

BREEDING AND GENETICS

Assessment of Day-Neutral Backcross Populations of Cotton Using AFLP Markers

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INTERPRETIVE SUMMARY

The collection of primitive cotton germplasm is a valuable source of genes for genetic improvement of cotton; however, this collection is underutilized in applied plant breeding programs because most of the accessions are photoperiodic in nature and do not flower in the temperate zone where most cotton breeding occurs. Day-neutral flowering genes can be introduced into these accessions by crossing them with a temperate-zone cultivar and selecting day-neutral flowering phenotypes in the segregating generation. When these selected day-neutral flowering plants are backcrossed to the photoperiodic parent, the expectation is that more of the photoperiodic parent genome will be introduced into the selected plants. We used four generations of backcrossed, selected day-neutral flowering plants and compared their similarity with the photoperiodic parent and the temperate-zone day-neutral flowering donor parent. We found that more of the amplified fragment length polymorphism (AFLP) molecular markers from the day-neutral parent than from the photoperiodic parent were present in the populations. We successfully developed day-neutral flowering plants from the crosses, but were not as successful in recovering the accession parental genome. This research showed that blocks of genes stay together during backcrossing in cotton and that selecting for types that flower in the temperate zone recovered much of the flowering cultivar parent genome. However, sufficient amounts of the accession genome were recovered so that these day-neutral populations could be useful for applied breeding

programs after one backcross to the accession parent.

ABSTRACT

The cotton (*Gossypium hirsutum* L.) collection of primitive accessions is underutilized for cotton improvement because they are photoperiodic. Day-neutrality can be achieved by crossing with a day-neutral cultivar and selection in the F_2 generation. Our objective was to determine the relationship among four backcross generations and parents using amplified fragment length polymorphism (AFLP) markers. We evaluated five populations (F_6 , BC_1F_6 , BC_2F_6 , BC_3F_6 , BC_4F_6) of day-neutral plants derived from crossing and backcrossing accessions T 78, T 174, T 326, and T 1149 as recurrent parents, respectively, with 'Deltapine 16' (DPL 16) as the donor day-neutral flowering parent. Genetic distances were determined using 43 AFLP primer combinations that produced 251 polymorphic AFLP markers among the five parents and 91 to 129 polymorphic markers within each of the five populations of the four sets of crosses and backcrosses. Among the 20 backcross populations, recovery of markers from accession parents was 27 to 92%, while the recovery of markers from DPL 16 was 71 to 91%. Genetic distance of populations from the recurrent parent was 0.35 to 0.75 among backcross populations. Genetic distance from the non-recurrent parent DPL 16 was 0.16 to 0.38. Many AFLP markers tended to stay together as linked blocks and were selected with the day-neutral flowering phenotype. This finding indicates that linkage drag was occurring during introgression of the day-neutral flowering trait. These results indicate that the day-neutral populations selected after one backcross to the accession parents could be used to add genetic diversity to applied breeding programs.

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Abbreviations: AFLP, amplified fragment length polymorphism; PCR, polymerase chain reaction; SSR, simple sequence repeat.

The USDA cotton germplasm collection currently contains more than 7000 accessions of *Gossypium hirsutum* L. (Percival et al., 1999). These accessions constitute a vast resource for genetic improvement of cotton for enhanced pest and pathogen resistance, tolerance for environmental stress, improved fiber quality, and yield (McCarty and Percy, 2001). A majority of the cotton accessions are short-day, photoperiodic plants of tropical or subtropical origin that fail to flower during the long summer days in the northern temperate regions where most of the cotton breeding is done. This fact presents a major impediment to their use. Systematic introduction of genes for day-neutrality into these accessions should make the germplasms more useful to applied cotton breeders in the temperate zone.

