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Genes and Phenotypes

QTL fine-mapping with recombinant-inbred heterogeneous stocks and in vitro heterogeneous stocks

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Abstract

We compared strategies to fine-map Quantitative Trait Loci (QTL) in mice with heterogeneous stocks (HS). We showed that a panel of about 100 Recombinant Inbred Lines (RIL) derived from an HS, and which we called an RIHS, was ideally suited to fine-map QTL to very high resolution, without the cost of additional genotyping. We also investigated a strategy based on in vitro fertilization of large numbers of F_1 offspring of HS males crossed with an inbred line (IVHS). This method required some additional genotyping but avoided the breeding delays and costs associated with the construction of an RI panel. We showed that QTL detection was higher by using RIHS than with IVHS and that it was independent of the number of RI lines, provided the total number of animals phenotyped was constant. However, fine-mapping accuracy was slightly better with IVHS. We also investigated the effects of varying the number of HS generations and using multiallelic microsatellites instead of SNPs. We found that quite modest generation times of 10–20 generations were optimal. Microsatellites were superior to SNPs only when the generation time was 30 or more and when the markers were widely spaced.

Inbred line crosses have proved invaluable for the detection of genetic loci that contribute to variation in quantitative phenotypes, but they have not been so useful in subsequent steps to identify the responsible molecular variants (Flint and Mott 2001). One obstacle to progress has been the difficulty of obtaining sufficiently high resolution genetic mapping to enable positional cloning strategies while at the same time minimizing costs.

Mapping resolution depends on the number of recombinants present in the mapping population and on the effect size of the quantitative trait locus (QTL). The latter is frequently small, accounting for less than 10% of the phenotypic variance for the majority of behavioral traits, so that high-resolution mapping of such QTL requires many thousands of recombinants. One way of obtaining sufficient numbers is to use populations which have accumulated recombinants over time. For instance, advanced intercross lines (AIL; Darvasi and Soller 1995) are formed by intercrossing two inbred lines for about ten generations until the resulting chromosomes are a mixture of the two founder haplotypes. AILs preserve the genetic simplicity of the intercross and backcross designs, since all alleles derive from one or other of the founding strains, but take years to breed and can only map QTL that happen to have different alleles in the founding strains.

Heterogeneous stocks (HS) (Demarest et al. 2001; McClearn et al. 1970; Talbot et al. 1999, 2003) are an alternative form of AIL, in which eight founder strains are intercrossed for a larger number of generations (between 30 and 60) while keeping a population of 40 mating pairs in each generation (to reduce the fixation of alleles by drift), resulting in chromosomes that are a very fine mosaic of the founders haplotypes. The average distance between recombinants in a 60-generation HS is only 100/60 = 1.7 cM. Like AIL, HS take time to be established, but their derivation from eight rather than two founding strains means there is a better chance that a QTL will segregate in an HS than an AIL. Therefore, one HS could be used to map many different traits.

Greater genetic complexity in the HS brings with it greater problems of analysis. Since the number of alleles segregating at any one locus is

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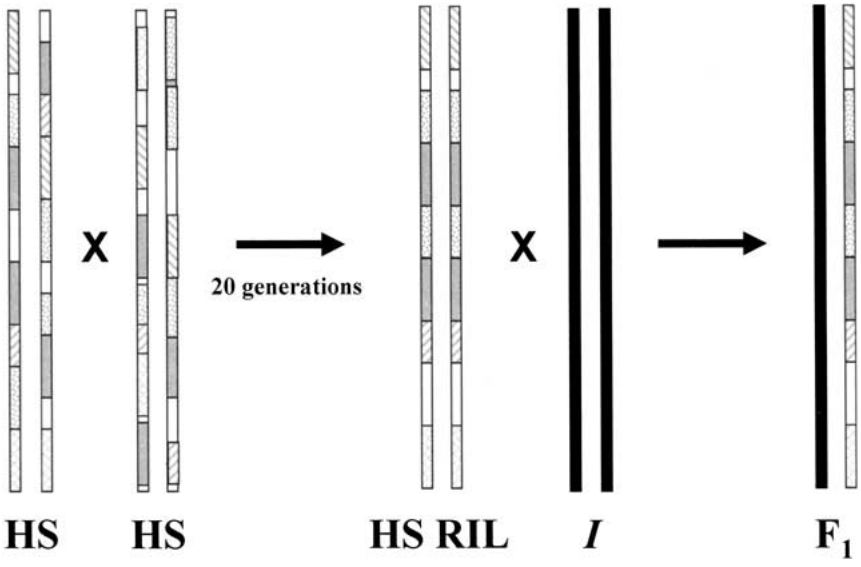

