Rapid evolutionary diversification of the flamenco locus in the *D. simulans* clade

Sarah Signor[1*], Jeffrey Vadanayagam[2], Filip Wierzbicki[3,4], Robert Kofler[3], and Eric Lai[2]

*Corresponding author: sarah.signor@ndsu.edu

[1]Biological Sciences, North Dakota State University, Fargo, North Dakota, USA

[2]Department of Developmental Biology, Sloan-Kettering Institute, 1275 York Ave, Box 252, New York, NY 10065, USA

[3]Institut für Populationsgenetik, Vetmeduni Vienna, Vienna, Austria

[4]Vienna Graduate School of Population Genetics, Vienna, Austria

Abstract

Maintaining genomic integrity through suppression of transposable elements (TEs) is paramount to organismal fitness. In *D. melanogaster flamenco* is the master regulator of TE suppression, preventing TEs from moving from the somatic support cells of the ovary to the germline. It is thought to operate as a trap, suppressing TEs upon their transposition into the locus, with a high representation of recent horizontally transferred TEs. We find that in the *simulans* clade *flamenco* has undergone physical and functional diversification. In *D. simulans flamenco* has duplicated and diverged from its original, maintaining no synteny within the locus but conserving its promotor. In *D. simulans* and *D. mauritiana*, *flamenco* acts as a dual stranded cluster with ping pong signals in some tissues. This expansion in function has been accompanied by an increase in the number of multicopy elements, as expected for regions operating as dual stranded clusters. In addition, we find that in *D. simulans* presence of a TE in either *flamenco* or a germline cluster, may be necessary for suppression of a TE but it may not be entirely sufficient. This physical and functional diversity of *flamenco* in the *simulans* clade redefines our understanding of this locus.

Introduction

In *Drosophila* there are two important fronts in the conflict between TEs and the host – the germline, and the somatic support cells of the ovary from which TEs can invade the germline (1, 2). The system by which TEs are suppressed in each of these tissues is thought to be different (3). What is in common between the two systems is the following – PIWI proteins of the Argonaute family bind 23-30 nt small RNAs and form protein complexes that recognize RNA transcripts cognate to the small RNA and initiate silencing transcriptionally and post-transcriptionally (4–7). piRNAs are derived from the processing of non-coding transcripts from piRNA clusters, repetitive regions densely populated with TEs (7–9). Where they diverge is exactly how those piRNAs are produced – in the germline the piRNAs are transcribed from both DNA strands through non-canonical Pol II transcription (6, 10–12). Transcription is initiated by chromatin marks rather than a particular sequence motif. In the somatic support cells of the ovary piRNA clusters are transcribed from a single strand, transcription is initiated by a promotor, and the transcript is alternatively spliced just like a protein coding gene (13–16). This general rule was formed through study of a few very productive and well characterized clusters (i.e. *42AB* and *flamenco*). For both types of piRNA clusters the ability to repress invading transposable elements comes through integration of a new transposon to the cluster site. As such, piRNA clusters are adaptive loci and they play an important role in the conflict between hosts and TEs.

The location and activation of germline piRNA clusters is somewhat stochastic and changeable, there are likely many redudant copies of TE families in different locations that may produce piRNAs (9, 17, 18) The model for the suppression of TEs in the somatic support cells is different and is exemplified by *flamenco*. piRNA clusters such as *flamenco* act as transposon traps for new TE invasions, such that any trapped TE inserted into *flamenco* should be silenced

70 and no further transposition events should occur (16, 19). Thus, *flamenco* should contain only
71 one copy per TE family (16). This expectation can be violated by local or segmental
72 duplications which are common at piRNA loci, but should generally hold for the locus as a
73 whole. These expectations hold true in the *flamenco* locus of *D. melanogaster* (16).

74 *flamenco* has been a model for understanding the piRNA pathway since the discovery of
75 piRNA mediated silencing of transposable elements (6). *flamenco* is a large stretch of repetitive
76 sequences spanning > 180kb in the *β*-heterochromatin of X chromosome (20). *flamenco* has
77 specifically been known to suppress the activity of *gypsy*, *ZAM*, and *Idefix* class elements,
78 though the mechanism was not known at the time (20–24). Additionally, analysis of *flamenco* in
79 various strains of *D. melanogaster* revealed that this locus acts as a trap for horizontally derived
80 TEs to achieve silencing of newly invaded TEs (16). Furthermore, *flamenco* exhibits synteny in
81 the *D. melanogaster* sub-group (25), however, the sequence composition of *flamenco* outside *D.*
82 *melanogaster* is not well-characterized (26).

83 In this study we compare the *flamenco* locus in 10 strains of *D. simulans*, *D. mauritiana*,
84 and *D. sechellia*. Using piRNA from ovaries of five genotypes of *D. simulans* we found that
85 *flamenco* is duplicated in *D. simulans*, and is even found in a third copy in one genotype. This
86 duplication is old enough that there is no sequence synteny between the two loci but the
87 promotor region and the adjacent *dip1* gene are conserved. We show that *flamenco* has also been
88 colonized by abundant copies of *R1* (>40), a TE that was thought to insert only at ribosomal
89 genes, and to evolve at the same rate as nuclear genes (27). Furthermore, between different
90 genotypes up to 64% of TE insertions are not shared within any given copy of *flamenco*. Despite
91 this, several full length TEs are shared between all genotypes in a similar sequence context. This

incredible diversity at the *flamenco* locus even from within a single species suggests there may be considerable variation in the ability to suppress transposable elements at this locus.

Comparisons amongst species suggest that the function of the *flamenco* locus may have diversified in different species. From embryonic and testis small RNA libraries in *D. simulans* and *D. mauritiana* we find that in some tissues *flamenco* may act as a dual strand cluster – piRNAs are produced from both strands and a ping pong signal is detected in these tissues. By contrast small RNA in these tissues from *D. sechellia* and *D. melanogaster* conform with the current understanding of *flamenco*, that it is not expressed and does not act like a germline cluster. We also find that the requirements for TE suppression – presence within *flamenco* or a germline cluster with ping pong signals, does not always entirely suppress the activity of TEs. Many questions remain – for example have the copies of *flamenco* in *D. simulans* sub-specialized, or are they entirely redundant? However, it is clear that while *flamenco* may be the master regulator of TE suppression in the somatic support cells of the ovary in *D. melanogaster* it is also a dynamic and rapidly evolving locus with other functions in other tissues.

