CHAPTER 5

APPLICATIONS OF LINKAGE DISEQUILIBRIUM AND ASSOCIATION MAPPING IN CROP PLANTS

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Abstract: The investigations of patterns of linkage disequilibrium for designing association-mapping studies are fast becoming a method of interest for complex trait dissection and improvement practices in many crop plants. The methodology and its applications to crop improvement, to date are discussed.

1. INTRODUCTION

Association mapping, also known as linkage disequilibrium mapping, is a relatively new and promising genetic method for complex trait dissection. Association mapping has the promise of higher mapping resolution through exploitation of historical recombination events at the population level, that may enable gene level mapping on non-model organisms where linkage based approaches would not be feasible (Nordborg and Tavare 2002; Risch and Merikangas 1996).

Association mapping utilizes ancestral recombinations and natural genetic diversity within a population to dissect quantitative traits and is built on the basis of linkage disequilibrium concept (Geiringer 1944; Lewontin and Kojima 1960). One of the working definitions of linkage disequilibrium (here on will be referred to as LD) is the non-random co-segregation of alleles at two loci.

In contrast to linkage based studies, linkage disequilibrium based genetic association studies offer a potentially powerful approach for mapping causal genes with modest effects (Hirschhorn and Daly 2005). While linkage analysis is based upon

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detection of non-random association between a genotype and a phenotype in well-characterized pedigrees, association mapping focuses on associations within populations of *unrelated* individuals. In general, chromosomes sampled from *unrelated* individuals in a population will be much more distantly related than those sampled from members of traditional pedigrees. In other words, the time to most recent common ancestor (MRCA) of any given two individuals from a population of unrelated individuals would be greater than that of a pedigree population. This is what makes LD mapping suitable for fine-scale mapping: there will have been more opportunities for recombination to take place over several generations, between many alleles, in a species, while there can be only a few generations of recombination present in pedigree populations. Increase in the rate of recombination will lead to reshuffling of the chromosomal segments into smaller pieces. This will lead to reduction of the LD in short distances around loci, and lead to significant co-occurrence (i.e. LD) between only loci physically close, allowing high resolution. Whereas pedigree studies work with recombination events in few generations that enable exchange between chromosomes at the order of megabases, association studies deal with segmental exchanges measured in kilobases (Paterson et al. 1990; Stuber et al. 1992; Thornsberry et al. 2001).

2. WHAT IS LINKAGE DISEQUILLIBRIUM AND HOW IS IT RELATED TO ASSOCIATION MAPPING STUDIES?

The term *linkage disequilibrium* was first introduced back in late 1940‘s to describe the degree of non-random association between pairs of loci. In the absence of demographic effects that might confound the linkage disequilibrium patterns, LD summary statistics such as r^2 can be used to define the level of co-occurrence of alleles at two loci (Hill and Robertson 1968). When r^2 is zero, alleles at two loci do not co-occur more frequently than would be expected under random sampling. r^2 approaches its maximum of 1 as alleles at two loci show more frequent co-occurrence within the population sample examined. There are various other linkage disequilibrium statistics that can be used for this purpose (Hedrick 1987) all of which aim to estimate the predictive value of a marker locus on another locus that is displaying non-zero LD with it (if LD statistic is zero, two loci examined have zero predictive value for each other).

Association mapping uses these properties of the measures of pairwise LD statistics to infer the predictive value of a marker locus for the association of the chromosomal region it resides with the phenotype. The high-LD chromosomal region around a marker locus defines the predictive range of a certain genetic marker. If LD within this genomic range is complete, any polymorphism within this range will have the same predictive value for the association with the phenotype. Hence, as a result of a significant marker-phenotype association, it can be concluded that the causative polymorphism resides within this high LD region around the marker locus.

With respect to association mapping, the most significant aspect of LD is its predictive properties over the haplotype it resides in. However, the extent of LD (in base pairs) within species and even within individual genomes are highly variable, and therefore most reliably estimated empirically (Long and Langley 1999). Theoretical estimation of the levels of LD for realistic population models that does not satisfy the assumptions of Wright-Fisher model is complex. The hardship is mostly due to the large number of interrelated factors involved in the formation of patterns of LD, including but not limited to genetic drift, population admixture, and natural selection (Pritchard and Przeworski 2001; Wall and Pritchard 2003).

The statistical power of associations is determined by the extent of LD with the causative polymorphism, as well as sample size used for the study (Long and Langley 1999; Wang and Rannala 2005). If LD decays too fast within a region, large number of markers would be required to scan target regions of a genome. On the other hand, if LD decays too slowly, the size of the haplotype blocks would be too large to unambiguously reveal underlying causative locus. In other words, the decay of LD over physical distance in the study population determines the marker density required and the level of resolution that may be obtained in an association study.

2.1. How to Estimate LD

There are several summary statistics proposed for estimation of linkage disequilibrium (Hedrick 1987), however the most commonly used summary statistic within the association study framework is known as r^2 (Hill and Robertson 1968; Lewontin 1988). Conceptually and mathematically r is the Pearson's (product moment) *correlation coefficient* of the correlation that describes the predictive value of the allelic state at one polymorphic locus on the allelic state at another polymorphic locus, where r^2 is the squared value of correlation coefficient that is also called *coefficient of determination*. r^2 explains the proportion of a sample variance of a response variable that is *explained* by the predictor variables when a linear regression is performed.

