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ORIGINAL ARTICLE

Underworld: evolution of blind mole rats in Eastern Europe

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Abstract

Large-bodied mole rats (*Spalax*) are a speciosus genus among obligate subterranean rodents, with seven currently recognized 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 morphologic characters in species delineation. We sequenced one mitochondrial (*cytb*) and one nuclear (*IRBP*) gene in six *Spalax* species, representing the most complete molecular dataset up to date. Both resulting phylogenies placed *S. graecus*, *S. antiquus* and *S. giganteus* at the base of the tree, while *S. microphtalmus*, *S. zemni* and *S. arenarius* appeared to have differentiated later in the evolutionary history of the genus. *Cytb* phylogeny supports monophyletic positions of all currently recognized species. According to the nuclear IRBP gene *S. zemni* and *S. arenarius* share similar haplotypes, which may represent either hybridization or recent separation from a common gene pool. The westernmost species *S. antiquus* and *S. graecus* represent the earliest split within the genus *Spalax*, indicating the possible origin of large-bodied blind mole rats from the South-West Europe. *S. giganteus* may represent the eastern relic of the ancient *Spalax* population. The central part of the genus distribution is inhabited by the most derived species: *S. zemni* + *S. arenarius* + *S. microphthalmus*. Large rivers of the Eastern Europe might have played a limited role in the distribution and speciation of mole rats and were crossed regularly by various genotypes.

Keywords *Cytochrome b* · *IRBP* · Geographic distribution · Isolation · Speciation · Relictualism

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

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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 entire 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 intriguing. Various hypothetical scenarios were proposed to describe the evolution within that genus. The earliest concept, based on the combination of morphologic characters, was proposed by Méhely (1909), and later adjusted by Reshetnik (1941). Both authors recognized 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 (Ognev 1947). 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.

The routes of colonization of *Splalax spp.* in the Northern Pontic Region (modern-day Ukraine, Moldova and Russia) remained controversial and unsettled. Both Ognev (1947) and Topachevskiy (1969) discussed two alternative routes of the dispersal of mole rats in the Northern Pontic regions. The first hypothesis suggested that the genus evolved in the Carpathian basin from where it dispersed to the steppes of Eastern Europe. In contrast, the second hypothesis proposed that *Spalax* sensu stricto separated from the common ancestor with *Nannospalax* (*Microspalax* according to Topachevskiy) either in the Caucasus or even further to the east of the modern Caspian Sea. According to the latter hypothesis *S. uralensis* and *S. giganteus* were considered to be the most basal species (Topachevskiy 1969).

In 1970-ies, a number of cytogenetic and allozyme studies were performed but did not bring much clarification to the phylogeny and taxonomy of the genus *Spalax*. It was shown that the 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 one to five 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 two easternmost species (*S. giganteus* and *S. uralensis* Titov and Usov, 1939), were included in any of the analyses. To solve the puzzle of phylogenetic

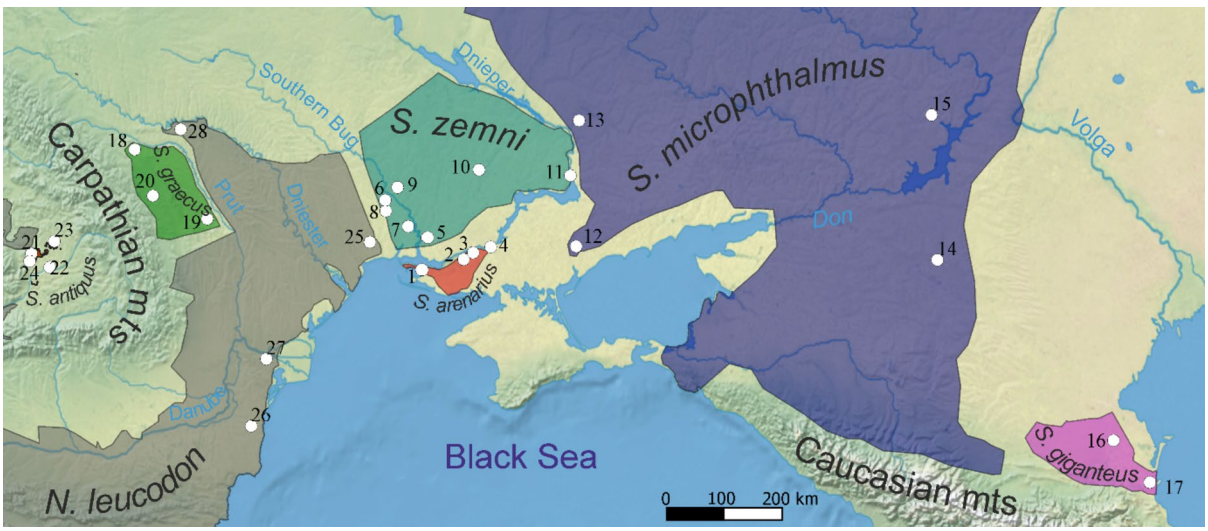

Fig. 1 Map of sampling locations of blind mole rats. Species distribution is based on IUCN and improved by our original survey data. Population numbers correspond to Table 1

relationship among the species of large-bodied mole rats in Eastern Europe, here we present the results of genetic investigation of six species in the *Spalax* genus. Using both mitochondrial and nuclear DNA on a large number of samples covering bigger part of the genus geographic range, we construct detailed phylogenies and suggest possible routes of speciation in this highly specialized rodent group.

Methods

Sampling

Our genetic analysis is based on DNA samples collected from six out of seven currently recognized *Spalax* species. We collected 35 samples of 6 species of *Spalax* and 6 samples of *Nannospalax leucodon* (Table 1). All tissue samples for DNA analyzes were stored in alcohol. Detailed sampling information is available at GBIF (Rusin 2024).