Materials and Methods

Fly strains

The four *D. simulans* lines *SZ232*, *SZ45*, *SZ244*, and *SZ129* were collected in California from the Zuma Organic Orchard in Los Angeles, CA on two consecutive weekends of February 2012 (28–32). *LNP-15-062* was collected in Zambia at the Luwangwa National Park by D. Matute and provided to us by J. Saltz (J. Saltz pers. comm., (33, 34)). *MD251*, *MD242*, *NS137*, and *NS40* were collected in Madagascar and Kenya (respectively) and are described in (35). The *D. simulans* strain wxD^1 was originally collected by M. Green, likely in California, but its

provenance has been lost (pers. comm. Jerry Coyne). *D. mauritiana (w12)* and *D. sechellia (Rob3c/Tucson 14021-0248.25)* are described in (36)

Long read DNA sequencing and assembly

MD242, four SZ lines and *LNP-15-062* were sequenced on a MinION platform at North Dakota State University (Oxford Nanopore Technologies (ONT), Oxford, GB), with base-calling using guppy (v4.4.2). *MD242*, the four SZ lines, and *LNP-15-062* were assembled with Canu (v2.1) (37) and two rounds of polishing with Racon (v1.4.3) (38). The SZ strains were additionally polished with short reads using Pilon (v1.23) (39)(SRR3585779, SRR3585440, SRR3585480, SRR3585391) (28). The first wxD^{1-1} assembly is described here (36). *MD251*, *NS137*, *NS40* and wxD^{1-2} were sequenced on a MinION platform by B. Kim at Stanford University. They were assembled with Flye (40), and polished with a round of Medaka (https://github.com/nanoporetech/medaka) followed by a round of pilon (39). Following this contaminants were removed with blobtools (https://zenodo.org/record/845347, (41)), soft masked with RepeatModeler and Repeatmasker (42, 43), then aligned to the wxD^{1} as a reference with Progressive Cactus (44). The assemblies were finished with reference based scaffolding using Ragout (45). *D. mauritiana* and *D. sechellia* were sequenced with PacBio and assembled with FALCON using default parameters (https://github.com/PacificBiosciences/FALCON)(36). The *D. melanogaster* assembly is described here (46). A summary of the assembly statistics is available in Supplementary Table 1. The quality of cluster assembly was evaluated using CUSCO as described in (17, 47) (Supplementary File 1).

Short read sequencing and mapping

Short read sequencing was performed by Beijing Genomics Institute (BGI) on approximately 50 dissected ovaries from adult female flies (*SZ45*, *SZ129*, *SZ232*, *SZ244*, *LNP-15-062*). Short read

libraries from 0-2 hour embryos were prepared from *D. melanogaster*, *wxD*$^{1-2}$, *D. sechellia*, and *D. mauritiana* (SRAXXX) (48). Small RNA from testis is described in (49, 50). Libraries were filtered for adapter contamination and short reads between 23-29 bp were retained for mapping with fastp (51). The RNA was then mapped to their respective genomes using bowtie (v1.2.3) and the following parameters (-q -v 1 -p 1 -S -a -m 50 --best --strata) (52, 53). The resulting bam files were processed using samtools (54). To obtain unique reads the bam files were filtered for reads with 1 mapping position. To obtain counts files with weighted mapping the bam files were processed using Rsubreads and the featureCounts function (55).

Long read sequencing and mapping

Long read Iso-seq was performed by Beijing Genomics Institute (BGI) on the same RNA used to produce the small read libraries using the PacBio Sequel II. Reads were processed with the Circular Consensus Sequencing workflow in SMRT link. Reads were mapped to individual genomes and TE libraries using minimap2 (56).

Defining and annotating piRNA clusters

piRNA clusters were defined using proTRAC (57). piRNA clusters were predicted with a minimum cluster size of 1 kb (option "-clsize 1000"), a P value for minimum read density of 0.07 (option "-pdens 0.07"), a minimum fraction of normalized reads that have 1T (1U) or 10A of 0.33 (option "-1Tor10A 0.33") and rejecting loci if the top 1% of reads account for more than 90% of the normalized piRNA cluster read counts (option "-distr 1-90"), and a minimal fraction of hits on the main strand of 0.25 (option "-clstrand 0.25"). Note that this ties the piRNA clusters to their function such that participation in the ping pong pathway can be inferred from these patterns. Clusters were annotated using RepeatMasker (v. 4.0.7) and the TE libraries described in Chakraborty et al. (2019) (42, 58). The position of *flamenco* was also evaluated based on the

position of the putative promotor, the *dip1* gene, and the enrichment of *gypsy* elements (13). Fragmented annotations were merged to form TE copies with onecodetofindthemall (59). Fragmented annotations were also manually curated in *flamenco*, particularly because TEs not present in the reference library often have their LTRs and internal sequences classified as different elements.

Aligning the flamenco promotor region

The region around the *flamenco* promotor was extracted from each genotype and species with bedtools getfasta (60). Sequences were aligned with clustal-omega and converted to nexus format (61). Trees were built using a GTR substitution model and gamma distributed rate variation across sites (62). The markov chain monte carlo chains were run until the standard deviation of split frequencies was below .01, around one million generations. The consensus trees were generated using sumt conformat=simple. The resulting trees were displayed with the R package ape (63).

Detecting ping pong signals in the small RNA data

Ping pong signals were detected using pingpongpro (64) This program detects the presence of RNA molecules that are offset by 10 nt, such that stacks of piRNA overlap by the first 10 nt from the 5' end. These stacks are a hallmark of piRNA mediated transposon silencing. The algorithm also takes into account local coverage and the presence of an adenine at the 10th position. The output includes a z-score between 0 and 1, the higher the z-score the more differentiated the ping pong stacks are from random local stacks.

Results

flamenco in the D. simulans clade

flamenco was initially identified in *D. simulans* based on piRNA cluster calls from proTRAC, and the identity of the cluster was verified based on the location of *dip1*, the putative *flamenco* promotor, and the enrichment of *gypsy* elements in the area (Figure 1; Supplementary Table 2). On average the *flamenco* locus is 376 kb in *D. simulans*. This is an expansion compared with *D. melanogaster*, where *flamenco* is only 156 kb (*Canton-S*). In *D. sechellia flamenco* is 363 kb, however in *D. mauritiana* the locus has expanded to 840 kb (Supplementary Table 2). This is a large expansion, and it is possible that the entire region does not act as the *flamenco* locus, however there is no evidence to support this conclusion. Uniquely mapping piRNAs are found throughout the region and *gypsy* enrichment is consistent with a *flamenco*-like locus (Supplementary Figure 1). There are no protein coding genes in the region, and while the neighboring genes on the downstream side of *flamenco* in *D. melanogaster* have moved in *D. mauritiana* (*CG40813- CG41562* at 21.5 MB), the following group of genes beginning with *CG14621* is present and flanks *flamenco* as it is annotated. Thus in *D. melanogaster* the borders of *flamenco* are flanked by *dip1* upstream and *CG40813* downstream, while in *D. mauritiana* they are *dip1* upstream and *CG14621* downstream. Between all species the *flamenco* promotor and surrounding region, including the *dip1* gene, are alignable and conserved (Figure 1).

Structure of the flamenco locus

In *D. melanogaster flamenco* has a particular structure, where the majority of transposable elements are of *gypsy*-class elements in the antisense orientation (79% antisense orientation, 85% of which are *gypsy* elements) (Figure 1; Supplementary Table 3). This is true of both the *iso-1* and *Canton-S* strains. In *D. simulans flamenco* has been colonized by large expansions of *R1* transposable element repeats such that on average the percent of antisense TEs is only 50% and the percent of the locus comprised of LTR elements is 55%. However, 76% of antisense

insertions are LTR insertions, thus the underlying *flamenco* structure is apparent when the *R1* insertions are disregarded (Figure 1). In *D. mauritiana flamenco* is 71% antisense, and of those antisense elements 85% are LTRs. Likewise in *D. sechellia* 78% of elements are antisense, and of those 81% are LTRs. *flamenco* retains the overall structure of a canonical *D. melanogaster*-like *flamenco* locus in all of these species, however in *D. simulans* the nature of the locus is somewhat altered by the abundant *R1* insertions (Figure 1).

flamenco is duplicated in D. simulans

In *D. simulans* the *flamenco* cluster has been duplicated at least once on the X chromosome, and the duplication was confirmed with PCR and a restriction digest (Supplementary Table 4). These duplications are associated with a conserved copy of the putative *flamenco* enhancer as well as copies of the *dip1* gene which is found proximal to *flamenco* in *D. melanogaster* (Figure 1, 2). While it is not clear which was the original copy, each genotype has two copies that align among genotypes, and they will be referred to as *flamenco* and the duplicate. This suggests that the duplication happened a single time in the *D. simulans* complex. The two copies of *flamenco* are not alignable with one another and do not share common synteny amongst transposable elements, suggesting either that the duplication is old, has evolved very rapidly, or consisted only of the promotor region and was subsequently colonized by TEs (Figure 1, 2). The *flamenco* duplicate is absent in the *D. simulans* reference strain, but present in wxD^1, suggesting it was polymorphic or absent between the collection of these strains (or was not assembled). The duplicate retains the structure of *flamenco*, with an average of 67% of TEs in the antisense orientation in the duplication of *flamenco*, and 91% of the TEs in the antisense orientation are LTRs. The duplicate of *flamenco* is less impacted by *R1*, with some genotypes having as few as 8 *R1* insertions (Figure 2).

