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ORIGINAL PAPER

QTL mapping with near-isogenic lines in maize

S. J. Szalma · B. M. Hostert · J. R. LeDeaux · C. W. Stuber · J. B. Holland

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Abstract A set of 89 near-isogenic lines (NILs) of maize was created using marker-assisted selection. Nineteen genomic regions, identified by restriction fragment length polymorphism loci and chosen to represent portions of all ten maize chromosomes, were introgressed by backcrossing three generations from donor line Tx303 into the B73 genetic background. NILs were genotyped at an additional 128 simple sequence repeat loci to estimate the size of introgressions and the amount of background introgression. Tx303 introgressions ranged in size from 10 to 150 cM, with an average of 60 cM. Across all NILs, 89% of the Tx303 genome is represented in targeted and background introgressions. The average proportion of background introgression was 2.5% (range 0–15%), significantly lower than the expected value of 9.4% for third backcross generation lines developed without marker-assisted selection. The NILs were grown in replicated field evaluations in two years to map QTLs for flowering time traits. A parallel experiment of testcrosses of each NIL to the unrelated inbred, Mo17, was conducted in the same environments to map QTLs in NIL testcross hybrids. QTLs affecting days to anthesis, days to silking, and anthesis-silk interval were detected in both inbreds and hybrids in both environments. The testing environments differed dramatically for drought stress, and different sets of QTLs were detected across environments. Furthermore, QTLs detected in inbreds were typically different from QTLs detected in hybrids, demonstrating the genetic complexity of flowering time. NILs can serve as a valuable genetic mapping resource for maize breeders and geneticists.

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S. J. Szalma · C. W. Stuber · J. B. Holland (✉)
USDA-ARS Plant Science Research Unit and Department of Crop Science, North Carolina State University, Raleigh, NC 27695, USA
e-mail: James_Holland@ncsu.edu

B. M. Hostert
Monsanto Co., Woodland, CA 95616, USA

J. R. LeDeaux
Monsanto Co., St Louis, MO 63167, USA

Introduction

Many population structures have been used for QTL detection and mapping in plants. Backcross (BC), F_2, doubled haploid, testcross progenies, half-sib and full-sib families, F_2 derived lines, recombinant inbred lines (RIL), and diverse inbred population structures have all demonstrated utility in QTL identification and confirmation (Byrne et al. 1996; Cowen 1988; Edwards et al. 1992, 1987; Ellis 1986; Knapp 1991; Knapp and Bridges 1990; Tanksley et al. 1982). Near-isogenic lines have also been used to verify and fine-map QTLs in maize (*Zea mays* L., Graham et al. 1997; Koester et al. 1993), tomato (*Lycopersion esculentum* L., Brouwer and St Clair 2004), soybean (*Glycine max* L., Muehlbauer et al. 1991), rice (*Oryza sativa* L., Yu et al. 1991), and lettuce (*Lactuca sativa* L., Paran et al. 1991). Typically, these studies have involved the creation of NILs to verify the effects of one or a few QTLs, and so only a relatively small proportion of the donor parent genome was represented among the NILs developed.

Although NILs have been mostly used to verify QTLs previously detected in other mapping population structures, Eshed and Zamir (1995) proposed the use of NILs to simultaneously map, verify, and incorporate QTLs into adapted, elite genetic backgrounds. Stuber et al. (1999) suggested a similar strategy for maize as a way to improve the practical utility of DNA marker and QTL mapping techniques. The NIL mapping strategy involves creating a set of lines in which each NIL carries only a small region of the donor parent genome, but across the whole set of NILs, a large proportion, or ideally, all, of the donor parent genome is represented. As many chromosomal regions as possible of the donor parent should be sampled in the NILs, to permit testing of their effects in near-isogenic backgrounds. For example, "introgression libraries" of NILs representing most of the genome of an exotic accession or a wild relative have been created in tomato (Canady et al. 2005; Eshed and Zamir 1995; Monforte and Tanksley 2000), *Brassica* (Ramsay et al. 1996), and melon (*Cucumis melo* L., Eduardo et al. 2005). The use of these "introgression libraries" may be a powerful tool for expanding the elite gene pools of crops (Zamir 2001).

Kaeppler (1997) demonstrated that NIL-based QTL tests have less statistical power for QTL detection than RILs, but this comparison assumed that each NIL is paired with the recurrent parent in the experimental design. More efficient NIL experimental designs, where the recurrent parent is compared to multiple NILs simultaneously, can improve the relative efficiency of NIL experiments. Furthermore, although power to detect a single QTL may be greater in RILs than NILs, NILs may still offer more accurate QTL effect estimates than RILs if multiple QTLs are segregating in the populations. Typical population sizes used in RIL mapping studies result in the confounding of effects of multiple segregating QTLs. This can lead to reduced power of QTL detection and overestimation of the effects of those QTLs that are detected (Beavis 1998; Melchinger et al. 1998). In contrast, phenotypic differences between the recurrent parent and a NIL should be due primarily to the allelic differences at the chromosomal region surrounding the introgressed target locus. Essentially, this should reduce much of the "noise" caused by the effects of genetic background. Homogenizing the genetic background eliminates associations, or colinearity, between QTLs that occur in typical RIL populations.

Employing a NIL-based approach to QTL discovery may also circumvent some of the obstacles faced in uniting the results from genetic investigation with germplasm development (Stuber et al. 1999). Despite the multitude of QTL identification experiments and resulting significant QTLs reported in the literature, the deployment of QTLs for germplasm improvement has been surprisingly uncommon (Holland 2004). The use of NILs would reduce the number of generations of backcrossing required to introgress favorable QTL alleles into an elite line (Stuber et al. 1999). NILs with elite inbreds as recurrent parents that display superior hybrid performance may be suitable for immediate release (Stuber et al. 1999). Finally, in NILs that display superior performance, epistatic interactions between the introgressed segment and the rest of the recurrent parent genome must either be favorable, or have only minor negative effects (Tanksley and Nelson 1996).

We created a set of 89 $BC_3F_{2:3}$ maize NILs using marker-assisted selection (MAS) to retain introgressions representing targeted regions for most of the 20 chromosome arms of the inbred Tx303 in the B73 genetic background. The recurrent parent B73 was chosen because it is historically one of the most important inbred lines for commercial maize in the United States (Mikel and Dudley 2006). Donor parent Tx303 was chosen because of previous studies indicated that it is genetically distinct from B73 and capable of improving the combining ability of B73 with appropriate testers (Stuber 1998). Regions targeted for introgression were selected to maximize representation of the donor genome in the NILs, rather than to test previously identified QTL regions, but this was somewhat limited by genome coverage and relatively low throughput available with restriction fragment length polymorphism markers, which were the best markers available at the initiation of this research. Nevertheless, this represents the most extensive marker-developed set of NILs reported in maize to date. These lines can directly serve as useful tools for QTL detection where the interest is in genome regions of Tx303 that alter phenotypes of the B73 genetic background. For example, Gonzalo et al. (2006) used a subset of these lines to characterize the effects of specific genome regions on density responses in maize.

The objectives of this study were to determine the size of introgressed segments around loci that were targeted during MAS, estimate the proportion of Tx303 donor-parent alleles at "non-target" loci in the set of maize NILs, and demonstrate the utility of the NIL set in QTL identification, using field evaluations of inbred NILs and hybrids created by testcrossing NILs to a common tester inbred. Many quantitative traits are segregating in the NIL set, but flowering time of inbred NILs and their testcross hybrids was chosen to demonstrate the NIL-QTL analysis because of its balance of moderate complexity and moderate heritability (Chardon et al. 2004).

Materials and methods

Genetic stocks

NILs were developed from a cross of unrelated inbred lines, B73 and Tx303 (Fig. 1). Progeny derived from this cross were backcrossed for three generations, using B73 as the recurrent parent. Plants from the BC_3F_1 generation were selfed to make BC_3F_2 seed, from which the $BC_3F_{2:3}$ generation NILs were derived. Marker-assisted selection using ten to 19 RFLP marker loci was performed during the NIL development process. The 19 target loci were chosen to represent regions on all 10 chromosomes and an attempt was made to sample both arms of each chromosome. Genotyping of RFLPs was accomplished according to the methods described in Helentjaris et al. (1985). In the BC_1F_1, BC_2F_1, BC_3F_1, and BC_3F_2 generations, plants or families heterozygous for the Tx303 allele at as few target loci as possible, and homozygous for the recurrent parent (B73) allele at as many other RFLP loci as possible were selected. Marker assisted selection was also used to select 89 of 378 BC_3F_2 plants homozygous for the Tx303 allele at one or two of 19 target loci with no Tx303 alleles at other RFLP loci. These plants were self-pollinated to form $BC_3F_{2:3}$ NILs. In addition to the 89 NILs, one "control" line was selected during the NIL development process. The control line was derived from the same cross and backcrossing procedures as described above, but was homozygous for the B73 allele at each of the 19 RFLP loci. Each of the 89 NILs, the control line, Tx303, and B73 was test crossed to the inbred line Mo17 for evaluation. Seed of the NILs will be distributed publicly (seed supplies permitting) and can be requested from J.B. Holland. To estimate the genetic map distances between loci used to genotype the NILs, 133 random B73 × Tx303 $F_{2:3}$ families were grown for tissue collection and DNA extraction.

