# UNIVERSIDADE ESTADUAL DE CAMPINAS

# INSTITUTO DE BIOLOGIA



**Luciane Mendes Hatadani**

## "Sistemática Molecular e Variação Morfométrica da Asa de Espécies de *Drosophila* da Radiação *Tripunctata*"



Tese apresentada ao Instituto de Biologia para obtenção do Título de Doutor em Genética e Biologia Molecular, na área de Genética Animal.

Orientador: Prof. Dr. Louis Bernard Klaczko

Co-Orientadora: Profa. Dra. Ana Maria L. de Azeredo-Espin

**Campinas, Ano 2007**

**FICHA CATALOGRÁFICA ELABORADA PELA BIBLIOTECA DO INSTITUTO DE BIOLOGIA – UNICAMP**

Hatadani, Luciane Mendes
**H28s** Sistemática molecular e variação morfométrica da asa de espécies de drosophila da radiação tripunctata / Luciane Mendes Hatadani. – Campinas, SP: [s.n.], 2007.

Orientador: Louis Bernard Klaczko.
Co-orientadora: Ana Maria Lima de Azeredo-Espin.
Tese (doutorado) – Universidade Estadual de Campinas, Instituto de Biologia.

1. Drosofila. 2. Filogenia. 3. Asas. 4. Inversão cromossômica. 5. Corpo - Temperatura. I. Klaczko, Louis Bernard. II. Azeredo-Espin, Ana Maria Lima de. III. Universidade Estadual de Campinas. Instituto de Biologia. IV. Título.

(rcdt/ib)

**Título em inglês:** Molecular systematics and wing morphometric variation in species of the Drosophila tripunctata radiation.
**Palavras-chave em inglês**: Drosophila; Phylogeny; Wings; Chromosome inversion; Body temperature.
**Área de concentração:** Genética Animal e Evolução.
**Titulação:** Doutora em Genética e Biologia Molecular.
**Banca examinadora**: Louis Bernard Klaczko, Sérgio Furtado dos Reis, André Victor Lucci Freitas, Antônio Bernardo de Carvalho, Sérgio Russo Matioli.
**Data da defesa**: 26/11/2007.
**Programa de Pós-Graduação:** Genética e Biologia Molecular.

Campinas, 26 de novembro de 2007.

**BANCA EXAMINADORA**

Prof. Dr. Louis Bernard Klaczko (Orientador)

Assinatura

Prof. Dr. Sérgio Furtado dos Reis

Assinatura

Prof. Dr. André Victor Lucci Freitas

Assinatura

Prof. Dr. Antônio Bernardo de Carvalho

Assinatura

Prof. Dr. Sérgio Russo Matioli

Assinatura

Profa. Dra. Maura Helena Manfrin

Assinatura

Prof. Dr. Arício Xavier Linhares

Assinatura

Prof. Dr. Michel Albert Georges Vincentz

Assinatura

À minha irmã, Izabella.

# AGRADECIMENTOS

Aos meus pais, Ruth e Arnaldo, por terem proporcionado todo o apoio necessário para que eu pudesse fazer o doutorado, sem terem perguntado, nem mesmo uma única vez: “E aí? Quando você vai defender a tese?”.

À Izabella, minha irmã, por ter ajudado sempre que possível com as minhas inúmeras dúvidas de estatística. E pela amizade e companhia.

À Salete, à Rosângela, às Alessandras, técnicas dos laboratórios, cuja ajuda foi fundamental para a realização dos meus trabalhos.

Ao Hermes, por ter acompanhado este trabalho desde o planejamento dos experimentos até a coleta de dados. Por sua ajuda durante as coletas, por ter me ensinado a identificar as espécies e por estar sempre disposto a dar sugestões e ajudar a resolver problemas.

Ao Prof. James McInerney, pela amizade e pela inestimável ajuda nas análises filogenéticas, sem a qual este trabalho não teria sido possível.

Aos meus colegas de laboratório de *Drosophila,* pela ajuda, sempre que foi possível.

Aos meus colegas do Laboratório de Genética Animal, por terem me acolhido de forma tão prestativa e carinhosa, desde o primeiro dia. Por estarem sempre dispostos a ajudar, pela companhia, pelas conversas, pelos almoços, pelos cafés, pelas piadas, pela amizade.

À Ana Carolina M. Junqueira, minha co-autora e amiga, sempre à disposição e cheia de entusiasmo pelo trabalho.

À Mariana Lyra, por ter me ajudado sempre a resolver todo e qualquer problema no laboratório, pelas sugestões e pela amizade mais altruísta que eu já conheci.

Ao Marco Antônio “Palocci”, pelas discussões infindáveis sobre inferência Bayesiana.

Aos profs. André Victor Lucci Freitas e Arício Xavier Linhares, membros da pré-banca, pelas sugestões.

Ao Prof. Sérgio Furtado dos Reis, pela ajuda no início da coleta de dados de sistemática molecular.

À Prof. Ana Maria Lima de Azeredo-Espin, minha co-orientadora, por ter me acolhido em seu laboratório e, assim, ter me socorrido em um momento de quase desespero. Por ter sempre facilitado meu trabalho, pela motivação, pela amizade e pela confiança. À ela, o meu mais sincero *muito obrigada!*

À CAPES e ao CNPq, pela bolsa de Doutorado.

Ao Prof. Louis Bernard Klaczko, meu orientador, por ter me proporcionado, desde a iniciação científica, durante o Mestrado e Doutorado, uma excelente formação. Pelo incentivo, pela confiança, pela amizade, pela paciência. Levo comigo as lembranças de um longo período de aprendizado e amor pela Ciência.

"Computers have a strong tendency to treat
data and models quite literally"
*J. P. Huelsenbeck, 2002.*

"But this song will play until the light
It's the sound of her trying to find something to like
The sound of her walking day and night
And this song may lead her far away
But tonight it seems to light the way
And she can almost see the future shine
And everything's in tune and everything's in time
It will play until the day Big Julie rules the world
Big Julie rules the world"
*Jarvis Cocker, 2007.*

## RESUMO

O grupo *tripunctata* é o segundo maior grupo neotropical de *Drosophila* em número de espécies e foi incluído na radiação *tripunctata* – que inclui outros grupos próximos. O segundo cromossomo de *Drosophila medipunctata* (espécie que pertence ao grupo *tripunctata*) é altamente polimórfico para inversões. Trabalhos anteriores sugeriram a presença de interação genótipo-ambiente para tamanho da asa, mas não para forma. Experimentos de laboratório foram realizados para testar os efeitos combinados de temperatura e inversão cromossômica no tamanho e forma da asa de *D. mediopuncta*. A morfologia da asa foi analisada por métodos de morfometria geométrica. Os resultados mostraram que tamanho e forma da asa são influeciados por temperatura, sexo e cariótipo. Também foram encontradas evidências que sugeriram a existência entre os efeitos de cariótipo e temperatura para forma da asa, mas não para tamanho, indicando a presença de interação genótipo-ambiente para forma da asa em *D. mediopunctata.* Sugerimos que outros fatores ecológicos – tais como densidade de larvas – ou variação sazonal de conteúdo genético das inversões poderia explicar os resultados anteriores. Além disso, uma nova hipótese filogenética para a radiação tripunctata é sugerida com base em dados de seqüências dos genes mitocondrias das subunidades I e II da citocromo oxidase, e a variação de tamanho e forma da asa é descrita com bases na nova hipótese filogenética. As árvores filogenéticas foram reconstruídas por métodos de parcimônia, máxima verossimilhança e inferência Bayesiana. Os resultados rejeitaram a hipótese de monofilia para o grupo tripunctata, enquanto esta hipótese não foi rejeitada para a radição *tripunctata* e outros grupos específicos dentro da radiação. Ambos tamanho e forma da asa apresentaram sinal filogenético e diferentes padrões de tamanho e forma puderam ser identificados para cada um dos agrupamentos mais importantes detectados pela análise filogenética.

## ABSTRACT

The *tripunctata* group is the second largest Neotropical group of *Drosophila* in number of species and was included in the *tripunctata* radiation – which comprises other closely related groups. The second chromosome of *Drosophila mediopunctata* (a species that belongs to the *tripunctata* group) is highly polymorphic for inversions. Previous work suggested the presence of genotype-environment interaction for wing size but not for shape. We performed experiments in the laboratory to test for the joint effects of temperature and chromosome inversions on size and shape of the wing in *D. mediopunctata.* Wing morphology was analyzed by methods of geometric morphometrics. Our findings show that wing size and shape are influenced by temperature, sex, and karyotype. We also found evidence suggestive of an interaction between the effects of karyotype and temperature on wing shape (but not for size), indicating the existence of genotype-environment interaction for wing shape in *D. mediopunctata*. We suggest that other ecological factors – such as larval crowding – or seasonal variation of genetic content within inversions may explain the previous results. Moreover, we suggest a new phylogenetic hypothesis for the *tripunctata* radiation based on sequences of mitochondrial genes of cytochrome oxidase subunits I and II, and describe wing size and shape variation in different species based on this new phylogenetic hypothesis. Phylogenetic trees were reconstructed by parsimony, maximum likelihood and bayesian inference. Results reject the monophyly hypothesis for the *tripunctata* group whereas monophyly is not rejected for the *tripunctata* radiation and other specific groups within the radiation. Both wing size and shape displayed phylogenetic signal and different patterns of size and shape could be identified for each of the most important clusters detected by phylogenetic analysis.

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# INTRODUÇÃO GERAL

## ORGANISMO MODELO: *DROSOPHILA*

Um dos modelos mais amplamente estudados em Biologia tem sido as espécies do gênero *Drosophila,* particularmente *Drosophila melanogaster.* Muitas das primeiras descobertas em genética clássica foram realizadas a partir de experimentos que envolviam esta espécie, além de seu importante papel na síntese da genética e da teoria evolutiva e, mais recentemente, no estudo do desenvolvimento e da biologia molecular (ver revisão em Powell, 1997). *D. melanogaster* foi o primeiro eucarioto complexo a ter o genoma completamente seqüenciado (Adams et al., 2000), levando em 2003 à publicação de um *White Paper* (Clark et al. 2003) propondo o seqüenciamento de outras espécies próximas. Atualmente estão sendo seqüenciados os genomas de outras 11 espécies (http://rana.lbl.gov/drosophila/index.html) – *D. ananassae*, *D. erecta*, *D. grimshawi, D. mojavensis*, *D. persimilis*, *D. pseudoobscura, D. sechelia, D. simulans, D. virilis, D. willistoni, D. yakuba* – o que fará com que o gênero *Drosophila* se torne um excelente modelo para estudos de genômica comparativa e favorecerá estudos de biologia comparativa entre espécies do gênero.

Espécies de *Drosophila* existem numa grande parte do mundo, em regiões temperadas, desertos e nos trópicos, onde se encontra sua maior diversidade (Powell, 1997), particularmente em matas e florestas (Throckmorton, 1975). Suas larvas alimentam-se de organismos que fazem fermentação ou em decomposição, desempenhando um importante papel em cadeias alimentares saprofíticas (Throckmorton, 1975). Os substratos de oviposição e os hábitos alimentares dos adultos são bastante diversificados, incluindo seiva e fungos, além de frutos, flores e vegetação em decomposição. Esta diversidade de hábitos alimentares tem em comum a presença de

organismos fermentadores, sendo que estes constituem verdadeiramente o alimento das espécies do gênero (Throckmorton, 1975).

## TAXONOMIA DO GÊNERO *DROSOPHILA*

TaxoDros (Bächli, 2007) é uma excelente base de dados de taxonomia de drosofilídeos *on-line*. Mantido por Gerhard Bächli, uma das maiores autoridades mundiais em taxonomia do gênero *Drosophila*, o TaxoDros é constantemente atualizado (a versão mais recente da base de dados é de junho de 2007) e fornece além das posições taxonômicas de espécies, referências às descrições originais e a trabalhos de taxonomia em geral, informações de sinonímia e de distribuição geográfica de espécies descritas. Todas as informações a respeito de números de espécies, subgrupos, grupos e subgêneros citadas nesta Introdução Geral foram retiradas da base de dados TaxoDros de junho de 2007 (Bächli, 2007), a não ser nos casos em que outra referência bibliográfica é citada.

A família Drosophilidae atualmente contém cerca de 3750 espécies descritas (Ashburner et al., 2005) e está dividida em duas subfamílias: Steganinae e Drosophilinae. Trinta e oito gêneros compõem a subfamília Drosophilinae (Bächli, 2007), entre os quais, o gênero *Drosophila*, no qual estão incluídas atualmente contém 1149 espécies descritas, subdividido em oito subgêneros (Bächli, 2007). Os subgêneros *Drosophila* e *Sophophora* abrigam mais de 90% das espécies do gênero (721 e 332, respectivamente, Bächli, 2007) e contêm as espécies mais amplamente estudadas, em particular *Drosophila melanogaster* (*Sophophora*). Em função do grande número de espécies, agrupamentos foram criados especificamente para o gênero *Drosophila* (Ashburner, 2005), tais como grupos, subgrupos e complexos de espécies, de modo que não constituem categorias taxonômicas reconhecidas pelo Código Internacional de

Nomenclatura Zoológica. O subgênero *Sophophora*, por exemplo, é subdividido em 8 subgrupos enquanto 43 subgrupos compõem o subgênero *Drosophila*.

Dentro do subgênero *Drosophila*, o grupo *immigrans* contém o maior número de espécies (101 espécies descritas atualmente, Bächli, 2007), seguido pelo grupo *repleta* (100 espécies descritas, Bächli, 2007) e pelo grupo *tripunctata* (79 espécies descritas, Bächli, 2007). Os demais grupos que pertecem a este subgênero são compostos por números significativamente mais baixos, em geral abaixo de vinte espécies descritas (Bächli, 2007).

### A RADIAÇÃO *TRIPUNCTATA*

Throckmorton (1962, 1975) propôs a primeira hipótese filogenética para a família Drosophilidae (Markow & O'Grady, 2006). A partir de caracteres morfológicos, ele organizou as espécies de drosofilídeos em radiações, que representam eventos de múltipla especiação, seguidos de diversificação (figura 1.1). Ainda que seus métodos de análise não sejam formalizados e que novos estudos tenham comprovado que algumas de suas conclusões estavam incorretas (Markow & O'Grady, 2006), seu trabalho continua a ser de extrema importância atualmente. Throckmorton (1962, 1975) incluiu o grupo *tripunctata* na radiação *tripunctata* (figura 1.1), juntamente com outros grupos (*calloptera, cardini, guarani, macroptera, pallidipennis, rubrifrons* e *sticta*). A radiação *tripunctata* teria se originado da radiação *immigrans-Hirtodrosophila*, que surgiu e se diversificou nos paleotrópicos, e teria enviado duas linhagens (*tripunctata* e *Hirtodrosophila*) à Região Neotropical, onde estas se diversificaram constituindo novas radiações (figura 1.1). Throckmorton (1975) também sugeriu que as espécies do grupo *tripunctata* não formam um grupo monofilético, concordando com observações feitas por Kastritsis et al. (1970) baseadas em dados citológicos. Recentemente, dois estudos

moleculares (Yotoko et al., 2003; Robe et al., 2005) também obtiveram resultados que rejeitaram a monofilia deste grupo.

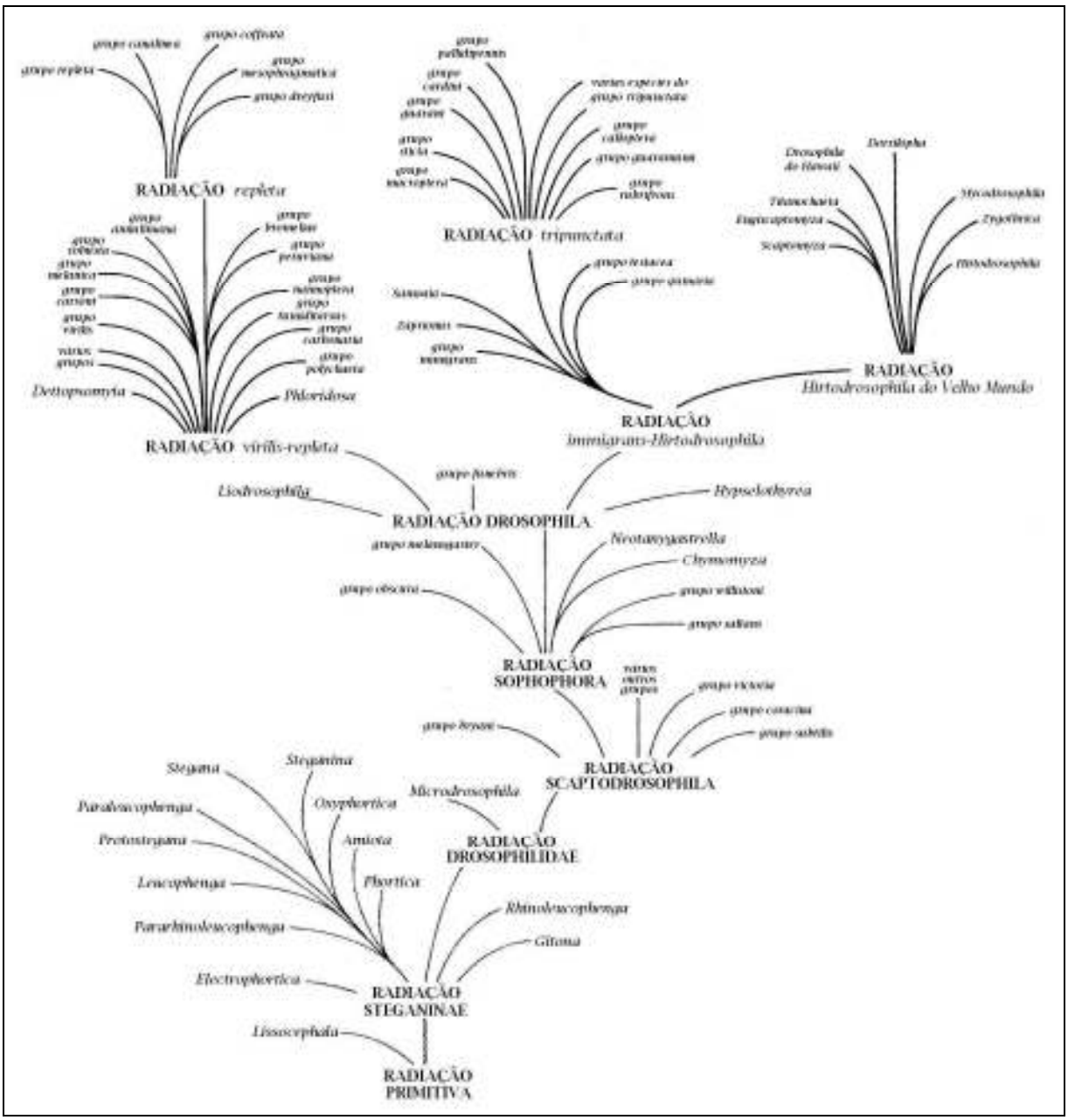


**Figura 1.1:** Radiações propostas como hipótese filogenética para a família Drosophilidae (baseado em Throckmorton, 1975 e Markow e O'Grady, 2006).

***O grupo calloptera***

O grupo *calloptera* foi proposto por Burla & Pavan (1953), que sugeriram que este grupo seria proximamente relacionado ao grupo *guarani* (o que também foi proposto por Remsen & O'Grady, 2002) e, ainda que de forma não tão próxima, aos grupos *tripunctata*, *quinaria* e *cardini.* Este grupo é composto atualmente por oito espécies, que se destacam por suas asas fortemente pigmentadas em manchas (Burla & Pavan, 1953).

***O grupo cardini***

O grupo *cardini* contém 16 espécies descritas e é subdividido em dois subgrupos: *cardini* e *dunni*. As espécies deste grupo são facilmente reconhecidas por possuírem o tórax marrom-avermelhado ou amarelado e bastante brilhante, e por apresentarem vários padrões de bandas pretas nos tergitos, caráter que é polimórfico em algumas espécies (Vilela et al., 2002).

A análise de dados de seqüências de genes mitocondriais e nucleares resultaram em alto suporte estatístico para a monofilia deste grupo (Brisson et al., 2006) e indicaram que *D. cardini* ocuparia a posição mais basal dentro do grupo.

***O grupo guarani***

Dezessete espécies estão incluídas no grupo *guarani,* das quais três pertencem ao subgrupo *guaramunu*, seis pertencem ao subgrupo *guarani* e as oito restantes não são classificadas em subgrupos. Kastritsis (1969) estudou os cromossomos politênicos de algumas espécies do grupo e sugeriu que os subgrupos *guarani* e *guaramunu* deveriam ser elevados à categoria de grupo. Esta sugestão foi baseada no fato de que os cromossomos de espécies do subgrupo *guaramunu* eram mais semelhantes aos cromossomos de espécies do grupo *tripunctata* do que aos cromossomos das espécies do subgrupo *guarani*. Entretanto, Vilela e Pereira (1985) argumentaram que o grupo *guarani* e seus dois subgrupos deveriam ser mantidos como tais até

que fossem encontradas mais evidências que sugerissem esta revisão taxonômica. Existe atualmente uma controvérsia em torno desta questão. Ainda que o grupo *guarani* seja apresentado como sendo composto por dois subgrupos por Bächli (2007), Markow e O'Grady (2006) adotaram a classificação sugerida por Kastritsis (1969) e mencionam a existência de espécies proximamente relacionadas aos dois grupos, cuja posição ainda não foi definida. Os mesmos autores apresentam uma tabela de espécies contidas em cada grupo da radiação *tripunctata* em que apenas o grupo *guarani* é mecionado, contendo as espécies dos dois subgrupos e as espécies não agrupadas, demonstrando a dificuldade dos autores em lidar com a conversão dos subgrupos *guarani* e *guaramunu* em grupos de espécies.

***O grupo macroptera***

As cinco espécies do grupo *macroptera* são pouco conhecidas e são possivelmente proximamente relacionadas às espécies do grupo *rubrifrons* (Vilela & Bächli, 2004).

***O grupo pallidipennis***

O grupo *pallidipennis* é formado por uma única espécie (*D. pallidipennis*), sobre a qual existem poucos estudos, a maior parte enfocando a caracterização de cromossomos politênicos (Freire-Maia & Engel, 1949; Pasteur & Kastritsis, 1971), além da existência de duas subespécies (Patterson & Dobzhansky, 1945).

***O grupo rubrifrons***

Este grupo também é pouco estudado e contém atualmente nove espécies descritas. Vilela & Bächli (2004) sugeriram que as espécies do grupo *rubrifrons* seriam proximamente relacionadas às espécies do grupo *macroptera*.

### *O grupo sticta*

Este grupo é composto por uma única espécie, muito pouco conhecida.

### *O grupo tripunctata*

O grupo *tripunctata* foi proposto por Sturtevant em 1942 e atualmente é composto por 79 espécies descritas. É um grupo quase exclusivamente neotropical, sendo *D. tripunctata* a única exceção, por ocorrer na América do Norte. Suas espécies são abundantes (Val et al. 1981), particularmente em áreas florestais (Sene et al. 1980), onde constituem o componente dominante da fauna de drosofilídeos (Ashburner et al., 2005).

Frota-Pessoa (1964) dividiu o grupo em quatro subgrupos (I, II, III e IV), o que foi questionado posteriormente por Vilela (1992), que apontou que, com exceção de algumas espécies cujo parentesco próximo era óbvio, esta seria uma subdivisão artificial. Recentemente, Yotoko et al. (2003) reforçaram estas observações com base em dados de seqüências de DNA mitocondrial.

## *DROSOPHILA MEDIOPUNCTATA*: UM MODELO NEOTROPICAL

*D. mediopunctata* pertence ao grupo *tripunctata* e tem uma ampla distribuição, tendo sido encontrada em localidades da América Central até o sul do Brasil (Val et al. 1981). A história de vida desta espécie é bastante diferente de outras espécies de *Drosophila* estudadas mais freqüentemente, tais como *D. melanogaster* e *D. pseudoobscura,* uma vez que o tempo de maturação é longo, a fecundidade é baixa e a longevidade é maior (Klaczko, 1995). Esta biologia peculiar da espécie poderia revelar aspectos não observados em outras espécies, o que motivou o início do estudo da espécie no Brasil, objetivando torná-la um organismo modelo (Klaczko, 1995; 2006). Desde então, *D. mediopunctata* tem sido estudada em diversos aspectos, tais como seus polimorfismos de inversões cromossômicas (Ananina *et al*., 2002), ocorrência de *sex-ratio*

(por exemplo: Carvalho *et al*., 1989; Carvalho & Klaczko, 1993 e 1994; Carvalho *et al.*, 1997; Varandas *et al.*, 1997), além de aspectos morfológicos da asa (Klaczko & Bitner Mathé, 1990; Bitner-Mathé *et al.*, 1995; Bitner-Mathé & Klaczko, 1999a; Bitner-Mathé & Klaczko, 1999b), polimorfismo de coloração (Hatadani et al., 2004) e biologia molecular (Loreto et al, 2001).

Esta espécie tem seis pares de cromossomos: cinco acrocêntricos e um pontual que não se politeniza. Os cromossomo II, IV e X apresentam polimorfismo de inversões (Peixoto & Klaczko, 1991; Ananina *et al*. 2002). O cromossomo II é o mais polimórfico e suas inversões podem ser divididas em dois grupos, de acordo com a região do cromossomo: existem oito inversões na região distal (*DA, DI, DV*, *DS* etc.) e nove na região proximal (*PA0*, *PB0*, *PC0, PC1, PC2* etc.) (Ananina *et al*., 2002). Há um forte desequilíbrio de ligação entre as inversões proximais e distais do cromossomo II, de modo que *PA0* está predominantemente ligada a *DA* enquanto *PC0* está preferencialmente ligada a *DV, DP* e *DS* (Peixoto & Klaczko, 1991). As freqüências das inversões do cromossomo II se alteram durante o ano: *PA0* tende a ser mais freqüente nos meses mais frios, enquanto o contrário ocorre para *PC0* (Klaczko, 1995; Ananina et al., 2004). Existe também uma correlação positiva entre a freqüência de *PA0* e altitude, enquanto a mesma correlação é negativa para *PC0* (Klaczko, 1995). Esses dados indicam que *PA0* está mais adaptada a temperaturas mais baixas e *PC0*, a temperaturas mais altas.

### VARIAÇÃO DE TAMANHO E FORMA DA ASA EM *DROSOPHILA*

Apesar de sua aparente simplicidade, as asas de insetos são estruturas complexas, o que é necessário, já que o vôo é um processo complexo em que as asas precisam executar uma série de movimentos diferentes (batimentos, curvas e deformações) que precisam estar sutilmente ajustados para permitir aos insetos a sua amplitude de velocidades e manobras (Wootton, 2001). As funções da asa em insetos não são apenas locomotoras, mas estão relacionadas a funções

sensorias, termorregulação, proteção e defesa, além de sinalização territorial e sexual (Wooton, 1992). De extrema importância, as veias das asas têm a função de suporte e de conduzir hemolinfa, oxigênio e informação sensorial, e de prevenção de que danos à membrana da asa se espalhem (Wooton, 1992; DeCelis & Diaz-Benjumea, 2003).

O padrão de venação da asa de *Drosophila* é relativamente simples em comparação com outros insetos. Geneticistas que estudavam mutações em *Drosophila* desenvolveram sua própria nomenclatura para as veias da asa, de modo que L1 corresponde à veia marginal ao longo da margem anterior da asa; L2 a L5 correspondem às quatro veias longitudinais principais; cv-a e cv-p, às duas veias tranversais; e L0 e L6, às duas veias curtas na região proximal da asa (Stark et al. 1999; DeCelis & Diaz-Benjumea, 2003).

Em geral, o tamanho da asa em *Drosophila* está relacionado com a temperatura de desenvolvimento das larvas. No que diz respeito à plasticidade fenotípica (por exemplo, Crill *et al.*, 1996; Imasheva *et al.*, 2000), moscas que se desenvolvem em temperaturas mais baixas tendem a ter asas maiores, inclusive em relação ao tamanho do resto do corpo (por exemplo, Robertson, 1987; Thomas, 1993; Pétavy *et al.*, 2001).

