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ORIGINAL PAPER

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Assessing temporal changes in genetic diversity of maize varieties using microsatellite markers

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Abstract To quantify genetic diversity among modern and earlier maize cultivars, 133 varieties, representative of the maize grown in France during the last five decades, were fingerprinted using 51 SSR. The varieties were grouped into four periods. For each period, allelic richness, genetic diversity and genetic differentiation among periods were computed. A total of 239 alleles were generated. Allelic richness, in terms of number of alleles per locus, for each period was 4.5, 3.6, 3.9 and 3.6 respectively. Genetic diversity corresponding to Nei's unbiased heterozygosity was calculated, based on allelic frequencies. Values ranged from 0.56 to 0.61. Period I presented the highest genetic diversity, whereas the three other periods all presented a similar value. A great proportion of the total genetic diversity ($H_T = 0.59$) was conserved within all periods ($H_S = 0.57$), rather than among periods ($G_{ST} = 0.04$). The analysis of molecular variance showed that the variation among periods represented only 10% of the total molecular variation. However, the differentiation among periods, although low, was significant, except for the last two periods. Our results showed that the genetic diversity has been reduced by about 10% in the maize cultivars bred before 1976 compared to those bred after 1985. The very low differentiation ($G_{ST} = 0.21\%$) observed among cultivars of the last two decades should alert French maize breeders to enlarge genetic basis in their variety breeding programmes.

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Introduction

With the advent of the first maize hybrids, in 1933 in the US and around 1950 in Europe, maize cultivation has undergone a complete change. Numerous open-pollinated landraces adapted to specific regions were substituted by a limited number of hybrids bred from a large genetic basis. Today, the main maize hybrids cultivated in the world involve a restricted number of key inbred lines. Therefore, genetic diversity of those cultivars is almost certainly limited, in comparison to the large genetic diversity available in genebanks (Gay 1984).

A few years ago, the threat of genetic erosion led to a significant interest in the assessment of genetic diversity in germplasm collections and a huge number of studies on various crops. Until now, numerous studies of maize genetic diversity have been carried out to analyse mainly populations (Dubreuil and Charcosset 1998) or inbreds (Dubreuil and Charcosset 1999). Isozyme, RFLP and more recently, SSR markers, were used (Senior et al. 1998; Gauthier et al. 2002; Labate et al. 2003, etc.). On the contrary, fewer investigations have been done on current breeding germplasm. However, as highlighted by Lu and Bernardo (2001) working on maize inbreds, breeders are worried about a possible reduction of the genetic base in current varieties. American breeders were already concerned by the genetic diversity among their maize hybrids after the Southern corn leaf blight of 1970 (Williams and Hallauer 2000). Maize breeders want to be assured that the genetic base of their cultivars has not become too narrow to face unexpected environmental stresses. Contrary to all expectations, genetic erosion in breeding material is not systematic. Indeed, as reported by Donini et al. (2000) working on UK wheat, no significant narrowing of genetic diversity was detected among winter wheat varieties cultivated between 1934 and 1994. The same results were presented by Manifesto et al. (2001) working on 105 Argentinean wheat cultivars released between 1932 and 1995. More surprisingly, Maccaferri et al. (2003) demonstrated that the level of

genetic diversity present in modern varieties of durum wheat was increasing over time.

In the present study, our objective was to evaluate the impact of the development of hybrid varieties upon maize genetic diversity and erosion, and to determine the proportion of the original landrace genepool transferred to modern hybrid varieties. For this purpose, we analysed genetic diversity among a large panel of French maize hybrids. In order to assess the way in which genetic diversity has been affected during the development of these varieties, the predominant varieties grown in France during the last five decades were fingerprinted with 51 SSR, using efficient semi-automated SSR analysis conditions developed by our laboratory.

Materials and methods

Plant material and DNA extraction

A representative subset of 133 maize varieties was chosen according two criteria: (1) the varieties most cultivated by farmers in France and (2) the impact on the development of maize cultivation in France between 1930 and 2001. Then, hybrids were selected according to their earliness, ranging from late to early and very early varieties (Table 1). As it was not possible to recover seeds for 23 historical hybrids, 45 lines were also analysed in order to deduce the genotypes of those cultivars, called theoretical hybrids. Seeds were provided by GEVES (Le Magneraud) from the French Maize reference collection for the lines and the hybrids and by INRA (Maugio) for all ten populations.

