UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

Estudo da DNA metiltransferase 2 (Dnmt2) e Metilação do DNA no Gênero *Drosophila*

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Sumário

Lista de Abreviaturas e Símbolos

µg micrograma

µL microlitro

µM micromolar

3D tridimensional

5mC 5-metil citosina

Adh Álcool Desidrogenase

BCIP 5-Bromo-4cloro-3-indolil fosfato

bp *base pairs*

cDNA DNA complementar

CH_3 grupamento metil

COII Citocromo Oxidase II

CpA citosina- fosfato-adenina

CpG citosina- fosfato-guanina

CpT citosina- fosfato-timina

Ct *Cycle threshold*

dATP desoxi-adenosina trifosfato

DEPC Di-Etil Pirocarbonato

DNA Ácido Desoxirribonucleico

DNase Desoxirribonuclaese

Dnmt/DNMT DNA-metiltransferase

dNTPs Desoxi-ribonucleotideos Trifosfatados

EIS *Environmental Inheritance System*

ETS *External Transcribed Sequence*

Gd-H4-1 linhagem Guadalupe-França, Tucson Center 14030-0811.94

GIS *Genetic Inheritance System*

H átomo de Hidrogênio

HP1 *Heterochromatin Protein 1*

HPLC *High-performance liquid chromatography*

ICF *Immunodeficiency, Centromere instability and Facial anomalies*

IGS *Intergenic Spacer*

ISH *In situ* Hybridization

ITS *Internal Transcribed Spacer*

kb quilobase

$MECP_2$ *Methyl CpG binding Protein*

$MgCl_2$ Cloreto de Magnésio

min minuto

mL mililitro

mM milimolar

M-MLV RT *Moloney Murine Leukemia Virus Reverse Transcriptase*

mRNA RNA mensageiro

MSRE *Methylation-Sensitive Restriction Enzyme*

Mt2 metiltransferase 2

NBT *Nitroblue tetrazol*

ng nanograma

NJ Neighbor-Joining

ºC graus Celsius

PBS *Phosphate Buffered Saline*

PcG *Polycomb group*

PCR *Polimerase Chain Reaction*

PEV *Position-Effect Variegation*

pmol picomol

PO_4 grupamento fosfato

PRE *Polycomb response elements*

r.h. *relative humidity*

rDNA DNA ribossomal

RNA Ácido Ribonucleico

RNase Ribonuclease

rRNA RNA ribossomal

RT-PCR Reação em Cadeia da Polimerase com Transcrição Reversa

SAP *Shrimp Alkaline Phosphatase*

SDS Dodecil sulfato de sódio

SETDB1 *SET domain protein with histone H3K9-specific methyltransferase activity*

SSC Tampão salina-citrato

Taq *Thermoaquaticus*

TE *Transposable Elements*

Tm *Melting Temperature*

TRD *Target Recognition Domain.*

tRNA RNA transportador

trxG *trithorax Group*

U unidade

w/v *weight/volume*

Resumo

Fenômenos epigenéticos têm sido amplamente caracterizados em genomas de vertebrados, especialmente mamíferos, e a metilação do DNA é um mecanismo chave para a regulação epigenética. Os sistemas de metilação do DNA de vertebrados e invertebrados apresentam diferenças marcantes, mas as implicações evolutivas dessas diferenças só recentemente começaram a ser estudadas. Nesta tese apresentamos primeiramente a investigação sobre a recorrência da metilação sexo-específica de genes de DNA ribossomal (*rDNA*), previamente descrita para adultos de *Drosophila willistoni*, estendendo a análise a outros grupos de espécies do subgênero *Sophophora* de *Drosophila*, ao qual o principal organismo modelo do gênero, *D. melanogaster*, também pertence. Utilizamos neste estudo, espécies dos grupos *willistoni*, *melanogaster*, *saltans* e *obscura* do gênero *Drosophila* e os resultados sugerem que a metilação diferencial entre os sexos, para o gene estudado, seja recorrente em *Drosophila tropicalis* e *D. insularis*, duas espécies do subgrupo críptico *willistoni*. Nossos achados indicam que a metilação pode apresentar importantes diferenças, mesmo dentro de grupos de espécies proximamente relacionadas, fornecendo dados interessantes para o conhecimento deste complexo grupo de espécies neotropicais.

Alguns estudos têm sido realizados no intuito de decifrar os mecanismos de ação da DNA metiltransferase 2 (Dnmt2), identificada como responsável pela metilação do DNA nos genomas de *Drosophila*, mas a grande maioria dos estudos no gênero, utiliza a espécie *Drosophila melanogaster*. Pouco se sabe sobre a conservação dos padrões de metilação do DNA e do quanto ela seria importante para a evolução de espécies relacionadas. Neste sentido, realizamos um estudo pioneiro buscando homólogos do gene *Dnmt2* em um grande número de espécies da família Drosophilidae, tentando traçar um perfil evolutivo do gene, uma vez que anteriormente encontramos diferenças em padrões de metilação de DNA entre espécies próximas. Nossos resultados mostram a conservação do gene *Dnmt2* entre as espécies, pois nossas análises filogenéticas recuperam os três principais clados da família Drosophilidae. No mesmo estudo, realizamos o mapeamento físico do gene *Dnmt2* por hibridação in situ em cromossomos politênicos de *D. willistoni*. Evidenciamos que a o gene *Dnmt2* foi mapeado próximo à inserção de elementos transponíveis, previamente descritos, e de um ponto de quebra de uma inversão. Estes resultados são promissores, pois

as relações entre rearranjos cromossômicos e expressão de genes próximos a eles têm sido alvo de importantes pesquisas. Além disso, por busca *in silico*, identificamos também a preservação da sintenia cromossômica descrita para as 12 espécies de *Drosophila* cujos genomas se encontram disponíveis. Em todas as espécies, assim como em *D. willistoni*, o gene se encontra nos braço cromossômicos correspondentes aos elementos B de Muller.

A caracterização da DNA metiltransferase 2 e estudos relacionados às prováveis funções desta enzima têm sido foco constante de investigações. *Drosophila* é um dos organismos que possuem somente a Dmnt2, os chamados *Dnmt2-only*. Homólogos para *Dnmt2* já foram identificados em praticamente todos os modelos biológicos utilizados, entretanto suas funções biológicas ainda não foram efetivamente determinadas. Devido a alguns trabalhos apontarem para um papel da Dnmt2 no início do desenvolvimento de alguns invertebrados, realizamos uma investigação para detectar os padrões de expressão de Dnmt2 em *D. willistoni*. Primeiramente, nós demonstramos a presença de transcritos não somente em embriões, como fora previamente descrito, mas em outros estágios de desenvolvimento e em tecidos somáticos e germinativos. Nas análises quantitativas, encontramos níveis mais altos de expressão durante a oogênese, o que pode ser indício de uma função importanteda Dnmt2 na regulação de genes de desenvolvimento e de diferenciação de ovócitos nesta espécie, atuando ainda em nível pré-zigótico.

Abstract

Epigenetic phenomena have been widely characterized in the genomes of vertebrates, especially mammals, and DNA methylation is a key mechanism for epigenetic regulation. DNA methylation systems of vertebrates and invertebrates differ markedly, but the evolutionary implications of these differences only recently begun to be studied. In this thesis we initially present an investigation about the recurrence of sex-specific methylation of DNA ribosomal genes (*rDNA*), previously described for adults in *Drosophila willistoni*. We spread out the analysis to other species groups of *Sophophora* subgenus of *Drosophila* to which the main model organism of the genus, *D. melanogaster*, also belongs. In this study we investigated species from *willistoni*, *melanogaster*, *obscura* and *saltans* groups of *Drosophila* genus and our results suggest that differential methylation between sexes in the gene studied, occurs only in *Drosophila tropicalis* and *D. insularis*, both species of the cryptic *willistoni* subgroup. Our findings indicate that DNA methylation may present important differences, even in closely related species, providing interesting data to the knowledge of this complex Neotropical group of species.

Studies have been conducted to understand the DNA methyltransferase 2 (Dnmt2) mechanisms, the enzyme identified as responsible for the DNA methylation in the genomes of *Drosophila*, but the most studies use *Drosophila melanogaster* representing the entire genus. Little is known about the conservation of DNA methylation patterns and whether it would be important in evolutionarily related species. In this sense, we conducted a pioneering and widespread study aiming to find *Dnmt2* gene homologs in a large number of species of the Drosophilidae family and thus try to trace an evolutionary profile of the gene, since there are differences in DNA methylation patterns among species. Our results demonstrated the Dnmt2 conservation among species, since our phylogenetic analysis recovered three major clades of the family Drosophilidae (*virilis-repleta* and *quinaria-tripunctata* radiations of *Drosophila* subgenus and *Sophophora* subgenus). Besides, we physically mapped the *Dnmt2* gene on polytene chromosomes of *D. willistoni* by *in situ* hybridization. It was also possible to observe that the section where *Dnmt2* is located is next to transposable elements insertions and to an inversion breakpoint, previously described. These results are promising because the relationship between chromosomal

rearrangements and gene expression positioned close to them has been subject to important discoveries. We also identified the preservation of chromosomal synteny described for the 12 species that have their genomes sequenced available. For *in silico* searches, we found that sequences homologs to the *Dnmt2* gene are on chromosomal arms corresponding to the Muller B element.

Characterization of DNA methyltransferase 2 activities and investigations about putative functions of this enzyme has been focus of recent studies. *Drosophila* is one of those organisms so-called "Dnmt2-only" since they only utilizes Dnmt2 enzyme. Dnmt2 homologs have been identified in virtually all biological models; however the biological function performed by this enzyme has not yet been determined. Since some studies indicate a Dnmt2 role in early development of some invertebrates, we conducted a study to analyze the *Dnmt2* expression patterns in *D. willistoni*. Our data demonstrated transcripts not only in embryos, as was previously described, but in other development stages, in somatic and germinative tissues. We found higher levels of expression during oogenesis, which may indicate an important role in gene regulation of development and differentiation of oocytes in this species, acting in pre-zygotic levels.

Capítulo I

Introdução Geral

Objetivos

Introdução Geral

Epigenética e metilação do DNA

Os mecanismos epigenéticos são componentes críticos no desenvolvimento normal e no crescimento das células e atuam para mudar a acessibilidade à cromatina para regulação transcricional por meio das modificações do DNA e pela alteração ou remodelagem de nucleossomos. Portanto, as modificações epigenéticas influenciam a expressão gênica, mas não mudam a sequência de DNA, podendo ser relativamente estáveis na mitose. Uma vez estabelecido, o estado epigenético é mantido ao longo da vida do indivíduo (Rakyan & Whitelaw, 2003). A herança epigenética, portanto, envolve a transmissão de informações que não são codificadas pelas sequências de DNA de uma célula para a célula filha ou de geração para geração.

A característica fundamental do fenômeno epigenético é que um genótipo pode apresentar fenótipos alternativos, os quais são baseados no estado epigenético de um ou mais lócus dentro do genoma. Muitos dos sistemas experimentais epigenéticos clássicos foram descobertos devido à aparente instabilidade genética ou a desvios nas proporções de herança mendelianas esperadas (Richards, 2006). A abordagem geral do genoma tendo como meta central definir as características pós-transcricionais das sequências de DNA relacionados a processos epigenéticos pode ser definida como epigenômica (revisão em Callinan & Feinberg, 2006).

Inúmeros processos epigenéticos já têm sido identificados e incluem fenômenos do tipo: a) metilação, onde ocorre a substituição de um átomo de hidrogênio (H) por um grupo metil (CH_3) e é catalisada por metiltransferases; b) fosforilação, principal participante nos mecanismos de regulação das proteínas, com a adição de um grupo fosfato (PO_4) a um aminoácido de uma cadeia protéica. As fosfatases são as enzimas responsáveis pela defosforilação e as quinases as reponsáveis pela fosforilação; c) a ubiquitinação, processo que ocorre nas células no qual uma molécula é marcada por moléculas de ubiquitina com o objetivo de ser reconhecida por um proteossoma onde é degradada; d) a acetilação, que é controlada por duas famílias de enzimas: as histonas-acetiltransferases (acetiladoras) e as

histonas-desacetilases (desacetiladoras). A acetilação está associada com a remodelação do nucleossomo e com a ativação da transcrição, enquanto que a desacetilação se associa com a repressão da transcrição, via condensação da cromatina (revisão em Malakhova *et al* 2003). As modificações covalentes do DNA ou seu empacotamento em histonas são os principais responsáveis pela transferência das informações epigenéticas (Fazzari & Greally, 2004).

Reconhecendo-se, então, os mecanismos citados anteriormente como capazes de promover variabilidade mesmo sem mudanças nas sequências de DNA, torna-se interessante reconsiderar uma antiga questão na biologia: "qual a origem da variação?". Mesmo sendo um tema aparentemente resolvido os estudos em epigenética fizeram com que dúvidas ressurgissem em torno dele, considerando-se duas questões principais: a variabilidade em populações aparece exclusivamente por mutações randômicas, como se pensava até pouco tempo a formação de novos caracteres pode ser induzida por forças ambientais externas? O conhecimento atual sobre o efeito das modificações epigenéticas nos genomas torna, portanto, possível que tal dúvida exista (Guerrero-Bosagna *et al*., 2005).

Atualmente, a epigenética surge como uma nova visão que requer muito estudo futuro no intuito de explicar os fatores que podem gerar variabilidade nas populações. Eva Jablonka e Marion Lamb em 1995, no livro "Epigenetic Inheritance and Evolution, The Lamarckian Dimension", descrevem os Sistemas de Herança Epigenética (EIS - *Epigenetic Inheritance Systems*) indicando-os como um canal adicional para transmissão de variação herdável entre gerações de células e sugerindo a interação desta variação com os Sistemas de Herança Genética (GIS - *Genetic Inheritance Systems*). Os EIS, segundo as autoras, podem responder mais rapidamente às mudanças ambientais em ciclos intermediários (com escalas de tempo menores), gerando alterações evolutivas mais rápidas do que as mutações randômicas e seleção de variação de sequência nucleotídica (revisão em Griesemer, 1998).

De acordo com a separação entre origem e fixação de uma novidade evolutiva, alguns autores afirmam que a evolução é sempre um processo de dois passos, primeiro envolvendo variação mediada pelo desenvolvimento, e após a seleção, cuja operação resulta nas mudanças de frequências gênicas (West-Eberhard, 1998). Nesse sentido, as mudanças ocorrem devido a alterações nos processos iniciais do desenvolvimento, os

quais, mais adiante, poderiam, em alguns casos, ser ambientalmente induzidos, e tais modificações seriam então fixadas e se perpetuariam na população. Portanto, a diversidade e a evolução das espécies poderiam ser explicadas não somente por processos seletivos impostos pelo ambiente, mas também pela ação do ambiente como um indutor de variação genotípica e fenotipica, variação esta que é matéria prima para a seleção (Guerrero-Bosagna *et al*., 2005).

Devido aos resultados de seus experimentos com *Drosophila*, Waddington, na década de 50, propôs dois novos conceitos relacionados à capacidade de influências ambientais induzirem a aparição de novos caracteres em organismos e a sua manutenção ao longo das gerações. O primeiro é a "canalização", ou seja, diante de distúrbios e influências externas estressantes, haveria uma perturbação das tendências do desenvolvimento normal do adulto em condições naturais. O outro é a "assimilação genética": enquanto existem essas tendências conflitantes, se um estímulo estressante é capaz de modificar o desenvolvimento de uma linhagem em um organismo, a população derivada pode evoluir exibindo a modificação mesmo na ausência do estresse (revisão em Guerrero-Bosagna *et al*., 2005).

Metilação do DNA e DNA metiltransferases

Dentre os processos epigenéticos conhecidos, e anteriormente citados, talvez o mais abordado seja a metilação do DNA (Weinhold, 2006), que consiste, basicamente, na adição de grupamentos metil na posição C5 de citosinas (Gruenbaum *et al*., 1981). Em eucariotos, as citosinas são convertidas a 5-metilcitosinas após a replicação do DNA sendo que em torno de 5% do genoma dos mamíferos é metilado. A metilação de citosinas do DNA desempenha um papel importante no processo de imprinting genômico, a estabilidade genômica e silenciamento irreversível de transposons e retrovírus endógenos (Bird, 2002). Além disso, sabe-se que muitas doenças humanas são consequências de desordens no controle epigenético. Os exemplos mais relevantes incluem a Síndrome ICF (imunodeficiência, instabilidade do centrômero e anomalias faciais), causada por mutações do DNA do gene metiltransferase DNMT3B e a síndrome de Rett, devido a mutações no gene que codifica a proteína $MECP_2$, que se liga a DNA melitado (Robertson & Wolffe,

2000).

Atualmente, sabe-se que a metilação do DNA é um dos principais mecanismos epigenéticos e hereditários que regulam a expressão genética em células de mamíferos (Khosla *et al*., 2001). Além disso, a metilação do DNA é capaz de ser modificada pela ação de agentes externamente aplicados (MacPhee, 1998). Alguns compostos particulares encontrados na natureza poderiam atuar como tais agentes. Dessa forma, eles poderiam ser capazes de afetar a evolução dos organismos, induzindo profundas mudanças em indivíduos e populações, talvez com consequências transgeracionais (Guerrero-Bosagna *et al*., 2005).

A metilação do DNA é realizada por um grupo conservado de DNA metiltransferases (Dnmts) usualmente classificadas em três diferentes famílias (Bestor, 2000; Li, 2002), que estão normalmente presentes nos genomas da maioria dos organismos eucariotos já estudados quanto a mecanismos epigenéticos. As enzimas da enzima Dnmt1 se ligam preferencialmente a DNA hemimetilado e são responsáveis pela manutenção da metilação do DNA após cada rodada de replicação (Bestor *et al*., 1988; Yoder *et al*., 1997; Margot *et al*., 2000). Proteínas do tipo Dnmt2 são similares às metiltransferases de procariotos, porém suas funções permanecem incertas nos organismos mais estudados, uma vez que elas parecem ser incapazes de metilar DNA *in vitro* (Li *et al*. 1992; Lei *et al*., 1996). A última família de DNA metiltransferases é constituída pelas DNA metiltransferases Dnmt3a e Dnmt3b que são as principais moléculas envolvidas na metilação *de novo* de vertebrados (Okano *et al*., 1998). Há um terceiro membro descrito para esta família, a Dnmt3L, que compartilha algumas homologias com Dnmt3a e Dnmt3b e desempenha um papel central no estabelecimento do *imprinting* genômico materno mesmo que sua atividade catalítica não tenha sido detectada *in vitro* até agora (Aapola *et al*., 2001; Deplus *et al.* 2002; Hata *et al*. 2002).

