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Strategies for the Design and Analysis of Introgression Libraries and Near-Isogenic Lines

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List of Abbreviations

BC, backcross
BH, Bonferroni-Holm
cM, centiMorgan
DCS, donor chromosome segment(s)
DH, doubled haploid
FDR, false discovery rate
IL, introgression library or line
LM, linear model
LOD, logarithm of odds
MAS, marker assisted selection
NIL, near-isogenic line
QTL, quantitative trait locus/loci
S, self
STAIRS, stepped aligned inbred recombinant strains

Chapter 1

General Introduction

Plant breeding, through domestication of wild landraces, led to modern day crop cultivars. The selection of plants for characteristics found to be useful to ancient plant breeders ultimately produced the predecessors of modern day high yielding crops. More recently, advanced breeding practices such as hybrid production have increased yields to levels never before seen in nature. Plant architecture in these crops have been vastly altered, both through domestication and in subsequent plant breeding over the last century. However, domestication and further selection have also narrowed the genetic base of the crops undergoing the breeding process. Removing deleterious, yield-damaging alleles from a population raises the yield potential, whether the crop is a hybrid or an open pollinated variety. It also lessens the populations ability to overcome susceptibility to disease or pest pressure and therefore adapt to new environments or persist in otherwise hospitable locations.

Restriction of diversity due to artificial selection in crop species is generally well-documented. Wright et al. (2005) described that around 1200 genes in maize were affected by selection during the domestication from teosinte. Within geographic regions there are also large selective sweeps (areas of reduced recombination) resulting from regional adaptation (Gore et al. 2009). Studies in wheat indicate a loss of diversity due to domestication, across multiple species/subspecies (Haudry et al. 2007). Genetic variation (Doi et al. 2008; McCouch et al. 2012) and domestication in rice (Izawa et al. 2009) have

also been the focus of multiple studies and reviews (Kovach and McCouch 2008; Sweeney and McCouch 2007). Importantly, the contraction of diversity through selection can have critical results for yield stability. A perfect example of selection-based susceptibility is the maize germplasm containing the Texas cytoplasm (*cms-T*), used for for male sterility. In addition to the sterility, it also conferred a susceptibility to Southern Corn Leaf Blight, discussed on a molecular basis by Levings (1990). Restriction of diversity can lead to negative consequences in yield and yield stability.

Crop improvement through selection requires phenotypic diversity. The ability for selection of phenotypic characteristics to improve a population over time implies heredity through parental genetic contribution. Limited diversity in a breeding pool lowers potential response to selection, and therefore continuously improving genetic diversity within breeding programs is a must. As selection constantly narrows the genetic base of the population, increasing diversity must be an ongoing process in plant breeding. To this end, there have been efforts to monitor the diversity level in crops in recent years (McCouch et al. 2012; Ford-Lloyd et al. 2009).

Plant breeding in the 21st century will increasingly rely on pulling genetic elements from alternative breeding pools in order to continuously improve key traits (Warschefsky et al. 2014). The main focus of improvement is aimed at yield components, but many other traits improve yield stability, such as resistance or plant architecture traits. Mining the diversity available in landraces and related breeding pools has proven significant as a way to find key genes and genetic elements to modify these traits (Tanksley and McCouch 1997). Plant breeders will continue to reach deeper and deeper into the genetic diversity contained in related pools available for various crops in order to find genetic variation for key traits. Often the parent supplying the beneficial alleles is unadapted and introgressing these genes and genetic elements into established breeding lines can require special breeding practices.

There are many examples of the enhancement of crop performance through the introduction of diverse germplasm. To improve drought resistance in rice, 160 cultivars from 25 countries were tested in drought environments and selected accessions were then backcrossed into elite recurrent parents (Lafitte

et al. 2006). In this way, the researchers looked to outside sources of genetic diversity in order to make breeding gains under drought conditions instead of recurrent selection of the same elite population. Wild accessions have also been used to improve other traits, such as aluminum tolerance in rice (Nguyen et al. 2003) or cold tolerance in sorghum (Knoll and Ejeta 2008). Mining the diversity of wild accessions has the potential to improve many traits (Flint-Garcia et al. 2009; Buckler et al. 2006; Kovach and McCouch 2008). Researchers are also designing specific experimental designs and methods which allow the influx and genetic variation to uncover beneficial alleles that may occur in diverse material (Johal et al. 2008; McMullen et al. 2009; Zhang et al. 2007).

It has been theorized that hybridizations between early crops and wild relatives occurred during domestication (Koornneef and Stam 2001). These random outcrosses increased the available genetic diversity available to early farmers and undoubtedly some beneficial traits were obtained and selected, however unlikely and infrequent. Early in the 20th century, the scientific community was introduced to the idea that domesticated crops could benefit from natural diversity in wild relatives (Bessey, 1906). Currently, under a more controlled setting, breeders are again turning to these wild relatives and crop progenitors to harness natural allelic diversity in the hopes of further crop improvement. A variety of methods are available for introgressing genetic elements into established crop lines, and perhaps chief among these is backcrossing.

Developing acceptable cultivars for commercial release takes years of crossing and testing, and any reasonable methods to reduce this time are often the subject of research. In particular, introgression of traits from unadapted or exotic germplasm can be particularly time-intensive, though can also be quite valuable as the selection in adapted materials can lack the genetic diversity required for advancement (Tanksley and Nelson, 1996). Backcrossing can substantially reduce this amount of time by maximizing the genome contribution of an elite parent (referred to as the recurrent parent) and minimizing the genome contribution of the donor parent (the unadapted or exotic line containing the trait of interest). This approach can therefore allow for introduction and testing of alleles from unadapted materials in already adapted elite germplasm, shortening the necessary time between detection and intro-

gression into breeding pools. The genetic distance between the recurrent and donor parent is a key factor in determining the number of backcrosses necessary to produce an acceptable cultivar. For instance, six backcrosses has been suggested as being sufficient for most programs though as the genetic unrelatedness of the parental lines increase, more backcrosses are potentially required (Allard 1960).

Molecular Breeding

With the development and subsequent use of molecular markers, plant breeders and researchers saw possibilities for a deeper understanding or trait inheritance as well as a method for reducing time required for line development. The first true molecular marker map was constructed for the tomato in 1987 (Bernatzky and Tanksley). Over the years, the techniques have evolved and improved and marker data points have become cheaper.

Marker-assisted selection (MAS) has emerged as a powerful technique, capable of using genotypic certainties, rather than relying on phenotypic ambiguities, to choose and advance superior lines in breeding programs of every crop (Lande and Thompson 1990). When using markers to recover the recurrent parent genome, the technique is referred to as marker-assisted backcrossing (MABC) and has been used extensively in introgression experiments (Steele et al. 2006, Iftekharuddaula et al. 2011, Zhao et al. 2011). It has also been explored via simulation study (Herzog and Frisch 2011) and the consensus is that MABC shortens the number of necessary generations to increase the recurrent parent genome to acceptable levels. However, an important step in MAS is the initial detection of marker linked quantitative trait loci (QTL) to be used as targets for population improvement through introgression.

Traditional QTL experiments have involved biparental populations, and the statistical methods behind the analysis of these designs are well developed (Haley and Knott 1992; Zeng 1994; Zeng 1999). These methods are often designed to encompass a biparental cross advanced to an F2 or more advanced selfed generation. When only two parents are used to create mapping popu-

lations, the two lines used as parents may not be segregating for a gene that can have a large effect, by is not detected in a given cross. The failure to detect a QTL for this reason has been referred to as a genetic drift error (Xu 1996). Despite the size of the mapping population or the size of effect, a non-segregating allele will not be detected in a QTL experiment. Using only a single F2 generation derived from a single cross to create a mapping population can further limit the finding of QTL (Xu 1998). These concepts have led to designs involving multiple strategies to enhance detection of QTL and reduce genetic drift error.

Previous strategies include using multiple families of line crosses (Xu 1998) or full-sib families with various designs and multiple parents (Muranty 1996). The Nested Association Mapping (NAM) design (McMullen et al. 2009) uses multiple diverse lines crossed to the same parent, and the individual populations of these crosses can be analyzed jointly (Li et al. 2011). Multi-parent advanced generation inter-cross (MAGIC) populations derive a diverse population from a set of staged intercrosses, which can then be used for fine-mapping genes (Johal et al. 2008; Bandillo et al. 2013). Likewise, the analysis methods have evolved to match the multi-parent nature of contemporary mapping populations. Methods used to analyze genome-wide association studies (GWAS), for instance, rely on mixed models which include population structure and kinship estimates between included genotypes (Yu et al. 2008; Kang et al. 2008; Zhang et al. 2010). This methodology has been used frequently in recent years to analyze diverse populations with cryptic substructure (Li et al. 2013; Cook et al. 2012; Brown et al. 2011). Even multi-location QTL designs have been created to explore allele by environment effects (van Eeuwijk et al. 2010), which can enhance detection of yield stabilizing (or destabilizing) genes in regionally adapted populations.

Studies have been performed regarding the repeatability of QTL results (Schön et al. 2004). There are indications that QTL can be population dependent, and therefore not show stable effects in different backgrounds. In many cases, introgression of QTL into breeding populations follows a two stage approach. First, QTL are detected in biparental mapping populations between parents that show a large difference in the trait of interest. Then in the second stage, large effect QTL are introgressed into a breeding pool for population

improvement.

Due to the possibility that detected QTL may be population or background dependent (Beavis et al. 1991; Orf et al. 1999; Schön et al. 2004), it has been suggested to couple QTL detection and introgression into a single step (Tanksley and Nelson 1996; Ramchiary et al. 2007). This involves crossing an established, stable elite cultivar with a variety showing favorable phenotypes for the trait of interest, though often poor agronomically performing otherwise. This can be extended to crosses between elite line and progenitors of modern populations (to recover variation lost to selection) and even wild relatives. When crosses are made between elite lines and wild relatives, it is important to regain a high percentage of elite parent genome as soon as possible, for evaluation and line development purposes. Molecular markers hasten this recovery, allowing breeders to select traits (via linked markers) from the unadapted wild relative genome with foreground selection while retaining if possible and then regaining a high percentage of the adapted elite parent genome (Tanksley and Nelson, 1996). The use of markers to keep the introgressions limited to only the essential genetic elements from the donor parent reduces the likelihood of introducing genes of negative effect, referred to as linkage drag (Tanksley et al. 1989).

Introgression Libraries

One specific method of assessing the genetic potential of wild relatives is to attempt to introgress entire genomes of wild relatives into established lines in small segments. This assembly of lines, commonly referred to as an introgression library, enables breeders to create a permanent resource for crop improvement. Using molecular markers, breeders can estimate the size of the donor parents genetic contribution. An elite breeding line utilized as the genetic background creates a readymade framework for line development. Libraries containing small donor introgressions also help to reduce sterility issues that can arise between wide crosses, or crosses containing genetically dissimilar wild relatives.

Introgression libraries were first proposed by Eshed and Zamir (1995). The concept was adapted from earlier work by Wehrhahn and Allard (1965) using backcross inbred lines (BILs) to measure the effects of individual QTL. In the Eshed and Zamir (1995) study, an introgression library was created which contained single *Lycopersicon pennellii* donor segments in a homozygous state, defined by RFLP markers, in a background of *Lycopersicon esculentum.* In this way, the entire *L. pennellii* genome, which is a wild species tomato relative, was represented in a cultivated tomato background. This approach allowed Eshed and Zamir to overcome fertility problems in general since each line contained only a comparatively small fragment of wild donor genome. Since the initial study, introgression libraries have been used to improve a variety of traits in many species. Such experiments include (but are not limited to) improving yield in rice (Cheema et al. 2008), disease resistance in wheat (Leonova et al. 2007) and barley (Schmalenbach et al. 2008), drought tolerance in wheat and barley (review, Nevo and Chen, 2010), quality traits in barley (Schmalenbach and Pillen, 2009) and rye (Falke et al. 2009b), and agronomic traits in barley (Schmalenbach et al. 2009) and rye (Falke et al. 2009b).

The popularity of introgression libraries, particularly for crop development and breeding, stems from two main benefits. The first reason involves the detection of QTL in introgression libraries. Significant effects from donor segments can often be unambiguously linked to a specific introgression line and hence a specific stretch of donor genomic introgression. Further testing is often needed to determine if the introgression in question is in fact contains the causative allele. This is because small introgressions may be present elsewhere in the line and not detected because of insufficient marker density. Subsequent generation testing can easily remedy this by determining the association strength between introgression and trait. The second reason is that once a beneficial donor segment is found, it can be easily assimilated into a breeding population or elite line. As stated above, often the recurrent parent is in fact an elite line. Further backcrossings and testing can further refine the position of the gene of interest. The locational information can help ensure that the causative allele is successfully transmitted to subsequent generations. It also can reduce the effects of linkage drag, or the introduction of negative or harmful alleles that reduce agronomic fitness of the line.

Introgression libraries often contain a series of lines, chosen according to introgression size and location, with little or no overlap of donor genome between lines. An alternate method to create introgression lines is to use advanced backcrossing. This method, combined with molecular markers, is more of a random approach to genome introgression than the creation of introgression libraries. By continually backcrossing to an advanced generation, BC3 for example, populations similar to a standard introgression library can be achieved. Markers can be used to validate the location and size of introgressed donor genome segments.

Objectives

Previous work regarding QTL detection has found that introgression libraries, in comparison to recombinant inbred line populations, are able to detect smaller QTL but suffer from problems of QTL localization (Keurentjes et al. 2007). However, QTL detection methods in introgression libraries are not as robust as those in traditional QTL populations. Oftentimes, the Dunnett test is used to compare each NIL with the recurrent parent, to determine if the phenotype for the trait of interest is significantly different. When each NIL contains only a single introgression, this produces clear results. However, often this is not the case, and each introgression line has several segments spread out over the recurrent parent genome background. Identifying the causal variants in this case can be challenging in most cases, and misleading depending on the layout of the donor segments. If another test procedure could be implemented that would retain the same high power but also increase the ability to locate QTL, this would be an overall improvement of QTL mapping in introgression libraries. In addition, the number of markers as well as the cost is increasing the amount of marker scores available to the average QTL analysis. The number of effects to estimate is rapidly outpacing the number of genotypes in QTL studies, due to cost, seed amounts, and available plot space.

The main goal of this research was to explore solutions for introgression library analysis. To accomplish this, we set the following objectives:

1. Compare the linear model method proposed in Falke and Frisch (2011) with the current standard analysis used in introgression library analysis (Dunnett test). Within this comparison, explore the performance of multiple tests on different introgression library designs, specifically libraries with non-overlapping and overlapping segments, as well as the STAIRS design.

2. Make the comparisons across a range of data, such as different simulated quantitative trait loci scenarios (QTL sizes and numbers) and heritabilities, as well as experimental data. When the method is determined, use it to analyze previously analyzed introgression libraries and compare the results.

3. Extend the principles of the analysis to introgression libraries with more markers than genotypes (over-parameterized libraries). Explore the possible methods available for estimating effects in over-parameterized data, such as mixed modelling. In addition to then selecting and testing methods, develop a test sufficient to supply p-values to the marker estimates.

4. Use the selected method to analyze experiments with high marker density. Compare the results with previous analyses if available.

Chapter 2

A comparison of tests for QTL mapping with introgression libraries containing overlapping and nonoverlapping donor segments

Mahone G., Borchardt D., Presterl T., Frisch M. (2012) Crop Sci 52:2198-2205

NOTE

A Comparison of Tests for QTL Mapping with Introgression Libraries Containing Overlapping and Nonoverlapping Donor Segments

Gregory S. Mahone, Dietrich Borchardt, Thomas Presterl, and Matthias Frisch*

ABSTRACT

Near-isogenic line (NIL) libraries can be used to detect beneficial trait variation in germplasm that is unadapted or has poor agronomic performance. The objectives of our study were to compare the *t* test, Dunnett test, and linear model test with regard to the power and false positive rate of quantitative trait loci (QTL) detection in NIL libraries of different design. We employed computer simulations with maize genome models to investigate nonoverlapping NIL libraries, overlapping NIL libraries, and stepped aligned inbred recombinant strains (STAIRS) libraries for traits with oligogenic inheritance. Quantitative trait loci detection power of the linear model and Dunnett tests were similar for nonoverlapping and STAIRS libraries; for overlapping NIL libraries the Dunnett test was slightly superior. False positives were greatest for the *t* test and lowest for the linear model test. False positive sums with the Dunnett test were generally higher than for the linear model test if the heritability was 0.9 or lower. We conclude that the linear model test is superior to the Dunnett test for nonoverlapping NIL libraries and for overlapping NIL libraries with heritabilities below 0.9, as usually occur. Analysis of a rapeseed (*Brassica napus* L.) library revealed two other major advantages of the linear model test. First, detection of positive and negative QTL effects present in the same line is possible. Second, for NILs with multiple donor segments, observed phenotypic differences can be assigned to individual chromosome segments.

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Abbreviations: BH, Bonferroni-Holm; DH, double haploid; FDR, false discovery rate; NIL, near-isogenic line; STAIRS, stepped aligned recombinant inbred strains; QTL, quantitative trait locus (or loci).

Crop domestication has had a narrowing effect on the genetic variation existing in many species, to the point that harnessing the natural variation prevalent in nonadapted exotic germplasm is increasingly important for improving yield, quality, and resistance (Gur and Zamir, 2004; McCouch, 2004). To uncover and exploit trait variation in exotic by elite crosses, near-isogenic line (NIL) libraries, also referred to as introgression libraries, are a powerful tool in plant breeding. Near-isogenic line libraries have proven useful for investigating yield in rice (*Oryza sativa* L.) (Cheema et al., 2008) and tomato (*Lycopersicon esculentum* Mill.) (Eshed and Zamir, 1995), disease resistance in wheat (*Triticum aestivum* L.) (Leonova et al., 2007) and barley (*Hordeum vulgare* L.) (Schmalenbach et al., 2008), drought tolerance in wheat and barley (review, Nevo and Chen, 2010), metabolites in tomato (Rousseaux et al., 2005) and maize (*Zea mays* L.) (Yang et al., 1995), quality traits in barley (Schmalenbach and Pillen, 2009) and rye (*Secale cereale* L.) (Falke et al., 2009), flowering time in maize (Szalma et al., 2007), and agronomic traits in barley (Schmalenbach et al., 2009) and rye (Falke et al., 2009).

Introgression libraries consist of NILs that contain donor segments in a background of recurrent parent genome. The

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introgressed segments are typically short stretches of donor genome, which may overlap in successive NILs depending on the aims of library construction. An alternative to typical NIL libraries is the stepped aligned inbred recombinant strains, or STAIRS, library (Koumproglou et al., 2002). The STAIRS library contains donor segments of increasing size, starting from small donor segments to entire donor chromosomes. This pattern is repeated for each chromosome. The advantage of this design is that it is easier to produce than typical NIL libraries. However, to our knowledge no one has investigated the performance of QTL analysis in STAIRS libraries compared with conventional NIL libraries with smaller targeted introgressions.

Analysis of introgression libraries typically involves a series of pairwise tests between the NILs and the recipient parent for the trait in question (Eshed and Zamir, 1995; Rousseaux et al., 2005; Eduardo et al., 2007; Schmalenbach and Pillen, 2009; Falke et al., 2009). A recent paper by Falke and Frisch (2011) proposed an alternative testing procedure, in which a linear model was used to estimate the effects of the segments directly. The study examined the differences in sums of correctly detected effects and false positive effects in NIL libraries with either nonoverlapping or overlapping segments. Results were based solely on the proposed linear model test but do not include a comparison with the pairwise tests that have been previously used. However, the efficiency of QTL detection might well depend on the statistical test used. While methods employing linear models and regression methods have been previously used to locate QTL in introgression libraries (Wang et al., 2006; Wang et al., 2007; Coles et al., 2011), the effect of the type of test used to identify QTL in NIL libraries has not yet been investigated.

The objectives of this study were to (i) compare the sums of correctly detected and false positive effects for pairwise *t* tests, the Dunnett test, and the linear model test in QTL detection with introgression libraries, (ii) compare the statistical properties of the tests for overlapping and nonoverlapping NIL libraries and STAIRS libraries, (iii) propose suitable tests that may enhance the precision of QTL detection in NIL libraries depending on the heritability and the amount of segment overlap, and (iv) validate our simulation results with experimental data of a rapeseed (*Brassica napus* L.) introgression library.

MATERIALS AND METHODS

Simulations

A model of the maize genome comprising 10 chromosomes of 160 cM length was used for our simulations. Linkage maps with marker distances (*d*) of 20, 10, and 5 cM were investigated for three types of introgression libraries: nonoverlapping libraries, overlapping libraries, and STAIRS libraries (Fig. 1). Nonoverlapping libraries contained donor segments that are contiguous but do not overlap. Overlapping libraries contain segments that are each present in two NIL lines. For STAIRS libraries, each chromosome was divided in parts of equal length. The first of the lines that covered the genome of a chromosome carried one such segment located at the telomere. The second line carried in addition the chromosome segment directly adjacent to the first one. For each subsequent line, a further segment was added, such that the last line contained the donor genome of the entire chromosome. Ten recipient parent plots per replication were included in phenotyping, as justified in Falke and Frisch (2011). The software Plabsoft (Maurer et al., 2008) was used for the simulations. Each simulation run was repeated with heritabilities of 0.5, 0.6, 0.7, 0.8, 0.9, and 0.9999. Therefore each simulation run incorporated the type of introgression library, marker distance *d*, quantitative genetic scenario, and heritability. All simulations were repeated 5000 times to ensure high numerical accuracy and reduce the effects of sampling.

Quantitative Genetic Models

We considered a polygenic trait and assumed that the genotypic value of the donor parent is 100 units superior to that of the recipient parent. The trait was controlled by major genes, minor genes, and genes with small effects. In all scenarios, 10 genes with small effects of size 1 were assumed. The remaining difference between donor and recipient was assigned to major and minor genes in four different scenarios (Table 1). These differed in the number n_a of major and n_i of minor genes, and their effect sizes s_a and s_i, respectively. The genes with small effects were included as background or stochastic noise, as it is unrealistic to assume that all genetic effects underlying a quantitative trait can be modeled and/or detected. The sizes of major and minor effects intended to model oligogenic resistance or quality traits. For each simulation run, genes were assigned to a different set of random locations in the genome.

QTL Detection

We employed pairwise tests and a linear model test to investigate the presence of QTL on donor segments in the NIL libraries. The pairwise testing methods consisted of comparisons between each NIL and the recipient parent. The rationale is that since each NIL contains a single donor segment, differences in phenotype between each NIL and the recipient parent can be attributed to the presence of the donor segment. Pairwise testing consisted of two methods, *t* tests and the Dunnett test (Dunnett, 1955). Pairwise *t* tests were performed with and without adjustment for multiple testing. In unadjusted tests, the per-comparison type I error rate was 0.05. Multiple comparison adjustment of tests followed two procedures: (i) the Bonferroni–Holm (BH) procedure proposed by Holm (1979) for an experiment-wise type I error rate of 0.05 and (ii) the procedure proposed by Benjamini and Hochberg (1995) for a false discovery rate (FDR) of 0.05. For STAIRS libraries, we used the standard error of a difference of treatment means for a Dunnett type comparison of two subsequent lines. Line genotypic values (and by extension the effect of the segment in the line) were calculated from genotypic values of previous lines in the library. To calculate *p* values for the Dunnett test, we used the density function of the multivariate normal distribution provided by the R package *mvtnorm* (Genz et al., 2011).