For each cultivar, 20 seeds were ground into fine powder. Bulk DNA extraction was done using the QIAGEN Plant DNeasy Mini Kit in order to obtain high DNA quality required for multiplexing SSR analysis.

SSR analysis

Amplification reactions were performed with a Gene Amp PCR system 9700 thermocycler (Applied Biosystems) in a 10-µl reaction mixture, using a tail primer strategy (Zhang et al. 2003). Each reaction contained 125 µM dNTPs, 3 mM MgCl2, 0.025 µM of primer tailed and 0.25 µM of primer non-tailed for each of the primer pairs used in the multiplex, 0.25 µM of tail M13 (5′-CAC-GAC-GTT-GTA-AAA-CGA- C-3′) or 35S (5′-GCT-CCT-ACA-AAT-GCC-ATC-A-3′) labelled with a fluorescent dye detected at 700 nm or 800 nm, 0.25 U of AmpliTaq Gold (Applied Biosystems) and 2 µl of genomic DNA at 5 ng/µl. The PCR reaction was carried out in a touchdown fashion, with a first denaturation at 94°C for 10 min, followed by ten cycles: denaturation at 94°C for 30 s, annealing at 64°C for 30 s and extension at 72°C for 30 s, the annealing temperature being reduced by 1°C per cycle. This procedure was followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s and a final extension at 72°C for 10 min. After 5 min at 94°C, 0.8 µl of the denaturated sample was loaded on a 5% denaturing acrylamide gel and electrophoresed using a LI-COR 4200 IR2 automated DNA analysis system. Gels were run in 64-well format at 2,000 V, 25 mA, for a maximum of 1 h, depending on the size of the PCR products. Sixty-two SSR proposed by the team of professor A. Melchinger, University of Hohenhein (UHOH) Germany, were tested in our conditions using six public lines. Fifty-five polymorphic SSR were selected according to the quality of PCR amplification. All of them had been mapped onto the ten maize chromosomes. Out of the 55 SSR, only one was not included in the original list proposed by UHOH (Table 2). All primer sequences are available at http://www.agron.missouri.edu/body/ssr. html. Primers were combined into 15 triplexes, eight duplexes, and only two loci were amplified separately. Due to the complexity of SSR profiling, the data generated by four SSR were not used in data analysis. Six SSR were employed in two or three different multiplexes to test the reliability of the PCR.

Data analysis

For each cultivar, allelic frequencies were visually estimated from the intensity of the band on the gel. For example, for one cultivar and one locus, the allelic frequency was 1.0 when there was only one band on the gel, 0.5 for each allele when two bands were detected with the same intensity and 0.25 versus 0.75 for each allele when the intensity of one of the two bands was higher. For a higher number of bands, the same frequency was given to each allele. All the cultivars were grouped into four periods (Table 1) and for each period, Nei's unbiased genetic diversity (Nei 1978) was calculated at each locus (H_{el}) and for all loci (H_e):

$$H_e = \frac{1}{L}\sum_{l=1}^{l=L} H_{el} \quad \text{and} \quad H_{el} = \frac{2n_l}{2n_l - 1}\left(1 - \sum_{a=l}^{a=A_l} (P_{al})^2\right),$$

where P_{al} is the frequency of allele a at locus l in each period, A_1 is the number of alleles detected at this locus, L is the total number of loci analysed and n_l is the number of cultivars characterised for locus l.

In order to compare the genetic diversity among the four periods, we computed population differentiation parameters as explained by Dubreuil and Charcosset (1998). For this purpose, we considered that one population corresponded to all the cultivars of one period. For each two periods, the total genetic diversity (H_T) was partitioned into within-population diversity (H_S) and among-population diversity (D_{ST}). The coefficient of genetic differentiation was also evaluated using $G_{ST} = D_{ST}/H_T$ (Nei 1973).