In 1970, the USDA-ARS research scientists at Mississippi State, Mississippi, initiated a program to introduce day-neutral flowering genes into these accessions (McCarty et al., 1979). In this program, plants are grown in Mexico and photoperiodic accessions from the collection are used as male parents in crosses with a U.S. cultivar. This cultivar provides genes for day-neutrality. The F_1 plants are grown in Mexico and self-pollinated. Day-neutral plants are backcrossed in the F_3 generation to the accession as recurrent parent.

MATERIALS AND METHODS

Plant Material

Four photoperiodic accessions of cottons were crossed with the day-neutral flowering donor parent 'Deltapine 16' (DPL 16). The accessions were T 78(PI 549140) race *latifolium*, T 174(PI 163647) race *latifolium*, T 326(PI 165326), and T 1149(PI 529966). T 326 and T 1149 have the appearance of race *palmeri* and race *latifolium*, respectively. Descriptive data for these accessions are provided by Percival (1987). Crosses and F_1 were made and grown in Mexico in a winter nursery where the male plants and the F_1 plants flower under the short-day environment. The F_2 populations of about 1000 plants each were grown at Mississippi State, Mississippi, where the populations segregate for flowering and nonflowering plants. In mid-August,

the nonflowering plants were cut from the plots. Flowering plants (day-neutral) were allowed to mature open-pollinated bolls. One F_2 flowering plant that set bolls over several fruiting branches was selected and 18 seeds were planted in a three-hill unit in Mexico. These F_3 plants were backcrossed as females to the recurrent photoperiodic accession parent that was planted as 30 seeds in five hills. Only five backcross bolls were harvested because of cost and space constraints in the winter nursery in Mexico; thus a maximum of five plants from one F_2 plant was used in each generation as female parents for the backcrosses. Twenty-eight BCF_1 seeds were planted in a seven-hill unit in Mexico and self-pollinated to produce the BC_nF_2 population. These crossing constraints plus the strong selection for day-neutral flowering types in each F_2 and subsequent generations could severely limit the recovery of the markers from the accession genome in successive backcrosses.

In each F_2 population, one open-pollinated boll was harvested from each day-neutral flowering plant that set bolls, and the bolls were bulked. A random sample of these F_2 seeds was planted in an F_3 progeny row and 25 open-pollinated bolls were harvested for the next generation. This protocol was repeated up to the F_6 generation following each cross and each backcross. Cotton normally self-pollinates unless a pollinating insect visits the flower and causes an outcross. In Mississippi there is a low percentage of outcrossing, especially when insecticides are used to control pest insects in the cotton field.

Thus we used four accessions as male parents and DPL 16 as a female parent. We developed five populations from each parental cross. These were the F_6 from the original cross and the BC_1F_6 through BC_4F_6 using the accession as the recurrent parent. These populations were developed over several years from 1972 through 1990. In each of these populations, the primary selection was for day-neutral flowering plants. Because of the manner in which these backcrosses and subsequent generations were handled, we wanted to determine the diversity among the two parents and the BC_nF_6 populations from each cross. The objective of this study was to assess the relationships among the 20 day-neutral populations and their four accession parents and the

DPL 16 cultivar parent. Amplified fragment length polymorphisms (AFLP) were used to access these relationships (Vos et al., 1995).

DNA Extraction

Seed from the 20 F₆ populations and the five parents were planted in the field to provide materials for the AFLP analyses. Young leaves from approximately 35 plants in each population were bulk sampled, crushed, and freeze-dried before storage at -20°C. Approximately 30 mg of crushed, freeze-dried tissue was used for DNA extraction. Isolation of DNA was by a modification of the protocol for plant tissue with the Dneasy Plant Mini Kit¹ (Qiagen, Chatsworth, CA).

AFLP Analyses

Primer pairs for the AFLP analysis were selected using DNA from a single plant from each of the four photoperiodic accessions and the cultivar 'Deltapine 50' (DPL 50), grown in the greenhouse. We used eight *Ecor1* and *Mse1* primers in 64 possible combinations. DPL 50 was used because DPL 16 did not germinate in the greenhouse experiment. We had DPL 50 growing in the greenhouse and it is of similar background as DPL 16. Forty-three primer pairs gave repeatable results and were chosen to use in the AFLP analyses of the 25 populations.

