Rapid evolutionary diversification of the *flamenco* locus across simulans clade *Drosophila* species

Sarah Signor[1*], Jeffrey Vedanayagam[2], Bernard Y. Kim[3], Filip Wierzbicki[4,5], Robert Kofler[4], and Eric C. Lai[2]

*Corresponding author: sarah.signor@ndsu.edu

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

[2]Developmental Biology Program, Sloan-Kettering Institute, 430 East 67th St, ROC-10, New York, NY 10065, USA

[3]Department of Biology, Stanford University, Stanford, CA, USA

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

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

Abstract

Effective suppression of transposable elements (TEs) is paramount to maintain genomic integrity and organismal fitness. In *D. melanogaster*, *flamenco* is a master suppressor of TEs, preventing their movement from somatic ovarian support cells to the germline. It is transcribed by Pol II as a long (100s of kb), single-stranded, primary transcript, that is metabolized into Piwi-interacting RNAs (piRNAs) that target active TEs via antisense complementarity. *flamenco* is thought to operate as a trap, owing to its high content of recent horizontally transferred TEs that are enriched in antisense orientation. Using newly-generated long read genome data, which is critical for accurate assembly of repetitive sequences, we find that *flamenco* has undergone radical transformations in sequence content and even copy number across *simulans* clade Drosophilid species. *D. simulans flamenco* has duplicated and diverged, and neither copy exhibits synteny with *D. melanogaster* beyond the core promoter. Moreover, *flamenco* organization is highly variable across *D. simulans* individuals. Next, we find that *D. simulans* and *D. mauritiana flamenco* display signatures of a dual-stranded cluster, with ping-pong signals in the testis and/or embryo. This is accompanied by increased copy numbers of germline TEs, consistent with these regions operating as functional dual stranded clusters. Overall, the physical and functional diversity of *flamenco* orthologs is testament to the extremely dynamic consequences of TE arms races on genome organization, not only amongst highly related species, but even amongst individuals.

Introduction

Drosophila gonads exemplify two important fronts in the conflict between transposable elements (TEs) and the host – the germline (which directly generates gametes), and somatic support cells (from which TEs can invade the germline) (1, 2). The strategies by which TEs are suppressed in these settings are distinct (3), but share their utilization of piwi-interacting RNAs (piRNAs). These are ~24-32 nt RNAs that are bound by the PIWI subclass of Argonaute proteins, and guide them and associated cofactors to targets for transcriptional and/or post-transcriptional silencing (4–7).

Mature piRNAs are processed from non-coding piRNA cluster transcripts, which derive from genomic regions that are densely populated with TE sequences (7–9). However, the mechanisms of piRNA biogenesis differ between gonadal cell types. In the germline, piRNA clusters are transcribed from both DNA strands through non-canonical Pol II activity (6, 10–12), which is initiated by chromatin marks rather than specific core promoter motifs. Moreover, co-transcriptional processes such as splicing and polyadenylation are suppressed within dual strand piRNA clusters (13, 14). On the other hand, in ovarian somatic support cells, piRNA clusters are transcribed from a typical promoter as a single stranded transcript, which can be alternatively spliced as with protein-coding mRNAs (15–18). These rules derive in large part from the study of model piRNA clusters (i.e. the germline *42AB* and somatic *flamenco* piRNA clusters). For both types, their capacity to repress invading TEs is thought to result from random integration of new transposons into the cluster (19). As such, piRNA clusters are adaptive loci that play central roles in the conflict between hosts and TEs.

The location and activity of germline piRNA clusters are stochastic and evolutionarily dynamic, as there are many copies of TE families in different locations that may produce

piRNAs (9, 20). By contrast, somatic piRNA clusters are not redundant and a single insertion of a TE into a somatic piRNA cluster should be sufficient to prevent that TE from further transposition (1, 18). Thus, *flamenco* should contain only one copy per TE family (18), which is true in the *flamenco* locus of *D. melanogaster* (18). *flamenco* is also the only piRNA cluster which produces a phenotypic effect when altered, as germline clusters can be deleted with no consequences (9).

flamenco has been a favored model for understanding the piRNA pathway since the discovery of piRNA mediated silencing of transposable elements (6). *flamenco* spans >180 kb of repetitive sequences located in *β*-heterochromatin of the X chromosome (21). Of note, *flamenco* was initially identified, prior to the formal recognition of piRNAs, via transposon insertions that de-repress *gypsy*, *ZAM*, and *Idefix* class elements (21–25). These mutant alleles disrupt the *flamenco* promoter, and consequently abrogate transcription and piRNA production from this locus. By contrast, the recent deletion of multiple model germline piRNA clusters, which eliminate the biogenesis of a bulk of cognate piRNAs, did not de-repress their cognate TEs (9). Thus, the analysis of *flamenco* evolution is presumably more consequential for TE dynamics. Analysis of *flamenco* in various strains of *D. melanogaster* supports that this locus traps horizontally derived TEs to achieve silencing of newly invaded TEs (18). The *flamenco* locus exhibits synteny across the *D. melanogaster* sub-group (26); however, the sequence composition of *flamenco* outside *D. melanogaster* has not been well-characterized (27).

In this study, we compare the *flamenco* locus across 10 strains of simulans-clade species, namely *D. simulans*, *D. mauritiana*, and *D. sechellia*. Analysis of piRNAs from ovaries of five genotypes of *D. simulans* found that *flamenco* is duplicated in *D. simulans*. This duplication is old enough that there is no sequence synteny across copies, even though their core promoter

93 regions and the adjacent *dip1* gene duplications are conserved. *flamenco* has also been colonized
94 by abundant (>40) copies of *R1*, a TE that was thought to insert only at ribosomal genes, and to
95 evolve at the same rate as nuclear genes (28). Furthermore, between different genotypes, up to
96 63% of TE insertions are not shared within any given copy of *flamenco*. Despite this, several full
97 length TEs are shared between all genotypes in a similar sequence context. This incredible
98 diversity at the *flamenco* locus, even within a single species, suggests there may be considerable
99 variation in its ability to suppress transposable elements across individuals.

100 Cross-species comparisons further indicate that functions of *flamenco* have diversified.
101 Data from *D. sechellia* and *D. melanogaster* conform with the current understanding of *flamenco*
102 as a uni-strand cluster. However, we find evidence that *D. simulans* and *D. mauritiana flamenco*
103 can act as a dual strand cluster in testis (*D. mauritiana*) and embryos (*D. mauritiana* and *D.*
104 *simulans*), yielding piRNAs from both strands with a ping pong signal. Overall, we infer that the
105 rapid evolution of *flamenco* alleles across individuals and species reflects highly adaptive
106 functions and dynamic biogenesis capacities.

107 **Materials and Methods**

108 *Fly strains*

109 The four *D. simulans* lines *SZ232*, *SZ45*, *SZ244*, and *SZ129* were collected in California from the
110 Zuma Organic Orchard in Los Angeles, CA on two consecutive weekends of February 2012 (29–
111 33). *LNP-15-062* was collected in Zambia at the Luwangwa National Park by D. Matute and
112 provided to us by J. Saltz (J. Saltz pers. comm., (34, 35)). *MD251*, *MD242*, *NS137*, and *NS40*
113 were collected in Madagascar and Kenya (respectively) and are described in (36). The *D.*
114 *simulans* strain *wxD*^{1} was originally collected by M. Green, likely in California, but its

115 provenance has been lost (pers. comm. Jerry Coyne). *D. mauritiana (w12)* and *D. sechellia*
116 *(Rob3c/Tucson 14021-0248.25)* are described in (37).

