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Genome diversity in the cyanobacterium *Nostoc linckia* at 'Evolution Canyon', Israel, revealed by inter-HIP1 size polymorphisms

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ABSTRACT

The relationship between genomic diversity and environmental heterogeneity and stress was assessed in the cyanobacterium *Nostoc linckia*, across the sharp microclimatic contrasts of opposing slopes in 'Evolution Canyon', Mt. Carmel, Israel. 'Evolution Canyon' is a microcosm model of life with south- and north-facing slopes (SFS and NFS) that share a limestone lithology but contrast biotically, primarily due to interslope differences in solar radiation, resulting in higher rock temperatures on the south-facing slopes. The cyanobacterium *N. linckia*, a sessile prokaryote that grows in the canyon on limestone rock surfaces, is exposed to fluctuations of solar radiation, temperature and desiccation.

We found remarkable differences in genomic diversity of *N. linckia* subpopulations between slopes (*interslope*) and within elevations of the south-facing slopes (*intraslope*). These differences were assessed by amplification of genomic DNA with primers based on the highly iterated palindrome (HIP1) (5′-GCGATCGC-3′), thereby revealing the diversity among the HIP1 sites (inter-HIP1 polymorphism) in the genome of *N. linckia*. The interslope divergence was demonstrated by significantly higher diversity (*He* and *v*) and polymorphism (*p*) indices on the heterogeneous south-facing slopes, due in particular to the upper and middle elevations (SFS 1 and SFS 2). The intraslope divergence on the south-facing slopes reflects an upslope increase in genetic diversity indices with xeric microclimate, across the slope. Correlations were found between *p* and *He* and variables influencing aridity stress: solar radiation, temperature and day–night temperature differences. This may imply that the major cause for the inter- and intraslope genetic divergence is the differences in their microclimatic conditions.

We show that the genetic diversity of *N. linckia* in 'Evolution Canyon' revealed by I-HIP1 is related to heterogeneity and stress at a microsite, and appears to be moulded by natural selection as a genomic adaptive strategy to cope with climatic stress. This could highlight the importance of ecological stress and selection in evolution and its remarkable effect on the genetic system across the prokaryotic genome. Our results indicate that I-HIP1 can be exploited

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not only as a genetic marker, but also as a genetic monitor for the response to climatic stress. This conclusion supports the suggestion that HIP1 is involved in promoting genome diversity in cyanobacteria.

Keywords: cyanobacteria, DNA, ecological heterogeneity and stress, 'Evolution Canyon', genetic polymorphism, HIP1.

INTRODUCTION

How environmental heterogeneity and stress are related to genome diversity as an adaptive strategy requires critical testing. Climate has been regarded as one of the most important evolutionary forces driving biodiversity in nature from local to global scales (Mattew, 1939). Recent understanding of the evolutionary process has shifted from a Darwinian view of competition as the major driving force of natural selection to the importance of environmental heterogeneity (Huston, 1994) and stress (Hoffmann and Parsons, 1991) on evolutionary dynamics.

Opposite canyon slopes differ drastically in their biotic aspects (Shreve, 1922). According to the niche-width variation hypothesis (Van Valen, 1965) and the environmental theory of genetic diversity (Soulé and Stewart, 1970; Nevo, 1988, 1998), forces generating and maintaining genetic diversity are expected to be higher in areas exposed to broader environmental heterogeneity and stress. In a Mediterranean climate, differences in slope orientation, soil water-holding capacity and evapotranspiration are accentuated under conditions of low rainfall. Ecologically sharply divided microsites, such as 'Evolution Canyon' in Mt. Carmel, are excellent ecological theatres for elucidating the effect of environmental heterogeneity and stress on the evolutionary process (Nevo, 2001). We have used two microsites for long-term studies in Israel: (1) Lower Nahal Oren, Mt. Carmel (Nevo, 1995, 1997; Fig. 1) and (2) Lower Nahal Keziv, Western Upper Galilee Mountains (Finkel *et al.*, in press). Our objectives in these long-term studies are to assess biodiversity (genes, genomes, populations, species, communities and biota) across life, from bacteria to mammals. We identified about 3000 species in both canyons and focused on some 20 model organisms for in-depth studies, including species richness and genome diversity, in tracking the twin evolutionary processes of *adaptation* and *speciation* at a microsite (Nevo, 2001).

We used natural populations of the filamentous cyanobacterium *Nostoc linckia*, as a model organism representing sedentary prokaryotes, for the study of the microclimate divergence effect of 'Evolution Canyon' on genetic diversity and genome evolution. Cyanobacteria constitute the most diverse group of photosynthetic organisms (Whitton and Potts, 2000). They have undergone multiple adaptations that enable them to live under extreme environmental stress and they can inhabit extreme environments, including hot springs, glaciers and rocky deserts. Ecological contrasts on regional and, in particular, local scales can help explain their heterogeneity and tolerance to stress (Whitton, 1987). Species of the cyanobacterium genus *Nostoc* are found on every continent on earth, in a wide range of terrestrial and aquatic ecosystems, where their growth can be conspicuous and abundant. Environments where water is scarce or absent for protracted periods are among the most extreme on earth, yet they support populations of the terrestrial desiccation-tolerant populations of *Nostoc* (Potts, 2000). The species *N. linckia* forms crusts on rocks, is widely distributed in wide ecological ranges of mesic and xeric habitats both regionally in Israel

(Vinogradova *et al.*, 2000) and locally on the opposing slopes of 'Evolution Canyon', and is one of 67 taxa of cyanobacteria found on Mt. Carmel (Vinogradova *et al.*, 1995).

