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Estabelecimento de relações evolutivas do grupo *willistoni* de *Drosophila* (Diptera, Drosophilidae) por meio de morfologia e marcadores moleculares combinados

Tese apresentada ao Programa de Pós-Graduação em Biologia Animal, Instituto de Biociências da Universidade Federal do Rio Grande do Sul, como requisito parcial à obtenção do título de Doutor em Biologia Animal.

Área de concentração: Biologia Comparada

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UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

PORTO ALEGRE

2015

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Aprovada em ____ de _____ de _____.

Dr. (a) Avaliador (a) 1.

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Dr. (a) Avaliador (a) 3.

O presente trabalho foi desenvolvido no Laboratório de *Drosophila* da Universidade Federal do Rio Grande do Sul, com apoio financeiro do CNPq, CAPES e FAPERGS.

“Nothing in biology makes sense, except in the light of evolution”.

(Theodosius Dobzhansky)

Dedico este trabalho ao Carlo, ao Guilherme e aos meus pais.

AGRADECIMENTOS

À minha querida orientadora Vera Valente, por acreditar em meu trabalho mesmo nos momentos em que nem eu mesma acreditei, por todo incentivo, apoio, confiança e pelo inabalável otimismo. Obrigada Vera, por tudo.

À minha querida co-orientadora, Maríndia, pelo seu esforço em fazer sempre o melhor, pela grande ajuda na elaboração dos capítulos dessa tese e pela amizade.

Aos queridos colegas do Laboratório de *Drosophila,* Lucas, Geórgia, Mário, Natália, Ju, Bibi, Paula e Brenda, pelos ótimos momentos que compartilhamos nesse laboratório.

Aos colegas, amigos e companheiros de discussão acerca da vida, do universo e de tudo mais, Gilberto e Carol.

À Helena, Dani e Berê, pelo apoio técnico e pelos momentos de descontração.

À equipe de apoio, Didi, Zizi e Maria Irene, que me ajudou a cuidar do pequeno filhote sempre que necessário.

Ao meu sogro, seu Wagner, que me acolheu como filha e nos ajudou de muitas formas nos últimos anos.

Aos meus ex-orientadores, Marcelo de Lacerda Grillo e Victor Hugo Valiati, que me ensinaram a ser a pesquisadora que sou hoje.

Aos meus amigos de toda uma vida e aos amigos recentes, que não vi tanto quanto gostaria nesse período de doutorado, mas que são parte importante da minha vida.

A minha vó Wilma, que mesmo não estando aqui, ficaria muito orgulhosa de ver o que conquistei.

Aos meus pais, por todo amor, dedicação, educação, apoio incondicional e por toda a ajuda, seja cuidando do Carlo ou apenas um ombro para me amparar nos momentos difíceis. Sem vocês eu não chegaria até aqui.

Ao Guilherme, por ter sido um pai tão presente para o Carlo, por todo apoio, por todas as revisões de inglês, por todas as deliciosas jantas, por todas as fotos de gatinhos, por todo amor.

Ao meu amado filho Carlo, por todo o amor, carinho, beijinhos, por ter sido tão paciente com a minha falta de tempo. Eu te amo muito, gatinho amado!

SUMÁRIO

APRESENTAÇÃO

O grupo *willistoni* de *Drosophila* é, juntamente com o grupo *saltans,* representante Neotropical do subgênero *Sophophora.* O grupo *willistoni* é formado por três subgrupos, *alagitans, bocainensis* e *willistoni* e vem sido amplamente estudado desde meados do século passado. Apesar da expressiva quantidade de trabalhos envolvendo o grupo, ainda existem lacunas a serem preenchidas, principalmente em relação à morfologia e evolução do grupo. Nesse contexto, a presente tese teve como foco o subgrupo *willistoni* de *Drosophila*, formado por seis espécies crípticas com uma história evolutiva complexa, e teve como objetivos a caracterização morfológica das espécies desse subgrupo e a busca pela reconstituição da história evolutiva do mesmo, utilizando para tal, ferramentas moleculares e análises morfológicas. Os capítulos II e III apresentaram uma atualização acerca da distribuição geográfica dos subgrupos *willistoni, bocainensis e alagitans.* Nos capítulos IV e V foram apresentados e discutidos aspectos morfológicos das espécies do grupo *willistoni.* Foi feita uma caracterização dos estágios imaturos do grupo *willistoni* baseando-se em imagens de Microscopia Eletrônica de Varredura (MEV) (Capítulo IV). Também foram caracterizadas e comparadas as genitálias masculinas das espécies do subgrupo *willistoni*, utilizando para tal MEV e microscopia de luz; os resultados obtidos demonstraram que é possível identificar as espécies desse subgrupo a partir da inspeção da genitália masculina (Capítulo V). No capítulo VI foram discutidas as relações evolutivas do subgrupo *willistoni* a partir de dados moleculares e morfológicos e os principais eventos foram datados.

RESUMO

O grupo *willistoni* de *Drosophila* tem origem e distribuição essencialmente Neotropical. A primeira descrição de espécies desse grupo data do final do século 19, sendo a mais recente nova espécie adicionada ao grupo em 2013. Dessa forma, apesar de uma longa trajetória de estudos, muito ainda precisa ser acrescentado acerca da diversidade e evolução das espécies desse grupo, que é o principal objetivo da presente Tese. Assim, revisamos os registros de distribuição geográfica das espécies dos subgrupos *willistoni*, *bocainensis* e *alagitans,* e reforçamos a *D. willistoni* como a de distribuição mais ampla do grupo. As lacunas quanto à distribuição das espécies do grupo, parecem ser devidas mais a escassez de estudos do que à ausência de espécies. A caracterização ultraestrutural dos estágios pré-adultos de espécies do grupo *willistoni*, mostrou que, com exceção da forma dos filamentos respiratórios dos ovos, que são variáveis, todas as outras estruturas apresentam-se de forma similar, tanto dentro quanto entre as espécies analisadas. Quanto à análise de estruturas morfológicas dos adultos das espécies crípticas do subgrupo *willistoni*, foram observadas diferenças essencialmente na genitália masculina. Essas diferenças permitem a diferenciação ou identificação de espécies, até mesmo daquelas entidades pertencentes ao *cluster* da *D. paulistorum*. As nossas análises filogenéticas combinando marcadores morfológicos e moleculares, sugerem a origem evolutiva do subgrupo no Mioceno, a partir da espécie *D. insularis*. *D. paulistorum* começou a divergir há cerca um milhão de anos, e parece estar ainda em processo de especiação. Tal sugestão tem respaldo na similaridade aqui observada com diferentes marcadores dentro do grande conjunto de espécies, incluindo as espécies incipientes da *D. paulistorum*.

ABSTRACT

The *willistoni* group of *Drosophila* has a distribution that is essentially Neotropical, as is its origin. The first species description of this group dates from the later 19th century, with the latest dating from 2013. Thus, even after many studies, a lot of information is still lacking about the diversity and evolution of the species in this group, a situation this work aims to improve. With that said, we revised the geographical distribution records for the species in the subgroups *willistoni, bocainensis* and *alagitans,* and reaffirmed *D. willistoni* as the one with the widest distribution within this group. Gaps in the distribution information, seems to be more due to lack of research than due to a lack of species. The ultrastructural characterization of the pre-adult stages of the *willistoni* group showed that, with the exception of the shape of the egg's respiratory filaments, which are variable, all other structures have a similar shape, either within or between the analyzed species. As for the the analisys of morphological structures in adults of the cryptic species in the subgroup *wlillistoni,* the differences were limited to the male genitalia. Those differences allowed us to differentiate or identify species, even those belonging to the *D. paulistorum* cluster. Our phylogenetic analyses, combining both morphologic and molecular markers, suggest an origin during the Miocene, from *D. insularis. D. paulistorum* started diverging around 1Mya, and it seems to be an ongoing process. This suggestion is backed by the similarities hereby observed with different markers within a big group of species, including the early *D. paulistorum* species.

CAPÍTULO I. INTRODUÇÃO

INTRODUÇÃO

1.1. O Gênero *Drosophila* (Diptera, Drosophilidae)

O gênero *Drosophila* FALLEN 1823 é o maior, mais diverso e mais amplamente distribuído dentre a família Drosophilidae, abrangendo cerca de um terço das espécies da família. Atualmente conta com 1367 espécies divididas em oito subgêneros: *Chusqueophila, Dorsilopha, Drosophila, Dudaica, Phloridosa, Psilodorha, Siphlodora* e *Sophophora* (Bächli, 2015). Dentre estes subgêneros, *Drosophila* e *Sophophora* são os que apresentam o maior número de representantes – juntos, correspondem a cerca de 90% da diversidade do gênero *Drosophila* (Markow e O'Grady, 2005). Estima-se que esses dois subgêneros tenham divergido entre 40 milhões de anos (Russo *et al*., 1995) e 63 milhões de anos (Tamura *et al*., 2003). Seus membros são encontrados desde o nível do mar até maiores altitudes, e dos Trópicos até regiões próximas à Tundra. A maior abundância de espécies ocorre em matas e florestas, porém também são encontradas em planícies, desertos, pântanos e savanas (Throckmorton, 1975).

Ainda que não se tenha feito nenhum estudo filogenético amostrando extensivamente todo o gênero, vários estudos parciais demonstraram que o gênero *Drosophila* não é monofilético e provavelmente deve ser dividido em vários clados (Markow e O'Grady, 2005). Throckmorton (1975), em sua revisão clássica da família Drosophilidae, reuniu grupos de espécies fortemente relacionadas em "radiações" (Figura 1). Esse tipo de arranjo taxonômico, que seria uma categoria entre subgênero e grupos de espécies, é exclusivo de Drosophilidae (Markow e O'Grady, 2007), e é utilizado como forma de tornar mais fácil a compreensão das relações filogenéticas dessa família tão grande e diversa.

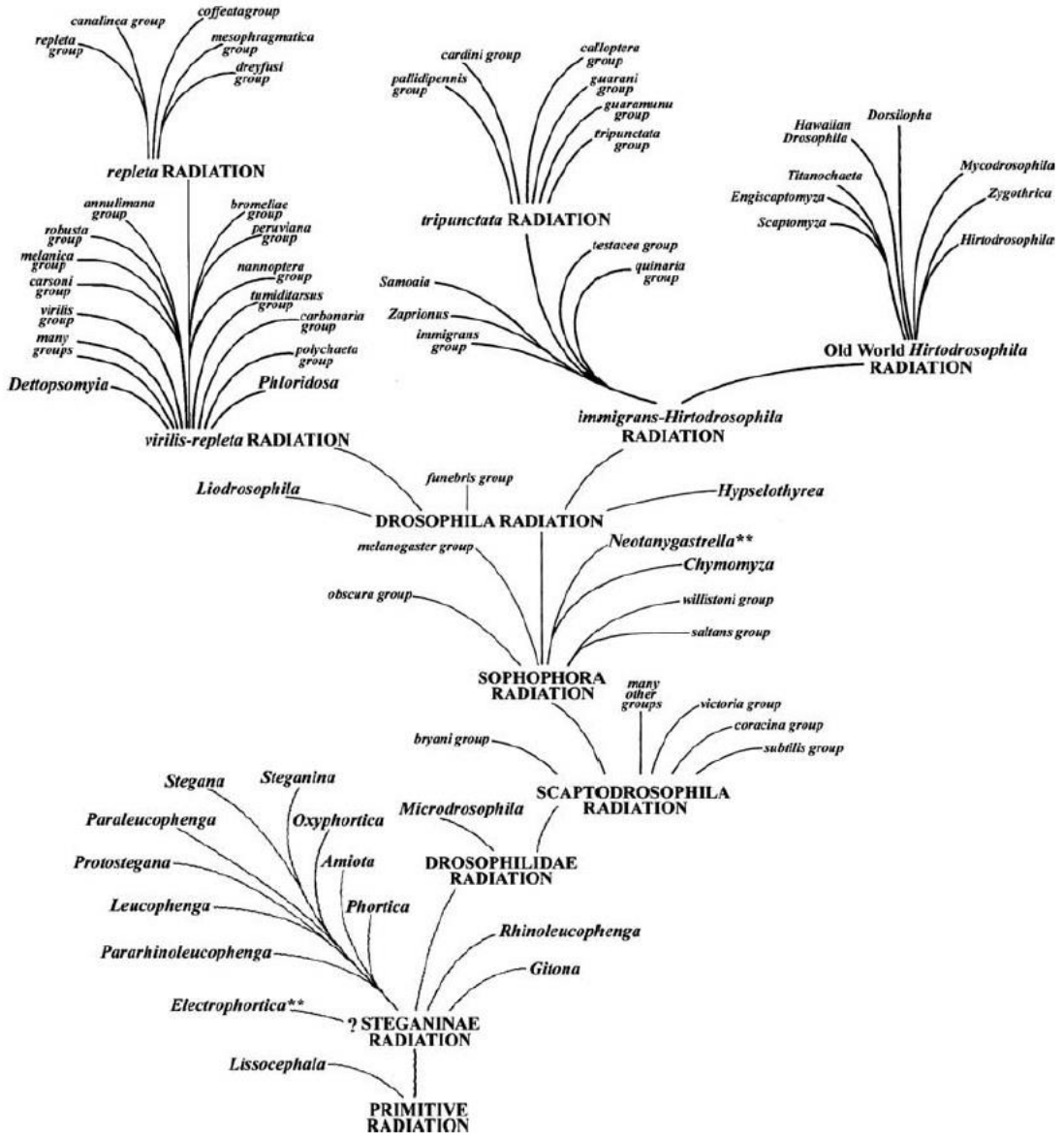

Figura 1. Filogenia de Drosophilidae, baseada em Trockmorton (1975). Figura retirada de Markow e O'Grady (2007).

Throckmorton (1975) propôs radiações representando eventos múltiplos de especiação com subsequente diversificação, baseado em caracteres morfológicos. De acordo com Markow e O'Grady (2005), existem duas grandes críticas quanto ao trabalho de Throckmorton (1975): primeiro, suas análises não foram baseadas em

algorítimos cladísticos e, por consequência, não podem ser repetidos; e segundo ele, não tentou manter qualquer conceito de monofilia nesse estudo, o que resulta em grupos de espécie do gênero *Drosophila* sendo mais fortemente relacionados com outros gêneros do que com outros grupos de espécies do próprio gênero.

A taxonomia de *Drosophila* utiliza alguns termos que não são comumente utilizados para outros taxa. Como já foi anteriormente mencionado, devido à complexidade do taxa e às complicadas relações entre seus membros, existe a necessidade de se utilizar termos que consigam abarcar todas as sutilezas dessas relações. Um dos conceitos mais utilizados entre os drosofilistas (pesquisadores que estudam a família Drosophilidae) é o de espécies-irmãs (*siblings*) ou espécies crípticas, que de acordo com Mayr *et al.* (1953) são duas ou mais populações Mendelianas ou espécies que não se intercruzam, cujos indivíduos são tão similares morfologicamente que a separação das espécies por um ou mais caracteres qualitativos não é possível.

Assim como o conceito de espécie, também não há um consenso quanto ao termo “espécie críptica”. Mayr (1963) classifica espécies crípticas como “populações naturais muito similares ou morfologicamente idênticas que são reprodutivamente isoladas”.

Beheregaray e Caccone (2007) consideram espécies crípticas como um sinônimo de *sibling species*, e as definem como espécies distintas cuja diferenciação morfológica é difícil ou algumas vezes impossível e, muitas vezes, são classificadas como um único táxon. Saez e Lozano (2005) também utilizam o termo “crípticas” como sinônimo de *siblings*. Knowlton (1986) considera que *siblings* apresentam um ancestral comum mais recente do que espécies crípticas, constituindo assim uma

relação de irmandade entre as espécies. Há autores que recategorizam *sibling* como “pseudo-sibling”, uma vez que há alguns caracteres diagnósticos (Saez e Lozano, 2005; Knowlton, 1993). Bickford *et al.* (2007) consideram que duas espécies são crípticas se elas são ou foram classificadas como apenas uma espécie nominal, visto que elas são, ao menos superficialmente, indistinguíveis morfologicamente. Ainda, há quem classifique espécies crípticas como espécies que apresentam camuflagem (Claridge *et al.,* 2004).

As espécies do subgrupo *willistoni* têm sido tradicionalmente classificadas como espécies crípticas ou *siblings*, sendo Burla *et al.* (1949) os primeiros a darem essa designação a esse subgrupo. Por questões históricas, usaremos o termo *siblings* como sinônimo de espécies crípticas.

Além de serem separadas em radiações e grupos de espécies, espécies de *Drosophila* também são classificadas em níveis infraespecíficos, que seriam as subespécies e semiespécies. Subespécie, de acordo com Mayr (1970), é um agregado de populações fenotipicamente similares, habitando uma subdivisão geográfica da distribuição da espécie. Ainda de acordo com Mayr (1970), semiespécies são populações naturais que adquiriram algumas características de espécie, cuja troca gênica não é tão frequente quanto em populações conspecíficas, e superespécie é o grupo monofilético formado pelas semiespécies, que devem ser inteira ou majoritariamente alopátricas e morfologicamente distintas ou reprodutivamente isoladas.

1.2. O Subgênero *Sophophora*

O Subgênero *Sophophora* foi consolidado por Sturtevant (1939) e é o segundo maior dentro do Gênero *Drosophila*, contando atualmente com 337 espécies, organizadas nos grupos de espécies *dentíssima, dispar, fima, melanogaster, obscura, populi, saltans, setifemur* e *willistoni* (Bachli, 2015). Algumas das espécies pertencentes ao Subgênero *Sophophora* não pertencem a nenhum dos grupos citados (Bachli, 2015).

Throckmorton (1975) considera que as espécies do Subgênero *Sophophora* façam parte de uma grande radiação de moscas, que também inclui os Gêneros *Chymomyza* e *Neotanygastrella*. Baseado em dados biogeográficos, Throckmorton (1975) sugeriu que os grupos *melanogaster* e *obscura* possuem um ancestral comum "protomelanogaster", que deu origem à ambos os grupos no Sudeste Asiático no Oligoceno médio. Ainda, esse mesmo autor teoriza que os grupos Neotropicais *willistoni* e *saltans* são fortemente relacionados entre si, porém distintos dos outros membros de *Sophophora* e que teriam se originado após a divergência dos grupos *melanogaster* e *obscura* a partir do ancestral "protomelanogaster".

Evidências morfológicas e análises filogenéticas com sequências de nucleotídeos provenientes de diversos genes e de diferentes genomas sugerem a monofilia do subgênero *Sophophora* (Throckmorton, 1975; Lemeunier *et al.,* 1986; Lachaise *et al.,*1988; Thomas e Hunt, 1991; DeSalle, 1992; Russo *et al.,* 1995), assim como de seus quatro grupos principais – *melanogaster, obscura, saltans* e *willistoni* (Powell, 1997). Por outro lado, Pelendakis *et al.* (1991) e Pelendakis e Solignac (1993), propõem que os grupos *melanogaster* e *willistoni* não são monofiléticos. O'Grady e Kidwell (2002) utilizaram sequências dos genes *Adh* (*Álcool desidrogenase*), *28S*

ribossômico e *COII* (*Citocromo Oxidase II*) mitocondrial para inferir a filogenia do subgênero. Os resultados sugeriram que os grupos *melanogaster* e *obscura* são monofiléticos e fortemente relacionados (O'Grady e Kidwell, 2002). Quanto ao clado Neotropical, o grupo *saltans* é recuperado como monofilético, enquanto o grupo *willistoni* é recuperado como parafilético em relação à *saltans* em várias análises.

1.3. O Grupo *willistoni* - aspectos históricos

Drosophila willistoni foi a primeira espécie do grupo a ser descrita. Em 1896, Samuel Williston publicou seu trabalho "On the Diptera of St. Vincent (West Indies)" e nele descreveu uma nova espécie denominada *Drosophila pallida*, desconhecendo que já havia uma espécie com esse nome. Anos mais tarde, o erro foi corrigido e a mosca descrita por Williston passou a ser denominada *Drosophila willistoni* (Sturtevant, 1916) em sua homenagem.

Em 1943, Dobzhansky e Pavan fizeram um estudo preliminar acerca da fauna de *Drosophila* no estado de São Paulo e no Distrito Federal e descreveram duas novas espécies que viriam a fazer parte do grupo *willistoni*: *D. paulista* e *D. capricorni. Drosophila paulista* foi descrita como muito similar à *D. willistoni*, porém com maior tamanho corporal e com algumas diferenças cromossômicas entre elas.

Dobzhansky (1946) descreveu *D. equinoxialis,* ressaltando sua alta similaridade com *D. willistoni* e a incluindo no grupo *willistoni,* naquele momento composto pelas espécies *D. capricorni, D. sucinea, D. nebulosa, D. fumipennis* e *D. subinfumata,* além de *D. paulista* e *D. willistoni.*

Dobzhansky e Pavan, em Burla *et al.* (1949), ao compararem as linhagens estudadas por Dobzhansky e Pavan (1943), perceberam que a nova espécie *D. paulista,* na realidade, pertencia à espécie *D. willistoni. Drosophila paulista* então se tornou sinônimo de *D. willistoni* e foi proposto um novo epíteto para a espécie recém descoberta que passou a se chamar *D. paulistorum*. Além da nova designação, Burla e Cunha, em Burla *et al*. (1949), descreveram uma nova espécie pertencente ao grupo, *D. tropicalis.*

Em 1954, Townsend observou o isolamento reprodutivo entre diferentes populações de *D. tropicalis* e estabeleceu uma divisão das mesmas em subespécies – *D. tropicalis tropicalis* e *D. tropicalis cubana*. As duas subespécies não vivem em simpatria - a subespécie *tropicalis* tem Rio Branco no Acre como sua distribuição mais ao Norte, e a Jamaica é o local de ocorrência de *cubana* localizado mais ao sul.

Uma nova espécie, maior e mais escura do que as demais espécies crípticas, foi encontrada e descrita por Dobzhansky (1957), em Dobzhansky *et al.* (1957). A nova espécie foi denominada *D. insularis,* cuja distribuição é restrita às Antilhas.

Posteriormente, propôs-se que *D. paulistorum* não é apenas uma espécie, mas sim um *cluster* formado por seis semiespécies ou espécies incipientes – Andino-Brasileira, Amazônica, Centroamericana, Guianense, Orinocana e Transicional (Dobzhansky e Spassky, 1959). As semiespécies foram descritas como morfologicamente idênticas, porém exibindo um forte isolamento etológico. Além disso, quando cruzadas, foi reportado que eram geradas apenas fêmeas férteis (Dobzhansky e Spassky, 1959). Kastritsis e Dobzhansky (1967) observaram diferenças suficientes na semiespécie Guianense para elevá-la à categoria de espécie, que passou então a ser denominada *D. pavlovskiana.* Uma nova

semiespécie, *D. paulistorum* Interior, foi descrita posteriormente por Pérez-Salas *et al.* (1970).

Ainda em relação a níveis infraespecíficos, Ayala *et al.* (1974) subdividiram *D. willistoni* nas subespécies *willistoni* e *quechua*, sendo que a última se encontra restrita ao oeste do Peru. Também separaram *D. equinoxialis* em duas subespécies, *equinoxialis* e *caribbensis,* sendo esta encontrada apenas nas ilhas de Porto Rico, Hispaniola e Costa Rica.

O subgrupo *alagitans* foi estabelecido por Patterson e Mainland (1944) e, inicialmente, foi incluído no subgênero *Drosophila.* Hsu (1949), no entanto, demonstrou que a genitália masculina das espécies desse grupo era muito similar a dos membros do grupo *willistoni,* sendo o grupo *alagitans* posteriormente transferido para o subgênero *Sophophora* (Wheeler, 1949).

Carson (1954) observou que haviam três diferentes espécimes registrados como *D. bocainensis* no Brasil; um dos espécimes foi confirmado como sendo *D. bocainensis* e os demais foram denominados *D. parabocainensis* e *D. bocainoides*. Esse complexo foi denominado subgrupo *bocainensis* e incluído no grupo *willistoni* (Wheeler e Magalhães, 1962).

Devido à similaridade entre *D. alagitans* e *D. bocainensis,* e respectivas espécies relacionadas, Wheeler e Magalhães (1952) concluíram que formavam um grupo natural de espécies e que deveriam ser classificadas em um ou dois subgrupos do grupo *willistoni*.

Recentemente foram descritas duas novas espécies pertencentes ao subgrupo *alagitans* – *D. pittieri* (Bächli e Vilela, 2002) e *D. neocapnoptera* (Figuero e Rafael, 2013).

Atualmente, o grupo *willistoni* reúne 24 espécies (Bächli, 2015) divididas em três subgrupos: *alagitans, bocainensis* e *willistoni*, cujas espécies estão relacionadas abaixo:

Subgrupo *alagitans*

- *D. alagitans* PETTERSON E MAILAND 1943

- *D. capnoptera* PETTERSON E MAINLAND 1944

- *D. megalagitans* WHEELER E MAGALHÃES 1962

- *D. neoalagitans* WHEELER E MAGALHÃES 1962

- *D. neocapnoptera* FIGUERO E RAFAEL 2013

- *D. pittieri* BÄCHLI E VILELA 2002

Subgrupo *bocainensis*

- *D. abregolineata* DUDA 1925

- *D. bocainensis* PAVAN E DA CUNHA 1947

- *D. bocainoides* CARSON 1954

- *D. capricorni* DOBZHANSKY E PAVAN 1943

- *D. changuinolae* WHEELER E MAGALHÃES 1962

- *D. fumipennis* DUDA 1925

- *D. mangabeirai* MALOGOLOWKIN 1951

- *D. nebulosa* STURTEVANT 1916

- *D. parabocainensis* CARSON 1954

- *D. pseudobocainensis* WHEELER E MAGALHÃES 1962

- *D. subinfumata* DUDA 1925

- *D. sucinea* PETTERSON E MAINLAND 1944

Subgrupo *willistoni*

- *D. equinoxialis* DOBZHANSKY 1946

    Subepécie *equinoxialis* AYALA *ET AL.* 1974

    Subespécie *caribbensis* AYALA *ET AL.* 1974

- *D. insularis* DOBZHANSKY 1957

- *D. paulistorum*

    Semiespécie Amazônica DOBZHANSKY E SPASSKY 1959

    Semiespécie Andino-Brasileira DOBZHANSKY E SPASSKY 1959

    Semiespécie Centroamericana DOBZHANSKY E SPASSKY 1959

    Semiespécie Interior PEREZ-SALAS *ET AL.* 1970

    Semiespécie Orinocana DOBZHANSKY E SPASSKY 1959

    Semiespécie Transicional DOBZHANSKY E SPASSKY 1959

- *D. pavlovskiana* KASTRITSIS E DOBZHANSKY 1967

- *D. tropicalis* BURLA E DA CUNHA 1949

Subespécie *tropicalis* TOWNSEND 1954

Subespécie *cubana* TOWNSEND 1954

- *D. willistoni* STURTEVANT 1916

Subespécie *willistoni* AYALA E TRACEY 1973

Subespécie *quechua* AYALA E TRACEY 1973

1.4. O Grupo *willistoni* – distribuição geográfica

As espécies dos subgrupos *alagitans* e *bocainensis* distribuem-se desde o sul da América do Norte, passando pela América Central continental e insular, até o Uruguai e norte da Argentina (Wheeler e Magalhães, 1962) (Figura 2). As espécies formam dois grupos distintos de distribuição geográfica, sendo o grupo mais ao norte formado pelas espécies do subgrupo *alagitans* e o grupo mais ao sul formado pelas espécies do subgrupo *bocainensis.* A distribuição de *D. bocainensis,* no entanto, se sobrepõe à distribuição norte, visto que essa espécie ocorre na Colômbia (Wheeler e Magalhães, 1962).

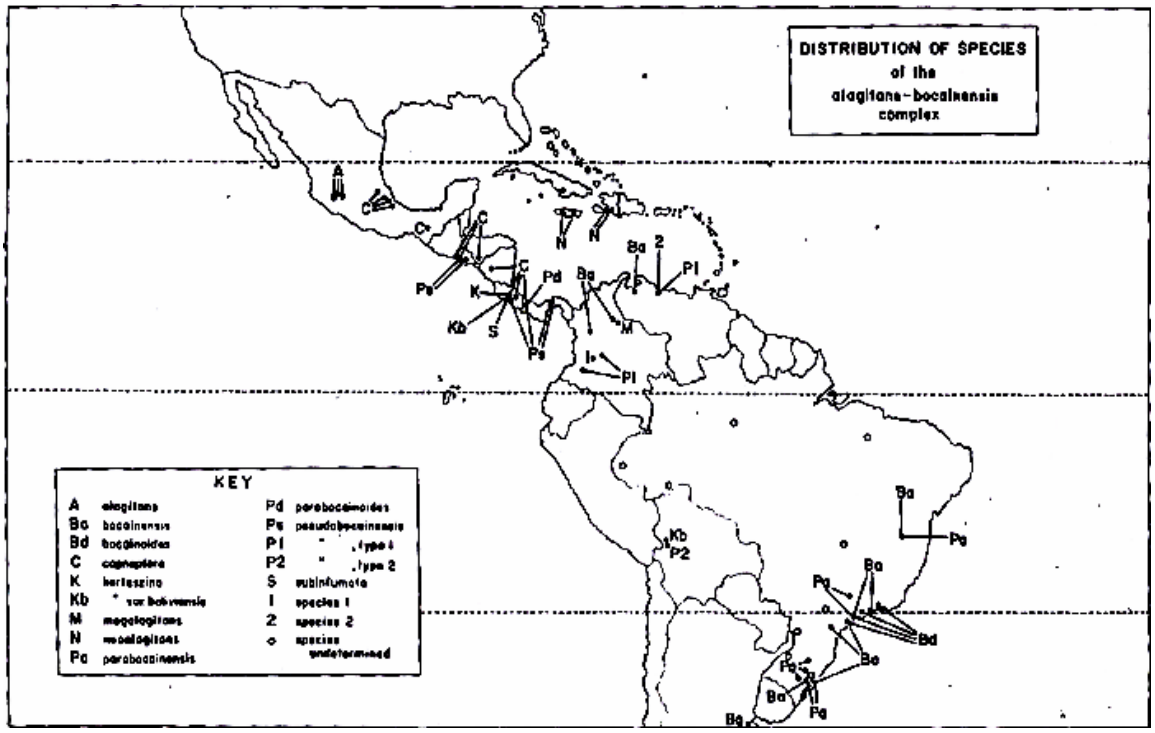

Figura 2. Distribuição das espécies dos subgrupos *alagitans* e *bocainensis* (em parte) (Figura retirada de Wheeler e Magalhães, 1962).

Drosophila sucinea distribui-se desde o México, passando pela América Central (El Salvador, Honduras, Nicarágua, Panamá) até o Norte da América do Sul (Equador, Peru e Venezuela) (revisão em Bächli, 2015 e Zanini *et al.,* 2015a).

Drosophila capricorni ocorre em várias localidades de diferentes Estados brasileiros (Rio Grande do Sul, Santa Catarina, São Paulo, Rio de Janeiro, Minas Gerais, Goiás, Distrito Federal, Maranhão e Bahia), assim como na Colômbia, México, Panamá, Peru e Venezuela (revisão em Bächli, 2015 e Zanini *et al.,* 2015a).

Drosophila fumipennis é encontrada na América Central, Índias Ocidentais (Antilhas e Bahamas), Peru, Colômbia e várias localidades dos Estados das regiões

Sul e Sudeste do Brasil, além do Amapá, Rondônia e Pernambuco (revisão em Bächli, 2015 e Zanini *et al.,* 2015a).

Drosophila nebulosa possui a distribuição mais ampla dentre as espécies do subgrupo *bocainensis*. A espécie ocorre desde os EUA, passando pelas ilhas do Caribe, México, América Central, Chile, Equador, Peru, Venezuela, diversas localidades brasileiras, com exceção de áreas mais úmidas como a Amazônia, até a Argentina e o Uruguai (Revisão em Bächli, 2015 e Zanini *et al.,* 2015a).

O subgrupo *willistoni* tem distribuição predominantemente Neotropical, ainda que algumas amostras de *Drosophila willistoni* tenham sido coletadas em florestas temperadas mistas de pinheiros e carvalhos no México (Ehrmann e Powell, 1982). A espécie *D. willistoni* tem a maior distribuição dentre as espécies desse subgrupo, sendo encontradas desde o México e Flórida até o sul do Brasil, Uruguai e Argentina do Oceano Atlântico ao Pacífico, exceto em áreas de grande altitude e desertos (Spassky *et al.,* 1971; Ehrmann e Powell, 1982), até mesmo em áreas urbanas (Spassky *et al.,* 1971*;* Valiati e Valente, 1997).

Na Flórida, Uruguai e Argentina e norte do México encontra-se apenas *D. willistoni* (Spassky *et al.,* 1971). Em outras localidades, no entanto, *D. willistoni* encontra-se em simpatria com outras espécies do subgrupo. Nas Antilhas Maiores (Cuba, Jamaica, Haiti e Porto Rico) vive juntamente com as espécies *D. tropicalis* e *D. equinoxialis*; nas Antilhas Menores, coabita apenas com *D. insularis* (Spassky *et al.,* 1971).

Drosophila insularis tem sua distribuição restrita a algumas ilhas das Antilhas Menores. Até o momento, foram coletadas amostras nas ilhas de St. Kitts, St. Lúcia e

Montserrat, porém, Spassky *et al.* (1971) acreditam que essa espécie possa ser encontrada em outras ilhas da cadeia das Antilhas Menores.

Drosophila pavlovskiana é endêmica da Guiana e Venezuela e foi coletada em apenas duas localidades próximas à capital Georgetown (Spassky *et al.,* 1971) e em três localidades da Venezuela (revisão em Bächli, 2015 e Zanini *et al.* (2015b), não sendo mais coletada desde a década de 70.

Drosophila tropicalis e *D. equinoxialis* têm preferência por habitats de florestas um pouco mais secas e savanas, como as encontradas na costa caribenha da Colômbia, Venezuela e Guiana (Spassky *et al.,* 1971).

As espécies *D. willistoni, D. tropicalis, D. equinoxialis* e *D. paulistorum* ocorrem em simpatria desde a Guatemala até o Brasil Central (Figura 3). Rhode *et al.* (2010) reportaram o primeiro registro de *D. equinoxialis* na região Nordeste do Brasil, em algumas localidades do Estado de Pernambuco.

Quanto às semiespécies de *D. paulistorum*, Andino-Brasileira é a que apresenta a mais ampla distribuição, sendo encontrada desde o Noroeste da Colômbia e parte adjacente da Venezuela, Trinidad e Guiana central até o sul do Brasil (Rio Grande do Sul) e dos Andes peruanos, colombianos, equatorianos e venezuelanos, até a costa Atlântica do Brasil. Ocorre em simpatria com Amazônica, Interior e Orinocana principalmente ao norte do rio Amazonas e na nascente do Orinoco (Spassky *et al.,* 1971) (Figura 4).

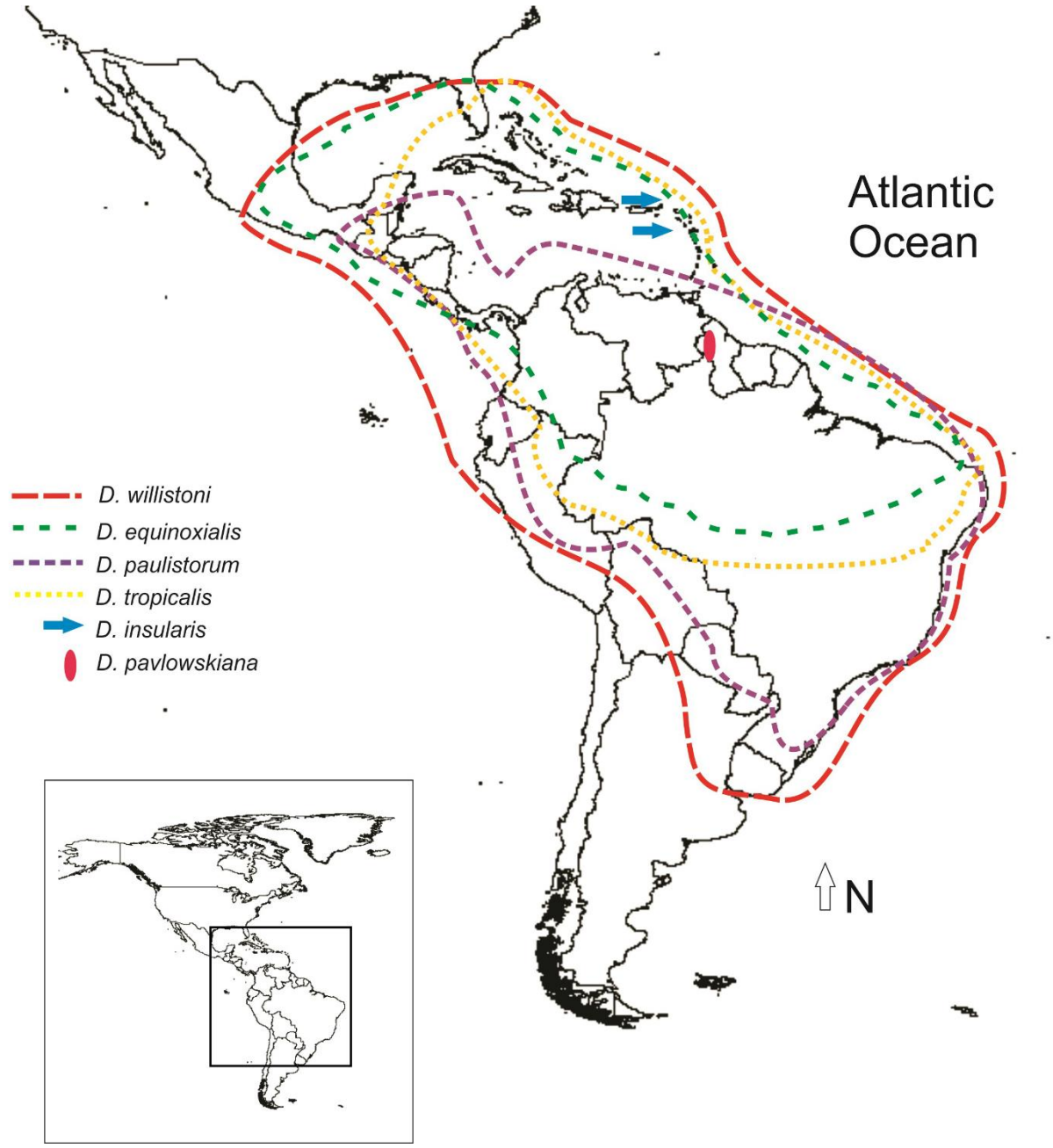

Figura 3. Distribuição geográfica do subgrupo *willistoni* (Figura retirada de Zanini *et al.,* 2015c).

A semiespécie *D. paulistorum* Amazônica se distribui do Panamá e Trinidad até o estuário do Amazonas e Tocantins. É a semiespécie que ocorre em maior

abundância nas bacias dos rios Negro e Orinoco, assim como ao longo do rio Amazonas. Vive em simpatria com Andino-Brasileira, Orinocana e Interior, e parece não ocorrer sozinha em nenhum ponto de sua distribuição (Spassky *et al.,* 1971) (Figura 4).

D. paulistorum Interior vive ao sul de Llanos, na Colômbia; nas proximidades do Rio Negro, na nascente do Orinoco e na nascente do Amazonas e seus afluentes. A semiespécie Orinocana parece estar confinada à costa caribenha do Norte da Colômbia e Venezuela e não ocorre em simpatria com Interior (Spassky *et al.,* 1971) (Figura 4).

A semiespécie Centroamericana ocorre sozinha na Guatemala, Honduras, El Salvador, Costa Rica e oeste do Panamá. Ocorre em simpatria com Amazônica e Orinocana na região central do Panamá (Zona do Canal e áreas adjacentes). A semiespécie Transicional é encontrada sozinha na costa Oeste úmida da Colômbia e juntamente com Orinocana no maciço de Santa Marta e com Andino-Brasileira e Amazônica no Norte da Venezuela (Figura 4).

Nos capítulos II e III serão apresentadas revisões mais completas da distribuição dos subgrupos *willistoni* e *alagitans-bocainensis*, respectivamente.