117 *Long read DNA sequencing and assembly*

118 *MD242*, four SZ lines and *LNP-15-062* were sequenced on a MinION platform at North Dakota
119 State University (Oxford Nanopore Technologies (ONT), Oxford, GB), with base-calling using
120 guppy (v4.4.2). *MD242*, the four SZ lines, and *LNP-15-062* were assembled with Canu (v2.1)
121 (38) and two rounds of polishing with Racon (v1.4.3) (39). The CA strains were additionally
122 polished with short reads using Pilon (v1.23) (40)(SRR3585779, SRR3585440, SRR3585480,
123 SRR3585391) (29). The first wxD^{1-1} assembly is described here (41). *MD251*, *NS137*, *NS40* and
124 wxD^{1-2} were sequenced on a MinION platform at Stanford University. They were assembled with
125 Flye (42), and polished with a round of Medaka followed by a round of pilon (40). Following
126 this contaminants were removed with blobtools (https://zenodo.org/record/845347, (43)), soft
127 masked with RepeatModeler and Repeatmasker (44, 45), then aligned to the wxD^{1} as a reference
128 with Progressive Cactus (46). The assemblies were finished with reference based scaffolding
129 using Ragout (47). *D. mauritiana* and *D. sechellia* were sequenced with PacBio and assembled
130 with FALCON using default parameters
131 (https://github.com/PacificBiosciences/FALCON)(37).The *D. melanogaster* assembly is
132 described here (48). A summary of the assembly statistics is available in Supplementary Table 1.
133 The quality of cluster assembly was evaluated using the coverage and soft clip quality as
134 described in (20, 49) (Supplementary File 1).

135 *Short read sequencing and mapping*

136 Short read sequencing was performed by Beijing Genomics Institute on approximately 50 dissected
137 ovaries from adult female flies (*SZ45*, *SZ129*, *SZ232*, *SZ244*, *LNP-15-062*). Short read libraries from 0-2
138 hour embryos were prepared from *D. melanogaster*, wxD^{1-2}, *D. sechellia*, and *D. mauritiana* (SRAXXX)

(50). Small RNA from testis is described in (51, 52). *D. melanogaster* OSC small RNA libraries were downloaded from the SRA (SRR11999160). Libraries were filtered for adapter contamination and short reads between 23-29 bp were retained for mapping with fastp (53). 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) (54, 55). The resulting bam files were processed using samtools (56). 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 (57).

Defining and annotating piRNA clusters

piRNA clusters were initially defined using proTRAC (58). 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) (41, 44). The position of *flamenco* was also evaluated based off of the position of the putative promoter, the *dip1* gene, and the enrichment of *gypsy* elements (15). Fragmented annotations were merged to form TE copies with onecodetofindthemall (59). Fragmented annotations were also manually curated within *flamenco*, particularly because TEs not present in the reference library often have their LTRs and internal sequences classified as different elements.

Aligning the flamenco promoter 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). Markov chain monte carlo iterations 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 10^{th} 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.

Annotating shared and unique TE insertions

To align the TE annotations of homologous piRNA clusters, we first extracted the sequences of the clusters and annotated TEs in these sequences using RepeatMasker (open-4.0.7) with a custom TE library and the parameters: -s (sensitive search), -nolow (disable masking of low complexity sequences), and -no_is (skip check for bacterial IS) (37, 65, 66). Finally, we aligned the resulting repeat annotations with Manna using the parameters -gap 0.09 (gap penalty), -mm 0.1 (mismatch penalty) -match 0.2 (match score) (20, 37). Manna can be used for aligning the annotations of the transposable elements by relying on synteny to determine insertion homology. Alignments were manually checked for inconsistencies arising from assignment to similar TEs

(i.e. *gypsy-3* versus *gypsy-5*) . TEs were considered to be full length if they were present in at least 70% of their reference length and contained internal sequence as well as two LTRs if applicable.

Results

flamenco in the D. simulans clade

We identified *D. simulans flamenco* from several lines of evidence: piRNA cluster calls from proTRAC, its location adjacent to divergently transcribed *dip1*, the existence of conserved core *flamenco* promoter sequences, and enrichment of *gypsy* elements (Figure 1 & 2); Supplementary Table 2). The *flamenco* locus is at least 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 at least 840 kb (Supplementary Table 2). This is a large expansion, and it is possible that the entire region does not act as a region controlling somatic TEs. However, evidence that is does include uniquely mapping piRNAs that are found throughout the region and *gypsy* enrichment consistent with a *flamenco*-like locus (Supplementary Figure 1). There are no protein coding genes within 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* promoter and surrounding region, including the *dip1* gene, are alignable and conserved (Figure 1D).

Structure of the flamenco locus

D. melanogaster flamenco bears a characteristic structure, in which the majority of TEs are *gypsy*-class elements in the antisense orientation (79% antisense orientation, 85% of which are *gypsy* elements) (Figure 2C; Supplementary Table 3). 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 2C). In *D. mauritiana* *flamenco* is 71% antisense, and of those antisense elements it is 85% 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 2C).

flamenco is duplicated in D. simulans

In *D. simulans*, we unexpectedly observed that *flamenco* is duplicated on the X chromosome; the duplication was confirmed with PCR and a restriction digest (Figure 1, Supplementary File 2). These duplications are associated with a conserved copy of the putative *flamenco* enhancer as well as copies of the *dip1* gene located proximal to *flamenco* in *D. melanogaster* (Figure 1, 3A). While it is unclear which copy is orthologous to *D. melanogaster flamenco*, all *D. simulans* lines bear one copy that aligns across genotypes. We refer to this copy as *D. simulans flamenco*, and the other copies as duplicates. Otherwise, *flamenco* duplicates do not align with one another and lack synteny amongst their resident TEs. Possible evolutionary scenarios are that the *flamenco* duplication occurred early in the *simulans* lineage, that the clusters evolved very rapidly, or that the duplication encompassed only the promoter region and

was subsequently colonized by TEs (Figure 1A, 3A).

The *flamenco* duplicate is absent in the *D. simulans* reference assembly, w^{501} (GCA_000754195.3), 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, 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 3C).

R1 LINE elements at the flamenco locus

R1 elements are well-known to insert into rDNA genes, are transmitted vertically, and evolve similarly to the genome background rate (28). They have also been found outside of rDNA genes, but only as fragments. *R1* elements are absent from *flamenco* in the *D. simulans* reference assembly, aside from a single fragmented *R1-1* element (w^{501}). However, as mentioned, *R1* elements are abundant within *flamenco* loci in the *simulans* clade. Outside of *flamenco*, *R1* elements in *D. simulans* are distributed according to expectation, with full length elements occurring only within rDNA (Supplementary File 3). Within *flamenco*, most copies of *R1* occur as tandem duplicates, creating large islands of fragmented *R1* copies (Figure 3A). They are on average 3.7% diverged from the reference R1 from *D. simulans*. Across individual *D. simulans* genomes, ~99 kb of *flamenco* loci consists of *R1* elements, i.e. 26% of their average total length. *SZ45, LNP-15-062, NS40, MD251,* and *MD242* contain 4-7 full length copies of *R1* in the sense orientation, even though all but *SZ45* bear fragmented *R1* copies on the antisense strand. (The *SZ45 flamenco* assembly is incomplete). As the antisense *R1* copies are expected to suppress *R1* transposition, *flamenco* may not suppress these elements effectively. Alternatively, it is possible

that *D. simulans flamenco* is still mostly active in the soma, while *R1* is active in the germline, and thus escapes host control by *flamenco*.

In *D. mauritiana*, *flamenco* harbors abundant fragments or copies of *R1* (19 on the reverse strand and 20 on the forward strand), and only one large island of *R1* elements. In total, *D. mauritiana* contains 84 kb of *R1* sequence within *flamenco*. In *D. mauritiana* there are 8 full length copies of *R1* at the *flamenco* locus, 7 in antisense, which are not obviously due to a segmental or local duplication. Finally, we find that *D. sechellia flamenco* lacks full length copies of *R1*, and it contains only 18 KB of *R1* sequence (16 fragments on the reverse strand). Yet, all the copies are on the sense strand, which would not produce fragments that can suppress R1 TEs. Essentially the antisense copies of *R1* in *D. mauritiana* should be suppressing the TE, but we see multiple full length antisense insertions, and *D. sechellia* has no antisense copies, but we see no evidence for recent *R1* insertions. From this it would appear that whatever is controlling the transposition of *R1* lies outside of *flamenco*.

The presence of long sense-strand *R1* elements within *flamenco* is a departure from expectation (18, 28). 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. Furthermore, the suppression of *R1* transposition does not appear to be controlled by *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. The only exception is the ovarian

277 sample from *SZ232* which is a large outlier at only 5%. However reads mapping to the reverse
278 strand account for an average of 84% of the piRNA being produced from the strand in every
279 genotype and tissue – maternal fraction, testis, or ovary. If unique mapping is considered instead
280 of weighted these percentages are reduced by approximately 20%, which is to be expected given
281 that *R1* is present in many repeated copies. Production of piRNA from the reverse strand seems
282 to be correlated with elements inserted in the sense orientation, of which the vast majority are *R1*
283 elements in *D. simulans* (Supplementary Figure 2). The production of large quantities of piRNA
284 cognate to the *R1* element is seemingly pointless – if *R1* only inserts at rDNA genes and are
285 vertically transmitted there is little reason to be producing the majority of piRNA in response to
286 this element.

