Ann. Zool. Fennici 57: 89–98
Helsinki 16 April 2020
ISSN 0003-455X (print), ISSN 1797-2450 (online)

Multi-locus genetic identification of a newly discovered population reveals a deep genetic divergence in European blind mole rats (Rodentia : Spalacidae : *Nannospalax*)

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Received 7 Jan. 2020, final version received 1 Apr. 2020, accepted 7 Apr. 2020

Németh. A., Csorba, G., Laczkó, L., Mizsei, E., Bereczki, J., Pásztor J. A., Petró P. & Sramkó, G. 2020: Multi-locus genetic identification of a newly discovered population reveals a deep genetic divergence in European blind mole rats (Rodentia : Spalacidae : *Nannospalax*). — *Ann. Zool. Fennici* 57: 89–98.

A new population of blind mole rat (genus *Nannospalax*) was discovered near the town of Albertirsa in north-central Hungary. We used newly designed primers to specifically amplify the whole mitochondrial cytochrome-*b* region and two nuclear DNA regions. Based on the most comprehensive taxonomic sampling to date, we compared this population with several other European blind mole-rat taxa. The results from both mitochondrial and nuclear regions have unequivocally placed the Albertirsa population into the monophyletic group of the Vojvodina blind mole rat (*N.* (*leucodon*) *montanosyrmiensis*), which turned out to be a sister clade to all other molecularly studied European *Nannospalax*. This study not only identified the fourth known population of an extremely rare rodent taxon but also calls for a taxonomic revision of European lesser blind mole rats (*N. leucodon* superspecies) to systematically evaluate the genetic structure of their populations and to understand the complex evolutionary history of these European rodents. The occurrence of the Vojvodina blind mole rat at this northern location helps to clarify the distribution area of this heavily data-deficient taxon. As currently understood, this lineage predominantly occurs in sandy grasslands of the Danube–Tisza Interfluve in Hungary and Serbia. Its distribution range and phylogenetic structure might reflect the importance of potential biogeographical barriers (e.g. large rivers) that shaped the blind mole rats' allopatric or peripatric speciation.

Introduction

According to the "unified species concept" (de Queiroz 2007), which defines species as "segments of separately evolving metapopulation lineages", all species criteria (i.e., morphological, phylogenetic, ecological, biological, etc.) may appear during the divergence of isolated lineages. Sometimes, the sequence of appearance of these criteria is different or their simple acquisition does not take place during the course of existence of the "separately evolving metapopulation lineages", still, they form species. The lack of morphological difference (i.e., the violation of the phenetic species concept) can lead to the existence of cryptic species, which are defined as "species that remain indistinguishable morphologically" (Bickford *et al*. 2007). Morphological stasis can easily be a result of stabilising selection posed by extreme environmental conditions if coupled with phylogenetic niche conservatism (i.e., the tendency of a lineage to retain ecological niche over evolutionary time) (Fišer *et al*. 2018). Genetic isolation between species, as a primary proxy of ongoing speciation (Bock 2004) and the emergence of separately evolving entities, can be best measured by DNA methods (Fišer *et al*. 2018).

Eurasian blind mole rats (Spalacidae : Spalacinae) form one of the least-known group of Palaearctic rodents. They live a subterranean life that is typical for the whole subfamily, thus, they nicely illustrate phylogenetic niche conservativism that has led to a decreased interspecific morphological variability compared with that in other rodents (Nevo 2000). They can also be typical examples of cryptic species and their systematics — despite more than hundred years of detailed systematic studies (e.g., Nehring 1897, Méhely 1909, Topachevskii 1969, Nevo *et al*. 2001) — is a source of long-standing disagreement (i.e. Savić & Nevo 1990, Harrison & Bates 1991, Musser & Carleton 2005, Kryštufek & Vohralík 2009, Norris 2017). The lesser blind mole rat *Nannospalax leucodon* Nordmann, 1840 superspecies is endemic to Europe and characterised by high chromosomal diversity (Kryštufek & Vohralík 2009), with 25 karyologically distinct forms reported so far (*see* Arslan *et al*. 2016). Although the species status of taxa described in the genus solely on chromosomal grounds has not been widely accepted (Kryštufek 1997, Kryštufek *et al*. 2012), breeding experiments on several chromosomal forms (Savić & Soldatović 1984, Savić *et al*. 2017), corroborated by the results of recent molecular phylogenetic investigations (Hadid *et al*. 2012, Kryštufek *et al*. 2012, Németh *et al*. 2013a), strongly suggest the possibility of the existence of cryptic species status of some of these forms.