Em relação ao componente genético que determina o tamanho da asa, também foram encontrados clines latitudinais em várias espécies (Pfriem, 1983; Hyytia *et al.*, 1985; Imasheva *et al.* 1994; Huey *et al.*, 2000), como correlações positivas com a latitude. Estes clines são causados quase certamente por seleção natural e o fator seletivo é provavelmente a temperatura (Imasheva *et al.*, 1994).

A forma da asa, também, parece estar relacionada à temperatura. Imasheva *et al.* (1995) detectaram padrões geográficos possivelmente adaptativos e correlacionados com a temperatura. Além disso, foi observado que a forma da asa em *D. melanogaster* estava sob seleção natural dependente de temperatura em laboratório (Cavicchi *et al.,* 1985).

Debat et al. (2003) separaram a variação na forma da asa de *D. simulans* em dois componentes: alométrico (correlacionado com o tamanho) e não-alométrico. Seus resultados mostraram que o componente alométrico (responsável por 23,8% da variação de forma) apresentava um variação direcional proporcional à variação de temperatura, enquanto o componente não-alométrico apresentou uma reversão na direção da mudança de forma em uma das temperaturas intermediárias. Entretanto, em ambos os componentes, os marcos anatômicos localizados na parte distal da asa foram mais sensíveis à variação de temperatura que os marcos anatômicos proximais.

Apesar do tamanho e da forma da asa de algumas espécies de *Drosophila* terem sido bastante estudados, poucas vezes foram feitas comparações entre as morfologias da asa entre diferentes espécies de *Drosophila*. Um dos poucos estudos (Imasheva *et al.*, 2000) comparou distâncias entre pontos da asa em *D. melanogaster* e *D. simulans*. Starmer & Wolf (1989) compararam diversas espécies com o objetivo de encontrar a melhor forma de estimar o *wing loading* (definido como a massa corporal dividido pela área da asa), o que representa uma forma de estimar variações no tamanho da asa em relação ao tamanho do animal. Portanto, a comparação da forma da asa entre espécies de *Drosophila* permanece pouco explorada.

No que diz respeito à morfologia da asa em *D. mediopunctata*, Bitner-Mathé & Klaczko (1999a) utilizaram um método que descreve a asa através da equação da elipse (Klaczko & Bitner-Mathé, 1990) e encontraram uma alta herdabilidade para a forma da asa em condições naturais. Utilizando métodos de morfometria tradicional, Bitner-Mathé & Klaczko (1999b) encontraram resultados que sugeriram que tamanho e forma da asa nesta espécie têm propriedades diferentes. A variância e a herdabilidade do tamanho parecem bastante sensíveis a pequenas variações ambientais, impossibilitando a obtenção de estimativas consistentes. Por

outro lado, no que diz respeito à forma, a variância é menos sensível às variações ambientais e a sua herdabilidade é alta, possibilitando estimativas similares em diferentes coletas e localidades. Em um trabalho anterior, Bitner-Mathé *et al.* (1995) analisaram uma população natural e encontraram uma correlação significativa entre a forma da asa e a altitude. Este mesmo trabalho detectou um efeito significativo do mês de coleta tanto no tamanho quanto na forma da asa. Também foram encontrados um efeito significativo do cariótipo do cromossomo II na forma da asa e uma interação significativa entre o cariótipo do cromossomo II e o mês de coleta em ambos forma e tamanho. Estas interações são fortes indícios da presença de interação genótipo-ambiente para a morfologia da asa em condições naturais nesta espécie.

## MORFOMETRIA GEOMÉTRICA

Nas décadas de 1960 e 1970, pesquisadores começaram a aplicar métodos de estatística multivariada para a análise de variáveis morfológicas, em geral, medidas lineares. Este conjunto de métodos é hoje chamado "morfometria tradicional". Um dos principais problemas encontrados foi obter medidas de forma, livres de tamanho. Vários métodos foram propostos, mas nenhum deles parecia suficientemente adequado ou eficiente. Outro problema da abordagem morfométrica tradicional era a identificação de homologia entre as distâncias que serviam como dados, uma vez que muitas das distâncias usadas não eram definidas por pontos homólogos. Além disso, estes métodos não possibilitavam a representação gráfica das mudanças de forma, já que as distâncias lineares não preservavam as relações geométricas entre as variáveis (ver revisões em Rohlf & Marcus, 1993; Adams et al., 2004).

No final da década de 1980 e início da década de1990, um novo conjunto de métodos de análise de forma biológica foi proposto. Estes métodos enfatizavam a preservação da geometria das formas ao longo da análise e foram, portanto, chamados de "morfometria geométrica". Vários

destes métodos são baseados em coordenadas de marcos anatômicos, definidos por Dryden & Mardia (1998) como "pontos de correspondência em cada objeto, compatíveis entre e dentro de populações". Uma característica fundamental dos marcos anatômicos é que devem ser homólogos entre todos os espécimes estudados. Além de apresentar uma definição clara de tamanho e uma maneira eficiente de removê-lo do conjunto de dados, uma das vantagens dos métodos de morfometria geométrica é permitir representações gráficas dos resultados, uma vez que a geometria da forma é preservada ao longo da análise (ver revisões em Rohlf & Marcus, 1993; Adams et al., 2004).

Kendall (1977) definiu forma como "toda a informação geométrica que permanece quando efeitos de localização, escala e rotação são removidos de um objeto". O tamanho, em morfometria geométrica, é descrito pelo tamanho do centróide, calculado pela raiz quadrada da soma dos quadrados das distâncias de cada marco anatômico ao centróide (Bookstein, 1991). A localização do centróide é calculada pela média das coordenadas de uma configuração de marcos anatômicos, ou seja, a coordenada X do centróide é a média das coordenadas X de todos os marcos anatômicos e a a coordenada Y do centróide é a média das coordenadas Y de todos os marcos anatômicos.

O método de superimposição de Procrustes ou GLS (*generalized least squares Procrustes superimposition*) (Rohlf, 1990) é o método mais usado atualmente para remover os efeitos de localização, escala (tamanho) e rotação dos objetos de análise. Os passos deste método podem ser descritos da seguinte forma (ver revisão em Zelditch et al, 2004):

1. Centrar cada configuração de marcos anatômicos na origem, subtraindo as coordenadas do centróide das coordenadas correspondentes de cada marco;

2. Padronizar as configurações para o tamanho unitário, dividindo as coordenadas de cada marco anatômico pelo tamanho do centróide.
3. Escolher uma configuração que será a referência, rotacionar todas as demais configurações de modo a minimizar a soma das distâncias ao quadrado entre marcos homólogos entre as configurações.
4. Calcular a forma média, que passa a ser a nova referência, e repetir o passo 3 até que a referência mais recente seja igual à anterior.

As novas coordenadas obtidas, livres de efeitos de translação, escala e rotação, descrevem apenas a forma de cada espécime. Entretanto, o número de variáveis geradas por GLS possui quatro dimensões a mais do que o espaço de forma, cujo número de dimensões é duas vezes o número de marcos anatômicos subtraídas quatro dimensões (uma perdida pela padronização do tamanho, uma perdida pela rotação e duas, pela translação). Uma explicação detalhada do espaço de forma, descrito com base nas teorias matemáticas de forma, pode ser encontrada em Zelditch et al. (2004). Este fato impossibiliza a utilização de métodos usuais de estatística multivariada para a análise dos dados. Uma das formas de lidar com este problema é o método de *thin-plate splines* (TPS), que gera um conjunto de variáveis de forma (chamadas deformações parciais), cujo número é igual ao número de dimensões do espaço de forma (Bookstein 1989, 1991), permitindo a análise por métodos usuais de estatística multivariada.

### MÉTODOS DE RECONSTRUÇÃO FILOGENÉTICA

Atualmente, *neighbour-joining* (Saitou & Nei, 1987), máxima parcimônia, máxima verossimilhança e, mais recentemente, inferência Bayesiana são os métodos de reconstrução filogenética mais usados para dados moleculares (Lewis, 2001) .

O método de *neighbour-joining* é baseado em uma matriz de distâncias entre os taxa e sua principal vantagem é a rapidez com que a análise pode ser feita em um computador comum. Essa matriz de distâncias pode ser calculada com base em um modelo específico de substituição de nucleotídeos, permitindo estimar o número de substituições de nucleotídeos que ocorreu durante a história evolutiva dos organismos, uma vez que múltiplas substituições no mesmo sítio ou substituições paralelas podem fazer com que o número de substituições observado seja uma subestimativa do número verdadeiro, causando o aparecimento de homoplasias (uma revisão dos diversos modelos de substituição pode ser encontrada em Felsenstein, 2004).

O objetivo do método de máxima parcimônia é encontrar a árvore em que o mínimo número de substituições seria necessário para produzir os dados. Este método, portanto, não leva em conta diferentes modelos de substituição nucleotídica. As vantagens oferecidas por este método é a relativa rapidez com que a análise pode ser realizada e sua robustez quando as sequências são provenientes de taxa relativamente próximos, ou seja, quando os ramos da árvore são curtos (Lewis, 2001). Entretanto, caso exista uma considerável variação nos comprimentos dos ramos, o método de parsimônia é particularmente sensível ao fenômeno de atração dos ramos longos (Felsenstein, 1978), em que ramos longos tendem a ser posicionados mais próximos um ao outro do que seriam na árvore verdadeira de relações filogenéticas.

Um dos métodos mais eficientes de reconstrução filogenética é o método de máxima verossimilhança (ver revisão em Felsenstein, 2004). Este método tem como objetivo encontrar a árvore de maior verossimilhança (no caso de inferência filogenética, a probabilidade de os dados terem sido produzidos por um determinada topologia e modelo de substituição de nucleotídeos). Estimativas de máxima verossimilhança possuem propriedades bastante favoráveis a medida em que a quantidade de dados aumenta, tais como consistência (convergência para o valor correto do parâmetro) e eficiência (mínima variância em torno do valor real do parâmetro) (Fisher, 1922). A

principal desvantagem deste método é a lentidão com que a análise é completada, o que pode tornar impossível a análise de um número de táxons relativamente alto. No entanto, Guindon & Gascuel (2003) propuseram um algoritimo que permite a estimativa de árvores filogenéticas pelo método de máxima verossimilhança, implementado no programa PHYML, que realiza a análise de grande número de espécies em um tempo razoavelmente curto (equivalente ao tempo necessário para análises usando métodos de distância ou de parsimônia) e cujo desempenho, de acordo com os autores, é equivalente a outros métodos implementados em programas bastante usados, tais como PAUP (Swofford, 2003) e PHYLIP (Felsenstein, 1993).

Rannala & Yang (1996) foram os primeiros a propor o uso de inferência Bayesiana para reconstrução filogenética. No mesmo ano, duas teses de doutorado (Mau, 1996; Li, 1996) propuseram métodos semelhantes. A inferência Bayesiana baseia-se no teorema de Bayes, usado para a obtenção de probabilidades posteriores, a partir da verossimilhança e de uma probabilidade *a priori* (ver revisões em Lewis, 2001; Huelsenbeck et al., 2001; Holder & Lewis, 2003). Em vez de ser um método de optimização (como máxima parcimônia ou máxima verossimilhança), a inferência Bayesiana faz uma amostragem de árvores, de acordo com suas probabilidades posteriores (Huelsenbeck et al., 2001). A mesma análise produz tanto a estimativa da árvore quando medidas de incerteza (probabilidades posteriores) para os agrupamentos (Holder & Lewis, 2003). Uma das vantagens da inferência Bayesiana apontadas por seus defensores é a interpretação direta das probabilidades posteriores dos agrupamentos: estas representam as probabilidades de que um agrupamento é verdadeiro, dados o modelo, as probabilidades a priori e o conjunto de dados (Huelsenbeck, 2002).

O cálculo das probabilidades posteriores, entretanto, envolve uma soma entre todas as árvores possíveis e, para cada árvore, a integral entre todas as combinações de comprimentos de ramos e valores de parâmetros do modelo de substituição. Este fato torna impossível uma solução

analítica para o problema e faz com que sejam necessários métodos numéricos para o cálculo das probabilidades posteriores. O método usado para obter uma aproximação destas probabilidades em inferência filogenética é *Markov Chain Monte Carlo* (MCMC) (revisão em Huelsenbeck et al., 2002). Este método permite a estimativa de hipóteses filogenéticas em um tempo razoavelmente curto e pode ser descrito pela seguinte sequência de passos:

1. Iniciar a cadeia de Markov com uma árvore;
2. Uma nova árvore é proposta através de uma perturbação aleatória;
3. A nova árvore é aceita ou rejeitada de acordo com a probabilidade descrita por Metropolis et al. (1953) e Hastings (1970);
4. Retornar ao passo 2.

Estes passos devem ser repetidos até que a cadeia de Markov atinja a convergência para a distribuição correta de probabilidades posteriores. O diagnóstico de convergência pode ser feito de duas formas. Uma delas é examinar os gráficos dos logarítimos de verossimilhança e verificar a ausência de tendências nos valores ao longo da cadeia. Uma alternativa, mais eficiente, é comparar cadeias independentes. A convergência das cadeias pode ser aceita se várias cadeias iniciadas de pontos diversos no espaço de parâmetros estiverem produzindo amostras aparentemente iguais (Huelsenbeck et al., 2002; Nylander et al., 2004).

Uma das vantagens freqüentemente apontadas para a inferência Bayesiana é a oportunidade que o pesquisador dispõe de incorporar na análise seu conhecimento prévio sobre o grupo de organismos de interesse através das probabilidades *a priori*. Por outro lado, uma das maiores críticas é a de que as probabilidades a priori seriam escolhidas de forma arbitrária ou inadequada. Entretanto, à medida em que mais dados são adicionados à análise, a influência das probabilidades a priori diminui de modo que em uma análise Bayesiana típica, os resultados são

pouco influenciados pelas probabilidades a priori (Huelsenbeck et al., 2002). Em geral, pesquisadores dão preferências a distribuições não-informativas de probabilidades a priori.

As medidas de suporte estatístico para os ramos de uma árvore filogenética mais usadas atualmente são os valores de *bootstrap* não-paramétrico (gerados por um processo de reamostratem, com reposição, dos dados) e as probabilidades posteriores calculadas por inferência Bayesiana. Empiricamente, as probabilidades posteriores são normalmente mais altas do que os valores de *bootstrap* correspondentes (Erixon et al., 2003).

O significado estatístico dos valores de *bootstrap* é controverso. Ainda que fortemente correlacionados com a probabilidade de que o ramo correspondente seja verdadeiro, os valores de *bootstrap* parecem ser uma subestimativa da confiabilidade dos ramos (Hillis & Bull, 1993).

Por outro lado, as probabilidades posteriores geradas por inferência Bayesiana tendem a ser bastante altas, de acordo com Suzuki et al., 2003 que, através do estudo de simulações, concluíram que estas probabilidades são superestimativas das probabilidades reais de se recuperar um determinado ramo. Os mesmos autores concluíram que os valores de *bootstrap* são uma estimativa conservadora de suporte filogenético e aconselharam o uso do *bootstrap* como medida de confiabilidade em árvores filogenéticas. Outro estudo envolvendo simulações (Wilcox et al., 2002) obteve conclusões ligeiramente diferentes: os valores de *bootstrap* seriam estimativas altamente conservadoras da exatidão das árvores filogenéticas, enquanto as probabilidades posteriores são estimativas mais próximas das probabilidades reais.

Douady et al. (2003) sugerem que, pelo fato de os valores de *bootstrap* serem medidas conservadoras enquanto as probabilidades posteriores geram superestimativas, estas duas medidas de suporte filogenético poderiam servir como limites inferior e superior (respectivamente) da confiabilidade dos ramos. Os autores ressaltam, no entanto, que estas duas medidas não são de forma alguma comparáveis. Erixon et al. (2003) obtiveram resultados

semelhantes e concluíram que "*altos valores de suporte não garantem conclusões corretas, mas apenas conclusões com alto suporte. Dados adicionais, se amostrados da mesma filogenia, fazem com que as conclusões sejam mais confiáveis e mais robustas*."

## DNA MITOCONDRIAL EM ANÁLISES FILOGENÉTICAS

O primeiro genoma mitocondrial completamente seqüenciado em *Drosophila* foi o de *D. yakuba* (Clary & Wolstenholme, 1985), constituindo também o primeiro invertebrado a ter o genoma mitocondrial completo. O genoma mitocondrial de *D. yakuba* é contituído por 16.019 pares de base e contém 13 genes codificadores de proteínas, dois genes de RNA ribossômico, e 22 genes de RNA transportador, e uma alta proporção de adeninas e timinas (32,3% e 44,4%, respectivamente) (Clary & Wolstenholme, 1985).

As características gerais que tornam seqüências de DNA mitocondrial particularmente atraente para estudos de genética populacional e de sistemática são as seguintes: herança materna; alto número de cópias; haplóide; não há recombinação; ausência de introns; as regiões intergênicas são pequenas ou ausentes (Moritz et al., 1987; Harrison, 1989; Wolstenholme, 1992; Simon, 1994). Além disso, O genoma mitocondrial animal é bastante conservado em termos de tamanho (cerca de 16 mil pares de base), em conteúdo e ordem de genes, mas apresenta uma alta taxa de substituição de nucletídeos (Avise, 1986). Estas características fazem com que sequências mitocondrias sejam as mais amplamente utilizadas para estudos filogenéticos de insetos e animais em geral (Caterino et al., 2000).

Outra vantagem do uso de seqüências mitocondriais em estudos filogenéticos é o fato de que quanto menor o tamanho efetivo da população, maior a probabilidade de a árvore filogenética do gene recuperar a árvore de espécies, uma vez que uma árvore construída a partir da seqüência de um gene reflete as relações filogenéticas entre os diferentes alelos deste gene e não

necessáriamente as relações filogenéticas entre as espécies estudadas (Pamilo & Nei, 1988; Doyle, 1997; Nichols, 2001). Já que o tamanho efetivo populacional de genes mitocondriais é um quarto do tamanho efetivo de genes nucleares, uma árvore filogenética construída a partir da seqüência de um gene mitocondrial tem uma probabilidade maior de recuperar a filogenia das espécies do que qualquer árvore construída a partir de um único gene nuclear (supondo que a árvore do gene tenha sido recuperada corretamente) (Moore, 1995). Entretanto, a alta taxa de substituição de nucletídeos de genes mitocondriais faz com que estes genes sejam inadequados para a reconstrução filogenética entre taxa com tempos longos de divergência (Graf & Sparks, 2000), gerando árvores de ramos internos curtos e ramos terminais longos, com baixo suporte estatístico dos nós, e favorecendo a ocorrência de atração de ramos longos (Felsenstein, 1978). Graf & Sparks (2000) sugeriram que, nestes casos, o simples acréscimo de dados de seqüências mitocondriais não é suficiente para melhorar a probabilidade de se recuperar a árvore correta de espécies (em função de outros genes mitocondriais sofrerem do mesmo problema de alto índice de homoplasia) e que seriam necessários dados de seqüências mais conservadas (genes nucleares).

O gene da citocromo oxidase subunidade I (COI) é o mais longo dos três genes mitocondriais que codificam subunidades da citocromo oxidase. Este gene possui regiões mais conservadas e outras mais variáveis, o que o torna particularmente útil para estudos evolutivos (Lunt et al., 1996). Este gene tem recebido bastante atenção recentemente por ter sido escolhido como o gene a ser usado como código de barras genético (*DNA barcoding*) em animais (Hebert et al., 2003a; Hebert et al., 2003b; Hajibabaei et al., 2007).

## O MÉTODO COMPARATIVO

Em geral, a morfologia, ecologia e comportamento são mais semelhantes entre espécies próximas do que entre espécies mais distantes. Hipóteses filogenéticas permitem identificar eventos evolutivos independentes e, conseqüentemente, são extremamente úteis em análises estatíticas de diferentes caracteres, uma vez que a maior parte destas análises partem da premissa de eventos independentes (Harvey & Pagel, 1991). De acordo com Harvey & Pagel (1991), existem pelo menos três razões pelas quais espécies proximamente relacionadas sejam semelhantes: conservacionismo filogenético do nicho, *time lag* filogenético e respostas adaptativas similares. O trabalho clássico de Felsenstein (1985) chamou a atenção para o fato de que as espécies, devido às relações filogenéticas, fazem parte de uma estrutura hieráquica e não poderiam, portanto, ser usadas em análise estatísticas na forma de eventos independentes provenientes de uma mesma distribuição, uma vez que poderiam levar à detecção de correlações inexistentes entre caracteres. Neste mesmo trabalho, Felsenstein (1985) propôs o método de contrastes independentes filogenéticos, cujo objetivo seria produzir um conjunto de dados contínuos de eventos independentes, adequado a análises estatíticas que partissem deste pressuposto. Este foi o primeiro trabalho a enfatizar a importância de se considerar as relações filogenéticas em biologia comparativa, em que o autor conclui que: "Filogenias são fundamentais em biologia comparativa; não há como fazê-lo sem levá-las em consideração." Desde então, vários métodos de análise comparativa foram propostos com o mesmo objetivo (ver revisões em Pagel, 1991 e Martins, 2000). Rohlf (2006) mostrou que o uso de correções filogenéticas não afeta de forma significativa o valor da estimativa dos coeficientes de correlação ou regressão. Entretanto, ignorar as relações filogenéticas aumenta o desvio padrão destas estimativas e a probabilidade de erro tipo I, ou seja, rejeitar uma hipótese que é verdadeira.

A existência de hipóteses filogenética também permite, a princípio, descrever o estado de caracteres em ancestrais hipotéticos e, a partir disto, inferir padrões de evolução e função de caracteres. Existem alguns métodos de reconstrução de caracteres ancestrais, entre os quais o critério de máxima parsimônia é um dos mais usados, além de métodos de máxima verossimilhança para caracteres discretos (Pagel, 1999).

Uma vez que a superimposição de Procrustes é baseada na superimposição de todos os espécimes em uma configuração escolhida como referência, os métodos de análise estatístistica não podem ser influenciados por diferentes escolhas da orientação da configuração de referência (ver revisão em Rohlf, 2002). Isso significa que métodos de parcimônia linear não devem ser usados para a reconstrução de configurações ancestrais, sendo preferíveis os métodos de *squared-change parsimony* (Huey & Bennet, 1987; Maddison, 1991) e de máxima verossimilhança para caracteres contínuos (Felsenstein, 1988). No método de *squared-change parsimony* (Huey & Bennet, 1987; Maddison, 1991), os estados ancestrais são estimados de forma que a soma dos quadrados dos comprimentos dos ramos sejam minimizados em uma árvore filogenética. A estimativa do valor de um caráter para um nó interno da árvore é simplesmente a média entre os valores dos nós imediatamente conectados a ele (Huey & Bennet, 1987; Maddison, 1991).

## ORGANIZAÇÃO DA TESE

Fazem parte desta tese dois artigos preparados da forma em que seriam submetidos à publicação, que constituem os capítulos 1 e 2, aos quais se segue uma compilação das conclusões gerais obtidas em cada trabalho. São apresentados os manuscritos, no formato requisitado por cada periódico ao qual seriam submetidos. Com a finalidade de facilitar a leitura da tese, as figuras e tabelas foram inseridas ao longo do texto, em vez de serem apresentadas ao final de cada manuscrito.

## OBJETIVOS

Os objetivos deste trabalho foram:

1. Estudar a variação morfométrica da asa de *Drosophila mediopunctata*, procurando verificar efeitos da temperatura, do cariótipo e a possível existência de interação genótipo-ambiente para o tamanho e forma da asa.
2. Caracterizar a variação morfométrica da asa entre espécies da radiação *tripunctata*.
3. Propor uma filogenia de espécies da radiação *tripunctata* com base nas seqüência do genes mitocondriais da citocromo oxidase subunidades I e II (COI e COII) e discutir, com base nesta filogenia, padrões de evolução da asa entre estas espécies.

***CAPÍTULO 1***

# SHAPE AND SIZE VARIATION ON THE WING OF *DROSOPHILA MEDIOPUNCTATA*: INFLUENCE OF CHROMOSOME INVERSIONS AND GENOTYPE-ENVIRONMENT INTERACTION

Luciane M. Hatadani and Louis B. Klaczko

Departamento de Genética e Evolução, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), Cx. Postal 6109, 13083-970. Campinas, SP, Brazil.

telephone: 55+19-3788-1150; FAX: 55+19-3788-6235.

*Artigo aceito para publicação pelo periódico **Genetica** em 5 de outubro de 2007.*

## ABSTRACT

The second chromosome of *Drosophila mediopunctata* is highly polymorphic for inversions. Previous work reported a significant interaction between these inversions and collecting date on wing size, suggesting the presence of genotype-environment interaction. We performed experiments in the laboratory to test for the joint effects of temperature and chromosome inversions on size and shape of the wing in *D. mediopunctata.* Size was measured as the centroid size, and shape was analyzed using the generalized least squares Procrustes superimposition followed by discriminant analysis and canonical variates analysis of partial warps and uniform components scores. Our findings show that wing size and shape are influenced by temperature, sex, and karyotype. We also found evidence suggestive of an interaction between the effects of karyotype and temperature on wing shape, indicating the existence of genotype-environment interaction for this trait in *D. mediopunctata*. In addition, the association between wing size and chromosome inversions is in agreement with previous results indicating that these inversions might be accumulating alleles adapted to different temperatures. However, no significant interaction between temperature and karyotype for size was found – in spite of the significant presence of temperature - genotype (cross) interaction. We suggest that other ecological factors – such as larval crowding – or seasonal variation of genetic content within inversions may explain the previous results.



KEYWORDS:

Chromosome inversions, wing, temperature, genotype-environment interaction, plasticity, geometric morphometrics.

## Introduction

Many traits have been associated with chromosome inversion polymorphisms in *Drosophila*. These include heat/cold resistance, female fecundity, male mating success and longevity (see review in Hoffmann et al., 2004). For morphometric traits, chromosome inversions have also been related to wing loading (Iriarte , Norry & Hasson, 2003), wing area and thorax length (Calboli, Gilchrist & Partridge, 2003), and wing size and shape (Bitner-Mathé, Peixoto & Klaczko, 1995; Orengo & Prevosti, 2002).

*Drosophila mediopunctata* Dobzhansky & Pavan 1943 has a distribution that ranges from southern South America to Central America (Val et al. 1981). Life history traits in this species are quite different than in other commonly studied *Drosophila* species (e.g. *D. melanogaster* and *D. pseudoobscura*), since maturation time is long, fecundity is low and longevity is extended. This species belongs to the *Drosophila tripunctata* group, the second largest Neotropical *Drosophila* group. Chromosome inversion polymorphisms appear to be common in this group. Kastritsis (1966) found polymorphic inversions in nine of eleven species, the second chromosome being the most polymorphic chromosome of the group. *D. mediopunctata* has six pairs of chromosomes: five acrocentrics and a dot that does not undergo polytenization. The X, second and fourth chromosomes are polymorphic for inversions. The second chromosome is the most polymorphic and its inversions can be divided in two groups according to their position with respect to the centromere: there are eight distal inversions (*DA, DP, DS, DV* etc.) and nine proximal (*PA0*, *PB0*, *PC0, PC1* etc.) (Ananina et al., 2002). Distal and proximal inversions are in intense linkage disequilibrium so that *DA* is associated with *PA0, DP* is associated with *PC0* and *DS* with *PC0* (Peixoto & Klaczko, 1991). Second chromosome inversion frequencies in *D. mediopunctata* change during the year so that *PA0* tends to be more frequent in colder months than in warmer months, whereas *PC0* varies inversely (Klaczko, 1995, 2006; Ananina et al.,

2004). These data suggest that *PA0* is more adapted to lower temperatures and *PC0* to higher temperatures. In addition, second chromosome inversions in this species have been previously associated with abdominal pigmentation polymorphism (Hatadani et al., 2004).