NIL genotyping

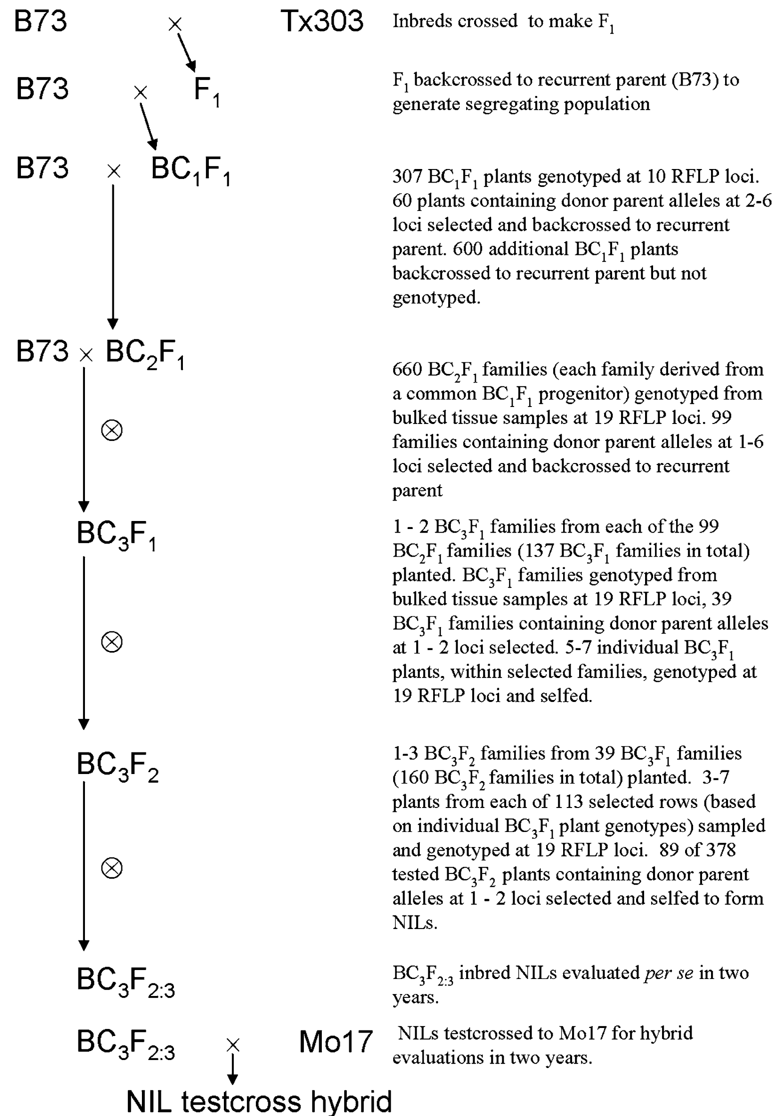
To obtain DNA, the 89 $BC_3F_{2:3}$ NILs, the "control" NIL, B73, and Tx303 were planted and grown to the two true-leaf stage. Tissue samples were harvested from approximately 10 plants per line, bulked, and DNA was extracted as described by Riede and Anderson (1996). Each line was genotyped with a core set of 90 simple sequence repeat (SSR) markers and the 19 RFLP markers used to select the NILs (Fig. 1). SSR markers comprising the core set were chosen at intervals of approximately 20–30 cM throughout the maize genome in order to estimate the proportion of Tx303 germplasm remaining in the B73 background for each of the NILs. While screening the genetic background of each NIL, additional SSR loci flanking each target locus were added to resolve the sizes of the introgressed Tx303 segments. Across all lines, a total of 128 SSR loci were used to genotype the NILs (Supplementary Table 1). SSR marker genotyping was conducted according to the methods reported by Senior et al. (1998).

The RFLP and SSR markers used to obtain genotype information for the NIL set were also used to generate a linkage map for the B73 × Tx303 F_2 population (Fig. 2). For each of 133 F_2 plants, leaf tissue was harvested from approximately 10 F_3 progeny, bulked, and used for DNA extraction for RFLP and SSR analyses. MAPMAKER/EXP version 3.0 (Lander et al. 1987) was used to generate a linkage map based on SSR and RFLP data from the F_2 generation. In some instances, flanking SSR markers tightly linked to RFLP loci used in MAS were used to estimate the position of RFLP loci. The linkage map served as a benchmark to determine the position of molecular markers in the NIL set. The size of introgressed regions from Tx303 into B73 were estimated using map distances from the Tx303 × B73 F_2 map. The ends of introgressed blocks were assumed to extend halfway between the last locus of the block at which a NIL carried the Tx303 allele and the nearest locus identified as homozygous for the B73 allele. Loci heterozygous or homozygous for the Tx303 allele and located within 50 cM of the contiguous block of introgressed loci surrounding the target locus were also considered to be part of the target introgression. In cases where genotypic scores were missing or ambiguous (e.g., more than one marker mapped to the same locus in the F_2 map but gave different scores in the NILs), the genotype that implied the fewest recombination was assumed. Unlinked markers were assumed to represent linkage blocks of 20 cM for the purposes of calculating genotypic composition. The proportion of heterozygous loci and proportion of Tx303 alleles maintained in the NILs were compared to their expected values in random $BC_2F_{2:3}$ lines using the nonparametric sign test (Steel and Torrie 1980).

Phenotypic evaluation

A subset of 65 $BC_3F_{2:3}$ NILs with the greatest seed supplies and the maize inbred lines B73, Tx303, and Mo17 were evaluated phenotypically in the field in years 2001 and 2002. In 2001, B73 was entered four times and Tx303 two times within each replication for a total of 72 entries in the experiment. The experimental design

Fig. 1 Procedure used to develop near-isogenic lines



was an 8 × 9 lattice design with three replications. In 2002, seed supply was limited on some NILs, so 56 NILs were tested, and B73 was entered five times and Tx303 twice per replication to make a total of 64 entries. The experimental design was an 8 × 8 lattice design with three replications, with an additional check entry, B73 purple, grown between every four experimental plots. In both years, 30 seeds were planted in each plot, and stands were thinned to 20 plants per plot at the four-leaf stage. Plots were single rows measuring 4.86 m long with a 1 m alley at the end of each plot, and 0.97 m between rows. All evaluations were conducted at the Central Crops Research Station, Clayton, NC.

In both years, a separate evaluation of testcrosses of each of the 89 NILs, B73, and Tx303 to the unrelated inbred line tester, Mo17, was conducted at a nearby field at the same research station. The entries for testcross evaluations in both years were the 89 NIL × Mo17 F_1 testcrosses, three replicate entries of B73 x Mo17 F_1, Tx303 x Mo17 F_1, and six commercial hybrids (Pioneer brand hybrids 3165, 3223, 32K61, and 3394; Northrup King brand hybrid N8811; and Dekalb brand hybrid 687), and the testcross hybrid made from one NIL that was later determined to be contaminated (see Results). Entries were arranged in 10 × 10 lattice designs with two replications. Plots were two rows of the same size and spacing as used for the inbred studies, sown at a density of 43,200 seeds ha^{-1} (44 seeds per plot).

