

Exploring the Phenotypic Space and the Evolutionary History of a Natural Mutation in *Drosophila melanogaster*

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Abstract

A major challenge of modern Biology is elucidating the functional consequences of natural mutations. Although we have a good understanding of the effects of laboratory-induced mutations on the molecular- and organismal-level phenotypes, the study of natural mutations has lagged behind. In this work, we explore the phenotypic space and the evolutionary history of a previously identified adaptive transposable element insertion. We first combined several tests that capture different signatures of selection to show that there is evidence of positive selection in the regions flanking *FBti0019386* insertion. We then explored several phenotypes related to known phenotypic effects of nearby genes, and having plausible connections to fitness variation in nature. We found that flies with *FBti0019386* insertion had a shorter developmental time and were more sensitive to stress, which are likely to be the adaptive effect and the cost of selection of this mutation, respectively. Interestingly, these phenotypic effects are not consistent with a role of *FBti0019386* in temperate adaptation as has been previously suggested. Indeed, a global analysis of the population frequency of *FBti0019386* showed that climatic variables explain well the *FBti0019386* frequency patterns only in Australia. Finally, although *FBti0019386* insertion could be inducing the formation of heterochromatin by recruiting HP1a (Heterochromatin Protein 1a) protein, the insertion is associated with upregulation of *sra* in adult females. Overall, our integrative approach allowed us to shed light on the evolutionary history, the relevant fitness effects, and the likely molecular mechanisms of an adaptive mutation and highlights the complexity of natural genetic variants.

Key words: transposable elements, selective sweep, gene regulation, fitness, adaptation.

Introduction

Understanding the functional consequences of naturally occurring mutations remains a largely open question in Biology. Most of our knowledge on the effect of mutations comes from the analyses of laboratory-induced mutations. However, it is not clear whether laboratory mutations are representative of mutations that arise and persist in natural populations (Kolaczowski et al. 2011; Rose et al. 2011). First, most laboratory mutations studied are loss-of-function mutations that are most likely rare in natural populations and/or their effects are masked by the presence of buffering mechanisms (Landry and Rifkin 2012). Additionally, laboratory-induced mutations tend to be highly pleiotropic and it is difficult to infer which of the phenotypes might be targets of selection in nature (Kolaczowski et al. 2011).

The recent explosion in the number of studies aimed at identifying natural adaptive mutations in several organisms allows us to study the effect of natural genetic variants at an unprecedented scale (González et al. 2008; Turner et al. 2010; Jones et al. 2012; Huang et al. 2014; Tobler et al. 2014). These studies are revealing that mapping genotype to phenotype is even more complex than previously thought due to the prevalence of gene-by-environment interactions, gene-by-gene interactions, and pleiotropy (Rockman 2012; Lehner 2013; Mackay 2014). First, being able to map a putatively adaptive mutation to its relevant phenotypic effect depends partly on finding the particular environmental conditions in which the

mutation is adaptive (Paaby and Schmidt 2008; Storz and Wheat 2010). Thus, taking into account environmental information of the populations where putative adaptive mutations are identified should help mapping the mutation to its relevant phenotype. Second, epistatic interactions also affect the phenotypic outcome of mutations. The phenotypic effect of mutations could be enhanced or suppressed depending on the background being analyzed (Huang et al. 2012). Additionally, several backgrounds should be analyzed to discard the effect of other mutations and reliably attribute the identified phenotypic effect to the candidate mutation (Burnett et al. 2011). Third, many genes are linked to several traits (Paaby and Rockman 2013). In some cases, mutations can have antagonistic effects, that is, beneficial effects in a trait/environment and deleterious effects on a different trait/environment. Pleiotropic mutations can also have beneficial effects on two different traits (McGee et al. 2014). Tradeoffs are prevalent when selection acts on a single trait, whereas payoffs arise when multiple traits are selected for simultaneously (McGee et al. 2014). Thus, if we want to fully characterize the effects of a given natural mutation, several phenotypes need to be studied (Mackay 2010; Guio et al. 2014).

Finally, a comprehensive understanding of adaptation goes beyond identifying fitness consequences of adaptive mutations. Pinpointing the molecular mechanisms underlying adaptation is needed to provide conclusive support for the

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Table 1. Summary of the Analyses Showing Evidence of Positive Selection in the 1-kb Region around *FBti0019386* Insertion.

	Observed		Neutral Simulations				Resampling of Strains	
	P	A	Mean (CI 95%)		P value		Mean (CI 95%)	P Value
			P	A	P	A		
π	0.43	4.51	3.92 (1.32, 7.81)	4.20 (1.33, 8.04)	0.001	> 0.05	3.35 (2.78, 3.87)	<0.001
Tajima's <i>D</i>	-1.77	0.68	-0.11 (-1.46, 1.62)	-0.04 (-1.41, 1.64)	0.007	> 0.05	0.4 (-0.19, 1.02)	<0.001
CL (log)	-5.95	-18.15	-18.69 (-29.67, -8.80)	-15.20 (-25.89, -6.82)	0.006	> 0.05	-12.18 (-15.23, -8.81)	<0.001

NOTE.—Neutral simulations were performed with MS program using the parameter $\theta = 4$. For simulations with $\theta = 5$, please see supplementary table S2, Supplementary Material online. P, data set of strains with *FBti0019386* insertion; A, data set of strains without *FBti0019386* insertion.

adaptive role of the mutation (Storz and Wheat 2010). Additionally, elucidating the evolutionary history of adaptive variation for fitness traits allows to start answering long-standing questions on the genetic basis of adaptation (Orr 2005).

In this work, we focused on characterizing the functional effects, the molecular mechanism, and the evolutionary history of a natural transposable element (TE)-induced mutation in *Drosophila melanogaster*: *FBti0019386* belonging to the *invader4* retrotransposon family (González et al. 2008, 2010; St Pierre et al. 2014). *FBti0019386* has been identified as a candidate adaptive TE insertion based on its population dynamics (González et al. 2008). González et al. (2010) further reported that *FBti0019386* shows parallel clinal frequency patterns in North America and Australia suggesting that it is involved in adaptation to temperate environments. *FBti0019386* is inserted in the 5'-untranslated region (UTR) intron of *sarah* (*sra*) and 2.5 kb upstream of *Bicoid-interacting protein 1* (*Bin1*) in the 3 R chromosomal arm (St Pierre et al. 2014). *sra* laboratory mutants affect several biological processes, such as egg activation, female meiosis, and long-term memory among others (Ejima et al. 2001, 2004; Chang et al. 2003; Horner et al. 2006; Takeo et al. 2006; Sakai and Aigaki 2010; Nakai et al. 2011). In most cases, these phenotypes are the result of the deregulation of *calcineurin*, which is inhibited by *sra* (Takeo et al. 2006; Sakai and Aigaki 2010; Nakai et al. 2011). Laboratory-induced mutations affecting *Bin1*, a highly conserved transcriptional corepressor, play a role during environmental stress response in *Arabidopsis* (Song and Galbraith 2006) and in *Drosophila* (Costa et al. 2011). Thus, to identify the phenotypic consequences of *FBti0019386* mutation, we explored several candidate phenotypes previously associated with *sra* and *Bin1* mutants in different developmental stages, in different environmental conditions, and in flies with different genetic backgrounds.

Our results showed that *FBti0019386* increased in frequency in out-of-Africa populations due to positive selection and is associated with shorter developmental time (DT) and increased sensitivity to cold-stress. These two phenotypic effects together with the lack of correlation between *FBti0019386* frequency and latitude in European populations raised doubts about the role of *FBti0019386* in temperate adaptation. Finally, although *FBti0019386* insertion could be inducing pi-RNA mediated heterochromatin assembly, the insertion is associated with upregulation of *sra* in adult females.

Results

FBti0019386 Flanking Regions Show Signatures of Positive Selection

We tested whether the region flanking *FBti0019386* showed signatures of positive selection (see Materials and Methods for a description of the different tests used). We found an extreme decrease of nucleotide diversity (π) in strains with *FBti0019386* insertion compared with strains without the insertion, which was accompanied by a decrease in Tajima's *D* statistic (table 1, supplementary fig. S1A and B and table S1, Supplementary Material online) (Hudson et al. 1992; Tajima 1989). The Composite Likelihood (CL) test, specifically designed to detect selective sweeps (Nielsen et al. 2005), was higher in flies with *FBti0019386* insertion compared with flies without the insertion, as expected if flies with the insertion show signatures of a selective sweep in the analyzed region (table 1). We confirmed that values of π , Tajima's *D*, and CL were statistically different from neutral simulated scenarios in flies with *FBti0019386* insertion but not in flies without the insertion (table 1 and supplementary table S2, Supplementary Material online).

