Original article

Myosin-heavy-chain DNA polymorphisms of subterranean mole rats of the *Spalax ehrenbergi* superspecies in Israel

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Summary – Restriction-fragment-length polymorphisms (RFLPs) of the sarcomeric-myosin heavy chain multigene family were studied in 13 populations of 4 chromosomal species ($2n = 52, 54, 58$ and 60) of the mole rat *Spalax ehrenbergi* superspecies in Israel. A minimum number of 6 hybridizing fragments occurred in the mole rat, corresponding to a minimum of 6, probably even 8, different loci of the sarcomeric myosin heavy chain. The level of polymorphism was $\approx 31\%$, with a low number of alleles in the polymorphic loci. All of the populations, including a desert isolate, were polymorphic at some level. Among-sample fragment variation was adequate to differentiate between some of the chromosomal species, and confirms earlier evidence that gene flow between the chromosomal species is limited.

myosin heavy chain / RFLP / *Spalax ehrenbergi* = mole rat

Résumé – Polymorphismes de l'ADN de la chaîne lourde de la myosine chez des rats-taupes souterrains de la superespèce *Spalax ehrenbergi* en Israël. *Les polymorphismes de restriction (RFLP) de la famille multigénique de la chaîne lourde de la myosine sarcomérique ont été étudiés dans 13 populations de 4 espèces chromosomiques (*2n = 52, 54, 58 et 60*) de la superespèce de rat-taupe* Spalax ehrenbergi *en Israël. Il existe chez le rat-taupe au moins 6 fragments qui peuvent s'hybrider, correspondant à au moins 6, probablement même 8, locus différents de la chaîne lourde de la myosine sarcomérique. Le niveau moyen de polymorphisme est de 31% environ, avec un faible nombre d'allèles aux locus polymorphes. Toutes les populations, y compris un isolat dans un désert, sont polymorphes à quelque degré. La variation des fragments entre échantillons est suffisante pour différencier quelques-unes des espèces, et confirme des résultats précédents quant à des flux géniques limités entre ces espèces chromosomiques.*

chaîne lourde myosine / RFLP / *Spalax ehrenbergi* = rat-taupe

INTRODUCTION

Subterranean mole rats of the *Spalax ehrenbergi* superspecies live in underground sealed runways most of their lives. Several reviews describe the multidisciplinary studies on the *S ehrenbergi* complex, both in terms of adaptation and speciation (Nevo, 1985, 1986a,b, 1989). The complex is comprised of 4 chromosomal species ($2n = 52, 54, 58$ and 60) displaying final stages of chromosomal speciation, as evidenced by narrow hybrid zones (Nevo and Bet-El, 1976), and assortative mating (Nevo, 1985). Hybrid zones along the species boundaries (fig 1) decrease in width progressively northward from 2.8 km (between $2n = 58 - 60$) to 0.7 km (between $2n = 54 - 58$) to 0.3 km (between $2n = 52 - 58$).

The adaptive radiation of the *S ehrenbergi* complex is closely associated with aridity gradients. Hence, this adaptive radiation is associated with distinct climatic diversity: $2n = 52$ radiated in the cool-humid upper Galilee mountains; $2n = 54$ in the cool-semi-dry Golan heights; $2n = 58$ in the warm-humid lower Galilee mountains, central Yizrael valley and coastal plains; and $2n = 60$ in the warm-dry mountains of Samaria, Judea, northern Negev and the southern part of the Jordan valley and coastal plain (fig 1).

Surveys of allozyme polymorphisms in the *S ehrenbergi* superspecies, as well as in other subterranean mammals (Nevo *et al*, 1984) revealed low levels of allozyme polymorphisms. This was explained as an adaptive strategy to the relatively constant microclimate underground. In general, DNA polymorphisms display far more variation, as compared with allozyme polymorphisms of metabolically vital enzymes.

Analyses of restriction fragment length polymorphisms (RFLPs) in *Spalax ehrenbergi* reveal, as expected, a higher level of polymorphism. Diversity was found in the non-transcribed spacer of ribosomal DNA (Suzuki *et al*, 1987) and in mitochondrial DNA (Nevo *et al*, 1993). A very high level of RFLPs and a high number of *per locus* alleles per loci were found in the major histocompatibility complex (Nizetic *et al*, 1985; Ben-Shlomo *et al*, 1988) and in the *Period*-homologous sequence (Ben-Shlomo *et al*, 1993). On the other hand, a low level of polymorphism was found in the haptoglobin gene (Nevo *et al*, 1989). In the present study, we examined the RFLPs of the sarcomeric myosin heavy chain (MHC) gene family.

Myosin is the major structural component of the contractile apparatus of the muscle. The myosins are proteins that interact with actin to convert chemical energy into mechanical work. A myosin molecule consists of 2 heavy chains ($\approx$ 200 kDa) and 2 pairs of light chains (for review on myosin structure, aggregation properties and its role in force generation, see Harrington and Rodgers 1984). Myosin developed very early in the evolution of eukaryotic organisms (Clarke and Spudich, 1977; Warrick *et al*, 1986).

