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Rediscovery of the Hungarian birch mouse (*Sicista subtilis trizona*) in Transylvania (Romania) with molecular characterisation of its phylogenetic affinities

Abstract: The Southern birch mouse (*Sicista subtilis*) is a small-sized rodent species characteristic of the Palearctic steppes with westernmost occurrences in central Europe. The species was considered to be extinct in Transylvania (central Romania), but in our field survey we captured three living individuals near the city of Cluj-Napoca. On the basis of nuclear interphotoreceptor retinoid-binding protein and mitochondrial cytochrome *c* oxidase subunit 1 sequences, we assessed the taxonomic status of the newly found *S. subtilis* population by comparing them to available sequences, including the sequences of its subspecies. The Transylvanian samples were found to be genetically closest to the Hungarian samples of *S. subtilis trizona*. These new records extend the known geographic range of this rediscovered species and provide additional information on its habitat preference and external morphological features. Moreover, our phylogenetic tree reconstruction for seven *Sicista* taxa provides a basic insight into the phylogenetic relationships of the genus, placing the northern Eurasian taxa (*S. betulina* and *S. subtilis*) at the crown of the tree and the central Asian taxa at the base of the tree. The Transylvanian occurrence of *S. subtilis trizona*, which is endemic to the Carpathian Basin, is of high faunistic value as a result of an increase in the number of known populations of this subspecies – one of the most endangered rodents of Europe – from one to two.

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Introduction

The Southern birch mouse (*Sicista subtilis*, Pallas, 1773 Sminthidae) is a small rodent characteristic of the Palearctic steppes. Its range extends from Kazakhstan to Hungary, where it reaches the western limit of its geographic distribution. Four subspecies are recognised in Europe: the eastern part of the distribution in Kazakhstan and adjacent Russian territories are occupied by *S. subtilis subtilis* Pallas, 1773 and *S. subtilis vaga* Pallas, 1779. These are replaced by *S. subtilis nordmanni* Keyserling and Blasius, 1840 in southern Ukraine and with extension of these subspecies further into southern and eastern Romania (Pucek 1982). However, chromosome differences between *S. subtilis s. str.* and *S. subtilis nordmanni* may indicate their reproductive isolation, and these two forms are considered as independent species by Kovalskaya et al. (2011). Moreover, Zagorodnyuk (2009) demonstrated that the name *S. loriger* (Nathusius, 1840) is an older synonym than *S. nordmanni* (Keyserling and Blasius, 1840), thus the former should be used as the taxon name by Principle of Priority. Nevertheless, here we will follow Holden and Musser (2005) because their classification is more widely accepted in the literature. The westernmost representative is *S. subtilis trizona* Frivaldszky, 1865, which is known from only one location in Hungary (Cserkész and Gubányi 2008). The species is also known to occur in Poland but its taxonomic status is unclear. Nevertheless, a morphometric study indicates close morphometric proximity of the specimens from Poland to S. subtilis trizona (Cserkész et al. 2009). In addition to the aforementioned records, there is one record

from Transylvania (central Romania) in 1900. This is testified by a single female specimen collected by Endre Orosz from Apahida (currently in Cluj County). The specimen is stored at the Mammalian Collection of the Hungarian Natural History Museum, Budapest. Given that the subspecies *trizona* and *nordmanni* can be distinguished only by examination of the male reproductive organs (Méhely 1913), it is impossible to identify this specimen at the subspecies level. The subspecific identification of this specimen and the Transylvanian *S. subtilis* population is further complicated by the geographically intermediate position between the Hungarian *trizona* and the east Romanian *nordmanni*. Méhely (1913) noted that "*the specimen found in Apahida may belong to other "race" because the Transylvanian Plain is not typical lowland, but rather a low plateau*".

The recent discovery of *Sicista* remains in owl pellets in the Apahida region has opened a new possibility in the identification of the Transylvanian Southern birch mouse. The remains of two specimens (one maxilla and a pair of mandibles) were found in owl pellets of unknown age collected within the vicinity of the villages Câmpeneşti and Juc Herghelie, 6 km away from Apahida (Aczél-Fridrich and Hegyeli 2009). After this first discovery, the remains of eight additional individuals had been identified in the region between 2009 and 2011 from barn owl (*Tyto alba* Scopoli, 1769) and little owl (*Athene noctua* Scopoli, 1769) pellets (unpublished data, Zs. Aczél-Fridrich). With these new data, we aimed to trap living Southern birch mice in the Apahida region and to determine the taxonomic position of the Transylvanian *subtilis* population with fresh material using molecular phylogenetic methods.

Surprisingly, very little is known about the genus-level molecular phylogenetics of the genus *Sicista*, which has 13 species in Eurasia (Holden and Musser 2005). Several studies dealing with the overall phylogeny of the order Rodentia (DeBry and Sagel 2001, DeBry 2003, Montgelard et al. 2008, Wu et al. 2012) or the family Dipodidae (Fan et al. 2009) and related groups (Jansa and Weksler 2004) included samples of *Sicista*. The only study that analysed four species of *Sicista* was that of Zhang et al. (2013), who also provided divergence times based on the Bayesian molecular clock approach. Although Kovalskaya et al. (2011) gained an important insight into the phylogenetic relationships of the genus based on karyological evidence, the molecular phylogeny of the genus is still lacking. Nonetheless, the phylogenetic position of the genus is assured; all higher-level studies agree in placing the genus at the basal branch of the phylogenetic tree of the superfamily Dipodoidea, sister to all remaining species of the family, which is in turn sister to family Muridae (DeBry and Sagel 2001, DeBry 2003, Jansa and Weksler 2004, Montgelard et al. 2008). Therefore, the genus *Sicista* represents an early diverged group of the superfamily showing unspecialised morphological adaptations. These characteristics can validate the separate classification of the genus into family Sminthidae. Sminthidae was considered as a separate family within the superfamily Dipodoidea by Shenbrot et al. (1995), and this position was supported by genetic analysis (Lebedev et al. 2013).