To test whether the observed differences were due to the *FBti0019386* insertion, we estimated the three statistics in random samples of the strains (see Materials and Methods). None of the randomized data sets had lower π , lower Tajima's *D*, or higher CL value compared with the data set of strains with *FBti0019386* insertion (table 1 and supplementary table S3, Supplementary Material online). Finally, we performed the Composite Likelihood Ratio (CLR; Nielsen et al. 2005) test comparing strains with and without the *FBti0019386* insertion, and we found that it was significant: CLR = 24.40 P value = 7.82×10^{-7} . Moreover, this CLR value is three times bigger than any of the CLR values calculated in a random sample of 1,000 1-kb-long regions from 3 R chromosome, where *FBti0019386* is located (supplementary table S4, Supplementary Material online). Note that estimates of π and Tajima's *D* in these 1,000 regions also showed that these two statistics did not significantly differ between strains with and without *FBti0019386* insertion (supplementary fig. S1C and D, Supplementary Material online).

Note that we checked whether polymorphisms other than TE were present in the flanking regions analyzed. No other polymorphisms were found that could potentially confound the results of our tests of selection suggesting that the TE is the causative mutation.

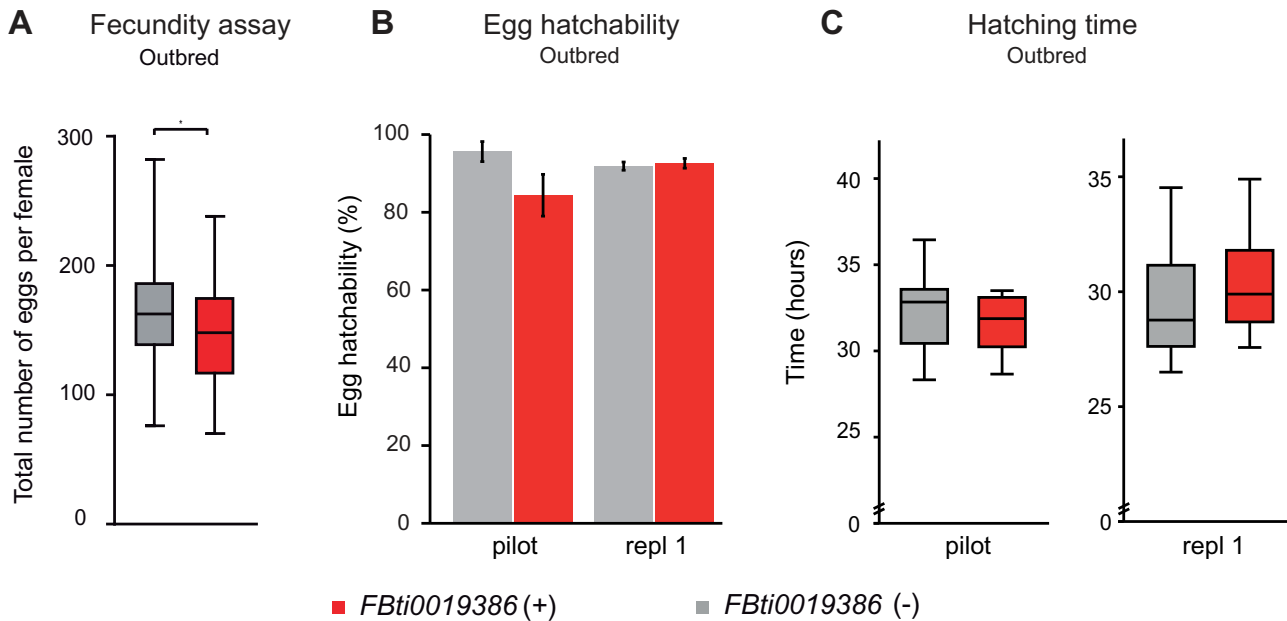


Fig. 1. *FBti0019386* does not affect fecundity (A), egg hatchability (B), or hatching time (C) in outbred populations. (A) Average number of eggs laid by outbred females without *FBti0019386* insertion (*FBti0019386* (-)) and with *FBti0019386* insertion (*FBti0019386* (+)). (B) Percentage of hatched embryos. (C) Average hatching time. In all cases, error bars represent standard error of the mean (SEM).

Table 2. Odds Ratios (OR) and Confidence Intervals (CI) for Phenotypic Experiments Performed with Embryos with and without *FBti0019386*.

Experiment	Strain	OR (CI)
Fecundity	Outbred	1.05 (0.67–1.64)
Hatching time in cold	Outbred pilot	7.07 (3.37–14.83)
	Outbred replica 1	2.21 (1.49–3.26)
DT	Outbred pilot	5.69 (2.72–11.94)
	Outbred replica 1	2.62 (1.88–3.66)
	Outbred replica 2	2.60 (1.94–5.88)
	Individual DGRP	1.95 (1.30–2.92)

Overall, we found evidence of positive selection in the region flanking *FBti0019386* insertion suggesting that *FBti0019386* is an adaptive insertion.

Exploring the Fitness Space of *FBti0019386*

To explore the phenotypic space of *FBti0019386* insertion, we investigated several traits related to the phenotypic effects of nearby genes: Fecundity and egg hatchability associated with *sra* mutant alleles. Related to egg hatchability, we also investigated egg hatching time, egg-to-adult viability, and DT. Additionally, we investigated cold stress, osmotic stress, and starvation stress as *Bin1* mutants have been shown to play a role in stress resistance.

Because *FBti0019386* is located 242.4 kb away from the distal breakpoint of *In(3R)Payne* inversion and inversions are known to be under selection, we checked whether this inversion was present in any of the six strains used to perform the different phenotypic analyses (see Materials and Methods). We found that none of the strains used in our analyses carries *In(3R)Payne* inversion.

We also checked whether polymorphisms other than the *FBti0019386* insertion were present in the genomic region

including *sra* and *Bin1* genes. We did not find any polymorphism linked to the *FBti0019386* that could potentially confound the results of the phenotypic assays performed.

FBti0019386 Insertion Does Not Affect Fecundity or Egg Hatching

Laboratory mutant flies in which *sra* is underexpressed lay less eggs than wild-type flies and most of the eggs do not hatch (Horner et al. 2006). To check whether *FBti0019386* insertion has an effect on fecundity, we compared the number of eggs laid per female in outbred populations with and without the insertion (see Materials and Methods). Our results showed that, on average, flies without the insertion laid slightly more eggs than flies with the insertion (*t*-test, $P = 0.047$) (fig. 1A). However, the size effect of the mutation was not significant (table 2). We also tested whether differences in fecundity were present early in life, as has been reported by Paaby et al. (2014). Although the mean number of eggs laid by flies with the insertion in the first 48 h of egg laying was bigger than the number laid by flies without the insertion (3.95 vs. 2.33 eggs), these differences were not statistically significant (*t*-test, $P = 0.06$).

We then checked whether outbred flies with and without *FBti0019386* differed in egg hatchability and/or hatching time. We first performed a pilot experiment using 150 embryos per strain and we found that flies with the insertion did not show significant differences compared with flies without the insertion in egg hatchability (*t*-test, $P > 0.05$) (fig. 1B) or hatching time (*t*-test, $P > 0.05$) (fig. 1C). Although differences were not significant, flies with the insertion showed a lower number of hatched eggs (fig. 1B) and a shorter hatching time (fig. 1C). We thus repeated the experiments using 500 embryos per strain and we found that flies with and without *FBti0019386* did not differ in egg hatchability (*t*-test,