DNA isolation and amplification

Genomic DNA was isolated from the alcohol-preserved tissue samples using commercial extraction kits. Two genes, one mitochondrial (cytochrome b, *cytb*) and one nuclear (part of first exon of Interphotoreceptor retinoid-binding protein, IRBP) were chosen for the analyses. IRBP was the only nuclear DNA marker available for large-bodied blind mole rats prior to our studies (Stanhope et al. 1992). For the *cytb*, a 1140 bp long fragment was amplified using the modified L14727-SP and H15497-SP primers (Irwin et al. 1991), as well as custom design primers F24-SP 5`-AGACCAATG ACATGAAAAATCATCGT-3` and R24-SP 5`-ATGATG AATGGGTGTTCAAC-3`. The consensus of three complete mitochondrial genomes of *Nannospalax galili* (Nevo, Ivanitskaya and Beiles, 2001) (NC_020754), *N. carmeli* (Nevo, Ivanitskaya and Beiles, 2001) (NC_020756) and *N. golani* (Nevo, Ivanitskaya and Beiles, 2001) (NC_020757) 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) using the Primer-BLAST online tool (Madden 2013), and no matching amplicons were found within < two 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, the Netherlands).

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 11 software (Tamura et al. 2021). We believe that the GenBank sequences JX455993-JX455996 had erroneous reads in ten 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 *cytb* and IRBP sequences were deposited in GenBank (accession no. OP882019 – OP882058 and OP882059 – OP882097, respectively).

Phylogenetic analyses

Phylogenies were reconstructed for *cytb* and IRBP genes separately. For both genes, the Maximum Likelihood (ML) and Bayesian Inference (BI) trees were constructed. ML trees were built in MEGA 11 (Tamura et al. 2021) and BI trees were estimated with MrBayes version 3.2.7a (Ronquist et al. 2012). The best fit substitution model was chosen in MEGA 11. HKY + I was used for reconstructing *cytb* gene. T92 had the lowest BIC score for IRBP gene and used for ML, but since this model is not available in MrBayes, the K2p model (second best fit) was used for Bayesian analyses of IRBP. BI was run with following parameters: 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 toward 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). All trees produced in ML and BI analyzes were visualized using midpoint root. Congruence of the maximum likelihood and maximum clade credibility Bayesian phylogenetic trees were estimated using Shimodaira—Hasegawa test (SH test, 10,000 bootstrap replicates) in package phangorn v. 2.11.1 (Schliep 2011) of R v. 4.3.0 software (R Core Team 2023).

Initial nucleotide diversity was estimated based on the full alignment, and further phylogenetic analyses were performed only on the unique haplotypes.

Since our sequences had different lengths, genetic diversity was calculated with method described by Fan et al. (2021). For this, we used Fan et al. R-script v. 1.0.1 presented in Supplementary files (archived on Zenodo: https://zenodo.org/records/6941867). Sequences were compared pairwise. The length of the overlapping regions, as well as the numbers of common and

Table 1 Population sampling used for the phylogenetic analyses

Species	Population	N	*cytb*		IRBP	
			Haplotype	Samples attributed	Genotype**	Samples attributed
Spalax arenarius	1. Solonoozernyi, Kherson Region, Ukraine	4	Scb1:	Soloz6, Soloz19, Soloz58, Soloz63	Si1: Si2: Si3: S-Oi:	Soloz6 Soloz19 Soloz58 Soloz63
	2. Oleshky, Kherson Region, Ukraine	5	Ocb:	Sagi 3, Sagi8, KF021263*, KF021255*, KF021262*	Oi: S-Oi:	Sagi8 Sagi3
	3. Krynky, Kherson Region, Ukraine	1	Krycb:	KRY1	Kryi:	KRY1
	4. Kakhovka, Kherson Region, Ukraine	1	Kacb:	Kakh1	Kai:	Kakh1
Spalax zemni	5. Mykolaiv, Mykolaiv Region, Ukraine	4	Mcb:	Nik1, Nik2, Nik3, 2018–60	Mi1: Mi2: Mi3:	Nik1 Nik2, Nik3 2018–60
	6. Prybuzhany, Mykoliav Region, Ukraine	1	Prcb:	2019–7	V-Pr-SLi:	2019–7
	7. Shyrokyi Lan, Mykolaiv Region, Ukraine	2	SLcb1: SLcb2:	2018–57 2018–58	V-Pr-SLi:	2018–57, 2018–58
	8. Veselynove, Mykolaiv Region, Ukraine	1	Vecb:	2018–51	V-Pr-SLi:	2018–51
	9. Voznesensk, Mykolaiv Region, Ukraine	3	Vcb:	2019–1, 2019–2, 2019–3	Kh-Vi:	2019–2
	10. Kryvyi Rih, Dnipro Region, Ukraine	2	KRcb:	KF021260*, KF021261*	–	–
	11. Khortytsia, Zaporizhia Region, Ukraine	2	Khcb:	2018–61, 2018–62	Kh-Vi:	2018–61
Spalax microphthalmus	12. Melitopol, Zaporizhia Region, Ukraine	1	–	–	Man-Sur-Meli:	2019–30
	13. Novomoskovsk, Dnipro Region, Ukraine	2	Dncb:	KF021258*, KF021259*	–	–
	14. Manych, Rostov Region, Russia	1	Mancb:	Man1	Man-Sur-Meli:	Man1
	15. Surovikino, Volgograd Region. Russia	1	Surcb:	Sur1	Man-Sur-Meli	Sur1
Spalax giganteus	16. Kizlyar, Dagestan, Russia	1	Kcb:	Kizlyar	Ki:	Kizlyar
	17. Caspiy, Dagestan, Russia	1	Ccb:	Caspiy	Ci:	Caspiy
Spalax graecus	18. Chernivtsi, Ukraine	4	Chcb:	2018–11,2018–12, 2018–13, 2018–14	Chi:	2018–11, 2018–12, 2018–13, 2018–14
	19. Iasi, Romania	3	Ia1cb: Ia2cb: Ia3cb:	KF021251* KF021252* KF021253*	–	–
	20. Suceava, Romania	4	Suc1cb: Suc2cb: Suc3cb: Suc4cb:	JX455993* JX455994* JX455995* JX455996*	–	–
Spalax antiquus	21. Aiton, Romania	4	A1cb: A2cb	A01, A02, R65 KF021256*	A-Bui:	A01, A02, R65
	22. Malaiesti, Romania	2	Bu-Macb:	R98, R99	A-Bui:	R98, R99
	23. Budesti, Romania	1	Bu-Macb:	KF021263*	–	–
	24. Sandulesti, Romania	1	Sancb:	KF021257*	–	–

Table 1 (continued)

