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A GENETIC LINKAGE MAP OF A FLINT MAIZE (*ZEA MAYS* VAR. *INDURATA* L.) ITALIAN LANDRACE USING A ONE-WAY PSEUDO-TESTCROSS STRATEGY AND MULTILOCUS PCR-BASED MARKERS

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ABSTRACT - We have used for the first time in maize a one-way pseudo-testcross mapping strategy in combination with different types of multi-locus PCR-based markers (RAPD, ISSR, AFLP, SAMPL) to construct a saturated genetic linkage map of the Italian flint maize (*Z. mays* var. *indurata* L.) landrace 'Nostrano di Storo'. This mapping strategy was compared with the traditional backcross one and SSR loci selected from Maize Genome Database were also assayed to associate linkage groups to those known from published map and to orient linkage groups or specific chromosome arms. A total of 326 markers, comprising 8 RAPD, 7 I-SSR, 259 AFLP, 28 SAMPL, 24 SSR, were scored in 64 F_1 individuals obtained from a cross between a highly heterozygous genotype of the landrace with the inbred line B37. Grouping of the markers at a LOD score of 5.0 resulted in 10 linkage groups and a framework map covering 1826 cM was assembled by using 282 markers that could be ordered with a LOD threshold of 2.5. The efficiency of pseudo-testcross strategy was concluded to be around twice compared to that of backcross because of its higher ability to detect the recombination events occurring between pairs of linked loci. Of the total markers, 12.5% showed segregation distortion in the F_1 population, whereas in the BC_1 the distortion was evident for 18.5% of loci. The results are discussed in terms of map use as a tool for characterizing the Italian maize germplasm and designing an appropriate conservation.

KEY WORDS: Molecular markers; Genetic map; Pseudo-testcross strategy; Maize landrace.

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INTRODUCTION

Until the mid of the twentieth century, when hybrids were introduced in Europe mainly from USA, several open-pollinated varieties of maize (*Zea mays* L.) were cultivated in Italy (BRANDOLINI *et al.*, 1967). Following hybrids introduction, local varieties were progressively abandoned. Few landraces of flint maize (*Z. mays* var. *indurata*) can still be found under peculiar agricultural situations or in marginal areas where an exclusive traditional utilization for human consumption limits the diffusion of modern hybrids.

The need to characterize crop surviving landraces to establish *in situ* conservation strategies has been recently stressed (BRUSH, 1995; LOUETTE *et al.*, 1997; MAXTED *et al.*, 1997; JARVIS *et al.*, 1998). A research project concerning an old flint local maize variety, known as 'Nostrano di Storo', still cultivated in hilly environments of the low Chiese River valley (Trentino, North-Eastern Italy), was started to assess the influence of different conservation methods on the genetic structure of 'Nostrano di Storo' farmer's populations.

This information becoming available allows to monitor changes in pattern of variation within landraces. In this perspective, the construction of a genetic map of the 'Nostrano di Storo' landrace could represent the tool to control population dynamics, gene flow and genetic erosion.

In the last fifteen years, highly detailed linkage maps based on restriction fragment length polymorphism (RFLP) and polymerase chain reaction (PCR)-derived markers have been developed for maize. In this species homozygous inbred lines, as well as large progenies from their crosses, allow to develop F_2 mapping populations which have been widely used (HELENTJARIS *et al.*, 1986; HOISINGTON and COE, 1989; BEAVIS and GRANT, 1991; CAUSSE *et al.*, 1996;

BEAUMONT *et al.*, 1996; AGRAMA *et al.*, 1997; CASTIGLIONI *et al.*, 1999; SIBOV *et al.*, 2003). On the contrary, BC_1 populations from an F_1 hybrid plant crossed to one of the two inbred parents were never adopted for linkage mapping purposes. Recombinant inbred lines (RILs) have been suggested by BURR *et al.* (1988) as an alternative mapping strategy. They can be indefinitely perpetuated through selfing and are suitable to successively enrich an existing map by additional marker loci (CAUSSE *et al.*, 1996; SENIOR *et al.*, 1996; TARAMINO and TINGEY, 1996; VUYLSTEKE *et al.*, 1999; COE *et al.*, 2002; SHAROPOVA *et al.*, 2002). GARDINER *et al.* (1993) have also developed a mapping strategy based on immortalized F_2 (IF_2) populations, later exploited by several groups (CHAO *et al.*, 1994; DAVIS *et al.*, 1999; VUYLSTEKE *et al.*, 1999; SHAROPOVA *et al.*, 2002).

Genetic linkage maps of maize were first based on RFLP markers (HELENTJARIS *et al.*, 1986; BURR *et al.*, 1988; HOISINGTON and COE, 1989; BEAVIS and GRANT, 1991; CHAO *et al.*, 1994; CAUSSE *et al.*, 1996). This type of DNA polymorphism requires Southern blot hybridization with selected probes, a procedure which is time consuming and expensive. The advent of PCR-based markers, such as simple sequence repeats (SSRs) or microsatellites, was effective in supplying a novel impetus to the development of genetic linkage maps of maize (SENIOR *et al.*, 1996; TARAMINO and TINGEY, 1996). Highly saturated genetic maps were constructed using both RFLP and SSR polymorphisms (DAVIS *et al.*, 1999; SHAROPOVA *et al.*, 2002), including markers with a known sequence and expressed sequence tags (ESTs). More recently, amplified fragment length polymorphisms (AFLPs) provided an efficient tool to detect DNA polymorphism and generate large sets of molecular markers for detailed genetic linkage mapping in maize (AGRAMA *et al.*, 1997; CASTIGLIONI *et al.*, 1999; VUYLSTEKE *et al.*, 1999).