As metiltransferases da família Dnmt2 chamam particularmente a atenção porque são as mais amplamente conservadas em vertebrados e plantas e também podem ser encontradas em várias espécies de insetos. Apesar de demonstrarem uma perfeita conservação dos motivos catalíticos, as enzimas Dnmt2 não eram consideradas DNA metiltransferases ativas (Dong *et al*., 2001). A conservação de motivos catalíticos de (citosina-5) DNA methiltransferase sugere fortemente uma atividade DNA metiltransferase

para as proteínas Dnmt2. Esta conservação foi primeiramente reconhecida no produto gênico de *pm1* de *Schizosaccharomyces pombe* (Wilkinson *et al*. 1995). Contudo, os autores não detectaram nenhuma atividade catalítica de metiltransferase para esta proteína, o que foi atribuído à inserção de um resíduo de serina em um dipeptídeo crítico prolina-cisteína que é essencial para a atividade DNA metiltransferase em outras enzimas. Um estudo posterior indicou que a atividade metiltransferase sítio-específica pode ser restaurada de forma significativa por remoção do resíduo inserido de serina (Pinarbasi *et al*., 1996). Entretanto esses resultados não se confirmaram por estudos independentes (Schaefer and Lyko, 2010).

Cabe salientar que as fortes similaridades entre Dnmt2 e DNA metiltransferases ativas também se estendem ao nível estrutural, uma vez que a DNMT2 de humanos possui significante similaridade estrutural com a DNA metiltransferase bacteriana M.*Hha*I (Dong *et al*., 2001). Este estudo mostrou que enzimas Dnmt2 podem formar complexos DNA-proteínas resistentes à desnaturação, o que sugere que Dnmt2 podem se ligar a DNA. Como consequência destas ideias, atualmente a atividade DNA metiltransferase de Dnmt2 vem sendo analisada em vários ensaios experimentais e organismos-modelo.

Metilação do DNA em vertebrados

Nos vertebrados, a metilação do DNA tem papel importante nos diferentes processos biológicos, incluindo a inativação do cromossomo X (Norris *et al*., 1994), *imprinting* genômico (Lloyd, 2000), silenciamento da expressão gênica (Busslinger *et al.*, 1983), e também é essencial no processo de desenvolvimento normal em mamíferos (Li *et al.*, 1992). Um exemplo bastante estudado sobre metilação dentro do processo de desenvolvimento é o que ocorre com os genes de globina das células sanguíneas de humanos e galinhas. A sequência dos promotores destes genes não apresenta metilação nas células sanguíneas, enquanto que os mesmos promotores em outras células que não produzem globina apresentam as citosinas metiladas (Gilbert, 2000).

A metilação no DNA em muitos organismos eucariotos também está envolvida no processo de modificação de sequências repetitivas as quais podem ter efeito deletério para o genoma hospedeiro, tais como elementos transponíveis (TEs) e vírus. Importantes evidências indicam que, em alguns casos, o papel da metilação do DNA está relacionado

ao silenciamento de transposons e retrovírus endógenos (Bird, 2002). A metilação de sequências repetitivas também pode suprimir recombinações entre repetições em diferentes posições no genoma, as quais poderiam levar a translocações ou a outros rearranjos cromosômicos desfavoráveis (Bender, 1998).

Ao contrário do que ocorre com os vertebrados, que tem seus genomas metilados bem estudados (Tweedie *et al*., 1997), pouco se sabe a respeito da metilação do DNA em invertebrados. Estudos recentes feitos com insetos, contudo, têm indicado uma aparente diversidade funcional que parece argumentar contra a conservação funcional estrita da metilação (Field *et al*., 2004).

Epigenética e metilação do DNA em Drosophila

Drosophila melanogaster é um consagrado organismo modelo para estudos genéticos e agora também vem sendo bastante utilizado como um modelo fundamental para a pesquisa no campo da epigenética. Observações de fenômenos epigenéticos em *Drosophila* foram feitas até mesmo décadas antes do termo epigenética ser descrito (Elgin & Reuter, 2007). A variegação por efeito de posição (PEV), que consiste na mudança no fenótipo devido à mudança de posição de um gene no genoma foi descrita pela primeira vez na década de 1930 através de observações da cor dos olhos (revisão em Girton & Johansen, 2008). Pesquisas posteriores com supressores e *enhancers* do PEV levaram à descoberta de mais fatores epigenéticos, tais como proteínas de remodelagen da cromatina e proteínas modificadoras de histonas. Os grupos de proteínas Polycomb (PcG) e Trithorax (trxG) foram originalmente descobertos em *Drosophila*, mas hoje são objetos de pesquisa desde fungos até humanos (Scheuttengruber *et al*., 2007) As proteínas PcG agem como repressoras da transcrição por se ligarem a Elementos de Resposta Polycomb (PREs). Diferentes complexos proteicos PcG metilam histonas ou se ligam a histonas metiladas, gerando transcricionalmente mudanças estruturais supressoras. Por outro lado, as proteínas trxG se ligam aos PREs, mas servem para ativar a transcrição através de modificação das histonas e remodelagem da cromatina (Tillib *et al*., 1999).

A questão da metilação do DNA em *Drosophila* vinha sendo discutida de modo controverso por muito tempo. Havia sido demonstrado que o DNA de embriões de

Drosophila aparentemente não apresentava metilação em dinucleotídeos CpG (Urieli-Shoval *et al*., 1982). Além disso, alguns trabalhos que demonstravam a ausência de 5-metilcitosinas ao analisar os estágios do desenvolvimento pupa e adultos (Patel and Gopinathan, 1987; Tweedie *et al*., 1999). No início da década de 2000, havia uma aparente incoerência, pois alguns trabalhos posteriores forneciam evidências de pequenos níveis de metilação do DNA em embriões e também em adultos de *Drosophila* (Gowher *et al*., 2000; Lyko *et al*., 2000b). Entretanto, esta contradição pôde ser parcialmente resolvida ao analisar o contexto das 5-metilcitosinas nas sequências no genoma de *Drosophila melanogaster* (Lyko *et al*., 2000b). As análises confirmaram que a metilação de resíduos CpG é praticamente ausente em *Drosophila melanogaster*. Estes trabalhos também demonstraram baixas, mas incontestáveis quantidades de metilação do DNA em nucleotídeos não CpG (CpA, CpT e CpC) em embriões de *D. melanogaster* (Lyko *et al*., 2000b).

A dificuldade em detectar a metilação do DNA em espécies de *Drosophila* provavelmente se devia ao fato de que os estudos se concentravam em estágios mais avançados do desenvolvimento, comparativamente aos estudos que vinham sendo realizados com mamíferos. No entanto, hoje se sabe que os mecanismos de metilação do DNA de *D. melanogaster* e mamíferos apresentam muitas diferenças importantes. Por exemplo, os estudos que primeiramente reportaram a metilação do DNA em *D. melanogaster*, descobriram que ela ocorre predominante durante o início do desenvolvimento embrionário (Lyko *et al*., 2000a). Nos mamíferos, a maior quantidade de metilação do DNA é detectada em estágios mais tardios do desenvolvimento. Ao contrário das células de vertebrados, que invariavelmente mantêm seus padrões de metilação ao longo do desenvolvimento, a metilação do DNA em *D. melanogaster* parece ser um sinal epigenético transiente observado basicamente em estágios iniciais da embriogênese (Hung *et al*., 1999; Tweedie *et al*., 1999; Lyko *et al*., 2000; Lyko, 2001; Kunert *et al*., 2003). Além do mais, a metilação do DNA em *D. melanogaster* está concentrada em dinucleotídeos CpA e CpT de forma assimétrica (Lyko *et al*., 2000a), enquanto que a metilação do DNA em mamíferos está concentrada em dinucleotídeos CpG simétricos, nas conhecidas Ilhas CpG.

A metiltransferase de Drosophila

Até então, a enzima que mediava a metilação do DNA nestes organismos permanecia desconhecida, apesar de alguns estudos apontarem para um único gene candidato a codificar uma DNA metiltransferase (Hung *et al*., 1999; Lyko, 2001; Tweedie *et al*., 1999). Este gene (*Mt2* – FlyBase) pertence à família amplamente conservada de Dnmt2, com homólogos conhecidos em humanos, ratos, insetos e fungos (Dong *et al*., 2001; Okano *et al*., 1998a; Wilkinson *et al*., 1995; Yoder and Bestor, 1998).

No entanto, não havia sido demonstrada nenhuma atividade catalítica para proteínas do tipo Dnmt2 e em estudos de indução de silenciamento do gene *Dnmt2* em camundongos não se demonstrava qualquer efeito detectável na metilação do DNA. (Okano *et al*., 1998a). Da mesma forma, investigações do gene *Dnmt2* em *Drosophila* falhavam em detectar atividade em ensaios padrão *in vitro*. (Tweedie *et al*., 1999). Desse modo, a função das enzimas da família Dnmt2 permanecia enigmática (Bestor, 2000).

Entretanto, com o avanço das tecnologias nas investigações em epigenética, começaram a surgir trabalhos relatando atividades enzimáticas das Dnmt2. O sequenciamento bissulfito do genoma de *Drosophila* sugeriu que a Dnmt2 metila resíduos isolados de citosinas sem um reconhecimento específico de uma sequência-alvo (Lyko *et al*. 2000). Neste contexto, Tang *et al*. (2003) apresentam evidências de que os produtos gênicos da Dnmt2 de *Drosophila* e camundongos são autênticas DNA metiltransferases. Além disso, outros estudos identificaram uma pequena, porém detectável atividade da DNA metiltransferase Dnmt2 (Hermann *et al*., 2003; Liu *et al*., 2003) em alguns organismos estudados. O trabalho de Kunert *et al*. (2003) evidenciou que a Dnmt2 é essencial para a metilação do DNA em embriões de *Drosophila*. Os autores demonstraram ainda que a super expressão da Dnmt2 resulta em níveis significantes de metilação em resíduos CpT e CpA, em relação às amostras controle, estabelecendo que a metilação do DNA em *Drosophila* é mediada por Dnmt2 (Kunert *et al*., 2003).

Há dados que sugerem uma especificidade das Dnmt2 por CpT e CpA, que as distinguem das demais DNA metiltransferases descritas e confirmam o predomínio de metilação "não-CpG" em *Drosophila* (Lyko *et al*., 2000b). A metilação do DNA em resíduos CpT/A também tem sido reportada em inúmeros sistemas de teste em mamíferos

(Clark *et al*., 1995; Lorincz *et al*., 2000; Toth *et al*., 1990; Woodcock *et al*., 1997). Portanto, é interessante analisar a metilação do DNA em sistemas que metilam sítios da CpT/A e possível sua relação com proteínas Dnmt2.

A Dnmt2 de *Drosophila* (como outras proteínas do tipo Dnmt2) contém todos os motivos catalíticos de DNA metiltransferases ativas. A distribuição da enzima Dnmt2 entre alguns insetos dípteros foi estudada mais recentemente por Marhold *et al*. (2004) os quais confirmaram que a sequência da proteína Dnmt2 é altamente conservada, essencialmente dentro dos motivos catalíticos 5-citosina-DNA metiltransferase. Além disso, o trabalho mostra que as proteínas Dnmt2 de *Drosophila* contêm um segmento de cerca de dez aminoácidos entre o domínio de reconhecimento do alvo (a ser metilado) e o motivo catalítico IX, que não está presente em proteínas de *Anopheles gambiae*, camundongos e humanos.

Sabe-se que os padrões de metilação do DNA precisam ser estabelecidos no DNA não metilado durante o desenvolvimento inicial. Por esta razão, a identificação de DNA metiltransferases com atividade de Dnmts *de novo* se tornou um tema importante no campo da epigenética. A recente publicação do sequenciamento genômico de 12 espécies do gênero *Drosophila* (Clark *et al*., 2007) em associação com a disponibilidade em bancos de dados do genoma de *A. gambiae* (Holt *et al*., 2002) e de outros insetos dípteros, torna possível a análise destes genomas quanto à presença de sequências relacionadas a genes *Dnmt2*.

Metilação do DNA em Drosophila willistoni

Além da reconhecida *Drosophila melanogaster*, o gênero *Drosophila* apresenta inúmeras espécies, que servem como organismos-modelo igualmente hábeis para estudos ecológicos, genéticos, e mais recentemente, epigenéticos. Mais recentemente, no nosso grupo de pesquisas foi evidenciada a metilação fêmea-específica do DNA em *Drosophila willistoni* (Garcia *et al*., 2007).

O grupo *willistoni* de *Drosophila* é constituído por seis espécies crípticas e por dezenove não crípticas, sendo o grupo de espécies mais bem representado nas

comunidades neotropicais do gênero (Val *et al*., 1981). As espécies crípticas são *D. willistoni, D. paulistorum, D. tropicalis, D. equinoxialis, D. insularis,* e *D. pavlovskiana*. A este grupo de espécies é atribuída origem no Brasil Central (Da Cunha *et al*., 1950) em associação com as florestas quentes e úmidas daquela região.

Drosophila willistoni chama particularmente a atenção dentro do grupo, por possuir uma grande "versatilidade ecológica" diversas vezes comprovada, o que se expressa pela capacidade de exploração bem sucedida de diversos tipos de ambientes, tais como matas, formações abertas (Da Cunha *et al*., 1950, 1959; Da Cunha & Dobzhansky 1954), cidades (Valente *et al*., 1989, 1993; Santos & Valente 1990; Valiati & Valente, 1997; Goñi *et al*., 1997, 1998), bem como de diferentes tipos de substratos (Valente & Araújo, 1986). Sua distribuição geográfica vai desde o sul dos Estados Unidos (Flórida) e México na América do Norte, até o norte da Argentina (Spassky *et al*., 1971; Ehrman & Powell, 1981).

Além desta versatilidade ecológica, *D. willistoni* apresenta uma grande variabilidade genética expressa através de diferentes marcadores, tais como polimorfismo cromossômico para inversões paracêntricas e polimorfismo enzimático (Ayala & Powell, 1972). Cerca de 42 inversões heterozigotas foram inicialmente descritas em populações naturais (Burla *et al*., 1949). Em outras espécies de *Drosophila*, tais como *D. pseudoobscura* e *D. nebulosa*, as inversões são concentradas em um único cromossomo, ficando os demais livres de inversão. As inversões de *D. willistoni*, no entanto, são distribuídas em todos os cinco braços dos cromossomos politênicos (Da Cunha *et al*., 1950) revelando a plasticidade de seu cariótipo.

Uma investigação sobre o envolvimento dos elementos transponíveis na reorganização do genoma de *D. willistoni* foi realizada em nosso laboratório por Regner *et al.* (1996), que, através da localização *in situ* do elemento transponível *P* no genoma desta espécie, verificaram a ocorrência de uma associação entre vários sítios de inserção do elemento com pontos de quebra de inversões nos braços cromossômicos.

De alguma forma, *D. willistoni* conseguiu manter um grande polimorfismo cromossômico que a tornou paradigma para muitos estudos evolutivos. Por outro lado, vem sendo cada vez mais demonstrado como os TEs são relacionados à formação de quebras de inversões no genoma dos organismos. Assim, estudar mecanismos epigenéticos em *D. willistoni* (e espécies do subgrupo *willistoni*) que regulem a expressão de sequências

genéticas móveis parece ser uma boa perspectiva para encontrar a explicação desta variabilidade.

Também em nosso laboratório, Sassi *et al* (2005), ao investigar a presença e integridade do elemento *P* em 18 populações de *D. willistoni*, observaram variações nos fragmentos gerados quando comparado aos fragmentos esperados para *D. melanogaster*. Uma das prováveis explicações seria a alteração nos sítios de restrição devida a modificações na porção genômica adjacente ao elemento. Por outro lado, os dados de Blauth *et al*. (2009) demonstraram atividade transcricional dos TEs *P*, *gypsy* e *412* em adultos de *D. willistoni*, sugerindo uma regulação pós-transcricional destes elementos. Esta autora, também do nosso grupo de pesquisas, detectou a presença de possíveis transcritos de transposase do elemento *P* em *D. willistoni* e sugeriu que os RNA antisenso detectados possam estar relacionados com a regulação da mobilidade deste transposon (Blauth *et al*., 2011). O papel destes transcritos tanto em membros de Classe II de TEs, como *P* e de Classe I, como *gypsy* e *412*, entretanto, permanece por ser esclarecido.

Em uma análise da estrutura dos cromossomos politênicos, usando técnicas de bandamentos com as enzimas de restrição *Alu*I e *Hae*III, que reconhecem regiões de DNA metilado (Garcia, 1997), revelou que em duas populações de *D. willistoni* são induzidos padrões bem definidos de bandas refletindo os blocos específicos de sequências nos braços cromossômicos e no cromocentro. Ao tentar explicar tais padrões, Garcia *et al*. (2007), em uma outra abordagem, submeteram amostras de DNA genômico de machos e fêmeas adultas de *D. willistoni* à técnica de clivagem por com enzimas de restrição sensíveis à metilação do DNA (Methylation Sensitive Restriction Endonuclease - MSRE), o que gerou padrões sexo-espefícicos de bandas e descreveram as primeiras evidências de metilação na espécie *D. willistoni*. A análise destes fragmentos revelou a presença de genes ribossomais entre eles (Garcia *et al*., 2007).

No mesmo estudo, os autores compararam as sequências de aminoácidos de Dnmt2 entre *D. willistoni* e *D. melanogaster*, em que a conservação da sequência ocorre no domínio responsável pela metilação do DNA, enquanto que o domínio responsável pelo reconhecimento da sequência alvo a ser metilada (TRD) é altamente variável, indicando que as diferenças substanciais podem ocorrer mesmo entre espécies do mesmo gênero (Garcia *et al*., 2007).

A função biológica da Dnmt2 em Drosophila

Recentemente, inúmeros esforços vêm sendo realizados no intuito de identificar o papel biológico da Dnmt2 nos organismos onde ela é responsável por metilar resíduos de citosina no DNA. Neste contexto, o estudo de Lin *et al.* (2005) demonstrou que a superexpressão da isoforma C da DNA metiltransferase 2 de *Drosophila* (dDnmt2c) aumenta a expectativa de vida das moscas, provavelmente devido ao aumento da expressão de pequenas proteínas de choque térmico tais como Hsp22 ou Hsp26. Adicionalmente, Roder (2007) evidenciou que a superexpressão da dDnmt2c também interage *in vivo* com a proteína Hsp70 endógena em células *Schneider 2* (S2) de *D. melanogaster*.