Novel TEs in flamenco

Full length TEs more than 5% diverged from the reference were investigated for identity with known TEs. While there were likely more fragments of novel TEs, we limited ourselves to full length TEs. Three novel TEs were identified. The first of these was inferred to be a copy of *Bel-6* by Repeatmasker, but was 10% diverged from the reference. We term this TE *Argyroneta.* It is an LTR element likely in the *Pao* group. The second element was designated as *Nomad* but it is 13.5% diverged from the reference. We will refer to this element as *Nomad-1*. Lastly, an element designed as *gypsy-53* was 23% diverged from the reference, which we will refer to as *gypsy-53A*. *gypsy-53A* and *Nomad-1* were present in every *flamenco*/duplicate combination, however *Argryoneta* is still in the process of colonizing *flamenco*. *Argryoneta* and *Nomad-1* are also found in *flamenco* in *D. mauritiana*.

R1 LINE elements at the flamenco locus

R1 elements are canonically known as inserting into rDNA genes, being vertically transmitted, and evolving at the same rate as the background nucleic acids (27). They may be present outside of rDNA genes, but only as fragments. However, as aforementioned, *R1* elements are abundant within *flamenco* in the *simulans* clade. Outside of *flamenco R1* elements in *D. simulans* are distributed according to expectation, with full length elements only occurring at rDNA genes (Supplementary File 6). Most copies of *R1* within *flamenco* occur as tandem duplicates, creating large islands of fragmented *R1* copies (Figure 2). They are on average 3.7% diverged from the reference R1 from *D. simulans*. In *D. simulans*, on average, 99 KB of the *flamenco* locus consists of *R1* elements, 26% of the total length of the locus. *SZ45, LNP-15-062, NS40, MD251*, and *MD242* contain full length copies of *R1* (between 4 and 7) in the sense orientation, despite all but *SZ45* containing fragmented copies on the antisense strand (The *SZ45 flamenco* assembly is not

complete). For a somatic cluster the copies on the antisense strand would be expected to suppress further *R1* transposition, suggesting *flamenco* is not effectively suppressing these elements or is functioning differently than expected.

There are abundant fragments or copies of *R1* on both strands of *D. mauritiana*, 19 on the reverse strand and 20 on the forward strand, and only one large island of *R1* elements. *D. mauritiana* contains 84 kb of *R1* sequence within the *flamenco* locus. In *D. mauritiana* there are 8 full length copies of *R1* at the *flamenco* locus which are not associated with any evidence of duplication events, 7 of which are inserted in the antisense orientation. There are no full length copies of *R1* in *D. sechellia*, and it contains only 18 KB of *R1* sequence (16 fragments on the reverse strand). Yet, there are no copies of *R1* found on the antisense strand which would suppress *R1* in this species.

The presence of long *R1* elements within *flamenco* is a departure from expectations for these transposable elements (16, 27). There is no evidence of an rDNA gene within the *flamenco* locus that would explain the insertion of *R1* elements there, nor is there precedence for the large expansion of *R1* fragments within the locus. In addition, the fragmented copies of *R1* on the antisense strand in *D. simulans* would be expected to prevent transposition into *flamenco*. The presence of full length *R1* elements on the sense strand suggests this is not the case. The same is true in *D. mauritiana*, with full length *R1* elements and fragments on both strands within *flamenco*.

piRNA production from R1

On average *R1* elements within the *flamenco* locus of *D. simulans* produce more piRNA than any other TE within *flamenco* (Supplementary Table 6). *R1* reads mapping to the forward strand constitute an average of 51% of the total piRNAs within the *flamenco* locus from the maternal

fraction, ovary, and testis using weighted mapping (counts are divided by the number of locations they map to, such that a read that maps to 100 locations is counted as 1/100 of a read). The only exception is the ovarian sample from *SZ232* which is a large outlier at only 5%. However reads mapping to the reverse strand account for an average of 84% of the piRNA being produced from the strand in every genotype and tissue – maternal fraction, testis, or ovary. If unique mapping is considered instead of weighted these percentages are reduced by approximately 20%, which is to be expected given that *R1* is present in many repeated copies. Production of piRNA from the reverse strand seems to be correlated with elements inserted in the sense orientation, of which the vast majority are *R1* elements in *D. simulans* (Supplementary Figure 2). The production of large quantities of piRNA cognate to the R1 element is seemingly pointless – if *R1* only inserts at rDNA genes and are vertically transmitted there is little reason to be producing the majority of piRNA in response to this element.

In *D. sechellia* there are very few piRNA produced from *flamenco* in these tissues, and there are no full length copies of *R1*. Likewise overall weighted piRNA production from *R1* elements on either strand is 2.8-5.9% of the total mapping piRNA. In contrast in *D. mauritiana* there are full length *R1* elements and abundant piRNA production in the maternal fraction and testis. In *D. mauritiana* an average of 28% of piRNAs mapping to the forward strand of *flamenco* are arising from *R1*, and 33% from the reverse strand. In *D. mauritiana R1* elements make up a smaller proportion of the total elements in the sense orientation (24%), versus *D. simulans* (55%).

Conservation of flamenco

The *dip1* gene and promotor region adjacent to each copy of *flamenco* are very conserved both within and between copies of *flamenco* (Figure 2). The phylogenetic tree of the area suggests

that we are correct in labeling the two copies as the original *flamenco* locus and the duplicate (Figure 2). Based on the upstream sequences the original *flamenco* locus is more diverged amongst copies while the duplicate clusters closely together with short branch lengths (Figure 2). They are also conserved and alignable between *D. melanogaster*, *D. sechellia*, *D. mauritiana*, and *D. simulans* (Figure 1). However, the same is not true of the *flamenco* locus itself. Approximately 3 KB from the promoter *flamenco* diverges amongst genotypes and species and is no longer alignable by traditional sequence-based algorithms, as the TEs are essentially a presence/absence that spans multiple KB. There is no conservation of *flamenco* between *D. melanogaster*, *D. simulans*, *D. sechellia*, and *D. mauritiana* (Figure 3). However, within the *simulans* clade many of the same TEs occupy the locus, suggesting that they are the current genomic invaders in each of these species (Figure 3).

In *D. simulans* the majority of full length TEs are singletons – 52% in *flamenco* and 64% in the duplicate. Copies that are full length in one genotype but fragmented in others are counted as shared, not singletons. Almost half of these singletons in the duplicate are due to a single genotype with a unique section of sequence, in this case *MD251*. Likewise a third of the singleton insertions in the duplicate are due to an *NS40* specific region of *flamenco*. singletons are the largest category of transposable element insertions, followed by fixed insertions. Thus even within a single population there is considerable diversity at the *flamenco* locus, and subsequently diversity in the ability to suppress transposable elements. For example, *gypsy-29* is present in three genotypes either in *flamenco* or the duplicate, which would suggest that these genotypes are able to suppress this transposable element in the somatic support cells of the ovary while the other genotypes are not. In contrast *gypsy-3* is present in more than one full length copy in *flamenco* and its duplicate in every genotype but one where it is present in a single copy.