The linear model test consisted of estimating the effects of donor segments with a linear model. An *F* test was subsequently

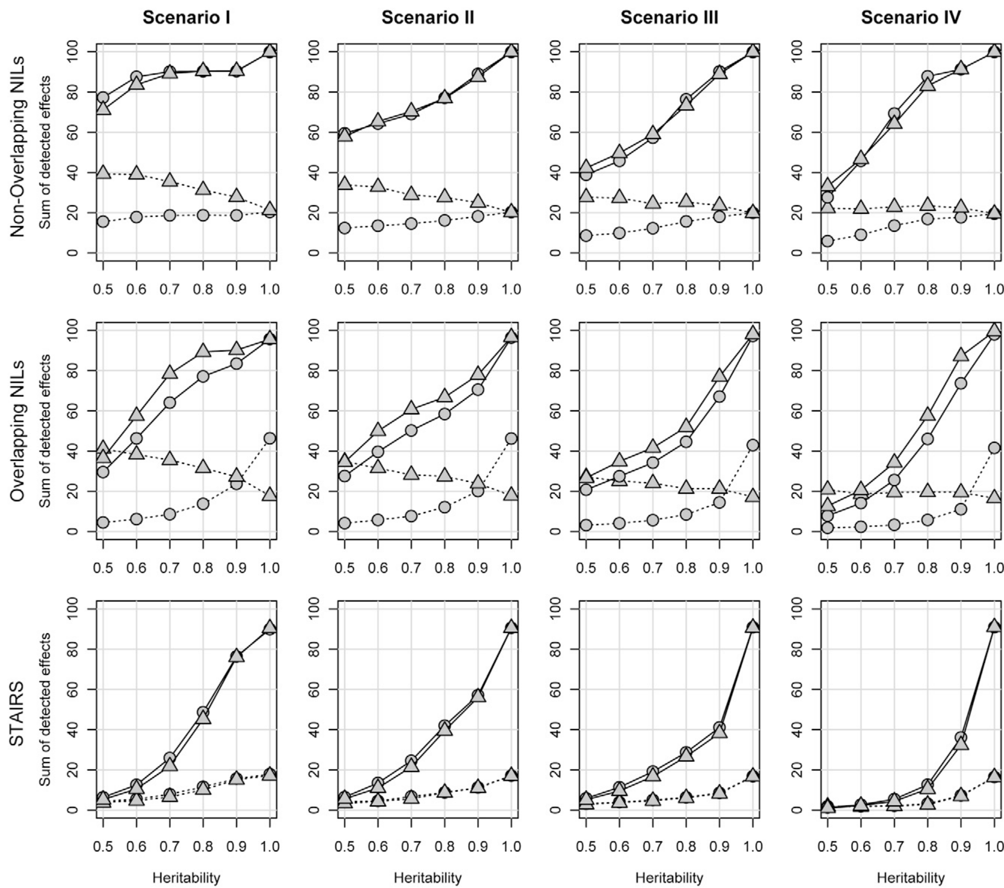

Figure 1. Genomic composition of the different types of near-isogenic lines libraries. Donor segments are indicated with black and recipient parent genome with gray lines. The dashed lines flanking the donor segments are genomic segments of unknown parental origin, located between markers at the end of the donor segment and flanking markers. Marker distance is 5 centiMorgans (*d*).

conducted for every segment to determine significance. Multiple testing adjustments for results from the linear model test were made using the BH procedure. The linear model test procedure was used in total as described by Falke and Frisch (2011).

For our analysis, we determined the sum of correctly detected effects and the sum of false positive effects (false positive rate) of each test to have measures of their efficiency. The sum of correctly detected effects, our measure of test detection power, was calculated by summation of the true QTL effects of segments for which the null hypothesis was correctly rejected and was collected for the total, major, minor, and small effect QTL. True QTL effects were used rather than the estimated effects to avoid bias due to overestimation of QTL effects. The false positive rate was calculated by the summation of all detected QTL effects of segments for which the null hypothesis was incorrectly rejected. A false positive for the Dunnett test was declared when a NIL was found significant despite containing no QTL. We avoided using the terms type I and type II error because not only statistical sampling contributes to these errors. In addition, "genetical sampling" due to QTL located on chromosomal segments adjacent to the target regions is also present in our measurements for success or failure of QTL detection.

Table 1. Quantitative genetic scenarios. Number (n_a and n_i) and effect size (s_a and s_i) of major and minor genes, respectively, for four scenarios.

Scenario	Major Genes		Minor Genes	
	n_a	s_a	n_i	s_i
I	3	30	–	–
II	2	30	3	10
III	1	30	6	10
IV	–	–	9	10

Validation with Experimental Data

To validate our results from simulations, we analyzed an introgression library in rapeseed using the Dunnett test and the linear model. The rapeseed introgression library was contributed by KWS SAAT AG, Einbeck, Germany. The library is a BC_4 double

haploid (DH) population created from the crossing of the winter rapeseed varieties 'Mansholt's Hamburger Raps' and 'Samourai' and was grown in randomized field trials over 3 yr. The same parental cross combination has been previously investigated for various trait QTL (Uzunova et al., 1995; Marwede et al., 2005). Glucosinolate content (μmol/g), measured using near-infrared spectroscopy, was collected from five locations each in 2006 and 2007 and a single location in 2008. The heritability was above 0.9, which is in accordance with previous studies (Marwede et al., 2004). The recipient parent was included repeatedly in field trials. The linkage map consisted of 176 amplified fragment length polymorphic markers and had a length of 1361 cM, resulting in an average marker distance of 6.8 cM. The NIL library contained 127 lines. Each NIL carried at least one donor segment, the average number of donor segments was between two and three. The average segment length was 21.6 cM and the donor genome coverage of the library was 87.7%. Regarding comparison with our simulation libraries, the rapeseed library would most resemble an overlapping library, though it also has aspects of the nonoverlapping (segments present in only a single line) and STAIRS (segments present in multiple lines) libraries.

The model used for the Dunnett analysis was:

$$Y_{ijk} = \mu + G_i + L_j + Y_k + e_{ijk}$$

where Y_{ijk} is the glucosinolate content of genotype G_i at location L_j in year Y_k, with a grand mean of μ and residual error e_{ijk}. The Dunnett test was incorporated using PROC GLM of SAS software version 9.2 (SAS Institute Inc.). A detailed description of the linear model that was used to estimate and test the effects of individual chromosome segments was presented by Falke and Frisch (2011). Calculations were performed with R (R Development Core Team, 2011).

RESULTS

Total detection power was similar for the linear model and Dunnett tests for each of our three simulation sets. The *t* tests, which were included only in the nonoverlapping set, also had similar detection power (results not shown). The Dunnett test generally had a higher power of detection in the overlapping library set. Overall, detection power was directly related to heritability and QTL effect size, an expected result. Power decreased overall as the QTL component of the libraries moved from a few large-effect QTL (major QTL; Scenario I) to many QTL with smaller effects (minor QTL; Scenario IV). Within each scenario, the presence of major QTL lowered the power of both tests at low heritabilities. As the number of major QTL decreased across scenarios, detection of major QTL increased at these low heritabilities. Detection power of minor QTL also increased overall as major QTL number decreased. For both tests, increasing degree of introgression overlap negatively affected power of detection.

In the nonoverlapping library set, false positives decreased across all tests with decreasing marker distances *d* (Table 2). Increasing heritabilities caused consistent decreases in the false positives for the FDR adjusted and the unadjusted pairwise tests across all marker distances and scenarios. At small marker distances (d = 5 cM), the pairwise tests showed decreasing false positives with increasing heritabilities. In contrast, the linear model test showed an increase in false positives as heritabilities increased, though these values were much lower than those of the pairwise tests at low heritabilities. At the highest heritabilities, false positive rates were similar for all tests. The *t* tests were excluded from comparison in the overlapping and STAIRS library sets because of their high false positive rates in the nonoverlapping library set.

Overall, false positives generally decreased with decreasing genetic variance, for example as QTL effect sizes decreased and as heritability increased, with the exception of the linear model test (Fig. 2). The linear model showed generally lower false positive rates than the Dunnett test in the nonoverlapping library and the overlapping library excluding high heritabilities, with similar rates as the Dunnett test found in the STAIRS library. Marker density also affected false positives, as the introgressed segments can be more clearly defined (Table 2). This lowers the chance that a QTL will be outside the marker-defined segment to which the QTL is ascribed.

In the rapeseed library, the Dunnett test detected 26 NILs that had a significantly different glucosinolate content than the recipient parent (Table 3). Eight of those carried a single donor introgression. The remaining carried between two and six introgressions, with the most common number of introgressions being three. All significant lines had glucosinolate contents greater than that of the recurrent parent, with an average difference in means of 22.6. The linear model test found 15 separate significant donor introgressions, varying in length from one to four markers. One to six introgressions were present in 54 NILs. On four occasions, positive and negative QTL located in close proximity were detected with the linear model test. Most of the lines containing these contrasting-effect QTL were not significant in the Dunnett test results.

DISCUSSION

Statistical Tests

Our results confirm that the Dunnett test is better suited for analyzing NIL libraries than pairwise *t* tests. Even with adjustment for multiple testing, the *t* tests had a considerably greater false positive rate (Table 2). A further increase in the precision of QTL detection is expected with the linear model analysis, in particular for libraries with some chromosome regions duplicated in more than one NIL, as in the libraries of previous studies (Eduardo et al., 2007; Falke et al., 2008). The advantage of the linear model test is likely due to a more precise estimation of the residual variance by using the entire library rather than the recipient parent and the introgression line under consideration.

Detection of a QTL depends on the amount of genetic variance that can be attributed to the QTL compared with

Table 2. Sum of false positive effects in maize (*Zea mays* L.) near-isogenic lines libraries with nonoverlapping donor segments for varying marker distances (d), heritabilities (h^2), and quantitative genetic scenarios (I–IV). The testing methods are as follows: LM, linear model test; DT, Dunnett test; PW_n, unadjusted pairwise t test; PW_{fdr}, pairwise t test adjusted using false discovery rate; PW_{bh}, pairwise t test adjusted using Bonferroni-Holm. Each sum of false positive effects is a mean value from 5000 simulation runs.

		h^2						h^2					
d	Test	0.5	0.6	0.7	0.8	0.9	1	0.5	0.6	0.7	0.8	0.9	1
		Scenario I						Scenario II					
20	LM	48.0	62.8	71.9	75.5	77.3	85.0	39.0	47.2	52.6	58.5	68.8	81.3
	DT	85.2	87.1	91.3	91.7	87.5	85.7	71.1	72.5	74.1	75.7	78.4	82.7
	PW_n	371.3	325.3	278.0	226.1	179.4	87.5	324.7	275.8	242.0	200.2	161.8	84.7
	PW_{fdr}	215.8	203.7	179.4	160.5	130.5	86.8	191.5	166.5	156.1	141.6	125.1	83.9
	PW_{bh}	92.1	95.6	98.9	94.2	90.8	84.3	82.9	80.3	82.8	81.8	83.7	83.7
10	LM	28.7	34.2	36.7	37.5	37.6	41.3	22.0	25.3	27.7	30.5	34.8	39.7
	DT	55.5	55.0	55.1	49.9	47.0	41.4	47.2	47.1	45.3	42.8	42.6	40.2
	PW_n	304.7	259.1	218.2	171.0	127.9	43.6	265.3	227.3	187.6	151.2	114.8	42.2
	PW_{fdr}	172.1	148.0	128.4	108.3	83.6	42.9	147.6	122.3	106.4	94.5	77.1	41.2
	PW_{bh}	67.9	61.7	59.3	54.5	49.0	41.1	56.9	54.8	50.7	47.4	45.4	40.3
5	LM	15.5	17.8	18.6	18.7	18.6	20.3	12.3	13.5	14.5	16.1	18.1	20.3
	DT	39.4	39.0	35.5	31.3	27.8	21.3	33.8	32.8	28.8	27.6	25.0	20.2
	PW_n	275.2	221.1	182.1	144.1	103.0	23.2	233.5	196.5	160.2	126.1	91.6	22.4
	PW_{fdr}	136.1	112.2	99.6	83.8	63.3	21.9	120.7	100.5	85.6	76.1	56.3	21.6
	PW_{bh}	50.8	45.0	42.8	36.8	30.6	21.4	40.6	39.2	34.7	31.4	27.8	20.4
		Scenario III						Scenario IV					
20	LM	27.9	33.6	41.2	52.9	68.5	80.4	17.6	27.1	40.9	58.6	69.7	78.8
	DT	54.4	57.9	60.9	65.7	74.7	80.6	42.3	48.5	56.5	66.5	74.7	78.6
	PW_n	274.5	233.0	208.4	176.6	143.8	82.9	218.3	194.1	168.9	148.1	123.1	79.9
	PW_{fdr}	152.6	141.5	132.3	124.4	115.0	82.6	124.3	123.6	120.1	115.8	105.4	79.4
	PW_{bh}	65.5	66.9	69.8	72.8	79.2	80.9	51.9	54.8	62.3	69.8	78.4	78.8
10	LM	15.1	17.7	21.6	28.1	33.9	38.3	10.1	15.9	23.8	31.6	34.5	38.3
	DT	38.1	36.8	37.8	38.9	41.0	39.7	29.4	31.0	34.4	37.4	39.1	38.3
	PW_n	221.0	187.1	155.1	130.3	99.0	40.6	173.0	149.3	124.2	106.4	82.3	39.3
	PW_{fdr}	117.4	102.7	93.7	84.5	72.5	40.5	95.3	87.8	83.3	75.1	64.0	38.8
	PW_{bh}	45.6	44.8	42.2	43.6	42.5	39.1	35.9	36.8	37.5	41.3	40.7	38.0
5	LM	8.5	9.8	12.2	15.6	18.0	19.9	5.8	9.0	13.5	16.8	17.6	19.4
	DT	27.7	27.3	24.5	25.3	23.6	19.8	22.3	21.9	22.9	23.4	22.4	19.5
	PW_n	192.6	162.3	135.4	106.2	77.4	21.6	150.7	123.6	103.7	84.8	62.6	20.5
	PW_{fdr}	101.9	84.0	76.9	65.4	49.6	20.5	83.2	71.3	66.1	56.6	44.3	20.1
	PW_{bh}	35.0	32.1	29.1	28.1	26.0	20.1	27.9	26.3	25.7	26.7	24.2	19.5

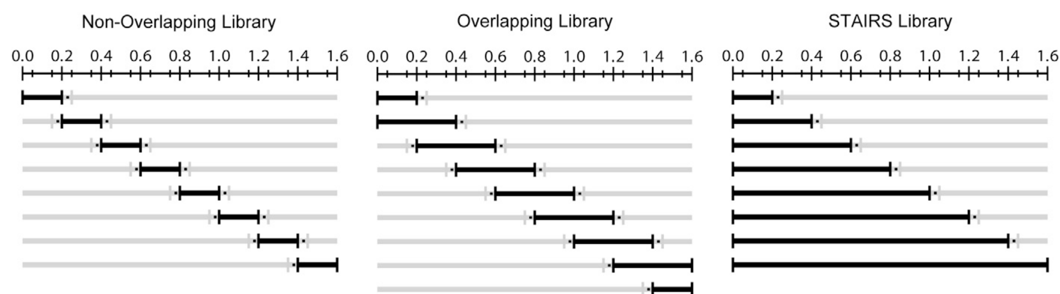

Figure 2. Sums of correctly detected effects (solid line) and false positive effects (dotted line) in different maize (*Zea mays* L.) introgression library types (d = 5 cM) and across four genetic scenarios. The graphs compare the linear model test (circle) with the Dunnett test (triangle).

the total variance in the experiment. The four scenarios (Table 1) show a progression from few QTL of large effect to many QTL of smaller effect. As the individual QTL decrease in effect size and increase in number, the variance explained by a single QTL decreases. Likewise, decreasing heritability also decreases the relative variance that a single

Table 3. Lines containing donor segments found to be significant for glucosinolate content in the rapeseed (*Brassica napus* L.) introgression library. Linear model: All lines carrying significant segments are listed and the significant segments are shown. Dunnett test: All significant lines are presented and all donor segments that are contained in those lines are shown.

Linear model		Dunnett test	
Line	Segments	Line	Segments
9	98,119,142	9	98,119–125,142
44	142	–	–
47	128	47	127–128
50	119,142	50	119–127,142
55	98,119,142	55	98,119–128,142
58	162–163,165–166	–	–
59	162,165–166	–	–
117	153,163,165–166	117	34–35,153,163–166
124	82	124	82
172	98	–	–
189	142	–	–
203	119	203	56–57,62,119–125
212	80–82	–	–
227	165–166	227	56–57,62,165–166
257	163,165–166	257	83–85,163–166
258	163,165–166	258	149,163–166
260	163,165–166	260	163–166
261	119,142	–	–
262	98,119,142	–	–
263	98,158	263	98,158
264	119,128	264	119–128
265	119	265	119
280	153	–	–
287	153	–	–
293	48,80–82,165–166	293	48,80–82,112–115,165–166
294	153,163,165–166	294	87–91,132,134,136–137,153,163–166
296	128,142	296	128,142
367	98	–	–
387	165–166	387	53,55–57,62,165–166
430	153,163,165–166	430	32,112,149,153,163–166
496	80–82	–	–
498	80–82,153,163,165–166	498	80–91,153,163–166
499	163,164–165	499	34–35,83–88,90–91,163–166
576	163,164–165	576	163–166
578	38–41	–	–
641	48	–	–
789	38–41	–	–
814	158	–	–
842	38–41	–	–
864	163,165–166	864	83–85,149,163–166
873	38–41	–	–
875	80–82	–	–
877	80–82,128	–	–
1036	48	1036	48,111,113–115
1150	4,39–40,48,60,103–106,108–110	–	–
1155	39–40,82,108–110	–	–
1157	103–106,108–110	–	–
1158	48,103–106,108–110	–	–
1196	48,80–82,128	–	–
1204	142	–	–
1332	48	–	–
1373	48	–	–
–	–	1395	159–161
–	–	1397	159–161
1433	142	–	–
1548	48,103–106,108–110	–	–

QTL explains. This decrease in the variance explained by individual QTL is a contributing reason for the observed decrease in power. Our results indicate that these factors, as well as the number of times a QTL is present in the library, all contribute to the variance and therefore affect detection. For instance, power of detection was highest with nonoverlapping NIL libraries and few major genes (Scenario I) for both the linear model test and the Dunnett test (Fig. 2). Although overlapping NIL libraries and STAIRS libraries may have advantages owing to the reduced efforts for establishing the library, we conclude that these advantages come at the cost of a considerably lower power of QTL detection. This is especially true for minor-effect QTL, which in some cases may be the focus of introgression line population development. Falke and Frisch (2011) reported a considerable lower power of QTL detection with overlapping rather than with nonoverlapping NIL libraries employing the linear model test, and our findings extend those results also to STAIRS libraries.

With increasing heritability, the false positive rate increased for the linear model test and decreased for the Dunnett test in nonoverlapping and overlapping NIL libraries. The increase observed for the linear model test is due in part to a higher power to detect QTL located between the marker at the end of the target segment and the first flanking marker at which selection is performed for the recipient genome, that is, QTL between known donor DNA and known recipient DNA. This trend may also reflect detection of adjoining segments that do not contain QTL but are being declared significant because of low residual variance present at high heritabilities. The decrease observed for the Dunnett test can be explained with the decrease in the residual variance caused by increasing heritability, which reduces spurious QTL detections. For low heritabilities in the nonoverlapping library and the overlapping library, the false positive rate of the Dunnett test was considerably higher than the linear model test. For instance, at marker distance of 0.05 cM, the false positive rate for the Dunnett test was more than twice as high as the linear model test for low heritabilities in the nonoverlapping library. In overlapping NIL libraries, Dunnett test power was slightly greater than the linear model test power, but cannot be exploited because of the inflated false positives. To further investigate false positive rates in the Dunnett test, additional simulations were run in overlapping libraries. In these simulations, false positives were only declared when both lines with non-QTL-containing donor segments were declared significant. While this lowered false positive rates, the linear model test generally still outperformed the Dunnett test regarding false positives.

Evaluating overall test performance by incorporating both the detection power and false positive rate provides a more definitive answer. One way to synthesize the results of type I and type II error rates is to calculate the ratio of

test power to false positive rate. We performed a similar calculation with our values of sum of correctly detected effects vs. the sum of false positive effects. The ratio of major and minor QTL detection power to false positives generally increased for both tests as genetic variance decreased, proceeding from Scenario I to Scenario IV. Within each scenario, major QTL detection ratio of the linear model test peaked at low heritabilities and decreased at high heritabilities while the Dunnett test peaked at high heritabilities. This was true in the nonoverlapping and overlapping libraries, with both tests peaking at high heritabilities in the STAIRS library. The ratio was higher for the linear model overall than for the Dunnett test, as both tests had similar power but the Dunnett test had generally higher false positives. The largest difference between the two tests occurred in the overlapping library. At the lowest heritabilities, the linear model test ratio was over 6:1 for detection power to false positive rate for Scenarios I to III and over 4:1 for Scenario IV. The Dunnett test was below 1:1 for Scenarios I and IV and slightly above 1:1 for Scenarios II and III at those same heritabilities.

To summarize, the sum of correctly detected effects identifies neither the Dunnett test nor the linear model test as the superior method in every case. Lower false positives may be regarded as an advantage of the linear model test in most instances. In overlapping NIL libraries, the Dunnett test is in particular not suitable if heritabilities are low; with heritabilities between 0.9 and 1 it can be a favorable alternative to the linear model test. An additional point to consider is the flexibility allowed by using a linear model approach. Model building is possible, as well as interactions of genetic effects. Using introgression libraries, linear model methods could uncover and investigate epistasis with precision that is hard to achieve in segregating populations. Using mixed models is also possible, as done in a recent publication by Coles et al. (2011).

Rapeseed Introgression Library

A principal difference between the Dunnett test and the linear model test is that the linear model is testing for the presence of QTL on individual chromosome segments, whereas the Dunnett test is testing NILs as a whole. For example, line 203 was found to be significant using the Dunnett test, and it contains three separate introgressed donor segments (Table 3). This includes segment 119, which was found to be significant using the linear model test. The remaining segments, however, were not declared significant with this test. Using the linear model test was able to provide much more information on the location of the QTL than could be determined with the Dunnett test. Similar results were obtained for lines 227, 387, and 1036. We conclude that the linear model test is of great advantage for NIL libraries with lines that carry multiple introgressions, because it can detect those introgressions that are responsible for the differences in the phenotype of the NIL and the recipient parent. Additional simulations support the results, indicating that the linear model test has higher power than the Dunnett test when multiple QTL are on separate introgressions in the same line.