287 In *D. sechellia* there are very few piRNA produced from *flamenco* in these tissues, and
288 there are no full length copies of *R1*. Likewise overall weighted piRNA production from *R1*
289 elements on either strand is 2.8-5.9% of the total mapping piRNA. In contrast in *D. mauritiana*
290 there are full length *R1* elements and abundant piRNA production in the maternal fraction and
291 testis. In *D. mauritiana* an average of 28% of piRNAs mapping to the forward strand of *flamenco*
292 are arising from *R1*, and 33% from the reverse strand. In *D. mauritiana R1* elements make up a
293 smaller proportion of the total elements in the sense orientation (24%), versus *D. simulans*
294 (55%).

295 *Conservation of flamenco*

296 The *dip1* gene and promoter region adjacent to each copy of *flamenco* are very conserved both
297 within and between copies of *flamenco* (Figure 3A). The phylogenetic tree of the area suggests
298 that we are correct in labeling the two copies as the original *flamenco* locus and the duplicate
299 (Figure 3A). The original *flamenco* locus is more diverged amongst copies while the duplicate

clusters closely together with short branch lengths (Figure 3A). The promotor region is also conserved and alignable between *D. melanogaster*, *D. sechellia*, *D. mauritiana*, and *D. simulans* (Figure 1D). 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 presence/absence polymorphisms that span multiple kb. There is no conservation of *flamenco* between *D. melanogaster*, *D. simulans*, *D. sechellia*, and *D. mauritiana* (Figure 4). 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 4).

In *D. simulans* the majority of full length TEs are singletons – 54% 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*. Singletons are the single 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 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 promoter are conserved.

It is notable that any full length TEs are shared across all genotypes, given that *wxD*^{1} was like collected 30-50 years prior to the others, and the collections span continents (Figure 3C). Two facts are relevant to this observation: (1) TEs were shown not to correlate with geography (67) and (2) *D. simulans* is more diverse within populations than between different populations (68–70). Other explanations are also plausible. 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 was 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 (18).

Here, we evaluate the following lines of evidence to determine if they support the trap model of transposable element suppression. (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* (18)

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 flanking the promoter, 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 14 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 present but missing an LTR. Full length TEs make up 19% of the *flamenco* locus.

In *D. simulans* there are 24 full length TEs present in any of the seven complete *flamenco* assemblies. Six of these are present in multiple antisense copies within a single genome – *INE1*, *Chimpo*, *gypsy-4*, *412*, *Tirant*, and *BEL-unknown*. 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 promoter as aforementioned. *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*, *BEL-unknown*, *Nomad-1*, *Chimpo*, *gypsy-53A*, *R1*, and *INE1* are all multicopy with respect to the original *flamenco* within a given genome. Some of these may have been inherited at the time of duplication, however are full length in both copies suggesting recent transposition. 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.

How many TEs have full length and fragmented insertions?

Full length elements are younger insertions than fragmented insertions. 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 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 (71). 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 (18, 28). 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 (18). Transfer appears to have occurred primarily between *D. melanogaster*, *D. sechellia*, and *D. willistoni*. This includes some known horizontal transfer events such as *Chimpo* and *Chouto* (72), and others which have not been recorded such as *gypys-29* (*D. willistoni*) and the *Max-element* (*D. sechellia*) (Supplementary File 4). The duplicate of *flamenco* is similar, with 53% of full length TEs originating from horizontal transfer. They are many of the same TEs, with a 46% overlap, thus *flamenco* and its duplicate are trapping

411 many of the same TEs. Both *flamenco* and the duplicate the region appears to serve as a trap for
412 TEs originating from horizontal transfer.

413 In *D. melanogaster* 85% of full length TEs appear to have arisen through horizontal
414 transfer, which is consistent with previous estimates (18). In *D. sechellia* 53% of full length TEs
415 have arisen from horizontal transfer, including some known to have moved by horizontal transfer
416 such as *GTWIN* (*D. melanogaster*/*D. erecta*) (72). *D. mauritiana* has 68% of its full length TEs
417 showing a closer relationship than expected by vertical descent with TEs from *D. sechellia*, *D.*
418 *melanogaster*, and *D. simulans*. The hypothesis that *flamenco* serves as a trap for TEs entering
419 the population through horizontal transfer holds throughout the *simulans* clade.

420 *Flamenco piRNA is expressed in the testis and the maternal fraction*

421 Canonically, *flamenco* piRNA is expressed in the somatic follicular cells of the ovary and
422 not in the germline, and also does not produce a ping pong signal (24). It was not thought to be
423 present in the maternal fraction of piRNAs or other tissues. However, that appears to be variable
424 in different species (Figure 5). We examined single mapping reads in the *flamenco* region from
425 testes and embryos (maternal fraction) in *D. simulans*, *D. mauritiana*, *D. sechellia*, and *D.*
426 *melanogaster*. As a control we also included *D. melanogaster* ovarian somatic cells, where Aub
427 and Ago3 are not expressed and therefore there should be no ping pong signals. In *D. simulans*
428 and *D. mauritiana flamenco* is expressed bidirectionally in the maternal fraction and the testis,
429 including ping pong signals on both strands (Figure 5A & C; Supplementary Figure 1). In *D.*
430 *sechellia*, there is no expression of *flamenco* in either of these tissues. Discarding multimappers
431 in the maternal fraction 63% (*D. mauritiana*) – 36% (*D. simulans*) of the ping pong signatures on
432 the X with a z-score of at least 0.9 are located within *flamenco* (Figure 5C). In the testis the
433 picture is more complicated – in *D. mauritiana* 50% of ping pong signals on the X with a z-score

of at least 0.9 are located within *flamenco*, which amounts to a substantial ping pong signature (Supplementary Figure 1). While mapping of piRNA to both strands was observed in *D. simulans* testis, there is very little apparent ping pong activity (5 positions in *flamenco* z > 0.9; 15 potential ping pong signals on the X). In *D. melanogaster*, there is uni-strand expression in the maternal fraction, but it is limited to the region close to the promoter. In *D. melanogaster* no ping pong signals have a z-score above 0.8 in the maternal fraction or the ovarian somatic cells. There are ping pong stacks in *flamenco* in the testis of D. melanogaster (2% of the total on the contig), however they are limited to a single region and are not abundant enough to be strong evidence of ping pong activity.

In the duplicate of *flamenco* in the maternal fraction 15% of the ping pong signals with a z-score above 0.9 on the X are within the *flamenco* duplicate. The *flamenco* duplicate does not have a strong signal of the ping pong pathway in the testis. In addition, *flamenco* in these species has been colonized by full length TEs thought to be active in the germline such as *blood*, *burdock*, *mdg-3*, *Transpac*, and *Bel* (73, 74). *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 are present. The differences in ping pong signals between species and the presence of germline TEs in *D. simulans* and *D. mauritiana* suggests that the role of *flamenco* in these tissues has evolved between species.

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 likely redundant (9).

457 However, *flamenco* is thought to be the master regulator of the somatic support cells of the
458 ovary, preventing *gypsy* elements from hopping into germline cells (18, 21, 23, 24, 75, 76). It is
459 not redundant to other clusters, and insertion of a single element into *flamenco* in *D.*
460 *melanogaster* is sufficient to initiate silencing. Here we show that the function of *flamenco*
461 appears to have diversified in the *D. simulans* clade, acting in at least some tissues as a germline
462 piRNA cluster.

