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REDISCOVERY OF THE BLIND MOLE RAT IN CROATIA WITH REVISION OF THE PHYLOGENETIC ANALYSIS OF THE *NANNOSPALAX MONTICOLA* COMPLEX IN EUROPE

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After being presumed extinct, the blind mole rat was rediscovered in Vučedol, Croatia. Phylogenetic analyses using mitochondrial markers (*cytochrome b* and *16S rRNA*) were conducted to classify the population. The results confirmed a distinct separation between the Lesser mole rats (*Nannospalax leucodon* clade) and Western mole rats (*N. monticola* clade). Within the *N. monticola* complex, six lineages were identified, which likely diverged during the Middle to Late Pleistocene. The Vučedol sample belongs to the *montanosyrmiensis* cytotype subclade, which was also found in Fruška Gora, Serbia. This subclade may represent a new species, showing K2P genetic distances of 4.3% to 5.7% from the "Pannonian Plain" subclade located on both banks of the Danube River. In two Pannonian localities, secondary contacts between populations of both subclades have been documented. One of them is the Kelebia – Subotička peščara population, where a unique subclade "Kelebia" was identified. Populations with *montanoserbicus* cytotype cluster within two distinct subclades, *montanoserbicus* A and *montanoserbicus* B, with K2P distances ranging from 4.6% to 5.3%, indicating potential species status for *montanoserbicus* A. In the most parsimonious network, *montanoserbicus* B exhibits a close relationship with the Pannonian Plain sublineage and a single sample from Bosnia and Herzegovina, showing K2P distances of 1.9% to 3.2%, suggesting that they may belong to the same species, *N. monticola*. Significant divergence between populations in Bosnia and Herzegovina highlights the need for further exploration of local variability and taxonomic status. Detailed analyses with additional markers from more localities are necessary before final species delimitation and taxonomic revision can occur. For now, *Nannospalax monticola* (Nehring, 1898) remains the sole valid name in the *N. monticola* complex.

Keywords: Spalacinae, molecular phylogeny, species delimitation, Pannonian area, Dinaric and Balkan mountains

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Nakon što se pretpostavljalo da je izumro u Hrvatskoj, sljepaš je ponovno pronađen u Vučedolu. Filogenetičkom analizom pomoću mitohondrijskih markera (*citokrom b* i *16S rRNA*) provedena je klasifikacija populacije. Rezultati su potvrdili jasnu odijeljenost između dviju grupa, one vrste *Nannospalax leucodon* i zapadne grupe *N. monticola*. Unutar *N. monticola* grupe identificirano je šest genetičkih linija koje su se razdvojile vjerojatno tijekom razdoblja srednjeg i gornjeg pleistocena. Uzorak iz Vučedola pripada liniji poznatoj iz Fruške Gore, odakle je opisan citotip "*montanosyrmiensis*". Ta podgrupa mogla bi predstavljati novu vrstu, na što ukazuju K2P genetske udaljenosti (4,3% do 5,7 %) prema liniji iz Panonske nizine nađenoj na obje obale Dunava. Na dva panonska nalazišta utvrđen je sekundarni kontakt između populacija obje linije. Jedno od njih je Kelebia – Subotička peščara gdje je identificirana i jedinstvena Kelebia linija. Populacije "*montanoserbicus*" citotipa razdvojene su u dvije različite genetske linije, *montanoserbicus* A i *montanoserbicus* B, a razlike u K2P genetskim udaljenostima od 4,6% do 5,3% ukazuju na potencijalni status *montanoserbicus* A linije kao zasebne vrste. U filogenetskoj mreži konstruiranoj metodom statističke parsimonije, *montanoserbicus* B pokazuje usku srodnost s linijom iz Panonske nizine i jednim uzorkom iz Bosne i Hercegovine, a njihove međusobne K2P genetske udaljenosti od 1,9% do 3,2% sugeriraju da svi ovi uzorci pripadaju istoj vrsti, *N. monticola*. Značajne genetske razlike između populacija iz Bosne i Hercegovine potcrtavaju potrebu za budućim istraživanjem lokalne varijabilnosti i taksonomskog statusa sljepaša. Neophodna je detaljna analiza s dodatnim markerima na više lokaliteta prije konačne odluke o evolucijskom statusu i taksonomskoj reviziji. Za sada je *Nannospalax monticola* (Nehring, 1898) jedino validno ime u *N. monticola* grupi.

Ključne riječi: Spalacinae, molekularna filogenija, razgraničavanje vrsta, panonsko područje, Dinaridi i Balkanske planine

INTRODUCTION

Blind mole rats (Spalacinae) represent a very old fossorial herbivore rodent group living in Eurasia and the eastern parts of Northern Africa (Muser & Carleton, 2005). They have adapted to life in the deep soil of steppe or forest-steppe habitats that are rich in underground parts of perenial plants such as bulbs, tubers and rhizomes (Savić, 1982). The early occurrence of spalacids was initially estimated on the basis of fossil remains to the Late Oligocene (Topachevskii, 1969). In Eastern Europe, spalacid fossils of *Vetusspalax progressus* are described in Banovići coal mine near Tuzla, Bosnia and Herzegowina (de Bruijn *et al.*, 2013), and *Pannoniamys paragovensis* in Paragovo mine, Fruška gora, Serbia (van de Weerd *et al.*, 2021), both dated arround 24 Ma. The major radiation began in the Late Miocene and Pliocene, with most of the recent species in genus *Nannospalax* Palmer, 1903. It is distributed in East Europe, Asia Minor, the Middle East and Northern Africa, resulting from Pleistocene events (Hadid *et al.*, 2012; Bugarski *et al.*, 2022). Due to their convergent morphology, it is difficult to delimit cryptic mole rat species (Kryštufek & Vohralik, 2009), but *Nannospalax* populations exhibit a variety of different cytotypes, as reviewed in Arslan *et al.* (2016). Some studies (e.g. Savić & Soldatović, 1984) have considered different cytotypes as separated species, but the simple generalization of a 'cytotype-equals-species' concept was rejected, and names never published according to ICZN rules were not accepted (Muser & Carleton, 2005; Kryštufek *et al*., 2012; Arslan *et al*., 2016), making them *nomina nuda.* However, karyology combined with a molecular approach can be a potential tool for delimiting morphologically cryptic species in diverse animal groups. For instance, recent complex genetic investigations in the Middle East confirmed chromosomal speciation in mole rats (Li *et al.*, 2020). Meanwhile, in Europe, we are just starting to unravel the puzzle of the proposed chromosomal taxa of *Nannospalax leucodon*.

