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Molecular mapping in tropical maize (*Zea mays* L.) using microsatellite markers. 1. Map construction and localization of loci showing distorted segregation

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Microsatellites have become the most important class of markers for mapping procedures. Primarily based on restriction fragment length polymorphism (RFLP) markers, several molecular genetic maps of maize have been developed, mainly using temperate inbred maize lines. To characterize the level of polymorphism of microsatellite loci and construct a genetic map in tropical maize, two elite inbred lines, L-08-05F and L-14-4B, were crossed to produce 400 F_2 individuals that were used as a mapping population. A survey of 859 primer pair sequences of microsatellites was used. The polymorphism screens of each microsatellite and genotype assignment were performed using high-resolution agarose gels. About 54 % of the primer sets gave clearly scorable amplification products, 13 % did not amplify and 33 % could not be scored on agarose gels. A total of 213 polymorphic markers were identified and used to genotype the mapping population. Among the polymorphic markers, 40 showed loci deviating from expected Mendelian ratios and clusters of deviating markers were located in three chromosome regions. Non-Mendelian scoring was present in 19 markers. The final genetic map with 117 markers spanned 1634 cM in length with an average interval of 14 cM between adjacent markers.

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Extensive genome mapping, based on restriction fragment length polymorphism (RFLP) markers, has been developed in maize (HELENTJARIS et al. 1986, 1988; BURR et al. 1988; WEBER and HELENTJARIS 1989; GARDINER et al. 1993; COE et al. 1995). These maps have been used for a number of applications in genetic research and breeding, including the mapping of several genes contributing to agronomically relevant traits, marker-assisted selection (MAS), quantitative trait loci (QTL) detection and positional cloning (TANKSLEY 1993; LEE 1995; KHAVKIN and COE 1997). However, there are several disadvantages regarding RFLP marker systems that make the analysis of large populations an expensive and time-consuming process. The development of the polymerase chain reaction (PCR – MULLIS and FALLONA 1987) and the advent of PCR-based markers, mainly microsatellites, provided new marker systems where the analyses are more efficient and inexpensive.

Microsatellites, also termed as simple sequence repeats (SSRs) (LITT and LUTY 1989) or short tandem repeats (STRs) (EDWARDS et al. 1991), are genomic regions which are comprised of highly variable tandem repeats of two to six DNA nucleotides. Microsatellite markers are highly polymorphic, co-dominant, widely disperse in diverse genomes, and mapped to a single locus. Moreover, they are easy to be assayed by polymerase chain reaction methodology and their dissemination is straightforward among laboratories, making them very valuable genetic markers for mapping analysis (POWELL et al. 1996). Similar to human and other mammalian genomes (JACOB et al. 1995; DIB et al. 1996; DIETRICH et al. 1996), microsatellite markers have been developed

and integrated into several existing plant linkage maps, such as rice (CHEN et al. 1997), wheat (RÖDER et al. 1998), potato (MILBOURNE et al. 1998), *Eucalyptus* (BRONDANI et al. 1998), soybean (CREGAN et al. 1999), and sorghum (KONG et al. 2000). In maize, microsatellites were very useful as genetic markers for determining genetic similarities and relationships (SMITH et al. 1997; SENIOR et al. 1998; BARBOSA et al. 2003) and for genetic mapping (SENIOR and HEUN 1993; SENIOR et al. 1996; TARAMINO and TINGEY 1996; DAVIS et al. 1999).

However, molecular markers mapped in such works, RFLPs or microsatellites, helped to further characterize the temperate maize genome, mainly representing a range of U.S. Corn Belt germplasm. Few studies have dealt with mapping on tropical maize material (BOHN et al. 1997; RIBAUT et al. 1997; GROH et al. 1998; KHAIRALLAH et al. 1998). The U.S. maize crop has a relatively narrow germplasm base because of the high levels of relatedness among the parents of current elite cultivars (SMITH 1988). In contrast, tropical maize populations have a broad genetic base, formed usually by composites with larger variability than temperate synthetic materials (LANZA et al. 1997). The development of a second-generation genetic map for tropical maize germplasm, based on a wide set of microsatellite markers in a large F_2 mapping population, should provide more information on tropical maize genome and the level of similarities and differences between divergent genetic backgrounds.

The common approach employed for the analysis of microsatellites is radioactive labeling of the PCR product, followed by electrophoresis on denaturing polyacrilamide gels. An alternative for radioactive labeling is the silver staining of polyacrilamide gels. The most recent method is the use of fluorescent dyes for the automated detection of alleles. These methods offer the best resolution as single repeat differences can be resolved and thus all possible alleles detected, but they require special equipment and/or quite a lot of manipulation, which makes the process time-consuming for large experiments, expensive and impossible to be carried out in non-radioactive laboratories.

For a quick polymorphism screen of each microsatellite and later genotyping, the approach employed for allele detection in our study was the high-resolution agarose gel system stained with ethidium bromide (SENIOR and HEUN 1993; SENIOR et al. 1998). This method has a lower resolution, but makes the process of scanning a large number of loci, in a large mapping population, faster and less expensive.