Lewontin's D, is another summary statistic for LD that is commonly used. D describes the difference between the coupling gamete frequencies and repulsion gamete frequencies at two loci. From D a second measure of linkage disequilibrium, that is normalized D' can also be estimated. Even in samples taken from populations at equilibrium under neutrality, variances of linkage disequilibrium summary statistics are typically large but D' has the lowest variance (Hedrick 1987). However, estimation using D' may generate erratic and unreliable results when low frequency alleles or small sample sizes are used for the analysis. It is advised to collapse the alleles using an allele frequency cut-off prior to estimation of linkage disequilibrium statistics D and D'.

Other than these commonly used summary statistics for LD, there are also likelihood-based methods that investigate probability of independence between pairs of sites using two-locus sampling distributions, rather than calculating a summary statistic for LD. These methods, usually referred to as model-based LD

estimators, also provide means of estimating population recombination parameter $4Nc$ under neutral equilibrium model from nucleotide sequence data (Golding 1984; Hudson 1985; Hudson 2001) or generating other model-based estimates of LD for comparisons with observed patterns (Mueller 2004) under various population structure and demographic history scenarios. Although the estimation of LD through these methods are more computationally intensive compared to the pairwise-LD estimation methods, they are extensively used for evolutionary and population genetic studies as well as investigations on the domestication of various crop plant species (Wright et al. 2005; Wright and Gaut 2005).

2.2. Interpretation of LD Data

Estimating LD from empirical data is a straightforward procedure; however interpretation of results of LD analysis and extrapolation of this information to the genome may be more complex. It is important to estimate the rate of decay of LD with physical distance to be able to extrapolate information gathered from a small collection of sampled loci to the whole genome investigated. This extrapolation is essential for association mapping study design since it may be used for determining the marker density required for scanning previously unexplored regions of the genome as well as determining the maximum resolution that can be achieved for genotype phenotype associations for the study population.

The levels of LD are expected to be highly variable across the genome, due to several factors such as variation in recombination rate and selection. For reliable results, this variation needs to be taken into account when designing experiments to exploit LD. Variation in rate of recombination across the genome is a key factor that contributes to the variance observed in patterns of LD. A number of researchers have focused on the distance at which average r^2 is reduced to 0.10, as a reasonable point to conclude there is minimal LD to detect associations with complex traits. The reasoning for this r^2cut-off is as follows: in a complex trait a large quantitative trait locus (QTL) may only explain approximately 10% of the phenotypic variation. If a marker only explains 10% of the total QTL variation, then the marker will only explain one percent of the phenotypic variation. Detection of locus effects that cause smaller than 1% phenotypic variation requires exponentially increasing population sizes therefore such small effects would be considered undetectable in a moderate size study population.

Sufficient power for association studies of complex traits requires LD blocks to be defined more strictly for greater LD as well as larger population sizes. Current human genetic studies focus on genome scans aiming for much higher LD (e.g. $r^2 > 0.80$) (Barrett 2006), and are developing haplotype based approaches that can help capture more variants (Pe'er et al. 2006).

2.3. LD in Plants

Studies on rates of decay of linkage disequilibrium in various plant taxa (Flint-Garcia et al. 2003) such as maize (*Zea mays* ssp. *mays*) (Ching et al. 2002;

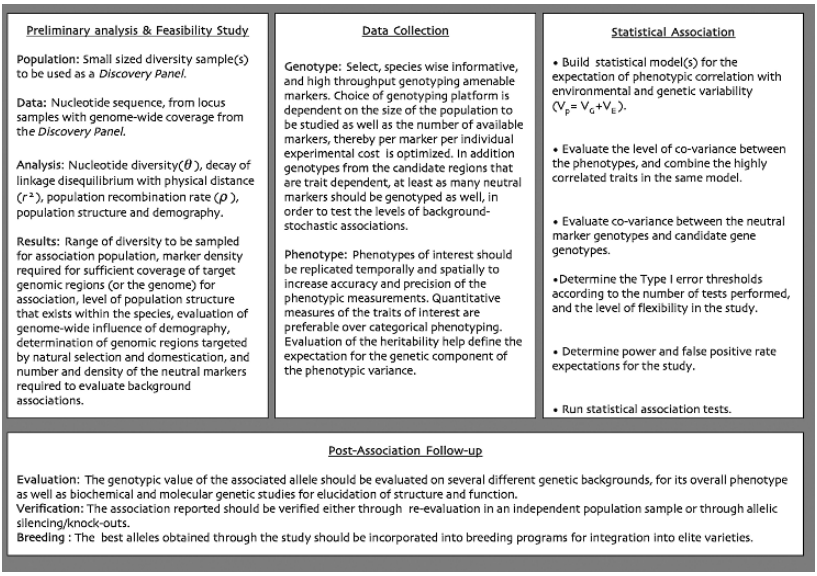
Palaisa et al. 2003; Remington et al. 2001a; Tenaillon et al. 2002), barley (*Hordeum vulgarae*) (Caldwell et al. 2004; Caldwell et al. 2006), *Arabidopsis thaliana* (Nordborg et al. 2002; Nordborg et al. 2005), and sorghum (*Sorghum bicolor*) (Hamblin et al. 2005) and durum wheat (*Triticum durum*) (Maccaferri et al. 2005), indicate tremendous variation in the extent of linkage disequilibrium. This variation is mostly due to founder effect followed by genetic drift that leads to unequal number of effective recombinations in species sub-populations. Furthermore, selfing also plays an important role (Nordborg 2000).

