Joint Analysis of Near-Isogenic and Recombinant Inbred Line Populations Yields Precise Positional Estimates for Quantitative Trait Loci

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Abstract

Data generated for initial quantitative trait loci (QTL) mapping using recombinant inbred line (RIL) populations are usually ignored during subsequent fine-mapping using near-isogenic lines (NILs). Combining both datasets would increase the number of recombination events sampled and generate better position and effect estimates. Previously, several QTL for resistance to southern leaf blight of maize were mapped in two RIL populations, each independently derived from a cross between the lines B73 and Mo17. In each case the largest QTL was in bin 3.04. Here, two NIL pairs differing for this QTL were derived and used to create two distinct $F_{2:3}$ family populations that were assessed for southern leaf blight (SLB) resistance. By accounting for segregation of the other QTL in the original RIL data, we were able to combine these data with the new genotypic and phenotypic data from the $F_{2:3}$ families. Joint analysis yielded a narrower QTL support interval compared to that derived from analysis of any one of the data sets alone, resulting in the localization of the QTL to a less than 0.5 cM interval. Candidate genes identified within this interval are discussed. This methodology allows combined QTL analysis in which data from RIL populations is combined with data derived from NIL populations segregating for the same pair of alleles. It improves mapping resolution over the conventional approach with virtually no additional resources. Because data sets of this type are commonly produced, this approach is likely to prove widely applicable.

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THE UTILITY of quantitative trait locus (QTL) mapping to identify specific genes affecting complex traits is limited by a lack of precision of QTL position estimates and biased estimates of their effects (Holland, 2007). Increasing the number of lines sampled, the number of markers genotyped, or number of replications grown will reduce these problems (Beavis, 1998). In addition, a difficulty of QTL analysis is the simultaneous segregation of multiple QTL within a test population, resulting in reduced detection power, and inflated effect estimates of those QTL detected, a problem that becomes severe in small population samples (Beavis, 1998; Schon et al., 2004).

To study more precisely the effect and position of a specific QTL, a uniform genetic background, differing only for the target QTL, should be constructed to eliminate all other sources of genetic variation. Near-isogenic lines (NILs), pairs of lines that are identical except for a single genomic segment, are ideal for this purpose (Szalma et al., 2007; Tanksley, 1993). Near-isogenic lines can be derived through repeated backcrossing to a recurrent parent. Molecular

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Abbreviations: AUDPC, area under disease progress curve; BLUP, best linear unbiased predictor; CTAB, cetyltrimethylammonium bromide; DTA, days to anthesis; HIF, heterogenous inbred family; HR, hypersensitive response; HST, host-selective toxin; IcM, IBM centiMorgan, IBM, intermated B73 × Mo17; LOD, log of odds; LRR, leucine-rich repeat; NBS, nucleotide binding site; NIL, near-isogenic line; PCR, polymerase chain reaction; QTL, quantitative trait locus/loci; RIL, recombinant inbred line; SLB, southern leaf blight; SNP, single nucleotide polymorphism; SSR, simple sequence repeat; STWMD, standardized weighted mean disease; TBE, Tris-Borate-EDTA; WMD, weighted mean disease.

markers are used to select lines with the donor parent allele at the QTL and to maximize contribution of the recurrent parent background outside of the QTL region (Paterson et al., 1990; Szalma et al., 2007; Tanksley, 1993). "Mendelizing" QTL by isolating their differences from background genomic segregation in NILs has been used as a key step in identifying causal sequence variation at some QTL (e.g., Frary et al., 2000; Salvi et al., 2007).

Another option used to derive NILs is identification of heterogeneous inbred families (HIFs) from recombinant inbred line (RIL) populations. Although the majority of the genetic background in a RIL is fixed, a small percentage of loci will be segregating. A RIL that is heterozygous at the QTL can be selfed and a set of HIFs, lines differing for alleles at that QTL with an otherwise homogeneous genetic background, can be developed (Tuinstra et al., 1997).

Near-isogenic lines are typically constructed after the target QTL have been identified in initial mapping experiments. This initial data for lines segregating at the QTL of interest and throughout the genome are usually ignored once NILs have been developed. Here, we demonstrate a method in which data from initial RIL studies is combined with new data collected from NIL experiments, which increases the number of recombination events sampled in the QTL region. We demonstrate that this leads to greater precision of position and effect estimates than if the NIL data alone had been used.

Southern leaf blight (SLB), a foliar disease of maize caused by the necrotrophic fungus *Cochliobolus heterostrophus*, is controlled partially by host quantitative resistance (Carson and White, 1999). Several QTL have been mapped that confer resistance to this disease in biparental segregating populations that do not segregate for major resistance genes, including the intermated B73 × Mo17 (IBM) population (Lee et al., 2002). The largest effect QTL identified in the IBM lies in bin 3.04 (Davis et al., 1999) between 163.7 and 165.9 IBM cM (IcM; Balint-Kurti et al., 2007) units on the chromosome 3 IBM map (Schaeffer et al., 2008). This QTL has also been detected in several other studies; however, its existence has not been validated or fine-mapped in a homogenized genetic background (Balint-Kurti et al., 2006, 2007, 2008; Jiang et al., 1999; Zwonitzer et al., 2009). Near-isogenic line pairs were constructed and crossed to obtain two distinct populations of $F_{2:3}$ families segregating for this QTL. The objectives of this research were to validate the 3.04 QTL and estimate its effects in uniform genetic backgrounds and to develop a method to analyze combined data from these $F_{2:3}$ families with two previously characterized B73 × Mo17 populations in attempt to more precisely position the QTL.

MATERIALS AND METHODS

Derivation of Populations Studied

A set of 204 B73 × Mo17 $F_{6:7}$ RILs were derived by C. Stuber and colleagues as described in Carson et al. (2004) and are here referred to as the Stuber RILs. The IBM population is a set of 302 advanced intercross lines derived from the cross between parental inbreds B73 and Mo17. In this population four cycles of intermating were conducted at the F_2 stage before the derivation of lines. This increased the number of recombination events captured within the population that, in turn, increased mapping resolution (Lee et al., 2002; Balint-Kurti et al., 2007). A highly significant southern leaf blight resistance QTL was detected in bin 3.04 in both Stuber RIL and IBM populations (Balint-Kurti et al., 2007; Carson et al., 2004). Based on this result, populations segregating only for the 3.04 region were created for validation and fine mapping studies.

A set of families segregating for the Mo17 resistance allele at bin 3.04 in an otherwise mostly homozygous B73 background was created. After the initial cross of B73 to Mo17, progeny underwent five cycles of marker assisted backcrossing. At each backcross generation, offspring heterozygous at the 3.04 region were selected via genotyping with the following simple sequence repeat (SSR) markers flanking the IBM 3.04 SLB resistance QTL support interval (Balint-Kurti et al., 2007; umc1886, bnlg1447, umc1030, and umc1495; http://www.maizegdb.org [verified 25 May 2010]). Outside of the 3.04 region, these lines are expected to be 98.4% homozygous for B73 alleles. BC_5F_1 lines were selfed to create the BC_5F_2 generation. Six BC_5F_2 heterozygous lines, 863-8, 874-1, 878-5, 880-4, 895-1, and 895-8, were selected and selfed to create six subpopulations of $BC_5F_{2:3}$ lines segregating for the 3.04 QTL. This set of 209 lines is referred to here as the B73 NIL $F_{2:3}$'s.

