

# Period-homologous sequence polymorphisms in subterranean mammals of the *Spalax ehrenbergi* superspecies in Israel

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Restriction fragment length polymorphism (RFLPs) of the mouse *period*-homologous sequence were studied in 13 populations of the four chromosomal species ( $2n = 52, 54, 58$  and  $60$ ) of the mole rat, *Spalax ehrenbergi* superspecies in Israel. The *period* locus of *Drosophila melanogaster* is implicated in controlling the circadian rhythm as well as the male courtship song rhythm. Multiple DNA homologies exist in the mole rat and correspond to more than 10 loci. The level of polymorphism is very high, with a large number of alleles per locus, increasing from the northern to the southern species along a gradient of increasing aridity. Variation was also found in an isolated desert population, with a unique fragment specific to this population. Fragment variation allows distinction between chromosomal species, and confirms earlier evidence that gene flow does not occur between them. A correlation was found between some allelic fragments and the number of apparent harmonics of the courtship calls. This finding suggests an interesting testable hypothesis that the existence of a locus (homology) is responsible for the courtship call parameters.

**Keywords:** mole rat, *Per*-homologous sequence, RFLP.

## Introduction

Daily rhythms in many behavioural, physiological and biochemical functions are generated by endogenous oscillators which function as internal 24-h clocks. Under natural conditions these oscillators are synchronized to the daily environmental cycle of light and darkness (reviewed in Takahashi & Zatz, 1982). Circadian rhythms are genetically determined (Hall, 1990). Mutations which alter the expression of rhythmicity have been described in *Drosophila melanogaster*, *D. pseudoobscura*, *Neurospora crassa*, and *Chlamydomonas reinhardtii* (reviewed in Feldman, 1982), and in hamsters (Ralph & Menaker, 1988), mice (Schwartz & Zimmerman, 1990) and the Brattleboro rat (Reppert *et al.*, 1987). In many cases, the mutants also show alterations to other time-dependent properties, such as their response to light and temperature.

The *period* (*per*) locus of *Drosophila melanogaster* is one of the best studied behavioural genes in any species. The gene is implicated in the control of both the circadian and male courtship song rhythms (Konopka & Benzer, 1971; Kyriacou & Hall, 1980). The *per* gene product is involved in the control of two very different neural oscillators: one in the brain controlling circadian rhythmicity and the second in the thoracic ganglia, which controls courtship song rhythmicity (Hall, 1984).

The structure of some segments of the predicted protein consists of uninterrupted tandem repetitions of the sequence ACNGGN. These tandem repeats code for poly-threonine-glycine (Thr-Gly) tracts which are up to 48 amino acids long (Shin *et al.*, 1985; Jackson *et al.*, 1986; Reddy *et al.*, 1986). In addition, a region beginning about 100 bp upstream from the Thr-Gly repeat is rich in serine-glycine (Ser-Gly) codons. Immunological and biochemical studies of the *per* protein indicate that it may have some proteoglycan-like qualities (Reddy *et al.*, 1986; Hall, 1990). Typically, proteoglycans are found to be associated with the cell surface, or in extracellular locations (Edmunds, 1988).

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Hybridization of the *Drosophila per* locus probe with genomic DNA can detect homologies among chickens, mice and humans (Shin *et al.*, 1985). The discovery of these homologies, as well as the immunological implications that the *per* protein has some proteoglycan qualities, both point to a possible role that this unusual protein segment may play. Such a segment composed of alternating Thr-Gly and Ser-Gly residues contributes to the organization of biological rhythms, including courtship songs in many species, and as a result, to mate selection (Yu *et al.*, 1987).

We tested the hybridization homologies and the restriction fragment length polymorphisms (RFLPs) of the mouse *per*-homologous sequence in the complex of four chromosomal species ( $2n = 52, 54, 58$  and  $60$ ) of the actively speciating blind subterranean mole rat, *Spalax ehrenbergi*, in Israel (overviewed in Nevo, 1991). This evolutionary model of the superspecies in Israel provides a unique potential test of both the *per*-homologous sequence function in rhythmicity and in mating calls in mammals. *S. ehrenbergi* is totally blind, but the animals do perceive photoperiodic changes (Haim *et al.*, 1983; Pevet *et al.*, 1984; Nevo, 1989). The chromosomal species differ in both levels and patterns of activity (Nevo *et al.*, 1982). Likewise, in the *S. ehrenbergi* superspecies, species-specific 'courtship' calls provide a major premating isolating mechanism in speciation (Nevo *et al.*, 1987).

The objective of the present research was to: (a) examine the pattern of homologies of the *per* sequence

in *Spalax*; (b) compare the RFLPs of these homologies among and within the four chromosomal species; and (c) assess whether this *per*-homologous sequence is associated with periodic rhythms, or with courtship call rhythms, as a mechanism of reproductive isolation.

