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Linkage Disequilibrium and Association Mapping in Elite Germplasm of European Maize

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Abbreviations

AFLP	amplified fragment length polymorphism
BSCB1	Iowa Corn Borer Synthetic #1
BSSS	Iowa Stiff Stalk Synthetic
cM	centiMorgan
DAM	diallel association mapping
DB	distance-based
LD	linkage disequilibrium
LP	line *per se* performance
LRRT	logistic regression ratio test
MRD	modified Rogers distance
NAM	nested association mapping
NSS	non-Stiff Stalk
OPV	open-pollinated variety
PFP	proportion of false positives
QIPDT	quantitative inbred pedigree disequilibrium test
QPDT	quantitative pedigree disequilibrium test
QTL	quantitative trait locus
RIL	recombinant inbred line
SNP	single nucleotide polymorphism
SRR	single round-robin
SS	Iowa Stiff Stalk
SSR	simple sequence repeat
TDT	transmission disequilibrium test
TILLING	targeting-induced local lesions in genomes
TP	testcross performance

1. General Introduction

Since the late 1980s, a large number of DNA markers are available for agriculturally important crops, and are heavily applied to dissect quantitative traits into their underlying genetic factors, called quantitative trait loci (QTL). The goals of these studies are to (i) estimate the number of QTL contributing to the genetic variation of these traits, (ii) localize their positions in the genome, (iii) estimate their gene effects, (iv) identify possible epistatic interactions between QTL, and (v) detect pleiotropy (Lander and Botstein 1989).

Linkage disequilibrium in European maize

Linkage mapping has become a routine tool for the identification of QTL in plants (for review, see Alpert and Tanksley 1996; Stuber et al. 1999). This method provides a high power to detect QTL in genome-wide approaches. However, its major limitations besides high costs (Parisseaux and Bernardo 2004) are the poor resolution in detecting QTL and that only two alleles at any given locus can be studied simultaneously with biparental crosses of inbred lines (Flint-Garcia et al. 2003). An alternative, promising approach is association mapping, which has been successfully applied in human genetics to detect QTL coding for simple as well as complex diseases (Corder et al. 1994; Kerem et al. 1989). This method uses the linkage disequilibrium

(LD) between DNA markers and genes underlying traits which is present in a germplasm set. Adopting association mapping to plant breeding promises to overcome the limitations of linkage mapping (Kraakman et al. 2004).

The resolution and applicability of association mapping depends on the extent of LD within the population under consideration. Furthermore, the applicability of association mapping is influenced by the forces generating and conserving LD, which potentially are population stratification, relatedness, selection, mutation, genetic drift, and linkage. Except for linkage, all other forces may cause spurious marker-trait associations in population-based association mapping approaches. Major differences between human and plant breeding populations are expected with respect to these forces, because in the latter (i) random mating is the exception rather than the rule and selfing is prevalent (Bernardo 2002), (ii) effective population sizes are generally small (Ching et al. 2002), and (iii) strong selection pressure is applied.

To our knowledge, no information is available on LD in European maize germplasm. Furthermore, no study examined genome-wide LD between amplified fragment length polymorphism (AFLP) markers in maize, despite the fact that AFLPs detect a large number of DNA polymorphisms more efficiently than any other DNA marker technique (Miyashita et al. 1999). In addition, no earlier study examined the forces generating and conserving LD in maize inbreds.

Family-based association mapping

Until now, the logistic regression ratio test (LRRT) has been applied as a population-based association mapping method accounting for population stratification (Pritchard et al. 2000; Thornsberry et al. 2001). However, the LRRT fails to correct for LD caused by relatedness, genetic drift, or selection and, thus, may show an increased rate of spurious marker-trait associations

if LD is attributable to these forces. In contrast, family-based association mapping methods suggested in human genetics, such as the transmission disequilibrium test (TDT) (Spielman et al. 1993) or the quantitative pedigree disequilibrium test (QPDT) (Zhang et al. 2001), adhere to the nominal α level even when LD is generated by population stratification, relatedness, genetic drift, or selection. While these disturbing forces are most likely active in plant breeding populations, a family-based association mapping test has neither been developed in a plant breeding context nor applied to data typically available from plant breeding programs.

Joint linkage and association mapping for detecting higher-order epistatic QTL

Both linkage and association mapping methods have merits and limitations for QTL mapping. While linkage mapping methods offer a high power to detect QTL in genome-wide approaches, association mapping methods have the merit of a high resolution to detect QTL (Remington et al. 2001). Wu and Zeng (2001) studied a joint linkage and LD mapping strategy for natural populations. Using data from a general complex pedigree of cattle, Blott et al. (2003) and Meuwissen et al. (2002) identified candidate-gene polymorphisms at previously mapped QTL by combining linkage and LD information.

Yu et al. (2006) examined a genome-wide QTL mapping strategy using genome sequence information of recombinant inbred lines (RILs) which were generated from several crosses of parental inbreds. This QTL mapping strategy is based on the idea that the genomes of RILs are mosaics of chromosomal segments of their parental genome. Consequently, within the chromosomal segments the LD information across the parental inbreds is maintained. Thus, if diverse parental inbreds are used as in the study of Yu et al. (2006), LD decays within the chromosomal segments over a short

physical distance (Wilson et al. 2004). Therefore, the new mapping strategy will show not only a high power to detect QTL in genome-wide approaches but also a high mapping resolution when both linkage and LD information are used.

Yu et al. (2006) focused on the detection of QTL with additive effects. However, results from model organisms suggest that epistatic interactions among loci also contribute substantially to the variation in complex traits (Carlborg and Haley 2004; Marchini et al. 2005). The power to detect two-way interactions by using different mating designs was examined by Verhoeven et al. (2006). Furthermore, Ritchie et al. (2003) assessed the power of multifactor dimensionality reduction to detect two-way interactions. However, several studies described QTL×genetic background interactions (*e.g.*, Alonso-Blanco et al. 1998; Doebley et al. 1995) which can be caused by higher-order epistatic interactions among QTL (Jannink and Jansen 2001). Furthermore, the metabolic pathways that presumably underlie quantitative traits involve multiple interacting gene products and regulatory loci that could generate higher-order epistatic interactions (McMullen et al. 1998). Information about the power for genome-wide detection of epistatic interactions among more than two QTL is still lacking.

Objectives

The goal of my thesis research was to examine the feasibility of association mapping in elite maize breeding populations and develop for this purpose appropriate biometric methods. In particular, the objectives were to

1. investigate the genetic diversity and population structure of elite European maize inbred lines;

2. examine the extent and genomic distribution of LD between pairs of simple sequence repeat (SSR) marker loci and compare these results with those obtained with AFLP markers;

3. compare the usefulness of SSR and AFLP markers for genome-wide association mapping in plant breeding populations;

4. examine the forces generating and conserving LD in plant breeding populations;

5. adapt the QPDT to typical pedigrees of inbred lines produced in plant breeding programs and demonstrate the use of the newly developed quantitative inbred pedigree disequilibrium test (QIPDT);

6. compare the QIPDT and the commonly employed LRRT with respect to the power and type I error rate of QTL detection;

7. compare by computer simulations three- *vs.* four-step genome scans to identify three-way epistatic interactions among QTL involved in a metabolic pathway; and

8. investigate the power and proportion of false positives (PFP) for detecting three-way epistatic interactions among QTL in RIL populations derived from a nested design and compare these estimates to those obtained for RIL populations derived from diallel and different partial diallel mating designs.

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ORIGINAL PAPER

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Linkage disequilibrium in European elite maize germplasm investigated with SSRs

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Abstract Information about the extent and genomic distribution of linkage disequilibrium (LD) is of fundamental importance for association mapping. The main objectives of this study were to (1) investigate genetic diversity within germplasm groups of elite European maize (*Zea mays* L.) inbred lines, (2) examine the population structure of elite European maize germplasm, and (3) determine the extent and genomic distribution of LD between pairs of simple sequence repeat (SSR) markers. We examined genetic diversity and LD in a cross section of European and US elite breeding material comprising 147 inbred lines genotyped with 100 SSR markers. For gene diversity within each group, significant ($P < 0.05$) differences existed among the groups. The LD was significant ($P < 0.05$) for 49% of the SSR marker pairs in the 80 flint lines and for 56% of the SSR marker pairs in the 57 dent lines. The ratio of linked to unlinked loci in LD was 1.1 for both germplasm groups. The high incidence of LD suggests that the extent of LD between SSR markers should allow the detection of marker-phenotype associations in a genome scan. However, our results also indicate that a high proportion of the observed LD is generated by forces, such as relatedness, population stratification, and genetic drift, which cause a high risk of detecting false positives in association mapping.

Introduction

Linkage mapping has become a routine tool for the identification of quantitative trait loci (QTL) in plants (for review, see Alpert and Tanksley 1996; Stuber et al. 1999). However, this procedure has major limitations, including high running costs (Parisseaux and Bernardo 2004) and a poor resolution in detecting QTL, and that with biparental crosses of inbred lines only two alleles at any given locus can be studied simultaneously (Flint-Garcia et al. 2003). An alternative, promising approach is association mapping, which has been successfully applied in human genetics to detect QTL coding for simple as well as complex diseases (Corder et al. 1994; Kerem et al. 1989). This method uses the linkage disequilibrium (LD) between DNA polymorphisms and genes underlying traits. The application of association mapping to plant breeding appears to be a promising approach to overcome the limitations of conventional linkage mapping (Kraakman et al. 2004).

The applicability and resolution of association mapping depends on the extent and structure of LD within the population under consideration. Major differences between human and plant breeding populations are expected, because in the latter (1) random mating is the exception rather than the rule and selfing is prevalent (Bernardo 2002), (2) effective population sizes are generally small (Ching et al. 2002), and (3) strong selection pressure is applied. In addition, association mapping requires detailed knowledge about genetic and phylogenetic relationships of the materials investigated because a disregarded population structure may cause spurious associations (Pritchard et al. 2000).

In contrast to animals and humans, little information is available on LD in plants, with most research being done in *Arabidopsis thaliana* and maize. Nordborg et al. (2002) examined patterns of LD of *A. thaliana* and reported that broad-based populations showed a lower level of LD than narrow-based populations. The same trend was observed in maize. In broad-based germplasm, intragenic LD decreased within 200 base pairs to r^2 values smaller than 0.25 (Tenaillon et al. 2001). Remington et al. (2001) obtained similar results with single nucleotide polymorphism (SNP) markers, and genome-wide LD for simple sequence repeat (SSR)

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markers was significant ($P < 0.01$) among 10% of the marker pairs. In narrow-based elite germplasm, no decay of LD within a few hundred basepairs was observed (Ching et al. 2002). In a very diverse set of elite germplasm, Liu et al. (2003) detected a high level of LD between 94 SSR markers. To our knowledge, no information is available on LD in European maize germplasm, and no earlier study has examined the forces conserving and generating LD in elite maize inbreds.

The objectives of our research were to (1) investigate genetic diversity within germplasm groups of elite European maize inbred lines, (2) examine the population structure of elite European maize germplasm, and (3) determine the extent and genomic distribution of LD between pairs of SSR markers.

Materials and methods

Plant material and molecular markers

A cross-section of European and US elite breeding material comprising 147 inbred lines was used in this study. The European inbreds belong to the flint and dent germplasm groups. The US lines originated from the Iowa stiff stalk (SS) and non-stiff stalk (NSS) germplasm groups (Table 1) (pedigree see supplemental table at http://www.uni-hohenheim.de/~stich/pedigree/Tab_S1.html). For a direct comparison of the genetic diversity and LD estimates with those of Liu et al. (2003), the same set of 100 SSR markers was analyzed by the same company (Celera, 1756 Picasso Avenue, Davis, CA 95616, USA) with identical protocols for PCR and allele calling procedure. As in the study of Liu et al. (2003), we dropped six loci, each which had either a mean within-line heterozygosity of more than 0.10 or a proportion of missing data surpassing 0.20. A list of the SSR loci with their chromosomal locations has been deposited as a supplemental table at http://www.uni-hohenheim.de/~stich/markers/Tab_S2.html. Map positions of all SSRs are based on the Celera linkage map (unpublished data).

Statistical analyses

The modified Rogers distance (MRD) was calculated according to Wright (1978). Associations among the groups were revealed with principal coordinate analysis (Gower 1966) based on MRD estimates between pairs of inbred lines. The average number of alleles per locus and the number of group-specific alleles were determined for the germplasm groups and for various subsets within this collection. The total gene diversity was decomposed into gene diversity estimates between individual lines within each germplasm group according to Nei (1987). Confidence intervals for gene diversity estimates were obtained by a bootstrap procedure with resampling across markers. Nei's minimum distance was calculated according to Nei (1987).

A model-based approach implemented in software package STRUCTURE (Pritchard et al. 2000) was used to subdivide the group of flint and dent inbreds. Because STRUCTURE overestimates the number of subgroups when examining inbred individuals (Pritchard and Wen 2004), it is more reliable to choose the number of subgroups based on prior information. We set the number of subgroups to two based on prior knowledge of the pedigree structure. The established subgroups were further subdivided using separate runs of STRUCTURE for each subgroup as proposed by Pritchard and Wen (2004). The number of clusters was set for each subgroup to two. For each run, the burn-in time was 50,000 and the number of replications was 100,000, following the suggestion of Pritchard and Wen (2004). The run with the maximum likelihood was used to assign lines with membership probabilities of 0.80 or more to subgroups. Lines with membership probabilities less than 0.80 for both subgroups were assigned to a mixed group.

A permutation test using a Monte-Carlo procedure was applied to test for LD between pairs of SSR loci on a genome-wide scale (Lewis and Zaykin 2002; Weir 1996). An adaptive permutation procedure was used to reduce the computational effort: if the P value was smaller than 0.3 after 2,500 permutations, another 14,500 permutations were performed. If the P value was

Table 1 Grouping of the 147 maize inbred lines

Group	Subgroup	Inbreds
Flint	Subgroup 1	L1, L2, L3, L4, L5, L6, L7, L8, L9, L10, L13, L55, L56, L57, L58, L59, L62, L83, L172, L173, L174, L175, L176, L177, L178, L179, L180, L181, L182, L183, L184, L185, L186, L187, L188, L189, L190, L191, L191, L192, L193, L194, L195, L196, L197, L198, L199, L200, L201, L205, L206, L207, L208, L209, L210, L211, L212, L213, L229
	Subgroup 2	L66, L95, L96, L97, L98, L99, L100, L101, L102, L103, L104, L105, L106, L107
	Mixed	L11, L12, L15, L54, L61, L74, L89, L269, L272
Dent	Subgroup 1	L28, L29, L31, L33, L34, L39, L41, L65, L108, L244, L271, L289, L291, L293, L295, L297, L299, L301
	Subgroup 2	L24, L46, L47, L49, L50, L51, L52, L53, L64, L67, L68, L70, L72, L92, L110
	Mixed	L21, L23, L25, L27, L32, L35, L36, L37, L38, L42, L43, L44, L85, L88, L93, L109, L111, L112, L245, L246, L247, L248, L249, L275
SS		B101, B105, B108, B109
NSS		B98, B100, B102, B106, B107, LO977

Table 2 Average number of alleles per locus, number of group-specific alleles, and gene diversity for the four maize germplasm groups

Statistics	Overall	Flint	Dent	SS	NSS
Sample size	147	80	57	4	6
Alleles per locus	9.8	5.1	6.0	2.9	3.5
Group-specific alleles		186	183	52	73
Gene diversity[a]	0.68	0.50b	0.56a	0.43c	0.50b

[a] Gene diversity values followed by the same letters are not different at the 0.05 significance level according to a bootstrap procedure

then smaller than 0.075, another 933,000 permutations were performed. Test statistic D' for LD was calculated according to Tenesa et al. (2003). Since the power of the test for LD depends on the number of inbred lines analyzed per group, we used a resampling strategy to obtain comparable estimates: random samples were drawn from the group with the higher number of lines using a sample size equal to the number of lines of the smaller group. This procedure was repeated 50 times and the results were averaged. The sequential Bonferoni procedure was used for testing the genome-wide null hypothesis "all marker loci pairs are in linkage equilibrium" taking the multiple test problem for independent tests into account (Stahel 1995).

The ratio of the percentage of linked to unlinked loci pairs in LD was calculated, whereas linked loci were defined to be located on the same chromosome and unlinked loci were defined to be located on different chromosomes. Spearman rank correlation was calculated between D' estimates and the genetic map distance between markers. If within a chromosome region all pairs of adjacent loci were in LD, this region was referred to as a LD block. Gene diversity and Nei's minimum distance were calculated with POWERMARKER (Liu 2002). All other analyses were performed with software PLABSOFT (Maurer et al. 2004), which is implemented as an extension of the statistical software R (Ihaka and Gentleman 1996).

Results

The total number of alleles detected for the 94 SSR loci was 923, with the number of alleles per locus ranging from 3 to 22. The mean number of alleles per locus ranged from 2.9 for the four SS lines to 6.0 for the 57 dent lines (Table 2). The number of group-specific alleles was lowest (52) for the SS lines and highest (186) for the 80 flint lines. We observed a total gene diversity of 0.68. For gene diversity within each group, significant ($P < 0.05$) differences existed among groups, with values ranging from 0.43 (SS) to 0.56 (dent).

In principal coordinate analysis based on MRD estimates of all inbred lines, the first two principal coordinates explained 20% and 6% of the molecular variance (Fig. 1). The flint lines were clearly separated from the other lines with respect to the first principal

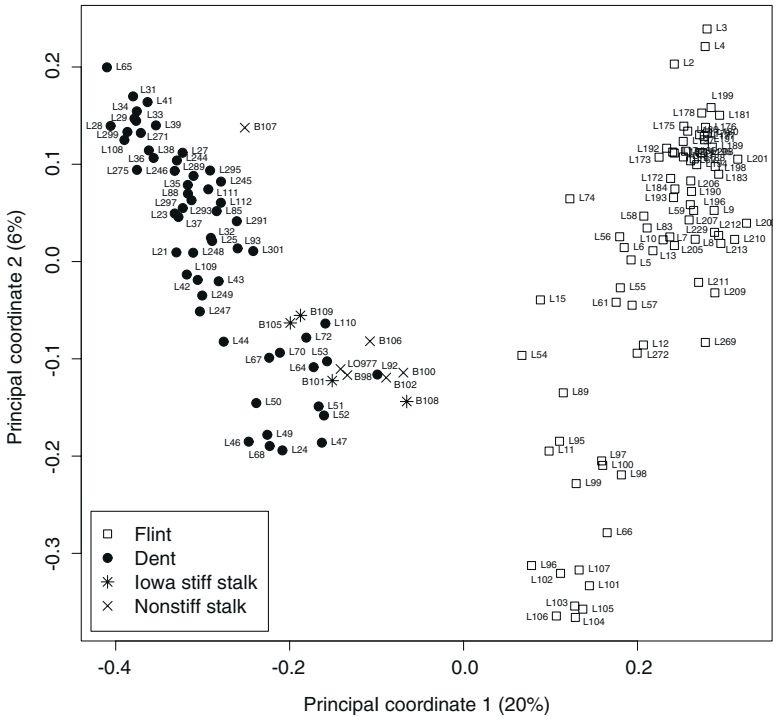

Fig. 1 Principal coordinate analysis of 147 maize inbred lines based on modified Rogers distance estimates. Numbers in parentheses refer to the proportion of variance explained by the principal coordinate

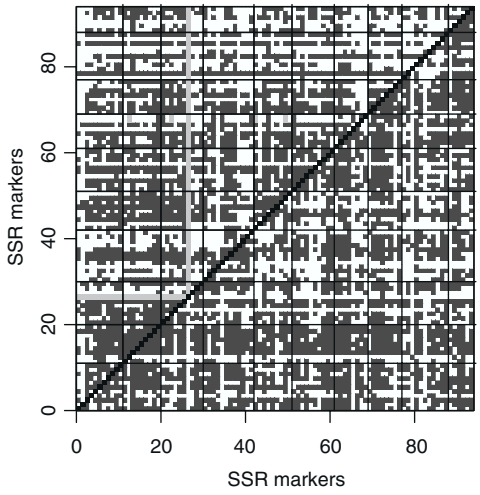

Fig. 2 Pairs of SSR loci in significant ($P < 0.05$) linkage disequilibrium (LD) in the flint group (80 lines, above the diagonal) and in the dent group (57 lines, below the diagonal). Dark-gray squares indicate pairs of loci in significant LD, light-gray comparisons were excluded because at least one locus was monomorphic. The thin horizontal and vertical lines mark off the chromosomes

coordinate. The US lines clustered together with one exception (B107) and were slightly separated from the dent cluster. Nei's minimum distance between pairs of groups was 0.18 (dent × NSS), 0.20 (dent × SS), 0.21 (SS × NSS), 0.27 (flint × dent, flint × NSS), and 0.31 (flint × SS).

The model-based approach of STRUCTURE clearly separated flint and dent lines. STRUCTURE further subdivided the flint group into two subgroups consisting of 57 and 14 inbreds. Nine inbred lines had for both subgroups membership probabilities of less than 0.80, and, thus were assigned to a mixed group. For the dent group, the STRUCTURE analysis revealed two subgroups with 18 and 15 inbred lines. Twenty-four dent lines were assigned to a mixed group.

The genome-wide null hypothesis "all SSR loci pairs are in linkage equilibrium" was rejected for all of the germplasm groups ($P < 0.05$). The percentage of SSR loci pairs with significant ($P < 0.05$) LD was 49% in the flint group and 56% in the dent group (Fig. 2). The ratio of linked to unlinked loci in LD was 1.1 in both groups.

Spearman rank correlation between D' and the genetic map distance of the respective marker loci was highly significant ($P < 0.001$) in both germplasm groups (Table 3). Loci pairs in LD on a given chromosome varied among the different chromosomes and ranged from 27% to 86% in the flint group and from 33% to 93% in the dent group. The proportion of significant pairwise LD tests was smaller within each model-based subgroup except for subgroup 2 of the dent lines. In addition, the ratio of the percentage of linked to unlinked loci in LD increased within the subgroups. For the group "flint unrelated", which consisted of lines not descending from common ancestors within the last two generations, we detected 17.7% of the loci pairs in LD. The percentage of loci pairs in LD was 22.1% in the group "flint size 9".

The total number of LD blocks was higher for the flint group than for the dent group (Table 4). Nevertheless, the dent group showed longer LD blocks than the flint group. In the flint group, the longest LD blocks were found on chromosome 2 (105 cM) (Fig. 3); in the dent group, on chromosome 8 (103 cM).

Discussion

Maize was first introduced into Europe by Columbus from the West Indies to southern Spain in 1493. Later on, maize germ plasm was also imported from various other regions of the New World. In particular, North American flint populations have played a key role in the adaptation of the crop to the cooler climatic conditions of Central Europe (Rebourg et al. 2003). This material is regarded as the origin of the European open-pollinated flint varieties. Hybrid maize breeding was started in

Table 3 Percentage of SSR loci pairs in significant ($P < 0.05$) linkage disequilibrium (LD) and Spearman rank correlation coefficient ρ between D' estimate and genetic map distance between markers in European maize germplasm groups

Group	No. of lines	Loci pairs in LD			Ratio linked: unlinked loci in LD	Spearman ρ
		Linked (%)	Unlinked (%)	Total (%)		
Flint	80	55	48	49	1.1	−0.25***
Subgroup 1	57	38	30	31	1.3	−0.18***
Subgroup 2	14	35	20	21	1.7	−0.20***
Flint size 57[a]	57	53	44	45	1.2	−0.22***
Flint size 14[a]	14	30	25	25	1.2	−0.12***
Dent	57	60	55	56	1.1	−0.33***
Subgroup 1	18	32	24	25	1.3	−0.12*
Subgroup 2	15	51	47	47	1.1	−0.19***
Dent size 18[a]	18	41	36	37	1.1	−0.22***
Dent size 15[a]	15	39	34	34	1.2	−0.20***

*,***Significant at the 0.05 and 0.001 probability level, respectively
[a] Random samples were drawn from the corresponding group 50 times, and the results were averaged

Table 4 Number of linkage disequilibrium (LD) blocks per chromosome and their average length per chromosome in centiMorgan (cM) in European maize germplasm groups

Chromosome	Group			
	Flint		Dent	
	Number of blocks[a]	Length (cM)	Number of blocks[a]	Length (cM)
1	3	33	2	69
2	1	105.0	1	49
3	3	9.3	3	10
4	2	33	2	53
5	2	32	1	52
6	2	41	2	48
7	2	11	1	73
8	2	27	1	103
9	3	10	4	13
10	2	8.0	1	44
Sum	22		18	
Mean		26		41

[a] An LD block consists of a sequence of markers for which all pairs of adjacent loci are in significant ($P < 0.05$) LD

Central Europe after World War II. As a promising heterotic pattern, high-yielding US dent lines were crossed with the adapted European flint lines (Schnell 1992). The steady influx of dent germplasm from North America to Europe has continued over the past 50 years. In contrast, the parental flint inbreds were developed by selfing from a few European open-pollinated varieties such as Lacaune, Lizargarote, Gelber Badischer Landmais, and Rheintaler (Messmer et al. 1992).

