Characterizing the transcriptional expression and in situ localization of the Dnmt2 gene in Drosophila willistoni

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Abstract

Background: Organisms that have only the DNA methyltransferase 2 (Dnmt2) to mediate the DNA methylation are called "Dnmt2-only" and they have been investigated in recent surveys. Drosophila is one of the “Dnmt2-only” organisms and is also an ideal model for Dnmt2 research. However, the biological function of the Dnmt2 protein is still uncertain. Some studies have pointed to a putative role during the early stages of invertebrate development. In this work, we present our findings on the Dnmt2 expression in D. willistoni, a neotropical species of large ecological versatility and peculiar molecular features. Results: By RT-PCR and in situ hybridization we demonstrate here the presence of transcripts not only in the early stages of development, but also during the oogenesis. Using qPCR analysis, we verify that Dnmt2 transcription level is higher during early stages of development, though transcription levels are subtly higher in D. willistoni adults than in D. melanogaster levels found in previous studies. We also mapped the Dnmt2 on the IIL chromosome arm (Muller’s B element) of D. willistoni, near at the end of the singular telomeric region. Conclusions: Our findings give insights on the possible biological function of Dnmt2-related processes associated with the development and differentiation of oocytes since germinative tissue formation seems to require a higher expression of Dnmt2. The Dnmt2 localization in the subtelomeric region brings up a series of issues that involve the peculiar characteristics of D. willistoni Dnmt2 enzyme, like evolutionary patterns and the epigenetic phenomena of sex-specific methylation.

Background

Cytosine-5 RNA methylation is one among more than 100 distinct RNA modifications that has been already detected in tRNA, rRNA and mRNA [1], representing an important epigenetic modification that regulates gene expression in eukaryotes, with a standing role on their development and for the etiology of human diseases and mediating chromatin organization [2–4]. Elucidation of the molecular mechanisms mediating RNA and DNA methylation is crucial to understanding the roles that diverse nucleic acids play in the regulation of genetic information.

Although DNA methylation appears to be a widespread epigenetic regulatory mechanism, genomes are methylated in different ways in diverse organisms. DNA methylation in plant genomes, for instance, can occur symmetrically at cytosine nucleotides in both CG and CHG (H= A, T or C) contexts, whereas DNA methylation systems are well characterized in vertebrates [5] and occur mostly symmetrically at the cytosine of a CG dinucleotide [6,7]. The cytosine methylation is established and maintained by a family of conserved methyltransferases. In eukaryotes, there are three distinct families of DNA methyltransferases: Dnmt1, Dnmt2, and Dnmt3. Whereas Dnmt3 enzymes seem to be responsible for establishing DNA methylation patterns (*de novo* methyltransferases), Dnmt1 enzymes are involved in the maintenance of the methylation patterns [8]. On the other hand, the biochemical activity and the biological function of Dnmt2 enzymes are still an open case.

Evidence for DNA methylation has been described and its importance demonstrated in different insect orders. The honeybee genome contains genes that encode orthologues of all vertebrate proteins required for DNA methylation [9,10]. In addition to Dnmt2 (also found in Diptera), two CpG-specific Dnmt family genes were identified: Dnmt1 and Dnmt3a/b genes [11]. The specificity for CpT and CpA nucleotide residues distinguishes Dnmt2 from all other known animal DNA methyltransferases and confirms our previous suggestion of predominant non-CpG methylation in *Drosophila* [12,13]. The *Apis mellifera* genome shows greater similarities to vertebrate genomes than *Drosophila* and *Anopheles* genomes for the genes involved in DNA methylation, among others (The Honeybee Genome Sequencing Consortium, 2006). For these reasons, methylation in *D. melanogaster* and other invertebrates remains a controversial research area.

Regarding the genus *Drosophila*, the species of this genus are so-called "*Dnmt2*-only" organisms, not containing any of canonical vertebrates DNA methyltransferases homologs (Dnmt1 and Dnmt3). For the species of this genus studied so far, methylation was demonstrated at non-CpG nucleotides [13–15], in contrast to most organisms already analyzed. Furthermore, the specificity for CpT, CpA, and CpC nucleotide residues distinguishes Dnmt2 from all other known animal DNA methyltransferases [13,16], and the functional role of DNA methylation in *Drosophila* remains unclear. However, adding to that discussion, a recent study describes the importance of the methyl-CpG binding domain proteins to inhibit interspecies courtship and promoting aggression in *Drosophila* [17]. Another important difference between DNA methylation in mammals and *Drosophila* is the level of the cytosine nucleotides methylated. Whereas mammals have 2 to 10% of all cytosine residues modified to 5-methylcytosine [18,19], *Drosophila melanogaster* has only about 0.1 - 0.6% [14,20], making it experimentally difficult to demonstrate unambiguously DNA methylation in this organism. Moreover, in contrast to the pattern of genome-wide DNA methylation in vertebrates, DNA methylation is relatively scattered in invertebrates [21].

Several attempts were made to demonstrate the DNA methylation activity of Dnmt2 in *D. melanogaster* [13,22] revealing a low, but significant activity by distinct experimental methods. Overexpression of *Dnmt2* in *Drosophila* species appears to enhance genome-wide DNA methylation from 0.2% to 0.4 – 0.7%, as determined by capillary electrophoresis [13,22]. Nevertheless, in a recent study reported that Dnmt2 controls DNA methylation in early *D. melanogaster* embryos and provide insight into its function in control of retrotransposon silencing and telomere integrity in somatic cells [23]. This report showed a clear-cut difference of methylation within Invader4 elements LTRs between wild type and Dnmt2-/- flies [23]. Dnmt2 appears to mediate methylation on *D. melanogaster* embryos genome, even though both this activity and its functional consequences remaining poorly understood. However, a recent study showed that cytosine methylation in the genome of *Drosophila melanogaster* probably is independent of Dnmt2 activity [24]. These findings show how elusive is the genomic methylation context in drosophilids.