Species	Population	N	*cytb*		IRBP	
			Haplotype	Samples attributed	Genotype**	Samples attributed
Nannospalax leucodon	25. Tiligul, Odesa, Ukraine	3	Tcb:	Tiligul4, Tiligul3, Tiligul2	Ti:	Tiligul4, Tiligul3, Tiligul2
	26. Constanta, Romania	1	Concb:	DOB1	Con-Tul-Khoti:	DOB1
	27. Tulcea, Romania	1	Tulcb	TUL1	Con-Tul-Khoti:	TUL1
	28. Khotyn, Chernivtsi, Ukraine	1	Khotcb:	2018–5	Con-Tul-Khoti:	2018–5
Nannospalax ehrenbergi	(Meredith et al. 2011)	1	-	–	–	JN414825*
	Whole genome, Fang et al. (2014)	1	-	–	–	XM_008834127*
	(Steppan and Schenk 2017)	1		KY754157*	–	–
	Hadid et al. (2011)	1		NC_020756*	–	–
	Spradling et al. (2001)	1		AF155871*	–	–

*Data from GenBank

**Unphased genotypes, including both homozygous and heterozygous variants

polymorphic sites were calculated. Haplotype diversity was then obtained as the number of pairwise comparisons with different sequences divided on the total amount of compared pairs. Nucleotide diversity was calculated as the mean frequency of pairwise differences in the overlapping regions. This way we preserved as much information about genetic diversity as possible. Unfolding of the IRBP sequences with ambiguity codes was conducted in DnaSP (Rozas et al. 2017). DnaSP simulated the pairs of homozygous or heterozygous alleles. Heterozygous positions randomly assigned to any of the two chromosomes. Thus, number of sequences was doubled.

Haplotype networks of *cytb* and IRBP genes were built in PopART v. 1.7 with median joining network (Bandelt et al. 1999). Only the fullest sequences representing each haplotype were used for the analysis. Haplotype network of *cytb* was built on 37 specimens. We phased 22 the most complete sequences which represented each haplotype of IRBP. We used PHASE v. 2.1.1 with parent-independent mutation model (Stephens et al. 2001; Stephens and Scheet 2005). The input and output files for PHASE were generated from FASTA-alignments in SeqPHASE (Flot 2010).

Neutrality statistics (such as Tajima's D test, Fu and Li's test) were not calculated due to low sample sizes, as these tests need a sample of at least 50–100 sequences to attain significant power (Simonsen et al. 1995).

Between species and within species genetic distances were calculated as uncorrected p-distances of *cytb* sequences. Raw divergence (Dxy) and net divergence (Da) were calculated in MEGA 11. Standard errors (SE) were estimated using 10,000 pseudoreplications in MEGA 11.

Results

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*.

Twelve sequences of the nuclear IRBP gene had ambiguity IUPAC codes, we considered these positions as heterozygous (Table 2). Haplotype network was built on 32 phased sequences. Almost all of the ambiguous positions were perfectly predicted (probability = 1.0). Site 24 in one specimen of *S. arenarius* was predicted with probability 0.59, PHASE produced 2 variants with C or T nucleotide in this position.

Specimen U48589 attributed to *S. zemni* (Stanhope et al. 1992) differed from other *S. zemni* by 55 – 68 sites, and was closest to *Nannospalax* XM_008834127 and JN414825 with 28–27 differing sites. We excluded this sequence from further phylogenetic analysis to reduce faux variability.

Nucleotide diversity of the studied populations was low for both genes (Tables 3 and 4). Haplotype diveristy in *cytb* was high in all species. We distinguished 37 haplotypes in *cytb*. IRBP gene had lower haplotype diversity, resulting in 22 haplotypes. All species were well separated in *cytb*

Table 2 Statistics of ambiguity positions in IRBP gene in the studied sequences

Species	Number of specimens with ambiguity positions	UIPAC ambiguity letter	Position
N. ehrenbergi	1	R	25
S. graecus	1	Y	680
S. arenarius	1	R Y	519, 617 43
S. arenarius	1	R Y	519, 617 24, 43
S. arenarius	1	Y	24
S. arenarius	1	R	1031
S. arenarius	1	R Y S	519, 577, 617, 1031 24, 43 175
S. zemni	2	M	255
S. zemni	2	Y	928
S. giganteus	1	R	177

Table 3 Polymorphism of the *cytb* gene

	Number of sequences	Number of pairwise comparisons	Common length of sequences in compared pairs (min – max)	Number of polymorphic sites in compared pairs (min – max)	Haplotype diversity, Hd	Nucleotide diversity, Pi
N. ehrenbergi	4	6	1140	3–104	1	0.066
N. leucodon	4	15	996–1032	0–17	0.8	0.105
S. antiquus	8	28	800–1140	0–5	0.786	0.003
S. graecus	11	55	800–1140	0–5	0.891	0.003
S. arenarius	11	55	1140	0–6	0.709	0.003
S. zemni	15	105	800–1140	0–24	0.895	0.012
S. microphthalmus	4	6	800–1140	0–3	0.833	0.002
S. giganteus	2	1	1140	9	1	0.008

Table 4 Polymorphism of the unfolded IRBP gene

	Number of unfolded sequences	Number of pairwise comparisons	Common length of sequences in compared pairs (min–max)	Number of polymorphic sites in compared pairs (min–max)	Haplotype diveristy, Hd	Nucleotide diversity, Pi
N. ehrenbergi	4	6	1077	0–4	0.833	0.002
N. leucodon	12	66	1077	0–1	0.545	0.0005
S. antiquus	10	45	1077	0	0	0
S. graecus	8	28	1052–1077	0–1	0.536	0.0005
S. arenarius	16	120	1076–1077	0–7	0.917	0.003
S. zemni	22	231	880–1077	0–3	0.762	0.011
S. microphthalmus	6	15	1054–1077	0	0	0
S. giganteus	4	6	1065–1076	0–2	0.833	0.001

network. IRBP network showed good separation for genera *Nannospalax* and *Spalax* (by 36 polymorphic sites), whereas between-species distance was rather low. Two IRBP haplotypes were shared between *S. zemni* (Mykolaiv and Khortytsia) and *S. arenarius* (Oleshky, Solonoozernyi).

Mitochondrial phylogeny

Phylogeny reconstructed in ML and Bayes resulted in nearly identical topologies (Fig. 2). According to Shimodaira – Hasegawa test ML and Bayesian trees of *cytb* were similar (Diff ln L = 9.78, $p = 0.259$).

