

Note

A Potential Regulatory Polymorphism Upstream of *hairy* Is Not Associated With Bristle Number Variation in Wild-Caught *Drosophila*

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ABSTRACT

To extend results from laboratory genetic mapping experiments to natural populations it is necessary to estimate the phenotypic effects attributable to laboratory-identified genetic factors in nature. We retested a polymorphism found to be strongly associated with an increase of 0.35 sternopleural bristles in laboratory strains in two large samples of wild-caught *Drosophila melanogaster*. Despite >90% power to detect effects as low as 0.27 bristles (<1% of the total variation in bristle number) we did not replicate the association in nature. Potential explanations for this result are explored.

THE community is accumulating a set of reports identifying quantitative trait loci (QTL) for various traits in *Drosophila* and other model organisms (MACKAY 2001). In these investigations, the study organism is generally cultured under standardized laboratory conditions and often variously genetically manipulated prior to genetic analysis (*e.g.*, by inbreeding, chromosome extraction, homogenizing the genetic background, and so on). This serves to improve the signal-to-noise ratio and allow detection of small- to moderate-effect genetic factors.

Conclusions about the evolutionary and ecological relevance of variants identified in such studies are predicated on the assumption that effects detected in the laboratory are similar to those present in natural populations. To address the question of how well laboratory associations hold up in the wild, we retested a particularly strong association between a polymorphism upstream of the transcription start site of the developmental gene *hairy* (*h*) and sternopleural bristle number (SBN) in laboratory-reared *Drosophila melanogaster* (ROBIN *et al.* 2002), in outbred *Drosophila* sampled from nature.

ROBIN *et al.* (2002) surveyed 39 variants in a 29-kb region encompassing the *h* locus in a panel of 57 natural alleles of *Drosophila* sampled from Raleigh, North Carolina. A single polymorphism, del2187in, was associated

with SBN ($F = 15.84$, $P = 0.000081$) and survived Bonferroni correction for multiple testing (P -values $< 0.05/32 = 0.001563$ are significant). del2187in is a complex insertion/deletion polymorphism, and the presence of the allele 2187in was associated with an increase in SBN across four genetic backgrounds, regardless of sex. The estimated effect of an allelic substitution at this locus is between 0.27 and 0.42 bristles (mean is 0.35 bristles; see legend to Figure 1).

We genotyped this candidate polymorphism, with seven other variants across the region (Table 1), in a sample of 2000 *D. melanogaster* collected in 2001 from a single locality in Napa Valley, California (the nv2001 population). To eliminate any possibility of sample-specific effects, we also genotyped del2187in in a second similarly large sample collected in 1996 in Sonoma Valley, California (the sv1996 population). All markers were in Hardy-Weinberg equilibrium, and abdominal and sternopleural bristle numbers, scored for each individual as previously described (LYMAN and MACKAY 1998), appeared normally distributed (*cf.* GENISSEL *et al.* 2004). Bristle number means (phenotypic variance) are 16.7 (4.64), 17.3 (4.79), 15.8 (5.63), and 18.2 (7.63), for male and female sternopleural and male and female abdominal bristle number, respectively, within the wild-caught nv2001 flies.

The following ANOVA models were applied separately to each population to assess the contribution of each polymorphism to the bristle number phenotypes: (1) additive model, which corresponds to a regression of the phenotypic data on the number of major alleles present in each individual and provides an estimate of the effect, a , of an allelic substitution; (2) additive by

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession nos. AY587211–AY587226.

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TABLE 1
Sternopleural bristle additive model F -ratio tests

Polymorphism ^a	Common allele frequency	Assay/population ^b	Males			Females		
			N^c	F^d	a (SE) ^e	N^c	F^d	a (SE) ^e
A127G	0.75	OLA/nv2001	940	0.172 (NS)	-0.047 (0.114)	937	0.122 (NS)	-0.041 (0.117)
AG646-7GC	0.69	OLA/nv2001	953	0.583 (NS)	0.081 (0.107)	959	2.057 (NS)	-0.158 (0.110)
G668A	0.71	OLA/nv2001	953	0.146 (NS)	0.042 (0.110)	948	2.789 (NS)	0.192 (0.115)
A775C	0.77	OLA/nv2001	957	1.419 (NS)	0.144 (0.121)	936	0.266 (NS)	-0.064 (0.125)
A864T	0.70	OLA/nv2001	961	0.284 (NS)	-0.056 (0.106)	908	0.696 (NS)	0.093 (0.112)
C2158T	0.57	OLA/nv2001	933	3.167 (NS)	0.173 (0.097)	902	0.181 (NS)	0.045 (0.105)
del2187in	0.74	PCR/nv2001	923	0.227 (NS)	0.055 (0.114)	912	0.721 (NS)	-0.096 (0.113)
del2187in	0.71	PCR/sv1996	792	0.487 (NS)	-0.082 (0.118)	719	0.008 (NS)	-0.010 (0.116)
G2759T	0.97	OLA/nv2001	919	0.489 (NS)	0.216 (0.309)	962	0.109 (NS)	0.097 (0.292)

^a Polymorphisms are labeled such that the common allele is followed by the position in the *hairy* alignment of ROBIN *et al.* (2002) and then by the rarer allele.

