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Genetic diversity among plants of non-transgenic and transgenic versions of a single cross maize hybrid

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ABSTRACT

Previous studies have documented that transgene introduction may alter the phenotypic expression of several traits (e.g., biomass production, grain yield). We hypothesized that genetic diversity could influence the phenotypic variation among hybrids of a same genetic background and also among plants of a hybrid. The objectives of this preliminary study were: (i) to quantify the genetic diversity between the non-transgenic (DK747) and transgenic versions (DK747MG, DK747RR and DK747MGRR) of a single-cross maize hybrid and among plants of each version, (ii) to observe the distribution of genetic diversity along the genome and (iii) to explore relationships between phenotypic variability and genetic diversity. Hybrids were cultivated at field conditions during two growing seasons and plants of each hybrid with high, intermediate and low biomass at physiological maturity were selected to perform a study of single nucleotide polymorphisms (SNP). Genetic diversity among plants of each version was greater than among versions and both sources of variation were significant ($\Phi_{ST} = 0.45$, $P < 0.01$). Genetic diversity of the non-transgenic DK747 was higher than those of the transgenic versions, probably reflecting the conventional breeding history of these iso-hybrids. Similarity coefficients indicate that the most homogeneous group was that composed by plants of DK747MGRR. A Fisher's exact test together with a principal component analysis identified certain SNPs related to the contrasting plant biomass of DK747, 747MG and DK747RR. Caution should be taken with these results, because of the small sample size for SNPs study and the narrow set of tested hybrids.

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1. Introduction

Previous studies in maize hybrids have documented that transgene introduction may alter the phenotypic expression of several traits (e.g., biomass production, grain yield) (Ma and Subedi, 2005; Subedi and Ma, 2007; Laserna et al., 2012; Shi et al., 2013). The incorporation of the event in transgenic hybrids is carried out by crossing a line source of the transgene followed by successive backcrosses to the recurrent parent. In these backcrosses, the linkage drag of the donor parent increases the likelihood of heterozygous loci in the converted line (Tanksley and Nelson, 1996). Therefore, the breeding process to obtain an inbred line (Liu et al., 2003) as well as the process of transgene conversion could contribute to increase the genetic diversity of a hybrid. In accordance with these hypotheses and after having observed significant phenotypic variability between non-transgenic and transgenic versions of a single cross maize hybrid and among plants of each version (Laserna et al., 2012), the question that arises is whether genetic diversity could contribute to the observed phenotypic variability. To answer that question we have sampled DNA of plants with contrasting total biomass at physiological maturity of the non-transgenic and the transgenic versions (single and stacked-events) of the single-cross maize hybrid DK747 cultivated under field conditions at high plant population density. The objectives of this study were: (i) to quantify the genetic diversity (by using SNPs) between versions and among plants of each version, (ii) to observe the distribution of genetic diversity along the genome, and (iii) to explore relationships between phenotypic variability and genetic diversity.

2. Materials and methods

Two field experiments were conducted in the experimental site of the Faculty of Agronomy of the University of Buenos Aires (34°35′S, 58°29′O). Experimental details are given in Laserna

Abbreviations: AMOVA, Analysis of molecular variance; SNP, single nucleotide polymorphism; D, dominant plants; d, dominated plants.

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et al. (2012). Tested genotypes were temperate single-cross maize hybrids produced by Monsanto Argentina, composed by the non-transgenic DK747 and its transgenic versions: Bt (DK747MG), RR (DK747RR) and Bt-RR (DK747MGRR). The trials were irrigated and fertilized (N) according to best practices, and weeds, pest and diseases were adequately controlled.

