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Comparative analysis of genetic similarity among maize inbred lines detected by RFLPs, RAPDs, SSRs, and AFLPs

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Abstract DNA-based fingerprinting technologies have proven useful in genetic similarity studies. RFLP is still most commonly used in the estimation of genetic diversity in plant species, but the recently developed PCR-based marker techniques, RAPDs, SSRs and AFLPs, are playing an increasingly important role in these investigations. Using a set of 33 maize inbred lines we report on a comparison of techniques to evaluate their informativeness and applicability for the study of genetic diversity. The four assays differed in the amount of polymorphism detected. The information content, measured by the expected heterozygosity and the average number of alleles, was higher for SSRs, while the lowest level of polymorphism was obtained with AFLPs. However, AFLPs were the most efficient marker system because of their capacity to reveal several bands in a single amplification. In fact, the assay efficiency index was more than ten-fold higher for AFLPs compared to the other methods. Except for RAPDs, the genetic similarity trees were highly correlated. SSR and AFLP technologies can replace RFLP marker in genetic similarity studies because of their comparable accuracy in genotyping inbred lines selected by pedigree. Bootstrap analysis revealed that, in the set of lines analysed, the number of markers used was sufficient for a reliable estimation of genetic similarity and for a meaningful comparison of marker technologies.

Key words *Zea mays* L. · Genetic relationship · Molecular markers · DNA-fingerprinting · Genetic diversity

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Introduction

Knowledge of germplasm diversity and of relationships among elite breeding materials has a significant impact on the improvement of crop plants (Hallauer et al. 1988). In maize, this information is useful in planning crosses for hybrid and line development, in assigning lines to heterotic groups, and in plant variety protection. It can be obtained from pedigree and heterosis data, from morphological traits or using molecular markers which detect variation at the DNA sequence level (Smith and Smith 1992). In particular, DNA-based polymorphisms are a powerful tool in the assessment of the genetic similarity between breeding stocks (reviewed in Lee 1995).

The discrimination power of restriction fragment length polymorphisms (RFLPs) has been extensively studied in maize, as has their use in establishing relationships with yield and heterosis (Melchinger 1993). However, there are several drawbacks to RFLPs that have stimulated the development of alternative marker systems: large quantities of DNA are in fact required for RFLP analysis, which is costly, and the technique is difficult to automate. Moreover, it requires sizeable laboratories and specialised equipment.

Various PCR-based marker techniques have recently been successfully introduced in the fingerprinting of plant genomes (Welsh and McClelland 1990; Kesseli

et al. 1994) and in genetic diversity studies (Tinker et al. 1993). Among them, random amplified polymorphic DNA (RAPD) analysis is quick (Welsh and McClelland 1990; Williams et al. 1990) and well adapted for the efficient non-radioactive DNA fingerprinting of genotypes (dos Santos et al. 1994; Thormann et al. 1994). Problems with the reproducibility of amplification and with the scoring of error data have been reported for RAPDs (Demeke et al. 1997; Karp et al. 1997).

Eukaryotic genomes are interspersed with tandem repeats of DNA, referred to as microsatellites or simple sequence repeats (SSRs). SSR polymorphisms have been extensively used as genetic markers in mammals (Tautz 1989); they occur frequently also in plant genomes, showing an extensive variation in different individuals and accessions (Akkaya et al. 1992; Senior and Heun 1993; Wu and Tanksley 1993). SSR loci are co-dominant markers more informative than RAPDs and RFLPs (Russell et al. 1997). Specific technical developments are underway (Mitchell et al. 1997) that should result in the provision of SSRs that will be faster, more standardised and more effective than RFLP technology.

Amplified fragment length polymorphism (AFLP™) is a multilocus marker technique developed by Vos et al. (1995). AFLP markers are genomic fragments detected after selective PCR amplification which provide a number of appealing features in the fingerprinting of genomes of different complexity, including that of maize (Vos et al. 1995). The AFLP technique has been used to identify markers linked to disease resistance loci (Becker et al. 1995; Cervera et al. 1996), to fingerprint DNAs (Vos et al. 1995; Sharma et al. 1996), and to assess relationships between molecular polymorphism and hybrid performance in maize (Ajmone-Marsan et al. 1998).

