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PUTATIVE LOCI ASSOCIATED TO POLYEMBRYONY IN MAIZE POPULATIONS

[LOCI TENTATIVOS ASOCIADOS A LA POLIEMBRIONIA EN POBLACIONES DE MAÍZ]

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SUMMARY

Polyembryony (PE) is a reproductive phenomenon that produces more than one plant per seed in maize and other species. The objective of this study was to identify polymorphic DNA bands associated to the genetic expression of this trait using AFLPs. The genetic materials used in this study were 19 Polyembryonic experimental maize lines from two populations, identified as BAP (dwarf, high PE frequency genotypes) and NAP (normal height, high PE frequency) and 16 non-polyembryonic commercial genotypes (NPE). Results showed that the PE seeds had a germination rate of 89.47 %, where 28.23 % were single seedlings and 71.76 % were polyembryonic seedlings. Most of the polyembryonic seeds produced double plants although some triple plants cases were also observed. In addition, twenty nine putative loci were identified associated to the maize polyembryony; this association may be related to the differential origins of the maize populations. In addition, the rate of genetic diversity between the groups PE and NPE was estimated at the locus level using the Shannon index, getting a range of polymorphism from 29% to 53%, this could be related to the origin of the tested samples and their phenotypic and genotypic traits. These results suggest that the polymorphic bands may be related to the expression of genes linked to polyembryony in maize.

Keywords: AFLP´s; Shannon entropy; *Zea mays*; polyembryony.

RESUMEN

La poliembrionía (PE) es un fenómeno reproductivo que produce más de una planta por semilla en maíz y otras especies vegetales. El objetivo de este estudio fue identificar bandas de ADN polimórfico asociadas a la expresión genética de esta característica mediante AFLP´s. Los materiales genéticos utilizados en este estudio, fueron 19 líneas experimentales de maíz, derivadas de dos poblaciones poliembriónicas, identificadas como BAP (genotipos enanos, de alta frecuencia de PE) y NAP (altura normal, de alta frecuencia PE) y 16 genotipos comerciales no poliembriónicos (NPE). Los resultados mostraron que las semillas PE presentaron una tasa de germinación de 89.47 %; de las plántulas germinadas 28.23 % fueron de una sola plántula y 71.76 % plántulas poliembriónicas. La mayoría de las semillas poliembriónicas producen plantas dobles, aunque en algunos casos también se observaron plantas triples. Además, veintinueve loci tentativos fueron identificados en relación a la poliembrionía del maíz; esta asociación puede estar relacionada con el origen diferencial de las poblaciones de maíz. También fue estimado el índice de diversidad genética entre los grupos PE y NPE a nivel de locus con el índice de Shannon. Dicho índice mostró una serie de polimorfismos de 29% a 53%, lo que puede estar relacionado con el origen de las muestras analizadas y sus características fenotípicas y genotípicas. Estos resultados sugieren que las bandas polimórficas puede estar relacionadas con la expresión de genes ligados con poliembrionía en el maíz.

Palabras clave: AFLP´s; entropía de Shannon; *Zea mays*; poliembrionía.

