**On the Evolution of Yeti, a Drosophila melanogaster Heterochromatin Gene**

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**Abstract**

Constitutive heterochromatin is a ubiquitous and still veiled component of eukaryotic genomes, within which it comprises large portions. Although constitutive heterochromatin is generally considered to be transcriptionally silent, it contains a significant variety of sequences that are expressed, among which about 300 single-copy coding genes have been identified by genetic and genomic analyses in the last decades. Here, we report the results of the evolutionary analysis of Yeti, an essential gene of Drosophila melanogaster located in the deep pericentromeric region of chromosome 2R. By FISH, we showed that Yeti maintains a heterochromatin location in both D. simulans and D. sechellia species, closely related to D. melanogaster, while in the more distant species e.g., D. pseudoobscura and D. virilis, it is found within euchromatin, in the syntenic chromosome Muller C, that corresponds to the 2R arm of D. melanogaster chromosome 2. Thus, over evolutionary time, Yeti has been resident on the same chromosomal element, but it progressively moved closer to the pericentric regions. Moreover, *in silico* reconstruction of the Yeti gene structure in 19 Drosophila species and in 5 non-drosophilid dipterans shows a rather stable organization during evolution. Accordingly, by PCR analysis and sequencing, we found that the single intron of Yeti does not undergo major intraspecies or interspecies size changes, unlike the introns of other essential Drosophila heterochromatin genes, such as *light* and *Dbp80*. This implicates diverse evolutionary forces in shaping the structural organization of genes found within heterochromatin. Finally, the results of *d_0* - *d_0* tests show that Yeti is under negative selection both in heterochromatin and euchromatin, and indicate that the change in genomic location did not affect significantly the molecular evolution of the gene. Together, the results of this work contribute to our understanding of the evolutionary dynamics of constitutive heterochromatin in the genomes of higher eukaryotes.

**Introduction**

Constitutive heterochromatin is commonly found in large blocks near centromeres and telomeres; it consists mostly of repetitive DNA sequences and maintains its characteristic organization on both homologous chromosomes. It is a ubiquitous component of eu- karyotic genomes and, in many species, comprises large chromosomal portions, or even entire chromosomes. For example, about 30% of the Drosophila and human genomes, and up to 70–90% of certain nematode and plant genomes, are made up of constitutive heterochromatin [1,2,3], yet the reasons for its widespread occurrence are still unclear.

Heterochromatin was originally defined at cytological level as the chromosome portion that stains deeply at prophase and maintains a compact organization throughout all stages of the mitotic cell cycle [4]. Historically, distinctive antagonistic properties compared to the rest of the genome were identified: 1) strongly reduced level of meiotic recombination; 2) low gene density; 3) mosaic inactivation of the expression of euchromatic genes when moved nearby (position effect variegation, PEV); 4) late replication during S phase; 5) transcriptional inactivity; 6) enrichment in the so-called “junk” repetitive DNA, such as satellite sequences and truncated transposable element remnants.

Together, these properties led to the view of constitutive heterochromatin as a “desert” of genetic functions [5]. In the last three decades, however, studies primarily conducted in Drosophila melanogaster have shown that constitutive heterochromatin does in fact play roles in important cellular functions, such as chromosome organization and inheritance [6,7,8,9,10,11]. Although generally regarded as transcriptionally silent, constitutive heterochromatin has been found to contain actively transcribed genes [3]. For example, in Drosophila melanogaster, more than 40 genes essential for viability or fertility have been mapped to pericentric heterochromatin [12,13,14,15,16,17].

In the last decade, the release of D. melanogaster heterochromatin sequence by the Berkeley Drosophila Genome Project (http://www.fruitfly.org/) and Drosophila Heterochromatin Genome Project (http://www.dhgp.org/index_release_notes.html) has greatly facilitated studies of mapping, molecular organization and function of genes located in pericentromeric heterochromatin [18].
More recently, an improved whole genome shotgun assembly [19] has been produced, which includes 20.7 Mb of draft-quality heterochromatin sequence. In the last years, 15 Mb of this sequence have been further improved or completed [20] and a BAC-based physical map of 13 Mb of pericentric heterochromatin, together with the cytogenetic map that locates some 11 Mb to specific heterochromatin regions, have been constructed [20]. About 250 protein-coding genes were defined in the release 5.1 annotation of the currently sequenced heterochromatin DNA [21]. According to these results, the number of active genes in constitutive heterochromatin of \textit{D. melanogaster} appears to be higher than defined by genetic analysis. Notably, these genes encode proteins involved in important cellular and developmental processes [3].

Further mapping of \textit{D. melanogaster} heterochromatin was performed by comparative genomic hybridization [22]. The transcription profiles of mapped sequences by microarray analysis also revealed region-specific temporal patterns of transcription within heterochromatin during oogenesis and in early embryonic development.

