

## Efficient QTL detection for nonhost resistance in wild lettuce: backcross inbred lines versus F<sub>2</sub> population

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**Abstract** In plants, several population types [F<sub>2</sub>, recombinant inbred lines, backcross inbred lines (BILs), etc.] are used for quantitative trait locus (QTL) analyses. However, dissection of the trait of interest and subsequent confirmation by introgression of QTLs for breeding purposes has not been as successful as that predicted from theoretical calculations. More practical knowledge of different QTL mapping approaches is needed. In this recent study, we describe the detection and mapping of quantitative resistances to downy mildew in a set of 29 BILs of cultivated lettuce (*L. sativa*) containing genome segments introgressed from wild lettuce (*L. saligna*). Introgression regions that are associated with quantitative resistance are considered to harbor a QTL. Furthermore, we compare this with results from an already existing F<sub>2</sub> population derived from the same parents. We identified six QTLs in our BIL approach compared to only three in the F<sub>2</sub> approach, while there were two QTLs in common. We performed a simulation study based on our actual data to help us interpret them. This revealed that two newly detected QTLs in the BILs had gone unnoticed in the F<sub>2</sub>, due to a combination of recessiveness of the trait and skewed segregation, causing a deficit of the wild species alleles. This study clearly illustrates the added value of extended genetic studies on two

different population types (BILs and F<sub>2</sub>) to dissect complex genetic traits.

### Introduction

In the search for more durable disease resistance in plants, scientists and breeders are now also focusing on exploring alternative resistances like quantitative resistance. Quantitative resistance exhibits properties that differ from the widely used qualitative resistances, which are explained by R genes that interact with Avr-genes (Lindhout 2002). Quantitative resistance is a complex trait, as it exhibits a continuous phenotypic variation and is mostly under polygenic control [quantitative trait loci (QTLs)]. Breeders are interested in detecting and introgression of QTLs for resistance and also QTLs for other quantitative traits like quality and yield (Varshney et al. 2006). However, the introgression of QTLs (not specifically QTLs for resistance) by marker-assisted selection (MAS) for breeding purposes has often shown inconsistent results and has not been as successful as that predicted from theoretical calculations (Schön et al. 2004). This is mainly caused by the lack of efficient and accurate QTL mapping approaches that depend on some critical key factors like population type, population size, marker density, the quality of the phenotypic evaluation, environment, number of QTLs, the magnitude of the effect, the inheritance of the QTL (dominant, recessive or additive), the genotype × environment interaction and the epistatic interaction between loci.

Several theoretical studies show the importance of the population size on the power of QTL detection (Beavis 1994, 1998; Van Ooijen 1999). For example, Beavis showed that when using a population of 100 plants, the

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chance of detecting a QTL with an explained phenotypic effect of <10% was only 3%, and the magnitude of estimated effect was 10-fold inflated. Furthermore, increasing the population size to 500 plants improved the accuracy considerably, and in a population of 1,000 plants the magnitude of the phenotypic effect was close to the actual value. These theoretical studies have been supported by field experiments in which an increase of sample sizes resulted in an increase in the number of detected QTLs explaining the trait (Schön et al. 2004; Vales et al. 2005).

Often, phenotypic evaluations and marker screenings on large population sizes of >500 are not used, as they are too laborious and costly. This especially holds true for wide crosses where the chance to obtain vital progenies is often very low.

Alternative approaches aiming to improve QTL mapping efficiency without large population sizes have been developed. Examples are linkage disequilibrium (LD) mapping (Flint-Garcia et al. 2003; Gupta et al. 2005; Kraakman et al. 2004; Yu et al. 2006), meta-analyses (Arcade et al. 2004; Guo et al. 2006) or QTL mapping on selected informative genotypes only (Peleman et al. 2005). Alternatively, a limited set of inbred lines [near-isogenic lines (NILs) or backcross inbred lines (BILs) or advanced backcross (AB) lines] can be used instead of a large  $F_2$  or recombinant inbred line (RIL) population (Keurentjes et al. 2007; Tanksley and Nelson 1996; Zamir 2001). Since such lines are homozygous, numerous genetically identical plants can be evaluated, which will increase the accuracy of phenotyping without increasing the efforts of genotyping. In addition, this also allows extended genotypes  $\times$  environment studies.

We choose this “Inbred Line” strategy for our research topic “Genetic dissection of the non-host resistance of wild lettuce” because of the above mentioned advantages and because the genetically wide cross between resistant wild lettuce (*L. saligna*) and the susceptible lettuce (*L. sativa*) limited the generation of a large population of vital and (self-)fertile  $F_2$  plants or even RILs.

We repeatedly backcrossed the parental *L. saligna*  $\times$  *L. sativa*-cross with the *L. sativa* parent until the  $BC_4$  generation. The  $BC_4S_1$  population and later generations, obtained by selfing, were screened by MAS, and this resulted in a set of 29 BILs. Each BIL contains one introgression of *L. saligna*, harboring on average 4% of the *L. saligna* genome. The set of 29 BILs together cover 96% of the *L. saligna* genome (Jeuken and Lindhout 2004).

In the present study, we describe the detection and mapping of QTLs for downy mildew resistance in this set of 29 BILs. Furthermore, we estimate the magnitude of the QTL effects and the inheritance of QTLs.

Previously, we have executed a QTL mapping study on a small  $F_2$  population of 126 plants (based on the identical parental cross; Jeuken and Lindhout 2002). This now

allows us to compare the two studies with regard to detection of the number and the position of the QTLs and to compare the actual efficiency of QTL mapping by both approaches. Because of the diminished genetic noise and the increased accuracy of phenotyping in the set of BILs, it was expected that this approach would result in the identification of more QTLs than when using the  $F_2$  approach. Only if positive, *trans*-acting epistatic interactions are involved might a QTL be identified in an  $F_2$  population but remain hidden in a set of BILs.

As the present study represents the results of a single experiment, which was a sample from many possible experimental results, we have performed a simulation study to assess the probability that a QTL is detected in the  $F_2$  and in the BILs. For a given set of values of biological and experimental parameters like population size, true QTL effect, marker density, skewness of segregation and accuracy of phenotypic evaluation, 50,000 replicate  $F_2$  populations or sets of BILs were sampled. By varying the parameter values, we were able to quantitatively study their influence on the chance of detecting a QTL. The “default” values of these parameters were based on the actual results of the mapping studies of the  $F_2$  and the BILs. These simulations help us to interpret mapping results of real data and to see the impact of segregation distortion on QTL detection power.

In the present study, the experimental QTL mapping approaches by using an  $F_2$  population and a set of BILs originating from the same parental cross are compared with each other and with *in silico* results. This state of affairs represents a unique combination of *experimental* and *theoretical* approaches to verify QTL mapping efficiencies, which to our knowledge, is a novelty in this research area. Although the actual results of the present study are valid for the lettuce-downy mildew model, our method can readily be applied to other quantitative traits and other plant species, and therefore, is of quite general value for developing QTL mapping strategies.