Table 1 Cultivars used in the current study, together with their year of release, type, earliness, and for populations the geographical origin

Period	Number	Year	Variety[a]	Type of cultivars	Earliness
I (< 1975)	1	1950	*W240*	DH	Very early
	2	1950	*W255*	DH	
	3	1957	*INRA 200*	DH	
	4	1958	*INRA 258*	DH	
	5	1971	*CP170*	TWH	
	6	1951	*W355*	DH	Early
	7	1951	*W416*	DH	
	8	1961	*INRA 260*	TWH	
	9	1962	*INRA 270*	DH	
	10	1970	*LG 11*	TWH	
	11	1973	*STAR 304*	TWH	
	12	1951	*IOWA 4417*	DH	Late
	13	1960	*INRA 640*	DH	
	14	1962	*INRA 430*	DH	
	15	1962	*ILLINOIS 3152*	DH	
	16	1969	*INRA 508*	SH	
	17	1962	*INRA 570*	DH	
	18		Bade	P-Alsace	
	19		Wagonville	P-Nord	
	20		Ain	P-Ain	
	21		Roux de Chalosse	P-Chalosse	
	22		Estarvielle	P-Htes Pyrénées	
	23		Landes	P-Landes	
	24		Grand roux Basque	P-Vallée de l'Adour	
	25		Millette du Lauragais	P-Lauragais	
	26		Millette du Finham	P-Tarn et Garonne	
	27		Ruffec	P-Charente	
II (1976–1985)	28	1978	KEO	TWH	Very early
	29	1980	LEADER	TWH	
	30	1982	EMA	TWH	
	31	1983	RAMI	TWH	
	32	1980	*Brulouis INRA 180*	TWH	
	33	1985	*Browning INRA 150*	TWH	
	34	1977	CUZCO 251	TWH	Early
	35	1980	DEA	SH	
	36	1982	DERBY	TWH	
	37	1980	BRUSSOL	TWH	
	38	1983	MONA	SH	
	39	1983	CELTIC	TWH	
	40	1984	ATHENA	TWH	
	41	1980	*BRUEX*	TWH	
	42	1981	EVA	SH	Late
	43	1985	COLT	SH	
	44	1977	*ROC*	SH	
	45	1980	*INRA 440*	TWH	
	46	1982	*MOHICAN*	TWH	
III (1986–1995)	47	1986	AVISO	TWH	Very early
	48	1987	ASTRID	TWH	
	49	1988	CORALIS	TWH	
	50	1989	AREM	TWH	
	51	1989	DK205	SH	
	52	1990	DK200	SH	
	53	1990	RIVAL	SH	
	54	1991	CARAIBE	TWH	
	55	1992	GRANAT	SH	
	56	1992	SIMBAD	SH	
	57	1993	ANTARES	SH	
	58	1993	LG2230	SH	
	59	1994	IMPACT	SH	
	60	1994	LOFT	SH	
	61	1995	EMIRIS	SH	
	62	1995	MANATAN	SH	
	63	1995	PASSI	TWH	
	64	1986	CARGIVOLT	TWH	Early
	65	1986	DK250	SH	
	66	1987	PRIAM	SH	
	67	1988	DK415	SH	
	68	1989	ANJOU37	SH	

Table 1 (Contd.)