The objectives of our study were: (I) the development of a second-generation genetic map based on microsatellite markers in a tropical maize F_2 mapping population; (II) to determine the level of polymorphism of microsatellite markers in a tropical maize genetic base; (III) to provide more information in terms of usefulness on the least costly and most widely available agarose gel system to solve microsatellite polymorphisms.

MATERIAL AND METHODS

Plant material

Two maize inbred lines showing contrasting behavior for grain yield were used: L-08-05F and L-14-4B. These inbreeds belong, respectively, to IG-1 and BR-106 maize populations and this crossing was selected because of differences between parents in terms of plant height, kernel type, maturity and yield. In addition, their cross produces a highly heterotic hybrid. By crossing these lines, F_1 plants were obtained which were selfed, generating the F_2 populations. As homozygous inbreds were used, every F_1 hybrid obtained from the crossing between the two parental lines was identical. In this way, to increase the number of seeds available for posterior evaluations, F_2 seeds from four selfed F_1 hybrid plants were used for generating the mapping population.

DNA extraction

Leaf material from parents, F_1 and F_2 generations were collected, freeze-dried, ground to powder and stored at $-20°C$ in individually labeled vials. DNA extraction followed the procedures described in HOISINGTON et al. (1994).

Microsatellite primer selection

The accumulated information on molecular markers in maize allows the designation of a set of core RFLP markers evenly spaced throughout the genome. These markers have facilitated the dissection of the maize genome into 100 evenly spaced "bins" of approximately 20 cM each (GARDINER et al. 1993). First, two or three microsatellites per bin were chosen for the polymorphism screen. Next, microsatellite selections were addressed to (1) bins where no polymorphic marker was found after tests; and (2) bins that contained polymorphic microsatellites but presented erratic amplification, fuzzy or indistinct bands during the genotyping of the mapping population.

Sequences of 859 primer pairs to amplify maize microsatellites were obtained from the MAIZE DATA BANK (2001), and synthesized by Gibco BRL (São Paulo, SP). When available, tetranucleotides repeats or even a greater number of repeats, were preferably selected to relate to di- or trinucleotide ones. Independently of the repeat type, microsatellite loci with

higher repeat counts and perfect motif were the first choice.

By means of agarose gels, the primer pairs were screened against the parents and the four F_1 plants. In this way, it was possible to confirm the homozygosous state of the parental inbred and the genetical identity of the F_1 hybrids used to originate the mapping population. Microsatellites showing the clearest polymorphism among the parents were then used for genotyping. Locus designation, chromosome location, primer sequence information, description of microsatellite motif, and size of PCR product amplified in reference lines, can be found in the MAIZE DATA BANK (2001).

Amplification conditions

The reactions were carried out in 96-well microtiter plates using a PTC-100 Thermal Cycler (MJ Research, Watertown, MA). The amplification consisted of a 2 min denaturation step at 94°C followed a by modified ''touchdown'' program (DON et al. 1991). This program began with two 1 min cycles at 94°C, 1 min at 65°C and 2 min at 72°C. The annealing temperature was then reduced by 1°C every two cycles until a final annealing temperature of 55°C was reached. The last cycle was repeated 20 times and was terminated with a continuous cycle at 15°C. The 20 µl reaction mix consisted of 1 × reaction buffer (20 mM Tris–HCl, 50 mM KCl; pH 8.4), 2 mM $MgCl_2$, 100 µM of each dNTP, 0.5 units of Taq DNA polymerase (GIBCO BRL, São Paulo, SP), 0.2 µM of each primer, 50 ng of template DNA and ddH_2O. Reactions were stopped with 2 µl loading-dye (500 µl/ml glycerol, 20 mM EDTA, 0.6 mg/ml bromophenol blue). After amplification, a total of 22 ul of the mix was loaded into the wells of a 1 mm wide comb and products were separated by electrophoresis in a horizontal gel system (Horizontal 20:25, GIBCO BRL, Gaithersburg, MD) using 0.5 × TBE (SAMBROOK et al. 1989) on 4 % agarose gel (2 % ultra pure agarose, GIBCO BRL, Gaithersburg, MD: 2 % Metaphor agarose; FMC BioProducts, Rockland, ME). The gel ran at approximately 170V for 1.5 h and was stained with 0.5 µg/ml ethidium bromide. Two different workers independently performed the visual score of the gel. Agarose gels were reused at least three times after running by remelting.

When the primers failed to amplify using the ''touchdown'' program, the amplification was performed with low stringency annealing temperatures. This program began with a 2 min denaturation step at 94°C, followed by 30 1 min cycles at 94°C, 1 min at 48°C to 52°C (according to each primer pair optimized annealing temperature) and 2 min at 72°C, and terminated with a continuous cycle at 15°C. When banding patterns were difficult to score accurately on agarose gels, electrophoresis was conducted at a lower voltage (100 V) and at an increased running time (5 to 8 h).

Segregation and genome composition

Segregation at each marker locus was checked for deviations from Mendelian segregation ratio 1:2:1 by chi-square tests. Taking into account that multiple tests were performed, appropriate type I error rates were determined by the sequential rejective Bonferroni procedure (RICE 1989). Estimates of the proportion of parental genome for each individual were obtained according to PATERSON et al. (1991).