A comparison of different marker techniques is timely, even though the utility of different molecular markers for soybean and barley germplasm has already been reported (Powell et al. 1996; Russell et al. 1997). The objectives of the present study were: (1) to compare the informativeness of different molecular markers and their applicability for genetic diversity analysis, genotype identification and variety protection purposes, (2) to determine the genetic similarity obtained with RFLP- and PCR-based techniques in a set of maize inbred lines, and (3) to compare their effectiveness in estimating genetic similarity among maize inbreds.

Materials and methods

Plant materials and DNA extraction

Thirty three inbred lines were chosen to explore the diversity of maize germplasm. All these inbreds have been extensively used in the production of hybrid seed and in maize breeding programs. Pedigree information was previously described in Livini et al. (1992). Based on available information and on the heterotic behaviour in crosses, 13 (A641, B14 A, B37, B73, B84, Cm109 Lo1016, Lo916, Lo950, Lo951, Lo964, Lo999, and N28) can be associated with the Iowa Stiff Stalk Synthetic (BSSS) heterotic group, 13 (A619, C103, C123, H99, Lo881, Lo924, Lo976, Lo1077, Mo17, Oh43, Va22, Va59, and Va85) with the Lancaster Sure Crop (LSC), two to Wf9 (WF9 and Pa91), three to W153R (W153R, Lo932, and Lo944), and two to HY (H55 and H96). Genomic DNA was isolated from a bulk of 20–30 shoots of 7–9-day old germinated seedlings and extracted using the CTAB method as previously described (Livini et al. 1992).

Nucleic-acid manipulation and molecular-marker assays

Conditions for restriction enzyme digestion, gel electrophoresis for RFLP, Southern transfer hybridisation, and autoradiography followed Livini et al. (1992). Forty seven genomic clones from the UMC and BNL collections and two restriction enzymes (*Eco*RI and *Hin*dIII) were used to characterise 53 RFLP loci in the 33 inbred lines. A total of 253 RFLP bands were binary coded as 1 or 0 for the presence or absence of such loci in each line, respectively.

RAPD amplification was performed as described by Ajmone-Marsan et al. (1993) using a Perkin Elmer 9600 Thermal Cycler. Reaction products were analysed by electrophoresis on a 2% agarose gel and stained with ethidium bromide. A total of 85 primers (Operon Technologies, California, USA) were surveyed in two inbred lines. Twenty five primers showing reproducible, and clearly scorable, polymorphic (present or absent) fragments, were used to fingerprint the 33 inbreds.

The primers for the SSR markers were synthesised according to the sequences published by Senior and Heun (1993) and Taramino and Tingey (1996). SSR procedures were those described by Taramino and Tingey (1996). Processed fragments, along with loading dye and internal size standards, were run out on a 6% acrylamide gel (Pfeiffer et al. 1997) using an Automated Laser Fluorescent sequencing electrophoresis unit (Pharmacia). Fragments were labelled with fluorescein by direct incorporation of F-12-dUTP (2-μM final concentration) during the PCR reaction. Un-incorporated labelled nucleotide was removed by ethanol precipitation prior to loading samples on the gel. Data were processed using Fragment Manager Software v. 1.1 (Pharmacia). SSR bands were sized first and then binary coded by 1 or 0 for their presence or absence in each line.

AFLP marker analysis was according to Vos et al. (1995). Briefly, total genomic DNA (400 ng) was restricted with 5 U of *Eco*RI (rare cutter) and 5 U of *Mse*I (frequent cutter) (Pharmacia), and double-stranded adapters ligated to the fragment ends. The structure of the adapter sequences, pre-amplification, amplification and polyacrylamide-gel electrophoresis conditions were as in Ajmone-Marsan et al. (1998). Polymorphic amplification products were visualised by autoradiography and scored manually. All AFLP polymorphisms were scored as dominant markers.

All names of the RFLP probes and the nucleotide sequences of the primers used for the amplification of AFLP, RAPD, and SSR markers are available on request.

