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Wheat phylogeny determined by RFLP analysis of nuclear DNA. 2. Wild tetraploid wheats

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Abstract Intra- and inter-specific variations in the nuclear DNA of *Triticum dicoccoides* Körn. (2n = 28, genome constitution AABB) and *T. araraticum* Jakubz. (2n = 28, AAGG), wild species, respectively, of the Emmer and Timopheevi group, were studied by restriction fragment length polymorphism (RFLP) analysis. Total DNAs of 32 *T. dicoccoides* and 24 *T. araraticum* accessions, collected from throughout the distribution areas of these species, were treated with two 6-bp cutters and hybridized with 30 nuclear DNA clones as probes to detect RFLPs. A total of 167 hybrid bands were observed per accession. All the enzyme-probe combinations showed RFLPs between accessions. The average genetic distance between the *T. dicoccoides* accessions was 0.0135 ± 0.0031 and that between the *T. araraticum* accessions 0.0036 ± 0.0015, indicative of about a four-fold intraspecific variation in *T. dicoccoides* as compared to *T. araraticum* in terms of genetic distance. No significant genetic differentiation was found for the geographical populations of these species, the genetic distance between the two species being 0.0482 ± 0.0022. The interspecific divergence corrected for intraspecific divergence was 0.0395, about three times that for *T. dicoccoides* and 11 times that for *T. araraticum*. The results show that in the wild state the Emmer and Timopheevi groups are clearly differentiated and that *T. dicoccoides* has much greater variation than *T. araraticum*, suggesting a relatively recent origin for the latter and therefore a diphyletic origin for these species.

Key words Wheat · RFLP · *Triticum dicoccoides* · *T. araraticum* · Genetic divergence

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Introduction

Four genomes, A, B, G and D, are known in polyploid species of the wheat genus *Triticum*, of which the A and D genomes respectively originated from Einkorn wheat and *Aegilops squarrosa* (Kihara 1924, 1944; McFadden and Sears 1946). The origin of the B and G genomes remains a matter of contention, but there is general agreement that they derive from species of the section Sitopsis of *Aegilops*.

Previous studies of the genetic homologies between the B or G genome and genomes in related diploid species have given meiotic chromosome pairing prime importance, but morphology, protein products (including enzymes), relative nuclear DNA contents, chromosome translocations, and cytoplasmic effects also have been considered. Restriction fragment length polymorphism (RFLP) analysis and genomic in situ hybridization are new methods developed for the study of nuclear DNA homology.

Respectively, the B and G genomes appear first in the wheat phylogeny in the wild tetraploid species *T. dicoccides* and *T. araraticum*. We have therefore attempted to clarify the intra- as well as the inter-specific divergence of the nuclear DNA of these two species. Our results show that the two species are distinct and greatly differentiated at the DNA level. This information should prove useful for elucidating the origins of the B and G genomes.

Materials and methods

Plant materials

The 32 accessions of *Triticum dicoccoides* Körn. and the 24 of *T. araraticum* Jakubz. used were collected at 42 sites in Palestine, southeast Turkey, northern Iraq, western Iran and the Transcaucasus (Fig. 1 and Table 1). One accession each of common wheat (*T. aestivum* cv 'Chinese Spring'), Emmer wheat (*T. durum* var. *reichenbachii*), cultivated Timopheevi wheat (*T. timopheevi* var. *typicum*), a cultivated Einkorn wheat (*T. monococcum* var. *flavescens*), and *Aegilops speltoides* were the references in the RFLP analysis.

The chromosomal locations of the DNA sequences which hybridized with the probe DNAs were determined by nulli-tetrasomic analysis using the 20 nulli-tetrasomics of *T. aestivum* cv 'Chinese Spring' produced by Sears (1966).

Plant DNA preparation and the probe DNAs

Total DNAs, prepared according to Liu et al. (1990), were used in the RFLP analysis. Thirty DNA clones (21 genomic, nine cDNA) were used as the probes.

