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Evolution of the *tan* Locus Contributed to Pigment Loss in *Drosophila santomea*: A Response to Matute et al.

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SUMMARY

We have shown previously that the loss of abdominal pigmentation in *D. santomea* relative to its sister species *D. yakuba* resulted, in part, from *cis*-regulatory mutations at the *tan* locus. Matute et al. claim, based solely upon extrapolation from genetic crosses of *D. santomea* and *D. melanogaster*, a much more divergent species, that at least four X chromosome regions but not *tan* are responsible for pigmentation differences. Here, we provide additional evidence from introgressions of *D. yakuba* genes into *D. santomea* that support a causative role for *tan* in the loss of pigmentation and present analyses that contradict Matute et al.'s claims. We discuss how the choice of parental species and other factors affect the ability to identify loci responsible for species divergence, and we affirm that all of our previously reported results and conclusions stand.

INTRODUCTION

The identification of genes and mutations involved in evolutionary change is a major goal of evolutionary biology. Previously, Jeong et al. (2008) implicated changes in the regulation of two major pigmentation genes in the evolutionary loss of pigmentation in *D. santomea*. Seven lines of evidence were presented that led to the conclusion that *cis*-regulatory mutations at the *D. santomea tan* locus contributed to the loss of pigmentation in *D. santomea* relative to its *D. yakuba* sister species. First, it was shown how the Tan and Yellow proteins collaborate to promote melanic pigmentation in *D. melanogaster*. Second, it was demonstrated that *tan* and *yellow* expression were greatly reduced in the most posterior segments of the *D. santomea* abdomen. Third, through examination of gene expression in hybrids of *D. santomea* and *D. yakuba*, it was shown that the loss of *yellow* expression was due to loci that act in *trans*, while the loss of *tan* expression was due to changes in *cis*. Fourth, a *cis*-regulatory element (CRE) necessary for *tan* expression and function in the developing abdomen in *D. melanogaster* was identified. Fifth, this CRE was shown to be functional in *D. yakuba* but mutationally inactivated in *D. santomea*. Sixth, it was found that reversion of just two mutations restored activity to the *D. santomea* CRE. Together, these results indicated that the *D. santomea* gene was inactive in the abdomen due to mutations in one specific *tan* CRE.

Since Tan activity is necessary for the full dark pigmentation of *D. melanogaster* or *D. yakuba*, Tan activity must be restored to *D. santomea* to restore *D. yakuba*-like pigmentation. In the seventh line of investigation, a functional, well-characterized genomic *tan* transgene (from *D. melanogaster*) with an intact CRE was introduced into *D. santomea* and the transgene partially restored pigmentation.

Matute et al. reach conclusions that conflict with one of the findings reported by Jeong et al. (2008), that *cis*-regulatory evolution at the *tan* locus contributes to the pigmentation difference between *D. santomea* and *D. yakuba*. In this response, we provide additional evidence supporting our conclusion, present analyses that argue against their conclusions, and discuss some of the biological and methodological factors that can undermine the detection and identification of loci involved in species divergence.

RESULTS

Localization of the Major X QTL to a Small Region that Includes *tan*

In addition to the developmental, molecular, and transgenic evidence implicating *tan* in pigmentation divergence, there are substantial supportive genetic data. Prior to publication, M.R.-W. and