The approach of molecular barcoding, i.e., utilising DNA sequences for identifying a specimen (Hebert et al. 2003a), is now well established. The sequence of the mitochondrial cytochrome *c* oxidase subunit 1 (*COI*) gene in animals is used (Hebert et al. 2003b) as the standard barcoding region, and this is the region of choice in mammals as well (Ivanova et al. 2012). Nevertheless, other DNA regions with comparably high mutation rates may also be used for molecular identification of unidentified biological specimens. The first exon (ca. 1200 bp) of the nuclear gene encoding the interphotoreceptor retinoid-binding protein (IRBP) is extensively utilised in rodent phylogenetics because of its ability to provide reliable, well-resolved, and robust results (e.g., Michaux et al. 2002, Jansa and Weksler 2004, Pagés et al. 2010, Barbosa et al. 2013). In addition, the use of nuclear regions are favoured in mammalian phylogenetics over mitochondrial regions as the former performs better than the latter in providing resolution and they are less affected by superimposed substitutions (Springer et al. 2001). In the present study, we present information on the Transylvanian Southern birch mouse and describe its current habitat and accompanying small mammal species. We also identify the species by molecular phylogenetic methods using a molecular barcoding approach utilising nuclear *IRBP* and mitochondrial *COI* genes. Finally, we present a preliminary molecular phylogeny of the genus *Sicista* on the basis of these gene regions.

Materials and methods

Field surveys and study area

Field surveys were conducted in July 2010 with a week-long trapping session within the vicinity of Câmpeneşti and Juc Herghelie (Cluj County, central Romania), where 180 pitfall traps were installed. Having been unsuccessful, we returned to the area on the 6–12 August 2012 with 350 pitfall traps and extending the study area further to the village of Feiurdeni (Figure 1). The unbaited pitfall traps were simple 5- and 7-litre buckets sunk to a depth

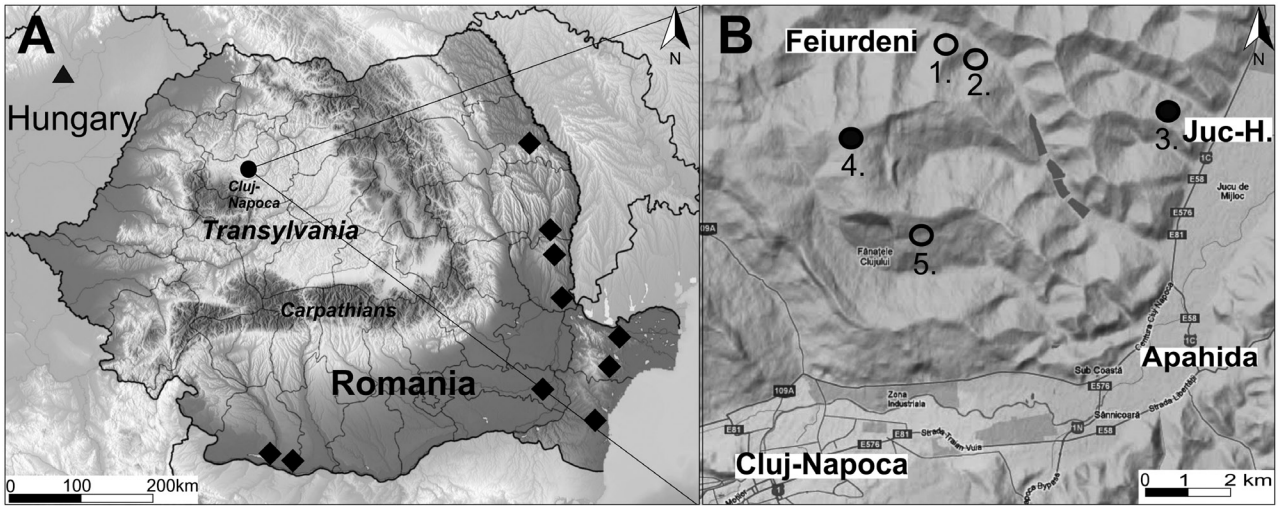

Figure 1 (A) Published and new records of the Southern birch mouse (*Sicista subtilis*) in Romania and Hungary. (B) Overview map of the study area, showing trapping sites in Transylvania. Legends: ◆– published records for *S. subtilis nordmanni* (Ausländer and Hellwing 1957, Hamar and Şutova 1965, Simionescu 1965, Marcheş 1970, Popescu et al. 1976, Petrache 1988). ▲– published records for *S. subtilis trizona* (Cserkész and Gubányi 2008). ●– new records for *S. subtilis trizona*. ○– trapping site without capture of the Southern birch mouse.

where the rim was even with the ground level. Small holes were bored into the bottom of the buckets to allow water to drain. Drift fences or ditches were not used to exert as little impact on the habitat as possible. In five trapping sites (70 traps/site) the traps were checked three times a day, and each individual captured was measured for the following characters: length of tail, body, hind foot, and total weight. Sex and age (determined as juvenile, subadult, or adult) were also noted. A genetic sample of each *Sicista subtilis* specimen was obtained by extracting a hair with the bulb attached. Samples and photographs were taken, and then the animals were released at the capture site after the measurements. Sites 1 and 2 were abandoned grasslands with shrubs; sites 3 and 4 were dry pasture swards grazed with cattle; site 5 was steppe-like grassland dominated by feather grass species (*Stipa* spp.). A weather dataset was downloaded from Tutiempo (www.tutiempo.net) (providing data from a weather station located in Cluj-Napoca, some 10 km away from the trapping sites).

The study area is located at the Some Plateau, a geographic region dominated by steppe and forest steppe habitats, intersected by rivers, steep or gently sloping hills and ridges. These steppes, together with those that remain in the Pannonian Basin, can be regarded as a western transition zone towards the large Eastern European steppes enclosed by the Carpathians (Bohn et al. 2000). On the southern exposure, landslides and undulate terraces reveal sunny bare slopes, whereas the northern slopes are more mesic. Intensive crop monoculture and plantations are typically found within the vicinity of main roads. Most of the Some Plateau lays within the Natura 2000 protected area "Dealurile Clujului Est" (site code: ROSCI0295). Nomenclature for plant species and communities follows that of Ciocârlan (2009), whereas those for mammals follows that of Wilson and Reeder (2005).