## Materials and methods

Summary of materials, methods and results on the  $F_2$  population

The  $F_2$  plant material, genetic linkage map and QTL analyses have been described earlier in Jeuken et al. (2001) and Jeuken and Lindhout (2002).

### Summary

The  $F_2$  population consisted of 126 plants and was genotyped with about 500 AFLP markers. A genetic linkage

map was constructed with a random marker distribution covering all nine chromosomes. The average marker spacing was 1.8 cM, and 65% of the marker distances lied between 1 and 8 cM (Jeuken et al. 2001).

Four disease tests on leaf discs of F<sub>2</sub> plants infected by *B. lactucae* races Bl:14 and Bl:16 were executed. Per disease test, six leaf discs per F<sub>2</sub> plant were scored for infection severity. Finally, a QTL analysis was executed with MapQTL software (Van Ooijen 2004), performing a Kruskal–Wallis test, simple Interval Mapping and approximate multiple QTL mapping (MQM) with all genotypic (markers) and phenotypic (disease test) data. This resulted in the detection of one race-specific *R*-gene-like resistance designated *R39* and three race-nonspecific QTLs designated *Rbq1*, *Rbq2* and *Rbq3*. These three QTLs were located at Chromosomes 7, 1 and 9, had a peak LOD score of 7, 3 and 4 and had a proportion of explained variance of 26, 13 and 12%, respectively (Jeuken and Lindhout 2002). The heritability of resistance to *B. lactucae* races Bl:14 and Bl:16 was high (>0.8) in the F<sub>2</sub> population, and the three QTLs explained only 51% of the quantitative resistance. This indicated that other resistance QTLs might segregate in the F<sub>2</sub> population.

## Plant material

### Tests on the set of 29 BILs

A set of 29 BILs plus the two parental lines were used for disease tests. The parental lines are the cultivar *L. sativa* cv. Olof, which is the recurrent parent that does not harbor any known *Dm* gene (*downy mildew R* gene) and is therefore susceptible to all described *B. lactucae* races, and *L. saligna* CGN05271 as the nonhost donor parent.

The set of 29 BILs has been developed earlier and details about the nomenclature, development or the genotypes of the set of BILs are described in (Jeuken and Lindhout 2004). Summarized, this set of BILs was genotyped with more than 700 DNA markers (AFLP, ESTs and SSRs) and covers 96% of the *L. saligna* genome. On average, each BIL contained one homozygous introgression fragment of the wild species of 33 cM in a *L. sativa* background (=4% of the *L. saligna* genome). An exception is four BILs that remain heterozygous for their introgression. The nomenclature of the BILs refers to the location of the introgression, for example, BIL4.3 stands for the third introgression on Chromosome 4.

Modifications on the description of BILs in (Jeuken and Lindhout 2004) are as follows: Chromosome 8 of *L. saligna* is completely represented by four BILs (since an AFLP-marker E48M59-197 was detected that showed an overlap between the introgression region of BIL8.2 and

BIL8.3); BIL1.1 is now homozygous *L. saligna* for the whole introgression region. A “doubleBIL” was added to the set of 28 BILs to improve resolution on Chromosome 7, designated “doubleBIL7.1,” which has one large and one small homozygous *L. saligna* introgression region; one on Chromosome 7 from 25 to 40 cM and one on Chromosome 1 from 76 to 87 cM. These 29 BILs represent 53 Bins. (Bins are arbitrarily-defined segments delimited by two fixed core markers. A Bin includes all loci from the left-most or top core marker to the next core marker.)

A “preBIL” is a line that has a single heterozygous *L. saligna* introgression, while a “BIL” carries a homozygous introgression from *L. saligna*. Plants were grown in the greenhouse in a randomized block design.

## Disease test

### Tests on the set of 29 BILs

Six disease tests with two downy mildew races were performed on selections of the set of 29 BILs between 2001 and 2006. The first and the fifth tests were performed with *B. lactucae* race Bl:16 and the other four tests with Bl:14. For practical reasons, we performed six incomplete tests with twenty BILs instead of using complete tests with all 29 BILs. In each of the six incomplete tests, a different combination of eleven BILs plus the same nine “reference BILs” plus the two parental lines were included. The reference BILs were selected after the first two disease tests. All BILs that showed significantly lower infection levels were selected and a few BILs with a similar infection level as the susceptible parental line were selected. The reference BILs are BIL1.2, 2.2, 4.2, 4.4, 5.1, 6.3, 8.2, 8.4 and 9.2. The other 20 BILs were rotated between the six incomplete tests and were tested in at least three out of six tests. In each disease test, seven plants were analyzed per BIL and per control parent line, with four evaluated leaf discs per plant. The infection severity level of each BIL was compared with that of the susceptible recurrent parent *L. sativa* Olof and the other BILs. A BIL was considered quantitatively resistant if its infection severity level was significantly ( $\alpha = 0.05$ ) lower than that of *L. sativa* Olof. A BIL that showed quantitative resistance was considered to harbor a QTL at the introgression region.

### Inheritance tests for BILs with resistance QTLs

A selected preBIL population of twenty plants segregating for the introgression and four or five plants of the corresponding resistant BIL progeny were tested for resistance to two *B. lactucae* races. The twenty plants per preBIL

were genotyped with codominant EST-markers based on lettuce EST sequences, one SSR marker and one phenotypic marker (Fig. 1, Table 1). DNA was extracted according to Jeuken and Lindhout (2004). Amplification of PCR-markers was performed in 25  $\mu$ l PCR reactions with 10 ng DNA, 0.5 U Taq polymerase, 10 pmol of both primers, 2.5  $\mu$ l 10 $\times$  reaction buffer and 5 mM of all four dNTPs. The following PCR program was used: 2' 94°C, 30" 94°C, 30" 52°C, 1' 72°C, 2' 72°C. Steps 2–4 were repeated for 40 cycles. For cleaved amplified polymorphic sequence digestion, we added 0.1 U restriction enzyme and 3  $\mu$ l specific enzyme buffer and incubated for at least 3 h at 37°C. PCR products were run on 1–2% agarose gels to separate products with larger size differences. Each introgression was specified by at least two markers that were less than 5 cM away from the ends of the introgression. Only BIL8.2 and BIL4.2 had a marker further away from the top end at 7 and 14 cM, respectively. The SSR marker LsB104 is described in (Van de Wiel et al. 1999). EST markers were developed from lettuce EST sequences by the Compositae Genome Project (<http://cgpdb.ucdavis.edu>); information for these markers is available from the project web site ([http://cgpdb.ucdavis.edu/database/genome\\_viewer/viewer/](http://cgpdb.ucdavis.edu/database/genome_viewer/viewer/)). The primer sequences for each marker are listed in Table 1. Marker loci were classified in one of the following three genotype classes: homozygous *L. sativa* (code "A"), heterozygous (code "H") or homozygous *L. saligna* (code "B"). Each genotype class was represented by five plants per preBIL population. Genotypes that showed a recombination event within the introgression region were excluded from analyses. If the genotype class

"homozygous *L. saligna*" contained fewer than five plants, it was filled up with plants of their counterpart BILs to equalize numbers of plants in the three genotype classes and to compensate for expected preference of *L. sativa* alleles for preBIL8.2 and preBIL6.3 population.