We assessed genetic diversity by amplifying genomic DNA with primers based on the highly iterated palindrome (HIP1) (5′-GCGATCGC-3′), thereby revealing the diversity among HIP1 sites, by analysing inter-HIP1 (I-HIP1) size polymorphism in the genome of *N. linckia* (Smith *et al.*, 1998). The HIP1 sequence was shown to constitute up to 2.5% of the DNA in some cyanobacteria (Gupta *et al.*, 1993; Robinson *et al.*, 1995; Robinson *et al.*, 1997; Kotani and Tabata, 1998). Molecular genetic techniques to identify genetic polymorphism, by analysing genomic DNA amplified by PCR with a variety of primers, have been described. These include random primers (random amplified polymorphic DNA) for the cyanobacterium *Anabena* (Neilan, 1996), specific primers for cyanobacteria using short tandem repeated repetitive (STRR) sequences, which create typical PCR-fingerprints (Zheng *et al.*, 1999), and PCR typing using short and long tandemly repeated repetitive sequences (Rasmussen and Svenning, 1998).

The main advantage of the HIP1 typing method over other molecular techniques is that it is specific for cyanobacteria and it provides information on the whole genome. Smith *et al*. (1998) showed that HIP1 primers do form some minor PCR products with bacteria; this required the purification of cyanobacterial isolates before PCR typing. Gupta *et al*. (1993) suggested that the over-expression of this motif in the genome of cyanobacteria has a major role in genome plasticity and cellular adaptation in these organisms. The HIP1 element is absent in rRNA and tRNA genes and, recently, it has been exploited for analysing global gene expression in cyanobacteria (Bhaya *et al.*, 2000b). It is distinct from previously reported prokaryotic repetitive sequences, such as STRR, in its small size, perfect palindromic structure, phylogenetic distribution, localization (also in protein coding regions) and its projected frequency of iteration. It has previously been hypothesized that HIP1 has a role in reorganizing and increasing genome diversity of cyanobacteria, thereby reinforcing their adaptation to multiple climatic stresses (Gupta *et al.*, 1993; Robinson *et al.*, 1997).

By using the successful microscale model of 'Evolution Canyon' in Mt. Carmel as a natural experiment and the I-HIP1 typing method, we demonstrate extraordinary inter- and intraslope genomic divergence within south-facing slopes. We show that the genetic polymorphism of *N. linckia* in 'Evolution Canyon' is related to heterogeneity and stress at a microsite, and appears to be moulded by natural selection as a genomic adaptive strategy to cope with climatic stress.

METHODS

'Evolution Canyon'

Lower Nahal Oren, Mt. Carmel, Israel (32°43′N; 34°58′E), dubbed 'Evolution Canyon', is a seasonally dry valley draining Mt. Carmel from east to west into the Mediterranean Sea. The opposing south-facing (SFS) and north-facing (NFS) slopes are 100 m apart at the valley bottom and 400 m apart at the valley top (Fig. 1). The slopes share an identical Plio-Pleistocene evolutionary history, presumably 3–5 million years ago, geology (Upper Cenomanian Limestone), pedology (Nevo *et al.*, 1998b) and regional Mediterranean climate (Nevo *et al*., 1999). The slopes diverge in topology (the south-facing slope dips 35° and the north-facing slope dips 25°) and in geographic orientation, resulting in different

