

Research article

Evidence for the adaptive significance of an LTR retrotransposon sequence in a *Drosophila* heterochromatic gene

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Abstract

Background: The potential adaptive significance of transposable elements (TEs) to the host genomes in which they reside is a topic that has been hotly debated by molecular evolutionists for more than two decades. Recent genomic analyses have demonstrated that TE fragments are associated with functional genes in plants and animals. These findings suggest that TEs may contribute significantly to gene evolution.

Results: We have analyzed two transposable elements associated with genes in the sequenced *Drosophila melanogaster* *y; cn bw sp* strain. A fragment of the *Antonia* long terminal repeat (LTR) retrotransposon is present in the intron of *Chitinase 3* (*Cht3*), a gene located within the constitutive heterochromatin of chromosome 2L. Within the euchromatin of chromosome 2R a full-length *Burdock* LTR retrotransposon is located immediately 3' to *cathD*, a gene encoding cathepsin D. We tested for the presence of these two TE/gene associations in strains representing 12 geographically diverse populations of *D. melanogaster*. While the *cathD* insertion variant was detected only in the sequenced *y; cn bw sp* strain, the insertion variant present in the heterochromatic *Cht3* gene was found to be fixed throughout twelve *D. melanogaster* populations and in a *D. mauritiana* strain suggesting that it maybe of adaptive significance. To further test this hypothesis, we sequenced a 685bp region spanning the LTR fragment in the intron of *Cht3* in strains representative of the two sibling species *D. melanogaster* and *D. mauritiana* (~2.7 million years divergent). The level of sequence divergence between the two species within this region was significantly lower than expected from the neutral substitution rate and lower than the divergence observed between a randomly selected intron of the *Drosophila Alcohol dehydrogenase* gene (*Adh*).

Conclusions: Our results suggest that a 359 bp fragment of an *Antonia* retrotransposon (complete LTR is 659 bp) located within the intron of the *Drosophila melanogaster* *Cht3* gene is of adaptive evolutionary significance. Our results are consistent with previous suggestions that the presence of TEs in constitutive heterochromatin may be of significance to the expression of heterochromatic genes.

Background

The potential adaptive significance of transposable elements (TEs) to the host genomes in which they reside is a topic that has been hotly debated by molecular evolutionists for more than two decades. While the biological importance of TEs seemed self-evident to those scientists involved in their initial discovery [e.g., [1,2]], the subsequent realization that TEs could be maintained in populations even while imparting slight selective disadvantage to their hosts [e.g., [3–5]] drew into question the presumption of adaptive significance. However, even if TEs can be maintained in populations on a day-to-day basis without providing selective advantage, it does not preclude the possibility that the insertion of TEs in or near genes may, in some instances, be of adaptive advantage.

If TE insertion variants have contributed to adaptive gene evolution, such variants might be expected to be in high frequency or fixed in populations and species. Initial surveys of natural populations of *Drosophila melanogaster* showing that TE insertion alleles are in uniformly low frequency seemed to negate the adaptive hypothesis [6]. However, the sporadic discovery of degenerate TEs or TE fragments as critical components of functional genes in both plants and animals was sufficient to keep the adaptive hypothesis alive throughout the pre-genomic era [7–11].

The current availability of the complete or nearly complete sequence of select genomes representing a variety of species is providing an unprecedented opportunity to examine the frequency and distribution of TEs in eukaryotic genomes. The results have been dramatic. TEs not only comprise a significant fraction of nearly all eukaryotic genomes thus far sequenced, they have been found to be components of the regulatory and/or coding regions of a surprisingly large number of genes [e.g., [12]]. For example, a recent genomic analysis of 13,799 human genes revealed that approximately 4% harbored retrotransposon sequences within protein-coding regions [13]. Similar results have been recently reported for the nematode *Caenorhabditis elegans*[14]. Here we analyze the polymorphism of two LTR retrotransposon / host gene associations across geographically widespread *D. melanogaster* populations and a representative population of the *D. melanogaster* sibling species, *Drosophila mauritiana*.