From the grasslands of the Pannonian (or Carpathian) Basin four *Nannospalax* taxa have been described: *N.* (*leucodon*) *transsylvanicus* Méhely, 1909; *N.* (*leucodon*) *hungaricus* Nehring, 1898; the most likely extinct *N.* (*leucodon*) *syrmiensis* Méhely, 1909 and *N.* (*leucodon*) *montanosyrmiensis* Savić and Soldatović, 1974 (Savić and Soldatović 1984, Németh *et al*. 2009, Csorba *et al*. 2015). The latter taxon (the Vojvodina blind mole rat) was originally described on karyological grounds by Savić and Soldatović (1974) as *Spalax montanosyrmiensis* from two neighbouring localities (Stražilovo and Čortanovci) on the Fruška gora in Vojvodina, Serbia, and proved to be reproductively isolated from other chromosomal forms of *Nannospalax* in the region (Savić & Soldatović 1984, Savić *et al*. 2017). During the research of blind mole rats of the Pannonian Basin, a small and fragmented population of blind mole rats was found in 2008 between Subotica (N Serbia) and Kelebia (S Hungary) (Németh *et al*. 2013a). Cytogenetic investigations identified the population as *N.* (*leucodon*) *montanosyrmiensis* (Németh *et al*. 2013a). In 2013, another population was discovered near Baja (S Hungary), which, based on molecular phylogenetic studies, also turned out to be *N.* (*leucodon*) *montanosyrmiensis* (Csorba *et al*. 2015).

In April 2017, a new population of blind mole-rats was discovered near Albertirsa (central Hungary). Although several localities were previously reported from this region, blind mole rats were considered extinct in the area because the last occurrence dated back to 1905 (Németh *et al*. 2009). As a consequence of the lack of observable morphological differences between Hungarian *Nannospalax* species, we used sequences of cytochrome *b* (*CYTB*) a widely used mitochondrial-DNA (mtDNA) region and

developed two nuclear regions to (i) identify the Albertirsa population using molecular genetic methods, and (ii) test the phylogenetic resolution of these markers in discriminating between cryptic species of the genus *Nannospalax*.

Material and methods

As all taxa of blind mole rats are strictly protected in Hungary, it was permitted to sample only two individuals from the newly discovered Albertirsa population. In May 2017, a male and a female individual were captured using a live-catching method (Németh *et al*. 2007), and were handled in the field in accordance with guidelines approved by the American Society of Mammalogists (Gannon *et al*. 2007). After biopsy of hind foot skin matrix applying topical and systemic anaesthesia and 70% alcohol disinfection following the protocol described by Sós *et al*. (2009), both individuals were immediately released at the site of capture back into their tunnel system. Samples from other populations in the Pannonian Basin and outgroup taxa were collected between 2008 and 2017 following the same protocol. Tissue samples were stored in 96% ethanol at –20 °C and housed in the Hungarian Natural History Museum until analysed.