Whole-plasmid DNA with the insert, or else the insert excised from the plasmid, was used as a probe. The insert DNA was labeled to a specific activity higher than 1×10^9 dpm/μg DNA by the use of a Random Primer kit (Boehringer Mannheim). Whole-plasmid DNA was labeled to a specific activity higher than 5×10^8 dpm/μg DNA by nick translation. After labeling, free deoxyribonucleoside triphosphates were removed by Sephadex G-50 column chromatography (Sambrook et al. 1989).

Southern hybridization

Ten micrograms of DNA per accession was treated with *Bam*HI or *Hin*dIII (both 6-bp cutters) according to the supplier's instructions (Takara Shuzo Co., Ltd.). The digests were electrophoresed in a 0.85% agarose gel for 18–22 h at 1.0 V/cm then blotted to a Hybond-N+ nylon membrane (Amersham) by alkaline blotting (Southern 1975). *Hin*dIII-treated lambda DNA (500 ng/lane) was used as the size marker. After hybridization by the technique of Liu et al. (1990), the membrane was washed at 65 °C, once with a mixture of 2 × SSC and 0.1% SDS for 15–20 min and then once with 0.1 × SSC and 0.1% SDS for 30 min.

Analysis of the RFLP data

After autoradiography, the hybrid fragment at each position was recorded as 0 (absence) or as 1 or 2 (presence as a single or double dose). Hybrid fragments were detected using a total of 60 enzyme-probe combinations. The total and commonly shared hybrid fragments were scored for all the accession pairs. The genetic distance (d) between each accession pair was estimated from the proportion of the common fragments to the total fragments by inserting this value in equations 5.53–5.55 of Nei (1987). From the estimated genetic distances, we constructed a dendrogram using the UPGMA (unweighed pair-group mean with arithmetical averages) method of Sneath and Sokal (1973) which showed the phylogenetic relationships among all the accessions.

Results

Distribution in wheat chromosomes of DNA sequences hybridized with the 30 probes

The results of the nulli-tetrasomic analysis of the chromosomal locations in common wheat (cv 'Chinese Spring') for the DNA sequences hybridized with the 30 probes are given in Table 2. The probed sequences are distributed evenly among the three genomes of common wheat; but, their distributions among the seven homoeologous groups of chromosomes are uneven, being abundant in groups 2 and 3 and scarce in group 1.

Intra- and inter-specific variations in the nuclear DNA of two wild tetraploid wheats

Two examples of Southern hybridization patterns from all the accessions, in which *Hin*dIII-digested total DNAs were probed with two wheat clones, Tag694 and Tac77, are given in Fig. 2. By comparing the Southern hybridization patterns of all the accessions that emerged from the 60 probe-enzyme combinations, the total and differ-

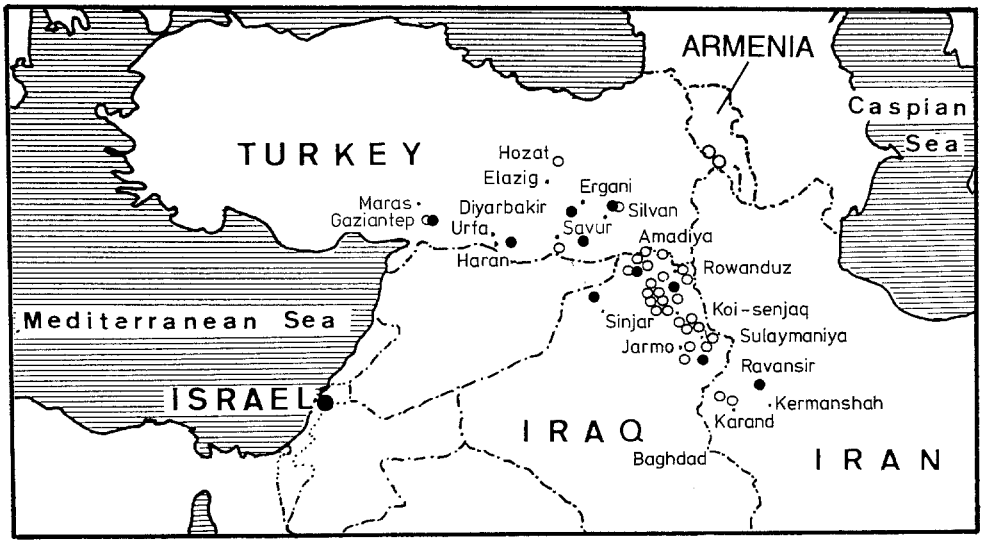


Fig. 1 Collection sites of the *T. dicoccoides* and *T. araraticum* accessions. ●: *T. dicoccoides*, ○: *T. araraticum*