Fig. 1. Outline of the RIHS scheme. Each vertical bar represents a chromosome. HS chromosomes are a mosaic of founder chromosomes. Inbred chromosomes are represented as black bars. Each pair of homologous chromosomes in the F_1 generation comprises one HS chromosome and one inbred chromosome. Each RIHS animal is mated to as many inbreds as necessary to achieve the desired sample size.

unlikely to equal the number of founding strains (most microsatellites have between three and four alleles in a typical HS), it is not straightforward to distinguish alleles that are identical by state from those that are identical by descent (for instance, two microsatellite alleles of 100 bp may derive from two different inbred strain progenitors). Fortunately this problem can be solved to some extent: HS chromosomes are mosaics derived from *known* inbred lines, so it is possible to infer the ancestry of any locus on the genome, expressed as the probability the locus is descended from each founder (Mott et al. 2000). To detect QTL in the HS, we type markers, whose alleles are known in the founder strains, at high density, and perform a multipoint analysis with the dynamic-programming algorithm implemented in the HAPPY package (http//www.well.ox.ac.uk/happy). We test for the presence of a QTL in each marker interval by analysis of variance.

Sufficient recombinants for high-resolution mapping may also be available through the analysis of a large number of recombinant inbred lines (RIL). The advantage of this approach is that once RILs have been genotyped, the costs of mapping the QTL are reduced to the cost of phenotyping animals, but sub-centiMorgan mapping of small-effect QTL will require the creation of an RI set numbering many hundreds.

Here we investigated and compared strategies for reducing genotyping costs in experiments where either HS, or RIL derived from HS (RIHS), was crossed with an inbred line. We were particularly interested in methods that fine-map genetic modifiers of a mutant or knock-out inbred line, and we investigated the effects of varying HS generation time, marker spacing, and choice of microsatellite or SNP marker.

We present simulation results for the scenario in which a QTL had previously been detected and localized to a 25-cM interval, for instance by an F_2 intercross, to fine-map the QTL to sub-centiMorgan resolution.

Experimental design

RIHS. In this scheme (see Fig. 1) a set of N recombinant inbred lines are constructed from a heterogeneous stock by taking N male-female pairs from the current HS generation and performing brother-sister matings until the N lines are inbred. Here we consider populations of $N = 40, 80, 120, 180$ RIHS constructed from a 30-generation HS maintained from 40 mating pairs.

Once the RIHS lines have been established, each line is genotyped once at very high density across the entire genome, and the data are made publicly available. No further genotyping is then required, but the advantage has to be offset against the cost of establishing and genotyping the inbred strains. RIHS allow finer-scale mapping with fewer animals compared with standard RI because each RIHS is a homozygous HS animal; consequently the data can be analyzed with the same methodology as an HS experiment, taking advantage of the multiple ancestral progenitors.

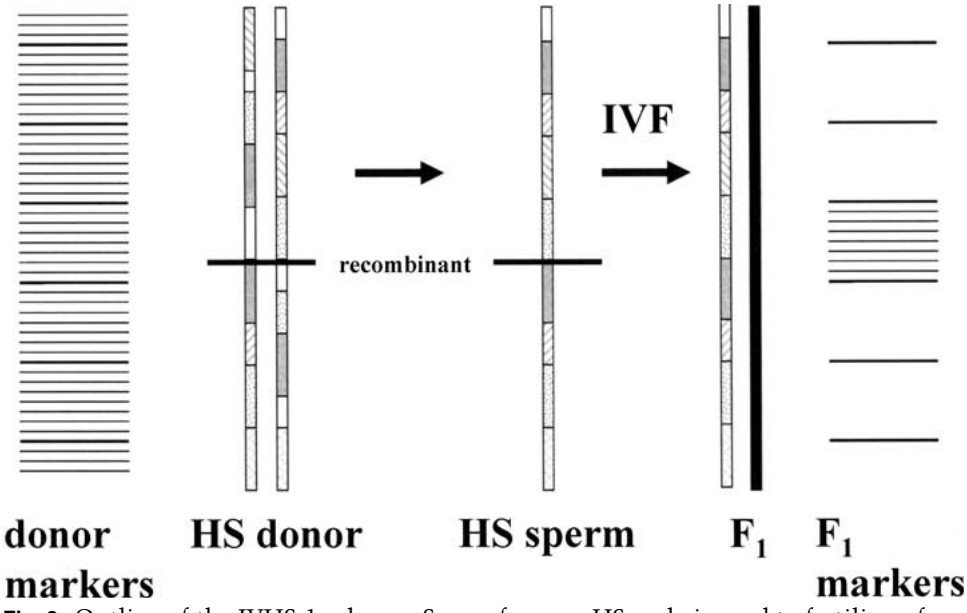

Fig. 2. Outline of the IVHS-1 scheme. Sperm from an HS male is used to fertilize a female inbred, by in vitro fertilization. The donor is genotyped at high resolution, but only a sparse skeleton of markers is typed on the F_1 generation in order to determine the location of recombinants.

