Underworld: Evolution of blind mole rats in Eastern Europe

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Underworld: Evolution of blind mole rats in Eastern Europe

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Abstract

Large-bodied mole rats (*Spalax*) are a specious genus among obligate subterranean rodents, with seven currently recognised species, ranging from the Carpathians to the North Caucasus and further into the Caspian basin. Several conflicting hypotheses were proposed to explain the phylogenetic relationship among these taxa, mostly based on the subjective interpretation of the importance of certain morphological characters in species delineation. We sequenced one mitochondrial (*cyt b*) and one nuclear (*IRBP*) gene in six *Spalax* species, representing the most complete molecular dataset up to date. Both resulting phylogenies placed (i) *S. graecus*, *S. antiquus* and *S. giganteus* at the base of the tree, while (ii) *S. microphtalmus*, *S. zemni* and *S. arenarius* appeared to have differentiated later in the evolutionary history of the genus. The reciprocal monophyly of the two latter species was resolved only in the *cyt b* gene sequence, but not in *IRBP*. We hypothesize that group (i) might represent the relics of an ancient *Spalax* population that used to have continuous distribution in the entire Ponto-Caspian steppe zone, while group (ii) experienced speciation and range expansion in more recent times. Geographical barriers, in particular large rivers, could have played a role in the speciation process, but to a varying degree.

Key Words: *cytochrome b*, *IRBP*, geographic distribution, isolation, speciation, relictualism

Statements and Declarations

Competing interests

The authors have no financial or proprietary interests in any material discussed in this article.

Author contribution

MR and AY designed the study; MR, MG and AS collected samples; OÇ performed laboratory experiments, supervised by AY; MG and MR analysed the data; MR, AY and MG wrote the paper and all authors contributed substantially to the discussion of the final version of the manuscript.

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Ethics approval

The study followed the best research practices and guidelines adopted in Schmalhausen Institute of Zoology of NAS of Ukraine. Permit no. 2021/5 approved during the project by the Ministry of Environmental Protection of Ukraine and followed the guidelines USAMV CN Bioethics Committee (reg.no 23/21-09-2010), the EU 2010/63 and National Directives Ord. 28/31-08-2011 and National Law 206/2004 and were performed in the framework of the CNCSIS IDEI PCCE 7/2010 project in in Romania. No permit is required for analyzing the DNA samples in Türkiye.

1. Introduction

Fossorial rodents occupy a highly specific ecological niche, that in turn affects their evolution and speciation pattern in a particular way (Begall et al. 2007). They are believed to have low dispersal rates, so one can expect isolation, by distance or due to physical barriers, to play a major role in the formation of new species. For the same reason, local environmental fluctuations, past and present, may exert significant effects on differential adaptations between isolated populations.

Eurasian blind mole rats (Spalacinae) are among the most specialized groups of obligatory subterranean rodents. The two distinct genera *Nannospalax* and *Spalax,* are thought to have split in the early Pliocene (Hadid et al. 2012), at a time when the climate in the European subcontinent was generally warmer, and they strived through the whole Pleistocene, despite the repeated and drastic cold periods. The modern distribution of the large-bodied blind mole rats (*Spalax*) coincides roughly with the Ponto-Caspian steppe (Fig. 1). With the exception of the Carpathian mountains, this entire region is notably free of major mountain systems that could lead to population isolation, with the only significant barriers to dispersal of terrestrial animals posed by a number of rivers running into the Black, Azov and Caspian Seas. The relatively high taxonomic diversity within the *Spalax* genus, which currently includes seven extant and one recently extinct species, with distinct parapatric ranges, is therefore somewhat surprising. Various hypothetical scenarios were proposed to describe the evolution within that genus. The earliest concept, based on the combination of morphological characters, was proposed by Méhely in 1909 (Méhely 1909), and later adjusted by Reshetnik (1941). Both authors recognised two major clades within the genus, one centered in the Carpathian basin and another eastwards on the Eastern European plain, but disagreed in regards to the position of *S. giganteus* Nehring, 1898, found only in a narrow area North-East of the Caucasus mountains (Kennerley et al. 2016). Ognev (1947) suggested minor changes to Reshetnik's phylogeny and was the first to recognize *S. arenarius* Reshetnik, 1939 as a separate species, placing it between *S. zemni* (Erxleben, 1777) and *S. microphthalmus* (Guldenstaedt, 1770). Topachevskiy (1969) constructed his own somewhat contradictory evolutionary tree of Spalacinae, with *S. giganteus* and *S. arenarius* placed in the same basal clade.

In 1970-ies, the new cytogenetic and allozyme studies did not bring much clarification to the phylogeny and taxonomy of genus *Spalax*. It was shown that large-bodied blind mole rats, in contrast to the small-bodied *Nannospalax*, have just two chromosomal forms that differ by a single Robertsonian rearrangement. All species in the genus had 2n=62, with the exception of *S. microphthalmus* having 2n=60 (Martynova et al. 1975). The karyological differences between the different 2n=62 species were minimal. Allozyme studies showed the presence of several electromorphs of blood proteins within Spalacinae, but results were not sufficient to draw any new taxonomic conclusion (Vorontsov et al. 1977).