There are a number of these conserved full length TEs that are present in all or nearly all genotypes, including *Chimpo*, *gypsy-2*, *Tirant*, and *gypsy-4*. In addition, the *INE1* elements adjacent to the promotor are always conserved.

It is surprising that full length TEs would be shared at all between all genotypes, given that *wxD*^{1} was likely collected 30-50 years prior to the other strains where the geographic location of collection span continents (Figure 2). There are two relevant facts to this observation 1) TEs have been shown previously to not correlate with geography (65) 2) *D. simulans* has been shown to have most of the variation within a species present within populations (66–68). There are also different possible explanations for this observation – Selection could be maintaining these full length TEs, *wxD*^{1} could have had introgression from other lab strains, or a combination of these explanations.

Suppression of TEs by the flamenco locus and the trap model of TE control

In *D. melanogaster* it has been proposed that while germline clusters may have many insertions of a single TE, the somatic 'master regulator' *flamenco* will have a single insertion of each transposon, after which they are silenced and no longer able to transpose (16). We will evaluate the following lines of evidence to determine if they support the trap model of TE suppression for somatic piRNA clusters: 1) How many TEs have antisense oriented multicopy elements within *flamenco*? 2) How many TEs have full length and fragmented insertions, suggesting the older fragments did not suppress the newer insertion? 3) How many *de novo* insertions of TEs in the *flamenco* duplicate of *D. simulans* are also present in the original *flamenco* copy?

<u>How many TEs have antisense oriented multicopy elements within *flamenco*?</u>

Due to the difficulty in classifying degraded elements accurately, for example between multiple classes of *gypsy* element, we will focus here on full length TEs, suggesting recent transposition.

In *D. melanogaster* there are 7 full length elements, none of which are present in more than one antisense copy. These elements make up 27% of the *flamenco* locus. Full length copies of five of these elements were also reported previously for other strains of *D. melanogaster* (16)

In *D. sechellia* there are 14 full length TEs within the *flamenco* locus, three of which are present in multiple copies. Two of these, *INE1* and *412*, are likely present due to local duplication. In particular the *INE1* elements flank the promotor, are in the sense orientation, and are conserved between *D. sechellia, D. mauritiana*, and *D. simulans*. The only element present in multiple antisense copies is *GTWIN*. Similar to *D. melanogaster* these elements make up 27% of the *flamenco* locus.

D. mauritiana contains 22 full length TEs within the *flamenco* locus. Four of these are present in multiple antisense full length copies – *INE1*, *R1*, *Stalker-4*, and *Cr1a*. While some of the five antisense copies of *R1* likely originated from local duplications – they are in the same general region and tend to be flanked by *gypsy-8*, not all of them show these patterns. Furthermore, as aforementioned, there also are full length sense copies of *R1* suggesting *R1* is not being suppressed by *flamenco*. *gypsy-12* and *gypsy-3* have a second antisense copy within *flamenco* that is just below the cutoff to be considered full length – in *gypsy-3* the second copy is 10% smaller, for *gypsy-12* it is 80% present but missing an LTR. Full length TEs make up 19% of the *flamenco* locus.

In *D. simulans* there are 29 full length TEs present in any of the seven complete *flamenco* assemblies. Eight of these are present in multiple antisense copies within a single genome – *INE1*, *Chimpo*, *copia*, *gypsy-3*, *gypsy-4*, *412*, *Tirant*, and *Argryoneta*. The two *Tirant* copies are likely a segmental duplication as they flank an *R1* repeat region. In addition, most *INE1* copies are present proximal to the promotor as aforementioned, however in *NS40* a copy is present in

antisense at the end of the locus. *Chimpo* is present in three full length copies within *MD242 flamenco*, with no evidence of local duplication. While there are no full length copies of *R1* inserted in antisense, *R1* is present in full length sense copies despite many genomes containing antisense fragments, suggesting *flamenco* is not suppressing *R1*. On average full length TEs constitute 20% of *flamenco* in *D. simulans*.

In the duplicate of *flamenco* in *D. simulans* there are 30 full length TEs present in any one of the five complete *flamenco* duplicate assemblies. However, none of them are multicopy in antisense. However, they are multicopy relative to the original copy of *flamenco*. *gypsy-3*, *Argyoneta*, *Nomad-1*, *Chimpo*, *gypsy-53A*, *R1*, and *INE1* are all multicopy with respect to the original *flamenco* within a given genome. In the duplicate of *flamenco* full length TEs occupy an average of 17% of the locus. *MD251* is an exception which weights the average, with 28% of the locus, while between 10 and 15% is found for the remaining copies. Thus *D. simulans* and *D. mauritiana* overall do not meet the expectation that *flamenco* will contain a single insertion of any given TE.

<u>How many TEs have full length and fragmented insertions?</u>

Assuming that TE insertions are degraded over time, e.g. due to a deletion bias in *Drosophila*, then full length elements are usually younger insertions than fragmented insertions (69). If a full length element is inserted in *flamenco* and there are fragments in the antisense orientation elsewhere in *flamenco* this indicates that *flamenco* did not successfully suppress the transposition of this element.

In *D. melanogaster* two elements have fragments in antisense and a full length TE – *Doc* and *Stalker-2*. *D. sechellia* has 9 elements that are present as a full length TE and a fragment in antisense (including *412*, *GTWIN*, *mdg-1*, and *nomad*) and 6 that are multicopy that are due to a

solo LTR (including *blood*, *297*, and *Stalker-4*). *D. mauritiana* has 21 elements that are present in full length and a fragment in antisense (including *blood*, *412*, *gypsy-10-13*, and *R1*), and four elements that are multicopy due to a solo LTR (*mdg-1*, *Idefix*, and *gypsy-7,10).*

In *D. simulans*, TEs that fit this criteria in *flamenco* include *gypsy-2*, *gypsy-3*, *gypsy-4*, *gypsy-5*, *Chimpo*, *412*, *INE1*, *R1*, *Tirant*, and *Zam*. *297* and *Nomad-1* are present in full length copies but only multi-copy in the context of solo LTRs. In the duplicate of *flamenco* in *D. simulans* this includes *gypsy-2*, *gypsy-3*, *gypsy-5*, *297*, *Stalker-4*, and *R1*. For example in *NS40* there are 7 full length copies of *R1* in the sense orientation that likely duplicated in place, as well as 12 partial copies in the antisense orientation. In the *simulans* clade either fragments of TEs are not sufficient to suppress transposable elements (i.e. fragments are no longer perfect matches to the TE) or some elements are able to transpose despite the hosts efforts to suppress them.

Is flamenco a trap for TEs entering through horizontal transfer?