Atualmente, existem fortes evidências mostrando os papéis da metilação por Dnmt2 tanto em DNA quanto em RNA. Dessa forma, além da Dnmt2 atuar na metilação do DNA em embriões de *Drosophila*, como descrito no estudo de Kunert *et al*. (2003), foi descoberto que tRNAAsp são metilados com alta eficiência por Dnmt2, nos sistemas modelo camundongo, *Drosophila* e *Arabidopsis* (Goll *et al*., 2006). O papel biológico da metilação de tRNAAsp, porém, continua obscuro. Cabe salientar que em *Drosophila* a atividade de Dnmt2 também tem sido associada a modificações covalentes das histonas (Kunert *et al.*, 2003), o que sugere que Dnmt2 tenha um papel na regulação epigenética. Se esta função está relacionada com a atividade DNA metiltransferase ou com a atividade de tRNAAsp metiltransferase da Dnmt2 ainda não se sabe.

Schaefer *et al*. (2008) verificou que a proteína Dnmt2 é tanto citoplasmática quanto nuclear e que há uma quantidade significativa de Dnmt2 está ligada à matriz nuclear. A análise da localização subcelular revelou que as proteínas Dnmt2 são abundantes em células ativamente em divisão e que a localização da Dnmt2 é muito dinâmica durante o ciclo celular. Os autores revelam também que Dnmt2 apresenta um padrão de localização fusiforme durante as divisões mitóticas. Experimentos adicionais sugerem que essa localização é microtúbulo-dependente e que Dnmt2 pode acessar o DNA durante a divisão celular mitótica. Estes resultados representam a primeira caracterização detalhada das proteínas Dnmt2 em nível celular e têm importantes implicações para a compreensão das atividades moleculares das Dnmt2.

O papel específico de Dnmt2 em vias de silenciamento epigenético foi

recentemente analisado em *Drosophila* por Phalke *et al*. (2009). Os resultados deste estudo confirmam o papel da Dnmt2 na metilação do DNA do retroelemento *Invader*4 durante a embriogênese inicial de *Drosophila melanogaster*, além de uma associação da Dnmt2 com a manutenção da integridade dos telômeros. Permanece a ser demonstrado ainda, no entanto, se o DNA de outros retrotransposons é igualmente modificado por Dnmt2 e se a metilação do DNA de retroelementos, mediada por Dnmt2, se confirma em outros organismos modelo.

Outra função da Dnmt2 em *Drosophila* também foi sugerida recentemente por Schaefer *et al*. (2010), durante a biogênese de pequenos RNAs derivados de tRNAs. Os autores mostram que, além de tRNAAsp-GTC, os tRNAVal-AAC e tRNAGly-GCC também são metilados pela Dnmt2 em *Drosophila*.

No mesmo ano, Gou *et al*. (2010) descobriram que a proteína SETDB1 de *Drosophila* (dSETDB1) catalisa a trimetilação do resíduo de lisina 9 na histona 3 (H3K9) e se liga a motivos CpA metilados. A trimetilação de H3K9 por dSETDB1 medeia o recrutamento da Dnmt2 e da Su(var)205, o ortólogo da HP1 (Heterochromatin Protein 1) de mamíferos, direcionando genes para a dSETDB1. Os resultados apontam o sistema de metilação do DNA pós-embrionário mediado por dSETDB1 como um modelo para o silenciamento de supressor tumoral e revelam um importante papel para metilação do DNA tipo-específica de células no desenvolvimento de *Drosophila* (Gou *et al*., 2010).

A partir, portanto, do novo e amplo campo de investigação surgido pelos diversos trabalhos que estudam metilação no DNA de *Drosophila*, e especialmente pelo estudo realizado por Garcia *et al.* (2007) no nosso grupo de pesquisas, abre-se uma nova perspectiva de pesquisa e se faz necessário avaliar o fenômeno de metilação no genoma de diferentes espécies do gênero *Drosophila*. Análises neste sentido poderão esclarecer se dentro da história evolutiva dos diferentes grupos de espécies do gênero *Drosophila* possa ter ocorrido uma diversificação na metilação do DNA. Nosso intuito é contribuir para a melhor compreensão deste fenômeno.

Objetivos

Geral:

O objetivo do presente trabalho foi estudar aspectos moleculares do gene e da enzima DNA metiltransferase 2 (Dnmt2), responsáveis pela metilação do DNA no gênero *Drosophila*,

Específicos:

- Verificar a conservação da metilação sexo-específica para genes de rDNA em espécies do subgênero *Sophophora* de *Drosophila* **(Capítulo II)**
- Avaliar a conservação do gene da metiltransferase 2 (*Dnmt2*) em espécies de Drosophilidae e localizar fisicamente o gene da metiltransferase 2 em cromossomos politênicos de *Drosophila willistoni* **(Capítulo III)**
- Analisar a presença de potenciais transcritos da Dnmt2 em diferentes tecidos e estágios de desenvolvimento e estabelecer o padrão tecido-temporal de expressão sua durante a oogênese e embriogênese de *D. willistoni* **(Capítulo IV)**

Capítulo II

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

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ORIGINAL RESEARCH

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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.

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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) (Khosla 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 *Alu*I and *Taq*I (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 1X and SDS 0.1%,

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

Group (origin)	Subgroup	Species	Origin	Habitat
willistoni (Neotropical)	*willistoni*	*D. willistoni*	Itaqui, Rio Grande do Sul, Brazil	Woods and town
		D. paulistorum[a]	Ribeirão Preto, São Paulo, Brazil	Woods and town
		D. equinoxialis	Mexico city, México	Only woods
		D. tropicalis[b]	San Salvador, El Salvador	Only woods
		D. insularis	Saint Kitts, Caribbean	Endemic (insular)
	bocainensis	*D. capricorni*	Florianópolis, Santa Catarina, Brazil	Only woods
		D. fumipennis[b]	Arima Valley, Trinidad and Tobago	Woods
		D. nebulosa[b]	Sinaloa, Mexico	Woods and clearings
saltans (Neotropical)	*sturtevanti*	*D. sturtevanti*	Florianópolis, Santa Catarina, Brazil	Woods and town
	elliptica	*D. neoeliptica*	Joinville, Santa Catarina, Brazil	Only woods
melanogaster (Afrotropical)	*melanogaster*	*D. melanogaster*	Zarate, Argentina	Cosmopolitan
		D. sechellia	Seychelles Islands	Endemic (insular)
obscura (Holarctic)	*obscura*	*D. pseudoobscura* (Nearctic)	Mesa Verde, Colorado, USA	Woods
		D. subobscura (Palearctic)	La Florida, Santiago, Chile	Woods and town

[a] 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)

[b] From Tucson Stock Center

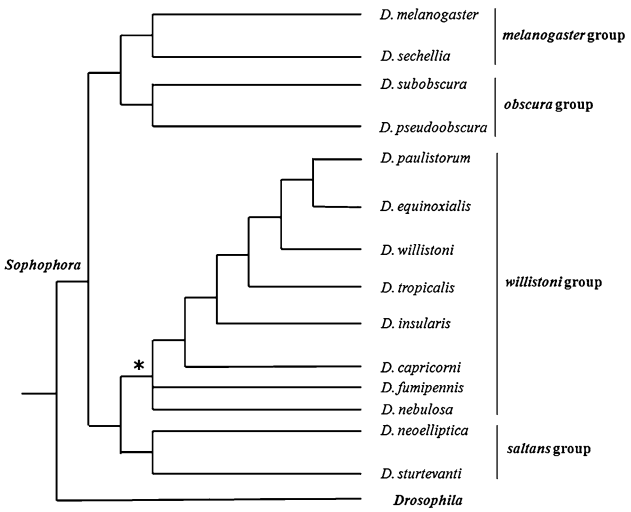

Fig. 1 Evolutionary relationship of the *Sophophora* subgenus species analyzed in this study, according to the phylogenies of O'Grady and Kidwell (2002), O'Grady et al. (1998), Markow and O'Grady (2005) and Robe et al. (2010). * Indicates that the relationship between *bocainensis* and *willistoni* subgroups have been inconclusive

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

Restriction endonucleases	
Sensitive to methylation	Non-sensitive to methylation
*Hae*III (5′GG↓**C**C3′)	*Rsa*I (5′GT↓**AC**3′)
*Alu*I (5′AG↓**C**T3′)	*Taq*I (5′T↓**C**GA3′)

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

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′AG↓CT3′) and *Hae*III (5′GG↓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*

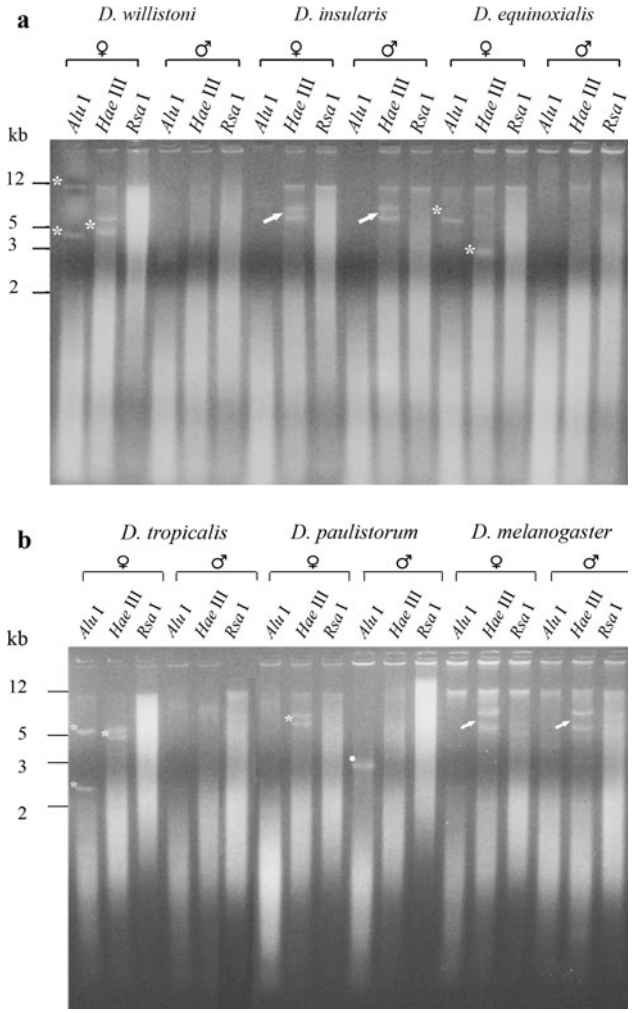

Fig. 2 Cleavage patterns obtained by the digestion of total genomic DNA of adult males (♂) and females (♀) of (**a**) *D. willistoni*, *D. insularis*, *D equinoxialis*; (**b**) *D. tropicalis*, *D. paulistorum*, *D. melanogaster* with enzymes *Alu*I, *Hae*III and *Rsa*I. *Arrows* indicate the same pattern 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

presented clearly different cleavage patterns–which were also observed for *D. willlistoni*. The cleavage pattern observed with the enzyme *Rsa*I (5′GT↓**AC**3′), 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 hybridizated 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).

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

	*Alu*I	*Hae*III	*Rsa*I
D. willistoni	+	+	−
D. insularis	−	?	−
D. equinoxialis	+	+	−
D. tropicalis	+	+	−
D. paulistorum	+	+	−
D. melanogaster	−	−	−

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

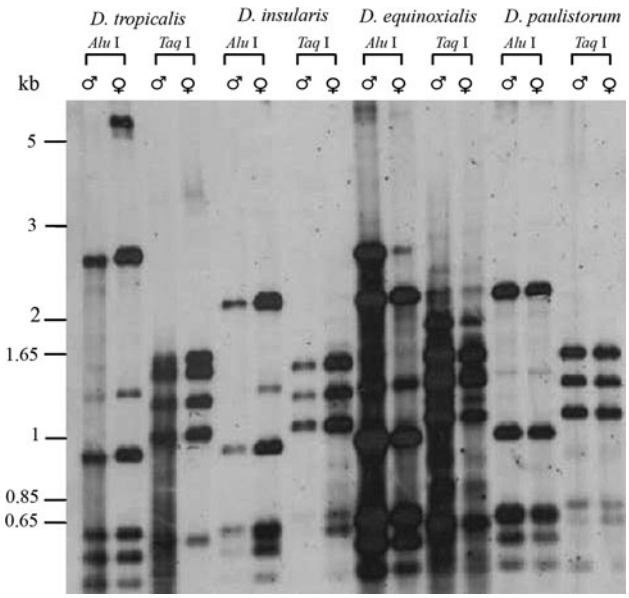

Fig. 3 Southern blot analysis of the genomic DNA of adult males (♂) and females (♀) of *D. tropicalis*, *D. insularis*, *D. equinoxialis* and *D. paulistorum* digested with *Alu*I and *Taq*I and hybridized with the pDm238 probe. The size of fragments, in kilobase pairs (kb), is indicated at the left

was observed only in female samples. When samples of male and female DNAs were cleaved with *Taq*I, 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 *Alu*I 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 *Alu*I and *Hae*III 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 *Alu*I (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 ($\sim$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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Capítulo III

Conservation of DNA methyltransferase Dnmt2 in Drosophilidae

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Abstract

Several studies have been performed aiming to characterize the DNA methyltransferase 2 (Dnmt2), responsible to DNA methylation in *Drosophila*, but the most data is described only for *Drosophila melanogaster*. Not much is known about conservation of DNA methylation in *Drosophila* related species and its evolutionary consequences. Here we present our findings of a widely searching by Dnmt2 homologs in sixty species from Drosophilidae family. In our *Dnmt2* phylogenetic analyses, the three main clades of the Drosophilidae species (*virilis-repleta* radiation, *quinaria-tripunctata* radiation and *Sophophora* subgenus) were recovered. Additionally, we mapped the Dnmt2 homologue on the IIR chromosome arm (Muller's B element) of *D. willistoni*, near to insertions of transposable elements and of an inversion breakpoint. By searches *in silico* in remaining *Drosophila* species whose genomes are available, we verified *Dnmt2* homologs also at chromosomal arms corresponding to Muller's B element. Then, we also confirmed the preservation of chromosomes synteny described to *Drosophila* genomes. Our results, as a whole, indicate a high conservation of *Dnmt2* in virtually all species analyzed.

Keywords: Dnmt2, DNA methyltransferase, Drosophilidae, physical gene mapping, inversion breakpoints.

Introduction

The Drosophilidae family is among the most diverse of the Diptera, encompassing more than 3,900 species (Bächli, 2009). Species of this family, especially the *Drosophila* genus species, are often used in many areas of contemporary biological research. More recently, studies have shown the occurrence of the phenomenon of DNA methylation in *Drosophila* species (Lyko *et al*., 2000; Kunert *et al*., 2003; Marhold *et al*., 2004).

DNA methylation is a covalent modification that occurs through the addition of a methyl group to DNA, almost exclusively at cytosine bases. This modification is accomplished by several key enzymes, evolutionary conserved among eukaryotes, known collectively as 5-cytosine DNA methyltransferases (Dnmts) (Goll and Bestor, 2005; Albalat, 2008). Three types of Dnmts have been found in metazoans and they are classified according to their activity: Dnmt1, Dnmt2 and Dnmt3 (review in Goll and Bestor, 2005). Dnmt3 enzymes methylate cytosine nucleotides within unmethylated double stranded DNA, and can thus act as *de novo* methyltransferases. Dnmt1 enzymes are maintenance methyltransferases because they present clear preference for hemimethylated DNA. The smallest eukaryotic methyltransferase, Dnmt2, is most widely distributed in animals, fungi, protists, and plants (Ponger and Li, 2005), but its catalytic activity on DNA is very weak according to analyses of the human and of the *Enthamoeba* enzymes (Hermann *et al*., 2003; Fisher *et al*., 2004). This enzyme is the only Dnmt found in dipterans, including *Drosophila*.

Dnmt2 proteins contain the consensus catalytic motifs of DNA 5-cytosine methyltransferases and have been strongly conserved during evolution of eukaryote organisms (Jeltsch *et al*., 2006). In addition, the 3D structure of human DNMT2 has been obtained by X-ray crystallography and is highly similar to that of bacterial DNA methyltransferases (Dong *et al*., 2001). In agreement with this structural conservation, Dnmt2 has been demonstrated, by different methods in various systems, to have DNA methyltransferase activity (Hermann *et al*., 2003; Tang *et al*., 2003; Fisher *et al*., 2004; Katoh *et al*., 2006). For instance, studies had associated Dnmt2 with RNA interference in *Dictyostelium* (Kuhlmann *et al*., 2005) and with covalent histone modifications in

Drosophila (Kunert *et al*., 2003), which suggests that Dnmt2 has a role in epigenetic regulation.

The distribution of the Dnmt2 enzymes in Diptera was explained by Marhold *et al*. (2004) and showed that Dnmt2 protein sequences are highly conserved in *D. virilis, D. hydei, D. simulans, D. melanogaster and D. pseudoobscura*, principally within the catalytic DNA methyltransferase motifs. Additionally, authors reported that *Drosophila* Dnmt2 proteins contain a stretch of roughly ten amino acids between the target recognition domain and the catalytic motif (so-called IX) which is not present in proteins from *Anopheles gambiae*, mouse and human. Focusing on the *Drosophila* genus, Garcia *et al*. (2007) compared *D. willistoni* and *D. melanogaster* Dnmt2 protein sequences. The authors found higher conservation at the domains responsible for methyl transfer catalysis and great variability in the region responsible for specific DNA target recognition domain (TRD), which could be an indicative of Dnmt2 function variability in different organisms, suggesting that targets of methylation in species of the same genus can be also variable. Accordingly, it raises the possibility that between different, but closely related, species it can occurred a selection for different targets to methylation (Garcia *et al*., 2007).

The *Drosophila* 12 Genomes Project (Clark *et al*., 2007) highlights the need to develop bioinformatics tools for assembly, annotation and analysis of groups of related taxa, as was done with the genomes of mammals. The knowledge of the limits in synteny blocks is a valuable bioinformatics tool to aid in the ordering of scaffolds that emerge from projects of the whole-genome shotgun (Schaeffer *et al*., 2008), to infer the history of chromosomal rearrangements, and to determine the rates of reuse of breakpoints. Scaffolds can be mapped to Muller elements and can be ordered using information from synteny blocks. The large number of states of gene-order allows the history of changes in gene-order to be retrieved, even when the breakpoints are reused. Furthermore, genes at breakpoint boundaries can be used to reconstruct ancestral states of common ancestors (Ma *et al*., 2006). Thus, the availability of the 12 genomes presents a unique opportunity to develop new tools for assembly, annotation and analysis of gene-order.