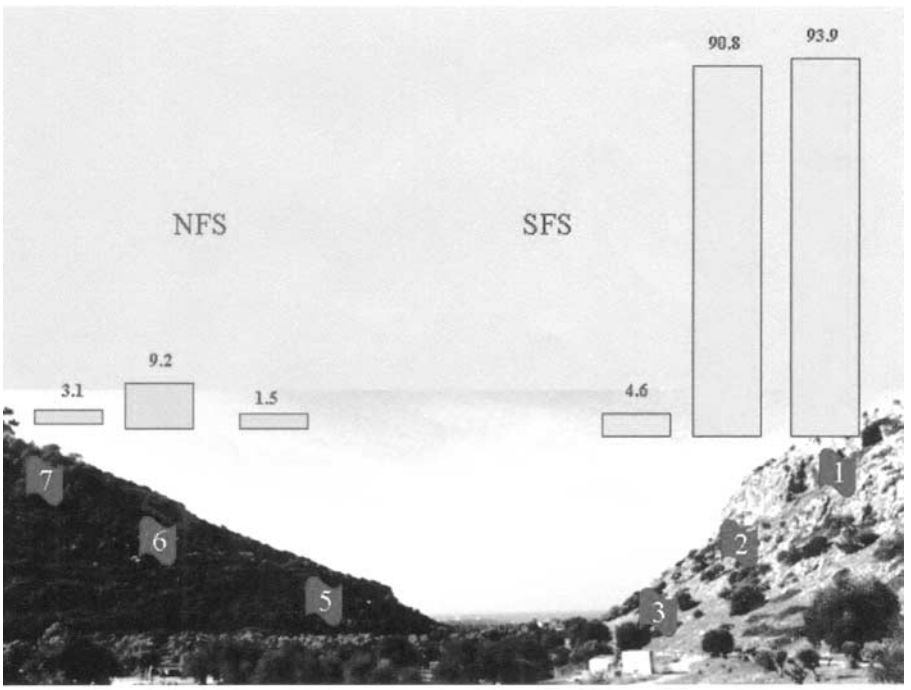

Fig. 1. Cross-section of 'Evolution Canyon' indicating polymorphism (*P*) at each of the collection sites SFS 1, 2 and 3 and NFS 5, 6 and 7.

input of solar radiation, interslope microclimatic and biotic differences (Nevo, 1995, 1997, 1998, 2001; Nevo *et al.*, 1999; T. Pavlicek, V. Kravchenco and E. Nevo, in prep.). *Nostoc linckia* was sampled in 'Evolution Canyon' at six collection sites at three elevations 30 m apart (60, 90 and 120 m above sea level) on each slope: SFS 3, 2 and 1 and NFS 5, 6 and 7, respectively.

Microclimatic data (Table 1) were recorded at two stations on each slope: the xeric SFS 1 and SFS 2 and the mesic NFS 6 and NFS 7 (described in detail by T. Pavlicek *et al.*, in prep.). For technical reasons (timing of the measurements), rock surface temperature was measured only at SFS 2 and NFS 6 (in sun and shade) by infrared thermometer (model M102HTL, Mikron Instrument Co. Inc., USA) (described by Satish *et al.*, in press).

Sampling, isolation and DNA extraction of *Nostoc linckia*

Cyanobacteria were sampled by scraping the limestone rock surface and washing off the fouling deposits. Mixed cultures were studied, beginning with the first appearance of algae and for 3 months during their growth. After obtaining mixed cultures, *N. linckia* was isolated and examined in pure cultures. Axenic cultures were obtained by cyanobacterial identification following Geitler (1932) and Desikachary (1959). Purification included removal of bacteria and fungi as described by Mitsui and Cao (1988). Single filaments were chosen at random for culturing biomass for genetic analysis. The tested *N. linckia* were cultured in BG-11-0 media (Sigma C-3186, pH 7.8) and agar (1.5%) was used for isolation

Table 1. Microclimatic measurements in 'Evolution Canyon', stations SFS 1, SFS 2, NFS 6, NFS 7

							Rock surface Tm	
Station	Sr	Hu	Hudd	Pc	Tdd	Tm	Sun	Shade
SFS 1	644 219	67.5	42.0	45	11.2	19.5		
SFS 2	894 212	70.3	41.5	55	11.4	19.4	45.0 ± 0.48	36.8 ± 0.21
NFS 6	101 813	71.4	30.7	140	7.6	18.4	35.7 ± 0.86	27.7 ± 0.33
NFS 7	148 565	70.7	38.2	70	9.1	18.7		

Note: Sum of solar radiation (Sr; $W \cdot m^{-2}$), average relative humidity (Hu; %), daily relative humidity difference (Hudd; %), daily temperature difference (Tdd; °C) and average ambient temperature (Tm; °C) are means for entire days (T. Pavlicek *et al.*, in prep.). General plant cover (Pc; %) is the mean for 2 years (Nevo *et al.*, 1999). Note that perennial species are divided into trees, shrubs and other life forms in different plant layers; hence, coverage can exceed 100%. Rock surface Tm was measured at SFS 2 and NFS 6 in sun and in shade.

and maintenance of the cultures of *N. linckia*. Cultures were grown in the laboratory at 25–28°C, illuminated by cool-white tubes. DNA was extracted by a modified version of the chromosomal miniprep procedure previously described (Golden *et al*., 1988; Satish *et al.*, in press).

HIP1-PCR analysis

PCR amplification

Four primers were used, each containing the HIP1 (5′-GCGATCGC-3′) sequence, but extended by different additional bases (Smith *et al.*, 1998): (1) HIP-TG (GCGATCGC-TG), (2) HIP-GC (GCGATCGC-GC), (3) HIP-CA (GCGATCGC-CA) and (4) a primer that was modified to have three additional nucleotides randomly selected at the 5′ as well as two at the 3′ ACT-HIP-CA (ACT-GCGATCGC-CA) (Table 2). The PCR reaction was conducted following Smith *et al*. (1998) with some modifications. Each reaction of 25 μl contained 0.5 units of DNA *Taq* polymerase (manufactured by Sigma, except HIP-TG, which was amplified by *Taq* manufactured by Promega), 25 ng genomic DNA, 5 ng primer (Genosys), 0.1 $mmol \cdot l^{-1}$ dNTPs mix, X10 PCR buffer supplied by the manufacturer and 2.5 $mmol \cdot l^{-1}$ $MgCl_2$. The PCR reactions were performed in a PE GeneAmp 9700: (1) denaturation at 95°C for 5 min; (2) 30 cycles of denaturation at 95°C for 30 s, annealing at 40°C for 30 s and elongation at 72°C for 1 min; (3) termination at 72°C for 5 min.