^b Genotypes for most polymorphisms were collected using an oligonucleotide ligation assay (OLA) approach as described previously (GENISSEL *et al.* 2004). del2187in is a complex insertion/deletion polymorphism (common allele ATAAAAAAAA; rare allele TATACATAGTATAGTATATATAGT) and as such is not amenable to this method. Instead we developed a fluorescent fragment size-based assay for an ABI 377 automated sequencer. Briefly, from gDNA we PCR amplified a short fragment about the polymorphism using the oligos del2187in.F, 5'-GACGTTGTAACG[N]CGCTGTAAACTCGCAACG-3', and del2187in.R, 5'-CAATTTACACAGG[N]TCTTAGATACTAACTATGTTGA-3', where the underlined sequences represent 14-nucleotide (nt) M13-specific tails for second-round amplification, and the region in square brackets a variable-length spacer (0, 2, or 8 nt for del2187in.F, and 0 or 4 nt for del2187in.R), allowing samples to be pooled. Second-round PCR with the oligos M13F.BRL, 5'-[6FAM]CCCAGTCACGACGTTGTAACG-3', and M13R.BRL, 5'-AGCGGATAACAATTTACACAGG-3', served to simultaneously amplify and fluorescently label the fragments.

^c Number of genotyped individuals used in the regression.

^d F -ratio computed from a linear regression of genotype on phenotype (NS, $P > 0.05$).

^e Effect (standard error) on bristle number of substituting a common allele with a rare allele (*i.e.*, AA to Aa, or Aa to aa).

sex model, which applies a factorial ANOVA to generate estimates of a , the effect of sex, s , and an estimate of the genotype-by-sex interaction, a^*s ; and (3) arbitrary dominance model, whose F -ratio statistic is mathematically equivalent to a one-way ANOVA with three levels, but provides estimates of a and the dominance deviation, d .

The candidate polymorphism del2187in showed no association with either bristle trait for any sex or population combination. Indeed, no polymorphism showed a significant effect of a at $P < 0.05$ for any test (Table 1). The arbitrary dominance model for variant AG646-7GC is significant for male abdominal bristle number (ABN) in nv2001 ($F = 4.49$, $P = 0.011$), largely because d is significant ($F = 8.72$, $P = 0.003$). We find no effect of this variant on female ABN and, in common with ROBIN *et al.* (2002), find no effect of AG646-7GC on SBN in either sex. Linkage disequilibrium (LD) is low between AG646-7GC and del2187in in both studies. Further work is required to determine if AG646-7GC represents a true bristle number QTL.

Figure 1 plots the estimated additive effect (a) of del2187in on SBN from the additive model for each sex and population, and the 95% confidence limits (a_{\max}) on the estimated effects. The number of standard errors between our estimates of the effect of del2187in, averaged over sex and population, and the three significant estimates from ROBIN *et al.* (2002) are 2.6, 3.8, and

5.0. We suggest that if del2187in does influence natural variation in SBN, its maximal effect and perhaps its sign are not in accord with the previous estimates from laboratory lines.

The upper bound on the effect of an allelic substitution at del2187in (a_{\max}) depends on the total observed phenotypic variation, such that as the sum of the variation due to other loci and the environment increases, a is estimated with less accuracy, and a_{\max} shows a corresponding increase. Since such variance in nature appears higher than that under controlled laboratory environments and genetic backgrounds, we employed very high sample sizes ($N \sim 2000$) to counteract its negative effect on our confidence in a and to obtain a narrow confidence interval. It is noteworthy that our estimate of a is not in any way conditional on the heritability of bristle number in nature. Irrespective of heritability of bristle number (in the laboratory or nature) we can accurately estimate a and place an upper bound on the effect of del2187in. Due to our large sample size the confidence interval on our estimate of a is smaller than previously reported laboratory estimates.