Myosin has been classified into 3 major categories according to their abundance in different tissues and cellular compartments: sarcomeric muscle, smooth muscle and non-muscle myosins. In vertebrates, the different sarcomeric MHC isoforms are encoded by a multigene family of closely related members (Nguyen *et al*, 1982; Strehler *et al*, 1986; Mahdavi *et al*, 1987). There is no cross-hybridization between sarcomeric MHC and smooth muscles and non-muscle MHC (Nguyen *et al*, 1982; Leinwand *et al*, 1983). Each MHC gene displays a pattern of expression
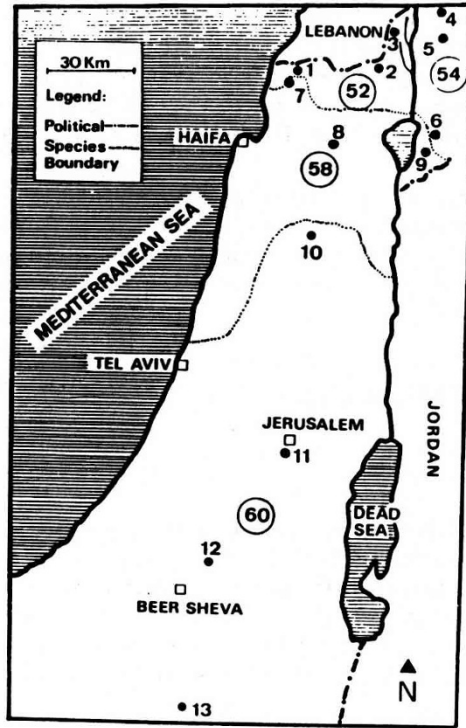
Fig 1. Geographic distribution of the chromosomal species of the *Spalax ehrenbergi* superspecies in Israel. Numbers in circles indicate chromosomal species. Other numbers identify sampling localities defined in table I.

that is tissue- and developmental-stage specific, both in cardiac and skeletal muscles (Mahdavi *et al*, 1987).

Different numbers of MHC genes have been found in different organisms. Extensive hybridization of the rat sarcomeric MHC gene probe was observed in DNA of all metazoan organisms tested from nematode to man (Nguyen *et al*, 1982). The simplest pattern of only one MHC sequence was observed in 2 species of sea urchins: *Strongylocentrotus purpuratus* and *Lytechinus pictus*. The most complex pattern was found in the goldfish *Carassius auratus* and in the chicken, with up to 30 hybridizing fragments (Nguyen *et al*, 1982). Mammalian species (human, mouse, chinese hamster, rat and rabbit) exhibit an intermediate complexity with 7 – 13 hybridizing bands (Nguyen *et al*, 1982; Maeda *et al*, 1987). The gene in the rat is comprised of 24×10^3 bases of DNA and is split into 41 exons (Strehler *et al*, 1986). In the rabbit, the gene is even longer, *ie* ≈ 25 kb (Friedman *et al*, 1984).

No populational analyses have yet been conducted on MHC polymorphism in wild mammals. A low level of RFLP, located in the flanking region, was found in human skeletal MHC (Leinwand *et al*, 1983; Schwartz *et al*, 1986). Digestion with *Msp*I endonuclease (4-base recognition site) yielded 3 different alleles. Some

allelic polymorphism of MHC genes was found in the rat (Nguyen *et al*, 1982). Different strains of laboratory rats exhibited distinct hybridization patterns both in cardiac- and skeletal-specific sequences. Weydert *et al* (1985) found RFLPs that differentiated between 2 mouse species; *Mus musculus* and *Mus spretus*. The markers were found in skeletal MHC (embryonic, perinatal and adult) as well as in cardiac MHC genes.

Spalax ehrenbergi superspecies may be an excellent model for examining patterns of genetic variation in mammals. The objectives of the present research are to estimate the number of sarcomeric myosin heavy chain genes in the mole rat and to estimate the extent of RFLP divergence within and between the 4 chromosomal species.

MATERIALS AND METHODS

Sampling

We examined DNA polymorphisms of the myosin heavy chain genes in 121 mole rats sampled from 13 populations representing all 4 chromosomal species of the *Spalax ehrenbergi* superspecies in Israel (localities are plotted in figure 1, and sample sizes and other relevant data are summarized in table I). Live animals were caught in their underground runways, brought to the laboratory and dissected. Tissues were instantly frozen in liquid nitrogen and then stored at $-80°C$.

Table I. Samples of *Spalax ehrenbergi* examined in this study; numbers (N) refer to localities plotted in figure 1.

