Old views and new insigths: taxonomic revision of the Bukovina blind mole rat, *Spalax graecus* (Rodentia: Spalacinae)

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As a result of their rather uniform external appearance and gross cranial morphology, the systematics of blind mole rats has been hotly debated over the last century; however, the separation of the large-bodied and small-bodied blind mole rats at the genus level (*Spalax* and *Nannospalax*, respectively), suggested earlier on morphological grounds, is strongly supported by recent molecular biological evidence. The species of *Spalax* have so far been distinguished from each other by cranial traits only, especially the outline of sutures of the cranium, and the shape and relative size of the nasal and parietal bones. Based on mitochondrial DNA sequences (with the widest taxonomic and geographic coverage so far) and detailed anatomical comparisons of museum specimens, we herewith provide a revision of the taxonomic and phylogenetic status of the westernmost representative of the genus, *Spalax graecus s.l.* We clarify that *antiquus* and *istricus* – presently regarded as synonyms of *graecus* – are well-defined species, and they together form a separate clade within *Spalax*. The robustness of our conclusions is supported by the combined evidence of morphology, multilocus phylogeny, species distribution, and taxon history (species congruence with past tectonic and climate events).

INTRODUCTION

The Eurasian blind mole rats (subfamily Spalacinae) are adapted exclusively to underground life (Méhely, 1909; Topachevskii, 1969; Savić & Nevo, 1990). Species of the group can be found in the Balkan Peninsula, in steppe grasslands in Central and Eastern Europe, in the Middle East (Asia Minor and the coastline of the Levant), and in a narrow coastal strip in north-east Africa (Musser & Carleton, 2005).

Because of their rather uniform external appearance and gross cranial morphology, the systematics of blind mole rats has been hotly debated over the last century. The first comprehensive work, a milestone in blind mole rat systematics, was published by Méhely (1909) who, based on his study of subtle differences in cranial and dental structures, recognized one genus with three subgenera and eight species, with 14 additional subspecies. His opinion was later regarded as overly 'splitting', whereas at the other extreme Ellerman & Morrison-Scott (1951) accepted only three species in one genus. It is worth noting that these

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latter authors conducted no detailed comparisons of the taxa they synonymized. The next baseline work in Spalacinae systematics was published by the outstanding Ukranian morphotaxonomist, Topachevskii (1969), who, after studying hundreds of specimens, basically came to the same conclusions as Méhely (1909) as regards the genus-group systematics. However, his concept of a basic taxonomic divide between 'small' and 'large' blind mole rats, although supported by craniodental differences, was not always followed in the literature published in English (e.g. Savić & Nevo, 1990; Nevo, Ivanitskaya & Beiles, 2001; Musser & Carleton, 2005), but traditionally was accepted in the Russian mammalogical works (e.g. Resetnik, 1941; Gromov *et al.*, 1963; Pavlinov & Rossolimo, 1987). Nevertheless, as cytogenetic (Lyapunova, Vorontsov & Martynova, 1971) and molecular genetic (Hadid *et al.*, 2012) works provided further support of deep taxonomic divergences within the subfamily, and support of the presence of two genera (*Spalax* and *Nannospalax*), this old-established classification scheme has finally been accepted in the most recent publications (Németh *et al.*, 2009; Arslan, Akan & Zima, 2011, Kryštufek *et al.*, 2012, Chişamera *et al.*, 2013).

Whereas the exclusively subterranean lifestyle of blind mole rats defines the phenotype, and effectively narrows the range of anatomical variations, meticulous morphological studies can reveal sound osteological and dental characters with taxonomic meaning, and the applicability of such traits in the taxonomy of the large-bodied blind mole rats has been widely accepted. The species of the genus *Spalax* have so far been distinguished from each other by cranial traits only, especially the outline of sutures of the cranium, and the shape and relative size of the nasal and parietal bones (Méhely, 1909; Topachevskii, 1969; Korobchenko & Zagorodnyuk, 2009). According to presently accepted views, six species distributed from Romania to Kazakhstan in the western part of the Eurasian steppe zone are recognized (IUCN, 2012; Fig. 1), namely *Spalax arenarius* Reshetnik, 1938; *Spalax giganteus* Nehring, 1898; *Spalax graecus* Nehring, 1898; *Spalax microphthalmus* Güldenstaedt, 1770; *Spalax uralensis* Tiflov and Usov, 1939, and *Spalax zemni* (Erxleben, 1777) (Musser & Carleton, 2005).