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 a sister to *S. zemni* + *S. arenarius* + *S. microphthlamus*. *S. antiquus* and *S. graecus* formed another well supported branch.

The neighbor-joining haplotype network analyses places *S. antiquus* and *S. graecus* as internal nodes to all other large-bodied blind mole rats (Fig. 3). *S. arenarius* similarly occupied internal position to non-Carpathian mole rats, whereas *S. zemni* is placed internally to *S. microphthalmus* and *S. giganteus*. All species are separated by a significant amount of substitutions.

At the level of the *Spalax* genus, the interspecific p-distances varied from ~ 0.05 (*S. zemni* – *S. arenarius*) to ~ 0.11 (*S. microphthalmus*—*S. antiquus*). These were still considerably smaller compared to the distances between *Nannospalax spp.* and *Spalax spp.* (min ~ 0.16, Table 5). Within species p-distances were significantly smaller (0.002–0.012, Table 6).

Nuclear phylogeny

The evolution of the IRBP gene was better explained by the ML tree rather than BI (diff ln L = 21.62, $p < 0.0001$), but the topologic differences were associated with nodes with low support, which did not reach the generally acceptable level of confidence during bootstrapping (< 70%).

The IRBP gene did not allow to separate *S. arenarius* from *S. zemni*. They are both placed in a single clade (Fig. 4). *S. microphthalmus* appeared to be sister to *S. zemni* + *S. arenarius*. Unlike Podolian and sandy mole rats, two closely related Carpathian species—*S. antiquus* and *S. graecus*—were well separated in our analyses. In contrast to mitochondrial marker, nuclear IRBP placed *S. giganteus* as sister to all other large-bodied blind mole rats.

In the haplotype network analyses (Fig. 5) *S. antiquus, S. graecus* and *S. giganteus* all branched from closely related potential ancestral haplotypes. *S. zemni* and *S. arenarius* were closely related with only a small number of substitutions between the haplotypes respectively attributed to each species. Moreover, two phased haplotypes (Mykolaiv3a and Khortytsia2a) were shared by both species (Table 7).

The sandy mole rat (*S. arenarius*) had the highest level of intraspecific variation in the nuclear IRBP gene (Table 4). In

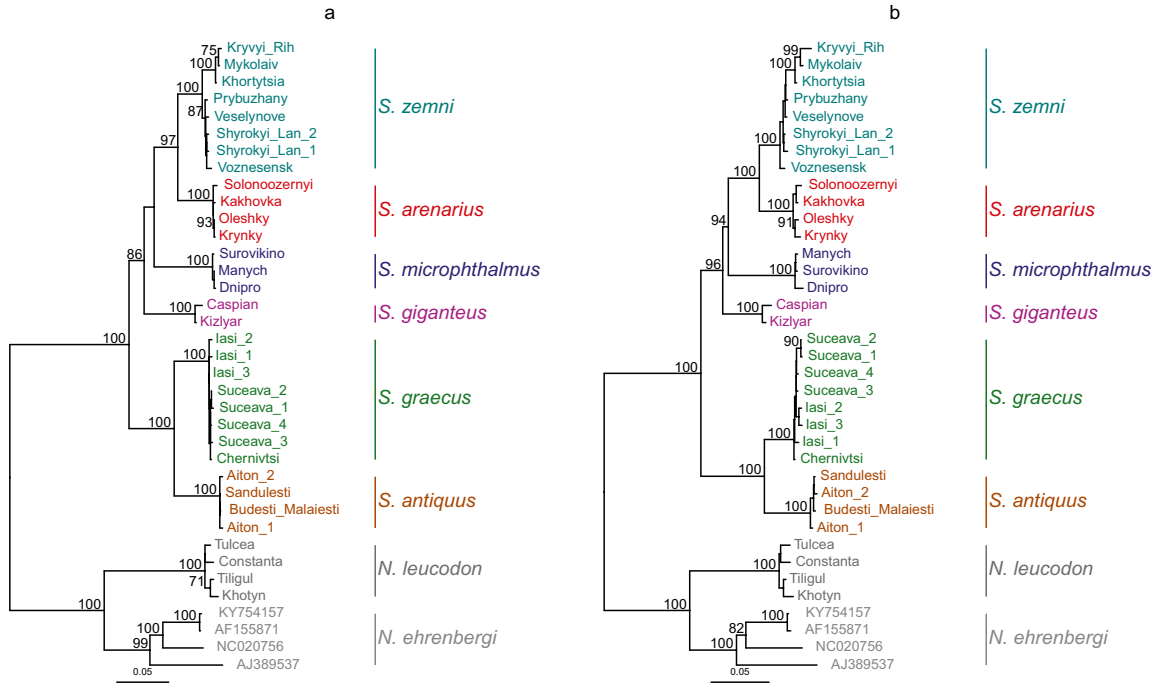

Fig. 2 Phylogeny of blind mole rats based on full mitochondrial *cytb* gene. **a**—ML tree, **b**—BI tree. Values at nodes represent bootstrap support (**a**) and posterior probability (**b**). Values only greater than 70% are shown

