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Spatiotemporal allozyme divergence caused by aridity stress in a natural population of wild wheat, *Triticum dicoccoides*, at the Ammiad microsite, Israel

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Abstract Spatiotemporal diversity at 35 allozyme loci was assayed over 6 years in 1,207 individuals of wild emmer wheat (*Triticum dicoccoides*) from a microgeographic microsite, Ammiad, north Israel. This analysis used new methods and two additional sample sets (1988 and 1993) and previous allozymic data (1984–1987). This microsite includes four major habitats (North-facing slope, Valley, Ridge, and Karst) that show topographic and ecological heterogeneity. Significant temporal and spatial variations in allele frequencies and levels of genetic diversity were detected in the four subpopulations. Significant associations were observed among allele frequencies and gene diversities at different loci, indicating that many allele frequencies change over time in the same or opposite directions. Multiple regression analysis showed that variation in soil-water content and rainfall distribution in the growing season significantly affected 10 allele frequencies, numbers of alleles at 8 loci, and gene diversity at 4 loci. Random genetic drift and hitchhiking models may not explain such locus-specific spatiotemporal divergence and strong allelic correlation or locus correlation as well as the functional importance of allozymes. Natural ecological selection, presumably through water stress, might be an important force adaptively directing spatiotemporal allozyme diversity and divergence in wild emmer wheat at the Ammiad microsite.

Keywords Temporal diversity · Microgeographic genetic diversity · Allozyme · Wild emmer wheat · *Triticum dicoccoides*

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Introduction

Environmental heterogeneity in space or time can maintain genetic polymorphisms. The existence of a protected polymorphism is more likely in more heterogeneous environments, and spatial variation is considered more effective than temporal in protecting a polymorphism (Karlin 1982). Spatial heterogeneity at the microgeographic level could contribute to the maintenance of polymorphisms reinforced by habitat selection (Shorrocks and Nigro 1981; Barker et al. 1986). Kirzhner *et al*. (1994, 1995) found that cyclical selection with a short period may induce auto-oscillations with a long period (supercycle). A multilocus system subjected to stabilizing selection with a cyclically moving optimum can generate ubiquitous complex limiting behavior including supercycles, T-cycles, and chaotic-like phenomena (Kirzhner et al. 1996). Genetic polymorphism on a local, regional and global scales was shown to be driven by ecological heterogeneity and stress (Nevo 1998). Temporal variation in the environment may be a universal factor maintaining comparatively high rates of recombination in natural populations, as well as the cause of rapid rearrangements of the recombination system at times of drastic changes in the environment (Korol et al. 1994). Temporal analyses may allow insights into the dynamics of natural populations beyond those offered by geographic surveys, unraveling spatial genetic variation. Such analyses not only permit the validation of genetic divergence in space at only one point in time but also provide information on forces responsible for genetic changes on a short time scale of a few generations. Three evolutionary forces are usually advanced to explain temporal variation in allelic frequencies: selection, random genetic drift, and migration. While some experimental or empirical studies have revealed selective effects on the temporal variation of genetic structure (Gyllenstein 1985; Mueller et al. 1985; Barker et al. 1986; Allard 1988; Saghai-Maroof et al.1994), but other studies have suggested that most or all of the temporal shifts in allele frequencies within populations appear to

represent random genetic drift (Jorde and Ryman 1996; Levy and Neal 1999) or gene flow (Viard et al. 1997; Vandewoestijne et al.1999). The inconsistent results, therefore, encourage further temporal analyses in different species at various geographic scales.

For a wide range of plant species the mating systems are known to affect patterns of genetic variability both within and among populations. Hamrick and Godt (1990) generalized that in outcrossing species most of the total genetic diversity resides within populations, while in selfing species more is found between populations. On the macrogeographic scale, colonization events are thought to be important in patterning this variation, as colonization, which is frequently associated with selfing populations (Schoen and Brown 1991), increases the among-population component of genetic diversity but decreases within-population heterogeneity and numbers of alleles (Waples 1989). In wild emmer, a selfer, 60% of the allozymic variation resides between populations (Nevo and Beiles 1989). On the microgeographic scale, gene flow can potentially decrease among-subpopulation variation through seed dispersal by animal and human activities, and occasional outcrossing, unless overridden by natural selection.

Wild emmer wheat has been intensively studied using molecular markers on macro- and microgeographic scales (e.g., Nevo et al. 1982; Nevo and Beiles 1989; Nevo 1998; Fahima et al.1999; Li et al.1999, 2000). Its spatiotemporal changes in allele frequency from 1984 to 1987 at the Ammiad microsite were analyzed using 43 allozymic markers (Nevo et al. 1991). Significant differentiation according to vegetationally and topographically defined habitats and subhabitats was found primarily in space over very short distances, and secondarily over time. The highest gene diversity (*He*) occurred in the Karst habitat (Nevo et al. 1991). The results suggested a primary effect of environmental factors related to topography and temporal climatic changes, probably through drought, i.e. aridity stress. In the study reported here, based on the original allozymic data of 1984–1987 in Nevo et al. (1991), we added 2 sampling years (1988 and 1993) and again analyzed spatial and temporal diversities of wild emmer using different approaches. This study not only analyzed changes in allele frequency, genetic diversity, and divergence over space and time but, most importantly, contributed direct estimates for a correlation between spatiotemporal variation and soil moisture, and rain distribution during the growing season of wild emmer, for coordinated changes between alleles/loci and for the effect of protein functional groups.

Materials and methods

Plant materials and the Ammiad microsite

Wild emmer wheat, *Triticum dicoccoides* (genomic constitution AABB), the progenitor of all bread wheats (Feldman 1976), grows as an annual, highly selfing grass in several steppe-like herbaceous formations in the *Quercus ithaburensis* open park forest belt (Zohary 1973). It grows primarily on basaltic and terra rossa soil types. The investigated site is situated in the center of a large area of a *T. dicoccoides* population, at Ammiad, on terra rossa soil weathered from Middle Eocene limestone rocks in the Upper Galilee Mountains, north of the Sea of Galilee, Israel. The sampling of *T. dicoccoides* for this study was conducted along four

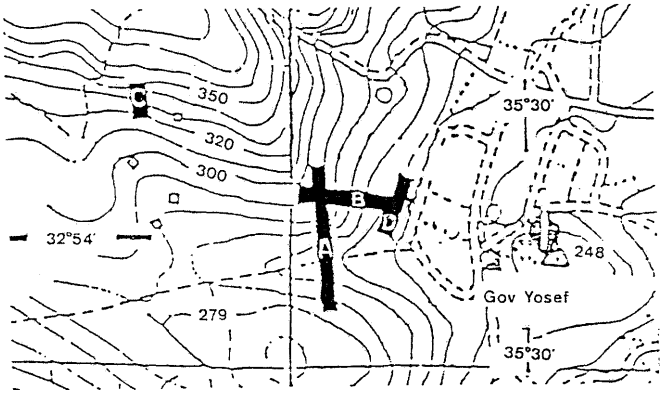

Fig. 1 The Ammiad study site showing transects A, B, C, and D. Scale = 1: 15,000 (from Nevo et al.1991)