Of the 30 NILs containing segments detected with the linear model but not determined to be significant with the Dunnett test, 17 carried QTL with both positive and negative effects. This includes nearly every NIL containing multiple significant segments detected with the linear model. For example, two QTL with different signs and similar effect size appear in segments 38 to 41. These segments are not present in any NILs detected with the Dunnett test. In conclusion, a second big advantage of the linear model test is that it is able to find QTL in lines that carry more than one QTL with different signs on different chromosome segments.

To investigate the transferability of our results we performed simulations with a model of the barley genome. The detection power and false positive rates differed, but the trends observed for different types of libraries, quantitative genetic scenarios, heritabilities, and the choice of tests were similar. We conclude that our results are robust with respect to the number and length of the chromosomes and should serve as reliable guidelines for introgression libraries in other crops.

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Chapter 3

Identification of quantitative trait loci in rye introgression lines carrying multiple donor chromosome segments

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ORIGINAL PAPER

Identification of quantitative trait loci in rye introgression lines carrying multiple donor chromosome segments

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Abstract Introgression libraries can be used to make favorable genetic variation of exotic donor genotypes available in the genetic background of elite breeding material. Our objective was to employ a combination of the Dunnett test and a linear model analysis to identify favorable donor alleles in introgression lines (ILs) that carry long or multiple donor chromosome segments (DCS). We reanalyzed a dataset of two rye introgression libraries that consisted of ILs carrying on average about four donor segments. After identifying ILs that had a significantly better per se or testcross performance than the recipient line with the Dunnett test, the linear model analysis was in most instances able to clearly identify the donor regions that were responsible for the superior performance. The precise localization of the favorable DCS allowed a detailed analysis of pleiotropic effects and the study of the consistency of effects for per se and testcross performance. We conclude that in many cases the linear model analysis allows the assignment of donor effects to individual DCS even for ILs with long or multiple donor segments. This may considerably increase the efficiency of producing sub-ILs, because only such segments need to be isolated that are known to have a significant effect on the phenotype.

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Introduction

Introgression libraries ideally consist of a set of homozygous lines, each of which carries a single marker-defined donor chromosome segment (DCS) in the genetic background of an elite line (Eshed et al. 1992; Eshed and Zamir 1994). These DCS are introduced into the genetic background of the recipient line by marker-assisted backcrossing and should cover the entire genome of the donor. The approach of introgression libraries was first demonstrated by Eshed et al. (1992) in tomato to broaden the restricted genetic variation of the breeding material and to exploit natural variation available in genetic resources.

Introgression libraries are an important resource for the identification of quantitative trait loci (QTL) and the discovery of genes (Zamir 2001; Kearsey 2002). From a practical point of view, introgression libraries might overcome the drawbacks of the classical QTL mapping approach, since they do not separate the process of QTL detection and their use in breeding. Thus, (1) QTL alleles will not lose their effects after being transferred into breeding material due to epistatic interactions with the genetic background and (2) the transfer of QTL alleles into breeding material does not require further extensive

marker-assisted backcrossing programs (Tanksley and Nelson 1996). Introgression libraries are, therefore, a very interesting approach for practical plant breeding as development time is a key factor in the efficacy of trait manipulation in seed companies.

Analysis of introgression libraries typically involves a series of pairwise tests between the introgression lines (ILs) and the recipient for the traits of interest. This procedure has proven to be useful for finding genomic regions that carry beneficial alleles including yield-related traits in tomato (Eshed and Zamir 1955), wheat (Pestsova et al. 2006), and barley (Schmalenbach et al. 2009), agronomic traits in barley (Matus et al. (2003, 2009, 2011), maize (Szalma et al. (2007), and rye (Falke et al. (2009a, b), quality traits in barley (Matus et al. 2003; Schmalenbach and Pillen 2009), tomato (Rosseaux et al. 2005), melon (Eduardo et al. 2007), and rye (Falke et al. (2008, 2009a, b) as well as biotic stress in tomato (Finkers et al. (2007), lettuce (Jeuken et al. (2008) and barley (Schmalenbach et al. (2008).

In practical experiments, however, the ideal introgression library with lines containing only a short single marker-defined chromosomal segment of the exotic parent is mostly not available; either multiple segments are present (e.g., Liu et al. (2006; Falke et al. 2008) and/or long segments (cf Eshed et al. 1992; Chetelat and Meglic 2000; Matus et al. 2003; Jeuken and Lindhout 2004; Eduardo et al. 2005; Keurentjes et al. 2007; Schmalenbach et al. 2011). Due to these unbalanced DCS, the following questions remain: (1) which segment carries the putative QTL and/or (2) where is the QTL on the DCS located? At present, further backcross generations and subsequent field tests are employed to answer this question. These isolate or shorten the individual DCS with the goal to locate the QTL. This is necessary because statistical procedures that are able to precisely detect the location of a QTL when an IL carries several and/or longer DCS are, to our knowledge, still lacking.

Using marker-assisted backcrossing, we developed two rye introgression libraries consisting each of 40 BC_2S_3 lines. Each line carries on average three–five DCS (Falke et al. 2008). In separate experiments, a two-sided Dunnett test (Dunnett 1955) was used to determine ILs carrying DCS with putative QTL regions for agronomic and quality traits for per se as well as for testcross performance (Falke et al. 2008, 2009a, b).

In the present study, we reanalysed these data by employing pairwise Dunnett tests for identification of ILs that differ from the recipient and subsequently a linear model to identify the precise location of QTL in the unbalanced introgression library. In particular, our objectives were to (1) develop an analysis procedure for identifying QTL more precisely in introgression libraries with unbalanced DCS, (2) apply it to our rye ILs to identify QTL for agronomic and quality traits, (3) compare the determined QTL with QTL regions found in previous analyses, (4) examine the consistency of QTL for per se and testcross performance, and (5) investigate the presence of pleiotropic QTL effects.

Materials and methods

Development of introgression lines

The development of the introgression libraries is described in detail by Falke et al. (2008). Briefly, two rye introgression libraries, A and B, consisting each of 40 BC_2S_3 lines were developed by marker-assisted backcrossing to introduce exotic DCS of the Iranian primitive rye population Altevogt 14160 (provided by the Botanical Garden Warsaw, Poland) into the genetic background of the elite line L2053-N from the Petkus gene pool (bred by Hybro GmbH & Co KG, Germany). For library A and B, 131 and 182 amplified fragment length polymorphism (AFLP), respectively, and 137 and 118 simple sequence repeat (SSR) markers, respectively, were used to characterize and select individual plants in each backcross and selfing generation from BC_1 to BC_2S_3, to produce a total of 40 lines for each introgression library.

Agronomic trials

The evaluation of the field experiments has been described in our companion articles (Falke et al. 2008, 2009a, b). Briefly, the experimental design at each location was a 10 × 9 α-design (Patterson and Williams 1976) with three replicates for assessing per se performance and two replicates for testcross performance. For evaluating the testcross performance, the ILs of both libraries were crossed with the unrelated cytoplasmatically male-sterile testers from the Petkus gene pool L2092-P × LY2130-N (T1; bred by Hybro GmbH & Co KG, Schenkenberg, Germany) and Lo55-P × Lo88-N (T2; bred by KWS LOCHOW GmbH, Bergen, Germany). Trait data were collected for the agronomic traits grain yield (per se: g m^{-2}; testcross: dt ha^{-1}) and plant height (cm). A representative sample of grain (per se: 200g; testcross: 500g) was taken for quality analyses to record thousand kernel weight (g), test weight (kg), falling number (s), pentosan, protein, and starch content in grain (%). The latter three were estimated by near-infrared reflectance spectroscopy.

The field trials were conducted in separate but adjacent experiments at five sites in Germany (Bergen, Eckartsweier, Hohenheim, Oberer Lindenhof, and Wulfsode) in 2 years. The per se performance at Oberer Lindenhof was

evaluated only for grain yield and plant height for 1 year. Testcross performance of the agronomic traits for T1 could not be recorded at Eckartsweier in both years and for T2 at Oberer Lindenhof only for 1 year. Testcross performance of the quality traits was assessed only for T1 at Bergen, Hohenheim, and Wulfsode in both years. Pentosan, protein, and starch content were measured only in 1 year.

Statistical analysis

Analyses of variance for per se and testcross performance have been reported previously by Falke et al. (2008, 2009a, b). Briefly, ordinary lattice analyses for all traits were performed for each experiment and location using software PLABSTAT (Utz 2001). Adjusted entry means were then used to compute combined analyses of variance across locations (Cochran and Cox (1957). Variance components were estimated based on adjusted entry means and effective error mean squares from the individual lattice analyses by restricted maximum likelihood estimation (REML), using PROC MIXED of SAS (SAS Institute 2004). Estimates of the genotypic variances were significant, indicating that new genetic variation was generated by the exotic donor.

Introgression lines with a significantly different performance than the recipient were detected with a two-sided Dunnett test (Dunnett 1955) employing a type I error rate of $\alpha = 0.05$. The model was fitted with PROC MIXED of the SAS system (SAS Institute 2004) as described by Falke et al. (2008, 2009a, b). Briefly, the following model was used:

$$Y = \mu + G_r + L_s + J_t + (GL)_{rs} + (GJ)_{rt} + (LJ)_{st} + (GLJ)_{rst} + e$$

where G_r (r = 1,...,78) are the genotypes, L_s (s = 1,...,5) the locations, and J_t (t = 1, 2) the years. In the testcross analysis, additional terms were included in the model to account for the tester and interactions effects. For the analyses, genotypes were considered fixed factors while the other factors were included as random factors in the above analyses.

In order to allocate QTL to specific DCS, a linear model was fitted employing the principle that was described in mathematical detail in the simulation study of Falke and Frisch (2011). Briefly, the chromosomes were divided into segments that correspond to the DCS present in the library. For each segment, the effect β_s of the donor genome was estimated and tested for being significantly different from zero with standard linear model methodology and a comparison-wise type I error rate of $\alpha = 0.05$. QTL were considered to be putatively pleiotropic if a QTL was found for two or more traits in close proximity. However, because QTL can only be resolved to DCS, or in some cases subsegments, putative pleiotropic QTL may be in fact separate genes located proximally in the genome.

The model used was:

$$Y = \mu + L_s + J_t + M_u + e$$

where M_u is a marker or non-segregating group of markers (introgressed segment). In the testcross analysis, an additional model factor T_w for the wth tester effect was included in the above model. The effect of each segment was estimated with the linear model using $\hat{\beta} = (X'X)^{-1}X'y$. The part of the design matrix that codes for the effects of the donor segments X_D consisted of a g by h matrix, where g was the number of phenotypes and $h = 1 + u$, the number of included markers plus the intercept. For the levels of marker factor M, donor parent genome received a 1, recipient parent marker scores received a 0, and heterozygous loci received a 0.5. This produced a vector β, consisting of the genotypic value of the recipient parent β_0 and an effect β_u for each marker segment.

Each M was then tested with the null hypothesis $H_0 : k'\beta = 0$, where $k_u = 1$ and $k_v = 0$ for all $v \neq u$ and the corresponding F statistic as $F(H_0) = Q/(\mathrm{SSE}/\mathrm{DFE})$ where $Q = (k'\hat{\beta})'[k'(X'X)^{-1}k]^{-1}(k'\hat{\beta})$, $\mathrm{SSE} = y'y - \hat{\beta}'X'y$, and $\mathrm{DFE} = N - \mathrm{rank}(X) - stw$. N is the total number of genotypes, s the number of locations, t the number of years, and w the number of testers (when applicable).

Results

The recipient had a higher per se performance for pentosan content and a shorter plant height than the donor, whereas grain yield was nearly equal for both. The Dunnett test detected 162 pairwise comparisons between the recipient and the ILs to be significant ($P < 0.05$), and in 20 % of these, the ILs showed a superior performance. The recipient had a higher testcross performance than the donor for grain yield, falling number, and pentosan content and showed a shorter plant height. With the Dunnett test, we found 58 significant ($P < 0.05$) pairwise comparisons between testcrosses of the recipient and ILs and thereof 59 % had a superior testcross performance.

For all considered traits, we investigated the ILs that were significantly better than the recipient. In addition, we investigated ILs with significantly lower starch content than the recipient, because starch content is known to be negatively correlated with the other traits. DCS with effects on per se performance were detected by the linear model in all ILs of introgression library A that were identified by the Dunnett test as being significantly different from the recipient (Fig. 1). In library B, DCS with effects on per se

performance were found in 13 out of 15 significant ILs (Fig. 3). DCS with effects on testcross performance were detected in 20 out of 22 significant ILs of introgression library A (Fig. 2) and in 3 out of 12 significant ILs of introgression library B (Fig. 4).

With the linear model, the regions carrying putative QTL were identified precisely in many cases. QTL with p values below 0.05 are listed in Tables 1, 2, 3, 4. The effects given in Tables 1, 2, 3, 4 are 2α, or two times the allelic substitution effect, in the per se and α for the testcross. These effects therefore represent the substitution of homozygous recipient genomic segments with homozygous DCS for per se and to heterozygous DCS for testcross. For per se performance, putative QTL for thousand kernel weight were detected on chromosomes 4R, 6R, and 7R (library A), for pentosan content on chromosomes 1R, 3R, and 5R−7R in library A and on chromosomes 3R−7R in library B, for starch content on all chromosomes in library A and chromosomes 1R and 3R to 7R in library B as well as for protein content on chromosomes 1R–3R and 5R–7R in library A and on chromosomes 1R and 3R to 5R in library B. For testcross performance, the linear model found putative QTL for thousand-kernel weight on chromosomes 1R, 4R–7R (library A), for test weight on chromosomes 1R and 4R–7R in library A and on chromosome 4R in library B, for pentosan content on chromosome 7R (library A), for starch content on chromosome 1R–3R, 5R, and 7R in library A and on chromosome 4R in library B as

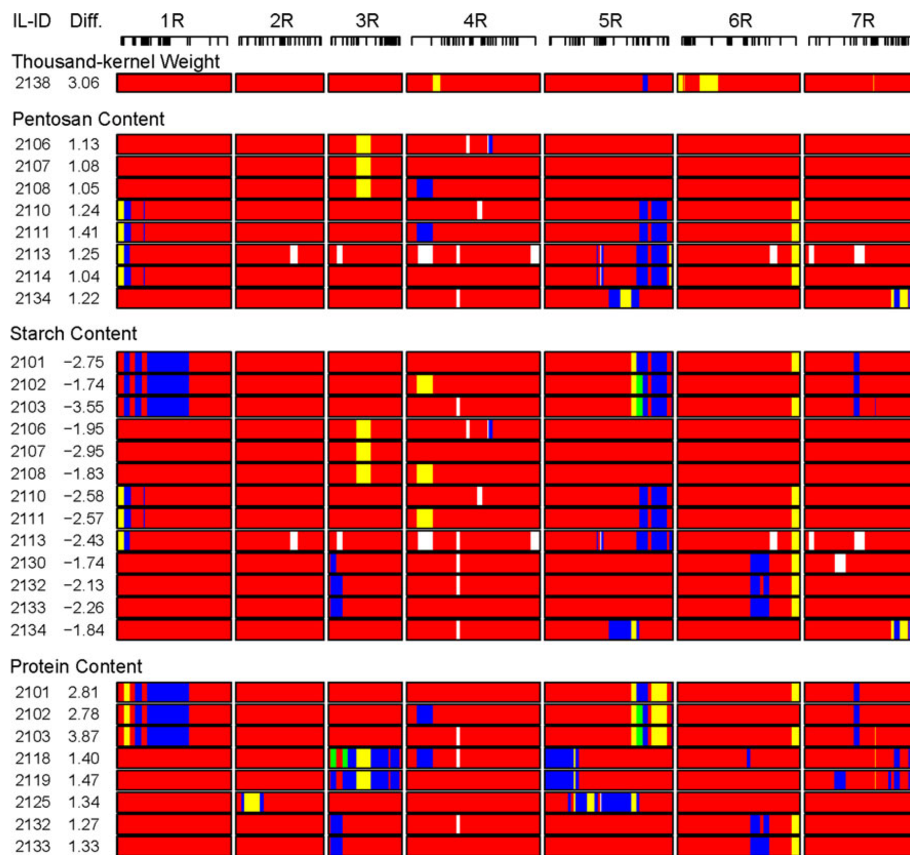

Fig. 1 Per se performance of introgression library A: differences in the performance between the recipient and introgression lines (ILs). Only ILs with significant ($P < 0.05$) differences of the Dunnett test were included. The respective chromosome and marker position (*vertical bars*) are presented above the figure; *blue coloring* denotes homozygous donor introgressions, *red coloring* indicates homozygous state of the recipient, *green coloring* denotes heterozygous state, *white coloring* denotes missing data, and *yellow coloring* indicates donor introgressions found to be significant with the linear model test (color figure online)

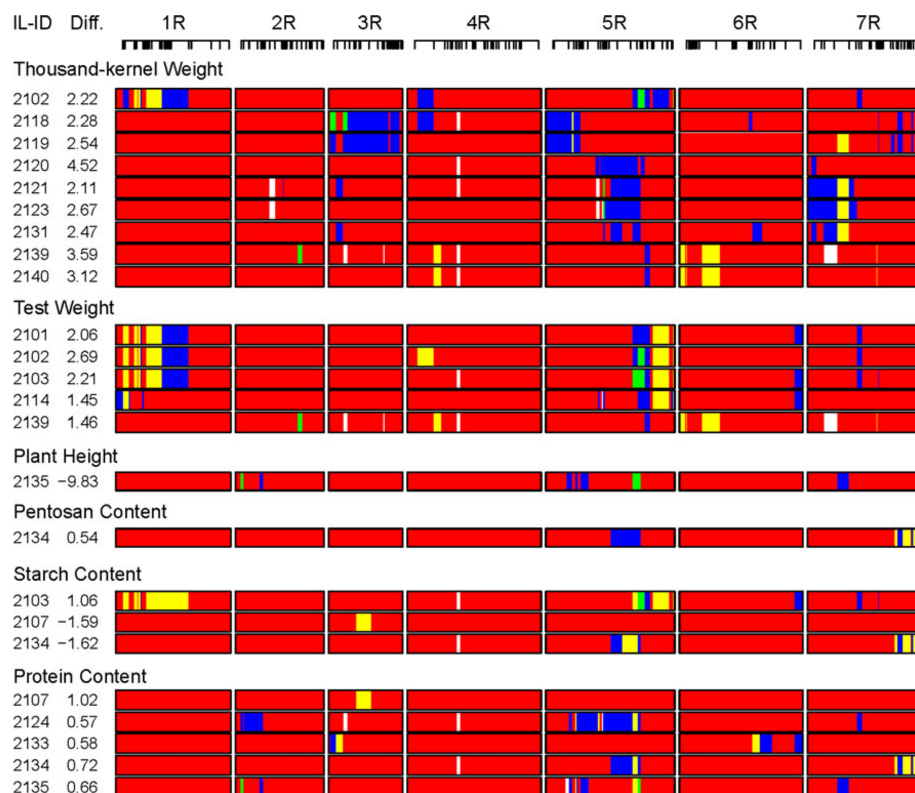

Fig. 2 Testcross performance of introgression library A: differences in the performance between the recipient and introgression lines (ILs). Only ILs with significant ($P < 0.05$) differences of the Dunnett test were included. The respective chromosome and marker position (*vertical bars*) are presented above the figure; *blue coloring* denotes homozygous donor introgressions, *red coloring* indicates homozygous state of the recipient, *green coloring* denotes heterozygous state, *white coloring* denotes missing data, and *yellow coloring* indicates donor introgressions found to be significant with the linear model test (color figure online)

well as for protein content on chromosome 3R and 5R–7R (library A).

Pleiotropic QTL were identified by the linear model in many instances. Results indicate that while pleiotropy between starch, pentosan, and protein content is not the general case, there were several QTL found that indicate a level of pleiotropy. In introgression library A, QTL for per se performance for pentosan, starch, and protein content were present on chromosomes 3R, 6R, and 7R, while QTL affecting two of the three traits occur on chromosomes 1R (pentosan and starch content) and 5R (starch and protein content) (Fig. 1). QTL detected for per se performance in introgression library B showed also pleiotropic effects. Chromosomes 3R, 4R, and 5R carried QTL for pentosan, starch, and protein content and chromosome 6R for pentosan and starch content. Some contradictory results for pleiotropic QTL were also seen. Introgression line 2166, for instance, while containing putative pleiotropic QTL for starch and protein content on chromosome 4R, was not declared significantly different from the recipient parent for pentosan content in the previous analysis, along with IL 2164 and 2165 for protein content.

Consistency between QTL for per se and testcross performance was observed in both introgression libraries. In introgression library A, QTL on chromosomes 3R (starch and protein content), 5R (starch and protein content), and 7R (pentosan, starch, and protein content) show pleiotropy consistently in both the per se and testcross performance (Figs. 1, 2). Similar results were found for introgression library B. A putative QTL for starch content on chromosome 4R were detected for both per se and testcross performance.