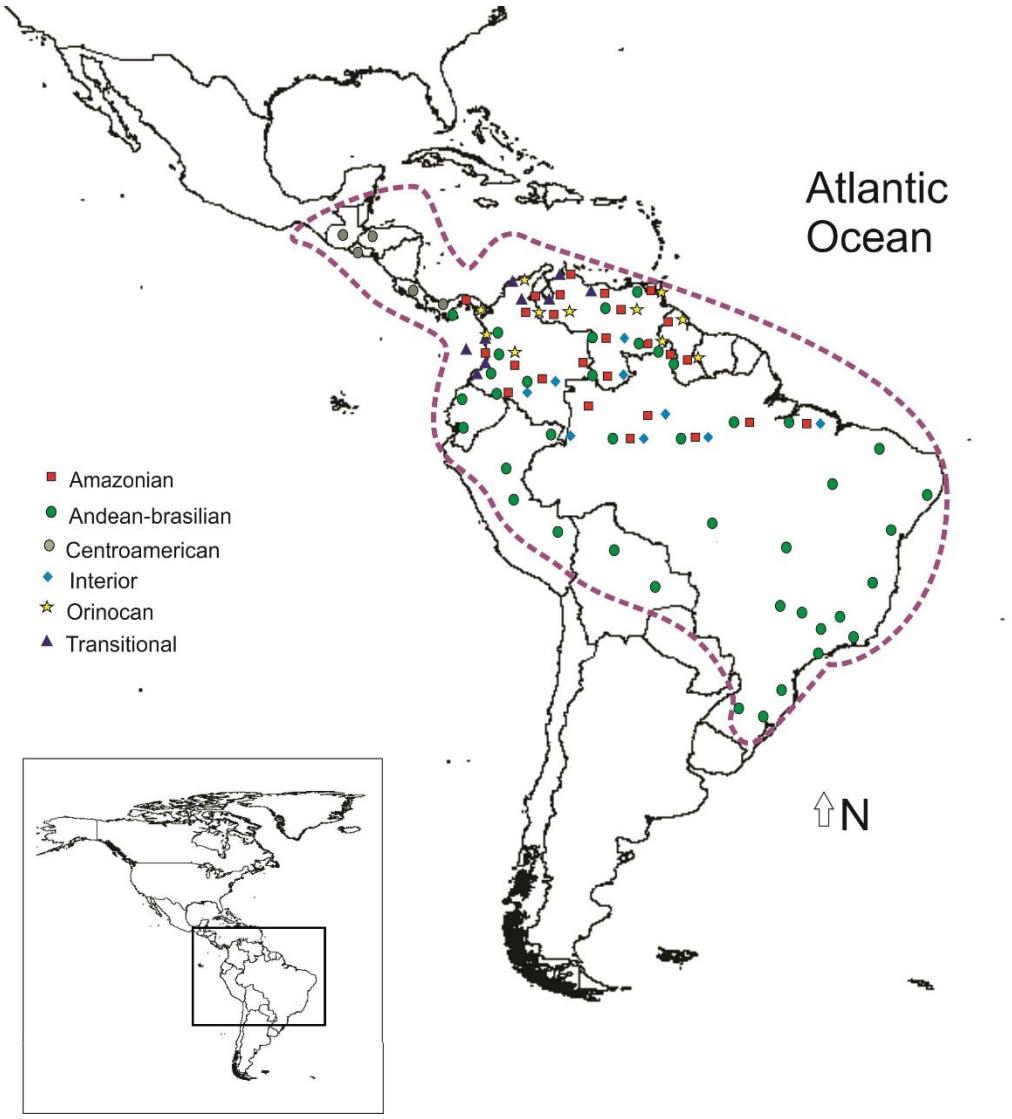

Figura 4. Distribuição geográfica das semiespécies do complexo *D. paulistorum,* de acordo com Spassky *et al*. (1971), Winge (1971), Dobzhansky e Powell (1975), Ehrman e Powell (1982), Santos e Valente (1990) e revisões de Bachli *et al*. (2015). Figura retirada de Zanini *et al.* (2015c).

1.5. Estudos evolutivos do grupo *willistoni*

1.5.1. Estudos cromossômicos

Os primeiros estudos genéticos acerca dos relacionamentos evolutivos entre as espécies foram realizados a partir da análise cromossômica.

A existência de cromossomos politênicos (cromossomos gigantes gerados a partir de sucessivos ciclos de endorreplicação) permite a comparação do padrão de bandas desses cromossomos entre diferentes espécies. Assim, a história evolutiva das espécies do grupo *willistoni* pôde ser sugerida através da determinação do número e tipos de rearranjos cromossômicos fixados durante os eventos de divergência a partir de seu último ancestral comum (Ehrmann e Powell, 1982).

O complemento cromossômico descrito para *D. willistoni* consiste de dois pares metacêntricos e um par acrocêntrico (Metz, 1916). Esse mesmo complemento é encontrado não apenas nas espécies do subgrupo *willistoni,* mas também em outros membros do grupo *willistoni,* como *D. nebulosa, D. fumipennis, D. capricorni* e *D. sucinea* e do grupo *saltans,* ambos pertencentes ao subgênero *Sophophora* (Dobzhansky e Powell, 1975).

A análise dos cromossomos politênicos é um dos métodos utilizados para diagnosticar as espécies crípticas do subgrupo *willistoni*. Um dos primeiros relatos acerca das características dos cromossomos politênicos do subgrupo foi feito por Burla *et al.* (1949). Burla *et al.* (1949) afirmaram que o padrão de bandeamento das porções terminais livres dos cromossomos XR, XL, 2R, 2L é bastante similar nas espécies *D. willistoni, D. paulistorum, D. tropicalis* e *D. equinoxialis.* Ainda, relatam

que o cromossomo III apresenta padrão característico para cada uma das espécies analisadas.

Dobzhansky *et al.* (1950) publicou um mapa dos cromossomos politênicos das glândulas salivares das larvas de *D. willistoni* e *siblings.* Kastristsis (1966) fez o mesmo para *D. paulistorum*. O arranjo dos cromossômicos politênicos de *D. insularis* foram apresentados por Dobzhansky *et al*. (1957)*;* os autores chamaram atenção para as semelhanças da porção terminal do braço direito do cromossomo X (XR) dessa espécie com *D. tropicalis*, porém, mostraram que essas duas espécies podem ser distinguidas também por outras regiões cromossômicas. O mapa dos cromossomos politênicos de *D. pavlovskiana* foi apresentado em Dobzhansky e Pavlovsky (1962) e atualizado em Kastritsis e Dobzhansky (1967).

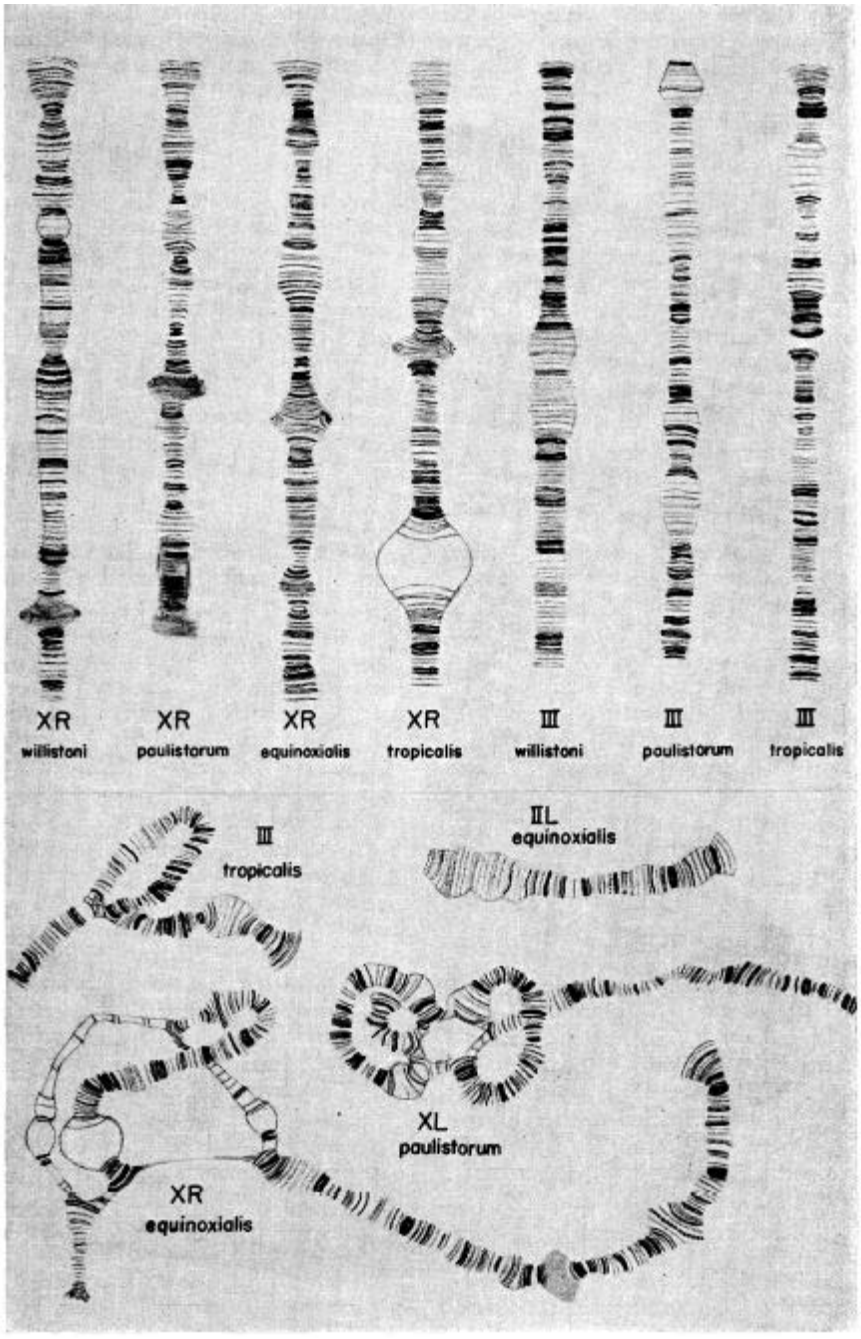

Figura 5. Porção distal do braço direito do cromossomo X, do cromossomo III e algumas inversões encontradas em populações naturais de *D. willistoni*, *D. paulistorum, D. equinoxialis* e *D. tropicalis.* (Figura retirada de Burla *et al.,* 1949).

1.5.2. Estudos morfológicos

Apesar de *D. willistoni* ter sido descrita em 1916, somente em 1949 foi apresentado o primeiro trabalho envolvendo uma descrição morfológica ilustrada e mais detalhada do grupo. Burla *et al.* (1949) apresentaram breves descrições e ilustrações de alguns caracteres morfológicos tradicionalmente utilizados na taxonomia de Drosophilidae – palpos maxilares, placas vaginais, espermateca e hipândrio, posição das cerdas orais e largura relativa da fronte (Figura 6). Nesse mesmo trabalho, Burla *et al.* (1949) afirmam que existiam caracteres que tornavam possível a identificação de cada uma das *siblings*, porém destacaram que existia uma variabilidade muito grande entre populações, tornando a identificação individual muito incerta.

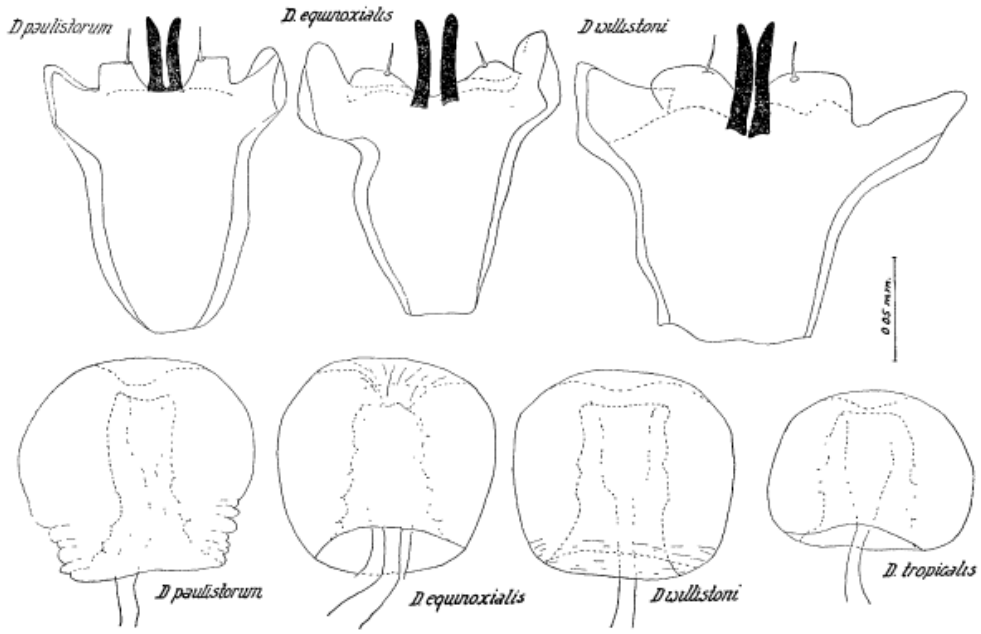

Figura 6. Hipândrios (acima) e espermatecas (abaixo) de *D. willistoni* e *siblings*. (Figura retirada de Burla *et al.*, 1949).

Ainda em 1949, Hsu faz uma breve descrição da genitália masculina de algumas espécies do grupo *willistoni – D. willistoni, D. equinoxialis, D. sucinea, D. nebulosa, D. fumipennis, D. alagitans* e *D. capnoptera.* Malogolowkin (1952) – Figura 7 - apresenta uma descrição bastante detalhada das genitálias masculina e feminina das espécies *D. willistoni, D. equinoxialis, D. tropicalis* e *D. paulistorum* do subgrupo *willistoni*, além de *D. bocainensis, D. capricorni, D. fumipennis, D. nebulosa* e *D. sucinea*, do subgrupo *bocainensis.*

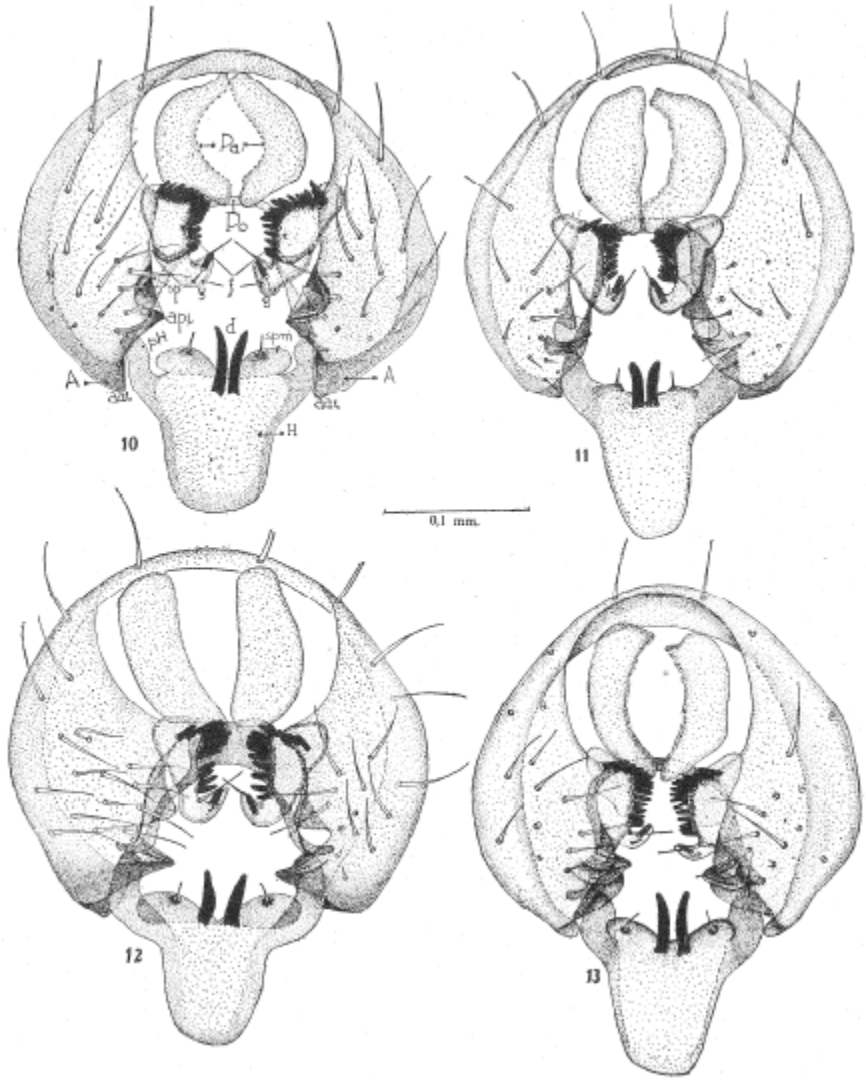

Figura 7. Genitália masculina de *D. willistoni* (10), *D. paulistorum* (11), *D. tropicalis* (12), *D. equinoxialis* (13). Figura retirada de Malogolowkin (1952).

Spassky (1957) apresentou ilustrações do hipândrio, edeago, surstilus e prensisetas de cinco siblings do subgrupo *willistoni – D. willistoni, D. tropicalis, D. equinoxialis, D. insularis* e *D. paulistorum* (Figura 8).

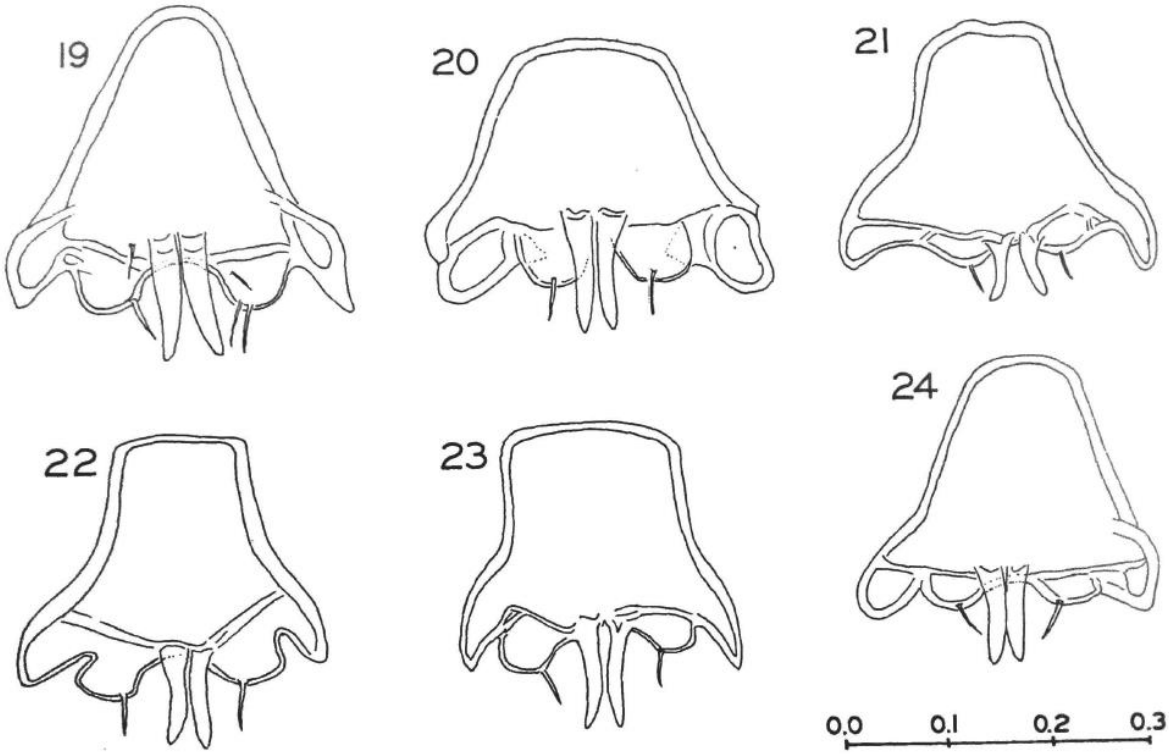

Figura 8. Hipândrios de *D. tropicalis* (19), *D. willistoni* (20), *D. insularis* (21), *D. paulistorum* do Brasil (22), *D. paulistorum* de Honduras (23), *D. equinoxialis* (24). Figura retirada de Spassky (1957).

Wheeler e Magalhães (1952) apresentaram uma série de ilustrações das genitálias masculinas de *D. alagitans, D. megalagitans, D. neoalagitans, D. bocainoides, D. parabocainoides* (Sinônimo de *D. subinfumata*), *D. bocainensis, D. pseudobocainensis, D. capnoptera* e *D. changuinolae*, sendo algumas delas parte da descrição de novas espécies.

Pasteur (1970) tentou diferenciar quatro semiespécies de *D. paulistorum* através da comparação biométrica, utilizando como parâmetros o comprimento das asas e da tíbia, a razão asa-tíbia, o tamanho da asa e o número de prensisetas do surstilus.

Vilela e Bächli (1990) redescreveram *D. abregolineata* (subgrupo *alagitans*), *D. fumipennis* e *D. subinfumata* (subgrupo *bocainensis*) e *D. willistoni* (subgrupo *willistoni).* Eberhard e Ramirez (2004) apresentaram várias fotomicrografias das genitálias masculina e feminina como parte de uma análise da teoria de chave e fechadura para espécies do gênero *Drosophila.*

Em 2010, Rohde e colaboradores apresentaram imagens do hipândrio de *D. willistoni, D. equinoxialis, D. tropicalis* e *D. paulistorum* em microscopia óptica, sugerindo a importância dessa estrutura para identificação das espécies do subgrupo (Figura 9). As semiespécies de *D. paulistorum* não foram identificadas.

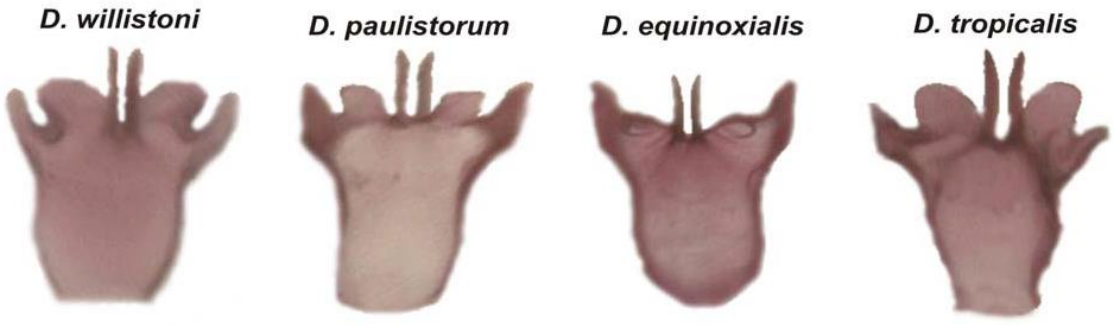

Figura 9. Hipândrios de espécies do subgrupo *willistoni* – *D. willistoni, D. paulistorum* e *D. equinoxialis* provenientes da Reserva Biológica de Saltinho, Tamandaré, Pernambuco e *D. tropicalis* do Stock Center (coletada em Jalisco, México). Imagem retirada de Rhode *et al.* (2010).

Recentemente, Souza *et al.* (2014) apresentaram imagens da genitália masculina de *D. willistoni* em Microscopia Eletrônica de Varredura (MEV), no entanto, esse trabalho tinha como foco um estudo filogenético do grupo *saltans* baseado em caracteres de genitália masculina, sendo *D. willistoni* utilizada apenas como grupo externo.

1.5.3. Estudos enzimáticos

Diversos trabalhos buscaram estabelecer uma maneira rápida e prática de identificar as espécies do subgrupo *willistoni*. Vários autores apresentaram diferentes ensaios enzimáticos como possibilidade de identificação mais rápida e confiável das espécies do que a partir de análises cromossômicas e inspeção da genitália masculina. Os pioneiros nesse tipo de estudo para o subgrupo *willistoni* foram Ayala *et al*. (1970), que analisaram os padrões eletroforéticos de migração de 14 enzimas em diversas populações de quatro *siblings* do subgrupo – *D. insularis* e *D. pavlovskiana* não foram analisadas.

Ayala *et al.* (1971) compararam polimorfismos cromossômicos e alozimáticos de populações continentais e insulares de *D. willistoni.* Richmond (1971) analisou as semiespécies de *D. paulistorum* quanto à presença de polimorfismos em 11 enzimas, e concluiu que, apesar de ter encontrado polimorfismos, nenhum dos loci analisados serve como ferramenta para a identificação das semiespécies. Também foi estudada a variação alélica de 28 loci gênicos de populações naturais de *D. willistoni* ao longo de sua distribuição geográfica (Ayala *et al.,* 1971) e observado que são encontrados

mais polimorfismos em populações da região central da distribuição do que nas populações marginais.

Ayala e Powell (1972) investigaram a possibilidade de diferenciação entre as espécies do subgrupo *willistoni* utilizando-se diferentes loci enzimáticos. Para isso, foram analisadas diversas populações de cada espécie, tentando abranger a distribuição das mesmas. Os autores concluíram que algumas das enzimas analisadas podem ajudar na diferenciação das espécies, quando comparadas par a par.

Ayala e Tracey (1973) analisaram a variabilidade enzimática e isolamento reprodutivo entre as duas subespécies de *D. willistoni – willistoni* e *quechua*, e afirmaram que "evidências disponíveis apresentadas indicam que ocorre uma substancial diferenciação genética na formação de subespécies". Os mesmos autores apresentaram um estudo comparando a diferenciação genética intra e interespecífica em espécies do grupo *willistoni* (Ayala e Tracey, 1974).

Foram investigados também aspectos genéticos e reprodutivos das subespécies de *D. equinoxialis – equinoxialis* e *caribbensis* (Ayala *et al.,* 1974). Os resultados encontrados foram semelhantes aos reportados por Ayala e Tracey (1973).

Ayala *et al.* (1975) analisaram a diferenciação genética baseada em 36 loci gênicos de 14 taxa pertencentes ao grupo *willistoni.* A partir dessas informações, construíram um dendrograma apresentando as possíveis relações filogenéticas do grupo *willistoni* (Figura 10).

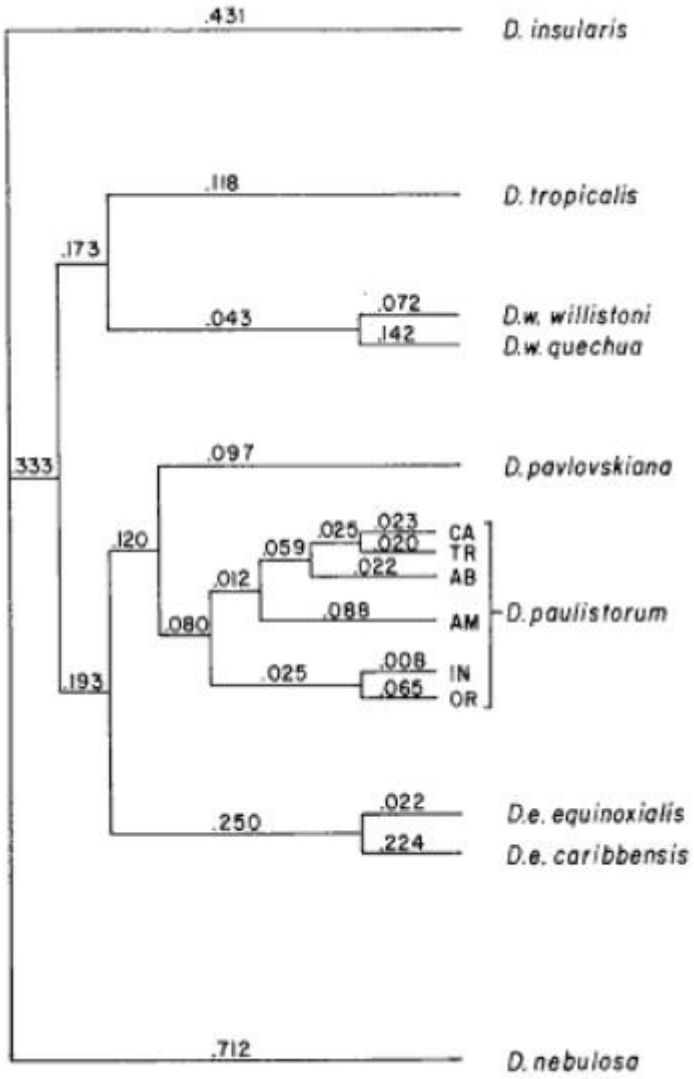

Figura 10. Dendrograma mostrando as prováveis relações filogenéticas baseadas na diferenciação genética de 36 loci gênicos de 14 taxa pertencentes ao grupo *D. willistoni*. Os números apresentados são as distâncias genéticas entre nós consecutivos ou entre um nó e um taxa. (Figura retirada de Ayala *et al.,* 1975).

Após um grande hiato na produção de trabalhos envolvendo estudos enzimáticos relacionados ao grupo *willistoni*, Garcia *et al.* (2006) obtiveram sucesso na identificação das espécies do subgrupo *willistoni* baseando-se na análise da mobilidade eletroforética da fosfatase ácida (*Acph-1*) (Figura 11). Esse trabalho

mostrou que é possível identificar de forma relativamente rápida os indivíduos pertencentes ao subgrupo *willistoni* a partir de machos ou fêmeas, não sendo possível, no entanto, diferenciar as semiespécies de *D. paulistorum.* Além disso, verificou-se que a espécie *D. equinoxialis* é polimórfica (Figura 11).

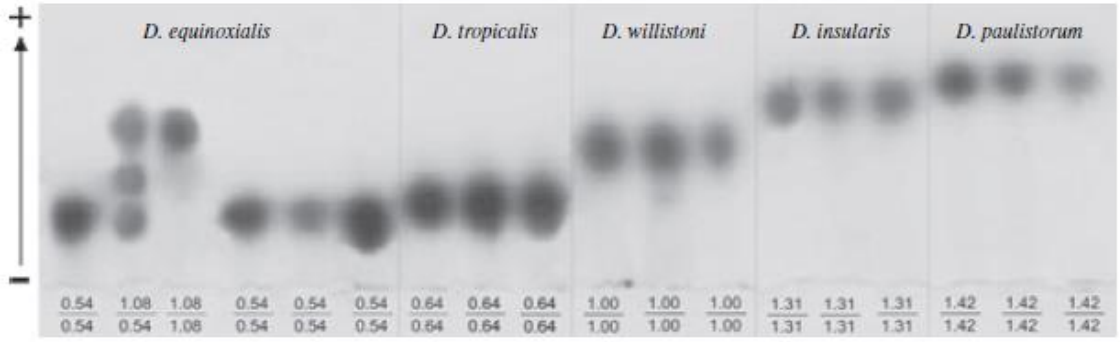

Figura 11. Zimograma dos padrões fenotípicos de *Acph-1* encontrados em cada uma das espécies do subgrupo *willistoni*. Diferentes genótipos homo e heterozigotos estão indicados. A direção de migração segue a direção da seta. Figura retirada de Garcia *et al.* (2006).

1.5.4. Estudos de cruzamentos

Desde a descrição das espécies do subgrupo *willistoni*, tem sido feitos diversos estudos acerca de cruzamentos interespecíficos entre as espécies desse subgrupo.

O primeiro estudo de cruzamentos envolveu as espécies *D. willistoni* e *D. equinoxialis* (Dobzhansky, 1946). Verificou-se que não houve produção de híbridos, que foi um dos fatores considerados ao se descrever *D. equinoxialis* como uma espécie diferente de *D. willistoni* (Dobzhansky, 1946).

Burla *et al.* (1949) apresentaram um estudo um pouco mais abrangente, no qual foram testados cruzamentos entre as quatro espécies do subgrupo *willistoni* descritas até o momento – *D. willistoni, D. equinoxialis, D. tropicalis* e *D. paulistorum.* Ao promoverem testes sem escolha, observaram que machos de *D. equinoxialis* não inseminaram fêmeas de *D. tropicalis* e *D. paulistorum;* o mesmo foi observado para machos *D. tropicalis* em relação à fêmeas *D. willistoni* e *D. equinoxialis.* Também verificaram um baixo nível de inseminação de machos *D. willistoni* com as demais espécies. Os machos de *D. paulistorum* foram os que apresentaram maior índice de inseminação, fato que os autores atribuíram a um maior grau de atividade desses machos ou uma maior aceitação desses machos por parte das fêmeas das demais espécies.

Dobzhansky e Spassky (1959) apresentaram uma série de cruzamentos entre diferentes linhagens de *D. paulistorum,* provenientes de localidades distintas. Foi observado que algumas dessas linhagens, quando cruzadas com linhagens de outros lugares, não produziam prole viável ou produziam apenas machos estéreis. A partir desses testes, separaram as linhagens de *D. paulistorum* em seis semiespécies – Amazônica, Andino-Brasileira, Centroamericana, Guianense e Transicional. Além disso, reportaram que o cruzamento da semiespécie Transicional com Andino-Brasileira e Centroamericana pode produzir prole fértil, ainda que o fenômeno não seja observado para todas as linhagens dessas semiespécies.

Alguns anos depois, Ehrman (1960a) relatou a obtenção de híbridos entre os pares de semiespécies Centroamericana e Amazônica, Centroamericana e Andino-Brasileira e entre Amazônica e Andino-Brasileira, e, a partir dos resultados, tentou esclarecer os mecanismos genéticos que promovem o isolamento sexual entre as semiespécies, baseando-se em estudos cromossômicos.

Ainda em 1960, Ehrman analisou a genética da esterilidade do híbrido entre as espécies incipientes Centroamericana e Transicional, também a partir de análises cromossômicas. Nesse estudo, a autora afirma que o cruzamento entre as semiespécies Centroamericana, Amazônica, Andino-Brasileira, Orinocana e Guianense sempre produzem fêmeas férteis e machos estéreis (Ehrman, 1960b).

Além de testes de cruzamentos envolvendo populações marginais de *D. willistoni* e *D. paulistorum,* Winge e Cordeiro (1963) caracterizaram as epermatecas e hipândrios dos híbridos gerados a partir do cruzamento entre essas duas espécies (Figura 12). Os autores observaram que as espermatecas apresentam um fenótipo intermediário entre os parentais (Figura 12). Quanto aos hipândrios, os autores observaram que a forma dos lobos do hipândrio varia muito entre os híbridos. A posição da seta dos lobos, nos híbridos, apresenta-se inserida na borda do lobo, assim como em *D. paulistorum.* Os dentes do hipândrio apresentam uma inserção mais baixa em *D. willlistoni* do que em *D. paulistorum*, sendo nos híbridos encontrados em posição intermediária.

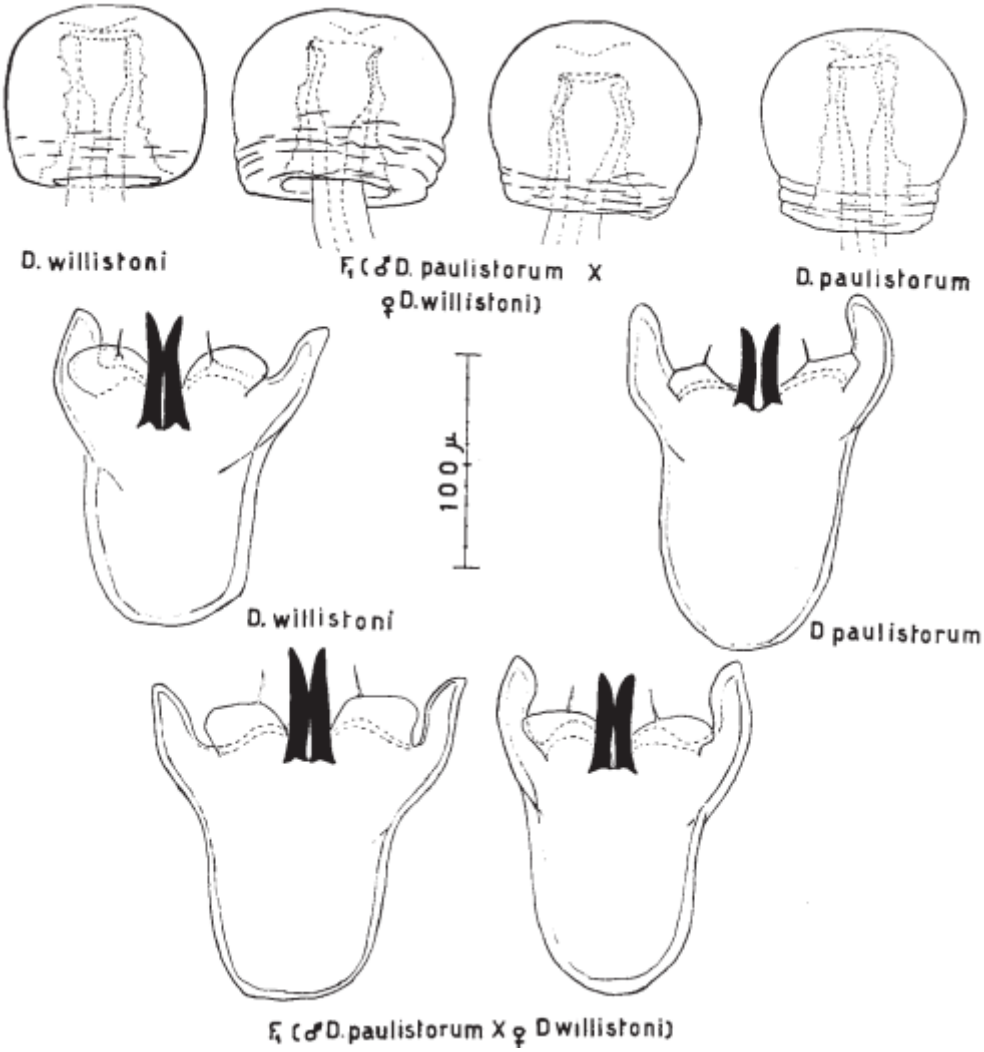

Figura 12. Hipândrios de *D. paulistorum, D. willistoni* e seus híbridos. Figura retirada de Winge e Cordeiro (1963).

Ehrman (1965) analisou o isolamento sexual entre linhagens simpátricas e alopátricas das semiespécies, e observou que os cruzamentos entre diferentes semiespécies ocorrem com muito menos frequência do que dentro de cada semiespécie. Ainda, verificou que pares de semiespécies que ocorrem em simpatria tendem a ser mais isolados reprodutivamente do que os mesmos pares em alopatria.

De acordo com os estudos de Ehrman (1960a, 1960b), o isolamento sexual é controlado por fatores distribuídos em todos os três cromossomos, sendo que esses genes possuem efeitos aditivos.

Os mecanismos de isolamento são discutidos em Ehrman e Powell (1982), que descrevem três mecanismos possíveis:

1. Isolamento etológico ou comportamental

2. Esterilidade do híbrido, no qual são relatadas três diferentes possibilidades:

- Híbridos machos da primeira geração (F1) são estéreis, como resultado de seu próprio genótipo;

- Alguns dos machos retrocruzados produzidos por fêmeas híbridas são estéreis, como resultado do genótipo materno;

- Efeito materno, que resulta de incompatibilidades entre o genótipo materno e um simbionte semelhante à Mycoplasma (Ehrman e Kernaghan, 1972).

3. Inviabilidade do híbrido devido a um conjunto de fatores, como efeito materno, efeito paterno e presença de organismos semelhantes à Mycoplasma no citoplasma do ovo.

Ainda em Ehrman e Powell (1982), os autores resumem os resultados de cruzamentos envolvendo o subgrupo *willistoni* e algumas espécies do subgrupo *bocainensis.* Os resultados variaram desde ausência de híbrido, o que ocorre na maioria dos cruzamentos, até a obtenção de híbridos viáveis. *Drosophila insularis* produz híbridos com *D. equinoxialis* e *D. paulistorum* Transicional, porém, estes morrem nos estágios pré-adultos. *Drosophila pavlovskiana* produz apenas fêmeas férteis quando cruzada com as seis diferentes semiespécies; o mesmo ocorre nos cruzamentos entre as subespécies *D. willistoni willistoni* e *D. willistoni quechua, D.*

equinoxialis equinoxialis e *D. equinoxialis caribbensis,* e *D. tropicalis tropicalis* e *D. tropicalis cubana.*

As relações entre as semiespécies de *D. paulistorum* variam desde isolamento completo, sem produção de híbridos (cruzamentos entre Andino-Brasileira e Interior; Amazônica e Orinocana; Amazônica e Transicional), passando pela produção de híbridos com morte no ovo ou em estágios pré-adultos, esterilidade apenas do macho até prole totalmente fértil (Ehrman e Powell, 1982). Como observado previamente por Dobzhansky e Spassky (1959), a semiespécie Transicional é a única que produz descendentes férteis, quando cruzada com Andino-Brasileira e Centroamericana (Ehrman e Powell, 1982).

Winge (1965) realizou testes de cruzamento envolvendo as espécies do subgrupo *willistoni*, e observou que machos de *D. willistoni* cruzados com fêmeas de *D. paulistorum* provenientes da mesma localidade, produzem híbridos estéreis; o cruzamento recíproco, no entanto, produz prole fértil. O cruzamento de *D. willistoni* com *D. equinoxialis* não produz híbridos viáveis. *Drosophila equinoxialis* produz híbridos viáveis apenas com *D. paulistorum,* porém com olhos, asas e cerdas anormais. O cruzamento entre machos de *D. paulistorum* e fêmeas de *D. tropicalis* pode produzir híbridos, dependendo da linhagem utilizada; não ocorrem híbridos no cruzamento recíproco. Machos de *D. tropicalis,* quando cruzados com fêmeas *D. insularis* podem produzir híbridos inviáveis ou inférteis, dependendo da linhagem, e não ocorrem híbridos com o cruzamento recíproco.

Cordeiro e Winge (1995) resumiram resultados de cruzamentos previamente realizados e destacaram a hibridização entre *D. paulistorum* e *D. willistoni*, que resultou em baixo número de híbridos, sendo em sua maioria fêmeas férteis e machos

estéreis, frequentemente com asas anormais. Os autores ainda relataram que linhagens de localidades próximas produziram um número maior de larvas e adultos, levantando a hipótese de que populações marginais geram mais híbridos interespecíficos.