A separate mapping population was also developed by intercrossing a HIF pair polymorphic at the QTL. One of the original Stuber RILs, segregating at the 3.04 QTL, was identified by genotyping at markers asg48 and phi036 (http://www.maizegdb.org [verified 25 May 2010]). This line was selfed and progeny were genotyped at four SSR markers flanking the IBM 3.04 SLB resistance QTL support interval (umc1886, bnlg1447, umc1030, bnlg1452; http://www.maizegdb.org [verified 25 May 2010]) to assess fixation for either the B73 or Mo17 allele. Two sublines, 844-1 and 844-6, were identified as homozygous for the Mo17 and B73 alleles, respectively, in the target region. Lines 844-1 and 844-6 were crossed, and the F_1 was selfed to the $F_{2:3}$ generation. This set of 144 $F_{2:3}$ families are referred to here as the Stuber NIL $F_{2:3}$'s.

Phenotyping for Southern Leaf Blight Resistance

Details of phenotyping the Stuber RIL and IBM populations are presented in Carson et al. (2004) and Balint-Kurti et al. (2007). Two experiments encompassing the set of 209 B73 and 144 Stuber NIL $F_{2:3}$'s were grown in Clayton, NC, during the summer of 2008. Lines were randomized into incomplete blocks of ten plants in an α lattice design with three complete replications using the software Alphagen (Scottish Agricultural Statistics Service, Edinburgh, UK). In the Stuber NIL $F_{2:3}$'s experiment, B73, Mo17, P39, and parental sublines 844-1 and

844-6 were included once in each replication as checks. In the B73 NIL $F_{2:3}$'s experiment, B73, Mo17, and P39 were included three times in each replication as checks. Ten seeds were planted per 2.44-m row, with 0.96 m between rows.

All plants were inoculated with the 2-16Bm isolate of *C. heterostrophus* at the six to eight leaf stage as described in Carson, (1998). Irrigation was immediately applied after inoculation to foster fungal growth. *Cochliobolus heterostrophus* is endemic to North Carolina and, as such, inoculum most likely constituted a mixed isolate population. Plants were rated for symptoms of SLB once per week for 4 wk starting approximately 1 mo after inoculation. A 1 to 9 scale was used, with 1 denoting a symptomless plant and 9 denoting a dead plant (Balint-Kurti et al., 2006). Values were recorded in half unit increments. Days to anthesis (DTA) were measured on each plot as the number of days from planting to 50% pollen shed.

Deriving Best Linear Unbiased Predictors for Marker-Association Tests

In the original analysis of the Stuber RIL population, Carson et al. (2004) calculated AUDPC (area under the disease progress curve) from raw percent disease severity data taken three times during 1995 and two times during 1996; these AUDPC values were used for QTL mapping. Raw data were used in calculating AUDPC values due to small replication effects and highly significant entry effects ($p < 0.0001$) (Carson et al., 2004). Intermated B73 × Mo17 lines were rated three times during 2005 and four times during 2006 on the 1 to 9 scale. These ratings were used to calculate a weighted mean disease (WMD) score, a weighted AUDPC value (Wilcoxson et al., 1974). Least square means of these WMD scores were estimated and used for QTL mapping (Balint-Kurti et al., 2007).

Data for the two segregating near-isogenic populations were analyzed with a multivariate mixed model that treated the four disease scores taken on different dates on each plot as dependent variables using ASREML (Gilmour et al. 2002). Random effects in the model were included for replications, blocks within replications, and entries. Separate error variances and covariances were fit for each disease score. Diagonal variance–covariance matrices were modeled for replication and blocking effects, allowing each disease score to have separate variances due to effects for replications and blocking but constraining covariances to zero to obtain model convergence. In addition, an unstructured variance–covariance matrix was modeled for entry effects, permitting each disease score to have a unique genetic variance and each pair of rating dates to have a unique genetic covariance.

Southern leaf blight symptoms increase most rapidly during and after flowering; consequently, earlier-flowering plants generally exhibit disease sooner than later-flowering plants and are scored higher. Days to anthesis was included as a cofactor in the analysis to minimize confounding between maturity effects and disease resistance. Because DTA and SLB score do not follow a perfectly linear relationship, a multiple regression analysis was performed using Proc Mixed in SAS software version 9.1.3 (SAS Institute, Inc., 2004). Southern leaf blight scores from the first through fourth weeks of evaluation were modeled as linear, quadratic, cubic, and quartic polynomials, and the Akaike Information Criterion (AIC) was used to select the best model (Supplementary Table S1). The quadratic plus linear polynomial model best accounted for the relationship between disease score and DTA. Therefore, linear plus quadratic fixed covariates for DTA were included in the SLB analysis model across all scoring dates. Predictions were made for each entry at each of the four scoring dates. A best linear unbiased predictor-weighted mean disease (BLUP-WMD) index was calculated for each entry through use of the following formula:

$$\begin{aligned}\text{WMD} = \{&[(BLUP1 + BLUP2)/2]d_{1,2} \\ &+ [(BLUP2 + BLUP3)/2]d_{2,3} \\ &+ [(BLUP3 + BLUP4)/2]d_{3,4}\} \\ &/(d_{1,2} + d_{2,3} + d_{3,4}),\end{aligned}$$

in which *BLUPn* refers to the best linear unbiased predictor (BLUP) for the SLB score of the entry in the *n*th week of evaluation and $d_{n,n+1}$ is the number of days that elapsed between scores in consecutive weeks.

To minimize differences in phenotype values across different populations due to different scoring methods and different environments, BLUP-WMD values for the two near-isogenic populations were standardized by subtracting the population mean from the BLUP-WMD value and dividing by the population standard deviation (Walling et al., 2000). The resulting value is referred to as standardized weighted mean disease (STWMD).

Heritability of family means for BLUP-WMD was estimated for an index in which BLUPs were weighted according to their relative contributions in the BLUP-WMD calculation. These index weights were 1/6, 1/3, 1/3, and 1/6 for BLUP1, BLUP2, BLUP3, and BLUP4, respectively. The following formula was used to calculate heritability:

$$h^2 = b'Gb/b'Pb,$$

in which b is the vector of index coefficients and G and P are the genetic and phenotypic variance–covariance matrices (Lin and Allaire, 1977). The phenotypic variance–covariance matrix was derived by summing the genetic variance covariance matrix and the residual variance–covariance matrix and dividing by the harmonic mean of the number of replications in which entries were scored (Holland et al., 2003). Family mean heritabilities were also calculated for individual scores by dividing the genotypic by phenotypic variance for that scoring date. Student's t tests were used to assess significance of BLUP-WMD differences between parent and inbred checks.

