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Quantitative Trait Locus Analysis Using Recombinant Inbred Intercrosses: Theoretical and Empirical Considerations

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ABSTRACT

We describe a new approach, called recombinant inbred intercross (RIX) mapping, that extends the power of recombinant inbred (RI) lines to provide sensitive detection of quantitative trait loci (QTL) responsible for complex genetic and nongenetic interactions. RIXs are generated by producing F_1 hybrids between all or a subset of parental RI lines. By dramatically extending the number of unique, reproducible genomes, RIXs share some of the best properties of both the parental RI and F_2 mapping panels. These attributes make the RIX method ideally suited for experiments requiring analysis of multiple parameters, under different environmental conditions and/or temporal sampling. However, since any pair of RIX genomes shares either one or no parental RIs, this cross introduces an unusual population structure requiring special computational approaches for analysis. Herein, we propose an efficient statistical procedure for QTL mapping with RIXs and describe a novel empirical permutation procedure to assess genome-wide significance. This procedure will also be applicable to diallel crosses. Extensive simulations using strain distribution patterns from CXB, AXB/BXA, and BXD mouse RI lines show the theoretical power of the RIX approach and the analysis of CXB RIXs demonstrates the limitations of this procedure when using small RI panels.

ALTHOUGH significant progress has been achieved in the identification of human genes underlying many pathological conditions, the vast majority of genes have been limited to simple Mendelian traits and well-defined quantitative traits with relatively large and consistent effects (Nadeau and Frankel 2000; Korstanje and Paigen 2002). However, the vast majority of mammalian phenotypic variation, whether it is morphological or susceptibility to various pathological conditions, is polygenic and influenced by complex interactions with environmental factors. Traits that have been historically difficult to analyze include those with incomplete penetrance or expressivity such as behavior, cancer susceptibility, and physiological responses to environmental stimuli as well as those traits that change with age. Complicating the analysis of these types of traits is the prediction that many are also controlled by genes that have small effects individually, but whose cumulative action is the cause of significant interindividual variation. Consequently, a single phenotypic measurement per unique genome is often not robust enough to accurately localize the underlying genetic differences associated with the traits under study. However, in both experimental and domesticated species, where large collections of molecular and genetic markers have been used to develop detailed genetic maps and from which large numbers of recombinant individuals can be generated, statistical analysis of the association between phenotype and genotype for the purpose of localizing genomic regions affecting complex traits is plausible. Nonetheless, the regions harboring quantitative trait loci (QTL) are usually mapped to broad intervals and identifying candidate genes after initial mapping has proven to be a difficult task.

Because of the genetic resources and manipulations available and because of the biological similarity to humans, the mouse has become the de facto model organism to genetically dissect medically important complex traits. However, the most widely used experimental mapping approaches, particularly intercrosses and backcrosses, lack the genetic reproducibility to efficiently perform multivariant analyses across traits and environmental conditions (Darvasi 1998). This is a particularly acute problem when one wants to examine numerous gene-environment interactions or study disease progression at many stages and ages. Chromosome substitution strains (CSS) were recently shown to be powerful re-

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sources to genetically dissect additive-effect loci (Nadeau *et al.* 2000; Singer *et al.* 2004). However, when used without additional crossbreeding, they lack the genetic complexity to detect genetic interactions between nonsyntenic genomic regions. Another powerful resource, recombinant congenic strains (RCS), has the ability to dissect nonsyntenic genetic interactions but lacks the reproducibility to efficiently investigate gene-environment interactions because of the backcrosses required to identify the interacting genomic intervals (van Zutphen *et al.* 1991; Groot *et al.* 1992).

Recombinant inbred (RI) lines are another of the major resources that have contributed to genetic dissection of simple and complex traits (Bailey 1971; Swank and Bailey 1973; Watson *et al.* 1977; Plomin *et al.* 1991b). A major advantage of RI panels over other commonly used mapping approaches is their ability to support genetic mapping and correlations among many traits, even under different environmental conditions (Plomin *et al.* 1991a). However, mouse RI panels generally have low power and precision compared to other resources because of their small size; typical mouse RI panels have only 15–35 strains from a single pair of parental inbred lines. The situation is significantly different in other species like plants and invertebrates where hundreds to thousands of RI lines may exist because of the quick generation time and ease of maintenance (Johnson and Wood 1982; Burr *et al.* 1988; Reiter *et al.* 1992; Fry *et al.* 1998).

We recently proposed a novel derivative of RI lines, called recombinant inbred intercrosses (RIX), that permits repeated interrogation of a fixed, but complex genotype to reduce nongenetic variance while increasing the power of the original RI panel (Threadgill *et al.* 2002). Although isogenic, a group of RIX individuals has a genetic structure that is remarkably similar to that of an F_2 intercross, except that individuals from the same RIX can be viewed as clones of F_2 individuals that inherit all the advantages of RI strains. Moreover, compared to RI, the advantages of RIX include twice the number of recombination sites in a single individual since each is derived from two parental RIs, albeit there are no new recombination sites; that dominance effects can be estimated; a large expansion of different RIX genomes over the parental RI; and, because of the buffering capacity of their heterogeneous genome structure, that RIX genomes should provide more reliable trait means than the parental RIs. However, the nonsyntenic associations present in RI panels, particularly those with a small number of lines (Williams *et al.* 2001), are retained and even exacerbated in the RIX. The RIX approach also has advantages over classical crosses like the F_2 design since each RIX has a higher recombination density because of the map expansion of the parental RI, averaging almost fourfold more recombination sites than a single F_2 individual when performing interval mapping (Williams *et al.* 2001); the genotypes will be known in advance by imputing from the parental RI lines; RIX are especially useful for long-term collaborative research because their genotypes are renewable, making the phenotypic data cumulative within the research community; and, since RIX genomes are easily replicated, experiments with different environmental variables or temporal relationships can be performed on the same genotypes.

In this study, the novel RIX method that builds upon classical RI panels is evaluated and tested. While subjects in traditional QTL mapping using backcross or intercross populations all have an identical genetic relatedness to one another, this is not the case for the RIX design; some RIXs share a common parental RI line, making them genetically more related to each other than those RIX that do not share parental RI lines. Specifically, RIX can be viewed as the last generation of a pedigree originating from two inbred founders or the diallel designs widely used in plant genetics. To control for this complex relationship structure, we adapted a mixed model for RIX mapping that was originally proposed to handle human pedigree data (Amos 1994). Similarly, we show that the widely used direct permutation procedure to assess significance in QTL mapping is not applicable to the RIX design but requires adaptation to maintain proper relationships among traits and polygenes. Using these new methods, we compare the relative power of RI panels ranging from 13 to 34 lines and demonstrate that, although small RI panels and their derivative RIXs suffer from a lack of power, the RIX approach adds significant power for larger RI panels.