In The Atlas of European Mammals (Mitchell-Jones *et al.* /eds./1999), only one *Nannospalax* species was listed for Eastern Europe: the Lesser mole rat *N. leucodon* (Nordmann, 1840). However, a taxonomic revision of the Eastern European populations is required (Kryštufek, 1999). Phylogenetic analysis based on five mitochondrial (mt) genes (3742 bp in total) revealed a basal branching of *montanosyrmiensis* within *N. leucodon* clade (Hadid *et al.,* 2012). Additionally, based on mt *cytochrome b* sequence, Németh *et al.* (2013) recommended that one sample of the newly investigated mole rat population from the dislocated Kelebia-Subotička peščara in the Pannonian plain (border region of Hungary and Serbia) belongs to the chromosomal race "montanosyrmiensis", originally described by Savić & Soldatović (1971) from Stražilovo and Čortanovci (Fruška gora, Serbia). Furthermore, it has been shown that the European *Nannospalax leucodon* superspecies, a complex of species and subspecies (ICZN 1999: Article 6.2), consists of two main clades. One is the *N. (leucodon) montanosyrmiensis* clade from Kelebia and Fruška gora Mountain (Hungary and Serbia) and the other is the *N. leucodon sensu stricto* clade, which includes *N. (l.) leucodon*, *N. (l.) hungaricus* (Nehring, 1898) and *N. (l.) transsylvanicus* (Méhely, 1909). These two deeply separated clades were also revealed in a larger sample by Németh *et al.* (2020) and Bugarski-Stanojević *et al.* (2022), using complete and partial *cytb* sequences, respectively. Aditionally, phylogenetic analyses conducted on mitochondrial *16s rRNA* gene sequences by Bugarski-Stanojević *et al.* (2020, 2022) corroborated their existence, but the authors designated both as a part of the *N. leucodon* aggregate clade. Furthermore, it was shown that the *montanosyrmiensis* clade (Fruška gora; Serbia) harboured a distinct subclade comprising populations of the *montanoserbicus* cytotype (Mačvanski Pričinović, Zlatibor, Jadovnik and Vlasina; Serbia). Németh *et al.* (2020) added new samples from more northern localities in Hungary between the Danube and Tisza rivers (Baja and Albertirsa). They observed unusually high *cytb* sequence diversity within the Hungarian tentative *montanosyrmiensis* clade, but did not provide any comments on this matter. Genetic analysis of two nuclear regions in the same study revealed either no differences or only a single base difference within this clade. Bugarski-Stanojević *et al.* (2024) in study about refinding the former taxon *Spalax monticola syrmiensis* Mehely, 1909 added new localities in Serbia for *N. l. montanosyrmiensis* (Krušedol) and *N. l. montanoserbicus* (Ševarice). Finally Németh *et al.* (2024) analyzed 16 samples of the "*N. monticola* superspecies" in addition to a larger sample of the "*leucodon* superspecies" which encompassed samples collected at previously unanalyzed localities: Čajetina in Serbia, Čakor in Montenegro, Çerem in Albania, Tomislavgrad in Bosnia and Hercegovina. Based on results obtained from a reduced sample that included both mitochondrial and nuclear markers, the authors recommended recognizing three species within this group, corresponding to chromosome form names as described in Savić & Soldatović (1984): *N. (monticola) montanoserbicus*, *N. (monticola) montanosyrmiensis*, and *N. (monticola) monticola*.

Our present study was initiated by the unexpected rediscovery of the blind mole rat in Croatia after more than 120 years (Korlević, 1903: *Spalax typhlus*, Vukovar, 14.7.1899, leg. Eugen Kamenar). In June 2023, Anđelko Ištuk in Vučedol, Vukovar City (Croatia), took a photo of one animal. Previous publications in Croatia reported a local blind mole rat as *S. leucodon* from another part of Srijem (Vukovarsko-Srijemska County). The findings from Andrijaševci, Nuštar, Tordinci and Antin (Savić, 1967) lacked documentation, and the record for the Zagreb locality (Hopkins & Rotschild, 1966) was determined to be an error on the label (Brelih & Trilar, 2004). In the Red Book of Mammals of Croatia (Tvrtković, 2006), under the name *Nannospalax leucodon*, it is noted that the mole rat in

Croatia is probably a Regionally Extinct species (RE?). We anticipated a close affinity between the Vučedol sample and the nearest known, 40 km distant population on Fruška gora (Serbia) since both are on the western bank of the Danube River. Another interesting population is located about 200 km southwest in Bosnia and Herzegovina, *N. (l.) monticola* (Nehring 1898), which exhibits a distinct karyotype (Soldatović, 1971). However, this population shares many similarities in chromosomal structure with the *montanosyriensis* karyotype (Savić & Soldatović, 1984: p.90). As the first step in the study, we compared the *16S rRNA* and *cytb* sequences of our samples from Vukovar and Tomislavgrad with available GeneBank records, followed by phylogenetic analyses.

MATERIAL AND METHODS

Material examined and DNA extraction

With permit KLASA: UP/I-352-04/23-08/151, URBROJ: 517-10-1-2-23-2 from the Department of Nature Conservation the Ministry of Economy and Sustainable Development of the Republic of Croatia, we revisited Vučedol, Vukovar (Fig. 1) several times. This locality likely hosts the last population of mole rats in Croatia. However, given

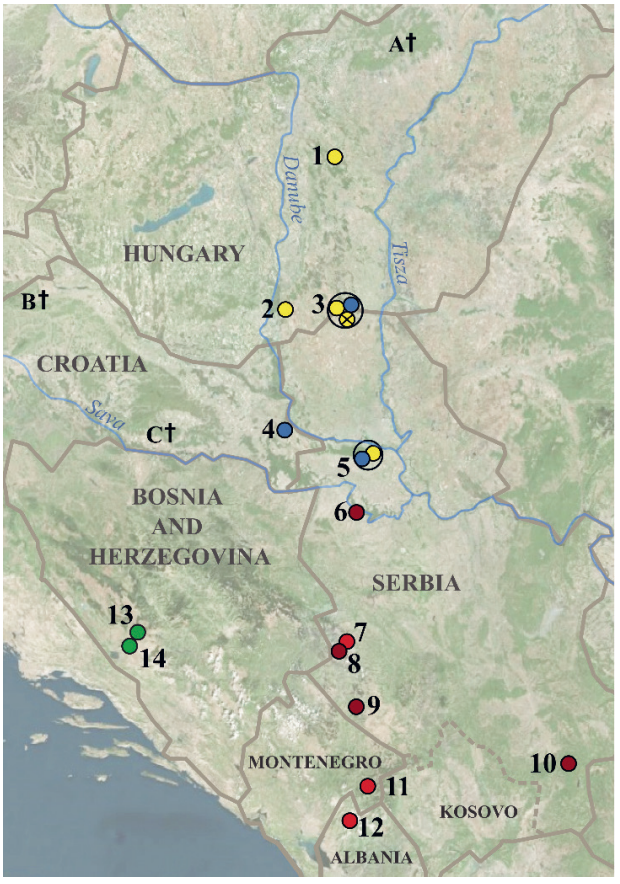

Fig. 1. Distribution of the analysed lineages of *Nannospalax monticola* (Nehring, 1898) complex. 1. Albertirsa; 2. Baja; 3. Kelebia - Subotička peščara; 4. Vučedol; 5. Fruška gora (Stražilovo, Čortanovci, Sremski Karlovci, Krušedol); 6. Mačva (Mačvanski Pričinović, Ševarice); 7. Čajetina; 8. Mt. Zlatibor; 9. Mt. Jadovnik; 10. Mt. Vlasina; 11.Mt. Čakor; 12. Çerem, Mts. Bjeshkët e Nemuna; 13. Ravanjsko polje; 14. Tomislavgrad. Coloured circles represent different lineages: ● = „Pannonian Plain" lineage; ⊗ =„Kelebia" lineage; ● = *montanosyrmiensis* lineage; ● = *montanoserbicus* A linege; ● = *montanoserbicus* B lineage; ● = *monticola* A and B lineage. Late Pleistocene *Nannospalax* remains sites: A† = Mts Bükk (Janossy, 1986); B† = Vindija cave (Smith *et al.*, 2024), C† = Kamenika semicave (Malez *et al.*, 1977).

the absence of typical mole-rat hills, we were fortunate that a domestic cat preyed upon one specimen (Fig. 2) in the garden of Anđelko Ištuk providing us with the opportunity to preserve the first tissue sample (Tab. 1). We also obtained two samples near Tomislavgrad in Bosnia and Hercegovina, from the gardens of local farmers. The first sample was from the northern border of the city, and the second was from southern border, towards the large karst Duvanjsko polje, both located in the highest Mediterranen submontane belt. Tissue samples were taken from fresh cadavers by Vinko Ljubas.

