

STRATEGIES FOR MAPPING AND CLONING QUANTITATIVE TRAIT GENES IN RODENTS

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Abstract | Over the past 15 years, more than 2,000 quantitative trait loci (QTLs) have been identified in crosses between inbred strains of mice and rats, but less than 1% have been characterized at a molecular level. However, new resources, such as chromosome substitution strains and the proposed Collaborative Cross, together with new analytical tools, including probabilistic ancestral haplotype reconstruction in outbred mice, Yin–Yang crosses and *in silico* analysis of sequence variants in many inbred strains, could make QTL cloning tractable. We review the potential of these strategies to identify genes that underlie QTLs in rodents.

In the 1990s, when it first became possible to map the chromosomal locations of quantitative trait loci (QTLs) in rodents, it looked as if QTL discovery would soon lead to the identification of genes involved in any medically important phenotype that could be modelled in mice or rats^{1–4}.

More than 10 years later, the field of QTL analysis is heading towards a crisis. The National Center for Biotechnology Information (NCBI) lists 700 QTLs that are found in rats (see the [Entrez Gene](#) web site in the Online links box), and the mouse genome database currently contains 2,050 mouse QTLs (see the [Mouse Genome Informatics](#) web site in the Online links box). Compare these figures with the number of candidate genes that are proposed to underlie QTLs in rodents (TABLES 1,2). If we uncritically accept all the claims as correct, only about 20 genes have been identified. Even if no more QTLs are mapped, at the present rate of progress (20 genes identified in 15 years) it will take 1,500 years to find all the genes that underlie known QTLs.

Several recent developments have been published that might make what is currently almost impossible more tractable. These include new genomic resources (for example, the availability of sequences and sequence variants), animal resources (for example, chromosome substitution strains and the proposed

Collaborative Cross), techniques (such as *in silico* mapping and whole-genome expression studies) and analytical tools (such as quantitative complementation tests and Yin–Yang crosses, see below for more information).

In this review, we discuss the realized or potential success of new approaches to identify genes that underlie QTLs. We focus on methods to identify genetic variants that have only a small effect on the phenotype (by which we mean that the variants account for 10% or less of the phenotypic variation of a quantitative trait). In the past, robust and reliable detection of small-effect QTLs has been a problem⁵; but, as should be evident from the number of QTLs that have been reported, difficulties in QTL detection are not impeding research. The methods might not be the most efficient, but there is no doubt that QTLs can be detected at high levels of significance and that the findings can be replicated, even for behavioural phenotypes^{6–9}. Although it is likely that only a small fraction of the total number of QTLs that segregate in inbred strain crosses are currently known to us (not to mention those in outbred stocks), without the ability to determine the genes they represent, their detection is of limited interest. We urgently need advances in gene, not QTL, identification.

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doi:10.1038/nrg1576

Table 1 | **Cloned quantitative trait loci**

Gene symbol	Gene name	Phenotype/function	Variation (%)	Gene identification method	Reference*
<i>Alox15</i>	Arachidonate 12/15 lipoxygenase	Abnormal bone mass	4%	Expression differences, analysis of knockout alleles, pharmacological inhibition	107
<i>Rgs2</i>	Regulator of G-protein signalling 2	Fear-related behaviour	5%	Outbred mice, QTL–knockout interaction	31
<i>Cyp11b1</i>	Cytochrome P450, subfamily 11B, polypeptide 1	Abnormal blood pressure	5%	Congenics, sequence variants	154
<i>Lipc</i>	Hepatic lipase	Obesity	7%	Multiple crosses, knockout interaction	155
<i>C5</i>	Complement component 5	Experimental allergic asthma	8%	Expression differences, loss-of-function mutation	106
<i>Ace2</i>	Angiotensin 1 converting enzyme 2 (peptidyl-dipeptidase A)	Abnormal blood pressure	12%	Knockout phenotype	156
<i>Apoa2</i>	Apolipoprotein A2	HDL cholesterol	23%	Multiple-strain haplotypes, coding sequence variants	88
<i>Mpdz</i>	Multiple PDZ domain protein	Pentobarbital drug withdrawal with seizures	25%	Genotype-dependent differences in coding sequence and expression	16
<i>Ncf1</i>	Neutrophil cytosolic factor 1	Severe arthritis	25%	Functional sequence change, pharmacological treatment	157
<i>Kcnj10</i>	Potassium inwardly-rectifying channel, subfamily J, member 10	Susceptibility to seizure	25%	Coding sequence variants, multiple-strain haplotypes	15
<i>Ptprj (Scc1)</i>	Protein tyrosine phosphatase receptor type, J polypeptide	Susceptibility to colon cancer	29%	Recombinant haplotypes, sequence differences, loss of heterozygosity in human cancer	158
<i>Tas1r3</i>	Taste receptor, type 1, member 4	Saccharin response	30%	Multiple-strain haplotypes, coding sequence variants	159
<i>Nppa</i>	Natriuretic peptide precursor A	Cardiac hypertrophy	44%	Promoter variants increase gene expression	160
<i>Cd36</i>	Fatty acid translocase	Abnormal fatty acid metabolism	50%	Expression differences, loss-of-function mutation, transgene complementation	161
<i>Pla2g2a</i>	Phospholipase A2, group IIA (platelets, synovial fluid)	Modifier of tumours in intestinal cancer	50%	Frameshift loss of function and transgene complementation (cosmid)	162
<i>Mtap1a</i>	Microtubule-associated protein 1a	Modifier of hearing loss	57%	Coding sequence variants, transgene complementation (cosmid)	163

*The first publication of this material. HDL, high density lipoprotein.

Before proceeding, we should also be clear about what we exclude from our discussion. In this paper, we do not consider artificially induced mutations that present as QTLs, such as those that are due to mutagenesis, although it has been suggested that mutagenesis in the mouse can be used to determine the genetic basis of quantitative traits instead of QTL mapping¹⁰. Screening mice for the quantitative effects of induced mutations is doubtless an efficient method for the identification of genes that are involved in the expression of a phenotype, quantitative or otherwise. However, our concern here is with the subset of polymorphic loci that give rise to phenotypic variation in natural populations (although we admit that laboratory mouse populations are in many respects unnatural). Mutagenesis does not distinguish between these loci and the large number of loci that are functionally invariant in nature, but in which mutations can be induced.

Lessons from previous success stories

Are there any lessons from success stories in the field of QTL mapping that might allow us to determine the

probable effectiveness of the new approaches? To answer this question, we need to define success, which is not so straightforward. Two recent reviews that attempt this task identify 21 genes in total, but agree about the identification of only four genes in mice and five in rats^{11,12}. As many have pointed out, there is no single proof that confirms a gene at a QTL and, to a large extent, each case has to be evaluated on its own strengths^{13,14}. Replacing one allele with another at a locus through targeted mutagenesis with subsequent functional analysis provides a stringent test, as does complementation, but these procedures might not be possible (or even necessary) in every case: alternative evidence can provide sufficient proof that the gene has been found.

There are also issues about what to include as a QTL. Strictly speaking, a QTL is any locus that contributes to a phenotype that is measured quantitatively. However, some investigators use a quantitative measure for a phenotype that others assess qualitatively (for example, susceptibility to cancer can be assessed as affected or not affected, or by a quantitative measure, such as the number of tumours). To be comprehensive, we have summarized data on candidate genes in

Table 2 | **Cloned susceptibility loci**

Gene symbol	Gene	Phenotype	Penetrance	Method of detection	Reference*
<i>Cblb</i>	Casitas B-lineage lymphoma b	Type 1 diabetes	High	Nonsense mutation, transgene complementation (cDNA)	164
<i>Stk6</i>	Serine–threonine kinase 6	Skin tumour susceptibility	Low	Multiple-strain haplotypes, outbred mice, expression difference	74
<i>Ctla4</i>	Cytotoxic T-lymphocyte-associated protein 4	Autoimmune disease	Low	Sequence variants, association studies in humans	165
<i>Ii2</i>	Interleukin 2	Type 1 diabetes	Low	Coding sequence variants, differences in electrophoretic patterns	166
<i>Pctr1</i>	Plasmacytoma resistance 1	Susceptibility to plasmacytoma	Low	Coding sequence variants, protein variant less active	167
<i>B2m</i>	β-2-Microglobulin	Type 1 diabetes	Low	Coding sequence variant, transgene complementation (plasmid)	168

*The first publication of this material.

