Evaluation of genetic diversity in diploid wheat : *Triticum urartu* using AFLP markers

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Abstract

AFLP markers were used to study genetic diversity of six accessions of wild wheat Triticum urartu collected from north (Aleppo) and south (Sweida) of Syria. AFLP data analysis on 18 genotypes of T. urartu, using different combination of selective primers Pst / Mse show a high informative banding pattern. Selective PCR amplification produce 50 to 100 bands. An average of 50 bands have molecular weight ranged between 50 to 300 bp. A total of 176 bands, with molecular weight 50 to 300 bp, were scored. Midpoint-rooted NJOIN and principal coordinate analysis of AFLP data show two distinct clusters of the six accessions. Analysis of molecular variance and Genstat package analysis were similar. Data AFLP analysis using individuals simplest of T.urartu show variability among the regions and within the populations with four primers Pst / Mse combinations. This variability suggesting that there are members of each distinguishes accessions.

Keys words : *Triticum urartu*, AFLP, genetic diversity

Résumé : Caractérisation moléculaire de la diversité génétique du blé diploïde : *Triticum urartu*, par l'utilisation de la technique AFLP

L'analyse de la diversité génétique de dix huit plants de six populations de blé diploïde Triticum urartu provenant du Nord et du Sud de la Syrie, précédemment caracterisées agromorphologiquement et biochimiquement a été réalisée. Les dendogrammes obtenus à l'aide de l'indice de similitude de Jaccard par les méthodes NJOIN et PCO ont révelé deux groupes distincts répartissant les six accessions étudiées. L'analyse de la variance moléculaire (AMOVA) a confirmé les résultats issus du calcul GENSTAT. L'analyse AFLP au niveau des individus de T. urartu a montré une variabilité parmi les régions et les populations testées par les quatre combinaisons d'amorces (Pst AA/Mse CCG, PstCC/Mse ACC, Pst CA/Mse ACA

et Pst CC/Mse CCG). Ces résultats ont montré clairement la répartition et l'appartenance des plantes individueles au niveau de chaque population.

Mots clés : *Triticum urartu*, AFLP, diversité génétique, empreintes génétique d'ADN

ملخص : التوصيف الجزيئي للتنوع الوراثي في القمح البري : *Triticum urartu* باستخدام البصمة الوراثية بطريقة AFLP

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إن تحليل تعدد أشكال طول كسرة الصبغي المضخمة (AFLP) 18 (طرازا) وراثيا من 6 مدخلات من القمح البري *Triticum urartu* (عبر أوائلها الطبيعية) التي جمعت في جنوبي و شمالي سورية، قد دعم التوزيع الذي حدد سابقا باستخدام الصفات المرفولوجية الزراعية و التوصيف الكيميائي الحيوي. وكشف UPGMA و التحليل الرئيسي المنسق لبيانات ALFP عنقودين محددين مطابقين للمدخلات الستة. و بالإضافة إلى ذلك، أكد تحليل التباين الجزيئي النتائج التي تم الحصول عليها بواسطة برنامج Genstat للتحليل الإحصائي. وأظهر إجراء تحليل AFLP على الطراز الوراثية الفردية ل *T. urartu* تباينا بين المناطق والعشائر مع وجود أربع بادئات نيوكليتيدية أساسية مما يوحي بأن هذه الأنواع تتوزع ضمن المدخلات الستة.

الكلمات المفتاحية : *Triticum urartu*، AFLP القمح البري، التنوع الوراثي، بصمة د.ن.أ. الوراثية

Introduction

The recently developed molecular marker systems based on PCR amplification have facilitated and enhanced fundamental and applied biological studies. Although DNA amplified-derived techniques are in general more advantageous than classical markers, all have their limitations and specific applications. Among the most important factors, they have the multiplex ratio and the information content of a marker technique. The choice of a particular method depends on the specific application. If the objective is to assess genetic diversity, method with a high multiplex ratio as AFLP is appropriate (Vos et al.1995). The Amplified Fragment Length Polymorphism (AFLP) is a powerful DNA fingerprinting technique, which is especially suitable to investigating new crops or natural populations. It was already adopted to characterize plant natural populations (Folkertsma et al.1996; Greef et al. 1997). It has been found to be a very fast and reliable technique avoiding the obstacles of previously used markers like

RFLPs and RAPDs. For the crop improvement program, plant breeders require a marker technology which is technically simple, cost-and time-effective, and which generates a high level of polymorphism. AFLPs offer an opportunity to perform detailed genetic studies in a large number of organisms (Sharma et al.1996; Hill et al.1996; Mueller et al.1996 and Manfred et al. 1997). The polymorphism detected per reaction is much higher than revealed by RFLPs or RAPDs because of the number of loci sampled in a single assay. In the present study, we have applied AFLPs to study genetic variation between and within populations of wild wheat (*T.urartu*).

