ALESSANDRA AUGUSTA BERNARDO

CARACTERIZAÇÃO DO PADRÃO DE ESTERASES DE ESPÉCIES DE *Drosophila* DO GRUPO *saltans* (SUBGÊNERO *Sophophora*) E SUA APLICAÇÃO AO ESTUDO DA FILOGENIA E À IDENTIFICAÇÃO DE ESPÉCIES

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
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Tese apresentada para a obtenção
do título de Doutor em Genética

Orientadora: Profa. Dra. Hermione Elly Melara de Campos Bicudo

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Hermione Elly Melara de Campos Bicudo

"Se eu vi mais longe, foi por estar de pé sobre ombros de gigantes".
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"Comece fazendo o que é necessário, depois o que é possível e de repente, você estará fazendo o impossível".

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I. INTRODUÇÃO GERAL

1. Evolução e filogenia: princípios

A teoria da evolução (DARWIN, 1859) foi resumida por Dobzhansky (1973) em quatro afirmações: 1) os seres que vivem agora são descendentes de seres muito diferentes que viveram no passado; 2) as mudanças evolutivas foram mais ou menos graduais, de tal modo que, se fosse possível reunir todos os habitantes da Terra, do passado e do presente, surgiria um conjunto de formas razoavelmente contínuo; 3) algumas das mudanças foram divergentes e muitas das espécies vivas descenderam de um número de espécies ancestrais que é tanto menor quanto mais longe se retrocede no passado; 4) todas as mudanças foram produtos de causas que continuam operando e que, por isso, podem ser estudadas experimentalmente. A aceitação desta teoria deixou claro que as relações de parentesco entre as espécies fornecia um critério para uma classificação hierárquica, consistente e única, dos organismos. Então, um sistema de classificação baseado nestes princípios deveria contemplar os eventos que ocorreram durante a história da vida no nosso planeta.

Hennig (1950 apud MIYAKI et al., 2001) propôs um método de reconstrução das relações de parentesco entre grupos de organismos, a sistemática filogenética. Conforme o autor, os organismos que compartilhassem condições derivadas (apomórficas) de caracteres poderiam ser hipoteticamente descendentes da espécie ancestral na qual a condição primitiva (plesiomórfica) passou, por mutação, à condição derivada. Desde então, este método vem sendo aperfeiçoado.

Segundo Futuyma (1992), a evolução consiste principalmente de anagênese – a mudança direcional dentro de uma única linhagem evolutiva – e de cladogênese, a ramificação da árvore filogenética por meio da especiação. Inferir a história da evolução de um grupo de organismos é inferir as relações filogenéticas entre as espécies – o padrão de ramificação – e descrever, para cada ramo da árvore, a velocidade e o padrão da mudança anagenética nas características que nos interessam. Uma árvore filogenética constitui uma

hipótese sobre a história evolutiva. Assim como outras hipóteses científicas, as árvores filogenéticas não podem ser provadas com certeza absoluta. É possível, porém, agrupar evidências favoráveis e contrárias a uma hipótese filogenética.

O papel da sistemática filogenética, portanto, é, além de realizar o trabalho tradicional da taxonomia, o de organizar o conhecimento sobre a diversidade biológica a partir das relações de parentesco entre grupos. Os resultados dos estudos sistemáticos são representados graficamente na forma de filogenias ou árvores filogenéticas, indicando a relação histórica entre os organismos.

Atualmente, há diversos critérios que podem ser utilizados nos estudos filogenéticos, abrangendo características morfológicas, comportamentais, ecológicas, fisiológicas, citogenéticas e moleculares das espécies ou grupos. Tendo em vista, porém, que geralmente os resultados quanto à filogenia, produzidos por diferentes características não são congruentes, permanece uma questão central: que critério escolher?

2. Variabilidade das populações e sua aplicação em estudos evolutivos

Mayr (1942) enunciou o conceito biológico de espécie como: “Espécies são grupos de populações real ou potencialmente intercruzantes que estão isoladas reprodutivamente de outros grupos”.

Segundo da Cunha et al. (1950), “no processo evolutivo foram produzidas muitas espécies de organismos como uma forma de manter o grande número de genótipos, adaptados aos mais variados ambientes, que não poderiam existir numa só população de indivíduos se intercruzando”. Como o processo evolutivo é contínuo e gradual, a espécie corresponderia a um estágio cuja importância reside no fato de ser unidade de diversificação.

A variabilidade genotípica, determinada pelas mutações em seu sentido amplo e manipulada pela seleção natural, foi um fator básico no desenrolar das transformações evolutivas, capacitando as populações à conquista de novos ambientes no espaço e no tempo, e permitindo o estabelecimento de um domínio cada vez maior sobre os ambientes já conquistados. Como resultado surgiram as numerosas e variadas espécies de organismos (BICUDO, 1967).

Netto e Menck (2001) definem mutações como mudanças permanentes que ocorrem nas seqüências de bases nitrogenadas dos genes podendo originar novas variações nos alelos.

Como as condições ambientais variam de forma não previsível, quanto maior o repertório de alelos diferentes entre os indivíduos de uma população, maior será a chance destes sobreviverem às variações ambientais. Os autores referem-se à seleção natural como um forte evento seletivo que reduz a diversidade biológica (representada pelo número de alelos) devido a uma pequena fração populacional apresentar vantagem adaptativa. Porém, a diversidade biológica continua a ser restaurada, permitindo a adaptação a novas variações ambientais.

Em uma ampla revisão, Bicudo (1984) propõe que um ponto crucial na evolução é como surgem os diferentes tipos de DNA, visto que sua duplicação, que precede a divisão celular e a formação dos gametas, é um processo altamente conservador. Ocasionalmente, ocorrem erros nessa duplicação, sendo que parte é corrigida por mecanismos celulares de reparo e parte, porém, permanece e constitui as chamadas mutações. Ainda, nos organismos sexuados é preciso considerar dois mecanismos que proporcionam a variabilidade: a segregação independente e o crossing-over. Segundo a autora, com o advento da tecnologia do DNA recombinante que permitiu a clonagem e a seqüenciação rápida do DNA, foi mostrado que a variabilidade genética é suprida por um número bem maior de mecanismos do que previamente se supunha. Esse conhecimento derivou da descoberta dos seguintes fatos principais: 1. muitos genes dos eucariotos são divididos em regiões codificadoras (exons) e não codificadoras (introns); 2. muitos genes dos eucariotos têm uma tendência para produzir cópias de si mesmos, fornecendo matéria prima para a evolução; 3. segmentos de DNA móveis migram de uma posição para outra no cromossomo, ou de um cromossomo para outro, causando mutações e rearranjos gênicos.

Conforme Solé-Cava (2001) a expressão diversidade biológica, desde a sua origem, já trazia a idéia do conjunto de variabilidade ecológica (número de espécies de uma comunidade e suas interações) e variabilidade genética (diversidade de alelos nos vários locos de uma espécie). A variabilidade genética proporciona várias formas de polimorfismo, que além de serem importantes para a evolução, podem ser usadas em investigações que comparam indivíduos, populações ou espécies. Para a pesquisadora, a escolha de uma característica polimórfica adequada permite realizar tanto estudos filogenéticos (relação de parentesco entre as entidades consideradas) como filogeográficos (histórico da distribuição geográfica de organismos).

Segundo Solferini e Selivon (2001) os estudos, até a década de 60, limitavam os organismos utilizados que além de possuírem os marcadores adequados, deviam ser adaptados às condições de laboratório, pois era necessário acompanhar as gerações consecutivas. Outra impossibilidade era a quantificação de diferenças interespecíficas.

A introdução da técnica de eletroforese foi de grande importância no estudo da variabilidade das populações. Esta técnica baseia-se na aplicação de uma corrente elétrica a um gel de amido ou poliacrilamida e na visualização das proteínas pela sua coloração no gel. A utilização desta técnica promoveu uma revolução para a genética de populações: pela primeira vez era possível ter acesso a um grande número de locos em qualquer organismo, dos mais variados ambientes, utilizando-se apenas uma amostra de tecido do organismo estudado. Porém, esta metodologia também apresenta algumas limitações: os níveis de variabilidade podem estar sendo subestimados, pois mutações no material genético nem sempre levam a alterações na estrutura protéica e nem toda alteração na seqüência de aminoácidos provoca diferença na mobilidade eletroforética.

Para McReynolds (1967), a análise de similaridade bioquímica e de diferenças entre espécies pode proporcionar dois tipos de informações. De um lado, tais análises podem ser vistas como um simples critério taxonômico adicional, e assim podem ser importantes auxiliares para distinguir entre grupos taxonômicos, particularmente quando a similaridade morfológica é grande. De outro lado, tais análises proporcionam um meio de calcular a quantidade de mudanças genéticas que ocorrem em um único loco, durante a evolução, que algumas informações morfológicas não podem proporcionar sozinhas.

Posteriormente, com a introdução das técnicas de biologia molecular, surgiram diversos métodos de detecção de polimorfismos genéticos diretamente no DNA, os quais permitem a obtenção de um grande número de marcadores cobrindo todo o genoma do organismo. Estes "marcadores moleculares" podem ser utilizados para estudos evolutivos, ecológicos, filogenéticos e taxonômicos (LYNCH e MILLIGAN, 1994; REINEKE e ZEBITZ, 1999).

Para Solé-Cava (2001) a escolha do método depende de vários critérios importantes, sendo que tanto métodos bioquímicos como moleculares apresentam algumas restrições. Em primeiro lugar, está a adequação do grau de variabilidade do marcador: marcadores moleculares que evoluem rapidamente são úteis para o estudo de indivíduos, famílias e populações, enquanto que aqueles que evoluem mais lentamente são mais indicados no estudo de espécies ou táxons supra-específicos. Outro critério é a disponibilidade do material: estudos com aloenzimas necessitam de quantidades maiores de material biológico, fresco ou congelado, sendo este um fator limitante quando se pretende estudar espécies raras. Já na PCR utilizam-se quantidades pequenas de tecido, incluindo aquelas preservadas em álcool ou desidratadas, porém estes estudos tornam-se onerosos quando se tem muitos indivíduos para analisar. Critérios não científicos também influenciam a escolha do marcador, como a tradição

do laboratório, o qual torna-se proficiente em algumas técnicas aplicando-as ao maior número de problemas.

3. Traçado da filogenia em *Drosophila*

A família de dípteros, Drosophilidae, dividida em duas subfamílias, a Drosophilinae e a Steganinae, apresenta aproximadamente 2.500 espécies descritas (WHEELER, 1986 apud KWIATOWSKI e AYALA, 1999). A variação morfológica é grande dentro desta família, destacando-se o dimorfismo sexual, o padrão das asas, modificações no aparelho bucal, dentre muitos outros caracteres. Sua distribuição mundial é caracterizada pela ocupação de uma variedade de nichos ecológicos. As espécies mais comuns se reproduzem em plantas em decomposição e material fungado (HEED, 1971). O gênero *Cladochaeta* é parasita juntamente com alguns membros de *Drosophila* (CARSON, 1974) e algumas larvas de drosofilideos são predadoras (ASHBURNER, 1989). A ampla variedade da família Drosophilidae tem atraído sistematas, ecologistas e taxonomistas, sendo que os estudos evolutivos têm envolvido várias espécies, procurando traçar as relações filogenéticas a partir de diferentes parâmetros biológicos.