Data analysis

The average number of alleles per locus, the allele frequency, the expected heterozygosity (He), and the effective number of alleles per locus were calculated as reported by Morgante et al. (1994). The total number of effective alleles (Ne) surveyed by RFLP, RAPD, SSR, and AFLP analyses was calculated by summing the number of effective alleles of all the analysed loci as $Ne = \sum ne\ (i)$. To compare the efficiency among the four methods, where RFLPs and SSRs generally detect multiple alleles and one band per assay, whereas RAPDs and AFLPs detect two alleles and multiple bands per assay,

an assay efficiency index (Ai) was calculated. Ai combines the effective number of alleles identified per locus and the number of the polymorphic bands detected in each assay as Ai = Ne/P, where Ne is the total number of effective alleles detected and P is the total number of assays performed for their detection.

The genetic similarities (GSs) from RFLP, RAPD, SSR, and AFLP data were calculated among all possible pairs of lines using the Dice similarity index as in Nei and Li (1979). The co-ancestry coefficient, *f*, between lines related by pedigree, was calculated as previously reported (Ajmone-Marsan et al. 1992). Cluster analyses were based on similarity matrices obtained with the unweighted pair group method using arithmetic averages (UPGMA) (Rohlf 1990) and relationships between inbred lines were visualised as dendrograms. For each dendrogram the co-phenetic coefficients between the matrix of genetic similarities and the matrix of co-phenetic values were computed using appropriate routines of the NTSYS-pc package. The significance of the co-phenetic correlation observed was tested using the Mantel matrix correspondence test (Mantel 1967).

The bootstrap procedure was employed to determine the sampling variance of the genetic similarities calculated from the data sets obtained with the different marker systems. All data, irrespective of the dual or multiallelic nature of the marker system, were scored in the form of a binary matrix. For each pair of inbreds, the Dice similarity index (GS) was calculated from the 2000 random subsamples at different sample sizes (10, 50, 100, 150, 200, and all bands when the total exceeded 200). Bootstrap standard deviation estimates were based on 2000 samples. The calculations were performed with the SAS macro "BOOT" (Jackknife and Bootstrap Analyses, SAS Institute Inc.).

Results

Levels of polymorphisms

The 33 inbred lines were surveyed with the four different marker systems. All of the molecular markers were able to uniquely fingerprint each of the inbred lines. The levels of polymorphism detected with each marker system and the index comparing their informativeness are reported in Table 1. The total number of assays ranged from only six primer combinations for AFLPs to 53 probe/enzyme combinations for RFLPs. The total number of polymorphic bands identified ranged from 90 for RAPDs to 253 for RFLPs. An average number of 4.8 alleles per locus, with an average effective number of 3.2 alleles per locus, ranging from 1.2 to 6.5, could be distinguished for each probe/enzyme combination using RFLPs. This value increased to 6.8 with SSRs, with an average number of effective alleles of 4.4 per locus, ranging from 1.1 to 6.6, while for RAPDs and AFLPs these values were lower (1.6 for both). This was reflected also in lower expected heterozygosity values. Overall the highest assay efficiency index was observed for AFLPs (61.9) and the lowest for RFLPs (3.2). RAPDs and SSRs (5.8 and 4.4, respectively) were comparable to RFLPs. In particular, for AFLPs the high assay efficiency index is due to the simultaneous detection of several polymorphic bands in a multiplex amplification per single reaction.

Genetic similarity

A summary of the genetic similarity estimates, calculated for each marker system, between pairs of lines of the various heterotic groups is shown in Table 2. All marker systems indicated that lines of BSSS origin were more similar in comparison to inbred lines of other heterotic groups. The mean value of the GS estimate was, as expected, lower for BSSS × LSC crosses than within the BSSS and LSC groups themselves. This is consistent with the common practice in maize breeding of preferentially developing hybrids between heterotic groups because they are expected to perform better than those from crosses within heterotic groups. The similarity ranged from 0.92 within LSC types, using AFLPs, to 0.00 within BSSS × miscellaneous types, using SSRs. The estimates of GS follow the same pattern across marker systems, i.e. higher estimates of similarity within the BSSS types and lower estimates within the LSC × miscellaneous types. Overall SSRs revealed the lowest similarity values and AFLPs the highest.