DNA samples were taken from leaves of plants at a plant population density = 12 pl m^{-2} (n = 30 per experiment and hybrid). Sampled leaves were lyophilized and milled. DNA was extracted from ~40 mg of the milled leaf tissue, following the protocol described by Shagai-Maroof et al. (1984). For the SNP analysis, twelve of the sixty sampled plants of each genotype were selected: four samples came from plants with high plant biomass at maturity (dominant plants, D), four of them from plants with biomass values close to the arithmetic mean (mean plants) and four of them from plants with low biomass (dominated plants, d). Biomass categories were based on the frequency distribution of the trait pooling together tagged plants of the two experiments (experiment x hybrid effect on plant biomass was not significant). The small number of plants used for SNP study was a compromise between studying a large number of plants with few molecular markers or few plants with a large number of markers. Because of the expected low genetic diversity among plants, we increased the number of molecular markers to detect variable SNPs. Thus, DNA of 48 plants was analysed by GeneSeek Inc. (Lincoln, NE) using the Maize SNP 50 BeadChip of Illumina®, containing 56110 bi-allelic SNPs selected from public and private databases, based on the B73 sequence and distributed along the whole maize genome. The 48 plants were analysed in two replicates. Even though the BeadChip is based on the B73 sequence, it has been successfully tested in several parent/F1 combinations (Ganal et al., 2011).

The GC value (GenCall confidence Score) of each SNP was used as an indicator of the accuracy of the data (Fan et al., 2003). From the 56110 SNPs, 7388 were excluded because of their low GC values or because they were not informative or replicable. The rest of the SNPs (48,722, herein termed variable SNPs) were quantified and compared among plants within each version and among versions. These SNPs were also located using maizegdb.org tools (Lawrence et al., 2004).

The significance of the genetic diversity was tested with the analysis of molecular variance (AMOVA) (Excoffier et al., 1992), performed from a distance matrix, which also allowed calculating the genetic diversity within each genotype. The Euclidean distances of Manhattan were used to build the matrix of genotypes x markers following the model described in Eq. (1) of the program GENES (Cruz, 2008).

$$\underline{x_{ij}} = x + \underline{a_i} + \underline{b_{ij}} \quad (1)$$

where, x_{ij} is the genetic distance between plant i and plant j, x is a general mean distance value (constant); a_i is the effect of subpopulation i, and b_{ij} is the effect of the distance between pairs of plants j in the subpopulation i.

Genetic diversity was also calculated as the contribution of each sub-population (i.e. hybrid version) to intra-population variance to determine whether the genetic diversity was due to inter-plant variation, inter-version variation, or both sources of variation. The Φ_{ST} index ($\Phi_{ST} = \sigma^2_a/\sigma^2$) was estimated using the mean squares ($\Phi_{ST} = MS_{among\ versions}/MS_{total}$), to describe the proportion of the total genetic diversity (σ^2) explained by differences among versions (σ^2_a). In addition, the similarity coefficient (SC) described by Rogers and Tanimoto (1960) and the UPGMA linkage method were used to describe genetic similarity among plants.

A two tailed Fisher's exact test, usually used when sample size is small (Larntz, 1978), was performed to study whether the genetic diversity was associated with biomass, comparing D and d plants of each version across the SNPs. For each variable SNP, this test compared the number of A and B alleles between groups with contrasting biomass, and gave the exact probability value for the relationship between two dichotomous variables. Finally, a principal component analysis (PCA) was carried out to identify if molecular markers, which had shown significant differences between D and d plants in Fisher's exact test, could classify plants in a bi-dimensional graph based on a singular value decomposition of the centered basic matrix by the column mean (Sneath and Sokal, 1973).

3. Results

Variable SNPs were mainly distributed on Chromosomes 2, 3, 4, 5 and 8, grouped within closed regions (Fig. 1). The DK747 had a higher number of variable SNPs (3432) than DK747RR (2460), DK747MG (2201) and DK747MGRR (1218). All hybrids shared 26% of the total number of variable SNPs, 56% were exclusively shared