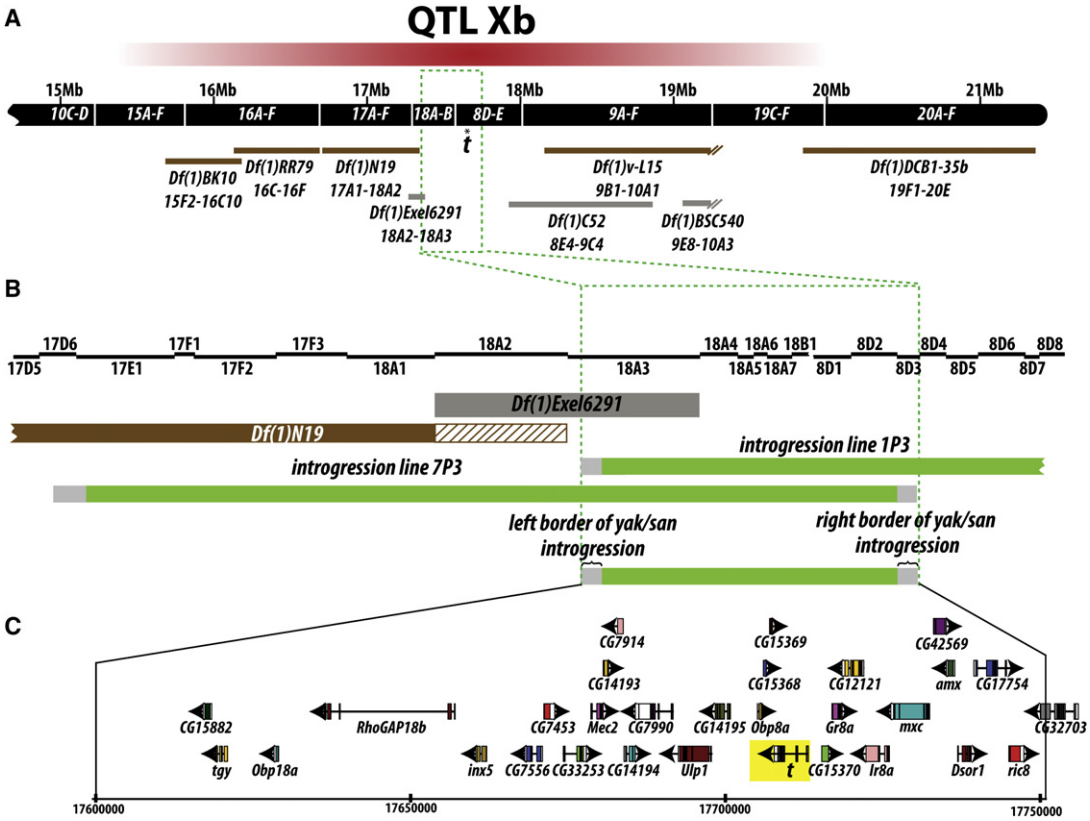

Figure 1. QTL Xb Contains the *tan* Locus and Excludes All Four Regions Identified by Matute et al. as Candidate QTLs

(A) Chromosomal view of QTL Xb (red shading), showing the locations of deficiencies that Matute et al. reported affected the *D. melanogaster*/*D. santomea* hybrid phenotype (brown lines), the region of the *D. santomea*/*D. yakuba* QTL Xb mapped by introgression (green dashed lines), and the *tan* gene (asterix). One deficiency shown [*Df(1)Exel6291*, gray line] was reported by Matute et al. as having no phenotypic effect, which indicated that the region of overlap with *Df(1)N19* had no effect. Cytological positions of the chromosomal regions from *D. melanogaster* that are syntenic with the *D. yakuba* chromosome are shown.

(B) Fine map of two introgression lines that define a 161 kb interval for QTL Xb. The left end of the interval (corresponding to the 18A3 syntenic region) is defined by introgression line 1P3 (green bar). Gray shading shows the region in which the recombination break point between *D. santomea* and *D. yakuba* resides. The right side of the interval is defined by an introgression line with a breakpoint in the 8D3 syntenic region. For a lower-resolution but more extensive view of the introgressed regions, see Figure S1. The breakpoint of the deficiency closest to QTL Xb, *Df(1)N19* (brown box), lies just outside of the minimal introgression interval and is overlapped by a second deficiency, *Df(1)Exel6297*, which had no hybrid pigmentation phenotype (overlap is striped brown) and excludes the overlap region from containing gene(s) affecting the hybrid phenotype.

(C) Location and orientation of 30 genes, including *tan* (*t*, yellow shaded box), contained within the interval defined by introgressions.

D.L.S. informed Jeong et al. that they had independently mapped an interval containing the major X chromosome quantitative trait locus (QTL) by introgression of segments of the *D. yakuba* X chromosome into the *D. santomea* X chromosome. This interval was localized to approximately 500 kilobases (kb), and in all introgression lines, the *tan* locus could not be separated from the QTL interval. This independent verification that *tan* resided in the major QTL interval was cited in Jeong et al. (p. 784–785). Here, we report these data in detail and at higher resolution.

Carbone et al. (2005) identified four QTLs that account for most of the difference in pigmentation between *D. yakuba* and *D. santomea*. One QTL spanning the *tan* locus, which we refer to as QTL Xb (Figure 1A), makes a major contribution to pigmentation in males and a much smaller contribution to pigmentation in females (see also Table 4 and Figure 1b in Carbone et al. [2005]). We isolated introgressions of QTL Xb by phenotypic selection on flies from multiple generations of backcrossing into a *D. santomea* background accompanied by genotyping with markers within the QTL regions identified by Carbone et al. (2005). Male hybrid progeny of this backcrossing scheme remained sterile for approximately seven generations, and females displayed little pigmentation for QTL Xb. In order to isolate introgressions of this region, we therefore backcrossed single females (whose brothers displayed pigmentation) to *D. santomea* males until, after many generations, males carrying the introgression became fertile. We isolated six introgression lines that carried *D. yakuba* DNA for only QTL Xb and mapped most of their breakpoints to approximately 10 kb resolution (Figure S1 available online).