## Materials and methods

### Sampling

We tested DNA polymorphisms of the *per*-like genes in 121 mole rats, comprising 13 populations and four chromosomal species of the *Spalax ehrenbergi* superspecies in Israel (Table 1 and Fig. 1). Live animals were brought to the laboratory, killed and dissected. Tissues were instantly frozen in liquid nitrogen and then stored at  $-80^{\circ}\text{C}$ .

### DNA extraction, digestion and blotting

High molecular weight genomic DNA was extracted from the kidneys, following Holland (1983). Two different six-base recognition endonucleases (*Bam*HI and *Eco*RI), and one four-base recognition enzyme (*Taq*I), were used for digestion. Twenty-five micrograms of genomic DNA were incubated overnight with 150 units of a given endonuclease, following the conditions recommended by the supplier (New England, BioLabs, U.S.A.). Fully digested DNA was precipitated in ethanol, followed by electrophoresis through both

**Table 1** Tested populations of *Spalax ehrenbergi*

Chromosomal species	Number*	Location	Number of animals	Type of population
$2n = 52$	1	Maalot	9	NHZ
	2	Kerem Ben Zimra	10	Central
	3	Qiryat Shemona	10	Marginal
$2n = 54$	4	Hermon	10	Marginal
	5	Quneitra	10	Central
	6	El-Al	9	NHZ
$2n = 58$	7	Kabri	10	NHZ
	8	Zippori	10	Central
	9	Afiq	9	NHZ and marginal
$2n = 60$	10	Anza	10	NHZ
	11	Jerusalem	11	Marginal
	12	Lahav	9	Central
	13	Sede Boqer	4	Desert isolate
Total		121		

\*Localities listed as in Fig. 1.  
NHZ = near hybrid zone.

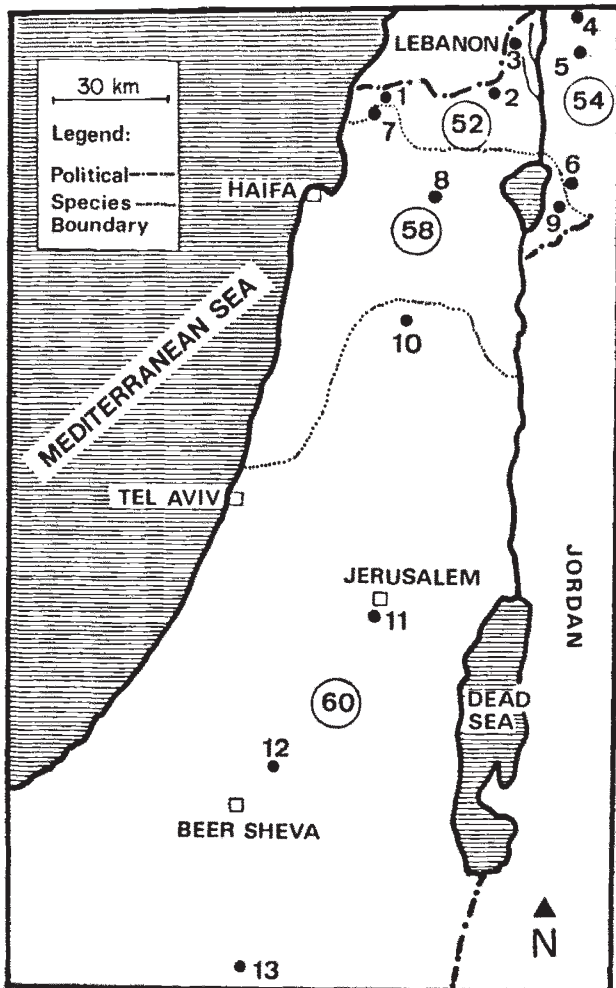


Fig. 1 Geographical distribution of the chromosomal species and the tested population of the *Spalax ehrenbergi* superspecies in Israel.

0.6 and 1.0 per cent agarose gels for 16 h at 30 V. The DNA was denatured and transferred to nylon filters (Hybond-N, Amersham, U.K.) following Southern (1975).

#### Hybridization

The probe used was a 2.5 kb *EcoRI* insert from a mouse cosmid, homologous to the *Drosophila per* sequence, containing a conceptual translation of a 2.1-kb open-reading frame. The translational frame was composed of two hexamer repeats, ACNGGN (Thr-Gly) and TACGGC (Ser-Gly) (Shin *et al.*, 1985). The DNA insert was radiolabelled by the random prime method of Feinberg & Voglestein (1984) (Amersham: 'Multiprime DNA labelling systems') overnight at room temperature. Hybridization of  $^{32}\text{P}$ -labelled probes with the DNA on the filters was performed in

the 50 per cent formamide solution overnight at 42°C, following the filter manufacturer's (Amersham, U.K.) recommended methods. Filters were washed under high-stringency conditions of  $0.1 \times \text{SSPE}$  at 64°C and were autoradiographed at -70°C using two intensifying screens (Kodak rapid or super rapid).