#### Protocol disease test

Disease tests were performed according to Jeuken and Lindhout (2002) with minor modifications. Nine to eleven days after inoculation, the leaf discs were assessed for growth of sporangiophores. Infection severity was scored visually as the percentage of leaf area covered with sporangiophores.

#### *B. lactucae* races

The maintenance and the inoculation of *B. lactucae* were described in (Jeuken and Lindhout 2002). We used identical *B. lactucae* races for this study. However, the names of these races (NL14 and NL16) are now designated according the International Bremia Evaluation Board as races BI:14 and BI:16.

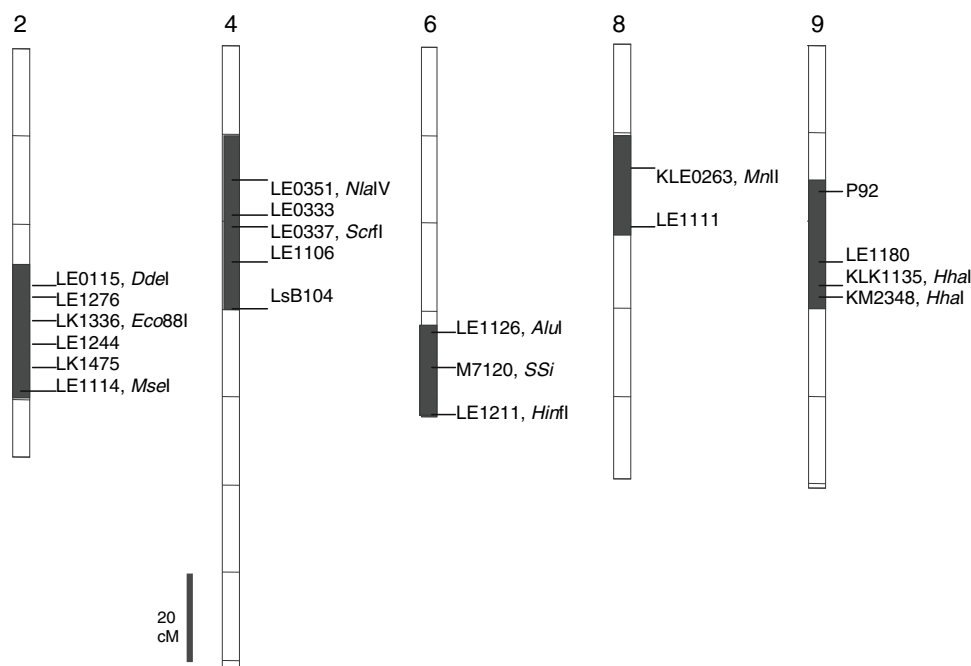
#### Data analysis

#### Tests on the set of 29 BILs

All infection severity scores from the disease tests were arcsine square-root-transformed to improve the

**Fig. 1** The position of EST and CAPs markers on introgressions of BIL2.2, 4.2, 6.3, 8.2 and 9.2 are shown per chromosome.

Black boxes represent introgressions on BILs. Restriction enzymes are shown with CAPS marker if necessary to reveal a polymorphism. P92 is typical leaf phenotype from BIL9.2



**Table 1** Primer sequences of EST markers

Marker name	Primer forward	Primer reverse
LE0115	ACTGCTCCACTACCCACCAC	CGACAAGGCTGACATCGTAA
LE1276	TTTGGGTTTCCTTCAGTTTGC	CACAGTTTGGGATGAACACG
LK1336	TGAGGAGTCCATGGATACGG	CGATGCAACAGCATGGATAC
LE1244	CATCCGCTTCTCTTCAGTC	ACGAGCACCTGCATCTACAA
LK1475	GGAGTTCAGGGCCTCTGTC	CCGATTCTGCGGTTATCTTC
LE1114	CAAGAGGTGAATGGGAAGGA	TACCACACAAAACAAGCGGAA
LE0351	GAATATGCGGCGGAGATAAG	AATCACATGAATGGATGCAAA
LE0333	GGACCGGGTTTTAAGTCGT	TTTCTCTGTATATATGCAATCTCCATT
LE0337	CCATGGCTAAAAAGCAAACC	ACATTAGCCAAGCGACAACA
LE1106	TGATTATGGAGGCGAAGAGG	CACAAAGATTACTACTTGCCATC
LE1126	CTTGCTCCAATTCCTCTCG	AATGCCATAGTGAAGCTGGG
M7120	ACAGCAACAGCCGACCG	CGCACATTATTCGGCTCAA
LE1211	CGGGTGATTACATCGGCTAT	CGCAACCAACCAAATTTACC
KLE0263	CAACCTCACGGAGTTTTGT	GCCGGAAAGTTTGTGTGTGT
LE1111	AATCACTCCACCACCGAAG	CTACGTCAAGTGCCTATGCCA
KLK1135	TAAACTTCGGGACGAACCAC	GCCAAAATGCGAAAGTTGC
LE1180	TTGAGGTTTATGGACCCGAG	CAAGCGCGCACTGATAGTAA
KM2348	TAAACTTCGGGACGAACCAC	GCCAAAATGCGAAAGTTGC
LE0115	ACTGCTCCACTACCCACCAC	CGACAAGGCTGACATCGTAA
LE1276	TTTGGGTTTCCTTCAGTTTGC	CACAGTTTGGGATGAACACG

homogeneity of residual variance before further calculations were performed. The infection severity level of one plant was calculated as the average of four leaf discs per plants per disease test.

Differences in mean infection severity levels of BILs over six tests (seven plants per line per test) were analyzed by a mixed-model procedure with “experiment” and “genotype” as fixed factors (Patterson and Thompson 1971). Tukey HSD (honestly significantly difference) multiple comparison procedure was used as a post hoc test to determine significant differences among 29 BILs and their parental lines ( $\alpha = 0.05$  and  $\alpha = 0.01$ ). In the Tukey HSD procedure, the error rate that is controlled is an experimentwise error rate over all pairwise comparisons.

As different seasons might influence plant conditions and resistance levels, the similarity between the six disease tests was measured by a two-way ANOVA with replication on the nine reference BILs plus *L. sativa* Olof. Race specificity was measured by two-way ANOVA with replication between disease tests 1 and 2 and 5 and 6 with *B. lactucae* race Bl:14 and race Bl:16 on separate leaf discs from the same set of plants.

