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RFLP analyses of early-maturing European maize germ plasm

I. Genetic diversity among flint and dent inbreds

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Summary. Thirty inbred lines representing a wide range of early-maturing European elite germ plasm of maize (*Zea mays* L.) were assayed for RFLPs using 203 clone-enzyme combinations (106 DNA clones with restriction enzymes *Eco*RI and *Hin*dIII). The genetic materials comprised 14 flint, 12 dent, and 4 lines of miscellaneous origin. Objectives were to (1) characterize the genetic diversity for RFLPs in these materials, (2) compare the level of genetic diversity found within and between the flint and the dent heterotic groups, and (3) examine the usefulness of RFLPs for assigning inbreds to heterotic groups. All but two DNA clones yielded polymorphism with at least one restriction enzyme. A total of 82 and 121 clone-enzyme combinations gave single-banded and multiple-banded RFLP patterns, respectively, with an average of 3.9 and 7.7 RFLP patterns per clone-enzyme combination across all 30 inbreds, respectively. Genetic similarity (GS) between lines, estimated from RFLP data as Dice's similarity coefficient, showed considerable variation (0.32 to 0.58) among unrelated inbreds. The mean GS for line combinations of type flint × dent (0.41) was significantly smaller than for unrelated flint lines (0.46) and dent lines (0.46), but there was considerable variation in GS estimates of individual line combinations within each group. Cluster and principal coordinate analyses based on GS values resulted in separate groupings of flint and dent lines in accordance with phylogenetic information. Positioning of lines of miscellaneous origin was generally consistent with expectations based on known breeding behavior and pedigrees. Results from this study corroborated that RFLP data can be used for assigning inbreds to heterotic groups and revealing pedigree relationships among inbreds.

Key words: *Zea mays* L. – RFLPs – Genetic diversity – Heterotic groups

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Introduction

Exploitation of heterotic patterns between different sources of germ plasm is of great concern to maize breeders. The current major heterotic patterns for hybrid maize grown in the temperate regions, Reid Yellow Dent by Lancaster Sure Crop in the U.S. Corn Belt and flint by dent in central Europe, have been established empirically by relating the heterosis observed in crosses with the origin of their parents (Hallauer et al. 1988). A more systematic and exact approach is needed for a clearer definition of established heterotic groups, identification of new heterotic patterns, and concerted introgression of new germ plasm into breeding populations.

Knowledge of the genetic diversity in the available germ plasm is fundamental for optimal design of breeding programs. Therefore, maize breeders have a keen interest in the characterization of the genetic diversity between and within heterotic groups. Besides the observed heterosis of varietal crosses as a measure of genetic divergence, various kinds of phenotypic and genotypic descriptors (e.g., morphological, cytological, biochemical, and DNA markers), as well as geographic origin and pedigree information, have been employed to study the phylogenetic relationships among and the genetic variation within different germ plasm sources. For these applications, restriction fragment length polymorphisms (RFLPs) presently seem to be the most promising class of markers, due to the high degree of polymorphism at the DNA level detected in maize (Bernatzky and Tanksley 1989).

The genetic diversity of inbred lines, commercial hybrids, and open-pollinated populations from the U.S. Corn Belt germ plasm has extensively been studied by using isozyme or zein data (for review, see Messmer et al. 1991) and, recently, RFLP data (Lee et al. 1989; Godshalk et al. 1990; Smith et al. 1990; Melchinger et al. 1991). By comparison, limited work has been done to characterize and classify the European maize germ plasm. In contrast to the dent × dent crosses grown in the U.S. Corn Belt, most of the maize hybrids cultivated as forage crop in central Europe are crosses between early-maturing flint inbreds and high-yielding dent inbreds. The flint lines were selected from European open-pollinated populations, which 'presumably trace back to the tropical flints from the West Indies and the Caribbean Islands' (Wallace and Brown 1956). The dent lines for the most part trace back to the U.S. Corn Belt germ plasm which, according to these authors, was developed by combining 'the early slender-stalked, flint corn of northeastern U.S. with the late heavy-stalked gourd seed corn of the south-central U.S.'.

Camussi et al. (1983) examined the genetic diversity between 20 Italian maize populations by multivariate statistical analyses of 18 quantitative traits and their heterosis observed in varietal crosses. Salanoubat and Pernes (1986) studied the genetic relationships among 21 populations from southern Europe by means of isozyme analyses. Smith (1989) used isozyme and zein data for characterization and assessment of the genetic diversity among 61 commericial maize hybrids of widespread usage in France. However, we are not aware of any published reports in which RFLPs have been employed to investigate the genetic variability among European flint and dent inbred lines.

Here we report an RFLP assay of a set of 30 flint and dent inbred lines that have been widely used for commercial hybrid seed production in Germany and France. The objectives of our study were to (1) characterize the genetic diversity for RFLPs in the early-maturing European maize breeding marterials, (2) compare the level of genetic variation found between and within the flint and dent heterotic groups, and (3) investigate the usefulness of RFLP data for assigning inbreds to heterotic groups and for revealing pedigree relationships among lines.

Materials and methods

Maize inbred lines

A total of 30 inbred lines (22 public and 8 private lines) representative for early-maturing European maize breeding materials was examined (Table 1). Fourteen inbreds belong to the flint and 12 to the dent germ plasm. Four inbreds, subsequently designated as inbreds of miscellaneous origin, were flint lines with various proportions of dent germ plasm. The flint lines trace back to different European sources: DK105 and D107 are second-cycle inbreds recovered from the German open-pollinated land variety 'Gelber Badischer Landmais' (GBL), F2 and F7 originated from the French population 'Lacaune', D102 is closely related (75%) to the French inbred F2, and EP1 originated from the Spanish population 'Lizargarote'. The dent lines are or trace back to early inbreds of Canadian (CG12, CO120, CO125, CO151, CO158, V3), Wisconsin (W41A, W59E, W401, WD), or Minnesota (A632) origin. B73 and Mo17 are two public U.S. dent inbreds that were included as standards because of their importance as parents for commercial hybrid seed production in the U.S. Corn Belt and southern Europe in the past. All lines were highly inbred by more than ten selfing generations.