The currently understood preconizes *D. willistoni* placement on *Drosophila* phylogenies, a species of the subgenus *Sophophora*, is very close to the split between the

two subgenera (*Sophophora* and *Drosophila*) of the genus *Drosophila* (Tamura *et al*., 2004). *D. willistoni* is known to have extensive gene arrangement polymorphisms on all chromosomes (Da Cunha *et al*., 1950, 1959; Da Cunha and Dobzhansky, 1954; Valente and Araújo, 1986; Valente *et al*., 1993; Rohde *et al*., 2005) as observed from chromosomal variability in natural populations. As a result of its higher rate of intraspecific polymorphism and significantly large independent evolutionary time compared to other species of *Drosophila* 12 Genomes Project, computational methods seem to demonstrate some ambiguity in its phylogenetic placement (Bhutkar *et al*., 2008). 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 out group on the basis of elevated 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 ~~a~~ additional markers 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 body of data from this species.

In this study, we extended the search for DNA metiltransferase 2 in *D. virilis, D. hydei, D. simulans, D. melanogaster, D. pseudoobscura*, *D. willistoni* and other dipteran insects (Marhold *et al*., 2004; Garcia et al., 2007) and in *D. willistoni* (Garcia *et al*., 2007) to include a great number of *Drosophila* species and some related species. Our results indicate a high conservation of *Dnmt2* in all species tested. In addition, in this investigation we localized *Dnmt2* homologs on chromosomes of *D. willistoni* and we tried to establish relations of cause and/or effect for the exact location of the gene with its adjacent sites and possible activity modulation by position.

Material and Methods

Fly stocks

To test the conservation of *Dnmt2* sequences on Drosophilidae, 56 *Drosophila* species were used in this study, in addition to *Zaprionus indianus*, *Z. tuberculatus*,

*Scaptodrosophila latifasci*aeformis and *S. lebanonensis* (Table 1). To the non-fluorescent *in situ* hybridization (ISH) assay, we used *D. willistoni* Gd-H4-1 strain, which genome was sequenced (Clark *et al*., 2007). All these species have been maintained in laboratory by mass crosses and cultivated in corn flour culture medium (Marques *et al.*, 1966), in a controlled chamber (17 ± 1°C, 60% r.h.).

PCR, cloning and sequencing

Genomic DNA was extracted from adult flies according to Sassi *et al*. (2005). The quality of samples were checked by usual restriction endonucleases and fractionated in 1% agarose gels, with 1kb Plus DNA ladder molecular marker (Invitrogen). Specific primers were designed based on the sequences of *D. melanogaster* (Marhold *et al*., 2004) and *D. willistoni* (according to sequence described by Garcia *et al*., 2007) *dnmt2* genes (wDnmt2A-F: 5' TCACCCACAACCTTGACATT 3' and wDnmt2C-R: 5' ACCTTCTCGCAGACACCAA 3'). The PCRs were performed in 25 µL using 20 ng of genomic DNA, 1 U Taq DNA Polymerase (Invitrogen), 1X reaction buffer, 200 µM of each nucleotide, 20 pmol of each primer and 1.5 mM $MgCl_2$. The employed amplification conditions were 95°C for 5 min, 30 cycles at 95°C for 40 s, 55°C to 60°C for 40 s and 72°C for 1 min, followed by a final extension cycle at 72°C for 5 min. Different conditions were tested in an attempt to obtain amplification in a larger number of species, though unfruitful. The more specific *Dnmt2* amplicons were directly purified by incubation at 37ºC for 30 min with *Exonuclease* I and *Shrimp Alcaline Phosphatase* (SAP) (both from USB) followed by a 15 min inactivation step at 80ºC. For *D. ornatipennis* and *D. tropicalis Dnmt2* amplicons, the fragments were excised and purified using Illustra GFX PCR DNA and gel band purification kit (GE Healthcare). The obtained bands were cloned into pCR4-TOPO plasmid (Invitrogen). DNA sequencing was performed by Macrogen Inc. (Korea) using the universal primers, M13 forward and M13 reverse.

Dot blot analyses

For *dot blot* hybridizations, denatured DNA samples (1 mg) were transferred on a nylon membrane Hybond-N+ (GE Healthcare). The investigated species are shown in Table 1. The hybridization and detection were according with AlkPhos direct labeling and CDP Star Detection system protocol (GE Healthcare). The filters were washed twice with

SSC 0.2X and 0.5% SDS (w/v) for 15 min at 65 °C. The *Dnmt2* PCR amplified fragment from *D. melanogaster* genomic DNA was used as probe.

Sequence analyses

To obtain the sequences for each clone, 2–4 readings were used to the assemblage, using the GAP 4 software of the Staden Package (Staden, 1996). Then, the sequences were aligned using the Muscle tool (Edgar, 2004). The phylogenetic relationship between the *Dnmt2* sequences was estimated using Neighbor-Joining (NJ) method (Saitou and Nei, 1987), with 5,000 bootstrap replicates in the Mega 5 program (Tamura *et al*., 2011), and Bayesian analysis implemented in the MrBayes (Ronquist and Huelsenbeck, 2003) program with evaluation of the at least 1,000,000 generations and a burning region of 2,500 trees. The Bayesian analysis for the nucleotide sequences was performed with the general time reversible (GTR) model using the ratio of invariable sites (I) and the gamma distribution of the variable sites (G) model, as suggested in the MrModel Test 2.3 program (Nylander, 2004). In the NJ, the Tamura-Nei model with a gamma distribution was implemented. In the phylogenetic analysis using amino acid, each sequence was individually translated to protein and aligned in the ClustalW2 tool (Larkin *et al*., 2007) with default parameter values. For evolutionary analysis of the amino acid sequences, the Jones-Taylor-Thornton (JTT+G) model was used, as suggested by ProtTest 2.2 (Abascal *et al*., 2005), in accordance to the Akaike information criterion (Akaike, 1974) to the Bayesian analysis. Neighbor-Joining (NJ) method (Saitou and Nei, 1987) was implemented with JTT+G in the Mega 5 program (Tamura *et al*., 2011). The sequences of the *Aedes aegypti* (accession number: XM_001657505.1) and *Culex quinquefasciatus* (accession number: XM_001867292.1) were used as out group.

For nucleotide and amino acid divergence analyses, sequences were clustered in species groups to perform a p-distance analysis using the Mega 5 program (Tamura *et al*., 2011).

Physical mapping of Dnmt2 gene

For the precise location of Dnmt2 sequences in polytene chromosomes of *D. willistoni*, it was used the non-fluorescent *in situ* hybridization technique, since it allows a

better visualization of the banding patterns and is recommended for precise physical mapping (Clark *et al*., 2007) according to Lim (1993) protocol. The DNA probe of *Dnmt2 D. willistoni* was constructed from a fragment of 949 bp by cloning into the vector TOPO PCR-4 (Invitrogen), which was obtained by PCR using the primers wDnmt2A-F and wDnmt2C-R (as described above). The probe was labeled with Bio-14-dATP by BioNick Kit DNA Labeling System (Invitrogen). The detection of hybridization signals was performed by Vectastain Elite ABC (Vector Laboratories). The slides were stained with 1% Acetic Orcein, with the blades assembled by Entellan (Merck). The best preparations obtained were registered in phase contrast photomicroscope and analyzed according to the photomap of *D. willistoni* (Schaeffer *et al*., 2008).

Results

Detection of the Dnmt2 sequences in the Drosophilidae species

In a preliminary screening for the presence of *Dnmt2* homolog sequences on *Drosophila* genomes, we tested 54 species by Dot Blot (Figure 1S – Supplementary Material). To confirm the homology found with the *Dnmt2* gene, we also performed a *Dnmt2* homologues search by PCR amplification in a large number of Drosophilidae species that belong to different *Drosophila* groups (Table 1). PCR results showed amplification in several species from the following groups: *guarani*, *guaramunu*, *tripunctata*, *calloptera*, *immigrans*, *mesophragmatica*, *flavopilosa*, *repleta*, *melanogaster* and *willistoni*. The length of the amplified fragments varies nearby 800 bp. Though, some amplicons did not represent de *Dnmt2* sequence. Dot blot results (Figure 1S of Supplementary Material) corroborated the PCR data, showing positive signals in all the species in which we obtained amplification. However, not all species whose samples showed hybridization signal by dot blot were amplified by PCR for the gene *Dnmt2*.

In this assay, we observed a strong hybridization signal mainly in the *melanogaster* group. Nevertheless, hybridization was detected in the other species, showing that the *Dnmt2* gene presents homologs related sequence in the analyzed species. Exceptions were

the *D. polymorpha*, in which no amplification by PCR was detected, and *D. orena*, that belong to the *melanogaster* group.

Table 1 List of Drosophilidae species used in this study.

Genus	Subgenus	Section	Group	Species	Dot blot	PCR
Drosophila	*Drosophila*	*quinaria tripunctata*	*guarani*	*D. ornatifrons*	+	**MC**
				D. subbadia	+	+
				D. guaru	+	+
			guaramunu	*D. griseolineata*	+	+
				D. maculifrons	+	+
			tripunctata	*D. nappae*	+	+
				D. paraguayensis	**ND**	-
				D. crocina	+	+
				D. paramediostriata	+	-
				D. tripunctata	+	+
				D. mediodiffusa	**ND**	+
				D. mediopictoides	+	-
			cardini	*D. neocardini*	+	**ND**
				D. polymorpha	**W**	**ND**
			pallidipennis	*D. pallidipennis*	**ND**	-
			calloptera	*D. ornatipennis*	+	+
			immigrans	*D. immigrans*	+	+
			funebris	*D. funebris*	+	-
		virilis repleta	*mesophragmatica*	*D. gasici*	+	+
				D. brncici	**ND**	-
				D. gaúcha	+	+
				D. pavani	**ND**	+
			repleta	*D. hydei*	+	+
				D. mercatorum	+	-
				D. mojavensis	+	**NS**
			mulleri	*D. buzzatii*	**ND**	-
			canalinea	*D. canalinea*	+	**W**
			flavopilosa	*D. cestri*	**ND**	**W**
				D. incompta	+	+
			virilis	*D. virilis*	+	**NS**
			robusta	*D. robusta*	+	-
	Sophophora		*melanogaster*	*D. melanogaster*	+	**G**
				D. simulans	+	**G**
				D. sechellia	**ND**	**G**
				D. mauritiana	+	+
				D. teissieri	+	**MC**
				D. santomea	+	+
				D. erecta	+	**G**
				D. yakuba	+	**G**
				D. kikkawai	+	-
				D. ananassae	+	**G**
				D. malerkotliana	+	-
				D. orena	**VW**	-
			obscura	*D. pseudoobscura*	+	-
			saltans	*D. prosaltans*	+	-
				D. saltans	+	-
				D. neoelliptica	+	-
				D. sturtevanti	+	-
			willistoni	*D. sucinea*	+	**NS**
				D. nebulosa	+	**W**
				D. willistoni	+	**G**
				*D. willistoni (Wip-4)**	+	**ND**
				*D. willistoni (17A2) **	+	**ND**
				D. paulistorum	+	**W**
				D. insularis	+	**W**
				D. tropicalis	+	+
				D. equinoxialis	+	**W**
				D. capricorni	+	-
				D. fumipennis	+	-

Table 1 Continued

Genus	Subgenus	Section	Group	Species	Dot blot	PCR
	Dorsilopha		*busckii*	*D. busckii*	+	**W**
Zaprionus				*Z. indianus*	+	-
				Z. tuberculatus	+	-
Scaptodrosophila				*S. latifasciaeformis*	+	-
				S. lebanonensis	+	-

W: weak band; **VW**: very weak signal; **+**: PCR amplification; **G**: Genome –GenBank sequence; **-**: no PCR amplification; **ND**: not determinated. * *D. willistoni* strains maintained in laboratory cultures.

Dnmt2 conservation

The availability of the genomic sequences provided us with an opportunity to search for open reading frames encoding *Dnmt2* in *Drosophila* genomes. Some sequences used in our analyses were obtained on database searches using the *D. melanogaster Dnmt2* nucleotide sequence as query sequence, retrieving an unambiguous *Dnmt2* homologue (the primary FlyBase identifier number of these sequences is shown in table 1S – Supplementary Material). All cloned sequences and those obtained by *in silico* searches were used in the phylogenetic analysis to understand the evolutionary pattern and conservation of *Dnmt2* in the Drosophilidae species. The sequences were aligned and approximately 750 nucleotides were used to build a phylogenetic matrix, from 30 sequences analyzed. The two phylogenetic inference methods used (Neighbor- Joining and Bayesian analysis) revealed that some clusters differ among the different trees generated from the nucleotide sequences.

Figure 1 shows a nucleotides neighbor-joining (NJ) tree of *Dnmt2*, which is essentially congruent with the tree obtained previously to Drosophilidae, using similar methods to the housekeeping gene *Adh* (Russo *et al.*, 1995). As shown in Figure 1, three main clades can be observed. The first clade is composed by the *guarani*, *guaramunu*, *calloptera* and *tripunctata* species groups, that represented the section *quinaria-tripunctata* (or *immigrans-hirtodrosophila* radiation) of the *Drosophila* subgenus. Another cluster was further subdivided into two main clades, one that includes *virilis, repleta, flavopilosa* and *mesophragmatica* species (*virilis-repleta* radiation) of the *Drosophila* subgenus, and a

second one composed by the *Sophophora* subgenus species. Nevertheless, this subdivision is supported by low bootstrap values, which could explain the *D. immigrans* position closer to *Sophophora* subgenus. The tree generated by Bayesian analysis can be seen in the Figure 2S -Supplementary Material.

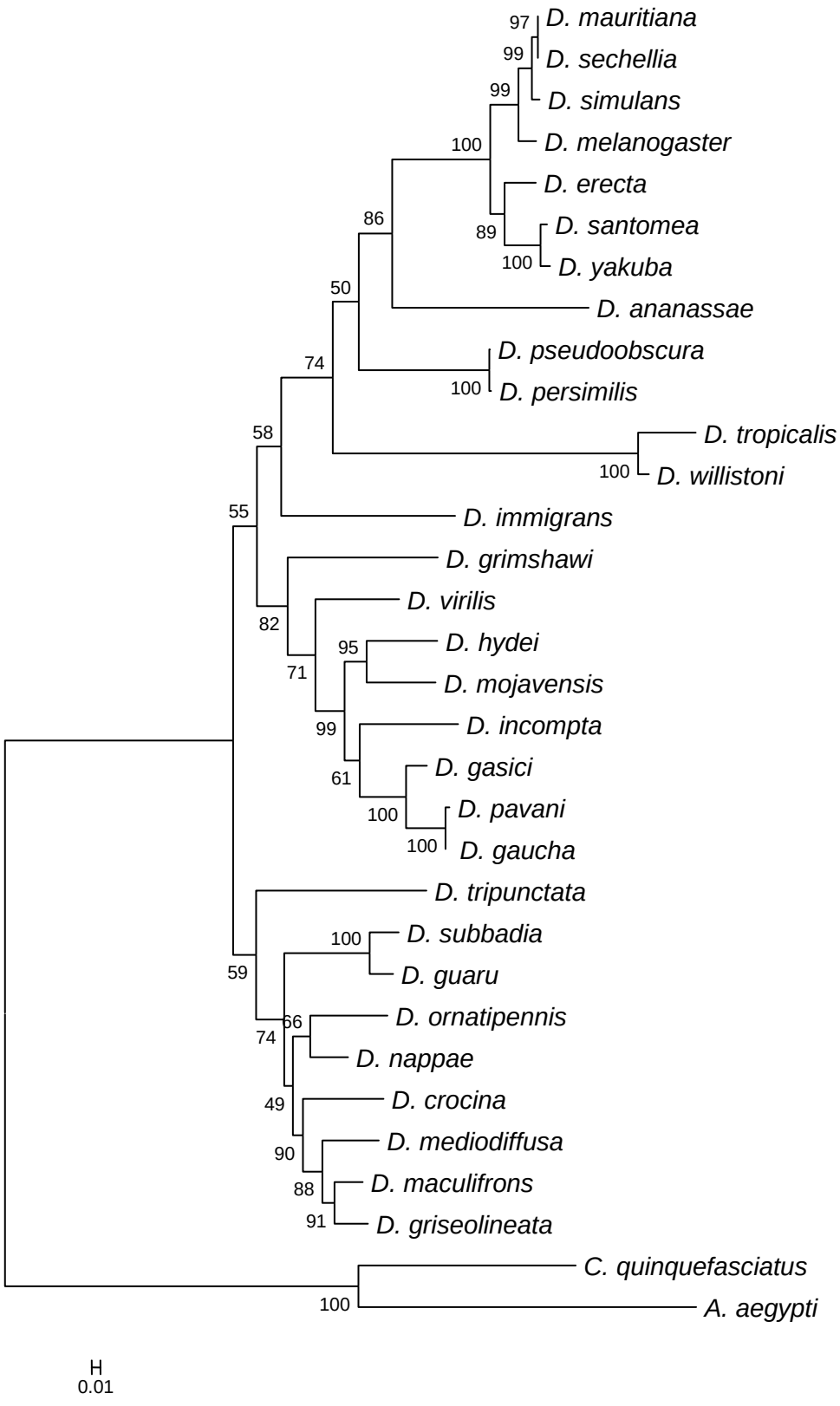
97
D. mauritiana
99
D. sechellia
99
D. simulans
100
D. melanogaster
D. erecta
86
89
D. santomea
100
D. yakuba
50
D. ananassae
D. pseudoobscura
74
100
D. persimilis
58
D. tropicalis
100
D. willistoni
55
D. immigrans
D. grimshawi
D. virilis
82
95
D. hydei
71
D. mojavensis
99
D. incompta
61
D. gasici
100
D. pavani
100
D. gaucha
D. tripunctata
100
D. subbadia
59
D. guaru
66
D. ornatipennis
74
D. nappae
49
D. crocina
90
D. mediodiffusa
88
D. maculifrons
91
D. griseolineata
C. quinquefasciatus
100
A. aegypti
0.01

Fig. 1: Phylogenetic analysis of *Dnmt2* gene based in nucleotide sequences alignment. The tree shown was generated using Neighbor-Joining distance method with Tamura-Nei model with gamma distribution. Numbers above branches are bootstrap values based on 5000 replications in the consensus tree. The *C. quinquefasciatus* and *A. aegypti* sequences were used as outgroup taxa.

The phylogenies obtained by amino acids analysis yielded essentially similar results in the Bayesian and NJ analysis. Thus, amino acid tree seems to better recover the relationship among the host species. Figure 2 shows an amino acids Bayesian inference of *Dnmt2* phylogeny, where the subgenus *Drosophila* was subdivided into two main clades, one composed of members of the *virilis-repleta* radiation and another composed of the *immigrans-hirtodrosophila* radiation species. Examining the *Sophophora* subgenus, the *melanogaster* group formed a sister-clade of all the remaining Drosophilidae species, with the *D. ananassae* as ancestral species. The *willistoni* and *obscura* groups did not show the expected basal position in relation to the *melanogaster* group, but this is not strongly supported. The NJ amino acids tree can be seen in the Figure 3S – Supplementary Material.