Linkage analysis

Linkage analysis was performed using the MAPMAKER/EXP v.3.0 program (LANDER et al. 1987; LINCOLN et al. 1992). Linkage was inferred using a LOD ($\log_{10}$ of the odds ratio) threshold of 3.0 and maximum distance between two loci of 50 cM for forming linkage groups. Two-point linkage analysis was conducted in order to estimate the maximum likelihood recombination frequency, and linkage groups were established using the ''group'' command. Linkage analyses were then conducted to determine the most likely order of loci within groups using ''compare'' command for smaller linkage groups, obtaining the likelihood of all possible orders. For large linkage groups, a framework was established and the ''ripple'' command was used to confirm marker order as determined by multipoint analysis. Data quality was checked using ''error detection'' command, and unlikely double crossovers, due to possible genotyping errors, were corrected by rechecking the data. Crossover units were converted into map distances (centiMorgans, cM) by applying the Kosambi map function (KOSAMBI 1944).

RESULTS

Polymorphism analysis

Out of the 859 microsatellites screened against the genomic DNA from the two parents and four F_1 individuals, 213 (25 %) yielded a good amplification pattern, showed polymorphism between the parents and were selected for further study; 251 (29 %) were not polymorphic; 113 (13 %) failed to produce any amplified products even after the optimization of annealing temperature and re-amplification; and 282 (33 %) lacked resolution on agarose gels. The ''touchdown'' program was able to amplify 66 % of the

primer pairs. For the remaining 34 %, the amplification was performed with low stringency annealing temperatures. Among the 464 (54 %) microsatellites which were solved on agarose gels, 85 % of the molecular weight of amplification products varied between 50 to 200 pb. For 15 % of these microsatellites, electrophoresis was conducted at a lower voltage and at an increased running time to separate fragments weighing over 200 pb.

Among the 213 polymorphic microsatellites, 119 (56 %) were useful for mapping analysis, 35 (16 %) have not shown clearly distinguishable bands during genotyping and could not be scored; 40 (19 %) presented segregation distortion after genotyping; and 19 (9 %) showed non-Mendelian scoring with absence of a parental band in one or more F_1 plants tested. All microsatellites mapped appear to be single locus markers, producing only one set of segregating bands.

Repeat type

These 859 microsatellites were grouped into five classes according to the repeat type: 392 dinucleotides, 230 trinucleotides, 66 tetranucleodites, 45 penta- or hexanucleotides and 26 non-perfect repeats (imperfect, interrupted and/or compound microsatellites). For 100 microsatellites, no information was found in the repeat type in this research. The level of polymorphism and resolution on agarose gels vary widely among such classes (Table 1, Fig. 1A–F).

Polymorphism. Similar levels of polymorphism were demonstrated between di- tetra- or non-perfect microsatellites. Penta- or hexanucleotides repeats had the lowest polymorphism. The proportion of non-polymorphic microsatellites for each class was directly related to the size of their repeat unit. Dinucleotides had the lowest proportion, penta- or hexanucleotides had the highest proportion of non-polymorphic microsatellites. Non-perfect repeat microsatellites showed intermediate proportion of non-polymorphic microsatellites.

Resolution. Microsatellites with tetranucleotides or more had the best resolution on agarose gels. Dinucleotide repeats had the lowest resolution. Trinucleotides and non-perfect microsatellites had intermediate levels of resolution between these two classes. Despite the repeat type differences, every class revealed similar levels of amplification failure.

Segregation

By applying the sequential rejective Bonferroni procedure, the critical threshold value for chi-square tests went up of 5.99 ($\alpha = 0.05$, 2 df) for 16.31 (for 170 tests). A total of 119 markers segregated into a 1:2:1 ratio among the F_2 individuals in agreement with the segregation of single locus co-dominant alleles. Deviation from the expected 1:2:1 genotype frequency was significant for 40 markers scored and they were not used in subsequent mapping analysis (Table 2). Seven out of the 40 excluded markers (1.00-umc1354, 1.06-umc1396, 2.01/2.02-umc1518, 2.03-bnlg0469, 2.03-bnlg0381, 5.02/5.03-umc1226 and 9.01-umc1867) showed non significative values of chi-square test. Nevertheless, because they were used for genotyping less than 300 plants from the mapping population, it was decided not to use those marker in the map construction. Twenty-three markers with distorted segregation ratios tended to cluster into four specific genomic regions on chromosomes 2, 4, 9 and 10. Adjacent loci in bins 2.03 and 10.07 had a much greater frequency of heterozygous and homozygous of L-08-05F alleles associated to a decrease of homozygous of L-14-4B alleles. In bin 4.07-4.08, a locus with a deficiency of homozygous L-08-05F genotypes and an excess of homozygous for L-14-4B alleles was observed. In bin 9.01, there was a deficiency of homozygous L-14-4B genotypes and an excess of homozygous of L-08-05F alleles.

