Molecular changes during intra and inter recurrent selection of two populations of maize: one adapted and one non adapted to the selection environment

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In several reciprocal recurrent selection (RRS) programs heterosis and favorable characteristics are achieved by means of one adapted and one non adapted population. We evaluated with molecular markers an intrapopulation selection followed by RRS of one Spanish population adapted to Mediterranean Spain and one US Corn Belt population non adapted to Spanish conditions. Results from other authors suggest that during recurrent selection, non adapted populations have higher loss of variability, genetic differentiation and lower effective population size than expected according to the number of families selected each generation. This could be due to natural selection which is not under the breeder’s control and is expected to mainly act on non adapted populations. The number of markers with convergent allelic change was similar to the number of markers with divergent allelic change which explains the lack of genetic differentiation and the failure to increase heterosis during RRS because the effects of both types of changes compensate. It seems that the predominant mode of gene action depends on the particular germplasm involved in the RRS. By evaluating the allelic changes during selection, we identified four regions (2.04, 4.06, 6.03, 9.02) that significantly changed during selection in our selection experiment and that have been associated to selection in other selection experiments and to multiple traits in QTL experiments.

**Keywords:** RRS, variability, genetic diversity, *Zea mays*

**Abbreviations:**
- RRS: Reciprocal recurrent selection
- SSR: Simple Sequence Repeat
- LD: Linkage disequilibrium
Introduction

Maize breeders only use a small part of the available genetic variability, generating a reduction in the expected rate of genetic improvement (Mikel, 2011) and an increase of the susceptibility to stresses (Reif et al., 2010). Only a small proportion of the genetic variation of the original open-pollinated varieties is used (Ho et al., 2005; Reif et al., 2005), and the recycling of elite inbreds is the cause that many of them have become genetically related (Mikel and Dudley, 2006). Consequently, one important objective for breeders is the introduction of new germplasm into temperate breeding programs to increase the narrow genetic base of the existing inbred lines and varieties (Taller and Bernardo, 2004). European landraces have much less variability than the American populations but present the advantage of many years of adaptation to temperate climates (Revilla et al., 2006). However, the poor agronomic performance of landraces, particularly low yield and high lodging, in comparison to elite varieties hampers their use on breeding programs which are mainly focus on short-term improvement.

With the aim of increase the genetic base of the Spanish germplasm used in breeding programs, two composites of populations, EZS1 and EZS2, which follow the heterotic pattern “U.S. Corn Belt (dent) x dry or Mediterranean Spain (flint)” (Ordás, 1991) have been developed at Aula Dei Experimental Station (Spain) in 1983. The dry Spain varieties have a special interest for increasing genetic variability because they present favorable alleles for adaptation to Mediterranean climatic conditions (Garay et al., 1996b). The composites were subjected to three cycles of S1 progeny intrapopulation recurrent selection for grain yield (Alvarez et al., 1993). After that, the composites (named EZS33 and EZS34) were subjected to three cycles of full-sib reciprocal recurrent selection (RRS) for grain yield. RRS significantly increased the yield of the population crosses (3.0% per cycle) and the yield of the crosses of the populations with different testers (Peña-Asín et al., 2012). On the other hand, some inbreeding depression, especially at the last part of the RRS was observed.

Our objective was to evaluate with molecular markers the changes in genetic variability and structure of EZS1-EZS33, the adapted population, and EZS2-EZS34, the non adapted population, during the selection program that combined intra and inter population recurrent selection. In addition, we studied the evolution of allele frequencies to identify regions associated to selection.
Materials and methods

Plant Material

Starting in 1983 two composites were developed in Zaragoza (Spain). EZS1 was formed by intercrossing four flint populations from dry or Mediterranean Spain, and, EZS2 by intercrossing four American dent populations of mainly Reid germplasm (Garay et al., 1996a). In this study, samples were obtained from these two maize composite populations (EZS1 and EZS2), their improved populations after three cycles of intrapopulation selection (EZS33C0 and EZS34C0, respectively), and all cycles from the interpopulation selection process of EZS33 [EZS33C1, EZS33C2 and EZS33C3] and EZS34 [EZS34C1, EZS34C2 and EZS34C3]. Intra and interpopulation selection processes were conducted at Zaragoza, Spain. Details of the different process of the breeding program are described by Alvarez et al. (1993), Garay et al. (1996b) and Peña-Asin et al. (2012).