Genetic diversity and population structure

We found a total gene diversity of 0.69. Our results are directly comparable to those of Liu et al. (2003) due to the fact that in both studies the same SSR marker set was analyzed by the same lab. Liu et al. (2003) detected a

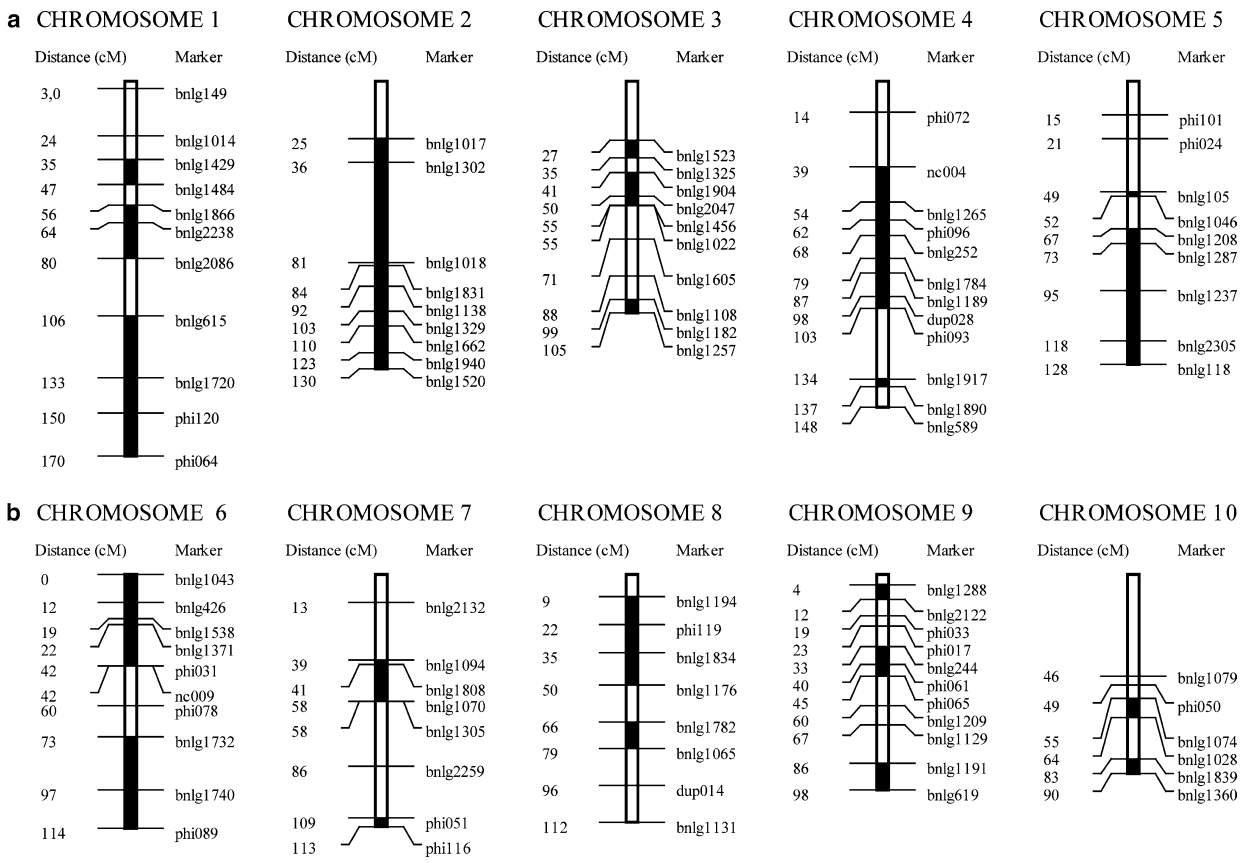

Fig. 3 Genome-wide distribution of the SSR linkage disequilibrium (*LD*) blocks in the flint lines. Black bars indicate LD blocks

higher gene diversity (0.82) when examining 260 inbreds, which can be explained by the inclusion of a broad range of germplasm adapted to tropical, subtropical, and temperate regions in their survey.

The principal coordinate analysis revealed a clear separation between the flint and dent lines (Fig. 1). This is in accordance with results of previous studies (e. g., Lübberstedt et al. 2000; Messmer et al. 1992) and can be explained by the breeding history described above. Nei's minimum distance between pairs of germplasm groups showed that SS and NSS lines clustered closely to the dent lines. This is consistent with the breeding history because the elite European dent germplasm was developed by breeders using both SS and NSS material. The model-based subgroups were also in accordance with pedigree data. The flint lines assigned to different subgroups were bred in different breeding programs. The dent lines of subgroup 1 are, as far as the pedigree is known, related to Iodent line L65, whereas the predominant parents of subgroup 2 are the inbred lines UH021 and UH022.

Nei's minimum distance between the SS and NSS group is in the present study based only on a small number of US lines. Therefore, Nei's minimum distance between the flint and dent group estimated in the present study was compared with the distance between the SS and NSS group reported by Liu et al. (2003). The divergence between the two heterotic pools of the US was of the same magnitude as that between the heterotic pools of Central Europe.

The comparison of the flint and dent germplasm based on the average number of alleles per locus and gene diversity (Table 2) reflected a higher genetic diversity among the dent lines than among the flint lines. This is in accordance with the experimental results of Lübberstedt et al. (2000) based on amplified fragment length polymorphic data and can be explained by the breeding history of the European germplasm groups.

The gene diversity of the dent group (0.56) was of the same magnitude as the gene diversity observed by Liu et al. (2003) for SS lines (0.59) but lower than that observed by Liu et al. (2003) for NSS (0.78) lines. As the European dent group was established by using SS and NSS germplasm, the low gene diversity was not expected from pedigree information. It was presumably caused by a rigid selection for adaption to the cooler growing conditions in Central Europe (Brandolini 1969).

Both European germplasm groups showed a low genetic diversity in comparison with the US heterotic groups. This indicates that new genetic material should be integrated in both European germplasm groups to ensure a long-term response to selection. The genetic basis of the flint group can be broadened by the introgression of European open-pollinated flint varieties (Reif et al. 2005). In addition, our results strongly suggest that both of the SS and NSS US dent pools represent a valuable source to broaden the European dent pool.

Linkage disequilibrium and its potential causes

LD was analyzed within the single germplasm groups and not within the set of all inbreds because the germplasm groups were clearly separated by the STRUCTURE analysis. In each germplasm group, about one-half of the loci pairs showed significant ($P < 0.05$) LD (Table 3), which is in accordance with the results of Liu et al. (2003) but considerably higher than those reported by Remington et al. (2001). This discrepancy between our results and those of the latter study is presumably attributable to two factors: (1) we used a much higher marker density than Remington et al. (2001); (2) their germplasm was chosen to avoid closely related lines (Liu et al. 2003), whereas we examined a cross-section of breeding material comprising both related and unrelated inbred lines.

Information about the causes generating LD is essential for drawing conclusions about the prospects of association mapping. LD conserved by linkage is useful for association mapping. The classification of selection and mutation forces generating useful and non-useful LD for association mapping depends on how the LD information is used. LD generated by relatedness, population stratification, and genetic drift causes spurious marker-phenotype associations.

LD was initially generated in the flint and dent heterotic groups when establishing them in the 1950s by using germplasm with differing allele frequencies. For the initial 25 years, both germplasm groups were bred without winter nurseries (W. Schipprack, personal communication), so that the number of completed breeding cycles is 7.78 when assuming a cycle length of 9 years without winter nurseries and 5 years with winter nurseries. Applying the formula of Haldane and Waddington (1931) yields an estimated number of effective crossovers for one breeding cycle of 1.31 per Morgan and, consequently, 10.19 effective crossovers per Morgan during the past 50 years. This implies that we can expect to detect significant ($D' > 0.25$) LD due to linkage between the SSR loci separated by less than 14.5 cM when assuming an initial D' estimate of 1. In contrast, no significant ($D' < 0.25$) LD is expected between loosely linked and unlinked loci. This indicates that the SSR marker density used in our study might be high enough for genome-wide association mapping, even if there were no forces other than linkage conserving or generating LD.

We observed for both germplasm groups a highly significant ($P < 0.001$) correlation between the D' estimate and the genetic map distance between SSR markers (Table 3), suggesting a decay of LD with distance. This has also been observed for *A. thaliana* (Nordborg et al. 2002), sugarcane (*Saccharum officinarum* L.) (Jannoo et al. 1999), and humans (Service et al. 2001). A decay of LD with distance indicates that linkage is a factor conserving LD. The weak association between D' and genetic map distance and the high proportion of unlinked loci in significant LD (Table 3) suggests,

however, the presence of further forces generating LD between both linked and unlinked loci.

Selection acting on a monogenic trait generates LD around the gene. If selection is performed on an oligo- or polygenic trait, LD is generated not only between linked genes but also between unlinked genes coding for the trait. Theoretical considerations suggest that this LD is useful for association mapping. Moreover, in plant breeding programs, selection is commonly performed simultaneously on several traits and, consequently, can generate also LD between genes influencing different traits. Thus, selection can complicate association mapping. In their experiments with maize, Robinson et al. (1960) observed no inversion of the Bulmer effect upon random mating. Despite the large standard errors of the variance components, this result suggested that the previously performed selection did not strongly reduce the additive variance. Consequently, selection may be an unlikely cause for LD.

Vigouroux et al. (2002) estimated the mutation rate for SSRs in maize to be 7.7×10^{-4} for loci with dinucleotide repeats and lower for loci with higher-nucleotide repeats. Because of the low mutation rate and due to the fact that a mutation must occur in a predominant parent to cause significant LD, we assume that mutation is at best a marginal factor causing LD in elite maize germplasm.

Theoretical considerations suggest that relatedness generates LD between linked loci. Only if predominant parents exist in a germplasm group LD between unlinked loci is expected to be generated by relatedness. The group "flint unrelated" consisted of lines not descending from common ancestors within the last two generations. The comparison of the percentage of loci pairs in LD identified of the group "flint unrelated" with that of the group "flint size 9" suggests that relatedness increases the extent of LD. Furthermore, we observed that relatedness causes LD between linked and unlinked loci in equal proportion. This is in accordance with the fact that there were predominant parents in the flint group. The higher Spearman rank correlation coefficient ρ between D' and genetic map distance in the group "flint unrelated" than in the group "flint size 9" (data not shown) reflects that the influence of linkage on the level of intrachromosomal LD in the group "flint size 9" is masked by relatedness.

For three out of four model-based subgroups the percentage of loci pairs in LD decreased (Table 3). Simultaneously, the ratio of linked to unlinked loci in LD increased. This result suggests that population stratification is a major factor causing LD in the inbred lines examined. In the subgroups, no clear trend was observed that linkage has an higher influence on the level of intrachromosomal LD than in the main group. This can be explained by the presence of other forces influencing intrachromosomal LD in the subgroups, such as relatedness and genetic drift, which mask this expected effect.

A small effective population size, commonly present in breeding programs (Ching et al. 2002), accelerates the decay of LD with distance (Hill and Robertson 1968). However, the variance of the observed D' estimates increases if the effective population size gets smaller. Therefore, a high percentage of loci pairs in LD can be observed due to genetic drift. In the present study, the percentage of loci pairs in LD did not show a uniform distribution among the chromosomes. This suggests that genetic drift is most likely a major force generating LD in the inbred lines examined (Huttley et al. 1999).

Linkage disequilibrium blocks

The length of chromosome regions in LD is crucial for application of association mapping because (1) regions in LD need to be present in order to detect marker-phenotype associations and (2) the length of the regions limits the resolution of association mapping. The LD blocks observed in the present study had an average length of 33 cM (Table 4), while those detected by Ching et al. (2002) were much shorter (500 basepairs). Possible reasons for the difference are (1) the different marker system used and (2) the much smaller average genetic map distance between markers in the study of Ching et al. (2002). A small average genetic map distance between markers reduces the observed average LD block length in two ways: (1) it allows the detection of short LD blocks when present and (2) if LD between two linked markers is not caused by linkage, additional markers in between the former loci pair are not expected to be in LD. Thus, additional markers can chop a large LD block into smaller pieces. In order to get detailed knowledge about the length of LD blocks, further research with a high marker density is needed.

Prospects for association mapping

Successful association mapping depends on the possibility of detecting LD between marker alleles and alleles affecting the expression of phenotypic traits. This is only feasible if LD is present in the breeding material to be studied. The results of our study suggest that the extent of LD between SSR markers should allow the detection of marker-phenotype associations in a genome scan. However, our results also indicate that a high proportion of the observed LD is caused by relatedness, population stratification, and genetic drift. The advantage of performing association mapping in such populations is that the number of markers needed is minimized due to the high level of LD. However, this approach causes a high risk of detecting false positives in association mapping. When performing family-based association mapping approaches, this problem could be overcome.

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Comparison of linkage disequilibrium in elite European maize inbred lines using AFLP and SSR markers

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Abstract

Application of association mapping to plant breeding populations has the potential to revolutionize plant genetics. The main objectives of this study were to (i) investigate the extent and genomic distribution of linkage disequilibrium (LD) between pairs of amplified fragment length polymorphism (AFLP) markers, (ii) compare these results with those obtained with simple sequence repeat (SSR) markers, and (iii) compare the usefulness of AFLP and SSR markers for genomewide association mapping in plant breeding populations. We examined LD in a cross-section of 72 European elite inbred lines genotyped with 452 AFLP and 93 SSR markers. LD was significant ($p < 0.05$) for about 15% of the AFLP marker pairs and for about 49% of the SSR marker pairs in each of the two germplasm groups, flint and dent. In both germplasm groups the ratio of linked to unlinked loci pairs in LD was higher for AFLPs than for SSRs. The observation of LD due to linkage for both marker types suggested that genome-wide association mapping should be possible using either AFLPs or SSRs. The results of our study indicated that SSRs should be favored over AFLPs but the opposite applies to populations with a long history of recombination.

Introduction

Association mapping exploits linkage disequilibrium (LD) between genes coding for a trait and closely linked markers to map these genes. In human genetics, it has been advocated as the method of choice to detect and estimate the effects of novel genes (Risch and Merikangas 1996). If association mapping methods can be adopted in plant breeding, they have the potential to revolutionize plant genetics. The limitations of classical quantitative trait locus (QTL) mapping such as the poor resolution of detecting QTL and the limited number of alleles per locus that can be studied simultaneously (Flint-Garcia et al. 2003) could then be overcome (Kraakman et al. 2004). Additionally, classical QTL mapping is very expensive (Parisseaux and Bernardo 2004). However, before association mapping methods can be applied in a plant breeding context the extent and genomic distribution of LD must be examined because of the major differences regarding the forces that generate and conserve LD in plant breeding versus human populations: (i) random mating is the

exception rather than the rule and selfing is prevalent (Bernardo 2002), (ii) effective population sizes are generally small (Ching et al. 2002), and (iii) strong selection pressure is applied.

In contrast to animals and humans, little information is available on LD in crop species with most research being done in maize (for review, see Flint-Garcia et al. 2003). The studies employing single nucleotide polymorphism (SNP) or simple sequence repeat (SSR) markers to study LD in maize observed a higher extent of LD in narrow-based than in broad-based germplasm (Tenaillon et al. 2001; Ching et al. 2002; Liu et al. 2003). Results from our companion study on European maize inbred lines (Stich et al. 2005) suggested that relatedness, population stratification, genetic drift, and linkage were important forces generating and conserving LD between pairs of genome-wide distributed SSR markers. In a set of 102 inbreds, Remington et al. (2001) compared the extent of LD between SNPs of six genes with that observed between genome-wide distributed SSR markers. A higher level of LD was observed between pairs of SSR markers than between interlocus pairs of SNPs. Nevertheless, a direct comparison of the extent of LD between different types of genome-wide distributed molecular markers is still lacking.

Amplified fragment length polymorphism (AFLP) is a multilocus marker techique that detects DNA fragments after selective PCR amplification (Vos et al. 1995). The AFLP technique has been widely used in maize either to construct genetic maps (e.g., Vuylsteke et al. 1999) or to study phylogenetic relationships and genetic diversity among lines (e.g., Lübberstedt et al. 2000). To our knowledge, no study examined genome-wide LD between AFLP markers in maize, despite the fact that AFLPs detect a large number of DNA polymorphisms more efficiently than any other DNA marker technique (Miyashita et al. 1999).

The objectives of our research were to (i) investigate the extent and genomic distribution of LD between pairs of AFLP marker loci in a set of 72 European elite maize inbred lines, (ii) compare these results with those obtained with SSR markers, and (iii) compare the usefulness of AFLP and SSR markers for genome-wide association mapping in plant breeding populations.

Materials and methods

Plant materials and molecular markers

A cross-section of European elite breeding material comprising 72 inbred lines (35 flint and 37 dent) was used in this study (Table 1, pedigree see supplemental table at *http://www.uni-hohenheim.de/~stich/pedigree/Table_S1.html)*. These inbreds represent a random sample of the germplasm investigated with SSRs and presented in a companion study (Stich et al. 2005). In terms of alleles per SSR locus the set of inbreds of the current study explains 80% of the genetic diversiy of the inbreds examined by Stich et al. (2005). The inbred lines were developed either by private companies or by the University of Hohenheim. A total of 452 polymorphic AFLP markers was scored by Keygene N.V. for all inbred lines using the set of 20 primer combinations described by Heckenberger et al. (2003). Map positions of these AFLP markers were obtained from a proprietary integrated map of maize (Peleman et al. 2000). In addition, all lines were genotyped by Celera with 93 polymorphic SSR markers uniformly covering the entire maize genome. Budget for fingerprinting SSRs was the same like for AFLPs. Map positions of the SSRs are based on the Celera linkage map (unpublished data). The average map distance between adjacent loci was 2.9 cM for AFLPs and 12.6 cM for SSRs. Sixty-six of the 93 SSR markers have been mapped by Keygene together with the AFLPs (unpublished data). The remaining 27 SSR markers were integrated into a joint map by fitting a linear model. For direct comparison of the AFLP and SSR linkage maps, the SSR map positions based on the integrated map were used.

Statistical analyses

The genetic dissimilarity d between pairs of inbred lines was calculated according to Nei and Li (1979) using the combined set of 452 AFLP and 93 SSR markers. Associations among germplasm groups were revealed with principal coordinate analysis (Gower 1966) based on the square root of genetic dissimilarity ($\sqrt{d}$), which is an Euclidean distance as required for principle coordinate analysis (Gower and Legendre 1986).

Table 1. Grouping of the 72 elite maize inbred lines revealed with STRUCTURE.

Group	Subgroup	Inbreds[a]
Flint	Subgroup 1	L66, L96, L100, L101, L102, L103, L104, L105, L106, L107
	Subgroup 2	L2, L3, L4, L5, L6, L7, L8, L9, L10, L55, L56, L58, L59
	Mixed	L11, L12, L15, L54, L61, L74, L83, L89, L95, L97, L98, L99
Dent	Subgroup 1	L28, L29, L31, L34, L38, L39, L41, L65, L108
	Subgroup 2	L24, L46, L49, L51, L67, L68, L70
	Mixed	L21, L23, L25, L27, L32, L33, L35, L36, L37, L42, L43, L44, L64, L72, L85, L88, L93, L109, L110, L111, L112

[a]Notation refers to the notation of Stich et al. (2005).

A model-based approach implemented in software package STRUCTURE (Pritchard et al. 2000) was used to subdivide the set of flint and dent inbreds. Because STRUCTURE overestimates the number of clusters when examining inbred individuals, it is more reliable to choose the number of clusters based on prior information (Pritchard and Wen 2004). We set the number of clusters to two, owing to prior knowledge of the pedigree structure. The established main groups were further subdivided into subgroups using separate runs of STRUCTURE for each main group as proposed by Pritchard and Wen (2004). The number of clusters was set to two for each main group based on pedigree information. For each run, the burn-in time was 50,000 and the number of replications was 100,000, following the suggestion of Pritchard and Wen (2004). The run with the highest likelihood was used to assign lines with membership probabilities ≥0.80 to subgroups. Lines that did not pass this threshold were assigned to a mixed subgroup.

For each marker pair we determined the extent of LD (r^2) according to Maruyama (1982). A permutation test using a Monte-Carlo procedure was applied to test for LD between pairs of marker loci (Weir 1996; Lewis and Zaykin 2002). An adaptive permutation procedure was used to reduce the computational effort: if the p value was smaller than 0.3 after 2500 permutations, another 14,500 permutations were performed. Since the power of the LD test depends on the number of inbred lines analyzed per main group or subgroup, we used a resampling strategy to obtain comparable estimates: the percentage of loci pairs in LD was estimated in random samples that were drawn from the main group using a sample size equal to the number of inbreds in the subgroup. This procedure was repeated 50 times and the results were averaged.

We calculated the ratio of the percentage of linked to unlinked loci pairs in LD, where linked loci were defined as located on the same chromosome and unlinked loci on different chromosomes. For linked loci, Spearman's rank correlation was calculated between the p value of the LD test and the genetic map distance between marker loci. If all pairs of adjacent loci within a chromosome region displayed significant LD, this region was referred to as an LD block.

In the main flint and dent groups, the number of alleles of each SSR locus was reduced to two (''flint biallelic SSRs'' and ''dent biallelic SSRs'') to examine the influence of the allele number on the power to detect LD. For each SSR locus, one allele was randomly chosen and the others were bulked to one allele class. This procedure was repeated 50 times and the average was calculated for the (i) percentage of loci pairs in LD, (ii) LD block length, and (iii) number of LD blocks. All statistical analyses were performed with software Plabsoft (Maurer et al. 2004), which is implemented as an extension of the statistical software R (R Development Core Team 2004).

Results

In principal coordinate analysis of the 72 maize inbred lines, the first two principal coordinates explained 18.3 and 9.2% of the molecular variance (Figure 1). The 35 flint lines were clearly separated from the dent lines with respect to the first principal coordinate. Likewise, the model-based approach of STRUCTURE clearly separated the flint and dent lines. The two flint subgroups that were established using STRUCTURE consisted of 13 and 10 inbreds, whereas the dent subgroups consisted of 9 and 7 inbred lines.