The first molecular DNA phylogenies that included some representatives (from 1 to 5 species) of the genus *Spalax* appeared only recently, and they were based solely on mitochondrial markers (Hadid et al. 2012; Németh et al. 2013; Chişamera et al. 2014). Moreover, none of the easternmost two species (*S. giganteus* and *S. uralensis* Titov & Usov, 1939), were included in any of the analyses. To solve the puzzle of phylogenetic relations among the species of large-bodied mole rats of Eastern Europe, here we present the results of genetic examination of six species of the *Spalax* genus. Using both mitochondrial and nuclear DNA of a large number of samples covering most populations of these species we constructed detailed phylogenies and suggest possible ways for speciation in this highly specialized rodent group.

2. Methods

2.1 Sampling

Our genetic analysis is based on DNA samples collected from six out of the currently recognised seven *Spalax* species. DNA was extracted from field collected blood or tissue samples, completed with *cyt*b sequences

accessed in GenBank. We collected 35 samples of six species of *Spalax* and 6 samples of *Nannospalax leucodon* (Table 1). All tissue samples for DNA analyzes were stored in alcohol.

Table 1. List of genetic samples (used in this study. For sequences accessed in GENBANK we provide full references.

Species	Sample ID	Location, lat-long, collection date, collector(s)	*cytb* acc.no.	*IRBP* acc.no.
S. antiquus	R98	Romania, Malaiesti, Ranta, N46.493006 E24.143998, 2017-06-22, col. AS	OP882054	OP882088
S. antiquus	R99	Romania, Malaiesti, Ranta, N46.493006 E24.143998, 2017-06-22, col. AS	OP882055	OP882087
S. antiquus	R65	Romania, Cluj, Aiton, N46.700365 E23.754098, 2015-05-06, col. AS	OP882056	OP882089
S. antiquus	A01	Romania, Cluj, Aiton, N46.692484 E23.756604, 2018-08-21, col. AS	OP882057	OP882091
S. antiquus	A02	Romania, Cluj, Aiton, N46.692484 E23.756604, 2018-08-21, col. AS	OP882058	OP882090
S. antiquus		Romania, Budesti, N46.87 E24.24, Nemeth et al. (2013)	KF021263*	–
S. antiquus		Romania, Sandulesti, N46.58 E23.72, Nemeth et al. (2013)	KF021257*	–
S. antiquus		Romania, Aiton, N46.69 E23.76, Nemeth et al. (2013)	KF021256*	–
S. arenarius	Kakh1	Ukraine, Kherson, Kakhovka, N46.7852 E33.43132, 2017-05-11, col. MR&MG	OP882027	OP882086
S. arenarius	Sagi3	Ukraine, Kherson, Oleshky, N46.60656 E32.85807, 2017-05-13, col. MR&MG	OP882029	OP882084
S. arenarius	Sagi8	Ukraine, Kherson, Oleshky, N46.60648 E32.85758, 2017-05-14, col. MR&MG	OP882030	OP882083
S. arenarius	KRY1	Ukraine, Kherson, Kazachi Lageri, N46.70376 E33.05971, 2016-04-18, col. MR&MG	OP882028	OP882085
S. arenarius	Soloz6	Ukraine, Kherson, Kinburn, N46.45546 E31.99734, 2017-05-18, MR&MG	OP882031	OP882082
S. arenarius	Soloz19	Ukraine, Kherson, Kinburn, N46.457 E31.988, 2017-05-19, MR&MG	OP882032	OP882081
S. arenarius	Soloz58	Ukraine, Kherson, Kinburn, N46.457 E31.988, 2017-05-23, MR&MG	OP882033	OP882080
S. arenarius	Soloz63	Ukraine, Kherson, Kinburn, N46.457 E31.988, 2017-05-24, MR&MG	OP882034	OP882079
S. arenarius	–	Ukraine, Kherson, Kazachi Lageri, N46.694 E33.0043, 2009-05-20, Nemeth et al. 2013	KF021254*	–
S. arenarius	–	Ukraine, Kherson, Kazachi Lageri, N46.63091 E32.91849, 2009-05-20, Nemeth et al. 2013	KF021255*	–
S. arenarius	–	Ukraine, Kherson, Kazachi Lageri, N46.694 E33.0043, 2009-05-20, Nemeth et al. (2013)	KF021262*	–
S. giganteus	Kizlyar	Russia, Dagestan, Kizlyar, N43.92412 E46.57245, 2016-06-01, col. MR&NN	OP882025	OP882077
S. giganteus	Caspiy	Russia, Dagestan, Sulak, N43.240	OP882026	OP882078