Simple Sequence Repeat Genotyping

Forty-eight individuals from each of the ten populations EZS1, EZS33C0, EZS33C1, EZS33C2, EZS33C3, and EZS2, EZS34C0, EZS34C1, EZS34C2 and EZS34C3 were randomly chosen for DNA extraction. The establishment of forty-eight individuals responds to previous works (Labate et al., 1997 and Hinze et al., 2005) where similar results were found under very different sampled size, 100 and 30 individuals, respectively. From each apex of an individual DNA was extracted according to Liu and Whittier (1994) with modifications, and diluted to a concentration of approximately 100 ng μl⁻¹. Simple sequence repeat (SSR) amplifications were performed as described by Butron et al. (2003), and SSR products were separated by capillary electrophoresis using 1x Tris base, boric acid (5.5 g l⁻¹) and ethylenediaminetetraacetic acid (EDTA) (2mM) on a polymerase TAQ (30,000 ud). A Beckman Coulter CEQ 8800 Genetic Analysis System (Beckman Coulter, Inc.) was used for all determinations.

First, seventy-seven SSR markers evenly distributed along the genome (approximately one marker per bin) were tested in the original populations to assess the variability at the beginning of the program, although finally, twenty-five markers were discarded because they were not polymorphic or because they had problems in one or more populations during the amplification process. Information about the final markers used (Online resource 1) can be
found at MaizeGDB (Andorf et al., 2010).

Statistical analysis

The following parameters were calculated: the proportion of polymorphic loci (P<sub>o</sub>), the mean number of alleles per locus (A), the mean number of alleles per polymorphic locus (A<sub>p</sub>), the observed heterozygosity (H<sub>o</sub>), the expected heterozygosity (H<sub>e</sub>) and the fixation index (F<sub>is</sub>).

Genetic Data Analysis software, developed by Lewis and Zaykin (2001) for genetic analysis of molecular data, was used to obtain the populations’ descriptive variables. Differences between cycles within populations EZS33 and EZS34 were studied by fitting a linear regression model for those variables using PROC REG of SAS program (SAS Institute, 2008).

The effective population size for the populations after the intrapopulation selection and after the reciprocal recurrent selection and its confidence intervals at 95% were calculated following the temporal method proposed by Waples (1989). According to Labate et al. (1999), the formula from Nei and Tajima (1981) was used to calculate the standardized variance in allele frequency change. Those loci with the most common allele frequency above 0.9 were discarded to avoid the possible bias that can be caused by alleles with initially high frequencies (Labate et al., 1999).

To study the change in relationships between populations during selection, genetic distance between populations was calculated using Nei’s distance (Nei, 1978). Genetic Data Analysis software (Lewis and Zaykin, 2001) was used to perform this analysis.

Genetic Data Analysis software was also used to calculate estimates of Fisher’s exact test to determine if the markers were in Hardy-Weinberg equilibrium. The test for the random union of the gametes was performed using 3,200 permutations and the null hypothesis was discarded when p ≤ 0.05. Independence between loci pairs was similarly tested. To prevent that within-locus disequilibrium affects the significance of this test, genotypes were preserved according to their impact in the exact tests (Butron et al., 2005).