#### Data analysis

The data were analysed as fragment and/or fragment combination frequencies by scoring the bands directly from the autoradiograph.

Genetic indices of hypervariable regions were estimated following Jeffreys *et al.* (1985) and Georges *et al.* (1988). The indices are:  $q$  — the mean population frequency of a resolvable allele, assuming that co-migrating bands are always the same allele;  $x$  — the mean probability that a fragment present in one individual is also present in another one taken randomly from the population:

$$x = q^2 + 2q(1 - q) = 2q - q^2;$$

and  $h$  — the mean probability for a resolvable fragment to be present in a heterozygous state:

$$h = 2q(1 - q)/(2q - q^2) = 2(1 - q)/(2 - q).$$

## Results

#### Digestion of *Spalax* DNA with *Bam*HI

Hybridization of *Spalax* DNA digested with *Bam*HI with the mouse *per*-homologous sequence yielded many bands (more than 15). All the strong hybridization bands were polymorphic (Fig. 2a). The distribution of the different fragments and their sizes appears in Table 2, and the frequency of the different fragment combinations appears in Table 3.

The chromosomal species vary in their *per* RFLPs. The  $2n=54$  species was monomorphic for fragments 1-7. The chromosomal species  $2n=52$  was polymorphic, whereby the main combination of fragments was either 1-7 or 1-7-8. In the  $2n=58$  species, population 9 (Afq), located near  $2n=54$  in the Golan Heights (Fig. 1), was polymorphic, but had the same predominant fragment combination as that of  $2n=54$ . The two other populations of  $2n=58$  were entirely different. Population 8 (Zippori) was monomorphic for fragment 3, and population 7 (Kabri) was polymorphic with mainly two fragments, 3 and 4, sharing fragment 3 with the Kabri (7) population, and fragment 4 with the Golan Heights, Afq (9) population. Thus, the species shared fragments although the differentiation between the populations was remarkable. The species  $2n=60$

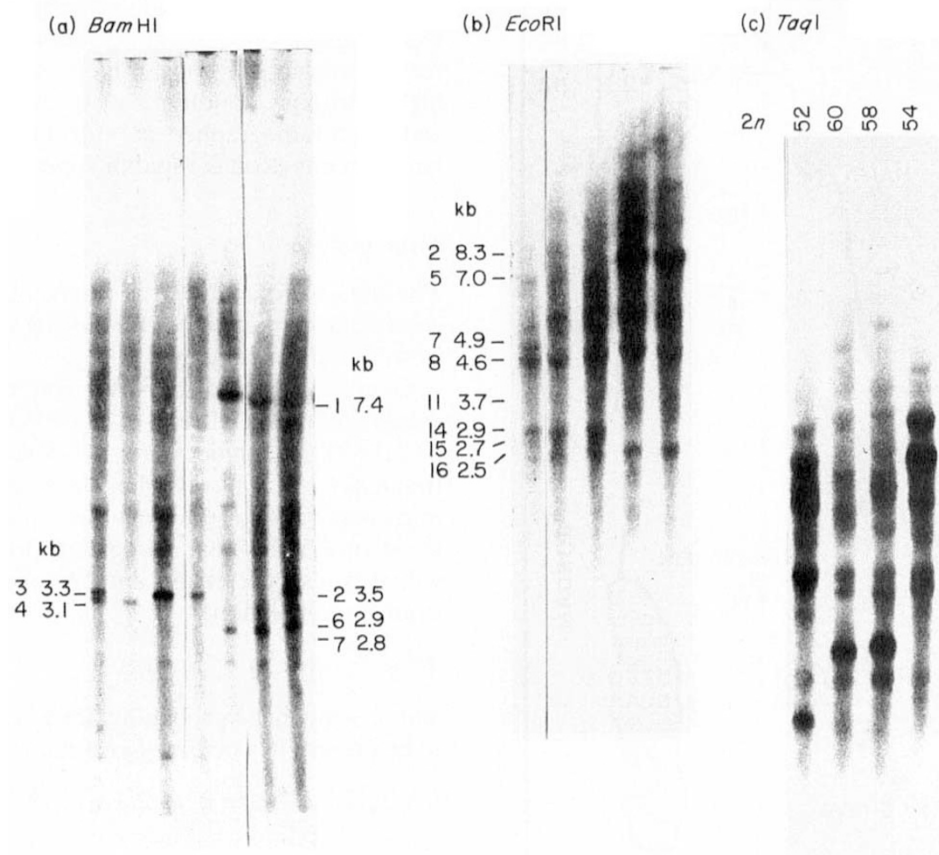


Fig. 2 Fragment polymorphism of *per*-homologous sequences resulting from digestion with (a) *Bam*HI, (b) *Eco*RI, (c) *Taq*I.