Os resultados alcançados por Winge (1965) e Cordeiro e Winge (1995) foram diferentes dos apresentados por Ehrman e Powell (1982). Cordeiro e Winge (1995) afirmaram que essas diferenças foram em decorrência de diferentes objetivos de seu trabalho. Cordeiro e Winge (1995) tinham como objetivo não somente o estudo dos mecanismos de isolamento reprodutivo, mas também a produção de híbridos interespecíficos para estudos cromossômicos.

Ainda, recentemente, Civetta e Gaudreu (2015) atribuíram a esterilidade dos machos híbridos das duas subespécies de *D. willistoni* – *D. willistoni willistoni* e *D. willistoni quechua* - à incapacidade do macho de primeira geração híbrida (F1) de transferir o esperma durante a cópula.

1.5.4.1. Especiação infecciosa no subgrupo *willistoni*

A presença de endossimbiontes atuando como fatores de isolamento reprodutivo em espécies do subgrupo *willistoni* tem sido amplamente discutida desde os primeiros trabalhos envolvendo análises dos fatores relacionados a isolamento reprodutivo, como em Ehrman e Kernagan (1975). A presença desses endossimbiontes foi discutida em Ehrman e Powell (1982), onde os autores sugerem que microorganismos semelhantes à Mycoplasma podem ser uma herança materna ou possivelmente citoplasmática, uma vez que estão inseridos nas células

germinativas do embrião (Daniels e Ehrman, 1974) e são transmitidos verticalmente entre gerações via citoplasma do ovo. Os autores observaram que os híbridos de *D. paulistorum* produzidos em laboratório são machos estéreis e fêmeas férteis, e que, quando essas fêmeas híbridas são retrocruzadas com machos parentais de ambas as semiespécies, a progênie gerada é composta também de machos estéreis e fêmeas férteis.

Em outro estudo, Somerson *et al.* (1984) verificaram a presença de formas L de *Streptococcus* em machos estéreis de *D. paulistorum.* Os autores atribuíram a esterilidade dos machos à presença deste endossimbionte.

Mais recentemente, Miller *et al*. (2010) apontam para o envolvimento de diferentes estirpes do endossimbionte *Wolbachia* como geradoras da incompatibilidade citoplasmática observada em cruzamentos no complexo de espécies de *D. paulistorum*. Apesar disso, pesquisas examinando especificamente a influência da bactéria intracelular *Wolbachia*, quer seja na geração de híbridos estéreis, incompatibilidades, ou mesmo nas preferências de parceiros no gênero *Drosophila*, ainda são escassos. Assim, a influência de *Wolbachia* atuando nos mecanismos de seleção sexual tem encontrado pouco apoio (Zuk e Wedell, 2014).

1.5.5. Estudos baseados em sequências de DNA

O primeiro trabalho envolvendo análise filogenética molecular do grupo *willistoni* foi o de Gleason *et al.* (1998), no qual foram incluídas todas as espécies e semiespécies do subgrupo *willistoni*, além de algumas espécies do subgrupo *bocainensis.* As hipóteses filogenéticas desse trabalho foram feitas a partir de sequências dos genes *Adh* (*Álcool desidrogenase*), *Per* (*Period*) e *COI* (*Citocromo Oxidade* subunidade I).

Tarrío *et al.* (2000) abordaram a problemática do enraizamento das árvores filogenéticas com grupo externo quando existem diferenças na composição nucleotídica entre os grupos interno e externo. Foram utilizados como estudo de caso os grupos *willistoni* e *saltans*, cujas relações filogenéticas foram estabelecidas utilizando-se sequências de *Xdh* (*Xantina desidrogenase*).

Em 2002, O'Grady e Kidwell propuseram uma filogenia para o subgênero *Sophophora* baseada em fragmentos dos genes *28s, Adh* e *COII* e utilizaram algumas espécies do subgrupo *willistoni* como parte dessa análise.

Finalmente, Robe *et al.* (2010) apresentaram uma análise filogenética do subgrupo *willistoni,* utilizando dois genes mitocondriais (*COI, COII*) e quatro genes nucleares - *Adh, Amd* (*Alfa-metildopa*), *Hb* (*Hunchback*) e *Per*, combinados em diferentes conjuntos de dados.

Os dados apresentados por Gleason *et al.* (1998) sugerem que *D. insularis* foi a primeira espécie do subgrupo *willistoni* a divergir a partir de um ancestral comum, seguida por *D. tropicalis* e *D. willistoni* aparecendo como espécie-irmã do clado

formado por *D. equinoxialis* e *D. paulistorum.* Resultado semelhante foi encontrado por Tarrío *et al.* (2000) e Robe *et al.* (2010).

O grupo *willistoni* é, até o momento, o único representante Neotropical a ter o genoma completo de uma de suas espécies sequenciado. *Drosophila willistoni* teve seu genoma totalmente sequenciado como parte do Consórcio 12 genomas (Clark *et al.,* 2007). Este amplo projeto multinacional envolveu especialistas em diferentes grupos de *Drosophila* e abriu amplas perspectivas para estudos evolutivos.

Os estudos evolutivos baseados em sequências de DNA serão discutidos de maneira mais aprofundada no capítulo VI.

CAPÍTULO II – On the geographic distribution of the *Drosophila willistoni* group (Diptera, Drosophilidae) – updated distribution of *willistoni* subgroup

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On the geographic distribution of the *Drosophila willistoni* group (Diptera, Drosophilidae) – updated geographic distribution of the Neotropical *willistoni* subgroup

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Artigo aceito para publicação no periódico *Drosophila Information Service* 98, 2015.

INTRODUCTION

Drosophila willistoni species group comprises 24 Neotropical species, divided into three subgroups – *alagitans, bocainensis* and *willistoni* (Bächli, 2015). The *willistoni* subgroup is composed of six sibling species: *D. willistoni, D. equinoxialis, D. tropicalis, D. insularis, D. pavlovskiana* and *D. paulistorum.* The latter is actually a species complex. *Drosophila willistoni* was described as *Drosophila pallida* (Williston, 1896). Since this nomenclature was already used, Sturtevant (1916) changed it to *D. willistoni.* Dobzhansky and Pavan (1943) found two *willistoni*-like species, one more common and slightly smaller than the other. They believed that the more common species was *D. willistoni* and nominated the larger and less frequent species as *D. paulista.* Later, the authors perceived that, in fact, *D. paulista* was a synonym of the *D. willistoni* and, since then, the smaller species was nominated *D. paulistorum* (Dobzhansky and Pavan in Burla *et al.,* 1949).

A few years later, three new siblings were described: *D. equinoxialis* (Dobzhansky, 1946), *D. tropicalis* (Burla and Cunha, 1949 in Burla *et al.,* 1949) and *D. insularis* (Dobzhansky, 1957 in Dobzhansky *et al.,* 1957). Also, Townsend (1954) found that *D. tropicalis* comprises two subspecies, *tropicalis* and *cubana*. *Drosophila tropicalis tropicalis* presents a southern distribution and the northernmost register is in Rio Branco, Brazil; while *D. tropicalis cubana* is a northern form and the southernmost register is in Jamaica (Townsend, 1954).

In 1959, Dobzhansky and Spassky discovered that *D. paulistorum* was not a unique species, but a cluster of six incipient species – Amazonian, Andean-Brazilian, Centroamerican, Guianan, Orinocan and Transitional. The denominated Guianan was

posteriorly elevated to species, *D. pavlovskiana* (Kastritsis and Dobzhansky, 1967) and another incipient species denominated Interior was added to *D. paulistorum* cluster by Pérez-Salas *et al.* (1970).

Similar to *D. tropicalis,* it was discovered that *D. willistoni* and *D. equinoxialis* also present subspecies. Ayala (1973) observed that populations of *D. willistoni* from Lima, Peru presented incipient reproductive isolation from flies collected in Colombia, Venezuela, Trinidad and Brazil (*D. willistoni willistoni*) and were assigned as a new subspecies, *D. willistoni quechua.* Populations of *D. equinoxialis* from Hispaniola, Puerto Rico and Costa Rica exhibited incipient reproductive isolation from flies from Panama and Continental South America. These subspecies were denominated *caribbensis* and *equinoxialis*, respectively.

Concerning the geographic distribution of *D. willistoni* and its siblings, Spassky *et al.* (1971) compiled previously published data and also added new information. This study was updated by Dobzhansky and Powell (1975) and Ehrmann and Powell (1982). Since then, these species have been collected in several new localities. Therefore, the objective of this study was to update the distribution map of *D. willistoni* subgroup, including *D. paulistorum* species complex.

MATERIAL AND METHODS

We gathered all distribution records in literature for each species and its synonyms of the *willistoni* subgroup. We searched all distribution records compiled in Taxodros and verified the data in the original material. We checked the geographic coordinates using Google Maps and plotted those in maps using QGis 2.10.1 software. All distribution records, coordinates and respective references are available in Taxodros (taxodros.uzh.ch) (Bächli, 2015).

RESULTS AND DISCUSSION

The *willistoni* subgroup is almost entirely Neotropical, except for the occurrences in USA and North Mexico.

Drosophila willistoni has the broadest distribution of this subgroup, spanning from Florida, Mexico and Caribbean Islands, in North America, to Argentina and Uruguay, in Southern South America (Figure 1A). This species has been reported in most of South America, except in Paraguay and Chile, while also being found in Galapagos Islands. The northernmost records for *D. willistoni* are in Hawaii and in Crater Lake, Oregon, USA.

Drosophila tropicalis occurs in Florida, Mexico, Caribbean Islands, Central America, North and central South America. The southernmost registered locality for

this species is São José do Rio Preto, in São Paulo state, Brazil (Figure 1B). *Drosophila equinoxialis* has a geographic distribution very similar to *D. tropicalis,* living in sympatry in the major part of its territory (Figure 1B).

Two species of this subgroups have a very restrict distribution. *Drosophila insularis,* endemic of the Antilles, was founded in five localities: Guadeloupe, Monkey Hill, Montserrat, Saint Kitts and Saint Lucia (Figure 1B). *Drosophila pavlovskiana* occurrence was registered in Apoteri and Georgetown, in Guyana, and in Ocamo, Porto Ayacucho and Rancho Grande, in Venezuela (Figure 1B). *D. pavlovskiana* has not been recently collected.

Regarding *Drosophila paulistorum* cluster, we can observe an interesting aspect. When considering *D. paulistorum* occurrences, not specifying the semispecies (Figure 2A), there are much more registers, and, consequently, the living area seems to be wider than when we indicate the semispecies (Figure 2B). *Drosophila paulistorum* has a highly similar distribution to *D. willistoni,* although not occurring in Uruguay and Argentina (Figure 2A). The southernmost records of *D. paulistorum* are in Porto Alegre, Rio Grande do Sul state, Southern Brazil.

Considering the semispecies of *D. paulistorum,* Andean-Brazilian is the most widespread, occurring in several localities of Brazil, Equador, Peru, Colombia and Venezuela (Figure 2B). This semispecies occurs alone in the largest part of its distribution, although it lives sympatrically with Amazonian, Orinocan or Interior along the Amazon river and the upper Orinoco.

Drosophila paulistorum Centroamerican has been found in Tical (Colombia), Lancetilla (Honduras), San Salvador (El Salvador), Turrialba (Costa Rica) and

Boquete, Almirante and the Central area of Panama (Figure 2B). In central Panama, *D. paulistorum* Orinocan occurs together with Amazonian and Orinocan (Figure 2B).

The Transitional semispecies presence was reported in Chocó Condoto, Santa Marta and Valle, in Colombia and in Perija, Vigia, Barinas, Sarare, Rancho Grande and Guatopo, in Venezuela (Figure 2B). It occurs together with Orinocan in Santa Marta and with Andean-Brazilian and Amazonian in Northern Venezuela (Figure 2B).

The Amazonian semispecies spans from Panama and Trinidad to Colombia, Venezuela, Guyana and Northern Brazil (Figure 2B). It lives in sympatry with Andean-Brazilian, Interior, Orinocan and Transitional in several localities (Figure 2B).

The Orinocan semispecies occur in Panama, Trinidad, Colombia, Venezuela and Guyana, mainly in the Caribbean coast (Figure 2B). The Interior semispecies distributes in Colombia, Venezuela and Northern Brazil. These semispecies had never been found together (Spassky *et al*., 1971).

Most of the species of the *willistoni* subgroup lives in sympatry in Colombia, Venezuela, Ecuador, Bolivia and Northern Brazil, while we can observe a large empty patch in central Brazil, Paraguay and Argentina (Figures 1A-B, 2A-B). This species also has not been reported in Chile, which might be explained by the presence of a great barrier: The Andes chain. This observation raises some questions: It is possible that none of this species really lives in this area? This could be an artifact because of a lack of studies in these specific areas or the species are not properly identified?

It has been reported that the species identification in this subgroup is difficult, due to the morphological similarity between them. Although many authors have published ecological studies with *D. willistoni* subgroup, only a few studies presented the identification at specific level. Many attempts to identify these species have been

made, using different approaches, such as morphological studies, allozymatic assays, crossing tests and chromosomic analysis (Burla *et al.,* 1949; Malogolowkin, 1952; Spassky, 1957; Dobzhansky and Spassky, 1959; Pasteur, 1970; Ayala *et al.,* 1970; Richmond, 1972; Ayala and Powell, 1972; Garcia *et al.,* 2006; and review of the main results in Ehrmann and Powell, 1982 and in Cordeiro and Winge, 1995). Recently, Zanini *et al.* (2015) has shown that it is possible to identify all species of the *willistoni* subgroup and even *D. paulistorum* incipient species based on morphological characters of the male genitalia, so this study could be helpful in further research.

The most reasonable explanation for those empty spaces is, in fact, a combination of not enough studies encompassing those areas and the misidentification or lack of identification of the *willistoni* subgroup members. Further studies and a more accurate identification of these species are necessary in order to improve its distribution records.

Acknowledgements:

We thank to CNPq, CAPES and FAPERGS for the scholarships and grants.

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FIGURE CAPTIONS:

Figure 1. Geographic distribution of the *willistoni* subgroup.

A. Distribution of *Drosophila willistoni*

C. Distribution of *Drosophila tropicalis, Drosophila equinoxialis, Drosophila insularis* and *Drosophila pavlovskiana*

Figure 2. Geographic distribution of the *willistoni* subgroup.

A. Distribution of *Drosophila paulistorum.*

B. Distribution of *Drosophila paulistorum* semispecies Amazonian, Andean-Brazilian, Centroamerican, Interior, Orinocan and Transitional.

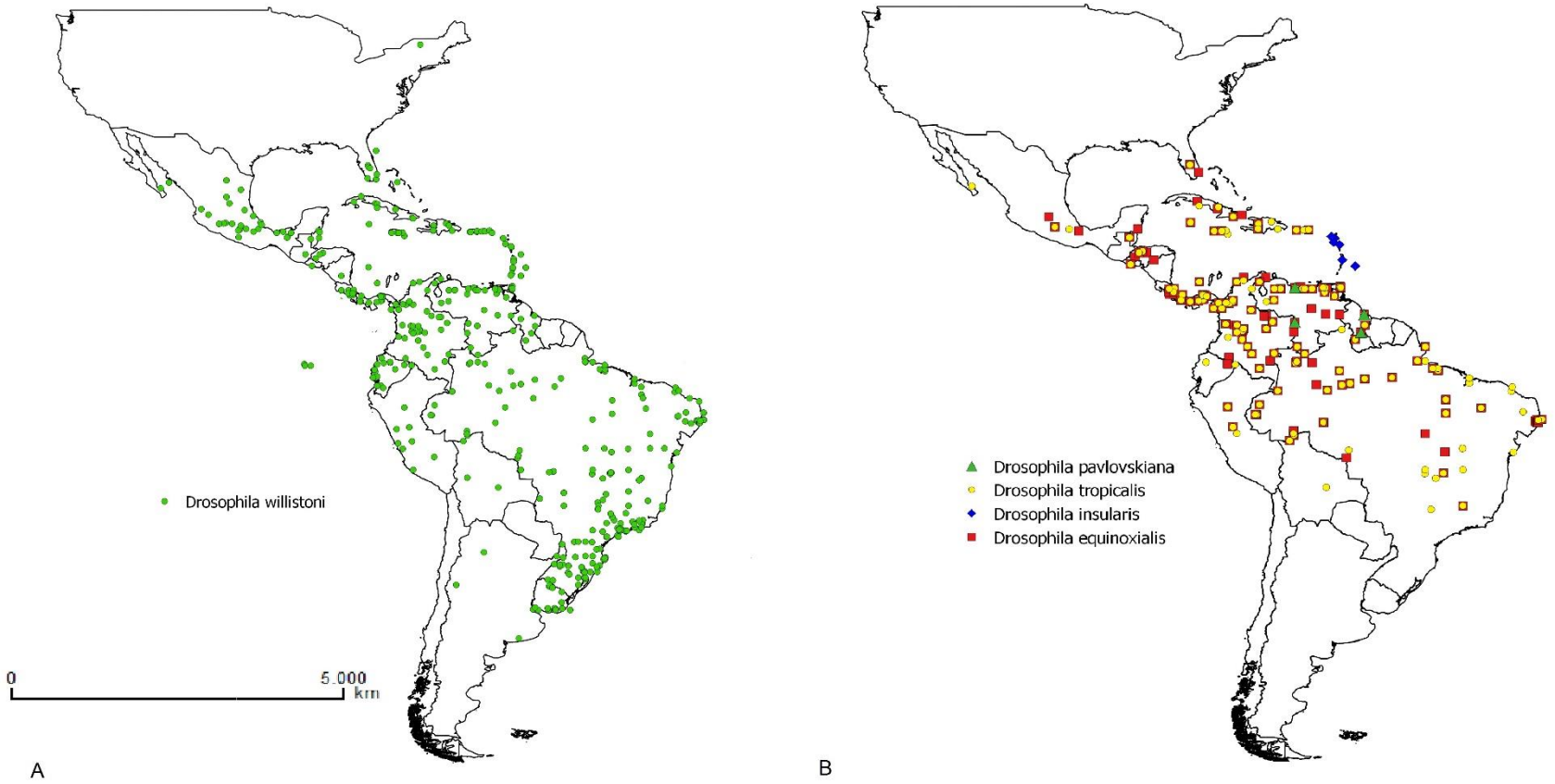
Drosophila willistoni
0
5.000
km
A
Drosophila pavlovskiana
Drosophila tropicalis
Drosophila insularis
Drosophila equinoxialis
B

Figure 1.


Drosophila paulistorum
0
5.000
km
A

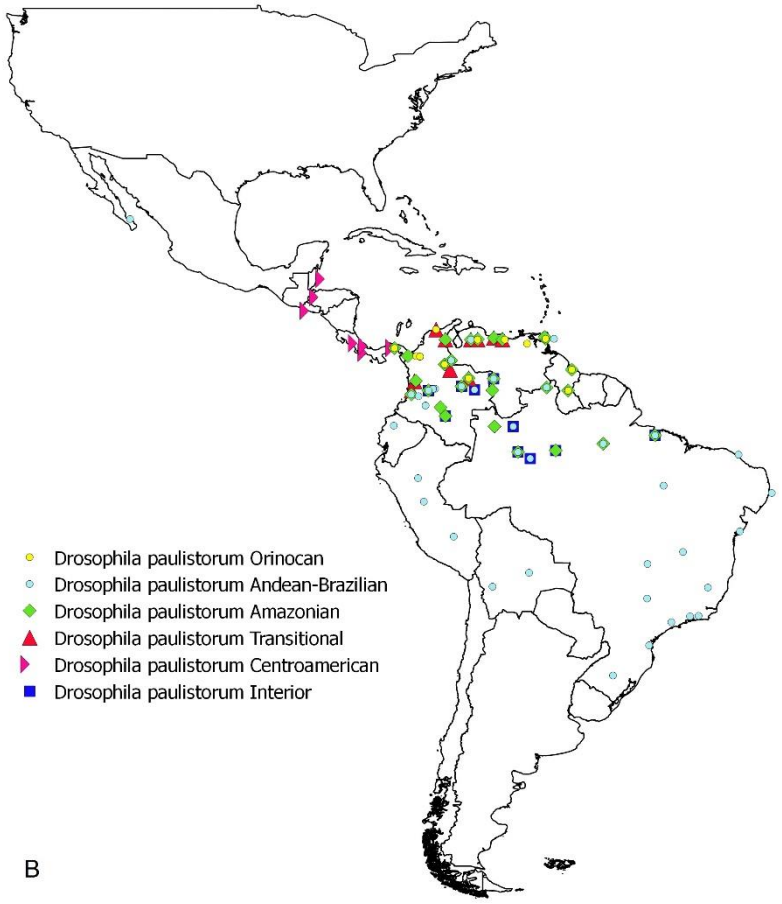
Drosophila paulistorum Orinocan
Drosophila paulistorum Andean-Brazilian
Drosophila paulistorum Amazonian
Drosophila paulistorum Transitional
Drosophila paulistorum Centroamerican
Drosophila paulistorum Interior
B

Figure 2.

CAPÍTULO III – On the geographic distribution of the *Drosophila willistoni* group (Diptera, Drosophilidae) – updated distribution of *alagitans* and *bocainensis* subgroups

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On the geographic distribution of the *Drosophila willistoni* group (Diptera, Drosophilidae) – updated distribution of *alagitans* and *bocainensis* subgroups.

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Artigo aceito para publicação no periódico *Drosophila Information Service* 98, 2015.

INTRODUCTION

Drosophila willistoni group (Diptera, Drosophilidae) is a Neotropical species group and is currently composed of 24 species distributed in three subgroups – *alagitans, bocainensis* and *willistoni* (Bächli, 2015).

The *alagitans* species group was established by Patterson and Mainland (1944), which reunited *D. alagitans* and *D. capnoptera* in a species group belonging to subgenus *Drosophila* (Wheeler and Magalhães, 1962). Later, Hsu (1949) observed that the male genitalia of these species were resemblant to the *willistoni* species group and the *alagitans* group was later transferred to subgenus Sophophora (Wheeler, 1949). Wheeler and Magalhães (1962) described two new species for this subgroup – *D. megalagitans* and *D. neoalagitans.*

Carson (1954) observed that three distinct specimens were designated as *D. bocainensis*; one of them was confirmed as *D. bocainensis* and the others redescribed as *D. parabocainensis* and *D. bocainoides,* establishing the *bocainensis* species complex (Carson, 1954). Salzano (1956) also reviewed the status of this species. Wheeler and Magalhães (1962) described some species belonging to this subgroup – *D. changuinolae, D. pseudobocainensis* and *D. parabocainoides.* The later were allocated as *D. subinfumata* synonym (Vilela and Bächli, 1990).

Drosophila mangabeirai was inserted in *melanogaster* group (Malogolowkin, 1952); its male genitalia, however, is compatible with those of *D. willistoni* group, to where this species was later transferred (Carson *et al.,* 1957). The *bocainensis*

subgroup also encompasses *Drosophila capricorni, D. fumipennis, D. nebulosa* and *D. sucinea.*

Later, Vilela and Bächli (1990) redescribed *D. abregolineata, D. fumipennis* and *D. subinfumata.* The authors also described *D. pittieri* (Bächli and Vilela, 2002). Recently, Figuero and Rafael (2013) described *D. neocapnoptera* in Ecuador. Both new reported species belong to *alagitans* subgroup.

Wheeler and Magalhães (1962) provided a distribution map of some *alagitans-bocainensis* species and asserted that the species fall into two clusters: northern distribution (*alagitans*-like forms) and Southern distribution (*bocainensis*-like forms). The geographic range of *D. nebulosa* was discussed in Ehrmann and Powell (1982).

The objective of this study was to update the current distribution map for *alagitans* and part of *bocainensis* subgroup as well as provide distribution maps for the species not included in the previously available map.

MATERIAL AND METHODS

We gathered all distribution records for each species of *alagitans* and *bocainensis* subgroup in literature and plotted those in maps using QGis 2.10.1 software. All entries and respective references are available in Taxodros (taxodros.uzh.ch) (Bächli, 2015).

RESULTS AND DISCUSSION

The *alagitans* subgroup distribution is restricted to Northern South America, Central and North America and Caribbean Islands (Figure 1A). The exception is *D. alagitans,* which is founded in some localities in Mexico and in Santa Catarina State, in Southern Brazil. *D. capnoptera* has the broadest distribution in this subgroup, ranging from Mexico through Central America countries. *D. neoalagitans* occurs in Jamaica and Hispaniola islands. The remaining species only have one distribution entry – *D. pittieri* in Rancho Grande, Northern Venezuela; *D. neocapnoptera* in Baeza-Teno, Ecuador and *D. megalagitans* in Bucaramanga, Colombia.

Regarding *bocainensis* subgroup, *D. nebulosa* seems to have the widest distribution (Figure 1B). The southernmost point of occurrence is in Grutas, Argentina and the northernmost is in Chatham, Canada. This species is also reported in USA, Mexico, Costa Rica, El Salvador, Panama, Colombia, Venezuela, Peru, Ecuador, Chile, Brazil, Uruguay, Galapagos Islands, Antilles, Hispaniola, Cuba and Bahamas. *D. sucinea* occurs in Northern South America, Central America and Mexico (Figure 1B) and lives in sympatry with *D. nebulosa* in Mexico, Panama, Ecuador and Peru.

Drosophila fumipennis distributes since Central America, through Colombia, Venezuela and Peru to Brazil, from North to South (Figure1C). *D. capricorni* has a similar distribution, except that it occurs in Mexico and is very uncommon in Northern and Northeast areas of Brazil (Figure 1C).

Some species have a very narrow known distribution - *D. abregolineata* was only found in the type locality, Turrialba (Costa Rica) (Figure 2A); *D. changuinolae*

occurs in the type locality Changuinola (Panama), in Leticia (Peru) and in Barreiro Rico (Brazil) (Figure 2B).

Drosophila mangabeirai is mostly found in Central America, but also occurs in Salvador, Brazil and in Antilles (Figure 2A). *D. bocainensis* is also found in South and Southeast Brazil, Colombia, Ecuador, Venezuela and Honduras (Figure 2A); *D. bocainoides* also lives in South and Southeast Brazil in a more restricted distribution.

Drosophila subinfumata presents a very discontinuous distribution, since inhabits South and Southeast Brazil, Panama and Costa Rica (Figure 2B). *D. pseudobocainensis* is found in Northern South America (Colombia, Bolivia and Venezuela) and in Central America (Costa Rica, El Salvador and Panama) (Figure 2B) whereas *D. parabocainensis* distributes in South and Southern Brazil and Colombia (Figure 2B)

Central America, Colombia, Venezuela, Ecuador and Southeast Brazil are the areas where most of the species of *willistoni* group lives in sympatry. The most widespread species are *D. nebulosa, D. capricorni* and *D. fumipennis.* Some species, especially belonging to *alagitans* subgroup, have a few or sometimes a single occurrence, which lead us to wonder if there is a lack of studies, the species are misidentified/ unidentified or these taxa have a very narrow or endemic distribution.

Aknowledgments:

We thank CNPq, CAPES and FAPERGS for the scholarships and grants.

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FIGURE CAPTIONS:

Figure 1. Geographic distribution of the *alagitans* and *bocainensis* subgroups.

A. Subgroup *alagitans* (*D. alagitans, D. capnoptera, D. megalagitans, D. neoalagitans, D. neocapnoptera* and *D. pittieri*).

B. Subgroup *bocainensis* (*D. nebulosa* and *D. sucinea*).

C. Subgroup *bocainensis* (*D. capricorni* and *D. fumipennis*)

Figure 2. Geographic distribution of the *bocainensis* subgroup.

A. *D. abregolineata, D. bocainensis, D. bocainoides* and *D. mangabeirai.*

B. *D. changuinolae, D. parabocainensis, D. pseudobocainensis* and *D. subinfumata.*

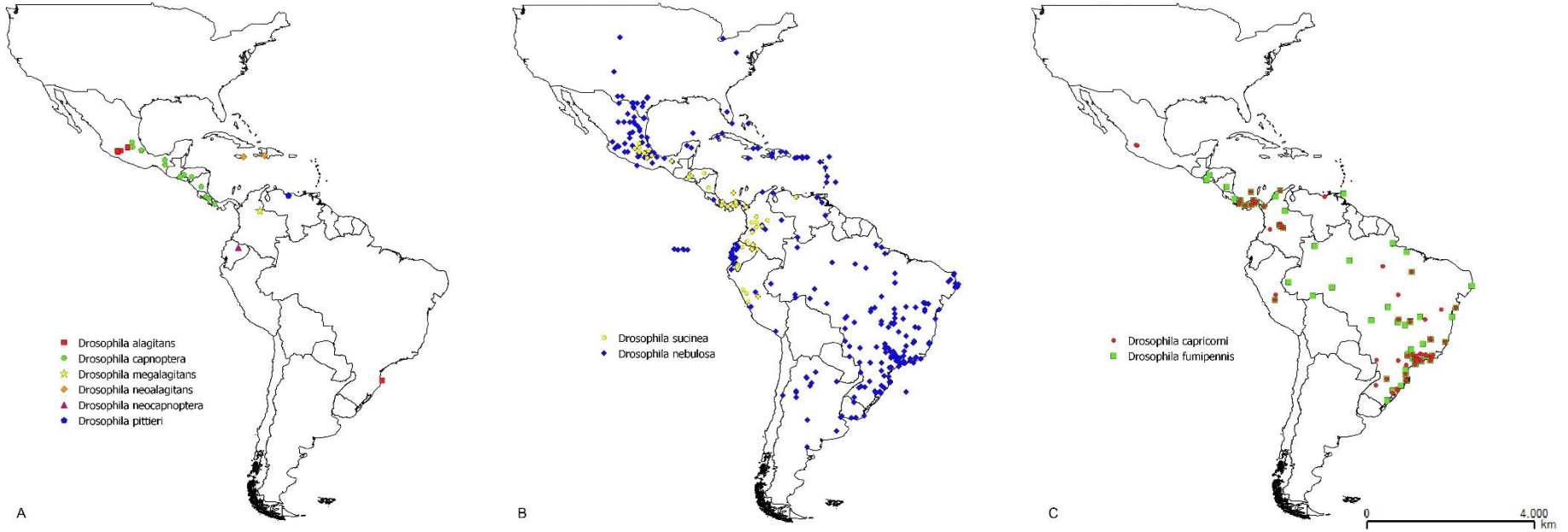
Drosophila alagitans
Drosophila capnoptera
Drosophila megalagitans
Drosophila neoalagitans
Drosophila neocapnoptera
Drosophila pittieri
A
Drosophila sucinea
Drosophila nebulosa
B
Drosophila capricorni
Drosophila fumipennis
C
0
4.000
km

Figure 1.

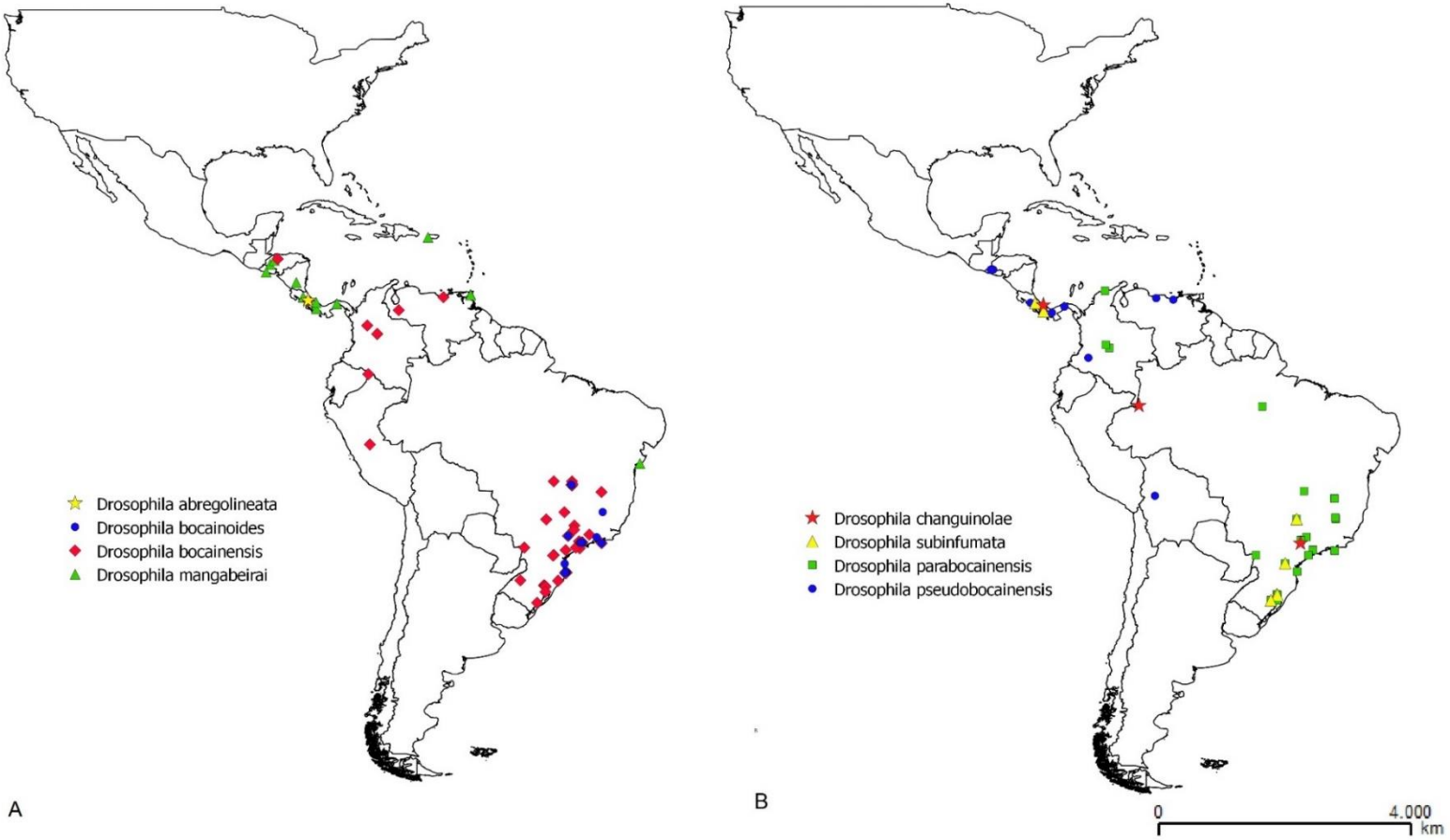
Drosophila abregolineata
Drosophila bocainoides
Drosophila bocainensis
Drosophila mangabeirai
A
Drosophila changuinolae
Drosophila subinfumata
Drosophila parabocainensis
Drosophila pseudobocainensis
B
0
4.000
km

Figure 2.

CAPÍTULO IV – Ultrastructural characterization of the pre-adult stages of Drosophila willistoni species group (Diptera, Drosophilidae)

Ultrastructural characterization of the pre-adult stages of *Drosophila willistoni* species group (Diptera, Drosophilidae)

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Artigo submetido ao periódico Trends in Entomology.

ABSTRACT

Drosophila willistoni group is a Neotropical model for evolutionary studies and has been explored since the latter half of the past century. The *willistoni* group encompasses 24 species divided into three subgroups: *alagitans, bocainensis* and *willistoni.* This group has a very interesting evolutionary and taxonomic history, represented by the different speciation levels of its species – some seem to be in process of speciation whereas others already have completed it. Despite the amount of studies with this group, there is a lack of morphological studies, especially concerning the pre-adult stages of development. Thus, the objective of this study was to characterize the external morphology of the eggs, larvae and pupae of the *Drosophila willistoni* species group based on Scanning Electron Microscopy. We verified that the morphology of this immature stages are very similar between the analyzed species. The respiratory filaments of the eggs, however, present variation even between the semispecies of the *D. paulistorum* complex. This morphological similarity is compatible with the relatively recent process of speciation and the pattern of substrate exploration.

Keywords: eggs, larvae, pupae, *Drosophila,* Neotropical

INTRODUCTION

The *Drosophila willistoni* species group (Diptera, Drosophilidae) is a Neotropical member of the *Sophophora* subgenus [1,2]. This group includes, in addition to *Drosophila willistoni* STURTEVANT 1916, another 23 species divided into *alagitans, bocainensis* and *willistoni* subgroups [3]. The *willistoni* group is known for its complex evolutionary history, characterized by the presence of distinct taxonomic levels – species, subspecies and semispecies [4,5]. Studies analyzing morphological characteristics of early developmental stage are still scarce. The study conducted by Kambysellis [6] is one of the main references in the characterization of the eggshell of Drosophilidae. In this study, the author compared the chorionic morphology of several Drosophilidae species, including *D. willistoni* and *D. paulistorum* DOBZHANSKY AND PAVAN 1949, and attempted to infer phylogenetic relationships based on eggshell characters. Markow *et al.* [7] presented a comparative study regarding the egg size and time of embrionary development of *D. willistoni* and the another species with fully sequenced genome.

Okada [8] characterized eggs, larvae and pupae of several genus and species of Drosophilidae and created an identification key based on pre-adults' characters.

The early stages of development of some species of this group were briefly characterized by Dobzhansky [9], who described superficially the external morphology of eggs and pupae of *D. equinoxialis* DOBZHANSKY 1946; Burla *et al*. [10] characterized eggs and pupae of *D. tropicalis* BURLA E CUNHA 1949 and *D. paulistorum*; Kastritsis e Dobzhansky [11] also described the general shape of eggs and pupae of *D.*

pavlovskiana KASTRITSIS AND DOBZHANSKY 1966; Dobzhansky *et al.* [12] highlighted the similarity between the eggs of *D. insularis* DOBZHANSKY 1957 and *D. willistoni.*

Several studies have aimed to unveil the formation routes for chorionic appendixes in *Drosophila* eggs, as well as the pathways that result in the patterns of cuticular denticles and trichomes in larvae. Even though several studies dealt with immature stages of *Drosophila,* only a few studied their external morphology, and therefore the aim of this study was to describe comparatively the external morphology of eggs, larvae and pupae of species in the *willistoni* group, based on scanning electron microscopy (SEM) analysis which could later result in evolutive studies.

MATERIAL AND METHODS

Fly stocks

The stocks were reared in a cornmeal medium [13], at constant temperature and humidity (17±1ºC; 60% rh), with controlled photoperiod. All species and strains used in this study are listed in Table 1.

At least 50 eggs and 10 3rd instar larvae and pupae of each species were observed. Were analyzed two strains of *D. paulistorum* Andean-Brazilian and five strains of *D. willistoni* from different localities, in order to verify intraspecific variation. Only one strain of each remaining species were analyzed.

Collecting eggs, larvae and pupae

For the egg collection, flies were transferred to vials containing oviposition medium [14], were they were kept during 3 hours. After this period, eggs were manually collected with a histological needle and stored at 4°C for 7 days in a fixative solution (12% Glutaraldehyde 25%, 50% Phosphate Buffer pH 7.4 and 38% distilled water).

Larval and pupal stages were obtained directly from the stock, after transferring the adults to another vial. Larvae were 3rd instar (approximately seven days after fertilization) and pupae 8-12 days after fertilization.

SEM preparation and visualization

Previously fixated eggs were washed three times in a 1:1 Phosphate Buffer (pH 7.4) and distilled water solution, 30 minutes each wash. Next, eggs were dehydrated with acetone washes in the following concentrations: 30%, 50%, 70%, 90% (2X), 100% (2X), for 10 minutes each. Finally, the eggs were critical point dried in a BALZERS CPD030 using CO_2 acetone 100% as a transition fluid.

Larvae and pupae were not fixated nor critical point dried. Eggs, larvae and pupae were mounted in stubs with carbon tape and metalized with gold in a BALZERS SCD050 sputter coater. Visualization and image capture were made in a JEOL JSM6060 scanning electron microscope, in Centro de Microscopia Eletrônica - UFRGS.

Images were processed with Adobe Photoshop CC 2015.

The terminology used in this study followed Kambysellis [6] (eggs), Wipfler *et al.* [15] (larvae) and Okada [8] (pupae).

RESULTS AND DISCUSSION

Eggs

The eggs of analyzed species of the *willistoni* group are whitish oblong structures, approximately 0.5mm long, covered with hexagonal follicular imprints (Figure 1A-B). The posterior area of the egg (posterior pole) presents follicular cells modified in porous (Figures 1C-D). The anterior pole presents two respiratory filaments, shorter than total egg length, distally expanded, projecting from the anterior dorsal surface (Figure 1A). The shape of these filaments is subtly different between the analyzed species (Figure 2 A-L). The number of respiratory filaments were previously reported for some of the analyzed species [6, 9, 10, 11]. According to Kambysellis [6], the number of respiratory filaments varies among the genus, but tends to be more consistent among each subgenus. The author also stated that all the species of *Sophophora* subgenus have just one pair of equally sized filaments. The presence of two respiratory filaments is pointed by Okada [8] as a diagnostic character of the eggs of *Sophophora* subgenus.

Presence, number and size of respiratory filaments are directly linked to the type of substrate used by each species as an oviposition site [6]. The species of *willistoni* subgroup usually oviposit in fallen and rotten fruits, leaving the respiratory filaments and eventually the micropyle in open air. The distal expansion observed in the

respiratory filaments of the eggs of this group (Figures 1A, 2A-L) seems to be befitting with the oviposition behavior, since it increases the available gas exchange surface and aids the egg anchor to the substrate.