For the joint analysis, data from both NIL populations plus the Stuber RIL and IBM populations were combined. To estimate the effects of genome positions specifically within the 3.04 QTL, data from the RIL

and IBM populations were adjusted for the effects of QTL outside the 3.04 QTL interval. Southern leaf blight AUDPC or WMD values for each RIL from Balint-Kurti et al. (2007) and Carson et al. (2004) were used but adjusted according to their genotypes at the QTL detected in these populations. Adjusted RIL phenotype values from each population were derived by fitting a multiple interval mapping model (MIM) in Windows QTL Cartographer version 2.5 (Wang et al., 2007) with the QTL that had been previously detected in these populations. For a population containing *n* QTL, a line's adjusted phenotypic value, *p*, was estimated with the following equation:

$$p = \mu + \sum_{i=1}^{n} Q_i \alpha_i + \varepsilon,$$

in which μ is the population mean, Q_i is a QTL genotype indicator variable that takes on the value of 0 if the genotype at the *i*th QTL is homozygous for the B73-derived allele and 1 if the genotype at the *i*th QTL is homozygous for the Mo17-derived allele, α_i is the effect of the homozygous Mo17 genotype in reference to the B73 genotype at the *i*th QTL, and ε is a residual term including error and all nongenetic variation. The adjusted genotype values should reflect as little of the segregating variation as possible other than that from segregation of the 3.04 QTL, so all of the $Q_i\alpha_i$'s besides the one for the 3.04 QTL were included in the adjustment equation. Separate equations were modeled for the Stuber RIL and IBM populations, based on the QTL mapped in those populations individually.

Because the QTL genotypes are unknown, the genotype at the closest flanking marker was used as an approximation. In the case of missing data, the probability that a RIL was homozygous for the Mo17 allele at the QTL was estimated according to the genotypes at the nearest flanking markers for which data were available and the genetic distance between these markers (Lander and Botstein, 1989). This probability was multiplied by the Mo17 allele effects at the QTL and used in the summation portion of the phenotypic adjustment equation. Finally, these adjusted values were standardized by subtracting the population mean from the Stuber RIL AUDPC or IBM WMD value and dividing by the population standard deviation. The resulting value on this standardized scale is referred to as STWMD.

DNA Extraction and Genotyping

Intermated B73 × Mo17 line genotypes were obtained from MaizeGDB (http://www.maizegdb.org/ibm302scores.html [verified 26 Oct. 2010]). Duplicate sets of tissue of the B73 and Stuber NIL F_2 plants were collected from the winter nursery in Homestead, FL. One set of tissue was sent to DuPont Crop Genetics Research for preliminary genotyping in the 3.04 region at the proprietary single nucleotide polymorphism (SNP) loci PHM8477, PHM12576, and PHM4145. Additional background screening at markers flanking highly significant QTL conditioning resistance to SLB in the IBM population (bins 1.10, 3.04, and 8.02–8.03; Balint-Kurti et al. 2007) was also undertaken with proprietary SNP markers (PHM5586, PHM11071, PHM16795, PHM14098, PHM13823, PHM6836, PHM12861, PHM13725, PHM5158, and PHM6523). DNA was extracted according to a modified cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle, 1987) and SNP data were generated using a polymerase chain reaction (PCR) and probe-based technology at Pioneer Hi-Bred International.

A duplicate set of leaf tissue of the B73 and Stuber NIL F_2 plants was extracted using the CTAB protocol (Doyle and Doyle, 1987) with the addition of 1.0% PVP w/v to the CTAB buffer (John, 1992). Tissue was extracted in sets of eight 1.1-mL strip tubes arranged in a 96 well format. A #4 stainless steel shot (washed with 1x TE [Tris-EDTA] buffer) was added to each well, and plates were suspended in liquid nitrogen. Grinding was performed using a Retsch Mixer Mill MM301 (Retsch GmbH & Co., Haan, Germany).

F_2 plants were genotyped with the following SSRs in the 3.04 QTL region: umc1772, umc1425, umc2000, umc2158, umc1495, and umc1392 (http://www.maizegdb.org [verified 25 May 2010]). These six SSR markers had already been genotyped in the IBM populations but not in the Stuber RIL population. Therefore, tissue of the Stuber RILs was obtained from seedlings germinated in greenhouse pots, extracted according to the protocol referenced above, and genotyped at these six SSR loci. Genomic DNA was diluted 1:5 with sterilized distilled water. For each 17.5-μL PCR reaction, 5 μL of diluted DNA was added to 1 μL each of.25 μM forward and reverse primer, 1.9 μL of 15 mM $MgCl_2$, 0.6 μL of dNTPs (with dATP, dCTP, dGTP, and dTTP at.25 mM), 3.78 μL of 5 M betaine, 1.82 μL of 1:2.04% (v/v) cresol red:glycerol, 1.9 μL of Tris-KCl buffer (.5 M Tris [pH = 8.4], 1 M KCl), and 1.5 U of Taq polymerase. Polymerase chain reactions were performed on the 384-well Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) according to the following protocol: 94°C for 4 min, 50 cycles of 94°C for 25 s, 55°C for 25 s, and 72°C for 35 s, followed by 72°C for 5 min. Fifteen microliters of each PCR product was loaded on to a 4.0% super fine resolution (SFR) Tris-Borate-EDTA (TBE) agarose gel and electrophoresed at 130 V in TBE buffer for 2 h on the Life Technologies Gibco BRL Sunrise 96 Gel Electrophoresis Apparatus (Life Technologies, Carlsbad, CA). Genotyping data indicating a double recombination in a small interval or manifesting inconsistencies with the preliminary SNP genotyping were discarded.

Single Marker Regression Analyses – Single Population and Combined Analyses

For use in regression analysis, genotype data for all of the populations were converted to indicator variables for additive and dominance effects according to the following scheme:

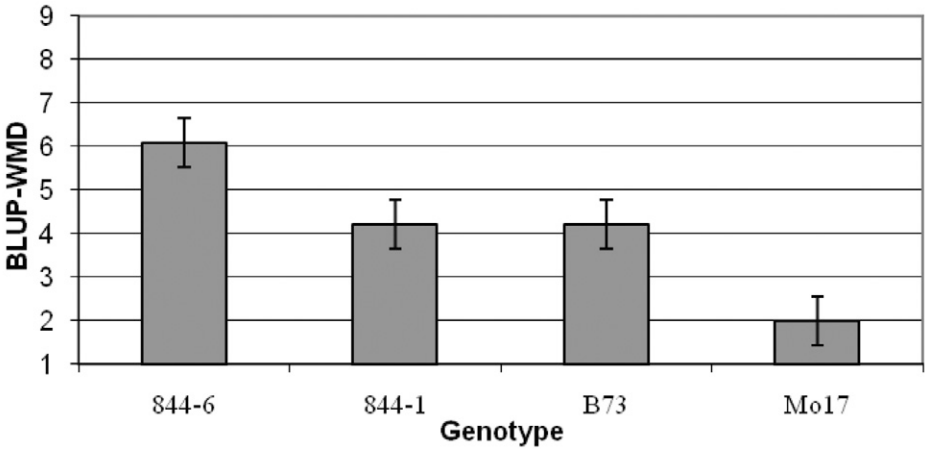

Figure 1. Best linear unbiased predictor weighted mean disease (BLUP-WMD) values for B73, Mo17, and Stuber near-isogenic line (NIL) $F_{2:3}$ population parents 844-1 and 844-6. Error bars reflect the standard error of the difference of means.

