Population Genetic Diversity in a Maize Reciprocal Recurrent Selection Program

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ABSTRACT

The genetic structures of the Iowa Corn Borer Synthetic #1 (CB) and Iowa Stiff Stalk Synthetic (SS) maize (*Zea mays* L.) populations are important because these populations serve as the model for development of modern commercial hybrids. In 1949, CB and SS were used to start a reciprocal recurrent selection (RRS) breeding program at Iowa State University. This study was conducted to analyze more thoroughly the genetic diversity within this RRS program and illustrate how the RRS program has changed over time at the molecular level. The progress of this program was measured by analyzing the variation at 86 SSR loci among 28 progenitor lines and 30 plants sampled from each of seven cycles (Cycle 0, Cycle 1, Cycle 3, Cycle 6, Cycle 9, Cycle 12, and Cycle 15) in each population. The progenitors of these populations show a high amount of variation on the basis of expected heterozygosity (0.557). As the RRS program proceeded, this variation decreased (Cycle 15, 0.245). In total, a larger amount of genetic variation was found among plants within cycles (66%) than among cycles (13%) or between populations (21%). The repartitioning of variation from within populations (96% in progenitors) to between populations (58% in Cycle 15) over time is consistent with theoretical expectations of divergence between the populations. By sampling intermediate time points, we gained a comprehensive genetic view of CB and SS permitting evaluation of the molecular-level changes occurring as a result of reciprocal recurrent selection.

Iowa corn borer Synthetic #1 and Iowa Stiff Stalk Synthetic maize populations are important for research because they characterize the basis of the modern hybrid corn industry (Senior et al., 1998). Both populations are involved in a reciprocal recurrent selection program and are thought to have complementary alleles fixed as a result (Keeratinijakal and Lamkey, 1993). Many phenotypic evaluations of this program have been made since the first cross between the CB and SS populations was made in 1949 (Penny and Eberhart, 1971). The goal of RRS is to improve the mean performance of the interpopulation cross while maintaining the variability within populations (Comstock et al., 1949). Results from Keeratinijakal and Lamkey (1993), Schnicker and Lamkey (1993), and Holthaus and Lamkey (1995) indicate that direct response (i.e., the value of the interpopulation cross)

to selection for yield has increased through Cycle 11, while genetic variance within populations showed a nonsignificant decrease.

Labate et al. (1997) evaluated several measures of genetic diversity of the progenitors and 100 plants each from Cycle 0 and Cycle 12 in both populations using 82 restriction fragment length polymorphism (RFLP) markers. Gene diversity (expected heterozygosity) and average number of alleles per locus decreased in both populations. Nei's (1978) unbiased genetic distance between populations increased from the progenitors to Cycle 0 and from Cycle 0 to Cycle 12. The progenitors of both populations were closely related (Nei's genetic distance = 0.07). Over time, the distance between the populations continued to increase (Cycle 0, Nei's genetic distance = 0.21; Cycle 12, Nei's genetic distance = 0.66). The genetic distance between the progenitors and Cycle 0 of CB was approximately zero (Nei's genetic distance = 0.02), while the same comparison in SS was larger (Nei's genetic distance = 0.13). Most loci did meet the expectation of random mating or, more specifically, independence of occurrence of pairs of alleles under Hardy-Weinberg equilibrium. Excess homozygosity was commonly a factor in deviations from random mating. The tendency to intermate plants with similar flowering times increases the chances of homozygosity (Labate et al., 2000).

The objective of this research was to answer questions remaining as a result of the work that has been reported previously (Labate et al., 1997, 2000). Our approach was to obtain a more thorough molecular characterization of the RRS program between CB and SS by evaluating an increased number of cycles of selection in each population using simple sequence repeat (SSR) markers. We wanted to see if a smaller sample size is adequate to characterize these populations, to observe the variations in allele frequency that occur in intermediate time points, to see if additional information could be gained from SSR versus RFLP analysis, and to more rapidly perform the laboratory procedures. The main questions we were interested in answering were (i) how has allelic diversity, measured with microsatellites, changed over time in the populations and (ii) has reciprocal recurrent selection changed the population structure or the relationships between the populations and among the cycles?

MATERIALS AND METHODS

Maize Populations

The plants genotyped in this study were from two maize populations, Iowa Corn Borer Synthetic #1 and Iowa Stiff Stalk Synthetic. These populations were formed from crosses of 12

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Abbreviations: CB, Iowa Corn Borer Synthetic #1; RRS, reciprocal recurrent selection; SS, Iowa Stiff Stalk Synthetic; SSR, simple sequence repeat.

