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Estimates of genetic similarities and DNA fingerprinting of wheats (*Tritium* species) and triticale cultivars using molecular markers

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ABSTRACT

An experiment was conducted during 1999–2000 to study the genetic similarities and DNA fingerprinting of bread wheat (*Triticum aestivum* L. emend. Fiori & Paol.), durum wheat (*Triticum durum* Desf.) and triticale cultivars using molecular markers). In this study 14 varieties of bread wheat, 1 of durum and 1 of *triticale* released for general cultivation since 1920, were analysed using amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD) and simple sequence repeat (SSR) markers. Since these varieties represent the spectrum of germplasm present in breeders' working crossing blocks, the objective of this study was to establish the magnitude of genetic diversity and to fingerprint the individual lines for intellectual property protection. Total number of assay units were 31 random primers for RAPDs, 6 primer combinations for AFLPs and 25 primer pairs for SSR markers. Highest number of polymorphic bands per assay unit was observed for AFLPs (39.7), followed by RAPDs (12.7) and SSRs (4.0) but polymorphic information content (PIC) range was highest for SSRs (0.13–0.86). Based on estimation of genetic similarities, more realistic dendrogram was obtained from SSR compared to that obtained from AFLP or RAPD data. Most of the cultivars could be uniquely identified (fingerprinted) with SSR and RAPD markers but not with AFLPs.

Key words: *Triticum aestivum*, *T. durum*, Triticale, genetic similarity, DNA fingerprinting, AFLP, RAPD, SSR

The information concerning diversity and relationship among germplasm lines can be obtained from morphological traits, pedigree data or DNA-based variation. The DNA-based markers are more powerful in assessing the genetic similarity (Paterson *et al.* 1992, Lee 1995) and for genetic fingerprinting (Smith and Smith 1992) among germplasm lines. Restriction fragment length polymorphic (RFLP) markers were initially used for estimating genetic distance and fingerprinting in wheat (McGuire and Qualset 1997) and other crops (Lee 1995). However, low level of polymorphism present in RFLPs in wheat has restricted the use of this technique.

The PCR-based markers like random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP^{TM}) and simple sequence repeats (SSRs- also termed as microsatellites) are simpler and cheaper compared to RFLPs. These are being routinely used for fingerprinting and estimation of genetic variability in several crops like rice (*Oryza sativa* L.) (Mackill *et al.* 1996, Garland *et al.* 1996), maize (*Zea mays* L.) (Pejic *et al.* 1998), soybean [*Glycine max* (L.) Merr.] (Powell *et al.* 1996); barley (*Hardeum vulgare* L.s. l.) (Russel *et al.* 1997), sorghum (*Sorghum bicolor.* (L.) Moench) (Bohn *et al.* 1999) and wheat (*Triticum aestivum* L. emend. Fiori & Paol.) (Dje *et al.* 2000).

At Punjab Agricultural University, several varieties have been developed since 1920 involving diverse germplasm like-land races, and spring and winter wheats introduced from other countries. Working crossing blocks of wheat breeding programmes have the germplasm composition matching the history of wheat breeding at the PAU. Therefore, estimates of genetic similarity or distances in a set of released varieties may be a reflection of the magnitude of genetic diversity available in breeders' crossing blocks. This can act as a guide to wheat breeders, as how to utilize the old wheat varieties for further improving wheat productivity. Also with the present intellectual property rights (IPR) scenario, it becomes imperative to fingerprint the released varieties for safeguarding the interests of public institutes. The present study was therefore aimed at (i) estimating the magnitude of genetic variability in a set of wheat and triticale cultivars released since 1920 and (ii) fingerprinting of these varieties. Relative efficiency of these marker systems for studying genetic variability and

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fingerprinting was also studied.

MATERIALS AND METHODS

Plant material

A set of 16 varieties of bread wheat, durum wheat and triticale released in Punjab for general cultivation since 1919 were used. These varieties along with their parentage are presented in Table 1. Out of these 16 varieties, a set of 11 varieties was analysed using all the 3 marker systems, RAPD, AFLP, and SSR. The complete set of 16 lines was analysed using SSR primers.

