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RESEARCH PAPER

Evaluating soil type as a barrier: spatial genetic structure and ecological speciation in the Upper Galilee Mountains blind mole rat (*Nannospalax galili*)

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Abstract. Ecological speciation is an evolutionary process driven by divergent natural selection in heterogeneous environments characterised by diverse resources and habitats. Increasing evidence supports the occurrence of this phenomenon in nature. One frequently cited example among mammals is the Upper Galilee Mountains blind mole rat, *Nannospalax galili*. Over a decade ago, it was proposed that this species is undergoing incipient ecological speciation due to the sharply contrasting ecological conditions resulting from the presence of pale rendzina and dark basaltic soils. In this study, we examined the population genetic structure and gene flow between mole rats inhabiting these two distinct soil types at two localities in Northern Israel, Rihaniya and Gush Halav, each containing sites on both rendzina and basaltic soil types. We used eight microsatellite markers to assess genetic differentiation. The results indicate that in Rihaniya, where blind mole rats from both soils were sampled in close proximity, the genetic divergence between animals from the different soil types was the lowest. In Gush Halav, the genetic differentiation increased with geographic distance between sampled sites, indicating an isolation-by-distance effect. The presence of migrants and first-generation hybrids in both soils at both localities suggests that blind mole rats migrate and mate relatively frequently between the two soil types. These findings imply that ecological speciation in *N. galili* may be in its very early stages, with no clear evidence of assortative mating yet. Further research is needed to understand this phenomenon in this study system.

Key words: subterranean rodent, *Nannospalax*, ecology, speciation with gene flow, habitat requirements, microsatellites

Introduction

Speciation generally occurs when a single population of organisms divides into two or more subpopulations that stop interbreeding, and these subpopulations may later evolve into distinct species (Mayr 1963). This process may only require geographical isolation and time (Turelli et al. 2001), during which accumulated mutations cause reproductive incompatibility (Orr & Turelli 2001). These changes may occur randomly (i.e. through genetic drift) or through natural selection. However, as demonstrated by growing evidence in various taxa, natural selection can also lead to the emergence of new species in overlapping (or even

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panmictic) populations that are not geographically isolated.

Speciation by natural selection can be divided into two main types: mutation-order speciation and ecological speciation (Schluter 2009). Mutation-order speciation is defined as the evolution of reproductive isolation through the random occurrence and fixation of different alleles between populations adapting to similar selection pressures (Schluter 2009). In contrast, ecological speciation is the evolution of reproductive isolation between populations, or subsets of a single population, due to ecologically based divergent natural selection (Schluter 2000, Rundle & Nosil 2005, Hoikkala & Poikela 2022).

Although ecological speciation may not be a common phenomenon in nature, there is increasing evidence of its existence in various animal taxa (e.g. Nosil 2012). In vertebrates, well-documented examples typically involve disruptive selection in contrasting environments, leading to reproductive isolation of diverging sympatric populations. For instance, *Anolis* lizards have adapted to different habitats (mesic and xeric) (Losos 2009, Muñoz et al. 2013), Darwin's ground finches (*Geospiza* spp.) to different seed types (Grant & Grant 2011), and freshwater fish have formed genetically distinct ecotypes differing in morphology, ecology and reproductive biology. For the latter, both lake- and river-dwelling cichlids have adapted to different trophic niches (e.g. Barluenga et al. 2006, Gante & Salzburger 2012, Piálek et al. 2019), which was also documented in Dolly Varden (*Salvelinus malma*) (Markevich et al. 2018) and European whitefish (*Coregonus lavaretus*) (Öhlund et al. 2020). It is important to note, however, that even when the necessary conditions for ecological speciation are present in nature, the progress towards or completion of speciation is not guaranteed (Gavrilets 2004, Hendry 2009). For instance, introgression is common in a species complex of *Tilapia*, which represents the earliest stages of speciation where morphological and ecological divergence are incomplete (Martin 2013).

Subterranean mammals are a compelling subject for studying speciation processes because they typically exhibit low vagility and highly territorial behaviour (Nevo 1999). Their speciation is generally considered to be allopatric or peripatric, with new species evolving from isolated peripheral populations (Nevo 1999, 2001). Interestingly, a suggestion of incipient ecological speciation in the Upper Galilee Mountains blind mole rat, *Nannospalax galili*, a strictly subterranean rodent, was made more than a decade ago (Hadid et al. 2013). This species also inhabits localities characterised by strongly contrasting physical and vegetational conditions due to adjacent but ecologically very different pale rendzina and dark basaltic soil (Grishkan et al. 2008, Lövy et al. 2015, 2017). Rendzina soil has higher $CaCO_3$ content but more than threefold lower organic matter content, twofold lower water, and a lower C/N ratio (1.5 in rendzina, 6.2 in basaltic soil) (Grishkan et al. 2008). The soil hardness and moisture are higher in basaltic soil, making it significantly harder when dry and stickier when wet and, therefore, likely more difficult to excavate year-round. The vegetation on basaltic soil is dominated by *Carlina hispanica*, while *Sarcopterium spinosum* on rendzina soil. *Sarcopterium* dwarf shrubs dramatically reduce the density and species richness of other vegetation, such as forbs and grasses, the main food of blind mole rats, who seem to avoid sites dominated by this plant (for details, see Lövy et al. 2015). Basaltic soil also has higher aboveground and underground biomass of plants and is richer in geophytes, which are a staple diet of mole rats (Lövy et al. 2015, 2017). For more information about the food supply in rendzina and basaltic soil, see Lövy et al. (2015, 2017).

Recent studies have examined various genetic, physiological and behavioural aspects that are supposed to be involved in the process of ecological speciation in *N. galili*. For instance, Li et al. (2015, 2016, 2020) found more positively selected genes related to energetics and musculature in mole rats from basaltic soil, where burrowing is energetically more demanding. Mole rats from both soils are able to use olfaction cues to distinguish between the two soil types when they are wet and prefer to dig in the soil of their origin (Lövy et al. 2017). However, when presented with a choice of potential sexual mates from different soils, females did not prefer males from their own soil type, contrary to the predictions that soil type may lead to reproductive isolation (Lövy et al. 2020). Finally, previous studies have detected a certain level of gene flow between mole rats from the two different soils (Hadid et al. 2013, Li et al. 2015, 2016).

In this study, we employed eight microsatellite markers to examine the population genetic structure of the Upper Galilee blind mole rat *N. galili* at two localities, each consisting of basaltic and rendzina soils (for ecological characteristics of localities Rihaniya and Gush Halav see Lövy et al. 2015, 2017). Our objective was to assess the rate of gene flow between mole rats from different soils at each locality.

