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Facilitating Complex Trait Analysis via Reduced Complexity Crosses

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Abstract

Genetically diverse inbred strains are frequently used in quantitative trait mapping to identify sequence variants underlying trait variation. Poor locus resolution and high genetic complexity impede variant discovery. As a solution, we explore reduced complexity crosses (RCCs) between phenotypically divergent, yet genetically similar, rodent substrains. RCCs accelerate functional variant discovery via decreasing the number of segregating variants by orders of magnitude. The simplified genetic architecture of RCCs often permit immediate identification of causal variants or rapid fine-mapping of broad loci to smaller intervals. Whole genome sequences of substrains make RCCs possible by supporting the development of array- and targeted sequencing-based genotyping

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platforms, coupled with rapid genome editing for variant validation. In summary, RCCs enhance discovery-based genetics of complex traits.

Keywords

GWAS; functional variant; rat genetics; positional cloning; substrain; QTL

Reduced Genetic Complexity Accelerates Variant Discovery

Genome-wide association studies (GWAS) and **quantitative trait locus** (**QTL**) mapping are discovery-based approaches to identify novel DNA variants, genes, and biological mechanisms underlying complex traits. While discovery-based genetics has been highly successful at identifying regions associated with trait variation, identification of the causal genes and variants remains a challenge[1]. There are two major impediments. First, several thousand genetically diverse individuals are often required to achieve sufficient power to detect loci responsible for heritable trait variation in a typical GWAS. Causal variants are rarely obvious. Second, classic linkage studies typically yield large loci containing hundreds of genes and thousands of potential variants. A surprising solution to these roadblocks has emerged as a byproduct of the cumulative fixation of residually heterozygous variants and spontaneous mutations (**genetic drift**, Box 1) in closely related, yet phenotypically divergent, **inbred substrains**. Advances in whole genome sequencing permit 1) identification of causal DNA variants underlying phenodeviation and 2) the development of high throughput genotyping platforms (targeted sequencing and custom DNA microarrays). The purpose of this review is to introduce **reduced complexity crosses** (**RCCs**) as a simple and powerful solution for rapid, high-confidence gene discovery for complex traits (Figure 1, Panel A).

RCCs between substrains consist of drastically reduced genetic diversity (complexity) in a segregating cross, yielding QTLs with far fewer candidate variants using a smaller number of individuals. Separation and maintenance of **substrains** that trace their descent from **inbred strains** has created a rich resource of nearly isogenic sets of substrains that vary genetically from less than 100 to up to ~ 100,000 sequence variants (Table 1). A rapidly growing number of genetically similar sets of substrains have now been fully sequenced, creating a database of variants that can be used both as genetic markers for mapping and positional candidate variants linked to trait variation. Several of these sets of substrains demonstrate robust and heritable differences in phenotypes, despite very low genetic diversity. For these traits, the RCC strategy is ideal for rapid identification of large-effect **quantitative trait genes** (**QTGs**) and **variants** (**QTVs**)[2,3]. QTLs in RCCs contain orders of magnitude fewer variants compared to crosses between divergent parental inbred strains, thus facilitating QTV identification and exemplifying that physical precision is not always necessary (Figure 1, Panels B and C). Validation of QTVs from a RCC is now easily and efficiently achieved via CRISPR-*Cas9* gene editing to demonstrate that a variant is both necessary (*i.e.* repair of the QTV in the mutant line) and sufficient (*i.e.* introduction of the QTV on the wild-type, ancestral line) for the predicted phenotype in both backgrounds.

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Theoretical Limits on Genetic Complexity in a RCC

How much should the segregating variant pool be “reduced” in order to be considered a RCC? Conversely, how “complex” can a cross be before it no longer affords the advantages of a RCC? The answers depend partly on the trait and its **genetic architecture** in a particular RCC. At the extremely low end of genetic diversity, there may not be a sufficient number of genome-wide variants to conduct genome-wide mapping and thus, identifying the causal variant(s) relies on deep, reliable whole genome sequencing, positional cloning, and gene editing. There is also an upper variant limit after which the RCC approach becomes equivalent to a standard F2 cross between divergent inbred strains and QTVs are no longer easily resolved. To address these questions, we consider a case of low versus moderate genomic complexity. A discussion regarding sources of genetic variation and mutation rates can be found in Box 1.

In the first example, several BXD Recombinant Inbred (RI) strains were separated at different breeding colonies, which led to genetic drift and new substrains. The BXD29/TyJ-*Tlr4*$^{lps-2J}$ (BXD29 mutant) substrain exhibits spontaneous bilateral subcortical heterotopias with partial callosal agenesis and deficits in auditory processing compared to the progenitor BXD29 strain[4]. The causal variants are unknown, but the heterotopia exhibits a two-locus autosomal recessive mode of inheritance. This is an extreme case of reduced complexity whereby sequencing identified ~1000 distinguishing variants. Although low, the number of variants is too high to identify causal QTVs by sequencing alone and mapping in a RCC is required.

The second example comes from the high demand for B6 mice that spawned multiple breeding locations and the creation of several B6 substrains currently separated by hundreds of generations at different institutions. In the 1950s, the C57BL/6N (N) lineage from NIH was separated from the C57BL/6J (J) breeding colony at The Jackson Laboratory at F32 which split the B6 lineage into two branches. Trait differences have been described, mainly between these lineages[5–7]. (Table 1). The number of variants distinguishing J versus N substrains is estimated between 10K and 20K (SNPs and indels)[6,8,9]. Causal gene variants for most phenotypic differences have yet to be identified and will require RCCs. Functional variants in *Nnt* and *Crb1* with a large impact on phenotypes were identified[6] and a *Cyfip2* variant demonstrated a pleiotropic impact on psychostimulant behaviors and binge eating[10,11]. Discovery of *Cyfip2* required only 100 to 200 RCC individuals. However, some traits in B6 RCCs could exhibit polygenic inheritance underlying trait variation. A reasonable strategy would be to assume a one-locus model and power the study based on the parental effect size using R/qtlDesign[12]. If no QTLs or suggestive QTLs [e.g., a **Logarithm of the Odds (LOD) score** greater than 3 [13] or the permissive, yet conventional “suggestive” genome-wide p-value of less than 0.63 based on permutation analysis[14]] are identified, then multiple, smaller effect loci could underlie parental substrain differences and a large sample size will be required.