DNA isolation

DNA was isolated from young leaves of field-grown plants at maximum tillering stage. A small quantity of DNA was isolated from each line using CTAB method as described by Murray *et al.* (1992).

SSR assay

Sequence tagged microsatellite (STMS) primers, developed and mapped by Roder *et at.* (1998) were used in this study. A set of 25 primer pairs, mapping throughout the genome, was selected. Polymerase chain reaction (PCR) was performed in 25µl reaction mixture as described by Roder *et al.* (1998) with little modifications. The initial denaturation was carried at 94°C for 3 min, followed by 30 cycles at 94°C for 1 min, annealing temperature 50–60°C for 1 min, extension at 72°C for 2 min and final extension at 72°C for 15 min. The amplification products were resolved on 3% high-resolution gel, prepared by mixing agarose and metaphore in equalproportions, in 0.5 X TBE buffer. These gels were routinely reused for 5–6 times.

Table 1 Pedigree of wheat and triticale lines used in the study

Cultivar	Pedigree	Year of release
Bread wheat		
'8 A'	Local selection	1919
'C 591'	Type 9/8B	1934
'C 273'	C 591/C 209	1957
'C 306'	RGN/CSK3/3*C591/3C217/N14/ C281	1965
'Kalyansona'	PJ'S'/GB55	1967
'Sonalika'	MIDA-U/K117A/2*TH/3/FN/ 4*TH/4/AN/5/YT54/N10B//LR/6/ B4946-A4-18-1/Y53//3*Y50	1967
'HD 2009'	LR64A/NA160	1974
'WL 711'	S309/CHR/KAL	1979
'HD 2329'	SL'S'/NP852/4/PJ'S'/P14/KT54B/3/ K65/5/SKA/6/UP262	1985
'PBW 138'	WG337'S'/HD2177	1986
'PBW 175'	HD2160/4/JN/GAGE//JN/KAL/ 3PV18/C273	1989
'CPAN 3004'	GLL/AUST.II 61–157//CNO/NO/ 3/VEE	1991
'WH 542'	JUP/BJY'S'//URES	1992
'PBW 343'	ND.VG9144//KAL/BB/3/YACO'S'/ 4/VEE'S'	1995
Durum wheat		
'PBW 34'	AA'S'/FGO'S'	1985
Triticale 'TL 1210'	CINNAMON/RAJ821//INIA/ TURKY60/3/ARM'S'	1986

RAPD assay

A set of 31 random primers was used for amplification. PCR amplification as described by Willian *et al.* (1990) was performed in a 25µl reaction mixture containing 3.9µl of 10 X PCR buffer, 2.3µl of 1mM dNTPs, 1.5µl primer, 0.8µl (IU µl) Ta polymerase, 1.2µl of DNA (30 ng/µl). The initial denaturation was carried out at 95°C for 3 min followed by 10 cycles of denaturation at 95°C for 30 sec, annealing of primers at 35°C for 2 min and primer extension at 72°C for 1 min 30 sec. PCR was further continued for another 39 cycles, following denaturation at 92°C for 30 sec, annealing at 40°C for 30 sec, primer extension at 72°C for 30 sec and final extension was carried at 72°C for 5 min.

AFLP analysis

AFLPs were generated using AFLP analysis system I following manufacturer's instructions and as described by Vos *et al.* (1995) using EcoRI/MseI enzyme combination. Selective amplication was performed using 6 primer combinations. The PCR was perfomed using aliquots of the pre-amplified fragments diluted 50 fold, using ^{32}P labelled *Eco*RI+3 primers and unlabelled *Mse*I+3 primers (AT/CTT, AT/CAA, AG/CAC, AC/CAC, AT/CTG, AG/CAG). Amplifications was carried out using 1 cycle at 94°C for 30 sec, 65°C for 30 sec and 72°C for 1 min, followed by lowering the annealing temperature, each cycle by 0.7°C for 12 cycles. Further amplication was carried out for 23 cycles at 94°C for 30 sec, 56°C for 30 sec, 72°C for 1 min. The PCR products were separated on 6% polyacrylamide denaturing gel contaning 7.5 M urea. Autoradiographs were taken by exposing BioMax MR-2 film to the dried gel overnight with intensifying screens.