RFLP analyses

For RFLP analyses of the 30 inbreds, equal quantities of leaf tissue from five seedlings per inbred, about 8–10 weeks old, were harvested, frozen in liquid nitrogen, and stored at −70 °C. About 10 g of frozen plant material from each inbred was ground by mortar and pestle. Isolation of genomic DNA proceeded according to the protocol of Dellaporta et al. (1983), with minor modifications. Upon separate digestion of DNA with restriction enzymes *Eco*RI and *Hin*dIII, DNA samples of 7.5 µg were loaded onto 0.75% agarose gels and electrophoresed in TAE buffer at 15 V for 16 h. There were two combs with 26 lanes per gel and at every eighth lane, a set of markers of known restriction fragment length was loaded, which was composed of lambda fragments of 2.3, 4.3, 6.7, 9.4, 13.3, and 21.3 kb obtained from single digests of lambda with restriction enzymes *Hin*dIII and *Bgl*II. DNA fragments were transferred from gels to Pall Biodyne B membranes according to the alkaline technique described by the manufacturer (Pall Filtrationstechnik GmbH, Dreieich, FRG). DNA probes were radiolabelled with ^{32}P-dCTP by random primer synthesis (Feinberg and Vogelstein 1983). Hybridization of probes to maize DNA fragments on membranes at 65 °C, autoradiography, and post-hybridization washes for stripping of DNA probes were performed as described by Jung et al. (1990). A total of 106 genomic DNA clones (Table 2), chosen from collections of mapped maize clones (Burr et al. 1988; Coe et al. 1988) kindley provided by Dave Hoisington (University of Missouri, Columbia/MO), was applied. DNA probes were selected on the basis of single-copy hybridization patterns and to warrant a good coverage of the genome, with at least eight probes per chromosome. The average map distance between adjacent markers was approximately 25 cM, based on published RFLP maps. Altogether, we analyzed RFLP data from 203 clone-enzyme combinations, because not all clones were hybridized against both restriction enzyme digests. Ninety-seven clones were assayed with both restriction enzymes (*Eco*RI and *Hin*dIII), seven clones were assayed only with *Eco*RI, and two only with *Hin*dIII.

RFLP profiles for inbreds on autoradiographs were visually scored by assigning a number to each band according to its approximate migration distance, estimated by comparison with the two adjacent lanes of lambda markers. Only full intensity bands were taken into consideration. Two bands were scored as different if their range did not overlap, i.e., if the position of band x across the lanes did not overlap with the position(s) of band y. In the case of ambiguous RFLP patterns due to low DNA concentration or problems with hybridization, data were recorded as missing values. However, if bands were absent in a lane on a good quality blot, a 'null' variant was assigned with an imaginary kilobase value of 0.0. This occurred only once and was verified with a second blot. Scoring was independently performed twice by two different people to minimize the risk of errors. Data were binary coded for subsequent numerical analysis, i.e., the presence or absence of a band in a line was coded by 1 or 0, respectively.

Table 1. Inbreds used in the analysis of RFLP data

Inbred[a]	Pedigree[b]
Flint lines (10 public and 4 private inbreds)	
D140	DK105 (75%), JF30sd[c] (25%)
D141	D107 (75%), D102 (25%)
D142	DK105 (50%), D102 (25%), D107 (25%)
D143	D102 (50%), DK105 (25%), G35.23 (12.5%)
D144	D102 (50%), FSPau (50%)
D146	DK105 (62.5%), FSPau (25%), D107 (12.5%)
D503	Synthetic PF75 (50%), D102 (25%), DK105 (12.5%), F6B.Scag (12.5%)
DK105	GB35/B (50%), GB101 (50%)
F192	F2 (50%), F7 (50%) (Henderson 1984)
F564	F7 (50%), F64 (50%) (Henderson 1984)
K1	Synthetic of European flints (W. Schmidt, personal communication)
K2	EP1 (37.5%), population Rheintaler (25%) (W. Schmidt, personal communication)
K3	EP1 (25%), F2 (25%), population Rheintaler (25%) (W. Schmidt, personal communication)
K4	EP1 (25%), F2 (25%), F7 (12.5%) (W. Schmidt, personal communication)
Dent lines (11 public and 1 private inbred)	
B73	BSSS(HT)C5 (Henderson (1984)
CG12	Selection from Pride 4 (Henderson 1984)
CO120	DeKalb 46 (Henderson 1984)
CO151	Early Butler (W. Schmidt, personal communication)
D01	CO125 (50%), CO158 (25%), NE1A (12.5%), W41A (6.3%), W59E (6.3%)
D02	A632 (50%), CO125 (12.5%), CO158 (12.5%), W401 (9.4%), V3 (6.3%), WD (6.3%), IHP (3.1%)
D05	A632 (50%), CO125 (12.5%), CO158 (12.5%), W401 (9.4%), V3 (6.3%), WD (6.3%), IHP (3.1%)
D06	A632 (50%), CO125 (12.5%), CO158 (12.5%), W401 (9.4%), V3 (6.3%), WD (6.3%), IHP (3.1%)
D403	CO125 (75%), IHP (25%)
D408	PD Synthetic (75%), Pride 4 (9.4%), IHP (6.3%), W41A (4.7%), W59E (4.7%)
Mo17	C103 (50%), CI.187-2 (50%) (Henderson 1984)
K5	CO125 (50%), B14 (25%), B37 (25%) (W. Schmidt, personal communication)
Lines of miscellaneous origin (1 public and 3 private inbreds)	
D145	D107 (62.5%), Mo17 (25%), D102 (12.5%)
K6	INRA258 (25%), population Rheintaler (25%), EP1 (12.5%) (W. Schmidt, personal communication)
K7	DK105 (50%), INRA258 (12.5%), population Rheintaler (12.5%), EP1 (6.3%) (W. Schmidt, personal communication)
K8	CO125 (50%), F2 (25%), F7 (25%) (W. Schmidt, personal communication)