Fig. 2: Bayesian phylogenetic inference of *Dnmt2* gene in Drosophilidae species based in amino acid sequences alignment. The tree shown was generated using the JTT model with gamma distribution. The *C. quinquefasciatus* and A. *aegypti* sequences were used as outgroup taxa.

In order to perform the analyses of the *Dnmt2* nucleotide and amino acid divergences, the sequences were pairwise compared (species were clustered by taxonomic group). The highest divergence can be seen between *willistoni* and *mesophragmatica* groups, with a p-distance value of 35.5%, while the lowest divergence (p-distance value of 14.1%) was detected between *guaramunu* and *calloptera* group (that is represented only by *D. ornatipennis*). Overall, the comparison of all nucleotide sequences between species groups shows that the sequences of *willistoni* and *melanogaster* groups are more divergent than the sequences from the remaining groups (Table 2). The highest divergence within *melanogaster* group (11.95% of the divergence) was also confirmed in additional analysis (Table 3). In the estimate of average evolutionary divergence over sequence pairs within groups, it was observed that the more divergent groups are the *tripunctata* (18.65% of the divergence) and *melanogaster* (11.95% of the divergence).

Table 2. Estimates of evolutionary divergence over sequence pairs between Drosophilidae species groups. The numbers of amino acid differences per site from averaging over all sequence pairs between groups are shown below the diagonal. The nucleotide evolutionary divergence is demonstrated above the diagonal.

	Groups	1	2	3	4	5	6	7	8	9	10	11	12	13
1	*willistoni*		0.350	0.351	0.345	0.338	0.322	0.316	0.335	0.355	0.340	0.348	0.332	0.336
2	*guaramunu*	0.309		0.145	0.141	0.173	0.262	0.226	0.259	0.245	0.250	0.270	0.269	0.302
3	*tripunctata*	0.311	0.108		0.169	0.190	0.261	0.239	0.270	0.256	0.265	0.279	0.284	0.308
4	*calloptera*	0.312	0.094	0.116		0.170	0.274	0.239	0.270	0.248	0.255	0.276	0.301	0.316
5	*guarani*	0.316	0.131	0.142	0.127		0.255	0.243	0.273	0.249	0.262	0.270	0.288	0.324
6	*immigrans*	0.279	0.201	0.214	0.205	0.201		0.273	0.283	0.268	0.290	0.265	0.293	0.301
7	*virilis*	0.293	0.170	0.189	0.188	0.201	0.183		0.174	0.175	0.192	0.211	0.259	0.311
8	*flavopilosa*	0.317	0.214	0.237	0.231	0.262	0.214	0.118		0.161	0.173	0.239	0.301	0.327
9	*mesophragmatica*	0.320	0.197	0.214	0.213	0.231	0.213	0.102	0.147		0.166	0.236	0.288	0.313
10	*repleta*	0.308	0.209	0.222	0.225	0.239	0.227	0.127	0.155	0.123		0.234	0.289	0.306
11	Hawaiian *Drosophila*	0.282	0.240	0.243	0.245	0.231	0.218	0.148	0.201	0.192	0.179		0.279	0.301
12	*obscura*	0.281	0.243	0.254	0.253	0.243	0.234	0.218	0.266	0.256	0.262	0.231		0.243
13	*melanogaster*	0.305	0.249	0.252	0.249	0.268	0.247	0.217	0.246	0.248	0.246	0.239	0.209	

Pairwise alignment of amino acid between groups (Table 2) revealed that sequences of *willistoni* and *melanogaster* groups are also more divergent related to the others groups. However, within the groups, the divergence was lower: 8.8% within the *melanogaster* group and 6.5% within the *willistoni* group (Table 3). Additionally, the sequences that

were individually translated to yield the corresponding protein sequence did not show premature stop codons, suggesting that they are putatively encoding sequences.

Table 3. Estimates of average evolutionary divergence over sequence pairs within groups. The number of amino acid and nucleotide differences per site from averaging over all sequence pairs within each group is shown. Standard error estimate(s) are shown. The presence of n/c in the results denotes cases in which it was not possible to estimate evolutionary distances because the group is represented by only one species.

	Amino acid		Nucleotide	
Group	Divergence	Error	Divergence	Error
willistoni	0.0655	0.0162	0.0711	0.0094
guaramunu	0.0611	0.0153	0.0644	0.0087
tripunctata	0.1405	0.0181	0.1865	0.0107
calloptera	n/c	n/c	n/c	n/c
guarani	0.048	0.0144	0.0560	0.0081
immigrans	n/c	n/c	n/c	n/c
virilis	n/c	n/c	n/c	n/c
flavopilosa	n/c	n/c	n/c	n/c
mesophragmatica	0.0335	0.0094	0.0446	0.0058
repleta	0.1092	0.0202	0.1311	0.0131
Hawaiian *Drosophila*	n/c	n/c	n/c	n/c
obscura	0.0087	0.0058	0.0028	0.0020
melanogaster	0.0887	0.0108	0.1195	0.0071

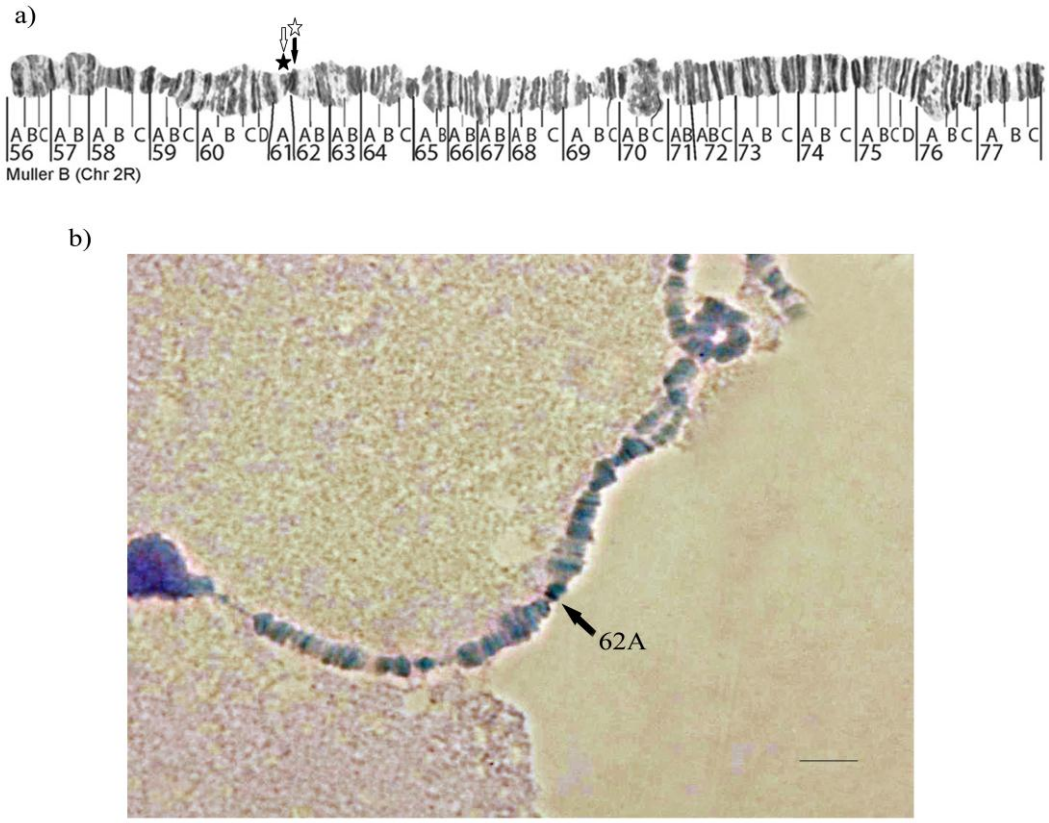

Fig. 4: Physical mapping of Dnmt2 gene in polytene chromosomes of *D.willistoni* **a)** IIR arm with the site of gene *Dnmt2* hybridizations (black arrow), the *P* element (empty asterisk), the element *Galileo* (filled asterisk) and proximal breakpoint of inversion IIR-M (empty arrow) are indicated. **b)** *In situ* hybridization signal of *Dnmt2* gene in chromosomal arm IIR in Gd-H4-1 strain of *D. willistoni*. Bar: 1µm

The location of the probe hybridization signal (80%) was determined at the subproximal region of the chromosome II right arm (IIR), specifically in section 62A (Figure 3). The homology was confirmed *in silico* by comparing the *D. willistoni Dmnt2* probe sequence with the *D. willistoni* Gd-H4-1 strain genome available on FlyBase GBrowse tool (Wilson *et al*., 2008). The IIR arm of *D. willistoni* corresponds to the Muller B element. On FlyBase search tool (Wilson et al., 2008), 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 localized also on B element. A schematic correspondence is shown in Figure 4S – Supplementary Material.

Discussion

Dnmt2 in Drosophilidae

Overall, our data suggest that *Dnmt2* is highly conserved in Drosophilidae insects. In our analyses on the occurrence of homologs of *Dnmt2* in Drosophilidae species (Dot blot, Figure 1S), we detected the hybridization signal in the most of the analyzed species. The degree of *Dnmt2* similarity among Drosophilidae species varies, since in not all the species was amplified by using *D. melanogaster* primers. However, the average of divergence values did not exceed 26.5% for the amino acid and 28.8% for the nucleotide sequences (data not shown).

In the *Dnmt2* phylogenetic analysis, the three main clades of the Drosophilidae species (*virilis-repleta* radiation, *quinaria-tripunctata* radiation and *Sophophora* subgenus) were recovered according to Robe *et al*. (2005). Moreover, on NJ nucleotides analysis, *D. immigrans* sequence was incorrectly positioned, grouping with *Sophophora* subgenus. However, there is no strong evidence to sustain this finding, and the data from Bayesian analysis confirm the correct positioning of this species, grouped with the rest of the clade of radiation *immigrans-tripunctata*, according to previous studies (Throckmorton, 1975; Russo *et al*., 1995; Kwiatowski and Ayala, 1999).

Other inconsistencies were observed on the Bayesian analysis. On the *Sophophora* subgenus clade, the *willistoni* and *obscura* group are not shown on the expected basal position related to the *melanogaster* group, even though this relationship has been not strongly supported. The monophyly of the subgenus *Sophophora* has been confirmed in the study of Tatarenkov *et al*. (1999) and Robe *et al*. (2005). Problems in the correct placement of the species within the clade are attributed to the molecular evolution of atypical species of the *willistoni* group (and its sister group *saltans*), characterized by high rates of nucleotide substitution and low portion G /C (DeSalle, 1992; Powell and DeSalle, 1995;

Remsen and O'Grady, 2002). By examining our NJ nucleotide tree for *Dnmt2* in *Sophophora* subgenus, the data suggest a more consistent position when compared with the literature available data.

Within the *melanogaster* group, in both analyses (Figure 1 and Figure 2) *D. ananassae* appears more externally positioned, what probably reflects the condition of the *ananassae* subgroup as the most primitive within the *melanogaster* group (Clark et al., 2007). This can be confirmed since the other analyzed species of the *melanogaster* group belong to the *melanogaster* subgroup and are closely related.

Drosophila belongs to the so-called ‘‘*Dnmt2*-only’’ organisms, and does not contain 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. For instance, about 1 in 600 (0.17%) cytosine nucleotides are methylated in the DNA of the *A. albopictus* mosquito (Adams *et al.,* 1979). Data for five other dipteran species were obtained by immunocytology, slot blot analysis, and capillary electrophoresis (Marhold *et al,* 2004). Recently, female-specific DNA methylation was reported for *D. willistoni* and related species (Garcia *et al.*, 2007; D’Avila *et al.,* 2010 – Chapter II of this thesis). These evidences highlight the importance of Dnmt2 studies to a better understanding of DNA methylation in ‘‘*Dnmt2*-only’’ systems, whose data are still vastly controversial and uncertain.

Whereas *Dnmt2* might represent the conserved core of the complex vertebrate DNA methylation systems, there are several characteristics that distinguish insect methylation from vertebrate methylation. For example, 0.5% or less of the cytosine residues were found to be methylated in *Drosophila* and *Anopheles* embryos, whereas about 4–5% of the cytosine nucleotides are usually methylated in vertebrates (Gama-Sosa *et al*., 1983). In addition, 5-methylcytosine appears to be distributed over large parts of insect metaphase chromosomes, whereas it is particularly enriched in pericentric DNA of vertebrate chromosomes (Miniou *et al.,* 1994). The significance of these differences remains unclear at present. Currently ongoing studies are uncovering the enzymatic mechanisms that mediate the roles of Dnmt2 enzyme in both DNA and RNA methylation and also provide

first insights into the biological functions of Dnmt2. Here, we verified the strong *Dnmt2* conservation among Drosophilidae family, thus suggesting an important function of Dnmt2-mediated DNA methylation on the evolution of these species.

Localization of Dnmt2 *gene*

The genome of *D. willistoni* was recently sequenced and, so far, few genes were accurately physically mapped in polytene chromosomes. Currently, there are limited numbers of genetic and physical markers for *D. willistoni*. Therefore, the orientation of the scaffolds should be viewed as provisional (Schaeffer *et al.*, 2008). Mapping genes on chromosomes is essential to confirm the correct assembly and orientation of scaffolds in the genome. Thus, our study consists in a first report, to our knowledge, of a physical gene location in the sequenced *D. willistoni* Gd-H4-1strain. The identity of the sequences and the localization at IIR arm, where the gene is allocated, were confirmed in the *Drosophila* database (Wilson *et al*., 2008).

In the genus *Drosophila*, the gene content of the five major chromosomal elements has been preserved during the evolution of most lineages (Powell, 1997; Schaeffer *et al*., 2008; Bhutkar *et al*., 2008). By contrast, the order of genes within each chromosomal element has been scrambled repeatedly via the fixation of numerous paracentric inversions (Segarra *et al*., 1995; Vieira *et al*., 1997a; 1997b; Ranz *et al*., 2001; 2007; Bhutkar *et al*., 2008).

The fact that the *Dnmt2* homologous sequences are also located in Muller's B element (standard nomenclature established by Muller, 1940) in the other 11 *Drosophila* species for which there are available genomic data, demonstrates preservation in the chromosome synteny. These data confirm the conservation of the gene within the genus *Drosophila*, not only at sequence level, but also at cytogenetic level. Some of these species have the approximate location of *Dnmt2* inferred by the chromosomal location of other genic markers previously described (Wilson *et al*., 2008). However, the exact *D. willistoni* chromosome section description was only possible based on our *in situ* hybridizations. This is central for chromosomal evolution studies, since this species is known to be very polymorphic for chromosomal inversions. Near the section 62A, where *Dnmt2* was

mapped, it has been previously identified a breakpoint to inversion IIR-M, recently described by C. Rohde and V.L.S. Valente (personal communication).

The analysis of the effects of natural inversions position has been troubled by the lack of molecular studies of their breakpoints and genes for monitoring, though there are evidences of adaptive significance of the inversion polymorphisms. However, the molecular mechanisms underlying the maintenance of inversions in natural populations remain unclear. It is hypothesized a position effect, suggesting that the location of inversion breakpoints near or within genes may affect their function or expression (Sperlich, 1966). Little is known about the result of mutations in the breakpoints of inversions and their consequences on the natural patterns of expression of nearby genes (Wesley and Eanes, 1994). Besides, a variety of position effects have been observed in spontaneous mutations generated by inversions in diverse organisms (Hough *et al*., 1998, Lakich *et al*., 1993, Lister *et al*., 1993), including an inversion in a chromosome segment affecting the expression pattern in the *Antp73b* mutation of *D. melanogaster* (Frischer *et al*., 1986). Inversions can also move genes to distant sites, where its expression is silenced by the proximity of centromeric heterochromatin (Henikoff, 1990).

Another factor that could contribute to the position effects of inversions is the presence of transposable elements (TEs) at their breakpoints. In the vicinities of the 62A section of the IIR arm in *D. willistoni*, besides the presence of the proximal breakpoint of inversion IIR-M (C. Rohde and V.L.S. Valente), it was also reported the presence of the *P* element (Regner *et al*., 1996). Thus, the hybridization signal of *Dnmt2* gene on chromosome band corresponds to the same *P* element. An association between inversion breakpoints and repetitive sequence has been well demonstrated in many *Drosophila* species (Lim, 1988; Cáceres *et al*., 1999; Evgen'ev *et al*., 2000; Richards *et al*., 2005).

An inversion in *D. buzzatii* was studied by Puig *et al*. (2004), whose described a single event caused by ectopic recombination between two oppositely oriented copies of the element *Galileo*, which contains different transposable elements at the breakpoints that are absent in non-inverted chromosomes (Cáceres *et al*., 1999; Cáceres *et al*., 2001). The authors present a clear example of a position effect associated with the breakpoints of a *Drosophila* natural inversion. Another important result of Puig *et al*. (2004) is that

silencing was not caused by the inversion itself, but by a TE inserted in the breakpoint junction, demonstrating that at least this *Drosophila* inversion seems to be adaptive since it alters the expression level of a nearby gene (Puig *et al*., 2004).

As our data indicate, the gene *Dnmt2* is located near a chromosomal breakpoint inversion where transposable elements *P* (Regner *et al*., 1996) and *Galileo* (C. F. Garcia, personal communication) were previously mapped. The significance of this location needs to be further elucidated, but it is tempting to propose the establishment of a promising relationship between inversions and the expression of genes adjacent to their breakpoints. Moreover, since the entire genome of *D. willistoni* is available, it makes possible a genome-walking around the *Dnmt2* gene looking for other genes and to further investigate their expressions in different tissues.

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Supplementary Material

Tamura K, Peterson D, Peterson N, Stecher G, Nei M and Kumar S (2011) MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol Biol Evol doi: 10.1093/molbev/msr121.

