Sex-specific methylation in *Drosophila*: an investigation of the *Sophophora* subgenus

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Abstract Epigenetic phenomena have been widely characterized in the genomes of vertebrates and DNA methylation is a key mechanism of epigenetic regulation. The DNA methylation systems of invertebrates and vertebrates show several notable differences. However, the evolutionary implications of those differences only recently began to be revealed. Our study investigated the recurrence of sex-specific methylation, as previously described for the species *Drosophila willistoni*, in other species of the *Sophophora* subgenus that present close evolutionary relationship. The MSRE and Southern blot techniques were used to analyze rDNA of some species of the *willistoni*, *melanogaster*, *saltans* and *obscura* groups of *Drosophila* and the results suggested that differential DNA methylation between sexes only occurs in *Drosophila tropicalis* and *D. insularis*, two sibling species of the *willistoni* subgroup. However, only using the MSRE technique we could detect sex-specific patterns of DNA methylation in all species of *willistoni* subgroup. These results indicate that DNA methylation may present important differences, even between closely related species, shedding new light on this Neotropical species complex.

Keywords *Drosophila* · *Sophophora* subgenus · DNA methylation · Epigenetics · rDNA

Introduction

Among the epigenetic processes of gene expression control in eukaryotes, the most well-known process is DNA methylation (Weinhold 2006), which essentially consists of the addition of a methyl group in the C5 position of cytosines (Gruenbaum et al. 1981). DNA methylation is one of the main epigenetic mechanisms that regulate gene expression in mammal cells (around 5% of the genomes) (Kholsa et al. 2001). Contrasting with several vertebrate groups, which have methylated DNA in their genomes (Tweedie et al. 1997), little is known about DNA methylation in invertebrates.

However, studies on the *Drosophila* genus have demonstrated the occurrence of DNA methylation in some species. Chromatography (HPLC) of the *D. melanogaster* genomic DNA in the first stages of embryo development has revealed low though significant levels of methylated cytosines (Gowher et al. 2000; Lyko et al. 2000), and most 5-methylcytosines have been found in CpT/A dinucleotides (Lyko et al. 2000). This finding indicates that DNA methylation in *Drosophila* is not sustained by the symmetric alteration in CpG dinucleotides, as opposed to what happens with the other organisms studied. Additionally, as little as 0.4% of the total *D. melanogaster* DNA is methylated, and even these low levels are detected only in embryos at an early development stage. Contrasting with vertebrate cells, which steadily maintain their methylation patterns throughout development, DNA methylation in *D. melanogaster* seems to be a transient epigenetic signal...
observed in the early development stages (Hung et al. 1999; Tweedie et al. 1999; Lyko et al. 2000; Lyko 2001; Kunert et al. 2003). A different picture was revealed by Garcia et al. (2007) in Drosophila willistoni. When the DNA of adult males and females of this species was cleaved with enzymes sensitive to methylation by MSRE, sex-specific patterns of fragments were observed. The analysis of the sequences forming these bands revealed the presence of ribosomal genes (Garcia et al. 2007).

Despite the current information on DNA methylation in Drosophila, its role in different genomes still remains to be clarified. Some studies, however, have suggested that the methylation of DNA sequences may be involved in regulation of transposable element expression (Salzberg et al. 2004) and of the development process (Mandrioli and Borsatti 2006). Overall, the data seem to point to diverse functions of DNA methylation in the genome of different Drosophila species. This idea may be corroborated by comparing the dDnmt2 amino acid sequences of D. willistoni and of D. melanogaster, in which the sequence conservation common to these both species only occurs in the domain responsible for DNA methylation. The domain responsible for the recognition of the target sequence to be methylated is highly variable, indicating that substantial variations may occur even between species of the same genus (Garcia et al. 2007).

But why do species of one genus (in this case, subgenus Sophophora) present different patterns in the DNA methylation status? Recent findings from Clark et al. (2007), Schaeffer et al. (2008), Bhutkar et al. (2008) and Vicario et al. (2007) point to the singularity of Drosophila willistoni, among the other 11 species that have their genomes completely sequenced, with respect to several characteristics. The methylation pattern found by Garcia et al. (2007) in D. willistoni seems to provide yet another piece of evidence of this uniqueness.