Evolutionary studies have shown that \textit{D. melanogaster} heterochromatin genes, such as \textit{light} and others, originated from progenitors that were originally located within euchromatin in the drosophilid lineage [23,24]. Here we have focussed our study on the evolutionary origin of Yeti, an essential heterochromatin gene of \textit{D. melanogaster}, which encodes a protein belonging to the evolutionarily conserved BCNT family of chromatin remodelers [25,26]. We report that Yeti locates in euchromatin in distant species, e.g. \textit{D. pseudoobscura} and \textit{D. virilis}, similarly to what has been found for \textit{light} and other genes [23,24]. Moreover, we found that the Yeti gene structure remains rather stable during the evolution of \textit{Drosophila} species. In particular, the second exon that

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Cytogenetic mapping of heterochromatin genes of chromosome 2. The map was modified from that shown in previous papers [3,45]. The diagram shows the essential genes defined by mutational analyses (below) and annotated genes defined by the heterochromatin genome project (above). Shades of blue correspond to the intensity of DAPI staining, with the darkest blue blocks representing regions with strong fluorescence intensity and open blocks representing non fluorescent regions. The different cytological regions are numbered. doi:10.1371/journal.pone.0113010.g001}
\end{figure}
encodes the last 30 amino acids of the conserved BCNT domain is invariably 91 bp-long. Finally, we found that the single intron of Yeti does not undergo major size changes in D. melanogaster and closely related species, unlike the introns of other essential D. pseudoobscura polytene chromosomes [25,26,28,29].

**Table 1. List of the Yeti orthologs and their encoded proteins.**

| Species          | Gene ID          | Database Location                  | GenBank A.C. (position) | Uniprot reference | Amino acids |
|------------------|------------------|------------------------------------|-------------------------|-------------------|-------------|
| D. melanogaster  | FBgn0128734      | 2Rh:1343403..1345119               | NW_001848856.1          | B4J7U2            | 241         |
| D. simulans      | FBgn0119193      | 3R:38,269..39,056 [-]              | NT_167061.1             | B4QUX4           | 241         |
| D. sechellia     | FBgn0166002      | 2R:170,38,673..3,460 [-]           | NW_001999885.1          | B4IM9             | 241         |
| D. yakuba        | FBgn0236265      | 2R:1,2v,1rh_random_005:668,463..673,172. | NW_002052891.1          | B4IT7S           | 215         |
| D. erecta        | FBgn0103193      | scaffold 4929                     | NW_001956548.1          | B3N457            | 236         |
| D. eugracilis    | not available    | not available                      | KB464511.1 (19716..18949) | not available     | 235         |
| D. biarmipes     | not available    | not available                      | KB462255.1 (18839..19580) | not available     | 239**       |
| D. takahashi     | not available    | not available                      | KB460683.1 (11044..10283) | not available     | 235**       |
| D. elegans       | not available    | not available                      | KB458177.1 (6204..6991)  | not available     | 244**       |
| D. ananassae     | FBgn0088065      | 2R euchromatic region scaffold 13266 | NW_001939294.1          | B3MBR1*           | 272*        |
| D. bipectinata   | not available    | not available                      | KB464371.1 (48012..48773) | not available     | 232         |
| D. pseudoobscura | FBgn0245984      | 2R euchromatic region               | NC_009906.2             | B5E0W2            | 275         |
| D. persimilis    | FBgn0148669      | not available                      | NW_001985955.1          | B4G8J4            | 275         |
| D. miranda       | not available    | not available                      | CM001519.2 (366522..367496) | not available   | 275         |
| D. willistoni    | FBgn0212772      | 2R euchromatic arm                 | NW_002032340.1          | B4JM08            | 273         |
| D. mojavensis    | FBgn0143134      | 2R euchromatic arm                 | NW_001979114.1          | B4KQ8             | 295         |
| D. viridis       | FBgn0209341      | 2R euchromatic arm                 | NW_002014420.1          | B4LK7             | 300         |
| D. albomicans    | not available    | not available                      | JH859027.1 (5894805..5895706) | not available   | 279         |
| D. grimshawi     | FBgn0128734      | 2R euchromatic arm                 | NW_001961673.1          | B4J7U2            | 285         |
| C. quinquefasciatus | CPU018830       | not available                      | NW_001888048.1          | B0XH12            | 284         |
| A. aegypti       | AAEI007422       | supercontig 1,25S, euchromatic region 2p25 [46] | NW_001810963.1          | Q0IF03            | 279         |
| A. darlingi      | not available    | not available                      | ADMH0200609.1 (45710..44829) | not available   | 295         |
| A. gambiae       | AGAP005152       | 2L euchromatic region 21E           | NT_078265.2             | Q7P9P4            | 293         |
| M. destructor    | not available    | not available                      | GLS01532.1 (36921..368309) | not available   | 300         |