#### Inheritance tests for QTLs

Deviations of normal segregation ratios (skewness) of the introgressions of the preBIL populations in the three genotype classes (homozygous *L. sativa*, heterozygous,

homozygous *L. saligna*) were tested by using a chi-square test.

In each preBIL population, the infection severity levels were tested per genotype class by one-way ANOVA and the means were compared by Tukey HSD test.

#### In silico QTL detection experiments

We have written a simulation program, which mimics QTL detection in an  $F_2$  population and in a set of BILs. The purpose of the in silico experiments carried out with this program was to investigate the role of several factors that determine the probability of detection of a QTL of given effect. The program calculates the probability of detecting a QTL in an  $F_2$  population by performing an approximate QTL mapping procedure on numerous (preferably >10,000) samples of  $F_2$  populations and BILs. The proportion of simulation runs in which the QTL is detected is then taken as the detection probability. When running the program, the following variables can be set: average score (phenotypic value) of the presumed QTL (i.e. the QTL effect),  $F_2$  population size, genetic distance between QTL and nearest marker (designated as “marker density”), segregation ratio at this marker locus (designated as “skewness”), number of QTLs (of equal effect) underlying the trait, total phenotypic variance in the  $F_2$  population and number of observations per  $F_2$  plant.



For simulation of the BILs, we used a two-sample *t* test for each BIL to be tested against the control (*L. sativa* cv. Olof), applying a Bonferroni correction for multiple testing. Mean values for BILs and control were the same as those used in the corresponding F<sub>2</sub> experiment. For the set of BILs, the following variables can be set: number of BILs to be tested, number of plants per line and number of samples per plant. A detailed description of the simulation program is given in Appendix A.

As a “standard configuration” to which all other simulation runs could be compared, we used parameter settings that correspond to our actual F<sub>2</sub> population. Thus, by changing one of the parameter values at a time (e.g. population size, segregation ratio, number of observations per plant, etc.), we were able to investigate the influence of these parameter values on the chance of detection in an actual experiment and how detection probability decreases or increases as one moves away from the “standard configuration.”

The standard parameter settings were as follows. Genotypic values of the presumed QTL were based on the average phenotypic scores at loci *Rbq1*, *Rbq2* and *Rbq3*, obtained from the actual QTL analysis on the F<sub>2</sub> population tested with *B. lactucae* race Bl:14. These values are as follows:

Homozygous *L. sativa*: 2.5  
 Homozygous *L. saligna*: 1.7  
 Heterozygous: 2.5, if QTL is dominant  
 Heterozygous: 1.7, if QTL is recessive

From the same QTL analyses on our F<sub>2</sub>, the total phenotypic variance was taken to be 1.3. The standard segregation ratio at the marker locus was 1:2:1 (*MM:Mm:mm*), and the standard number of observations per plant was 6. For the standard genetic distance between QTL and nearest marker, we choose 5 cM, a representative

value, since 65% of the marker distances were between 1 and 8 cM. The default number of segregating QTLs was taken to be 7, i.e. the number of QTLs detected altogether in the F<sub>2</sub> population and the set of BILs.

Although the simulation program has an option to mimic interval mapping (IM) as well as composite interval mapping (CIM), we only used IM. The reason for this is that mimicking CIM requires knowledge of the proportion of the variance that is explained by markers near the background QTLs, a quantity that cannot be known beforehand. The number of simulation runs was 50,000 for each configuration of parameter values. The program was written in C++; an executable, with graphical interface running under OS Windows, is available from P.S. upon request.

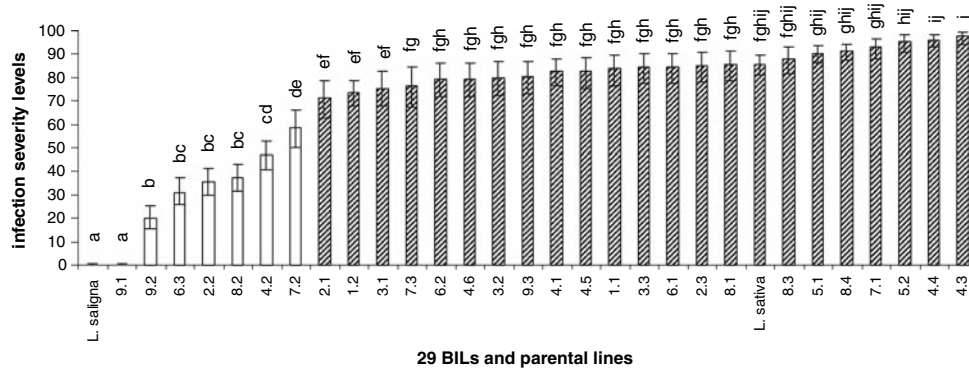
## Results

A summary of the former F<sub>2</sub> analyses and results is presented in the “Materials and methods.”

### Disease tests on a set of BILs

The objectives of these experiments were to detect quantitative resistance loci (QTLs) in the set of BILs and to quantify their resistance levels. The infection severity of the parental lines in six tests was always 0% for *L. saligna* and ranged from 80 to 94% for *L. sativa* Olof, indicating a high reproducibility between the six tests. Similarly, the infection severity levels of the nine reference BILs were similar in all six tests.

A multiple comparison statistical test classified the 29 BILs in groups based on the infection severity levels over six tests (Fig. 2). Twenty-two BILs had a similar infection level as the susceptible parent *L. sativa* Olof and were



**Fig. 2** Infection severity levels of twenty-nine BILs and parental lines over six disease tests with *B. lactucae* are shown. Ninety-five percent confidence intervals are shown by lines on top of the bars. Letters in common indicate that the values are not significantly

different ( $\alpha = 0.01$ , Tukey HSD procedure). Striped bars represent lines not significantly different from *L. sativa* Olof. White bars represent lines that are significantly different from *L. sativa* Olof

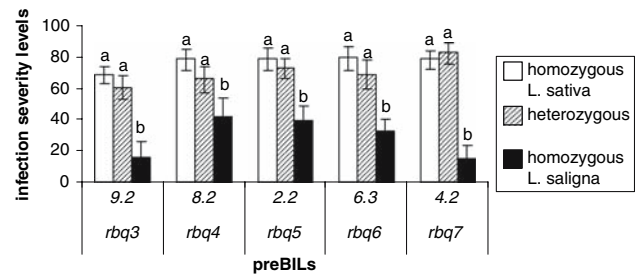
designated “susceptible.” Six BILs were significantly different from the susceptible parent and were designated as “quantitatively resistant” ( $\alpha = 0.05$  and  $0.01$ ). The quantitatively resistant group consists of BILs 9.2, 6.3, 2.2, 8.2, 4.2 and 7.2. Their infection severity levels ranged from 20 to 58% (Fig. 2). The susceptible group consists of the other twenty-two BILs and their infection levels ranged from 71 to 98%. Line preBIL9.1 could not be classified in one of these groups, as it segregated into plants that were as susceptible as *L. sativa* Olof and plants that showed no infection. No race-specific interaction was detected for any of the BILs.