Several analyses support the monophyly of the Old World melanogaster–obscura and Neotropical saltans-willistoni clades (review in Powell 1997) of the Sophophora subgenus. Furthermore, the Neotropical Drosophila willistoni subgroup is a complex at various taxonomic levels, and hence it can be considered an ideal material for evolutionary studies (review in Robe et al. 2010).

Considering the variation detected in DNA methylation between Drosophila melanogaster and D. willistoni, both members of Sophophora subgenus, this study investigates whether sex-specific patterns of DNA methylation are conserved between other species of the same subgenus, genetically related (willistoni subgroup) or not (saltans, obscura and melanogaster groups). We also tried to contribute to the understanding of the evolutionary implications of these differences.

### Materials and methods

#### Species and strains

All species analyzed (Table 1) are members of the subgenus Sophophora, according to Ashburner (1989), but they are ranked under distinct clades–groups of holarctic (obscura group), afrotropical (melanogaster group), and neotropical (willistoni and saltans groups) origin—which exploit distinct environments (Table 1). The relationships among those species shown in Fig. 1 are in accordance with the combined data by O’Grady and Kidwell (2002), O’Grady et al. (1998), Markow and O’Grady (2005), and Robe et al. (2010). All the fly strains studied were reared in flour medium (Marques et al. 1966 or Burdick 1954) and kept in chambers at 17°C ± 1°C and 60% RH.

#### Genomic DNA isolation and analysis using Methylation-Sensitive Restriction Endonuclease (MSRE)

Genomic DNA of adult males and females were extracted according to the methods described in Sassi et al. (2005) and Lodhi et al. (1994). The restriction endonucleases sensitive and non-sensitive to methylation are listed in Table 2. Digestion was performed according to the manufacturers’ instructions. The amount of DNA used in each experiment was roughly 10 µg. All samples were electrophoresed on 1% agarose gels stained with ethidium bromide.

#### MSRE: Southern blot analyses

The genomic DNAs of adult males and females of all the species (Table 1) were digested using the restriction endonucleases AluI and TaqI (Table 2), which were chosen according with the results of Garcia et al. (2007). These enzymes do not form a pair of isoschizomers, but both have similar restriction sites and are sensitive and non-sensitive to methylation, respectively. The probe used in the experiments was the plasmid pDm 238 (Tautz et al. 1988), which contains one complete ribosomal DNA unit of D. melanogaster (12 kb), including the genes 28S, 18S, 5.8S, and 2S, apart from the spacer regions ITS, IGS, ETS (GenBank M21017 and M299800). The MSRE technique, combined with Southern blot, used a probe labeled with the random primer method, with the Gene Images™ kit (GE Healthcare). DNA of samples was electrophoresed on 0.8% agarose gels, transferred to a Hybond N+ membrane (GE Healthcare) and hybridized according to the protocol described by the manufacturer at 60°C. The membrane was washed twice at 60°C, first with SCC IX and SDS 0.1%, then...
and then with SSC 0.5X and SDS 1%, under gentle agitation for 15 min, in both cases. The CPDStar™ kit (GE Healthcare) was used for labeling.

**Results**

The MSRE technique revealed different cleavage patterns in the *Drosophila* species analyzed—*D. willistoni*, *D. insularis*, *D. equinoxialis*, *D. tropicalis*, *D. paulistorum* (*willistoni* subgroup species) and *D. melanogaster*. In the *willistoni* subgroup species, both male and female genomic DNAs cleaved with *Alu* I (5'0AG; 3'CT3) and *Hae* III (5'0GG; 3'CC3), enzymes sensitive to methylation (Table 2), produced differential sex-specific fragment patterns after fractionation in 1% agarose gels; the exception was the samples of *D. insularis* DNA (Fig. 2a). A more intensive digestion was observed for all male DNA samples as compared to those of females; however, exclusive bands for each sex were identified, varying approximately from 2 to 12 kb (Fig. 2a, b, e). Below 2 kb, only a smear with low molecular weight was detected in both sexes (Fig. 2). For *D. melanogaster* there is no sex-specific banding pattern in DNA samples digested with the enzymes *Alu* I and *Hae* III (Fig. 2b).