DNA-extraction, PCR-amplification and sequence analyses

Whole genomic DNA was extracted from hairs with bulbs collected from animals trapped in the fields (Table 1) using manual lysis and extraction method. Tissue was placed in 100 µl lysis puffer (containing NaCl, EDTA, and SDS), treated with proteinase K at 55°C overnight, and then proteins were removed by adding 0.5 volume ammonium acetate. Isopropanol was added to extract the DNA, which was pelletised at full speed in a microcentrifuge, followed by washing it twice by 70% ethanol and resuspending it in 10 mM Tris. Some 1 µl of unquantified amount of this DNA-extract was used as template in a polymerase chain reaction (PCR) to specifically amplify a 1.1 kb long part of the first exon of the *IRBP* gene using newly devised primers (forward: 5′-AGC AGG CCA TGA AGA GTC G-3′; reverse: 5′-TCA TTA TCA CGG AGG CAT CAG C-3′) based on publicly available *Sicista* sequences (GenBank accession numbers: AF297288, FM200058). The PCR-reaction mixture contained 0.6 mM dNTP, 2 mM Mg, 0.02 µM of each primer, 0.2 U DreamTaq Green DNA polymerase (Thermo Fisher Scientific Ltd., Waltham,

Table 1 Samples and sequences used in this study.

Sample code	GenBank acc. no. IRBP/COI	Species	Location	Date of capture	Collectors	Source
SSN01	KF854235/KF854243	*Sicista subtilis nordmanni*	Kherson, Ukraine 48°29′N 32°33′E	01.08.2009	M. Rusin	This study
SSU68	KF854236/KF854244	*S. subtilis nordmanni*	Iaşi, Romania 47°11′N 27°27′E	12.09.2013	Authors	This study
SSU57	KF854237/KF854245	*S. subtilis trizona*	Mezőcsát, Hungary 47°45′N 20°47′E	22.09.2010	Authors	This study
SSU58	KF854238/KF854246	*S. subtilis trizona*	Mezőcsát, Hungary 47°45′N 20°47′E	24.09.2010	Authors	This study
SSU64	KF854239/KF854247	*S. subtilis*	Juc-Herghelie, Romania 46°52′N 23°45′E	11.08.2012	Authors	This study
SSU65	KF854240/KF854248	*S. subtilis*	Feiurdeni, Romania 46°51′N 23°36′E	11.08.2012	Authors	This study
SBE02	KF854241/KF854249	*S. betulina*	Suseni, Romania 46°37′N 25°35′E	17.07.2010	Authors	This study
STR01	KF854242/ –	*S. strandi*	Luhansk, Ukraine 48°07′N 39°48′E	10.07.2009	M. Rusin	This study
–	JF835089/ –	*S. concolor*	–	–		(Zhang et al. 2013)
	FM200058/ –	*S. kazbegica*	–	–		(Montgelard et al. 2008)
	AF297288/ –	*S. tianshanica*	–	–		(DeBry and Sagel 2001)
–	AF126968/ –	*Mus musculus*	–	–		(Stanhope et al. 1992)

MA, USA), and 2× of the corresponding reaction buffer. Amplification was performed in an Abi Veriti 9600 thermocycler (Applied Biosystem, Grand Island, New York, USA) programmed for initial denaturation at 94°C for 3 min, followed by 25 cycles of denaturation at 94°C for 15 s, annealing at 58°C for 30 s, extension at 72°C for 1 min, and a final extension at 72°C for 7 min. PCR amplification of the mitochondrial *COI* region followed the description given by Ivanova et al. (2012) using the universal primers BatL5310 (fw) and R6036R (rv). The amplification success was checked on 1% agarose gel stained with ethidium bromide. Successfully amplified products were submitted to be sequenced from both directions by a commercially available service provider (Macrogen Inc., Seoul, South-Korea) using the original primers for sequencing. The sequences of the same sample were manually checked for errors, and then continuous reads ("contigs") were made by hand using the software Chromas Lite v.2.1 (Technelysium Pty Ltd., South Brisbane, Australia). All sequences were submitted to GenBank (accession numbers: KF854235–KF854249). Besides our own sequences, we downloaded publicly available IRBP sequences from GenBank (Table 1). Given the scarcity of publicly available *Sicista COI* sequences, the mitochondrial dataset was not expanded by GenBank sequences. The sequences were aligned manually in BioEdit v.7.1.3 (Hall 1999). Phylogenetic analysis of the sequences relied on maximum parsimony search conducted in Paup v.4.0b*10 (Swofford 2003). Given that the number of sequences allowed an exhaustive search (i.e., examine all possible trees in the virtual tree-space) to be conducted on both datasets, this analysis was performed with defining publicly available sequences of *Mus musculus* Linnaeus, 1758 (*IRBP*: AF126968; *COI*: NC_005089) as outgroup, and treating every character as unordered and unweighted. The reliability of topology was checked in a bootstrap test with 1000 pseudoreplications. To check for an alternative phylogenetic hypothesis when using different tree-building criteria, we conducted maximum likelihood search on the RaxML server (Stamatakis et al. 2008) with default settings and searching for best-scoring tree after 100 bootstrap. Finally, we compared genetic distances between the analysed specimens based on Kimura 2-p distances of the *COI* region calculated in Paup v.4.0b×10. The raw distances were transformed into percentages to illustrate genetic divergence.

Results

Field surveys

During the survey in August 2012, three specimens of the Southern birch mouse were captured from Sites 3 and 4 (Figure 1) within a single 24-h period after the trapping had been ongoing for 5 days (Table 2). The first two animals were found in pitfall traps during the afternoon indicating that mice had entered the traps during the day; the third

Table 2 External measurements (mm) and weight (g) for the three specimens of *Sicista subtilis* captured in Transylvania in August 2012.

ID	Species	Location	Date and time of capture	Sex	Age	Weight (g)	Body[a]	Tail[a]	Foot[a]
SSU64	*S. subtilis*	Juc-Herghelie	11.08.2012, 18:00 h	Male	Adult	–	60	78	14.5
SSU65	*S. subtilis*	Feiurdeni	11.08.2012, 18:00 h	Female	Subadult	5.75	49	68	15
SSU66	*S. subtilis*	Juc-Herghelie	12.08.2012, 09:00 h	Male	Adult	6.5	54	72	14

[a]Length of body/tail/hind foot in mm.

mouse was captured the next morning. The capture sites were close to Apahida (6 and 11 km away, respectively).