Gel electrophoresis

The PCR products amplified by HIP-TG, HIP-GC and HIP-CA were separated in 3% agarose gel. The ACT-HIP-CA PCR products were separated in 4% polyacrylamide gel (Acryl-Bis 29:1, 40%).

Statistical analysis

The I-HIP1 fragments were regarded as loci with alleles *present* (1) or *absent* (0). Genetic diversity indices were estimated by locus by locus polymorphism and haplotype polymorphism.

Locus by locus polymorphism

The proportion of polymorphic loci (*P*-5%), gene diversity (*He*; Nei, 1978), homogeneity of allele frequency, genetic distance (*D*) and the relative differentiation of genetic diversity (G_{ST}) were calculated for each station and slope by POPGENE 1.31 (http://www.ualberta.ca/~fyeh/), analysed as a haploid organism (Yeh *et al.*, 1999). The differences in *He* between stations were tested by analysis of variance with Duncan's multiple range test for each station over all loci. Significance was set at $p < 0.05$ (SAS, 1996).

Haplotype polymorphism

We determined the *total* and *unique* number of haplotypes for each station or slope (i.e. haplotype is the combination of sets of fragments amplified by the four HIP1 primers). The probability of sampling two different haplotypes simultaneously (*h*) and the proportion of loci differences between two randomly chosen haplotypes (*v*) were derived from the equations of Nei and Tajima (1981).

Comparisons of genetic diversity were conducted between all subpopulations within (*intraslope*) and between (*interslope*) slopes. The interslope comparison was conducted by pooling the I-HIP1 data obtained from the upper and drier stations on each slope (SFS 1, SFS 2, NFS 6, NFS 7) and by pooling data from whole slopes (SFS 1–3 and NFS 5–7).

Pearson correlation coefficients (*r*) were estimated between genetic indices and climatic variables at four stations: SFS 1, SFS 2, NFS 6 and NFS 7. Stepwise multiple regression analysis was used to test for the best predictors of *P* and *He* in the four stations using these features as dependent variables and the climatic factors as independent variables (SAS, 1996).

RESULTS

I-HIP1 size polymorphism

The HIP1-based primers amplify DNA sequences *between* adjacent HIP1 repeated sequences, present in a suitable orientation and a distance apart across the genome, revealing inter-HIP1 (I-HIP1) size polymorphism. Using four different oligonucleotides (Table 2), 65 fragments were amplified in the range 270–1250 bp (example of fragments amplified by HIP1-TG are shown in Fig. 2). The mean numbers of amplified fragments per genotype varied between 3.8 for HIP-GC to 9.2 for HIP-CA.

Table 2. Description of I-HIP1 fragments generated by different HIP1-based primers used to examine genome diversity in *Nostoc linckia*

Primer	Sequence (5′-3′)	Mean no. of fragments	No. of loci	Range of band size (bp)
HIP-TG	GCGATCGC-TG	7.5	17	270–1220
HIP-GC	GCGATCGC-GC	3.8	13	420–1140
HIP-CA	GCGATCGC-CA	9.2	17	500–1140
ACT-HIP-CA	ACT-GCGATCGC-CA	8.0	18	500–1250

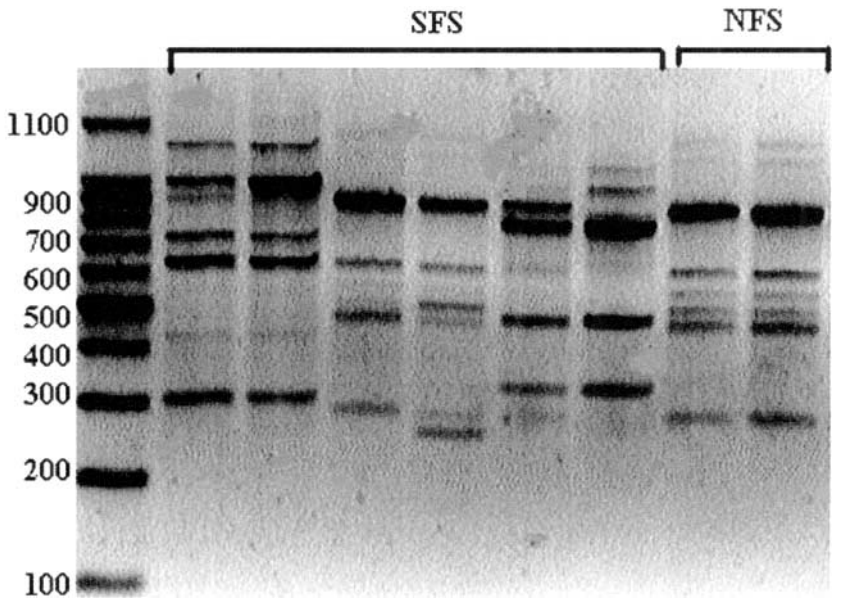

Fig. 2. PCR fingerprint patterns generated from genomic DNA of *Nostoc linckia* by HIP1-TG primer, separated on 3% agarose gel. Lane 1: DNA molecular weight marker in bp. Lanes 2–7: samples from SFS 1 and 2. Lanes 8 and 9: samples from NFS 5 and 6.