The present paper reports the construction of a saturated genetic linkage map of the Italian flint maize landrace 'Nostrano di Storo' by using different types of multi-locus PCR-based markers. This work is part of a program aimed at characterizing and maintaining through *in situ* conservation old maize landraces still cultivated in Northeastern Italy. An one-way pseudo-testcross mapping strategy (GRATTAPAGLIA and SEDEROFF, 1994; DE SIMONE *et al.*, 1997) has been adopted for the first time in maize and compared with a traditional back-cross mapping strategy. A saturated genetic map covering the whole genome is now available.

MATERIALS AND METHODS

Plant material

The genetic map of the flint maize Italian landrace 'Nostrano di Storo' (NSt) was constructed using two different segregating populations: an F_1 population of 64 individuals obtained by crossing a highly heterozygous genotype (NSt15/8) with the inbred line B37, and a BC_1 population of 70 individuals generated by backcrossing a random F_1 plant (F_1/5) with the same inbred line. Crosses were carried out in greenhouse at the experimental farm of the University of Padova (Italy) in 1999. Seeds were germinated in jiffy pots and plantlets were grown in the greenhouse.

DNA extraction

Genomic DNA of single F_1 and BC_1 plants and parents was isolated from fresh leaf tissue of 1-month-old plants using the Nucleon Phytopure Plant DNA Extraction Kit (Pharmacia Biotech) with the addition of β-mercaptoethanol to the extraction buffer and RNase treatment to the nucleic acid samples. The quality of DNA samples was assessed by electrophoresis on 1% gels, and its concentration was determined by optical density reading (DU650 spectrophotometer, Beckman) at 260 nm (1 O.D.=50 µg/ml). The purity was calculated by the $O.D._{260}/O.D._{280}$ ratio and by $O.D._{210}$-$O.D._{310}$ pattern (SAMBROOK *et al.*, 1989).

Molecular markers

Preliminary analyses defined the optimum PCR conditions for molecular marker survey to select the most efficient primer (RAPD, ISSR) or primer combinations (AFLP) to reveal molecular polymorphisms between NSt15/8 and the B37 inbred line.

RAPD markers

PCR reactions were carried out following BARCACCIA *et al.* (1997) in a 25 µl total volume, including 20 ng genomic DNA, 1.5 mM $MgCl_2$, 1.25 U Taq DNA polymerase (Amersham Pharmacia Biotech) and 1 µM primer. In addition to the universal primer M13, six 10-mer primers from Operon Technologies Inc. (OP-B4, OP-B9, OP-B13, OP-C14, OP-C15 and OP-F15) were selected on the basis of the number and size of polymorphic bands generated, and reproducibility of banding patterns. PCR reactions were performed in a 9700 Thermal Cycler (Perkin Elmer, Norvalk, CT, USA) and amplification products were separated by electrophoresis in 1.5% agarose gels run with 1× TBE buffer (45 mM Tris-HCl, 45 mM ortoboric acid and 1 mM EDTA).

ISSR markers

Inter-microsatellite markers were assayed by using ten different I-SSR primers (synthesized by Life Technologies, Inc.) anchored at 3' or 5' terminus of the simple repeat and extended into the flanking sequence by one to four nucleotide residues, i.e. I14 $CAGC(AC)_7$, I18 $GTGC(TC)_7$, I20 $GAG(TC)_8$, I22 $CCA(TG)_8$, I24 $GGA(TG)_8$, I28 $(GT)_6CG$, I33 $(AGC)_4T$, I34 $(AGC)_4GG$, I36 $(AGC)_4GC$, I37 $(AGC)_4GT$. The PCR protocol for ISSR polymorphisms is described in BARCACCIA *et al.* (2003). Inter-microsatellite DNA fragments were separated by electrophoresis in 2% agarose gels run with 1× TBE buffer (45 mM Tris-HCl, 45 mM orthoboric acid and 1 mM EDTA).

AFLP markers

Restriction-ligation, preamplification and hot-PCR experiments were performed according to BARCACCIA *et al.* (1998). The analysis of DNA polymorphisms was based on the use of ten

*Eco*RI/*Mse*I primer combinations each with three different selective bases: E-CCA/M-AGG, E-CAA/M-AGG, E-CAC/M-ATC, E-CAC/M-AAG, E-CCA/M-AAG, E-CAA/M-AAG, E-CCA/M-AGC, E-CCA/M-ATC, E-CAC/M-AGC, E-CAA/M-ATC. Further seven *Pst*I primers having two selective bases in combination with *Mse*I primers with three selective bases were used to construct the saturated linkage map according to the pseudo-testcross strategy: P-AG/M-CAC, P-AG/M-CAT, P-AT/M-CAT, P-AT/M-CAG, P-AT/M-CCA, P-AA/M-CAC, P-AA/M-CAA. The AFLP products were separated by 6% denaturing polyacrylamide gel (Acrylamide/Bis 19:1 solution) electrophoresis run with 1× TBE (45 mM Tris-HCl, 45 mM orthoboric acid and 1 mM EDTA) buffer using a sequencing cell apparatus. Gels were blotted on Whatmann 3 MM paper, dried at 75°C for 1 h and visualized by autoradiogram after 12 hrs exposure at -80°C using intensifying screens.

SAMPL markers

Template DNA samples were prepared as for the AFLP assay. PCR amplification was performed using three combinations of a SAMPL primer *As* with an AFLP primer *Mse*I with three selective bases: As1/M-ATC, As2/M-ATC, As2/M-AAG. The SAMPL primer was labeled by phosphorylating the 5' end with [γ^{33}P] ATP. All the successive procedure for gel electrophoresis and autoradiography were identical to those used for AFLP detection.