Total genomic DNA was extracted from approximately 1 mm^3 of ethanol-preserved muscle tissue of those three animals (Tab. 1) using the NucleoSpin Tissue kit (Macherey-Nagel), following the manufacturer's instructions and eluted in 100 µl of elution buffer.

PCR Amplification and Sequencing

Two mitochondrial markers, the *cytochrome b* (*cytb*) and the *16S rRNA* (*16S*) gene, were employed.

For all samples, the entire *cytb* gene was amplified using L14727 (Nikaido *et al.*, 2000) and H15915mod (5′ TCATTTCTGGTTTACAAGAC 3′, modified after Ducroz *et al.* (2001) primers, while the fragment of *16S* was amplified using the universal primers pair 16Sar (LR-N-13398) / 16Sbr (LR-J-12887) (Simon *et al.*, 1994). For both markers, the

Fig. 2. Blind mole rat from Vučedol, Vukovar (Croatia), photo Anđelko Ištuk.

Tab. 1. New sampling localites and sequence codes of the Western blind mole rat complex

Taxon	Locality, altitude a.s.l., Coordinates	Date sex	Sample code	16S rRNA	Cyt b	Cyt b numt
Nannospalax (*monticola*) *monticola*	Tomislavgrad, BiH, 910 m 43°42'5,29,3"N; 17°13'11,8"E	V.2018, juv. f	NMONT1	PP889583	PP896878	
Nannospalax (*monticola*) *monticola*	Tomislavgrad, BiH; 880 m 43°43'7,4748"N;17°13'25,1364"E	16.X.2022, subad. f	NMONT2	PP889582	PP896879	
Nannospalax (*monticola*) sp. candidate	Vučedol, Vukovar, HR; 100 m 45°19'58,75"N; 19°2'48,06"E	21.VIi.2023 subad. m	NMONT3	PP889584	PP896877	PP896880

PCR mix (20 μl) included 0.625 U of DreamTaq DNA Polymerase (Thermo Fisher Scientific), 1× DreamTaq Green Buffer, 200 μM of each dNTP, 0.4 μM of each primer, and 1 μl of DNA eluate. The cycling parameters were as follows: an initial denaturation at 95°C for 2 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for either 60 or 90 seconds for the amplification of *16S* or *cytb*, respectively. The final extension step was performed at 72°C for 7 minutes.

Additionally, due to the substantial divergence (uncorrected p-distances of up to 4.3% based on the complete gene sequence), revealed in *cytb* sequences of *Nannospalax (leucodon) montanosyrmiensis* from the same Hungarian locality (Kelebia FK157, FK162, FK228, FK229, vs Kelebia FK061) in the study by Németh *et al.* (2020), we explored the possibility that one variant might represent a nuclear mitochondrial pseudogene (*numt*). To investigate this, based on published sequences, we designed two internal (forward) PCR primers specific for each variant. The forward primers were NannoCYTFK061_L (5′ GCTACAAATCATCACAGGAC 3′) and NannoCYTFK070_L (5′ ACTACAAATTATCACCGGGT 3′), while the reverse primer, NannoCYT1060_H (5′ TGAGAAATATAGGATTGAGGC 3′), was 100% specific for both variants.

PCR amplification of the *cytb* fragment was attempted using both combinations of the aforementioned primer pairs across a range of dilution (Calvignac *et al.*, 2011) of the original NMONT3 genomic DNA extract (10x, 100x, 1000x and 10000x dilution). The reagents and cycling conditions matched those described for the amplification of the entire *cytb* gene, and 1 μl of different dilutions served as PCR template. The sequencing was performed by Macrogen Europe (Amsterdam, The Netherlands). For the sequencing of *16S* fragments, amplification primers were used, while *cytb* sequencing was carried out using amplification primer H15915mod in addition to internal primers L-SisCyt497 (Pavlinić *et al.*, 2012) and nanseqH (5′ TGTAGAAATAGAAGGTGAAC 3′). The sequencing of PCR products obtained with *cytb* variant-specific primers was carried out using the reverse amplification primer (NannoCYT1060_H). GenBank accession numbers are listed in Tab. 1.

Sequence analysis and alignment

The sequences were assembled and visually inspected using BioEdit v. 7.2.5 Sequence Alignment Editor (HALL, 1999) and evaluated using the Basic Local Alignment Search Tool (BLAST, Altschul *et al.*, 1990).

Datasets and sequence alignments

All available *Nannospalax cytb* and *16S* sequences were retrieved from GenBank.

Cytb sequences were organized into three datasets. Along with sequences obtained in this study, dataset CYTB1 contained the representatives of all *Nannospalax* subclades as revealed by Németh *et al.* 2024. Additionally, we included several previously published *N. montanosyrmiensis* sequences (GenBank accession numbers: JN656386, JN656389 and JN656390 – Németh *et al.*, 2013 and MN497975 (FK061) – Németh *et al.*, 2020) that were omitted from phylogenetic analyses of Németh *et al.*, 2024. We also included two longer *N. montanoserbicus* sequences (800 bp) from the study by Bugarski-Stanojević *et al.*, 2020. *Spalax antiquus* and *N. xanthodon* sequences were used as outgroups.

CYTB2 dataset comprised all available *Nannospalax montanoserbicus*, *N. montanosyrmiensis* and *N. monticola* sequences, each at least 423 bp in length, except the *N. monticola* (Šuica, OM714869) and *N. montanoserbicus* (Zlatibor, OM714866) sequences, which exhibited peculiar phylogenetic placement in the original study (Bugarski-Stanojević *et al.*, 2020) and were therefore excluded. The sampling localities are shown in Fig. 1.

The CYTB3 dataset, designed for genetic distance calculation, consisted solely of sequences from the CYTB1 dataset that were at least 800 base pairs (bp) in length. These sequences were then trimmed to an equal length of 800 bp to avoid potential differences in the evolutionary rate of individual gene segments.

The *16S* dataset included the *16S* sequences obtained here and the representative haplotypes of all subclades within the *"Nannospalax leucodon"* clade from the study by Bugarski-Stanojević *et al.* (2020).

Sequence alignments were conducted online using multiple sequence alignment software MAFFT version 7 (Katoh & Standley, 2013). The "auto" option was employed for *cytb* sequence alignments, while the FFT-NS-i method was utilized for aligning *16S* sequences.