463 *Dual stranded expression of flamenco*

464 In this work, we showed that piRNAs of the *flamenco* locus in *D.simulans* and *D.*
465 *mauritiana* are deposited maternally, align to both strands, and exhibit ping-pong signatures.
466 This is in contrast to *D. melanogaster*, where *flamenco* acts as a uni-strand cluster in the soma
467 (3), our data thus suggest that the *flamenco* locus in *D. simulans* and *D. mauritiana* acts as a
468 dual-strand cluster in the germline. In *D. sechellia* the attributes of *flamenco* uncovered in *D.*
469 *melanogaster* appear to be conserved – no expression in the maternal fraction and the testis and
470 no ping pong signals. Given that *flamenco* is likely a somatic uni-strand cluster in *D. erecta*, we
471 speculate that the conversion into a germline cluster happened in the *simulans* clade (3). Such a
472 conversion of a cluster between the somatic and the germline piRNA pathway is not
473 unprecedented. For example, a single insertion of a reporter transgene triggered the conversion
474 of the uni-stranded cluster *20A* in *D. melanogaster* into a dual-strand cluster (77).

475 The role of *flamenco* in *D. simulans* and *D. mauritiana* as the master regulator of piRNA
476 in somatic support cells may still well be true – the promoter region of the *flamenco* cluster is
477 conserved between species and between copies of *flamenco* within species. This suggests that in
478 at least some contexts (or all) the cluster is still serving as a uni-strand cluster transcribed from a
479 traditional RNA Pol II site (15). However it has acquired additional roles, producing dual strand

480 piRNA and ping pong signals, in these two species, in at least the germline. However, in *D.*
481 *simulans*, the majority of these reverse stranded piRNAs are emerging from the *R1* insertions
482 within *flamenco*. There is no evidence at present that *R1* has undergone an expansion in function
483 in *D. simulans*, thus it is unclear what, if any, functional impact the reverse stranded piRNAs
484 have at the *flamenco* locus.

485 *Duplication of flamenco in D. simulans*

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

494 *Rapid evolution of piRNA clusters*

495 A previous work showed that dual- and uni-strand clusters evolve rapidly in *Drosophila*
496 (20). In agreement with this work we also found that the *flamenco*-locus is rapidly evolving
497 between and within species (Fig. 1C, 3B). A major open question remains whether this rapid
498 turnover is driven by selection (positive or negative) or an outcome of neutral processes (eg. high
499 TE activity or insertion bias of TEs). These rapid evolutionary changes at the *flamenco* locus, a
500 piRNA master locus, suggest that there is a constant turnover in patterns of piRNA biogenesis
501 that potentially leads to changes in the level of transposition control between individuals in a
502 population.

Funding

This work was supported by the National Science Foundation Established Program to Stimulate Competitive Research (NSF-EPSCoR-1826834 and NSF-EPSCoR-2032756) to SS and the Austrian Science Fund FWF (https://www.fwf.ac.at/;) grant P35093 to RK. JV was supported by a Pathway to Independence award from the National Institute of General Medical Sciences (K99-GM137077). BYK was supported by the NIH-NRSA F32GM135998. ECL was supported by the National Institute of General Medical Sciences (R01-GM083300) and National Institutes of Health MSK Core Grant (P30-CA008748).

Competing interests

We declare that we have no competing interests.

Acknowledgements

Thanks to Colin Meiklejohn for providing some of the fly strains used in this manuscript. We would also like to thank Dimitri Petrov and his lab for providing logistical support to BYK. SS would like to thank Jeff Kittilson for assistance in the laboratory. SS would also like to thank C & F & S Emery for insightful commentary on the manuscript.

Authors' contributions

SS 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. ECL drafted portions of the manuscript and provided data. BYK generated and contributed sequence data.

Availability of data and materials

All data has been made available in the following repositories:

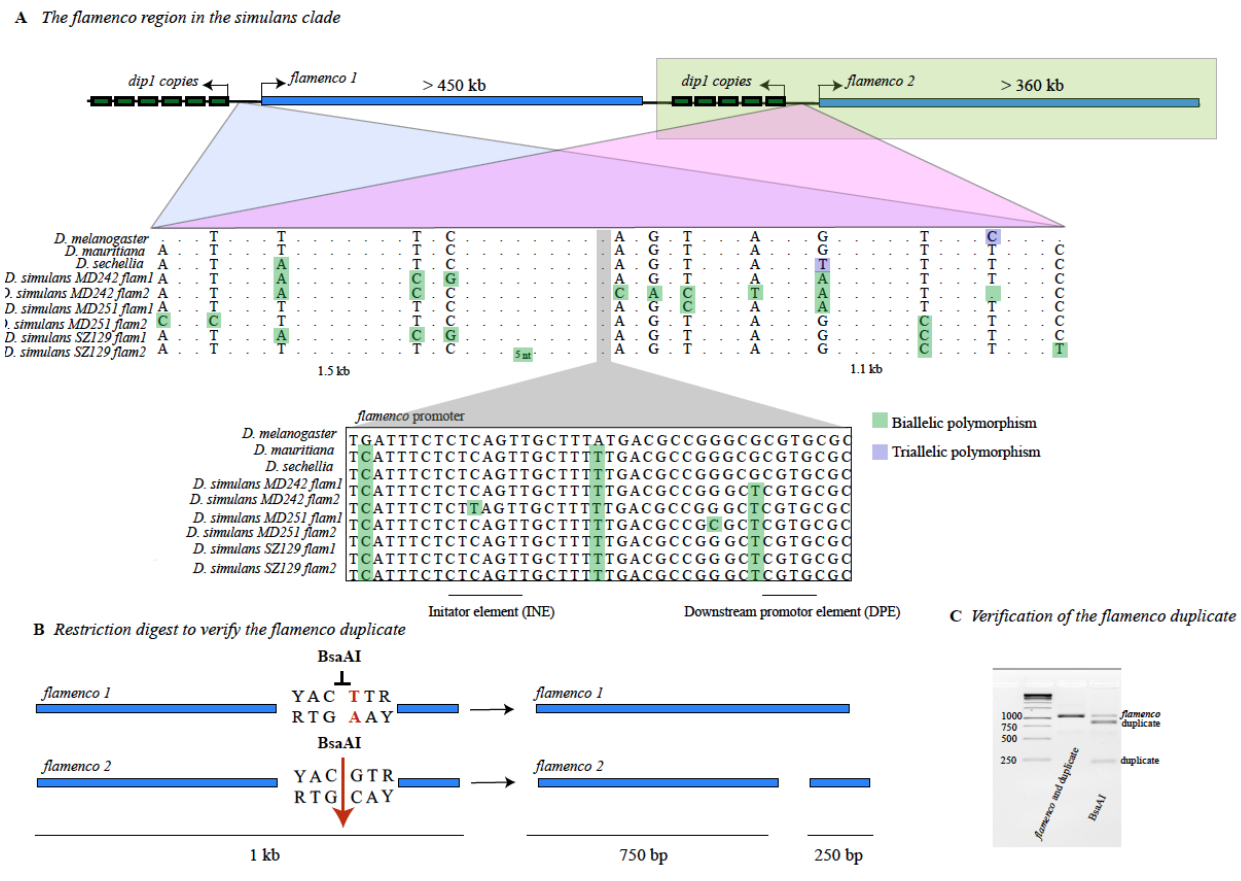
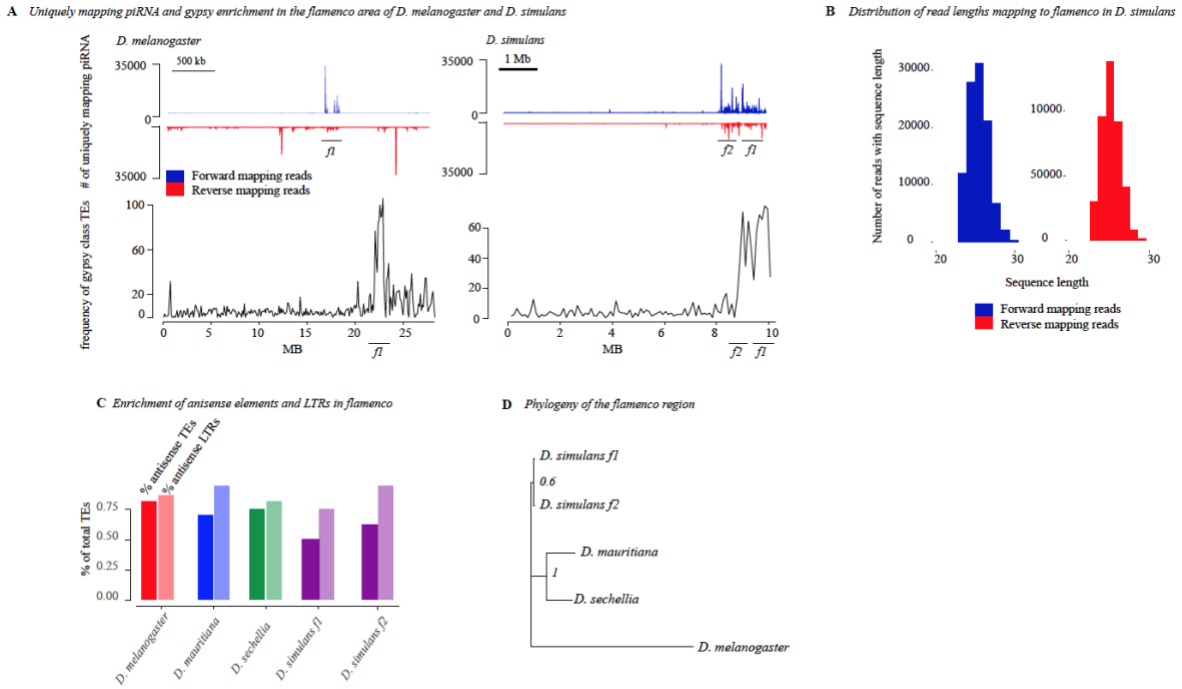