Previous studies reported by our research group in the neotropical *D. willistoni* and its related species (*willistoni* subgroup) shows a distinct scenario. Adult flies show sex-specific patterns of rDNA genes investigated by Methylation Restriction Sensitive Endonucleases [15,25]. Comparisons of *D. willistoni* and *D. melanogaster* Dnmt2 protein sequences indicated higher primary structure conservation on motifs

responsible for the catalysis of methyl transfer and great variability in the region related to specific recognition of target DNA sequences. These outcomes from the *willistoni* subgroup species are encouraging due to the previously reported peculiarities of this species when compared to other species of the *Drosophila* genus that have their genomes sequenced [26]. *D. willistoni* singularities correspond to overall genome size, distribution of transposable element classes, patterns of codon usage, dot chromosome lacking and unclear phylogenetic clustering placement, among others [26–28].

In addition to the peculiarity of having sex-specific methylation [15,25], *D. willistoni* is known to have extensive gene arrangement polymorphisms on all chromosomes [29–32] as observed from chromosomal variability in natural populations. Because of the high rate of intraspecific polymorphism and and a deep evolutionary time that separates *D. willistoni* from the other species of *Drosophila 12* Genomes Project, several methods of analysis have demonstrated difficulty in establishing the correct phylogenetic positioning of this clade [33]. The ambiguity arises as a consequence of the elevated level of sequence and gene-order evolution that leads to a significantly long lineage leading to *D. willistoni*. Most phylogenetic reconstruction software tends to force *D. willistoni* as an outgroup, based on the high evolutionary rates. In light of these reasons, studies are needed to map more genes in the genome of *D. willistoni* in order to elucidate the discrepancies found in the previous reports. New approaches, using an additional marker will not only identify more genes to confirm the ordering of scaffolds assembled so far but will also facilitate further comparative studies by increasing the database from this species.

To improve the Dnmt2 knowledge about its biological function and importance, more assays for characterization and detection of Dnmt2-dependent DNA/RNA methylation will have to be established. Genomic DNA methylation patterns need to be characterized mostly in "*Dnmt2*-only" model systems, like *Drosophila*. In the present study, we detected and quantified the expression of *D. willistoni Dnmt2* in different development stages. Accordingly, we also detected the expression of Dnmt2 on oogenesis and embryogenesis, indicating a possible contribution of *Dnmt2* expression during development. In addition, we localized *Dnmt2* homologs on polytene chromosomes of *D. willistoni* and we attempted to establish cause and/or effect relationships for the exact location of the gene with its adjacent sites and possible activity modulated by position.

Material And Methods

Detection of Dnmt2 transcripts by Reverse Transcription PCR (RT-PCR)

RNA was obtained from *D. willistoni* adult males, adult fertilized females, adult females without ovaries, pupae, larvae and 0-3h dechorionated embryos using Trizol, according to the manufacturer's protocol (Invitrogen). Extracted RNA was further treated with DNaseI (Promega) to eliminate DNA contamination. cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher) with random primers. After cDNA synthesis, *Dnmt2* fragment was amplified by PCRs with the following primers wDnmt2AF: 5'-CAGGCTCGCCAACAGTTTAT-3' and wDnmt2BR: 5'-CCTTGTCCTTGCGTGCTAAA-3'. Primers were designed based on the *D. willistoni* Dnmt2 sequence [15]. The PCR reaction was performed in 25μL reactions using 20ng of cDNA, 1U Platinum Taq DNA Polymerase (Invitrogen), 1x reaction buffer,

200μM dNTPs, 20pmol of each primer and 1.5mM MgCl2. The amplification conditions were 95°C for 5 min and 30 cycles of 95°C for 40s, 55°C for 40s and 72°C for 1 min, followed by a final extension cycle at 72°C for 5 min. A fragment of 280 bp was expected as a result of the amplification reactions. The *β-actin* housekeeping gene was used as a control for amplification. PCR products were verified by electrophoresis on a 1% agarose gel and stained with GelRed GelRed™ (Sigma-Aldrich).

Expression analysis by Quantitative Real-Time PCR

The relative abundance of *D. willistoni* Dnmt2 mRNA transcripts was measured by quantitative real-time PCR (qPCR) using an Applied Biosystems (ABI) 7500 Real-Time PCR System with the same primers of conventional RT-PCR (*Dnmt2* and *β-actin* gene). Samples of cDNA from *D. willistoni*, produced as previously described, were used. The qPCR conditions were: 94°C for 5 min followed by 40 cycles at 95°C for 15s, 60°C for 10s, 72°C for 15s and 35s at 60°C to measure fluorescence. Next, samples were heated from 55ºC to 99°C at a 0.1°C/s temperature gradient to construct the denaturation curve of the amplified products. Relative quantifications of amplified products were made by the 2−ΔΔCt method [34] and Ct values were obtained in the SDS software. SYBR-green (Molecular Probes) was used to detect amplification and to estimate Ct values, as well as to determine specificity of the amplicons by denaturing curves and melting temperatures (Tm). The *β-actin* gene was used as the internal control gene for all relative expression calculations.

Inspecting *Dnmt2* mRNA transcripts by *in situ* hybridization in embryos

For *Dnmt2* riboprobe synthesis, the fragment of 949-bp of the gene was obtained from genomic DNA of adult flies by PCR using the primers wDnmt2C-F: 5' TCACCCACAACCTTGACATT 3' and wDnmt2D-R: 5' ACCTTCTCGCAGACACCAA 3'. Resulting PCR products were cloned into pCR4-TOPO vector (Invitrogen) and submitted to automatic nucleotide sequencing performed by Macrogen Inc. (Korea), to assign insert orientation. Resulting plasmids were then linearized according to the vector map and digoxigenin-labeled using a DIG-labeled dNTP mix (Roche). The antisense riboprobe was labeled by T3 RNA Polymerase in vitro transcription of the *NotI*-linearized plasmid. *In situ* hybridization assays were performed in embryos collected in different developmental stages [35]. Prior to *in situ* hybridization, probes (200ng in 50μL) were mixed with *in situ* hybridization solution as follows: 50% formamide, 5x SSC, 100μg/mL of herring sperm DNA, 50μg/mL of heparin and 1% of Tween 20. The hybridization mixture was added to the embryos at 55 °C and incubated overnight. After hybridization embryos were washed several times with PBS and color development was performed with BCIP/NBT 75 (Promega). Embryonic stages were identified according to the outlined criteria [36].