$$a = \begin{cases} 0, \text{ if homozygous for the B73 allele} \\ 1, \text{ if heterozygous} \\ 2, \text{ if homozygous for the Mo17 allele} \end{cases}$$

$$d = \begin{cases} 0, \text{ if homozygous for the B73 allele} \\ 2, \text{ if heterozygous} \\ 0, \text{ if homozygous for the Mo17 allele} \end{cases}$$

Missing marker data were imputed if immediately adjacent flanking marker data were available. Marker order and positions were assumed to be equivalent to the IBM2008 Neighbors Map (Schaeffer et al., 2008). Single nucleotide polymorphism marker positions were provided by DuPont Crop Genetics and validated by prior genetic and physical mapping. Quantitative trait loci regression tests were conducted at each marker position and at the midpoint of each marker interval (by using average values of flanking marker *a* and *d* coefficients for each line). Proc GLM of SAS software v. 9.1.3 (SAS Institute, Inc., 2004) was used to model STWMD as a function of the additive and dominance variables at both markers and virtual markers. The log of odds (LOD) score at each test position was calculated according to Haley et al. (1994):

$$\text{LOD} = n \ln (SSE_{reduced}/SSE_{full})/2 \ln 10,$$

in which SSE_{full} is the error sum of squares for the full model in which a marker is fit and $SSE_{reduced}$ is the error sum of squares for the reduced model in which no marker is fit. The location of the QTL was taken to be the marker or virtual marker at which the highest LOD score occurred. 2-LOD support intervals were identified as the smallest interval between two positions surrounding the LOD peak for which the LOD score dropped at least two points below the interval maximum (van Ooijen, 1992). Because LOD scores are affected by the number of observations, only lines with a full complement of marker data, either by genotyping or imputation, were included in LOD score calculations. The additive effect was estimated as the partial regression coefficient of *a*.

A combined data set consisting of the data from the B73 NIL $F_{2:3}$, Stuber NIL $F_{2:3}$, IBM, and Stuber RIL populations was constructed and analyzed with this same method. To more precisely estimate the ends of the 2-LOD support interval for the QTL in the combined analysis, interval mapping was also conducted at positions spaced 0.1 IcM apart within each interval defining the support interval. Interval mapping was performed using the same regression model as single marker analysis, but coefficients of *a* and *d* at each position were obtained from a weighted average of the flanking marker coefficients:

$$[(1 - r_{LQ})c_L + r_{LQ}c_R]/r,$$

in which r_{LQ} is the map distance in IcM between the left flanking marker and the interval test position, c_L is the respective coefficient at the left flanking marker, c_R is the coefficient at the right flanking marker, and *r* is the total map distance of the interval.

Searching for Candidate Genes

The B73 genome sequence (http://www.maizesequence.org [verified 26 Oct. 2010]) between the two markers flanking the support interval was examined. CLUSTALW (http://www.ebi.ac.uk/Tools/clustalw2 [verified 26 Oct. 2010]) was used to align the amino acid sequences of the three candidate genes identified (Larkin et al., 2007). BLAST searches of these three candidate genes against proteins in the nonredundant National Center for Biotechnology Information (NCBI) database were also performed (http://blast.ncbi.nlm.nih.gov [verified 10 Nov. 2010]).

RESULTS

Variation among entries was significant for both B73 NIL $F_{2:3}$ and Stuber NIL $F_{2:3}$ populations (Supplementary Tables S2 and S3). 844-1 and 844-6, the Stuber NIL $F_{2:3}$ parental lines, had BLUP-WMD values of 4.2 and 6.1 and were significantly different ($p < 0.0001$) from one another. B73 (BLUP-WMD = 4.2) had the same level of resistance

Table 1. Results from single marker regression analyses of adjusted southern leaf blight phenotypic values on marker genotypes. Positions of markers are given in intermated B73 × Mo17 (IBM) cM units. For each marker tested, the R^2 value, additive effect (a), and dominance effect (d) are given. Values are in terms of the standardized scores calculated as number of standard deviations from the mean (see Materials and Methods). Negative additive effects indicate that the Mo17 allele increased resistance. Positive dominance effects indicate that heterozygotes have lower resistance than the midparent value. p values associated with a and d are given in parentheses. Empty cells indicate unavailable marker information.

Marker	Position	B73 NIL† $F_{2:3}$'s	Stuber NIL $F_{2:3}$'s	IBM‡	Stuber RILs§	All populations combined
umc1772	163.5	R^2 = 0.52 a = −0.89 (<0.0001) d = 1.36 (<0.0001)	R^2 = 0.38 a = −0.78 (<0.0001) d = 0.60 (0.0487)	R^2 = 0.19 a = −0.44 (<0.0001)	R^2 = 0.060 a = −0.23 (0.0093) d = 0.61 (0.2268)	R^2 = 0.21 a = −0.48 (<0.0001) d = 0.66 (<0.0001)
PHM12576	165	R^2 = 0.58 a = −0.96 (<0.0001) d = 1.30 (<0.0001)	R^2 = 0.47 a = −0.87 (<0.0001) d = 0.76 (0.0123)			R^2 = 0.25 a = −0.54 (<0.0001) d = 0.74 (<0.0001)
umc1425	165	R^2 = 0.55 a = −0.93 (<0.0001) d = 1.32 (<0.0001)	R^2 = 0.41 a = −0.82 (<0.0001) d = 0.58 (0.0388)	R^2 = 0.22 a = −0.49 (<0.0001)	R^2 = 0.10 a = −0.32 (0.0003) d = 0.46 (0.0944)	R^2 = 0.23 a = −0.53 (<0.0001) d = 0.70 (<0.0001)
umc2000	166	R^2 = 0.55 a = −0.95 (<0.0001) d = 1.24 (<0.0001)	R^2 = 0.37 a = −0.77 (<0.0001) d = 0.66 (0.0214)	R^2 = 0.20 a = −0.44 (<0.0001)	R^2 = 0.084 a = −0.28 (0.0021) d = 0.65 (0.1925)	R^2 = 0.22 a = −0.50 (<0.0001) d = 0.70 (<0.0001)
umc2158	176.6	R^2 = 0.50 a = −0.90(<0.0001) d = 1.12 (<0.0001)	R^2 = 0.36 a = −0.76 (<0.0001) d = 0.72 (0.0144)	R^2 = 0.083 a = −0.30 (<0.0001)	R^2 = 0.047 a = −0.19 (0.0377) d = 0.90 (0.1242)	R^2 = 0.16 a = −0.41 (<0.0001) d = 0.68 (<0.0001)
umc1392	181.1	R^2 = 0.50 a = −0.90 (<0.0001) d = 1.12 (<0.0001)	R^2 = 0.35 a = −0.75 (<0.0001) d = 0.68 (0.0170)	R^2 = 0.068 a = −0.26 (<0.0001)	R^2 = 0.044 a = −0.19 (0.0385) d = 0.44 (0.1797)	R^2 = 0.14 a = −0.39 (<0.0001) d = 0.68 (<0.0001)
PHM4145	181.7	R^2 = 0.50 a = −0.93 (<0.0001) d = 1.10 (<0.0001)	R^2 = 0.30 a = −0.67 (<0.0001) d = 0.70 (0.0223)			
PHM14098	190.8	R^2 = 0.28 a = −0.67 (<0.0001) d = 1.32 (<0.0001)	R^2 = 0.18 a = −0.59 (<0.0001) d = 0.14 (0.6704)			
PHM13823	213.6	R^2 = 0.068 a = −0.37 (0.0032) d = 1.34 (0.0016)	R^2 = 0.24 a = 0.76 (<0.0001) d = 0.26 (0.5821)			
PHM6836	260.1	R^2 = 0.015 a = −0.16 (0.2081) d = 0.64 (0.1180)	R^2 = 0.30 a = 0.83 (<0.0001) d = 0.08 (0.8649)			
PHM12861	305.8	R^2 = 0.018 a = −0.20 (0.3361) d = −0.96 (0.3687)	R^2 = 0.021 a = 0.02 (0.8664) d = −0.56 (0.1339)			

