

RFLP and AFLP polymorphism analysis in sugarcane varieties.

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Palabras claves: AFLP, RFLP, polimorfismo, *Saccharum* spp, caña de azúcar.

Key words: AFLP, RFLP, polymorphism, *Saccharum* spp., sugarcane.

ABSTRACT: The effectiveness of the sugarcane breeding programs could be improved by a comprehensive knowledge of the amount of genetic diversity between parental cultivars. In this study, fifteen commercial varieties, regularly used in the Cuban sugarcane breeding program, were assessed using restriction fragment length polymorphism (RFLP) revealed by twelve dispersed low copy probes in combination with *Eco*RI, *Hind*III and *Bam*HI enzymes restriction for its nuclear genetic diversity and amplified fragment length polymorphism (AFLP) obtained by fourteen primer combinations. The level of polymorphism generated by RFLP and AFLP techniques were compared. Sixty-four RFLP bands and 309 AFLP bands were clearly polymorphic among the studied genotypes, representing respectively a 77.1 and 46.7 % of the bands assessed. In order to distinguish the more informative markers the resolving power values (Rp) were calculated. Eight probe enzyme RFLP and twelve AFLP primer combinations were recommended based on their polymorphism and Rp values for individual identification. The varieties were clustered in three molecular diversity groups on UPGMA dendrograms constructed separately using RFLP and AFLP pairwise Dice distance estimate.

RESUMEN: La efectividad de los programas de mejoramiento de caña de azúcar podrían ser mejorados mediante el conocimiento de la diversidad genética entre los cultivares parentales. En este estudio, se evaluaron 15 variedades, regularmente utilizadas en el programa cubano de mejoramiento de la caña de azúcar, mediante el polimorfismo de la longitud de los fragmentos de restricción (RFLP) revelado por doce sondas de bajo número de copias en combinación con las enzimas de restricción *Eco*RI, *Hind*III y *Bam*HI para estudiar su diversidad genética nuclear y mediante el polimorfismo de la longitud de los fragmentos amplificados (AFLP) obtenidos por catorce combinaciones de cebadores. Se comparó el nivel de polimorfismo generado por las técnicas RFLP y AFLP. Entre los genotipos estudiados se detectaron 64 bandas claramente polimórficas de RFLP y 309 bandas polimórficas de AFLP, representando el 77.1 % y 46.7 % respectivamente, de las bandas

analizadas. Para distinguir los marcadores más informativos se calcularon los valores del poder de resolución (Rp). Se recomendaron para la identificación individual, 8 combinaciones enzima-sonda para RFLP y 12 combinaciones de cebadores para AFLP basado en su polimorfismo y el valor Rp. Las variedades fueron agrupadas en tres grupos de diversidad según dendrogramas UPGMA construidos separadamente usando los pares de los estimados de distancia de Dice.

INTRODUCTION

The knowledge about the amount and distribution of the genetic variation among cultivated species and their wild relatives is necessary to improve the efficiency of breeding and genetic conservation programs.

A major limitation of sugarcane breeding is its reduced genetic base, modern cultivars are essentially hybrids among a few clones of *Saccharum officinarum* and *Saccharum spontaneum*. This narrow genetic base of the majority of hybrids is one factor limiting progress in sugarcane breeding programs.

The success of the breeding program depends on the knowledge and understanding of the genetic diversity

available on the germplasm. Increase in breeding program efficiency has been also supported by the development of molecular markers. These can be employed as auxiliary tools in genotype characterization, due to the high polymorphism and genome coverage, that are unaffected by environment and free of epistatic interactions.