Physical mapping of *Dnmt2* gene

Non-fluorescent *in situ* hybridization technique for the precise location of *Dnmt2* sequences in polytene chromosomes of *D. willistoni* was used since it allows better visualization of the banding patterns and is recommended for precise physical mapping [26]. DNA probe of *Dnmt2* of *D. willistoni* was constructed from a fragment of 949 bp cloned into the vector TOPO PCR-4 (Invitrogen). The probe was obtained by PCR, from the sequenced Gd-H4-1 line, as well as the photos of the polytene chromosomes. The probe was labeled with a Biotin PCR Labeling Core Kit (Jena Bioscience) following the manufacturer

instructions, with minor adjustments. The labeled products were purified with a NucleoSpin Gel and PCR Clean-up Kit (MACHEREY-NAGEL). Vectastain Elite ABC (Vector Laboratories) and DAB Substrate (Roche) performed the detection and revelation of hybridization signals, respectively. Slides were stained with Giemsa solution (5%) and coverslips were assembled with Entellan (Merck). Polytene chromosomes images were acquired in phase contrast photomicroscope and analyzed according to the photomap of *D. willistoni* [33].

Results

Transcriptional expression of *D. willistoni Dnmt2* is developmental delimited

We performed RT-PCR analysis to verify the levels of *Dnmt2* transcriptional expression. The expression of *Dnmt2* transcripts was detected for all cDNA samples used: *D. willistoni* adult males, females, females without ovaries, pupae, larvae, and 0-3h dechorionated embryos. The amplification of a single fragment with an expected size of 280 bp, corresponding to processed mRNA, confirmed the absence of genomic DNA contaminants in all samples (**Figure 1**). In order to infer the amount of *Dnmt2* transcripts at different stages of development and samples analyzed, further investigations were carried out by qPCR.

The qPCR showed that *Dnmt2* expression has different levels along *D. willistoni* development (**Figure 2**). The analysis showed a pattern of *Dnmt2* expression where embryos rank higher than the other developmental stages (approximately 2 to 3-fold). The *Dnmt2* transcription decreases along the development, although the adult stages showed a slight increase. Females, females without ovaries and males showed no substantial differences between the levels of *Dnmt2* expression.

Dnmt2 transcripts follow the dynamics of embryonic development

In situ hybridization experiments were conducted at different developmental oogenesis and embryogenesis stages to determine the spatial and temporal pattern of the *Dnmt2* transcriptional expression of the *D. willistoni*. Initially, the *Dnmt2* transcripts were detected as uniform and widespread in all stages of oogenesis (**Figure 3**). The staining pattern indicates early expression at the anterior end and in the ventral position of the oocyte (**Figure 3A-3H**).

In the initial embryonic developmental stages, *Dnmt2* transcripts appear as superficial and later as diffuse internal granules, a pattern similar to those found in maternal transcripts (**Figure 4A-4C**). This pattern persists until the cellular blastoderm stage. Throughout development, *Dnmt2* transcripts accumulate at the embryonic ventral periphery **(Figure 4D**), and subsequent expression can be detected on the anterior portion, coinciding with the moment when the cephalic furrow is formed (**Figure 4E**). The expression of *Dnmt2* was detected from the cephalic region expanding through the ventral region to the embryo's posterior pole (**Figure 4F-4H**). The anterior and posterior expression levels of staining decreased and became a basal signal from then on (**Figure 4I**). During late embryogenesis, at the germ-band retraction stage, transcript accumulation persisted as a basal expression in the ectoderm layer.

The *Dnmt2* gene resides in the arm IIL of the *D. willistoni* chromosome

The location of the probe hybridization signal was determined to be at the distal region of the chromosome II left arm (IIL), specifically in section 55C (**Figure 5A and 5B**). The IIL arm of *D. willistoni* corresponds to the Muller B element, according to a previous study [37]. On FlyBase search tool [38], we could verify that in the other eleven species (*D. melanogaster*, *D. simulans*, *D. sechellia*, *D. erecta*, *D. yakuba*, *D. ananassae*, *D. pseudoobscura*, *D. persimilis*, *D. virilis*, *D. mojavensis,* and *D. grimshawi*) whose genomes are available, the sequences homologs to *Dnmt2* are also localized on B element.

Discussion

Drosophila are so-called "*Dnmt2*-only" organisms, that is, they do not have any of the canonical DNA methyltransferases (*Dnmt1* and *Dnmt3*). DNA methylation was also observed in other "*Dnmt2*-only" organisms, especially in other dipterans, though direct evidence of *Dnmt2* involvement is still enigmatic. Interestingly, there is a diverse group of animal species that have retained Dnmt2 as their only DNA methyltransferase candidate, like *Schizosaccharomyces pombe, Dictyostelium discoideum, Entamoeba histolytica*, *Schistosoma mansoni*, and *D. melanogaster*. For instance, about 1 in 600 (0.17%) cytosine nucleotides are methylated in the DNA of the *Aedes albopictus* mosquito [39]. However, although such species have genomic methylation, this is still at low global levels and *Dnmt2* biological function has been fervently discussed [40,41].

There are accumulating findings that instigate investigations in "*Dnmt2*-only" organisms, such as female-specific DNA methylation that was reported for *D. willistoni* and related species [15,25]. Recently, males that have reduced expression of dMBD-R2 (a methylated cytosine binding protein), specifically octopamine (equivalent to norepinephrine) neurons, have exhibited mating behavior toward divergent interspecies such as *D. virilis* and *D. yakuba* and a decrease in the success of co-specific mating [17]. These data strongly suggest the existence of methylation-dependent chromatin structures in *Drosophila*, that is, the role of methylation of genomic cytosines appears to be fundamental at various levels in these organisms. Thus, *Dnmt2* studies is of great importance towards a better understanding of DNA and RNA methylation in "*Dnmt2*-only" systems, whose data are still vastly controversial and uncertain.

Our quantitative analyses on different development stages showed differences in *Dnmt2* expression (**Figure 2**), with the highest expression being observed in embryos, in the *D. willistoni*, in which *Dnmt2* expression was 2-fold higher than in the larvae stage. Essentially, on *Drosophila* genus, DNA methylation is described as predominant during embryonic development. In *D. melanogaster*, the DNA methylation is prevalent in young (1–2 h) embryos, but less marked in older (15–16h) embryos and only trace amounts of 5-methylcytosine were found in isolated ovaries (oocytes), and *Dnmt2* transcripts present similar levels corresponding to DNA methylation stages [12]. The data herein showed a very close pattern of *Dnmt2* expression in *D. willistoni*, similar to previously founded in *D. melanogaster*. Interestingly, the *Dnmt2* expression in females and males of *D. willistoni* revealed a slightly higher value when compared with the pupae stage, and this relative expression levels in the *D. willistoni* adults are higher than what is found in *D. melanogaster* adults (**Figure 2**) [12]. Thus, this *Dnmt2* expression can be relevant, since recently we had reported evidence of DNA methylation in adult flies of *D. willistoni* and closely relates species [15,25].