The operculum and micropyle are prominent chorionic structures and are located in the anterior region of the egg. The operculum is the chorionic region surrounded by the basis of respiratory filaments, where the larvae emerges. The operculum presents a reticulated surface, which became more globular with the proximity of larvae emergency (Figure 1F). The micropyle is a chorionic structure where the spermatozoa penetrate the egg. This structure is situated in the anterior region of the operculum and is not covered with follicular imprints (Figures 1E, 1F). These structures do not show variation between the analyzed species.

Kambysellis [6] reported differences in the follicular imprints: in some species, they are tall and lacy and in other species, they are short and narrow. The follicular imprints of *D. willistoni* species group are short and narrow and do not exhibit differences between the species.

Larvae

Third instar larvae of *willistoni* group reach 3-4mm in length (Figure 3A) and the cuticle shows little sclerotization. The body is composed of a pseudocephalon, three thoracic segments and eight abdominal segments (Figure 3A). The thoracic and abdominal segments do not present legs or protolegs (Figure 3A).

The cephalic capsule is reduced and internalized (Figures 3A and 3C). The pseudocephalon region presents a pair of maxillary sensorial organs and a pair of

antennal organs (Figure 3C). The antennal organs are globular structures and are located between the pair of sensorial organs. The sensorial organs are composed of two sets of sensillae, surrounded by a ring shaped cuticular salience (Figure 3C). The larvae present a pair of sickle-shaped, serrated, heavily sclerotized oral hooks in the ventral region (Figure 3B). The larvae of *willistoni* group presents well-developed oral hooks, compared to those of *D. melanogaster,* shown in Wipfler *et al.* [15]. In the oral hook area, we also can observe the cirri, organized in 6-8 parallel rows (Figures 3B, 3C, 3H).

The thoracic segments and the seven first abdominal segments are very similar; each segment present a transversal band composed of several irregular stripes of cuticular denticles or spines (Figure 3G). This transversal bands, in *willistoni* group, exhibit triangular cuticular denticles and spines and do not present trichomas (Figure 3G), as can be observed in some species of *melanogaster* group [16]. This pattern founded in *willistoni* group is similar to those of *D. sechellia* [16] and do not present visible differentiation between the analyzed species, contrasting with *melanogaster* subgroup, in which patterns are species-specific.

The last abdominal segment (A8), the anal segment, is distinctly different from the other segments. This segment is covered with microthrichia, except in the anal organs and in the spiracular tubes (Figures 3A, 3D-F). Anal organs or anal pads are oblong oval structures, with a transversal furrow in the central area (Figures 3A, 3D-F). The anus is located between the anal organs, both of which are in the ventral face of the larvae (Figures 3A, 3D-F).

The abdominal spiracle is formed by spiracular tubes, which are two cylindrical structures connected to the anal segment through the peritrma and are surrounded by

six pairs of anal papillae (Figures 3A, 3D-F). Peritrema and spiracular tubes can be everted (Figure 3D) or retracted (Figure 3F). The spiracular tubes present a spiracular scar and spiracular openings, located laterally to the scar (Figure 3E). Two pairs of anal papillae are adjacent to anal organs and the remaining pairs surround the spiracles (Figures 3C and 3E).

Pupae

The pupae of *willistoni* group are approximately 2.5mm in length. The coloration varies from light brown, in the beginning of pupal stage, to dark brown, in the end. Pupae are elliptic shaped, slightly dorsal and ventrally convex in the anterior region (Figures 4A and 4C). The pupae also present bands of denticles and spines alternated with bands without cuticular ornamentations (Figure 4A, 4C, 4F, 4H).

The species in *willistoni* group present a pair of anterior spiracles, composed of eight ramifications covered with tiny spines (Figure 4D e 4G). According to Okada [8], the number of ramifications of the anterior spiracles is the main distinguishing feature of pupae when comparing different species. The anal segment of the pupae is very similar to that in the larvae, and it is possible to observe the anterior spiracles and anal papillae (Figures 4B, 4C. 4E and 4F).

As we observed for larvae, the external morphology of the pupae apparently does not vary among the *willistoni* group, even between the *bocainensis* and *willistoni* subgroups.

CONCLUSIONS

Coming from the obtained results, we can infer that the analysis of the immature stages can be useful to differentiate *willistoni* subgroup from other species groups. However, those cannot be considered diagnostic characters of species when analyzed alone, since the level of differentiation is very low and seems to be restrict to the distal portion of the respiratory filaments of the eggs.

Evidences of intraspecific variation were not found, the same way as Zanini *et al.* [17] did not find intraspecific variation in the characters of male genitalia of *willistoni* subgroup. Intraspecific variation of the morphological characters of the immature stages were tested for two species and extrapolated for the remaining. Considering that even the interspecific differences were subtle (based on the shape of respiratory filaments), it is unlikely that with more lineages in the analysis, intraspecific differences would be found. The *D. willistoni* lineages included were from many different points from the distribution and therefore a gradient of phenotypes should be evident in case of intraspecific differences, which did not occur. Furthermore, besides the lack of marked differences within the *willistoni* subgroup and between this group and the species analyzed from *bocainensis* subgroup, we cannot state that this pattern is also true for the rest of the *bocainensis* subgroup or the *alagitans* subgroup, since those two were not analyzed in their entirety. In a further study, the immature stages of the remaining species of the *bocainensis* subgroup and species of *alagitans* subgroup will be analyzed.

ACKNOWLEDGMENTS

We would like to thank CNPq, CAPES and FAPERGS for scholarships and grants. We thank all the collectors for the provided strains and we also thank Centro de Microscopia Eletrônica - UFRGS.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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Table 1. Species and strains of *Drosophila* used in this study.

Subgroup	Species	Semispecies	Locality	Colector
willistoni	*D. equinoxialis*		Apazapán/ México	Lee Ehrmann and Yong Kyu Kim
	D. insularis		St. Lucia/ Pequenas Antilhas	Jeffrey R. Powell
	D. paulistorum	Andino Brasileira	Florianópolis/ Brasil	Marco Gottschalk
		Andino Brasileira	Ribeirão Preto/ Brasil	Vera L. S. Valente
		Amazônica	Belém do Pará/ Brasil	Lee Ehrmann and Yong Kyu Kim
		Centroamericana	Lancetilla/ Honduras	Lee Ehrmann and Yong Kyu Kim
		Interior	Llanos/ Colômbia	Lee Ehrmann and Yong Kyu Kim
		Orinocana	Georgetown/ Guiana	Lee Ehrmann and Yong Kyu Kim
		Transicional	Santa Marta/ Colômbia	Lee Ehrmann and Yong Kyu Kim
	D. tropicalis		San Salvador/ El Salvador	Tucson Stock Center
	D. willistoni		Salvador/ Brasil (1)	Helga Winge e Antonio Cordeiro
			Guadalupe/ Caribe (2)	Tucson Stock Center
			Coronilla/ Uruguai (3)	Beatriz Goñi
			Apazapán/ México	Margaret Kidwell
			Ribeirão Preto/ Brasil	Cládia Rohde
bocainensis	*D. capricorni*		Porto Alegre/ Brasil	Maríndia Deprá and Brenda Alexandre
	D. fumipennis		Joinville/Brasil	
	D. nebulosa		UFPE	Cláudia Rohde
	D. sucinea		México	Stock Center

Figure captions:

Figure 1. Scanning electron microscopy of the eggs of the *Drosophila willistoni* species group.

A. Egg in dorsal view (*D. willistoni*); B. Follicular imprints (*D. tropicalis*)*;* C. Posterior pole (*D. paulistorum* Centroamerican); D. Aeropyles (*D. paulistorum* Centroamerican); E-F Operculum and micropyle (*D. willistoni* and *D. tropicalis,* respectively).

(MI) Micropyle; (RF) Respiratory filaments; (OP) Operculum

Figure 2. Scanning electron microscopy of the respiratory filaments of the eggs of *Drosophila willistoni* species group.

A. *D. equinoxialis;* B. *D. nebulosa;* C. *D. paulistorum* Amazonian; D. *D. paulistorum* Andean-Brazilian; E. *D. paulistorum* Centroamerican; F. *D. paulistorum* Interior; G. *D. paulistorum* Orinocan; H. *D. paulistorum* Transitional; I. *D. tropicalis*; J. *D. willistoni* (1); K. *D. willistoni* (2); L. *D. willistoni* (3).

Figure 3. Scanning electron microscopy of the larvae of *Drosophila willistoni* species group.

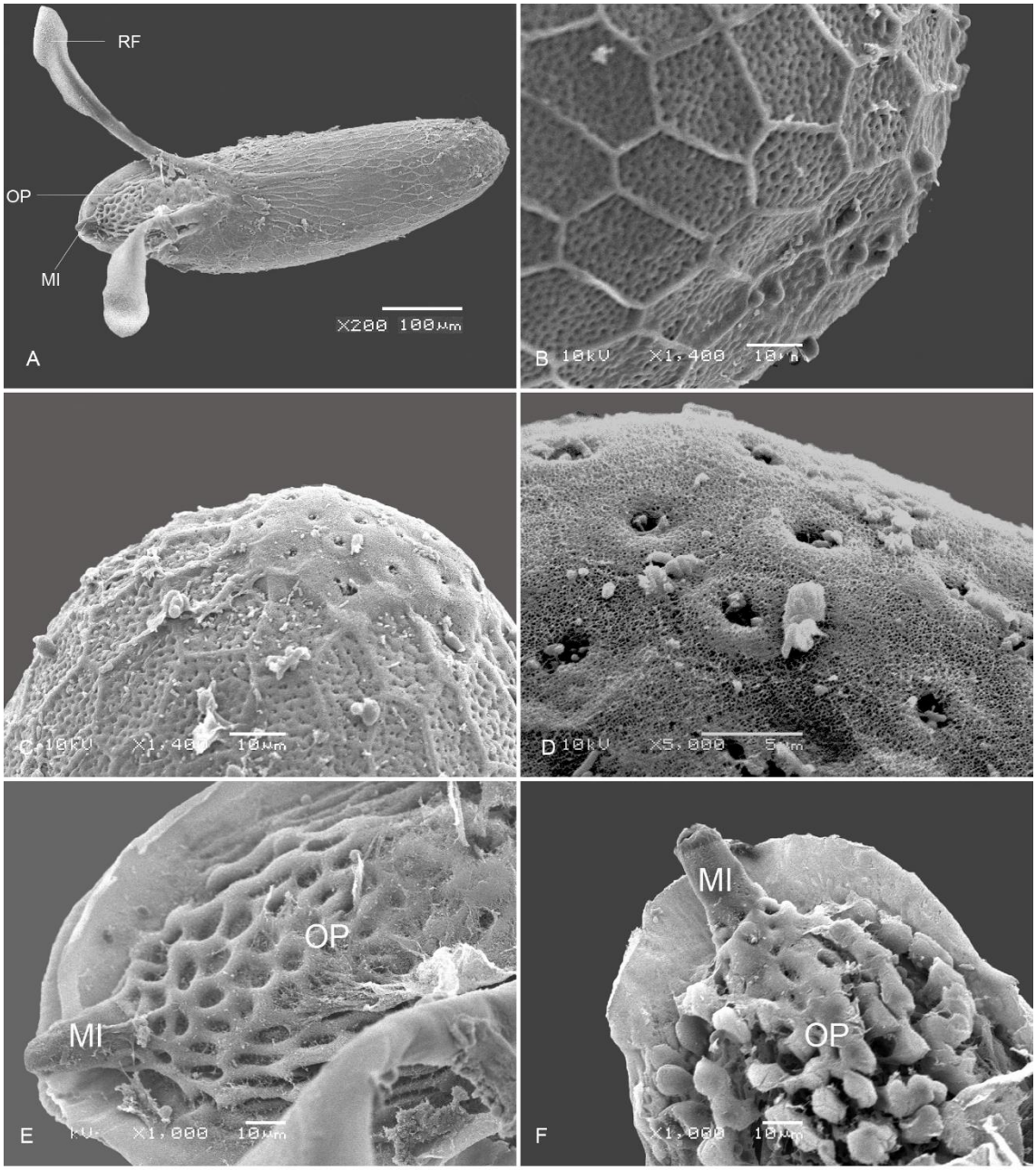
A. Larvae in lateral view (*D. sucinea*); B. Oral hooks (*D. paulistorum* Amazonian); C. Anterior spiracles and oral hooks (*D. paulistorum* Amazonian); D. VIII. Abdominal segment, lateroventral view (*D. insularis*). E. Posterior spiracles (*D. willistoni*); F. VIII. Abdominal segment, lateral view (*D. willistoni*); G. Cuticular denticles and hooks (*D. paulistorum* Amazonian); H. Cirri (*D. sucinea*).

(as) Abdominal spiracle; (pce) Pseudocephalon; (mh) Mouth hooks; (ci) Cirri; (ao) Antennal organs; (mso) Maxillar sensorial organs; (st) Spiracular tube; (pe) Peritrema; (ap) Anal papilla; (ano) Anal organs; (an) Anus; (so) Spiracular opening; (ss) Spiracular scar.

Figure 4. Pupae of *Drosophila willistoni* group.

A. Pupae in lateral view (*D. paulistorum* Andean-Brazilian); B.VIII Abdominal segment (D. paulistorum Andean-Brazilian); C. Pupae in ventral view (*D. paulistorum* Amazonian); D. Anterior spiracles (*D. capricorni*); E. Posterior spiracles, lateral view (*D. capricorni*); F. Pupae in dorsal (*D. paulistorum* Amazonian); G. Detail of anterior spiracles (*D. paulistorum* Interior); H. Cuticular denticles (*D. sucinea*).

(as) abdominal spiracle; (ans) anterior spiracle; (ap) anal papillae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OP
MI
X200 100µm
A
B 10kV X1,400 10µm
C 10kV X1,400 10µm
D 10kV X5,000 5µm
OP
MI
E kV X1,000 10µm
MI
OP
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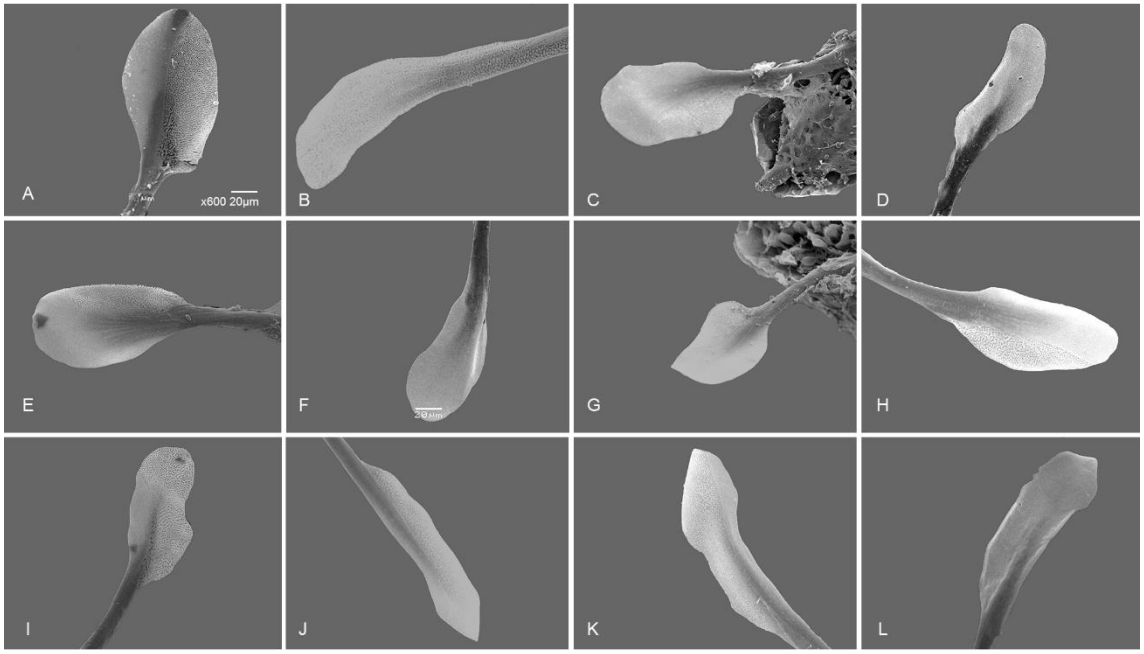
Figure 1.



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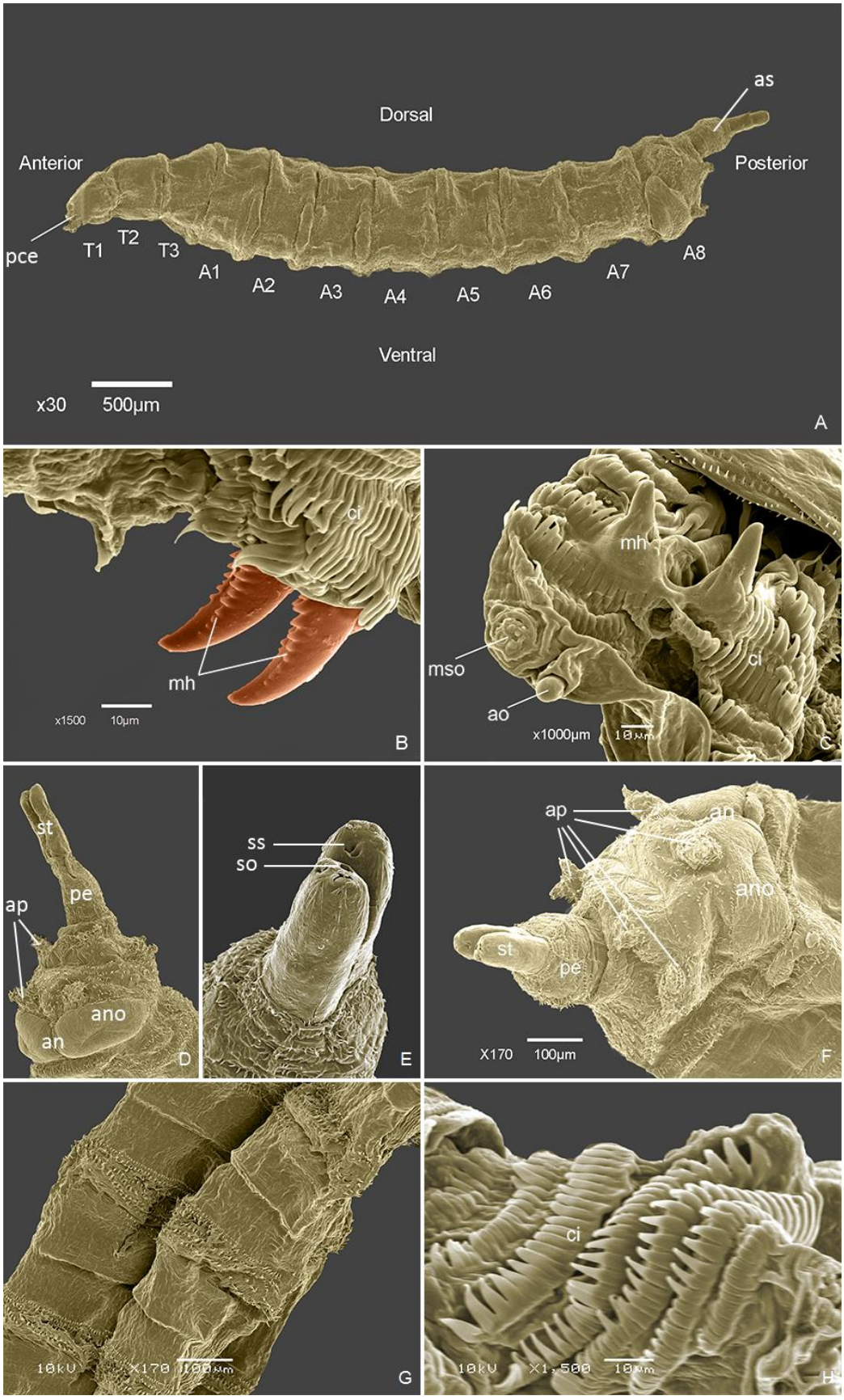
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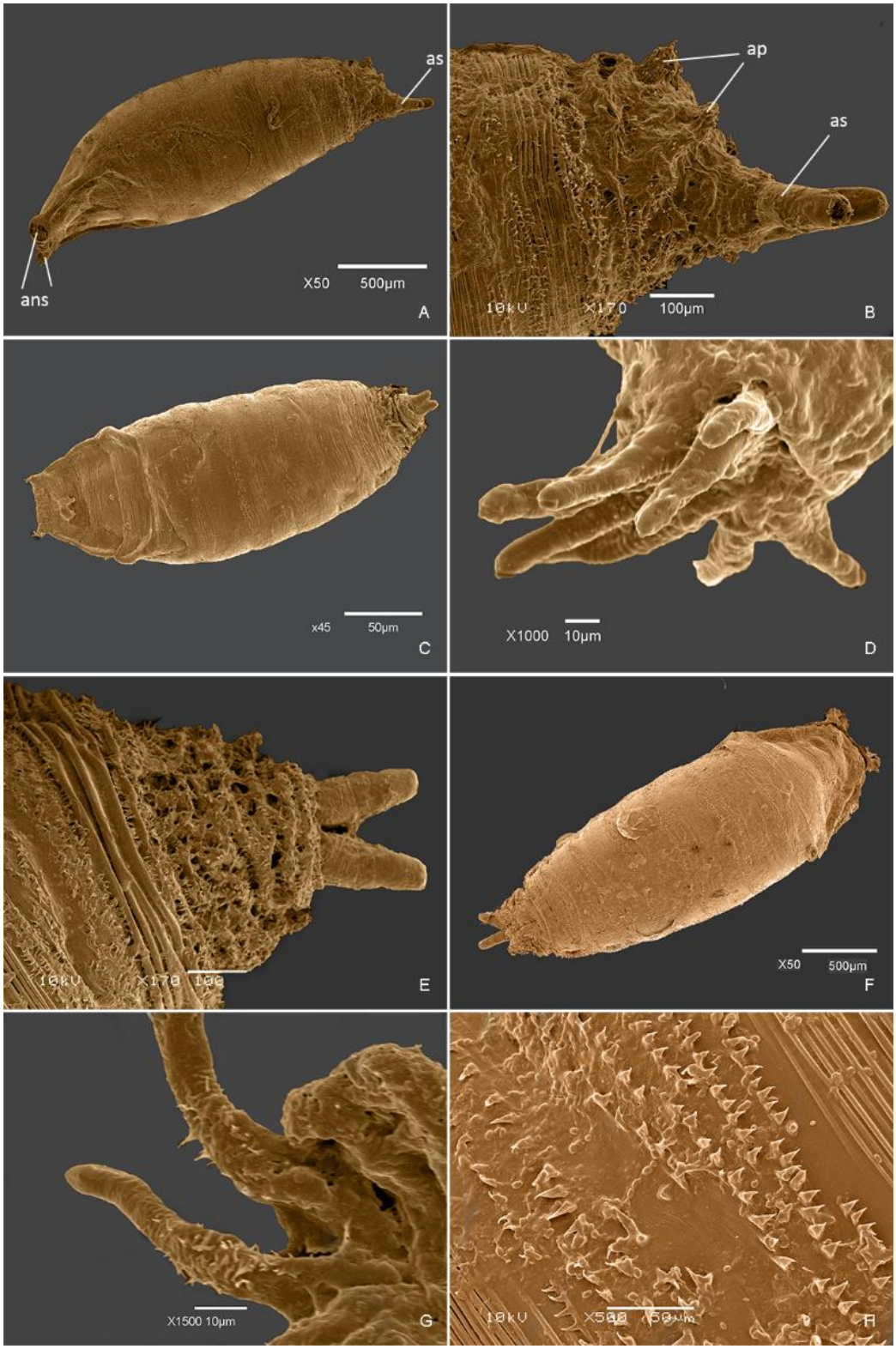
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10kV X500 50µm
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Figure 4.

CAPÍTULO V - Can sibling species of the *Drosophila willistoni* subgroup be recognized through combined microscopy techniques?

Can sibling species of the *Drosophila willistoni* subgroup be recognized through combined microscopy techniques?

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Artigo publicado na Revista Brasileira de Entomologia 59(4): 323-331.

Revista Brasileira de Entomologia 59 (2015) 323–331

REVISTA BRASILEIRA DE
Entomologia
A Journal on Insect Diversity and Evolution
www.rbentomologia.com

Systematics, Morphology and Biogeography

Can sibling species of the *Drosophila willistoni* subgroup be recognized through combined microscopy techniques?

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ARTICLE INFO

Article history:
Received 6 April 2015
Accepted 4 August 2015
Available online 9 October 2015
Associate Editor: Márcia S. Couri

Keywords:
Cryptic species
Drosophila willistoni subgroup
Drosophila paulistorum complex
Male genitalia
Semispecies

ABSTRACT

In several arthropod groups, male genitalia is the most important feature for species identification, especially in cryptic species. Cryptic species are very common in the *Drosophila* genus, and the Neotropical *Drosophila willistoni* species group is a good example. This group currently includes 24 species divided into three subgroups: *alagitans*, *bocainensis* and *willistoni*. There are six sibling species in the *willistoni* subgroup – *D. willistoni*, *D. insularis*, *D. tropicalis*, *D. equinoxialis*, *D. pavlovskiana* and *D. paulistorum*, which is a species complex composed of six semispecies – Amazonian, Andean-Brazilian, Centroamerican, Interior, Orinocan and Transitional. The objective of this study was to characterize male genitalia of the *willistoni* subgroup, including the *D. paulistorum* species complex, using scanning electron microscopy and light microscopy. We also tried to contribute to the identification of these cryptic species and to add some comments about evolutionary history, based on male genitalia characters. Despite being cryptic species, some differences were found among the siblings, including the *Drosophila paulistorum* semispecies.

Introduction

Male terminalia is one of the most important traits used to identify cryptic species, which are very common in the *Drosophila* Fallén genus. The Neotropical *Drosophila willistoni* species group is a good example of cryptic speciation. This group includes 24 species, which are divided into three subgroups: *alagitans*, *bocainensis* and *willistoni* (Bachli, 2015); the last of them showing various taxonomic levels with successive degrees of reproductive isolation (Robe et al., 2010). The *willistoni* subgroup includes six sibling species: *D. willistoni* Sturtevant, 1916, *D. equinoxialis* Dobzhansky, 1946, *D. insularis* Dobzhansky, 1957, *D. tropicalis* Burla and Da Cunha, 1949, *D. pavlovskiana* Katritsis and Dobzhansky, 1967 and *D. paulistorum* Dobzhansky and Pavan, 1949. These species are almost morphologically indistinguishable based on external morphology, exhibit varying degrees of premating isolation and usually do not cross-hybridize (Ehrmann and Powell, 1982). Within the subgroup, *Drosophila paulistorum* is a species complex, or also referred as a superspecies, composed of six semispecies (Dobzhansky and Spassky, 1959; Perez-Salas et al., 1970). The *willistoni* subgroup also shows taxonomic differentiation at the subspecies level: *D. willistoni* differentiates into the *willistoni* and *quechua* subspecies (Ayala and Tracey, 1973); *D. tropicalis* contains the *tropicalis* and *cubana* subspecies (Townsend, 1954); and *D. equinoxialis* is divided into the *equinoxialis* and *caribbensis* subspecies (Ayala et al., 1974).

According the review of Cordeiro and Winge (1995), the sibling group is still in an active process of speciation and all levels of this process can be observed. The authors suggest two steps of speciation: incipient isolation, represented by the subspecies. The second step of speciation is exemplified by the semispecies, which show several degrees of reproductive isolation ranging from complete isolation to the presence of fertile offspring (Cordeiro and Winge, 1995) which was observed in the crossings of the Transitional semispecies with the Andean-Brazilian and the Centroamerican semispecies (Ehrman, 1961, 1965).

D. willistoni has the broader distribution of the group (Fig. 1), spanning from Central Mexico and Florida to Southern Brazil and Northern Argentina, and from the Atlantic to the Pacific Ocean (Dobzhansky and Powell, 1975; Ehrmann and Powell, 1982), even in areas of human disturbance (Valiati and Valente, 1996). *D. willistoni* is uninterruptedly distributed over this area, except in deserts and high altitudes (Ehrmann and Powell, 1982). Other sibling species have narrower distributions within the distribution of *D. willistoni*, except *D. insularis*, which is endemic to Saint Kitts and Saint Lucia of the Antilles Islands (Dobzhansky et al., 1957), and *D. pavlovskiana*, which has been found only once in

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http://dx.doi.org/10.1016/j.rbe.2015.09.006

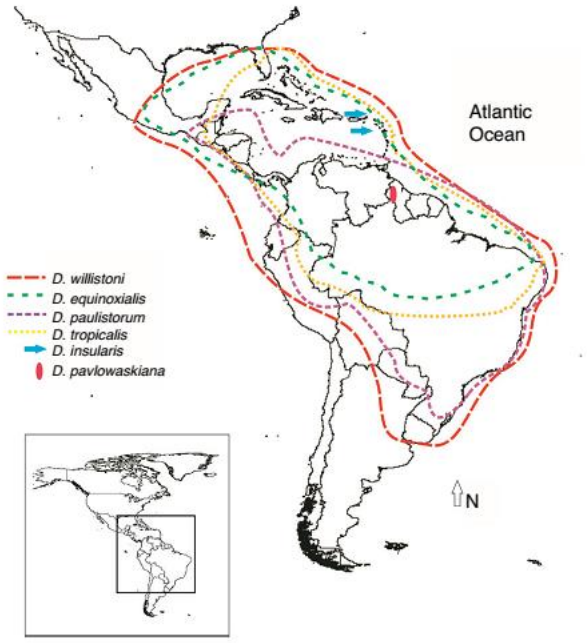

Fig. 1. Geographic distribution of the *D. willistoni* species subgroup, according to Spassky et al. (1971), Winge (1971), Dobzhansky and Powell (1975), Ehrmann and Powell (1982), Santos and Valente (1990).

Guyana (Spassky et al., 1971) and has not been collected since then (Fig. 1).

The *D. paulistorum* semispecies occur from Southern Brazil to Central America (Guatemala) and Trinidad (Dobzhansky and Spassky, 1959) (Fig. 2). According to Dobzhansky et al. (1964),

Fig. 2. Geographic distribution of the *D. paulistorum* superespecies, according to Spassky et al. (1971), Winge (1971), Dobzhansky and Powell (1975), Ehrmann and Powell (1982), Santos and Valente (1990).

when the semispecies' territories overlap, they apparently do not interbreed. Previous studies suggested that the differences among morphological, physiological and ecological traits within the semispecies are too small to distinguish each semispecies (Pasteur, 1970; Perez-Salas and Ehrmann, 1971) and could only be recognized by examination of the gene arrangements on their chromosomes (Kastritsis, 1967; Rohde et al., 2006) and by crossing tests (Perez-Salas and Ehrmann, 1971).

Despite being a traditionally studied group with an exciting evolutionary history, there is a lack of studies concerning the morphology in the early stages of development and also in adults. Some morphological studies of adults were made after the species were described. Burla et al. (1949) presented illustrations of maxillar palpi, vaginal plates, spermatechae and hypandria of *D. willistoni*, *D. tropicalis*, *D. equinoxialis* and *D. paulistorum*. Hsu (1949) briefly described *D. willistoni* and *D. equinoxialis* male genitalia, in addition of some species of the *alagitans* and *bocainensis* subgroups. Malogolowkin (1952) provided a very detailed description of the male and female genitalia of *D. willistoni*, *D. equinoxialis*, *D. tropicalis*, *D. paulistorum* and some species of the bocainensis subgroup. Spassky (1957) shown illustrations of hypandria, aedeagi, surstily and prensiseta of five sibling species – *D. willistoni*, *D. tropicalis*, *D. equinoxialis*, *D. paulistorum* and *D. insularis*. Pasteur (1970) made a biometrical comparison of four semispecies of *D. paulistorum* regarding wing and tibia lengths, wing-to-tibia ratios, wing size and number of prensiseta on surstylus. Vilela and Bächli (1990) redescribed *D. willistoni* and provided several illustrations of the male terminalia of this species. Eberhard and Ramirez (2004) presented several Scanning Electron Microscopy (SEM) images of male and female terminalia of *D. willistoni*. Rohde et al. (2010) provided photos of the hypandria of *D. willistoni*, *D. equinoxialis*, *D. tropicalis* and *D. paulistorum* without indicating the semispecies while suggesting the importance of this structure for their identification. Recently, Souza et al. (2014) provided SEM images of *D. willistoni*, which was used as outgroup of the phylogenetic reconstruction of the *D. saltans* group. Civetta and Gaudreau (2015) shown photos of external male genitalia and aedeagus of *D. willistoni* and the subspecies *D. willistoni quechua*. Also, there are SEM images of male terminalia of four sibling species (*D. willistoni*, *D. tropicalis*, *D. equinoxialis* and *D. paulistorum*) available in Emilio Goeldi Museum database (marte.museu-goeldi.br).

Burla et al. (1949) only found slight morphological differences that were insufficient for the identification of single individuals, while Spassky (1957) noted differences in the male genitalia that did permit such identification. In other way, Malogolowkin (1952) described the general morphology of the male genitalia of four sibling species (*D. willistoni*, *D. equinoxialis*, *D. tropicalis* and *D. paulistorum*) as very similar but completely different from non-sibling species. In addition, this author found no differences in the penises of these four sibling species. Spassky (1957), however, observed that the shapes of the penises and their gonapophyses differed in the sibling species.

With respect to *D. insularis*, only a few illustrations were presented by Spassky (1957). The previous descriptions and illustrations of *D. paulistorum* were based on Andean-Brazilian specimens once the specimens were collected in areas where only this semispecies is found (Mogi das Cruzes, São Paulo, Brazil in Burla et al. (1949) and Malogolowkin (1952); Tamandaré, Pernambuco, Brazil in Rohde et al. (2010)). The remaining species, *D. pavlovskiana*, has not been collected and is no longer available in Stock Centers. There is no description in the literature of the male genitalia of this species.

In this scenario, our objective was to characterize and compare the male terminalia of the species of the *D. willistoni* subgroup, including the *D. paulistorum* semispecies complex, using

Table 1
Species and strains used in this study. The numbered strains are represented in the figures.

Species	Semispecies	Population sample	Localities	Source
D. equinoxialis [1]		Mexico	Apazapán/Mexico	Lee Ehrman and Yong Kyu Kim
D. equinoxialis		FNT12	Belterra/Brazil	Mário Josias Müller
D. equinoxialis		FNT19	Belterra/Brazil	Mário Josias Müller
D. equinoxialis		FNT32	Belterra/Brazil	Mário Josias Müller
D. equinoxialis		Tape03	Santarém/Brazil	Mário Josias Müller
D. equinoxialis		Tape31	Santarém/Brazil	Mário Josias Müller
D. equinoxialis		Tape32	Santarém/Brazil	Mário Josias Müller
D. equinoxialis		Tape63	Santarém/Brazil	Mário Josias Müller
D. equinoxialis		Tape89	Santarém/Brazil	Mário Josias Müller
D. equinoxialis		207018	Belém do Pará/Brazil	Marlucia Martins
D. insularis [2]		SL03	Saint Lucia/Lesser Antilles	Jeffrey R. Powell
D. insularis		SL15	Saint Lucia/Lesser Antilles	Jeffrey R. Powell
D. tropicalis [3]		0801	San Salvador/El Salvador	Tucson Stock Center
D. willistoni [4]		WIP4	Salvador/Brazil	Antônio R. Cordeiro and Helga Winge
D. willistoni		Gdh4-1	Guadalupe/Caribe	Tucson Stock Center
D. willistoni		Ribeirão Preto	Ribeirão Preto/Brazil	Cláudia Rohde
D. willistoni		Mexico	Apazapán/Mexico	Margaret Kidwell
D. willistoni		DW-Pool Ita	Itapuã/Brazil	André Schnorr
D. willistoni		DW-Pool Faz	Serra do Cipó/Brazil	Carlos Vilela
D. willistoni		CIP	Serra do Cipó/Brasil	Carlos Vilela
D. willistoni		COR	Coronilla/Uruguay	Beatriz Goñi
D. willistoni		ISC	Santa Catarina/Brazil	Daniela de Toni
D. willistoni		DLA	Dois Lajeados/Brazil	Cláudia Rohde
D. willistoni		MAS	Morro Santana/Brazil	Ana Lauer Garcia
D. willistoni		Joinville	Joinville/Brazil	Jonas Döge
D. willistoni		17A2	Eldorado do Sul/Brazil	Vera L.S. Valente
D. paulistorum [5]	Amazonian	Pará q3	Belém do Pará/Brazil	Lee Ehrmann and Yong Kiu Kim
D. paulistorum		Tape 60	Santarém/Brazil	Mário Josias Müller
D. paulistorum [6]	Andean-Brazilian	MLC	Santa Catarina/Brazil	Marco Gottschalk
D. paulistorum		RIB	Ribeirão Preto/Brazil	Vera L.S. Valente
D. paulistorum		RMT	Porto Alegre/Brazil	Ana Lauer Garcia
D. paulistorum		Pool-Bur	Brasília/Brazil	Rosana Tidon
D. paulistorum		Játon-Sacha	Jaton Sacha/Equador	Margaret Kidwell
D. paulistorum [7]	Centroamerican	Lancetilla	Lancetilla/Honduras	Lee Ehrman and Yong Kyu Kim
D. paulistorum [8]	Interior	INT	Llanos/Colombia	Lee Ehrman and Yong Kyu Kim
D. paulistorum [9]	Orinocan	ORI	Georgetown/Guyana	Lee Ehrman and Yong Kyu Kim
D. paulistorum [10]	Transitional	Santa Marta	Santa Marta/Colombia	Lee Ehrman and Yong Kyu Kim

Scanning Electron Microscopy and Light Microscopy. We attempt to describe this morphological trait within species and find features that differentiate the sibling species and semispecies of the *willistoni* subgroup and verify if these species can be recognized based on characters of the male terminalia.

Material and methods

Fly stocks

The stocks were reared in a cornmeal medium (Marques et al., 1966) at a constant temperature and humidity (17 ± 1 °C; 60% rh). Some individuals were preserved in 70% ethanol, and mounted stubs and slides will be deposited in Fundação Oswaldo Cruz (Fiocruz) Collection. All strains used in this study are listed in Table 1.

Species recognition

Species were confirmed with the *Acph*-1 (Acid Phosphatase) electrophoresis protocol (Garcia et al., 2006). Also, DNA sequences were generated and compared with sequences available in *GenBank* – mitochondrial gene fragments *COI* (Cytochrome oxidase I) (deposited by Gleason et al., 1998), *COII* (Cytochrome oxidase II) (deposited by Robe et al., 2010), and nuclear genes fragment *Adh* (Alcohol dehydrogenase) (deposited by Gleason et al., 1998 and Robe et al., 2010). The sequences obtained will be presented in a further study.

Scanning electron microscopy (SEM) preparation and observation

Male terminalia were treated with 10% KOH (Wheeler and Kambysellis, 1966 modified by Bächli et al., 2004) and dissected in glycerol. Terminalia were dehydrated for 20–30 s with acetone washes in the following concentrations: 30%, 50%, 75% and 100%. The entire terminalia and separated parts were mounted in stubs with carbon tape and metalized with gold in a Balzers SCD050 sputter coater. Visualization and image capture were performed in JSM6060 Scanning Electron Microscope in Centro de Microscopia da Universidade Federal do Rio Grande do Sul. We observed approximately 50 seven-day-old specimens of each species and semispecies.

Light microscopy preparation and observation

Male terminalia were prepared as previously described. The terminalia, aedeagi and hypandria were mounted in a non-permanent glycerin jelly medium (Klaus et al., 2003). The slides were observed and photographed with a Carl-Zeiss Standard phase contrast microscope. We analyzed the genital structures of 7-day-old males of each species and semispecies (750 individuals).

Terminology and references

The morphological terminology used in this study follow McAlpine (1981), Grimaldi (1990), Vilela and Bächli (1990), Bächli et al. (2004) and Souza et al. (2014). Figures of external male

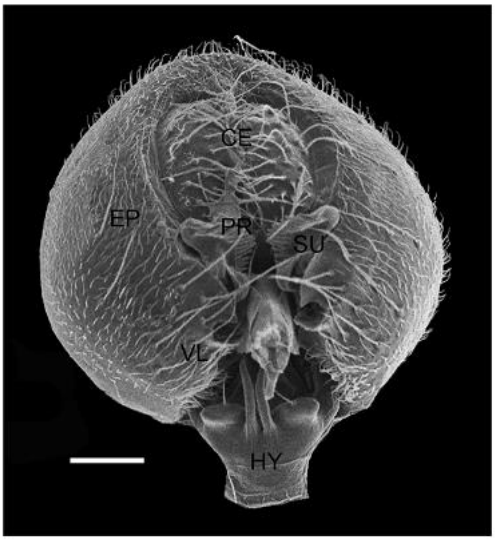

Fig. 3. General example of external male genitalia of the *D. willistoni* subgroup (picture of *D. paulistorum* Amazonian [5]). Scale bar 50 μm. EP, epandrium; CE, cerci; SU, surstylus; PR, prensisetae; AE, aedeagus; VL, ventral lobe; HY, hypandrium.

genitalia of *D. willistoni* subgroup, under light microscopy, are shown in Supplementary material 1 (S1).