SSR markers

Microsatellite (SSR) loci analysis was according to Barcaccia *et al.* (2003). Polymorphisms were visualized by labeling one of the primers with γ-[^{33}P] ATP (Amersham, Life Science) using T4 polynucleotide kinase (Pharmacia Biotech). One SSR locus for each of the 10 maize chromosome complements was assayed in both F_1 and BC_1 mapping populations to associate linkage groups to those known from published maps. For the construction of the final map, 40 SSR loci were used to orient linkage groups on specific chromosome arms. Primer pairs for the detection of SSR markers were selected among those available at the Maize Genome Database of the University of Missouri (http://www.agron.missouri.edu) on the basis of the *bin* value (Coe *et al.*, 1995) and polymorphism information content at each locus (Table 1).

Statistical analysis and map construction

Segregating markers in the F_1 and BC_1 mapping populations were scored for presence *vs.* absence of specific alleles. Data were recorded as binary matrices and genetic similarity (GS) was estimated between individuals in all possible pair-wise combinations using the Simple Matching coefficient. Markers polymorphic between parents and segregating in the progeny, being heterozygous in one parent and null in the other, were tested by chi-square analysis for goodness-of-fit to the expected 1:1 ratio. Linkage maps were constructed using the software JoinMap 3.0 (Van Oijen and Voorrips, 2001) by treating segregation data of markers as a 'cross-population' (CP) or a 'Backcross-population' (BC), respectively for F_1 and BC_1 populations. For the identification of linkage groups with selected markers, the grouping module was applied by setting a minimum LOD score of 5 and a maximum recombination frequency *r* of 0.40. Subsequently, the splitting module was employed to order marker loci within each linkage group. Markers that could be ordered with a LOD ≥ 2.5 were adopted as framework map, and the remaining marker loci located at their most probable positions. The parameter ripple was applied to improve the final order of the marker loci. Map distances, expressed in centiMorgans (cM), were calculated using the Kosambi function (Kosambi, 1944).

Individual RAPD locus labels refer to the 10-mer primer nomenclature and the relative marker number, whereas ISSR locus labels refer to the repeated motif with 3'- or 5'-anchored bases and the relative marker number. AFLP and SAMPL locus designations refer to the selective bases of *Eco*RI, *Pst*I and *Mse*I primer combinations, or *As*1 and *As*2 primers. Microsatellite locus labels refer to the original SSR locus name as published in the Maize Genome Database of the University of Missouri. All markers were numbered consecutively from the largest to the smallest size. In each linkage group, the upper and the lower part refer, respectively, to the short and the long arms of the chromosome.

RESULTS

Segregation pattern analysis in mapping populations

A total of 199 marker loci were defined according to the segregations observed in the F_1 and BC_1 populations using 27 selected primers and primer combinations. In particular, the segregation patterns of 118 polymorphic marker alleles between NSt15/8 and B37 in the F_1 population, and 81 in the BC_1 population were studied. In the F_1 population, 98 AFLP (14.0 per primer combination), 8 RAPD (1.1 per primer) and 7 ISSR (1.0 per primer) polymorphisms were detected, while the BC_1 population segregated for 52 AFLP (5.2 per primer combination), 8 RAPD (1.6 per primer), and 16 ISSR (2.0 per primer) polymorphisms. In both populations, only 5 of the SSR loci tested were shown to segregate.

Among AFLP markers, the highest number of marker alleles (22) was produced by the primer combination E-CAC/M-ATC in the F_1 population, and by E-CCA/M-AAG in the BC_1 population. The 10-mer and inter-microsatellite primers in general generated only one polymorphic locus each, with the exception of the RAPD OP–F15 and ISSR I34 primers, which gave 3 and 5 segregating marker alleles, respectively in the BC_1 population, and ISSR I14, I34, and RAPD M13 primers with two segregating alleles in the F_1 population. Most of the heterozygous loci were detected in the NSt15/8 landrace parent (95.8% in F_1 and 90.1% in BC_1), even if segregating marker alleles from the inbred line B37 were identified in both the F_1 (4 AFLPs and 1 ISSR, 4.2% of the total) and BC_1 populations (2 AFLPs, 1 RAPD, and 5 ISSRs, 9.9% of the total), thus indicating that not all genomic loci in the inbred parent were in a homozygous condition.

Six pairs of marker alleles in the F_1 population (4 of maternal and 2 of paternal origin) were inher-

TABLE 1 - *List of SSR loci, along with information concerning chromosome position, repeated sequence and name of segregating loci revealed by markers.*