Dentre os primeiros trabalhos que abordavam apenas o grau de semelhança morfológica, destaca-se o de Malogolowkin (1953) que procurou estabelecer linhas filogenéticas entre 17 grupos, comparados quanto a 62 caracteres envolvendo a genitália dos machos. Grimaldi (1990 apud RENSEN e O'GRADY, 2002) utilizando um conjunto de 208 caracteres dividiu *Drosophilidae* em tribos, subtribos e grupos de gêneros. Seus resultados mostraram que *Hirtodrosophila*, *Lordiphosa* e *Scaptodrosophila*, previamente consideradas subgênero dentro de *Drosophila*, poderiam ser promovidas a uma posição genérica. O estudo de características morfológicas continua sendo importante ferramenta para estudos filogenéticos, como também para descrever novas espécies.

Trabalhos baseados em polimorfismo cromossômico, também têm contribuído para inferências filogenéticas. Ruiz et al. (1997) trabalhando com 86 inversões paracêntricas observadas no grupo *repleta*, obtiveram resultados consistentes com as homologias cromossômicas correntemente aceitas entre *D. repleta* e *D. melanogaster*. Diniz e Sene (2004) analisando cromossomos politênicos, propuzeram que o subgrupo *fasciola* não seria uma porção filogenética secundária dentro do subgrupo *mulleri*, mas que haveria um ancestral

comum do grupo *repleta*. Também concluíram que *D. onca* e *D. carolinae* são espécies próximas, assim como *D. fascioloides* e *D. ellisoni*, enquanto *D. rosinae* está separada em relação ao ancestral comum do subgrupo *fasciola*. Ananina et al. (2004) analisaram o polimorfismo do cromossomo II de *D. mediopunctata*, observando que nos espécimes do Parque Nacional do Itatiaia (Sudeste brasileiro) as freqüências de 3 inversões distais variam sazonalmente. Ainda, notaram diferença entre moscas de Campinas e da Serra do Japi, as quais são separadas por 50 km. Em contraste, *D. mediopunctata* da Serra do Japi são mais similares aos espécimes do Parque Nacional do Itatiaia, locais que se distanciam 200km. Rhode et al. (2006) estudaram a evolução cromossômica de espécies do grupo *willistoni* utilizando o cromossomo IIR. As análises revelaram que *D. willistoni*, *D. tropicalis* e semiespécies de *D. paulistorum* da América Central podem estar envolvidas diretamente por um ancestral em comum que provavelmente originou todas as espécies do grupo. Um segundo grupo divergente inclui as demais semiespécies de *D. paulistorum* (andinas e do Orenoco) e *D. equinixialis*, porém não foi possível posicionar *D. insularis*.

Estudos ao nível do DNA têm complementado questões relativas à variabilidade das populações. Castro e Madi-Ravazzi (2000) utilizando RAPD confirmaram a diferenciação das linhagens de *D. serido* tipo B (hoje, *D. gouveiae*, TIDON-SKLORZ e SENE, 2001) e tipo D (hoje, *D. antonietae*, TIDON-SKLORZ e SENE, 2001) em relação às demais espécies do grupo *buzzatii*. Entretanto, aproximaram a linhagem A55 (*D. serido* tipo B), do cerrado brasileiro, às linhagens de *D. koepferae* do Chaco Argentino. Goto e Kimura (2001) estudaram o gene mitocondrial *COI* (subunidade 1 da citocromo oxidase) e o gene nuclear *Gpdh* (glicerol 3- fosfato dehidrogenase) no grupo *melanogaster*. As análises baseadas nesse gene revelaram que o subgrupo *ananassae* dividiu-se primeiro no grupo *melanogaster* seguido pelo subgrupo *montium* e mais distante pelo subgrupo *melanogaster*. Como os dados obtidos pelo gene mitocondrial foram inconsistentes, os autores sugeriram que sua utilidade filogenética poderia ser variável entre os diferentes grupos de *Drosophila*. Remsen e O'Grady (2002) apresentaram uma hipótese filogenética para 41 taxa de Drosophilinae, baseada em 2 genes mitocondriais (*rDNA 16S* e *COII*) e 3 nucleares (*rDNA 28S*, *Adh* e *Sod*) combinando-os com características morfológicas. Segundo os autores, a diversidade de dados gerados mostra a importância de analisar os dados juntos e separadamente, como também escolher vários testes, para propiciar mais segurança nas hipóteses. Bonacum et al (2005) estudaram 17 taxa do grupo *planibia* de espécies de *D. hawaiana* baseando-se em dois genes mitocondriais (*16S* e *COII*) e quatro nucleares (*Adh*, *Gpdh*, *YpI*, e *Yp2*), sendo que seus resultados sugeriram que a base de diversificação ocorreu na ilha Kauai, a mais velha. Kopp et al (2006) propuzeram a

utilidade filogenética dos genes do cromossomo Y estudando espécies dos complexos *D. bipectinata* e *D. simulans*. Conforme os autores, as vantagens do uso deste cromossomo incluem ausência de recombinação, coalescência rápida e redução da oportunidade para a introgressão interespecífica devido à esterilidade do macho híbrido.

A técnica de eletroforese tem se mostrado importante no estudo de enzimas e outras proteínas de diferentes populações ou espécies, no sentido de observar mudanças na constituição gênica. Os trabalhos pioneiros utilizaram *D. melanogaster*, destacando-se Wright (1963), que estudou a esterase 6, e Beckman e Johnson (1964), que estudaram a esterase –C. O aprimoramento desta técnica também tem permitido detectar diferenças entre proteínas. Enquanto Whight utilizou gel de amido e detectou 10 bandas com atividade esterásica em *D. melanogaster*, Healy et al. (1991) usando uma combinação de gel de poliacrilamida e focalização isoelétrica, detectaram 22 bandas diferentes na mesma espécie.

As esterases são um grupo de enzimas que participam da hidrólise dos ésteres. Além dos ésteres, as esterases têm a capacidade de atuar sobre outros substratos, hidrolizando peptídeos, amidas e halinas. Estas enzimas tem sido estudadas em bactérias (SHIMADA et al., 1993), fungos (NISCHIKAWA e LIZUKA, 1993), plantas (CHO et al., 1994; CARVALHO et al, 2003; TEIXEIRA et al, 2004; SOUZA et al, 2005) e animais (CHAKRABORTI et al., 1993; LIMA-CATELANI, et al., 2004; GLYNN et al, 2005). Trabalhos utilizando insetos mostraram sua participação em processos digestivos (KAPIN e AHMAD, 1980; SREERAMA e VEERAHADRAPPA, 1991; KERLIN e HUGUES, 1992; ARGENTINE e JAMES, 1995), reprodutivos (RICHMOND e SENIOR, 1991; COSTA et al., 1983; SCOTT, 1986; KAROTAM et al., 1993, MIKHAILOV e TORRADO, 2000), regulação dos níveis do hormônio juvenil (KORT e GRANGER, 1991; ZERA e HOLTMEIER, 1992; GU e ZERA, 1994; NASCIMENTO e BICUDO, 2002; KETHIDI et al, 2005) e degradação de inseticidas (MUTERO et al., 1994; WHYARD et al., 1994; FEYEREISEN, 1995; WANG et al, 2004; GUPTA et al, 2005).

Algumas esterases têm sido extensivamente estudadas, sendo suas seqüências de aminoácidos estabelecidas e os genes responsáveis seqüenciados e localizados cromossomicamente (ROBIN et al, 2000; BALAKIREV, 2003; GOMES e HASSON, 2003). Por apresentarem uma enorme diferenciação em seus padrões enzimáticos, as esterases têm sido amplamente utilizadas para estudos evolutivos e taxonômicos. Johnson et al. (1966) estudaram esterases, com base na especificidade ao substrato e na inibição e ativação da enzima pelo álcool, em várias espécies do gênero *Drosophila* e verificaram que as diferenças observadas entre as espécies estão correlacionadas com o estabelecimento de categorias

taxonômicas. Lapenta et al. (1995) trabalharam com linhagens isofêmeas, sendo 4 de *Drosophila serido*, 2 de *D. koepferae*, 1 de *D. seriema* e 1 de *D. buzzatii*. Foram observadas 43 bandas em géis de poliacrilamida, com variações nas reações específicas com α e β-naftil acetatos, variações de espessura das bandas, intensidade de coloração, diferenças individuais, diferenças entre os sexos, entre linhagens e espécies. Foi possível a separação das linhagens em 5 classes, sendo que cada espécie tinha um padrão próprio de bandas, mas algumas eram compartilhadas. Este trabalho mostrou uma forte evidência de que a linhagem A_{95} não pertenceria a *D. serido*, mas seria uma nova espécie, que foi posteriormente descrita como *D. seriema*, por Tidon-Sklorz e Sene (1995). Lapenta et al. (1995) também destacam que Madi-Ravazzi e Bicudo (1992) observaram que a linhagem A_{95} exibia isolamento reprodutivo para o acasalamento e extensiva assinapse nos cromossomos politênicos dos híbridos de cruzamentos com $B_{20}D_2$ (*D. koepferae*). Mais tarde, Lapenta et al. (1998) utilizaram as mesmas linhagens e outras 10 do complexo *buzzatii* para estudar os padrões de esterases em machos e fêmeas, e em partes do corpo (cabeça, tórax e abdome) observando suas afinidades por α e β naftil-acetatos e comportamento em relação aos inibidores. As 69 bandas detectadas possibilitaram a confirmação de alta similaridade entre *D. seriema* e *D. koepferae*, mas indicaram uma diferenciação entre as linhagens de *D. serido*. A utilização da técnica de eletroforese permitiu que os resultados dos trabalhos de Lapenta et al. (1995, 1998) fossem importantes auxiliares para o reconhecimento do *status* de novas espécies.

4. Estudos filogenéticos no grupo *saltans*

A radiação do subgênero *Sophophora* de *Drosophila* produziu quatro grandes linhagens, reconhecidas como os grupos *melanogaster* nos trópicos do Velho Mundo, o grupo *obscura* no Holártico, e os grupos *willistoni* e *saltans* no Novo Mundo. Os dois grupos do Velho Mundo, *melanogaster* e *obscura* são mais próximos entre si do que dos dois grupos do Novo Mundo, *willistoni* e *saltans* (STURTEVANT, 1942; PATERSON e STONE, 1952; THROCKMORTON, 1975 apud RODRÍGUES-TRELLES et al, 1999).

