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Fine-mapping natural alleles: quantitative complementation to the rescue

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Abstract

Mapping the genes responsible for natural variation and divergence is a challenging task. Many studies have mapped genes to genomic regions, or generated lists of candidates, but few studies have implicated specific genes with a high standard of evidence. I propose that combining recent advances in genomic engineering with a modified version of the quantitative complementation test will help turn candidate genes into causal genes. By creating loss-of-function mutations in natural strains, and using these mutations to quantitatively fail-to-complement natural alleles, fine mapping should be greatly facilitated. As an example, I propose that the CRISPR/Cas9 system could be combined with the FLP/FRT system to fine-map genes in the numerous systems where inversions have frustrated these efforts.

Keywords

quantitative complementation; QTL mapping; inversions; CRISPR

Introduction

Linking genotypic and phenotypic variation would allow progress to be made on long standing evolutionary questions (Dean & Thornton 2007; Wray 2013). Our understanding of the maintenance of genetic variation, the relevance of this variation for adaptation, and the complexity of adaptive change at the genetic level would all be improved by building functional links between genotype and phenotype. Functional analysis of natural variation also holds great promise for illuminating biological processes, because selection samples from an enormous pool of mutations over very long time periods, and may therefore produce natural variants with different (and useful) properties compared to mutations created in the lab. A strategy of understanding which genes alter traits in nature may be more productive than understanding all the genes that could, if altered, affect traits: if certain pathways are consistently used by natural selection to alter traits, then in one sense these pathways are the ones that "control" the expression of those traits. As we will never understand all aspects of biological complexity, focusing on these pathways seems like a productive venture.

Mapping trait variation to the gene level remains a daunting task despite the tremendously reduced cost of DNA sequencing and a growing number of success stories (Stern &

Orgogozo 2011; Martin & Orgogozo 2013; Nunes *et al.* 2013). Supporting this view is the simple observation that there are many more publications performing QTL mapping, comparing gene expression profiles, or mapping genomic differentiation than there are studies identifying causal alleles. Moreover, mapped alleles mostly have major effects on phenotype, and may come from the extreme tail of effect sizes found in nature (Rockman 2012). In addition, many attempts to map divergent traits or reproductive isolation have been thwarted by the caprice of recombination rate variation across the genome. Inversions, in particular, have been found to span many alleles of interest and these inversions can frustrate fine-mapping (Riesberg *et al.* 1999; Noor *et al.* 2001; Coluzzi *et al.* 2002; Feder *et al.* 2003; Anderson *et al.* 2005; Sawamura *et al.* 2008; Wang *et al.* 2013). Other regions of low recombination, like centromeres, have also proved recalcitrant (Henikoff *et al.* 2001; White *et al.* 2010). Some, but not all, of these problems can be overcome by using association studies in outbred populations rather than controlled crosses, but these approaches have additional complications such as low statistical power (Long & Langley 1999).

Below, I argue that new approaches to genomic engineering can be applied to partially alleviate these difficulties. Homologous replacement of natural alleles into a common background is sometimes thought of as the 'gold standard' of evidence for mapping natural variation, and new approaches such as the CRISPR/Cas9 system should make replacement a more high-throughput endeavor (Cong *et al.* 2013; Mali *et al.* 2013; Tzur *et al.* 2013). However, I argue that advances in genomic manipulation will have much more impact in evolutionary and ecological models if they are also used in quantitative complementation strategies. Though this approach has been somewhat maligned in the literature (Service 2004), new tools circumvent its primary weakness and may allow major progress in mapping and characterizing natural alleles.