Results

We have initiated a genomic analysis of LTR retrotransposons present in the *Drosophila melanogaster* genome [e.g., [15]]. Of particular interest is identification of genes harboring TEs and determining if these insertion alleles are in high frequency or fixed among natural populations as would be expected from the adaptive hypothesis. We report here the results of an analysis of two LTR retrotrans-

poson-containing genes located on the second chromosome of the sequenced *D. melanogaster* *y; cn bw sp* strain. These two genes present an interesting contrast in that one of them, *Chitinase 3* (*Cht3*), is located within constitutive heterochromatin (Genbank accession: AE002743) while the other, *cathD*, is located in a euchromatic region of the chromosome (Genbank accession: AE003839). Our findings demonstrate that while the euchromatic *cathD* insertion variant was not detected in any of the natural populations examined, the insertion variant present in the heterochromatic *Cht3* gene was found to be apparently fixed throughout the species. These results are consistent with the view that the presence of TEs in constitutive heterochromatin may have relevance to the expression of heterochromatic genes [e.g., [16,17]].

Table 1: Presence or absence of retroelement sequence associated with *cathD* and *Cht3* genes in strains representing 12 natural populations of *D. melanogaster*.

Geographic area	Strain	<i>cathD</i> / Burdock	<i>Cht3</i> / Antonia
Americas	Lab stock	+	+
	<i>y; cn bw sp</i>	-	+
	Athens	-	+
	California	-	+
Europe	Antilles	-	+
	Germany	-	+
	Italy	-	+
Africa	Capetown	-	+
	Cotonake	-	+
	Dimonika	-	+
	Kenia	-	+
	Niamey	-	+
	Swaziland	-	+
Asia	India	-	+

Males and females from each strain were tested. (+) indicates presence of retroelement sequence, (-) indicates absence of retroelement sequence.

Genomic analysis of the sequenced *y; cn bw sp* strain of *Drosophila melanogaster* identified a full-length *Burdock* LTR retrotransposon located just 3' to the *cathD* gene and a 359bp LTR fragment (complete LTR is 659 bp) of an *Antonia* LTR retrotransposon [15] located within an intron of the *Cht3* gene (Figure 1). A set of PCR primers were designed to amplify regions of both genes and retrotransposon sequences. Appropriate pairs of gene and element primers were used to detect the presence or absence of the respective retrotransposon inserts associated with each gene in strains representing 12 geographically dispersed populations of *D. melanogaster*. The results presented in Figure 2 and Table 1 demonstrate that while the *Burdock* insertion located just 3' to *cathD* gene is not present in any