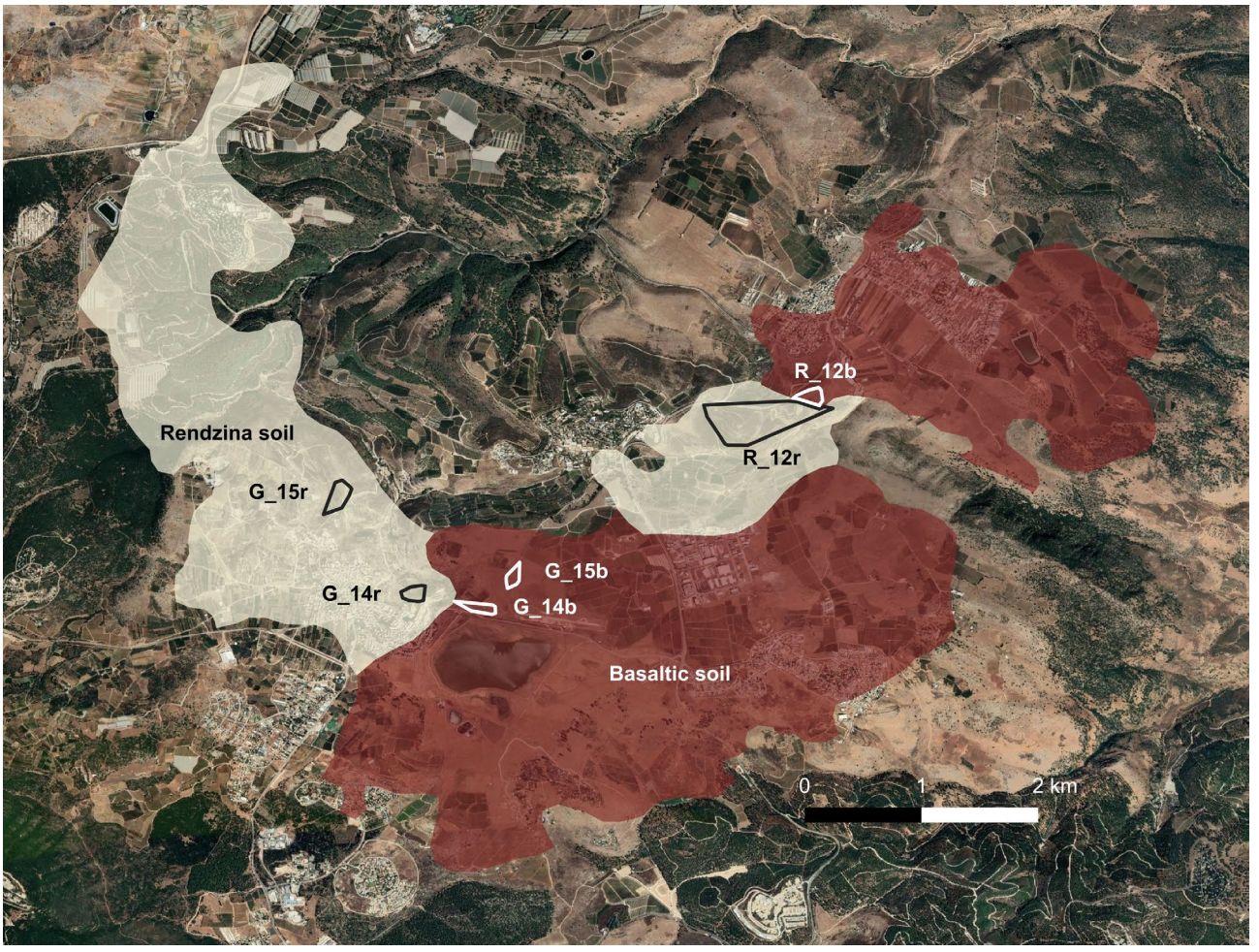

Fig. 1. The map of the study site depicting two sampled localities in the Upper Galilee Mountains in Northern Israel. White and black rectangles depict the sampling micropopulations in basaltic and rendzina soils at each locality (R – Rihaniya, G – Gush Halav).

If a reproductive barrier has developed between mole rats from the two soil habitats, we predict that i) there will be a low rate of migration between the soils and ii) a low occurrence of hybrid individuals in either of the soils.

Material and Methods

Sampling

A total of 189 individuals of the blind mole rat *N. galili* were captured from six sampling sites (hereafter referred to as micropopulations R_12r, R_12b, G_14r, G_14b, G_15r and G_15b) across two localities (Rihaniya and Gush Halav) in the Upper Galilee Mountains in northern Israel. The sampling was conducted using Hickman traps during two periods: 2011-2012 in Rihaniya (33°02.5′ N, 35°29.2′ E, altitude 760 m) and 2014-2015 in Gush Halav (33.023° N, 35.454° E, altitude 800 m). A total of 60 blind mole rats were captured in Rihaniya, 35 individuals from one sampling site in the basaltic soil (R_12b) and 25 from one sampling site in the rendzina soil (R_12r). In Gush Halav, 35 and 26 individuals were captured in 2014 and 2015 in the basaltic soil (G_14b and G_15b, respectively), and 40 and 28 individuals were captured in 2014 and 2015 in the rendzina soil (G_14r and G_15r, respectively) (Fig. 1; refer to Lövy at al. 2017 for detailed information about the localities). For each individual, body mass, sex, and geographical coordinates of the capture site were recorded (Tables S1, S2 and S3).

DNA extraction and genotyping

Tissue samples were obtained by cutting a small piece of skin and preserved in 70% ethanol. Total genomic DNA was extracted using the DNeasy Blood and Tissue Kit (QIAGEN) according to the manufacturer's protocol. Eight microsatellite loci originally developed for *Nannospalax ehrenbergi* (Karanth et al. 2004) were used in this study. These markers were amplified in two multiplex PCRs (multiplex 1 – CA22, CA19, 4b12, 4a15, CA21, multiplex 2 – 4b11, CA86, 4b18) using Qiagen multiplex PCR kit. PCR was performed in a total volume of 10 μL containing 5μL of the Multiplex PCR Kit, 1μL of DNA, 0.05 μM (CA21), 0.10 μM (CA22, CA19, 4b11, CA86), 0.20 μM

Table 1. Average number of alleles (Na), allelic richness (AR), observed (Ho) and expected (He) heterozygosity, test for Hardy-Weinberg equilibrium (P), coefficient of interbreeding (Fis), and effective populations size (Ne-LD) with parametric 95% confidence interval (CI).

Locality	Micropopulation	Soil type	Na	AR	Ho	He	P	Fis	Ne-LD	95% CI
Rihaniya	R_12r	rendzina	15.38 ± 5.24	14.815 ± 3.01	0.76 ± 0.15	0.87 ± 0.07	0.129	0.05 ± 0.07	25.9	18.3-39.1
	R_12b	basaltic	12.03 ± 4.13	11.325 ± 4.02	0.65 ± 0.12	0.75 ± 0.04	0.178	0.04 ± 0.04	39.8	23.9-89.7
Gush Halav	G_14r	rendzina	11.00 ± 3.34	10.556 ± 2.05	0.71 ± 0.16	0.80 ± 0.10	0.169	0.058 ± 0.02	17.9	12.5-27.1
	G_15r	rendzina	12.50 ± 3.82	12.013 ± 2.85	0.71 ± 0.22	0.81 ± 0.08	0.048	0.07 ± 0.04	65.6	39.0-153.0
	G_14b	basaltic	10.00 ± 3.93	9.201 ± 3.03	0.64 ± 0.16	0.79 ± 0.09	0.123	0.05 ± 0.06	60.0	34.5-153.7
	G_15b	basaltic	10.63 ± 4.10	9.899 ± 3.98	0.68 ± 0.19	0.79 ± 0.06	0.209	–0.07 ± 0.05	19.8	12.9-34.0

(4b12, 4a15) or 0.25 μM (4b18) of each primer pair (forward primers were fluorescently labelled with FAM, VIC, NED and PET) and deionised water to the final volume of 10 μL.

The cycling procedure started with an initial denaturation at 95 °C for 15 min, followed by 35 cycles of 94 °C for 30 s, 57 °C for 1.5 min and 72 °C for 10 min. PCR products were run on an ABI 3130 genetic analyser (Applied Biosystems) with a LIZ-500 size standard. Peaks were visualised using the software GeneMarker and scored manually by a single observer (L. Dovičicová).

Population genetic analysis

The genotypes were checked for stuttering, large allele dropout, and null alleles using Micro-Checker 2.2.3 (Van Oosterhout et al. 2004). Observed heterozygosity (Ho) and expected heterozygosity (He), number of alleles (Na), their frequencies, and allelic richness (AR) were calculated using the program GenAlEx 6.1 (Peakall & Smouse 2012). Effective population sizes (Ne) were estimated using the molecular co-ancestry method of Nomura (2008), as implemented in NeEstimator V2.1 (Do et al. 2014).

In the first step, the genetic relationships among the studied individuals were explored by factorial correspondence analysis (FCA) in Genetix 4.05 (Belkhir et al. 1996-2004). FCA projects each individual into a multidimensional space and visualises it as a point positioned between two axes that account for most of the variation based on the genotypes of the analysed individuals.

