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Molecular changes in the maize composite EPS12 during selection for resistance to pink stem borer

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Abstract The pink stem borer (*Sesamia nonagrioides* Lefèvbre) is the most important pest of maize (*Zea mays* L.) throughout the Mediterranean area. The maize composite EPS12 has been chosen as the base population for a breeding program based on its resistance to pink stem borer, with the main selection criterion being resistance to stem tunneling. Yield was taken as a secondary selection criterion to avoid any unwanted negatively correlated response on this character. The aims of investigation were: (1) to monitor the effects of selection for resistance to pink stem borer on allele frequency at 70 simple sequence repeat (SSR) markers and their impact on the genetic structure of EPS12 and (2) to identify loci at which allelic frequencies changed significantly due to directional selection. Genetic diversity was reduced during the selection process (as expected since random genetic drift as well as selection could reduce genetic variability), but not significantly so. Although the loss of genetic variation was generally consistent with that expected in a model in which random genetic drift acts alone on neutral alleles, the changes observed in the frequency of five alleles were significantly greater than expected. Further, the linear trend of the departure from the random genetic drift model was significant for some allelic versions of two SSR markers, *umc1329* and *phi076*; directional selection was therefore acting on these loci. The significant effect of directional selection on those markers suggests the presence of quantitative trait loci (QTLs) for tunnel length and/or for yield under artificial infestation with *Sesamia nonagrioides* on the long arm of chromosome 4.

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Introduction

The pink stem borer (*Sesamia nonagrioides* Lefèvbre) is the main pest of maize (*Zea mays* L.) throughout the Mediterranean area (Cordero et al. 1998). Several maize varieties have been assessed for resistance to this insect: among the best are the landraces from the Ebro valley (Spain) and the composite EPS7 (Malvar et al. 1993, 2004). EPS7 has been formed from four landraces originating from the Ebro valley and eastern Spain (Ordás 1991) and has undergone three cycles of S_1 family selection for increasing its yield (Vales et al. 2001). EPS7(S)C3 was renamed EPS12 and chosen as the base population for a new breeding program for resistance to pink stem borer. Since the larvae tunnel into the stem, resistance to this activity was the main selection criterion. Yield was taken as a secondary selection trait to avoid any negatively correlated responses, given that such effects have been reported by other breeders (Russell et al. 1979; Klenke et al. 1988; Butrón et al. 2002).

The assessment of selection cycles for determining the efficiency of a selection scheme requires field experiments in which phenotypes are evaluated. However, when allele frequencies rather than individuals are the focus, molecular markers are more reliable tools (Labate 2000). Knowing which molecular changes occur in populations during selection might provide information on the genetic components underlying agronomic responses to selection. Further, the use of molecular markers might help identify specific regions of the genome that have been favored by selection (Heredia-Díaz et al. 1996). While isozymes have been used in the past to assess allelic frequency changes during selection (Stuber and Moll 1972; Stuber et al. 1980; Khaler 1983; Pollak et al. 1984; Revilla et al. 1997), nowadays DNA markers offer a better coverage of the genome and can detect greater

polymorphism (Dubreuil and Charcosset 1998). In this context, restriction fragment length polymorphism (RFLP) markers have been used to study changes in allelic frequency in maize populations in response to different programs of recurrent selection (Heredia-Díaz et al. 1996; Labate et al. 1999; Landi et al. 2002) and for determining the intensity of selection effectively applied (Labate et al. 1999).

Genotype frequencies at molecular marker loci also provide useful information on the genetic structure of populations. In the absence of migration, mutation, and selection, randomly mated large populations should show genotype frequencies in a Hardy-Weinberg equilibrium. Deviations from these proportions indicate the influence of these three phenomena, either singly or in combination. In maize, migration is prevented by artificial hand pollination, and the mutation rate is generally very low. Selection and non-random mating are therefore the most likely candidates for explaining any deviations from the Hardy-Weinberg equilibrium (Khaler et al. 1986). The existence of significant deviations provides information on the efficiency of maize population management during seed conservation and multiplication. Another critical decision in the selection process concerns the number of generations of random mating needed for the selected progenies to reach linkage equilibrium. In routine selection programs, two cycles of random mating are normally used instead of the four to five recommended (Hanson 1959).