Employing whole *D. willistoni* embryos hybridization with *Dntm2* riboprobe, we could observe transcriptional expression along different embryonic stages. The oocytes showed the most prominent hybridization signals, suggesting a higher activity of the enzyme on this stage of oogenesis (**Figure 3**). The early stages of embryogenesis (**Figure 4**) showed hybridization staining with distinct patterns, in which the expression pattern seems to decrease from syncytial blastoderm to late development. Interestingly, the expression patterns ranged from a granular composition throughout the whole embryo on syncytial blastoderm to a peripheral occurrence on cellular blastoderm. During gastrulation, we observed that the *Dnmt2* expression seems to suffer compartmentalization from the anterior portion in the ventral region, reaching the posterior portion. Moreover, it showed only a fading basal signal in the head region and in the position of the developing central nervous system (CNS). All the presented results with embryos of *D. willistoni* are in agreement with those previously found concerning the genomic DNA methylation that predominates during early embryonic development and decreases at later stages [12], presumably as a result of reduced methyltransferase expression.

The importance of DNA methylation in *Drosophila* still is an elusive case, and there are some attempts to understand the biological function in *Drosophila* genomes. However, when we are talking about a genus with more than 4.000 species, peculiarities may arise, like the sex-specific methylation described in *D. willistoni* [15,25], so generalization must be avoided. It was observed that overexpression of *D. melanogaster Dnmt2* results in an extended fly life span and in overexpression of several genes [42]. Also, it is discussed whether Dnmt2 acts as DNA or tRNA methyltransferase. If Dnmt2 was described associated to a nuclear matrix, and if *Drosophila* is a "*Dnmt2*-only" organism, and if *D. willistoni* has sex-specific methylation in its genome, we cannot deny that the evidence suggests a duality in the activity of that enzyme in these organisms. The peculiarities of Dnmt2 enzyme rise in different organisms; in humans, for example, it is located in cytoplasmic regions [43], whereas in *Dyctiostelium discoideum* and *Entamoeba histolytica*, Dnmt2 is located only in the nuclear matrix [27,44], and guaranteeing the structural integrity of chromatin and silencing retrotransposons in *Drosophila* [23]. Generating Dnmt2 null mutations allowed us to correlate loss of DNA methylation, identified in early *D. melanogaster* embryos by bisulfite sequencing and restriction enzyme analysis. Besides that, these results suggest the Dnmt2-dependent DNA methylation during early embryonic development of *D. melanogaster* has a key function in control of retrotransposon silencing in somatic cells, specifically as an initial step of the process [23]. This feature contrasts with the high local specificity of the Dnmt1 and Dnmt3 to the cell nucleus region.

On the other hand, the HIV virus is able to increase its survival in the host cell using the RNA methylation activity of Dnmt2, increasing the stability of its own genome. Through infection, Dnmt2 relocates from the nucleus to the cytoplasmic stress granules and methylates the RNA virus, promoting the post-transcriptional stability of viral RNA [45]. RNAs interference (RNAi) have a strong association with different epigenetic mechanisms, such as histone methylation/acetylation and genomic DNA methylation [46]. Double-stranded RNAs (dsRNA) trigger the process of post-transcriptional homology-dependent gene interference (RNAi) closely related to the co-suppression of viral exogenous transcripts. The dsRNAs are processed by a Dicer family of enzymes into small dsRNA sequences having 21-25 nucleotides, termed small interfering RNAs (or siRNAs). Interestingly, it is known that Dnmt2 also interacts with Dicer-2 in

response to heat shock treatments [47,48], controlling the expression and regulation of various Heat Shock proteins [49–51]. Finally, the activity of Dnmt2 in conjunction with NSun2 is important to promote the stability of tRNAs and protein synthesis [52]. That is, we can assume that the Dnmt2 has a multilevel biological role, inserted in an extensive network of interactions in the genetic machinery of the gene expression control.

In the present study, we performed the *in situ* localization of the *D. willistoni Dnmt2* (**Figure 5**). The gene is located in the arm IIL (Muller B element), like in *D. melanogaster* and *D. pseudoobscura*, where it was possible to ascertain the position of the gene, it is present in B element. This is a very important aspect because we present here the determination of another gene marker in *D. willistoni*, following previous work [37], which will help in the organization of the *D. willistoni* scaffolds regarding the physical position of the genes in the chromosomes.

Surprisingly, *D. willistoni Dnmt2* is found in the subtelomeric region of the chromosome IIL (Muller B element), whereas *D. melanogaster* and *D. pseudoobscura Dnmt2* are found, approximately, in the central region of the chromosome IIL (Muller B element) (**Figure 6**). Most likely, the differences found for the Muller element and the location of the *Dnmt2* in the chromosome are related to translocation events followed by breakage and inversion of the region where the gene is found.
It is known that heterochromatin regions (centromeric and telomeric) have different patterns of evolution than those found in the euchromatin regions (gene-rich). Comparative analyses between telomeric regions of humans and chimpanzees have shown that both are very distinct, in that the human chromosomes present a large scale of rearrangements and differences in the repetitive elements present [53]. Interestingly, the chromosome ends in *Drosophila melanogaster* have the peculiarity of presenting transposable elements (*HeT-A* and *TART*) [54–57], contrasting with the human chromosome ends that are structured from the activity of the enzyme telomerase. Another interesting aspect is that most-distal regions of *Drosophila* subtelomeres seem to evolve rapidly between *Drosophila melanogaster* and its close relatives, *D. simulans* and *D. yakuba* [58]. In this region, the mutation rate of the most-distal portion is so high that the structure is likely to be different among cells of the same individual [59]. However, what is observed in terms of recombination in the telomeric regions of *Drosophila melanogaster* is that there is a great suppression of crossing over events, within and in regions proximal to the telomeres [60]. These aspects are in agreement with previous findings of our research group, in which *D. willistoni Dnmt2* shows the highest rate of nucleotide substitution per codon in relation to the other drosophilids and different selective pressures at specific sites involved in an interaction-driven co-evolution with other genes [61].