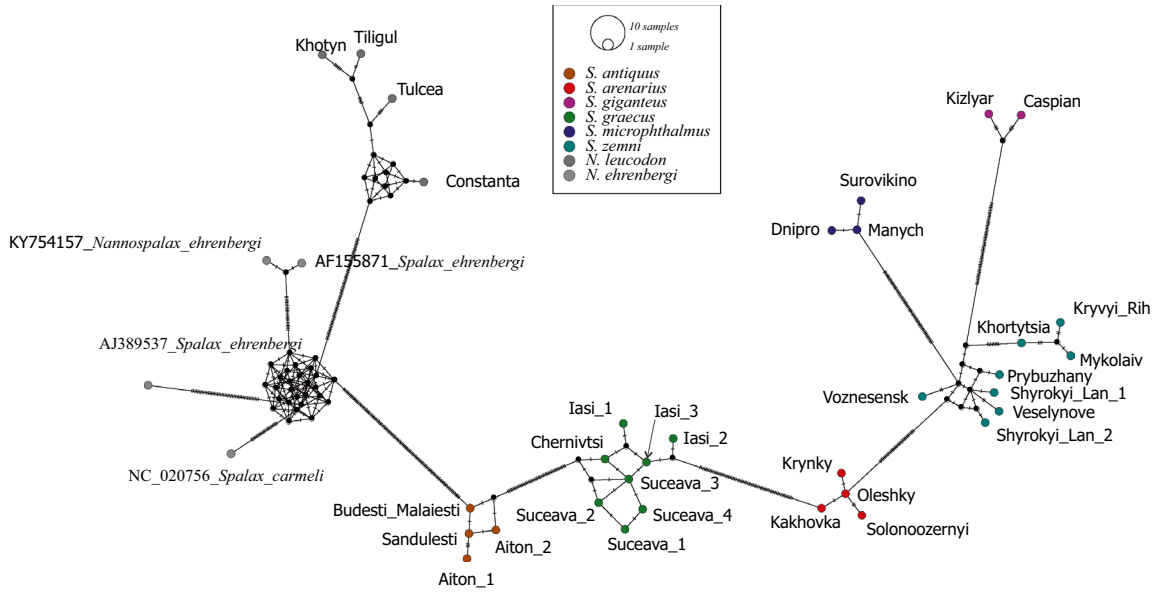

Fig. 3 Haplotype network of blind mole rats based on mitochondrial *cytb* gene

Table 5 Genetic distances between species of mole rats based on *cytb*

Species 1	Species 2	Raw p-distance (Dxy)	SE	Net p-distance (Da)	SE
S. arenarius	*S. zemni*	0.0568	0.0064	0.0492	0.0061
S. antiquus	*S. graecus*	0.0617	0.0073	0.0588	0.0071
S. arenarius	*S. microphthalmus*	0.0810	0.0083	0.0787	0.0083
S. microphthalmus	*S. giganteus*	0.0848	0.0086	0.0800	0.0085
S. zemni	*S. microphthalmus*	0.0864	0.0086	0.0794	0.0083
S. zemni	*S. giganteus*	0.0872	0.0080	0.0771	0.0077
S. graecus	*S. arenarius*	0.0929	0.0086	0.0901	0.0086
S. arenarius	*S. giganteus*	0.0937	0.0085	0.0884	0.0083
S. graecus	*S. zemni*	0.0966	0.0088	0.0890	0.0086
S. graecus	*S. giganteus*	0.1012	0.0090	0.0958	0.0087
S. antiquus	*S. zemni*	0.1042	0.0090	0.0966	0.0087
S. antiquus	*S. arenarius*	0.1051	0.0091	0.1023	0.0089
S. antiquus	*S. giganteus*	0.1065	0.0092	0.1012	0.0090
S. graecus	*S. microphthalmus*	0.1134	0.0102	0.1112	0.0101
S. antiquus	*S. microphthalmus*	0.1150	0.0102	0.1128	0.0101
N. ehrenbergi	*N. leucodon*	0.1260	0.0091	0.0876	0.0080
N. leucodon	*S. zemni*	0.1648	0.0112	0.1534	0.0110
N. leucodon	*S. giganteus*	0.1656	0.0114	0.1564	0.0110
N. ehrenbergi	*S. arenarius*	0.1668	0.0102	0.1322	0.0093
N. ehrenbergi	*S. graecus*	0.1677	0.0104	0.1331	0.0095
N. ehrenbergi	*S. zemni*	0.1698	0.0101	0.1305	0.0093
N. ehrenbergi	*S. antiquus*	0.1707	0.0105	0.1361	0.0096
N. ehrenbergi	*S. giganteus*	0.1721	0.0104	0.1350	0.0095
N. leucodon	*S. arenarius*	0.1744	0.0117	0.1677	0.0114
N. leucodon	*S. antiquus*	0.1755	0.0119	0.1688	0.0115
N. leucodon	*S. graecus*	0.1787	0.0119	0.1720	0.0115
N. ehrenbergi	*S. microphthalmus*	0.1813	0.0112	0.1473	0.0104
N. leucodon	*S. microphthalmus*	0.1835	0.0124	0.1774	0.0122

Table 6 Genetic distances within species based on *cytb* sequences

Species	p-distance	SE
N. ehrenbergi	0.0664	0.0054
N. leucodon	0.0105	0.0021
S. antiquus	0.0028	0.0012
S. graecus	0.0028	0.001
S. arenarius	0.0027	0.0011
S. zemni	0.0124	0.0023
S. microphthalmus	0.0017	0.001
S. giganteus	0.0079	0.0026

contrast, *S. microphthalmus* had no intraspecific variation, despite the fact that it currently has the largest distribution area of all *Spalax* species and the distance between the furthermost locations of *S. microphthalmus* in our sampling was almost 600 km.

By choosing a nuclear 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 (Fang et al. 2014) in the annotated reference genome assembly of the Palestinian blind mole rat *N. galili* has a 12 bp 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. Pseudogenization was recently suggested to play an important role in the evolution of subterranean mammals (Zheng et al. 2022).

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. giganteus*, in the analysis, allowed us to resolve a