Chromosomal species	N	Locality	No of animals	Type of population
$2n = 52$	1	Maalot	8 – 9	Near hybrid zone (NHZ)
	2	Kerem Ben Zimra	8 – 10	Central
	3	Qiryat Shemona	8 – 10	Marginal
$2n = 54$	4	Hermon	8 – 10	Marginal
	5	Quneitra	8 – 10	Central
	6	El-Al	6 – 9	NHZ
$2n = 58$	7	Kabri	7 – 10	NHZ
	8	Zippori	6 – 10	Central
	9	Afiq	6 – 9	NHZ and marginal
$2n = 60$	10	Anza	7 – 10	NHZ
	11	Jerusalem	7 – 11	Marginal
	12	Lahav	5 – 9	Central
	13	Sede Boqer	1 – 4	Desert isolate
Total			121	

DNA extraction, digestion and blotting

High molecular weight genomic DNA was extracted from the kidneys, following Holland (1983). Three different 6-base recognition endonucleases (*Bst*EII, *Kpn*I, *Bam*HI) and one 4-base recognition enzyme (*Taq*I) were used for digestion. Twenty-five µg of genomic DNA were incubated overnight with 150 units of a given endonuclease, as recommended by the supplier (BioLabs, New England, USA). Fully digested DNA was precipitated in ethanol, followed by electrophoresis, through both 0.6% and 1.0% agarose gels for 16 h at 30 v. Thus a single sample contained about 10 µg DNA. DNA was denatured and transferred to nylon (Hybond-N, Amersham, UK) filters following Southern (1975).

The specific probe used – pA81 (L Garfinkel, Bio-technology General, Rehovot, Israel; personal communication) was a specific skeletal myosin heavy chain of a rat adult, G-C tailed into the *Pst*I site of pBR322. The cDNA library was prepared from RNA which was extracted from hind leg muscle of adult rats (Garfinkel *et al*, 1982). The clone hybridized to mRNA from adult skeletal muscle only. Since the insert included several *Pst* I sites, restriction of plasmid resulted in some fragments. The 2 longer fragments detected the same *Spalax* DNA fragments. We used mainly the longest fragment of ≈ 0.8 kb as a probe to detect the *Spalax* myosin heavy chain genes. The exact location of the probe within the myosin gene in unknown.

The DNA fragment was radiolabelled by the random priming method of Feinberg and Voglestein (1984) (Amersham: 'Multiprime DNA labelling systems') overnight at room temperature. Hybridization of ^{32}P-labelled probes with the DNA on filters was performed in 50% formamide solution (consisting of 5 × SSPE, 5× Denhardt's solution, 0.5% SDS and 10% dextran sulphate) overnight at 42°C, following the filter manufacturer's (Amersham, UK) recommendations. Filters were washed at stringency conditions of 2 × SSPE and 0.1% SDS at 63°C for 30 min. We then autoradiographed 24–48 h at −70°C, using 2 intensifying screens (Kodak rapid or super rapid).

Data analysis

The data were analyzed as allele fragment or fragment frequencies, by scoring the bands directly from the autoradiographs. We recorded a minimum number of 6 hybridizing fragments. These fragments were obtained from hybridization of both the larger *Pst*I fragments of the probe, suggesting the existence of at least 6 different loci. Some of these loci were monomorphic, and the others polymorphic. Two fragments were considered as allelic fragments if they appeared either each alone, or both together. However, there was no case in which none appeared, *eg* codominant system. The identification was relatively simple, since for each restriction enzyme there were only a few polymorphic loci (tables II, III). Identification of homozygous and heterozygous genotypes of the polymorphic loci was made directly from the autoradiographs. Observed heterozygosity (Ho) and expected heterozygosity (He) (Nei, 1973) were calculated as the proportion of heterozygous individuals, per population and per locus (per restriction enzyme). Expected heterozygosity was computed from the observed frequencies of codominant alleles under the assumption that the population was in Hardy–Weinberg equilibrium.

In some cases we could not identify or find the alternative allele(s). It seems that a portion of the fragments was undetectable by the cloned probe used in this experiment. We define the portion that could not be detected as 'none-allele' (similar to the null alleles of isozymes). In these cases we analyzed the presence of the allele and its absence (none-allele). Therefore the frequency of the present fragment might be an overestimate of the allele frequency, since possible heterozygotes were scored as homozygotes. Consequently, the none-allele frequency might be an underestimate.

Table II. Myosin heavy chain fragments in *Spalax ehrenbergi.*

Restriction enzyme	Fragment	Allele	Size (kb)
BamHI	I		12.1
	II		11.5
	III		9.6
	IV	a	7.2
		b	4.8
	V		5.3
	VI		3.4
	VII		2.7
BstEII	I		15.1
	II	a	14.0
		b	11.1
	III		12.4
	IV		10.6
	V		4.2
	VI		2.5
KpnI	I		16.5
	II		14.6
	III		12.2
	IV		11.5
TaqI	III		5.5
	IV		4.3
	V		3.5
	VI		2.7
	VII		2.2
	VIII		2.0
	IX		1.6
	XI		1.4
	XII		1.3

We assume that most, if not all, of the mutations which caused polymorphisms are located within the myosin heavy chain genes. The gene in all mammals tested is very long, comprising > 20 kb. There is no reason to suggest smaller genes in the mole rats. Most of the polymorphic fragments we recorded were small (< 10 kb and in many cases, even < 5 kb – table II). Thus the probability that the polymorphism was within the 20 kb of the gene was very high. However, we could not specify

Table III. Frequency and distribution of myosin heavy chain (MHC) allelic fragments or phenotypes in 4 chromosomal species of *Spalax ehrenbergi*.