Bitner-Mathé & Klaczko (1999) estimated "natural" heritabilities of size and shape of the wing of *D. mediopunctata* and found a high heritability for shape and a lower, but still significant, heritability for size. As these authors also found that size variation was much greater in nature than in the laboratory, whereas variation in shape remained approximately the same in both conditions, they concluded that size and shape could have different genetic properties in this species. In another study, Bitner-Mathé, Peixoto & Klaczko (1995) observed an altitudinal cline for wing shape in which distances between landmarks on the distal margin of the wing were responsible for most of the shape variation. Moreover, they found a significant effect of second chromosome inversions on wing shape – also caused primarily by variation in distances between landmarks on the distal margin of the wing – and a significant interaction between second chromosome inversions and collection date on wing size. According to the authors, this interaction indicated the presence of genotype-environment interaction for wing morphology in this species.

In the present study, we performed laboratory experiments to test for effects of temperature and second chromosome inversions on wing size and shape in *D. mediopunctata.* Our findings show that wing size and shape are influenced by temperature, sex, and karyotype. Moreover, our results suggest the presence of the interaction of temperature and karyotype on wing shape, indicative of genotype-environment interaction.

**Materials and Methods**

In order to test for the effect of second chromosome inversions on wing morphology, we produced strains which differed from each other on the second chromosome but shared the same genetic background. We used isofemale lines obtained from two collections from Serra do Japi, SP, Brazil (23°11'S, 46°40'W) in July 1994 and May 1995. These strains were crossed with marker strains to replace all chromosomes except the second, which was made autozygous by inbreeding. Whenever the second chromosome from the isofemale line carried a recessive lethal allele, heterokaryotipic males and females were crossed and the strain was kept balanced against a visible mutation, which was also recessive and lethal. Thus, we obtained strains that were differentiated in the second chromosome, but not in the other chromosomes, that is, they shared the same genetic background. In addition, no strains were lost due to lethal alleles on the second chromosome. The detailed description of the procedure used to produce these strains can be found in Hatadani et al. (2004). Overall, 27 strains were produced (14 *PA0* and 13 *PC0*) using this approach. In order to avoid autozygosis for an entire chromosome, crosses were made between strains with the same karyotype, so that the resulting offspring would simultaneously be homokaryotypic and allozygous (two alleles of independent origin) for the proximal inversions of the second chromosome. The crosses were chosen randomly among all possibilities, with the condition that each strain would be part of only one cross. This procedure was used to ensure that the results would be independent among crosses. Crosses were made with males from one strain and females from another strain and reciprocal crosses were not made. First instar larvae were collected from the offspring of each cross and placed in vials containing 10 ml of culture medium. These larvae were kept at 16.5°C or 20°C, twelve larvae per vial.

Left wings were prepared on microscope slides and images were captured using a digital camera attached to a microscope. Approximately 30 wings of each sex per cross were measured, resulting in a total of 1216 wings. For morphometric analysis, we used geometric morphometrics methods (Bookstein, 1991; Rohlf & Marcus, 1993; Dryden & Mardia, 1998 and Adams et al., 2004). Eleven landmarks were used (figure 1) located at points of vein intersections or at their extremities, ensuring that all landmarks would be homologous and easily recognized.

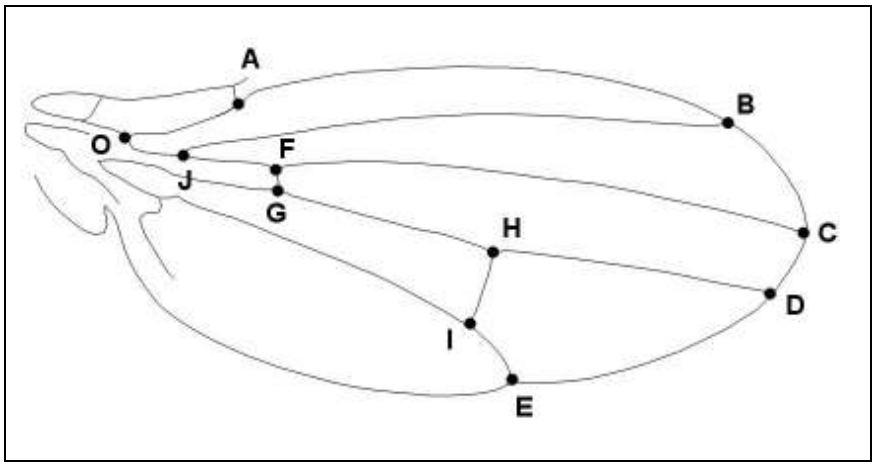


**Figure 1:** Wing of *Drosophila mediopunctata* with the eleven landmarks used for the analysis.

Size was measured as the centroid size (CS), calculated as the square root of the sum of the squared distances of each landmark to the centroid (Bookstein, 1991). Temperature, karyotype, cross and sex effects on CS were assessed by analysis of variance (ANOVA). Effects of temperature, sex and karyotype were considered fixed and the effect of cross was considered to be a random effect.

For the analysis of shape, the landmark coordinates were aligned by generalized least squares Procrustes superimposition (Rohlf, 1990). The aligned coordinates were used to compute the matrix of partial warps and uniform components, as described in Bookstein (1989, 1991). This method uses the fitting of an interpolating function, called the thin-plate spline, to the coordinates of the landmarks of the individuals in the sample. Partial warps and uniform

components scores were calculated by the program TPS Relw 1.29 (F. James Rohlf, 2003, SUNY at Stony Brook), with the scaling option $\alpha=0$ (i.e. no weights were assigned to any of the landmarks). The simplest way to evaluate effects of temperature, karyotype, sex and interactions on wing shape would be a multivariate analysis of variance (MANOVA) to the partial warps and uniform components scores. However, as our data are the result of a nested experimental design – the effect of cross is nested within karyotype and should be treated as a random factor in the model – it was not possible to perform a MANOVA correctly (i.e. considering cross as a random effect) due to insufficient degrees of freedom. Hence, partial warps and uniform component scores were analyzed by canonical variates analysis (CVA) in which 95% confidence regions were calculated for each of the eight groups formed by classifying individuals by sex, karyotype and temperature. The 95% confidence regions were computed with MATLAB 6.5, as described in Von Zuben et al. (1998). The same shape variables were also analyzed by discriminant analysis, separated by sex, with four groups in each analysis, determined by temperature and karyotype.

In order to estimate repeatability, 30 wings of each sex, temperature and karyotype (240 wings in total) were digitized twice. Repeatability was computed for the coordinates of each landmark and for centroid size as the intraclass correlation (Falconer, 1981). We specifically avoided overestimating repeatabilities caused by using the mean squares as variance components as described in Lessells & Boag (1987).

In order to account for allometric effects, we performed a multivariate regression of landmark aligned coordinates on CS with the program NTSYSpc 2.11(Rohlf, 1998). Size-dependent variation was removed by adding the residuals to the mean landmark configuration. Consequently, we obtained a standardized shape dataset, not correlated to CS. The standardized

Procrustes coordinates were used to produce the partial warps and uniform components matrix, which was analyzed by canonical variates analysis and discriminant analysis as described previously.

**Results**

All repeatabilities for coordinates of each landmark and centroid size were higher than 90% and did not vary considerably among landmarks (table 1). Analysis of variance of centroid size (table 2) resulted in significant effects of temperature, sex, karyotype and a significant interaction between temperature and cross (within karyotype). As expected, flies raised at 20°C have smaller wings (CS=3.46±0.009mm) than flies that developed at 16.5°C (CS=3.65±0.010mm), a pattern known as the temperature-size rule (reviewed in Atkinson, 1994). Male wings were smaller (CS=3.33±0.006mm) than those of females (CS=3.76±0.006mm), and flies carrying the *PA0* karyotype had larger wings (CS=3.59±0.010mm) when compared to flies carrying *PC0* (CS=3.51±0.011mm).

**Table 1:** Repeatabilities of coordinates of each landmark and centroid size (CS).

| Variable | Repeatability |
|---|---|
| 1x | 0.9980 |
| 1y | 0.9988 |
| 2x | 0.9989 |
| 2y | 0.9992 |
| 3x | 0.9994 |
| 3y | 0.9994 |
| 4x | 0.9991 |
| 4y | 0.9994 |
| 5x | 0.9987 |
| 5y | 0.9992 |
| 6x | 0.9140 |
| 6y | 0.9945 |
| 7x | 0.9278 |
| 7y | 0.9821 |
| 8x | 0.9083 |
| 8y | 0.9937 |
| 9x | 0.9226 |
| 9y | 0.9822 |
| 10x | 0.9981 |
| 10y | 0.9995 |
| 11x | 0.9982 |
| 11y | 0.9991 |
| CS | 0.9994 |

Multivariate regression of shape variables on centroid size was significant by the generalized Goodall F-test ($F=74.92$, $P<0.0001$), although the percentage of explained variation was only 5.81%. Discriminant analysis of total shape variation correctly classified 80.1% of females and 78.0% of males (table 3) whilst 74.2% of females and 77.0% were classified correctly when size-dependent effects on shape were removed (table 4). The first and second canonical variates were responsible for 75.02% of total shape variation and 94.02% of non-allometric shape variation (figure 2). Regarding total shape variation (figure 2a), there was no overlap among confidence regions, except for *PA0* males and *PC0* females reared at 20°C. However, when size-dependent effects were removed (figure 2b), no confidence intervals overlapped among any of the eight groups separated by temperature, karyotype and sex. In both cases, the first canonical variate separated groups by temperature and the second canonical variate separated karyotypes with the exception of *PA0* males and *PC0* females reared at 20°C. Males and females were separated by CV1 and CV2 combined. Although this kind of analysis does not allow for a direct test for interactions between independent variables, an indication of different response rates of each karyotype to temperature can be obtained by observing whether Mahalanobis distances between average shapes of each temperature differ according to each karyotype. An interaction of these two variables would be suggested if the distance between average shapes of the two temperatures differed according to karyotype. Our results show that distances are larger when comparing between temperatures for inversion *PC0* than for *PA0* (table 5). The Mahalanobis distance between temperatures for *PA0* is approximately half of the same distance for *PC0*. This indicates that the same temperature change causes flies carrying the *PC0* karyotype to undergo a larger shape change than flies carrying *PA0*. This is true whether size

effects on shape are removed or not. However, since these comparisons are not based on confidence intervals, these results should be considered carefully.

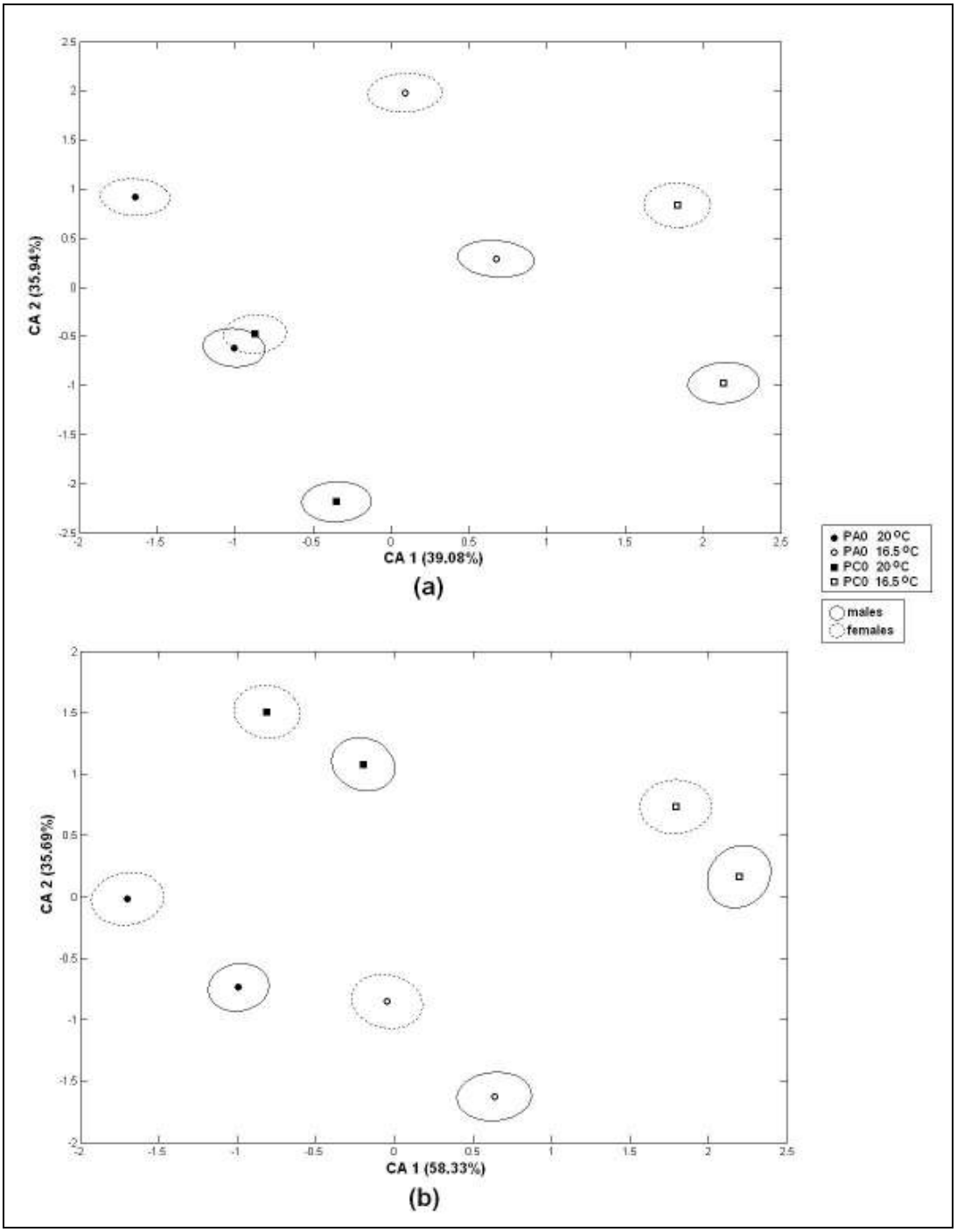


**Figure 2:** Confidence regions (95%) of first and second canonical variates obtained from partial warps and uniform components scores. The percentage of variance explained by each variate is shown in the label of each axis. (a) total shape variation; (b) non-allometric shape variation.

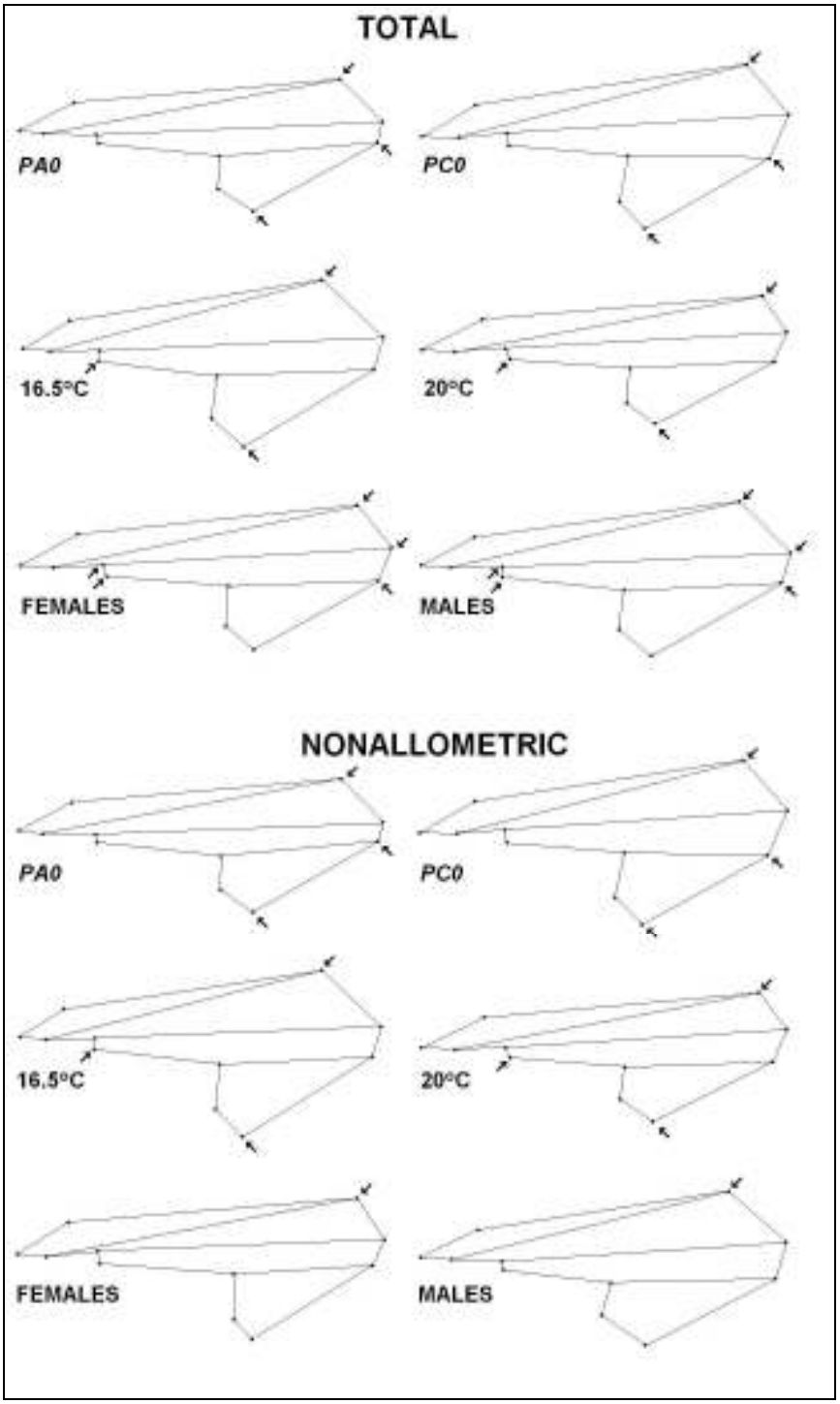


**Figure 3:** Pair-wise comparisons between consensus configurations of each karyotype, temperature and sex. Total shape variation is represented in the upper half of the figure: the first pair of configurations represents shape differences between karyotypes *PA0* and *PC0*; the second pair represents differences between temperatures 16.5°C and 20°C; and the third pair represents differences between females and males. Nonallometric shape variation is represented in the lower half of the figure in the same order as above. The arrows indicate landmarks responsible for most of the shape variation according to each variable.

**Table 2:** ANOVA of centroid size with effects of temperature, karyotype, cross, sex and interactions.

| Source | DF | MS | F | P |
|---|---|---|---|---|
| Temperature (T) | 1 | 10.8805 | 158.33 | <0.001 |
| Karyotype (K) | 1 | 1.9289 | 17.74 | 0.002 |
| Cross (C) | 9 | 0.1087 | 1.65 | 0.243 |
| Sex (S) | 1 | 55.3085 | 15582.87 | 0.000 |
| T x K | 1 | 0.0961 | 1.40 | 0.267 |
| T x C | 9 | 0.0687 | 11.05 | <0.001 |
| K x S | 1 | 0.0005 | 0.13 | 0.724 |
| S x C | 9 | 0.0035 | 0.57 | 0.822 |
| Error | 1183 | 0.0062 | | |

In addition, we also conducted ANOVAs on the first five relative warps (RWs) scores in order to test for the effects of temperature, karyotype, sex, cross (not analyzed by CVA or discriminant analysis), and interactions (analyses not shown). Results from these analyses confirmed significant effects of temperature (RW1, RW2, RW3 and RW5), sex (RW1 RW2, RW4 and RW5), and karyotype (RW1 and RW3). A significant interaction between temperature and karyotype (for RW1) was also found.

**Table 3:** Classification matrix derived from discriminant function analysis based on partial warp scores and uniform components (total shape variation).

| Predicted group | Correct group | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Females | | | | Males | | | |
| | 16.5°C | | 20°C | | 16.5°C | | 20°C | |
| | PA0 | PC0 | PA0 | PC0 | PA0 | PC0 | PA0 | PC0 |
| 16.5°C / PA0 | 134 | 17 | 11 | 2 | 130 | 18 | 22 | 2 |
| 16.5°C / PC0 | 24 | 118 | 3 | 6 | 23 | 114 | 3 | 11 |
| 20°C / PA0 | 21 | 3 | 122 | 11 | 17 | 3 | 114 | 9 |
| 20°C / PC0 | 0 | 8 | 16 | 118 | 3 | 11 | 10 | 110 |
| Total N | 179 | 146 | 152 | 137 | 173 | 146 | 149 | 132 |
| Proportion correct | 0.749 | 0.808 | 0.803 | 0.861 | 0.751 | 0.781 | 0.765 | 0.833 |

**Table 4:** Classification matrix derived from discriminant function analysis based on partial warp scores and uniform components (non-allometric variation).

| Predicted group | Correct group | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Females | | | | Males | | | |
| | 16.5°C | | 20°C | | 16.5°C | | 20°C | |
| | PA0 | PC0 | PA0 | PC0 | PA0 | PC0 | PA0 | PC0 |
| 16.5°C / PA0 | 114 | 14 | 29 | 9 | 111 | 9 | 19 | 4 |
| 16.5°C / PC0 | 13 | 114 | 0 | 14 | 10 | 110 | 2 | 12 |
| 20°C / PA0 | 17 | 2 | 127 | 23 | 24 | 1 | 133 | 21 |
| 20°C / PC0 | 8 | 7 | 23 | 102 | 4 | 13 | 19 | 108 |
| Total N | 152 | 137 | 179 | 148 | 149 | 133 | 173 | 145 |
| Proportion correct | 0.750 | 0.832 | 0.709 | 0.689 | 0.745 | 0.827 | 0.769 | 0.745 |

**Table 5:** Mahalanobis distances among group average shapes for total shape variation (above diagonal) and non-allometric shape variation (below diagonal). Distances between temperatures for each karyotype are in bold.

| | | | Females | | | | Males | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | PA0 | | PC0 | | PA0 | | PC0 | |
| | | | 16.5°C | 20°C | 16.5°C | 20°C | 16.5°C | 20°C | 16.5°C | 20°C |
| Females | *PA0* | 16.5°C | - | **0.00388** | 0.00524 | 0.00786 | 0.00463 | 0.00740 | 0.01099 | 0.01471 |
| | | 20°C | **0.003148** | - | 0.01087 | 0.00354 | 0.00812 | 0.00383 | 0.01495 | 0.00961 |
| | *PC0* | 16.5°C | 0.005090 | 0.010745 | - | **0.00797** | 0.00811 | 0.01244 | 0.00450 | 0.01226 |
| | | 20°C | 0.005327 | 0.003059 | **0.006582** | - | 0.00985 | 0.00515 | 0.00992 | 0.00393 |
| Males | *PA0* | 16.5°C | 0.001137 | 0.007092 | 0.006137 | 0.009930 | - | **0.00374** | 0.00536 | 0.00918 |
| | | 20°C | 0.001010 | 0.001074 | 0.008282 | 0.004723 | **0.003362** | - | 0.00916 | 0.00402 |
| | *PC0* | 16.5°C | 0.005338 | 0.012645 | 0.000744 | 0.009398 | 0.005121 | 0.009326 | - | **0.00684** |
| | | 20°C | 0.003260 | 0.003217 | 0.003608 | 0.000888 | 0.006904 | 0.003502 | **0.005827** | - |

Comparisons of consensus configurations of each treatment indicated that, regarding total shape variation, temperature seemed to affect mainly landmarks B, E and G, whereas karyotype affected mainly landmarks B, D and E (figure 3). Temperature and karyotype seemed to affect nonallometric shape variation in a very similar way. Shape differences between sexes, on the other hand, are due to mostly allometric variation, since landmarks B, C, D, F and G are affected by sex when total shape variation is considered, whereas only landmark B is influenced by sex when size correlation is removed (figure 3). Thus, most of the shape variation observed in this study was caused by displacement of landmarks located at the distal region of the wing.

## Discussion

*Wing size*

Our results revealed a significant association between wing size and chromosome inversions, similar to other studies of chromosome arrangement associated morphological variation in *Drosophila* species, e.g. *D. subobscura* (Orengo & Prevosti 2002), *D. melanogaster* (Weeks et al, 2002; Calboli, Kennington & Partridge, 2003), *D. ananassae* (Yadav & Singh, 2003) and *D. buzzatii* (Fanara, Hasson & Rodriguez 1997; Ruiz et al., 1991; Hasson et al. 1992). Previously, a chromosome inversion effect in *D. mediopunctata* was detected only as a

significant interaction with collecting date (Bitner-Mathé, Peixoto & Klaczko (1995). In the present study, inversion *PA0* was clearly associated with larger wings, and inversion *PC0* was associated with smaller wings. This is consistent with previous evidence that inversion *PA0* is more adapted to lower temperatures (Ananina et al., 2004; Klaczko, 2006) and *PC0*, to higher temperatures as suggested by a significantly positive altitudinal cline for *PA0,* and a negative altitudinal cline for *PC0*.

Even though our results are consistent with the hypothesis of natural selection favoring larger wings under lower temperatures, the adaptive nature of this relationship is still not clear. Santos et al. (2006) argued that clines formed in North and South America for inversion frequencies and body size in *D. subobscura* were not related, based on laboratory populations, although natural populations in Europe exhibit a positive association between chromosome inversions and wing size along a latitudinal cline (Orengo & Prevosti 2002). Santos et al. (2006) also proposed that clines in North and South America could not only be explained by temperature variation and suggested that other factors, including larval crowding, may have an important role in the formation of these clines. This hypothesis is consistent with our results: there was an overall effect of temperature on size because individuals developing under lower temperatures are larger than those growing under higher temperatures (Robertson, 1987; Thomas, 1993; Partridge et al., 1994; Crill et al., 1996; Pétavy et al., 2001; Azevedo, French & Partridge, 2002; David et al., 1994, 1997, 2006); there was an interaction between genotype (cross) and temperature, the inversions analyzed showed average differences in size (*PA0* larger than *PC0*), and no interaction between inversion and temperature was found. This suggests that other factors – perhaps larval density – may be responsible for the karyotype x collecting date interaction observed by Bitner-Mathé, Peixoto & Klaczko (1995).

*Wing shape*

Temperature also seems to influence wing shape in *Drosophila* (Imasheva et al., 1995; Cavicchi et al., 1985), although there are fewer studies focusing the effect of temperature on wing shape than on size and Griffiths et al. (2005) did not detect a latitudinal cline for this trait in *D. birchii*.

Our results suggested that temperature influences wing shape in *D. mediopunctata* due mostly to differences in the placement of landmarks located at the distal region of the wing (Figure 3) consistent with previous results (Bitner-Mathé, Peixoto & Klaczko 1995). They suggested that wing shape variation among flies collected at different times of the year, possibly due to temperature changes, was caused mainly by variation in distances between landmarks B, C and D (figure 3). Distal landmarks for both total and non-allometric shape variation were responsible for most of the shape differences between temperatures (Figure 3). Similarly, Debat et al. (2003) found that distal wing landmarks were more strongly influenced by temperature than proximal ones in *D. simulans*. These authors decomposed wing shape variation in allometric and non-allometric components and found that both components were influenced by temperature in different ways; the allometric component displayed a directional change proportional to temperature, and the non-allometric component showed a reversal in the direction of shape change at an intermediary temperature. In both components, however, distal landmarks were the most sensitive to temperature changes, similar to our results with *D. mediopunctata*.

*Genotype-environment interaction*

Our results suggest the existence of genotype-environment interaction for wing shape in *D. mediopunctata*, but not size whereas the opposite was found by Bitner-Mathé, Peixoto & Klaczko (1995). One possibility for this apparent disagreement is that wing morphology is

affected by environmental factors other than temperature (e. g. larval crowding, as suggested by Santos et al., 2006).

As an alternate explanation, the genetic contents influencing wing size and shape could vary for the same chromosome inversion. Our observations support this hypothesis for size. In that case, the strains used in our study could have differed genetically from the flies that were observed by Bitner-Mathé, Peixoto & Klaczko (1995) in natural populations, leading to different conclusions. One of the possibilities is that seasonal fluctuations of alleles occur within each karyotype. Such seasonal cycles of gametic disequilibrium between allozyme and inversions were observed in *Drosophila subobscura* (Rodríguez-Trelles, 2003), suggested by the authors to be caused most likely by natural selection. We should note that our results revealed a strong significant interaction between cross (within karyotype) and temperature for wing size showing that each karyotype is heterogeneous in its response for size in different temperatures. Actually, a similar result of interaction between temperature and line for wing size was detected in *D. subobscura* by Iriarte, Céspedes & Santos (2003).

