DNA Sequence Variation in the Mitochondrial Control Region of Subterranean Mole Rats, *Spalax ehrenbergi* Superspecies, in Israel

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The complete mitochondrial control region was sequenced for 60 individuals representing different populations for each of the four species of the subterranean mole rat *Spalax ehrenbergi* superspecies in Israel: *Spalax galili* (2n = 52), *S. golani* (2n = 54), *S. carmeli* (2n = 58), and *S. judaei* (2n = 60). The control region of all species and populations is very similar both in length (979 to 983 bp) and in base composition. As in agreement with previous surveys on mitochondrial control regions on mammals, the mole rat control region can be divided into a central domain and two flanking domains, ETAS (extended termination associated sequences) and CSB (conserved sequence blocks). Along with the common conserved blocks found in these domains (ETAS1, ETAS2, CSB1, CSB2, and CSB3), we have also detected in all individuals an ETAS1-like and a CSB1-like element, both in the ETAS domain. The most conserved region was the central domain, followed by the CSB and ETAS domains, showing important differences in the four species analyzed. Phylogenetic analysis supported the existence of two clades. One clade contained individuals belonging to *Spalax galili* (2n = 52) and *S. golani* (2n = 54), separated in two different branches depending on the species. The other clade contained individuals belonging to *S. carmeli* (2n = 58) and *S. judaei* (2n = 60) mixed together, suggesting a more recent event of speciation. Within species we have observed a southward trend of increasing variability. These results have been explained as a consequence of the adaptation of the species to ecological factors such as aridity and temperature stresses.

Introduction

Mammalian mitochondrial genome is extremely compact, with some overlapping genes, a few intergenic bases, and two noncoding regions that account for less than 7% of the entire genome. The main noncoding region, also called the D-loop region, has been the subject of several surveys that have provided a detailed characterization of the overall structure in mammals and other vertebrates (e.g., Saccone, Attimonelli, and Sbisà 1987; Lee et al. 1995; Sbisà et al. 1997; Randi and Lucchini 1998; Sumida et al. 2000; Larizza et al. 2002). Three major regions or domains have been described in the mammalian control region: (1) the Central domain, (2) the region containing the extended termination associated sequences (ETAS domain), and (3) the region including the conserved sequence blocks (CSB domain). The central domain is the most conserved region, even between distantly related species. The ETAS region is associated with the termination of the nascent H strand during replication and includes two conserved blocks, ETAS1 and ETAS2 (Sbisà et al. 1997), that contain the TAS elements previously described by Doda, Wright, and Clayton (1981). The CSB domain contains the replication origin of the heavy H strand, the promoters for the transcription of the heavy and light strand, and three conserved blocks, CSB1, CSB2, and CSB3, presumably involved in priming H-strand replication (Walberg and Clayton 1981).

Comparative studies carried out in the control region of mammals have revealed that each of the three domains presents a distinct pattern of variation, with some regions evolving rapidly, such as the ETAS and CSB domains, and others maintaining a high degree of conservation across taxa, such as the central domain (Brown et al. 1986; Sbisà et al. 1997; Pesole et al. 1999). This rate heterogeneity allows the use of the control region for the study of the patterns of genetic variation at different levels of divergence within mammals. Thus, the most variable regions have been used for detailed studies of population and phylogeographic structure (Goldberg and Ruvolo 1997; Matsuhashi et al. 1999; Rosel et al. 1999; Koh, Lee, and Kocher 2000; Matson and Baker 2001), whereas conserved regions may prove to be useful for phylogenetic studies of more divergent taxa (Douzery and Randi 1997; Larizza et al. 2002). In spite of this, just a few studies have been carried out on the structure and patterns of variation in rodent species (Faulkes et al. 1997; Prager, Orrego, and Sage 1998; Matson and Baker 2001).

The subterranean mole rats of the *Spalax ehrenbergi* superspecies in Israel include four species that only very recently have been named formally: *Spalax galili* (2n = 52), *S. golani* (2n = 54), *S. carmeli* (2n = 58), and *S. judaei* (2n = 60) (Nevo, Ivanitskaya, and Beiles 2001). The oldest fossil record of this superspecies is dated 1.4 MYA from the "Ubeidiya Formation," an Early Pleistocene hominid site in the Jordan Valley (Tchernov 1987). Since then, a prolific diversification of the *S. ehrenbergi* superspecies has taken place, but the past 120,000 years of the Upper Pleistocene probably better reflect the active speciation process (Tchernov 1968). Thus, nowadays, these four species represent progressive stages of late speciation and constitute an excellent example of ecological speciation and adaptive radiation. The adaptive radiation of mole rats in Israel, from the Early Pleistocene to recent times, is closely associated with fossoriality, increasing aridity and progressive deforestation, and savannization, and hence with distinct climatic diversity in both the Mediterranean and steppic climatic regimes

Key words: *Spalax ehrenbergi*, mitochondrial DNA, control region, speciation, adaptation, molecular evolution.

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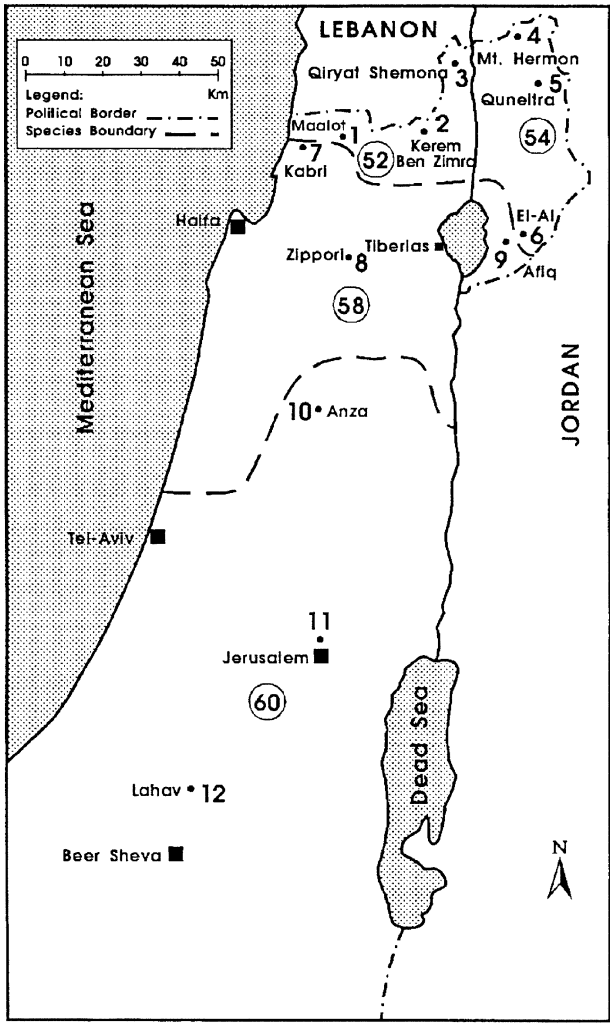

FIG. 1.—Geographical distribution of the four species belonging to *Spalax ehrenbergi* superspecies in Israel: *Spalax galili* (2n = 52), *S. golani* (2n = 54), *S. carmeli* (2n = 58), and *S. judaei* (2n = 60). The location of the 12 populations examined herein for the mitochondrial control region is also reported.

(Nevo 1991, 1999; Nevo, Ivanitskaya, and Beiles 2001). *S. galili* (2n = 52) is present in the cool and humid Upper Galilee Mountains; *S. golani* (2n = 54) ranges in the cool and semidry Golan Heights; *S. carmeli* (2n = 58) is distributed in the warm and humid Lower Galilee Mountains, Central Yizreel, and the coastal plain; and *S. judaei* (2n = 60) colonizes the warm and dry mountains of Samaria, Judea, the northern Negev, the southern part of the Jordan Valley, and the coastal plain (fig. 1). Several different studies, especially those based on DNA-DNA hybridization (Catzeflis et al. 1989; Nevo 1991), indicate that *S. golani* (2n = 54) is the oldest species, originating about 1.6 MYA. The split to *S. galili* (2n = 52) occurred next, about 0.35 MYA. *S. carmeli* (2n = 58) and *S. judaei* (2n = 60) are the most recent derivatives, with speciation event estimated at 0.2 MYA.