Schaffer’s test (Schaffer et al., 1977) was carried out to detect if the changes in allele frequencies observed during reciprocal recurrent selection program could be explained only by genetic drift or not. To perform the test a SAS program was used (SAS Institute, 2008) and a significance level for the family of tests (0.05) was set.
Results and discussion

In coincidence with the progenitor populations of the BSSS x BSCB1 RRS program (Labate et al., 1999), the distribution of the alleles frequency in the original populations EZS1 and EZS2 had an exponential shape with large proportion of the alleles at low frequency (Figure 1). The distribution of the allele frequency after six cycles of intra and interpopulation selection was still exponential. The distribution of the allele frequency in the populations of the BSSS x BSCB1 RRS program, in particular BSCB1, after 12 cycles changed from an exponential distribution to a distribution which is uniform at intermediate allele frequencies, but with large proportion of alleles at the extreme values (0 and 1). This is a typical distribution caused by genetic drift (Falconer and Mackay, 1996) which the populations of our program did not present due, probably, to the lower number of cycles of selection. The alleles at 0.1 frequency were more common in EZS1, EZS33C0, EZS2 and EZS34C0 than in EZS33C3 and EZS34C3, however the opposite occurred for alleles close to 0.0 frequency. This suggests that several of the alleles that were lost between C0-C3 were rare types (Labate et al., 1999; Pinto et al., 2003; Solomon et al., 2010) which is otherwise expected from random drift (alleles at extreme frequencies tend to be fixed earlier than alleles at intermediate frequencies).

The purpose of selection of the two original composites was to maximize the variability and the heterosis of their cross. EZS1-EZS33 was chosen for its adaptation to Mediterranean areas, while EZS2-EZS34 was selected for its high yield and low lodging. In the molecular characterization of the original composites we detected 239 (67%) alleles in EZS1 and its derivatives, and 254 (71%) alleles in EZS2 and its derivatives (data not shown). The genetic diversity (proportion of polymorphic loci, alleles per locus, etc) of the original composites (Table 1) was higher than or similar to the genetic diversity found in temperate or tropical populations (Labate et al., 2003; Reif et al., 2006) and in the original populations of other RRS programs (Labate et al., 1997; Romay et al., 2012) relative to later cycles. This confirms that EZS1 and EZS2 have a high variability and, therefore, are very appropriate as initial populations for a recurrent selection program, particularly if the main objective is to increase the genetic base of the germplasm used in elite breeding. In addition, we found that 82 alleles were not shared between the two reciprocal populations: 67 alleles were unique in EZS1 (28%) and 15 alleles in EZS2 (6%) (data not shown). On the opposite, Romay et al. (2012) found a similar number of not shared alleles in the two reciprocal populations of their RRS. EZS1 is made of varieties that did not contribute to
maize elite breeding, while some of the varieties of EZS2 made important contributions to
maize elite breeding. The presence of a relatively high number of unique alleles in EZS1
confirms that this population has an original variability that was not exploited before. This
novel population has never been improved and it is expected to contain both desirable and
undesirable traits.

Regarding the diversity parameters, we found that, for EZS1 and EZS2, the proportion of
polymorphic loci kept stable during intrapopulation selection. For EZS2, the number of alleles
per locus and the observed heterozygosity decreased during the intrapopulation selection from
4.8 and 0.64 to 4.1 and 0.57, respectively (Table 1). In contrast, for EZS1 the number of alleles per
locus and the observed heterozygosity remained fairly constant during the intrapopulation
selection (4.5 and 0.58, respectively). The variability decreased during the interpopulation
selection at higher rate than during the intrapopulation selection. Thus, 8% of the alleles, 5 % of
the observed heterozygosity and 1% of the polymorphic loci were approximately lost per cycle
in each population. The loss of alleles of our RRS program was higher than the loss of alleles of
other RRS programs (about 2-7% per cycle; Labate et al., 1997; Pinto et al., 2003; Romay et al.,
2012). The loss of observed heterozygosity in our program was also slightly higher to that found
in other temperate RRS programs (3%; Labate et al., 1997; Romay et al., 2012), but lower than
the decline in heterozygosity found in some tropical RRS programs (15%; Pinto et al., 2003;
Solomon et al., 2010). The discrepancies between the studies could be due to the origin and
characteristics of the breeding populations: temperate or tropical, adapted or not to the selection
environment, etc.