Differential methylation patterns were observed to occur between sexes in *D. willistoni*, *D. tropicalis*, *D. equinoxialis* and *D. paulistorum*, but the pattern of fragments generated by restriction enzymes *Alu* I and *Hae* III (both sensitive to methylation) differed between these sibling species. *D. tropicalis*, *D. equinoxialis* and *D. paulistorum*

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**Table 1** Species and strains of the *Sophophora* subgenus of *Drosophila* analyzed in the present study (according to Ashburner 1989)

<table>
<thead>
<tr>
<th>Group (origin)</th>
<th>Subgroup</th>
<th>Species</th>
<th>Origin</th>
<th>Habitat</th>
</tr>
</thead>
<tbody>
<tr>
<td>willistoni</td>
<td>willistoni</td>
<td><em>D. willistoni</em></td>
<td>Itaqui, Rio Grande do Sul, Brazil</td>
<td>Woods and town</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>D. paulistorum</em></td>
<td>Ribeirão Preto, São Paulo, Brazil</td>
<td>Woods and town</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>D. equinoxialis</em></td>
<td>Mexico city, México</td>
<td>Only woods</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>D. tropicalis</em></td>
<td>San Salvador, El Salvador</td>
<td>Only woods</td>
</tr>
<tr>
<td>bocainensis</td>
<td></td>
<td><em>D. capricorni</em></td>
<td>Florianópolis, Santa Catarina, Brazil</td>
<td>Only woods</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>D. fumipennis</em></td>
<td>Arima Valley, Trinidad and Tobago</td>
<td>Woods</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>D. nebulosa</em></td>
<td>Sinaloa, Mexico</td>
<td>Woods and clearings</td>
</tr>
<tr>
<td>saltans</td>
<td></td>
<td><em>D. sturtevanti</em></td>
<td>Florianópolis, Santa Catarina, Brazil</td>
<td>Woods and town</td>
</tr>
<tr>
<td></td>
<td>elliptica</td>
<td><em>D. neoelliptica</em></td>
<td>Joinville, Santa Catarina, Brazil</td>
<td>Only woods</td>
</tr>
<tr>
<td>melanogaster</td>
<td></td>
<td><em>D. melanogaster</em></td>
<td>Zarate, Argentina</td>
<td>Cosmopolitan</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>D. sechellia</em></td>
<td>Seychelles Islands</td>
<td>Endemic (insular)</td>
</tr>
<tr>
<td>obscura</td>
<td>obscura</td>
<td><em>D. pseudoobscura</em></td>
<td>Mesa Verde, Colorado, USA</td>
<td>Woods</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>D. subobscura</em></td>
<td>La Florida, Santiago, Chile</td>
<td>Woods and town</td>
</tr>
</tbody>
</table>

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**Table 2** Restriction endonucleases used in the digestion of genomic DNA of the species analyzed in this study

<table>
<thead>
<tr>
<th>Restriction endonucleases</th>
<th>Sensitive to methylation</th>
<th>Non-sensitive to methylation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Hae</em> III (5'GG</td>
<td>CC3')</td>
<td><em>Rsa</em> I (5'GT</td>
</tr>
<tr>
<td><em>Alu</em> I (5'AG</td>
<td>CT3')</td>
<td><em>Taq</em> I (5'T</td>
</tr>
</tbody>
</table>

The letter in C bold indicates that this site, when methylated, prevents cleavage

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*Andean Brazilian semi-species, elected due to its wider geographical distribution. *-D. paulistorum* Amazonica, Interior and Orinocana semi-species were also analyzed and the results were the same obtained for Andean Brazilian semi-species (data not shown).

*From Tucson Stock Center.*
presented clearly different cleavage patterns—which were also observed for *D. willistoni*. The cleavage pattern observed with the enzyme *Rsa*I (5'GT3') non-sensitive to methylation, however, was identical in all species analyzed, and between sexes (Fig. 2a, b). The results of these experiments are summarized in Table 3.

For the MSRE technique combined with Southern blot, the genomic DNAs of adult males and females of all the species of *Sophophora* subgenus studied (Table 1) were cleaved only using the restriction enzymes *Alu*I and *Taq*I and hybridized with the labeled rDNA probe. No differences were observed between the sexes for the species *D. sturtevanti* and *D. neoelliptica* (*saltans* group), *D. melanogaster* and *D. sechellia* (*melanogaster* group), and *D. pseudoobscura* and *D. subobscura* (*obscura* group).