One important application is to map modifiers of, for example, a gene knockout. This may be accomplished by crossing the RIHS with an inbred line *I* carrying the knockout to form an F_1 generation, which are phenotyped (Fig. 1). The genotypes of the F_1 can be deduced from the known genotypes of the RIHS and *I* without further cost.

IVHS. We consider two alternatives to RIHS. Both strategies use in vitro fertilization by HS males (hence IVHS) of females from an inbred line (Nakagata 2000), or simply mating fully genotyped HS males to multiple females. The choice of in vitro fertilization over mating will depend on the availability of densely genotyped HS males. We envisage a situation where HS animals are genotyped, their sperm collected and distributed to participating laboratories, and for this reason describe the approach as IVHS.

The F_1 offspring of IVSH are phenotyped. The first IVHS strategy involves genotyping the F_1 chromosomes and is referred to here as IVHS-1; the second strategy requires no genotyping and is termed IVHS-2.

IVHS-1 (see Fig. 2) is a form of progeny testing in which QTL segregating in the F_1 generation are mapped. We assume here that we have sperm from 40 HS mice (i.e., 80 sets of chromosomes) and can generate up to 40 offspring from each sperm sample (to give a maximum sample size of 1,600 animals).

A pair of homologous F_1 chromosomes comprise a fixed chromosome, *I*, descended from the inbred dam (and which can, therefore, be ignored as it contributes no genetic variation) and a variable HS chromosome, so an F_1's genotypes can be determined from that of the sperm. Furthermore, the genotype at a haploid sperm locus must be one of two possible diploid HS sperm donor haplotypes; to determine which one, it is only necessary to type markers at high density on the sire together with its parents. Then each sperm/F_1 genotype can be inferred by performing a few additional skeleton genotypes on the F_1 to identify the locations of recombinants in the meiosis that produced the sperm, in order to tell which sire chromosome is present at a given locus in the sperm.

Skeleton markers spaced approximately 10–20 cM apart are typed on the F_1 individuals across the region of interest defined in a previous QTL detection experiment, such as an F_2 intercross. Markers should be chosen so that they are heterozygous in the sire and can, therefore, identify a recombinant in the sperm, hereafter referred to as a new recombinant. Inconsistent flanking genotypes reveal the new recombinant. Alleles at the intervening markers can then be deduced without further genotyping, except in those intervals containing a new recombinant, where further genotyping is required to identify the position of the recombinant.

The optimal marker spacing can be estimated by the following argument. In the sires, in a region of length *G* centiMorgans, equally spaced markers, *r* cM apart, are typed at very high density ($r = 0.2$ cM, for instance). Genotyping in the F_1s occurs in two rounds: initially a skeleton of equally spaced mark-

ers are typed at lower density spaced, say, R cM apart; i.e., the region is divided into about G/R intervals of length R. Under the assumption of the Haldane mapping function, the probability that such an interval contains at least one recombinant, and therefore requires retyping at higher density r, is $p = (1-e^{-R/100})$. Therefore, the expected number of intervals that contain a recombinant is Gp/R, and the average total number of markers typed in an F_1 animal will be the sum of the number of skeleton markers and the number of recombinant intervals, multiplied by the number of high density markers per interval.

$$G/R + (Gp/R)(R/r) = G(1/R + p/r)$$

Treating r as fixed, simple calculus shows this expression is minimized when R satisfies

$$R^2 e^{-R/100} = r$$

Using this equation, we find that a 10-cM intermarker spacing is near-optimal in most cases.

Our second strategy, IVHS-2, avoids genotyping the F_1 animals by treating the choice of paternal chromosome as missing data. However, it does still require the male sperm donors to have been fully genotyped. The simplest analysis, adopted here, is to equate the expected trait value at a given locus in an F_1 animal to the mean of the locus trait values in the donor. Suppose that at a particular locus x, the HS donor sire n of an F_1 mouse has two known haplotypes, and that the probability that each haplotype is descended from founder strain s is p_{1nxs}, p_{2nxs} respectively. These probabilities may be estimated from nearby marker genotypes by using a dynamic-programming algorithm. Then, in the absence of genotype data, the probability that either HS sire chromosome is transmitted is ½, so the probability that the HS-derived chromosome in the F_1 is descended from founder strain s is

$$q_{nxs} = (p_{1nxs} + p_{2nxs})/2$$

One can test for differences between the founder strain effects at the locus by ANOVA.