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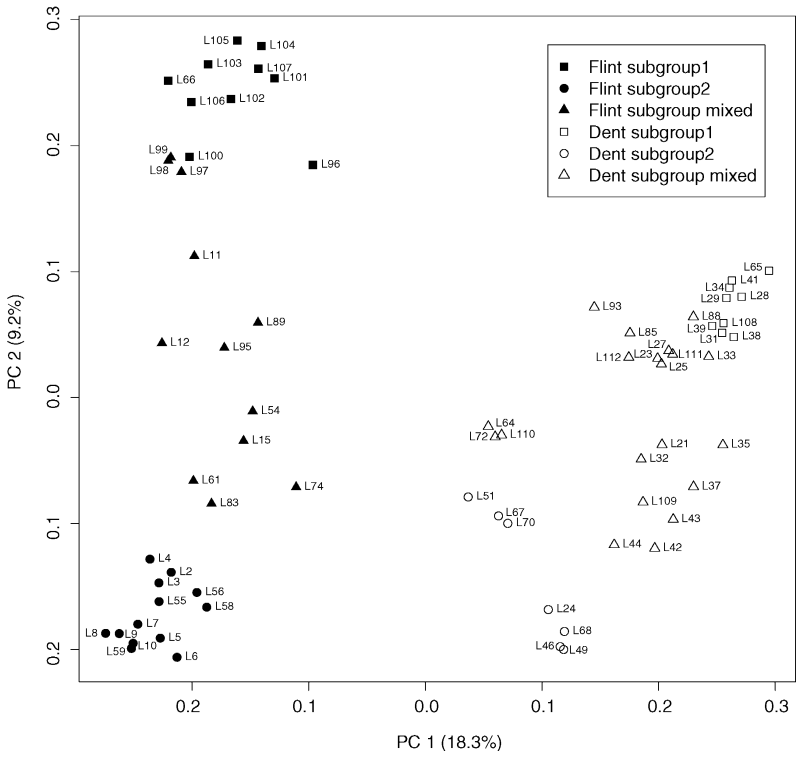

Figure 1. Principal coordinate analysis of 72 European elite maize inbred lines based on the square root of genetic dissimilarity estimates calculated from a combined set of AFLP (452) and SSR (93) markers. PC1 and PC2 are the first and second principal coordinate. Numbers in parentheses refer to the proportion of molecular variance explained by the principal coordinate. Subgroups were identified using STRUCTURE.

For the group of 35 flint lines we observed 14.6% of the AFLP loci pairs and 50.2% of the SSR loci pairs in significant ($p < 0.05$) LD (Table 2). For the group of 37 dent lines these percentages were 14.6 and 47.1. In both main groups the ratio of linked to unlinked loci pairs in LD was higher for AFLPs than for SSRs. The observed ratio of linked to unlinked AFLP loci pairs was higher in three of the four subgroups than in the two main groups. For the SSR markers the same trend was observed in two subgroups. With increasing genetic map distance between marker loci, the p value of the LD test and the extent of LD (r^2) decreased in all germplasm groups for both marker types (Figure 2).

On individual chromosomes, the percentage of loci pairs in LD in the main flint group ranged from 8.0 to 28.2% for AFLPs and from 23.6 to 85.7% for SSRs. In the main dent group, this percentage varied between 6.5 and 25.6% for AFLPs and between 27.1 and 83.3% for SSRs. Bulking the SSR alleles to "biallelic SSRs" resulted in a decrease in the percentage of loci pairs in significant LD compared with the unbulked SSRs. In both main groups, shorter and more LD blocks were observed between AFLPs than between SSRs (Table 3). The longest AFLP LD blocks were detected in the flint group on chromosome 6 and in the dent group on chromosome 4, whereas the longest SSR LD blocks were observed in the flint group on chromosome 6 and in the dent group on chromosome 7 (Figure 3). On average, the same LD block length (13.2 vs. 13.6 cM) but different total numbers (11.6 vs. 9.0) were observed in the flint and dent groups when examining "biallelic SSRs".

Discussion

SSR markers detect codominantly inherited length polymorphisms of repetitive DNA sequences and,

Table 2. Number of European elite maize inbred lines, mean number of alleles, percentage of loci pairs in significant ($p < 0.05$) LD, and Spearman's rank correlation coefficient ρ between p value of the LD test and genetic map distance between markers.

Group	No. of lines	Mean no. of alleles	Loci pairs in LD (%)			Ratio linked: unlinked loci in LD	Spearman's ρ
			Linked	Unlinked	Total		
AFLP markers							
Flint	35	2.0	20.2	13.9	14.6	1.5	0.16***
Subgroup 1	10	1.5	31.6	14.2	16.0	2.2	0.32***
Subgroup 2	13	1.6	21.2	13.7	14.6	1.5	0.19***
Flint size 10[a]	10	1.8	13.5	9.9	10.3	1.4	0.12***
Flint size 13[a]	13	1.9	15.8	11.3	11.8	1.4	0.13***
Dent	37	2.0	21.8	13.8	14.6	1.6	0.11***
Subgroup 1	9	1.4	19.3	10.9	11.9	1.8	0.26***
Subgroup 2	7	1.6	25.3	22.0	22.3	1.1	0.04*
Dent size 9[a]	9	1.8	16.5	11.2	11.7	1.5	0.13***
Dent size 7[a]	7	1.7	17.5	12.4	13.0	1.4	0.11***
SSR markers							
Flint	35	4.3	52.6	50.0	50.2	1.1	0.18***
Subgroup 1	10	1.9	25.3	13.1	14.2	1.9	0.28***
Subgroup 2	13	2.5	30.6	30.0	30.0	1.0	0.26***
Flint size 10[a]	10	3.2	35.6	31.0	31.4	1.1	0.11*
Flint size 13[a]	13	3.4	39.8	36.2	36.5	1.1	0.17**
Dent	37	4.8	51.4	46.7	47.1	1.1	0.23***
Subgroup 1	9	2.2	25.5	11.8	13.1	2.2	0.47***
Subgroup 2	7	2.6	40.1	37.3	37.5	1.1	0.12*
Dent size 9[a]	9	3.1	36.0	29.9	30.4	1.2	0.20***
Dent size 7[a]	7	2.9	31.4	25.2	25.8	1.2	0.18**
Flint biallelic SSRs[b]	35	2.0	13.2	10.9	11.1	1.2	0.05
Dent biallelic SSRs[b]	37	2.0	8.6	6.0	6.8	1.4	0.03

*, **, ***Significant at the 0.05, 0.01, and 0.001 probability levels, respectively.

[a]Random samples were drawn from the corresponding germplasm group 50 times and the results were averaged.

[b]The number of alleles of each SSR locus was reduced to two, by randomly choosing one allele and pooling the others to one allele class. The average percentage of loci pairs in LD was calculated for 50 repetitions.

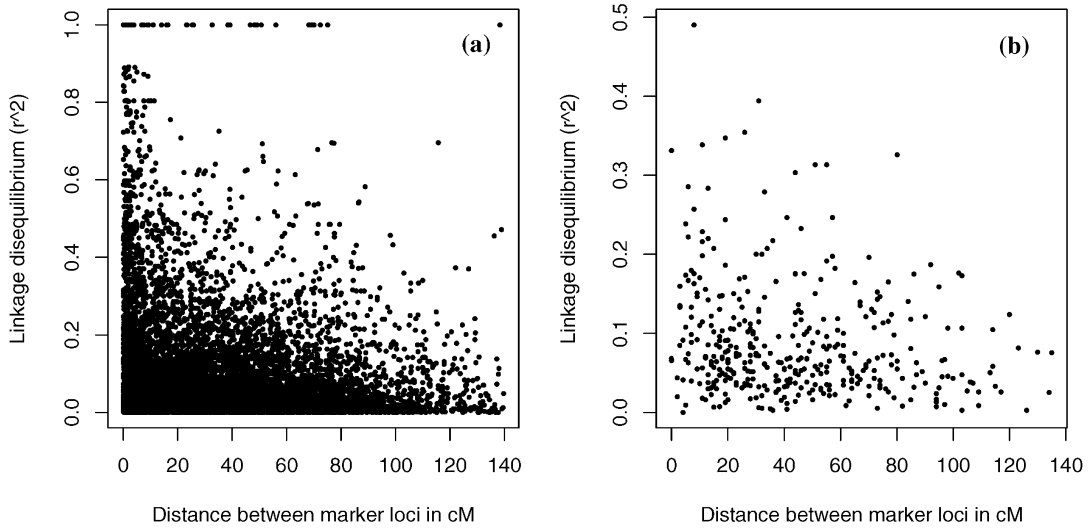

Figure 2. LD (r^2) between (a) AFLP and (b) SSR markers in the 35 flint lines as a function of the genetic map distance between the markers.

Table 3. Number of LD blocks per chromosome (*B*) and their average length (*l*) in centimorgan (cM) in the 35 flint and 37 dent maize inbred lines.

Chromosome	AFLP				SSR			
	Flint		Dent		Flint		Dent	
	B No.	*l* cM	*B* No.	*l* cM	*B* No.	*l* cM	*B* No.	*l* cM
1	12	3.5	10	1.4	1	29.0	2	58.5
2	10	6.4	11	3.4	1	105.0	1	49.0
3	12	5.7	10	2.7	2	17.0	3	10.3
4	11	3.2	7	8.2	1	59.0	2	39.0
5	12	1.2	10	2.9	2	46.0	2	17.0
6	11	5.3	7	5.9	2	14.5	4	13.0
7	9	5.2	10	3.7	2	11.5	1	96.0
8	8	4.9	7	3.3	1	70.0	1	90.0
9	5	0.8	4	5.9	3	9.3	3	12.3
10	7	2.1	4	6.9	3	6.3	1	39.0
Sum	97		80		18		20	
Mean		4.0		4.0		30.1		31.2

An LD block consists of a sequence of markers for which all pairs of adjacent loci displayed significant ($p < 0.05$) LD.

thus, can discriminate between a large number of alleles (Matsuoka et al. 2002). An AFLP band of a given fragment length can be observed in the gel if a specific DNA sequence is present at the target sequence of the restriction enzymes as well as at the target sequence of the typically three selective primer nucleotides (Vos et al. 1995). In contrast, the absence of a band at a given position in the gel can be caused by several reasons, such as (i) indels at the target sequence of the restriction enzymes and/or (ii) at least one point mutation at the target sequence of the restriction enzymes and/or target sequence to of the selective nucleotides. Therefore, the absence of a band is less informative as it may contain several alleles based on DNA sequence difference. Furthermore, a specific AFLP fragment can arise from more than one locus, a phenomenon known as homoplasy (Vekemans et al. 2002). In the present study, homoplasy was disregarded as only AFLP loci with known map positions were used. During the mapping procedure, bands that show an abnormal segregation ratio due to homoplasy were removed.

The clustering of the AFLP loci in the genome is in agreement with findings of Vuylsteke et al. (1999) (Figure 3). The reasons for the clustering can be (i) the presence of adenine- and thymine-rich DNA sequence blocks, which are more likely to be recognized by restriction enzymes EcoRI and MseI (Wang et al. 2004) and (ii) supression of recombination in centromeric or telomeric regions of the genome (van Eck et al. 1995). The clustering of AFLPs suggests that the AFLP marker density cannot be increased uniformly across all genome regions by simply raising the number of EcoRI/MseI primer combinations but rather by using other primer combinations (Vuylsteke et al. 1999).

Population structure

The principal coordinate analysis revealed a clear separation between flint and dent lines (Figure 1). This is in accordance with results of previous studies (Messmer et al. 1992; Lübberstedt et al. 2000) and can be explained by the breeding history of European maize (Reif et al. 2005). The subgroups identified by STRUCTURE were also in accordance with the pedigree data. The flint lines assigned to different subgroups were developed in separate breeding programs. The dent lines of subgroup 1 are, as far as the pedigree is known, related to the inbred line L65, whereas the predominant parents of dent subgroup 2 are inbreds UH021 and UH022. The population pattern detected with STRUCTURE indicated that LD has

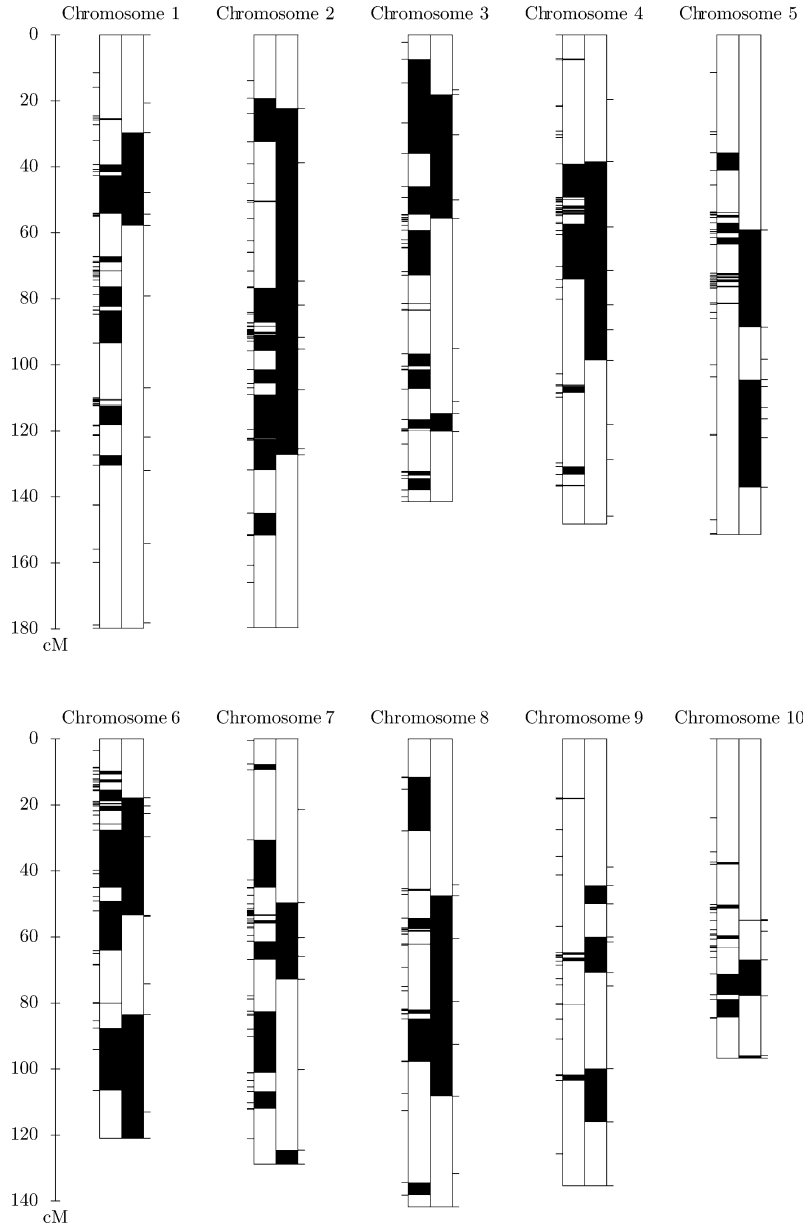

Figure 3. Genome-wide distribution of LD blocks for AFLP and SSR markers in the 35 flint lines. The black bars on the left and right half of each chromosome designate the AFLP and SSR LD blocks, respectively, in which all pairs of adjacent loci displayed significant ($p < 0.05$) LD. The tick marks on both sides of each chromosome indicate the position of the molecular markers based on an integrated map.

to be examined within the main flint and dent germplasm groups but also within their subgroups because LD generated by population stratification causes spurious marker-phenotype associations.

Extent of LD between AFLPs and SSRs

The percentage of AFLP loci pairs in LD was about one third of the percentage of SSR loci pairs

in LD. This can be explained by the fact that for a large number of AFLP loci pairs, LD could not be detected as it exists between alleles based on DNA sequence difference that are bulked for band absence. The lower percentage of loci pairs in LD for ''biallelic SSRs'' compared with that observed for the AFLPs supported this explanation.

The LD blocks between AFLPs were shorter than those between SSR markers. This can be explained by the bulking of alleles for AFLP band absence, which prevents detecting LD, and the shorter genetic map distance between AFLPs than between SSRs (Figure 3). A high marker density reduces the average LD block length in two ways: (i) it allows detection of short LD blocks when present and (ii) if LD between two markers on a chromosome is not caused by linkage, additional markers in between the two markers are not expected to be in LD and, thus, chop a large LD block into smaller pieces. These two reasons explain the detection of chromosome regions with SSR LD blocks but without AFLP LD blocks and vice versa (Figure 3).

Potential causes of LD

Information about the forces generating and conserving LD is essential for drawing conclusions about the prospects of association mapping. LD conserved by linkage is useful for genome-wide association mapping, whereas LD generated by selection, mutation, and relatedness can be advantageous or disadvantageous for association mapping, depending on the particular situation. LD generated by population stratification and genetic drift can result in spurious marker-phenotype associations.

Application of the formula of Haldane and Waddington (1931) yields an estimated number of effective cross overs of 1.31 per Morgan for one breeding cycle in maize (Stich et al. 2005). This indicates that only a low number of effective cross overs occurred in the European flint and dent germplasm groups since their establishment after World War II. Thus, we expected to detect significant LD in today's flint and dent group due to linkage between pairs of AFLP and pairs of SSR markers (Stich et al. 2005). Moreover, if there were no forces other than linkage generating or conserving LD, the marker density in the present study for SSRs but even more so for AFLPs is expected to be sufficient for genome-wide association mapping.

For both germplasm groups, the p value of the LD test and the extent of LD (r^2) decreased with increasing genetic map distance between AFLP and SSR markers (Figure 2). This suggests linkage as a force conserving LD. The ratio of linked to unlinked loci in significant LD was in most instances higher for AFLPs than for SSRs, which can be explained by the smaller genetic map distance between AFLPs than between SSRs (Figure 3).

The high proportion of unlinked AFLP and SSR loci in significant LD (Table 2) suggests the presence of other forces generating LD. A detailed analysis in our companion study suggested that (i) selection is an unlikely cause for LD whereas (ii) relatedness is a cause for LD between SSR loci. Because LD is a property of the examined plant material, relatedness is presumed to be also a force generating LD between AFLP markers. Mutation is assumed to be at best a marginal factor causing LD between pairs of AFLP or SSR markers, as the mutation rates are too low to strongly influence the level of LD in elite maize inbred lines. In addition, a mutation has to occur in a predominant parent to cause significant LD.

In several of the subgroups, we observed a higher ratio of linked to unlinked loci pairs in LD than in the main groups for both AFLPs and SSRs. In addition, in three subgroups we detected a higher correlation coefficient ρ between the p value of the LD test and the genetic map distance between AFLP and SSR markers, than in the main groups. These results suggest population stratification as a force generating LD in the main flint and dent germplasm groups. However, applying association mapping methods to the identified subgroups is no solution to overcome the problem of population stratification because the power to detect marker-trait associations decreases with decreasing size of the examined population. In our study, the percentage of AFLP and SSR loci pairs in LD showed no uniform distribution among the chromosomes, suggesting genetic drift as a major force generating LD (Huttley et al. 1999).

Summarizing, our results suggested that beside linkage, relatedness, population stratification, and genetic drift were important forces generating and conserving LD in the examined germplasm groups. However, when population-based associ-

ation mapping tests are applied to germplasm in which LD is generated by the latter three forces, the proportion of spurious marker-trait associations is expected to be much higher than the nominal type I error rate (Pritchard et al. 2000). This problem could be overcome by applying family-based association mapping approaches.

Prospects of genome-wide association mapping with AFLPs and SSRs

The favorite marker system for genome-wide association mapping is expected to show a high correlation coefficient between the p value of the LD test and the genetic map distance between marker loci as well as a high power to detect LD. However, no difference was observed between the correlation coefficient of the AFLP and SSR markers. The results of our study indicated that the power in association mapping depends on several factors such as (i) the average map distance and (ii) the number of alleles of the marker loci. Under the assumption of a fixed budget, AFLPs are superior to SSRs with respect to the first criterion as a higher marker density can be generated with AFLPs than with SSRs. However, our findings show that AFLPs are only marginally superior to ''biallelic'' SSRs in their power of detecting LD. This is because the genome coverage provided by both types of markers is expected to be sufficient for genome-wide association mapping (Stich et al. 2005). With respect to the number of alleles per marker locus, however, our results showed a clear advantage for SSRs.

In conclusion, SSRs should be favored over AFLPs in populations with a short history of recombination, because then their higher power to detect LD can be used. In contrast, in populations. with a long history of recombination, for which no LD is expected between pairs of SSR markers, and under the assumption of a fixed budget, AFLP markers should be favored over SSRs, despite the bulking of alleles based on DNA sequence differences for AFLP band absence.

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Potential Causes of Linkage Disequilibrium in a European Maize Breeding Program Investigated with Computer Simulations

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ABSTRACT

Knowledge about the forces generating and conserving linkage disequilibrium (LD) is important for drawing conclusions about the prospects and limitations of association mapping. The objectives of our research were to examine the importance of (i) selection, (ii) mutation, and (iii) genetic drift for generating LD in plant breeding programs. We conducted computer simulations based on genotypic data of Central European maize open-pollinated varieties which have played an important role as founders of the European flint heterotic group. Results indicated that in plant breeding populations genetic drift and selection are major forces generating LD. The currently used population-based association mapping tests do not correct for LD caused by these two forces. Therefore, increased type I error rates are expected if these tests are applied to plant breeding populations. As a consequence, we recommend to use family-based association tests for association mapping approaches in plant breeding populations.

HYBRID maize breeding in Central Europe started in the 1950s (Schnell 1992). As a promising heterotic pattern, adapted flint lines were extracted from a few European open-pollinated flint varieties and were crossed with high yielding U.S. dent lines. During the establishment of the corresponding heterotic groups, LD was generated because the founders of each heterotic group differed in their allele frequencies (Reif et al. 2005a).

Subsequently, new lines were developed primarily by second-cycle breeding, *i.e.*, from crosses among elite inbreds within heterotic groups. The extent of LD present in each heterotic group was reduced by genetic recombinations associated with this intermating. Based on the formula of Haldane and Waddington (1931), Stich et al. (2005) estimated the number of effective crossovers to be 1.31 per Morgan for one breeding cycle in maize. This result implies that only about 10 effective crossovers occurred per Morgan in the European heterotic groups since their establishment. Therefore, linkage is expected to be a force conserving considerable LD between markers that are around 15 cM or less distant.

Such LD can be used for association mapping, which has been successfully applied in human genetics to identify polymorphisms coding for cystic fibrosis (Kerem et al. 1989) and Alzheimer's disease (Corder et al. 1994). In plant genetics, association mapping has the potential to overcome the limitations of linkage mapping such as the poor resolution of detecting quantitative trait loci (QTL) and the limited number of alleles per locus that can be studied simultaneously (Flint-Garcia et al. 2003). Furthermore, in comparison with linkage mapping (Parisseaux and Bernardo 2004), association mapping is less expensive because data routinely collected in plant breeding programs can be used.

In addition to linkage, the extent and distribution of LD in plant breeding populations is presumed to be also influenced by population stratification, relatedness, selection, mutation, and genetic drift (Stich et al. 2005). Knowledge about the forces generating and conserving LD is of fundamental importance for drawing conclusions about the prospects and limitations of association mapping, because many forces other than linkage may cause spurious marker-trait associations in population-based association mapping approaches. Stich et al. (2005) examined the forces generating and conserving LD in European elite maize inbreds based on experimental data. This approach provides only conclusions about the relevance of population stratification, relatedness, and linkage. However, no information about the importance of selection, mutation, and genetic drift for the generation of LD in plant breeding populations is available. The objectives of our research were to examine the importance of (i) selection, (ii) mutation, and (iii) genetic drift for generating LD in plant breeding programs based on computer simulations.

MATERIALS AND METHODS

Breeding scheme

Five Central European maize open-pollinated varieties (OPVs): Gelber Badischer Landmais, Mahndorfer, Maleksberger, Rheintaler, and Strenzfelder played an important role as ancestors of the first-cycle flint inbreds in Germany (Oettler et al. 1976).