Data analysis

Scoring of gels : Gels were scored in a binary format with the presence of bands scored as unity and its absence scored as zero. The binary data were used for computing similarity matrices and generation of dendrograms.

Estimation of polymorphic information content : Allelic polymorphic information content (PIC) was calculated using formula :

$$PIC = 1 - \Sigma (P_i)^2$$

where P_i is the proportion of the population carrying ith allele, calculated for each locus (Botstein *et al.* 1980).

Estimation of genetic similarity : Genetic similarity (GS) was calculated for each marker system as per the formula given by Nei and Li (1979).

$$G_{sij} = 2N_{ij}/(N_i + N_j)$$

where N_{ij} is the number of bands in common between lines i and j and N_i and N_j are total number of bands in lines i and j respectively. Thus G_{sij} reflects proportion of bands common between 2 lines and may vary from 0 (no common band) to 1 (identical profile of 2 lines). Bands that were monomorphic across the entire set of genotypes were omitted from the analysis. A similarity matrix for the whole set of genotypes was thus obtained.

Cluster Anlaysis : The similarity matrices obtained using Nei and Li (1979) formula were used for cluster analysis, using unweighted pair group method with arithmetic averages (UPGMA) algorithm on NTSYS-PC version 2.02. Dendrograms were obtained using graphics module of NTSYS. Cophenetic correlation coefficients for each cluster were also estimated using MXCOMP module of NTSYS.

RESULTS AND DISCUSSION

Potential applicatitons of molecular markers in plant breeding are (*i*) assesing the genetic similarity among parents and (*ii*) fingerpinting of genotypes for variety indentification and protection. Several marker systems like AFLPs, RAPDs and SSRs are being used routinely for estimating magnitude of genetic variability and fingerprinting. Of these, RFLPs are based on southern hybridization and are time consuming and expensive. We used PCR-based marker system, viz RAPDs, AFLPs and SSRs, for assessing genetic variability and fingerprinting of wheat varieties released and recommended for general cultivation by the PAU. Here we report overall genetic variability in wheat varieties released since 1920 and specific fingerprints that could be used for their identification and protection.

Comparison of marker systems

Marker systems, viz RAPDs, AFLPs and SSRs were used for studying variability in 11wheat varieties. The SSRs, however, were used for studying variability in 16 varieties that included pre-green-revolution releases as well. These marker system were compared (Table 2). The total number of assay units used were 31 random primers (10 mers) for RAPDs 6 primer combinations for AFLPs and 25 primer pairs for SSRs. Total polymophic bands observed were 393 for RAPDs, 238 for AFLPs and only 100 for SSRs. The polymorphic bands per assay unit were 4 for SSRs, 12.7 for RAPDs and 39.7 for AFLPs. The AFLPs thus generated highest number of polymorphic bands per primer combination.

The average PIC values were also highest for AFLPs (0.97) compared with RAPDs (0.87) and for SSRs (0.53). However, the range was wider for SSRs, being 0.13 to 0.86 compared to a very narrow range for AFLPs (0.96–0.99) and RAPDs (0.81–0.98). This highest PIC value for AFLPs is attributable to large numebr of loci detected per AFLP primer combination. The highest PIC value for AFLPs and lowest for SSRs is in agreement with results reported for rice (Mackil *et al.* 1996), soybean (Powel *et al.* 1996), barley (Russell *et al.* 1997), maize (Pejic *et al.* 1998) and wheat (Bohn *et al.* 1999).