		E47.490, 2018-09-09, col. Yuriy Yarovenko		
S. graecus	2018-11	Ukraine, Chernivtsi, Zavoloka, N48.25273 E25.88968, 2018-05-05, MR&MG	OP882050	OP882076
S. graecus	2018-12	Ukraine, Chernivtsi, Zavoloka, N48.25268 E25.88861, 2018-05-05, MR&MG	OP882051	OP882075
S. graecus	2018-13	Ukraine, Chernivtsi, Zavoloka, N48.25130 E25.88861, 2018-05-05, MR&MG	OP882052	OP882074
S. graecus	2018-14	Ukraine, Chernivtsi, Zavoloka, N48.25394 E25.88949, 2018-05-05, MR&MG	OP882054	OP882073
S. graecus	–	Romania, Suceava, Bunesti, N47.52 E26.32, 2011-05-13/16, Chisamera et al. (2014)	JX455993*	–
S. graecus	–	Romania, Suceava, Bunesti, N47.52 E26.32, 2011-05-13/16, Chisamera et al. (2014)	JX455994*	–
S. graecus	–	Romania, Suceava, Bunesti, N47.52 E26.32, 2011-05-13/16, Chisamera et al. (2014)	JX455995*	–
S. graecus	–	Romania, Suceava, Bunesti, N47.52 E26.32, 2011-05-13/16, Chisamera et al. (2014)	JX455996*	–
S. graecus	–	Romania, Iasi, N47.191 E27.46697, 2009-05-15, Nemeth el al. (2013)	KF021251*	–
S. graecus	–	Romania, Iasi, N47.191 E27.46697, 2009-05-15, Nemeth el al. (2013)	KF021252*	–
S. graecus	–	Romania, Iasi, N47.191 E27.46697, 2009-05-15, Nemeth el al. (2013)	KF021253*	–
S. microphthalmus	2019-30	Ukraine, Zaporizhia, Melitopol, N46.76983 E35.27356, 2019-04-09, col. MR&NN	—	OP882072
S. microphthalmus	Manych1	Russia, Rostov, Manych, N46.59999 E42.85662, 2016-05-18, col. MR&NN	OP882035	OP882071
S. microphthalmus	Surov1	Russia, Volgograd, Surovikino, N48.65725 E42.73218, 2016-05-15, col. MR&NN	OP882036	OP882070
S. microphthalmus	–	Ukraine, Dnipro, Pischanka, N48.5787 E35.2946, 2009-05-22, Nemeth et al. (2013)	KF021258*	–
S. microphthalmus	–	Ukraine, Dnipro, Pischanka, N48.5787 E35.2946, 2009-05-22, Nemeth et al. (2013)	KF021259*	–
S. zemni	2018-51	Ukraine, Mykolaiv, Veselinovo, N47.34086 E31.22342, 2018-06-16, col. MR&MG	OP882059	OP882069
S. zemni	2018-57	Ukraine, Mykolaiv, Krynychky, N47.08242 E31.70550, 2018-06-18, col. MR&MG	OP882060	OP882068
S. zemni	2018-58	Ukraine, Mykolaiv, Novoselivka, N47.11966 E31.65884, 2018-06-16, col. MR&MG	OP882062	OP882067
S. zemni	2018-60	Ukraine, Mykolaiv, Kulbakine N46.95366 E32.11404, 2018-06-19, col. MR&MG	OP882061	OP882066
S. zemni	Nik1	Ukraine, Mykolaiv, Kulbakine, N46.95160 E32.09758, 2017-05-27,	OP882047	OP882061

		col. MR&MG&NN		
S. zemni	Nik2	Ukraine, Mykolaiv, Kulbakine, N46.93458 E32.11068, 2017-05-28, col. MR&MG&NN	OP882048	OP882060
S. zemni	Nik3	Ukraine, Mykolaiv, Kulbakine, N46.92225 E32.10244, 2017-07-30, col. MR&MG	OP882049	OP882059
S. zemni	2018-61	Ukraine, Zaporizhia, Khortytsia, N47.80609 E35.11081, 2018-06-23, col. MR&MG	OP882041	OP882065
S. zemni	2018-62	Ukraine, Zaporizhia, Khortytsia, N47.79637 E35.12049, 2018-06-23, col. MR&MG	OP882042	OP882064
S. zemni	2019-1	Ukraine, Mykolaiv, Voznesensk, N47.60918 E31.33728, 2019-04-03, col. MR&NN	OP882043	–
S. zemni	2019-2	Ukraine, Mykolaiv, Voznesensk, N47.6086 E31.33666, 2019-04-03, col. MR&NN	OP882044	OP882063
S. zemni	2019-3	Ukraine, Mykolaiv, Voznesensk, N47.61108 E31.33824, 2019-04-04, col. MR&NN	OP882045	–
S. zemni	2019-7	Ukraine, Mykolaiv, Prybuzhany, N47.51853 E31.31263, 2019-04-04, col. MR&NN	OP882046	OP882062
S. zemni	–	Ukraine, Dnipro, Kryvyi Rig, N47.88736 E33.18344, 2009-05-21, Nemeth et al. (2013)	KF021260*	–
S. zemni	–	Ukraine, Dnipro, Kryvyi Rig, N47.88736 E33.18344, 2009-05-22, Nemeth et al. (2013)	KF021261*	–
S. zemni	–	Stanhope et al. 1992	—	U48589*
N. leucodon	2018-5	Ukraine, Chernivtsi, Babyn, N48.51591 E26.81664, 2018-05-02, col. MR&MG	–	OP882092
N. leucodon	Tiligul2	Ukraine, Odesa, Kalynivka, N46.89796 E31.01615, 2017-06-02, col. MR&MG	–	OP882095
N. leucodon	Tiligul3	Ukraine, Odesa, Kalynivka, N46.89637 E31.01129, 2017-06-02, col. MR&MG	–	OP882096
N. leucodon	Tiligul4	Ukraine, Odesa, Kalynivka, N46.89659 E31.01236, 2017-06-03, col. MR&MG	–	OP882097
N. leucodon	TUL1	Romania, Tulcea, Somova, N45.1470 E28.7140, 2016-10-01, col. SS&MR	–	OP882093
N. leucodon	DOB1	Romania, Constanta, Murfatlar, N44.14226 E28.39587, 2016-09-15, col. SS&MR	–	OP882094
N. ehrenbergi	–	(Meredith et al. 2011)	–	JN414825*
N. galili (ehrenbergi)	–	Whole genome, Fang et al. (2014)	–	XM_008834127*
N. ehrenbergi	–	(Steppan and Schenk 2017)	KY754157*	–
N. carmeli	–	Hadid et al. (2011) Mitochondrion, Direct submission to GenBank	NC_020756*	–
N. ehrenbergi	–	Spradling et al. (2001) (Spradling et al. 2001)	AF155871*	–