The AFLP analyses were carried out using modifications of an AFLP selective Amplification Module for large plant genomes from PE Applied Biosystems (Foster City, CA). The AFLP reactions were resolved by the automated PE Applied Biosystem ABIPRISM 310 Genetic Analyzer. This apparatus is a capillary electrophoretic system that separates fluorescent-labeled amplified DNA markers, which are visualized as peaks or electropherograms.

Results were analyzed by the GeneScan analysis software (Perkin Elmer, Norwalk, CT), which displays amplification products as peaks and determines the size and amount of these products. All

electropherograms were also visually examined and scored. Each AFLP marker was scored as 1 for presence and 0 for absence. The markers had a size range from 90 to 450 bp and only markers with peak heights >35 were scored as present. All AFLP fragments were scored as dominant markers.

Genetic distance (GD) was calculated according to Nei and Li's (1979) definition of genetic similarity, $GS_{ij} = 2a/(2a + b + c)$, where GS_{ij} is the similarity between two individuals, i and j; a is the number of markers present in both i and j; b is the number of markers present in i and absent in j; and c is the number of markers present in j and absent in i. Genetic distance was obtained by the equation $GD_{ij} = 1 - GS_{ij}$. The jackknife method was used to estimate the confidence intervals for GD. Only the polymorphic markers were used in determining the percentage of markers from each parent that showed up in the populations from the various generations.

RESULTS

Guidelines for Primer Combinations

The evaluation of 64 possible primer pair combinations on the four accession parents and the cultivar parent is shown in Table 1. There were 43 primer combinations selected to use in the AFLP analysis on the basis of reproducibility and the polymorphic nature of the markers for the experiment.

Diversity Among Parents Grown in the Field

The AFLP data using 43 primer pair combinations detected 251 polymorphic markers among DPL 16 and the four photoperiodic accessions. A marker was counted as polymorphic in

Table 1. Primer pairs selected to use in AFLP analyses.

	-----Mse 1-----							
EcoR1	CAA	CAC	CAG	CAT	CTA	CTC	CTG	CTT
AAC	X	X	X	X	X		X	
AAG		X						
ACA	X	X	X	X	X	X		X
ACC			X	X	X	X	X	X
ACG		X	X			X		
ACT	X	X	X	X	X	X	X	X
AGC	X		X	X		X		
AGG	X	X	X	X	X	X	X	X

¹ Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

Table 2. Genetic distance matrix among four race stocks and DPL 16 based on 251 polymorphic markers.

Parent	T 78	T 174	T 326	T 1149	DPL 16
T 78	0.00				
T 174	0.53	0.00			
T 326	0.59	0.37	0.00		
T 1149	0.63	0.53	0.56	0.00	
DPL 16	0.51	0.46	0.50	0.35	0.00

the analysis of parents if there was at least one polymorphism among the five parents for that marker. Genetic distances among the five parents ranged from 0.35 to 0.63 (Table 2). The greatest distance was between T 78 and T 1149, and the smallest distance was between T 1149 and DPL 16, followed by T 174 and T 326. Liu et al. (2000), using simple sequence repeat (SSR) markers, ranked the BC₄ day-neutral versions of these four accessions in the same order in relation to a cultivar as we ranked the accession parents. This similarity provided us with confidence that AFLP data were useful for comparison purposes among the populations.