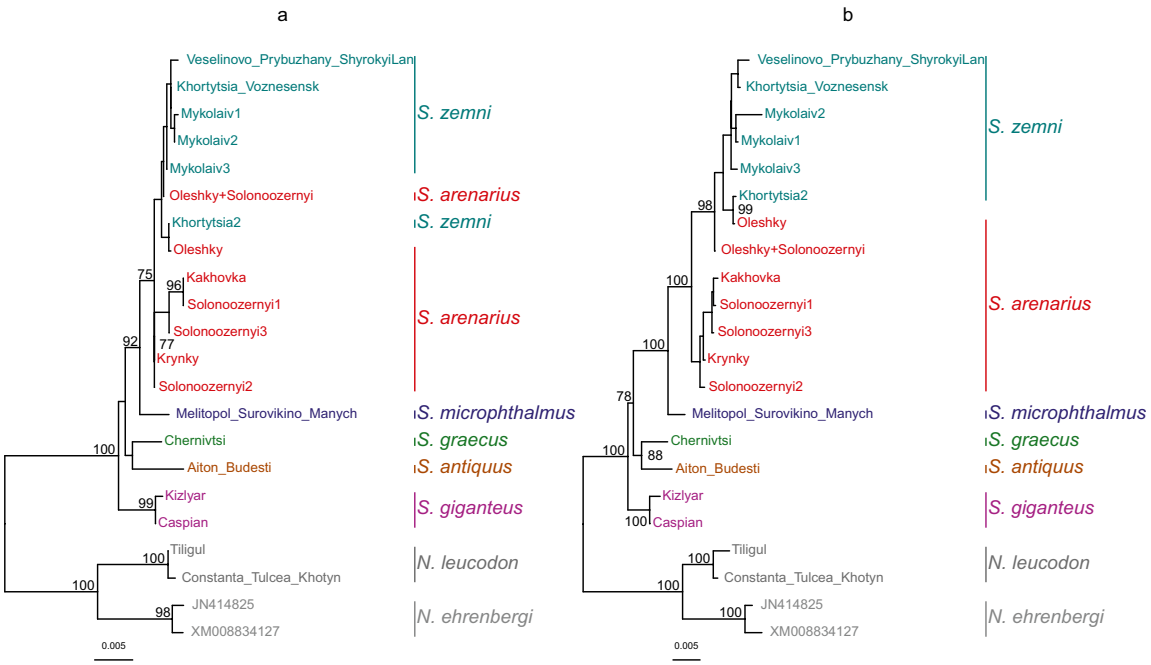

Fig. 4 Phylogeny of blind mole rats based on nuclear IRBP gene. **a**—ML tree, **b**—BI tree. Values at nodes represent bootstrap support (**b**) and posterior probability (**a**). Values only greater than 70% are shown

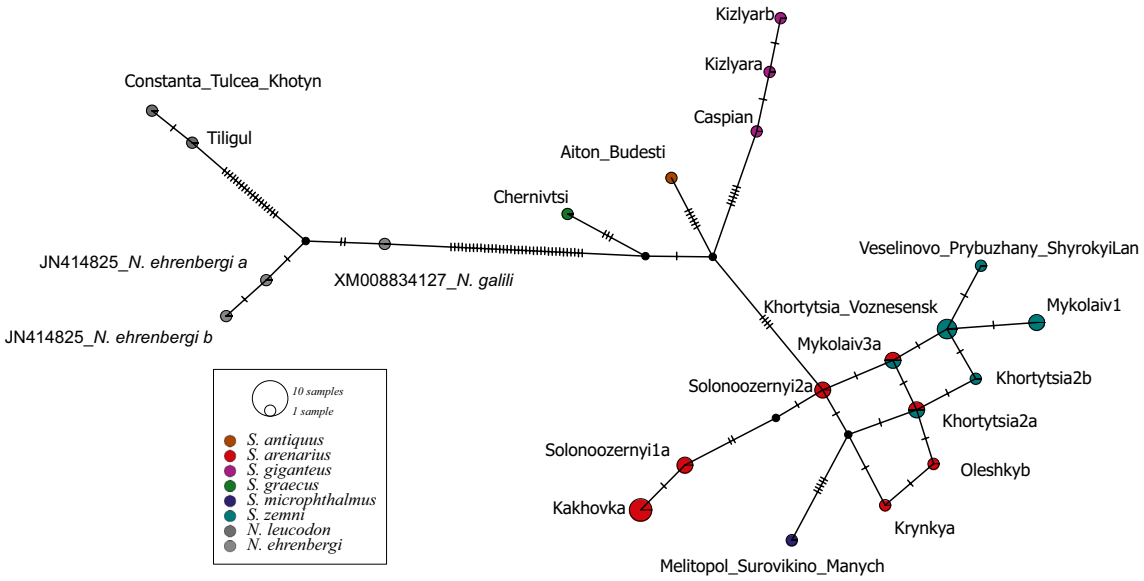

Fig. 5 Haplotype network of blind mole rats based on nuclear IRBP gene

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 time it was described. Reshetnik (1941) designated 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 morphologic 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 species within the genus that does not appear monophyletic in the nuclear IRBP gene tree, but instead is placed entirely within *S. zemni*. Still, more nuclear markers are required to answer whether *S. arenarius* represents the valid species.

Spalax graecus was proposed to be the basal species among all large-bodied blind mole rats since earliest studies (Méhely 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, the two species are clearly separated at the level of the nuclear IRBP gene.

The most problematic taxonomic situation occurs in Southern Ukraine where several species of mole rats occur close to each other. While mtDNA clearly separates *S. arenarius* and *S. zemni* as separate matrilineages, the nuclear gene shows more complicated relationships (Fig. 5). Several animals collected from Khortytsia (a rocky island at the middle of the Dnieper River in the center of Zaporizhia City), near Mykolaiv City on the right bank of the Dnieper river, from Solonoozernyi (Kinburn Spit in Kherson Region) and near the town of Oleshky from the left bank of the Dnieper river, had heterozygous genotypes, with one haplotype unique to their respective locations and one haplotype shared with other localities (Fig. 6). This observation could be explained by: (i) hybridization between species; or (ii) recent divergence from the common ancestor. The low sampling size did not allow to answer how widespread is this phenomenon, and denser genome marker coverage would be crucial to understand the situation with these two forms. We conclude that the species status of *S. arenarius* is so far dubious and could not be explicitly confirmed by our current results.

Table 7 Allele distribution of IRBP across *S. arenarius* and *S. zemni*

Species	Genotype	Phased IRBP alleles	IRBP haplotypes										
			Hap 03	Hap 04	Hap 07	Hap 15	Hap 17	Hap 05	Hap 06	Hap 01	Hap 12	Hap 02	Hap 19
S. arenarius	Kai	Kakhovka			■								
	Kryi	KrynkyA				■							
		KrynkyB			■								
	Oi	OleshkyA	■										
		OleshkyB					■						
	S-Oi	Oleshky+Solonoozernyi		■									
	Si1	Solonoozernyi1A							■				
		Solonoozernyi1B			■								
	Si2	Solonoozernyi2A						■					
		Solonoozernyi2B							■				
	Si3	Solonoozernyi3A						■					
		Solonoozernyi3B			■								
S. zemni	Kh-Vi	Khortytsia_Voznesensk								■			
		Khortytsia2A	■										
		Khortytsia2B									■		
	Mi1	Mykolaiv1										■	
	Mi2	Mykolaiv2A								■			
		Mykolaiv2B										■	
	Mi3	Mykolaiv3A		■									
		Mykolaiv3B								■			
	V-Pr-SLi	Veselinovo_Prybuzhany_ShyrokyiLan											■

Surprisingly enough, *S. arenarius* despite having one of the smallest distribution range (only *S. antiquus* is found within an even smaller area) is characterized by a high haplotype diversity. Whereas in the mitochondrial *cytb* we recorded four haplotypes, the nuclear IRBP gene showed a significant diversity with seven haplotypes present in *S. arenarius* (Table 5). Four alleles were found within the Solonoozernyi population at the Kinburn Spit, with all animals collected in few hundred meters from each other. Four alleles of IRBP were also recorded in the outskirts of the Mykolaiv City, with Hap04 shared between these two populations. At the same time both populations were invariable at the mtDNA level.