Among molecular markers, RFLP is one of the most used in sugarcane and was employed to assess the polymorphism at nuclear ribosomal DNA level; ¹ nuclear DNA; ^{2,3,4,5,6,7,8} cytoplasmic DNA. ^{9,10,11,12}

The advent of PCR based marker systems overcame most of the limitations of the RFLP technique AFLP molecular markers, which reveal a high number of polymorphic bands in a multiplex pattern, offer several advantages over a multitude of other markers ^{13,14,15} and were first used to estimate the genetic distances in maize lines, ^{16,17} these results were followed up by studies to investigate the genetic diversity in several plant species, ^{18,19,20,21,22,23} including sugarcane. ²⁴

AFLP analysis detects large numbers of polymorphics genetic loci in a single PCR reaction. Multiple AFLP analysis can detect thousands of genetic loci in a short period of time. AFLP technique is quick, robust, requires minimal preliminary work. ¹⁴

The present study have the following objective: to compare the polymorphism level revealed by RFLP and AFLP methods; to detect the RFLP and AFLP patterns most suitable for their identification and to determine the probe-enzyme and primers combinations more appropriated for mapping studies purposes.

MATERIALS AND METHODS

Plant Material

Fifteen sugarcane varieties, regularly employed as parents in breeding programs were studied (Table 1). The materials surveyed were obtained from the germplasm bank of the National Institute for Sugarcane Research (INICA) in Cuba.

RFLP analysis

Total genomic DNA was extracted from freeze-dried leaves according to Hoisington (1992).²⁵ DNA (10 µg) was

Table 1. Genotypes used for RFLP and AFLP analysis.

Code	Genotypes	Origin	Ripening	Code	Genotypes	Origin	Ripening
1	CP 74-2005	USA	early	9	My 5514	Cuba	late
2	C 568-75	Cuba	late	10	Mex 68P23	México	early
3	CP 72-2086	USA	early	11	Ja 60-5	Cuba	middle
4	C 323-68	Cuba	middle	12	C 87-51	Cuba	early
5	Ja 64-19	Cuba	early	13	C 1051-73	Cuba	early
6	Mex 66-1235	México	non data	14	Mex 57-473	México	early
7	Mex 69-290	México	early	15	CP 52-43	USA	early
8	PR 980	P. Rico	late				

digested by the restriction enzymes *EcoR* I, *BamH* I and *Hind* III, according to the supplier's (Amersham) recommendation. Restricted DNA was fractionated by electrophoresis in 0.8 % agar gels in 1 X TAE buffer (40 mM Tris acetate, 1 mM EDTA) and transferred to a nylon membrane (Hybond N+, Amersham) by alkaline transfer (0.4 N NaOH). Probes were amplified using standard polymerase chain reaction procedures and labeled by [³²P] - dATP using a random primer labeling Kit (Amersham). Hybridizations were performed according to Hoisington (1992). Molecular weights were determined by comparison to molecular weight marker, Raoul I (Appligene).

Probes

Probe/enzyme combinations (PEC) of 12 single copy probes from different *Saccharum* linkage groups²⁶ and the 3 restriction enzymes were selected according to their polymorphism and autoradiographic quality (data not shown). The probes were provided by the maize genomic library of the University of Missouri, Columbia, USA (UMC), the maize genomic library of the Brookhaven National Laboratory, USA (BNL), and the sugarcane library SSCIR from CIRAD, France. The linkage group according to Grivet et al., 1996 are: I (SSCIR 69, BNL 5.09), II:(UMC 93), III: SSCIR 60, V (SSCIR 217), VI (SSCIR 256), VII (SSCIR 194), VIII (UMC 44, BNL 12.06), IX (UMC 113, SSCIR 76) and the un linkage UMC 58.

AFLP analysis

The AFLP methods was performed by using the AFLP Analysis System I Kit (Instruction manual; GIBCO BRL, Life Technologies). Genomic DNA was digested with an *EcoR*/*MseI* enzyme combination. The preamplification step was carried out with AFLP primers having one selective nucleotide (*EcoRI*+A, *MseI*+C). Selective amplification was performed with three selective nucleotide (*EcoRI*+ANN, *MseI*+CNN). Fourteen primer combinations were assessed (Table 2). PCR samples were denaturated by adding an equal volume of formamide buffer (98% formamide (v/v), 10 mM EDTA, pH 8.0, 0.05% bromo-phenol blue (w/v), and 0.05% xylene cyanol (w/v)), heating for 5 min at 93 °C and chilled on ice. The samples were loaded on 6.5 % polyacrilamide gel under standard sequencing conditions. AFLP fingerprints were visualized using silver nitrate staining method according to the manufacturer's instruction (Promega Cat. # TMD005). Each primer combination was scored by eyes for number of polymorphic fragments detected and overall sharpness and intensity of polymorphic fragments. The scored fragments ranged in size from 200 to 700 bp. The size of the fragments was determined by comparing sequencing ladders of control template DNA to AFLP patterns.