At the beginning of the trapping session the weather was warmer and drier than the average, but the temperature decreased as the trapping continued. At the time of the captures the air pressure decreased while the humidity sharply increased. When the third mouse was captured it had also started to rain. Both capture sites were dry swards, which are typical near streams, but in these examples they are part of a swamp bed. The formation of the swamp bed is caused by the presence of an insulation layer in the soil that prevents drainage. Therefore, the site experiences high levels of groundwater especially in spring, favouring the development of high and dense vegetation. None of the habitats had been ploughed in the previous year (or perhaps never), but they are slightly disturbed causing the apparent spread of weeds.

Site 3 near to Juc-Herghelie is a slope with intense shrub encroachment (*Prunus spinosa, Rosa canina*) situated between 325–352 m asl. It had been extensively grazed by cattle and horses, but it was not in use during the trapping period. This 19-hectare mosaic grassland habitat with a smaller mesophilous part, classified as *Cirsio cani-Festucetum pratensis* (Sanda et al. 2008), is bordered by extensive arable fields, meadows and a built-up area, and there is a busy traffic road just 500 m away from the capture site. The apparent spread of weeds was probably due to grazing during the previous periods. Abundant plant species in the site were *Alopecurus pratensis* L., *Carex hirta* L., *Arrhenatherum elatius* (L.) P.Beauv. ex J.Presl & C.Presl., *Poa angustifolia* L., *Festuca pratensis* Huds., *Dactylis glomerata* L., *Cirsium arvense* (L.) Scop., *Mentha longifolia* (L.) Huds., and *Cirsium canum* L. This site lies beyond the borders of the Natura 2000 protected area. Together with the two birch mice, we captured the following small mammals (number of captured individuals in parentheses): *Microtus arvalis* Pallas, 1778 (12), *Micromys minutus* Pallas, 1771 (2), *Sorex araneus* (2), *S. minutus* Linnaeus, 1758 (1), and *Crocidura leucodon* Hermann, 1780 (1).

Site 4 is located on a plateau between 603–615 m in altitude, near to the village of Feiurdeni. Like Site 3, it had been extensively grazed by cattle but the pasture land was not operational during the trapping period. According to the locals, it was the only field around the area that had never been ploughed because it was always used as pasture and the bush was eradicated annually. This 73-hectare dry sward habitat is classified as *Deschampsietum caespitosae* (Sanda et al. 2008) and is bordered by newly ploughed grassland, oak forest, and meadow intensively grazed by sheep. Weeds were apparently gaining territory in the area probably because of grazing. Abundant plant species found in the site were *Deschampsia caespitosa* (L.) P.Beauv., *Agrostis stolonifera* L., *Juncus conglomeratus* L., *Carex* sp., *Festuca arundinacea* Schreb., 1771, *Phleum pratense* L., *Lythrum salicaria* L., *Cirsium arvense*, and *Centaurea jacea* L. This site is part of the Natura 2000 protected area. Some small mammals captured along with the birch mouse were *Microtus arvalis* (5), *Nannopalax leucodon* Nordmann, 1840 (1), *Sorex minutus* Linnaeus, 1766 (6), *S. araneus* (1), and *Crocidura leucodon* (1). This list has generated surprising new data; to our knowledge, this is the first report of a lesser blind mole rat (*Nannospalax leucodon*) captured by pitfall trap.

DNA-based molecular barcoding and phylogenetic tree reconstruction

The newly devised primers specifically amplified an 1100 bp long region of the first exon of the *IRBP* gene, which was possible to align unambiguously without the need of introducing gaps in the alignment. The alignment matrix had 215 variable positions, which decreases to 58 sites if the outgroup is disregarded. In this matrix, the Transylvanian samples were 0–2 steps segregated from the Hungarian samples of *Sicista subtilis trizona* (the Transylvanian sample SSU64 was actually identical with the Hungarian sample SSU58).

The universal primers and method given by Ivanova et al. (2012) provided 706 bp long reads of the mitochondrial *COI*, the conventional barcoding region of mammals, from the 5′ end of the gene (spanning positions 5328–6872 in *Mus musculus* reference genome NC_005089). The alignment did not require the introduction of gaps and it provided 195 variable positions, 120 if we only regard the ingroup (i.e., *Sicista* samples), on 706 bp length.

Unfortunately, we were unable to obtain reliable reads of the sample 'STR01'. Nevertheless, we believe that this sample is not highly important for this study, thus we omitted this sample from the *COI* dataset.

Based on the *IRBP* sequences, the exhaustive search under maximum parsimony (MP) criterion identified two equally parsimonious phylogenetic trees at length 234 [consistency index (CI) =0.9573, homoplasy index =0.0427 (HI), retention index (RI)=0.8592], which differ from each other in the placement of the species *Sicista concolor* (Büchner, 1892) and *S. tianshanica* (Salensky, 1903) – a placement that received no bootstrap support in 1000 pseudoreplicates. Otherwise, all branch received strong (>85%) bootstrap support (bs) (Figure 2A). The best scoring maximum likelihood (ML) phylogenetic tree found by RaxML had identical topology (data not shown) to that of the MP-tree presented with the branch leading to *S. concolor* again receiving no bootstrap support (Figure 2A).