The aims of the study reported here were: (1) to monitor the effects of selection for resistance to pink stem borer on allele frequency at 70 simple sequence repeat (SSR) markers and their impact on the genetic structure of EPS12 and (2) to identify loci at which the allelic frequencies changed significantly due to directional selection.

Materials and methods

Plant material

The experimental plant material used in this study was the maize (*Zea mays* L.) synthetic EPS12 population and the populations developed from it following two and three cycles of selection [PS12(S)C2 and EPS12(S)C3].

The selection procedure involved intra-populational recurrent selection of S_1 families; the selection intensity was 10%. One hundred S_1 lines were evaluated for resistance to pink stem borer (*Sesamia nonagrioides* Lefèvbre) following artificial infestation of the insect's eggs. At harvest, tunnel length and yield were measured and the ten families with the shortest tunnels chosen. However, when the yield of one family was lower than the 100-family mean, it was removed and the next S_1 family with short tunnels but high yield chosen. In the following year, the ten selected S_1 lines were inter-mated to produce the EPS12(S)C1-syn1 population as follows: (1) in each family, five plants acted as males and other five plants as females—the pollen from males was bulked across families and used to pollinate all female plants; (2) a bulk of 1,000 (20 seeds per ear) was established and named EPS12(S)C1-syn1; (3) plants from EPS12(S)C1-syn1 were self-crossed the following year to continue selection, as described above, until three cycles were completed; (4) after each cycle of selection, EPS12(S)C1-syn1, EPS12(S)C2-syn1, and EPS12(S)C3-syn1 were recombined again. For each cycle, 300 seeds were sown in ten rows of 15 two-kernel hills per row. After thinning, 150 plants were left, and random plant-to-plant crosses (using each plant only once as male or female) resulted in at least 50 ears. A bulk of 1,000 seeds using equal numbers of seeds per ear was established. The resultant populations were named EPS12(S)C1, EPS12(S)C2, and EPS12(S)C3, respectively. After step (4) was completed, seeds from another maize population were accidentally mixed with EPS12(S)C1. As at this point EPS12(S)C1-syn1 had been discarded, EPS12(S)C1 was therefore not included in this study.

SSR genotyping

DNA was extracted from approximately 96 plants from each population (EPS12, EPS12(S)C2, and EPS12(S)C3) according to Liu and Whittier (1994) with modifications. SSR amplifications were performed as described by Butrón et al. (2003). After amplification, SSR products were separated by electrophoresis using 1× TBE on a 6% non-denaturing acrylamide gel (approximately 250 V for 3 h) (Shi et al. 2001). Seventy SSR loci distributed throughout the genome were examined. The aim of the study was not to characterize the populations but to assess the allelic changes caused by selection. Fragments for each SSR locus were ordered and numbered according to increasing size.

Statistical analysis

The GDA software (http://lewis.eeb.uconn.edu/lewishome/), which was developed by Lewis and Zaykin (1999) for population genetic analyses of molecular marker data, was used to obtain population-descriptive variables for EPS12, EPS12(S)C2, and EPS12(S)C3. These included the numbers of polymorphic loci, of alleles per locus, and of alleles per polymorphic locus, the allelic frequencies, the expected (Nei 1978) and observed heterozygosity, and the fixation index.