Locus by locus polymorphism

Comparisons between stations. A summary of genetic diversity of the I-HIP1 data for *N. linckia* for each of the six subpopulations is provided in Table 3. The highest values of polymorphism and gene diversity were obtained at SFS 1 with 61 polymorphic fragments ($P = 93.85\%$, $He = 0.353$) and SFS 2 with 59 polymorphic fragments ($P = 90.77\%$, $He = 0.2622$). Both subpopulations had significantly ($p < 0.001$) higher polymorphism and gene diversity than the lower (30 m below) SFS 3 ($P = 4.62\%$, $He = 0.0097$) and all three NSF subpopulations with only 1–6 polymorphic loci (NFS 5: $P = 1.54\%$, $He = 0.0034$; NFS 6: $P = 9.23\%$, $He = 0.0282$; NFS 7: $P = 3.08\%$, $He = 0.0077$) (Table 3). The level of genetic polymorphism was significantly different among the subpopulations ($\chi^2_5 = 301.2$, $p < 0.001$). Differences in *He* between stations were also significant ($F = 192.2$, $p < 0.0001$). The highest *He* value was recorded at SFS 1, which was significantly different from that at SFS 2; both these values were significantly different from those at SFS 3 and all NFS stations.

Comparison between slopes. Of the 65 amplified fragments on the south-facing slopes, 64 ($P = 98.46\%$) were polymorphic and 30 were *uniquely* amplified. On the north-facing slope, only eight fragments were polymorphic ($P = 12.31\%$); of the 57 monomorphic loci, 30 were null allele (no amplification product) (Table 3). A comparison of SFS 1 and 2 with NFS 6 and 7 shows that, on the south-facing slope, *He* was higher ($He = 0.342$) than when the lower subpopulation was included ($He = 0.277$), reflecting the high polymorphism of SFS 1 and SFS 2. Differences in *He* between the two slopes was assessed using analysis

Table 3. Genetic polymorphism (*P*) and gene diversity (*He*) of *Nostoc linckia*, based on 65 I-HIP1 loci, and haplotype diversity of six stations and two slopes in 'Evolution Canyon'

	Isolates analysed (*n*)	Polymorphism *P*-5%[1]	*He*[2]	Haplotype diversity			
				Total	Unique	*h*[3]	*v*[4]
Stations							
SFS 1	12	93.85	0.353[a]	11	9	0.984	0.394
SFS 2	13	90.77	0.262[b]	10	8	0.974	0.377
SFS 3	11	4.62	0.010[c]	3	1	0.345	0.026
NFS 5	8	1.54	0.003[c]	2	1	0.250	0.015
NFS 6	10	9.23	0.028[c]	7	5	0.911	0.032
NFS 7	10	3.08	0.008[c]	3	2	0.511	0.020
Slopes*							
SFS 1 and 2	25	98.46	0.342	21	20	0.993	0.359
NFS 6 and 7	20	10.77	0.022	9	8	0.853	0.036
SFS 1–3	36	98.46	0.277	22	21	0.904	0.345
NFS 5–7	28	12.31	0.025	10	9	0.852	0.038

* Interslope comparisons were performed by pooling the I-HIP1 data obtained from the genotypes of (1) the two upper stations on each type of slope (SFS 1, 2 *vs* NFS 6, 7), and (2) all three stations for each type of slope (SFS 1–3 *vs* NFS 5–7).
[1] Proportion of polymorphic loci-5%. [2] Gene diversity (Nei, 1973). [3] The probability of sampling two different haplotypes simultaneously. [4] The proportion of loci differences between two randomly chosen haplotypes.
[a,b,c] Significant differences using Duncan's multiple range test (SAS, 1996): $p < 0.05$.

of variance. Higher *F*-values were obtained when comparing SFS 1 and 2 with NFS 6 and 7 ($F = 374$, $p < 0.0001$) than when including the relatively homogeneous SFS 3 and NFS 5 in the analysis ($F = 257$, $p < 0.0001$).

Homogeneity of allele frequencies across the two slopes was assessed by χ^2. Interslope divergence was seen in 30 of 65 loci that were significantly different between the slopes (χ^2 or Fisher exact test with $p < 0.05$). This total proportion of significant loci exceeded the 5% level expected by chance (binomial test, $p < 0.000001$) (Sokal and Rohlf, 1981), indicating significant differences in allele frequencies between the slopes.

Haplotype polymorphism

Comparisons between stations. All parameters of haplotype diversity (i.e. total and unique numbers of haplotypes, *h* and *v*; Table 3) were very high on SFS 1 and SFS 2, while low values were recorded in all other subpopulations. Although a high value (0.911) of *h* was found in NFS 6, it was accompanied by a low value of *v* (0.032); that is, the number of haplotypes was high but the mean proportion of loci that differed between haplotypes in this subpopulation was very low.

