Botanical Journal of the Linnean Society, 2005, **148**, 305–310. With 8 figures

PCR-based RFLP analysis of an intergenic spacer region in cpDNA of some wild wheat species

SİBEL ÜNLÜ* and SİBEL SÜMER

Hacettepe University, Faculty of Science, Department of Biology, Molecular Biology Section, 06532 Beytepe, Ankara, Turkey

Received August 2004; accepted for publication November 2004

Wheat is one of the basic food materials for humans and other animals. Continuing studies into breeding new species, which are suitable for agriculture, are based on the contention that the restricting factors are the effects of the nuclear genes. Such focusing of studies on the nuclear genome leads to restriction on the information on organelle DNAs, although cytoplasmic genetic factors are effective on the morphological, physiological and reproductive features of plants. Restriction fragment length polymorphism analysis (RFLP) of chloroplast DNA (cpDNA) has become a powerful tool for studying phylogenetic relationships. In this study, seven wheat species, belonging to *Aegilops* and *Triticum*, were studied. An intergenic spacer region of cpDNA was digested by six different restriction endonuclease enzymes, and fragments so obtained were investigated using the agarose gel electrophoresis technique. Discussion on the phylogenetic relationships among *Triticum* and *Aegilops* species is based on the resultant data.

ADDITIONAL KEYWORDS: *Aegilops* – phylogenetic relationship – *Triticum*.

INTRODUCTION

Wheat is foremost in the planting and harvesting of cultivated plants, which are used for human nutrition. It is the staple food of 35 per cent of the world's population and is grown on 240 million hectares annually (Knott, 1987). During the twentieth century, scientists developed many new kinds of wheat that can produce large quantities of grain able to resist cold, disease, insects, and other threats. As a result, wheat production around the world has risen dramatically.

Wheat, a member of Gramineae (Poaceae) and the tribe Triticeae (= Hordeae) (Briggle & Reitz, 1963), has one to several flowered spikelets that are sessile and alternate on opposite sides of the rachis forming a true spike. Wheats (*Triticum*) and ryes (*Secale*) together with *Aegilops, Agropyron, Eremopyron*, and *Haynaldia* form the subtribe *Triticinae* (Simmonds, 1976).

Linnaeus (1753) first classified wheat. Sakamura (1918) reported the chromosome number sets for each commonly recognized type; this was a turning point in *Triticum* classification because it separated wheat into three groups. Diploids had 14 chromosomes ($n = 7$), tetraploids had 28 ($n = 14$) and hexaploids had 42 ($n = 21$). Bowden (1959) includes *Aegilops* with *Triticum*. The wild diploid species are presumably monophyletic in origin although they have diverged from each other.

The wheats known today are cereals that evolved in the Middle East through repeated hybridizations of *Triticum* spp. with members of a closely related grass genus, *Aegilops*. The process, which began some 10 000 years BP, involved the following major steps. Wild einkorn *T. urartru* crossed spontaneously with *Aegilops speltoides* to produce wild emmer *T. dicoccoides*. Bread wheat finally evolved when cultivated emmer interbred with *A. tauschii* in southern Caspian plains. This evolution was accelerated by an expanding geographical range of cultivation and by human selection, and had produced bread wheats as early as the sixth millennium BC (Simmonds, 1976).

The chloroplast genome (cpDNA) of plants has been a focus of research in plant molecular evolution and systematics (Clegg & Curtis, 1984; Golenberg *et al*., 1993; Morton & Clegg, 1993). Several features of this

*Corresponding author. E-mail: sozsoy@hacettepe.edu.tr

genome have facilitated molecular evolutionary analysis (Clegg *et al*., 1994). It is present in high copy number in the cell, and is thus easy to detect on gels. Conservation of gene content and a relatively slow rate of nucleotide substitution in protein-coding genes have made the chloroplast genome an ideal focus for studies of plant evolutionary history. Despite its conservative nature, as revealed by genome size, restriction fragment analysis, and nucleotide sequencing, a number of mutations in the chloroplast genome has been observed. These include inversions (Howe *et al*., 1988; Hiratsuka *et al*., 1989), rearrangements of gene order (Ogihara, Terachi & Sasakuma, 1988), and insertions/deletions (Palmer, 1991), as well as base substitutions.