A test of the null hypothesis regarding the random union of gametes was performed for all polymorphic markers in EPS12, EPS12(S)C2, and EPS12(S)C3 to determine whether these markers were in Hardy-Weinberg equilibrium. The GDA software provided estimates of the Fisher's exact significance via shuffling tests (number of shufflings = 3,200). Exact tests calculate the probability that the observed sample (and others less probable than the observed one) could be drawn from

the population by chance if the null hypothesis held true (Lessios 1992). Exact tests are advised when the sample size is small and some alleles are rare (Louis and Dempster 1987). Fisher's exact test forces a success to be defined as the event in which a particular shuffling results in a data set that is as probable or less probable than the original data. Missing data were discarded. The null hypothesis was rejected when $P \leq 0.05$. For loci not in Hardy-Weinberg equilibrium, the proportion of excess homozygosity was estimated as the difference between the expected number of heterozygotes under Hardy-Weinberg equilibrium and the observed number of heterozygotes divided by the sample size and multiplied by 100.

For EPS12 and EPS12(S)C3, GDA was used to test for genotypic independence between pair of loci by performing exact Fisher tests. To prevent the within-locus disequilibrium from affecting the significance of disequilibrium in pairwise, we preserved genotypes when performing the shuffling tests.

SAS software (SAS 2000) was used to carry out Schaffer's (Schaffer et al. 1977) test. This test relies on the fact that if directional selection is acting on the locus studied, then there should be an approximately linear directional trend in the gene frequency data that can be tested for significant deviations from the null hypothesis, i.e., random genetic drift acting alone. The model uses the transformed allelic data ($2 \sin^{-1}$ allelic frequency$^{1/2}$) expressed in radians and the variance-covariance matrix defined in Schaffer et al. (1977). The model tests whether the changes in allelic frequency observed in the selection cycles could be the result of random drift acting alone. The sum of squares of the deviation calculated to test the null hypothesis (changes due to random genetic drift) had a central χ^2 distribution with one degree of freedom less than the number of cycles evaluated. In addition, the sum of squares due to all departures from the model based solely on random genetic drift is partitioned in two components, one of which has a central χ^2 distribution with one degree of freedom that accounts for any linear trend in the gene frequency. Therefore, the significance of this linear component determines whether the allelic frequency changes due to directional selection were important. The tabulated χ^2 value (2 *df*, $P \leq 0.01$) critical for declaring a significant deviation was 9.21, and the critical χ^2 value (1 *df*, $P \leq 0.01$) for declaring a significant linear trend was 6.63.

Empirical estimates for the effective population size were obtained as well as their 95% confidence intervals using Waples' (1989) temporal method, with EPS12 and EPS12(S)C3 as generations 1 and 3, respectively. The standardized variance in allele frequency change (F_c) was calculated following the method of Nei and Tajima (1981) for all loci—neutral loci and non-neutral loci (as identified by Schaffer's test). Our choice of the method of Nei and Tajima (1981) was based on comparisons among different estimators presented by Labate et al. (1999). To avoid the possible bias in the estimation of the effective population size caused by alleles at initially high frequencies, loci with the frequency of the most common allele in EPS12 larger than 0.90 were removed (Labate et al. 1999).

Results

The mean number of alleles per locus ranged from 2.53 in EPS12(S)C3 to 3.29 in EPS12 (Table 1). The mean number of alleles per polymorphic locus was slightly higher. The number of alleles per locus varied from two to eight (data not shown). The number of polymorphic loci, alleles per locus, alleles per polymorphic locus, and the mean expected heterozygosity (H_e) or genetic diversity diminished with the selection process (Table 1), although the reduction in the expected heterozygosity over selection was not significant.

The theoretical expectation of the heterozygosity in EPS12(S)C3, in a model in which random genetic drift acts alone on selectively neutral alleles for t generations, was computed as follows:

$$H_t = H_{e(EPS12)}(1 - 1/2N_e)^t$$

When an effective population size (N_e) equal to ten (i.e., the number of S_1 families selected at each generation) was used, the theoretical expectation of the heterozygosity in EPS12(S)C3 was 0.3977 [which is not significantly different to the hetetozygosity calculated for EPS12(S)C3; see Table 1)]

Only 14, 10, and 7 SSR marker loci (Table 2) of the 70, 68, and 64 polymorphic loci in EPS12, EPS12(S)C2,