The phylogenetic tree based on nuclear *IRBP* and rooted with *Mus musculus* (Figure 2A) placed central Asian *Sicista tianshanica* at the basal clade, followed by the Chinese species *S. concolor*. Nonetheless, the phylogenetic relationship is unsupported (i.e., the basal clades can be collapsed into a trichotomy), and we have to say

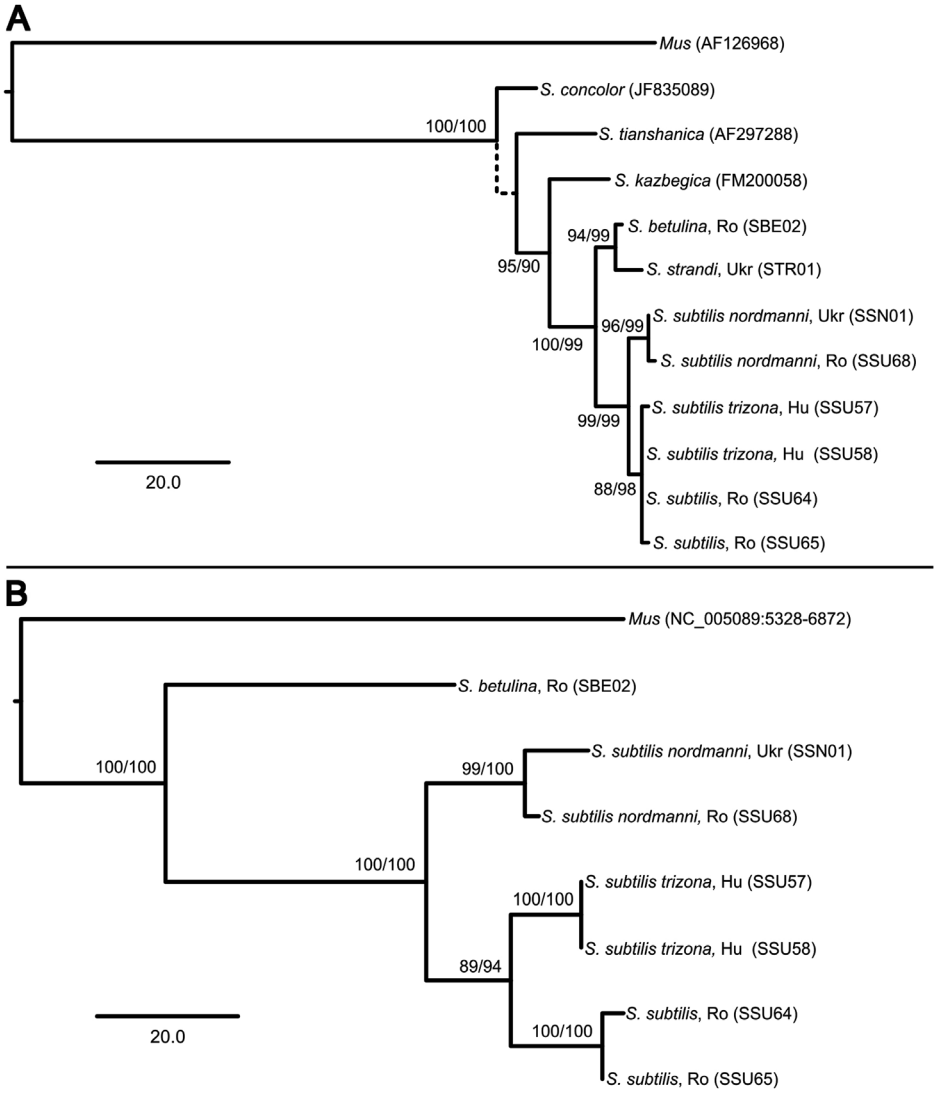

Figure 2 Phylogenetic relationships of selected *Sicista* species based on nuclear *IRBP* (A) and mitochondrial *COI* (B) sequences. The trees were obtained in exhaustive searches under maximum parsimony criterion. Here presented are one of the two equally maximum parsimonious trees (A), and the single most parsimonious tree (B) drawn as a phylogram (the scale bar represents 20 mutation steps) with bootstrap values of 1000 pseudoreplicates at corresponding branches. Given that a maximum likelihood search on RaxML found a ML tree with identical topology, only bootstrap values of that analysis are presented here after the slash. The dashed branch received no bootstrap support in either analysis.

that the topology of the basal clades represented by these two species remained unresolved. All other relationships are clear: the basal clades are followed by the Caucasian species *S. kazbegica* Sokolov, Baskevich & Kovalskaya 1986 (bs: 95/90%), and above this node, the tree has two main branches – the first clade (bs: 94/99%) contains two species as a monophyletic group comprising samples of the boreal species *S. betulina* Pallas, 1779 and *S. strandi* Formozov, 1931. The second clade (bs: 100/99%) is the monophyletic clade of samples of the widespread Eurasian species *S. subtilis*, where we can see a clear taxonomic pattern: the eastern Romanian and southern Ukrainian samples of subspecies *nordmanni* forms a well-supported (bs: 96%/99%) diverged clade, whereas the Hungarian *S. subtilis trizona* and the Transylvanian samples (SSU64 & 65) are found on the same branch (bs: 88/98%) without significant genetic difference between the samples.

The exhaustive search under MP criterion found only one most parsimonious tree (CI=0.9028, HI=0.0972, RI=0.7895) at length 247 based on the mitochondrial *COI* region. Although this single most parsimonious tree (Figure 2B) echoed the tree based on *IRBP* in topology, it provided some more insights into the phylogenetic relationships. The higher resolution of the *COI*-tree comes as no surprise because in animals mitochondrial *COI* is one of the most rapidly evolving, highly resolving marker (Hebert et al. 2003b). *Sicista* samples formed a well-supported (bs: 100%) monophyletic group, where *S. betulina* branches again first. Within this branch we find samples of the *S. subtilis* aggregate, where the subspecies *S. s. nordmanni* samples branch first (bs: 99/100%). Sister to this clade we find the monophyletic group (bs: 89/94%) of the Carpathian Basin samples with both pair of samples from the same population forming highly supported (bs: 100%) terminal nodes. At this particular part of the tree, we found more resolution compared to the *IRBP*-tree, which did not provide resolution between the Hungarian and Transylvanian samples. Additionally, it is notable that, unlike the Hungarian population, the Transylvanian population displays some within-population variation because the individuals examined do not share the same mitochondrial haplotype. When we repeated the same analysis with the inclusion of the corresponding *IRBP* sequences, we found only slight increment in bootstrap support (data not shown).

In terms of *COI* sequence divergence, we found differences from 0–24% (Table 3). The *Sicista* species showed an average divergence of 23.4% from *Mus*, whereas the well-diverged species *S. betulina* showed 14.7% average divergence from *S. subtilis s.l.* Within this latter taxon, we found 6.2% and 6.4% of average sequence difference between *S. subtilis nordmanni* and the Hungarian and Transylvanian *S. subtilis* populations, respectively. There is a considerable 3.6% genetic distance between the Transylvanian and Hungarian populations of *S. subtilis*.