* GenBank aquisisions

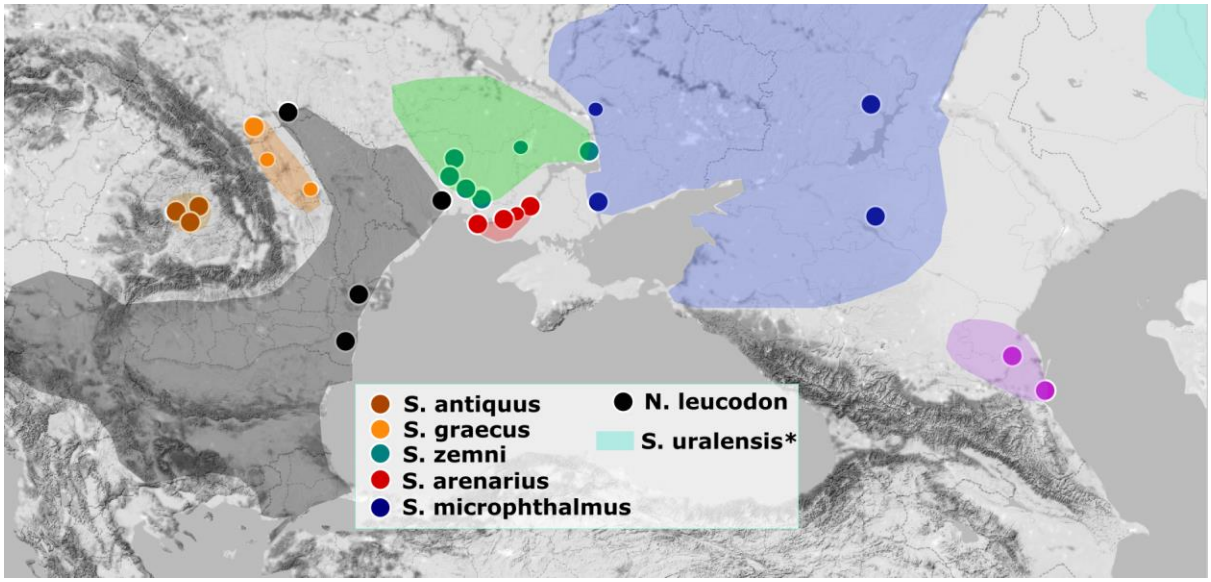

Fig. 1 Map of sampling locations (larger circles) and geographical origin of GenBank sequences (smaller circles) used in the study. The outlines of species ranges (sourced from IUCN and further improved by our surveys) are shown in respective colors. *Athough no sample of *S. uralensis* was included into the present study, hereby we show the westernmost part of its range for comparison.

2.2 DNA isolation and amplification

Genomic DNA was isolated from the alcohol-preserved tissue samples using commercial extraction kits. Two genes, one mitochondrial (*cytochrome b, cyt b*) and one nuclear (part of first exon of *Interphotoreceptor retinoid-binding protein, IRBP*) were chosen for the analyses. For the *cytb*, a 1140 bp long fragment was amplified using the modified L14727-SP and H15497-SP primers published earlier (Kryštufek et al. 2012), as well as custom design primers F24-SP 5`-AGACCAATGACATGAAAAATCATCGT-3` and R24-SP 5`-ATGATGAATGGGTGTTCAAC-3`. The consensus of 3 complete mitochondrial genomes of *Nannospalax galili* (Nevo, Ivanitskaya & Beiles, 2001) (NC_020754.1), *N. carmeli* (Nevo, Ivanitskaya & Beiles, 2001) (NC_020756) and *N. golani* (Nevo, Ivanitskaya & Beiles, 2001) (NC_020757.1) was used as a template for primer design and modification. A previously published primer-pair *+irbp217* and *-irbp1531* was used to amplify the complete (1082 bp) IRBP gene (Stanhope et al. 1992). All primers were tested for specificity against the full genome reference assembly of *N. galili* (GCA_000622305.1) using the Primer-BLAST online tool (Madden 2013), and no matching amplicons were found within < 2 nucleotide substitutions in each primer.

PCR was performed using a standard protocol. The PCR products were purified using a column-based kit (AMBRD Laboratories, Istanbul, Türkiye) and outsourced for sequencing to Macrogen Europe (Amsterdam, Netherlands).

2.3 Sequence alignment

Sequence chromatograms were verified visually and assembled in Geneious Prime 2020.0.4 (https://www.geneious.com). They were aligned by Muscle algorithm (Edgar 2004) and adjusted manually in MEGA X software (Kumar et al. 2018). We believe that the GenBank sequences JX455993-JX455996 had erroneous reads in 10 positions of *cytb* gene (10, 21, 27, 30, 33, 42, 54, 57, 60, 61), thus the ambiguity codes were placed in those positions during our analyses. All new *cyt b* and IRBP sequences were deposited in GenBank (accession no. OP882019 – OP882058 and OP882059 – OP882097, respectively).