In the present paper, we have carried out a detailed characterization of the control region structure of the *S. ehrenbergi* superspecies. Patterns of variation across the major control region domains were also examined both within and between species. In addition, the observed diversity has been used to establish (1) the phylogenetic relationships among populations and species and (2) the correlations between genetic variability and ecogeographical factors that could be involved in the speciation and adaptation processes.

Materials and Methods

Specimens Examined

The complete mitochondrial control region was sequenced for 60 individuals representing different populations for each of the four species of the mole rat *Spalax ehrenbergi* superspecies in Israel: *Spalax galili* (2n = 52), *S. golani* (2n = 54), *S. carmeli* (2n = 58), and *S. judaei* (2n = 60). Three populations have been sampled for each species, and at least five individuals per population have been tested in almost all populations. Figure 1 shows the geographic distribution of the four species and the location of the 12 populations sampled in this survey, while table 1 summarizes some ecogeographic characteristics of these populations.

DNA Extraction, Amplification, and Sequencing

Genomic DNA was isolated from rodent liver or kidney tissues according to the technique outlined by Hillis et al. (1990). Using the polymerase chain reaction (PCR), the entire mitochondrial DNA (mtDNA) control region plus tRNAPro and tRNAPhe genes were directly amplified with primers 1FR (5′-TAATTACCC-TGGTCTTGTAA-3′) and 4RV (5′-CTAATAATAAGG-CCAGGACC-3′), annealing in the tRNAThr and 12S rRNA genes, respectively (fig. 2). Primers were designed on the basis of the complete mitochondrial DNA sequence of *Spalax judaei* (2n = 60) (unpublished data).

Double-stranded PCR amplifications were performed in 100 μl reaction volumes containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0,001% (w/v) gelatin, 0.25 mM of each dNTP, 0.5 μM of each primer, and 2.5 U of TaqGold polymerase (PerkinElmer). PCR cycling conditions were 10 min of hot start at 95°C for the activation of the enzyme, followed by 30 amplification cycles (45 s of denaturation at 95°C, 45 s of primer annealing at 50°C, and 1 min 30 s of extension at 72°C), followed by a final cycle of 7 min at 72°C. A single amplification product of about 1,200 bp was always obtained. Before sequencing, these products were purified using the Amicon Microcon-PCR Centrifugal Filter Devices (Millipore) following the manufacturer's instructions.

The complete mtDNA control region (979 to 983 bp) was double-strand sequenced using primers 1FR and 4RV described above and four additional primers (fig. 2), 2FR (5′-TACCATCCTCCGTGAAACCA-3′), 2RV (5′-TGGTTTCACGGAGGATGGTA-3′), 3FR (5′-AG-TCTAGCTGGACTTGTATG-3′), and 3RV (5′CATACAAGTCCAGCTAGACT-3′), designed on the basis of the complete mitochondrial genome of *Spalax judaei* (2n = 60) (unpublished data). Sequencing reactions were performed using the Thermo Sequenase Cy 5.5 Dye

Table 1
Ecogeographical and Climatological Data for 12 Populations of *Spalax ehrenbergi* Superspecies in Israel

			Geographical[c]		Temperatures[d]				Water Availability[e]			Eolaphic[f]	Biotic Factors[g]		
Species	Population[a]	Type[b]	Ln	Lt	Tm	Td	Tdd	Sh	Ra	Rd	Huan	So	Cv	Dn	Te
S. galili	1. Maalot	NHZ	35.27	33.00	16.8	15.0	8.8	63	785	53	64	1	85	31	59
(2n = 52)	2. Kerem-Ben-Zimra	C	35.47	33.03	16.5	16.3	8.5	85	650	59	60	1, 3	88	40	52
	3. Qiryat Shemona	M	35.57	33.22	19.0	16.5	10.3	65	655	60	61	1, 4	90	23	52
S. golani	4. Mt. Hermon	M	35.73	33.30	12.4	17.1	5.0	80	1450	65	61	1	80	32	56
(2n = 54)	5. Quneitra	C	35.83	33.12	14.9	16.6	9.6	71	857	65	61	3	40	22	54
	6. El Al	NHZ	35.75	32.80	18.7	16.4	11.1	24	464	52	57	3	100	39	54
S. carmeli	7. Kabri	NHZ	35.15	33.02	20.0	13.5	10.1	35	600	50	66	1, 2	75	30	54
(2n = 58)	8. Zippori	C	35.28	32.74	18.5	15.4	12.0	50	500	53	63	2	75	26	44
	9. Afiq	NHZ, M	35.70	32.77	18.8	16.2	11.0	24	460	51	57	3	70	17	47
S. judaei	10. Anza	NHZ	35.22	32.35	18.0	14.9	9.6	65	630	46	62	2	65	22	74
(2n = 60)	11. Jerusalem	M	35.23	31.78	17.5	15.2	9.0	104	500	42	62	1	45	24	91
	12. Lahav	C	34.87	31.38	18.8	14.6	12.0	60	303	33	58	5	20	15	92

[a] Populations are numbered according to figure 1.
[b] Type of population: C, central; M, marginal; NHZ, near hybrid zone.
[c] Geographical data: Ln, longitude in decimals; Lt, latitude in decimals.
[d] Temperatures: Tm, mean annual temperature (°C); Td, season temperature difference (°C); Tdd, day-night temperature difference (°C); Sh, mean number of hot and dry days.
[e] Water availability: Ra, mean annual rainfall (mm); Rd, mean number of rainy days; Huan, mean annual humidity (%).
[f] Eolaphic: So, soil type; 1, terra rossa; 2, rendzina; 3, basalt; 4, alluvium; 5, loess.
[g] Biotic data: Cv, plant cover (%); Dn, mole rat population density (animals per 10,000 m^2); Te, territory size (m^2).

Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech) in 8 μl reaction volumes and following the manufacturer's instructions. After purification, DNA sequences were analyzed on a Seq4X4 automated sequencer (Amersham Pharmacia Biotech). Complete control region sequences have been deposited in EMBL under accession numbers AJ440417 to AJ440466.

Data Analyses

Complete mtDNA control regions were multialigned using the PILEUP program from GCG (1994). The alignment of the sequences was corrected by eye inspection, even though it was quite straightforward due to the high degree of similarity of the sequences and the low number of indels (table 2).

Control regions domains (ETAS, Central, and CSB domains) were identified and demarcated on the basis of the data obtained from a comprehensive survey carried out on several mammals (Sbisà et al. 1997). The presence of conserved blocks such as ETAS1, ETAS2, CSB1, CSB2, and CSB3 was investigated using the PatSearch program (Pesole, Liuni, and D'Souza 2000).

The positions undergoing variation between sequences were evidenced and variability at these sites was calculated using the SiteVar program (Pesole and Saccone 2001). In order to determine the nature of the genetic change (i.e., the number and type of transitions and transversions), the data were analyzed using the PAUP program (Swofford 1998).

To investigate the origin, genetic structure, and relationships between and within the four species, several algorithms available in the ARLEQUIN package (Schneider, Roessli, and Excoffier 2000) were used. In particular, estimates of the nucleotide diversity and the analysis of molecular variance (AMOVA) that allows the partitioning of the observed variability in a hierarchical fashion were carried out with this package.

Phylogenetic relationships between individuals were established on the basis of a Neighbor-Joining tree applied to a distance matrix obtained using the Markov Stationary Method (also called the GTR method) implemented in the PAUP 4.0b10 package (Swofford 1998). Another Neighbor-Joining tree was obtained from a GTR distance matrix using a proportion of invariant sites (I) and a gamma distribution for variable sites (Γ), being both parameters estimated from the data. Maximum Parsimony analysis was performed using PAUP 4.0b10 (Swofford 1998). Bootstrap values were based on 1,000 replicates.

Results

Control Region Organization

As in agreement with previous surveys on mitochondrial control regions on mammals, the mole rat control

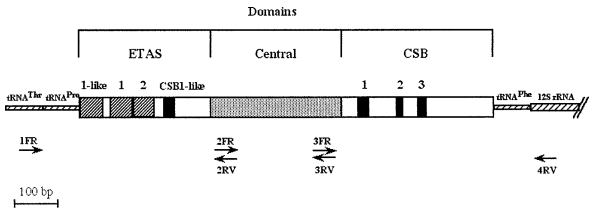

FIG. 2.—Schematic diagram of the mitochondrial control region of *Spalax ehrenbergi* superspecies. Locations of the extended termination associated sequence (ETAS), central, and conserved sequence block (CSB) domains are delimited. Conserved elements across mammalian control regions, such as ETAS1 and ETAS2 (dark gray box), CSB1, CSB2, and CSB3 (black box), and characteristics of the ETAS and CSB domains, respectively, are presented. ETAS1-like and CSB1-like repeated elements, present in the ETAS domain, are also shown. Arrows identify the location and direction of the amplification and sequencing primers.