Ultimately, the lower limit of genetic complexity in a RCC with regard to variant load depends on genomic sequencing and bioinformatics to resolve the causal variant(s). Approaches such as ENU mutagenesis employed a similar mapping strategy for decades and

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genomic sequencing now provides the ability to map causal variants[15]. If parental substrains do not phenotypically deviate, then ENU mutagenesis followed by a RCC can be used to map the phenodeviant followed by sequencing to identify the causal QTV[16]. Validation of predicted high impact variants (*e.g.*, variants within/near genes possessing *cis-* **eQTLs**) is then conducted using gene editing (Figure 1, Panel A). If a candidate causal variant is not revealed by sequencing, then generation and targeted mapping with a cohort of RCC individuals using candidate variants and locus-specific markers is required to resolve candidate QTGs and QTVs[17].

RCC with tens of thousands of variants have yielded clear causal variants, suggesting that the upper limit of genetic complexity providing RCC advantages has not been reached, However, at some point, increasing genetic complexity defeats the advantages of a RCC. As a case in point, consider the number of variants per 20 Mb interval (indicated in parentheses; assuming a genome size of 2.6 billion bases and random placement of variants) when the variant load is 1,000 (8), 10,000 (76), 100,000 (756), or 1 million (7,564). Once the number of segregating variants distinguishing progenitor strains swells into hundreds of thousands or millions, the number of variants within a candidate interval becomes intractable. As the number of segregating variants increases, so does the genetic complexity of a locus and the overall genetic architecture of a trait, such that additional additive or non-additive loci contribute to trait variation and obscure single-gene/variant identification. Thus, theoretical consideration of the total number of variants is worthwhile. For lower variant limits in a RCC, investigators should assess the likelihood that sequencing alone can identify causal variants with high confidence among nearly isogenic strains. If there are one or two obvious candidate variants, it is worth considering skipping the mapping step and moving straight to gene validation. For upper limits, investigators should consider trait and QTL effect size, possible underlying genetic architecture, QTL precision (the size of candidate loci) and the number of genes and variants likely to be harbored by a QTL.

RCC Design and Exemplary Behavioral Differences among Mouse and Rat Substrains

A successful RCC experiment requires heritable trait differences between substrains, a compendium of sequence variants, a marker panel and genotyping strategy, and QTL mapping software to link marker genotypes with trait variation to identify high confidence genomic intervals containing QTGs and QTVs (Figure 1, Panel A).

The first critical step is to identify phenotypic differences between substrains. Judicious selection of parental strains with desired trait differences can be difficult. The most efficient strategy is to screen related sets of substrains for reported or suspected phenotypic differences and then focus on the most closely related pair exhibiting robust trait divergence. Identification of the most genetically similar phenodeviant pair is useful because the resulting RCC will segregate fewer genetic variants, making causal variant identification easier.

Mouse substrains displaying robust phenotypic differences such as sequenced C57BL/6 substrains (Table 1), are ripe for constructing RCCs and span multiple facets of biology and

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diseases, including alcohol [18], opioid[19], cocaine[10], nicotine[20], and methamphetamine behaviors[21–23], eating disorders[11], pain[5,24], stroke[25,26], circadian activity[27], and brain morphology, including corpus callosum[28].

Inbred rat strains have also been housed for hundreds of generations at over 20 breeding facilities worldwide which spawned several rat substrains (Table 1). Recent progress with rat genomic sequencing (Box 2) has made rat RCCs feasible and more efficient. Several rat substrains (genomic sequencing in progress) exhibit robust, heritable differences in addiction-, stress- and depression related traits. Two examples, SHR substrains and Wistar Kyoto (WKY)-derived substrains, are discussed below.

Cocaine self-administration in the SHR/NCrl substrain was characterized on several indices of addiction liability[29–33]. SHR/NCrl acquired cocaine self-administration faster, exhibited escalated operant cocaine intake across doses; displayed greater reinforcement and motivation for cocaine, and greater reactivity to cocaine cues in reinstatement of cocaine seeking, compared to inbred WKY/NCrl and outbred Wistar (WIS/Crl) rats. SHR/NCrl also self-administered more heroin[34], d-amphetamine[35], and methylphenidate[36] compared to WKY/NCrl and/or WIS/Crl. Additionally, adolescent SHR/NCrl females consumed more ethanol than Sprague Dawley (SD)/Crl control rats[37]. These behaviors in SHR/NCrl capture distinct hallmarks of increased drug abuse liability. Critically, the SHR/NHsd substrain does not self-administer more drugs of abuse versus WKY/NHsd, WIS/Hsd, and/or SD/Hsd, including d-amphetamine or methylphenidate compared to WKY/NHsd, WIS/Hsd, and/or SD/Hsd controls[38–40]. With regard to nicotine, SHR/NHsd rats self-administered more nicotine via lever presses than WKY/NHsd at a dose of 30 but not 15 μg/kg[40,41]. Nicotine responses in SHR/NCrl have not been tested. High historical phenotypic diversity and low genetic complexity between SHR/NCrl and SHR/NHsd are ideal for a RCC to map the genetic basis of addiction vulnerability traits.