CONCLUSION

Epigenetic mechanisms are fundamental in the coordination of the ontogeny of organisms and in the intermediation of information from the environment to the nucleus and from the nucleus to the environment. The understanding of such mechanisms is of paramount importance in order to understand the ecological, physiological and evolutionary aspects of the different species. Among the epigenetic

phenomena, cytosine methylation is the most widely studied and known. However, its role is still elusive in organisms known as "*Dnmt2*-only", among them *Drosophila*.

In the present work, we verified that the expression of *Dnmt2* in *D. willistoni* is closely related to its ontogenetic stages. In addition, it has been found that expression levels in adults of *D. willistoni Dnmt2* are apparently slightly higher than in *D. melanogaster*. It is suggested, therefore, that this difference may be closely related to the phenomenon of sex-specific methylation, a peculiar and restricted phenomenon to the species of the subgroup *willistoni*. We also conduct the *in situ* localization of the *Dnmt2* gene, revealing its presence in the arm IIL (Muller B element). The *Dnmt2* is located in the subtelomeric region, and this brings up a series of issues that involve the peculiar characteristics of *D. willistoni* Dnmt2 enzyme, like evolutionary pattern in nucleotide substitutions rates [61], the high prevalence of basic amino acids residues in its target recognition domain surface [62] and the epigenetic phenomena of sex-specific methylation [15,25].

With that, fortunately further questions are being raised about such peculiarities in this unique group of Neotropical species which are *D. willistoni*. Future studies are needed for a more in-depth understanding of the importance of sex-specific methylation in development and survival, as well as the impact related to the subtelomeric position occupied by the *Dnmt2*.

Abbreviations

tRNA: transfer ribonucleic acid; mRNA: messenger ribonucleic acid; rRNA: ribossmal ribonucleic acid; DNA: deoxyribonucleic acid; C: cytosine; G: guanine; A: adenine; T: thymine; Dnmt: DNA methyltransfarese; CpG: Cytosine-phosphate-Guanine; CpA: Cytosine-phosphate-Adenine; CpC: Cytosine-phosphate-Cytosine; RT-PCR: reverse transcription polymerase chain reaction; cDNA: complementary DNA; qPCR: quantitative polymerase chain reaction; CNS: central nervous system; HIV: human immunodeficiency virus; RNAi: RNA interference; dsRNA: double-stranded RNA; siRNA: small interfering RNA

Declarations

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Availability of data and materials

All the data supporting the results of this article are included within the article. All data can be provided by request.

Authors' contributions

GCV, MFD and MD conceived the project and designed the experiments. GCV, RZ and PSO conducted the RT-PCR and qPCR analysis. CFG and GCV conducted the *in situ* hybridization analysis. MD and GCV conducted the *in situ* hybridization in oocytes and embryos analysis. VLSV provided expertise and feedback. GCV, CFG and RZ wrote the manuscript with input from all authors. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

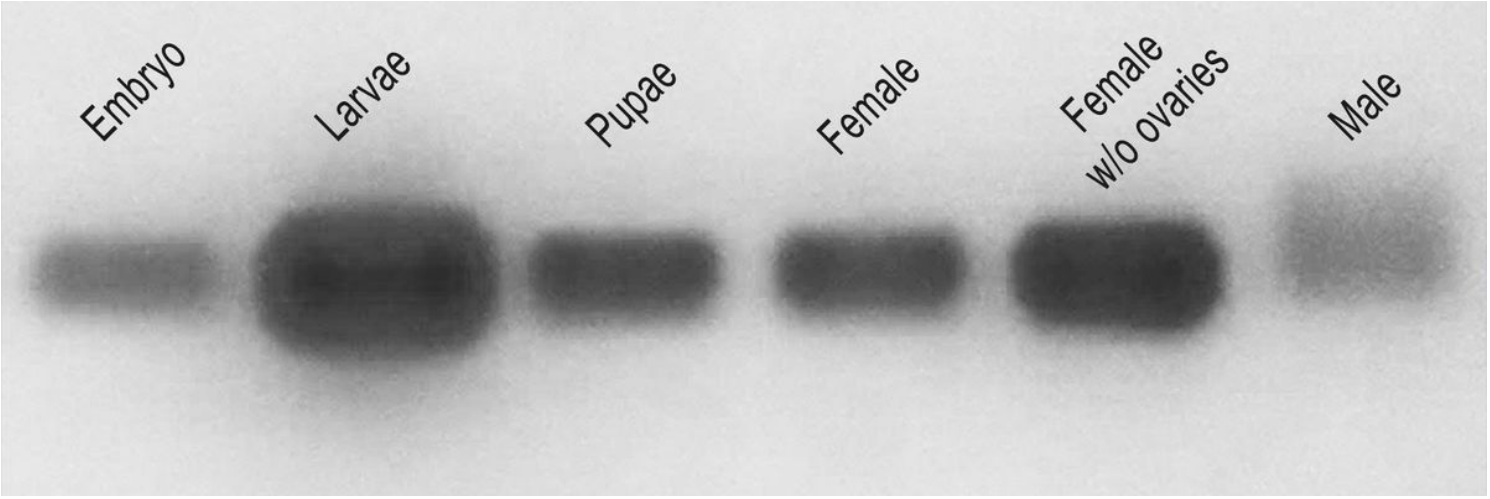

Figure 1

Detection of Dnmt2 by conventional RT-PCR. All the RT-PCR products had the expected size (280 bp) on 1% agarose gel and stained with GelRed™ (Sigma-Aldrich).