Period	Number	Year	Variety[a]	Type of cultivars	Earliness
	69	1989	NOBILIS	SH	
	70	1990	MAGDA	SH	
	71	1990	VDH295	TWH	
	72	1991	FANION	SH	
	73	1991	MAGISTER	SH	
	74	1991	TIKI	SH	
	75	1992	BANGUY	SH	
	76	1992	NOELLA	SH	
	77	1992	PACTOL	SH	
	78	1993	BEMOL	SH	
	79	1993	CHERIF	SH	
	80	1994	ANJOU 285	SH	
	81	1994	AQUI	SH	
	82	1994	TOTEM	TWH	
	83	1995	CLARICA	SH	
	84	1995	TWIN	SH	
	85	1986	DK524	SH	Late
	86	1987	VOLGA	SH	
	87	1988	ARIANE	SH	
	88	1988	FURIO	SH	
	89	1989	AMPLOR	SH	
	90	1990	MARISTA	SH	
	91	1991	MONDAIN	SH	
	92	1991	RANDA	SH	
	93	1992	CECILIA	SH	
	94	1992	PERCEVAL	SH	
	95	1993	CERVIA	SH	
	96	1993	SAMSARA	SH	
	97	1994	DK479	SH	
	98	1994	DURANDAL	SH	
	99	1995	EDEN	SH	
	100	1995	ALVINA	SH	
IV(> 1996)	101	1996	PASTORAL	TWH	Very early
	102	1996	SEMIRA	SH	
	103	1997	DK217	SH	
	104	1997	RAFALE	TWH	
	105	1998	RICHMOND	SH	
	106	1999	KLEOPATRA	SH	
	107	1999	ANJOU220	TWH	
	108	2000	DK255	SH	
	109	2000	SISSI	SH	
	110	1996	DJANGO	SH	Early
	111	1996	IVOIRIS	SH	
	112	1996	PRINZ	SH	
	113	1997	DK246	SH	
	114	1997	FLORES	SH	
	115	1998	HIFI	SH	
	116	1998	LG 2280	TWH	
	117	1999	KUXXAR	SH	
	118	1999	TEXTO	SH	
	119	2000	EUROSTAR	SH	
	120	2000	MONUMENT	SH	
	121	2001	CHANTILLI	SH	
	122	1996	MANAGUA	SH	Late
	123	1996	SALSA	SH	
	124	1997	CIGAL	SH	
	125	1997	DAHIR	SH	
	126	1998	NAUDI	SH	
	127	1998	REMIA	SH	
	128	1999	PANAWAX	SH	
	129	1999	FIDJI	SH	
	130	2000	OPEN	SH	
	131	2000	ENERGETIC	SH	
	132	2001	ANDRIS CL	SH	
	133	2001	GIBSI	SH	

[a]Varieties in *italics* are theoretical hybrids: *SH* single hybrid, *DH* double hybrid, *TWH* three-way hybrid, *P* population

Table 2 SSR markers used to genotype 100 hybrids, 10 populations and 45 inbred lines. SSR loci amplified in different multiplexes appear in *italics*. *H* Hybrids, *P* populations