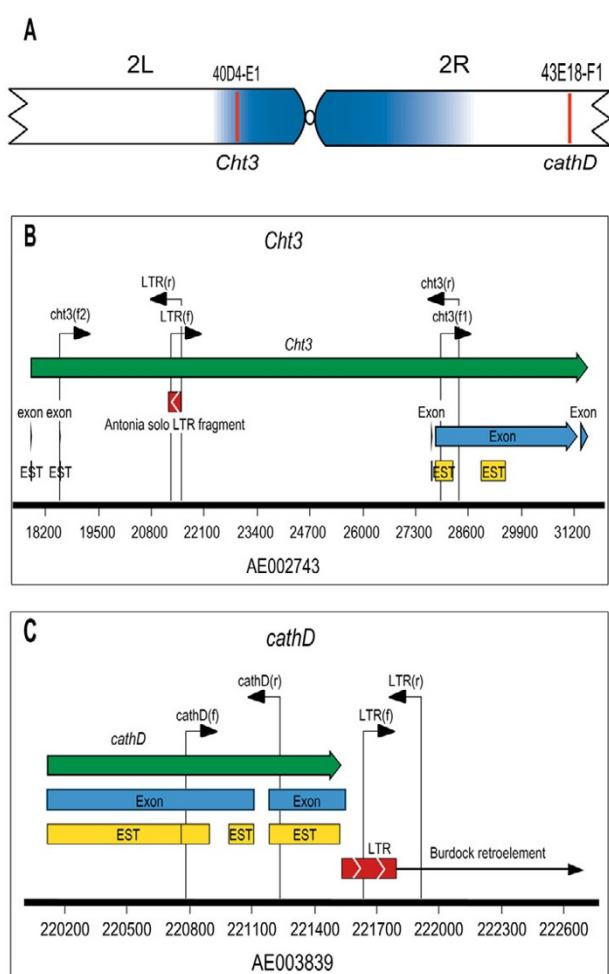


Figure 1
Genomic structure of the *Cht3* and *cathD* genes in the *Drosophila melanogaster* genome. **(A)** Chromosome 2 illustrating location of *Cht3* and *cathD* genes (red lines) in reference to constitutive heterochromatin (in blue) [34]. Numbers above each red line refer to Flybase cytogenetic placement. (Chromosome not drawn to scale). **(B & C)** Green arrows represent Flybase-predicted gene regions with corresponding identification. Yellow blocks depict ESTs concordant to the predicted gene region. Blue boxes are predicted exon regions. Red boxes denote LTR position and internal arrows indicate orientation of retroelement. The black line and numbers represent position along the genomic clone sequence which is identified below the figure. Black arrows indicate direction and location of forward (f) or reverse(r) PCR primers. **(B)** An Antonia LTR fragment (359nt) is inserted in an intron of *Cht3* in 12 geographically distinct *Drosophila melanogaster* strains. **(C)** A full-length Burdock retroelement, only present in the sequenced *y; cn bw sp* strain, overlaps the predicted exon boundaries of the *cathD* gene by 6nt.