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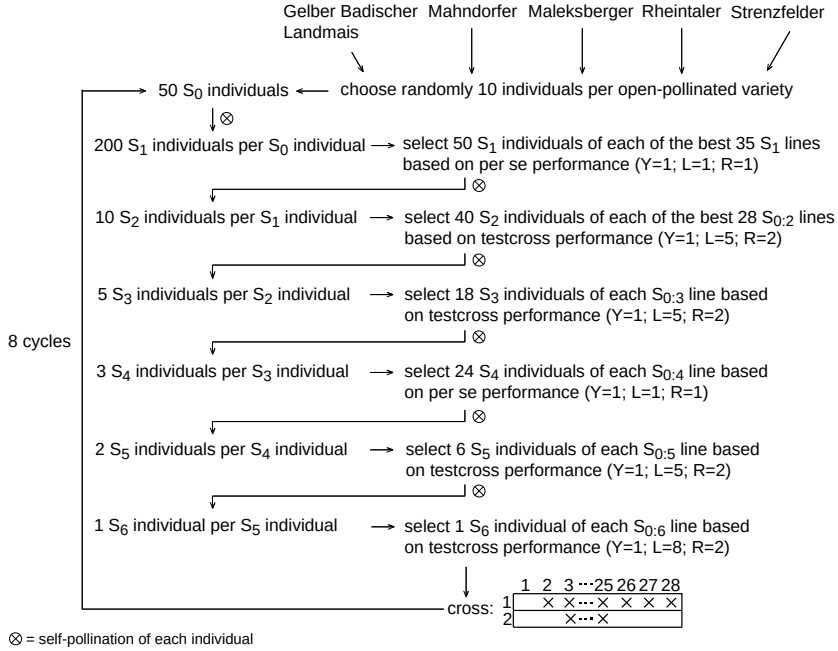

Figure 1 Model for simulating 55 years of breeding history in the European flint heterotic group. Y, L, and R denote the number of years, locations, and replications in performance trials, respectively.

In the framework of the EU project GEDIFLUX (QLRT-2000-00934), the population structure of these OPVs was examined using 55 genome-wide distributed simple sequence repeat (SSR) markers (Reif et al. 2005a). These data served as basis of our computer simulations. The breeding scheme and the dimensioning underlying our simulations reflect essentially the maize breeding program of the University of Hohenheim (Figure 1). In the first breeding cycle, 10 individuals, further referred to as S_0 individuals, were sampled out of each of the five OPVs. A total of 28 S_6 individuals, derived by selfing the 50 S_0 individuals, was selected based on a multi-stage selection procedure. The best S_6 individual was crossed with the other 27 S_6 individuals and the second best S_6 individual was crossed with the third to 25th best S_6 individuals to generate the 50 S_0 individuals for the next breeding cycle. We simulated eight breeding cycles corresponding to 55 years of hybrid maize breeding in Europe (Schnell 1992).

Genetic model

Testcross performance: We assumed 220 QTL influencing testcross performance (TP), which were randomly positioned in the maize genome. To ensure that OPVs showing a large genetic distance based on molecular markers also strongly differ in their alleles at QTL, for each of the five OPVs the allele frequencies of each of the 55 SSR loci were assumed for four QTL. Likewise, linkage equilibrium among QTL as well as between QTL and SSRs was assumed for each OPV as suggested by the results based on European maize OPVs (Reif et al. 2005a).

At each QTL the m alleles showed an average testcross effect of allele substitution of 0, 1/(m-1), 2/(m-1), ..., 1 as suggested by quantitative genetic theory (Falconer and Mackay 1996). Changes in these effects attributable to alteration in allele frequencies of QTL were ignored. The genotypic value of each individual regarding TP was determined by summing up the effects of its alleles.

The phenotypic values of the individuals were generated by adding a normally distributed variable $N(0, {\sigma_n}^2)$ to the genotypic values, where

$$ {\sigma_n}^2 = \frac{\sigma^2_{gy}}{Y} + \frac{\sigma^2_{gl}}{L} + \frac{\sigma^2_{gyl}}{YL} + \frac{\sigma^2_e}{YLR} $$

represents the non-genetic variance. Y, L, and R denote the number of years, locations, and replications in performance trials, respectively. In our simulations, a ratio of variance components σ^2_g:σ^2_{gy}:σ^2_{gl}:σ^2_{gyl}:σ^2_e of 1:1:1:2:4 was assumed for TP (Longin et al. 2006), where σ^2_g refers to the genotypic variance, σ^2_{gy} to the variance of genotype × year interactions, σ^2_{gl} to the variance of genotype × location interactions, σ^2_{gyl} to the variance of genotype × year × location interactions, and σ^2_e to the error variance.

Line per se performance: The genotypic values of the lines for line *per se* performance (LP) were estimated based on the 220 QTL underlying TP, assuming a genotypic correlation between LP and TP of 0.5 (Mihaljevic et al. 2005). For the generation of phenotypic values for LP we assumed a ratio of variance components σ^2_g:σ^2_{gy}:σ^2_{gl}:σ^2_{gyl}:σ^2_e of 1:0.2:0.2:0.5:0.5 (Mihaljevic et al. 2005).

Mutation model: According to Calafell et al. (2001), a symmetric stepwise mutation model, which allows the gain and loss of more than one repeat, was implemented for the SSR loci. We assumed (i) a variance of change in the number of repeats of 3.2 and (ii) a mutation rate of 5.1×10^{-5} (Vigouroux et al. 2002). Because of the extremely low mutation rate of eucaryotic genomes, no mutations were assumed for the QTL (Drake et al. 1998).

Examined simulation scenarios

In addition to the standard scenario described above, four modifications were examined. In variant A, a migration rate per breeding cycle of 1/14 was assumed for the set of selected S_6 individuals based on breeding history. The population

from which the migrating individuals were randomly drawn showed the same alleles as the OPVs but each breeding cycle 10 new QTL alleles and two new SSR alleles were added. In variant B, we assumed the absence of SSR mutations. In variants C and D, individuals were not selected based on TP or LP but chosen at random. In variant D, the population size at each stage was tripled in comparison with the other simulation scenarios. The simulation of each scenario was replicated 50 times.

Estimation of linkage disequilibrium

Because of the complexity of our simulations we do not know a priori which loci pairs are in LD. Therefore, after each breeding cycle, the LD in the set of selected S_6 individuals must be examined. For assessing the feasibility of association mapping the statistical significance of this LD is more important than its actual amount of LD (Maurer et al. 2006). Thus, Fisher's exact test, which provides a significance test for LD between a pair of loci, *i.e.*, determines whether two-locus genotypic frequencies can be represented as products of one-locus genotypic frequencies, seems to be an appropriate tool for this purpose. To facilitate the comparison of our results with those of Reif et al. (2005a; 2005b) we chose a significance level of $\alpha = 0.01$ and determined for each pair of loci the probability of occurence of less probable contingency tables with the same marginal totals as the ones observed with a Monte Carlo method using 2500 replications (Guo and Thompson 1992). The applied test, which adheres to the nominal α level, does not assume Hardy-Weinberg equilibrium at the loci under consideration (Zaykin et al. 1995). Percentage of significant LD was calculated between loci located on the same chromosome (linked) and between loci located on different chromosomes (unlinked). In variant D, we used a resampling strategy described in detail by Stich et al. (2005), to obtain comparable power for detecting significant LD in populations of different sizes. All simulations and analyses were performed with software Plabsoft (Maurer et al. 2004), which is implemented as an extension of the statistical software R (R Development Core Team 2004).

RESULTS

The average selection gain per breeding cycle was 5.8, 4.9, 5.8, 0, and 0 % in the standard scenario and variants A, B, C, and D, respectively. In cycle zero of all five examined simulation scenarios, the number of alleles ranged for both SSRs and QTL from 2.0 to about 14.1 and was in average 5.0 (Figure 2). With increasing number of completed breeding cycles, the average number of alleles per locus decreased, whereas the percentage of monomorphic loci increased. After eight breeding cycles, the average number of alleles per locus ranged from 1.5 (variant B) to 2.3 (variant A) for SSRs and from 1.3 (variant B) to 1.9 (variant A) for QTL.

After one breeding cycle, the percentage of SSR, SSR-QTL, and QTL pairs in significant ($P < 0.01$) LD was about 4 % in the standard scenario and variants A and B (Table 1). In contrast, a percentage of about 3.3 % was detected in variants C and D. With increasing number of completed breeding cycles, the percentage of linked and unlinked SSR, SSR-QTL, and QTL pairs in significant LD increased in all simulation scenarios, up to a maximum between cycles two and four. In the subsequent breeding cycles, the percentage of loci pairs in significant LD decreased. After eight breeding cycles, the percentage of SSR pairs in significant LD ranged from 4.2 (variant D) to 6.8 % (variant B) for linked loci and from 1.0 (variant D) to 1.7 % (variant A) for unlinked loci. After eight cycles the percentage of pairs of SSRs, each of which was within a 2 cM interval from a QTL, was 2.2 % in the standard scenario and 1.9 % in variant C. The percentage of SSR-QTL pairs in significant LD after eight breeding cycles was much lower, and varied between 0.8 (standard scenario) and 3.2 % (variant A) for linked loci and between 0.2 (variant B) and 0.8 % (variant A) for unlinked loci. Finally, the percentage of QTL pairs in significant LD after eight breeding cycles ranged from 7.0 (variant A) to 12.2 % (variant B) for linked loci and from 1.0 (variant D) to 2.5 % (standard scenario) for unlinked loci.

DISCUSSION

Comparison of simulated with experimental data

In the present study we determined a selection gain per breeding cycle of about 5.5 % in the standard scenario and variants A and B. This is in good accordance with the selection gain of about 4 % per breeding cycle, observed in selection experiments using open-pollinated and synthetic base populations (Hallauer and Miranda 1981; Pandey et al. 1987; Stromberg and Compton 1989).

In the standard scenario in our study, absence of migration was assumed. Therefore, results of this scenario can be directly compared with those of the selection experiment performed with the Iowa Stiff Stalk Synthetic (BSSS) and Iowa Corn Borer Synthetic #1 (BSCB1). In a comparison of inbreds from cycle 0 and cycle 12 of BSSS and BSCB1, Labate et al. (1999) observed a strong decrease in the number of alleles per marker locus with an increasing number of completed cycles of selection, which is in agreement with our findings from simulations.

After eight breeding cycles, about 50 % of the SSR loci were fixed in the standard scenario. In contrast, only about 32 % of the restriction fragment length polymorphism markers were fixed after 12 cycles of reciprocal recurrent selection in the study of Labate et al. (1999). This difference can be explained by the longer period under consideration in our study (55 years) compared to the latter one (24 years).

Our computer simulations were designed to model the breeding history of the flint heterotic group. Reif et al. (2005b) examined flint inbreds arranged in four groups according to the decade of release and genotyped with the same 55 SSRs as in our study. These results can be compared directly with those of variant A. The higher extent of significant LD between SSRs in the newly established heterotic group, as well as its subsequent decrease in heterotic groups were detected in both studies.

The extent of significant LD found in our study was considerably lower than that described by Reif et al. (2005b). As the same SSR linkage map underlies both studies, the observed difference cannot be explained by differences in the average map distances between SSRs. A more likely reason is that the first-cycle flint inbreds were developed not only from the OPVs considered in our study but from additional source populations such as Lacaune and Lizargárate (Cartea et al. 1999). This increases the allele frequency differences among the ancestors

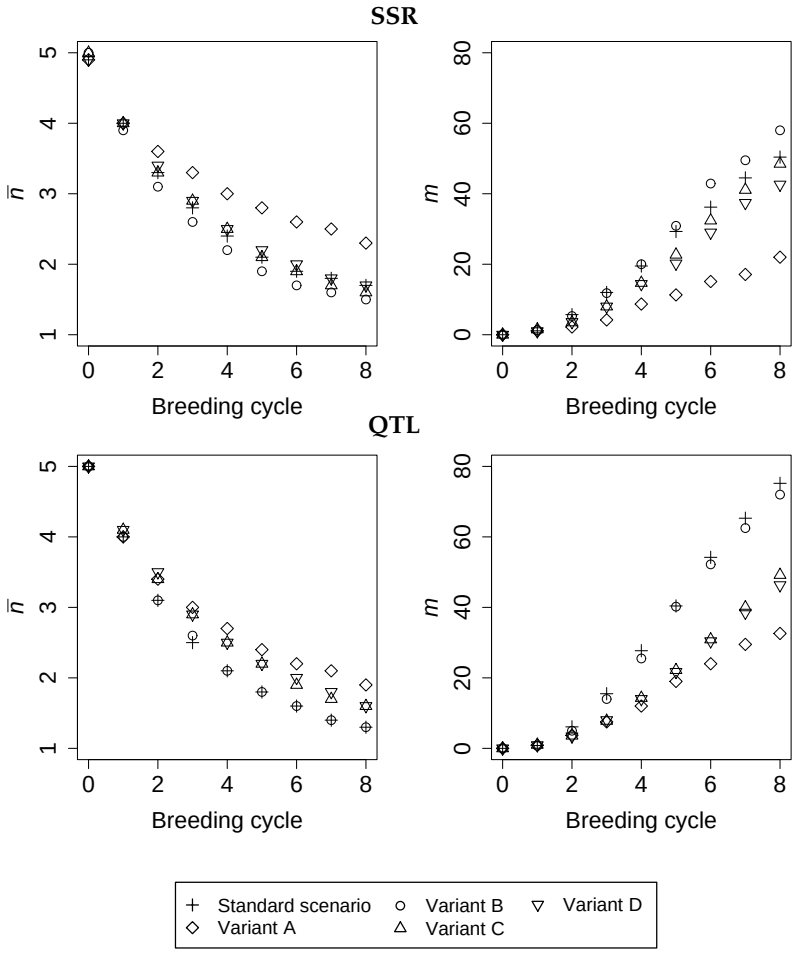

Figure 2 Average number of alleles per locus ($\overline{n}$) and percentage of monomorphic loci (m) for SSRs and QTL in the different simulation scenarios adjusted for a population size of 28. For a detailed description of the variants see Materials and Methods.

of a heterotic group as well as the extent of LD in a heterotic group.

Furthermore, inbreds in the study of Reif et al. (2005b) were sampled from several breeding programs, whereas we assumed only a single breeding program. Therefore, population stratification is likely to generate a high extent of LD in the former study. This explanation is supported by the observation of (i) a lower ratio of the percentage of linked to unlinked SSR loci pairs in significant LD and (ii) a higher number of alleles per SSR locus after 55 years of hybrid maize breeding in Europe in the study of Reif et al. (2005b).

In summary, our simulation results on the selection gain, trends in allelic diversity, and linkage disequilibrium are in most instances in good accordance with results of experimental studies, thus, confirming the assumptions and models underlying our simulations.

Potential causes of LD

The cost- and time-effectiveness of genome sequencing improves exponentially (Shendure et al. 2004). Therefore, it may be possible to sequence genomes of all individuals of an association mapping population in the future. In this case funtional markers will be available for the QTL itself (Andersen and Lübberstedt 2003) and, thus, association mapping approaches will no longer be based on LD between QTL and adjacent molecular markers. In this scenario, knowledge about the forces generating and conserving LD will not be of importance. However, with today's genome sequencing technology, such approaches are not feasible.

The influence of relatedness, population stratification, and linkage as causes of LD in plant breeding populations can be examined with experimental data (*e.g.*, Stich et al. 2005). However, basing investigations for selection, mutation, and genetic drift on experimental data is difficult (Farnir et al. 2000). Hence, we applied computer simulations to assess the relevance of these forces for generating and conserving LD in plant breeding populations.

Selection: No clear temporal trend was observed for the difference in the percentage of linked and unlinked SSR loci pairs in significant LD between the standard scenario and variant C. In the latter case, the selection of individuals during the breeding process was not based on their TP or LP, but was made at

Table 1 Percentage of loci pairs in significant ($P < 0.01$) linkage disequilibrium (LD) in different simulation scenarios adjusted for a population size of 28. For a detailed description of the variants see Materials and Methods.

	Standard scenario Loci pairs in LD			Variant A Loci pairs in LD			Variant B Loci pairs in LD			Variant C Loci pairs in LD			Variant D Loci pairs in LD		
Cycle	Linked	Unlinked	Total	Linked	Unlinked	Total	Linked	Unlinked	Total	Linked	Unlinked	Total	Linked	Unlinked	Total
	%			%			%			%			%		
SSR															
1	3.4	3.8	3.7	3.2	3.9	3.8	3.5	4.0	3.9	2.9	3.4	3.3	3.0	3.3	3.2
2	9.6	4.0	4.4	9.5	4.3	4.8	11.5	5.0	5.5	5.0	2.7	2.9	5.7	2.9	3.1
3	7.8	3.2	3.6	10.4	4.3	4.8	9.4	2.8	3.3	7.1	2.3	2.7	6.6	2.2	2.6
4	6.8	2.2	2.6	9.4	2.7	3.3	8.7	2.2	2.8	8.6	2.3	2.8	6.1	2.0	2.4
5	6.1	2.1	2.4	6.8	2.3	2.7	6.8	2.4	2.7	7.1	1.8	2.2	5.7	1.5	1.9
6	6.5	1.0	1.5	8.0	2.4	2.9	7.1	1.6	2.0	6.4	1.4	1.9	5.3	1.3	1.7
7	6.8	1.9	2.2	6.9	1.9	2.4	7.0	1.7	2.1	8.7	1.2	1.9	5.2	1.5	1.8
8	5.1	1.2	1.4	5.8	1.7	2.1	6.8	1.4	1.7	5.4	1.1	1.5	4.2	1.0	1.4
SSR-QTL															
1	4.0	3.8	3.8	3.8	3.8	3.8	4.3	4.1	4.1	3.2	3.2	3.2	3.3	3.3	3.3
2	11.6	4.2	5.0	11.6	3.9	4.7	11.2	4.1	4.8	10.3	3.4	4.1	7.8	2.6	3.2
3	9.0	2.5	3.2	12.9	4.1	5.0	8.8	2.3	3.0	9.9	2.2	3.0	7.7	1.9	2.5
4	5.7	1.0	1.5	9.2	2.0	2.8	6.5	1.4	1.9	8.4	1.7	2.4	6.9	1.5	2.0
5	4.7	0.9	1.3	7.3	1.7	2.3	4.2	0.8	1.2	6.1	1.0	1.5	5.0	1.0	1.4
6	2.5	0.5	0.7	6.5	1.3	1.8	2.8	0.5	0.7	4.6	0.6	1.0	3.7	0.7	1.1
7	1.5	0.2	0.4	4.4	0.9	1.3	1.7	0.3	0.4	3.3	0.5	0.8	3.3	0.4	0.7
8	0.8	0.2	0.2	3.2	0.8	1.0	1.0	0.2	0.2	2.3	0.3	0.5	2.3	0.3	0.5
QTL															
1	4.1	4.0	4.0	4.0	4.0	4.0	4.5	4.4	4.4	3.3	3.4	3.4	3.5	3.4	3.4
2	13.6	5.0	5.9	12.6	4.3	5.2	12.8	4.7	5.5	11.7	3.9	4.7	8.9	2.9	3.5
3	13.0	3.5	4.5	15.2	4.9	6.0	12.1	3.2	4.1	12.0	2.7	3.6	9.3	2.3	3.0
4	11.1	2.0	2.9	11.9	2.7	3.6	11.7	2.5	3.4	11.7	2.4	3.4	9.6	2.0	2.8
5	12.9	2.4	3.6	11.2	2.6	3.6	11.6	2.3	3.3	10.5	1.6	2.5	8.2	1.6	2.3
6	11.4	2.3	3.4	11.2	2.2	3.1	12.2	2.1	3.2	9.7	1.3	2.2	8.6	1.6	2.3
7	11.4	1.7	2.8	8.4	1.9	2.6	11.2	1.8	2.8	9.2	1.4	2.2	8.6	1.2	2.0
8	11.5	2.5	3.5	7.0	1.8	2.4	12.2	1.9	3.0	9.1	1.1	2.0	8.2	1.1	1.9

random. Therefore, our results suggested that on a genome-wide scale selection is a neglectable cause of significant LD between neutral markers in the examined marker density (35 cM). This finding is in accordance with the observation of Farnir et al. (2000) based on genome-wide distributed SSR markers in cattle. Furthermore, the results of the present study indicate that selection generates no significant LD between SSR-QTL pairs on a genome-wide scale.

In contrast, we observed a considerably higher extent of significant LD between QTL pairs in the standard scenario than in variant C. This result indicated that selection generates LD between pairs of QTL and thereby reduces the additive variance. This phenomenon is also known as the Bulmer effect (Bulmer 1971). Our observation is contrary to findings from an experimental study on maize in which no Bulmer effect was detected (Robinson et al. 1960). However, since the variance components reported by these authors showed large standard errors, the conclusions drawn are afflicted with uncertainty.

Our findings of significant LD between QTL, which is generated by selection, indicates that in plant breeding populations, which typically undergo selection for several traits, significant LD will be generated between QTL for different traits. Furthermore, the extent of significant LD between pairs of SSRs, each of which was separated by less than 2 cM from a QTL, was slightly higher in the standard scenario than in variant C. This result suggested that selection may generate significant LD not only between QTL for different traits but also between markers linked to QTL for different traits. In candidate-gene association mapping approaches, molecular markers are closely linked to the QTL. Therefore, an increased rate of false positive associations between markers and unlinked QTL may be detected if population-based association mapping methods are used in candidate-gene approaches with populations undergoing selection for several traits.

Theoretical considerations suggest that the distribution of the size of QTL effects influences the importance of selection as LD-generating force. However, in a scenario in which the size of the QTL effects were defined based on the geometric series $220(1-a)[1, a, a^2, a^3, ..., a^{219}]$, with $a = 0.95$, we detected about the same percentage of SSRs, QTL, and SSR-QTL pairs in LD (data not shown), as observed in the scenario with a uniform distribution of the size of QTL effects. Therefore, our conclusions about the influence of selection on LD seem insensitive with respect to the distribution of the size of QTL effects.

Mutation: The percentage of linked and unlinked SSR loci pairs in significant LD was lower in the standard scenario than in variant B. Because absence of mutation was assumed in the latter scenario, this result suggested that mutation reduces the extent of significant LD. This observation was supported by the findings of Calafell et al. (2001) and Terwilliger et al. (1998) that the LD present in a population eroded with an increasing mutation rate. In contrast, Iles and Bishop (1998) observed for populations without LD a generation of LD by mutation. However, in plant breeding populations, LD is continuously generated by crossing individuals with differing allele frequencies. Therefore, in plant breeding populations mutation is expected to reduce the extent of significant LD between SSRs rather than increasing it.

Genetic drift: The formulas of Hill and Robertson (1968) indicate that in populations with small effective population size (N_e), LD is expected to decay faster than in populations with large N_e. However, the variance of the LD measure increases if N_e is reduced. Therefore, genetic drift increases the extent of LD in a population. This was supported by the observations of Nord-

borg et al. (2002) that local populations of *Arabidopsis thaliana* showed a higher extent of LD than global populations.

The percentage of linked and unlinked SSRs and QTL pairs in significant LD were slightly higher in variant C than in variant D. The population size applied in variant C corresponds to that typically applied in plant breeding programs, whereas the population size of variant D was three times larger. Therefore, our findings indicate that for the population sizes typically applied in plant breeding programs, genetic drift is a major force generating significant LD between linked but also between unlinked loci. This result was supported by the findings of Stich et al. (2005). These authors observed for experimental data of European elite maize inbreds a non-uniform distribution of significant LD along the chromosomes suggesting that genetic drift is a major force generating LD.

Implications for association mapping

The results of our study suggested that mutation reduces the extent of significant LD in plant breeding populations. In contrast, our results indicated that in plant breeding populations genetic drift is a major force that generates significant LD between linked as well as unlinked loci. Furthermore, selection is a cause of significant LD between QTL but also between markers closely linked to QTL. The existing population-based association mapping tests do not correct for LD caused by the latter two forces. Therefore, increased type I error rates are expected if these tests are applied to plant breeding populations. In contrast, family-based association tests are valid tests of associations in the presence of linkage (Zhang et al., 2001) and, thus, adhere also to the nominal α level if LD is generated by population stratification, relatedness, genetic drift, and selection. Therefore, such tests are strongly recommended for association mapping approaches in plant breeding populations.