Importantly, each of these lines had the same pigmentation phenotype in the posterior abdomen, an inverted "T" pattern in males (Figure S2). The similar phenotypes are most consistent with a model in which the region they share in common contains one or more genes that constitute the QTL. One of these

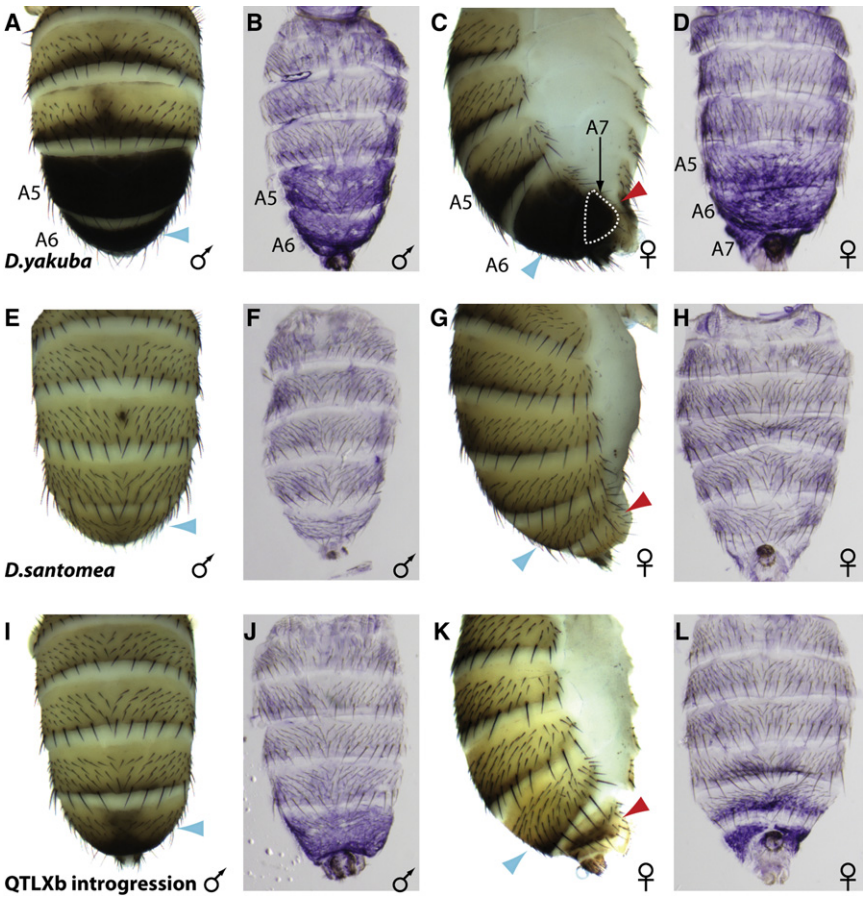

Figure 2. The Phenotype of QTL Xb and *tan* Gene Expression Are Strongly Correlated

Pigmentation and *tan* gene expression in *D. yakuba* (A–D), wild-type *D. santomea* (E–H), and *D. santomea* animals carrying the 1P3 introgression (I–L) are shown. The sex of each animal is indicated.

(A) Wild-type male *D. yakuba*, note the intense pigmentation of segments A5 and A6.

(B) *tan* is expressed at high levels in segments A5 and A6 in *D. yakuba* males.

(C) Wild-type *D. yakuba* female. Note that segment A7 is also pigmented (red arrow).

(D) *tan* is expressed in segments A5–A7 in *D. yakuba* females.

(E) Wild-type *D. santomea* male is largely unpigmented.

(F) *tan* expression is lost from the posterior abdomen in *D. santomea* males.

(G) Wild-type *D. santomea* female lacks strong posterior pigmentation (blue and red arrows).

(H) *tan* expression is lost from the posterior abdomen in *D. santomea* females.

(I) *D. santomea* male carrying the 1P3 introgression exhibits strong pigmentation of segment A6 (blue arrow).

(J) *tan* expression is restored in segment A6 of *D. santomea* male carrying the 1P3 introgression, which correlates with the pigmentation phenotype.

(K) *D. santomea* female carrying the 1P3 introgression exhibits strong pigmentation of segment A7 (red arrow) and part of segment A6 (blue arrow).

(L) *tan* expression is restored to segment A7 and part of segment A6 in *D. santomea* female carrying the IP3 introgression, which correlates with the pigmentation phenotype.

introgressions was only ~410–443 kb (Figure 1C), indicating that DNA outside this introgression does not contribute to QTL Xb. This region has been further subdivided and refined by the overlap of all introgressions which define an ~149–161 kb minimal region containing QTL Xb (green bars in Figures 1B and 1C; Figure S1) that includes the *tan* locus (shaded yellow in Figure 1C), as well as the CRE discovered by Jeong et al. (2008). Twenty-nine other genes are included in the minimal region (Figure 1C), none of which have known effects on pigmentation.

For all introgressions, hemizygous males produce strong posterior abdominal pigmentation (Figure 2I) that is similar to the pigmentation observed in *D. santomea* males homozygous for a *tan* transgene (Jeong et al., 2008). Homozygous females carrying these introgressions produce noticeable but lower levels of pigmentation (Figure 2K), while heterozygous females produce much lower levels of pigmentation (data not shown). Carbone et al. (2005) also observed this strong sex difference in the effect of QTL Xb.