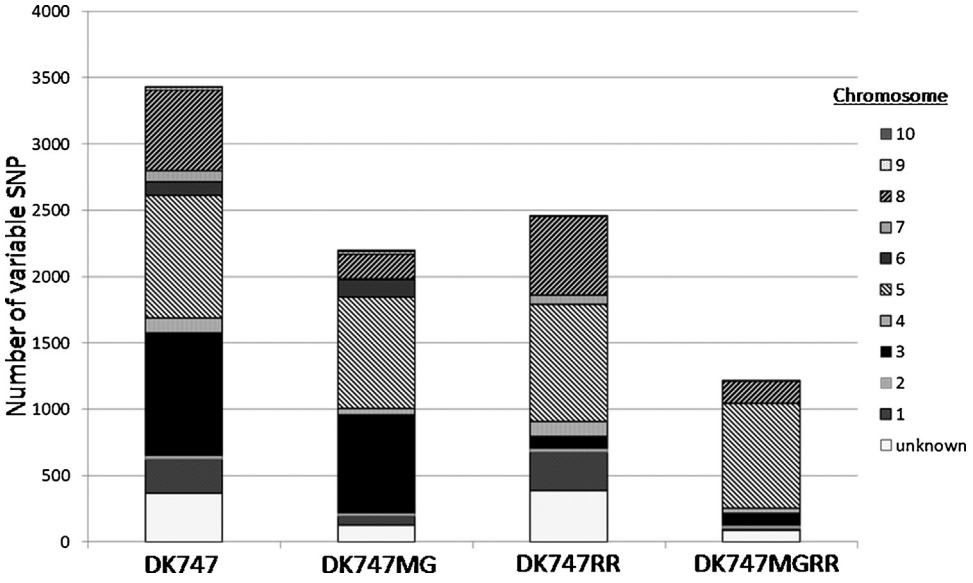

Fig. 1. Number of variable SNPs located at each chromosome, for each genotype. "Unknown" represents the number of variable SNPs with no information of map location.

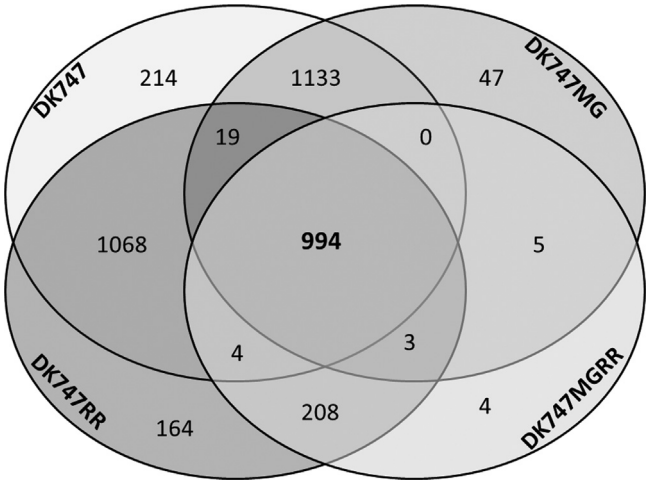

Fig. 2. Venn diagram showing the number of variable SNPs exclusive for each genotype and those shared by the different versions.

by DK747 and DK747MG, 54% by DK747 and DK747RR and 26% by DK747 and DK747MGRR (Fig. 2).

Genetic diversity within versions (54.9%) was higher than that among versions (45.1%), though both sources of variation were significant ($\Phi_{ST} = 0.45$, $P < 0.01$, Table 1). 97% of variable SNPs within a version were associated to homozygous (AA or BB) and heterozygous plants (AB). The genetic diversity of the DK747 (2.74) was higher than those of the transgenic versions (1.05 for DK747MGRR, 1.72 for DK747MG and 2.17 for DK747RR). The analysis of genetic diversity among plants by the Rogers and Tanimoto similarity coefficient also showed the lowest similarity among plants for DK747 and the highest for DK747MGRR (data not shown).

From the two tailed-Fisher's exact test, no SNPs were significantly ($P > 0.05$) associated with biomass production for DK747MGRR. For the other versions, the significant SNPs ($P < 0.05$) whose allele frequency was associated to the biomass of D and d plants, did not coincide among versions (Table 2) and were located in Chr. 3 (one SNP for the DK747 and three SNPs for DK747MG) and Chr. 5 (one SNP for DK747, four SNPs for DK747MG and five SNPs for DK747RR). These SNPs were usually surrounded by other SNPs significantly ($P < 0.08$) associated with biomass production (Table 2). The PCA analysis classified D and d plants mainly by the PC1 across the significant SNPs of each version (Fig. 3). These SNPs shared a similar genetic pattern: when D plants were homozygous, d plants were heterozygous, or vice versa and the pattern BB for D plants and AB for d plants was the most frequent (24.24% for the DK747, 31.83% for the DK747MG and 18.18% for the DK747RR). Finally, the PC2 divided D plants in two groups: one of these groups shared by AA and the other one by BB genotypes.