Table 2
Main Characteristics of the Control Regions of 12 Populations of *Spalax ehrenbergi* Superspecies

Species	Population[a]	N(hap)[b]	Length	%A	%C	%G	%T	Var. site[c]	TS[d]	TS Pur[e]	TS Pyr[f]	TSPyr/Pur	TV[g]	TS/TV	π^h ($\times 10^{-2}$)
S. galili	1. Maalot	5(4)	982	32.99	22.71	12.59	31.71	19	18	9	9	1.00	1	18.00	1.04 ± 0.67
(2n = 52)	2. Kerem Ben Zimra	6(4)	982	33.40	22.88	12.14	31.59	12	11	5	6	1.20	1	11.00	0.46 ± 0.30
	3. Qiryat Shemona	4(3)	982	33.45	22.84	12.25	31.47	15	13	6	7	1.17	1	13.00	0.81 ± 0.57
S. golani	4. Hermon	5(3)	981	32.72	22.83	12.56	31.89	15	12	4	8	2.00	3	4.00	0.61 ± 0.41
(2n = 54)	5. Quneitra	6(4)	981	32.94	22.94	12.30	31.82	18	15	2	13	6.50	3	5.00	0.99 ± 0.61
	6. El Al	5(5)	981	32.78	23.08	12.58	31.56	21	14	5	9	1.80	7	2.00	1.06 ± 0.68
S. carmeli	7. Kabri	6(6)	979–981	33.08	22.20	12.26	32.45	21	13	7	6	0.86	8	1.63	0.99 ± 0.61
(2n = 58)	8. Zippori	5(5)	981–983	32.88	22.34	12.45	32.33	53	45	15	30	2.00	9	5.00	2.61 ± 1.62
	9. Afiq	2(1)	981	33.44	22.7	12.33	31.50	—	—	—	—	—	—	—	—
S. judaei	10. Anza	5(5)	981	32.80	22.37	12.48	32.35	43	34	14	20	1.43	9	3.78	2.39 ± 1.48
(2n = 60)	11. Jerusalem	5(5)	981	32.84	22.53	12.42	32.21	50	45	18	27	1.50	9	5.00	2.64 ± 1.64
	12. Lahav	6(6)	981	32.82	22.44	12.37	32.36	18	15	3	12	4.00	5	3.00	0.91 ± 0.57

[a] Populations are numbered according to figure 1.
[b] Number of individuals sampled per population; hap, number of different haplotypes found per population.
[c] Number of observed variable sites.
[d] Number of observed transitions.
[e] Number of observed purine transitions.
[f] Number of observed pyrimidine transitions.
[g] Number of observed transversions.
[h] Nucleotide diversity.

region can be divided in three domains: a highly conserved central domain and the ETAS and CSB flanking domains (figs. 2 and 3).

The central domain ranges from position 313 to position 626 of the multialignment. This is the most conserved part of the whole control region, not only between different mole rat species but also when all rodents are taken into consideration. Indeed, only 24 (7.7%) of the 313 nucleotide positions were variable when all four species are considered. At species level, six, seven, 12, and 12 variable positions were found in *S. galili* (2n = 52), *S. golani* (2n = 54), *S. carmeli* (2n = 58), and *S. judaei* (2n = 60), respectively (fig. 3), with an average sequence dissimilarity of 1.05%. In the case of all complete rodent control regions so far available, the mean pairwise dissimilarity was 22.6%, considering only interspecies comparisons.

In the ETAS domain, ranging from position 1 to 312 of the alignment, three conserved blocks have been found: ETAS1, ETAS2, and ETAS1-like. The ETAS1 is located between positions 81 and 139 of the multialignment and shows 15 variable positions out of a total of 59 (25.42%) for all species and four, four, five, and five variable positions in *S. galili* (2n = 52), *S. golani* (2n = 54), *S. carmeli* (2n = 58), and *S. judaei* (2n = 60), respectively (fig. 3). The consensus ETAS1 shows 18.05% dissimilarity with the consensus obtained from rodent species (data not shown). An ETAS1-like sequence is found to be present from position 1 to 59 of the multialignment (fig. 3). The consensus of the ETAS1-like sequence shows a 32.43% dissimilarity with the consensus ETAS1 of mole rats and a 36.07% dissimilarity with other ETAS1 from rodents (data not shown). The degree of variation of this ETAS1-like sequence is similar to that of ETAS1, with 14 variable positions out of 59 (23.72%) for all species and four, four, seven and seven variable positions in 2n = 52, 54, 58, and 60 species, respectively (fig. 3). ETAS2 is located between positions 141 and 197 of the multialignment and shows a higher degree of variability than the previous elements. Indeed, 23 of 57 (40.35%) nucleotide positions were variable for the whole data set and nine, 10, nine, and 11 variable positions in *S. galili* (2n = 52), *S. golani* (2n = 54), *S. carmeli* (2n = 58) and *S. judaei* (2n = 60), respectively (fig. 3). In agreement with the higher degree of variation, the dissimilarity of the consensus ETAS2 with that of rodent species is 22.41% (data not shown).

In the CSB domain, ranging from position 627 to position 983 of the multialignment, three conserved blocks, CSB1, CSB2, and CSB3, have been found. CSB1 is located between positions 665 and 688 of the alignment and shows only two variable positions out of 24 (8.33%), in *S. carmeli* (2n = 58) and *S. judaei* (2n = 60), exclusively (fig. 3). The high degree of conservation of this block is also stressed by the fact that the consensus sequence for all species shows just a 12.00% dissimilarity with the consensus obtained for all rodent species (data not shown). The other two conserved blocks, CSB2 and CSB3, placed at positions 754 to 770 and 805 to 822 of the multialignment, respectively, displayed no variable positions in mole rats (fig. 3). The dissimilarity with the