TABLES 1,2. TABLE 1 lists candidate genes at loci where the effects are expressed as the percentage of the total phenotypic variance. TABLE 2 lists candidate genes at susceptibility loci, the effects of which are expressed as differences in penetrance.

The weight of evidence in favour of each gene varies considerably. For example, Ferraro and colleagues have proposed *Kcnj10* as a candidate gene that is involved at a seizure-susceptibility locus on the basis of finding sequence variants in coding regions and gene expression in the relevant tissue, but had to consider data for 120 genes within the region of study¹⁵. Shirley and colleagues applied similar criteria for the

candidacy of *Mpdz*, but had a much smaller interval, with only three known and three predicted genes to validate, and so were able to make a more convincing case for gene identification¹⁶. Although TABLES 1,2 should not be interpreted as definitive lists, they do allow us to draw two conclusions of general importance.

Effect size. First, the QTLs that have yielded genes have had exceptionally large EFFECT SIZES. To appreciate how large, we ideally need an unbiased estimate of the typical QTL effect size. Unfortunately this is difficult to obtain because, in cases of low statistical power to detect effect sizes, the contributions of detected QTLs are

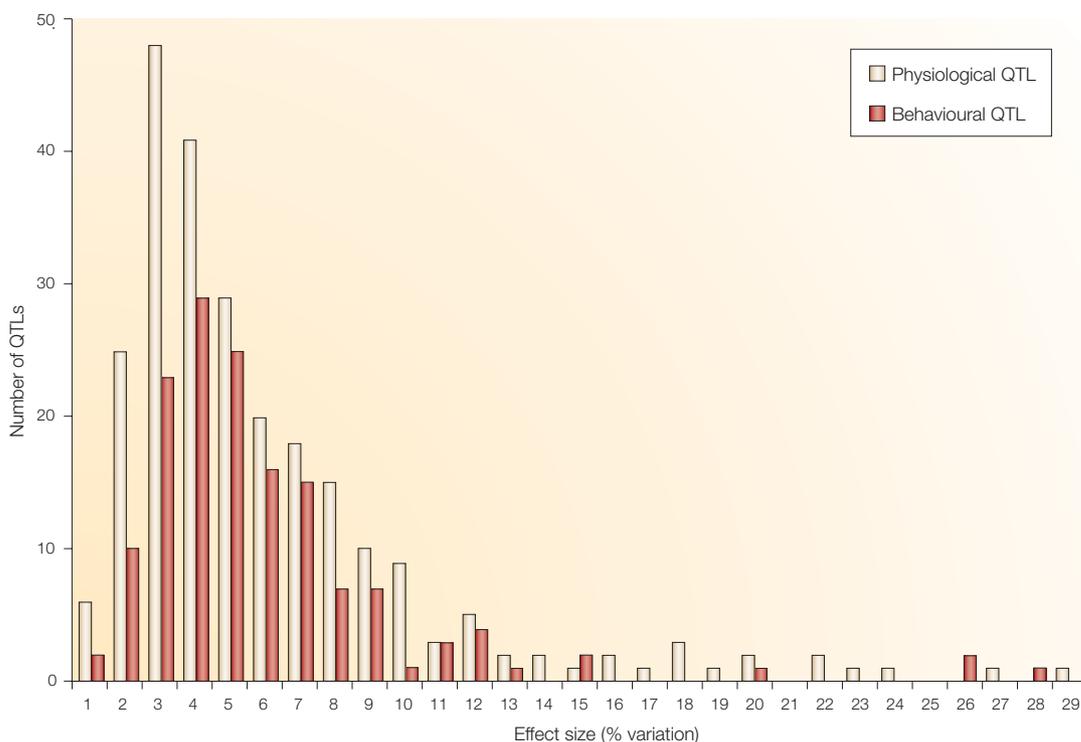


Figure 1 | **The distribution of QTL effect sizes.** Data are from 14 recent quantitative trait loci (QTL) mapping experiments (including 244 significant QTLs). Behavioural QTL data are from REF. 116. Data for physiological QTLs are from REFS 140–153.

EFFECT SIZE
The percentage of the total phenotypic variation that is attributable to a QTL.

Box 1 | **How QTL effect size depends on the mapping population**

The effect size of a quantitative trait locus (QTL) depends on the population in which the QTL alleles segregate. Here, we give a quantitative treatment and show that a genetic effect that produces a QTL with a 5% effect in a recombinant inbred (RI) population will produce a QTL that explains ~2.6% of the phenotypic variance in an F2 generation and ~1.3% in a backcross (BC). Consider a diallelic QTL at which a major allele has frequency p and physiological effect g , and a minor allele has frequency $q = (1 - p)$. Let G be the genetic effect of the QTL on the phenotype for a given animal. The genetic variance, $Var(G)$, in each population is $4g^2pq$ for RI, $2g^2pq$ for F2, and g^2pq for BC (REF 5). This shows that the genetic variance coming from the QTL — that is, the proportion of the phenotypic variance it explains — decreases in the ratio 4:2:1 for RI:F2:BC (FIG. 2). What does this do to the effect size of the QTL in these populations? In the simplest case where there are no other genetic components, the environmental variance $Var(E)$ is constant and the effect size, θ , is shown in equation 1.

$$\theta = \frac{Var(G)}{Var(G) + Var(E)} \tag{1}$$

The top part of the table shows θ for each population and how the effect of a QTL in an RI becomes smaller in F2 and BC populations¹³². (Note that ‘|’ in the table means ‘conditional on’.)

Measure	RI	F2	BC
Single QTL system			
θ	$\frac{4g^2pq}{4g^2pq + Var(E)}$	$\frac{2g^2pq}{2g^2pq + Var(E)}$	$\frac{g^2pq}{g^2pq + Var(E)}$
$\theta \theta_{RI}$	θ_{RI}	$\frac{\theta_{RI}}{2 - \theta_{RI}}$	$\frac{\theta_{RI}}{4 - 3\theta_{RI}}$
$\theta\{\theta_{RI} = 5\%\}$	5%	2.56%	1.30%
Many QTLs system			
θ_1	$\frac{4\lambda\Gamma}{4\Gamma + Var(E)}$	$\frac{2\lambda\Gamma}{2\Gamma + Var(E)}$	$\frac{\lambda\Gamma}{\Gamma + Var(E)}$
$\theta_1 \theta_{1,RI}$	$\theta_{1,RI}$	$\frac{\lambda\theta_{1,RI}}{2\lambda - \theta_{1,RI}}$	$\frac{\lambda\theta_{RI}}{4\lambda - 3\theta_{RI}}$

But what if there are k QTLs that affect the trait? We take the simple case of k — unlinked, diallelic, independent, additive QTLs. The genetic variance coming from the i th QTL in, for example, an RI, will be $4g_i^2p_iq_i$, and the total genetic variance $Var(G)$ in the RI will be

$$Var(G) = \sum_i^k Var(G_i) = 4 \sum_i^k g_i^2p_iq_i \tag{2}$$

The bottom part of the table gives the effect size, θ_1 , of QTL 1, (which accounts for a fraction λ of the genetic variance) in RI, F2 and BC populations, as well as conditional effect sizes. In the table, we make the substitution defined in equation 3 for brevity.

$$\Gamma = \sum_i^k g_i^2p_iq_i \tag{3}$$

The drop in effect size moving from RI–F2–BC is shallower when other genetic components have a large role in the phenotype. In the extreme case of no environmental variance (when other QTLs account for all the remaining phenotypic variance), there is no drop at all. At the other extreme, when $\lambda = 1$, the formulae are the same as in the top part of the table.

over-estimated^{17,18}. With this caveat in mind, FIG. 1 shows a comparison of effect sizes from a reasonably complete survey of behavioural QTLs (94 significant QTLs) and a compendium of physiological phenotypes (such as growth, weight, blood pressure and gall stones; note that we are using data from uncloned QTLs). It is important to realize that effect sizes depend on the mapping population in which they are measured (see BOX 1 for a detailed discussion; see also FIG. 2). Here, and in the rest of this review, we use effect size to mean the percentage of the total phenotypic variance that is explained by the QTL segregating in an F2 intercross.