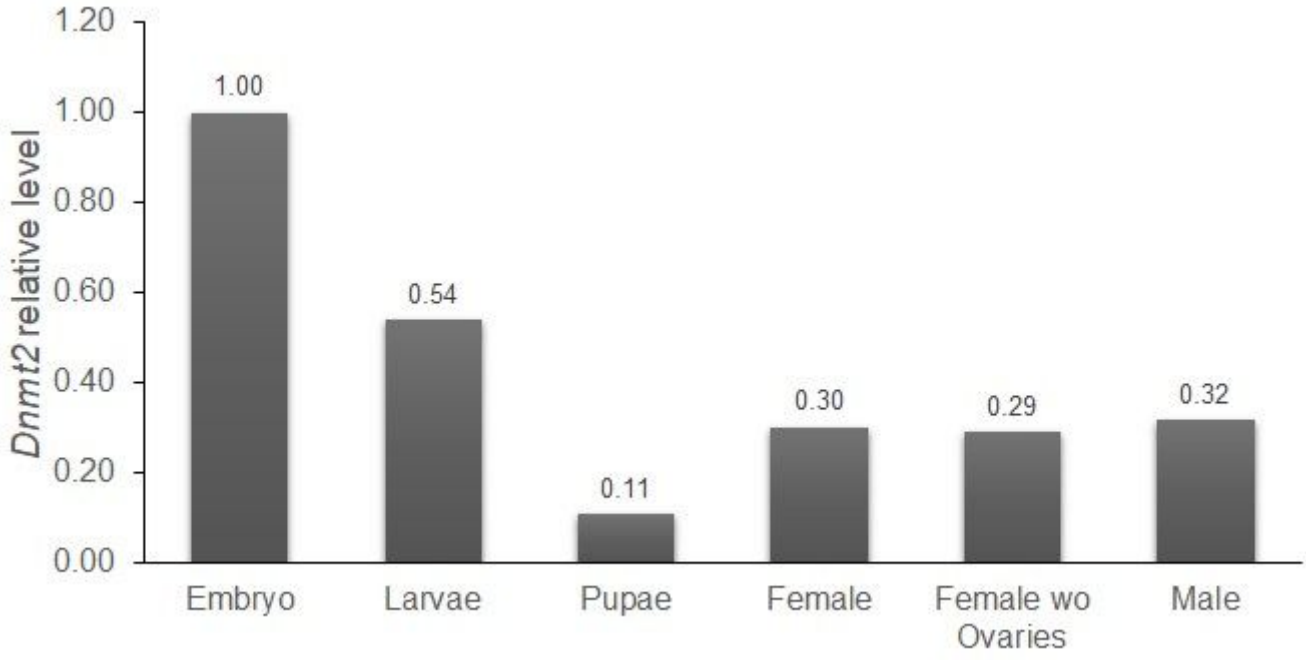

Figure 2

Estimate of Dnmt2 transcriptional expression levels in the D. willistoni samples. The results were normalized with β-actin expression. All data were obtained from triplicates.

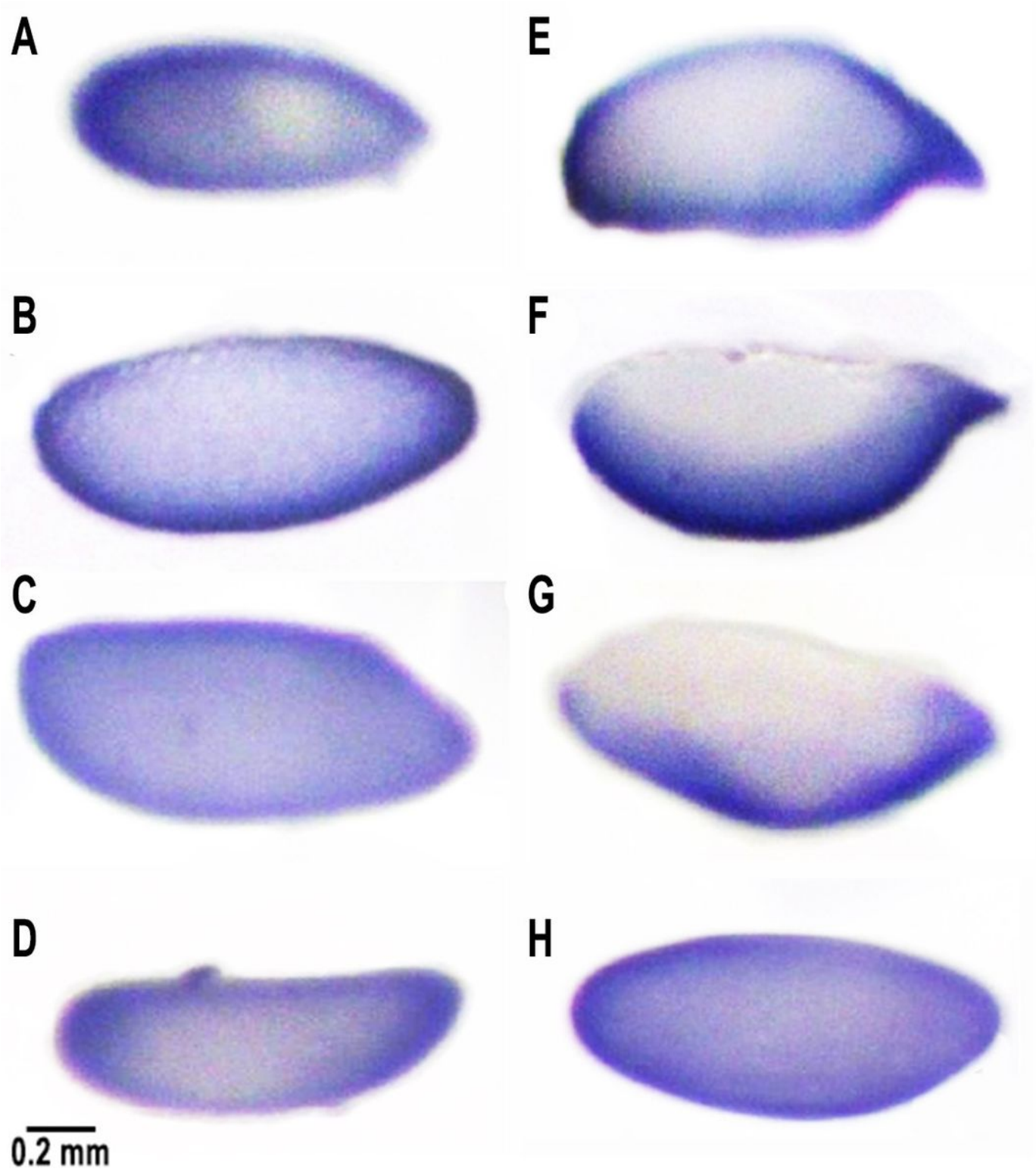

Figure 3

Spatial expression patterns of Dnmt2 transcripts on D. willistoni during oogenesis, showing a widespread pattern during differentiation, which gradually reaches an evident pattern on its ventral surface. Orientation of oocytes: anterior is to the left. Bar: 0.2mm.