CROP SCIENCE, VOL. 45, NOVEMBER–DECEMBER 2005

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and 16 inbred lines, respectively, as described by Labate et al. (1999). The 28 inbred lines used to develop these populations will hereafter be referred to as progenitor (PR) lines. Two of the progenitor lines of SS (*CI617* and *F1B1*) were not available for sampling. However, both parents (*Fe* and *IndB2*) of *F1B1* have been included in this study. One progenitor line, *Illinois Hy*, was common to both populations.

Reciprocal recurrent selection is a method of interpopulation improvement proposed by Comstock et al. (1949) to take advantage of both additive and nonadditive genetic effects for traits under selection. A detailed outline of the selection procedure in CB and SS from formation of the populations through Cycle 5 is found in Penny and Eberhart (1971). Keeratinijakal and Lamkey (1993) provided a description of the program from Cycle 6 through Cycle 11. The selection methodology has not changed from Cycle 11 to Cycle 15.

Microsatellite Genotyping

Thirty plants were randomly selected from seven cycles of selection (Cycles 0, 1, 3, 6, 9, 12, and 15) in both CB and SS. A single plant of each of the 28 progenitor lines was sampled to give a total of 448 plants genotyped. After about 2 wk growth in a greenhouse, a 5-cm lengthwise section of a single leaf with the midrib removed was collected from all plants and stored at −80°C until extraction. Genomic DNA was extracted from leaf samples via a CTAB (cetyltrimethyl ammonium bromide) miniprep protocol (Mitchell et al., 1997), keeping the DNA from each plant separate.

One hundred five SSRs were chosen for analysis on the basis of presence of polymorphism among the progenitor lines and their coverage of the maize genome (Table S1, which is published as supporting information on the *Crop Science* website). After analysis, 19 SSRs were discarded because of low amplification or ambiguous results. SSR primers were fluorescently labeled and multiplexed before the polymerase chain reaction (PCR). Multiplex PCR was performed in 20-μL volumes containing 25-ng template DNA for samples of the progenitors and CB cycles and 7.5-ng template DNA for samples of SS cycles. The remainder of the volume contained $1\times$ PCR buffer, 1.5 mM MgCl$_2$, 0.2 mM dNTPs, 1 U *Taq* polymerase, and 4 pmoles of each forward and reverse primer in the multiplex set. The PCR protocol began with a denaturing step at 95°C for 4 min; followed by 30 cycles of 95°C for 1 min (denature), 55°C for 2 min (anneal), and 72°C for 2 min (extend), and ended with a final extension step at 72°C for 1 h.

Following this amplification, 0.1-μL GENESCAN 500XL size standard (PE Applied Biosystems, Foster City, CA) and 1.0-μL loading buffer were added to 0.5 μL of each PCR product. The samples were denatured at 95°C for 5 min and placed on ice. A multichannel syringe was used to load 0.5 μL of each sample into 96-well 5% (w/v) polyacrylamide gels. During electrophoresis by an automated DNA sequencer (PE Applied Biosystems, model 377), GENESCAN 3.1 software (PE Applied Biosystems) recorded the fragment sizes in base pairs for both the PCR products and the internal size standard as they migrated through the gel. If no amplification product was seen in the gel image, PCR was rerun for that particular plant DNA–SSR primer combination. GENOTYPER 2.1 software (PE Applied Biosystems) combined size and fluorescence information to identify the different markers and their specific allelic products. All data were verified for accuracy. Alleles at each SSR locus were then "binned" on the basis of natural breaks in the distribution of allele sizes (Matsuoka et al., 2002). When this criterion was not met, additional criteria such as observed heterozygotes were used to identify bins. Binning was performed

for all loci because alleles did not tend to follow a discrete, stepwise distribution pattern on the basis of repeat sizes.

Statistical Analysis

Diversity of the SSR markers and genetic variability within each cycle and population were calculated by PopGene (Yeh and Boyle, 1997) as the number of polymorphic loci, average number of alleles per locus, and expected mean heterozygosity. Differences among cycles within populations and between populations were tested with linear regressions of diversity estimates by SAS Proc GLM. We identified unique alleles (those that appear only once) in the progenitors and traced the fate of those alleles through Cycle 15. Likelihood-ratio *G* tests (Sokal and Rohlf, 1995) in PopGene were used to adjust for the effects of markers that are linked together on chromosomes (nonindependent markers) and detect significant deviations from Hardy-Weinberg Equilibrium (HWE).

Genetic distances were estimated from Nei's unbiased measure (Nei, 1978) in PopGene to account for small sample sizes. Relationships among maize populations were evaluated by principal component analysis (PCA) of the variance-covariance matrix of genotype data in NTSYSpc (Rohlf, 2000). Since allele frequencies within loci and populations sum to zero, the original genotypic data matrix contains many linear dependencies. These dependencies were removed without loss of information for the PCA by dropping one allele at each locus (Smouse et al., 1982).