High sequence similarity between TEs in different species suggests horizontal transfer (70). However, because sequence similarity can also exist due to vertical transmission we will use sequence similarity between R1 elements (inserted at rDNA genes) as a baseline for differentiating horizontal versus vertical transfer. There has never been any evidence found for horizontal transfer of *R1* and it is thought to evolve at the same rate as nuclear genes in the *melanogaster* subgroup (16, 27). Of the full length elements present in any genome at *flamenco* 62% of them appear to have originated from horizontal transfer. This is similar to previous estimates for *D. melanogaster* in other studies (16). Transfer appears to have occurred primarily between *D. melanogaster*, *D. sechellia*, and *D. willistoni* (agnostic to the direction of transfer). This includes some known horizontal transfer events such as *Chimpo* and *Chouto* (71), and others which have not been recorded such as *gypys-29* (*D. willistoni*) and the *Max-element* (*D.*

sechellia) (Supplemental File 3). The duplicate of *flamenco* is similar, with 53% of full length TEs involved in horizontal transfer. They are many of the same TEs, with a 46% overlap, thus *flamenco* and its duplicate are trapping many of the same TEs. Both *flamenco* and the duplicate the region appears to serve as a trap for TEs originating from horizontal transfer.

In *D. melanogaster* 85% of full length TEs appear to have been involved in horizontal transfer, primarily with *D. yakuba* and *D. sechellia* (16). In D. sechellia 53% of full length TEs been involved in horizontal transfer, including some known to have moved by horizontal transfer such as *GTWIN* (*D. melanogaster*/*D. erecta*) (71). *D. mauritiana* has 68% of its full length TEs showing a closer relationship than expected by vertical descent with TEs from *D. sechellia*, *D. melanogaster*, and *D. simulans*. The hypothesis that *flamenco* serves as a trap for TEs entering the population through horizontal transfer holds throughout the *simulans* clade.

flamenco is expressed in the testis and the maternal fraction

Canonically, *flamenco* is expressed in the somatic follicular cells of the ovary and not in the germline, and also does not produce a ping pong signal (23). It was not thought to be present in the maternal fraction of piRNAs or other tissues. However, that appears to be variable in different species (Figure 4). We examined single mapping reads in the *flamenco* region from testes and embryos (maternal fraction) in *D. simulans*, *D. mauritiana*, *D. sechellia*, and *D. melanogaster*. In *D. simulans* and *D. mauritiana flamenco* is expressed bidirectionally in the maternal fraction and the testis, including ping pong signals on both strands (Figure 4). In *D. sechellia*, there is no expression of *flamenco* in either of these tissues. Using weighted mapping in the maternal fraction 63% (*D. mauritiana*) – 36% (*D. simulans*) of the ping pong signatures on the X with a z-score of at least 0.9 are located within *flamenco* (Figure 4). Similar patterns are seen in the testis, with 50% (*D. mauritiana*) to 40% (*D. simulans*) of ping pong signals on the X

with a z-score of at least 0.9 being located within *flamenco*. In *D. melanogaster*, there is uni-strand expression in the maternal fraction, but it is limited to the region close to the promotor. In *D. melanogaster* no ping pong signals have a z-score of 0.9, however of those with a z-score of at least 0.8 only 2.3% of those on the X are potentially located within *flamenco*, suggesting that the role of *flamenco* in these tissues has evolved between species.

In the duplicate of *flamenco* in the maternal fraction 18% of the ping pong signals on the X are within the *flamenco* duplicate, while in the testis this is 13%. While overall expression of unique piRNAs is lower, proportionally the locus appears to behave the same in each tissue as the original copy of *flamenco*. In addition, *flamenco* in these species has been colonized by full length TEs thought to be germline TEs such as *blood*, *burdock*, *mdg-3*, *Transpac*, and *Bel* (72, 73). *blood* is also present in *D. melanogaster* in a full length copy while there is no evidence of germline activity for *flamenco* in *D. melanogaster*, though no other putative germline TEs.

Silencing of transposable elements

Canonically, once a TE is present in either a germline or somatic cluster it will be silenced. For example, *ZAM* is controlled by *flamenco* and prevents it from transposing from the somatic support cells of the ovary to the germline (74). However, if *ZAM* is deleted from *flamenco*, it will rapidly insert into a germline cluster and recommence suppression (72). Most TEs do not produce full length transcripts in the genotypes for which we have long read data (*SZ45*, *SZ129*, *SZ232*, *SZ244*, *LNP-15-062*). Exceptions are *P*, *R2*, *mariner2*, *Bari1*, *Transib2*, *INE1*, *Copia*, *Blastopia*, *Burdock*, *Gyspy*, *Idefix*, and *diver*. Many of these are expressed despite full length insertions in *flamenco*, such as *Blastopia*, *Burdock*, and *gypsy*. *R2* is the most highly expressed, and is typically only targeted by the ping pong cycle in a limited region of its sequence. Due to

the limited number of possible insertion sites it may be that expression of this TE is not detrimental to the host (27).

We propose that silencing is not absolute, and that some copies of TEs can escape silencing by the piRNA pathway. Differences in the expression and insertion locations of *Blastopia* exemplify this assertion. *Blastopia* is only present within the *flamenco* locus or its duplicate in *LNP-15-062*. However, it is present in multiple full length copies in every genome. *LNP-15-062* and *SZ232* produce full length *Blastopia* transcripts, while *SZ45* does not (Figure 5). All copies of *Blastopia* in each of these genomes show evidence of the ping pong cycle (Supplementary File X). In addition, abundant piRNAs are produced against *Blastopia* in every genotype (Supplementary File X). However, all long RNA reads arising from *Blastopia* can be traced to a single insertion shared between *LNP-15-062* and *SZ232*, but lacking in *SZ45*. The region is present and assembled in *SZ45*, just lacking a *Blastopia* insertion. The region containing this *Blastopia* insertion in *LNP-15-062* is transposon rich, but there is no evidence of general transcription from the area. There is no transcription from any of the other full length copies of *Blastopia* in any of the three genomes. This does not guarantee that *Blastopia* is able to transpose, but it is not silenced. Thus for unknown reasons some copies of TEs seem able to escape silencing despite robust evidence that they should be silenced.

Discussion

The piRNA pathway is the organisms primary mechanism of transposon suppression. While the piRNA pathway is conserved, the regions of the genome that produce piRNA are labile, particularly in double stranded germline piRNA clusters (9). The necessity of any single cluster for TE suppression in the germline piRNA pathway is unclear, but the clusters are likely redundant (9, 18). However, *flamenco* is thought to be the master regulator of the somatic

support cells of the ovary, preventing *gypsy* elements from hopping into germline cells (16, 20, 22, 23, 75, 76). It is not redundant to other clusters, and insertion of a single element into *flamenco* in *D. melanogaster* is sufficient to initiate silencing. Here we show that the function of *flamenco* appears to have diversified in the *D. simulans* clade, acting in at least some tissues as a germline piRNA cluster.

Dual stranded expression of flamenco

In this work we showed that piRNAs of the *flamenco* locus in *D. simulans* and *D. mauritiana* are maternally deposited, are aligning to both strands and show ping-pong signatures. This is in contrast to *D. melanogaster* where *flamenco* acts as a uni-strand cluster in the soma (3), our data thus suggest that the *flamenco* locus in *D. simulans* and *D. mauritiana* acts as a dual-strand cluster in the germline. In *D. sechellia* the attributes of *flamenco* uncovered in *D. melanogaster* appear to be conserved – no expression in the maternal fraction and the testis and no ping pong signals. Given that *flamenco* is likely a somatic uni-strand cluster in *D. erecta* we speculate that the conversion into a germline cluster happened in the *simulans* clade (3). The fact that it remains a unistrand cluster in *D. sechellia* suggests two independent conversion events in *D. simulans* and *D. mauritiana*. Such a conversion of a cluster between the somatic and the germline piRNA pathway is not implausible; for example a single insertion of a reporter construct triggered the conversion of the uni-stranded cluster *20A* in *D. melanogaster* into a dual-strand cluster (77).