From the group of six quantitatively resistant lines, preBIL7.2 and BIL9.2 carry an introgression in which a QTL has been mapped previously (Jeuken and Lindhout 2002). The introgression region of preBIL7.2 coincides with *Rbq1*, and the introgression region of BIL9.2 corresponds with *Rbq3*. The introgression regions of BIL8.2, BIL 2.2, BIL6.3 and BIL4.2 do not carry known QTLs, and their detected quantitative resistances, which are considered as QTLs, are successively numbered from *Rbq4* to *Rbq7*.

PreBIL9.1 differed clearly from all other lines. PreBIL9.1 is heterozygous for its introgression region that contains the locus of *R39*, a race-specific R-gene detected in the  $F_2$  population (Jeuken and Lindhout 2002). Without *R39*, the plants were as susceptible as *L. sativa* Olof, while the presence of *R39* was associated with complete resistance to *B. lactucae* Bl:16 and Bl:14. An autonecrosis phenotype was exhibited by preBIL9.1 plants with *R39*.

#### Disease tests to measure inheritance of five QTLs for resistance

We measured the size of the allelic effect of *Rbq3* through *Rbq7* in populations of twenty plants segregating for the corresponding *Rbqs*. To this end, five segregating populations (“pre-BILs”) were used that were the ancestors of the corresponding BILs. In each of these preBIL populations, the genotype class “homozygous *L. saligna*” showed the expected low infection severity level that was significantly different from the two other genotype classes (“heterozygous” and “homozygous *L. sativa*”) that were similar to the susceptible cv. Olof (Fig. 3). Consequently, *Rbq3* through *Rbq7* are recessive and are designated *rbq3* through *rbq7* according to the *B. lactucae* resistance nomenclature. The locations of the seven QTLs and *R39* do not coincide with known R-gene clusters in the lettuce genome, except for *Rbq2* and *rbq4*, which show some overlap with *Dm5/8* and *Dm10* on Chromosome 1 and *Dm13* on Chromosome 8, respectively (Fig. 4). The segregation of four of the preBIL populations was not



**Fig. 3** The allelic effects of five QTLs were measured in a disease test with *B. lactucae* Bl:14 on preBIL populations. Infection severity levels and 95% confidence intervals are shown per QTL per genotype class that was represented by five plants. Letters in common within preBIL populations indicate that the values for genotype classes are not significantly different ( $\alpha = 0.05$ , Tukey HSD procedure)

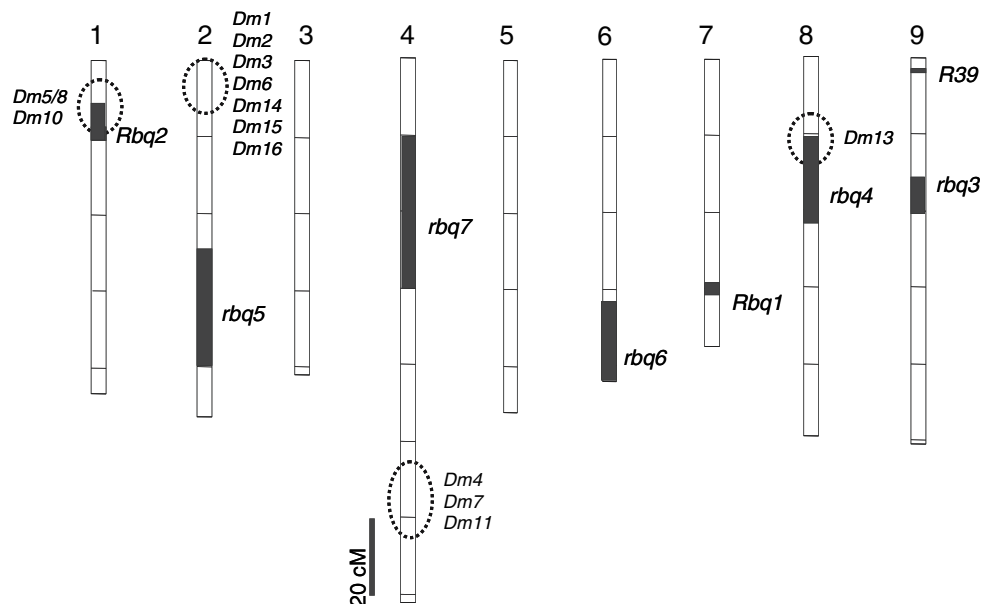
significantly different ( $\alpha = 0.05$ ) from a monogenic (1:2:1) segregation, while preBIL population 8.2 showed a 10:8:1 segregation, indicating skewed inheritance.

#### Sensitivity test for QTL detection in $F_2$ and in BILs

Since we identified six QTLs in our BIL QTL mapping approach compared to only three in an  $F_2$  mapping approach (with two QTLs in common), we wondered whether this result could be expected given the experimental conditions. Therefore, we designed a genetic model with seven QTLs and their allelic effects as estimated from the disease tests on the 29 BILs and the  $F_2$  population. Subsequently, we estimated the chance of identifying a QTL in the  $F_2$  and in the BIL mapping approach given the experimental conditions used (marker density, population size and skewness; see “Materials and methods”).

As a result, the average chance for detecting any of the seven QTLs in this  $F_2$  population detection was 0.7 (Fig. 5) and in the BILs was 0.98 (Table 2, last column). This model allowed us to estimate the effect of changing the experimental conditions used in the  $F_2$ . For instance, the parameters that caused the most important effect on the detection chance were “ $F_2$  population size” and “number of QTLs explaining the trait.” As the density of the genetic map was already quite high, the marker distance had the least influence on the detection chance. Surprisingly, the segregation ratio also had an important effect on the chance of detecting a QTL. For example, when the frequency for the recessive genotype (homozygous *L. saligna*) varied from 0.25 to 0.06, the QTL detection chance decreased from 0.70 to 0.17. In contrast, when the frequency of a dominant QTL varied similarly, the detection chance increased only from 0.70 to 0.84 (Fig. 5). For both recessive and dominant QTLs, the decrease in detection chance due to skewness towards a certain parental genotype was much stronger than the increase of the detection chance

**Fig. 4** The position of QTLs and *R39* compared to known resistance gene clusters in lettuce according to Kesseli et al. (1994), Truco et al. (2007) and <http://cgpd.ucdavis.edu/>