Methods

We evaluated the strategies RIHS, IVHS-1, and IVHS-2 by simulation. We considered a 25-cM chromosomal region, containing 100 diallelic SNP markers spaced 0.25 cM apart. A diallelic additive QTL was placed randomly, midway between two adjacent SNPs. An HS population was simulated, derived from eight inbred lines intercrossed for 30 generations, with 40 mating pairs selected at each generation; sibling matings were avoided. One-half of the HS founders, chosen at random, carried an increaser QTL allele, and the other half a decreaser allele. For construction of the RIHS, we simulated brother-sister mating in HS families for 20 generations.

From the final generation, 40 non-siblings were selected and mated with an inbred line to produce an F_1 generation, which was phenotyped. The percentage of additive genetic variance attributable to the QTL was fixed by adjusting the effect size of the QTL allele; if the proportion of HS chromosomes carrying an increaser allele of size $+a$ in the F_1 generation is p, then the additive variance is $V = p(1-p)a^2$. Those simulation runs where the QTL went to fixation (about 5%) were discarded (note that we expect a similar proportion of experiments to suffer this fate). Simulations were performed for which V = 1%, 2%... 10%, 20%... 50% of the total variance; the environmental variance component was sampled from a normal distribution. The number of F_1 individuals varied from 400 to 2000 in steps of 400. RIHS were performed for 40, 80 and 120 lines; a small number of runs with larger numbers of lines was also investigated.

For each simulation a HAPPY analysis (Mott et al. 2000) was performed to identify the marker interval with the most significant ANOVA P-value. In the HAPPY analysis of both RIHS and IVHS-1, the genotype data can be thought of as being homozygous at every marker locus, since the inbred line contributes nothing to additive genetic effects. For IVHS-2, in order to reflect the uncertainty in the genotypes, each F_1 genotype was set equal to that of the sire. The procedure was repeated 1000 times for each parameter combination.

As in Mott and Flint (2002), simulations were assessed in two stages. First, the detection rate was measured as the percentage of simulations in which the most significant marker interval had a P-value <0.0001, i.e., an overall region-wide P-value <0.01. This procedure is conservative in that the tests are not independent; however, results with a zero QTL effect size imply the pass rate is close to 1% in the case of no QTL. For those runs in which a QTL was detected, accuracy was measured both as the 90th percentile of the distribution of the distance between the true and predicted QTL interval, and as the mean distance between the true and predicted trait location. In passing, we point out that the HAPPY program includes an option for estimating the confidence intervals of a QTL by bootstrapping.

To investigate factors affecting mapping accuracy, we performed a three-way simulation experiment to fine-map a QTL in a 25-cM interval, using the

IVHS-1 design (i.e., all F_1 animals genotyped) with (i) either SNP (diallelic) markers or multiallelic microsatellites, modeled to have strain/allele frequencies typical of those observed in HS data with a mean of 3.80 alleles per marker; (ii) markers spaced 0.25, 0.20, 0.15, 0.10, 0.05, 0.025 cM apart; (iii) HS populations of 40 mating pairs that were outbred for 5, 6,..., 15, 20, 30, 60 generations. The QTL effect size was held constant at 5%, and the number of F_1 individuals was fixed at 1200. One thousand simulations were performed for each parameter combination.

Results

Detection rates and mapping accuracy. We show results from an HS that contained 40 mating pairs. For IVHS this means that sperm from one male of each pair was used, allowing for up to 40 offspring from each insemination. In the RIHS strategy, 40, 80 or 120 inbred strains were derived from the 40 HS pairs. Then equal numbers of offspring from each inbred line were bred up for phenotyping.

Because of space limitations, we present only a subset of our data. Full results of the simulations are available as supplemental data from http://www.well.ox.ac.uk/happy/strategies.shtml

Figure 3 shows the detection rate for QTL explaining from 5 to 10% of the phenotypic variance. We show simulations for three different sample sizes of 400, 800, and 1,600 animals. Three important results are demonstrated. First, it is clear that the recombinant inbred strategies perform better than the IVHS, although typing the F_1 does give results close to those of the RIHS. Second, the results are relatively insensitive to the increase in sample sizes shown here, at least for QTL accounting for more than 5% of the total variance. The RIHS methods have detection rates of over 90% with 400 animals. Third, increasing the number of RIHS lines above 40 does not give a noticeable increase in power. Together, the results indicate that 400 F_1 animals from a cross using 40 RIHS lines have adequate power to detect relatively modest QTL effects (less than 10%).