The analysis of restriction fragment patterns of cpDNA is most useful for population samples or for the study of evolutionary relationships among closely related species (Clegg & Curtis, 1984). Most studies of chloroplast, however, have concentrated on coding regions. As a result, a great deal is known about evolution of chloroplast genes and genome structure but little about the evolution of noncoding sequences of the chloroplast genome (Morton & Clegg, 1993).

In this study, an intergenic region of seven *Triticum* and *Aegilops* species was investigated. This is a hypervariable region and these species harbour different types of length mutations at this position. The purpose of the investigation was to estimate the evolutionary divergence between these species.

MATERIAL AND METHODS

Aegilops and *Triticum* species (*A. tauschii*, *A. speltoides* var. *ligustica*, *T. monococcum* var. *boeoticum*, *T. urartu*, *T. dicoccoides*, *T. turgidum* var. *dicoccon*, *T. aestivum*) used in this study were obtained from a field study in the south-eastern Anatolian region and from ICARDA. Total DNA was isolated following the procedure of Doyle & Doyle (1987) from fresh leaf materials that were frozen by liquid nitrogen. The collected DNA was used directly for PCR amplification. The amplification reaction was carried out with primer SU-3 (TTCGAGTTCGAGCCGGTAGATA), located at the 1236 position of the *rbcL* gene, and primer SU-1′ (CTAAGCCTACTAAAGGCACGA), located at 3274 position of the *psaI* gene. Each PCR was performed in a 100 µL reaction mixture containing 1 µg of total DNA extract, 10 µL of 10XPCR buffer (100 mM Tris-HCl, pH 8.8, 500 mM KCl, %0.8 Nanidet P40) supplied with the enzyme, 50 µM each of dNTPs, 2 mM $MgCl_2$, 100 pmol of each primer, and 2.5 units of Taq DNA Polymerase. The reaction volume was made up to 100 µL using PCR grade water. Thermal cycling was carried out in a Biometra thermal cycler. The reaction was 30 cycles of 50 s at 94 °C, 50 s at 64 °C, and 90 s at 72 °C, and a final elongation cycle of 7 min at 72 °C. PCR products were fractionated in a 1% agarose gel and samples were stored at –20 °C. The amplification products were digested with *Hpa*II, *Alu*I, *Hinc*II, *Ava*III, *Nde*I, and *Hae*III enzymes. Digestion reactions were performed in a 20 µL reaction mix containing 5 µg of amplification product, 2 µL of 10× restriction buffer, 5 U of restriction enzyme and water. Resultant fragments were separated in 1.5% agarose gels. Gels, which were screened by SYNGENE, Gene Genius image analyser system, were analysed using Cross Checker Fingerprint analysis software v.2.9 (Buntjer, 1999). Based on resultant data, phenograms were plotted using NTSYSpc v.2.10 (Rohlf, 2000).

RESULTS AND DISCUSSION

The region of chloroplast genome bounded by *rbcL* and *psaI* genes was amplified by PCR reaction. Results are given in Figure 1.