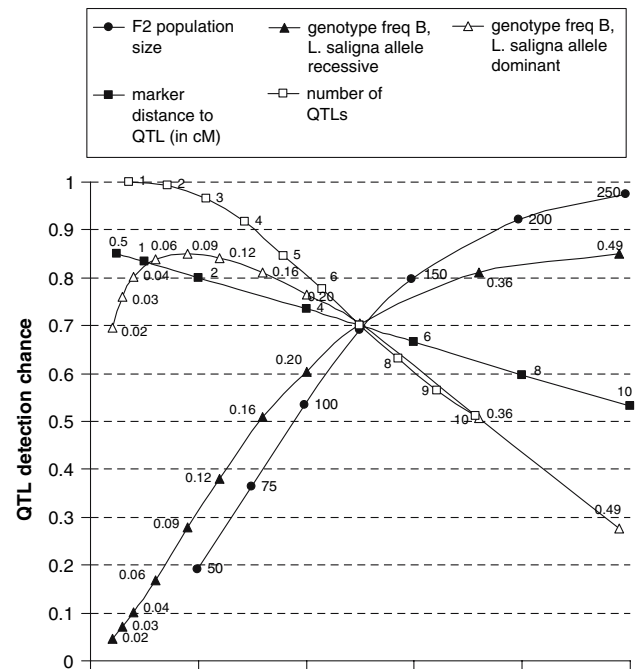
with skewness of the same value but in favor of the other parental genotype (Fig. 5).

#### Detection chances for the observed QTLs

Up to now, we did not differentiate between the QTLs in the sensitivity studies. However, we had real data on each of the QTLs, which allowed us to estimate the chance of identifying each QTL individually. This was based on three variables: (1) the inheritance (allelic effect), (2) the skewness and (3) the marker density (Table 2). Remarkably, by performing these simulation studies, we could explain the results of the  $F_2$  mapping approach very well. The detection chances were highest (0.81–0.91) for the three QTLs detected in the  $F_2$  (*Rbq1*, *Rbq2* and *Rbq3*), while the detection chance of the other QTLs was lower to extremely low (0.15–0.79). These extremely low detection chances were a result of the recessive character of the QTLs in combination with skewness against *L. saligna* alleles.

To have a detection chance of 0.8 for *rbq3*, *rbq4* and *rbq6* with the same values for skewness, upwards of 250 to 550  $F_2$  plants should have been needed for a detection chance of 95% (Fig. 6). This illustrates very well the great effect of distorted segregations on QTL detection chances. Recessive QTLs from the wild species became almost undetectable when they are located in a region with skewness against the wild species alleles.

With the size of the QTL effects observed in our experiment the detection chance in BILs was invariably high (0.98, last column in Table 2). Nevertheless, this



**Fig. 5** Simulation studies show the chance of QTL detection in an  $F_2$  population. The starting point is an  $F_2$  population with properties identical to the in vivo practical  $F_2$  population data set, “default settings” (Jeuken and Lindhout 2002). The effects of five parameters on the starting-point  $F_2$  population are presented. Visually, the starting point is the middle point where all lines come together. Parameters of starting-point:  $F_2$  size = 126, genotype frequency homozygous *L. saligna* = 0.25, marker distance to QTL = 5 cM, number of QTLs explaining the trait = 7. For other parameters, see “Materials and methods.” Genotype freq B means the genotype frequency of plants that are homozygous *L. saligna* at the specific QTL locus



**Table 2** Estimation of detection chance per QTL

QTL	Detected in	Allelic effect <sup>b</sup>	Average marker distance <sup>c</sup>	Segregation ratio in F <sub>2</sub> <sup>d</sup>	Frequency genotype “homozygous <i>L. saligna</i> ” <sup>e</sup>	Detection chance in F <sub>2</sub>	Detection chance in BILs <sup>e</sup>
<i>Rbq1</i>	F <sub>2</sub> + pBIL7.2	dom	2.1	37:44:8	0.09	0.91	0.98
<i>Rbq3/</i> <i>rbq3</i> <sup>a</sup>	F <sub>2</sub> + BIL9.2	dom/rec	2.7	29:49:9	0.11	0.86/ 0.38	0.98
<i>Rbq2</i>	F <sub>2</sub>	dom	2	1:2:1	0.25	0.81	0.98
<i>rbq7</i>	BIL4.2	rec	2.5	1:2:1	0.25	0.79	0.98
<i>rbq5</i>	BIL2.2	rec	3.9	1:2:1	0.25	0.74	0.98
<i>rbq6</i>	BIL6.3	rec	3.3	36:46:9	0.10	0.34	0.98
<i>rbq4</i>	BIL8.2	rec	2.3	30:90:6	0.05	0.98	

<sup>a</sup> The inheritance of *rbq3* is still ambiguous

<sup>b</sup> dom = dominant, rec = recessive

<sup>c</sup> Average marker distance in QTL mapping region

<sup>d</sup> The segregation ratio in F<sub>2</sub> between genotypes (*a:h:b*); *a* = homozygous *L. sativa*, *h* = heterozygous, *b* = homozygous *L. saligna*

<sup>e</sup> Note that the detection chance in BILs does not depend on any of the factors that vary between the F<sub>2</sub>-detected QTLs

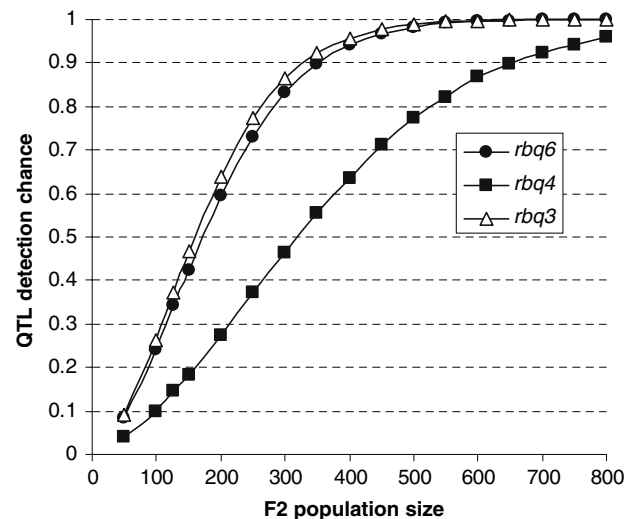
indicates that there is still a small probability that a QTL, which was detected in an F<sub>2</sub>, may go unnoticed in a BIL experiment. Our simulation program is a useful tool to quickly investigate these probabilities for given QTL effects and experimental conditions.

## Discussion

Description of QTLs and their contribution to non-host resistance

Seven QTLs have been detected in an F<sub>2</sub> and a BIL strategy together: *Rbq1*, *Rbq2*, *rbq3*, *rbq4*, *rbq5*, *rbq6* and *rbq7*. Two QTLs are dominant, four QTLs are recessive and the inheritance for one QTL (*rbq3*) is ambiguous. As the majority of the locations of the seven QTLs do not coincide with known *R*-gene clusters in the lettuce genome and the majority of the QTLs are recessive, these QTLs are not likely to be alleles of known *R*-genes or newly detected *R*-genes. Indications of candidate genes for the QTLs and their possible functions will be described elsewhere.