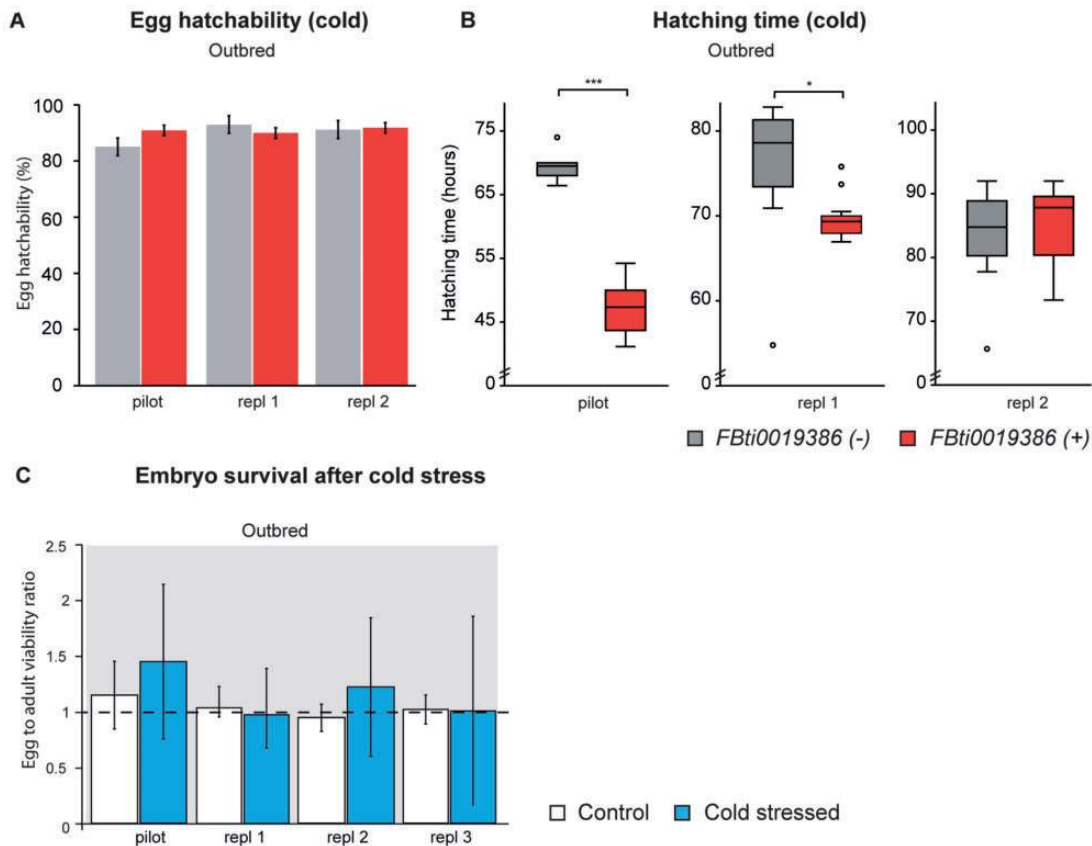


Fig. 2. *FBti0019386* does not affect embryo hatching or survival in cold stress conditions in outbred populations. (A) Percentage of embryos that hatched during cold-stress periods (see Materials and Methods). (B) Average egg hatching time. (C) Egg-to-adult survival after a single cold stress period during embryonic stage (cold stressed) and under control conditions (control). Bars represent the survival ratio between flies with *FBti0019386* and flies without *FBti0019386* and error bars represent SEM.

$P > 0.05$) (fig. 1B) or hatching time (t -test, $P > 0.05$) (fig. 1C).

Overall, we did not find significant differences in fecundity, egg hatchability, or egg hatching time in flies with and without *FBti0019386* insertion. These results suggest that *FBti0019386* does not have a significant effect on these phenotypes.

FBti0019386 Insertion Does Not Affect Egg Hatching or Egg-To-Adult Viability under Cold Stress Conditions

As mentioned above, *Bin1* plays a role in general environmental stress response in *Drosophila* (Costa et al. 2011). We thus screened several phenotypes in embryos under cold stress conditions: Egg hatching, egg hatching time, and egg-to-adult viability.

We performed egg hatchability and egg-hatching time assays in outbred populations under repeated cold stress exposure (see Materials and Methods). We did not detect differences in egg hatchability between flies with and without the insertion in any of the three replicas performed (t -test, $P > 0.05$) (fig. 2A). However, flies with *FBti0019386* insertion from the pilot experiment and the first replica hatched significantly before flies without the element (t -test, $P < 0.001$ and $P = 0.011$, respectively) (table 2) whereas no differences were observed in the second replica (t -test, $P > 0.05$) (fig. 2B).

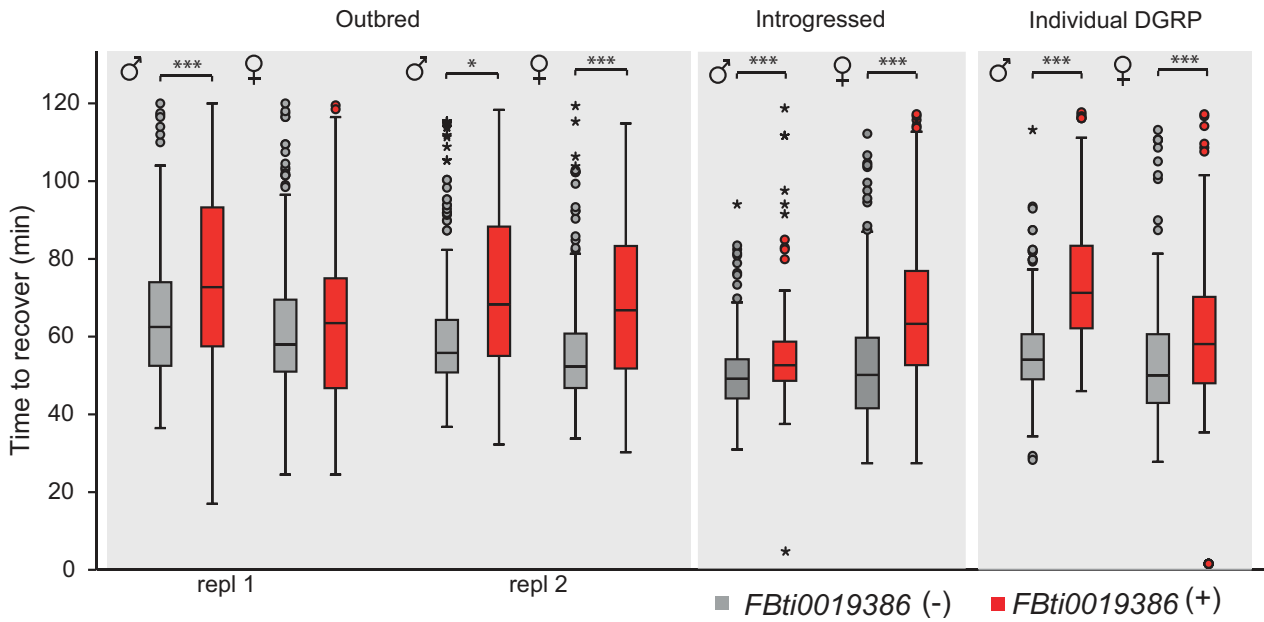
We further tested whether flies with and without *FBti0019386* differed in the egg-to-adult viability after exposing outbred flies to a single cold-stress period during early embryonic stages. Our results showed that there are no differences in survival between flies with and without the insertion in control conditions or under cold-stress (two-way ANOVA [analysis of variance], $P > 0.05$, fig. 2C).

Overall, and although variability in hatching time was observed in some of the experiments performed, our results suggest that *FBti0019386* insertion does not affect cold-tolerance during the embryonic stage.

FBti0019386 Is Associated with Increased Sensitivity to Cold Stress in Adults

Because we could not find any significant difference between strains with and without *FBti0019386* in embryonic stage, we decided to test whether differences between the two strains were present in adult flies. We first tested whether adult flies with and without *FBti0019386* insertion differed in chill-coma recovery time (CCRT) and survival after cold stress. CCRT is used as a reliable measure of cold tolerance in *Drosophila* (Gibert et al. 2001; Macdonald et al. 2004). We observed that flies with the insertion showed significantly longer recovery time compared with flies without the insertion suggesting that they were more sensitive to cold stress (Mann–Whitney test, $P < 0.001$) (fig. 3A and table 3). We