In addition to consistency between per se and testcross performance, there were six instances where QTL were

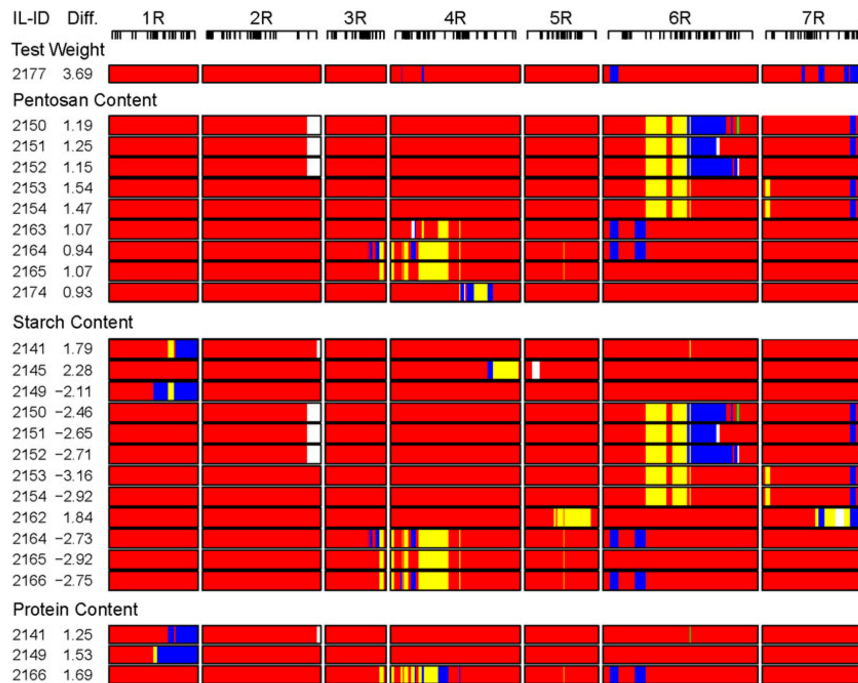

Fig. 3 Per se performance of introgression library B: differences in the performance between the recipient and introgression lines (ILs). Only ILs with significant ($P < 0.05$) differences of the Dunnett test were included. The respective chromosome and marker position (*vertical bars*) are presented above the figure; *blue coloring* denotes homozygous donor introgressions, *red coloring* indicates homozygous state of the recipient, *green coloring* denotes heterozygous state, *white coloring* denotes missing data, and *yellow coloring* indicates donor introgressions found to be significant with the linear model test (color figure online)

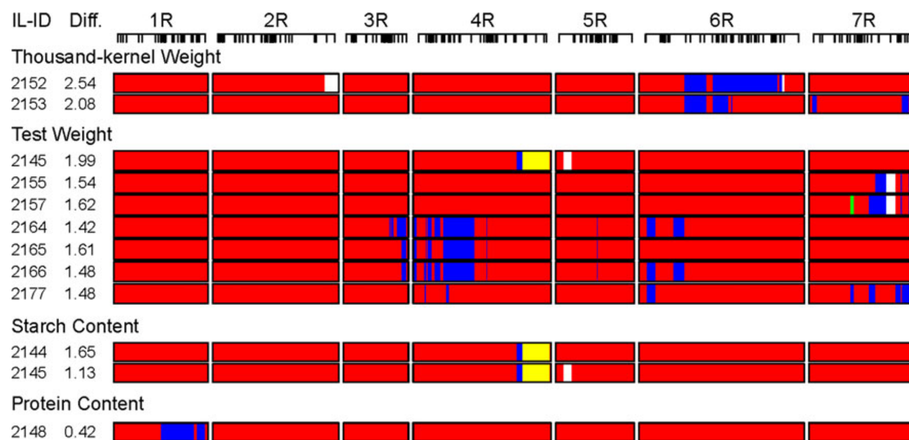

Fig. 4 Testcross performance of introgression library B: differences in the performance between the recipient and introgression lines (ILs). Only ILs with significant ($P < 0.05$) differences of the Dunnett test were included. The respective chromosome and marker position (*vertical bars*) are presented above the figure; *blue coloring* denotes homozygous donor introgressions, *red coloring* indicates homozygous state of the recipient, *green coloring* denotes heterozygous state, *white coloring* denotes missing data, and *yellow coloring* indicates donor introgressions found to be significant with the linear model test (color figure online)

Table 1 QTL detected for different traits in the per se data of introgression library A

Trait	Location	QTL effect
Pentosan content	Chr.3 (27.8–36.7)	0.937
	Chr.7 (84.5–107.5)	0.669
	Chr.6 (116.7)	0.553
	Chr.1 (0.3–2.4)	0.440
	Chr.5 (120.5)	0.289
	Chr.5 (79.0)	0.282
Protein content	Chr.5 (85.6)	1.312
	Chr.3 (27.8–36.7)	1.202
	Chr.1 (0.0)	1.186
	Chr.5 (29.1)	1.178
	Chr.6 (116.7)	0.908
	Chr.7 (67.5)	0.643
	Chr.2 (45.2)	0.641
	Chr.1 (11.8), Chr.5 (102.2)	0.382
	Chr.2 (8.3–22.3), Chr.5 (46.0–46.1)	0.283
Starch content	Chr.4 (19.7)	1.011
	Chr.2 (45.2)	0.462
	Chr.1 (0.3–2.4)	−0.648
	Chr.7 (84.5–107.5)	−0.978
	Chr.1 (0.0)	−1.026
	Chr.6 (116.7)	−1.448
	Chr.5 (85.6)	−1.471
	Chr.3 (27.8–36.7)	−3.076
Thousand-kernel weight	Chr.4 (30.5), Chr.6 (2.3–6.7), Chr.6 (30.1–30.5)	1.057
	Chr.7 (66.1)	0.816

For the traits listed, the location of QTL (with approximate position or interval in cM) and their corresponding estimated effects are given. When multiple chromosomes are given for the same QTL, the segments containing these QTL are confounded

present in both introgression libraries. Though the maps were created separately for each library, comparing centiMorgan (cM) locations of QTL in both libraries enables a rough comparison to judge overlap. Putative QTL for kernel composition traits (pentosan, starch, and/or protein content) found in common between the two introgression libraries were located on chromosomes 1R, 5R, 6R, and 7R. Another potential common QTL lies on chromosome 4R, however here the cM locations did not overlap exactly.

Discussion

Introgression libraries were usually analyzed with a series of pairwise tests to detect whether the recipient and the ILs differ with respect to the investigated traits (Eshed and Zamir 1995; Matus et al. 2003; Rosseaux et al. 2005; Eduardo et al. 2007; Finkers et al. 2007; Szalma et al. 2007; Jeuken et al. 2008; Falke et al. 2008, 2009a, b; Schmalenbach et al. 2008, 2009, 2011); Schmalenbach and Pillen (2009). However, pairwise tests that detect phenotypic differences between the ILs and the recipient, such as the Dunnett test, are unable to identify the precise location of a QTL when multiple or long DCS are present in an IL.

The two introgression libraries investigated in our study consisted each of 40 ILs. 39 of the 40 ILs of introgression library A contained multiple DCS, as well as 25 of the ILs of library B. In most instances, the original Dunnett

Table 2 QTL detected for different traits in the testcross data of introgression library A

Trait	Location	QTL effect
Pentosan content	Chr.7 (84.5–107.5)	0.320
Protein content	Chr.3 (27.8–36.7)	1.543
	Chr.5 (85.6)	1.020
	Chr.5 (29.1)	0.843
	Chr.3 (11.5)	0.477
	Chr.6 (71.8–74.4)	0.392
	Chr.7 (84.5–107.5)	0.358
	Chr.5 (50.7)	0.150
Starch content	Chr.2 (85.0)	1.451
	Chr.1 (20.6–43.0)	1.260
	Chr.1 (44.3–67.7)	0.635
	Chr.1 (11.8), Chr.5 (102.2–116.1), Chr. 5 (121.7)	0.476
	Chr.5 (120.6)	0.452
	Chr.5 (79.0)	−0.463
	Chr.7 (84.5–107.5)	−0.861
	Chr.5 (85.6)	−1.110
	Chr.3 (27.8–36.7)	−2.318
Thousand-kernel weight	Chr.7 (35.9)	2.682
	Chr.1 (20.6–43.0)	1.911
	Chr.4 (30.5), Chr.6 (2.3–6.7), Chr.6 (30.1–30.5)	1.542
	Chr.5 (120.6)	1.370
	Chr.7 (66.1)	1.092
	Chr.5 (26.2)	1.091
Test weight	Chr.1 (20.6–43.0)	1.782
	Chr.4 (19.7)	1.191
	Chr.5 (120.6)	1.057
	Chr.1 (11.8), Chr.5 (102.2–116.1), Chr. 5 (121.7)	0.685
	Chr.7 (66.1)	0.557
	Chr.4 (30.5), Chr.6 (2.3–6.7), Chr.6 (30.1–30.5)	0.497

For the traits listed, the location of QTL (with approximate position or interval in cM) and their corresponding estimated effects are given. When multiple chromosomes are given for the same QTL, the segments containing these QTL are confounded

Table 3 QTL detected for different traits in the per se data of introgression library B

Trait	Location	QTL effect
Pentosan content	Chr.6 (75.2–75.6)	0.853
	Chr.4 (76.0–83.5)	0.795
	Chr.4 (27.8)	0.740
	Chr.4 (46.0)	0.710
	Chr.6 (39.6–63.6)	0.621
	Chr.6 (66.8–70.5)	0.555
	Chr.7 (5.7–6.0)	0.435
	Chr.6 (60.3)	0.427
	Chr.3 (50.9), Chr.4 (0.0–26.3), Chr.4 (30.7–36.4), Chr.5 (35.0)	0.294
Protein content	Chr.3 (50.9), Chr.4 (0.0), Chr.4 (10.7–15.2), Chr.4 (26.3–36.4), Chr.5 (35)	0.547
	Chr.4 (20.3)	0.531
	Chr.1 (39.4–41.7)	0.492
	Chr.4 (9.4)	0.475
	Chr.4 (10.2)	0.239
Starch content	Chr.7 (87.2)	1.524
	Chr.4 (92.9–109.8)	1.344
	Chr.7 (74.6–75.4), Chr.7 (85.1)	0.925
	Chr.5 (28.0), Chr.5 (32.5), Chr.5 (34.5), Chr.5 (35.5–51.9), Chr.7 (47.6)	0.768
	Chr.5 (30), Chr.5 (32.9)	0.760
	Chr.7 (75.8)	−0.663
	Chr.7 (57.7–58.2)	−0.671
	Chr.4 (60.3)	−0.751
	Chr.7 (5.7–6.0)	−0.813
	Chr.6 (66.8–70.5)	−0.847
	Chr.1 (53.9–56.3)	−0.913
	Chr.4 (27.8)	−1.175
	Chr.6 (39.1–63.6)	−1.214
	Chr.3 (50.9), Chr.4 (0.0), Chr.4 (30.7–36.4), Chr.5 (35.0)	−1.254
	Chr.4 (46.0)	−1.339
	Chr.6 (75.2–75.6)	−1.592

For the traits listed, the location of QTL (with approximate position or interval in cM) and their corresponding estimated effects are given. When multiple chromosomes are given for the same QTL, the segments containing these QTL are confounded

analysis was unable to point towards single donor introgressions that were responsible for the detected phenotypic differences. In such situations, further experimental work can help to locate the position of QTL (Rousseaux et al. 2005). To accomplish this, the DCS of a significant IL are split up into several sub-ILs by further backcrosses. Then the sub-ILs are compared with the recipient. However this procedure is time and cost intensive.

Table 4 QTL detected for different traits in the testcross data of introgression library B

Trait	Location	QTL effect
Starch content	Chr.4 (92.9–109.8)	0.946
	Chr.4 (85.0)	0.354
Test weight	Chr.4 (92.9–109.8)	1.572

For the traits listed, the location of QTL (with approximate position or interval in cM) and their corresponding estimated effects are given. When multiple chromosomes are given for the same QTL, the segments containing these QTL are confounded

Instead of generating sub-ILs, employing a linear model analysis after having carried out the Dunnett test can help to identify QTL in ILs with multiple DCS. For example, in introgression library A, ILs 2121, 2123, and 2131 contain multiple DCS across several chromosomes. The testcross performance for thousand-kernel weight of all of these lines was detected as being significantly different from the recipient by the Dunnett test, but the location of the causative alleles could not be exactly determined. The linear model analysis pointed to the common DCS on chromosome 7R in all three ILs, thus lessening the potential length of DCS for fine-mapping from around 50 to under 20 cM. Hence, the linear model analysis allowed us to identify genomic regions carrying putative QTL much more precisely than the Dunnett test alone. We therefore conclude that the combination of the Dunnett test with a linear model analysis provides a valuable tool to identify and localize QTL, and may help to reduce the need for further splitting of the DCS in ILs with multiple segments.

The linear model analysis further allowed a much more detailed analysis of the pleiotropic effects of DCS than the Dunnett test alone. For example, the analysis revealed that putative QTL found on the DCS on chromosomes 4R and 6R which increase the per se performance for pentosan and protein content in introgression library B were also responsible for a decrease in starch content. Similar observations can be made throughout both libraries for per se and testcross performance. In practical breeding programs such results on pleiotropy might help to identify DCS that increase the performance of one of two negatively correlated traits without negative effects on the second trait. Fine-mapping and/or further sub-IL generation would help to determine if the pleiotropic QTL detected in this study are the result of single QTL or several linked QTL. For the purposes of this study, we can only localize QTL to DCS and assume that they are either a single QTL or two or more tightly linked QTL.

The more precise assignment of QTL to individual DCS with the linear model also allowed investigation of the consistency between QTL for per se and testcross performance. The rather low consistency observed in our analysis

suggests that testcross experiments are essential to assess the usefulness of introgressed DCS in hybrid rye breeding. In general, such analyses might assist the breeder in deciding on intensity of pre-selection among lines before going to the more resource demanding testcross phase. Additionally, the extensibility of this technique can allow for detection of gene interactions (epistasis) as well as model building. The utility and extensibility of regression for use in IL analysis has been demonstrated, for example, in rice (Wang et al. 2006, 2007) and maize (Coles et al. (2011).

To summarize, we conclude that employing a linear model test is a very promising method that allows the detection of favorable DCS in introgression libraries consisting of ILs that carry long or multiple DCS. It has the potential to greatly enhance the efficiency of producing sub-ILs, because only segments with a significant effect need to be isolated.

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Chapter 4

Genome-wide prediction methods for detecting genetic effects of donor chromosome segments in introgression populations

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Genome-wide prediction methods for detecting genetic effects of donor chromosome segments in introgression populations

Karen Christin Falke[1,4*†], Gregory S Mahone[1†], Eva Bauer[2], Grit Haseneyer[2], Thomas Miedaner[3], Frank Breuer[5] and Matthias Frisch[1]

Abstract

Background: Introgression populations are used to make the genetic variation of unadapted germplasm or wild relatives of crops available for plant breeding. They consist of introgression lines that carry small chromosome segments from an exotic donor in the genetic background of an elite line. The goal of our study was to investigate the detection of favorable donor chromosome segments in introgression lines with statistical methods developed for genome-wide prediction.

Results: Computer simulations showed that genome-wide prediction employing heteroscedastic marker variances had a greater power and a lower false positive rate compared with homoscedastic marker variances when the phenotypic difference between the donor and recipient lines was controlled by few genes. The simulations helped to interpret the analyses of glycosinolate and linolenic acid content in a rapeseed introgression population and plant height in a rye introgression population. These analyses support the superiority of genome-wide prediction approaches that use heteroscedastic marker variances.

Conclusions: We conclude that genome-wide prediction methods in combination with permutation tests can be employed for analysis of introgression populations. They are particularly useful when introgression lines carry several donor segments or when the donor segments of different introgression lines are overlapping.

Background

If the genetic variability for traits of agronomical interest is limited, plant breeders attempt to make available favorable alleles from exotic material in breeding programs. A main problem is that lines derived from crosses of elite and exotic parents lack adaptation and their agronomic performance is so poor that they cannot be directly used in the breeding process. So called introgression libraries or introgression populations [1] are a concept that tries to overcome the problem by establishing introgression lines, of which the genome originates in large part from an elite line and only small chromosome segments originate from an exotic donor. The goal of this concept is to generate lines that have the adaptation and agronomic performance of the elite parent, and are enhanced by small chromosome segments from the exotic donor, which provide favorable alleles for specific traits that should be improved.

Introgression populations have been developed first in tomato [2] and subsequently in other crops [3-6]. In most experiments [5-13] the Dunnett test [14] was used to detect whether an introgression line differs significantly from the recipient elite line. If a line, that is significantly better than the recipient with respect to a certain trait, contains only one single donor chromosome segment, then such an analysis is able to identify this segment as affecting the trait. However, the lines of an introgression

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populations typically carry more than one donor segment [5,15]. For such introgression lines, the Dunnett test is not able to identify which of the donor segments affects the trait.

A linear model in which each donor segment has a fixed effect [16], can be used to analyse introgression popualtions with lines that carry more than one donor segment. It can be employed, if the number of donor segments in the introgression library does not surpass the number of introgression lines, i.e., if the design matrix of the linear model has full rank. For introgression populations, in which the number of donor segments exceeds the number of introgression lines, the donor segment effects are not estimable with a fixed linear model. Statistical analysis methods for such situations were not yet investigated.

The goal of our study was to investigate the usefulness of statistical methods developed in the context of genome-wide prediction for the analysis of introgression populations. In particular, our objectives were to (1) apply the BLUP [17] and RMLV [18] methods to simulated and experimental data, (2) investigate their power of detecting donor chromosome segments that have effects on the phenotype of an introgression line, as well as their false positive rate, and to (3) draw conclusions on their potential application for the analysis of introgression populations.

Methods

Estimating donor segment effects

The genetic effects of the donor segments on a phenotypic trait were estimated with the linear model $\mathbf{y} = \mathbf{1}\beta_0 + \mathbf{Z}\mathbf{u} + \mathbf{e}$. Here, $\mathbf{y}$ is the vector of the phenotypic values of N introgression lines, β_0 a fixed intercept, $\mathbf{Z}$ the design matrix relating the donor segments to the introgression lines, $\mathbf{u}$ the vector of the donor segment effects, and $\mathbf{e}$ the vector of residuals.

To construct the the design matrix $\mathbf{Z}$, markers for which the alleles were in complete linkage disequilibrium in the introgression population were combined to donor segments. The elements of $\mathbf{Z}$ are coded in the design matrix such that the number represents the donor segment zygosity, i.e., as 0, 1, 2. The structure of the design matrix $\mathbf{Z}$ is illustrated in Figure 1B for the two hypothetical introgression populations shown in Figure 1A.

For estimation of the donor segment effects, we used (a) least squares estimation (LSQ) assuming fixed donor segment effects, (b) best linear unbiased prediction (BLUP) assuming that the donor segment effects were random [17], or (c) the RMLV method suggested for genome-wide prediction [18]. For the LSQ analysis the intercept β_0 was removed from the model. Calculations were carried out with the software SelectionTools (www.uni-giessen.de/population-genetics/downloads).

Testing donor segment effects

For the LSQ analysis, the significance of the donor segment effects was tested with F-tests for linear contrasts. For the BLUP and RMLV analyses, we adopted a permutation test similar to that suggested by [19] for QTL mapping. For carrying out the permutation test for the effect u_i of the ith donor segment, entries of the ith column of $\mathbf{Z}$ were randomly permuted and u_i was estimated for the random permutations. The distribution of the u_i from r random permutations was used to approximate the distribution under the null-hypothesis that 'the segment has no effect on the phenotype'. Comparison of the effect estimate obtained for the actually observed phenotypic data with the approximated distribution of effects under the null hypothesis was used to assign p-values to the donor effect estimates. The p-values from testing linear contrasts and from the permutation test were adjusted with a modified Bonferroni procedure [20].

Sample data sets

For investigating effect estimation in introgression populations with genome-wide prediction methods, we considered two hypothetical introgression populations of different genetic structure. The genome considered for the simulations consisted of three chromosomes of length 120 cM. The introgression population 1 was an ideal introgression population consisting of 9 lines, each carrying a donor segment of length 40 cM. The donor segments were not overlapping. In introgression population 2 the donor segments had varying length, were overlapping, and several donor chromosome regions were present in more than one line. The graphical genotypes of both introgression populations are shown in Figure 1A.

For a first analysis we considered one major gene located in the center of chromosome 1 with an additive effect of size 0.5. An observation vector $\mathbf{y}$ that results from this genetic effect and a random error is shown in Figure 1B.

Simulations for comparing power and false positive rate

We carried out computer simulations with the introgression populations 1 and 2 to determine the power and false positive rate of the LSQ, BLUP, and RMLV analyses. We simulated a quantitative trait, controlled by 2, 4, or 6 loci with additive gene action. The donor had a performance that was 100 units better than the recipient, hence, the effect of a favorable allele was 25, 12.5, and $8.\bar{3}$, respectively. The genes were assigned to random positions in the genome. Heritabilities between 0.50 and 0.99 were assumed. For introgression population 1 ($\mathbf{Z}$ has full column rank), LSQ, BLUP, and RMLV analyses were carried out. For introgression population 2 ($\mathbf{Z}$ doesn't have full column rank), BLUP and RMLV analyses were carried out. The sum of correctly detected effects and the sum of false positive effects was recorded for 5000 simulation

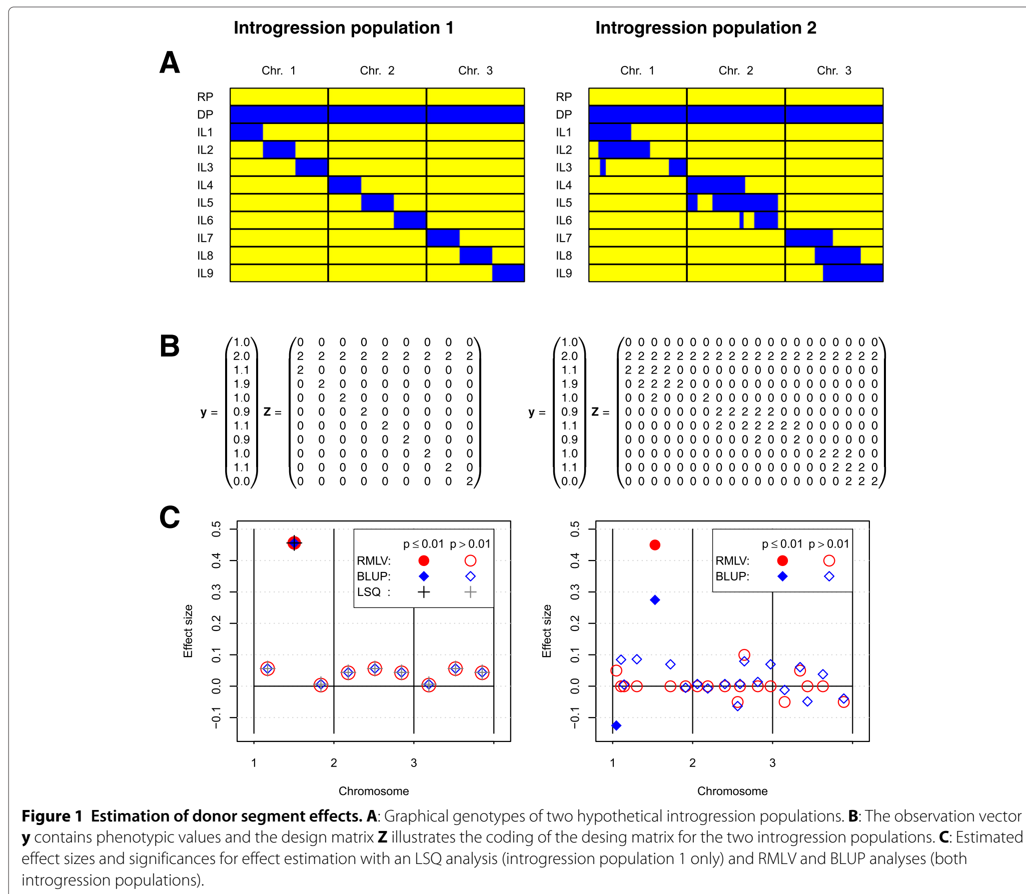

Figure 1 Estimation of donor segment effects. A: Graphical genotypes of two hypothetical introgression populations. **B**: The observation vector **y** contains phenotypic values and the design matrix **Z** illustrates the coding of the desing matrix for the two introgression populations. **C**: Estimated effect sizes and significances for effect estimation with an LSQ analysis (introgression population 1 only) and RMLV and BLUP analyses (both introgression populations).

runs with different random positions of the genes underlying the trait. For the permutation tests $r = 1000$ random permutations were used.