Genomic DNA was extracted as detailed in Cserkész *et al*. (2017). The mitochondrion-encoded *CYTB* was amplified using *de novo* designed primers (SpalaxCBfw2: 5′-TGA CAT GAA AAA TCA TCG-3′; SpalaxCBrv2: 5′-CGA GAA GAG AGG TAC TA-3′) based on publicly available sequences of *Nannospalax carmeli* (GenBank accession number: NC_020756) and *Nannospalax ehrenbergi* (GenBank accession number: AJ416891). As a single gene can be misleading in identification and because of the common occurrence of mito-nuclear discordance via hybridisation (Toews & Brelsford 2012), the sequence information of the maternally inherited (mitochondrial) gene was supplemented with two nuclear-DNA (nDNA) regions using specific primers designed here for the first time. We amplified and sequenced fragments of two nuclear coding genes, the Lecithin–Cholesterol Acyltransferase (*LCAT*) gene and the Interphotoreceptor Retinoid Binding Protein (*IRBP*). The first fragment of *LCAT* was amplified using primers LCAT1fw (5′-GGA CTT CTT CAC CAT CTG GC-3′) and LCAT1rv (5′-AGG TTG ATG CAG TAA GAA ATA GAG C-3′), whereas primers LCAT3fw (5′-CGC ATA ACG ACG ACT TCT CC-3′) and LCAT3rv (5′-ATG TTG AGG TGT TGT GTC CC-3′) were used to amplify the second fragment; both primer pairs were designed based on genomic sequence of *Nannospalax galili* (GenBank accession number: NW_008343085). The coding gene *IRBP* was amplified and sequenced using primers SpalaxIRBPfw (5′-ATG ATG AGA CAA TGG CTC CTG C-3′) and SpalaxIRBPrv (5′-TGA GGT GCT CTG TGT TCT GC-3′) designed based on available sequences of *Spalax zemni* (GenBank accession number: U48589) and *Nannospalax ehrenbergi* (GenBank accession number: JN414825). Polymerase Chain Reactions (PCRs) were performed in 25 µl volume using 1× Reaction Buffer (Thermo Scientific), 0.2 mM of each dNTP (Thermo Scientific), 2 mM $MgCl_2$ (Thermo Scientific), 0.25 mg Bovine Serum Albumin (Invitrogen), 0.2 µM of each primer, 0.03 U of Phusion II Hot Start Polymerase (Thermo Scientific), and 1 µl unquantified DNA template. PCR conditions for *CYTB* were: initial denaturation for 3 min at 98 °C followed by 40 cycles of denaturation for 10 sec at 98 °C, 30 sec annealing at 57 °C, 1 min 30 sec extension at 72 °C, and final extension for 7 min at 72 °C. For the *LCAT* fragments, this cycling was modified by setting annealing temperature to 62 °C and extension time to 1 min. The *IRBP* gene was amplified using the same cycling as described for *CYTB*, except annealing temperature set to 60 °C. Sequences were checked for errors and aligned by MUSCLE ver. 3.8.31 (Edgar 2004). All newly generated sequences were deposited in GenBank (Table 1; GenBank accession numbers: MN497962–MN498009).

We compiled a data set of newly generated *CYTB* and two nuclear gene sequences covering all known taxa and populations of *Nannospalax* from the Pannonian Basin (Table 1 and Fig. 1), most of which have never been studied in terms of molecular phylogeny. In the analyses we included sequences published in the most comprehensive phylogenetic study of European *Nannospalax* to date (Kryštufek *et al*. 2012) in

Table 1. List and GenBank accession numbers of newly generated sequences. Additional sequences used in this study were downloaded from GenBank.