O grupo *saltans* foi estabelecido por Sturtevant (1942) como um membro do subgênero *Sophophora*, abrangendo apenas espécies escuras que eram divididas em dois subgrupos. Estudos posteriores incluíram nesse grupo espécies amarelas (MAGALHÃES, 1956), como também estabeleceram cinco subgrupos ao invés de dois (MAGALHÃES e

BJORNBERG, 1957). Atualmente o grupo é constituído de 21 espécies, que estão distribuídas do México ao Rio Grande do Sul e Paraguai.

Magalhães (1962) realizou uma revisão do grupo *saltans* de *Drosophila* quanto à taxonomia, morfologia e distribuição. As espécies do grupo foram caracterizadas pela morfologia do 6º e 7º esternitos, pelas espermatecas das fêmeas e edeagos dos machos. Posteriormente, Mourão e Bicudo (1967) descreveram duas novas espécies, *D. magalhaensi* (Rio Grande do Sul) e *D. dacunhai* (Kingston, Jamaica) incluindo-as no subgrupo *sturtevanti*. Assim, foram apresentados os seguintes subgrupos e respectivas espécies:

1. Subgrupo *saltans* (espécies escuras): *D. saltans*, *D. lusaltans*, *D. prosaltans*, *D. nigrosaltans*, *D. septentriosaltans*, *D. austrosaltans* e *D. pseudosaltans*.
2. Subgrupo *sturtevanti* (espécies escuras): *D. sturtevanti*, *D. milleri*, *D. rectangularis*, *D. magalhaensi* e *D. dacunhai*.
3. Subgrupo *parasaltans* (espécies amarelas): *D. parasaltans*, *D. subsaltans* e *D. pulchella*.
4. Subgrupo *elliptica* (espécies escuras): *D. elliptica*, *D. emarginata*, *D. neolliptica* e *D. neosaltans*.
5. Subgrupo *cordata* (espécies escuras): *D. cordata* e *D. neocordata*.

Throckmorton e Magalhães (1962) analisaram as espécies do referido grupo quanto ao acúmulo do pigmento pteridina. Os resultados permitiram estabelecer uma primeira filogenia, na qual o subgrupo *saltans* pareceu representar o ápice de uma população tronco do grupo. Segundo os autores, eventos evolutivos mais recentes foram os que envolveram a subdivisão dessa população tronco, no aglomerado de espécies crípticas que compõem os subgrupos do grupo *saltans*. Para os autores, a maioria dessas espécies apresenta uma distribuição restrita, sugerindo sua derivação como isolados regionais a partir da população tronco mais expandida. Ainda, *D. prosaltans* mostrou a maior variabilidade quanto ao acúmulo de pteridinas no corpo e também a mais alta concentração de drosopterina D, podendo ser considerada a própria população tronco. Contudo, os autores destacam que uma ou duas características analisadas não constituem um indicativo adequado da diversidade total.

Bicudo (1973a) analisou o isolamento reprodutivo em espécies do subgrupo *saltans*. O resultado deste estudo permitiu verificar que o intercruzamento das espécies do subgrupo só produz cruzamentos férteis quando realizado entre *D. prosaltans* e *D. saltans*, *D. prosaltans* e *D. septentriosaltans*, *D. prosaltans* e *D. lusaltans*, *D. saltans* e *D. lusaltans* e *D lusaltans* e *D. septentriosaltans*. Nestes casos, os híbridos machos são estéreis em uma ou nas duas direções do cruzamento. Considerando as medidas de isolamento, a autora estabeleceu uma relação de proximidade filogenética entre as espécies do subgrupo. Um primeiro grupo de maior

proximidade foi constituído por *D. prosaltans*, *D. saltans* e *D. lusaltans*, o segundo por *D. austrosaltans* e *D. nigrosaltans*. *Drosophila septentriosaltans* foi considerada mais próxima do primeiro do que do segundo grupo e *D. pseudosaltans*, a mais distante das demais.

Em outro trabalho sobre isolamento reprodutivo, Bicudo (1978) utilizou 18 linhagens de *D. prosaltans* procedentes da América Central até o sul do Brasil. Esse trabalho mostrou que devido ao isolamento incipiente, essas linhagens poderiam ser diferenciadas compondo três conjuntos de isolamento denominados: A, B e C. O conjunto de isolamento A incluiu as linhagens da América Central, o conjunto B, as linhagens da América do Sul, ao norte do Rio Amazonas e o conjunto C, as linhagens brasileiras, ao sul do Rio Amazonas. Os mecanismos de isolamento encontrados operando entre os três conjuntos foram isolamento sexual e esterilidade dos híbridos atuando principalmente entre A e B e entre A e C. Como seus resultados são comparáveis àqueles das semiespécies de *D. paulistorum*, a autora concluiu que os três conjuntos de isolamento poderiam ser classificados como 3 semiespécies. Ainda, a análise do polimorfismo cromossômico das linhagens indicou que os conjuntos B e C são ancestrais do conjunto A, sugerindo que *D. prosaltans* originou-se na América do Sul e dispersou-se para a América Central.

Paralelamente ao estudo do isolamento reprodutivo, Bicudo (1973b) investigou a variabilidade cromossômica devida a inversões. Neste segundo trabalho, observou que as espécies mostraram 23 inversões, sendo *D. prosaltans* a que apresentou maior variabilidade, seguida de *D. saltans*. As demais espécies, com uma distribuição geográfica mais restrita apresentaram polimorfismo reduzido ou ausente. As variabilidades intraespecífica e interespecífica permitiram traçar a relação filogenética das sete espécies com base na inversão PS3 do braço IIL, de forma que, em um extremo, temos *D. prosaltans*, no outro, cinco espécies partilhando as mesmas inversões básicas (*D. austrosaltans*, *D. lusaltans*, *D. nigrosaltans*, *D. saltans* e *D. septentriosaltans*) e, entre os dois extremos, a espécie *D. pseudosaltans*. Essa disposição filogenética não indica a direção de evolução dentro do subgrupo. Considerando as inversões dos cromossomos II e III, a seqüência permaneceu a mesma e o que se obteve foi apenas o desmembramento das cinco espécies do extremo acima referido.

Targa (1973) realizou comparações de arranjos em quatro espécies do grupo *saltans*. Com relação ao cromossomo IIL, a inversão PS3, é comum a *D. austrosaltans*, *D. saltans* e *D. septentriosaltans*. Duas novas inversões, II e III, incluídas em PS3, foram encontradas na linhagem de *D. austrosaltans*. Os resultados obtidos concordam com os de Bicudo (1967 e 1973b), mostrando que *D. saltans* e *D. septentriosaltans* formam um grupo de espécies

próximas e que *D. austrosaltans* e *D. septentriosaltans* poderiam ser consideradas como membros de dois ramos evolutivos originados de *D. saltans*.

O' Grady et al. (1998) submeteram as espécies do grupo *saltans* a um estudo de duas seqüências de DNA mitocondrial (*COI*- citocromo oxidase I e *COII*- citocromo oxidase II) e duas nucleares (*Adh*- álcool desidrogenase e *ITS1*- espaçador 1 interno transcrito do DNAr nuclear). A análise combinada dos dados permitiu construir uma filogenia na qual os subgrupos *saltans* e *parasaltans* ocupam a posição derivada mais recentemente. Concluíram também que, da mesma forma que os resultados morfológicos anteriores, as relações filogenéticas dentro do subgrupo *saltans* não foram satisfatoriamente resolvidas pelos dados moleculares. Por exemplo, os dados de *ITS1* e *COI* colocam *D. austrosaltans* dentro do subgrupo *saltans* como esperado, enquanto *COII* coloca essa espécie como um táxon irmão, separado das demais espécies do subgrupo. Por outro lado os autores consideraram que os resultados obtidos por essa análise molecular foram incongruentes quando comparados tanto com os dados obtidos pelo isolamento reprodutivo (BICUDO, 1973a), quanto com os dados de inversão cromossômica (BICUDO, 1973b).

Rodríguez-Trelles et al. (1999) estudaram a evolução molecular e a filogenia em seis espécies, sendo pelo menos uma de cada subgrupo do grupo *saltans*, inferidas pelo gene *Xdh* (xantina desidrogenase). A árvore obtida mostrou que o subgrupo *parasaltans* (espécie estudada: *D. subsaltans*) é o mais antigo, seguido do subgrupo *sturtevanti* (espécie estudada: *D. sturtevanti*), do *elliptica* (*D. emarginata*), do *cordata* (*D. neocordata*) e do *saltans* (*D. saltans* e *D. prosaltans*). De acordo com os autores, caracteres são filogeneticamente informativos quando sua taxa de evolução concorda com os tempos de divergência. Neste sentido, o gene *Xdh*, como marcador nuclear, mostrou-se particularmente útil para estudos filogenéticos de espécies ou subgrupos recentemente derivados, como é o caso do grupo *saltans*.

Uma comparação entre *D. prosaltans* e *D. saltans* foi realizada com base na fecundidade em cruzamentos de idades, densidades e temperaturas diferentes por Bernardo (2000). A linhagem P_7 (*D. prosaltans*) procedente do Rio Grande do Sul (Brasil) produziu mais ovos e foi mais fecunda apenas na temperatura menor quando comparada à linhagem S_6 (*D. saltans*) procedente de Huichiuayan (México), sugerindo que estas linhagens tenham mantido sua variabilidade genética, mesmo sendo mantidas por aproximadamente 40 anos no laboratório. Os dados também mostraram que o padrão de oviposição foi semelhante, com pico máximo na primeira semana e posterior declínio, propondo que a proximidade filogenética das espécies (crípticas) poderia explicar a semelhança deste padrão. Comparando

seus resultados com os de Nagel (1989) que estudou seis linhagens de *D. sturtevanti*, observou proximidade entre os padrões de oviposição destas espécies, podendo ser estes característicos do grupo *saltans*. Posteriormente, Freschi et al. (2002) trabalhando com AFLP (Amplified Fragment Length Polymorfism) na mesma linhagem de *D. saltans* e na linhagem P_{76} de *D. prosaltans*, observaram bandas comuns entre as espécies, porém, notaram que o padrão de bandas é distinto entre ambas.

O'Grady e Kidwell (2002) estudaram as relações filogenéticas no subgênero *Sophophora* utilizando dois genes nucleares (*28S* e *Adh*) e um mitocondrial (*COII*). Seus resultados sugeriram que os grupos de espécies *melanogaster* e *obscura* são monofiléticos e formam um clado estreitamente relacionado. Enquanto as espécies do grupo *saltans* suportam que ele é monofilético, os resultados de várias análises indicam que as espécies do grupo *willistoni* podem ser parafiléticas com respeito às espécies do grupo *saltans*.

