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Determination of phylogenetic relationships between some wild wheat species using amplified fragment length polymorphism (AFLP) markers

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This study reports the molecular characterization, polymorphism, and phylogenetic relationships of *Triticum aestivum*, *T. dicoccoides*, *T. urartu*, and *T. monococcum* ssp. *boeoticum*, obtained from different locations in Anatolia, using 33 primer combinations to generate amplified fragment length polymorphism (AFLP) patterns in 31 individual plant samples. The objectives of this work were to estimate the phylogenetic relationships between these species and to investigate the genetic distance as a result of ecological and climatic factors. The origin of the A genome of polyploid wheats is also discussed. Eight hundred and seventy-five AFLP fragments had polymorphic loci, 133 of which were unique to *T. monococcum* ssp. *boeoticum*, 66 were unique to *T. urartu*, and 141 were unique to *T. dicoccoides*. Analysis using the program POPGENE showed polymorphism levels of *T. monococcum* ssp. *boeoticum*, *T. urartu*, and *T. dicoccoides* of 42.63, 32.34, and 27.71%, respectively. No correlation between genetic distance and ecological or climatic factors was recorded in this study. Our results support the hypothesis that *T. urartu* is a diploid ancestor of *T. dicoccoides* and *T. aestivum*. *Botanical Journal of the Linnean Society*, 2007, **153**, 67–72.

ADDITIONAL KEYWORDS: A genome – *Triticum* – Turkey.

INTRODUCTION

The genetic structure of various germplasms, including wheat found in the Mediterranean basin, varies largely, from traditional landraces and cultivars characterized by high versatility, to modern varieties characterized by high-yield potential, wide adaptation, and technological quality (Bozzini *et al*., 1998). Molecular markers have provided a powerful approach for the analysis of genetic relationships between different accessions of many crop species. They are a useful complement to morphological and physiological characterization of cultivars because they are plentiful, independent of plant tissue or environmental effects, and allow cultivar identification very early on in plant development (Manifesto *et al*., 2001). Molecular characterization of cultivars is also useful for the evaluation of potential genetic erosion, such as a decrease in genetic diversity during the breeding process. DNA-based markers are particularly useful in wheat and other crops with an apparently narrow genetic background, and are helpful in evaluating genetic and genome studies and, more recently, gene cloning and practical breeding.

An important condition for the use of wild species as potential gene donors is to define closely related species. DNA markers, such as random amplified polymorphic DNA (RAPD), microsatellites, and single-strand repeats (SSRs), are the most convenient data sources in the search for these relationships. In studies related to the evolution of wheat, the origin of the A genome of polyploid wheats has been the subject of considerable controversy. Two diploid species, *T. monococcum* ssp. *boeoticum* and *T. urartu*, have been proposed as possible A genome donors of tetrap-

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loid wheats, on the basis of the C-banding pattern of their chromosomes and the isozyme and seed storage protein profiles (Gill & Kimber, 1974; Caldwell & Kasarda, 1978). Konarev *et al*. (1979) and Konarev (1983) proposed that the A genome of *T. dicoccoides* and *T. aestivum* was derived from *T. urartu,* whereas that of *T. timopheevii* was derived from *T. monococcum*. Basing their conclusions on immunochemical and electrophoretic studies of seed storage proteins, Dvorak, McGuire & Cassidy (1988), using restriction fragment length polymorphism (RFLP), determined that the A genome of *T. aestivum*, *T. dicoccoides*, *T. turgidum* ssp. *durum*, and *T. timopheevii* ssp. *araraticum* originated from *T. urartu* and not from *T. monococcum* ssp. *boeoticum* (*aegilopoides*). By contrast, Nishikawa *et al*. (1992) suggested that the A genome of *T. turgidum* was possibly derived from both *T. monococcum* ssp. *boeoticum* and *T. urartu*.

The efficiency of polymorphism detection by amplified fragment length polymorphism (AFLP) in wheat is high compared with that of other available marker systems (Soleimani, Baum & Jonson, 2002; Tuberosa, Gill & Quarrie, 2002; Almanza-Pinzon *et al*., 2003), as the AFLP technique combines the reliability of RFLP with the power of polymerase chain reaction (PCR) to amplify many restriction fragments simultaneously (Vos *et al*., 1995).