The primary motivation behind efforts to identify QTL under laboratory conditions is to improve the signal-to-noise ratio: reducing variation due to environmental and other loci segregating bristle number QTL will increase power to detect any desired genetic factor. Hence, one might suspect that power to detect moder-

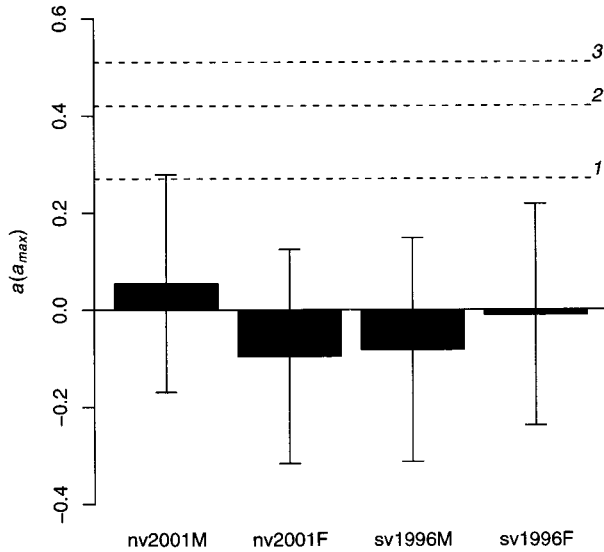


FIGURE 1.—Estimated additive genetic effect from a linear regression of genotype at the candidate polymorphism del2187in on SBN. a is the effect of substituting the common deletion allele with the rare insertion allele, and a_{max} is the 95% confidence limit on this estimate. The dashed lines represent independent estimates of the effect of del2187in from ROBIN *et al.* (2002), measured as the difference in mean SBN between 2187in and del2187 marker genotypes among natural h alleles in three genetic backgrounds (four backgrounds were tested but only three showed a significant effect of del2187in on SBN). (1) Among homozygous h near-isogenic lines (NIL) the effect of del2187in was 0.54. With no dominance, $a = 0.54/2 = 0.27$ (as plotted), but if 2187in is dominant to del2187, as suggested by ROBIN *et al.* (2002), $0.27 < a \leq 0.54$. (2) For the set of h NIL made heterozygous against wild-type h from the *Sam* homozygous genetic background, $a = 0.42$. (3) The effect of del2187in was estimated as $a = 0.51$ from the set of h NIL heterozygous against an h' null allele introgressed into the *Sam* background. However, since the natural h alleles are combined with a null allele it is difficult to estimate the true value of a in this background.

ate effects in natural populations is low. Figure 2 refutes this suggestion, showing that with just one sex ($N \sim 1000$) we have 90% power to detect effects as low as 0.38 sternopleural bristles, or $\sim 1.2\%$ of the natural variation in SBN (*i.e.*, $2pqa^2/V_p \approx 1.2\%$, where V_p is the total phenotypic variation; FALCONER and MACKAY 1996), and by including both sexes ($N \sim 2000$) we are able to identify effects as small as 0.27 sternopleural bristles ($\approx 0.8\% V_p$). Since heritability of bristle number in nature has been estimated to be perhaps 50% (RISKA *et al.* 1989), we have high power to detect sites contributing 1–2% to the total genetic variation in bristle number. Such effects would be considered subtle by the standards of the QTL mapping community (see TANKSLEY 1993, Table 1).

It is important to appreciate that in failing to replicate the previously reported laboratory association in nature, we are not implying that the original report was a false positive. The association was highly significant and passed a rigorous Bonferroni correction for multiple