Table 2 Frequency and distribution of *Per*-homologous sequence fragments in the four chromosomal species of *Spalax ehrenbergi*

Chromosomal species		2n = 52			2n = 54			2n = 58			2n = 60				
Localities*		1	2	3	4	5	6	7	8	9	10	11	12	13	
Enzyme	Fragment	Size													
<i>Bam</i> HI	1	7.4	1.00	0.90	1.00	1.00	1.00	1.00	—	—	0.86	0.11	0.55	1.00	1.00
	2	3.5	—	—	—	—	—	—	—	—	—	0.33	—	—	—
	3	3.3	—	0.10	—	—	—	0.77	1.00	—	0.66	0.88	1.00	1.00	
	4	3.1	—	—	—	—	—	0.55	—	0.28	0.11	—	0.20	—	
	5	3.0	—	—	—	—	—	—	—	—	—	0.11	—	—	
	6	2.9	—	0.10	—	—	—	—	—	—	0.33	—	—	—	
	7	2.8	1.00	0.90	1.00	1.00	1.00	1.00	—	0.86	—	0.11	—	—	
	8	2.7	0.44	0.30	—	—	—	0.11	—	—	—	—	—	—	
Probability of band Sharing Present in the heterozygous state		<i>x</i>	0.72	0.57	1.00	1.00	1.00	1.00	0.65	1.00	0.81	0.48	0.55	0.79	1.00
		<i>h</i>	0.41	0.62	0.00	0.00	0.00	0.00	0.65	0.00	0.44	0.80	0.68	0.30	0.00

\*Localities as in Fig. 1 and Table 1. Fragment sizes are measured in kilobases.

**Table 3** Frequency and distribution of *per*-homologous sequence fragments combinations in the four chromosomal species of *Spalax ehrenbergi*

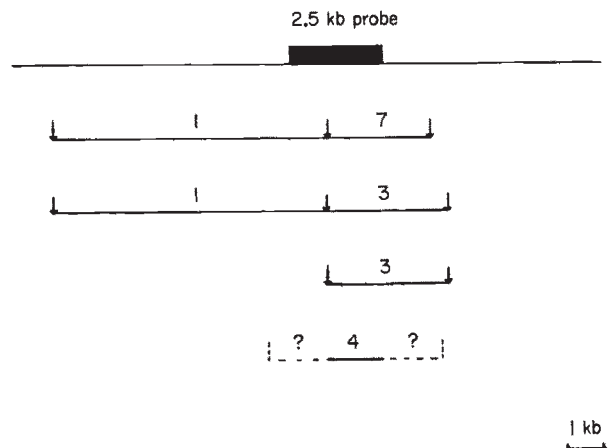
Chromosomal species	2n = 52			2n = 54			2n = 58			2n = 60			
Localities*	1	2	3	4	5	6	7	8	9	10	11	12	13
Enzyme fragment combinations													
<i>Bam</i> HI 1-3	—	—	—	—	—	—	—	—	—	—	0.44	0.80	1.00
1-7	0.44	0.60	0.70	1.00	1.00	1.00	—	—	0.72	—	0.11	—	—
1-7-8	0.44	0.30	0.30	—	—	—	—	—	—	—	—	—	—
3	—	—	—	—	—	—	0.33	1.00	—	0.44	0.33	—	—
4	—	—	—	—	—	—	0.22	—	0.14	—	—	—	—
3-4	—	—	—	—	—	—	0.33	—	—	—	—	—	—
1-3-4	—	—	—	—	—	—	—	—	—	0.11	—	0.20	—
1-3-7	0.11	—	—	—	—	—	—	—	—	—	—	—	—
1-4-7	—	—	—	—	—	—	—	—	0.14	—	—	—	—
2	—	—	—	—	—	—	—	—	—	0.11	—	—	—
2-6	—	—	—	—	—	—	—	—	—	0.11	—	—	—
2-3-6	—	—	—	—	—	—	—	—	—	0.11	—	—	—
6	—	—	—	—	—	—	—	—	—	0.11	—	—	—
3-6†	—	0.10	—	—	—	—	—	—	—	—	—	—	—
3-5	—	—	—	—	—	—	—	—	—	—	0.11	—	—
3-8†	—	—	—	—	—	—	0.11	—	—	—	—	—	—
Number of combinations	3	3	2	1	1	1	4	1	3	6	4	2	1

\*Localities as in Fig. 1 and Table 1.

†Due to very small differences in size it was impossible to determine whether two rare fragments were identical or only similar in size.

showed greater polymorphism than the other three species. It shared fragment 3 with the populations of 2n = 58 and showed a clinal increase southward in the combination of fragments 1-3, which reached fixation in the Sede Boqer desert isolate. The frequency of the 1-3 'allele' among populations 10-13 was very strongly correlated with decreasing latitude (latitude range: 30.87-32.35;  $r^2 = 0.9765$ ,  $P = 0.0118$ ).