A Chill-coma recovery time



B Survival after chill-coma

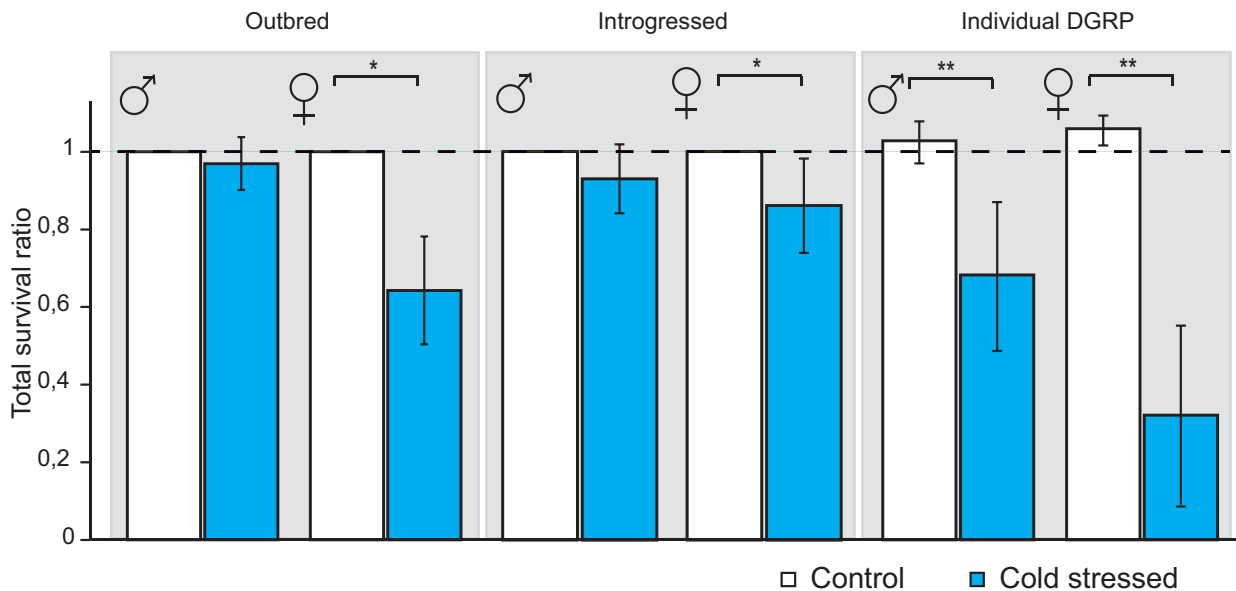


Fig. 3. Flies with *FBti0019386* insertion are more sensitive to cold stress. (A) Average time to recover after chill coma in adult flies from outbred populations, introgressed strains, and inbred DGRP strains (RAL-857 and RAL-802). (B) Survival ratio between flies with *FBti0019386* insertion and flies without the insertion after chill coma exposure (cold stress) and in control conditions (control) in the three genetic backgrounds. Error bars represent SEM.

replicated this result in flies with the same genetic background (Mann–Whitney test, $P < 0.05$) and in flies with two other genetic backgrounds: The introgressed strains generated in our laboratory (Mann–Whitney test, $P < < 0.001$) and a couple of inbred strains from the DGRP (Drosophila Genetic Reference Panel) project (Mann–Whitney test, $P < < 0.001$) (fig. 3A and table 3) (see Materials and Methods).

In accordance with this increased cold sensitivity, flies with the insertion also showed an increased mortality following

chill-coma exposure, although these differences were not always significant (fig. 3B and table 3).

Finally, we also tested whether flies with *FBti0019386* insertion were more sensitive to osmotic stress and starvation stress. We found that outbred females with the insertion were more sensitive to high salt concentrations (Kaplan–Meyer, log rank $P < 0.001$) (supplementary fig. S2A, Supplementary Material online, and table 3), and outbred males with the insertion were more sensitive to starvation stress (Kaplan–Meyer, log rank $P < 0.001$)

(supplementary fig. S2B, Supplementary Material online, and table 3).

Overall, longer CCRT and lower cold-stress survival in flies with *FBti0019386* insertion across backgrounds suggested that this mutation is negatively affecting adult cold-stress response. This high sensitivity to cold stress likely represents the cost of selection of this TE mutation. Furthermore, preliminary results are suggestive but not conclusive of a negative role of *FBti0019386* in general response to stress.

FBti0019386 Insertion Is Associated with Shorter DT

During the course of the experiments, we noticed that flies with *FBti0019386* showed a shorter DT than flies without the insertion. Because DT is relevant to fitness in all organisms, and especially for those such as *D. melanogaster* that occupy ephemeral habitats (Chippindale et al. 1997), we tested this observation. We found that outbred flies (Mann–Whitney test, pilot experiment $P=0.006$ and replica 1 and 2 $P < 0.001$) and introgressed DGRP flies (t -test, $P=0.02$) with the insertion developed faster compared with flies without the TE insertion (fig. 4 and table 2). On average, flies with *FBti0019386* insertion developed 9.4–17.9 h before compared with flies without the insertion. However, we could not detect

Table 3. Odds Ratios (OR) and Confidence Intervals (CI) for Phenotypic Experiments Performed with Male and Female Adult Flies with and without *FBti0019386*.

Experiment	Strain	Males OR (CI)	Females OR (CI)
CCRT	Outbred replica 1	3.44 (2.31–5.18)	N/A ^a
	Outbred replica 2	3.79 (2.54–5.67)	5.18 (3.43–7.82)
	Introgressed	2.44 (1.64–3.62)	4.16 (2.69–6.41)
	Individual DGRP	11.63 (6.79–19.93)	2.26 (1.54–3.33)
Survival after chill-coma	Outbred	N/A	7.80 (3.27–18.60)
	Introgressed	N/A	1.89 (0.99–3.62)
	Individual DGRP	9.94 (5.49–18)	6.88 (3.43–13.82)
Osmotic stress	Outbred	N/A	1.61 (1.21–2.13)
Starvation stress	Outbred	1.52 (1.15–2.01)	N/A

^aN/A (OR was estimated when differences between flies with and without *FBti0019386* were statistically significant).

significant DT differences in the introgressed strains differing by the presence/absence of *FBti0019386* (t -test, $P > 0.05$) (fig. 4), suggesting that polymorphisms other than the TE influence DT in this background. Note that the effect size of the mutation on the other phenotypes studied also varies depending on the background being analyzed (tables 2 and 3). This suggests that polymorphisms other than *FBti0019386* play a role not only in DT but also in other phenotypes.

FBti0019386 Frequency Showed Clinal Patterns in North America and Australia but No Correlation between Frequency and Latitude Is Found in Europe

Shorter DT and increased sensitivity to cold stress are not consistent with a role of *FBti0019386* in temperate adaptation (González et al. 2010). However, previous evidence for a role in temperate adaptation was based on the analysis of only two North American and five Australian populations (González et al. 2010). To further test these results, we estimated *FBti0019386* frequencies in additional populations from North America, Australia, Europe, and Africa (supplementary table S5, Supplementary Material online) using *T-lex2* pipeline (Fiston-Lavier et al. 2014). We found that *FBti0019386* insertion is present at 10% frequency in a Rwanda population confirming its low frequency in Africa (supplementary table S5, Supplementary Material online). We confirmed that the TE is present at intermediate to high frequencies in 15 additional out-of-Africa populations (fig. 5 and supplementary table S5, Supplementary Material online). We also confirmed that the TE frequency varies clinally with latitude in North America and Australia (Pearson correlation $P=0.011$ and $P=0.002$, respectively; supplementary table S6, Supplementary Material online). However, when we analyzed the *FBti0019386* frequency in six European populations we did not find any significant correlation between frequency and latitude (Pearson correlation $P=0.313$; supplementary table S6, Supplementary Material online).

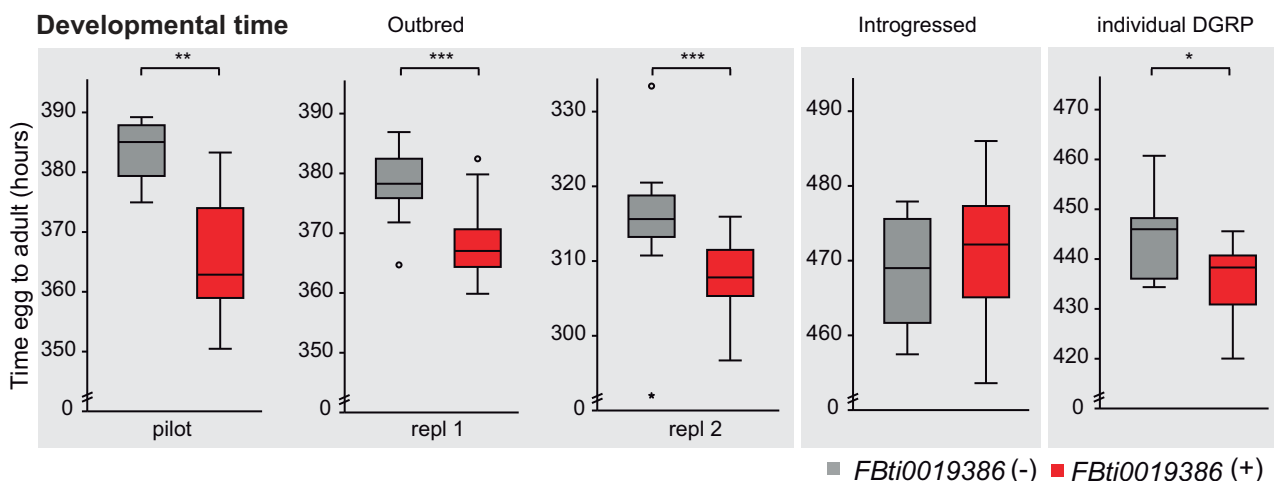


Fig. 4. *FBti0019386* is associated with shorter DT. Average egg-to-adult DT in populations without *FBti0019386* insertion and with the insertion. Error bars represent SEM.