SSR combination	Tail	Locus	Repeat motif	Number of alleles per locus					
				Period I			Period II	Period III	Period IV
				H + P	H	P			
Triplex 1	35S	phi 015	AAAC	4	4	4	4	4	3
		phi 109275	AGCT	5	4	4	4	4	4
		phi 053	ATAC	6	5	5	7	4	4
Triplex 2	M 13	umc 1143	AAAAT	5	5	4	6	6	6
		phi 423796	AGATG	6	5	4	3	5	5
		phi 448880	AAG	4	3	4	2	3	3
Triplex 3	M 13	*phi 333597*	AAG	3	3	3	3	3	3
		phi 448880	AAG	4	3	4	2	3	3
		umc 1161	(GCTGGG)5	5	5	3	4	5	4
Triplex 4	M 13	*phi 333597*	AAG	3	3	3	3	3	3
		phi 448880	AAG	4	3	4	2	3	3
		phi 233376	CCG	6	5	5	3	5	6
Triplex 5	M 13	*phi 333597*	AAG	3	3	3	3	3	3
		phi 452693	AGCC	7	4	5	5	4	3
		umc 1152	(ATAG)6	7	7	4	6	6	5
Triplex 6	35S	umc 1489	(GCG)5	3	3	2	3	3	2
		umc 1180	(CATG)5	2	2	2	2	2	2
		phi 084	GAA	2	2	1	2	2	2
Triplex 7	35S	phi 308090	AGC	2	2	2	2	2	2
		umc 1122[a]	(CGT)7						
		umc 1153	(TCA)4	4	4	3	4	4	4
Triplex 8	35S	phi 374118[a]	ACC						
		phi 079	AGATG	5	4	4	3	4	3
		phi 127	AGAC	6	5	4	4	4	4
Triplex 9	35S	*phi 079*	AGATG	5	4	4	3	4	3
		phi 128	AAGCG	4	4	3	4	4	3
		phi 072	AAAC	4	3	4	3	5	4
Triplex 10	M 13	phi 069	GAC	5	5	3	4	4	4
		phi 116	ACTG/ACG	5	4	4	4	5	3
		umc 1887	(CGA)4	4	4	4	4	4	4
Triplex 11	35S	phi 96100[a]	ACCT						
		phi 084	GAA	2	2	1	2	2	2
		phi 083	AGCT	5	4	5	4	5	4
Triplex 12	35S	phi 213984	ACC	2	2	2	3	3	3
		phi 032	AAAG	4	3	3	4	3	3
		phi 079	AGATG	5	4	4	3	4	3
Triplex 13	35S	umc 1641	(TCGCC)4	7	7	4	6	6	6
		nc 130	AGC	3	3	2	3	2	2
		phi 064	ATCC	8	6	8	7	8	7
Triplex 14	M 13	*phi 089*	ATGC	3	3	2	2	2	2
		phi 123	AAAG	3	3	3	3	3	3
		phi 396160	AGGCG	3	3	3	3	4	3
Triplex 15	35S	phi 101049	AGAT	9	7	6	4	7	7
		phi 104127	ACCG	2	2	2	2	2	2
		phi 331888	AAG	4	4	1	3	2	2
Duplex 1	35S	phi 427913	ACG	4	4	4	3	4	4
		umc 1329	(GCC)7	3	3	3	3	4	4
Duplex 2	M 13	phi 114	GCCT	6	6	5	4	5	4
		umc 1061	(TCG)6	4	4	3	2	2	2
Duplex 3	M 13	umc 1675	(CGCC)4	4	4	3	3	3	3
		phi 050	AAGC	3	2	1	3	4	3
Duplex 4	35S	phi 093	AGCT	5	5	3	3	4	3
		phi 011	AGC	5	4	4	4	3	3
Duplex 5	M 13	umc 1545	(AAGA)4	4	4	4	4	4	4
		umc 1304	(TCGA)4	5	3	4	2	3	4
Duplex 6	M 13	*phi 065*	CACTT	5	4	4	4	4	4
		umc 1279	(CCT)6	3	3	3	2	3	3
Duplex 7	M 13	*phi 065*	CACTT	5	4	4	4	4	4
		phi 420701	CCG	6	5	4	5	6	6
Duplex 8	M 13	*phi 089*	ATGC	3	3	2	2	2	2
		phi 100175	AAGC	4	4	2	4	3	3
Simplex 1	M 13	phi 041	AGCC	4	4	3	4	5	5
Simplex 2	35S	phi 102228	AAGC	3	3	3	3	3	3
Simplex 3	35S	umc 1169[a]	(TTA)4						

[a]SSR not retained for analysis

To statistically assess genetic variation within and among periods, we performed an analysis of molecular variance (AMOVA, see Excoffier et al. 1992) with the software package Arlequin, version 2000 (Schneider et al. 2000). A classical analysis of variance on the Euclidean squared distances was done among haplotypes grouped into the four periods. Probabilities of variance components were estimated from 1,000 random permutations.

To investigate relationships among cultivars, principal coordinate analysis (PCoA) was carried out on a matrix of Sokal and Micheners' distances, using Darwin, version 4.0, software (Perrier et al. 2003). For computations with Arlequin and Darwin as for most of the population genetic analysis software, each cultivar was coded in a biallelic way when working with codominant markers. Some bulks displayed more than two alleles per locus due to the fact that we worked with double and three-way hybrids, which are not homogeneous cultivars, and with inbred lines presenting a residual non-uniformity for some loci. Thus, it was not possible to compute a data file with this raw information ready to use with the software. To deal with this problem, we relied on the presence and absence of alleles at each locus and computed a 0/1 matrix. In this case, data were considered as dominant markers.

Results

Allelic richness–allelic diversity

A total of 239 alleles were observed by analysing 51 loci. The number of alleles ranged from two to nine, with an average value of 4.68 (Table 3). Cultivars from period I exhibited the highest allelic richness, with 4.45 alleles per locus. Rare alleles (i.e. frequency lower than 5% in one period) were not found for the cultivars of the period I (when looking at the populations and the hybrids separately) and for period II, whereas 34 and 20 rare alleles were detected for periods III and IV, respectively. When looking at the number of alleles specific to period I versus period IV, 22% of the total number of alleles observed in period I was not recovered in period IV, whereas ten new alleles were detected in cultivars of period IV (data not presented).