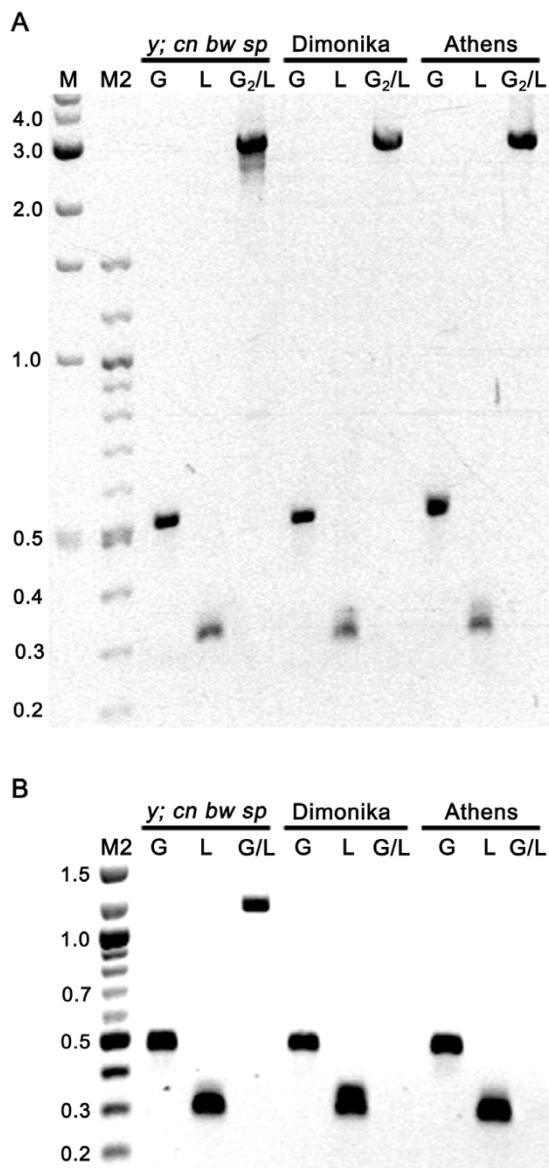


Figure 2
PCR analysis testing for the presence of an LTR retroelement feature in two genes, *Cht3* and *cathD*, across three representative *Drosophila* strains. A negative image is presented for visual clarity. Three PCR reactions were performed per strain, per gene. M = 1 kb ladder, M2 = 0.1 kb ladder. **(A)** An Antonia LTR fragment is fixed in the intron of the heterochromatic *Cht3* gene in all 12 tested strains (only three shown). *Cht3-G* = *cht3* primers (f+r), expected product= 488 bp. L = Antonia LTR primers (f+r), expected product= 272 bp. *G₂/L* = *cht3(f2)* + Antonia LTR (r) primers, expected product= 3022 bp. **(B)** A full-length Burdock LTR retrotransposon is found to be associated with *cathD* only in the sequenced *y; cn bw sp* strain. *cathD - G* = *cathD* primers (f+r), expected product = 461 bp. L = Burdock primers (f+r), expected product = 280 bp. *G/L* = *cathD(f)* and Burdock element (r), expected product= 1139 bp.

of 12 strains representing a geographically diverse sampling of natural populations, the *Antonia* LTR fragment located in the intron of the heterochromatic *Cht3* gene is fixed in all 12 strains tested.

It is formally possible that the presence of the *Antonia* LTR within the *Cht3* intron was the result of a chance fixation event prior to the expansion of *D. melanogaster* around the world. Thus, to further test the adaptive hypothesis we compared the level of sequence divergence within the LTR and its flanking intronic sequence between the two sibling species *Drosophila melanogaster* and *Drosophila mauritiana*. If the LTR-containing intron is under stabilizing selection, a lower than neutral rate of substitution would be expected. A total of 685 bp of the *Cht3* intron was sequenced. This region spans 264 bp of the 359 bp *Antonia* LTR fragment. The sequence of this region in a *D. melanogaster* (Dimonika, Africa) and *D. mauritiana* (Mauritius, Africa) strain was aligned with the homologous region in the sequenced *D. melanogaster* *y; cn bw sp* strain (Figure 3). The two *melanogaster* strains were 100% identical. The *melanogaster* sequences were found to be only 1.3% (9 substitutions/685 nucleotide sites) diverged from that of *D. mauritiana*. This value is significantly less than half of the expected 4.3% (± 2.7) divergence based on the *Drosophila* neutral substitution rate of 0.016 (± 0.005) substitutions/site/million year [18] over the estimated 2.7 million years separating the two species [19].

To directly compare the substitution rate for the *Cht3* intron with that of another *Drosophila* gene intron, we randomly selected intron 1 of the *Drosophila* alcohol dehydrogenase (*Adh*) gene. *Adh* is a widely studied *Drosophila* gene and it has been sequenced in several *Drosophila* species including *D. melanogaster*, accession X60793 [20] and *D. mauritiana*, accession M19264 [21]. The sequence divergence between *D. melanogaster* and *D. mauritiana* in the *Adh* intron 1 (7.9%, Figure 4), is higher than that for the LTR containing *Cht3* intron (1.3%). These results strongly suggest that conservative selection has been operating on the LTR containing intron associated with the *Drosophila Cht3* gene over the past 2.7 million years.

Discussion

For many years, constitutive heterochromatin was considered to be of little or no functional significance [22]. This view seemed to be supported by early molecular studies showing that heterochromatin consists almost exclusively of highly repeated and middle repetitive DNA [e.g., [23,24]]. The middle repetitive fraction was viewed as the descendant of once active TEs that had the misfortune of inserting into transcriptionally inert heterochromatin at some point in their evolutionary history [e.g., [6,20]]. The view of heterochromatin as a genetic wasteland gradually changed with the mapping of a number of functionally

important *Drosophila* genes to constitutive heterochromatin [e.g., [24–31]]. Reexamination of *Drosophila* constitutive heterochromatin revealed that long stretches of highly repetitive DNA are interrupted by "islands" of retrotransposon sequences [e.g., [32,33]]. *Drosophila* genes in heterochromatin are typically associated with these islands of retrotransposons [2,31,34–36]. It has been suggested that transposable elements inserted into heterochromatin may locally alter chromatin structure [e.g., [16]]. Our results suggest that in at least some instances, the association of heterochromatic genes with transposable element sequences may be of adaptive significance.