INTRODUCTION

Polyembryony is a natural trait that can be used as an alternative tool in the development of maize varieties for special uses (Espinoza *et al*., 1998). It has been reported that kernels of certain kinds of polyembryonic maize lines contain a higher nutritional value than their normal counterpart, because of their enhanced quantity and quality in oil and embryonic proteins (Valdez *et al*., 2004); in addition, this kind of maize varieties would save the number of seed per unit of agricultural land (Espinoza *et al*., 1998; Valdez *et al*., 2004). One of the first reports on the maize polyembryony was presented in 1951 by Morgan and Rappleye, whom after irradiating pollen at different concentrations of X-rays, obtained a range of polyembryony from 1.65% to 18.18%. On the other hand, Pesev *et al*. (1976) reported the development of maize inbred lines with multiple embryos which had levels of polyembryony between 2.1% and 25.3%. Two polyembryonic maize populations have been developed at the Instituto Mexicano del Maíz (IMM) of the Universidad Autonoma Agraria Antonio Narro (UAAAN), during the last thirty five years. A maize population was developed with a broad- genetic basis, with the brachyitic condition (dwarf plants) as a central feature. Among the segregating progenies from this special population, a small group of plants (1.5%) showing the twin condition: one seed, two stems was identified. Later, schemes of recurrent selection were applied to increase the polyembryony frequency, achieving levels of 47% by 1991**;** after this year the base population was spread into two: dwarf, and normal height plants; during 1996, the two populations had reached more than 60% of polyembryony. Reproductive management of the polyembryonic groups has been through fraternal crosses with mixed pollen, leading polyembryonic seeds with a germination of 60% in direct sown and about 90% under greenhouses conditions (Espinoza *et al*., 1998). In recent years, molecular markers such as RAPD'S, and microsatellites have been used to determine genetic differences between non polyembryonic and polyembryonic plants (Ravishankara *et al*., 2004, Andrade-Rodriguez *et al*., 2005). In addition, some genes associated to polyembryony in cereals have been reported per example *Ig1* in maize (Evans, 2007); *OsCem* (Yang and Hwa, 2008) and *OsPE* in rice (Puri *et al*., 2009). The last one was even cloned. However, difficulties to incorporate the polyembryony trait to maize varieties and some environmental effects associated to expression of this trait, suggest that in maize more than one gene is associated to polyembryony. In this context, and assuming that the maize polyembryony is useful characteristics with potential to enhance efficient production of this cereal, the objectives of this study were: to determine the polyembryony frequency in polyembryonic and non-polyembryonic maize populations; to identify related loci to polyembryony based on the AFLPs technique; and to evaluate the genetic diversity among and within those maize populations.

MATERIALS AND METHODS

In order to obtain vegetal material (leaf tissue), 19 polyembryonic experimental lines (PE) and 16 non-polyembryonic maize commercial genotypes were sown under greenhouse conditions (Table 1). The polyembryonic maize lines were provided by the IMM-UAAAN; those lines were named in accordance to the population where they came from: BAP or NAP. The genotypes 1f to 11f belong to BAP and the 12f to19f belong to NAP. The commercial non- polyembryonic genotypes, codified as NPE, were provided by different seed companies. The genotypes were represented by a random sample of 5 seeds, and sowed in polyurethane germination cages, 200 cavities each.

DNA isolation was based on the protocol reported by Dellaporta *et al*. (1983). DNA integrity was tested in an agarose (1%) gel electrophoresis. Approximately 100 ng of genomic DNA from each sample were used to determine the amplified fragment length polymorphism (AFLP's) following the "IRDyeTM Fluorescent AFLP ® Kit for Large Plant Genome Analysis LI - COR (Biosciences)" protocol. Once obtained the selective PCR amplification products, they were denatured, after that the DNA segments were fractioned by polyacrylamide gel electrophoresis (6.5%); using an automatic DNA sequencer Model 4300 Li-Cor. Electrophoresis was carried out for three hours. The images obtained from the gel were analyzed manually. Thirty four primer combinations were evaluated. The combinations that gave the highest genetic polymorphism were based on the primer combinations M - CAT/ E - AAG and M - CAT / E – ACG. Once the best AFLPs primer combination was selected, the AFLPs test was performed, obtaining the DNA amplifications visualized in 700 and 800 nm. The fragment sizes ranged from 50 to 354 bp, based on the molecular size marker 50 - 750 bp (LiCor). To determine the similarity index, a binary matrix was generated; assigning a value of 1 or 0 according to the presence or absence of a band; in addition, the Nei and Li (1979) formula was applied.

To test the possible association between AFLP loci and polyembryony, a test statistic for

association was used. The central statistic is the correlation between two qualitative traits. The square of this coefficient multiplied by the sample size has an approximate chi square distribution. Since several AFLP loci were tested, to calculate the threshold value for hypothesis testing the loci were assumed to be independent and the type II error probability was calculated as 0.05/n, where n was the number of polymorphic loci. The area under the right tail of the chi square distribution was equated to this value and solved for the corresponding correlation value with the aid of the mathematical system Wolfram. This correlation value was then used as the threshold to reject the hypothesis of no association between AFLP loci and polyembryony.