Experimental data sets

We investigated two experimental data sets. The first data set was a rapeseed (*Brassica napus* L.) introgression population consisting of of 350 DH lines. It originates from a cross between the elite line variety Express and the resynthesized line RS239 as donor. The introgression population was genotyped with 484 amplified fragment length polymorphism (AFLP) markers that spanned 1885 cM with an average marker distance of 4 cM. The introgression population covered 100% of the genome of the donor. The lines carried on average 2.8 donor segments, with a mean length of 17 cM. Field trials were conducted at 4 locations in the year 2008/09. Trait data were collected for glucosinolate content (μmol/g) and linolenic acid content (%) measured by using near-infrared spectroscopy. Adjusted entry means were determined with a mixed linear model. The chromosomes in this data set were randomized because the data set is proprietary and the goal of our study is to investigate the analysis methods and not to report QTL for the two traits under consideration.

The second data set was a rye (*Secale cereale* L.) introgression population consisting of 37 introgression lines. It originates from a cross between the elite inbred line L2053-N and the Iranian primitive rye population Altevogt 14160 as donor. The plant height was assessed in two years at five locations with two testers. A detailed description of the experiment is available in earlier publications [5,12,21] where the data used in this study is referred to as 'Library A'. The lines were genotyped with

the Rye5K SNP array containing 5,234 markers [22]. The introgression population covered 94% of the genome of the donor. The lines carried on average 4.6 donor segments, with a mean length of 27 cM. This is a public data set, the marker and field data are provided together with the analysis software SelectionTools.

Results

For introgression population 1 (Figure 1A) and the observation vector shown in Figure 1B, the LSQ, BLUP, and RMLV analyses estimated effects of similar size for all donor segments (Figure 1C). The F-tests for the LSQ analysis as well as the permutation tests for the BLUP and RMLV analyses correctly detected the effect in the center of chromosome 1 as significant and all other donor effects as not significant (Type 1 error rate: 0.01). For introgression population 2, the position of the donor segment underlying the trait was detected correctly by the BLUP and RMLV analyses. However, the BLUP analysis underestimated the effect size considerably. In contrast, the RMLV analysis was able to provide a more precise estimate of the donor segment effect also with the non full-rank design matrix $\mathbf{Z}$ of introgression population 2.

In the simulations with the introgression population 1, the LSQ analysis resulted in a false positive rate that was near the nominal type I error rate (Figure 2). The BLUP and RMLV analyses showed greater false positives rates. For heritabilities between 0.6 and 0.8 and four or six loci underlying the trait, the sum of correctly detected effects was considerably greater for the BLUP and RMLV analyses than for the LSQ analysis.

In the simulations with introgression population 2, the RMLV analysis had a greater rate of correctly detected effects than the BLUP analysis for all scenarios with the exception of heritabilities ≥ 0.9 and 6 loci underlying the trait. For increasing heritabilities, the sum of false positive effects increased for the BLUP analysis while it decreased for the RMLV analysis. The false positive rate of the BLUP analysis was particularly high when only two genes were underlying the trait.

For both introgression populations and all three quantitative genetic scenarios, the RMLV analysis had a considerably greater rate of correctly detected effects than the LSQ or BLUP analysis if the heritability was only 0.5. For introgression population 2 and a heritability of 0.5, the rates of correctly detected effects of the BLUP analysis were below 10%.

The RMLV analysis detected that 8 of the 223 donor segments in the rapeseed introgression population were significant ($p < 0.01$) for glucosinolate content, the BLUP analysis detected 69 significant segments (Figure 3). For linolenic acid content the RMLV analysis found 25 donor segments, and the BLUP analysis 81 (Figure 4). For both traits the BLUP analysis estimated many small effects, whereas the RMLV analysis estimated a few large effects and many effects near zero.

In the rye introgression population the RMLV estimation of effects for plant height showed a good model fit, the correlation between observed and predicted values was 0.96 (Figure 5). Three donor segments were detected that significantly increased plant height, and one that significantly reduced plant height. The donor segment that reduced plant height had an additive effect of 2 cm.

Discussion

Genome-wide prediction models for the analysis of introgression populations

Combining markers of which the alleles are in complete linkage disequilibrium to donor segments results in a design matrix $\mathbf{Z}$ with full column rank if (1) the donor segments are non-overlapping, (2) each donor allele occurs exactly in one introgression line, and (3) the donor coverage is 100%. (All three conditions are fulfilled by introgression population 1 in Figure 1.) As a consequence, $\mathbf{Z}^T\mathbf{Z}$ is regular and can be inverted. Hence, in a linear model without intercept the donor segment effects u_i are estimable and can be tested with F-tests for linear contrasts.

For introgression populations that do not fulfill the above conditions (1) to (3), the number of donor segment effects (columns of $\mathbf{Z}$) can be greater than the number of lines in the introgression population (rows of $\mathbf{Z}$). Because the row rank is smaller or equal to the number of rows, those matrices do not have full column rank, resulting in singular $\mathbf{Z}^T\mathbf{Z}$ matrices. While for such situations the genetic effects u_i are not estimable with ordinary least squares, ridge regression can be employed. Both, the BLUP and the RMLV analyses can be regarded as ridge regression models, BLUP with an equal shrinkage factor for all markers, and RMLV with shrinkage factors, that differ depending on the marker.

Collinearity of the columns of $\mathbf{Z}$ may occur if conditions (1) to (3) are not fulfilled, and collinearity of the rows of $\mathbf{Z}$ may occur if strongly related sister lines are among the lines of the introgression population. Such collinearity can increase the false positive rate above the nominal type 1 error rate used for construction of the permutation test. The strength of this departure depends on the strength of the collinearity of the row and column vectors of the $\mathbf{Z}$. In conclusion, it can not be expected that the permutation test adheres to its nominal type I error rate, if collinearity is present in $\mathbf{Z}$. However, even if the permutation tests are only approximate, they provide a means of analyzing introgression populations that depart from conditions (1) to (3), as do most of the introgression populations that were constructed so far in crops [5,6,10,15,23,24].

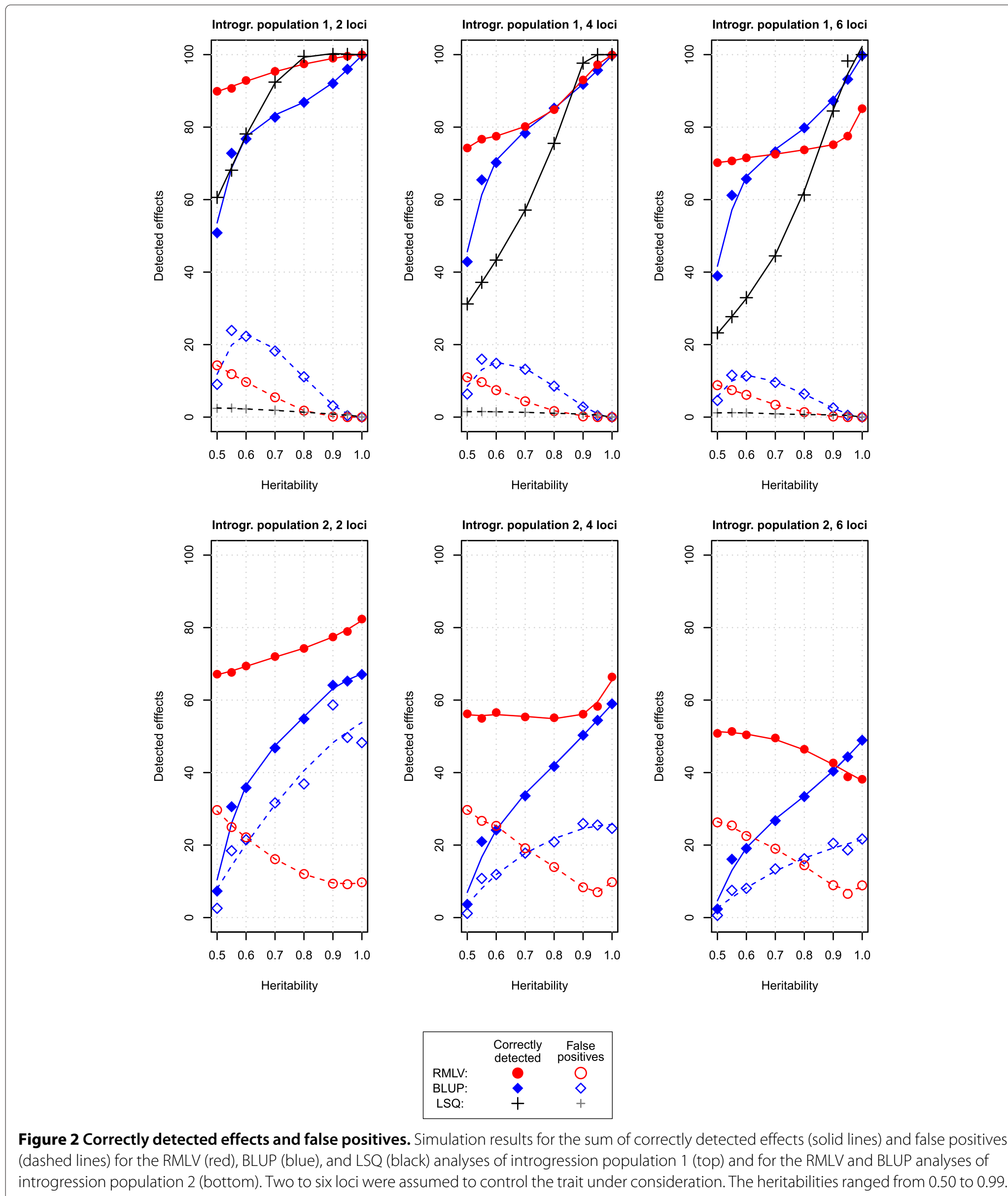

Figure 2 Correctly detected effects and false positives. Simulation results for the sum of correctly detected effects (solid lines) and false positives (dashed lines) for the RMLV (red), BLUP (blue), and LSQ (black) analyses of introgression population 1 (top) and for the RMLV and BLUP analyses of introgression population 2 (bottom). Two to six loci were assumed to control the trait under consideration. The heritabilities ranged from 0.50 to 0.99.

Typically the vector of phenotypic values **y** in genome-wide prediction models consists of phenotypic means or of adjusted entry means from incomplete block designs. Therefore the residual variance used for the significance tests of the donor segments is only that which is unexplained by the genetic composition, not the full residual variance due to the experimental error of the field trial. This means that the pure experimental error of the plot

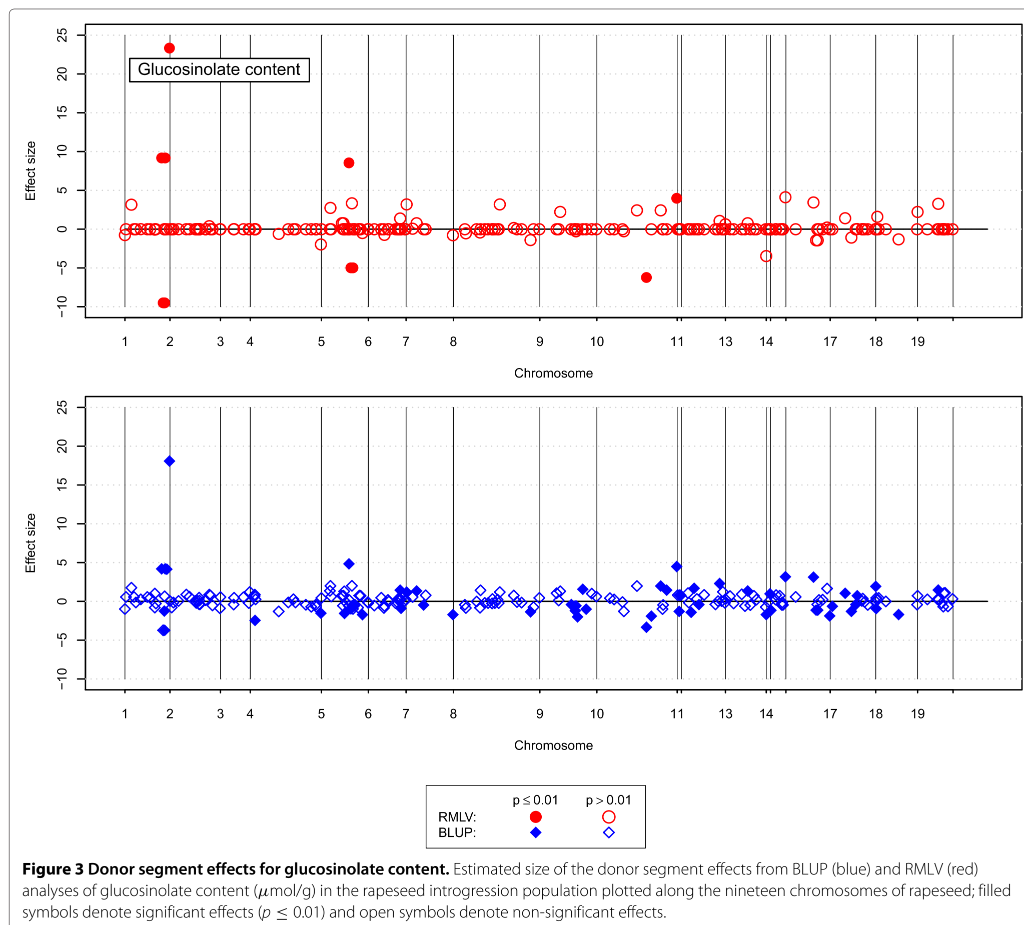

Figure 3 Donor segment effects for glucosinolate content. Estimated size of the donor segment effects from BLUP (blue) and RMLV (red) analyses of glucosinolate content (μmol/g) in the rapeseed introgression population plotted along the nineteen chromosomes of rapeseed; filled symbols denote significant effects ($p \leq 0.01$) and open symbols denote non-significant effects.

values is ignored, and the residual variance used in the tests is underestimated. An alternative approach is to adjust the plot values for the effects of the factors that are determined by the experimental design, such as replication, year, or location. Using such adjusted plot values in the genome-wide prediction model results in a more precise estimate of the residual variance. This procedure makes it possible to include the trial design in the analysis, even if the statistical model for genome-wide prediction does not allow to include directly factors for the field design. We applied this approach for our rye data set.

Power of detecting favorable donor segments and false positive rate

The LSQ analysis adhered in our simulations with introgression library 1 to the nominal type I error rate. However, this was accompanied with a lower power of detecting significant donor segments than the BLUP and RMLV analyses for heritabilities between 0.6 and 0.8 and four or six genes controlling the trait. Hence, with full rank design matrices, the LSQ analysis seems the most suitable method when it can be assumed that the trait is controlled by one or two major genes and the heritabilities are 0.8 or greater. For situations with low heritabilities and in situations where the trait is assumed to be polygenic, the genome-wide prediction approaches might be advantageous for the detection of donor effects, even for full-rank design matrices. The higher type I error rate, however, requires subsequent verification of the detected donor segment effects.

The BLUP analysis showed a very high false positive rate in the simulations with introgression population 2

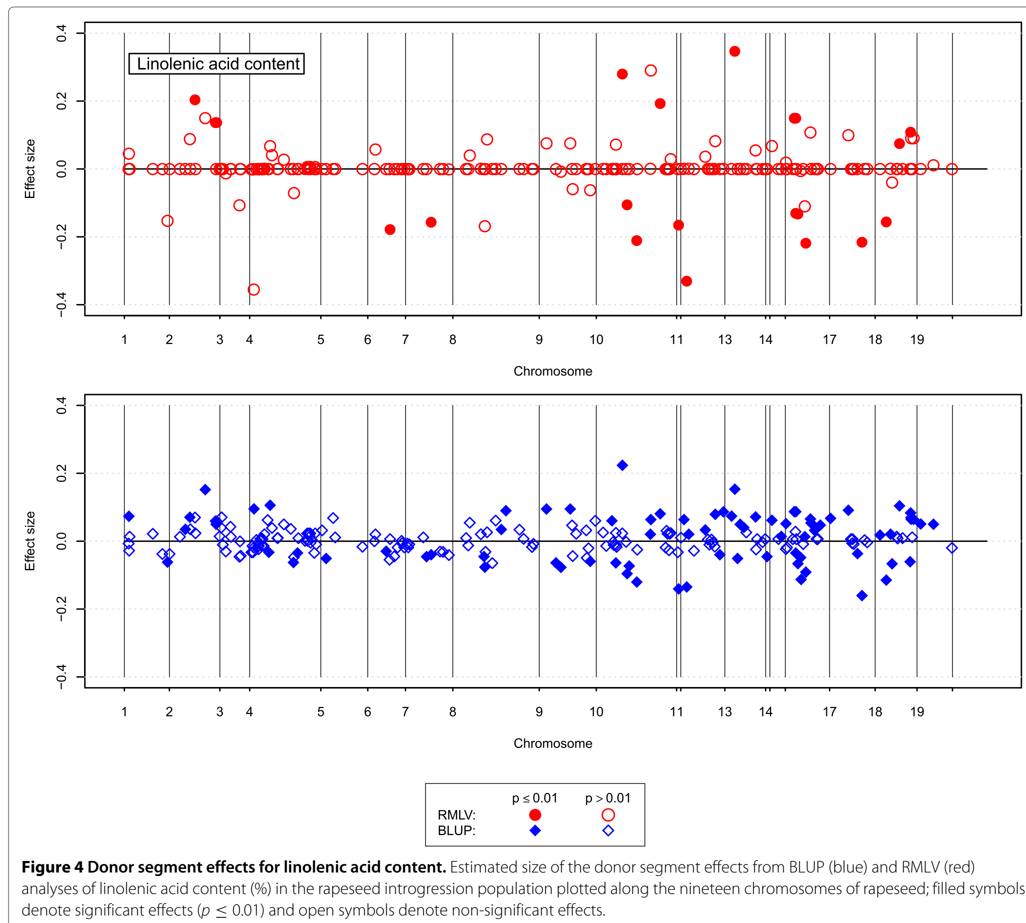

Figure 4 Donor segment effects for linolenic acid content. Estimated size of the donor segment effects from BLUP (blue) and RMLV (red) analyses of linolenic acid content (%) in the rapeseed introgression population plotted along the nineteen chromosomes of rapeseed; filled symbols denote significant effects ($p \leq 0.01$) and open symbols denote non-significant effects.

when two loci controlled the trait. A possible explanation is that the model underlying the BLUP analysis assumes that each donor segment contributes equally to the genetic variance, i.e., the donor segment variances are homoscedastic. This assumption is severely violated if only two genes control the trait under consideration. As a consequence, large effects are underestimated and small or zero effects are overestimated. This systematic estimation error can be observed for the BLUP analysis of introgression population 2 in Figure 1B. The overestimation of small effects is likely the cause for the high false positive rate in the permutation test of the BLUP analysis with non-polygenic inheritance.

The RMLV analysis showed a considerably greater rate of correctly detected effects than the BLUP analysis for low heritabilities. This suggests that an RMLV analysis is an option to detect donor segment effects, which would otherwise remain undetected. Due to the high false positive rate, subsequently a thorough verification of the detected segments is mandatory.

In general, the focus of introgression populations lies on identifying donor segments that have a considerable effect on the trait under consideration. Hence, the traits to be improved are typically oligogenic and are controlled by few major genes. Our simulations have shown that for few genes an RMLV analysis is superior to a BLUP analysis. This is in accordance with the theoretical expectations, because the BLUP approach employs homoscedastic genetic variances at all markers, which can be assumed for highly polygenic traits, but not for oligogenic traits. We conclude that for most applications of introgression populations, where few genes are assumed to control

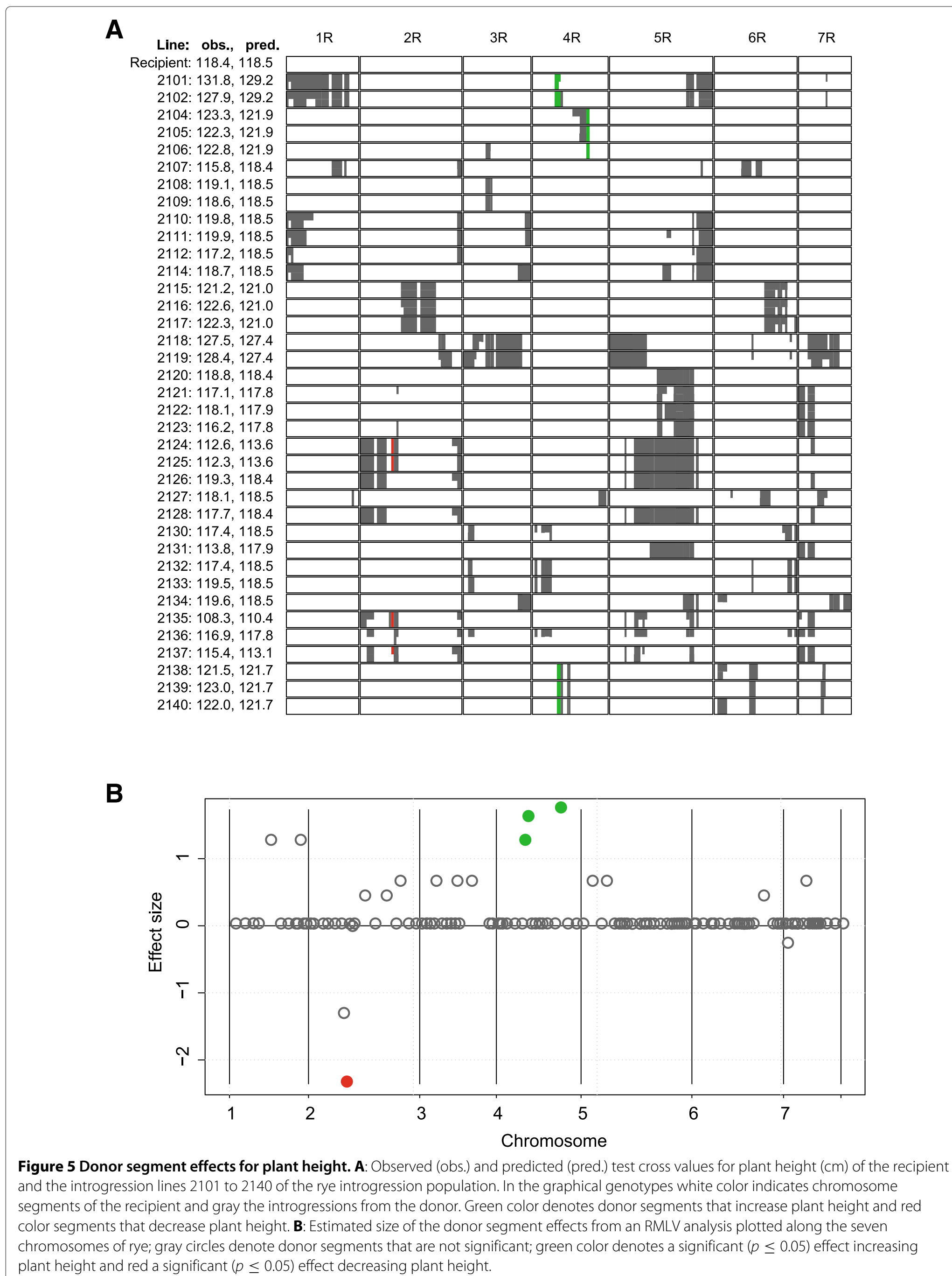

Figure 5 Donor segment effects for plant height. A: Observed (obs.) and predicted (pred.) test cross values for plant height (cm) of the recipient and the introgression lines 2101 to 2140 of the rye introgression population. In the graphical genotypes white color indicates chromosome segments of the recipient and gray the introgressions from the donor. Green color denotes donor segments that increase plant height and red color segments that decrease plant height. **B**: Estimated size of the donor segment effects from an RMLV analysis plotted along the seven chromosomes of rye; gray circles denote donor segments that are not significant; green color denotes a significant ($p \leq 0.05$) effect increasing plant height and red a significant ($p \leq 0.05$) effect decreasing plant height.

the trait, a BLUP analysis is expected to be inferior to models with heteroscedastic marker variances, such as an RMLV analysis. It remains open to further research how well other heteroscedastic approaches for genome-wide prediction, such as Bayesian methods [17] or the HEM method [25] perform when applied to introgression populations.

