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Restriction fragment length polymorphism in pearl millet, *Pennisetum glaucum*

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Résumé : L'analyse de 19 génotypes différents de mil a l'aide de 200 sondes d'ADN génomique à simple ou nombre réduit de copies a permis de mettre en évidence l'existence d'un polymorphisme important au sein de l'espèce, mais aussi le rôle prépondérant des phenomenes de mutation de paires de bases par rapport aux processus de délétion insertion, dans la présence de RFLP.

La comparaison intervarietale souligne, avant même l'élaboration d'une carte génétique, l'intérêt d'une utilisation des marqueurs moleculaires pour répondre à certaines interrogations fondamentales du sélectionneur. Ainsi la comparaison des cultivars ICMB 841 et 81B (ICMB 1) a révélé l'inexactitude des pedigrees publies. De meme, la comparaison de quatre hybrides F1 avec leur parents respectifs a montre que le niveau d'hetérozygotie peut être un predicteur de la performance des hybrides de mil particuliérement utile.

Mots-clés : *Pennisetum*, RFLP, sélection.

Abstract : Analysis of 19 diverse pearl millet genotypes with 200 single or low copy genomic DNA probes showed the species to be extremely polymorphic and that RFLP was due, in large part, to base-pair mutations rather than deletion insertion events.

The intervarietal comparisons indicated that, even before a genetic map is constructed, the molecular markers can be employed to resolve questions of importance for breeders. For example, comparisons involving ICMB 841 and 81B (ICMB 1) showed clearly that their published pedigrees are incorrect, and comparisons among four F_1 hybrids and their parents indicated that heterozygosity level may be a useful predictor of performance in hybrid millet.

Key-words : *Pennisetum*, RFLP, plant breeding.

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Introduction

The utility of DNA markers to construct detailed genetic maps has been demonstrated in many crop species. The application of these maps and DNA probes in breeding research has been demonstrated for parental evaluation in F1 hybrid maize breeding (Walton and Helentjaris, 1987), analysis of genetic control of quantitative characters in tomato (Paterson *et al.*, 1988), and the provision of selection 'tags' for important qualitative genes in rice (Yu *et al.*, 1991). The new dense genetic maps have also found application in the resolution of some of the outstanding questions from classical genetic studies, such as demonstrating intergenomic relationships between maize and sorghum (Hulbert *et al.*, 1990), and between tomato and potato (Bonierbale *et al.*, 1988 ; Gebhardt *et al.*, 1991), the quantification of sex differences in recombination rates in tomato (De Vicente and Tanksley, 1991) and the description of evolutionary translocations in wheat and rye (Liu *et al.*, 1992). The use of maps in gene isolation, by providing landmarks for DNA walking, will soon be demonstrated in crop plants as has already been achieved with mammalian genes (Orkin, 1986).

The strategy for rapid development of a new map of maximum utility in breeding depends on the degree and nature of the polymorphism available. For example, a relative lack of polymorphism among tomato varieties necessitated the use of an interspecific cross (Helentjaris *et al.*, 1986 ; Bernatzky and Tanksley, 1986), while the high levels of RFLP available in maize allowed a choice of mapping populations of direct utility to breeders (Helentjaris, 1987). The predominant source of RFLP, either base-pair mutations such as in common bean (Chase *et al.*, 1991) or deletion/insertion such as in rice (McCouch *et al.*, 1988) will also determine screening strategies during map development. The experiments described below were carried out to determine these statistics in pearl millet, *Pennisetum glaucum*

Pearl millet is of major importance in low rainfall areas in Asia and Africa and there are many breeding and research applications awaiting the development of maps and polymorphic genetic marker systems.