FIGURE 1 shows that the distribution of effect sizes for both behavioural and physiological phenotypes are almost identical, as are the average effects of the uncloned QTLs for these phenotypes, which are 5.8% and 5.3%, respectively. The average effect size of a cloned QTL is 26% (TABLE 1). If we restrict our attention to the QTL genes that have been most stringently confirmed (by complementation), then the average effect size is 50%. Clearly, it is easier to identify genes when the QTL effect sizes are large.

In fact, the true effect sizes of most uncloned QTLs are almost certainly much smaller than the 5–6% average mentioned above, and this is not just because of

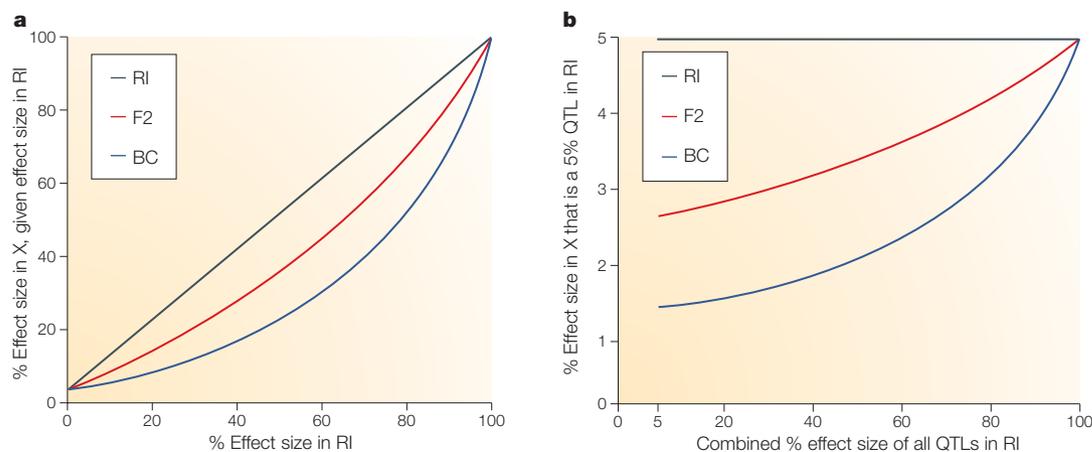


Figure 2 | **Effect size in F2 and BC relative to RI.** **a** | Illustrates how a QTL's effect size in F2 and BC populations relate to its effect size for a monogenic trait in an RI population. **b** | Shows the effect sizes in F2 and BC populations of what, in RI populations, is a 5% QTL for a single QTL that affects a multigenic trait (heritability is shown on the horizontal axis). Epistatic and gene–environment interactions require a more sophisticated treatment than is presented here. BC, backcross; RI, recombinant inbred.

statistical problems in their estimation. Perhaps the most important finding to emerge from attempts to clone genes that underlie rodent QTLs is that a single genetic effect, as detected in an inbred strain cross, turns out in most cases to be due to several physically linked small effects. There are many examples of this phenomenon, including QTLs that influence seizures¹⁹, obesity²⁰, growth²¹, blood pressure^{22–26}, diabetes²⁷, antibody production²⁸ and infection²⁹. Classical approaches to gene identification (BOX 2) generally assume that a single QTL is just that, a single genetic effect. By not allowing for the fractionation of the QTL, gene identification becomes much more frustrating.

Unfortunately, we still do not have a precise picture of the genetic architecture of QTLs in rodents, or indeed in any organism³⁰. Just how small are the effects likely to be? At the rarely achieved level of mapping where genetic effects can be resolved into intervals of a hundred kilobases or less, QTLs might further disintegrate. This has been found in studies of a behavioural QTLs in mice³¹, and has been seen in similar work in other species^{32,33}. Further high-resolution information at other QTLs might drive the estimate of average effect size below 5%.

In short, QTLs are predominantly loci with small effects, so the problem of identifying the genes that underlie them is a problem of identifying the molecular basis of small genetic effects. Whether we call the locus a QTL or not is of little importance; at the moment, the distinction that makes cloning tractable is between large and small effect sizes. Unless we have methods that can identify the genes at QTLs that contribute 1 or 2% to the phenotypic variation, the backlog of uncloned QTLs will remain.

Molecular signature. The second lesson from the success stories in TABLE 2 is that finding a recognizable molecular signature at a QTL, such as a sequence variant that introduces a stop codon or that changes protein function, makes QTL cloning tractable^{34,35}. In such cases, not

only does the sequence tell us which gene is the most likely candidate, it also indicates what experiments are needed to confirm the candidacy. For example, complementation will rescue the function of a null allele, or a biochemical or pharmacological intervention will determine whether an aberrant protein is involved. Unfortunately, possessing a recognizable molecular signature might be the exception rather than the rule for QTLs (although, admittedly, we have only a small sample of cloned QTLs to assess).

Although SNPs that cause monogenic disease have a strong tendency to occur at highly conserved amino acids, this does not seem to be true of variants in complex diseases. At least in human studies, the pattern of disease-associated coding variants is so far indistinguishable from the distribution that is found in the normal population³⁶. Moreover, in many cases, no coding-sequence variant is found; instead many sequence variants are detected in non-coding DNA in regions, the function of which (if any) is unclear^{31,37}. In these cases, we do not yet have an easy method to prove the involvement of a gene at a QTL.

It is important to emphasize that identifying a gene is not the same as isolating a sequence variant (or quantitative trait nucleotide; QTN). Discovering a QTN does not guarantee gene identification because the position of sequence variants does not necessarily coincide with the position of the genes on which the QTNs have an effect. For example, a QTN could lie within a regulatory region that is megabases away from its cognate gene³⁸. Note also that it is a QTN, not a gene, that segregates in a cross, so genetic strategies for QTL dissection focus on sequence variants, not genes.

The measure of success. Before we look at new methods, we should be clear about how well the old methods perform (BOX 2). The relevant points (taken from a theoretical literature that is almost as extensive as the reports on QTL mapping) are as follows: 300 F2 animals can be used to map a QTL with an effect size of 5% onto a 40-cM

CONGENIC

A strain produced by a breeding strategy that delineates a genomic region containing a trait locus. Recombinants between two inbred strains are backcrossed to produce a strain that carries a single segment from one strain on the genetic background of the other.

Box 2 | Existing strategies for QTL mapping based on inbred strain crosses

F2 intercrosses and backcrosses

Two parental inbred strains are crossed to produce an F1, the starting point for the two most common mapping strategies. Intercrossing the F1 generates an F2 and backcrossing to one or other of the parental strains produces a backcross. The number of animals (*n*) needed to map an additive QTL in an F2 can be calculated using equation 1 (REFS 5,42).

$$n = \left(\frac{1 - V_{\text{QTL}}}{V_{\text{QTL}}} \right) \left(\frac{Z_{1-\alpha/2}}{(1 - V_{\text{QTL}})^{1/2}} + Z_{1-\beta} \right)^2 \quad (1)$$

$Z_{1-\alpha/2}$ is the cut-off point in a standard normal distribution for a probability of $1 - \alpha/2$ and α is the significance threshold. If we use a genome-wide significance threshold of $\log P 4.3$ (REF. 133) from normal tables, the cut-off point is 3.89. If we set the power at 90% (in other words, 10% chance of missing a true association) then we need a $Z_{1-\beta}$ of 1.28 (from normal tables). V_{QTL} is the proportion of variance that is attributable to the QTL. For a purely additive QTL that explains 5% of the variance, the calculation yields equation 2.

$$\left(\frac{0.96}{0.05} \right) \left(\frac{3.89}{0.97} + 1.28 \right)^2 = 527 \quad (2)$$

Recombinant inbred (RI) lines

A pair of inbred strains is intercrossed and their progeny is inbred to generate a panel of inbred animals, each with a different combination of the progenitor genomes. Once a set of RI lines has been genotyped, the marker data are available for all subsequent mapping experiments, which take place by determining the strains that are present at a marker and by correlating this strain distribution pattern with phenotypic differences. Using the terminology given above for power in an F2 intercross, the relationship between the number of animals (*n*) and the effect size of the QTL is given by equation 3 (REF. 134).

$$n - 2 = \frac{(1 - V_{\text{QTL}})(Z_{1-\alpha/2} + Z_{1-\beta})^2}{(V_{\text{QTL}})} \quad (3)$$

Congenic and interval-specific congenics

Congenic are still the mainstay of fine-mapping QTLs in rodents. By repeatedly backcrossing one strain onto another, it is possible to create animals that have a particular genomic region from one strain and the remainder of their genome from the other; subsequent intercrossing makes the genomic segment homozygous and the mouse fully inbred. However, inadequate or incorrect assumptions about the distribution of chromosome segments, the population structure, the marker spacing and the selection strategy might mean that the breeding does not go as predicted⁵⁹. Flaherty and colleagues point out that transgenic mice, which are routinely created on a 129 background and then backcrossed onto another strain (usually C57BL/6), can be used as congenics for the interval around the engineered mutation¹³⁵. An interval-specific congenic is made by first identifying an animal with a recombinant chromosome in a region of interest and then backcrossing it for several generations to remove other QTLs. Resultant animals are intercrossed, and homozygotes for the recombinant haplotype are selected.