The WKY rat strain is also a well-established model for depression as its behavior mirrors symptoms of human major depression and anxiety including depressed mood, excessive anxiety, loss of interest or pleasure, disturbed sleep and appetite, and low energy[42–50]. Chronic treatments with antidepressants[51], electroshock administration (model for electroconvulsive therapy)[52] and deep-brain stimulation[53] can all reverse these depression-like behaviors.

The WKY strain was developed as the normotensive control for SHR strains. Louis and Howes[54] demonstrated that the WKY strain was distributed to different vendors and universities between F12 and F17 generations of inbreeding. RH segregating in WKY rats at these filial generations likely contributed to the genetic divergence within and between WKY colonies (see Box 1). Indeed, WKY demonstrated genetic and behavioral differences[55,56], including in the forced swim test (FST; a model for antidepressant efficacy in rodents). These results motivated bi-directional breeding in FST immobility[57]. Rats with the highest immobility and lowest climbing scores in the FST were bred, producing the WKY More Immobile (WMI) line. Rats with the lowest immobility and highest climbing scores were bred, producing the WKY Less Immobile (WLI) line. Rats showing the most extreme FST behavior within each line were selected for breeding,

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specifically avoiding sibling mating until F5, when sibling mating was initiated. These strains have surpassed 40 generations of inbreeding.

WMIs reliably show greater FST immobility than the WLIs[58]. With the exception of fluoxetine, antidepressant treatment (and an enriched environment in adulthood) alleviates depressive-like behavior of WMIs[57,59]. Behavioral and hormonal responsiveness to acute and chronic stress also differ between substrains[59]. In addition, stress-reactive WMIs showed greater stress-enhanced fear learning and alcohol consumption versus WLIs in a model for post-traumatic stress disorder[60]. Importantly, WMI and WLI differ by only ~4,500 SNPs and ~40 large structural variants based on combined ~100x coverage of WGS on three different sequencing platforms. Most variants are located in noncoding regions but there are some intriguing mutations in exons (e.g. *Pclo*) or splicing sites (e.g., *Rab1a*, *Slc01a2*, *Ryr3*, *Lyg1*, and *Nap1l1*). Taken together, WMI and WLI substrains can be used as RCCs to evaluate genetic factors mediating susceptibility to depression, stress reactivity, comorbidity between stress reactivity and motivation, learning, depression, anxiety and aging.

Sequenced genomes and pre-existing genotyping strategies accelerate QTG and QTV identification. The increasing availability of whole genome sequences for phenodeviant mouse and rat substrains necessitates efficient and affordable genotyping platforms for new RCCs. Only 200 or so genetic markers are required to conduct an F2 mapping study (spacing every 20 to 40 Mb or 10 to 20 cM). Optimal markers should be chosen based on pilot experiments and flanking DNA sequences. Two genotyping strategies include array-based technologies and targeted DNA sequencing. The miniMUGA DNA microarray was designed by Fernando P.M. de Villena and colleagues at UNC Chapel Hill and is sold commercially by NeoGen Genomics, Inc. (Lincoln, NE USA). This array contains complementary probes targeting between 200 and 400 SNPs for crosses between any two of over 40 inbred mouse substrains originating from 10 different parental strains[61] (Table 1). Microarrays can also be custom-designed (e.g., with rat substrains) to suit individual investigator needs.

Genotyping via multiplexed, targeted DNA re-sequencing is a high throughput and cost-effective genotyping strategy[62]. In this approach, specific genomic regions are selected and amplified using region-specific primers and multiplexed PCR followed by next-generation sequencing. Hundreds of markers can be profiled in hundreds of individuals simultaneously. Primers are designed to amplify each genomic interval containing the marker (SNP or indel). Each marker region from every RCC individual is amplified by PCR, barcoded, and sequenced at sufficient read coverage (at least 100 reads per targeted variant locus) using a next generation sequencing platform[62]. For every marker and each individual, the genotype is determined based on variant calling following read alignment to a reference genome. Commercial options are available (e.g. MonsterPlex from Floodlight Genomics LLC, Knoxville, TN USA).

Once heritable substrain differences in a trait are identified and a genotyping strategy is selected, the RCC is generated by a classic backcross or F2 intercross. Marker genotypes and trait values (e.g., behavior and gene expression) are measured for each RCC individual.

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Power analysis (e.g. the R package qtlDesign[12]) takes into account the estimated trait heritability and the additive effect of a locus in order to determine the sample size required to detect a single QTL of a given effect size and a set power level. The R package R/qtl2[63] and GeneNetwork[64] permit inclusion of additive and/or interactive covariates (e.g., Treatment, Sex, Cohort, etc.)[65] to statistically define genomic intervals containing variants that influence trait expression. The strength of linkage across the genome is represented by LOD scores, significance thresholds are generated via permutation analysis, and confidence intervals (e.g., 1.5-LOD or Bayesian credible) define the locus size[3,66]. In many cases the simple genetic architecture of RCCs enables efficient identification of large-effect QTGs and QTVs. For more complex RCCs and/or QTL intervals with no obvious candidate variant(s), rapid fine mapping can be applied to overcome low QTL resolution (Box 3).