Markers per bin

The 859 microsatellites assayed were distributed to all 100 bins. Seventeen bins remained uncovered because no polymorphic markers were found for them. The

Table 1. *Comparisons of microsatellite amplification results as detected in five classes of repeat types and among a number of microsatellites in which repeat type is unknown.*

Repeat type	Polymorphic	Non-polymorphic	Not amplified	Non-resolution	Total	%
Di-	102	84	57	149	392	45
Tri-	47	71	30	82	230	27
Tetra-	19	34	7	6	66	8
Penta- or Hexa-	7	33	5	0	45	5
Non-perfect	7	8	4	7	26	3
Unknown	31	21	10	38	100	12
Total	213	251	113	282	859	100

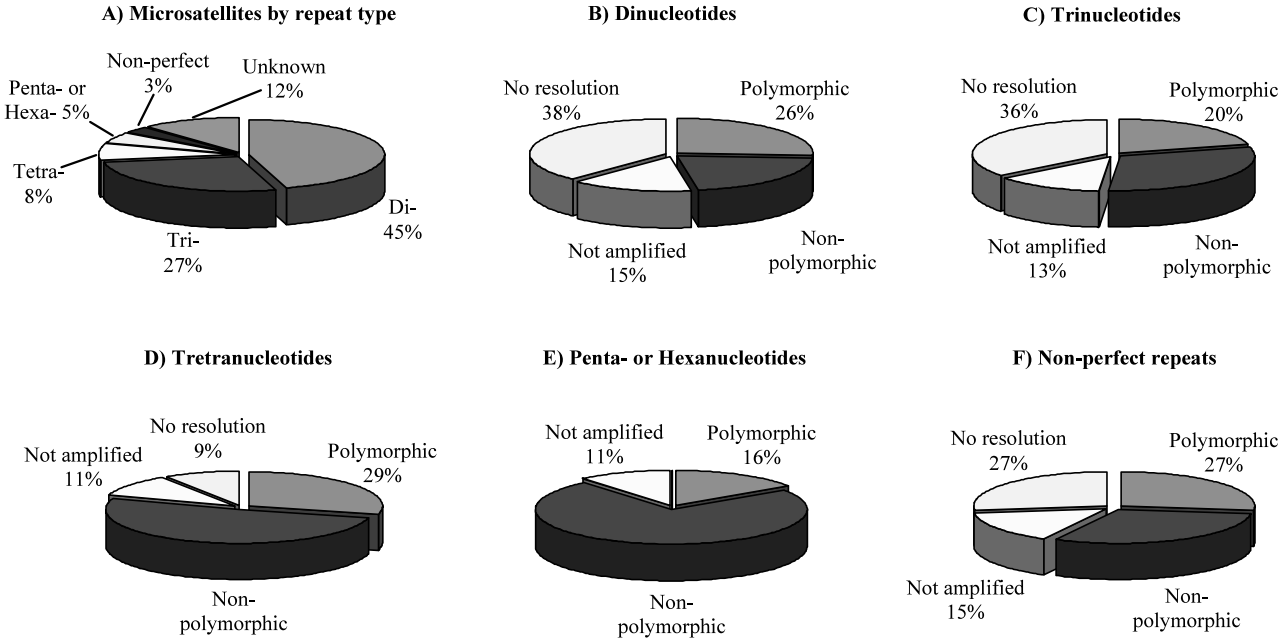

Fig. 1. Frequency distribution of amplification results after polymorphism screen among the five classes of known repeat types. (**A**) Distribution of repeat types among the 859 microsatellites tested. (**B**–**F**): results of dinucleotides, trinucleotides, tetranucleotides, penta- or hexanucleotides and non-perfect repeats (imperfect, interrupted and/or compound microsatellites), respectively.

119 polymorphic microsatellite loci useful in mapping analysis fell into 83 bins. Chromosome 5 contains the largest number of microsatellite/bin with four microsatellite loci found in bin 5.06 (Fig. 2). To reach these 119 microsatellites, 2 to 10 markers were tested in order to find one polymorphic marker for 62 bins, and 11 to over 20 markers were tested to find one polymorphic marker for 21 bins (Table 3). On average, 5.57 microsatellites per bin were tested in order to find a polymorphic marker useful for mapping analysis, that is to say, a polymorphic marker with no problems during genotyping of the mapping population.

Genetic map

The 119 loci data set was evaluated using MAPMAKER/EXP, resulting in 2 unlinked loci excluded in the map: bnlg2057 in bin 1.06 and bnlg1863 in bin 8.04, and 11 distinct linkage groups. Eleven linkage groups were obtained due to the failure in an attempt to establish linkage between the four most distal markers on chromosome 2 (umc1560, umc1633, umc1230 and bnlg1520) located in bins 2.07 to 2.09 and bnlg1396 in bin 2.06. The microsatellite linkage map of the F_2 population with 117 microsatellites covered 1634 cM and the average distance between the markers on the map was 14 cM. The marker order on this F_2 map was in good agreement with the microsatellite loci mapped, which have been published for temperate maize germoplasm in the MAIZE DATA BANK (2001). A few exceptions were observed: 1) the initial linked markers umc1106 (bin 1.00) and umc1177 (bin 1.01) on chromosome 1 and phi420701 (bin 8.00) and umc1139 (bin 8.01) on chromosome 8 were reversed in an order based on the mapping analysis carried out in this work; 2) on chromosome 4, the marker bnlg0589 (bin 4.11) was mapped onto a position close to the marker umc1989 (bin 4.09), but was assigned to the lower end of chromosome 4 in previous maps (Fig. 2).