Recombinant congenics and genome-tagged mice

Recombinant congenics are a type of RI line made by backcrossing and randomly fixing parts of the genome by inbreeding, so that each contains an average of 87.5% genes of a common background strain and 12.5% of a common donor¹³⁶. Genome-tagged mice are sets of overlapping congenics with sufficient material from one strain crossed onto the other so that the whole genome is represented¹³⁷. The 60 strains created by West and colleagues contain segments of about 23 cM that are INTROGRESSED from either DBA/2 or CAST/Ei onto C57BL/6 (REF. 137).

Recombinant progeny testing

Animals that have a recombinant chromosome in a region of interest are backcrossed to a parental strain to determine the location of the QTL relative to the recombination point.

Advanced intercross lines

Two parental strains are crossed to produce an F1 that is intercrossed to produce an F2. Subsequent generations are produced by intercrossing, so that animals in an advanced intercross line accumulate new recombinants. Animals are maintained according to a pseudo-random breeding protocol to reduce loss of genetic variation in the population. An appropriate advanced intercross line could take at least 5 years to make, but it can be used to map many loci.

Heterogeneous stocks

Genetically, heterogeneous stocks are derived from inbred strains through a series of progressive intercrosses. Existing heterogeneous stocks are derived from eight strains, but any number can be used (an advanced intercross line is a heterogeneous stock derived from two strains). By subsequently maintaining the stock for many generations, using a pseudo-random breeding protocol, recombinants are introduced that allow high-resolution mapping. Traits are mapped by associating allelic variants with a phenotype, so that genotyping must be carried out for each experiment. Two heterogeneous stocks have been used for mouse mapping experiments: the older of the two (the Boulder heterogeneous stock), has been breeding for more than 60 generations and is derived from the C57BL/6, BALB/c, RIII, AKR, DBA/2, I, A/J and C3H strains¹³⁸. The second heterogeneous stock (the Northport heterogeneous stock) has passed its fortieth generation¹³⁹, and is derived from the A/J, AKR/J, BALB/cJ, C3H/HeJ, C57BL/6J, CBA/J, DBA/2J and LP/J strains. Genetic variation from many strains complicates analysis, but robust and powerful QTL detection is possible by estimating the probability that an allele descends from each progenitor strain⁷⁰ (BOX 3).

INTROGRESSION
Introduction of a chromosomal segment from one strain into another by interbreeding.

interval with 50% power, using markers that are spaced every 20 cM across the genome³⁹. Increasing the marker density does not appreciably increase mapping resolution for small-effect QTLs⁴⁰. Increasing the number of animals increases the likelihood of QTL detection (so that with 800 animals there is a greater than 99% chance of detecting a QTL with an effect size of 5%; BOX 2), but does not increase resolution sufficiently to identify genes — even 1,000 animals will only map a 5% QTL onto an interval of about 10 cM (REF. 41). In summary, a standard inbred-strain mapping experiment will need about 400 animals and will use 100 markers.

Starting from a cross between two inbred animals, it could take less than 6 months to map a QTL to a 40-cM region. It will take a few more years to isolate a QTL by creating CONGENICS⁴² (BOX 2). Alternatively, by continuing to intercross beyond the F2 generation, fine mapping can be carried out in an advanced intercross line or a heterogeneous stock⁴³ (BOX 2). After 6 years, we can reasonably expect traditional strategies to have resolved a 5% QTL into a region of about 1 cM, which in the mouse corresponds to 2 Mb on average. The average gene density in the mouse is about 10 genes per megabase, so a 1 cM QTL interval will contain about 20 genes (note that gene density varies significantly across the genome). Keeping this in mind, we now turn to the new strategies to see how they fare in comparison.

Chromosome substitution strains

Chromosome substitution strains (CSSs) consist of a set of animals in which one chromosome is derived from one strain and all the rest are derived from another. For mice, a panel of 22 CSS strains is required (for 19 autosomes, 2 sex chromosomes and mitochondria), or 44 if a reciprocal set (with both parents as progenitor donors) is used. QTL mapping is carried out through the relatively simple process of comparing the phenotypes of each strain with the parental background strain. CSS strains were first used to map QTLs in mice in 1999 (REF. 44); theoretical aspects of the mapping process were subsequently described for mice⁴⁵ and rats^{46,47}. The method has a long history in plant⁴⁸ and *Drosophila* genetics⁴⁹. The first complete CSS set, created from A/J and C57BL/6 strains, was produced in 2004 and was used to detect QTLs across the mouse genome^{50,51}.

The ease of QTL detection using CSS strains results from two features: first, the background genetic variance is reduced so that each QTL explains a greater proportion of the total phenotypic variation (BOX 1). Second, a lower significance level is needed for QTL detection because, compared with the ~100 markers tested in an F2 generation, only 21 comparisons need to be made. Singer and colleagues point out that the F2 intercross requires at least 35% more animals for QTL detection⁵¹. Nevertheless, Belknap estimates that to detect a QTL with an effect size of 6% and with 50% power will require 20 CSS strains and 20 parental animals for each comparison, or between 3 and 400 animals (depending on how many background animals are used) for a genome scan⁵². Belknap's estimate of 3 to 400 animals agrees with the actual figure of 435 animals used to map

QTLs in the first mouse CSS experiment⁵¹. Notably, this is not a substantial saving on the numbers used for an F2 intercross (BOX 2).

A comparison between parental and CSS strains will only map a QTL to a chromosome. For higher-resolution mapping, CSS strains allow the rapid creation of a congenic strain, either by using interval-specific congenic strains or by recombinant progeny testing⁴² (BOX 2). Because of the relative increase in effect size, congenic construction and recombinant progeny testing will require 3–4 generations to reduce the interval to 1 cM, rather than the 9–10 generations that are required when starting from an F2 intercross⁵².

QTL mapping in a CSS delivers researchers to the same point faster than classical strategies have led them, but no farther. The main drawback of the method is that it makes no allowances for the fractionation of a large QTL effect into many loci with smaller effects. This is the problem that has for so long beset the use of congenics for QTL dissection and gene identification. CSS mapping is a powerful method for the identification of small-effect QTLs, but it does not offer advantages over other methods for the identification of genes.

Collaborative Cross: a proposal

Would the difficulties of QTL cloning be overcome if we had 1,000 recombinant inbred (RI) lines, as suggested by the Complex Trait Consortium⁵³? Although the Collaborative Cross — a panel of 1,000 RIs derived from 8 parental strains — is only a proposal, not an existing resource, we discuss it here because it might provide a tool for gene identification (although this is not the sole reason for its creation⁵³).

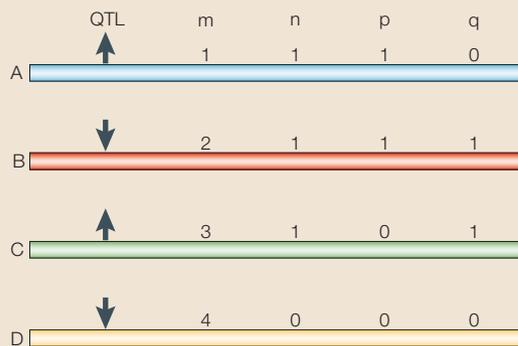
The use of RI lines for QTL mapping in rodents has so far been limited by the relatively small size of each set. The BXD RI set (which is derived from C57BL/6 and DBA/2 strains) consisting (until recently) of 26 lines has 90% power to detect a QTL that explains half the phenotypic variance in a trait⁵⁴. The comparable effect of a QTL segregating in an F2 generation is half this value (because the F2 animals are not inbred), or 25% of the phenotypic variance (BOX 1). What happens to power and mapping resolution if we have 100 lines?