Besides latitude, we also tested whether other geographical and climatic variables showed significant correlations with *FBti0019386* frequency. We found significant correlations between frequency and temperature-related variables in North America and between frequency and both temperature-related and precipitation-related variables in Australia (supplementary table S6, Supplementary Material online). No significant correlation was found in Europe (supplementary table S6, Supplementary Material online). Because most of the climatic variables are significantly correlated among them and with latitude (supplementary table S7, Supplementary Material online), we performed a Principal Component Analysis (PCA) to disentangle the relationships between the variables. In North America, climate variables were grouped in two components, in Australia in three and in Europe in two (supplementary table S8, Supplementary Material online). As expected based on the correlation analyses, only in North America and in Australia, some of the climatic variables grouped with latitude and frequency (supplementary fig. S3A, Supplementary Material online). In North America, the first component accounted for 46% of climatic variation (supplementary table S9, Supplementary Material online) and explained 54% of the variation in *FBti0019386* frequency (supplementary fig. S3B, Supplementary Material online). In Australia, the first component accounted for 68% of climatic variation (supplementary table S9, Supplementary Material online) and explained 86% of the frequency variation (supplementary fig. S3B, Supplementary Material online). Finally in Europe, the first principal component explained 54% of the climatic variation (supplementary table S9, Supplementary Material online) but was not significantly correlated with *FBti0019386* frequency (supplementary fig. S3B, Supplementary Material online).

Overall, although we were able to confirm the clinal pattern of *FBti0019386* in North America and Australia, our results did not provide evidence for the presence of a clinal pattern in Europe. In Australia, the clinal pattern is well explained by the observed climatic variation, whereas in North

America climatic variation did not fully explain the observed correlation between *FBti0019386* frequency and latitude, suggesting that other factors might be involved in the observed clinal pattern. As expected, none of the climatic variables significantly correlated with TE frequency in Europe.

FBti0019386 Is Associated with Upregulation of *sra* in Female Flies

To shed light on the molecular mechanism of *FBti0019386* insertion, we measured the expression of *sra* and *Bin1* in nonstress conditions in embryos and in nonstress and cold-stress conditions in female flies with and without *FBti0019386* insertion.

We did not observe significant differences in *sra* or *Bin1* expression in embryos differing by the presence/absence of *FBti0019386* insertion (*t*-test, $P > 0.05$) (fig. 6A and B). However, we observed that adult female flies with *FBti0019386* insertion showed an increase of *sra* expression compared with flies without the insertion both in control conditions and after cold-stress conditions, although results were only significant under control conditions (*t*-test, $P = 0.03$) (fig. 6C). On the other hand, no significant differences in expression level between flies with and without *FBti0019386* were observed for *Bin1* (*t*-test, $P > 0.05$) (fig. 6D).

Interestingly, we observed a change in *sra* and *Bin1* expression after cold stress in flies with and without *FBti0019386* insertion: *sra* is upregulated in cold stress conditions (*t*-test, $P < 0.05$ in both cases) (fig. 6C) whereas *Bin1* is downregulated (*t*-test, $P < 0.05$ in both cases) (fig. 6D).

Overall, we did not observe any change in expression of *sra* and *Bin1* in embryos, in agreement with the lack of phenotypic consequences of *FBti0019386* in this developmental stage. However, we observed an upregulation of *sra* in flies with *FBti0019386* insertion that was significant under non-stress conditions. Moreover, we showed that both *sra* and *Bin1* changed their expression in response to cold stress.

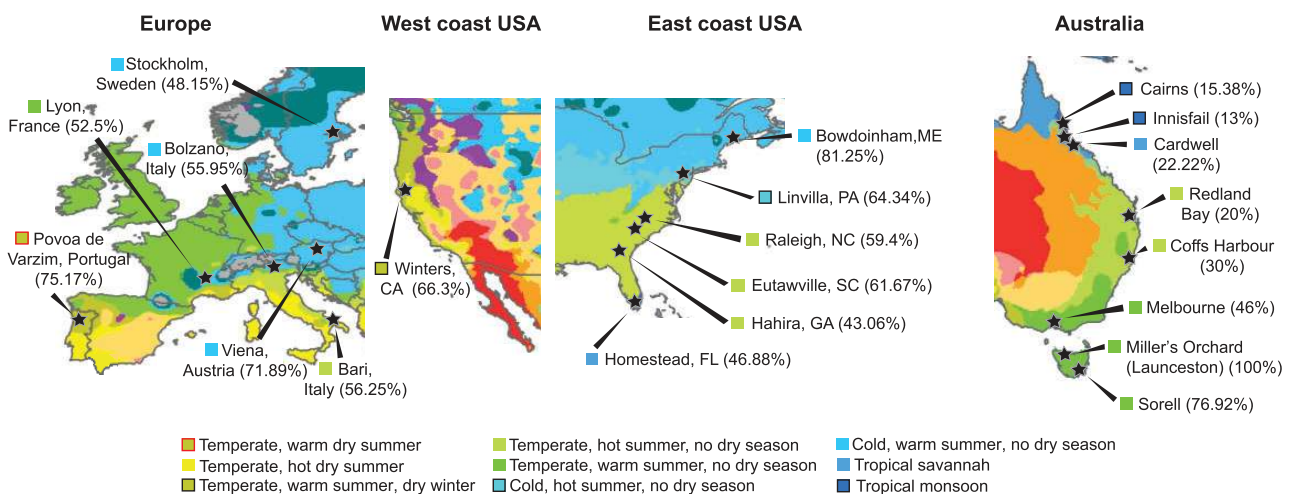


FIG. 5. Climate map with *Drosophila melanogaster* population samples analyzed with T-lex2. The frequency of *FBti0019386* in each population is shown in brackets. Climate maps are modified from Peel et al. (2007).

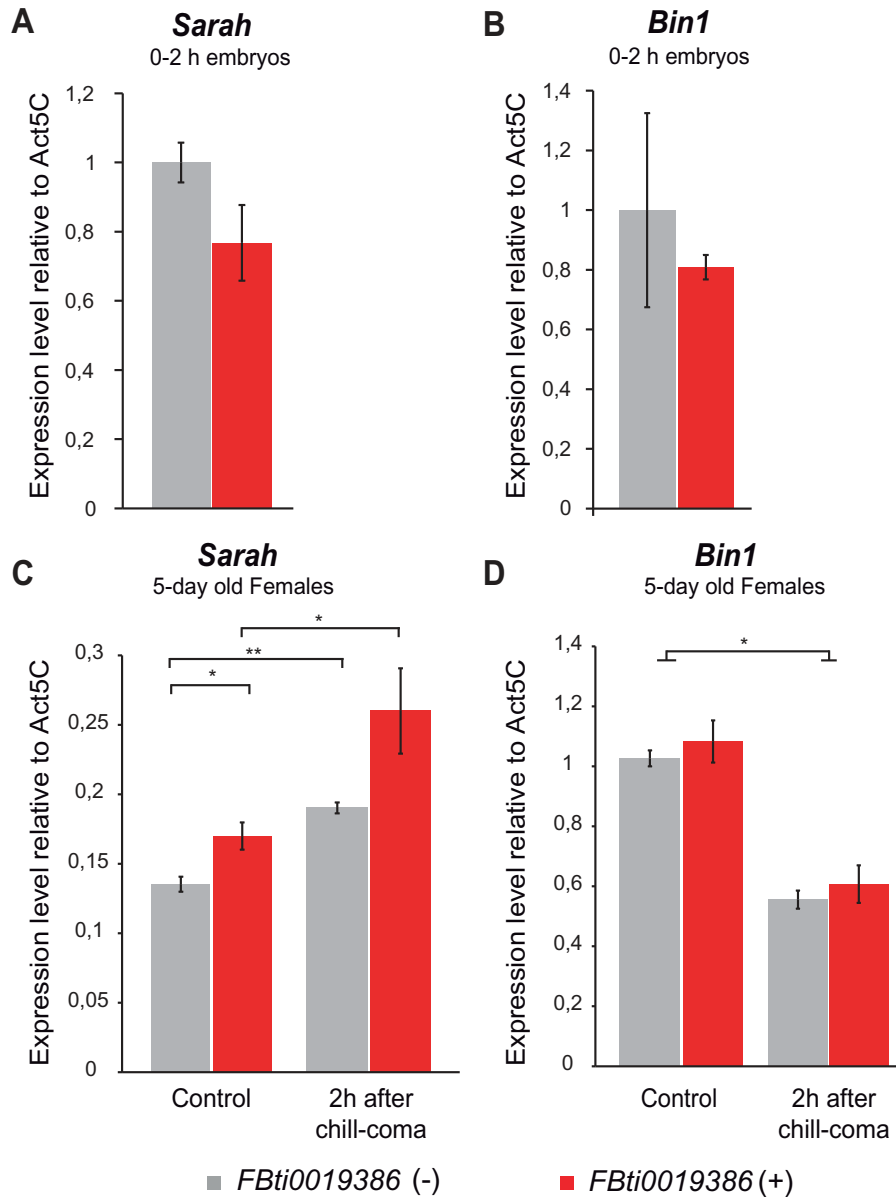


FIG. 6. Flies with *FBti0019386* insertion showed *sra* upregulation. Real-time polymerase chain reaction quantification of *sra* and *Bin1* transcript levels in outbred flies without *FBti0019386* insertion and with *FBti0019386* insertion. We represented the average expression level of *sra* (A and C) and *Bin1* (B and D) relative to *Act5C* with SEM error bars for three biological replicates in 0–2 h embryos and in 5-day-old females. Normalized expression measured 2 h after chill-coma for *sra* and *Bin1* is depicted in (C) and (D), respectively.