Table 3 Matrix of genetic divergence expressed as percent difference between the studied samples based on Kimura 2-p distance of 706 bp long mitochondrial *COI* sequences.

	Mus	SBE02	SSN01	SSU68	SSU57	SSU58	SSU64	SSU65
Mus	0							
SBE02	23.4	0						
SSN01	24	14.4	0					
SSU68	23	14.4	1.6	0				
SSU57	23	14.4	6.6	5.7	0			
SSU58	23	14.4	6.6	5.7	0	0		
SSU64	23.8	15.5	6.8	6.4	3.8	3.8	0	
SSU65	23.4	15.3	6.3	6	3.4	3.4	0.4	0

SBE2, *S. betulina*; SSN01, *S. subtilis nordmanni* from Ukraine; SSU68, *S.s. nordmanni* from Romania; SSU57 and SSU58, *S.s. trizona* from Hungary; SSU64 and SSU65, *S.s. trizona* from Romania.

Discussion

Molecular identification of the Transylvanian samples

Our nuclear *IRBP* and mitochondrial *COI* sequence-based molecular barcoding approach to identify the newly rediscovered Transylvanian population of birch mouse identified *Sicista subtilis trizona* as the closest genetic relative. Additionally, the other subspecies *S. subtilis nordmanni*, represented by the eastern Romanian and the southern Ukrainian samples, was placed on a separate clade. Thus, we can exclude the possibility that this subspecies, which is otherwise native to eastern Romania, was found in Transylvania. Given the favorable molecular characteristics (confidence in homology, base-compositional stationarity, high resolution power) of the *IRBP* region (Jansa and Weksler 2004), and the general utility of the *COI* region as barcoding marker, we conclude that the Juc-Herghelie and Feiurdeni specimens can be taxonomically identified as *S. subtilis trizona*. Indeed, one of the Transylvanian samples shared the same *IRBP* haplotype with the Hungarian samples, which – by also considering the significant difference of the other subspecies– can only be explained if these two individuals had retained the common *IRBP* haplotype once characteristic to their common ancestor at the subspecies level. Moreover, there is a substantial 3.6% genetic divergence between the two *S. subtilis trizona* populations, which is greater than what we can observe

between the *S. subtilis nordmanni* populations (1.6%). This genetic divergence can be the result of long isolation of the Hungarian and Transylvanian populations, but still less than what is found between the subspecies (mean 6.3%).

Habitat characteristics, conservation issues and activity of the re-discovered population in Transylvania

The results confirm the occurrence of *Sicista subtilis trizona* in Romania. Our study has increased the number of known populations of this extremely rare subspecies from one to two. So far the only known population has been located in Hungary (Cserkész and Gubányi 2008), 233 km far away from this new location. The phylogenetic structure of this geographically isolated subspecies should be further investigated using more samples from several locations. Further research is also needed to define its known geographical range more clearly and to derive estimates of population density. Although the Natura 2000 area ["Dealurile Clujului Est" site of community interest (SCI)] was designated, among others, for the conservation of the Southern birch mouse based on previous owl pellet occurrences (Aczél-Fridrich and Hegyeli 2009), limits of this site require important modifications given that one of our trapping sites (Site 4) was located outside of the SCI. Vegetation structure in the Transylvanian study area corresponded to the structure of the Hungarian study area particularly because they both have a weedy flora. In Hungary, the birch mouse habitat's indicator plants are *Carduus acanthoides* L. and *Cirsium arvense* (L.) Scop. (Cserkész and Gubányi 2008). Likewise in Transylvania, *C. arvense* was present at both locations. The Hungarian birch mouse feed on the small seeds of these weeds (Cserkész 2011).

The result of the study has high conservation significance because *S. subtilis trizona* is one of the most endangered rodents in Europe and it is endemic to the Carpathian Basin. The range of the Transylvanian population has not yet been established, but presumably it may be widespread with isolated subpopulations possibly occurring far from the locations of current records. Conversely, it is clear that populations of this species are threatened because the area of its preferred habitats is decreasing owing to the spread of intensive agriculture and to the unbroken popularity of sheep farming. Habitats covered by high and dense vegetation, which are preferred by the birch mouse, are often ploughed up by local farmers or overgrazed by sheep. In Hungary, the recommended management method for the birch mouse habitats is cattle grazing (Cserkész et al. 2010). In Romania, extensive grazing has been the dominant method of grassland farming over large areas for many centuries. In recent years, grasslands have either been fertilised or abandoned, mainly as a result of the altered socioeconomic situation since 1989, and the agricultural landscape is increasingly dominated by intensively cultivated fields and fragmented by roads (Cremene et al. 2005). The Transylvanian steppe-like grasslands harbor a variety of plant and insect species of continental steppe origin, which was likely to have been colonised during postglacial warm periods (Rákosy and Kovács 2001). Therefore, conservation efforts should also consider the isolation of these habitats by improving the connectedness between remnants of steppe-like grasslands (Cremene et al. 2005).

It may be unprecedented in the densely populated Europe of the 21st century that a mammal could have remained undetected for more than a century despite its proximity to people and settlements. Another such discovery is known among rodents: the Bavarian pine vole (*Microtus bavaricus* König, 1962) was not recorded after 1962 and it was thought to be extinct, but a population apparently belonging to this species was discovered in 2000 in Austria (Spitzenberger et al. 2000, 2008).

Beyond its rareness, there may be ethological causes in the background of the scarce occurrence of Southern birch mouse. As suggested by terrarium observation and Hungarian capture data, the weather has a significant influence on the surface activity of the mammal and it may aestivate in summer (Cserkész 2011). Our new observation of meteorological conditions also suggests that birch mice are less active during the dry, warmer periods of the study period. As high temperatures fell and the humidity increased, the individuals were captured probably because of their increased activity.