Comparison between slopes. Thirty-one different haplotypes were found on the south- and north-facing slopes (Table 3). Twenty-two haplotypes were found on the south-facing

slope, mainly on SFS 1 and SFS 2; of these, 21 were *unique*. Ten haplotypes were found on the north-facing slope, nine of which were *unique*; these were found mainly on NFS 6 and NFS 7. Only one haplotype was common to both slopes. As described above, the divergence between the north- and south-facing slopes was evident from the higher divergence among haplotypes (v) of the south-facing slope.

Genetic differentiation (G_{ST}) within and among subpopulations

Gene diversity relative to total gene diversity (G_{ST}) (Nei, 1973) was estimated for all 65 loci, between the six subpopulations and between the slopes. For two alleles at a locus, the G_{ST} is identical to the F_{ST} (Wright, 1946). According to Hartl (1980), a range of 0.05–0.15 for F_{ST} indicates moderate differentiation, whereas one of 0.15–0.25 indicates large differentiation. Comparison between stations showed significant differentiation at 43 loci, moderate differentiation at 21 loci and negligible differentiation at one locus (Wright, 1978). We found that 66% of the total genetic diversity was within the six stations, whereas 34% (i.e. $G_{ST} = 0.340$) was *between* the stations. To assess the significance of these results, a permutation test was applied using 5000 randomized data sets produced by random shuffling of the genotypes within the various stations. Significantly ($p < 0.001$) lower G_{ST} values (average 0.083, range 0.033–0.197) were obtained for the randomized than the real data.

When comparing the xeric upper and middle stations of each slope (SFS 1 and 2 *vs* NFS 6 and 7), large differentiation was obtained at 28 loci, moderate differentiation at 27 loci and only negligible differentiation at 10 loci. We found that 81.3% of the total genetic diversity was *within* and 18.7% (i.e. $G_{ST} = 0.187$) was *between* the slopes. The average G_{ST} value obtained with the randomized data sets was 0.023 (range 0.004–0.123), which was lower than for the real data ($p < 0.001$). In conclusion, the partition of the genetic diversity showed a *real* and *strong* differentiation between the *N. linckia* subpopulations at the six stations and between the two slopes.

Genetic distance and cluster analysis

Unbiased coefficients of genetic distance (D; Nei, 1978) were calculated for paired comparisons of the six subpopulations (Table 4) based on the normalized identity of each locus in each of the subpopulations. The genetic distance, D, between SFS 1–3 and NSF 5–7 was 0.034, whereas a higher value was obtained ($D = 0.082$) between the xeric SFS 1–2 and mesic NFS 6–7 as described above. Intraslope divergence on the south-facing slope was demonstrated by a high genetic distance (0.201) between SFS 1 and SFS 3 and lesser distances between SFS 1 and SFS 2 and between SFS 2 and SFS 3 (0.084 and 0.036, respectively). Short distances were found ($D = 0.007$–0.032) among all stations on the north-facing slope, indicating low intraslope divergence of this slope.

A dendrogram based on Nei's (1978) calculated genetic distance (D) values, using the UPGMA method (PHYLIP package v. 3.5), is shown in Fig. 3. The highly polymorphic SFS 1 is located far from the other five stations. A cluster that consists of five stations is further subdivided into SFS 2, which is seen as a separate branch; a cluster consisting of SFS 3 and NFS 5, which are the most similar populations; and a cluster consisting of NFS 6 and NFS 7, reflecting their close positions on the north-facing slope and their genetic similarity.

Table 4. Genetic distances (Nei, 1978) between six subpopulations of *Nostoc linckia* on south- and north-facing slopes of 'Evolution Canyon'

	SFS 1	SFS 2	SFS 3	NFS 5	NFS 6	NFS 7
SFS 1	****					
SFS 2	0.084	****				
SFS 3	0.201	0.036	****			
NFS 5	0.205	0.042	0.001	****		
NFS 6	0.189	0.035	0.012	0.016	****	
NFS 7	0.190	0.037	0.026	0.032	0.007	****

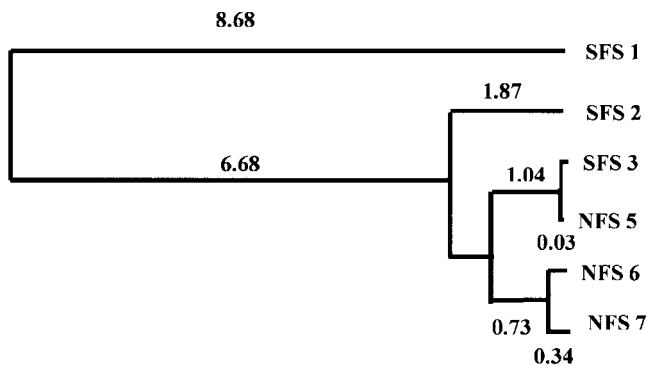

Fig. 3. Dendrogram of the genetic relationships of six subpopulations of *Nostoc linckia* in 'Evolution Canyon' based on Nei's (1978) calculated genetic distance (D) values, using the UPGMA method (PHYLIP package v.3.5). Numbers on branches are $D \times 100$.