Table 1 *T. dicoccoides* (*dcc*) and *T. araraticum* (*arr*) accessions used for the RFLP analysis of nuclear DNA

Acc. no.	Species	Country	Collection site[a]	Source[b]
1	*arr*	Russia	?	KU 196-1
4	*arr*	Armenia	8 km W of Garni	KU 1908A
10	*arr*	Armenia	8 km W of Garni	KU 1927
12	*arr*	Turkey	5 km SE of Maras	KU 1943
17	*arr*	Turkey	5 km SE of Maras	KU 1986
18	*arr*	Turkey	26.3 km NE from Mardin to Midyat	KU 8912
19	*arr*	Turkey	17.3 km E from Silvan to Bitlis	KU 8924
20	*arr*	Turkey	39.9 km N from Elazig to Hozat	KU 8940
21	*arr*	Iraq	13.2 km S from Sulaymaniyah to Quara Dagh	KU 8456
23	*arr*	Iraq	22.1 km S from Sulaymaniyah	KU 8528A
24	*arr*	Iraq	19.3 km S from Sulaymaniyah to Quara Dagh	KU 8543
25	*arr*	Iraq	14 km S from Sulaymaniyah to Quara Dagh	KU 8561
26	*arr*	Iraq	35.3 km NNE from Sulaymaniyah to Chwarta	KU 8593
30	*arr*	Iraq	53 km NW from Sulaymaniyah to Dukan Dam	KU 8682
31	*arr*	Iraq	5.5 km ENE from Koi Sanjaq to Ranya	KU 8697
32	*arr*	Iraq	11.4 km ENE from Koi Sanjaq to Ranya	KU 8707
34	*arr*	Iraq	19.1 km W from Shaqlawa to Arbil	KU 8713
37	*arr*	Iraq	S of Shaqlawa	KU 8720
38	*arr*	Iraq	7.1 km NE from Shaqlawa to Rowanduz	KU 8725
44	*arr*	Iraq	4.8 km NNE from Shaqlawa to Rowanduz	KU 8784
46	*arr*	Iraq	15.3 km ENE from Dohuk to Amadiyah	KU 8819
48	*arr*	Iraq	4.4 km NW from Amadiyah, Mzorka Gorge	KU 8831
50	*arr*	Iraq	13.4 km W from Amadiyah to Bamarni	KU 8880
51	*arr*	Iraq	21.9 km W from Amadiyah to Dohuk	KU 8884
56	*dcc*	Turkey	45 km SE of Maras	KU 1945
57	*dcc*	Turkey	4.5 km SE of Maras	KU 1952
58	*dcc*	Turkey	4.5 km SE of Maras	KU 1959B
59	*dcc*	Turkey	4.5 km SE of Maras	KU 1978B
60	*dcc*	Turkey	17.3 km E from Silvan to Bitlis	KU 8915A
61	*dcc*	Turkey	9.3 km SE from Ergani to Diyarbakir	KU 8935
62	*dcc*	Iraq	20.3 km from Sulaymaniyah to Quara Dagh	KU 8536
63	*dcc*	Iraq	SSW of Rowanduz	KU 8736A
64	*dcc*	Iraq	North slope of Jabal Sinjar, N of Kursi	KU 8840
65	*dcc*	Iraq	North slope of Jabal Sinjar, N of Kursi	KU 8817
66	*dcc*	Iraq	15.3 km ENE from Dohuk to Amadiyah	KU 8821C
68	*dcc*	Israel	Quatzrin (Pop. 2)	N
78	*dcc*	Israel	Quatzrin (Pop. 2)	N
83	*dcc*	Israel	Yehudiya (Pop. 3)	N
91	*dcc*	Israel	Yehudiya (Pop. 3)	N
95	*dcc*	Israel	Rosh Pinna (Pop. 4)	N
106	*dcc*	Israel	Rosh Pinna (Pop. 4)	N
108	*dcc*	Israel	Sanhedriyya (Pop. 11)	N
116	*dcc*	Israel	Sanhedriyya (Pop. 11)	N
118	*dcc*	Israel	Bet Meir (Pop. 12)	N
129	*dcc*	Israel	Bet Meir (Pop. 12)	N
134	*dcc*	Israel	Mt. Hermon (Pop. 1)	N
138	*dcc*	Israel	Mt. Hermon (Pop. 1)	N
143	*dcc*	Israel	Tabigha (Pop. 5)	N
149	*dcc*	Israel	Tabigha (Pop. 5)	N
156	*dcc*	Israel	Tabigha (Pop. 5)	N
167	*dcc*	Israel	Bat Shelomo (Pop. 6)	N
174	*dcc*	Israel	Taiyba (Pop. 10)	N
181	*dcc*	Israel	Taiyba (Pop. 10)	N
200	*dcc*	Israel	Mt. Gilboa (Pop. 7)	N
201	*dcc*	Israel	Mt. Gilboa (Pop. 7)	N
202	*dcc*	Israel	Mt. Gerizin (Pop. 8)	N