Genetic Distance Among T 78, Five Day-Neutral Populations, and DPL 16

Between T 78 and DPL 16, 129 polymorphic and 62 monomorphic markers were detected with 60 of the polymorphic markers from T 78 and 69 from DPL 16. In the F₆ of the first cross, 40% of the markers from T 78 were present; however, 91% of the markers from DPL 16 were present (Table 3). Following successive backcross generations, the percentage of markers from DPL 16 remained between 80 and 88%, while the percentage of markers from T 78 ranged from 68 to 92% with the greatest number of markers from T 78 in the third backcross generation. In the BC₁, there was an increase in the number of markers recovered from T 78 compared with the original cross. However, there was not a consistent increase in the percentage of

Table 3. Percentage of polymorphic fragments recovered from accession parents and DPL 16 for the five populations in each cross.†

	T 78	DPL 16	T 174	DPL 16	T 326	DPL 16	T 1149	DPL 16
	-----%							
F ₆	40	91	49	86	33	87	27	87
BC ₁ F ₆	75	88	38	91	33	91	48	91
BC ₂ F ₆	77	83	40	89	38	90	27	85
BC ₃ F ₆	92	80	42	85	47	87	45	81
BC ₄ F ₆	68	83	64	71	51	87	41	79

† Number of polymorphic markers in parents: T 78=60; DPL 16=69; T 174=45; DPL 16=66; T 326=55; DPL 16=69; T 1149=44; DPL 16=47.

markers from T 78 recovered with succeeding backcross generations. Comparing T 78 with the original cross and the four backcross-derived populations showed a genetic distance of 0.67 in the original F₆ and a range of 0.35 to 0.48 in the four backcross-derived populations (Table 4). After the first backcross, there was not a significant gain in the number of markers recovered from T 78, the recurrent parent. Similarly, the genetic distance between DPL 16 and successive backcross-derived populations was smallest in the F₆ and remained relatively constant in backcross generations.

Genetic Distance Among T 174, Five Day-Neutral Populations, and DPL 16

We detected 111 polymorphic and 65 monomorphic markers comparing T 174 and DPL 16, with 45 of the polymorphic markers from T 174 and 65 from DPL 16 (Table 3). In the F₆ generation of the first cross, 49% of the markers from T 174 and 86% from DPL 16 were present (Table 3). In the first three backcross populations, 38 to 42% of the T 174 markers were recovered. In the four backcross-derived populations, 64% of the T 174 markers were recovered, which was a significant increase over the other three backcross-derived populations. Among the four backcross-derived populations, 71 to 91% of the markers from DPL 16 were recovered. The genetic distance from T 174 in the five populations

Table 4. Genetic distance and ±95% confidence interval from accession and DPL 16 parents in the five populations for each accession with the distance between each accession and DPL 16 set as 1.00.

Population	T 78	DPL 16	T 174	DPL 16	T 326	DPL 16	T 1149	DPL 16
F ₆	0.67 ± 0.10	0.19 ± 0.07	0.64 ± 0.10	0.21 ± 0.08	0.73 ± 0.11	0.18 ± 0.06	0.75 ± 0.12	0.18 ± 0.18
BC ₁ F ₆	0.46 ± 0.09	0.30 ± 0.08	0.72 ± 0.11	0.16 ± 0.17	0.73 ± 0.11	0.16 ± 0.17	0.61 ± 0.12	0.22 ± 0.07
BC ₂ F ₆	0.43 ± 0.09	0.34 ± 0.08	0.70 ± 0.11	0.17 ± 0.07	0.69 ± 0.10	0.18 ± 0.06	0.75 ± 0.11	0.19 ± 0.09
BC ₃ F ₆	0.35 ± 0.08	0.38 ± 0.08	0.68 ± 0.11	0.20 ± 0.08	0.63 ± 0.11	0.22 ± 0.07	0.61 ± 0.12	0.27 ± 0.10
BC ₄ F ₆	0.48 ± 0.10	0.32 ± 0.08	0.52 ± 0.11	0.34 ± 0.09	0.61 ± 0.10	0.23 ± 0.07	0.63 ± 0.13	0.27 ± 0.10

ranged from 0.52 to 0.72, with the smallest distance in the fourth backcross population (Table 4). The genetic distance from DPL 16 ranged from 0.16 to 0.34, with the distance of 0.34 in the fourth backcross-derived population being significantly greater than the other populations. The data show that the successive backcross populations had more markers in common with DPL 16 than with T 174.