Localities Enzyme/Fragment	2n = 52			2n = 54			2n = 58			2n = 60			
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*
BamHI													
IV a – 7.2 kb	0.75	0.63	0.37	0.88	0.94	0.88	1.00	1.00	1.00	1.00	0.93	1.00	1.00
b – 4.8 kb	0.25	0.37	0.63	0.12	0.06	0.12	–	–	–	–	0.07	–	–
Ho	0.25	0.50	0.75	–	0.13	0.25	–	–	–	–	0.14	–	–
He	0.38	0.47	0.47	0.21	0.11	0.21	–	–	–	–	0.13	–	–
BstEII													
II a – 14.0 kb	0.39	0.39	0.20	0.83	0.90	0.81	0.56	0.44	1.00	1.00	0.91	1.00	1.00
b – 11.1 kb	0.61	0.61	0.80	0.17	0.10	0.19	0.44	0.56	–	–	0.09	–	–
Ho	0.11	0.11	–	0.33	–	0.13	0.38	0.38	–	–	–	–	–
He	0.48	0.48	0.32	0.28	0.18	0.31	0.49	0.49	–	–	0.16	–	–
V 4.2 kb	1.00	1.00	1.00	0.44	0.70	0.25	0.56	0.75	0.75	0.78	0.91	0.63	0.75
None V –	–	–	–	0.56	0.30	0.75	0.44	0.25	0.25	0.22	0.09	0.37	0.25
VI 2.0 kb	0.78	0.78	0.70	0.88	0.80	1.00	0.89	0.75	0.75	0.89	0.91	0.88	1.00
None VI –	0.22	0.22	0.30	0.12	0.20	–	0.11	0.25	0.25	0.11	0.09	0.12	–
KpnI													
III 12.2 kb	1.00	1.00	1.00	–	–	–	1.00	1.00	–	0.56	0.22	0.50	1.00
None III –	–	–	–	1.00	1.00	1.00	–	–	1.00	0.44	0.78	0.50	–
TaqI													
IX 1.6 kb	1.00	1.00	1.00	1.00	1.00	0.88	1.00	0.67	1.00	1.00	0.33	1.00	0.75
None IX –	–	–	–	–	–	0.12	–	0.33	–	–	0.67	–	0.25
XI 1.4 kb	–	–	–	–	0.20	–	–	0.50	–	–	0.22	0.33	–
None XI	1.00	1.00	1.00	1.00	0.80	1.00	1.00	0.50	1.00	1.00	0.78	0.67	1.00
XII 1.3 kb	1.00	1.00	0.90	–	–	0.37	1.00	1.00	0.33	1.00	1.00	0.67	1.00
None XII –	–	–	0.10	1.00	1.00	0.63	–	–	0.67	–	–	0.33	–

Numbers identify localities plotted in figure 1; Ho = observed heterozygosity; He = expected heterozygosity.

whether a mutation was in the exons or in the introns. We suggest that all the mutations we scored were located outside the region of the probe since the 2 probes tested showed the same pattern of bands, and we found either only 1 different allelic band, or no band in the polymorphic fragment. If the mutation occurred within the probe region, each change in a restriction site should have affected the 2 corresponding fragments. Further study is needed to map locations of restriction sites to the specific myosin heavy chain genes, as well as to specific regions inside the gene.