Table 2. Primers combinations employed for AFLP analyses.

Primer combination	3' Selective nucleotide
E1/M1	E-ACT/M-CTA
E2/M2	E-ACA/M-CAT
E2/M1	E-ACA/M-CTA
E1/M2	E-ACT/M-CAT
E3/M2	E-AAC/M-CAT
E6/M4	E-ACG/M-CAC
E4/M7	E-AAG/M-CTG
E5/M1	E-ACC/M-CTA
E7/M3	E-AGC/M-CAA
E6/M6	E-ACG/M-CTC
E7/M8	E-AGC/M-CTT
E8/M6	E-AGG/M-CTC
E3/M6	E-AAC/M-CTC
E5/M7	E-ACC/M-CTG

Data analysis

Each polymorphic RFLP and AFLP fragment was scored 1 for presence and 0 for absence. Individual bands and patterns were considered as units of polymorphisms for each primer or probe-enzyme combinations.

Totally correlated variants were considered once. In both molecular techniques, the genetic similarity (S_{ij}) between clones was calculated according to the formula proposed by Dice (1945) and Nei and Li (1979).^{27,28}

In order to distinguish the more informative probe/enzyme combinations for RFLP and primer combinations for AFLP, the resolving power values (R_p) were calculated (Prevost and Wilkinson, 1999; Gilbert, 1999) by the formula $R_p = \varepsilon / I_b$, where $I_b = 1 - [2 \times |0.5 - p|]$ and p is the proportion of genotypes containing band. It depends on the relation of 0 and 1 of each combination, the highest values correspond to the combination where the proportion of 0 and 1 is closed to 50%.^{29,30}

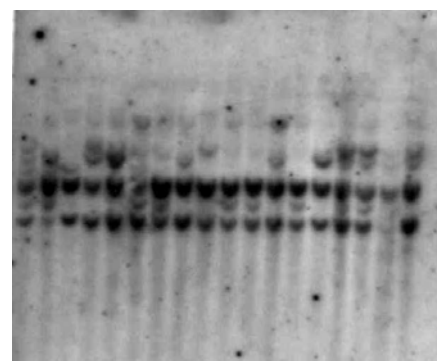
To determine clonal diversity groups, a cluster analysis was performed using unweighted pair-group method with arithmetic mean (UPGMA) linkage algorithm, based on each matrix of distance estimates. These analysis were performed using the software package NTSYS-pc (1998).

RESULTS AND DISCUSSION

RFLP diversity revealed 64 polymorphic fragments, with 16 probe-enzyme combination with an average number of 4 polymorphic fragments per genotype, ranging from 1 to 12. Figure 1 shows the southern blot hybridization results of BNL 509-*Bam*HI combination as an example of the polymorphism revealed by RFLP.

The ability of the PEC to diagnose sugarcane accessions was assessed on the basis of Resolving power (R_p). The R_p values varied between 0.4 for PEC SSCIR 69-*Bam*HI and 6.92 for PEC SSCIR 217-*Bam*HI. PEC with higher R_p values were generally able to distinguish more genotypes with unique bands pattern (Table 3).

Eight probe-enzyme combinations (codes 4, 5, 7, 8, 9, 11, 12 and 16) shown higher level of polymorphism and R_p values. Bands shown poor correlation, because RFLP markers monitor different points on the genome. The clonal specificity was very low because it was revealed only eight unique bands. Band patterns considered as unit of polymorphism allow the identification of the genotypes. Likewise, these PEC revealed a high closed specificity because of the higher number of genotypes with unique pattern. SSCIR-217-*Bam*HI combination allowed the identification of all genotypes.

**Fig 1.** Southern blot hybridization results of BNL 509 – *Bam*HI, exhibiting patterns of the fifteen sugarcane varieties studied.