We next investigated the mapping accuracy and again report results for three sample sizes (400, 800, and 1,600). Figure 4 displays average mapping error rather than the 90% confidence interval tabulated in Mott and Flint (2002); although the two measures are similar, the mean measure is more sensitive to the effects of varying the simulation parameters.

The simulation results indicate that a moderately large set of RIHS (120 or more) or the IVHS-1 strategy will deliver sub-centiMorgan mapping. For QTL accounting for more than 5% of the total variance, sample size does not greatly influence mapping resolution, so that it will be possible to fine-map relatively small effect QTL with a few hundred animals. However, even with a sample size of 1,600 animals, an RIHS of 40 animals does not achieve a mean mapping error under 1 centiMorgan. Accuracy increases as the number of RI lines increases, with an almost twofold improvement from 40 to 120 lines. Importantly, the simulations indicate that, in those cases where the QTL is detected, even without genotyping the F1 (IVHS-2), we obtain equivalent resolution to the recombinant inbred lines.

Marker type and marker density. For investigations into marker accuracy, we used the IVHS-1 strategy (all F_1 animals genotyped), fixed the QTL size at 5%, and restricted the search to a 2.5-cM interval. The number of individuals for these simulations is fixed at 1200, providing extremely high resolution: as Fig. 4 and results presented above show, the mean mapping error associated with this choice of parameters is less than 0.5 cM. Figures 5 and 6 show the results of the investigation into factors affecting mapping accuracy.

We found that microsatellites, that is to say, markers with more than two alleles, provide more accurate map locations than SNPs, but that the difference vanishes when the inter-marker distance is less than 0.05 cM (Fig. 5). Accuracy improves with increasing marker density, as expected: markers spaced 0.025 cM apart should give accuracy of 0.05–0.1 cM. However, we were surprised to see that increasing the number of generations for HS production beyond 15 has a detrimental effect on mapping accuracy: for the first 10 generations, mapping accuracy increased, remained relatively constant for the succeeding five generations, and thereafter deteriorated (Fig. 6). The optimum time is 10–20 generations, although even a five-generation HS performs surprisingly well.

Discussion

We have presented a new method suitable for fine-mapping the small effects that are likely to be responsible for genetic modifiers in rodent inbred strain crosses. For fine-mapping we show that it is possible to achieve considerable savings in genotyping costs without losing mapping resolution by using either recombinant inbred lines derived from heterogeneous stocks or sperm from outbred HS. Both these strategies are quick, requiring the analysis of the F_1 generation, assuming that appropriate RI animals or sperm from genotyped HS animals is available. Both strategies can deliver