We have investigated this question using a simulation⁵⁵. Fine mapping of a QTL with a 5% effect is efficient: 100 RI lines provide 80% power for QTL detection at a 5% significance level. Mapping resolution is also good: the 95% confidence interval is 7 cM. Fortunately, RI panels approaching this size are now available: the LXS panel consists of 77 lines⁵⁶ and the expanded BXD set consists of 90 lines⁵⁷. Therefore, the new RI sets can deliver high resolution, although this is still insufficiently high to identify genes at small-effect QTLs.

The proposed Collaborative Cross differs in two ways from standard RI lines. It is created from eight progenitors, not two as is the case with RI lines, so that the set would contain more genetic diversity than a standard RI. Second, the proposal is to create 1,000 lines, ten times more than the largest available set. What would be the advantage, for gene identification, in having such an RI set? Mapping resolution improves to

Box 3 | Probabilistic ancestral haplotype reconstruction

Single-marker association mapping is an inefficient method when the allele frequencies of the variant and the quantitative trait locus (QTL) do not coincide and when contrasting QTL alleles are in linkage disequilibrium (LD) with the same marker allele, as illustrated in the figure. In this example, the population from which all chromosomes descended consisted of four inbred strains. Inbred strains A and C contain a QTL allele that increases the phenotype, and strains B and D contain an allele that decreases the phenotype. A polymorphic marker, m, that is in complete LD with the QTL possessing four distinct alleles (labelled 1,2,3,4 in the figure) will show evidence of association. Consider what happens with



a diallelic marker, n, that, in the ancestral population, has one allele '1' that is physically linked to strains A, B and C and the other allele '0' linked to strain D. Evidence of association will now be reduced because the marker fails to partition the genotypes into two equally contrasting groups. Things are even worse at markers p and q where both alleles 0 and 1 are linked to both increaser and decreaser QTL alleles; there is no detectable association at all. But the haplotypes that are defined by the markers p and q distinguish all four strains and so are surrogates for the efficient marker m. Therefore, multi-point haplotype-based mapping is generally more powerful than single-point mapping.

Heterogeneous stocks are mosaics of eight inbred founder strains, and QTL mapping in these stocks is best approached by reconstructing the ancestral haplotype mosaic. However, the strain distribution patterns of neighbouring markers rarely distinguish between all founder strains, so instead we compute the probability that an animal is descended from a given pair of strains at a particular locus. A useful statistical model of the genome mosaic of a diploid heterogeneous stock animal is a pair of HIDDEN MARKOV MODELS, in which the hidden states of the model are the ancestral strains, and the observed data are the marker genotypes. A DYNAMIC PROGRAMMING ALGORITHM is then used to compute the probabilities of descent using all the available information. The marker alleles must be known in the founder strains for the method to work. Recently, we have shown that other outbred mice of unknown ancestry can be modelled and analysed as mosaics of standard inbred strains in the same way³¹.

about 4 cM when the number of lines increases to 1,000 and further lines make it possible to map effects that are smaller than 5%. As we have seen, QTLs have a habit of disintegrating when they are dissected and we need the ability to find the pieces.

If the Collaborative Cross were to be created, it would enable us to map many of the 2,000 uncloned QTLs into small genetic intervals. This is an attractive proposition but it must be balanced against two important disadvantages. First, maintaining and distributing an RI set of 1,000 lines poses significant problems. Even a laboratory devoted to providing animals to the research community, such as the Jackson Laboratory, is able to maintain only a few hundred strains as live stock. Second, the resolution of the Collaborative Cross is not sufficient to identify genes: a 4-cM interval is roughly equivalent to 8 Mb of DNA, which will contain approximately 80 genes. Unfortunately, the Collaborative Cross would give us no help with the final goal of gene discovery. However, one solution might lie in the application of recombination inbred segregation tests (RISTs) and its generalization, Yin-Yang crosses (YYCs).

Yin-Yang crosses

RISTs and YYCs, applied to a large number of RIs, will increase mapping resolution to the point where individual genes can be identified. YYCs take advantage of the abundance of recombination events in RI or inbred strains to improve mapping resolution⁵⁸. To

understand the principle, we first explain RISTs, which are a particular example of YYCs⁴².

Assume that a QTL has been mapped in an inbred-strain cross. RI strains that have recombinants within the QTL interval are crossed with both parental inbred strains to produce two segregating populations. In one cross, the RI and the parental line share the QTL allele, so no QTL segregates; in the other cross, the RI and the parental line have a different allele at the QTL, and the QTL segregates (hence the name segregation test). Consequently, the QTL will be mapped to a position either above or below the recombination point in the RI that is used for the two crosses. The overall results from a selected set of RIs will locate the QTL to a small interval.

Resolution is determined solely by the number of recombinations in the interval. For example, with 100 RI lines, each having approximately 3.3 recombinations per 100 cM (REF. 56), the total number of recombinations is 3.3 multiplied by the number of centimorgans in the genome (~1,400) to give 4,620 recombinations; so, the expected resolution is 1,400/4,620 = 0.30 cM. This corresponds to an interval of 0.6 Mb. With 1,000 RI, the expected accuracy is 0.03cM (potentially a few hundred kilobases). However, recombinations are not distributed evenly in the genome, making the above calculation an approximation only⁵⁹.

RIST, as with congenic construction and other strategies that seek to treat QTLs as Mendelian genetic factors, will have problems with multiple linked QTLs;

HIDDEN MARKOV MODEL
A probabilistic description of a system in which the observed data depends on the hidden internal state of the system. The objective is usually to infer the likelihood that the system is in a particular hidden state, given the observed data.

DYNAMIC PROGRAMMING ALGORITHM
An algorithm that finds the optimum solution to a problem involving *N* objects in terms of the solutions to a series of smaller problems that involve subsets of the objects.

but unlike the construction of a congenic line, a RIST analysis is quick to carry out, so if multiple QTLs are suspected, it will be possible to test that hypothesis with further RISTs (assuming appropriate recombinant lines are available). Indeed, with 1,000 RI lines, it would be possible to resolve most QTLs onto an interval that contains a few genes in just two generations; an efficient and relatively economical strategy. In some instances, the resolution will be good enough to identify a gene underlying a QTL.

The YYC approach is a generalization of the RIST method that treats inbred strains as if they were RI lines, on the grounds that inbred strains descend from a relatively small number of founders. In this respect, YYCs make the same assumption as *in silico* mapping, which is discussed below. The large number of recombination events that have accumulated in a set of inbred strains since their origin can be used to map the QTL to a small region⁵⁸. Similar to RISTs, YYCs are used as a fine mapping tool that is applied after the initial mapping of the QTL. The allelic state of the QTL in multiple strains is revealed by crossing the two strains that were used initially to map the QTL and any new strain. YYCs establish the strain distribution pattern of the QTL, which is used to identify the list of variants in the region that follows this distribution.

The YYC method is based on the assumption of a single biallelic variant or haplotype that affects the trait in the different strains. Therefore, as for the RIST approach, the main limitation of YYC analysis with inbred strains is that it is not suitable for multiple QTLs in a region. Furthermore, QTLs that primarily have epistatic effects will not be suitable for this kind of analysis. They will result in inconclusive results of the Yin–Yang analysis, but this is unlikely to produce false negative results.

Outbred stocks

Outbred stocks accumulate recombinants over time, so that they offer high mapping resolution; potentially high enough to identify candidate genes. The effects of individual genes can be mapped by association, as has been successfully shown in human populations^{60–67}. In a few cases, QTLs have been mapped in mouse populations: small-effect QTLs have been mapped to sub-centimorgan resolution using heterogeneous stock mice^{68–71} (BOX 2); crosses using outbred *Mus spretus* have been used first to map^{72,73} and then to identify serine–threonine kinase 6 (*Stk6*) as a candidate skin-tumour susceptibility gene⁷⁴; outbred CD1 mice have been used to map a susceptibility locus for pulmonary adenoma⁷⁵; and outbred MF1 strains were used to identify regulator of G-protein signalling 2 (*Rgs2*) as a gene that influences anxiety in mice³¹.