FBti0019386 Could Be Affecting gene Expression by Ectopically Assembling Heterochromatin

TEs from the *invader4* family contain sites with homology to PIWI interacting RNAs (piRNAs) that act as *cis*-acting targets for heterochromatin assembly by recruiting Heterochromatin Protein 1a (HP1a) (Sentmanat and Elgin 2012). Specifically, these piRNA binding sites are located in the long terminal repeat (LTR) sequences. Because *FBti0019386* is a 347-bp solo-LTR, we hypothesized that it could be inducing the ectopic assembly of heterochromatin. We analyzed the 14.6-kb region containing *Bin1*, *sra*, and *FBti0019386* and found that both sense and antisense piRNAs bind specifically to *FBti0019386* (fig. 7A) (see Materials and Methods). Second, we tested

whether there is evidence for the presence of HP1a binding to *FBti0019386* sequence. We found that HP1a specifically binds to *FBti0019386* sequence (fig. 7B) (see Materials and Methods). Thus, these results suggest that *FBti0019386* could be affecting gene expression by inducing the ectopic assembly of heterochromatin.

Discussion

In this work, we explored the plausible phenotypic space of the putatively adaptive *FBti0019386* insertion in different developmental stages, embryo and adult, and in different environmental conditions, nonstress conditions and cold, osmotic, and starvation stress conditions. Overall, we found

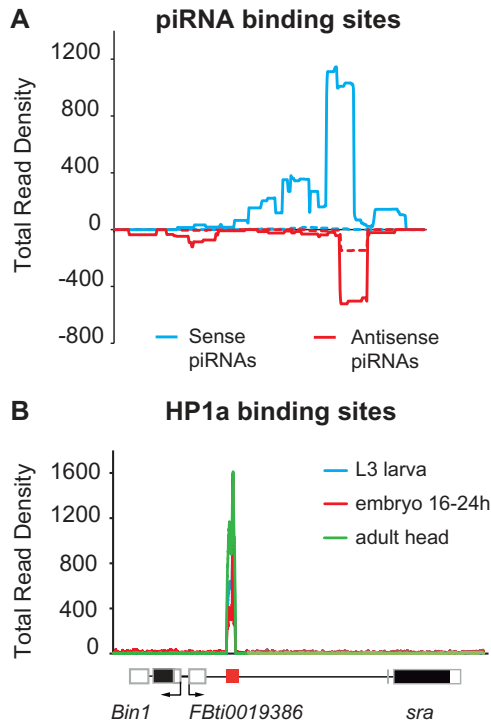


FIG. 7. *FBti0019386* could bind piRNA and HP1a protein. (A) Mapping of piRNA sense and antisense RNA-seq reads against *FBti0019386* sequence. Data from Li et al. (2009) are depicted in dashed lines and data from Satyaki et al. (2014) are represented in continuous lines. (B) Mapping of reads coming from HP1a ChIP-Seq experimental data against the genome region containing *Bin1*, *FBti0019386*, and *sra*. Experimental data from L3 larva, 16–24h embryo, and adult heads are given.

that *FBti0019386* mediates sensitivity to cold stress conditions (fig. 3) and is associated with faster DT (fig. 4). These two phenotypic effects have plausible fitness consequences in nature that could explain why the mutation increased in frequency in natural populations but has not reached fixation. Increased sensitivity to cold stress conditions is likely to reduce fitness of the flies that carry *FBti0019386* insertion, and may represent the cost of selection of this mutation. On the other hand, faster DT is likely to increase the fitness of flies with *FBti0019386* insertion. In nature, quick development favors *D. melanogaster* individuals for several reasons. First, larvae feed on rotting fruits that are ephemeral. Thus, quick development allows larvae to pupate before the food source is exhausted. Second, competition increases as more and more eggs are laid on a piece of fruit, also favoring individuals with faster DT (Nunney 1990). Third, breeding sites in nature can be destroyed by physical factors and predation, individuals that develop faster are thus more likely to escape microhabitat destruction. And fourth, faster DT accelerates the age of first breeding, which is relevant for the organism if most reproduction happens in expanding populations. This is the case for *D. melanogaster* populations that expand their population size every spring. Thus, it is plausible that *FBti0019386* increased in frequency in natural populations because of its positive effect on DT whereas it did not reach fixation because of its negative effect on cold-stress

resistance. Our results emphasize the importance of exploring different phenotypes to fully characterize the effects of natural mutations, as have been suggested before (Mackay 2010; Guio et al. 2014). Although our results provide a plausible explanation for the effect of *FBti0019386* insertion in natural populations, experiments under natural conditions are needed to unequivocally identify the effect of this insertion in nature.

By combining several tests that capture different signatures of selection at the DNA level, we demonstrate that *FBti0019386* shows signatures of positive selection suggesting that it is an adaptive mutation (table 1). However, our results are not entirely consistent with a role of *FBti0019386* in temperate adaptation as has been previously proposed (González et al. 2010). First, adaptation to temperate climates has been associated with increased stress resistance, increased DT, and decreased fecundity (Stanley and Parsons 1981; Hoffmann et al. 2003; Schmidt et al. 2005; Folguera et al. 2008; Schmidt and Paaby 2008; but see also James and Partridge 1995; James et al. 1997; Trotta et al. 2006). However, we found that *FBti0019386* is associated with increased sensitivity to cold stress (fig. 3), with shorter DT (fig. 4) and does not significantly affect fecundity (fig. 1). Thus, the phenotypic effects of *FBti0019386* are not consistent with a role of this insertion in temperate adaptation. Second, our global analyses of *FBti0019386* population frequency showed that *FBti0019386* frequency correlates with latitude and with climatic variables in North America and in Australia but not in Europe (fig. 5 and supplementary table S6, Supplementary Material online). We suggest that the clinal frequency patterns in North America and in Australia could be due to the dual colonization of these two continents by European and African populations rather than to the operation of spatially varying selection (Caracristi and Schlotterer 2003; Rouault et al. 2004; Duchon et al. 2013; Bergland et al. 2014). The lack of clinal frequency patterns in Europe would support this conclusion. However, it is also possible that phenotypic effects of *FBti0019386* not yet characterized could be consistent with a role of this natural mutation in temperate adaptation. Additionally, although there is evidence for the presence of clinal variation in European populations (David et al. 1985, 1986, 1989; Costa et al. 1992), other works have shown that clines are weaker in Europe compared with other continents (Oakeshott, Chambers, et al. 1983; Oakeshott, Gibson, et al. 1983). This could be partly due to differences in the latitudinal ranges spanned by populations analyzed in the different continents. In this work, the latitudinal range spanned by North American (25.82°–45.06°) and Australian (–16.88° to –42.83°) populations is larger than the range spanned by European populations (41.13°–59.33°). In any case, genome-wide scan studies that identify loci that are differentiated between populations should be taken as a first step toward the identification of loci that are subject to spatially varying selection (González et al. 2010; Kolaczkowski et al. 2011; Fabian et al. 2012; Reinhardt et al. 2014). Further functional validation should be gathered before concluding that the candidate loci are under spatially varying selection (Bergland et al. 2014).