Supplemental irrigation was applied to both experiments as needed. Maximum irrigation rates were approximately 25 mm of water applied via overhead sprinklers every 5 days. Total amounts of water applied as irrigation to the inbred experiment were 102 and 257 mm in years 2001 and 2002, respectively. Total amounts of water applied as irrigation to the hybrid

Table 1 RFLP locus targeted for introgression, size of introgressed segment, size of segregating segments within target introgression, total proportion of segregating loci, and proportion of donor parent genome recovered outside of targeted introgressions for each NIL, based on genetic distances

NIL designation	RFLP locus targeted for introgression		Target introgression size (cM)	Size of segregating segments within target introgression (cM)	Total segregating genome regions (%)	Proportion of donor parent genome outside of target introgression (%)
	Chromosome arm	Locus				
TBBC3-68	1S	UMC76A	22	0	1.2	1.2
TBBC3-69	1S	UMC76A	54	0	0.3	1.2
TBBC3-14	1L	UMC107A	73	36	2.3	0.8
TBBC3-15	1L	UMC107A	60	0	1.9	1.9
TBBC3-17	1L	UMC107A	132	55	6.7	4.5
TBBC3-60	1L	UMC107A	132	0	2.4	5.7
TBBC3-61	1L	UMC107A	88	70	5.3	2.5
TBBC3-04	2L	UMC122	71	21	0.8	1.0
TBBC3-05	2L	UMC122	71	0	0.5	0.5
TBBC3-06	2L	UMC122	121	0	0.7	0.7
TBBC3-07	2L	UMC122	73	0	0.0	0.0
TBBC3-43	2L	UMC122	107	0	0.0	2.3
TBBC3-44	2L	UMC122	105	78	4.2	2.3
TBBC3-45	2L	UMC122	100	13	0.5	2.3
TBBC3-49	2L	UMC122	94	21	1.9	2.4
TBBC3-50	2L	UMC122	71	21	1.9	1.1
TBBC3-53	2L	UMC122	71	0	0.0	0.0
TBBC3-55	2L	UMC122	94	19	1.9	1.1
TBBC3-57	2L	UMC122	71	0	1.8	6.4
TBBC3-82	2L	UMC122	107	0	0.7	0.7
TBBC3-89	2L	UMC122	71	0	0.7	1.6
TBBC3-90	2L	UMC122	50	0	2.1	2.1
TBBC3-65	3S	UMC32A	24	0	0.0	0.0
TBBC3-81	3S	UMC32A	24	0	0.0	0.9
TBBC3-16	4S	BNL5.46	27	0	1.2	3.2
TBBC3-28	4S	BNL5.46	81	0	2.0	2.0
TBBC3-29	4S	BNL5.46	109	0	0.0	1.7
TBBC3-30	4S	BNL5.46	109	55	4.1	3.2
TBBC3-41	4L	BNL8.45B	26	0	2.2	2.2
TBBC3-13	4L	BNL8.23	20	0	3.8	7.5
TBBC3-64	4L	BNL8.23	20	0	0.0	0.0
TBBC3-78	4L	BNL8.23	20	0	7.0	9.0
TBBC3-01	5S	UMC147A	89	0	0.4	0.4
TBBC3-02	5S	UMC147A	44	0	0.0	1.8
TBBC3-31	5S	UMC147A	23	0	0.5	2.7
TBBC3-32	5S	UMC147A	95	39	4.1	3.7
TBBC3-33	5S	UMC147A	61	39	2.5	1.4
TBBC3-34	5S	UMC147A	78	11	1.4	3.1
TBBC3-83	5S	UMC147A	23	0	2.1	2.9
TBBC3-84	5S	UMC147A	33	0	1.2	2.8
TBBC3-85	5S	UMC147A	23	0	1.5	2.2
TBBC3-37	5L	UMC068	22	0	2.1	6.8
TBBC3-71	5L	UMC068	22	0	0.0	0.0
TBBC3-86	5L	UMC068	22	0	3.9	3.9
TBBC3-09	6S	UMC085	49	0	1.2	1.8
TBBC3-63	6S	UMC085	149	63	5.6	6.4
TBBC3-51	6L	UMC021	83	0	2.7	3.9
TBBC3-52	6L	UMC021	83	32	1.3	2.7
TBBC3-75	6L	UMC021	50	0	0.0	0.0
TBBC3-76	6L	UMC021	50	0	1.7	3.1
TBBC3-03	7L	UMC116	38	0	3.9	6.6
TBBC3-24	7L	UMC116	38	0	1.8	1.8
TBBC3-25	7L	UMC116	109	0	2.0	3.5
TBBC3-08	7L	UMC168	10	0	0.5	0.5
TBBC3-18	7L	UMC168	10	0	0.0	1.4
TBBC3-19	7L	UMC168	57	0	0.9	1.2

Table 1 continued

NIL designation	RFLP locus targeted for introgression		Target introgression size (cM)	Size of segregating segments within target introgression (cM)	Total segregating genome regions (%)	Proportion of donor parent genome outside of target introgression (%)
	Chromosome arm	Locus				
TBBC3-22	7L	UMC168	34	0	1.3	1.3
TBBC3-23	7L	UMC168	34	0	0.8	2.1
TBBC3-26	7L	UMC168	10	0	0.0	0.0
TBBC3-40	7L	UMC168	34	0	0.0	1.5
TBBC3-70	7L	UMC168	34	0	0.8	0.8
TBBC3-72	7L	UMC168	34	0	0.0	1.9
TBBC3-73	7L	UMC168	34	0	0.5	1.3
TBBC3-42	8S	UMC032B	37	0	3.0	5.1
TBBC3-20	9S	UMC113	52	0	0.4	1.3
TBBC3-21	9S	UMC113	40	0	2.2	2.2
TBBC3-47	9S	UMC113	79	0	0.8	0.8
TBBC3-48	9S	UMC113	52	0	0.0	0.9
TBBC3-58	9S	UMC113	52	0	0.0	0.9
TBBC3-59	9L	BNL14.28	140	27	10.5	15.1
TBBC3-66	9L	BNL14.28	113	0	2.6	4.4
TBBC3-67	9L	BNL14.28	92	22	3.7	3.5
TBBC3-87	9L	BNL14.28	70	0	0.7	0.7
TBBC3-88	9L	BNL14.28	92	0	1.3	2.1
TBBC3-10	10S	UMC155	55	17	3.2	2.5
TBBC3-11	10S	UMC155	55	29	4.5	4.2
TBBC3-46	10S	UMC155	38	0	2.3	3.7
TBBC3-62	10L	UMC044A	22	0	1.6	3.5
TBBC3-79	10L	UMC044A	22	0	1.0	3.6
TBBC3-80	10L	UMC044A	22	0	1.6	4.8
TBBC3-74	NA	None, “control”	NA	NA	1.5	1.9
TBBC3-38	1S, 5L	UMC76A, UMC068	54, 22	0, 0	0.6	3.8
TBBC3-39	1S, 5L	UMC76A, UMC068	22, 22	0, 0	1.4	2.2
TBBC3-77	1S, 6L	UMC76A, UMC021	81, 50	0, 0	1.7	1.7
TBBC3-35	1S, 8L	UMC76A, NPI107	132, 20	21, 0	1.8	1.0
TBBC3-36	1S, 8L	UMC76A, NPI107	132, 20	52, 0	2.6	1.0
TBBC3-54	2L, 9S	UMC122, UMC113	71, 101	44, 22	3.5	0.8
TBBC3-12	3S, 10S	UMC032A, UMC155	24, 38	0, 0	4.1	4.7
TBBC3-27	4S, 7L	BNL5.46, UMC168	150, 57	82, 24	4.2	0.0
Mean			60	9	1.8	2.5
Minimum			10	0	0.0	0.0
Maximum			150	82	10.5	15.1

NA not applicable

experiment were 76 and 203 mm in years 2001 and 2002, respectively.

Days after planting to 50% anthesis (DTA) and silking (DTS) were recorded on each plot as the date when 50% of the plants in a plot were observed to be shedding pollen or displaying visible silks, respectively. Anthesis-silk interval (ASI) was determined by subtracting DTA from DTS.

Data analysis

Mixed models analyses were employed using PROC MIXED of SAS Version 8.0 (Littell et al. 1996). Each environment and experiment (inbred or hybrid) was analyzed separately, considering entry to be a fixed factor and replications and incomplete blocks to be random. This lattice model was used to analyze the 2001 inbred NIL experiment and the hybrid experiment in both years.

In the inbred study in 2002, however, substantial spatial variation in the trial due to soil heterogeneity was observed. For that experiment alone, a check inbred line, B73 purple, was grown between every four experimental plots. Plant height was measured on every repeated B73 purple check plot, and the deviation of plant height of each B73 purple plot from the overall mean of B73 purple was computed. A covariate was then constructed for each experimental plot as a weighted mean of the two flanking B73 purple plot deviations from the overall B73 purple mean. The weights on the two means were the relative distances from the experimental plot to each flanking repeated