Mapping in outbreds is not as straightforward as mapping in inbred crosses: it requires higher density genotyping and larger numbers of animals. At first sight, the method seems to have not only the advantages, but also to suffer from the same drawbacks, as human genetic association studies, where robust, replicable results have been hard to obtain⁷⁶ and sample sizes of

many thousands are required⁷⁷. However, QTL analysis in outbred mouse populations is likely to be easier than its human equivalent. In fact, it would be as simple and powerful as mapping in inbreds if each allele at every locus could be traced back to a unique progenitor strain, so that ANALYSIS OF VARIANCE (ANOVA) at a marker extracted maximal information for QTL detection (BOX 3).

In contrast to human populations, in which progenitor alleles are unknown (although there are a few exceptions, such as ADMIXTURE MAPPING⁷⁸), one type of outbred mouse (heterogeneous stock) is derived from known ancestral inbred strains. This makes it possible, with the help of some mathematics, to derive the origin of each allele and map QTLs at sub-centimorgan resolution (BOX 3). Unfortunately, mapping a QTL to a megabase interval is still insufficient to identify a single gene. Other outbred stocks might offer better resolution, but they lack the advantages of progenitor information from the heterogeneous stock.

Several methods have been developed that deal with this problem in its various incarnations^{79–84}. So far, there has been only one application of this method in mouse genetics. We have shown that sufficient recombinants have accumulated in a commercially available outbred stock (known as MF1) to map genetic effects onto regions of only a few hundred kilobases or less³¹. Sequence analysis showed that variants in the MF1 strain were identical to those found in inbred strains, and haplotype reconstruction revealed that more than 95% of the haplotypes could be derived from known inbred strains. Therefore, we were able to map the MF1 strain by probabilistic ancestral haplotype reconstruction (BOX 3). It is important to realize that our approach neither required us to recover the correct haplotypes, nor to identify the progenitor strains correctly. We found that a QTL for anxiety-like behaviour, previously mapped to an interval of less than 1 cM, resolved into three smaller independent QTLs, each about 100–200 kb wide. It would also be possible to combine pedigree and LINKAGE DISEQUILIBRIUM (LD) data for fine mapping in the MF1 strain and, indeed, in other outbred mice^{79,81,85}.

Potentially, outbred animals could deliver a resolution that would be sufficient to guarantee candidature of a single gene. The disadvantages of the method lie in the complexities of the analysis and the need for large numbers of animals and high-density genotyping. For example, genome-wide mapping in heterogeneous stocks (BOX 2) requires at least 6,000 SNP markers. To reduce false-positive results to acceptable levels with this number of markers means imposing stringent significance thresholds: by permutation, we estimate that the 5% and 1% significance levels correspond to *P*-values, expressed as negative logarithms, of 4.4 and 5.1 (REF. 86), and will require about 1,000 animals to provide 80% power to detect a 5% QTL⁸⁷. More markers, and more animals, will be needed for whole-genome analysis of more complex outbred stocks. These features militate against whole-genome analyses using outbreds, but developments in high-throughput genotyping will make it feasible.

ANALYSIS OF VARIANCE

A statistical method to test the null hypothesis that the mean values of two or more groups are equal. The variance around the mean in groups is compared with the variance of the group mean. In genetic applications, the variance between families is compared with the variance within families. A significant *F*-ratio implies that variance between families is larger than within families.

ADMIXTURE MAPPING

Genetic mapping using individuals whose genomes are mosaics of fragments that are descended from genetically distinct populations. This method exploits differences in allele frequencies in the founders to determine ancestry at a locus in order to map traits, in a way that is broadly similar to an advanced intercross.

LINKAGE DISEQUILIBRIUM

The tendency for markers to have correlated genotypes when they are physically close together. Over several generations, recombination will break down linkage between markers and a QTL, so that linkage disequilibrium will only occur between markers that are close to a QTL. This explains why outbred animals can provide high-mapping resolution.

Box 4 | **QTL mapping using recombinant inbred lines versus *in silico* mapping**

Because recombinant inbred (RI) panels are descended from (usually) two inbred strains, their genomes are random mosaics of these founder haplotypes. Once established, an RI panel need only be genotyped once, using markers that distinguish between the founder strains. At a given marker, the pattern of genotypes across the panel is called a strain distribution pattern. For example, in a panel of eight RI strains, the SDP 0100111 means that the first, third and fourth strains carry the allele '0' and the remainder the allele '1' at the marker in question. To map a quantitative trait locus, RI animals across the panel are phenotyped (if possible, in replicate, to reduce non-genetic variance), and the values are correlated with the strain distribution patterns at each marker (see the [Gene Network](#) web site in the Online links box) to identify markers with statistically significant associations. Mapping resolution depends on the size of the panel, but is roughly equivalent to that of an intercross.

Superficially, *in silico* mapping looks similar. A set of inbred strains (that have an unclear ancestry) replaces the panel of RI strains of known ancestry. An important difference is that regions of HAPLOTYPE SHARING between the inbred strains must first be predicted from runs of contiguous markers with shared genotypes. The inbred strains are phenotyped and correlated with the predicted haplotype distribution patterns as before. Because the blocks of haplotype sharing between inbreds are smaller than in RI lines, theoretically *in silico* mapping resolution should be greater for the same number of strains. The key difference is that mapping using RI lines is by descent, but is by inferred state for *in silico* mapping. RI mapping is uncontroversial; by contrast, the merits of *in silico* mapping are hotly debated.

Haplotype analysis and *in silico* mapping

Many have pointed out that a QTL must be contained in a region where sequence divergence corresponds to genetic action. So, when QTLs have been mapped in different combinations of inbred-strain crosses, the strain distribution pattern can be combined with mapping data to refine the region that contains the functional variant^{68,88–90}. For example, a QTL identified in a cross between strain A and strain B that does not segregate in a cross between strains A and C will be located where sequences from strains A and C are the same, but those from strains A and B are different. This approach has been used to carry out mapping at an extremely high resolution and to identify candidate genes^{68,88–90}.

A development of this idea is *in silico* mapping (BOX 4). It relies on known phenotypic differences between inbred strains (such as those being collected as part of the Mouse Phenome Project⁹¹; see the [Mouse Phenome Database](#) in the Online links box) and uses a high density of markers to derive a strain distribution pattern that can be used for mapping⁹². The proponents of this method argue that it reduces the time required for analysis of genetic models of complex disease “from many months to milliseconds”⁹².

In principle, *in silico* mapping is similar to mapping using RI lines — by establishing phylogenetic relationships between inbred strains from sequence information it should be possible to identify regions that are identical by descent. The problem is that in contrast to a set of RI strains (or indeed all mapping experiments that are derived from crosses between inbreds), in which genotyping unambiguously defines descent so that genetic identities can be associated with phenotypic similarities, finding sequence variants that are shared by a set of inbreds does not guarantee their descent from a common ancestor.

Nevertheless, several observations support the treatment of inbred strains as if they were derived from a set of founder strains. For example, the genealogies of many inbred strains can be traced back to a relatively small number of animals^{93,94}. Moreover, the pattern of sequence variation between inbred strains initially indicated that the distribution of polymorphisms has a

mosaic structure, which is consistent with their derivation from a relatively small number of Asian and European stocks^{95–97}.

There is little doubt that *in silico* mapping is effective for monogenic, highly penetrant mutations^{95,98,99}. The crucial issue is whether *in silico* mapping performs well in detecting small-effect loci. Wiltshire and colleagues have gone the furthest towards this goal. They used a dense map (of 10,990 SNPs) that was typed in 48 strains and applied a permutation method to determine the significance of their results⁹⁹. They demonstrated the effectiveness of the method for localizing large effects (on coat colour and taste preference) and then showed that for two multigenic complex traits (high-density lipoprotein cholesterol levels and gallstone formation) they could find loci that corresponded to some previously identified QTLs. These results are encouraging, although further work is required to distinguish between true- and false-positive findings.