To this point, we have strong evidence that temperature and chromosome inversions influence both wing shape and size in *D. mediopunctata.* As for the existence of karyotype-environment interaction, the apparent conflict between our results and those obtained by previous works suggest that additional experimentation, including other environmental factor may clarify the field observations.

**ACKNOWLEDGEMENTS**

We thank Wilma N. Souza and Maria Aparecida Ramos Libânio for technical help. Mariana Lúcio Lyra helped us with figure drawing and Ted Hogan reviewed the English version of the manuscript. We thank Izabella Mendes Hatadani for important help with the statistical

analysis; Fernando Von Zuben for statistical advices and help on the use of Matlab software; and Prof. F. James Rohlf for statistical suggestions. We also thank two anonymous reviewers and particularly Dr. William Etges (the Associate Editor) for suggestions that greatly improved this paper. This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa de São Paulo (FAPESP) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

**REFERENCES**

Adams DC, Rohlf FJ, Slice DE (2004) Geometric morphometrics: Ten years of progress following the revolution. Ital J Zool 71: 5-16

Ananina G, Peixoto AA, Souza WN et al (2002) Polytene chromosome map and inversion polymorphism in *Drosophila mediopunctata*. Mem Inst Oswaldo Cruz 97: 691- 694

Ananina G, Peixoto AA, Bitner-Mathé BC et al (2004) Chromosomal inversion polymorphism in *Drosophila mediopunctata*: seasonal, altitudinal, and latitudinal variation. Genet and Molec Biol 27: 61-69

Atkinson D, Sibly, RM (1997) Why are organisms usually bigger in colder environments? Making sense of a life history puzzle. Trends Ecol Evol 12: 235-239

Azevedo RB, French V, Partridge L (2002) Temperature modulates epidermal cell size in Drosophila melanogaster. J. Insect Physiol. 48: 231-237

Bitner-Mathé BC, Klaczko LB (1999) Size and shape heritability in natural populations of *Drosophila mediopunctata*: temporal and microgeographical variation. Genetica 105: 35-42

Bitner-Mathé BC, Peixoto AA, Klaczko LB (1995) Morphological variation in a natural population of *Drosophila mediopunctata*: altitudinal cline, temporal changes and influence of chromosome inversions. Heredity 75: 54-61

Bookstein FL (1989) Principal warps: Thin-plate splines and the decomposition of deformations. IEEE Trans Patt Anal Mach Intell 11: 567-585

Bookstein FL (1991) Morphometric tools for landmark data. Cambridge University Press, Cambridge

Calboli FC, Gilchrist GW, Partridge L (2003) Different cell size and cell number contribution in two newly established and one ancient body size cline of *Drosophila subobscura.* Evolution 57: 566-73

Calboli FCF, Kennington WJ, Partridge L (2003) QTL mapping reveals a striking coincidence in the positions of genomic regions associated with adaptive variation in body size in parallel clines of *Drosophila melanogaster* on different continents. Evolution 57: 2653-2658

Cavicchi S, Guerra D, Giorgi, G et al (1985) Temperature-related divergence in experimental populations of *Drosophila melanogaster.* I. Genetic and developmental basis of wing size and shape variation. Genetics 109: 665-689

Crill WD, Huey RB, Gilchrist GH (1996) Within and between-generation effects of temperature on the morphology and physiology of *Drosophila melanogaster*. Evolution 50: 1205-1218

David JR, Moreteau B, Gauthier JR et al (1994) Reaction norms of size characters in relation to growth temperature in Drosophila melanogaster: an isofemale lines analysis. Genet Sel Evol 26: 229-251

David JR, Gibert P, Gravot et al (1997) Phenotypic plasticity and developmental temperature in *Drosophila*: analysis and significance of reaction norms of morphometrical traits. J Therm Biol 22: 441-451

David JR, Legout H, Moreteau B (2006) Phenotypic plasticity of body size in a temperate population of *Drosophila melanogaster*: when the temperature-size rule does not apply. J Genet 85: 9-23

Debat V, Begin M, Legout H, David JR (2003) Allometric and nonallometric components of *Drosophila* wing shape respond differently to developmental temperature. Evolution 57: 2773-2784

Dobzhansky Th, Pavan C (1943) Studies on Brazilian Species of *Drosophila.* Bol Faculd Fil Cienc Letr S Paulo 36: 7-72

Dryden IL, Mardia KV (1998) Statistical Shape Analysis. John Wiley & Sons, Chichester

Falconer DS (1981) Introduction to quantitative genetics. 2nd edn. Longman, London

Fanara JJ, Hasson E, Rodriguez C (1997) The effect of polymorphic inversions on body size in two natural populations of *Drosophila buzzatii* from Argentina. Hereditas 126: 233-237

Griffiths JA, Schiffer M, Hoffmann AA (2005) Clinal variation and laboratory adaptation in the rainforest species *Drosophila birchii* for stress resistance, wing size, wing shape and development time. J. Evol. Biol. 18: 213-22

Hasson E, Fanara JJ, Rodriguez C et al (1992) The evolutionary history of *Drosophila buzzatii*. XXIV. Second chromosome inversions have different average effects on thorax length. Heredity 68: 557-563

Hatadani LM, Baptista JC, Souza WN et al (2004) Colour polymorphism in *Drosophila mediopunctata*: genetic (chromosomal) analysis and nonrandom association with chromosome inversions. Heredity 93: 525-534

Hoffmann AA, Sgrò CM, Weeks AR (2004) Chromosomal inversion polymorphisms and adaptation. Trends Ecol Evol 19: 482-488

Imasheva AG, Bubli OA, Lazebny OE et al (1995) Geographic differentiation in wing shape in *Drosophila melanogaster*. Genetica 96: 303-306

Iriarte PF, Céspedes W, Santos M (2003) Quantitative-genetic analysis of wing form and bilateral asymmetry in isochromosomal lines of *Drosophila subobscura* using Procrustes methods. J Genet 82: 95-113

Iriarte PF, Norry FM, Hasson ER (2003) Chromosomal inversions effect body size and shape in different breeding resources in *Drosophila buzzatii*. Heredity 91: 51-59

Kastritsis CD (1966) Cytological studies on some species of the *tripunctata* group of Drosophila. Univ Texas Publs Stud Genet 6615: 413-474.

Klaczko LB (1995) Population genetics of *Drosophila mediopunctata*. In: Levine L (Ed.) Genetics of Natural Populations: the Continuing importance of Theodosius Dobzhansky. Columbia University Press. New York

Klaczko LB (2006) Evolutionary genetics of *Drosophila mediopunctata*. Genetica 126: 43-55

Lessells CM, Boag PT (1987) Unrepeatable repeatabilities: a commom mistake. Auk 104: 116-121.

Orengo DJ, Prevosti A (2002) Relationship between chromosomal polymorphism and wing size in a natural population of *Drosophila subobscura*. Genetica 115: 311-318

Partridge L, Barrie B, Fowler K et al (1994). Evolution and development of body size and cell size in *Drosophila melanogaster* in response to temperature. Evolution 48: 1269-1276

Peixoto AA, Klaczko LB (1991) Linkage disequilibrium analysis of chromosomal inversion polymorphisms of *Drosophila*. Genetics 129: 773-777

Petavy G, Moreteau B, Gibert P et al (2001) Phenotypic plasticity of body size in *Drosophila*: effects of a daily periodicity of growth temperature in two sibling species. Physiol Ent 26: 351-361

Robertson FW (1987) Variation of body size within and between wild populations of *Drosophila buzzatii*. Genetica, 72: 111-125

Rodríguez-Trelles F (2003) Seasonal cycles of allozyme-by-chromosomal-inversion gametic disequilibrium in *Drosophila subobscura*. Evolution 57: 839-848

Rohlf FJ (1990) Rotational fit (Procrustes) methods. In: Rohlf FJ & Bookstein FL (eds) Proceedings of the Michigan Morphometrics Workshop, Special Publication No 2. University of Michigan Museum of Zoology, Ann Arbor

Rohlf FJ (1998) NTSYSpc numerical taxonomy and multivariate analysis system version 2.10. Exeter Software, Setauket

Rohlf, F. J. 2003. tpsRelw, relative warps analysis, version 1.36. Department of Ecology and Evolution, State University of New York at Stony Brook.

Rohlf FJ, Marcus, LF (1993) A revolution in morphometrics. Trends Ecol Evol 8: 129-132

Ruiz A, Santos M, Barbadilla A et al (1991) Genetic variance for body size in a natural population of *Drosophila buzzatii*. Genetics 128: 739-750

Santos M, Céspedes W, Balanyà J et al (2006). Temperature-related genetic changes in laboratory populations of *Drosophila subobscura:* evidence against simple climatic-based explanations for latitudinal clines. Am Nat 165: 258-273

Thomas RH (1993) Ecology of body size in *Drosophila buzzatii*: untangling the effects of temperature and nutrition. Ecol Entomol 18: 84-90

Val FC, Vilela CR, Marques MD (1981). Drosophilidae of neotropical region. In: Ashburner M, Carson HC, Thompson, Jr JN. (eds.), The genetics and Biology of Drosophila, vol 3a. Academic Press. London

Von Zuben FJ, Duarte LC, Stangenhaus G, Pessôa LM, Reis SF (1998) Bootstrap confidence regions for canonical variates: application to studies of evolutionary differentiation. Biom J 40: 327-339

Yadav JP, Singh BN (2003) Population genetics of *Drosophila ananassae*: inversion polymorphism and body size in Indian geographical populations. J Syst Evol Res 41: 217-226

Weeks AR, McKechnie SW, Hoffmann AA (2002) Dissecting adaptive clinal variation: markers, inversions and size/stress associations in *Drosophila melanogaster* from a central field population. Ecol Lett 5: 756-763

***CAPÍTULO 2***

# MOLECULAR PHYLOGENY AND WING SIZE AND SHAPE VARIATION OF THE *TRIPUNCTATA* GROUP AND CLOSELY RELATED SPECIES GROUPS (*DROSOPHILA* – DROSOPHILIDAE)

Luciane Mendes Hatadani[1], James O. McInerney[2], Hermes Fonseca de Medeiros[1], Ana Carolina Martins Junqueira[3], Ana Maria de Azeredo-Espin[3] and Louis Bernard Klaczko[1]

[1] Departamento de Genética e Evolução, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), P.O. Box 6109, Campinas, 13083-970 SP, Brazil.

[2] Bioinformatics Laboratory, Department of Biology, National University of Ireland, Maynooth, Co. Kildare, Ireland.

[3] Laboratório de Genética Animal. Centro de Biologia Molecular e Engenharia Genética e Instituto de Biologia. Universidade Estadual de Campinas (UNICAMP). P.O. Box 6010. Campinas, 13083-875 SP, Brazil.

## ABSTRACT

The *tripunctata* group is the second largest Neotropical group of *Drosophila* in number of species and was included in the *tripunctata* radiation – which comprises other closely related groups. Our objectives were: suggest a new phylogenetic hypothesis for the *tripunctata* radiation based on CO1 and CO2 sequences, and describe wing size and shape variation based on this new phylogenetic hypothesis. Phylogenetic trees were reconstructed by parsimony, maximum likelihood and bayesian methods. In addition, tests of monophyly were performed in order to test for specific hypothesis. Wing size and shape were described by geometric morphometrics methods and patterns of variation among species were evaluated through character reconstruction. Results reject the monophyly hypothesis for the *tripunctata* group whereas monophyly is not rejected for the *tripunctata* radiation and other specific groups within the radiation. Both wing size and shape displayed strong phylogenetic signal and different patterns of size and shape could be identified for each of the most important monophyletic groups detected by phylogenetic analysis. Most of the shape variation among species involved landmarks responsible for the placement of the two major crossveins.

## 1. Introduction

The *Drosophila tripunctata* species group was proposed by Sturtevant in 1942. Several other species have been added since, so that this group is presently the second largest Neotropical group of *Drosophila* (surpassed by the *D. repleta* group), comprising 79 species according to Taxodros database of June 2007 (Bächli, 2007). With the exception of *D. tripunctata* (which occurs in North America) the *D. tripunctata* group is endemic to the Neotropics (Throckmorton, 1975), where its species are abundant (Val et al., 1981) and are a dominant component of the drosophilid fauna (Ashburner et al., 2005), particularly in forest areas (Sene et al., 1980).

The *D. tripunctata* group was included by Throckmorton (1975) in the *tripunctata* radiation, as well as other groups (*calloptera, cardini, guarani, macroptera, pallidipennis, rubrifrons* and *sticta*). According to this author, a radiation which he called *immigrans-Hirtodrosophila* originated in the Paleotropics, where it initially diversified and from where it sent two separate lineages to the Neotropics: *tripunctata* and *Hirtodrosophila.* The groups mentioned previously originated from the first lineage, composing the *tripunctata* radiation. Throckmorton (1975) also suggested that the *tripunctata* group itself should not be considered a monophyletic group. This statement is in agreement with cytological observations (Kastritsis et al., 1970) and recent molecular studies (Yotoko et al., 2003; Robe et al., 2005). The monophyly of *tripunctata* radiation as a whole has also been questioned. Remsen & O'Grady (2002) analyzed molecular and morphological data and their results did not recover this radiation as a monophyletic group, whereas the opposite was suggested by Robe et al. (2005), based on mitochondrial and nuclear sequences. Yotoko et al. (2002) presented evidence of the monophyly of what they called the "*tripunctata* lineage", a group composed by the *tripunctata* radiation in addition to species of the *D. testacea* and the *D. funebris* groups.

In general, phylogenetic relationships among species belonging to the *tripunctata* radiation have been poorly studied, a fact pointed out by Markow and O'Grady (2006), who stated the need of such studies, particularly for the *D. tripunctata* and *D. guarani* groups. Even though it seems clear that the *tripunctata* group is not monophyletic, the monophyly of the *tripunctata* radiation is still unresolved. Moreover, the studies mentioned previously were unable to recover a well supported phylogenetic hypothesis for relationships among the species groups within the radiation.

A reliable phylogenetic hypothesis is essential to any comparative study (Felsenstein, 1981). The use of phylogenies allows for the identification of independent evolutionary events (Harvey & Pagel, 1991). Phylogenies are also useful for the reconstruction of ancestral character states and therefore for the inference of evolutionary patterns (Pagel, 1999).

Compared with other insects, *Drosophila* venation pattern is relatively simple. It is formed by a marginal vein running all along the anterior wing margin, four main longitudinal veins, two short crossveins, and two short veins in the basis of the wing (DeCelis & Diaz-Benjumea, 2003). The functions of wing veins include strengthening of the wing, the presence of conducts in which the haemolymph can circulate and that may carry trachea and axons (DeCelis & Diaz-Benjumea, 2003). They also prevent rips from spreading across the membrane (Wooton, 1992). In insects, in addition to locomotion, wing characters are involved in self maintenance; folding; sensation; thermoregulation; protection and defense, both physical and visual; and territorial and sexual signaling (Wooton, 1992).

To our knowledge only one study has focused on the comparative analysis of wing shape of more than two species in *Drosophila.* Houle et al. (2003) analyzed 25 species in the subfamily Drosophilinae and concluded that wing shape and centroid size could be used to diagnose different species.

In this paper we propose a new phylogenetic hypothesis for species of the *tripunctata* radiation of *Drosophila* based on sequences of mitochondrial genes of citochrome oxidase subunits 1 and 2 (COI and COII). Our goal was to improve the results obtained by Yotoko et al. (2003) by adding both taxa and characters. In addition, we tested for monophyly of taxonomic groups (*calloptera*, *cardini*, *guarani* and *tripunctata*), of the *tripunctata* radiation, and of specific clades that appeared on the phylogenetic trees as monophyletic. In order to investigate wing morphology evolution in the *tripunctata* radiation, we also describe wing size and shape variation and perform ancestral state reconstruction and tests for phylogenetic signal on these characters based on the phylogenetic reconstruction obtained from COI and COII sequences.

## 2. Materials and Methods

### *2.1. COI and COII sequence data*

The largest part of the sequences included in this experiment was obtained from flies collected in the state of São Paulo, Brazil, during the years of 2003 and 2004. Only one species (*D. cardinoides*) was collected in Brasilia, Brazil. Specific location of collection and taxonomic placement of each species is given in Appendix B. Adult flies were collected with traps (Medeiros & Klaczko, 1999) containing banana bait or by net sweeping over banana bait or natural substrates. All individuals included in the analysis were adult males, either obtained directly from the field or from isofemale lines established from the same collections. Males were identified by the aedeagus, the most reliable method of identification of these species (Vilela, 1992). In addition, prior to DNA extraction, the terminalia of each male was removed and preserved in alcohol 70%. This procedure would allow for future confirmation of species identification, reevaluation in case of taxonomic revisions and for storage of voucher specimens.

Total DNA of each individual was extracted using a phenol-chloroform protocol (Azeredo-Espin et al., 1991). PCR for amplification of COI consisted of 35 cycles, each formed by 94°C denaturing (60s), 50°C annealing (75s), and 72°C elongation (90s). The same protocol was conducted for amplification of COII, except for the annealing temperature, modified to 55°C. The primers used for amplification were TL2-N-3014 and C1-J-2195 (COI), and TL-2-J3037 and TK-N-3785 (COII), described in Simon et al. (1994). The amplified products were purified with the QIAquick PCR purification kit. With the exceptions of the 5' fragment of COI of *D. guaru, D. trifilum*, *D. maculifrons* and *D. setula,* and COII of *D. mediostriata* – in which case PCR products were cloned into the PCR2.1 cloning vector using a TA Cloning Kit (Invitrogen) – all PCR products were directly sequenced. Sequencing was performed using BigDye (Applied Biosystems) chemistry on either an ABI377A or an ABI3700 automatic sequencer. At least two sequences of each fragment were obtained for each individual to ensure high quality of sequences. Except for the cloned samples, for which proper primers were used, the primers used for sequencing were the same as in PCR. All resulting sequence chromatograms were evaluated and edited with the programs Phred (Ewing et al. 1998), Phrap e Consed (Gordon et al., 1998).

Additional sequences were obtained from GenBank whereas individuals of *D. ornatipenis* were obtained from Tucson Fly Stock Center and *D. tripunctata* was kindly provided by Dr. Jean R. David. Accession numbers of all sequences are listed on Appendix A.

For the analysis of COI, we obtained fragments of 1413 bp whereas a fragment of 663 bp was obtained for COII. The phylogenetic analyses included 48 taxa, of which 36 sequences of COI and 21 sequences of COII were newly obtained in this study. Sixteen taxa belonging to the *tripunctata* radiation were added – as an attempt to improve phylogenetic resolution – compared

with the sequence data used by Robe et al. (2005), the most inclusive molecular study focusing on these groups to date.

**Table 1:** *Drosophila* species used for the wing morphometric analyses.

| Group | Species | Abbreviation |
|---|---|---|
| *Cardini* | *D. cardini* | CD |
| | *D. cardinoides* | CO |
| | *D. neocardini* | NO |
| | *D. polymorpha* | PO |
| *Guarani* | *D. guaraja* | GA |
| | *D. griseolineata* | GR |
| | *D. maculifrons* | MA |
| | *D. ornatifrons* | OR |
| *tripunctata* | *D. bandeirantorum* | BA |
| | *D. cuaso* | CUA |
| | *D. frotapessoai* | FP |
| | *D. mediopicta* | MI |
| | *D. mediosignata* | MN |
| | *D. mediopunctata* | MPT |
| | *D. metzii* | MZ |
| | *D. nappae* | NA |
| | *D. paraguayensis* | PG |
| | *D. paramediostriata* | PM |
| | *D. setula* | SE |
| | SP22* | SP22 |
| *pallidipennis* | *D. pallidipennis* | PD |
| *immigrans* | *D. immigrans* | IM |

*Non-described species. SP22 and *D. nappae* are sibling species.

Outgroups included species belonging to the *quinaria* (*D. innubila*, *D. falleni, D. quinaria, D. recens* and *D. subquinaria*,), *immigrans* (*D. immigrans*) and *repleta* (*D. eohydei* and *D. hydei*) groups – in order to assess the monophyly of the *tripunctata* radiation – as well as species from the *melanogaster* group (*D. mauritiana*, *D. melanogaster*, *D. sechellia*, *D. simulans* and *D. yakuba*) – used to root the trees.

*2.2 Phylogenetic analysis and hypothesis testing*

Sequences were aligned with ClustalW (Thompson et al., 1994), implemented as a tool in MEGA 3.1 (Kumar et al. 2004), followed by translation into amino acids for confirmation of alignment and assignment of codon positions.

PTP (permutation tail probability) tests were conducted in order to detect phylogenetic signal on the sequence data. Base composition heterogeneity among taxa was also tested.

Uncorrected distances – considering all codon positions, only first and second, and only third – were computed separately in order to evaluate the amount of variation and homoplasy depending on codon position. Distances were calculated on PAUP 4.0 beta 10 (Swofford, 2003) among all taxa and among ingroup taxa.

The maximum parsimony (MP) tree was obtained with PAUP, using TBR heuristic search, whereas maximum likelihood (ML) trees were generated by Phyml (Guindon & Gascuel, 2003). In order to perform the ML analysis, we allowed the program to estimate both the proportion of invariant sites and the gamma distribution parameter. In addition, base frequencies were estimated by maximum likelihood. Branch support for both MP and ML trees was computed as bootstrap values (1000 replicates) either on PAUP (MP tree) or Phyml (ML tree).

MrBayes 3.1 (Huelsenbeck and Ronquist, 2001) was used to obtain the Bayesian tree. In order to account for differences in nucleotide substitution parameters in each codon position, we used a model partitioned by gene and codon position. This model allows for independent estimates of parameters for each codon position of each gene and is considered to result in better phylogenetic reconstructions (Nylander et al., 2004). Posterior probabilities were based on two independent MCMC runs, each composed of four chains (three heated chains and one cold chain), with sample frequency of 1000 generations of a total of 70 million generations. We used a flat Dirichlet prior (non-informative) and the first 25% generations of each run were discarded as

burn-in. Average standard deviation of split frequencies of the cold chain likelihoods between the two independent MCMC runs was used as convergence diagnostic. We considered that convergence had been achieved when this value dropped below 0.01, as suggested by MrBayes 3.1 manual.

The program Modeltest (Posada and Crandall, 1998) was used to select the appropriate model of nucleotide substitution for ML and bayesian analyses. The model suggested by Modeltest with the Akaike information criterion (AIC) for each of the genes separately was the general time-reversible model with a gamma distribution of substitution rates across sites and proportion of invariant sites (GTR+I+G). The Tamura-Nei model was suggested by AIC for the combined data set, also with a gamma distribution of substitution rates across sites and proportion of invariant sites (TN93+I+G). As GTR and TN93 are very similar models, we reconstructed trees on Phyml with the both models and tested for significant differences between topologies produced by the two models with the sequences of each separate gene and the combined data. We used a one-tailed SH topology test on PAUP (10000 RELL bootstrap replicates) for this test. Parameters suggested by Modeltest for each model were not used since Phyml estimates them prior to tree reconstruction. The SH test did not detect significant differences between the GTR and TN93 models for any of the comparisons: COI ($p=0.408$), COII ($p=0.256$) and combined data ($p=0.418$). We therefore opted to analyze the combined dataset and use the TN93+I+G model of substitution for the ML analysis. However, as it is not possible to specify this model of nucleotide substitution in MrBayes, we used the GTR+I+G for the bayesian reconstruction. This should not be a problem, since bayesian inference is relatively robust to model overparameterization (Nylander et al., 2004).

All trees were reconstructed as unrooted trees and the five species belonging to the *melanogaster* group were later used to root the trees obtained by the three methods of phylogenetic reconstruction.

In order to test for specific hypotheses of monophyly we compared constrained and unconstrained trees. For each hypothesis, trees were reconstructed with the constraint to enforce a specific group of taxa to be a monophyletic group. Tests of monophyly used were Templeton (Wilcoxon-rank) and winning-sites tests.

*2.3 Wing morphometric data*

Isofemale lines were established from the same collections made to obtain individuals for the phylogenetic analysis. Since we were not able to rear in the laboratory all the species included in the phylogenetic analysis, we analyzed wings of 22 species of the *tripunctata* radiation (table 1). Two isofemale lines per species and 30 individuals of each sex per isofemale line were analyzed, summing up 2640 wings.

First instar larvae were collected from each isofemale line kept under controlled conditions of temperature and larval density. Temperature of 20°C was chosen for being an adequate growth temperature for all species included in the experiment, and density of 20 larvae per vial (containing 10ml of culture medium) was selected in order to avoid crowding effects. Left wings were prepared on microscope slides and images were produced by a digital camera attached to a microscope.

Even though we detected variation in shape and size among isofemale lines (not shown), we opted to pool the data of both isofemale lines to describe each species, in order to simplify the analysis. This is justified by the fact of variation among species being expected to be much greater than variation within each species (confirmed by our preliminary analysis, not shown).

Wing morphology was analyzed by methods of geometric morphometrics (see reviews in Rohlf & Marcus, 1993; Adams, Rohlf & Slice, 2004). Coordinates of eleven easily recognizable and homologous landmarks (figure 1) were obtained with the program TPSdig 1.4 (F. James Rohlf, 2004, SUNY at Stony Brook). Size was measured as the centroid size – calculated as the square root of the sum of the squared distances of each landmark to the centroid (Bookstein, 1991) – and examined by analysis of variance (ANOVA). Size sexual dimorphism was computed as the ratio between the sizes of females and males, which, according to David et al. 2003) is a better index of size sexual dimorphism, since it avoids scaling effects among species.

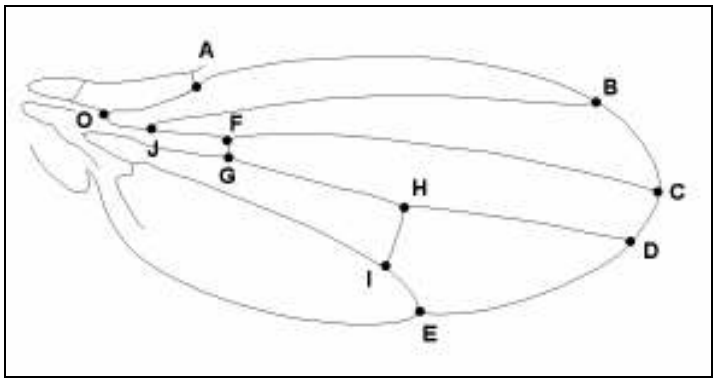


**Figure 1:** Wing of *Drosophila mediopunctata* with the eleven landmarks used for morphometric analysis.

For the analysis of shape, the landmark coordinates of all individuals were aligned using the generalized least squares Procrustes superimposition (Rohlf, 1990). The aligned coordinates were used to compute the partial warps and uniform components matrix with the program TPS Relw 1.29 (Rohlf, 2003). A discriminant analysis of these shape variables, in addition to a multivariate analysis of variance (MANOVA), was carried out in MINITAB 1.4.

A multivariate regression of landmark aligned coordinates on CS with the program NTSYSpc 2.11 was performed in order to account for allometric effects. Size-dependent variation was removed by adding the residuals to the mean landmark configuration. Consequently, we obtained a standardized shape dataset, not correlated to CS.

TPS Relw was also used to compute relative warps (RWs) of the aligned coordinates of the mean shape configurations (consensus) of each species, for both total and nonallometric shape variation. The relative warp analysis (RWA), as described in Bookstein (1989, 1991), is analogous to a principal components analysis (PCA) and can be used to describe major trends in shape variation as deformations in shape (Rohlf, 1993). When all landmarks are weighted equally, RWA is PCA (Zelditch et al., 2004). We used the scaling option $\alpha=0$ (i.e. no particular weights were given to any of the landmarks) in TPS Relw to compute the relative warps.

*2.4. Ancestral state reconstruction and tests for phylogenetic signal*

As both MP and ML trees obtained here presented low resolution, ancestral character reconstruction on those trees would have almost no value for discussion either due to very low branch support or to politomies (in case we decided to consider only branches supported by high bootstrap values). Hence, we chose to use the bayesian tree for ancestral character reconstruction and consider for discussion only branches with posterior probabilities higher than 0.80. Taxa for which we did not have wing data had their branches pruned so that the phylogenetic tree would have the same taxa as those for which we had morphometric data.