Candidate Variant Validation in a RCC: Necessity and Sufficiency

Validation of candidate QTGs/QTVs in a RCC is straightforward because the two highly similar genetic backgrounds provide the unique opportunity to demonstrate both necessity (via mutation correction[67] and sufficiency of the QTV (via mutation induction) with little risk that epistatic interactions will obscure the results. In contrast, nominating and validating QTVs is more difficult when using populations that emphasize increased genetic complexity[68]. When there are orders of magnitude more variants to sift through in nominating candidates, it is not always clear which genetic background is appropriate to demonstrate causality – this is especially true for the Diversity Outbred (DO) population that is composed of eight segregating genetic backgrounds[69]. The potential for epistasis and modifier loci to obfuscate predicted results is a genuine concern and is not reliably predicted based on typical sample sizes that are underpowered to detect pairwise epistasis. A seminal study exemplifying the power of epistasis showed that the detection, effect size and the directional effect of heterozygous knockout alleles for two GWAS genes (*TCF7L2* and *CACNA1C*) differed across 30 different F1 backgrounds on which the heterozygous mutation was bred[70]. Even in a simpler scenario with a two-strain F2 cross, the potential is real for epistasis to obscure an allelic effect when placed on one of the parental strains (differing in millions of variants). The historical gold standard that approximates proof of QTG causality involves genetic complementation whereby mice heterozygous for a null mutation on one genetic background are crossed to the second background. If the effect of the knockout allele is rescued toward the wild-type ancestral level by the alternate strain allele, this demonstrates genetic complementation. A failure to complement as indicated by the retention of the mutant phenotype with the alternate allele is strong evidence that the causal QTG has been identified[71].

In the age of gene editing, one can provide stronger evidence for proof of the causal QTG[72] and/or QTV[67]. A major strength of RCCs is that they provide two similar genetic backgrounds with which to test for QTG/QTV causality. On the one hand, necessity can be demonstrated whereby the mutant allele (e.g., loss-of-function) is replaced (“corrected”) with the ancestral allele (e.g., regain of function) on the genetic background that normally harbors the mutation. For example, we identified a single intronic nucleotide deletion in *Gabra2* (gene coding for the alpha 2 subunit of the GABA-A receptor) in the C57BL/6J substrain that caused a decrease in transcript and protein levels (gene expression

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is the quantitative trait exhibiting partial loss-of-function with the hypomorphic C57BL/6J allele) and was reversed by insertion of the corrected allele (regain of function) onto the same genetic background[67]. In a second example, a nonsynonymous missense SNP (proline->threonine) within the trace amine-associated receptor 1 (*Taar1*) of the DBA/2J strain was strongly implicated in higher methamphetamine intake in a subset of mice from the DBA/2J strain generated from 2001–2003 in oral intake of methamphetamine[21]. Replacement of the mutant allele in the high-selected line demonstrating high methamphetamine intake with the ancestral allele reduced intake toward wild-type (ancestral allele) levels, indicating that the mutant allele was necessary for increased methamphetamine intake[22]. On the other hand, sufficiency can be demonstrated whereby the mutant allele is edited onto the nearly identical genetic background that normally harbors the ancestral allele to show that the phenotype associated with the mutant allele can be induced on the genetic background that does not normally show it. To our knowledge, demonstration of both necessity and sufficiency of a QTV from a RCC has yet to be reported.

Reduced Complexity Advanced Intercrosses

Despite the advantages of RCCs, F2 intercrosses provide imprecise localization of QTLs and “rapid” fine mapping to resolve QTLs is still quite laborious (Box 3). A separate notion to improve QTL resolution in RCCs is the **Reduced Complexity Advanced Intercross** (**RCAI**) (Box 4) which involves continual intercrossing of unrelated individuals starting in F3 offspring and continuing for an infinite number of generations to introduce new recombination events and genetically unique individuals at each generation[73–76] (Figure 1, Panel D).

Decreased diversity of RCAIs, combined with accumulation of numerous meiotic breakpoints for fine mapping of QTVs permits a nearly “comprehensive” genetic analysis of complex traits, in the sense that the contribution of all segregating variants to a phenotype within a population can be evaluated. The resulting insights will address several conundrums in mammalian genetics, including sources of missing heritability[77,78], percentage of variants that have an impact on complex traits[79,80], whether truly neutral variants exist[81], pleiotropy of QTVs among complex disease-relevant traits[82] and the omnigenic hypothesis[83]. Reduced complexity of the RCAIs also means reduced diversity, and so the resource may not answer these current issues in a generally applicable way. Nevertheless, deep phenotyping of the RCAIs, including molecular phenotypes and omics level analyses, combined with comprehensive QTV mapping of clinical traits, at the very least offers the prospect of a complete and satisfying evaluation of these issues within a simple, genetically defined segregating population[17], a prospect far beyond the reach of approaches with higher levels of genetic diversity. Another advantage of RCAIs relates to the observation that most QTVs are found in *cis*-regulatory regions and can be located at a considerable distance from the corresponding gene with many interposed “silent” variants[84,85], thus hindering QTG identification. QTLs derived from RCOs can quickly zero in on small regions with greater than two orders of magnitude reduced complexity which simplifies QTG/QTV identification. Epistasis contributes significantly to complex traits, but the large number of pair-wise combinations blunts statistical power to detect reliable interactions[86]. Decreased

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genetic complexity of RCOs makes epistatic analysis more tractable in the correspondingly rarer cases in which it clearly exists.

Concluding Remarks

RCCs offer key advantages over alternative mapping approaches that boost genetic complexity[69]. RCCs increase the speed and strength of evidence of QTG/QTV identification. However, RCCs are limited in the number of genetic factors that can be identified in a single cross as well as the number of phenotypes that can be measured. These disadvantages are offset by the rich array of existing rodent inbred substrains and the possibility of purposefully establishing new substrains. Low QTL yield and poor resolution in RCCs are mitigated by genetically simple loci, efficiency of fine mapping, and strength of evidence for causal identification. Ultimately, crosses comprising both decreased and increased genetic diversity offer unique, complementary advantages to complex trait analysis that should be fully pursued, given the emerging number of genome sequences and tools available for both approaches (see Outstanding Questions).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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GLOSSARY BOX

Expression QTL

A chromosomal region associated with variance in gene expression. A *cis*-eQTL is located near or within the cognate gene. A *trans*-eQTL is located far away from the position of the cognate gene on the same chromosome or on a different chromosome.