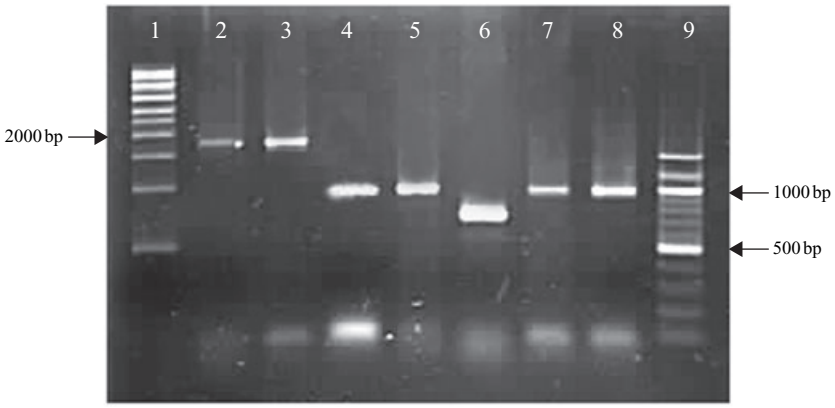

Figure 1. The PCR results. 1, 1-kbp DNA ladder; 2, *Triticum monococcum* var. *boeticum*; 3, *T. urartu*; 4, *T. dicoccoides*; 5, *T. turgidum* var. *dicoccon*; 6, *Aegilops tauschii*; 7, *A. speltoides* var. *ligustica*; 8, *T. aestivum*; 9, 100-bp DNA ladder.

PCR products were digested using six restriction endonucleases. In all experiments *T. aestivum* was used as a control group, because, in a previous study, Ogihara, Terachi & Sasakuma (1991) achieved this region's sequence analysis. The first enzyme used for digestion was *Hpa*II. Nine bands (H1–H9) were generated at the end of the digestion (Fig. 2). *Triticum dicoccoides*, *T. turgidum* var. *dicoccon*, *A. speltoides* var. *ligustica*, and *T. aestivum* showed the same banding pattern (H5, H8). *Triticum monococcum* var. *boeoticum* showed three bands (H3, H6, H7). In the banding pattern of *A. tauschii*, there was only one band (H4). The other fragment was not big enough to detect on agarose gel. *T. urartu* showed three bands (H1, H2, H9).

The second enzyme used for digestion was *Alu*I. Ten bands (A1–A10) were generated by digestion (Fig. 3). According to Ogihara *et al.* (1991) there is one recognition site for this region in *T. aestivum*. *Triticum dicoccoides*, *A. speltoides* var. *ligustica*, and *T. aestivum* showed the same banding pattern (A6), that represents two fragments of almost equal size. *Triticum monococcum* var. *boeoticum* showed three bands (A1, A4, A5). *Aegilops tauschii* showed a different banding pattern (A9, A10). *Triticum urartu* and *T. turgidum* var. *dicoccon* showed a common band with *A. tauschii* (A10). *T. urartu* showed three other bands (A3, A7, A8), and *T. turgidum* showed another band (A2).

The third enzyme was *Hin*cII (*Hin*dII). Six bands (Hi1–Hi6) were generated by digestion (Fig. 4). There is only one recognition site of *Hin*cII for this region in *T. aestivum* as shown by Ogihara *et al*. (1991). *Triticum aestivum*, *A. speltoides* var. *ligustica*, *T. turgidum* var. *dicoccon* and *T. dicoccoides* showed the same banding pattern (Hi4, Hi5). *Aegilops tauschii* shares a fragment with these species (Hi4), so there is no recognition site for this enzyme. *T. monococcum* var. *boeoticum* showed two bands (Hi2, Hi3). *T. urartu*, also, showed two bands (Hi1, Hi6).

The fourth enzyme used for digestion was *Nde*I. Seven bands (N1–N7) were generated by digestion (Fig. 5). There is no recognition site for this region in