Figure 1. A) The duplication of *flamenco* in the *D. simulans*. Both copies are flanked by copies of the *dip1* gene and copies of the putative *flamenco* promoter. The top portion of the alignment shows ~ 2 kb around the promoter. SNPs are shown if they differentiate copies of *flamenco* within a single genotype of *D. simulans*. Dots do not indicate a single nucleotide, but rather a sequence region where no SNPs differentiate the two copies of *flamenco* within a single genotype. The lower portion illustrates the promoter region with all SNPs illustrated in *D. melanogaster*, *D. sechellia*, *D. mauritiana*, and *D. simulans*. B) A schematic of the restriction digest used to verify the duplicate of *flamenco*. The targeted region is a 1 kb fragment adjacent to the promotor of *flamenco*. Within this region the original *flamenco* copy does not contain a YACGTR site and is not cut by the restriction enzyme BsaAI. The duplicate of *flamenco* is cut into two pieces (750 bp and 250 bp). C) A gel showing the fragments of the original and

545 duplicated copy of *flamenco* before and after digestion with BsaAI. Both copies of *flamenco* are
546 amplified by the primers, in column two of the gel (Supplemental File 2). In column three of the
547 gel, the original copy of *flamenco* is uncut (band 1), while the duplicate of *flamenco* forms two
548 bands at 750 bp (band 2) and 250 bp (band 3).
549



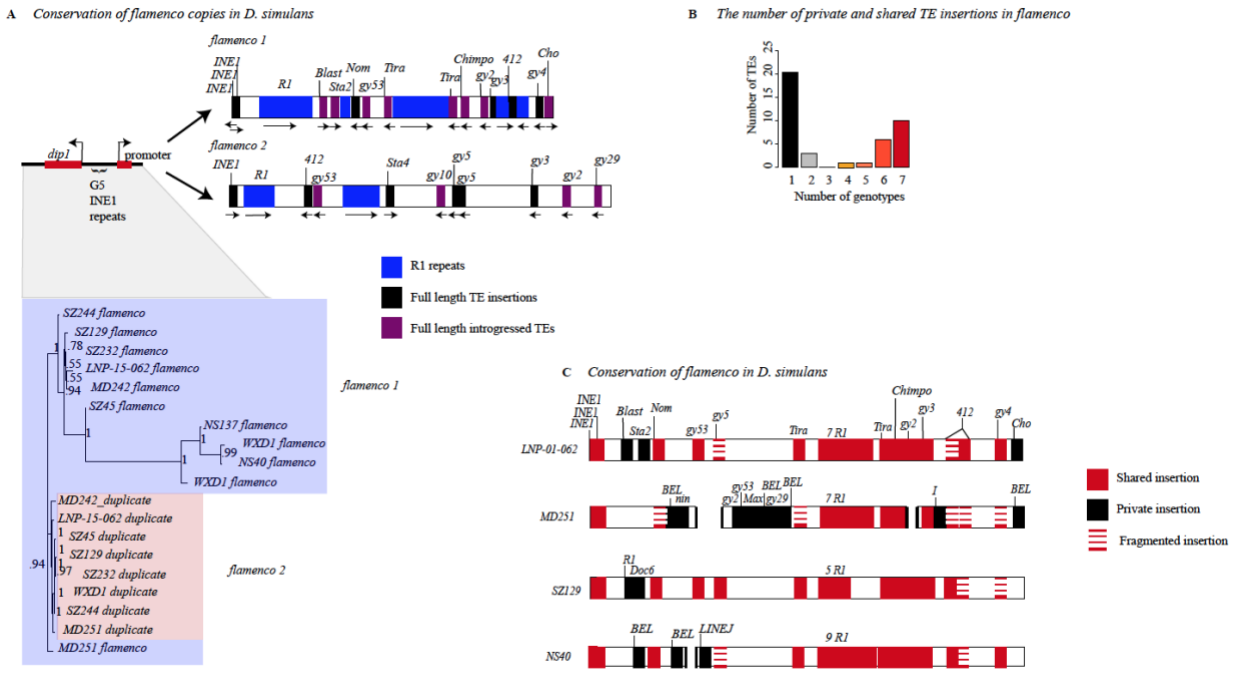
550

551 **Figure 2**. A) Unique piRNA from the ovary and *gypsy* enrichment around *flamenco* and its
552 duplicate in *D. simulans* and *D. melanogaster*. piRNA mapping to the entire contig that contains
553 *flamenco* is shown for both species. The top of the panel shows piRNA mapping to f*lamenco* and
554 is split by antisense (blue) and sense (red) piRNA . The bottom panel shows the frequency of
555 *gypsy*-type transposon annotations across the contig containing *flamenco*, counted in 100 kb
556 windows. There is a clear enrichment of *gypsy* in the area of *flamenco* and, in *D. simulans*, its
557 duplicate compared to the rest of the contig. B) The distribution of read size for small RNA
558 mapping to *flamenco*. The peak is at approximately 26 bp, within the expected range for piRNA.

559 C) The percent of TEs in *flamenco* in each species which are in the antisense orientation (first
560 bar) and the percent of TEs in the antisense orientation that are also LTR class elements (second
561 bar). D) A phylogenetic tree of the *dip1* and *flamenco* enhancer region for *D. melanogaster* and
562 the *simulans* clade. This region is conserved and alignable between all species. The tree was
563 generated with Mr. Bayes (62).

564

565
566
567
568
569



570
571
572

573

574 Figure 3. A) Divergence between copies of flamenco. Proximal is a phylogenetic tree of *dip1* and
575 the *flamenco* promoter region from each genome. In between *dip1* and the promoter are a series
576 of *G5/INE1* repeats that are found in every genome. Overall this region is fairly conserved, with

577 the duplicate copies all grouping together with short branch lengths (shown in pink). The original
578 copy of *flamenco* is more diverse with some outliers (shown in light blue) but there is good
579 branch support for all the deep branches of the tree. Distal is a representation of *flamenco* and its
580 duplicate. R1 repeat regions are shown in blue. Full length transposable elements are labeled.
581 There is no synteny conservation between *flamenco* and its duplicate. B) The proportion of
582 insertions that are shared by one through seven genotypes (genotypes with complete *flamenco*
583 assemblies). C) Divergence of flamenco within *D. simulans*. Labeled TEs correspond to
584 elements which are present in a full length copy in at least one genome. If they are shared
585 between genomes they are labeled in red, if they are unique they are black. If they are full length
586 in one genome and degraded in other genomes they are represented by stacked dashes. If they are
587 present in the majority of genomes but missing in one, it is represented as a missing that TE,
588 which is agnostic to whether it is a deletion or the element was never present
589
590