The genetic similarity trees produced from each marker system are presented in Fig. 1. In these trees inbreds were ordered as expected, though with exceptions, into the major groups BSSS and LSC.

Table 1 Level of polymorphism and comparison of informativeness obtained with RFLP, RAPD, SSR and AFLP markers in 33 maize inbred lines

Parameters	Marker system			
	RFLP	RAPD	SSR	AFLP
Number of assay units	53 (probe/enzymes)	25 (primers)	27 (primer pairs)	6 (primer combination)
Number of polymorphic bands	253	90	183	232
Number of loci	53	90[a]	27	232[a]
Average number of alleles per locus	4.8	2.0	6.8	2.0
Expected heterozygosity	0.63	0.36	0.72	0.34
Effective number of alleles per locus	3.2	1.6	4.4	1.6
Assay efficiency index	3.2	5.8	4.4	61.9

[a] Theoretical maximum number of loci

Table 2 Mean, minimum and maximum of the Dice genetic similarity coefficient (GS) calculated from different molecular marker systems for various groups of maize inbred lines

Marker system		GS value						
		Within heterotic groups			Between heterotic groups			Between all lines
		BSSS ($n = 78$)	LSC ($n = 78$)	MISC ($n = 21$)	BSSS × LSC ($n = 169$)	BSSS × MISC ($n = 91$)	LSC × MISC ($n = 91$)	ALL ($n = 528$)
RFLP	Min.	0.28	0.25	0.23	0.18	0.20	0.18	0.18
	Max.	0.79	0.86	0.90	0.59	0.49	0.56	0.90
	Mean	0.48	0.40	0.41	0.36	0.36	0.35	0.37
RAPD	Min.	0.34	0.38	0.31	0.37	0.36	0.29	0.29
	Max.	0.90	0.80	0.89	0.76	0.65	0.68	0.91
	Mean	0.64	0.57	0.53	0.58	0.53	0.53	0.56
SSR	Min.	0.13	0.08	0.08	0.07	0.00	0.04	0.00
	Max.	0.84	0.88	0.82	0.52	0.52	0.40	0.88
	Mean	0.38	0.31	0.28	0.27	0.22	0.20	0.26
AFLP	Min.	0.45	0.43	0.43	0.36	0.43	0.39	0.36
	Max.	0.89	0.92	0.89	0.61	0.64	0.62	0.92
	Mean	0.62	0.59	0.55	0.48	0.52	0.51	0.53

Discrepancies in forming subgroups within the major groups were observed as well as in the clustering of inbred lines of miscellaneous origins. Considering the BSSS-related lines the topology of each tree is unique with some evident similarity: the clustering of B14, B37, and B73 is, for example, fully conserved. On the LSC side, clustering was consistently reported by all methods with the exception of Va22, a line derived from C103, indicating that all methods aggregated lines of different origin. The Oh43-related lines (Oh43 and A619) were positioned within the Lancaster group only by RAPDs and AFLPs, while SSRs and RFLPs clustered these with BSSS lines (although Oh43 is usually considered a Lancaster type). Similarly to the RAPD-based tree, the clustering based on AFLP data produced a tree with a relatively narrow range of similarity values between the more-related and the more-distant pairs of inbreds. In spite of this, all the main clusters were confirmed by AFLP data.

Four pairs of very similar inbreds (B14 A, Cm109; Lo932, Lo944; A619, Oh43; and H55, H96) were clustered together by all marker systems, while in one additional case similar lines (Lo916 and Lo999) were consistently grouped in AFLP, SSR and RAPD trees.