For dendrogram construction the PHYLIP program version 3.63 (Felsenstein, 2000) was used. Genetic distances were calculated using restriction site formulae (Nei and Li, 1979) in the Rest Dist routine. The genetic distance matrix was then used as the input of the Neighbor routine in the same program to construct a Neighbor–Joining based dendrogram. The coded dendrogram was interpreted by Tree view 1.66 to draw a rectangular cladogram. Shannon entropy (Reyes-Valdes, 2006) was developed to measure the amount of information that can be transmitted in a code. Applied to polymorphic loci it can be expressed as follows:

$$H = - \Sigma \, \delta i \, Log_{2} \, \delta i$$

Where: H = Shannon entropy and δi = frequency of the i-th genotype.

RESULTS

Polyembryony frequency in polyembryonic and non-polyembryonic maize populations.

Once the plants were 25 days-old, the number of plants per seed were counted in both, polyembryonic and not polyembryonic maize groups (Table 1); given a case of PE in certain genotype, it was quantified the number of seedlings (two, three or more) per seed. Results showed that the PE seeds had a germination rate of 89.47 %, where 28.23 % were single seedlings and 71.76 % were polyembryonic seedlings. In both BAP and NAP populations, most of the polyembryonic seeds produced double plants although some triple plants cases were also observed. The average seed germination rate in the NPE genotypes was higher (92.5%). This high germination was expected because of the commercial nature of the seed. According to the expected performance of the commercial genotypes, it was not found any case of polyembryony in the NPE group.

Identification of loci associated to polyembryony

Correlation tests were performed in order to determine the association among qualitative characteristics in all loci. It was necessary to obtain the critical values of the AFLP's bands obtained in both analyzed absorbances (700 and 800 nm), whose values were 0.309 and 0.292, respectively. A total of 11 candidates loci were found in the absorbance of 700 nm and 18 candidates loci in the absorbance at 800 nm. Only two out of the 33 analyzed loci were 100% monomorphic bands in both PE and NPE groups, which may indicate that the region of these two loci is very stable in the maize genome (Table 2). On the other hand it was obtained a range from 29% to 53% of polymorphism within maize genotypes this could be associated to the origin source of the maize genotypes, their phenotypic and genotypic characteristics. In this last point, it should be stressed that could be related to the expression of genes that are associated to polyembryony in both analyzed absorbances.

Genetic diversity among maize PE and PE genotypes

In the obtained dendrogram, it can be appreciated clusters of polyembryonic and non- polyembryonic genotypes (Figure 1). The genetic differences, as expected between NPE and PE groups, were very high; it may be so because all the analyzed genotypes come from different maize breeding programs, therefore, bands with a greater presence in the PE group, and absent or poorly frequent in the NPE group are potential candidates to be related to polyembryony.

Table 1. Seed germination and identification of polyembryony in the NPE and in the PE genotypes.

Code PEG	NGS	Non PP	Polyembryonic plants		Code NPE Commercial Hybrids	NGS	NNGS
			Double	Triple or more			
1F	5	1	4	0	RX776W (A1)	5	0
2F	5	1	4	0	RX708 (A2)	5	0
3F	3	1	2	0	HCF2330 (WS)	3	2
4F	5	2	3	0	RX897 (M)	5	0
5F	5	2	2	1	TG8535 (TM)	3	2
6F	5	2	2	1	DK697 (D)	5	0
7F	5	1	4	0	Pague Agri 4000 (VS)	4	1
8F	4	0	3	1	9703 (GH)	5	0
9F	5	2	3	0	31698 – N907 (PM1)	5	0
10F	4	2	2	0	TG7900W (SC)	5	0
11F	4	2	2	0	33J56 (PM2)	5	0
12F	4	1	3	0	GEI9850 (G1)	5	0
13F	5	1	3	1	30688 (PM3)	5	0
14F	3	1	2	0	1851W (SA)	4	1
15F	4	0	4	0	AN388T1–FMH209 (AN2)	5	0
16F	5	1	4	0	AN388T2–FMH210 (AN3)	5	0
17F	5	3	2	0			
18F	5	1	4	0			
19F	4	0	3	1			
Total	85	24	56	5		74	6

PEG=Polyembryonic genotypes, F=Family number, NGS= number of germinated seeds, PP= polyembryonic plants, NPE= non polyembryonic, NNGS= number of non-germinated seeds.