Chromosome	Centromere position	Locus SSR	Arm	Bin	SSR sequences	Locus detected
1 (1.00/1.12)	1.06	phi 056*	S	1.01	GCC	tub1
		bnlg1556*	L	1.06	$(AG)_{18}$	bnlg1556
		phi 037*	L	1.08	AG	umc128
		phi055	L	1.09	GAA	glb1
2 (2.00/2.10)	2.05	phi 083*	S	2.04	CTAG	prp2
		dupssr24*	L	2.08	$(GA)_{16}$	dupssr24
		phi090	L	2.08	ATATC	npi298
		phi127	L	2.08	AGAC	phi127
		umc1464	L	2.08	$(CCA)_6$	umc1464
3 (3.00/3.10)	3.05	phi 049	S	3.01	ACT	umc32a
		umc1057	S	3.01	$(CGG)_6$	cko1
		umc1458	S	3.02	$(GCT)_5$	umc1458
		nc030	S	3.04	CT	tpi4
		phi029	S	3.04	CCCT-CT	tpi4
		bnlg1452*	S	3.04	$(AG)_{22}$	bnlg1452
		phi073	L	3.05	CAG	gst4
		phi 053*	L	3.05	ATGT	umc102
4 (4.00/4.11)	4.05	phi 021*	S	4.03	AG	adh2
		phi096	S	4.04	GAGGT	zp1
		phi079	S	4.05	CATCT	gpc1
		phi 076*	L	4.11	GAGCGG	cat33
5 (5.00/5.09)	5.04	phi024	S	5.01	CCT	Ohp2
		phi 113*	S	5.03	GTCT	ole3
		umc1019*	L	5.05	$(CT)_{17}$	umc126a
		phi128*	L	5.07	AAGCG	asg85b
6 (6.00/6.08)	6.01	umc1143*	S	6.00	AAAAT	umc1143
		phi 031*	L	6.04	GTAC	pl1
		bnlg1740*	L	6.07	$(AG)_{21}$	bnlg1740
7 (7.00/7.06)	7.02	phi 057*	S	7.00	GCC	o2
		phi 116*	L	7.06	TGAC-GAC	phi 116
8 (8.00/8.09)	8.03	umc1075*	S	8.00	$(ATTGC)_5$	umc1075
		phi 115	L	8.03	AT-ATAC	act1
		phi 015*	L	8.08	TTTG	gst1
		phi080	L	8.08	AGGAG	gst1
9 (9.00/9.08)	9.03	phi 027*	S	9.03	GCGCT	wx1
						waxy1
		phi 016*	L	9.04	GGT	sus1
10 (10.00/10.07)	10.03	phi 063*	S	10.02	TATC	phi 063
		Phi084	L	10.04	GAA	nacl
		phi 071*	L	10.04	GGA	hsp90
		dupssr7*	-	-	$(CA)_{25}$	dupssr7

* SSR loci mapped in the final genetic linkage map.

TABLE 2 - *Segregation patterns observed for different markers in the F_1 and BC_1 mapping populations.*

Markers	Parent polymorphisms*		F_1		BC_1	
	NSt15/8	B37	1:1 ratio $P \geq 0.05$	Distorted markers	1:1 ratio $P \geq 0.05$	Distorted markers
AFLP	1	0	86	8	41	9
	0	1	4	0	2	0
RAPD	1	0	8	0	6	1
	0	1	0	0	1	0
ISSR	1	0	4	2	8	3
	0	1	0	1	3	2
SSR	1	0	4	1	5	0
	0	1	0	0	0	0
Total			106	12	66	15

* 1=presence of marker allele, 0=absence of marker allele.

ited as co-dominant. No marker alleles inherited in a co-dominant way were found in the BC_1 population.

Of the 10 SSR loci tested, only 5 were heterozygous in NSt15/8 and therefore adopted for studying segregation patterns and assigning linkage groups. In all cases, segregating marker alleles were of maternal origin.

Concerning segregation patterns, 89.8% (106/118) in the F_1 and 81.5% (66/81) in the BC_1, of the polymorphic marker loci segregated with the expected ratios (Table 2). On the whole, segregation distortion was low in the F_1 mapping population, and more evident in the BC_1.

On the basis of the marker alleles tested, the genetic variability observed in the segregating populations revealed similar. The average index of genetic diversity (H) of Nei was 0.374 in the F_1 and 0.368 in the BC_1 population. Although the backcross to the inbred line B37 would involve inbreeding, in the BC_1 population a reduction of genetic variability compared to the F_1 population was not evident. The hypothetical heterozygous condition at a given locus of the NSt15/8 genotype, in fact, supports a theoretical loss of the related marker allele in 50% of the gametes, and therefore in 50% of the loci, of the hybrid F_1/5 plant crossed with the inbred line B37.

Although the backcross led on average to the loss of half of the alleles of NSt15/8, the analysis of the genome with dominant markers did not point out in the BC_1 population the expected progress in terms of genetic similarity towards the parental line B37. The average value of genetic similarity (GS) of the progeny with respect to the parents, was comparable in the F_1 and BC_1 populations, being at 49.4% and 50.5%, for B37, and 50.6% and 49.5%, for NSt15/8, respectively. However, the interval of variation of this coefficient was greater in the BC_1 (27.8%-74.1%) than in the F_1 (40.2%-59.8%).

Linkage mapping with a pseudo-testcross strategy

The genetic map constructed following the pseudo-testcross strategy based on the F_1 population included 108 marker loci and altogether spans 399.5 cM. Eight main linkage groups were identified, each with a number of marker loci varying between four and 22, and three groups each consisting of two marker loci. The 108 loci (9.8 per linkage group) represented 91.5% of segregating marker loci (118). Of these, 93 were AFLP (86.1%), 6 RAPD (5.6%), 5 ISSR (4.6%) and 4 SSR (3.7%) alleles. It was possible to assign four linkage groups to those listed in published maps, including the SSR loci phi056 (linkage group 1), phi031 (linkage group 6), umc1075 (linkage group 8) and phi027 (linkage group 9), whereas the SSR locus phi057 was not mapped, probably because positioned on linkage groups represented in our experiments by few markers.