The currently known localities of *S. graecus* (Fig. 2), the westernmost representative of the genus, can be found in three areas separated by the Carpathians (Zagorodnyuk & Coroiu, 2008; see map in Chişamera *et al.*, 2013). These isolated populations can be assigned to described taxa, the nomenclatural history of which is summarized by Chişamera *et al.* (2013). Accordingly, the nominotypical form *graecus graecus* Nehring, 1898 occurs in Ukranian and
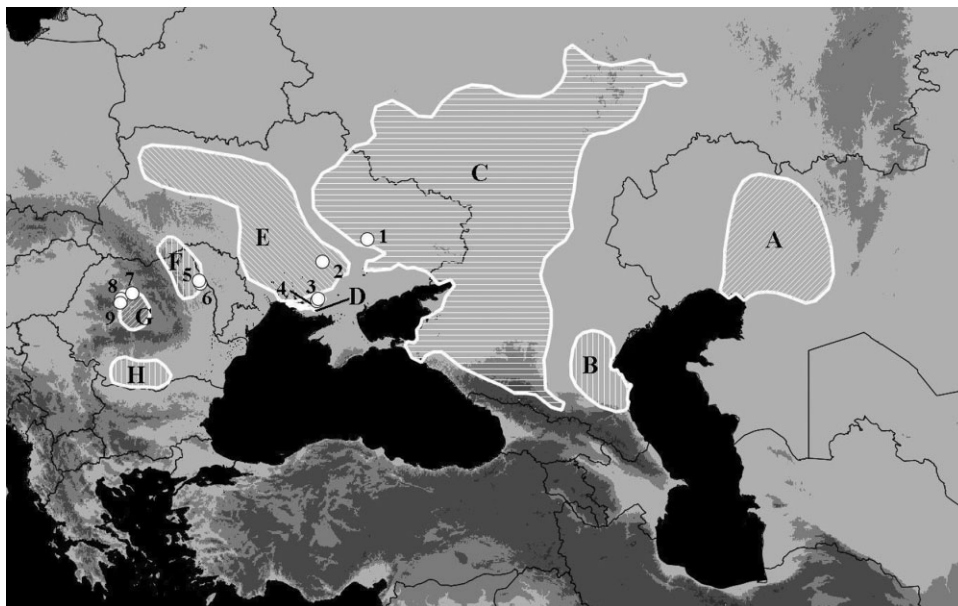

Figure 1. Distribution of *Spalax* species (capitals) and the geographic origin of the investigated tissue samples (numerals). Distribution areas are based on the International Union for Conservation of Nature (IUCN) Red List maps, Chişamera *et al.* (2013), and our own data. A, *Spalax uralensis*; B, *Spalax giganteus*; C, *Spalax microphthalmus*; D, *Spalax arenarius*; E, *Spalax zemni*; F, *Spalax graecus s.s.*; G, *Spalax antiquus*; H, *Spalax istricus*. 1, Novomoszkovsz; 2, Krivij Rig; 3, Kherson Province; 4, Tzurupinsk District; 5, David Valley, Iasi; 6, Dealul lui Dumnezeu; 7, Budeşti; 8, Aiton; 9, Sânduleşti.

Figure 2. Live specimen of *Spalax graecus* from David Valley, Iasi, Romania (photo: G. Csorba).

Romanian Bukovina, *istricus* Méhely, 1909 is known from Oltenia and Muntenia, Romania, and two forms, *antiquus* Méhely, 1909 and *mezosegiensis* Szunyoghy, 1937, were described from Transylvania, Romania. The last three names mentioned are generally regarded as synonyms of *graecus*, although their taxonomic status is usually not discussed (Pavlinov & Rossolimo, 1987, 1998; Gromov & Erbaeva, 1995; Pavlinov, Yakhontov & Agadjanian, 1995; Musser & Carleton, 2005). Interestingly, Topachevskii (1969) recognized *istricus* as a valid subspecies of *graecus*, and Murariu & Torcea (1984) found it specifically distinct from other species recorded from Romania. However, these conslusions were questioned because they were based on the small sample size of the specimens studied (Musser & Carleton, 2005).

Apart from the morphological approaches, only two papers deal with the phylogenetic relationships of *graecus* (and *Spalax* species in general) based on molecular methods. In their consensus tree (based on five mitochondrial sequences), Hadid *et al*. (2012) showed that within the *Spalax* lineage the Bukovinian and Transylvanian samples formed two separate clades. The average difference separating these two clades is similar to the genetic difference found between two well-established species, *arenarius* and *zemni*. Chişamera *et al*. (2013) examined the phylogenetic relationship of *graecus graecus* with the three species (each regarded as superspecies by, for example: Musser & Carleton, 2005; Németh *et al*., 2009) of *Nannospalax*, and regarding the relationship of the two genera arrived at the same conclusion as Hadid *et al*. (2012). Nevertheless, because of the lack of available material from other populations of *S. graecus* – although their investigation was regarded as a crucial issue – Chişamera *et al*. (2013) had to restrict their study to the nominotypical form.

The aim of the present paper is to fill this gap in our knowledge, and based on mitochondrial DNA (mtDNA) sequences of *Spalax* species (with the widest taxonomic and geographic coverage so far) and detailed anatomical comparisons of museum specimens – including the types of *mezosegiensis* and *istricus* and historical topotype material of *antiquus* – to provide a revision of the taxonomic and phylogenetic status of *S. graecus* and its subspecies.

MATERIAL AND METHODS

SAMPLES

The museum specimens investigated are held in the following collections: 'Grigore Antipa' National Museum of Natural History, Romania (GAM); Hungarian Geological and Geophysical Institute, Hungary (MÁFI); Hungarian Natural History Museum, Hungary (HNHM); Department of Evolutional and Genetical Systematics of Schmalhausen Institute of Zoology, Ukrainian Academy of Sciences, Ukraine (SIZUAS); Székely National Museum, Romania (SZNM); Palaeontological Museum, Ukrainian Academy of Sciences, Ukraine (PMUAS); Zoological Museum of Moscow State University, Russia (ZMMU); and Zoological Museum, Ukrainian Academy of Sciences, Ukraine (ZMUAS).