Materials and methods

Plant material

Eighteen genotypes of six accessions (three samples by accession) were collected from different parts of Syria (table 1).

Table 1. List of the genotypes and populations used for AFLP analysis

Population code	Single plant code	IG number	Province of Syria	Latitude	Longitude
S1	S11, S12, S23	45284	Sweida	N32 38	E36 49
S2	S21, S22, S23	45287	Sweida	N32 42	E36 50
S3	S31, S32, S33	45282	Sweida	N32 41	E53 49
S4	S41, S42, S43	45283	Sweida	N32 38	E36 49
A1	A11, A12, A13	45298	Aleppo	N36 25	E37 00
A2	A21, A22, A23	45299	Aleppo	N36 30	E36 57

DNA extraction

DNA was extracted from leaf tissue, frozen in liquid nitrogen, of three-week-old seedlings according to the modified CTAB technique (Saghai Maroof et al.1984).

AFLP

Digestion of DNA

The AFLP procedure was performed as described by the protocol of Keygene N.V (Vos et al. 1995) with some minor modifications. Five hundred nanograms of DNA from each sample were double digested with 5 U MseI and 10 U PstI; the One-Phor-All buffer (10x, Pharmacia) in a final volume of 40 µl was used incubating two hours at 37 °C.

Adaptor-ligation

Dynal beads procedure

The DNA fragments were then ligated with Pst.I adapters, which are 5'biotinylated, and MseI adapters. DNA fragments containing the biotin-labeled PstI adapters were separated from the reaction using streptavidin beads (DYNAL.Inc. New Hyde Park. N.Y), thus decreasing the complexity of the DNA by removing the more abundant MseI-MseI. Adaptor ligation was achieved by adding 50 pmol of MseI-adapter, 5 pmol of PstI-adapter, 1µl of 10 mM ATP, 1,4 U of T4-DNA ligase. 1µl of One-Phor-Buffer (10x) and sterile distilled water to the double-digested DNA sample (50 µl final volume) and incubating for 3h more at 37 °C. All primer adapter sequences used are given in table 2. 10 µl of Digested-restricted DNA was run on a 1 % agarose gel to check for complete digestion-ligation.

Table 2. Sequence of primers and adapters used.

Adapters primers	
MseI adapter	5' – GAC GAT GAG TCC TGA-3'
	5'-TAC TCA GGA CTC AT-3'
PstI adapter	5'-biotin-CTC GTA GAC TGC GTA CAT GCA-3'
	5' –TGT ACG CAG TCT AC- 3'
Selective primers	
PstI primers (18 mers)	
PstI + 2 primers	5'-GAC TGC GTA CAT GCA GAC-3'
	5'GAC TGC GTA CAT GCA GAA-3'
	5'GAC TGC GTA CAT GCA GCA-3'
	5'-GAC TGC GTA CAT GCA GCC-3'
MseI primers (19 mers)	
MseI + 3 primers	5'-GAT GAG TCC TGA GTA AGA A-3'
	5'-GAT GAG TCC TGA GTA AAA T-3'
	5'-GAT GAG TCC TGA GTA AAC A-3'
	5' GAT GAG TCC TGA GTA AAC C-3'

Pre-amplification

Pre-amplification of prepared template was performed with primers complementary to the core of the adaptor sequences (table 2). Biotinylated restriction fragments in each ligation mixture was used as template for polymerase chain reaction amplifications. Thus, dilution 5-10x of digested and ligated was mixed with 50 ng primer P+00, 50 ng primer M+00, 5U Taq DNA polymerase (boehringer, Germany), 2 µl of 10x PCR buffer (boehringer, Germany) and 5mM dNTPs, in a final volume of 25 µl. The PCR reaction was performed in a Perkin Elmer-9600 thermal cycler (Perkin Elmer, USA) using the following temperature : 30 cycles of 30 s at

94 °C, 30 s at 56 °C, 60 s at 72 °C. After pre-amplification, 5 µl of this pre-amplified DNA was checked on 1% agarose gel where a smear (0.2 kb) was visible.