Table 3 General statistics on 133 cultivars

Period	Cultivars	Sample size	Number of alleles	Mean number of alleles per locus
I	H	17	204	3.83
	P	10	176	3.40
	Total	27	225	4.45
II	H	19	183	3.59
III	H	54	199	3.90
IV	H	33	185	3.63
All periods		133	239	4.68

Genetic diversity within periods

The genetic diversity (H_e) of the four periods was high on average (0.59) and varied from 0.56 to 0.61 (Table 4). It also varied greatly among loci. It was slightly higher for period I and was very similar for the three other periods.

Genetic differentiation among periods

The comparison of the gene diversity among the four periods showed that the total gene diversity (H_T) of two different periods essentially originated from the gene diversity within a period (H_S); the gene diversity among periods (D_{ST}) accounted for less than 10% of the total gene diversity for all pairs of periods (Table 5). The diversity among two periods was low, ranging from 0.0021 to 0.0622 for periods III/IV and periods I/IV, respectively. AMOVA showed that the molecular diversity was significantly different ($P < 0.001$) among all periods and also between two periods, except for the periods III and IV (Table 6). As previously shown, the genetic differentiation increased progressively with time, the most important values being obtained for period I and periods III/IV.

Associations among the cultivars revealed by PCoA were represented in Fig. 1.

The first three components explain about 24% of the total variation, with 11.1, 8.2 and 4.5% for the first, the second and the third component, respectively. For cultivars of the period II, III and IV, the first axis exhibits a separation according to earliness, with only a few exceptions. Axis 2 highlights differentiation according to time. Cultivars of the period I are widely dispersed in the right part of the scatter plot. The populations are clustered in the upper part of the plot. Hybrids of the period II are intermediate between historical and modern hybrids whereas for the cultivars of the last two periods, the overlapping nature of the diversity is clear.

Discussion

Microsatellite loci have proven their efficiency as genetic markers to assess genetic diversity in numerous plant species. Until now, SSRs have been used on maize for mapping (Senior and Heun 1993; Taramino and Tingey 1996), genetic fingerprinting (Smith et al. 1997; Senior et al. 1998) and to assess genetic diversity among inbred lines (Lu and Bernardo 2001; Enoki et al. 2002; Liu et al. 2003). Apart from Matsuoka et al. (2002) realizing multiplex PCR for evolutionary studies, all other studies were conducted with SSR loci which were amplified and

Table 4 Average Nei's genetic diversity and standard deviation (*SD*) calculated for each period

Period	Genetic diversity (H_e)	SD	Genetic diversity per locus (H_{el})	
			Minimum value	Maximum value
I	0.61	0.14	0.15	0.84
II	0.57	0.14	0.10	0.80
III	0.56	0.12	0.25	0.76
IV	0.56	0.13	0.20	0.94
Total	0.59	0.10	0.31	0.79

Table 5 Population differentiation parameters for subsets of maize cultivars belonging to four different periods

	Gene diversity			
	Total (H_T)	Within (H_S)	Among (D_{ST})	(G_{ST})
Periods I–II	0.6005	0.5911	0.0095	0.0158
Periods I–III	0.5933	0.5747	0.0186	0.0313
Periods I–IV	0.6183	0.5797	0.0386	0.0624
Periods II–III	0.5653	0.5592	0.0062	0.0109
Periods II–IV	0.5730	0.5588	0.0142	0.0248
Periods III–IV	0.5600	0.5562	0.0037	0.0021
All periods	0.5912	0.5686	0.023	0.0382

Table 6 Partition of variation from analysis of molecular variance (Arlequin, version 2.000) among four periods on 229 markers. Φ_{ST} values correspond to the fraction of the molecular diversity accounted by the factor 'period' (significance level is *above* the diagonal). NB Φ_{ST} value among periods I and II, I and III calculated on 234 markers 1 and 4:222 markers