The effects of these seven single QTLs on resistance to *B. lactucae* are rather large, as each of them lowers the infection level to 31–76% of the infection severity of the susceptible parent line *L. sativa* Olof. Stacking two or three QTLs with an additive effect is probably already enough to reach 0% infection severity. Therefore, harboring seven QTLs seems redundant, but can also be an explanation for nonhost resistance: accumulation of several resistance layers by several QTLs with a rather strong effect and possibly present at high (favorable) allele frequencies.



**Fig. 6** The effect of F<sub>2</sub> population size on the QTL detection chances for recessive QTLs that are located in regions with severe distorted segregations. QTLs: *rbq6* = segregation ratio 36:46:9, average marker distance: 3.3. *rbq4* = segregation ratio 30:90:6, average marker distance: 2.3. *rbq3* = segregation ratio 29:49:9, average marker distance: 2.7

## Efficiency of QTL mapping

### Detection of additional QTLs in a set of BILs

The results between the six disease tests on the selections of BILs are very similar, despite the variations between the different experiments that were done over different seasons. This indicates that resistance governed

by these QTLs is stable and rather insensitive to environmental conditions.

Six out of the set of 29 BILs showed a quantitative resistance to both *B. lactucae* races. One QTL is dominant, four QTLs are recessive and the inheritance for one QTL is ambiguous. The four recessive QTLs were mapped at new positions and the other two QTLs were mapped at positions detected earlier in the  $F_2$  population. The quantitative resistances lower the infection level to 31–76% of the infection severity level of the susceptible parent line *L. sativa* Olof. This is remarkable, as each of the three QTLs detected in the  $F_2$  population only showed an average reduction to 68%. This confirms that QTL effects can be measured more precisely in BILs because of the increased accuracy of phenotyping and diminished genetic noise.

Although the heritability was high ( $>0.8$ ) and the environmental variance was low (0.3) in the  $F_2$  population, the three QTLs explained only 51% of the quantitative resistance, indicating that other resistance QTLs still were segregating in the  $F_2$  population. The four newly detected QTLs in the BILs may represent QTLs that went unnoticed in the  $F_2$  population.

#### *Skewness severely decreases detection chance of recessive QTLs in an $F_2$ population*

Usually, it is impossible to verify the causes of the failure to identify QTLs in mapping studies. However, our data are very suitable to study the effect of the most important parameters on the power of QTL mapping. Therefore, we simulated QTL detection on  $F_2$  populations and did a sensitivity analysis on the size of the effects of the most important parameters. By doing so, we showed that the parameters “population size” and “number of QTLs explaining the trait” had the largest effects, while the parameter “skewness” had only a large negative effect if the wild recessive resistance enhancing allele was underrepresented (deficit of *L. saligna* alleles, Fig. 5). This is not surprising, as in this case skewness will decrease the number of recessive homozygotes to far below the proportion of 25% (the average frequency in the case of no segregation distortion). With only a few plants in this marker class, a test for a difference between phenotypic means of marker genotype classes will lose much power. The same underrepresentation of wild alleles (deficit of *L. saligna* alleles) had a slight positive effect on detection chance of dominant QTLs. Taken together, the effects of direction of dominance and segregation distortion explain why we only detected dominant QTLs in the  $F_2$  population (Table 2).

#### *Backcross inbred lines are more efficient and powerful in QTL mapping than an $F_2$ population*

In this study, the set of BILs reveals more QTLs for downy mildew resistance than the  $F_2$  population, and its detection chances for QTLs were higher than that in the  $F_2$  (Table 2). This is in agreement with several comparative studies between AB lines and classic segregating populations (like  $BC_1$ ,  $F_2$ , DH and RILs) in tomato for fruit quality traits (Grandillo and Tanksley 1996; Tanksley et al. 1996) and in barley for agronomic and yield quality traits (Pillen et al. 2004). In a comparative study between NILs and RILs in Arabidopsis for developmental traits, similar numbers of total QTLs were detected between the two populations, although different loci were detected (Keurentjes et al. 2007). Unique QTLs that showed epistatic interactions were detected in the RILs, and unique small-effect QTLs were detected in the NILs. A comparative study between AB lines and RILs in barley for seedling resistance traits showed the same number of QTLs (Yun et al. 2006). Generalizing, we may state that Inbred Line strategies most of the time reveal more QTLs than classical population studies, which is in agreement with theoretical studies (Tanksley et al. 1996).

Most comparative studies cannot address specific reasons for this difference in QTL detection. Based on our simulation study, we can, for two out of four newly detected QTLs, address a reason for being unnoticed in the  $F_2$ : the combination of recessiveness of the trait with skewness against the recessive wild species alleles. A similar reason was suggested for detection of a heading date QTL in a BIL and being unnoticed in the  $F_2$  in a study in rice (Lin et al. 1998). Skewness occurs in many segregation populations, which suggests that in QTL mapping experiments on relatively small segregating populations ( $N < 200$ ), several QTLs may remain undetected. The abundance and degree of skewness in a genetic mapping population is thought to be associated with the level of genomic divergence between parental species (Jenczewski et al. 1997; Taylor and Ingvarsson 2003; Truco et al. 2007). This indicates that in QTL mapping studies, the size of the  $F_2$  or RIL population should increase as the genetic distance between the parents gets larger. This supports the choice for the development of a set of BILs in wide crosses. A set of BILs has more power to detect, especially recessive QTLs, because of its advantageous segregation ratio compared to  $F_2$  or RILs.

The reason why two QTLs that were identified in the BILs were not detected in the  $F_2$  is less obvious. Theoretically, there are three possible causes: (a) a negative *trans*-acting epistatic interaction, (b) the magnitude of the effect is too small to raise the LOD value above the set threshold and (c) just “bad luck.”

By its very nature, epistasis of the type mentioned earlier cannot be removed by experimental set up nor can it be detected by means of a special statistical analysis. Therefore, some QTLs may always be hidden in the “genetic background noise.” Reasons (b) and (c) mentioned earlier are, of course, not mutually exclusive. With an a posteriori detection probability of around 70% (cf. Table 2), the chance to miss two QTLs is 0.09. On one hand this may be considered “bad luck,” but on the other hand this is not extremely unlikely. The reason why one QTL was detected in the  $F_2$  only, and not in the BILs, might theoretically be explained by a positive *trans*-acting epistatic interaction in the  $F_2$ . This study clearly shows the added value of extended genetic studies on two types of populations (BILs and  $F_2$ ) instead of one type only in dissecting complex genetic traits.