End labelling

Only MseI compatible primer was radiolabeled with [γ^{33}P] ATP (Amersham redivue AH 9968). 0.16 µl [γ^{33}P ATP] (50 µCi), 10x OPA buffer, 0.017 µl (0.1 U) T4 polynucleotide Kinase (6.100 U ml^{-1}; Pharmacia) , 0.1 Mse I.X (50 ng/µl) samples were incubated at 37 °C for 30 min then heated to 68 °C for 10 min to stop the reaction.

Selective-amplification

For selective PCR, Mse I were end-labeled with [γ^{33}P] ATP (Amersham) using a T4 polynucleotide kinase reaction (Pharmacia). Selective amplification was performed using selective primers specific Pst and Mse I. Each primer contained two or three selective nucleotides at the 3'end. AFLP fingerprints were generated in selective amplification in the 20 µl of PCR mix composed by 5 µl of the diluted 5.10x pre-selective amplification, 5mM dNTPs, 0.5 U of Taq polymerase, 30 ng each of two primers (table 2) in PCR buffer. The following cycle profile ensured optimal primer selectivity : 10 cycles of 30 s at 94 °C, 30 s at 62 °C, 60 s at 72 °C followed by 25 cycles of 30 s at 94 °C, 30 s at 56 °C , 60 s at 72 °C and 1 cycle of 30 min at 72 °C . Sixteen different primer combinations were used. Products were visualized by autoradiography and scored manually as described by Vos et al. (1995). All AFLP polymorphisms were scored as dominant markers (presence or absence of a band).

Data analysis

Assigning a number to each band visually scored the fingerprinting profiles. Only full intensity bands were scored. Polymorphism was scored as either present (1) or absent (0) across all genotypes. Calculation of the distance matrix and cluster were carried out using the statistical package NTSYS Version 2.00 (1997). Data from the four combinations were combined and the pairwise similarities calculated between samples using the Jaccard coefficient. The resultant similarity matrix was input into a NJOIN cluster analysis and a principal coordinate analysis. AMOVA (Analysis of Molecular Variance) has been used to determine the percentage of variation among and between the populations and the region to complete Genstat analysis.

Results

AFLPs performed on 18 *T. urartu* genotypes show highly informative banding patterns. Selective PCR amplification using primer Pst+2 nucleotides and Mse+3 nucleotides produce 50 to 100 bands, when AFLP analysis was performed on a *T. urartu*, on a sequencing gel. Among sixteen primer combinations, four have given an interesting informative results. Among the four combinations used, a primer combination Pst+ CC / Mse+ACC (Figure 1) was most informative. An average of 50 bands with molecular weight 50 to 300 bp was obtained for

each genotype. The total 176 bands were scored across the 18 genotypes using four combinations of Pst and Mse primers.