Estimates of genetic similarities

Genetic similarities estimates obtained from Nei and Li (1979) formula were used for generating dendrograms based on UPGMA. Dendrograms generated based on binary data obtained from RAPD, AFLP and SSRs are presented in Fig 1. The cophenetic correlation coefficients provided for each marker system indicate the extent to which the clustering of genotypes depicted in the trees accurately represents the estimates of genetic similarity between the lines obtained with that marker system. Overall cophenetic coefficients were higher, being 0.91 for RAPDs, 0.98 for AFLPs and 0.90 for SSRs (Fig 1) The dendrograms generated revealed that these marker systems were able to differentiate all the

Table 2 Variation in banding patterns generated by RAPDs, AFLPs and SSRs for wheat and triticale cultivars

Marker system	No. of assay units	No. of polymorphic bands	Polymorphic bands per assy unit	PIC[a] value
RAPDs	31	393	12.7	0.87 (0.81–0.98)
AFLPs	06	238	39.7	0.97 (0.96–0.99)
SSRs	25	100	4.0	0.53 (0.13–0.86)

[a]Figures in parentheses represent the range in PIC values obtained for different marker systems

Table 3 The STMS primers that can fingerprint wheat varieties uniquely

Wheat variety	Distinguishing primer
'C 951'	gwm136 (278)[a]
'C 306'	gwm382 (86, 184 108), gwm 136 (278)
'Kalyansona'	gwm292 (214)
'HD 2009'	gwm136 (278)
'WL 711'	gwm617 (154/133), gwm 292 (214)
'PBW 175'	gwm140 (223)
'CPAN 3004'	gwm251 (110)
'WH 542'	gwm340 (159)
'PBW 34'	gwm335 (203), gwm 382 (86, 184, 103), gwm136 (278), gwm140 (223), gwm340 (159)
'TL 1210'	gwm639 (141, 166, 130), gwm121 (107/141), gwm369 (184), gwm60 (190), gwm617 (154, 133)

[a]Figures in parentheses are the approximate molecular weights of the variety specific bands