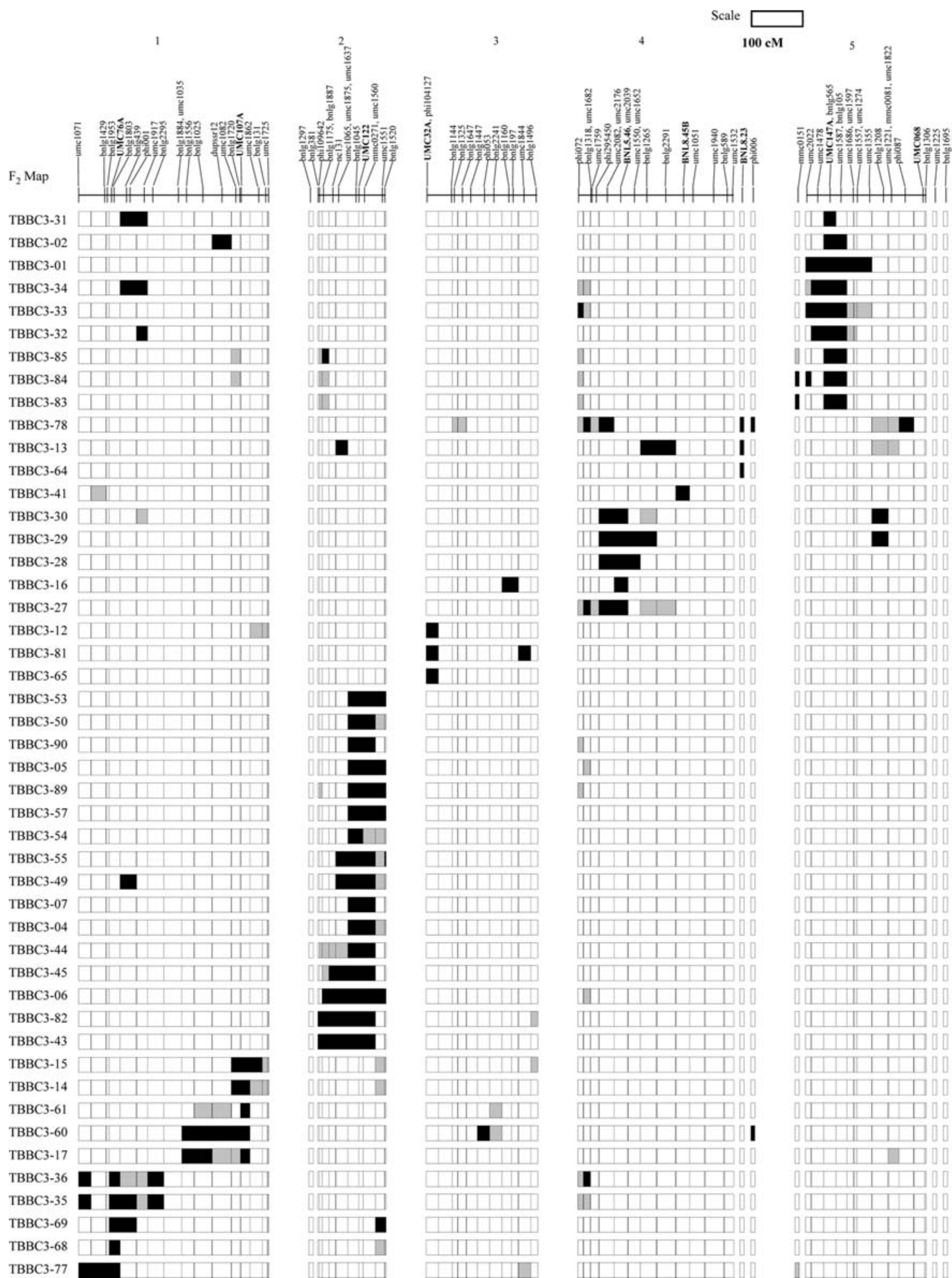


Fig. 2 Representation of Tx303 introgressions in NIL set relative to B73 × Tx303 F_2 genetic map. NIL identifiers are presented along the top of the figure. The F_2 generation linkage map with SSR and RFLP marker positions is presented on the right-hand side. RFLP loci are indicated in bold font and capital letters. Unlinked loci are placed at the ends of the relevant linkage groups, in the order predicted by the IBM2 2004 Neighbors Map (www.maizegdb.org). White blocks represent genome regions homozygous for the recurrent parent (B73) allele, gray blocks represent segregating genome regions, and black blocks represent genome regions homozygous for the donor parent (Tx303 allele)

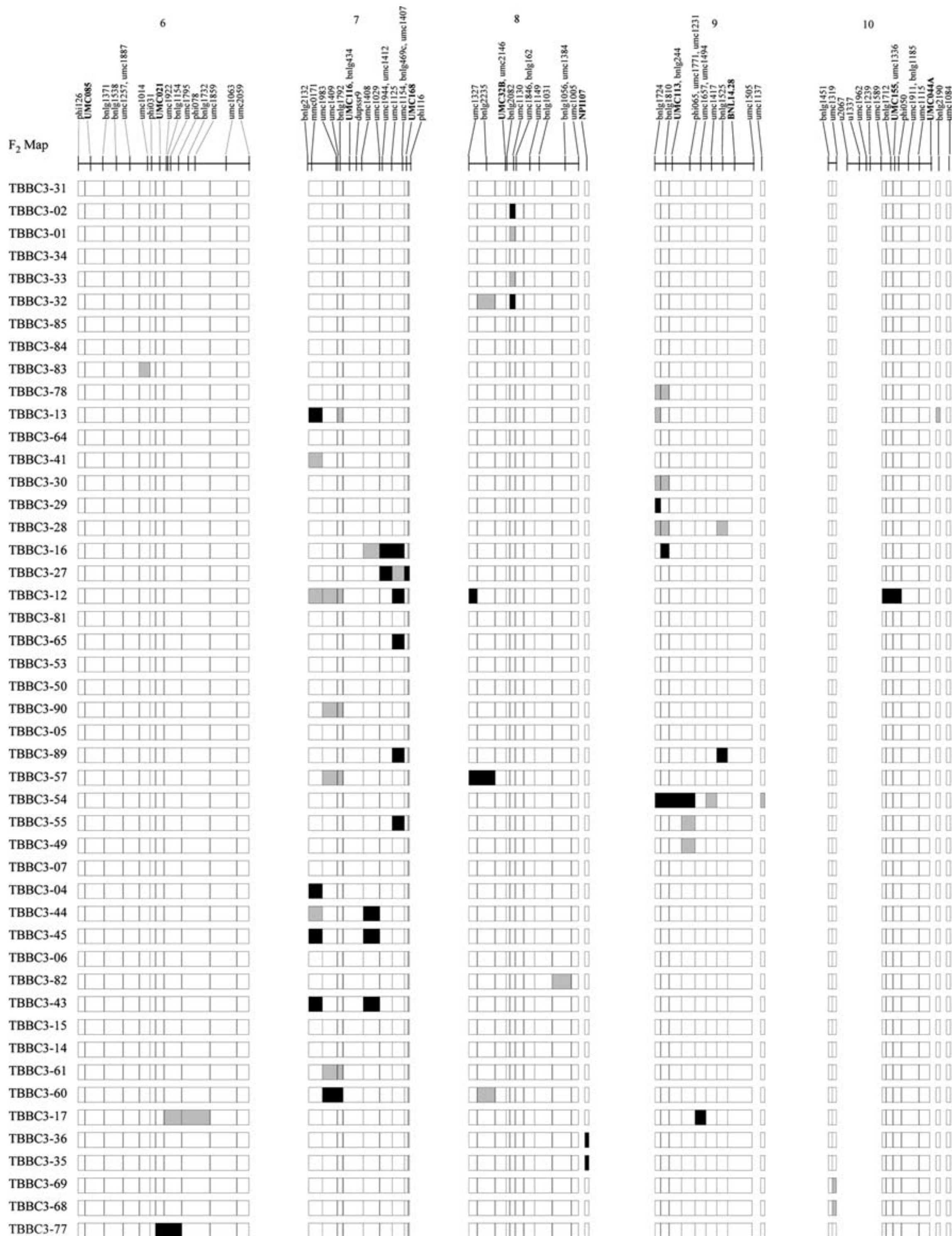


Fig. 2 continued

check plot. Lattice, trend analysis, correlated errors, and covariate models were tested for each trait in this experiment, and the model with lowest Akaike Information Criterion was selected as the most appropriate model for QTL discovery (Brownie et al. 1993; Tarter et al. 2003).