Genetic Distance Among T 326, Five Day-Neutral Populations, and DPL 16

When comparing T 326 and DPL 16, 124 polymorphic and 62 monomorphic markers were detected between T 326 and DPL 16, with 55 polymorphic markers specific to T 326 and 69 to DPL 16. In the F_6 of the original cross, 33% of the markers from T 326 and 87% of the DPL 16 markers were recovered (Table 3). Among the four backcross-derived populations, the markers from T 326 increased from 33 to 51%, while the percentage of markers from DPL 16 ranged from 87 to 91%. The genetic distance from T 326 (0.61 to 0.73) was greater than from DPL 16 (0.16 to 0.23) (Table 4). The genetic distances of the five populations from T 326 were not significantly different; neither were the five populations different in distance from DPL 16. The five populations had more markers in common with DPL 16 than with the recurrent parent T 326.

Genetic Distance Among T 1149, the Five Populations, and DPL 16

There were 91 polymorphic and 84 monomorphic markers between T 1149 and DPL 16, with 44 polymorphic markers specific to T 1149 and 47 specific to DPL 16. These parents showed the smallest amount of polymorphism. The percentage of markers recovered from T 1149 in various backcross generations ranged from 27 to 48%. Markers recovered from DPL 16 ranged from 79 to 91% (Table 3). The later backcross-derived populations had a slightly smaller percentage of the markers from DPL 16. The genetic distance from T 1149 ranged from 0.61 to 0.75; however there was no significant difference among the five populations (Table 4). Genetic distance from DPL 16 ranged from 0.18 to 0.27, with no significant difference among the five populations. Thus these five populations were also

closer to DPL 16 than to the recurrent parent T 1149.

DISCUSSION

These 20 populations were derived over an 18-yr period from 1972 to 1990. The objective in developing the backcross-derived populations was to recover increasing amounts of accession germplasm in each successive backcross generation, while introducing day-neutral flowering from the donor DPL 16 parent. We easily selected for the day-neutral flowering trait; however, we did not recover increasing numbers of markers from the recurrent parent in the day-neutral flowering plants selected in successive backcrosses (Tables 3 and 4). The data deviated significantly from the theoretical recovery of one-half of the recurrent parent from each backcross generation. A higher percentage of the markers from the cultivar parent than from the recurrent, photoperiodic, accession parent were recovered in the 20 day-neutral populations. Several things probably contributed to this. Lui et al. (2000), in an examination of 97 BC_4F_4 converted line populations, suggested that linkage drag adjacent to gene(s) controlling day-neutrality coming from the donor parent may be a factor. Also, we had a range of only 91 to 129 polymorphic markers among parents in these populations. Since cotton has 26 linkage groups, we may not have had a sufficient number of markers. We found six markers present in all backcross generations so there may be at least six loci involved in day-neutrality. With six possible loci independently segregating for day-neutrality, we could speculate that 6 of 26 loci, or 23% of the genome, could be involved in linkage drag. The breeding procedure we used also affected the recovery of recurrent parent genes. By harvesting one boll from each flowering plant in the F_2 for advancing to the F_6 , a high selection pressure was placed on the ability to flower. We also chose only one plant in each F_2 to cross back in the F_3 to the recurrent accession parent. This limitation was a constraint placed on the program because of space availability and cost in the winter nursery.

Since we bulked leaves from 35 plants in each population for DNA extraction, the number of recovered markers in each day-neutral population should represent the maximum recovery, and

individual plants may have fewer markers than we report here for the population. Individual plants may also have different combinations of parental markers than reported for the populations. The individual plant used for the backcross would not necessarily have all the recurrent parent markers that were in the population sample. Consequently, the number of recurrent parent markers recovered in the backcrosses populations varied, with some advanced backcross populations having fewer recurrent parent markers than earlier backcross-generation populations.

