizona*.

Our results on *Sicista severtzovi* and *S. subtilis subtilis* revealed little genetic differentiation between the two taxa; on the nuclear IRBP-tree S. *severtzovi* is nested within the *S. subtilis subtilis* specimens, while the more variable mtCytB sequences resolve them as sister to each other, but with very little resolution between *S. severtzovi* accessions. Taking the chromosomal variability also into consideration, it seems that the cytogenetically heterogeneous *S. severtzovi* has a surprisingly homogeneous genotype (see Figs. 1 and 2) but involving more sampling sites would be desired to extend this statement for the unsampled range of *Sicista severtzovi.* In the lack of nuclear and mitochondrial sequence divergence, craniometric and genital differences, we conclude the conspecific status of *S. severtzovi* and *S. subtilis subtilis*, and only accept this taxon as a subspecies of *S. subtilis*, as it was regarded before 1986 (Ognev 1935). Therefore, we classify it as *S. subtilis severtzovi*. Similarly, the taxonomic separateness of *S. severtzovi cimlanica* is also doubtful; most probably it is a distinct *severtzovi* karyotype because the level of genetic divergence is low and presently there are no morphological diagnostic characters that distinguish *S. severtzovi cimlanica* from *S. severtzovi*.

On the contrary to *S. severtzovi*, a very significant genetic distance was found between the 'western' and 'eastern' clades of the *Sicista subtilis* group. This distance is much larger than what is usually found below the species-level (Bradley & Baker 2001); moreover, it corresponds to 'geographically discreet phylogroups typical of different biological species'. Taking this information into consideration, and adding that genital, and phylogenetic differences exist between the two clades, plus recognizing that 27% of sister-species have no karyological differences indicating that not all speciation events are accompanied by detectable chromosomal rearrangements (Castiglia 2014), we conclude the separate specific status of *Sicista subtilis trizona* and *S. subtilis nordmanni*. Therefore, we elevate these species at the rank of species with the names *S. trizona* (Frivaldszky, 1865) and *S. nordmanni* (Keyserling and Blasius, 1840) respectively. As a consequence of this taxonomic treatment, we introduce the usage of *Sicista subtilis* sensu stricto to refer to the clade named 'eastern' on our Fig. 2.

This taxonomic treatment, what we regard the only acceptable in light of our data presented above, warrants for the re-evaluation of conservation status of the species, formerly only accepted at the subspecific level by IUCN (Kryštufek et al. 2008). As the extent of occurrence of *S. trizona* is much smaller than 5,000 km$^2$, and is known from a small number of locations (<5), we propose the IUCN 'endangered' status [EN – B1a+b(i,ii,iii)] to be applied for this species. As for *S. nordmanni*, the area of occupancy is estimated to be less than 2,000 km2 and the number of currently known populations are nine, we propose

for this species the 'vulnerable' status [VU – B2a+b(iii)] to be applied. Clearly, we need more field surveys to obtain more data on the exact occurrences of the taxa, but we can confidently state right now that as the result of our taxonomic rearrangement in the genus, the western members of the *Sicista subtilis* group deserve high conservation attention.

Finally, we point out the unusually large genetic distance between the *S. trizona* population of the Pannonian (#7) and Transylvanian (#8) Basins. This is far larger than what is usual at the intra-specific level; however, very little is known on these populations, as the Pannonian population is – as the sole representative of the lineage in the region – critically endangered and too small to have access to enough biological data on them (Cserkész & Gubányi 2008); while the Transylvanian population has only recently been re-discovered after more than 100 years (Cserkész et al. 2015). Nevertheless, the very significant genetic distance between these two populations warrants taxonomic recognition, and we describe here the Transylvanian population as separate from *S. trizona* at the subspecific level. Further studies have to establish morphological and cytogenetic differences between the two races, if exist.