[a] N, S, E, W and ?: North, south, east, west and unknown
[b] KU: Plant Germplasm Institute, Faculty of Agriculture, Kyoto University, Kyoto 617, Japan, N: E. Nevo, Institute of Evolution, University of Haifa, Haifa 31999, Israel

ential hybrid fragments between each pair of accessions could be enumerated. The average total number (range given in parentheses) of hybrid fragments was 351.5 (341–359) between the *T. dicoccoides* accession pairs, 338.4 (330–358) between the *T. araraticum* accession pairs, and 344.6 (336–361) between the *T. dicoccoides* and *T. araraticum* accession pairs. In contrast, the average number (range given in parentheses) of the differential fragments was 87.0 (9–118) between the *T. dicoccoides* accession pairs, 23.9 (9–73) between the *T. araraticum* accession pairs, and 208.2 (164–228) between the *T. dicoccoides* and *T. araraticum* accession pairs. From these values the genetic distance (d), defined as the number of nucleotide substitutions per site, was estimated for all the accession pairs by the method of Nei (1987).

The distribution areas of the two species comprised three regions: from the edge of the Taurus Mountains in

Table 2 Number of restriction fragments in 21 wheat chromosomes, probed with 21 genomic and nine cDNA clones

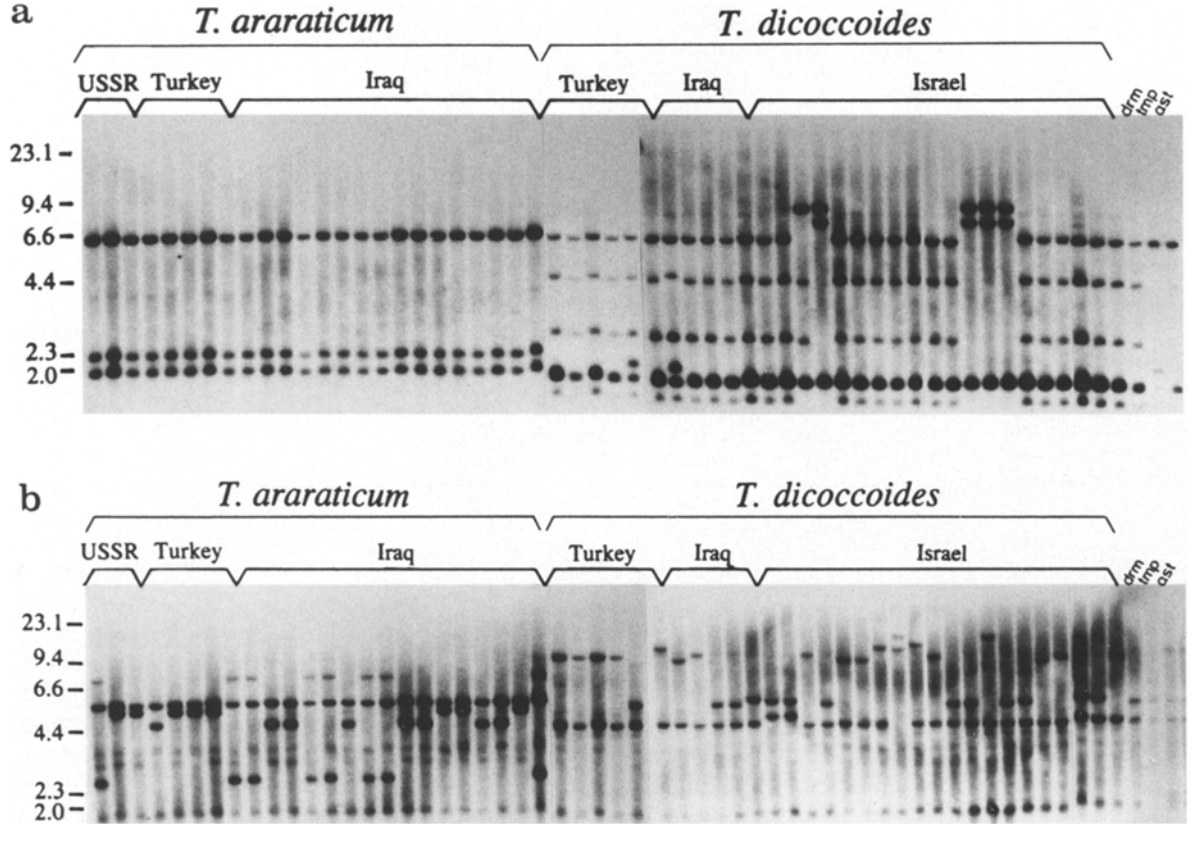
Homoeologous group	Genome			Total
	A	B	D	
1	1	1	0	2
2	6	6	10	22
3	5	4	6	15
4	3	5	3	11
5	3	3	3	9
6	4	3	3	10
7	2	3	2	7
Total	24	25	27	76