Moreover, we found that in animals carrying these introgressions, *tan* expression was restored to the posterior segments displaying increased pigmentation (Figures 2J and 2L, compare with Figures 2F and 2H). This correlation between the trait and *tan* expression suggests that the phenotype of QTL Xb is largely, if not entirely, due to *tan* expression.

Thus, all direct evidence concerning the *D. yakuba-D. santomea* divergence including the concordance between the gene introgression and *tan* gene expression phenotypes, coupled with the prior molecular, developmental, and transgenic analysis of Jeong et al. (2008), strongly support the inference that *tan* is QTL Xb. This combination of evidence satisfies multiple consensus criteria for the identification of causative QTL (The Complex Trait Consortium, 2003). We cannot yet rule out that one or more other loci in this 161 kb region also contribute to QTL Xb.

We now turn to our analysis of how and why Matute et al. (2009) reached different, and in our view erroneous, conclusions.

The Genetic Architecture of Pigmentation in *D. melanogaster-D. santomea* Hybrids Is Different from that of *D. yakuba-D. santomea* Hybrids

Matute et al. attempted to measure the effect of *tan* mutations and chromosomal deficiencies from *D. melanogaster* on pigmentation of *D. melanogaster-D. santomea* hybrids. To draw conclusions from these experiments with respect to the genetic basis of the differences between *D. santomea* and *D. yakuba* requires the assumption that the quantitative and qualitative genetic basis of pigmentation differences between each species pair is the same. This assumption is not justified. The genetic architecture

of pigmentation differences is not the same in the two different species hybrids with *D. santomea*.

This is demonstrated by the fact that *melanogaster-santomea* hybrids are much less pigmented than *yakuba-santomea* hybrids. On a 12 point scale, the mean pigmentation score of the *D. santomea* female parent is 0.221 and of the *D. melanogaster* female parent is 4.61 (Matute et al., Table 1), but the mean pigmentation score of *melanogaster-santomea* female hybrids is only 0.477 (Matute et al., Table 1). The hybrids thus exhibit just 5.8% of the mean pigmentation difference ([0.477−0.221]/ [4.61−0.221] = 0.058) between the parental species. In contrast, previous work reported that the pigmentation of *yakuba-santomea* hybrid females is much greater and more intermediate (39% of the difference between the parental species; see Table 1 in Carbone et al. [2005]).

The much weaker pigmentation of *melanogaster-santomea* female hybrids relative to *yakuba-santomea* hybrids immediately indicates that caution is required in interpreting the results from the *melanogaster-santomea* hybrids. All *melanogaster* genes combined in a heterozygous state generated a phenotype of only 0.256 pigmentation units. In contrast, one copy of QTL Xb alone contributed 6.75 pigmentation units in males and 1.44 units in females in *yakuba-santomea* crosses (Carbone et al. [2005], Table 4). The weak pigmentation of *melanogaster-santomea* hybrids indicates that *D. yakuba* and *D. melanogaster* have diverged at an unknown number of loci affecting pigmentation and that the loci exhibit significant epistasis and/or different patterns of dominance in a *D. santomea* hybrid.

Furthermore, there is the important matter of phylogenetic distance. *D. melanogaster* is far more diverged from *D. santomea* (>10 million years) than is *D. yakuba* (<500,000 years). It is likely that differences at numerous loci have accumulated between *D. santomea* and *D. melanogaster* during their long divergence, loci that are not pertinent to the much more recent *yakuba-santomea* divergence that we have analyzed. Inferences that such loci identified in crosses between *D. melanogaster* and *D. santomea* play a role in the *yakuba-santomea* divergence would be erroneous.

The Four X Chromosome Regions Detected by Matute et al. Lie Outside the Minimal Interval Defined by Introgressions

Matute et al. employed X chromosomal deficiencies in quantitative complementation tests to identify four genomic regions that appeared to contribute to the pigmentation difference between *D. melanogaster-D. santomea* female hybrids and *D. santomea* females. We therefore compared the cytological locations of intervals defined by these deficiencies (9C4-10A1, 15F2-16C10, 17A1-18A1, and 19F1-20E; brown bars in Figure 1A) with the molecular interval defined by our introgression analysis of QTL Xb (green bars in Figures 1B and 1C). All four intervals defined by deficiency mapping fall outside of the minimal interval defined by the overlap among introgressions (Figure 1C; see expanded view for boundary of closest deficiency). While no single introgression lacks all four regions, introgression 7P3 lacks three of the four regions, and most of the fourth (Figure 1B), and introgression 1P3 lacks two regions, including the region not excluded by introgression 7P3 (Figure 1B).

The inference that these four regions comprise essential parts of QTL Xb in the *yakuba-santomea* divergence is, therefore, not supported by gene introgressions. There are several possible explanations for this discrepancy. These explanations differ in the genetic models for QTL Xb.