The population sample effect is clearly observed in maize, where LD decays within 1kb in land races (Tenaillon et al. 2001), in approximately 2 kb in diverse inbred lines (Remington et al. 2001a) and can extend up to 100 kb in commercial elite inbred lines (Ching et al. 2002). In barley, in a study of four loci Caldwell et al. (2006) shows that LD might extend up to 212 Kb in elite lines while it might decay below $r^2 = 0.2$ within 0.4 kb for the same region in wild lines. In wild barley (*Hordeum spontaneum*) the results on analysis of LD over 18 loci suggests that LD decay displays a pattern quite similar to that of maize at some loci, that decays below significant levels within 2 kb (Morrell et al. 2005). However, there are a proportion of the loci that show more extensive LD, which may be the result of admixture. In European Aspen (*Populus tremula*), Ingvarsson (2005) shows that there is substantial variation not only across populations but also across loci, and estimates the range of decay of LD to an expected value of r^2 to less than 0.05 within a few hundred basepairs. In a comparison of nine loci across two population samples of loblolly pine (*Pinus taeda* L.), Gonzalez-Martinez et al. (2006a) shows that the rates of decay of linkage disequilibrium are fast; decays below the level of $r^2 < 0.2$ within 2 kb but is variable and not significantly different for the independent population samples investigated for loblolly pine.

In predominantly selfing *Arabidopsis*, LD at a key flowering time locus (*FRI*) extends beyond 250 Kb (Nordborg et al. 2002). However, in large genomic surveys, the decay of LD was reported to be much faster genome-wide: below the level of $r^2 < 0.2$ within about 30 Kb (Nordborg et al. 2005). In another selfing species, soybean (*Glycine max*), Zhu et al. (2003) studied the patterns of LD in 143 short amplicons that spans approximately 12.5 cM of the genome. The study reports that significant decay of LD was detectable within approximately 2–2.5 cM that roughly equals to 1–1.5 Mb. There are few studies that investigate LD in rice (*Orzya sativa*) to date; at a disease resistance locus it was reported that substantial LD extends beyond 100 kb (Garris et al. 2003) and even further at the *waxy* domestication locus (Olsen et al. 2006). For the rice genome, more comprehensive studies are underway.

3. ASSOCIATION POPULATIONS AND STATISTICS

There are five main stages for association studies: (1) Selection of population samples, (2) Determination of the level and influence of population structure on the sample, (3) Phenotyping the population sample for traits of interest (4) Genotyping



Box 1. The steps employed during an association study

the population, for either candidate genes/regions or as a genome-wide scan and (5) Testing the genotypes and phenotypes for their associations (Box 1).

The choice of association test is the last step of the study and is mostly dependent on the previous steps according to the characteristics of the population that was used to collect the genotypic and phenotypic data (Breseghello and Sorrells 2006a; Breseghello and Sorrells 2006b; Lewis 2002). Furthermore, possible complications due to population structure in the study sample may adversely affect the association test results. The influence of population structure on each association study depends on the relatedness between sampled individuals in the studied population. Therefore, the populations amenable for association studies may be classified according to the level of relatedness between the individuals forming the association population.

In the following subsections, we will first discuss the influences of population structure on various association study designs, followed by examples of control for its influences by accounting for the relatedness between individuals forming the association population.

3.1. Population Structure

Most important constraint for the use of association mapping for crop plants is unidentified population substructuring and admixture due to factors such as adaptation or domestication (Thornsberry et al. 2001; Wright and Gaut 2005). Population structure creates genome-wide linkage disequilibrium between unlinked loci. When the allele

frequencies between sub-populations of a species is significantly different, due to factors such as genetic drift, domestication or background selection, genetic loci that do not have any effect whatsoever on the trait may demonstrate statistical significance for their co-segregations with a trait of interest. Provided that a large number of neutral markers are available for estimation of genome wide effects of structure, it is possible to statistically account for such effects in association data analysis (Yu et al. 2006b).

In cases where the population structuring is mostly due to population stratification (Bamshad et al. 2004; Pritchard 2001) three methods are often acknowledged to be suitable for statistically controlling the effects of population stratification on association tests: (1) genomic control (GC) (Devlin et al. 2004; Devlin and Roeder 1999; Devlin et al. 2001), (2) structured association (SA) method including two extensions that are modified for the type of association study as case-control (SA-model) (Pritchard et al. 2000b) or quantitative trait association study (Q-model) (Camus-Kulandaivelu et al. 2006; Thornsberry et al. 2001), (3) unified mixed model approach (Q+K) (Yu et al. 2006b).

First method suggested for statistically controlling population structure was GC that assumes population structuring has equivalent effects on all loci genome-wide. In GC method, a small random set of markers (e.g., polymorphisms unlikely to affect the trait of interest) are used to estimate influence of population structure on the association test statistics *(inflation factor)*, such that the significance of the association statistic (P value) estimated is adjusted to account for population structure. The general principle of GC is to use individual genomes from the sample, to estimate levels of confounding due to substructure and more direct relatedness such as familial relationship in the study and scale the final significance level of the association reported accordingly (Devlin et al. 2001).

Structured association methodology, utilizes marker loci unlinked to the candidate genes under investigation to infer *subpopulation membership*. The application of structured association to qualitative and quantitative traits is done using the appropriate model depending on the trait and population type, with either SA or Q models respectively. In application of SA for quantitative trait association (Q-model), a two stage procedure is constructed where for the first stage each subject's probability of membership in each subpopulation is estimated (Pritchard et al. 2000a; Pritchard et al. 2000b) and then in the next stage, a test of association is conducted using subpopulation membership as a variable for the anociation model tested (Pritchard et al. 2000b). In case-control studies, the probability of the SNP frequency distribution based on population structure is compared between the case and control samples. For quantitative traits, the population structure estimates are used as covariates in the regression model that defines the correlation of the genotype with the phenotype (Camus-Kulandaivelu et al. 2006; Thornsberry et al. 2001).

