African Journal of Biotechnology Vol. 5 (22), pp. 2058-2061, 16 November, 2006
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2006 Academic Journals

Full Length Research Paper

RFLP of analyses of an intergenic spacer region of chloroplast DNA in some wild wheat species

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Accepted 18 October, 2006

Several studies are being made to get high productive wheats throughout the world because they provide the most of human energy and protein needs. In this study, 11 wheat species of *Triticum* and *Aegilops* were investigated. One of the intergenic regions of cpDNA was studied. This region was amplified with PCR and digested with 6 restriction endonucleases (*Hpa* II, *Alu* I, *Hinc* II, *Ava* III, *Nde* I and *Hae* III). According to results, amplified products can be divided into the three groups which are Group 1 (~2000 bp), Group 2 (~1000 bp) and Group 3 (~700 bp). Group 1 contains *Ae. neglecta* Req. ex Bertol. (syn.:*Ae. triaristata*), *Ae. triuncialis*, *Ae. mutica* Boiss. and *T. monococcum* L. var. *monococcum*. Group 2 contains *T. aestivum* L., *Ae. biuncialis* Vis., *T. turgidum* L. (Syn.: *T. turgidum* var. *turgidum*) and *T. carthlicum* Nevski. Group 3 contains *Ae. tauschii* Cosson. subsp. *tauschii* and *Ae. cylindrica* Host. Data obtained from the restriction enzyme digestions were evaluated by using numerical taxonomy system and a phenogram was constructed using SAHN clustering unweighted pair group method using arithmetic averages.

Key words: *Aegilops*, cpDNA, intergenic spacer region, RFLP, *Triticum*.

INTRODUCTION

The genetic variation found in the wild progenitors of crops is very important. It is essential to maintain and safeguard germplasm for improving crop production. The value of wild species as a genetic resource for crop improvement depends on the amount of genetic variability they represent relative to cultivated crops. The protein based techniques which include the usage of morphological and biochemical markers are influenced by the environment, but DNA based techniques represent reliable tools and do not have many of the standard problems associated with other techniques.

The chloroplast genome of flowering plants is a single circular molecule mostly within a range of 120-150 kb in length. It is organized into two single-copy regions (large and small) separated by an inverted repeat. Most of the variation of genome size between species is accounted for by variation in the size of the inverted repeat. The entire structure of the chloroplast, along with the gene content, is well conserved among the higher plants, in spite of the loss of inverted repeat in some species and existence of some variations in gene content (Downie and Palmer, 1992). Sequence data show that the rate of nucleotide substitutions of chloroplast genes is conservative relative to plant nuclear genes (Wolf et al., 1987). This conservatism of chloroplast genome allows phylogenetic studies at higher taxonomic levels using both RFLP and sequence comparison (Palmer et al., 1988; Clegg, 1993).

The small amount of diversity observed within cpDNA genomes, relative to nuclear genomes, led some workers to suggest that cpDNA restriction fragment variants could be useful in constructing moleculer phylogenies (Palmer and Zamir 1982; Palmer et al., 1985; Sytsma and Gottlieb, 1986). Restriction enzyme digestion of chloroplast DNA is useful technique for studying inter and intraspecific phylogenetic relationships in plants (Palmer, 1985; Liston, 1992; Amane et al., 2000).

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The genera *Triticum* and *Aegilops* are taxonomically placed in the tribe Triticeae of the Poaceae. They are biologically congeneric and consist of more than 30 species mainly distributed over the Mediterranean region. Since *Aegilops* has the closest relationship with cultivated wheat (*Triticum*), its use for wheat breeding has attracted a wide attention for a long time (Kimber and Feldman, 1987; Wang et al., 2000). One of the features of these genera is the polyploid formation following interspecific hybridization. Polyploid evolution of *Aegilops* species is one of the most investigated subject in plant genetics. cpDNAs from 29 species of *Triticum* and *Aegilops* were compared by the restriction fragment analysis and demonstrated for its usefulness in clarifying interspecific relationship (Ogihara and Tsunewaki, 1982). In another study, they described the classification of chloroplast genomes in almost all *Triticum* and *Aegilops* species and the mode of chloroplast DNA evolution of these species by using RFLP analysis (Ogihara and Tsunewaki, 1982). Dvorak et al. (1988), demonstrated the utility of RFLP in repeated nucleotide sequence in the investigation of the origin of genomes in wheats. Soreng et al. (1990) compared a cladistic analysis of chloroplast DNA restrict-tion sites with current subgeneric classification of Poa-ceae and used chloroplast DNA restriction sites as mar-kers of geographic radiation. In another study, restriction map variation of two cpDNA regions was investigated by using five diploid *Aegilops* and two tetraploid wheat species (Miyashita et al., 1994).

This investigation was carried out in order to evaluate the usefulness of restriction site analysis of a PCR amplified cpDNA product for measuring levels of cpDNA divergence in closely related species of wheat.