[a] Lines designated by K1 through K8 are proprietary to Kleinwanzlebener Saatzucht (KWS) AG, W-3352 Einbeck, Germany. Lines with initials D or DK were developed by W. G. Pollmer and are proprietary to the University of Hohenheim, W-7000 Stuttgart 70, Germany

[b] W. G. Pollmer (personal communication) unless stated otherwise

[c] sd = semi-dwarf

Statistical analyses

Estimates of genetic similarity (GS) were calculated for all 435 pairs of inbreds according to the following equation (Dice 1945; Nei and Li 1979):

$$GS(i,j) = 2N(i,j)/[N(i)+N(j)],$$

where $GS(i,j)$ is the measure of genetic similarity between inbreds i and j, $N(i,j)$ is the number of bands common between i and j, and $N(i)$ and $N(j)$ are the number of bands in i and j, respectively, with regard to all clone-enzyme combinations considered. Hence, the GS value reflects the proportion of RFLP bands that cannot be distinguished between two inbreds. A GS value of zero indicates maximum RFLP diversity for the respective inbreds (i.e., no bands in common), whereas a GS value of one indicates identical RFLP profiles for all clone-enzyme combinations in the two inbreds. Provided the inbreds assayed are homozygous, as in the present study, and all clone-enzyme combinations yield single-banded RFLP patterns for all inbreds, then the Dice's GS coefficient is equivalent to one minus the Rogers' distance (Rogers 1972) used in earlier RFLP studies (Melchinger et al. 1990a, b). Standards errors of GS estimates were obtained by the jackknife procedure (Miller 1974).

Graphical representations of the associations among the 30 inbreds were obtained by standard procedures of numerical taxonomy. Average linkage (UPGMA) cluster analysis and principal coordinate analysis (PCOA) (Gower 1972) were performed with the matrix of RFLP-based GS estimates by using appropriate procedures of program NTSYS-pc (Rohlf 1989). Coancestry coefficients (f) were calculated for pairs of inbreds with known pedigree relationship according to the rules described in Falconer (1981) and by using the assumptions listed by Melchinger et al. (1991).

Results

Genetic variation for RFLPs

All but 2 of the 106 DNA clones employed in this study revealed RFLPs among the 30 inbreds with at least one of the two restriction enzymes used; only 7 (3.4%) of the 203 clone-enzyme combinations assayed were monomorphic (Table 2). A total of 1,039 RFLP bands (including 'null' patterns) were detected among the 30 inbreds for all clone-enzyme combinations. Eighty-two (40.4%) clone-enzyme combinations yielded single-banded RFLP patterns (i.e., precisely one band or a 'null' pattern for each inbred) and 121 (59.6%) clone-enzyme combinations yielded multiple-banded RFLP patterns (i.e., more than one band for at least one of the 30 inbreds). The number of RFLP patterns per clone-enzyme combination ranged from one to nine in the former case and from 2–26 in the latter case, with an average of 3.94 and 7.71, respectively (Fig. 1).

Sixty-two of the 97 DNA clones simultaneously assayed with restriction enzymes *Eco*RI and *Hin*dIII yielded either single-banded (22) or multiple-banded (40) RFLP patterns with both enzymes, whereas 35 clones gave single-banded RFLP patterns with one enzyme but multiple-banded RFLP patterns with the other enzyme (Table 2). The average number of RFLP patterns among inbreds per clone-enzyme combination was slightly greater for *Eco*RI (6.29) than for *Hin*dIII (6.01).

Inbred D01 had a 'null' pattern for clone UMC6 with both restriction enzymes, suggesting a deletion in this line at the corresponding map position on the long arm of chromosome 2.

Genetic similarities among unrelated inbreds

The comparison of the genetic similarity of inbreds from the same and different heterotic groups was restricted to the 370 pairs of unrelated inbreds with coancestry coefficient $f<0.1$. GS values for the remaining 65 pairs of related ($f\geq 0.1$) inbreds and their relationship to the corresponding f values will be presented in a separate paper (Brunklaus-Jung et al., in preparation).

Figure 2 shows the histograms for genetic similarities between unrelated lines from the flint and dent heterotic groups. GS estimates for line combinations of type flint × flint and dent × dent had identical means (0.46) and similar ranges (0.40–0.57 and 0.35–0.58, respectively). By comparison, GS estimates for line combinations of type flint × dent had a significantly ($P<0.01$) smaller mean (0.41) and ranged from 0.32 to 0.51. Standard errors of individual GS estimates ranged from 0.033 to 0.035.

GS estimates for individual line combinations between the 10 public flint and 11 public dent inbreds are given in Table 3. Flint inbreds varied in their mean GS to dent inbreds from 0.39 (D144) to 0.43 (D140). Conversely, dent inbreds varied in their mean GS to flint inbreds twice as much, from 0.38 (B73) to 0.45 (D408, CO120). Among dent inbreds, the mean GS to flint inbreds was significantly ($P<0.01$) correlated ($r=0.82$) with the mean GS to the other dent lines, but no such relationship was found for the flint lines.