The lost of variability continued the tendency observed in the intrapopulation selection of being
higher in EZS34 than in EZS33; for example, the observed heterozygosity decreased at 4.7 % per
cycle in EZS33 and at 6.3% per cycle in EZS34. Therefore, the reduction in variability was more
important in the composite with germplasm from an area different from the evaluation
environment (EZS2-EZS34) than in the adapted composite (EZS1-EZS33). A similar
phenomenon was observed in other RRS, for example, in the RRS of BSSS and BSCB1, carried
out in Iowa, the reduction of variability of BSSS which is more adapted to US Corn Belt was
lower than in BSCB1 (Labate et al., 1997). In the RRS of BR-106 and BR-105, carried out in South
America, the lower reduction of variability occurred in the composite best adapted to those
conditions (BR-106) (Pinto et al., 2003). In the intrapopulation selection of EPS13 and EPS14,
carried out in Northern Spain, the reduction in variability was less important for the population
with germplam from the same area from the selection environment (EPS13) (Romay et al., 2012).

The population size is the number of breeding individuals which is directly related to the rate of inbreeding and the rate of fixation of alleles in an idealized population (random mating, no selection, no mutation). However, when the population deviates from the idealized breeding structure is most convenient to express the situation in terms of the effective population size (Ne). This parameter is the number of individuals that would give rise to the rate of inbreeding if they bred in the manner of an idealized population (Falconer and Mackay, 1996). Following the formula proposed by Sprague and Eberhart (1977), we expect that the effective population size in a selection program based on Si families would be between the number of recombined Si families and twice that number. As the number of Si families used for recombination was 10 in both programs (intra and interpopulation selection), we expected a Ne between 10 and 20. The estimates of Ne based on the changes in allele frequency were in this interval for both programs, except for the interpopulation selection in EZS34 (6.54) (Table 2). The values of Ne were higher in the inrapopulation selection than in the interpopulation selection in spite of the same number of families selected in both selection programs. This could be due to the accumulate effect of selection during generations (Walsh, 2004) because the interpopulation selection was carried out after the inrapopulation selection.

EZS1-EZS33, the adapted population, had higher Ne than EZS2-EZS34 in both the intra and the inter population selection. Solomon et al. (2010) and Labate et al. (1999) also found that Ne was higher in the adapted population, particularly when neutral loci were used to estimate the parameter. Romay et al. (2012) found that Ne was higher in the adapted population during the inrapopulation selection, but not during the interpopulation selection that followed the inrapopulation selection. Our molecular results agree with other authors. Results suggest that non adapted populations could lose more variability and have a lower Ne than expected according to the number of families selected during RRS. In congruence with this, it was observed that when selection is performed in one adapted and one non-adapted population, full-sib RRS is more effective improving the yield of the adapted population (Peña-Asin et al., 2012). This could be due to the inbreeding depression that counterbalances the effect of selection in the non adapted population. Some evidence of higher inbreeding depression of the non adapted population EZS34 with respect to the adapted population EZS33 was obtained, for yield, by crossing the selected populations (C1, C2 and C3) to the original population (C0) (Peña-Asin et al., 2012).
The number of families selected each cycle is usually the same in both reciprocal populations in RRS. However, the natural selection has probably eliminated some families in the non adapted population, making the families selected related to each other by ancestry. This effect, in our experiment, was particularly important during the interpopulation selection in which $Ne$, for EZS34, was equal to 6.5 indicating that, although we selected 10 families of EZS34 each cycle, the rate of inbreeding and the lost of variability would have been the same if we had selected only 6 or 7 families in an idealized population.