Sample ID	Taxon name	Locality	Coordinates		GenBank accession number		
			Lat. °N	Long. °E	*CYTB*	*LCAT*	*IRBP*
Nannospalax leucodon							
FK138	*hungaricus*	Tompapuszta (HU)*	46.35359	20.978302	MN497968		
FK117	*hungaricus*	Kunmadaras (HU)	47.02029	20.79089	MN497967		
FK068	*hungaricus*	Mezőtúr (HU)	47.01611	20.60574	MN497965		
FK062	*hungaricus*	Battonya (HU)	46.30139	21.02513	MN497963	MN498003	MN497999
FK066	*hungaricus*	Battonya (HU)	46.30139	21.02513	MN497964		
FK047	*hungaricus*	Vajdahunyad (RO)	45.75443	22.92848	MN497962		
FK139	*hungaricus*	Deliblato (RS)	44.87538	21.03073	MN497966	MN498005	MN498000
FK136	*transsylvanicus*	Apahida (RO)*	46.82085	23.68355	MN497972		
FK007	*transsylvanicus*	Hajdúhadház (HU)	47.66638	21.72861	MN497969		
FK178	*transsylvanicus*	Hajdúhadház (HU)	47.66638	21.72861	MN497973		
FK210	*transsylvanicus*	Debrecen (HU)	47.56336	21.59638	MN497974	MN498007	MN498001
FK015	*transsylvanicus*	Józsa (HU)	47.59253	21.58745	MN497971		
FK009	*transsylvanicus*	Hajdúbagos (HU)	47.39247	21.67091	MN497970		
FK106	*montanosyrmiensis*	Cortanovci (RS)*	45.15916	19.97033	MN497979		
FK099	*montanosyrmiensis*	Strazilovo (RS)*	45.15816	19.92861	MN497978		
FK070	*montanosyrmiensis*	Subotika (RS)	46.17498	19.70428	MN497977		
FK133	*montanosyrmiensis*	Subotika (RS)	46.17498	19.70428	MN497980		
FK063	*montanosyrmiensis*	Kelebia (HU)	46.19650	19.67618	MN497976	MN498004	MN497996
FK061	*montanosyrmiensis*	Kelebia (HU)	46.19650	19.67618	MN497975		
FK229	*montanosyrmiensis*	Kelebia (HU)	46.19650	19.67618	MN497988		
FK157	*montanosyrmiensis*	Kelebia (HU)	46.19631	19.70763	MN497982		
FK223	*montanosyrmiensis*	Kelebia (HU)	46.19631	19.70763	MN497986		
FK228	*montanosyrmiensis*	Kelebia (HU)	46.19631	19.70763	MN497987		
FK134	*montanosyrmiensis*	Kelebia (HU)	46.22233	19.67121	MN497981		
FK162	*montanosyrmiensis*	Kelebia (HU)	46.22233	19.67121	MN497983		
FK171	*montanosyrmiensis*	Baja (HU)	46.19264	18.98965	MN497984	MN498006	MN497997
FK191	*montanosyrmiensis*	Baja (HU)	46.19264	18.98965	MN497985		
FK272	*montanosyrmiensis*	Baja (HU)	46.19264	18.98965	MN497991		
FK261		Albertirsa (HU)	47.24169	19.63655	MN497989	MN498009	MN497998
FK262		Albertirsa (HU)	47.24047	19.63491	MN497990		
Outgroup							
FK217	*Nannospalax ehrenbergi*	Ariz (LB)	34.24156	36.04806	MN497993	MN498008	MN497995
FK173	*Spalax microphtalmus*	Stircovskaja (UA)	49.28901	40.06986	MN497992	MN498002	MN497994

* sampled at or near the type locality

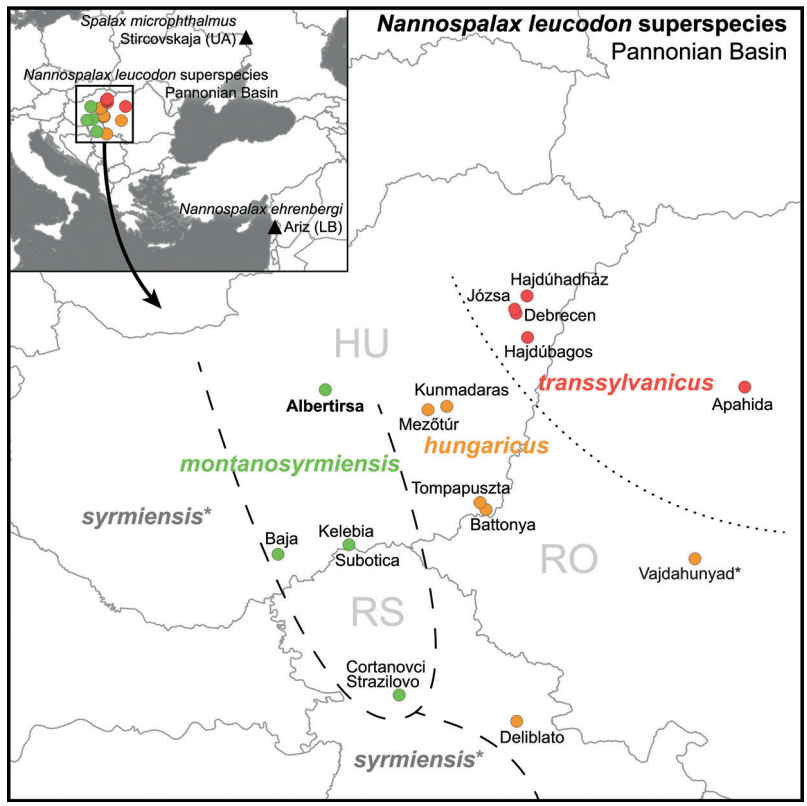

Fig. 1. Approximate distribution and location of the studied populations of blind mole-rats in the Carpathian Basin. Main rivers are shown in blue. Triangles in the upper left inset indicate locations of samples included as outgroups. * extinct taxon/population.

our *CYTB* data set (GenBank accession numbers JX451833–JX451848) to place our results into a comprehensive phylogenetic context.