Comparison between marker systems

All the estimates of correlation coefficients (*r*s) among available co-ancestry coefficients (*f*s) and genetic similarity (GS) data were highly significant ($P < 0.01$). RAPDs showed the lowest correlation ($r = 0.40$) with *f* values, RFLPs and SSRs intermediate values ($r = 0.57$ and $r = 0.53$, respectively), while AFLPs showed the highest value ($r = 0.62$). The *r*s among similarity data were also significant. Correlation coefficients of RAPD marker data ($r = 0.51$, $r = 0.57$, and $r = 0.52$ with RFLP, SSR and AFLP, respectively) with those obtained using other marker systems were lower than those among similarity estimates based on AFLPs, RFLPs, and SSRs ($r = 0.70$, $r = 0.67$ and $r = 0.59$, respectively between AFLP and RFLP, AFLP and SSR, and RFLP and SSR). The extent to which similarities were correlated varied considerably across the whole data set. When the set of pairwise data (528) was divided into two groups (according to the arithmetic mean of the observed GS range based on RFLP data: "more similar" lines with GS > 0.37 and "less similar" lines with GS < 0.37), it became evident that the genetic similarities estimated by different marker systems were mainly correlated only among similar lines, while the relationships among dissimilar lines were low and not significant. The GS values plotted against the estimate of co-ancestry value based on pedigree data followed the same pattern.

The co-phenetic 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 inbreds obtained with that marker system. Overall the co-phenetic coefficients were medium to high, with the RFLP (0.84) and AFLP (0.83) data resulting in the highest correlation, SSR (0.80) showing an intermediate value, and the RAPD (0.72) assay producing the lowest correlation.

All methods could clearly distinguish all 33 inbred lines, although the SSR data provided the highest level of discrimination between any pair of inbreds. In general, the grouping agreed with the pedigree information of the lines, although some discrepancies were