Pearson correlation and multiple regression with climatic variables

Pearson correlation

Analysis of the Pearson correlation coefficients between climatic parameters (Table 1) with polymorphism (P) and gene diversity (He) are presented in Table 5. Significant correlations were detected between genetic polymorphism and several variables influencing aridity stress: solar radiation ($r = 0.952$, $p < 0.05$), temperature ($r = 0.959$, $p < 0.05$) and day–night temperature difference ($r = 0.920$, $p = 0.08$). Non-significant inverse correlations were found between relative humidity/plant coverage and P and He, showing the same trend of high humidity conditions with lower genetic polymorphism.

Multiple regression analysis

To determine the best predictors of P and He at the four stations, we conducted stepwise multiple regression analysis, using these features as dependent variables and the climatic factors as independent variables. Average temperature explained 92% ($p = 0.04$) of the variance of polymorphism (P) and 90% ($p = 0.04$) of the variance of gene diversity (He). Two-variable combination predictors of genetic polymorphism (P) were average temperature and percent of plant cover, which explained 99.8% ($p = 0.04$) of the variance of P.

Table 5. Pearson correlation coefficients (r) between genetic indices of four subpopulations of *Nostoc linckia* and climatic variables (T. Pavlicek *et al.*, in prep.) at four stations in 'Evolution Canyon'

Climatic variable	*P*	*He*
Sum of solar radiation ($W \cdot m^{-2}$)	0.952*	0.879
Average ambient temperature (°C)	0.959*	0.951*
Daily temperature difference (°C)	0.920[@]	0.889
Average relative humidity (%)	–0.732	–0.843
Daily relative humidity difference (%)	0.778	0.767
General plant cover (%)	–0.691	–0.691

[@] $P < 0.1$, * $P < 0.05$.

DISCUSSION

Genomic divergence of *Nostoc linckia* revealed by I-HIP1

The genomic diversity and divergence of the cyanobacterium *Nostoc linckia* was examined in 'Evolution Canyon' in relation to a microscale evolutionary model of biodiversity across life (Nevo, 1995, 1997, 2001). The interslope (between the south- and north-facing slopes) and intraslope (on the south-facing slope) genome divergence of *N. linckia*, revealed by size polymorphism of I-HIP1, is probably related to climatic heterogeneity caused by interslope daily, seasonal and inter-annual environment fluctuations of solar radiation, temperature and drought.

The level of genetic diversity and divergence was well demonstrated by both the locus by locus and haplotype polymorphisms. The partition of the genetic diversity (G_{ST}) showed significant real differentiation between the *N. linckia* subpopulations and between the two slopes. The intraslope divergence on the south-facing slope reflects an upslope increase in genetic diversity indices of *N. linckia* subpopulations with xeric microclimate, across the slope. The highest polymorphism and gene diversity on the south-facing slopes was found in the upper and middle stations, which are the most xeric stations (Table 3).

Interslope divergence was demonstrated by significantly higher polymorphism (P) and diversity indices (*He* and v) on the south-facing slope, which receives the most solar radiation, resulting in remarkably higher rock temperatures. The highly significant differences in *He* between the extreme upper and middle subpopulations on the south-facing slope reflect the high polymorphism of SFS 1 and SFS 2. Interslope divergence was also confirmed by a homogeneity test of allele frequencies across the two slopes, with 46% of the loci being significantly different between the slopes.

The major reason for the *inter*- and SFS *intra*-slope genetic divergence is the differences in their microclimatic conditions, as demonstrated by the correlations between *P* and *He* with variables influencing aridity stress: solar radiation, temperature and day–night temperature differences (Table 5). The higher the environmental heterogeneity and stress on the south-facing slope in its two upper stations, the higher the I-HIP1 polymorphism and diversity.

'Isolation by distance' alone (Wright, 1931) cannot explain the genetic divergence between the six subpopulations, as the distances between the stations are not great and there is no physical barrier between them. Furthermore, there is no correspondence between

genetic distance and geographical distance. For example, the genetic distance between SFS 1 and SFS 3 (60 m apart) is 0.201, whereas the genetic distance between SFS 3 and NFS 5 (130 m apart) is only 0.001 (Table 4, Fig. 3). The genetic similarity between SFS 3 and NFS 5 can be explained by the microclimate in the lower parts of the canyon: a deep soil profile, higher moisture due to greater water run-off on the entire south-facing slope to the lowest station, and increased plant coverage, which provides some protection to *N. linckia* against solar radiation and the accompanying stress conditions of high temperatures and drought (Nevo *et al.*, 1999).