In unified mixed model approach (aka Q+K model) of Yu and Pressoir et al.(2006b), a large set of random markers that can provide genome-wide coverage are used to estimate population structure (Q) and relative kinship matrix (K), which are fit into a mixed-model framework to test for marker-trait association. In the

unified mixed-model approach, each of the factors that may confound association analysis, that is, familial relatedness between individuals (K) and relatedness due to population structure (Q) are considered as independent variables within the species population. In order to account for the combined affects of such relatedness factors, they are included as covariates into the regression model that defines the correlation between genotype and the phenotype during association testing.

The genetic makeup of the study population that was used to collect genotype and phenotype data defines the model and type of association statistics to be used for association tests. This will be discussed further in the next section.

3.2. Classic Association Populations

If the individuals forming the study population are *effectively* unrelated, the study population may be considered a random sample of individuals from species population and is therefore equivalent to any natural population. The relatedness amongst the individuals forming the population can be either estimated using pedigrees (Emik and Terrill 1949) or inferred using molecular markers (Blouin 2003; Lynch and Ritland 1999; Oliehoek et al. 2006; Wang 2002). These individuals can either be selected from originally natural populations, or subselected from selections included in breeding programs, to form a classic association population. Selecting individuals from breeding programs offers the advantage of easy incorporation into future breeding programs, however the number of lineages incorporated in the association study becomes limited (Breseghello and Sorrells 2006a; Breseghello and Sorrells 2006b).

All the previously mentioned statistical methods for population structure inferences are applicable to the classic association populations; however Q+K model has the widest base of applicability across all structured association study designs in natural populations.

In plants, so far the focus has been on quantitative traits in natural populations. In maize, using diverse inbred lines it was possible to select a sample of 102 lines with relatively few closely related individuals by sampling across the world's breeding programs (Remington et al. 2001a; Thornsberry et al. 2001). However, as larger samples were gathered to increase statistical power to over 300 maize lines it became extremely difficult to find samples that match the structure expected in natural populations (Flint-Garcia et al. 2005). These are the cases where the combined natural and family based approaches are most powerful (Yu et al. 2006a). In *Arabidopsis* (Nordborg et al. 2005), natural samples were collected from around the world but because of strong population structure and selfing, these samples in many respects behave more like families for association mapping purposes (Aranzana et al. 2005). Association studies with some tree species are more likely to fall into the model of effectively unrelated individuals (Gonzaléz-Martinéz et al. 2006b; Thumma et al. 2005). Most crop plant studies will probably fall on a continuum between natural and family-based association populations.

3.3. Family Based Association Populations

If the association population is a collection of unrelated families, instead of single unrelated individuals, it is possible to perform a joint linkage and association analysis on the population, that potentially can be more informative on the trait of interest than either approach alone (Holte et al. 1997; Karayiorgou et al. 1999). For instance, in human genetics, where the association populations are collections of parent-offspring trios, two types of study design is considered: transmission disequilibrium tests (TDTs) (Allison 1997; Fulker et al. 1999; Monks et al. 1998; Rabinowitz 1997; Spielman et al. 1993) , family based association tests (FBATs) (Herbert et al. 2006; Horvath et al. 2001; Laird et al. 2000; Laird and Lange 2006; Lake et al. 2000; Lange et al. 2003). Stich et al. (2006) modified the QTDT algorithm to test its applicability to inbred plant populations, and developed a model named Quantitative Inbred Pedigree Disequilibrium Test (QIPDT), for analysis of joint linkage and association data from crop plant populations. Another family based population design that was essentially developed for crop and livestock breeding is the Henderson's Mixed Model Approach (Henderson 1975), generally known for its applications in Best Linear Unbiased Predictors (BLUPs). Family based association study design investigates co-segregation and linkage simultaneously (Spielman et al. 1994).

A long standing mixed model method has been used by animal scientists to analyze the data from extended pedigree in dairy or cattle breeding programs (Henderson 1975; Henderson 1976; Henderson 1984). The superiority of the mixed model lies in its incorporation of the phenotypic observations from relatives of an individual into the estimation of the breeding value of that individual. The amount of information that is incorporated depends on the heritability of the trait and the genetic relationships (traditionally defined by pedigree information) among individuals. Naturally, this method has been extended to quantify the single gene effect while accounting for the pedigree relationship (Kennedy et al. 1992) and is applicable to association mapping with family based association populations. Taking this mixed model framework, Yu et al. (2006b) suggested to replace the pedigree-based co-ancestry with a marker-based relative kinship (K) to account for the relatedness among individuals.

This unified mixed model approach is demonstrated to be the most powerful statistic compared to all the rest of the statistics, for the family based association studies and those studies falling between classical and family-based designs. The flexibility and generality of this approach allow association studies to be carried out on any population without the restriction on the specific family structure.