Phylogenetic relationships

Our results provided a primary insight into the phylogenetic relationships of the genus *Sicista,* although the taxonomic representation of our phylogenetic trees is far from complete: only six species are present in the more densely sampled *IRBP* dataset out of the 13 species recognised in the genus (Holden and Musser 2005). Moreover, the congruence of the trees obtained by two different optimality criteria, and the general high statistical support of the branches allow us to draw some conclusions on the phylogenetic relationships of the studied taxa. Firstly, our *IRBP* tree is congruent with the phylogenetic tree of Zhang et al. (2013), who included four species of *Sicista* in their broader study. This finding is not surprising because they used the

same region in a very similar analysis. Nonetheless, we can add to their tree, which does not show support values, that the relationship between *S. tianshanica* and *S. concolor* is in fact unresolved. Despite this uncertainty, these two eastern Asian species likely represent an early lineage within the genus. The next branching lineage is represented by the Caucasian species *S. kazbegica*, which again can represent a relict lineage of the mountain range characterised by many relict species of both plants and animals.

The two-crown lineage of our *IRBP*-based phylogenetic tree is represented by the monophyletic group of the species pair *Sicista betulina* and *S. strandi*, as well as by the species *S. subtilis s.l.* This group of steppe- and taiga-inhabiting birch mice may suggest a common origin of these species (i.e., those adapted to more northern latitudes) or relatively recent expansion to these regions. The placement of *S. strandi* as sister to *S. betulina* corroborates the recent separation of this species from the latter (Sokolov et al. 1989), and confirms the karyological data (Kovalskaya et al. 2011). By including two subspecies of *S. subtilis* in our work, we could examine the monophyly of the species. According to our results, the Southern birch mouse is indeed a phylogenetically cohesive, monophyletic species, which likely shows separation at the subspecific level. This question, together with others related to the phylogeny of *Sicista*, can be addressed in a future study that samples the genus more densely. Nonetheless, we can readily report the first exon of the *IRBP* gene as a good candidate to be used in the molecular phylogenetics of *Sicista*.

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References

Aczél-Fridrich, Zs. and Zs. Hegyeli. 2009. New finding of the southern birch mouse in Transylvania. Abstracts of the 10th Biology Days, Cluj, pp. 5.

Ausländer, D. and S. Hellwing. 1957. Observations écologiques sur les petits mammiferes des ecrans forestiers de protection de "Valul Traian"; références spéciales concernant leur dynamique. Trav. du Mus. d'hist. nat. "Gr. Antipa" 1: 111–139.

Barbosa, S., J. Pauperio, J.B. Searle and P.C. Alves. 2013. Genetic identification of Iberian rodent species using both mitochondrial and nuclear loci: application to non-invasive sampling. Mol. Ecol. Resour. 13: 43–56.

Bohn, U., G. Gollub, C. Hettwer, Z. Neuhäuslová, T. Raus, H. Schlüter and H. Weber. 2000. Map of the Natural vegetation of Europe. Scale 1: 2 500 000. Bundesamt für Naturschutz, Bonn.

Ciocârlan, V. 2009. Flora ilustrată a României: Pteridophyta et Spermatophyta. Editura Ceres, Bucureşti.

Cremene, C., G. Groza, L. Rákosy, A. Schileyko, A. Baur, A. Erhardt and B. Baur. 2005. Alterations of steppe-like grasslands in Eastern Europe: a threat to regional biodiversity hotspots. Conserv. Biol. 19: 1606–1618.

Cserkész, T. 2011. Ecological, taxonomical and conservation biological investigation of Southern Birch Mouse (*Sicista subtilis trizona*). Doctoral (PhD) dissertation, Eötvös Loránd University.

Cserkész, T. and A. Gubányi. 2008. New record of Southern birch mouse (*Sicista subtilis trizona*) in Hungary. Fol. Zool. 57: 308–312.

Cserkész, T., I. Kitowski, K. Czochra and M. Rusin. 2009. Distribution of the Southern birch mouse (*Sicista subtilis*) in East-Poland. Morphometric variations in discrete European populations of superspecies *S. subtilis*. Mammalia 73: 221–229.

Cserkész, T., H.A. Török, J. Farkas, M. Bodnár and N. Seres. 2010. Southern birch mouse (*Sicista subtilis trizona*) action plan. Bükk Mammalogical Society, Eger.

DeBry, R.W. 2003. Identifying conflicting signal in a multigene analysis reveals a highly resolved tree: the phylogeny of Rodentia (Mammalia). Syst. Biol. 52: 604–617.

DeBry, R.W. and R.M. Sagel. 2001. Phylogeny of Rodentia (Mammalia) inferred from the nuclear-encoded gene IRBP. Mol. Phylogenet. Evol. 19: 290–301.

Fan, Z., S. Liu, Y. Liu, B. Zeng, X. Zhang, C. Guo and B. Yue. 2009. Molecular phylogeny and taxonomic reconsideration of the subfamily Zapodinae (Rodentia: Dipodidae), with an emphasis on Chinese species. Mol. Phylogenet. Evol. 51: 447–453.

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

Hamar, M. and M. Şutova. 1965. Studiul ecologic al mamiferelor (Mammalia) din agrobiocenozele din Dobrogea şi Bărăgan. Comunicări de Zoologie-Societatea de Ştiinţe Naturale şi Geografice din R.P.R. 3: 37–66.

Hebert, P.D.N., A. Cywinska, S.L. Ball and J.R. deWaard. 2003a. Biological identifications through DNA barcodes. Proc. Roy. Soc. B-Biol. Sci. 270: 313–321.

Hebert, P.D.N., S. Ratnasingham and J.R. de Waard. 2003b. Barcoding animal life: cytochrome *c* oxidase subunit 1 divergences among closely related species. Proc. Roy. Soc. B-Biol. Sci. 270: 96–99.

Holden, M.E. and G.G. Musser. 2005. Family Dipodidae. In: (D.E. Wilson and D.M. Reeder, eds.) Mammal species of the world. A taxonomic and geographic reference. 3rd edn. Johns Hopkins University Press. pp. 2142.

Ivanova, N.V., E. L. Clare and A.V. Borisenko. 2012. DNA barcoding in Mammals. In: (W.J. Kress and D.L. Erickson, eds.) DNA Barcodes: Methods and Protocols, Methods in Molecular Biology, vol. 858. Springer Science+Business Media. pp. 153–182.

Jansa, S.A. and M. Weksler. 2004. Phylogeny of muroid rodents: relationships within and among major lineages as determined by IRBP gene sequences. Mol. Phylogenet. Evol. 31: 256–276.