Quantitative complementation: the next generation

Genetic complementation is a standard approach in molecular genetics that is used to determine if recessive mutations with the same phenotype are alleles of the same gene (Hawley & Gilliland 2006). If the two mutations are each recessive, but are found in different genes, then they will each be "complemented" by the corresponding wild type allele in an F1 cross. If the recessive phenotype is still seen in the F1, the mutations "fail to complement", supporting a hypothesis that they are alleles of the same gene. There are some conditions where a failure to complement can be due to epistasis rather than allelism, but when the mutations are in a common background these cases are the exception rather than the rule (Yook *et al.* 2001; Badano & Katsanis 2002; Hawley & Gilliland 2006). Imagine, for example, that a phenotype can be produced by a homozygous recessive mutation at genes A or B, but that being heterozygous for both simultaneously can produce this same phenotype. This is expected to be rare unless these two genes interact directly, or are at least in the same pathway, so this failure to complement is potentially confusing but can also reveal interactions and pathways (Yook *et al.* 2001; Badano & Katsanis 2002; Hawley & Gilliland 2006).

The quantitative complementation test (QCT) was devised as an application of this approach for quantitative traits (Long *et al.* 1996; Mackay & Fry 1996). In this case, any two natural

strains are expected to have different alleles at multiple loci affecting the trait of interest, and these alleles could have any dominance relationships. In an F1 cross between strains, the effects of these various alleles will be averaged if codominant or masked if recessive. If each natural strain is hybridized to a strain with a known loss-of-function allele, however, the natural allele at that locus will not be averaged with or masked by any other allele. As originally devised, the QCT crossed two or more natural isolates to a strain with an induced loss-of-function mutation and a control allele (fig. 1). If there is a significant statistical interaction between the loss-of-function mutation and the natural strain, this supports a hypothesis that natural variation at that locus affects the trait of interest (fig. 2).

The QCT is a clever way to use molecular genetic resources to study population genetics. In *Drosophila melanogaster*, where the test was initially employed, many loss-of-function mutations can be simply ordered in the mail from stock centers. Additionally, many stocks have been produced with large deletions spanning many genes (Cook et al. 2012; Ryder et al. 2004). If the QCT was reliable, these deletions would be exceptionally useful for mapping, because the scale of deleted regions is independent of the natural rate of crossing over at a given locus. If a QTL is mapped to a 10 Mb interval, for example, overlapping deletions spanning that interval could be used to screen this locus and fine map the alleles (e.g. De Luca et al. 2003). Once the candidate region is reduced to a handful of genes, loss-of-function alleles at these genes could then be used to determine which gene(s) harbor causal alleles.

There are two roadblocks to implementing the quantitative complementation test in emerging model systems. First, gene knock-outs and deletions are generally not delivered via the postal service. This challenge has suddenly become less intimidating due to breakthroughs in genomic manipulation -- especially the CRISPR/Cas9 system (Cong et al. 2013; Mali et al. 2013). After a fever-paced year of development (a Pubmed search for 'CRISPR' delivers 314 results for 2013), there is optimism that this technique will enable targeted gene knock-out across the tree of life (e.g. Chang et al. 2013; Gratz et al. 2013; Shan et al. 2013; Wang et al. 2013b). CRISPR, short for "clustered regularly interspaced short palindromic repeats", was recently discovered to be a component of the immune responses of bacteria and archaea (Barrangou et al. 2007). The RNAs produced from these repeats associate with CRISPR-associated (Cas) proteins to target specific DNA sequences for degradation. This machinery has now been engineered to produce mutations in a targeted way in other organisms (Jinek et al. 2012; Mali et al. 2013), and seems to work in a wide variety of taxa (Pennisi 2013). The Cas9 protein and targeting RNA can be produced *in vivo* by incorporating transgenes into the target genome (Kondo and Ueda 2014), but they can also be produced *in vitro* and coinjected into the target organism (Gratz et al. 2014). Multiple targeting RNAs can be used simultaneously, allowing multiplexed genome editing (Jao et al. 2013; Cong et al. 2013). CRISPR can also be used to insert engineered constructs (Cong et al. 2013; Mali et al. 2013; Tzur et al. 2013), such as those used in *D. melanogaster* to create deletions (Cook et al. 2012; Ryder et al. 2004). In a similar way, CRISPR could be used to swap natural alleles among genomic backgrounds, which would obviate the need for complementation tests. I suspect that replacement of gene-sized loci will remain more labor-intensive than producing knock-outs for the foreseeable future, however. Even in a

compact genome like *D. melanogaster*, many genes span considerable intervals: transcripts of *tenascin accessory* can exceed 200 kb, as annotated in flybase.org. If this gene was suspected of harboring natural variation, this variation might lie within this 200 kb interval or in regulatory regions outside it. Unless such huge pieces of DNA can be exchanged between natural isolates, such a candidate gene would have to be investigated in many pieces; two protein coding genes (thus far) are also annotated to lie within the introns of *tenascin accessory*, and would complicate inference. Quantitative complementation is therefore likely to provide a useful addition to genetic toolkits for some time.