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ORIGINAL PAPER

A new test for family-based association mapping with inbred lines from plant breeding programs

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Abstract Association mapping holds great promise for the detection of quantitative trait loci (QTL) in plant breeding populations. The main objectives of this study were to (1) adapt the quantitative pedigree disequilibrium test to typical pedigrees of inbred lines produced in plant breeding programs, (2) compare the newly developed quantitative inbred pedigree disequilibrium test (QIPDT) with the commonly employed logistic regression ratio test (LRRT), with respect to the power and type I error rate of QTL detection, and (3) demonstrate the use of the QIPDT by applying it to flowering data of European elite maize inbreds. QIPDT and LRRT were compared based on computer simulations modeling 55 years of hybrid maize breeding in Central Europe. Furthermore, we applied QIPDT to a cross-section of 49 European elite maize inbred lines genotyped with 722 amplified fragment length polymorphism markers and phenotyped in four environments for days to anthesis. Compared to LRRT, the power to detect QTL was higher with QIPDT when using data collected routinely in plant breeding programs. Application of QIPDT to the 49 European maize inbreds resulted in a significant ($P < 0.05$) association located at a position for which a consensus QTL was detected in a previous study. The results of our study suggested that QIPDT is a promising QTL detection method for data collected routinely in plant breeding programs.

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Introduction

Estimation of the positions and effects of quantitative trait loci (QTL) is of central importance for marker-assisted selection. Up to now, this has been accomplished by classical QTL mapping approaches (Lander and Botstein 1989). The necessary experiments require establishment as well as pheno- and genotyping of large mapping populations and, thus, are very cost- and time intensive (Parisseaux and Bernardo 2004). These limitations could be overcome by applying association mapping methods in elite germplasm, using phenotypic and genotypic data routinely collected in plant breeding programs (Jansen et al. 2003). Moreover, results from association mapping would be of direct use in breeding, because allelic variation present in the entire elite germplasm is investigated.

With association mapping methods, detection of markers closely linked to QTL requires linkage disequilibrium (LD) caused by linkage being present between QTL and markers. Results of a companion study (Stich et al. 2006) on Central European elite maize germplasm suggested that LD between amplified fragment length polymorphism (AFLP) markers attributable to physical linkage could be expected between markers spaced at not more than 3 cM. However, it was concluded that also relatedness, population

stratification, and genetic drift are important causes of LD. When population-based association mapping tests are applied to germplasm in which LD is generated by the latter forces, the proportion of spurious marker-trait associations is expected to be much higher than the nominal type I error rate α (Pritchard et al. 2000b).

So far, the logistic regression ratio test (LRRT) has been applied as a population-based association mapping method accounting for population stratification (Pritchard et al. 2000a; Thornsberry et al. 2001). This test is based on the calculation of two hypotheses: first, that the distribution of the molecular marker under consideration was associated with population structure and phenotypic variation, and second, that the molecular marker distribution was associated only with population structure. The test statistic of LRRT is the ratio of the two likelihoods. However, the LRRT fails to correct for LD caused by relatedness or genetic drift and, thus, may show an increased rate of spurious marker-trait associations. In contrast, family-based association mapping methods suggested in human genetics, such as transmission disequilibrium test (TDT) (Spielman et al. 1993) or quantitative pedigree disequilibrium test (QPDT) (Zhang et al. 2001), adhere to the nominal α level even when LD is generated by population stratification, relatedness, or genetic drift. While these disturbing forces are most likely active in plant breeding populations, to our knowledge a family-based association mapping test has neither been developed in a plant breeding context nor applied to data typically available from plant breeding programs.

The objectives of our pilot study were to (1) adapt the QPDT to typical pedigrees of inbred lines produced in plant breeding programs, (2) compare the newly developed quantitative inbred pedigree disequilibrium test (QIPDT) with the commonly employed LRRT, with respect to the power and type I error rate of QTL detection, and (3) demonstrate the use of the QIPDT by applying it to flowering data of European elite maize inbreds.

Materials and methods

QIPDT, a family-based association test with inbred offsprings

In this treatise, we describe the QIPDT, a family-based association test applicable to genotypic information of parental inbred lines and geno- and phenotypic information of their offspring inbreds. This information is typically available from plant breeding programs with inbred line development such as line breeding or hybrid breeding. Nuclear families consisting of two parental inbred lines and at least one offspring inbred line can be combined to extended pedigrees, the basis of the QIPDT, if the parental lines of different nuclear families are related (Fig. 1). The QIPDT extends the QPDT, a family-based association test developed in the context of human genetics, which is in contrast to the quantitative transmission disequilibrium test (Abecasis et al. 2000) a valid test of association in the presence of linkage when some of the nuclear families are related (Zhang et al. 2001). Furthermore, the QIPDT takes into account the correction of Martin et al. (2001) regarding the pedigree disequilibrium test.

For a given nuclear family j_k with t_{jk} offspring inbreds belonging to the kth independent extended pedigree, let Y_{ijk} denote the phenotypic value of the i_{jk}th offspring inbred line for a quantitative trait of interest. Assume the molecular marker locus under study is bi-allelic with alleles M and N. Define $X_{ijk} = 0$ if the two parental inbreds possess the same allele and

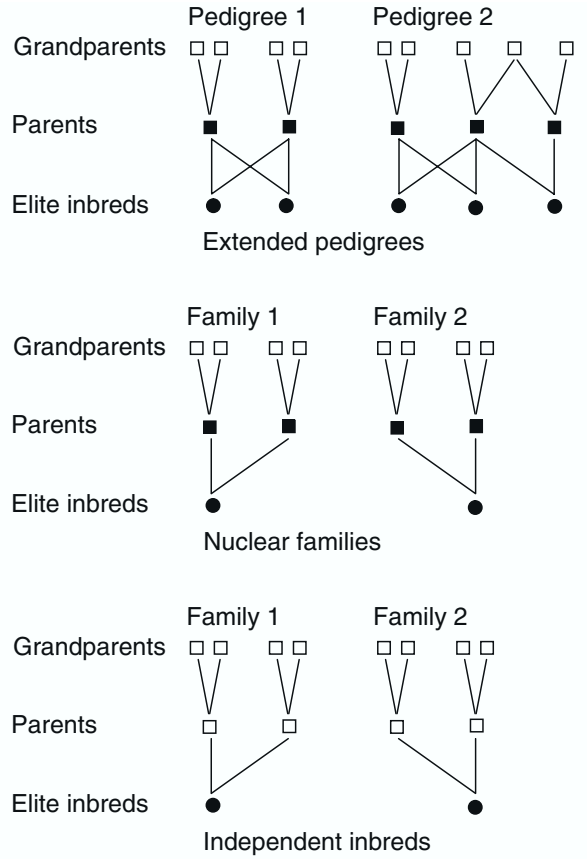

Fig. 1 Illustration of the simulated structures of inbred lines. Genotyped and phenotyped inbreds are presented as *filled circles*, genotyped but not phenotyped inbreds as *filled squares*, and inbreds neither genotyped nor phenotyped as *open squares*

$X_{ijk} = 1$ (or -1) if the two parental inbreds possess different alleles and transmit allele M (or N) to the i_{jk}th offspring inbred. Define the random variable U_{jk} for each nuclear family j_k of the extended pedigree k as:

$$U_{jk} = \sum_{i=1}^{t_{jk}} (Y_{ijk} - \overline{Y_k}) X_{ijk}, \quad (1)$$

where $\overline{Y_k}$ is the mean phenotypic value of all offspring inbreds in the kth extended pedigree. If the number of offspring in the kth extended pedigree was only 1, $\overline{Y_k}$ was replaced by $\overline{Y}$, the mean phenotypic value of all offspring inbreds in all extended pedigrees, following the original test of Zhang et al. (2001).

For the kth of p independent extended pedigrees, let n_k denote the number of the nuclear families. Define

$$D_k = \sum_{j=1}^{n_k} U_{jk}. \quad (2)$$

Under the null hypothesis of no marker-trait association, both alleles M and N are transmitted within each extended pedigree with equal probability to offspring inbreds with phenotypic values higher or lower than $\overline{Y_k}$ resulting in $E(D_k) = 0$. Therefore,

$$\mu = E\left(\sum_{k=1}^{p} D_k\right) = 0 \quad (3)$$

and

$$\sigma^2 = Var\left(\sum_{k=1}^{p} D_k\right) = \sum_{k=1}^{p} Var(D_k) = E\left(\sum_{k=1}^{p} {D_k}^2\right). \quad (4)$$

Hence, if we define the test statistic of the QIPDT as

$$T = \frac{\sum_{k=1}^{p} D_k}{\sqrt{\sum_{k=1}^{p} {D_k}^2}}, \quad (5)$$

under the null hypothesis of no LD between marker locus and QTL, T is asymptotically normally distributed with mean zero and variance one. A statistical test for the null hypothesis based on T can be determined by comparison with a standard normal distribution.

LRRT

The LRRT, described in detail by Thornsberry et al. (2001) and implemented in software package TASSEL (http://www.maizegenetics.net), is the population-based association mapping approach most frequently applied in plant populations. We compared it with the QIPDT with respect to the power and type I error rate of QTL detection. The LRRT requires information about the population structure, which is commonly determined using the model-based approach implemented in software package STRUCTURE (Pritchard et al. 2000a; Falush et al. 2003). In our investigations, the set of inbred lines under study was analyzed by setting the number of clusters from 1 to 12 in each of five repetitions. For each run of STRUCTURE, the burn-in time as well as the iteration number for the Markov Chain Monte Carlo algorithm were set to 100000, following the suggestion of Whitt and Buckler (2003). The population structure matrix with the highest likelihood was used for the LRRT.

Simulations

Detailed surveys about the breeding history of the Central European elite maize germplasm reported in two companion studies (Reif et al. 2005; Stich et al. 2005) provided the basis for choosing realistic assumptions and parameters in the simulations described below. Because the results of Stich et al. (2005) indicated that selection was an unlikely cause of LD, it was neglected in our simulations. Furthermore, our simulations were based on two existing AFLP linkage maps with 722 or 1925 loci with map positions, according to a proprietary integrated map (Peleman et al. 2000). In the first case, the map positions of the 722 AFLPs are identical to those used to genotype the set of inbred lines described below.

Modeling the breeding history

Hybrid maize breeding was initiated in Europe in the 1950s. Therefore, a breeding period of 55 years was assumed in our simulations (Fig. 2). For hybrid maize breeding in Central Europe two heterotic groups with similar breeding histories, flint and dent, are used as parental pools. Thus, we simulated the breeding history for two distinct heterotic groups. The modified Rogers' distance (Wright 1978) between them was assumed to be 0.50, based on experimental data from the current flint and dent heterotic groups analyzed in a companion study (Stich et al. 2005). The predominant ancestor inbreds F2, F7, and EP1 of the European flint heterotic group trace back to two European open-pollinated varieties Lacaune and Lizargarote (Cartea et al. 1999). Hence, for each heterotic group, we sampled 250 individuals out of each of two base populations in

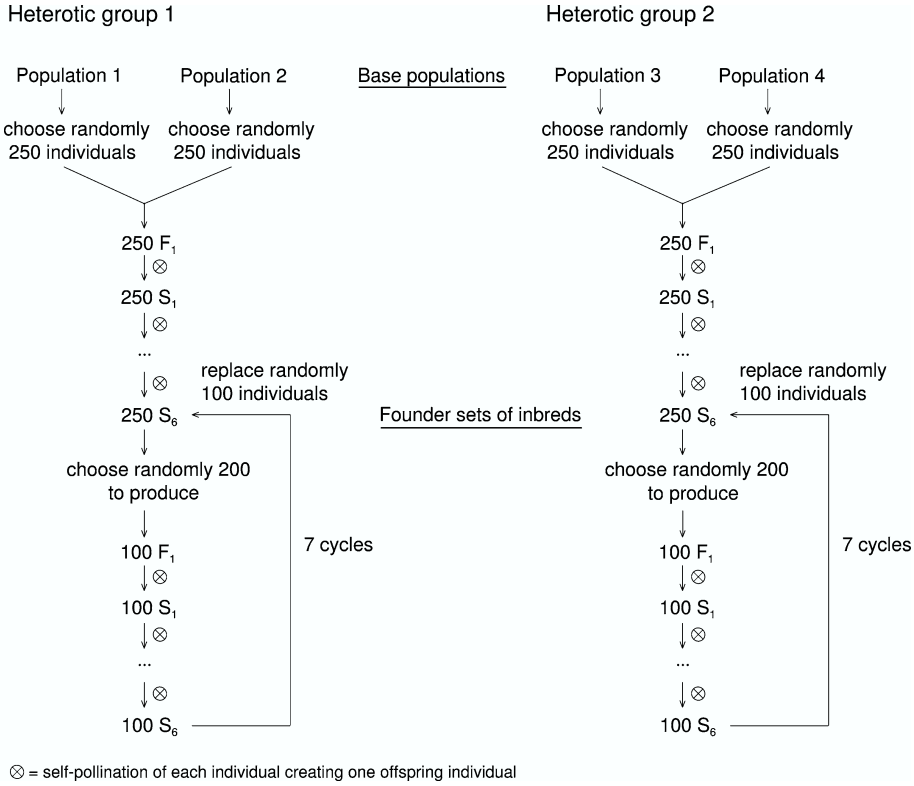

Fig. 2 Model for simulating 55 years of breeding history in the European maize germplasm groups

Hardy–Weinberg and linkage equilibriums. Different base populations were used for the two heterotic groups. The modified Rogers' distance between base populations of each heterotic group was assumed to be 0.30, based on experimental estimates between European open-pollinated varieties (Reif et al. 2005). The average modified Rogers' distance between individuals from the same base population was assumed to be 0.45.

For each heterotic group, 250 F_1 individuals were produced by crossing each of the 250 individuals chosen from each base population with one individual from the other base population. Subsequently, the F_1 individuals were selfed for six generations, resulting for each heterotic group in 250 S_6 individuals that formed the founder set of inbred lines for the next breeding cycle. The recycling breeding following the establishment of the founder set of inbreds was simulated such that for each heterotic group a total of 200 inbreds was randomly drawn out of the founder set. These 200 inbreds were crossed pairwisely to generate 100 F_1 individuals. All 100 F_1 individuals were self pollinated for six generations, resulting for each heterotic group in 100 S_6 individuals, which replaced 100 randomly chosen inbreds of the founder set of inbreds. This scheme of crossing, selfing, and replacing lines of the founder set was repeated seven times for each heterotic group.

Population structure of inbred lines examined with QIPDT and LRRT

After completing seven cycles of recycling breeding, the 500 inbred lines from the founder sets of both heterotic groups were designated as current elite inbreds. These inbreds, together with their parental inbreds, formed the nuclear families. Because all offspring inbreds can be traced back to common ancestors, the independence assumption is violated. The criterion employed to warrant an approximative stochastic independency of D_k estimates was that nuclear families having at least one common grandparental inbred were combined to extended pedigrees. By applying this criterion, we obtained 75 independent extended pedigrees. Groups of 25 and 75 independent nuclear families were generated by sampling nuclear families from the 75 independent extended pedigrees. The offspring inbreds of the 75 independent nuclear families were examined with LRRT.

Definition of phenotypic values and estimated parameters

One, 10, and 50 AFLP markers sampled at random from the linkage map were defined as QTL with equal

genetic effects. At each QTL, one allele was assigned the genotypic effect one and the other the genotypic effect zero. The genotypic value of each inbred was calculated by summing up the effects of the individual alleles. The phenotypic value used for association tests was generated by adding a normally distributed variable $N(0, \sigma_E^2)$ to the genotypic value. The error variance was then calculated as $\sigma_E^2 = \sigma_G^2/h^2 - \sigma_G^2$, where σ_G^2 denotes the genotypic variance estimated in the group of offspring inbreds, and h^2 denotes the corresponding heritability. We examined h^2 values of 0.50, 0.75, and 1.00.

The proportion $1 - \beta^*$ of significant associations detected between a QTL and at least one adjacent AFLP marker, further designated as the power of QTL detection, was determined for both QIPDT and LRRT. For scenarios with more than one QTL, the average of the power estimates observed for each individual QTL was calculated. Furthermore, we estimated the rate of false positives, α^*, as the proportion of significant associations observed between a QTL and a marker locus, when at least one marker located between them showed a nonsignificant association with the trait.

One-QTL-one-marker simulations

In addition to the simulations based on the breeding history, simulations solely based on one QTL and one marker locus were performed for the QIPDT to vary exclusively selected parameters influencing the power of QTL detection. In the set of 75 pairs of parental inbreds used to generate 75 F_1 individuals, both loci were assumed to have an allele frequency of 0.5 and to be tightly linked (0.000001 cM). The F_1 individuals were selfed for six generations. The 75 pairs of parental inbreds and the 75 descendant S_6 individuals were used to estimate the power of the QIPDT to detect QTL if (1) LD between QTL and marker locus, measured as r^2 (Hill and Robertson 1968), ranged from 0 to 0.5, and (2) h^2 estimates of the phenotypic trait were 0.50, 0.75, or 1.00. The QTL explained 10% of the genotypic variance.

Application of QIPDT to data from elite maize inbreds

A cross-section of 49 elite European maize inbreds was examined in this study. The inbred lines belong to four independent extended pedigrees (Table 1) and represent a subset of the materials investigated by Heckenberger et al. (2005a, b). In total, 722 polymorphic AFLP markers were scored by Keygene N.V. (P.O. Box 216, 6700 AE Wageningen, The Netherlands), using 20 primer combinations described by Heckenberger et al. (2005b). Map positions of the AFLP markers were obtained from a proprietary integrated map (Peleman et al. 2000). Field experiments with the inbreds were conducted in 2000 and 2001 at two locations in South Germany, with two replications per location, as described in detail by Heckenberger et al. (2005a). Briefly, adjusted-entry means were calculated for each environment for days to anthesis. Heritability was calculated on an entry-mean basis using software Plabstat (Utz 2001). The average of adjusted entry means over environments was used for the QIPDT. To avoid a reduction of the power to detect QTL, we did not correct for the multiple test problem. All simulations and calculations for QIPDT were performed with Plabsoft (Maurer et al. 2004), which is implemented as an extension of the statistical software R (R Development Core Team 2004).

Results

Simulations based on breeding history

In the LRRT of the 75 independent inbred lines, which corresponds to 54 150 AFLP data points, the power to detect QTL increased with decreasing number of QTL, and ranged from 0.126 (50 QTL) to 0.280 (1 QTL) for $h^2 = 0.50$ (Table 2). The corresponding estimates of α^* surpassed the nominal α level of 0.05 throughout all

Table 1 Pedigree structure of the examined 49 inbred lines

Independent pedigree[a]	Nuclear family	Parents[b]	Offspring[b]
A	1	L61, L87	L83
B	1	L54, L66	L11
C	1	L55, L56	L5, L6, L7
	2	L55, L59	L8, L9, L10
	3	L59, L74	L2, L3, L4
D	1	L24, L49	L46
	2	L28, L39	L41
	3	L28, L49	L42
	4	L33, L49	L44
	5	L36, L49	L43
	6	L49, L65	L21
	7	L51, L65	L25, L29
	8	L64, L65	L28
	9	L65, L67	L33, L34
	10	L65, L68	L32, L35, L36, L37, L38
	11	L65, L72	L39
	12	L81, L82	L17

[a] Inbreds from independent pedigrees have no common grandparents

[b] Notation refers to the notation of Stich et al. (2005, 2006)

scenarios examined. Increasing h^2 from 0.50 to 1.00 resulted in a minor increase in the power to detect QTL for all numbers of QTL examined.

In QIPDT, the power of QTL detection with 25 independent nuclear families (54 150 AFLP data points) was just above half as high as in the LRRT with 75 independent inbreds (Table 2). Compared with the latter, the power of QIPDT was still lower with 75 independent nuclear families (162 450 AFLP data points), but higher with 75 independent extended pedigrees, which corresponds to 313 348 AFLP data points. With 200 independent nuclear families, the QIPDT exceeded the power of the LRRT with 75 independent inbreds by 50–120%. In QIPDT with 75 independent nuclear families, increasing the marker density from 722 to 1925 AFLP loci resulted in a considerable increase in $1 - \beta^*$ for small numbers of QTL (1 and 10 QTL). However, only marginal increases were observed for 50 QTL, irrespective of the heritability. When increasing h^2 from 0.50 to 1.00, a substantial increase in the power to detect QTL was observed only for high numbers of examined nuclear families or extended pedigrees. In contrast to the LRRT, the QIPDT adhered in all scenarios except for the 200 independent nuclear families to the nominal α level.

One-QTL-one-marker simulations

In the one-QTL-one-marker simulations, the power $1 - \beta^*$ of the QIPDT increased almost linearly with increasing LD between the marker locus and the QTL (Fig. 3). The slope of the increase was dependent on the h^2 estimate. Hence, the largest differences between estimates of $1 - \beta^*$ for different values of h^2 were found for r^2 values between 0.3 and 0.6.

Application of QIPDT to flowering data of elite maize inbreds

For the set of 49 European maize inbreds, days to anthesis showed a highly significant ($P < 0.01$) genotypic variance, and a h^2 estimate of 0.97. Entry means across environments for the 29 offspring inbreds ranged from 69.0 to 87.3 days. The QIPDT detected one AFLP marker mapping to bin 5.03 as significantly ($P < 0.05$) associated with days to anthesis (Fig. 4).

Discussion

To our knowledge, the simulation study of Pritchard et al. (2000b) is the only investigation comparing the LRRT with the classical family-based association test TDT. However, their simulations are based on a standard human coalescent model assuming random mating. Because this mating system is the exception rather than the rule in plant breeding, the results of Pritchard et al. (2000b) do not apply to most situations in plant breeding (Bernardo 2002). We based computer simulations on the 55 years of hybrid maize breeding in

Table 2 Power of QTL detection ($1 - \beta^*$) and rate of false positives (α^*) for the quantitative inbred pedigree disequilibrium test (QIPDT) and logistic regression ratio test (LRRT) at a nominal α level of 0.05 for different simulation scenarios

h^2	722 AFLP loci										1925 AFLP loci	
	LRRT		QIPDT								QIPDT	
	75 independent inbreds		25 independent nuclear families		75 independent nuclear families		75 independent extended pedigrees		200 independent nuclear families		75 independent nuclear families	
	$1 - \beta^*$	α^*	$1 - \beta^*$	α^*	$1 - \beta^*$	α^*	$1 - \beta^*$	α^*	$1 - \beta^*$	α^*	$1 - \beta^*$	α^*
1 locus coding for a trait												
1.00	0.300	0.054	0.140	0.046	0.240	0.050	0.440	0.049	0.760	0.056	0.820	0.052
0.75	0.280	0.055	0.140	0.046	0.180	0.051	0.340	0.048	0.740	0.054	0.800	0.051
0.50	0.280	0.053	0.120	0.045	0.160	0.049	0.300	0.048	0.680	0.053	0.760	0.049
10 loci coding for a trait												
1.00	0.146	0.056	0.074	0.043	0.112	0.050	0.152	0.052	0.470	0.054	0.372	0.050
0.75	0.142	0.056	0.074	0.042	0.106	0.049	0.146	0.051	0.450	0.054	0.332	0.049
0.50	0.134	0.054	0.074	0.041	0.102	0.049	0.138	0.051	0.304	0.052	0.218	0.048
50 loci coding for a trait												
1.00	0.144	0.057	0.081	0.042	0.116	0.051	0.147	0.048	0.239	0.053	0.152	0.050
0.75	0.135	0.056	0.082	0.041	0.112	0.050	0.142	0.048	0.223	0.052	0.149	0.049
0.50	0.126	0.053	0.082	0.040	0.102	0.050	0.110	0.047	0.174	0.050	0.122	0.050
Number of phenotyped/genotyped inbreds (number of AFLP data points)												
	75/75 (54 150)		25/75 (54 150)		75/225 (162 450)		248/434 (313 348)		200/600 (433 200)		75/225 (433 125)	

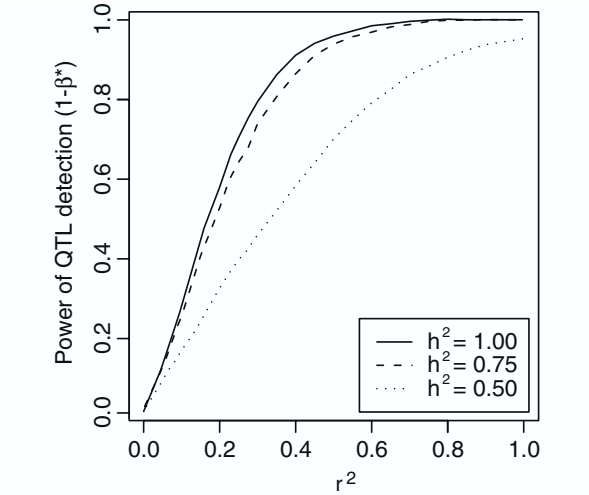

Fig. 3 Power of QTL detection ($1 - \beta^*$) by the quantitative inbred pedigree disequilibrium test (QIPDT) for different heritabilities (h^2) as a function of the extent of LD (r^2) between the marker locus and the QTL using one-QTL-one-marker simulations. The number of nuclear families was 75 and the allele frequencies of the two tightly linked loci were 0.5. The QTL explained 10% of the genotypic variance

Central Europe, to approach a comparison of family-based with population-based association mapping in a scenario in which LD is generated by the same forces as those observed for European maize breeding populations (Fig. 2).