2.4 Phylogenetic analyses

Phylogeny was reconstructed for *cytb* and IRBP genes separately. For both genes, the Maximum Likelihood (ML) and Bayesian trees were constructed. Model selection was performed in MEGA X (Kumar et al. 2018): substitution models with the lowest Bayesian information criterion (BIC) were chosen (HKY+G+I for *cytb* and T92+G for IRBP). The ML trees were built in MEGA X. Bayesian trees were estimated with MrBayes version 3.2.7a (Ronquist et al. 2012). Four chains were run twice with 5 million generations and the sample frequency of 1000. The MrBayes analysis was performed on the CIPRES Science Gateway resource (Miller et al.2010). The output log files were analyzed with Tracer version 1.7.1 (Rambaut et al. 2018). Both runs converged towards the same joint density and had sufficient effective sample size (ESS larger than 1000) for all trace statistics. The burn-in rate was set at 25%. The maximum clade credibility tree with median node heights was constructed using TreeAnnotator version 1.10.5 (Suchard et al. 2018) for the first run with burn-in of the first 1000 trees. Intra- and interspecific genetic distances (Kimura 2-parameter - K2p) were calculated in MEGA X.

3. Results

3.1 Nucleotide composition

The final multiple alignment of *cytb* gene included 61 sequences: 40 from our field-collected samples and 21 downloaded from GenBank. The sequence length in our analyses varied from 800 bp (some GenBank acquisitions) to 1140 bp. The mean nucleotide composition was A=31%, T=31%, G=13%, C=25%. The number of variable positions in the whole dataset was 380 (352 parsimony-informative), of which 246 (239 parsimony informative) represented the variation within the genus *Spalax*. Out of 380 amino acids in the *cytochrome b* protein sequence, 64 were variable when analyzing the entire dataset (*Spalax* and *Nannospalax*), and 29 remained variable only within the *Spalax* samples.

Alignment of *IRBP* consisted of 41 sequences: 39 original and 2 downloaded from the GenBank. The length varied from 880 to 1077 bp. The mean nucleotide composition was A=21%, T=23%, G=29%, C=27%. The number of variable positions in the whole dataset was 82 (77 parsimony-informative), of which 34 (30 parsimony informative) represented the variation within the genus *Spalax*.

3.2 Mitochondrial phylogeny

Phylogeny reconstructed in ML and Bayes resulted in nearly identical topology (Fig. 2). The two most related species, *S. arenarius* and *S. zemni*, were placed in the crown of the tree, while the giant mole rat, *S. giganteus* was an outgroup to *S. zemni* + *S. arenarius* + *S. microphthlamus* in a position firmly supported in the Bayesian tree. In the ML tree, *S. giganteus* appeared as a sister taxon to *S. microphthalmus*, but this association was weakly supported by the bootstrap (0.47) compared to the Bayesian topology (0.97).

Table 2. Kimura 2-parameter distance between and within the studied taxa, based on the *cyt b* sequences. Estimates of intraspecific variation are indicated in bold.

	N. ehrenbergi	*N. leucodon*	*S. giganteus*	*S. arenarius*	*S. microphthalmus*	*S. zemni*	*S. graecus*	*S. antiquus*
N.ehrenbergi	**0.0715**							
N.leucodon	0.1416	**0.0109**						
S.giganteus	0.1990	0.1900	**0.008**					
S.arenarius	0.1939	0.1994	0.0999	**0.0023**				
S.microphthalmus	0.2114	0.2133	0.0918	0.0864	**0.0017**			
S.zemni	0.1958	0.1882	0.0950	0.0580	0.0939	**0.0126**		
S.graecus	0.1927	0.2084	0.1111	0.0997	0.1257	0.1052	**0.0028**	
S.antiquus	0.1942	0.2035	0.1175	0.1129	0.1281	0.1146	0.0652	**0.003**

The K2p distances estimated within each *Spalax* species were, on average, an order of magnitude smaller than the distances between species (Table 2). At the level of the *Spalax* genus, the interspecific K2p distances varied from ~0.06 (*S. zemni* - *S. arenarius*) to ~0.128 (*S. microphthalmus* - *S. antiquus*). These were still considerably smaller compared to the distances between *Nannospalax* and *Spalax* (min ~0.189, Table 2).