3.4. Special Association Populations

Recently, the field of plant association genetics pioneered the use of a new type of association population, designed to incorporate advantages of both linkage based and linkage disequilibrium based quantitative trait dissection approaches in association studies, in a stronger design than Transmission-Disequilibrium Test (TDT)

design. This builds off of some of the joint linkage-association approaches encountered in cattle breeding (Blott et al. 2003; Meuwissen and Goddard 1997). The *Nested Association Populations* (NAM) are developed through controlled crosses between a diverse selection of unrelated individuals according to a breeding scheme that aims shuffling of alleles in diverse samples either across backgrounds or against a reference background while keeping track of number and locations of the recombination events that shuffle the parental chromosomes (Yu et al. 2006a). The subsequent generations of progeny of the crosses can then used as association populations. A population generated according to this described scheme not only provides tremendous power to the statistical tests of association, but also enables the projection of genotype information from the parents to the progeny optimizing genotyping cost for large studies. The cross design is expected to effectively reduce many of the affects of admixture and population structure on the association population. For such populations, a two step procedure for associations is suggested.

The two stage study design of nested association mapping requires deep sequencing or genotyping of the parents for SNP identification across the genome followed by lower density genotyping in the progeny in order to infer the locations of the recombination breakpoints during the crosses. Once the recombination breakpoints are localized and the recombination blocks are traced back to the contributing parent, the haplotype information from the parents can be directly projected on the progeny genome, without further need for genotyping within these blocks.

This design scheme enables the researcher to utilize the advantages of both linkage based and linkage disequilibrium based genetic mapping approaches. It provides genome wide coverage, with high resolution and is performed on an experimental cross that is robust to genetic heterogeneity with representation of several alleles per loci in a large population.

Because of the balanced design, straightforward multiple regression approaches can be applied (Yu et al. 2006a) for association testing. Currently, availability of such nested association populations are reported for maize (Yu et al. 2006a) and loblolly pine (Baltunis 2005; Ersoz 2006; Kayihan et al. 2005). Further statistical methods that are going to utilize and combine information from both parent and progeny generations for NAM type populations are currently under development.

These mentioned association population structures represent the continuum of LD levels from low in classic association populations towards high in biparental breeding populations. Nested association populations that are similar to heterogenous intermated populations (Niebur et al. 2004) fall in the mid-range of this continuum with moderate levels of LD and linkage.

4. FALSE POSITIVES AND POWER OF ASSOCIATION

One of the major concerns in the association mapping studies is the statistical power of the association testing, since as it stands, there is a trade off between the power and accuracy for reporting associations due to false positives. The major

determinant of the levels of false positives and power of associations is the level of population structure in the association population.

A false positive (Type I error) occurs when a test incorrectly reports that it has found a positive result where none really exists. The classical definition of Type I error is an incorrect rejection of the null hypothesis - accepting the alternative hypothesis even though the null hypothesis was true. The second functional biological definition of false positives is also used in association studies. In this framework, false positives do not only arise due to the failure of the statistical test performed, but also in cases where the statistical test is valid and the association exists but it is an association with population structure instead of the trait of interest. Population structure can lead to identification of loci that generate statistically significant but biologically invalid associations solely due to their tight correlation with population structure. However, if the population structure in an association study is properly dealt with, this is not expected to be a source of false positives.

Traditionally, Type I error rate (α) for multiple testing is controlled with the Bonferroni correction. The Bonferroni correction in general is conservative and leads to power loss for detection if the polymorphisms are in linkage disequilibrium and/or the traits are correlated with one another.

Another statistical method suggested for control for multiple testing is False Discovery Rate (FDR) procedure. The FDR is the proportion of positive results that are actually false positives to the whole set of positive results obtained from a statistical test. The procedure can be used to estimate a cutoff for a particular FDR (Benjamini and Hochberg 1995), or estimate an FDR for a particular cutoff (Storey 2002; Storey and Tibshirani 2003). The FDR approaches may be most appropriate when multiple traits are being compared or when the markers are not in extensive LD (Chen and Storey 2006). Essentially based on the relative costs of false positives on further follow-up research, appropriate false discovery rates should be determined and be used.

A third procedure that can be applied for multiple testing correction is the permutation test (Churchill and Doerge 1994; Doerge and Churchill 1996), which controls for the genome-wide error rate (GWER). The permutation test has the ability to estimate effects on significance levels caused by the use of correlated markers as well as correlated traits. In this approach, the trait values are permuted relative to the genotypic data. These permutation approaches are appropriate ways to control the GWER, however, they can be quite conservative if one expects numerous QTLs. Recently, the $GWER_k$ approach of Chen and Storey (2006) incorporates a method for a more liberal balance of true and false positives provides a reasonable avenue.

Other than these statistical methods proposed, it is also possible to non-parametrically estimate the false discovery rate through comparison of distributions of P values, against a set of markers of known influence and a set of random markers scored on the same association population, with simulations. The probability of false associations is simply the ratio of the proportion of significant associations detected in the random set to the proportion of significant associations detected

in the simulated set of known influence loci. This method provides a fast and rigorous way of estimating FDR, if a set of random markers has been scored on the association population. Since random markers are required to estimate population structure, this method should be applicable for association testing in most cases.

The power of a statistical test is the probability that the test will reject a false null hypothesis. Some of the relevant parameters that can effect the power of association studies are, but not limited to (1) The type of association test, single marker or haplotype based, (2) The multiplicity control method, (3) Population-Structure control method, (4) Genetic architecture of the trait, (5) Population size, (6) Marker density, (7) Type of populations used for associations, family based or effectively unrelated (Long and Langley 1999).