MATERIALS AND METHODS

Mouse breeding and sample collection: CXB1 through CXB13 RI breeding stock, originally produced from BALB/cByJ crossed to C57BL/6ByJ (Dux *et al.* 1978), were obtained from The Jackson Laboratory (www.jax.org). The F_1 intercrosses between pairs of CXB RI lines were set up to generate all 78 nonreciprocal matings by crossing low-numbered female strains by higher-numbered male strains. This simple low-by-high breeding scheme results in a systematic bias: CXB1 is always used as a maternal strain and CXB13 is always used as a paternal strain. To assess the role of parental effect we generated 14 pairs of reciprocal RIXs. Progeny for each RIX were produced from at least two litters for each cross. We did not use cross-fostering of litters or standardize the numbers of animals within litters. All RIX mice were produced in a pathogen-free barrier facility at one site (University of Tennessee Health Science Center) over a 1-year period. Mice between 50 and 100 days of age were weighed, anesthetized, and perfused transcardially with 0.1 M phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in 0.1 M PBS. Bodies were stored in 50-ml conical tubes in 1% PFA at 4° until dissection. Data on body and brain weight, age, sex, litter size, and parity were collected. For the parental RI mice we often did not have data on litter size or parity. Body and brain weights were log-transformed and adjusted for log(age) and sex for body weight or adjusted for log(age) and sex as well as log(body weight) for brain weight. All mice were housed in an Associa-

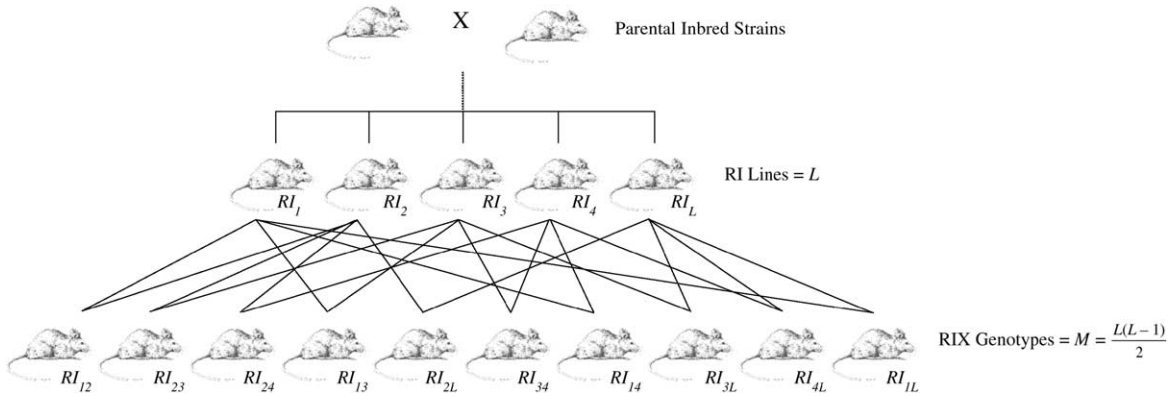

Figure 1.—Production of RIX hybrids. The relationship between the parental strains and the derivative RIs along with the relationships between RIXs is shown.

tion for Assessment and Accreditation of Laboratory Animal Care-approved facility under specific pathogen-free conditions.

Crosses to generate an F_2 population were between BALB/cByJ and C57BL/6ByJ. A total of 184 animals, between 49 and 89 days of age, were collected. Measurements were obtained for body and brain weights, log-transformed, and adjusted for covariates as described above. Interval mapping, using R/qtl (Broman *et al.* 2003), was performed to detect loci modulating body and brain weights for comparison to the CXB RI and RIX results.

Genotypes: The genotypes of all RI lines used in the simulation studies were previously reported (Taylor and Phillips 1995; Williams *et al.* 2001). For analysis of the CXB RIs, 382 markers, representing unique strain distribution patterns (SDPs), were used. The RIX genotypes were imputed from the RI genotypes automatically with QTX (Manly *et al.* 2001) while the CXB F_2 progeny were genotyped for 72 simple sequence length polymorphism (SSLP) markers.

Three pairs of the AXB/BXA RI strains have highly similar SDPs (Williams *et al.* 2001); the high degree of identity is strikingly different from the 50% expected for independently derived RI strains. We therefore used only one representative from each of these pairs in the simulation studies. Otherwise, false declaration of linkages and spuriously high and low recombination frequency estimates may be produced.

RIX mapping requires a unique statistical approach: Genetic mapping algorithms using experimental populations, such as backcrosses, intercrosses, or RI panels, to localize QTL are well developed. Many excellent open source software packages, such as QTLCart (Basten *et al.* 1994), MapManager (Manly *et al.* 2001), and MAPMAKER/QTL (Lincoln *et al.* 1992), are available. Due to the similarity of RIX and F_2 genome structures, it would appear that methods developed for F_2 intercrosses could be directly applicable to RIXs. However, the relationship between different RIXs is complicated. For F_2 individuals, the relationship between any pair will on average be the same with each individual sharing, on average, 50% of its genetic composition. However, this is not the case for RIX genomes. Pairs of RIXs sharing one parent are more closely related than those RIXs that do not share a parent. For example, a RIX produced by crossing RI_1 and RI_2 (RIX_{12}) is expected to be more similar to a RIX produced by crossing RI_1 and RI_3 (RIX_{13}) than to a RIX from crosses between RI_3 and RI_4 (RIX_{34}) since RIX_{12} and RIX_{13} share a parental RI (RI_1) while RIX_{12} and RIX_{34} do not share any parental RI lines (Figure 1). From a statistical point, if the relationship among individuals is complex and is not modeled, or modeled incorrectly, high false-positive rates are likely to result (Zou *et al.* 2001). Thus any appropriate analysis should take this special structure of RIX genomes into consideration. Furthermore, in some situations there is interest in estimating parental contributions to traits, which also requires consideration of the special architecture of RIX genomes.

Calculating thresholds and power are important practical issues in the design and analysis of any QTL study. However, the usual pointwise significance level based on chi-square approximation is inadequate because the entire genome is tested for the presence of a QTL. Empirical permutation procedures to estimate genome-wide threshold values for traditional interval mapping have been proposed (Churchill and Doerge 1994). These procedures can be easily extended to other, more complicated situations such as combined crosses where the permutation can be restricted within each class (Liu and Zeng 2000). However, the appropriate procedure to extend permutation analysis to RIXs is not straightforward because the mating scheme used to produce different RIX genomes introduces correlation between related RIXs (those sharing a parental RI line). Ignoring the correlation between related RIXs is problematic and leads to large biases in estimating thresholds. Intuitively, the data should be permuted such that the parental contribution is preserved while the major gene effect on the trait is destroyed. To achieve this, we developed a novel permutation procedure.

Calculations for the RIX design: The following notations are introduced for later use. Suppose there are L RI lines that produce $M = (L(L-1))/2$ nonreciprocal RIXs. Then suppose the RI lines are numbered as $RI_1, RI_2, \ldots, RI_L$ and denote the nonreciprocal RIXs derived from parental lines RI_i and RI_j as RIX_{ij}, where $i < j = 1, 2, \ldots, L$ or, alternatively, as RIX_k, where $k = 1, 2, \ldots, M$ for ease of notation.

For quantitative traits, it is often assumed that traits are controlled by both poly- and oligogenes, genes with small and intermediate effects, respectively. The effects of polygenes on the ability to map oligogenes have been documented and taken into account in algorithms used for commonly used genetic crosses (Visscher and Haley 1996). Within single crosses, such as backcrosses or intercrosses, the progeny have identical relationships given the QTL genotypes, resulting in a compound symmetry structure (Yandell 1997). Thus, unbiased estimates of QTL effects are obtained even when polygenic effects are ignored. Nonetheless, the power to detect QTL is influenced by the magnitude of the polygenic effect. The situation becomes problematic for complicated pedigree structures. Methods using Wright's relationship matrix A to accommodate

different correlations between related individuals have been developed for analyzing human pedigrees and diallel mating designs (Goldgar 1990; Amos 1994; Zhu and Weir 1996; Xu 1998). For the RIX design, a similar approach can be used since RIX can be viewed as the last generation of a pedigree originating from two inbred founders or the diallel designs widely used in plant genetics.