The specimens used in the morphological comparisons were as follows:

Spalax arenarius Ukraine: ZMUAS ($N = 1$, paralectotype), HNHM ($N = 2$)

Spalax giganteus Russia: ZMUAS ($N = 10$), GAM ($N = 1$), HNHM ($N = 1$)

Spalax graecus antiquus Romania: SZNM ($N = 2$), MÁFI ($N = 1$), HNHM ($N = 3$)

Spalax graecus graecus Ukraine: SIZUAS ($N = 1$), ZMUAS ($N = 13$), Romania: HNHM ($N = 1$)

Spalax graecus istricus Romania: HNHM ($N = 2$, lectotype and paralectotype)

Spalax graecus mezosegiensis Romania: HNHM ($N = 3$, lectotype and paralectotypes)

Spalax microphthalmus Ukraine: SIZUAS ($N = 4$), ZMUAS ($N = 27$), HNHM ($N = 3$) Russia: HNHM ($N = 4$)

Spalax uralensis Kazakhstan: ZMMU ($N = 3$), HNHM ($N = 1$)

Spalax zemni Ukraine: ZMUAS ($N = 2$), PMUAS ($N = 10$), HNHM ($N = 4$)

Live animals (Table 1) captured for genetic sampling were handled in the field in accordance with guidelines approved by the American Society of Mammalogists (Gannon, Sikes & the Animal Care and Use Committee of the American Society of Mammalogists, 2007). The animals were caught by breaking open the tunnel system and capturing the animal trying to mend the

Table 1. Blind mole rat taxa, origin of tissue samples, and GenBank accession numbers of all sequences used in the phylogenetic reconstructions

Taxon	Locality	Voucher no.	Accession numbers					
			NADH1	12S RNA	16S RNA	tRNA-Val	t-RNA Leu	cytochrome b
arenarius	Kherson, Ukraine	23215	HQ652218	HQ652149	HQ652193	HQ652325	HQ652281	KF021254
arenarius	Tzurupinsk, Ukraine	23216	HQ652218	HQ652149	HQ652193	HQ652325	HQ652281	KF021255
arenarius	Kherson, Ukraine	23217	–	–	–	–	–	KF021262
graecus s.s.	David Valley, Romania	23202	HQ652219	HQ652150	HQ652194	HQ652326	HQ652282	KF021251
graecus s.s.	David Valley, Romania	23204	–	–	–	–	–	KF021252
graecus s.s.	Dealul lui Dumnezeu, Romania	23206	–	–	–	–	–	KF021253
antiquus	Aiton, Romania	23003	HQ652214	HQ652147	HQ652191	HQ652323	HQ652279	KF021256
antiquus	Săndulești, Romania	23189	–	–	–	–	–	KF021257
antiquus	Budești, Romania	23532	–	–	–	–	–	KF021263
microphthalmus	Novomoszkovsz, Ukraine	23221	HQ652220	HQ652127	HQ652172	HQ652305	HQ652260	KF021258
microphthalmus	Novomoszkovsz, Ukraine	23222	HQ652220	HQ652127	HQ652172	HQ652305	HQ652260	KF021259
zemni	Krivij Rig, Ukraine	23219	HQ652219	HQ652150	HQ652194	HQ652326	HQ652282	KF021260
zemni	Krivij Rig, Ukraine	23220	HQ652219	HQ652150	HQ652194	HQ652326	HQ652282	KF021261
Nannospalax judaei	Anza, Israel	–	HQ652226	HQ652133	HQ652177	HQ652310	HQ652265	JN571135
Acomys cahirinus	–	–	HQ652223	HQ652130	HQ652063	HQ652285	HQ652251	AJ012017

Vouchers are stored in the Hungarian Natural History Museum). (HNHM).

damage (Németh *et al*., 2007). After biopsy of hindfoot skin matrix (applying topical and systemic anaesthesia and 70% alcohol disinfection) for DNA analysis, individuals were released at the site of capture straight into their own tunnel. Tissue samples were kept in 96% ethanol and stored at –20 °C. The museum specimens of *giganteus*, *istricus*, and *uralensis* yielded no useable genetic material.