A combined analysis across environments was conducted for each experiment (inbred and hybrid),

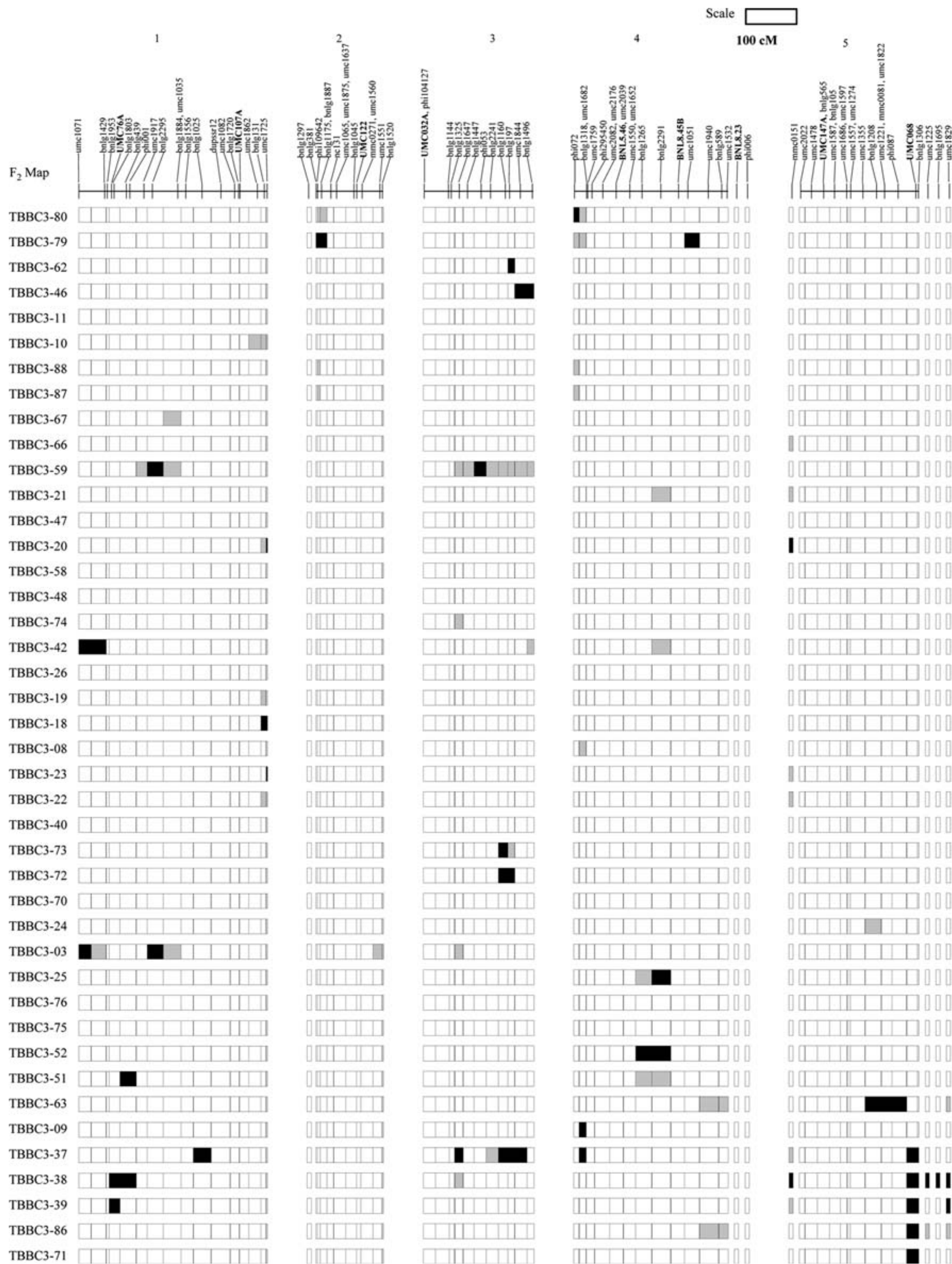


Fig. 2 continued

considering years and genotype-by-year interactions to be random effects. For each trait, a full model, including genotype-by-environment interaction, and a reduced model without genotype-by-environment interaction were analyzed. The significance of genotype-by-environment interaction was tested using a likelihood ratio computed as the difference between the $-2 \times \log$ likelihoods of the reduced and full

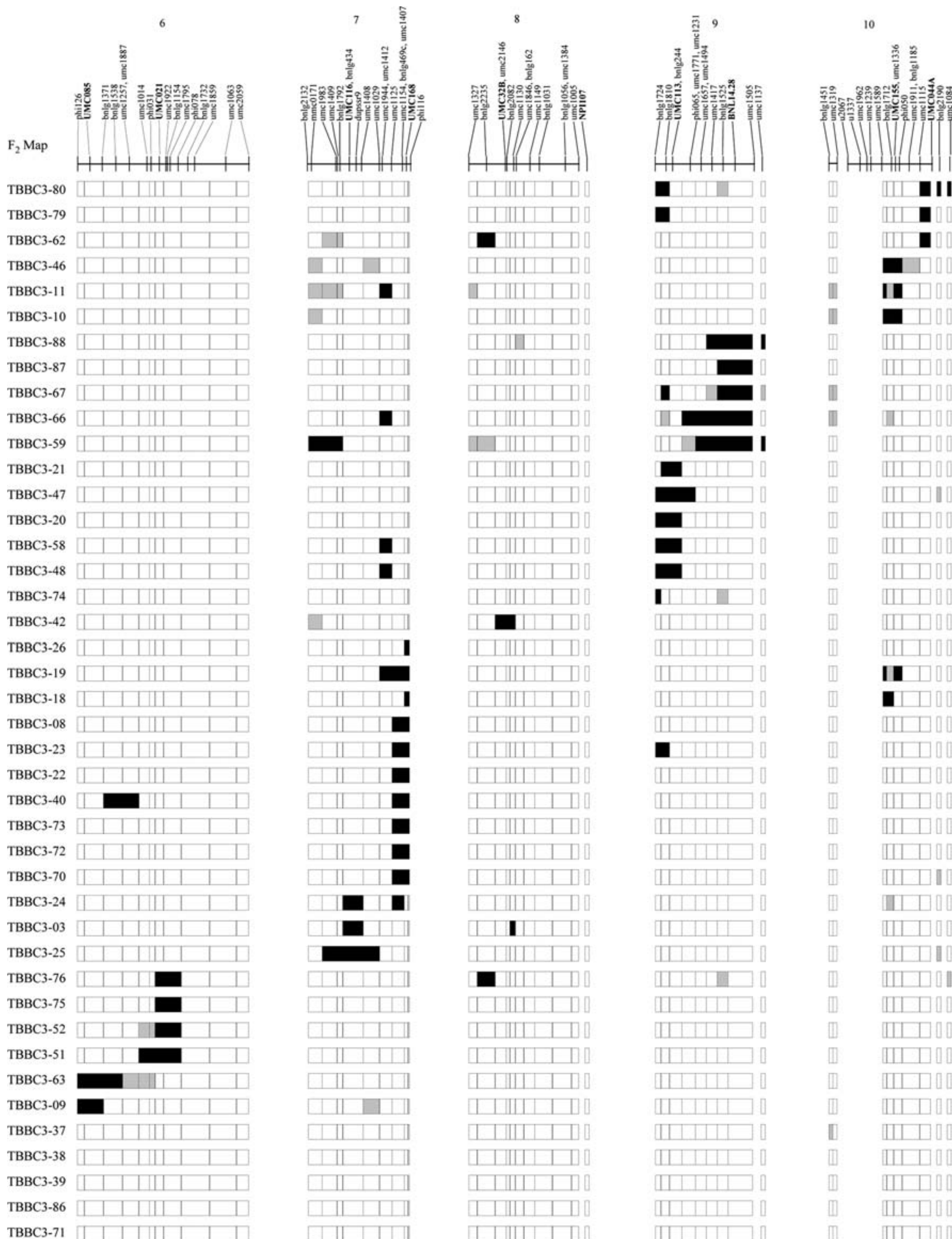


Fig. 2 continued

models. This statistic is approximately distributed as a chi-square with one degree of freedom, and the *P*-value is obtained by dividing the tabular *P*-value in half (Littell et al. 1996).

Effects of each locus in NIL inbreds, whether part of a target introgression or not, were tested by comparing the mean of all NILs homozygous for the Tx303 allele at a marker locus to the mean of inbred B73 using "estimate" statements in PROC MIXED. Similar tests were used to estimate effects in hybrids by comparing the testcross mean of all NILs homozygous for the Tx303 allele at a marker locus to the B73 × Mo17