transects (A, B, C, and D) about 800 m in length at the Ammiad microsite (Fig. 1). Transect A consists of a north-facing slope, is then dissected by a valley, continues along a south-facing slope, and ends on a ridge. Transect B begins on the top of the ridge and continues along an east-facing slope, which gradually levels out to a valley intersecting with Transect D. Transect C to the west of Transect A has a mainly southern exposure with an extremely steep incline in its central part. This C transect is 0.5-km distant from the westernmost point of Transect B and differs from the other three transects in its deeply creviced karstic rock formations and the relatively abundant soil moisture supply. This richer moisture supply is indicated by the occurrence of a reed (*Arundo donax*) in this habitat. Transect D is located in a gently sloping valley below the east-facing slope and is less rocky (Anikster and Noy-Meir 1991; Noy-Meir et al. 1991). Across the four transects, four major habitats were distinguished as 'Valley' (including lower slopes in Transects A, B, and D), 'Ridge' (including the south-facing slope, ridge top, and shoulder in Transect A, and ridge top east-facing slope in Transect B), 'North' (a north-facing slope in Transect A), and 'Karst' (with deeply dissected rock relief in Transect C). The four habitats showed strong and highly significant differentiation in ecological factors, in particular with respect to cover, proximity and height of rocks, and surface soil moisture after early rains of the growing season (Table 1, and see Noy-Meir et al. 1991). The Karst habitat appears to be the most favorable environment for plant growth, and the Valley habitat is the microenvironment most in contrast to the Karst one (Noy-Meir et al.1991).

An annual rainfall of 400–500 mm seems to be optimal for wild emmer populations, but within this range, a scarcity of early (Oct.-Nov.) and late (March-April) rains in the growing season has a negative effect on the population of *T. dicoccoides* (e.g., seed germination, population density: Noy-Meir et al. 1991; Noy-Meir 1999). The total annual rainfall and its distribution fluctuated between 1984 and 1993, as shown in Table 1. The present study tested 1,207 individuals of wild emmer wheat sampled from the Ammiad microsite from 1984 to 1993, and partly (1984–1987) analyzed earlier for spatiotemporal allozymic diversity and divergence (Nevo et al. 1991).

Allozyme data

The allozyme data analyzed here were extracted from the original data set for the 1984–1987 collections (Nevo et al.1991), with the addition of data detected for the 1988 and 1993 collections. Thirty-five allozymic loci were tested; locus and allele designations are the same as those used for the wild emmer wheat (Nevo and Beiles 1989; Nevo et al.1991). There are 12 allozymic groups (loci with the same code, such as *Aat* and *Adh* groups). These allozymic groups can be susdivided into four metabolic groups (with similar metabolic mechanism) of enzymes: the transferase, oxido-

Table 1 Rainfall (mm) distribution, surface (0–5 cm) soil-moisture content (%) after early rains in the growing season of wild emmer, and sample sizes in different years at the Ammiad microsite (1984–1988 data from Noy-Meir et al. 1991)

Year	Rainfall (mm)				Soil-moisture content (%)				Sample size				
	Annual	Oct-Nov	Dec-Feb	Mar-Apr	North	Valley	Ridge	Karst	North	Valley	Ridge	Karst	Total
1983–84	505	97	203	205					30	89	86	28	233
1984–85	623	124	416	83					24	75	61	22	182
1985–86	415	43	321	25	30.8b[a]	29.0c	32.7a	31.9a,b	23	62	74	18	177
1986–87	792	288	365	139	23.6b	19.9c	21.3b,c	30.3a	28	81	89	24	222
1987–88	598	33	468	97	25.7b	23.6c	27.8b	29.9a	30	68	99	21	218
1992–93	649	116	476	49					23	61	72	19	175
Total									158	436	481	132	1207

[a] Duncan's multiple range test among microhabitats ($P<0.05$) for each of 3-year measurements, separately. Values with a common letter are not significantly different. The analysis of variance for soil-moisture content among microhabitats showed high significance ($P<0.0001$, see Noy-Meir et al.1991)

reductase, hydrolase, and isomerase groups. (1) The transferase group included one allozymic code for aspartate aminotransferase (E.C.2.6.1.1), 6 loci (*Aat-1A, -B; Aat-2A, -B; Aat-3A, -B*). (2) The oxidoreductase group included seven allozyme codes for: alcohol dehydrogenase (E.C. 1.1.1.1), 4 loci (*Adh-1A, -B; Adh-2A, -B*); glutamate dehydrogenase (E.C. 1.4.1.2), 2 loci (*Gdh-A,-B*); indophenol oxidase in root (E.C. 1.10.3.1), 2 loci (*Ipor-A, -B*); malate dehydrogenase (E.C. 1.1.1.37), 3 loci (*Mdh-1A, -B; Mdh-2*); lipoamide diaphorases (E.C. 1.6.4.3), 4 loci (*Nadh-1A, B*; *Nadh-2A, -B*); phospho-glucomutases (E.C. 2.7.5.1), 2 loci (*Pgm-A, -B*); and 6-phosphogluconate dehydrogenase (E.C. 1.1.1.44), 3 loci (*6Pgd-1A, -B*; *6Pgd-2*). (3) The hydrolase group included three allozymes containing esterase (E.C. 3.1.1.2), 2 loci (*Est-5A, -B*); glucosidase (E.C. 3.2.1.21), 2 loci (*Gluc-A, -B*); and peptidase (E.C. 3.4.13.11), 3 loci (*Pept-1A, -B*; *Pept-3*). (4) The isomerase group included only one allozymic code, glucose-phosphate isomerase (E.C. 5.3.1.9), 2 loci (*Pgi-A, -B*).

Data analysis

For each subpopulation from the four habitats and sampling year, allele frequencies, number of alleles, gene diversity (*H*e, Nei 1973), and gene differentiation ($G_{ST,}$ Nei 1973) among the four subpopulations and sampling years were computed using the *POPGENE* program (Yeh et al. 1997). Multiple regression and other statistical tests were performed with the *STATISTICA* program (Statsoft 1996).

Results

Spatial and temporal diversity in allele frequencies

This study included 35 allozymic loci which were analyzed electrophoretically from 1984 to 1993. The means and standard deviations of allele frequencies over the 6 years are presented in Table 2. Out of the 35 allozymic

Table 2 Temporal and spatial variations in allele frequencies at polymorphic allozymic loci over 6 years in four natural subpopulations of *T. dicoccoides* at the Ammiad microsite