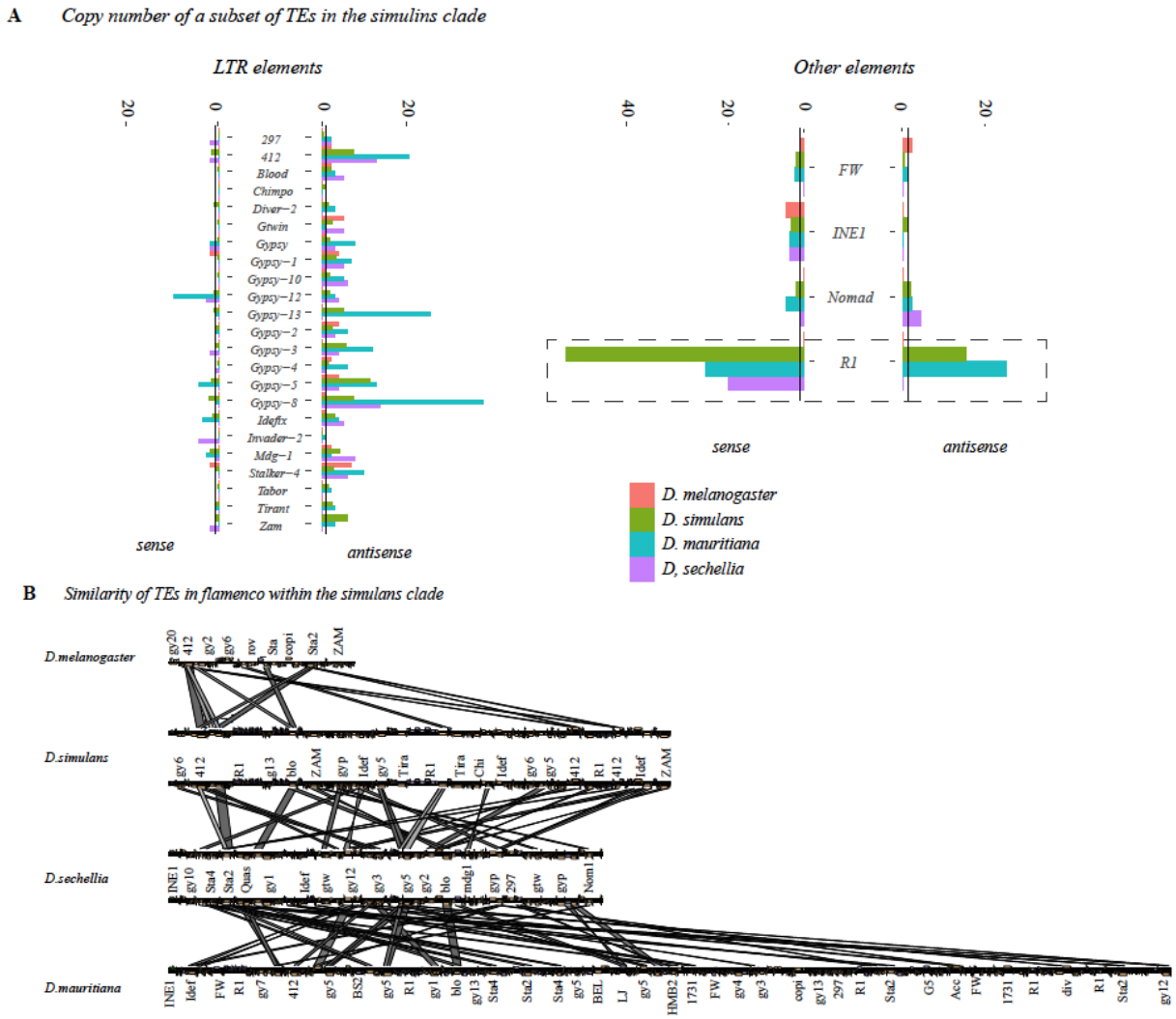
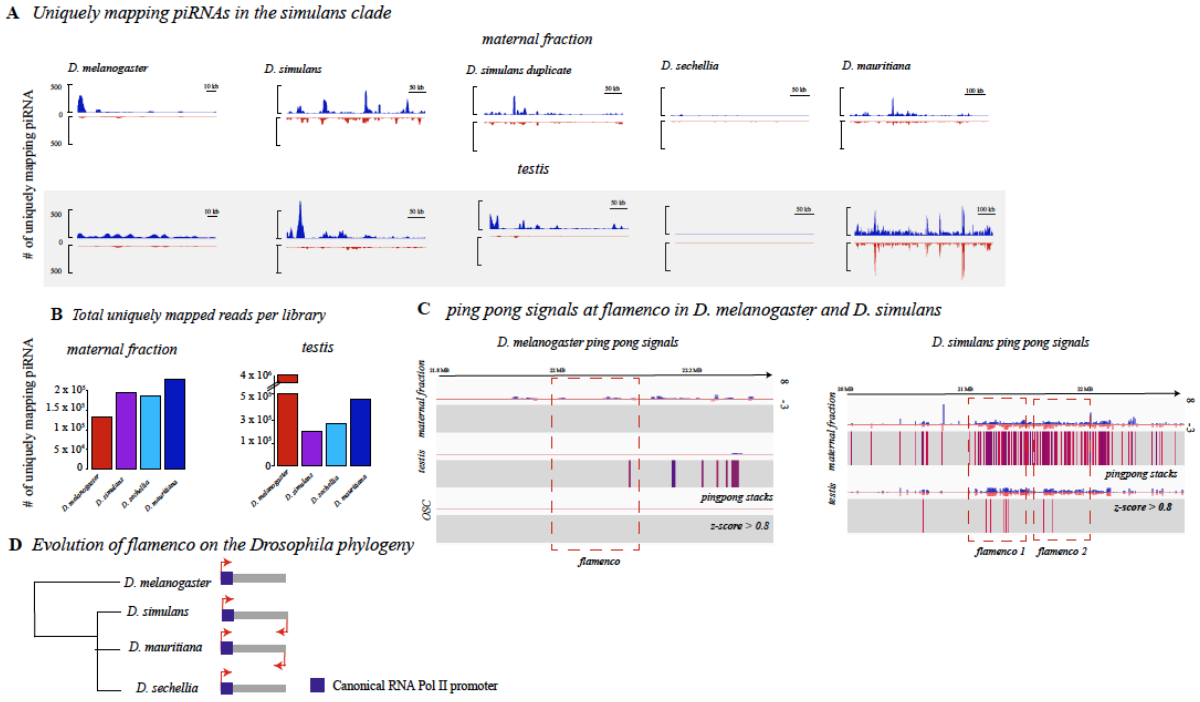


Figure 4) A. Copy number of a subset of transposable elements at *flamenco*. Solo LTRs are indicated by in a lighter shade at the top of the bar. The black line on each bar graph indicates a copy number of one. Values for *D. simulans* are the average for all genotypes with a complete *flamenco* assembly. 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 with a dotted box. Many elements within *flamenco* are multicopy in the *simulans* clade. While

600 some of this is likely due to local duplications it is clearly a different pattern than *D.*
601 *melanogaster*. Enrichment of LTR elements on the antisense strand is clear for all species. **B.**
602 Alignment of *flamenco* in *D. melanogaster*, *D. simulans*, *D. sechellia*, and *D. mauritiana*. There
603 is no conserved synteny between species but there are clearly shared TEs, particularly within the
604 *simulans* clade. The expansion of *D. mauritiana* compared to the other species is apparent.



605
606 -
607
608 **Figure 5) A**. Expression of single mapping piRNAs in the maternal fraction and testis (gray) of
609 *D. melanogaster* and the *simulans* clade. Antisense mapping reads are shown in blue, sense in
610 red. Libraries are RPM normalized and scaled across library type. *D. sechellia* has no expression
611 of *flamenco* in the maternal fraction or the testis. *D. melanogaster* has low expression in the
612 maternal fraction and very little ping pong activity. *D. simulans* and *D. mauritiana* show dual
613 stranded expression in the testis and maternal fraction. **B**. The total number of uniquely mapping
614 reads for each of the libraries illustrated in A. This is included to demonstrate that a low number
615 of mapping reads does not explain the patterns seen in *D. sechellia* versus *D. mauritiana*. **C.** The

height of 10 nt pingpong stacks at *flamenco* in *D. melanogaster* maternal fraction, testis and ovarian somatic cells is shown on the left. Below each schematic of the height of the stacks is the position of z-scores over 0.8, indicating the likelihood that this is a real ping pong signal as opposed to an artifact. Signals move from red to blue as they approach 1. In the testis a few ping pong signals reach this threshold but not enough to indicate convincingly that there is ping pong activity. On the right are the ping pong stacks and z-scores for the maternal fraction and testis in *D. simulans*. Only in the maternal fraction are the density of z-scores over 0.8 convincing enough to indicate an active ping pong cycle in the *flamenco* region. However, the presence of stacks is enriched in testis, thus this may warrant further investigation. *D. mauritiana* also has convincing ping pong signals in this region (Supplementary Figure 1). **D.** A schematic of the evolution of *flamenco* and its mode expression in the *simulans* and *melanogaster* clade.