One model is that the introgressions define a minimal interval containing all of the genes that constitute QTL Xb. If this is the case, then it is possible that along the evolutionary lineages leading to *D. melanogaster* and the common ancestor of *D. yakuba* and *D. santomea* additional QTLs have evolved that cause differences in the way pigmentation is generated in females. It is possible then that Matute et al. have discovered regions that contribute to the difference in pigmentation between *D. melanogaster* and *D. santomea*, but not to the recently evolved difference between *D. yakuba* and *D. santomea*.

An alternative model is that QTL Xb is comprised of multiple regions in addition to the minimal interval defined by the introgressions. If this is the case, then Matute et al. may have discovered QTLs outside of the QTL Xb interval we have mapped that contribute to the *D. yakuba- D. santomea* pigmentation divergence but that were not detected by introgression analysis. We believe that this model is unlikely for two reasons. First, introgressions that lack two or three of these regions have essentially the same pigmentation pattern. This would not be expected if each region contributed to the overall phenotype. Second, while it is possible that these regions contain weak QTLs that were not detected in our introgressions, we think this is unlikely because we have identified introgressions elsewhere in the genome with weaker effects than QTL Xb (M.R.-W. and D.L.S., unpublished data).

A third possible explanation why Matute et al. have detected four regions that were not detected by our introgression study is that they may not in fact be detecting allelic effects with the quantitative complementation tests, but rather epistatic interactions. That is, deficiencies for D. *melanogaster* regions may not be only uncovering alleles at orthologous *D. santomea* loci; these deficiencies may be also exposing epistatic interactions with loci anywhere in the genome whose activity is altered when gene dosage for the many loci in each deficiency is reduced. The deficiency test therefore constitutes simultaneously both a complementation test and a genetic modifier screen in a sensitized, hypomorphic background. We note that each of the four deficiencies are reported to reduce pigmentation by 45%–84% (Matute et al., Table 1) and that the sum of effects of just these four regions of the X chromosome total more than 270% of the difference in pigmentation between hybrid females bearing the deficiency and balancer chromosomes. The magnitude of effects of these deficiencies, which exceeds the total increment of pigmentation difference between the hybrid and *D. santomea*, is not consistent with the relative magnitude of effect of QTL Xb nor with the fact that additional QTLs are present elsewhere in the genome. Such larger than expected relative effects are consistent with the deficiencies acting epistatically in the *melanogaster-santomea* hybrid background.

Complementation tests for qualitative traits are a standard genetic tool that usually, but not always, provides evidence for allelism (Hawley and Gilliland 2006). This test can work well in interspecies crosses when the difference is caused by a loss of

function at a single locus in one species (Sucena and Stern, 2000). But, it is well known that complementation tests can mislead when mutations in the transheterozygous state generate a phenotype through epistasis that resembles failure to complement (Hawley and Gilliland, 2006). The specter of epistasis becomes an increasing concern when a phenotypic difference between parental lines results from changes at multiple genes, as is the case here. Some authors have promoted use of quantitative complementation tests using deficiencies to help localize loci contributing to quantitative traits (Mackay 2001), as Matute et al. have done. However, as Service (2004) has explained, these tests are susceptible to generating many false positives through epistatic interactions between loci within the deficiency and with loci throughout the genome. This problem can be overcome by the use of reciprocal complementation tests, where the quantitative complementation tests are performed separately with null alleles from each parental line (Stern, 1998). It remains to be determined whether loci in the regions that Matute et al. identified as affecting pigmentation in *melanogaster-santomea* hybrid females have played any role in the evolved differences between these two species.

Furthermore, the possibility that many loci contribute to the pigmentation difference between *D. melanogaster* and *D. santomea* suggests that it may be difficult to detect the effects of individual loci, simply because each locus may contribute a small amount to the overall species difference. As the number of QTLs increases, the fraction of phenotypic divergence attributable to genetic divergence at any one locus is expected to decrease. In practical terms, the very small increment of increased pigmentation in *melanogaster-santomea* hybrids relative to *D. santomea* means that detection of many of these loci would be challenging. It is thus easy to envision how differences at other genetic loci could dilute or mask the effect of the *tan* locus to a greater degree in the *melanogaster-santomea* hybrids than in the *yakuba-santomea* hybrids. This possibility raises the issue of the power of the complementation tests employed by Matute et al. and whether they could have detected, or in fact did detect, an effect of the *tan* locus.

Support for *tan*'s Contribution to Pigmentation of *D. melanogaster-D. santomea* Hybrids

Matute et al. tested for effects of six *tan* alleles on pigmentation levels in female hybrids relative to a standard balancer chromosome (*Basc*; see Matute et al., Table 1). They report that "these crosses showed no statistically significant effect on the interspecific differences. In the combined data, the effect of genotype is not significant," and report a p value of 0.0654. They further state that "clearly, *tan* does not have a large (or even statistically significant) effect on the pigmentation of *D. santomea* females" (note that they should say here "*melanogaster-santomea* hybrid females").

However, the p value obtained is very close to a common standard of significance (at the 5% level). We also noted that in each of the six crosses reported in their Table 1, the animals bearing the *tan* allele were lighter than those bearing the wild-type *Basc* chromosome. If one applies a simple sign test to the direction of the effect in all six crosses, one obtains p = 0.03, indicating a significant overall effect of *tan* mutants on pigmentation.