Marker distribution was not random, since 76%

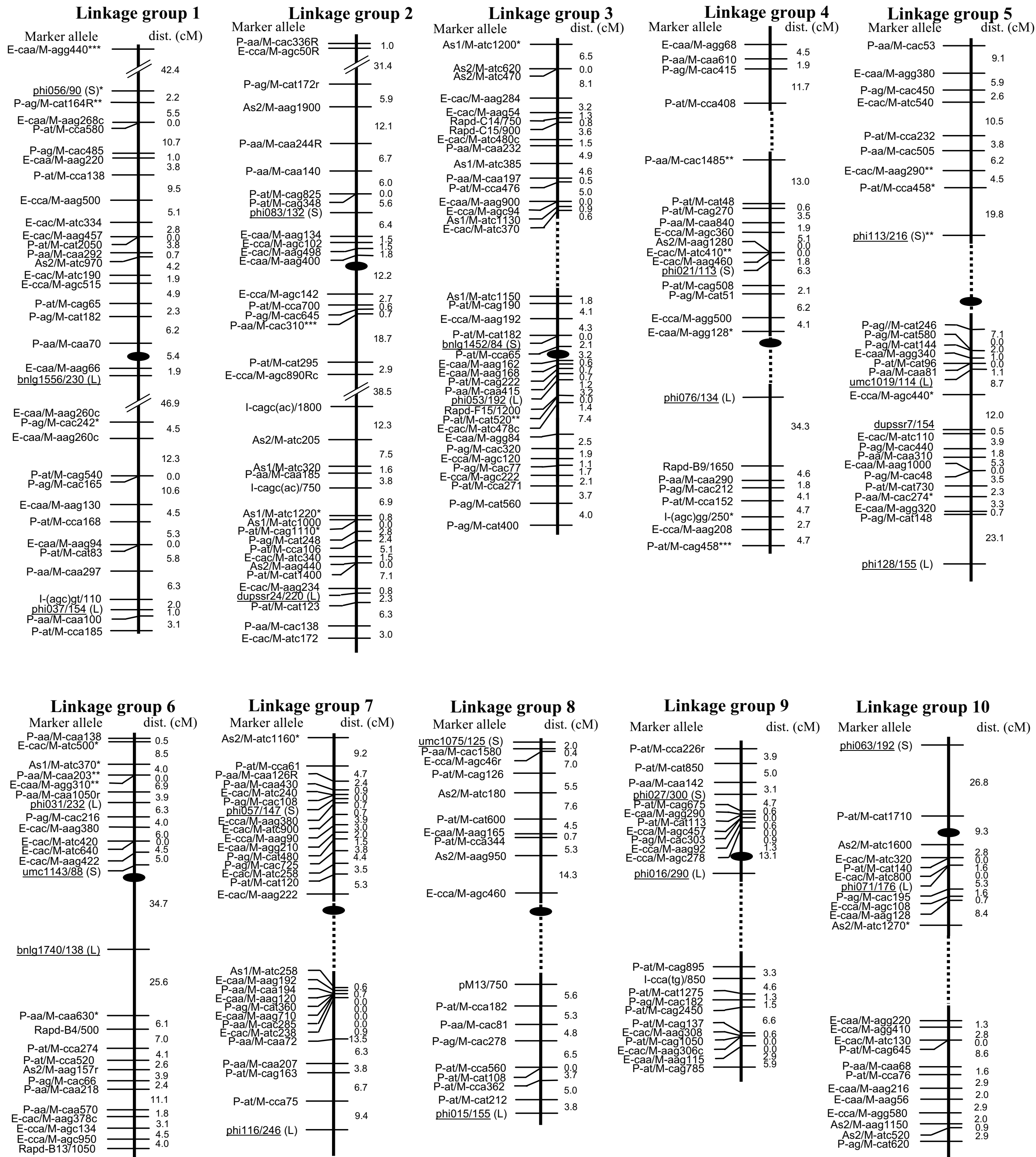

FIGURE 1 - Saturated linkage map of the maize landrace 'Nostrano di Storo' based upon AFLP, RAPD, ISSR and SSR markers. The framework map showing the linear order and relative distance in cM between marker loci was constructed according to a multipoint analysis with a threshold LOD score of 5.0 and a recombination frequency of 0.40. AFLP and SAMPL loci are identified by primer combinations (P, E, and M, respectively for *Pst*, *Eco*, *Mse,* As1 and As2), whereas SSR loci designations (underlined) refer to the locus name as published in the Maize Genome Database of the University of Missouri (for details see Table 1). I- prefixes, followed by the repeated motif with 3' or 5' anchored bases indicate ISSR loci. Markers showing a significant level of segregation distortion are indicated by asterisks (*: P≤ 0.05, **: P ≤ 0.01, ***: P≤ 0.001). The centromere position in the linkage group has to be considered purely as indicative because it has been defined on the basis of the *bin* value of SSR loci.

of the markers (82 out of 108) mapped in five linkage groups. A clustering of closely associated AFLP marker loci was noted for linkage groups 1 and 4, with 17 AFLP loci out of the 18 mapped. A similar situation was evident for linkage groups 6, 7 and 10.

Of the six co-dominant AFLP loci, the four of maternal origin (NSt15/8) mapped to linkage groups 1, 6, 7 and 10. Of the two of paternal origin (B37), one mapped in linkage group 6.

Four of the five ISSR mapped loci occupied a distal position in the linkage group to which they belong (evident for loci I37/110 in group 1, I14/750 in group 4 and I22/850 in group 9) while five of the six RAPD mapped loci were associated to two linkage groups (in group 6, loci RAPD OP-B4/500 and RAPD OP-B13/1050 were positioned at position 0).

Linkage mapping with a backcross (BC_1) strategy

The map constructed on the basis of the segregation data of the BC_1 population included 69 marker loci and covered 511.6 cM. These loci were assigned to nine linkage groups, each containing from five to 10 loci, and to three linkage groups containing two markers each. Linkage group 3 had the highest number of marker loci (10). Altogether, 45 AFLP (65.2% of the mapped marker loci), 7 RAPD (10.1%), 12 ISSR (17.4%) and 5 SSR (7.3%) loci were mapped. The percentage of mapped markers was lower (85.2%) than that of the F_1 population.