Although the number of microsatellite markers mapped onto individual chromosomes is roughly proportional to their cytogenetic length (COE et al. 1995), some genomic regions do not have complete coverage. For example, all attempts to identify more polymorphic loci in the 76.6 cM interval on chromosome 2 failed. Therefore, the two linkage groups were placed together based on previously published maps on the MAIZE DATA BANK (2001). Also, due to the lack of polymorphic loci, relatively little coverage was obtained for chromosomes regions 4, 5, 9, and 10.

Genome composition

According to the genotyping of 117 microsatellites mapped, the average heterozygosity percent per plant in the F_2 population was 52.09 ± 4.6 %, with a range from 35 % to 60 %. The average homozygosity percent was 23.0 ± 3.2 % (range: 13.50-29.25 %) for L-

Table 2. *Microsatellite loci showing distorted segregation.*

Bin	Locus	L08/L08[a] Obs.	%	L08/L14[a] Obs.	%	L14/L14[a] Obs.	%	Total	χ^2	p-value
1.00	umc1354	60	(38)	85	(-2)	29	(-33)	174	11.14	3.81×10^{-3}
1.06	umc1396	36	(-30)	121	(17)	50	(-3)	207	7.81	2.01×10^{-2}
1.06	umc1508	65	(-35)	226	(13)	109	(9)	400	16.44	2.70×10^{-4}
2.01-2.02	umc1518	73	(9)	149	(12)	45	(-33)	267	9.47	8.78×10^{-3}
2.02	bnlg2277	98	(5)	216	(16)	58	(-38)	372	18.28	1.10×10^{-4}
2.02	umc1823	68	(-23)	222	(25)	64	(-28)	354	22.97	1.10×10^{-5}
2.03	**bnlg0469**	55	**(-26)**	180	**(20)**	64	**(-14)**	299	12.99	1.51×10^{-3}
2.03	**dupssr27**	87	**(-4)**	217	**(20)**	57	**(-37)**	361	19.75	5.00×10^{-5}
2.03	**mmc0111**	88	**(4)**	199	**(17)**	52	**(-39)**	339	17.91	1.30×10^{-4}
2.03	**bnlg0381**	79	**(11)**	158	**(11)**	48	**(-33)**	285	10.12	6.35×10^{-3}
2.03	**bnlg2248**	112	**(12)**	225	**(13)**	62	**(-38)**	399	19.05	7.00×10^{-5}
2.03	**umc1776**	112	**(12)**	224	**(12)**	64	**(-36)**	400	17.28	1.80×10^{-4}
2.07	umc1637	41	(-42)	134	(-5)	106	(51)	281	30.67	2.20×10^{-7}
2.08	umc1464	53	(-37)	176	(5)	106	(27)	335	17.63	1.50×10^{-4}
2.08	umc2005	96	(-4)	238	(19)	66	(-34)	400	18.94	8.00×10^{-5}
3.10	bnlg1098	57	(-39)	211	(13)	105	(13)	373	18.79	8.00×10^{-5}
3.10	umc1136	70	(13)	175	(42)	2	(-97)	247	80.39	3.50×10^{-18}
4.07	dupssr34	66	(-33)	203	(3)	124	(26)	393	17.55	1.50×10^{-4}
4.08	**bnlg2162**	56	**(-43)**	228	**(16)**	110	**(12)**	394	24.56	4.00×10^{-6}
4.08	**umc1086**	53	**(-41)**	195	**(9)**	111	**(24)**	359	21.42	2.00×10^{-5}
4.08	**bnlg2244**	63	**(-37)**	220	**(10)**	117	**(17)**	400	18.58	9.00×10^{-5}
4.08	**umc1051**	57	**(-42)**	212	**(8)**	122	**(25)**	391	24.40	1.00×10^{-5}
5.02-5.03	umc1226	49	(-30)	160	(15)	70	(0)	279	9.19	1.01×10^{-2}
5.04	dupssr10	123	(59)	118	(-24)	69	(-11)	310	36.48	1.20×10^{-8}
5.05-5.06	bnlg0278	106	(6)	161	(-20)	133	(33)	400	18.86	8.00×10^{-5}
6.02	bnlg1371	87	(-13)	163	(-19)	150	(50)	400	33.54	5.20×10^{-8}
9.01	**umc1867**	41	**(46)**	58	**(4)**	13	**(-54)**	112	14.14	8.50×10^{-4}
9.01	**umc1040**	51	**(82)**	42	**(-25)**	19	**(-32)**	112	25.29	3.20×10^{-6}
9.01	**phi0028**	115	**(52)**	154	**(2)**	33	**(-56)**	302	44.65	2.00×10^{-10}
9.02	**bnlg1401**	119	**(19)**	220	**(10)**	61	**(-39)**	400	20.82	3.00×10^{-5}
9.02	**dupssr06**	136	**(38)**	203	**(3)**	55	**(-44)**	394	33.67	4.90×10^{-8}
9.02	**umc1170**	136	**(39)**	202	**(4)**	52	**(-47)**	390	36.69	1.10×10^{-8}
10.05	umc1506	121	(27)	206	(8)	54	(-43)	381	26.09	2.20×10^{-6}
10.07	**umc1084**	62	**(22)**	119	**(17)**	22	**(-57)**	203	21.80	2.00×10^{-5}
10.07	**umc1344**	72	**(20)**	142	**(18)**	26	**(-57)**	240	25.70	2.60×10^{-6}
10.07	**umc1569**	103	**(16)**	214	**(21)**	37	**(-58)**	354	40.08	2.00×10^{-9}
10.07	**umc1038**	113	**(22)**	214	**(16)**	43	**(-54)**	370	35.58	1.90×10^{-8}
10.07	**umc1196**	115	**(18)**	231	**(18)**	45	**(-54)**	391	37.96	5.70×10^{-9}
10.07	**umc2021**	119	**(27)**	212	**(13)**	43	**(-54)**	374	37.57	6.90×10^{-9}
10.07	**bnlg1839**	121	**(30)**	205	**(10)**	46	**(-51)**	372	34.12	3.90×10^{-8}