The program Mesquite 1.11 (Maddison & Maddison, 2006) was used for ancestral character state reconstruction of centroid size using the squared change parsimony method (Maddison, 1991), a method for reconstructing ancestral character states that minimizes the sum of squared changes along the branches of the phylogenetic tree. TPStree 1.18 (Rohlf, 2003) was used for character reconstruction of wing shape. This software fits shape data to a tree by estimating the shape of the nodes in the tree. The shape of each node was estimated using the squared change parsimony procedure based on McArdle & Rodrigo (1994).

Tests for phylogenetic signal on size and size sexual dimorphism were performed on PHYSIG (Bloomberg et al., 2003). This test compares the fit of tip data of a given tree with the fit obtained when the data have been randomly permuted across the tips of the tree and gives the probability of the existence of phylogenetic signal that can be interpreted as significant at the 0.05 level (Bloomberg et al., 2003).

## 3. Results

### *3.1. COI and COII data description*

The complete data set included 2076 bp, of which 1301 were constant, 97 were variable parsimony-uninformative and 678 were parsimony informative characters.

As expected, we found a low GC content for both genes (A: 30.3%; C: 15.2%; G: 15.3%; T: 39.2%), a common finding in insect mitochondrial sequences. No bias in base composition among taxa was detected by the chi-square test (P-value=0.999) whereas the PTP test detected the presence of phylogenetic signal on the sequence data (p=0.001).

Distances among taxa when the third codon position was excluded were much lower than those obtained when second and first positions were excluded (table 2), indicating a high level of homoplasy on the third codon position and a low amount of variation on the first and second positions.

**Table 2:** Uncorrected distances among taxa for all codon positions; only first and second positions; and only third position.

| | | 1st and 2nd codon positions | 3rd codon position | all codon positions |
|---|---|---|---|---|
| all taxa | mean ± standard error | 0.0278 ± 0.0002 | 0.2949 ± 0.0013 | 0.1168 ± 0.0005 |
| | maximum | 0.0434 | 0.3829 | 0.1527 |
| | minimum | 0 | 0.0275 | 0.0092 |
| ingroup | mean ± standard error | 0.0252 ± 0.000164 | 0.2912 ± 0.0012 | 0.1139±0.0005 |
| | maximum | 0.0362 | 0.3714 | 0.1450 |
| | minimum | 0 | 0.0275 | 0.0091 |

*3.2. Phylogenetic analysis*

Maximum parsimony analysis resulted in only one most parsimonious tree, with 5358 steps (consistency index= 0.226; retention index= 0.396; homoplasy index=0.774). The phylogenetic tree obtained by this method resulted, as expected, in the *repleta* and *immigrans* groups diverging first (figure 2a). Within the clade formed by species of the *quinaria* group and the *tripunctata* radiation, *D. ornatipenis* is the first do diverge, followed by a clade composed by species of the *D. tripunctata* group (*D. frotapessoai, D. paramediostriata* and *D. mediostriata*), *D. guaramunu* subgroup (*D. maculifrons* and *D. griseolineata*) and *D. calloptera* group (*D. schildi*). A basal polytomy is formed within this clade in which three pairs of obviously closely related species are placed together. In addition, the *quinaria*, *cardini* and a clade of species belonging to the *tripunctata* group (including also *D. pallidipennis* and *D. sticta*) appear as monophyletic. However, the bootstrap 50% majority consensus tree (figure 2b) resulted in a basal polytomy in the ingroup, with reliable support values only for the monophyly of *cardini* group, three species of the *quinaria* group, and a few pairs of sibling species. Hence, the maximum parsimony reconstruction presented very low resolution, most of the basal branches were not resolved and only a few clades presented bootstrap values higher than 50%. This analysis per se does not allow for the discussion of hypotheses of monophyly of either the *tripunctata* radiation or the *tripunctata* group. As a consequence, the relationship among groups of the *tripunctata* radiation is also unresolved.

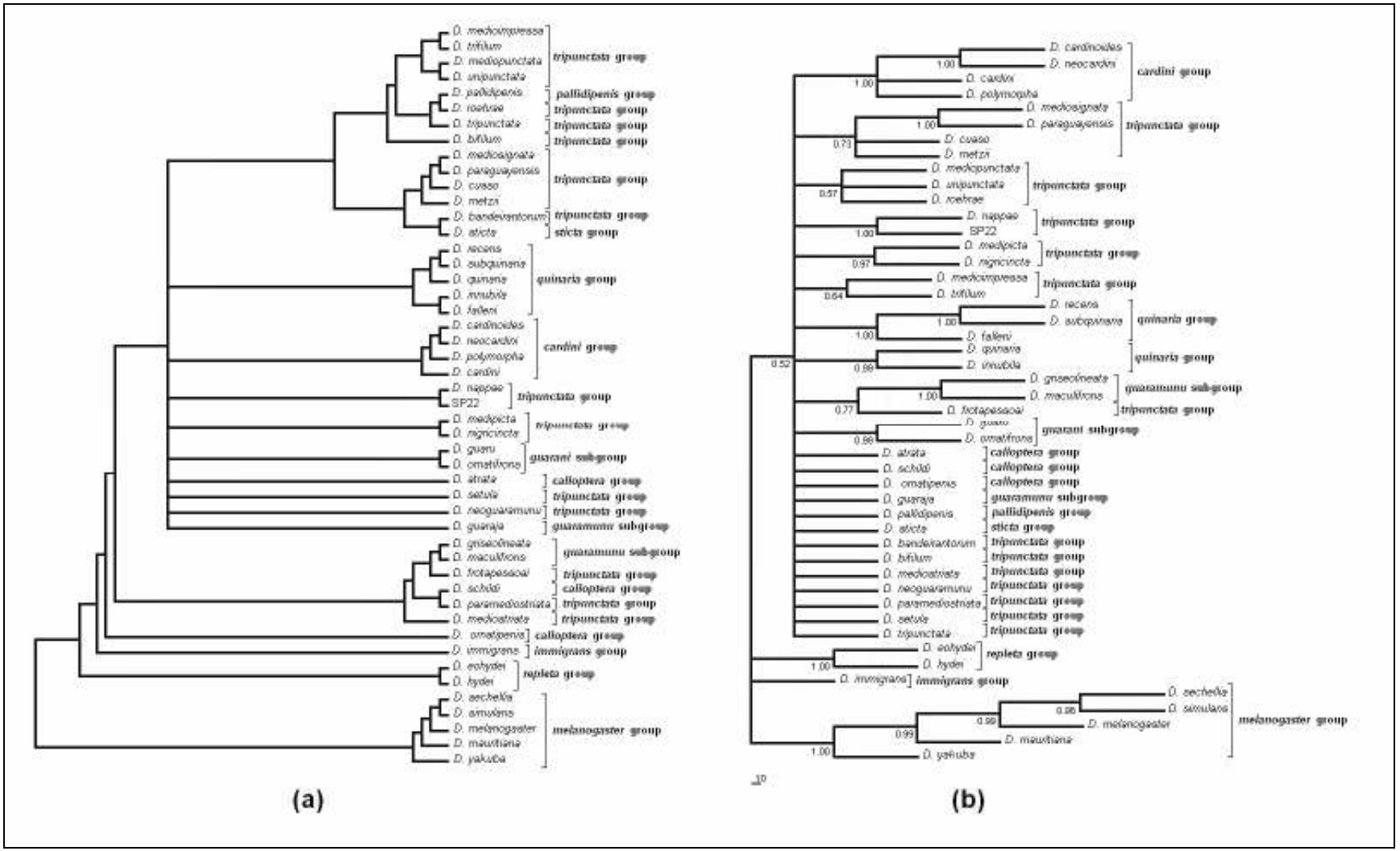


**Figure 2:** Phylogenetic reconstruction of the *tripunctata* radiation by maximum parsimony on PAUP. (a) most parsimonious tree; (b) bootstrap 50% majority consensus tree - numbers below branches represent bootstrap values.

Maximum likelihood reconstruction resulted in a tree very similar to the one obtained by MP, with analogous clusters (figure 3). Again, we observe a large clade formed by species of the *tripunctata* group (in addition to *D. sticta* and *D. pallidipennis*), monophyletic clades composed by the taxa of the *cardini* and *quinaria* groups, and another clade formed by species of the *tripunctata* group (*D. frotapessoai, D. paramediostriata* and *D. mediostriata*) and *guaramunu* subgroup (*D. maculifrons* and *D. griseolineata*). Bootstrap values, however, give low support to most of the basal nodes, with high branch support only to the same clades produced with high support by MP reconstruction.

In agreement with the two previous methods, bayesian analysis revealed an early divergence of the *repleta* and *immigrans* groups (figure 4). However, in the bayesian tree, the *quinaria* group diverges earlier than the remaining taxa, even though posterior probability for monophyly of the *tripunctata* radiation is low (0.62). A basal polytomy is observed within the *tripunctata* radiation, within which clades with high posterior probabilities are: (1) a monophyletic *cardini* group (1.00); (2) the same large *tripunctata* clade observed in MP and ML analysis (0.97); (3) a clade composed by *guaramunu* subgroup, *tripunctata* and *calloptera* groups (0.98), within which a clade with high posterior probability (1.00) is formed by *D. griseolineata*, *D. maculifrons*, *D. frotapessoai* and *D. paramediostriata*; (4) there is also relatively high support (0.89) to a clade formed by *D. nappae,* SP22 (a pair of sibling species), and *D. setula*.

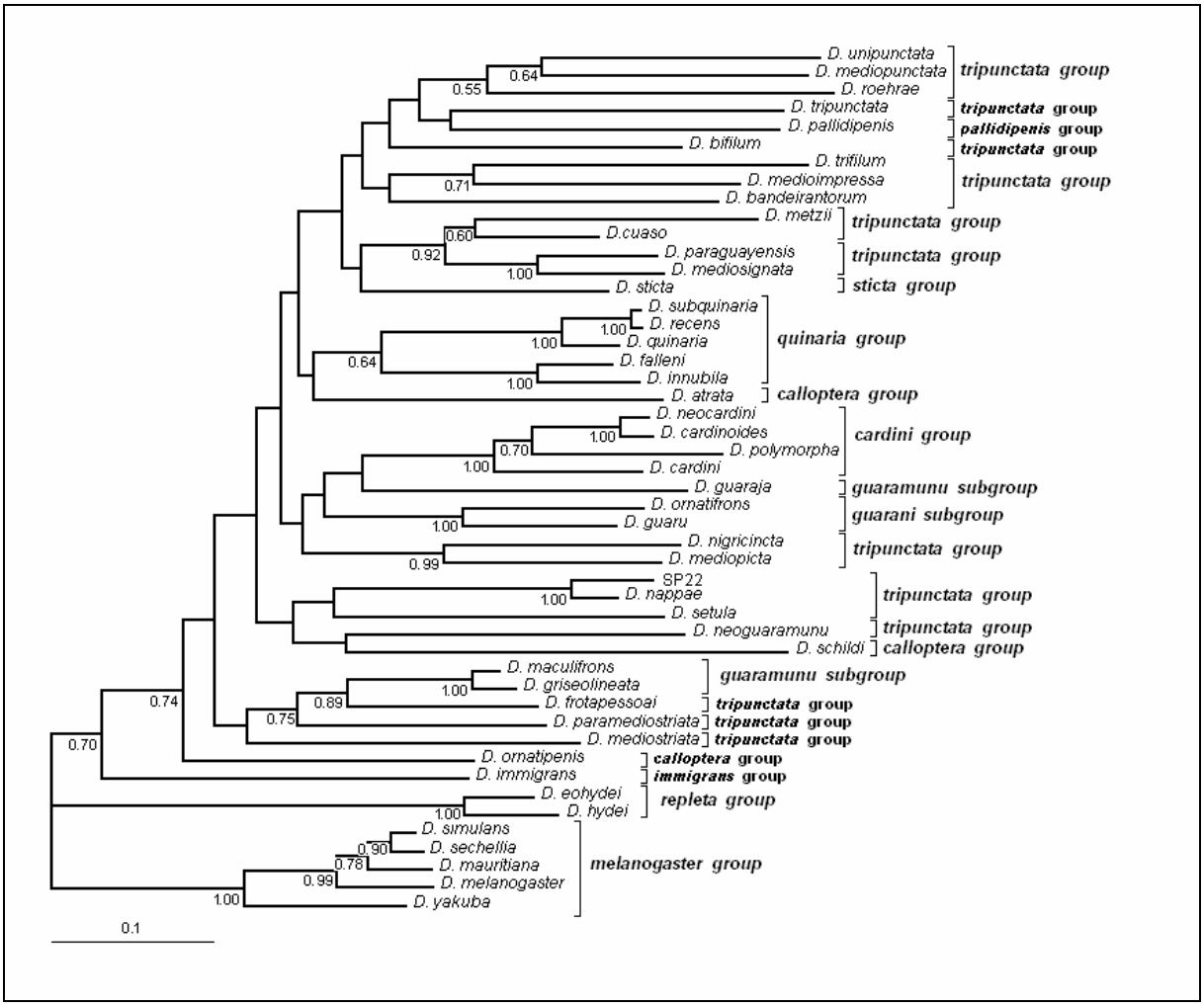


**Figure 3:** Phylogenetic reconstruction of the *tripunctata* radiation by maximum likelihood on PHYML. Numbers below branches represent bootstrap values (values below 0.50 are not shown).

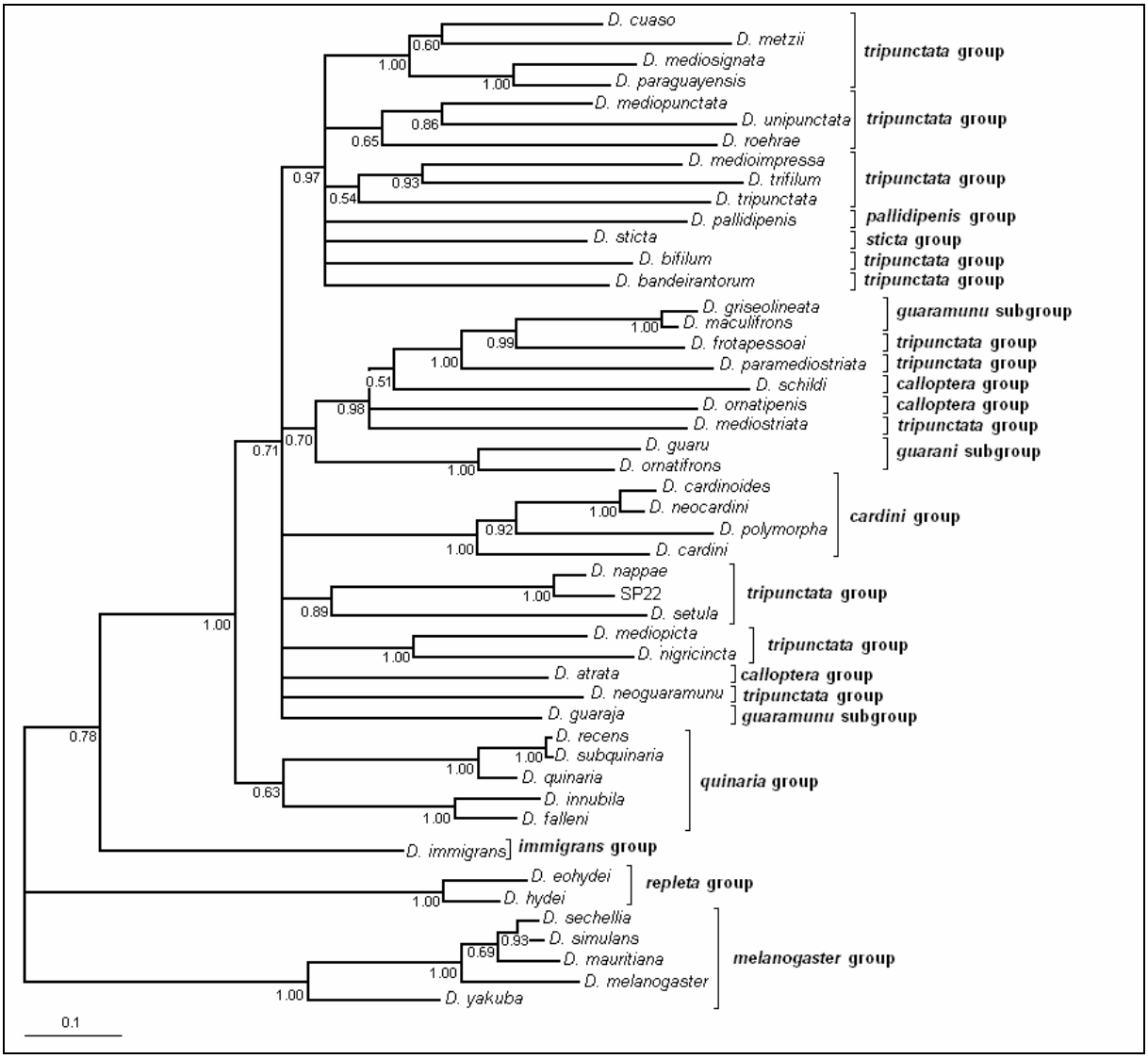


**Figure 4:** Phylogenetic reconstruction of the *tripunctata* radiation by bayesian analysis on MrBayes. Values below branches represent posterior probabilities. Branches were collapsed whenever posterior probabilities were lower than 0.50.

### *3.3. Tests of monophyly*

Based on the results obtained by the three methods of phylogenetic reconstruction, and due to low support values to most of the basal nodes, we performed tests of the following specific hypotheses of monophyly of taxonomic groups:

i. *tripunctata* group

ii. *calloptera* group

iii. *cardini* group

iv. *guarani* group

v. *guaramunu* subgroup

vi. *quinaria* group

In addition, we also tested for the monophyly of the following clades:

vii. *tripunctata* radiation

viii. "*tripunctata* restricted lineage" (*D. bifilum*, *D. cuaso*, *D. medioimpressa*, *D. mediopicta*, *D. mediopunctata*, *D. mediosignata, D. metzii*, *D. pallidipennis*, *D. paraguayensis*, *D. roehrae*, *D. sticta*, *D. trifilum*, *D. tripunctata* and *D. unipunctata*)

ix. "*nappae* lineage" (*D. nappae*, *D. setula* and SP22)

x. "*tripunctata-guaramunu* lineage" (*D. mediostriata*, *D. paramediostriata*, *D. frotapessoai*, *D. maculifrons* and *D. griseolineata*)

xi. "*tripunctata-guaramunu* alternative lineage" (*D. paramediostriata*, *D. frotapessoai*, *D. maculifrons* and *D. griseolineata*)

xii. "*guarani* alternative lineage" (all species belonging to the *guarani* group, excluding *D. guaraja*)

The reason for testing for the last hypothesis is that monophyly of the *guarani* group would consequently be rejected in case the monophyly of the *guaramunu* subgroup was also rejected due to *D. guaraja* not being closely related to *D. griseolineata* and *D. maculifrons*, as suggested by our phylogenetic reconstructions. It is worth noticing that the cluster we called "*tripunctata* restricted lineage" does not correspond to the *tripunctata* lineage proposed by

Yotoko et al. (2002), which comprised the entire *tripunctata* radiation added to two other species groups.

Winning-sites and Templeton tests, comparing strict consensus trees produced according to each hypothesis, rejected monophyly for the *tripunctata* group, for both constrained trees by the *guarani* group (either including or not *D. guaraja*), and for the *guaramunu* subgroup (table 3). Monophyly of the "*tripunctata*-*guaramunu* alternative lineage" was also rejected by the winning-sites test whereas this hypothesis was not rejected by the Templeton test.

**Table 3:** P-values for Templeton (Wilcoxon-rank) and winning-sites tests on most parsimonious trees obtained by each enforced constraint. The unconstrained MP tree presented a tree length of 5358. Significant values (<0.05) are in bold.

| Constraint | Tree length | Templeton | Winning-sites |
|---|---|---|---|
| *calloptera* group | 5376 | 0.1837 | 0.1036 |
| *cardini* group | 5358 | - | - |
| *guarani* group | 5398 | <0.0001 | <0.0001 |
| *guaramunu* subgroup | 5429 | 0.0387 | 0.2065 |
| *tripunctata* group | 5359 | 0.0035 | 0.0107 |
| *tripunctata* radiation | 5359 | 0.9202 | 1.0000 |
| *tripunctata* restricted lineage | 5359 | 0.9202 | 1.0000 |
| *nappae* lineage | 5358 | 0.9202 | 1.0000 |
| *tripunctata*-guaramunu lineage | 5358 | - | - |
| *tripunctata*-guaramunu alternative | 5789 | <0.0001 | <0.0001 |
| *guarani* alternative lineage | 5750 | <0.0001 | <0.0001 |

### *3.4. Wing size*

ANOVA of centroid size (table 4) resulted in significant effects of species, sex and the interaction between these two factors. Sex had the stronger effect on size, females being larger than males, as expected. In general, species of the *cardini* group have smaller wings whereas wings of species of the *tripunctata* group are larger (figure 5).

**Table 4:** ANOVA of centroid size with effects of species, sex and interaction between the two factors.

| Source | d. f. | Mean squares | F | P-value |
|---|---|---|---|---|
| species | 21 | 14.8201 | 1955.83 | <0.0001 |
| sex | 1 | 74.0324 | 9770.15 | <0.0001 |
| species × sex | 21 | 0.3014 | 39.78 | <0.0001 |
| Error | 2596 | | | 0.0076 |
| Total | 2639 | | | |

Since we observed a significant interaction between species and sex, ancestral state reconstructions and tests for phylogenetic signal were done separately for each sex. Tests for phylogenetic signal were significant for both female and male size (p=0.0008 and p=0.0104, respectively). Ancestral state reconstructions (figure 6) confirm that flies belonging to the *cardini* group and *tripunctata-guaramunu* lineage have small wings whereas species of the *tripunctata* restricted lineage have much larger wings. The *nappae* lineage appears to be very homogeneous, displaying large wings. These results suggest that the ancestral states were: small wings for the *cardini* group and *tripunctata-guaramunu* lineage, large wings for the tripunctata restricted lineage and intermediary size for the *nappae* lineage. Results were similar for both females and males.

On the other hand, we did not detect phylogenetic signal on sexual size dimorphism (p=0.4320). Ancestral state reconstruction for this character resulted in no easily recognizable pattern and consequently ancestral states appear as intermediary values for all four lineages (figure 6).

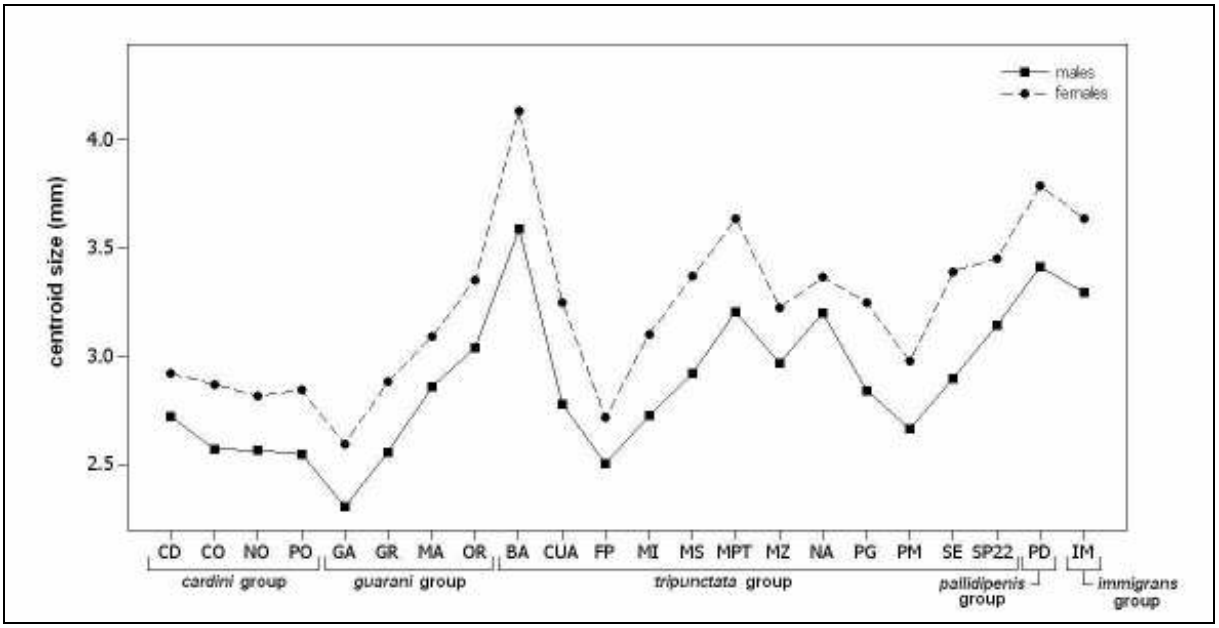


**Figure 5:** Mean centroid size of males and females of each species separated by species group.

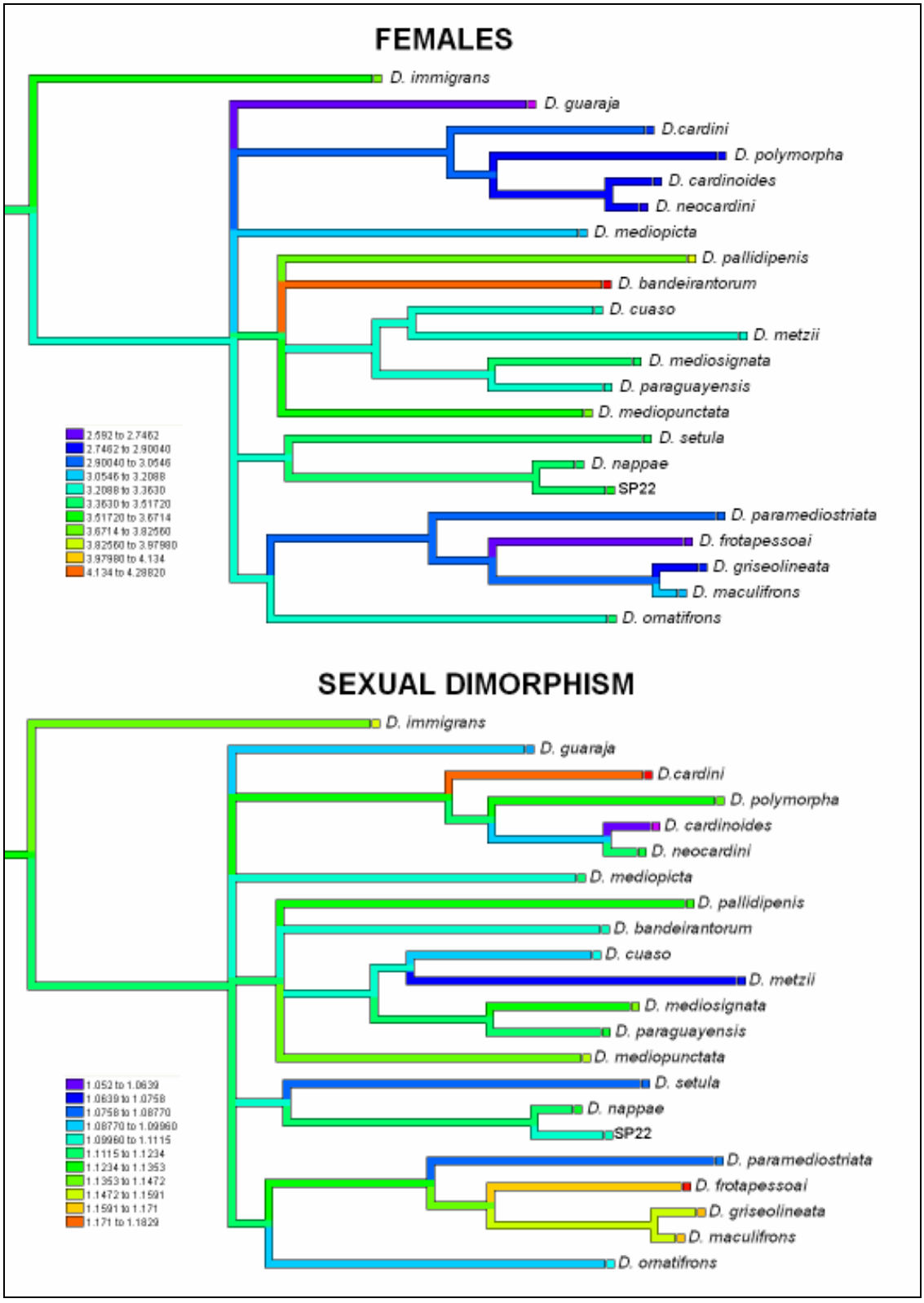


**Figure 6:** Ancestral state reconstruction of females centroid size and sexual size dimorphism on species of the *tripunctata* radiation. Brackets point out phylogenetic lineages proposed previously.