To achieve more efficient and comprehensive utilization of the conserved wild gene pool, it is essential to be able to predict, screen, and evaluate promising genetic diversity and resources in natural populations and in genotypes of wild wheat relatives. Thus, the present study was designed to reveal the levels of polymorphism and phylogenetic relationships of *T. aestivum*, *T. dicoccoides, T. urartu*, and *T. monococcum* ssp. *boeoticum* in Anatolia using AFLP. In particular, the research focused on whether there was any correlation between the genetic distance and ecological and climatic factors. The study also evaluated the origin of the A genome of polyploid wheats.

MATERIAL AND METHODS

Plant material

Thirty-one individuals of four *Triticum* species were used in the present research (Table 1). They were collected from different eco-geographical localities of central and south-east Anatolia in Turkey (Table 2).

DNA preparation

Total DNA was extracted from fresh leaf tissue of 2-week-old seedlings and frozen in liquid nitrogen (Doyle & Doyle, 1987). The DNA concentration was determined by the gel documentation system (UVP).

AFLP protocol

The procedure was performed essentially as described by Vos *et al*. (1995), with some minor modifications. About 0.5 ng μL^{-1} of DNA was digested with 5 U of each of the restriction enzymes *Pst*I and *Mse*I at 37 °C for 3 h. Next, 10 μL of a solution containing 10 pmol *Pst*I adapters and 100 pmol *Mse*I adapters (Table 3), 1 U T4 DNA-ligase, 1 mM adenosine triphosphate (ATP) in 10 mM Tris-HCl, pH 7.5, 10 mM magnesium acetate (MgAc), 50 mM potassium acetate (KAc), 5 mM dithiothreitol (DTT), and 50 ng μL^{-1} bovine serum albumin (BSA) was added to the digested DNA fragments, and incubation was continued for 4 h at 37 °C.

For pre-amplification, a 50 μL PCR mixture containing 10 μL of re-suspended ligated DNA template, 10 pmol of each of the two primers (*Pst*I and *Mse*I) without selective nucleotides, 10 × PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 2.5 U of *Taq* polymerase, and 250 μM of each dNTP was used. The following cycle profile was used for PCR amplification: 26 cycles of 94 °C for 60 s; 60 °C for 30 s; 72 °C for 60 s; this was followed by extension at 72 °C for 7 min. The pre-amplified samples were diluted tenfold in water and used as a template for selective amplification.

Selective amplification was conducted using 33 primer combinations (Table 3). Each primer contained three selective nucleotide extensions at the 3′ end. Selective amplification was carried out in 20 μL reaction volumes using 3 μL of template, 0.5 μL of γ-[^{33}P]-end-labelled *Pst*I primer, 5 pmol of unlabelled *Mse*I primer, 250 μM of dNTPs, 10 × PCR buffer, 2.5 U of *Taq* polymerase, and 3 μL of the pre-amplified and diluted DNA template. The selective amplification was performed for 36 cycles with the following cycle profile:

Table 1. Wheat species used in this study

Species	Abbreviation	Genome symbol	2*n*
Triticum monococcum L. ssp. *boeoticum* (Boiss) C. Yen	TRMB	AA	14
T. urartu Thumanjan ex Gandilyan.	TRUR	AA	14
T. turgidum L. ssp. *dicoccoides* Korn.	TRDI	AABB	28
T. aestivum L.	TRAE	AABBDD	42

Table 2. *Triticum* genotypes used for amplified fragment length polymorphism (AFLP) analysis and selected eco-geographical data of the sites of origin