Analysis of molecular variance (AMOVA; Excoffier et al., 1992) was performed by Arlequin (Schneider et al., 2000) to detect differences in the distribution of multilocus genotypes with respect to the following patterns of variation: among populations, among groups within populations, and among plants within groups. The sources of variation include two populations (i.e., CB and SS), eight groups (i.e., progenitors and Cycles 0, 1, 3, 6, 9, 12, and 15), and 448 plants that represent the total sample.

AMOVA was performed to evaluate variation in multilocus genotypes between and within the two populations for each of the eight groups. The test statistics of this analysis are composites of the expected mean squares associated with each source of variation (hierarchical level; Excoffier et al., 1992). They are indirectly related to each source.

Φ_{CT} = correlation among random SSR genotypes within *populations (C)* relative to the correlation of random pairs drawn from the *total sample (T)*;

Φ_{SC} = correlation among random SSR genotypes within *groups (S)* relative to the correlation of random pairs drawn from the respective *populations*;

Φ_{ST} = correlation among random SSR genotypes within *groups* relative to the correlation of random pairs drawn from the *total sample*.

These Φ statistics are similar to the *F* coefficients (e.g., F_{ST}) proposed by Wright (1951), commonly used to estimate the amount of differentiation in population subdivisions. However, the Φ statistics were calculated by a multivariate method from geometric distances rather than a univariate method of deviations from an average (Excoffier, 2001). The null distribution was determined from 1000 permutations of all possible distance measures across the different hierarchical levels. The significance of the Φ statistic was determined by measuring the difference between itself and the null distribution (Excoffier et al., 1992).

RESULTS AND DISCUSSION

Genetic Diversity

Three hundred seventy-two alleles were amplified by the 86 SSR markers to give a mean number of alleles per

locus equal to 4.33. The percent of polymorphic loci within each cycle and population decreased significantly over time through Cycle 15 ($P < 0.0001$; Fig. 1a). There was no difference between CB and SS populations in the rate at which polymorphic loci were lost ($P = 0.55$). By Cycle 15, the CB population had 73% and the SS population had 59% polymorphic loci remaining. This was expected, given the greater number of polymorphic