Deciphering the underlying genetic basis of the *Bam*HI polymorphism was difficult owing to the multiple homologies. However, the common fragments were distributed as a minimum of one fragment, and a maximum of three per individual. This suggests that *Bam*HI polymorphism involves only one gene, which apparently has a *Bam*HI recognition site inside the probe region, yielding two fragments. One of the fragments might be no. 1 and the other might be either 7 or 3 (Fig. 3). The appearance of fragment 3 alone may have been the result of a mutation which caused an additional restriction site in fragment 1, shortening and possibly excluding it from the gel. Fragment 4 is probably allelic to fragment 3, but it could be allelic to 1+3 as well. Further study is required to determine decisively the different alleles and genetics of the *Bam*HI polymorphism in *Spalax*.



**Fig. 3** A possible restriction map of *per*-homologous sequence hybridizing to *Spalax* DNA digested with *Bam*HI.

***Eco*RI polymorphism**

*Per-Eco*RI polymorphism yielded at least 16 bands, ranging in size from 9.0 to 2.5 kb (Fig. 2b and Table 4). All the bands were polymorphic. Some of the fragments were rare and appeared only in one or two individuals. Ten fragments were common in at least some

**Table 4** Frequency and distribution of *per*-homologous sequence fragments in the four chromosomal species of *Spalax ehrenbergi*

Chromosomal species			$2n=52$			$2n=54$			$2n=58$			$2n=60$				
Localities*			1	2	3	4	5	6	7	8	9	10	11	12	13	
Enzyme	Fragment	Size														
<i>EcoRI</i>																
	1	9.0	—	—	—	0.14	—	—	—	—	—	—	—	—	—	
	2	8.3	1.00	1.00	1.00	1.00	1.00	1.00	—	—	0.75	0.83	0.38	0.86	1.00	
	3	7.7	—	—	—	—	—	—	—	—	0.25	0.33	0.63	0.14	—	
	4	7.5	—	—	—	—	—	—	—	0.17	—	—	—	—	—	
	5	7.0	—	—	—	—	—	—	1.00	1.00	0.13	—	—	—	—	
	6	6.6	—	0.10	—	—	—	—	—	—	—	—	—	—	—	
	7	4.9	—	—	—	—	—	—	0.13	—	0.13	—	—	—	—	
	8	4.6	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.88	1.00	0.90	0.67	1.00	
	9	4.4	—	—	—	—	—	—	—	—	—	—	0.10	—	—	
	10	4.2	—	0.10	—	—	—	—	—	—	0.75	0.13	0.50	0.33	1.00	
	11	3.7	—	—	—	—	—	—	0.50	0.17	0.25	—	0.10	0.17	—	
	12	3.1	—	—	—	—	—	—	—	0.17	—	—	—	0.17	—	
	13	3.0	—	—	—	—	—	—	—	—	—	—	—	—	1.00	
	14	2.9	0.14	—	—	—	0.10	—	1.00	1.00	—	0.80	0.75	0.86	0.50	
	15	2.7	0.43	1.00	0.60	0.14	—	—	0.38	0.17	0.38	0.40	0.25	0.29	—	
	16	2.5	0.86	1.00	0.60	1.00	1.00	1.00	0.25	—	0.75	0.40	0.25	0.29	0.50	
Probability of band																
Sharing			<i>x</i>	0.78	0.73	0.92	0.70	0.80	1.00	0.72	0.61	0.64	0.71	0.60	0.59	0.92
Present in the heterozygous state			<i>h</i>	0.38	0.32	0.29	0.37	0.25	0.00	0.46	0.52	0.64	0.55	0.68	0.69	0.22

\*Localities as in Fig. 1 and Table 1.

Fragment sizes are measured in kilobases.

of the populations. The distribution of the common fragments varied in frequency among species. Fragment 2 was fixed in the species  $2n=52$  and  $2n=54$ , and its frequency was high in most of the  $2n=60$  populations. It appeared in population 9 (Afiq) but was totally absent in population 7 and 8 (Kabri and Zippori), of  $2n=58$ . Populations 7 and 8 harboured another fragment, no. 5, probably allelic to fragment 2, which appeared in every tested individual, and in a lower frequency in population 9. Thus, fragment 5 is species-specific to  $2n=58$ . Fragment 3 may also be allelic to these two fragments (2 and 5); this fragment only appeared in population 9 of  $2n=58$  and in the main population of  $2n=60$ . Fragment 11 (and less so, fragment 10) may distinguish the southern ( $2n=58$  and 60) from the northern ( $2n=52$  and 54) species.