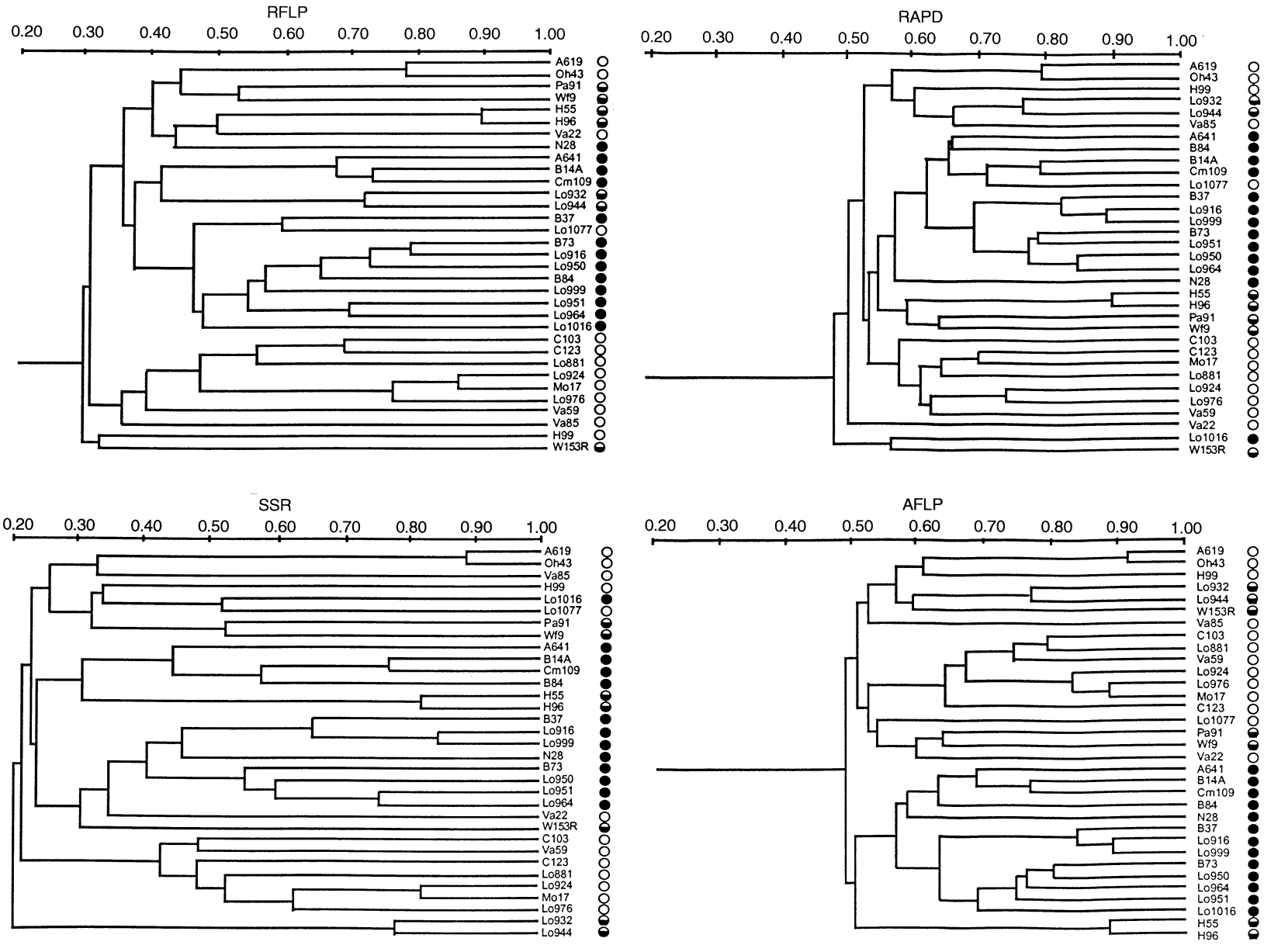

Fig. 1 Dendrograms of 33 inbred lines obtained using the RFLP, RAPD, SSR and AFLP marker systems (● = BSSS, ○ = LSC, ◒ = MISC).

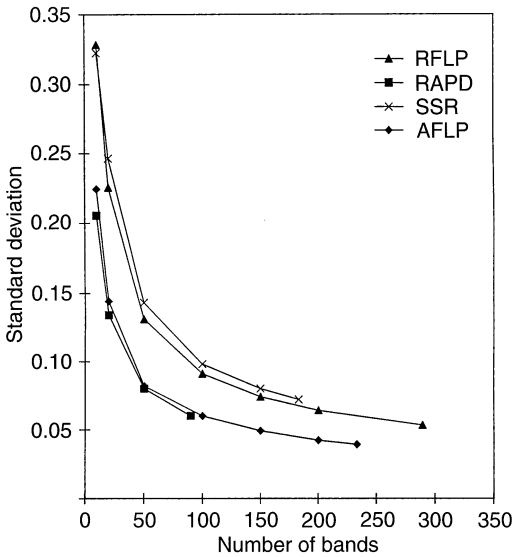

Fig. 2 Variation assessed by bootstrap sampling of genetic similarity between maize inbreds across different marker systems due to the different number of markers (bands)

observed. In particular, genetic similarities based on AFLP data had the highest correlation with pedigree data, while those based on RAPDs had the lowest one.

Bootstrap analysis

To determine the sampling variance of genetic similarities calculated from different molecular marker data sets, bootstrap analysis with a declining number of bands was performed. The relationships between number of bands and the sampling variance of the genetic similarity among all pairs of inbred lines for each method is presented in Fig. 2. The results indicated that above 150 bands there was a diminishing return in the precision gained by adding additional bands. As the number of bands moves below these thresholds the standard deviation begins to increase (and precision decreases) at a greater rate.

Discussion

In this paper we have shown that the number of alleles detectable in maize by SSRs is higher in comparison to other methods. This high level of polymorphism is to be expected because of the unique mechanism responsible for generating SSR allelic diversity by replication slippage (Tautz et al. 1986). It is also known that when SSRs have been compared to other marker systems they have revealed the highest level of polymorphism (Wu and Tanskley 1993; Morgante et al. 1994; Powell et al. 1996). The present data indicate that on average SSRs carry two-fold more information than AFLPs and RAPDs, and 40% more information than RFLPs, when the number of alleles per locus is the target.