loci in the CB progenitors and similar rate of loss by both populations. Average number of alleles per locus and expected heterozygosity also decreased over time in both CB and SS populations ($P < 0.0001$; Fig. 1b, c). The progenitor lines had higher average number of alleles per locus (avg = 3.38) relative to Cycle 15 (avg = 1.88). Similarly, expected heterozygosity was high in the progenitors (avg = 0.56) and decreased by greater than
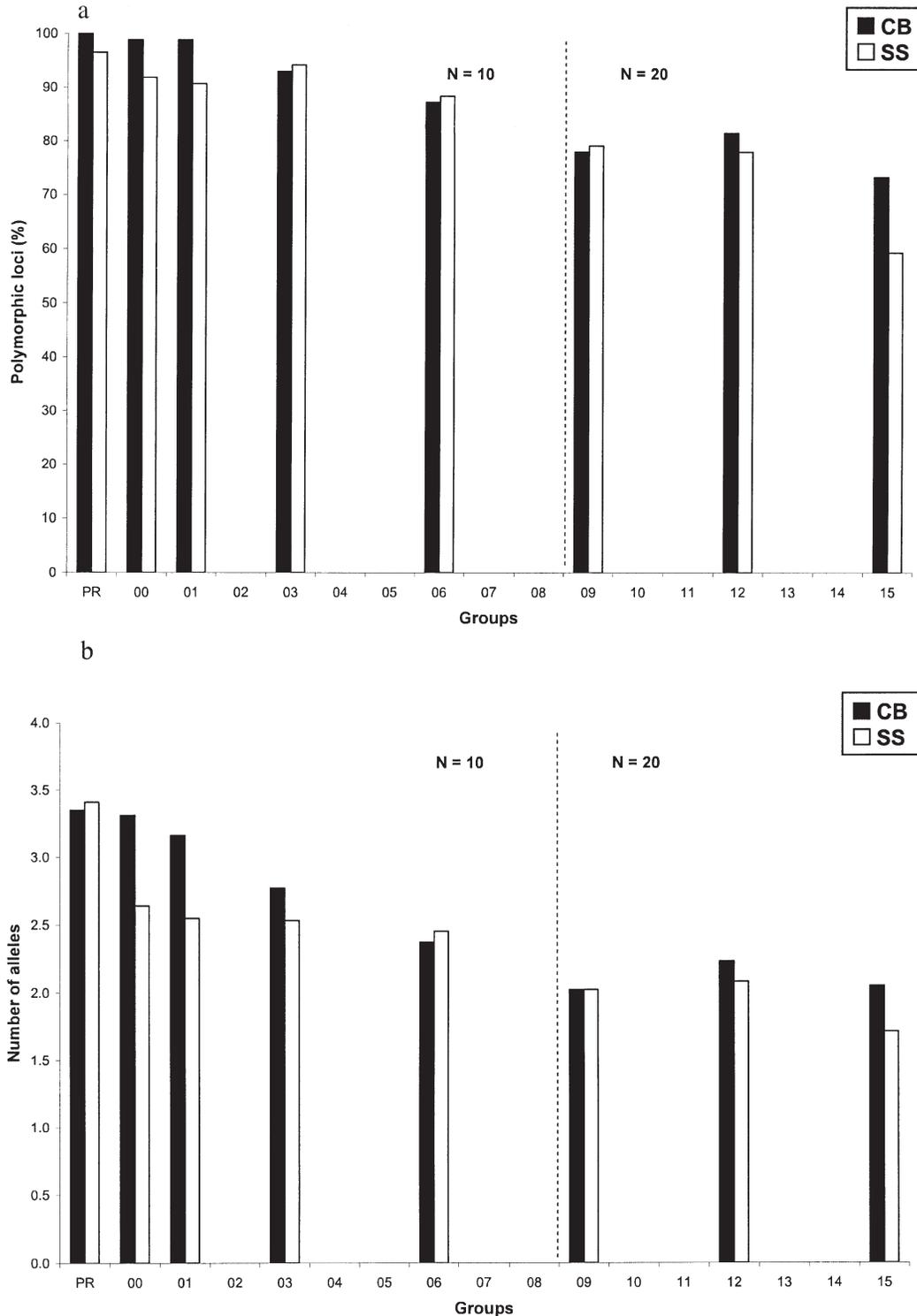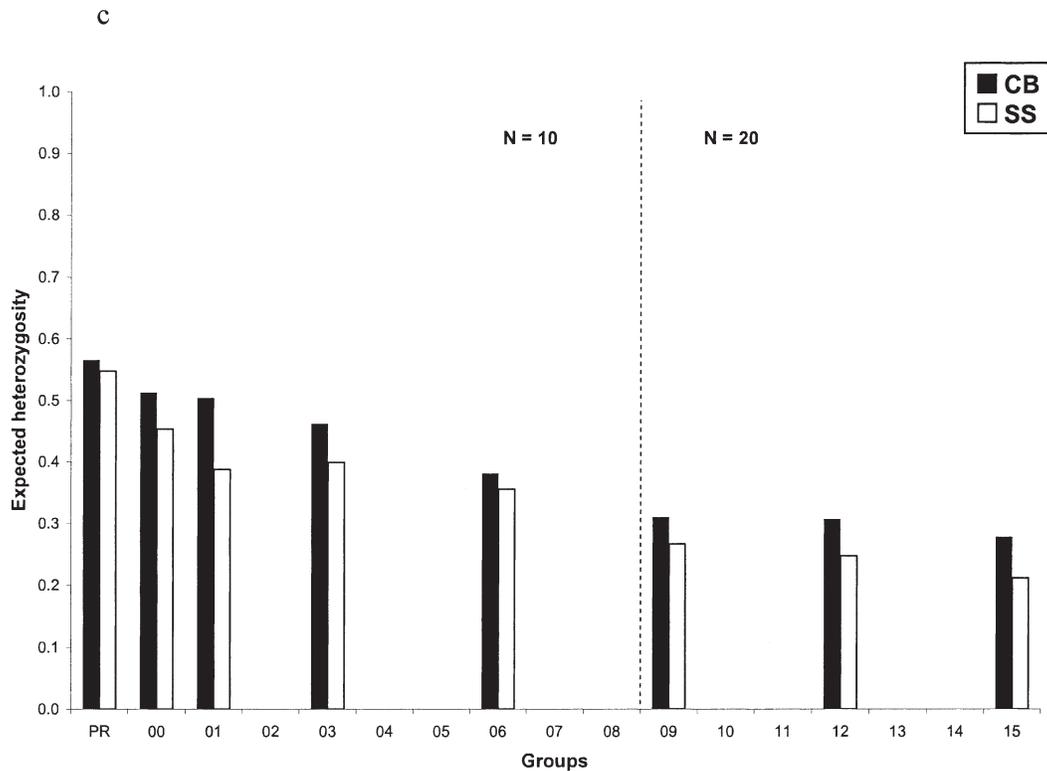

Fig. 1. Continued on next page.

Fig. 1. Genetic variation of 86 SSR loci in Iowa Corn Borer Synthetic #1 (CB) and Iowa Stiff Stalk Synthetic (SS) calculated for each group in each population. Dashed vertical line indicates change when 20 selections (N), instead of 10, were chosen from Cycle 8 to form Cycle 9. (a). Percentage polymorphic loci. (b). Average number of alleles per locus. (c). Expected heterozygosity.

one-half at Cycle 15 (avg = 0.25). There was no significant difference between CB and SS for these two diversity estimates.