The role of *flamenco* in *D. simulans* and *D. mauritiana* as the master regulator of piRNA in somatic support cells may still be true – the promotor region of the *flamenco* cluster is conserved between species and between copies of *flamenco* within species. The overall structure of flamenco, where the majority of the locus is comprised of antisense oriented LTRs, is still

conserved. This suggests that in at least some contexts the cluster is still serving as a unistrand cluster transcribed from a traditional RNA Pol II site (13). However it has acquired additional roles, producing dual strand piRNA and ping pong signals, in these two species, in at least the germline and testis. However, in *D. simulans*, the majority of these reverse stranded piRNAs are emerging from the *R1* insertions within *flamenco*. There is no evidence at present that *R1* has undergone an expansion in function in *D. simulans*, thus it is unclear what, if any, functional impact the reverse stranded piRNAs have at the *flamenco* locus.

Duplication of flamenco in D. simulans

In *D. simulans* flamenco is present in 2-3 copies, and this duplication is present in all sequenced *D. simulans* lines. The *dip1* gene and putative *flamenco* promoter flanking the duplication also has a high similarity in all sequenced lines (Fig. 2B). This raises the possibility that the duplication of *flamenco* in *D. simulans* was positively selected. Such a duplication may be beneficial as it increases the ability of an organism to rapidly silence TEs. Individuals with large piRNA clusters (or duplicated ones) will accumulate fewer deleterious TE insertions than individuals with small clusters (or non-duplicated ones), and duplicated clusters may therefore confer a selective advantage (78).

Rapid evolution of piRNA clusters

A previous work showed that dual- and uni-strand clusters are rapidly evolving in *Drosophila* (17). In agreement with this work we also found that the *flamenco*-locus is rapidly evolving between and within species (Fig. 1C, 3B). A major open question remains whether this rapid turnover is driven by selection (positive or negative) or an outcome of neutral processes (eg. high TE activity or insertion bias of TEs). These rapid evolutionary changes at the *flamenco* locus, a piRNA master locus, suggest that there is a constant turnover in patterns of piRNA biogenesis

that potentially leads to changes in the level of transposition control between individuals in a population.

Escaping transposon silencing

We have shown here that not all transposons are completely silenced despite all of the expected signals of somatic or germline repression. This does not necessarily mean they are able to successfully transpose, but production of a full length transcript is the first necessary step by which to do so. For example, they could be secondarily silenced by splicing as has been shown in the *P*-element (79). They are also not all recent invaders, for example *INE1*, which likely invaded *D. melanogaster* 2-4 million years ago, is the most abundant transposable elements in *Drosophila* (80, 81). Thus silencing by the piRNA pathway may be necessary for TE suppression, but not always entirely sufficient.

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Competing interests
We declare that we have no competing interests.

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Authors' contributions
S.S. conceived the study, performed bioinformatics and drafted portions of the manuscript. FW and RK performed bioinformatics and drafted portions of the manuscript. JV contributed data and bioinformatic analysis. EL drafted portions of the manuscript and provided data.

Availability of data and materials
All data has been made available in the following repositories:

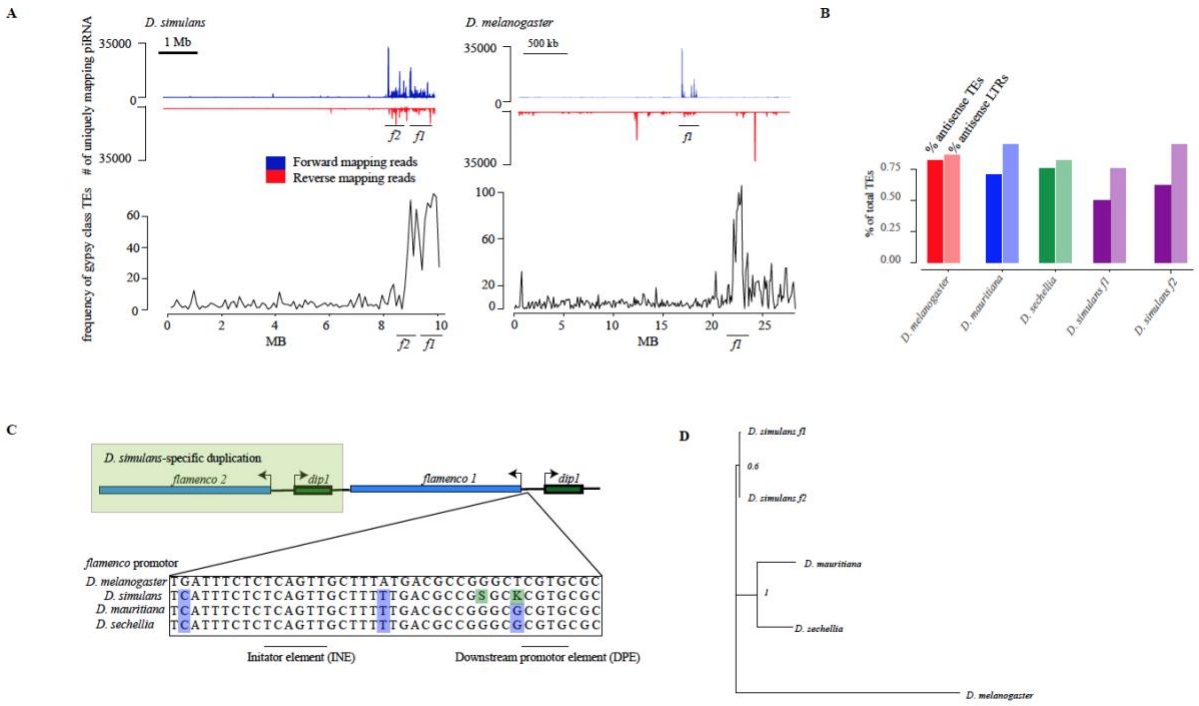

Figure 1. A) Unique piRNA from the ovary and *gypsy* enrichment around *flamenco* and its duplicate in *D. simulans* and *D. melanogaster*. piRNA mapping to the entire contig that contains *flamenco* is shown for both species. The top of the panel shows piRNA mapping to f*lamenco* and is split by antisense (blue) and sense (red) piRNA . The bottom panel shows the frequency of *gypsy*-type transposon annotations across the contig containing *flamenco*, counted in 100 kb windows. There is a clear enrichment of *gypsy* in the area of *flamenco* and, in *D. simulans*, its duplicate compared to the rest of the contig. B) The percent of TEs in *flamenco* in each species which are in the antisense orientation (first bar) and the percent of TEs in the antisense orientation that are also LTR class elements (second bar). C) The duplication of *flamenco* in the *D. simulans*. Both copies are flanked by the *dip1* gene and copies of the putative *flamenco* promotor. Polymorphisms within the promotor that are shared within the *simulans* clade are shown in blue, *D. simulans* specific polymorphisms are shown in green. The region around the

promotor is very conserved across species. D) A phylogenetic tree of the *dip1* and *flamenco* enhancer region for *D. melanogaster* and the *simulans* clade. This region is conserved and alignable between all species. The tree was generated with Mr. Bayes (62).