Five linkage groups were assigned to those listed in published maps (1, 6, 7, 8 and 9), and it was possible to map also the SSR locus phi057 on linkage group 7.

The AFLP loci mapped all along the different linkage groups. The number of loci per linkage group ranged from three (linkage groups 4, 5, 8) to nine (linkage group 3). Only in the latter case the tendency was observed for AFLP loci to cluster in a region showing low recombination frequency.

Within each linkage group, the majority of mapped ISSR loci were placed in a distal position or away from the other marker loci, with the exception of locus I37/450, which mapped on linkage group 1 at 0 cM from the SSR locus phi056, and locus I33/450R, linkage group 7 at 2.7 cM from the AFLP locus E-CCA/M-AGG910 and at 3.4 cM from the SSR locus phi057.

The assessment of the co-linearity between the F_1 and the BC_1 maps was not possible given the limited number of common markers. Of nine genomic loci segregating in both populations, six were located on both maps. Of these, two mapped on the same linkage group 1, and other two on linkage group 10. The remaining two loci mapped on linkage group 4 in the F_1 population and in the separate linkage groups, 2 and 4, in the BC_1 population.

Construction of a saturated genetic map

On the basis of the results obtained with the two mapping strategies, the genetic map constructed from the F_1 population was saturated. Further, 161 AFLP (23.0 per primer combination) and 28 SAMPL (9.3 per primer combination) polymorphisms were obtained. Of the 326 marker loci assayed, 16 AFLP, 1 SAMPL and 1 ISSR, corresponding to 5.5% of the total markers, proved heterozygous in the inbred line B37 and segregated 1:1 in the mapping population.

The genetic map was constructed using a LOD threshold of 5 to assign the marker loci to the single linkage groups, and a minimum LOD threshold of 2.5 was adopted to establish the order of the markers within each linkage group.

The saturated genetic map included 282 marker loci, ordered into 10 linkage groups covering 1826 cM (Fig. 1). It also included 20 SSR loci as well as the four already located in the initial genetic map. These 24 SSR loci allowed all 10 linkage groups to be assigned and to orient single linkage group maps by positioning the part corresponding to the short chromosomal arm on top and the long one at the bottom. The mapped marker loci (28.2 markers per group on average) represented 86.5% of all segregating markers (326) Most of these were AFLP (222 equal to 78.7%), 24 SAMPL (8.5%), 7 RAPD (2.5%) and 5 ISSR (1.8%) loci. Among the AFLP loci, 132 (46.8% of total mapped loci) and 90 (31.9%) were detected using *Pst*I/*Mse*I and *Eco*RI/*Mse*I primer combinations. On the whole, the highest number of mapped marker loci per experiment (18.9) was recorded for the *Pst*I/*Mse*I primer combinations. This parameter was much lower for *Eco*RI/*Mse*I primer combinations (12.9) and for SAMPL markers (8.0). Eight AFLP markers showed a co-dominant inheritance and six of these were mapped. A summary of map length, number of mapped loci per each class of molecular markers and per linkage group is reported in Table 3.

19 *Pst*I/*Mse*I, 8 SAMPL and 1 SSR markers identified during map saturation presented a distorted

TABLE 3 - *Map length, number of marker loci mapped per linkage group and total length of the saturated map.*

	Linkage group										Total
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	
Map length (cM)	216.6	220.4	165.6	260.2	172.2	160.7	172.8	150.3	142.0	165.2	1826.0
No. AFLP (*Eco*RI/*Mse*I)	12	10	13	7	8	9	11	3	7	10	90
No. AFLP (*Pst*I/*Mse*I)	17	17	14	13	16	10	14	11	13	7	132
No. SAMPL	1	6	6	1	0	2	2	2	0	4	24
No. RAPD	0	0	3	1	0	2	0	1	0	0	7
No. ISSR	1	2	0	1	0	0	0	0	1	0	5
No. SSR	3	2	2	2	4	3	2	2	2	2	24
Total marker loci	34	37	38	25	28	26	29	19	23	23	282
Average map density	6.4	6.0	4.4	10.4	6.2	6.2	6.0	7.9	6.2	7.2	6.5

TABLE 4 - *Expected segregation patterns and their significance for classes of molecular markers used for the construction of the saturated genetic linkage map of the 'Nostrano di Storo' based on the pseudo-testcross strategy.*

Molecular markers	Parental genotypes* (NSt15/8 x B37)	$P \geq 0.05$		$0.05 \geq P \geq 0.01$		$0.01 \geq P \geq 0.001$		$P \geq 0.001$	
		No. alleles	Mean ± s.e.	No. alleles	Mean ± s.e.	No. alleles	Mean ± s.e.	No. alleless	Mean ± s.e.
AFLP	*a*/- x -/-	220	0.818± 0.063	10	5.289± 0.172	7	8.283± 0.437	6	18.995± 1.513
	-/- x *a*/-	12	0.674± 0.188	1	4.000	3	9.042± 0.866	0	-
SAMPL	*a*/- x -/-	19	0.804± 0.236	8	5.141± 0.294	0	–	0	-
	-/- x *a*/-	1	3.189	0	–	0	–	0	-
RAPD	*a*/- x -/-	8	0.438± 0.302	0	–	0	–	0	-
	-/- x *a*/-	0	–	0	–	0	–	0	-
ISSR	*a*/- x -/-	4	2.008± 0.462	2	5.659± 0.259	0	–	0	-
	-/- x *a*/-	0	–	1	4.091	0	–	0	-
SSR	*a*/- x -/-	22	0.727± 0.194	1	5.226	1	9.931	0	-
	-/- x *a*/-	0	–	0	–	0	–	0	-
Total		286	0.827± 0.056	23	5.127± 0.160	11	8.640± 0.376	6	18.995± 1.513