Number of individuals observed and the percent of the deviations from the expected ratio are shown for: homozygous for L-08-05F alleles (L08/L08), heterozygous (L08/L14) and homozygous for L-14-4B alleles (L14/L14). Obs.: Numbers in black and shaded strings highlight the clusters of segregation distortion loci.

08-05F/L-08-05F genotypes and 23.6 ± 4.0 % (range 13.50-32.25 %) for L-14-4B/L-14-4B genotypes. The parental genome ratio was 49.03 % L-08-05F and 49.64 % L-14-4B, which places the genotypic class mean values within the theoretical expectations for F_2 populations.

DISCUSSION

The first genetic map of the tropical maize genome based on microsatellite markers is presented here. The 117 mapped loci in the genetic map cover 83 % of the bins from the maize genome. However, some genomic regions present large intervals among markers. The intervals up to 40 cM on chromosomes 4 and 5 are related to bins without polymorphic markers: bins 4.02, 4.03 and 4.08 on chromosome 4 and bins 5.01 and 5.08 on chromosome 5. The interval of 76.6 cM on chromosome 2, between bnlg1396 (2.06) and umc1560 (2.07), two markers mapped on adjacent bins, is not expected. Since the average size of each bin is 20 cM, if two linked markers were mapped on

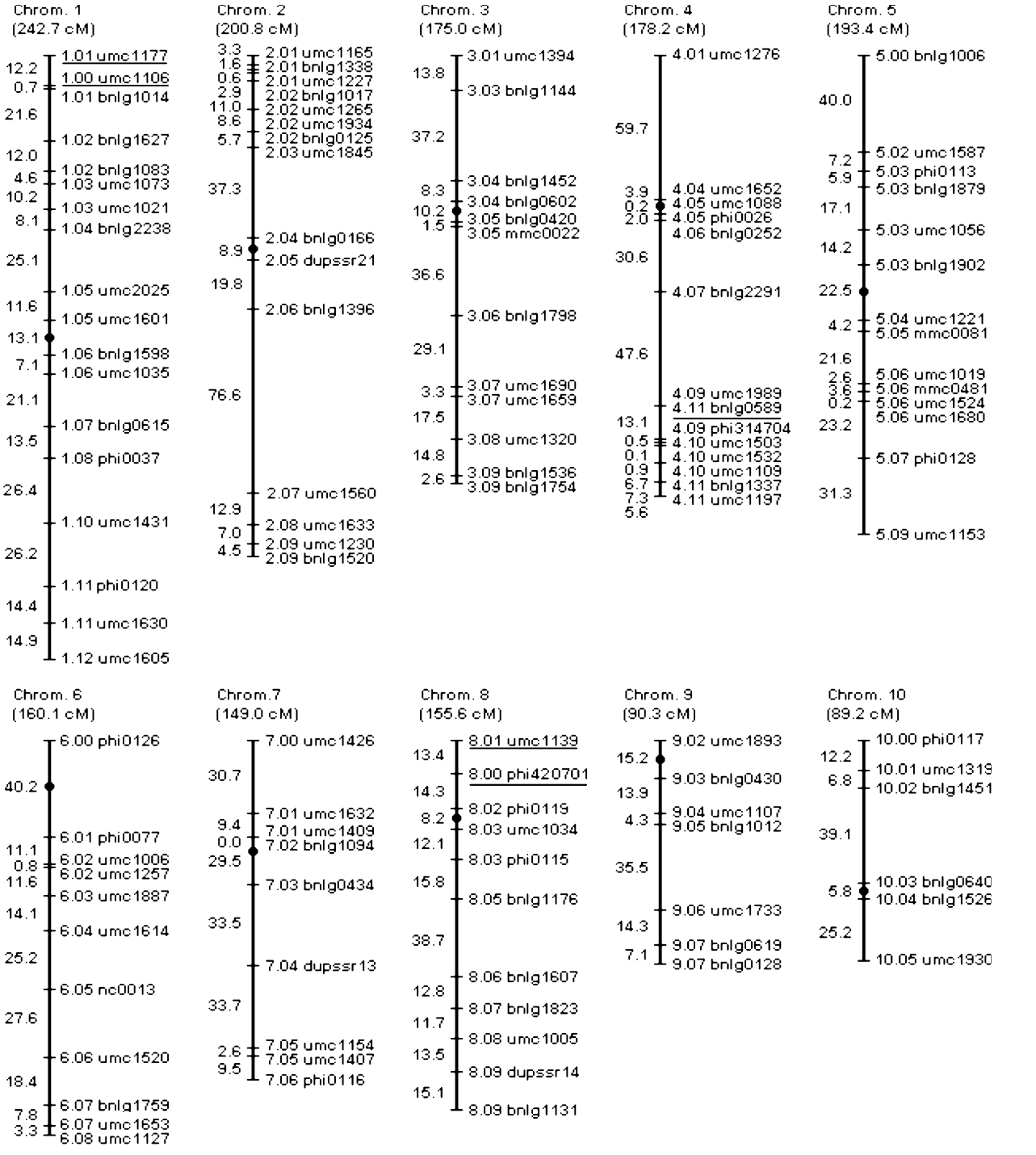

Fig. 2. Genetic map of tropical maize showing the locations of 117 microsatellite loci based on 400 F_2 individuals from a cross between L-08-05F and L-14-4B. Loci names are to the right of each chromosome with the corresponding maize bin. Map distances between adjacent markers, on the left side of the chromosomes, are in centiMorgans (cM) calculated using the Kosambi map function. Dark circles indicate estimated centromere positions, obtained from COE et al. (1995). Underlined marker loci were mapped to different position than those reported in literature (MAIZE DATA BANK 2001). Map length is 1634 cM and average interval length is 14 cM. All markers are linked to the map with LOD scores of 3.0 or greater. Total centiMorgan length for each chromosome is indicated below the chromosome number.

Table 3. *Number of microsatellites tested per bin to find the 119 polymorphic microsatellite loci useful for mapping analysis.*

Number of microsatellites tested	Number of bins covered
from 1 to 5	27 (33 %)
from 6 to 10	35 (42 %)
from 11 to 15	14 (17 %)
from 16 to 20	4 (5 %)
more than 20	3 (4 %)
Total[a]	83 (100 %)

[a] Non-polymorphic markers found or problems during genotyping maintained 17 bins uncovered. The remainder 83 bins are covered by one or more polymorphic microsatellites.