Simulation studies that investigate the power of the association tests for candidate gene association approach report that 300 individuals in a natural population provide enough power to detect *repeatable* associations when population structure is controlled properly (Camus-Kulandaivelu et al. 2006; Long and Langley 1999; Thornsberry et al. 2001; Yu et al. 2006a). These power estimates are based on candidate gene studies, where there are few SNPs being evaluated relative to the entire genome. Genome scan type association studies rapidly becoming feasible, but for such studies the population sample size required to obtain sufficient power will be larger. The exact population size required will depend on the LD structure for the population. Population sizes of 1000 to 5000 genotypes will likely be sufficient in most cases.

The power of association will be low, if the trait is highly correlated with population structure. Statistical controls for population structure, under such circumstances would result in false negatives. An example of such a case is demonstrated for maize and *Arabidopsis* flowering time traits (Aranzana et al. 2005; Flint-Garcia et al. 2005). The reason for flowering time and population structure to be correlated is that flowering time is an adaptive trait that largely defines the structure. The Q+K model can produce somewhat better results in these situations (Yu et al. 2006b), but in general a different sample or genetic design is required to work with traits that are tightly correlated with population structure. From a study of 60 traits on a maize diversity panel of 302 inbred lines, the only traits that showed strong relationship with structure were two flowering time related traits.

Three studies using different germplasm have analyzed maize flowering time and the *dwarf8* (*d8*) gene (Andersen et al. 2005; Camus-Kulandaivelu et al. 2006; Thornsberry et al. 2001). These studies highlight the difficulties of studying traits related to population structure. In all three studies, when population structure is ignored; highly significant associations between the traits and polymorphisms in *d8* are detected that are often much more significant than any of the random markers. It is clear that the putatively functional allele is segregating with a very high allele frequency in some populations while it is represented at very low frequencies in other populations. This is exactly what would be expected if flowering time is under diversifying selection between the various sub-populations. Furthermore, upon application of standard corrections for managing population structure (Q) the *d8*-flowering time association is significant for some samples but not for others in all three studies.

Essentially, there is low statistical power to evaluate candidate genes that are involved in the clinal adaptation and/or creation of population structure. While empirical significance estimates obtained through contrasting the significances of the candidates with large numbers of random markers, the most effective approach for this type of trait may be specially constructed association populations, with balanced designs.

5. PHENOTYPING AND GENOTYPING STRATEGIES FOR ASSOCIATION TESTING

As in all other quantitative genetic studies, the success of an association study is heavily dependent on the accurate evaluation of the phenotype of interest. The within population variation observed for genotypes and phenotypes for an association is much greater than that found in most bi-parental mapping populations. While greater variation is preferable while aiming for higher resolution and allele mining, it can pose problems for accurate evaluation of this variation in a meaningful way in a single environment.

The inherent variation observed in phenotypic trait measurement, when combined with the substantial genetic variation included in some association studies, requires careful experimental design to acquire quality data. In addition, evaluations in multiple environments with controls and unbalanced designs may be required. In our experience with maize, we found that evaluating the germplasm in short day environments has facilitated some trait evaluation by reducing photoperiod effects between lines. Additionally, we found that evaluating the germplasm in testcrosses (F1 hybrids) has reduced the phenotypic range into a manageable level. Since each of these approaches interact with the genetic architectures of the traits, future studies will be needed to fully understand the tradeoffs of various study design approaches.

In the association study design, genotyping is required for both inferences on the genotype/phenotype associations and on the population structure and demography. The first aim of querying candidate regions for polymorphisms is best achieved by genotyping SNPs within these candidate regions. The second aim of gathering information on population specific phenomenon like structure, linkage, demography, and kinship can be achieved through genotyping neutral background markers, such as SNPs on non-coding regions or SSRs (simple sequence repeats) distributed evenly throughout the genome.

All genetic markers can be used for investigating association; however, SNPs potentially have the most utility compared to rest of the genetic markers. Various assays were developed for detection of known and unknown SNPs. Some are relatively easy to implement and low in cost, others are developed for high volume screening at substantial cost. As the cost of genotyping reduces, genome-wide scans of all available polymorphisms in a species genome are becoming rapidly feasible and preferable over targeted SNP genotyping approaches. SSR markers have historically been useful in association studies and do have high information content, but they may be difficult to find in candidate gene regions and they are several fold more expensive to score than SNPs.

For the purposes of inferences on the population history, genotype information from a large number of neutral marker loci is required. We are using the term neutral marker loosely here, to indicate the non-candidate loci, i.e. the loci that were *not* designated as candidate loci that can putatively influence a trait of interest. The density of the markers required should be scaled to provide genome-wide coverage. Simulation studies suggest 100 SSR or 200 SNP markers would suffice to get a reasonable estimate of population structure and relatedness for most crop plants (Yu and Buckler unpublished results).

When targeting candidate loci for association studies, the greatest statistical power is achieved when the marker and QTL have equal allele frequencies (Abecasis et al. 2001) in the study population. This is due to opportunity created for maximal linkage and LD since robust detection of associations requires the marker and trait loci are in phase. If there is no knowledge of the QTL frequency distribution *a priori*, the best alternative is to choose markers with a wide range of allele frequencies that are likely to mimic the QTL mutation rate. Some SSRs probably mutate faster and have a different frequency distribution than QTL, which may make them less useful for association mapping. SNPs with a wide range of allele frequencies are most likely to be informative. In order to maximize the information content of SNPs, a large number of them can be chosen to scan a particular genomic region, and this can be achieved with numerous algorithms available for choosing SNPs. (Ackerman et al. 2003; Daly et al. 2001; Forton et al. 2005; Gabriel et al. 2002; Halldorsson et al. 2004; Johnson et al. 2001; Ke and Cardon 2003; Patil et al. 2001; Sebastiani et al. 2003; Zhang and Jin 2003).