In order to visualize affinity among the clones, a hierarchical ascending classification was performed on the whole sample based on the 53 independent polymorphic fragments. The dendro-gram produced is presented in Fig. 2 and no clear clustering into distinctive groups was visible.

AFLP analysis effectively detects large numbers of polymorphisms genetic loci in a single PCR reaction. Fourteen primer combinations produced a total of 1523 bands, fragments sizes ranged from approximately 50 to 850 base pairs (bp). From which 661, ranging from 200 to 700 bp, had a good sharpness and intensity able to discriminate the polymorphic bands. Of these, 309 bands were clearly polymorphic between two or more genotypes for 46.7 % of polymorphism. Examples of partial AFLP fingerprint are showed in figure 3. On average, 22.1 polymorphism were scored per primer pair, with a range of 8-54 scored polymorphism. Based on the percentage of polymorphic fragments, primer pairs also detected different levels of polymorphism, ranging from 18.45 to 69 % (Table 4). Moreover, individual polymorphic fragments (unique bands) were observed on twelve primer combinations, which varied from 2 to 7 bands. The R_p values varied between 3.6 and 25.46. The degree of AFLP polymorphism does not appear to be very large in the set of sugarcane genotypes we used. However, the majority of primer combinations were able to distinguish unique bands in some genotypes.

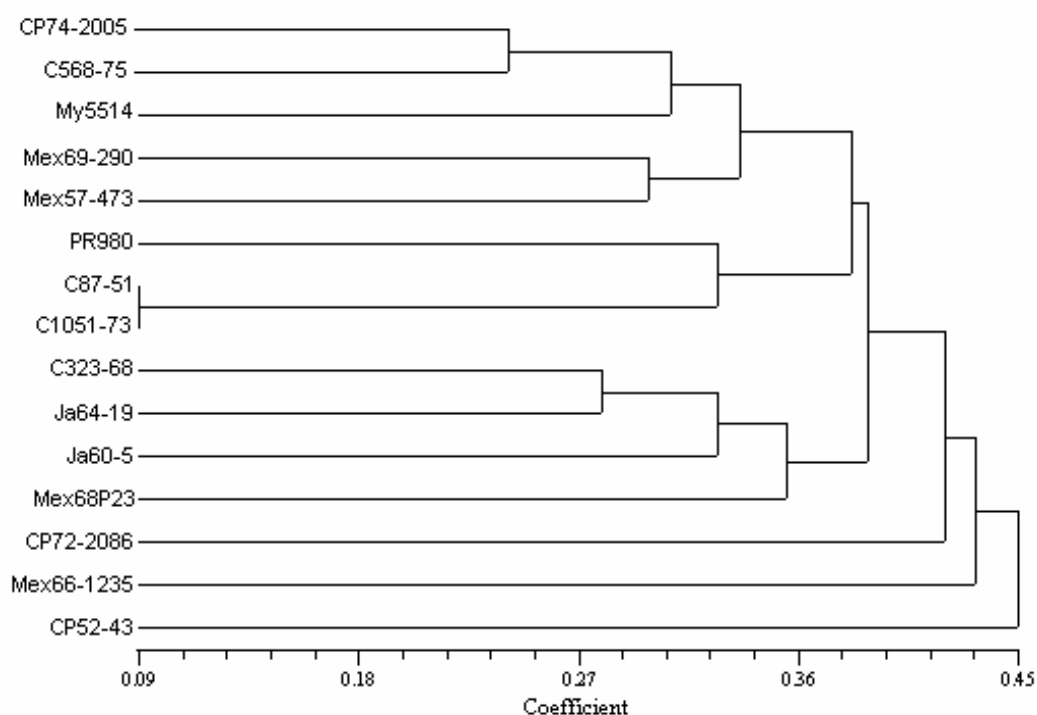


Fig.2. UPGMA cluster analysis of Dice similarity coefficients calculated on the basis of 64 RFLP comparisons among fifteen sugarcane varieties.