Mixed-model analysis: Assume the existence of major QTL and polygenes, all affecting a trait of interest. In aggregate, the polygenic effect is normally distributed and acts independently of the major QTL. For simplicity, a model with one major QTL is considered; an extension to a multiple-QTL model is straightforward. We fit the following mixed-effect model,

$$Y = X_1 a_1 + X_2 a_2 + Z\alpha + e, \quad (1)$$

where a_1 is a fixed effect due to nongenetic factors such as age; $a_2 = (a, d)$ is a fixed effect with a and d corresponding to the additive and dominant effects of the major QTL, respectively; $\alpha(L \times 1)$ is a random effect due to polygenes and other nonmodeled QTL and is $\sim N(0, \sigma_a^2)$; and e is a random error and is $\sim N(0, \sigma_e^2)$. Z is an $M \times L$ matrix with

$$z_{kj} = \begin{cases} 1 & \text{if one of the } k\text{th RIX individual's parents is } R_j \\ 0 & \text{otherwise,} \end{cases}$$

$$\text{for } k = 1, 2, \ldots, M,\ j = 1, 2, \ldots, L.$$

Obviously, $\Sigma_j z_{kj} \equiv 2$ for all $k = 1, 2, \ldots, M$ since each individual has two and only two parents. Although this model can be extended to parental effects through the generation of genetically identical RIXs using reciprocal RI crosses as noted later, we assumed no parental effects in our analyses.

The hypotheses for whether any major QTL exists at a given locus are

$$H_0: a = d = 0 \quad vs. \quad H_1: a \neq 0 \text{ and } d \neq 0.$$

An F-statistic or likelihood-ratio test statistic or equivalent LOD score can be used to test this model. In all subsequent analyses, the model was tested in SAS with Proc Mixed (SAS code is provided as a supplement at http://www.genetics.org/supplemental/ or can be downloaded at http://www.mouselab.org; SAS Institute, Cary, NC).

Permutation test: Obtaining appropriate threshold values for RIX analysis using model (1) is quite complicated. We have found that the threshold depends on the magnitude of the background polygenic effects when all else is equal, especially when the number of parental RI strains is small, such as with the CXB set where only 13 parental RI lines are available. Thus, to minimize genome-wide type I errors, appropriate permutation procedures must be used to control for polygenic effects when detecting major QTL.

Ideally, when testing the existence of major QTL, the permutation procedure should not destroy the relationship between the trait and the polygenic effect, but only the relationship between the trait and the major QTL. If data are permuted directly (Churchill and Doerge 1994), the relationship not only between the major QTL and the trait but also between the polygenes and the trait is destroyed. Since this relationship is destroyed with the RIX, permutations performed according to Churchill and Doerge (1994) give artificially low thresholds, resulting in enormously high false-positive rates in the presence of polygenic effects.

To avoid this problem, we extended the permutation method of Churchill and Doerge in such a way that the special correlation structure of the data is maintained after permutation. We first permute $1, 2, \ldots, L$, the parental strain number, and then suppose we get $\phi(1), \ldots, \phi(L)$. Then the permuted marker genotypes of RIX_{ij} will be the corresponding marker genotypes of $RIX_{\min(\phi(i),\phi(j))\max(\phi(i),\phi(j))}$.

Consider a toy example where there are four parental RI lines, RI_1, RI_2, RI_3, and RI_4, which produce six nonreciprocal RIXs: RIX_{12}, RIX_{13}, RIX_{14}, RIX_{23}, RIX_{24}, and RIX_{34}. Now suppose we get RI_3, RI_1, RI_2, and RI_4 after permuting RI_1, RI_2, RI_3, and RI_4; then the permuted marker genotypes of RIX_{12}, RIX_{13}, RIX_{14}, RIX_{23}, RIX_{24}, and RIX_{34} are the corresponding genotypes of RIX_{13}, RIX_{23}, RIX_{34}, RIX_{12}, RIX_{14}, and RIX_{24}, respectively.

Note that instead of permuting the genotypes of RIXs directly, we permute the genotypes of the parental RIs and then create the new genotypes for each RIX; this preserves the original relatedness between RIXs, which is equivalent to maintaining the relationship between the trait and the polygenes. After we randomly reassign marker genotypes to RIXs, we can treat the permuted data sets in the same way as the original data and repeat the analysis using model (1).

RESULTS

Power analysis of RIXs: Extensive simulations were performed to investigate the properties of the RIX mapping method. Rather than simulating hypothetical genotypes of parental RI lines, we choose three panels of existing, widely used mouse RI lines and their associated genotypes for the simulations to more accurately reflect those in practice. The three RI panels are CXB (13 lines derived from a BALB/cByJ × C57BL/6ByJ cross), AXB/BXA (22 lines derived from an A/J × C57BL/6J cross and the reciprocal C57BL/6J × A/J cross), and BXD (37 lines derived from a C57BL/6J × DBA/2J cross); these three provide a good range of RI panel sizes. Most RI panels have well-documented nonsyntenic linkage associations that are caused by correlated genotypes that make correct QTL localization impossible when by chance one of the highly correlated markers is linked to the QTL (Williams *et al.* 2001). When this occurs, other follow-up studies are required to determine which region is actually linked to the QTL. As would be expected, the smaller RI panels are more severely affected by the problem of high nonsyntenic correlation. Furthermore, having a small number of parental RI lines makes it difficult to separate major QTL effects from polygenic effects. Conversely, we would expect that the larger the RI panel, the greater the power is for mapping major QTL and for separating major QTL effects from polygenic effects. Thus, the three RI panel sizes can be used to investigate the effect of the number of parental RI lines on QTL mapping using the RIX method.

The 13 extant CXB RI lines can produce 78 nonreciprocal RIX genomes, while the 22 AXB/BXA (after strains whose genotypes are highly correlated with other strains are excluded) and the 34 BXD RI lines will allow the generation of 231 and 561 RIX unique genomes, respectively. The total markers used in our simulations were 382, 591, and 552 for CXB, AXB/BXA, and BXD, respectively (Williams *et al.* 2001).

Our simulations were intended to answer the following questions: (a) How does the proposed model perform under different scenarios?, (b) How do the parental RI and derivative RIX designs differ in QTL mapping

power?, and (c) How does the empirical permutation procedure perform?

To achieve these, two general scenarios were simulated: (a) no major QTL, with polygenes and random error; and (b) one major QTL, with polygenes and random error. Scenario a can be viewed as the null of b. For all simulations, $\sigma_e^2 = 1$ and σ_a^2 is set to 0.25. A series of additive and dominant effects of major QTL were simulated, which are explained in subsequent tables and figures.

For CXB and AXB/BXA, the sample sizes of RIXs are 78 and 231, respectively, which is equal to the maximal number of unique, nonreciprocal RIXs that can be produced from the parental RIs. For BXD, in practice, using all 561 RIXs may be too large so we decided to set the RIX sample size to 340 with each sample generated by a clockwise mating scheme. That is, RI_1 was mated with the following 10 RI lines, $RI_2, \ldots, RI_{11}$; RI_2 was mated with the next 10 RI lines following it, $RI_3, \ldots, RI_{12}$; and RI_L was mated with $RI_{L+1}, \ldots, RI_{L+10}$. To compare RIXs with RIs, the same number of RI animals was used. Thus, for the CXB simulations, we used a single RIX sample for each of the 78 possible RIX genomes but six replicas for each parental RI line, giving 78 total individuals for both populations. The same phenotype-generating mechanism used for RIXs was applied to the parental RIs. Instead of analyzing phenotypes from individual RIs, we averaged the phenotypes within each line and used the RI line means for all analyses. For RIs, the following model was fit:

$$Y = X_1 a_1 + Xa + e_{\text{new}}. \quad (2)$$

The symbols used both in this equation and in Equation 1 have the same meaning. The differences between the two models are that (a) for RIs, the polygenic effects are nonestimable and have been lumped into the random error e_{new}, that is, $\sigma_{e_{\text{new}}}^2 = \sigma_e^2 + \sigma_a^2$, and thus only the fixed-effect model is necessary; and (b) in RIs, only the additive effect a can be tested and $X = 1$ or -1, an indicator for the two homozygous genotypes.