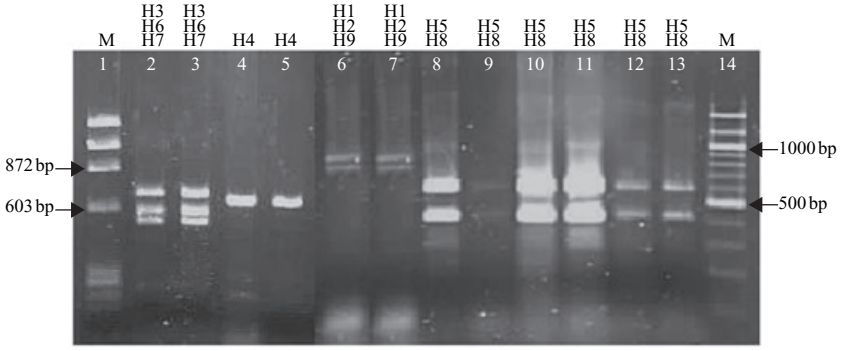

Figure 2. *Hpa*II digestion. 1, ØX 174/*Hae*III; 2, 3, *Triticum monococcum*, 4, 5, *Aegilops tauschii*; 6, 7, *T. urartu*; 8, 9, *T. dicoccoides*; 10, 11, *T. turgidum* var. *dicoccon*; 12, *A. speltoides* var. *ligustica*; 13, *T. aestivum*; 14, 100-bp DNA ladder.

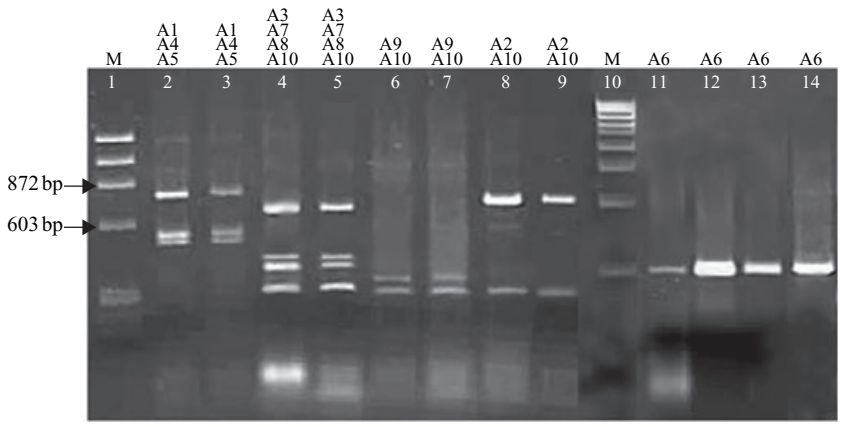

Figure 3. *Alu*I digestion. 1, ØX174/*Hae*III; 2, 3, *Triticum monococcum* var. *boeoticum*; 4, 5, *T. urartu*; 6, 7, *Aegilops tauschii*; 8, 9, *T. turgidum* var. *dicoccon*; 10, 1-kbp DNA ladder; 11, 12, *T. dicoccoides*; 13, *T. aestivum*; 14, *A. speltoides* var. *ligustica*.

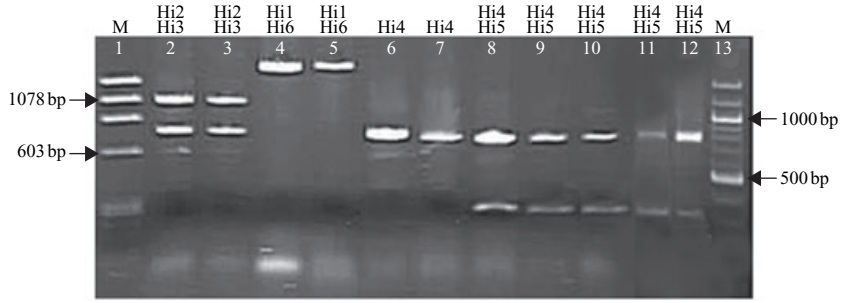

Figure 4. *Hinc*II (*Hind*II) digestion. 1, ØX174/*Hae*III; 2, 3, *Triticum monococcum* var. *boeoticum*; 4, 5, *T. urartu*; 6, 7, *Aegilops tauschii*; 8, 9, *T. turgidum* var. *dicoccon*; 10, *A. speltoides* var. *ligustica*; 11, *T. dicoccoides*; 12, *T. aestivum*; 13, 1-kbp DNA ladder.