Phylogenetic analysis

A neighbor-joining analysis of *cytochrome b* sequences (CYTB1 dataset plus three additional *N. montanosyrmiensis* sequences: OM714893, OM714894 and OMZ14865) was performed in MEGA version XI (Tamura *et al.*, 2021) using the p-distance method and pairwise deletion option for the treatment of ambiguous sites. Branch support was obtained through bootstrapping (2000 replicates).

Bayesian phylogenetic analyses were performed on 16S and CYTB1 datasets using MrBayes (v.3.2.7a, Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) at CIPRES Science Gateway (Miller *et al.*, 2010), employing the best-fit model of sequence evolution as selected by jModelTest 2.1.7 (Darriba *et al.*, 2012) under the Bayesian information criterion (BIC). Two independent parallel runs, each comprising four Monte Carlo Markov chains (MCMCs), were executed for 10,000,000 generations, with sampling conducted every 1000th generation. Tracer v1.7.1 (Rambaut *et al.*, 2018) was utilized to assess the convergence of two runs and determine the effective sample size (ESS) values. The initial 25% of sampled trees was discarded (burn-in), and a 50% majority-rule consensus tree was generated, with nodal values representing the posterior probabilities.

Since phylogenetic networks are more suitable for the analysis of intraspecific datasets (Posada & Crandall, 2001), a statistical parsimony haplotype network based on CYTB2 dataset was constructed under a 95% parsimony connection limit using TCS v. 1.21 software (Clement *et al.*, 2000).

Distance analysis

The uncorrected pairwise distances (p-distances) as well as Kimura 2-parameter (K2P; Kimura, 1980) pairwise distances were calculated for CYTB3 and 16S dataset by using MEGA version XI (Tamura *et al.*, 2021), with missing data treated using the complete deletion option.

RESULTS

SEQUENCE ANALYSIS

The available *Nannospalax* sequences omitted from phylogenetic analyses

A thorough alignment inspection revealed that the *N. montanoserbicus* (OM714866) sequence is chimeric. The first 303 bp (5′ end) correspond to *N. montanoserbicus*, exhibiting 100% sequence identity with OM714884, OM714895 and OM714867 *montanoserbicus* sequences, while showing only 95% identity with the most similar available *N. montanosyrmiensis* sequences (MN497991 and MN497986). The last 497 bp (3′ end) matches *N. montanosyrmiensis* displaying 100% sequence identity with JN656389 *montanosyrmiensis* sequence while showing only 93–95% sequence identity with available *N. montanoserbicus* sequences. As the here excluded *N. monticola* (Šuica, OM714869) sequence is concerned, with 99.75 % sequence identity (over 800 bp) to sequences JX451848, OR751022 and OR751021 (Kryštufek *et al.*, 2012, Németh *et al.*, 2024), a BLAST search matched it to *N. serbicus thermaicus* (*makedonicus*).

Cytb pseudogene

Using NannoCYTFK061_L primer the *cytb* fragment was successfully amplified via PCR amplification across the whole range of dilution of the original NMONT3 genomic DNA extract (10x, 100x, 1000x and 10000x dilution). In the overlapping region (838 bp), the sequence obtained was identical to sequences obtained with L14727 and H15915mod amplification primers. On the other hand, when NannoCYTFK070_L was used as forward amplification primer, the PCR amplification was successful only for the 10X and 100X template dilutions. This suggests that the amplified sequence is likely a nuclear mitochondrial sequence (*numt*), as nuclear sequences are present in the sample in much lower quantities than in mitochondrial samples. However, the sequence analysis revealed no premature STOP codons typical for *numt*s. Also, it did not match another Kelebia variant as was originally assumed: the uncorrected p-distance values were in the range 5.4–5.5%. The p-distance between *numt* and FK061 Kelebia variant was 6.5%, and between *numt* and mitochondrial sequence obtained from the same sample, NMONT3, 7.3%.

PHYLOGENETIC ANALYSES AND GENETIC DISTANCES

The alignments of datasets CYTB1, CYTB2, CYTB3 and 16S were 1140, 423, 800 and 559 bp long, respectively.

All phylogenetic analyses resulted in a similar overall tree topology (Fig. 3) characterized by two well-supported clades designated as *Nannospalax leucodon* complex and *N. monticola* complex. The first one is further subdivided into two well-supported subclades: *N. turcicus* and a subclade comprising all remaining *N. leucodon* complex taxa.

Four of the six distinct *cytb* lineages observed within *N. monticola* complex clade are named after known cytotypes: *montanoserbicus* A (with two sublineages A1 and A2), *montanoserbicus* B, *montanosyrmiensis*, *monticola* (with two sublineages A and B). The samples belonging to two remaining sublineages, "Pannonian Plain" and "Kelebia", originated from populations that have never been karyotyped. These lineages were incorrectly designated as *montanosyrmiensis* in the studies of Csorba *et al.* (2015), Né-

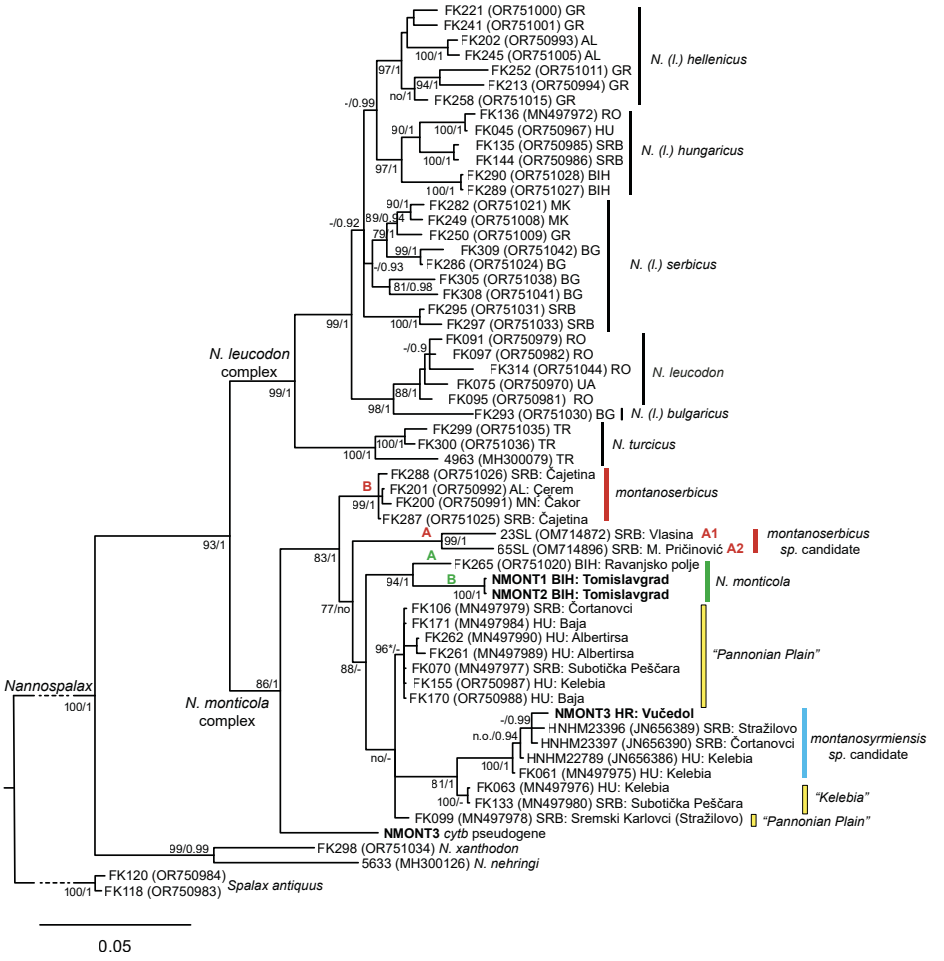

Fig. 3. Bayesian phylogenetic tree reconstructed from *cytochrome b* sequences (CYTB1 data set). Numbers at the nodes indicate Neighbor-Joining bootstrap values (BS, 2000 replicates) and Bayesian posterior probabilities (BPP). BS values lower than 70 and BPP lover than 0.9 are not shown. The label „no" at the nodes indicates „clade not obtained" and * signifies that in NJ analysis corresponding clade contained also FK099 sequence. Tentative cytotypes of different lineages are presented in the same colours as in Fig. 1, while lineages with unknown cytotypes are labeled in yellow.