MATERIALS AND METHODS

Plant materials

Triticum turgidum L. (Syn.: *T .turgidum* var. *turgidum*), *T. carthlicum* Nevski, *T. aestivum* L., *Aegilops biuncialis* Vis., *Ae. cylindrica* Host., *Ae. tauschii* Cosson. subsp. *tauschii*, *Ae. neglecta* Req. ex Bertol. (syn.:*Ae. triaristata*), *T. monococcum* L. var. monococcum, Ae. mutica Boiss and *Ae. triuncialis* used (Table 1) were supplied by the Institute of Aegean Agricultural Research, Ministry of Agriculture and Rural Affairs, , Izmir, Turkey. *Triticum aestivum* was used as reference.

PCR

Total DNA was isolated from two week old seedlings, after two days etiolation in dark using 1-1.5 g of each individual plant following protocol of Doyle and Doyle (1987).

The region amplified by polymerase chain reaction lies at the end of *rbcL* gene and beginning of *psaI* gene. The primers homologous CCG GCT CGA ACT CGA A-3') and primer SU-1' (5'-CTA AGC CTA CTA AAG GCA CGA -3'). The amplification reaction was performed in 25 µL reaction mix composed of 100 pmol of each primer, 50 µM each dNTP, 2.5 µL 10XPCR buffer (10 mM Tris-HCl pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.8% Nanidet P40), 2.5 unit *Taq* DNA Polymerase (Appligen Deutschland) and 1 µg total DNA. The reaction volume was completed to 25 µL by PCR grade water.

Samples were covered with 100 µL of mineral oil. Amplifications were performed in a Perkin Elmer Cetus DNA Thermal Cycler 2400 programmed for one cycle of 1-2 min at 85ºC, 30 cycles of 50 s at 94°C, 50 s at 64°C, 1.5 min at 72°C and one cycle 7 min at 72°C. After the amplification reactions, the samples were stored at 4°C (or –20°C for long term).

Restriction of PCR products

The amplification products were digested with the following restriction enzymes: *Hpa* I, *Alu* I, *Hinc* II (*Hind* II), *Mph 11031* (*Ava* III), *Nde* I and *BsuR* I (*Hae* III). The restriction reactions were performed in 20 µL total volume: 5 µg amplification product, 2 µL 10X restrict-tion buffer, 5 U restriction enzyme and water. Restriction fragments were separated by electrophoresis in 1.5-2% agarose gels and detected by staining with ethidium bromide.

Analyses of RFLP data

For the statistical analyses of the results NTSYS-pc 1.80 (Rohlf, 1992) were used and a phenogram were constructed by using SAHN clustering UPGMA method, euclidian distance (Sneath and Sokal, 1973).

RESULTS

We obtained three different groups according to the lengths of PCR products. The lengths of these products are ~2000 bp, ~1000 bp, ~700 bp. 1.Group: The group contains 2000 bp: *Ae. neglecta*, *Ae. triuncialis*, *Ae. mutica* and *T. monococcum* var. *monococcum*. 2nd Group: The group contains 1000 bp: Group 2 contains *T. aestivum*, *Ae. biuncialis*, *T. turgidum* and *T. carthlicum.* 3rdGroup: The group contain 700 bp: *Ae. tauschii* subsp. *tauschii* and *Ae. cylindrica.*

With the use of six restriction endonucleases (*Hpa*II, *Hae*III, *Alu*I, *Hinc*II, *Ava*III, and *Nde*I) restriction fragment patterns of cpDNA were obtained from species and subspecies studied (Table 1) and compared with each other. *T. aestivum* was used as a control species in all experiments, because this region was already sequenced by Ogihara et al. (1991). The data were summarized with a phenogram (Figure 1).

DISCUSSION

Restriction enzyme digestion of cpDNA has been used since 1980s to analyse interspecific, phylogenetic relationships in plants. By using this technique, it is possible to construct a physical map and to determine the gene orders on the chromosomes in wheat (Bowman et al., 1983; Miyashita et al., 1994, Mori et al., 1997).

When the data were analysed statistically, a phenogram was constructed. According to this phenogram (Figure 1), it was shown that *Ae. cylindrica* and *Ae. Tauschii* subsp. *tauschii* were clustered together as a separate group. Both contain D genome; *Ae. tauschii* subsp. *tauschii* contains DD and *Ae. Cylindrica* contains CD genomes.

Table 1. Wheat species and six restriction endonucleases which have recognation sites in the studied region of wheat cpDNA.