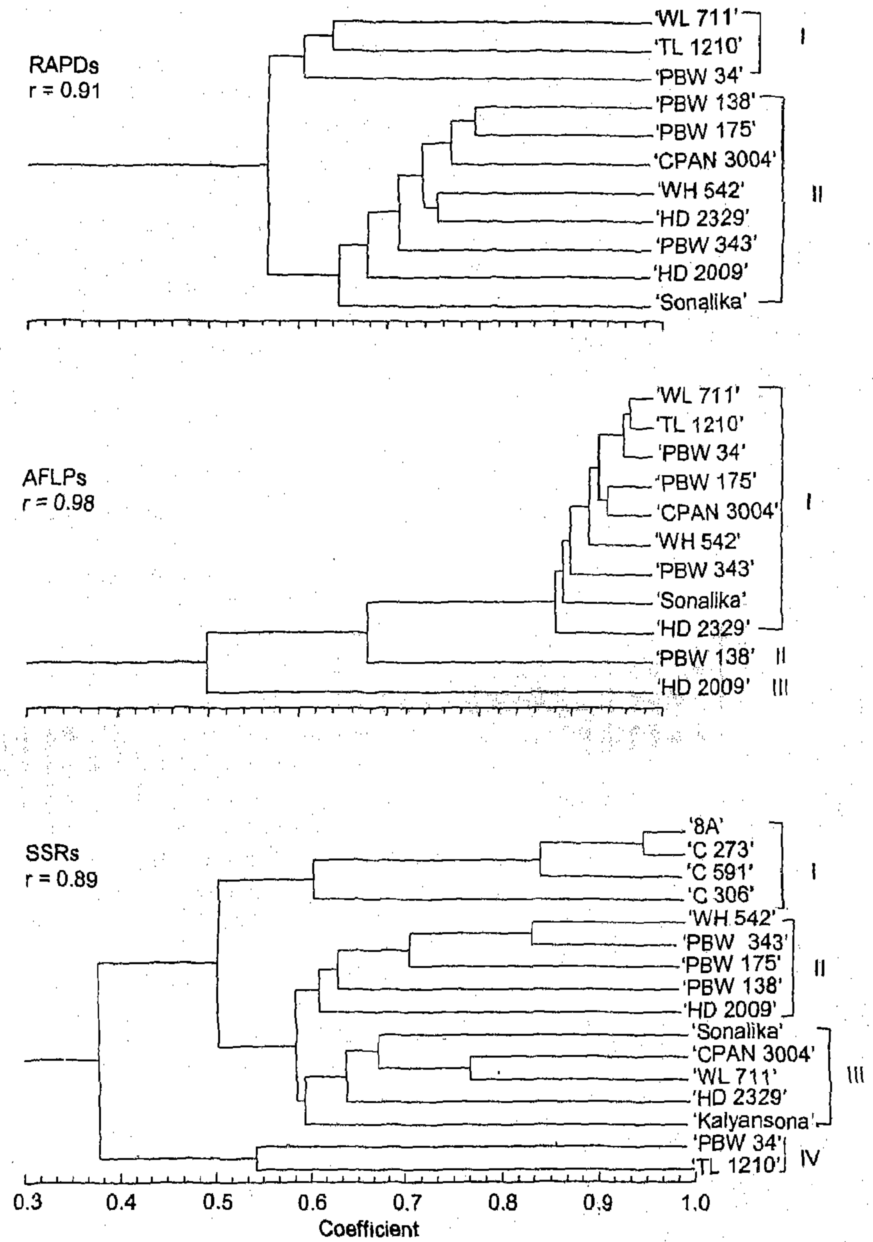

Fig 1 Dendrograms of wheat and triticale varieties generated, using RAPD, AFLP and SSR marker systems. Scale at the bottom is genetic distances from Nei's coefficient of similarities

lines. However, the 3 marker systems showed some discrepancies in clustering these genotypes (Fig 1). For example, 'TL 1210' and 'WL 711' were grouped in the same cluster when analysed using RAPD and AFLP markers but were grouped in different clusters when analysed with SSR markers. These 2 lines showed 62% and 96% similarity, respectively when analysed with RAPDs and AFLP markers.

At 70% similarity, SSRs produced 9 distinct clusters, thus differentiating all but 'PB 343' and 'WH 542' and "CPAN 3004' and 'WL 711' from each other. Both 'PBW 343' and 'WH 542' derived from spring × winter wheat crosses and 'PBW 343' has 'Veery' (=WH 542) as one of the parents in its pedigree. AFLPs on the other hand produced only 3 clusters at 70% similarity, but were able to separate 9 out of 11 lines from each other. RAPDs however, produced 6 clusters at 70% similarity. The AFLP-based genetic similarity estimates grouped most of the genotypes very close to each other but it was not the case with RAPD-and SSR-based genetic similarity estimates. Overall RAPDs revealed the lowest similarly values, followed by SSRs and AFLPs, but RAPD-and SSR-based clusters seem more realistic as

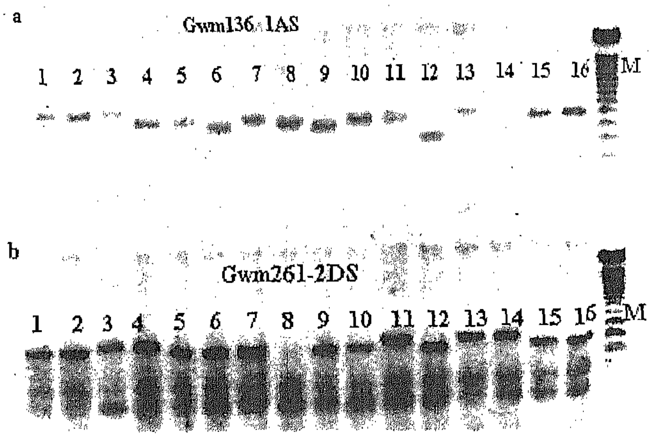

Fig 2 Banding pattern of 14 varieties of bread wheat, 1 of durum wheat and 1 of triticale generated by microsatellite primers, gwm 136 and gwm 261. Lanes 1-16 are, '8 A', 'C 273', 'C 591', 'WH 542' 'BW 343', 'PBW 138', 'PBW 175', 'PBW 34', 'T 1210', 'Sonalika', 'Kalyansona', 'HD 2009', 'C 306', 'HD 2329', 'CPAN 3004', and 'WL 711' in that order

corroborated by pedigree records of these varieties.