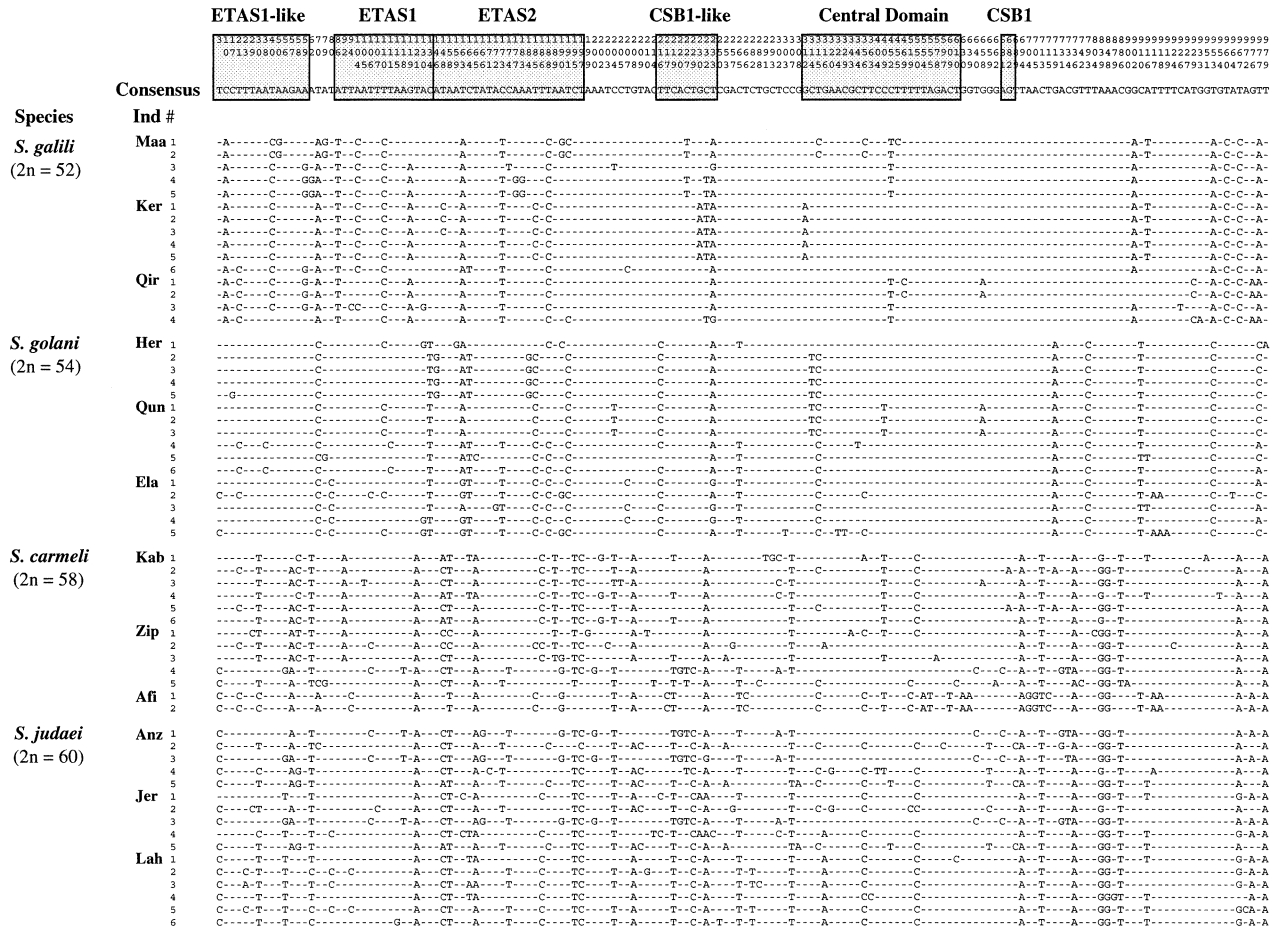

FIG. 3.—Sequence alignment for the 60 complete mitochondrial control regions of the four species of *Spalax ehrenbergi* superspecies in Israel: *Spalax galili* (2n = 52), *S. golani* (2n = 54), *S. carmeli* (2n = 58), and *S. judaei* (2n = 60). Only polymorphic sites are represented, and they refer to a majority-rule consensus sequence. Locations of the different conserved elements across mammalian control regions are shaded.

consensus sequence for rodent species was 11.76% and 0.00% for CSB2 and CSB3, respectively (data not shown). In addition to the previously documented CSB elements, we have also identified a CSB1-like element. This element, however, is not located in the CSB domain, but in the ETAS domain, between positions 215 and 239. This block shows a relatively low degree of dissimilarity with CSB1 both from mole rats (28%) and from the consensus of other rodent species (20%). However, it is less conserved than the CSB1, and nine of 25 (36%) positions were variable (fig. 3).

Control Region Length and Base Composition

The control region of all species and populations is very similar both in length and base composition. The length of the control region of most sequences is 981 bp. It is noteworthy that all individuals from *S. galili* (2n = 52) present a 1-bp insertion in position 60 of the alignment, resulting in control regions of 982 bp. Only three other individuals show length variation: one from Zippori, which presented a 2-bp insertion in position 59 of the alignment (983 bp), and two from Kabri, which showed a 2-bp deletion in position 61 of the alignment (979 bp).

Regarding base composition, only small differences are detected between populations and species (table 2). All taxa showed similar biased base composition in the three domains of the control region: A>T>C>G in the ETAS domain (38.50%, 33.95%, 18.78%, and 8.77%, respectively), T>C>A>G in the central domain (30.68%, 26.65%, 23.60%, and 19.07%, respectively), and A>T>C>G in the CSB domain (36.46%, 31.39%, 22.48%, and 9.6%, respectively). As observed, G is strongly avoided in the two peripheral domains, but it is surprisingly high in the central domain, which is in agreement with previous observations made in other rodent species and in mammals in general.

Site Variability Heterogeneity Between Populations

The number of variable sites differed significantly across the control region, the central domain being the most conserved, followed by the CSB and the ETAS domains (figs. 3 and 4). This general pattern of variability distribution was observed in all four species, but important differences between species were detected (fig. 4). *S. galili* (2n = 52) represents the species with the lowest site variability, regarding both the number of variable sites and

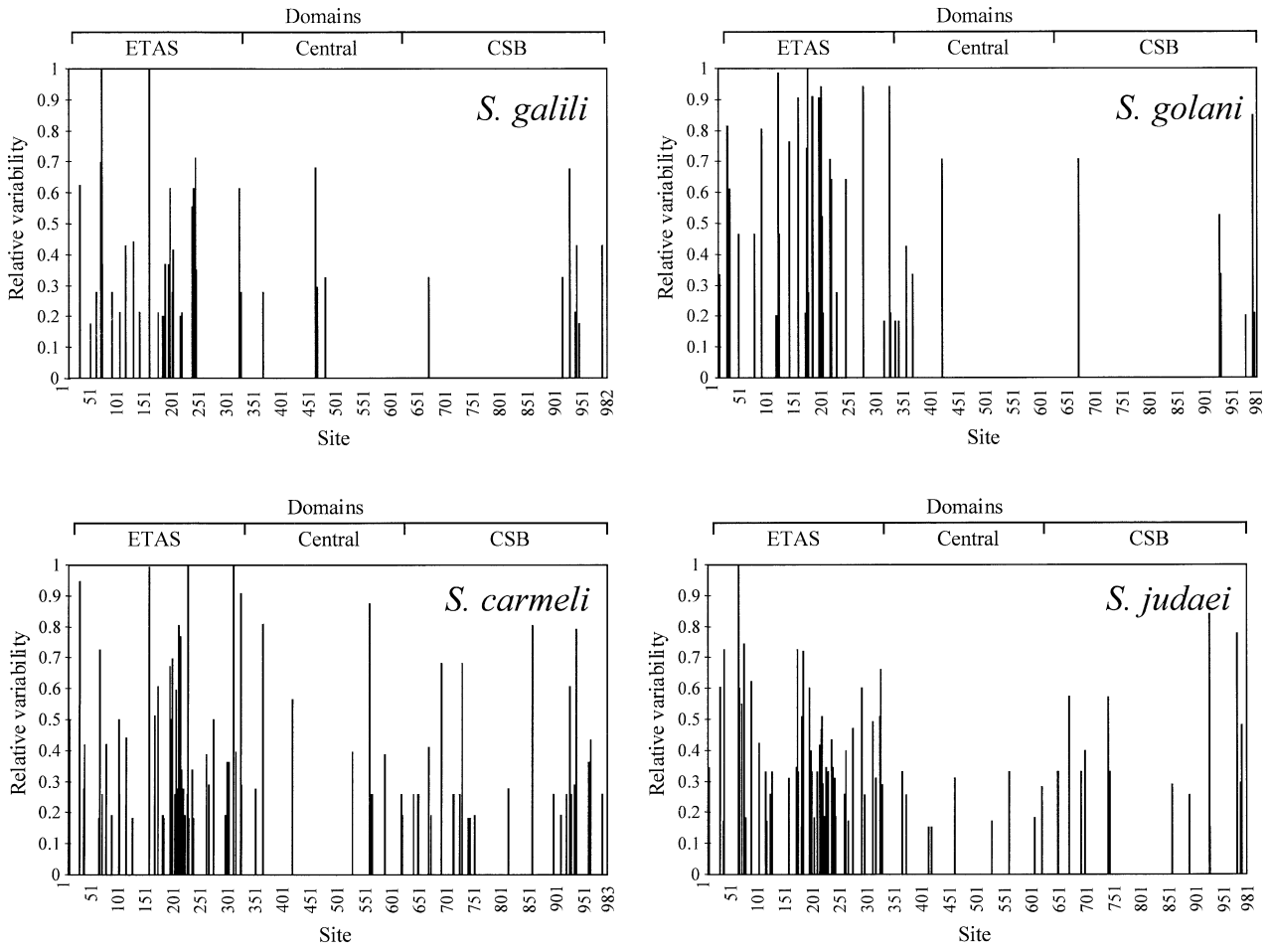

FIG. 4.—Site-specific relative variability calculated using the SiteVar software on the complete mitochondrial control regions of the four species of *Spalax ehrenbergi*: *Spalax galili* (2n = 52), *S. golani* (2n = 54), *S. carmeli* (2n = 58), and *S. judaei* (2n = 60). Locations of the ETAS, central, and CSB domains are also presented.

the relative variability per site. *S. golani* (2n = 54) shows a slight higher degree of variability in the ETAS domain, mainly due to higher values of relative variability rather than to a higher number of variable sites (fig. 4 and table 2). In the case of *S. carmeli* (2n = 58) and *S. judaei* (2n = 60), we detected a higher number of variable sites in all three domains with respect to the other two species (fig. 4 and table 2).