Nascimento e Bicudo (2002) trabalhando com 8 linhagens de *D. prosaltans*, 6 de *D. saltans*, 1 de *D. austrosaltans* e 1 de *D septentriosaltans*, pertencentes ao subgrupo *saltans*, utilizaram a eletroforese em gel de poliacrilamida para o estudo do padrão de esterases. A relação filogenética com base no padrão das bandas esterásicas (34 bandas observadas no total) mostrou proximidade entre *D. saltans*, *D. septentriosaltans* e *D. austrosaltans* e que *D. prosaltans* está mais distante. Conforme as autoras, seus resultados são compatíveis com os do estudo do polimorfismo de inversões (BICUDO, 1973b), mas são diferentes dos baseados no isolamento reprodutivo (BICUDO, 1973a) e DNA nuclear e mitocondrial (O'GRADY et al, 1998).

Castro e Carareto (2004) analisando 10 espécies do grupo *saltans* com relação aos elementos *P*, sugeriram que *D. neocordata* e *D. emarginata*, isolaram-se do ancestral do grupo, anteriormente às espécies dos demais subgrupos e que *D. parasaltans* diferenciou-se antes das espécies dos subgrupos irmãos *sturtevanti* e *saltans*.

Nascimento e Bicudo (2006) examinaram adultos, larvas, pupa e casulos vazios de *D. prosaltans*, *D. saltans* e *D. austrosaltans* observando esterases específicas para cada estágio de desenvolvimento, freqüência específica e grau de expressão variável de bandas compartilhadas entre os estágios. As autoras destacam que a caracterização dos padrões de esterases no grupo *saltans* está começando. As informações obtidas no seu trabalho sobre o subgrupo indicam que ele é uma boa ferramenta para o entendimento da expressão e função das esterases, bem como das mudanças envolvendo os genes responsáveis, as quais ocorreram com a divergência evolutiva do subgrupo.

Para O' Grady et al. (1998) a ordem de ramificação do subgrupo *saltans* não está bem definida, devido à divergência relativamente recente destas espécies. Segundo Kopp et al (2006), a reconstrução de relações filogenéticas entre espécies recentemente divergentes apresenta três problemas gerais: segregação de polimorfismos que pré-datam a divergência de espécies; fluxo gênico durante e após a especiação e recombinação intra-locus.

Muito ainda deverá ser estudado com relação à variabilidade das espécies do grupo *saltans* procurando estabelecer possíveis inferências filogenéticas. Cada técnica de estudo tem vantagens e desvantagens. Independente do caráter considerado, cada tipo de trabalho deixa sua contribuição para aumentar as informações sobre o subgrupo, como também novas questões.

II. OBJETIVOS

II. Objetivos

No presente trabalho, foram estudados os padrões de esterases em 19 linhagens de 10 espécies do grupo *saltans* de *Drosophila*, objetivando:

1. Complementar o estudo realizado por Nascimento e Bicudo (2002) quanto às esterases do subgrupo *saltans*, um dos cinco subgrupos que compõem o grupo *saltans*, estendendo-o aos demais de modo a ter uma visão geral dessa característica, no grupo. Para tanto, as bandas esterásicas obtidas por eletroforese em géis de poliacrilamida foram caracterizadas quanto à posição no gel, à afinidade pelos substratos utilizados (α e β-naftil acetatos) e à resposta aos inibidores Malathion e sulfato de eserina.
2. Analisar separadamente a cabeça, o tórax e o abdome das moscas e verificar quais bandas se expressam em cada uma dessas partes, para obter informações preliminares quanto à função das diferentes bandas esterásicas.
3. Obter outras informações, como diferenças entre machos e fêmeas quanto à presença de bandas sexo-específicas, ou diferenças quanto ao grau de expressão das bandas, que é indicado pela intensidade de coloração e espessura.
4. Utilizar os padrões obtidos para as linhagens e espécies, em programas computacionais específicos, para estabelecer a proximidade filogenética entre elas e comparar os resultados com os obtidos em trabalhos filogenéticos baseados em outras características.
5. Verificar a existência de bandas que ocorrem exclusivamente em uma espécie e que pelas suas características, como alta freqüência de expressão e facilidade de reconhecimento, possam servir como marcadores para facilitar o reconhecimento da espécie.

Os resultados obtidos estão reunidos em dois trabalhos preparados para publicação, sob os títulos:

1- Esterase pattern variability in adult flies of *Drosophila* species from the five subgroups of the *saltans* group (*Sophophora* subgenus) and its use as indicator of phylogeny

2- Esterase patterns used as markers for identification of *Drosophila* species from the *saltans* group

III. ARTIGO 1

III. ARTIGO 1

Esterase pattern variability in adult flies of *Drosophila* species from the five subgroups of the *saltans* group (*Sophophora* subgenus) and its use as indicator of phylogeny

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Key words: Drosophila saltans group, sex-specific esterases, esterase expression in body parts, phylogenetic trees

Short Title: Esterase patterns and phylogeny in *Drosophila saltans* group

Resumo

Os padrões de esterases de 19 linhagens de 10 espécies do grupo *saltans* foram analisados usando eletroforese em gel de poliacrilamida e α e β-naftil acetatos como substratos. Cinqüenta e uma bandas esterásicas foram detectadas e classificadas como 31 α-esterases, 18 β-esterases e duas α/β-esterases. Com base nos padrões de inibição usando Malathion e sulfato de eserina, 34 bandas foram classificadas como carboxilesterases, 14 como acetilesterases e três como colinesterases. Dez loci gênicos foram tentativamente estabelecidos com base nos dados de posição da banda no gel, preferência ao substrato e padrões de inibição. Vinte bandas foram espécie-específicas, as restantes foram compartilhadas por espécie de um mesmo ou diferentes subgrupos. Sete bandas α-esterásicas foram observadas exclusivamente em machos. Bandas com diferentes freqüências ou grau de expressão entre os sexos também foram detectadas. Nos géis preparados para análise da expressão dos genes nas partes do corpo (cabeça, tórax e abdome), o grau de expressão das β-esterases foi alto no tórax, enquanto as α-esterases expressaram-se predominantemente no abdome e tórax. Uma visão global dos dados, atualmente disponíveis, quanto às esterases das espécies do grupo *saltans* é apresentada neste trabalho. As hipóteses filogenéticas geradas à partir dos dados deste estudo foram comparadas com filogenias anteriores baseadas na morfologia, bioquímica e características moleculares.

Abstract

The esterase patterns of 19 strains from 10 species in the *saltans* group were analyzed using polyacrylamide gel electrophoresis and α- and β-naphthyl acetates as substrates. Fifty-one esterase bands were detected and classified as 31 α-esterases, 18 β-esterases and two α/β-esterases. On the basis of the inhibition patterns using Malathion and eserine sulfate, 34 bands were classified as carboxylesterases, 14 as acethylesterases and three as cholinesterases. Ten gene loci were tentatively established on the basis of data on band position in the gel, substrate preference and inhibition pattern. Twenty bands were species-specific, the remaining being shared by species from the same or different subgroups. Seven α-esterase bands were observed exclusively in males. Bands with a different frequency or degree of expression between sexes were also detected. In the gels prepared for analysis of gene expression in the body parts (head, thorax and abdomen), the degree of expression of the β-esterases was higher in the thorax, while the α-esterases were expressed predominantly in the abdomen and thorax. A global view of the data available at present on the esterases of species from the *saltans* group is presented. Phylogenetic hypotheses generated from data in this study are compared with prior phylogenies based on morphological, biochemical and molecular characteristics.

Introduction

The *saltans* group was established by Sturtevanti (1942) as a member of the *Sophophora* subgenus, including only dark species divided into two subgroups. Yellow species were later included in the group by Magalhães (1956). Soon afterwards, five subgroups were recognized in the group on the basis of male genitalia characteristics and external morphology of the body (Magalhães & Bjornberg, 1957).

Magalhães (1962) presented a review of the *saltans* group as to the taxonomy, morphology and distribution of the 19 species known at the time. The five subgroups were maintained but they were renamed, as follows: (1). *saltans* subgroup (dark species): *D. saltans*, *D. lusaltans*, *D. prosaltans*, *D. nigrosaltans*, *D. septentriosaltans*, *D. austrosaltans and D. pseudosaltans*; (2) *sturtevanti* subgroup (dark species): *D. sturtevanti*, *D. milleri*, *D. rectangularis*, *D. magalhaensi* and *D. dacunhai*; (3) *parasaltans* subgroup (yellow species): *D. parasaltans*, *D. subsaltans* and *D. pulchella*; (4) *elliptica* subgroup (dark species): *D. elliptica*, *D. emarginata*, *D. neolliptica and D. neosaltans*; and (5) *cordata* subgroup (dark species): *D. cordata* and *D. neocordata*. *Drosophila magalhaesi* and *D. dacunhai* were later added to the *sturtevanti* subgroup by Mourão & Bicudo (1967) completing the total of 21 species now included in the group.

The results of studies on reproductive isolation support the present distribution of the species in the five subgroups. Species from different subgroups do not intercross (Bicudo, unpublished data). However, hybrids were produced in laboratory in intercrosses of species from the same subgroup. This was observed in the *saltans* subgroup, involving the species *D. prosaltans* in crosses with *D. saltans*, *D. septentriosaltans* and *D. lusaltans*; in crosses involving *D. saltans* X *D. lusaltans* and *D. lusaltans* X *D. septentriosaltans*. Male F_1 hybrids were sterile in one or both directions of crosses (Bicudo, 1973a). In the *sturtevanti* subgroup, hybrids were obtained in a single or both directions of intercrosses involving *D. sturtevanti* with *D. milleri, D. magalhaesi and D. dacunhai* and involving *D. magalhaesi* X *D. dacunhai (*Mourão & Bicudo, 1967; Bicudo, 1979). From the other subgroups, only *parasaltans* was analyzed as to reproductive isolation. The two species available in the laboratory, *D. parasaltans* and *D. subsaltans*, showed complete isolation (Bicudo & Prioli, 1978).

The species from the *saltans* group are exclusively found in the Nearctic and Neotropical regions, preponderantly in the latter (Magalhães, 1962). The presently known distribution area of the species in the *saltans* group is variable. In the *saltans* subgroup, *D.*

prosaltans is the species with the greatest distribution area, being found from Costa Rica to the south of Brazil, Paraguay (Magalhães, 1962; Bicudo et al., 1978) and Uruguay (collected by Dr. Beatriz Goñi), followed by *D. saltans*, from Mexico to Costa Rica (Magalhães, 1962) and *D. austrosaltans*, found in Maranhão and several localities in the State of São Paulo, Brazil (Magalhães, 1962; Bicudo, Hozaki & Machado, 1974). The distribution of the remaining species from this subgroup is restrict to one or two collection places (Magalhães, 1962).

Drosophila sturtevanti is the most widespread species from the *sturtevanti* subgroup and also from the *saltans* group, occupying almost the entire range of distribution of the group, from Mexico and Caribbean Islands to the south of Brazil (Magalhães, 1962; Kobaiashi & Bicudo, 1997). The distribution of the other species of this subgroup is geographically restricted (Magalhães, 1962; Mourão & Bicudo, 1967).