Wether the phenotype of interest has a binary or quantitative phenotype is also of interest for the association study design. When a binary trait is being investigated, case-control type populations are required for association analysis, where equivalent sized sub-populations of individuals that display the phenotype of interest (cases) and do not display the phenotype of interest (controls) are querried for allelic association of genetic loci with the case and control phenotypes in a statistically significant manner. The statistical test performed is simply a hypothesis test, that asks weather or not the allelic frequency distribution of a locus is the same or different for a given locus between the two sub-populations. Bulk Segregant Analysis (BSA) type (Michelmore et al. 1991) bulked sample genotype screening methods for all the available marker loci may facilitate the candidate gene and association discovery, for binary traits (Shaw et al. 1998). The challange of case-control type studies is to make sure that the case and control groups are comparable in terms of their genetic makeup. Most of the statistical methods aim to detect and correct for the affects of population statification and ancestry differences between the case and control groups (Price et al. 2006; Pritchard et al. 2000b).

6. ASSOCIATION MAPPING IN CROP PLANTS

The motivations for attempting association mapping in different crop plants are highly variable. For historically well studied crop plants, such as maize and rice, the major motivation for association approach is dissection of complex traits at

very high-level resolution, as well as allele mining from natural genetic diversity resources. For other organisms where there is insufficient or little genetic resources the major motivation is functional marker development and identification of molecular markers tightly linked to the trait locus for marker assisted selection and breeding practices. Thus, each association study stands alone for their own motivations and should be evaluated for its utility and success based on their initial motivations and aims.

Association mapping approach requires extensive infrastructure development and preliminary studies to determine population structure and LD (Box 1). Once the preliminary data and infrastructure for association mapping for a species is available, several association studies on various plant taxa report successful results for tests of associations between candidate locus genotypes and various complex phenotypes (Table 1).

In model organism *Arabidopsis*, the association mapping practice is mostly motivated by generating proof of concept, identification of QTL involved in adaptation, and additional alleles to supplement other mutagenesis approaches. The candidate-gene association study at the *CRY2-Cryptochrome2* locus reported diverse functional alleles (Olsen et al. 2004). In their first attempt for a genome-wide association study in *Arabidopsis*, Aranzana et al. (2005) reports identification of previously known flowering time (*FRI* locus) and three known pathogen resistance genes.

In maize, all reported association studies so far have targeted candidate genes with known mutant phenotypes and are motivated by high resolution mapping and allele mining purposes. For instance, *d8* locus with flowering time (Andersen et al. 2005; Camus-Kulandaivelu et al. 2006; Thornsberry et al. 2001), *bt2(brittle2)*, *sh1(shrunken1)* and *sh2(shrunken2)* with kernel composition, *ae1(amylose extender1)* and *sh2(shrunken2)* with starch pasting properties (Wilson et al. 2004) and sweet taste (Tracy et al. 2006), *a1(anthocyaninless1)* and *whp1(whitepollen1)* genes with maysin synthesis (Szalma et al. 2005), *lyc-e (lycopene epsilon cyclase)* gene with carotenoid content (Harjes et al. 2006) are studies that report very high resolution associations, as well as localizing the causative polymorphism within 1–2 Kb of the marker loci reported. In maize, very little is known about association mapping from a genomic scale, mostly due to incomplete genomic sequence and very rapid decay of LD. At the *Y1* locus a relatively large genomic context was examined. *Y1* is a key gene in carotenoid production in maize (Buckner et al. 1990; Buckner et al. 1996), and through an association study (Palaisa et al. 2003) the allelic variation was traced down to multiple independent insertions in the *Y1* promoter region that cause up regulation of the downstream *Y1* gene. At this locus, associations were also shown to extend to neighboring genes (Palaisa et al. 2004) albeit with weaker significances. This extended LD is mostly the result of breeding efforts in the 20th century that specifically targeted this simple Mendelian inherited trait. The extended LD at *Y1* locus is likely to be one of the most extensive in the maize genome; effective over 100s of kb, while other domestication loci *tb1* (*teosinte branched 1*) (Lukens and Doebley 2001) and *tga (teosinte glume architechture)* (Wang et al. 2005) show LD that extends over 10s of kb. However, it should be emphasized that *tb1* and *tga* domestication loci demonstrate patterns of reduced diversity as well as extended LD,

Table 1. Association studies that report significant results. SA: Structured Association, GLM: General Linear Model, MLM: Mixed Linear Model, DRR: Double Round Robin, FR: Fusiform Rust, PC: Pitch Canker. PB-AM: Pedigree Based Association Mapping and FB-AM: Family Based Association Mapping are two special applications of Nested Association Mapping (NAM) applications described in the text