A main difficulty of applying genome-wide prediction methods to introgression populations is the rather high false positive rate. It depends on the degree to which the assumptions underlying the statistical models are violated and can not be corrected by adjusting p-values for multiple testing. We therefore conclude that genome-wide prediction methods have the potential to detect favorable alleles, but a validation of the effects in subsequently conducted well-designed trials with a reduced set of lines is mandatory.

Application to experimental data sets

We applied the BLUP and RMLV analyses to two experimental data sets to derive guidelines for the application of genome-wide prediction methods to introgression populations. In the analysis of the rapeseed introgression population a major gene for glucosinolate content was found, that controls the phenotypic difference between the donor and the recipient (Figure 3). The RMLV analysis estimated an effect size of 23 and the BLUP analysis an effect size of 18. The BLUP analysis detected in addition a large number of significant donor segments with small effects. Many of these were shrunken near zero in the RMLV analysis. The results presented in Figure 1C suggest that the true effect size might be more closely to the RMLV estimate than to the BLUP estimate, because the differences between donor and recipient can mainly be attributed to a single major gene.

For linolenic acid content the BLUP analysis detected considerably more significant donor segments with small effects than the RMLV analysis (Figure 3). Linolenic acid content showed an oligogenic, but not a highly polygenic inheritance in QTL studies [26]. Therefore it can be expected that also here the results of the RMLV analysis are closer to reality than the results of the BLUP analysis.

Plant height in rye showed a polygenic inheritance, but large parts of the genetic variance are controlled by major genes [27,28]. Therefore, we employed an RMLV analysis for the rye introgression population. The graphical genotypes of the rye introgression lines (Figure 5) indicate that in this data set the rows of the design matrix $\mathbf{Z}$ show a strong collinearity, because obviously sister lines are included in the introgression population. This might severely violate the assumptions underlying the permutation test. Nevertheless, the RMLV analysis was able to detect a donor segment on chromosome 2 as responsible for the considerably shorter plant height of the lines 2124, 2125, and 2135.

A shorter plant height is a key agronomic property that distinguishes modern rye lines from older breeding material. The exotic donor had a considerably greater plant height than the elite recipient [12,13,27]. Hence, the donor segment that reduced plant height found by the RMLV analysis may serve as a proof of concept that favorable alleles can be found in exotic donors, even if the exotic donor itself is inferior to the recipient for a certain trait.

Conclusions

We conclude that genome-wide prediction methods can be employed to detect favorable donor segments in introgression populations. In particular they can, in contrast to the typically employed Dunnett test [14], identify favorable donor segments when introgression lines carry more than one donor segment and when the segments present in different introgression lines are overlapping. In contrast to fixed linear models, genome-wide prediction methods can also be applied to over-parametrized data sets with non full-rank design matrices.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

EB, GH, TM collected the data for the rye introgression populations, FB collected the data for the rapeseed introgression populations GSM, KCF, MF performed the analyses, KCF, GSM, MF wrote the manuscript. All authors read and approved the final manuscript.

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Chapter 5

Detection of donor effects in a rye introgression population with genome-wide prediction

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Detection of donor effects in a rye introgression population with genome-wide prediction

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With 5 figures

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Abstract

Introgression populations are developed to make genetic resources for breeding purposes available. In the case that the number of donor segments exceeds the number of lines, genome-wide prediction (GWP) methods are suggested as promising for the analysis of such populations. Our objectives were to characterize a rye introgression population with the Rye5K SNP assay and to apply a GWP model with a modification of the restricted maximum likelihood procedure that yields heteroscedastic variances to detect significant donor effects. The introgression lines (ILs) carried on average 4.6 donor segments with a mean length of 27 cM and represented 94% of the donor genome. Two donor effects were detected that significantly increased thousand-kernel weight. We found four donor effects for protein, total pentosan and starch content that can improve baking quality. Three donor effects for protein content were observed for improving feeding purposes and one donor effect for starch content to improve ethanol production. The effects were localized to small genomic regions. Consequently, these ILs can improve rye breeding by directly employing them in breeding programmes for variety development.

Key words: *Secale cereale* L. — introgression population — introgression line — Rye5K SNP array — genome-wide prediction — heteroscedastic marker variances — RMLV

Introgression libraries or introgression populations employ the strategy of incorporating chromosome segments of mainly exotic donors into elite backgrounds by marker-assisted backcrossing. Ideally, such populations represent a set of homozygous lines each carrying a single marker-defined donor segment in the background of an elite recipient (Zamir 2001). In this way, phenotypic variation of a specific line compared to the recipient can be attributed specifically to the introgressed segment. The principle of detecting donor segments affecting specific traits with introgression populations was first established in tomato (Eshed and Zamir 1994, 1995) and has been well proven in various cereals for diverse traits (*cf. e.g.* Ishikawa et al. 2005, Pestsova et al. 2006, Szalma et al. 2007, Falke et al. 2008, Schmalenbach et al. 2009).

The development and analysis of introgression populations has so far been mainly based on a limited number of molecular markers generated from anonymous genomic regions such as restriction fragment length polymorphisms (RFLPs; *cf.* Eshed and Zamir 1994, Szalma et al. 2007), amplified fragment length polymorphisms (AFLPs; *cf.* Finkers et al. 2007, Jeuken et al. 2008) or simple sequence repeats (SSRs; *cf.* Falke et al. 2008, Schmalenbach et al. 2009). Dense genetic linkage maps, however, are essential for the precise identification of donor segments carrying the putative favourable alleles. Today, single nucleotide polymorphisms (SNPs) have become the marker system of choice for plant geneticists and breeders (Rafalski 2002, Ponting et al. 2007) due to their (i) high abundance in the genome, (ii) suitability for multiple assays and (iii) low cost per data point. Up to now, the potential of high-resolution genotyping with SNP arrays has been demonstrated for many genomic approaches, but they are rarely applied for the analysis of introgression populations (Schmalenbach et al. 2011).

In practice, introgression populations typically consist of a set of introgression lines (ILs) which contain several and/or overlapping donor segments (Liu et al. 2006, Falke et al. 2008). This hinders pairwise testing to find the specific donor segments affecting the traits of interest. For these cases, linear model analysis with fixed effects has been suggested (Falke and Frisch 2011, Mahone et al. 2013). However, if the number of donor segments exceeds the number of ILs, the donor effects are not estimable with such models. Recently, genome-wide prediction (GWP) approaches are proposed as promising to this problem (Falke et al. 2014).

Rye (*Secale cereale* L.) is an economically important and widely cultivated crop for bread, feed and as a renewable energy source in Middle and Eastern Europe. Of all small-grain cereals, it has the highest winter hardiness and is outstanding with regard to biotic and abiotic stresses. As an outcrossing species, considerable heterosis can be exploited in hybrid breeding programmes. The lack of genomic resources in rye has been solved with the recently developed Rye5K SNP array (Haseneyer et al. 2011).

We developed a BC_2S_3 introgression population based on a cross between the elite line L2053-N and the primitive rye population Altevogt 14160 by marker-assisted backcrossing using AFLP and SSR markers (Falke et al. 2008). First attempts to find the ILs differing significantly from the recipient and to detect the responsible donor segments were performed by applying a two-sided Dunnett test (Dunnett 1955, Falke et al. 2008, 2009a,b, 2010) and linear model analysis (Mahone et al. 2013).

In this study, we re-analysed a set of rye ILs with the high-resolution Rye5K SNP array to precisely characterize our rye introgression population. Subsequently a GWP model with a modification of the restricted maximum likelihood procedure that yields heteroscedastic variances (RMLV; Hofheinz and Frisch 2014) was used to detect the specific donor effects that affected the traits of interest.

Materials and Methods

Plant material and agronomic trials: A rye (*Secale cereale* L.) introgression population originating from a cross between the inbred line

L2053-N (bred by Hybro GmbH & Co KG, Schenkenberg, Germany), as recipient, and the Iranian primitive rye population Altevogt 14160 (provided by the Botanical Garden, Warsaw, Poland), as donor, was used for our analyses. A set of BC_2S_3 lines (previously mentioned as introgression library A) was derived by marker-assisted backcrossing with AFLP and SSR markers (Falke et al. 2008).

The performance *per se* was assessed in field trials (Falke et al. 2008, 2009a). Briefly, the field trials were conducted in 2 years at five sites in Germany (Bergen, Eckartsweier, Hohenheim, Oberer Lindenhof and Wulfsode). We analysed the performance of the ILs together with the recipient L2053-N (ten plots per replicate) and the donor Altevogt 14160 (three plots per replicate). The experimental design at each location was a 10 × 9 α-design (Patterson and Williams 1976) with three replicates. Data were recorded for plant height (cm), thousand-kernel weight (g), protein, total pentosan and starch content in grain (%), the latter three estimated by near-infrared reflectance spectroscopy (NIRS) from milled grain. Near-infrared spectra were recorded with a FT-NIRS instrument (Bruker MPA, reflectance mode, 850–2500 nm). The samples were scanned twice in duplicate repacking using two different petri dishes of 8.7 cm diameter and 1 cm height as sampling cups on rotating device (average of 32 scans in 10 s, two spectra per sample). Prediction models were calculated with OPUS software from Bruker (Bruker Optic GmbH, Ettlingen, Germany), version 6.5. Calculations were carried out with a modified partial least squares (PLS) procedure using a validation and a scatter correction of the spectra (SNV). Spectra were tested as original and as 1st derivatives. Two sets of samples were prepared for calibration and prediction. The samples were randomly distributed among the calibration and validation sets. Suitability of the models was controlled with the validation set. Prediction quality was calculated as determination coefficient, standard error of prediction and as RPD value, which relates the standard error of prediction to the standard deviation of the original data (SEP/SD). The relevant statistics for calibration and validation are given in Table S1.

We focused in this study on the traits plant height and the yield component thousand-kernel weight due to their importance for plant breeders as well as on the quality traits protein, total pentosan and starch content as a relevant factor for baking quality, feeding purposes and ethanol production. For baking quality, low protein content combined with high pentosan and starch content is required, while for feeding, high protein and low pentosan content are favoured. For ethanol production, starch content should be maximized.

Genotypic analysis and characterization of the introgression lines: Genotyping of the subset of 37 ILs and the recipient was performed with the Rye5K SNP array containing 5234 markers (Haseneyer et al. 2011). Out of these, the chromosomal positions of 3272 SNP markers were determined according to the rye consensus genetic linkage map as reported by Martis et al. (2013).

Estimation and test of the effects from the donor segments: The genetic effects of the donor segments on a phenotypic trait were estimated with the linear model:

$$\boldsymbol{y} = \boldsymbol{1}\boldsymbol{\beta_0} + \boldsymbol{Z}\boldsymbol{u} + \boldsymbol{e}.$$

Here, $\boldsymbol{y}$ is the vector of the phenotypic values of N introgression lines, $\boldsymbol{\beta_0}$ is a fixed intercept, $\boldsymbol{Z}$ is the design matrix relating the donor segments to the introgression lines, $\mathbf{u}$ is the vector of the donor segment effects, and $\mathbf{e}$ is the vector of residuals.

To construct the design matrix $\boldsymbol{Z}$, markers for which the alleles were in complete linkage disequilibrium in the introgression population were combined to donor segments. The elements of $\boldsymbol{Z}$ are coded in the design matrix such that the number represents the donor segment zygosity, that is as 0,1,2. Details on the structure of the design matrix $\mathbf{Z}$ are described by Falke et al. (2014).

For estimation of the donor segment effects, we used the RMLV method suggested for GWP (Hofheinz and Frisch 2014). The calculations were carried out with the software SelectionTools (www.uni-giessen.de/population-genetics/downloads). Subsequently, we adopted a permutation test similar to that suggested by Churchil and Doerge (1994) for QTL mapping. For carrying out the permutation test for the effect u_i of the ith donor segment, entries of the ith column of $\mathbf{Z}$ were randomly permuted and u_i was estimated for the random permutations. The distribution of the u_i from r random permutations was used to approximate the distribution under the null hypothesis that 'the segment has no effect on the phenotype'. Comparison of the effect estimate obtained for the actually observed phenotypic data with the approximated distribution of effects under the null hypothesis was used to assign p-values to the donor effect estimates. The p-values from testing linear contrasts and from the permutation test were adjusted with a modified Bonferroni procedure (Hochberg 1988).

Results

High-resolution genotyping revealed that the BC_2S_3 lines represented 94% of the donor genome. No large gaps were observed on any chromosome (Figs 1–5). The ILs carried on average 4.6 donor segments with a mean length of 27 cM (Table S2). Most of the donor segments were in the homozygous state.

The results of the field trials have been reported in detail previously (Falke et al. 2008, 2009a). The performance of the donor Altevogt 14160 exceeded the recipient L2053-N for thousand-kernel weight, protein and starch content, while the recipient showed a higher total pentosan content and a considerably shorter plant height. The ILs had the tendency to be more similar to the recipient. REML estimates of the genotypic variance were significant ($P < 0.01$) for all traits indicating that there is genetic variation between the ILs.

The RMLV method detected seven donor effects that significantly ($P < 0.05$) increased the plant height (Fig. 1, Table S3). The respective donor segments were distributed over the whole genome. Almost every IL carried a donor segment significantly ($P < 0.05$) affecting the plant height.

For thousand-kernel weight, we found two donor effects that significantly ($P < 0.05$) increased and six donor effects that significantly ($P < 0.05$) reduced the performance (Fig. 2, Table S3). The favourable donor segments were located on chromosomes 5R and 7R, while the unfavourable ones on chromosomes 1R, 3R, 5R and 6R. Eight ILs carried donor segments with significant ($P < 0.05$) favourable and unfavourable effects. If these ILs carried only one favourable donor segment, the unfavourable overcame the favourable one and the performance was reduced (2124, 2128, 2135 and 2136). If the ILs (2118 and 2119) carried two favourable donor segments, the positive effect overcame the negative and the ILs showed a significant ($P < 0.05$) higher thousand-kernel weight than the recipient.

For protein content, three donor effects were detected that significantly ($P < 0.05$) increased the performance, and one that significantly ($P < 0.05$) reduced the performance (Fig. 3, Table S3). The donor segments increasing the protein content were found on chromosomes 1R, 5R and 6R, and the segment reducing the performance was found on chromosome 7R. The two ILs (2131 and 2136) carrying a significantly ($P < 0.05$) positive and negative donor segment resulted in an increased protein content.

For total pentosan content, the RMLV method found two donor effects significantly ($P < 0.05$) increasing the pentosan content (Fig. 4, Table S3). Both donor segments were detected on chromosome 3R.

For starch content, we detected one donor effect with a significant ($P < 0.05$) positive effect on the starch performance and three donor segments with a significant ($P < 0.05$) negative

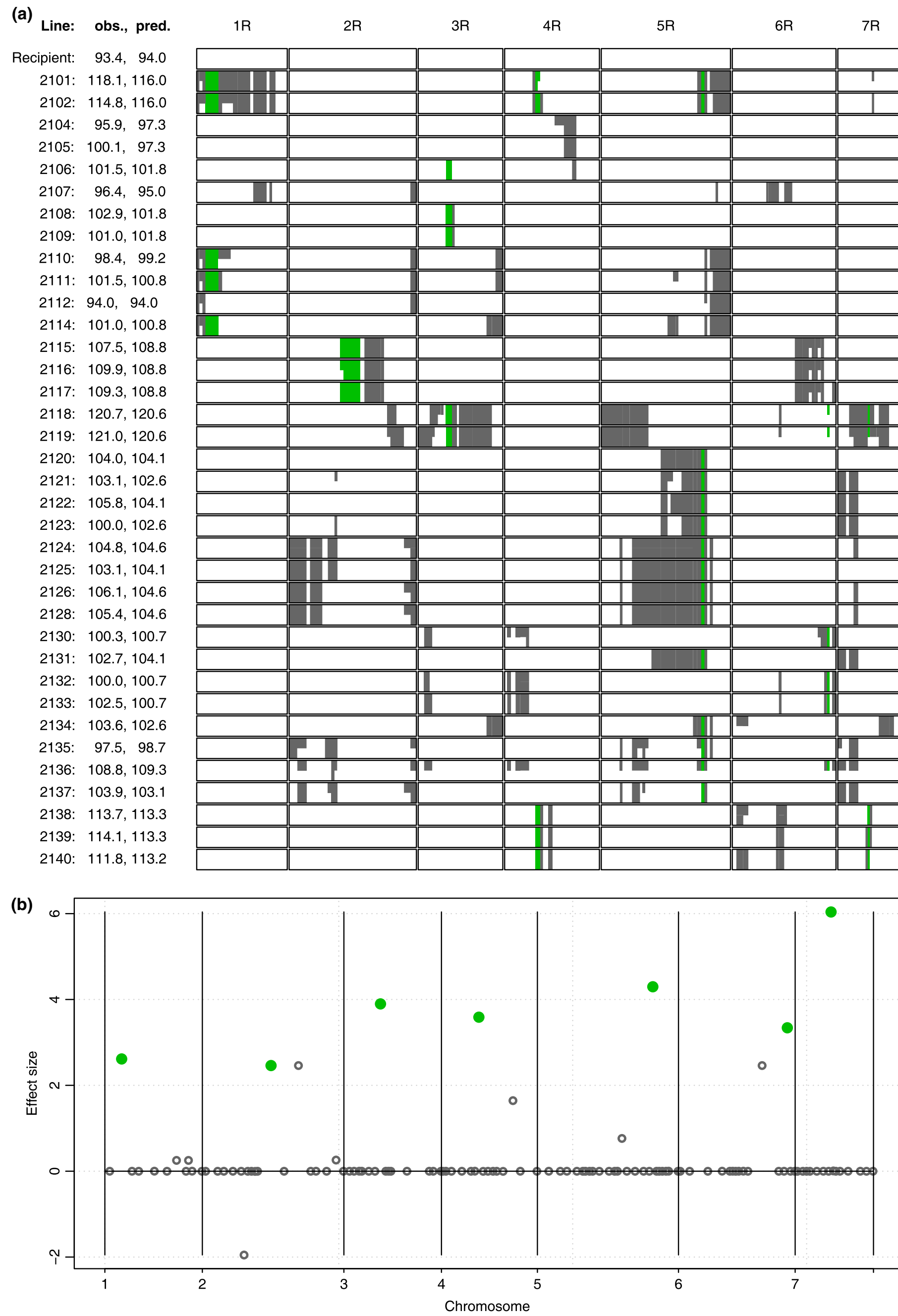

Fig. 1: Donor segment effects for plant height. (a) Observed (obs.) and predicted (pred.) plant height (cm) of the recipient and the ILs 2001–2040 of the rye introgression population. In the graphical genotypes, white colour indicates chromosome segments of the recipient and grey the introgressions from the donor. Green colour denotes donor segments that increase plant height. (b) Estimated size of the donor segment effects from the RMLV analysis plotted along the seven chromosomes of rye; grey circles denote donor segments that are not significant and green colour denotes significant ($P < 0.05$) effects increasing plant height

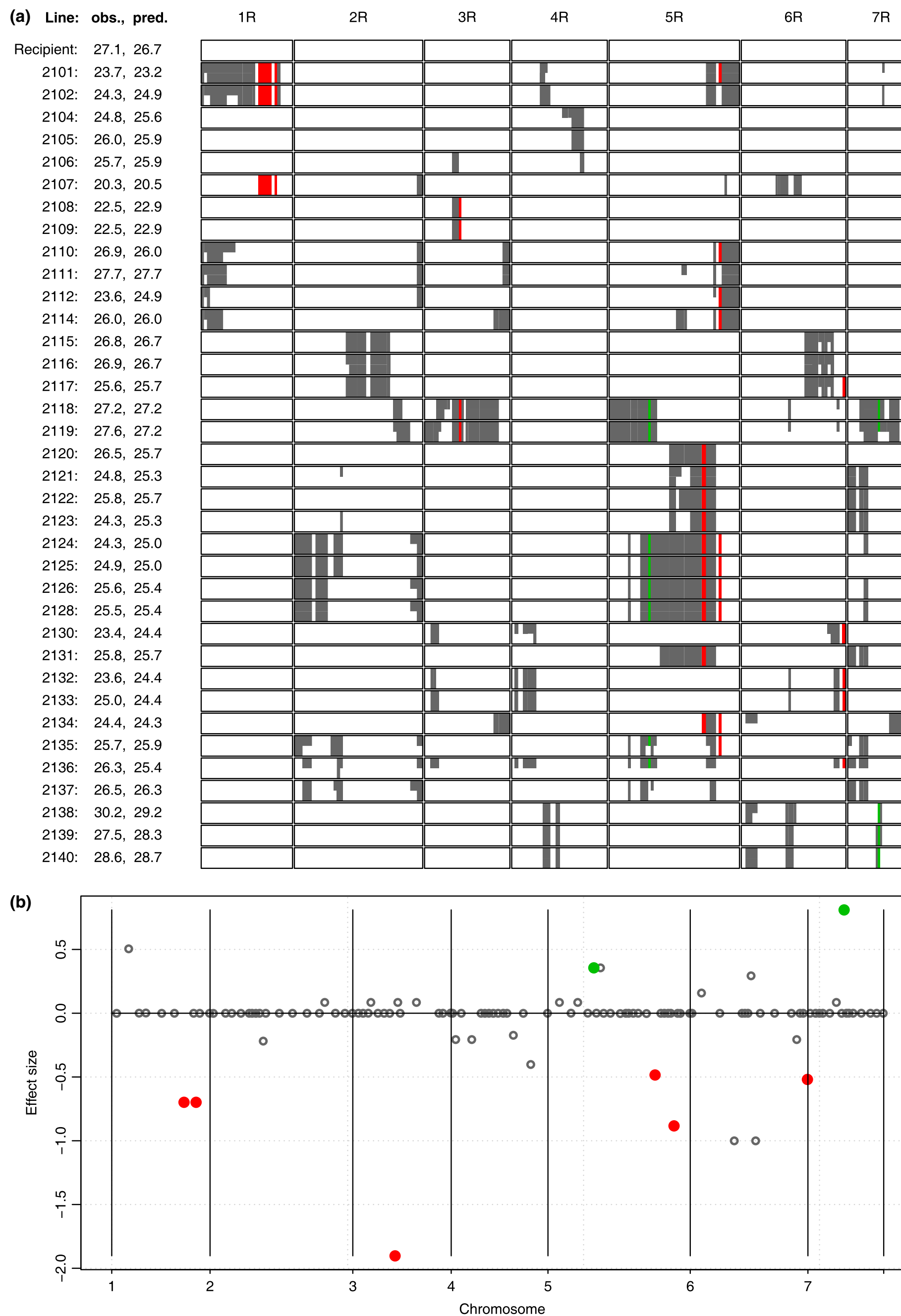

Fig. 2: Donor segment effects for thousand-kernel weight. (a) Observed (obs.) and predicted (pred.) thousand-kernel weight (g) of the recipient and the ILs 2001–2040 of the rye introgression population. In the graphical genotypes, white colour indicates chromosome segments of the recipient and grey the introgressions from the donor. Green colour denotes donor segments that increase thousand-kernel weight and red colour segments that decrease thousand-kernel weight. (b) Estimated size of the donor segment effects from the RMLV analysis plotted along the seven chromosomes of rye; grey circles denote donor segments that are not significant, green colour denotes a significant ($P < 0.05$) effect increasing and red a significant ($P < 0.05$) effect decreasing thousand-kernel weight