In the CB and SS populations, 333 and 330 different alleles, respectively, were detected. Eighty alleles (24%) were unique in CB progenitors, and in the SS progenitors, 78 alleles (24%) were unique. Thirteen (16%) and seven (9%) of the alleles unique in the CB and SS progenitors, respectively, were present in Cycle 0. One (0.01%) of the unique alleles in the CB progenitors was still detected in CB Cycle 15 while none of the unique alleles from the SS progenitors were detected in SS Cycle 15. In the previous study, 25% of alleles detected with RFLPs were unique in the progenitors, and 10% remained in Cycle 12 (Labate et al., 1997). In comparison, essentially all unique alleles in the current study were no longer detected in Cycle 15. This difference in fate of the unique alleles could partly be a result of sample size [200 gametes sampled per cycle in Labate et al. (1997) vs. 60 gametes here]. With the larger sample size, there is a greater opportunity to detect unique or rare alleles.

The percent of loci deviating from HWE tended to decrease from Cycle 0 to Cycle 15 within both populations (Fig. 2). Fewer loci deviate from HWE over time because more loci become fixed as the RRS program progresses. This decrease is significant across the SS cycles ($P = 0.03$) but nonsignificant across the CB cycles ($P = 0.12$). The deviations from HWE were in the direction of excess homozygosity. A potential source of homozygosity is assortative mating. Assortative mating is

a type of non-random mating that occurred when crosses were made on the basis of pollination and silking synchrony (i.e., among early lines and among late lines; Labate et al., 2000). Homozygosity could also increase because of null alleles. Null alleles are those which are present but do not amplify (Hedrick, 2000); therefore, a heterozygote may appear as a homozygote or an entire genotype may be missing after PCR analysis. This would result in a higher apparent frequency of homozygotes and possibly entire genotypic classes missing from the data. Any of these factors (i.e., assortative mating or genotyping errors) may have caused the observed deviations from HWE.

Beginning with the formation of Cycle 9, 20 selections, instead of 10, were used to form new cycles in the RRS program (Keeratinijakal and Lamkey, 1993). Researchers implemented this change, which lowered the selection intensity from 10 to 20%, hoping to reduce random genetic drift (Schnicker and Lamkey, 1993). This modification to the breeding procedure was observed in terms of its effect on molecular diversity. The rate of loss of diversity reported above appears highest between Cycles 6 and 9 and shows signs of slowing thereafter when the number of selections was increased. Because of these apparent differences, we tested the significance of diversity separately for Cycles 0 to 6, 6 to 9, and 9 to 15. When the regression analysis was conducted in this manner, only the change between Cycle 6 and Cycle 9 was significant, indicating this period of selection to be pivotal in affecting diversity estimates over time. There-
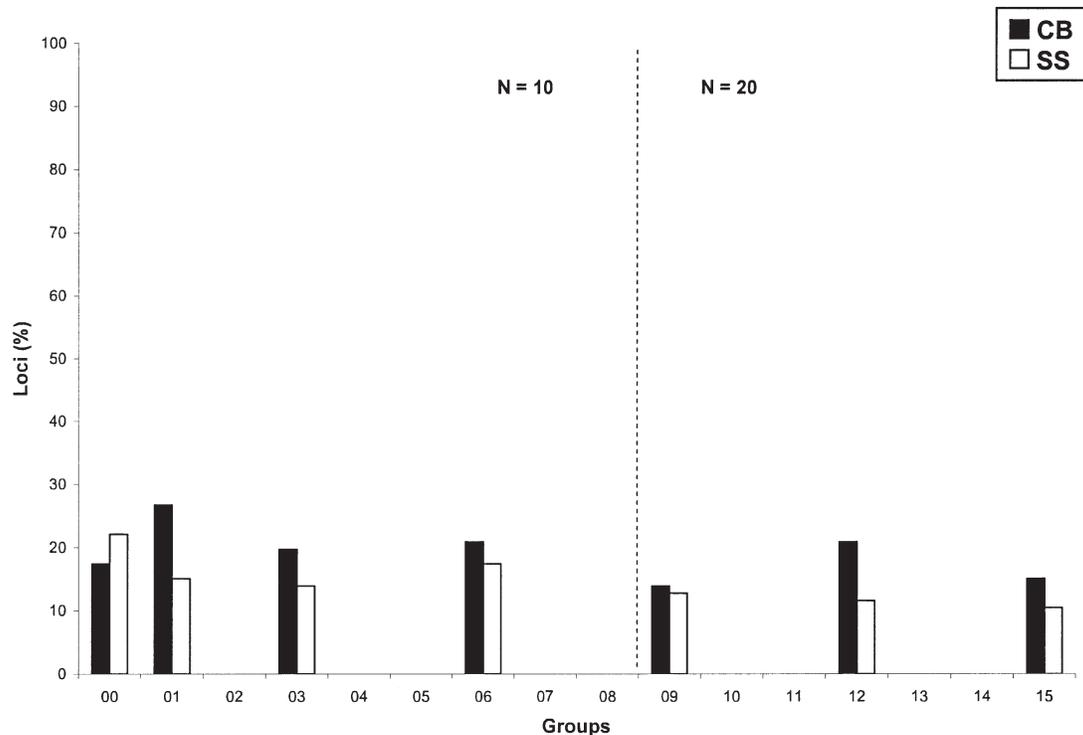
Fig. 2. Percentage of 86 SSR loci deviating from Hardy-Weinberg Equilibrium in Iowa Corn Borer Synthetic #1 (CB) and Iowa Stiff Stalk Synthetic (SS) calculated for each group in each population. Dashed vertical line indicates change when 20 selections (N), instead of 10, were chosen from Cycle 8 to form Cycle 9.