METH *et al.* (2024) after NÉMETH *et al.* (2013). In Kelebia, only one sample (HNHM22789) was karyotyped, and this one coincidentally corresponded to the true montanosyrmiensis lineage.

The *N. monticola* subclade appears as monophyletic, comprising two lineages: *N. monticola* A represented by the previously published FK265 (OR751020) sequence end *N. monticola* B which includes the two sequences from this study (NMONT1 and NMONT2,). The uncorrected intraspecific p-distances between the latter one and *N. (m.) monticola* sequences from this study are high (2.8%, Tab. 2) although the samples originate from geographically adjacent regions (Fig. 1).

Tab. 2. The ranges of uncorrected pairwise genetic distances (p-distances, above values) and Kimura 2-Parameter (K2P distances, lower values) based on the 795 bp long 5′ portion of the cytochrome *b* gene.

	leucodon complex	*turcicus*	*monticola* complex
leucodon complex	0.3-6.0 0.1-6.4		
turcicus	4.5-7.5 4.7-8.1	1.8 1.8	
monticola complex	5.5-10.8 5.8-11.9	6.5-10.4 6.9-11.4	0.1-7.7 0.1-8.2

	montanoserbicus A	*montanoserbicus* B	*montanosyrmiensis*	"Kelebia"	"Pannonian Plain"	*monticola*	*leucodon*	*turcicus*
montanoserbicus A (A1 + A2)	3.0 3.1							
montanoserbicus B	4.4-5.0 4.6-5.3	0.1-0.6 0.1-0.6						
montanosyrmiensis	6.7-7.7 7.1-8.2	5.3-6.0 5.5-6.4	0.4-0.8 0.4-0.8					
"Kelebia"	5.8-5.9 6.1-6.2	4.2-4.5 4.3-4.7	2.3-3.0 2.3-3.1	0.0 0.0				
"Pannonian Plain"	4.4-5.3 4.6-5.5	1.9-2.9 1.9-3.0	4.2-5.4 4.3-5.7	2.3-2.6 2.3-2.7	0.3-1.0 0.3-1.0			
monticola A + B	5.3-7.3 5.6-7.8	3.1-5.3 3.2-5.5	4.8-5.5 5.0-5.8	4.2-5.0 4.3-5.3	2.9-5.2 3.0-5.4	2.8 2.8		
leucodon	8.2-9.6 8.8-10.4	6.7-7.4 7.1-7.9	8.6-10.3 9.2-11.3	8.1-8.8 8.6-9.5	6.8-7.8 7.1-9.5	7.9-10.3 8.5-11.3	0.3-2.0 0.3-2.1	
turcicus	8.8-9.6 9.5-10.4	6.5-6.8 6.9-7.2	9.3-10.4 10.1-11.4	8.6-8.9 9.2-9.6	6.5-7.5 6.9-8.1	8.2-9.8 8.8-10.7	5.7-6.9 6.0-7.4	1.8 1.8

Montanoserbicus sequences do not form a monophyletic group, but appear within two distinct well supported subclades, one encompassing the *montanoserbicus* sequences published by Bugarski-Stanojević *et al.* (2022) (*montanoserbicus* A), and the other (*montanoserbicus* B) containing the sequences published by Németh *et al.* (2024). The K2P distances between those two clades are 4.6–5.3% which falls within the range of interspecific distances (Tab. 2).

Bayesian analysis placed all *montanosyrmiensis sensu lato* sequences within a single, albeit unsupported subclade which can be further subdivided (Fig. 3). The majority of the former *montanosyrmiensis* sequences published by Németh *et al.* (2020 and 2024) appeared as the basal or clustered within basal, unsupported "Pannonian Plain" subclade, while the remaining sequences are encompassed within a strongly supported subclade. The latter can be further subdivided into one well-supported subclade with *montanosyrmiensis* cytotypes from Savić & Soldatović (1984) and the moderately supported "Kelebia" subclade recorded only locally in Kelebia – Subotička peščara. The *montanosyrmiensis* subclade includes the Vučedol sequence from this study, along with all *montanosyrmiensis* sequences published in 2013 by Németh *et al.*, as well as a single sequence from the Németh *et al.* (2020) study (FK061). Meanwhile, all sequences within the "Kelebia" subclade come from the Németh *et al.* (2020) study. The K2P genetic distances found between these three subclades are also high, ranging from 2.3% to 5.7%. The largest difference, 4.3% to 5.7%, accounts for genetic variations between the "Pannonian Plain" subclade and our *montanosyrmiensis* subclade (Tab. 2). Contrary to the Bayesian approach, in the neighbor-joining analysis *montanosyrmiensis sensu late*

sequences do not cluster within a monophyletic clade, but are instead resolved as three independent, monophyletic lineages placed in an unresolved tetratomy with *N. monticola* lineage (Fig. 3). The same closer phylogenetic relationship of *montanosyrmiensis* and *monticola* in respect to *montanoserbicus* was also revealed in Bayesian analysis based on *16S* sequences (Fig. 4). In this analysis, the problematic Šuica (BIH) sample, designated in Bugarski-Stanojević *et al.* (2022) as *N. l. monticola* clustered within the *N. (l.) serbicus* clade, therefore the only *16S* sequences in *N. monticola clade* are those from this study.The Vučedol 16S sequence from this study is comprised within *montanosyrmiensis,* differing by 0.2% from the remaining available *montanosyrmiensis 16S* sequences (Bugarski-Stanojević *et al.*, 2022; 2024). In the same fragment, the uncorrected p-distances between *monticola* and *montanosyrmiensis* ranged from 0.5–0.9%, while distances of both of them to *montanoserbicus* were in the range of 1.6–2%.