From the *parasaltans* subgroup, *D. parasaltans* was collected in Colombia and several localities in the State of São Paulo, Brazil (Magalhães, 1962; Bicudo & Prioli, 1978). *Drosophila subsaltans* was collected in a single place in Brazil and *D. pulchella* is known exclusively from the island of St. Vincent, B. W. I. (Magalhães, 1962).

From the *elliptica* subgroup, *D. emarginata* shows the largest distribution area, from Mexico to the North of South America, including Colombia, Venezuela, Ecuador and Peru. *Drosophila neolliptica* was collected in four Brazilian States, *D. elliptica* only in Mexico and *D. neosaltans*, in a single place of the State of São Paulo, Brazil (Magalhães, 1962).

From the *cordata* subgroup, *D. cordata* has only been collected in Guatemala, and *D. neocordata* in Mexico and the State of Minas Gerais, Brazil (a single collection place in each country) (Magalhães, 1962).

Several studies of the genetic variability of species in the *saltans* group have been carried out, using different characteristics. They involved species from several subgroups such as the studies on pteridine and drosopterin-D accumulations (Throckmorton & Magalhães, 1962); the study on variability as to the presence of transposable elements (Clark et al., 1995; Clark & Kidwell, 1997; Castro & Carareto, 2004) and on the sequence variation as to mitochondrial and nuclear genes (Tarrío, Rodríguez-Trelles & Ayala, 1998; O'Grady, Clark & Kidwell, 1998; Rodríguez-Trelles, Tarrío & Ayala, 1999). Other studies involved only species from the *saltans* subgroup, such as inversion polymorphism (Bicudo, 1973b; Targa, 1973; Bicudo et al., 1978; Kobayashi & Bicudo, 1997; Tamarozzi & Bicudo, 2002) and the variability as to AFLP (Amplified Fragment Length Polymorphism) (Freschi et al., 2002).

The variability of esterase patterns was analyzed in adult flies of four species from the *saltans* subgroup (*D. prosaltans*, *D. saltans*, *D. austrosaltans* e *D septentriosaltans*) (Nascimento & Bicudo, 2002). In that study, 34 bands were detected, being 18 α-esterases, 15 β-esterases and one α/β-esterase. The 34 bands were tentatively included in 9 loci. Biochemically, these enzymes were classified as 21 carboxylesterases, 9 acethylesterases and 2 cholinesterases. Another study on the esterase patterns in the *saltans* subgroup was carried out during development of *D. prosaltans*, *D. saltans* and *D. austrosaltans*, showing greater esterase polymorphism in the adult stage and differences among stages as to the presence of specific esterases and as to the frequency and degree of bands expression (Nascimento & Bicudo, 2006).

In the present study, the analysis of esterase variability was extended to include species from the other subgroups from the *saltans* group. The aim was primarily to obtain more detailed information regarding the esterase patterns, enabling species and subgroups to be compared for the purpose of studies on their phylogenetic relationships.

Materials and methods

Geografic origin of strains used and maintenance in laboratory

The subgroups, species and strains from the *Drosophila saltans* group used in the present study are set out in Table 1 and Figure 1. A total of 19 strains of 10 species from different subgroups were analyzed as to the esterase patterns. They were maintained in culture medium of corn meal- agar, at the temperature of 20°C ±1, 0°C.

Sample preparations for electrophoresis

For sample preparations, 10 days old virgin males and females were used. Two same strain and sex flies were homogenized at 0°C, in 25 µl of buffer solution (0.1 M Tris plus 10% glycerol at pH 8.8). Ten µl of the homogenate were used in each sample. Two flies were used in the sample preparations for a better visualization of the bands in the gel and also for following the same procedure used by Nascimento & Bicudo (2002; 2006). The number of samples analyzed per strain is also shown in Table 1. The total number of adult flies analyzed was 3,668, being 1,794 males and 1,874 females.

Preparation of gels and identification of esterases

Esterase patterns were analyzed in polyacrylamide gels (size 0.14 X 0.195 m), using a separating 12% gel and a 4% stacking gel (Laemmli, 1970). After the sample application (10 µl), the gels were subjected to electrophoresis for 4 h at ~25°C, using a running buffer (0.1 M Tris pH-glycine at pH 8.3) and a constant 200V.

Esterases were identified in the gels following basically the technique described by Johnson et al (1966) and Steiner & Johnson (1973), involving preincubation (45 min) at room temperature (~25°C) in 50 mL 0.1 M sodium phosphate, pH 6.2, followed by a staining reaction in the dark (1h) with a solution containing 20 mg of α-and 15 mg de β- naphtil acetate dissolved in 1 mL of acetone, used as substrates, 60 mg of Fast Blue RR and 5 mL of *N*-propanol in 50 mL of sodium phosphate buffer solution. After this treatment, when the α-naphtil acetate is preferentially hydrolyzed, the bands in the gels become black and are named α-esterases. When the β-naphtil acetate is preferentially hydrolyzed, the bands in the gels become red and are named β-esterases. The bands that hydrolyze both α-and β-naphtil acetates stain magenta and are named α/β-esterases. The gels were destained (24-48 h) in a solution containing 250 mL of ethyl alcohol, 100 mL of acetic acid and 650 mL of distilled water. The gels were air dried at room temperature, following the technique of Ceron, Santos & Bicudo (1992).

Biochemical characterization of esterases

Tests for biochemical characterization of esterases involved the use of the inhibitors organophosphate Malathion (0.7 mM) and eserine sulfate (0,1 mM) (Holmes & Masters, 1967). In these tests the gels were preincubated for 1h in 50 mL of 0.1 M aqueous sodium phosphate solution containing one of the inhibitors and then treated with the staining solution also containing the inhibitor. Using these tests, the esterase bands in the gels could be classified as carboxylesterases (those inhibited by Malathion), cholinesterases (those inhibited by Malathion and eserine sulphate) and acethylesterases (those which are not affected by inhibitors) (Oakeshott et al., 1993).

Evaluation of the esterase activity

The activity of esterases in polyacrylamide gels was evaluated using the Global Lab Image Program (SP0550, Data Translation, Inc., MA, USA, 1995), a Windows® - based monochrome image processing and analysis package. In this method the intensity analysis is

displayed as linear profiles and histograms, the profiling tool graphing the gray values (in the present study equating to the intensity of band staining) along a line segment in an image of the horizontal or vertical sequence of bands in the gel. The horizontal axis of the resultant profile graph represents the points along the line segment while the vertical axis represents the pixel value at each point. The full range of gray-scales values is from 0 (completely black) to 255 (the absence of color), between which lie intermediate shades of gray. In the present study, the grayscales values given to bands by the program were used for comparison of the expression degree of a band between sexes in a same strain or among different strains or species.

Analysis of esterases in body parts

The gels for analysis of the body parts expressing the esterases (head, thorax and abdomen) were also prepared as described for the entire flies, using the parts of five flies to prepare the samples. The species analyzed were *D. prosaltans* (strain P_5), *D. sturtevanti* (strain JAB), *D. parasaltans* (strain TAP-1), *D. subsaltans* (strain SUB) and *D. neocordata* (strain NEO).

Phylogenetic relationships

Differences of esterase patterns among strains (presence and absence of bands) were used to infer phylogenetic relationships. Each strain was scored as 0 (absence of the band) or 1 (presence of the band). Trees were obtained using the PAUP (Phylogenetic Analysis Using Parsimony) Program, v. 4.0b10 (Swofford, 2001), according criteria of distance and parsimony; for distance we have used neighbor-joining search with a bootstrap analysis (100 replicates); for parsimony, a heuristic search (1000 replicates), with tree-bisection-reconnection, branch-swapping algorithm, addition sequence random, with a bootstrap analysis (1000 replicates). The parsimony trees were rooted using *Drosophila melanogaster* strain Canton S as an outgroup, and the esterase data used for this strain were those obtained by Nascimento and Bicudo (2002).

Results

Number of esterase bands detected in the saltans group and their characterization

Esterase bands were characterized by their position on gels (due to their mobility degree) and by their staining color (due to their substrate specificity). With the same

procedure used previously by Nascimento & Bicudo (2002; 2006) for species in the *saltans* subgroup, the esterase bands detected in the present study were numbered successively according to the position in the gel, increasing the number with the increase in negative charge.

Fifty-one esterase bands were detected in the adult flies examined, in the present study, from 10 species (Figure 2). Among them, 28 bands (indicated in the Figure 2 by letter N from new) were not detected by Nascimento & Bicudo (2002). Conversely, 11 bands detected by those authors were not observed in the present study.

On the basis of their affinity for α- and β-naphthyl acetates used as substrates, the 51 bands detected in this study were classified as 31 α-esterases, 18 β-esterases and two α/β-esterases, recognized in the gels by their black, red and magenta colors, respectively, as illustrated in Figure 2. The distribution of α-, β- and α/β-esterases in the strains is shown in the Table 2.

On the basis of the inhibition pattern using Malathion and eserine sulfate, 34 bands were classified as carboxylesterases, 14 as acetylesterases and three as cholinesterases. The band EST-31a was not classified due to its absence in the gels submitted to the treatment (Table 3).

Trying to include the bands into loci

The number of gene loci was tentatively established on the basis of data on substrate preference, position in the gel and inhibition pattern, being 10 the number suggested. The characteristics of each locus are described in Table 3. In this Table are included all the bands described till now in the adult flies of species in the *saltans* group. The bands previously described by Nascimento & Bicudo (2002), which were not detected in the present study are underlined.

Loci 1 to 9 were previously established by Nascimento & Bicudo (2002). Now we described a new locus named 7/8 because the esterase bands included in it are located between bands from locus 7 and 8.

Nascimento & Bicudo (2002) included two β-esterase bands in the locus 1 (EST-1 and EST-2), but their biochemical classification could not be established. In the present study, those bands were not detected, but another β-esterase band, EST-5a, detected in the same region of the gel, was classified as acethylesterase. We are tentatively including the three bands in this locus 1.

Also two new bands, the α-esterases EST-3a and EST-31a were not present in the inhibition gels. However we included tentatively the first band in the locus 2, and the second in the locus 7/8, with other α-esterases found in the same regions of the gel.

Shared and exclusive bands in the subgroups and species

Considering the shared and exclusive bands, the total number of bands detected in each of the subgroups was 36 in both the *saltans* and the *sturtevanti* subgroups, 15 in the *parasaltans*, 15 in the *cordata* and 11 in the *elliptica* subgroups. In the species, the number varied from 11 (*D. subsaltans* and *D. emarginata*) to 27 (*D. austrosaltans* and *D. sturtevanti*) (Figure 2, Table 2).