Results

Epandrium, surstylus and prensisetae

The cerci are not fused to the epandrium (Figs. 3 and 4; Fig. S1) in all the species analyzed. The surstylus is elongated into a hook at the bottom, is not micropubescent, has up to 12 prensiseta (also called primary teeth) in *D. paulistorum* Orinocan and *D. paulistorum* Interior, and up to 18 prensiseta in *D. equinoxialis* (Fig. 5) in addition to one large prensisetae and one or two seta on the ventral hook.

Drosophila equinoxialis presents 18 prensiseta, nine smaller and nine larger, and one setae on the ventral hook (Figs. 4A and 5A). *D. insularis* has approximately 17 prensiseta of equal size and one setae in the ventral hook (Figs. 4B and 5B). *D. tropicalis* has 13–14 crescent size prensiseta and one setae in the ventral hook (Figs. 4C and 5C). *D. willistoni* also has 13 crescent sized prensiseta and one setae in the ventral hook (Figs. 4D and 5D).

Among the *D. paulistorum* semispecies, we found that *Drosophila paulistorum* Amazonian has a surstylus with 15 prensiseta, nine longer and six shorter, and one setae in the ventral hook (Figs. 4E and 5E). *D. paulistorum* Andean-Brazilian has a surstylus with 15 prensiseta, eight longer and seven shorter, and two seta in the ventral hook (Figs. 4F and 5F). *D. paulistorum* Centroamerican has a surstylus with 15 prensiseta, eight larger and seven smaller, and two seta in the ventral hook (Figs. 4G and 5G). *D. paulistorum* Interior has a surstylus with 12 crescent size prensisetae and two seta in the ventral hook (Figs. 4H and 5H). *D. paulistorum* Orinocan has a surstylus with 12 prensiseta of approximately the same size and two seta in the ventral hook (Figs. 4I and 5I). *D. paulistorum* Transitional has a surstylus with 12 prensisetae of approximately the same size, with one larger prensisetae in the middle and two seta in the ventral hook (Figs. 4J and 5J). The distance between the sides of surstylus and hooks could be an artifact of the SEM preparation and is not considered a diagnostic character for species identification.

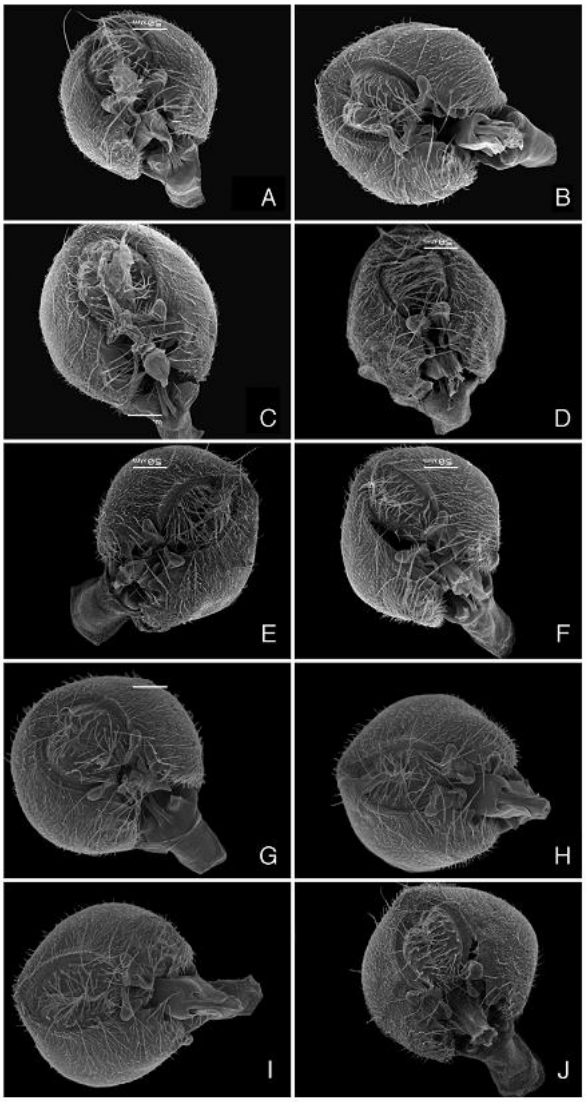

Fig. 4. Scanning electron microscopy of *D. willistoni* species subgroup external male genitalia. Scale bar 50 μm. The strains are in brackets and listed in Table 1. (A) *D. equinoxialis* [1]; (B) *D. insularis* [2]; (C) *D. tropiacalis* [3]; (D) *D. willistoni* [4]; (E) *D. paulistorum* Amazonian [5]; (F) *D. paulistorum* Andean-Brasilian [6]; (G) *D. paulistorum Centroamerican* [7]; (H) *D. paulistorum* Interior [8]; (I) *D. paulistorum* Orinocan [9] and (J) *D. paulistorum* Transitional [10].

Hypandrium

The hypandrium is smaller than the epandrium; it is approximately 1/4 to 1/3 of the size of the epandrium. The hypandria in all of the species analyzed have, in the apical region, one pair of heavily sclerotized median teeth, lobes with paramedian seta on the apex and lateral extensions (Figs. 6 and 7). The relative size and thickness of the median teeth, as well as the size and shape of the lobes, vary within the species (Figs. 6 and 7).

In *D. equinoxialis*, the hypandrium is triangular, with well-developed trapezoidal lobes and lobe seta convergent to teeth. The teeth are large and thick, twice the height of the lobes, aligned with the lobes, and do not touch each other. The lateral extensions are very prominent toward the top (Figs. 6A and 7A). *D. insularis* has

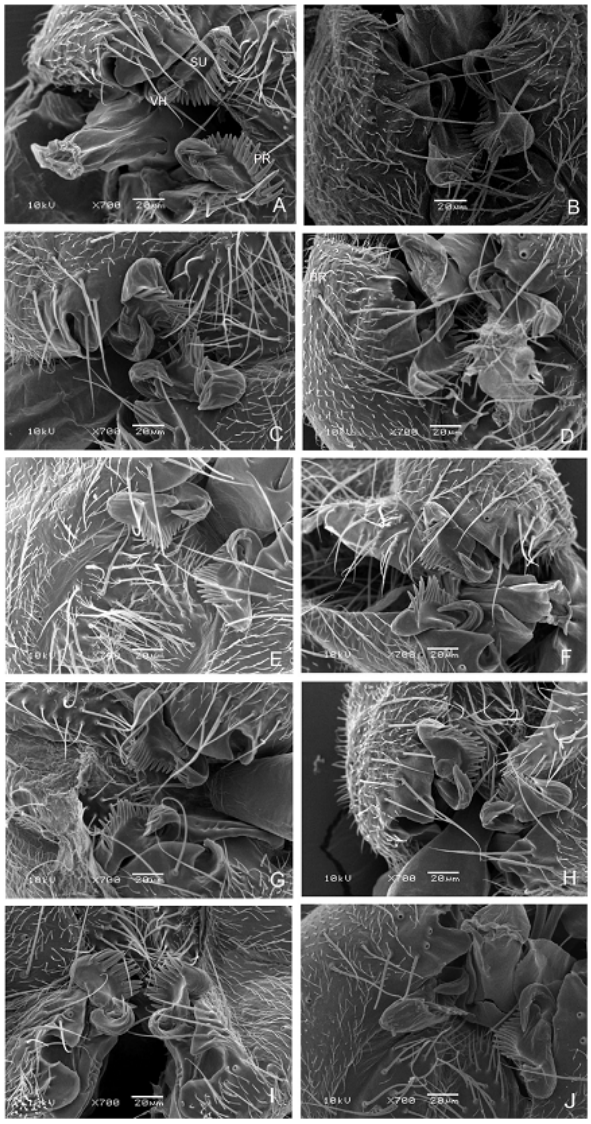

Fig. 5. Scanning electron microscopy of *D. willistoni* species *subgroup* surstyli and prensiseta. Scale bar 20 μm. The strains are in brackets and listed in Table 1. SU, Surstylus; PR, Prensisetaes; VH, Ventral hook. (A) *D. equinoxialis* [1]; (B) *D. insularis* [2]; (C) *D. tropicalis* [3]; (D) *D. willistoni* [4]; (E) *D. paulistorum* Amazonian [5]; (F) *D. paulistorum* Andean-Brasilian [6]; (G) *D. paulistorum* Centroamerican [7]; (H) *D. paulistorum* Interior [8]; (I) *D. paulistorum* Orinocan [9] and (J) *D. paulistorum* Transitional [10].

a triangular hypandrium, with almost absent lobes. This species presents one or two paramedian seta in the lobes and presents the smaller and more separated teeth that occur in the *willistoni* subgroup (Figs. 6B and 7B). *D. tropicalis* presents a triangular hypandrium, with very large round lobes and seta convergent to teeth and almost touching them. It also has large thick teeth, twice the height of the lobes, inserted slightly below the lobe line. The lateral extensions are located under the lobes (Figs. 6C and 7C). *D. willistoni* also has a triangular hypandrium, with subtle round lobes. Its very close large, thick teeth are thrice the height of the lobes and are inserted far below the lobe line. The lateral extensions are similar to those of *D. tropicalis*, but are adjacent to the lobes (Figs. 6D and 7D).

In the *D. paulistorum* complex, *D. paulistorum* Amazonian presents a square-shaped hypandrium, dome-shaped lobes, and slightly convergent seta. The lobes almost touch each other and the teeth are large and slightly separated, inserted in the lobe line. The lateral extensions are prominent toward the top, but less than in *D. equinoxialis* (Figs. 6E and 7E). *D. paulistorum* Andean-Brazilian presents a square-shaped hypandrium, slightly square-shaped lobes, convergent seta and very close, medium-sized, thin teeth that are twice the height of the lobes and inserted in the lobe line. There is a visible gap between the lobes and teeth. The lateral extensions are almost continuous with the lobes (Figs. 6F and 7F). *D. paulistorum* Centroamerican presents a rectangular hypandrium, which is the most elongated in *D. willistoni* subgroup, irregular-shaped lobes, convergent seta and very close, medium-sized, thin teeth that are twice the height of the lobes and inserted below the lobe line. The lateral extensions are similar to *D. willistoni* but nearer to the lobes (Figs. 6G and 7G). *D. paulistorum* Interior is very similar to *D. paulistorum* Andean-Brazilian, but the lateral extensions are not continuous with the lobes and there is no gap between the teeth and lobes (Figs. 6H and 7H). *D. paulistorum* Orinocan hypandrium is the smallest of the subgroup, is square-shaped with small round lobes, convergent seta and medium-sized, thin teeth that are thrice the height of the lobes and inserted a little below the lobe line. Lateral extensions are expanded to external sides of the lobes (Figs. 6I and 7I). *D. paulistorum* Transitional presents a square-shaped hypandrium, small round lobes very close to the teeth, convergent seta and large thick teeth that are almost thrice the height of the lobes and inserted in the lobe line. Lateral extensions are prominent toward the top, higher than the lobes (Figs. 6J and 7J).

Aedeagus, aedeagal apodeme, paramere and lateral projections

In all of the species of the *D. willistoni* subgroup that have been analyzed, the aedeagus is dorsally membranous and ventrally directed downwards, as a bird beak-like protusion at the distal end (distiphallus), with two lateral projections at the anterior half, covered with some tiny spines that are not always visible in preparations. The aedeagal apodeme is as long as the aedeagus, is bar shaped and is linked to the aedeagus by a membranous tissue. The parameres are smooth and are also linked to the apodeme by a membranous tissue. The paramere anchors the aedeagus to the hypandrium through the lateral expansions of hypandrium.

The most noticeable difference within the *willistoni* subgroup is the distal portion of the aedeagus (distiphallus). This is very prominent and curved in *Drosophila tropicalis* (Figs. 8C, 9C and 10C), long and straight in *D. willistoni* (Figs. 8D, 9D and 10D), and straight and shorter than *D. willistoni* in *D. equinoxialis* (Figs. 8A, 9A and 10A). In *D. insularis*, this structure is the shortest among the siblings (Figs. 8B, 9B and 10B). There are small variations in size within the *D. paulistorum* semispecies, but the shape of the distiphallus is unique in each incipient species (Figs. 8E–J, 9E–J and 10E–J).

The lateral projections exhibit some differences within the subgroup, which are more notable in *D. willistoni*, *D. tropicalis*, *D. equinoxialis* and *D. insularis*. In *D. equinoxialis* and *D. paulistorum* Orinocan the distal portion of lateral expansions is rounded; in *D. insularis*, *D. tropicalis* and *D. willistoni* it is pointy; and in the remaining *D. paulistorum* semispecies, it is slightly pointy.

The aedeagal apodeme also shows variation; however, it is not species specific. In *D. equinoxialis*, *D. insularis*, *D. tropicalis* and *D. willistoni*, the aedeagal apodeme is rod shaped, without ornamentation in the distal portion (Figs. 9A–D and 10A–D). In the Amazonian, Andean-Brazilian, Interior and Transitional semispecies, the aedeagal apodeme is also rod shaped but with a small rounded expansion in the distal portion (Figs. 9E,F,H,J and 10E,F,H,J), and in the Centroamerican and Orinocan semispecies, there is a fan-like expansion in the distal area (Figs. 9G,I and 10G,I).

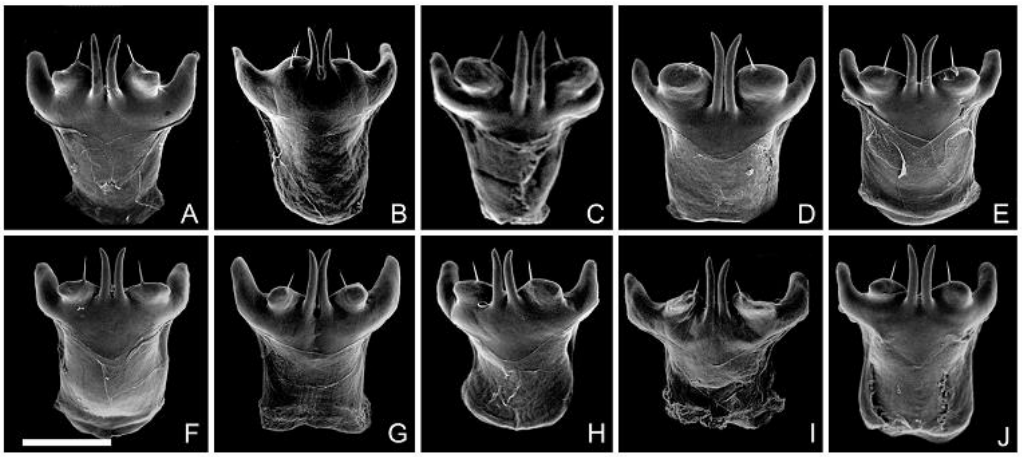

Fig. 6. *Scanning Electron Microscopy* of the *D. willistoni* species subgroup hypandria. Scale bar 50 μm. The strains are in brackets and listed in Table 1. LO, lobes; TE, teeth; LE, lateral extensions; SE, seta. (A) *D. equinoxialis* [1]; (B) *D. insularis* [2]; (C) *D. tropicalis* [3]; (D) *D. willistoni* [4]; (E) *D. paulistorum* Amazonian [5]; (F) *D. paulistorum* Andean-Brasilian [6]; (G) *D. paulistorum* Centroamerican [7]; (H) *D. paulistorum* Interior [8]; (I) *D. paulistorum* Orinocan [9] and (J) *D. paulistorum* Transitional [10].

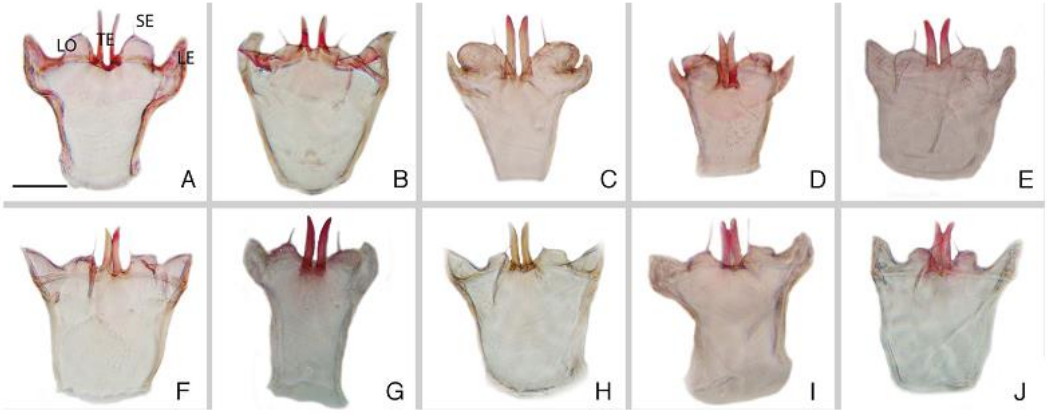

Fig. 7. Hypandria of *D. willistoni* species group. Scale bar 0.1 mm. The strains are in brackets and listed in Table 1. LO, lobes; TE, teeth; LE, lateral extensions; SE, seta. (A) *D. equinoxialis* [1]; (B) *D. insularis* [2]; (C) *D. tropicalis* [3]; (D) *D. willistoni* [4]; (E) *D. paulistorum* Amazonian [5]; (F) *D. paulistorum* Andean-Brasilian [6]; (G) *D. paulistorum* Centroamerican [7]; (H) *D. paulistorum* Interior [8]; (I) *D. paulistorum* Orinocan [9] and (J) *D. paulistorum* Transitional [10].

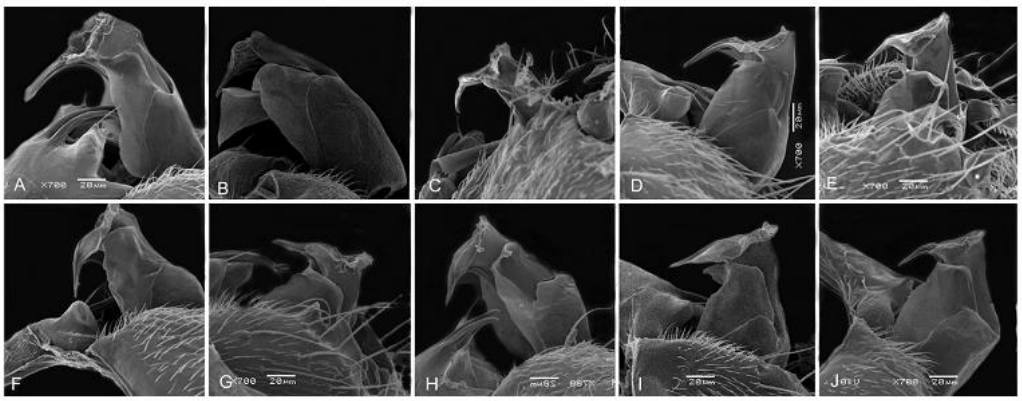

Fig. 8. Scanning electron microscopy of *D. willistoni* species *subgroup* aedeagi. Scale bar 20 μm. The strains are in brackets and listed in Table 1. (A) *D. equinoxialis* [1]; (B) *D. insularis* [2]; (C) *D. tropicalis* [3]; (D) *D. willistoni* [4]; (E) *D. paulistorum* Amazonian [5]; (F) *D. paulistorum* Andean-Brasilian [6]; (G) *D. paulistorum* Centroamerican [7]; (H) *D. paulistorum* Interior [8]; (I) *D. paulistorum* Orinocan [9] and (J) *D. paulistorum* Transitional [10].

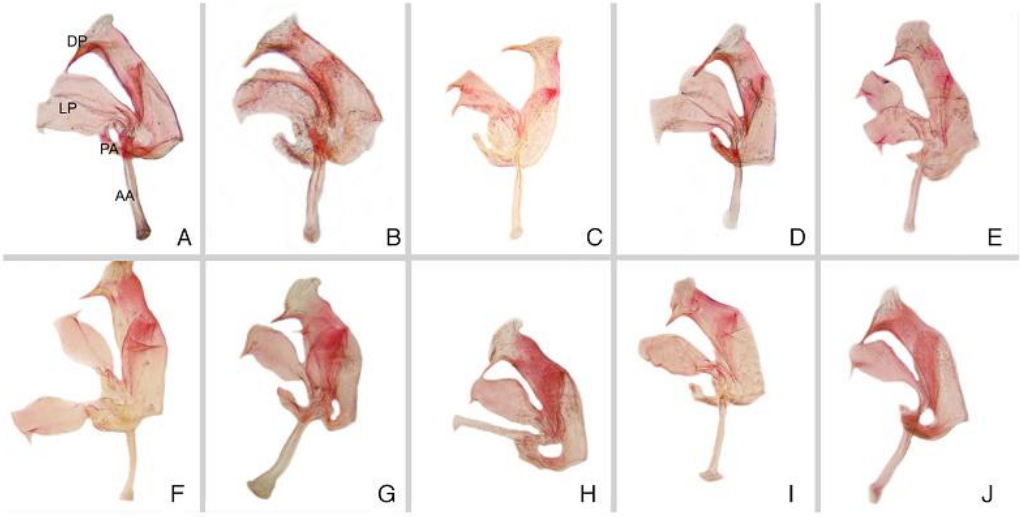

Fig. 9. Aedeagi, aedeagal apodeme and parameres of the *D. willistoni* species subgroup; lateral view. Scale bar 0.1 mm. The strains are in brackets and listed in Table 1. AA, Aedeagal apodeme; DP, Distiphallus; LP, Lateral projections; PA, Paramere. (A) *D. equinoxialis* [1]; (B) *D. insularis* [2]; (C) *D. tropicalis* [3]; (D) *D. willistoni* [4]; (E) *D. paulistorum* Amazonian [5]; (F) *D. paulistorum* Andean-Brasilian [6]; (G) *D. paulistorum* Centroamerican [7]; (H) *D. paulistorum* Interior [8]; (I) *D. paulistorum* Orinocan [9] and (J) *D. paulistorum* Transitional [10].

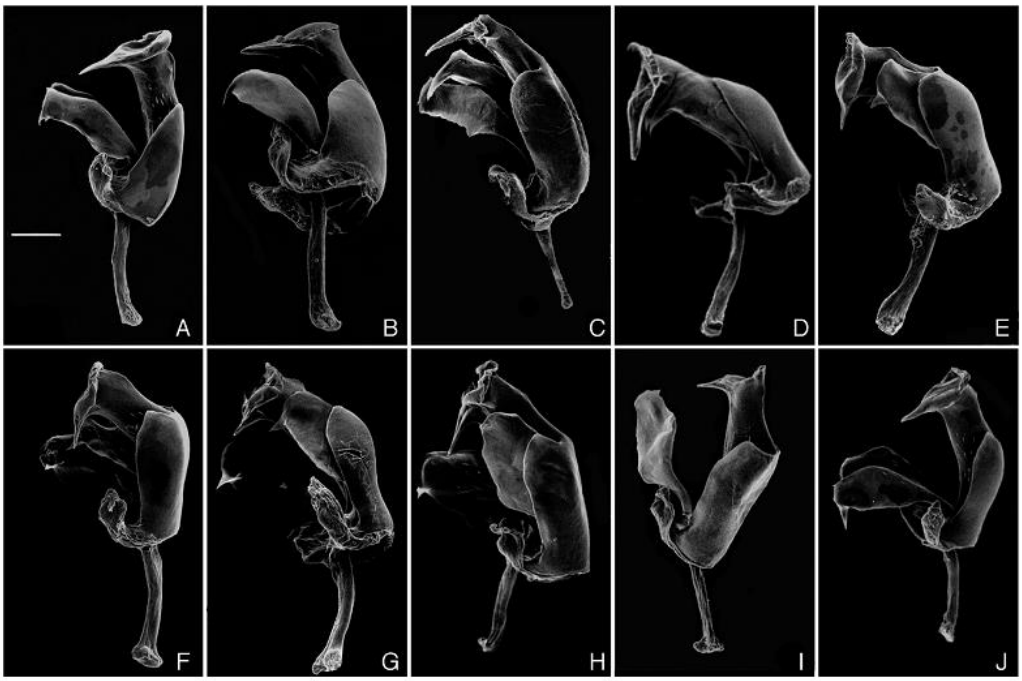

Fig. 10. SEM of aedeagi, aedeagal apodeme and parameres of the *D. willistoni* species subgroup; lateral view. Scale bar 50 μm. The strains are in brackets and listed in Table 1. AA, Aedeagal apodeme; DP, Distiphallus; LP, Lateral projections; PA, Paramere. (A) *D. equinoxialis*; (B) *D. insularis*; (C) *D. tropicalis* [3]; (D) *D. willistoni* [4]; (E) *D. paulistorum* Amazonian [5]; (F) *D. paulistorum* Andean-Brasilian [6]; (G) *D. paulistorum* Centroamerican [7]; (H) *D. paulistorum* Interior [8]; (I) *D. paulistorum* Orinocan [9] and (J) D. paulistorum Transitional [10].

Discussion

This study sheds new light on the identification of the *D. willistoni* sibling species subgroup, especially regarding the *D. paulistorum* complex. We presented here for the first time images of the male terminalia of the six semispecies of the *D. paulistorum*. Now, the male identification of the cryptic species of this subgroup could be easier and quicker than enzymatic, molecular and chromosomal approaches.

We found major differences especially in *D. willistoni*, *D. tropicalis*, *D. equinoxialis* and *D. insularis*. While there is a strong sexual isolation within these species, it has been reported that they occasionally interbreed (Dobzhansky et al., 1957; Winge and Cordeiro, 1963; Winge, 1965; Cordeiro and Winge, 1995), and several degrees

of reproductive affinity are present between the sibling species group (reviewed in Cordeiro and Winge, 1995).

Our findings are consistent with the results presented in Spassky (1957) regarding *D. equinoxialis*, *D. insularis*, *D. tropicalis*, *D. willistoni* and *D. paulistorum* Andean-Brazilian. However, a notable aspect of this comparison is that intraspecific variation was not observed in our results. We analyzed the male genitalia of *D. equinoxialis*, *D. insularis*, *D. willistoni* and *D. paulistorum* Andean-Brazilian from several localities (Table 1), including some recently collected strains, and no remarkable character variation was found (data not shown). This fact does not imply that there are no intraspecific variation in these species and in other species and semispecies of the *willistoni* subgroup. Also, laboratory strains may have less character variation than found in nature. Burla et al. (1949), however, concluded that "the variability is great enough to make identification of single individuals hazardous".

Burla et al. (1949) found differences in the hypandria of four of the sibling species – *D. willistoni*, *D. paulistorum*, *D. tropicalis* and *D. equinoxialis* – and described the *D. tropicalis* hypandrium as similar to that of *D. paulistorum* in shape, although larger. These authors also stated that the *D. willistoni* hypandrium is the most distinctive. In contrast with the findings of Burla et al. (1949) we observed that the hypandria of *D. insularis* and *D. tropicalis* are the most distinctive with respect to the remaining species. *D. willistoni* seems to be more similar to the *D. paulistorum* semispecies than to the other species. Some features could only be observed in SEM: *D. willistoni* and *D. insularis* presented two seta in the apex of the lobes, only in one side (Fig. 6B); Despite the low frequency of this modification (1:50 in *D. willistoni* and 2:50 in *D. insularis*), it is an interesting feature. The specimens with this characteristic did not present any other peculiarity.

Pasteur (1970) considered the teeth of the claspers (the prensisetae in the surstylus) to be the single character of the male genitalia that differentiates the *D. paulistorum* incipient species. In our results (Fig. 5), the number of prensisetae is consistent with the range of values previously observed by Pasteur (1970). We observed two groups regarding the number of prensisetae – the semispecies Amazonian, Andean-Brazilian and Centroamerican with 15 prensisetaes each and Centroamerican, Interior and Transitional semispecies with 12 prensisetaes each, although the size and arrangement of the prensisetae are not the same for each one. Pasteur (1970) found variation in the number of prensisetaes in each semispecies from different localities. In this study, we observed *D. paulistorum* Andean-Brazilian from several localities and the number of prensisetae was constant, even in the recently collected strains.

In the present report, we show that there are several diagnostic characters of the male genitalia useful for differentiating the species and even the semispecies of the *D. willistoni* species subgroup. However, the aedeagus is not the most important trait for identifying the subgroup's cryptic species, as is common in other *Drosophila* species groups. In the studied species, the hypandrium seems to be the main character for species identification. In addition to this, we suggest the visualization of the aedeagus under light microscopy for identification purposes, since this is a membranous structure and may be deformed in SEM.

Based on visual observations of hypandrium *D. insularis* seems to be the most distinctive species, related to the other sibling species. Within the *D. paulistorum* semispecies, the most dissimilar is the transitional, especially with respect to the hypandrium shape, surstylus and prensisetae. Such variation is in accord with the assumptions of Spassky et al. (1971) and Dobzhansky (1970), stating that the diversification of the semispecies is apparently still in progress.

Concerning the evolutionary relationship among the *willistoni* subgroup, an attempt to phylogenetic reconstruction was made using the characters of the male terminalia observed and described in this study. The reconstruction, however, were inconclusive, since the generated tree presented some polytomies and low support values for the characters (data not shown). Despite of the differences observed, it is possible that these characters did not accumulated enough differences to represent the evolutionary history of the subgroup. Nevertheless, these characters could be useful in a combined phylogeny and will be presented in a further study.

Although complete reproductive isolation is not present within the species and semispecies of the *D. willistoni* subgroup (reviews in Ehrmann and Powell (1982) and Cordeiro and Winge (1995)), the number of differences among the male genitalia found in our observations is relevant, especially between the *D. paulistorum* semispecies. In insects, the rapid divergence in male genitalia is so pronounced that even recently diverged sibling species show a high degree of variation in the male genitalia (Richards, 1927; Liu et al., 1996; Song, 2009), as we have observed in the *D. willistoni* species subgroup. Finally, in response to our own question, sibling species of the *D. willistoni* subgroup can be recognized through combined microscopy techniques.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

We thank to CNPq, CAPES and FAPERGS (process 10/0028-7) for financial support. We thank to Dr. Hermes José Schmitz, Dr. Marco Gottschalk and MsC. Jean Lucas Poppe for precious suggestions. We thank to Dr. Yong Kyu Kim, Dr. Lee Ehrmann, Dr. Jeffrey R. Powell and all the other collectors for the provided strains. We also thank the anonymous reviewers for the suggestions.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.rbe.2015.09.006.

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Supplementary material:

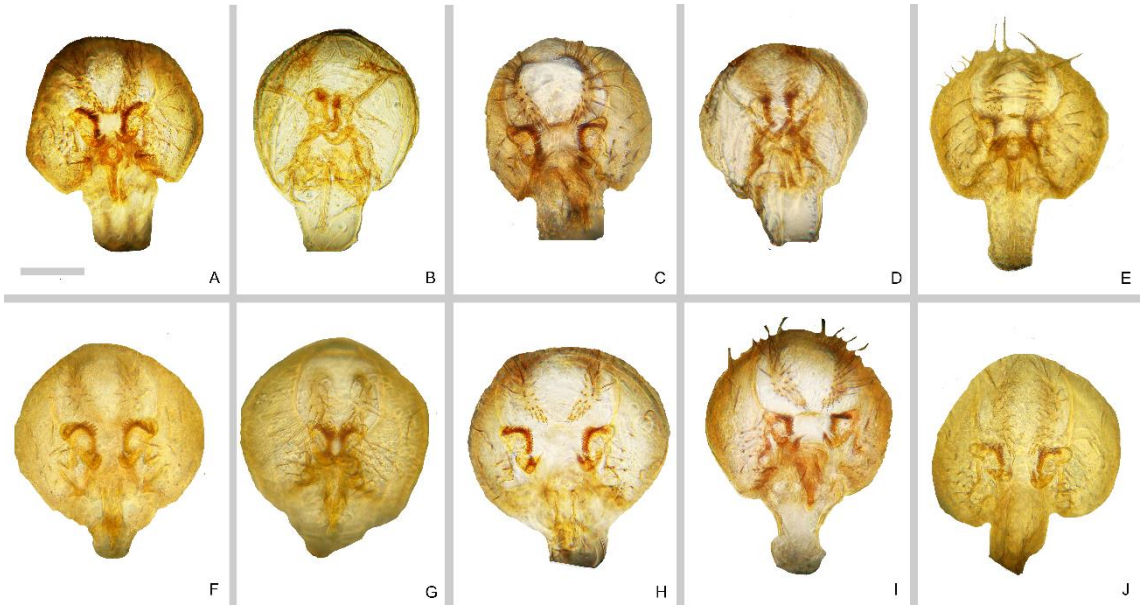

Figure S1. External male genitalia of *willistoni* subgroup under Light Microscopy.

(A) *D. equinoxialis;* (B) *D. insularis;* (C) *D. tropicalis;* (D) *D. willistoni*; (E) *D. paulistorum* Amazonian; (F) *D. paulistorum* Andean-Brazilian; (G) *D. paulistorum* Centroamerican; (H) *D. paulistorum* Interior; (I) *D. paulistorum* Orinocan; (J) *D. paulistorum* Transitional.

CAPÍTULO VI - Still *Drosophila willistoni* species group: combining morphological and molecular data to establish evolutionary relationships

Still *Drosophila willistoni* species group: combining morphological and molecular data to establish evolutionary relationships

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Artigo em preparação para ser submetido ao periódico *Molecular Phylogenetics and Evolution*

ABSTRACT

The Neotropical group *willistoni* of *Drosophila* has been used as a model for evolutionary studies since the last century. This group of flies comprises three subgroups: *alagitans, bocainensis* and *willistoni*. The *willistoni* subgroup is very peculiar, given that there are many levels of reproductive isolation and different evolutionary stages occurring within them. Despite being a well-studied subgroup, some questions remain unclear, especially regarding phylogenetic relationships within the *willistoni* subgroup. In order to shine a new light about the phylogenetic aspects, we used 3 mitochondrial gene fragments, 5 nuclear gene fragments and 45 morphological characters to infer the phylogenetic relationships and to estimate the divergence time of these species with more accuracy. We found some variations in the tree topology with the different datasets, but the bulk of the evidences obtained reflects the evolutionary history of this group of flies.

Keywords: *Drosophila willistoni* subgroup, morphology, molecular markers, combined phylogeny

1. INTRODUCTION

The *Drosophila willistoni* group has been studied since the later half of the last century. This Neotropical group includes 24 species, divided in three subgroups: *alagitans, bocainensis* and *willistoni* (Bächli, 2015). The *willistoni* subgroup includes *D. willistoni* Sturtevant 1916 and its five siblings: *D. equinoxialis* Dobzhansky 1946, *D. tropicalis* Burla and Da Cunha 1949, *D. insularis* Dobzhansky *et al.* 1957, *D. pavlovskiana* Kastritsis and Dobzhansky 1967 and *D. paulistorum* Dobzhansky and Pavan 1949. *Drosophila paulistorum* has traditionally been defined as a superspecies (Dobzhansky *et al.,* 1964), which is composed of six semispecies: Amazonian, Andean Brazilian, Centroamerican, Interior, Orinocan and Transitional (Dobzhansky and Spassky, 1959; Pérez-Salas *et al.,* 1970).

The *Drosophila willistoni* subgroup is mostly Neotropical, spanning from Mexico, Florida and Central America to a large portion of South America, namely Northern Argentina, Uruguay and Brazil, with Rio Grande do Sul as its Southern most distribution limit. This species also occurs in Caribbean and Galapagos Islands (Review in Zanini *et al.* 2015a). *Drosophila tropicalis* and *D. equinoxialis* is found from Florida, Mexico, Caribbean Islands, Central America to North and Central South America (Review in Zanini *et al.,* 2015a). *Drosophila insularis* is endemic to Saint Kitts, Saint Lucia and Montserrat islands, in the Antilles and *D. pavlovskiana* has been found only once in Guyana (Dobzhansky *et al.,* 1957; Spassky *et al.,* 1971; Review in Bächli, 2015 and Zanini *et al.* 2015a). Regarding the semispecies, they have a geographic distribution similar to *D. willistoni*, except for Argentina and Uruguay, where there are no records for those semispecies (Review in Bächli, 2015). Even if living in sympatry, it is

traditionally considered that the semispecies usually do not interbreed in nature. However, in laboratory tests, *D. paulistorum* Transitional semispecies mates with *D. paulistorum* Andean-Brazilian and *D. paulistorum* Centroamerican and is able to produce a fertile offspring (Review in Ehrmann and Powell, 1982 and Cordeiro and Winge, 1995).

In addition to species and semispecies, there is also taxonomic differentiation at subspecific level within the *willistoni* subgroup: *D. tropicalis* is subdivided into *tropicalis* and *cubana* subspecies (Townsend, 1954); *D. willistoni* is subdivided into *willistoni* and *quechua* subspecies (Ayala and Tracey, 1973); and *D. equinoxialis* subdivided into *equinoxialis* and *caribbensis* subspecies (Ayala *et al.,* 1974). These subspecies are not sympatric and when crossed produce mainly sterile males (Ehrmann and Powell, 1982). However, Civetta and Gaudreau (2015) reported successful crossings between *D. willistoni willistoni* and *D. willistoni quechua* subspecies, producing fertile offspring. Since presenting several taxonomic levels and a range of evolutionary stages characterized by successive degrees of reproductive isolation, the *willistoni* subgroup is pointed as an interesting model for evolutionary studies (Robe *et al.,* 2010).

Up until now, few studies have been published on the *Drosophila willistoni* subgroup phylogeny. Spassky *et al.* (1971) provided a schematic diagram representing the evolutionary relationships based on cytological, biogeographical, genetic and biochemical data. Ayala *et al.* (1974) constructed a dendrogram based on the genetic differentiation of the species at 36 allozymes loci. In these previous studies, the relationship of the five siblings was inferred: *D. willistoni, D. equinoxialis, D. tropicalis, D. insularis* and *D. paulistorum,* without considering the semispecies. Gleason *et al.* (1998) presented the first DNA based phylogeny of the *willistoni* subgroup, using three

marker genes – *Adh* (*Alcohol Dehydrogenase*), *Per* (*Period*) and *COI* (*Cytochrome c Oxidase* subunit *I*), and included all siblings and semispecies as well as some species of *D. bocainensis* subgroup as an outgroup. Later, Tarrío *et al.* (2000) used the *Xdh* (*Xanthine Dehydrogenase*) gene fragment to clarify some aspects of the evolutionary relationships of the *D. willistoni* group. O'Grady and Kidwell (2002) proposed a phylogeny of the *Sophophora* subgenus and included some species of *D. willistoni* group. More recently, Robe *et al*. (2010) presented a phylogenetic analysis including two mitochondrial gene fragments – *COI* and *COII* (*Cytochrome c Oxidase* subunit *II*) and four nuclear genes – *Adh, Amd* (*Alpha methyldopa*), *Hb* (*Hunchback*) and *Per,* arranged in datasets.

Despite all these studies, some questions remain not completely clarified, mainly those concerning the *D. paulistorum* complex. In the preceding studies, some of the relationships are incongruent between data partitions and barely supported. Additionally, until now, there is no phylogenetic reconstruction of the *willistoni* subgroup based on morphological characters.

Similarity in the external morphology led to the use of different markers for the identification or differentiation of these siblings species. Chromosomal studies (Burla *et al.,* 1949; Rhode *et al*., 2006), crossing tests (review in Cordeiro and Winge, 1995 and Ehrman and Powell, 1982) and morphological analysis of the male terminalia (Burla *et al.*, 1949; Hsu, 1949; Malogolowkin, 1952; Spassky, 1957; Rhode *et al.,* 2010, Zanini *et al.,* 2015b). To differentiate the semispecies of *D. paulistorum* in particular, some attempts were made by Pasteur (1970), which used a morphological approach and by Kim *et al.* (2004), which analyzed the hydrocarbon composition of the cuticle. Just recently, Zanini *et al*. (2015b) demonstrated that is possible to identify each one of the semispecies based on characters of the male terminalia.

Drosophila willistoni species subgroup is inserted in a complex evolutionary scenario where either speciation or diversification of species was not completely clarified. This background makes *D. willistoni* subgroup a suitable model for evolutionary and biogeographical studies. Accordingly, in the present study we attempt to contribute to a better understanding of the evolutionary history of the *willistoni* species subgroup, with emphasis on the *D. paulistorum* species complex. The main goal of this study is enhancing the knowledge of phylogenetic relationships and the divergence time of the subgroup, combining molecular and morphological datasets.

2. MATERIAL AND METHODS

2.1. Species

Were used five of the six siblings of the *willistoni* subgroup, including the six semispecies of the *D. paulistorum* complex. *D. pavlovskiana* has not been collected since its description and is no longer available in stock centers. Four species of the *bocainensis* subgroup and *D. sturtevanti*, a member of *saltans* group, were used as outgroup. The list of employed species is in Table 1.