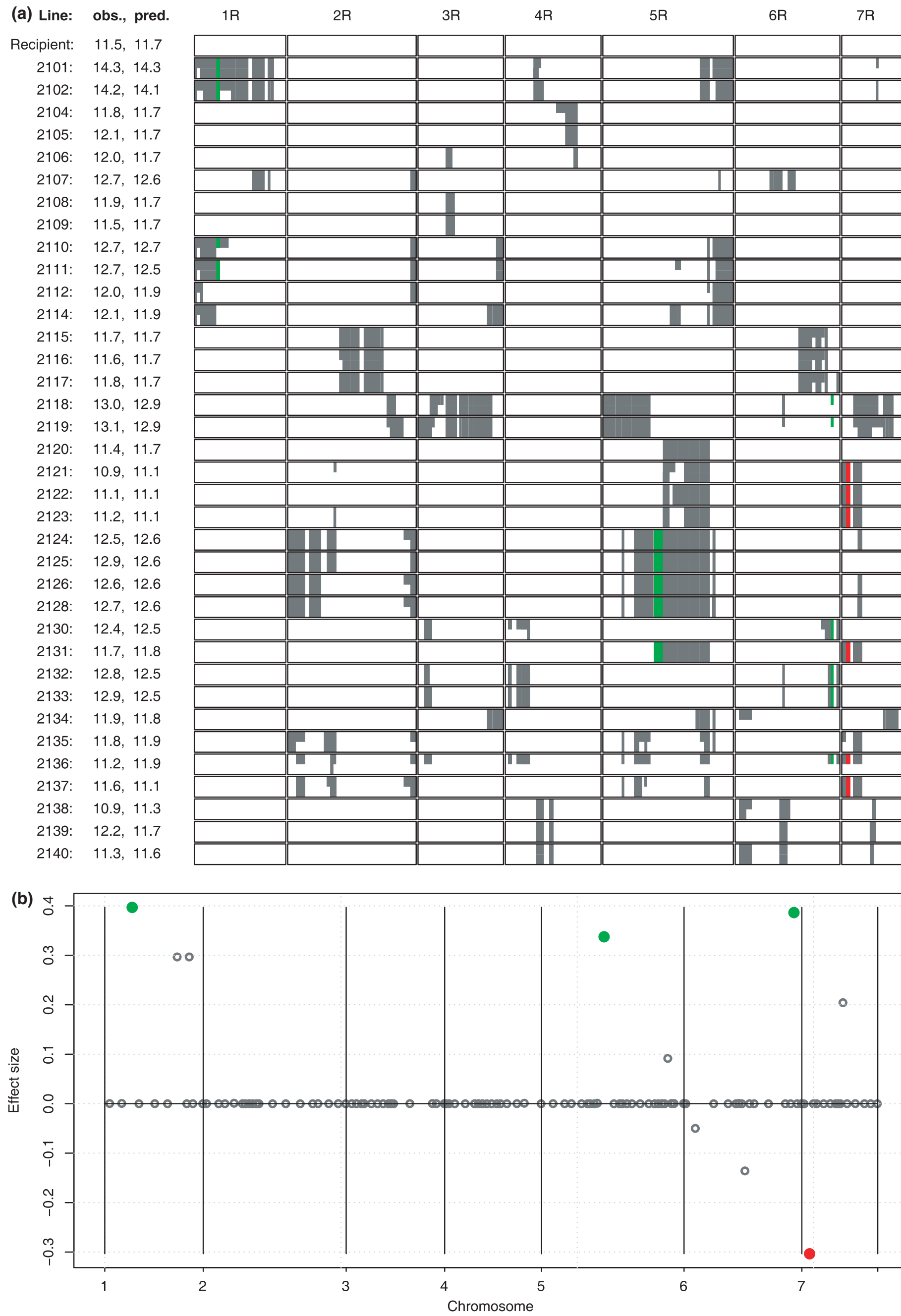

Fig. 3: Donor segment effects for protein content. (a) Observed (obs.) and predicted (pred.) protein content (%) of the recipient and the ILs 2001–2040 of the rye introgression population. In the graphical genotypes, white colour indicates chromosome segments of the recipient and grey the introgressions from the donor. Green colour denotes donor segments that increase protein content and red colour segments that decrease protein content. (b) Estimated size of the donor segment effects from the RMLV analysis plotted along the seven chromosomes of rye; grey circles denote donor segments that are not significant, green colour denotes a significant ($P < 0.05$) effect increasing and red a significant ($P < 0.05$) effect decreasing protein content

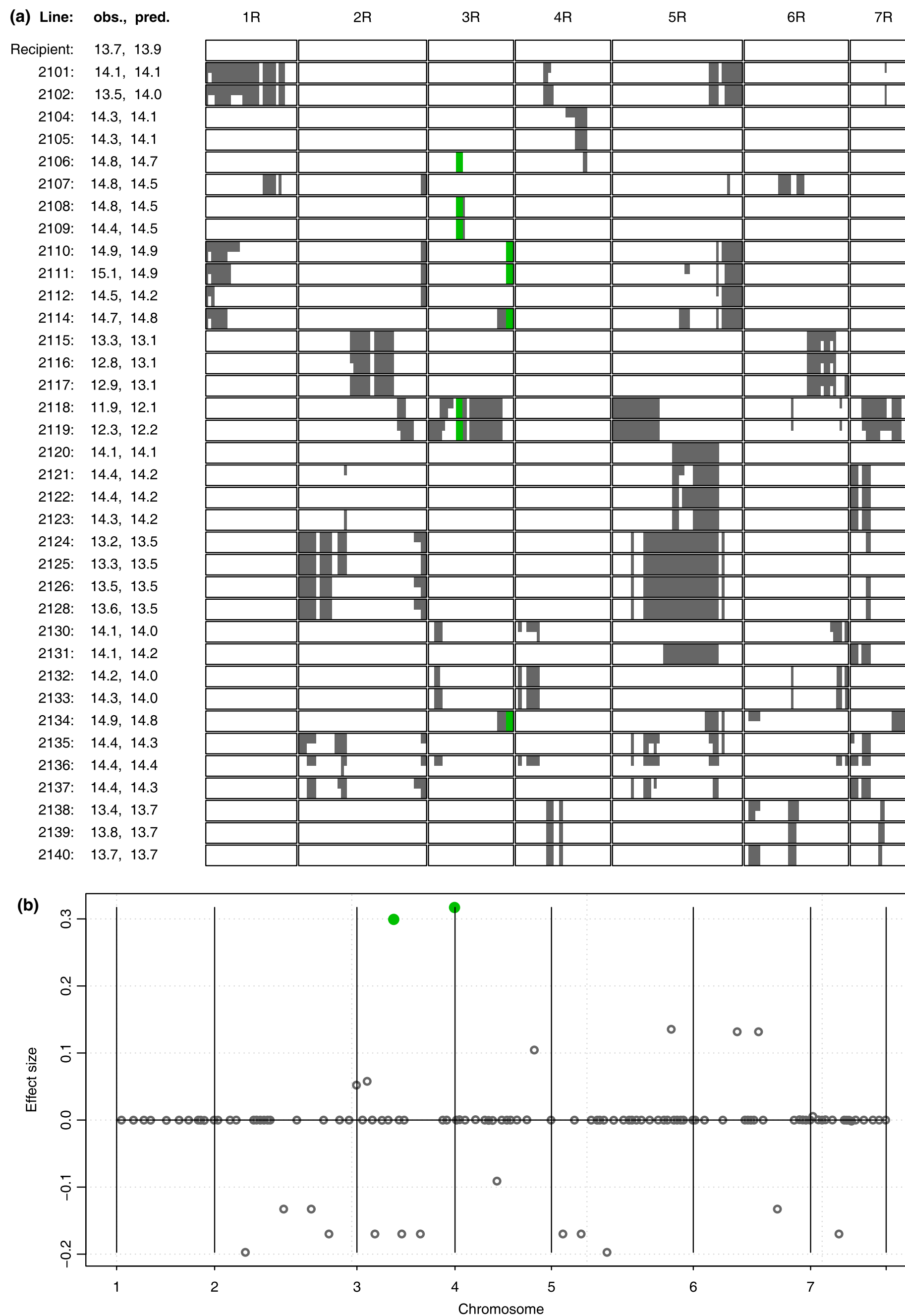

Fig. 4: Donor segment effects for total pentosan content. (a) Observed (obs.) and predicted (pred.) pentosan content (%) of the recipient and the ILs 2001–2040 of the rye introgression population. In the graphical genotypes, white colour indicates chromosome segments of the recipient and grey the introgressions from the donor. Green colour denotes donor segments that increase pentosan content and red colour segments that decrease pentosan content. (b) Estimated size of the donor segment effects from the RMLV analysis plotted along the seven chromosomes of rye; grey circles denote donor segments that are not significant, green colour denotes a significant ($P < 0.05$) effect increasing pentosan content

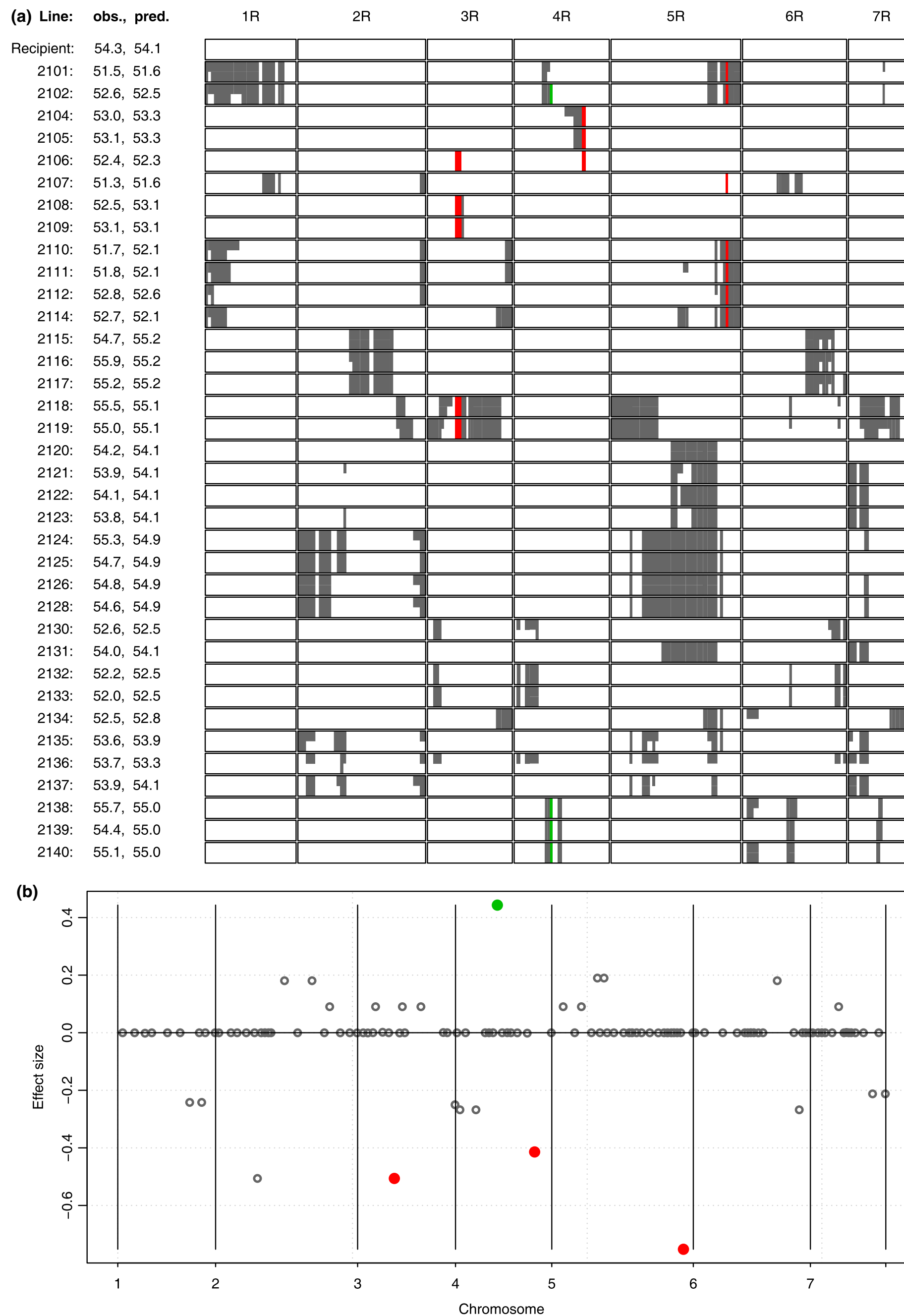

Fig. 5: Donor segment effects for starch content. (a) Observed (obs.) and predicted (pred.) starch content (%) of the recipient and the ILs 2001–2040 of the rye introgression population. In the graphical genotypes, white colour indicates chromosome segments of the recipient and grey the introgressions from the donor. Green colour denotes donor segments that increase starch content and red colour segments that decrease starch content. (b) Estimated size of the donor segment effects from the RMLV analysis plotted along the seven chromosomes of rye; grey circles denote donor segments that are not significant, green colour denotes a significant ($P < 0.05$) effect increasing and red a significant ($P < 0.05$) effect decreasing starch content

effect on the starch performance (Fig. 5, Table S3). The donor segment with a positive effect was located on chromosome 4R, while those with negative effects on chromosomes 3R, 4R and 5R. IL 2102 contained the positive and the negative donor segment from chromosome 5R. Here, the negative effect exceeded the positive which lead to a reduced starch content compared to the recipient.

Discussion

Characterization of the introgression population with high-density mapping

The rye introgression population was initially developed and characterized with up to 137 SSR markers and 14 AFLP primer combinations (Falke et al. 2008). The marker-assisted backcrossing resulted in BC_2S_3 ILs carrying on average 4.7 introgressions with a mean length of 13 cM. The total population covered 74% of the donor genome. In this study, the introgression population was re-analysed with the high-density Rye5K SNP array and the chromosomal positions of the SNP markers were determined according to a rye consensus genetic linkage map (Martis et al. 2013). In general, consensus genetic mapping is more complex than mapping based on single data sets. Therefore, limitations such as differences in recombination rate, exchange distribution along chromosomes or variation in dominance of the used markers can occur (Ronin et al. 2012). The re-analysis of our introgression population validated mainly our previous results but also revealed that the donor genome coverage is considerably higher with 94% and that additional donor segments exist. For example, new individual introgressions were found on chromosome 2R in several ILs (2107, 2110–2112, 2124–2126, 2128 and 2135–2137), on chromosome 3R (ILs 2110, 2111, 2114 and 2134) and on chromosome 4R (ILs 2130, 2132, 2133 and 2136). The detection of new additional donor segments when using high-resolution SNP arrays is in close agreement with results found for a barley introgression library (Schmalenbach et al. 2011) and can be attributed to the higher mapping accuracy of the SNP arrays. Accurately characterized introgression populations are a prerequisite for precise donor effect detection. Our results suggest that introgression populations can be better characterized with high-resolution genotyping assays than with a limited number of markers.

Detection of donor effects

The detection of donor segments with favourable effects has initially been developed in tomato by Eshed and Zamir (1994). The interest of this approach has been growing as these introgression populations allow the simultaneous detection of favourable effects and variety development in nearly one step. Thus, it facilitates the successful use of these effects in the breeding process and reduces the time required for variety development. So far, mainly pairwise testing is used to determine whether an IL differs significantly from the recipient. Here, it is advantageous that the ILs carry only single donor segments to assign the effect to the specific segment. In practice, however, the development of introgression populations is size limited by the number of concurrent backcross programmes and field space, and thus, the ILs carry mainly multiple donor segments. The situation can easily occur that the introgression population contains more donor segments than lines. In this case, the donor effects are not estimable with fixed linear models. Integrating GWP methods can overcome the drawbacks of pairwise testing and fixed linear models. In combination with permutation tests, the RMLV model (Hofheinz and Frisch 2014) is particularly recommended to detect donor effects in introgression populations with multiple or overlapping introgressions and provides the detection of positive and negative effects in individual ILs (Falke et al. 2014). Our rye introgression population contains 168 disjunct chromosome segments and therefore more donor segments than ILs. Consequently, the RMLV model seems here the appropriate tool of choice. However, the effects detected in this study are not yet validated. Due to the small sample size, cross-validation is not an option. We plan experimental validation of the effects in an independent validation experiment.

Plant height is a trait affecting the fitness in natural populations and plays an essential role in plant breeding programmes as selection criterion. Its inheritance is expected to be complex, controlled by many loci distributed over the whole genome (Schön et al. 2004, Wang et al. 2006, Miedaner et al. 2011, 2012). In this study, RMLV detected on each chromosome a significant donor effect (Fig. 1) and, thus, confirmed the results from the literature. All of the significant donor effects were associated with an increase of plant height which agrees with other studies in cereals using exotic germplasm in introgression populations (Pillen et al. 2003, Septiningsih et al. 2003, Liu et al. 2006, Von Korff et al. 2006, Falke et al. 2009a,b, Miedaner et al. 2011). The analysis of our introgression population with the Dunnett test (Dunnett 1955) showed that nearly every IL had a significantly increased plant height compared to the recipient (Falke et al. 2009a). RMLV confirmed these results, but additionally enabled the precise localization of seven donor effects which were responsible for the increased plant height (Fig. 1). In conclusion, our results support the assumption of the very complex inheritance of plant height.

Grain yield is proposed to follow the infinitesimal model of quantitative genetics (Fisher 1918), and thus, it is not expected that marker-assisted selection can be successfully employed. We therefore focused on the yield component thousand-kernel weight. Two donor segments with effects significantly increasing the thousand-kernel weight were detected on chromosomes 5R and 7R (Fig. 2). Both effects correspond well with large effect QTL found with classical QTL mapping (Miedaner et al. 2012) and major genes (Wricke 2002). The high effects of these QTL were explained as an indication of single genes. Our results strengthened this assumption. The Dunnett test (Dunnett 1955) found eight ILs with a significant decreased and one IL with a significant increased thousand-kernel weight compared to the recipient (Falke et al. 2009a). These results were confirmed by the RMLV method. However, even more ILs with significant donor effects were found with RMLV than with pairwise testing. Interestingly, many of these ILs carried both a significant favourable and an unfavourable donor effect (Fig. 2). Here, mainly the unfavourable dominated the favourable effect and a lower thousand-kernel weight was observed. We explain this by the fact that these ILs carried mostly two unfavourable and only one favourable donor segment. In conclusion, the confirmation of the major genes and the possibility to detect positive and negative donor effects in individual ILs support the high power of the used GWP model.

Plant height and yield components are among the most important traits in rye breeding. Quality traits in rye, however, vary depending on the end-use purpose of the breeding programme. We focused in our study on protein, total pentosan and starch content as they are all of crucial importance for baking quality, feeding and ethanol production.

For protein content, we detected three donor segments with a significant effect that resulted in a increased protein content compared to the recipient (Fig. 3). These segments were located on chromosomes 1R, 5R and 6R. Miedaner et al. (2012) detected QTL on chromosomes 1R and 6R with classical QTL mapping, too. However, these QTL detected in other backgrounds were located on different positions on the chromosomes. Moreover, one donor segment with a significant negative effect was found on chromosome 7R, which has not been described in the literature yet. We rate these results as an indication that we found here new alleles for protein content from the exotic donor. We therefore conclude that the donor segments with significant effects on chromosomes 1R, 5R and 6R are good starting points for improving feed quality and the donor effect on chromosome 7R for improving baking quality. For protein content, all ILs detected by the Dunnett test as significantly different from the recipient were also found with RMLV. However, here occurred the same situation as for thousand-kernel weight, if an IL carried both, positive and negative donor effects, only RMLV enabled their detection. In this situation, the positive effect dominated the negative effect and a higher protein content was observed. We explain this by the higher *per se* performance of the donor compared to the recipient (Falke et al. 2009a).

For total pentosan content, two donor effects that significantly increase the pentosan content were found by RMLV on chromosome 3R (Fig. 4). This result confirmed the results from the Dunnett test and additionally identified the two responsible segments. One of these two donor segments corresponded well with a QTL for total pentosan content detected on chromosome 3R in a segregating population with a different genetic background (Miedaner et al. 2012). The other significant donor segment might be an indication for new favourable alleles introduced through the exotic donor. Hence, the eight ILs carrying the two donor segments can directly be used for improving baking quality of elite material.

For starch content, RMLV detected one donor segment on chromosome 4R with a significant positive effect (Fig. 5). Miedaner et al. (2012) found also QTL on chromosome 4R with classical mapping but on other positions. This can indicate that our exotic germplasm contributes new favourable alleles to improve starch content. We therefore conclude that the detected donor segments in ILs 2102, 2138, 2139 and 2140 might be valuable for improving baking quality and ethanol production.