1. C. Duc, *et al.*, Trapping a somatic endogenous retrovirus into a germline piRNA cluster immunizes the germline against further invasion. *Genome Biol* 20, 127 (2019).

2. B. Barckmann, *et al.*, The somatic piRNA pathway controls germline transposition over generations. *Nucleic Acids Res* 46, gky761- (2018).

3. C. D. Malone, *et al.*, Specialized piRNA Pathways Act in Germline and Somatic Tissues of the Drosophila Ovary. *Cell* 137, 522–535 (2009).

4. L. S. Gunawardane, *et al.*, A Slicer-Mediated Mechanism for Repeat-Associated siRNA 5' End Formation in Drosophila. *Science* 315, 1587–1590 (2007).

5. S. H. Wang, S. C. R. Elgin, Drosophila Piwi functions downstream of piRNA production mediating a chromatin-based transposon silencing mechanism in female germ line. *Proc National Acad Sci* 108, 21164–21169 (2011).

6. J. Brennecke, *et al.*, Discrete Small RNA-Generating Loci as Master Regulators of Transposon Activity in Drosophila. *Cell* 128, 1089–1103 (2007).

644 7. A. A. Aravin, *et al.*, The Small RNA Profile during Drosophila melanogaster Development.
645 *Developmental Cell* 5, 337–350 (2003).

646 8. G. Chirn, *et al.*, Conserved piRNA Expression from a Distinct Set of piRNA Cluster Loci in
647 Eutherian Mammals. *Plos Genet* 11, e1005652 (2015).

648 9. D. Gebert, *et al.*, Large Drosophila germline piRNA clusters are evolutionarily labile and
649 dispensable for transposon regulation. *Mol Cell* 81, 3965-3978.e5 (2021).

650 10. P. R. Andersen, L. Tirian, M. Vunjak, J. Brennecke, A heterochromatin-dependent
651 transcription machinery drives piRNA expression. *Nature* 549, 54–59 (2017).

652 11. C. Klattenhoff, *et al.*, The Drosophila HP1 Homolog Rhino Is Required for Transposon
653 Silencing and piRNA Production by Dual-Strand Clusters. *Cell* 138, 1137–1149 (2009).

654 12. F. Mohn, G. Sienski, D. Handler, J. Brennecke, The Rhino-Deadlock-Cutoff Complex
655 Licenses Noncanonical Transcription of Dual-Strand piRNA Clusters in Drosophila. *Cell* 157,
656 1364–1379 (2014).

657 13. Y.-C. A. Chen, *et al.*, Cutoff Suppresses RNA Polymerase II Termination to Ensure
658 Expression of piRNA Precursors. *Mol Cell* 63, 97–109 (2016).

659 14. F. Mohn, G. Sienski, D. Handler, J. Brennecke, The Rhino-Deadlock-Cutoff Complex
660 Licenses Noncanonical Transcription of Dual-Strand piRNA Clusters in Drosophila. *Cell* 157,
661 1364–1379 (2014).

662 15. C. Goriaux, S. Desset, Y. Renaud, C. Vaury, E. Brasset, Transcriptional properties and
663 splicing of the flamencopi RNAcluster. *EMBO reports* 15, 411–418 (2014).

664 16. G. Sienski, D. Dönertas, J. Brennecke, Transcriptional Silencing of Transposons by Piwi and
665 Maelstrom and Its Impact on Chromatin State and Gene Expression. *Cell* 151, 964–980 (2012).

666 17. C. Dennis, E. Brasset, C. Vaury, flam piRNA precursors channel from the nucleus to the
667 cytoplasm in a temporally regulated manner along Drosophila oogenesis. *Mobile DNA* 10, 203–9
668 (2019).

669 18. V. Zanni, A. Eymery, M. C. P. of the, 2013, Distribution, evolution, and diversity of
670 retrotransposons at the flamenco locus reflect the regulatory properties of piRNA clusters.
671 *National Acad Sciences* https:/doi.org/10.1073/pnas.1313677110/-/dcsupplemental.

672 19. C. M. Bergman, H. Quesneville, D. Anxolabéhère, M. Ashburner, Recurrent insertion and
673 duplication generate networks of transposable element sequences in the Drosophila melanogaster
674 genome. *Genome Biology* 7, R112-21 (2006).

675 20. F. Wierzbicki, R. Kofler, S. Signor, Evolutionary dynamics of piRNA clusters in Drosophila.
676 *Mol Ecol* (2021) https:/doi.org/10.1111/mec.16311.

677 21. N. Prud'homme, M. Gans, M. Masson, C. Terzian, A. Bucheton, Flamenco, a gene
678 controlling the gypsy retrovirus of Drosophila melanogaster. *Genetics* 139, 697–711 (1995).

679 22. S. U. Song, T. Gerasimova, M. Kurkulos, J. D. Boeke, V. G. Corces, An env-like protein
680 encoded by a Drosophila retroelement: evidence that gypsy is an infectious retrovirus. *Genes &*
681 *development* 8, 2046–2057 (1994).

682 23. M. Mével-Ninio, A. Pelisson, J. Kinder, A. R. Campos, A. Bucheton, The flamenco Locus
683 Controls the gypsy and ZAM Retroviruses and Is Required for Drosophila Oogenesis. *Genetics*
684 175, 1615–1624 (2007).

685 24. A. Pelisson, *et al.*, Gypsy transposition correlates with the production of a retroviral
686 envelope-like protein under the tissue-specific control of the Drosophila flamenco gene. *The*
687 *EMBO Journal* 13, 4401–4411 (1995).

688 25. A. Bucheton, The relationship between the flamenco gene and gypsy in Drosophila: how to
689 tame a retrovirus. *Trends Genet* 11, 349–353 (1995).

690 26. C. D. Malone, G. J. Hannon, Molecular Evolution of piRNA and Transposon Control
691 Pathways in Drosophila. *Cold Spring Harbor Symposia on Quantitative Biology* 74, 225–234
692 (2010).

693 27. A. G. Clark, *et al.*, Evolution of genes and genomes on the Drosophila phylogeny. *Nature*
694 450, 203–218 (2007).

695 28. D. G. Eickbush, W. C. Lathe, M. P. Francino, T. H. Eickbush, R1 and R2 retrotransposable
696 elements of Drosophila evolve at rates similar to those of nuclear genes. *Genetics* 139, 685–695
697 (1995).

698 29. S. A. Signor, F. N. New, S. Nuzhdin, A Large Panel of Drosophila simulans Reveals an
699 Abundance of Common Variants. *Genome Biology and Evolution* 10, 189–206 (2017).

700 30. S. Signor, S. Nuzhdin, Dynamic changes in gene expression and alternative splicing mediate
701 the response to acute alcohol exposure in Drosophila melanogaster. *Heredity* (2018).

702 31. S. Signor, Population genomics of Wolbachia and mtDNA in Drosophila simulans from
703 California. *Scientific Reports*, 1–11 (2017).

704 32. S. A. Signor, M. Abbasi, P. Marjoram, S. V. Nuzhdin, Social effects for locomotion vary
705 between environments in Drosophila melanogaster females. *Evolution* 71, 1765–1775 (2017).

706 33. S. Signor, Transposable elements in individual genotypes of Drosophila simulans. *Ecology*
707 *and Evolution* 130, 499–11 (2020).

708 34. D. R. Matute, J. Gavin-Smyth, G. Liu, Variable post-zygotic isolation in Drosophila
709 melanogaster/D. simulanshybrids. *Journal of Evolutionary Biology* 27, 1691–1705 (2014).

710 35. D. R. Schrider, J. Ayroles, D. R. Matute, A. D. Kern, Supervised machine learning reveals
711 introgressed loci in the genomes of Drosophila simulans and D. sechellia. *PLoS Genetics* 14,
712 e1007341-29 (2018).

713 36. R. L. Rogers, *et al.*, Landscape of Standing Variation for Tandem Duplications in Drosophila
714 yakuba and Drosophila simulans. *Molecular Biology and Evolution* 31, 1750–1766 (2014).