The role of HIP1 in genome diversity

What is the role of HIP1 in promoting genome diversity in *N. linckia*? The HIP1 genotyping method reveals genome diversity by analysis of inter-HIP1 (I-HIP1) size polymorphism (Smith *et al.*, 1998). It has previously been hypothesized that HIP1 has a role in reorganizing and increasing genome diversity of cyanobacteria, thereby reinforcing their adaptation to multiple climatic stresses (Gupta *et al.*, 1993; Robinson *et al.*, 1997). Although it is unclear why HIP1 has proliferated in the genomic DNA of some cyanobacteria, it is known to promote chromosomal gene rearrangements that could be adaptive in stressful environments (Robinson *et al.*, 1995; Robinson *et al.*, 1997). Gupta *et al.* (1993) suggested that the over-expression of this motif in the genome of cyanobacteria has a widespread role in genome plasticity and cellular adaptation in these organisms.

Similar patterns of *inter*-slope and *intra*-slope divergence were found in our previous study of genetic analysis of *N. linckia* in 'Evolution Canyon' (Satish *et al.*, in press) by amplified fragment length polymorphism (AFLP). In contrast to the similar polymorphisms found on the south-facing slope by AFLP and I-HIP1 ($P = 99.0\%$ and 98.4%, respectively), considerably higher polymorphisms were found on the north-facing slope using AFLP ($P = 85.7\%$) than I-HIP1 ($P = 12.3\%$). Hence, I-HIP1 polymorphism is more sensitive than AFLP to environmental heterogeneity at a microsite. The results obtained in this study indicate that I-HIP1 polymorphism can be exploited not only as a genetic marker but also as a genetic monitor for the response to climatic stress. This conclusion is supported by a previous suggestion that HIP1 is involved in promoting genome diversity in cyanobacteria (Gupta *et al.*, 1993; Robinson *et al.*, 1997).

Comparison with other organisms in 'Evolution Canyon'

Our results support previous findings of adaptive microgeographic differentiation of allozyme and DNA polymorphism in 'Evolution Canyon', in which higher polymorphism occurs mainly on the south-facing slope: land snails (Nevo *et al.*, 1982a), diplopods (Pavlicek and Nevo, 1996), beetles (Pavlicek and Nevo, 1995a,b), wild barley and lichens (Nevo *et al.*, 1997).

The *inter*-slope and *intra*-slope results obtained in the present study appear to be more remarkable, perhaps because *N. linckia*, in contrast to all other taxa tested (except the lichen *Caloplaca aurantia*), is completely sessile and permanently exposed throughout the year to high solar radiation, temperature and drought, with no opportunity for habitat choice. In their natural environment, cyanobacteria generally live under growth-limiting conditions, with frequent changes in several environmental parameters, including light,

salinity, humidity, nutrient availability and temperature (Whitton and Potts, 2000). Cyanobacteria owe their remarkable capacity to adapt to environmental heterogeneity and stress to the range of morphological and physiological properties they have acquired (Stanier and Cohen-Bezire, 1977; Bhaya *et al.*, 2000a). They respond to stress with physiological adaptations (acclimatization) and, depending on the kind and intensity of the stress, the response can persist for hours or days. Successful acclimatization can confer increased resistance to inhospitable conditions (Hagemann and Erdmann, 1997). Our preliminary observations on the growth rate of *N. linckia* from 'Evolution Canyon' under high temperature stress indicate that a reduction of growth rate of NFS cultures in higher temperatures is more pronounced than that of SFS cultures (unpublished data). Hence, the cultures originating from the xeric south-facing slope are more able to resist inhospitable conditions.

Stress and genetic polymorphism

The idea of a positive correlation between genetic diversity and environmental heterogeneity is widespread in evolutionary biology (Levins, 1968; Nevo, 1998). A promising approach for understanding the mechanisms maintaining genetic polymorphism is the search for a direct correlation of diversity with environment and with physiological function (Nevo *et al*., 1977). Various examples include the response of *Drosophila melanogaster* to environmental stress (acetic acid, ethanol, starvation and thermal stress) and the significant increase in phenotypic variation of *D. arista* under temperature stress in all morphological characters studied (Clark and Fucito, 1988). In 'Evolution Canyon', Rankevich *et al*. (1996) found genetic and physiological adaptations to microclimatic stress of land snails, and distinct interslope adaptive complexes have been described for *D. melanogaster* (Nevo *et al.*, 1998a).

Genetic diversity and divergence studies have encompassed local (Nevo *et al*., 1988; Owuor *et al.*, 1997; Li *et al.*, 2000), regional (Nevo *et al.*, 1979, 1982b; Fahima *et al.*, 1999) and global (Nevo *et al.*, 1984) scales, demonstrating that multilocus DNA diversities are non-randomly distributed and correlated in space and/or time with environmental factors. These observations, together with our results for *N. linckia*, which is constantly exposed to unfavourable environmental conditions, support the hypothesis that genetic diversity increases in stressful environments (Imasheva *et al.*, 1988).

The most likely mechanisms for maintaining genetic polymorphism are *natural diversifying or balancing selection* in spatially and temporally heterogeneous environments (Levene, 1953; Bryant, 1976; Gillespie, 1978; Hedrick, 1986), particularly in cycling environments (Kirzhner *et al.*, 1996; Korol *et al.*, 1998). Future work should focus on the direct effect of individual stressors, or combinations of stressors, on the differential expression of candidate stress genes in *N. linckia* from 'Evolution Canyon' and compare structural and functional genomics.

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