EST-33 was the only band detected in every fly of every species and subgroup analyzed. Nascimento & Bicudo (2002) had already mentioned the occurrence of this band in every fly from four species from the *saltans* subgroup. EST-12 and EST-34 were detected in every strain of every species and subgroup, but not in 100% of the flies. EST-10 was detected in every species, but not in every strain. EST-13 and EST-15 were present in every subgroup but not in every species. Other 25 bands were shared by two or more species of different subgroups.

As shown in Figure 2, all the subgroups showed exclusive bands, being nine in the *saltans* subgroup (EST-32, EST-31a, EST-20, EST-17; EST-12a, EST-11b, EST-7a, EST-7, and EST-4), seven in the *sturtevanti* subgroup (EST-32d, EST-32b, EST-32a, EST-9b, EST-6c, EST-5c, EST-5b), three in the *parasaltans* subgroup (EST-33a, EST-32c EST-10a), two in the *cordata* subgroup (EST-30a, EST-3a) and one in the *elliptica* subgroup (EST-29a). A total of 20 bands were species-specific, being two in *D. prosaltans* (EST-32 and EST-31a), one in *D. saltans* (EST-4), five in *D. austrosaltans* (EST-17, EST-12a, EST-11b, EST-7a and EST-7), three in D. *sturtevanti* (EST-32d, EST-32a, and EST-5b), one in *D. milleri* (EST-32b), two in *D. dacunhai* (EST-6c and EST-5c), one in *D. parasaltans* (EST-33a), two in *D. subsaltans* (EST-32c and EST-10a), two in *D. neocordata* (EST-30a and EST-3a) and one in *D. emarginata* (EST-29a).

Bands found exclusively in males

Seven bands (all of them α-esterases) were observed exclusively in males, suggesting that at least some of them are male-specific (some probably occurred only in males by random, due to their low frequency of expression in both sexes). The seven bands are EST-

32e, EST-9a, EST-8a, EST-3a (classified as carboxylesterases) and EST-18, EST-12a EST-11 (classified as acethylesterases). Among them, EST-32e, EST-18 and EST-8a were the most widespread, the first two occurring in the species *D. prosaltans*, *D. saltans*, *D. austrosaltans*, *D. sturtevanti*, *D. milleri* and *D. neocordata* and EST-8a in *D. prosaltans*, *D. austrosaltans*, *D. sturtevanti* and *D. neocordata*. EST-11 and EST-9a were found in *D. saltans* and *D. dacunhai*. The band EST-12a was observed exclusively in *D. austrosaltans* and EST-3a in *D. neocordata*.

Bands with different frequency of expression between sexes

Some bands showed different frequency of expression between sexes (number of males and females showing the band). This is the case of the EST-33b band, which was found in *D. prosaltans*, *D. saltans*, *D. austrosaltans*, *D. sturtevanti, D. dacunhai and D. subsaltans,* showing higher frequency of expression in females than in males. For example, in *D. austrosaltans* (strain RP), among 74 analyzed samples (37 from each sex), 18 female samples and three male samples expressed the band (49% and 8%, respectively).

Bands with different degree of expression between sexes

Esterase bands with different intensity of color denoting difference between sexes as to the degrees of gene expression were detected, predominating higher-level of expression in males than in females. The α-esterases, carboxylesterases EST-5 and EST-7 bands showed such differential expression in *D. prosaltans*, *D. saltans* and *D. austrosaltans*, respectively, difference which had already been mentioned by Nascimento & Bicudo (2002). In the present study, EST-5 was also found in *D sturtevanti* (strains MAC and TAP), showing the same difference of expression.

EST-12 (α-esterase, acetylesterase) also showed higher expression in males, in every strain except EVC (*D. emarginata*) where, conversely, the expression was higher in females (Figure 3a, b). The expression of the α-esterases, acetylesterases EST-27b and EST-28 both from the strain IC (*D. austrosaltans*) was also higher in females than in males (Figure 3c, d).

EST-33c, a new cholinesterase in the locus 9

Nascimento & Bicudo (2002) described the α/β-esterase, cholinesterase EST-34 band located in the most anodic region of the gel. In that paper, this band was found in adults of 16 strains of species *D. prosaltans* (8 strains), *D. saltans* (6 strains), *D. septentriosaltans* and *D. austrosaltans* (a single strain each), with frequencies varying from 12% to 49%. In the present

study, EST-34 was detected in the 17 new strains from the subgroups *saltans* (*D. prosaltans, D. saltans* and *D. austrosaltans*), *sturtevanti*, *parasaltans*, *cordata* and *elliptica*, with frequencies varying among the strains from 2% to 41%. However, a second band α/β-esterase, cholinesterase was now detected, located very close to EST-34, in a less anodic position; we named it EST-33c (Figure 2). This second band was detected in seven strains, being three from *D. austrosaltans* and one from *D. prosaltans*, *D. saltans*, *D. milleri* and *D. dacunhai,* with frequencies ranging from 2 to 36%.

EST-34 and EST-33c occurred simultaneously in gels of five of the six strains, with frequencies varying from 2% to 10%. In general, the frequency of the simultaneous presence of the two bands was low, independent of the frequency of each band alone. For example, in the strain RP (*D. austrosaltans*) the separate frequencies of the bands were 11% and 15%, respectively, but only 4% of the samples showed the two bands. The extreme case was the BB strain (*D. austrosaltans*), where the two bands were detected separately with a relatively high frequency (29% and 20%, respectively) and no sample showed the two bands occurring simultaneously. We do not discard the possibility that this is a case of alternative splicing.

Esterase patterns in body parts

The β-esterases present in the gels for analysis of gene expression in the body parts were those from loci 5, 6 and 7, all of them classified as carboxylesterases (Table 4). They showed three different patterns of expression, according to the species, but in every case the degree of expression was higher in the thorax: (a) in *D. prosaltans* they were present in gels of head and thorax; (b) in *D. sturtevanti* and *D. neocordata* they were detected in the three parts; and (c) in *D. parasaltans* and *D. subsaltans* they were detected in the thorax and abdomen.

As to the α-esterases, the body parts expressing their codifying genes varied among the bands but in general they were the same for different species, considering the same band (Table 5). Nine α-esterase bands, being three carboxylesterases, five acethylesterases and one cholinesterase could be analyzed as to their expression, showing six patterns: (1) expression exclusively in the abdomen (EST-13); (2) low expression in the thorax and high in the abdomen (EST-12); (3) low expression in the head and high in the thorax and abdomen (EST-27b, EST-28 and EST-29); (4) high expression in the thorax and low in the abdomen (EST-30); (5) high expression in the thorax and abdomen (EST-31a); (6) high expression in the

head and abdomen and low expression in the thorax (EST-5) an (7) high expression in the head and in the thorax and low expression in the abdomen (EST-33).

Phylogenetic analysis

In the phylogenetic tree shown in Figure 4, obtained by neighbor-joining search, the bootstrap supported nodes (ratios above 50%) involved (1) the three strains of *D. prosaltans* (P_5, POV, PSH); (2) the three strains of *D. austrosaltans* (RP, BB, IC); (3) the strains SRP (*D. sturtevanti*) and MI (*D. milleri*), both from the *sturtevanti* subgroup; (4) SRP, MI, and *D. neocordata* (NEO), from the *cordata* subgroup; (5) the two strains of *D. parasaltans* (TAP-1 and $B_{17\text{-}5}$); (6) an unexpected, bootstrap supported sister clade involved *D. saltans* (strain S_3, from *saltans* subgroup) and *D. dacunhai* (strain DAC, from *sturtevanti* subgroup). The suspicion of stock contamination was carefully analyzed but not confirmed. The two strains exhibited the morphological characteristics mentioned in the original descriptions and did not intercross in four mass crosses of more than 10 pairs, prepared in both cross directions. A seventh supported relationship involves S_3 plus DAC and the three *D. austrosaltans* strains.

It is interesting to observe that the strains of *D. sturtevanti* are forming sister clades with species from different subgroups. In a single case, already mentioned, involving *D. neocordata* (NEO) and *D. sturtevanti* (SRP) plus *D. milleri* (MI), the relationship is bootstrap supported. Other cases, not bootstrap supported, places (1) *D. emarginata* (EVC, *elliptica* subgroup) as sister taxon to NEO, SRP and MI, (2) the *D. sturtevanti* strains MAC and TAP as sister taxon to *D. prosaltans* (which appears in this tree as the most derivative species), and (3) the *D. sturtevanti* strains J_8 and JAB as sister taxon to all other *saltans* group species, except *D. subsaltans* (strain SUB, *parasaltans* subgroup), which appears at the base of this monophyletic tree.

In the phylogenetic tree set out in Figure 5, obtained by heuristic search, many of the nodes bootstrap supported involve the same strains as the tree in Figure 4: (1) The *D. prosaltans* strains (P_5, PSH, POV), (2) the *D. austrosaltans* strains (RP, IC, BB), (3) the *D. parasaltans* strains (TAP-1, $B_{17\text{-}5}$), (4) the strains SRP and MI, from *D. sturtevanti* and *D. milleri*, respectively, (5) SRP, MI and the strain NEO, from *D. neocordata*, (6), the same unexpected sister clade formed by S_3 and DAC, and (7) the association between S_3 plus DAC to the *D. austrosaltans* strains.

In the strains in Figure 5, the *D. sturtevanti* strains also appear in different associations along the tree. MAC and TAP strains remain as sister taxon to *D. prosaltans*, but now this

relationship is bootstrap supported. The J_8 and JAB strains from *D. sturtevanti* are also close to *D. parasaltans* and *D. neocordata*, and EVC (*D. emarginata*) is the sister taxon to NEO, SRP and MI. However, compared to the previous one, this tree presents relocations of the species, in the sequence of associations. Now EVC and NEO are at the base of the tree, as the less derived species, followed by *D. parasaltans* and *D. subsaltans* and then by the species in the *saltans* subgroup. *Drosophila prosaltans* remains as the most derivative species in the group.

Discussion

About forty years of studies on esterases revealed a high degree of polymorphism involving these isozymes, in many organisms. In *Drosophila*, during the time elapsed, many studies were carried out aiming to detect and characterize the esterases in different species. Biological, biochemical and molecular studies were performed and used for comparing related species for evaluating changes occurred in the evolutionary process. Among the first of these comparative studies is the one which involved comparison of the esterases in three sibling species from the *virilis* group (*D. virilis*, *D. americana* and *D. novamexicana*). Two different esterases were detected in each species and considered homologous on the basis of their substrate specificity. However, they were neither identical in the three species nor identical among strains from the same species. In addition, the number of alleles found in each species differed (Mc Reynolds, 1967).