715 37. M. Chakraborty, *et al.*, Evolution of genome structure in the Drosophila simulansspecies
716 complex. 139, 1067–63 (2020).

717 38. , Genome Res.-2017-Koren-gr.215087.116.

718 39. R. Vaser, I. Sović, N. Nagarajan, M. Šikić, Fast and accurate de novo genome assembly from
719 long uncorrected reads. *Genome Res* 27, 737–746 (2017).

720 40. B. J. Walker, *et al.*, Pilon: An Integrated Tool for Comprehensive Microbial Variant
721 Detection and Genome Assembly Improvement. *Plos One* 9, e112963 (2014).

722 41. M. Chakraborty, J. J. Emerson, S. J. Macdonald, A. D. Long, Structural variants exhibit
723 widespread allelic heterogeneity and shape variation in complex traits. *Nature Communications*,
724 1–11 (2019).

725 42. M. Kolmogorov, J. Yuan, Y. Lin, P. A. Pevzner, Assembly of long, error-prone reads using
726 repeat graphs. *Nat Biotechnol* 37, 540–546 (2019).

727 43. D. R. Laetsch, M. L. Blaxter, BlobTools: Interrogation of genome assemblies.
728 *F1000research* 6, 1287 (2017).

729 44. M. Tarailo-Graovac, N. Chen, Using RepeatMasker to Identify Repetitive Elements in
730 Genomic Sequences. *Current Protocols in Bioinformatics*, 1–14 (2009).

731 45. J. M. Flynn, *et al.*, RepeatModeler2 for automated genomic discovery of transposable
732 element families. *Proc National Acad Sci* 117, 9451–9457 (2020).

733 46. J. Armstrong, *et al.*, Progressive Cactus is a multiple-genome aligner for the thousand-
734 genome era. *Nature* 587, 246–251 (2020).

735 47. M. Kolmogorov, *et al.*, Chromosome assembly of large and complex genomes using multiple
736 references. *Genome Res* 28, 1720–1732 (2018).

737 48. F. Wierzbicki, F. Schwarz, O. Cannalonga, R. Kofler, Generating high quality assemblies for
738 genomic analysis of transposable elements. *Biorxiv*, 2020.03.27.011312 (2020).

739 49. F. Wierzbicki, F. Schwarz, O. Cannalonga, R. Kofler, Novel quality metrics allow
740 identifying and generating high-quality assemblies of piRNA clusters. *Mol Ecol Resour* 22, 102–
741 121 (2022).

742 50. Vedanayagam, Jeffrey, "Evolutionary Genomics of piRNA Mediated Transposon Silencing
743 in Drosophila," University of Rochester. (2016).

744 51. J. Vedanayagam, *et al.*, Endogenous RNAi silences a burgeoning sex chromosome arms race.
745 *Biorxiv*, 2022.08.22.504821 (2022).

746 52. J. Vedanayagam, C.-J. Lin, E. C. Lai, Rapid evolutionary dynamics of an expanding family
747 of meiotic drive factors and their hpRNA suppressors. *Nat Ecol Evol* 5, 1613–1623 (2021).

748 53. S. Chen, Y. Zhou, Y. Chen, J. Gu, fastp: an ultra-fast all-in-one FASTQ preprocessor.
749 *Biorxiv*, 274100 (2018).

750 54. M. J. Axtell, ShortStack: Comprehensive annotation and quantification of small RNA genes.
751 *RNA* 19, 740–751 (2013).

752 55. B. Langmead, C. Trapnell, M. Pop, S. L. Salzberg, Ultrafast and memory-efficient alignment
753 of short DNA sequences to the human genome. *Genome Biol* 10, R25 (2009).

754 56. H. Li, *et al.*, The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 25, 2078–
755 2079 (2009).

756 57. Y. Liao, G. K. Smyth, W. Shi, The R package Rsubread is easier, faster, cheaper and better
757 for alignment and quantification of RNA sequencing reads. *Nucleic Acids Res* 47, gkz114-
758 (2019).

759 58. D. Rosenkranz, H. Zischler, proTRAC - a software for probabilistic piRNA cluster detection,
760 visualization and analysis. *Bmc Bioinformatics* 13, 5 (2012).

761 59. M. Bailly-Bechet, A. Haudry, E. Lerat, "One code to find them all": a perl tool to
762 conveniently parse RepeatMasker output files. *Mobile Dna-uk* 5, 13 (2014).

763 60. A. R. Quinlan, I. M. Hall, BEDTools: a flexible suite of utilities for comparing genomic
764 features. *Bioinformatics* 26, 841–842 (2010).

765 61. F. Sievers, D. G. Higgins, Clustal Omega for making accurate alignments of many protein
766 sequences. *Protein Sci* 27, 135–145 (2018).

767 62. F. Ronquist, *et al.*, MrBayes 3.2: Efficient Bayesian Phylogenetic Inference and Model
768 Choice Across a Large Model Space. *Systematic Biology* 61, 539–542 (2012).

769 63. E. Paradis, J. Claude, K. Strimmer, APE: Analyses of Phylogenetics and Evolution in R
770 language. *Bioinformatics* 20, 289–290 (2004).

771 64. S. Uhrig, H. Klein, PingPongPro: a tool for the detection of piRNA-mediated transposon-
772 silencing in small RNA-Seq data. *Bioinformatics* 35, 335–336 (2018).

773 65. Smit, A. and Hubley, R. and Green, P, *RepeatMasker Open-4.0. 2013--2015* (2005).

774 66. H. Quesneville, *et al.*, Combined Evidence Annotation of Transposable Elements in Genome
775 Sequences. *Plos Comput Biol* 1, e22 (2005).

776 67. E. Lerat, *et al.*, Population specific dynamics and selection patterns of transposable element
777 insertions in European natural populations. *Molecular Ecology*, 1–42 (2018).

778 68. R. S. Singh, Population genetics and evolution of species related to Drosophila melanogaster.
779 *Annual Review of Genetics* 23, 425–453 (1989).

780 69. H. E. Machado, *et al.*, Comparative population genomics of latitudinal variation in
781 Drosophila simulans and Drosophila melanogaster. *Molecular Ecology* 25, 723–740 (2016).

782 70. A. Sedghifar, P. Saelao, D. J. Begun, Genomic patterns of geographic differentiation in
783 Drosophila simulans. *Genetics* (2016) https:/doi.org/10.1534/genetics.115.185496.

784 71. E. L. S. Loreto, C. M. A. Carareto, P. Capy, Revisiting horizontal transfer of transposable
785 elements in Drosophila. *Heredity* 100, 545–554 (2008).

786 72. N. Bargues, E. Lerat, Evolutionary history of LTR-retrotransposons among 20 Drosophila
787 species. *Mobile Dna-uk* 8, 7 (2017).

788 73. Z. Durdevic, R. S. Pillai, A. Ephrussi, Transposon silencing in the Drosophila female
789 germline is essential for genome stability in progeny embryos. *Life Sci Alliance* 1, e201800179
790 (2018).

791 74. B. Czech, J. B. Preall, J. McGinn, G. J. Hannon, A Transcriptome-wide RNAi Screen in the
792 Drosophila Ovary Reveals Factors of the Germline piRNA Pathway. *Mol Cell* 50, 749–761
793 (2013).

794 75. A. and T. Pélisson, About the origin of retroviruses and the co-evolution of the gypsy
795 retrovirus with the Drosophila flamenco host gene. 29–37 (1997).

796 76. C. Duc, *et al.*, Trapping a somatic endogenous retrovirus into a germline piRNA cluster
797 immunizes the germline against further invasion. *Genome Biol* 20, 127 (2019).

798 77. Y. Luo, P. He, N. Kanrar, K. F. Toth, A. Aravin, Maternally inherited siRNAs initiate piRNA
799 cluster formation https:/doi.org/10.1101/2022.02.08.479612.

800 78. R. Kofler, piRNA Clusters Need a Minimum Size to Control Transposable Element
801 Invasions. *Genome Biology and Evolution* 12, 736–749 (2020).

802 79. A. M. Waterhouse, J. B. Procter, D. M. A. Martin, M. Clamp, G. J. Barton, Jalview Version
803 2—a multiple sequence alignment editor and analysis workbench. *Bioinformatics* 25, 1189–1191
804 (2009).

805