* The letter *a* indicates the presence of a distinct marker allele, while – indicates its absence.

segregation. On the whole, 40 (12.3%) of the polymorphic loci of the F_1 population (27 AFLP, 8 SAMPL, 3 ISSR and 2 SSR), 26 of which were mapped, showed a significant distortion (Table 4). The distribution of the markers with distorted segregation among and along the chromosomes was not uniform. For instance, in linkage groups 8 and 9 no marker loci with distorted segregation were mapped, and only one locus was mapped in linkage groups 7 and 10. Furthermore, regions with three to four consecutive loci with distorted segregation ratios were identified. In particular, three marker loci characterized by segregation distortion of their alleles were mapped on linkage group 1, and 3, and four on linkage groups 5 and 6, respectively. Distorted segregation ratios were also observed for five of the mapped loci on linkage group 4 and two loci on 3. The distribution of AFLP markers was not uniform across linkage groups: clusters of marker loci were found on linkage groups 3, 7 and 9, on the lower part of linkage group 5 and on the upper part of linkage groups 4 and 6.

Few markers were not mapped (13.5%), well under the figures reported by other authors, *e.g.* 23.1% (AGRAMA *et al.*, 1997) and 27.6% (CASTIGLIONI *et al.*, 1999). The mapping of 24 SSR loci (8.5% of the total marker loci) allowed all 10 linkage groups to be numbered as in published maize maps.

DISCUSSION

The 'Nostrano di Storo' landrace, has been the basis to construct a linkage map of maize using segregation patterns of marker alleles detected in an F_1 mapping population. This map is made up of 282 marker loci (222 AFLP, 24 SAMPL, 7 RAPD, 5 ISSR and 24 SSR) positioned on 10 linkage groups and covers a total length of 1826 cM.

This mapping based on the pseudo-testcross allows a higher level of parental genomic polymorphism (118 marker loci vs. 81 in the BC_1 strategy) to be mapped. This is true, at least, when dominant molecular markers are considered. This is consistent with the ability of the two strategies to detect the recombination events occurring between pairs of linked loci. Theoretically, in the F_1 plant backcrossed to the inbred line, half of the marker loci polymorphic between parents and segregating in the mapping population, *i.e.* those present in a heterozygote state in the landrace genotype NSt15/8 and absent in the inbred line B37, become homozygote null for the marker allele, thus limiting the possibility to allow segregating marker alleles in the BC_1 generation. The frequency of recombination in the BC_1 population, in fact, resulted on average half of that observed in the F_1 population (3.7% *vs.* 7.4%). It is concluded that the efficiency of the pseudo-testcross strategy is around twice compared to that of the backcross. A consistent demonstration of this different efficiency is that the genetic map constructed on the basis of the F_1 segregation data includes 108 loci and covers almost 400 cM, whereas that constructed on the basis of the BC_1 data includes 69 loci but covers more than 500 cM. It is worth mentioning that, for maize, no genetic map had previously been constructed following a pseudo-testcross strategy, nor on the basis of the segregation data of BC_1 populations.

It was not however possible to integrate the two maps given the few markers in common: of the nine shared markers, only six were positioned on both maps and, of these, one (E-CAC/M-ATC340) was assigned to different linkage groups: 2 in the F_1 map and 4 in the BC_1. A consistent percentage (more than 7%) of marker loci located on different chromosomes of two different genetic maps was also observed by CASTIGLIONI *et al.* (1999), VUYLSTEKE *et al.* (1999) and SIBOV *et al.* (2003). Such results can be explained by the fact that two PCR-derived markers with the same molecular weight may represent different DNA sequences and, therefore, be considered alleles of two different genomic loci. A second possibility is that these markers represent duplicated sequences of paralogous genomic loci as supported by the paleotetraploid nature of maize genome (GAUT and DOEBLEY, 1997).

It is worth noting that in this paper the locus *dupssr*7, whose location site was unknown, was assigned to linkage group 5, and that the position of locus *umc*1143 in linkage group 6 is not compatible with that expected on the basis of the reported *bin* value (COE *et al.*, 1995). Discrepancies in terms of marker order and position for SSR loci were also found in the linkage map recently developed by SIBOV *et al.* (2003).

In accordance with what already observed by CASTIGLIONI *et al.* (1999) and VUYLSTEKE *et al.* (1999), the primer combinations of *Pst*I/*Mse*I were more informative than the *Eco*RI/*Mse*I ones for the construction of the genetic maps, both in terms of the average number of polymorphisms identified (23.0 *vs.* 14.0) and mapped marker alleles (18.9 *vs.* 12.9). CASTIGLIONI *et al.* (1999) observed an average num-

ber of polymorphisms equal to 19,6 and 14,9, respectively, while VUYLSTEKE *et al.* (1999) of, respectively, 27 and 19 using RILs and 26 and 16 using immortalized F_2 populations. Most of the studies using AFLP markers are based on *Eco*RI and *Mse*I as restriction enzymes. In this research, both *Eco*RI/*Mse*I and *Pst*I/*Mse*I primer combinations were used. The advantage of using *Pst*I/*Mse*I primer combinations was evident and arose mainly from a nearly twofold higher frequency of polymorphism over *Eco*RI/*Mse*I combinations. Moreover, the *Eco*RI and *Pst*I enzymes sample different regions of the genome: *Pst*I is methylation-sensitive and cuts mainly in unmethylated regions of the genome, containing expressed and mainly single-copy genes. *Eco*RI, in contrast, is methylation-insensitive and cuts DNA throughout the genome.