CYTOCHROME *B* SEQUENCE ANALYSIS

Total DNA was extracted from recently collected tissue samples (Table 1) using DNeasy Blood and Tissue Kit (Qiagen) according to the standardized extraction protocol. The sequences of cytochrome *b* were amplified with polymerase chain reaction (PCR) using F-muarso (5′-ATGACATGAAAAATCATYGTTG T-3′) and R-muarso (5′-GAAATATCATTCKGGTTT AATRTG-3′) primers (Pfunder, Holzgang & Frey, 2004). The polymerase chain reaction was performed in a final reaction volume of 25 µL containing 30 ng template DNA, 1 µM of each oligonucleotide primer, 1.5 µM of $MgCl_2$, 0.16 µM of deoxinucleoside triphosphates (dNTPs), and 0.5 U of AmpliTaq DNA polymerase (Applied Biosystems). PCR amplification was conducted in a DNA Engine Dyad (MJ Research) machine: 94 °C for 2 min, followed by 45 cycles of 94 °C for 45 s, 48 °C for 15 s (ramp speed: $1°C \ s^{-1}$), 60 °C for 1 s (ramp speed: $0.5°C \ s^{-1}$), and 72 °C for 2 min (ramp speed: $1°C \ s^{-1}$), with a final extension step of 72 °C for 7 min. The PCR product was checked on 1.6% agarose gel stained with ethidium bromide and cleaned with High Pure PCR Product Purification Kit (Roche). The PCR product with AmpliTaq generated 3′ overhangs was ligated into pGEM Easy vector (Promega) and transformed into JM109-competent cells (supplied with pGEM Easy Vector Systems II). The positive white colonies were used for colony PCR with the T7 and SP6 promoter primer sites of the pGEM Easy vector. The positivity of the products was checked on 1.6% agarose and then cleaned with the Pure PCR Product Purification Kit (Roche). To minimize the risk of nucleotide substitutions during the PCR and sequencing reaction, sequences of three clones from each sample were determined. The sequencing reaction was accomplished in GeneAmp PCR System 9700 according to the thermal profile of 94 °C for 4 min followed by 25 cycles of 94 °C for 30 s, 50 °C for 15 s, and 60 °C for 4 min. The sequencing reaction was carried out in 10 µL of 80 ng template DNA, 2 µL of BigDye v3.1 Terminator, 2 µL of 5× buffer, and 2 µL of T7 and SP6 primer (5 µM). We used the BigDye XTerminator Purification Kit for cleaning. Sequences for both directions were obtained by using the AB3130 Genetic Analyser.

PHYLOGENETIC RECONSTRUCTION

Phylogenetic and molecular evolutionary analyses were constructed using MEGA 5 (Tamura *et al.*, 2011). For a combined sequence data set (4482–4486 bp in total), besides the cytochrome *b* data a further five mitochondrial sequences (*NADH1*, *12S* rRNA, *16S* rRNA, tRNA-Leu (UUR), tRNA-Val) investigated by Hadid *et al.* (2012) were downloaded from GenBank. Both cytochrome *b* sequences and the combined sequence data set were used for evolutionary divergence estimation using Kimura's two-parameter model of evolution (Kimura, 1980). A phylogenetic dendrogram was constructed by using the maximum-likelihood method. To confirm tree topology, neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony analyses were also carried out. Bootstrap analysis was based on 10 000 resamplings (Felsenstein, 1985). Sequences of *Acomys cahirinus* (spiny mouse) and *Nannospalax judaei* were used as out-groups.

RESULTS

MOLECULAR PHYLOGENY BASED ON CYTOCHROME *B*

Forward and reverse sequence alignments, for each individual, and multiple sequence alignments were made using Vector NTI Advance 9.0 sequence analysis software (InforMax; Invitrogen). All sequences were checked for the presence of the stop codon with the Open Reading Frame Finder tool of NCBI. Sequences generated in the present study are deposited in GenBank under accession numbers KF021251–KF021263 (Table 1).

Phylogenetic reconstruction based on an 844-bp alignment of cytochrome *b* sequences of *Spalax* samples resulted in the separation of five discrete phylogenetic groups. The presence of five phylogenetic groups was supported by all of the analytical methods tested: maximum-likelihood (Fig. 3), maximum-parsimony, and neighbour-joining analyses (not shown here). The well-supported separation of these five groups could be seen in the matrix of the estimated evolutionary divergence (Table 2). Within-group genetic divergences calculated by Kimura's two-parameter model ranged between 0.00 and 0.60%, whereas values among groups varied between 5.23 and 12.76%. Four out of the five groups are considered as different species (*arenarius*, *graecus*, *microphthalmus*, and *zemni*; Musser & Carleton, 2005). Evolutionary divergence values within the fifth group containing the Transylvanian samples (representing three populations) ranged from 0.12 to 0.36%, which corresponds to the range of the intraspecies values of the other four species. Samples of the recognized fifth group formed a distinct sister group of *graecus s.s.* (Fig. 3), and evolutionary divergence among samples within these two groups were estimated between 6.25 and 6.65% (Table 2).
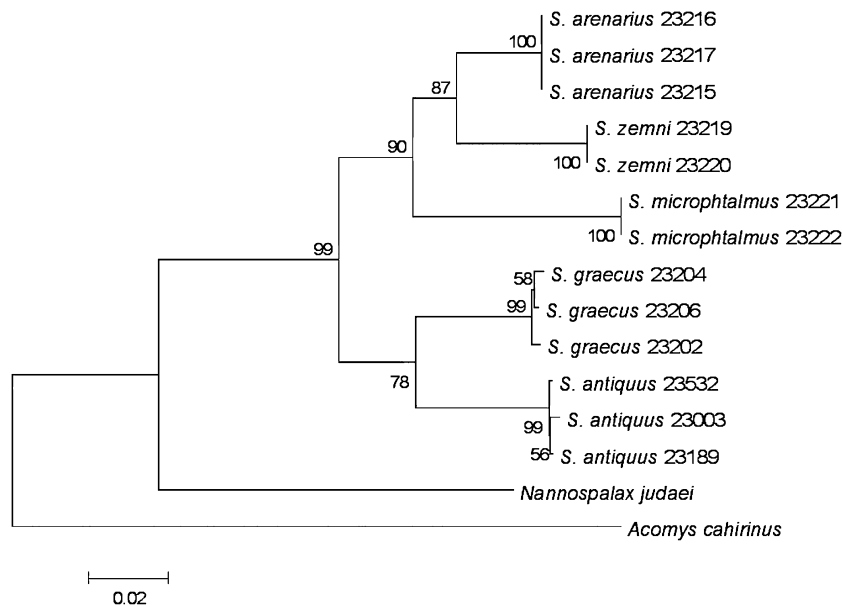