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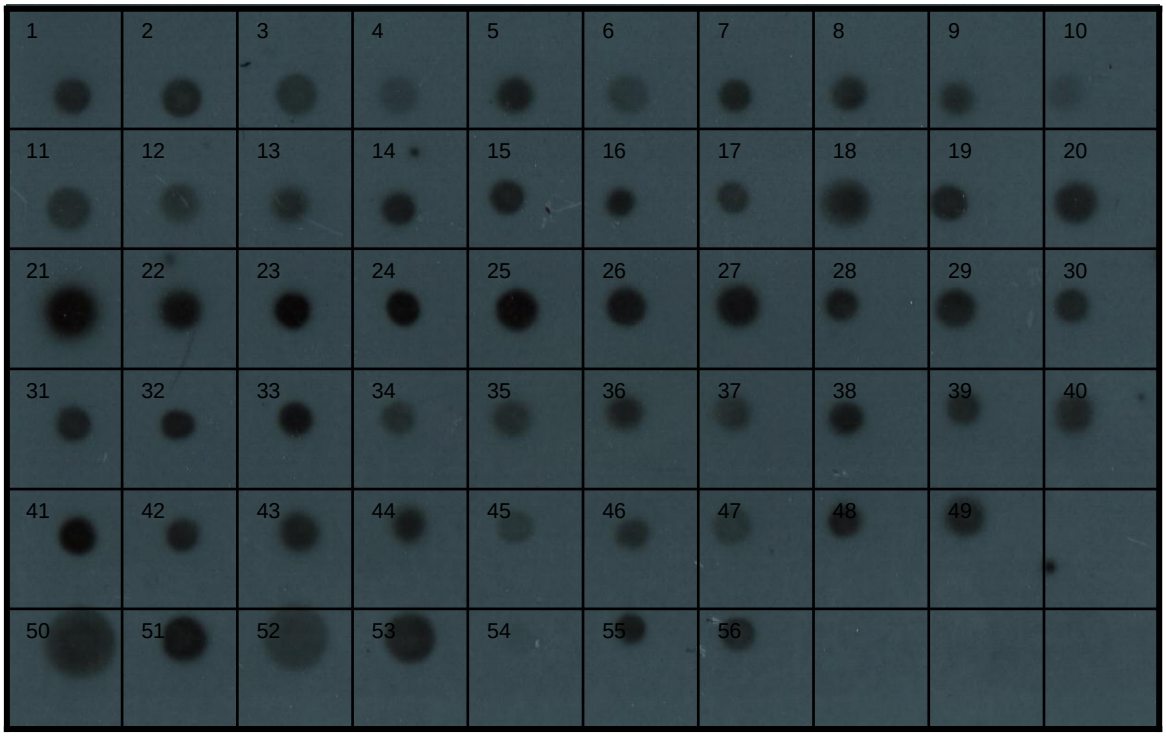

Fig. 1S: Dot blot screening for *Dnmt2* sequences homologous to the *Drosophila melanogaster Dnmt2* gene. The species tested are the following: 1- *Drosophila ornatifrons*; 2- *D. subbadia*; 3- *D. guaru*; 4- *D. griseolineata*; 5- *D. nappae*; 6- *D. paramedioestriata;* 7- *D. tripunctata*; 8- *D. medipictoides*; 9- *D. neocardini*; 10- *D. polymorpha*; 11- *D. ornatipennis*; 12- *D. immigrans*; 13- *D. funebris*; 14- *D. gasici*; 15- *D. gaucha*; 16- *D. mercatorum*; 17- *D. mojavensis*; 18- *D. incompta*; 19- *D. virilis*; 20- *D. robusta*; 21- *D. melanogaster*; 22- *D. simulans*; 23- *D. mauritiana*; 24- *D. teissieri*; 25- *D. santomea*; 26- *D. erecta*; 27- *D. yakuba*; 28- *D. kikkawai*; 29- *D. ananassae*; 30- D. *malerkotliana*; 31- *D. pseudoobscura*; 32- *D. prosaltans*; 33- *D. saltans*; 34- *D. neoelliptica*; 35- *D. sturtevanti*; 36- *D. sucinea*; 37- *D. nebulosa*; 38- *D. willistoni* (ww strain); 39- *D. paulistorum Orinocan*; 40- *D. insularis*; 41- *D. tropicalis*; 42- *D. equinoxialis*; 43- *D. capricorni*; 44- *D. fumipennis*; 45- *D. busckii*; 46- *Zaprionus indianus*; 47- *Z. tuberculatus*; 48- *S. latifasciaeformis*; 49- *S. lebanonensis*; 50- *D. maculifrons*; 51- *D. crocina*; 52- *D. hydei;* 53- *D. canalinea*; 54- *D. orena*; 55- *D. willistoni* (Wip-4 strain); 56- *D. willistoni* (17A2 strain).

Table 1S: Primary FlyBase identifier number (FlyBase ID) of the *Dnmt2* homologous sequences.

	FlyBase ID
D. melanogaster	FBgn0028707
D. simulans	FBgn0195186
D. sechellia	FBgn0181579
D. erecta	FBgn0115904
D. yakuba	FBgn0235978
D. ananassae	FBgn0098561
D. persimilis	FBgn0153821
D. pseudoobscura	FBgn0070556
D. mojavensis	FBgn0139811
D. virilis	FBgn0261343
D. grimshawi	FBgn0119056
D. willistoni	FBgn0223078

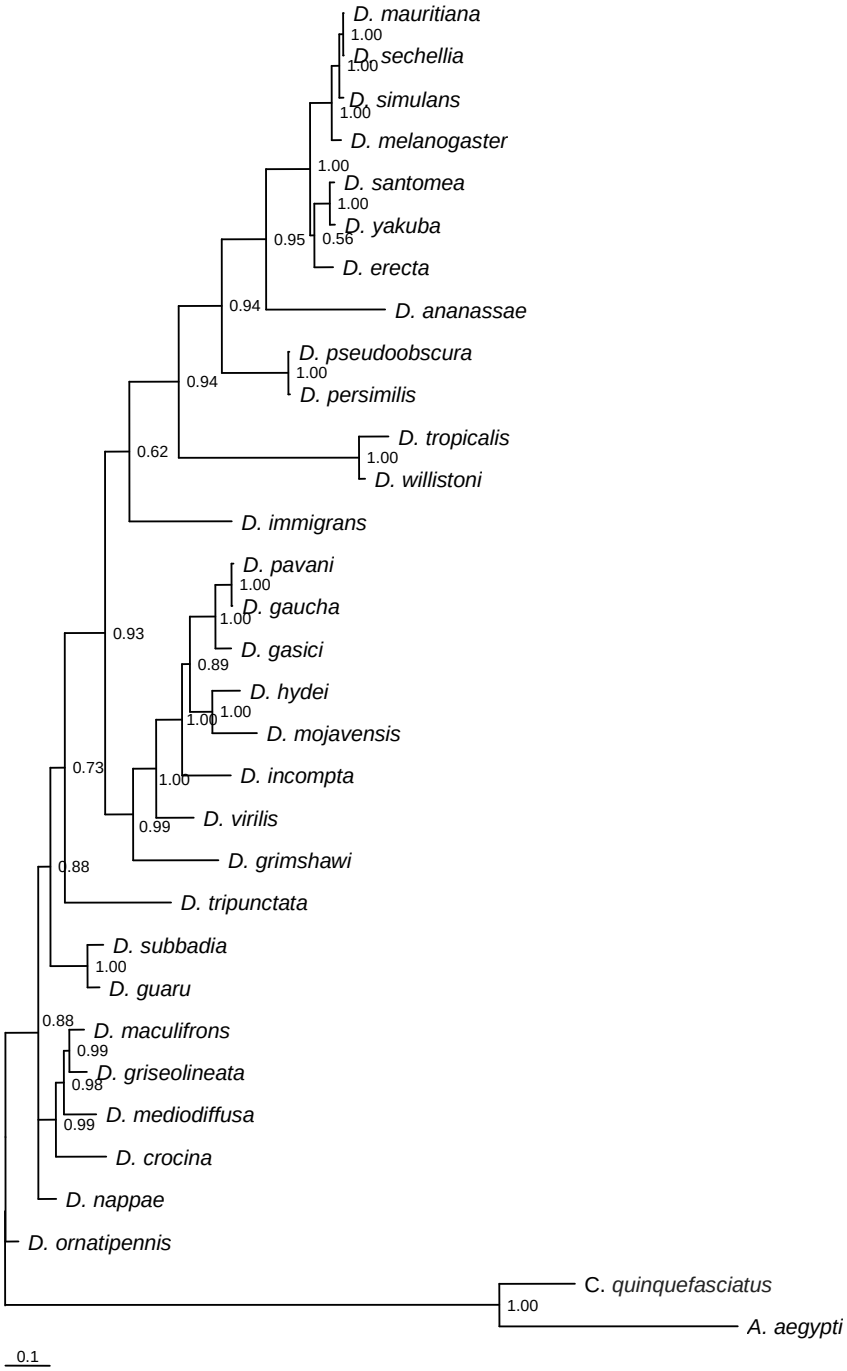

Fig. 2S: Bayesian phylogenetic analysis of *Dnmt2* using nucleotide sequences alignment with the GTR + I + G model. The C. *quinquefasciatus* and A. *aegypti* sequences were used as outgroup taxa.

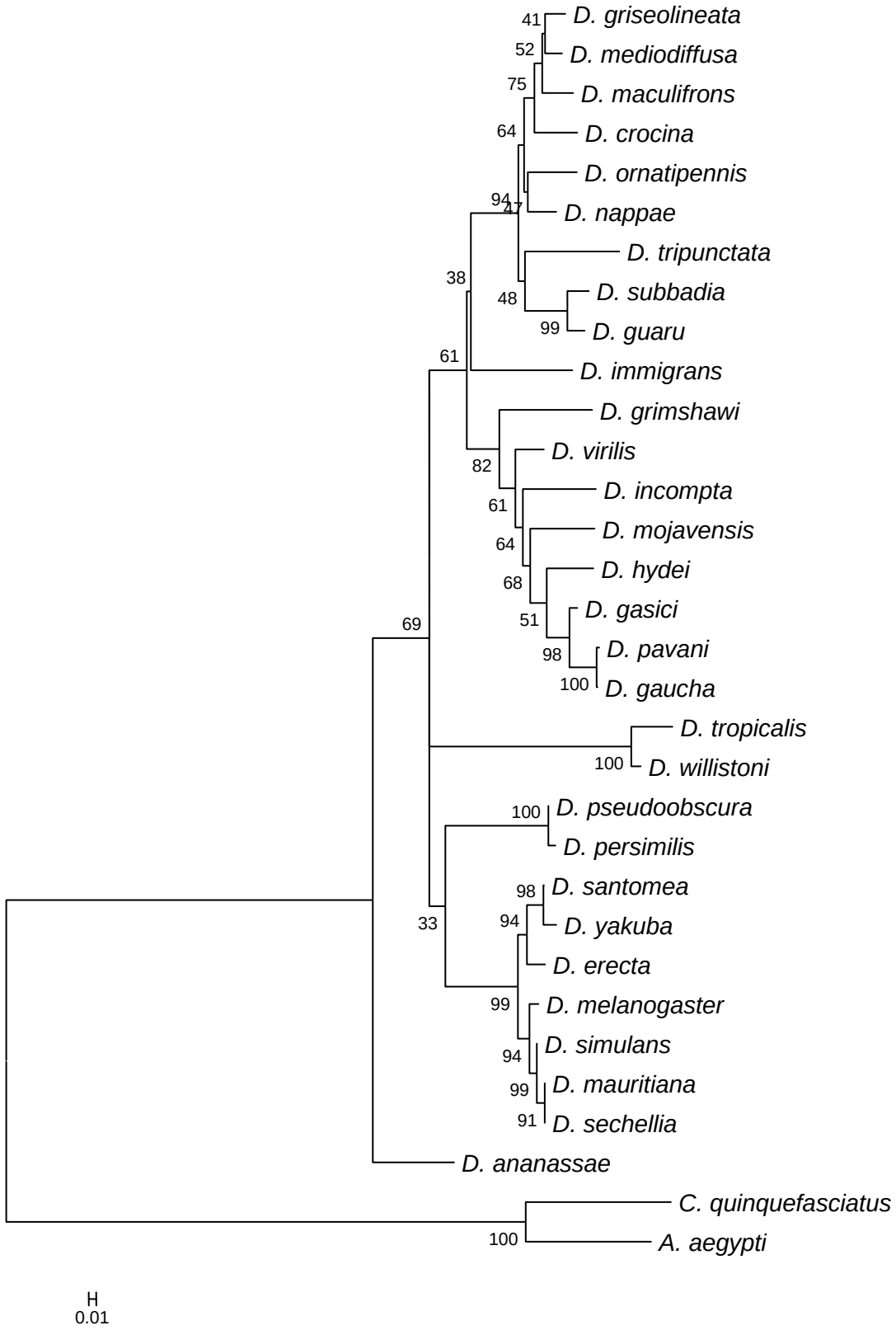
D. griseolineata
D. mediodiffusa
D. maculifrons
D. crocina
D. ornatipennis
D. nappae
D. tripunctata
D. subbadia
D. guaru
D. immigrans
D. grimshawi
D. virilis
D. incompta
D. mojavensis
D. hydei
D. gasici
D. pavani
D. gaucha
D. tropicalis
D. willistoni
D. pseudoobscura
D. persimilis
D. santomea
D. yakuba
D. erecta
D. melanogaster
D. simulans
D. mauritiana
D. sechellia
D. ananassae
C. quinquefasciatus
A. aegypti
41
52
75
64
94
47
38
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Fig. 3S: Phylogenetic analysis of the *Dnmt2* using amino acid sequences alignment by Neighbor-Joining distance method with the JTT model with gamma distribution. Numbers above branches are bootstrap values based on 5000 replications in the consensus tree.

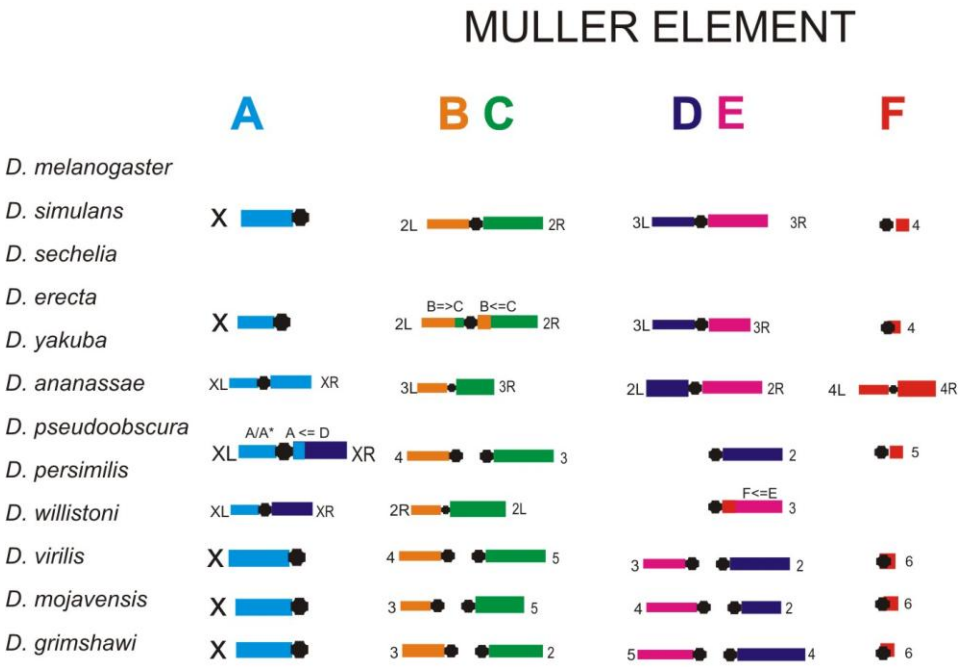

Fig. 4S: Schematic correspondence of rearrangement for the six Muller elements among twelve species of *Drosophila*. The centromeres are indicated with solid black circles. Each Muller element is shown in shades of a single color: A, light blue; B, orange; C, green; D, deep blue; E, magenta; F, red. Modified from Schaeffer *et al.* (2008).

Capítulo IV

Expression of the DNA methyltransferase Dnmt2 in *Drosophila willistoni*

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Abstract

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 researches. Homologs for this enzyme are found in virtually all organisms already analyzed for the DNA methylation, including *Drosophila melanogaster*, but 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 findings on the Dnmt2 expression in *D. willistoni*, a neotropical species of large ecological versatility with peculiar molecular features, in comparison to other species of same genus. We demonstrate here the presence of transcripts not only in the early stages of development, as described for other species, but also during the oogenesis. Our findings shed some light on the possible biological function of Dnmt2-related processes associated with the development and differentiation of oocytes, since the expression of Dnmt2 seems to be more required to germinative tissues formation.

Keywords: Dnmt2, DNA methylation, *Drosophila willistoni*, embryogenesis, oogenesis.

Introduction

Cytosine-5 RNA methylation is one among many different RNA modifications that has been already detected in tRNA, rRNA and mRNA (Rozenski *et al.*, 1999), representing an important epigenetic modification that regulate gene expression in eukaryotes, with a standing role on their development and for the etiology of human diseases (Klose and Bird, 2006; Jones and Baylin, 2007). Elucidation of the molecular mechanisms mediating RNA and DNA methylation is crucial to understand 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 (Bird, 2002) and occurs mostly symmetrically at the cytosine of a CG dinucleotide (Lee *et al.*, 2010). Evidence for DNA methylation has been demonstrated in several different orders of insects. The honeybee genome contains genes that encode orthologues of all vertebrate proteins required for DNA methylation. In addition to Dnmt2 (also found in Diptera), three CpG-specific DNMT family genes were identified: two Dnmt1 genes and one Dnmt3a/b gene (Wang *et al.*, 2006). 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* (Lyko *et al.*, 2000b; Kunert *et al.*, 2003). 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.

Despite the distinct methylation sequence contexts, 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 (reviewed in Goll and Bestor, 2005). By contrast, the biochemical activity and the biological function of Dnmt2 enzymes are still controversial. While a tRNA methyltransferase activity has been documented for the *Drosophila*, *Arabidopsis*, mouse and human Dnmt2 enzymes (Goll *et al.*, 2006; Jurkowski *et al.*, 2008), it still remains to be proved whether Dnmt2 can also function as a nuclear DNA methytransferase *in vivo*.

Drosophila genus belongs to the so-called "Dnmt2-only" organisms, and does not contain 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 (Lyko *et al.*, 2000b; Kunert *et al.*, 2003; Garcia *et al.*, 2007), in contrast to most organisms already analyzed. Furthermore, the specificity for CpT and CpA nucleotide residues distinguishes Dnmt2 from all other known animal DNA methyltransferases (Kunert *et al.*, 2003), and the functional role of DNA methylation in *Drosophila* remains unclear.

Whereas in mammalian DNA, 2 to 10% of all cytosine residues are modified to 5-methylcytosine (Ehrlich and Wang, 1981), in *Drosophila melanogaster* only about 0.1 - 0.6% of all cytosine nucleotides use to be methylated (Gowher *et al.*, 2000; Lyko *et al.*, 2000), 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 disperse in invertebrates (Suzuki and Bird, 2008).