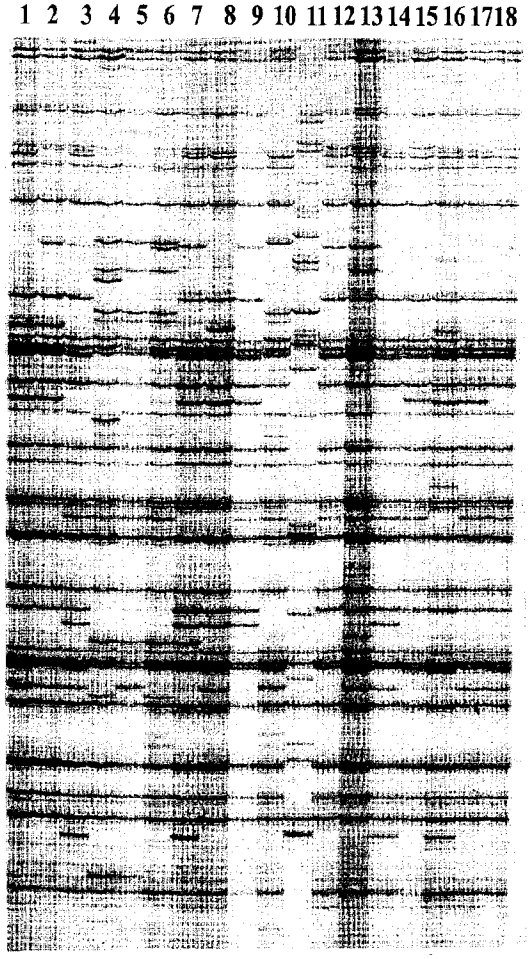

Figure 1. Amplified fragement length polymorphism profile obtained with the primer combination Pst + CC and Mse + ACC

Statistical analysis

Cluster analysis

When NTSYS analysis was performed with each couple of primer, the major groups obtained were similar to those with four mixed primers using a similarity matrix produced by Genstat. The dendrograms based on the neighbor-joining method for cluster analysis using Jaccard Similarity Coefficients are showed in the Figure 2 and 3. The *T. urartu* pool is arranged

in two geographic groups, from Aleppo (North of Syria) and Sweida (South) respectively. Both populations from Aleppo region were more diverse than the populations from the southern parts. However, the accessions from the South showed a high diversity between them. The high correlation between pair of combination of primers suggests that each combination pro vided different complementary information.

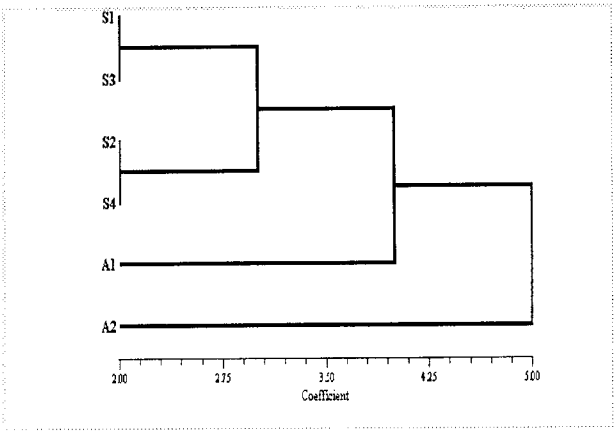

Figure 2. Midpoint-rooted NJOIN tree for six *T. urartu* populations

S : Populations from Sweida region ; A : populations from Aleppo region

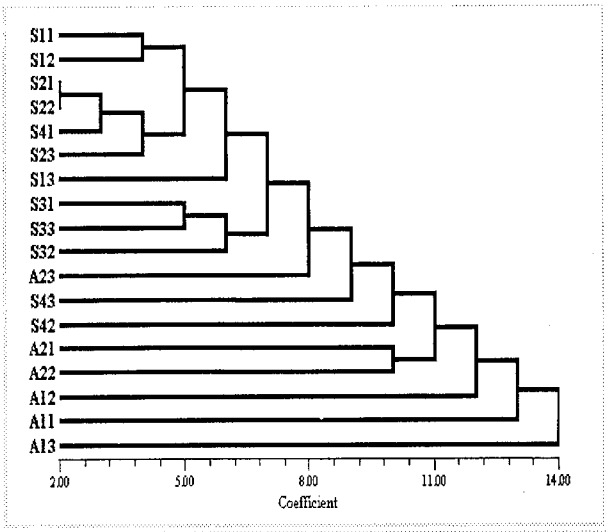

Figure 3. Midpoint-rooted NJOIN tree for 18 *T. urartu* plants

S : Populations from Sweida region ; A : populations from Aleppo region

Principal coordinate analysis

The structure of the genetic diversity is demonstrated by the principal coordinate analysis presented in figure 4. The first and second principal co-ordinates explained 32 % and 22 % respectively of a total variation in the AFLP data. The results suggest that the first principal coordinate clearly separate the two regions. The second principal coordinate show a distinct separation between Aleppo accessions.