†NIL, near-isogenic line.

‡IBM, intermated B73 × Mo17.

§RIL, recombinant inbred line.

as 844-1. 844-1, 844-6, and B73 were all significantly different ($p < 0.0001$) from Mo17 (BLUP-WMD = 2.0; Fig. 1).

Heritability of BLUP-WMD was estimated to be 89.4% in the B73 NIL $F_{2:3}$ experiment and 86.6% in the Stuber NIL $F_{2:3}$ experiment. For individual scoring dates, heritability estimates were 81.3, 82.5, 76.0, and 82.9% for the first through fourth weeks, respectively, in the B73 NIL $F_{2:3}$ population. For the Stuber NIL $F_{2:3}$ population, these individual scoring date heritability estimates were 76.8, 79.0, 82.0, and 84.4%.

In the single population marker association analyses, the B73 NIL $F_{2:3}$ population exhibited a peak R^2 value (R^2 = 0.58) at PHM12576 (165 IcM); however, its peak LOD score (26.6) occurred at umc2000 (166.9 IcM) (Tables 1 and 2; Fig. 2). This inconsistency is most likely due to the omission of lines with missing marker data from the LOD calculation. At this QTL, the estimated additive effect was $a = -0.96$ ($p < 0.0001$) and the dominance effect was $d = 1.3$ on the standardized scale ($p < 0.0001$; Table 1). On the original 1 to 9 scale, the additive effect was $a = -0.38$ and the dominance effect was $d = 0.52$. Thus, at the bin 3.04 SLB resistance QTL, the Mo17 homozygote is on average 0.76 points more resistant than the B73 homozygote. The significant dominance effect

Table 2. Log of odds (LOD) scores for both real and virtual markers in both combined and single population analyses. Virtual markers are located at the midpoints of adjacent real markers and at 0.1 cM intervals for the purpose of defining support intervals.

Marker	Position	All populations combined	B73 NIL† $F_{2:3}$'s‡	Stuber NIL $F_{2:3}$'s‡	IBM§	Stuber RILs¶
umc1772	163.5	37.5	24.6#	14.1#	13.5	2.0
umc1772/PHM12576 midpoint	164.25	42.3#	25.8#	14.5#	15.5#	2.9#
PHM12576	165	44.4#††	25.9#	14.7#††	16.4#††	3.6#††
PHM12576/umc1425 midpoint	165	44.4#	25.9#	14.7#	16.4#††	3.6#††
umc1425	165	44.4#	25.8#	14.7#	16.4#††	3.6#††
umc1425/umc2000 midpoint	165.95	42.9#	26.3#	13.6#	16.1#	3.1#
umc1425/umc2000 interval position	166.1	42.4#				
umc2000	166.9	38.7	26.6#††	12.3	14.5#	2.3#
umc2000/umc2158 midpoint	171.75	35.9	26.2#	13.0	10.7	2.7
umc2158	176.6	27.8	23.3	13.1	5.5	2.8
umc2158/umc1495 midpoint	177	27.8	23.3	13.1	5.4	2.9
umc1495	177.4	26.8	23.3	13.1	5.2	2.6
umc1495/umc1392 midpoint	179.25	26.8	23.3	12.8	5.4	2.1
umc1392	181.1	24.0	23.1	12.4	5.2	1.3

†NIL, near-isogenic line.

‡The 2-LOD (log of odds) interval in this population extends past the markers tested.

§Intermated B73 × Mo17.

¶RIL, recombinant inbred line.

#Marker positions that fall within the 2-LOD interval calculated for the population.

††Maximum LOD score for population.

indicates that heterozygotes for the QTL are less resistant than the average of the B73 and Mo17 homozygotes. In other words, the Mo17 SLB resistance allele in bin 3.04 is largely recessive to the B73-derived susceptibility allele.

The Stuber NIL $F_{2:3}$ population similarly exhibited a peak R^2 value (R^2 = 0.47) at PHM12576 (165 IcM; Table 1). Neither the B73 nor the Stuber NIL $F_{2:3}$ populations was segregating for the four markers tested in bins 1.10 or 8.02 through 8.03; however, the Stuber NIL F_2 plants, as well as two of six B73 NIL BC_5F_2 lines, were segregating in the region of a second, smaller effect SLB resistance QTL in bin 3.04 that had been previously reported in the IBM population (Fig. 3). This second QTL was identified in the Stuber NIL $F_{2:3}$ population, where single marker analysis revealed a local maximum R^2 value at 260 IcM, 94 IcM from the main 3.04 QTL, with B73 contributing the more resistant allele. Although both $F_{2:3}$ populations were derived from near-isogenic parents, a smaller proportion of the phenotypic variation in the Stuber NIL $F_{2:3}$ population was explained by PHM12576 because of segregation at this additional bin 3.04 QTL. The peak LOD score for the major 3.04 QTL in the Stuber NIL $F_{2:3}$ population (14.7) also occurred at PHM12576 (Table 2; Fig. 2), consistent with the position for the maximum R^2 value. For this population, the additive effect of the major QTL was estimated as −0.87 and the dominance effect