In light of these observations, we thought that further exploration of their data set was warranted. We were provided their raw data and have examined them and Matute et al.'s analysis in more detail. Matute et al. employed a one-way ANOVA approach (with allele effects) that assumes pigmentation scores are normally distributed. However, application of a Shapiro-Wilk test of normality (implemented in the R statistical package, http://www.r-project.org/) to the distributions of pigmentation scores for *tan/san and Basc/san* progeny reveals that both distributions depart considerably from normality (W = 0.8571, p = 5e-16 and W = 0.9219, p = 2e-11, respectively). A routine transformation of the data [i.e., log(X+constant)] failed to correct this issue, strongly suggesting that application of a nonparametric test would be more appropriate.

To test for significant effects of *tan* mutants on pigmentation levels, we compared *tan/san* and *Basc/san* progeny using a Wilcoxon two-sample test (also implemented in R). This test suggests that *tan* mutants do have a small effect on pigmentation in female *melanogaster-santomea* hybrids (W = 41130, p = 0.033, one tailed).

Furthermore, of the six alleles tested, some are likely null for *tan* function, while others probably retain some level of *tan* function (True et al., 2005). In a quantitative complementation test, it would be expected that hypomorphic alleles would have less of an effect than null alleles. Since it is biologically plausible that *tan* mutants that produce any kind of protein may still partially rescue pigmentation, one should distinguish null from hypomorphic alleles. For example, while the tan^{07784} allele reduces *tan* mRNA levels (data not shown), the tan^{20A} allele is the only allele that produces no detectable mRNA (due to a deletion of the *tan* promoter region; True et al. [2005]). Application of a Wilcoxon two-sample test to the tan^{20A} versus *Basc* comparison suggests that this *tan* allele has a strong effect on pigmentation in female *melanogaster-santomea* hybrids (W = 900.5, p = 0.0076, one tailed).

The difference between the effect of the *tan* null (tan^{20A}) and the wild-type allele of 0.122 pigmentation units in hybrid females constitutes 38% of the total difference between the *santomea* and hybrid genotypes. The effects of other tan alleles ranged from 10%–28% (average 23%; see calculations in the right-most column of our Table 1), but these values were not statistically significant (p > 0.1). However, it is premature to conclude that an allele has no statistically significant effect, as Matute et al. did, without a supporting analysis of the power to detect effects given sample sizes and the expected distribution of effect sizes.

A key limitation in the Matute et al. study is the very small increment of pigmentation in female hybrids. In the complementation assay in the hybrid, the most any gene could contribute (on average) was 0.256 units. But if multiple genes are involved (as is certainly the case), then any one gene would contribute only a fraction of 0.256 units. A compounding problem is that in the Matute et al. complementation assays, the standard deviation is typically about 0.25–0.40 units (see our Table 1)—that is, of the same magnitude as the entire increment of pigmentation increase in the female hybrids relative to *D. santomea*. The range of *tan* allele effects observed in Matute et al.'s complementation tests was 10%–38%, and the range of QTL Xb effects in *yakuba-santomea* backcrosses was 6%–43%

Table 1. The Effects of *tan* Mutations on Pigmentation of *D. melanogaster-D. santomea* Female Hybrids

Genotype	*B/san*[a]	Standard Deviation	*tan/san*[a]	Standard Deviation	*tan* Delta[b]	*B/san* − *san*[c]	Total Impact of Hybrid Normalized to *mel-san* Difference[d]	Influence of *tan* Normalized to Hybrid-*san* Difference[e]
scp^1t^1	0.470	0.398	0.401	0.259	0.069	0.249	5.67%	27.71%
$t^2v^1f^1$	0.408	0.314	0.371	0.234	0.037	0.187	4.26%	19.79%
t^3	0.412	0.356	0.383	0.256	0.029	0.191	4.35%	15.18%
$t^5v^1r^1$	0.503	0.403	0.423	0.303	0.080	0.282	6.43%	28.37%
$P\{XP\}t^{d07784}$	0.522	0.257	0.493	0.397	0.029	0.301	6.86%	9.63%
Df(1)t20A	0.544	0.352	0.422	0.304	0.122	0.323	7.36%	37.77%
Average	0.477	0.346	0.416	0.292	0.061	0.256	5.82%	23.08%

[a] Data from Matute et al., Table 1, row 1.
[b] The difference in pigmentation score between wild-type *melanogaster/santomea* hybrids and *tan* mutant hybrids (*B/san* − *tan/san*).
[c] The difference in pigmentation score between wild-type melanogaster/santomea hybrids and the average pigmentation score of *D. santomea* females (*B/san* - .221).
[d] The pigmentation effect of *melanogaster santomea* hybrids expressed as a percentage of the total difference in pigmentation of the parental species: (*B/san* – *san*) / 4.389).
[e] Our calculation of the mean influence of *tan* mutant on F1 Hybrids between *D. melanogaster* and *D. santomea* expressed as a percentage of the pigmentation difference between hybrids and pure santomea: *tan* delta / (*B/san* – *san*).