Isolation by distance (IBD), as described by Slatkin (1987, 1993), refers to a pattern of population differentiation where genetic differences between populations increase with geographic distance. In this study, we analysed the relationship between genetic isolation and geographic distance for six pairs of sampling sites from two localities, Rihaniya and Gush Halav. Subsequently, we focused on sampling sites from Gush Halav using the program GenePop (Raymond & Rousset 1995, Rousset 2008). For each sampling site data set, we used the Mantel test (Mantel 1967) to assess the significance of the relationship between genetic distance and geographic distance among all sampling sites. We estimated geographic distances between sampling sites as the shortest linear distance between them in km.

To assess the genetic structure of the studied individuals, we applied the Bayesian clustering

method implemented in the program Structure 2.3.1 (Pritchard et al. 2000). The program uses the Markov chain Monte Carlo (MCMC) process to assign the individuals to different numbers of clusters (K) while minimising Hardy-Weinberg and linkage disequilibria. We ran the program with ten independent simulations for each K, using the following parameters: 500,000 MCMC iterations, an admixture ancestry model, and an independent allele frequency model, with K-values from 1 to 10. The most suitable K was selected based on the highest ΔK and mean likelihood probability of the data (Evanno et al. 2005). Initially, we used the default mode for STRUCTURE, which uses only genetic information to learn about population structure. Then, geographic sampling location was incorporated into the inference procedure, and the USEPOPINFO model was run to test for any individuals in the sample who may be immigrants to their supposed micropopulations or have recent immigrant ancestors. The outputs were then explored in the STRUCTURE HARVESTER program (Earl & Vonholdt 2012) to determine the most likely K value using the ΔK method.

Alternatively, we used the program NEWHYBRIDS 1.1 beta (Anderson & Thompson 2002), another statistical model-based Bayesian method, to identify possible hybrids among groups of mole rats from pre-defined micropopulations. The software considers six genotype categories: pure species P1, pure species P2, F1 hybrid (first filial generation of offspring of a pair of parents), F2 hybrid (second generation of offspring of a pair of parents of F1s), and the backcrosses (offspring of F1 to pure species P1 or pure species P2). The estimated posterior probability is used to assign each individual to one of the six genotypic categories.

Results

Genetic diversity

The frequency of null alleles was less than 5% for all loci, with individual locus frequencies ranging from 0.003 (locus 4a15) to 0.048 (locus CA22) (Table S4). Thus, all were used in further analysis.

The micropopulation G_15r from the rendzina soil deviated significantly from Hardy-Weinberg equilibrium (HWE) based on exact tests in GENEPOP ($P < 0.05$). The allelic richness of the studied micropopulations ranged from 14.325 to 9.201, with the highest values found for animals from the R_12r and G_15r micropopulations (Table 1). The highest values of genetic diversity (Na, AR, He) were found for the micropopulations in the rendzina soil (R_12r and G_15r). The average Na, AR, Ho, He and coefficient of inbreeding (Fis) are shown in Table 1.

Table 2. Pairwise fixation index FST values between six micropopulations of *Nannospalax galili*.

	R_12r	R_12b	G_14r	G_14b	G_15r
R_12b	0.016				
G_14r	0.044	0.050			
G_14b	0.043	0.039	0.039		
G_15r	0.045	0.068	0.042	0.060	
G_15b	0.065	0.043	0.038	0.030	0.051

The effective population size estimates for rendzina-soil micropopulations (R_12r, G_14r, G_15r) were similar to those from the basaltic soil (R_12b, G_14b, G_15b) (Table 1). However, there was a considerably lower value in Ne in 2014 for G_14r (rendzina soil) and in 2015 for G_15b (basaltic soil) in Gush Halav. The micropopulations with the highest LD-based Ne were G_15r and G_14b (Table 1).

The pairwise fixation index FST values between micropopulations ranged from 0.016 to 0.068, with a mean of 0.044 ± 0.015, all significantly different from zero ($P < 0.05$). The highest FST value was between R_12b and G_15r, while the lowest was between R_12b and R_12r (Table 2).

Genetic structure of mole rat populations

The FCA plot separated individuals into two main groups based on their geographical origin from Rihaniya (R_12b, R_12r) and Gush Halav (G_14r, G_15r, G_14b, G_15b) along the first axis (31.9%). The second axis (22.5%) further separated Gush Halav individuals into two clusters, the first cluster comprising two basaltic-soil micropopulations (G_14b and G_15b) and one rendzina-soil micropopulation (G_14r) and the second cluster containing only the rendzina-soil micropopulation G_15r (Fig. S1).

Genetic distances were correlated with geographic distances for all six micropopulations combined (t-value = 3.012, $P = 0.003$, $R^2 = 0.139$; Fig. 2A). This correlation was also observed for the four micropopulations from Gush Halav when tested separately (t-value = 2.011, $P = 0.006$, R2 = 0.764; Fig. 2B).

The STRUCTURE analysis based on all individuals from the six micropopulations of both Rihaniya and Gush Halav assigned individuals into two clusters, separating individuals from Rihaniya and Gush