Based on SSR data, the pre green-revolution releases ('8A', C 273', 'C 591' and 'C 306') formed separate cluster and post-green-revolution releases formed 2 clusters (cluster II and III Fig 1) at 55% similarity.

Similarly, durum variety 'PBW 34' and triticale variety 'TL 1210' also clubbed together at 55% similarity. This is expected because triticale was synthesized by crossing *T. durum* with rye (*Secale cereale*). 'TL 1210' although a secondary triticale, is expected to have higher similarity with the 'A' and 'B' genomes of durum wheat. Marker systems showing differences in clustering a set of genotypes has been observed by other groups for wheat (Bohn *et al.* 1989), maize (Pejic *et al.* 1998) and rice (Mackill *et al.* 1996) as well.

Although highest PIC values were obtained in RAPD and AFLP markers but the range was widest in SSR markers (Table 2). This indicates that SSRs are more informative than either AFLPs or RAPDs. This is in good agreement with the other studies in barley (Becker *et al.* 1995), pea (Lu *et al.* 1996), soybean (Powell *et al.* 1996), rice (Mackill *et al.* 1996), maize (Pejic *et al.* 1998) and wheat (Bohn *et al.* 1998). Not only the level of polymorphism was high in SSRs, but these also gave more realistic clustering of the wheat varieties. Our analysis of a small sample of released varieties indicated that the traditional wheat lines form a distinct group that is presently stored in gene banks and is not a part of active crossing blocks of wheat breeders because of their tall stature and susceptibility to diseases. Involving these lines in crosses with modern varieties, using molecular breeding techniques may help in pyramiding of desirable alleles for quantitative trait loci (QTL) in one line.

DNA fingerprinting of the genotypes

Fingerprinting of genotypes for variety indentification and protection has now become necessary for all the institutes. Three marker systems RAPDs, AFLPs and SSRs were used for obtaining unique fingerprints of each variety. These markers systems differ in their ability to uniquely identify all the varieties. For 6 AFLP primer combinations no variety specific bands were obtained. In RAPDs, 31 random primers yielded 59 variety specific bands, thus differentiating all the 11 varieties studied. Similarly in SSRs, out of 25 primers pairs 11 yielded 20 variety specific bands. Results show that a single microsatellite marker *gwm* 136 was able to differentiate 4 genotypes (Table 3, Fig 2). Two microsatellite markers, *gwn* 136 and *gwm* 617 together could differentiate 6 genotypes. Similarly, additional 4 microsatellite markers, making a total of 6, could dofferentiate only 10 genotypes but not all the 16. A complete set of 25 markers did not make any difference, ie only 10 genotypes could be given a unique fingeprint. The number of alleles per locus for microsatellites varied from 2 to 6 with an average of 3 (Fig 2).

Although RAPDs also yielded variety specific bands, but reproducibility of RAPD bands makes its use questionable. AFLPs yielded largest number of bands, but no variety specific pattern was obtained. This, however, is in contrast to the results of Bohn *et al.* (1999), who could identify each of the 11 wheat cultivars with a single AFLP unit and recommended AFLPs for fingerprinting and plant variety protection. As per this study the SSRs remain the best choice for fingerprinting, as well as for estimating magnitude of genetic variability.

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REFERENCES

Becker J, Vos P, Kuiper M, Solamini F and Heun M. 1995. Combined mapping of AFLP and RFLP markers in barley. *Molecular General Genetics* **249** : 65–73.