$Locus_{allele}$[a]	Temporal variation								Spatial variation	
	North (n = 158)		Valley (n = 436)		Ridge (n = 481)		Karst (n = 132)		Of mean[b] H(3, 24)	Of variance Bartlett's test(df = 3)
	Mean	SD	Mean	SD	Mean	SD	Mean	SD		
Aat-3A$_a$	0.97	0.034	0.97	0.029	0.99	0.006	1.00	0.000	7.36	0.20
Adh-1A$_a$	0.93	0.117	0.93	0.092****	0.92	0.105****	0.60	0.332***	3.45	4.96
Adh-1B$_a$	0.95	0.033	1.00	0.000	1.00	0.000	0.93	0.167**	11.42**	–
Adh-2A$_a$	1.00	0.000	0.99	0.011	0.99	0.006	1.00	0.000	2.09	–
Est-5A$_a$	0.90	0.045	0.71	0.102***	0.52	0.089***	0.98	0.024	20.80****	0.88
Est-5B$_{null}$	0.58	0.209****[c]	0.86	0.110***	0.87	0.094****	0.56	0.408****	9.01*	9.03*
Gluc-A$_b$	0.95	0.061	0.77	0.025	0.94	0.039	0.34	0.217**	19.80****	17.74**
Gluc-B$_b$	0.95	0.054	0.75	0.033	0.93	0.042	0.31	0.187*	19.46****	14.00**
Ipor-B$_a$	1.00	0.000	0.95	0.124***	0.96	0.086***	0.96	0.067	2.50	–
Mdh-1A$_b$	0.97	0.032	0.92	0.062	0.92	0.022	0.36	0.198**	16.46****	10.62*
Nadh-1A$_a$	0.81	0.260****	0.48	0.305****	0.56	0.230****	0.80	0.137	6.27	1.65
Nadh-1B$_a$	0.50	0.234***	0.65	0.241****	0.57	0.130****	0.86	0.114	9.17*	2.57
Nadh-2A$_a$	0.59	0.200****	0.98	0.028	0.99	0.017	0.98	0.023	14.82**	4.36
Nadh-2B$_a$	0.61	0.210***	0.95	0.090****	0.89	0.189****	0.82	0.402***	9.11*	7.85*
Pept-1B$_a$	0.97	0.047	0.96	0.030	0.98	0.020	1.00	0.000	7.40	–
Pept-3$_a$	0.97	0.054	0.96	0.029	0.82	0.208***	0.72	0.327****	3.58	15.60**
Pgi-A$_b$	0.40	0.208****	0.57	0.283***	0.55	0.277****	0.16	0.120****	9.46*	1.92
Pgi-B$_a$	0.65	0.336****	0.14	0.085****	0.09	0.048****	0.56	0.313****	10.13*	10.78*
6Pgd-2$_a$	0.98	0.022	0.95	0.037	0.88	0.076**	0.44	0.321****	10.62*	12.65**

[a] The following loci were monomorphic in all samples collected in different years: *Aat-1A, Aat-1B, Aat-2B, Aat-3B, Adh-2B, Mdh-1B, Pept-1A.* The loci *Pgm-A, Pgm-B, 6Pgd-1A, 6Pgd-1B* were monomorphic in all except the 1988 collection

[b] Difference in the means among the four subpopulations was tested using the nonparametric Kruskal-Wallis test

[c] The χ^2 test for homogeneity of allele frequency: *, **, ***, ****, $P<0.05$, 0.01, 0.001, 0.0001, respectively

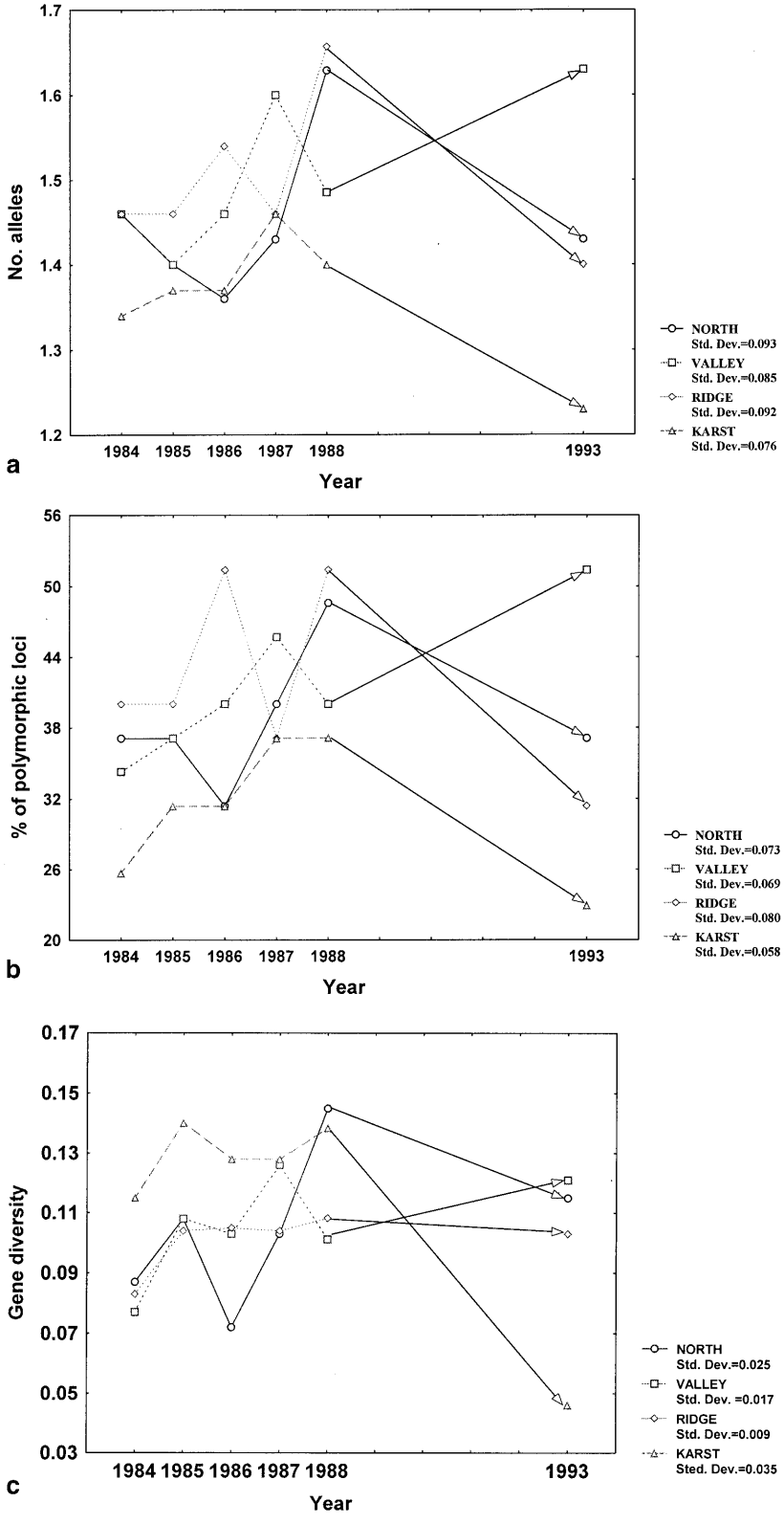

Fig. 2 Temporal variation in average number of alleles per locus (*A*, **Fig. 2a**), percentage of polymorphic loci (P-5%, **Fig. 2b**), and gene diversity (*He*, **Fig. 2c**) averaged over 35 allozyme loci of *T. dicoccoides* during the 1984–1988, and 1993 sampling years in the four habitats at the Ammiad microsite

loci, allele frequencies varied significantly (χ^2-test, P<0.05–0.00005) over time at 7, 9, 11, and 11 allozymic loci in the North, Valley, Ridge and Karst subpopulations, respectively (Table 2). Based on the binomial test (P = 0.05, assuming independence of loci), these numbers of the loci with significant temporal diversity in allele frequencies out of the 35 loci were significantly (P<0.0015–0.00005: Aiken 1955) larger than those expected by 5% chance in each habitat. For alleles *Est-5B*$_{null}$, *Nadh-2B*$_a$, *Pgi-A*$_b$, *Pgi-B*$_a$, temporal variations

Table 3 Partition of genetic differentiation (G_{ST}) among the North, Valley, Ridge, and Karst microhabitats in samples of *T. dicoccoides* collected at Ammiad in the 1984–1993 sampling years