Genetic architecture

All DNA variants that influence trait variation, including the number, effect size, mode of inheritance, and their additive or epistatic interactions.

Genetic drift

A change in allele frequency caused by the cumulative fixation of residually heterozygous variants (inbred strains) and spontaneous, *de novo* mutations following prolonged physical separation (vendors, institutes). Generations at earlier stages of inbreeding will have more residual heterozygosity and thus a larger number of variants that become fixed. The greater number of generations separating each population, the greater number of spontaneous mutations that will become fixed.

Inbred strain

An isogenic, or genetically identical, strain with homozygous alleles at every DNA nucleotide. A strain is considered 98% inbred after 20 generations of strict brother/sister mating[87].

Inbred substrain

Distinct, isogenic strains that arose via genetic drift following separation from a common lineage, typically at different institutions or breeding facilities. The level of substrain divergence depends on the number of generations of inbreeding that separates the last common ancestor and the number of generations separating each substrain. Substrains are differentiated by abbreviations that indicate their origin from different labs or commercial vendors. For example, the designations J, N, Crl and Tac represent sublines from The Jackson Laboratory, the NIH, Charles River Laboratories, and Taconic Biosciences, respectively.

Logarithm of the Odds (LOD)

Statistical measure of the probability that a polymorphic genetic marker is physically linked to, and thus co-inherited with one or more causal variants contributing to overall phenotypic variance of a complex trait. A higher LOD score indicates a stronger statistical signal of linkage. A LOD score is akin to a negative logP value and thus, a LOD score of 3 indicates that the odds that the genetic marker and the causal variant(s) are linked are 1000 greater than the odds that they are not.

Quantitative trait locus (QTL)

A statistically defined region of the genome for which there is a strong association between genotype and trait variation such that inheritance of one set of parental alleles in a genetic population is associated with higher trait expression.

Quantitative trait gene (QTG)

The causal gene underlying a QTL.

Quantitative trait variant (QTV)

The causal variant underlying a QTL.

Reduced Complexity Cross (RCC)

A forward genetic cross (typically an F2 cross) between individuals from two substrains that contain very low genetic diversity that is used to generate a population of recombinant individuals for QTL mapping.

Reduced Complexity Advanced Intercross (RCAI)

Animals obtained by intercrossing progeny originating from two closely related strains over multiple generations.

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TEXT BOX 1

Origin of substrains.

Two sources of genetic variation underlie genetic drift, enabling RCCs for variant mapping. First, residual heterozygosity (RH) describes unfixed, heterozygous genetic loci in a progenitor strain. If founders are separated prior to complete inbreeding (< 20 generations), RH makes a larger contribution to substrain variation. RH can linger for up to 40 generations of inbreeding[87]. The number of RH variants that become fixed depends on the filial (F) generation at which the populations diverged, the number of founders (more breeder pairs equals more RH loci), the number of heterozygous variants within segregating RH loci, and the founder breeding scheme. Strict sib-sib mating within founder lineages will rapidly generate new substrains; deliberate outcrossing of offspring derived from a large number of breeder pairs will delay, although not prevent, establishment of a substrain. Mutation rates in rats (per single site base pair substitution per generation) have been estimated at 4.2×10^{-9} from wild brown rats [88] and 2.96×10^{-9} based on evolutionary divergence from mice[89].

A second source of variation is *de novo* mutations. The rate at which mutations are acquired and fixed contributes to genetic drift. Early calculations of mutation rates in rodents were based on reporter constructs at loci in single mouse lines. Meta-analysis estimated the per generation mutation rate as 38×10^{-9} per single site base pair substitution per generation (200 mutations per diploid genome per generation, assuming a 2.6 billion nucleotide genome[90]). Next-generation sequencing facilitates unbiased identification of *de novo* mutations and estimation of mutation rates in rodents is an ongoing endeavor. The mutation rate for C57BL/6J following 20 generations of sibling mating was estimated as 5.4×10^{-9} or 28 per diploid genome and generation for single nucleotide variants using DNA sequencing [91]. Notably, the mutation rate estimated by sequencing was an order of magnitude less than estimated from reporter constructs. Leveraging sequences permitted estimation of the mutation rate for homozygous (line average of 4.25×10^{-9}) and heterozygous (line average $5.45 \times 10\text{-}^{9}$) single nucleotide variants and the mutation rate of insertions and deletions (indels; 3.1×10^{-9})[91]. Mutation rates for homozygous and heterozygous variants were similar and the strategy of sibling breeding at each generation possibly contributed to greater genetic fixation of *de novo* mutations. Additional DNA sequencing studies are needed to obtain an unbiased, mechanistic understanding of rodent mutation rates, drift, and impact on trait variation.

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TEXT BOX 2

Genomic Tool Development in Rats.