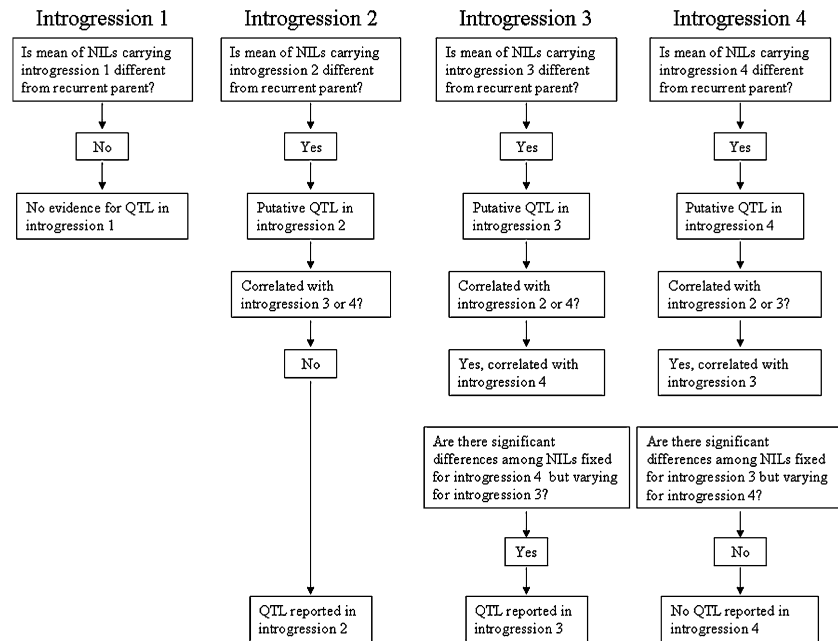


Fig. 3 Example of hypothesis testing flowchart for declaring presence of a QTL in an introgression region. Four introgression regions are described in this example. Introgression 1 does not exhibit a significant effect on the mean of NILs carrying the introgression, so no QTL is reported in this region. Introgression 2 has a significant effect on the mean of NILs carrying the introgression and it is not correlated with other significant introgressions, so it is reported as carrying a QTL in the region. Introgressions 3 and 4 also have significant effects in the initial QTL tests, but they are correlated because at least one NIL carries both introgressions. To determine which of the two regions carries the QTL, tests of pair wise differences among individual NILs that all carry introgression 4 but vary for introgression 3 are made. Differences are observed among NILs fixed for introgression 4 but varying for introgression 3, so a QTL in introgression 3 is reported as the cause of this difference. No significant differences are observed among NILs fixed for introgression 3 but varying for introgression 4, so no QTL is reported for introgression 4

mean. To make QTL tests in the hybrid study comparable to the inbred experiment, the coefficients of means of hybrids of all NIL that were not included in the inbred experiment were set to zero in the “estimate” statements. This was the most efficient analysis, as it permitted inclusion of all testcrosses and check hybrids in the analysis, providing maximum information on replication, block, and error effects, even if they did not provide information about genetic effects. A significance threshold of $\alpha = 0.05$ was used, since only pre-planned comparisons were made.

The procedure to refine QTL locations is outlined in Fig. 3. The first step was to conduct the analysis comparing the mean of all NILs carrying Tx303 alleles at a locus to the mean of B73 (or the B73 testcross, as appropriate), as just described. Next, the pair wise incidence correlation matrix of all significant loci was inspected to identify unlinked groups of loci that were correlated in the NIL set and also linked loci that had low or zero correlations. This allowed identification of independent loci and groups of non-independent loci. Within linked genome blocks, the locus with greatest statistical significance was chosen as the most likely position of a QTL. Finally, any pairs of the remaining loci that were correlated were tested for within-group pair wise differences to determine which locus of a correlated set was most likely linked to a QTL (see below). If this test could not resolve which region carried the QTL, the locus with greatest statistical significance was reported as the most likely QTL position. In some cases, two or more loci were completely correlated in the NIL set, so they were reported as a correlated block of loci to indicate the uncertainty of the QTL position.

Resolving QTL positions required accounting for the correlation between some groups of unlinked loci in the NIL set. For example, in some cases, the initial QTL tests described above suggested that two unlinked genome regions contained putative QTLs, but the two tests were partly confounded in the data set because one or more NILs carried both introgressions. Therefore, we inspected the marker genotype correlation matrix among NILs to identify unlinked pairs of significant loci at which introgressions occurred together in one or more NILs. To test whether there was evidence that both genome regions carried a QTL, the group of NILs that all carried the first introgression but varied at the second introgression were compared

in all pair wise combinations. The test of the null hypothesis of no pair wise differences within this group is a test that no QTL exists in the second introgression region. Similarly, the group of NILs fixed for the second introgression but varying for the first introgression were compared in all pair wise combinations. If the null hypothesis is not rejected for either group (or if the test could not be performed because the Tx303 allele at both loci was represented by a single NIL), this indicates that the two introgression regions are too highly correlated to determine the true QTL position. If the null hypothesis is rejected for both groups, this indicates that both regions carry QTLs. If the null hypothesis is rejected for the first group, but not the second, this is evidence that there is a QTL in the introgression that the second group shares in common, but there is no QTL at the introgression represented by the first group of lines (Fig. 3).

Results

Genomic characterization of germplasm

Among the 89 NILs developed, 80 contain a single introgression at an RFLP locus used in MAS and eight have introgressions at two target RFLP loci. In addition, TBBC3-74 was considered a "control" line, as it did not contain a Tx303 introgression at any RFLP locus originally targeted for introgression, but it does possess Tx303 introgressions at some non-target loci. The set originally contained 90 NILs, but one line, TBBC3-56, was discovered to be contaminated with nonparental alleles, and was dropped from all further analyses and is not included in the 89 NILs discussed here.

Target RFLP loci were located on both arms of every chromosome with the exception of 2S, 3L, 7S, and 8L, and target introgressions account for coverage of 61% of the maize genome, based on genetic distances (Fig. 2). Including both targeted and non-targeted introgressions, 89% of the donor genome is sampled in this NIL set. Different introgression events are represented by different numbers of entries due to the random chance of identifying lines with a given target introgression. The number of NILs representing each target introgression region ranged from one to 15, with an average of about five NILs per introgression (Table 1). Among the 80 NILs with single RFLP introgressions, introgressed Tx303 segment sizes around the target RFLP loci ranged from 10 to 150 cM with an average of 60 cM (Fig. 2, Table 1).

Lines within the NIL set contain an average of 26 cM of genome homozygous for the Tx303 allele and 36 cM of segregating loci outside of the targeted introgression regions (Fig. 2). This represents an average of 63 cM (2.5%) of the donor parent genome recovered outside of the targeted introgression regions due to background introgression (Table 1). The distribution of non-target introgressions ranged from 0 to 15.1% (Table 1). Only three of the NILs (TBBC3-07, TBBC3-26, and TBBC3-75) are truly isogenic for the chromosome region targeted by the RFLP locus used for selection. The proportion of non-target loci with Tx303 alleles in the "control" line was 1.9% (Table 1). These results can be compared to the expectation of 9.4% of loci containing Tx303 alleles at non-target loci in BC_3F_2-derived lines created without selection (3.13% of loci homozygous for Tx303 alleles plus 6.25% of loci segregating). The statistically significant ($P < 0.0001$) reduction in the observed mean proportion of Tx303 alleles across all NILs compared to the expected proportion of non-target Tx303 alleles is a result of MAS for B73 at non-target RFLP loci during NIL development.

The mean proportion of segregating loci for all NILs was 1.8%, and ranged from 0 to 10.5% (Table 1). This was significantly lower than the expectation of 6.25% of loci segregating in BC_3F_2-derived lines created without selection ($P < 0.0001$).

Flowering time QTLs mapped in NIL inbreds

From the combined analysis of variance across environments, significant ($P < 0.05$) genotype effects were detected for DTA and DTS, but not for ASI in NIL inbreds per se. Genotype-by-environment interaction was significant for all three flowering traits ($P < 0.001$ for DTA and DTS, $P < 0.01$ for ASI). More heat and drought stress occurred during the 2002 than the 2001 growing season. Before the median anthesis date for the inbred experiment, 14 days with maximum temperatures greater than 32°C occurred in 2002, compared to only four in 2001, and 56 mm of precipitation fell in 2002, compared to 84 mm in 2001. More irrigation was applied in 2002, but it was not sufficient to compensate for the lower rainfall and higher temperatures, as leaf rolling, an indicator of drought stress, was observed frequently in the 2002 experiment. Because of the large differences in drought stress and strong genotype-by-environment interaction observed across years, QTL analysis was conducted on each environment separately.

Within each environment, significant overall genetic differences ($P < 0.05$) were observed for all three flowering traits. During the 2001 season, Tx303 shed pollen and produced silks 6.6 and 9.1 days later than B73, respectively, with a 2.5 days increase in ASI (Table 2).