2.2. DNA extraction and amplification

DNA was isolated following Sassi *et al.* (2005). Phylogenetic information was evaluated with sequences obtained from five nuclear loci - *Alcohol Dehydrogenase* (*Adh*), *Dopa-Decarboxylase* (*Ddc*), *Hunchback* (*Hb*), *Kl-3* (male fertility factors Kl-3 located in the short arm of the Y chromosome) and *Period* (*Per*). Additionally, three mitochondrial markers were employed - *Cytochrome oxidase subunit I* (*COI*), *Cytochrome Oxidase subunit II* (*COII*), *Cytochrome Oxidase b* (*Cytb*). We generated new sequences for *COI, Cytb, Ddc, Kl3* for all species analyzed, and *Adh* and *COII* for some species. *Hb*, *Per* and *COII* (in part) were downloaded from GenBank (Table 1). The amplification conditions as well as the primers used to amplify the sequences of molecular markers are in Supplementary material S1. Following the PCR, amplicons were purified with Exonuclease I (10U/µl) and Shrimp Alkaline Phosphatase (1U/µl). Macrogen (Seoul, South Korea) carried out the sequencing, using BigDye technology, in both forward and reverse directions using the same primers as those used in amplification. All generated amplicons will be submitted to the GenBank.

2.3. Sequence analysis

The electropherograms obtained were analyzed, assembled and the consensus sequences generated were edited and visualized using Staden Package (Staden, 1996). Consensus sequences were aligned using the Clustal W algorithm, implemented in MEGA version 6.0 (Tamura *et al*., 2013).

Each loci were tested for substitution saturation in Dambe 5.3.109 (Xia, 2013) (Supplementary material S2). Pairwise genetic divergences were estimated with Kimura two parameter (K2P) model in MEGA 6.0 (Tamura *et al*., 2013), considering each loci and total evidence dataset (Supplementary material S3).

Patterns of nucleotide substitution for each locus and combined loci were evaluated using the evolutionary models suggested by the Akaike Information Criterion (AIC test) (Akaike, 1974) performed in JmodelTest (Posada, 2008). Descriptive parameters were obtained in MEGA 6.0 (Tamura *et al*., 2013) and in DAMBE 5.3.109 (Xia, 2013), and can be visualized in Supplementary material S4.

Pairwise Partition Homogeneity Test (Farris, 1994, 1995) implemented in PAUP 4.0b10 (Swofford, 2003) was performed to test the incongruences between the different loci (Table 2). This test was performed using the parsimony criterion with 1,000 replications. We generated 100 starting trees per replica by random stepwise addition followed by TBR in order to reach the null distribution.

2.4. Morphological Analysis

We observed and coded 45 morphological characters (Supplementary material – S3). Four species of *D. bocainensis* subgroup and *D. sturtevanti* were used as outgroup. The morphological characters were chosen based on previous studies with *Drosophila* species (Yassin, 2009; Souza *et al.,* 2014) and adapted to our study. For general morphology characters, 10 individuals of each species and semispecies of *willistoni* and *bocainensis* subgroups were studied. We analyzed different strains of *D. willistoni, D. equinoxialis, D. insularis, D. paulistorum* Andean-Brazilian and *D. nebulosa* from several localities. Male genitalia characters of *willistoni* subgroup were obtained from a previous work (Zanini *et al.,* 2015b). For the *bocainensis* subgroup, we observed at least 20 individuals of each species, using Scanning Electron Microscopy and Light Microscopy preparations following Zanini *et al.* (2015b).

2.5. Phylogenetic Analysis

Gene trees and combined molecular trees were constructed using two methods: Bayesian Analysis (BA) performed in MrBayes 3.1.2 (Huenselback and Ronquist, 2001) and Maximum Likelihood (ML) performed in PhyML (Guindon *et al*., 2010). Both BA and ML followed the evolutionary model suggested by the AIC test.

Morphological trees were constructed using BA, with the Standard Model in MrBayes and with Maximum Parsimony (MP) in PAUP (Swofford, 2003). The MP analysis was performed using the branch and bound algorithm. All characters had equal weight. Character optimization was performed using ACCTRAN (accelerated transformation), and the analysis was repeated after successively weighting the characters on the initial cladogram. Confidence values for each internal node were

assigned after 1,000 bootstrap iterations. Consistency Index (CI) (Kluge and Farris, 1969), Retention Index (RI) (Farris, 1989a), Homoplasy Index (HI) and the Reescaled Consistency Index (RC) (Farris, 1989b) were estimated to evaluate the fit of the character to the 50% bootstrap consensus tree.

For the BA with combined datasets (Mitochondrial, Nuclear, Total Molecular, Mitochondrial+Morphological, Nuclear+Morphological and Total Evidence), the matrix was partitioned into morphological data and into different gene sets, and a nucleotide substitution model was used for each partition. The Markov Chain Monte Carlo (MCMC) was run for at least 1,000,000 generations, sampling trees every 100 generations and using four chains with heating default values. The runs were stopped when the convergence values declined below 0.01 and applied a burn-in of 25% of the generations before summarizing trees and parameters.

The ML and MP node support were based on 1,000 bootstrap replications and in BA node support reflected the posterior probabilities of each clade.

2.6. Divergence time estimation

Divergence time was estimated with BEAST software (Drummond and Rambaut, 2007) with parameters defined in BEAUTi. Running parameters resumed in Tracer. Trees summarized with Tree Anotator and visualized and edited with FigTree. Three independent runnings were performed with 20,000,000 generations, with a log-normal relaxed clock. We used the substitution parameters generated on the analyzed datasets (Supplementary material 2 - S2). We used two different approaches for the divergence time estimation: in the first approach, two calibration points were used, based on known events of speciation previously datated. The first calibration point

was the split between *D. willistoni* and *D. melanogaster* groups and the second point was the split of subgenus *Drosophila* and *Sophophora,* both based on De Salle and Powell (2003) and Russo *et al.* (1995). In the second approach, were used a single calibration point, the split between *willistoni* and *saltans* groups (Powell *et al*., 2003). Nuclear dataset and molecular total evidence (combined nuclear and mitochondrial datasets) were employed for divergence time estimation purpose.

3. RESULTS

3.1. Analysis of the dataset

For molecular data, we analyzed partial sequences of eight loci, covering more than 5,800 base pairs in lenght. The strains employed, as well as the properties of each loci datasets analyzed and the concatenated datasets are presented in Table 1 and Supplementary material S4, respectively.

The genetic distances comparisons at different taxonomic levels within *D. willistoni* species group were obtained for each of the eight molecular data partitions (Figure 1) and pairwise genetic divergence from total evidence dataset and from each gene are summarized in Supplementary material S3. The genetic divergence percentual, based on the total evidence matrix, ranges from 0.2% between *D. paulistorum* Orinocan and *D. paulistorum* Andean-Brazilian and 13.8% between *D. sturtevanti* and *D. willistoni*. Considering only the *willistoni* subgroup, genetic divergence fluctuates from 2.4% between *D. insularis* and *D. tropicalis* to 4.6% between *D. willistoni* and *D. equinoxialis*. Within the *bocainensis* subgroup, divergence

levels range from 3.5% between *D. capricorni* and *D. sucinea* to 10.2% between *D. fumipennis* and *D. sucinea.* Regarding the *D. paulistorum* complex, the divergence varies between 0.2% between Orinocan and Andean-Brazilian semispecies and 2.1%, between Transitional and Orinocan semispecies. Transitional semispecies also present the highest divergence level when compared to the other semispecies, always above 1.3% (Supplemetary material S3).

Considering pairwise genetic divergence in each single gene, some *D. paulistorum* semispecies share the identical gene sequence, in both mitochondrial and nuclear genes. The Centroamerican and Transitional semispecies share *COI* and *Cytb* nucleotidic sequences; Orinocan and Transitional semispecies have identical *Adh* nucleotidic sequence; Interior, Orinocan and Andean-Brazilian semispecies present the same sequence of *Kl3*, even as Transitional and Amazonian. The *D. equinoxialis* and *D. insularis* siblings share a haplotype of *Hb* sequence.

When we compare the divergence level among each loci (Figure 1), we can observe that all nuclear genes presented almost the same pattern, with the lowest levels of divergence within *D. paulistorum* complex and the highest between *bocainensis* subgroup and siblings of *willistoni* subgroup. Were found high divergence values within *bocainensis* subgroup, and regarding *Adh* locus, this value was higher than the distance of *bocainensis* subgroup and *willistoni* siblings.

Analyzing mitochondrial loci we verified the same pattern of the nuclear ones, except that, despite of the lowest values of divergence within *D. paulistorum* complex, this values are much higher than those of nuclear loci. *COII* locus, however, presented genetic divergence level comparable to the nuclear genes values.

The chi-square test did not found any evidence of base composition heterogeneity between taxa, despite the significant nucleotide frequency bias in some partitions, especially in the 3rd codon position and a deviation in the transitions versus transversions ratio. All matrices exhibited significant levels of heterogeneity in substitution rates among sites, which were adjusted through continuous gamma distribution (G) and/or proportion of variable sites (I) implementation (Supplementary material S4).

In the Partition Homogeneity Test, mitochondrial markers presented conflicting phylogenetic signals when combined with themselves or with nuclear markers (Table 2), except for *Cyt b* loci, which does not show conflict with *Adh* and *Kl3* fragments. Nuclear markers work well together, except for *Hb*, which highly disagrees with the other nuclear markers and also with the mitochondrial ones.

The morphological data was obtained among 45 analyzed characters. From these, there are 16 general morphology characters and 29 characters of the male genitalia. Female genitalia was not included because they do not present remarkable or informative differences between the analyzed taxa. Four characters were parsimoniously uninformative (abdominal color, scutum stripes presence, prensisetae of surstylus arrangement and decasternum shape); these characters only separated the outgroup species *D. sturtevanti* from the remaining group. Details can be seen in Supplementary material S5).

3.2. Molecular phylogenies - mitochondrial data partitions

The three mitochondrial data partitions resulted in phylogenetic trees with distinct relationships among the species of the *willistoni* group, reflecting the PHT results (Figure 2 A-C, Figure 3, Table 2). The *bocainensis* subgroup were recovered as polyphiletic in all mitochondrial data partitions and all reconstruction methods (Figures 2 A-C, Figure 3); *D. capricorni - D. sucinea* and *D. nebulosa – D. fumipennis* clades were recovered in *COI* and *Cytb* analysis, with all reconstruction methods. However, the relationship between the clades was not the same. The reconstruction with *COII* was the most dissimilar – *D. sucinea* was recovered together with the species in *willistoni* subgroup. The complex *Drosophila paulistorum* species was recovered as a polyphiletic clade with all mitochondrial data. *D. paulistorum* Centroamerican and Transitional formed a clade in all analysis (Figure 2B).

The *COI* partition shown some well supported clades (PP>0.95) (Figure 2A) as those formed by: *D. insularis* and *D. willistoni* along *D. tropicalis* clade*; D. fumipennis* and *D. nebulosa* clade*; D. capricorni* and *D. sucinea* clade*.* Concerning *D. paulistorum* species complex, we noticed that *D. equinoxialis* clustered with Transitional and Centroamerican semispecies, in a closer relationship than within other semispecies.

Regarding the *COII* data partition, some unusual relationships were presented, such as the clade formed by *D. sucinea* and this clade placed together with Centroamerican and Transitional semispecies (Figure 2B). The *D. willistoni* and *D. insularis* plus *D. tropicalis* clade was recovered in BA with high PP (>0.98), however it was not supported in ML and NJ reconstructions. This gene partition did not clarify the subgroup relationships and particularly the *D. paulistorum* cluster, reflecting the low divergence values observed (Figure 1 and Supplementary material S3). In contrast,

the *COII* data supported the polyphily of the *bocainensis* subgroup and also the monophily of the *willistoni* subgroup.

Using the *Cytb* data partition, most of the relationships were recovers even with different reconstruction methods (Figure 2C). This partition also agrees with *bocainensis* subgroup polyphily and with the formation of two clades: *D. sucinea* with *D. capricorni,* and *D. nebulosa* with *D. fumipennis.* Regarding the *willistoni* subgroup, the clade including *D. willistoni, D. insularis* and *D. tropicalis* is recovered once again. The cluster *D. paulistorum* Centroamerican and *D. paulistorum* Transitional grouped with *D. equinoxialis* was recovered once again. The *willistoni* subgroup seems to be monophyletic, but the *D. paulistorum* complex apparently is not.

The concatenated analysis of the three mitochondrial genes presented high Bayesian PP values for most relationships (Figure 3). Exactly as in the individual analysis, *bocainensis* subgroup was polyphyletic, maintaining the previously mentionated clades (Figures 2 A-C). The *willistoni* subgroup was divided in two main clades: one encompassing the siblings *D. willistoni, D. insularis* and *D. tropicalis* and the other comprising *D. equinoxialis* and *D. paulistorum* semispecies. The latter was further subdivided in a clade composed of *D. equinoxialis* and Transitional semispecies and another clade with the remaining semispecies.

Analyzing the saturation plots presented above of each gene trees (Figure 2A-C), we can verify the transitional saturation in the three *COI, COII* and *Cytb* genes. However, this saturation levels were verified only in higher divergence levels (above 6% in *COII* and above 9% in *COI* and *Cytb*) and, according to Xia's Index of Substitutional Saturation test result (Supplementary material S2), these are low levels of substitutional saturation.

3.3. Molecular phylogenies – nuclear partitions

Phylogenetic inferences based on nuclear data partitions did not present substitutional saturation and were robust regarding statistical support (Figure 4 A-E). Most relationships were recovered with all markers, except for *Hb* tree reconstruction, which did not reach the same topology.

As observed in mitochondrial partitions, *bocainensis* subgroup presented some unresolved taxonomic conflicts. Despite the well-supported relationship between *D. sucinea* and *D. capricorni* recovered in all reconstructions, the *D. fumipennis* and *D. nebulosa* positions in the trees were variable. Among the siblings, except in *Hb* reconstruction, there is a branching order observed with the remaining nuclear gene fragment reconstructions. *D. insularis* is the first to branch out from a common ancestor, followed by *D. tropicalis, D. willistoni* and *D. equinoxialis,* which apparently is closely related to *D. paulistorum* incipient species complex. Regarding all nuclear genes trees, we verified the lack of resolution of the evolutionary relationships among the *D. paulistorum* superspecies – all the reconstructions presented some polytomic clades and even the resolved clades were not congruent in all gene partitions (Figure 4 A-E).

The concatenated gene trees generated with all nuclear partitions (Figure 5) recovered the general tree topology similar to individual gene trees, except for the previously pointed incongruences. The bocainensis subgroup presented two diversification fronts: *D. sucinea* and *D. capricorni* clade branched out first, followed by *D. fumipennis* and *D. nebulosa* clade. The relationships among the siblings were the same founded with the genes trees (except *Hb* gene). The best improvement was

regarding the relationship among the semispecies of *D. paulistorum*. The Amazonian semispecies was the first to branch out, followed by a two clade division – one of them formed by Centroamerican and Transitional semispecies and the other one composed of Interior, Orinocan and Andean-Brazilian semispecies, with Orinocan and Andean-Brazilian a sister species clade (Figure 6).

3.4. Morphological phylogeny

The analysis of the morphological characters resulted in a more parsimonious phylogenetic consensus tree with 74 steps in length (Figure 6A). The *bocainensis* subgroup branched out first. The remaining species formed two sister clades. One clade includes the siblings *D. insularis, D. equinoxialis, D. tropicalis* and *D. willistoni* and the other is composed of *D. paulistorum* semispecies. However, inside of the main clades, some relationships are weakly supported (bootstrap <60%).

In the *bocainensis* subgroup, the species grouped as two clades – one containing *D. sucinea* and *D. capricorni* and another containing *D. nebulosa* and *D. fumipennis.* Regarding the siblings, *D. insularis* was the first to diverge from a common ancestor, followed by *D. equinoxialis.* The remaining siblings - *D. tropicalis* and *D. willistoni* were recovered as sister species. *D. paulistorum* species complex clade presented a politomy; however, some relationships were recovered – Amazonian and Transitional formed a clade, the same for Andean Brazilian and Interior semispecies.

Bayesian morphological reconstruction result in a tree that recovered the three major clades as previously supported by MP (Figure 6B). The relationships within species of *bocainensis* subgroup were maintained. The sibling species were the next to branch out, in a polytomic clade formed by *D. insularis, D. equinoxialis* and *D.*

willistoni and *D. tropicalis* grouped one more time as sister-species. The *D. paulistorum* cluster was recovered with a polytomy between the semispecies Amazonian, Transitional and a possible common ancestor of the remaining semispecies, which branched out in two distinct clades: Interior and Andean-Brazilian and Orinocan and Centroamerican. However, all these relationships were poorly supported (pp<0.95).

3.5. Mixed data

Despite the different size of the datasets, the addition of the morphological data to nuclear and mitochondrial datasets generated interesting results, especially concerning node support and definition of the relationships in *bocainensis* subgroup and among the semispecies.

In mitochondrial and morphological combined data (Figure 7), the *bocainensis* subgroup was recover as previous shown: two clades, one reuniting *D. sucinea* and *D. capricorni,* and another *D. nebulosa* e *D. fumipennis.* The *willistoni* subgroup was recovered separately in two clades, one composed of *D. tropicalis, D. insularis* e *D. willistoni*, while the other clade reunited *D. equinoxialis* and *D. paulistorum* cluster, branching in the following sequence: Transitional, Centroamerican, Interior, Orinocan and Andean-Brazilian and Amazonian grouped as sister-species.

Combined morphological and nuclear data reconstruction recovered a topology very similar to the combined nuclear reconstruction, except for the relationships among *D. paulistorum* semispecies, better defined and well supported in this approach (Figure 8). With this method, semispecies branched out sequentially in the following order: Amazonian, Transitional, Centroamerican, Interior, and Orinocan and Andean-Brazilian grouped as sister-species.

The results of combined mitochondrial and nuclear analysis are shown in Figure 9. The *bocainensis* subgroup is polyphyletic. However, the same clades found in the other combined analysis were recovered (Figure 9). Within *willistoni* subgroup, *D. insularis* diverged first, followed by *D. tropicalis, D. willistoni* and *D. equinoxialis.* Concerning *D. paulistorum* complex, Transitional semispecies diverge first, with the remaining semispecies split in two clades; Interior and Centroamerican (but weakly supported) and a clade containing the other semispecies (strongly supported).

The most complete analysis of this study encompassed all data partitions, generating a total evidence tree (Figure 10). The total evidence tree presented well-supported relationships, did not presented unresolved polytomies and summarized the results of all analysis. The *bocainensis* subgroup is ancestral to *willistoni* subgroup and divided in two clades – *D. sucinea* and *D. capricorni,* and *D. nebulosa* and *D. fumipennis* (Figure 10). Regarding *willistoni* subgroup, the divergence sequence founded in the other combined reconstructions is the same: *D. insularis, D. tropicalis, D. willistoni* e *D. equinoxialis,* followed by *D. paulistorum* semispecies Transitional, Centroamerican, Interior, Amazonian, and the sister-species Orinocan and Andean-Brazilian. Concerning to *D. paulistorum* cluster, only the split between *D. paulistorum* Interior and remain species showed a low support value: (PP<0.6).

3.6. Divergence Time Estimation

We used two approaches to estimate the divergence time of *willistoni* subgroup. In the first approach, we used a single calibration point (the split of *willistoni* and *saltans* groups) and tested combined nuclear datasets (Figure 11). In the second approach, we used two calibration points (the split between *Drosophila* and *Sophophora*

subgenus and the split between *willistoni* and *melanogaster* subgroups), also with combined nuclear datasets (Figure 12).

Regarding one calibration point approach employing combined nuclear dataset (Figure 11), were verified that differentiation in the *willistoni* subgroup began around 7.88Mya, with the branching of *D. insularis.* The *D. tropicalis* branched out around 5.16Mya; *D. willistoni* around 4.2Mya; *D. equinoxialis* branched out at approximately 2.51Mya and *D. paulistorum* complex began their divergence process around 1.09Mya, with *D. paulistorum* Amazonian draft. According to this data the latest split occurred around 0.4Mya, between *D. paulistorum* Orinocan e *D. paulistorum* Andean-Brazilian species. The analysis with two calibration points and with the same nuclear dataset recovered a tree with the same topology as observed in the previous analysis, with very similar divergence times but with smaller standard deviation (Figure 12): *D. insularis* (6.41Mya); *D. tropicalis* (4.28Mya); *D. willistoni* (3.49Mya); *D. equinoxialis* (2.07Mya); *D. paulistorum* Amazonian (0.89Mya); *D. paulistorum* Orinocan e Andean-Brazilian (0.32Mya).

DISCUSSION

This paper presented innovations in respect to the evolutionary relationships between members of the subgroup *willistoni* of *Drosophila*. Here, we employed a large molecular dataset including some genes never before used in the reconstructions of this group. *Cytb, Ddc* and Kl3, from different genomes, were not included in the

previous phylogenetic reconstruction. Additionally, we present for the first time a morphological reconstruction for this species subgroup and combined morphological-molecular phylogenies. Trockmorton (1975), Grimaldi (1990), Souza *et al*. (2014), Hu e Toda (2011), Yassin (2009) used morphological data to establish evolutionary relationships in Drosophilidae, in less abrangent levels. However, none of these studies covered all *willistoni* subgroup.

The entire *willistoni* group, including non sibling species, is in distinct evolutionary levels. The *bocainensis* subgroup appears to have already completed the evolutionary process, which can be observed by high divergence levels between species (Supplementary material S3, Figure 1) and by the consistency of the recovered phylogenetic relationships (Figures 3, 5-10). Considering just the species analyzed in this study, *bocainensis* subgroup seems to have had a polyphyletic origin (Figures 3, 5, 6, 7, 8, 9, 10). *D. sucinea* and *D. capricorni* seems closely related, since this relationship was recovered using different gene markers (Figures 2-10). This relationship corroborates O'Grady and Kidwell (2002). Neverthereless, more members of *bocainensis* subgroup must be included to accurately estimate evolutionary relationships within this subgroup.

Concerning gene trees, the same evolutionary relationships were not recovered with all markers. Mitochondrial genes trees were less congruent, as previously verified for this subgroup (Robe *et al.,* 2010). The nuclear genes trees (Figure 4A-E) were more consonant and in general, they only differ in respect to the *D. paulistorum* semispecies relationships. In this case, the saturation levels cannot explain the observed difference, since there is no evidence of saturation in the nuclear genes (Figure 4A-E, Supplementary material S2). For the mitochondrial genes we observed that there is saturation only when the difference between sequences is higher than 6%, in COII,

and higher than 9% in COI and Cytb. Meanwhile, this level of divergence was not observed among the *willistoni* sibling species (Fig 2A-C).

Still, some events could explain the incongruent topologies obtained with different datasets, like incomplete sorting of polymorphic ancestral lineages, introgressive hybridization, gene duplication and paralogous sampling, horizontal gene transfer (Zachos, 2009), or as an effect of maternally inherited symbionts (Hurst and Jiggins, 2005).

Introgression events seem to be one of the main sources of incongruence observed among the different markers, since interespecific crossings were already reported (review in Cordeiro and Winge, 1995; Ehrman and Powell, 1982). Ehrman and Powell (1982) summarize possible crossings between species, subspecies and semispecies of the *willistoni* subgroup and point out that most of this crossings do not produce viable offspring. Some crossings, however, can produce sterile hybrids or even fertile viable hybrids. This sucessful crossing seems to be restrict to *D. paulistorum* semispecies – Transitional semispecies crosses with Andean-Brazilian and Centroamerican, which could partially explain observed incongruences, mainly among mitochondrial markers. Corroborating with this, Mallet (2005) stated that hybridization may occur in a great portion of younger animals (10%) and plants (25%), since complete reproductive isolation takes a long time.

Sharing same nucleotidic sequences in mitochondrial genome is not an uncommon phenomenon. Some studies reported that *D. yakuba* and *D. santomea,* despite nuclear differentiation, present identical mitochondrial genome (Turissini *et al.* 2015, reviewing Lachaise *et al.,* 2000; Llopart *et al*., 2005a,b; Bachtrog *et al.,* 2006 and Llopart *et al.,* 2014). The lower values of genetic divergence that we found in *COII*

locus may be the result of gene flow (Monerrot *et al.,* 1990, Bachtrog *et al.,* 2006, Turissini *et al.,* 2015).

Finally, we have to consider that gene trees tell the particular history of each gene. *Adh* gene, for example, does not have high divergence levels, therefore this gene is well conserved in *Drosophila* genus, and even more in a species group. This group explores the same substract and is subject to the same selective pressures. *Hb* gene also has low genetic divergence levels, which can be explained by its role as a developmental gene, with a homeobox (review in Wolpert and Tickle, 2010).

Considering the relative recent divergence time of the *willistoni* subgroup, the low levels of morphological difference can be justified. The most evident morphological differences between the *willistoni* subgroup siblings are in the male genitalia, which can be explained by the distinct evolutionary rate of genital characters or morphological characters in general. Masly and Presgraves (2007) stated that the characters of the external genitalia evolve faster than other morphological character. According to Richards (1927), Liu *et al.* (1996) and Song (2009), the rapid divergence of the male genitalia in insects is so striking that recently diverged species can exhibit pronounced differences in the male genitalia. Richmond *et al.* (2012), resuming McPeek *et al.* (2008, 2009) said that sexual characteristics go through substantial shifts during speciation events, due to alternating selective pressures coming from directional and stabilizing selection on mate recognition systems. According to the authors, a correspondent shift in female genital morphology is not necessary, since females are not necessarily impaired by structural variation that gives copulatory advantage to males. The lack of definition in the phylogenetic relationships obtained through morphological characters' analysis can be reflection of the recent events of speciation. It is possible that these characters did not accumulated enough differences for a strong

phylogenetic signal, although the differences are enough to identify sibling species and even the *D. paulistorum* semispecies (Zanini *et al.,* 2015b). Conversely, in a recent study employing high-resolution, multiscale microcomputed tomography (CT) scans to visualize and measure changes in situ in the Drosophila female before, during, and after mating, Mattei *et al*. (2015) showed that, despite the apparent morphological conservation or small morphological differentiation in female genitalia, certain seminal components may mediate morphological modifications inside the female genitalia.

Some relationships found in our study were previously described. We found that *D. insularis* was the first sibling to diverge from a common ancestor. This species has been considered as basal in relation to the remaining subgroup. Spassky *et al*. (1971) considered *D. insularis* a probable insular offshoot of the continental *D. tropicalis.* Ayala *et al.* (1974) considered *D. insularis* as the oldest lineage of the *willistoni* subgroup. Gleason *et al*. (1998) also found that *D. insularis* seems to be the first species of this subgroup to diverge, with the same hypothesis also presented by Robe *et al.* (2010). Still, Rhode *et al.* (2006) established that *D. insularis* chromosomes are the most rearranged, corroborating the molecular findings.

The sequence in which *D. tropicalis, D. willistoni, D. equinoxialis* and *D. paulistorum* split was previously found by Gleason *et al*. (1998) with *Per* and *Adh* genes and by Robe *et al*. (2010), with *Adh.* According to Zhang *et al.* (2015) (reviewing Page and Charleston,1997; Sang *et al.* 1997; Pamilo and Nei, 1988 and Calvino *et al.,* 2008), despite the improvement in the resolution of phylogenetic relationships, larger DNA datasets (specially different loci that are not genetically linked or from from a different genome) may present some discordant phylogenetic information.

Zachos (2009) stated that different characters may have non-concordant histories and one correct character phylogeny does not necessarily reflect the true phylogeny of the taxa of interest. Ayala and Kwiatowski (1999) also stated that a gene phylogeny may not represent a species phylogeny. However, they believe that more reliable phylogenetic relationships will be reached when unrelated genes yield similar phylogenies.

Diversification of the *willistoni* subgroup, according to our estimation, apparently began around 8-6.5 MYA. We hypothesize that the origin and further diversification of the *willistoni* subgroup took place in the North region of South America, at Amazonian area. Most of the species of the subgroup lives in sympatry in this area, except for *D. insularis*, which occurs in the Antilles and the semispecies *D. paulistorum* Centroamerican, which lives in Central America (Spassky *et al.,* 1971). The geographic distribution corroborates our hypothesis of diversification. Moreover, Haffer (1997) proposed some hypotheses to explain the vertebrate speciation in Amazonia and this explanation could be extrapolated to *willistoni* subgroup speciation process, as already suggest by Spassky *et al*. (1971) and reviewed in Ehrmann and Powell (1982).

D. paulistorum semispecies diversification began around 1.0 MYA, during the Pleistocene. Haffer (1967, 1969) theorized a historical explanation to geographical distribution patterns of some closely related bird species in Tropical America. According to Spassky *et al*. (1971), there is a resemblance between those bird species and *D. paulistorum* semispecies. During dry periods of the Pleistocene, the Amazonian forest was divided into smaller patches of forest, surrounded by areas of non-forest open vegetation (Haffer, 1967, 1969). These forest areas acted as “refuge areas” for taxa dependent on the humidity, promoting a rapid differentiation of the Amazonian fauna in a very recent geological time (Haffer, 1967, 1969). Spassky *et al*. (1971)

related *D. paulistorum* semispecies distribution with known Haffer's refuges. Transitional semispecies occurs alone in Chocó refuge and in the mountains near the Caribbean coast of Colombia and Venezuela. Centroamerican semispecies distribution coincides with Haffer's Centroamerican refuge. Orinocan and Amazonian semispecies may be derived from Catatumbo, Imeri, Guyana or Belém refuges. Interior distribution corresponds to Napo or Imori refuges. Andean-Brazilian may have expanded from the East Peruvian refuge (Spassky *et al*., 1971).

Morphological characters and the different genes evolve at distinct rates. A group of species also do not evolve as a single unit, each species and incipient species evolve at its own rate. Considering this, we could infer that the best approach to analyze the phylogenetic relationships in a heterogeneous group, which includes taxa with the speciation process already completed and taxa in active process of evolution, is total evidence. For this reason, we proposed that, to this species group, more conserved genes and or with a slower evolutionary rate will help establish and explain the relationships between the species which diverged first and the faster evolving genes and genitalia characters are useful to separate the recent speciated and incipient species.

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Figure 1. Average and standard deviations of Kimura 2-parameters genetic distances obtained for each of the eight data partitions at different taxonomic levels within *D. willistoni* species group.

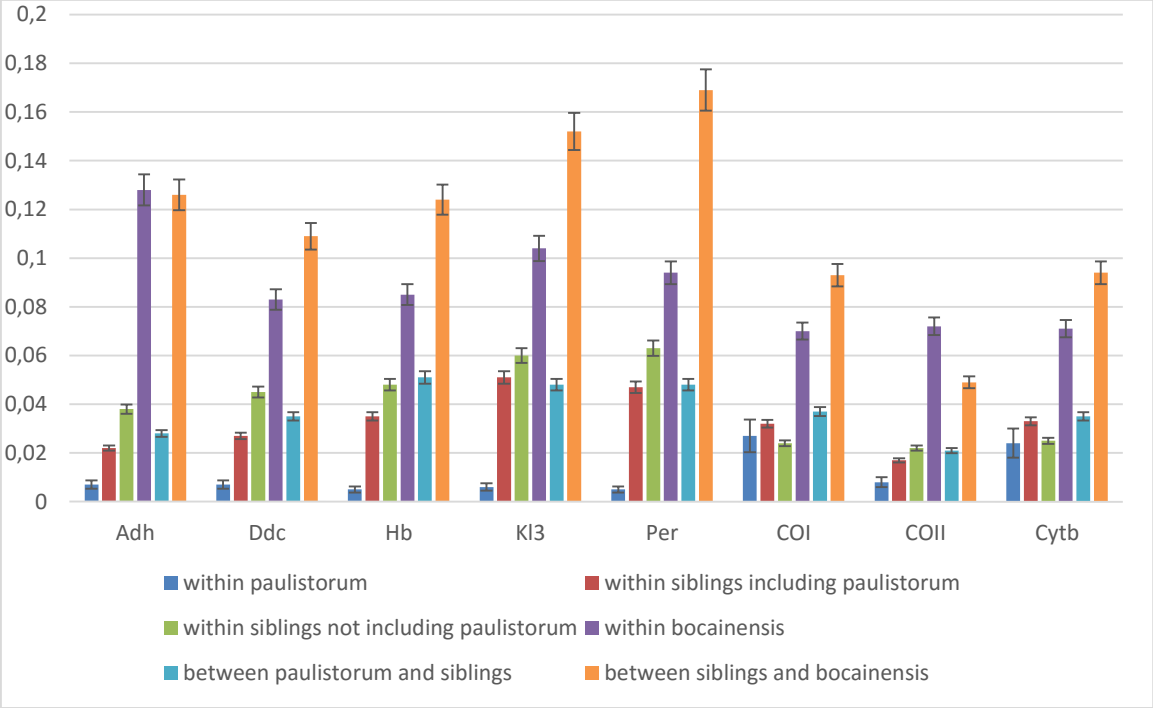
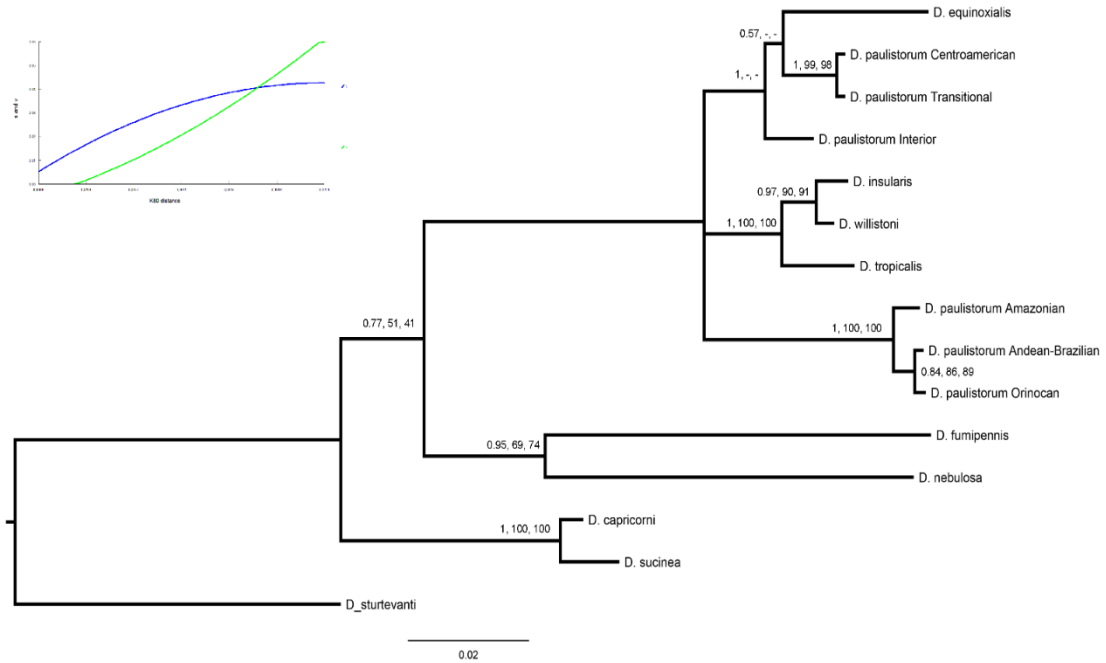
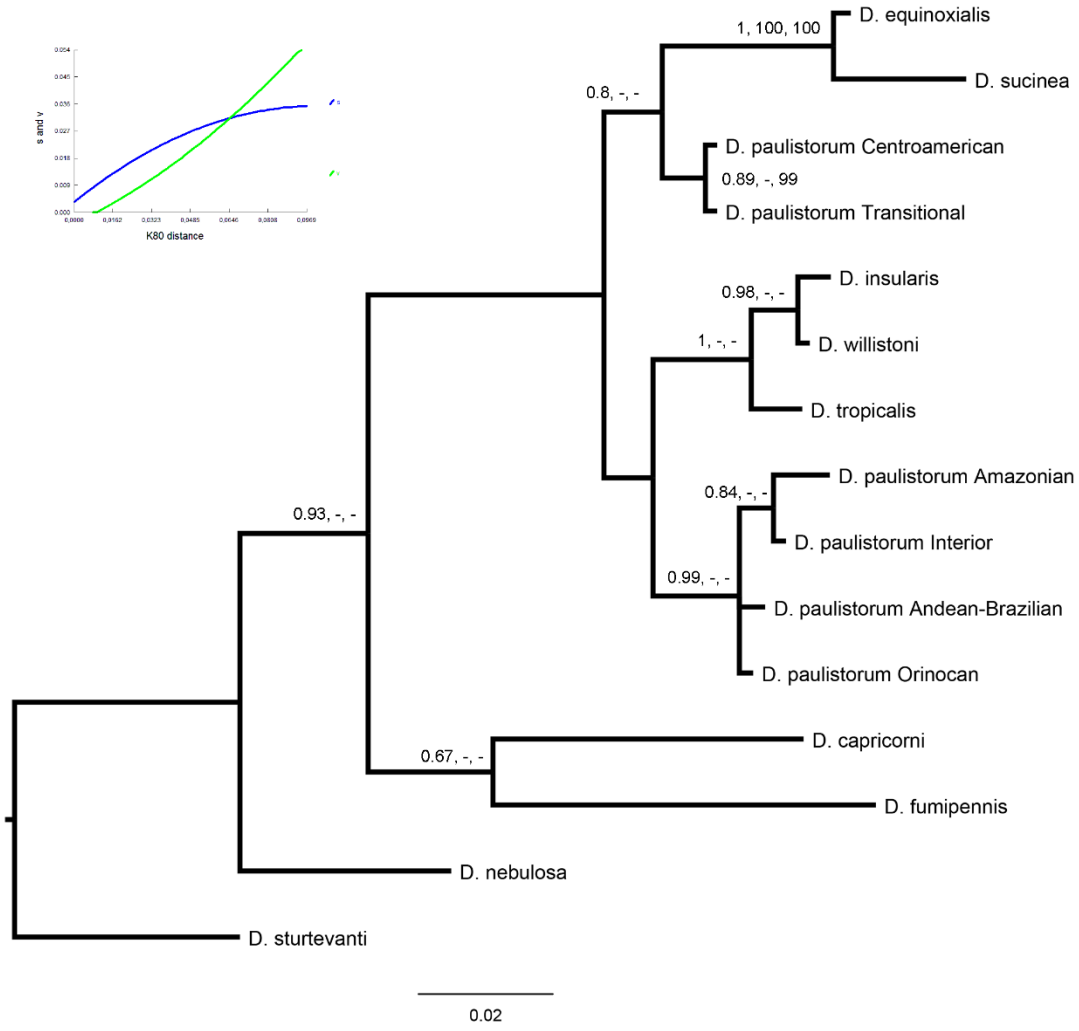


Figure 2. Bayesian trees obtained with mitochondrial genes and saturation plots presented for each individual partition (A. *COI;* B. *COII;* C.*Cytb*). The numbers above the nodes are Bayesian Posterior Probabilities (PP) and -bootstrap values for Maximum Likelihood and Neighbor-Joining trees.

A.



B.


s and v
K80 distance
1, 100, 100
D. equinoxialis
D. sucinea
0.8, -, -
D. paulistorum Centroamerican
0.89, -, 99
D. paulistorum Transitional
0.98, -, -
D. insularis
1, -, -
D. willistoni
D. tropicalis
0.84, -, -
D. paulistorum Amazonian
D. paulistorum Interior
0.99, -, -
D. paulistorum Andean-Brazilian
D. paulistorum Orinocan
0.93, -, -
0.67, -, -
D. capricorni
D. fumipennis
D. nebulosa
D. sturtevanti
0.02

C.