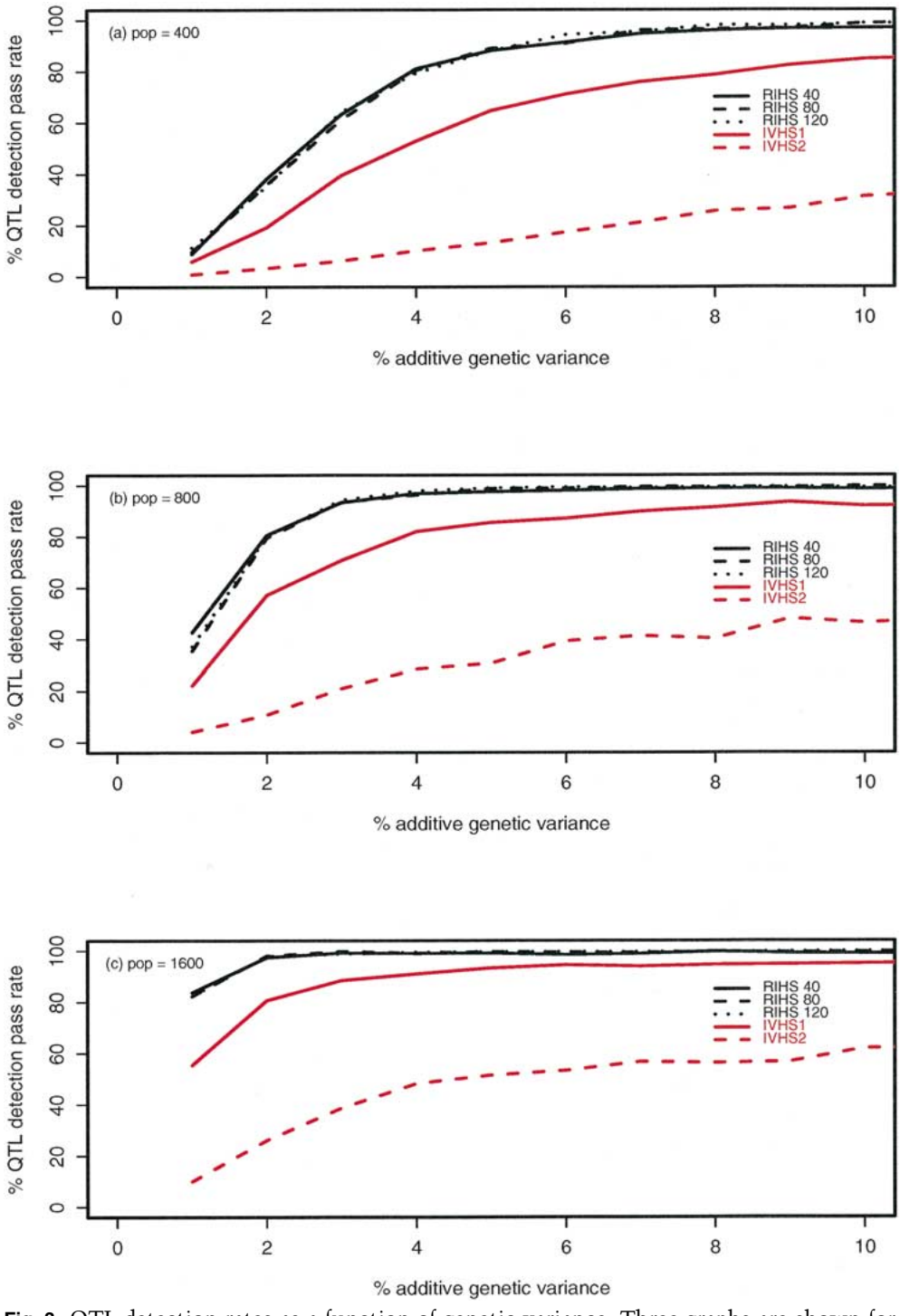

Fig. 3. QTL detection rates as a function of genetic variance. Three graphs are shown for 400 (a), 800 (b), and 1,600 (c) F_1 animals. The percentage additive variance that the QTL contributes to the phenotype is shown on the horizontal axis and the detection rate on the vertical access. We show results for RIHS of 40, 80, 120 lines and for the two IVHS strategies (IVHS-1 and IVHS-2). Each data point is the mean pass rate from 1000 simulations at region-wide significance level of <1%.

sub-centiMorgan mapping resolution. We caution that these conclusions are based on the assumption that an interval contains a single QTL; in practice, fine-scale mapping may reveal a more complex genetic architecture that will be difficult to dissect with the strategies presented here.

Although the strategies presented here are intended to fine-map QTL previously detected in other