Species*-sample	Location	Latitude, longitude	Altitude (m)	Mean temperature (°C)			Annual humidity at 14:00 h (%)	Mean annual rainfall (mm)	Soil†
				Year	Aug	Jan			
TRAE-1	Kirikkale, Balişeyh	39°59′N, 31°51′E	1300	10.3	21.8	–1.1	42.0	397.3	Bn
TRMB-2	Ankara, Beytepe	39°52′N, 32°44′E	900	11.2	22.3	–0.6	48.2	402.2	C
TRMB-3	S. Urfa, Suruc	37°04′N, 38°31′E	670	18.1	31.1	5.4	38.3	305.0	Rb
TRMB-4	S. Urfa, Hilvan	37°33′N, 38°55′E	590	15.9	29.7	3.7	37.0	469.7	Rb
TRMB-5	Diyarbakir, Karamuz	37°36′N, 39°07′E	600	15.9	29.7	3.7	37.0	469.7	Rb
TRMB-6	Diyarbakir, Karamuz	37°46′N, 40°14′E	720	15.7	30.2	1.7	41.9	261.8	B
TRMB-7	Viranşehir, Botas	37°02′N, 39°46′E	630	17.3	29.7	4.7	40.0	372.0	B
TRMB-8	S. Urfa, Viranşehir	37°13′N, 39°39′E	600	17.3	29.7	4.3	40.0	372.0	Rb
TRMB-9	S. Urfa, Maloren	37°36′N, 38°55′E	550	15.9	29.7	3.7	37.0	469.7	Rb
TRMB-10	Ankara, Haymana	39°37′N, 32°40′E	1100	9.3	18.4	–2.0	59.0	413.6	Rb
TRMB-11	S. Urfa, Hilvan	37°45′N, 38°46′E	575	15.9	29.7	3.7	37.0	469.7	Rb
TRUR-12	Oguzeli, Ekizkoyun	36°52′N, 37°03′E	650	15.3	27.3	3.5	38.0	423.8	Rb
TRUR-13	S. Urfa, Arat	37°03′N, 38°08′E	760	18.7	30.3	5.5	38.0	254.0	Rb
TRUR-14	S. Urfa, Ezgil	37°03′N, 38°18′E	570	18.7	30.2	5.5	38.0	254.0	Rb
TRUR-15	S. Urfa, Suruc	37°04′N, 38°31′E	670	18.1	31.1	5.4	38.3	305.0	Rb
TRUR-16	Diyarbakir, Hilvan	37°36′N, 39°07′E	600	15.9	29.7	3.7	37.0	469.7	Rb
TRUR-17	Diyarbakir, Karamuz	37°46′N, 40°14′E	720	15.7	30.2	1.7	41.9	261.8	B
TRUR-18	Ayele, Hilvan	37°36′N, 38°49′E	550	15.9	29.7	3.7	37.0	469.9	Rb
TRUR-19	S.Urfa, Agizhan	37°26′N, 38°55′E	600	15.9	29.7	3.7	37.0	469.7	Rb
TRDI-20	Gaziantep, Elmali	36°52′N, 37°22′E	730	15.8	27.1	2.7	44.5	381.1	B
TRDI-21	Gaziantep, Zagabazca	36°52′N, 37°23′E	730	15.8	27.1	2.7	44.5	381.1	B
TRDI-22	Gaziantep, Caltalsu	36°05′N, 37°23′E	700	15.8	27.1	2.7	44.5	381.1	B
TRDI-23	Diyarbakir, Siverek	37°45′N, 39°25′E	910	17.2	29.8	3.3	42.0	349.2	B
TRDI-24	Diyarbakir, A. Karabahce	37°48′N, 39°44′E	1100	15.7	30.2	1.7	41.9	261.8	B
TRDI-25	Karamuz, Ovabag	37°47′N, 40°11′E	750	15.7	30.2	1.7	41.9	261.8	B
TRDI-26	Karamuz, Ovabag	37°46′N, 40°08′E	810	15.7	30.2	1.7	41.9	261.8	B
TRDI-27	Diyarbakir, Karacadag	37°28′N, 39°48′E	1010	12.5	27.0	–1.7	40.0	615.3	B
TRDI-28	Diyarbakir, Viransehir	37°02′N, 39°46′E	630	17.3	29.7	4.7	40.0	372.0	B
TRDI-29	Diyarbakir, Karakeci	37°27′N, 39°26′E	810	17.2	29.8	3.3	42.0	349.2	Rb
TRDI-30	Diyarbakir, A.Dilimli	37°17′N, 39°29′E	670	17.2	29.8	3.3	42.0	349.2	Rb
TRDI-31	Diyarbakir, Siverek	37°54′N, 39°52′E	900	17.2	29.8	3.3	42.0	349.2	B

*Abbreviations of species are given in Table 1.
†B, basaltic; Bn, brown; C, calcareous; Rb, red–brown.

a 30 s DNA denaturation step at 94 °C, a 30 s annealing step at 65 °C, and a 1 min extension step at 72 °C. The annealing temperature of 65 °C in the first cycle was subsequently reduced in each cycle by 0.7 °C for the next 12 cycles, and was continued at 56 °C for the remaining 23 cycles. All amplification reactions were performed in a Biometra T-personal thermocycler.