Table 1 Genetic diversity of the maize composite EPS12 population and of two populations derived from it [EPS12(S)C2 and EPS12(S)C3] following two selection cycles

Selection cycles	Polymorphic loci	Alleles per loci	Alleles per polymorphic loci	H_e^a	H_o^b	f^c
EPS12	70	3.29	3.29	0.4639	0.4471	0.0364*
EPS12(S)C2	68	2.87	2.95	0.4078	0.3977	0.0248
EPS12(S)C3	64	2.53	2.76	0.3846	0.3832	0.0037

* Significantly different from zero at 0.05 probability level

[a] Mean expected heterozygosity. Standard deviations for the mean expected heterozygosities in EPS12, EPS12(S)C2, and EPS12(S)C3 were 0.2108, 0.2229, and 0.2268, respectively

[b] Mean observed heterozygosity

[c] Fixation index. Confidence intervals for the fixation index in EPS12, EPS12(S)C2, and EPS12(S)C3 were (0.0006, 0.0781), (−0.0113, 0.0678), and (−0.0359, 0.0453), respectively

Table 2 Percentage of excess of homozygosity for SSR markers not in Hardy-Weinberg proportions in the EPS12 maize composite and in two cycles of selection derived from it [EPS12(S)C2 and EPS12(S)C3]

Marker location	Marker name	Populations		
		EPS12	EPS12(S)C2	EPS12(S)C3
1.00	*phi109275*	–	–	3.81
1.10	*bnlg1347*	9.09	–	–
2.02	*umc1265*	9.88	14.79	–
2.03	*umc1185*	14.62	–	–
2.09	*bnlg1520*	−1.77	1.72	
3.04	*phi036*	–	8.61	–
4.00	*phi072*	6.81	–	–
4.03	*phi021*	8.59	3.55	–
4.04	*umc1963*	10.51	–	–
5.00	*umc1097*	–	–	2.31
5.07	*phi128*	–	8.58	–
6.01	*phi077*	–	–	−18.89
6.02	*umc1006*	11.53	–	–
6.05	*bnlg1154*	7.95	−1.46	–
6.07	*bnlg1740*	−5.61	–	–
8.00	*umc1327*	6.87	–	–
8.03	*umc1984*	–	8.96	–
8.04	*bnlg1863*	4.22	9.8	–
9.05	*umc1078*	–	–	−16.17
10.00	*phi118*	–	12.45	25.61
10.04	*umc1453*	42.83	36.47	24.13
10.05	*umc1930*	–	–	−3.88
10.07	*bnlg1360*	22.89	–	–
Mean		10.60	10.35	2.42

and EPS12(S)C3, respectively, were not in Hardy-Weinberg equilibrium ($P \leq 0.05$). The number of loci in disequilibrium diminished with the selection process but did not vary widely among maize chromosomes. An excess of homozygotes was observed in most cases of single-locus disequilibrium (25 of 31 cases of disequilibrium, although the excess was not the same in all cycles)(Table 2). In EPS12(S)C3, three out of seven cases of single-locus disequilibrium showed an excess of heterozygotes. In addition, the fixation index (f) was only significantly different from zero in EPS12 (Table 1), suggesting a significant excess of homozygotes in this population compared to the proportion expected under conditions of Hardy-Weinberg equilibrium.

Linkage disequilibrium for more than one-half the number of pairs of loci in linkage disequilibrium was significant at the 0.05 probability level, while a small proportion was significant at $P \leq 0.001$ (Table 3). The number of pairs of loci in linkage disequilibrium was greater in EPS12(S)C3 than in the original composite EPS12, although the number of loci involved in disequilibrium was similar. Disequilibrium was not more likely between loci on the same chromosome than between loci on different chromosomes (data not shown). In general, loci near fixation showed less disequilibrium with other loci than did loci with intermediate allelic frequencies (data not shown).