**Description of *Sicista trizona transylvanica* ssp. nov. (Cserkész et al. 2016)**

*Holotype*

HNHM2459 (adult female), body in alcohol, skull extracted (Fig. 9), collected in Apahida (Romania; Transylvania; Cluj country, the former Kolozs country) by Endre OROSZ on August 1900. The specimen was determined by Lajos MÉHELY and deposited in the Hungarian Natural History Museum.

*Type locality*

Juc-Herghelie (Zsukiménes), Cluj county, central Romania (Transylvania), in the vicinity of Cluj-Napoca (Kolozsvár) and Apahida, 46° 52'N, 23° 45'E, 348 m above sea level, #8 in Fig. 1. [Detailed description of the habitat is given in (Cserkész et al. 2015) and description in (Cserkész et al. 2016)].

*Diagnosis*

The subspecies is clearly different from *S. trizona trizona* by having genetically different mitochondrial genome as exemplified by sequences of the cytochrome-b (CytB) and cytochrome c oxidase subunit I (COI) genes (Cserkész et al. 2015). The subspecies has a unique motive at the 5' end of CytB: 5'-ATTTCCTCATGATGAAATTTTGGCTCCCTACTAGGAATCTGCTTAATCATTCAAA-3'; whereas the unique motive at the 5' end of COI is: 5'- CGAGCTGAATTAGGTCAACCAGGTGCCCTATTAGGGGACGAC-3'. Typical CytB/COI sequence of specimen SSU64 is deposited in GenBank under the accession numbers: KP715874/KF854247, respectively. We refer to SSU64 as representative of the genetic characters described for *Sicista trizona transylvanica*.


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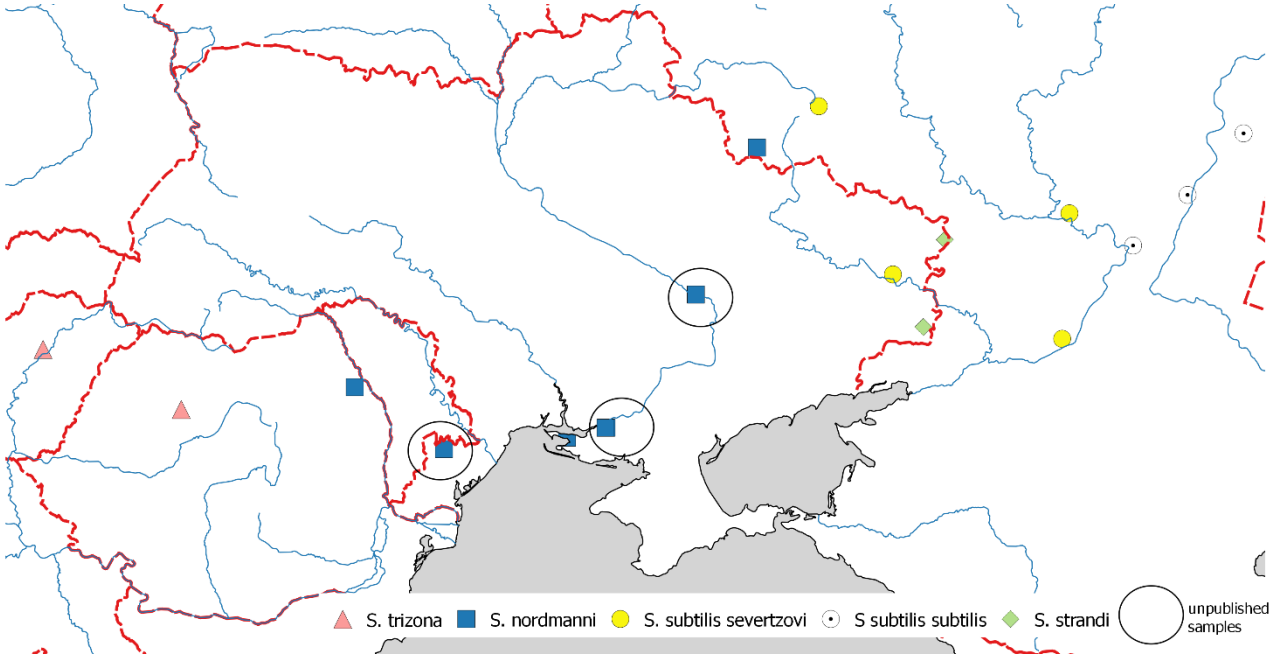
**APPENDIX**

Sampling sites in Hungary, Romania, Ukraine and Russia.