Furthermore, important differences are detected not only at the level of number of variable sites and relative variability but also at the level of type and pattern of mutations. Thus, variation in the control region was split into transitions (TS) and transversions (TV), and then pyrimidine (Pyr) and purine (Pur) transitions were scored (table 2). Important differences in the number of transitions, the ratio of TS-Pyr/TS-Pur, and the number of transversion are observed between and within species. *S. galili* (2n = 52) is characterized by a TS-Pyr/TS-Pur ratio close to 1.00 and significant high values of TS/TV ratio, compared with the other three species ($P = 0.001$ to 0.018). In the case of *S. golani* (2n = 54), similar values of transitions are detected, but the ratio TS-Pyr/TS-Pur is significantly biased toward higher TS-Pyr ($P = 0.049$), and also higher values of transversions are found ($P = 0.021$). In *S. carmeli* (2n = 58) and *S. judaei* (2n = 60), we detected populations with a number of transitions similar to those found in *S. golani* (2n = 54) and other populations displaying higher values but always significantly higher than in *S. galili* (2n = 52) ($P = 0.018$–0.024). In any case, the TS-Pyr/TS-Pur and TS/TV ratios are maintained close to those of *S. golani* (2n = 54) (table 2).

The pattern of transition and transversion accumulation between populations and species was further investigated by plotting the number of TS against the number of TV (fig. 5). Linear regression and correlation between TS and TV is detected ($y = 2.32x + 6.44$; $r = 0.88$, $P < 0.001$) for comparisons involving individuals of the same population (open circles), populations of the same species (filled circles), and species with similar chromosomal number (open triangles), that is, *S. galili* (2n = 52) against *S. golani* (2n = 54) and *S. carmeli* (2n = 58) against *S. judaei* (2n = 60). However, for comparisons involving populations with different chromosomal number (filled triangles), that is, *S. galili* (2n = 52) and *S. golani* (2n = 54) against *S. carmeli* (2n = 58) and *S. judaei* (2n = 60), the slope of the curve decreases ($y = 1.22x + 12.28$; $r = 0.84$, $P < 0.0001$), most likely reflecting the

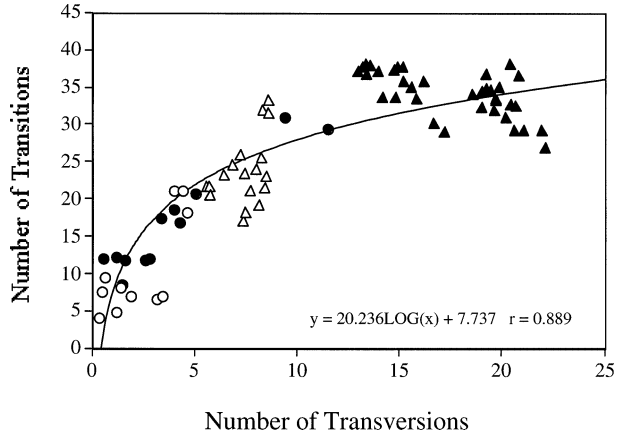

FIG. 5.—Evolution of the total percentage of transitions relative to the total percentage of transversions. The points correspond to mean values involving intrapopulation comparisons (open circle); intraspecies comparisons (filled circle); closely related species comparisons (open triangle), that is, *S. galili* (2n = 52) against *S. golani* (2n = 54) and *S. carmeli* (2n = 58) against *S. judaei* (2n = 60); and distantly related species comparisons (filled triangle), that is, *S. galili* (2n = 52) and *S. golani* (2n = 54) against *S. carmeli* (2n = 58) and *S. judaei* (2n = 60). The nonlinear regression through these points is a logarithmic type, y = 20.34logx + 7.74 ($r = 0.89$, $P < 0.001$). The curve can be approximated by the intersection of two linear regression plots: the first involving intrapopulation, intraspecies, and closely related species comparisons and the second involving distantly related species. This shows the beginning of saturation transitions relative to transversions.

beginning of the saturation of TS relative to TV. The curve that better explains all points is a logarithmic type, y = 20.34logx + 7.74 ($r = 0.89$, $P < 0.001$), which clearly shows the beginning of saturation transitions relative to transversions.

Phylogenetic Analysis

Fifty-one haplotypes were identified from the analysis of 60 individuals representing four species of *S. ehrenbergi*. The phylogenetic tree constructed using either distance or parsimony methods revealed the clustering of individuals into three groups supported by high bootstrap values (fig. 6). Individuals belonging to the *S. galili* (2n = 52) species are clustered together in a monophyletic group (bootstrap value, 100%). *S. golani* (2n = 54) specimens are also clustered together in a monophyletic group (bootstrap value, 78% to 94%), and they are placed as a sister group of *S. galili*. In both cases, individuals are clustered in a bushlike fashion, and branch lengths are relatively short, suggesting little sequence divergence within each species. Regarding *S. carmeli* (2n = 58) and *S. judaei* (2n = 60) individuals, there is no clear separation of populations on the basis of the chromosomal number, but they are clearly separated from the other two species (bootstrap value, 100%). In addition, branch lengths are higher than in the other species, and relationships between individuals are better resolved, suggesting a higher degree of sequence divergence.

Results of the AMOVA analysis have revealed that 27.04% of the total observed variation is due to within-populations variation, which is in agreement with a moderate level of variability within populations observed

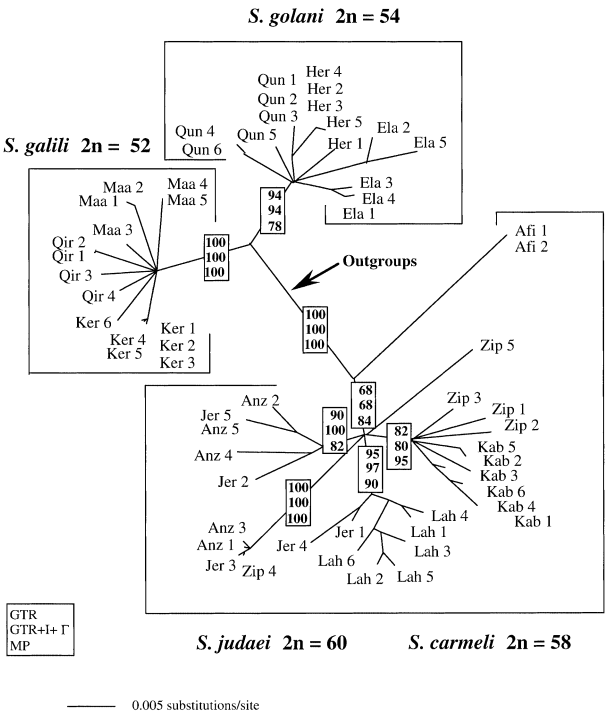

FIG. 6.—Phylogenetic tree describing the evolutionary relationships between 60 individuals belonging to *Spalax ehrenbergi* superspecies in Israel: *Spalax galili* (2n = 52), *S. golani* (2n = 54), *S. carmeli* (2n = 58), and *S. judaei* (2n = 60), as deduced from the comparison of complete mitochondrial control regions. Branch lengths are proportional to the distances as derived from the distance matrix obtained using the GTR method from PAUP. Bootstrap values, based on 1,000 replications, for Neighbor-Joining GTR (GTR), Neighbor-Joining GTR plus invariant sites and gamma distribution (GTR+I+G), and Maximum Parsimony (MP) are shown. The arrow indicates the position where the outgroups (rat and mouse) cluster when using a different mitochondrial molecular marker such as cytochrome b (Nevo, Beiles, and Spradling 1999).

at the level of number of different haplotypes or nucleotide diversity (table 2). Differences among populations within species account for only 15.13% of the variation, and, thus, individuals from the same population are not clustered together in monophyletic groups (fig. 6). The highest percentage of the variance (57.83%) is due to differences among species. This is in agreement with the existence of three different clusters, two of them corresponding to each of the two species *S. galili* (2n = 52) and *S. golani* (2n = 54), and the third cluster including the other two species, *S. carmeli* (2n = 58) and *S. judaei* (2n = 60) (fig. 6).