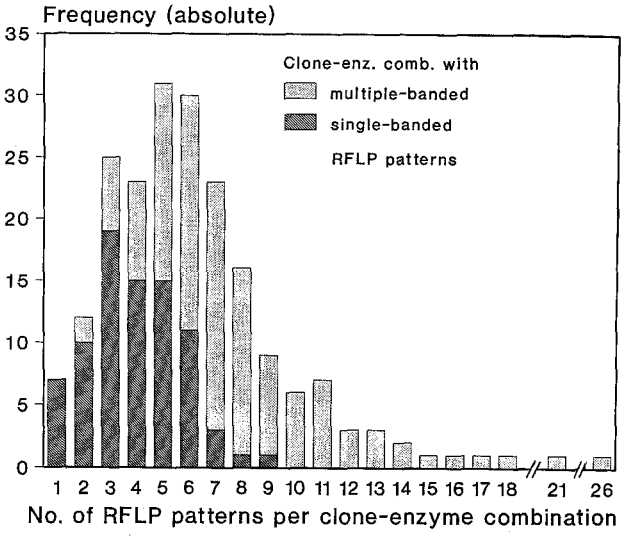

Fig. 1. Histogram of number of RFLP patterns detected among 30 maize inbreds by 82 and 121 clone-enzyme combinations that yielded single-banded and multiple-banded RFLP patterns, respectively

Means and ranges of GS of the four inbreds of miscellaneous origin to unrelated flint and dent inbreds are presented in Fig. 3. Inbreds D145, K6, and K7, which are flint lines with an estimated proportion of 25, 9, and 5% of dent germ plasm, respectively, had a significantly ($P<0.01$) greater mean and a smaller range of GSs to flint than to dent inbreds. Line K8 had almost identical mean and range of GSs to flint (0.41) as to dent (0.42) inbreds, in accordance with its genetic background being 50% flint and 50% dent.

Cluster analysis of RFLP data

Associations among the 30 inbreds revealed by UPGMA cluster analysis based on GS estimates of all 435 line combinations are presented in Fig. 4. Flint and dent inbreds (designated by pyramids and cylinders, respectively) were classified into two main clusters. The flint cluster was subdivided into subclusters of public and private lines. Lower hierarchy clusters traced back to common ancestors, viz., lines related to (1) DK105 (D140, D142, DK105, D143, D146), (2) D107 and F2 (D141, D145), (3) F2 (D144, F192, D503), and (4) EP1 (K2, K3, K4, K6, K7) The dent cluster consisted of three subgroups, viz., (1) eight CO125-related lines (K8, K5, D01, D408, D403) including the subset of A632-related lines (D05, D06,

Table 2. Chromosomal location of DNA clones assayed

Chromosome	Clone designation[a]	No. of clone-enz.	
		Single-banded[b]	Multiple-banded[b]
1	BNL5.62, UMC11, UMC13, UMC119, UMC58, UMC23, UMC37, UMC83, UMC140, UMC129, UMC106, UMC84, BNL6.32	10	16
2	UMC53, UMC5, UMC61, UMC34, UMC131, UMC55, UMC6, UMC98, UMC4, UMC49, UMC36	6	16
3	UMC32, UMC92, UMC10, UMC50, UMC102, UMC18, UMC60, UMC3, UMC16, UMC39[E], UMC63, UMC96, UMC2	10	15
4	UMC87, UMC31[E], UMC47[d], UMC42, UMC66, UMC19, UMC133, UMC15, UMC111	10	7
5	BNL6.25, UMC90, UMC27, UMC1, UMC67, UMC40[Hd], UMC126, UMC54[d], UMC51, UMC108[E], UMC68, UMC35[Ec]	13	8
6	UMC85, UMC59, UMC65, UMC21, UMC46, UMC38, UMC138, UMC132[c], UMC134, UMC28	11	9
7	UMC136, UMC116, UMC110, UMC56[H], UMC45[E], UMC80, UMC151, BNL8.44	6	8
8	BNL13.05, BNL9.11, UMC124, UMC89, UMC12, UMC117[c], UMC30[E], UMC48, UMC93, UMC7	7	12
9	UMC109, UMC113, UMC94, UMC127, UMC81, UMC20, UMC153, UMC114, UMC95, BNL14.28, BNL5.09	4	18
10	BNL3.04[E], UMC130[d], UMC155, UMC64, UMC159, BNL10.13, UMC57, UMC44, BNL7.49	5	12
Total	106	82	121

[a] Clone designation according to the RFLP map of Coe et al. (1988)
[b] RFLP patterns for a given clone-enzyme combination were designated as single-banded, if all inbreds had precisely one RFLP band or a 'null' pattern on autoradiographs; otherwise, they were designated as multiple-banded
[c] Clone yielded monomorphic RFLP pattern in combination with *Eco*RI
[d] Clone yielded monomorphic RFLP pattern in combination with *Hin*dIII
[E] Clone only used in combination with *Eco*RI
[H] Clone only used in combination with *Hin*dIII

Table 3. Dice's similarity coefficient (GS × 100) calculated from RFLP data of 203 clone-enzyme combinations for line combinations between 10 public flint and 11 public dent inbreds