opposite extremes of adjacent bins, theoretically, the distance could reach a maximum of 40 cM. This fact could explain the interval ranges from 30 to 40 cM along the genome. However, the greatest interval on chromosome 2 reveals one region where a great rate of crossing-over could hinder linkage. The rate of crossing-over is known to differ from one species to another, as well as among genotypes and among genomic regions (TANKSLEY et al. 1992, LUKASZEWSKI and CURTIS 1993). Other markers mapped on this interval could solve this question.

Four genomic segments with segregation ratios that are distorted in favor of heterozygous and L-08-05F alleles (bins 2.03 and 10.07), L-08-05F alleles (bin 9.01), and L-14-4B alleles (bins 4.07-4.08) were observed (Table 2). Deviations from expected Mendelian segregation ratios of molecular markers have been reported in maize (BENTOLILA et al. 1992; GARDINER et al. 1993; MURIGNEUX et al. 1993; PEREIRA and LEE 1995), as well as in many other plant species. The reasons for this distortion may result from a selection process during gametogenesis, fertilization or germination (LYTTLE 1991). Errors in marker genotyping may also cause systematic deviations from the expected segregation ratio. However, this could lead to misscoring of only a single marker, and it is relatively easy to detect errors in marker genotyping using a appropriated statistical test (LINCOLN et al. 1992). In contrast, if segregation distortion is caused by segregation-distortion loci (SDL), all markers in the vicinity of the SDL will be affected (VOGL and XU 2000). Therefore, pre or post zygotic selection at one or more genes in the neighborhood of these loci is a possible explanation for these clusters with distorted ratios.

The frequency of non-Mendelian scoring was low: 9 % among the polymorphic microsatellite tested, but was greater than the frequencies that have been observed for microsatellite markers in maize. SMITH et al. (1997), checking the scoring and the inheritance of polymorphisms of 131 microsatellite loci among 13 sets of triplets (a progeny line and both parents), found that 2.2 % of microsatellites segregated in a non-Mendelian fashion. Such non-Mendelian scoring is generally attributed to residual heterozygosity remaining within an inbred line used as parents, outcrossing or a mutation within the binding site for a DNA primer. In the present work, the heterozygosity remaining within the parental inbred lines or the outcrossing cannot explain the results obtained since the whole microsatellite loci were genotyped in the parental lines and F_1 hybrids. The results indicate that there was no heterozygosity in the inbreds (only homozigous loci was observed) or outcrossing in the 4 F_1 hybrids that originated the mapping population. Besides that, the absence of the PCR product of one homologue in the heterozygous, made this marker unsuitable for correct genotyping and the marker was discarded from the mapping experiment. This fact indicates that the presence of null alleles also does not explain the segregation deviation.