Table 2. Shannon diversity index of 700 and 800 nm AFLP's band patterns.

700 nm				800 nm			
Loci	**Shanon**	**Loci**	**Shanon**	**Loci**	**Shanon**	**Loci**	**Shanon**
1	0.53	16	0.46	1	0.39	14	0.32
2	0.37	17	0.29	2	0.51	15	0.49
3	0.52	18	0.46	3	0.53	16	0.20
4	0.48	19	0.32	4	0.47	17	0.07
5	0.50	20	0.53	5	0.47	18	0.14
6	0.29	21	0.53	6	0.43	19	0.00
7	0.40	22	0.48	7	0.29	20	0.53
8	0.42	23	0.44	8	0.32	21	0.53
9	0.29	24	0.53	9	0.14	22	0.51
10	0.35	25	0.52	10	0.47	23	0.53
100	0.49	27	0.53	11	0.47	24	0.47
11	0.46	28	0.37	12	0.41	25	0.52
12	0.48	30	0.44	13	0.53	26	0.51
13	0.53	31	0.29				
14	0.00	32	0.52				
15	0.42	34	0.46				
15[a]	0.51						

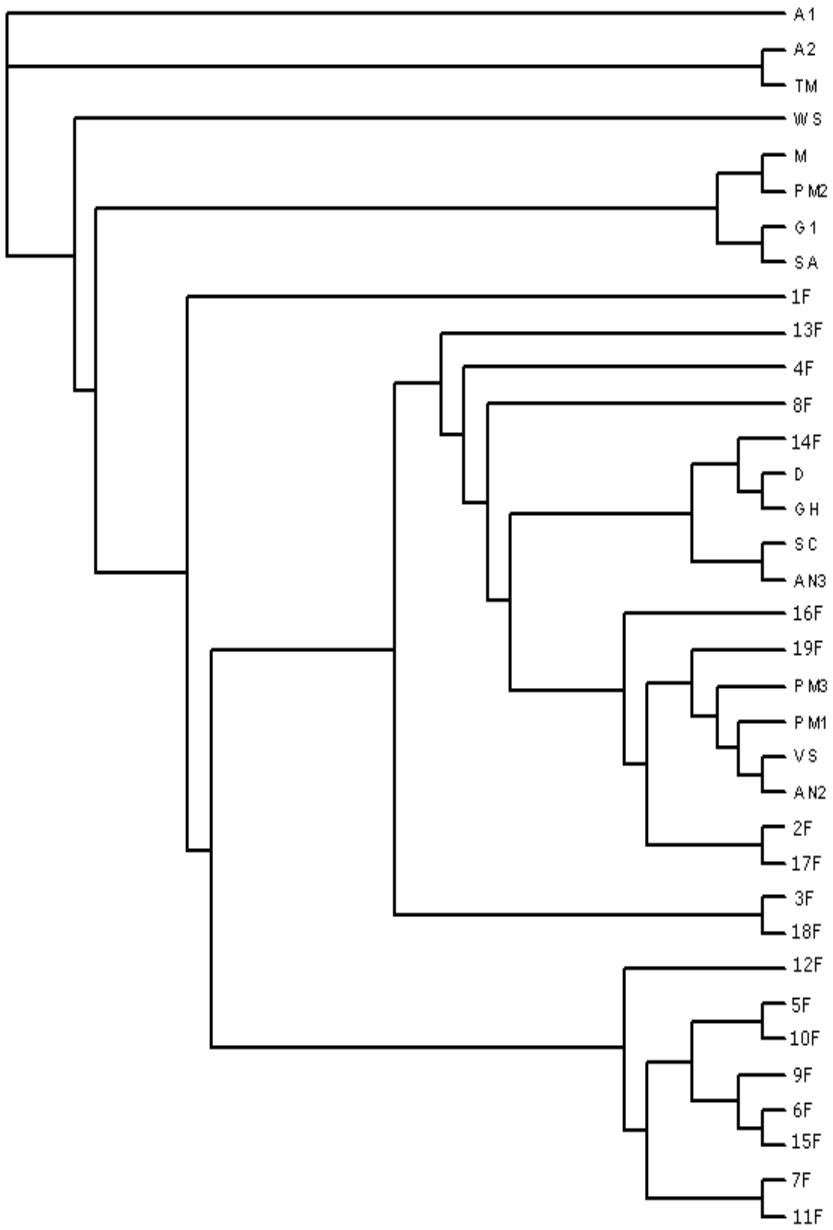

Figure 1. Dendrogram of the polyembryony (NAP and BAP) and non polyembryonic (commercial maize) groups. F=Family number

However, differences may not obey to polyembryony but to contrasting origins. Genetic distances between the two polyembryonic groups were from 0.01238 to 0.002332, showing small genetic distances between BAP and NAP populations. This may be due to two main causes; 1) both populations share a common gene pool, and had been selected in the same manner to increase the PE frequency up to 60 to 70% now days; and 2) some part of their genetic information contains the same sequence that could express polyembryony.

DISCUSSION

Polyembryony frequency in polyembryonic and non-polyembryonic maize populations.

For decades different authors have searched the causes of polyembryony; Reiser *et al.,* (1993) attributed the polyembryony to genetic causes as meiotic and / or mitotic irregularities, polyploidy and hybridization. On the other hand, it has been proposed that various factors might be associated with the expression of polyembryony, such as: type of pollinator, the amount of pollen available,

plant nutrition, environmental temperature, moisture of soil and environment, however there are no sufficient studies to verify this (Andrade-Rodriguez *et al*., 2005). The polyembryony percentages observed in the BAP and NAP populations, may be attributed to the proper reproductive management applied to the polyembryonic groups which has been done through fraternal crosses with mixed pollen (Espinoza *et al*.,1998).

Identification of loci associated to polyembryony