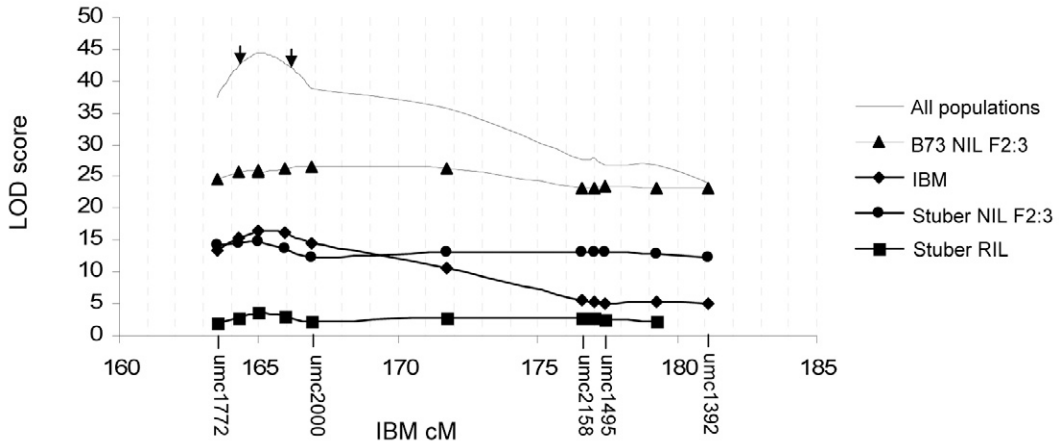

Figure 2. Log of odds (LOD) profiles of the four populations studied over the major southern leaf blight (SLB) resistance quantitative trait loci (QTL) in bin 3.04. The endpoints of the 2-LOD support interval for the joint analysis are indicated by arrows. IBM, intermated B73 × Mo17; NIL, near-isogenic line; RIL, recombinant inbred line.

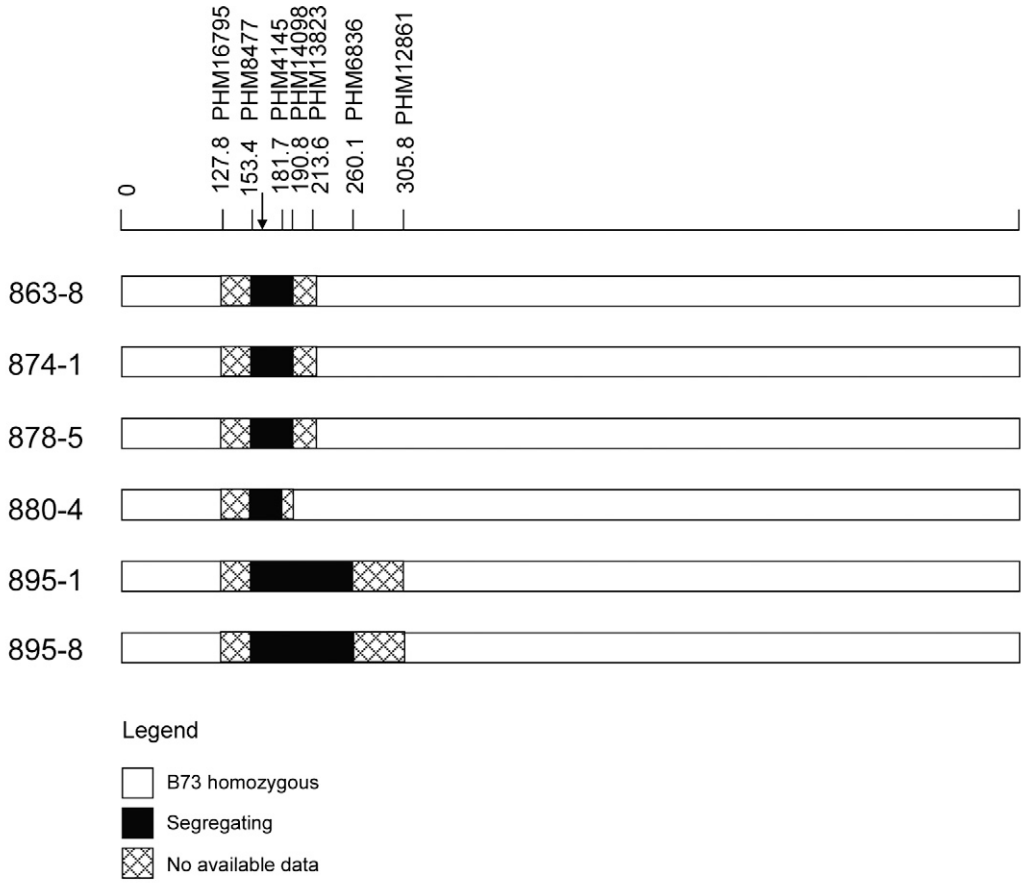

Figure 3. Chromosome 3 genotypes of the six B73 near-isogenic line (NIL) $F_{2:3}$ subpopulations derived from the six BC_5F_2 lines listed. Map positions are in IBM2008 units (Schaeffer et al., 2008). Arrow indicates position of the bin 3.04 southern leaf blight (SLB) resistance quantitative trait loci (QTL).

was estimated as 0.76 (Table 1). Back transformed to the original 1 to 9 scale, the estimates were $a = -0.43$ and $d = 0.38$. Although two of six B73 NIL F_2 subpopulations were segregating at the smaller effect QTL, its effect was not significant (Table 1; PHM6836), likely due to limited sample size of lines carrying the Mo17 allele (Table 1).

After accounting for the segregation of previously detected QTL other than the major effect QTL in bin 3.04, the IBM and Stuber RIL populations both had maximum R^2 values (0.22 and 0.10, respectively) and LOD scores (16.4 and 3.6, respectively) for SLB resistance at umc1425 (Tables 1 and 2; Fig. 2). Their estimated additive effects were −0.49 and −0.32, respectively (Table 1). On the 1 to 9 scale, the additive effect of the major bin 3.04 QTL in the IBM population was −0.33 points. The Stuber RILs were originally rated on a percent disease severity scale rather than the 1 to 9 scale used for the other populations; this value was used to calculate an AUDPC value. On this scale, the additive effect for the Stuber RIL population was −50.3. The results of the IBM and Stuber RIL analyses are consistent with the original studies of these populations; however, the R^2 values are higher due to adjustment for effects of other segregating QTL in these populations. The 2-LOD support interval for the IBM spanned 163.5 to 166.9 IcM (Table 2) in this analysis, slightly larger than the support interval obtained directly from multiple interval mapping (Balint-Kurti et al., 2007). Peak LOD scores increased when data were combined across all four populations in the joint analysis. The maximum LOD score (44.4) in the joint analysis occurred at PHM12576 (Table 2; Fig. 2). This position also had the maximum R^2 value (0.25; Table 1). The 2-LOD support interval of the QTL in the combined analysis encompassed 164.25 to 166.1IcM. The standardized additive effect estimated across all four populations was −0.54; the dominance effect was 0.74 (Table 1).