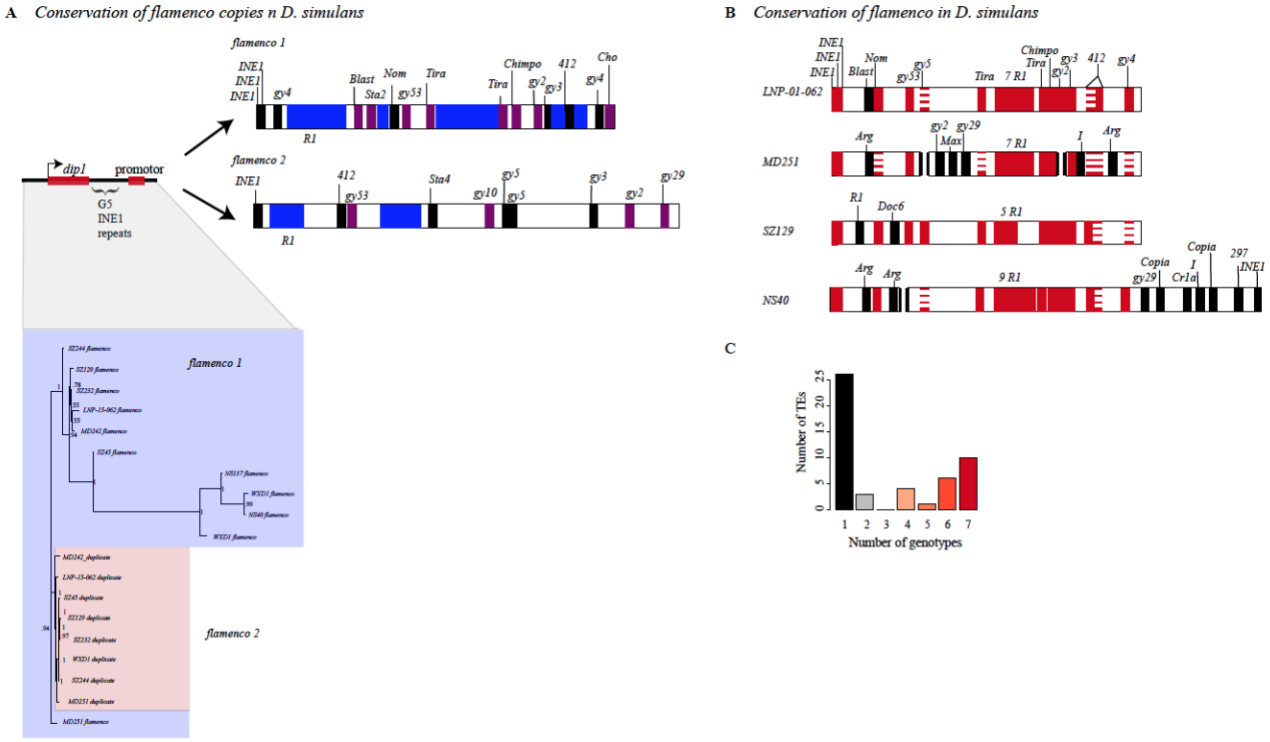

Figure 2. A) Divergence between copies of flamenco. Proximal is a phylogenetic tree of *dip1* and the *flamenco* promotor region from each genome. In between *dip1* and the promotor are a series of *G5/INE1* repeats that are found in every genome. Overall this region is fairly conserved, with the duplicate copies all grouping together with short branch lengths (shown in pink). The original copy of *flamenco* is more diverse with some outliers (shown in light blue) but there is good

branch support for all the deep branches of the tree. Distal is a representation of *flamenco* and its duplicate. R1 repeat regions are shown in blue. Full length transposable elements are labeled. There is no synteny conservation between *flamenco* and its duplicate. B) Divergence of flamenco within *D. simulans*. Labeled TEs correspond to elements which are present in a full length copy in at least one genome. If they are shared between genomes they are labeled in red, if they are unique they are black. If they are full length in one genome and degraded in other genomes they are represented by stacked dashes. If they are present in the majority of genomes but missing in one, it is represented as a missing that TE, which is agnostic to whether it is a deletion or the element was never present. C. The proportion of insertions that are shared by one through seven genotypes.

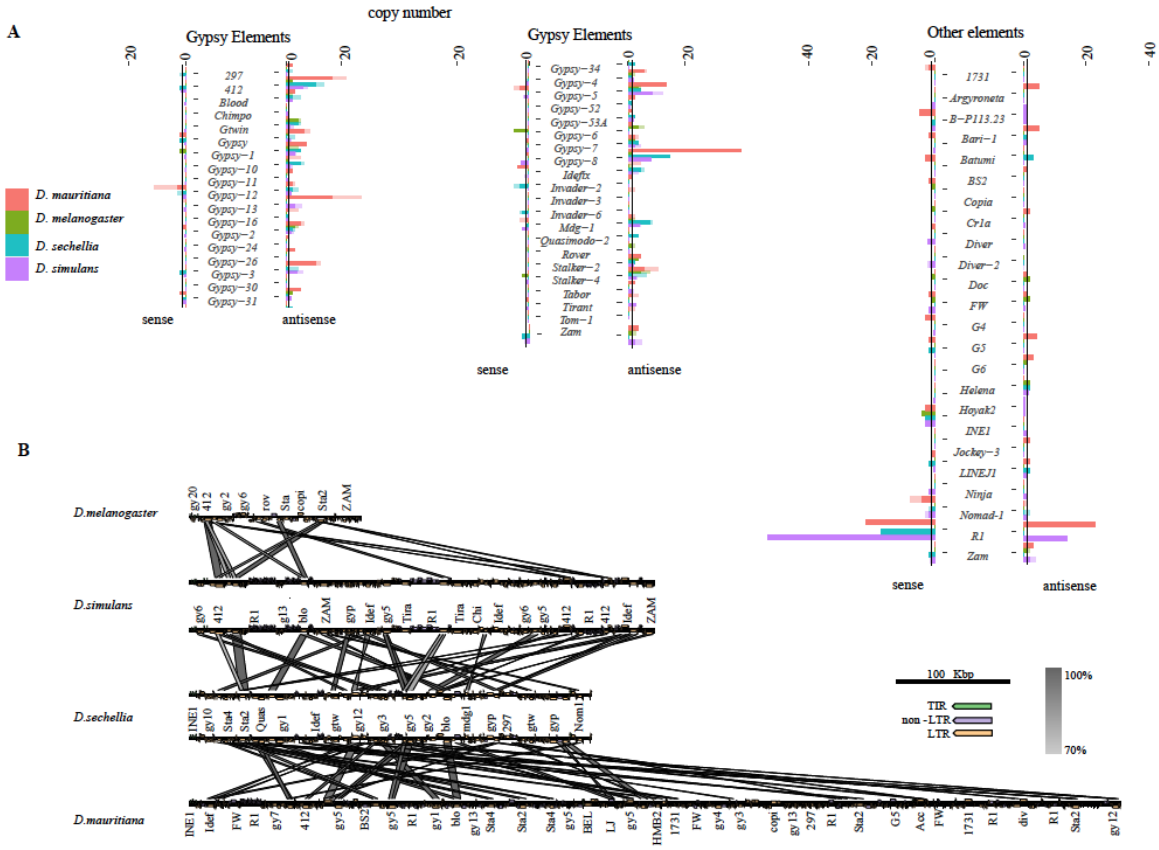

Figure 3) A. Copy number of transposable elements at *flamenco*. Solo LTRs are indicated by in a lighter shade at the top of the bar. The black line on each bar graph indicates a copy number of one. Values for *D. simulans* are the average for all genotypes with a complete *flamenco* assembly. Note that in *D. melanogaster* (green) most TEs have a low copy number. The expansion of *R1* elements in the *simulans* clade is clearly indicated on the right hand panel. Many elements within *flamenco* are multicopy in the *simulans* clade. While some of this is likely due to local duplications it is clearly a different pattern than *D. melanogaster*. Enrichment of LTR elements on the antisense strand is clear for all species. **B.** Alignment of *flamenco* in *D. melanogaster*, *D. simulans*, *D. sechellia*, and *D. mauritiana*. There is no conserved synteny

between species but there are clearly shared TEs, particularly within the *simulans* clade. The expansion of *D. mauritiana* compared to the other species is apparent.