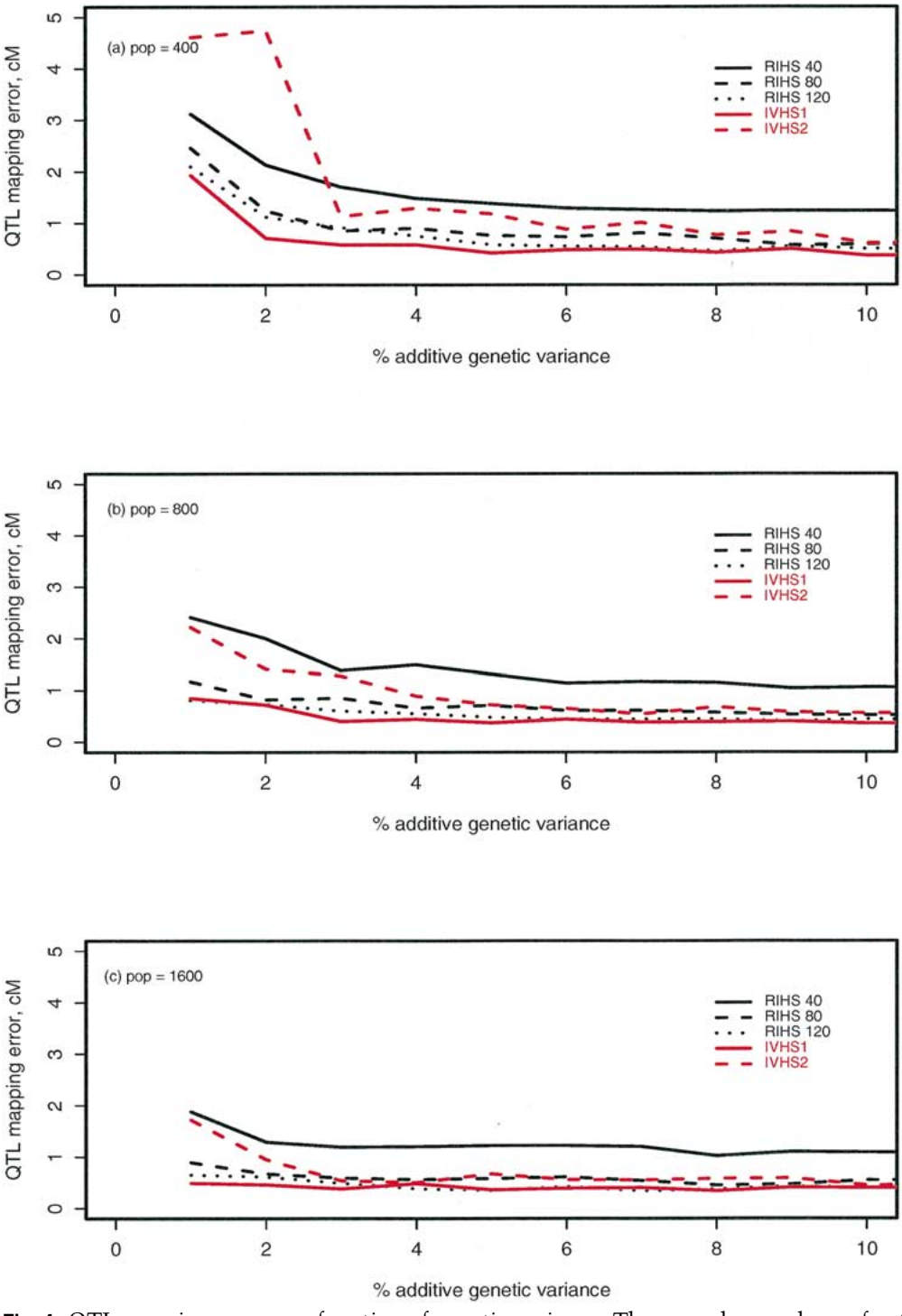

Fig. 4. QTL mapping error as a function of genetic variance. Three graphs are shown for 400 (a), 800 (b), and 1,600 (c) F_1 animals, for RIHS (40, 80, 120 lines), IVHS-1, and IVHS-2. In all cases, 1200 F_1 animals were phenotyped. The measure of mapping error displayed is the mean error over 1000 simulations, measured in cM intervals.

experiments, we note that it would be possible to carry out a genome-wide search, provided the number of animals were increased to around 2,000 to account for the increased number of markers tested. However, even with large numbers of animals, the method would be limited to the detection of QTL with relatively large effects.

A critical question is: which of the methods we have discussed is most appropriate for fine-mapping? The ideal resource for a large number of mapping ex-

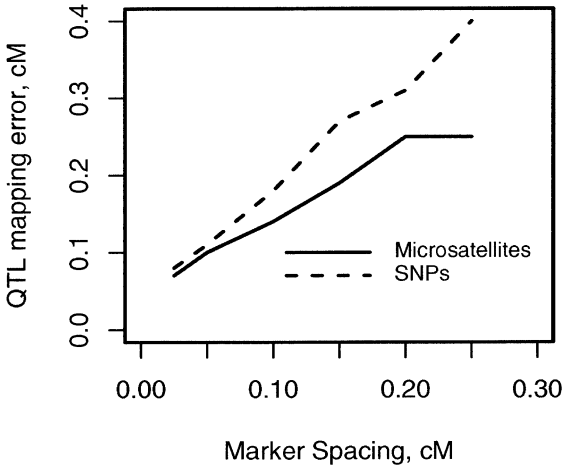

Fig. 5. The effect of marker spacing (horizontal axis) on mapping accuracy. Results are shown for a microsatellites (black line) and SNPs (dotted line). The analysis is for an IVHS-1 design, using 1200 animals, a QTL accounting for 5% of the variance and markers spaced [- - -] between 0.025 cM and 0.25 cM over a 3 cM region.

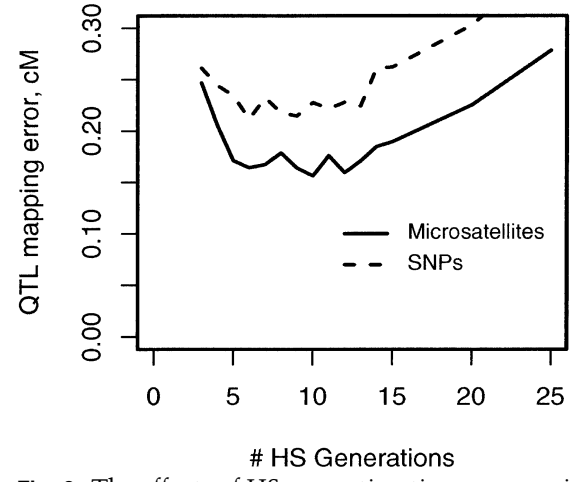

Fig. 6. The effects of HS generation time on mapping accuracy. The number of generations used to create the HS is shown on the horizontal axis and the mean mapping error on the vertical axis. The analysis is for an IVHS-1 design, using 1200 animals, a QTL accounting for 5% of the variance and markers spaced 0.025 cM over a 2.5 cM region. Results are shown for a microsatellites (black line) and SNPs (dotted line). Each data point is based on 1000 simulations. QTL effect size was 5%.