Statistical assumptions for LRRT and QIPDT

LRRT requires independent genotypes whereas QIPDT requires independent nuclear families or extended pedigrees. For both tests ignoring the assumption of independence, results in α level higher than the nominal one (Pritchard and Rosenberg 1999; Monks and Kaplan 2000). For LRRT, this result was observed by Thornsberry et al. (2001) when applying this test to a worldwide sample of partially related maize inbred lines. The rejection rate at SSR markers, for which no association with the QTL was expected was higher (0.081) than the nominal α level of 0.05. The same observation is expected for the QIPDT because treating dependent extended pedigrees as independent causes an underestimation of the variance of the D_k estimates in the denominator of the test statistic T. However, in plant breeding populations, all inbreds trace back to common ancestors and, thus, only approximate independence can be achieved with any criterion.

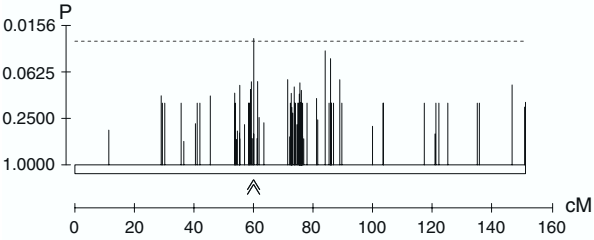

Fig. 4 Distribution of the *P* values of the AFLP-days to anthesis associations on chromosome 5 detected in European elite maize inbreds using the QIPDT

In a companion study, Stich et al. (2005) observed a significant reduction in the extent of LD caused by relatedness when examining only individuals with no common grandparents. This criterion was chosen for the LRRT to define independent inbreds, and for the QIPDT to define independent extended pedigrees or nuclear families. The observation that the QIPDT adhered to the nominal α level suggested that the applied criterion warrants sufficient independency of extended pedigrees and nuclear families. Nevertheless, further research is needed to gain a detailed knowledge on the required independency of extended pedigrees and nuclear families in populations with a breeding history different from the described one.

Comparison of LRRT with QIPDT

In every plant breeding program, large numbers of inbred lines with known pedigree relationships are routinely phenotyped with high efforts as a basis for selection decisions. Our basic idea is to complement this information with genotypic data and perform association mapping. Here, two cases must be distinguished.

If genotypic data are not routinely collected and must be generated anew, candidate-gene but also genome-wide association mapping approaches are possible. For association mapping using a candidate-gene approach, the QIPDT is recommended because the LRRT requires a substantially higher genotyping effort to examine the same number of inbred lines. This is because an independent genome-wide marker set is additionally needed to reliably uncover the population structure. This argument does not apply to association mapping via a genome-wide approach, because one genome-wide marker data set is sufficient to (1) estimate the population structure and (2) map QTL with the LRRT (Pritchard et al. 2000b). With a given budget for genotyping a fixed number of lines (e.g., $N = 75$), the power $1 - \beta^*$ showed higher estimates for LRRT, examining 75 independent inbreds, than for QIPDT, examining 25 independent nuclear families

(Table 2). This result clearly indicated the superiority of the LRRT for genome-wide association mapping if genotypic data must be newly generated and a large number of independent inbred lines is available.

In contrast to the situation described above, in many maize breeding programs genotypic data from genome-wide distributed marker loci are collected routinely for various reasons, such as plant variety protection or choice of parents for establishing new base populations. In addition, a large proportion of these inbred lines are full sibs or half sibs of independent parental inbreds that were also genotyped. Consequently, extended pedigrees as the basis of the QIPDT reflect the typical data structure available from plant breeding programs. This outcome allows exploitation of routinely collected data for the purpose of QTL detection. On the other hand, the LRRT requires independent genotypes. Therefore, we compared QIPDT and LRRT based on 75 independent extended pedigrees and 75 independent inbreds, respectively. While the type I error rate of LRRT surpassed the nominal α level of 0.05, the QIPDT adhered to it and yielded higher estimates of $1 - \beta^*$ in all scenarios except in one. These results suggest that the QIPDT is superior to the LRRT for genome-wide association mapping if data collected routinely in plant breeding programs are available.

In contrast to the LRRT, dependent inbreds can be included in association mapping approaches using the unified mixed model method, recently described by Yu et al. (2006), without increasing the type I error rate. Therefore, a higher power to detect QTL is expected for the unified mixed model method than for the LRRT when using data from plant breeding programs. However, a comparison of the former and the QIPDT with respect to their power to detect QTL in a plant breeding context is still lacking. In contrast to QIPDT, the unified mixed model method is no test of association in the presence of linkage. Therefore, based on the various forces generating LD in plant breeding programs (Stich et al. 2005), an increased type I error rate is expected for the latter when examining data from elite breeding programs.

Factors influencing the power of QIPDT in QTL detection

The power $1 - \beta^*$ of QIPDT is influenced by (1) the genetic architecture of the trait, (2) the number of independent nuclear families or extended pedigrees, (3) the genetic map distance between QTL and marker loci, (4) the extent of LD between QTL and marker loci, (5) the heritability of the trait examined, (6) the allele frequencies of QTL and marker loci, and (7) selection for the trait under consideration in plant breeding programs.

For QTL with equal effects, the power $1 - \beta^*$ increases with decreasing number of QTL coding for a trait, because the proportion of variance explained by a single QTL increases. Thus, for QTL with unequal effect the power $1 - \beta^*$ is expected to be higher for loci explaining a large proportion of the variance than for loci explaining a small proportion of the variance.

For previous association mapping studies in plants about 90 lines were examined (e.g., Thornsberry et al. 2001). A data set of comparable size with 75 nuclear families is expected to be available from any ordinary breeding program. The QIPDT of such a data set resulted in a very low power ($1 - \beta^* = 0.112$) for complex traits (50 QTL, $h^2 = 0.75$). This result is in accordance with findings for classical QTL mapping (Schön et al. 2004). The QTL detected for grain yield, using 122 families evaluated in 19 environments ($h^2 = 0.64$), explained about 10% of the genotypic variance. Under the assumption of QTL with equal genetic effects this is equivalent to an $1 - \beta^*$ estimate of 0.100. Examining 200 instead of 75 independent nuclear families with QIPDT almost doubled the power to detect QTL for complex traits (50 QTL). However, the proportion of QTL detected was still too low to be used for marker-assisted selection (Schön et al. 2004). Nevertheless, for mono- and oligogenic traits (≤10 QTL) the power of QIPDT examining 200 independent nuclear families was sufficiently high, so that 75% of 1 QTL and 50% of 10 QTL could be detected. Our results therefore give first guidelines for the necessary population size required in the case of mono- and oligogenic traits. Marker-assisted selection for such traits is particularly appealing if collection of phenotypic data is difficult and/or expensive, which is the case for many resistance and quality traits (Young 1999).

An increased power $1 - \beta^*$ was observed with an increased marker density (Table 2). This is attributable to the increased probability of substantial LD between QTL and marker loci as well as to the reduced recombination frequency between these loci when generating the offspring inbreds from the cross of the parental inbreds. Despite a comparable genotyping effort, the estimates of $1 - \beta^*$ observed for the scenarios with 10 and 50 QTL and 200 independent nuclear families genotyped with 722 AFLPs were higher than those of the 75 independent nuclear families genotyped with 1925 AFLPs. This finding corroborates the conclusion of our previous study (Stich et al. 2006) that an AFLP map with an average marker spacing of

3 cM should be sufficient for genome-wide association mapping in European elite maize germplasm.

Increasing h^2 from 0.50 to 1.00 led in the one-QTL-one-marker simulations to a substantial increase in the power $1 - \beta^*$ of QIPDT only for r^2 values between 0.3 and 0.6 (Fig. 3). This observation suggested that increasing the power to detect QTL by increasing the h^2 estimates of a phenotypic trait is only a promising approach for studies applying a moderate marker density.

The QIPDT has a higher power to detect QTL if both QTL and marker locus show allele frequencies of 0.5. The reason being the minimized probability that some QTL or marker haplotypes have only a very small class size. However, the allele frequency is in contrast to the above factors inappropriate to increase the $1 - \beta^*$ level of association mapping approaches for plant breeding populations.

Selection in plant breeding programs leads to fast genetic fixation of the favorable allele at those QTL which explain a large proportion of the genotypic variance. These fixed QTL cannot be detected in segregating progenies of elite crosses. However, even in elite materials significant genetic variance is still found that could be further analyzed by the QIPDT, which is also valid in populations that have undergone selection. In such populations, too, the QTL alleles are transmitted under the null hypothesis within each extended pedigree with equal probability to inbreds with high or low phenotypic values, as assumed in the definition of the test statistic.

Extension of QIPDT to multiallelic markers

The simulations of the present study as well as the application to a data set of maize were based on AFLP markers. Because in some maize breeding programs inbreds are routinely genotyped with multiallelic markers, we propose an extension of the QIPDT that is commonly used in multiallelic versions of related tests. A global test can be constructed by summing the squared QIPDT statistics for each of the alleles and multiplying the sum by $(m - 1)/m$, where m is the number of marker alleles (Martin et al. 2000). A statistical test for the null hypothesis can be determined by comparison to a chi-square distribution with $m - 1$ degrees of freedom.

Application of QIPDT to a data set from maize

For the application of the QIPDT to the data set comprising 49 European elite maize inbreds, a low power to detect QTL was expected because low $1 - \beta^*$ estimates were observed in the simulations based on the breeding history for data sets of comparable size (25 nuclear families). Nevertheless, we detected one AFLP marker significantly ($P < 0.05$) associated with days to anthesis. The identified QTL region was located at a position for which a consensus QTL was detected in the meta-analysis of Chardon et al. (2004). This QTL provides strong support for the significant association detected in the present study. Furthermore, based on the local maize-rice synteny conservation a flowering time gene was predicted at the QTL region identified in the current study (Chardon et al., 2004). In conclusion, the results of this pilot study suggest that the QIPDT is a promising QTL detection method for data routinely collected in plant breeding programs.

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Power to Detect Higher-Order Epistatic Interactions in a Metabolic Pathway Using a New Mapping Strategy

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ABSTRACT

Epistatic interactions among quantitative trait loci (QTL) contribute substantially to the variation in complex traits. The main objectives of this study were to (i) compare three- *vs.* four-step genome scans to identify three-way epistatic interactions among QTL belonging to a metabolic pathway, (ii) investigate by computer simulations the power and proportion of false positives (PFP) for detecting three-way interactions among QTL in recombinant inbred line (RIL) populations derived from a nested mating design, and (iii) compare these estimates to those obtained for detecting three-way interactions among QTL in RIL populations derived from diallel and different partial diallel mating designs. The single nucleotide polymorphism haplotype data of B73 and 25 diverse maize inbreds were used to simulate the production of various RIL populations. Compared to the three-step genome scan, the power to detect three-way interactions was higher with the four-step genome scan. Higher power to detect three-way interactions was observed for RILs derived from optimally allocated distance-based designs than from nested designs or diallel designs. The power and PFP to detect three-way interactions using a nested design with 5000 RILs was for both the 4 QTL and the 12 QTL scenario of a magnitude that seems promising for their identification.

Up to now estimation of the positions of quantitative trait loci (QTL) was accomplished in plant genetics by classical linkage mapping (Lander and Botstein 1989). Recently, the adaption of association mapping in plant genetics has been proposed by several authors (*e.g.*, Vuylsteke et al. 2000; Thornsberry et al. 2001). Both linkage and association mapping methods have merits and limitations for QTL mapping. While linkage mapping methods offer a high power to detect QTL in genome-wide approaches, association mapping methods have the merit of a high resolution to detect QTL (Remington et al. 2001). Wu and Zeng (2001) studied a joint linkage and linkage disequilibrium (LD) mapping strategy for natural populations. Using data from a general complex pedigree of cattle, Blott et al. (2003) and Meuwissen et al. (2002) identified candidate-gene polymorphisms at previously mapped QTL by combining linkage and LD information.

In a companion paper (Yu et al. 2006) and the present study, we examine a genome-wide QTL mapping strategy using genome sequence information of recombinant inbred lines (RILs) which were generated from several crosses of parental inbreds. This QTL mapping strategy is based on the idea that the genomes of RILs are mosaics of chromosomal segments of their parental genome. Consequently, within the chromosomal segments the LD information across the parental inbreds is maintained. Thus, if diverse parental inbreds are used like in the present study, LD decays within the chromosomal segments over a short physical distance (Wilson et al. 2004). Therefore, the new mapping strategy will show not only a high power to detect QTL in genome-wide approaches but also a high mapping resolution when both linkage and LD information are used.

Yu et al. (2006) focused on the detection of QTL with additive effects. However, results from model organisms suggest that epistatic interactions among loci also contribute substantially to the variation in complex traits (Carlborg and Haley 2004; Marchini et al. 2005). The power to detect two-way interactions by using different mating designs was examined by Verhoeven et al. (2006). Furthermore, Ritchie et al. (2003) assessed the power of multifactor dimensionality reduction to detect two-way interactions. However, several studies described QTL×genetic background interactions (*e.g.*, Doebley et al. 1995; Alonso-Blanco et al. 1998), which can be caused by higher-order epistatic interactions among QTL (Jannink and Jansen 2001). Furthermore, the metabolic pathways that presumably underlie quantitative traits involve multiple interacting gene products and regulatory loci that could generate higher-order epistatic interactions (McMullen et al. 1998). Information about the power for genome-wide detection of epistatic interactions among more than two QTL is still lacking.

The objectives of our research were to (i) compare three- *vs.* four-step genome scans to identify three-way interactions

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among QTL involved in a metabolic pathway, (ii) investigate by computer simulations the power and proportion of false positives (PFP) for detecting three-way interactions among QTL in RIL populations derived from a nested mating design, and (iii) compare these estimates to those obtained for detecting three-way interactions among QTL using RIL populations derived from diallel and different partial diallel mating designs.

MATERIALS AND METHODS

Simulations

Data underlying the simulations: Our computer simulations were based on single nucleotide polymorphism (SNP) haplotype data, comprising 653 loci, of B73 and 25 diverse maize inbreds B97, CML52, CML69, CML103, CML228, CML247, CML277, CML322, CML333, Hp301, IL14H, Ki3, Ki11, Ky21, M37W, M162W, Mo18W, MS71, NC350, NC358, Oh7b, Oh43, P39, Tx303, and Tzi8. The 25 diverse inbreds were selected based on 100 simple sequence repeat markers out of a world-wide sample of 260 inbreds to capture the maximum genetic diversity (Liu et al. 2003). Details about SNP discovery, detection, and mapping were described in our companion study (Yu et al. 2006). The 26 inbreds were used to simulate the production of various RIL populations.

Examined mating designs: The nested association mapping (NAM) data set was established in accordance with the crossing scheme applied in the project "Molecular and Functional Diversity of the Maize Genome" (Yu et al. 2006). From each cross of the 25 diverse inbreds with B73, a segregating population with 200 RILs was developed. The diallel association mapping (DAM) data set DAM4875 was generated by deriving RIL populations with 15 RILs from each of the 325 crosses in the diallel (method 4; Griffing 1956) among all 26 inbreds.

The distance-based (DB) data sets DBc×r were created by selecting out of the 325 crosses in a diallel the c combinations of parental inbreds which show, based on all marker loci, the maximum genetic dissimilarity calculated according to Nei and Li (1979). For the c combinations of parental inbreds r RILs were derived from each combination. In the current study the data sets DB75×65, DB125×39, and DB195×25 were examined. For single round-robin (SRR) (Verhoeven et al. 2006), 188 RILs were derived from each of the 26 chain crosses, *i.e.*, inbred 1×inbred 2, inbred 2×inbred 3, ..., inbred 26×inbred 1. The data sets DAM900, DB25×36, DB50×18, DB100×9, DB150×6 were only examined in combination with the NAM data set and were therefore based on the 300 crosses in a diallel among the 25 diverse inbreds.

Definition of phenotypic values: For each of the simulated 50 replications, four SNPs were sampled at random from the linkage map and defined as QTL of a four-locus pathway (Figure 1). The genotypic values assigned to the inbreds were based on their allelic states at the four QTL and chosen in such a way, that a combination of complementary and duplicate molecular interaction existed among the QTL (Table 1)(Jayaram and Peterson 1990). Based on the F_∞–metric model (Yang 2004) the corresponding additive effects of QTL1, QTL2, QTL3, and QTL4 were 1.375, 0.375, 0.500, and 0.250, respectively. Furthermore, the digenic additive×additive epistatic effects QTL1*QTL2, QTL1*QTL3, QTL1*QTL4, QTL2*QTL3, QTL2*QTL4, QTL3*QTL4 were 0.375, 0.500, 0.250, 0.750, 0.250, and 0.125, respectively. Higher-order epistatic effects involving three and four QTL QTL1*QTL2*QTL3, QTL1*QTL2*QTL4, QTL1*QTL3*QTL4, QTL2*QTL3*QTL4, and QTL1*QTL2*QTL3 *QTL4 were 0.750, 0.250, 0.125, 0.625, and 0.625, respectively. Under the assumption of allele frequencies of 0.5 and linkage equilibrium among the QTL, the assumed genotypic values correspond to variances σ^2_A, σ^2_{AA}, σ^2_{AAA}, and σ^2_{AAAA} of 1.172, 0.273, 0.129, and 0.024, respectively (Wricke and Weber 1986). In the NAM data set, σ^2_A, σ^2_{AA}, σ^2_{AAA}, and σ^2_{AAAA} amounted 0.669, 0.087, 0.022, and 0.002, respectively, due to the deviations of allele frequencies from 0.5.

In a second scenario, 12 QTL, organized in three four-locus pathways, were assumed. Genotypic values of the inbreds were determined by summing up the effects caused by the individual pathways. In this scenario σ^2_A, σ^2_{AA}, σ^2_{AAA}, and σ^2_{AAAA} were 3.516, 0.820, 0.387, and 0.073, respectively, for a population with allele frequencies of 0.5, whereas for NAM data set the corresponding variances were 2.034, 0.248, 0.064, and 0.007, respectively.

The phenotypic values of the RILs were generated by adding a normally distributed variable $N(0, {\sigma_E}^2)$ to the genotypic values. The error variance was calculated as

$$\sigma^2_E = (\frac{1-h^2}{h^2}){\sigma_G}^2,$$

where ${\sigma_G}^2$ denotes the genetic variance and h^2 denotes the heritability on an entry-mean basis. Based on previous empirical studies, we examined h^2 values of 0.5 and 0.8 (Flint-Garcia et al. 2005). All simulations were performed with software Plabsoft (Maurer et al. 2004), which is implemented as an extension of the statistical software R (R Development Core Team 2004).

Statistical analyses

Due to their vast number, it is intractable to detect three-way interactions using a three-dimensional genome scan. Therefore, we used two different model selection approaches for identifying three-way interactions which considerably reduce the number of models to be evaluated during the model selection process. For both approaches PROC GLMSELECT of the statistical software SAS (SAS Institute 2005) was used.

Several authors suggested the use of information criteria such as the Akaike information criterion or Schwarz Bayesian criterion (Piepho and Gauch 2001) or modifications thereof (Baierl et al. 2006; Bogdan et al. 2004) to circumvent the problems connected with multiple likelihood-ratio tests for model selection. In preliminary simulations, however, we used the Schwarz Bayesian criterion and observed a high PFP (data not shown). Therefore, in the current study we used the model selection criteria P-to-enter and P-to-stay (Miller 2002) which allows the use of a more conservative threshold.

All SNPs, also those treated as QTL, were included in both approaches. Hence, QTL detection is not based on LD between QTL and adjacent molecular markers and, thus, the correlation structure among the RILs can be ignored.

Three-step genome-scan: The three-step genome scan applied in the current study to identify three-way interactions was based on the one-dimensional genome scan described by Jannink and

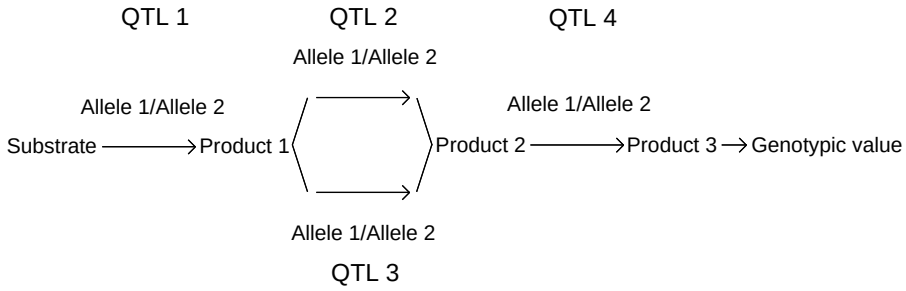

Figure 1 Metabolic pathway underlying the simulations.

Jansen (2001). In the first step, stepwise multiple linear regression (Efroymson 1960) was performed on y, the phenotypic values of the RILs, as dependent variable and w_1, w_2, ..., w_{653}, the SNP loci, and x, the affiliation of each RIL to a cross of parental inbreds, as independent variables. Independent variables showing a P-to-enter or P-to-stay < 1×10^{-8} were added or kept in the model.

Table 1 Genotypic values for the four-locus genotypes underlying the simulations.

Genotype				Genotypic value
QTL1	QTL2	QTL3	QTL4	
1	1	1	1	4
1	1	1	2	4
1	1	2	1	4
1	1	2	2	4
1	2	1	1	4
1	2	1	2	4
1	2	2	1	4
1	2	2	2	4
2	1	1	1	10
2	1	1	2	6
2	1	2	1	7
2	1	2	2	7
2	2	1	1	2
2	2	1	2	5
2	2	2	1	10
2	2	2	2	7

In addition to the variables identified in the first step, the variables $w_1\times x$, $w_2\times x$, ..., $w_{653}\times x$ were used as independent variables in the stepwise multiple linear regression of the second step, where variable selection was only performed on $w_1\times x$, $w_2\times x$, ..., $w_{653}\times x$. Variables showing a P-to-enter or P-to-stay < 1×10^{-5} were added or kept in the model.

The i variables w out of the i $w\times x$ interactions identified in the second step were used in the backward elimination procedure of the third step together with the $\binom{i}{2}$ and $\binom{i}{3}$ possible two- and three-locus interactions among them as independent variables. Variables showing a P-to-stay < 1×10^{-5} were kept in the model. The model resulting from the third step was designated as the final model.