The second challenge confronting the QCT is confounding from epistasis. Any two genotypes sampled from nature are different at myriad loci, and if any of these differences interact epistatically with the loss-of-function allele, this could produce a false positive (Service 2004). This approach has been used successfully despite this problem (e.g. Coyne *et al.* 1999; De Luca *et al.* 2003; Presgraves 2003; Kopp *et al.* 2003; Meiklejohn *et al.* 2013), but the test fails to produce a high standard of evidence on its own. This challenge is also surmountable: a similar but less problematic version of the QCT can be performed when loss-of-function mutations can be produced within the strains under study rather than in a reference strain (fig. 1). With this modified QCT, the trait value of a heterozygous genotype can be compared to both hemizygous genotypes, all in a common genetic background. This compares favorably with gene replacement: if alleles were swapped between wild-type backgrounds, the trait values of the heterozygote could be compared to both homozygous in a common genetic background. In both cases, any epistatic interactions between dosage and genomic background would be maintained across the genotypes under comparison. In the QCT, it is possible that the alleles being compared would behave abnormally in a hemizygous state if there were some non-transitive dominance, but this problem seems analogous to the limited potential for epistatic confounding in the traditional complementation test. Epistatic interactions with genomic background might lead to results that do not replicate in other backgrounds, but this is also true of allelic replacement (Chandler *et al.* 2013). If replacement is done in inbred lines, this may lead to greater background effects compared to the F1 background in the modified QCT (though in interspecies crosses, results in the hybrid background might not be repeatable within species; Rebeiz *et al.* 2009; Maheshwari and Barbash 2011). This modified QCT has previously been used to map morphological divergence between *D. melanogaster* and *D. simulans* to the hox gene *Ubx* (Stern 1998).

A conceptually equivalent approach is available even using knock-outs in reference strains: if the genetic background can't be controlled, randomize it. In the special case of recombinant inbred lines, for example, alleles are present in many genomic backgrounds (e.g. King *et al.* 2012). By crossing a set of recombinant inbred lines to a knock-out and control allele, effects of local alleles can be averaged across genomic backgrounds (Kopp *et al.* 2003; Mezey *et al.* 2005). The same is true for validating genes from genome-wide association studies (GWAS). To validate the hypothesis that variation at a given gene affects the trait under study, the knock-out and control alleles could be crossed to a set of individuals sampled directly from nature. After quantifying the trait values of the F1 individuals, DNA could be extracted to determine the genotype class of each individual

(AX, AC, BX, BC in figures 1 and 2). Unless there were alleles elsewhere in the genome that 1) interact epistatically with the loss-of-function mutation and 2) are in disequilibrium with the natural alleles under study, this test would be comparable to the modified QCT. Note also that this method estimates the effect of an allele across the gene pool rather than in a single background, which may lead to high reproducibility.

Mapping within inversions as a potential application

Suppression of recombination between inversion karyotypes has stymied mapping efforts in many fascinating systems. Engineering mutations and using them in the QCT may present a solution. First, large deletions could be generated and used to roughly map traits of interest. In *D. melanogaster*, "deletion kits" have been systematically created using engineered transposable elements to deliver recognition (FRT) sites for the *Saccharomyces cerevisiae* site-specific recombinase (FLP) (Ryder *et al.* 2004). When strains with FRT sites are crossed in the presence of the FLP enzyme, non-homologous recombination between FRT sites can generate deletions with specific breakpoints (in other applications, the FLP/FRT system could also be used to induce homologous recombination at specific base pairs to facilitate fine mapping; Cox 1988; Golic & Golic 1996).