southeastern Turkey to western Iran through northern Iraq (region A), Palestine (region B) and the Transcaucasus (region C). *T. dicoccoides* is found in regions A and B, and *T. araraticum* in A and C. As shown in Table 3, the total variation in terms of the number of net nucleotide substitutions per site (d_A) is partitioned into the within- and between-regional variation for each species and the between-species variation (after Nei 1987). *T. dicoccoides* had much larger within- and between-regional variations than *T. araraticum*, but their distribution areas generally overlap. Interspecific variation was much greater than the intraspecific variations.

Table 3 Average genetic distances (d) and number of net nucleotide substitutions (d_A) estimated between the *T. dicoccoides* and *T. araraticum* accessions

Source of variation	No. comparisons	Average genetic distance ($d \times 10^2$)	No. net nucleotide substitutions ($d_A \times 10^2$)
Within region			
T. dicoccoides			
Region A	55	1.04 ± 0.35	1.04
Region B	210	1.27 ± 0.31	1.27
Pooled	–	1.22	1.22
T. araraticum			
Region A	210	0.36 ± 0.19	0.36
Region C	3	0.21 ± 0.09	0.21
Pooled	–	0.36	0.36
Between regions			
T. dicoccoides	231	1.50 ± 0.19	0.28
T. araraticum	63	0.36 ± 0.15	0.00
Within species			
T. dicoccoides	496	1.35 ± 0.31	1.35
T. araraticum	63	0.36 ± 0.15	0.36
Between species	768	4.82 ± 0.22	3.97

Fig. 2a, b Autoradiographs of Southern blots of the total DNAs isolated from 55 wild tetraploid wheat accessions and three reference species. The *Hin*dIII digests were probed with two clones; Tag694 (**a**) and Tac77 (**b**). The one Russian and two Armenian accessions are shown as USSR