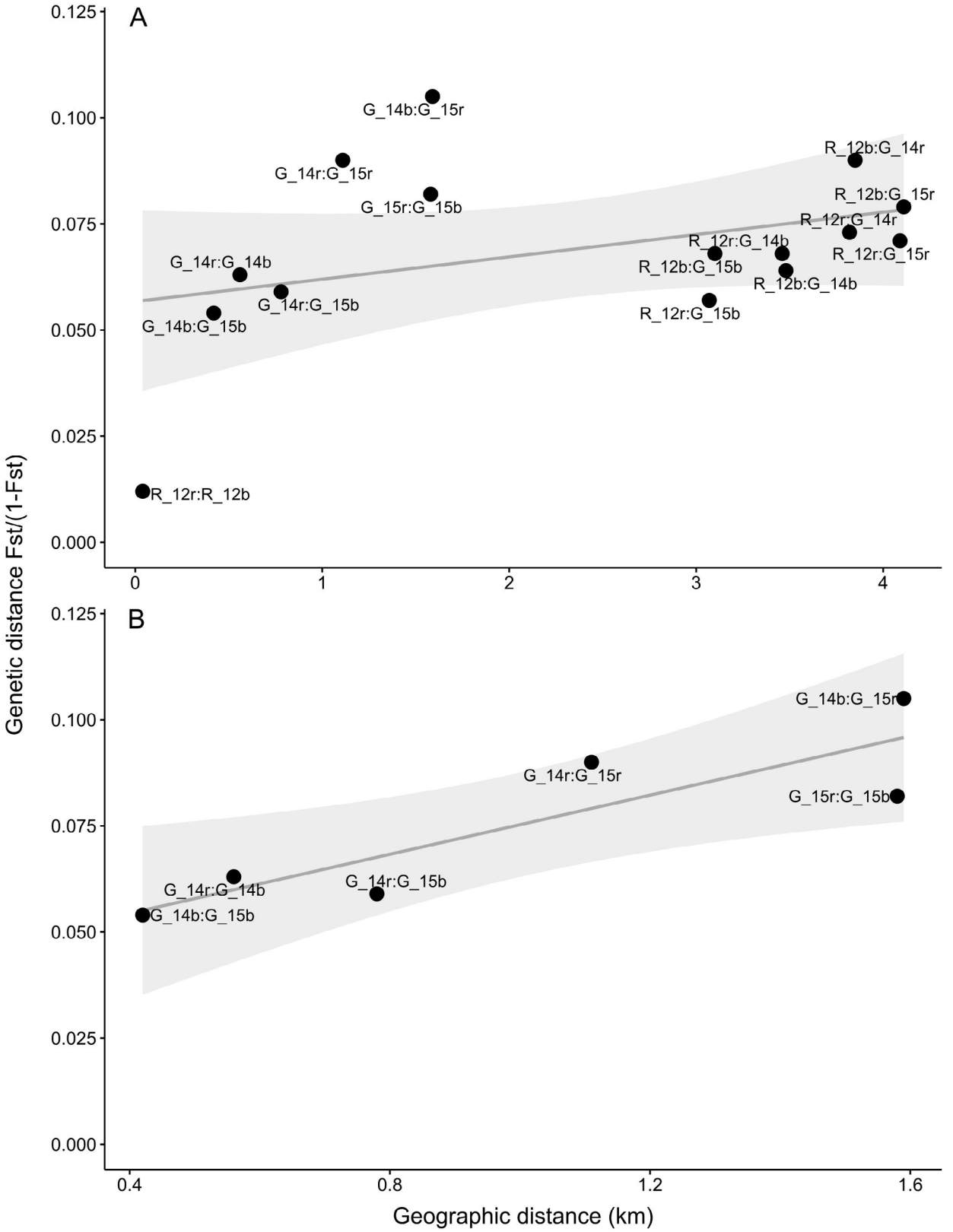

Fig. 2. Mantel test of isolation by distance for eight microsatellite loci for blind mole rats from localities Rihaniya and Gush Halav. Each point represents one population pairwise FST/(1-FST) plotted against geographic distance between a respective pair of populations. A) six populations from Rihaniya and Gush Halav together; B) four populations from Gush Halav only.

Halav (K = 2; Fig. 3A). A separate STRUCTURE analysis for Rihaniya identified the best number of clusters to be K = 2, separating most of the individuals based on their sampling micropopulation, i.e. into the basaltic-soil and rendzina-soil, with some individuals showing mixed origins (Fig. 3B). Other K clusters

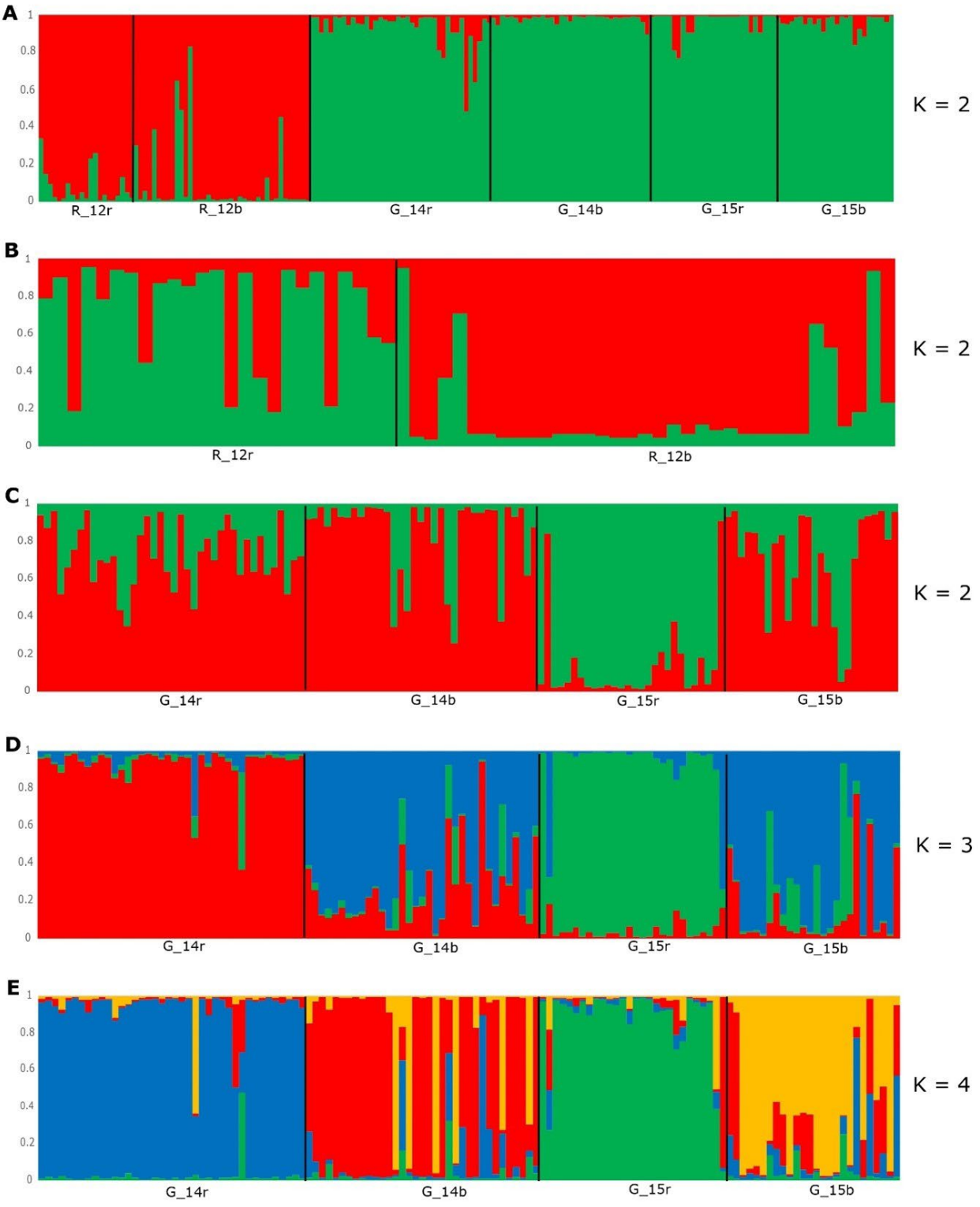

Fig. 3. Population structure inferred by the STRUCTURE analysis. Each individual is represented by a vertically stacked column of genetic components proportions shown in colour (R_12r, R_12b, G_14r, G_14b, G_15r and G_15b). A) The genetic structure of blind mole rats from six populations in Rihaniya and Gush Halav, analyzed together for K = 2; B) The genetic structure of blind mole rats in Rihaniya for K = 2; C); D); and E) The genetic structure of blind mole rats in Gush Halav for the models K = 2, K = 3 and K = 4 respectively.

were not preferred based on ΔK for detecting the most likely number of clusters inferred by STRUCTURE according to the method of Evanno et al. (2005) (Fig. S2). A separate STRUCTURE analysis for Gush Halav showed the best result for K = 4, assigning each micropopulation into a separate subcluster (Fig. 3E).

Table 3. Population assignment of admixed individuals from Rihaniya using STRUCTURE (USEPOPINFO model) and NewHybrids. Q value generated by STRUCTURE software with USEPOPINFO model using sampling locations to test for migrants or hybrids. NewHybrids R_12r – rendzina soil, R_12b – basaltic soil, F1– first generation hybrid, F2 – second generation hybrid, BC_R_12r – backcross to R_12r, BC_R_12b – backcross to R_12b. Underlined results indicate a higher association with micropopulation/group.

Individual (sex)	Distance from the soil boundary	Sampling micropopulation	STRUCTURE assignment	STRUCTURE Q value		NewHybrids					
				R_12r	R_12b	R_12r	R_12b	F1	F2	BC_R_12r	BC_R_12b
M01 (m)	53	R_12r	R_12b	0.40	0.60	13.48	85.00	1.52	0.00	0.00	0.00
S09 (m)	139	R_12r	R_12r	0.58	0.42	0.00	0.33	85.17	14.50	0.00	0.00
S22 (m)	268	R_12r	R_12r	0.62	0.38	3.00	1.00	89.00	3.00	3.00	1.00
S28 (f)	198	R_12r	R_12r	0.69	0.31	0.28	0.13	0.01	90.23	8.84	0.51
S06 (m)	64	R_12r	R_12b	0.35	0.65	1.00	96.00	0.00	2.00	1.00	0.00
S13 (f)	179	R_12r	R_12b	0.30	0.70	0.00	81.00	0.00	11.00	8.00	0.00
S16 (f)	5	R_12r	R_12b	0.24	0.76	0.00	83.10	0.31	7.19	9.06	0.34
M17(f)	534	R_12r	R_12r	0.83	0.17	0.18	0.89	0.05	0.95	91.29	6.64
S35 (f)	53	R_12r	R_12b	0.14	0.86	2.00	89.00	0.00	2.00	2.00	5.00
S37 (f)	110	R_12r	R_12b	0.09	0.91	5.00	94.00	1.00	0.00	0.00	0.00
M02 (f)	7	R_12b	R_12r	0.80	0.20	0.00	0.75	0.01	88.40	0.03	10.81
S04 (f)	37	R_12b	R_12r	0.67	0.33	88.00	5.00	2.00	1.00	3.00	1.00
M08 (f)	54	R_12b	R_12r	0.71	0.29	91.00	6.00	0.00	0.00	0.00	3.00
M24 (m)	30	R_12b	R_12b	0.21	0.79	10.44	0.00	1.51	71.45	15.70	0.90
S34 (f)	59	R_12b	R_12b	0.28	0.72	3.00	15.00	0.00	79.00	1.00	2.00
S19 (f)	43	R_12b	R_12b	0.30	0.70	4.09	7.71	0.60	79.90	3.76	3.94