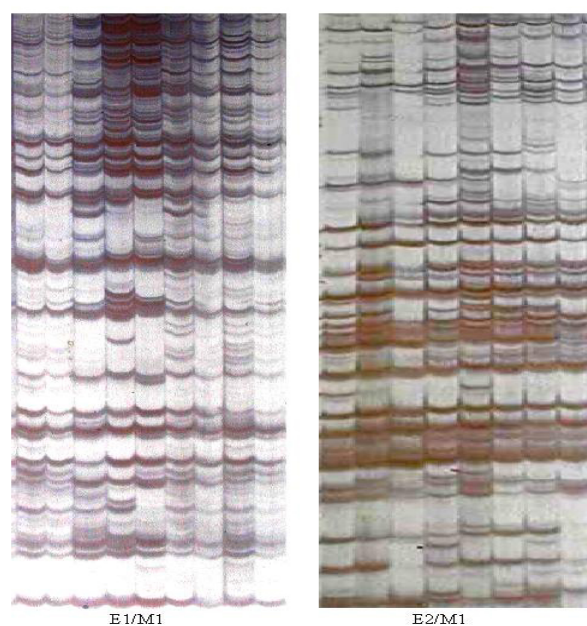


Fig. 3. Examples of partial AFLP fingerprint obtained with primer combinations E1/M1 and E2/M1. Only part of the gels, with fragments between 250 and 700 basepairs are shown

The base composition of the primer selective extensions had a significant effect on the number of segregating AFLP fragments. *EcoR* I-selective primer rich in guanine and cytosine provided the great number of fragments because on average it yielded 26.75 polymorphic fragments and the R_p was

11.45. AT-rich *EcoR* I-selective primer yielded only 15.8 polymorphic fragments with an R_p value of 8.18.

Based on this AFLP screening, we selected twelve primer combinations (E2/M1, E1/M2, E3/M2, E6/M4, E4/M7, E5/M1, E7/M3, E6/M6, E7/M8, E8/M6, E3/M6,

E5/M7) based on the polymorphism level, ranging between 28.3% and 67.4% and the higher values of R_p for use in further mapping. The collective R_p value for AFLP (140.7) was higher than the obtained from RFLP (34.82).

The dendrogram (Fig.4) depicts the clustering of sugarcane genotypes

into three groups of individuals showed a clear distinction between genotypes according by their ripening degree. Cluster A is composed of the two middle ripening genotypes with genetic similarity of 64%.

The early ripening genotypes appear as a compact group (cluster B) with greater similarity among its members than in the remaining groups. The cophenetic value for AFLP analysis was 0.82.

The limited genetic diversity detected is congruent with earlier study based on isozymes³¹ and nuclear data^{1,2,3} where also a relatively low number of accessions were used.