Also, individual plants in a population could have fewer cultivar parent markers and these individual plants would be a greater distance from the cultivar parent than the population sample. Thus individual plants in the population should vary from the population values, and there may be some plants that have recurrent parent markers equal to the population value and also have fewer cultivar parent markers than the population value. These individual plants would be those with maximum diversity from the cultivar parent and thus should be candidate sources of novel alleles.

Liu et al. (2000) used SSR markers to compared 97 BC₄F₄ day-neutral versions of 97 accessions with a day-neutral cultivar. They reported the majority had genetic distances less than 0.25 from the *G. hirsutum* standard TM-1. They genotyped 10 plants within the most diverse nine accessions and found that in some families, the photoperiodic parent was recovered, while in others, there was extensive linkage drag from the day-neutral donor parent. They suggested that day-neutral conversion of race lines could benefit from careful use of marker-assisted selection during the backcross process. When the ligo lintless trait was backcrossed six times to TM-1, the backcross line still retained 18% polymorphism with TM-1 (Karaca et al., 2002). Molecular markers would be helpful to monitor the average proportion of the genome from the donor parent, perhaps making backcross breeding more successful.

Young and Tanksley (1989) reported that when the gene for resistance to tobacco mosaic virus was introduced into tomato by repeated backcrossing, it was flanked by introgressed segments of DNA from the donor parent. They estimated the length of the introgressed segment, using RFLP markers, to be

from 4 cM to more than 51 cM. They also reported that the backcross breeding method was largely ineffective in reducing the size (amount) of linked DNA around the resistant gene. They proposed that monitoring recombination around genes of interest with linked RFLP markers would allow one to quickly and efficiently reduce the amount of linkage drag associated with introgression.

The parents and the 20 derived populations were grown in 1999 in the field in Mississippi, and considerable variation was noted within each population. The genetic distance from the recurrent parents ranged from 0.45 to 0.75 (Table 4), and a considerable number of accession DNA markers (and linked genetic material) were recovered while retaining a large percentage of the DPL 16 markers (genetic similarity ranged from 0.62 to 0.84). Thus these 25 populations from the four accessions should be good material from which to make individual plant selections that would have a good mixture of accession and DPL 16 genes. Since each population is in the F₆, the individual plants should be nearly homozygous.

Swindle (1993) used these same 25 populations as parents in crosses with four cultivars. He collected agronomic and fiber data in the F₂ following the crosses. No general trend was found as to which population made the best parent in the crosses with the cultivars. In our study, there was not a general trend toward increasing amounts of marker genes from the recurrent parent as the number of backcrosses increased. The results of our study support Swindle's conclusions. The backcross-derived populations from T 78 were more similar to the recurrent parent than were the populations derived from the other three accessions.

We conclude that linkage drag exists in these day-neutral selections. During backcrossing, large blocks of chromatin apparently stayed together. Alternately, the AFLP markers may be closely linked with blocks of genes for day-neutrality and productivity, or perhaps the selected AFLP markers are not scattered at random across all 26 pairs of chromosomes in upland cotton. Shappley et al. (1998a,b) found that some linkage groups have more RFLP markers than others. In a joint map involving RFLP markers, two linkage groups (Ulloa et al., 2000), later shown to be on chromosomes 16 and 26, were shown to have considerably more markers than

other chromosomes or linkage groups. Thus these data suggest that the markers used are not randomly distributed and that there are some “hot spots” within the cotton genome. If this is true, then our genetic distance data may apply only to selected regions of the upland cotton genome and in particular to regions that are associated with day-neutrality and productivity. We also observed that six polymorphic AFLP markers (Zhong, 2001) were present from the donor parent in all backcross generations of all of the accessions, indicating that they may be candidate markers for day-neutral genes.

A practical finding from this study is that after one backcross to the accession parent, the day-neutral populations can be used to introduce genetic diversity into applied cotton breeding programs.

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