Table 4. Population assignment of admixed individuals from Gush Halav using STRUCTURE (USEPOPINFO model) and NewHybrids. Q value generated by STRUCTURE software with USEPOPINFO model using sampling locations to test for migrants or hybrids. NewHybrids G_14r – rendzina soil 2014, G_14b – basaltic soil 2014, G_15r – rendzina soil 2015, G_15b – basaltic soil 2015, F1 – first generation hybrid, F2 – second generation hybrid, BC_G14r – backcross to G_14r, BC_G_14b – backcross to G_14b, BC_G15r – backcross to G_15r, BC_G15b – backcross to G_15b. Underlined results indicate a higher association with micropopulation/group.

Indiv.	Sampling micropopul.	STRUCTURE assignment	STRUCTURE Q value				NewHybrids								
			G_14r	G_14b	G_15r	G_15b	G_14r	G_14b	G_15r	G_15b	F1	F2	BC_ G14r	BC_ G14b	BC_ G15r	BC_ G15b
S8 (f)	G_14r	G_14r	0.83	0.05	0.01	0.11	0.00	2.00	0.40	6.40	5.00	2.00	83.20	1.00	0.00	0.00
S24 (f)	G_14r	G_14r	0.64	0.01	0.00	0.35	0.79	0.40	0.40	10.20	86.29	1.70	0.22	0.00	0.00	0.00
S30 (f)	G_14r	G_14r	0.59	0.30	0.01	0.10	0.00	0.00	0.00	0.00	99.96	0.01	0.03	0.00	0.00	0.00
S31 (f)	G_14r	G_14b	0.34	0.62	0.04	0.00	0.00	96.40	2.60	0.50	0.03	0.45	0.01	0.01	0.00	0.00
B15 (m)	G_14b	G_14b	0.03	0.67	0.03	0.27	2.89	27.00	2.90	0.00	0.00	0.01	0.00	67.20	0.00	0.00
B20 (f)	G_14b	G_15b	0.00	0.01	0.00	0.99	0.00	0.80	0.00	99.16	0.00	0.00	0.00	0.04	0.00	0.00
C02 (f)	G_15r	G_15r	0.37	0.12	0.43	0.08	0.00	1.10	0.00	7.41	0.08	91.30	0.00	0.11	0.00	0.00
C21 (m)	G_15r	G_15r	0.05	0.39	0.55	0.01	0.00	0.00	0.01	0.00	99.48	0.50	0.00	0.01	0.00	0.00
C28 (m)	G_15r	G_14b	0.00	0.90	0.10	0.00	0.20	90.10	9.04	0.30	0.03	0.33	0.00	0.00	0.00	0.00
T8 (f)	G_15b	G_15b	0.02	0.34	0.01	0.63	8.50	2.30	0.60	0.20	84.50	2.80	0.00	0.00	0.00	1.10

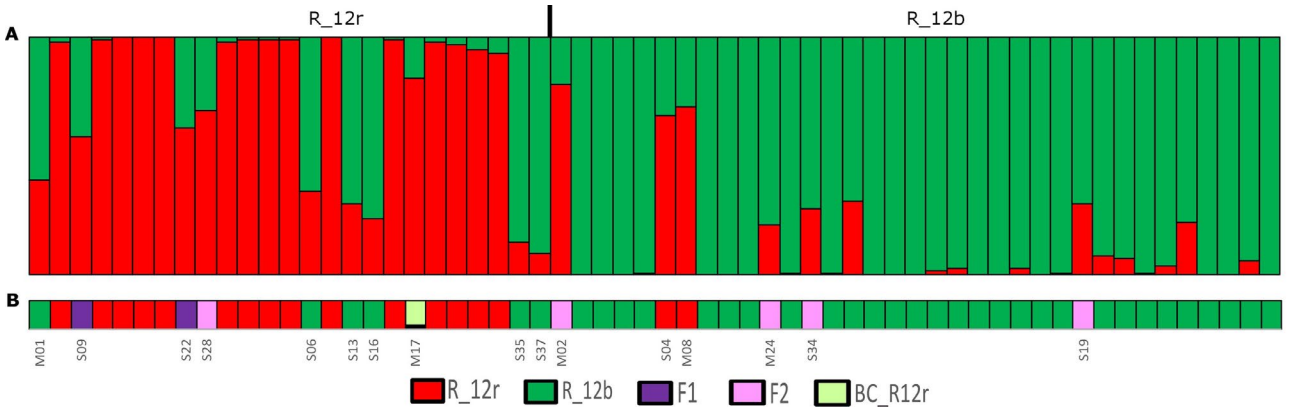

Fig. 4. Genetic structure and assignment of individuals into classes as assessed by STRUCTURE USEPOPINFO model A) and NEWHYBRIDS B) for Rihaniya. Each individual is represented by a single vertical bar. For STRUCTURE, red and green colours represent rendzina-soil and basaltic-soil animal clusters, respectively; for NEWHYBRIDS, each colour represents a genealogical class: red – purebred R_12r, green – purebred R_12b, violet – F1 generation hybrid, pink – F2 generation hybrid, light green – backcross to R_12r. Backcrosses to R_12b were not identified. The length of the coloured bar indicates the individual's estimated posterior probability of assignment to a given class.

Other K clusters (Figs. 3C, D) were not preferred based on ΔK (Fig. S3).

To reveal admixed individuals within the studied micropopulations, USEPOPINFO analysis, NewHybrids analysis, and STRUCTURE models with K = 2 for Rihaniya and K = 4 for Gush Halav were used (summarised in Tables 3 and 4). In Rihaniya, six individuals captured in the rendzina soil (two males, four females) had genetic admixture of both soils with Q > 50% of the basaltic-soil genetic component (two individuals had Q > 80%; Table 3 and Figs. 4 and 5A). Only three admixed individuals (all females) with Q > 50% of the rendzina-soil genetic component were revealed in the basaltic-soil micropopulation. A NewHybrids analysis identified six individuals in rendzina R_12r as migrants from the basaltic soil (two males and four females); two males were identified as F1 hybrids, one female as a F2 hybrid, and one female as a backcross to rendzina R_12r. In the basaltic site R_12b, two females were identified as migrants from the rendzina soil, and four individuals as F2 hybrids (three females and one male) (Table 3, Figs. 4 and 5A). All admixed individuals were captured closer to the inter-soil boundary than non-admixed ones in both micropopulations (R_12b: 38 ± 19 m and 80 ± 41 m; R_12r: 160 ± 154 m and 405 ± 195 m).