4. Discussion

This work analyzed the genetic diversity among non-transgenic (DK747) and transgenic versions (DK747MG, DK747RR and DK747MGRR) of a single-cross maize hybrid and among plants of each version. First, it was necessary to define a criterion for the selection of individuals to be included in the molecular marker study. Based on plant biomass variation at R_6, the most contrasting plant types (i.e. D and d plants) and the mean plants of each genotype were selected.

Variable SNPs were located along the whole genome of these iso-hybrids and 97.4% of them exhibited a common pattern in which part of the population was homozygous for one of the alleles, and the remaining population, heterozygous. This result suggests that parental lines could have presented residual heterozygosis for these loci due to multiple conventional breeding steps rather than to the transgene introduction (Harrigan et al., 2010; Venkatesh et al., 2014). Moreover, the Bt-RR version (DK747MGRR) exhibited the lowest genetic diversity value despite the fact that both, the MON810 event and the NK603 event, were independently inserted in the maize genome and stacked by backcrossing (CERA, 2012). Additionally as transgenic hybrids of this study have a single copy of the MON810 event (DK747MG and DK747MGRR) and a single copy of the NK603 event (DK747RR and DK747MGRR) (CERA, 2012), homologous recombination between copies as source of genetic diversity is also discarded (Weber et al., 2012).

The largest number of variable SNPs among plants of the non-transgenic and the smallest of DK747MGRR is probably related to the increasing number of molecular markers available to assist in the selection and/or backcross process at the releasing time of each hybrid. The DK747 was released in 2004, followed by DK747MG in 2005, DK747MGRR in 2007 and DK747RR in 2008. Despite the overall reduction of the genetic diversity of these versions over time, high proportions of variable SNPs (ca. 26%) were conserved especially on Chr.5 of all versions. These SNPs were located near the centromere (maizegdb.org, Lawrence et al., 2004), probably as a part of a heterochromatic region. That feature could explain their maintenance over the time of production of the different versions, since the pericentromeric regions are highly heterozygous, with high diversity and restricted recombination frequency (McMullen et al., 2009). Interestingly, these regions of maize genome possess a significant number of genes (Gore et al., 2009) that can affect the phenotypic expression of several traits.

As genetic diversity within versions was significant, we explored the association between genotypic and phenotypic variability. Fisher's exact test and the PCA determined that plants of the same hierarchy (i.e. d or D plants) of DK747, DK747MG and DK747RR could be associated with SNP allele variation, allowing us to define regions probably related to biomass production. Some QTLs previously detected for biomass traits (Ajmone-Marsan et al., 1994, 1995; Austin and Lee, 1996; Beavis et al., 1994; Chen et al., 2011) co-localized with regions detected in the present study with the Fisher's exact test (those regions on Chromosomes 1 and 5 on bin 1.05 and 5.03; respectively). Additionally, significant SNPs contained in Chr.3 (bin 3.04) of DK747 and DK747MG, are in regions where no QTL for biomass production have been reported (maizegdb.org). These regions would deserve to be explored because are sufficiently broad to encompass several genes.

Our data show that genetic variability exists among versions of the same hybrid and that its origin is more likely to be related to the process of conventional breeding than to the introduction of

Table 1
AMOVA of variable SNPs (i.e. genotype data).

Source of Variation	DF	SS	MS	Variance component	% variance	Fixation index
Among versions	3	5.68	1.89	0.1433*	45.08	$\Phi_{ST} = 0.451$*
Within versions	44	7.68	0.17	0.1745*	54.92	
Total	47	13.36	0.28	0.318	100	

DF = degrees of freedom; SS = sum of squares; MS = mean squares, Φ_{ST} = fixation index.
* Significance at $P < 0.01$.