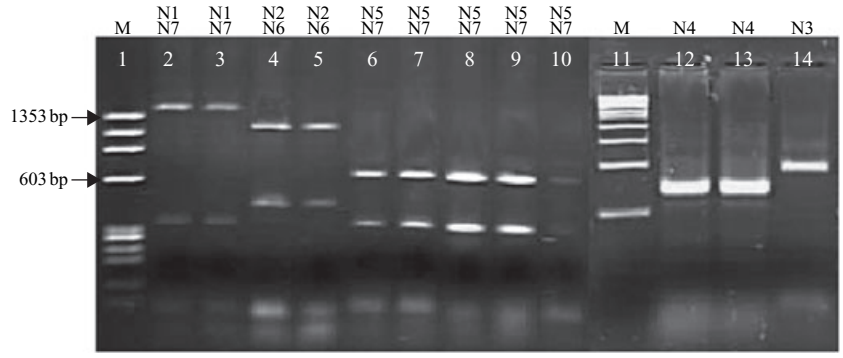

Figure 5. *Nde*I digestion. 1, ØX174/*Hae*III; 2, 3, *Triticum monococcum* var. *boeoticum*; 4, 5, *T. urartu*; 6, 7, *T. dicoccoides*; 8, 9, *T. turgidum* var. *dicoccon*; 10, *A. speltoides* var. *ligustica*; 11, 1-kbp DNA ladder; 12, 13, *A. tauschii*; 14, *T. aestivum*.

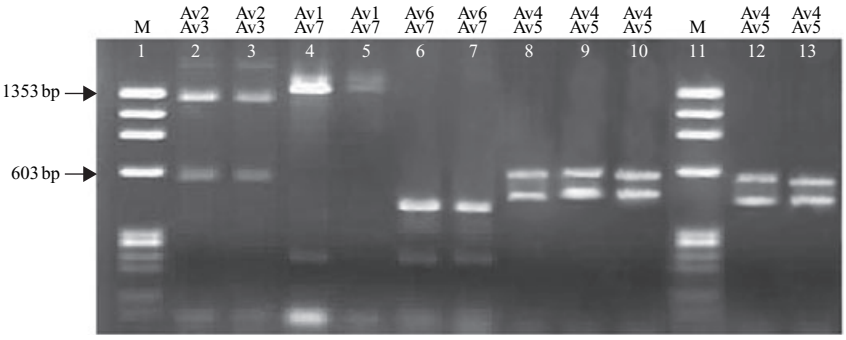

Figure 6. *Ava*III digestion. 1, ØX174/*Hae*III; 2, 3, *Triticum monococcum* var. *boeoticum*; 4, 5, *T. urartu*; 6, 7, *Aegilops tauschii*; 8, 9, *T. turgidum* var. *dicoccon*; 10, *A. speltoides* var. *ligustica*; 11, 1-kbp DNA ladder; 12, *T. dicoccoides*; 13, *T. aestivum*.

T. aestivum (N3) (Ogihara *et al*., 1991). *Aegilops tauschii* also has no recognition site for this region (N4). *Triticum diccoccoides*, *T. turgidum* var. *dicoccon*, and *A. speltoides* var. *ligustica* showed the same banding pattern (N5, N7). *Triticum monococcum boeoticum* showed one common band with these species and another band (N1, N7). *Triticum urartu* also showed two bands (N2, N6).

The fifth enzyme used for digestion was *Ava*III. Seven bands (Av1–Av7) were generated by digestion (Fig. 6). There is only one recognition site of *Ava*III for this region in *T. aestivum* (Ogihara *et al*., 1991). *Triticum aestivum*, *T. dicoccoides*, *T. turgidum* var. *dicoccon*, and *A. speltoides* var. *ligustica* showed the same banding pattern (Av4, Av5). *Triticum monococcum* var. *boeoticum*, *A. tauschii*, and *T. urartu* all showed two