In the present study, the esterase patterns of species included in the five subgroups from the *saltans* group (*saltans*, *sturtevanti*, *parasaltans*, *elliptica* and *cordata*) were detected by electrophoresis in polyacrylamide gels. A high degree of polymorphism was observed, confirming data in literature which showed the great involvement of esterases in the physiology of *Drosophila* and many other organisms. Processes such as digestion (Kapin & Ahmad, 1980; Kerlin & Hugues, 1992; Argentine & James, 1995; Kozaric et al., 2006); reproduction (Costa, Nigro & Danieli, 1983; Richmond & Senior, 1991; Karotam & Oakeshott, 1993, Mikhailov & Torrado, 2000; Odgers et al., 2002), regulation of juvenile hormone levels (Gu & Zera, 1994; Furiatti & Lazzari, 2003; Kethidi, Xi & Palli, 2005; Wogulis et al., 2006), insecticide degradation and resistance (Feyereisen, 1995; Wang et al, 2004; Gupta et al, 2005; Cui et al., 2006) are related to esterase activities.

In a previous work using 16 strains of four species from the *saltans* subgroup (*D. prosaltans*, *D. saltans*, *D. austrosaltans* and *D. septentriosaltans*), a high number of esterases had already been detected in each species, being represented by 15, 25, 14 and 15 bands in the gels,

respectively; these bands were tentatively included into nine loci. On the basis of the band mobility in the gel and substrate-specificity a total of 34 bands were described, being seven shared by the four species, 12 shared by two or three of them and 15 were exclusive (Nascimento & Bicudo, 2002).

In this work, 19 strains from 10 species (apparently the only species from the *saltans* group presently available in laboratories around the world) were studied. Among them, only two strains, being one from *D. prosaltans* (P_5) and the other from *D. saltans* (S_3), both from the *saltans* subgroup, were analyzed as to the esterase patterns by Nascimento & Bicudo (2002). They were also included in this study to facilitate the recognition of the previously described bands, in the gels.

The 10 species presently studied are included in the subgroups as follows: three species in the *saltans* subgroup (*D. prosaltans*, *D. saltans* and *D. austrosaltans*); three species in the *sturtevanti* subgroup (*D. sturtevanti*, *D. milleri* and *D. dacunhai*); two species in the *parasaltans* subgroup (*D. parasaltans* and *D. subsaltans*) and one species in each of the remaining subgroups (*cordata* subgroup- *D. neocordata*; *elliptica* subgroup-*D. emarginata*). Fifty-one esterase bands were detected, being 28 new. That number added to the eleven bands described by Nascimento & Bicudo (2002), which were not found in the strains examined in the present study, gives a total of 62 esterase bands in the *saltans* group.

Considering the data from both studies and the specificity to α- and β-naphtyl acetates, used as substrates, 58% of the 62 bands (36) were classified as α-esterases, 39% (24 bands) as β-esterases and 3% (2 bands) as α/β-esterases. Taking the strains individually, the number of α-esterases varied from 5 (JAB-*D. sturtevanti*) to 17 (IC-*D. austrosaltans*) and that of β-esterases, from 1 (JAB-*D. sturtevanti*) to 6 (IC and RP-*D. austrosaltans*). The biochemical classification of the 62 bands, on the basis of tests with inhibitors, was 42 carboxylesterases, 17 acethylesterases and three cholinesterases.

Considering together (a) the substrate-specificity, (b) the effect of inhibitors and (c) the position of the bands in the gels, the 62 bands were tentatively included in 10 loci, one more than the number proposed by Nascimento & Bicudo (2002). The locus 2 was the most polymorphic, with 18 bands (α-esterases, carboxylesterases), followed in decreasing order by locus 3 (10 bands-α-esterases, acethylesterases), locus 7 (nine bands β-esterases, carboxylesterases) and locus 5 (seven bands-β-esterases, carboxylesterases) and. The remaining showed from one band (locus 8) to five bands (locus 4).

The subgroups *saltans* and *sturtevanti*, from which we studied the greatest number of species (three species each) and strains (seven strains each), showed the greatest numbers of bands (36 bands each). However, the *parasaltans* subgroup from which two species and three strains were analyzed showed 15 bands, the same number detected in the *cordata* subgroup, from which a single species and strain was analyzed. The lowest number of bands was detected in the *elliptica* subgroup (11), from which also a single species and strain was studied.

Drosophila austrosaltans and *D. sturtevanti*, from the *saltans* and the *sturtevanti* subgroups, respectively, were the most polymorphic species, with 27 bands each, followed in decreasing order by *D. prosaltans* (22 bands), *D. saltans* (20 bands) and *D. neocordata* (15 bands). The remaining species showed 17 bands (*D. dacunhai*), 12 (*D. milleri* and *D. parasaltans*) and 11 (*D. subsaltans* and *D. emarginata*). The greatest number of bands was observed in the species with the greatest number of strains studied (*D. prosaltans*, *D. austrosaltans* and *D. sturtevanti*), but among *D. saltans*, *D. dacunhai*, *D. neocordata* and *D. emarginata*, with a single strain analyzed, the number of bands was, in the sequence of species, 20, 17, 15 and 11 bands. In its turn *D. parasaltans*, with two strains analyzed showed 12 bands.

The number of bands described in the literature for *D. melanogaster* (included in the *melanogaster* group, which like the *saltans* group belongs to the subgenus *Sophophora*) is similar to the number detected for the most polymorphic species in the *saltans* group. Oakeshott et al. (1993) mentioned about 30 distinct esterases in *D. melanogaster* detected by electrophoresis, most of them classified as carboxylesterases and cholinesterases. Also according to data in the literature, *D. melanogaster* genome allows foreseeing from 25 to 30 enzymatically active genes in the carboxyl/cholinesterase family. From them, 15 to 20 carboxylesterase isozymes were detected in eletrophoretic zymograms (Robin et al., 2000; Ranson et al., 2002).

A difference between *D. melanogaster* and the species in the *saltans* subgroup is that while in the last species the carboxylesterases and acethylesterases predominate, in *D. melanogaster* carboxyl and cholinesterases predominate (Healy, Dumancic & Oakeshott, 1991; Oakeshott et al., 1993). Healy, Dumancic & Oakeshott (1991) identified 22 esterases in 4 strains of *D. melanogaster*, which were classified in 10 carboxylesterases, six cholinesterases and three acethylesterases (three were not classified).

One challenging finding in the present study of esterases in the *saltans* group is the high prevalence of α-esterases over β-esterases. What would be the biological meaning of this

observation? This aspect is not emphasized in the literature. However, the same prevalence seems to occur in *D. melanogaster*. In this species, the α-esterase cluster includes 10 carboxyl/cholinesterase genes, located in the 3R chromosome, which diverged strongly from each other as to their expression profile (Campbell et al., 2003), in their sequence of bases (Robin et al., 1996; 2000) and in the aminoacid sequence of their product (from 37 to 66% aminoacid similarity) (Balakirev & Ayala, 2003). According to Campbell et al. (2003), the divergence in expression and in bases sequence was already indicative of a substantial divergence of esterases function in this species. In turn, the β-esterase cluster in *D. melanogaster*, located in the 3E chromosome, includes only two genes duplicated in tandem, which were already analyzed as to the sequence (Richmond et al., 1990; Collet et al., 1990; Oakeshott et al., 1995; Balakirev & Ayala, 2003).

One idea to explain the prevalence of α-esterases could be the differential requirement of α- and β-esterases in specific physiological processes. However, this does not seem to be the case. Campbell et al. (2003) studied two of the 10 active genes from the α-esterase cluster of *D. melanogaster* (EST-9 and EST-23). Their products confirmed previous suggestions of involvement of the cluster in digestion and detoxification of xenobiotics. However, the same authors mentioned that many other genes from the cluster showed tissue-specific expression in the development, apparently inconsistent with those functions.

Another hypothesis is that the genes coding for α-esterases, for some reason (for example, presence of mobile elements) could be more liable to events of duplication and or divergence of duplicated genes, originating a greater number of new functions adopted during evolution. The involvement of mobile elements originating mutations is well known (Voelker et al. 1990; Mack, Smith & Kuhn, 1997; Pomerantseva et al., 2006). However, by now this remains a hypothesis.

Since the presence of esterases in certain tissues and organs suggest their general biological functions, providing an added tool for comparisons (Kambysellys, Johnson & Richardson, 1968), a study of the esterase gene expression in body parts (head, thorax and abdomen) of five species from four subgroups was carried out (*D. prosaltans*, *D. sturtevanti*, *D. parasaltans*, *D. subsaltans* and *D. neocordata*). The β-esterases present in the gels of body parts were those from loci 5, 6 and 7, all of them classified as carboxylesterases. According to the species, these bands were present in gels of head and thorax (*D. prosaltans*), in the thorax and abdomen (*D. parasaltans* and *D. subsaltans*) or in the three parts (*D. sturtevanti* and *D. neocordata*), but in every case the degree of expression was higher in the thorax. The pattern

of expression was the same for the set of β-esterases, in every case. For *Drosophila* alcohol desidrogenase (Adh) it has been shown that species-specific variation of expression in sets of tissues is mediated by interaction of cis-acting sequences (Fischer & Maniatis, 1988; Cy & Brennan, 1993).

As to the α-esterases, the body part expressing the codifying genes varied among the bands, but in most cases, the pattern of expression of a band was the same in different species. The nine α-esterase bands analyzed in the gels showed six different patterns but the expression in the abdomen or in the abdomen and thorax predominated. The cholinesterase EST-33 was the only band which showed high expression in the head and thorax (higher in the first than in the second part). The expression of this band was previously described by Nascimento & Bicudo (2006) in *D. prosaltans*, *D. saltans* and *D. austrosaltans*, suggesting its involvement in the physiology of the nervous system.

On the other hand, bands expressed in the abdomen may be related to digestion or reproduction. The EST-12 band, which is expressed predominantly in the abdomen, and shows higher level of expression in males than in females of every strain except *D. emarginata* is a candidate to be involved in the reproduction, such as the esterase 6 in *D. melanogaster* and its sibling species *D. simulans* and *D. mauritiana* and the esterase S of *D. virilis*. Both esterase genes are mainly expressed in the ejaculatory bulbs of males (Richmond et al, 1980; Karotam, Delves & Oakeshott, 1993; Sergeev et al., 1993; Saad et al., 1994; Odgers et al., 2002).

Qualitative and quantitative differences in gene expression have been considered indicative of difference of function and important sources of adaptive phenotypic variation (Oakeshott et al.1993; Odgers et al. 2002). In addition to specific expression variation between sexes and in parts of the body, detected in the present study, variation of esterase expression during development of species in the *saltans* group were also described, showing that, besides the presence of stage-specific bands, the expression of the bands from loci 1 to 3 predominated in pupae, while, in larvae, bands from loci 4 to 9 were predominantly produced, with some variation among strains and species (Nascimento & Bicudo, 2006). Taken together the observations on the esterases of species from the *saltans* group may be considered a good tool for studies on gene structure divergence in the evolutionary process and corresponding functional changes.

Phylogenetic relationships

The observation of the phylogenetic trees generated for esterase variability using criteria distance (Figure 4) or parsimony (Figure 5) shows that they agree in several results. In both trees, the monophyletic *saltans* subgroup is placed in the more recently derived location, and among species in this subgroup, *D. saltans* and *D. austrosaltans* are closer to each other than to *D. prosaltans*.