Flint inbred	Dent inbred											
	B73	CG12	CO120	CO151	D01	D02	D05	D06	D403	D408	Mo17	Mean
D140	35.2[a]	39.7	51.5	35.8	43.6	42.5	45.0	44.2	44.0	45.2	41.3	42.5
D141	34.7	40.4	51.5	42.0	48.2	42.3	46.6	48.6	41.7	51.3	38.7	41.2
D142	37.4	40.6	48.2	33.8	41.6	40.5	44.8	45.5	44.0	45.7	42.1	42.2
D143	37.6	42.3	41.9	37.0	42.5	41.8	42.5	43.0	43.6	44.2	40.0	41.5
D144	37.5	39.7	39.5	33.6	41.5	37.7	41.3	38.7	39.7	41.4	39.8	39.1
D146	40.4	35.2	43.5	35.8	39.7	41.9	44.1	43.1	41.1	47.2	39.3	41.0
D503	39.3	41.5	43.5	39.9	36.0	38.8	44.9	43.6	45.4	46.0	44.2	42.1
DK105	37.4	38.2	42.4	32.3	38.1	39.6	44.1	44.3	46.2	46.5	43.8	41.2
F192	38.6	43.9	44.4	38.2	42.2	40.1	44.4	46.1	41.5	45.4	42.1	42.4
F564	37.4	42.5	43.1	38.1	40.0	38.9	44.6	40.8	39.2	39.8	41.7	40.6
Mean	37.6	40.4	45.0	36.7	41.3	40.4	44.2	43.8	42.6	45.3	41.3	41.7

[a] Standard errors for individual GS estimates calculated by the jackknife method (Miller 1974) ranged between 3.3 and 3.5

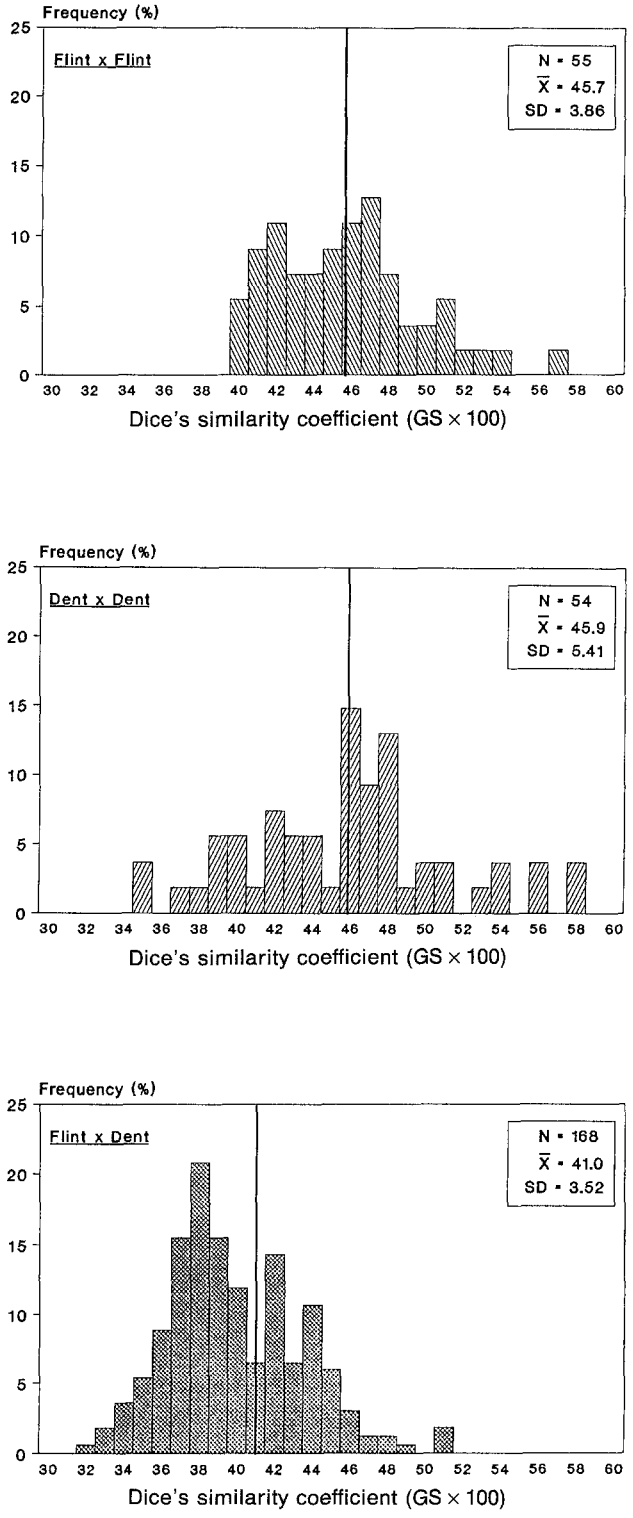

Fig. 2. Histograms of Dice's similarity coefficients (GS x 100), calculated from RFLP data of 203 clone-enzyme combinations, between unrelated ($f<0.1$) inbreds from the European flint and dent heterotic groups. *N* indicates the number of line combinations in each category. Mean is indicated by *solid line*

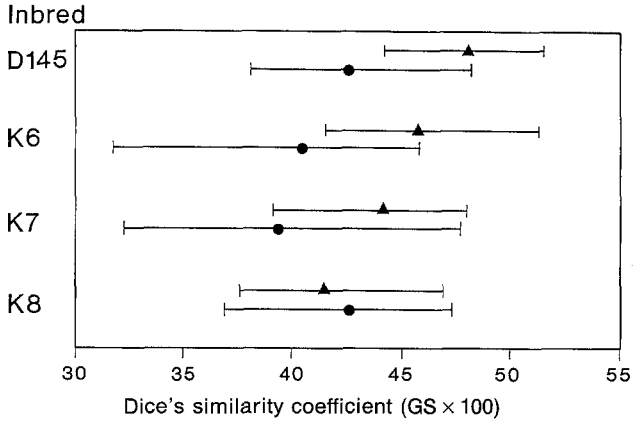

Fig. 3. Mean (indicated by symbols) and range of Dice's similarity coefficients (GS x 100), calculated from RFLP data of 203 clone-enzyme combinations, between four inbreds of miscellaneous origin and unrelated ($f<0.1$) flint (*triangle*) and dent (*circle*) inbreds