Materials and methods

Genetic materials

A diverse range of 19 genotypes were studied, including (a) five maintainer lines, all with Tift 23B (registered as Tift 23DB$_1$, Burton 1969) in their pedigrees : Tift 23DB (registered as Tift 23 DB$_1$, Burton 1969) is a backcross derivative of Tift 23B ; 5141B, a backcross of Tift 23B with a downy mildew resistant source (Pokhriyal *et al.*, 1976 ; Dave, 1987) ; ICMB 841, reported to be a selection from variability within 5141B (Singh *et al.*, 1990) ; 81B (= ICMB 1), reported to be a mutation of Tift 23DB (registered as ICMB-1, Kumar *et al.*, 1984) ; and 843B (= ICMB 2), derived from crossing Tift 23DB and [illegible] 185642 (ICRISAT, 1985) ; (b) five restorer lines : BJ 104, H 77/833-2, ICMP 423, ICMP 451, and K 560 ; (c) four hybrids : ICMH 423 from parents ICMA 841 and ICMP 423, ICMH 451 from parents 81A (= ICMA-1) and ICMP 451, BK 560 from parents 5141A and K 560 ; and HHB 67 from parents 843A and H 77 833-2 ; and (e) five other genotypes : 700651-1, P7-3, 7042(S)-1, ICMP 85410, and (LGDI-B-10)-1 (abbreviated to LGD-1 below), selected as potential parents for mapping populations and to assess the level of polymorphism in very diverse genotypes.

Probes

A genomic library of 1000 clones was constructed with total plant DNA extracted from leaves of the pearl millet genotype 7042(S)-11. 50 µg DNA was digested with 100 U PstI to completion and subjected to electrophoresis on 1 % agarose gel. The DNA fragments in the size range of 500-3000 bp were collected using DEAE membrane. The purified fragments were ligated into the PstI site of PUC18 plasmid vectors. The *E. coli* bacterial strain DH5a was transformed and plated out on Xgal IPTG ampicillin LB plates.

Individual colonies were picked out and grown in 5 ml LB containing 10 µg ml carbenicillin. Those clones containing highly repeated sequences were identified by transferring PstI digested plasmid mini-preparations from 1 % agarose gels of Hybond N$^+$ nylon membranes, and hybridizing these with HaeIII restricted total genomic DNA. The remaining putative low copy inserts were PCR-amplified directly.

RFLP procedures

Methods for plant DNA isolation, restriction enzyme reactions, gel electrophoresis, Southern transfer, probe labelling and filter hybridization were as described by Sharp *et al.* (1988), with the modification that Hybond N$^+$ nylon membranes were used. After hybridization the membranes were first washed twice in 2xSSC and 1 % SDS for 15 min each and then followed by two washes in 0.2xSSC and 1 % SDS for 15 min each at 65 °C.

Results and discussions

Genomic clones

The use of PstI to generate the genomic DNA fragments for cloning follows the demonstration that CG-rich methylation sensitive sites are particularly effective in enriching libraries for low copy clones in several species, e.g. PstI in maize (Burr *et al.*, 1988) and rice (McCouch *et al.*, 1988) and HpaII in wheat (Cheung *et al.*, 1992). In pearl millet this strategy is particularly effective since only 11 % of the clones give significant signal when hybridized with total genomic DNA. The remaining clones, of which 200 were analyzed, detected single copy sequences (83 %), 2 to 4 copy se-

quences (8 %) and 5 to 10 copy sequences (9 %). These results indicated that this strategy for library production is of similar efficacy as in tomato (92 % single copy, Tanksley *et al.*, 1987), and much more effective than in rice (58 %, McCouch *et al.*, 1988), or wheat (49 %, Devos *et al.*, 1992). Since pearl millet has a larger genome, 1C = 2.5 pg (Bennett and Smith, 1976), than rice, 1C = 0.5 (Arumuganathan and Earle, 1991), the frequency of single copy sequences generated by PstI would appear not to be directly related to genome size.

Level and nature of RFLP

Of all the 200 low-copy probes, only 15 % failed to reveal any RFLP among the 19 genotypes with the use of two restriction enzymes. The remainder revealed polymorphism among these genotypes with at least one of two enzymes, with an average pair-wise polymorphism between all the probe-enzyme combinations of 56 % (35609/63249 comparisons). This percentage is much higher than that found in hexaploid wheat (Chao *et al.*, 1989 ; Devos *et al.*, 1992) and barley (Heun *et al.*, 1991 ; Graner *et al.*, 1991), and similar to maize (Helentjaris *et al.*, 1985 ; Evola *et al.*, 1986).