*3.5. Wing shape*

Size-dependant effects explained 26.67% of shape variation in females and 26.25% in males. Regarding total shape variation, MANOVA of partial warps and uniform components scores was significant for species (Wilks' $\lambda$=0.00002, F=127.510, P<0.001), sex (Wilks' $\lambda$=0.75071, F=48.501, p<0.001) and the interaction between species and sex (Wilks' $\lambda$=0.66673, F=3.664, P<0.001). Considering nonallometric variation, MANOVA also resulted in significant effects for species (Wilks' $\lambda$=0.00003, F=106.312, P<0.001), sex (Wilks' $\lambda$=0.074483, F=49.086, p<0.001) although no interaction between species and sex was detected (Wilks' $\lambda$=0.99963, F=0.002, P=1.000).

Discriminant analysis of partial warps and uniform components considering total shape variation resulted in correct classification of 93.7% of females and 92.3% of males (tables 5 and 6) whereas 92.7% of both males and females were correctly classified when the allometric component was removed (results not shown). These results indicate that wing shape displays enough variation to distinguish among the studied species with confidence higher than 90%, for both total and nonallometric variation. In addition, among the individuals incorrectly classified, most of them were grouped with closely related species, e.g. within the *cardini* group (CD, CO, NO and PO) and with the *paraguayensis* complex (CUA, MN and PG).

The first two relative warps explained 70.13% of total shape variation in females and 70.56% in males whereas, 60.51% of nonallometric variation was explained in females and 60.51% in males. RW1 separates most species belonging to the *tripunctata* restricted lineage from species of the other three lineages, whereas RW2 separates species from the *cardini* group and the *nappae* lineage (figure 7). The *tripunctata-guaramunu* lineage could not be differentiated

from the others by either RW1 or RW2. In contrast, when only nonallometric shape variation is considered (figure 7), it is not possible to separate the lineages by either RW1 or RW2.

**Table 5:** Classification matrix derived from discriminant function analysis based on partial warp scores and uniform components of total shape variation of females.

| Predicted species | Correct species | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CD | CO | NO | PO | GR | GA | MA | OR | BA | CUA | FP | MI | MPT | MN | MZ | NA | PG | PM | SE | SP22 | PD | IM |
| CD | 55 | 1 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| CO | 0 | 50 | 5 | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| NO | 0 | 4 | 55 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| PO | 5 | 3 | 0 | 48 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| GR | 0 | 0 | 0 | 0 | 60 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| GA | 0 | 1 | 0 | 0 | 0 | 60 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| MA | 0 | 0 | 0 | 0 | 0 | 0 | 60 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| OR | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 60 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| BA | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 59 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| CUA | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 53 | 0 | 0 | 0 | 3 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 |
| FP | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 55 | 0 | 0 | 0 | 0 | 0 | 0 | 8 | 1 | 0 | 0 | 0 |
| MI | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 58 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| MPT | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 56 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| MN | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 0 | 0 | 0 | 55 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 |
| MZ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 60 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| NA | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 56 | 0 | 0 | 0 | 3 | 0 | 0 |
| PG | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 2 | 0 | 0 | 55 | 0 | 0 | 0 | 0 | 0 |
| PM | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 2 | 0 | 0 | 0 | 1 | 0 | 52 | 0 | 0 | 0 | 0 |
| SE | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 58 | 0 | 0 | 0 |
| SP22 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 57 | 0 | 0 |
| PD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 60 | 0 |
| IM | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 60 |
| % correct | 0.92 | 0.83 | 0.92 | 0.80 | 1.00 | 1.00 | 1.00 | 1.00 | 0.98 | 0.88 | 0.92 | 0.97 | 0.93 | 0.92 | 1.00 | 0.93 | 0.92 | 0.87 | 0.97 | 0.95 | 1.00 | 1.00 |

In general, most of the variation in total shape among the four lineages is due to the displacement of landmarks F, G, H and I, which determine the position of the crossveins (figure 8). In the *cardini* group, these landmarks are displaced so that the two crossveins are closer to each other than in the mean shape of all species, whereas in the *tripunctata* restricted lineage, the crossveins are more separate from each other when compared to the mean shape. Landmarks F, G, H and I are also responsible for differences in species of the *nappae* lineage, although in this case, these four landmarks are displaced towards the tip of the wing. In the *tripunctata-guaramunu* lineage, the lack of noticeable displacement of landmarks of the lineage consensus

suggests that this lineage did not present an easily recognizable pattern of shape changes. Details of total shape variation between each species and the consensus can be visualized in figure 9.

**Table 6:** Classification matrix derived from discriminant function analysis based on partial warp scores and uniform components of shape variation of males.

| Predicted species | Correct species | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CD | CO | NO | PO | GR | GA | MA | OR | BA | CUA | FP | MI | MPT | MN | MZ | NA | PG | PM | SE | SP22 | PD | IM |
| CD | 58 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| CO | 1 | 49 | 1 | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| NO | 0 | 3 | 59 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| PO | 1 | 8 | 0 | 47 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| GR | 0 | 0 | 0 | 0 | 60 | 1 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| GA | 0 | 0 | 0 | 0 | 0 | 58 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| MA | 0 | 0 | 0 | 0 | 0 | 0 | 58 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| OR | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 60 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| BA | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 58 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| CUA | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 56 | 0 | 1 | 4 | 0 | 1 | 0 | 5 | 0 | 0 | 0 | 0 | 0 |
| FP | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 51 | 1 | 0 | 0 | 0 | 0 | 0 | 7 | 0 | 0 | 0 | 0 |
| MI | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 57 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| MPT | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 55 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| MN | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 50 | 1 | 0 | 3 | 1 | 1 | 0 | 0 | 0 |
| MZ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 56 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| NA | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 57 | 0 | 0 | 0 | 0 | 0 | 0 |
| PG | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 5 | 0 | 0 | 51 | 0 | 0 | 0 | 0 | 0 |
| PM | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5 | 0 | 0 | 0 | 0 | 0 | 1 | 52 | 0 | 0 | 0 | 0 |
| SE | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 59 | 0 | 0 | 0 |
| SP22 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 60 | 0 | 0 |
| PD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 60 | 0 |
| IM | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 60 |
| % correct | 0.97 | 0.82 | 0.98 | 0.78 | 1.00 | 0.97 | 0.97 | 1.00 | 0.97 | 0.93 | 0.85 | 0.95 | 0.92 | 0.83 | 0.93 | 0.95 | 0.85 | 0.87 | 0.98 | 1.00 | 1.00 | 1.00 |

Nonallometric shape variation involved mostly landmarks G and H (figure 8). However, little difference is recognizable between the consensus configuration of all species and the mean shapes of the *cardini* group and the *tripunctata-guaramunu* lineage. Displacement of landmarks G and H are more visible in the *nappae* lineage, moving the anterior crossvein towards the tip of the wing. Landmarks B and C are also affected in this lineage. The *tripunctata restricted lineage* displayed a difference in the coordinates of landmarks G and H, although much smaller than in the *nappae* lineage. In regard to nonallometric wing shape variation, the details of landmark coordinates differences between each species and the consensus configuration (figure 10) do not present trends as strong as the ones observed for total shape variation (figure 9).

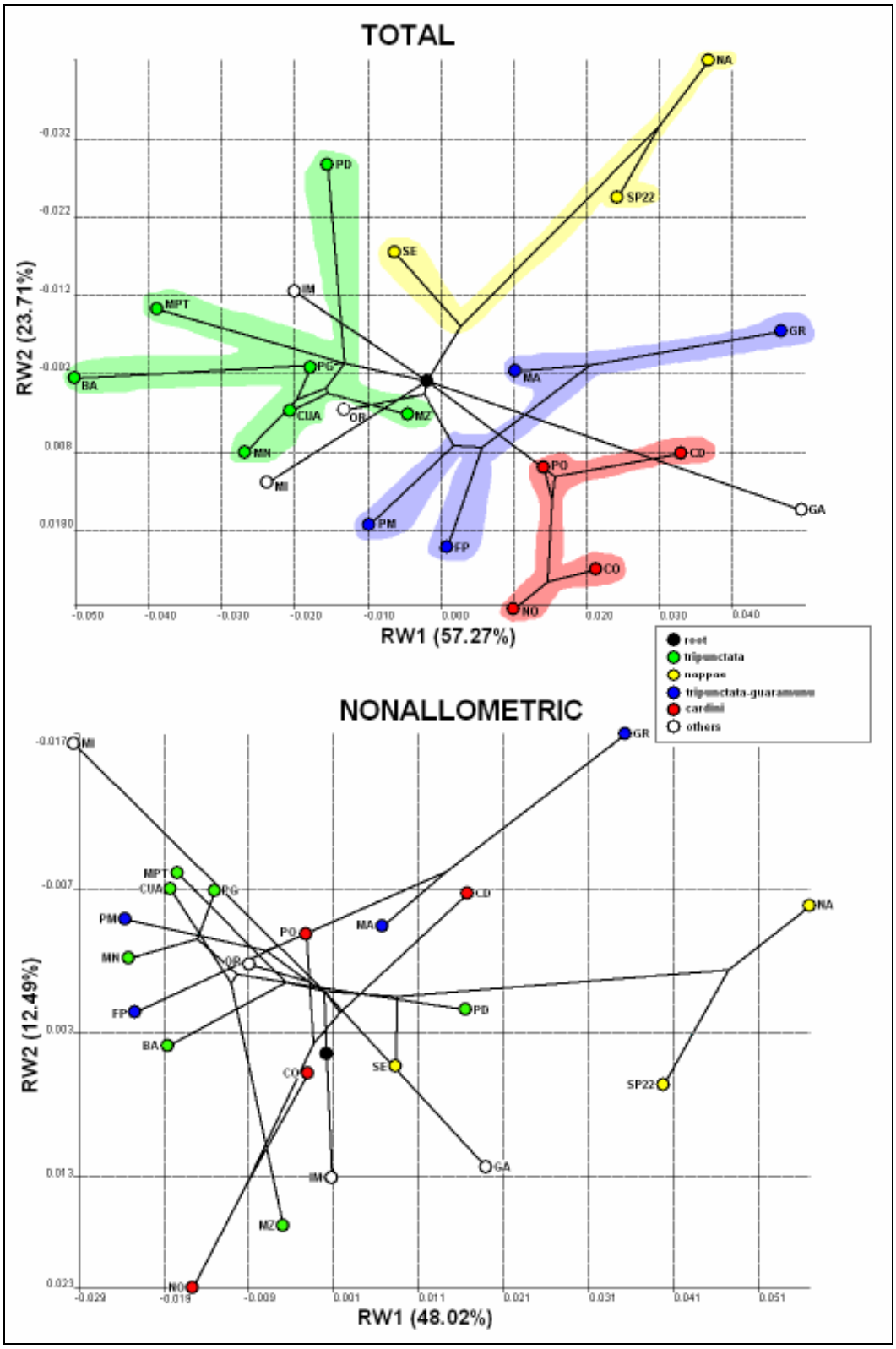


**Figure 7:** Ancestral state reconstruction of the first and second relative warps (for total and nonallometric shape variation of females) plotted against and the second relative warp.

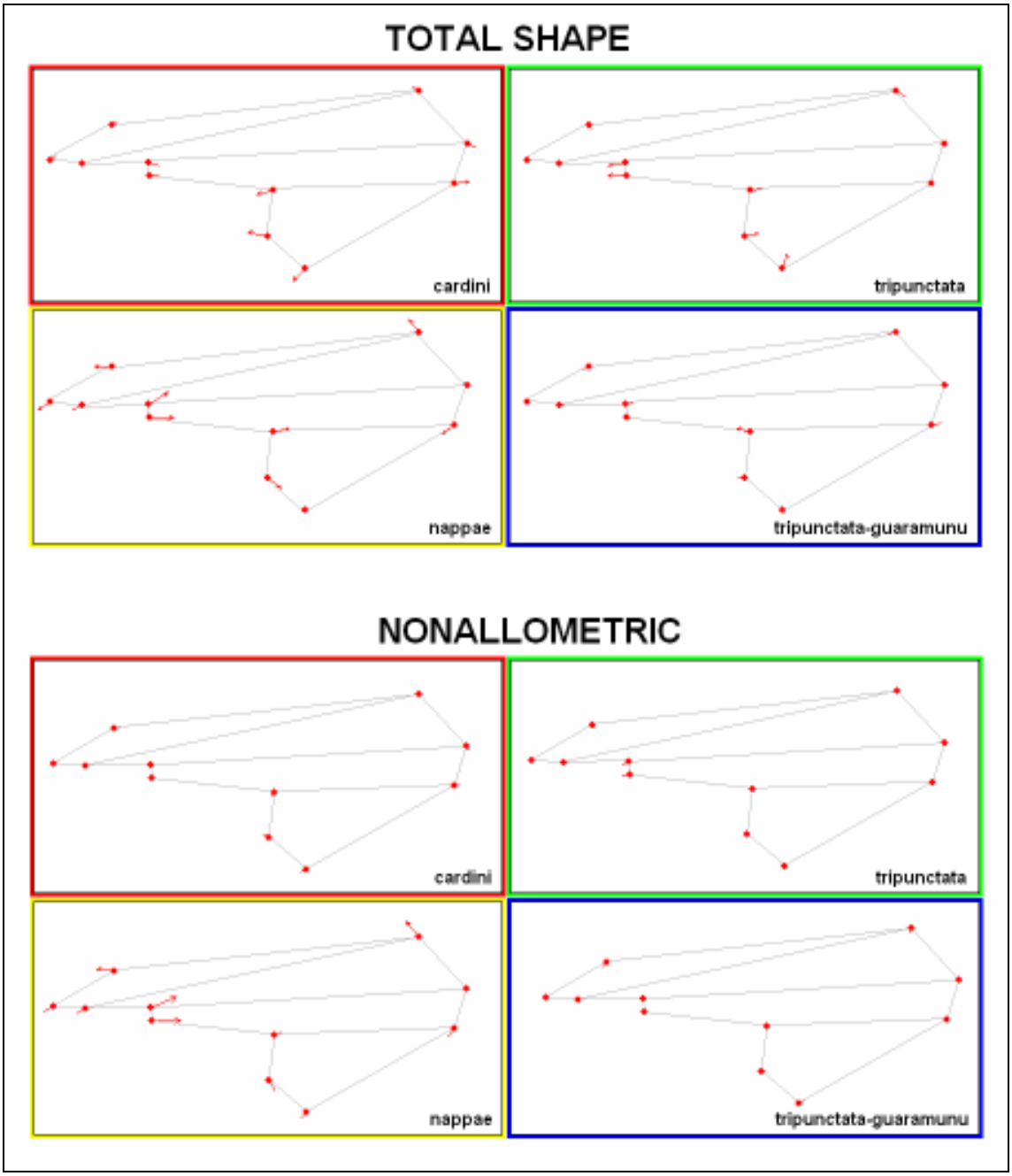


**Figure 8:** Vectors indicating major shape deformations among mean shapes of females of each phylogenetic lineage (total and nonallometric shape). Each consensus also represents the reconstruction of wing shape of the ancestral of each lineage. Shape changes are exaggerated three times for better visualization.

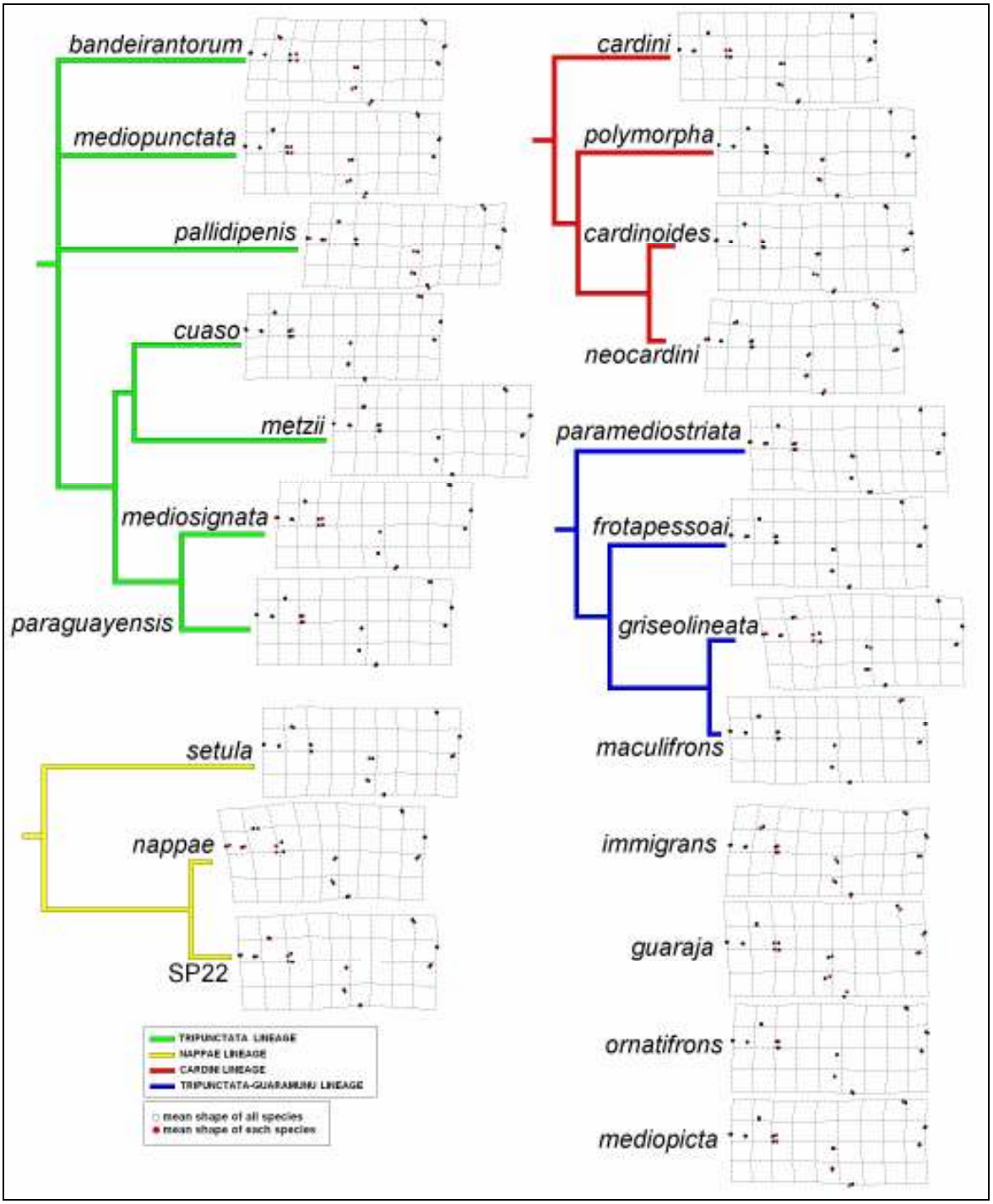


**Figure 9:** Shape deformations in females of each species in relation to the mean shape of all species (total shape variation). Black dots represent the mean of each species landmark configuration and red dots represent the mean shape of all species.

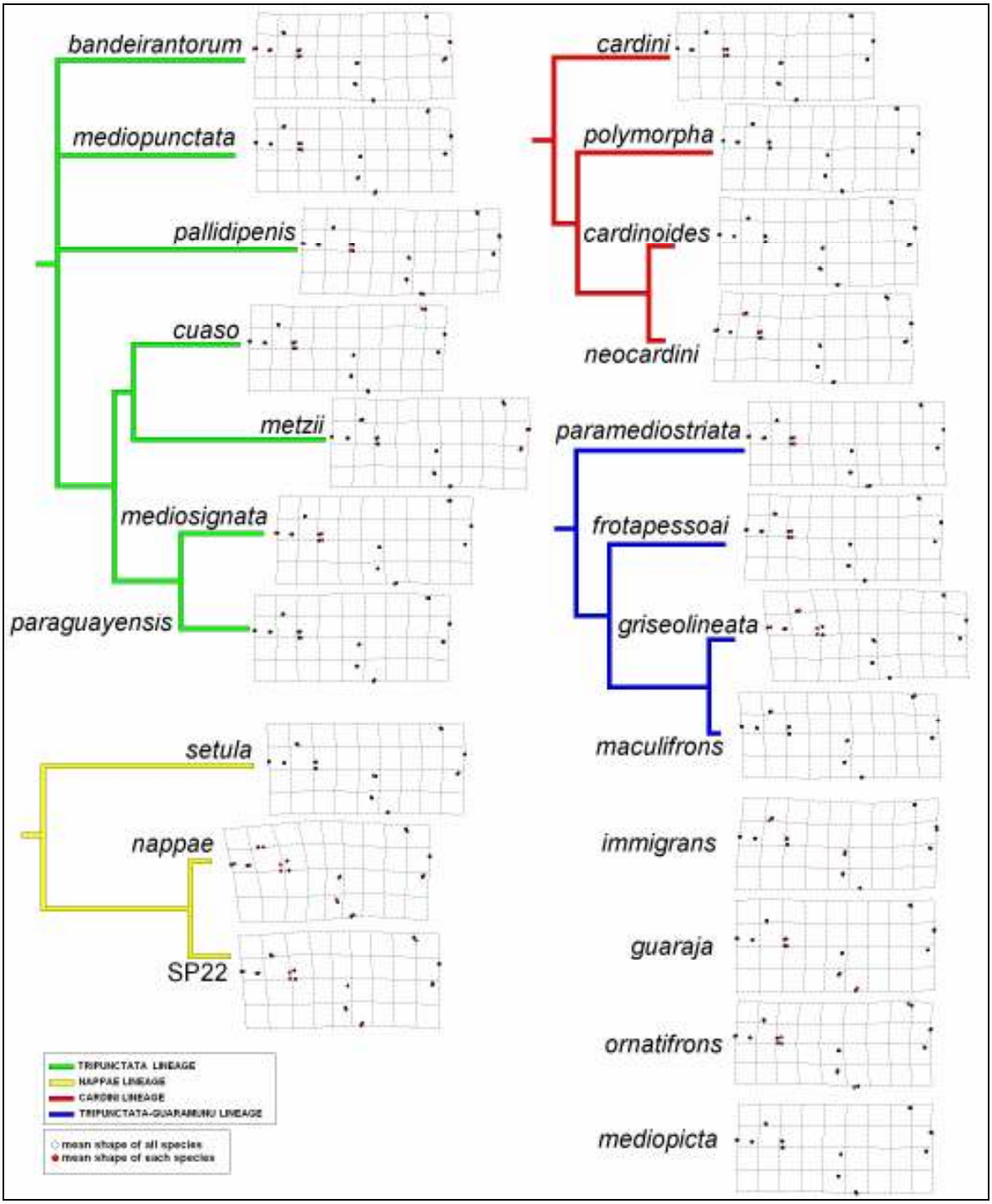


**Figure 10:** Shape deformations in females of each species in relation to the mean shape of all species (nonallometric shape variation). Black dots represent the mean of each species landmark configuration and red dots represent the mean shape of all species.

## 4. Discussion

### *4.1. Phylogenetic analysis*

All three methods of phylogenetic reconstruction (MP, ML and bayesian) resulted, as expected, in the early divergence of the *D. repleta* and *D. immigrans* groups. Although the posterior probability was low (0.62), the *tripunctata* radiation was recovered as monophyletic by bayesian reconstruction. Moreover, winning-sites and Templeton tests did not reject the monophyly of this clade. This result disagrees with the results obtained by Remsen & O'Grady (2002), and supports the hypothesis of monophyly of the *tripunctata* radiation, suggested earlier by Throckmorton (1975) and later reinforced by molecular studies (Yotoko et al., 2003 and Robe et al., 2005). On the other hand, the *D. tripunctata* group itself did not appear as monophyletic and both tests rejected its monophyly, confirming previous suggestions (Throckmorton, 1975; Kastritsis, 1970; Yotoko et al., 2003; Robe et al., 2005).

Our results suggest the existence of four monophyletic groups within the *tripunctata* radiation: the *D. cardini* group and three clades which we called "*tripunctata* restricted lineage", "*nappae* lineage", and "*tripunctata-guaramunu* lineage". All three methods of phylogenetic reconstruction recovered these four clades. Even though MP and ML analyses gave low support values (with the exception of the *D. cardini* group), these groups were supported by posterior probabilities higher than 0.85 in the bayesian reconstruction. It is worth mentioning that, in general, posterior probabilities are higher that their correspondent bootstrap values (Erixon et al., 2003). These two measures of phylogenetic accuracy are not comparable (Douady et al., 2003; Erixon et al., 2003). The statistical meaning of bootstrapping is controversial. Hillis & Bull (2003) argue that "at best bootstrap estimates should provide an indication only of the degree of support of a particular clade" and should not be treated as probabilities of that clade being a real

historical group. In contrast, posterior probabilities have a simple interpretation: they represent the probability that a particular clade is true conditional on the model, the priors and the data (Huelsenbeck, 2002). Simulations studies showed that bootstrap values are usually an underestimate of the accuracy of branches (Hillis & Bull, 2003; Suzuki et al., 2003; Wilcox et al., 2002; Douady et al., 2003) whereas posterior probabilities are overestimates (Suzuki et al., 2003). Douady et al. (2003) suggested that, even though bootstrap support and posterior probabilities cannot be directly compared, they could be treated as potential lower and upper bounds of node support.

The *D. cardini* group is composed of 16 described species and is subdivided in two subgroups: *cardini* and *dunni*. Brisson et al. (2006) analyzed sequence data of all 16 species for four mitochondrial and two nuclear genes and obtained a tree for which strong support was given to the monophyly of *D. cardini* group as a whole, although the *D. cardini* subgroup was not monophyletic. Our dataset included only four species of this group, all belonging to the *D. cardini* subgroup. In agreement with the previous results obtained by Brisson et al. (2006), our trees recovered a monophyletic *D. cardini* group, with *D. cardini* as the most ancestral species within the group.

The *D. guarani* group currently comprises 17 species, three of which belong to the *D. guaramunu* subgroup, six belong to the *D. guarani* subgroup and the eight remaining species are ungrouped. Kastritsis (1969) suggested that this group should be split into two groups (*guarani* and *guaramunu*), based on polytene chromosomal banding analysis. Vilela & Pereira (1985) disagreed and proposed that the *D. guarani* group (containing two subgroups) should be maintained until more detailed information was available. Kastritsis (1969) noticed that the chromosomes of species of the *D. guaramunu* subgroup were more similar to some of the *D. tripunctata* group than to those of the *D. guarani* subgroup and a recent molecular study by Robe

et al. (2002) was unable to divide the *D. guarani* group into two subgroups. In addition, Kastritsis et al. (1970) compared chromosomes of *D. griseolineata* and *D. mediostriata* and concluded that "the polytene chromosomes of the species studied are more similar among themselves than they are found to be when compared to the chromosomes of species of their own group". Our results recovered the relationships suggested by Kastritsis (1969) and Kastritsis et al. (1970): we found an apparently monophyletic clade composed by *D. griseolineata* and its sibling species *D. maculifrons,* and the pair of sibling species *D. mediostriata* and *D. paramediostriata*, in addition to *D. frotapessoai*.

*D. guaraja* with *D. maculifrons* and *D. griseolineata* compose the *D. guaramunu* subgroup. However, according to our results, the phylogenetic placement of *D. guaraja* is uncertain and there is no evidence of a close relationship of this species and the clade which we named the *tripunctata-guaramunu* lineage.