For Gush Halav, ten individuals showed different assignments than their sampling site based on their Q > 50% (Table 4 and Figs. 5B and 6). The results of USEPOPINFO indicated that female s31 from the rendzina-soil micropopulation G_14r and male c28 from the rendzina-soil micropopulation G_15r, both had a high percentage of the genetic component from the basaltic-soil micropopulation G_14b (Q = 62% and 90%, respectively). The female b20 from the basaltic-soil micropopulation G_14b had a high percentage (Q = 99%) of the genetic component from the basaltic-soil micropopulation G_15b. New Hybrids results confirmed that the three individuals identified by USEPOPINFO were migrants from the basaltic-soil micropopulations. One individual (male b20) migrated from the basaltic micropopulation G_15b to the basaltic micropopulation G_14b. Four other individuals were identified as F1 hybrids between the soils (male s30 and female s24 from G_14r, male c21 from G_15r and female t8 from G_15b), one F2 hybrid (female c02 from G_15r), and two were backcrosses (female s08 backcross to G_14r and male b15 backcross to G_14b). Migrants from the basaltic soil to the rendzina soil were captured 278 m (female s31, migrated from G_14b to G_14r) and 848 m (male c28, migrated from G_14b to G_15r) from the inter-soil boundary.

Discussion

We have demonstrated that six *N. galili* micropopulations from two localities with two ecologically distinct soil types and no physical barrier between them exhibit a relatively high observed heterozygosity and genetic diversity. The lowest genetic difference in blind mole rats was observed between two sampling micropopulations from Rihaniya, where mole rats from the rendzina and basaltic soil directly adjoin each other. Although mole rats from this locality were clearly divided into two clusters based on their soil origin, several individuals showed a mixture of genotypes from both soils (see

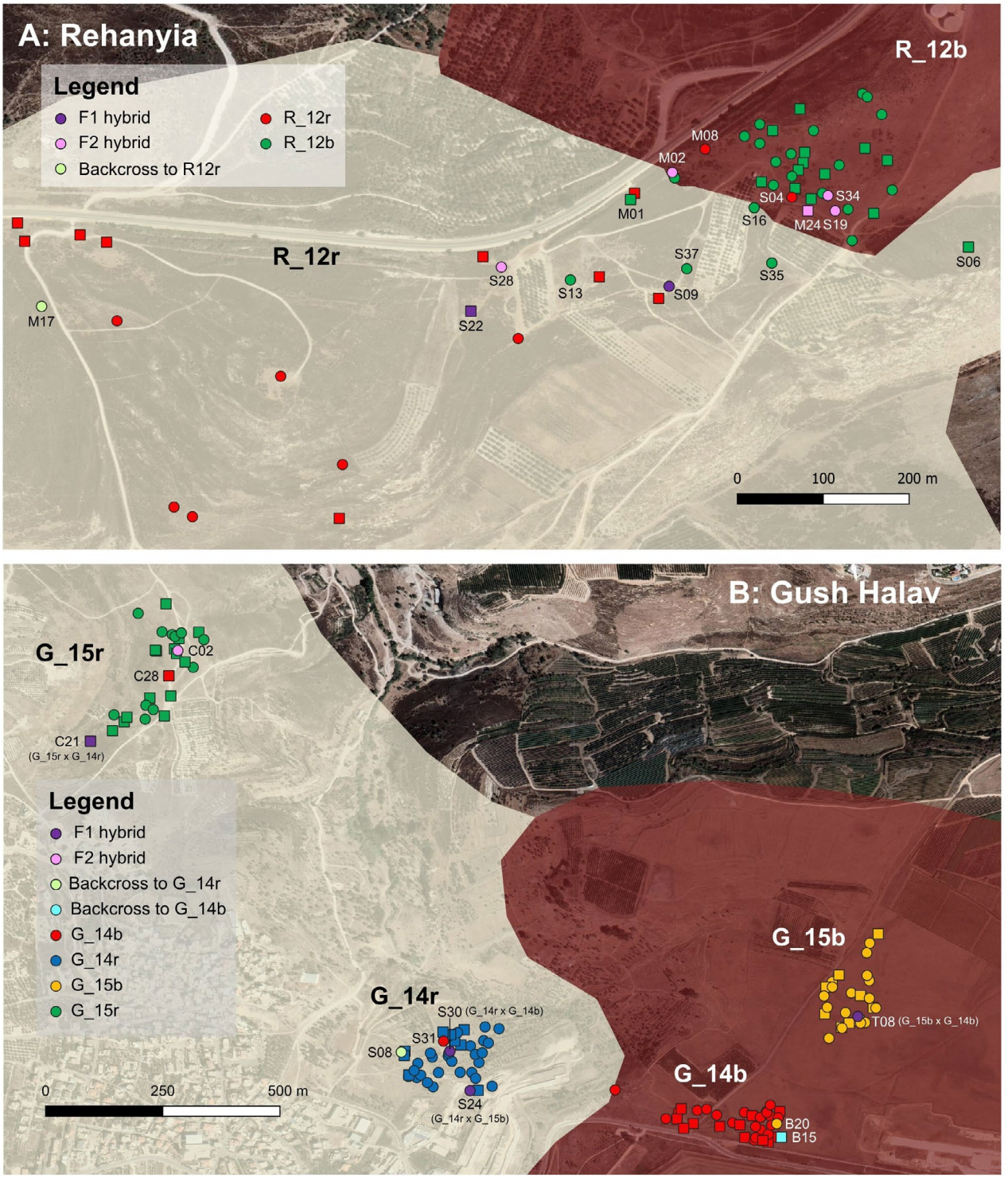

Fig. 5. The two study localities with positions of the genotyped female (circles) and male (squares) blind mole rats clustered into two populations based on K = 2 in Rihaniya (A) and into four populations based on K = 4 in Gush Halav (B). Within each locality, each population is depicted by its own colour; admixed individuals revealed by the NewHybrids analysis are depicted with different colours (see the Legend), with migrants being represented by the colour of the source population. Beige and red polygons delimit rendzina and basaltic soil, respectively (soil distribution adapted after Ravikovitch 1969 and own unpublished data).

Figs. 3B, 4 and 5A). Additionally, there were migrants between the soils in both directions. In Gush Halav, each of the four sampling micropopulations, differing in their distance from the inter-soil boundary, formed a separate genetic cluster, with fewer individuals of mixed origin than in Rihaniya. Only two individuals were identified as migrants from basaltic to rendzina soil (see Figs. 3E, 5B and 6). We found a positive correlation between geographic distance and genetic distance, indicating isolation by distance

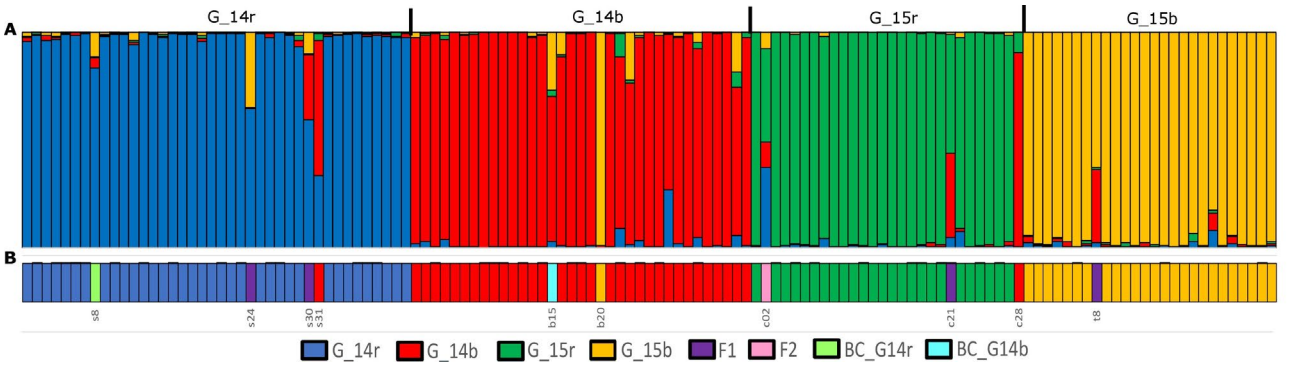

Fig. 6. Genetic structure and assignment of individuals into classes as assessed by STRUCTURE USEPOPINFO model A) and NEWHYBRIDS B) for Gush Halav. Each individual is represented by a single vertical bar. For STRUCTURE, each colour (blue, red, green, yellow) represents a cluster (G_14r, G_14b, G_15r, G_15b). For NEWHYBRIDS, each colour represents a genealogical class: blue – purebred G_14r, red – purebred G_14b, green – purebred G_15r, yellow – purebred G_15b, violet – F1 generation hybrid, pink – F2 generation hybrid, light green – backcross to G_14r, light blue – backcross to G14b. Backcrosses to G_15b and G_15r were not identified. The length of the coloured bar indicates the individual´s estimated posterior probability of assignment to each class.

for all six micropopulations combined and the four micropopulations from Gush Halav when analysed separately.