Bohn M, Ultz H F and Melchinger A E. 1999. Genetic similarities among winter wheat cultivars determined on the basis of RFLPs, AFLPs and SSRs and their use for predicting progeny variance. *Crop Science* **39** : 228–37.

Botstein D, White R L, Skolruck M and Davis R W. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *American Journal of Human Genetics* **32** : 314–31.

Dje Y, Heuertz M, Lefebvre C and Vekemans X. 2000. Assessment of genetic diversity within and among germplasm accessions in cultivated sorghum using microsatellite markers. *Theoretical and Applied Genetics* **100** : 918–25.

Garland S H, Lewin L, Abedinia M, Hinky R and Blakeney A.

1999. The use of microsatellite polymorphism for the identification of Australian breeding lines of rice (*Oryza sativa* L.). *Euphytica* **108** : 53–63.

Jain K B L. 1994. *Wheat cultivars in India : Names, Pedigrees, Origins and Adaptations*. Research Bulletin, 272. Directorate of Wheat Research, Karnal, Haryana, India.

Lee M. 1995. DNA markers and plant breeding programmes. *Advances in Agronomy* **55** : 265–344.

Lu J, Knox M R, Ambrose M J, Brown J K M and Ellis T H N. 1996. Comparative analysis of genetic diversity in pea assessed by AFLP and PCR based methods. *Theoretical and Applied Genetics* **93** : 1 103–11.

Mackill D J, Zhang Z, Redona E D and Colowit P M. 1996. Level of polymorphism and genetic mapping of AFLP markers in rice. *Genome* **39** : 969–77.

McGuire P E and Qualset C O. 1997. Progress in genome mapping of wheat and related species. (*in*) *Joint Proceedings of the 5th and 6th Public Workshops of the International Triticeae Mapping Initiative*, Norwich, UK, 1–3 September 1995, and Sydney, Australia, 30–31 August 1995. Report 18, University of California Genetic Resource Conservation Program, Davis.

Murray M G, Subudi P K, Domingo J, Magpantay G and Huang N. 1995. Rapid DNA isolation of high molecular weight plant DNA. *Nucleic Acids Research* **8** : 4 321–5.

Nei M and Li W H. 1979. Mathematical model for studying genetic variation in terms of restriction endonuclease. *Proceedings, National Academy of Sciences, USA* **79** : 5 269–73.

Paterson A H, Tanksley S D and Sorrells M E. 1992. DNA markers in plant improvement. *Advances in Agronomy* **46** : 39–90.

Powell W, Morgante M, Andre C, Hanafey M, Vogel J, Tingey S and Rafalsky A. 1996. The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. *Molecular Breeding* **2** : 225–38.

Pejic I, Ajmone-Marshan P, Morgante M, Kozumplick V, Castiglioni P, Toramino G and Motto M 1998. Comparative analysis of genetic similarity among maize inbred lines detected by RFLPs, RAPDs, SSRs and AFLPs. *Theoretical and Applied Genetics* **97** : 1 248–55.

Roder M S, Korzun V, Wendehake K, Plaschke J, Tixier M, Leroy P and Ganal M W. 1998. A microsatellite map of wheat. *Genetics* **149** : 2007–23.

Russell J R, Fuller J D, Macaulay M, Hatz B G, Jahoor A, Powell W and Waugh R. 1997. Direct comparison of levels of genetic variation among barley accessions detected by RFLPs, AFLPs, SSRs and RAPDs. *Theoretical and Applied Genetics* **95** : 714–22.

Smith J S C and Smith O S. 1992. Fingerprinting crop varieties, *Advances in Agronomy* **47** : 85–140.

Vos P, Hogers R, Bleeker M, Reijans M, Van De Lee T, Homes M, Frijters A, Pot J, Peleman J, Kuiper M and Zabeau M. 1995. AFLP : A new technique for DNA fingerprinting. *Nucleic Acids Research* **23** : 4 407–14.

Williams J G K, Kubelik A R, Livak K J, Rafulski J A and Tingey S V. 1990. DNA polymorphism amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research* **18** : 6 531–5.