Deciphering genes and allelic fragments in this system was complex, more than in the *Bam*HI polymorphism. However, the number of bands per individual ranged from 3 to 5, suggesting the existence of a few (probably 3) multiallelic genes (possible allelism of fragments 2, 3, 5).

The second probable gene represented by fragment 8 was nearly monomorphic in  $2n=52$  and 54. However, in the other two species, its frequency was lower, and fragments 11 in  $2n=58$  and fragment 10 of  $2n=60$  might possibly be allelic to it. This classification is problematic owing to the complete absence of fragment 11 'homozygotes', as well as a deficiency in fragment 10 'homozygotes'. Further study on a larger sample size is required to define the exact genetics of the multiband patterns of *Eco*RI polymorphism in *Spalax*.

The desert isolate population 13 (Sede Boqer) of  $2n=60$  exhibited a very interesting pattern. An additional new fragment — 13 (3.0 kb), appeared in the two tested individuals, suggesting a high frequency (or fixation of an additional fragment) in that population.

#### *Taq*I polymorphism

The *per*-homologous sequence polymorphism yielded many bands, as expected from the digestion of genomic DNA, with a four-base recognition endonuclease.

Although most of the polymorphic fragments were unscorable on a population level, it seemed that there were two major different patterns. One represented the species  $2n=52$ , 54 and population 9 (Afiq) of  $2n=58$ , and the other represented the other two populations of  $2n=58$  and all of the  $2n=60$  populations (Fig. 2c). Once again, in this analysis, population 9 (Afiq) of  $2n=58$ , displayed a pattern entirely different from the other populations of its species.

## Discussion

### Number of genes/homologies

The hybridization of the *Drosophila per* locus probe to genomic DNA detected different numbers of homologies in different unrelated taxa, such as the chicken, mouse, and man (Shin *et al.*, 1985). Three different *per* RNA transcripts, generated by alternative splicing events of the *per* gene, differing in only about 10 per cent of their sequences, were found in *Drosophila* (Citri *et al.*, 1987). In mammalian cells a repeating Ser-Gly sequence has been found in the proteoglycans: heparin sulfate (Robinson *et al.*, 1978), dermatan sulfate (Chopra *et al.*, 1985), chondroitin sulfate (Bourdon *et al.*, 1985; Miller *et al.*, 1988) and in bovine cartilage (Oldberg *et al.*, 1987).

The results of the present study indicate the existence of multiple DNA homologies in the four species of subterranean mammals of the *Spalax ehrenbergi* superspecies, similar to those of the mouse pattern (Shin *et al.*, 1985). The probe used is characterized by the presence of tandem repetitions of short motifs (ACNGGN and TCAGGC), and was shown to allow the detection of DNA fingerprints in men, dogs, horses and pigs (Georges *et al.*, 1987, 1988). The determination of the genetic basis of the different fragments described proved to be complex owing to the high level of polymorphism, the low frequency of some fragments, and the difficulty in establishing allelism. Nevertheless it seems that the probe detected a multigene family with more than 10 different homologies. Only three to four of them showed a stronger homology. It was possible to assign some of the bands that showed strong hybridization to specific loci, unlike DNA fingerprints. Moreover, the high stringency conditions used in hybridization and in washing the filters (much higher than those conducted by Georges *et al.*, 1987, 1988), suggest very strong homologies between *Spalax* DNA and the mouse *per*-homologous sequence. One or more of these loci may code for proteoglycan(s) which may be involved in biological rhythmicity. This speculation, however, needs further critical testing.

### The level of polymorphism

The level of polymorphism detected by hybridizing *Spalax* DNA with the mouse *per*-homologous sequence was very high, presumably with many alleles per locus. However, the variation within the population is much lower than that described by Jeffreys *et al.* (1985) for DNA fingerprints in human populations, or by Georges *et al.* (1988) for DNA fingerprints of *per*-homologous sequences of horses, chickens and fish. Many populations of *Spalax* are totally monomorphic (within populations), so that the mean probability that a fragment present in one individual is also present in another of the same population is  $x=1.0$  (Tables 2 and 4). Another well known minisatellite probes (Jeffreys — 33.15) shows DNA fingerprinting in the most monomorphic populations of *Spalax*, with fragment-sharing among individuals of the same population at only 26 per cent (E. Nevo *et al.*, in preparation).

Polymorphism was found in the three tested restriction enzymes, in all loci detected. Such a high level of polymorphism and a high number of alleles per locus (within the superspecies) are very interesting in view of the fact that subterranean mammals are generally characterized by a low level of protein polymorphism as compared with above-ground mammals (Nevo, 1978; Nevo *et al.*, 1984; Nevo *et al.*, 1990). A low level of DNA polymorphism and a low number of alleles were found in the RFLPs of haptoglobin (Nevo *et al.*, 1989), where only one out of six restriction enzymes detect polymorphism, with a maximum of three alleles per locus. Moreover in the RFLPs of the myosin heavy chain, a maximum of two alleles per locus (per restriction enzyme) was detectable (R. Ben-Shlomo & E. Nevo, in preparation). A high number of alleles per locus was found only in the RFLPs of the class II major histocompatibility complex, *Mhc* (Ben-Shlomo *et al.*, 1988). Likewise, a high number of different haplotypes was found in mtDNA (E. Nevo *et al.*, 1993).