**ORF defective. Amino acids deducted from bestfit alignments.**

**Table 1.** The results of this analysis are shown in Figure 2. In D. melanogaster, D. simulans and D. sechellia the Yeti cDNA probe produces a signal mapping to the base of division 41A, in the right arm of chromosome 2 (Figure 2A,B,C). Notably, the signals show a large diffuse structure very different from the sharp hybridization signals usually seen with euchromatic probes; such a morphology is a distinctive mark for sequences derived from partially polytenized heterochromatin regions [30,31]. Together, our FISH results indicate that Yeti maintains a heterochromatic location in D. simulans and D. sechellia. The FlyBase localization of Yeti in D. sechellia is in 2R (scaffold_170:38,673..3,460; Table 1), in accord with our mapping results, while that in D. simulans in 3R (38,269..39,056; Table 1) is apparently conflicting and may reflect an assembly error in the D. simulans genome sequences (see discussion), as reported by Schaffer et al. [32]. In both D. pseudoobscura and D. viridis a single FISH signal was observed in the euchromatic arms of polytene chromosomes, in agreement with FlyBase (Figure 2D, E; Table 1). In D. pseudoobscura, the Yeti PCR probe produced a sharp signal that maps to region 63C in the proximal euchromatin of chromosome 3, while in D. Viridis the Yeti signal is found at region 33E, in the distal euchromatin of chromosome 5. Thus, independently of their genome localization (heterochromatin or euchromatin), in the analysed species Yeti lies

**Results**

Evolutionary repositioning of the Yeti gene from euchromatin to pericentric heterochromatin

The single-copy Yeti gene of D. melanogaster maps to the region h41 of chromosome 2R mitotic heterochromatin (Figure 1; Table 1), which corresponds to division 41A of salivary gland polytene chromosomes [25,26,28,29].

To characterize the chromosomal location of Yeti among Drosophila genus species, we performed fluorescent in situ hybridization (FISH) experiments on polytene chromosomes of D. simulans, D. sechellia, two sibling species of D. melanogaster, and on two distantly related species: D. pseudoobscura, belonging to the Sophophora subgenus and D. viridis, belonging to the Drosophila subgenus. These species cover nearly a 40 million years divergence time and thus represent a wide spectrum of the evolutionary history of Yeti.

To map Yeti in D. simulans and D. sechellia, we used the D. melanogaster Yeti cDNA probe (RE36623), while PCR species-specific probes were used in D. pseudoobscura and D. viridis. PCR

probes were amplified over a less conserved region located outside the C-terminal BCNT coding domain of YETI protein (see Materials and Methods).

The results of this analysis are shown in Figure 2. In D. melanogaster, D. simulans and D. sechellia the Yeti cDNA probe produces a signal mapping to the base of division 41A, in the right arm of chromosome 2 (Figure 2A,B,C). Notably, the signals show a large diffuse structure very different from the sharp hybridization signals usually seen with euchromatic probes; such a morphology is a distinctive mark for sequences derived from partially polytenized heterochromatin regions [30,31]. Together, our FISH results indicate that Yeti maintains a heterochromatic location in D. simulans and D. sechellia. The FlyBase localization of Yeti in D. sechellia is in 2R (scaffold_170:38,673..3,460; Table 1), in accord with our mapping results, while that in D. simulans in 3R (38,269..39,056; Table 1) is apparently conflicting and may reflect an assembly error in the D. simulans genome sequences (see discussion), as reported by Schaffer et al. [32]. In both D. pseudoobscura and D. viridis a single FISH signal was observed in the euchromatic arms of polytene chromosomes, in agreement with FlyBase (Figure 2D, E; Table 1). In D. pseudoobscura, the Yeti PCR probe produced a sharp signal that maps to region 63C in the proximal euchromatin of chromosome 3, while in D. Viridis the Yeti signal is found at region 33E, in the distal euchromatin of chromosome 5. Thus, independently of their genome localization (heterochromatin or euchromatin), in the analysed species Yeti lies
in the syntenic chromosome Muller C, that corresponds to the 2R arm of D. melanogaster chromosome 2.

In silico reconstruction of Yeti gene organization in different sequenced genomes

To study the evolutionary conservation of the Yeti gene organization, we have characterized the structure of the Yeti orthologs in 19 Drosophila species (D. melanogaster, D. simulans, D. sechellia, D. yakuba, D. erecta, D. eugracilis, D. biarmipes, D. takahashi, D. elegans, D. ananassae, D. bipectinata, D. pseudoobscura, D. persimilis, D. miranda, D. willistoni, D. mojavenisii, D. virilis, D. albomicans, and D. grimshawi) and in five non-drosophilid dipterans (C. quinquefasciatus, A. Aegypti, A. darlingi, A. gambiae and M. destructor pest crop).

All the Yeti DNA sequences were retrieved from FlyBase. In case of annotated genes, the Yeti protein sequences were extracted from the Ortho DB database [33], where they are reported as orthologous sequences belonging to the BCNT family complex [26,34]. For the recently sequenced genomes, the Yeti DNA sequences were recovered by the TblastN procedure, using the D. melanogaster Yeti protein sequence. The alignments of the retrieved Yeti orthologs are shown in Figure 3 and their coordinates are reported in Table 1.