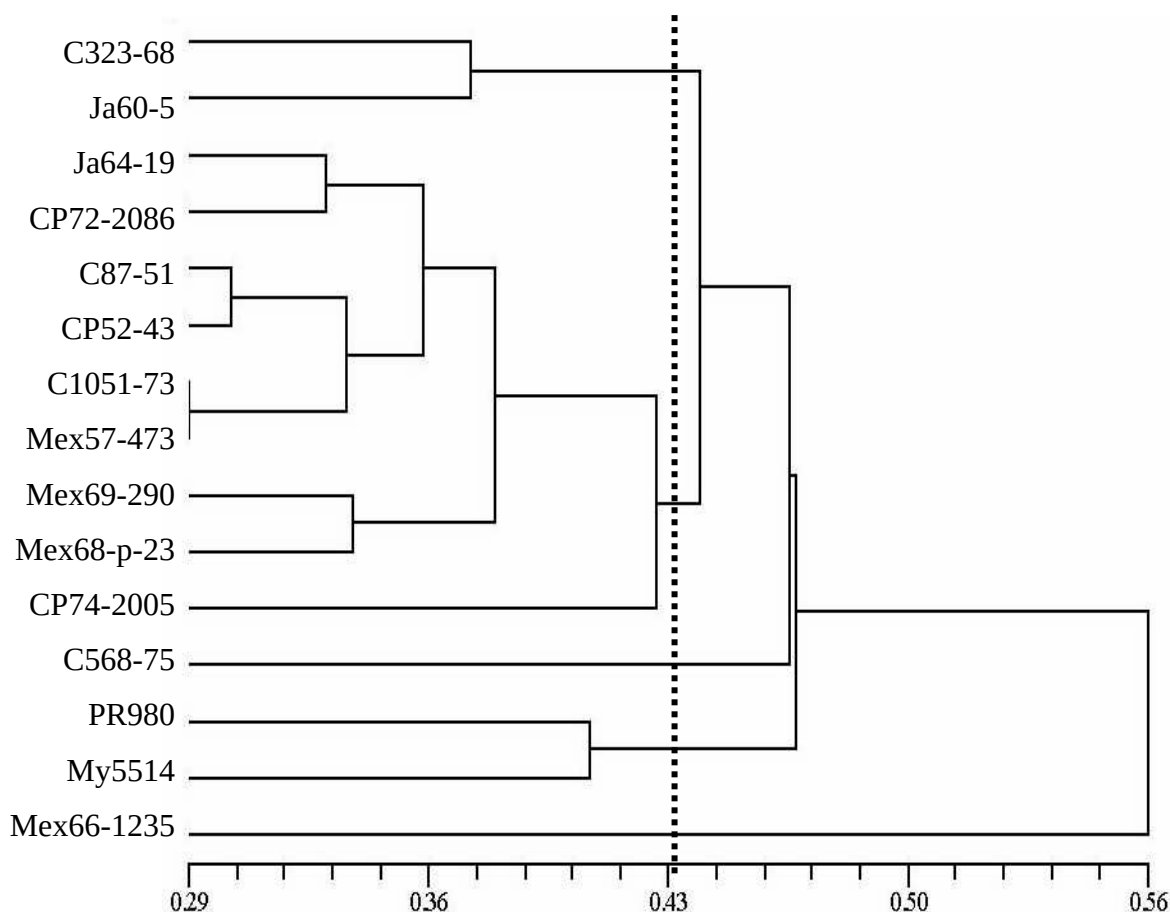


Fig. 4. UPGMA cluster analysis of Dice similarity coefficients calculated on the basis of 309 AFLP comparisons among fifteen sugarcane varieties.

CONCLUSIONS

Both, RFLP and AFLP were efficient in detecting polymorphisms among the studied genotypes, showing their utility in the characterization of sugarcane genotypes. We identified PEC for RFLP and AFLP primer combinations that produce unique banding patterns of the genotypes studied. The AFLP analysis produced 201 bands pattern from which the 96.05% was unique pattern. RFLP produced 103 pattern of bands for 52.4%. The degree of AFLP polymorphism does not appear to be very large in the set of sugarcane

genotypes we used. However, the majority of primer combinations were able to distinguish unique bands in some genotypes.

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Table 3. Nuclear polymorphism associated to each probe-enzyme combination. PECs evaluated and visible, polymorphic bands and unique patterns revealed among the parents.