The same approach revealed sex-specific methylation patterns for rDNA of the sibling species *D. tropicalis* and *D. insularis* (*willistoni* subgroup) as previously described for *D. willistoni* (Garcia et al. 2007). However, no differences were observed in these patterns between DNA samples of males and females of the other sibling species, *D. equinoxialis* and *D. paulistorum* (Fig. 3).

After hybridization, the genomic DNA of female *D. tropicalis* digested with the enzyme *Alu*I generated a fragment above 5 kb that is not visualized in the sample of DNA of males. The remaining fragments below 3 kb were similar for both sexes. Five fragments below 1.65 kb were also produced after digestion with *Taq*I for samples of both males and females of *D. tropicalis* (Fig. 3).

Samples of genomic DNA of males and females of *D. insularis* cleaved with *Alu*I produced one fragment higher than 2 kb, a band of approximately 1 kb and fragments smaller than 0.65 kb. A fragment between 1.65 and 1 kb

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**Table 3** Presence (+) or absence (−) of sex-specific methylated fragments on females genomic DNA of the species analyzed by Methylation-Sensitive Restriction Endonuclease (MSRE)

<table>
<thead>
<tr>
<th>Species</th>
<th><em>Alu</em>I</th>
<th><em>Hae</em>III</th>
<th><em>Rsa</em>I</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. willistoni</em></td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td><em>D. insularis</em></td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td><em>D. equinoxialis</em></td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td><em>D. tropicalis</em></td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td><em>D. paulistorum</em></td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td><em>D. melanogaster</em></td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

---

**Fig. 2** Cleavage patterns obtained by the digestion of total genomic DNA of adult males (♂) and females (♀) of (a) *D. willistoni*, *D. insularis*, *D. equinoxialis*; (b) *D. tropicalis*, *D. paulistorum*, *D. melanogaster* with enzymes *Alu*I, *Hae*III and *Rsa*I. Arrows indicate the same pattern in females and males fragments; stars indicate differential patterns obtained in the fragments of females. The size of fragments, in kilobase pairs (kb), is indicated at the left.

**Fig. 3** Southern blot analysis of the genomic DNA of adult males (♂) and females (♀) of *D. tropicalis*, *D. insularis*, *D. equinoxialis* and *D. paulistorum* digested with *Alu*I and *Taq*I and hybridized with the pDm238 probe. The size of fragments, in kilobase pairs (kb), is indicated at the left.
was observed only in female samples. When samples of male and female DNAs were cleaved with TaqI, the fragments generated were equal (between 1.65 and 1 kb) in both sexes (Fig. 3).

**Discussion**

The findings here obtained using the Methylation-Sensitive Restriction Endonuclease (MSRE) and MSRE-Southern blot analyses raise some questions about the appearance and divergence of DNA methylation, even in genetically related *Drosophila* species. Furthermore, our results are in agreement with those of previous studies, which revealed that DNA methylation does not occur exclusively in sites CpG in the species of the genus *Drosophila* (Lyko et al. 2000; Kunert et al. 2003; Garcia et al. 2007). Nevertheless, the present study revealed the occurrence of sex-specific patterns of DNA methylation only in some species of the *willistoni* subgroup using the same methodological approaches (cleavage with AluI and Southern blot with rDNA probe) of Garcia et al. (2007) for *D. willistoni*. This phenomenon, however, did not occur in pairs of species representative of other groups of species members of the *Sophophora* subgenus, as *D. pseudoobscura* and *D. subobscura*, *D.melanogaster* and *D.sechellia*, *D.sturtevanti* and *D. neoelliptica*.

Partial methylation in rDNA genes has been described for several organisms, such as fish and amphibians (Tweedie et al. 1997), rats (Santoro and Grummt 2005), the parasitic protozoan *Entamoeba histolytica* (Fisher et al. 2004), and humans (Ghoshal et al. 2004). Studies about regulatory elements and transcribed sequences in vertebrate rDNA (Bird et al. 1981; Santoro and Grummt 2001) revealed an intriguing correlation between the proportion of active and inactive genes versus non methylated and methylated rDNA, and the fraction of methylated sequences that corresponds to silent repeats. Moreover, active and silent rDNA clusters can be differentiated in terms of their pattern of DNA methylation, of specific histone modifications, and distinct nucleosome positions (McStay and Grummt 2008). However, except for *D. willistoni*, and now *D. tropicalis* and *D. insularis*, no studies showing differential methylation patterns of ribosomal genes between the sexes, have been published.