Ecogeographic Pattern of Diversity

Levels of genetic diversity have been found to vary between and within species of *S. ehrenbergi*. The clearest difference is the one observed between northern, *S. galili* (2n = 52) and *S. golani* (2n = 54), and southern, *S. carmeli* (2n = 58) and *S. judaei* (2n = 60), populations. As mentioned above, the number of variable sites and the relative variability per site are higher in southern than in northern populations (fig. 4). Furthermore, the levels of nucleotide diversity (table 2) are also on the average higher in southern populations. This trend is detected even within

each species. For example, in the case of *S. golani* (2n = 54), nucleotide diversity from northern to southern Golan populations are 0.0061 in Hermon, 0.0099 in Quneitra, and 0.0106 in El Al (table 2). Two particular cases are Afiq, which, due to the low sample size, presents only one haplotype, and Lahav, which, compared with previous estimates, displays a lower level of diversity than expected, most likely due to sampling variations. Thus, regarding the general trend, population nucleotide diversity is highly and significantly correlated with latitude ($y = -45.62x + 33.46$; $r = -0.84$, $P = 0.0025$) when Afiq and Lahav populations are not considered in the analysis (table 1 and 2).

Climatic conditions in Israel are highly determined by the latitude, and thus, the lower the latitude, the lower the mean annual rainfall and the number of rainy days, and the higher the temperature and the day-night temperature differential. Therefore, when we searched for correlations between climatic parameters and levels of genetic variability (table 1 and 2), we found a significant correlation between nucleotide diversity and mean number of rainy days ($y = -664.84x + 63.54$; $r = -0.73$, $P = 0.0164$) and moderate correlations, even though not significant, with both day-night and season temperature differences ($r = -0.41$, $P = 0.2341$ and $r = 0.41$, $P = 0.2336$), always with the exclusion of Afiq and Lahav populations.

Furthermore, observed nucleotide diversity in the mitochondrial control region is significantly correlated with estimates of cytochrome b variability ($y = 335.62x - 2.46$; $r = 0.83$, $P = 0.003$) (Nevo, Beiles, and Spradling 1999), all four nuclear allozyme diversity measures, *A*, *P*, *H*, and *He* ($r = 0.67$ to 0.82, $P = 0.033$ to 0.0033) (Nevo, Filippucci, and Beiles 1994), and RAPD number of polymorphic loci ($y = 143.53x + 3.75$; $r = 0.66$, $P = 0.0371$) (Ben-Shlomo, Fahima, and Nevo 1996), with the exclusion of Afiq and Lahav populations.

Discussion

Conserved Features of the *Spalax ehrenbergi* Control Region

The control region of *Spalax ehrenbergi* superspecies follows the general structure described in mammals in general and in other species of rodents in particular (Brown et al. 1986; Saccone, Attimonelli, and Sbisà 1987; Sbisà et al. 1997; Matson and Baker 2001; Larizza et al. 2002). The division of this region into three domains, ETAS, Central, and CSB, is highly supported by our data of base composition, the presence of conserved blocks, and site variability heterogeneity (figs. 2–4).

Important differences in base composition have been detected in the three different domains. However, they follow the general trend described for mammals (Sbisà et al. 1997) and the same base content within statistical fluctuations in *Clethrionomys* species (Matson and Baker 2001). It is noteworthy to mention the relatively high G content of the central domain (19.07%), which is higher than that observed in both the other two domains and the whole mitochondrial genome of mammals (Reyes et al. 1998). Moreover, the presence of the dinucleotide and trinucleotide GG and GGG (14 and four, respectively) is higher than expected at random (11 and two, respectively), as also described for other rodent species (Larizza et al. 2002). The presence of such motifs suggests that this region could be involved in the interaction between mitochondria and cytoskeletal elements (Jackson, Barlett, and Cook 1996).

In addition to the differences in base composition, the three described domains show important differences in site variability (figs. 3 and 4). The central domain is the most conserved region (only 7.7% variable sites), which is in agreement with previous studies (Sbisà et al. 1997; Larizza et al. 2002), and its level of conservation has been shown to be similar to that of mitochondrial 12S and 16S rRNAs, tRNAs, and nonsynonymous sites of protein-coding genes (Pesole et al. 1999). This high degree of conservation contrasts with the apparent lack of function of this region, even though its possible involvement in the interaction with cytoskeletal elements (Jackson, Barlett, and Cook 1996) could partially explain such conservation. The two peripheral domains, ETAS and CSB, are less conserved (28.53% and 13.17% variable sites, respectively), the former being more variable than the latter (fig. 3 and 4) and almost reaching the level of variability found in the synonymous positions of protein-coding genes (Pesole et al. 1999). However, within these regions, highly conserved blocks are found: ETAS1, ETAS2, ETAS1-like, CSB1, CSB2, CSB3, and CBS1-like (figs. 2 and 3). Some of these elements have been reported to be conserved across different mammalian orders (Brown et al. 1986; Saccone, Attimonelli, and Sbisà 1987; Sbisà et al. 1997), and different functions have also been proposed based on experimental evidence (Doda, Wright, and Clayton 1981; Chang and Clayton 1987; Ghivizzani et al. 1993; Tullo et al. 1995). ETAS1 has been proposed as the region containing multiple processing and/or termination signals functioning bidirectionally, whereas ETAS2 could contain the binding sites for termination factors (Sbisà et al. 1997). Nevertheless, a recent survey carried out in rodents has showed that only ETAS1 is present in all species and that ETAS2 could not be present or be much less conserved (Larizza et al. 2002). In the case of *Spalax* species, ETAS1 is much more conserved than ETAS2 (25.42% and 40.35% variable sites, respectively), as also found in closely related species of *Clethrionomys* (Matson and Baker 2001), giving further support to the idea that ETAS2 could be functionally less important or shows a species-specific evolutionary pattern. A similar pattern is observed in the case of CSB blocks, where CSB1 is the least conserved block even though it has been suggested to be the most important from the point of view of functionality, since it is the only one present in all species of mammals examined so far (Sbisà et al. 1997; Matson and Baker 2001; Larizza et al. 2002).

An ETAS1-like and a CSB1-like element have been identified in the ETAS domain (fig. 2). The presence of ETAS1-like elements has been described in different species of mammals and even within rodent species (Sbisà et al. 1997; Larizza et al. 2002). The relatively low degree of dissimilarity with ETAS1 (36.06% in the case of *Spalax*

species) suggests that they could be derived from this element by a slippage event. All four species of *Spalax* present this ETAS1-like element, and it is quite conserved in all of them, supporting the idea of a single slippage event before the speciation. It is important to note that the position of the ETAS1-like element corresponds with the position where indels have been found. Regarding the CSB1-like element, it shows a low degree of dissimilarity with CSB1 (28.0%) but is located in the ETAS domain. CSB1-like elements have been described in different mammals, always in the CSB domain (Sbisà et al. 1997); only in the case of *Clethrionomys* species have they been found in the ETAS domain (Matson and Baker 2001). While the most likely hypothesis for their origin is slippage event, the underlying mechanism that led to the location of this CSB1-like element in the ETAS domain remains unclear.

Phylogenetic Content and Pattern of Diversity of the Control Region

Transitions were shown to be at the beginning of saturation with respect to transversions when distantly related populations are involved in the comparison (fig. 5), as previously observed for the control regions of other mammals (e.g., Wills 1995; Douzery and Randi 1997). At the species level, a bias toward transitions over transversions was observed, but important differences were detected in the four *Spalax ehrenbergi* species, with ratio values ranging from 18 to 1.63 (table 2). This bias has also been observed in other rodent species such as *Rattus* (Brown et al. 1986) and *Clethrionomys* (Matson and Baker 2001). Moreover, within transition, we have detected a significant bias toward pyrymidine over purine transitions in most populations that reach a ratio of 6.5 to 1, as in the case of Quneitra (table 2). These results are in agreement with those found in *Clethrionomys* (Matson and Baker 2001), where a significant bias toward pyrymidine over purine transitions are also observed. However, they differ from the results obtained in *Rattus*, where no pyrimidine transitional bias is detected (Brown et al. 1986). This different behavior of murid rodents, when compared with other rodent species, has also been observed at the level of change of nucleotide composition in both whole mitochondrial genome (Karlin and Mrazek 1997) and nuclear genes (e.g., Cortopassi and Wang 1996; Gissi et al. 2000; Michaux, Reyes, and Catzeflis 2001).