We also discovered clear phylogeographic intraspecific structure within *S. zemni*. At least two lineages were identified. The South-Eastern lineage includes *cytb* haplotypes from Khortytsia Island, Mykolaiv and Kryvyi Rih. While the IRBP phylogenetic analysis did not support combining all South-Eastern populations into the same clade, at least one haplotype had a wide distribution from Mykolaiv to Voznesensk and to Khortytsia (Fig. 6).

The South-Western lineage of *S. zemni* seems to have a limited distribution in the narrow strip between the Southern Bug River and Tiligul Estuary (eastern border of the species distribution, where it is replaced by *N. leucodon*). The four animals were collected from three different populations and they all had unique *cytb* haplotypes. At the same time, nuclear IRBP gene in all these samples was represented with a same homozygous genotype. The mtDNA of the mole rats from the town of Voznesensk clustered together with the South-Western lineage in ML analyses with 87% bootstrap support, yet this clustering was not supported by the BI. The

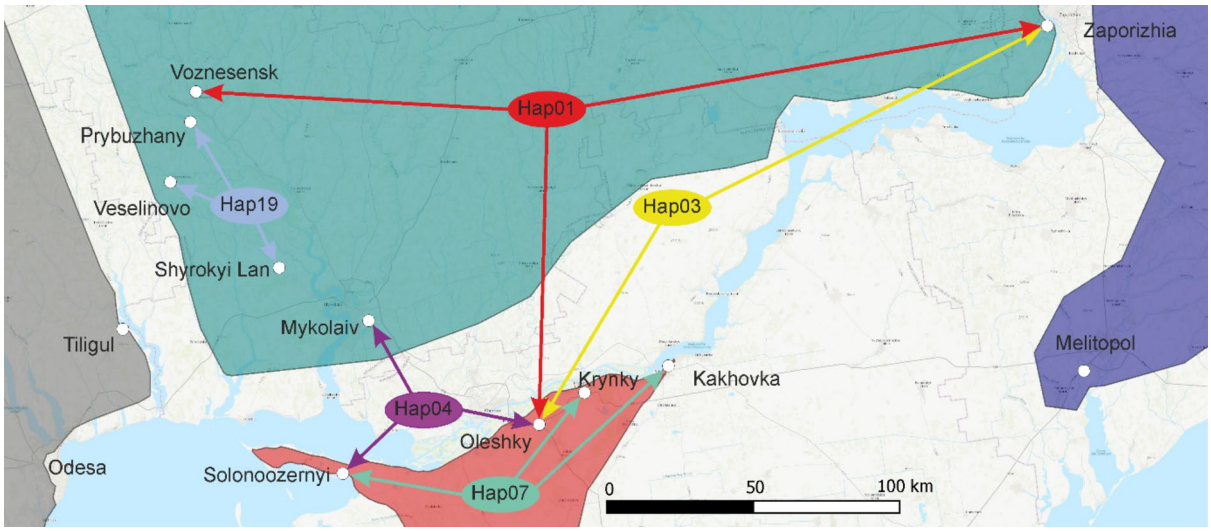

Fig. 6 Distribution of shared haplotypes of nuclear IRBP gene between populations of *S. zemni* and *S. arenarius*

IRBP results were even more puzzling, as the haplotype carried by the Voznesensk mole rat was shared with Khortytsia Island from the easternmost distribution locality. Therefore, Voznesensk may represent an intermediate population between the South-Eastern and the South-Western lineages.

The phylogeography of Podolian mole rat further to the north is unknown, especially that in most of Northern and Western Ukraine the species became extinct in the past decades (M. Rusin, original data). In the past *S. zemni* range extended to the north of Kyiv City and to the west near Lviv City. There is a single active population from Ivano-Frankivsk Region in the western Ukraine known today (M. Rusin, original data).

Despite having large geographic distances between our sampling sites for *S. microphthalmus*, this species shows high degree of 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, and they share the same homozygous genotype in nuclear IRBP gene. Similar results were obtained in earlier studies by Matveeva et al. (2019). Using control region of the mtDNA they estimated minimum variations for populations from Kharkiv and Kursk and Samara regions (distance between furthest localities app. 1000 km). Our sample from Ciscaucasia (South of the Don River) did not show separate lineage and was similar to populations from the western part of the whole species distribution. This is in contrast to several other species of small mammals, which have separate Ciscaucasian lineages, f.e. *Sicista subtilis* (Lebedev et al. 2020) or *Dryomys nitedula* (Mohammadi et al. 2021).

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* (Ognev 1947). Later, Dzuev and Shogenov (2003) described animals from Kabardino-Balkaria as having the chromosom number $2n = 62$, similar to all other species in the genus, but different to other populations of *S. microphthalmus* ($2n = 60$). More so, they described the chromosomal formula to be significantly different from that found by Martynova et al. (1975) for all species of *Spalax*. Unfortunately, authors 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 sister to *S. zemni* + *S. arenarius* + *S. microphthalmus* (*cytb* tree); (b) it forms a sister group with *S. microphthalmus* (both IRBP and *cytb* haplotype networks); (c) or it is placed at basal position to all other species in the genus (IRBP tree). 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 (Ognev 1947). It remains to be seen whether this morphologic similarity is confirmed by the molecular data, too.

North-Western Pontic Steppes as well as the Carpathian basin appears to be the primary evolutionary arena for

the evolution of large-bodied blind mole rats. Two conflicting hypotheses were earlier introduced to discuss the origin and dispersal of mole rats to Eastern Europe (Ognev 1947; Topachevskiy 1969). One hypothesis suggests that the *Spalax* s. str. separated from the common ancestor with *Nannospalax* in the Balkans and its distribution eastwards led to speciation events. The other alternative hypothesis suggests that the *Spalax* separated from *Nannospalax* in the east, either in the Caucasus, or even further above the Caspian Sea and it dispersed to Europe in the westward direction. In the latter hypothesis *S. uralensis* and *S. giganteus* represent the basal species.