At least one of the mutations that caused the polymorphism described in this study is located inside the probe region. As shown in Fig. 3, *Bam*HI restriction enzyme divided the gene into fragment 1 and either fragment 3 or 7. It is interesting that the recognition sequence of *Bam*HI is GGATCC, which can translate into Gly-Ser amino acids. A poly-(Ser-Gly) acceptor site has been verified directly in the instance of the heparin sulfate proteoglycan (Robinson *et al.*, 1978). Although the probe contained the Thr-Gly and the Ser-Gly encoding minisatellites could show homology with non-transcribed regions of the DNA, it is possible that the mutation occurred inside the acceptor site of the proteoglycan. It is reasonable that at least some of the other mutations also occurred inside the gene or in

its near vicinity, especially those mutations that yielded the smaller fragments.

In *S. ehrenbergi*, the *per*-homologous sequence polymorphism increased in the following order:  $54 < 52 < 58 < 60$ ; chromosomal species  $2n = 54$  and  $2n = 52$  were only slightly polymorphic. By contrast,  $2n = 58$  was highly polymorphic and  $2n = 60$  was even more so. These results concur with previous analyses of genetic diversity based on allozymes, in many unrelated species, which increase southwards with increasing aridity and unpredictability (Nevo, 1983; Nevo & Beiles, 1988, 1989). The geographical variation of the *per*-homologous sequence polymorphism may indicate the underlying evolutionary forces molding it. If indeed the *per*-homologous sequence is related to behavioural factors associated with rhythmicity, then controlling rhythmicity in a climatically unpredictable region may be more complex, and may select for a higher level in the fine tuning of the regulatory polymorphic signals.

#### *Polymorphisms in isolated populations*

The Sede Boqer desert isolate (population 13) of  $2n = 60$  exhibits a very interesting pattern. The Sede Boqer population is isolated by ~40 km of harsh desert from the main range of  $2n = 60$ . This isolation may have persisted off and on during at least part of the last 10,000 years (Nevo, 1989). The current population size is very small: ~100 individuals.

The effect of the population size reduction (bottleneck effect) on average heterozygosity is expected to last for hundreds of thousands of years after the recovery of the population size (Nei *et al.*, 1975). Likewise, it has been suggested that while allele numbers showed significant excesses when the population rapidly expanded from the homoallelic state, population size construction caused allelic deficiencies (Maruyama & Fuerst, 1985). These neutral considerations suggest that in the Sede Boqer isolate of *Spalax*, where the bottleneck effect predominated 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, which harbours a substantial *per*-homologous sequence polymorphism.

An additional unique *EcoRI* fragment of 3.0 kb (no. 13) appeared only in this population. Although its frequency (100 per cent) may change because only two individuals were tested in this system, it should still be quite high. The existence of an allele of a gene that is unique to an isolated population is rare. We have previously found such a phenomenon in RFLPs of an

unidentified muscle protein (R. Ben-Shlomo & E. Nevo, unpublished data).

Genetic variability in this isolated population has been found in some allozymes, mtDNA, and chromosomal polymorphisms (Nevo, 1989); *Mhc* (Ben-Shlomo *et al.*, 1988), and in the myosin heavy chain (R. Ben-Shlomo & E. Nevo, in preparation). The relatively high level of genetic variation may contribute to successful speciation (Barton & Charlesworth, 1984; Carson and Templeton, 1984; Nevo, 1989) of the desert isolate. A unique variation in a locus that might be connected to the courtship calls, hence to reproductive isolation, may support successful speciation.

Population 9 (Afiq) of  $2n = 58$  also revealed interesting attributes. Afiq is a marginal population that is located in the southern Golan Heights (Fig. 1), south of the hybrid zone with  $2n = 54$ , and only 10 km from population 6 (El-AI,  $2n = 54$ ). Geographically, this population is an isolated relic of  $2n = 58$ , surrounded by the Yarmouk river to the south-east, and by the Sea of Galilee and the Jordan river to the west.

The results of the present study place population 9 in an intermediate position concerning its genetic differentiation. In both *EcoRI* and *BamHI* polymorphisms, this population exhibited a mixture of all the species, although a low level of the  $2n = 58$  specific fragment was found. It seems that the Afiq population (9) reflects its origin from the  $2n = 58$  species, as well as some alleles which indicate xeric ecological correlates that appear in the species  $2n = 54$  and  $2n = 60$ .