Rat genome annotation and genomic tools are rapidly improving[92,93]. Whole genome sequencing (WGS) of additional inbred rat strains and substrains[94] will increase translational relevance and expand the number of complex disease and neurobehavioral traits evaluated with the RCC approach[95]. There is a coordinated effort from several NIH-funded projects (PIs: Chen and Williams, University of Tennessee Health Science Center; Dwinell, Medical College of Wisconsin; Akil and Li, University of Michigan) to sequence 100 inbred rat strains, including two of the rat RI panels (HXB and LEXF). Sequencing substrains is also part of this effort and several substrains such as WLI/Eer, WMI/Eer, LEW/Crl, LEW/SSNHsd, F344/NCrl, F344/DuCrl, and F344/NHsd have already been sequenced (Table 1). The SHR substrains, including SHRSP (stroke-prone) will be sequenced in the near future using both Illumina short reads and high molecular weight linked-read libraries, which barcodes long DNA molecules (e.g. 50–100 kb) and thus has the advantage of more accurately identifying large *structural variants. This sequencing effort also revealed assembly errors in the current rat reference genome, rn6 (H.Chen et al., Complex Trait Consortium and Rat Genome, 2019, San Diego, CA USA). A coordinated effort with the Vertebrate Genomes Sequencing project (Sanger) is generating data from one male inbred Brown Norway rat (BN/NHsdMcwi) to construct a new reference genome for the rat.

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TEXT BOX 3

Fine Mapping in RCCs.

Because RCC traits exhibit a simple genetic inheritance on a nearly isogenic F2 background[10,11,67], fine mapping (if necessary) can be implemented immediately to overcome the coarse resolution of F2 QTLs. Fine mapping involves backcrossing and the introduction of new recombination events within a QTL interval to narrow down which subregions capture the phenotypic variance and thus, capture the causal QTGs and QTVs. Existing tools for fine-resolution QTL mapping such as advanced intercross lines, outcrosses (e.g., Diversity Outbred[96] but see Reduced Complexity Outcrosses section below), and interval-specific congenics are used to increase recombination events and improve QTL resolution, sometimes to near-single gene resolution[97–100]. Implementation of these fine mapping tools can take several years as they require several generations of breeding and unique genotyping assays. In contrast, fine mapping of a QTL in a RCC can begin with the very next generation of crossing, owing to a highly similar background and simplified genetic architecture of complex traits. Once a major QTL is identified in a RCC, F2 recombinant mice within the QTL interval are selected for immediate backcrossing and phenotypic screening at each consecutive generation containing new recombination events[3]. Because of the sparse density of informative markers, one limitation of fine mapping in the RCC is the potential to "run out" of markers before obtaining single-gene or, e.g.,1 Mb resolution. In this case, functional molecular analyses (e.g., transcriptome or proteome) can help further narrow the list of causal candidate genes before moving to validation. In other words, QTL mapping and DNA sequence are not always sufficient to pinpoint likely causal QTGs and QTVs. A systems genetic approach that employs *cis*- and *trans*-eQTL analysis (e.g., at the transcript or protein level) as well as transcript covariance analysis is always a welcome addition in establishing causality.

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TEXT BOX 4

Advantages and Disadvantages of Reduced Complexity Advanced Intercrosses.

The RCAIs are advanced intercross lines produced from two closely related progenitor strains. We suggest that this approach will provide several advantages. First, cost-effective genotyping approaches are available, including exon capture and multiplexed PCR and sequencing[62]. Commercially available RCAIs would save the costs and time needed to generate, genotype, phenotype, and backcross F2 mice in order to fine map loci (if necessary). In addition, advanced intercrossing can improve mapping resolution to sub-Mb levels, depending on recombination frequency and the presence of markers that distinguish the progenitor strains. Random intercrossing of unrelated individuals in generating and maintaining RCAIs will yield a sufficient number of meiotic breaks within 10 to 15 generations (depending on the locus) to resolve the majority of individual QTVs [101]. Because of the large number of variants in genetically diverse crosses, only ~0.003% of variants are usually genotyped[96,102]. In contrast, the limited number of variants in an RCAI means that all variants can be evaluated cost-effectively. Genotyping ~100 animals will adequately interrogate the accumulated breakpoints in the RCAIs. Reduced complexity outcross strains (RCOs) are created by intercrossing animals from several closely related parental stocks. Compared to the RCAIs, the RCOs offer more opportunity for discovery but at the price of added genetic complexity. Two examples of genetically complex populations include Diversity Outbred (**DO**) mice originating from eight diverse founder strains and segregating for ~60 million variants[96] and the LG/J x SM/J Advanced Intercross Line (AIL) comprising two founder strains that have been continually outcrossed up to generation G56 at the time of the latest publication[99]. Each individual within these lines is genetically unique and thus, must be genotyped in order to conduct QTL mapping.

There are some limitations to the RCAI approach. First, sufficient power to detect phenotypic differences may require additional animals, depending on the trait variance and effect sizes. In addition, a lack of markers at narrowed loci can limit QTL resolution of RCAIs[3]. As an example, we mapped and narrowed a 30 Mb QTL to 3.7 Mb via fine mapping (**Box 2**) but we were unable to validate additional markers to detect additional recombination events. Some variants may also not be separable because of the presence of intervening recombination “cold spots”, strong selective advantage of linked variants, or transmission ratio distortion[103]. The final limitation (and perhaps the most challenging one) is that the resource has to be created for each pair or pairs of closely related strains or substrains.

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OUTSTANDING QUESTIONS

- Will the molecular mechanisms and biological pathways perturbed by gene variants in the RCC inform complex traits and diseases in humans?
- How does genetic variation in the mutation spectrum and underlying mutational mechanisms differ among inbred rodent strains and how might this impact: germline mutation rate; generation of substrains; and the types of variants that drift to fixation?
- What is the upper variant limit that defines the maximum utility of an RCC?
- Can RCCs facilitate our understanding of the highly complex role that noncoding variants play in gene regulation?
- As we discover new functional variants in B6 and other substrains, could "correcting" a portion or all of these mutations in a single parental inbred line serve as a useful resource?
- What is the optimal number of outcrosses in a RCAI and how does this relate to the degree of genetic complexity across pairs of substrains?
- Can we iteratively increase RCC complexity beyond a single major locus to improve our understanding of additive and epistatic interactions among genetic variants that influence biological pathways underlying complex traits and diseases?