At present, two difficulties confront *in silico* mapping: low power and the complex structure of the genomes of laboratory strains. At first sight, the problem of power seems to be insuperable: how can the genetic basis of complex traits be dissected with just a few dozen animals, or even a few 100, when we know that successful association studies in humans requires thousands of individuals⁷⁷? The truth of this analogy depends on the extent to which the inbred strains are related by descent. If the inbred strains are completely unrelated, the analogy is correct and thousands of animals will be needed for genetic mapping. This is partly because there will be less LD between loci in unrelated individuals, so that more markers are needed to detect the QTL⁷⁷. More unlinked markers will require an increase in the stringency of the significance threshold to avoid false-positive results, which in turn requires an increase in the number of animals to maintain power for QTL detection. However, more animals are also needed because, in fully outbred populations, more loci are likely to contribute to the genetic variance. Therefore, the relative contribution to the phenotypic variance that is attributable to a single locus will be less than for the same locus in a population

HAPLOTYPE SHARING
Sets of closely linked genetic variants in different individuals that are identical by descent around a locus.



Figure 3 | Haplotype complexity in inbred strains of mice. 700 variants with a strain distribution pattern frequency >1% between eight inbred strains across a 2-Mb region of mouse chromosome 1 are shown. The region is represented along the horizontal axis and scaled so that the n th coordinate from the left edge corresponds to the n th variant. The alternating grey and white tracks show the spatial arrangement of the 13 most common strain distribution patterns. Each track represents one strain distribution pattern, a bar on the track shows where the corresponding variant has that strain distribution pattern. The strains are A/J, AKR, BALB/cJ, C3H, C57BL/6J, DBA/2J, I and RIII. The data presented are from REF. 37.

of individuals who have common ancestors (BOX 1). However, as we have seen, inbred strains do share ancestors, so their degree of genetic relatedness is not equivalent to that in outbred humans.

To obtain a lower limit on the number of animals needed to detect a genetic effect by *in silico* mapping, assume that all inbreds are derived from just two progenitors; in other words, assume the inbreds are a set of RI lines. Using a standard equation to calculate the number of RI lines required to map a QTL, Darvasi argued that between 40 and 150 inbred strains would provide 50% power to detect a QTL that explains 5–20% of phenotypic variation in an F2 cross⁴². So, the smallest number needed to obtain sufficient power will be about 50 animals. However, it will almost certainly be more than that, because inbreds are not as closely related to each other as a set of RI lines.

Nevertheless, Peltz and colleagues claimed that they could detect QTLs using data from just eight inbred strains. They make the point that by phenotyping more animals it is possible to increase the heritability by reducing measurement error¹⁰⁰. The drawback to this argument is that, even if all environmental noise could be removed in this way, the individual effect of each QTL will still be limited by the number of other QTLs that contribute to phenotypic variation. Good estimates of the average number of QTLs and their relative contributions to a typical complex trait are hard to come by, but assuming an exponential distribution of effect sizes, our own data on behavioural measures yield a mean of 7 QTLs (with an upper limit of 14 using a 95% confidence interval (REF. 101)). So, given the known distribution and effect sizes of QTLs, it is extremely unlikely that a single effect accounting for more than 20% of the phenotypic variance will occur in a comparison of inbred strains. Therefore, we can be reasonably certain that to tackle genetically complex phenotypes successfully, *in silico* mapping will require much larger numbers of strains than have so far been analysed.

The second difficulty for *in silico* mapping concerns the structure of the genomes of inbred strains. Is the degree of mosaicism sufficient to satisfy the assumptions of the mapping method? The idea that large segments of the genomes of inbred mice maintained their sequence identity over many megabases has recently been modified by several publications that describe a much more complex picture^{37,102,103}.

The power of *in silico* mapping will vary along the genome depending on the complexity of the pattern of sequence variation. There will be effects on both power and resolution, depending on the number of progenitor genomes that are assumed to give rise to the inbred strains and on the size of the blocks of sequence identity. Although initial surveys of polymorphisms have revealed an apparently simple pattern of relatively large blocks of sequence similarity and difference, detailed analyses of multiple strains show that contiguous strain distribution patterns are different, but consistent with the same phylogenetic tree.

FIGURE 3 shows a representative example of the complexities that have been discovered in a single 2-Mb region on mouse chromosome 1 (REF. 38). In the right half of the figure, the structure is relatively simple; without too much loss of information, the distribution of sequence variants can be modelled as if the genomes were descended from just two progenitor strains, with one strain distribution pattern accounting for 93% of the variants. In this case, *in silico* mapping could detect QTLs (assuming that an adequate number of strains has been analysed). However, the method cannot resolve QTLs into an interval that is smaller than the block (which in this case is about 1 Mb). By contrast, the left half of the figure shows a much more complex structure, with several strain distribution patterns that alternate in an unpredictable fashion, but are still consistent with the same phylogenetic tree. Much higher mapping resolution will be obtained in this area, but with some loss in the power to detect QTLs.

The analysis of sequence variation described above was carried out using just eight strains, but we expect that the results can be extrapolated to accommodate more strains. Importantly, the full complexity of the structure was only revealed when near-complete sequence information became available. If the structure is mis-specified, as is likely with a low density of markers (which in this particular region would be more than about 10 kb apart), differences between strains will not be identified and so the power to detect QTLs will be reduced.

In silico mapping is an intriguing and attractive method, but it does require the ability to infer identical sequences unequivocally when neighbouring genotypes are the same. The recent announcement that 15 inbred mouse strains are to be resequenced

means that questions about appropriate marker density will soon be addressed (see the [US National Institute of Environmental Health Sciences Press Releases](#) web site), but the potential of *in silico* mapping will only materialize when we have the complete sequences of more than 50 inbred strains.

Gene-expression profiling

Few strategies even attempt to turn a QTL from a locus into a molecular variant, which highlights the difficulty in confirming the role of a gene in a complex phenotype. Gene-expression profiling is not a method for QTL detection by itself, but when combined with genetic mapping data it can help to identify candidates, even for behavioural phenotypes¹⁰⁴.

In most examples, researchers have compared the gene-expression profiles of inbreds and congenics, to reduce the number of background strain differences and to leave just a small number of genes for further consideration. The good news is that relatively few differences are found, even when a large chromosome segment is derived from two genetically distant strains¹⁰⁵. Gene-expression profiling identified the fatty-acid translocase gene *CD36* as a QTL that affects insulin fatty-acid metabolism, an example that other researchers have attempted to emulate, with varying degrees of success: complement factor 5 has been suggested as a gene at a QTL that influences susceptibility in a model of asthma¹⁰⁶, lipoxygenase (*Alox15*) has been proposed as a candidate gene for bone mass¹⁰⁷, an interferon-inducible gene for susceptibility to an autoimmune condition (systemic lupus)¹⁰⁸ and glutathione *S*-transferase M2 has been identified as a gene that might be involved in hypertension¹⁰⁹.

Although this method has strengths, there are also several caveats. First, differential gene expression is not always a marker of a QTL. Variants that alter protein structure might not alter expression levels, or there could be compensatory mechanisms that obscure the effect of a QTL on expression. Todd and colleagues used microarray analysis to study a model of type I diabetes and reported that disease protection that is conferred by each of three known loci was not reflected in global effects on the non-induced immune system, and that gene-regulatory systems seemed to be remarkably robust to genetic variation¹⁰⁵.

Second, expression differences might be restricted to certain tissues or developmental stages. For example, expression of 5HT_{1a} receptors (*Htr1a*) in the forebrain is required to modulate anxiety during embryonic and fetal life, but it is not required for the same task in adult animals¹¹⁰. Therefore, expression differences for genes that encode the serotonin receptor that are relevant to anxiety would not be detected in the adult brain, although the phenotype is assessed in the adult animal. Gene-expression analyses will have to be carried out across a large number of tissues at different developmental times to determine whether the gene is differentially expressed.

Third, finding a gene-expression difference within a relevant tissue in a relevant biochemical pathway does not prove the gene's candidacy at the QTL. There is no

guarantee that the physical coincidence of gene expression and QTL mapping data mean the two are causally related. This is a problem for Peltz and colleagues, who claim to have identified an allele-specific functional genomic element that regulates the expression of the histocompatibility 2, class II antigen E- α gene (*H2-Ea*)⁹⁸. *In silico* mapping was used to map *H2-Ea* expression variation to a 1-kb block that lies within the first intron of the gene, followed by *in vitro* assays to identify a putative 45-bp regulatory element. But differential expression of *H2-Ea* is caused by several mechanisms, including a loss-of-function deletion of 600 bp in the promoter and first exon of the *H2-Ea* gene^{111,112}.