Genetic distances between the wild tetraploids and their relatives

Genetic distances were calculated between all the wild tetraploid accessions and the reference accessions (Table 4). The average distance between the *T. dicoccoides* and *T. durum* accessions was almost the same as that between *T. araraticum* and *T. timopheevi*. Moreover, the distances between the two Emmer (*T. dicoccoides* and *T. durum*) and two Timopheevi (*T. araraticm* and *T. timopheevi*) species were similar, about four times the intra-group distances. A common wheat accession (*T. aestivum*) showed a much closer relation to Emmer than to Timopheevi wheat. The diploid species, *T. monococcum* and *Ae. speltoides*, showed a very distant relation to each other and to all polyploid wheats.

Construction of a phylogenetic tree

On the basis of the genetic distances, we constructed a dendrogram that shows the phylogenetic relationships for the 60 *Triticum* and the *Ae. speltoides* accessions (Fig. 3). Its most characteristic features are that all of the 32 *T. dicoccoides* accessions and a *T. durum* accession form a distinct cluster, whilst all 24 *T. araraticum* accessions and a *T. timopheevi* accession form another cluster. These two clusters are only distantly related. The dendrogram clearly shows very close relationships between the wild and cultivated species in both the Emmer and Timopheevi groups. There is a relatively close relationship between Emmer and common wheat ($d = 0.0191$–0.0239) in comparison to the distant relationship between Timopheevi and common wheat ($d = 0.0535$–0.0616). These facts suggest that the evolutionary history of the Timopheevi group differs from that of the Emmer and common wheat groups.

Both diploid species, *T. monococcum* and *Ae. speltoides*, are only distantly related to all polyploid wheat species, the average genetic distance between them being 0.1462 (Fig. 3). A matter for caution, however, is the presence of an additional genome(s) in the polyploid species which gives extra hybrid bands that may cause overestimation of the genetic distance between the diploid and polyploid species.

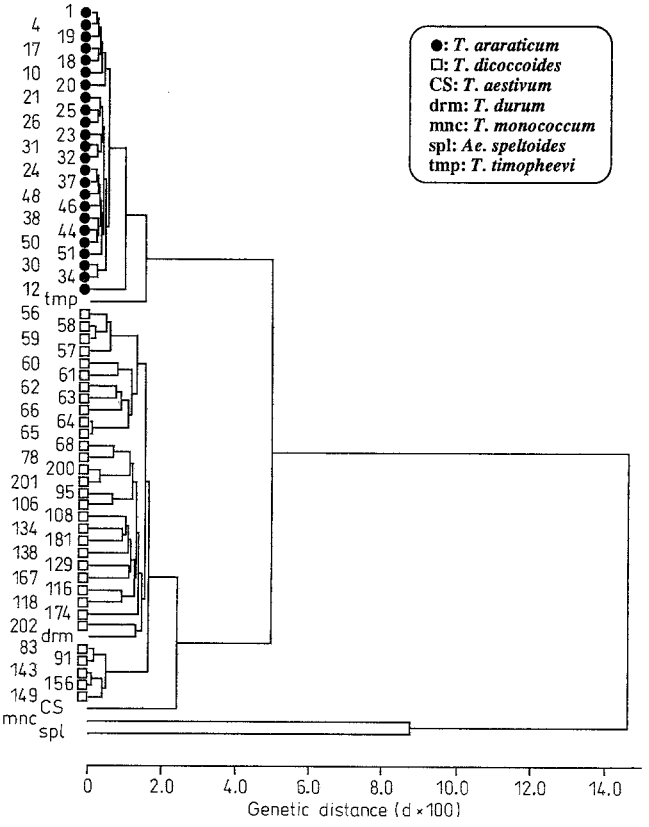


Fig. 3 Dendrogram showing the genetic relationships for 60 wheat accessions and an *Ae. speltoides* accession.