Since the parental RI lines are fixed, it is more reasonable to fix the polygenic effect for a specific trait than to allow it to be totally random each time in the simulation. It is also important to generate different polygenic effects to investigate how these effects influence the genome-wide thresholds to obtain a more generalized picture because polygenic effects vary for different traits. Thus, in all simulations 10 different realizations (or 10 sets of α's) of the polygenic effect were generated. These α's were held fixed within the CXB (or AXB/BXA or BXD) cases for comparisons between RIXs and RIs. Thresholds and power were determined by simulating 10,000 data sets under the null and 200 data sets under the alternative (one major QTL) hypothesis for each set of α's. For data simulated under the null, the highest LOD score among all markers was recorded and the empirical threshold was set to the 95th percentile of the highest LOD score among the 10,000 simulated data sets. The empirical threshold can be, in some degree, viewed as the true threshold. For power calculations under the alternative, if any of the 20 markers adjacent to the QTL had a LOD score greater than the empirical threshold, the QTL was considered detected. The overall power was the average power across the 10 sets of α's.

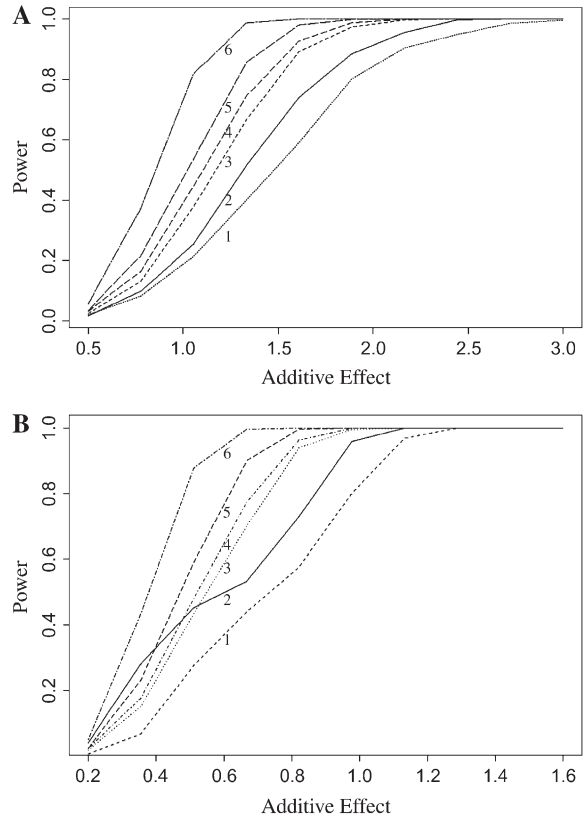

FIGURE 2.—Power comparison of RI and RIX. A QTL with a series of additive (a) and dominant (d) effects was simulated using genotypes from (A) CXB and (B) BXD. Thresholds were determined using 10,000 simulations and 2000 experiments were performed for each level of additive effect. The solid line (2) is the RI power curve and dashed lines are the RIX power curves corresponding to different dominant effects: (1) no dominant effect; (3) $d = a/2$; (4) $d = a/\sqrt{3}$; (5) $d = a/\sqrt{2}$; and (6) d = a.

A direct comparison of the power of the RI compared to the RIX under the model with one major additive QTL and a polygenic effect shows that the RI has slightly more power than the RIX to detect additive QTL for both CXB and BXD (Figure 2). However, in the presence of QTL with dominant effects the power of the RIX is higher; the intermediate-sized AXB/BXA RI set gives similar power curves (data not shown). Due to the lack of power to detect dominant effects using the RI, it is expected that with increasing dominant effects, the power of the RIX increases and we have observed that the RIX has substantially higher power than the RI when dominant effects are large.

To evaluate the performance of the permutation procedure in assessing the genome-wide significance level, we randomly generated 10 data sets under the null for

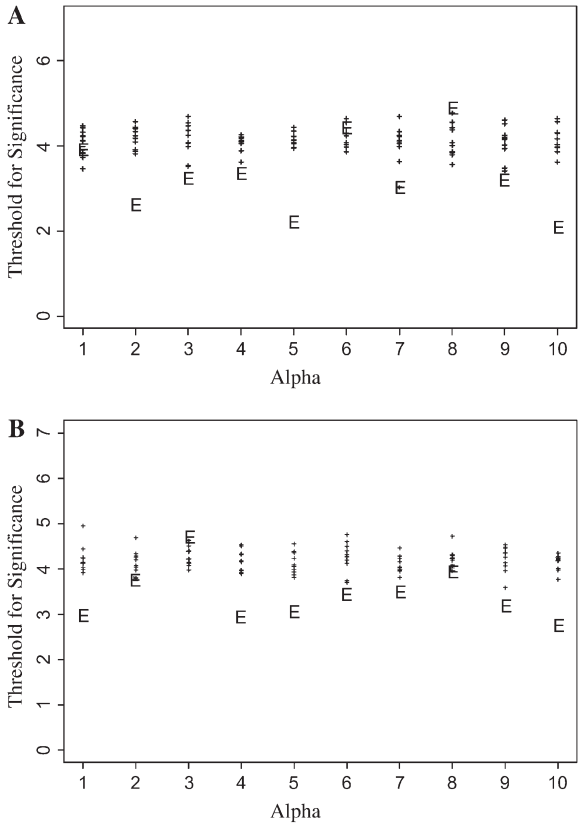

FIGURE 3.—Comparison of permuted and empirical thresholds for RIXs. Ten different realizations of the polygenic effect (*x*-axis) were simulated for RIXs generated from the (A) AXB/BXA and (B) BXD RI sets. The empirical 95th percentile threshold was estimated from the maximal LOD score obtained from 10,000 simulations where data were simulated under the null with every realization of the polygenic effect. For permutation, 10 data sets for each realization of the polygenic effect and their 95th percentile permuted thresholds were calculated. +, permuted thresholds of 1000 simulated data sets under different realizations of the polygenic effect; E, empirical thresholds under different realizations of the polygenic effect.

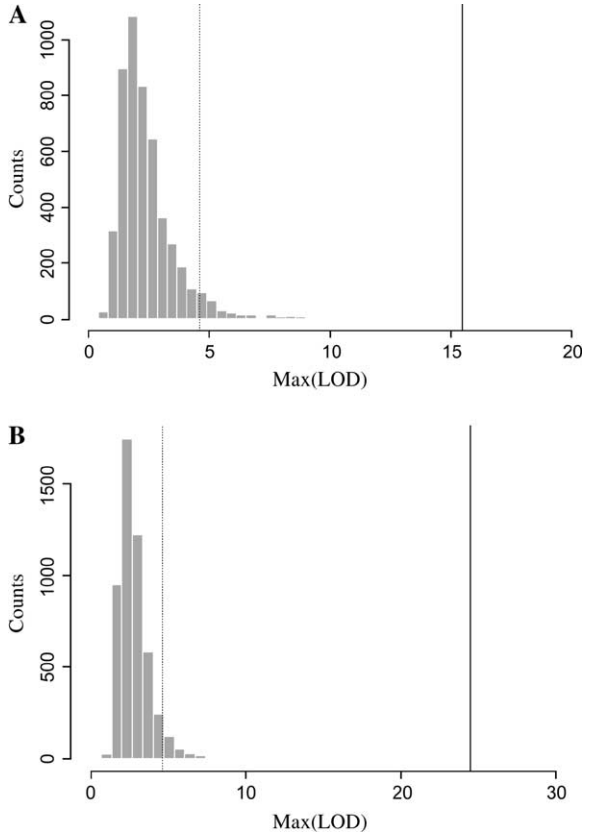

FIGURE 4.—Significance thresholds and permutation distribution of LOD scores for RIXs. Distributions are shown of maximal LOD scores of the data set used to generate Figure 3 for RIXs generated from (A) AXB/BXA and (B) BXD using 5000 permutations of one data set (additive effect = 1.7) that was simulated with one major QTL (results from other simulated data sets show similar patterns). The solid line is the maximal unpermuted LOD score; the dotted line is the 95th percentile of the permuted maximal LOD scores.