Statistical analysis

The differences between species and populations were tested by chi-square (χ^2) tests. In those cases where sample sizes were too small and the expected values of a cell were < 5, we used the Fisher exact probability test (Sokal and Rohlf, 1981). We doubled the calculated signifiance level for a 2-tailed significance test.
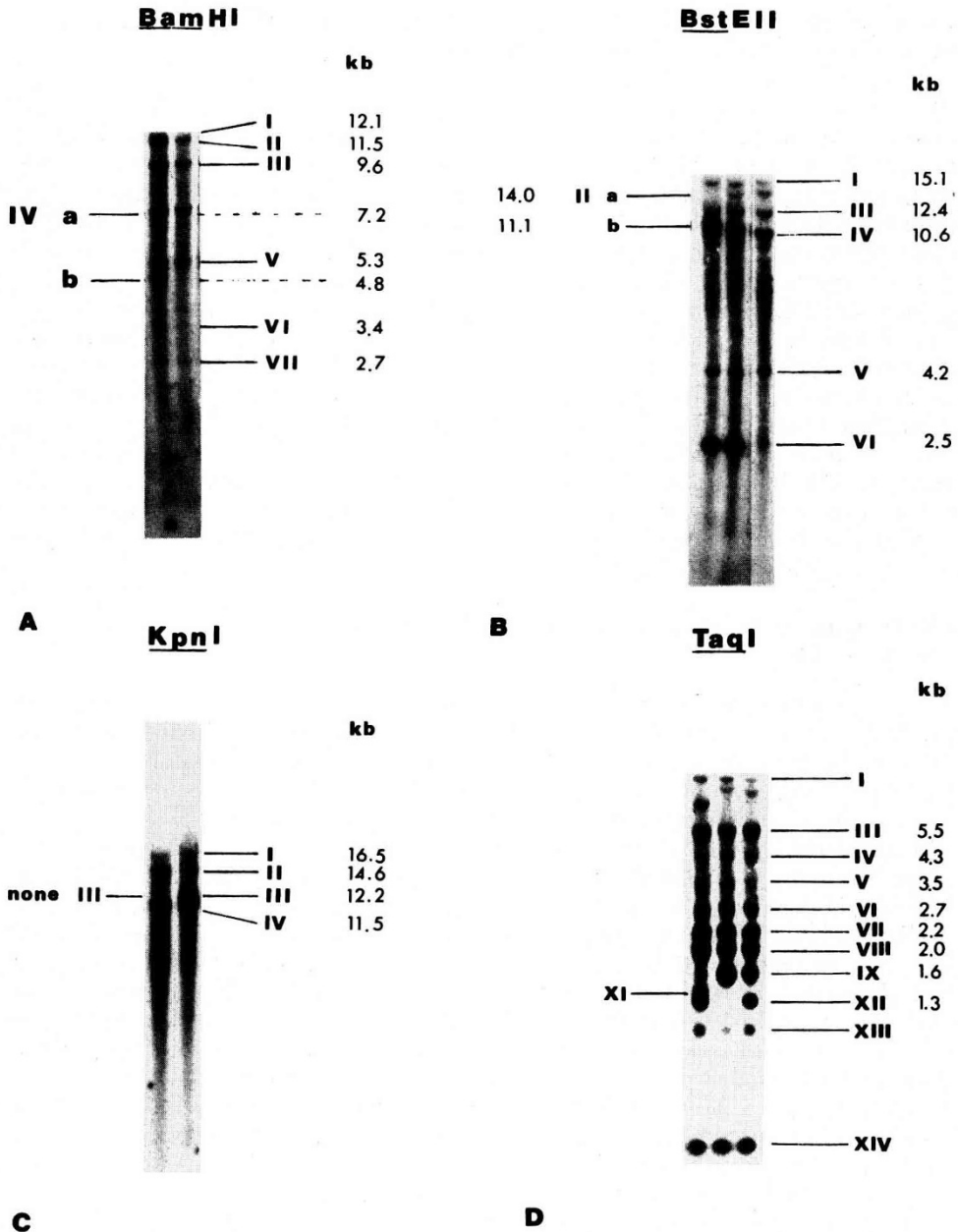

RESULTS

The results of hybridizing the MHC gene probe with *Spalax* DNA digested with several restriction enzymes are summarized in tables II, III and figure 2. In *Spalax*, as in other mammals, the MHC is a multigene family comprised of a minimum number of 6 loci, detected with the rat gene probe (see fig 2). We considered the number of large and strongly hybridizing bands obtained from digestion of 6-base-recognition endonuclease as the number of genes. The rationale for that consideration relies on: a) the fact that the 2 different probes yielded the same fragments – *ie*, there was no case where the endonuclease cleaved the DNA inside the probe homology region; and b) the MHC gene is much larger than the obtained fragments, so there is no reason to assume that one band corresponds to 2 linked genes. Six was the minimum number of strongly hybridizing bands.

A total number of 26 scoreable (on a population level) frequent fragments were produced by the 4 restriction endonucleases used in this study (table II). There were some bands that could not be consistently detected in all samples, and were therefore excluded from the analysis. Most of the detected fragments are monomorphic in the 4 chromosomal species and in the 13 populations examined. Some of them, however, are polymorphic and are summarized in table III. Out of the 26 fragments, 8 (31%) presumptive loci were found to be polymorphic, with no more than 2 alleles observed per locus.

Codominance with alternative allelic fragments was found only in 2 loci. In the other 6 loci, we detected only the appearance or absence of the fragments. We considered the missing alternative alleles as non-allele. The lack of alternative allele could result from several causes. First, the experimental conditions: small fragments could run out of the 0.6% agarose gels. Second, there is the possibility of dominance. This is unlikely in our case because the phenomeon is usually scarce yet absence of the fragment is common in our data set. Third, the possibility exists that gene duplication appears in some of the individuals tested. However, since the phenomenon appears across all 4 chromosomal species it must pre-date speciation. Short of family analysis, this possibility cannot be ruled out.

Fig 2. Fragment polymorphisms of myosin heavy chain multigene family, resulting from digestion with (A) *Bam*HI, (B) *Bst*EII, (C) *Kpn*I, and (D) *Taq*I; Roman numbers indicate the different loci; the letters a and b indicate different alleles; each lane represents 1 individual.