	Period I	Period II	Period III	Period IV
Period I		***	***	***
Period II	0.094		***	***
Period III	0.162	0.061		**NS**
Period IV	0.169	0.100	0.001	
		Φ_{ST}	Prob.	
Among all periods		0.097	<0.001	

run individually. In the present study, we relate the use of SSR multiplexing in maize for both PCR amplification and gel electrophoresis. Using triplexes in a PCR reaction (i.e. combination of three primer pairs) and reloading the same gel three times consecutively, we were able to analyse nine microsatellite loci per gel. This gives a very low cost for routine SSR analysis in maize. The mean number of alleles detected on the 178 cultivars (4.7) was similar to the one obtained by Lu and Bernardo on 40 US maize inbreds (4.9) and slightly lower than previously determined by Senior et al. (1998) on 94 US inbreds (5.2) or Matsuoka et al. (2002) on 101 inbreds (6.9). According to Senior et al. (1998), their results may be explained by the use of dinucleotide repeats, which in general displayed a higher number of alleles than tri- and tetranucleotide repeats. For example, five dinucleotide repeat SSR markers allowed the detection of between 10 and 23 alleles per locus. If the dinucleotide repeat-based SSRs are removed, the number of alleles comprises between two and nine per locus, with an average of 4.2, which is consistent with our results. This feature was also highlighted by Liu et al. (2003). The amplification with dinucleotide repeats may result in scoring problems because of artifactual 'stutter' bands. Therefore, we decided to not use them in our study.

The allelic richness of periods II, III and IV was very similar and lower compared with that of period I, and reflects changes occurred in French maize breeding. Indeed, with the advent of hybrids, populations were progressively replaced. Consequently, the maize varieties became more and more homogeneous. As explained by Dubreuil and Charcosset (1999) regarding the number of alleles specific to lines and populations, the obvious deficit of alleles within lines can partly be explained by genetic improvement. During the last decades, double hybrids were replaced by three-way hybrids and single hybrids. Therefore, the most important reduction in allelic richness was observed between historical cultivars of period I and modern cultivars of period IV. As suggested by Allard (1996), the reduction in allelic diversity was not only due to plant breeding, but also largely to the elimination of deleterious alleles by selection rather than erosion. The mean genetic diversity estimated in the present research was 0.59, which is very close to that determined by Senior et al. (1998). Again, values were similar for periods II, III and IV and slightly higher for period I. Therefore, we state that no drastic reduction in genetic diversity has occurred during the last five decades. Moreover, the advent of new alleles in modern cultivars gives evidence of the introduction of new genetic material in breeding programmes. Most of the total genetic diversity ($H_T = 0.5912$) was explained by the genetic diversity within period ($H_S = 0.5686$), showing that a great proportion of the genetic diversity was maintained in each period. Some differentiations were perceptible in cultivars of period I compared to those of period IV, whereas very low differentiation was found among cultivars of the periods III and IV. As explained previously, the main forms of cultivars for the last two decades have been single hybrids (more than 80%), whereas before 1975, populations followed by double hybrids were predominant. Even if absolute values for the Φ_{ST} parameter, analogous to F_{ST} (Wright 1951) and G_{ST} (Nei 1973) parameters were higher than those obtained for G_{ST}, the general trend was the same. AMOVA showed that only 10% of the total molecular variation was explained by the variation among periods. Even if low, the differentiation among periods was significant, except for the last two periods. According to the factorial analysis, if we connect the extremes of each period to assess the extent of the diversity, as previously done by Donini et al. (2000) on wheat, the size of the shape was not very