each of the 10 α's with a total of 100 simulated data sets. Within each data set, an additive model is fit and 1000 permutations were performed, from which the 95th percentile permutation threshold was calculated. The conservative nature of the permutation scheme for BXD and AXB/BXA panels is demonstrated (Figure 3). For the two largest RIX panels tested, the permutation thresholds for significance are generally greater than the empirical thresholds, indicating conservativeness of the permutation procedure in controlling the type I error rate. However, for the CXB panel where the number of parental strain is small, the permutation procedure is too conservative and essentially has no power to detect QTL.

For AXB/BXA and BXD, where the number of parental strains is relatively large, the conservativeness of the permutation procedure does not prevent the detection of QTL (Figure 4); the conservativeness goes down as the number of parental RIs goes up. The unpermuted maximal LOD score for the data set that was simulated with one major QTL was compared to the maximal LOD scores for the 5000 permutated data sets. Since the original maximal LOD score exceeds all of the 5000 permutation maximal LOD scores, one would reject the null hypothesis at the 0.05 level.

However, the permutation procedure fails for the case of the CXB panel (Figure 5A). As can be observed, the 95th percentile of the permuted data sets exceeds the maximal LOD score of the unpermuted data. This indicates that the permutation procedure is too conservative and one cannot reject the null hypothesis at the 0.05 level. To show that the problem is not specific to RIXs, we also ran the permutation for the parental CXB RI panel, showing that the potential RI power suffers from the same conservativeness (data not shown).

The permutation algorithm reveals lack of power for small RI panels: For the CXB (both RI and RIX), the permutation test is found to be overly conservative at the 0.05 level. The maximal LOD scores have a banded pattern when plotted across different simulations under the one major QTL alternative (H_{A1}). Since the banding

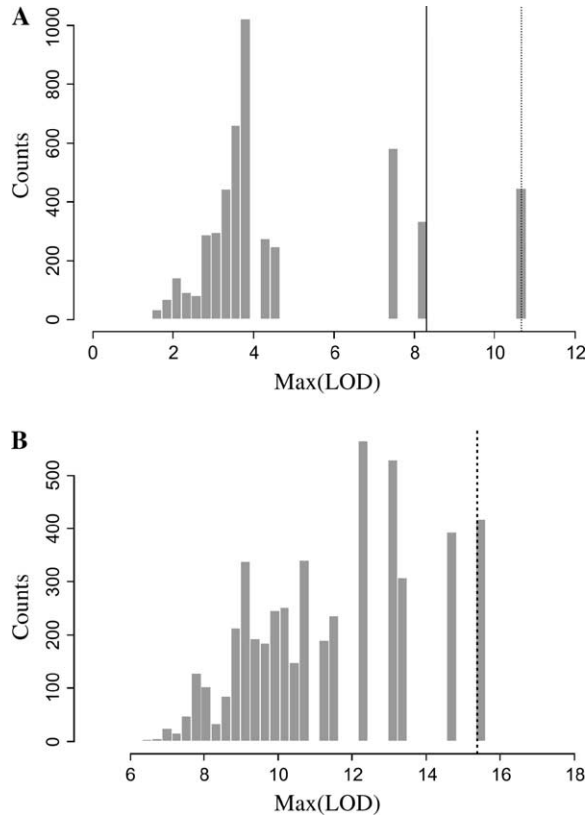

FIGURE 5.—Significance thresholds and permutation distribution of LOD scores for CXB RIXs. (A) Simulations performed for RIXs generated from CXB as described in Figure 4. (B) Permutation results using body weight to identify the 95th percentile threshold. The solid line is the maximal unpermuted LOD score. The dotted line is the 95th percentile. Solid and dotted lines overlap in B.

pattern appears not to depend on the particular values of σ_a^2 or σ_e^2, we considered the case where σ_a^2 or σ_e^2 is very small. This scenario corresponds to the case in which there is no polygenic or random error, an ideal situation with only one QTL and no error. Under this scenario, the response $Y_{n\times 1}$ (n is the number of lines) is a binary vector for the RI case where $Y_i = aM_i^{\text{qtl}}$ and where $M_i^{\text{qtl}} \in \{-1, 1\}$ is the QTL genotype of strain i. Without loss of generality, we let $a = 1$ so that $Y_i \in \{-1, 1\}$. Now we permute Y and fit Y to each of the markers individually. The measure of association (like LOD scores or correlations) will be maximized when any marker M is a perfect predictor of Y. M is a perfect predictor of Y when the value of Y_i completely determines M_i. That is, if $Y_i = M_i$ for all i or if $Y_i = -M_i$ for all i, then marker M is a perfect predictor of Y. If Y is a random vector of independent Bernoulli random variables with $P = 0.5$, then the probability of a perfect match between any likewise random marker would be $2^{-(n-1)}$. If the markers were both random and uncorrelated, then the probability of Y being a perfect match with any one of m makers would be $1 - (1 - 2^{-(n-1)})^m$. For CXB with 382 markers, the resulting probability is 0.09 while for AXB/BXA and BXD with 591 and 552 markers, the probability is 6×10^{-4} and 6×10^{-8}, respectively.

The probability of a perfect match of a permuted response vector is ≥ 0.05 for the CXB, whereas the probabilities for the AXB/BXA and BXD are much less than the stated 0.05 nominal level. This suggests that the permutation test will have positive power for the AXB/BXA and the BXD panels, but the CXB would yield a 95th percentile threshold under the permutation no less than the observed maximal LOD score. Therefore, no QTL can be declared at the 0.05 level for the CXB panel and RI sets of this small size ($n = 13$) lack power to distinguish true genetic signals from random associations. However, it is worth mentioning that the CXB panel still reserves power for candidate gene testing where the regions studied are small.

The probabilities presented above for a genome-wide perfect match may be conservative since the true markers are correlated rather than independent; the probabilities for a genome-wide match may be less than those where all markers are independent. To be more realistic, simulations were performed in which a marker picked at random from the genome was permuted and tested for being a perfect match with all other markers. However, similar conclusions are drawn and the CXB panel still shows lack of power to distinguish true genetic signals from random associations while the AXB/BXA and the BXD reserve the power.

Empirical analysis of CXB RIs and RIXs: To provide experimental support for the power of RIX analysis, as well as the problems associated with small RI panels, we generated a complete nonreciprocal set of 78 RIXs along with 14 reciprocal RIX hybrid genomes from the 13 CXB RI lines (Table 1). We phenotyped 2891 individuals (900 RIs, $\overline{X} = 69$; 1714 RIXs, $\overline{X} = 22$; 277 reciprocal RIXs, $\overline{X} = 20$) for adult body and brain weight, both known to be under complex genetic control. Additionally, 184 CXB F_2 mice were generated and phenotyped for experimental comparison with the RI/RIX results.