Several attempts were made to demonstrate the DNA methylation activity of Dnmt2 in *D. melanogaster* (Tang *et al.*, 2003; Kunert *et al.*, 2003; Mund *et al.*, 2004) 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 (Kunert *et al.*, 2003; Mund *et al.*, 2004), a result qualitatively corroborated by bisulfite sequencing data (Tang *et al.*, 2003; Kunert *et al.*, 2003). Nevertheless, in a recent study, Phalke *et al.* (2009) reported that Dnmt2 controls DNA methylation in early *D. melanogaster* embryos and provide the insight into its function in control of retrotransposon silencing and telomere integrity in

somatic cells (Phalke *et al.*, 2009). This report showed a clear-cut difference of methylation within *Invader4* elements LTRs between wild type and Dnmt2$^{-/-}$ flies (Phalke *et al.*, 2009). Dnmt2 appears to mediate methylation on *D. melanogaster* embryos genome, even though both this activity and its functional consequences remaining poorly understood.

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 (Garcia *et al.*, 2007; D'Ávila *et al.*, 2010). Comparisons of *D. willistoni* and *D. melanogaster* Dnmt2 protein sequences Garcia *et al.* (2007) 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 by *Drosophila* 12 Genomes Consortium (Clark *et al.*, 2007). *D. willistoni* singularities correspond to overall genome size, distribution of transposable element classes, patterns of codon usage, number of structural changes and rearrangements of genes, dot chromosome lacking, unclear phylogenetic clustering placement, among others (Clark *et al.*, 2007; Schaeffer *et al.*, 2008; Bhutkar *et al.*, 2008; Vicario *et al.*, 2007).

While the role of other Dnmt proteins has been extensively characterized, little is known about Dnmt2 biological role. This is surprising since Dnmt2 is the most widely conserved Dnmt protein with homologs already identified among protists, plants, fungi, and animals. To improve the Dnmt2 knowledge, more assays for characterization and better detection of Dnmt2-dependent DNA methylation will have to be established. In addition, genomic DNA methylation patterns need to be characterized mostly in "Dnmt2-only" models systems, like *Drosophila*.

In this study we detected and quantified the expression of *D. willistoni* Dnmt2 in different tissues and development stages (including adult flies). Accordingly, we also

detected the expression of Dnmt2 on oogenesis and embryogenesis, indicating a possible contribution of Dnmt2 expression during development.

Material and Methods

Drosophila strains

The strains used in this study were: *D. willistoni* TS (recently collected in Tangará da Serra/MT, Brazil - 14°37'08"S 57°29'09"O) and *D. melanogaster* Oregon R (control).

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

RNA was obtained from *D. willistoni* and *D. melanogaster* (control) adult males, adult fertilized females, adult virgin females, adult females without ovaries, ovaries and embryos 0-3h, using Trizol reagent according to the manufacturer's protocol (Invitrogen). Extracted RNA was further treated with *DNase*I (Promega) to eliminate DNA contamination. cDNA synthesis was performed using the M-MLV enzyme (Invitrogen) with random primers. After synthesis of the first cDNA strand, PCRs with primers wDnmt2B-F: 5' GAACCAATTGGGAACGACTG 3' and wDnmt2C-R: 5' TCACCCACAACCTTGACATT 3' based on *D. willistoni Dnmt2* sequence (Garcia *et al.*, 2007) were used to detect *D. willistoni Dnmt2* transcripts. The PCR reaction was performed in 25µL reactions using 20 ng of cDNA, 1 U Taq DNA Polymerase (Invitrogen), 1x reaction buffer, 200 µM dNTPs, 20 pmol of each primer and 1.5 mM $MgCl_2$. The amplification conditions were 95 °C for 5 min and 30 cycles of 95 °C for 40 s, 58 °C for 40 s and 72 °C for 1 min, followed by a final extension cycle at 72 °C for 5 min. A fragment of 153 bp was expected as result of the amplifications reactions. The *α-tubulina* housekeeping gene was used as a control for amplification. An additional primer forward designed for *D. willistoni* wDnmt2A-F (5' ACCTTCTCGCAGACACCAA 3'), in combination with reverse primer wDnmt2C-R were employed as negative control for DNA contamination (Figure S1). PCR products were separated by electrophoresis on a 1% agarose gel and stained with ethidium bromide.

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 *α-tubulin* gene). Samples of cDNA from *D. willistoni* TS strain, produced as previously described, were used. The qPCR conditions were: 94 °C for 5 min followed by 40 cycles at 95 °C for 15 s, 60 °C for 10 s, 72 °C for 15 s and 35 s at 60 °C to measure fluorescence. Next, samples were heated from 55 to 99 °C at a 0.1 °C/s temperature gradient to construct the denaturing curve of the amplified products. Relative quantifications of amplified products were made by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) 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 amplicons by denaturing curves and melting temperatures (Tm). The *α-tubulin* 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 wDnmt2A-F: 5' and wDnmt2C-R, as described before. 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 with 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 *Not*I-linearized plasmid. *In situ* hybridization assays were performed in embryos collected in different developmental stages, as described by Deprá *et al.* (2009). Prior to *in situ* hybridization, the probes (200 ng in 50 μL) were mixed with *in situ* hybridization solution as follows: 50% formamide, 5x SSC, 100 μg/mL of herring sperm DNA, 50 μg/mL of heparin and 1% of Tween 20. The hybridization mixture was added to the embryos at 55 °C and incubated overnight. After hybridization, the embryos were washed several times with PBS and color development was performed with BCIP/NBT

(Promega). The embryonic stages were identified according to the criteria outlined by Campos-Ortega and Hartenstein (1985).

Results

1. *Transcriptional expression of Dnmt2*

RT-PCR analysis was performed to verify the transcriptional expression of Dnmt2 in each sample. Using specific primers wDnmt2B-F and wDnmt2C-R for cDNA synthesis, expression of Dnmt2 transcripts was detected for all cDNA samples used: *D. willistoni* adult males, *D. willistoni* adult fertilized females, *D. willistoni* adult virgin females, *D. willistoni* adult females without ovaries, *D. willistoni* ovaries and *D. willistoni* embryos 0-3h (Figure 1). The *D. melanogaster* samples do not show any transcript amplification, certifying the specificity of primers to *D. willistoni* (Figure 1S - Supplementary Material). The amplification of a single fragment with expected size of 153 bp, corresponding to processed mRNA, confirmed the absence of genomic DNA contaminants in all samples. The apparent differences on the quantities of products are probably due the differences of the initial amount of cDNA samples added into the PCR reaction. In order to infer whether there are significant differences in the amount of Dnmt2 transcripts on the samples analyzed, further investigations were carried out by qPCR.

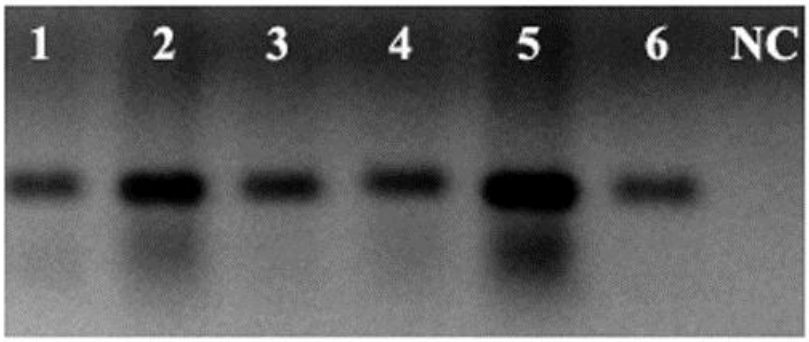

Fig. 1: Detection of Dnmt2 by conventional RT-PCR. All the RT-PCR products had the expected size (153 bp) on ethidium bromide stained agarose gels. **1.** *D. willistoni* adult males; **2.** *D. willistoni* adult fertilizated females; **3.** *D. willistoni* adult virgins females; **4.** *D. willistoni* adult females without ovaries; **5.** *D. willistoni* ovaries; **6.** *D.*

willistoni 0-3h embryos; **NC.** Negative Control. Molecular weight marker: 1 Kb Plus DNA ladder (Invitrogen).

The quantitative PCR showed that the Dnmt2 is transcribed at different levels in the samples from distinct developmental stages and tissues examined (Figure 2 and Table 1S – Supplementary Material). The analysis indicated that the embryos sample showed a basal expression level, therefore being used as calibrator of the samples. The results for oocytes sample showed a comparatively higher level of Dnmt2 expression (2.084) times higher than that of the embryos), while the amount of transcripts in samples of adult males and virgin or fertilized females of *D. willistoni* showed an average value 1.63 times higher than that of the embryos sample. Also, there is no substantial difference between the level of Dnmt2 expression for the females without ovaries sample (only somatic tissues) and the male sample.

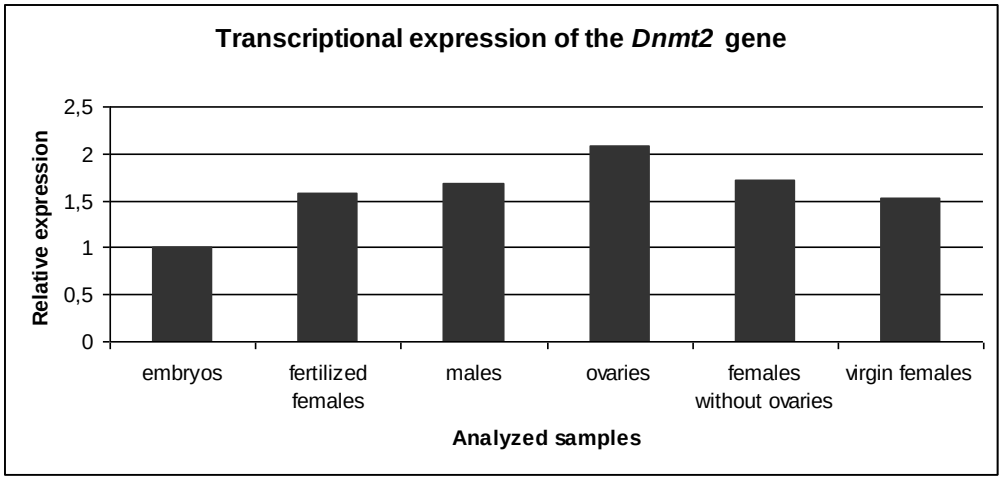

Fig. 2: Estimative of *Dnmt2* transcriptional expression levels in the *Drosophila willistoni* samples.

2. In situ Dnmt2 hybridization of whole mount embryos

To determine the spatial and temporal pattern of the transcriptional expression of *Drosophila willistoni* Dnmt2, *in situ* hybridization experiments were conducted at different developmental oogenesis and embryogenesis stages. The expression pattern of *Dnmt2*

sense transcripts was initially detected as uniform and widespread in all stages of oogenesis (Figure 3). The hybridization can be seen scattered throughout the tissues. Staining suggests early expression at the anterior extremity and further in the ventral position of the oocyte (Figure 3a-3h).

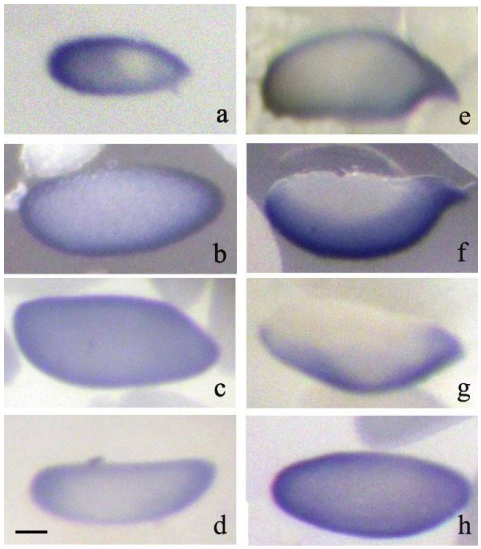

Fig. 3: Spatial expression patterns of *Dnmt2* transcripts on *Drosophila willistoni* during oogenesis, showing a widespread pattern during differentiation, which gradually reaches a evident pattern on its ventral surface. Stages ordered according to Mahowald and Kambysellis (1980). Orientation of oocytes: anterior is to the left. Bar: 0.2 mm.

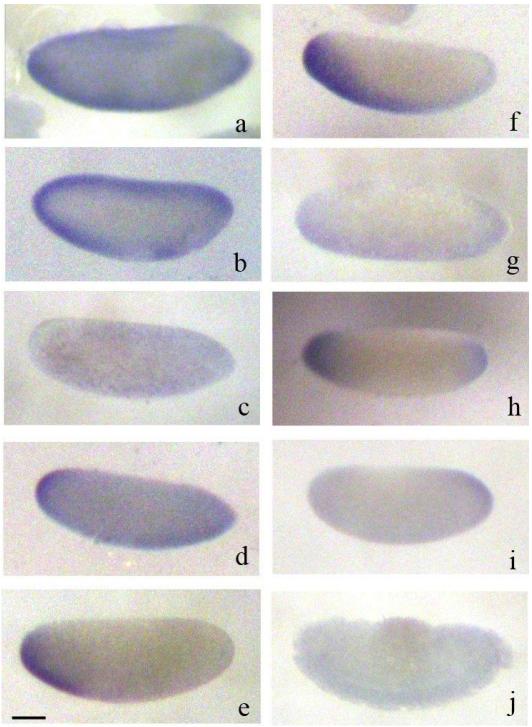

Fig. 4: Spatial expression patterns of *Dnmt2* transcripts on *Drosophila willistoni* during embryogenesis showing. **a-d:** first embryogenesis stages showing global staining patterns. **e-i**: signal expansion from anterior to posterior embryo pole; **j:** late embryogenesis showing germ-band retraction and weak staining. Orientation of embryos: anterior is to the left. Bar: 0.2 mm.

In the first embryonic developmental stages, an accumulation of *Dnmt2* transcripts appear initially as superficial staining patterns and later as diffuse internal granules, similar to those of maternal transcripts (Figure 4a-4c). This pattern of staining persists until the cellular blastoderm stage. Throughout development, transcripts expression accumulated at the ventral periphery of the embryos (Figure 4d) and subsequent expression was detected on anterior portion approximately coincident with the moment when the cephalic furrow is formed (Figure 4e). The expression of *Dnmt2* was detected from the cephalic region expanding through the region ventral to the posterior embryo pole (Figure 4f-4h). The anterior and posterior expression levels of staining decreased and became a basal signal from then (Figure 4i). During late embryogenesis, at the germ-band retraction stage, transcript accumulation persisted as basal expression in the ectoderm layer.

Discussion

In animals, each cell has an identity gained during the development and maintained over time. The determination of this identity results from the choice to express, or suppress, a panel of genes, as well as the maintenance of this choice. The memory cell is embedded in the core by the application and reading of epigenetic marks on DNA (Filion and Defossez, 2004) and histones (Ray-Gallet *et al.*, 2005). Because genomic imprinting is regulated by differential DNA methylation patterns of the sperm and egg genomes and through development, attention has been directed to the role of DNA metyltransferases.

Our quantitative analyses on different development stages and tissues showed a difference in Dnmt2 expression (Table 1), with highest expression being observed in oocytes, hence, in the *D. willistoni* pre-zygotic development (oogenesis), in which Dnmt2 expression was 2 times higher than in the embryos sample. Essentially, on *Drosophila* genus DNA methylation is described as predominant during embryonic development. *Drosophila* Dnmt2 expression was first reported by Hung *et al.* (1999), suggesting that transcripts were most abundant in larvae, with weak expression in embryos and undetectable expression at the adult stage in *D. melanogaster*, indicating that the expression of Dnmt2 is developmentally regulated (Hung *et al.*, 1999). Furthermore, Lyko *et al.* (2000b) reported DNA methylation in *D. melanogaster* that was 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).

In our experiments with *D. willistoni* employing whole embryos hybridization with Dntm2 riboprobe, we observed transcriptional expression in distinct embryonic stages. Hybridization signals of oocytes were most prominent, suggesting a higher activity of the enzyme on this stage of oogenesis. In addition, the embryos in the early stages of embryogenesis have shown hybridization staining with distinct patterns. In them, the expression pattern seems to decrease from syncytial blastoderm to late development. The expression patterns ranged from a granular expression throughout the whole embryo on syncytial blastoderm to a peripheral occurrence on cellular blastoderm. A compartmentalization of expression was observed from the anterior portion, from the ventral region, reaching the posterior portion at the time of the gastrulation. Furthermore, it

shows only a fading basal signal in the head region and in the position of the developing central nervous system (CNS). Our results with embryos of *D. willistoni* are in agreement with those found by Lyko *et al.* (2000b) concerning the genomic DNA methylation that predominates during early embryonic development and decreases at later stages, presumably as a result of reduced methyltransferase expression in *D. melanogaster* (Lyko *et al.*, 2000a).

Our data on Dnmt2 expression in *D. willistoni* adult tissues revealed a slightly higher value when compared with embryos pool sample, reaching values that are similar to each other, showing a weak but existing gene activity during the adult stage of flies' life cycle. Although being low, this expression can be relevant since recently we had reported evidences of DNA methylation in adult flies of *D. willistoni* and closely relates species (Garcia *et al.*, 2007; D'Ávila *et al.*, 2010 - Chapter II of this Thesis). The identification of methylated sequences from the genome of adult *D. melanogaster* using anti-5-methylcytosine antibodies in affinity columns also was previously reported (Salzberg *et al.*, 2004; Mandrioli and Borsatti, 2006). Efforts to demonstrate Dnmt2 DNA-methyltransferase activity on gametogenesis and embryogenesis are essential to increase knowledge on this unclear research field. Moreover, on previous studies, tiny amounts of 5- methylcytosine had been observed in adult *Drosophila* DNA (Lyko *et al.*, 2000b; Achwal *et al.*, 1984) and the significance of these findings were unclear until now.

It seems to be unrealistic to expect that any general and unified theory will encompass all the biological consequences of DNA methylation in all organisms. Currently, there are some attempts aiming to identify the DNA methylation role in *Drosophila* genomes. It has been observed that overexpression of *D. melanogaster* Dnmt2 results in an extended fly life span and in overexpression of several genes (Lin *et al.*, 2005). In a recent work, Schaefer *et al.* (2008) described that Dnmt2 is associated with the nuclear matrix and so it can access DNA during mitosis. These results represent the first comprehensive characterization of Dnmt2 proteins on the cellular level and have important implications for our understanding of the molecular activities of Dnmt2. In another recent study, Phalke *et al.* (2009) also have identified a function of Dnmt2 in early embryonic DNA methylation of *D. melanogaster*. The newly generated Dnmt2 null mutations allowed them to correlate loss of DNA methylation, identified in early *D. melanogaster* embryos by

bisulfite sequencing and restriction enzyme analysis, with the function of the Dnmt2 enzyme. Collectively, their results suggest that 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.