(depending upon genetic background; Carbone et al. [2005]). So a key question is, given these effect sizes, assay parameters, and the sample sizes of individual crosses (n = 50), what is the probability that Matute et al. could detect various sizes of effect in their assay at $p < 0.05$? A simple power calculation reveals that Matute et al. had only a 54% probability of detecting a 43% effect, a 19% probability of detecting a 20% effect, a 10% probability of detecting a 10% effect, and a 7% probability of detecting a 5% effect. All of these power estimates are below the common standard of 80%. Therefore, Matute et al.'s experimental design lacks the statistical power to reliably detect the effects of *tan*.

At various points in their analysis and discussion, Matute et al. state that there is "no significant effect" or "no effect" of *tan* or "no evidence" for tan's role in the pigmentation difference between *D. melanogaster* and *D. santomea* and "by inference on the difference between *D. yakuba and D. santomea*." These negative statements are not supported by our analysis of their data, which indicates that *tan* does appear to have an effect on pigmentation of *melanogaster-santomea* hybrids, even though their experimental design had limited power to detect such effects of individual alleles. Therefore, Matute et al.'s inference (by extrapolation) that *tan* does not contribute to the *yakuba-santomea* divergence is not supported by their own data.

Matute et al. have allowed in their title that *tan* may have a "little" effect on female *melanogaster-santomea* hybrids. Of course, given the assay parameters, a "little" effect is the most that one could have expected. If *tan* does have some effect in their complementation tests with *tan* alleles, as suggested by our analysis of their data, then there is no substantive discrepancy concerning the contribution of *tan* to the loss of pigmentation in *D. santomea*.

DISCUSSION

Understanding the evolution of phenotypes requires knowledge of the developmental, genetic, and molecular basis of trait formation and divergence, which in turn requires selection of suitable experimental models and methodology. The discrepancy between our conclusions and those of Matute et al. is therefore instructive.

Our conclusions were based first on a detailed molecular characterization of the candidate gene *tan* in the pigmentation difference between *D. yakuba* and *D. santomea*, and are consistent with gene introgression data, an independent method of assessing the contribution of loci to species divergence. Matute et al. used a fundamentally different experimental approach in a different species pair to reject the role of *tan* and to implicate at least four other loci in the region as the basis of QTL Xb.

We believe that the main reasons for these discrepancies stem from the selection by Matute et al. of a different parental species, *D. melanogaster*, for comparison with *D. santomea*. Since *D. melanogaster* would appear to offer certain advantages for genetic analysis (such as the availability of deficiencies), it is thus important to identify the major reasons why, in this case, the use of this species did not provide sound inferences about the divergence between *D. santomea* and *D. yakuba.*

As we have shown, the experimental approach of Matute et al. was handicapped from the outset by the very small increment of increased pigmentation in *melanogaster-santomea* female hybrids. This handicap, as well as related issues with statistical power and methods, led Matute et al. to the inference that *tan* was not involved in pigmentation differences between *D. yakuba* and *D. santomea*, an inference that we have shown is not supported.

Their approach was also confounded by the phylogenetic distance of *D. melanogaster* from *D. santomea*, which is likely to be the cause of several key limitations in the Matute et al. study design. First, the anomalously weak pigmentation of female hybrids may result, in part, from the greater evolutionary distance between *D. melanogaster* and *D. santomea* than between *D. yakuba* and *D. santomea*. Such anomalous phenotypes may be the result of aberrant gene regulation. Indeed, disruptions of gene regulation are well documented in *Drosophila* species

hybrids, where the abundance of many mRNA species is not intermediate between the parental species (see Landry et al. [2007] and references therein). Hybrids of *D. melanogaster* and *D. santomea* may experience many such "regulatory incompatibilities," since these are among the most divergent species hybridized to date.

Second, we suggest that the greater distance between *D. melanogaster* and *D. santomea* has allowed differences to accumulate at many loci that may have nothing to do with the divergence between *D. yakuba* and *D. santomea*. Usually, in order to minimize potential complications from such extraneous loci, genetic crosses for QTL studies aim to utilize the two most closely related parental lines that differ in the trait of interest. This is why, for example, Beadle (1980) and other workers (Doebley and Stec, 1993) chose primitive landraces of maize in now-classic studies to identify the minimum number of loci involved in maize-teosinte divergence. Modern "elite" types of maize differ at additional loci from primitive maize that would have complicated those analyses. In this instance, *D. santomea* is most closely related to *D. yakuba*, so clearly they are the most desirable species pair to analyze.