Due to the large mapping population and high number of microsatellites, a simple methodology for amplification and detection of all primer pairs (''touchdown'' program and agarose gels), with little optimization of these conditions, was adopted. The aim was the quick polymorphism screen and genotype assignment of each marker. Certainly, among the 113 microsatellites for which non-amplification was observed, a number of them could be recovered by modifying concentrations of the PCR components or amplification program. However, considerable time had to be spent in this optimization for each microsatellite. It was observed that 87 % of the microsatellites tested amplified either by ''touchdown'' program (66 %), or simply by decreasing annealing temperature (34 %) showing that this methodology was effective.

SENIOR and HEUN (1993) reported that Metaphor agarose was effective in separating microsatellite alleles. The same approach was used for SENIOR et al. (1998) for determining genetic relationships in maize using microsatellites. Preliminary tests in our lab (data not shown) showed those 4 % gels with 2 % ultra-pure agarose: 2 % Metaphor has a degree of resolution equal to that of 4 % gels with only Metaphor agarose. This way, a mix of 2 % ultra pure agarose and 2 % Metaphor agarose, was used. This methodology was able to solve PCR products for 62 % (464 microsatellites) of the 746 microsatellites that amplified. For 38 % (282 microsatellites), the agarose gel methodology was not effective to solve the PCR products. High-resolution agarose gels have

a resolution limit of 4 pb, and alleles are more difficult to solve when the total allele length exceeds 200 bp. This limitation could be overcome by selecting microsatellites the amplification products of which are easier to score on agarose gels.

Microsatellite polymorphism is due to mutation events that change the number of copies of repeat units. These mutations might be generated by polymerase slippage during DNA replication (LEVINSON and GUTMAN 1987; SCHLOTTERER and TAUTZ 1992; WEBER and WONG 1993). The mutation rate varies substantially among microsatellite loci and depends on structural factors of repeat unit such as size, type, and composition. WEBER (1990) found that the polymorphism of (CA)n markers increased when the average number of repeats increased. For a given size of repeat unit, the presence of non-perfect repeats (imperfect repeats with one or more interruptions and/or compound repeats with adjacent tandem repeats of different sequences) is correlated with the stability of a microsatellite, reducing mutation rates (GOLDSTEIN and CLARK 1995; PETES et al. 1997). Finally, it has been observed that dinucleotide repeats showed mutation rates higher than tetranucleotides, with trinucleotides intermediate between the di and tetra (CHAKRABORTY et al. 1997).

Based on these results, the best microsatellites for agarose gel systems are the perfect tetranucleotides (or greater) repeats, the sizes of which had good allele separation after a long run. However, the most common microsatellite found in genomes is the dinucleotide repeat. Trinucleotide repeats are less common than dinucleotides and tetranucleotide repeats are the less abundant microsatellite (WANG et al. 1994; GUPTA et al. 1996). Moreover, our results on agarose gels showed that the number of polymorphic microsatellites was inversely related to the size of their repeat unit. It means that tetranucleotides were less polymorphic than tri- or dinucleotides, and the results are in good agreement with the estimates of CHAKRABORTY et al. (1997).

Thus, in order to explore the microsatellite polymorphism on agarose gels, a balance between the abundance of the repeat type and their resolution in this genotyping system, must be considered. Tetranucleotides microsatellites or greater, have the best resolution, but are less abundant and less polymorphic. Dinucleotides have the worst resolution, but are the most abundant and are more polymorphic. According to the results here, if equal amounts of di- and tetranucleotides are assayed, the number of polymorphic microsatellites resolved on agarose gels will be approximately the same.

Conclusion

Microsatellites have several features that make them increasingly useful as DNA markers. What limits their widespread use is the need of sequence information for the development of microsatellite primers, which is an expensive and intensive labor process. However, once developed, the use of specifically designed primers based upon the flanking sequences allows the easy dissemination of these sequences from public databases. With the primer sequences available and the relatively low price of custom-made oligonucleotides, more attention can be drawn to a faster and less expensive microsatellite detection. The agarose gel system has been demonstrated to be effective to solve amplification products of microsatellites, thus avoiding the radioactive or fluorescent labeling or the development of high throughput automation systems. This simple methodology has become an important option for analysis for a large number of samples in laboratories unable to undertake expensive and laborious processes.

The comparisons of maps in different genetic backgrounds confirm that microsatellites are highly reproducible between laboratories and maintain the marker order of loci mapped on previous maps. Although 117 microsatellite loci have been mapped, this genetic map represents the first step towards the development of a more fully saturated map of the tropical maize genome based on microsatellite markers. Work is presently under way to map additional microsatellite markers to close the gaps on poorly represented chromosome regions and to provide more information about the segregating distortion loci and genomic regions presenting high levels of crossing-over events discovered.

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