Locus	G_{ST} among the four subpopulations						Overall H_T	Relative gene diversity (G_{ST})[a]	
	1984	1985	1986	1987	1988	1993		Between years	Between habitats
Aat-3A	0.026	0.010	0.010	0.016	0.045	0.041	0.026	0.008	0.011**
Adh-1A	–	0.320****	–	0.269***	0.089**	–	0.298	0.096****[b]	0.141****
Adh-1B	0.033	–	–	0.291***	0.028	0.070	0.066	0.026****	0.051****
Adh-2A	–	0.020	0.010	–	–	–	0.004	0.006	0.002
Est-5A	0.170****	0.242****	0.171****	0.198****	0.256****	0.150*	0.363	0.057****	0.167****
Est-5B	0.055**	0.373****	0.260****	0.089***	0.097***	0.466****	0.456	0.054****	0.095****
Gluc-A	0.190****	0.122**	0.279****	0.431****	0.304****	0.810****	0.373	0.003	0.299****
Gluc-B	0.260****	0.121**	0.268****	0.418****	0.314****	0.810****	0.388	0.003	0.315****
Ipor-B	–	0.024	0.087*	0.019	–	–	0.054	0.151****	0.010
Mdh-1A	0.132***	0.392****	0.404****	0.446****	0.310	0.712****	0.330	0.011**	0.368****
Mdh-2	0.026	0.020	–	0.028	0.015	0.024	0.019	0.005	0.009*
Nadh-1A	–	0.119*	0.282***	0.316****	0.161****	0.126*	0.466	0.174****	0.107****
Nadh-1B	0.246***	0.081*	0.225****	0.190****	0.068	0.221***	0.498	0.071****	0.080****
Nadh-2A	0.244****	0.465****	0.111	0.418****	0.142****	0.433****	0.202	0.033****	0.231****
Nadh-2B	0.101**	0.465****	0.111	0.418***	0.142****	0.322****	0.296	0.195****	0.114****
Pept-1B	0.025	0.027	0.015	0.038	–	0.012	0.041	0.014**	0.011*
Pept-3	0.168**	0.395**	0.015	0.013	0.222****	0.521****	0.209	0.086****	0.078****
Pgi-A	0.046	0.236****	0.123***	0.199***	–	0.267****	0.664	0.312****	0.057****
Pgi-B	0.444****	0.316****	0.340****	0.423****	0.032	0.438****	0.658	0.462***	0.158****
Pgm-A	–	–	–	–	0.368****	–	0.060	0.086****	0.043****
Pgm-B	–	–	–	–	0.168****	–	0.127	0.257***	0.019****
6Pgd-1A	–	–	–	–	0.161****	–	0.199	0.703***	0.021****
6pgd-2	0.164****	0.397****	0.442****	0.431****	–	0.564****	0.300	0.039***	0.296****
Mean	0.182	0.264	0.249	0.306	0.196	0.428	0.175	0.173	0.152

[a] Calculated over all 35 allozymic loci

[b] Significance level of the relative gene differentiation was obtained from χ^2 test for allele frequency heterogeneity at each hierarchical level *, **, ***, **** P<0.05, 0.01, 0.001, 0.0001, respectively

in their frequencies were significant in all four subpopulations. For 4 other alleles, *Adh-1B*$_a$, *Gluc-A*$_b$, *Gluc-B*$_b$, *Mdh-1A*$_b$, significant temporal changes in frequencies were only found in the Karst subpopulation (Table 2).

Among the four subpopulations from the North, Valley, Ridge, and Karst habitats, the mean allele frequencies over the 6 years significantly varied at 12 loci (Kruskal-Wallis test, P<0.05–0.0001). Likewise, significant spatial variation was found for between-year variance in allele frequencies at 8 loci (Bartlett's test using transformation data by ''ArcSin (√allele-frequency)'' (P<0.05–0.01, the last column of Table 2). According to the binomial test (P = 0.05), the 8 and 12 loci with significant spatial differentiation in variances and means of allele frequencies were significantly (P<0.00027, 0.00005, respectively: Aiken 1955) larger than those expected by 5% chance. The largest differences were found not only between the farthest Karst and the other three habitats (e.g., *Gluc-A*$_b$, *Gluc-B*$_b$, *Mdh-1A*$_b$), but also among the neighboring North, Valley, and Ridge subpopulations (e.g., at loci *Pgi-B*$_a$, *Nadh-2A*$_a$). The results suggested that allele distributions at allozymic loci were nonrandom on the microgeographic scale as demonstrated for only 4 years (1984–1987) using 45 allozymic loci in Nevo et al. (1991).

Genetic diversity in allozymic loci in space and time

The level of genetic diversity, including number of alleles per locus (A) averaged over the 35 loci, proportion of polymorphic loci (P), and gene diversity (He), fluctuated over the 6 years in the four subpopulations (Fig. 2a-c). Different amounts of temporal variation were found for A, P, and He. The variation in A (Fig. 2a) and P (Fig. 2b) over the 6 years in the Karst subpopulation was relatively less (SD = 0.076, 0.058 for A and P, respectively) than in the other three subpopulations (SD = 0.085–0.093, 0.069–0.080 for A and P, respectively). However, variation in gene diversity over sampling years was larger in the Karst subpopulation (SD = 0.035) than in the other three (SD = 0.009–0.025). In particular, the *He* value for Karst became much lower than those of the other subpopulations in the 1993 sample collections (Fig. 2a-c); such a pattern was inconsistent with those found in 1984–1988 by Nevo et al. (1991) and the present study (Fig. 2c). From these figures, it was obvious that some yearly changes were parallel in some habitats, while some differed considerably.

Partition of genetic diversity

Significant gene differentiation (G_{ST}) among sampling years was observed at 18 allozymic loci (χ^2-test,

P<0.05–0.0001, Table 3). In particular, at locus *Ipor*-B, significant gene differentiation (G_{ST} = 0.151, P<0.0001) was found only among sampling years, not among microgeographic habitats, and in 1984, 1988 and 1993, locus *Ipor*-B was monomorphic with the same allele *Ipor*-B_a. Over all 35 loci, variation due to differences in allele frequency among sampling years within habitats accounted for 17.3% of the total gene diversity (Table 3).

Over the 6 years, the Karst subpopulation showed relatively lower A (1.8) and P (0.54) than the North (A = 2.0, P = 0.66), Valley (A = 2.0, P = 0.66) and Ridge subpopulations (A = 2.0, P = 0.77). But the He over all loci and 6 years was relatively higher in the Karst subpopulation (He = 0.180) than those in the other three subpopulations (He = 0.136–0.138). Significant gene differentiations (G_{ST}) at 11–14 loci were found among the four subpopulations in each of the sampling years. The values of G_{ST} among the four subpopulations fluctuated through the sampling years. At 4 loci, *Est-5A*, *Est-5B*, *Gluc-A*, and *Gluc-B*, gene differentiations among the four subpopulations were highly significant (χ^2-test, P<0.05–0.0001) in all 6 sampling years. Over all the polymorphic loci, a significant correlation was found between the amount of early rains (Oct.-Nov., Table 1) and G_{ST} value (Pearson's r = 0.82, n = 6, P<0.05). This correlation may suggest that early seed germination due to early rains and microgeographic differences in soil moisture after early rains (see Noy-Meir et al. 1991) influence genetic divergence among habitats. Averaged over the 6 sampling years, variation among the four habitats accounted for 15.2% of the total gene diversity (Table 3).