Conclusion

The analysis of our rye introgression population using RMLV confirmed many QTL described in the literature. Moreover, for the quality traits, segments with donor effects with obviously new and particularly favourable alleles were detected. It is remarkable that such results can be found in genetic resources having such an inferior *per se* performance like the applied donor Altevogt 14 160 (Falke et al. 2009a,b). These donor effects can directly be exploited in breeding programmes for improving baking and feed quality, and ethanol production. Thus, this should encourage geneticists and plant breeders to invest more time and work in genetic resources. Compared to our previous studies using pairwise testing with the very conservative Dunnett test (Falke et al. 2008, 2009a), we found more segments with significant donor effects using the RMLV method. We explain this by the fact that the GWP model allows the detection of positive and negative effects in individual ILs. These donor effects might cancel each other out if using pairwise testing, and thus, there were no significant donor effects detected. For utilizing favourable donor effects without getting the unfavourable ones, typically, further backcrosses are recommended to split the different donor segments into several sub-ILs by marker-assisted selection. An advantage of our rye introgression population here is that the significant donor segments are relatively small when further backcrossed into elite lines. Thus, linkage drag can be drastically reduced due to the sharper localization of the effects to smaller genomic regions. We therefore conclude that the application of RMLV opened a new possibility for plant breeders and geneticists when working with introgression populations.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Statistics for calibration and validation.

Table S2. Donor segments.

Table S3. Donor effects.

Chapter 6

General Discussion

Statistical Tests

Introgression libraries have been typically analyzed with a series of pairwise tests to detect whether the recipient and the ILs differ with respect to the investigated traits (Eshed and Zamir 1995; Matus et al. 2003; Rosseaux et al. 2005; Eduardo et al. 2007; Finkers et al. 2007; Szalma et al. 2007; Jeuken et al. 2008; Falke et al. 2008, 2009a, b; Schmalenbach et al. 2008, 2009, 2011; Schmalenbach and Pillen 2009). Results from Mahone et al. (2012) confirm that the Dunnett test is better suited for analyzing NIL libraries than pairwise t-tests. Even with adjustment for multiple testing, the t-tests had a considerably greater false positive rate. Pairwise tests, such as the Dunnett test, used to detect phenotypic differences between ILs and the recipient parent are unable to identify the precise location of a QTL when multiple or long DCS are present in the ILs. Using the linear model analysis, an increase in the precision of QTL detection is expected. This particularly true for libraries with some chromosome regions duplicated in more than one NIL, as in the libraries of previous studies (Eduardo et al. 2007; Falke et al. 2008). The advantage of the linear model test is likely due to a more precise estimation of the residual variance, which is accomplished by using the entire library rather than only the recipient parent and the introgression line under consideration.

The two introgression libraries investigated in Mahone et al. (2013) consisted each of 40 ILs. Multiple DCS are found in 39 of the 40 ILs of introgression library A and 25 of the ILs of library B. The original Dunnett analysis was generally unable to show the single donor introgressions putatively causing the detected phenotypic differences. Further experimental work can help to locate the position of QTL (Rousseaux et al. 2005) in such situations. To accomplish this, the DCS of a significant IL are split up into several sub-ILs by further backcrosses, which can then again be tested against the recipient parent. As an alternative to the creation of sub-ILs, employing a linear model analysis after performing a Dunnett test can aid the identification of QTL in ILs with multiple DCS. The results of the Dunnett test and the linear model method can be therefore combined to point towards promising DCS. The results of the analysis in Mahone et al. (2013) illustrated this. In that analysis, introgression library A, ILs 2121, 2123, and 2131 contain multiple DCS across several chromosomes. The Dunnett test results of testcross thousand-kernel weight showed these ILs as being significantly different from the recipient, but the location of the causative alleles could not be exactly determined. The linear model analysis pointed to the common DCS on chromosome 7R in all three ILs, and thus the potential length of DCS for fine-mapping shrank from around 50 to under 20 cM. The use of the linear model analysis therefore allowed us to identify genomic regions carrying putative QTL, improving the precision achieved with the Dunnett test alone. We can conclude that the combination of the Dunnett test with a linear model analysis provides a valuable tool to identify and localize QTL, potentially reducing the need for further splitting of the DCS in ILs with multiple segments.

The linear model analysis can potentially provide a much more detailed analysis of the pleiotropic effects of DCS than a pairwise test such as the Dunnett test. For example, the analysis from Mahone et al. (2013) revealed that putative QTL found on the DCS on chromosomes 4R and 6R which increase the per se performance for pentosan and protein content in introgression library B were also responsible for a decrease in starch content. Similar observations can be made throughout both libraries of that study for per se and testcross performance. In practical breeding programs such indications of pleiotropy might help to identify DCS that increase the performance of one of

two negatively correlated traits without negative effects on the second trait. Fine-mapping and/or further sub-IL generation would be necessary to determine if the pleiotropic QTL detected in this study are the result of individual QTL or several linked QTL. There is an inherent limitation for the analyis and for the purposes of our research, we can only localize QTL to DCS and assume that they are either a single QTL or two or more tightly linked QTL.

QTL Detection

In our research, we evaluated overall test performance with simulations. We used both the detection power and false positive rate in order to benchmark test performance. The sum of correctly detected effects was the measure of the detection power, while the false positive rate was estimated via the sum of false positive effects. Mahone et al. (2012) described in detail the comparison of the Dunnett test and the linear model method in regards to correct detection and false positive rate. The ratio of major and minor QTL detection power to false positives generally increased for both tests as genetic variance decreased, proceeding from few large effect QTL to many small effect QTL. Within each scenario, major QTL detection ratio for the linear model test was highest at low heritabilities and decreased at high heritabilities while the Dunnett test peaked at high heritabilities. This was true in the both overlapping and nonoverlapping libraries, while in the STAIRS library both tests peaked at high heritabilities. The ratio of correct detections to false positives was higher for the linear model overall than for the Dunnett test. This is explained by both tests having shown similar power but the Dunnett test had generally higher false positives. The ratio of correct detections to false positives was highest for the linear model test, peaking at low heritabilities. The Dunnett test, by comparison, had somewhat equal correctly detected and false positive effects at the same heritabilities. The sum of correctly detected effects in general identifies neither the Dunnett test nor the linear model test as the superior method in every case. However, lower false positives in most instances may be regarded as an advantage of the linear model test. An additional point to consider is the flexibility allowed by using a linear model approach.

Additional features such as model building is possible, as well as the potential to model interactions of genetic effects. Using introgression libraries, linear model methods could uncover and investigate epistasis with precision that is hard to achieve in segregating populations.

From the results of Falke et al. (2014), the linear model analysis successfully controlled the false positive rate in introgression library 1. This was accompanied with a lower power of detecting significant donor segments than the BLUP and RMLV analyses for heritabilities between 0.6 and 0.8 when several QTL controlled the trait. For this reason, the linear model analysis is the more suitable method when it can be assumed that the trait has a high heritability and is controlled by a small number of major genes. In situations where traits have low heritabilities and/or they can be assumed to be polygenic, the genome-wide prediction approaches might be advantageous for the detection of donor effects. The higher false positive rate however means that subsequent verification experiments are necessary.

Also from Falke et al. (2014), the BLUP analysis showed a very high false positive rate in the simulations with introgression population 2 when two loci controlled the trait. A possible explanation is that the model underlying the BLUP analysis assumes that each donor segment contributes equally to the genetic variance, i.e., the donor segment variances are homoscedastic. Obviously if only two genes control the trait under consideration, this assumption is wrong. Large effects are consequently underestimated and small or zero effects are overestimated. The overestimation of small effects is likely the cause for the high false positive rate in the permutation test of the BLUP analysis with non-polygenic inheritance. The RMLV analysis showed a considerably greater rate of correctly detected effects than the BLUP analysis for low heritabilities, which suggests that an RMLV analysis is an option to detect donor segment effects that would otherwise go undetected.

From the previously described results, it is clear that a main difficulty of applying genome-wide prediction methods to introgression populations is the rather high rate of false positives. This depends directly on the degree to which the assumptions underlying the statistical models are violated and can not be corrected by adjusting p-values for multiple testing. It can be therefore

concluded that genome-wide prediction methods have the potential to detect favorable alleles, but a validation of the effects in subsequent trials is necessary.

Introgression Library Design

From our research, we can take away some information that can be considered when creating introgression libraries. Additionally, we can make assumptions regarding the analysis based on the structure of the donor chromosome segments in the library under investigation. We saw the largest difference between the linear model method and the Dunnett test in introgression libraries with overlapping donor segments (Mahone et al. 2012). The that study, the linear model analysis provided better results with low heritabilities in the overlapping libraries, but as heritabilities increase (to 0.9 and above) both the linear model method and the Dunnett test provided satisfactory results. The false positive rate increased for the linear model test and decreased for the Dunnett test as heritability increased in nonoverlapping and overlapping NIL libraries. An explanation for the increase observed for the linear model test is a higher power to detect QTL located between known donor DNA and known recipient DNA. Additionally, this trend may also reflect detection of adjoining segments that do not contain QTL but are being declared significant because of low residual variance present at high heritabilities.

For low heritabilities in the nonoverlapping library and the overlapping library, the false positive rate of the Dunnett test was considerably higher than the linear model test (Mahone et al. 2012). From that study, at marker distance of 0.05 cM, the false positive rate for the Dunnett test was more than twice that of the linear model test for low heritabilities in the nonoverlapping library. Dunnett test power was slightly greater than the linear model test power in overlapping NIL libraries, but this increased power cannot be exploited because of the inflated false positives. Additional simulations were performed in overlapping libraries to assess the false positive rate difference between the Dunnett test and the linear model method. In these additional simulations, false positives were only declared for the Dunnett test when both lines with overlapping donor segments were declared significant. The linear model test

generally outperformed the Dunnett test in regards to the sum of false positive effects even in these situations. The linear model method is therefore more appropriate for introgression libraries with overlapping donor segments, which in experimental datasets constitutes the vast majority of introgression libraries.

The quantitative genetic scenario of the trait also has important implications for QTL detection, and this is in turn also impacted by the structure of the introgression library. Detection of a QTL depends on the amount of genetic variance that can be attributed to the QTL compared with the total variance in the experiment. The four scenarios used in the simulations of Mahone et al. (2012) show a progression from few QTL of large effect to many QTL of smaller effect. As the individual QTL decrease in effect size and increase in number, the variance explained by a single QTL decreases. Decreasing heritability also decreases the relative variance explained by single QTL. This decrease in the variance explained by individual QTL is a contributing reason for the decrease in power observed in the study. The results from Mahone et al. (2012) indicate that these factors, as well as the number of times a QTL is present in the library, all contribute to the variance and therefore affect detection. The highest power of correct detection occurred with non-overlapping NIL libraries with a trait composed of few major genes, for both the linear model test and the Dunnet test. The reduced efforts in establishing a library with overlapping donor chromosome segments (including STAIRS libraries) are certainly advantageous, but there was a clear cost in terms of lower power of QTL detection. Obviously this cost disproportionately affected the minor effect QTL, which would constitute a major weakness since a major goal of introgression library development is to find those QTL that are potentially masked in traditional QTL populations. Falke and Frisch (2011) reported a considerable lower power of QTL detection with overlapping rather than with nonoverlapping NIL libraries employing the linear model test. The research in Mahone et al. (2012) reinforces those findings and also extended the comparison to the STAIRS library design.

In our research of overparameterized libraries (Falke et al. 2014), it was determined that from the two genomic prediction models tested, RMLV was the superior method. This result is in agreement with theoretical considerations regarding the QTL detection for the oligogenic traits, which encompass

many traits important to breeders. The BLUP approach used in Falke et al. (2014) employs homoscedastic genetic variances across donor chromosome segments, which can be assumed for highly polygenic traits. However, the assumption of homoscedastic genetic variances does not fit for oligogenic traits. In general, the focus of introgression populations lies on identifying donor segments that have a considerable effect on the trait under consideration. The traits to be improved are therefore generally oligogenic and thus primarily caused by a small number of major genes. From other studies utilizing over-parameterized library data, it is clear that some traits have multiple potential causal QTL (Mahone et al. 2015). From the research of Falke et al. (2014) and Mahone et al. (2015), it can be concluded that the RMLV is more useful as a general method because of the unequal rationing of variance to the donor chromosome segments, and therein conforming to the oligogenic nature of many traits of interest.

Application to Experimental Datasets

Simulations of many scenarios were utilized in order to test the introgression library analysis methods. The use of a range of simulation scenarios was an important step in investigating the transferability of our results to general experimental results. In the comparison of the linear model method and the Dunnett test (Mahone et al. 2012), the detection power and false positive rates differed but the trends observed for different types of libraries, quantitative genetic scenarios, heritabilities, and the choice of tests were similar. It can be concluded from that study that the results are robust with respect to the number and length of the chromosomes and can potentially serve as reliable guidelines for introgression libraries in any crop species.

A principal difference between the Dunnett test and the linear model test is that the linear model is testing for the presence of QTL on individual chromosome segments, whereas the Dunnett test is testing NILs as a whole. From the previous research, we can point to many cases where the linear model was able to separate and test donor segment effects in lines declared significant by the Dunnett test. Of course, this is limited by the arrangement of

donor segments in the introgression library. In Mahone et al. (2012), a rapeseed introgression library was analyzed with both the Dunnett test and then later with the linear model method. Introgression line 203 was found to be significant using the Dunnett test, and it contains three separate introgressed donor segments. This includes segment 119, which was found to be significant using the linear model test. The remaining segments were not declared significant with the linear model test, however. Additional QTL information was also gained for introgression lines 227, 387, and 1036. Using the linear model test in this case was able to provide more information on the location of the QTL compared to the Dunnett test. We conclude that the linear model test is of great advantage for NIL libraries with lines that carry multiple introgressions, because it has the potential to detect specific introgressions that are responsible for the differences in the phenotype of the NIL and the recipient parent. Additional simulations support the results, indicating that the linear model test has higher power than the Dunnett test when multiple QTL are on separate introgressions in the same line.

The linear model method also allows the detection of positive and negative QTL within the same line. The introgressions would have to be present in additional lines separately in order to separate their effects. In Mahone et al. (2012), this was observed in the analysis of the rapeseed introgression library. In the 30 introgression lines where significant donor segments were detected by the linear model test but not with the Dunnett test, 17 carried QTL with both positive and negative effects. In multiple instances, QTL with both positive and negative effects were detected in the same introgression line. In some cases these QTL fall on donor segments in introgression lines that were not declared significant by the Dunnett test. We can therefore conclude that when those donor segment effects are in fact separable, the linear model test has the advantage that it is able to find QTL in ILs that carry more than one QTL with different signs on different chromosome segments.

In Mahone et al. (2013), results of the linear model method across two populations with both per se and testcross performance data were compared. Although a rather low consistency for QTL was seen between the per se and testcross dataset, such an analysis was useful in finding stable QTL across both sets. In this way, sub-ILs could be created containing important QTL

that were validated in both sets. Allowing the dissection of phenotype-affecting donor segments within introgression lines using the linear model test ensures that resources can be attributed to promising lines.

The Dunnett test supplies researchers only with the a significance level of the difference between each introgression line and the recipient parent. The linear model method, in contrast, can be extended in many ways. As previously mentioned, the extensibility of this technique can allow for detection of gene interactions (epistasis) as well as model building. The utility of regression approaches has been demonstrated in previous studies (Wang et al. 2006, 2007; Coles et al. 2011). From the presented research, we can conclude that employing a linear model test is a very promising method that allows the detection of favorable DCS in introgression libraries consisting of ILs that carry long or multiple DCS. Additionally, it has the potential to greatly enhance the efficiency of producing sub-ILs, because only segments contributing a significant effect need to be isolated.

In experimental datasets, particularly those that were produced recently, there are typically many more markers available than there are genotypes to test. As previously discussed, a major drawback of the linear model method, in comparison to the Dunnett test, is that using it with such datasets produce unreliable results. Therefore the application of the genomic prediction models are available to provide the same benefits of the linear model method to datasets that are overparameterized. In Falke et al. (2014), genomic prediction models were applied to the rapeseed intorgression library in order to test the transferability of these methods. The results were then used to compare two methods, the BLUP method and the RMLV method. As discussed in detail in Falke et al. (2014), the BLUP method is likely to underestimate donor segment effects compared with the RMLV method. This was seen with a major QTL detected for glucosinolate content, in which the RMLV estimated effect was approximately 125% of the BLUP estimated effect. The RMLV analysis also showed a number of QTL for linolenic acid content that likely represents the true reality of the trait better than the BLUP analysis. Another RMLV analysis also detected QTL for yield and quality traits, such as protein and pentosan content, in the rye introgression libraries (Mahone et al. 2015). Many QTL found via this method were validated from previous studies.

The RMLV analysis also performed well for the analysis of the previously analyzed two rye libraries. For the trait plant height in the rye libraries, the RMLV analysis was able to detect a donor segment on chromosome 2 that considerably decreased plant height of the lines 2124, 2125, and 2135. A shorter plant height distinguishes modern rye lines from older breeding material and is a key agronomic property. The plant height for the exotic donor of the rye introgression library was larger than that of the recurrent parent (Falke et al. 2009a, 2009b; Miedaner et al. 2011). The donor segment that reduced plant height found by the RMLV analysis may therefore serve as an important reminder that beneficial alleles may be found in exotic donors, even when agronomic performance is not on the same level as an elite donor. Further work was done with the rye introgression libraries using the RMLV method, which showed that donor segments can also potentially improve protein, pentosan, and starch content (Mahone et al. 2015). Therefore elite varieties can potentially be improved for a variety of traits via introgression of exotic germplasm when beneficial donor segments can be identified.

Conclusions

The previous body of research is intended to supply a set of “best practice” guidelines for analyzing introgression libraries. The research has shown that composition of the introgression library is an important consideration for analysis. Additionally, the number of markers available compared with the number of NILs present should inform the decision of which method to employ. For those libraries where there are more NILs than markers, the linear model method of Falke and Frisch (2011) is, based on evidence presented previously, a more discerning approach than the Dunnett test. When there are more markers than genotypes, such as in an over-parameterized library, then the research suggests that using genomic prediction models is sufficient to provide estimates of marker effects. Adding permutations to this analysis allows the discovery of QTL because the null hypothesis can be tested, separating real marker trait associations from spurious associations arising by chance. Taken together, this collection of methods should give proper guidelines to researchers undertaking

an introgression library analysis. This work should inform the researchers to understand the extent and limits of analysis possibilities based on the structure of the introgression library in question.

Chapter 7

Summary

Introgression populations are used to make the genetic variation of unadapted germplasm or wild relatives of crops available for plant breeding. The libraries consist of near-isogenic lines (NILs) that carry small chromosome segments from an exotic donor in the genetic background of an elite line. The NILs can be used to detect beneficial trait variation in germplasm that is unadapted or has poor agronomic performance. Post-detection, introgression libraries can be used to make favorable genetic variation of exotic donor genotypes available in the genetic background of elite breeding material. In this way, introgression libraries or populations can help bridge the gap between elite and wild varieties, making further genetic resources available for breeding purposes. A key question is the detection aspect, since the detection of beneficial donor segments is the crux of the potential for introgression libraries to improve breeding material. In many cases, the number of donor segments exceeds the number of lines, and more specialized statistical methods are required in this case. The objective of this research was therefore to compare tests for QTL detection in NIL libraries regarding detection power and false positive rate, as well as propose best practice for their use regarding known aspects of trait architecture and heritability. Additionally, statistical tests were adapted and tested for use in introgression libraries that have more markers than lines. To accomplish these objectives, introgression libraries with different configurations of donor segments (overlapping, nonoverlapping, and stepped aligned inbred recombinant straits (STAIRS) libraries) were simulated and experimental data from

rapeseed and rye were used.

Computer simulations with maize genome models were employed to investigate nonoverlapping NIL libraries, overlapping NIL libraries, and STAIRS libraries for traits with oligogenic inheritance. Quantitative trait loci detection power of the linear model and Dunnett tests were similar for nonoverlapping and STAIRS libraries; for overlapping NIL libraries the Dunnett test was slightly superior. False positives were greatest for the t test and lowest for the linear model test. False positive sums with the Dunnett test were generally higher than for the linear model test if the heritability was 0.9 or lower. The linear model test outperformed the Dunnett test in nonoverlapping introgression libraries and for overlapping introgression libraries where trait heritabilities are below 0.9. As these constitute the majority of cases, the linear model test can be regarded as an improvement compared to the Dunnett test. Analysis of the rapeseed library additionally revealed that QTL localization ability using the linear model test has a higher potential. The linear model test has the potential to reveal a causative donor segment in lines containing several segments, and can discriminate between positive and negative QTL in the same line.

A dataset of two rye introgression libraries that consisted of ILs carrying multiple donor segments was reanalyzed. After identifying ILs that had a significantly better per se or testcross performance than the recipient line with the Dunnett test, the linear model analysis was in most instances able to clearly identify the donor regions that were responsible for the superior performance. The precise localization of the favorable DCS allowed a detailed analysis of pleiotropic effects and the study of the consistency of effects for per se and testcross performance. These analyses also highlighted the potential power of the linear model test to localize QTL beyond what is possible using only the Dunnett test.

Extending the linear model test to over-parameterized introgression libraries required adapted genome-wide prediction methods in order to achieve marker effects estimates when there are more markers than lines. Computer simulations showed that genome-wide prediction employing heteroscedastic marker variances had a greater power and a lower false positive rate compared

with homoscedastic marker variances when the phenotypic difference between the donor and recipient lines was controlled by few genes. The simulations helped to interpret the analyses of glycosinolate and linolenic acid content in a rapeseed introgression population and plant height in a rye introgression population. These analyses support the superiority of genome-wide prediction approaches that use heteroscedastic marker variances. When coupled with permutation tests, genome-wide prediction methods can be usefully applied to introgression populations.

In a more detailed analysis of rye introgression libraries, genome-wide prediction was used to attempt QTL detection. For several traits, GWP enabled the detection of positive and negative donor effects in individual ILs. Two donor effects were detected that significantly increased thousand-kernel weight. We found four donor effects for protein, pentosan and starch content that can improve baking quality. Three donor effects for protein content were observed for improving feeding purposes and one donor effect for starch content to improve ethanol production. The effects were localized to small genomic regions.

It can be concluded that the linear model test has many advantages over the Dunnett test, and can enhance the results of QTL analysis in introgression libraries in many ways. The linear model test has the potential to provide comparatively high levels of correct detection with low false positive rates in introgression libraries with various configurations. Also it allows a more accurate localization of the QTL effects compared to pairwise tests. However, it can only be used in cases where the number of lines exceeds the number of donor segment effects to estimate. In cases where there are more donor segments than lines, genome-wide prediction methods are able to detect QTL when employed with permutation tests. The highest performance was achieved with genome-wide prediction methods using heteroscedastic marker variances. These methods, as a whole, compose a toolbox for researchers to extract the most usefulness from introgression libraries while providing guidelines to the expected outcomes depending on the structure of the library.

Chapter 8

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Declaration

Ich erkläre:
Ich habe die vorgelegte Dissertation selbständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe.
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Gregory Stewart Mahone — Gießen, 18. Dezember 2015