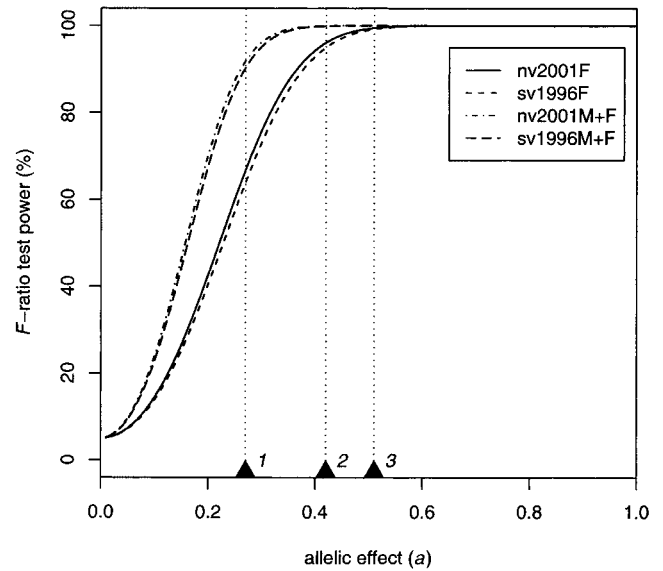


FIGURE 2.—Power to detect association between del2187in and SBN. Using parameters derived from our tests of association between del2187in and SBN in nv2001 and sv1996 (allele frequency, sample size, and F -ratio test error mean square), we calculated the power to detect arbitrary allelic effects, a (at $\alpha = 0.05$). For the single-sex additive model F -ratio test, power is defined as $1 - F_{\alpha(1, N-2, \phi^2)}$ using the general noncentrality parameter $\phi^2 = [(\sum n_i(\mu_i - \mu)^2)/(\sigma_e^2)]$, where n_i is size of the genotypic class i , μ is grand mean, μ_i is mean of class i , i indexes the three genotypes, AA , Aa , and aa , and σ_e^2 is the error mean square. Very similar estimates of power (at $\alpha = 0.05$) are obtained from a Monte Carlo simulation using the observed genotypic class frequencies and sample sizes, additive gene action, and scaling the allelic and Gaussian residual variation so that the site accounts for $x\%$ of the total phenotypic variation. We carried out 1000 Monte Carlo replicates for each value of x , with a linear regression of phenotype on genotype performed on each replicate, using the statistical computing language R (<http://www.R-project.org>). Triangles represent the same estimates of the effect of del2187in on SBN from ROBIN *et al.* (2002) described in the legend to Figure 1.

tests, was robust to genetic background, was based on a moderate number of natural alleles, and was consistent with previous quantitative complementation results showing that variation at h influenced SBN but not ABN (LONG *et al.* 1996; GURGANUS *et al.* 1999). Below we outline some alternative explanations and highlight some testable predictions. Note that these hypotheses are not mutually exclusive and could all play a role.

Larval competition: It has been shown that with severe competition among larvae for resources there is stabilizing selection for bristle number, such that the bristle number variance is reduced as larval competition is increased, resulting from a selective elimination of the extreme phenotypes (KEARSEY and BARNES 1970). If the populations from which we sampled were subject to stabilizing selection due to larval competition, the power of the F -ratio tests may have been reduced. This hypothesis predicts that heterozygotes would have a se-

lective advantage and thus be in excess of their expectation under Hardy-Weinberg equilibrium. However, neither of our populations show deviation from Hardy-Weinberg equilibrium with respect to the genotype at del2187in ($\chi^2 P > 0.05$), and the observed genotypic counts are just 12 (43) individuals from the expected counts under Hardy-Weinberg for the nv2001 (sv1996) population, with both populations showing a very slight dearth of heterozygotes.

Population structure: If a species is genetically divided into subpopulations one may not expect to find quantitatively or qualitatively similar associations among demes. The 10 East Coast North American chromosomes sequenced for *hairy* by ROBIN *et al.* (2002) appear similar to a short region about del2187in sequenced in 16 West Coast alleles (accession nos. AY587211–AY58726); however, neither sample is sufficiently large to detect small differences in allele frequency.

A better data set is provided by 196 biallelic single nucleotide polymorphisms (SNPs) in the *Epidermal growth factor receptor (Egfr)* gene region in 140 lines from Davis, California, and 86 lines from West End, North Carolina (DWORKIN *et al.* 2003). Using Fisher's exact tests only one SNP showed a significant frequency difference between populations at the 5% level after applying a permutation test (CHURCHILL and DOERGE 1994). The genetic homogeneity between East and West Coast samples of *Drosophila* for the *h* and *Egfr* loci matches previous observations for the alcohol dehydrogenase region (KREITMAN and AGUADÉ 1986), suggesting that source population differences are unlikely to explain the discordance in association.

Linkage disequilibrium: As ROBIN *et al.* (2002) point out, since they did not genotype all polymorphisms in the *h* region it is possible that del2187in is not causal and instead is in strong LD with an ungenotyped causal QTL. In this case the phenotypic effect at del2187in would be lower than the effect of the causal QTL by a factor proportional to the amount of LD between the sites (*cf.* LAI *et al.* 1994). Under such a scenario any reduction in the level of LD between del2187in and the true QTL in our outbred populations would reduce the ability to detect del2187in.

Since the set of SNPs genotyped in this study are different from those typed by ROBIN *et al.* (2002), we are unable to assess any population-specific differences in LD structure at the *h* locus. Instead, to gauge the level of any such differences, we used data provided in KREITMAN and AGUADÉ (1986) and DWORKIN *et al.* (2003) to assess the homogeneity of LD estimates between populations (WEIR 1996, p. 137). These two studies provide 53 and 14,143, respectively, informative estimates of LD (considering only SNPs showing >5% minor allele frequency within both populations), of which only 2 showed a significant difference between populations after Bonferroni correction.