In our first analysis, we selected eight populations to represent each taxon of the Pannonian Basin (Fig. 1) each of which included two individuals (Table 1), except for the outgroup taxa and *N. (leucodon) transsylvanicus*, for which only single individuals were included in the nuclear (*LCAT* and *IRBP*) data set. To investigate genetic distances between the samples, neighbour-networks (Bryant & Moulton 2004) were reconstructed separately for each locus using the Kimura-2-parameter genetic distance matrix as implemented in SplitsTree ver. 4.14.4. (Huson & Bryant 2006). Branch support values were assessed by applying 1000 bootstrap replications.

A phylogenetic reconstruction was based on *CYTB* sequences using both maximum likelihood and Bayesian approaches. Maximum likelihood (ML) phylogenetic tree reconstruction was performed using PhyML ver. 3.0. (Guindon *et al*. 2010) with smart model selection (SMS) turned on (Lefort *et al*. 2017). As outgroups, we sequenced *Spalax microphtalmus* and *Nannospalax ehrenbergi* (Table 1), with the inclusion of *CYTB* sequence of *N. (ehrenbergi) carmeli* (GenBank: NC_020756). To assess branch support values, an approximate likelihood ratio test (aLRT) was applied in the ML search. Bayesian phylogenetic reconstruction was performed using MrBayes ver. 3.2.7 (Ronquist *et al*. 2012), where the GTR + G + I nucleotide substitution model was applied, which was found by SMS as the best model of sequence evolution for this dataset. This analysis was run for 25 million generations sampling every 10 000th step after which convergence and effective sample sizes were checked using Tracer ver. 1.6 (Rambaut *et al*. 2014).

Results

The primers for the *CYTB* region yielded 1604 bp long sequences that include the entire *CYTB* gene from position 14 151 to 15 290 bp

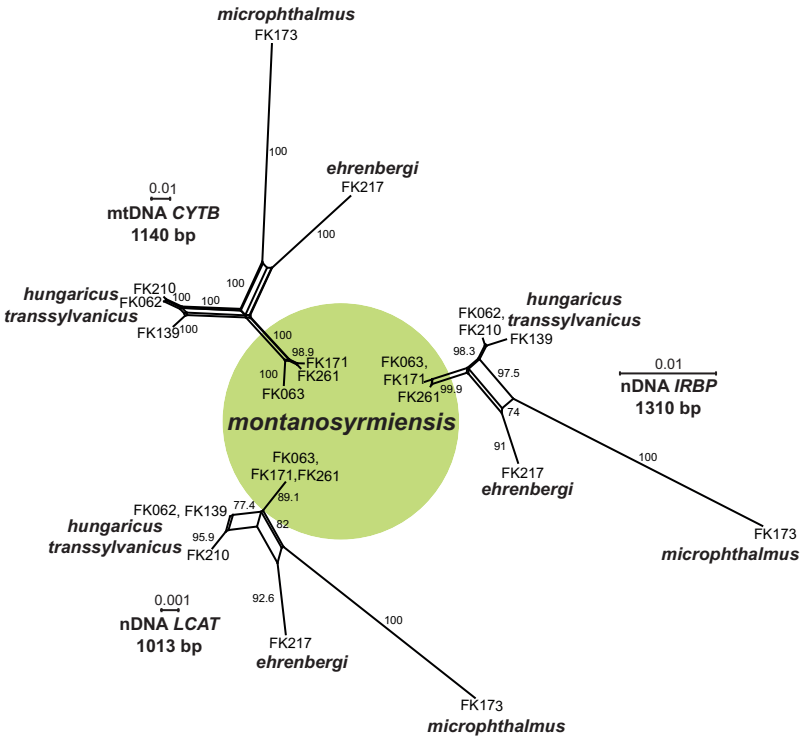

Fig. 2. Neighbour-joining networks of one mtDNA (*CYTB*) and two nDNA (*LCAT* and *IRBP*) genes of blind mole-rats in the Pannonian Basin and outgroups (*N. ehrenbergi*, *S. microphthalmus*).