Conclusions

The results presented here are consistent with the hypothesis that a 359 bp fragment of the *Antonia* retrotransposon located within the intron of the heterochromatic *Drosophila melanogaster Cht3* gene may be of adaptive evolutionary significance. Further genomic and molecular analyses will be required to assess the general importance of LTR retrotransposon sequences to the evolution of heterochromatic gene structure and function.

Materials and Methods

Gene Region Annotation

BLASTs of sequenced DNA turned up several instances of genes proximal to an LTR retrotransposon. Sequence retrieval was initiated via BLASTN searches (default parameters- [37]) against the BDGP [<http://www.fruitfly.org>] and GenBank [<http://www.ncbi.nlm.nih.gov>] databases using LTRs from previously identified *Drosophila* retroelements as queries [15]. Results with E-values $< e^{-10}$ were annotated on the corresponding clone, whereupon visual inspection of several annotations confirmed the presence of retroelements proximal to known genes. Selected genes were BLASTed against NCBI's EST database and mapped along with predicted transcript structures from Flybase [<http://www.flybase.org>] . Chromosomal location of clones was also determined from Flybase.

PCR

D. melanogaster strains from Dimonika, Niamey, Swaziland, Kenia, Capetown, Cotonake, and India were obtained from Charles F. Aquadro, Cornell University. Germany, Italy, and Antilles strains were obtained from Nikolaj Junakovic, Università la Sapienza, Rome, Italy. California and Athens strains are from Daniel Promislow, University of Georgia. *D. melanogaster* *y; cn bw sp* strain was obtained from the Bloomington, IN, stock center. The *D. mauritiana* (241.0) strain was provided by the Bowling Green, OH, *Drosophila* stock center.

PCR primers were designed with MacVector 7.0 [<http://www.gcg.com>] and synthesized by Integrated DNA Technologies (Coralville, LA) (Table 2). Three PCR reactions