Correlation of allele frequencies and *H*e among loci

Using the data of the four subpopulations taken over 6 years (1984–1993), we calculated correlations of allele frequencies and gene diversities at different loci, including only the most common alleles at polymorphic loci. A large number of correlation coefficients between allele frequencies (26.8% of the 153 cases examined) at different loci were significant (P<0.05–0.001, Table 4). Very close correlations were observed between some loci that belong to the same allozymic groups (loci that have the same enzymatic name), e.g., alleles *Gluc-*A_b and *Gluc-*B_b (r = 0.99, P<0.001). Likewise, very strong associations were found between allozymic groups, such as r = 0.91 (P<0.001) between *Mdh-1*A_b and the *Gluc* group. In general, however, allele-frequency changes within the same allozymic groups showed a higher likelihood (χ^2 = 4.65, df = 1, P<0.05) of being significantly (P<0.05) associated with each other (55% of 11 cases examined) than those among different allozymic groups (33% of 142 cases examined).

For gene diversity (He), 15.7% of the 153 cases examined were significantly correlated between a variety of loci (Table 4). Obviously, locus-pairs *within* the same allozymic group (57% of 11 cases examined) showed more significant correlations (P<0.01) than those *between* allozymic groups (4% of the 142 cases examined).

Table 4 Correlation coefficients among the most common allele frequencies (below the diagonal) and gene diversity (*He*, above the diagonal) for 18 pairs of polymorphic loci over the 1984–1988 and 1993 years, across the four subpopulations of *T. dicoccoides* at the Ammiad microsite

Allele	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1. *Aat-3*A_a		–0.11	–0.08	0.04	0.11	–0.15	–0.16	–0.16	–0.35	0.15	0.11	0.24	0.35	0.33	–0.06	–0.33	–0.10	0.13
2. *Adh-1*A_a	–0.26		0.44*	–0.29	–0.01	0.50*	0.46*	0.02	0.59**	0.38	–0.07	–0.18	–0.34	–0.30	–0.08	0.07	–0.06	0.26
3. *Adh-1*B_a	0.05	0.56*		–0.32	0.19	0.07	0.04	–0.14	0.11	–0.12	–0.29	0.15	0.06	–0.10	–0.20	0.30	0.10	0.12
4. *Est-5*A_a	–0.07	–0.33	–0.33		–0.33	–0.21	–0.17	0.11	–0.45*	0.18	0.50*	–0.25	–0.05	0.39	–0.00	–0.16	–0.46*	–0.24
5. *Est-5*B_{null}	0.14	0.01	0.19	–0.55**		–0.07	0.08	–0.49*	–0.07	–0.14	–0.32	0.37	0.37	–0.11	–0.23	0.46*	0.30	0.04
6. *Gluc-*A_b	–0.32	0.55**	0.29	–0.56**	0.27		0.99***	0.25	0.66**	0.18	–0.23	–0.50*	–0.59**	–0.12	0.03	0.13	0.17	0.32
7. *Gluc-*B_b	–0.34	0.53**	0.27	–0.57**	0.28	0.99***		0.26	0.66**	0.14	–0.23	–0.51*	–0.59**	–0.04	–0.01	0.09	0.18	0.33
8. *Ipor-*B_a	–0.18	0.04	–0.15	0.10	–0.34	0.03	0.02		0.13	0.30	0.33	–0.32	–0.38	–0.01	–0.20	0.10	0.13	0.26
9. *Mdh-1*A_b	–0.36	0.33	0.36	–0.57**	0.43*	0.92***	0.91***	0.00		0.12	–0.33	–0.45*	–0.51*	–0.18	0.26	–0.09	0.20	0.60**
10. *Nadh-1*A_a	0.29	0.03	–0.17	0.52*	–0.27	–0.21	–0.24	0.25	–0.28		0.57**	–0.25	–0.18	–0.20	0.08	–0.05	–0.19	0.06
11. *Nadh-1*B_a	0.41	–0.37	–0.18	0.22	–0.07	–0.66**	–0.69**	0.30	–0.60**	0.43*		0.15	0.24	0.11	–0.18	–0.09	–0.27	–0.27
12. *Nadh-2*A_a	0.46*	–0.25	0.15	–0.36	0.32	–0.40	–0.41	–0.25	–0.32	–0.25	0.57**		0.84***	0.22	–0.31	0.33	0.24	–0.48*
13. *Nadh-2*B_a	0.23	–0.38	0.01	–0.37	0.60**	0.06	0.04	–0.30	0.11	–0.31	0.24	0.59**		0.21	0.27	–0.31	0.14	–0.39
14. *Pept-1*B_b	–0.03	–0.33	–0.16	0.16	–0.18	–0.36	–0.34	–0.03	–0.36	–0.19	0.15	0.34	0.11		–0.18	0.09	0.14	–0.17
15. *Pept-3*$_a$	–0.32	–0.09	–0.20	–0.03	0.13	0.50*	–0.49*	–0.19	0.40	–0.06	–0.30	–0.27	0.24	–0.24		–0.39	–0.39	–0.06
16. *Pgi-*A_b	–0.13	0.52**	0.18	–0.29	0.22	0.54**	–0.53**	–0.20	0.56**	0.03	–0.26	0.03	0.08	–0.44*	0.39		0.54**	0.26
17. *Pgi-*B_a	–0.14	–0.12	–0.41	0.71***	–0.64**	–0.31	–0.32	0.12	–0.45*	0.61**	0.05	–0.52*	–0.55**	–0.02	0.06	–0.17		0.46*
18. *6Pgd-*2_a	–0.35	0.56**	0.40	–0.46*	0.47*	0.76***	0.76***	0.10	0.88***	–0.33	–0.53**	–0.31	0.14	–0.34	0.17	0.34	–0.54**	

*, **, ***, P<0.05, 0.01, 0.001, respectively

These two proportions were significantly different (χ^2 = 19.89, *df* = 1, *P*<0.00005). For instance, very strong correlations were observed between *He* for *Gluc-A*$_b$ and *Gluc-B*$_b$ (*r* = 0.99, *df* = 22, *P*<0.001) and between *Nadh-2A*$_a$ and *Nadh-2B*$_a$ (*r* = 0.84, *df* = 22, *P*<0.001).

No obvious pattern was found when we attempted to extend the foregoing group-related test to the level of protein groups, like transferase, oxidoreductase, hydrolase, and isomerase. This suggests that the factors causing the similarity of within-allozymic groups are highly specific.

ANOVA test for environmental and protein effects

The relative effects of environmental and protein effects were further analyzed using the method of analysis of variance (ANOVA) for frequency of the commonest allele and *He* at polymorphic loci. The allele-frequency data were transformed using 'ArcSin(√x)' before the ANOVA analysis was carried out. Loci coding for proteins with similar functions are defined as an allozymic group, and proteins with similar metabolic function belong to a metabolic group (showing similar metabolic mechanism, such as transferase, oxidoreductase, hydrolase, and isomerase groups). Table 5 summarizes this analysis. The analysis showed that habitat, allozymic group, metabolic group, and interaction of habitat × allozymic group and year × allozymic group significantly affected allele frequencies and *He* over all 21 polymorphic loci (*P*<0.05–0.00005, Table 5). Yearly effect seems to be low over all 21 polymorphic loci. This finding may imply that possible allozymic-specific effects of temporal environmental fluctuation were not taken into account.