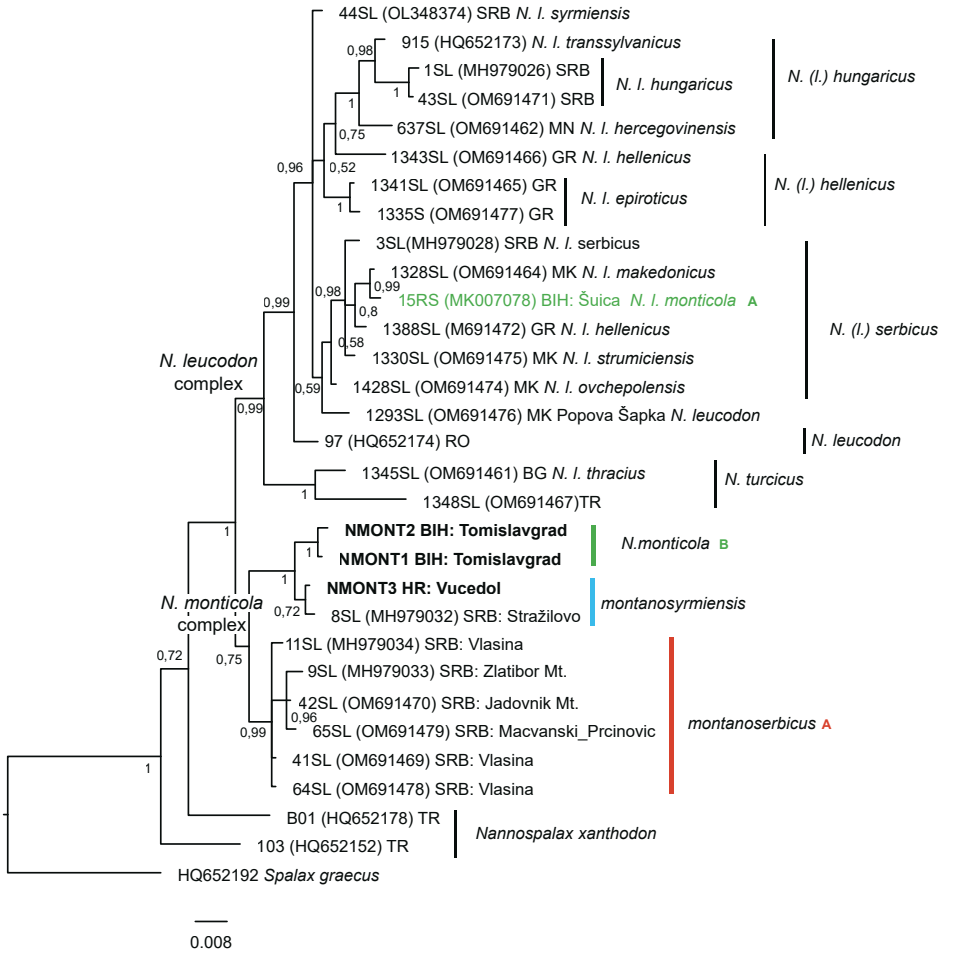

Fig. 4. Bayesian phylogram inferred from *16s rRNA* sequences. Numbers at nodes represent Bayesian posterior probabilities.

The statistical parsimony haplotype network based on CYTB2 dataset is shown in **Fig. 5**. At the 95% probability limit, haplotypes could not be connected within a single network but fell apart into 5 unconnected subnetworks. The *montanosyrmiensis* and "Kelebia" subclades, as well as the *montanoserbicus* A, correspond to the phylogenetic relationships revealed by neighbor-joining and Bayesian analysis (Fig. 3). However, the *monticola* subclade split into two disconnected haplotypes: one obtained from the two samples from this study (NMONT1, NMONT2), and the other from the sample FK265 from Németh *et al.* (2024). The latter haplotype appeared to be connected within the same network as the *montanoserbicus* B and "Pannonian Plain" sequences.

Vučedol sequence (NMONT3) clustered within *montanosyrmiensis* subclade together with sequences obtained from samples from Stražilovo, Čortanovci, Krušedol, Sremski Karlovci and Kelebia by Bugarski-Stanojević *et al.* (2022, 2024); Németh *et al.* (2013, 2020).

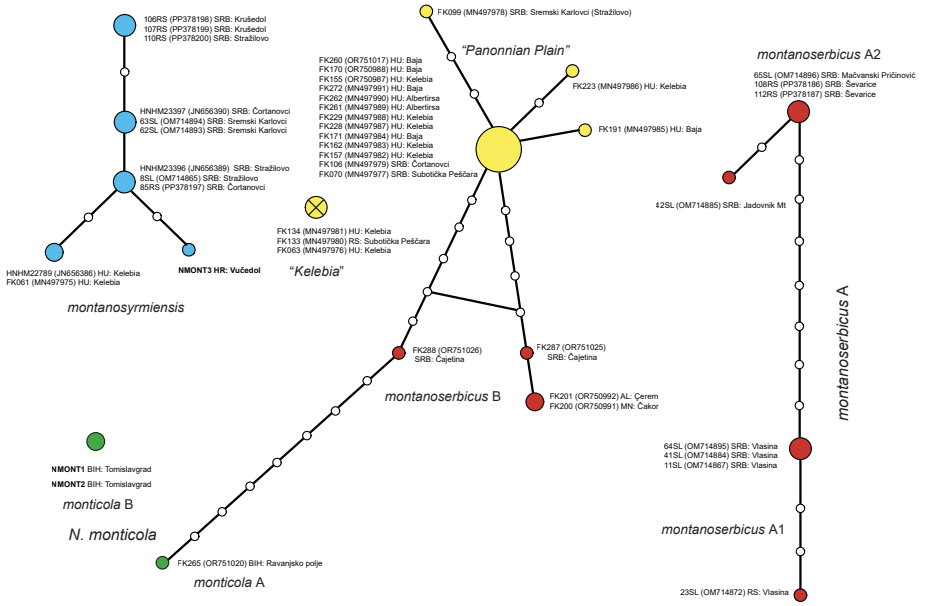

Fig. 5. Statistical 95% parsimony network (TCS) based on *cytochrome b* sequences of the *Nannospalax monticola complex* (CYTB2 data set). Small white circles indicate missing intermediates. Coloured circles represent different haplotypes, with their size corresponding to haplotypes frequencies.

In several cases, *montanosyrmiensis* samples from the same location exhibit highly diverse haplotypes and cluster within up to three different subnetworks: Kelebia/Subotička Peščara samples FK155, FK229, FK228, FK162, FK157, FK223 and FK070 (Németh *et al.*, 2020; 2024) are comprised within the "Pannonian Plain" subclade, samples FK133, FK134 and FK063 (Németh *et al.*, 2020) within the "Kelebia" subclade while the samples HNHM22789 (Németh *et al.*, 2013) and FK061 (Németh *et al.*, 2020) are placed within the *montanosyrmiensis* subnetwork. The K2P distances within the Kelebia locality reach values of up to 5.3%, consistent with interspecific distances recorded for distinct rodent species (Baker & Bradly, 2006). Similarly, samples from Stražilovo/

Sremski Karlovci obtained in the studies of Bugarski-Stanojević *et al.* (2022, 2024) and Németh *et al.* (2013) clustered within the *montanosyrmiensis* subnetwork, and sample FK099 obtained by Németh *et al.* (2022) within the "Pannonian Plain" subnetwork. The K2P distance between them is 4.7%. Finally, the same is true for Čortanovci, the sequences of the samples published in Németh *et al.* (2013) and Bugarski-Stanojević *et al.* (2022) belong to *montanosyrmiensis*, and FK106 sequence of Németh *et al.* (2022) is comprised within *montanosyrmiensis*, too. The K2P distance between 800 bp long sequences is 5.4%.