Despite these differences in the mutational pattern in the different species of mole rats, the analysis of the control region has proven to be suitable for establishing phylogenetic relationships among individuals, populations, and species (fig. 6). According to these figures, populations can be divided into two groups: northern populations belonging to *Spalax galili* (2n = 52) and *S. golani* (2n = 54) showing a clear separation of these two species, and southern populations from *S. carmeli* (2n = 58) and *S. judaei* (2n = 60) where the degree of speciation is less evident. These results are in agreement with the postulated speciation process for these species, that is, a progressive southward trend of late speciation (Nevo 1991, 1999; Nevo, Ivanitskaya, and Beiles 2001). According to this, northern species represent the oldest stages of speciation, whereas southern species represent more recent stages of speciation. Indeed, we can see that northern populations are nonoverlappingly clustered according to the species they belong to, whereas southern populations are mixed, suggesting a more recent speciation event (fig. 6). These results are also confirmed on the basis of other molecular markers (Catzeflis et al. 1989; Nevo et al. 1993; Nevo, Filippucci, and Beiles 1994; Ben-Shlomo, Fahima, and Nevo 1996; Nevo, Beiles, and Spradling 1999).

According to the neutral theory, older species are expected to show higher levels of genetic variability than younger ones. However, we have found higher nucleotide diversity values, number of haplotypes, and relative variability (table 2, fig. 4) in southern young species (*S. carmeli* [2n = 58] and *S. judaei* [2n = 60]) than in northern old species (*Spalax galili* [2n = 52] and *S. golani* [2n = 54]). These results are also supported by allozyme, DNA-DNA hybridization, mtDNA RFLPs, DNA minisatellite fingerprinting, RAPD, and cytochrome b sequence data (Catzeflis et al. 1989; Nevo et al. 1993; Nevo, Filippucci, and Beiles 1994; Ben-Shlomo, Fahima, and Nevo 1996; Nevo, Beiles, and Spradling 1999). Therefore, we can say that the observed pattern of distribution of gene variability is nonrandom, and it has been explained as a consequence of the adaptation of the species to ecological factors (Nevo 1991, 1999; Nevo, Ivanitskaya, and Beiles 2001). Aridity and temperature show a clinal distribution in Israel, with higher values in the south than in the north (table 1). Adaptation of *Spalax* species and populations to these ecologically different environments from the Pleistocene up to the present might have resulted in a north to south trend of variability. Thus, a significant correlation between nucleotide diversity in the control region of *Spalax* populations and latitude has been found and subsequently with the mean number of rainy days and to some extent with seasonal temperature differences.

This adaptation process has also involved many other variables regarding morphology (skull and body variables) (Nevo, Tchernov, and Beiles 1988; Nevo 1991) and physiology (basic metabolic rates, nonshivering thermogenesis, thermoregulation, respiration, heartbeat and breathing frequency, and hematocrite and hemoglobin concentration) (Nevo and Shkolnik 1974; Arieli et al. 1984; Arieli et al. 1986*a*, 1986*b*; Haim et al. 1984; Haim, Heth, and Nevo 1985). All these adaptations would be directed to the optimization of the energetic balance across the geographic range of *Spalax* species (Nevo 1991, 1999; Nevo, Ivanitskaya, and Beiles 2001). On one hand, adaptation is directed to the reduction of water expenditure and overheating in xeric environments and in environments that are hot during the daytime and the summer. On the other hand, thermoregulation enables better resistance to the cold environment during the winter and the night. Behavioral adaptations, including activity, exploratory, habitat selection, and aggression patterns also contribute to the optimization of the energy. Thus, for example, species living in a cooler climate, *S. galili* (2n = 52) and *S. golani* (2n = 54), display less rest periods than those living in warmer climates, that is, *S. carmeli* (2n = 58) and *S. judaei* (2n = 60) (Nevo et al. 1982). Aggression intensity also

varies geographically and, at least partly, in accordance with aridity and temperature. Aggressive phenotype frequency decreases southward: *S. galili* (2n = 52) > *S. golani* (2n = 54) > *S. carmeli* (2n = 58) > *S. judaei* (2n = 60) (Nevo 1986; Nevo, Heth, and Beiles 1986).

The importance of adaptation to the clinal distribution of ecological factors found in Israel is also highlighted by the fact that many unrelated species of plants, invertebrates, and vertebrates show the same north to south trend of variability (Nevo 1988). In these cases, southern populations display higher levels of genetic variability. The fact that diverse species sharing similar ecological distribution show a genetic parallelism gives further support to the idea that selection is involved in the genetic differentiation of populations in Israel, including the control region of *Spalax* mitochondrial genome.

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Literature Cited

Arieli, R., M. Arieli, G. Heth, and E. Nevo. 1984. Adaptive respiratory variation in 4 chromosomal species of mole rats. Experientia **40**:512–514.

Arieli, R., G. Heth, E. Nevo, and D. Hoch. 1986*a*. Hematocrit and hemoglobin concentration in four chromosomal species and some isolated populations of actively speciating subterranean mole rats in Israel. Experientia **42**:441–443.

Arieli, R., G. Heth, E. Nevo, Y. Zamir, and O. Neutra. 1986*b*. Adaptive heart and breathing frequencies in 4 ecologically differentiating chromosomal species of mole rats in Israel. Experientia **42**:131–133.

Ben-Shlomo, R., T. Fahima, and E. Nevo. 1996. Random amplified polymorphic DNA of the *Spalax ehrenbergi* superspecies in Israel. Isr. J. Zool. **42**:317–326.

Brown, G., G. Gadaleta, G. Pepe, C. Saccone, and E. Sbisà. 1986. Structural conservation and variation in the D-loop-containing region of vertebrate mitochondrial DNA. J. Mol. Biol. **192**:503–511.

Catzeflis, F. M., E. Nevo, J. E. Ahlquist, and C. G. Sibley. 1989. Relationships of the chromosomal species in the Eurasian mole rats of the *Spalax ehrenbergi* group as determined by DNA-DNA hybridisation, and an estimate of the spalacid-murid divergence time. J. Mol. Evol. **29**:223–232.

Chang, D., and D. A. Clayton. 1987. A novel endoribonuclease cleaves at a priming site of mouse mitochondrial DNA replication. EMBO J. **6**:409–417.

Cortopassi, G. A., and E. Wang. 1996. There is substantial agreement among interspecies estimates of DNA repair activity. Mech. Ageing Dev. **91**:211–218.

Doda, J. N., C. T. Wright, and D. A. Clayton. 1981. Elongation of displacement-loop strands in human and mouse mitochondrial DNA is arrested near specific template sequences. Proc. Natl. Acad. Sci. USA **78**:6116–6120.

Douzery, E., and E. Randi. 1997. The mitochondrial control region of Cervidae: evolutionary patterns and phylogenetic content. Mol. Biol. Evol. **14**:1154–1166.

Faulkes, C. G., D. H. Abbott, H. P. O'Brien, L. Lau, M. R. Roy, R. K. Wayne, and M. W. Bruford. 1997. Micro- and macro-geographical genetic structure of colonies of naked mole-rats *Heterocephalus glaber*. Mol. Ecol. **6**:615–628.

GCG. 1994. Program manual for the GCG package, Madison, Wis.

Ghivizzani, S. C., S. L. D. Mackay, C. S. Madsen, P. J. Laipis, and W. W. Hauswirth. 1993. Transcribed heteroplasmic repeated sequences in porcine mitochondrial DNA D-loop region. J. Mol. Evol. **37**:36–47.

Gissi, C., A. Reyes, G. Pesole, and C. Saccone. 2000. Lineage-specific evolutionary rate in mammalian mtDNA. Mol. Biol. Evol. **17**:1022–1031.

Goldberg, T. L., and M. Ruvolo. 1997. The geographic apportionment of mitochondrial genetic diversity in east African chimpanzees, *Pan troglodytes schweinfurthii*. Mol. Biol. Evol. **14**:976–984.

Haim, A., G. Heth, Z. Avnon, and E. Nevo. 1984. Adaptive physiological variation in nonshivering thermogenesis and its significance in speciation. J. Comp. Physiol. B **154**:145–147.