This implies that patterns of LD are similar among East and West Coast North American populations. As-

suming that this result also applies to the *h* gene region, even if del2187in is not itself the causal QTL, it should have maintained similar LD with the actual QTL in our population as it did in the lines used by ROBIN *et al.* (2002).

Genotype-by-environment interaction: Environmental conditions encountered by flies sampled directly from nature cannot be controlled, and a measured allelic effect represents a weighted average of the effect over environments. Therefore, to the extent that a genotypic effect is modulated by environmental factors, the ability to detect a variant in a natural population may be diminished.

The magnitude and generality of genotype-by-environment interaction (GEI) at the level of individual QTL is unclear. In one of the best studies GURGANUS *et al.* (1998) identified significant heterogeneity in bristle number QTL effect across thermal and sexual environments in *Drosophila*. However, when we consider errors in measuring the effects of QTL in different environments (simulation data not shown), although there is significant GEI (as observed by GURGANUS *et al.* 1998), it is difficult to precisely gauge its magnitude. Unfortunately, the pattern of GEI is still largely unknown for quantitative traits, especially when the environments of interest are laboratory *vs.* nature. Thus, we cannot reliably discount or support the possibility that GEI contributes to the discrepancy between the del2187in association in the laboratory and in nature.

Laboratory effects: Under some forms of epistasis the use of isogenic laboratory strains could result in estimates of effects in the laboratory that would be larger than those observed in outbred populations. Under a two-locus model (based on CROW and KIMURA 1970, p. 79) with no epistasis, where both a genotyped locus (*B*) and an ungenotyped unknown locus (*U*) have phenotypic effects, estimates of the phenotypic effect of *B* are identical in outbred and inbred populations regardless of frequency or the degree of dominance at either locus. As an example, however, in a population where the rare double homozygote *bbuu* has effect $-\epsilon$ and the other eight genotypes $+\epsilon$, the phenotypic effect associated with locus *B* will always be inflated in the laboratory (isogenic) population.

Although this is a fairly extreme case of synergistic or reinforcing epistasis, it is the type of epistasis expected under models of mutation-selection balance in which only the rare double homozygote genotypes produce extreme phenotypic effects visible to purifying selection. The evidence for synergistic epistasis (SE) is equivocal—compare WHITLOCK and BOURGUET (2000) and PETERS and KEIGHTLEY (2000)—however, such experiments have tended to look at SE between randomly induced mutations on fitness-related traits, and it is conceivable that SE is a more general phenomenon within genes, or pathways of genes, or on traits with small pleiotropic effects on fitness. For example, SHEPARD *et al.* (1989) demonstrated extensive interactions between the neuro-

genic loci *Notch*, *Delta*, and *Enhancer of split* on *Drosophila* eye morphogenesis, while DWORKIN *et al.* (2003) detected a synergistic interaction of photoreceptor determination between two sites within the same *Egfr* exon in *Drosophila*.

Choice of isogenic background may be particularly important if del2187in epistatically interacts with other loci. ROBIN *et al.* (2002) generated a set of nearly isogenic lines each having a small section of natural chromosome about *h*, but otherwise a completely isogenic *Samarkand* (*Sam*) genetic background. If a genotyped putative causal SNP epistatically interacts with an allele rare in natural populations but fixed in *Sam*, the estimated effect at the genotyped SNP will not necessarily be indicative of its effect in nature.

Future work: The contrast between the laboratory identification of del2187in as a strong bristle number QTL and the findings of this study suggests that laboratory associations may not always translate directly to average effects (*sensu stricto* FALCONER and MACKAY 1996, p. 112) in nature. The most viable hypotheses discussed above make testable predictions regarding the relationship between QTL in the laboratory and in nature, and while the experiments are certainly not trivial, the *D. melanogaster*/bristle number model will allow eventual resolution of the observed difference. To test the hypothesis that uncontrolled environmental variation prevents detection of the effect in our large natural population samples, associations could be examined in a large sample of wild-caught females and in their laboratory-reared progeny. Alternately, if the laboratory association were conditional on an epistatic interaction with an unidentified homozygous site in the *Sam* isogenic background, repeating the chromosome substitution scheme of ROBIN *et al.* (2002) in different genetic backgrounds would eliminate the effect.

It is possible that the discrepancy reported here represents an isolated case, where perhaps the initial result was due to a highly significant epistatic interaction with a particular genetic background or where GEI effects are more important than we suggest. Fortunately, the potential explanations for the observed differences yield testable predictions, and it is within our reach to understand the architecture of a complex model character in terms of the individual nucleotides governing the trait in nature.

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