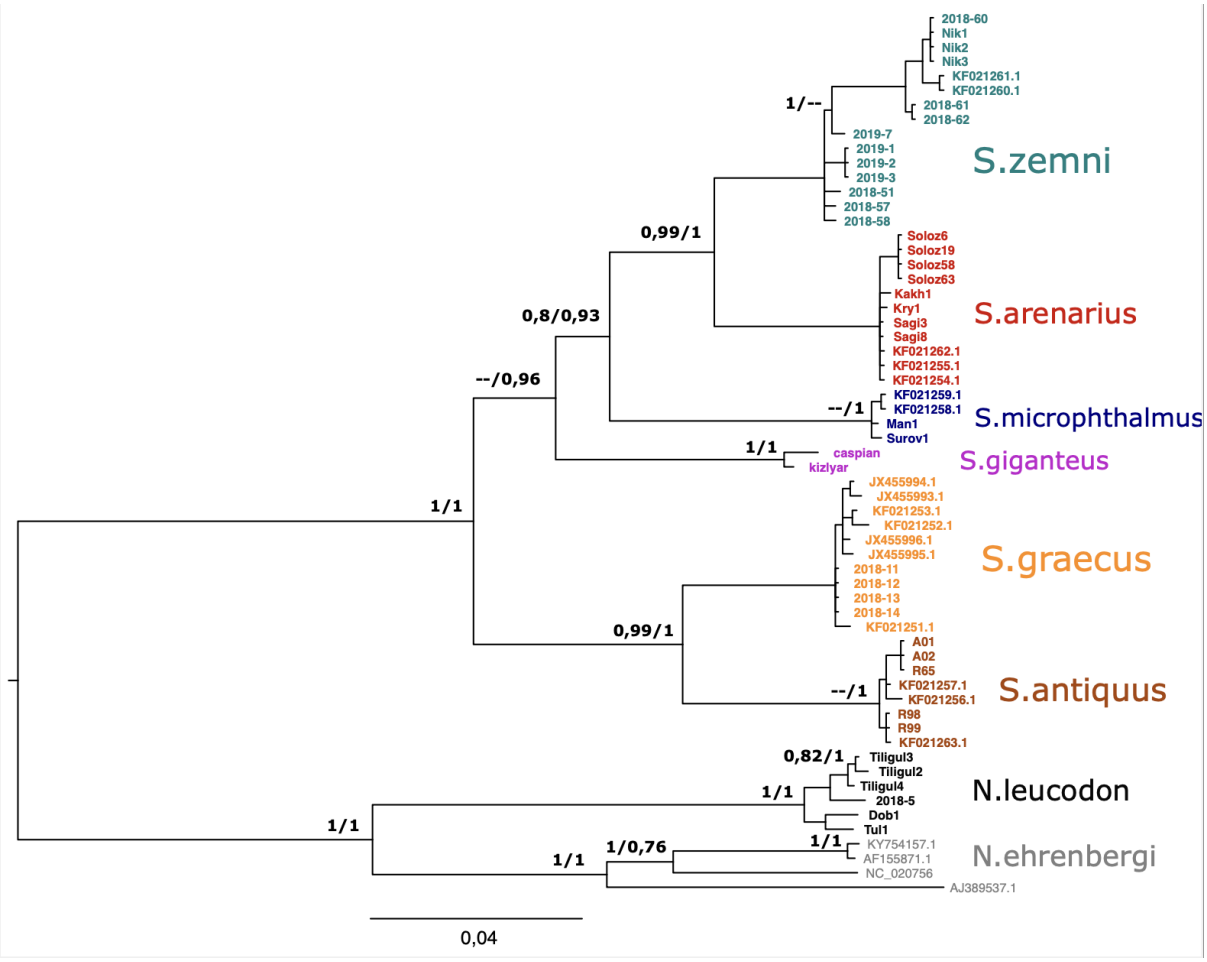

Fig. 2 Phylogenetic tree of *Spalax* spp., based on *cyt b* gene. ML Bayesian support values higher than 0.7 are shown next to the nodes. *S. giganteus* appears as a sister taxon to *S. microphthalmus* in the ML tree, however, only the Bayesian topology reached significant support level and is shown on the figure.

3.3 Nuclear phylogeny

Surprisingly, the species with the smallest current distribution range, the sandy mole rat (*S. arenarius*), had the highest level of intraspecific variation in the nuclear *IRBP* gene. In contrast, *S. microphthalmus* had the lowest intraspecific variation, despite the fact that currently it has the largest distribution area of all *Spalax* species and the distance between the furthest locations in our sampling was almost 600 km.

The two most related species - *S. arenarius* and *S. zemni* were not fully resolved using only *IRBP* sequences. At the same time, *S. antiquus* and *S. graecus* formed a distinct clade. In both the *IRBP* and the mitochondrial tree, *S. microphthalmus* appears as a sister clade to *S. zemni* + *S. arenarius*. In contrast to the *cytb* gene phylogeny, the *IRBP* tree has *S. giganteus* at the most basal position within the genus *Spalax* (Fig. 3).

The only previously available GenBank sequence of *S. zemni* (acc. n. U48589) clustered together with *N. ehrenbergi*. One can assume that this may be a result of an erroneous read and this sequence should not be attributed to *S. zemni*.