Our results also shed light on the molecular processes that lead from genotype to phenotype. We found that *FBti0019386* is associated with upregulation of *sra* (fig. 6C). As previously described for other elements from the *invader4* family, we showed that *FBti0019386* has piRNA binding sites (fig. 7A) (Sentmanat and Elgin 2012). We also showed that HP1a binds specifically to the *FBti0019386* sequence, further suggesting that *FBti0019386* could be inducing the ectopic assembly of heterochromatin (fig. 7B). These results highlight the potential role of TE remnants as silencing signals to be used by piRNAs to direct heterochromatin formation (Sentmanat et al. 2013). Although we observed an upregulation of *sra* in adult females, we can not discard that heterochromatin assembly induced by *FBti0019386* could be affecting gene expression in other developmental stages and/or specific tissues.

A recent update of FlyBase, the database of *Drosophila* genes and genomes, annotated two new *Bin1* transcripts that have their transcription start site inside *FBti0019386* (St Pierre et al. 2014). As a consequence, these two new transcripts would only be produced in strains with the insertion, and could contribute to differences in the level of *Bin1* expression in flies with and without the insertion. Although we did not find differences in *Bin1* expression, we cannot discard that differences in the level of expression of *Bin1* are present in developmental stages, tissues, or environmental conditions that we have not investigated.

Although *sra* and *Bin1* have not been associated with DT, both genes play important roles during development and have been associated with a wide range of processes (Chang et al. 2003; Ejima et al. 2004; Horner et al. 2006; Takeo et al. 2006, 2010; Chang and Min 2009; Matyash et al. 2009; Costa et al. 2011; Nakai et al. 2011). A genome-wide screening looking for genes influencing DT in *D. melanogaster* has shown that the many candidate genes were involved in a wide range of biological processes such as cellular metabolic processes, organismal development, and response to stress (Mensch et al. 2008). More recently, developmental timing in insects has been associated with hormonal and circadian control (Di Cara and King-Jones 2013; Yadav et al. 2014). Interestingly, *sra* is regulated by *Shaggy/GSK-3 β* (*sgg*), a Ser–Thr kinase involved in the regulation of circadian rhythmicity (Martinek et al. 2001). On the other hand, both *Bin1* and *sra* are stress-response genes: *Bin1* is upregulated in response to stress and *sra* is downregulated (fig. 6). *Bin1* is a known key player in transcriptional response to environmental stress (Costa et al. 2011). Although there was no previous evidence for a direct role of *sra* in response to stress, *sra* could be affecting stress response through its role in the calcium pathway (Takeuchi et al. 2009; Teets et al. 2013; Davies et al. 2014). *sra* inhibits *calcineurin*, a highly conserved protein in eukaryotes that has the ability to sense calcium (Hogan et al. 2003). Although it is not deeply understood, calcium pathways play a role during general cell-stress response including cold stress response (Takeuchi et al. 2009; Teets et al. 2013; Davies et al. 2014). Note that many genes that affect complex traits in *Drosophila* had well-characterized

roles in early development and were not previously annotated to affect adult quantitative traits (Mackay 2010).

FBti0019386 adds to the growing list of TE-induced adaptive mutations that have been linked to their fitness effects and their underlying molecular mechanisms (Schmidt et al. 2010; Magwire et al. 2011; Guio et al. 2014; Mateo et al. 2014; Sun et al. 2014). Overall, these examples highlight the variety of mechanisms underlying adaptive mutations and point toward a significant role of TEs in response to stress (Casacuberta and González 2013). However, the number of characterized mutations is still too small to obtain an overall picture of adaptation. In depth, characterization of a representative set of adaptive mutations in natural populations will allow us to start answering long-standing questions in the field such as which traits are more relevant for adaptation? What is the effect-size distribution of adaptive mutations? and What evolutionary processes underlie adaptive evolution?

Materials and Methods

Sequence Analysis of the *FBti0019386* Flanking Regions

Single nucleotide polymorphism (SNP) data were downloaded from the DGRP2 webpage (<https://www.hgsc.bcm.edu/arthropods/drosophila-genetic-reference-panel>) in vcf format. Strains with ($N=65$) and without ($N=38$) *FBti0019386* insertion were filtered using vcfTools v_0.1.10 (<http://vcftools.sourceforge.net/>).

We used three different statistics to detect positive selection: Nucleotide diversity (π), Tajima's D , and the CL of SNPs. Positive selection results in the elimination of standing genetic variation that is linked to the adaptive mutation. Thus, if *FBti0019386* has increased in frequency due to positive selection, we expect a decrease in π in flies with the insertion compared with flies without the insertion. π is calculated as the mean number of pairwise differences between two given sequences (Hudson et al. 1992). Tajima's D statistic is calculated as the ratio between the mean number of pairwise differences and the number of segregating sites (Tajima 1989). This ratio is expected to be 0 in a neutrally evolving population whereas negative values of Tajima's D can be taken as evidence of positive selection (Tajima 1989). Finally, CL test is calculated by multiplying the marginal likelihoods for each site along the studied sequences (Nielsen et al. 2005).

π , Tajima's D , and CL were calculated for the two sets of sequences, with and without the insertion, using the PopGenome package in R (Pfeifer et al. 2014). Sliding windows analyses were performed for 200-bp-size windows spanning 1 and 2-kb regions flanking the insertion. Differences between strains with and without the insertion were more drastic for the 1-kb region flanking the insertion; therefore, we focused our analysis in this region.

Simulations were performed using the MS program (Hudson 2002). Theta values were estimated using the 205 DGRP2 strains for the 2-kb region around *FBti0019386* ($\theta = 4.77/\text{kb}$) and for the 3R chromosomal arm ($\theta = 4.5/\text{kb}$). Thus, simulations were performed for theta

values of 4/kb and 5/kb, which are frequently used as neutral values in *D. melanogaster*.

Ad hoc perl scripts were used for the resampling analyses. In total, 1,000 random samples of the 103 DGRP strains analyzed were obtained keeping the same proportion as in the original present and absent data sets (60%/40%, respectively) and a sample size of nearly 50% of the total data set.

We also computed CLR as $2 * (\log \text{CL} (\text{present}) - \log \text{CL} (\text{absent}))$, for a 1-kb region around the TE insertion. Because demography could produce similar patterns as positive selection, we performed a random sampling of 1,000 1-kb-long regions from the 3R chromosome for the absent and present data sets and calculated π , Tajima's *D*, CL, and CLR tests in each one of them.

Fly Strains

Outbred Strains

We selected six inbred strains from the *Drosophila* Genetic Reference Panel (Mackay et al. 2012; Huang et al. 2014) homozygous for the presence of *FBti0019386* insertion (RAL-21, RAL-40, RAL-177, RAL-402, RAL-405, and RAL-857). We placed ten virgin females and ten males of each strain in a fly chamber to create an outbred population sharing the TE insertion. We also selected six inbred strains without the insertion (RAL-75, RAL-138, RAL-383, RAL-461, RAL-822, and RAL-908) and created an outbred strain following the same procedure explained above. Each outbred population was maintained by random mating ($N \approx 800$ flies per generation) for at least ten generations before starting the experiments.

Introgressed Strains

We selected two DGRP strains: One homozygous for the presence of *FBti0019386* insertion (RAL-177) and one homozygous for the absence (RAL-802). We crossed RAL-177 virgin females with RAL-802 males and backcrossed the virgin females that carry *FBti0019386* insertion from the following generations with RAL-802 males for 12 generations. After that, we did brother–sister crosses until we obtained homozygous strains for the absence and homozygous strains for the presence of *FBti0019386*.

Individual DGRP Strains

We used a couple of individual DGRP strains differing by the presence/absence of *FBti0019386* insertion to perform our phenotypic assays. We used RAL-857 (homozygous for the presence of *FBti0019386* insertion) and RAL-802 (homozygous for the absence).

Presence/Absence of *In(3R)Payne* in the Analyzed Strains

To discard the effect of *In(3R)Payne* inversion on *FBti0019386* phenotypic effects, we genotyped the strains analyzed to detect the presence/absence of this inversion: The two outbred, the two introgressed, and the two individual DGRP strains. We used the primer sequences described in Matzkin et al. (2005). As a positive control, we used a strain that was previously genotyped in our laboratory and that carries the *In(3R)Payne* inversion.

Phenotypic Assays

All experiments were performed using outbred populations. Additionally, we used introgressed and individual DGRP strains to perform CCRT assay, survival after chill-coma, and DT assays.

Fecundity

In total, 40 virgin females from each strain were placed individually in vials with one male from the same strain. During 17 days flies were moved to new vials every 2 days and the number of eggs laid per female during that period was counted. Total fecundity, that is, average of the total number of eggs laid per female during the 17 days, and early fecundity, that is, average of the total number of eggs laid per female during the first 48 h of egg laying, was compared between flies with and without *FBti0019386*.