And third, the large evolutionary distance between *D. melanogaster* and *D. santomea* also precluded Matute et al. from examining hybrid males. QTL Xb has an almost 5-fold greater effect in males than in females (Carbone et al. 2005). Jeong et al. (2008) and the introgression data presented here focused almost entirely on male pigmentation, which is more pronounced and regulated differently than female pigmentation in the *melanogaster* species group. Several pigmentation genes including *tan* are regulated in a sexually dimorphic fashion (Jeong et al., 2008; Kopp et al., 2000; Williams et al., 2008), and the inactivated *tan* CRE we identified in *D. santomea* governs sexually dimorphic *tan* expression. Matute et al. reject the role of *tan* in pigmentation differences of both males and females from studies of hybrid females only. Their experiments do not address the role of *tan* in the evolution of male pigmentation.

Finally, in weighing the merits of our results and conclusions relative to Matute et al.'s claims, we refer to widely accepted standards of evidence for causative loci. In order to develop a consensus view with respect to the definition and identification of QTL affecting complex traits, a consortium of eighty investigators has enumerated several possible lines of evidence that could be used to identify causative QTL and recommended that, ideally, more than one condition should be met (The Complex Trait Consortium, 2003). These conditions include the following:

(1) "Sequences that lead to changes in either the structure or regulation of a gene product should be detected between the strains that are used for mapping."
(2) "Some evidence should support a link between the function of the gene and the expression of the quantitative trait being analyzed, either by involvement in an appropriate pathway and/or by expression in the appropriate target tissue."
(3) "Transgenesis with . . . large chromosomal segments can be used to confirm the identity of the candidate gene . . . if there are several genes on the BAC, rescue . . . might require further experiments to confirm which gene is responsible."

Jeong et al. (2008) met each of these conditions, and the introgression data presented here further add to the weight of evidence that *cis*-regulatory changes at *tan* have contributed to the loss of pigmentation in *D. santomea*.

Despite the previously published evidence, Matute et al. state that *tan* "cannot be considered a convincing example of the effect of *cis*-regulatory mutations on a major phenotypic difference." The merit of such statements should be weighed against the established standards of evidence in the field. The indirect and statistically unsupported inferences Matute et al. have drawn from hybrids with a different parental species do not alter any of the conclusions made by Jeong et al. (2008).

EXPERIMENTAL PROCEDURES

Drosophila Strains

We used one strain for each species obtained through the Tucson Stock Center (stock number 14021-0261.00 for *D. yakuba*, and stock number 14021-0271.00 for *D. santomea*). All flies were maintained on standard cornmeal agar media enriched with live yeast at room temperature.

Generation of Introgression Lines

To isolate the genetic regions associated with variation in abdominal pigmentation between *D. santomea* and *D. yakuba*, we generated multiple independent lines through repeated backcrossing of hybrid females to *D. santomea* males coupled with selection of pigmented flies (see the Supplemental Data).

Genotyping

DNA was extracted with the Quick Fly genomic DNA extraction protocol (Gloor and Engels, 1992) from individual flies of interest. We used three different techniques to map introgression breakpoints: scoring of known restriction enzyme polymorphisms in PCR products, single-stranded conformation polymorphism of PCR products with the Phast Gel system (Amersham Biosciences, Separation Technique File No. 131), and allele-specific PCR followed by melting curve analysis (Papp et al. 2003, Gupta et al. 2005). Further details are presented in the Supplemental Data.

Imaging

Individual flies (aged to 3–4 days old) were pinned to a small apple juice plate filled with a 2% Triton-X solution using #000 insect pins. We captured three to five pictures at different depths of field with a digital camera (Photometrics Coolsnap cf) connected to a Nikon E1000 microscope at 40×. Light conditions were kept constant with a ring light attached to the microscope's objective. We used the 3D extended focus function of IPlab software (version 3.9.4 r2) to reduce image stacks into a single image showing the whole abdomen in focus. We did not use mounting and imaging techniques as in Jeong et al. (2008), as cutting the abdomen along the dorsal midline to mount it conceals subtle details of the variation in abdominal pigmentation.

Power Analysis

Mean pigmentation in *Basc/san* hybrid females is 0.476 with a standard deviation of 0.351. The mean pigmentation level of *D. santomea* females is 0.221 (Matute et al., Figure S2), implying that a QTL can decrease pigmentation at most by 0.476 − 0.221 = 0.255. According to Carbone et al. (2005) (Table 4), the QTL interval including *tan* explains about 6%–43% of the phenotypic variance in yakuba/santomea hybrid females. Thus, the maximum expected decrease in pigmentation of female hybrids due to *tan* would be 43% of 0.255 = 0.1097, implying that expected pigmentation of hybrid females would be 0.476 − 0.1097 = 0.366. We estimated the statistical power to detect a difference between these means as well as 20%, 10%, or 5% effects on pigmentation in sample sizes of n = 50 using the tools available at http://www.quantitativeskills.com/sisa/calculations/power.htm.

In Situ Hybridization

In situ hybridization to newly eclosed adults was performed with a riboprobe derived from a full-length *D. santomea tan* cDNA, as previously described (Jeong et al., 2008).

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, two figures, and one table and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(09)01377-4.

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