Divergence between population 9 (Afiq) of  $2n = 58$  and conspecific populations 7 and 8 has been seen in other genetic systems, such as the myosin heavy chain (R. Ben-Shlomo and E. Nevo, in preparation), rDNA spacer polymorphism (Suzuki *et al.*, 1987) and mtDNA (E. Nevo *et al.*, 1993). Notably, this population is very polymorphic, more than the other two populations of  $2n = 58$ . The population is much more polymorphic than the nearby population 6 (El-AI) of  $2n = 54$ , which was monomorphic in all the tested endonucleases. The high level of polymorphism of the Afiq population is reminiscent of the high level of polymorphism of the species  $2n = 60$ , suggesting, intraspecifically, a positive correlation between the level of polymorphism and the climatic unpredictability within  $2n = 58$ .

#### *Fragment differences between and within chromosomal species*

Fragment variation allows us to distinguish between chromosomal species (Table 2 and Fig. 2). There are three main patterns. The first characterizes the species  $2n = 52$  and  $54$ , the second represents population 7 and 8 of  $2n = 58$ , and the third that of  $2n = 60$ . Popula-



tion 9 of  $2n = 58$  shows an intermediate pattern which is dependent on the restriction enzyme and gene.

The most distinct species demarcation is the one expressed in fragment 5 of *EcoRI*. This diagnostic species-specific fragment appeared only in the three populations of  $2n = 58$  (Table 2). This is the first case that such an alternative fixation of alleles was found within the species of the *S. ehrenbergi* complex in Israel. Similarly, 93 per cent of the haplotypes of mtDNA are species-specific (E. Nevo *et al.*, 1993.) The uniqueness of  $2n = 58$  is expressed in other cases, such as the combination of 8–10 or fragment 4 homozygotes of *EcoRI*, although it does not display complete discrimination. Other fragments that were specific to the species  $2n = 58$  and 60 are fragment 3 of *BamHI* and fragment 3 of *EcoRI*.

#### *Does the per-homologous sequence code for rhythmicity in Spalax?*

The best described clock gene is the *per* gene of *Drosophila melanogaster* (Hall, 1990). The similarities between the properties of the *per* gene of *Drosophila* and another identified clock gene, the *frequency (frq)* gene of *Neurospora*, suggest that *per* and *frq* might code for similar proteins (Feldman, 1988). DNA sequence comparisons between *frq* and *per* (McClung *et al.*, 1989), suggest the possibility of a common element in the time mechanisms of these two organisms. The chloroplast genome of *Acetabularia*, which is assumed to encode the p230 clock protein, shares homology with the *per* locus of *Drosophila* (Li-Weber *et al.*, 1987). The specific *per* protein antibody (anti-*per*) labelled putative circadian pacemaker neurons and fibres in the eyes of two marine gastropods, *Aplysia* and *Bulla* (Siwicki *et al.*, 1989). Genetic studies of factors underlying circadian pacemaking are beginning to indicate that time mechanisms may also be ancient entities that have been highly conserved in evolution (Hall, 1990).

The biochemistry and physiology of proteins such as those encoded by the *period* genes of *Drosophila* may be understood by characterizing and manipulating *per* protein relatives in other organisms, in which circadian pacemakers have been identified at tissue locations and are accessible in cellular terms (Hall, 1990). A different approach to the question of the similarities between the *per* gene product and rhythmicity in other organisms could be attempted by correlating variability in the *per*-homologous sequence with different rhythmicity parameters. Although there is no evidence that the RFLPs have anything to do with time-dependent genes, positive significant correlations can provide another strategy and direction for further investigation.

Such a correlation was found in the *Spalax* complex among different fragments of the *per*-homologous sequence found in this research, and the mean number of the apparent harmonics (or additional frequencies) parameter of the courtship calls (Nevo *et al.*, 1987; E. Nevo *et al.*, personal communication). A highly significant correlation was found between the low number of harmonics and fragments 7 ( $r = -0.90$ ,  $P < 0.01$ ) and 1 of *BamHI* ( $r = -0.91$ ,  $P < 0.001$ ), and fragment 2 of *EcoRI* ( $r = -0.84$ ,  $P < 0.01$ ), as well as between the high number of harmonics and fragments 3 and 4 of *BamHI* ( $r = +0.84$ ,  $P < 0.01$  and  $r = +0.067$ ,  $P < 0.05$ , respectively). This correlation suggests the possibility of gene(s) (homology) that are responsible for courtship call (the apparent number of harmonics) parameters. Only further research can shed light on the significance of this correlation. If the correlation is substantiated, and if female discrimination is affected by this parameter, then the above-mentioned correlation may indicate that the *per*-homologous sequence is associated with vocalization. As courtship calls are associated with mate selection, they do contribute to the essential behaviour mechanisms involved in the critical aspects of reproductive isolation in the actively speciating *Spalax* complex of species in Israel. In this case, *per* variation may affect speciation.

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