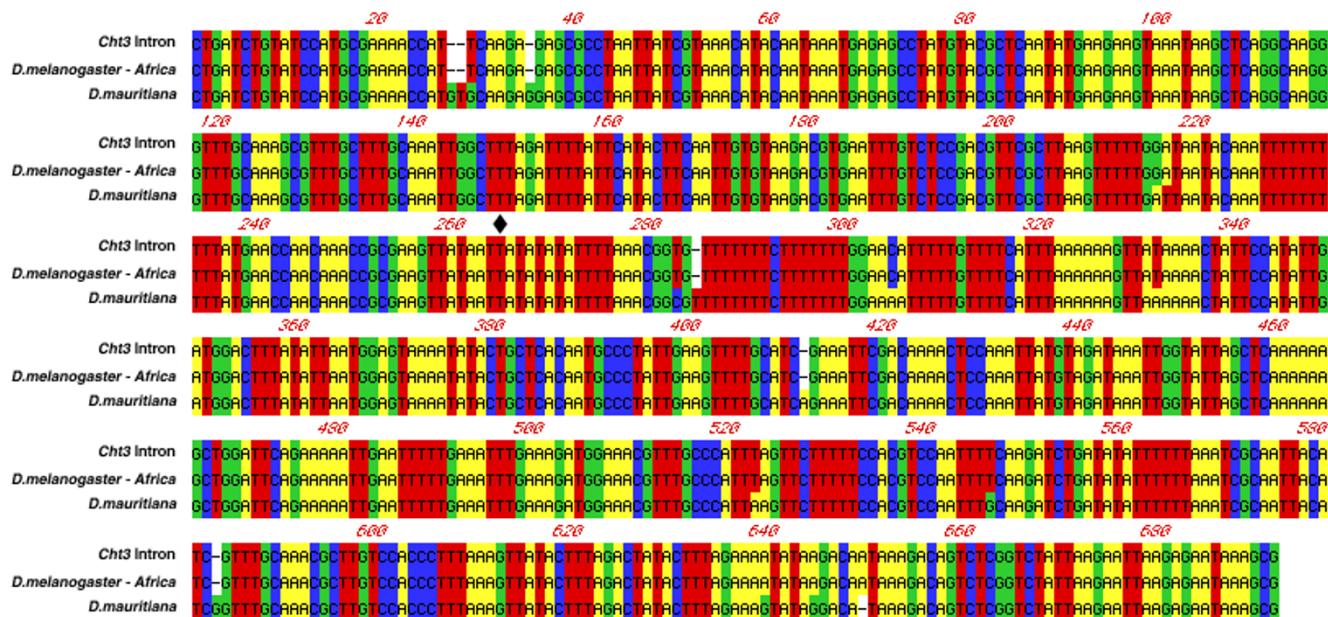


Figure 3
Nucleotide alignment of a 685 bp Cht3 intron fragment in *D.melanogaster* and *D.mauritiana*. Cht3 intron sequence from the *Drosophila melanogaster* y; cn bw sp strain (accession AE002743). The Antonia LTR stretches from bp 1 – 264, where a black diamond (◆) indicates the end of LTR sequence. Strains representing the *D. melanogaster*, Africa (Dimonika) population and a strain representing the *D. mauritiana*, Mauritius population were sequenced. Sequences were aligned using MacVector (See Materials and Methods for details).

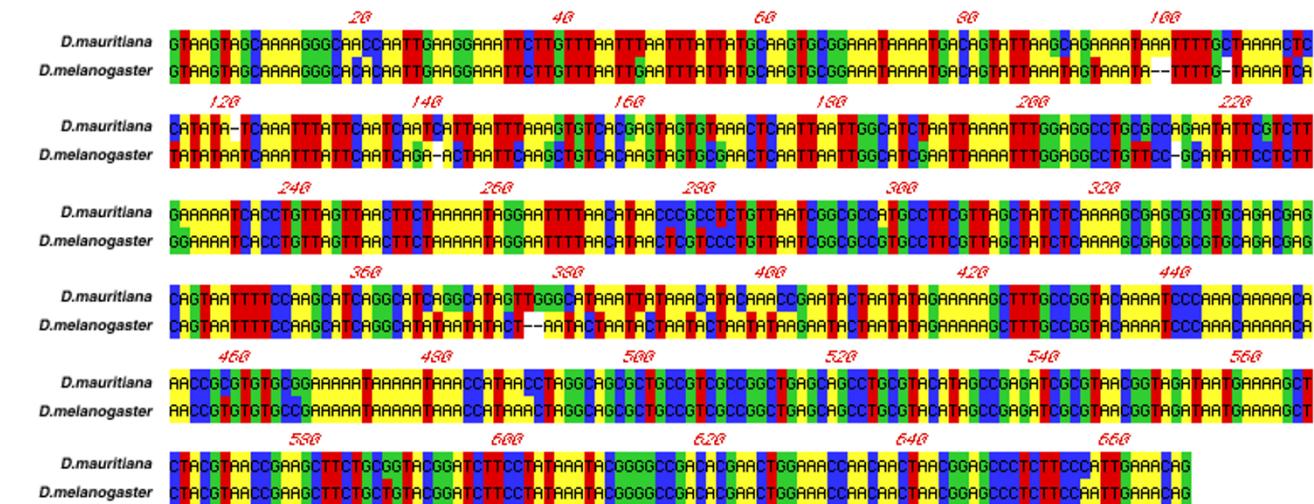


Figure 4
Nucleotide alignment of the 659 bp intron I of the Adh gene in *Drosophila melanogaster* and *Drosophila mauritiana*. Sequences obtained through GenBank for *D.melanogaster* (accession: X60793, [20] and *D.mauritiana* (accession: M19264, [21]). Sequences were aligned using MacVector (See Materials and Methods for details).

Table 2: Primers used for PCR analysis.