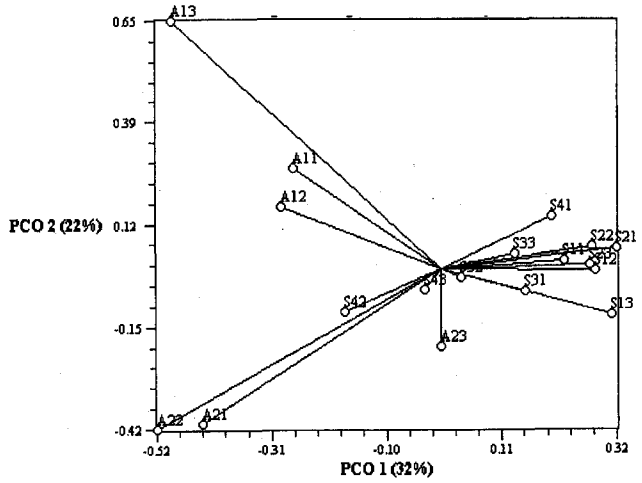

Figure 4. Principal coordinate anlysis

Analysis of molecular variance (AMOVA)

The variation estimates within and between regions are presented in Table 3. Nested the lowest variance within a region was obtained from northern Syria, which was different from the Southern region. Accessions from southern Syria were more diverse than those of the Northern parts. This result could result from different mutations or recombinations during the process of evolution. Also, it is possible that populations have a high proportion of heterozygotes. The variation within populations and regions was respectively 67.8 % and 78.7 %.

Table 3. Variance components from AMOVA analysis

Nested Analysis

Variance among regions	V(A) :	(17.13 %)
Variance among populations within regions	V(B) :	(15.05 %)
Variance within populations	V(C) :	(67.81 %)

PHI-statistics : PHIst = 0.322 PHIsc = 0.182 PHIct = 0.171

Analysis Among Populations

Variance among populations	V(A) :	(26.29 %)
Variance within populations	V(B) :	(73.71 %)

Analysis Among Regions

Variance among regions	V(A) :	(21.27 %)
Variance within regions	V(B) :	(78.73 %)

Discussion

The genetic variability of the 18 genotypes of wild wheat *T. urartu* derived from natural populations as revealed by AFLP technique showed highly informative banding patterns that were polymorphic both at plant and population level. Using AFLPs all the 18 plants and six populations were easily distinguished. The four primer combinations gave some putative specific bands as Pst+CC/Mse+CCG where a high polymorphism has been obtained. When Genstat 5 package has been used to determine which best of couple of primer would be retained among the four pair of combinations for the screening of *Triticum urartu*, no difference has been found. Each combination of primers could be used for distinguishing the geographic origin like Pst+CC/Mse+CCG or as Pst+AA/Mse+CCG should be used for fingerprinting. The *T. urartu* from the north and south of Syria clustered differently in the PCO plot . This agrees with the previous results obtained with multivariate statistical analysis of agro-morphological quantitative data (GRU AR, 1993). This grouping probably reflects the difference in ecological characteristics of the respective habitats. Our result indicates a high intrapopulational diversity for the populations from Aleppo, which was also found in a previous study based on seed protein polymorphism analysis (GRU Annual report, 1991). The South groups were more dispersed on the PCO plot (fig 4), which reflects a wider genetic variation. AFLP appears to represent an additional molecular polymorphism assay, which can be applied to analysis of genetic diversity and population genetics in wild wheat. The AFLP technique is expected to yield more reproducible results because the polymorphism is based on the presence or absence of band reflecting dominant mutations at the DNA level (Vos et al.1995). Repeatability of AFLP banding patterns was very high; thus showing credibility to the conclusions derived from the analysis. AFLP displayed a high rate of polymorphism compared to that obtained with RAPD (K.Chabane and J.Valkoun,1998). The present study was based on the preliminary set of *T. urartu* accessions that has undergone biochemical or DNA analysis. The number of accessions studied help to have preliminary conclusions about genetic structure of wild wheat *T. urartu* germplasm and to answer different questions such as which/how many markers must be used for genetic study. The high frequency of identifiable AFLP polymorphism's make AFLP DNA analysis an attractive technique for identifying polymorphism for plant germplasm characterization and for determining linkages by analyzing individuals from a segregation population. An AFLP data analysis with AMOVA of natural populations of wild

Triticum species may be a useful tool for the optimization of strategy for the collection and in situ conservation of this important component of wheat gene pool.

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