DISCUSSION

One of the peculiarities in recent studies on blind mole rats in Eastern Europe regarding the *montanosyrmiensis* lineage and *cytochrome b* mitochondrial marker is the presence of extremely different *cytochrome b* haplotypes (p-distances up to 5.4%, K2P up to 5.7%). These haplotypes have been obtained from samples from the same locality in different studies (Németh *et al.*, 2013; 2020; 2024, Bugarski-Stanojević *et al.*, 2022; 2024) or, in one case, from the same study (Németh *et al.*, 2020). Interestingly, although this situation occurs in studies by the same research group (i.e., Németh *et al.*, 2013; 2020), the phenomenon was never commented upon. Moreover, the two mentioned research groups avoided including each other's sequences (Bugarski-Stanojević *et al.*, 2022, Németh *et al.*, 2024), while in the papers of Németh *et al.* (2022, 2024) the divergent sequences from previous studies by the same authors were omitted without explanation. The background of the observed genetic pattern could be explained in at least two ways. First, through the existence and amplification of numerous nuclear pseudogenes, and second by the secondary contact of previously long isolated lineages. In this study we revealed the sequence of the nuclear pseudogene present in Vučedol sample, which does not match any of the three *montanosyrmiensis* s.l. subclades. Although the presence of multiple different *numt*s is also possible, we argue that the *cytb* sequence from Vučedol, Croatia published here (NMONT3) represents the genuine mitochondrial sequence for the following reasons: 1) In this study, unlike *numt* sequences, it was successfully amplified from a highly diluted DNA template. 2) It clustered within the *montanosyrmiensis* subclade alongside sequences obtained from old, archived samples (teeth, Bugarski-Stanojević *et al.*, 2022), where DNA degradation is expected, increasing the likelihood of amplifying mitochondrial sequences that are orders of magnitude more abundant.

In a former phylogenetic study using two species delimitation methods, Németh *et al.* (2024) evaluated only three subclades: the first subclade from Bosnia as *monticola*, the basal "Pannonian Plain" subclade together with the "Kelebia" subclade, and the *montanoserbicus* B subclade from Čajetina in Serbia, Čakor in Montenegro and Çerem erem in Albania. The Bayes factor delimitation (BFD) approach did not support the recommended species delineation; however, based on the lineage trough time (LTT) plot approach, the authors concluded that these are three independent species. Nevertheless, the crucial divergence times are similar to the divergence of taxonomically unresolved taxa N. (*leucodon*) *hungaricus*, *N.* (*leucodon*) *serbicus* and *N.* (*leucodon*) *hellenicus*, which in other studies (Bugarski-Stanojević *et al.*, (2022, 2024) are all considered to be *N. leucodon* subspecies.

The sample from Vučedol is placed within the *montanosyrmiensis* subclade (Fig. 3, Fig. 4, Fig. 5). This sublineage likely originated from the westernmost glacial refugium

area of the larger Pleistocene European blind mole rat populations. The ancestral population of the *montanosyrmiensis* subclade inhabited the area along the Drava and Sava rivers, as indicated by Late Pleistocene *Nannospalax* fossil remains from northern Croatian sites such as Vindija Cave and Kamenika Cave (Fig. 1), alongside remains of *Cricetus cricetus* (Malez *et al.*, 1977, Smith *et al.*, 2024). The fossil remains in the Bükk mountains, northern Hungary (Jánossy, 1986), represent another extra-Mediterranean climate glacial refugium (Schmitt, 2007), but for the ancestral Hungarian Pannonian Plain population between Danube and Tisza rivers. The *montanosyrmiensis* subclade is one of six subclades of the *monticola* complex revealed in this study (Fig. 3), being one of the three most differentiated lineages within the clade. Actually, specimens belonging to the *montanosyrmiensis* and "Pannonian Plain" lineages were found at the same location on two occasions: Kelebia-Subotička peščara and Fruška gora, localities on different sides of the river Danube, which before it was channelized, functioned as a permeable barrier, similar to other plains rivers. Kimura 2-parameter (K2P) genetic distances between these subclades range from 4.3 to 5.7% (≥ 5.07%), falling within the putative range of interspecies variation as proposed by Beker & Bradly (2006) and are, for example, notably higher than distances between *Arvicola amphibius* and *A. italicus* (4.4 ± 0.7 %, Chevret *et al*., 2020).

Alongside the two previously mentioned subclades, a third subclade (the "Kelebia" subclade) was recorded exclusively in samples from Kelebia – Subotička peščara. The K2P distances between the members of this third subclade and both, "Pannonian Plain" and *montanosyrmiensis* subclades fall within tentatively intraspecific variation (2.3–2.7% and 2.3–3.1%). Observed distances could indicate multiple secondary contacts following the original one. Kelebia – Subotička peščara, the locality comprising all three distinct *cytb sub*clades (7 samples belonging to "Pannonian plain", 3 to "Kelebia" and 2 to the *montsyrmyensis subclade*), has been mapped and estimated to have a population of 150–200 individuals (Csorba *et al.*, 2015). As a contact zone, this locality warrants further, more detailed investigation utilizing a combination of mitochondrial and nuclear markers such as microsatellites or genome-wide SNPs data (Vences *et al.*, 2024) on a larger sample size. This approach will provide deeper insights into historical evolutionary processes within the Pannonian populations and aid in determining the taxonomic status of different sublineages.

Montanosyrmiensis karyotypes, characterized by polymorphism in Y chromosomes (mostly small acrocentric or rare small metacentric types), were identified in the Fruška gora (Stražilovo, Čortanovci) population (sample size n= 31) (Savić & Soldatović, 1984). Currently, the karyotypes of the individuals belonging to thw "Kelebia" and "Pannonian Plain" subclades from Kelebia-Baja-Albertirsa are unknown. There is only a brief mention of a single male from Kelebia (HNHM22789) having medium-sized acrocentric Y chromosome (Németh *et al.*, 2013), which probably slightly differes from those observed in the Fruška gora population. However, the karyogram was not provided in the publication. Based on our study, this specimen belongs to the *montanosyrmiens*is subclade (Fig. 3, Fig. 4). Following the publication of the aforementioned karyotype, Csorba *et al.* (2015) erroneously classified the whole, genetically very diverse populations of Kelebia and Baja as belonging to the *montanosyrmiensis* cytotype group. Analysis of other traits, such as skull morphology after Topachevskii (1969) failed.