in AJ416891 and the 5′ part of the control region from position 15 291 to 15 749 in AJ416891. In the current analyses, only the 1140-bp-long *CYTB* region was used. The new primers for the amplification and sequencing of the first fragment of the *LCAT* region yielded 630-bp-long sequences that included (compared with the reference sequence of AH005252) the partial first exon (incomplete at 5′ end), three complete introns and two complete exon regions, plus a near complete fourth exon (incomplete at 3′ end). The second fragment of the *LCAT* region produced 370-bp-long reads that covered the fifth exon (incomplete at both ends) of the gene. For the downstream analysis of this gene region, we concatenated the above two non-overlapping fragments which contained no double-peaks (i.e., sign of heterozygote state) in the electropherograms. The new primers for the amplification and sequencing of the *IRBP* gene produced 1305 bp long sequences that covered a large proportion of the 3′ part of the *IRBP* gene (as compared with the XM_008834127 sequence as reference) but it was incomplete at both ends. Again, our sample set did not contain signs of the presence of different alleles (i.e., double-peaks in the electropherograms) in this diploid marker.

Neighbor networks of *CYTB*, *LCAT* and *IRBP* placed the samples from the newly discovered population of Albertirsa in the monophyletic group of *N.* (*leucodon*) *montanosyrmiensis* with high statistical support (Fig. 2). Based on our results it is unlikely that the Albertirsa population is a hybrid between *N.* (*leucodon*) *montanosyrmiensis* and *N.* (*leucodon*) *hungaricus*. The latter one, however, based on cranial and dental traits was previously reported from some nearby localities by Méhely (1909). The phylogenetic analyses of the mitochondrial *CYTB* gene sequences also placed the two individuals from the Albertirsa population within the well-supported (ML aLRT: 0.99; Bayesian inference posterior probability (PP): 1.0) lineage of *N. (leucodon) montanosyrmiensis* (Fig. 3). The assignment of this lineage to this taxon is confirmed by the inclusion of two individuals (FK099 and FK106) from the type locality of *N.* (*leucodon*) *montanosyrmiensis*.

The phylogenetic tree reconstruction based on the ML and Bayesian inference (BI) criteria