The Schaffer test detected that some allelic frequency changes were significantly greater than those expected from random genetic drift acting alone (Table 4). Such deviation from the latter model was significant for five alleles (Table 4). In addition, the sum of squares of the deviation was partitioned into two components, one of which had one degree of freedom and which accounted for all the effects of any potential linear trend in the allelic frequencies. This component provides information on changes in allelic frequency as a result of directional selection and was significant for four alleles (Table 4). The χ^2 of the directional selection effect (χ^2 lineal) was highly significant for the two alleles of the marker *umc1329* located on the long arm of the chromosome 4. Two allelic versions of an SSR marker (*phi076*) located on another region of the long arm of chromosome 4 showed significantly different frequencies than expected; the linear component of that change, unbiased by random genetic drift effects, was significant.

The number of selected families per cycle was ten and was within the 95% confidence intervals for the empirical estimates of the effective population size computed with all loci [$N_e = 11.99$, CI = (9.26, 15.17)]. When only neutral loci (loci whose allelic frequencies did not significantly change more than expected under the influence of drift alone), were considered, the effective population size [$N_e = 13.20$, CI = (10.12, 16.81)] was greater and differed significantly from ten. The empirical estimate for the effective population size considering only non-neutral loci [$N_e = 3.95$, CI = (0.80, 9.28)] was significantly lower than ten.

Discussion

The number of polymorphic loci, alleles per locus, alleles per polymorphic locus, and the mean expected heterozygosity diminished during the selection process since both random genetic drift and selection are able to reduce genetic variability. Nevertheless, the reduction of

Table 3 Number of pairs of loci in significant linkage disequilibrium in the original composite EPS12 and after three cycles of selection for resistance to pink stem borer [EPS12(S)C3]

Population	$P \leq 0.001$		$0.001 < P \leq 0.01$		$0.01 < P \leq 0.05$	
	Number of pairs	Number of loci involved	Number of pairs	Number of loci involved	Number of pairs	Number of loci involved
EPS12[a]	30	35	72	35	191	34
EPS12(S)C3[a]	53	31	96	32	230	34

[a] Total number of pairs = $n(n-1)/2$ for n polymorphic loci

Table 4 χ^2 values for deviations from random genetic drift (deviations χ^2) and for directional selection (linear χ^2) of significant allelic frequency changes at SSR markers in EPS12 during selection for resistance to pink stem borer

Location	SSR marker	Allele	Deviations χ^2	Linear χ^2
4.06	*umc1329*	1	9.380*	9.373*
4.06	*umc1329*	2	9.380*	9.373*
4.11	*phi076*	1	9.780*	6.760*
4.11	*phi076*	3	9.780*	6.760*
6.03	*umc1887*	1	9.380*	4.758

* Significant at 0.01 probability level

genetic variability was not significant ($P \leq 0.05$). This agrees with the results of Revilla et al. (1997), who studied the selection process for developing EPS12 (in that study named EPS7 C3) from the composite EPS7, and who found no significant reduction in heterozygosity during the selection process. In general, and in agreement with Labate et al. (1997), the loss of genetic variation was consistent with the theoretical expectation in a model in which random genetic drift acts alone on neutral alleles.

If we assume the absence of migration, mutation, and selection, and that two generations of random mating recombination were accomplished before sampling in EPS12, EPS12(S)C2, and EPS12(S)C3, the mean expected and observed heterozygosities should have been similar. Nevertheless, in EPS12, the fixation index was significantly different from zero, suggesting a significant excess of homozygotes compared to the number expected under conditions of Hardy-Weinberg equilibrium. In the original composite, EPS12, genotype frequencies for a larger number of loci significantly differed from those expected under Hardy-Weinberg conditions compared to EPS12(S)C2 and EPS12(S)C3. The expected mean heterozygosity decreased with selection. The loss of genetic diversity observed in EPS12(S)C2 and EPS12(S)C3 with respect to EPS12 could have prevented the observation of significant deviations from the expected proportions, even though one or more of the preconditions for Hardy-Weinberg equilibrium could not hold true (Lessios 1992).