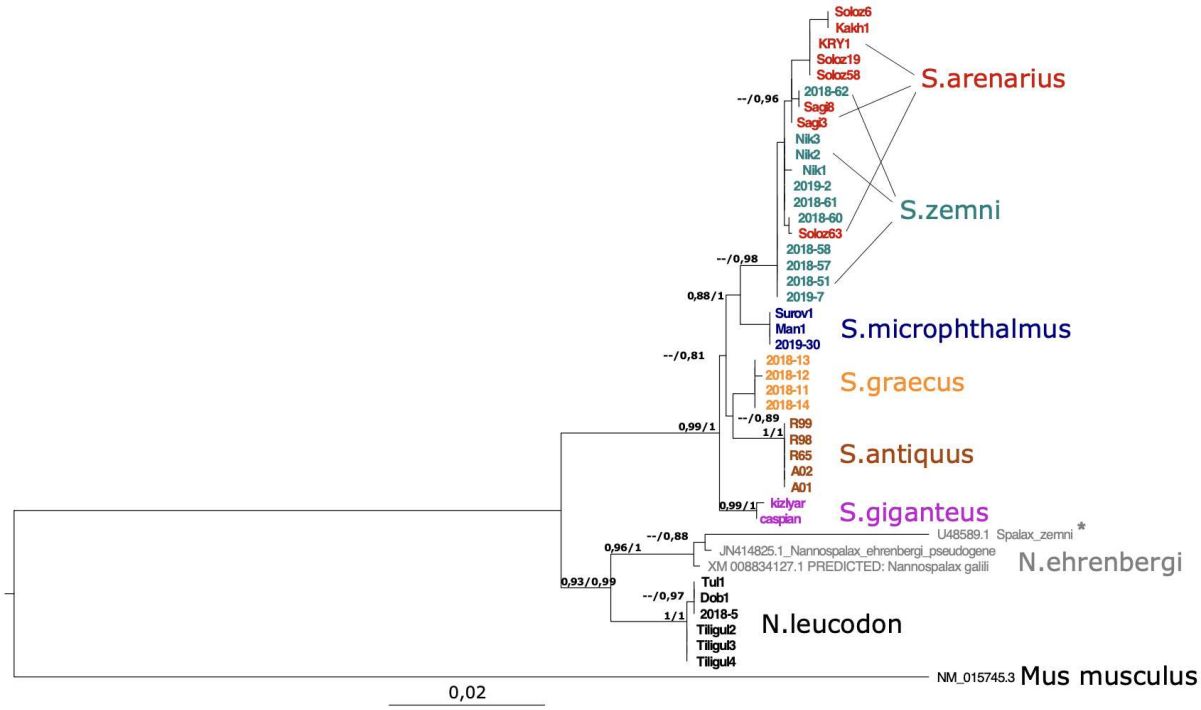

Fig. 3 Phylogenetic tree of *Spalax* based on IRBP gene. ML / Bayesian support values higher than 0.7 are shown next to the nodes. An apparently erroneous or chimeric sequence U48589.1 assigned in the GenBank to *Spalax zemni* is marked with an asterisk.

4 Discussion

To date, our study presents the most complete picture of the phylogenetic history and speciation in large-bodies blind mole rats (*Spalax*). A much denser sampling of several taxa, for which some molecular data was previously available, and, most importantly, the inclusion of the giant blind mole rat, *S. gigantenus* in the analysis, allowed us to resolve a number of open questions in regards to the evolution within this complex group.

The taxonomic position of *S. arenarius* was unclear and disputed since the very first years of its description. Reshetnik (1941) described it as a subspecies of *S. zemni*. Ognev (1947) elevated it to the species level based on morphology, but still acknowledged that it shares certain traits with both *S. zemni* and *S. microphthalmus*, and thus could be a transitional form between the latter two. Topachevskiy (1969) agreed with Ognev on the species status of *S. arenarius*, but at the same time suggested that its closest relative is *S. giganteus,* instead of *S. zemni*. Our results confirm that *S. arenarius* is indeed a sister species to *S. zemni*, thus supporting the original hypothesis by Reshetnik. Notably, *S. arenarius* is the only currently recognized species within the genus that does not appear monophyletic in the nuclear *IRBP* gene tree, but instead is placed entirely within *S. zemni*.

Spalax graecus was proposed to be the basal species among all large-bodied blind mole rats (Mehely 1909, Reshetnik 1941, Ognev 1947), and only Topachevskiy (1969) placed this species at the tip of the tree. The karyotype of *S. graecus* is indistinguishable from the other 2n=62 species of *Spalax* (Martynova et al., 1975). The electrophoretic analysis of albumins showed little to no differences between *S. zemni, S. microphthalmus, S. arenarius* and *S. giganteus* but separated *S. graecus* (Vorontsov et al., 1977). The same authors reported that *S. zemni*, *S. arenarius* and *S. giganteus* had one hemoglobin electromoprh, and *S. microphthalmus* had the second variant; while *S. graecus* had both types of hemoglobin (Vorontsov et al., 1977). These early molecular studies also supported the hypothesis of a basal rather than derived position of *S. graecus*.

Previous mtDNA-based reconstructions have shown that the taxon formerly described as *S. graecus* is actually composed of two lineages, which were then given the species status - *S. graecus* s. str. and *S. antiquus* Méhely, 1909 (Németh et al. 2013). Our results provide even stronger evidence for this split. While the distance in *cytb* between *S. graecus* and *S. antiquus* is not high (0.065 K2P), the two species are clearly separated at the level of the nuclear *IRBP* gene.

Surprisingly, we discovered pronounced phylogeographic structure in *S. zemni*. The south-eastern populations from Mykolaiv city, Kryvyi Rih and Khortytsia island, located in the middle of the Dnieper river within the city of Zaporizhzhia, form a single lineage with little differentiation. Though we do not have samples from the right bank of the Dnieper river from the nearby Zaporizhzhia city, we expect the populations there to be very close to the one from Khortytsia. We hypothesize that Khortytsia island was populated by the mole rats relatively recently, either at the time when the island was connected to the right bank, by human-assisted migration (i.e. via a bridge), or even across and in spite of the river barrier.

The second lineage within *S. zemni* consists of populations from both southern and central populations of Mykolaiv Region. Notably, this lineage is found on both sides of Southern Bug river. The border between the South-Eastern and South-Western lineages of *S. zemni* is yet to be defined. Unfortunately, *S. zemni* became extremely rare in the northern and western parts of its distribution range, so it is a challenging task to obtain enough samples for the reconstruction of phylogeny of this species over its entire historical distribution range.

Despite having large geographic distances between our sampling sites for *S. microphthalmus*, this species shows high genetic homogeneity. The westernmost populations from the left bank of the Dnieper river have minor variations compared to the south-eastern populations from both sides of the Don river. The same low level diversity has been identified by Matveeva et al. (2019) for populations from Kharkiv and Kursk and Samara regions (distance between furthest localities app. 1000 km). Unlike some other mammal species (i.e. *Sicista subtilis* or *Dryomys nitedula*) that have distinct Ciscaucasian lineages (Lebedev et al. 2020; Mohammadi et al. 2021), this is not the case for *S. microphthalmus*. In our results, the population from Manych had little differentiation from all other samples.