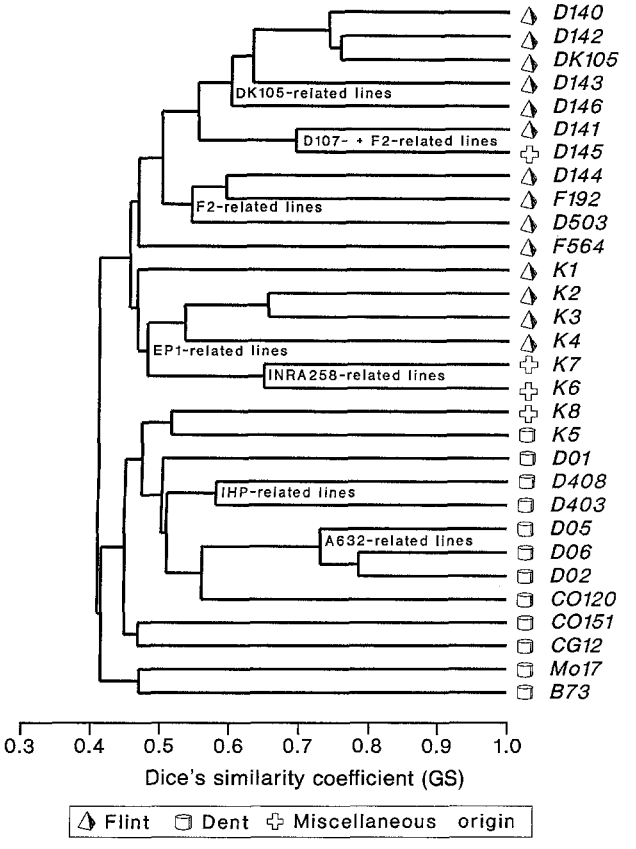

Fig. 4. Dendrogram of 30 maize inbreds revealed by UPGMA cluster analysis based on Dice's similarity (GS) coefficients, calculated from RFLP data of 203 clone-enzyme combinations. Inbreds of flint, dent, and miscellaneous origin are designated by *pyramids*, *cylinders*, and *crosses*, respectively

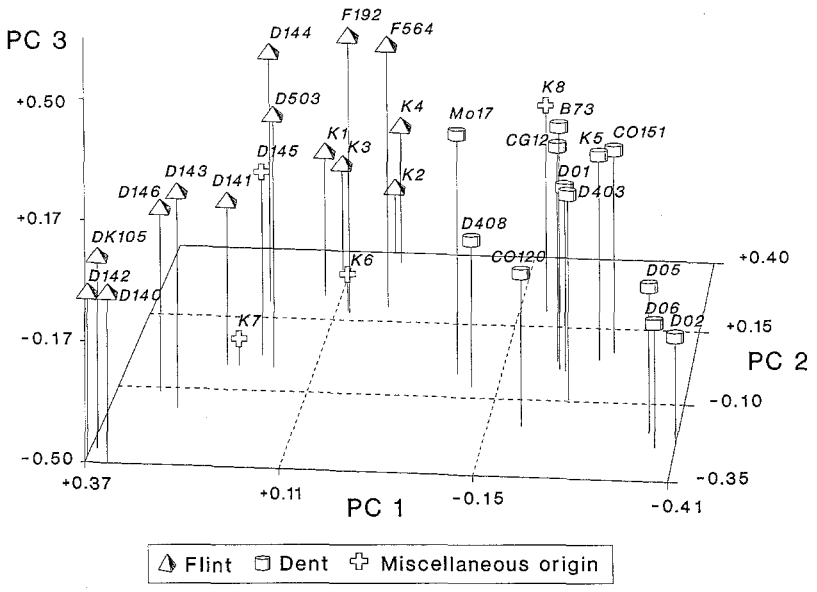

Fig. 5. Associations between 30 maize inbreds revealed by principal coordinate analysis (PCOA) of Dice's similarity (GS) coefficients, calculated from RFLP data of 203 clone-enzyme combinations. Inbreds of flint, dent, and miscellaneous origin are designated by *pyramids*, *cylinders*, and *crosses*, respectively

D02), (2) two Canadian lines (CO151, CG12), and (3) two lines from the U.S. Corn Belt (Mo17, B73). Inbreds of miscellaneous origin (designated by crosses) with predominant flint ancestry (D145, K6, K7) clustered to the flint lines, whereas inbred K8, with equal proportions of flint and dent progenitors, clustered to the dent lines. All lines merged beyond GS > 0.58 were related by pedigree ($f \geq 0.1$).

Principal coordinate analysis of RFLP data

Figure 5 shows associations among the 30 inbreds revealed by principal coordinate analysis (PCOA), based on GS estimates of all 435 line combinations. The first, second, and third principal coordinate (designated as PC1, PC2, and PC3, respectively) explained 11.0, 7.3, and 6.1% of the total variation in RFLP data, respectively. With only a few exceptions, PCOA revealed associations among lines largely consistent with known phylogenetic relationships. Flint lines (pyramids) were clearly separated from dent lines (cylinders) with respect to PC1, but each group was widely spread with regard to PC2 and PC3. Within flint inbreds, three loose groupings were apparent: (a) lines related to GBL (D140, D142, DK105, D143, D146, D141), (b) lines related to French inbreds F2 and F7 (D144, F192, F564), and (c) lines related to EP1 (K2, K3, K4). Lines D503 and D145 were positioned in between the first and second group, in accordance with their pedigree relatedness to each group. Inbreds K6 and K7 of miscellaneous origin were adjacent to the EP1-related and GBL-related lines, respectively, but they deviated from pure flint lines with regard to PC3, suggesting that at least one of their ancestral sources (population 'Rheintaler,' INRA258) is genetically diverse from other flint germ plasm included in the present study.