In the present study, positive assortative mating due to differences in days to flowering was the most likely cause for the lack of Hardy-Weinberg equilibrium. The observed genotype frequencies for the marker *umc1453* were different to those expected under conditions of Hardy-Weinberg equilibrium, and this SRR marker mapped to a region of maize chromosome 10, where Rebaï et al. (1997) have detected an important quantitative trait locus (QTL) for flowering time. Khaler et al. (1986) attributed the significant fixation index for 12 U.S. Corn Belt open-populations and five adapted exotic populations of maize to non-random mating and/or natural selection favoring homozygotes. Pollak et al. (1984) and Khaler et al. (1984) showed that non-random mating resulted in excess homozygosity in the offspring, although the Hardy-Weinberg equilibrium was recovered at the adult stage, probably because natural selection favored the heterozygotes. Dubreuil and Charcosset (1998) suggested assortative mating as the most likely cause of heterozygote deficiency in 20 maize populations. Therefore, the artificial mating system should be improved to avoid assortative mating.

The number of pairs of loci in linkage disequilibrium (either intra-gametic or inter-gametic in origin) was greater in EPS12(S)C3 than in EPS12. This disagrees with results reported by Brown and Allard (1971) who found that linkage disequilibrium was reduced compared to the progenitor generation after two cycles of selection. These authors concluded that genetic drift was the most probable cause for the linkage disequilibrium observed. Labate et al. (2000) reported that natural selection for epistatic effects was the main contributor to the linkage disequilibrium in two synthetics, both before selection and after 12 cycles of reciprocal recurrent selection. In the present study, since the rate of linkage disequilibrium was similar between pairs of loci on the same chromosome and between pairs of loci on different chromosomes, genetic drift and the hitchhiking of linked neutral alleles with selected alleles did not seem to be the primary cause of disequilibrium. In addition, in the present study, the migration or admixture of populations was prevented by artificial pollination and seed handling, and there was no evidence of these phenomena during selection: only a few alleles present in EPS12(S)C2 (in four markers on chromosome 1, one on chromosome 1, and four on chromosome 7) were not found in the original composite and were not supplied by the same individuals; their frequencies in EPS12(S)C2 were generally 0.01, except for one that was 0.06. Therefore, these new alleles could be the result of sampling error giving rise to a small number of rare alleles in EPS12(S)C2. The most suitable explanation for the linkage disequilibrium observed in EPS12 and EPS12(S)C3 was, therefore, the selection for epistatic effects between unlinked loci. Agronomic traits could be involved in this since linkage disequilibrium slightly increased in EPS12(S)C3 compared to EPS12, the original composite. The rate of tight linkage disequilibrium was very low; two generations of recombination may therefore be enough for breeding purposes.

Although the loss of genetic variation was generally consistent with that expected for a model in which random genetic drift acts alone on neutral alleles, the changes observed in the frequency of five alleles were significantly greater from that which might be expected under such conditions. For one of these alleles, the deviation from the null hypothesis was due not to the linear component, but to the residual that accounts for the action of any erratic factor, such as non-directional selection combined with random genetic drift and/or sampling error. However, the linear trend of the departure from the random genetic drift model was significant for some allelic versions of two SSR markers (*umc1329* and *phi076*), meaning that directional selection was acting on those loci (Schaffer et al. 1977).