When the parental RI lines were used to map QTL regulating body or brain weight, specific loci were detected (Figures 6 and 7). As would be expected from the small size of the CXB panel, numerous loci are strongly associated with the phenotypes. However, there is a general correlation between the body and brain mapping results using RIs and those using RIXs, with the RIXs providing significantly higher LOD scores. Although some of these loci are predicted to be false positives as described above, many of the body weight QTL do colocalize with locations of verified QTL regulating body weight (POMP and NIELSEN 1999).

Interestingly, a comparison between the RI and RIX results with an F_2 validation cross revealed significant similarities but also differences. For body weight, a major locus on chromosome (chr.) 4 is detected with all three approaches. However, two highly significant QTL detected in the F_2, on chrs. 6 and 12, were not detected

TABLE 1

Numbers of individual CXB RI and RIX genomes used in the analyses

Maternal genome	Paternal genome												
	1	2	3	4	5	6	7	8	9	10	11	12	13
1	29	26	8	16	10	22	22	11	18	16	17	18	25
2	14	42	24	20	5	27	18	13	18	16	34	21	22
3	0	0	103	29	23	22	17	33	15	35	10	24	15
4	51	38	0	37	19	25	14	15	31	36	39	16	44
5	6	0	0	0	72	32	18	19	30	17	18	15	18
6	0	0	48	0	16	93	17	21	16	53	20	25	26
7	0	0	0	0	15	2	104	26	23	13	25	9	14
8	0	0	0	0	0	0	0	44	33	20	12	29	25
9	0	0	0	0	0	0	0	0	48	35	28	25	21
10	0	0	18	0	0	21	0	0	0	105	35	31	20
11	0	0	0	0	0	0	0	0	0	12	52	18	22
12	0	0	0	0	0	5	22	0	0	11	0	108	16
13	0	0	0	0	0	0	0	0	4	0	0	0	63

Underlines indicate RI strains. Numbers below the RI diagonal are for reciprocal crosses.

by the RI or RIX analyses. Likewise, several significant loci were detected in the RI and RIX that were not detected in the F_2. A similar finding occurred with brain weights, where the single significant locus detected in the F_2 was also detected in the RI and RIX. Contrastingly, numerous highly significant loci were detected for brain weight in both the RI and the RIX, with the RIX detecting more putative loci that were not replicated in the F_2.

Permutation results using the body weight data demonstrate that the CXB RIX lacks power to distinguish true QTL from random associations since the maximal unpermuted LOD score is identical to the 95th percentile of the permuted LOD scores (Figure 5B); this verifies the simulation results previously described. Furthermore, these results show that the banded pattern produced by the permutations and observed in the simulated data is not an artifact of the simulation but rather due to the inadequate power of the CXB set. As such, the additional loci detected in the CXB RI and RIX, although potentially valid, may also be due to spurious associations with random markers. However, it is also important to realize that although the same parental strains were used for the F_2 validation cross as were used to generate the CXB RI set, they have been separated by an interval of >25 years. Thus, the failure to exactly replicate all positive signals could also be related to genetic drift since the development of the CXB RI lines.

Parental origin effects: Fourteen reciprocal RIX hybrids were also tested to determine the power to detect parental origin effects. We found that parental effects, contributed by either maternal uterine or nursing environments or parental origin of alleles, are a particularly important determinant as highlighted by the substantial differences in body and brain weights of genetically identical reciprocal RIXs. For example, CXB1 × CXB2 RIX animals are typically 2.2 g heavier than CXB2 × CXB1 RIX animals, even after correction for litter size and the mother's parity (data not shown). In contrast, body weights of the CXB1 and CXB2 mothers do not differ significantly.

The 10 reciprocal RIXs with at least 10 offspring from each reciprocal cross were tested for parental effects (Table 2). For body weight, four reciprocal RIXs gave highly significant differences ($P < 0.005$) while for brain weight, three reciprocal RIXs were highly significant. Unlike conventional F_1 hybrids between two inbred strains, the reciprocal RIX hybrids have identical mitochondrial genomes and also share the same sex chromosomes. The conclusion that emerges from this comparison is that trait means derived from conventional inbred strains can be modulated to a great extent by parental origin effects. The RIX design exposes this parental effect and also makes it possible to reduce its impact on a mapping study by using means derived from the two reciprocal RIXs. Consequently, if specific loci are contributing to the parental effect, they should be mappable in a set of reciprocal RIXs.

Comparison of inbred and hybrid trait means: Previous studies comparing inbred lines and their hybrid offspring have shown that environmental variance increases with inbreeding, where decreased heterozygosity likely causes increased developmental sensitivity or decreased environmental buffering capacity (Leamy 1982a,b). Consistent with these results, the variance for the body and brain weights in RIX hybrids is 10–20% lower, on average, when compared to the parental RI lines (Figure 8). After adjusting for sex and age, we found that the mean standard deviations of body weights for RIs and RIXs