Lack of effective gene flow and differences in MHC polymorphism between the oldest species 2n = 52 and 2n = 54

An alternative fixation between $2n = 52$ and $2n = 54$ is evident at the *Kpn*I markers. All samples of $2n = 52$ were fixed for fragment III, while none occurred in $2n = 54$. It is possible that fragment III does exist in $2n = 54$ as a rare allele (with frequency lower than 0.1). The probability of finding no individuals with *Kpn*I fragment III phenotype in a sample of 27 individuals is $(1 - 0.1)^{27} = 0.058$. The differences between the 2 species were highly significant $(\chi^2_1 = 55.98, p \ll 0.001)$. These results suggest that there is no effective gene flow between the oldest chromosomal species $2n = 52$ and $2n = 54$. Similar results, though less pronounced, can be seen in the *Taq*I fragment XII. Most individuals of $2n = 52$ showed this fragment which was rare or absent in $2n = 54$. The difference between the 2 species in this locus was highly significant $(\chi^2_1 = 39.31; p \ll 0.001)$. Some interspecies divergence is also evident in *Bst*EII. In fragment II the average frequency of allele a is 32% in $2n = 52$ and 85% in $2n = 54$ $(\chi^2_1) = 31.9, p \ll 0.001)$. The frequency of fragment V is 100% in $2n = 52$ and only 48% in $2n = 54$. Yet again the differences between the 2 species were highly significant $(\chi^2_1 = 19.5, p \ll 0.001)$. These results suggest that there is either no gene flow between $2n = 52$ and $2n = 54$, or if there is, it is eliminated by selection.

Differences in MHC polymorphism between the species 2n = 52 and 2n = 58

Significant differences in allele frequencies are evident between the species $2n = 52$ and $2n = 58$. All individuals of $2n = 52$ showed the *Bst*EII fragment V, and only 68% exhibited the fragment in $2n = 58$ (2-tailed Fisher exact probability test, $p = 0.0012$). Similarly, variation in *Bam*HI markers showed that the frequency of allele b is 42% in $2n = 52$ and 0% in $2n = 58$ $(\chi^2_1 = 24.37, p \ll 0.001)$.

Populations 1 and 7 of $2n = 52$ and $2n = 58$ straddle a hybrid zone, 10 km apart. Fragment V of *Bst*EII was present in all individuals of population 1 (Maalot, $2n = 52$) and in only 56% of the individuals from population 7 (Kabri, $2n = 58$). The differences between the populations were not statistically significant (2-tailed Fisher exact test, $p = 0.08$) due to a small sample size. Similarly, the variation in *Bam*HI markers showed that the frequency of allele b (4.8 kb) in fragment IV was 25% in population 1 ($2n = 52$) and 0% in population 7 ($2n = 58$) (2-tailed Fisher exact test, $p = 0.08$). However, the combined probabilities from tests of significance between the 2 populations straddling the hybrid zone (Sokal and Rohlf, 1981) were significant $(\chi^2_4 = -2\Sigma \ln P = 10.04; p < 0.05)$. These results suggest that different selective pressure and possibly no effective gene flow operates across the hybrid zone separating $2n = 52$ and $2n = 58$.

Differences in MHC polymorphism between the species 2n = 54 and 2n = 58

Differences in allelic fragment frequencies, although not statistically significant, can also be seen between $2n = 54$ population 6 (El-Al) and $2n = 58$ population 9 (Afiq). These are 10 km apart, across a hybrid zone 0.7 km wide, which separates the

species in the southern Golan Heights (fig 1) (Nevo and Bar-El, 1976; Nevo, 1985). Three loci: *Bst*EII – fragment II, *Bam*HI – fragment IV, and *Taq*I – fragment IX, were polymorphic in population 6 of $2n = 54$ and monomorphic in population 9 of $2n = 58$ (table III). These results may indicate that there is a possible lack of effective gene flow from $2n = 54$ to $2n = 58$, and possible different selective pressure that operate on these geographically close populations.

Limited amount of gene flow between the younger species 2n = 58 and 2n = 60

Reduction or absence of gene flow between the 2 younger $2n = 58$ and $2n = 60$ species is also suggested by several markers. Population 10 (Anza, $2n = 60$) is located south of a $2n = 58 \times 60$ hybrid zone, while sample 8 (Zippori, $2n = 58$) was collected from the center of the $2n = 58$ range. *Bst*EII fragment II variation showed 2 alleles in population 8 with a frequency of b at 56%, and the absence of this allele in population 10. The differences between the 2 populations were highly significant (2-tailed Fisher-exact test, $p \ll 0.001$). The opposite pattern was displayed by *Kpn*I variation. The frequency of fragment III was 100% in population 8 but 56% in 10. Although population 8 is not located near the hybrid zone, these patterns suggest no widespread effective gene flow between the $2n = 58$ and $2n = 60$ populations.

Differences in MHC polymorphism within the 2n = 58 chromosomal species

Population 9 (Afiq) of the $2n = 58$ chromosomal species exhibits a pattern of variation strikingly different from the pattern of the other 2 populations. These differences appear in 3 out of the 8 polymorphic loci. The most distinguished pattern is in *Kpn*I fragment III where the fragment fixed in populations 7 and 8 is totally absent in population 9 (2-tailed Fisher exact probability test, $p \ll 0.001$). Similarly, a quantitative rather than a qualitative pattern can be detected in the *Taq*I variation of fragment XII. The fragment is fixed in populations 7 and 8, and appeared at a frequency of only 33% in population 9. The differences between populations 7,8 and population 9 were statistically significant (2-tailed Fisher exact probability test, $p < 0.005$). In *Bst*EII fragment II variation, populations 7 and 8 are polymorphic with similar frequency of both allele a and b. Population 9 is monomorphic, consisting of only allele a (2-tailed Fisher exact probability test, $p < 0.02$). The differences described may indicate the existence of a gene flow barrier between the populations.