The B73 physical map and genome sequence between the two markers flanking this interval, umc1772 (163.5 IcM) and umc2000 (166.9 IcM), were examined for predicted and verified genes (http://maizegdb.org [verified 11 Nov. 2010]). This interval comprises 2.0 Mb of sequence predicted to contain 40 genes. The only obvious candidate genes representing previously identified classes of recessive resistance genes within this interval were two different genes each encoding adenosine triphosphate (ATP) binding sites, leucine rich repeats (LRRs), and serine threonine kinase domains (GRMZM2G463574 and GRMZM2G463580; http://www.maizesequence.org [verified 24 May 2009]). GRMZM2G463574 (1029 residues) and GRMZM2G463580 (928 residues) were 63% identical. Both of these LRR-kinase genes possessed

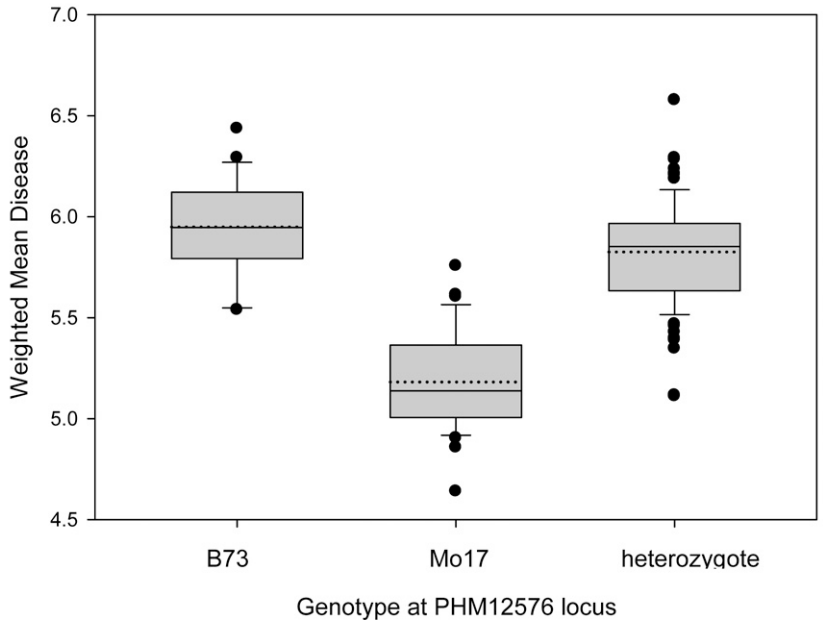

Figure 4. Boxplot of best linear unbiased predictor weighted mean disease (BLUP-WMD) scores for B73 near-isogenic line (NIL) $F_{2:3}$ lines according to genotype at the PHM12576 locus. Boxes incorporate the 25th to 75th percentile of data, while dotted lines represent means and connected lines represent medians. Outliers are represented as circles.

significant homology (E = 0) to a putative *Xa21* disease resistance gene in rice (dbj BAB03631.1; Altschul et al., 1997). The putative *Xa21* gene was 56 and 51% identical to GRMZM2G463574 and GRMZM2G463580, respectively.

DISCUSSION

In this study we have validated and fine-mapped a previously identified SLB resistance QTL on maize chromosome 3 using a novel approach involving the combined analysis of distinct generations of lines comprising four discrete populations segregating for a common pair of alleles. Two NIL pairs differing for this QTL were derived and used to create two distinct $F_{2:3}$ family populations that were assessed for SLB resistance. Importantly, we were able to "reuse" the data generated in the initial QTL mapping of this locus in the original RIL populations. By accounting for segregation of the other QTL in the RILs, we minimized the influence of background genetic variation on the adjusted RIL phenotypic scores. Limiting the genetic variation observed in the original RIL data to the

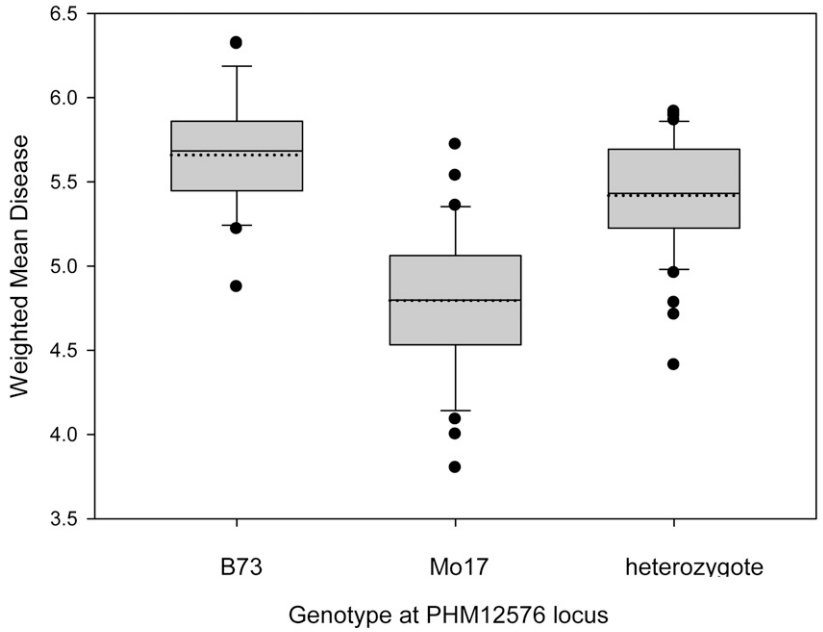

Figure 5. Boxplot of best linear unbiased predictor weighted mean disease (BLUP-WMD) scores for Stuber near-isogenic line (NIL) $F_{2:3}$ lines according to genotype at the PHM12576 locus. Boxes incorporate the 25th to 75th percentile of data, while dotted lines represent means and connected lines represent medians. Outliers are represented as circles

Table 3. Matrix of Pearson correlation coefficients between entry effect estimates for scores from weeks 1 through 4 in the B73 near-isogenic line (NIL) $F_{2:3}$ population.

	Week 2	Week 3	Week 4
Week 1	0.94	0.92	0.92
Week 2		0.99	1.0
Week 3			1.0

Table 4. Matrix of Pearson correlation coefficients between entry effect estimates for scores from weeks 1 through 4 in the Stuber near-isogenic line (NIL) $F_{2:3}$ population.

	Week 2	Week 3	Week 4
Week 1	0.96	0.99	0.90
Week 2		1.0	0.94
Week 3			0.95

chromosome 3 QTL region permitted combining these original data with the new genotypic and phenotypic data from the $F_{2:3}$ families. We have further examined the genes present in the 2-LOD support interval of the QTL peak and have identified some strong candidate genes in the region.

For precise mapping we wanted to "Mendelize" the response to SLB conferred by the target QTL in bin 3.04, that is, provide conditions under which the different phenotypes reliably differentiated genotypic classes. However there was still significant phenotypic overlap among genotypic classes (Fig. 4 and 5). At the time of flowering, the difference in SLB symptoms between plants homozygous for the Mo17 3.04 allele and plants homozygous for the B73 3.04 allele is almost imperceptible but grows to approximately 1.5 points on the 1 to 9 scale by the time of senescence. Because WMD averages these scores, the additive effect is a more modest estimate of the 3.04 Mo17 allele's contribution to SLB resistance over the plant's lifetime. Nevertheless, we validated the 3.04 QTL in the uniform genetic backgrounds of two $F_{2:3}$ populations derived from crosses between NIL pairs. In these populations, the QTL explained 58% (B73 NIL $F_{2:3}$'s) and 47% (Stuber NIL $F_{2:3}$'s) of the otherwise limited genetic variation for SLB resistance.