In Serbia, south of the rivers Sava and Danube, downstream of Belgrade, several allopatric populations (i.e. Mt. Zlatibor, Čajetina, Mt. Vlasina, and in Montenegro from

Mt. Čakor) with the described *montanoserbicus* karyotype exists (Savić & Soldatović, 1984). Unexpectedly, phylogenetic analysis based on *cytb,* revealed that some populations clustered within very divergent *cytb* subclades: *montanoserbicus* A (e.g. Vlasina, Bugarski-Stanojević *et al*., 2022) and *montanoserbicus* B (e.g. Čajetina, Čakor, Németh *et al*., 2024). The existence of different *cytb* subclades within the same chromosomal race suggests that chromosomal rearrangements may not necessarily correlate with *cytb* differentiation (Kryštufek *et al*., 2012). Similar phenomena have been reported in other mammals, such as the Common shrew *Sorex araneus* (Taberlet *et al*., 1994; Horn *et al*., 2012). Sequence divergence between these subclades (K2P distances range 4.6–5.3%) falls within the putative range of interspecies variation. However, due to the small sample size, they remain within the so called „grey zone" (Barbosa *et al*., 2013), between intraspecific and putative interspecific variation. Between the northern lowland population of Mačvanski Pričinović (Bugarski-Stanojević *et al*., 2024) and the high-altitude Mt. Vlasina population (Bugarski-Stanojević *et al*., 2022) of the *montanoserbicus* A subclade, divergence is relatively high, with K2P distances of 3.1% (Tab. 2). Taking all this into account, this subclade could be a candidate for a separate species, with some isolated populations possibly classified as subspecies. However, the final decision should not be made before conducting a more detailed analysis on a larger sample and employing an integrative (molecular, morphological and cytological) approach. Skull samples are currently available only from the Zlatibor, Kopaonik, and Tara mountains (Savić, 1982; Savić & Soldatović, 1984). The *Montanoserbicus* B subclade was found in Čajetina (Serbia), Čakor (Montenegro) and Çerem (Albania) (Németh *et al*., 2024). The latter two locations are in the Prokletije Mountains at the southeastern edge of Dinaric Alps. The *cytb* K2P distances to the "Pannonian Plain" subclade are only 1.9–3.0%. These genetic distances likely result from the recent split of a formerly homogeneous population, possibly occurring through several separations in the Late Pleistocene and from tentative strong competitive pressure from the *N. leucodon* complex southern of river Danube.

K2P distances of the real Tomislavgrad population (*monticola* B) to the *montanosyrmien*s subclade range from 4.8–5.4%, and 5.3–5.5% to the *montanoserbicus* A subclade. However, specimens from the "Pannonian Plain" subclade showe K2P distances of 3.0–3.5% to the first analysed sample of *monticola* A from Bosnia and Herzegovina (Németh *et al*., 2024), which is lower range of tentative intraspecific variation for rodents (Baker & Bradly, 2006). At the same time, the K2P distance ranges between the "Pannonian Plain", *montanoserbicus* B and *N. monticola* A subclades (1.9–2.8; 3.2–3.9; 3.0–3.5), which are connected within a single TCS subnetwork (Fig. 5), would support the conclusion that not only the first two subclades, but all three allopatric populations probably belong to a single diverged species, similar to the European and Asian allopatric populations of Snow vole *Chionomys nivalis* (Castiglia *et al*., 2009; Mahmoudi *et al*., 2017). In our case this is, however, in sharp contrast to the result of Bayesian and NJ analysis, which revealed a strongly supported monophyletic *N. monticola* clade (see below). Therefore, the unusual TCS clustering of *N. monticola* samples may be an artifact resulting from the analysis of short sequences. In any case, these subclades may belong to the same species, but it is also possible that they represent independent species, because they are in the "grey zone" with possible different levels of reproductive isolation (Vences *et al*., 2024).

The phylogenetic analyses (both Bayesian and NJ, Fig. 3) revealed *N. montanoserbicus* B as a distinct, well supported clade. The same is true for the well-supported *N.*

monticola clade comprising *N. monticola* A (1120 m a.s.l.) close to species *locus typicus*. Samples from Tomislavgrad in the present study originated from the Tomislavgrad City area, at the foot of the mountain slope (approximately 880–910 m a.s.l.). The latter population, like adjacent locations of mole rats between mountains Vran and Čvrsnica (Risovac) and south of Blidinje Lake (Svinjača) (Bolkay, 1928; Kryštufek & Tvrtković, 1988; Brelih & Trilar, 2004), inhabits submontane Mediterranean grasslands, not the "Alpine grasslands on mountain tops" noted in Németh *et al*., (2024). In the study by Németh *et al*. (2024), the geographic origin of the *N. monticola* sample is incorrectly designated as "Tomislavgrad". The published coordinates point to a more northern location at Ravanjsko polje, near the road, at a higher altitude (1140 m a.s.l.). The fact that they represent two distinct, probably allopatric populations, might explain the observed genetic distances between them (2.8%). The entire central Dinaric area has generally been poorly investigated, and more extensive sampling is required for better insight into the genetic composition and taxonomic status of the populations in this region.

Contrary to Németh *et al*. (2024), our final conclusion is that the *N. monticola* complex (or superspecies after Németh *et al*., 2024) represents actually only one species, *Nannospalax monticola* (Nehring, 1898). The observed intraspecific genetic diversity in a small sample suggests that it comprises at least three highly distinct subclades, which are likely to be nearing or in the final stages of speciation (*monticola* and two species candidates: *montanosyrmiensis*, and *montanoserbicus* A). However, before any taxonomic revision, these findings must be corroborated through further comprehensive studies and a multi-locus molecular approach based on genome-wide markers, which will also allow for the detection of possible hybridization between phylogroups within the complex.

Ancestral populations of *N. monticola* complex diverged from *N. turcicus*/*N. leucodon* complex approximately 2.07 (3.4 – 0.7) Mya ago (Németh *et al*., 2024), in the Early Pleistocene. Subsequent surviving subclades diverged later, likely in the Middle Pleistocene, around 0.7 Mya, coinciding with the divergence of the sister species *Arvicola amphibius* and *A. italicus* which have infraspecific fossorial lineages distributed in Western Europe (Mahmoudi *et al.*, 2020; Chevret *et al*., 2020). *N. monticola* exhibits substantial genetic divergencefrom the *Nannospalax leucodon* complex with K2P distances of 5.8 – 11.9% between *N. monticola* and *N. leucodon*, and 6.9–11.4%. between *N. monticola* and *N. turcicus.* The distinct species status of *N. monticola* from taxa in *N. leucodon* complex confirmed previous attempts with crossing experiments (Savić & Soldatović, 1984) in which embryos in females were never found in cases of crossings between today's *N. monticola* complex and *N. leucodon* complex pairs. Clear morphological differences are evident between *N. monticola* and specimens of *N. leucodon complex* in Bosnia and Herzegovina (Bolkay, 1928). The northernmost Pannonian Plain population of *Microspalax leucodon monticola* s.l. in Hungary distributed western of Tisza River was documented by Topačevskii (1969: 186). He detailed morphological differences in skull structure compared to *M. leucodon leucodon* (recently *N. leucodon hungaricus*) which is distributed east of the Tisza River.

CONCLUSION

The blind mole rat, previously believed to be extinct, has been rediscovered in Croatia's Pannonian region at Vučedol. Phylogenetic analyses based on mitochondrial

cytochrome b and *16S rRNA* markers were conducted to classify this population taxonomically. A clear distinction between Lesser mole rats (*Nannospalax leucodon*) and Western mole rats (*N. monticola*) was confirmed. Within the *N. monticola* complex, six lineages were identified, with the Vučedol population belonging to the *montanosyrmiensis* cytotype subclade, a candidate for new species status. Probable secondary contact between Pannonian populations has been documented. To clarify the taxonomic status of the lineages within *N. monticola* complex, more comprehensive studies, as well as a multi-locus approach utilizing genome-wide molecular markers are needed. Until then, in the whole *N. monticola* complex the only valid name remains *Nannospalax monticola* (Nehring, 1898).

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