Code	Probe-Enzyme Combination	Total bands scored	Polymorphic bands	Poly-morphism (%)	Within correlated bands ⁽¹⁾		Between correlated bands ⁽²⁾		Rp	Unique bands	Total number of patterns	Unique patterns (%)	Genotypes with unique patterns
					r = + 1	r = -1	r = + 1	r = -1					
1	UMC 113 – <i>EcoRI</i>	4	3	75	0	0	0	0	2.54	-	6	-	-
2	SSCIR 76 – <i>EcoR I</i>	3	2	66.6	0	0	0	0	1.32	-	4	-	-
3	BNL 1206 – <i>EcoR I</i>	3	1	33.3	0	0	0	0	0.94	-	2	-	-
4	BNL 509 – <i>EcoRI</i>	6	4	66.6	0	0	0	0	2.8	-	6	50	1, 3, 11
5	SSCIR 256 – <i>EcoRI</i>	3	3	100	0	0	0	0	1.46	-	5	40	14, 15
6	SSCIR 69 – <i>EcoRI</i>	4	1	25	0	0	0	0	0.8	-	2	-	-
7	UMC 58 – <i>BamHI</i>	6	6	100	0	0	1	1	2.68	2	10	80	1, 4, 6, 7, 9, 11, 14, 15
8	BNL 5.09 – <i>BamHI</i>	7	4	57.1	0	0	0	0	1.86	-	6	50	3, 6, 10
9	SSCIR 217 – <i>BamHI</i>	12	12	100	0	0	1	0	6.92	-	15	100	all
10	UMC 44 – <i>BamHI</i>	3	2	66.6	0	0	0	0	0.54	1	3	33.3	6
11	SSCIR 194 – <i>BamHI</i>	7	7	100	0	0	1	0	4.14	1	11	63.6	3, 4, 7, 8, 10, 11, 15
12	SSCIR 60 – <i>BamHI</i>	5	4	80	0	0	0	0	2.14	-	9	66.6	1, 2, 6, 9, 12, 15
13	SSCIR 69 – <i>BamHI</i>	4	2	50	0	0	0	0	0.4	1	3	33.3	14
14	UMC 93 – <i>BamHI</i>	2	2	100	0	0	0	0	0.8	-	3	-	-
15	SSCIR 256 – <i>BamHI</i>	6	3	50	0	0	0	0	0.94	2	4	50	3, 8
16	SSCIR 69 – <i>HindIII</i>	8	8	100	0	0	1	1	4.54	1	10	37.5	2, 6, 8, 9, 11, 15
Total	16	83	64	77.1					34.82	8	103	52.4	

(1,2): Correlated bands revealed by one or more PEC, respectively.

(1): Number of band pairs, totally correlated (r = + 1; r = -1) within PEC.

(2): Number of bands totally correlated (r = + 1; r = -1) with other ones revealed by other PECs

Table 4. AFLP primer combinations evaluated, visible and polymorphics bands and unique patterns revealed among the parents

Primer combination	Total bands	Bands scored 200-700 bp	Polymorphic bands ¹	Polymorphism (%)	Within correlated bands ⁽²⁾		Between correlated bands ⁽³⁾		Rp	Unique bands	Total number of patterns	Unique patterns (%)	Genotypes with unique patterns
					r = + 1	r = -1	r = + 1	r = -1					
E1/M1	124	35	8	23	0	0	2	2	4.46	0	13	84.6	1,3,4,5,6,7,8,9,12,13,15
E2/M2	100	48	9	18.45	0	0	15	5	3.60	2	11	81.8	2,3,5,7,9,10,11,12,15
E2/M1	110	53	32	60.37	6	0	16	8	10.18	7	15	100	All
E1/M2	81	46	20	43.47	0	0	19	1	8.14	3	14	92.8	1,2,3,4,5,6,7,8,9,10,11,12,15
E3/M2	101	37	15	40.54	1	0	11	1	7.06	2	15	100	All
E6/M4	105	39	25	64.1	2	3	17	11	12.18	6	15	100	All
E4/M7	122	53	15	28.3	0	0	11	3	6.96	3	15	100	All
E5/M1	129	51	26	51	1	0	12	1	10.42	2	14	92.8	1,2,4,5,6,7,8,10,11,12,13,14,15
E7/M3	184	85	54	63.5	7	0	15	8	25.46	7	15	100	All
E6/M6	125	42	29	69	2	3	16	2	12.8	3	15	100	All
E7/M8	78	51	23	45.1	2	0	16	4	11.1	6	15	100	All
E8/M6	96	40	13	32.5	0	0	10	1	5.22	5	14	92.8	1,2,3,4,5,7,8,10,11,12,13,14,15
E3/M6	82	38	11	29	0	0	3	0	15.76	0	15	100	All
E5/M7	86	43	29	67.4	3	0	10	1	7.36	4	15	100	All
Total	1523	661	309	46.7					140.7	48	201	96.05	

(1): Polymorphism according to the range scored (200-700 bp).

(2,3): Correlated bands amplified by one or more primer combinations, respectively.

(2): Number of band pairs, totally correlated (r = + 1; r = -1) within primer combination.

(3): Number of bands totally correlated (r = + 1; r = -1) with other ones amplified by other primer combination.