In this study, 29 candidates loci were associated to polyembryony in maize (11 in the absorbance at 700 nm, and 18 at 800 nm). Until now, there are no studies to determine the sequences or genes that are associated with polyembryony in maize. Some attempts in this direction have been done in other crops; for instance, two single genes has been associated to polyembryony in rice (Yang and Hwa, 2008; Puri *et al*., 2009). However at the present time, there is not evidence to demonstrate that maize polyembryony is due to a single gene; observation indicates that a set of genes interacting with each other may be the responsible of this trait. The difficulty to widely incorporate the polyembryony in maize, and the fact that a greater level of polyembryony is observed when the polyembryonic seeds are sown under greenhouse rather than in field conditions, suggest that polyembryony is conditioned by several genes, and environment has somehow an influence in the trait expression (Espinoza *et al*., 1998).

Genetic diversity among maize PE and PE genotypes

Analyzing the polyembryonic groups in the dendogram (Figure 1), it was observed that plants of the two populations were not pooled, and neither grouped plants of the same population, which reflects their genetic differences within and between the polyembryonic groups. This diversity reflects the success of the breeding program in avoiding inbreeding in the polyembryonic populations; it can be said that genetic diversity increases the chances of raising the percentage of the polyembryony within populations by breeding methods such as the recurrent selection applied to both populations. On the other hand, using the Shannon index it was possible to determine how much diversity exists between the PE and NPE groups at the locus level; in doing so, when the index is zero, it indicates that there is no diversity between genotypes according to the locus, *i.e.*, the obtained bands are monomorphic; and when the value is between zero and one, indicates that there is genetic diversity among the tested samples for that specific locus. Over the years, this index has been applied for diversity studies on ecology and agro forestry (Greig-Smith, 1983). Currently, it has been recognized for its applications in genetics, such as a comprehensive approach to molecular biology, evolution of complexity, linkage disequilibrium, mapping with genetic mixtures and genetic map information (Reyes, 2006).

CONCLUSIONS

Twenty nine putative loci were associated to the maize polyembryony; this association may be related to the differential origins of the maize populations. These results suggest that the polymorphic bands may be related to the expression of genes linked to polyembryony in maize.

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