Quantitative complementation

Quantitative complementation, or QTL-knockout interaction, which was designed originally for QTL work in *Drosophila* by Trudy MacKay¹¹³, is a method for testing the candidacy of a gene at a QTL. It is oblivious to the nature and position of the responsible sequence variant, a feature that is both its strength and weakness, because whereas one interpretation of a positive result is allelism to the QTL, another is epistasis. Strictly speaking, the test is not complementation in its classical form because it is testing for an interaction between the null allele and the QTL, rather than for a main effect of either. For this reason, the name QTL-knockout interaction test is preferable, as suggested by Darvasi¹¹⁴.

The experimental design is simple — it requires offspring from four crosses. An inbred animal bearing one QTL allele (for example 'high') is mated to an inbred animal with a null allele of the gene of interest ('m') and also to the co-isogenic wild-type animal ('wt'). A similar pair of crosses is established, but this time using an inbred strain with the alternative QTL allele ('low'). If the difference in mean phenotype between the high/m and low/m genotypes is greater than that between the high/wt and low/wt genotypes then we have evidence of quantitative failure of the mutation to complement the QTL alleles. This is detected as a statistical 'cross' (m or wt) by 'line' (high or low) interaction in a two-way analysis of variance. One biological interpretation of a significant interaction is that the expression of the wild-type (that is, functional) gene is modulated by a QTL allele on the homologous chromosome. The test does not implicate any particular QTL, which could be anywhere on the genome where the high and low strains differ.

QTL-knockout interaction tests require co-isogenic wild types, which can be difficult to obtain in mice. Knockouts created in a 129 strain are usually backcrossed onto a different strain (typically C57BL/6) so that often, no pure co-isogenic wild type is available. However, when the experimenter has only the hybrid to work with, the problem of mixed background can sometimes be overcome by taking advantage of the mosaic nature of the mouse genome: some regions of the 129 strain will be identical to the strain onto which it has been backcrossed. Where the targeted gene occurs in such a region (or in a region that is known from genetic crosses not to carry QTLs that

influence the trait of interest), and the rest of the 129 strain has been removed by repeated backcrossing, then it should be possible to find an appropriate co-isogenic wild type. For example, by extensive resequencing of the 129, C57BL/6J and DBA/2J strains, we showed that inbred C57BL/6 could be combined with a targeted mutation of the *Rgs2* gene in a quantitative complementation test³¹. This arduous task can be avoided in the future: once the relevant strains have been fully resequenced. Alternatively, knockouts could be made and maintained on a single background or obtained by screening the DNA of mutagenized inbred mice¹¹⁵.

Conclusion

Throughout this review we have emphasized two points: the importance of being able to resolve QTLs that contribute to less than 5% of the phenotypic variance into small regions and the need for methods that identify genes, not sequence variants, at the QTL.

We stress the first point because success stories in QTL cloning have identified genetic loci with atypically large effects that have obviously functional sequence changes. Therefore, an expectation has arisen that loci with smaller effects can be characterized using extensions of the same strategies: more mice, more congenics and more expression arrays. However, methods that work for large-effect QTLs are not well suited to finding genes that underlie most QTLs. Not only do most QTLs typically have small effects¹¹⁶, but the type of sequence variant responsible, even when it occurs in a coding region, is also hard to recognize³⁶, and the expression differences, if present, are subtle¹⁰⁵.

Given its importance for determining the success of QTL cloning, it is perhaps surprising that so little attention has been paid to emerging clues that reveal considerable variation in the genetic architecture of phenotypes. It seems that the number of loci, their relative effects, and how they interact with each other and the environment, might vary considerably between phenotypes, indicating that some phenotypes might be more tractable to QTL cloning than others. For example, it is striking that susceptibility to infectious disease in inbred strains has frequently turned out to be due to large genetic effects, and, where characterized, to null alleles or coding-sequence mutations^{117–122}. Why this should be the case, when other apparently equally complex phenotypes (such as hypertension, drug responses and obesity) have much more complex genetic architecture, is unclear, but it might be a consequence of the nature and extent of the selective pressure that is exerted by infectious agents. There are also differences in the extent to which gene-by-gene interactions (epistasis) shape a phenotype. Pervasive epistatic effects have been documented in some autoimmune conditions¹²³, morphology¹²⁴ and in susceptibility to some cancers^{125,126}, but despite intensive searches, the genetic architecture underlying fear-related phenotypes consists almost entirely of additive effects¹²⁷. QTL cloning strategies need to accommodate all these possible types of

genetic architecture, rather than considering whether there is a strategy that can reduce the genetic basis of any phenotype to a monogenic condition.

High-resolution mapping of small-effect loci will undoubtedly benefit from some of the new resources we have reviewed. Although some, such as the use of CSS, do little more than accelerate QTL discovery, and others, such as *in silico* mapping, make claims that have yet to be realized, there are grounds for optimism. The use of high-resolution mapping in outbred mice and the construction of a large set of RI strains (the Collaborative Cross), combined with RISTs, will make high-resolution mapping routine; and once the sequences of many inbred strains are available, *in silico* mapping could become a powerful tool. Nevertheless, even the ability to map down to under a kilobase will not always deliver genes into the hands of researchers. The availability of whole-genome analyses, at the level of both sequence variation and gene expression, can induce a hubris which blinds us to the fact that complex phenotypes continue to frustrate genetic analysis.

This is one reason why we have stressed gene identification as a goal for QTL mapping — we lack methods that deliver genes. Almost all the methods we discuss are genetic mapping strategies that chase the segregating sequence variants that are responsible for a QTL; they do not directly find genes. It is not our intention to diminish the importance of characterizing sequence variants, as there are cases where we will need to know the causal SNP (or whatever other form the molecular pathology takes). Instead, we wish to make it clear that the two goals are not necessarily the same. Stated simply, finding the QTN does not necessarily give us the quantitative trait gene. One explanation for the subtle effects of QTLs is that they lie in regulatory regions (the sequence features of which are poorly understood) that can lie at a considerable distance from cognate transcriptional units. For example, in the polydactylous mouse mutant Sasquatch, sonic hedgehog (*Shh*) is expressed at an ectopic site. Characterization of the mutant led to the identification of an *Shh* enhancer element that lies within intron 5 of a novel gene, and is also involved in limb development (limb region 1 (*Lmbr1*)¹²⁸), that is situated 1 Mb from *Shh*^{29,130}. Consider what could happen if a QTL had been found at this regulatory region: the obvious, but erroneous, conclusion would be that the *Lmbr1* gene was the quantitative trait gene.

The transition from locus to gene remains a formidable task. None of the strategies we have reviewed here provide a comprehensive solution to gene identification after QTL mapping. Simple approaches, such as investigating the phenotype of the knockout, will add weight to the candidature of the gene, but will not prove it. Definitive studies, such as the targeted interchange of nucleotides between two strains, will prove that a sequence variant operates as a QTL, but will not unambiguously identify a gene. For that, we still need functional assays that make few assumptions about the biology of candidate genes (for example, where and when they are expressed, and at what levels).

Among the approaches described here, only the gene–knockout interaction test comes close to meeting the necessary requirements. Where a null allele of the relevant gene can be found, and an appropriate co-isogenic strain is available, the test provides a simple test of candidacy and interpretation of a positive result, allelism or epistasis provides important information. The current drawback is the lack of appropriate mutants, but this might change with plans to knock out all mouse genes¹³¹. Furthermore, developments

in RNAi technology might aid functional investigation of those cases in which QTLs operate by altering expression. It might soon be possible to reproduce naturally occurring variation, to abrogate the expression of either parental mRNA, or to generate null alleles on the appropriate background for gene–knockout interaction testing. The challenge for the coming years is to develop new technologies that will accelerate gene identification after detection of a QTL.

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Acknowledgements

The authors are supported by the Wellcome Trust. We would like to thank C. Benoist for comments on the manuscript.

Competing interests statement

The authors declare no competing financial interests.

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