The multivariate analysis using individual scores permitted more efficient use of the phenotypic data than ANOVA with WMD values. Later scoring dates had proportions of missing data due to the inability to distinguish leaf death caused by disease from that caused by senescence. For the B73 NIL $F_{2:3}$ experiments, 10.0% of the data were missing across both scores, and for the Stuber NIL $F_{2:3}$ experiment 2.4% of the data across both scores were missing. Weighted mean disease cannot be calculated for plots with missing values; thus, multivariate analysis makes most efficient use of the available information by permitting prediction of plot values even at time points where data were missing for that plot. Information sharing across time points was extensive in this experiment due to the high correlations among genotypic effects at different scoring dates (r = 0.90 to 1.0; Tables 3 and 4) and residual error effects at different dates (r = 0.22 to 0.74; Tables 5 and 6). In general, correlations among genetic effect estimates decreased as time increased between scoring dates in both populations (Supplementary Tables S2 and S3; Tables 3, and 4). As a result, in both experiments, heritability was greater for BLUP-WMD values than for disease scores at any single time point.

Analysis of the two new fine-mapping populations identified a positive dominance effect, indicating the resistance imparted by the Mo17 allele at the 3.04 QTL is recessive. Iyer-Pascuzzi and McCouch (2007) hypothesized that recessive resistance may be caused by mutations at loci that otherwise normally condition susceptibility to the pathogen via interaction of their gene products with pathogen effector proteins in a gene-for-gene manner. Dominant genes for susceptibility exist in many plants infected by pathogens that produce host-selective toxins (HSTs; Friesen et al., 2008a; Wolpert et al., 2002). Host-selective toxins are pathogen effectors that, when introduced into the susceptible genotype of the host, induce tissue necrosis and disease. Race O of *C. heterostrophus* has been hypothesized to produce toxin(s) based on the results of culture filtrate experiments (Lim and Hooker, 1971). While susceptibility to HSTs is often conditioned by single dominant genes, toxin susceptibility genes sometimes condition only quantitative resistance depending on genetic background, (e.g., Liu et al., 2004; Friesen et al., 2007, 2008b; Singh et al., 2008). It is therefore possible that the dominant, B73-derived allele of the major 3.04 QTL is quantitatively conditioning susceptibility to a toxin produced by *C. heterostrophus*.

Recently several plant toxin susceptibility genes have been shown to be members of the nucleotide binding site (NBS)-LRR gene family (Lorang et al., 2007; Nagy and Bennetzen, 2008; Faris et al., 2010), a set of genes heretofore associated with disease resistance. Nucleotide binding site-leucine-rich repeat genes often mediate a rapid, localized programmed cell death called the hypersensitive response (HR) in response to attempted pathogen ingress. It is thought that some necrotrophic pathogens may "subvert" this mechanism by producing toxins that trigger NBS-LRR mediated HR. The two LRR-kinase candidate genes identified within the support interval of the major 3.04 QTL are both homologous to a putative *Xa21* gene, a resistance gene that imparts resistance to *Xanthomonas oryzae* pv. *oryzae*, a bacterial pathogen of rice (Song et al., 1995). One or both of these maize LRR-kinase genes could impart dominant, quantitative susceptibility to SLB by interacting with a fungal-derived protein and activating a pro-cell death signaling cascade and promoting growth of the necrotrophic fungus that causes SLB.

By accounting for the segregation of the QTL other than the major effect QTL in bin 3.04 in the original RIL mapping populations, we were able to combine this earlier data with the new genotypic and phenotypic data derived from the $F_{2:3}$ families. Combining data across the four populations yielded a higher LOD score (44.4 vs. 26.6, 14.7,

16.4, and 3.6 for the B73 NIL $F_{2:3}$, Stuber NIL $F_{2:3}$, IBM, and Stuber RIL populations, respectively; Table 2) at the QTL peak in bin 3.04 than when populations were analyzed individually. The joint analysis provided higher statistical significance for the QTL peak because of the larger sample size obtained from pooling information from multiple independent families. In addition, the QTL support interval was also smaller for the combined data set than for any of the single data sets. The QTL had previously been mapped to 163.7 to 165.9 IcM in the IBM population (Balint-Kurti et al., 2007), whereas the joint analysis identified a 2-LOD support interval encompassing 164.25 to 166.1 IcM. This represents a reduction in 0.35 IcM, or 23% over the previously reported interval. Because mapping units in the IBM are based on multiple meiotic generations, the joint analysis interval of 1.85 IcM encompasses approximately 0.46 cM (Winkler et al., 2003). The larger number of recombination events sampled in the joint analysis provides the basis for greater precision in localizing QTL.

Reductions in QTL support interval sizes were observed in previous meta-analyses and pooled analyses of families derived from multiple parents (Blanc et al., 2006; Chardon et al., 2004; Coles et al., 2010; Khatkar et al., 2004; Walling et al., 2000). However, this is the first study of which we are aware to combine data across discrete generations segregating for a common pair of alleles. Because only two alleles were segregating per locus, our method of analysis avoided several issues that complicate other combined analysis approaches. Allelic effects are known to be shared across generations in our design, leading to greater power of detection compared to analyses of nested allelic effects within populations. Common markers can be used across all generations, simplifying map construction and reducing the potential for differential recombination among populations to confound results. In our design, common QTL reflect common underlying genes, whereas multiple population analyses may fuse distinct but tightly linked QTL segregating in different populations.

Our method is also applicable to many extant sets of experimental data. Recombinant inbred line and NIL data from the same founder cross could be retrospectively joined in a combined analysis as proposed here to provide higher resolution mapping results. Our method addresses differences in segregating QTL, experimental environments, phenotyping protocols, and availability of marker information among populations. Adjustment of RIL phenotypic data based on initial QTL effect estimates and line genotypes allows for minimization of variation due to additional segregating QTL in RIL populations. Subtraction of the experiment mean and division by the standard deviation accounts for potential differences in experimental environments and phenotyping protocols among populations. Differences in availability of genotypic data between populations are addressed through imputation of marker genotypes.

In summary, this approach therefore allows us to "recycle" data that is usually discarded and thereby to increase the precision of QTL estimates without additional phenotypic or genotypic evaluation costs. It is furthermore widely applicable and could be retrospectively applied to a large number of studies in a wide variety of biological systems.

Table 5. Matrix of Pearson correlation coefficients between residuals for scores from weeks 1 through 4 in the B73 near-isogenic line (NIL) $F_{2:3}$ population.

	Week 2	Week 3	Week 4
Week 1	0.38	0.25	0.22
Week 2		0.57	0.57
Week 3			0.73

Table 6. Matrix of Pearson correlation coefficients between residuals for scores from weeks 1 through 4 in the Stuber near-isogenic line (NIL) $F_{2:3}$ population.

	Week 2	Week 3	Week 4
Week 1	0.74	0.51	0.49
Week 2		0.69	0.58
Week 3			0.73

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