Accordingly, further analysis was performed for each allozymic group. The results showed that sampling years significantly affected allele frequency and/or *He* at the *Gluc*, *Pgi*, *Pgm*, and *Nadh* loci (*P*<0.05–0.00005) and that habitat effect and the interaction of year × habitat also significantly affected allele frequency and/or *He* at the *Gluc* and *Pgm* loci (*P*<0.00005, Table 5). At loci *Gluc-A* and *Gluc-B*, the most extremely different year was 1993 for both allele frequency and gene diversity in all four habitats. At loci of *Pgi* and *Pgm*, the most contrast in allele frequencies and *He* values was found in 1988 in all four habitats. At loci *Nadh-1A* and *Nadh-1B*, the extremes in allele frequency and *He* value were found in 1993 in the North subpopulation and in 1984 in the Ridge and Karst subpopulations. In the Valley subpopulation, the extremes in allele frequency and *He* value were found in 1988 at the locus *Nadh-1A* and in 1986 at locus *Nadh-1B*.

Genetic diversity-environment associations

To determine the existence of any significant associations between genetic variables and environmental water supply, we conducted stepwise multiple regression using

Table 5 Results of ANOVA test for temporal and spatial effects on frequencies of the most common alleles (AF) and gene diversities (*He*) at polymorphic loci in the North, Valley, Ridge, and Karst subpopulations of *T. dicoccoides* at Ammiad for the 6 years (1984–1988 and 1993)

Variables[a]	*F* ratio											
	Over 21 polymorphic loci			df1, df2	*Gluc*		*Pgi*		*Pgm*		df1, df2	*Nadh*	
	df1, df2	AF	*He*		AF	*He*	AF	*He*	AF	*He*		AF	*He*
1. Year	5, 238	1.15	0.61	5, 24	13.41****	158.35****	4.79**	2.50@	18.77****	19.01****	5, 72	2.95*	1.92
2. Habitat	3, 238	9.85****	2.18	3, 24	2244.86****	816.01****	1.13	2.47@	1.94	0.67	3, 72	3.25*	1.41
3. Allozymic group[b]	10, 238	34.76****	18.32****										
4. Metabolic group[c]	3, 406	60.88****	21.60****										
1 × 2	15, 238	0.37	0.93	15, 24	110.52****	48.56****	0.07	1.19	6.13****	0.67	15, 72	0.33	0.63
1 × 3	50, 406	1.52*	1.91**										
2 × 3	30, 238	3.92****	3.16****										
1 × 2 × 3	150,238	0.38	0.39										
1 × 4	15, 406	1.69	1.51										
2 × 4	9, 406	1.16	1.44										
1 × 2 × 4	45,406	0.28	0.39										

@, *, **, **** *P*<0.10, 0.05, 0.01, 0.00005, respectively

[a] Variables include genome (A vs. B), but genome effect and its interaction with other four factors are not presented due to non-significant effect for AF and *He*

[b] Allozymic group, Proteins with the same enzymatic name, such as esterase group, peptidase group

[c] Metabolic group, Enzymes with similar metabolic mechanisms, such as oxidoreductase, hydrolase, isomerase groups

Table 6 Significant adjusted coefficients of stepwise multiple regression (R^2) of allele frequencies, number of alleles, and gene diversity (as the dependent variable) and water conditions (as independent variables) in the four habitats of *T. dicoccoides* in three consecutive growing years (1986–1988) at the Ammiad microsite

$Locus_{allele}$	Variable	R^2	Variables	R^2
Allele frequency:				
Adh-1A$_a$	Soil moisture[a]	0.007	Soil moisture, late rain	0.317*
Adh-1B$_a$	Early rain[b]	0.124	Early rain, soil moisture	0.421**
Mdh-1A$_b$	Soil moisture	0.067	Soil moisture, late rain	0.434*
Nadh-1A$_a$	Soil moisture	0.043	Soil moisture, early rain	0.355*
Ipor-B$_a$	Late rain[c]	0.460**	Late rain, early rain[c]	0.515*
Pept-1B$_b$	Early rain	0.311*		
Pept-3$_a$	Early rain	0.040	Early and late rain	0.392*
Pgi-A$_b$	Early rain	0.120	Early and late rain	0.578**
Pgi-B$_a$	Early rain	0.025	Early rain, soil moisture	0.411*
6Pgd-2$_a$	Soil moisture	0.027	Soil moisture, early rain	0.407*
Number of alleles				
Adh-1A	Early rain	0.083	Early rain, soil moisture	0.407*
Nadh-1B	Late rain	0.381**		
Pept-3	Soil moisture	0.233*	Soil moisture, early rain	0.507*
6Pgd-2	Early rain	0.517**	Late rain, early rain	0.658**
Pgi-A	Early rain	0.116	Late rain, early rain	0.633**
He				
Adh-1B	Early rain	0.165	Early rain, soil moisture	0.415*
Est-5A	Soil moisture	0.231@	Soil moisture, total rain	0.381*
Ipor-B	Late rain	0.477**	Late rain, annual rain	0.543*
Pept-1B	Early rain	0.308*		
Pept-3	Early rain	0.057	Early and late rains	0.524*
Pgi-B	Early rain	0.018	Early and late rains	0.586**

*, **, $P<0.05$, 0.01 respectively, @ = $P<0.10$

[a] Surface (0—5 cm) soil-moisture content (%) after early rains (data from Noy-Meir et al. 1991)

[b] Early rain, sum of rainfall during October and November

[c] Late rain, sum of rainfall during March and April

amounts of early and late rains and soil-moisture content for 1986–1988 (data for other years are not available) as independent variables and allele frequencies, *A* and *He* as dependent variables. The results showed that 10 allele frequencies were significantly ($P<0.05$–0.01) correlated with rain distribution in the growing season and soil-water content (Table 6). Early rain and soil moisture during 1986–1988 in the four habitats appear to be the most important ecological factors in determining 4 allele distributions (*Adh-1B*$_a$, *Nadh-1A*$_a$, *Pgi-B*$_a$, *6Pgd-2*$_a$) and could predict 35.5%–42.1% of the variation in allele frequencies. Early and late rains in the growing season seem to be important factors for 3 other alleles (*Ipor-B*$_a$, *Pgi-A*$_b$, *Pept-3*$_a$) (Table 6). Soil moisture and late rain could account for 31.7%–43.4% of the variation in the frequencies of alleles *Adh-1A*$_a$, *Mdh-1A*$_b$. Early rain alone could account for 31.1% of the variation in the frequency of allele *Pept-1B*$_b$.

The numbers of alleles per locus (*A*) at 5 loci were significantly correlated with yearly rain distribution and soil moisture in the four habitats (Table 6). Early rain and soil moisture significantly affected *A* at loci *Adh-1A* ($R^2 = 0.407$, $P<0.05$) and *Pept-3* ($R^2 = 0.507$, $P<0.05$). Early and late rains could significantly predict 65.8% and 63.3% of the variation in *A* at loci *Pgi-A* and *6Pgd-2*, respectively. Late rain alone also significantly affected the number of alleles at locus *Nadh-1B* ($R^2 = 0.381$, $P<0.01$).

Annual rainfall, and its early and late distribution in the growing season, and soil moisture also affected gene diversities at 6 loci (*Adh-1B*, *Est-5A*, *Ipor-B*, *Pept-1B*, *Pept-3*, *Pgi-B*, Table 6). One or two of these ecological factors could predict 30.8%–58.6% variation of gene diversity (*He*) at the 6 loci. For example, during 1986–1987, when the early and late rains were extremely high relative to levels over the 6 years, gene diversity was the lowest at locus *Ipor-B* and the highest at locus *Pept-1B*.