DISCUSSION

Number of genes

Similar to other mammals examined (Nguyen *et al*, 1982; Leinwand *et al*, 1983; Maeda *et al*, 1987), we have recorded in the mole rat a minimum number of 6 large hybridizing fragments and a maximum of 14, corresponding to at least 6 but probably 8 genes. The complexity of the MHC pattern in southern blots

is most probably an indication of the number of sarcomeric MHC genes in the organism (Nguyen *et al*, 1982). The complex structure of the rat MHC gene and the conservation of intron locations are indicative of a highly split ancestral sarcomeric MHC gene (Strehler *et al*, 1986). Once the split ancestral MHC gene evolved, it is likely to have undergone duplication(s) as an entire genetic unit in order to give rise to the multi-member sarcomeric MHC gene families (Strehler *et al*, 1986). The rat sarcomeric MHC gene family has diverged enough from smooth muscle and non-muscle MHC that there is no cross-hybridization between them (Nguyen *et al*, 1982; Leinward *et al*, 1983).

In the present analysis, we used rat DNA as a probe to the mole rat MHC genes. Although MHC appeared to be highly conserved during the evolution of straight muscles (Nguyen *et al*, 1982), we used medium stringency conditions of $2 \times$ SSPE and 0.1% SDS at 63°to detect the *Spalax* genes. These conditions suggest that the homology between the rat and the mole rat is not very high. Further study is needed to determine both the exact number of genes and their characteristics.

The level of polymorphism

Polymorphisms of some of the MHC genes were found with all the tested restriction enzymes. Although relatively high levels of polymorphism (31%) were found in MHC genes of *Spalax ehrenbergi*, the number of alleles identified was low. Table III shows that in the scoreable cases only 2 alleles were found. The differences between the observed and expected heterozygosity were not large, considering the small sample size. However, in *Bst*EII fragment II variation, Ho were smaller than He in 8 out of 9 polymorphic populations (sign test, $p < 0.04$). These results may suggest an existence of selection operating against heterozygotes in this locus.

In other loci, we could not identify the alternative allele(s), though there is no reason to suggest the presence of more than one unidentifiable allele. Existence of a restriction site polymorphism was found in the rat (Nguyen *et al*, 1982), and in the human (Nguyen *et al*, 1982; Leinwand *et al*, 1983; Schwartz *et al*, 1986). Although no population analyses have been conducted, it seems that in these species, as in *Spalax*, the number of alleles per locus is low. It is possible that genetic polymorphism in the MHC genes plays a role in generating the complex MHC patterns (Nguyen *et al*, 1982).

The low number of alleles in *Spalax* is similar to levels of isozyme polymorphism as determined by horizontal starch gel electrophoresis (Nevo and Shaw 1972, Nevo and Cleve, 1978). The most polymorphic protein locus had only 3 alleles, and most of the other loci had only 2 alleles. In contrast, the major histocompatibility complex showed a very high number of alleles at the RFLP level (Ben-Shlomo *et al*, 1988). In other words, while structural genes coding for basic metabolic enzymes and for heavy myosin chain are relatively conservative, those of the immune systems are extremely polymorphic.

Low levels of allozyme polymorphism appear to be the general phenomenon in fossorial and subterranean mammals (Nevo *et al*, 1984, Nevo, Fillipucci, Capanna and Hickman, in preparation). In the myosin heavy chain, the level of polymorphism is $\approx 31\%$ with few alleles (2) segregating at each polymorphic locus. Since equivalent population surveys on mammals living above ground have not yet been made, we

cannot compare the level of polymorphism and the number of alleles between under and above ground mammals. Additional comparative studies are in order.

Differences in myosin heavy chain polymorphism between the chromosomal species

RFLP variation in the sarcomeric myosin heavy chain multigene family allows one to differentiate among the chromosomal species, particularly among the oldest species $2n = 52, 54$ and 58 (table III). Similar results suggesting the possible lack of gene flow across the $2n = 52 \times 54$ and $2n = 52 \times 58$ borders were also found in analyses of major histocompatibility complex loci (Ben-Shlomo et al, 1988), rDNA nontranscribed spacer polymorphisms (Suzuki et al, 1987), and mtDNA haplotypes (Nevo et al, 1993). The narrowness of the hybrid zones, the lower than expected proportion of hybrids, the nonrandom distribution of karyotypes (Nevo, 1985) and the genetic differences among the chromosomal species indicate that genetic isolation is established, and supports the case for species status of these races. Moreover, it suggests that natural selection operates against migrants.