Table 2 Parental line means and differences and NIL means and ranges for days to anthesis (DTA), days to silking (DTS), or anthesis-silking interval (ASI), measured in NILs or F_1 hybrids of crosses between NILs and inbred Mo17 evaluated in years 2001 and 2002

	DTA				DTS				ASI			
	2001		2002		2001		2002		2001		2002	
	NIL[a] (days)	HYB[b] (days)	NIL (days)	HYB (days)	NIL (days)	HYB (days)	NIL (days)	HYB (days)	NIL (days)	HYB (days)	NIL (days)	HYB (days)
Parental means and differences												
Tx303	75.2	72.3	72.5	63.2	78.7	73.2	73.6	69.4	3.5	1.0	1.1	6.1
B73	68.7	68.9	70.3	61.4	69.7	70.6	72.1	64.5	1.0	1.7	1.9	3.1
Tx303—B73	6.6***	3.4***	2.0*	1.9*	9.1***	2.6**	1.5*	4.9***	2.5***	−0.7	−0.7	3.0**
NIL means and ranges												
NIL mean	68.9	69.6	71.2	61.3	69.9	71.0	72.8	65.0	1.1	1.4	1.7	3.6
NIL lower range	68.0	67.2	69.2	59.6*	68.6	68.9*	70.7	62.5	0.0**	0.0	−0.2	1.4
NIL upper range	70.5	71.7	76.0*	63.3	72.8	72.7	76.7**	68.6	2.3	2.5	3.4	7.4

[a] Near-isogenic line experiments

[b] Testcross hybrid experiments between NILs and Mo17

*,**,*** Significant difference between parental means or between extreme NIL range and most similar parent at the $P = 0.05$, 0.01, and 0.001 levels, respectively

In 2002, when water availability was limited, the differences in flowering traits between Tx303 and B73 were reduced, as Tx303 shed pollen 2.0 days later and silked 1.5 days later than B73, with no significant difference in ASI (Table 2). The overall mean of all NILs tended to be similar to B73, as expected because of their close genetic relationship to B73 (Table 2). The range of NIL mean values in 2001 tended to be within the range of the two parental lines, except for ASI in 2001, where a single NILs exhibited 0 ASI, significantly less than B73 (Table 2). However, several NILs flowered significantly later than Tx303 in the 2002 environment (Table 2).

Tx303 introgressions that were significantly associated with changes in both DTA and DTS were detected on chromosomes 1L, 3L, 5C, 6S, 7S, and 10S, but only one introgression, on chromosome 3L, was significant for ASI in 2001 (Table 3). Days to anthesis and days to silking were highly correlated, ($r = 0.82$ and $P < 0.001$), so similar results for DTA and DTS were expected. In the 2002 environment, however, the QTL results for DTA and DTS were less similar. Two common QTL regions (on 3L and 5S) were detected for both DTA and DTS, whereas introgressions on 1L, 2L, 3S, 4L affected only DTA and an introgression on 7S affected only DTS. Again, genetic control of ASI appeared largely independent of DTA and DTS, with QTL effects detected on 1L, 4L, 9S, and 10S in 2002.

Even though flowering traits were affected by similar regions within environments, there was little consistency between putative QTL effects estimated in different years. No similarity existed between QTLs for DTS or ASI detected in the two different years, and only two genomic regions, 1.08 and 3.07, affected DTA consistently in both years. Tx303 had lower ASI than B73 in 2002, although the difference was not significant (Table 2). Nevertheless, alleles contributed by Tx303 at all four QTL regions significantly reduced ASI (Table 3).

Flowering time QTLs mapped in NIL testcross hybrids

Significant ($P < 0.05$) genotype effects were detected for all three flowering traits within both years of the hybrid evaluation. Genotype means across years were significantly different for DTA and DTS ($P < 0.001$), and genotype-by-environment interaction was not observed for either of these traits. In contrast, genotype-by-year interaction was significant for ASI ($P < 0.01$), and, consequently there were no significant differences for ASI for the two years combined (results not shown). To be consistent with the inbred analyses, results within environments are presented separately.

The NIL hybrids tended to be within the range of the parents, except that some NIL hybrids shed pollen up to 1.8 days earlier than B73 × Mo17 in 2001 and some silked up to 1.7 days earlier than B73 × Mo17 in 2002 (Table 2). The relative differences between ASI of the parental lines varied substantially between the two years. During the 2001 season when precipitation was adequate, Tx303 × Mo17 shed pollen 3.4 days later and silked 2.6 days later than B73 × Mo17, with no significant difference in ASI (Table 2). In contrast, in the very dry 2002 environment, Tx303 × Mo17 shed pollen only 1.9 days later but silked 4.9 days later than B73 × Mo17, resulting in a highly significant difference between ASI values of 6.1 days for Tx303 × Mo17 and 3.1 for B73 × Mo17 (Table 2). Both hybrids had

Table 3 Most likely positions of QTL affecting days to anthesis (DTA), days to silking (DTS), or anthesis-silking interval (ASI), measured in NILs or F_1 hybrids of crosses between NILs and inbred Mo17 evaluated in years 2001 and 2002

		DTA[c]				DTS[c]				ASI[c]			
		2001		2002		2001		2002		2001		2002	
Chrom. bin[a]	Marker[b]	NIL[d] (days[f])	HYB[e] (days[f])	NIL (days[f])	HYB (days[f])	NIL (days[f])	HYB (days[f])	NIL (days[f])	HYB (days[f])	NIL (days[f])	HYB (days[f])	NIL (days[f])	HYB (days[f])
1.08	*dupssr12*	1.4**	2.8**	2.0*		1.4*	2.1*						
1.11	*umc1862*	0.9*			1.7**								
1.11	*bnlg131*											−1.6*	
2.08	*UMC122*			1.6**									
2.09	*bnlg1520*								1.7*				1.8*
3.01	*UMC032A*			1.9*									
3.06	*bnlg1160*								3.2***				3.2***
3.07	*bnlg197*	1.3***		1.9**		1.8***				0.7**			
3.08	*umc1844*							2.6*					
4.00	*phi072*		2.6**								−1.2*		
4.04	*umc1652*						−1.5*				−1.2**		
4.08	*BNL8.45B*		2.1*										
4.10	*BNL8.23*										−1.4*		
5.00	*mmc0151*								1.6*				1.6*
5.03	*umc1557*			4.0***				3.0**					
5.04	*bnlg1208*				−1.4**								
Completely correlated loci:													
5.04	*umc1221*	1.4**				1.8**							3.0**
5.05	*mmc0081*	1.4**				1.8**							3.0**
5.05	*umc1822*	1.4**				1.8**							3.0**
6.01	*bnlg1538*	1.4**				1.8**							3.0**
5.06	*phi087*												3.0**
5.07	*UMC068*										−1.2*		
6.01	*UMC085*				−1.4*								
7.00	*mmc0171*					1.0*							
7.03	*bnlg434*							1.8*					
7.04	*umc1029*	0.7*											
9.02	*bnlg244*											−1.1*	
9.05	*umc1231*								2.9**				2.8**
9.05	*umc1494*										−1.2*		
Completely correlated loci:													
9.05	*umc1417*						2.9***			2.5**			
9.07	*umc1137*								2.9***				2.5**
10.03	*bnlg1712*											−1.6*	
10.03	*UMC155*	1.6***				1.8**							

Refined QTL positions were determined by accounting for correlations among introgressed segments with significant effects

[a] Bin indicates relative chromosomal position according to the IBM2 2004 Neighbors Map (www.maizegdb.org)

[b] Marker indicates the SSR or RFLP locus at which Tx303 homozygous genotypes were significantly different from B73 homozygous genotypes

[c] Days to anthesis (DTA), days to silking (DTS), and anthesis-silk interval (ASI)

[d] Near-isogenic line experiments

[e] Testcross experiments between NILs and Mo17

[f] Estimates are represented as day differences relative to the maize inbred line, B73, in the case of inbred experiments, or to the hybrid B73 × Mo17 in hybrid experiments

*, **, *** Significant at the $P = 0.05$, 0.01, and 0.001 levels, respectively. No estimates are presented for non-significant comparisons

increased ASI in 2002, however, ASI increased much more in Tx303 × Mo17 than in B73 × Mo17 in 2002.

This difference in environments was reflected in different sets of putative QTLs detected for flowering traits in the hybrids across the 2 years (Table 3). Only one region, in bin 9.05, exhibited significant effects on hybrid flowering time in both years, however, it had opposite effects on ASI in the 2 years. In 2001, the Tx303 introgression in this region was associated with decreased ASI, whereas in 2002 it was associated with increased ASI. Moreover, Tx303 alleles at all QTLs detected for ASI in 2001 (on chromosomes 4S, 5L, and

9L) were associated with decreased ASI in 2001, whereas Tx303 alleles at all QTLs detected for ASI in 2002 (on chromosomes 2L, 3L, 5S, 5L, and 9L) had opposite effects of increasing ASI, reflecting the relative reduction of ASI in Tx303 × Mo17 compared to B73 × Mo17 in 2001 and its increase in 2002 (Table 2). In 2002, there was good correspondence between QTL for ASI and DTS. For example, Tx303 introgressions on chromosomes 2L, 3L, 5S, and 9L in 2002 increased DTS, but did not alter DTA, resulting in significant increases in ASI (Table 3).