We were able to recover a deducted complete protein sequence except for the recently added genomes of D. biarmipes, D. takahashi and D. elegans, where frame-shift mutations were found in the 5' end of the gene, probably due to errors in sequencing that needs to be improved. However, in each case the 3' end of the gene, containing the BCNT domain-coding region, was detected (Figure 5). The protein sequence alignments show a strong conservation of the BCNT domain in the Drosophila genus and in non-drosophilid dipterans (Figure 4).

We next compared the reconstructed molecular organization of the Yeti gene in the above-mentioned species, to study whether it underwent substantial structural changes during evolution. The results of this analysis are shown in Figure 5. It appears that the structure of the Yeti orthologs, with two exons and one intron remained highly conserved during the divergence of the lineages in the Drosophila genus, the only exception being D. willistoni, where an additional intron of 75 bp is present. The gene size is identical among D. melanogaster, D. simulans and D. sechellia, the only detectable difference is represented by the intron that in D. melanogaster is 7 bp longer. In general, in the Drosophila genus both the first exon and the single intron undergo changes in size: the exon varies from 557 bp (D. yakuba) to 812 bp (D. virilis), while the intron spans from 51 bp (D. biarmipes) to 70 bp (D. willistoni). Notably, the size of the second exon, that encodes the last portion of the BCNT domain, shows a striking conservation in the Drosophila genus, being invariably 91 bp long. Finally, in the five genome species of non-drosophilid dipterans (C. quinquefasciatus, A. Aegypti, A. darlingi, A. gambiae and M. destructor) the organization of Yeti differs in that the intron disappears and a single exon is present.

Characterization of the Yeti intron in Drosophila species

Intraspecific and interspecific size polymorphisms of heterochromatin gene introns have been found in Drosophila, which are likely to be associated with de novo insertions of TE-related sequences [27]. We then asked whether the intron of Yeti is prone to TE insertions or to other gross changes in length. To answer this question, we PCR amplified a region of about 180 bp comprising the Yeti intron from genomic DNAs extracted from D. melanogaster and D. simulans strains. Most of the analyzed strains derived from geographically distant natural populations (see materials and methods for the complete list). We also included in the analysis a single strain of D. sechellia and D. teissieri. The rationale of these experiments is that if an insertion have targeted the intron, it would have in turn increased the expected size of the amplified region.

We analysed 25 wild type strains and 4 laboratory stocks of D. melanogaster, as well as 9 wild type strains of D. simulans (see materials and methods). As shown in Figure 6, PCR amplification of Yeti produces a prominent band of the expected size in all the different strains of D. melanogaster (Figure 6A) and of D. simulans (Figure 6B) and in both D. sechellia and D. teissieri strains (Figure 6C). Sequencing of the purified PCR products from Iso and Scansano (D. melanogaster), Chicharo and Death Valley (D. simulans), D. sechellia and D. teissieri confirmed that they correspond to the Yeti intron-containing region (Figure 7). In conclusion, the results of this analysis suggest that the small intron of Yeti does not frequently undergo significant increase in size, unlike other essential heterochromatin genes of Drosophila [27]. This conclusion is in agreement with the observation that the Yeti gene structure tends to be stable during the evolution of the Drosophila genus, with the single intron that retains its short size (Figure 5).

Yeti is under negative selection

We next asked whether Yeti has evolved under negative (purifying) or positive selection, and whether the change in
location from euchromatin to heterochromatin may have affected the molecular evolution of the gene. To this aim we performed a $d_s - d_\text{K}$ test, a codon-based test of selection (for details see material and methods), using DNA sequences from five representative Yeti genes: three located in heterochromatin ($Dmel$ Yeti, $Dsec$ Yeti and $Dere$ Yeti) and two in euchromatin ($Dpse$ Yeti and $Dvir$ Yeti). The results suggest that Yeti is under purifying (negative) selection when present both in heterochromatin and euchromatin. Thus, the change in genomic location does not appear to have affected significantly the molecular evolution/function of Yeti (Table 2).

**Discussion**

In this paper, we have studied the evolutionary origin of Yeti, an essential gene of *Drosophila melanogaster* (Figure 1) located in the...
deep heterochromatin of chromosome 2 [25,26,28,29] and found that it has evolved from a euchromatic ancestor in drosophilids.