periments will be access to a large number of RIHS: our simulations show that of the order of 100 lines will be needed to provide sub-centiMorgan mapping resolution. Making and genotyping such a resource will require a considerable investment in time and resources and is unlikely to be justified for a single fine-mapping experiment. However, once the RIHS are available and have been genotyped, they provide a way of fine-mapping QTL with no additional genotyping.

A much cheaper and quicker strategy is to genotype a set of 40 HS mating pairs, collect the sperm, and then use this resource for mapping. Alternatively, depending on the costs of acquiring HS animals, the appropriate F_1 can be obtained by breeding from genotyped HS males. The major investment is the genotyping costs. Sperm freezing, followed by the production of large numbers of half-sibs though in vitro fertilization, is common in commercial animal breeding, and indeed a QTL for milk yield in cattle has been mapped by exploiting this fact (Coppieters et al. 1998; Riquet et al. 1999).

The IVHS design proposed here is ideal for fine-mapping modifiers of knockouts, in which the knockout is used as the inbred line. We have investigated two versions, one where two rounds of genotyping (full genotyping of the sires and limited regenotyping of the F_1 to identify recombinants) are required, and the other in which no offspring genotyping is performed, at the cost of a considerable reduction in power. However, its lower cost may make it attractive.

We investigated three variables that affect mapping accuracy: marker choice, marker density, and generation time in the production of HS. Microsatellites have been the traditional choice of marker in the mouse, although diallelic single nucleotide polymorphisms (SNPs) are likely to become the standard because SNP-typing technology is now as cheap, and superior in throughput and allele-calling accuracy, as microsatellite typing. Microsatellite markers are thought to be superior to SNPs for HS analysis because they contain more alleles and can distinguish between the eight HS founders more easily; the microsatellite markers we have used contain about four alleles on average. On the other hand, a multipoint analysis, such as that performed by HAPPY, can combine information from neighboring markers, so the circumstances under which SNPs or microsatellites are preferable are unclear. We have shown that microsatellite markers perform better than SNPs, but that at very high marker densities this advantage is lost; the optimum marker density will depend on the experimental design, particularly the number of generations required for the HS generation; under the conditions examined here, we find that SNPs and microsatellites provide equivalent mapping resolution at a 0.025 cM density.

Our investigation of the effect of generation time on mapping resolution indicates that there is an optimal generation time for creating a suitable HS. Simulations in which HS generation time was varied (Fig. 5) suggest that only modest generation times (10–15) are required to obtain high mapping precision; moreover, that large generation times actually result in a reduction in accuracy. The latter phenomenon is

most probably due to genetic drift, tending to fix the marker alleles, because using microsatellite markers instead of diallelic SNPs offsets the effect to some extent. Consistent with Darvasi's work on AIL (Darvasi and Soller 1995), we find that most of the improvement in mapping accuracy occurs during the first 10 generations; in order to obtain the very high resolution mapping (less than 0.5 cM) that is available with the strategies described here, it will be necessary to obtain an HS of the appropriate generation number. However, for less stringent mapping applications, this restriction on the use of HS may not be so important.

Despite the attractions of the IVHS strategy, in the longer term the RIHS design is preferable. Our simulations demonstrate the utility of constructing a panel of recombinant inbred lines from an HS population. The strategy is attractive in that a relatively small number of lines is required: 120 lines should be more than sufficient both for QTL detection and fine-mapping, and indeed 80 lines would be useful. However it is important to note that we have focused on mapping individual QTL, and have not considered the effects of epistasis or of closely linked QTL. Traits in which strong epistasis is suspected might be better studied with a larger number of RIL; for instance, Williams et al. (2001) suggest using 1000 lines.

Our work extends available experimental designs to map QTL into regions small enough to identify candidate genes for subsequent functional analyses. We have concentrated on designing strategies that can identify relatively small effect QTL (those explaining 10% or less of the variance of a trait), since there is accumulating evidence that such effect sizes are common and are difficult to fine-map using simple inbred strain crosses or via the construction of congenics. The methods we have presented here have the advantage of speed (requiring a single generation of breeding) and are relatively cheap to implement. It remains to be seen how successful they will be in practice.

Acknowledgments

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