Discussion

Intraspecific DNA divergence in *T. dicoccoides* and *T. araraticum*

The two wild tetraploid wheats investigated were collected across their entire natural distribution areas. To gain insight into within-species variations, we estimated the number of net nucleotide substitutions (d_A) within and between the regional populations in each species (Table 3). In both species, the inter-regional variation was much smaller than the within-regional variation, evidence that no prominent geographical differentiation has yet occurred for either species.

Table 4 Genetic distances (d) estimated between all pairs of the six *Triticum* and one *Aegilops* species[a]

Species	dcc	drm	arr	tmp	ast	mnc
drm	0.0139					
arr	0.0482	0.0475				
tmp	0.0583	0.0572	0.0147			
ast	0.0239	0.0191	0.0535	0.0616		
mnc	0.1232	0.1187	0.1339	0.2876	0.1324	
spl	0.1271	0.1209	0.1540	1.0000	0.1454	0.0872

[a] arr, *T. araraticum*; ast, *T. aestivum*; dcc, *T. dicoccoides*; drm, *T. durum*; mnc; *T. monococcum*; spl, *Ae. speltoides*; tmp, *T. timopheevi*

A comparison of the two species shows that *T. dicoccoides* has much greater nuclear DNA variation than *T. araraticum* at all levels (the within-regional, inter-regional, and overall within-species variation) in terms of the number of net nucleotide substitutions. This greater genetic variation of *T. dicoccoides* is explained by one, or a combination, of the following factors: (1) difference in the effective population size, (2) difference in the mutation rate, and (3) difference in the time of origin. We have no definite information about the effective population size and mutation rate. Taking into account their similar breeding behavior as self-fertilizers and their genetic make-up as allotetraploids, as well as their similar distribution areas, we posit a much earlier origin for *T. dicoccoides*. A similar conclusion has been reached by Jiang and Gill (1994) in their study of *T. araraticum*, *T. timopheevi* and *T. durum* accessions by genomic in situ hybridization.

If the rate of nucleotide substitution per site per year (λ) is the same between two DNA sequences, and the time of their divergence is T, the expected number of nucleotide substitutions per site (d) between the two DNAs is given by the equation, $d = 2\lambda T$ (Nei 1987). Wolfe et al. (1987) estimated the average synonymous substitution rate in plant nuclear genes to be $0.5–3.0 \times 10^{-8}$ per site per year. Inserting these λ and d values in Table 3 in the above equation, the respective times of the intra-specific divergence of *T. dicoccoides* and *T. araraticum* is about $2.3–13.5 \times 10^5$ and $0.6–3.6 \times 10^5$ years, which values support an earlier origin for the former species.

Origins of *T. dicoccoides* and *T. araraticum*

The genetic distance between these two species, corrected for intra-specific variations, is much greater than the within-species distances (Table 3). Furthermore, all the *T. dicoccoides* accessions form a distinct cluster that differs from that of the *T. araraticum* accessions (Fig. 3). These facts indicate that the nuclear genomes of these two species differ markedly at the DNA level.

The estimated within-species divergence time of *T. dicoccoides* seems to be about four times older than that of *T. araraticum*, indicative of an earlier origin for the former species. This is supported by the fact that the plasmon (cytoplasmic genome) of some *Ae. speltoides* accessions is identical to that of *T. araraticum*; whereas, no *Ae. speltoides* accession had a plasmon identical to *T. dicoccoides*, even though their plasmons are closely related (Ogihara and Tsunewaki 1988).

There are two different hypotheses for the origins of the Emmer and Timopheevi groups of wheat. One assumes them to be of monophyletic origin, from a common tetraploid wheat (Sachs 1953; Tanaka et al. 1978). The other postulates a diphyletic origin, from different parental combinations (Kihara 1963; Tsunewaki 1989; Noda and Koulin 1989). The results reported here, which suggest that *T. dicoccoides* originated much earlier than *T. araraticum*, clearly favor a diphyletic origin for the Emmer and Timopheevi groups of wheat. A diphyletic origin also is given strong support by the recent research of Jiang and Gill (1994), in which common cyclic chromosome translocations found in six accessions of *T. araraticum* and *T. timopheevi* differ from those known in durum and common wheat.

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