Here we also demonstrated that the sex-specific patterns of rDNA methylation observed in *D. willistoni* (Garcia et al. 2007) are present only in some of the closely related sibling species of the *willistoni* subgroup. This phenomenon varies from total similarity with *D. willistoni*, in *D. tropicalis* (patterns of fragments observed on MSRE-Southern blot), to the total absence of differences in the sex-specific pattern, as seen for *D. equinoxialis* and *D. paulistorum* Andean Brazilian semi-species (Fig. 3). *D. paulistorum* Amazonica, *D. paulistorum* Interior and *D. paulistorum* Orinocana semi-species were also analyzed and the results were the same as those obtained for Andean Brazilian.

It is important to point out two particularities of the phenomenon here reported: (1) there are sex-specific fragments obtained by MSRE with the enzymes AluI and HaeIII in *D. paulistorum* and *D. equinoxialis*, similarly to what was observed for *D. willistoni* (Fig. 2a, b), although the Southern blot results show that the differences between sexes are not linked or restricted to ribosomal genes; (2) *D. insularis* males and females have similar DNA cleavage pattern obtained with enzyme AluI (Fig. 2a), which at first sight can suggest that no sex-specific differences occur; yet, the Southern blot analysis revealed that a sex-specific banding pattern was present in smaller DNA fragments (~1.2 kb) of the rDNA (Fig. 3) not detected in agarose gels. Overall, the data obtained using the MRSE technique show that there are patterns of sex-specific methylation in all species of the *willistoni* subgroup evaluated; however, the methylation of rDNA genes, analyzed by Southern blot, only occurs in the species *D. willistoni*, *D. tropicalis* and *D. insularis*.

The phylogenetic relationships available for the sibling species of the subgroup *willistoni* by Ayala et al. (1974), O’Grady and Kidwell (2002) and Robe et al. (2010) (Fig. 1) indicated *D. willistoni* and *D. tropicalis* as the most closely related species inside the *willistoni* subgroup. O’Grady and Kidwell (2002) studied the 28SrDNA, Adh (alcohol dehydrogenase) and COII (cytochrome oxidase II) genes to construct the phylogeny that has assembled in a branch *D. willistoni* and *D. tropicalis*, with both species being closer to *D. insularis*, and putting *D. equinoxialis* as more derived and closer to *D. paulistorum*. More recently, Robe et al. (2010), reconstructed the phylogeny of the *willistoni* subgroup combining taxonomic, biogeography and molecular data revealing that *D. insularis*, *D.tropicalis*, *D. willistoni* and *D.equinoxialis* successively branched off from the *willistoni* subgroup stem, which more recently was subdivided to produce *D. paulistorum* and *D. pavlovskiana*. Our results agree with those findings, showing that *D. insularis* and *D. tropicalis* share similar sex-specific methylation patterns in rDNA genes and that the *D. tropicalis* cleavage patterns are similar to those obtained by Garcia et al. (2007) for *D. willistoni* (Fig. 3). They also suggest that the acquisition of the DNA methylation process occurred at different times during the evolution of the *Sophophora* subgenus. For instance, the species that belong to the *bocainensis* subgroup (in *willistoni* group) are more basal than those of the *willistoni* subgroup, according to the consensus phylogenies cited (Ayala et al. 1974; Gleason et al. 1998; O’Grady and Kidwell 2002; Robe et al. 2010). So, the absence of differential methylation between sexes
in the *bocainensis* subgroup species is suggestive that this phenomenon emerged later, in members of the *willistoni* subgroup.

In the light of all these findings, it also need to be mentioned that the occurrence and the fixation of such changes in one strain may take place in a shorter time span as compared to those observed for random mutations and selection of nucleotide sequences, as reviewed by Jablonka (2004).

Furthermore, different patterns of methylation between sexes in insects were described only in mealybugs (Nur 1990), in which differential sex-specific methylation acts as an epigenetic marker and hypomethylated regions are indicative of chromosomal inactivation (Field et al. 2004). So far, for the *Drosophila* genus, for which sex-specific DNA methylation patterns are detectable by using MSRE-Southern blot in closely related species, there are no records of any phenomenon similar to that here demonstrated for the *willistoni* subgroup.

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