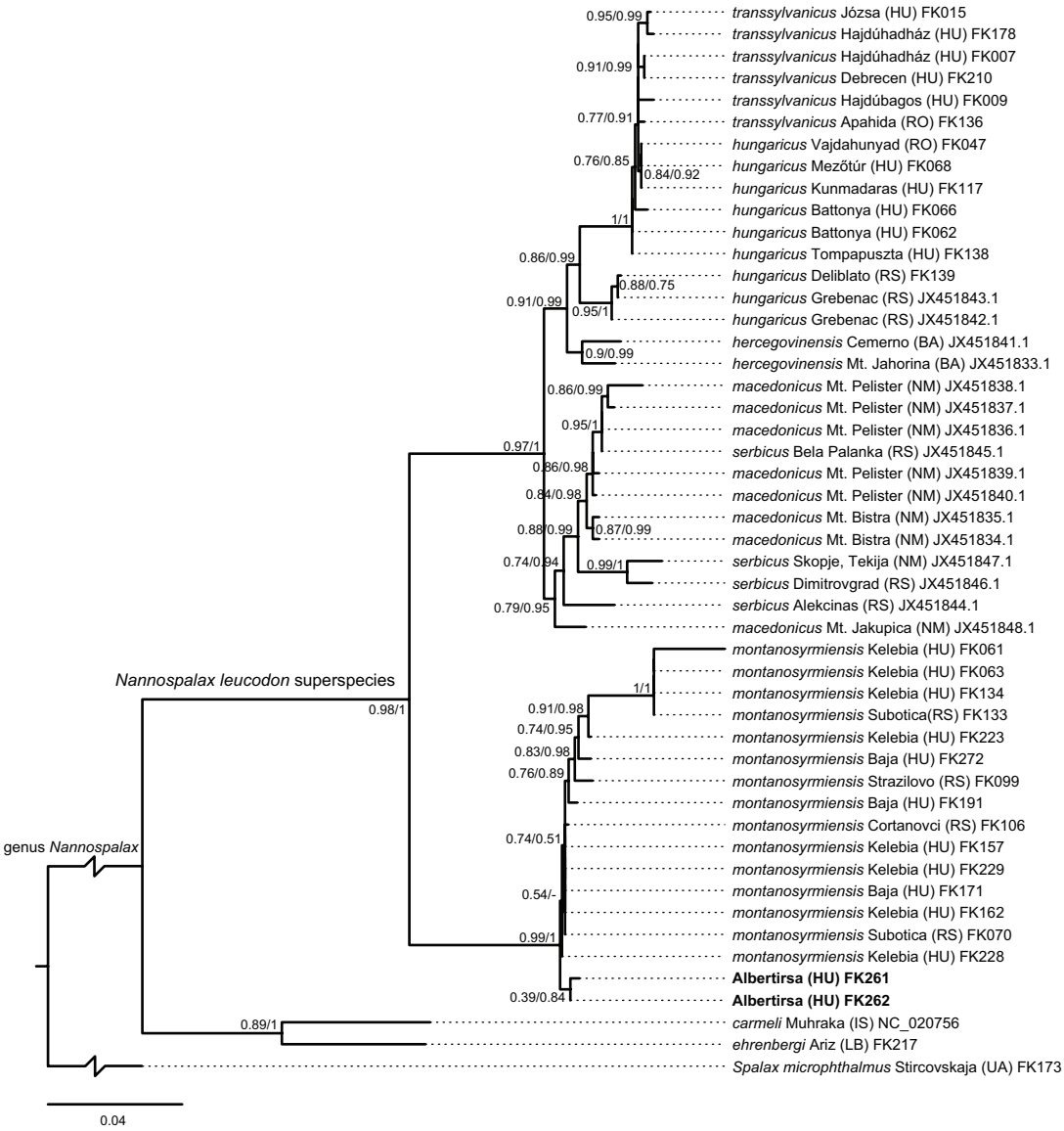

Fig. 3. Phylogenetic relationships of European *Nannospalax* species based on all currently available whole *CYTB* region sequences. The tree is a phylogram coming from a maximum likelihood tree reconstruction in PhyML-SMS. A phylogenetic tree with similar topology was recovered using the Bayesian inference. Support values (at the nodes) based on the approximate likelihood ratio test in PhyML followed by Bayesian posterior probability values assessed by MrBayes after a slash.

identified the best phylogenetic trees with the same topology. Surprisingly, the phylogenetic reconstructions showed a well-supported (ML aLRT: 0.98; BI PP: 1.0) split between *Nannospalax* from the Pannonian Basin and the Balkan Peninsula, where *N.* (*leucodon*) *montanosyrmiensis* forms a sister lineage to all other studied blind mole rats (Fig. 3). The proper systematic evaluation of this unexpectedly deep (i.e., genetically distant) split would only be possible with the inclusion of topotypic material representing other European chromosomal forms and other lineages of the genus regarded as superspecies (i.e., Anatolian *N. xanthodon*). However, the branch leading to *N.* (*leucodon*) *montanosyrmiensis* is comparable in length to the one leading

to all other remaining sampled taxa and also to the branch leading to the two outgroup samples.

It is also worth noting the phylogenetic position of *Nannospalax (leucodon) hungaricus* and *N. (leucodon) transsylvanicus* samples in our network (Fig. 2) and on our phylogenetic tree (Fig. 3). The sample of *N. (leucodon) hungaricus* from Serbia (FK139) shows divergence from the sample of the supposedly same species from Hungary (FK062). In turn, this latter sample shares the same DNA sequences in the nuclear region *IRBP* with the sample of *N. (leucodon) transsylvanicus* (FK210) (Fig. 2). This structure is even more clearly expressed on the phylogenetic tree more densely sampled for mitochondrial *CYTB* (Fig. 3) where the Serbian samples form a monophyletic group diverged from the sister-lineage composed of samples of Hungarian samples of *N. (leucodon) hungaricus* and Hungarian and Romanian *N. (leucodon) transsylvanicus*. Such a relatively deep split, which is comparable to splits between geographically distinct group of taxa within the genus, hints at taxonomic instability of the taxa involved, and their taxonomic position warrants further, more detailed research.