Our results support an earlier conclusion (Nevo 1985) that reproductive isolation increases progressively northward. While reproductive isolation is still weak between $2n = 58$ and $2n = 60$, it becomes much stronger between $2n = 52 - 54$, and $2n = 52 - 58$. Dispersal of hybrids into parental territories is restricted, presumably by a combination of genetic, cytogenetic, ethological, immunological and ecological incompatibilities (Nevo 1985), whereas hybrids appear inferior to the parental types.

Genetic differences within the $2n = 58$ species

Existence of a gene flow barrier between population 9 and populations 7 and 8 of $2n = 58$ is a possible explanation of the differences and an alternative fixation among these populations. Population 9 (Afiq) is a marginal population that is located in the southern Golan heights (fig 1), south of the $2n = 54 \times 58$ hybrid zone. Geographically this population of $2n = 58$ is surrounded by the Yarmouk river in the south-east (the political border between Israel and Jordan) and by the sea of Galilee and the Jordan river in the west. These geographical barriers may cause the isolation of population 9 in a small area and thus create an isolated relict. Alternative fixation of alleles between populations 7 and 8 and population 9 was found in Period gene homologous sequence (Ben-Shlomo et al, 1993). The unique pattern of population 9 can also be seen in mtDNA haplotypes (Nevo et al, 1993) where some haplotypes exist only in this population.

As shown in the previous section, there appears to be a possibility of no effective gene flow between the 2 chromosomal species $2n = 54$ and 58. Nevertheless, KpnI variation showed that population 9 of $2n = 58$ exhibits a similar pattern to that of the neighboring populations of $2n = 54$, by being fixed for the none-III fragment, in contrast to samples 7 and 8 (Kabri and Zippori) of $2n = 58$. This convergent genetic pattern may suggest a common selection pressure that operates similarly on all populations in the Golan heights. Such a selection pressure could be mediated for example by the type of soil (basalt) characterizing the area.

A possible operation of genetic drift on small isolated demes within the $2n = 58$ cannot be dismissed, although it seems less probable. While genetic drift gradually

increases the interpopulation variation of gene frequency, the genetic variability within population gradually declines (Nei, 1987). Since the proposed isolation of population 9 has probably existed for thousands of generations, the genetic diversity should be very low. In contrast to the expected low values, the diversity of this population is similar to the other populations of the same chromosomal species, in the MHC genes as well as in other genetically examined systems, such as the major histocompatibility complex (Ben-Shlomo *et al*, 1988) or rDNA nontranscribed spacer (Suzuki *et al*, 1987). Moreover, in *Per*-homologous sequence (Ben-Shlomo *et al*, 1993) and in the mtDNA haplotype (Nevo *et al*, 1993) the number of different types in population 9 is higher than in the other 2 populations.

The considerations mentioned above strengthen the possibility that parallel genetic patterns due to balancing natural selection operate on the MHC genes in the Golan heights.

Polymorphism in isolated populations

Sede Boqer (population 13) is an isolated population in the northern Negev desert (fig 1). The $2n = 60$ chromosomal species in the Israeli range persisted during 70 000 years in a continuous range connecting the coastal plains of the Negev and Sinai deserts with northern Africa (Nevo, 1989). This species became disjunct from its North African extension in the post-Wurm period (Lay and Nadler, 1972). The isolated population (13) studied here may have therefore been isolated > 10 000 years from the main range of $2n = 60$. The separation from the main range by inhospitable kilometers of steppic and desert environment resulted in a low effective population size, of the order of only about 100 individuals (Nevo, 1989). This relictual situation and small effective population size seem to have lasted for thousands of generations.

Nei *et al* (1975) have shown that the effect of population size reduction (bottleneck effect) on average heterozygosity is expected to last for hundreds of thousands of years after the recovery of population size. Maruyama and Fuerst (1985) claimed that while allele numbers showed significant excesses when the population rapidly expanded from a homoallelic state, population size contraction caused allelelic deficiencies. These neutral considerations would suggest that in the isolated population of *Spalax*, where the bottleneck effect was sustained for many generations, with no population recovery, the level of variability would be extremely low. The opposite pattern is displayed in the Sede Boqer isolate.

The results obtained in the present study indicate that there is no remarkable difference between population 13 (Sede Boqer) and the other population of $2n = 60$. In the case of *Taq*I, fragment IX (table III), the isolated population is polymorphic while most of the examined populations were monomorphic. Notably, due to the small population size of this isolate, we analyzed only up to 4 individuals from this population, while in all other populations we analyzed ≈ 10. More individuals from this isolated population may increase the level of polymorphism.

Similar results were found in the Sede Boqer isolate in other genetic systems, for example the major histocompatibility complex (Ben-Shlomo *et al*, 1988) and several allozyme systems, mtDNA and chromosomal polymorphisms reviewed in Nevo (1989). Apparently, an extreme homozygosity is unlikely even in a very small and isolated population.

The variability found in several genetic systems is due presumably to a very strong balancing natural selection. If this isolate may ultimately evolve into a new species rather than become extinct, then it involves a relatively high level of genetic variation which may lead to successful speciation (Barton and Charlesworth, 1984; Carson and Templeton, 1984; Nevo; 1989).

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