	*Hpa*II	*Alu*I	*Hinc*II	*Ava*III	*Nde*I	*Hae*III	
T. turgidum turgidum	+	+	+	+	+	+	
T. turgidum var. *carthlicum*	+	+	+	+	+	+	
Ae. cylindrica	+	-	-	-	-	+	
Ae. triaristata	+	+	+	+	-	+	
Ae. squarrosa	+	-	-	-	+	+	
T. turgidum var. *dicoccoides*	+	+	+	+	-	+	
Ae .triuncialis	+	+	+	+	+	+	
Ae. biuncialis	+	+	+	+	+	+	
T. aestivum	+	+	+	+	+	+	
Ae. mutica	+	+	+	+	+	+	
T. monococcum var. *monococcum*	+	+	+	+	+	+	

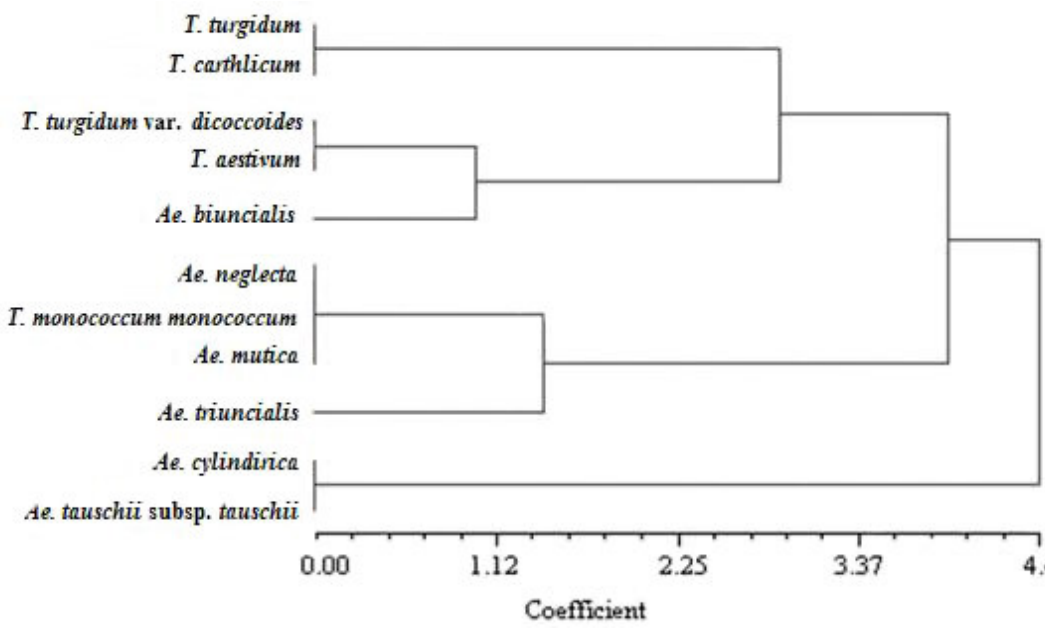

Figure 1. Phenogram of wheat species constructed by UPGMA clustering method, euclidian distance.

Ae. tauschii is considered as the D genome parent of *Ae. cylindrica* (Dubcovsky and Dvorak, 1994). The other species were showed another cluster. In this cluster *Ae. neglecta*, *T. monococcum* var. *monococcum*, *Ae. mutica* and *Ae. triuncialis* having UM, A, M^t, and UC genomes respectively, clustered together. Some taxonomists have argued that, because it has unique morphological characteristics, *Ae. mutica* should be classified as a monotypic genus or subgenus ie, *Amblyopyrum* (Eig, 1929; Hammer, 1980). However, previous molecular and cytological studies did not indicate any evidence of generic or subgeneric divergence between *Ae. mutica* and the rest of the *Aegilops* species (Kihara, 1954). The results of this study showed, *T. turgidum*, *T. carthlicum*, *T aestivum* and *Ae. biuncialis* as a separate cluster. In this group, *T. turgidum*, *T. carthlicum*, *T. turgidum* var. *dicoccoides* have AB genomes while *T aestivum* has ABD. This agrees with the fact that *T. aestivum* originated from hybridization of *T. turgidum* with *Ae. tauchii* (Kihara, 1954; Mcfadden and Sears, 1946). The positions of the species on the tree except *T. monococcum* var. *monococcum* and *T. aestivum*, were compatible with previous studies, but the positions of these two species were controversial. This result may be due to the region studied on.

Recent studies have shown that differences in chloroplast DNA restriction patterns can provide important clues concerning evolutionary relationships among plant species (Gordon et al., 1982; Bowman et al., 1983; Tsunewaki and Ogihara, 1983). In addition, strong infere-

nces can be made concerning interspecific relationships and extend of the intraspecific divergence within wheat cytoplasms (Palmer et al., 1985).

Although a significant amount of information is already available, many aspects of wheat evolution remain unknown or require independent verification (Huang et al., 2002). Our study has shown that RFLP analysis of non-coding regions of chloroplast DNA provides important information for systematic and genetic relationships among these species.

ACKNOWLEDGEMENT

The present work was financed by Turkish Scientific and Technical Research Counsil of Turkey (Project No: TBAG-1689). The authors are grateful to Assoc. Prof. Dr. Khalid Mahmood Khawar, Institute of Biotechnology, University of Ankara, Ankara, Turkey for his valuable contributions and guidance towards the preparation and publication of this paper.

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