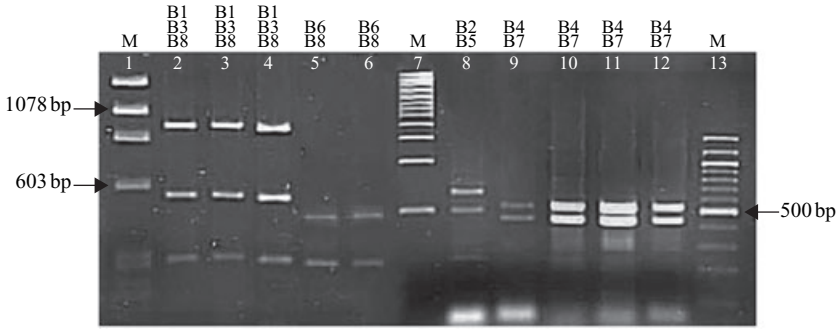

Figure 7. *Bsu*RI digestion. 1, ØX174/*Hae*III; 2, 3, *Triticum monococcum* var. *boeoticum*; 4, *T. urartu*; 5, 6, *Aegilops tauschii*; 7, 1-kbp DNA ladder; 8, *A. speltoides* var. *ligustica*; 9, *T. dicoccoides*; 10, 11, *T. turgidum* var. *dicoccon*; 12, *T. aestivum*; 13, 100-bp DNA ladder.

bands (Av2, Av3 for *T. monococcum* var. *boeoticum*, Av6, Av7 for *A. tauschii* and Av1, Av7 for *T. urartu*).

The last enzyme used for digestion was *Bsu*RI (*Hae*III). Eight bands (B1-B8) were generated by digestion (Fig. 7). There is one recognition site of *Bsu*RI for this region in *T. aestivum* as shown by Ogihara *et al*. (1991). *Triticum aestivum*, *T. turgidum* var. *dicoccon*, and *T. dicoccoides* showed the same banding pattern (B4, B7). *Triticum monococcum* var. *boeoticum* and *T. urartu* also showed the same banding pattern (B1, B3, B8). *Aegilops tauschii* showed one common band with these species (B8) and another band (B6). In the banding pattern of *A. speltoides* var. *ligustica* there were two bands (B2, B5).

According to data obtained from these results, a statistical analysis was performed using NTSYSpc v.2.10. A phenogram was constructed using the UPGMA method (Fig. 8).

According to the phenogram (Fig. 8) *T. urartu* (AA genome) clustered as a separate group from the other species. *Triticum monococcum* var. *boeoticum* (A^mA^m genome) formed a group, which was connected to the cluster formed by other species. *T. monococcum* is believed to be the cultivated form of *T. boeoticum,* not of *T. urartu* (Dvorak, McGuire & Cassidy, 1988; Takumi *et al*., 1993). In another study performed by Bowman, Bonnard & Dyer (1983), cytoplasmic DNA analysis showed that *T. monococcum* and *T. urartu* belong to different cytoplasm types. *T. dicoccoides* and *T. turgidum* have AABB genome. They were clustered together and they were connected to the cluster formed by *T. aestivum* (AABBDD genome). They all together formed a cluster and connected to *A. speltoides* var. *ligustica* (SS genome). *Aegilops speltoides* shows a closer relationship to tetraploid and hexaploid wheat species (Ishii, Mori & Ogihara, 2001). This is in agreement with the fact that *A. speltoides* was the possible cytoplasmic donor of these wheat species (Ogihara & Tsunewaki, 1988; Tsunewaki, 1996). This cluster was grouped with *A. tauschii* (DD

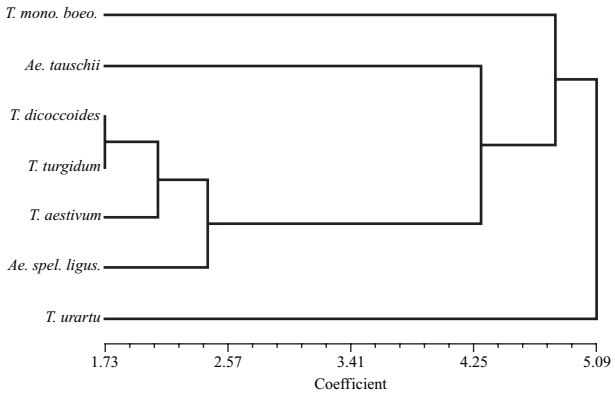

Figure 8. Phenogram constructed using the UPGMA method.

genome) and they all together grouped with *T. monococcum* var. *boeoticum*. In another study, the cytoplasm of *A. tauschii* was found to be unique (Bowman *et al*., 1983). This finding supports our results.