Discussion

The identification of the Albertirsa population as *N*. (*leucodon*) *montanosyrmiensis* helps to understand the distributional patterns of the taxon, which is currently data deficient and worryingly threatened by extinction (Csorba *et al*. 2015). The first population discovered was found in the southernmost part of its area, as currently understood, at the northeastern slopes of the Fruška gora (Stražilovo and Čortanovci) (Fig. 1). Further to the south and the west, it is replaced by another chromosomal form of the *N. leucodon* superspecies, namely the possibly extinct *N*. (*leucodon*) *syrmiensis* (Savić & Soldatović 1984). Until recently, only two additional populations of *N*. (*leucodon*) *montanosyrmiensis* were known from southern Hungary and northern Serbia. With the discovery of the fourth population, the known distribution of the taxon has expanded considerably northwards and now includes the northern part of the Kiskunság Sand Ridge, an important forest-steppe area of Hungary (Erdős *et al*. 2014, Bátori *et al*. 2018). The present finding raises the possibility that the taxon was once widely distributed in the large territory between the Danube and Tisza rivers in Hungary and Serbia but is nowadays restricted to isolated patches of remnant steppes. The historical presence of *N*. (*leucodon*) *hungaricus* in regions further north suggests that Albertirsa might define the northern limit of the Vojvodina blind mole rat's distribution.

Species of Spalacinae can hardly cross large rivers, therefore, such a barrier can easily trigger allopatric divergence (Pyron & Burbrink 2010); moreover, all taxa with unique chromosomal arrangement investigated so far are known to be reproductively isolated from the neighbouring taxa (Savić & Soldatović 1984, Savić *et al*. 2017). Geographic barriers, especially large rivers are known to be effective barriers for animal species with a reduced mobility and can have an isolating effect on populations (e.g., Trizio *et al*. 2005, Kennis *et al*. 2011) and species (e.g. Tóth *et al*. 2019). For example, the effective isolation of European ground-squirrel (*Spermophilus citellus*) populations was observed by Ćosić *et al*. (2013) in the southern part of the Danube–Tisza Interfluve. This model of allopatric speciation should be further studied in European blind mole rats of the genus *Nannospalax* as was undertaken in Israeli species of *Nannospalax* (Nevo 1982, Nevo *et al*. 2001). In *Spalax* species, it is known that the Carpathians (Németh *et al*. 2013b) and large rivers like Dnieper (Topachevskii 1969, Zagorodniuk *et al*. 2018) play important role in the speciation processes. The long-awaited taxonomic revision of *N. leucodon* superspecies could serve as a firm base for evolutionary mechanisms to be revealed, which resulted in the extreme karyotypic variability of this group of European rodents.

The combination of the novel markers reported here can effectively distinguish between morphologically indistinguishable cryptic species of the *Nannospalax leucodon* superspecies (Fig. 2) and holds promise to be used on a larger scale to identify species-level taxonomic units within this group of rodents. Notably, if phylogenetic analyses of these markers can be combined with species-delimitation methods (*see* Fišer *et*

al. 2018), one can then begin to objectively assess species boundaries in this group with several supposed cryptic species. Our case study can serve as a first step towards the understanding of the diversity of European lesser blind mole rats.

Acknowledgements

We thank the Hungarian and Serbian nature conservation authorities, the Department of Environmental Protection and Nature Conservation of the Government Office for Pest County, Kiskunság and Duna-Ipoly National Park Directorates and the Institute for Nature Conservation of Vojvodina Province for providing permits (14/1840-3/2008, 14/01439-9/2012, PE-KTF/5215-22/2017) and for their various supports during our field work. We are very grateful to David Michael Scantlebury for his comments and linguistic corrections. Part of the work reported in this study is linked to and was developed within the frame of the LIFE IP project supported by the grant LIFE 17 IPE/HU/000018.

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