Our FISH analysis shows that Yeti maintains a heterochromatin location in 2R, at the base of division 41A, in both D. simulans and D. sechellia sibling species of D. melanogaster (Figure 2). The FlyBase localization of Yeti in D. sechellia scaffold, 170:38,673..3,460, is in accord with our mapping results, while D. simulans Yeti is reported to map to the 3R arm (3,765..73,764; Table 1) in a 70 kb gene-poor genomic region. However, we are confident about the heterochromatin location of Yeti in D. simulans for the following reasons: First, as discussed in the result section, the FISH signal morphology produced by the Yeti probe is different from that usually seen with euchromatic probes and represents a distinctive mark for sequences derived from polytenized heterochromatin [30,31]. Second, our FISH mapping of Yeti in D. simulans is based on three reproducible experiments, each carried out on several polytene chromosome figures obtained from at least 10 larvae. Finally, the paucity of genes around D. simulans Yeti is per se highly suggestive of heterochromatin localization. Thus, it is possible that the apparent discrepancy between our data and FlyBase may reflect an assembly error that occurred in the D. simulans genome sequence assembly, as reported by Schaffer et al. [32].

Our FISH analysis show that in two distantly related species, D. pseudoobscura and D. virilis, Yeti is located in euchromatin (Figure 2). In D. pseudoobscura, Yeti maps to chromosome 3 at polytene division 63C, while in D. virilis it is found in chromosome 5, at polytene division 53E. Interestingly, Yeti lies in the syntenic chromosome Muller C that corresponds to the 2R arm of D. melanogaster chromosome 2. Together, the results of our analysis indicate that during the evolution of the Drosophila genus, Yeti has been resident on the same chromosomal element, but over time it progressively moved closer to the pericentric regions. Such movements would have occurred in about 40 million years, the estimated divergence time between D. melanogaster and D. virilis (Figure 8). A similar evolutionary trend was reported for light and other neighboring genes in 2L heterochromatin [23] and for other genes of chromosome 3 heterochromatin [24].

![Figure 4. Alignment of the BCNT domain of YETI proteins among species. The arrow points the intron position in the corresponding coding region of Drosophila species.](https://doi.org/10.1371/journal.pone.0113010.g004)

| ATG | STOP |
|-----|------|
| $\$ | 635 |
| $\$ | 635 |
| $\$ | 635 |
| $\$ | 557 |
| $\$ | 620 |
| # | 617 |
| # | 617 |
| # | 616 |
| # | 643 |
| $\$ | 620 |
| # | 608 |
| # | 737 |
| # | 737 |
| $\$ | 155 |
| $\$ | 576 |
| $\$ | 797 |
| $\$ | 812 |
| # | 749 |
| $\$ | 757 |
| $\$ | 855 |
| $\$ | 840 |
| # | 882 |
| $\$ | 882 |
| # | 903 |

![Figure 5. Comparison of the Yeti gene structure among sequenced genomes. Only the coding regions are showed. Exons are in boxes and numbers refer to nucleotides. Symbols: $\$,$ annotated genes; #, this study; **, defective ORF. The grey area at the 3’ end represents the conserved BCNT-C domain in the protein.](https://doi.org/10.1371/journal.pone.0113010.g005)
A striking difference between heterochromatin and euchromatin genes lies in the generally larger size and complex molecular structure of the former compared to the latter. The example of the ‘giant’ Y-chromosome fertility factors of *D. melanogaster* mentioned above is paradigmatic in this respect [12]. Some of the essential heterochromatin genes of chromosomes 2 and 3 are also large due to the presence of large introns that harbour truncated TE copies (or TE “remnants”) [27,35,36,37]. In this context, the *Yeti* gene of *D. melanogaster* with a 900 bp-long genomic region represents an exception [26]. The same is true for *RpL38, RpL5* and *RpL15*, three essential ribosomal protein-coding genes located in the heterochromatin of chromosomes 2 and 3, all of which are of relatively small size [24,26,37,38]. How might these observations be explained?

![Figure 6. PCR amplification of the genomic region containing the Yeti intron in Drosophila species.](image)

A single PCR product of about 180 bp was found, in *D. melanogaster* (A) and *D. simulans* (B) *D. sechellia* (C) and *D. tessieri* (C) related species. M = Marker; Frib = Friburgo; Mali = Mali; Scan = Scansano; Iso = *y*; cn’ bw’sp’ isogenic strain; OR = Oregon-R; Bej = Bejin; Chi = Chicharo; DV = Death Valley; Gen = Genoa; Mor = Moruya; Kyo = Kyogle; Arm = Armidale; Can = Canaries; Sech = *D. sechellia*; Tes = *D. tessieri*. Molecular weight is in bps.

![Figure 7. Sequencing of the purified PCR products from Drosophila species.](image)

Sequence alignments from Iso and Scansano (*D. melanogaster*), Chicharo and Death Valley (*D. simulans*), *D. sechellia* and *D. tessieri*. Sequence analysis confirmed that they correspond to the *Yeti* intron containing region. The *Yeti* intron is shown in normal text, the flanking exons are in bold. The *D. simulans* and *D. sechellia* intron lacks a 7 bp stretch (see the gap), in agreement with the genome sequence data (see results and Figure 5). The *D. Tessieri* intron sequence is identical to that of *D. simulans* and *D. sechellia*.