Many studies have shown that differences in chloroplast DNA restriction patterns can provide important clues concerning evolutionary relationships among plant species (Timothy *et al*., 1979; Gordon *et al*., 1982; Kung, Zhu & Shen, 1982; Bowman *et al*., 1983; Tsunewaki & Ogihara, 1983). In addition, strong inferences can be made concerning interspecific relationships and extend of the intraspecific divergence within wheat cytoplasm (Palmer, Jorgensen & Thompson, 1985).

Numerous phylogenetic studies have been based on chloroplast DNA (cpDNA) sequences. However, the mutation rate of conserved genes are generally insufficient for defining relationships at low taxonomic levels. Noncoding regions of cpDNA are more rapidly evolving, thus exhibiting a higher rate of informative sites such as insertion/deletion (indels) (Gielly & Taberlet, 1994). The major advantage of these regions is that they show sufficient variation and short enough

to allow for rapid sequencing of numerous taxa at the intrafamily level.

Our study has shown that RFLP analysis of noncoding regions of chloroplast DNA provides important information for systematic and genetic relationships among these species.

REFERENCES

Bowden WM. 1959. The taxonomy and nomenclature of the wheats, barleys, and ryes and their wild relatives. *Canadian Journal of Botany* **37:** 130–136.

Bowman CM, Bonnard G, Dyer TA. 1983. Chloroplast DNA variation between species of *Triticum* and *Aegilops*: location of the variation on the chloroplast genome and its relevance to the inheritance and classification of the cytoplasm. *Theoretical and Applied Genetics* **65:** 247–262.

Briggle LW, Reitz LP. 1963. Classification of *Triticum* species and of wheat varieties grown in the United States. *U.S. Department of Agriculture Bulletin* 1278.

Buntjer JB. 1999. *Cross Checker v.2.91*. Department of Plant Breeding. Wageningen University and Research Center, The Netherlands.

Clegg MT, Curtis SE. 1984. Molecular evolution of chloroplast DNA sequences. *Molecular Biology and Evolution* **1**(4)**:** 291–301.

Clegg MT, Gaut BS, Learn JRGH, Morton B. 1994. Rates and patterns of chloroplast DNA evolution. *Proceedings of the National Academy of Sciences, USA* **91:** 6795–6801.

Doyle JT, Doyle JL. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* **19**(1)**:** 11–15.

Dvorak J, McGuire PE, Cassidy B. 1988. Apparent sources of the A genomes of wheats inferred from polymorphism in abundance and restriction fragment length of repeated nucleotide sequences. *Genome* **30:** 680–689.

Gielly L, Taberlet P. 1994. The use of chloroplast DNA to resolve plant phylogenies: noncoding versus rbcL sequences. *Molecular Biology and Evolution* **11:** 769–777.

Golenberg EM, Clegg MT, Durbin ML, Doebley J, Ma DP. 1993. Evolution of a noncoding region of the chloroplast genome. *Molecular Phylogenetics and Evolution* **2:** 52–64.

Gordon KH, Crouse EJ, Bohnert HJ, Herrman RG. 1982. Physical mapping of differences in chloroplast DNA of the five wild-type plastosomes in *Oenothera* subsection *Euoenthera*. *Theoretical and Applied Genetics* **61:** 373–384.