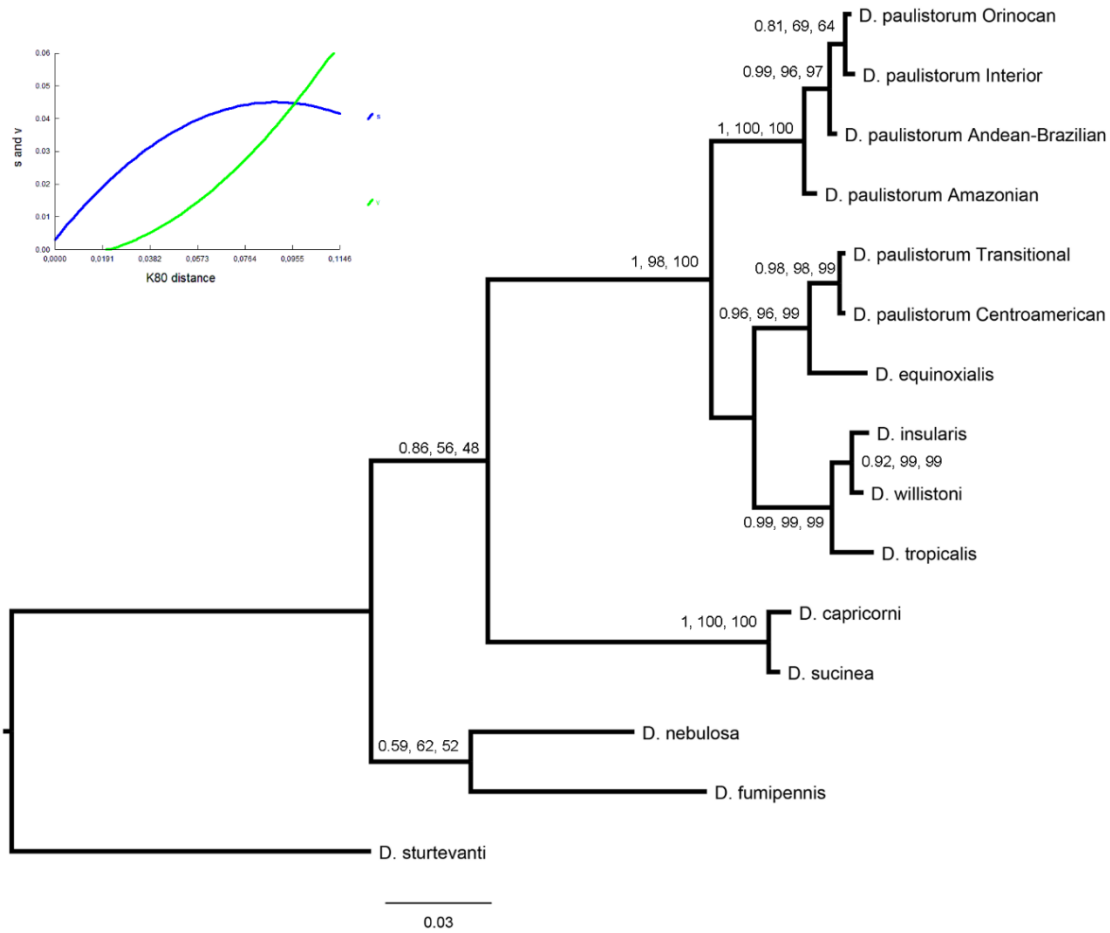

s and v
K80 distance
0.81, 69, 64
D. paulistorum Orinocan
0.99, 96, 97
D. paulistorum Interior
1, 100, 100
D. paulistorum Andean-Brazilian
D. paulistorum Amazonian
1, 98, 100
0.98, 98, 99
D. paulistorum Transitional
0.96, 96, 99
D. paulistorum Centroamerican
D. equinoxialis
D. insularis
0.92, 99, 99
D. willistoni
0.99, 99, 99
D. tropicalis
0.86, 56, 48
1, 100, 100
D. capricorni
D. sucinea
0.59, 62, 52
D. nebulosa
D. fumipennis
D. sturtevanti
0.03

Figure 3. Bayesian trees obtained with combined mitochondrial genes dataset (*COI, COII, Cytb*). The numbers above the nodes are Bayesian Posterior Probabilities (PP) and - bootstrap values for Maximum Likelihood and Neighbor-Joining trees.

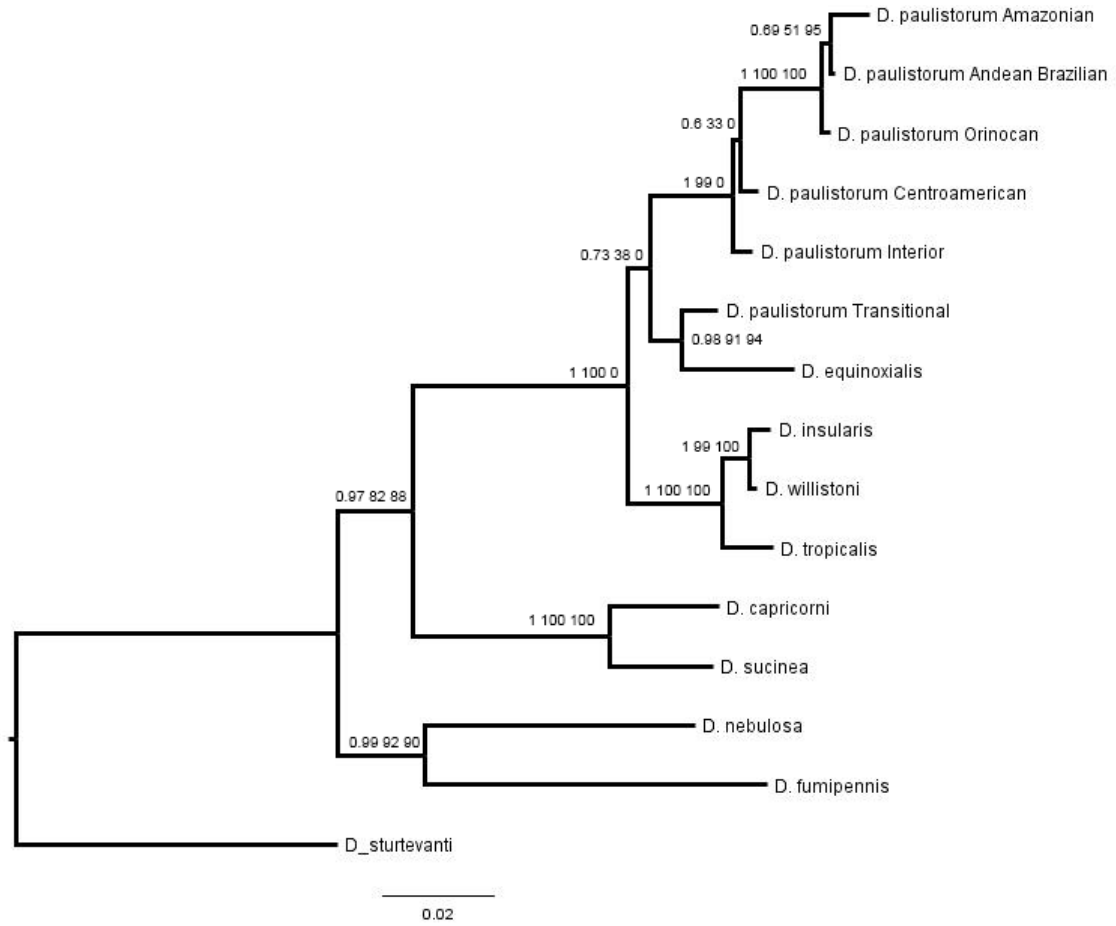
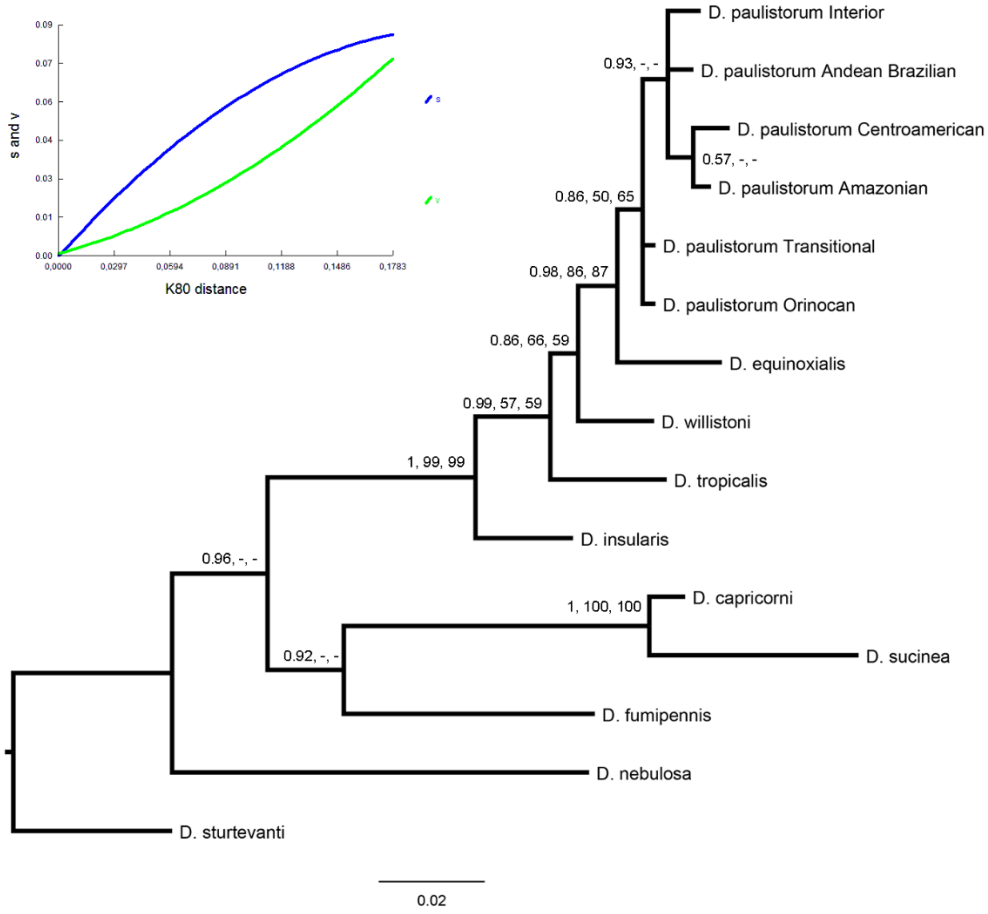
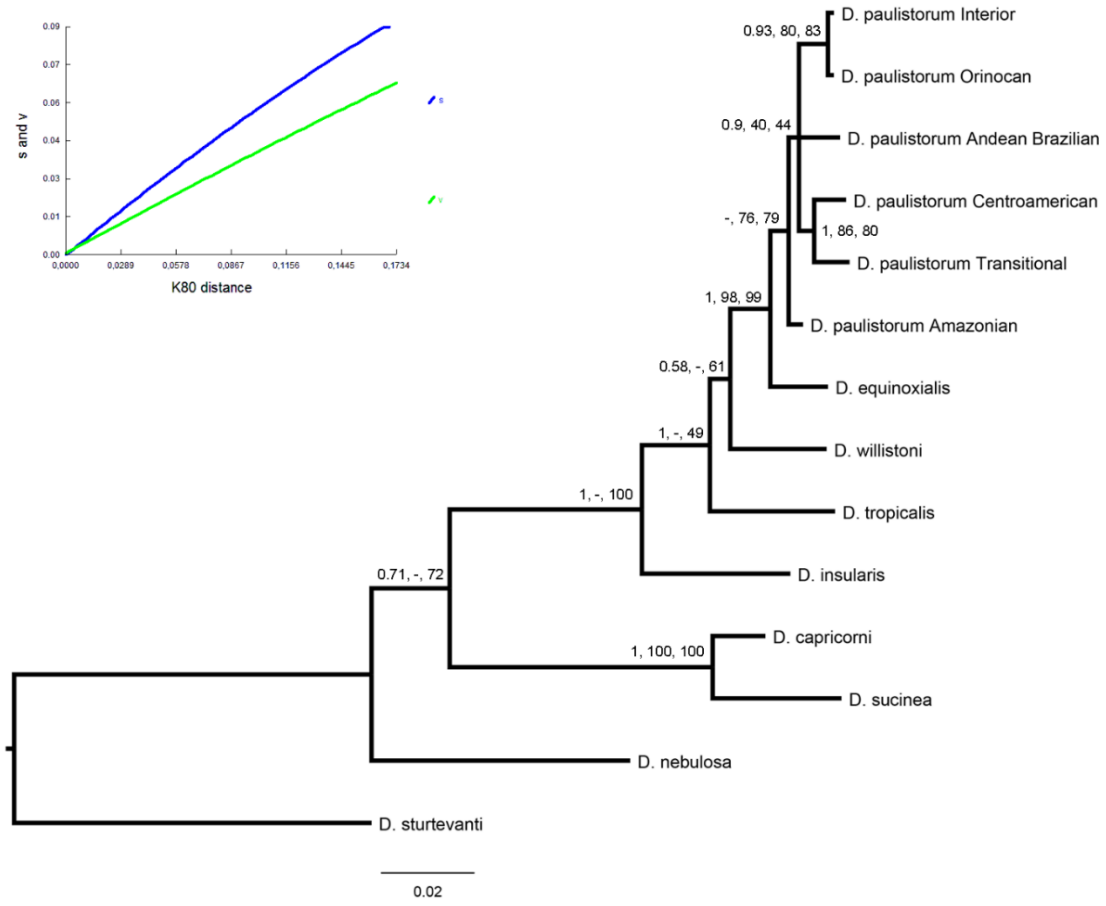


Figure 4. Bayesian trees obtained with nuclear genes and saturation plots presented for each individual partition (A. *Adh;* B. *Ddc;* C.*Hb;* D. Kl3; E. *Per*). The numbers above the nodes are Bayesian Posterior Probabilities (PP) and -bootstrap values for Maximum Likelihood and Neighbor-Joining trees.

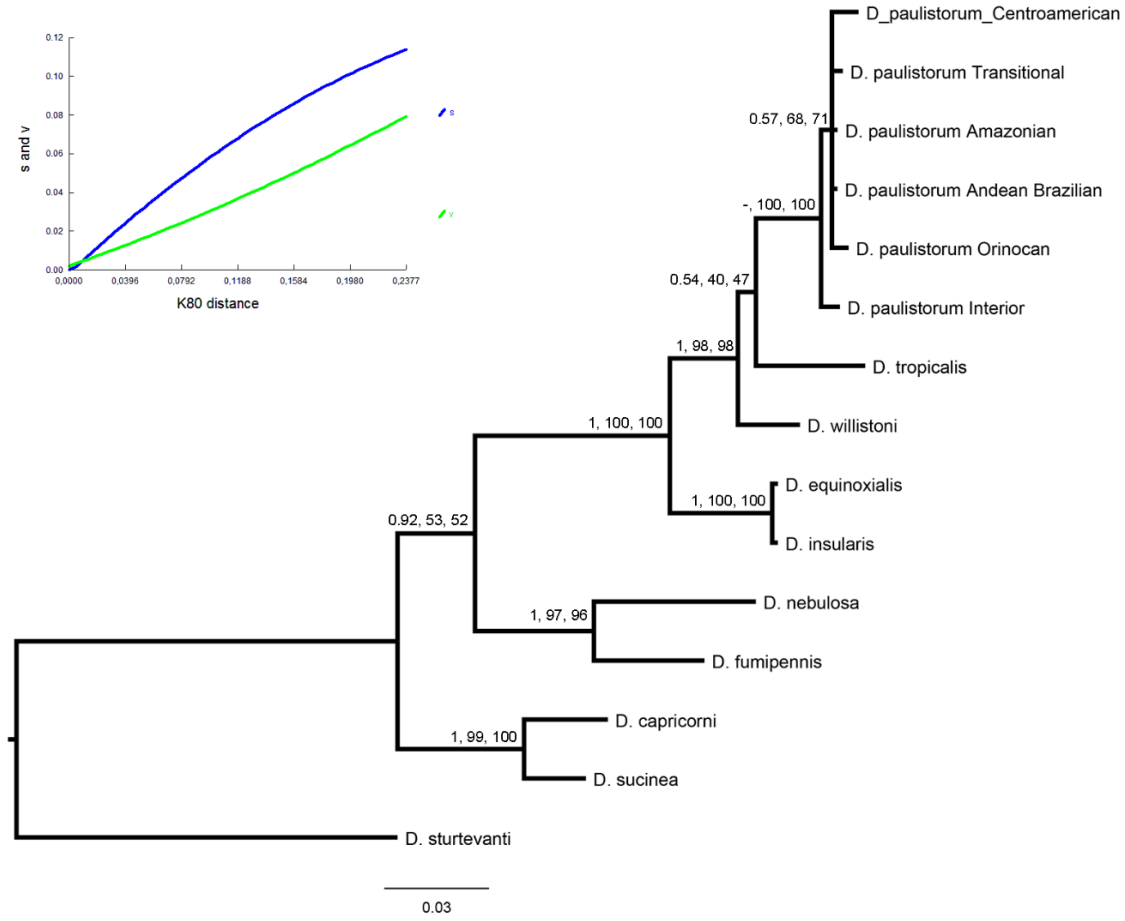
A.



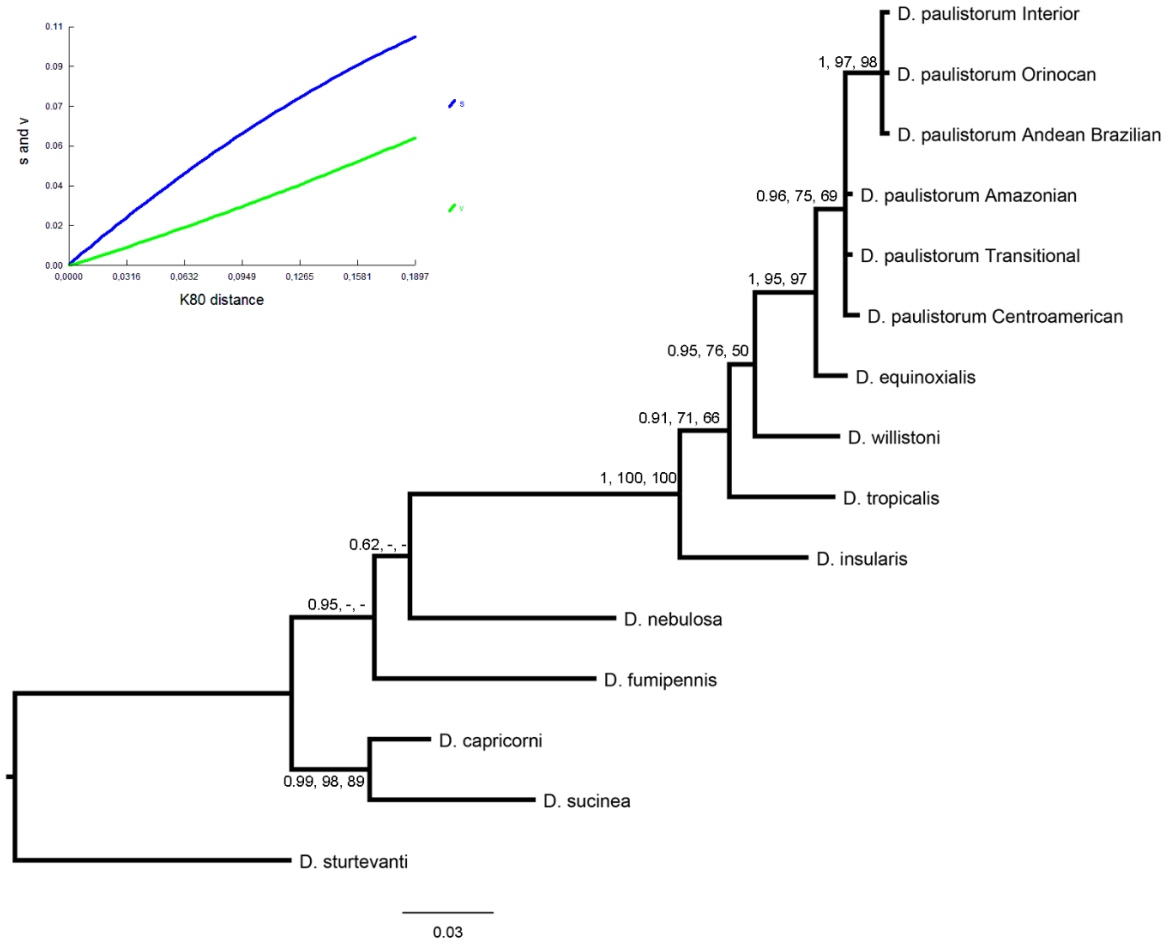
B.


s and v
K80 distance
0.00
0.01
0.03
0.04
0.06
0.07
0.09
0,0000
0,0289
0,0578
0,0867
0,1156
0,1445
0,1734
D. paulistorum Interior
D. paulistorum Orinocan
D. paulistorum Andean Brazilian
D. paulistorum Centroamerican
D. paulistorum Transitional
D. paulistorum Amazonian
D. equinoxialis
D. willistoni
D. tropicalis
D. insularis
D. capricorni
D. sucinea
D. nebulosa
D. sturtevanti
0.93, 80, 83
0.9, 40, 44
-, 76, 79
1, 86, 80
1, 98, 99
0.58, -, 61
1, -, 49
1, -, 100
0.71, -, 72
1, 100, 100
0.02

C.


0.12
0.10
0.08
0.06
0.04
0.02
0.00
s and v
0,0000
0,0396
0,0792
0,1188
0,1584
0,1980
0,2377
K80 distance
s
v
D_paulistorum_Centroamerican
D. paulistorum Transitional
0.57, 68, 71
D. paulistorum Amazonian
-, 100, 100
D. paulistorum Andean Brazilian
D. paulistorum Orinocan
0.54, 40, 47
D. paulistorum Interior
1, 98, 98
D. tropicalis
1, 100, 100
D. willistoni
D. equinoxialis
1, 100, 100
0.92, 53, 52
D. insularis
D. nebulosa
1, 97, 96
D. fumipennis
D. capricorni
1, 99, 100
D. sucinea
D. sturtevanti
0.03

D.


s and v
K80 distance
0.0000
0.0316
0.0632
0.0949
0.1265
0.1581
0.1897
0.00
0.02
0.04
0.06
0.07
0.09
0.11
s
v
D. paulistorum Interior
D. paulistorum Orinocan
D. paulistorum Andean Brazilian
D. paulistorum Amazonian
D. paulistorum Transitional
D. paulistorum Centroamerican
D. equinoxialis
D. willistoni
D. tropicalis
D. insularis
D. nebulosa
D. fumipennis
D. capricorni
D. sucinea
D. sturtevanti
1, 97, 98
0.96, 75, 69
1, 95, 97
0.95, 76, 50
0.91, 71, 66
1, 100, 100
0.62, -, -
0.95, -, -
0.99, 98, 89
0.03

E.

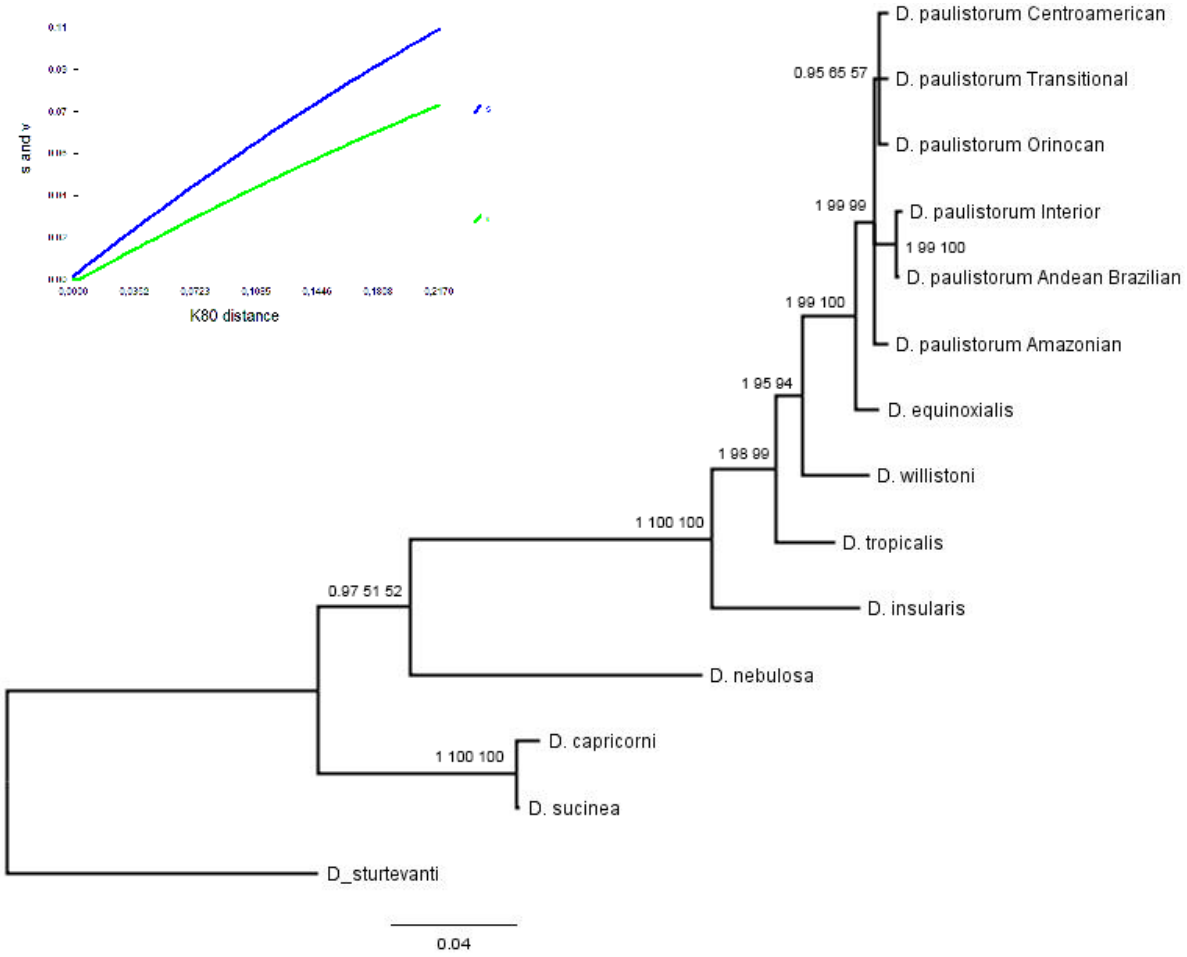

s and v
K80 distance
D. paulistorum Centroamerican
0.95 65 57
D. paulistorum Transitional
D. paulistorum Orinocan
1 99 99
D. paulistorum Interior
1 99 100
D. paulistorum Andean Brazilian
1 99 100
D. paulistorum Amazonian
1 95 94
D. equinoxialis
1 98 99
D. willistoni
1 100 100
D. tropicalis
0.97 51 52
D. insularis
D. nebulosa
1 100 100
D. capricorni
D. sucinea
D_sturtevanti
0.04

Figure 5. Bayesian trees obtained with combined nuclear genes dataset A- (*Adh, Kl3, Hb, Ddc, Per*). The numbers above the nodes are Bayesian Posterior Probabilities (PP) and -bootstrap values for Maximum Likelihood and Neighbor-Joining trees.

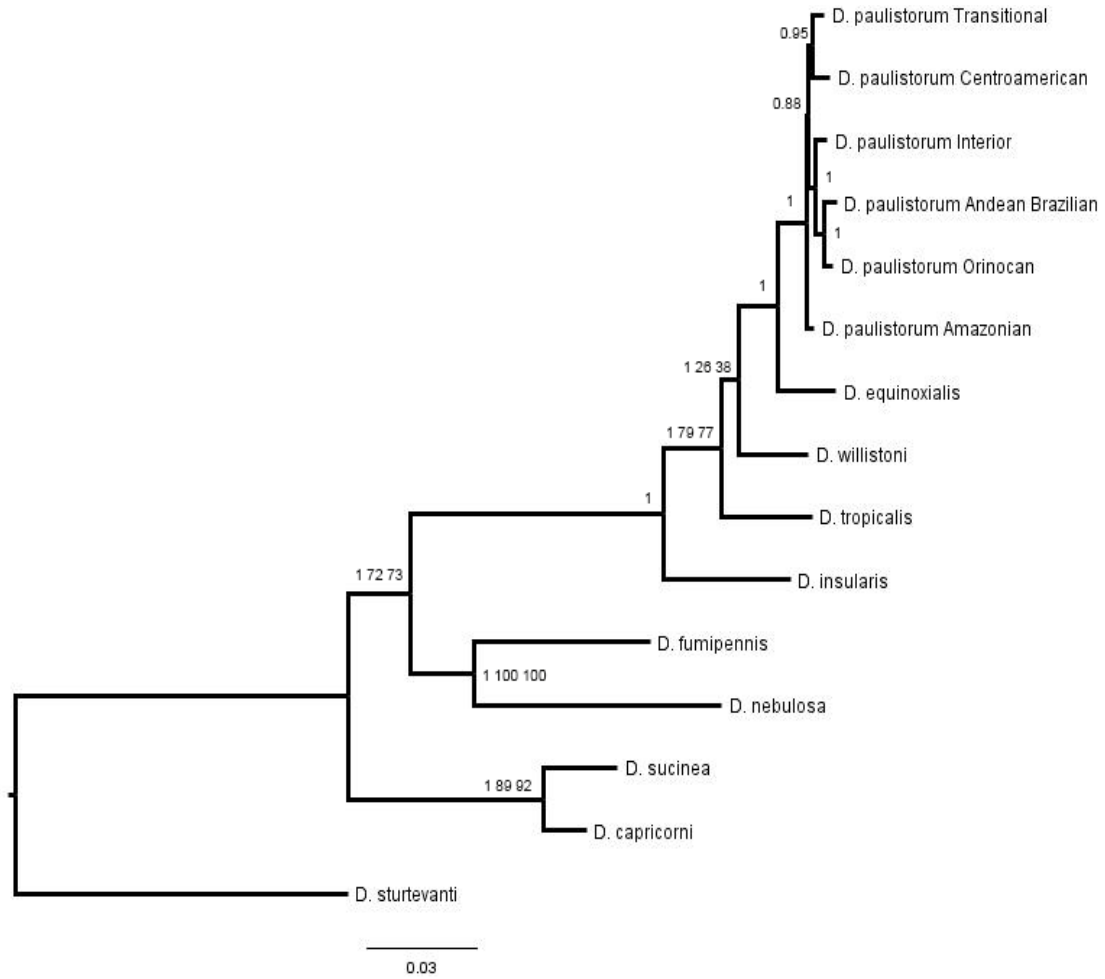
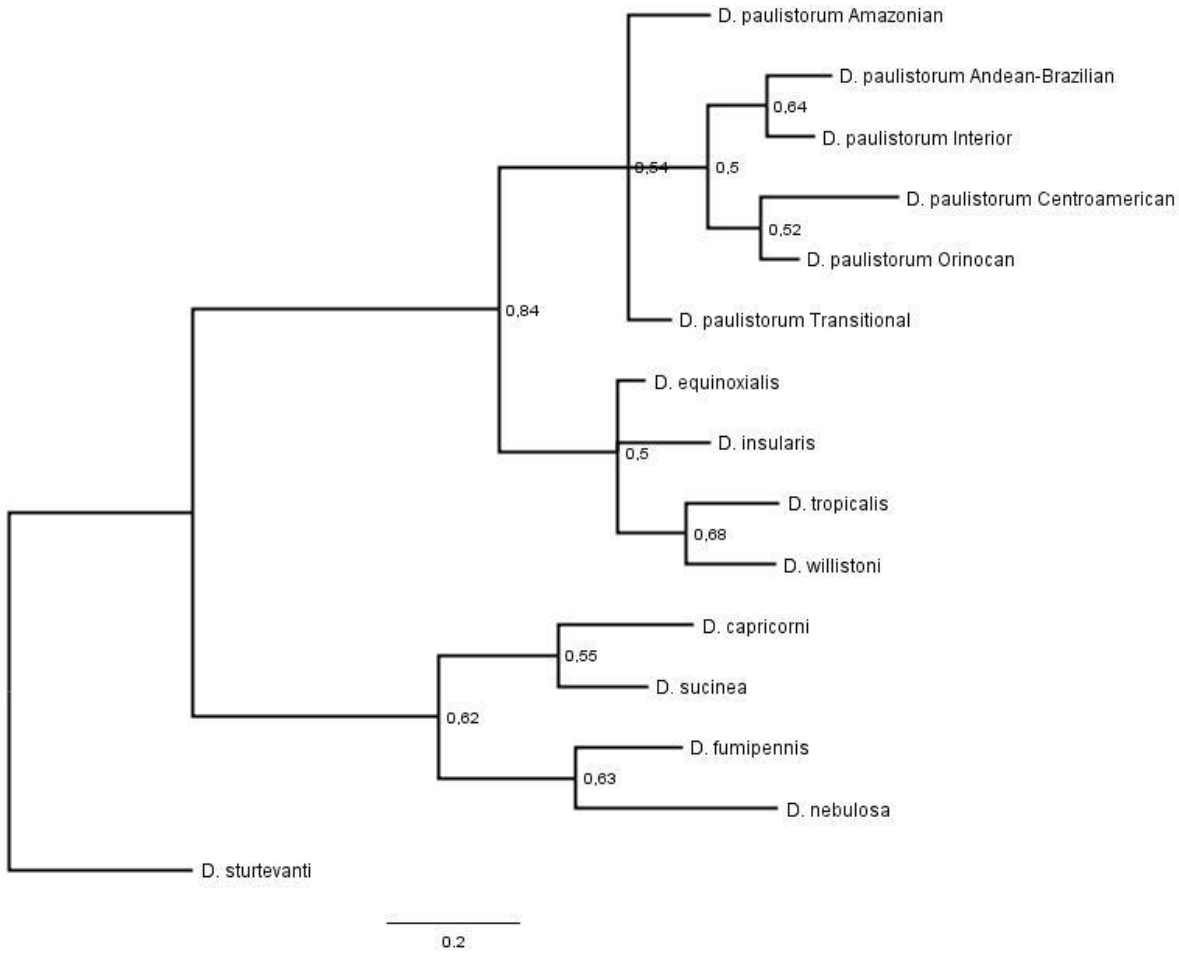


Figure 6. Morphological trees. A. Bayesian tree obtained with 45 morphological characters dataset. The numbers above the nodes are Bayesian Posterior Probabilities (PP). B. Maximum Parsimony tree obtained with morphological characters. The numbers above the nodes are bootstrap values.

A.



B.

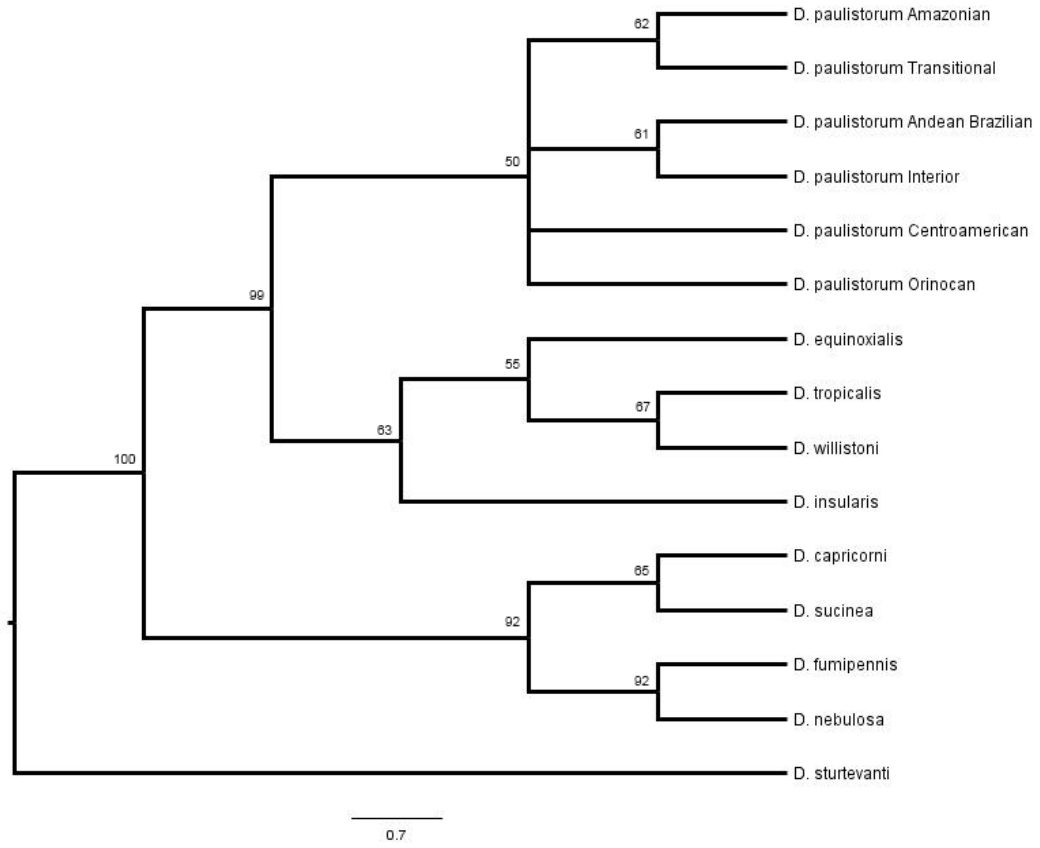

D. paulistorum Amazonian
D. paulistorum Transitional
D. paulistorum Andean Brazilian
D. paulistorum Interior
D. paulistorum Centroamerican
D. paulistorum Orinocan
D. equinoxialis
D. tropicalis
D. willistoni
D. insularis
D. capricorni
D. sucinea
D. fumipennis
D. nebulosa
D. sturtevanti
62
61
50
99
55
67
63
100
65
92
92
0.7

Figure 7. Bayesian trees obtained with mixed dataset – combined morphological characters and mitochondrial genes dataset (*COI, COII, Cytb*). The numbers above the nodes are Bayesian Posterior Probabilities (PP).

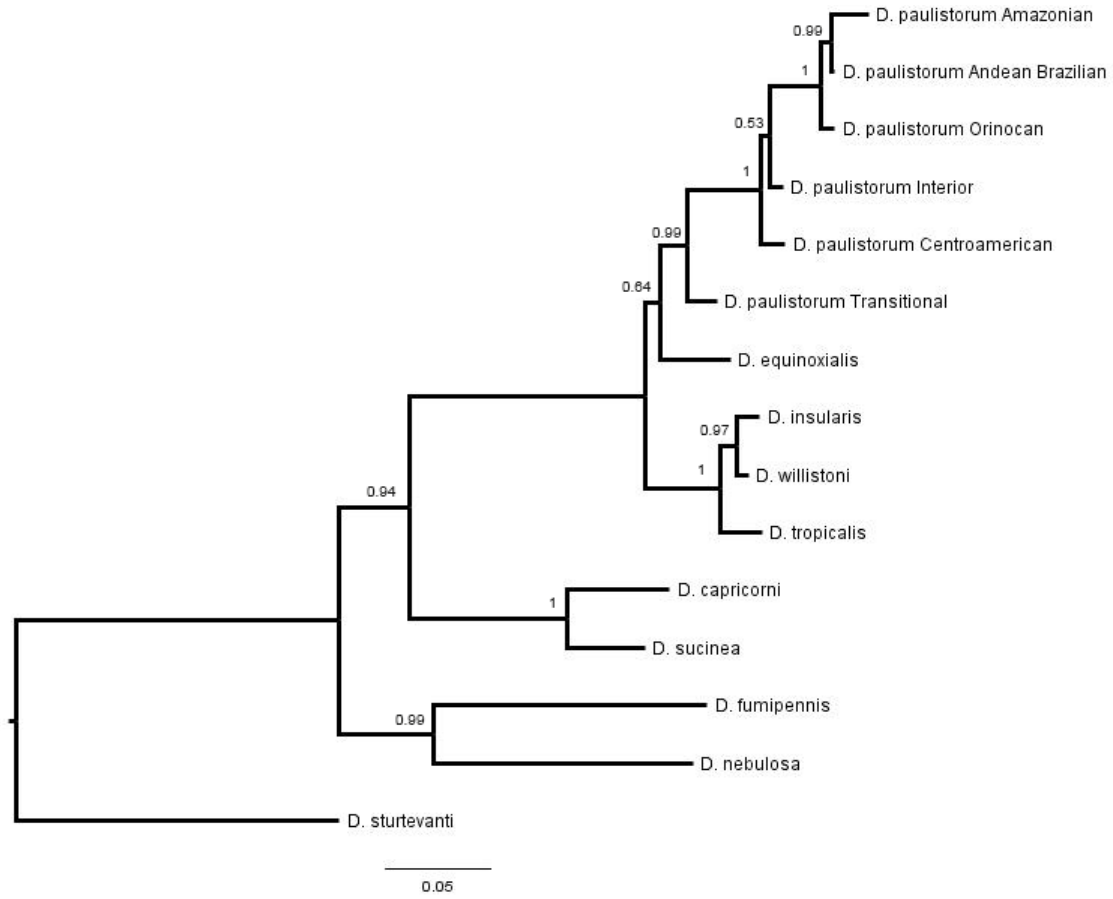


Figure 8. Bayesian trees obtained with mixed dataset – combined morphological characters and nuclear genes dataset (*Adh, Kl3, Hb, Ddc, Per*). The numbers above the nodes are Bayesian Posterior Probabilities (PP).