![A diagram showing PCR results](diagram)

![A table of sequences](table)
One may imagine that, during evolution, genes increased their size by becoming targets for reiterated transposable-element insertions in the intronic regions, depending on their time of residence in heterochromatin. This, however, does not seem to have been the case. In fact, the light and Yeti genes, although having likely resided in heterochromatin for a comparable evolutionary time (less than 30 million of years), underwent a different molecular architecture; the light gene structure dramatically changed during the evolutionary transition from euchromatin to heterochromatin, due to a remarkable increase in the size of introns targeted by TEs [23]; Yeti retained its original organization in all analysed species, with a short genomic region carrying a single short intron (Figure 5). In addition, by PCR analysis we found that the Yeti intron does not undergo significant interspecies or intraspecies changes of its physical size (Figure 6). Similarly, the Rpl15 gene shows a conserved structure among Drosophila species, independently from its genomic location [24].

How to explain the different behaviour of heterochromatin genes? In particular, there might have been a selective pressure to maintain some genes of short size (with few, short introns) despite of their genomic location, owing to their particular functional requirement: interestingly in that respect, highly expressed genes are known to harbour substantially shorter introns than genes that are expressed at low levels [39]. This may be the case of Yeti and Rpl38, Rpl5 and Rpl15 heterochromatic genes of Drosophila melanogaster, which are all highly expressed and have all indeed short size and carry short introns [26,37,38]. Yeti itself encodes an important chromatin-remodeling factor required for development [26] and ribosomal protein coding genes are also essential for proper development. It is not unreasonable to speculate that these genes maintained the original structure, in spite of their transition to heterochromatin during massive chromosomal rearrangements that occurred over time, because of the requirement for their efficient expression during early development. In addition, it is possible that once in heterochromatin, a given sequence might be differentially targeted by transposable elements, with some sequences being more refractory than others. These observations suggest that the evolutionary forces that acted in shaping the structural organization of genes currently found in D. melanogaster heterochromatin are molecularly diverse.

Finally, the results of our dS - dK tests, showing that Yeti is under negative selection both in heterochromatin and euchromatin (Table 2), are in accord with its evolutionary conserved function and suggest that the change in genomic location did not affected significantly the molecular evolution of the gene.

Together, these results contribute to expand our understanding of the molecular dynamics driving the evolution of the heterochromatin genome in higher eukaryotes.

### Materials and Methods

**Drosophila strains**

Fly cultures were carried out at 25°C in standard cornmeal yeast medium. D. melanogaster strains derived from natural populations: Altamura I and Altamura II (South Italy), Beijing (China), California and California I (USA), Charolles (France), Charolles 1999 (France), Friburgu 1997 (Germany), HJ30 (France), Hobo (Greece) Israel-4 (Israel), Lanuvio (Italy), Luminy (France), Mali and Mali I (West Africa), Marrakesh (North Africa), Scansano (Central Italy), Vallesc (Spain), W15 (USA), W30 (USA), W90 (USA), W130 (USA), W135 (USA), Zimbabwe (Africa), Gaiano (North Italy). D. melanogaster laboratory strains (separated by comma): isogenized y'; cn^1,bw^1,sp^1 strain (iso) [40]. l(2) LP2/SIM1, Cy. Cy/Sp; Sh, Delta2-3, ry^106/TM6, ry^106, l(2)EMS31/SIM1, Cy. D. simulans strains derived from natural populations: Armidale (New South Wales, Australia), Canaries (Atlantic Spain), Can River (Australia) Chantal (France), Chicharo (Portugal), Death Valley (USA), Genoa (Italy), Kyogle (Australia) and Moruya (New South Wales, Australia). D. melanogaster and D. simulans wild-type strains were derived from natural populations collected in the wild before the end of year 2000 and are gifts of Sergio Pimpinelli, Nikolaj Junakovic, Chantal Vaury and Pierre Capy.

**Cytology and fluorescent in situ hybridization**

Polytene chromosomes prepared according to Pardue [41] were stained with DAPI. The D. melanogaster RE36623 cDNA Yeti probe was labelled by nick-translation with Cy3-dCTP (Amersham). Species-specific PCR probes were used for FISH in D. pseudobscura and D. viridis (see below). FISH procedures were performed according to Dimitri [42]. Digital images were obtained...
Nucleic acid manipulation and sequence alignments

Genomic DNA extraction and PCR were performed according to the protocol of Berkeley Drosophila Genome Project (http://www.fruitfly.org/about/methods/index.html). To PCR amplify Yeti genomic fragments from D. melanogaster, D. simulans strains, D. sechellia and D. erecta the following primers were used: F: 5’- TTAGTATGGCTGGAGAGCA-3’; R: 5’-TGTGCCCTGGCA- TAACAAAGGC-3’.