Hiratsuka J, Shimada H, Whittier R, Ishibashi T, Sakamoto M, Mori M, Kondo C, Honji Y, Sun CR, Meng BY, Li YQ, Kanno A, Nishizawa Y, Hirai A, Shinozaki K, Sugiura M. 1989. The complete sequence of the rice (*Oryza sativa*) chloroplast genome: intermolecular recombination between distinct tRNA genes accounts for a major plastid DNA inversion during the evolution of the cereals. *Molecular and General Genetics* **217:** 185–194.

Howe CJ, Barker RF, Bowman CM, Dyer TA. 1988. Common features of three inversions in wheat chloroplast DNA. *Current Genetics* **13:** 343–349.

Ishii T, Mori N, Ogihara Y. 2001. Evaluation of allelic diversity at chloroplast microsatellite loci among common wheat and its ancestral species. *Theoretical and Applied Genetics* **103:** 896–904.

Knott R. 1987. Novel approaches to wheat breeding. In: Smith E, ed. *Genetic improvement in yield of wheat.* CSSA Publication 13. Madison, WI: Crop Science Society of America, 25–40.

Kung SD, Zhu YS, Shen GF. 1982. *Nicotiana* chloroplast genome. III. Chloroplast DNA evolution. *Theoretical and Applied Genetics* **61:** 73–79.

Morton BR, Clegg MT. 1993. A chloroplast DNA mutational hotspot and gene conversion in a noncoding region near *rbcL* in the grass family (Poaceae). *Current Genetics* **24:** 357–365.

Ogihara Y, Tsunewaki K. 1988. Diversity and evolution of chloroplast DNA in *Triticum* and *Aegilops* as revealed by restriction fragment analysis. *Theoretical and Applied Genetics* **76:** 321–332.

Ogihara Y, Terachi T, Sasakuma T. 1988. Intramolecular recombination of chloroplast genome mediated by a short direct-repeat sequence in wheat species. *Proceedings of the National Academy of Sciences, USA* **85:** 8573–8577.

Ogihara Y, Terachi T, Sasakuma T. 1991. Molecular analysis of the hot spot region related to length mutations in wheat chloroplast DNAs. I. Nucleotide divergence of genes and intergenic spacer regions located in the hot spot region. *Genetics* **129:** 873–884.

Palmer JD. 1991. Plastid chromosomes: structure and evolution. In: Bogorad L, Vasil IK, eds. *The molecular biology of plastids*. San Diego: Academic Press, 55–53.

Palmer JD, Jorgensen RA, Thompson WF. 1985. Chloroplast DNA variation and evolution in *Pisum*: patterns of change and phylogenetic analysis. *Genetics* **109:** 195–213.

Rohlf FJ. 2000. *NTSYSpc v.2.10*. New York: Applied Biostatistics Inc/Exeter Software.

Sakamura T. 1918. Kruze Mitteilung über die Chromosomenzahlen und die Verwandschaftsverhaltnisse der *Triticum*-Arten. *Botanical Magazine, Tokyo* **32:** 151–154.

Simmonds NW. 1976. *Evolution of crop plants*. London: Longman, 49.

Takumi S, Nasuda S, Liu YG, Tsunewaki K. 1993. Wheat phylogeny determined by RFLP analysis of nuclear DNA. 1. Einkorn wheat. *Japanese Journal of Genetics* **68:** 73–79.

Timothy DH, Levings CS III, Pring DR, Conde MF, Kermicle JL. 1979. Organelle DNA variation and systematic relationships in the genus *Zea*: Teosinte. *Proceedings of the National Academy of Sciences, USA* **6:** 4220–4224.

Tsunewaki K. 1996. Plasmon analysis as the counterpart of genome analysis. In: Jauhar PP, ed. *Methods of genome analysis in plants*. New York: CRC Press, 271–299.

Tsunewaki K, Ogihara Y. 1983. The molecular basis of genetic diversity among cytoplasms of *Triticum* and *Aegilops* species. II. On the origin of polyploidy wheat cytoplasms as suggested by chloroplast DNA restriction fragment patterns. *Genetics* **104:** 155–171.