Constructing a deletion kit in an organism with few genetic resources or a small research community is an intimidating problem, but new techniques may make it possible. If only a subset of the genome is targeted (e.g. an inverted segment), the number of FRT insertions required will be greatly reduced. If these FRT sites were incorporated into the genome at specific intervals using CRISPR rather than through random mutagenesis, the number of transformations required would also be greatly reduced. Incorporating a visible marker such as a fluorescent protein would facilitate screening of individuals both during the FRT incorporation stage and the FLP recombination phase, as is done with eye color in *D. melanogaster* (Ryder *et al.* 2004). It would be possible to maintain stocks with deletions in a heterozygous state by inducing a recessive lethal mutation in the alternative karyotype: this would turn the inconvenience of the inversion into a useful balancer for the target chromosomal interval. Additional FRT sites could then be incorporated and used for targeting sub-regions for deletion, eventually resulting in single-gene loss-of-function mutations. Using the QCT during these efforts could potentially map variation as far as the gene level. If mapping to sub-genic variants was desired, CRISPR could then be used to homologously recombine sub-regions of target genes.

By combining the population genetic study of natural variation with the liberal application of genomic engineering, major obstacles may be surmountable. Mapping of moderate effect alleles requires very sensitive assays, but the QCT is expected to be very sensitive when performed in ways that reduce epistatic confounding. The creation of resources like those described here may never be as high-throughput as DNA sequencing, and many potential challenges would confront the application of these methods to non-model systems (e.g. the ability to deliver CRISPR in a way that causes stable germline transformation, the lack of balancer chromosomes, and difficulty creating stable isogenic lines). If these obstacles can be overcome, advances in genomic manipulation may now be efficient enough to characterize numerous alleles of moderate effect in a variety of taxa. In addition to

addressing evolutionary questions of general utility, the functions of natural alleles are likely to be useful in the study of human health, agricultural improvement, pest control, and many other applications, so investment in this area should be a high priority.

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Box 1**CRISPR: where do I start?**

The literature on genomic engineering with CRISPR is growing explosively, making it hard to navigate. Though any specific method will likely be outdated rapidly, here are a few online resources to get started.

Addgene has a CRISPR site that can help you shop for the right plasmids for your goal: www.addgene.org/CRISPR/

To target a specific gene, a small piece of the guide RNA will need to be customized. There is an online tool to choose a target site from a sequence file at crispr.mit.edu, though only a small number of organisms are supported by this tool so far. For more information on RNA design, see Hsu *et al* 2013.

Protocols explaining how to customize the plasmid can be browsed at flycrispr.molbio.wisc.edu/protocols.

There is a very active Google Group for updates and troubleshooting: groups.google.com/forum/#!forum/crispr

Figure 1.

Schemes for performing quantitative complementation. The test as originally devised is shown in part A. Natural strains (red and blue) with different alleles at the target locus (A and B) are crossed to a control strain (grey) heterozygous for a loss of function allele (X) and a control allele (C). The trait values of four genotypes are compared: AC, BC, AX, and BX. The alternative test, shown in part B, can be performed when loss-of-function mutations can be produced within natural strains. The primary comparison is then between only AX

and BX, as these strains have the same genetic background; addition of an AB genotype adds information about dosage effects.

Figure 2.

Alternative outcomes of the quantitative complementation test. The original test, comparing four genotypes, is shown in part A (left). The outcome shown on the top is a negative result, though there appears to be an effect of genetic background (A vs. B) and gene dosage (X vs. C). The result below shows an interaction between background (A vs. B) and presence of the control allele (X vs. C). This could be due to alleles at the target locus or an interaction between the gene dosage and the other alleles differing between A and B backgrounds. The alternative version is shown in part B (right). The result on top shows a dosage effect but no evidence of allelic variation; below is a positive result for additive alleles. Unlike in A, an interaction between genetic background and dosage would produce the negative result on top, not the positive result below, because the AX and BX genotypes have the same genetic background.