Genetic diversity of basalt and rendzina mole rats

Genetic polymorphism was slightly higher in animals from the rendzina soil than in those from the basaltic soil, although the difference was not significant. It is worth noting that blind mole rats living in the rendzina soil are subjected to much higher ecological stress due to the drier habitat and lower density of geophytes, which are their primary food resource (Lövy et al. 2015). Previous studies have shown that rendzina-soil mole rats exhibit higher genetic diversity in mtDNA genome (Hadid et al. 2013), whole genome genetic diversity (Li et al. 2015), and AFLP (Amplified Fragment Length Polymorphism) loci genetic polymorphism (Polyakov et al. 2004) compared to basaltic-soil mole rats. These results support the hypothesis that higher genetic diversity may be related to environments where blind mole rats are likely exposed to higher environmental stress (Nevo 1998, 2014, Hadid et al. 2014). The general pattern of increased genetic variability with environmental stress, such as higher aridity and climatic unpredictability, was also found in 12 populations of blind mole rats belonging to four species of the *N. ehrenbergi* superspecies from Israel (Karanth et al. 2004).

Compared to other subterranean rodents, *N. galili* shows intermediate to relatively high levels of heterozygosity, with mean values ranging from 0.639 to 0.755. Estimates of genetic diversity in subterranean rodents vary widely, from complete homozygosity found in *Bathyergus janetta* (Nevo et al. 1987), *Ctenomys argentinus* (Sage et al. 1986) and *Geomys tropicalis* (Selander et al. 1974) to low heterozygosity in *Ctenomys sociabilis* (mean Ho = 0.039, Lacey 2001), *Ctenomys porteousi* (mean Ho = 0.42, Mapelli et al. 2012), *Bathyergus suillus* (mean Ho = 0.349, Bray et al. 2011), low haplotype diversity in *Georychus capensis* (mean Haplotype diversity, Hd = 0.385, Visser et al. 2018), or *Ctenomys lami* (mean Ho = 0.410, El Jundi & De Freitas 2004). Intermediate to high diversity was found in *Ctenomys australis* (mean Ho from three sampling sites varies between 0.51 and 0.61, Mora et al. 2010), *Ctenomys haigi* (mean Ho = 0.665, Lacey 2001) and *Fukomys damarensis* (mean Ho = 0.78, Mynhardt et al. 2021). Low heterozygosity in subterranean mammal taxa is explained by low environmental heterogeneity in their subterranean environment (Nevo 1976, 1979, Nevo et al. 1997). Therefore, the relatively high level of heterozygosity in *N. galili* is unexpected but consistent with earlier estimates for this species (Polyakov et al. 2004, Hadid et al. 2013, Li et al. 2015). Higher heterozygosity for *N. ehrenbergi* superspecies in Israel corresponds with higher genetic diversity of populations facing higher ecological stress and wider temporal fluctuations, which selects for higher heterozygosity (Nevo 1998).

The mean FST value for population differentiation in *N. galili* was 0.044 ± 0.015, which falls within the range observed in other subterranean rodents. For example, genetic differentiation among three populations of *C. australis* in a fragmented landscape yielded FST values ranging from 0.029 to 0.081 (Mora et al. 2010). Similarly, in *B. suillus*, a comparison of ten populations on a regional scale showed FST values ranging from 0.048 to 0.517 (Visser et al. 2014). Higher FST values were observed between parapatric *C. sociabilis* and *C.*

haigi (0.15), indicating relatively high differentiation (Tammone et al. 2018). In a recent study on the genetic diversity of the genus *Bathyergus*, most FST values were higher than 0.33 (e.g. *B. janetta vs*. *B. suillus*-West), suggesting relatively high differentiation (Šumbera et al. 2024). Therefore, the population differentiation observed among the studied micropopulations of *N. galili* appears to be more comparable to intraspecific variation than to interspecific differentiation when compared to various subterranean taxa.

Population structure of *N. galili*

Genetic differences observed in *N. galili* among the studied micropopulations (see Fig. 2) are consistent with the IBD population genetic model, similar to other solitary subterranean species such as in African mole-rats (*G. capensis*, Visser et al. 2018; *B. suillus*, Visser et al. 2014; *Heliophobius argenteocinereus*, Patzenhauerová et al. 2010) and in *C. australis* on a local scale (Mora et al. 2010). However, the IBD was not observed in *Ctenomys rionegrensis* (Wlasiuk et al. 2003, Kittlein & Gaggiotti 2008) or *C. australis* at the regional level (Mora et al. 2010).

The STRUCTURE analysis, which included all individuals from the six micropopulations, clearly differentiated the individuals from Rihaniya from those from Gush Halav. A separate analysis of Rihaniya revealed that most individuals were divided according to the soil they were captured from. However, a few animals with mixed origin from different soils were also found in both soils (Fig. 3B). Li et al. (2015) performed a genome-wide divergence analysis on five and six blind mole rats from the rendzina and basaltic soils in Rihaniya and found one individual likely to be a recombinant from both soil types. Based on a much larger sample size, we identified several recombinants of studied categories, including migrants, F1, F2 hybrids, and backcrosses, in both soil types (see Fig. 4B). Among the migrants, six individuals out of 25 animals in rendzina soil (24%) were confirmed to be migrants from basaltic soil while only two individuals out of 35 animals in the basaltic soil (6%) were identified as migrants from rendzina soil (Fig. 4B, 5A).

Animals from four micropopulations in Gush Halav were somewhat divided by their location of capture (see Fig. 3E). Nevertheless, we found that the genetic profiles of ten out of 129 individuals suggested a genetic mixture of both the rendzina-soil and basaltic-soil genotypes. Two females were confirmed to have migrated from nearby sampling micropopulations, with approximate distances of 650 m and 380 m from their original micropopulations. In contrast, one male dispersed approximately 1,700 m from his original micropopulation (Fig. 5B). Migration between neighbouring micropopulations is not uncommon, as they are near each other (see Fig. 5B). In this context, the dispersal abilities of particular species are of utmost importance. Although *N. galili* typically disperses underground (Rado et al. 1992), there is evidence that it can also disperse aboveground for relatively long distances. For example, Tzur et al. (2009) discovered that at the same locality, half of the genetically related individuals (half-siblings and full siblings) were separated by at least 1,500 m, or even by more than 2,000 m, which is clear evidence of aboveground dispersal. Aboveground dispersal over relatively long distances has been documented in many other subterranean rodents for several hundred meters or even a few kilometres (Bray et al. 2013, Šklíba et al. 2020, Finn et al. 2022). The relatively low number of hybrids in our study could also be related to the limited chances of successful dispersal in subterranean rodents. Their morphological and ecological adaptations to underground life may hinder their ability to disperse successfully, especially when travelling longer distances above the ground, where they inevitably face a higher risk of predation (e.g. Rado et al. 1992, Waser et al. 1994, Braude 2000, but see Finn et al. 2022 for successful long-distance dispersal in Damaraland mole-rats).