In the two genetic maps the large majority of marker loci were grouped with a LOD≥5 and ordered with a LOD≥3; only in some cases it was necessary to reduce the LOD score to a value between 2 and 3 to assign markers to specific linkage groups. Segregation of AFLP, RAPD, ISSR and SSR markers in the F_1 population followed the mendelian inheritance patterns in the large majority of cases, with a frequency of distorted segregation lower or similar to those reported by other authors working in the F_2 populations and comparable to those observed in populations of recombinant inbred lines. In the BC_1 population the distortion was evident for 18.5% of loci, value higher than that observed in other populations (HELENTJARIS *et al.*, 1986; BEAVIS and GRANT, 1991; BEAMONT *et al.*, 1996; SMITH *et al.*, 1997; CASTIGLIONI *et al.*, 1999). In particular, using F_2 populations and RFLP markers, HELENTJARIS *et al.* (1986) and BEAVIS and GRANT (1991) reported a distortion ranging from 7% to 15%, while BEAUMONT *et al.* (1996) and AGRAMA *et al.* (1997) using F_2 populations and RAPD markers obtained values of 17% and 46%, respectively. With AFLP markers, CASTIGLIONI *et al.* (1999) scored segregation distortion in 9,7% of the marker loci assayed.

In the saturated F_1 genetic map, both the number of markers per linkage group and the length of each linkage group, genetically measured by the overall recombination frequency, resulted proportional to the physical length of the corresponding chromosomes, as assessed in cytological maps (DAWE *et al.*, 1992; NEUFFER *et al.*, 1997), with some exceptions. The highest number of marker alleles was, in fact, observed for linkage group 3 (38, with a length of 165.6 cM), whereas the lowest number was for linkage group 8 (19, with a length of 150.3 cM). Fewer markers than expected were located in linkage group 4 (25, with a length of 260.2 cM), whereas in linkage group 7 the opposite occurred (29, with a length of 172.8 cM). Also DAVIS *et al.* (1999) have found exceptions both for the length of linkage group 7 and the number of mapped loci in linkage groups 2, 6 and 7.

In our saturated map, the distribution of the marker loci with distorted segregation was not uniform. In particular, a higher number of distorted markers were observed in the upper part of linkage groups 1, 5, and 6 and in linkage group 4 for the entire length. Regions with distorted segregation in linkage group 5 were also observed by CHAO *et al.* (1994) and CAUSSE *et al.* (1996). Three loci of the upper part of linkage group 4 and one locus of the lower part of linkage group 5 with distorted segregation were also identified, in agreement with SENIOR *et al.* (1996) and GARDINER *et al.* (1993), respectively. In these parts of linkage groups marker loci with distorted segregation were mapped in the region containing the *gametophytic factors* 1 and 2 (*ga1* and *ga2*) that affect the development of pollen tubes and the degree of chromosome transmission. Microsatellite marker alleles with distorted segregation were also detected by SHAROPOVA *et al.* (2002) in the same region of linkage group 5. Only two marker loci with distorted segregation were identified in linkage group 3. For the whole length of this linkage group also VUYLSTEKE *et al*. (1999) have reported marker loci with distorted segregation, as well as CHAO *et al.* (1994) and CAUSSE *et al.* (1996).

Clustering of markers in different chromosomal regions is in agreement with CASTIGLIONI *et al.* (1999). Also DUFOUR *et al.* (2001) found that the distribution of AFLP markers was not uniform in their linkage map: the centromeric regions had numerous *Eco*RI/*Mse*I loci, and, in addition, several genomic regions were not mapped by AFLP markers. Thus, *Pst*I/*Mse*I primer combinations should be preferred to finely saturate the genetic mapping of specific linkage groups.

In the past, saturated genetic maps of maize were most often constructed using single-locus codominant markers such as RFLP and SSR. The use of multilocus PCR-derived markers allows the acquisition of more data in a shorter time. Although the relative position of the marker loci in a genetic map can be influenced by the error inherent to the estimation of recombination frequencies - due to methodological difficulties and to the type of segre-

gating population - the AFLP technique represents an inexhaustible source of genomic markers. The high number of polymorphic markers obtained per experiment, and the large percentage of loci mapped out of those considered, along with the high genomic variability of maize, make the AFLP technique a valuable tool for improving the efficiency of genetic map construction in this species.

This paper anticipates the need of a serious and thorough work of exploration and collection of local maize varieties. When this approach is extended to the entire country, it will be the basis for protecting what remains of the Italian maize germplasm and designing an appropriate conservation. To this aim, the characterization and precise identification of landraces still cultivated is necessary. The genetic map developed for 'Nostrano di Storo' landrace can be an efficient and easily applicable tool for the acquisition of information related to other possible sources of local maize germplasm. The map allows to select primer combinations revealing the highest number of marker alleles to be used in the characterization of the gene pool of the farmer's populations which make up the 'Nostrano di Storo' landrace as a whole. They can also be profitably used to optimize the sample size of individuals to be utilized for deciding on conservation methods (*on farm*, *in situ* and *ex situ*) and foresee the consequences of conservation protocols on the genetic structure of the resulting populations. In particular, the availability of the map will allow to monitor genetic diversity and gene flow between farmer's populations and to verify the substructure, if existing, of the 'Nostrano di Storo' population (LUCCHIN *et al.*, 2003; BARCACCIA *et al.*, 2003).

A project dealing with the exploitation of a set of the available mapped molecular markers has been started and completed to investigate the influence of the conservation strategy on the genetic structure of farmer's populations belonging to the landrace 'Nostrano di Storo' (still unpublished). On the basis of the genetic differentiation that exist both within and between farmer's populations, it should be possible to identify core populations to be used for conservation of the variety.

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