Stuber et al. (1980) found that for two synthetics the changes in allozyme frequencies at one locus significantly deviated from those expected. This deviation was accompanied by significant linear trends. Landi et al. (2002) also found significant linear trends ($P \leq 0.01$) for two markers during recurrent selection for plant regeneration from maize callus cultures. Heredia-Díaz et al. (1996) detected eight markers that significantly changed their allelic frequencies due to directional selection for rind penetrometer resistance. The higher power of that study for detecting directional selection effects on allelic frequencies could partly be due to the better coverage of the maize genome, but mostly to the use of six cycles of bi-directional selection for a single trait. The cycles evaluated in the present study were the result of selecting for two traits (reduced tunnel length and higher grain yield). Both are complex traits and could be negatively correlated to some degree since selection for increased resistance can result in reduced yield (Russell et al. 1979; Klenke et al. 1986, 1988). Therefore, in addition to the slower rate of progress expected because of using two selection traits instead of one, the possible existence of QTLs with opposite effects on both traits, or of different QTLs for each trait linked in repulsion, would hinder the search for significant allelic changes owed to directional selection. Evidence of this kind of unfavorable relationship between insect resistance and yield was found in a search for QTLs for insect resistance and agronomic traits. Two regions involved in the resistance to leaf feeding damage by *Diatraea grandiosella* had negative effects on traits related to yield (Groh et al. 1998). Since marker-assisted selection (MAS) has been successfully used to improve lines for resistance to *Diatraea grandiosella* (Willcox et al. 2002), the future evaluation of these materials for grain yield might give an idea of the strength of the negative relationship between insect resistance and yield.

High coefficients of correlation between allelic frequencies significantly modified by directional selection and the trait under selection in the composites EPS12, EPS12(S)C2 and EPS12(S)C3 (whose phenotypic differences were maximized by selection) would allow the identification of SSR markers that could be used in marker-assisted breeding programs (Heredia-Díaz et al. 1996). As the phenotypic evaluation of the selection cycles is still in course, the usefulness of those markers in MAS for resistance to pink stem borer cannot yet be tested. In any event, the significant effect of directional selection on some markers suggests the presence of QTLs controlling the selection trait or traits linked to these markers. Many studies have been undertaken to find QTLs for resistance to *Ostrinia nubilalis* (Schön et al. 1993; Bohn et al. 2000; Cardinal et al. 2001; Papst et al. 2001; Jampatong et al. 2002; Krakowsky et al. 2002), another corn borer species with similar behavior to the pink stem borer, and a QTL on the long arm of chromosome 4 has been found (Papts et al. 2001). QTLs involved in resistance to *Ostrinia nubilalis* might also be involved in resistance to pink stem borer since such resistance is not totally independent (Velasco et al. 1999). QTLs for grain yield were found on the long arm of chromosome 4 (Zehr et al. 1992; Veldboom and Lee 1996; Ajmone Marsan et al. 2001).

Estimation of the empirical effective population size provides information on the number of individuals needed to produce the observed sampling variance or the rate of inbreeding (Falconer 1960). Sprague and Eberhart (1977) established that, in S_1 family selection, the effective population size is equal to twice the number of S_1 families selected per cycle divided by the addition of one to the coefficient of inbreeding of the parental plants of the lines being recombined. Since directional selection as well as random genetic drift generated by sampling could increase the coefficient of inbreeding, the expected effective population size should be between the number of S_1 selected at each cycle and twice that number (Labate et al. 1999). As hypothesized, the effective population size was close to the number of S_1 families selected when all loci were considered, which is in agreement with Labate et al. (1999). Nevertheless, when only neutral or non-neutral loci were considered, the effective population sizes were slightly smaller than those obtained by Labate et al. (1999). We obtained this result because in the present study the probability level required to declare a loci non-neutral was more conservative. Therefore, empirical estimations for population effective size confirmed expectations based on the breeding method.

In general, deviations from Hardy-Weinberg proportions and linkage disequilibrium were not important. Therefore, the population management protocol currently under test seems to be acceptable, although some level of assortative mating should be prevented. The loss of genetic variation was consistent with that expected in a model in which random genetic drift acts alone on neutral alleles, although changes in the frequency of five alleles was significantly greater than might be thus expected. In addition, the linear trend of the departure from the random genetic drift model was significant for some allelic versions of two SSR markers, *umc1329* and *phi076*, meaning that directional selection was acting on those loci.

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