Figure 3. Maximum-likelihood tree reconstructed from an 844-bp alignment of cytochrome *b* sequences of *Spalax* species. *Acomys cahirinus* and *Nannospalax judaei* were used as out-groups. The percentage of trees in which the associated taxa clustered together (after 10 000 replications) is shown next to the branches. The bar represents the number of substitutions per site.

Table 2. Estimates of evolutionary divergence between *Spalax* species based on 844 positions of cytochrome *b* sequences

	graecus 23202	*graecus* 23204	*graecus* 23206	*arenarius* 23215	*arenarius* 23216	*arenarius* 23217	*antiquus* 23532	*antiquus* 23003	*antiquus* 23189	*microphtalmus* 23221	*microphtalmus* 23222	*zemni* 23219	*zemni* 23220	*Nannospalax judaei*	*Aconys cahirinus*
graecus 23202															
graecus 23204	0.60														
graecus 23206	0.24	0.36													
arenarius 23215	9.46	9.05	9.18												
arenarius 23216	9.32	9.19	9.04	0.12											
arenarius 23217	9.46	9.05	9.18	0.00	0.12										
antiquus 23532	6.26	6.52	6.26	10.73	10.59	10.73									
antiquus 23003	6.25	6.51	6.25	10.72	10.58	10.72	0.36								
antiquus 23189	6.39	6.65	6.39	10.59	10.44	10.59	0.12	0.24							
microphtalmus 23221	12.01	12.02	12.30	7.99	8.13	7.99	12.76	12.75	12.61						
microphtalmus 23222	11.86	12.16	12.15	8.13	7.99	8.13	12.61	12.60	12.46	0.12					
zemni 23219	10.72	10.87	10.72	5.36	5.23	5.36	11.31	11.59	11.45	9.67	9.53				
zemni 23220	10.72	10.87	10.72	5.36	5.23	5.36	11.31	11.59	11.45	9.67	9.53	0.00			
Nannospalax judaei	18.70	18.84	18.54	18.60	18.44	18.60	16.81	16.96	16.66	20.15	19.99	18.93	18.93		
Aconys cahirinus	27.53	27.54	27.72	27.51	27.51	27.51	25.85	26.40	26.04	26.62	26.62	27.70	27.70	26.02	

Values were calculated using Kimura's two-parameter model and are given as percentages.

MOLECULAR PHYLOGENY BASED ON COMBINED DATA SET OF SIX mtDNA SEQUENCES

The phylogenetic reconstruction based on the alignment of 4507 bp and representing six mitochondrial genes resulted in congruent topologies with maximum-likelihood, maximum-parsimony, and neighbour-joining methods. Therefore, only the maximum-likelihood tree is discussed here (Fig. 4). Similar to the results of the cytochrome *b* analysis (Fig. 3), the evolutionary divergence value estimated based on the data set of the six mitochondrial sequences confirmed the distinction of five different groups of samples (Table 3). Interspecific divergence values among these samples varied between 3.57 and 8.65%, whereas intraspecific values were two orders lower: 0.02–0.04%. The divergence between the samples 23202 and 23003 (*graecus s.s.* and *antiquus*, respectively; see Table 1) was estimated as 4.98%, which confirms the divergence of the Transylvanian samples at the species level.

MORPHOLOGICAL COMPARISONS

The anatomical nomenclature used (Figs 5, 6) follows that of Méhely (1909) and Topachevskii (1969); the characters are applicable to fully grown adult specimens.

The definition of the graecus group

The clade on both the cytochrome *b* and the consensus trees comprising *graecus s.s.* and Transylvanian *antiquus*, is strongly supported by all analytical methods. This monophyletic clade is designated herewith as the *graecus* group, which can be separated morphologically from all the remaining *Spalax* species by the following unique cranial traits: (1) the length of the nasal bone equals or exceeds the total length of the frontal and parietal bones measured alongside the main axis of the skull (Fig. 7A, B) (versus nasal length relatively shorter in all other species, Fig. 7D); and (2) sella externa of the mandible always situated higher than sella interna. Beside *graecus s.s.* and *antiquus*, the type material of *mezosegiensis* and *istricus* (Fig. 7C) also show these distinguishing morphological features, therefore the *graecus* group is extended for these named forms as well.

Comparison between antiquus and graecus s.s.