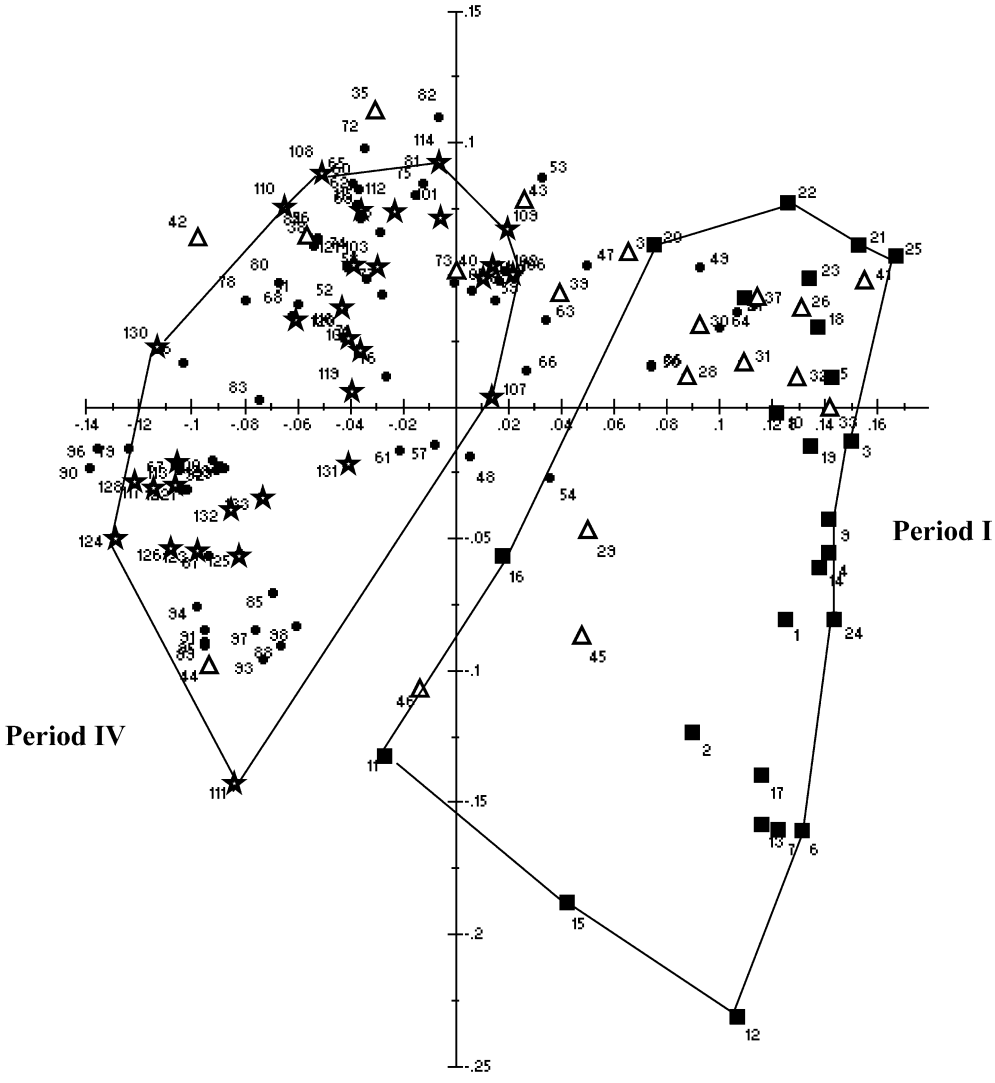

Fig. 1 Plot of the first two components derived from the principal coordinate analysis on the SSR data. For clarity, the *lines* join the extremes of periods I and IV. A cultivar is referenced by its number contained in Table 1 and a symbol: *black squares* period I, *triangles* period II, *rounds* period III, *stars* period IV

different from one period to another. One striking fact was that late cultivars of the period III and IV, respectively, were more closely related than the early and very early cultivars of the same periods. This leads us to imagine that the genetic basis employed for the selection of late cultivars is narrower than that used for early ones.

In conclusion, results obtained from allelic richness, genetic diversity, differentiation parameters, AMOVA and PCoA are consistent. The genetic diversity has been reduced by about 10% from the maize cultivars bred before 1976 to those bred after 1985. However, a great proportion of the genetic diversity is conserved in each period. The genetic diversity maintained in the historical cultivars is not exactly the same as the one conserved in the modern cultivars. Nevertheless, temporal changes are more qualitative than quantitative. The very low differentiation observed among cultivars of the last two decades could be worrying. Therefore, it seems reasonable to enlarge the breeder's genetic basis as already done in the past, with the introduction of French material in breeding programmes since 1960. It is important to mention that the present analysed genetic diversity was only expected to be representative of the major varieties grown in France (utilised diversity) and not representative of the maize diversity available in gene banks.

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