Emerging evidence from the field of epigenetics offers a compelling mechanistic explanation for developmental programming, nonetheless leaving many questions still to be answered. For example, although the DNA sequence is the same in all cells of an organism, histone modifications and DNA methylation determine cell type specificity by inactivating particular regions of the genome (Gohlkea, 2011). These epigenetic patterns are established *de novo* during critical developmental stages. In eukaryotes with high levels of 5-methylcytosine, the function of DNA methylation in epigenetic control of gene silencing is firmly established (Tamaru and Selker, 2001; Lehnertz *et al.,* 2003). However, in other eukaryotes, the occurrence of DNA methylation has being recently discovered, and its understanding is still slight.

Analysis of the *Dnmt2* gene (and its protein encoded product) in different species of *Drosophila* genus are highly relevant since previous approaches revealed prominent divergences among species of the genus *Drosophila* (Garcia *et al.*, 2007 and D'Avila *et al.*, 2010), making not clear whether the findings on *D. melanogaster* can be applied to the other species. Results reported herein enrich the body of evidences on protein Dnmt2 biological role, which is known to be the best preserved of the methyltransferases in all organisms studied so far.

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Supplementary Material

Table 1S. Estimative of the *Dnmt2* transcriptional expression levels for *D. willistoni* samples (absolute numbers).

Samples	Relative expression
embryos	1
fertilized females	1.58823
Males	1.687814
ovaries	2.084482
females without ovaries	1.715249
virgin females	1.520197

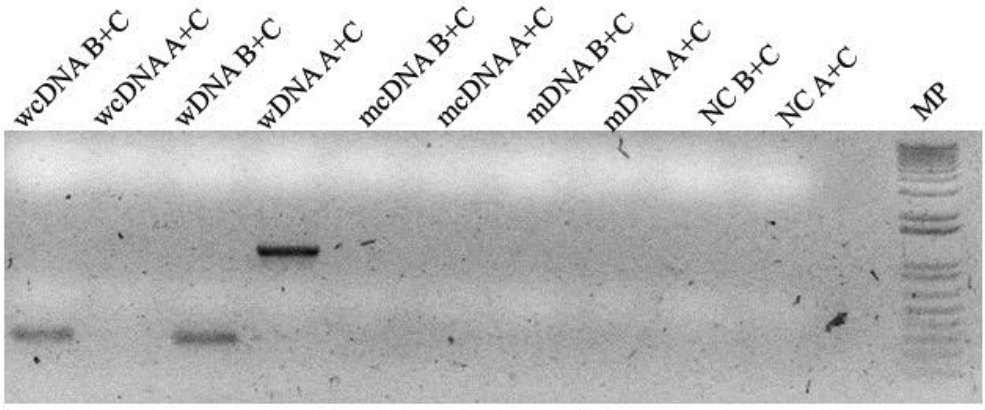

Fig. S1: DNA contamination control PCR and primers specificity PCR using DNA and cDNA females samples from *Drosophila willistoni*, *D. melanogaster* and wDnmt2 set of primers (designed for *D. willistoni Dnmt2* gene sequence). **w:** *D. willistoni* samples. **m:** *D. melanogaster* samples. **NC:** negative control. **B+C:** amplification by combined wDnmt2-B Forward and wDnmt2-C Reverse primers. **A+C:** amplification by combined wDnmt2A Forward and wDnmt2C Reverse. **MP:** Molecular weight marker: 1 Kb Plus DNA ladder (Invitrogen).

Capítulo V

Discussão Geral

Perspectivas

Referências Bibliográficas

Discussão Geral

A epigenética tem se tornado um campo importante de pesquisa tanto para a identificação das causas de algumas doenças (Paz *et al*, 2003; Ehrlich *et al*., 2006) quanto para o esclarecimento de questões evolutivas (revisão em Jablonka, 2004). Por isso, a relativamente recente abordagem de temas ligados às modificações epigenéticas dos genomas abriu um amplo campo para novas descobertas.

Os trabalhos que apresentamos aqui enfocam principalmente a metilação de resíduos de citosina no DNA, uma importante modificação epigenética relacionada à regulação da expressão gênica, imprescindível para o desenvolvimento em eucariotos e que também tem sido investigada em estudos de etiologia de doenças humanas (Klose and Bird 2006; Jones and Baylin, 2007). Além disso, a metilação do DNA é considerada um fator chave na formação da memória e identidade celular; mudanças em padrões de metilação do DNA durante o desenvolvimento embrionário e processos de diferenciação celular são frequentemente associadas à plasticidade transcricional dos genomas (Cohen *et al*., 2009). Portanto, elucidar mecanismos moleculares associados à metilação do DNA se torna essencial para a melhor compreensão das funções deste processo epigenético nos diferentes organismos onde ela ocorre.

Os estudos da metilação do DNA no gênero *Drosophila* começaram há pouco mais de uma década, pois se acreditava que a metilação nas espécies do gênero fosse inexistente ou que seus níveis fossem tão baixos a ponto de não serem identificados (Urieli-Shoval *et al*., 1982). Entretanto houve um avanço tecnológico rápido nos métodos para detecção de alterações epigenéticas, muito em função da grande concentração de estudos relacionando metilação do DNA com instabilidade genômica (revisão em Ehrlich *et al*., 2006) e com desenvolvimento de doenças neoplásicas em mamíferos (revisão em Paz *et al*., 2003). Assim sendo, as novas metodologias permitiram a descoberta dos primeiros indícios de metilação no DNA de embriões de *D. melanogaster* (Lyko *et al*., 2000a), além da identificação de uma única proteína metiltransferase no genoma deste organismo: a Dnmt2 (Kunert *et al*., 2003). O gênero *Drosophila*, portanto, pertence ao recém-denominado

grupo de organismos "*Dnmt2-only*", por não conter nenhum homólogo das metiltransferases canônicas descritas em outros eucariotos (Dnmt1 e Dnmt3).

Iniciamos nossas investigações a partir das questões levantadas por estudos realizados anteriormente pelo nosso grupo de pesquisa, os quais geraram resultados inesperados em análises de elementos transponíveis na espécie neotropical *Drosophila willistoni* ao compará-la com *D. melanogaster*, indicando algum tipo de regulação pós transcricional diferencial para essas peculiaridades (Sassi *et al*., 2005; Blauth *et al*., 2011). Além disso, também no nosso grupo, técnicas de bandamento cromossômico com enzimas de restrição que reconhecem regiões de DNA metilado mostraram a presença de padrões bem definidos de bandas refletindo blocos específicos de sequências nos cromossomos politênicos (Garcia, 1997). Para explicar estes padrões, num estudo anterior, nós estabelecemos as primeiras evidências de metilação no genoma de *D. willistoni*, identificando um padrão fêmea-específico de metilação do DNA em moscas adultas (Garcia *et al*., 2007). Dentre as sequências diferencialmente metiladas no genoma de machos e fêmeas, nós identificamos genes de DNA ribossomal (rDNA).

Pouco se sabe sobre a evolução da conservação dos padrões da metilação do DNA o qual impacto evolutivo de diferenças epigenéticas entre espécies proximamente relacionadas. Deste modo, na intenção de ampliar o conhecimento da metilação do DNA e de sua possível influência no estabelecimento de condições evolutivamente diferenciadas em determinadas espécies, partimos para outra investigação que nos pareceu bastante promissora. No intuito de verificar a recorrência destes padrões de metilação sexo-específicos do DNA em adultos em outras espécies do gênero *Drosophila*, além da *D. willistoni*, realizamos então um novo estudo com várias espécies do subgênero *Sophophora*, ao qual *D. willistoni* e *D. melanogaster* pertencem.

A proposta deste estudo foi verificar se os padrões encontrados para *D. willistoni* se repetiam em espécies evolutivamente próximas a ela e é apresentado no **Capítulo II** desta tese (D'Ávila *et al*., 2010). Demonstramos, por meio de nossos resultados, que os padrões sexo-específicos da metilação de genes de rDNA descritos para *D. willistoni* somente ocorrem em duas espécies pertencentes ao mesmo subgrupo de espécies crípticas

(subgrupo *willistoni*) – *D. tropicalis* e *D. insularis*, sendo que na primeira, os padrões são praticamente idênticos aos descritos por Garcia *et al*. (2007) em *D. willistoni*.

Em conjunto, os nossos resultados (Garcia *et al*., 2007; D'Ávila *et al*., 2010) indicam que a metilação do genoma das espécies do subgrupo *willistoni* estudadas poderia estar envolvida em um processo funcional específico. E esta visão é interessante, uma vez que *D. willistoni* vem cada vez mais sendo identificada como detentora de singularidades em aspectos genéticos e ecológicos (Stark *et al*., 2007, Schaeffer *et al*., 2008, Bhutkar *et al*, 2008, Vicario *et al*., 2007), as quais novamente a tornam um paradigma para estudos evolutivos.

Atualmente, vários grupos de pesquisa chamam a atenção para as enzimas metiltransferases responsáveis pela metilação nos genomas de invertebrados. A enzima identificada como responsável pela metilação nos genomas de *Drosophila* é a DNA metiltransferase 2 (Dnmt2). As funções das demais proteínas Dnmt encontradas nos vertebrados tem sido extensivamente caracterizados. Entretanto, pouco se sabe sobre o papel da Dnmt2 nos organismos para os quais ela é a única enzima capaz de realizar a modificação de citosinas a 5-metilcitosinas. E isto é surpreendente já que a Dnmt2 é a mais amplamente conservada das metiltransferases de DNA, com homólogos em protistas, plantas, fungos e animais (revisão em Schaefer & Lyko, 2010).

Como, no trabalho de Garcia *et al*. (2007) nós também fizemos uma breve abordagem sobre a conservação da Dnmt2 de *D. willistoni*, comparando sua sequência com a sequência conhecida de *D. melanogaster*, concentramos nossos esforços no estudo da Dnmt2. Nos organismos "*Dnmt2-only*", os padrões de metilação do DNA apresentam expressivas diferenças em relação aos eucariotos mais estudados e, por este motivo, estudos de análise da Dnmt2, e de suas prováveis funções biológicas, vêm ganhando especial atenção mais recentemente (Lin *et al*., 2005; Goll *et al*., 2006; Phalke *et al*., 2009; Schaefer *et al*., 2008, 2010; Gou *et al*., 2010).

A partir dos dados obtidos para o subgênero *Sophophora* (D'Ávila *et al*., 2010 – **Capítulo II**) decidimos estender nossas análises para outros grupos de espécies, de modo a expandir o conhecimento acerca de aspectos evolutivos de conservação da metilação do DNA, uma vez que os padrões estabelecidos para *D. melanogaster*, a espécie do gênero *Drosophila* que possui maior representatividade nos estudos epigenéticos realizados até

agora, não refletem o que acontece em todo o gênero.

Neste contexto, procuramos elucidar questões referentes à evolução do gene *Dnmt2* não só em espécies do gênero *Drosophila,* mas de outros gêneros relacionados a ele, pertencentes à família Drosophilidae. Assim sendo, analisamos a presença e a conservação de homólogos da enzima Dnmt2 (**Capítulo III** desta tese) em um grande número de espécies. Inicialmente, nós investigamos 54 espécies de Drosophilidae (gêneros *Drosophila*, *Scaptodrosophila* e *Zaprionus*) quanto à presença de homólogos ao gene *Dnmt2* de *D. melanogaster* e detectamos sinais de hibridização em praticamente todas elas. Em análises filogenéticas, utilizamos sequências de *Dnmt2* em 60 espécies dos mesmos gêneros de Drosophilidae. De uma forma geral, os principais clados foram recuperados (a radiação *virilis-repleta*, a radiação *quinaria-tripunctata* e o subgênero *Sophophora*) e nossos dados estão em concordância com estudos prévios, como o de Robe *et al*. (2005). Neste estudo sugerimos, portanto, que a Dnmt2 é altamente conservada na família Drosophilidae, indicando que há alguma função importante da metilação do DNA mediada por Dnmt2 para a evolução destas espécies (**Capítulo III).**

No mesmo estudo, verificamos também que há conservação quanto à localização do gene *Dnmt2* entre espécies do gênero *Drosophila* (**Capítulo III)** representantes de diferentes grupos. Ao mapearmos fisicamente o gene *Dnmt2* por hibridação *in situ* em cromossomos politênicos, nós localizamos o gene no braço direito do cromossomo II de *D. willistoni* (que corresponde ao elemento B de Muller). Comparando sua localização com os dados disponíveis para as demais 11 espécies com genomas sequenciados disponíveis (Wilson *et al*., 2008) - *D. melanogaster*, *D. simulans*, *D. sechellia*, *D. yakuba*, *D. erecta*, *D. ananassae*, *D. pseudoobscura*, *D. persimilis*, *D. virilis*, *D. mojavensis* e *D. grimshawi* – usando a sequência homóloga à *Dnmt2,* nós encontramos este gene nos braços cromossômicos correspondentes aos elementos B de Muller (**Capítulo III**) em todas elas, corroborando a proposta de preservação da sintenia de elementos cromossômica de Bhutkar *et al*. (2008). Esta conservação, segundo os autores, poderia estar refletindo uma vantagem evolutiva.

Em um estudo anterior, Spellman & Rubin (2002) sugeriram que genes adjacentes poderiam ter a necessidade de serem transcritos juntos. Assim, se blocos contendo genes

com padrões de expressão correlacionados são conservados entre diferentes espécies, isso deve refletir alguma vantagem evolutiva (Spellman & Rubin, 2002). Resta saber se há genes importantes para algum processo biológico de *Drosophila* próximos ao gene *Dnmt2* e se há expressão coordenada entre eles.

Outro resultado relevante no mapeamento físico do gene *Dnmt2* (**Capítulo III**) é sua localização próxima a um ponto de quebra de inversão cromossômica, que foi recentemente descrito (Rohde & Valente, no prelo) e onde já haviam sido mapeados o elementos transponíveis *P* (Regner *et al*., 1996) e *Galileo* (C. F. Garcia, comunicação pessoal). A hipótese de efeito de posição (Sperlich, 1966) propõe que a localização de pontos de quebra de inversões comossômicas perto ou dentro de genes poderia afetar seu perfil de expressão e sua função. Um exemplo de efeito de posição associado com pontos de quebra de uma inversão em *Drosophila* foi demonstrada por Puig *et al*., (2004), onde os autores descrevem o silenciamento de um gene adjacente ao rearranjo causado pela transcrição de RNA *antisenso* do elemento *Kepler*. Nossos achados nos permitem sugerir o gene *Dnmt2* como marcador para estudos futuros de efeitos de posição, usando-o como isca na busca de genes adjacentes ao ponto de quebra de inversão onde ele se encontra (Rohde & Valente, no prelo).

Estudos prévios descrevem a metilação do DNA apenas durante os estágios iniciais do ciclo de vida em *Drosophila* e em outros organismos modelo (Kunert *et al*., 2003; Hung *et al*., 1999; Lyko *et al*., 2000a), o que poderia sugerir que a Dnmt2 tenha alguma função ligada ao desenvolvimento. Por isso, torna-se interessante avaliar eventuais mudanças nos padrões de metilação de genes de desenvolvimento de modo espaço-temporal. No intuito de inferir prováveis funções para o gene *Dnmt2*, começamos uma investigação para traçar seus perfis de expressão em *Drosophila willistoni*. Assim, evidenciamos a presença de transcritos de *Dnmt2* em adultos, embriões, em tecidos germinativos e em tecidos somáticos. (**Capítulo IV** desta tese).

Em nossas análises quantitativas, evidenciamos ainda que a expressão da Dnmt2 é mais alta em ovócitos, ou seja, em estágios pré-zigóticos de *D. willistoni*. Esses dados foram confirmados ao realizarmos a hibridização *in situ* de ovócitos e embriões, com uma ribosonda de *Dnmt2* (**Capítulo IV**). Os resultados foram promissores e têm suporte em

um estudo recente que identificou uma relação inesperada entre fatores essenciais para o desenvolvimento da linhagem germinativa em *Drosophila melanogaster* (Anne *et al*., 2007). Outros estudos genéticos têm identificado genes maternos (e os produtos gênicos) necessários para a especificação de células germinativas (revisão em Kirino *et al*., 2010) e neste sentido, futuros estudos são necessários descobrir se a expressão de Dnmt2 na gametogênese de *Drosophila willistoni* pode estar refletindo controle de genes maternos de desenvolvimento.

De uma forma geral, nossos dados confirmam as ideias que têm sido levantadas por diversos autores de que o fenômeno da metilação do DNA é amplamente distribuído nos insetos e outros invertebrados, mas com mecanismos e funções bastante distintas. Apresentamos nesta tese um conjunto de resultados que contribuem para o conhecimento das modificações epigenéticas e da complexa evolução dos organismos eucariotos.

Perspectivas

A partir, portanto, do vasto campo de investigação proporcionado pelos trabalhos anteriormente realizados surge a necessidade de explorar as funções da metilação nos genomas de outras espécies do gênero *Drosophila*, uma vez que o papel da metilação no DNA nos diferentes genomas das espécies ainda precisa ser esclarecido.

Listamos a seguir alguns dos próximos passos que poderemos realizar com base nos dados gerados pelos nossos estudos sobre a DNA metiltransferase (Dnmt2) e a metilação do DNA em *Drosophila*:

- Buscar genes adjacentes ao *Dnmt2* nos cromossomos de *D. willistoni*, por meio de experimentos de *genome-walking*, utilizando as sequências deste gene e do próprio elemento *P* como ponto de partida e verificar se existe associação entre os perfis de expressão de genes e rearranjos cromossômicos.

- Determinar o *status* global da metilação do DNA genômico de embriões e adultos das espécies com o uso de anticorpos anti-5metilcitosinas em análises de *Slot blot* de acordo com o protocolo descrito por Marhold *et al*. (2004).

- Avaliar quantitativamente os níveis de 5-metilcitosinas nas espécies de *Drosophila*, no DNA genômico cujos níveis de 5-metilcitosinas sejam detectados por *Slot blot* por meio de experimentos de Eletroforese Capilar, conforme protocolo descrito previamente por Stach *et al*. (2003).

- Analisar a distribuição espaço-temporal de 5-mC em cromossomos metafásicos de embriões das espécies cujo experimento de *Slot blot* indique a presença de metilação em embriões, conforme protocolo específico para dupla imunomarcação fluorescente (Kunert *et al*., 2003) com anticorpos contra 5- metilcitosinas (affinity-purified, Megabase Research) e DNA (Natutec).

- Identificar sequências metiladas por cromatografia de afinidade em *D. willistoni* e

espécies relacionadas, utilizando colunas de ligação ao DNA metilado que contêm anticorpos específicos 5-metilcitosinas como ligantes, conforme descrito por Salzberg *et al*. (2004), ao descrever as porções metiladas no genoma de *D. melanogaster*.

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