In agreement with previous observations (Becker et al. 1995), the lowest degree of polymorphism was associated with AFLPs. Conversely, the information measured as the assay efficiency index, which correlates with the number of effective alleles identified per assay, was more than ten-fold higher for AFLPs compared to the other methods. These findings are in good agreement with previous germplasm analysis carried out in several crop species (Lu et al. 1996; Powell et al. 1996). It can be concluded that SSRs are capable of revealing the highest level of information per single marker and that AFLPs detect the highest number of polymorphisms in a single assay. This high assay efficiency index is a reflection of the efficiency of AFLPs to simultaneously analyse a large number of bands rather than the levels of polymorphism detected at each locus. The assay efficiency index for SSRs can, however, be considerably higher if multiplex PCR and gel-running procedures are adopted, where several microsatellites are simultaneously amplified and co-electrophoresed using multicolour fluorescent technologies (Lindqvist et al. 1996; Heyen et al. 1997). An additional advantage of SSRs over AFLPs is only relevant when mapping populations are derived from outcrossing heterozygous individuals, where the multi-allelism of SSR markers increases the number of informative genotypic classes in the progenies compared to the binary AFLP markers.

The results shown by genetic similarity trees indicate that, except for RAPDs, they are highly similar. In addition, trees from these molecular methods agree with the information obtained from pedigree data. Similarly, Powell et al. (1996) found the lowest correlations among RAPDs and other marker systems. In this respect, it has been shown that RAPD analysis, based on the use of random primers, is likely to suffer from a lack of reproducibility due to mismatch annealing (Neale and Harry 1994). In the trees obtained from cluster analysis, all lines with defined affiliation to one of the heterotic groups were assigned to their specific main clusters, in agreement with the available data for maize (Ajmone-Marsan et al. 1992; Livini et al. 1992; Mumm and Dudley 1994; Smith et al. 1997). A second observation is that, within the clusters, the grouping of more distantly related lines does not match precisely with the expectations based upon pedigree data. Differences among marker techniques in grouping genetically more distant lines have been previously reported (Powell et al. 1996). Other studies in *Brassica* and from pea accessions show that molecular marker-based similarities and trees were significantly correlated across a wide range of germplasms (Thormann et al. 1994; Lu et al. 1996). Many potential reasons for these discrepancies exist, including underlying assumptions in calculating pedigree data (Messmer et al. 1993), genome sampling

(Nei 1987), and the numbers of markers or probes employed (Tivang et al. 1994).

The number of loci required for a reliable estimate of genetic similarity has been shown to vary from 15 RFLP probes, giving 56 bands in *Brassica* sp. (dos Santos et al. 1994), to 100 RFLP clone-enzyme combinations (Messmer et al. 1993). Similarly, Tivang et al. (1994), investigating in maize the sampling variance of a RFLP data set in maize, found that the number of bands required for a CV of 10% was 388, 150, and 38 for closely, intermediately, and distantly related inbreds, respectively. Our results using the bootstrap procedure suggest that 150 bands are sufficient for reliable estimates of genetic similarity. Accordingly, the average number of assays that could have been used in this study to attain such a precision in the estimate were 30–40 clone-enzyme combinations for RFLPs, 40–50 primers for RAPDs, 20–30 primers for SSRs, and 4–5 enzyme combinations for AFLPs. Based on these estimates, the disagreement of the RAPD results in comparison to the other types of markers might be explained by the insufficient number of primers used.

In conclusion, the results of this study indicate that, with the exception of RAPDs, the other DNA markers provide consistent information for germplasm identification and pedigree validation. We have shown that SSR and AFLP profiling technologies can be good candidates to replace RFLP markers in genetic similarity estimates and variety description, and that they have comparable accuracy in grouping inbred lines selected by pedigree. They are generally much simpler to apply and more sensitive than the traditional morphological and biochemical methods or the RFLP-based fingerprinting techniques; yet they provide results correlated with those from RFLP analyses. A major advantage of the SSR and AFLP methods is that they can be automated. While SSRs, thanks to their multi-allelism and co-dominance, appear to be suited for the analysis of outcrossing heterozygous individuals, AFLPs, with their high multiplex ratio, offer a distinctive advantage when genome coverage is a major issue due to the presence of linkage disequilibrium, such as in inbred lines and breeding materials.

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