Within dent lines, two main groupings were apparent: (a) lines recovered from a complex cross with A632 as one parent (D02, D05, D06), and (b) lines highly related with CO125 (D01, D403, K5). Inbreds B73, CG12, and CO151 were positioned adjacent to the latter group despite the absence of any known pedigree relatedness among these inbreds. Dent inbred Mo17 was closest to the flint inbreds, partly because of its relatedness to D145. CO120 and D408 were positioned fairly remotely from the other dent inbreds towards the flint inbreds, although CO125 is a progenitor of PD Synthetic, the main germ plasm source of D408. Line K8 of miscellaneous origin was positioned adjacent to the CO125-related cluster of lines in the direction of F2/F7-related flint lines, reflecting its pedigree relatedness to all three lines.

Discussion

Genetic variation among maize inbreds for RFLPs

Results of recent studies with inbreds from the U.S. Corn Belt (Smith et al. 1990; Melchinger et al. 1991) demonstrated that the number of polymorphisms detectable in maize by RFLPs is high. The present RFLP analyses of 30 early-maturing European maize inbreds (Table 2) confirmed these findings. More than 96% of all 203 examined clone-enzyme combinations revealed RFLPs. The average number of RFLP patterns detected per clone-enzyme combination with single-banded (3.9) RFLP patterns was slightly smaller than for the respective number (4.3) reported for 32 U.S. inbreds (Melchinger et al. 1991). However, unlike in the present study, these authors selected for each clone that restriction enzyme (either *Eco*RI or *Hin*dIII) that provided the greater number of different RFLP patterns.

Clone-enzyme combinations with multiple-banded RFLP patterns revealed about twice as many RFLP patterns and about 50% more distinct RFLP bands than clone-enzyme combinations with single-banded RFLP patterns. Hence, with an equal effort in the lab, the former clone-enzyme combinations have a greater discriminatory power for line identification and for testing of seed purity of inbreds and hybrids. However, their genetic basis is ambiguous. Additionally, unequal numbers of RFLP bands in two inbreds for a given clone-enzyme combination complicate the genetic interpretation of Dice's similarity (GS) coefficients. More than one band (restriction fragment) per inbred on an autoradigraph can be due to (1) repetition of the binding sequence of the DNA probe in the genome, (2) restriction site(s) within the binding sequence, and/or (3) heterozygosity or heterogeneity in the lines. In the first case, the clone hybridizes with more than one RFLP locus, and multiple-banded RFLP patterns should be detected with both restriction enzymes, as was true for more than 50% of the clones yielding multiple-banded RFLP patterns. In fact, duplicate isozyme and RFLP loci, providing evidence for duplicate regions in the maize genome, were reported by Wendel et al. (1986) and Helentjaris et al. (1988). In the second case, the entire RFLP pattern represents an allele of a single RFLP locus. The third case should be of no practical significance in the present study because the lines used were all highly inbred.

Restriction enzymes *Eco*RI and *Hin*dIII revealed both a high level of polymorphism, and are therefore equally well-suited for RFLP analyses in maize. If RFLPs are generated by chromosome mutations, matches or mismatches of hybridization patterns of two lines obtained for a given clone might be associated for two different restriction enzymes. When GS values were calculated separately for each enzyme from RFLP data of the common set of 97 DNA clones, they were significantly ($P<0.01$) correlated with 0.57, indicating that the information obtained from different restriction enzymes is largely independent. Messmer et al. (1991) reported a similar correlation ($r=0.61$) between GS estimates based on RFLP data of 54 clones obtained with *Eco*RI and *Hin*dIII for 21 U.S. inbreds. Deviating from the previous practice of selecting the most polymorphic restriction enzyme for each DNA clone (Godshalk et al. 1990; Melchinger et al. 1990a, b; 1991), Messmer et al. (1991) recommended using all assayed clone-enzyme combinations for calculating genetic similarity or distance coefficients, in order to increase the precision of the corresponding estimates.

Molecular diversity of inbreds within and between the flint and dent heterotic groups

Modern maize hybrids usually represent crosses of inbreds from different heterotic groups, to take advantage of the increased heterosis attributed to increased heterozygosity in inter-pool compared with intra-pool crosses. In agreement with the expectation that lines from different heterotic groups are, on average, less similar (i.e., hybrids are more heterozygous) than those originating from the same heterotic group, the RFLP data showed a smaller mean GS for line combinations of type flint × dent (0.41) than for unrelated line combinations of type flint × flint (0.46) and dent × dent (0.46) (Fig. 2). A similar decrease in similarity of lines from different heterotic groups was reported in a recent RFLP study with the Iowa Stiff Stalk Synthetic (BSSS) and Lancaster Sure Crop (LSC) heterotic groups (Melchinger et al. 1991). In that study, the GS between B73 (a BSSS derivative) and Mo17 (a LSC derivative) was identical to the average GS between BSSS and LSC lines, whereas in the present study, B73 × Mo17 had a considerably greater GS (0.47) than the mean GS (0.41) between flint and dent inbreds. This suggests that the European flint and dent heterotic groups are genetically more divergent than the two main heterotic groups in the U.S. Corn Belt, BSSS and LSC, although further research is needed to corroborate this hypothesis.