Discussion

Spatial and temporal diversity of *T. dicoccoides* at Ammiad

This spatial and temporal genetic survey provides instrumental information on the relationship between genetic structure and population dynamics in wild emmer, *T. dicoccoides*. Allozymic diversity in proteins encoded by 43 allozymic loci was previously analyzed in 812 individuals of wild emmer wheat at the Ammiad microsite using data obtained over 4 consecutive growing seasons (1984–1987) (Nevo et al.1991). Significant genetic differentiation among habitats and subhabitats was found primarily in space and secondarily over time. The present study reanalyzed the spatial and temporal variations found for wild emmer wheat in the four habitats (North, Valley, Ridge and Karst) using new methods and the addition of 2 extra years of data on the 35 allozymic loci analyzed for all samples from the 1984–1993 collections. Our present results also show a similar pattern of genetic diversity in the four habitats over the 5 years from 1984 to 1988 – as Nevo et al. (1991) showed in the first 4 years – and significant genetic divergence over space and time. Our major new findings may be summarized as follow: (1) In the 1993 collection, except for the Valley subpopulation, the level of genetic diversity and allele frequencies at many loci (see Appendix 1) in the North,

Ridge, and Karst subpopulations obviously decreased. In particular, in the Karst habitat, the level of genetic diversity (*A*, *P* and *He*) and allele frequencies largely decreased to levels lower than those of the other three habitats. This pattern is inconsistent with the result obtained during 1984–1987 by Nevo et al. (1991). However, it is consistent with the simple-sequence repeat (SSR) pattern found in the same 1993 sample set (Li et al. 2000). The SSR diversity was significantly correlated with aridity indices (e.g., soil-water content) in microhabitats; the drier the microhabitat, the higher the SSR diversity (Li et al. 2000) (2) Allele frequencies and gene differentiation (G_{ST}) among the four habitats varied significantly among sampling years at a variety of allozymic loci. (3) The G_{ST} values among the four habitats tend to increase with an increase in the amount of early rains during Oct.-Nov. in each year. (4) Allele frequencies and gene diversities at some loci, particularly those within each allozymic group, changed coordinately with respect to the commonest alleles; others changed in the opposite direction and some changed independently. (5) Significant effects of sampling year, habitat, allozymic group, metabolic group, and/or their interactions were observed for allele frequency and *He* at a variety of polymorphic loci. (6) Soil moisture and rain distributions during the growing season of wild emmer seem to be the significant environmental factors affecting spatial and temporal diversity and divergence.

Neutral theory: can it explain the results?

The neutral theory (Kimura 1983) and nearly neutral model (Ohta 1992) emphasize the predominant effect of random genetic drift on electrophoretic protein polymorphisms and postulate that no selection or only weak selection act upon proteins (Kimura 1983; 1991a,b; Ohta 1992, 1997). Random genetic drift (Jorde and Ryman 1996; Levy and Neal 1999) and sampling error (Viard et al.1997) have been considered to be a main factor in temporal allozymic and DNA variations. Stochastic effects predict random and independent weak fluctuation in allele frequencies over time. However, we found high significant spatiotemporal changes in allele frequency, *He*, and G_{ST}, and these variations were associated with yearly soil moisture and rain distribution. We also found dependent or coordinated spatiotemporal changes in allele frequency and *He* between different loci. These observed patterns appear unlikely to be due to random genetic drift and sampling error.

Migration: can it explain the results?

Migration is another factor used to explain temporal variation (Viard et al. 1997; Vandewoestijne et al.1999). Without natural selection, even low migration could easily eliminate genetic divergence in a few generations. Although the low outcrossing rate of *T. dicoccoides* (1%: Golenberg 1986) limits gene flow by pollen exchange, seed dispersal can be caused by ants, animal and human activities in this sampling area. If migration were important among the four microhabitats in our study site, gene differentiation (G_{ST}) among the four subpopulations would decrease with generation-increase along the sampling years. However, our results show a significant correlation between G_{ST} among the four subpopulations and rainfall during the seed germination stage of wild emmer wheat (Oct.-Nov.). This finding may reflect the fact that the amount of October-November rains affects soil moisture, and then seed germination. The four habitats each have a different ability to hold water under the same amount of rainfall, and this causes different rates of wild emmer seed germination and influences adult population density and genetic differentiation. Without selection, migration cannot explain significant locus-association, and associations between aridity indices and allele frequencies, number of alleles, and *He* at a variety of allozymic loci (Table 6). Clearly, despite migration, other stronger forces, such as natural selection, appear to override the effect of migration and direct the observed variation.

Natural selection

Natural selection has proven to be an important factor affecting the temporal variation of a population gene pool (Nevo et al.1984, 1991; Gyllenstein 1985; Mueller et al. 1985; Barker et al. 1986; Allard 1988; Saghai-Maroof et al.1994). According to the Hardy-Weinberg rule, one can expect that the same allele frequencies should successively be unchanged from generation to generation, unless some forces are causing changes. In large populations, stochastic processes are very weak. Environmental selection is able to cause microspatial divergence in spite of a considerable gene flow. Assuming that the environmental selection affects 2 loci in a similar manner, one would expect the temporal variation in allele frequencies to be correlated (Mueller et al.1985). In the present study, strong correlations between allele frequencies at a variety of loci, particularly at those loci within the same allozymic group, were observed over space and time (Table 4), suggesting that spatial and temporal diversities at these loci are not independent but coordinated.

Different loci within an allozymic group are not linked on the same chromosome but are located separately in genome A and B (e.g., *Nadh-1A* and *Nadh-1B* are located on 4A and 4B chromosomes, respectively; *Est-5A* and *Est-5B* are on the 3A and 3B chromosomes; the *Gluc-A* and *Gluc-B* loci are separately located in genome A and B). Such locus distributions indicate that linkage can not explain the locus-association within allozymic groups here. Such an association in spatial and temporal changes in allele frequencies and *He* within allozymic groups cannot be explained by stochastic factors and seem to reflect highly specific effects of natural selection. Proteins within the same allozymic group, such as

the loci *Gluc-A* and *Gluc-B* in the glucosidase group, *Pgm-A* and *Pgm-B* in the phosphoglucomutase group (see Materials and Methods), have similar functional roles in the plant's metabolic process. Accordingly, the proteins with similar functions at unlinked loci, even those located in different genomes, can be selected coordinately. Multiple regression showed that some associated alleles at different loci correspond to similar environmental variables, soil moisture, and rainfall distribution; for example, between *Pgi-B*$_a$ and *6Pgd-2*$_a$, between *Adh-1A*$_a$ and *6Pgd-2*$_a$, and between *Mdh-1A*$_b$ and *6Pgd-2*$_a$. Both the *Pgi* and *6Pgd* enzymes are involved in glycolysis, a key metabolic feature of the respiratory process of the plant cell. We interpret such a significant correlation among allele frequencies and gene diversity between different loci as an indicator of environmental selection on allozymic systems in *T. dicoccoides* at Ammiad, similar to that in the *Drosophila* study (Mueller et al.1985).

Kimura (1983) assumed that because all allelic proteins perform the same biochemical function they are a priori neutral. However, the functions of allelic proteins may not be exactly the same. For example, it has been proven that allelic polymorphism at the *Pgi* locus influences viability, flight time, mating success, and fecundity in *Colias* butterflies (see Mitton 1997, for review). A significant association of allelic polymorphism at locus *Adh-1* with latitude was found in natural populations of wild barley, *Hordeum spontaneum* (Nevo et al. 1979). In 37 populations of *T. dicoccoides* from Israel and Turkey, *Adh-1A* polymorphism significantly correlated with environmental water and temperature conditions (Nevo and Beiles 1989). Our present study also demonstrates that soil moisture and rain distributions in the early and late growing season significantly affect allele frequencies, number of alleles and gene diversity at loci *Adh-1A* and *Adh-1B* (Table 5). Such a functional difference is against Kimura's assumption and neutral expectation.