Table 2
Upper Table. Summary results of Fisher's exact test for variable SNPs with significant differences ($P < 0.05$) between dominated (d) and dominant (D) plants of three maize hybrids (DK747, DK747MG and DK747RR). The chromosome position of variable SNPs and the allele frequency (*A*; *B*) for each plant hierarchy are indicated. Lower Table. Chromosome intervals and number of variable SNPs showing segregation differences ($P < 0.08$) between dominant and dominated plants.

			DK747				DK747MG				DK747RR			
	SNP	Plant hierarchy	d		D		d		D		d		D	
		Location	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*
SNP ($P < 0.05$)	PZE-103044028	Chr. 3_ 43.05 Mb (bin 3.04)	0.75	0.25	0	1	–	–	–	–	–	–	–	–
	PZE-103065531	Chr. 3_ 102.00 Mb (bin 3.04)	–	–	–	–	1	0	0	1	–	–	–	–
	PZE-103065533	Chr. 3_ 102.00 Mb (bin 3.04)	–	–	–	–	1	0	0	1	–	–	–	–
	SYN594	Chr. 3_ 102.54 Mb (bin 3.04)	–	–	–	–	1	0	0	1	–	–	–	–
	PZE-105039629	Chr. 5_ 24.48 Mb (bin 5.03)	–	–	–	–	–	–	–	–	0.25	0.75	1	0
	PZE-105039934	Chr. 5_ 25.12 Mb (bin 5.03)	–	–	–	–	–	–	–	–	0.25	0.75	1	0
	PZE-105069428	Chr. 5_ 72.41 Mb (bin 5.03)	0	1	0.75	0.25	–	–	–	–	–	–	–	–
	PZE-105074139	Chr. 5_ 80.18 Mb (bin 5.03)	–	–	–	–	0	1	0.75	0.25	–	–	–	–
	PZE-105098697	Chr. 5_ 144.97 Mb (bin 5.04)	–	–	–	–	–	–	–	–	0.75	0.25	0	1
SNP ($P < 0.08$)		Chr. 1_ 166.66- 170.06 Mb (bin 1.05)	–								19 SNPs			
		Chr. 3_ 43.00- 102.55 Mb (bin 3.04)	28 SNPs	–										
		Chr. 3_ 66.93- 115.13 Mb (bin 3.04)	–				229 SNPs							
		Chr. 5_ 72.41- 85.15 Mb (bin 5.03- 5.04)	–				15 SNPs				2 SNPs			
		Chr. 5_ 185.46- 198.29 Mb (bin 5.05)	–								13 SNPs			
		Chr. 7_ 24.79- 24.94 Mb (bin 7.02)	–				3 SNPs							

Fig. 3. Principal component analysis (PCA) performed with variable SNPs showing a significant ($P < 0.08$) association with plant biomass, according to the Fisher's exact test for each version. DK747MGRR did not show differences in SNPs segregation between dominant and dominated plants in Fisher's exact test and it is therefore omitted.

transgenes. This is an important finding, because if such genetic variation is properly channeled it could be used to sustain further genetic gains. However, it is critical to mention that our data should be consider as preliminary results because of the small sample size for SNPs study and the narrow set of tested hybrids.

5. Conclusions

This study provided preliminary evidence of genetic diversity within and among non-transgenic and transgenic versions of a single-cross maize hybrid. Non-transgenic DK747 presented the largest genetic variation among plants, and the stacked DK747MGRR, the smallest one. Additionally, variable SNPs were located along the whole genome, suggesting that transgene introduction was not the main cause of genetic diversity, which probably reflects the conventional breeding history of these hybrids. Based on Fisher's exact test and PCA, plants of the same hierarchy (i.e. d or D plants) of DK747, DK747MG and DK747RR were probably associated with SNP allele variation. Future studies should explore in more detail the chromosome regions associated with plant biomass and the potential candidate genes involved in the development of plant hierarchies.

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