fore, doubling the number of selections did appear to slow the loss of diversity in these populations.

We observed an increase in genetic distance (GD) between populations with each subsequent cycle and an increase in GD from the progenitors at each cycle in both populations (Table 1). By Cycle 15, CB and SS diverged further from each other (GD = 0.6286) than either of the Cycle 15 populations had diverged from their respective progenitors (CB, GD = 0.2446; SS, GD = 0.3389). The genetic distance between progenitors and Cycle 0 in the SS population (Table 1; GD = 0.1235) was larger than the same interval in CB (GD = 0.0406). Labate et al. (1997) observed a similar spread between SS progenitors and Cycle 0 (GD = 0.13). There was no intentional selection between the PR and Cycle 0; therefore, drift is the most likely explanation. However,

natural selection during maintenance or contamination could have also contributed to the observed differences in genetic distance.

Labate et al. (1997) theorized that the large GD between the SS progenitors and its Cycle 0 was not inherent in the original population, but formed as a result of the continual random mating necessary to maintain the cycles. The plants for this study were sampled from this regenerated material, not the original material. Pedigree records do not indicate how many generations of recombination have occurred in the CB and SS cycles before Cycle 2. All Cycle 3 plants were sampled from the fourth generation synthetic (i.e., four generations of recombination). Iowa Stiff Stalk Synthetic Cycle 6 and Cycle 12 were sampled from the third generation, and all other cycles had been sampled from the second generation

Table 1. Genetic distance measured with 86 microsatellite loci for eight groups, progenitors (PR) and selected cycles between Cycle 0 (00) and Cycle 15 (15), measured in Iowa Corn Borer Synthetic #1 (CB) and Iowa Stiff Stalk Synthetic (SS) populations.