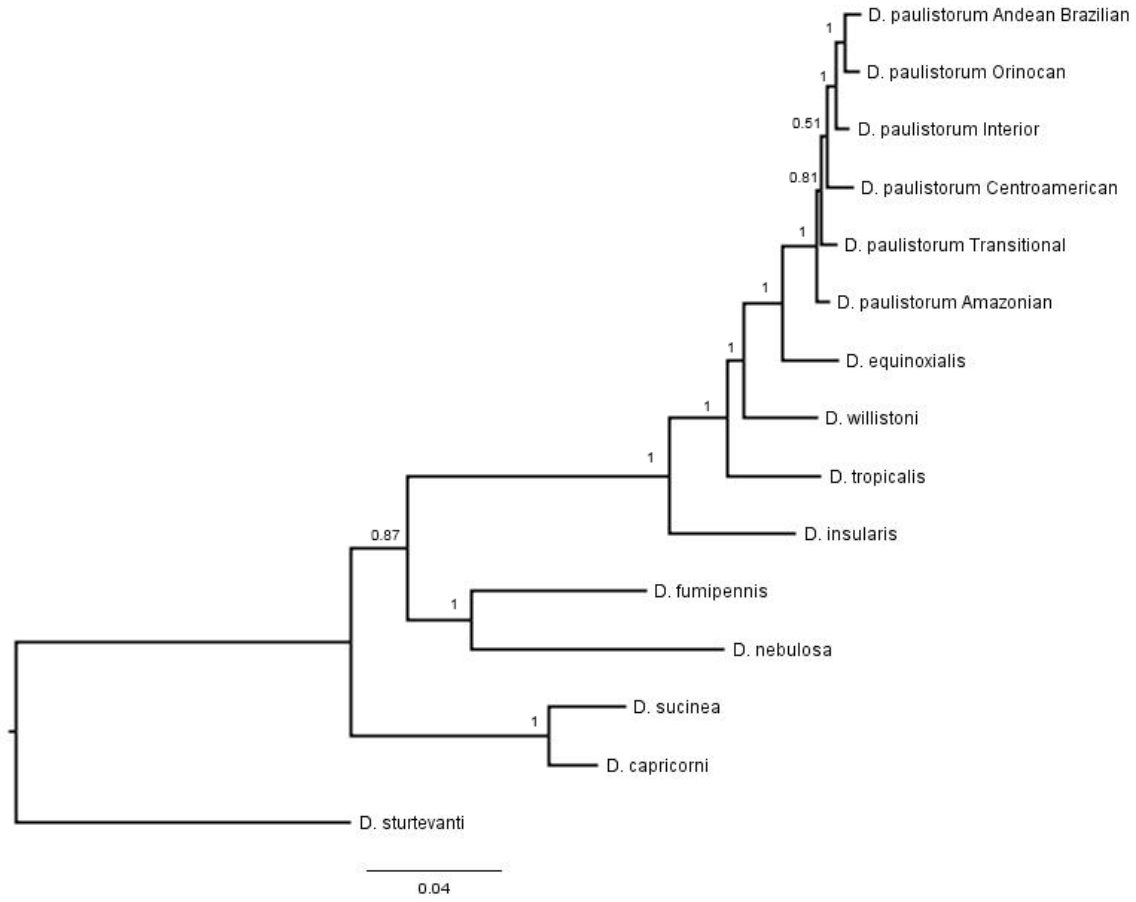


Figure 9. Bayesian trees obtained with Total Evidence Molecular Dataset, including the nuclear genes (*Adh, Kl3, Hb, Ddc* and *Per*) and the mitochondrial genes (*COI, COII* and *Cytb*). The numbers above the nodes are Bayesian Posterior Probabilities (PP), and bootstrap values for Maximum Likelihood and Neighbor-Joining trees respectively.

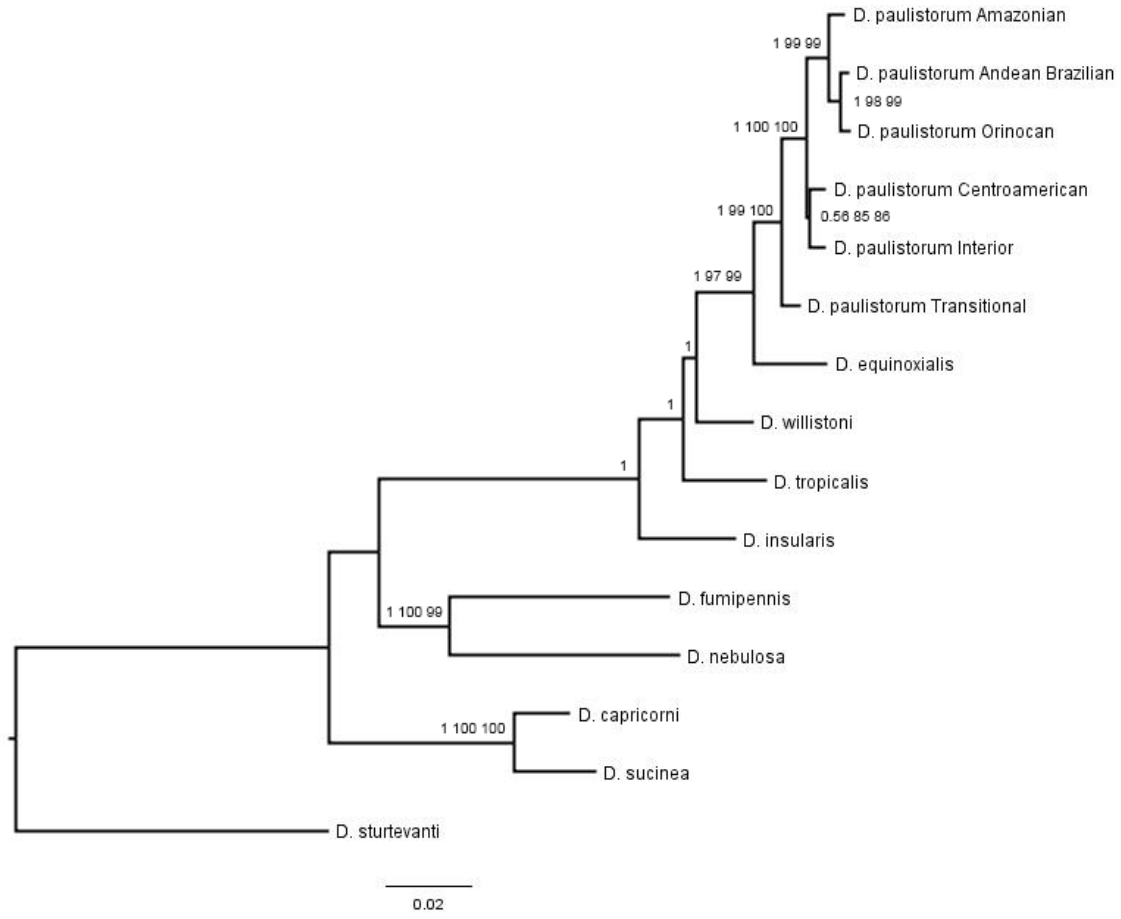


Figure 10. Total evidence tree - Bayesian tree obtained with mixed dataset – combined morphological characters, nuclear genes dataset (*Adh, Kl3, Hb, Ddc, Per*) and mitochondrial genes dataset (*COI, COII, Cytb*). The numbers above the nodes are Bayesian Posterior Probabilities (PP) and bootstrap values for Maximum Likelihood and Neighbor-Joining trees.

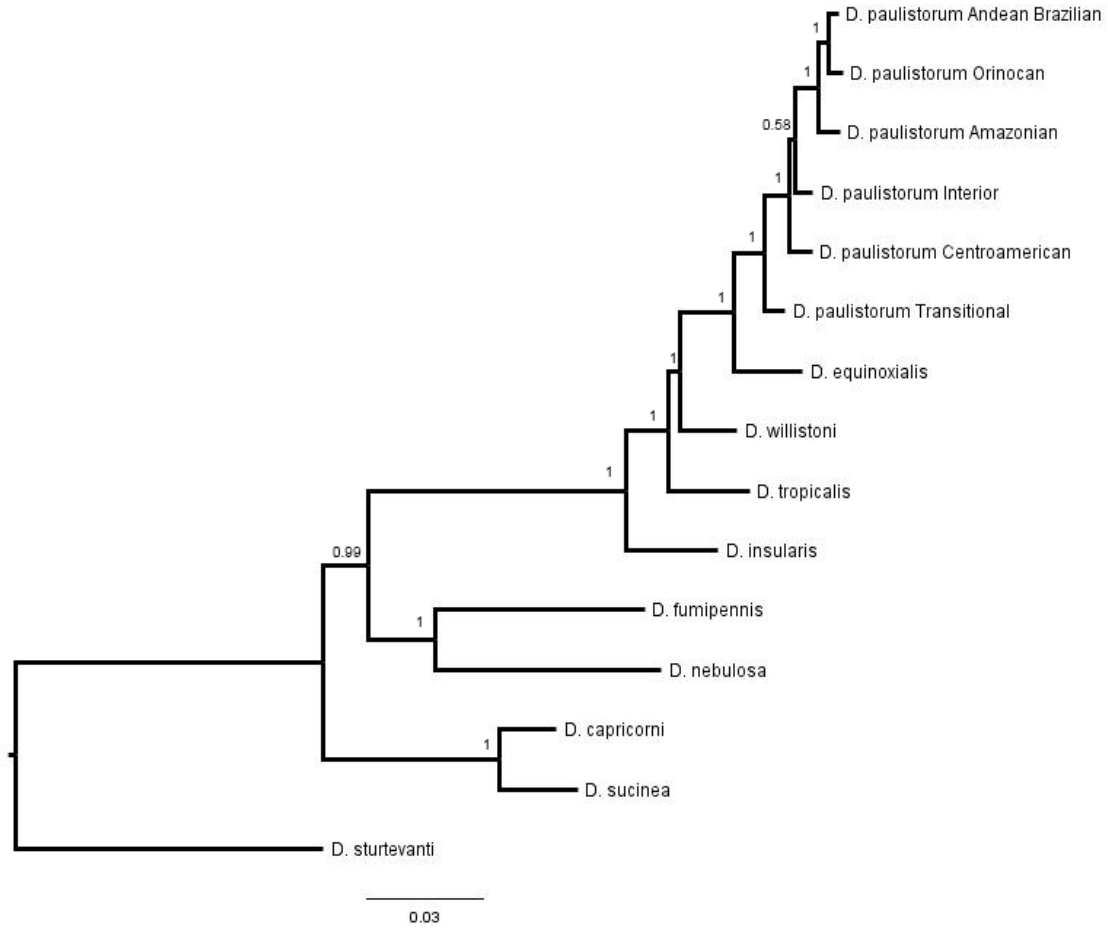


Figure 11. Divergence time estimation with one calibration point based on nuclear data partition. The values above the branches are the ages of the nodes.

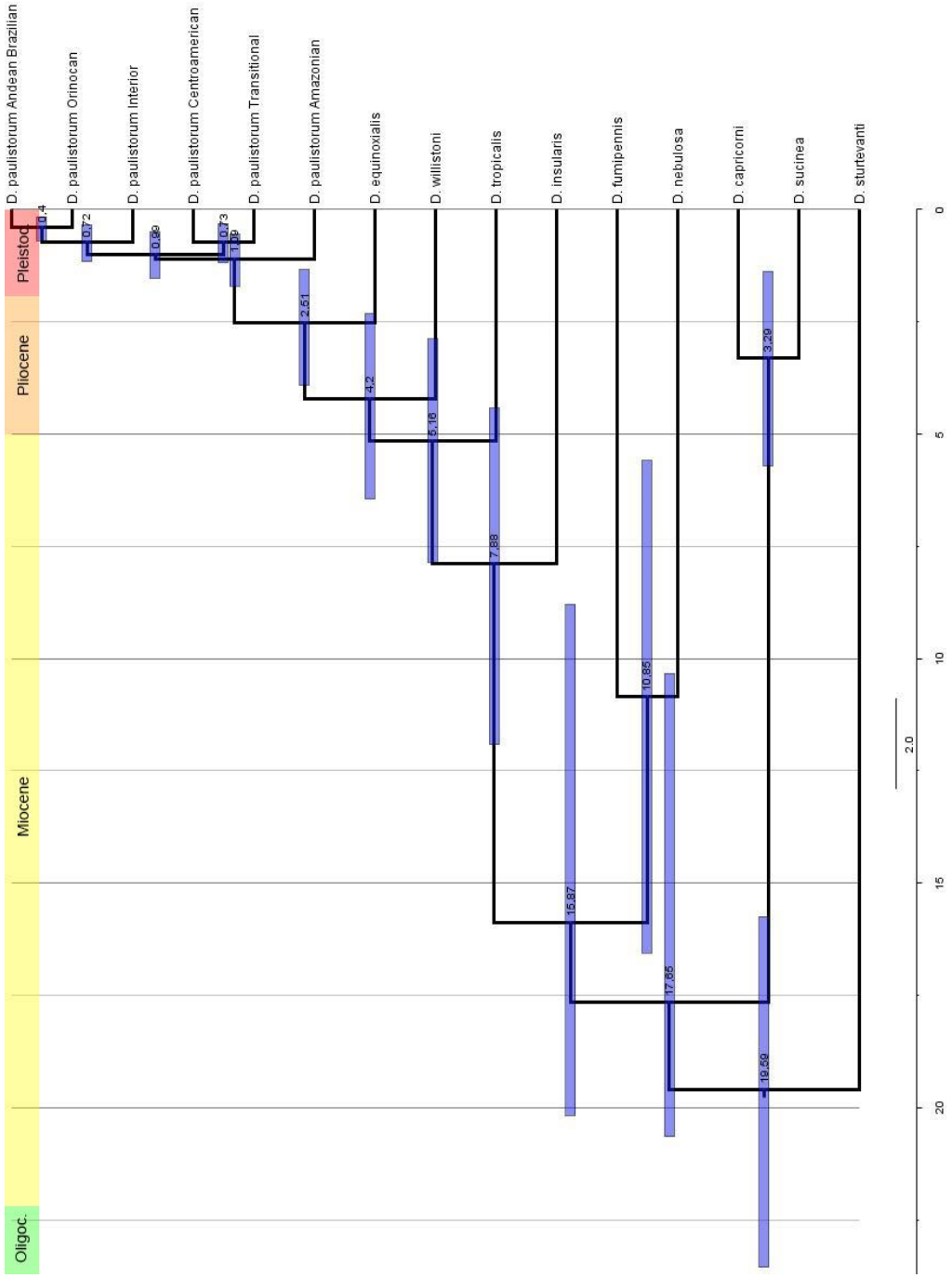


Figure 12. Divergence time estimation with two calibration points based on nuclear data partition. The values above the branches are the ages of the nodes.

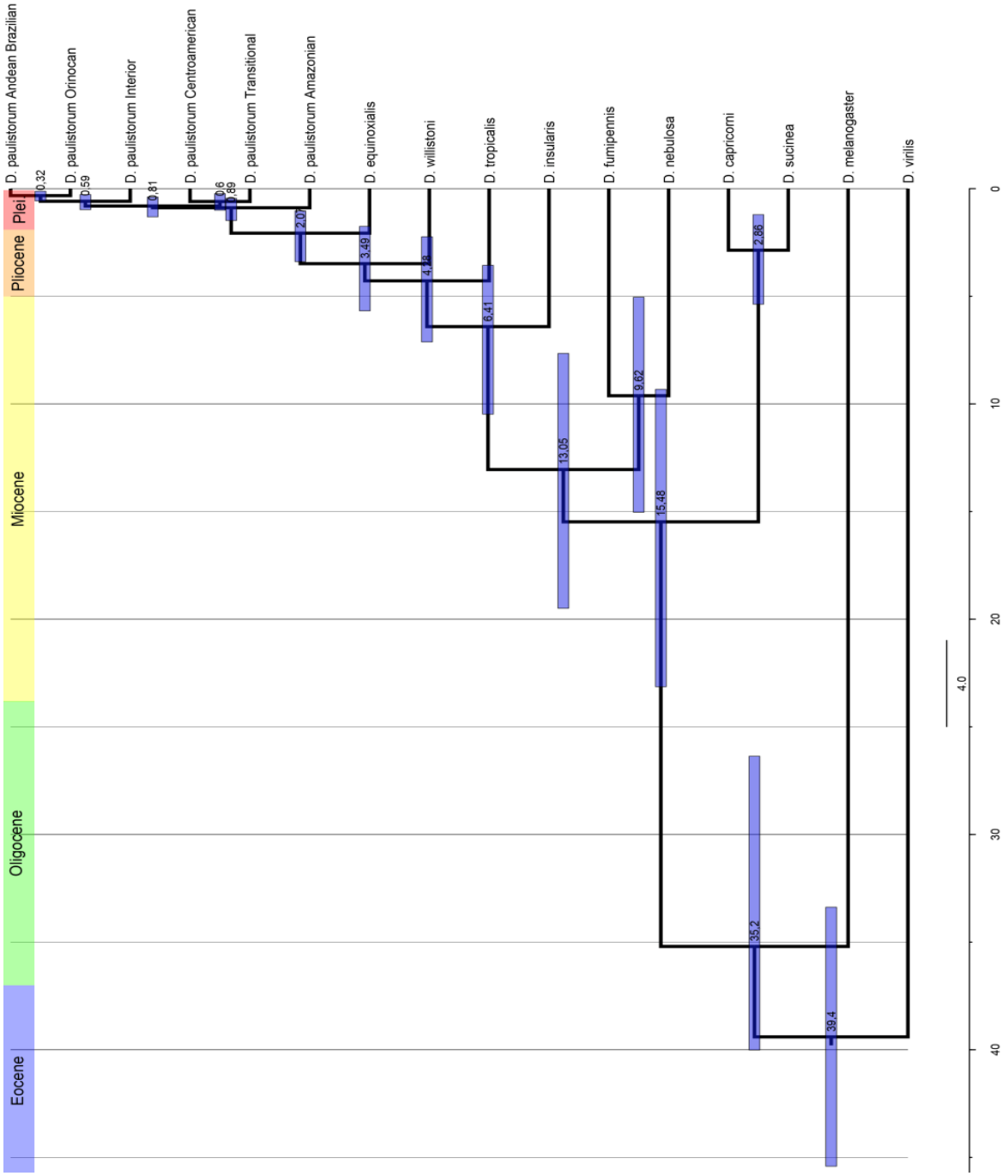


Table 1. Species and strains used in this study, with source and GenBank accession numbers.

Subgroup	Species	Semispecies	Group	Source	*Adh*	*Ddc*	*Kl3-Y*	*Hb*	*Per*	*COI*	*COII*	*Cytb*
willistoni	*D. equinoxialis*		*willistoni*	Apazapán/Mexico	X	X	X	-	-	X	X	X
				Tefé/Brazil				EU532101	U51074			
	D. insularis			St. Lucia/Lesser Antilles	-	X	X	-	-	X	X	X
				St. Kitts/Lesser Antilles	EU532120	-	-	EU532098	-	-	-	-
				Lesser Antilles					U51088			
	D. tropicalis			San Salvador/El Salvador	EU532131	X	X	EU532100	U51087	X	EU532095	X
	D. willistoni			Salvador/Brazil	X	X	X	-	-	X	X	X
				Belém do Pará/Brazil				EU532112	-			
				Cuernavaca/Mexico					U51057			
	D. paulistorum	Amazonian		Belém do Pará/Brazil	EU532122	X	X	EU532110	U51080	X	EU532089	X
		Andean Brazilian		Florianópolis/Brazil	EU532126	X	X	EU532111	-	X	EU532085	X
				Mesitas/Colombia					U51081			
		Centroamerican		Lancetilla/Honduras	EU532123	X	X	EU532102	U51082	X	EU532086	X
		Interior		Llanos/Colombia	EU532124	X	X	EU532103	U51083	X	EU532087	X
		Orinocan		Georgetown/Guyana	EU532127	X	X	EU532106	U51084	X	EU532090	X
		Transitional		Santa Marta/Colombia	EU532130	X	X	EU532104	U51085	X	EU532093	X
bocainensis	*D. capricorni*			Joinville/Brazil	-	X	X	EU532113	-	X	EU532079	X
					AF260073	-	-	-	-	-	-	-
				Florianópolis/Brazil				EU532079	-			
				Palmira/Colombia					U51092			
	D. fumipennis			Florianópolis/Brazil	X	-	X	EU532115	-	X	EU532081	X
	D. nebulosa			Pernanbuco/Brazil		X	X	-	-	X	EU532083	X
				Palmira/Colombia	U95275	-	-	-	U51090	-	-	-
				Porto Alegre/Brazil				EU532116	-			

D. sucinea		Mexico City/Mexico	-	X	X	EU532114	-	X	EU532094	X
		Teziutlan/Mexico					U51091			
		Unknown	AY335197	-	-	-	-	-	-	-
D. sturtevanti	*saltans*	Unknown	AY335201	X	X	EU532117	AY335219	X	AF045084	X

Table 2. P values obtained from pairwise PHT performed between each of the eight analyzed molecular data partitions.

	COI	*COII*	*Cytb*	*Adh*	*Ddc*	*Hb*	*Kl3*
COII	0.01*						
Cytb	0.001**	0.001**					
Adh	0.001**	0.001**	0.029^{ns}				
Ddc	0.001**	0.001**	0.001**	0.307^{ns}			
Hb	0.001**	0.001**	0.001**	0.001**	0.001**		
Kl3	0.001**	0.001**	0.014^{ns}	0.193^{ns}	0.940^{ns}	0.001**	
Per	0.001**	0.001**	0.001**	0.289^{ns}	0.154^{ns}	0.001**	0.564^{ns}

ns not significant at 0.01 level

*significant at 0.01 level

**significant at 0.001 level

Supplementary material 1 (S1): Genes and primers used in this study

Gene	Primer	Annealing temperature	Reference
Adh	4682- 5' ACA TYC AGC CAI GAG TTG AAY TTG TG 3'ACA TYC AGC CAI GAG TTG AAY TTGTG 3' 4683 - 5' CTG GGI GGC ATT GGI YTS GAC ACC AC 3'	55ºC	Griffith and Powell (1997)
COI	TY-J-1460 - 5' TAC AAT CTA TCG CCT AAA CTT CAG CC 3' C1-N-2329 - 5' ACT GTA AAT ATA TGA TGA GCT GA 3'	55ºC	Simon *et al.* (1994)
COII	TL2-J-3037 - 5' ATC GCA GAT TAG TGC AAT GG 3' TK-N-3785 - 5' GTT TAA GAG ACC AGT ACT TG 3'	55ºC	Simon *et al.* (1994)
Cytb	CB-J-10933 5' TAT GTT TTA CCT TGA GGA CAA ATA TC 3' TS1-N-11683 5' AAA TTC TAT CTT ATG TTT TTC AAA AC 3'	55ºC	Simon *et al.* (1994)
Ddc	BPF: 5' GAY ATY GAR CGN GTS ATC ATG CCK GG 3' BPR: 5' TSR GTG AAT CGN GAR CAD AYK GCC AT 3'	Touchdown 60º-55ºC	Tatarenkov *et al.* (1999)
Kl3	Kl-3Y_F 5'-AGCCAGGCGTTCAGTAACAC-3' Kl-3Y_R 5'-GATGCCATTGCTACAGTTGACTT-3'	Touchdown 60º-52ºC	Müller (2013)

For the *Hb* gene, see Robe *et al.* (2010) and for *Per* gene, see Gleason and Powell (1997).

Supplementary material S2. Xia's test (Index of substitution saturation)

	Adh	*Ddc*	*Hb*	*Kl3*	*Per*	*COI*	*COII*	*Cytb*
Iss	0.0959	0.1916	0.1412	0.1148	0.1437	0.1237	0.1059	0.0884
Iss.c	0.6981	0.7607	0.7393	0.7045	0.7722	0.7516	0.7384	0.7429
T	35.3674	43.0746	40.1986	35.5082	52.5227	36.6624	49.8726	59.9821
DF	404	947	675	440	1157	818	671	716
Probability	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

(Interpretation of results:

	Significant Difference	
	Yes	No
Iss < Iss.c	Little saturation	Substantial saturation
Iss > Iss.c	Useless sequences	Very poor for phylogenetics

Supplementary material (S3): Estimates of Evolutionary Divergence between Sequences considering total evidence dataset.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. D capricorni		0.015	0.013	0.014	0.014	0.015	0.015	0.014	0.014	0.015	0.014	0.019	0.006	0.015	0.014
2. D equinoxialis	0.102		0.015	0.006	0.015	0.006	0.006	0.005	0.006	0.006	0.004	0.020	0.014	0.007	0.006
3. D fumipennis	0.089	0.105		0.015	0.011	0.015	0.015	0.016	0.015	0.015	0.015	0.020	0.015	0.015	0.014
4. D insularis	0.095	0.034	0.102		0.015	0.007	0.007	0.007	0.007	0.007	0.006	0.020	0.014	0.006	0.004
5. D nebulosa	0.092	0.106	0.079	0.103		0.015	0.014	0.014	0.015	0.015	0.014	0.019	0.015	0.015	0.015
6. D paulistorum Amazonian	0.102	0.038	0.103	0.043	0.102		0.001	0.003	0.003	0.002	0.004	0.020	0.015	0.006	0.006
7. D paulistorum AndeanBrazilian	0.101	0.039	0.103	0.044	0.101	0.005		0.002	0.002	0.001	0.004	0.020	0.015	0.007	0.006
8. D paulistorum Centroamerican	0.100	0.033	0.106	0.043	0.102	0.013	0.012		0.002	0.003	0.003	0.020	0.014	0.006	0.006
9. D paulistorum Interior	0.100	0.035	0.104	0.042	0.102	0.014	0.011	0.007		0.002	0.003	0.020	0.014	0.006	0.005
10. D paulistorum Orinocan	0.102	0.039	0.103	0.044	0.102	0.006	0.002	0.012	0.011		0.004	0.020	0.015	0.007	0.006
11. D paulistorum Transitional	0.097	0.026	0.101	0.038	0.101	0.019	0.020	0.013	0.016	0.021		0.019	0.014	0.006	0.005
12. D sturtevanti	0.130	0.136	0.136	0.137	0.131	0.137	0.135	0.135	0.134	0.135	0.132		0.020	0.020	0.019
13. D sucinea	0.035	0.097	0.102	0.098	0.099	0.101	0.100	0.098	0.099	0.101	0.097	0.137		0.015	0.015
14. D tropicalis	0.104	0.046	0.108	0.034	0.107	0.040	0.041	0.039	0.039	0.041	0.035	0.138	0.105		0.004
15. D willistoni	0.100	0.039	0.101	0.024	0.102	0.032	0.034	0.033	0.032	0.035	0.028	0.133	0.101	0.026	

The number of base substitutions per site from between sequences are shown. Standard error estimate(s) are shown above the diagonal. Analyses were conducted using the Maximum Composite Likelihood model [1]. The analysis involved 15 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 3350 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

Supplementary material (S4). Molecular datasets properties.

	Adh	*Ddc*	*Kl3-Y*	*hb*	*per*	*COI*	*COII*	*Cytb*
N positions in alignment	405	948	441	675	1158	819	672	717
N of OTU's	15	14	15	15	14	15	15	15
N of variable sites	107	219	139	207	374	170	105	147
N of parsimony informative sites	57	120	70	112	213	97	53	96
Average nucleotidic frequency								
A	24.5	21.4	33.3	25.9	25.2	29.8	32.5	32.3
C	24.6	21.8	15.7	30.4	28.9	14.5	13.3	13.5
G	22.0	28.8	21.2	23.4	26.1	16.5	13.2	11.5
T	28.9	28.0	29.8	20.3	19.8	39.2	41.0	42.7
Average nucleotidic frequency 3rd position								
A	12.3	12.7	37.7	18.0	17.0	46.0	42.5	44.9
C	31.9	25.0	14.9	33.0	32.9	5.0	4.0	5.3
G	19.6	28.9	16.9	25.8	23.0	2.4	1.6	1.5
T	36.0	33.0	30.0	23.0	27.0	47.0	52.0	48.0
Transitions/transversions ratio	2.11	2.45	2.23	2.04	2.0	1.45	1.14	3.09
$\chi 2$ test	8.33 (42 df), ns	17.10 (36 df), ns	4.09 (42 df), ns	26.70 (42 df), ns	30.67 (39 df)	5.11 (42 df), ns	6.79 (42 df), ns	6.25 (42 df), ns
Nucleotidic diversity	0.0726±0.0351	0.0734±0.0350	0.0899±0.0432	0.0850±0.0407	0.0895±0.042	0.0611±0.0292	0.0418±0.0203	0.0612±0.0294
Jmodeltest AIC test best fit model	GTR+I+G	GTR+G	GTR+G	GTR+G	HKY+I	GTR+I+G	GTR+G	GTR+I
Proportion of invariable sites	0.4180	-	-	-	0.5530	0.4530	-	0.7050
Gamma distribution shape parameter	0.7190	0.1810	0.3560	0.3730	-	0.5160	0.06440	-
Rate matrix								
AC	0.6360	3.4607	1.8279	1.5547		0.1376	4.1098	3329.3632
AG	2.9794	5.2192	5.3772	3.7227		39.4976	9.3075	10830.0848
AT	1.4936	2.4911	1.6594	1.1062		24.0264	7.5741	5400.5233
CG	1.7426	2.7978	1.031	0.9216		0.0102	0.0011	1.0000
CT	5.6641	12.0137	11.1121	5.8384		86.5936	26.6808	62593.3929
GT	1.0000	1.0000	1.0000	1.0000		1.0000	1.0000	1.0000

	Combined Nuclear	Combined Nuclear -HB	Combined Mitochondrial	Total Evidence Molecular
N positions in alignment	3627	2952	2208	5835
N of OTU's	15	15	15	15
N of variable sites	1046	839	429	1476
N of parsimony informative sites	572	460	253	825
Average nucleotidic frequency				
A	25.3	25.1	31.4	27.7
C	25.2	24.0	13.8	20.8
G	25.2	25.6	13.9	20.8
T	24.3	25.3	40.9	30.8
Average nucleotidic frequency 3rd position				
A	24.6	18.3	44.5	32.3
C	24.1	27.4	4.8	16.6
G	26.3	23.4	1.9	16.9
T	25.0	31.0	49.0	34.0
Transitions/transversions ratio	2.12	2.19	2.03	1.82
$\chi 2$ test	42.23 (42 df),ns	43.13 (42 df) P=0.4227 (ns)	18.02 (42 df), ns	80.25 (42 df),
Nucleotidic diversity	0.0840±0.0397	0.081±0.039	0.0563±0.0267	0.07284±0.0344
Jmodeltest AIC test best fit model	GTR+I+G	GTR+I+G	GTR+I+G	GTR+I+G
Proportion of invariable sites	0.4120	0.4110	0.2530	0.4940
Gamma distribution shape parameter	0.9210	0.9050	0.2030	0.9370
Rate matrix				
AC	1.5854	1.5103	5.6997	2.1755
AG	4.2047	4.2582	33.4687	6.7246
AT	1.5016	1.6003	18.1989	3.0344
CG	1.3341	1.4627	0.0139	2.4697
CT	6.7171	6.9521	92.5101	10.7857
GT	1.0000	1.0000	1.0000	1.0000

Supplemetary material (S5). Morphological character list and character states.

Body

1. Body color: (0) dark; (1) tan; (2) yellow.

Head

2. Aristae, number: (0) 9 or less; (1) 10 or more
3. Oceli, color: (0) dark; (1) light.
4. Carina, shape: (0) short and narrow; (1) flat; (2) elongated, prominent.
5. First flagelomere, color: (0) brown; (1) tan; (2) yellow.
6. First flagelomere, pilosity, size: (0) short; (1) long.
7. First flagelomere, pilosity, density: (0) dense (1) sparse
8. Oral setae, number: (0) 3; (1) 2.

Thorax

9. Thorax, color: (0) brown; (1) tan; (2) yellow.
10. Achrostical setulae, number of rows: (0) 6 or less (1) 7 (2) 8.
11. Achrostical setulae, row shape: (0) irregular; (1) regular.
12. Scutum, stripes: (0) presence; (1) absence.
13. Scutellar setae, position: (0) convergent; (1) parallel; (2) divergent.

Abdomen

14. Abdomen, color: (0) brow/black; (1) yellow.

Wings

15. Wings, coloration: (0) clear; (1) smoked.

Male terminalia

16. Epandrium, size: (0) longer than hypandrium; (1) shorter than hypandrium.
17. Epandrium, pubescence: (0) micropubescent; (1) highly pubescent.
18. Epandrium, setae, number: (0) 18 or more; (1) 17 or less.
19. Epandrium, ventral lobe, shape: (0) very prominent; (1) straight; (2) truncated ou lobated.
20. Hypandrium, size: (0) short; (1) long.
21. Hypandrium, shape: (0) triangular; (1) squared; (2) rectangular.
22. Hypandrium, lobes: (0) absent; (1) present.
23. Hypandrium, lobes, size: (0) large; (1) medium; (2) small.
24. Hypandrium, lobes, shape: (0) elongated; (1) triangular; (2) rounded; (3) trapezoidal; (4) squared; (5) dome; (6) irregular.
25. Hypandrium, teeth: (0) absence; (1) presence.
26. Hypandrium, teeth, size: (0) large (1) medium; (2) small.
27. Hypandrium, size, thickness: (0) thick; (1) thin.
28. Hypandrium, teeth, distance between: (0) appart; (1) close.
29. Hypandrium, teeth, insertion: (0) lobe line; (1) below lobe line; (2) far below the lobe line
30. Hypandrium, chitinous projections: (0) absence, (1) presence
31. Hypandrium, lateral extension: (0) not bifurcated; (1) bifurcated.
32. Hypandrium, lateral extensions: (0) large; (1) small.
33. Hypandrium, lateral extensions, projection: (0) toward; (1) adjacent; (2) below the lobes.
34. Hypandrium, paramedian setae: (0) present; (1) absent.
35. Hypandrium, paramedian setae, position: (0) parallel; (1) divergent; (2) convergent
36. Surstylus, shape: (0) semi-eliptical; (1) straight; (2) sinuous
37. Surstylus, prensisetae, number: (0) 17 or more; (1) 15-16; (2)13-14; (3) 12 or less

38. Surstylus, prensisetae, size: (0) equal; (1) two sizes; (2) crecent sizes.

39. Surstylus, prensisetae, arrengement: (0) more than one row; (1) one row.

40. Surstylus, ventral hook, number of setae: (0) 4; (1) 3; (2) 2; 3 (1)

41. Decasternum, shape: (0) Very large and quitinose; (1) small and thin.

42. Aedeagus, pincer-like paraphysis: (0) absent; (1) present

43. Aedeagus, expansion, localization: (0) dorso-ventral; (1) laterodorsal; (2) lateral.

44. Aedeagus, distiphallus, shape: (0) deeply bifid; (1) rounded; (2) bird beak like.

45. Aedeagal apodeme, distal end, shape: (0) fan-like; (1) rouded; (2) straight.

CAPÍTULO VII. CONCLUSÕES

CONCLUSÕES

O grupo *willistoni* de *Drosophila* é um dos mais representativos da região Neotropical como um todo e também do Brasil, sendo encontrados nos mais diversos biomas brasileiros, tendo como exemplos: Pampa (Poppe *et al*. 2013), Caatinga (Garcia *et al.,* 2014), Amazônia (Martins e Oliveira, 2007), Cerrado (Chaves e Tidon, 2008), Mata Atlântica (De Toni *et al.,* 2007) e Pantanal. Ainda, algumas espécies do grupo *willistoni* são encontradas em ambientes urbanos, como relatado por Valiati *et al.* (1999) e Garcia *et al*. (2012).

Ainda que seja um grupo bastante estudado e com ampla representatividade, pondera-se que sua distribuição e registros de ocorrência sejam subestimados. Diversos levantamentos de distribuição e estudos ecológicos relatam a presença de espécies do grupo *willistoni*, porém não fazem a identificação taxonômica até o nível de espécie (Tidon *et al.,* 1994; De Toni e Hoffmann, 1995; Saavedra *et al.*, 1995; Martins, 2001; Silva *et al.,* 2005). Esse viés ocorre principalmente em relação ao subgrupo *willistoni* de *Drosophila*, cuja identificação à nível de espécie tem sido reportada como difícil ou imprecisa devido à grande similaridade entre as espécies e a variação intraespecífica. A distribuição das espécies incipientes de *D. paulistorum* é especialmente subestimada, uma vez que até pouco tempo não se considerava possível identifica-las exceto por meio de testes de cruzamentos e análises cromossômicas, que não são técnicas aplicadas rotineiramente a material proveniente de coleta, por demandarem muito tempo e esforço. Parte dessa dificuldade é devida, possivelmente, ao fato das descrições e estudos clássicos do subgrupo *willistoni* terem sido feitos por geneticistas e não por zoólogos.

Mesmo assim, foram feitos grandes avanços em relação à identificação das espécies incipientes do complexo *D. paulistorum.* Os primeiros relatos baseavam-se apenas em análises de cruzamentos entre linhagens provenientes de diferentes localidades (Burla *et al.*, 1949). Estudos posteriores avaliaram possíveis caracteres morfológicos úteis para a identificação de cada uma das semiespécies (Spassky, 1957; Pasteur, 1970), estudos cromossômicos (Burla *et al.,* 1949; Rhode *et al.,* 2006), ensaios enzimáticos (Ayala *et al.,* 1970; Richmond *et al.,* 1971; Ayala e Powell, 1972; Garcia et al. 2006), avaliação da presença do endossimbionte *Wolbachia* e seu possível papel na especiação (Miller *et al.* 2010). Uma das grandes contribuições para uma identificação rápida das espécies do subgrupo críptico, incluindo as semi-espécies do complexo *D. paulistorum,* foi apresentada nesta tese por Zanini *et al*. (2015c), no qual são apresentadas diferenças morfológicas entre as genitálias masculinas das espécies, observadas em Microscopia de Luz e Microscopia Eletrônica de Varredura.

Quanto à análise de estágios pré-adultos do grupo, observou-se que, embora existam algumas diferenças entre as espécies e semi-espécies do grupo *willistoni* de *Drosophila* elas parecem estar restritas ao formato dos filamentos respiratórios. Em relação aos estágios de larva e pupa, não foram observadas diferenças marcantes nem entre as espécies dos subgrupos *willistoni* e *bocainensis*, diferindo do que é observado entre espécies próximas pertencentes ao grupo *melanogaster* (Sucena e Stern, 2000; Wipfler *et al.*, 2013).

Os nossos estudos filogenéticos considerando tanto sequências de DNA como caracteres morfológicos também são inéditos para esse grupo de espécies. Eles solidificaram relações entre as espécies do grupo já previamente relatadas e ajudaram na resolução de alguns relacionamentos evolutivos, principalmente entre as semi-

espécies de *D. paulistorum*. De acordo com os resultados obtidos, verificou-se que os eventos de especiação no subgrupo *willistoni* são muito recentes, variando de 6,4-7,9 MYA (divergência de *D. insularis* a partir de um ancestral comum) a 0.32-0.4 MYA (separação das semiespécies *D. paulistorum* Orinocana e Andino-Brasileira). A diversificação do complexo *D. paulistorum* iniciou-se há mais ou menos 1 milhão de anos. O pouco tempo de divergência entre as espécies do subgrupo *willistoni* explica a pouca diferenciação morfológica até então encontrada. A maior parte dos caracteres morfológicos utilizados para a identificação das espécies desse subgrupo faz parte da genitália masculina, o que corrobora estudos que afirmam que os órgãos copuladores evoluem a uma taxa mais rápida do que os demais órgãos (Masly e Presgraves, 2007; Richardson, 1927; Liu *et al.,* 1996; Song, 2009). Ainda, observou-se que o aparato genital externo feminino não apresenta diferenciação explícita e espécie-específica, estando de acordo com Richmond *et al.* (2012) e McPeek *et al.* (2008, 2009), que afirmam que não é necessária uma mudança correspondente na genitália feminina, uma vez que as fêmeas não necessitam da mesma variação estrutural que garante vantagem copulatória aos machos.

O tema especiação e subespeciação no subgrupo *willistoni* de *Drosophila*, entretanto, é bastante polêmico. Os vários grupos de pesquisa que o estudaram tem discordado em vários aspectos, desde que Theodosius Dobzhansky o elegeu como modelo para o estudo de evolução, quando da sua estadia no Brasil.

Assim, os achados obtidos na presente tese nos levam à questão: *D. paulistorum* é uma espécie única?

Diante dos resultados obtidos em relação à morfologia da genitália masculina e análises moleculares, além de estudos cromossômicos prévios (Rhode *et al.,* 2006),

podemos propor que as semi-espécies de *D. paulistorum* possam ser elevadas à categoria de espécies. Encontramos respaldo para tal proposição na elevação da semi-espécie Guianense à D. pavlovskiana, que se baseou apenas em estudos cromossômicos e de cruzamentos (Kastritsis e Dobzhansky, 1967). Ainda, de acordo com David Grimaldi, em comunicação pessoal, “essas semiespécies apresentam características morfológicas suficientes para serem consideradas espécies” e “algumas espécies de *Drosophila* foram diferenciadas apenas baseando-se em estudos comportamentais”.

Ainda que as seis componentes do complexo *D. paulistorum* possam ser consideradas espécies, elas provavelmente ainda estão em processo de isolamento reprodutivo. A geração de híbridos férteis em determinados cruzamentos pode ser um processo natural que decorre do longo tempo requerido antes do isolamento reprodutivo ser completo.

Ainda em relação à classificação de *D. paulistorum* como uma superespécie composta de semi-espécies, como conceituado por Mayr (1970) vai de encontro a essa classificação, uma vez que o autor afirma que as semi-espécies de uma superespécie devem ser majoritariamente alopátricas, o que não se verifica para *D. paulistorum,* visto que a maior parte delas vive em simpatria no Norte da América do Sul e na região Amazônica (Spassky *et al.,* 1971; Zanini *et al.,* 2015b).

Quanto ao tempo necessário para o processo de especiação, é sugerido cerca de 1 milhão de anos como a duração máxima do processo (Etienne et al., 2014), o que nos levaria a acreditar que algumas das espécies do complexo *D. paulistorum* ainda não completaram esse processo. Estes últimos autores ressaltam que esse é o tempo requerido para a divergência entre espécies-irmãs, o que não é o mesmo que

o tempo requerido para que o isolamento reprodutivo esteja completo e que pode ser muito maior do que 4 milhões de anos, o que pode explicar os cruzamentos interespecíficos entre espécies de *Drosophila.*

Cabe salientar, entretanto, que certamente vários fatores estão envolvidos na geração de isolamento reprodutivo, como fatores ecológicos, climáticos (incluindo paleoclimas), comportamentais, presença de endossimbiontes e a própria morfologia da genitália.

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