Species	Population type	Association method	Trait	Reference(s)
Zea mays	Diverse Inbred Lines	SA(Q model)	Flowering Time	**Thornsberry et al. 2001 Andersen et al. 2005 Camus-Kulandaivelu et al. 2006**
		SA(Q model)	Kernel Composition Starch Pasting properties	**Wilson et al. 2005**
		SA(Q model)	Maysin Synthesis	**Szalma et al. 2005**
		Case-Control	Carotenoid Content	**Palaisa et al. 2004**
		MLM (Q+K model)	Caretenoid Content	**Harjes et al. 2006**
Zea mays	Diverse Inbred lines	Haplotype TreeScanning	Sweet Taste	**Tracy et al. 2006**
Arabidopsis thaliana	Diverse Ecotypes	SA(Q Model)	Flowering Time	**Olsen et al. 2004**
			Disease Resistance	**Aranzana et al. 2005**
			Flowering Time	
Eucalyptus spp.	Unstructured Natural Population	Regression (GLM)	Microfibril Angle	**Thumma et al. 2005**
Triticum aestivum	Diverse Cultivars	PB-AM (Q Model)	Kernel Size Milling Quality	**Breseghello and Sorrells 2006b**
Oryza sativa	Diverse Land Races	Haplotype Tree Scanning	Glutinous Phenotype	**Olsen and Purugganan 2002**
Pinus taeda	DRR-Cross of Diverse Parents	Case-Control FB-AM (GLM)	FR Resistance PC Tolerance	**Ersoz 2006**
Pinus taeda	Unstructured Natural Population	Regression(GLM) & MLM (K model)	Wood Specific Gravity% Late Wood Microfibril Angle Cellulose Content	**Gonzaléz-Martinéz et al. 2006a**
Lolium perenne	Diverse Natural Populations	SA(GLM)	Heading Date	**Skøt et al. 2005**

indicating that the estimates of LD is not as efficient as they are at *Y1*. Furthermore it is plausible to assume that not all of the selection events may have similar LD patterns to that of *Y1* locus.

Rice is another crop plant that was extensively studied and has whole genome sequence available. Association studies in rice are mostly motivated by allele mining for economically important traits. An example of such a study is the associations reported between *WAXY* locus of and glutinous phenotype that is commonly known as the *sticky rice* (Olsen and Purugganan 2002).

In many important plant species such as forest trees, the generation time of the organism presents a tribulation for the complex trait dissection through genetic analysis. In these species, association-mapping approach offers the opportunity to overcome the limitations of organismal systems, and enables fast trait improvement. Several successful results in candidate gene based association studies have recently been reported from forestry crop species eucalyptus (*Eucalyptus nitens* and *Eucalyptus globulus*) and loblolly pine (*Pinus taeda* L.). For instance, a study by Thumma et al. (2005) reports an association between the Cinnamoyl-CoA-Reductase (*CCR*) gene and microfibril angle in *Eucalyptus spp*. In a loblolly pine candidate gene joint-linkage and association study, associations of several candidate regions with fungal disease resistance traits are reported (Ersoz 2006). Also in loblolly pine wood quality candidate gene association study for association of chemical and physical wood property traits *cad* and *sams2* genes with early wood formation, *lp3-1* gene with percent late wood, *4CL* with juvenile and mature wood, *α-tubulin* with microfibril angle, and *CesA3* with cellulose content are reported (Gonzaléz-Martinéz et al. 2006b).

Another motivation for association approach is the opportunity to unify the elite germplasm resource of an organism through investigation of the breeding material. In an association study, Breseghello and Sorrells (2006b) investigate the wheat kernel size and milling quality in an elite germplasm collection of soft-winter wheat from eastern US. It identifies, three candidate regions on chromosomes 2D, 5A and 5B that are significantly associated with traits (Breseghello and Sorrells 2006b). This study clearly demonstrates the utility of association mapping as a powerful method that can provide a bridge for closing the gap between the implementation of the genetic trait dissection results to marker-assisted selection.

Several AFLP based genome scan studies have also been successful in discovering associations in germplasm samples with high LD. In perennial rye grass *Lolium perenne* (Skøt 2005) successful associations for underlying major flowering time (heading date) QTL were identified. In sea beet (*Beta vulgaris* ssp. *maritime*) (Hansen et al. 2001) identification of several AFLP markers that show significant associations with another flowering time trait (bolting date) is also reported.

7. CONCLUSIONS

So far, map based cloning approaches are reported to successfully clone 12 major effect QTL and nine small effect QTL (Price 2006). The time scale from QTL mapping to positional cloning practice is estimated to be between 5 to 10 years,

while sufficient resolution for QTL cloning through association mapping can be achieved within 2–3 years. Furthermore, there is a substantial lag between the QTL discovery to marker assisted crop improvement practices, dedicated to verification of the presence and stability of QTL, in the traditional linkage based studies. In a well-designed association study, some of the results can be immediately applied to marker-assisted improvement.

The true large scale applications of association mapping will become apparent as multiple species began to have marker densities sufficiently high for whole genome scan by association mapping. Currently, several research groups are working on whole genome scan approaches in half a dozen species that have whole genome sequences available, and there are at least 50 more species whose genome sequences are being completed in the near future.

The goal of association mapping in many crop plants is to identify key genes controlling various traits and mine the best alleles from diverse germplasm to be incorporated in elite breeding material. Traditionally genetic markers were mostly used for trait improvement through several breeding based approaches such as Marker Assisted Selection (MAS), Marker Assisted Breeding (MAB) and Mapping As You Go (MAYG) (Podlich 2004) as well as QTL cloning/transformation based approaches (Remington et al. 2001b). Association mapping has the potential to provide numerous useful alleles to these marker assisted breeding programs. These markers assisted breeding programs using association data are now underway in numerous plant breeding companies. In the next few years, we will also witness the applications of association mapping and MAS for public breeding programs.

Association mapping holds an important and rapidly expanding niche in quantitative trait mapping studies along with linkage mapping and positional cloning, and it is likely that this niche will continue to expand over the next decade.

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