The taxon *antiquus* differs markedly from *graecus s.s.* in the following characters: (1) anterior width of the nasal bone is more than twice the posterior width (versus anterior width exceeds less than twice the posterior width); (2) the nasal bones shorter posteriorly than the premaxillae (the nasale always extends posteriorly beyond the premaxillae in *graecus s.s.*)
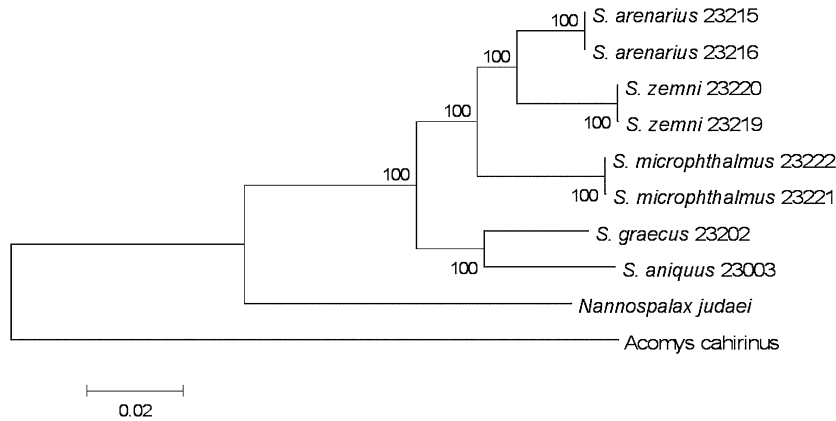
Figure 4. Maximum-likelihood tree reconstructed from a 4507-bp alignment of six mitochondrial sequencesof *Spalax* species [cytochrome *b*, *NADH1*, *12S* rRNA, *16S* rRNA, tRNA-Leu (UUR), tRNA-Val]. *Acomys cahirinus* and *Nannospalax judaei* were used as out-groups. The percentage of trees in which the associated taxa clustered together (after 10 000 replications) is shown next to the branches. The bar represents the number of substitutions per site.
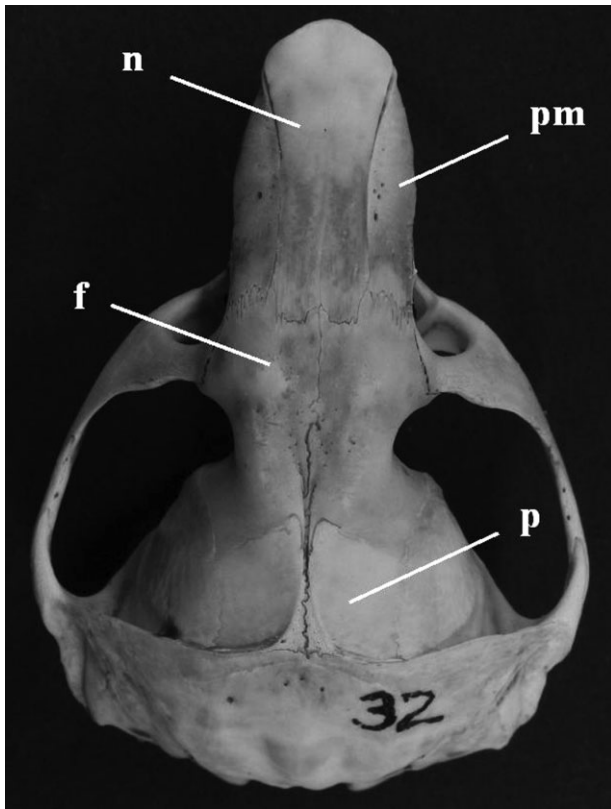

Figure 5. Dorsal view of skull of *Spalax graecus s.s.* (topotype from Chernovcy, Ukraine, ZMUAS 32). Abbreviations: f, frontal bone; p, parietal bone; pm, premaxilla; n, nasal bone.

Figure 6. Caudal view of left mandible of *Spalax antiquus* (Leorint, Romania, HNHM 23530). The plane is determined by the axis of the coronoid process. Abbreviations: cp, coronoid process; se, sella externa; si = sella interna.

(Fig. 7A, B, respectively); and (3) on the mandible the bottom of the incisura corono-alveolaris (the groove between the coronoid and alveolar processes) is domed (whereas this structure is flat in *graecus s.s.*).
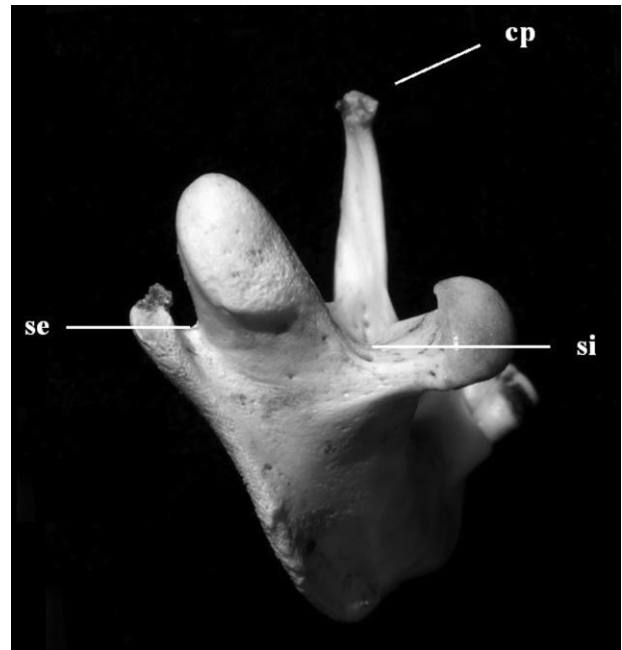
Comparison between antiquus and mezosegiensis
When describing the subspecies *graecus mezosegiensis*, Szunyoghy (1937) only had three recent skulls (all in fragmented condition) and used just a single subfossil skull specimen of *antiquus* for comparisons. He acknowledged that 'the separation [of *mezosegiensis*] from *graecus antiquus* is quite difficult' (translated from Hungarian), and that the characters