As has been demonstrated in other species, the observation of an RFLP in any pair-wise genotype comparison in any one restriction digest was likely to be repeated when another restriction enzyme was used. These relationships are shown by the significant correlation coefficients in Table I (note that a r value of 0.44 indicates that an RFLP with EcoRI is associated with an RFLP detected with another enzyme 19 % (r^2) of the time). These relationships were not, however, always due to the different enzymes revealing the same deletion/insertion event. Such a case is shown in Figure 1 where, for example, although both EcoRI and EcoRV detected a difference between LGD-1 and Tift 23DB, the two RFLPs are likely to be due to different base pair mutation/substitution events since Tift 23DB has the larger hybridizing band with EcoRI and a smaller sum of hybridizing bands with EcoRV. If the RFLPs were due to the same insertion/deletion event the band size difference would have been expected to be similar with both enzymes

Table I : Average fragment sizes and pair-wise polymorphisms detected by each restriction enzyme and correlation coefficients between the polymorphisms detected by a given enzyme and the average detected by all the other enzymes with the same probes

Enzyme	Fragment size	Polymorphism	r
EcoRI	6.5 kb	61 %	0.44
EcoRV	8.5 kb	69 %	0.67
DraI	11.0 kb	62 %	0.76
HindIII	8.8 kb	73 %	0.54

A survey of RFLP observed with a sample of genotypes and probes showed that on 73 % of occasions either RFLP was detected with only one enzyme or that large differences in the relative fragment sizes were observed with different enzymes. This value of 73 % « base-pair » situations is likely

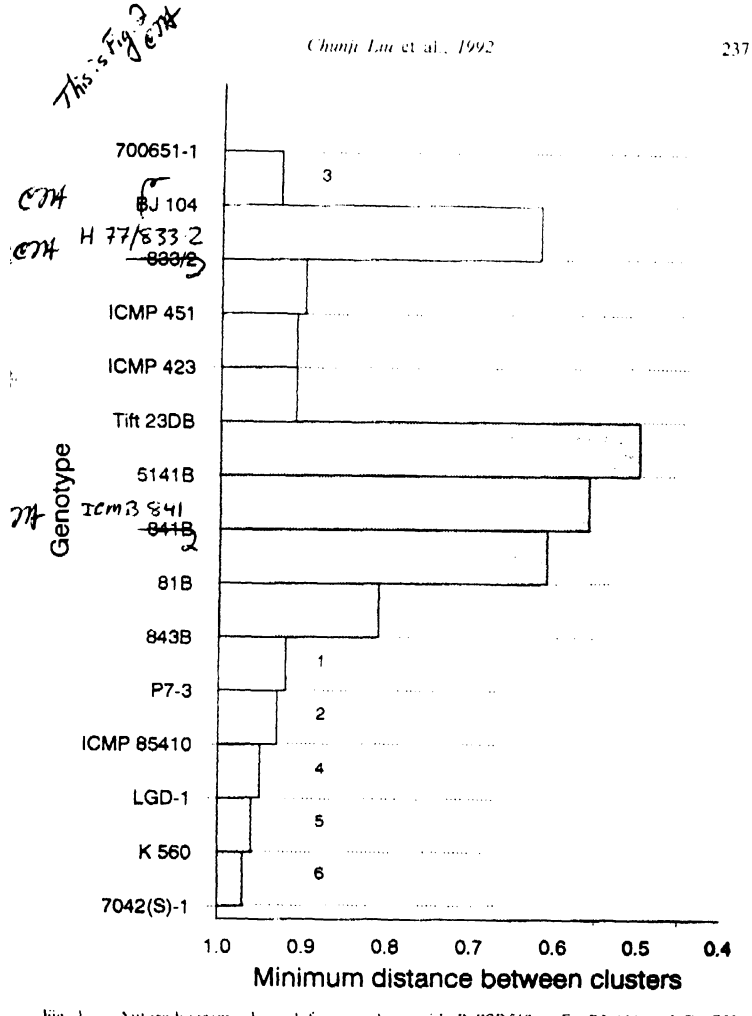

Fig. 1 — Autoradiograms derived from probing with PgPSR510 to EcoRI (A) and EcoRV (B) digested DNA from nine pearl millet genotypes (see text for explanation).

to be an overestimate but does indicate that if any probe is considered critical in an investigation but no RFLP was observed in a first screen with a limited number of enzymes then the use of further enzymes to reveal RFLP is likely to be rewarding. This conclusion is supported by the lack

of relationship between average fragment size and RFLP level detected by different restriction enzymes (Table 1), while such a relationship has been demonstrated in several other species (McCouch *et al.*, 1988 ; Graner *et al.*, 1990 ; Miller and Tanksley, 1990).