The function of these QTLs in nonhost resistance is still completely obscure and awaits further detailed molecular phytopathological studies. This will contribute to our knowledge of nonhost resistance and how it may have evolved during evolution. For the time being, plant breeders can already use these QTLs in their continuous fight to control downy mildew in lettuce.

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## Appendix A

The simulation program to carry out in silico QTL detection experiments was written to answer the following question: What is the probability that a QTL of given effect will be detected by means of a statistical test in an  $F_2$  population or in a set of BILs? To answer this question one has to assume (or to know) the following quantities:

- The genetic variance caused by other QTLs that affect the trait
- The size of the  $F_2$  population and the number of BILs
- The environmental (or error) variance per observation
- The number of observations per  $F_2$  or BIL plant
- The number of plants per BIL and the number of plants per control genotype

## Simulation of $F_2$ experiments

Since QTL detection is based on the notion that a marker closely linked to a QTL will “explain” part of the variance caused by that QTL, one also needs to know the genetic distance between the QTL and the nearest marker. Since we also wished to account for skewed segregation ratios at marker loci (and nearby QTLs), we decided to simulate the QTL mapping procedure rather than following an analytic approach to the question of detection power. Table 3 presents the parameters that are required to run a simulation. Using the parameters mentioned in Table 3, we first partition the variance as follows, using standard results from quantitative genetics.

The additive and dominance effects at the QTL read

$$a = \frac{1}{2}(\mu_{QQ} - \mu_{qq})$$

and

$$d = \mu_{Qq} - \frac{1}{2}(\mu_{QQ} + \mu_{qq}),$$

respectively.

This results in a genotypic variance caused by this single QTL of

$$V_{G(QTL)} = \frac{1}{2}a^2 + \frac{1}{4}d^2.$$

Since we have  $n$  QTL of this same effect, the total genetic variance in the  $F_2$  equals

$$V_{G(\text{tot})} = nV_{G(QTL)}.$$

The genetic background variance caused by the  $(n - 1)$  remaining QTLs equals

$$V_{G(\text{rest})} = \left(1 - \frac{1}{n}\right)V_{G(\text{tot})} = (n - 1)V_{G(QTL)}.$$

Thus, the nongenetic (or error) variance equals

**Table 3** Parameters that are required to run a simulation

Symbol	Description
$\mu_{QQ}, \mu_{Qq}, \mu_{qq}$	Genotypic values of QTL genotypes
$n$	Number of segregating QTLs
$V_e$	Error variance per observation
$k$	Number of observations per plant
$r$	Recombination frequency between QTL and nearest marker
$V_P$	Total phenotypic variance in the $F_2$ population
$f_{MM}, f_{Mm}, f_{mm}$	Frequencies at marker locus (possibly skewed)

$$V_e = V_P - V_{G(\text{tot})},$$

and the error variance attached to the mean of  $k$  observations per plant reads

$$V_E = \frac{V_e}{k}.$$

In the procedure, described step-by-step below, we first sample marker genotypes and then, depending on the marker genotype ( $MM$ ,  $Mm$  or  $mm$ ), we sample the QTL genotype ( $QQ$ ,  $Qq$  or  $qq$ ). These conditional probabilities read

$$\begin{aligned} P(QQ|MM) &= (1-r)^2 \\ P(Qq|MM) &= 2r(1-r) \\ P(qq|MM) &= r^2 \\ P(QQ|Mm) &= r(1-r) \\ P(Qq|Mm) &= 1-2r(1-r) \\ P(qq|Mm) &= r(1-r) \\ P(QQ|mm) &= r^2 \\ P(Qq|mm) &= 2r(1-r) \\ P(qq|mm) &= (1-r)^2. \end{aligned}$$

(Notice that the above implies that in the parents the alleles  $M$  and  $Q$  occur together, as well as  $m$  and  $q$ ).

Marker genotypes are sampled from the given segregation ratio. For each individual sampled, the marker genotype as well as its phenotypic score is recorded. After having sampled the required number of  $F_2$  plants, an analysis of variance is performed, and a test statistic is calculated to test whether or not the differences between the means of the marker classes are significant. To correct for multiple testing (that is to control the type 2 error), we have set the significance threshold corresponding to  $P = 0.005$  (rather than 0.05).

In the following,  $N(\mu, \sigma^2)$  represents a sample from a normal distribution with mean  $\mu$  and variance  $\sigma^2$ .

A single simulation “case” comprises the following steps:

1. Sample a marker genotype from the distribution  $\{f_{MM}, f_{Mm}, f_{mm}\}$  (result is  $MM$ ,  $Mm$  or  $mm$ ).
2. Sample a QTL genotype, conditional on the marker genotype (result is  $QQ$ ,  $Qq$  or  $qq$ ).
3. Set genotypic value (result is  $gv = \mu_{QQ}, \mu_{Qq}, \mu_{qq}$ , according to the result at step 2).
4. Add a random term to  $gv$ , representing the joint effect of the other QTLs:  $G = gv + (0, V_{G(\text{rest})})$ .
5. Add a random term to obtain the phenotypic score (average of  $k$  observations):  $y = G + N(0, V_E)$ .
6. Store marker genotype and phenotypic score.
7. Repeat steps 1–6  $N$  times ( $N = \text{population size}$ ).
8. Test significance between scores of marker genotypic classes.

9. Store outcome of test.
10. Repeat steps 1–9 50,000 times.
11. Calculate relative frequency of significant test results.

### Simulation of BIL experiment

In simulating a BIL experiment, we proceed as follows. Using the same genotypic values for the homozygous *L. sativa* and *L. saligna* genotypes and the appropriate environmental variances, two series of phenotypic values, representing the BIL and control observations, are generated. Note that these two samples are not necessarily of the same size. The difference between the two sample means is tested by a two-sample  $t$  test, using a Bonferroni correction for multiple testing to set the required  $P$ -value (the number independent test being equal to the number of BILs being tested). This procedure is repeated 50,000 times (or as many as the user judges appropriate) and the proportion of significant tests is taken as the detection probability.

### Discussion

In our simulations, we have used an analysis of variance (ANOVA) per marker rather than the widely used methods of interval mapping (Lander and Botstein 1989) or composite interval mapping (Jansen and Stam 1994; Zeng 1994). Although the latter are known to be more powerful than ANOVA at single markers, the difference in power between the two approaches rapidly decreases as marker density on the linkage map increases.

A more important and more practical reason to use the ANOVA approach in the simulations is its relative simplicity as compared to the maximum likelihood procedure of interval mapping. This simplicity also enabled us to provide an easy-to-use simulation program that is available to the scientific and plant breeding community, enabling a very quick exploration of the statistical power of QTL detection in  $F_2$  populations and sets of BILs with various settings of the factors involved.

The program, powQTL, was written in C++; an executable is available from P.S. upon request.

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