Haim, A., G. Heth, and E. Nevo. 1985. Adaptive thermoregulatory patterns in speciating mole rats. Acta Zool. Fennica **170**: 137–140.

Hillis, D. M., A. Larson, S. K. Davis, and E. A. Zimmer. 1990. Nucleic acids III: sequencing. Pp. 318–370 *in* D. M. Hillis and C. Moritz, eds. Molecular systematics. Sinauer Associates, Sunderland, Mass.

Jackson, D. A., J. Barlett, and P. R. Cook. 1996. Sequences attaching loops of nuclear and mitochondrial DNA to underlying structures in human cells: the role of transcription units. Nucleic Acids Res. **24**:1212–1219.

Karlin, S., and J. Mrazek. 1997. Compositional differences within and between eukaryotic genomes. Proc. Natl. Acad. Sci. USA **94**:10227–10232.

Koh, H. S., W. J. Lee, and T. D. Kocher. 2000. The genetic relationships of two subspecies of striped field mice, *Apodemus agrarius coreae* and *Apodemus agrarius chejuensis*. Heredity **85**:30–36.

Larizza, A., G. Pesole, A. Reyes, E. Sbisà, and C. Saccone. 2002. Lineage specificity of the evolutionary dynamics of the mtDNA D-loop region in rodents. J. Mol. Evol. **54**:145–155.

Lee, W. J., J. Conroy, W. H. Howell, and T. D. Kocher. 1995. Structure and evolution of teleost mitochondrial control regions. J. Mol. Evol. **41**:54–66.

Matson, C. W., and R. J. Baker. 2001. DNA sequence variation in the mitochondrial control region of red-backed voles (*Clethrionomys*). Mol. Biol. Evol. **18**:1494–1501.

Matsuhashi, T., R. Masuda, T. Mano, and M. C. Yoshida. 1999. Microevolution of the mitochondrial DNA control region in the Japanese brown bear (*Ursus arctos*) populations. Mol. Biol. Evol. **16**:676–684.

Michaux, J., A. Reyes, and F. Catzeflis. 2001. Evolutionary history of the most speciose mammals: molecular phylogeny of muroid rodents. Mol. Biol. Evol. **18**:2017–2031.

Nevo, E. 1986. Evolutionary behavior genetics in active speciation and adaptation of fossorial mole rats. Acad. Naz. Lincei **259**:39–109.

———. 1988. Natural selection in action: the interface of ecology and genetics in adaptation and speciation at the molecular and organismal levels. Pp. 411–438 *in* Y. Yom-Tov and E. Tchernov, eds. The zoogeography of Israel. Dr. Junk Publishers, Dordrecht, Netherlands.

———. 1991. Evolutionary theory and processes of active speciation and adaptive radiation in subterranean mole rats, *Spalax ehrenbergi*, superspecies in Israel. Evol. Biol. **25**:1–125.

———. 1999. Mosaic evolution of subterranean mammals: regression, progression, and global convergence. Oxford University Press, Oxford.

Nevo, E., A. Beiles and T. Spradling. 1999. Molecular evolution

of cytochrome b of subterranean mole rats, *Spalax ehrenbergi* superspecies, in Israel. J. Mol. Evol. **49**:215–226.

Nevo, E., M. G. Filippucci, and A. Beiles. 1994. Genetic polymorphisms in subterranean mammals (*Spalax ehrenbergi* superspecies) in the Near East revisited: patterns and theory. Heredity **72**:465–487.

Nevo, E., R. Guttman, M. Haber, and E. Erez. 1982. Activity patterns of evolving mole rats. J. Mammal. **63**:453–463.

Nevo, E., G. Heth, and A. Beiles. 1986. Aggression patterns in adaptation and speciation of subterranean mole rats. J. Genet. **65**:65–78.

Nevo, E., R. L. Honeycutt, H. Yonekawa, K. Nelson, and N. Hanzawa. 1993. Mitochondrial DNA polymorphisms in the subterranean mole rats of the *Spalax ehrenbergi* superspecies in Israel and its peripheral isolates. Mol. Biol. Evol. **10**: 590–604.

Nevo, E., E. Ivanitskaya, and A. Beiles. 2001. Adaptive radiation of blind subterranean mole rats: naming and revisiting the four sibling species in Israel: *Spalax galili* (2n = 52), *S. golani* (2n = 54), *S. carmeli* (2n = 58); *S. judaei* (2n = 60). Backhuys Publishers, Leiden, The Netherlands.

Nevo, E., and A. Shkolnik. 1974. Adaptive metabolic variation of chromosome forms in mole rats, *Spalax*. Experientia **30**: 724–726.

Nevo, E., E. Tchernov, and A. Beiles. 1988. Morphometrics of speciating mole rats: adaptive differentiation in ecological speciation. Zeit. Zool. Syst. Evol. **26**:286–314.

Pesole, G., C. Gissi, A. De Chirico, and C. Saccone. 1999. Nucleotide substitution rate of mammalian mitochondrial genomes. J. Mol. Evol. **48**:427–434.

Pesole, G., S. Liuni, and M. D'Souza. 2000. PatSearch: A pattern matcher software to find functional elements in nucleotide and protein sequences and to assess statistical significance. Bioinformatics **16**:439–450.

Pesole, G., and C. Saccone. 2001. A novel method for estimating substitution rate variation among sites in a large dataset of homologous DNA sequences. Genetics **157**: 859–865.

Prager, E. M., C. Orrego, and R. D. Sage. 1998. Genetic variation and phylogeography of Central Asian and other house mice, including a major new mitochondrial lineage in Yemen. Genetics **150**:835–861.

Randi, E., and V. Lucchini. 1998. Organization and evolution of the mitochondrial DNA control region in the avian genus *Alectoris*. J. Mol. Evol. **47**:449–462.

Reyes, A., C. Gissi, G. Pesole, and C. Saccone. 1998. Asymmetrical directional mutation pressure in the mitochondrial genome of mammals. Mol. Biol. Evol. **15**: 957–966.

Rosel, P. E., S. C. France, J. Y. Wang, and T. D. Kocher. 1999. Genetic structure of harbour porpoise *Phocoena phocoena* populations in the northwest Atlantic based on mitochondrial and nuclear markers. Mol. Ecol. **8**:S41–S54.

Saccone, C., M. Attimonelli, and E. Sbisà. 1987. Structural elements highly preserved during the evolution of the D-loop-containing region in vertebrate mitochondrial DNA. J. Mol. Evol. **26**:205–211.

Sbisà, E., F. Tanzariello, A. Reyes, G. Pesole, and C. Saccone. 1997. Mammalian mitochondrial D-loop region structural analysis: identification of new conserved sequences and their functional and evolutionary implications. Gene **205**: 125–440.

Schneider, S., D. Roessli, and L. Excoffier. 2000. Arlequin: a software for population genetics data analysis. Version 2.000, Genetics and Biometry Laboratory, Department of Anthropology, University of Geneva, Geneva, Switzerland.

Sumida, M., H. Kaneda, Y. Kato, Y. Kanamori, H. Yonekawa, and M. Nishioka. 2000. Sequence variation and structural conservation in the D-loop region and flanking genes of mitochondrial DNA from Japanese pond frogs. Genes Genet. Syst. **75**:79–92.

Swofford, D. L. 1998. PAUP*: phylogenetic analysis using parsimony (*and other methods). Version 4. Sinauer Associates, Sunderland, Mass.

Tchernov, E. 1968. Succession of rodent faunas during the Upper Pleistocene of Israel. Paul Parey, Hamburg, Germany.

———. 1987. The age of the "Ubeidiya Formation," an Early Pleistocene hominid site in the Jordan Valley, Israel. Isr. J. Earth Sci. **36**:3–30.

Tullo, A., W. Rossmanith, E. M. Imre, E. Sbisà, C. Saccone, and R. M. Karwan. 1995. RNase mitochondrial RNA processing cleaves RNA from the rat mitochondrial displacement loop at the origin of heavy-strand DNA replication. Eur. J. Biochem. **227**:657–662.

Walberg, M. W., and D. A. Clayton. 1981. Sequence and properties of the human KB cell and mouse L cell D-loop regions of the mitochondrial DNA. Nucleic Acids Res. **9**:5411–5421.

Wills, C. 1995. When did Eve live? An evolutionary detective story. Evolution **49**:593–607.

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