The *Mdh* enzymes in plants play a crucial role in several mictochondrial pathways and in photosynthetic pathways (Gietl 1992). The *6Pgd* enzyme is involved in the association between glycolysis in non-photosynthetic cells and the oxidative pentose phosphate pathway. In our study, 2 alleles *Mdh-1B*$_b$ and *6pgd-2*$_a$, were strongly coordinated in temporal frequency changes, and these temporal changes in both alleles were significantly associated with soil moisture and rain distribution during the growing season. Environmental water conditions significantly affected allele frequencies and gene diversity at the *Pept-1B* and *Pept-3* loci. Such differences in allozymic functions and their association with ecological factors may suggest an effect of natural selection rather than hitchhiking, random drift, or migration. Moreover, when different allozymic loci exhibit contrasting patterns of diversity, as shown in our study, such contrasts require selection, as has been argued previously (Christiansen and Frydenberg 1974; Nevo et al.1979).

In conclusion, our results emphasize the role of natural climatic selection in the observed temporal and microgeographic variations at the Ammiad microsite. Random genetic drift, sampling error, and migration may influence the observed estimates to some extent, but environmental effect, namely natural selection, appears to be the major factor responsible for the observed spatiotemporal pattern and dynamics of allozyme variation, overriding all other factors.

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Appendix 1 Allele frequencies at polymorphic allozymic loci of *T. dicoccoides* in the North, Valley, Ridge, and Karst subpopulations of the added 1988 and 1993 collection from the Ammiad microsite with the χ^2 test among subpopulations in each years

Locus	Allele[a]	1988				χ^2 test (df)	1993				χ^2 test (df)
		North	Valley	Ridge	Karst		North	Valley	Ridge	Karst	
Aat-3A	a	1.00	0.94	1.00	1.00	8.81(3)*	0.91	0.93	1.00	1.00	No test[c]
	null	–[b]	0.06	–	–		0.09	0.07	–	–	
Adh-1A	a	0.73	0.80	0.88	0.52	14.4(3)**	1.00	1.00	1.00	1.00	–
	b	0.27	0.20	0.12	0.48						
Adh-1B	a	0.04	–	–	–	6.57(3)	0.09	–	–	–	No test
	b	0.96	1.00	1.00	1.00		0.91	1.00	1.00	1.00	
Est-5A	a	0.83	0.54	0.38	1.00	42.0(6)****	0.96	0.80	0.64	1.00	38.5(3)****
	b	0.06	0.04	0.02	–		–	0.02	–	–	
	c	0.11	0.42	0.59	–		0.04	0.18	0.36	–	
Est-5B	a	0.10	–	0.04	–	21.1(3)**	0.04	–	–	–	67.7(3)****
	b	0.03	–	0.12	–		0.52	0.06	0.15	–	
	c	0.20	0.11	–	0.05		0.26	0.07	0.08	0.94	
	null	0.67	0.99	0.84	0.95		0.17	0.87	0.76	0.06	
Gdh-A	a	1.00	0.97	0.99	1.00	2.04(3)	0.04	0.03	–	–	No test
	b	–	–	–	–		0.96	0.97	1.00	1.00	
	null	–	0.03	0.01	–						
Gdh-B	a	0.03	1.00	1.00	1.00	6.3(3)	–	0.02	–	–	No test
	b	0.97	–	–	–		1.00	0.98	1.00	1.00	

Appendix 1 (continued)

Locus	Allele[a]	1988				χ^2 test (df)	1993				χ^2 test (df)
		North	Valley	Ridge	Karst		North	Valley	Ridge	Karst	
Gluc-A	a	0.83	0.76	0.94	0.29	51.0(6)****	1.00	0.80	1.00	–	96.2(3)****[d]
	b	0.03	–	–	–		–	–	–	–	
	null	0.13	0.24	0.06	0.71		–	0.20	–	1.00	
Gluc-B	a	0.13	0.25	0.06	0.71	49.6(3)****	–	0.20	–	1.00	96.2(3)****
	b	0.87	0.75	0.94	0.29		1.00	0.80	1.00	–	
Mdh-1A	a	0.03	0.06	0.11	0.57	42.5(3)****	–	0.07	0.07	0.89	94.5(3)****
	b	0.97	0.94	0.89	0.43		1.00	0.93	0.93	0.11	
Mdh-2	a	1.00	0.97	0.99	1.00	No test	0.96	0.95	1.00	1.00	No test
	b	–	0.03	0.01	–		0.04	0.05	–	–	
Nadh-1A	a	0.52	0.89	0.69	0.37	27.3(3)****	0.56	0.44	0.49	0.11	10.2(3)*
	b	0.48	0.11	0.31	0.62		0.44	0.56	0.51	0.89	
Nadh-1B	a	0.77	0.63	0.48	0.81	11.0(6)	0.22	0.54	0.51	0.89	17.1(3)***
	b	0.23	0.35	0.52	0.19		0.78	0.31	0.37	0.11	
	null	–	0.02	–	–		–	0.15	0.11	–	
Nadh-2A	a	0.77	1.00	1.00	0.95	No test	0.38	0.93	0.96	1.00	No test
	b	0.23	–	–	0.05		–	–	–	–	
	null	–	–	–	–		0.62	0.07	0.04	–	
Nadh-2B	a	0.77	1.00	1.00	0.95	No test	0.38	0.77	0.54	–	37.5(3)****
	b	0.23		–	0.05		0.62	0.23	0.46	1.00	
Pept-3	a	0.87	0.97	0.63	0.43	39.2(3)****	1.00	0.95	0.90	0.26	65.6(3)****
	b	0.13	0.03	0.37	0.57		–	0.05	0.10	0.74	
Pgi-A	a	1.00	1.00	1.00	1.00	–	0.48	0.39	0.26	0.95	31.7(6)****
	b	–	–	–	–		0.52	0.61	0.74	0.05	
Pgi-B	a	0.07	–	0.03	–	No test	0.65	0.25	0.06	0.89	68.0(3)****
	b	0.93	1.00	0.97	1.00		0.35	0.75	0.94	0.11	
Pgm-A	a	0.90	1.00	0.93	0.40	63.4(3)****	1.00	1.00	1.00	1.00	–
	b	0.10	–	0.07	0.60		–	–	–	–	
Pgm-B	a	0.20	0.01	–	0.57	8.4(3)	–	–	–	–	
	b	0.73	0.30	0.34	0.05		1.00	1.00	1.00	1.00	–
	c	0.07	0.69	0.66	0.38		–	–	–	–	
6pgd-1A	a	0.54	0.30	0.09	0.57	36.8(3)****	1.00	1.00	1.00	1.00	–
	b	0.46	0.70	0.91	0.43		–	–	–	–	
6pgd-2	a	1.00	1.00	1.00	1.00	–	0.96	0.97	0.83	0.16	69.2(3)****
	b	–	–	–	–		0.04	0.03	0.17	0.84	

*, **, ***, **** $P<0.05$, 0.01, 0.0001, 0.00005

[a] Alleles were labeled alphabetically in order of decreasing mobility of the enzymes

[b] Allele was not found in samples

[c] Data are not suitable for the χ^2-test

[d] The underlined result of the χ^2-test was slightly overestimated since one or two expected values were less than 5. However, the test was still significant even if the entries with small (<5) expected values were excluded