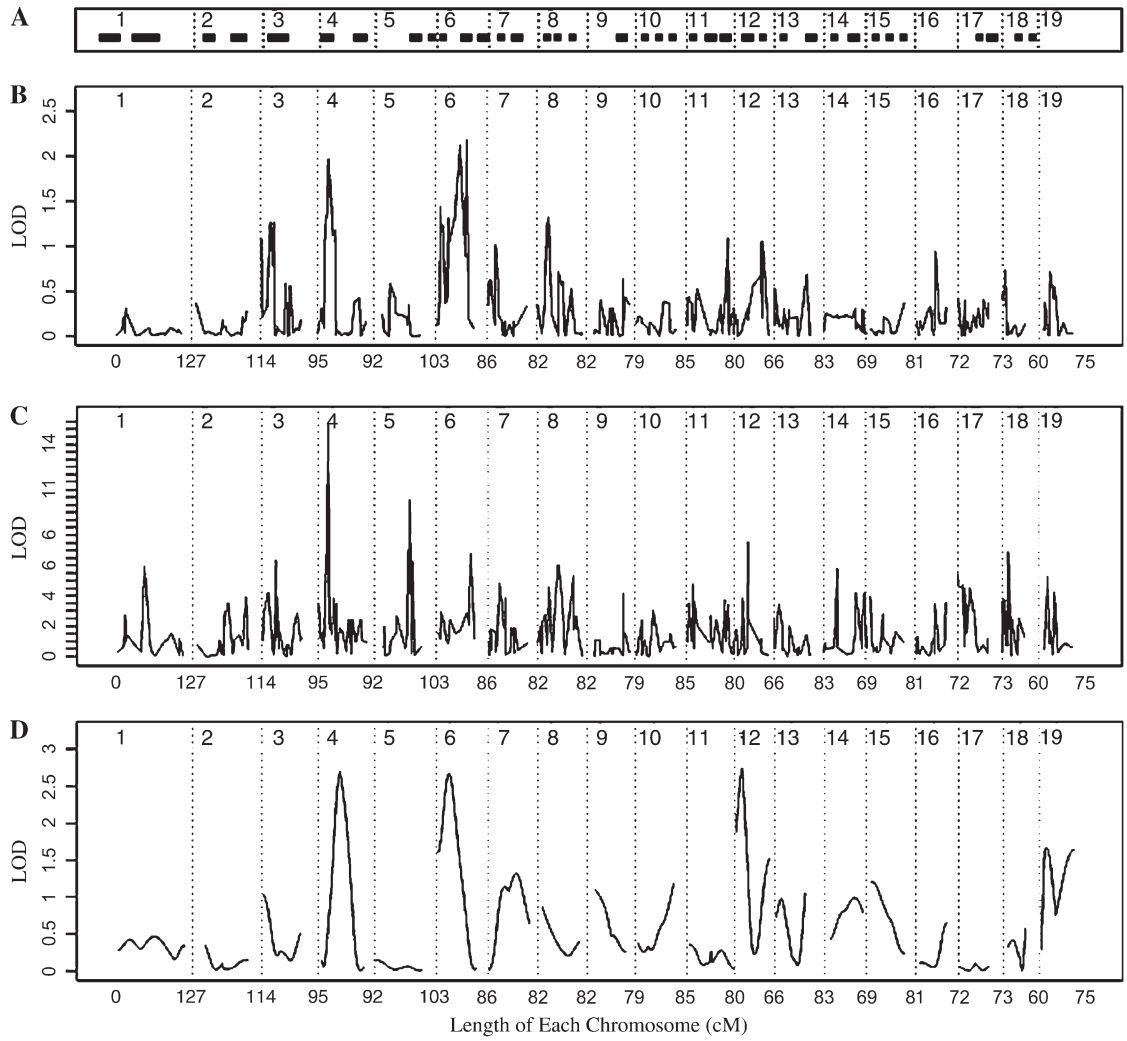

FIGURE 6.—Localization of body weight QTL. Results for are shown (B) RIs and (C) RIXs generated from CXB and (D) F_2's from the same parental strains compared to (A) locations of known body weight QTL. Body weight data were adjusted for age, sex, and the interaction between age and sex. Lines in A are regions known to harbor body weight QTL detected in crosses from many different strains. Lines in B–D represent LOD scores. Dotted lines distinguish individual chromosomes. The significance thresholds determined from permutations are not marked since they are higher than any of the resulting curves.

are 0.094 and 0.076, respectively. Similarly, for brain weight, the standard deviations for RIs and RIXs are 0.030 and 0.026, respectively, after adjusting for the effects of sex, age, and body weight. The difference in trait variation between RIs and RIXs suggests that fewer RIX individuals are needed compared to RIs to minimize nongenetic variance (CRUSIO 2004). Thus maternal modulation and developmental noise will have a greater impact on standard RI line means than on the hybrid RIX progeny, probably because the hybrid F_1's as described are demonstrably better buffered against nongenetic sources of variation.

DISCUSSION

Recombinant inbred intercrosses, produced by generating all or a subset of the potential F_1 hybrids between pairs of RI lines, increase the number of available genotypes from L RIs to $L(L-1)/2$ nonreciprocal RIXs or $L(L-1)$ using the reciprocal RIXs. RIXs do not need to be genotyped since their genotypes can be inferred from the parental RIs. Similar to the parental RIs, experimental error and environmental variance can be greatly reduced by testing many isogenic RIX animals and data are cumulative, enabling multivariate analyses across phenotypes, environmental conditions, and developmental timing. Unlike the parental RIs, the genetic structure of an RIX resembles that of an F_2 animal, reducing phenotypic anomalies associated with inbred genomes. Likewise, a set of RIXs closely resembles a set of F_2 progeny, with a 1:2:1 segregation ratio of genotypes permitting both additive and dominance effects to be detected and measured. Unlike either RI or F_2 populations, parental origin effects on phenotypic variance can be easily de-

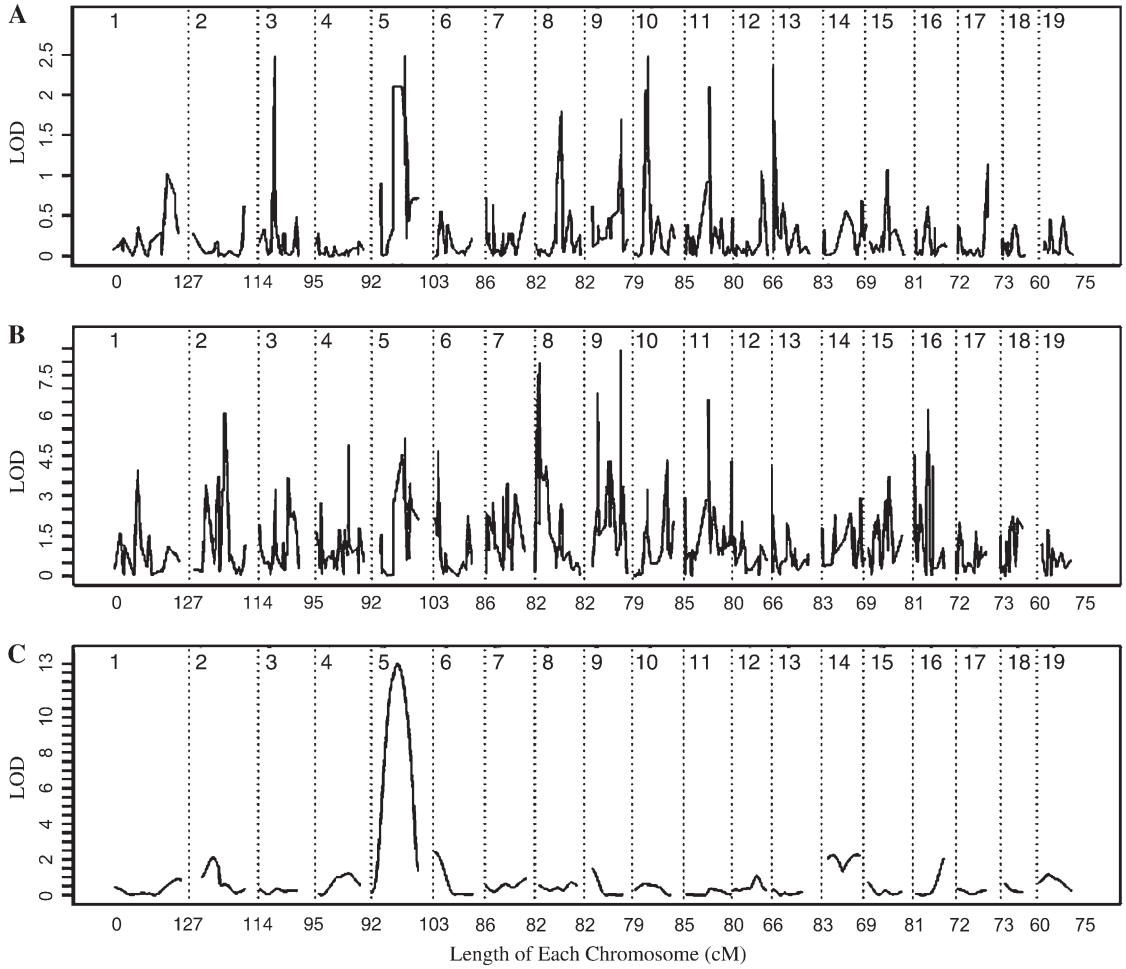

FIGURE 7.—Localization of brain weight QTL. Results for (A) RIs and (B) RIXs generated from CXB and (C) F_2's from the same parental strains are shown. Brain weight data were adjusted for age, body weight, sex, and the interactions between age and sex and sex and body weight. Lines represent LOD scores. Dotted lines distinguish individual chromosomes. The significance thresholds determined from permutations are not marked since they are higher than any of the resulting curves.

tected using RIXs generated from reciprocal crosses between RI pairs. All these attributes suggest that the RIX approach will be highly useful for many traits, particularly those that cannot be genetically dissected with other mapping populations.

A similar approach using RI hybrids to generate immortal F_2 populations has been performed in rice (HUA *et al.* 2003). However, unlike the situation in mice where limited numbers of RI lines are available, immortal F_2's can be generated from combinations of rice RI lines that are randomly mated such that no parental sharing occurs in the RIXs. The analysis of this type of population structure is identical to that for an F_2 population and, as such, does not require crosses with parental sharing or a unique permutation analysis like that proposed here.