PCR cycle were: 40 × at 98°C, 40 x (10° 98°C, 30° 58°C, 8° 72°C). Amplified fragments were gel purified and sequenced by Bio-Fab research s.r.l. The Yeti probes from D. pseudoobscura and D. viridis were generated by PCR over genomic DNA with the following primers:

Dps-F 5’-GGGACGGATGAGCATCAAT-3’; Dps-R 5’- GTGAGTGTCCGCTGCCTAT-3’

Dvir-F 5’-AGCTAAACGTAGCACGCGTC-3’; Dvir-R 5’- TGTGTACGCAGATCCTCGTC-3’

PCR cycle were: 4’ at 94°C, 35 x (30° 95°C, 45° 60°C, 30° 72°C). Amplified fragments were cloned in pGEM-T vector (Promega) and verified by DNA sequencing.

Multiple sequence alignment were performed by ClustalW procedure available at EMBL-EBI (http://www.ebi.ac.uk) or with the multalin interface at http://multalin.toulouse.inra.fr.

References

1. Moreitz KB, Roth GE (1976) Complexity of germline and somatic DNA in Ascaris. Nature 259: 55–57.
2. Peterson DG, Pearson WR, Stack SM (1998) Characterization of the tomato (Lycopersicon esculentum) genome using in vitro and in situ DNA reassociation. Genome 41: 346–356.
3. Dimitri P, Caizzi R, Giordano E, Carmela Accardo M, Lattanzi, et al. (2009) Constitutive heterochromatin: a surprising variety of expressed sequences. Chromosoma 118: 419–435.
4. Heitz E (1928) Das Heterochromatin der Moose. Jb Wiss Bot 69: 762–818.
5. John B (1980) The biology of heterochromatin. In: Heterochromatin: Molecular and Structural aspects of V. R. S., Cambridge University Press. p. 1–128.
6. Williams SM, Robbins LG (1992) Molecular genetic analysis of Drosophila rRNA arrays. Trends Genet 8: 335–340.
7. Dernburg AF, Sedat JW, Hawley RS (1996). Direct evidence of a role for heterochromatin in meiotic chromosome segregation. Cell 86: 135–146.
8. Elgin SCR (1996) Heterochromatin and gene regulation in Drosophila. Current Opinion in Genetics and Development 6: 193–200.
9. Karpen GH, Le MG, Le H (1996) Centric heterochromatin and the efficiency of achiasmate disjunction in Drosophila female meiosis. Science 273: 118–122.
10. Parich ML (2007) Following the chromosome path to the garden of the genome. Annu Rev Cell Dev Biol. 2007; 23: 1–22.
11. Villasante A, Méndez-Lago M, Abad JP, Montejo de García E (2007) The birth of the centromere. Cell Cycle 6: 2072–2076.
12. Gatti M, Pimpinelli S (1992) Functional elements in Drosophila melanogaster heterochromatin. Annu Rev Genet 26: 239–275.
13. Hilliker AJ (1976) Genetic analysis of the centromeric heterochromatin of chromosome 2 of Drosophila melanogaster: deficiency mapping of EMS-induced lethal complementation groups. Genetics 83: 765–782.
14. Marchant GE, Holm DG (1980) Genetic analysis of the heterochromatin of chromosome 3 in Drosophila melanogaster. II. Vital loci identified through EMS mutagenesis. Genetics 120: 519–532.
15. Dimitri P (1991) Cytogenetic analysis of the second chromosome heterochromatin of Drosophila melanogaster. Genetics 127: 533–564.
16. Koryakov DE, Zhimulev IF, Dimitri P (2002) Cytogenetic analysis of the third chromosome heterochromatin of Drosophila melanogaster. Genetics 160: 509–517.
17. Fitzpatrick KA, Sinclair DA, Schulze SR, Syszykowa M, Honda BM (2005) A genetic and molecular profile of third chromosome centric heterochromatin in Drosophila melanogaster. Genome 48: 571–584.
18. Adams MD, Celahker SE, Holt RA, Evans CA, Gocayne JD, et al. (2000) The genome sequence of Drosophila melanogaster. Science 287: 2185–2193.
19. Hoskins RA, Smith CD, Carlsson JW, Carvalho AB, Halpern A, et al. (2002) Heterochromatic sequences in a Drosophila whole-genome shotgun assembly. Genome Biology 3: research0085.1–0085.16.
20. Hoskins RA, Carlsson JW, Kennedy C, Acevedo D, Evans-Holm M, et al. (2007) Sequence finishing and mapping of Drosophila melanogaster heterochromatin. Science 316: 1625–1628.
21. Smith CD, Shea S, Mungall CJ, Karpen GH (2007) The Release 3.1 annotation of Drosophila melanogaster heterochromatin. Science 316: 1586–1591.
22. He B, Caudy A, Parsons L, Rossebrock A, Pane A, et al. (2012) Mapping the pericentric heterochromatin by comparative genomic hybridization analysis and chromosome deletions in Drosophila melanogaster. Genome Res 22: 2107–2119.
23. Yasuhara JC, DeCresce CH, Wakiimoto BT (2005) Evolution of heterochromatic genes of Drosophila. Proc Natl Acad Sci USA 102: 10958–10963.
24. Schulze SR, McAllister BF, Sinclair DA, Fitzpatrick KA, Marchetti M, et al. (2006) Heterochromatic genes in Drosophila: a comparative analysis of two genomes. Genetics 173: 1433–1445.
25. Cenci G, Belloni G, Dimitri P (2003) 1/241Aa, a heterochromatic gene of Drosophila melanogaster, is required for mitotic and meiotic chromosome condensation. Genet Res 81: 15–24.
26. Messina G, Damia E, Fanti L, Atteratto MT, Celauro E, et al. (2014) Yeti, an essential Drosophila melanogaster gene, encodes a protein required for chromatin organization. J Cell Sci 127: 2577–2580. doi:10.1242/jcs.150243
27. Dimitri P, Junakovic N, Arca B (2003) Colonization of heterochromatic genes by transposable elements in Drosophila. Mol Biol Evol 20: 303–512.
28. Dimitri P, Corradini N, Rossi F, Verini F, Geneci G, et al. (2003) Vital genes in the heterochromatin of chromosomes 2 and 3 of Drosophila melanogaster. Genetics. 2006: 209–215.
29. Rossi F, Moschetti R, Caizzi R, Corradini N, Dimitri P (2007) Cytogenetic and molecular characterization of heterochromatin gene models in Drosophila melanogaster. Genetics 175: 595–607.
30. Zhang P, Sprodling AC (1993) The Drosophila salivary gland chromocenter contains highly polytenized subdomains of mitotic heterochromatin. Genetics. 1995: Feb; 139(2): 659–70.
31. Berghola L, Dimitri P (1996) The heterochromatic rolled gene of Drosophila melanogaster is extensively polytenized and transcriptionally active in the salivary gland chromocenter. Genetics. 1996 Sep; 144(1): 117–25.
32. Schaefer SW, Bhutkar A, McAllister BF, Matsuda M, Matzkin LM, et al. (2008) Polytene chromosomal maps of 11 Drosophila species: the order of genomic scaffolds inferred from genetic and physical maps. Genetics 178: 1601–55.
33. Waterhouse RM, Zdobnov EM, Tegenfeldt F, Li J, Kriventseva EV (2011) OrthoDB: the hierarchical catalog of eukaryotic orthologs in 2011. Nucleic Acids Res. 39(Database issue): D283–8.