At both sites, the migration of mole rats from basaltic soil to rendzina soil is more frequent than in the opposite direction. This finding seems to be a typical case of metapopulation theory (Levins 1969), which suggests that metapopulations are spatially structured populations consisting of discrete units (subpopulations) that are separated by space or barriers and linked through dispersal movements (Levins 1969, Hanski 1998). The metapopulation concept revolves around source-sink dynamics, where the balance between subpopulation demographic and dispersal rates determines source and sink subpopulations (Pulliam 1988). Source subpopulations are typically located in areas of high habitat quality where birth rates exceed death rates. On the other hand, sink subpopulations are usually found in areas of reduced habitat quality where death rates exceed birth rates. The metapopulation concept in subterranean rodents is recognised in the genus *Ctenomys* (tuco-tucos). These animals have limited mobility and are typically found in patches with low local effective population numbers (Lacey et al. 2000). Several studies have described the metapopulation pattern in tuco-tucos, with a focus

on habitat fragmentation and their dispersal abilities (Mapelli & Kittlein 2009, Gómez Fernández et al. 2016, Mapelli et al. 2020). In this context, the five-fold higher population density of mole rats in basaltic soil than in rendzina soil (10 ind./ha *vs*. 2 ind./ha, Lövy et al. 2015) suggests that mole rats living in basaltic soil may serve as a source population for those living in rendzina soil. Indeed, Li et al. (2015) and our observations documented the crossing of the rendzina/basalt interface through the observation of a mound row extending from the basaltic to rendzina soil (see Li et al. 2015, our observation). To better understand factors influencing population dynamics in *N. galili* between the two soil types, further investigations are needed based on long-term capture-recapture or radio-tracking studies focused on population growth and survival rates.

Detection of hybrids

Hybrid individuals between mole rats from different soils were found at both study localities. Hybridisation between closely related species can result in reduced fitness (Barton 2001) or even sterility (Cabot et al. 1994, Forejt 1996, Widmayer et al. 2020), which can limit the exchange of genetic variants between species. Our samples support the presence of not only F1 hybrid individuals but also a few F2 hybrids. The existence of the F2 hybrids demonstrates that F1 hybrids are capable of reproduction. The presence of hybrid *N. galili* animals coexisting with genetically pure individuals raises the question of the mechanisms that control potential ecological speciation and if such a mechanism exists.

In tephritid fruit flies (*Rhagoletis mendax*), hybrids coexist in sympatry with both parental species but are adapted to different microenvironments, feeding and mating on different flowering plants. This adaptation helps them avoid competition with parental species, maintain fitness, and achieve reproductive isolation (Schwarz et al. 2005). Studies on crater lake cichlid fishes have shown that reproductive isolation during sympatric ecological speciation develops gradually (Kautt et al. 2020, Olave et al. 2022). Olave et al. (2022) identified a rare case of homoploid hybrid speciation (i.e. hybrid speciation without a change in ploidy) in sympatry in Midas cichlid fishes. The hybrid lineage is genomically and phenotypically diverged from both parental species, despite being at an early stage of speciation. This study found that, similarly to *N. galili*, hybrids and backcrosses were present. The authors (Olave et al. 2022) suggest that postzygotic rather than prezygotic isolation mechanisms may play an important role in maintaining the distinctness of the hybrid lineage. The occurrence of hybridisation in *N. galili* may not be frequent, as evidenced by a relatively low number of hybrids and backcrosses found in the sample (Gush Halav: four hybrids and two backcrosses from 129 individuals, Rihaniya: five hybrids and one backcross from 60 individuals). Further investigation into the hybrid individuals is necessary.

Implication for ecological speciation

Recent research has suggested incipient ecological speciation in *N. galili* based on genetic, ecological, and behavioural parameters related to their occurrence in different soil types (e.g. Hadid et al. 2013, Li et al. 2020, Lövy et al. 2017). The evolution of reproductive isolation is a fundamental part of each speciation event. Assortative mating in relationship to a particular soil type is believed to evolve due to divergent selection acting on resource use, niche, food, and mate choices, thus facilitating ecological speciation (Hadid et al. 2013). Although assortative mating based on female mate choice in *N. galili* has not been proven (Lövy et al. 2020), genetic studies suggest the existence of incomplete reproductive isolation due to reduced gene flow between mole rats from the two soil types (Hadid et al. 2013, Li et al. 2015, 2020). Nevertheless, Li et al. (2015) used a smaller sampling size, with only 11 individuals, one of which was identified as a recombinant. As our study includes 189 individuals, the chances of identifying more recombinants also increase. Indeed, the presence of 15 hybrids observed in the present study suggests that gene flow and recombination might be sufficiently high to homogenise genetic variation.

The studies mentioned above (Hadid et al. 2013, Li et al. 2015, 2020) demonstrated genetic and genomic divergence in blind mole rats, which may suggest the existence of adaptive genetic diversification through divergent natural selection operating directly on the mtDNA genome or genomic loci. Significant differences in mtDNA were found between rendzina- and basaltic-soil mole rats, with up to 40% of the mtDNA diversity being dependent on soil type (Hadid et al. 2013). It has been established that changes in mtDNA may be subject to divergent ecological selection driven by differences between the two soil types. If the groups are distinguishable by mtDNA formed recently, the longer coalescence times of nuclear loci (microsatellites) will prevent them from corroborating mtDNA patterns (Moore 1995, Zink & Barrowclough 2008). Adaptive genetic changes in *N. galili* may be masked by relatively high gene flow at

the level of neutral loci, such as microsatellite markers. Muñoz et al. (2013) described a porous genome where adaptive portions remain fixed while neutral portions are homogenised by gene flow in anoles from the Lesser Antilles. In addition, several studies have reported that ecological speciation in anoles appears to have stalled at different stages (Losos 2009, Thorpe et al. 2010), presumably because gene flow among populations inhibits further progress towards speciation. It is possible that the loci affecting fitness in different habitats are not the same as loci affecting traits involved in reproductive isolation (Muñoz et al. 2013). Further research is required to understand the mechanisms behind the population divergence in *N. galili* at the studied localities.

Conclusions

This study employed population genetic structure analysis to gain insight into the genetic divergence in *N. galili* living in two ecologically distinct soil types, which may drive ecological speciation. Based on microsatellite analyses, we detected a certain level of gene flow between the two soil-associated micropopulations, along with the presence of fertile inter-soil hybrids. Our findings suggest that assortative mating between mole rats from the two soil types is likely incompletely developed. If ecological speciation occurs in *N. galili*, it may be driven by other mechanisms, such as divergent natural selection acting directly on the mtDNA genome or specific genomic loci. Since reduced gene flow between potentially interbreeding populations is the key signature of speciation, genetic and genomic methods are valuable tools for studying reproductive isolation and decreasing gene flow in the studied species. Therefore, examining the entire genome of *N. galili* from different soil habitats at focal sites is essential. This information would help determine whether gene flow reduction between animals from the two soil types is linked to specific loci under positive selection or affects the entire genome, potentially contributing to speciation. In addition to genomic approaches, further research on morphological and physiological traits and their roles in potential ecological speciation in *N. galili* is also needed.

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Author Contributions

L. Dovičicová: writing – original draft, data analyses, review & editing; M. Lövy: conceptualisation, data collection, review & editing; J. Bryja: conceptualisation, data curation, review & editing; E. Nevo: conceptualisation, review & editing; R. Šumbera: conceptualisation, writing – original draft, data collection, review & editing.

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Supplementary online material

Table S1. Rihaniya, list of captured animals (ID, GPS, Sex, Body mass, Soil type).

Table S2. Gush Halav 2014, list of captured animals (ID, GPS, Sex, Body mass, Soil type).

Table S3. Gush Halav 2015, list of captured animals (ID, GPS, Sex, Body mass, Soil type).

Table S4. The number of alleles (Na) in microsatellite loci and the frequency of null alleles.

Fig. S1. A three-dimensional plot of the FCA performed using GENETIX. Blind mole rats from different populations are indicated with different colours: Blue- R_12b, Yellow- R_12c, Grey- G_14b, Green- G_15b, White- G_14r, Pink- G_15r.

Fig. S2. Result from Structure Harvester to determine the most likely K value using the ΔK method in Rihaniya.

Fig. S3. Result from Structure Harvester to determine the most likely K value using the ΔK method in Gush Halav.

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