Genotypic comparisons

The most informative probe-enzyme combination for each of the 200 probes was used for genotypic comparisons by cluster and dissimilarity analysis (Sas, 1989 ; Florek *et al.*, 1951).

Pedigree analysis

The five maintainer lines, which are all reported to have Tift 23B in their ancestries, fall, as expected, into one cluster (Figure 2). The dissimilarity coefficients for this group are shown in Table 2.

Table 2 : Dissimilarity coefficients between the set of male-sterile maintainer lines

	Tift 23DB	5141B	ICMB 841	81B (ICMB 1)	843B (ICMB 2)
Tift 23DB	0				
5141B	0.32	0			
ICMB 841	0.42	0.37	0		
81B (ICMB 1)	0.40	0.52	0.62	0	
843B (ICMB 2)	0.53	0.60	0.71	0.52	0

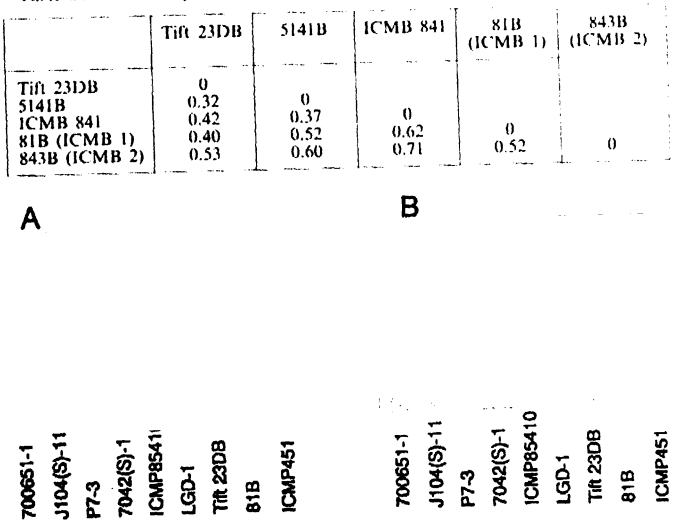

Fig. 2. – Dendrogram showing the clusters formed by the genotypes using single linkage cluster analysis. The 15 possible single member clusters are shown by the dotted lines. Linkages between these clusters are indicated by the bars. The cluster of five male-sterile line maintainers (shaded in grey), and the cluster of four restorer lines (no shading) eventually join to form the cluster. The entry of other genotypes into this large cluster is indicated by the figure to the right of the bar.

The low value for 5141B vs Tift 23DB is consistent with 5141B having been derived by backcrossing with Tift 23B. The value of 0.53 is consistent with 843B (ICMB 2) being the product of a single cross involving Tift 23DB. However the value of 0.37 is not consistent with the published derivation of ICMB 841 as a selection from variability within 5141B. It is more likely that ICMB 841 was selected from an outcross onto 5141B followed, possibly, by intercrossing with true-to-type 5141B plants. Similarly the value of 0.42 between 81B (ICMB 1) and Tift 23DB is not at all consistent with the published pedigree of the former being a simple mutation of the latter (Kumar *et al.*, 1984). An outcross was plainly involved in the origin of 81B (ICMB 1), and accounts for the large morphological differences between 81B (ICMB 1) and Tift 23DB (Rai and Hanna, 1990).

Comparisons of F1 hybrid and their parents

The greatest dissimilarity coefficient observed between two lines in this study was in the case of 81B and ICMP 451 (0.76), which are the parents of the released high-yielding hybrid ICMH 451. The coefficients of dissimilarity between the parental comparisons of HHB 67 and BK 560 were also much higher than the average over all the 19 genotypes in the study (0.64). The only exception was in the case of ICMH 423, where dissimilarity between its two parents was similar to the overall average. In view of the fact that the sample of varieties studied was extremely diverse, including lines of both Indian and African origin, the relatively high values observed between the parents of released hybrids, in particular the high yielding ICMH 451, indicate that the use of a test, based on a sample of informative DNA probes, to predict hybrid performance may be extremely rewarding.

Conclusions

The present study has shown clearly that a molecular map can be constructed efficiently in pearl millet using genomic DNA clones. The results have been used to select (LGD-1B-10)-1 and ICMP 85410 as the parents of an initial mapping population and this phase of the project is well underway.

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