34. Iwashita S, Osada N, Itoh T, Sazaki M, Osawa E, et al. (2003) A transposable element-mediated gene divergence that directly produces a novel type bovine Bcnt protein including the endonuclease domain of RTE-1. Mol Biol Evol 20: 1556–1563.

35. Devlin RH, Bingham B, Wakimoto BT (1990a) The organization and expression of the light gene, a heterochromatic gene of Drosophila melanogaster. Genetics 125: 129–140.

36. Tulin A, Stewart D, Spradling AC (2002) The Drosophila heterochromatic gene encoding poly(ADP-ribose) polymerase (PARP) is required to modulate chromatin structure during development. Genes and Development 16: 2108–2119.

37. Schulze SR, Sinclair DA, Fitzpatrick KA, Honda BM (2005) A genetic and molecular characterization of two proximal heterochromatic genes on chromosome 3 of Drosophila melanogaster. Genetics 169: 2165–2177.

38. Marygold SJ, Coelho CM, Leevers SJ (2005) Genetic analysis of RpL38 and RpL5, two minute genes located in the centric heterochromatin of chromosome 2 of Drosophila melanogaster. Genetics 169: 683–695.

39. Castillo-Davis CI, Melkote SL, Hartl DL, Koonin EV, Kondrashov FA (2002) Selection for short introns in highly expressed genes. Nat Genet 31: 415–418.

40. Brinza EJ, Efstratiou L, Ballard J, Tamkun JW, Kennison JA (1994) Genetic analysis of the brachia gene of Drosophila melanogaster and polytene chromosome subdivisions 72AB. Genetics 137: 803–813.

41. Pardue ML (1986) In situ hybridization to DNA of chromosome and nuclei. In: Roberts DB (ed) Drosophila: a practical approach. IRL Press, Oxford, pp. 111–137.

42. Dimitri P (2004) Fluorescent in situ hybridization with transposable element probes to mitotic chromosome heterochromatin of Drosophila. Methods Mol Biol 260: 29–39.

43. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution 30: 2725–2729.

44. Nei M, Gojobori T (1986) Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Molecular Biology and Evolution 3: 418–426.

45. Coulthard AB, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution 30: 2725–2729.

46. Timoshevskiy VA, Kinney NA, deBruyn BS, Mao C, Tu Z, et al. (2014) Genomic composition and evolution of Aedes aegypti chromosomes revealed by the analysis of physically mapped supercontigs. BMC Biol 12: 27. doi:10.1186/1741-7007-12-27.