| Group	CBPR	CB00	CB01	CB03	CB06	CB09	CB12	CB15	SSPR	SS00	SS01	SS03	SS06	SS09	SS12
CB00	0.0406														
CB01	0.0742	0.0692													
CB03	0.1059	0.1020	0.0759												
CB06	0.1699	0.1850	0.1328	0.0963											
CB09	0.2260	0.2575	0.1901	0.1574	0.0680										
CB12	0.2265	0.2746	0.2031	0.1922	0.0939	0.0421									
CB15	0.2446	0.2938	0.2265	0.1947	0.1027	0.0539	0.0236								
SSPR	0.0845	0.1128	0.1404	0.1815	0.2574	0.2952	0.3164	0.3249							
SS00	0.1547	0.1610	0.1629	0.2086	0.2852	0.3075	0.3356	0.3629	0.1235						
SS01	0.2136	0.1974	0.2024	0.2490	0.3346	0.3608	0.4018	0.4389	0.1824	0.0629					
SS03	0.2132	0.2120	0.2113	0.2464	0.3371	0.3798	0.4032	0.4431	0.2051	0.0804	0.0492				
SS06	0.2360	0.2308	0.2347	0.2796	0.3476	0.3890	0.4094	0.4573	0.2250	0.1120	0.0887	0.0563			
SS09	0.3275	0.3146	0.3066	0.3560	0.4542	0.4907	0.4996	0.5607	0.2894	0.1646	0.1353	0.1036	0.0610		
SS12	0.3558	0.3376	0.3357	0.3820	0.4876	0.5297	0.5376	0.5980	0.3181	0.1750	0.1405	0.1092	0.0793	0.0169	
SS15	0.3831	0.3631	0.3596	0.4195	0.5176	0.5561	0.5708	0.6286	0.3389	0.1816	0.1495	0.1268	0.1011	0.0464	0.0285
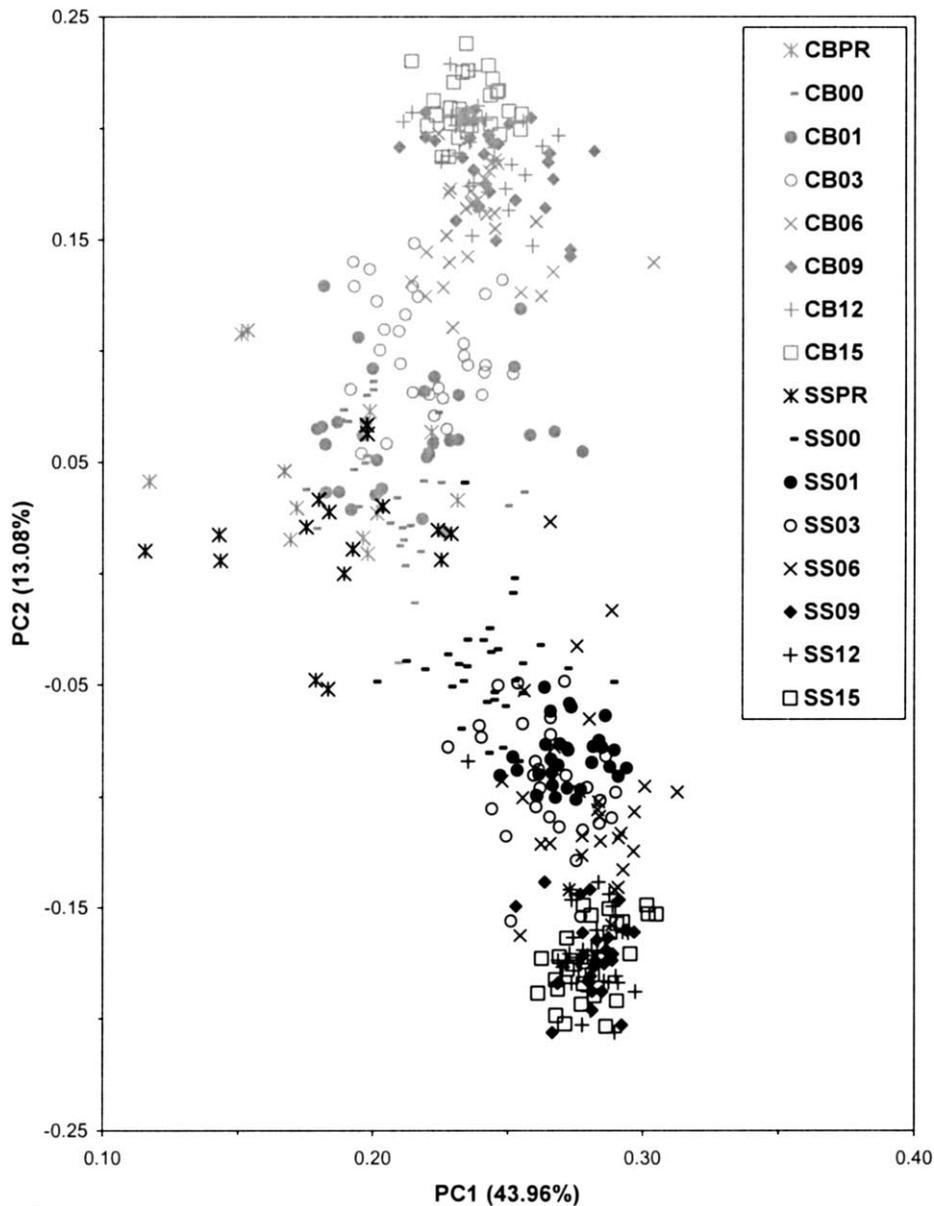
Fig. 3. Principal component analysis for the progenitor lines and cycles of selection in Iowa Corn Borer Synthetic #1 (CB) and Iowa Stiff Stalk Synthetic (SS) based on 86 SSR loci. The first two principal components (PC1 and PC2) are plotted against each other with the amount of variation explained by each in parenthesis.

synthetic. More cycles of random mating to maintain SS Cycle 0 would have allowed opportunity for genetic drift from the progenitors. This is feasible since SS was developed from 1934–35 (Sprague, 1946) while CB was not developed until the 1940s (Hallauer et al., 1974). While the SS cycles show this large GD, the CB cycles show a steady progression away from their progenitors. From Cycle 6 onward, both populations align in distance from their progenitors.

Genetic Structure

In the PCA, the first two principal components (PCs) explained 44 and 13% of the total variation, respectively (Fig. 3). The first PC separated plants on the basis of groups while the second PC separated the CB from the

SS population. Plants from the cycles of selection generally separated into their respective CB or SS population. Plants within individual CB cycles tended to be more similar, therefore grouping together, while SS plants were inclined to overlap more with plants of neighboring and non-neighboring groups. There was no difference in the mating designs for the CB and SS populations to explain this pattern. Therefore, the difference could be due to a greater resolution of the genetic diversity in CB given the larger number of polymorphic loci in CB present for analysis. Alternatively, this pattern could be due to the breeding and selection program applied to these two populations. The progenitors were highly polymorphic at the SSR loci studied yet were very close to one another in terms of genetic distance (GD = 0.0845). As the RRS program began testing the

Table 2. Analysis of molecular variance for plants sampled from eight groups (progenitors and seven cycles of selection) within two populations of maize (Iowa Corn Borer Synthetic #1 and Iowa Stiff Stalk Synthetic) over 86 SSR loci. Test statistics are defined in the text.