Polyacrylamide gel electrophoresis

Amplification products were mixed with an equal volume of formamide dye [98% (v/v) formamide, 100 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0, 0.025% (v/v) bromophenol blue, and 0.025% (v/v) xylene cyanol], which served as a tracking dye. The resulting mixture was heated for 3 min at 94 °C, and then quickly cooled on ice. Each sample was loaded on ice. After electrophoresis for 2 h at 80 W (constant power), the gel was dried and exposed to X-ray film for 1–4 days depending on the signal intensity.

Data analysis

All genotypes were scored for the presence or absence of polymorphic AFLP fragments, and the data were entered into a binary matrix as discrete variables ('1' for presence and '0' for absence of a homologous fragment). Only distinct, reproducible, well-resolved

fragments were scored. Data were analysed using POPGENE 32 (Yeh, Yang & Boyle, 1999).

RESULTS

AFLP analysis using 33 primer combinations to generate AFLP patterns with 31 individual samples of the four taxa showed more amplifications of some primer combinations than others, resulting in a total of 875 polymorphic loci. Of these, 133 were unique to *T. monococcum* ssp. *boeoticum*, 66 were unique to *T. urartu*, and 141 were unique to *T. dicoccoides*. Using the POPGENE program, and considering the genetic diversities of all loci, the polymorphism levels of *T. monococcum* ssp. *boeoticum*, *T. urartu*, and *T. dicoccoides* were 42.63, 32.34, and 27.71%, respectively. The genetic distances between *T. monococcum* ssp. *boeoticum* and the other two species, *T. dicoccoides* and *T. aestivum*, were 0.8382 and 0.6304, respectively (Table 4). The unbiased measurements of genetic identity and the genetic distance matrix of Nei (1978) showed genetic distances of 0.5856, 0.5784, and 0.5790 between *T. urartu* and *T. monococcum* ssp. *boeoticum*, *T. dicoccoides*, and *T. aestivum*, respectively. A dendrogram of populations based on this matrix is shown in Figure 1.

Table 3. Adapters and primers used for pre-amplification and selective amplification

Name of primer/adapter	Sequence of primers and adapters used in AFLP reactions (5′–3′)
*Pst*I adapter	CTCGTAGACTGCGTACATGCA-3′
	CATCTGACGCATGT-5′
*Mse*I adapter	GACGATGAGTCCTGAG-3′
	TACTCAGGACTCAT-5′
*Pst*I primer	GACTGCGTACATGCAG-3′
*Mse*I primer	GATGAGTCCTGAGTAA-3′
P55 + CGA	GACTGCGTACATGCAG + CGA
P56 + CGC	GACTGCGTACATGCAG + CGC
P57 + CGG	GACTGCGTACATGCAG + CGG
M49 + CAG	GATGAGTCCTGAGTAA + CAG
M50 + CAT	GATGAGTCCTGAGTAA + CAT
M51 + CCA	GATGAGTCCTGAGTAA + CCA
M52 + CCC	GATGAGTCCTGAGTAA + CCC
M53 + CCG	GATGAGTCCTGAGTAA + CCG
M55 + CGA	GATGAGTCCTGAGTAA + CGA
M56 + CGC	GATGAGTCCTGAGTAA + CGC
M57 + CGG	GATGAGTCCTGAGTAA + CGG
M60 + CTC	GATGAGTCCTGAGTAA + CTC
M61 + CTG	GATGAGTCCTGAGTAA + CTG
M62 + CTT	GATGAGTCCTGAGTAA + CTT

AFLP, amplified fragment length polymorphism.

DISCUSSION

An advantage of AFLP-based DNA fingerprinting is its potential for exposing high levels of genetic polymorphism, giving an almost complete coverage of the whole genome. In the study presented here, we analysed 31 samples belonging to four taxa from different localities using 14 primer pairs with 33 combinations.

One of the aims of this study was to investigate any difference between the genomes of the collected samples which could be related to their localities. The highest level of genetic diversity between plants from different localities was found in *T. monococcum* ssp. *boeoticum* (42.63%), followed by *T. urartu* (32.34%) and *T. dicoccoides* (27.71%), but these values were

Table 4. Nei's unbiased measurements (Nei, 1978) of genetic identity and genetic distance for genetic relationship analysis

Species*	TRAE	TRMB	TRUR	TRDI
TRAE	–	0.5324†	0.5605†	0.7595†
TRMB	0.6304‡	–	0.5567†	0.4325†
TRUR	0.5790‡	0.5856‡	–	0.5608†
TRDI	0.2751‡	0.8382‡	0.5784‡	–

*Abbreviations of species are given in Table 1.
†Genetic identity.
‡Genetic distance.