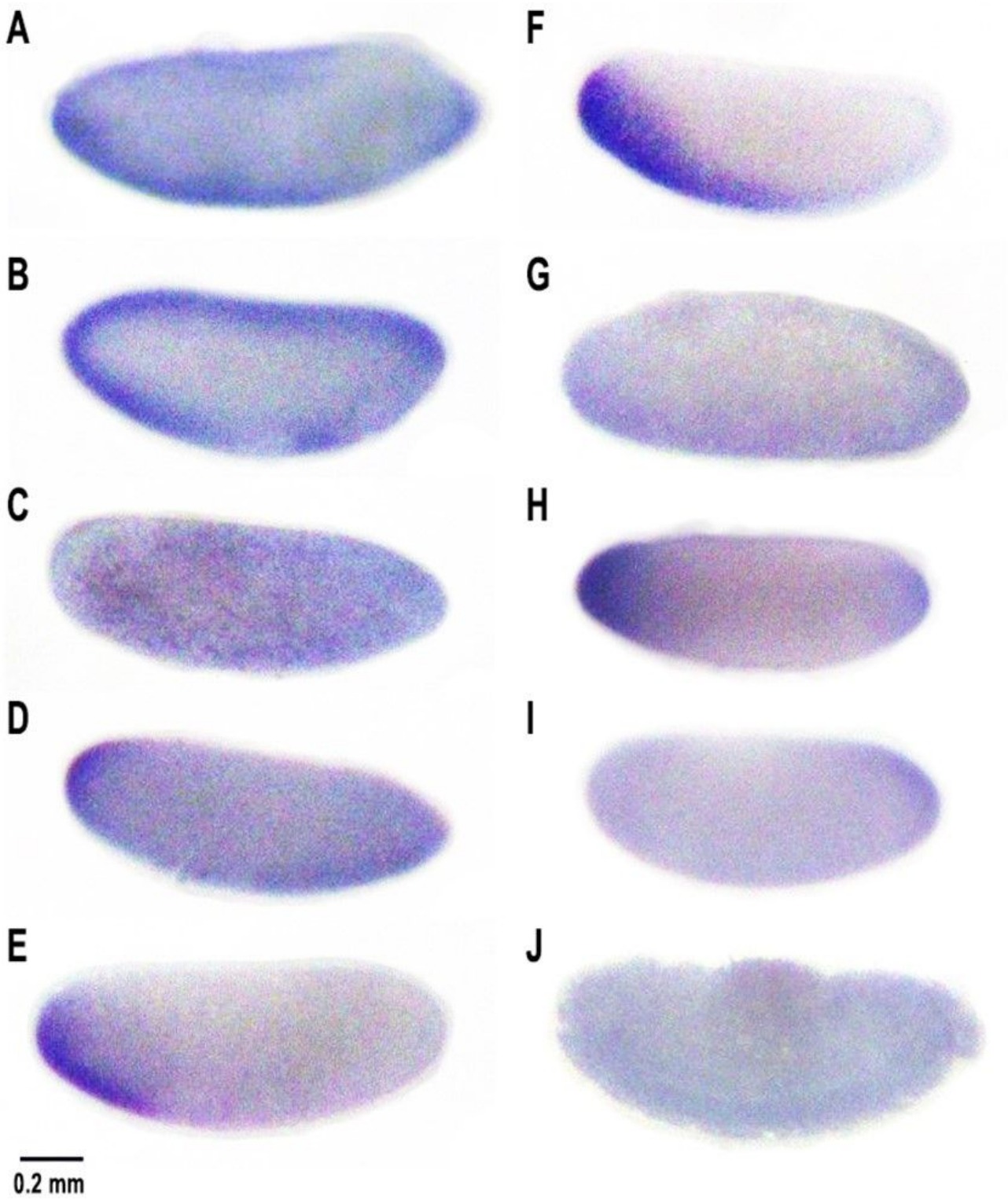

Figure 4

Spatial expression patterns of Dnmt2 transcripts on D. willistoni during embryogenesis showing. A-D: first embryogenesis stages showing global staining patterns. E-I: signal expansion from anterior to posterior embryo pole; J: late embryogenesis showing germ-band retraction and weak staining. Orientation of embryos: anterior is to the left. Bar: 0.2 mm.