For future breeding progress, it is important to maintain or even broaden the genetic variation within the elite flint and dent germ plasm, by developing new inbreds from improved populations undergoing recurrent selection or by introgressing unrelated lines. The danger of genetic erosion in elite breeding materials is best illustrated by the fact that 40% of the flint × flint and 18% of the dent × dent line combinations were related by pedigree ($f\geq 0.1$), because most of the elite lines used in the present study trace back to a few widely used flint (DK105, F2, EP1) and dent (CO125, A632) ancestors. Nevertheless, the low mean and wide range of GS estimates for unrelated flint and unrelated dent inbreds (Fig. 2) indicated substantial genetic variation at the molecular level within each heterotic group.

The wider range of GS values among unrelated dent inbreds compared with unrelated flint inbreds (Fig. 2) was due to the small mean GS of Mo17 (0.42), B73 (0.43), and CO151 (0.44) with other dent inbreds. Mo17 and B73 and their respective parental source populations, LSC and BSSS, are not adapted to cooler climatic conditions, in contrast to the early-maturing dent inbreds used in Europe. Consequently, the genetic diversity of the European dent heterotic group can be broadened by introgressing high-yielding U.S. Corn Belt dent lines into adapted materials and selecting for early vigor and early maturity. Assuming a positive association between genetic dissimilarity at the molecular level and heterosis, this should also increase the yield heterosis of flint × dent crosses, because Mo17 and especially B73 showed a small mean GS (0.40 and 0.37, respectively) to European flint inbreds. Examples of dent inbreds developed by intro-

gressing BSSS germ plasm into adapted materials are line K5 (with 25% B14 and 25% B37 germ plasm) and the three sister lines D02, D05, and D06 (with 47% B14 germ plasm). As expected line K5 had a relatively small mean GS (0.44) to other dent inbreds, whereas the corresponding values for D02, D05, and D06 were fairly large (0.47, 0.50, and 0.49, respectively), suggesting that selection for earliness may have resulted in a lower percentage of B14 genome in the latter lines than expected on the basis of their pedigree.

Within the group of flint lines, inbreds K2 and K4 had a fairly unique RFLP genotype, as indicated by their small mean GS (0.43 and 0.44, respectively) to other flint inbreds. On the other hand, D141 had an exceptionally large mean GS (0.52) to unrelated flint lines. Inbred D141 was derived from a first backcross of line D107 (which was developed from a flint synthetic composed of germ plasm originating mainly from GBL but also from F2 and EP1) with line D102 (containing 75% F2 germ plasm) and, hence, is related to several of the predominant ancestors in the flint heterotic group.

The mean GS of the four lines of miscellaneous origin to unrelated flint and dent lines (Fig. 3) was in agreement with their pedigree background, except for D145. According to pedigree information, D145 should have 25% dent (Mo17) genome. However, based on breeders' experience, D145 behaves more like a 'pure' flint inbred (W. G. Pollmer, personal communication). The RFLP data confirmed this observation in that the GS of D145 × Mo17 (0.48) was considerably smaller than expected from pedigree, suggesting that selection or genetic drift during line development of D145 may have eliminated a greater proportion of the Mo17 genome than expected from pedigree.

Usefulness of RFLPs for assignment of maize inbreds to heterotic groups

Melchinger et al. (1991) pointed out that the usefulness of RFLPs for classification of inbreds to heterotic groups depends on (a) the difference in the mean GS of line combinations between versus those within heterotic groups, and (b) the range of GS for individual line combinations within each heterotic group. In the present study, flint × dent line combinations had a significantly smaller mean GS than unrelated line combinations of type flint × flint and dent × dent, but the range of individual GS estimates in each category overlapped considerably (Fig. 2). Nevertheless, cluster analysis and PCOA performed with RFLP-based GS resulted in a clear separation of flint and dent inbreds (Figs. 4 and 5), consistent with their prior classification based on phylogenetic information (Table 1). Furthermore, each method of multivariate analysis revealed subgroups of lines, such as the DK105-, F2-, and EP1-related inbreds within the flint group and the CO125- and A632-related inbreds within the dent group, that were largely consistent with known pedigree information.

Assignment of inbreds to heterotic groups is especially problematic if lines have been developed from crosses between heterotic groups. Based on RFLP data, it was possible beyond doubt to assign three of the four lines of miscellaneous origin (D145, K6, K7) to the flint heterotic group (Fig. 5) in accordance with their pedigree (Table 1). By comparing the mean GS to each heterotic group (Fig. 3) and considering the GS to each ancestor (e.g., D145 × Mo17), it seems possible to determine the prevalent ancestral sources and even to assess the actual genomic contribution of individual progenitors. This information should be useful for the choice of unrelated testers to evaluate the combining ability of lines of miscellaneous origin.

Cluster analysis proved to be very sensitive in detecting pedigree relatedness among inbreds (Fig. 4). PCOA was less reliable in this regard because inbreds adjacently positioned in the three-dimensional graph do not necessarily have large GS estimates (e.g., B73 × CG12 with GS = 0.35), the main reason being that the first three principal coordinates explained only a small proportion (24.4%) of the total variation. On the other hand, PCOA was more informative than cluster analysis in visualizing the genetic distances between unrelated subgroups of inbreds with similar genetic background. In addition, PCOA accurately portrayed the relationships of the four lines of miscellaneous origin to the pure flint or dent inbreds. Hence, our results suggest that PCOA and cluster analysis should be considered as complementary rather than competitive tools for extracting a maximum of information from RFLP data.

In summary, our findings corroborate the conclusions of recent studies with U.S. Corn Belt maize germ plasm (Lee et al. 1989; Melchinger et al. 1991) that RFLPs are useful for (1) a clearer definition of existing heterotic groups (2) establishment of new heterotic groups, (3) assignment of maize inbreds of unknown genetic background to established heterotic groups, and (4) investigating pedigree relationships among inbred lines. Considering these potential applications, RFLP technology promises to contribute towards an increased efficiency of breeding programs for hybrid maize.

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