Among species belonging to the *D. tripunctata* group we found evidence for the monophyly of a group formed by *D. nappae* and SP22 (a pair of sibling species, in which the latter is an undescribed species), and *D. setula*. This is in agreement with the original description of *D. nappae* (Vilela et al., 2004), that suggested that this species could be closely related to *D. setula*.

Even though the *D. tripunctata* group was not recovered as monophyletic in our study, we found a cluster with high posterior probability in which several species of this group were included in addition to *D. pallidipennis* and *D. sticta.* Each of the *D. pallidipennis* and *D. sticta* species groups are formed by a single species and are morphologically similar to species from the *D. tripunctata* species group (Throckmorton, 1975). The remaining species placed in this cluster belong to subgroups II, III and IV, including the *paraguayensis* complex (composed by the *D. paraguayensis, D. mediosignata and D. cuaso,* Bächli et al., 2000) and *D. metzii* forming a clade

with high support value; *D. roehrae* and *D. unipunctata* (sibling species, Pipkin & Heed, 1964), *D. mediopunctata*, *D. medioimpressa*, *D. trifilum*, *D. bifilum* and *D. bandeirantorum*. Therefore, there is some evidence for the existence of a monophyletic group within the *D. tripunctata* species group.

Hence, our results suggest the need of a thorough taxonomic revision of groups belonging to the *tripunctata* radiation. However, the apparent monophyly of the *tripunctata* restricted lineage may turn out useful if the *D. tripunctata* group is maintained and redefined.

The case of the *guarani* group is more difficult, since we have evidence that species belonging to the *guaramunu* subgroup are more closely related to *D. frotapessoai*, *D. mediostriata* and *D. paramediostriata* than to species of the *guarani* subgroup. It is worth mentioning that this group comprises 17 species and several of them are not placed in either subgroup, reflecting the difficulty of taxonomists to classify species in this subdivision. More inclusive phylogenetic studies are necessary before the controversy whether this group should be split into two (or more) groups is resolved.

The *D. calloptera* group is another group that deserves further studies. Burla & Pavan (1953), who originally proposed the group, suggested that this group was closely related to the *D. tripunctata*, *D. quinaria*, *D. cardini* and *D. guarani* groups, being closest to the latest. Our bayesian tree suggests a close relationship of species of the *guarani* group to *D. schildi* and *D. ornatipenis* whereas *D. atrata* is not placed within the same cluster. MP and ML methods place these three species in different positions, always with low support values. Therefore, our results were unable to determine phylogenetic positions of species of the calloptera group and even its monophyly (strongly suggested by morphological evidence) was rejected.

Both MP and ML methods of phylogenetic reconstruction resulted in trees with very low resolution, particularly for basal nodes, even though the PTP test detected significant

phylogenetic signal on the data. However, PTP test results present high type I error probabilities (in this case, detecting phylogenetic signal when there is none) (Peres-Neto & Marques, 2000). Slowinsky & Crother (1998) suggested that "if a data set fails the PTP test, it should not be used in a phylogenetic analysis" but that this test merely detected that the data set is not random, instead of detecting significant phylogenetic signal.

Bayesian reconstruction resulted in better support values than ML and MP but most of the basal nodes remain unresolved. The pattern for the trees obtained by all three methods is the existence of short internal branches and long terminal branches, a pattern also observed by Yotoko et al. (2003). This kind of branching pattern is often interpreted as evidence of periods of rapid speciation. This hypothesis has been suggested by Throckmorton (1975) but it is not easy to confirm, since a polytomy on a gene tree does not necessarily mean a corresponding polytomy on the species tree (Slowinski, 2001). This is due to differences between gene trees and species trees (Pamilo & Nei, 1988; Doyle, 1997; Nichols, 2001). Most tests designed to detect politomies do not make this distinction and therefore are not useful for testing the hypothesis of rapid speciation in the *tripunctata* radiation (see examples in Slowinski et al., 2001). Slowinski et al. (2001) proposed a test for the detection of species politomies. However, this test requires independent gene trees. As both genes analyzed in this study were mitochondrial genes, and therefore not independently inherited, this test cannot be applied unless more data (nuclear sequence data) is collected. In addition, our data revealed very low variation on the first and second codon positions and high homoplasy on the third codon position and that could be causing deeper relationships to be more difficult to recover. Hence, perhaps the analysis of nuclear genes – with more appropriate nucleotide substitution rates – will be necessary before any consistent conclusions about the patterns of speciation of the *D. tripunctata* radiation can be properly discussed.

*4.2. Morphometric analysis and ancestral character reconstruction*

Our results detected significant phylogenetic signal for both female and male size, whereas no phylogenetic signal was detected for sexual dimorphism. These results suggest the existence of species adaptations for size sexual dimorphism that is independent of the phylogeny. Sexual size dimorphism might reflect different selection pressures acting on male and female body-sizes (Stamps, 1993) and are usually caused by differences in growth patterns between males and females (Shine, 1990).

Teder & Tammaru (2005) examined data from more than 150 species of different orders of insects and concluded that sexual size dimorphism was mostly female-biased, females being larger in 81.6% of the sampled species. They also suggested that sexual differences in size tended to increase with increasing body size. Our data was consistent with their results in the sense that females had larger wings than males in all species examined. However, we did not detect a significant correlation between body size and size sexual dimorphism (analysis not shown).

Discriminant analysis of shape variables revealed that species of the *tripunctata* radiation are reasonably diagnosable by wing shape. Houle et al. (2003) obtained similar results in a comparative analysis of 25 species in the subfamily Drosophilinae but the authors suggested that, because their data set was widely sampled across the subfamily, other sets of species could be less diagnosable. Our dataset was focused only on species of the *tripunctata* radiation and we could still correctly classify more than 90% of the individuals, indicating that wing shape may be used to discriminate even among closely related species.

Moreover, our results displayed an apparent pattern of wing total shape variation among different lineages. Most of the wing shape variation was due to the displacement of the two crossveins. In the *cardini* group they are closer to each other; in the *tripunctata* restricted lineage the distance between these veins is larger whereas in the *nappae* lineage both crossveins are

displaced towards the tip of the wing. This pattern was not present in the nonallometric shape variation, reflecting the possible absence of phylogenetic signal on this trait. It is possible that total shape variation, which includes the allometric component of wing shape, is subject to stronger constraints than nonallometric variation. Hence nonallometric aspects of wing variation would be more likely to respond to specific selective pressures or to diverge randomly by genetic drift. However, the fact that most of the total shape variation concerned the position of crossveins indicate that these veins could be involved in adaptations specific to each lineage. Crossveins are important in determining the flexural stiffness of the wing (Ennos, 1989). The number, placement, thickness, or flexibility of the crossveins are important mechanisms of altering the amount of lift a wing can generate (Ennos, 1989). Nevertheless, not all wing characters are related to flight. Some are concerned with sensory functions, thermoregulation and territorial or sexual signaling (Wooton, 1992), which should be considered when an adaptive explanation is to be proposed about wing shape patterns of evolution. In addition, the possibility that these patterns have been generated by random events in the ancestors of each lineage cannot be disregarded.

*4.3. Conclusions*

The results we obtained by the phylogenetic reconstructions based on the sequences of COI and COII, although not able to completely resolve the phylogenetic relationships among the species of the *tripunctata* radiation, allowed the identification of monophyletic groups that may shed some light over the patterns of speciation in this radiation. Our work may also motivate future phylogenetic studies on these species and a possible taxonomic revision, which appears to be necessary since we can now state with reasonable confidence that the *tripunctata* group is not monophyletic, as probably some other groups belonging to the *tripunctata* radiation. The existence of consistent patterns of wing shape variation among the phylogenetic lineages

identified by the molecular data reinforces the hypothesis of monophyly of these groups. However, many questions remain. In order to identify patterns of evolution of wing shape, we would have to know the phylogenetic relationships among these groups, which was not possible since most of the basal nodes in our analysis presented extremely poor statistical support. The collection of additional molecular data (preferably nuclear genes) is obligatory before we can establish a reliable phylogenetic hypothesis or conclude that the *tripunctata* radiation in fact originated from episodes of rapid or multiple speciation.

## 5. Acknowledgements

We thank administrators of Serra do Japi, Bosque dos Jequitibás and Mata Ribeirão Cachoeira for authorization for collection. We also thank Dr. Jean R. David and Dr. Antonio B. de Carvalho for providing specimens of *D. tripunctata* and *D. ornatipenis*, respectively. We also thank Maria Salete C. Campos and Rosângela A. Rodrigues for technical support. This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa de São Paulo (FAPESP) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

## 6. References

Adams, D.C., Rohlf, F.J. and Slice, D.E. 2004. Geometric morphometrics: Ten years of progress following the revolution. Ital. J. Zool. 71: 5-16.

Ashburner, M., Golic, K. G., Hawley, R. S. 2005. Drosophila. A laboratory handbook. Second edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Azeredo-Espin, A.M.L., Schroder, R.F.W., Huettel, M.D., Sheppard, W.S., 1991. Mitochondrial-DNA variation in geographic populations of Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera, Chrisomelidae). Experientia 47, 483–485.

Bächli, G. 2007. TaxoDros: The Database on taxonomy of Drosophilidae, version June 2007. http://taxodros.unizh.ch/

Bächli, G., Vilela, C.R., Ratcov, V. Morphological differences among Drosophila paraguayensis Duda, 1927 and its close relatives (Diptera, Drosophilidae). Mitt. Scheiz. Ent. Ges. 73: 67-92.

Bloomberg, S.P., Garland, Jr., T. and Ives, A.R. 2003. Testing for phylogenetic signal in comparative data: behavioral traits are more labile. Evolution 57: 717-745.

Bookstein, F.L. 1989. Principal warps: Thin-plate splines and the decomposition of deformations. IEEE Trans. Patt. Anal. Mach. Intell. 11: 567-585.

Bookstein, F. L. 1991. *Morphometric tools for landmark data*. Cambridge University Press, Cambridge.

Brisson, J.A., Wilder, J., Hollocher, H. 2006. Phylogenetic analysis of the *cardini* group of *Drosophila* with respect to changes in pigmentation. Evolution 60: 1228-1241.

Burla, H., Pavan, C. 1953. The calloptera group of species (Drosophila, Diptera). Revta. Bras. Biol. 13: 291-314.

David, J.R., Gibert, P., Mignon-Grasteau, S., Legout, H., Petavy, G., Beaumont, C., Moreteau, B. 2003. Genetic variability of sexual size dimorphism in a natural population of *Drosophila melanogaster*: an isofemale-line approach. J. Genet. 82: 79-88.

DeCelis, J.F., Diaz-Benjumea, F.J. 2003. Developmental basis for vein pattern variations in insect wings. Int. J. Dev. Biol. 47: 653-663.

Douady, C.J., Delsuc, F., Boucher, Y., Doolittle, W.F., Douzery, E.J.P. 2003. Comparison of Bayesian and maximum likelihood bootstrap measures of phylogenetic reliability. Mol Biol Evol 20:248-254.

Doyle, J.J. 1997. Trees within trees: genes and species, molecules and morphology. Syst. Biol. 46: 537-553.

Ennos, A.R. 1989. Comparative functional morphology of the wings in Diptera. Zool. J. Linn. Soc. 96: 27-47.

Erixon, P., Svennblad, B., Britton, T., Oxelman, B. 2003. Reliability of Bayesian posterior probabilities and bootstrap frequencies in phylogenetics. Syst. Biol. 52: 665-673.

Ewing, B., Hillier, L., Wendl, M., Green P. 1998. Basecalling of automated sequencer traces using phred. I. Accuracy assessment. Genome Research 8: 175-185.

Frota-Pessoa, O. 1954. Revision of the *tripunctata* group of *Drosophila* with description of fifteen new species (Drosophilidae, Diptera). *Arquivos do Museu Paranaense*, 10: 253-304.

Gordon, D., Abajian, C., Green, P. 1998. Consed: a graphical tool for sequence finishing. Genome Research 8: 195-202.

Guindon, S., Gascuel, O. 2003. A simple, fast and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. 52: 696-704.

Hillis, D.M., Bull, J.J. 1993. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. Syst. Biol. 42: 182-192.

Houle, D., Mezey, J., Galpern, P., Carter, A. 2003. Automated measurement of *Drosophila* wings. BMC Evol. Biol. 3: 25.

Huelsenbeck, J.P., Ronquist, F. 2001. MRBAYES: Bayesian inference of phylogeny. Bioinformatics 17:754-755.

Huelsenbeck, J.P., Larget, B., Miller, R.E., Ronquist, F. 2002. Potential applications and pitfalls of Bayesian inference of phylogeny. Syst. Biol. 51: 673-88.

Kass, R.E., Raftery A.E. 1995. Bayes Factors. J. Am. Stat. Assoc. 90 : 773-795.

Kastritsis, C.D., 1969. The chromosomes of some species of the *guarani* group of *Drosophila*. J. Heredity 60: 50-57.

Kastritsis, C.D., Pasteur, G., Quick, J. 1970. Relationships of the polytene chromosomes of *Drosophila mediostriata* and *Drosophila griseolineata*. *Canad. J. Genet. Cytol.* 12: 952-959.

Kumar, S., Tamura, K., Nei, M. 2004. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Briefings in Bioinformatics 5:150-163.

Maddison, W.P. 1991. Squared-change parsimony reconstructions of ancestral states for continuous-valued characters on a phylogenetic tree. Syst. Zool. 40: 304-314.

Maddison, W. P. and D.R. Maddison. 2006. Mesquite: a modular system for evolutionary analysis. Version 1.11 http://mesquiteproject.org

Markow, T. A., O'Grady, P. 2006. Drosophila: a guide to species identification and use. Academic Press, London.

McArdle, B. and Rodrigo, A.G.. 1994. Estimating the ancestral states of a continuous-valued character using squared-change parsimony: An analytical solution. Syst. Biol. 43: 573-578.

Medeiros, H.F., Klaczko, L.B. 1999. A weakly biased *Drosophila* trap. Dros. Inf. Serv. 82: 100-102.

Nichols, R. 2001. Gene trees and species trees are not the same. Trends Ecol. Evol. 16: 358-364.

Nylander, J.A.A., Ronquist, F., Huelsenbeck, J.P., Nieves-Aldrey, J.L. 2004. Bayesian phylogenetic analysis of combined data. Syst. Biol. 53: 47-67.

Pamilo, P., Nei, M. 1988. Relatioships between gene trees and species trees. Mol. Biol. Evol. 5: 568-583.

Peres-Neto, P.R., Marques, F. 2000. When are random data not random, or is the PTP test useful? Cladistics 16: 420-424.

Pipkin, S. B., Heed, W.B. 1964. Nine new members of the *Drosophila tripunctata* species group. Pacific Insects 6: 256-273.

Posada, D., Crandall, K.A. 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14: 817-818.

Remsen, J., O'Grady, P. 2002. Phylogeny of Drosophilinae (Diptera: Drosophilidae), with comments on combined analysis and character support. Molec. Phylog. Evol. 24: 249-264.

Robe, L.J., Basso da Silva, L.,, Loreto, E. L. 2002. Phylogenetic relationships among four species of the *guarani* group of Drosophila (Diptera, Drosophilidae) as inferred by molecular and morphological analyses. Revta. Bras. Entomol. 46: 515-519.

Robe, L.J., Valente, V. L., Budnik, M., Loreto, E. L. 2005. Molecular phylogeny of the subgenus *Drosophila* (Diptera, Drosophilidae) with an emphasis on Neotropical species and groups: a nuclear versus mitochondrial gene approach. Molec. Phylog. Evol. 36: 623-640.

Rohlf, F.J. 1990. Rotational fit (Procrustes) methods. In: Rohlf, F. J. & Bookstein, F. L. (eds) Proceedings of the Michigan Morphometrics Workshop, Special Publication No 2. University of Michigan Museum of Zoology, Ann Arbor.

Rohlf, F.J. 1993. Relative warp analysis and an example of its application to mosquito wings. In: Marcus, L. F., Bello & E., Garcia-Valdecasas, A. (eds) A Contributions to Morphometrics. Museo Nacional de Ciencias Naturales (CSIC), Madrid.

Rohlf, F.J. 2003a. tpsRelw, relative warps analysis, version 1.36. Department of Ecology and Evolution, State University of New York at Stony Brook.

Rohlf, F.J. 2003b. TpsTree, fitting shapes to trees, version 1.17. Department of Ecology and Evolution, State University of New York at Stony Brook.

Rohlf, F. J., Marcus, L. F. 1993. A revolution in morphometrics. Trends Ecol. Evol. 8: 129-132.

Sene, F.M., Val, F. C., Vilela, C. R., Rodrigues-Pereira, M. A. Q. R. 1980. Preliminary data on the geographical distribution of Drosophila species within morphoclimatic domains of Brazil. Pap. Avul. Zool. 33: 315-326.

Shine, R. 1990. Proximate determinants of sexual differences in adult body size. Am. Nat. 135: 278-283.

Slowinski, J.B. 2001. Molecular politomies. Mol. Phylogenet. Evol. 19: 114-120.

Slowinski, J.B., Crother, B.I. 1998. Is the PTP test useful? Cladistics 14: 297-302.

Stamps, J.A. 1993. Sexual size dimorphism in species with asymptotic growth after maturity. Biol. J. Linn. Soc. 50: 123-145

Sturtevant, A. H. 1942. The classification of the genus Drosophila, with descriptions of nine new species. Univ. Tex. Publs 4213: 5-51.

Suzuki, Y., Glazko, G.V., Nei, M. 2002. Overcredibility of molecular phylogenies obtained by Bayesian phylogenetics. Proc. Natl. Acad. Sci. 99: 16138-16143.

Swofford, D.L. 2003. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

Teder, T., Tammaru, T. 2005. Sexual size dimorphism within species increases with body size. Oikos 108: 321-334.

Thompson, J.D., Higgins, D.G., Gibson, T.J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22: 4673-4680.

Throckmorton, L.H. 1975. The phylogeny, ecology and geography of *Drosophila.* In: *Handbook of Genetics*. Ed. King, R. C. Plenum, New York. Pp. 421-459.

Val, F.C., Vilela, C.R., Marques, M.D. 1981. Drosophilidae of the Neotropical region. In: Ashburner, M., Carson, H.L., Thompson, J.N. (eds) The Genetics and Biology of Drosophila. Academic Press, London. Vol 3a, 123-168.

Vilela, C.R., Pereira, M. A. Q. R. 1985. Notes on two species of spot-thoraxed *Drosophila* belonging to the *guarani* group (Diptera, Drosophilidae) Revta. Bras. Ent. 29: 435-442.

Vilela, C.R., Valente, V. L. S., Basso da Silva, L. 2004. *Drosophila angustibucca* Duda sensu Frota-Pessoa is an undescribed species (Diptera, Drosophilidae) Revta. Bras. Entomol. 48: 233-238.

Vilela. C.R. 1992. On the *tripunctata* species group (Diptera, Drosophilidae) Revta. Bras. Ent. 36: 197-221.

Wilcox, T.P., Zwickl, D.J., Death, T.A., Hillis, D.M. 2002. Phylogenetic relationships of the dwarf boas and a comparison of Bayesian and bootstrap measures of phylogenetic support. Mol. Phylogenet. Evol. 25: 361-371.

Wooton, R.J. 1992. Functional morphology of insect wings. Annu. Rev. Entomol. 37: 113-140.

Yotoko, K.S.C., Medeiros, H.F., Solferini, V.N., Klaczko, L.B. 2003. A molecular study of the systematics of the *Drosophila tripunctata* group and the *tripunctata* radiation. *Mol. Phylog. Evol.* 28: 614-619.

Zelditch, M. L., Swiderski, D. L., Sheets, H.D. & Fink, W. 2004. Geometric Morphometrics for Biologists. Elsevier Academic Press, San Diego.

## APPENDIX A

Accession numbers of all sequences included in the phylogenetic analyses.

| Species | Accession number | |
|---|---|---|
| | CO1 | CO2 |
| *D. atrata* | **EF569988** * | **EF570024** * |
| *D. ornatipenis* | **EF570010** * | **EF570038** * |
| *D. schildi* | **EF570016** * | **AY162973** |
| *D. cardini* | **EF569991** * | **AY162974** |
| *D. cardinoides* | **EF569992** * | **AY162975** |
| *D. neocardini* | **EF570006** * | **EF570034** * |
| *D. polymorpha* | **EF570014** * | **EF570040** * |
| *D. griseolineata* | **EF569995** * | **EF570029** * |
| *D. guaraja* | **EF569996** * | **EF570030** * |
| *D. maculifrons* | **EF569998** * | **EF570031** * |
| *D. guaru* | **EF569997** * | **EF570031** * |
| *D. ornatifrons* | **EF570009** * | **AY162978** |
| *D. pallidipennis* | **EF570011** * | **AY162982** |
| *D. sticta* | **EF570018** * | **EF570044** * |
| *D. bandeirantorum* | **EF569989** * | **EF570025** * |
| *D. bifilum* | **EF569990** * | **EF570026** * |
| *D. cuaso* | **EF569993** * | **EF570027** * |
| *D. frotapessoai* | **EF569994** * | **EF570028** * |
| *D. medioimpressa* | **EF569999** * | **AY162994** |
| *D. mediopicta* | **EF570000** * | **EF570033** * |
| *D. mediopunctata* | **EF570001** * | **AY162988** |
| *D. mediosignata* | **EF570002** * | **AY162985** |
| *D. mediostriata* | **EF570003** * | **EF570034** * |
| *D. metzii* | **EF570004** * | **AY162992** |
| *D. nappae* | **EF570005** * | **AY162983** |
| *D. neoguaramunu* | **EF570007** * | **EF570036** * |
| *D. nigricincta* | **EF570008** * | **EF570037** * |
| *D. paraguayensis* | **EF570012** * | **EF570039** * |
| *D. paramediostriata* | **EF570013** * | **AY162995** |
| *D. roehrae* | **EF570015** * | **EF570041** * |
| *D. setula* | **EF570022** * | **EF570042** * |
| SP22** | **EF570017** * | **EF570043** * |
| *D. trifilum* | **EF570019** * | **EF570046** * |
| *D. tripunctata* | **EF570023** * | **AF519343** |
| *D. unipunctata* | **EF570020** * | **EF570047** * |
| *D. falleni* | **AY541136** | **AF147117** |
| *D. innubila* | **AY541192** | **AY541211** |
| *D. quinaria* | **AY154400** | **AF478428** |
| *D. recens* | **AY154456** | **AF147123** |
| *D. subquinaria* | **AY154457** | **AY154457** |
| *D. immigrans* | **EF570021** | **AY162993** |
| *D. eohydei* | **DQ471601** | **AF145889** |
| *D. hydei* | **DQ471602** | **DQ202020** |
| *D. melanogaster* | **NC001709** | **NC005779** |
| *D. mauritiana* | **NC005779** | **NC001709** |
| *D. sechellia* | **NC005780** | **NC005780** |
| *D. simulans* | **NC005781** | **AF474082** |
| *D. yakuba* | **NC001322** | **NC001322** |

* new sequences obtained in this study

**APPENDIX B**

Taxonomic placement and collection site of each species collected for DNA extraction.

| Subgenus | Group | Subgroup | Species | Collection site* |
|---|---|---|---|---|
| *Drosophila* | *calloptera* | | *D. atrata* | Mata Ribeirão Cachoeira |
| | | | *D. schildi* | Bosque dos Jequitibás |
| | *cardini* | *cardini* | *D. cardini* | Serra do Japi |
| | | | *D. cardinoides* | Brasília |
| | | | *D. neocardini* | Bosque dos Jequitibás |
| | | | *D. polymorpha* | Serra do Japi |
| | *guarani* | *guarani* | *D. guaru* | Mata Ribeirão Cachoeira |
| | | | *D. ornatifrons* | Serra do Japi |
| | | *guaramunu* | *D. griseolineata* | Serra do Japi |
| | | | *D. guaraja* | Serra do Japi |
| | | | *D. maculifrons* | Serra do Japi |
| | *sticta* | | *D. sticta* | Mata Ribeirão Cachoeira |
| | *pallidipennis* | | *D. pallidipennis* | Serra do Japi |
| | *tripunctata* | I | *D. nappae* | Mata Ribeirão Cachoeira |
| | | | *D. neoguaramunu* | Mata Ribeirão Cachoeira |
| | | | *D. setula* | Bosque dos Jequitibás |
| | | | SP22** | Serra do Japi |
| | | II | *D. cuaso* | Serra do Japi |
| | | | *D. medioimpressa* | Mata Ribeirão Cachoeira |
| | | | *D. mediopunctata* | Serra do Japi |
| | | | *D. mediosignata* | Serra do Japi |
| | | | *D. paraguayensis* | Serra do Japi |
| | | | *D. roehrae* | Serra do Japi |
| | | | *D. unipunctata* | Mata Ribeirão Cachoeira |
| | | III | *D. bandeirantorum* | Serra do Japi |
| | | | *D. bifilum* | Bosque dos Jequitibás |
| | | | *D. frotapessoai* | Mata Ribeirão Cachoeira |
| | | | *D. mediopicta* | Serra do Japi |
| | | | *D. mediostriata* | Serra do Japi |
| | | | *D. nigricincta* | Mata Ribeirão Cachoeira |
| | | | *D. paramediostriata* | Serra do Japi |
| | | | *D. trifilum* | Mata Ribeirão Cachoeira |
| | | IV | *D. metzii* | Bosque dos Jequitibás |
| | *immigrans* | *immigrans* | *D. immigrans* | Mata Ribeirão Cachoeira |

*Coordinates for each collection site are: 22º55' S, 47º03' W (Bosque dos Jequitibás, Campinas, SP); 15°46′ S 47°55′ W (Brasília, DF); 22°50' S, 46°55' W (Mata Ribeirão Cachoeira, Campinas, SP); 23°13' S, 46°53' W (Serra do Japi, Jundiaí, SP).

**Undescribed species. SP22 and *D. nappae* are sibling species.

## CONCLUSÕES GERAIS

1. A temperatura de desenvolvimento das larvas, as inversões cromossômicas e o sexo influenciam tanto o tamanho quanto a forma da asa de *D. mediopunctata*.
2. Existe uma interação de temperatura e cariótipo apenas para forma da asa, o que não foi detectado para o tamanho.
3. Outros fatores, talvez a densidade de desenvolvimento das larvas, ou mudanças sazonais no conteúdo de genes dentro das inversões cromossômicas, podem influenciar a morfologia da asa em populações naturais, causando a diferença observada entre nossos resultados e trabalhos anteriores.
4. A radiação *tripunctata* pode ser considerada um grupo monofilético.
5. O grupo *tripunctata* não constitui um grupo monofilético.
6. Encontramos evidências a favor de Kastritsis (1969, 1970) em relação ao grupo *guarani,* ou seja, as espécies do subgrupo *guaramunu* parecem ser mais próximas de algumas espécies do grupo *tripunctata* do que às espécies do subgrupo *guarani*.
7. Foram encontrados quatro agrupamentos dentro da radiação *tripunctata*, que podem constituir grupos monofiléticos dentro desta radiação.
8. A forma da asa permite diagnosticar, com 90% de certeza, as espécies pertencentes à radiação *tripunctata.*
9. Foram encontrados padrões de variação morfológica da asa nestes agrupamentos, causados principalmente pelo deslocamento das veias transversais da asa, além da variação de tamanho.

## LITERATURA CITADA


Adams, D. C., Rohlf, F. J. and Slice, D. E. 2004. Geometric morphometrics: Ten years of progress following the revolution. Ital. J. Zool. 71: 5-16.

Ananina, G., Peixoto, A.A., Bitner-Mathe, B.C., Souza, W.N., da Silva, L.B., Valente, V.L.S., Klaczko, L.B. 2004. Chromosomal inversion polymorphism in *Drosophila mediopunctata*: seasonal, altitudinal, and latitudinal variation. Genet. and Molec. Biol. 27: 61-69

Ananina, G., Peixoto, A.A., Souza, W.N., Klaczko, L.B. 2002. Polytene chromosome map and inversion polymorphism in *Drosophila mediopunctata*. Mem. Inst. Oswaldo Cruz 97: 691-694.

Ashburner, M., Golic, K.G., Hawley, R.S. 2005. Drosophila. A Laboratory Handbook. 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Atkinson, D., Sibly, R.M. 1997. Why are organisms usually bigger in colder environments? Making sense of a life history puzzle. Trends Ecol. Evol. 12: 235-239.