Four-step genome-scan: Stepwise multiple linear regression was also used for the four-step genome scan, where in the first step y was used as dependent variable and w_1, w_2, ..., w_{653}, the SNP loci, as independent variables. The j loci identified in the first step were used together with the two-way interactions, which were constructed by combining the j loci with all loci, as independent variables in the stepwise multiple linear regression of the second step, where variable selection was only performed on the two-way interactions. The single loci and two-way interactions identified in the first and second step, respectively, were used together with the three-way interactions, which were constructed by combining the two-way interactions with all loci, as independent variables in the stepwise multiple linear regression of the third step. Variable selection was only performed on the three-way interactions. Only two of the three two-way interactions belonging to each three-way interaction were examined in the previous steps. To ensure the detection of three-way epistatic interactions we applied in the fourth step backward elimination on all variables contained in the model resulting from the third step and the missing two-way interactions. In this step, variable selection was only performed on the three-way interactions and the missing two-way interaction. The model resulting from the fourth step was designated as the final model. In each of the four steps, variables showing a P-to-enter or P-to-stay < 1×10^{-8} were added or kept in the model. This conservative threshold was chosen to warrant an acceptable PFP. To observe an acceptable PFP in studies based on empirical data we suggest using computer simulations to estimate the corresponding P-to-enter and P-to-stay.

The power 1-β^* to detect three-way interactions was calculated as the proportion of three-way interactions correctly identified in the final model out of the total number of three-way interactions simulated. We estimated PFP (Fernando et al. 2004) as the proportion of three-way interactions for which at least one locus is no QTL out of the total number of three-way interactions identified in the final model. Averages were calculated across the simulated 50 replications to determine 1-β^* and PFP.

RESULTS

The average map distance between the 653 SNP markers was 2.6 cM. The pairwise genetic dissimilarity among the 26 inbreds ranged from 0.58 to 0.75 (Figure 2). The average frequency of the B73 allele was 0.81 in the RILs of the NAM data set and 0.64 in the RILs of data sets DAM4875 and SRR.

In the three-step genome scan, the power and PFP to detect three-way interactions were for the NAM data set 0.05 and 0.35 (4 QTL; h^2 = 0.5), respectively (data not shown). For 12 QTL and h^2 = 0.5, the power to detect three-way interactions using

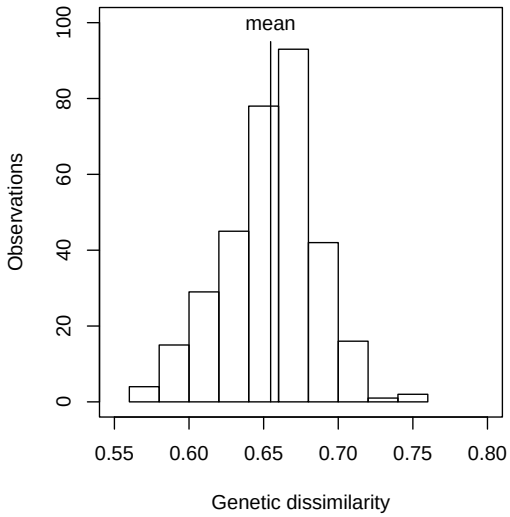

Figure 2 Distribution of pairwise genetic dissimilarities among the 26 parental inbreds based on 653 single nucleotide polymorphism markers.

the NAM data set decreased to 0.00 and PFP increased to 1.00. A power 1-β^* of 0.08 (4 QTL; h^2 = 0.5) was observed for data sets DAM4875 and DB125×39, whereas the PFP was 0.40 and 0.35, respectively.

Using the four-step genome scan, a power 1-β^* to detect three-way interactions of 0.28 (4 QTL; h^2 = 0.5) was found for the NAM data set (Table 2). In the scenario with 12 QTL, the power 1-β^* was 0.18. Lower PFP was detected for the 12 QTL scenario (0.29) than for the 4 QTL scenario (0.54). One and a fifth and one and a half times higher power estimates were observed for the 4 and 12 QTL scenario of the NAM data set, respectively, when increasing h^2 from 0.5 to 0.8.

The power 1-β^* using DAM4875, SRR, or DB data sets ranged in the four-step genome scan from 0.29 to 0.45 (4 QTL; h^2 = 0.5) and from 0.24 to 0.32 (12 QTL, h^2 = 0.5). PFP varied for these data sets between 0.39 and 0.56 (4 QTL, h^2 = 0.5). For the scenario with 12 QTL, lower PFP values were obtained.

For both levels of h^2, about one and a half times higher power estimates were observed for the combined data set of NAM and DAM900 than for NAM. For the former, PFP was for both levels of h^2 about 0.50. The power 1-β^* for combined NAM and DB data sets ranged for 4 QTL and h^2 = 0.5 from 0.27 to 0.42 and for h^2 = 0.8 from 0.33 to 0.60. For these data sets PFP varied for 4 QTL and h^2 = 0.5 between 0.46 and 0.52 and for h^2 = 0.8 between 0.51 and 0.64.

DISCUSSION

The comparison of power 1-β^* of different statistical analyses requires an equal PFP. However, model selection procedures such as those applied in the current study do not stringently adhere to a specified PFP (Miller 2002). Nevertheless, in the current study similar PFP values were obtained for all mating designs of each simulation scenario under the four-step genome scan and, thus, power estimates can be compared.

Owing to technical reasons, we were not able to ascertain similar PFP values for the four-step genome scan as for the three-step genome scan. However, differences in 1-β^* between both approaches were of such size that the four-step genome scan seems more promising for detecting epistatic QTL in the assumed metabolic pathway than the three-step genome scan irrespective of the mating design. Therefore, the comparison between the examined mating designs was based only on results of the former.

Power to detect three-way interactions under different mating designs

Despite a comparable number of RILs in the two data sets NAM and DAM4875, higher power estimates were found for the latter (Table 2). This observation is attributable to the average frequency of the B73 allele, which is closer to 0.5 for DAM4875 than for NAM. Crossing schemes resulting in RILs with an average allele frequency of 0.5 have a high power to detect QTL because the probability that some QTL haplotypes have only a very small class size is minimized (Verhoeven et al. 2006). For the detection of higher-order epistatic QTL this issue is even more important because the number of possible QTL haplotypes increases with an increasing number of QTL. But this reduces the probability that all QTL haplotypes are present in the data set.

The average frequency of the B73 allele was the same for the RILs of DAM4875 and SRR. Nevertheless, in the present study a higher power to detect three-way interactions was found for the former. This is in contrast to a result of Verhoeven et al. (2006), who observed for SRR a considerably higher power to detect epistatic QTL than for the same number of RILs derived from a diallel. The different findings can be explained by the different assumptions underlying the simulations. Verhoeven et al. (2006) assumed a distinct allele for each parental inbred. In this case large numbers of small populations show, due to the increased probability that some QTL haplotypes have only very small class size, a lower power to detect epistatic QTL than a small number of large populations. However, the assumption made by Verhoeven et al. (2006) ignores the consequences of genetic drift that for real data not all QTL segregate in every population (Xu 1996). In the present study this fact was considered by using SNP haplotype data of 26 inbreds as a basis of the simulations. Consequently, the mating designs resulting in a large number of small populations have indeed the above mentioned disadvantage but this is compensated by the large number of individuals within populations segregating for the QTL.

The probability that QTL are segregating is increased in individual line crosses by using parental inbreds which are phenotypically the opposite extremes for the trait of interest (Xu 1998). However, this may not be helpful for detecting QTL for other traits. Furthermore, results of Burkhamer et al. (1998) suggest that inbreds showing a large genetic distance based on molecular markers also strongly differ in their alleles at QTL.

Table 2 Power to detect three-way interactions (1-β^*) and proportion of false positives (PFP) using the four-step genome scan for different mating designs to establish recombinant inbred lines (RIL): Nested (NAM), diallel (DAM), single round-robin (SRR), and distance-based (DB) designs.

		Crite-	4 QTL		12 QTL	
Mating design	no. RIL	rion	$h^2 = 0.5$	$h^2 = 0.8$	$h^2 = 0.5$	$h^2 = 0.8$
NAM	5000	1-β^*	0.28	0.33	0.18	0.29
		PFP	0.54	0.64	0.29	0.41
DAM4875	4875	1-β^*	0.44	0.54	0.30	0.54
		PFP	0.44	0.46	0.16	0.30
SRR	4888	1-β^*	0.29	0.38	0.24	0.34
		PFP	0.56	0.51	0.26	0.47
DB75×65	4875	1-β^*	0.38	0.41	0.29	0.49
		PFP	0.47	0.63	0.19	0.37
DB125×39	4875	1-β^*	0.45	0.56	0.31	0.54
		PFP	0.39	0.56	0.19	0.35
DB195×25	4875	1-β^*	0.45	0.58	0.32	0.57
		PFP	0.45	0.64	0.17	0.34
NAM & DAM900	5900	1-β^*	0.40	0.60		
		PFP	0.47	0.51		
NAM & DB25×36	5900	1-β^*	0.27	0.33		
		PFP	0.52	0.64		
NAM & DB50×18	5900	1-β^*	0.38	0.53		
		PFP	0.46	0.58		
NAM & DB100×9	5900	1-β^*	0.42	0.60		
		PFP	0.46	0.51		
NAM & DB150×6	5900	1-β^*	0.39	0.57		
		PFP	0.46	0.59		

Therefore, we examined the DB approach by using only those parental combinations of the diallel to establish RILs which show, based on all marker loci, the maximum genetic dissimilarity. A higher power to detect three-way interactions was observed for DB125×39 and DB195×25 than found for NAM, SRR, and DAM4875. This indicated that the DB design is promising to increase the probability that QTL are segregating in populations.

Nevertheless, we observed for DB75×65 a lower power to detect three-way interactions than for DB125×39 and DB195×25. The opposite result was expected, based on the average genetic dissimilarity among the parental inbreds and the higher number of RILs per segregating population (Xie et al. 1998). Presumably, the reason for our observation is the insufficient sampling of QTL alleles of the base population if the number of selected parental combinations is too low (Muranty 1996; Wu and Jannink 2004).

In summary, the results of our study indicated that for a genetic dissimilarity among the parental inbreds as that observed in the current study the crossing schemes underlying the data sets DB125×39 and DB195×25 are the most promising designs to detect three-way interactions. However, our results also suggest, that only RILs derived from optimally allocated DB designs show an increased power to detect three-way interactions. Nevertheless, the project "Molecular and Functional Diversity of the Maize Genome" applies the crossing scheme underlying the NAM data set to construct RIL populations. The reasons are: (i) The common reference parent B73 has been the subject of extensive genetic and genomic studies (*e.g.*, Morgante et al. 2005). (ii) Crossing the 25 diverse inbreds to the well-adapted inbred B73 facilitates both the development and the phenotypic evaluation of RILs.

Factors influencing power and FDR to detect higher-order epistatic QTL in NAM

Genetic architecture of the trait: An higher power 1-β^* of detecting three-way interactions was observed with a single pathway influencing the phenotypic trait than with three pathways influencing the phenotypic trait (Table 2). This is due to the increased proportion of variance explained by a single pathway. Thus, if pathways explain unequal proportions of the genotypic variance, the power to detect epistatic interactions is higher for pathways explaining a large proportion of the genotypic variance than for pathways explaining a small proportion of the genotypic variance. Higher PFP was observed for the one pathway scenario than for the three pathway scenario. This finding can be explained by the higher power to detect three-way interactions in the former than in the latter scenario.

In the present study the genotypic values for the four-locus genotypes were arranged in such a way that they are interpretable as molecular interactions among genes (Figure 1) (Jayaram and Peterson 1990). Nevertheless, for each individual pathway genic effects of four single loci, six two-way interactions, four three-way interactions, and one four-way interaction can be estimated (Yang 2004). In addition, the variances σ^2_A, σ^2_{AA}, σ^2_{AAA}, and σ^2_{AAAA} can be calculated. The variances σ^2_A and σ^2_{AA} determined in the current study under the assumption of allele frequencies of 0.5 agree well with those reported by Wolf et al. (2000) for various traits in an F_2 maize population. Thus, we conclude that the assumptions underlying our simulations were realistic.

For pathways consisting of k QTL and showing mono-, di-,

and trigenic effects similar to those of the current study, a higher power to detect three-way interactions is expected if $k = 3$. This is because a lower number of genic effects is influencing the genotypic value and, thus, each single genic effect is explaining a higher proportion of the genotypic variance. The opposite is expected for $k > 4$.

Detection method: For the detection of three-way interactions, the four-step genome scan requires that the genic effect of at least one single locus, and one two-way interaction are different from zero in addition to the genic effect of the three-way interaction. In contrast, using the three-step genome scan for the detection of three-way interactions the main effect of the QTL does not matter and only the interaction effect of all three QTL with the genetic background must be different from zero. Epistatic interactions among QTLs cause QTL×genetic background interactions (Jannink and Jansen 2001) and, thus, the effect of the latter increases in the present study with the size of the genic effects of two-, three-, and four-way interactions. Therefore, for both QTL detection approaches a higher power to detect three-way interactions is expected if genotypic values are assumed for the four-locus genotypes such that all genic effects have a higher absolute value than in the current study. The opposite result is expected if all genic effects show a lower absolute value than in the current study.

Different trends of the power to detect three-way interactions are expected for the two QTL detection approaches, if all genic effects of at least one of the steps, single locus or two-way interactions, decrease. While a strong reduction in power to detect three-way interactions is expected by using the four-step genome scan, only a weak reduction is expected for the three-step genome scan. In the extreme, if all genic effects of one of these steps are zero, three-way interactions can only be detected by using the three-step genome scan. But this case is very unlikely (Marchini et al. 2005). However, further research is needed concerning the most promising detection method for epistatic interactions under different genetic architectures.

Probability of QTL segregation: The probability of QTL segregation is influenced by the (i) average and (ii) variance of the genetic dissimilarity among the parental inbreds of the RILs. The higher the average genetic dissimilarity among the parental inbreds, the higher the power to detect three-way interactions for RILs derived from all mating designs. For a low average genetic dissimilarity the opposite result is expected. The optimal number of populations and RILs per population is not influenced by the average genetic dissimilarity among the parental inbreds. In contrast, the higher the variance of the genetic dissimilarity among the parental inbreds, the larger the difference in power 1-β^* between mating designs considering genetic dissimilarities, like DB designs, and mating designs neglecting this information. For a low variance of the genetic dissimilarity the opposite result is expected.

Genetic map distance between marker loci and QTL: An increased power to detect three-way interactions and a reduced PFP are expected if RILs are genotyped for a high number of SNPs. This is attributable to the increased probability of substantial LD between QTL and marker loci (Marchini et al. 2005). In the current study all SNPs, also those treated as QTL, were included in the statistical analyses. This implies that markers are available for the QTL itself, which is true if the genome sequence of all RILs is known. Due to the fast progress of genome sequencing techniques (Churchill et al. 2004; Shendure et al. 2004) this is a realistic assumption which maximizes the power to detect three-way interactions. In comparison with results of the current study, a lower power and a higher PFP to detect three-way interactions are expected for studies with unknown genome sequence of the RILs.

Heritability of the trait: Increasing h^2 from 0.5 to 0.8 resulted for all examined data sets and both numbers of QTL in a considerably higher power to detect three-way interactions. This is because for $h^2 = 0.8$ the environmental influence on the phenotypic trait is reduced in comparison with $h^2 = 0.5$. Hence, increasing h^2 by conducting field experiments with several replications in several environments is a promising approach to increase the power to detect three-way interactions. However, in studies with a fixed budget this implies a reduction of the number of RILs to be tested in field experiments. Further research is needed concerning the optimal allocation of resources with respect to the number of RILs and the intensity of their phenotypic evaluation.

Number of examined RILs: Up to now, published QTL mapping experiments with replicated trials mostly employed between 100 and 200 progenies (Melchinger et al. 1998). Experiments of this size have a low power to detect epistatic QTL (Mihaljevic et al. 2005). However, in the present study the power to detect three-way interactions with 5000 RILs derived from a nested desgn was relatively high for both the 4 QTL and the 12 QTL scenario. Also the observed PFP of about 0.54 (4 QTL) and 0.29 (12 QTL) was at an acceptable level considering the complex genetic architecture. Nevertheless, near isogenic lines should be used to validate the identified epistatic interactions. For the validation of each identified three-way interaction, eight near isogenic lines are required.

For detection of three-way interactions in pathways being more complex than that of the current study, the NAM data set must be complemented with additional RILs. Furthermore, our results suggest that the optimally allocated DB approach is more appropriate for complementing the NAM data set than deriving the additional RILs from a diallel.

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7. General Discussion

LD is the non-random association of alleles at different loci (Flint-Garcia et al. 2003). Association mapping uses the LD between DNA markers and sequence variants underlying the trait, which is present in a set of germplasm. The applicability and resolution of association mapping in the germplasm set under consideration can be explored by examining the extent of LD among DNA markers. However, to interpret results of empirical studies of LD knowledge about the properties of the applied measures of LD is of crucial importance.

Measures of linkage disequilibrium

The two classical measures of LD are r^2 and D', which consider only biallelic loci (Hill and Robertson 1968; Lewontin 1964). An extension of r^2 to multiallelic loci is R (Maruyama 1982):

$$R = \frac{\sum_{i=1}^{a_i} \sum_{j=1}^{b_j} D_{ij}^2}{(1 - \sum_{i=1}^{a_i} p_i^2)(1 - \sum_{j=1}^{b_j} p_j^2)},$$

where a_i and b_j are the number of alleles occuring at locus A and B, respectively, p_i and p_j are the frequencies of the ith and jth allele at locus A and B, respectively, and $D_{ij} = p_{ij} - p_i p_j$ is the deviation between the

observed frequency p_{ij} of gamete A_iB_j and its expected frequency p_ip_j, assuming no statistical association between the alleles (Lewontin and Kojima 1960). Furthermore, D'_m is the extension of D' to multiallelic loci (Hedrick 1987):

$$D'_m = \sum_{i=1}^{a_i} \sum_{j=1}^{b_j} p_i p_j |D'_{ij}|,$$

where $|D'_{ij}|$ is the absolute value of Lewontin's normalized measure D'_{ij} (Lewontin 1964) with $D'_{ij} = \frac{D_{ij}}{D_{ij}^{max}}$, where D_{ij}^{max} is the maximum amout of disequilibrium possible between the ith allele at locus A and the jth allele at locus B that equals:

$$D_{ij}^{max} = \begin{cases} min(p_ip_j, (1-p_i)(1-p_j)), & \text{if } D_{ij} < 0 \\ min(p_i(1-p_j), (1-p_i)p_j), & \text{otherwise.} \end{cases}$$

The measures R and D'_m reflect different aspects of LD and perform differently under various conditions. R summarizes both recombinational and mutational history and can be interpreted as the squared correlation coefficient between the allele frequencies at locus A and B (Devlin and Risch 1995). In contrast, D'_m measures only the recombinational history between two loci (Jorde 2000) and is therefore the more accurate statistic for estimating recombination differences (Flint-Garcia et al. 2001). With each generation of random mating using a population of infinite size, the rate of decay is $(1-c)^2$ for R and $(1-c)$ for D'_m, where c is the recombination frequency between locus A and B (Hedrick 1987). R is affected by the allele frequencies and the number of alleles at locus A and B whereas D'_m is unaffected by these factors (Devlin and Risch 1995).

Both LD measures R and D'_m estimate the amount of LD present between a pair of loci. However, for assessing the feasibility of association mapping the statistical significance of the LD is more important than its actual amount of LD (Maurer et al. 2006). Therefore, Fisher's exact test, which tests association between alleles of two loci, seems to provide an appropriate tool for this purpose.

The percentage of loci pairs in significant LD, determined with Fisher's exact test, depends strongly on the population size. This is because the power to detect significant LD increases with increasing sample size. To obtain comparable power for detecting significant LD in populations of different size, we proposed the following procedure: estimate the percentage of loci pairs in significant LD in a random sample that was drawn from each population using a sample size equal to the population size of smallest population under consideration. This procedure was repeated k times and the results were averaged. High numbers must be chosen for k if the original populations are large and/or the smallest population comprises only a low number of individuals. The reason being the high number of possible, different samples in this situation. The above resampling strategy was applied in our studies on LD.

Using elite breeding germplasm for association mapping

As long as statistical differences can be detected among the phenotypic values of elite breeding germplasm this can be successfully applied for QTL detection using association mapping methods. However, the use of elite breeding germplasm for association mapping has several disadvantages in comparison with the use of diverse germplasm. Selection applied during the development of elite breeding germplasm from it's base population leads to the fixation of QTL. However, these QTL cannot be detected using elite breeding germplasm. Furthermore, the fixed QTL are those explaining a large proportion of the genetic variation of the traits underlying selection (Yin et al. 2003). In addition, because of the narrow genetic base of elite breeding germplasm a slow decay of LD along the chromosomes (Ching et al. 2002) and therewith a low resolution of QTL detection will be observed for association mapping approaches using elite breeding germplasm.

The use of elite breeding germplasm for association mapping approaches also has several advantages. The high extent of LD in elite breeding germplasm facilitates genome-wide QTL detection with a low number of DNA markers. In addition, elite breeding germplasm is phenotyped in plant breeding programs with high effort as a basis for selection decisions. Furthermore, in many plant breeding programs also genotypic data from genome-wide distributed DNA markers are collected routinely for various reasons such as plant variety protection or choice of parents for establishing new base populations. Consequently, both the pheno- and genotypic data are available for QTL detection at no extra costs (Rafalski 2002). Therefore, association mapping using data from plant breeding programs is an appealing way for plant breeders to detect QTL (Jannink et al. 2001).

Causes of linkage disequilibrium

Association mapping was advocated in human genetics as the method of choice to detect and estimate the effects of novel genes (Risch and Merikangas 1996). Major differences regarding the forces generating and conserving LD are expected between human and plant breeding populations. Therefore, plant breeding populations must be characterized with respect to the forces generating and conserving LD before association mapping methods can be applied in a plant breeding context.

Our theoretical considerations about the number of effective crossovers which occurred in the European flint and dent germplasm groups since their establishment in the 1950s (Schnell 1992) indicated that significant LD due to linkage can be expected in these germplasm groups between DNA marker loci separated by less than 14.5 cM. Therefore, the AFLP and SSR marker densities underlying our empirical studies on LD are expected to be sufficient for genome-wide association mapping. Furthermore, no significant LD is expected between DNA markers separated by more than 14.5 cM.

The observed high proportion of unlinked AFLP and SSR loci in significant LD suggest other forces generating LD in addition to linkage. The results of our computer simulations indicated that mutation reduces the extent of significant LD between DNA markers in plant breeding populations. Furthermore, the results of our computer simulations indicated that selection is an unlikely cause for LD between DNA markers showing a map distance of 35 cM or more. However, our results suggested that in plant breeding populations selection may cause LD between DNA markers which are tightly linked to QTL. Based on empirical data and computer simulations we found genetic drift being an important cause for LD between linked and unlinked loci. The results of our empirical studies on LD further suggested that relatedness and population stratification are important forces generating LD between DNA markers in plant breeding populations.

Prospects of genome-wide association mapping with AFLPs and SSRs

The favorite DNA marker system for genome-wide association mapping is expected to show a high correlation coefficient between the P value of the LD test and the genetic map distance between DNA marker loci as well as a high power to detect LD. However, we observed no difference between the correlation coefficient of the AFLP and SSR markers. The results of our study indicated that the power in association mapping depends on several factors such as (i) the average map distance and (ii) the number of alleles of the DNA marker loci. Under the assumption of a fixed budget, AFLPs are superior to SSRs with respect to the first criterion as a higher marker density can be generated with AFLPs than with SSRs. However, our findings show that with respect to their power of detecting LD AFLPs are only marginally superior to SSRs, for which the number of alleles of each SSR locus was reduced to two. This is because the genome coverage provided by both types of markers is expected to be sufficient for genome-wide association mapping.

With respect to the number of alleles per marker locus, however, our results showed a clear advantage for SSRs.