Refining QTL estimation

To determine which introgressions were stronger candidates for flowering QTLs, we evaluated the NIL genotypic data to identify significant loci at which introgressions occurred together in at least one NIL, resulting in correlated QTL tests. For example, the region on 5.04–5.05 defined by the SSR loci *bnlg1208-umc1822* and the region containing RFLP marker *umc085* on the short arm of chromosome 6 both had significant effects on DTA in NILs per se in 2001 (Table 3). However, this result alone does not demonstrate that there are two independent QTLs, because the effects of the two regions are partly confounded; line TBBC3-63 carries introgressions in both regions (Fig. 2). In fact, the four loci with greatest statistical significance in each region (*umc1221, mmc0081*, and *umc1822* on 5.04–5.05, and *bnlg1538* on 6.01, Table 3) were completely correlated in the NIL set. That is, each NIL that carried an introgression at *umc1221* on chromosome 5 also by chance carried an introgression at *bnlg1538* on chromosome 6). Therefore, it was not possible to distinguish which region carried the QTL, or if both regions carried QTLs.

In contrast, although markers in chromosomal bins 1.08 and 1.11 had significant effects on both DTA and DTS in NILs per se in 2001 and are on the same chromosome arm, they were independent in the NIL set. No NILs were homozygous for introgressions at both of these positions (Fig. 2). Therefore, we reported two separate QTLs for flowering time in these regions (Table 3).

Discussion

We recommend a combination of analytic approaches to detect QTLs using NILs. The primary approach is to compare the mean effect of all NILs that carry an introgression at the tested locus to the recurrent parent. By combining information across NILs, this test has increased power relative to comparisons of individual NILs to the recurrent parent. Tests at unlinked loci may still be correlated, however, because their introgressions may occur together in common NILs. Therefore, inspection of the matrix of correlations of incidence of introgressions among loci was conducted to identify genome regions with significant effects that were correlated with each other. A test of variation among NILs within each group carrying a common introgressed region tested for the effects of QTL outside of that common region. Therefore, we used this test to judge which of a pair of correlated loci were most likely to be linked to a QTL.

In typical mapping populations, correlations among genome positions are usually accounted for by multiple regression model selection or related techniques (Kao et al. 1999). A similar approach was not possible in this case, because marker loci were not used directly as factors in the analysis model. Instead, lines were used as model factors, and specific comparisons among groups of line means were used to test for QTLs. This had the advantage of permitting full specification of non-genetic effects, including incomplete blocks and covariates, in the model, and using information from check lines and hybrids that were not part of the mapping population to model the non-genetic and error effects. This is the most efficient analysis of the genetic variation, and is compatible with modeling of heterogeneous variances (Gonzalo et al. 2006). The drawback to this approach is that there is no way to directly estimate the effect of one locus after accounting for the effects of other loci, as is needed to efficiently conduct multiple locus model selection (Kao et al. 1999). Instead, we identified dependencies among putative QTL regions and selected independent sets of QTL. This was effective at refining QTL tests, but was time-consuming and not easily automated.

The difficulties introduced by correlations among introgressed segments could be avoided in future populations by developing NIL sets containing only non-overlapping genome introgressions. The NIL set described here has significantly fewer recurrent parent alleles outside of targeted genome regions and less segregation within lines compared to a random set of BC_3F_2-derived lines. Marker-assisted selection for recurrent parent alleles at non-target RFLP aided the recovery of lines with greater genetic similarity to the recurrent parent. However, when the NILs were developed, the number of markers available for genotypic selection was limited (19 RFLP loci in this case), and, consequently, enough non-target introgressions remain in the set to complicate the QTL analysis.

The current set of NILs can serve as the source for developing improved NIL sets by additional backcrossing

and marker-selection. We have performed an additional two generations of backcrossing with many of these lines, in an attempt to recover NILs with only a single introgression each. Using the available SSR genotype data, we are attempting to recover introgressions in as many genome regions as possible, including regions not originally targeted in the BC_3-derived NIL set. Genotyping and selection of these lines is not yet complete.

Substantially improved NIL sets could be developed using current high-throughput genotypic technologies and dense simple sequence repeat (SSR) and single nucleotide polymorphism (SNP) maps available in maize (Sharopova et al. 2002; Vroh Bi et al. 2006). Using modern capabilities, we suggest that substantially larger numbers of marker loci be used to select early generation backcross progeny most similar to the recurrent parent. In addition, four or five backcross generations should be used to decrease the number of progenies that need to be screened to recover NILs (Tanksley and Nelson 1996). Finally, if a mapping population with the two parents of interest already exists, a set of recombinant inbred lines or doubled haploids that collectively represent the donor parent genome, but individually have mostly recurrent parent alleles, can be selected to make the initial backcrosses (Eduardo et al. 2005).

Modern high-throughput DNA marker technologies also offer the opportunity to make introgression libraries that capture more allelic variation than is found in two-parent mapping populations. In maize, allelic diversity among public inbred lines has been well-studied (Liu et al. 2003). This information provides a means to select a subset of inbreds that represent most of the molecular marker variation present in the overall set of lines. This approach was used to select 26 maize inbreds that capture a large proportion of the marker variation to use as parents for developing 25 related RIL populations (Zhao et al. 2006). Each of these populations has B73 as one parent and one of 25 diverse maize inbreds as the other parent. It would be fruitful to develop NIL sets representing introgressions from these same 25 parents into the B73 background to serve as a means to validate QTL identified in the RILs. In addition, such lines would allow rapid identification of useful exotic alleles in a widely adapted genetic background, which should prove useful to broaden the relatively narrow genetic base of commercial US maize (Tallury and Goodman 1999).

Multiple introgressions had significant effects on all three flowering traits, but their effects, particularly on DTS and ASI, were not consistent across years (Table 3). The contrasting levels of drought stress between the two environments may explain this difference; experiments in 2001 received adequate rainfall and irrigation, whereas the 2002 growing season was a drought stress environment. ASI of most lines increased in the drought-stressed 2002 environment compared to the 2001 environment, as expected based on previous reports in maize (Bolaños and Edmeades 1996). Tx303 alleles at most QTLs contributed to later flowering time and greater ASI. The general inconsistency of QTLs across the two environments studied indicates the complexity of flowering time, and suggests that QTLs mapped in any one environment are not likely to provide reliable response to marker-assisted selection.

Chardon et al. (2004) conducted a meta-analysis of 67 maize flowering time QTL mapping studies and found QTLs on all chromosome arms except 4S and 7S, demonstrating the complexity of genetic control of flowering time. Strongest evidence for QTLs was found on chromosomes 1, 8, 9, and 10 (Chardon et al. 2004). We found reliable evidence for QTLs on every chromosome (including arms 4S and 7S) except chromosome 8 (Table 3), and the QTL identified on chromosome 10 near UMC155 maps very close to one of the regions with strongest evidence for flowering time effects in the Chardon et al. (2004) study.

Examination of the results from this study indicates striking differences between QTLs detected in NIL inbreds and NIL topcrosses to Mo17. For example, DTS and ASI of inbreds Tx303 and B73 were more similar in 2002 than in 2001, whereas the opposite was true for the hybrids Tx303 × Mo17 and B73 × Mo17 (Table 2). As a result, at QTLs detected for ASI in inbreds, Tx303 alleles increased ASI in 2001, but decreased ASI in 2002, whereas at QTLs detected for ASI in hybrids, the opposite relationship occurred: Tx303 alleles decreased ASI in 2001 but increased ASI in 2002 (Table 3). This observation suggests caution in interpreting the value of QTLs identified in inbred line mapping studies in maize. The estimates of QTL effects in inbreds may have little or no predictive value for the development of hybrid cultivars.

Analyses of NIL inbreds per se resulted in detection of more QTL regions for DTA and DTS than in the testcross experiment. Some QTLs detected in inbreds may have effects that are masked when crossed to unrelated testers. In our study, Mo17 alleles may have been dominant over both Tx303 and B73 alleles at some loci, eliminating the difference between the comparison of Tx303/Mo17 heterozygotes and B73/Mo17 heterozygotes. Unfortunately, even with genotypic information on Mo17, dominance relationships of the three parental alleles cannot be directly evaluated from our experimental design; to do so would require a mapping

population containing all possible homozygous and heterozygous genotypes in a common (fixed or segregating) genetic background. Overall physiological effects of plant vigor and epistatic interactions between QTLs expressed in inbreds and Mo17 alleles at other loci in hybrids may contribute to differences between effects observed in inbreds and hybrids. Epistatic interactions between the B73 genetic background and the introgression blocks from Tx303 could also result in differences between genetic effects estimated in these NILs and effects that could potentially be estimated by reciprocally transferring homologous regions from B73 into the Tx303 genetic background. Our objective was to identify genome regions from Tx303 that can improve the B73 inbred or B73 × Mo17 hybrid, therefore we did not pursue reciprocal transfers. This limits our inference on genetic effects to comparisons with the B73 genetic background, but for practical purposes, this is precisely the inference of interest.

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