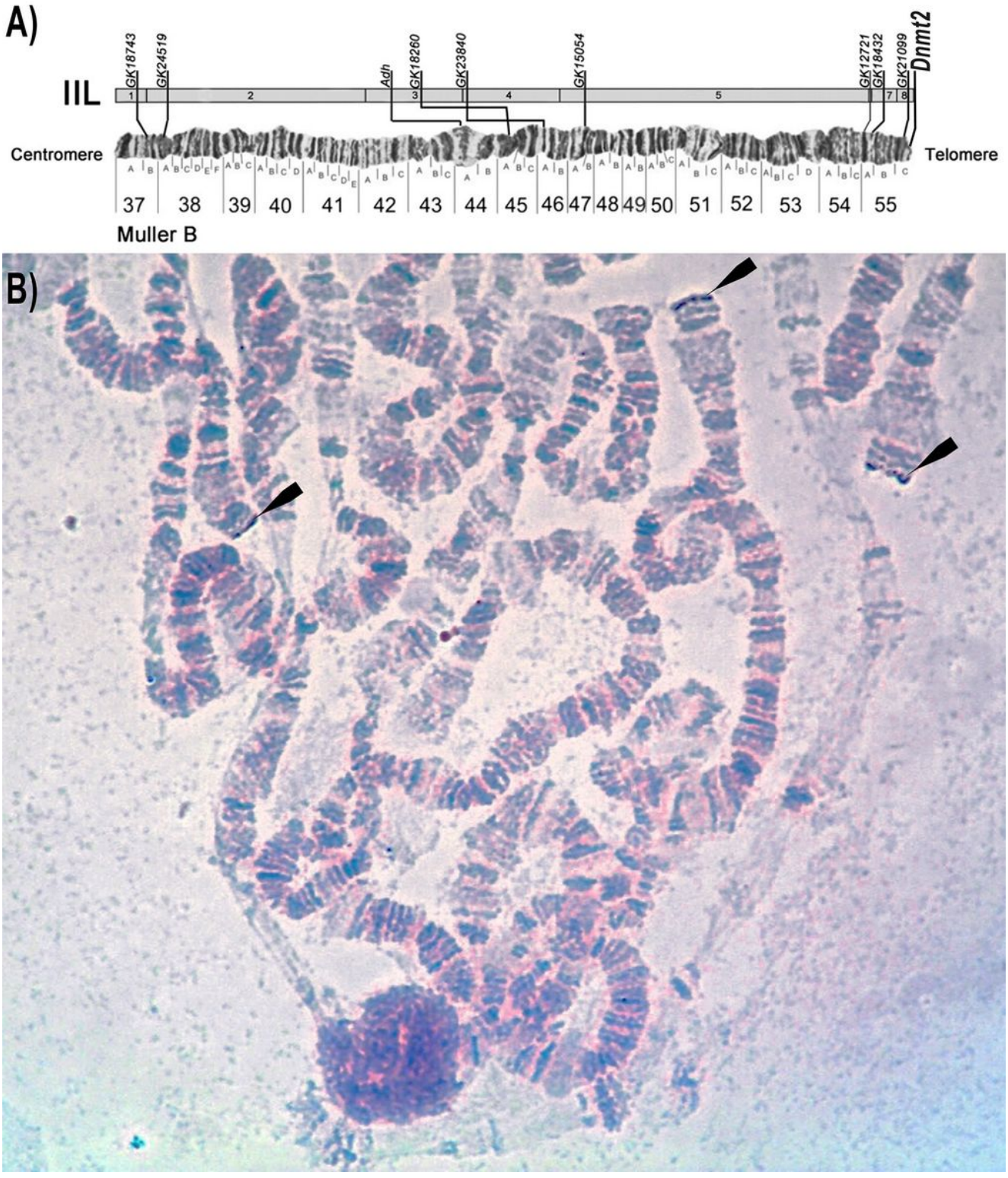

Figure 5

Physical mapping of Dnmt2 gene in polytene chromosomes of D. willistoni. A) IIL arm with the site of gene Dnmt2 hybridizations. B) In situ hybridization signal of Dnmt2 gene in chromosomal arm IIL in Gd-H4-1 strain of D. willistoni (black arrows).

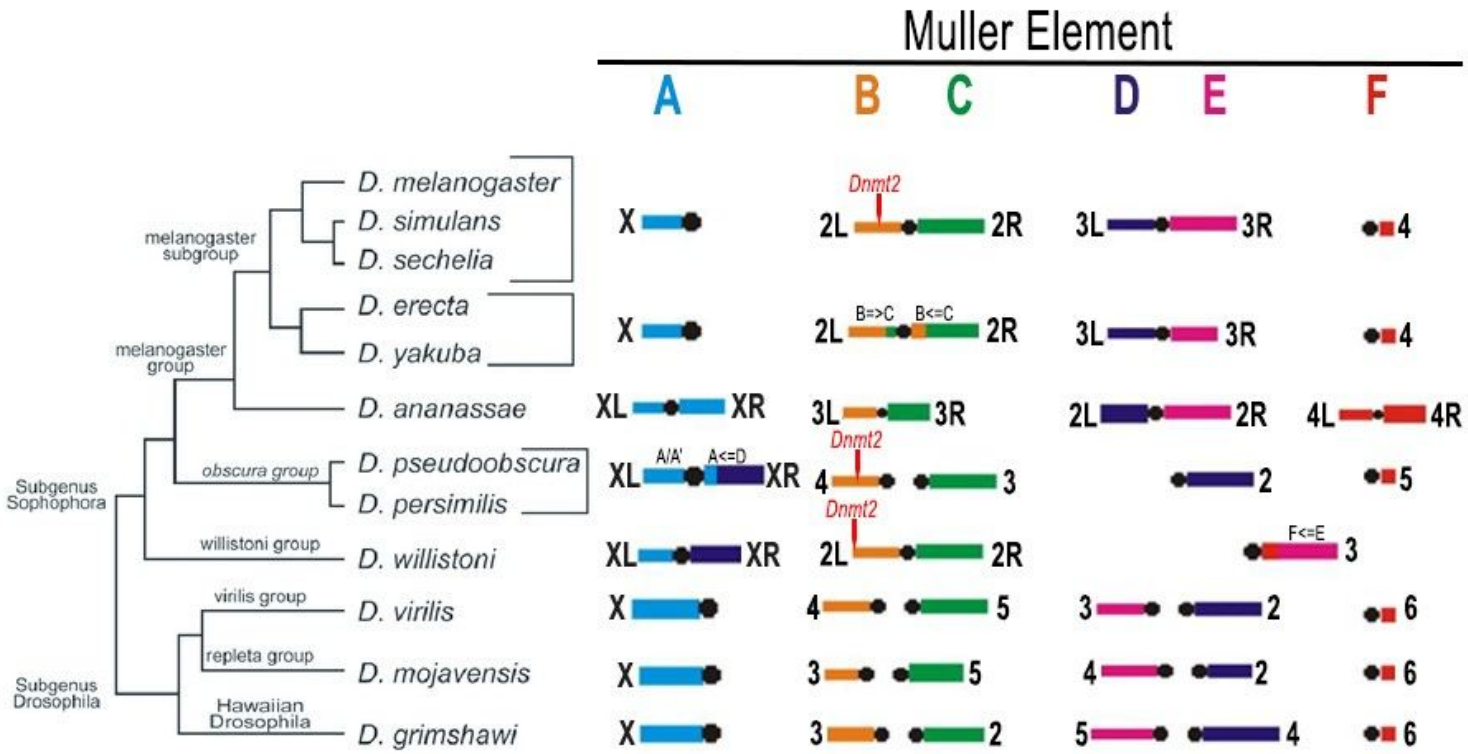

Figure 6

Schematic correspondence of rearrangement for the six Muller elements among twelve species of Drosophila. The centromeres are indicated with solid black circles. The approximate localization of the Dnmt2 in the arms is in indicated by a red arrow. Modified from Schaeffer et al. (2008) with corrections by Garcia et al. (2015).