In conclusion, SSRs should be favored over AFLPs in populations with a short history of recombination, because then one can take advantage of their higher power to detect LD. In contrast, in populations with a long history of recombination, for which no LD is expected between pairs of SSR markers, and under the assumption of a fixed budget, AFLP markers should be favored over SSRs.

Definition of false positive associations

In association mapping studies typically individual DNA markers are tested for their association with the phenotypic trait under consideration using a certain biometric approach. Different null and alternative hypotheses are underlying the various association tests. For example, the null hypothesis of the LRRT is that the DNA marker under consideration is independent of the phenotypic trait, whereas under the alternative hypothesis the DNA marker is associated with the phenotype (Thornsberry et al. 2001). In contrast, the null hypothesis of the QIPDT is that the DNA marker under consideration is independent of the phenotypic trait, whereas under the alternative hypothesis the DNA marker is associated with the phenotype and that this association is due to linkage between the DNA marker under consideration and a QTL (Martin et al. 2000). Thus, the null hypothesis using QIPDT should be true for a DNA marker which is unlinked to a QTL. However, if for such a DNA marker the alternative hypothesis is true, this is a false positive association.

In our computer simulations on QIPDT we defined a false positive association as a significant association detected between a QTL and a DNA marker, when at least one DNA marker locus located between them showed

a nonsignificant association with the phenotypic trait. This definition of a false positive association based on statistical tests for adjacent DNA markers may cause an increased rate of false positives, because for some DNA markers a type I error is made only because of a type II error at adjacent DNA markers. In our simulations on QIPDT, however, no increased rate of false positive associations was observed. Nevertheless, the definition of a false positive association between a DNA marker and a phenotypic trait based on a threshold for the map distance between the DNA marker and the QTL, *i.e.*, independent of statistical tests for adjacent DNA markers, is more appropriate than the definition based on statistical tests.

Association mapping using data routinely collected in plant breeding programs

So far, in plant genetics the LRRT has been applied as a population-based association mapping method accounting for population stratification (Pritchard et al. 2000b; Thornsberry et al. 2001). Because LRRT does not correct for LD caused by relatedness it requires independent genotypes. Ignoring the assumption of independence results in a type I error rate higher than the nominal one (Pritchard and Rosenberg 1999). This was observed by Thornsberry et al. (2001) when applying LRRT to a world-wide sample of partially related maize inbred lines. The rejection rate at SSR markers, for which no association with the QTL was expected, was higher (0.081) than the nominal α level of 0.05.

In plant breeding populations, all inbreds trace back to common ancestors and, thus, are half sibs or full sibs of some independent genotypes. Therefore, the above property of the LRRT limits its use for QTL mapping using routinely collected data. We adapted the QPDT (Zhang et al. 2001), a family-based association test, to typical pedigrees of inbred lines produced in

plant breeding programs. The newly developed QIPDT allows exploitation of routinely collected data for QTL detection.

To approach a comparison of QIPDT and LRRT in a scenario in which LD is generated by the same forces as those observed for European elite maize germplasm, we based our computer simulations on the 55 years of hybrid maize breeding in Central Europe. In contrast to QIPDT, LRRT did not adhere to the nominal α level of 0.05 even if independent inbreds were analyzed. This is because LRRT does not account for LD due to genetic drift, which is most likely active in plant breeding populations. In contrast, the QIPDT is a valid test of association caused by linkage and therefore adheres by all means to the nominal α level. Furthermore, in all simulation scenarios except one, QIPDT yielded higher power estimates than LRRT. Therefore, our results suggested that the QIPDT is superior to the LRRT for genome-wide association mapping using data routinely collected in plant breeding programs.

Recently, Yu et al. (2006b) proposed a new population-based association mapping approach called unified mixed-model method. This method accounts for multiple levels of relatedness as detected by random genetic markers. The mixed-model equation can be expressed as:

$$y = X\beta + S\alpha + Qv + Zu + e,$$

where y is a vector of phenotypic observations; β is a vector of fixed effects other than DNA markers or population group effects; α is a vector of effects of the DNA markers to be tested for association; v is a vector of population effects; u is a vector of polygene background effects, e is a vector of residual effects; Q is a matrix from STRUCTURE (Pritchard et al. 2000a) relating y to v; and X, S, and Z are incidence matrices of 1s and 0s relating y to β, α, and u, respectively. The variances of the random effects are assumed to be $\mathrm{Var}(u) = 2KV_g$, and $\mathrm{Var}(e) = RV_r$, where K is an $n \times n$ matrix of relative kinship coefficients that define the degree of genetic covariance between a pair of individuals. The relative kinship coefficients were estimated from

genom-wide distributed DNA markers. R is an $n \times n$ matrix in which the off-diagonal elements are 0 and the diagonal elements are the reciprocal of the number of phenotypic observations available for each individual. V_g is the genetic variance and V_r is the residual variance. Best linear unbiased estimates of β, α, and v and best linear unbiased predictions of u were obtained by solving the mixed-model equations (Henderson 1984; Kennedy et al. 1992).

In contrast to LRRT, dependent inbreds can be included in association mapping approaches using the unified mixed-model method without increasing the type I error rate. Therefore, a higher power to detect QTL is expected for the latter than for the former when using data routinely collected in plant breeding programs. However, a comparison of the unified mixed-model method and the QIPDT with respect to their power to detect QTL in a plant breeding context is still lacking. The unified mixed-model method is a valid test of association, however, in contrast to QIPDT it does not test if an association is due to linkage. Therefore, an increased type I error rate might be observed for the latter when examining data from plant breeding programs. Further research is needed for developing mixed-model methods which are not based on the Q matrix. This is because the establishment of the Q matrix using STRUCTURE (Pritchard et al. 2000a) requires a high computational effort and the estimation of the number of subgroups is ambiguous (Kraakman et al. 2004). In addition, the information content of the two matrices Q and K is expected to be highly correlated, because both are calculated from the same DNA markers.

Nested association mapping and diallel association mapping

The QIPDT is appropriate for joint linkage and association mapping. Because this test requires genotypic information of parental inbreds it is a suitable test for inbreds from plant breeding programs. However, as mentioned

before, such germplasm has several disadvantages for association mapping. Therefore, in a companion paper (Yu et al. 2006a) and our study a genome-wide QTL mapping strategy was proposed which can be used for joint linkage and association mapping in diverse germplasm. This approach is based on genome sequence information of RILs which were generated from several crosses of parental inbreds (Fig. 1). The new QTL mapping strategy simultaneously exploits the merits of linkage mapping and association mapping which are (i) high power to detect QTL in genome-wide approaches and (ii) high resolution to detect QTL.

By crossing two parental inbreds, complete LD is generated in the F_1 individual between all loci being polymorphic between the parental inbreds. When developing RILs from the F_1 individual, the extent of LD is reduced by recombination. Therefore, the genome of the RILs, which were derived from the same cross, consist of segments in LD, whereas no LD is present between these segments. Such a population can be used for linkage mapping. Because only a low number of recombinations occurred during the establishment of the RILs, the resolution of QTL detection will be low.

The resolution of QTL detection will be high if the RILs of several RIL populations, which were generated from a certain number of parental inbreds, were analyzed together. This is because across the populations, the segments in LD are short. Furthermore, we examined the extreme case in which the RIL populations were developed from a set of 25 diverse maize inbreds, which were selected based on 100 SSR markers out of a world-wide sample of 260 inbreds to maximally capture the genetic diversity (Liu et al. 2003). Because of the fact that for a set of diverse inbreds LD decays rapidly along the chromosomes (Wilson et al. 2004), an extremly short range LD will be observed for the RILs which underly our study. Therefore, beside a high power to detect QTL in genome-wide approaches the QTL mapping strategies nested association mapping (NAM) and diallel association mapping (DAM) have a mapping resolution, which is comparable to those of association mapping populations comprising diverse germplasm.

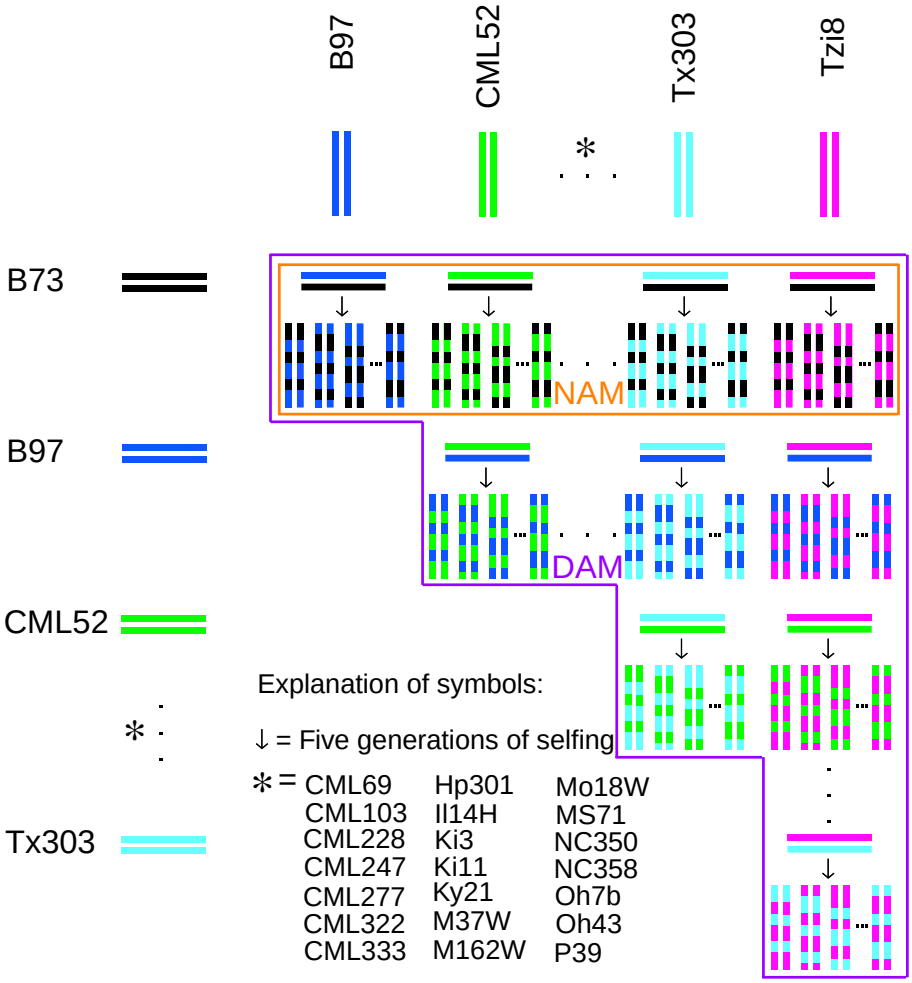

Figure 1. Crossing schemes underlying the nested association mapping (NAM) strategy and diallel association mapping (DAM) strategy.

Confirmation of QTL detected in association mapping approaches

Association mapping was proposed by Meuwissen and Goddard (2000) to confirm QTL, which were detected in linkage mapping experiments, across different genetic backgrounds. However, even the validation of earlier identified associations between DNA markers within candidate-genes and phenotypic traits using association mapping methods was impossible (*e.g.*, Thorns-

berry et al. 2001; Andersen et al. 2005). This failure of validation may be due to gene×environment interactions or gene×genetic background interactions. A further reason for this failure may be a low power to detect DNA marker-phenotype associations in the validation population. This lack of power may be due to a small experiment size or a low heritability on an entry-mean basis of the phenotypic values. Nevertheless, the observation of non-repeatable marker-phenotype associations may be due to spurious marker-trait associations in the initial population caused by statistical overinterpretation of study results (Munafó and Flint 2004), incorrect assumptions about the underlying genetic architecture (Cardon and Bell 2001), and LD between unlinked DNA marker-trait coding locus pairs generated by population stratification, relatedness, genetic drift, or selection. Because of these spurious DNA marker-phenotype associations and the fact that association studies provide only statistical, *i.e.*, indirect evidence for the function of the identified genome region (Andersen and Lübberstedt 2003), a direct proof of allele function is necessary.

Different methods for validation must be applied depending on the length of the genome region identified to be associated with the phenotype. The generation of near isogenic lines or heterogeneous inbred families (Borevitz and Chory 2004) by crossing individuals are appropriate methods for a direct proof of allele function if the length of the genome region to be validated is one to several cM. Near isogenic lines contain a small introgressed fragment in an isogenic background, whereas heterogeneous inbred families are derived from a single recombination inbred line that segregates at a single QTL region in an inbred background consisting of a mixture of the two parents.

Also the direct proof of allele function of DNA markers within candidate-genes can be obtained be comparing isogenic genotypes. However, transferring one DNA marker within a candidate-gene into an isogenic background but not adjacent DNA markers within the same candidate-gene is impossible by crossing individuals, the reason being the extremely low probability of recombination between the DNA markers within a candidate-gene in a single

meiosis. However, in this case isogenic genotypes can be produced by (i) induced mutation followed by screening of large populations by targeting-induced local lesions in genomes (TILLING) or (ii) homologous recombination.

TILLING was developed in *Arabidopsis thaliana* for efficient screening of ethylmethane sulfonate induced mutations (McCallum et al. 2000). Consequently, an allelic series of mis-sense mutations can be obtained for each target gene. The method is currently being transferred to crop plants (Andersen and Lübberstedt 2003). Alternatively, transformation of allelic series into isogenic background using homologous recombination-based, locus-targeted integration of alleles can confirm the function of individual sequence motifs (Hanin and Paszkoski 2003). Recently, Shaked et al. (2005) reported that expression of a yeast chromatin remodeling gene enhances gene targeting in *Arabidopsis thaliana* to 10^{-2}. This level of gene targeting frequency will allow the efficient DNA integration by homologous recombination (Puchta and Hohn 2005). Nevertheless, there are likely to be major hurdles ahead until this technique can be applied in crop plants.

Conclusions

With association mapping methods, detection of DNA markers closely linked to QTL requires LD caused by linkage being present between QTL and DNA markers. The results of our study suggested that in European elite maize germplasm LD attributable to physical linkage can be expected between DNA markers spaced at not more than 14.5 cM. However, our results also suggested that relatedness, population stratification, genetic drift, and selection are causes for LD in plant breeding programs. Because the existing population-based association tests do not correct for LD caused by the latter two forces, an increased type I error rate will be observed if these tests are applied to plant breeding populations. However, the familiy-based association test QIPDT developed in our study is a valid test of association due to

linkage and therefore adheres by all means to the nominal α level. Furthermore, our results suggested that the QIPDT is a promising QTL detection method for data routinely collected in plant breeding programs. Besides a high power to detect QTL in genome-wide approaches the genome-wide QTL mapping strategies NAM and DAM, which were proposed in our study, have a mapping resolution, which is comparable to those of association mapping populations comprising diverse germplasm.

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8. Summary

Linkage mapping has become a routine tool for the identification of quantitative trait loci (QTL) in plants. An alternative, promising approach is association mapping, which has been successfully applied in human genetics to detect QTL coding for diseases. The objectives of this research were to examine the feasibility of association mapping in elite maize breeding populations and develop for this purpose appropriate biometric methods.

The feasibility of association mapping depends on the extent of linkage disequilibrium (LD) as well as on the forces generating and conserving LD in the population under consideration. The objectives of our studies were to (i) examine the extent and genomic distribution of LD between pairs of simple sequence repeat (SSR) marker loci, (ii) compare these results with those obtained with amplified fragment length polymorphism (AFLP) markers, and (iii) investigate the forces generating and conserving LD in plant breeding populations. Our studies were based on experimental data of European elite maize inbreds as well as on computer simulations modeling the breeding history of the European flint heterotic group.

The experimental results on European elite maize germplasm suggested that the extent of LD between SSR markers as well as AFLP markers are encouraging for the detection of marker-phenotype associations in genome-wide scans. In populations with a short history of recombination, SSRs are advantageous over AFLPs in that they have a higher power to detect LD. In contrast, in populations with a long history of recombination, for

which no LD is expected between pairs of SSR markers, AFLP markers should be favored over SSRs because then their higher marker density that is generated with a fixed budget can be used. Furthermore, the results of our experimental and simulation studies indicated that not only physical linkage is a cause of LD in plant breeding populations, but also relatedness, population stratification, genetic drift, and selection.

So far, in plant genetics the logistic regression ratio test (LRRT) has been applied as a population-based association mapping approach. However, this test does only correct for LD caused by population stratification. The objectives of the presented study were to (i) adapt the quantitative pedigree disequilibrium test to typical pedigrees of inbred lines produced in plant breeding programs and (ii) compare the newly developed quantitative inbred pedigree disequilibrium test (QIPDT) and the commonly employed LRRT with respect to the power and type I error rate of QTL detection. This study was based on computer simulations modeling the breeding history of the European maize heterotic groups.

In QIPDT the power of QTL detection was higher with 75 extended pedigrees than in LRRT with 75 independent inbreds. Furthermore, while the type I error rate of LRRT surpassed the nominal α level, the QIPDT adhered to it. These results suggested that the QIPDT is superior to the LRRT for genome-wide association mapping if data collected routinely in plant breeding programs are available.

Epistatic interactions among QTL contribute substantially to the genetic variation in complex traits. The main objectives of our study were to (i) investigate by computer simulations the power and proportion of false positives for detecting three-way interactions among QTL involved in a metabolic pathway in populations of recombinant inbred lines (RILs) derived from a nested design and (ii) compare these estimates to those obtained for detecting three-way interactions among QTL in RIL populations derived from diallel and different partial diallel mating designs. The computer simulations of this

study were based on single nucleotide polymorphism haplotype data of 26 diverse maize inbreds.

The power and proportion of false positives to detect three-way interactions with 5000 RILs derived from a nested design was relatively high for both the 4 QTL and the 12 QTL scenario. Higher power to detect three-way interactions was observed for RILs derived from optimally allocated distance-based designs than for RILs derived from a nested or diallel design.

Our results suggested that association mapping methods adapted to the special features of plant breeding populations have the potential to overcome the limitations of classical linkage mapping methods.

9. Zusammenfassung

In der Pflanzengenetik ist die Kopplungsanalyse das Standardwerkzeug um Genloci zu identifizieren, die für quantitative Merkmale kodieren (QTL). Ein alternativer und vielversprechender Ansatz, der in der Humangenetik bereits erfolgreich dazu eingesetzt wurde, um QTL für Mukoviszidose und Alzheimer-Krankheit aufzufinden, ist die Assoziationskartierung. In der vorliegenden Arbeit werden die Anwendbarkeit von Assoziationskartierungsmethoden zum Auffinden von QTL in Maiselitezüchtungsmaterial untersucht sowie für diesen Zweck geeignete biometrische Methoden entwickelt.

Ob Assoziationskartierungsansätze auch in Pflanzenzüchtungspopulationen zum Auffinden von QTL eingesetzt werden können, hängt vom Ausmaß des Gametenphasenungleichgewichtes (GPU) in der interessierenden Population ab. Des weiteren wird die Anwendbarkeit von Assoziationskartierungsmethoden von den Kräften beeinflußt, die in der betreffenden Population GPU verursachen und aufrechterhalten. Die Ziele unserer Studien waren (i) die Erfassung des Ausmaßes und der genomweiten Verteilung des GPU zwischen Mikrosatelliten (SSR) Markern, (ii) der Vergleich dieser Ergebnisse mit denjenigen für Amplifizierte-Fragment-Längen-Polymorphismus (AFLP) Markern sowie (iii) die Untersuchung von Kräften, die in Maiselitezüchtungsmaterial GPU verursachen und aufrechterhalten. Unsere Studien basierten sowohl auf experimentellen Daten von europäischen Maiseliteinzuchtlinien als auch auf Computersimulationen.

Unsere Ergebnisse legen nahe, dass in europäischem Maiselitezüchtungsmaterial das Ausmaß an GPU sowohl zwischen SSR Markern als auch zwischen AFLP Markern ausreichend ist, um mit Hilfe der Assoziationskartierung genomweit QTL auffinden zu können. Da die Güte von SSR Markern GPU aufzufinden höher ist als diejenige von AFLP Markern, sind in Populationen mit kurzer Rekombinationsgeschichte SSR Marker geeigneter für Assoziationskartierungen als AFLP Marker. In Populationen mit langer Rekombinationsgeschichte, für die kein signifikantes GPU zwischen SSR Markern zu erwarten ist, sind AFLP Marker geeigneter für Assoziationskartierungen, da in diesem Fall ihre mit gleichem finanziellem Aufwand erzeugte höhere Markerdichte genutzt werden kann. Die Ergebnisse unserer experimentellen Untersuchungen und Simulationsstudien deuten darauf hin, dass in Pflanzenzüchtungspopulationen nicht nur physikalische Kopplung eine Ursache für GPU ist, sondern auch Verwandschaft, Populationsstratifizierung, genetische Drift und Selektion.

Der Assoziationstest, der bislang in der Pflanzengenetik eingesetzt wurde, ist der "logistic regression ratio test" (LRRT). Dieser Test korrigiert allerdings nur für GPU, das durch Populationsstratifizierung verursacht wird. Aus diesem Grund wird erwartet, dass der LRRT das α-Niveau nicht einhält, wenn er zur Assoziationskartierung in Populationen eingesetzt wird, in denen GPU vorhanden ist, das durch Verwandschaft, genetische Drift oder Selektion verursacht wird. Die Ziele unserer Studie waren (i) den "quantitative pedigree disequilibrium test" so zu modifizieren, dass er zur Assoziationskartierung in Pflanzenzüchtungspopulationen eingesetzt werden kann und (ii) den neuentwickelten Test (QIPDT) hinsichtlich seiner Güte und Typ I Fehlerrate bei der Detektion von QTL zu untersuchen und mit dem bislang eingesetzten LRRT zu vergleichen. Diese Studie basierte auf Computersimulationen.

Wir beobachteten für den QIPDT eine höhere Güte, QTL aufzufinden, als für den LRRT, wenn Daten verwendet wurden, die routinemäßig in Pflanzenzüchtungsprogrammen erhoben wurden. Im Gegensatz zum LRRT

hielt der QIPDT stets das α-Niveau ein. Diese Ergebnisse belegen, dass der QIPDT zur genomweiten Assoziationskartierung mit Daten, die routinemäßig in Pflanzenzüchtungsprogrammen erhoben werden, geeigneter ist als der LRRT.

Epistatische Interaktionen zwischen QTL tragen wesentlich zur genetischen Variation komplexer Merkmale bei. Die Ziele unserer Studie waren (i) Populationen rekombinanter Inzuchtlinien (RIL), die von einem geschachtelten Kreuzungsschema abgeleitet wurden, hinsichtlich ihrer Güte und Rate an falsch Positiven beim Auffinden epistatischer Interaktionen zwischen drei Loci, zu untersuchen und (ii) diese Ergebnisse mit jenen zu vergleichen, die beobachtet werden, wenn RIL von vollständigen oder unvollständigen Kreuzungsdiallelen abgeleitet wurden. Die Computersimulationen dieser Studie basierten auf Haplotypdaten von 26 Maisinzuchtlinien.

Sowohl die Güte als auch die Rate an falsch Positiven beim Auffinden von epistatischen Interaktionen zwischen drei Loci waren für 5000 RIL, die von einem geschachtelten Kreuzungsschema abgeleitet wurden, auf einem Niveau, das vielversprechend für deren Detektion ist. Für RIL, die von optimal allozierten distanz-basierten Kreuzungsschemata abgeleitet wurden, wurde eine höhere Güte, epistatische Interaktionen zwischen drei Loci aufzufinden, beobachtet, als für die gleiche Anzahl an RIL, die von einem geschachtelten Kreuzungsschema oder Kreuzungsdiallelen abgeleitet wurden.

Unsere Ergebnisse belegen, dass Assoziationskartierungsmethoden, die an die Besonderheiten von Pflanzenzüchtungspopulationen angepasst sind, in diesen erfolgreich zum Auffinden von QTL eingesetzt werden können.

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