Primer	Sequence	Position ¹
cht3(f)	5'-TGATGCCATACTCCTACTCCGTAAAC-3'	27910:27934
cht3(f2)	5'-ATGAAAAACGGATGGACAGCC-3'	18549:18569
cht3(r)	5'-CATTCCCTGTTGCCAACCCC-3'	28395:28376
Antonia LTR(f)	5'-TTAAGCGAACGTCGGAGAC-3'	21299:21317
Antonia LTR(r)	5'-CCACTAGAAGGGTGAAAATGC-3'	21570:21549
cathD(f)	5'-GGTGAAGCCGCCATTACG-3'	220780:220799
cathD(r)	5'-CGCCCAAGCACAAACTTGATTAC-3'	221240:221219
Burdock LTR(f)	5'-TGACCGACGCTTCTAATCTTCC-3'	221638:221659
Burdock element(r)	5'-GGTTGGCAGTATGGAACTTAGTG-3'	221918:221895

¹ Position is relative to location of first nucleotide within clone, AE002743 for cht3 or AE003839 for cathD.

were performed per strain, per gene. For all PCR reactions, 1.0 μl of a single fly DNA prep [38] was used and amplification was performed in a Hot Top equipped Robocycler Gradient 96 (Stratagene, La Jolla, CA). 10 μl of product was separated on a 1% agarose gel in 0.5× TBE running buffer containing 0.25 μg mL⁻¹ ethidium bromide. Gel images were visualized by UV transillumination.

Cht3 PCR

The PCR products for primer set cht3(f) and cht3(r) and primer set Antonia LTR(f) and Antonia LTR(r) were amplified in a 25 μl reaction containing 3 mM MgCl₂, 10X PCR buffer supplied by Pierce (Rockford, IL), 5% DMSO, 0.2 mM dNTPs, 0.5 μM of each primer, and 0.5 U of Taq DNA polymerase supplied by Pierce [Rockford, IL]. The program consisted of an initial incubation at 94°C for 3 min for 1 cycle, a 30 cycle extension at 94°C for 30 sec, 56°C for cht3(f)/cht3(r) primer set or 57°C for Antonia LTR(f)/Antonia LTR(r) primer set for 30 sec, 72°C for 1 min 30 sec, and a 1 cycle final extension of 72°C for 5 min. The PCR products for primer set cht3(f2) and LTR(r) were amplified in a 25 μl reaction containing Expand Long Template PCR System 10X PCR buffer #1 supplied by Roche (Indianapolis, IN), 0.35 mM dNTPs, 0.32 μM of each primer, and 1.3 U of Expand Long Template PCR System DNA polymerase mix supplied by Roche (Indianapolis, IN). The program consisted of an initial incubation at 94°C for 3 min for 1 cycle, a 30 cycle extension at 94°C for 30 sec, 52°C for 30 sec, 68°C for 3 min, and a 1 cycle final extension at 68°C for 5 min.

cathD PCR

The reaction mix and program used for all sets of primers are the same as those described for primer set cht3(f) and cht3(r) and primer set Antonia LTR(f) and Antonia LTR(r) in the Cht3 PCR (above). The annealing temperature for primer set cathD(f) and cathD(r) is 58°C, for primer set

Burdock LTR(f) and Burdock element(r) is 59°C, and for primer set cathD(f) and Burdock element(r) is 56°C.

Sequencing

PCR products of the Cht3 intron were sequenced in the Molecular Genetics Instrumentation Facility at the University of Georgia. Sequences were aligned with Mac Vector 7.0 and compared to the published y; cn bw sp strain. Substitutions and insertion/deletion sites (indels) were summed for each sequence product and compared to the expected divergence based upon the neutral substitution rate. The expected number of polymorphisms between *D. melanogaster* and *D. mauritiana* was calculated based on the *Drosophila* neutral substitution rate of .016 (± 0.005) substitutions per site/million years [18] on 685 bp over a divergence time of 2.7 million years [19].

Note added in proof

The two Cht3 intron fragments described in Figure 3 have the following provisional accession numbers in GenBank:

D. melanogaster, Africa - AY081055

D. mauritiana - AY081054

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