Table 3. Estimates of evolutionary divergence between *Spalax* species based on 4507 positions of six mitochondrial sequences [cytochrome *b*, *NADH1*, *12S* rRNA, *16S* rRNA, tRNA-Leu (UUR), tRNA-Val]

	Acomys cahirinus 23202	graecus 23202	aniquus 23003	arenarius 23215	arenarius 23216	microphthalmus 23222	microphthalmus 23221	zemni 23220	zemni 23219	Nannospalax judaei
Acomys cahirinus										
graecus 23202	24.39									
antiquus 23003	24.15	4.98								
arenarius 23215	24.00	7.20	8.09							
arenarius 23216	24.00	7.18	8.06	0.02						
microphthalmus 23222	24.35	7.50	8.32	5.04	5.01					
microphthalmus 23221	24.38	7.56	8.37	5.04	5.06	0.04				
zemni 23220	24.79	7.81	8.62	3.59	3.57	5.70	5.75			
zemni 23219	24.82	7.84	8.65	3.61	3.59	5.72	5.72	0.02		
Nannospalax judaei	23.31	14.36	13.80	13.78	13.75	14.36	14.42	14.23	14.26	

Values were calculated using Kimura's two-parameter model and are given as percentages.

thought to be diagnostic might prove to be individual differences. These characters include the parallel edges at the proximal part of the nasal bones, the wider upper zygomatic process of the maxilla, the relatively more elongated nasal bones, the shape of the frontonasal suture, slight differences in the shape of mandibular processes, and the occlusal pattern of the lower molars in the subadult specimen. After critical examination of these traits in the larger series of Transylvanian *Spalax* specimens (nine individuals, including subfossil and recent materials), we have come to the conclusion that none of these mentioned features can serve as distinguishing characters between the two forms, but indeed represent individual or age-related variations. As a consequence, because of the lack of differentiating features *mezosegiensis* is regarded herewith as the junior subjective synonym of *antiquus*.

Comparison of istricus with antiquus and graecus
The original description of *istricus* was based on three specimens from three different locations in Oltenia and Muntenia (Méhely, 1909). These were the only known exemplars of the taxon until Murariu & Torcea (1984) reported three further individuals collected in an unspecified locality from the Oltenia region. The authors provided no registration numbers for the material, but stated that it is 'deposited in the scientific collection of the "Grigore Antipa" Natural History Museum of Bucharest' (Murariu & Torcea, 1984: 248). Although the specimens, subsequently shared between the collections of GAM and the Muzeul Olteniei, Craiova, cannot be traced and are now regarded as lost (D. Murariu, pers. comm. 2010), a drawing of one of the skulls (Murariu & Torcea, 1984: 249, fig. 1A) and a generalized description of the material is given in the paper, and can be used for comparisons. Murariu & Torcea (1984) compared their newly acquired specimens with '*microphthalmus*' (without any reference to a collection or inventory number) from southern Moldavia. Certainly, '*microphthalmus*' does not occur in that area (its western distributional limit is the Dnieper River) but the drawing (Murariu & Torcea, 1984: 249, fig. 1B) depicting this specimen clearly shows the typical characteristics of the rostral part of *graecus s.s.* The features described of the GAM *istricus* material compare favourably with those of the type series. Based on the descriptions and direct investigations (altogether six individuals), *istricus* could be separated from the other species of the *graecus* group by the following characters: (1) the rostrum is narrowed above, the width at the foramen infraorbitale is less than the basal rostral width measured at the lower zygomatic process of the maxilla (whereas the rostrum is wider, foramen

Figure 7. Dorsal view of skulls showing the rostral structure of (A) *Spalax antiquus* (from Leorint, Romania, HNHM 23530), (B) *Spalax graecus s.s.* (topotype from Chernovcy, Ukraine, ZMUAS 11214), (C) *Spalax istricus* (paralectotype from Horezu Poenari, Romania, HNHM 2522.4), and (D) *Spalax zemni* (from Krivij Rig, Ukraine, HNHM 2009.37.9.). Not to scale.

infraorbitale width equals the basal rostral width in *antiquus* and *graecus s.s.*); (2) the anterior width of the nasal bone exceeds less than twice the posterior width (versus anterior width more than twice of posterior width in *antiquus*); (3) the nasal bones shorter posteriorly than the premaxillae (in sharp contrast with *graecus s.s.* where the nasale always extends posteriorly beyond the premaxillae) (Fig. 7B, C); and (4) the bottom of the incisura corono-alveolaris is domed (flat in *graecus s.s.*).