The genetic distance between the original and the improved populations was increasing along the selection process in both populations and with both selection methods (Table 3). The increase was larger for the non adapted population, EZS34, (0.15 and 0.22 in the intra a inter selection program) than for the adapted population, EZS33 (0.11 and 0.13 in the intra a inter selection program) (Table 3). This is in agreement with the greater loss of variability and lower Ne observed in the non adapted population, probably due to natural selection, which led to a higher genetic differentiation in the populations. Hinze et al. (2005) also found a higher genetic differentiation in the non adapted populations, but Romay et al. (2012) did not.

The genetic distance between the original populations (0.37) was higher than the genetic distance between the original populations of other RRS program; for example, the genetic distance between BSSS and BSCB1 was 0.21. This confirms the appropriate choice of the original populations that belong to the American dent × European flint heterotic pattern, for RRS. However, the genetic distance between the reciprocal populations did not increase with selection (Table 3). This result is in concordance with the lack of increment of the heterosis between the reciprocal populations during RRS, according to the agronomic data (Peña-Asin et al., 2012). On the opposite, the genetic distance between the reciprocal populations clearly increased along the selection cycles in the RRS program of BSSS and BSCB1 (Hinze et al., 2005) and EPS13 and EPS14 (Romay et al., 2012).

According to the Shaffer’s test, the changes of all allele frequencies during the intrapopulation selection can be explained by random drift, except for 3 and 5 loci in EZS1 and EZS2, respectively (data not shown). In the case of RRS, from a total number of 52 loci (258 alleles) evaluated, 35 loci (76 alleles) rejected the null hypothesis that its allele frequency changes were solely due to genetic drift. From these, 10 alleles that were at low frequency were deleted (data
not shown). Seven of the low frequency alleles were only present in one population. It seems that the selection eliminated some deleterious alleles that were still segregating, but at low frequency due to its deleterious nature. On the other hand, 17 loci (26 alleles) had a total change of allele frequency higher than 0.30 during RRS (Online resource 2). From these, 10 alleles had a significant change only in EZS34, while 5 alleles had a significant change only in EZS33. Under the solely action of the artificial selection we expected a similar intensity of selection in both populations because the number of families selected was identical. Consequently, under the solely action of artificial selection we expected similar allelic changes in both populations which is in contradiction to our results. The higher number of loci detected in the non adapted population could be explained by the natural selection which can act simultaneously to artificial selection and cannot be controlled by the breeder in field conditions, particularly in the case of non adapted materials.

From the 17 significant markers, 8 markers (umc1887-6.03, umc1545-7.00, umc1131-9.02, etc) changed its frequency toward a convergence in the two reciprocal populations, while 7 markers changed its frequency toward a divergence (umc1135-1.06, phi083-2.04, umc1963-4.04, etc). The loci in which the frequency tended to converge in the reciprocal populations are probably associated to additive effects, while the markers in which the frequency tended to diverge could be associated to dominance or overdominance effects. The divergent changes increase the heterosis and the genetic differentiation between reciprocal populations, while the convergent changes have the opposite effects. Because we have, approximately, the same number of convergent and divergent changes we expected that the effects of both types of changes will compensate and the overall effect of selection on heterosis and genetic differentiation will be low. The agronomic data (Peña-Asin et al., 2012) and the genetic distances between the reciprocal populations estimated with molecular markers confirm this expectation. Stuber et al. (1980) observed similar changes in allelic frequencies for interpopulation and intrapopulation selection, which these authors suggests an additive model of gene action. Butruille et al. (2004) also found that selection response was due to additive effects in the RRS of Golden Glow. On the contrary, the increment of heterosis and genetic differentiation between reciprocal populations in some RRS suggests a dominance or overdominance gen action (Romay et al., 2011; Romay et al., 2012). It seems from our and previous results that the predominant mode of gene action depends on the particular germplasm involved in the RRS. In the RRS of BSSS and BSCB1, Keeratinijakal and Lamkey (1993) found that improvement in BSSS was due to both additive and dominance effects and the improvement in BSCB1 was only due to dominance.
During RRS, the allele 2 of umc1887 (6.03) changed from low (0.16-0.20) in the initial cycle to high frequency (0.78-0.97) in the final cycle of both reciprocal populations (Online resource 2). Furthermore, the increment of allele frequency occurred in all cycles of selection, that is, the frequency of later cycles of selection was always higher than the frequency of earlier cycles. The markers umc1131 (9.02) and umc1505 (9.08) had also significant allelic changes, although the less magnitude than umc1887 (6.03), in both reciprocal populations during RRS. The regions 6.03 and 9.01-9.03 significantly changed in selection programs carried out in Spain (Butron et al., 2005; Romay et al., 2012) and have been associated to multiple biochemical and/or developmental traits (Causse et al., 1995). Additionally, in our experiment, some markers had large allelic changes in one of the two populations, for example the markers in the region 4.04-4.06 (umc1963 and umc1329) and 2.04 (phi083). These regions were also affected by selection in other selection experiments (Labate et al., 1999; Butron et al., 2005; Caicedo, 2010) and have been associated to multiple traits (Beavis et al., 1994; Goldman et al., 1993; Veldboom et al., 1994; Veldboom and Lee, 1996; Ajmone-Marsan et al., 1995, 1995; Schön et al., 1994).