Egg Hatchability and Hatching Time

In total, 800 4- to 8-day-old flies were allowed to lay eggs for 3 h on apple juice-agar medium with fresh yeast. Embryos were separated in groups of 20 or 50 and placed into food vials. Vials were kept at room temperature (19–22°C) and checked during the following hours for hatched eggs (2–5 times per day). We analyzed the average time over the midpoint of each successive interval in order to estimate the hatching time. Two experiments were performed following this protocol: A first pilot experiment with 150 embryos per strain, and one replica with 500 embryos per strain.

Egg hatchability and egg hatching time were also analyzed under cold stress conditions. Embryos were placed at 1°C overnight for 14 h and at 18°C during the day, and this cycle was maintained until all the eggs had hatched. We performed a pilot experiment with 100 embryos per strain and additional experiments with 240 and 160 embryos per strain, respectively.

Cold Stress in Embryos

In total, 800 7- to 10-day-old flies were allowed to lay eggs for 3 h on apple juice-agar medium with fresh yeast. Embryos were collected following the methodology described in Schou (2013), and placed into food vials in groups of 50. When embryos were 3–6 h old, vials were placed at 1°C for 14 h, and maintained at 18°C until adult emergence. Simultaneously, control vials were always maintained at 18°C and not cold-exposed to control for other variables affecting egg to adult survival. We performed a first pilot experiment using 280 embryos per strain and three biological replicas using 350 embryos per strain (replica 1) and 750 embryos per strain (replica 2 and replica 3, respectively). In all cases, we analyzed egg to adult survival after all the adults had emerged.

Chill-Coma Recovery Time

In total, 500 3- to 5-day-old flies were separated by sex and by strain and placed into five empty vials in groups of 50. We allowed flies to recover from CO₂ anesthesia for 1 h and then vials were put in ice and kept in a 4°C chamber for 16 h as described in David et al. (1998). After the cold shock, adults were transferred to Petri dishes at room temperature (22–24°C), and recovery time was monitored for successive

intervals of 30 s during 2 h. We considered as recovered flies those that were able to stand on their legs. As a control, we monitored survival of flies that were kept at room temperature: Three vials of 20 flies each, by sex and strain.

Survival after Chill-Coma

In total, 400 5- to 8-day-old flies were separated by sex and strain and placed into six food vials in groups of 20. We allowed flies to recover from CO₂ anesthesia for at least 2 days. After that, flies were changed to empty food vials and were put in ice, and kept in a 4°C chamber for 16 h. When adults were recovered from chill-coma, we transferred them to food vials and we monitored mortality during the next 5 days. As a control, we monitored survival of flies that were kept at room temperature: Three vials of 20 flies each, by sex and strain.

Osmotic Stress

In total, 2,000 4- to 7-day-old flies were separated by sex and strain and placed in groups of 20 into 20 food vials containing 3% of NaCl, and into five vials with normal food as a control. Flies were maintained at room temperature (22–24°C) and dead flies were counted every 12–24 h until all the treated flies were dead.

Starvation Stress

In total, 2,000 3- to 4-day-old flies per strain were separated by sex and strain and placed in groups of 20 into 20 food vials containing only 1.5% agar, and into five vials with normal food as a control. Flies were maintained at room temperature (22–24°C) and dead flies were counted three times a day until all the treated flies were dead.

Developmental Time

In total, 800 7- to 10-day-old flies were allowed to lay eggs for 3 h. A total of 500 embryos per strain were collected and distributed in groups of 50 per food vial and were maintained at 18°C. Vials were checked every 6–8 h for emerging adults until all flies had emerged. We estimated the average DT over the midpoint of each successive interval.

Statistical Analyses of the Phenotypic Assays

Analyses were performed with SPSS v21. We first tested whether data followed a normal distribution by performing Kolmogorov–Smirnov test. *t*-Test was performed for normal data and Mann–Whitney test for nonnormal data. Survival curves were compared with log-rank test. When the statistical test was significant, we estimated the size effect of the mutation by calculating the odds-ratio and its confidence interval.

FBti0019386 Frequency Estimation for Natural Populations

To obtain FBti0019386 frequency, we run *T-lex2* (Fiston-Lavier et al. 2014) using *Drosophila* whole-genome sequences available from a total of 23 populations from North America, Australia, Europe, and Africa (supplementary table S5, Supplementary Material online).

The accuracy of TE frequency estimates using *T-lex2* is affected by coverage. However, coverage for all samples was

higher than 20× except for Lyon (France) and California (USA), which had 8× and 4.7× coverage respectively, suggesting that overall frequency estimates are accurate.

Correlation Analysis of FBti0019386 Frequency with Geographic and Climate Variables

We analyzed whether the frequency of FBti0019386 insertion correlated with different geographical and climate variables in North America, Australia, and Europe using Pearson product–moment correlations. We also performed a PCA to disentangle the relationships between the climatic variables using Statistica (v8.0, StatSoft, Inc. 2007). Climatic data were obtained from the weather stations adjacent to collection sites of each population, available in Peel et al. (2007). When necessary, data were transformed as described in Sokal and Rohlf (2012) (see pages 411–422).

mRNA Transcript Levels Analysis (quantitative reverse transcription polymerase chain reaction)

Total RNA was extracted from three biological samples of 40 adult females (5-day old) from outbred populations differing by the presence/absence of FBti0019386 insertion using Trizol reagent and PureLink RNA Mini kit (Ambion). RNA was treated on-column with DNase I (Trizol) and after RNA purification. Reverse transcription was carried out using 1 µg of total RNA, Anchored-oligo(dT) primer, and Transcription First Strand cDNA Synthesis Kit (Roche). The resulting cDNA was used for quantitative reverse transcription polymerase chain reaction with SYBR Green (BioRad) on an iQ5 Thermal cycler. *sra* total expression was measured using a pair of primers specific to a 124-bp cDNA amplicon spanning the 5'-UTR/exon junction of the gene (5'-ACAACAACGGTGGAGAAGAGCCGT-3' and 5'-GGTGCATCGGCGGACGCA TTG-3'). For *Bin1*, we measured the 66-bp cDNA amplicon spanning the 5'-UTR/exon junction using specific primers (5'-TGTCGTCCCGTAGAGCAGAA-3' and 5'-CAGCAGATTGACCGCGAGA-3'). In both cases, we normalized the expression with *Act5C* (5'-GCGCCCTTA CTCTTTACCA-3' and 5'-ATGTCACGGACGATTTCACG-3'). Expression was measured in nonstress conditions and in cold-stress conditions: 16 h at 4°C and 2 h at room temperature to allow flies to recover.

We also analyzed the expression of both genes in 0–2 h embryos using the same procedure. We collected the embryos from population cages containing approximately 800 flies from outbred populations differing by the presence/absence of FBti0019386 insertion. Briefly, 4- to 8-day-old flies were allowed to lay eggs for 2 h on apple juice-agar medium with fresh yeast. Then, embryos were collected using a small brush and cleaned with water. Embryos were dechorionized by submerging them for 5 min in 50% bleach. After that, embryos were placed in a microcentrifuge tube, the excess of water was eliminated, and the samples were froze at –80°C until RNA extraction.

Detection of piRNA Reads Binding to *FBti0019386* Sequence

We used small RNA sequencing data to check whether piRNAs reads mapped to *FBti0019386* sequence, following a methodology similar to that described in Sentmanat and Elgin (2012). Briefly, we obtained the small RNA reads from Oregon R ovaries (accession number SRP000458) (Li et al. 2009), and from wild type ovaries (accession number: SRX470700) (Satyaki et al. 2014). We aligned the reads by using BWA-MEM package version 0.7.5-a-r405 (Li 2013) to the 14.6-kb sequence obtained from *Drosophila* reference genome, containing *Bin1* and *sra* genes, and *FBti0019386* (release five chromosomal coordinates 3R: 12,010,721–12,025,306). Then, we used samtools and bamtools (Barnett et al. 2011) to index and filter by sense/antisense reads. Finally, we obtained the total read density using R (Rstudio v0.98.507).

Detection of HP1a Protein Binding in *FBti0019386* Sequence

We downloaded all available raw data from modEncode HP1a protein ChIP-Seq experiments: Embryos (ID 3391 and 3392), third instar larvae (ID 4936), and adult heads (ID 5592) (<http://data.modencode.org>). Then, we mapped the reads against the 14.6-kb region described above. We performed the alignments following the same methodology as for the piRNA reads analysis.

Supplementary Material

Supplementary tables S1–S9 and figures S1–S3 are available at *Molecular Biology and Evolution* online (<http://www.mbe.oxfordjournals.org/>).

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