Sample details, IRBP, CytB sequences used in the genetic analyses of *Sicista* samples and published in Cserkész et al. 2015 and 2016.

| Sample code | Map code | GenBank acc. no. IRBP/ CytB | Taxon | Location | Date of capture |
|---|---|---|---|---|---|
| SS2 | 1 | KP715880/KP715864 | *S. severtzovi* | Trokhizbenka, Ukraine 48°48'N 38°57'E | 01.06.2013 |
| SS3 | 2 | KP715881/KP715865 | *S. severtzovi* | Serafimovich, Russia 49°39'N 42°43'E | 05.06.2013 |
| SS9 | 3 | KP715885/KP715869 | *S. severtzovi* | Yamskaya steppe, Russia 50°11'N 37°38'E | 13.06.2013 |
| SS10 | 15 | KP715886/KP715870 | *S. severtzovi cimlanica* | Tsimlyansk sands, Russia 47°49'N 42°39'E | 12.06.2014 |
| SSN01 | 4 | KF854235/KP715878 | *S. subtilis nordmanni* | Black-sea reserve, Ukraine 48°27'N 32°0'E | 01.08.2009 |
| SSU68 | 5 | KF854236/KP715876 | *S. subtilis nordmanni* | Iaşi, Romania 47°11'N 27°27'E | 12.09.2013 |
| SS7 | 6 | KP715887/KP715877 | *S subtilis nordmanni* | Borisovka, Russia 50°33'N 36°03'E | 12.06.2013 |
| SS8 | 6 | -/- | *S subtilis nordmanni* | Borisovka, Russia 50°33'N 36°03'E | 12.06.2013 |
| SSU56 | | -/KP715871 | *S. subtilis trizona* | Mezőcsát, Hungary 47°45'N 20°47'E | 22.09.2010 |
| SSU57 | 7 | KF854237/KP715872 | *S. subtilis trizona* | Mezőcsát, Hungary 47°45'N 20°47'E | 22.09.2010 |
| SSU58 | 7 | KF854238/KP715873 | *S. subtilis trizona* | Mezőcsát, Hungary 47°45'N 20°47'E | 24.09.2010 |
| SSU64 | 8 | KF854239/KP715874 | *S. subtilis trizona* | Juc-Herghelie, Romania 46°52'N 23°45'E | 11.08.2012 |
| SSU65 | 8 | KF854240/ KP715875 | *S. trizona trizona* | Feiurdeni, Romania 46°51'N 23°36'E | 11.08.2012 |
| SS5 | 9 | KP715883/KP715867 | *S. subtilis subtilis* | Kamyshin, Russia 49°55'N 45°14'E | 08.06.2013 |
| SS6 | 10 | KP715884/KP715868 | *S. subtilis subtilis* | Novokamenka, Russia 50°45'N 46°27'E | 09.06.2013 |
| SS4 | 11 | KP715882/KP715866 | *S. subtilis subtilis* | Ilovlya, Russia 49°14'N 44°07'E | 07.06.2013 |
| SBE02 | 12 | KF854241/KP715861 | *S. betulina* | Suseni, Romania 46°37'N 25°35'E | 17.07.2010 |
| STR01 | 13 | KF854242/KP715863 | *S. strandi* | Provallye, Ukraine 48°07'N 39°48'E | 10.07.2009 |
| SS1 | 14 | KP715879/KP715862 | *S. strandi* | Stricovskaya steppe, Ukraine 49°17'N 40°04'E | 29.05.2013 |