At the same time, the position of *Spalax* from the North-Western Caucasus remains an open question. Traditionally, mole rats from that region were attributed to *S. microphthalmus*. Later, Dzuev (2003) described animals from Kabardino-Balkaria as having the chromosomal formula 2n=62, similar to all other species, but different to other populations of *S. microphthalmus* (2n=60). Even more, he described the chromosomal formula to be significantly different from that found by Martynova et al. (1975) for all species of *Spalax*. Unfortunately, the author did not provide the photographs of the mitotic plates to verify this description. While this case led the later authors to reassess the Western Caucasian mole rats as belonging to *S. giganteus* (Arslan et al. 2016), until genetic data becomes available, the species identity of *Spalax* from the North-Western Caucasus cannot be established.

The position of *S. giganteus* remained unclear until the present study. For many years, the dominant hypothesis was that of Topachevskiy's (1969), who placed *S. giganteus* in the same basal branch with *S. arenarius*. Our molecular findings do not support this topology. Based on the molecular DNA evidence, the position of *S. giganteus* is uncertain: (a) it may be basal to *S. zemni*+*S. arenarius*+*S. microphthalmus*; (b) it forms a sister group with *S. microphthalmus*; (c) or it is placed at basal position to all other species in the genus. More

sophisticated approach with a larger number of genetic markers should resolve this question unambiguously. The only species not included in our analysis, *S. uralensis*, is phenotypically very similar to *S. giganteus* and is found north-east of the Caspian sea (Tsytsulina 2016). It remains to be seen whether this morphological similarity is confirmed by the molecular data, too.

By choosing anuclear gene sequence (*IRBP*) to reconstruct the phylogeny of *Spalax*, we were also able to gain some insight into the evolution of the gene itself. It was suggested earlier that *IRBP* in both mole rat genera *Nannospalax* and *Spalax* does not contain any indels or stop codons (David-Gray et al. 2002). However, the *IRBP* sequence XM_008834127.1 (Fang et al. 2014) in the annotated reference genome assembly of the Palestinian blind mole rat *N. galili* has a 12bp deletion (positions #842-853) in the first exon. Moreover, both *N. galili (Fang et al. 2014)* and *N. leucodon* (our results) have an amber stop codon at position #1012-1014, suggesting that the *IRBP* gene in *Nannospalax* may not be fully functional. All other blind mole rats, as well as *Mus musculus* have glutamine in this position. While neither of the large-bodied *Spalax* species has this stop, we found another amber stop codon in *S. antiquus* at the #298-300 position. This second position in all other species (including *Nannospalax*) also codes for glutamine. As it was shown in the experiments on the *IRBP* knock-out mice, the malfunction of this gene mainly affects cone photoreceptors (Jin et al. 2009), which are crucial for color vision and vision in the bright light. One can hypothesize that the ability to differentiate colors is not of uttermost importance to blind mole rats, so their *IRBP* gene may be under relaxed selection and perhaps on its way to becoming a pseudogene. Pseudogenisation was recently suggested to play an important role in the evolution of subterranean mammals (Zheng et al. 2022).

Based on our findings, the North-Western Pontic Steppes as well the Carpathian basin appears to be the main evolutionary arena for the evolution of large-bodied blind mole rats. The densely forested Carpathian mountain range and the large rivers on the Eastern European plain are the main geographic barriers in this area: they indeed appeared to have played a role in the differentiation between *S. graecus* and *S. antiquus*, and between *S. zemni*, *S. arenarius* and *S. microphthalmus*, respectively. The role of large rivers as barriers for dispersal of blind mole rats has been already discussed (Hadid et al. 2012). At the same time, we notice that the strength of the river barriers varies when different pairs of species / populations are considered. For example, the Dnieper river clearly delineates the ranges of *S. zemni* and *S. microphthalmus*, both species also well differentiated genetically. At the same time, the *S. arenarius* and *S. zemni* pair living on two sides of the Dnieper river are genetically very close to each other. The south-western lineage of *S. zemni* occupies both sides of the deep canyon of Southern Bug river, while the south-eastern lineage of the same species colonized Khortytsia island in the middle of the Dnieper river. We found almost no variations between populations of *S. microphthalmus* west and east of the Don river. It is possible that the varying genetic distances within different pairs of populations/species had been affected by the multiple changes in the rivers' course during the Quaternary period, which could have caused isolation as well as merging of different *Spalax* populations/species. Factors of speciation other than geographic isolation might also be considered, or, perhaps, the large rivers are not as impenetrable to the occasional dispersal of the blind mole rats as previously believed.

We can speculate on the historical range dynamics of the six species studied here, based solely on the comparison of their phylogeny and the current geographical ranges. In both the *cyt b* and *IRBP* trees, the most basal positions are occupied by three species: *S. graecus*, *S. antiquus* and *S. giganteus*. This is in contrast with *S. giganteus* being the most geographically isolated taxon from the first two. Provided that the current distribution areas of all three species are very narrow, we suggest they could represent the relics of a much larger and continuous range of the ancient group ancestral to all current *Spalax* species. Subsequent range fragmentation of this "proto-*Spalax*" group, possibly aided by the climatic changes and geographical barriers, could have led to the more recent emergence of the two species that currently occupy the Eastern European plain, *S. zemni* and *S. microphthalmus*. Finally, the sandy blind mole rat *S. arenarius* could be the youngest group currently still in the process of divergence from its progenitor, *S. zemni*. Future studies involving larger genomic datasets and a combination of molecular clock and demographic modeling (Li et al. 2020) for dating methods, may add further details on this general scenario or correct this speciation process among large-bodied blind mole rats.

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