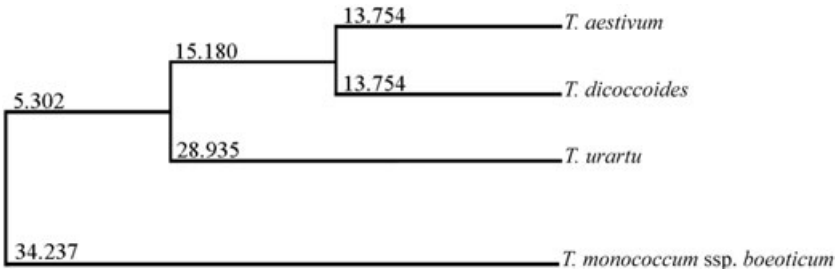

Figure 1. Dendrogram of populations based on Nei's genetic distance (Nei, 1978) and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) modified from NEIGHBOUR procedure of PHYLIP Version 3.5. Numbers shown on the branches represent the branch length.

lower in a starch gel enzyme electrophoresis study of *T. monococcum* ssp. *boeoticum* and *T. urartu* by Smith-Huerta *et al*. (1989). This is not surprising, as AFLP produces a large number of polymorphic DNA fragments throughout a genome. Low levels of genetic diversity are expected between self-pollinating plants, such as *Triticum* species. The polymorphism levels detected in other self-pollinating plants, such as tef (Bai *et al*., 1999), azuki (Yee *et al*., 1999), rice (Maheswaran *et al*., 1997), sugar beet (Schondelmaier, Steinrucken & Jung, 1996), and wild hordeum (Pakniyat *et al*., 1997), were 4, 18, 22, 50, and 76%, respectively. In our study, the diploid species *T. monococcum* ssp. *boeoticum* and *T. urartu* were found to be more polymorphic than the polyploid species *T. dicoccoides* and *T. aestivum*, supporting the results of RFLP analysis by Galili *et al*. (2000).

The lowest polymorphism level was found in *T. dicoccoides*, a result consistent with previous studies. RAPD analysis of *T. dicoccoides* collected from Israel and Turkey showed that the percentages of polymorphic loci were 81.4 and 18.6%, respectively (Fahima *et al*., 1999). Allozyme analyses of *T. dicoccoides* also showed levels of polymorphism that were higher in samples collected from Israel than in those from Turkey (Nevo & Beiles, 1989).

We did not find any correlation between genetic distance and eco-climatic factors in this study.

ORIGIN OF THE A GENOME

According to Nei's unbiased measures of genetic distance for genetic relationship analysis (Nei, 1978), the lowest genetic distance was observed between *T. aestivum* and *T. dicoccoides* (0.2751; Table 4). This result indicates that hexaploid *T. aestivum* (AABBDD) and tetraploid *T. dicoccoides* (AABB) are closer to each other than to the other species studied. It might have been expected that the genetic distance between the diploids *T. monococcum* ssp. *boeoticum* (AA) and *T. urartu* (AA) would have been lower than that between the other species, but our results were contrary to this expectation (genetic distance was 0.5856 according to the matrix data of Nei's unbiased measurements of genetic distance). Our results show that diploid *T. urartu* shares more similarities with hexaploid *T. aestivum* (0.5790) and tetraploid *T. dicoccoides* (0.5784) than with *T. monococcum* ssp. *boeoticum*. The genetic distance values between *T. monococcum* ssp. *boeoticum* and the other two species, *T. aestivum* (0.6304) and *T. dicoccoides* (0.8382), were higher than that of *T. urartu*. The results obtained from the dendrogram (Fig. 1) and the genetic distance matrix (Table 4) support the hypothesis that *T. urartu* is the diploid ancestor of *T. dicoccoides* and *T. aestivum*. Our results are consistent with the conclusions based on RFLP analysis of repeated DNA (Dvorak *et al*., 1988), immunochemical and electrophoretic studies of seed storage proteins (Konarev *et al*., 1979; Konarev, 1983), α-amylase analysis (Nishikawa *et al*., 1992), 5S rDNA analysis (Allaby & Brown, 2000), RbcS multigene family analysis (Galili *et al*., 2000), and microsatellite analysis (Sourdille *et al*., 2001). Studies on the sequencing of the internal transcribed spacer (ITS)-2 region of nuclear ribosomal DNA showed that the A genome of *T. dicoccoides* originated from *T. monococcum* (Zhang *et al*., 2002).

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