Kovalskaya, Y.M., V.M. Aniskin, P.L. Bogomolov, A.V. Surov, I.A. Tikhonov, G.N. Tikhonova, T.J. Robinson and V.T. Volobouev. 2011. Karyotype reorganisation in the *subtilis* group of birch mice (Rodentia, Dipodidae, *Sicista*): unexpected taxonomic diversity within a limited distribution. Cytogenet. Genome Res. 132: 271–288.

Lebedev, V.S., A.A. Bannikova, M. Pagès, J. Pisano, J.R. Michaux and G.I. Shenbrot. 2013. Molecular phylogeny and systematics of Dipodoidea: a test of morphology-based hypotheses. Zool. Scr. 42: 231–249.

Marcheş, G. 1970. Date privind răspîndirea şi importanţa ştiinţifică şi practică a unor mamifere din Dobrogea. Ocrotirea Naturii 14: 165–180.

Méhely, L. 1913. Magyarország csíkos egerei. Mathematikai és Term. tud. Közl. 31: 3–45.

Michaux, J.R., P. Chevret, M.G. Filippucci and M. Macholán. 2002. Phylogeny of the genus *Apodemus* with a special emphasis to the subgenus *Sylvaemus* using the nuclear IRBP gene and two mitochondrial markers: cytochrome b and 12S rRNA. Mol. Phylogenet. Evol. 23: 123–136.

Montgelard, C., E. Forty, V. Arnal and C. Matthee. 2008. Suprafamilial relationships among Rodentia and the phylogenetic effect of removing fast-evolving nucleotides in mitochondrial, exon and intron fragments. BMC Evol. Biol. 8: 321.

Pagés, M., Y. Chaval, V. Herbreteau, S. Waengsothorn, J.F. Cosson, J.P. Hugot, S. Morand and J. Michaux. 2010. Revisiting the taxonomy of the Rattini tribe: a phylogeny-based delimitation of species boundaries. BMC Evol. Biol. 10: 184–211.

Petrache, O. 1988. Studiul dinamicii populaţiilor de mamifere mici din sud-estul Olteniei (zona de hidroameliorare Sadova-Corabia) şi importanţa acestora în echilibrul naturii. Synopsis of the doctorate thesis, Universitatea din Bucureşti.

Popescu, A., P. Barbu and M. Cociu. 1976. Sur la présence de l'espece *Sicista subtilis* (Pallas, 1773) (Rodentia, Dipodidae) dans la réserve des dunes du complexe des irrigations Sadova-Corabia. Trav. du Mus. D'hist. nat. "Gr. Antipa" 17: 467–469.

Pucek, Z. 1982. *Sicista subtilis* (Pallas, 1773) – Steppenbirkenmaus. In: (J. Neithammer and F. Krapp eds.) Handbuch der Säugetiere Europas. Band 2./I. Nagetiere II. Akademische Verlagsgesellschaft, Weisbaden. pp. 501–515.

Rákosy, L. and S. Kovács eds. 2001. The nature reserve "Butterfly hill" in Viisoara. Societatea Lepidopterologica Romana, Cluj-Napoca.

Sanda, V., K. Öllerer and P. Burescu. 2008. Fitocenozele din Romania. Editura Ars Docendi-Universitatea din Bucuresti.

Shenbrot, G.I., V.E. Sokolov, V.G. Heptner and Y.M. Kovalskaya. 1995. The Mammals of Russia and Adjacent Regions. Dipodoidea. Nauka Press, Moscow, Russia.

Simionescu, V. 1965. Contribuţii la cunoaşterea sistematicii şi răspîndirii geografice a faunei de rozătoare (Glires) din Moldova. Analele Ştiinţifice ale Universităţii "Al. I. Cuza", Iaşi, Secţiunea 3-a, 11: 127–142.

Sokolov, V.E., Y.M. Kovalskaya and M.I. Baskevich. 1989. On species status of Northern birch mice Sicista strandi (Rodentia, Dipodidae). Zool. Zhur. 68: 95–106.

Spitzenberger, F., P. Brunet-Lecomte, A. Nadachowski and K. Bauer. 2000. Comparative morphometrics of the first lower molar in *Microtus* (*Terricola*) cf. *liechtensteini* of the Eastern Alps. Acta Therio. 45: 471–483.

Spitzenberger, F., J. Zima, H. Meinig and V. Vohralík. 2008. *Microtus bavaricus*. In: (IUCN 2012.) IUCN Red List of Threatened Species. Version 2012.2. <www.iucnredlist.org>. Accessed 09 February 2013.

Springer M.S., R.W. DeBry, C. Douady, H.M. Amrine, O. Madsen, W.W. de Jong, M.J. and Stanhope. 2001. Mitochondrial versus nuclear gene sequences in deep-level mammalian phylogeny reconstruction. Mol. Biol. Evol. 18: 132–143.

Stamatakis, A., P. Hoover and J. Rougemont. 2008. A Rapid Bootstrap Algorithm for the RAxML Web Servers. Syst. Biol. 57: 758–771.

Stanhope, M.J., J. Czelusniak, J.S. Si, J. Nickerson and M. Goodman. 1992. A molecular perspective on mammalian evolution from the gene encoding interphotoreceptor retinoid binding protein, with convincing evidence for bat monophyly. Mol. Phylogen. Evol. 1: 148–160.

Swofford, D.L. 2003. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

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

Wu, S., W. Wu, F. Zhang, J. Ye, X. Ni, J. Sun, S.V. Edwards, J. Meng and C.L. Organ. 2012. Molecular and paleontological evidence for a Post-Cretaceous origin of Rodents. PLoS ONE 7: e46445.

Zagorodnyuk, I. 2009. Taxonomy and nomenclature of the non-Muroidea rodents of Ukraine. Zbirnyk Prats' Zoologichnoho Muzeyu, Kiyev 40: 147–185.

Zhang, Q., L. Xia, Y. Kimura, G. Shenbrot, Z. Zhang, D. Ge, Q. Yang. 2013. Tracing the origin and diversification of Dipodoidea (Order: Rodentia): evidence from fossil record and molecular phylogeny. Evol. Biol. 40: 32–44.