Avise, J.C. 1986. Mitochondrial DNA and the evolutionary genetics of higher animals. Phil. Trans. R. Soc. Lond. B 312: 325-342.

Azeredo-Espin, A.M.L., Schroder, R.F.W., Huettel, M.D., Sheppard, W.S., 1991. Mitochondrial-DNA variation in geographic populations of Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera, Chrisomelidae). Experientia 47, 483–485.

Azevedo, R.B., French, V., Partridge, L. 2002. Temperature modulates epidermal cell size in *Drosophila melanogaster*. J. Insect. Physiol. 48: 231-237.

Bächli, G. 2007. TaxoDros: The Database on taxonomy of Drosophilidae, version June 2007. http://taxodros.unizh.ch/

Bächli, G., Vilela, C. R., Ratcov, V. Morphological differences among Drosophila paraguayensis Duda, 1927 and its close relatives (Diptera, Drosophilidae). Mitt. Scheiz. Ent. Ges. 73: 67-92.

Bitner-Mathé, B.C., Klaczko, L.B. 1999a. Heritability, phenotypic and genetic correlations of size and shape of *Drosophila mediopunctata* wings. *Heredity* 83: 688-696.

Bitner-Mathé, B.C., Klaczko, L.B.. 1999b. Size and shape heritability in natural populations of Drosophila mediopunctata: temporal and microgeographical variation. *Genetica* 105: 35-42.

Bitner-Mathé, B.C., Peixoto, A.A., Klaczko, L.B. 1995. Morphological variation in a natural population of *Drosophila mediopunctata*: altitudinal cline, temporal changes and influence of chromosome inversions. Heredity 75: 54-61.

Bloomberg, S.P., Garland, Jr., T. and Ives, A.R. 2003. Testing for phylogenetic signal in comparative data: behavioral traits are more labile. Evolution 57: 717-745.

Bookstein, F.L. 1989. Principal warps: Thin-plate splines and the decomposition of deformations. IEEE Trans. Patt. Anal. Mach. Intell. 11: 567-585.

Bookstein, F.L. 1991. Morphometric tools for landmark data. Cambridge University Press, Cambridge.

Brisson, J. A., Wilder, J., Hollocher, H. 2006. Phylogenetic analysis of the *cardini* group of *Drosophila* with respect to changes in pigmentation. Evolution 60: 1228-1241.

Burla, H., Pavan, C. 1953. The calloptera group of species (Drosophila, Diptera). Revta. Bras. Biol. 13: 291-314.

Calboli, F.C., Gilchrist, G.W., Partridge, L. 2003. Different cell size and cell number contribution in two newly established and one ancient body size cline of *Drosophila subobscura.* Evolution 57: 566-73.

Calboli, F.C.F., Kennington, W.J., Partridge, L. 2003. QTL mapping reveals a striking coincidence in the positions of genomic regions associated with adaptive variation in body size in parallel clines of *Drosophila melanogaster* on different continents. Evolution 57: 2653-2658.

Carvalho, A.B., Klaczko, L.B. 1993. Autosomal suppressors of sex-ratio in *Drosophila mediopunctata*. *Heredity* 71: 546-551.

Carvalho, A.B., Klaczko, L.B. 1994. Y-linked suppressors of the sex-ratio trait in *Drosophila mediopunctata*. *Heredity* 73: 573-579.

Carvalho, A.B., Peixoto, A.A., Klaczko, L.B. Sex-ratio in *Drosophila mediopunctata*. 1989. *Heredity* 62: 425-428.

Carvalho, A.B., Vaz, S.C., Klaczko, L.B. 1997. Polymorphism for Y-linked suppressors of sex-ratio in two natural populations of *Drosophila mediopunctata*. *Genetics* 146: 891-902.

Caterino, M.S., Cho, S., Sperling, F.A.H. 2000. The current state of insect molecular systematics: a thriving tower of Babel. Annu. Rev. Entomol. 45: 1-54.

Cavicchi, S., Guerra, D., Giorgi, G., Pezzoli, C. 1985. Temperature-related divergence in experimental populations of *Drosophila melanogaster.* I. Genetic and developmental basis of wing size and shape variation. Genetics 109: 665-689.

Clark, A., Gibson, G., Kaufman, T., McAllisteru, B., Myers, E., O'Grady, P. 2003. Proposal for *Drosophila* as a Model System for Comparative Genomics. Genomes White Paper.

Clary, D.O., Wolstenholme, D.R. 1985. The mitochondrial DNA molecule of Drosophila yakuba: nucleotide sequence, gene organization and genetic code. J. Mol. Evol. 22: 252-271.

Crill, W.D., Huey, R.B., Gilchrist, G.H. 1996. Within and between-generation effects of temperature on the morphology and physiology of *Drosophila melanogaster*. Evolution 50: 1205-1218.

David, J.R., Gibert, P., Gravot, E., Petavy, G., Morin, J.P., Karan, D., Moreteau, B. 1997. Phenotypic plasticity and developmental temperature in *Drosophila*: analysis and significance of reaction norms of morphometrical traits. J. Therm. Biol. 22: 441-451.

David, J.R., Gibert, P., Mignon-Grasteau, S., Legout, H., Petavy, G., Beaumont, C., Moreteau, B. 2003. Genetic variability of sexual size dimorphism in a natural population of *Drosophila melanogaster*: an isofemale-line approach. J. Genet. 82: 79-88.

David, J.R., Legout, H., Moreteau, B. 2006. Phenotypic plasticity of body size in a temperate population of *Drosophila melanogaster*: when the temperature-size rule does not apply. J Genet 85: 9-23.

David, J.R., Moreteau, B., Gauthier, J.P., Petavy, G., Stockel, A., Imasheva, A.G. 1994. Reaction norms of size characters in relation to growth temperature in *Drosophila melanogaster*: an isofemale lines analysis. Genet. Sel. Evol. 26: 229-251.

Debat, V., Begin, M., Legout, H., David, J.R. 2003. Allometric and nonallometric components of *Drosophila* wing shape respond differently to developmental temperature. Evolution 57: 2773-2784

DeCelis, J.F., Diaz-Benjumea, F.J. 2003. Developmental basis for vein pattern variations in insect wings. Int. J. Dev. Biol. 47: 653-663.

Dobzhansky, Th., Pavan, C. 1943. Studies on Brazilian Species of *Drosophila.* Bol. Faculd. Fil. Cienc. Letr. S. Paulo 36: 7-72.

Douady, C.J., Delsuc, F., Boucher, Y., Doolittle, W.F., Douzery, E.J.P. 2003. Comparison of Bayesian and maximum likelihood bootstrap measures of phylogenetic reliability. Mol Biol Evol 20:248-254.

Doyle, J.J. 1997. Trees within trees: genes and species, molecules and morphology. Syst. Biol. 46: 537-553.

Dryden, I.L., Mardia, K.V. 1998. Statistical Shape Analysis. John Wiley & Sons, Chichester.

Ennos, AR. 1989. Comparative functional morphology of the wings in Diptera. Zool. J. Linn. Soc. 96: 27-47.

Erixon, P., Svennblad, B., Britton, T., Oxelman, B. 2003. Reliability of Bayesian posterior probabilities and bootstrap frequencies in phylogenetics. Syst. Biol. 52: 665-673.

Ewing, B., Hillier, L., Wendl, M., Green P. 1998. Basecalling of automated sequencer traces using phred. I. Accuracy assessment. Genome Research 8: 175-185.

Falconer, D.S. 1981. Introduction to quantitative genetics. 2nd edn. Longman, London.

Fanara, J.J., Hasson, E., Rodriguez, C. 1997. The effect of polymorphic inversions on body size in two natural populations of *Drosophila buzzatii* from Argentina. Hereditas 126: 233-237.

Felsenstein, J. 1978. Cases in which parsimony and compatibility methods will be positively mislead. Syst. Biol. 27: 401-410.

Felsenstein, J. 2004. Inferring Phylogenies. Sinauer Associates, Inc. Sunderland, Massachusetts.

Felsenstein, J. 2005. PHYLIP (Phylogeny Inference Package) version 3.6. Distribuído pelo autor. Department of Genome Sciences, University of Washington, Seattle.

Fisher, RA. 1922. On the mathematical foundations of theoretical statistics. Phil. Trans. R. Soc. London A 222: 309-368.

Freire-Maia, N., Engel, H. 1949. Variação cromosômica em *Drosophila pallidipennis*. Cienc. e Cult. 1: 204-207.

Frota-Pessoa, O. 1954. Revision of the *tripunctata* group of *Drosophila* with description of fifteen new species (Drosophilidae, Diptera). *Arquivos do Museu Paranaense*, 10: 253-304.

Gordon, D., Abajian, C., Green, P. 1998. Consed: a graphical tool for sequence finishing. Genome Research 8: 195-202.

Graf, D.L, Sparks, J.S. 2000. Sometimes too much is not enough: a comment on the use of mtDNA to recover ancient divergences. Naïve Verificationist 1: 1-13.

Griffiths, J.A., Schiffer, M., Hoffmann, A.A. 2005. Clinal variation and laboratory adaptation in the rainforest species *Drosophila birchii* for stress resistance, wing size, wing shape and development time. J. Evol. Biol. 18: 213-22.

Guindon, S., Gascuel, O. 2003. A simple, fast and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. 52: 696-704.

Hajibabaei, M., Singer, G.A.C., Hebert, P.D.N., Hickey, D.A. 2007. DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics. Trends in Genet. 23: 167-171.

Harrison, RG. 1989. Animal mtDNA as a genetic marker in population and evolutionary biology. Trends Ecol. Evol. 4: 6-11.

Harvey, P.H. , Pagel, M.D. 1991. The comparative method in evolutionary biology. Oxford Univ. Press.

Hasson, E., Fanara, J.J., Rodriguez, C., Vilardi, J.C., Reig, O.A., Fontdevila, A. 1992. The evolutionary history of *Drosophila buzzatii*. XXIV. Second chromosome inversions have different average effects on thorax length. Heredity 68: 557-563.

Hastings, W. 1970. Monte Carlo sampling methods using Markov chains and their applications. Biometrika 57: 97–109.

Hatadani, L.M., Baptista, J.C., Souza, W.N, Klaczko, L.B. 2004. Colour polymorphism in *Drosophila mediopunctata*: genetic (chromosomal) analysis and nonrandom association with chromosome inversions. Heredity 93: 525-534.

Hebert, P.D.N., Cywinska, A., Ball, S.L., deWaard, J.R. 2003a. Biological identifications through DNA barcodes. Proc. R. Soc. Lond. B 270: 313-321.

Hebert, P.D.N., Ratnasingham, S., deWaard, J.R. 2003a. Barcoding animal life: citochrome c oxidase subunit 1 divergences among closely related species. Proc. R. Soc. Lond. B (Suppl. 1): S96-S99.

Hillis, D.M., Bull, J.J. 1993. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. Syst. Biol. 42: 182-192.

Hoffmann, A.A., Sgrò, C.M., Weeks, A.R. 2004. Chromosomal inversion polymorphisms and adaptation. Trends Ecol. Evol. 19: 482-488.

Holder, M., Lewis, P.O. 2003. Phylogeny estimation: traditional and Bayesian approaches. Nature Reviews Genetics 4: 275-284.

Houle, D., Mezey, J., Galpern, P., Carter, A. 2003. Automated measurement of *Drosophila* wings. BMC Evol. Biol. 3: 25.

Huelsenbeck, J.P., Ronquist, F. 2001. MRBAYES: Bayesian inference of phylogeny. Bioinformatics 17:754-755.

Huelsenbeck, J.P., Larget, B., Miller, R.E., Ronquist, F. 2002. Potential applications and pitfalls of Bayesian inference of phylogeny. Syst. Biol. 51: 673-88.

Huey, R. B., Bennett, A. F. 1987. Phylogenetic studies of coadaptation: preferred temperature versus optimal performance temperatures of lizards. Evolution 41:1098-1115.

Huey, R. B, Gilchrist, G. W., Carlson, M. L., Berrigan, D., Serra, L. 2000. Rapid evolution of a geographic cline in size in an introduced fly. *Science* 287: 308-309.

Hyytia, P., Capy, P., David, J. R., Singh R. S. 1985. Enzymatic and quantitative variation in European and African populations of *Drosophila simulans. Heredity* 54: 209-217.

Imasheva, A. G., Bubli, O. A., Lazebny, O. E. 1994. Variation in wing length in Eurasian natural populations of *Drosophila melanogaster. Heredity* 72: 508-514.

Imasheva, A.G., Bubli, O.A., Lazebny, O.E., Zhivotovsky, L.A. 1995. Geographic differentiation in wing shape in *Drosophila melanogaster*. Genetica 96: 303-306.

Imasheva, A.G., Moreteau, B., David, J.R. 2000. Growth temperature and genetic variability of wing dimensions in Drosophila: opposite trends in two sibling species. Genet. Res. 76: 237-247.

Iriarte, P.F., Céspedes, W., Santos, M. 2003. Quantitative-genetic analysis of wing form and bilateral asymmetry in isochromosomal lines of *Drosophila subobscura* using Procrustes methods. J. Genet. 82: 95-113.

Iriarte, P.F., Norry, F.M., Hasson, E.R. 2003. Chromosomal inversions effect body size and shape in different breeding resources in *Drosophila buzzatii*. Heredity 91: 51-59.

Kastritsis, C.D. 1966. Cytological studies on some species of the *tripunctata* group of *Drosophila*. Univ. Texas Publs. Stud. Genet. 6615: 413-474.

Kastritsis, C.D., 1969. The chromosomes of some species of the guarani group of Drosophila. J. Heredity 60: 50-57.

Kastritsis, C.D., Pasteur, G., Quick, J. 1970. Relationships of the polytene chromosomes of *Drosophila mediostriata* and *Drosophila griseolineata*. *Canad. J. Genet. Cytol.* 12: 952-959.

Kendall, D. 1977. The diffusion of shape. Advances in Applied Probability 9: 428-430.

Klaczko, L.B. 1995. Population genetics of *Drosophila mediopunctata*. In: Levine L (Ed.) Genetics of Natural Populations: the Continuing importance of Theodosius Dobzhansky. Columbia University Press. New York.

Klaczko, L.B. 2006. Evolutionary genetics of *Drosophila mediopunctata*. Genetica 126: 43-55.

Klaczko, L.B., Bitner-Mathe, B.C. 1990. On the edge of a wing. Nature 346: 321.

Kumar, S., Tamura, K., Nei, M. 2004. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Briefings in Bioinformatics 5:150-163.

Lessells, C.M., Boag, P.T. 1987. Unrepeatable repeatabilities: a commom mistake. Auk 104: 116-121.

Lewis, P.O. 2001. Phylogenetic systematics turns over a new leaf. Trends Ecol. Evol. 16: 30-37.

Li, S. 1996. Phylogenetic tree construction using Markov chain Monte Carlo. Ph.D. Dissertation, Ohio State Univ., Columbus.

Loreto, E.L., Valente, V.L., Zaha, A., Silva, J.C., Kidwell, M.G. 2001. *Drosophila mediopunctata* P elements: a new example of horizontal transfer. *J. Hered.* 92: 375-381

Lunt, D.H., Zhang, D.X., Szymura, J.M., Hewitt, G.M. 1996. The insect cytochrome oxidase I gene: evolutionary patterns and conserved primers for phylogenetic studies. Insect Mol. Biol. 5: 153-165.

Maddison, W.P. 1991. Squared-change parsimony reconstructions of ancestral states for continuous-valued characters on a phylogenetic tree. Syst. Zool. 40: 304-314.

Maddison, W.P. and D.R. Maddison. 2006. Mesquite: a modular system for evolutionary analysis. Version 1.11 http://mesquiteproject.org

Markow, T.A., O'Grady, P. 2006. Drosophila: a guide to species identification and use. Academic Press, London.

Martins, E. 2000. Adaptation and the comparative method. Trends Ecol. Evol. 296-299.

Mau, B. 1996. Bayesian phylogenetic inference via Markow chain Monte Carlo methods. Ph.D. Dissertation, Univ. Wisconsin, Madison.

McArdle, B. and A. G. Rodrigo. 1994. Estimating the ancestral states of a continuous-valued character using squared-change parsimony: An analytical solution. Syst. Biol. 43: 573-578.

Medeiros, H.F., Klaczko, L.B. 1999. A weakly biased *Drosophila* trap. Dros. Inf. Serv. 82: 100-102.

Metropolis, N., Rosenbluth, A., Rosenbluth, M.,Teller, A., Teller, E. 1953. Equations of state calculations by fast computing machines. J. Chem. Phys. 21:1087–1092.

Moore, W.S. 1995. Inferring phylogenies from mtDNA variation: the mitochondrial-gene trees versus nuclear gene-trees. Evolution 49: 718-726.

Moritz, C., Dowling, T.E., Brown, W.M. 1987. Evolution of animal mitochondrial DNA: relevance for population biology and systematics. Ann. Rev. Ecol. Syst. 18: 269-192.

Nichols, R. 2001. Gene trees and species trees are not the same. Trends Ecol. Evol. 16: 358-364.

Nylander, J.A.A., Ronquist, F., Huelsenbeck, J.P., Nieves-Aldrey, J.L. 2004. Bayesian phylogenetic analysis of combined data. Syst. Biol. 53: 47-67.

Orengo, D.J., Prevosti, A. 2002. Relationship between chromosomal polymorphism and wing size in a natural population of *Drosophila subobscura*. Genetica 115: 311-318.

Pagel, M. 1999. Inferring the historical patterns of biological evolution. Nature 401: 877-884.

Pamilo, P., Nei, M. 1988. Relatioships between gene trees and species trees. Mol. Biol. Evol. 5: 568-583.

Partridge, L., Barrie, B., Fowler, K., French, V. 1994. Evolution and development of body size and cell size in *Drosophila melanogaster* in response to temperature. Evolution 48: 1269-1276.

Pasteur, G., Kastritsis, C.D. 1971. Studies on *Drosophila pallidipennis* Dobzhansky and Pavan. Canad. J. Genet. Cytol. 13: 29-41.

Patterson, J.T., Dobzhansky, T. 1945. Incipient reproductive isolation between two subspecies of *Drosophila pallidipennis*. Genetics 30: 429-438.

Peixoto, A.A., Klaczko, L.B. 1991. Linkage disequilibrium analysis of chromosomal inversion polymorphisms of *Drosophila*. Genetics 129: 773-777.

Peres-Neto, PR, Marques, F. 2000. When are random data not random, or is the PTP test useful? Cladistics 16: 420-424.

Petavy, G., Moreteau, B., Gibert, P., Morin, J.P., David, J.R. 2001. Phenotypic plasticity of body size in *Drosophila*: effects of a daily periodicity of growth temperature in two sibling species. Physiol. Ent. 26: 351-361.

Pfriem, P. 1983. Latitudinal variation in wing size in *Drosophila subobscura* and its dependence on polygenes of chromosome O. Genetica 61: 221-232.

Pipkin, S. B., Heed, W. B. 1964. Nine new members of the *Drosophila tripunctata* species group. Pacific Insects 6: 256-273.

Posada, D., Crandall, K.A. 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14: 817-818.

Powell, J. R. 1997. *Progress and Prospects in Evolutionary Biology: The Drosophila Model.* Oxford University Press, New York.

Rannala, B., and Z. Yang. 1996. Probability distribution of molecular evolutionary trees: a new method of phylogenetic inference. J. Mol. Evol. 43:304-311.

Remsen, J., O'Grady, P. 2002. Phylogeny of Drosophilinae (Diptera: Drosophilidae), with comments on combined analysis and character support. Molec. Phylog. Evol. 24: 249-264.

Robe, L. J., Valente, V. L., Budnik, M., Loreto, E. L. 2005. Molecular phylogeny of the subgenus *Drosophila* (Diptera, Drosophilidae) with an emphasis on Neotropical species and groups: a nuclear versus mitochondrial gene approach. Molec. Phylog. Evol. 36: 623-640.

Robertson, F.W. 1987. Variation of body size within and between wild populations of *Drosophila buzzatii*. Genetica, 72: 111-125.

Rodríguez-Trelles, F. 2003. Seasonal cycles of allozyme-by-chromosomal-inversion gametic disequilibrium in *Drosophila subobscura*. Evolution 57: 839-848.

Rohlf, F.J. 1990. Rotational fit (Procrustes) methods. In: Rohlf, F. J. & Bookstein, F. L. (eds) Proceedings of the Michigan Morphometrics Workshop, Special Publication No 2. University of Michigan Museum of Zoology, Ann Arbor.

Rohlf, F.J. 1993. Relative warp analysis and an example of its application to mosquito wings. In: Marcus, L. F., Bello & E., Garcia-Valdecasas, A. (eds) A Contributions to Morphometrics. Museo Nacional de Ciencias Naturales (CSIC), Madrid.

Rohlf, F.J. 1998. NTSYSpc numerical taxonomy and multivariate analysis system version 2.10. Exeter Software, Setauket.

Rohlf, F.J. 2002. Geometric morphometrics in phylogeny. In Forey, P. and N. Macleod (eds.) Morphology, shape and phylogenetics. Francis & Taylor: London. Pp. 175-193.

Rohlf, F.J. 2003a. tpsRelw, relative warps analysis, version 1.36. Department of Ecology and Evolution, State University of New York at Stony Brook.

Rohlf, F.J. 2003b. TpsTree, fitting shapes to trees, version 1.17. Department of Ecology and Evolution, State University of New York at Stony Brook.

Rohlf, F.J., Marcus, L.F. 1993. A revolution in morphometrics. Trends Ecol. Evol. 8: 129-132.

Ruiz, A., Santos, M., Barbadilla, A., Quezada-Diaz, J.E., Hasson, E., Fontdevila, A. 1991. Genetic variance for body size in a natural population of *Drosophila buzzatii*. Genetics 128: 739-750.

Saitou, N., Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406-25.

Santos, M., Céspedes, W., Balanya, J., Trotta, V., Calboli, F.C.F., Fontdevila, A., Serra, L. 2005. Temperature-related genetic changes in laboratory populations of *Drosophila subobscura:* evidence against simple climatic-based explanations for latitudinal clines. Am. Nat. 165: 258-273.

Sene, F. M., Val, F. C., Vilela, C. R., Rodrigues-Pereira, M. A. Q. R. 1980. Preliminary data on the geographical distribution of Drosophila species within morphoclimatic domains of Brazil. Pap. Avul. Zool. 33: 315-326.

Shine, R. 1990. Proximate determinants of sexual differences in adult body size. Am. Nat. 135: 278-283.

Simon, C., Frati, F, Beckenbach, A., Crespi, B., Liu, H, Flook, P. 1994. Evolution, weighting and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Ann. Entomol. Soc. Am*. 87: 651-702.

Slowinski, J.B. 2001. Molecular politomies. Mol. Phylogenet. Evol. 19: 114-120.

Slowinski, J.B., Crother, B.I. 1998. Is the PTP test useful? Cladistics 14: 297-302.

Stamps, J.A. 1993. Sexual size dimorphism in species with asymptotic growth after maturity. Biol. J. Linn. Soc. 50: 123-145

Starmer, W.T., Wolf, L.L. 1989. Causes of variation in wing loading among *Drosophila* species. Biol. J. Linn. Soc. 37: 247-261.

Sturtevant, A.H. 1942. The classification of the genus Drosophila, with descriptions of nine new species. Univ. Tex. Publs 4213: 5-51.

Suzuki, Y., Glazko, G.V., Nei, M. 2002. Overcredibility of molecular phylogenies obtained by Bayesian phylogenetics. Proc. Natl. Acad. Sci. 99: 16138-16143.

Swofford, D. L. 2003. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

Teder, T., Tammaru, T. 2005. Sexual size dimorphism within species increases with body size. Oikos 108: 321-334.

Thomas, R.H. 1993. Ecology of body size in *Drosophila buzzatii*: untangling the effects of temperature and nutrition. Ecol. Entomol. 18: 84-90.

Thompson, J.D., Higgins, D.G., Gibson, T.J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22: 4673-4680.

Throckmorton, L.H. 1962. The problem of phylogeny in the genus Drosophila. Univ. Texas Publs Stud. Genet. 2: 207-343.

Throckmorton, L. H. 1975. The phylogeny, ecology and geography of *Drosophila.* In: *Handbook of Genetics*. Ed. King, R. C. Plenum, New York. Pp. 421-459.

Val, F.C., Vilela, C.R., Marques, M.D. 1981. Drosophilidae of neotropical region. In: Ashburner M, Carson HC, Thompson, Jr JN. (eds.), The genetics and Biology of Drosophila, vol 3a. Academic Press. London.

Val, F.C., Vilela, C.R., Marques, M.D. 1981. Drosophilidae of the Neotropical region. In: Ashburner, M., Carson, H.L., Thompson, J.N. (eds) The Genetics and Biology of Drosophila. Academic Press, London. Vol 3a, 123-168.

Varandas, F.R., Sampaio, M.C., Carvalho, A.B. 1997. Heritability of sexual proportion in experimental sex-ratio populations of *Drosophila mediopunctata*. Heredity 79: 104-112.

Vilela, C.R., Pereira, M.A.Q.R. 1985. Notes on two species of spot-thoraxed *Drosophila* belonging to the guarani group (Diptera, Drosophilidae) Revta. Bras. Ent. 29: 435-442.

Vilela. C.R. 1992. On the *tripunctata* species group (Diptera, Drosophilidae) Revta. Bras. Ent. 36: 197-221.

Vilela, C.R., Bächli, G. 2004. Revisions of the *Drosophila macroptera* and *D. rubrifrons* species groups, with descriptions of a new Neotropical group (Diptera, Drosophilidae). Mitt. Schweiz. Entomol. Ges. 77: 1-68

Vilela, C.R., Silva, A.F.G., Sene, F.M. 2002. Preliminary data on the geographical distribution of *Drosophila* species within morphoclimatic domains of Brazil. III. The cardini group. Rvta. Bras. Entomol. 46: 139-148.

Vilela, C.R., Valente, V.L.S., Basso da Silva, L. 2004. *Drosophila angustibucca* Duda sensu Frota-Pessoa is an undescribed species (Diptera, Drosophilidae) Revta. Bras. Entomol. 48: 233-238.

Von Zuben, F.J., Duarte, L.C., Stangenhaus, G., Pessôa, L.M., Reis, S.F. 1998. Bootstrap confidence regions for canonical variates: application to studies of evolutionary differentiation. Biom. J. 40: 327-339.

Weeks, A.R., McKechnie, S.W., Hoffmann, A.A. 2002. Dissecting adaptive clinal variation: markers, inversions and size/stress associations in *Drosophila melanogaster* from a central field population. Ecol. Lett. 5: 756-763.

Wilcox, T.P., Zwickl, D.J., Death, T.A., Hillis, D.M. 2002. Phylogenetic relationships of the dwarf boas and a comparison of Bayesian and bootstrap measures of phylogenetic support. Mol. Phylogenet. Evol. 25: 361-371.

Wolstenholme, D.R. 1992. Animal mitochondrial DNA: structure and evolution. Int Rev Cytol 141: 173-216.

Wooton, R.J. 1992. Functional morphology of insect wings. Annu. Rev. Entomol. 37: 113-140.

Wootton, R. J. 2001. How Insect Wings Evolved. In: Insect Movement: Mechanisms and Consequences. Woiwod, I. P., Reynolds, D.R., Thomas, C. D. (Eds.) CAB International, Wallingford, UK. Pp. 43-64.

Yadav, J.P., Singh, B.N. 2003. Population genetics of *Drosophila ananassae*: inversion polymorphism and body size in Indian geographical populations. J. Syst. Evol. Res. 41: 217-226.

Yotoko, K. S. C., Medeiros, H. F., Solferini, V. N., Klaczko, L. B. 2003. A molecular study of the systematics of the *Drosophila tripunctata* group and the *tripunctata* radiation. *Molec. Phylog. Evol.* 28: 614-619.

Zelditch, M. L., Swiderski, D. L., Sheets, H.D., Fink, W. 2004. Geometric Morphometrics for Biologists. Elsevier Academic Press, San Diego.