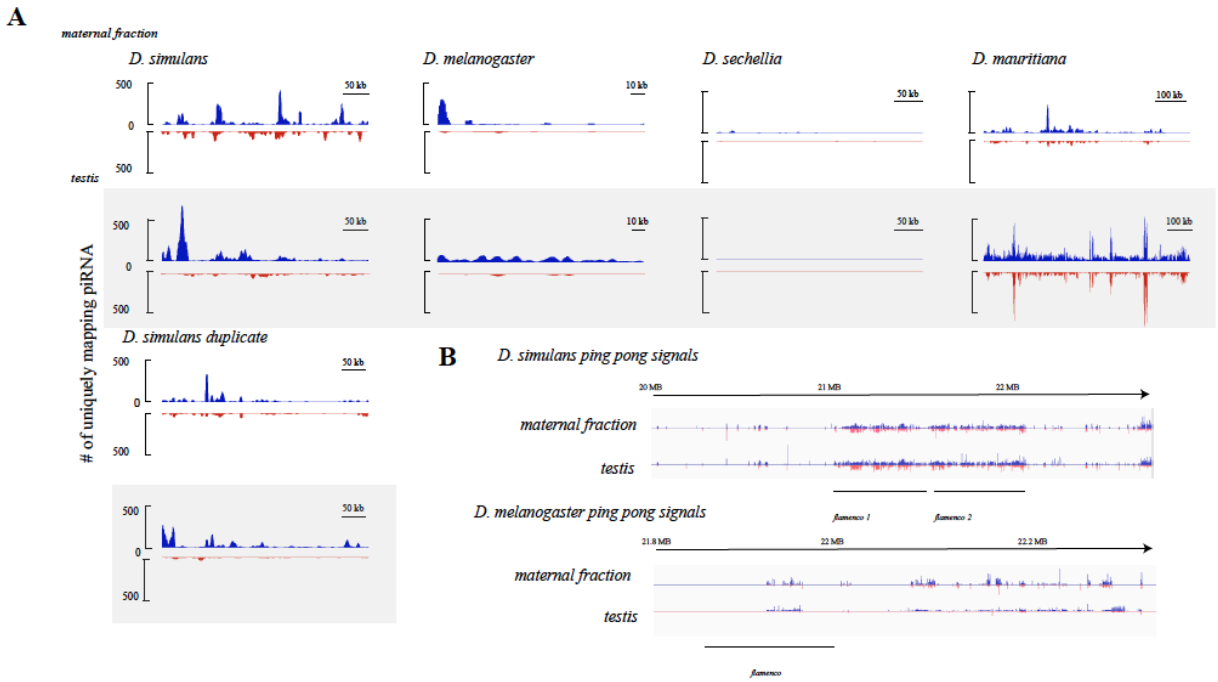

Figure 4) A. Expression of single mapping piRNAs in the maternal fraction and testis (gray) of *D. melanogaster* and the *simulans* clade. Antisense mapping reads are shown in blue, sense in red. Libraries are RPM normalized and scaled across library type. *D. sechellia* has no expression of *flamenco* in the maternal fraction or the testis. *D. melanogaster* has low expression in the maternal fraction and very little ping pong activity. *D. simulans* and *D. mauritiana* show dual stranded expression in the testis and maternal fraction. **B.** *D. simulans* and *D. mauritiana* (Supplementary File) have ping pong singles at *flamenco* in the testis and maternal fraction, while *D. melanogaster* does not.

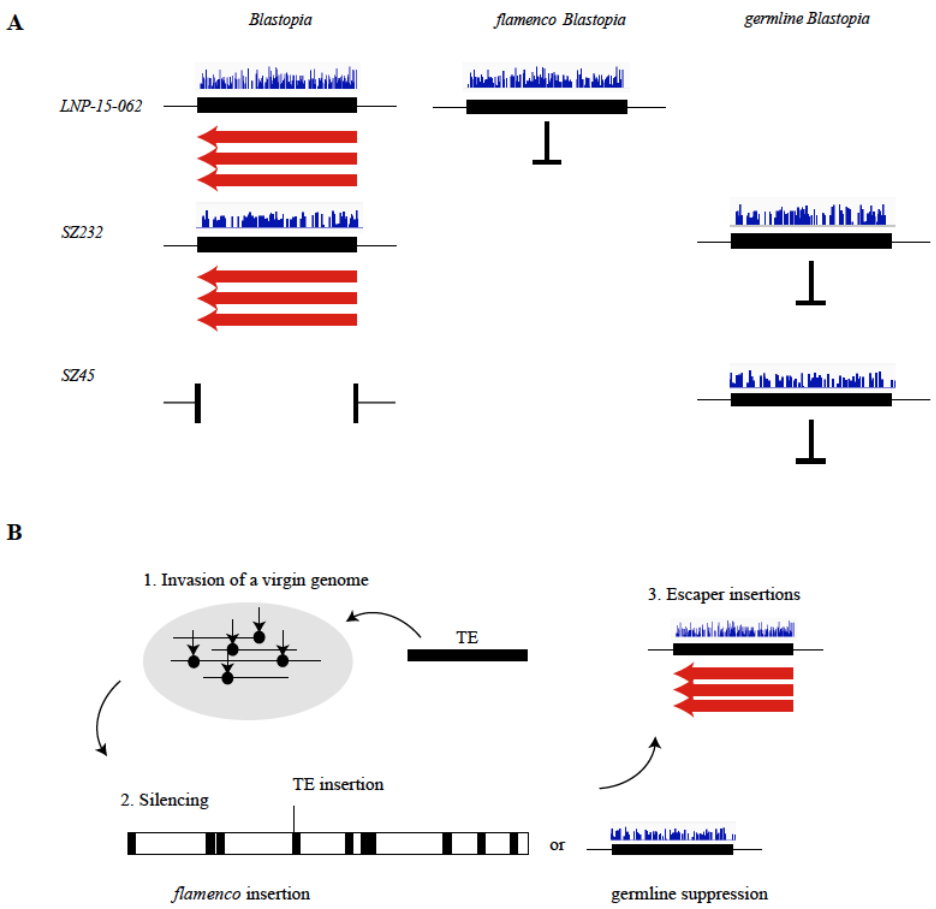

Figure 5) A. A schematic of the expression of *Blastopia* in three genotypes of *D. simulans*. Pingpong singals are shown in blue above the TEs, and transcripts are shown in red. In two genotypes *Blastopia* expresses full length transcripts from a single insertion, despite the presence of silencing from either *flamenco* or germline insertions of *Blastopia*. The third genotype lacks this particular insertion (the region is assembled) and does not express any transcripts from *Blastopia*. This raises the possibility that despite repression some TEs may escape silencing at particular insertions or in particular genotype/insertion combinations. **B.** A possible schematic for the life cycle of a TE invasion. TEs invade a virgin genome, either become silenced by the somatic or germline pathway, but are occasionally able to escape silencing. This must be comfirmed, as expression of a transcript is not the same as transposition, only the first step.

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