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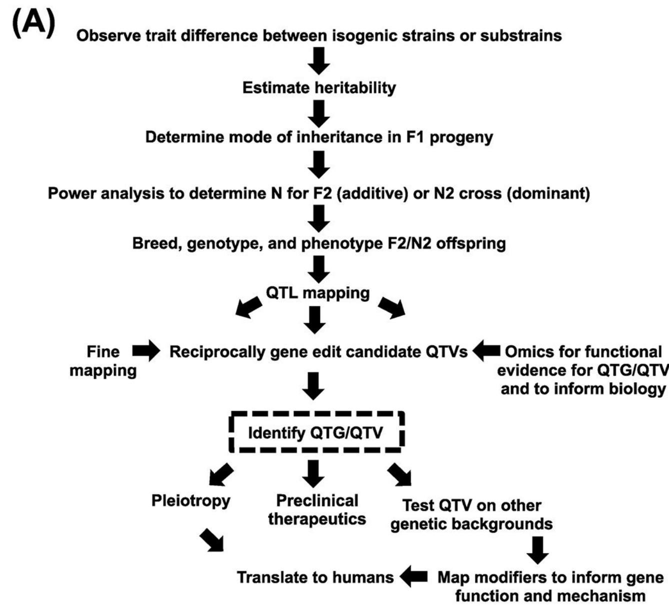
HIGHLIGHTS

- Discovery of causal genes and variants underlying complex trait variation using traditional rodent crosses is limited by the combination of high genetic complexity and modest locus resolution.
- Whole genome sequencing of nearly isogenic rodent substrains now enables a new type of cross— a reduced complexity cross (RCC) that can be highly efficient for variant discovery.
- Residual heterozygosity and spontaneous mutations between rodent breeding colonies make RCCs an efficient approach for identifying quantitative trait genes and variants.
- Compared to traditional crosses, RCCs segregate orders of magnitude fewer variants and accelerate causal quantitative trait gene and variant identification.
- Gene editing strengthens causal gene discovery by providing an efficient means to demonstrate both necessity and sufficiency of variants on substrain backgrounds.



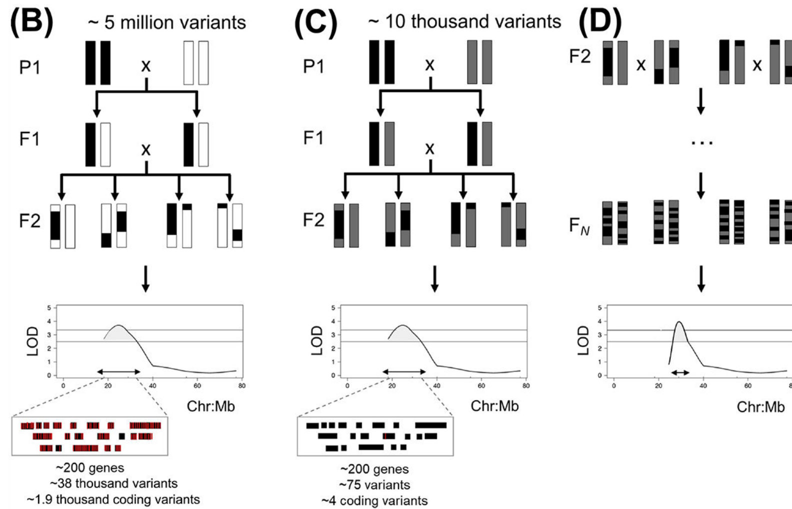


Figure 1Key Figure. Reduced Complexity Crosses (RCCs) for Complex Trait Analysis.

(A) RCC flow chart. (B) A classical F2 intercross between two inbred strains. Isogenic F1 offspring are generated by crossing two inbred strains. Every F1 individual has one chromatid from each parental strain for each chromosome. F1 offspring are intercrossed to generate recombinant F2 individuals. Historically, progenitor inbred strains segregate hundreds of thousands to millions of variants (5 million variants is used as an example here). (C) A reduced complexity F2 intercross. RCCs are generated by intercrossing strains the genomes of which are similar and, thus, segregate orders of magnitude fewer variants compared with more divergent inbred strains. As an example, a RCC between C57BL/6 substrains segregates between 10 000 and 20 000 variants (SNPs plus indels), or in other words, 250 000–500 000-fold fewer variants than a classical F2 cross. Even though the number of historical recombination events and the QTL resolution in an F2 cross are low (~20–40 Mb), the number of candidate causal variants underlying each locus is much

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smaller. For (B) and (C), the number of genes and variants within each QTL interval was determined based on a genome size of 2.64 B bases, a gene model that included 26 000 genes, and a random distribution model of genes and variants across the genome. (D) A reduced complexity advanced intercross (RCAI). The reduced complexity outcross strain (RCO) addresses the low resolution of an F2 cross by increasing the number of recombination events, which yields a narrower quantitative trait locus (QTL) interval and eliminates the need for fine mapping. The required number of generations for intercrossing to provide sufficient quantitative trait gene (QTG)/quantitative trait variant (QTV) resolution increases as genetic complexity increases.

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Table 1.

Exemplary mouse and rat substrains and phenotypes that can be subjected to RCC analysis.