DISCUSSION

The phylogenetic separation of the large-bodied and small-bodied blind mole rats at the genus-group level (*Spalax* and *Nannospalax*), as suggested earlier on morphological grounds (Méhely, 1909; Ognev, 1947; Topachevskii, 1969), and strongly supported by the recent molecular biological evidence (Hadid *et al*., 2012; Chişamera *et al*., 2013), is further corroborated by the results of the present investigation. Based on the fossil records (Nevo & Bar-El, 1976; Catzeflis *et al*., 1989), the separation of *Spalax* and *Nannospalax* happened during the dry late Miocene (8.24–7.10 Mya), and could be attributed to the establishment of a marine barrier between Asia Minor and the Balkan Peninsula during the Tortonian (11.6–7.2 Mya; Popov *et al*., 2006; Akkiraz *et al*., 2011). The colonization of the Balkan Peninsula by *Nannospalax* from Asia Minor took place at a later date (Hadid

et al., 2012), and was possibly responsible for the push of *Spalax* from the lowlands to less hospitable regions in the peninsula, where they today survive in small and scattered populations.

The topology of both the cytochrome *b* and the consensus trees shows deep bifurcation within the *Spalax* lineage: one clade includes two taxa of the *graecus* group (as defined above), whereas *arenarius*, *microphthalmus*, and *zemni* constitute the second clade. Based on phenetic markers, Méhely (1909) and Resetnik (1941) also hypothesized a similar branching within the large-bodied blind mole rats. According to molecular dating, this event occured 3.5–1.8 Mya (average 2.7 Mya), which corresponds to periods of strong climate change caused by changes in the orbital eccentricity (Hadid *et al.*, 2012).

Based on the 844-bp long partial sequences of the cytochrome *b* gene, interspecific divergence within *Spalax* was found to range between 5.23 and 12.76% (estimated using Kimura's two-parameter model). The sequence divergence between *antiquus* and *graecus s.s.* ranged between 6.25 and 6.65%, which is much higher than the estimated 2.7% average sequence divergence between sister species among Rodentia (Bradley & Baker, 2001). As the average degree of sequence divergence between *antiquus* populations, calculated with Kimura's two-parameter model, was 0.3%, we concluded that the sequence analyses confirm the taxonomic identity of the three populations. The high level of congruence found between cytochrome *b* tree and the tree constructed on the basis of six mitochondrial sequences (4507 bp in total) strongly supports the specific distinctiveness of *antiquus* from *graecus s.s.* The estimated divergence time of these clades (*c.* 1 Mya, range 0.81–1.9 Mya) coincides with the uplift of the Eastern Carpathians (Hadid *et al.*, 2012), which effectively separated the two taxa and halted gene flow between them. As a consequence, taking into consideration the genetic and morphological evidence, *antiquus* is regarded herewith as a separate species within the *graecus* group of *Spalax*.

The analysis of craniodental traits (Méhely, 1909; Murariu & Torcea, 1984; and direct comparisons of the lectotype and paralectotype specimens with large series of all accepted *Spalax* species) undoubtedly shows that *istricus* belongs to the *graecus* group. Although our attempts to extract useable genetic material from the type specimens have failed, cranial features readily distinguish *istricus* from the closely related *antiquus* and *graecus*, expressing the same magnitude of anatomical differences as observed between other species of the same genus (Méhely, 1909; Topachevskii, 1969; Korobchenko & Zagorodnyuk, 2009). The known occurences of *istricus*

also imply long-term genetic isolation. Therefore, in accordance with Méhely's opinion and the findings of Csorba (2010), we regard this taxon as a separate species.

Based on the discussion above, the following recent species of *Spalax* (type localities in parenthesis), with their (proposed) English names, are accepted herewith:

Spalax antiquus Méhely, 1909 (Transylvanian Plain, Romania) – Méhely's blind mole rat [The geographically reasonable epithet 'Transylvanian' is already in use to name the *Nannospalax* (*leucodon*) *transylvanicus* blind mole rat species. The suggested name is in recognition of Méhely's baseline work on Spalacinae systematics.];

Spalax arenarius Reshetnik, 1938 (Golaya Pristan, Ukraine) – sandy blind mole rat;

Spalax giganteus Nehring, 1898 (Makhuch-Kala, Russia) – giant blind mole rat;

Spalax graecus Nehring, 1898 (Chernovcy, Ukraine) – Bukovina blind mole rat;

Spalax istricus Méhely, 1909 (Bârza, Romania) – Oltenia blind mole rat;

Spalax microphthalmus Güldenstaedt, 1770 (Novokhoper steppe, Russia) – greater blind mole rat;

Spalax uralensis Tiflov and Usov, 1939 (Chingerlauz region, Kazahstan) – Kazakhstan blind mole rat;

Spalax zemni (Erxleben, 1777) (Ternopolsk region, Ukraine) – Podolsk blind mole rat.

These taxonomic and systematic results clarified that both *S. antiquus* and *S. istricus* are poorly known endemic mammal species of Europe, recorded only from the territory of Romania. They have restricted and fragmented ranges, and in spite of recent research efforts *S. istricus* has not been recorded in the last 30 years (Németh *et al.*, 2011). As a consequence, similar to the suggestion of Chişamera *et al.* (2013) for the inclusion of *S. graecus s.s.* in annexes II and IV of the EU Habitats Directive, we believe it is extremely important that the experts of the International Union for Conservation of Nature (IUCN) Species Survival Commission, Small Mammal Specialist Group, re-evaluate the status of *S. antiquus* and *S. istricus*, and possibly also bring their conservation status to the attention of decision-makers at the EU level.

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