From the 52 total markers, 32 were not in Hardy-Weinberg equilibrium in some of the populations (Online resource 3). In EZS1, EZS33C0 and EZS33C3, 10, 19 and 13 markers, respectively, were not in equilibrium, meanwhile in EZS2, EZS34C0 and EZS34C3, 16, 21 and 16 markers, respectively, were not in equilibrium. In general, markers in disequilibrium showed an excess of heterozygotes. This could be due to natural selection favoring the heterozygous against the homozygous individuals. At this respect, the marker umc1692 (5.03) presented a Hardy-Weinberg disequilibrium toward an excess of heterozygotes in all populations. This region is adjacent to a large QTL for yield heterosis which is congruent in more than one population (Schön et al., 2010).

Linkage disequilibrium (LD) between pair of markers was significant for 9.1% and 6.8% of the pairs of all combinations possible markers for populations EZS1 and EZS2, respectively. The significant LD of the pairs was 22.1% and 19.8% for EZS33C0 and EZS34C0, respectively, and 12.1% and 27.0% for EZS33C3 and EZS34C3, respectively. All markers occurred at least once in a pair in disequilibrium, and no differences between close marker pairs or pairs of markers in different chromosomes were found (data not shown). According to Labate et al. (2000) LD can be generated by genetic drift, hitchhiking, epistasis or admixture of populations, although genetic drift and hitchhiking are unlikely to generate LD between loosely linked loci. Because
we did not detect more pairs with LD on the same chromosome than in different chromosomes. According to our molecular data, EZS1 and EZS2 maintain some of the LD that was generated by crossing different populations during the development of the composites. During the selection program the increase in LD was probably due to selection for favorable combination of alleles, since genetic drift and hitchhiking are unlikely to generate LD in our populations. The increment in LD is more apparent in the non adapted population, EZS34, which was the population that was influenced by natural selection according to several evidences (greater loss of variability, more genetic differentiation and more significant changes in allele frequencies). Therefore, the increment of LD could be, at least in EZS34, the result of natural selection for epistatic effect between different loci.

Conclusions

We found, in agreement with other experiments, higher loss of variability, lower Ne and higher genetic differentiation than expected in non adapted population during recurrent selection. For this reason, we propose to increase the number of families selected when the populations are not adapted to compensate the potential loss of variability due to natural selection. In addition, we identified four regions (2.04, 4.06, 6.03, 9.02) that were related to selection and that could deserve more in deep studies because they were also identified in independent (different germplam and environment) selection and QTL experiments.
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