Mouse progenitor strains	Sequenced Mouse Substrains (on miniMUGA array)	Behavioral differences	Physiological, disease model differences	Oellular differences	Molecular differences
A	A/J, A/JOlaHsd		Muscle dysfunction [T1]		
BALB/c	BALB/cJ, BALB/cByJ	Aggressio n[T2], alcohol preference[T3], anxiety-like behavior[T4], cognitive flexibility[T5], inhibitory control[T6], epilepsy and neuroanatomical abnormalities[28],[T7]	Allergic orchitis and encephalomyelitis [T8,T9], immune response to infection[T10], Grave's hyperthyroidism[T11], experimental arthritis and spondylitis[T12], GABA transmission and anterior cingulate volume[T13,T14], cardiac calcinosis[T15], dexamethasone-induced osteonecrosis[T16], diet-induced fatty liver[T17], streptozotocin-induced diabetes[T18]	Sperm abnormalities[T19], antibody-mediated immunity[T20], hepatocyte invasion following infection[T21, virus-induced demyelination[T22]	Copy number variants[T23], amino acid and monoamine neurotransmitter content in caudate[T24]
C3H	C3H/HeJ, C3H/HeNCrl, C3H/HeNRj, C3H/HeH, C3H/HeNHsd, C3H/HeNTac	Nest building[T25], paw preferenc e[T26]	Skeletal[T25], immune reactivity[T27], LPS responsiveness[T28], experimental leprosy[T29], spontaneous colitis[T30], experimental arthritis and spondylitis[T31], absence seizures[T32]	Cytotoxic activity of lymphocytes in cancer model[T33],	Toll-like receptor 4[T34], Gpr179[T35]
C57BL/6	C57BL/6NJ, C57BL/6NCrl, C57BL/6JBom Tac, C57BL/6ByJ, C57BL/6JOlaH sd, C57BL/6NTyr<c>/BrdCrC rl, C57BL/6NJRj	Several. Reviewed by [5]. See also [6], [T36], and main text, corticoster one-induced depressive-like behaviors[T37],	Several. Stroke[25], metabolic traits[T38], immune response[T39]. See also[6], kidney stones[T40], severity of Dravet syndrome model with Scn1a +/− [T41], circadian disruptive effects on behavior[T42], ocular lesions[T43], liver production of reactive oxygen species[T44], pain[5,24], viral-induced inflammation[T45], hindlimb unloading-induced bone loss[T46], inflammation-induced neutrophil recruitment[T47], high fat diet-induced obesity[T48], and metabolic and skeletal dysfunction[T49], impaired glucose secretion[T50], auditory physiology and pathology[T51], blood pressure[T52]	Several[6], cardiac fibrogenic response to angiotensin[T53], acetaminophen-induced hepatotoxicity[T54], hypoxic-ischemic brain injury[T55]	*Gabra2*[67], *Cyfip2*[10,11], Crb1[T56], Nlrp12[T57]
DBA/1	DBA/1LacJ, DBA/1OlaHsd		Giant lysosomes in the kidney proximal tubules[T58], collagen-induced arthritis[T59]		C5[T60]

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Mouse progenitor strains	**Sequenced Mouse Substrains (on miniMUGA array)**	**Behavioral differences**	**Physiological, disease model differences**	**Oellular differences**	**Molecular differences**
DBA/2		Methamph etamine intake[21], acoustic startle [T61, T62]	Viral infection susceptibility[T63]		Klrd1 (CD94)[T64], Taar1[21]
FVB	FVB/NJ, FVB/NCrl, FVB/NRj, FVB/NHsd, FVB/NTac		Breast cancer[T65], pituitary abnormalities[T66], T-cell dysfunction and cutaneous pathology[T67]		
NOD	NOD/ShiLtJ, NOD/MrkTac		Cataracts[T68], diabetes resistance[T69]		Structural variants in chromosome3 and Icam2 (chromosome 11)[T70]
129	129Ps/OlaHsd, 129S1/SvImJ, 129S2/SvHsd, 129S2/SvPas OrlRj, 129S5/SvEvBrd, 129T2/SvEmJ, 129X1/SvJ	Opioid addiction-related behaviors[T71,T72], fear conditioning[T73,T74], anxiety-like behavior[T74,T75,T76], habituation[T74], reversal learning[T77], spatial learning/memory[T77], forced swim test[T76], m, k,.cocaine motivation [T78]		Inhibition of NaCl response in chorda tympani[T79]	Disc1 deletion[T80]
Rat progenitor strains	Sequenced Rat Substrains	Behavioral differences	Physiological differences	Cellular differences	Molecular differences
WKY	WKY/NCrl vs WKY/NHsd	Attention[T81,T82], anxiety-like behavior[T83, social interaction [T83]			Tyrosine hydroxylase, dopamine transporter[T84], hippocampal gene expression[T85]
SHR	SHRSP vs SHRSP5/Dmcr			progression of fibrosis induced by high fat diet[T86]	
SHR	SHR vs SHRSP	cerebral stroke[T87]			
WKY	WLI/Eer	Open field[T88], FST[T89], stress-enhanced fear conditioning and alcohol consumption[60], response to chronic stress[59], Premature memory decline[T90]	Resting state functional connectivity[T91], baseline and stress corticosterone levels[T92]		Blood transcriptome[T92], amygdala transcriptome[T89], hippocampal transcriptome[T89, T93]
WKY SS	WMI/Eer SS/Jr vs SS/JrHsdMcwi vs SR/Jr	Open field[T88], FST[T89], stress-enhanced fear conditioning[60], alcohol consumption[60], response to chronic stress [59], premature memory decline[T90]	Baseline and stress corticosterone levels[60], blood pressure[T94]		Blood transcriptome[104], hippocampal transcriptome [T89,T93], and amygdala transcriptome[T89]
F344	F344/NHsd vs F344/NCrHsd		Body weight gain, neuropeptide Y and agouti-related protein[T95]		