Although single-marker analysis was used in our simulations, the relative high marker density of the parental RI, and thus RIX, supports results similar to those that would be obtained using more complicated mapping methods, such as traditional interval mapping (LANDER and BOTSTEIN 1989) and regression interval mapping (HALEY and KNOTT 1992). Also in our simulations, we assume no maternal or paternal effects and thus only nonreciprocal RIXs are simulated. However, if maternal or paternal effects are suspected, reciprocal crosses can be generated and tested for those effects.

From our simulations, we can conclude that the higher the number of parental RI strains, the greater is the chance to separate the major QTL effects from polygenic effects. Furthermore, due to the low number of parental CXB lines, we find that the polygenic effects frequently correlate with unlinked markers and largely elevate the *F*-statistic or likelihood-ratio statistic under the null hypothesis, largely a result of nonsyntenic associations observed in small RI panels that will also be present in RIX progeny (WILLIAMS *et al.* 2001); thus a more stringent threshold is needed to control type I error. However, the low number of parental strains also interferes with the permutation procedure, producing a very high threshold and essentially making the mapping method have zero power. On the other hand, with the

TABLE 2

Comparison of phenotypes from reciprocal RIX offspring

RIX cross	No. of mice	Reciprocal RIX cross	No. of mice	*P*-value body weight	*P*-value brain weight
1 × 2	26	2 × 1	14	*0.005*	0.997
1 × 4	16	4 × 1	51	0.094	0.503
2 × 4	20	4 × 2	38	0.120	0.288
3 × 6	22	6 × 3	48	*0.0004*	0.166
3 × 10	35	10 × 3	18	*0.000006*	*0.0002*
5 × 6	32	6 × 5	16	0.614	*0.0005*
5 × 7	18	7 × 5	15	0.302	*0.000007*
6 × 10	53	10 × 6	21	*0.005*	0.046
10 × 11	35	11 × 10	12	0.382	0.082
10 × 12	31	12 × 10	11	0.514	0.101

Only RIX crosses with at least 10 offspring from each reciprocal cross are shown. Italics indicate *P*-values <0.005.

larger number of parental AXB/BXA and BXD lines, the influence of polygenic effects on the LOD score under the null hypothesis is much smaller. Additionally, the permutation procedure is slightly conservative and appropriately controls the type I error.

The difference in strategy represented by RIX and RI lines was anticipated by Knapp and Bridges (1990). Their work primarily pertained to plant genetics, where sets of RI lines exist that number in the hundreds; this provides a choice between changing the number of RI strains in a QTL mapping experiment and changing the number of individuals per strain. Knapp and Bridges argued that, for any given QTL model consisting of a specified number of QTL at specified locations, the trait variance can be divided into three components: (1) the variance explained by the QTL in the model, (2) the variance explained by QTL not in the model, and (3) nongenetic variance. Furthermore, they showed that increasing the number of RI strains would decrease variances 2 and 3, whereas, increasing the number of individuals per strain would decrease only variance 3. Further work suggested that the number of F_2 individuals required to produce a similar power provided by a panel of RIs is inversely proportional to the heritability of the trait in the RI lines (Belknap 1998). Consequently, a major benefit of RIXs is expected for QTL with low heritabilities.

Previous studies have suggested that the effectiveness of RI strains in identifying and mapping QTL is limited. Our simulations imply that with RIXs, caution is needed as well, especially when starting with small numbers of parental RI lines, because of nonsyntenic associations between independent RI lines as described above. For example, CXB may not be a good source for genome-wide QTL mapping, using either RI or RIX. Nonetheless, the RIX approach, even for small numbers of RI lines, is still suitable for ad hoc testing of specific allele combinations to support other genetic data; this is achieved by making virtual, segregating congenics in the target interval. In general, the larger the number of parental RIs, the greater the power is for mapping QTL and with RI panels equal to or larger in size to the 22 AXB/BXA lines, RIXs can provide substantially increased power, particularly in the presence of dominance and most likely also with complex epistatic interactions as previously demonstrated with the immortal F_2 populations in rice (Hua *et al.* 2003).

A variation of the immortal F_2 is to use combined crosses sharing at least one parent in common and that generally improve the power of QTL mapping (Liu and Zeng 2000; Zou *et al.* 2001). Since all three RI panels used in our simulation studies share one common parent, C57BL/6, we plan to extend the mixed model described here to handle RIX crosses generated from multiple RI panels. We predict that by using multiple RI sets, the increase in the number of parental RIs will better differentiate the major QTL from the polygenic effects.

Another major use of the RIX approach will be with the collaborative cross (CC) proposed by the Complex

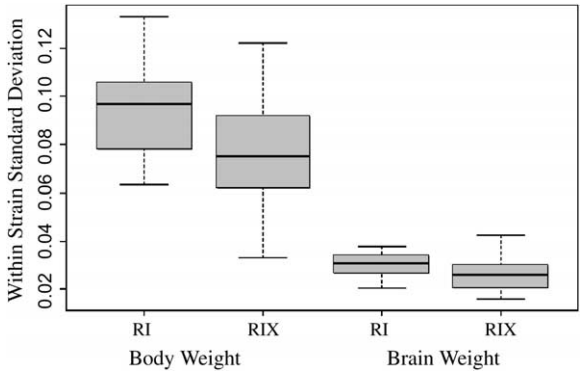

Figure 8.—Distribution of phenotypic variance. Within-strain variance for body and brain weights from RIs and RIXs generated using the CXB phenotypic data. Data were adjusted as described in Figures 6 and 7. Plots represent the range of maximal standard deviations within each representative set while shaded boxes show mid-50th percentiles and boldface lines show the means.

Trait Consortium (CTC) (Threadgill *et al.* 2002). A major goal of the CTC is to establish a community resource that consists of 1000 multiparental RI lines that will support complex trait analysis. With such a large pool of RI lines, immortal F_2's could also be considered.

In addition to the examples provided above, RIX panels will significantly improve our ability to genetically dissect complex gene-environment interactions. The ability to replicate large numbers of different genomes will facilitate the genetic dissection of epidemiological characteristics that until now could be described only at the population level. Rather, the identification of specific genomic regions interacting with environmental variables will allow population partitioning to test the level of the interaction on defined groups on the basis of genotype. Classical uses of recombinant model organisms have primarily been used to study very coarse traits like morphometric characteristics or response to nongenetic factors. However, with the development of sophisticated quantitative molecular tools like gene expression profiling with microarrays and proteome analysis with mass spectrometry, traits can be dissected at the molecular level through genetical genomics (Jansen and Nap 2001). Because of the innate noise present between individuals using these high-throughput approaches, having the ability to replicate individuals, such as can be done with RI and RIX genotypes, will dramatically improve the sensitivity for detecting and genetically defining *cis*- and *trans*-regulated gene interaction networks. Other studies that are currently not possible because of the limited number of replicable genomes (RI, inbred) or because of high phenotypic variation include traits with low heritabilities and those that have multivariant characteristics.

Although not computed here, the marginal averages of each RI strain could be used to generate haplome phenotypes to test the robustness and influence of genetic background on specific allele combinations. This could be achieved by using the mean phenotypic values for each parental RI line averaged across all RIX progeny of the RI instead of the actual RI strain value. For example, the average of RIX_{12}, RIX_{13}, . . . , RIX_{1L} could be used as the trait mean for RI_1.

No other population structure, such as CSS lines, RCS, or heterogeneous stock lines, provide the robust breadth of unique genomes in combination with the ability for genome replication. Like RIXs, these other mapping populations have particular strengths and weaknesses. However, the global advantage of RIXs over these other populations for complex genetic structures is due to their unique combination of replicability and broad genetic representation of a defined genetic background that will be critical when expanded studies are brought to fruition to address complex genetic and nongenetic trait architectures.

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