While our findings cannot decisively support neither of these hypotheses, there are more evidences toward the South-European origin of the genus *Spalax*. The haplotype network analyses of both *cytb* and IRBP suggest that *S. antiquus* and *S. graecus* are closer to the stem leading to the potential common ancestor with the *Nannospalax*. Certainly, while the interior nodes within haplotype network should not be directly interpreted as the ancestral state (Huson and Bryant 2006), the basal position of both Carpathian species is still highly probable.

Our IRBP results suggest relatively quick dispersal of the ancient *Spalax* lineage from the Carpathian region. It is likely that *S. giganteus* in the Caucasian region represents the relic of this early dispersal. Similar scenario has been suggested in the case of *Sicista*, with the westernmost species from the Caucasian mountains being the closest to the easternmost species from the Sakhalin Island in the Pacific Ocean (Lebedev et al. 2021). We can hypothesize that such early dispersal followed by range fragmentation of populations at the outer rims of the former distribution may be found in more species groups, in particular small mammals.

Quite unusual is the presence of several closely related species of *Spalax* in the Eastern European Plain. Large rivers were hypothesized to be the geographic barriers that led to the speciation events (Topachevskiy 1969; Hadid et al. 2012). While rivers indeed may play a certain role as barriers for dispersal of small mammals, especially for subterranean rodents, in light of our results they appear not as important as previously believed. All large rivers of Eastern Europe were crossed by the mole rats at least several times and often animals from both sides of the river do not show any significant differentiation, including the dispersal of same haplotypes on both banks. Here, we summarize each large river of Eastern Europe and its potential role for mole rats' evolution.

(1) The Danube does not appear to pose a barrier to *N. leucodon* since populations from as far North as Khotyn (Chernivtsi Region of Ukraine) to the south of Constanta (Romania) have the same haplotype in IRBP.

(2) The Prut river was often believed to be the border between *S. graecus* and *N. leucodon* (Chişamera et al. 2014). Yet, this may be true only for the northern and eastern borders between the species. At the south there are no river barriers to separate these two species.

(3) The Dniester river was believed to be a border between *N. leucodon* and *S. zemni* in the upper flow (northern part), yet *N. leucodon* occurs on both sides of the Dniester in the south within the lower flow.

(4) The Tiligul river and its estuary were believed to separate *N. leucodon* and *S. zemni* in the south, yet there are no recent localities of mole rats on the eastern shore of the Tiligul Estuary. Yet, according to Topachevskiy (1969) fossil remains attributed to *S.* cf *zemni* were found west of Tiligul.

(5) The Southern Bug was never earlier suggested to affect the range distribution of the mole rats. Yet, we identified the presence of a separate lineage of Podolian mole rats west of this river. Still, it seems that there is a certain geneflow between both sides of the Bug River, as animals from Voznesensk to the east of Bug were related to this western lineage.

(6) The Dnieper river is often speculated to be a major barrier for many terrestrial species in Eastern Europe. Yet, it is true only to some extent. The most obvious example is *S. zemni* from the western bank of the Dnieper and *S. arenarius* from the eastern bank. Both forms likely represent common gene pool and have shared alleles crossing the Dnieper several times. Khortytsia Island is populated by mole rats that have at least three alleles of IRBP, one 'local', second shared with Voznesensk, and the third shared with *S. arenarius* from Oleshky. The limited-available sampling does not allow to draw accurate maps of distribution of each allele. While the Dnieper river is often treated as a border between *S. zemni* and *S. microphthalmus*, and indeed two samples from Novomoskovsk (eastern Dnipro Region) belong to *S. microphthalmus* (Hadid et al. 2012), there are no genetically verified samples available from the western bank of the Dnieper river in that region. Moreover, the Dnieper did not act as a barrier for dispersal to other small mammals, such as *Apodemus sylvaticus* (Jeremy et al. 2017), *Allactaga major* (Shenbrot et al. 1995), *Ellobius talpinus* (M. Rusin, unpublished data), the latter representing another case of subterranean mammal crossing the Dnieper.

(7) The Don River does not pose a barrier for mole rats: populations from both sides of the river (Surovikino and Manych) have little differences in *cytb* and share the same IRBP haplotype.

We therefore must reconsider the role of large rivers for dispersal of *Spalax* spp. in Eastern Europe. Certainly, rivers may represent barriers for dispersal, but to a very limited extend. Noteworthy, all of the mole rat species represent allopatric forms. More so, it is often that while one species may occur on one side of the river, the other species does not occur immediately on the opposite side. *S. zemni* and *S. arenarius* pair is a notable example. The sandy mole rats are recorded close to the Dnieper's left bank (Rusin 2023). At the same time the opposite site of the river is free of mole rats and the Podolian mole rat always have a 50 km gap between its active localities and the localities of *S. arenarius* (see Fig. 6 for actual species ranges within the area), regardless of any geographic barriers (Rusin and Ghazali 2022). Similarly, while we were able to capture *N. leucodon* at the western shore of the Tiligul Estuary, we failed to record the presence of mole rats on the eastern side of the same Estuary.

Future studies involving larger sampling and additional markers, potentially genomic data (Li et al. 2020) may add further details on the evolutionary history of large-bodied blind mole rats and answer the remaining questions not resolved in our study. We have to note that since the Russian large-scale aggression against Ukraine started in 2022 turned the vast parts of the blind mole rats' distribution ranges (in particular, potential hybridization/ancestral area of *S. zemni* and *S. arenarius*) into a war zone, additional sampling efforts seem to be impossible for years to come. This makes the samples collected in course of our study a very precious material for in-depth genotyping efforts, and, ultimately, a unique starting point for the comparison of the effect of the modern war on the small mammals' populations dynamics, genetic variation and survivability.

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Author contributions MR and AY designed the study; MR, MG and ADS collected samples; OÇ performed laboratory experiments, supervised by AY; MG and MR analyzed 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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Data availability Sampling data available at GBIF: https://doi.org/https://doi.org/10.15468/hfr4gw. Nucleotide sequences available at NCBI GenBank with accession numbers: OP882019 – OP882097.

Declarations

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

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 Romania. No permit is required for analyzing the DNA samples in Türkiye.

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