Level of variation	df	Variance component absolute	%	Test statistic
Between populations	1	4.456	20.68	$\Phi_{CT} = 0.2068$***
Among groups within populations	14	2.816	13.07	$\Phi_{SC} = 0.1648$***
Among plants within groups	880	14.272	66.25	$\Phi_{ST} = 0.3375$***
Total	895	21.545		

*** Significant at the 0.001 probability level.

CB and SS populations against one another, the effects of selecting the highest yielding intercross progeny from the two populations are seen. The RRS program resulted in selection of different alleles in the CB and SS populations. While the progenitors may have had alleles in common, selection during the RRS program decreased the genetic similarity between the two populations while increasing similarity within them. The frequency of the SSR alleles should only be changing by drift unless the allele is located in a selected gene or is hitchhiking with a selected gene. In Labate et al. (2000), 25% of SSRs showed linkage disequilibrium, thus indicating the SSRs are not behaving in a selectively neutral manner. In a subsequent manuscript, we are evaluating the neutrality of SSR loci and testing whether divergence of the populations is due to selection, genetic drift, or a combination of both.

Analysis of molecular variance revealed significant structure across all hierarchical levels. When structure among plants was tested, the greatest correlation was observed among pairs of plants within groups when compared with pairs of plants from across the total sample ($\Phi_{ST} = 0.3375$, $P < 0.001$; Table 2). Under the analysis, 66% of the variance was explained by within group variation, while 21% of the total variance was partitioned between the CB and SS populations. The amount of variation found among groups within populations was the smallest (13%) of the three sources of variation. We observed more variation for individual groups than for populations when determining structure in this collection of maize germplasm. In addition to testing the structure of the total sample, we partitioned the sample into the eight groups and used AMOVA to analyze each separately. With this approach, there are two levels, between and within populations, used to describe the variance distribution. Estimates of between population variance increased from 4% in the progenitors to 58% in Cycle 15 (Table 3) with a complementary decrease in variation within populations over time. These results supplement our previous estimates of genetic diversity showing how allelic variation has changed in these populations.

With AMOVA, variation is calculated on the basis of hierarchical levels that are set a priori. Under ordination analysis, partitioning of variation and clustering into similar groups is made a posteriori on the basis of an observed data structure. These separate evaluations independently showed stronger evidence for group- compared with population-based data structuring.

Since different types of markers were used, our results and the results of Labate et al. (1997) do not allow for a direct comparison because the marker systems themselves could cause some of the observed differences. However, this research complements the work of Labate et al. (1997) and presents more evidence regarding the number of plants necessary for diversity studies. We obtained comparable results at the time points in common to the two studies (progenitors, Cycles 0 and 12), suggesting that our sample size of 30, rather than Labate et al.'s (1997) 100, would be adequate for characterizing cycles of selection within these populations. Labate et al. (1997) did show higher estimates for average number of alleles per locus, expected heterozygosity (gene diversity), heterozygous plants, and unique alleles when compared with our data. In addition to possible differences because of marker type, these higher values from the RFLP data could also reflect the power of a larger sample size in identifying less common alleles. Using our current strategy, we were able to sample more time points in the RRS program and obtain additional information on the molecular changes that had occurred.

This RRS program has created a distinct structure within and between CB and SS populations. Our analyses confirm that the partitioning of variance in this breeding program has changed over time. The progenitor lines were highly variable. As time passed, some of that variation moved from within each cycle to between respective cycles in each population. This repartitioning of the variance could explain why phenotypic studies have observed a decrease in variance of these populations over time (Holthaus and Lamkey, 1995). Adding to our knowledge of these populations allows us to better evaluate how a plant breeding strategy that has been in practice for over 50 yr has and continues to affect molecular genetic variation.

Table 3. Percent variation in gene frequencies over 86 SSR loci for Iowa Corn Borer Synthetic #1 and Iowa Stiff Stalk Synthetic maize populations. Differences between populations and within populations are calculated at the group level using analysis of molecular variance. Variation was significant between populations for all groups except the progenitors (PR).

Level of variation	PR	00	01	03	06	09	12	15
				%				
Between populations	4.19	13.11	18.53	21.60	33.28	48.66	51.26	58.33
Within populations	95.81	86.89	81.47	78.40	66.72	51.34	48.74	41.67

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