Phylogenetical relationships of *D. sturtevanti* subgroup vary among species and strains but also remain the same in both trees: the *D. sturtevanti* strains TAP and MAC appear as sister taxon to *D. prosaltans*; *D. dacunhai* appears in a sister clade with *D. saltans; D. neocordata* as a sister taxon of *D. milleri and* SRP (*D. sturtevanti* strain); and J_8 and JAB (*D. sturtevanti* strains) close to *D. parasaltans* (TAP-1 and $B_{17\text{-}5}$). We consider that the phylogenetic position of the *sturtevanti* subgroup is not resolved by the present data.

Both trees are not congruent to each other when the problem is the species which diverged early in the evolutionary history of the group. In the tree based on the criterion distance, *D. subsaltans* and *D. parasaltans*, species from the *parasaltans* subgroup, are at the base, while in the tree based on the criterion parsimony, EVC (*D. emarginata*, *elliptica* subgroup) is at the base, followed by *D. neocordata* (*cordata* subgroup).

As mentioned before, phylogenetical hypothesis based on different characteristics for species from the *saltans* group are often at most partially concordant. The hypothesis that the *saltans* subgroup is the most recently derived, as shown by the present data, is probably prevalent among the results from different phylogenies. It was presented by Throckmorton & Magalhães (1962), based on the accumulation of fluorescent pigments, and by Rodriguez-Trelles, Tarrío & Ayala (1999) based on the nucleotide sequence of the codifying region of the gene *Xdh.* For O´Grady, Clark & Kidwell (1998), *D. saltans* and *D. parasaltans* subgroups are sister clades that occupy the most recently derived portion of the phylogeny; and Castro & Carareto (2004) on the basis of presence of *P* elements suggested that *neocordata*, *emarginata* and *parasaltans* subgroups are older than the *sturtevanti* and *saltans* subgroups, considered in that study sister subgroups.

The main discordance among phylogenies on the *saltans* group are related to the subgroup which diverged early in its evolutionary history and consequently also in the intermediate positions. Throckmorton & Magalhães (1962) considered that the *cordata* subgroup is at the base of the phylogeny, followed in the position by the *elliptica*, *sturtevanti* and *parasaltans* subgroups. For O´Grady, Clark & Kidwell (1998) the *cordata* and *elliptica* subgroups occupy this position, followed in the intermediate position by the *sturtevanti* subgroup. For Castro & Carareto (2004) the *cordata* and *elliptica* subgroups also occupy this

position, followed in the intermediate position by *parasaltans* subgroup, while for Rodriguez-Trelles, Tarrío & Ayala (1999), *parasaltans* was the first derived subgroup, followed by *sturtevanti* and then by *elliptica* plus *cordata* (unresolved position).

The present study on esterase patterns showed different results in the two generated trees. The distance analysis indicated the *parasaltans* subgroup as derived first, supporting data from Rodriguez-Trelles, Tarrío & Ayala (1999), but differently from that study, in the present, the early derived subgroup was followed by the *elliptica* and then by the *cordata* subgroups. The parcimony analysis showed the *elliptica* subgroup at the base, followed by *cordata* and then by the *parasaltans* subgroups. This finding is more similar to data from Castro & Carareto (2004).

Finally, in relation to the branching inside the *saltans* subgroup, the species relationships shown in this study reinforce previously data based on inversion polymorphism (Bicudo, 1973b) and on esterase patterns (Nascimento & Bicudo, 2002), showing that *D. saltans* and *D. austrosaltans* are closer to each other than to *D. prosaltans*.

Unfortunately, at least in part the evolutionary history of the *saltans* group remains a problem to be resolved. In the present study, *D. sturtevanti* strains showed very variable esterase patterns. As mentioned before, *D. sturtevanti* is the species from the *saltans* group which has the greatest distribution area and is the only that is normally collected in great numbers. In this phylogeny study it showed similarity to different species along the trees, making difficult to define its position in the evolutionary history of the group. However, the similarity of the body pigmentation pattern of the flies in *D. sturtevanti* to that of flies in the *saltans* subgroup suggests that they are closer to each other than to the other subgroups; in this case, both subgroups would be the most recently derived in the group. This suggestion is reinforced by data from Castro & Carareto (2004) based on similarity of *P* elements. Even in the present study, two *D. sturtevanti* strains and the strain *D. dacunhai* show a close relationship with the *saltans* subgroup species, in the two trees.

The difficulties with reconstruction of phylogenetic relationships among recently diverged species were discussed in a short review by Kopp, Frank & Barmina (2006). Among other problems, they mention that, when the time since speciation is short, gene trees reconstructed from individual loci are unlikely to be an accurate reflection of species phylogeny. The loci are expected to have different genealogies and there is no single dichotomous tree that applies to the entire genome. In addition, the reconstruction of species phylogeny may be further complicated by the possibility of post-speciation gene flow before reproductive isolation is achieved.

The accumulation of data from different characteristics may be useful to clarify the evolutionary history of the *saltans* group species and thus, new studies on the subject are welcome.

Table 1. Subgroup, species and origin of the strains used. The numbers of samples used for electrophoresis are also included, separated by sex. Two flies were used in each sample.

Subgroup	Species	Strains	Geographical origin	N° of samples	
				♂	♀
saltans	*D. prosaltans*	P_5	Bucaramanga- Colombia	54	58
		PSH	Engo Schmidt (SP)-Brazil	33	37
		POV	Onda Verde (SP)- Brazil	42	47
	D. saltans	S_3	Guatemala- Guatemala	64	57
	D. austrosaltans	RP	S. J. do Rio Preto (SP)- Brazil	37	37
		BB	Barra Bonita (SP)- Brazil	48	48
		IC	Icém (SP)- Brazil	49	50
sturtevanti	*D. sturtevanti*	MAC	Macapá (AM)- Brazil	47	58
		J_8	Brotas (SP)- Brazil	43	44
		SRP	S. J. do Rio Preto (SP)- Brazil	48	53
		TAP	Tapuruquara (AM)- Brazil	39	44
		JAB	Jaboticabal (SP)- Brazil	42	45
	D. milleri	MI	El Yunque- Porto Rico	48	52
	D. dacunhai	DAC	Petionville- Haiti	60	53
parasaltans	*D. parasaltans*	TAP-1	Tapuruquara (AM)- Brazil	47	65
		$B_{17\text{-}5}$	Belém (PA)- Brazil	52	45
	D. subsaltans	SUB	Belém (PA)- Brazil	58	56
Cordata	*D. neocordata*	NEO	Montes Claros (MG)- Brazil	41	47
Elliptica	*D. emarginata*	EVC	Huatusco (Vera Cruz)- México	45	41
TOTAL				897	937

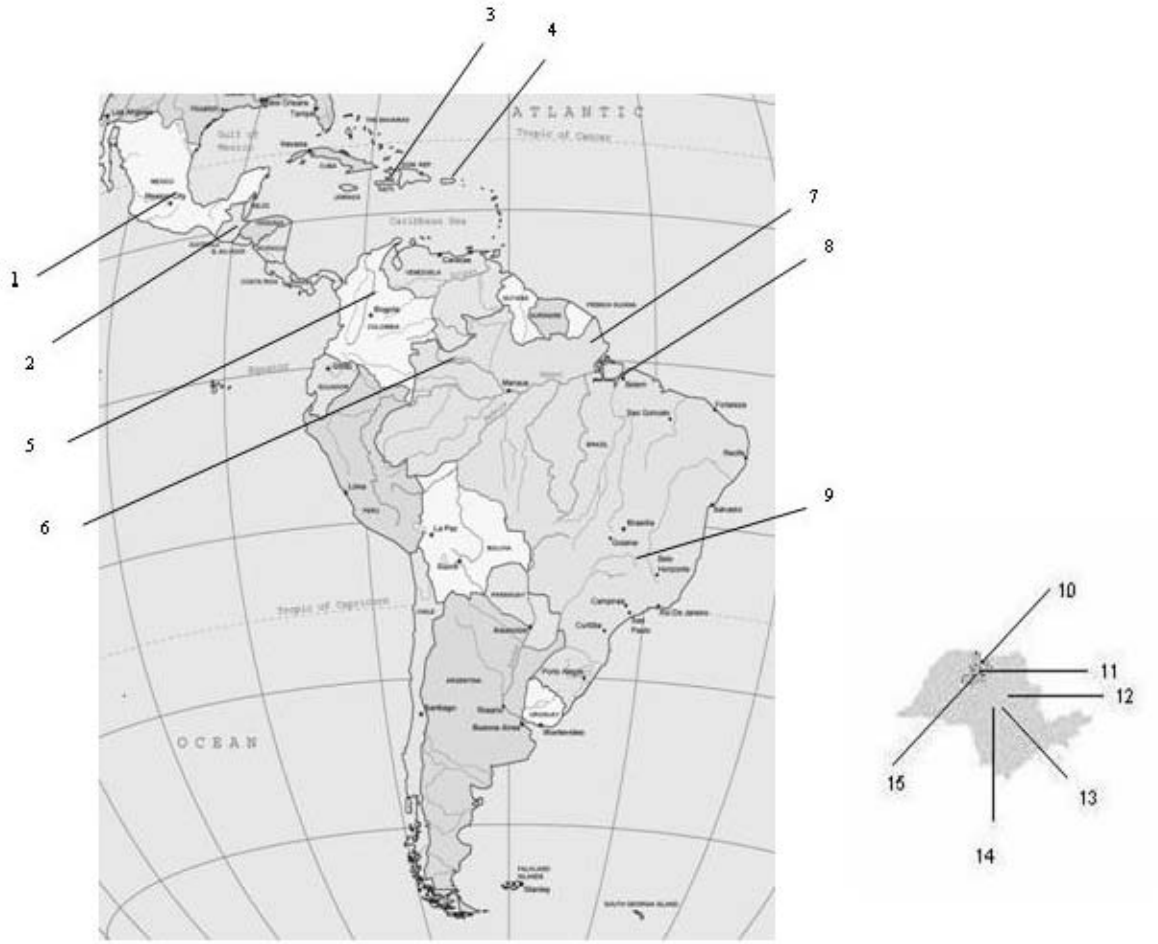

Figure 1. Location of the strains from the *saltans* group used in the present study. 1= Huatusco (Mexico), 2= Guatemala (Guatemala), 3= Petionvile (Haiti), 4= El Yunque (Porto Rico), 5= Bucaramanga (Colombia), 6= Tapuruquara (AM), 7= Macapá (AP), 8= Belém (PA), 9= Montes Claros (MG), 10= Icém (SP), 11= Onda Verde (SP), 12= Jaboticabal (SP), 13= Brotas (SP), 14= Barra Bonita (SP) and 15= São José do Rio Preto and Engenheiro Schmidt (SP).

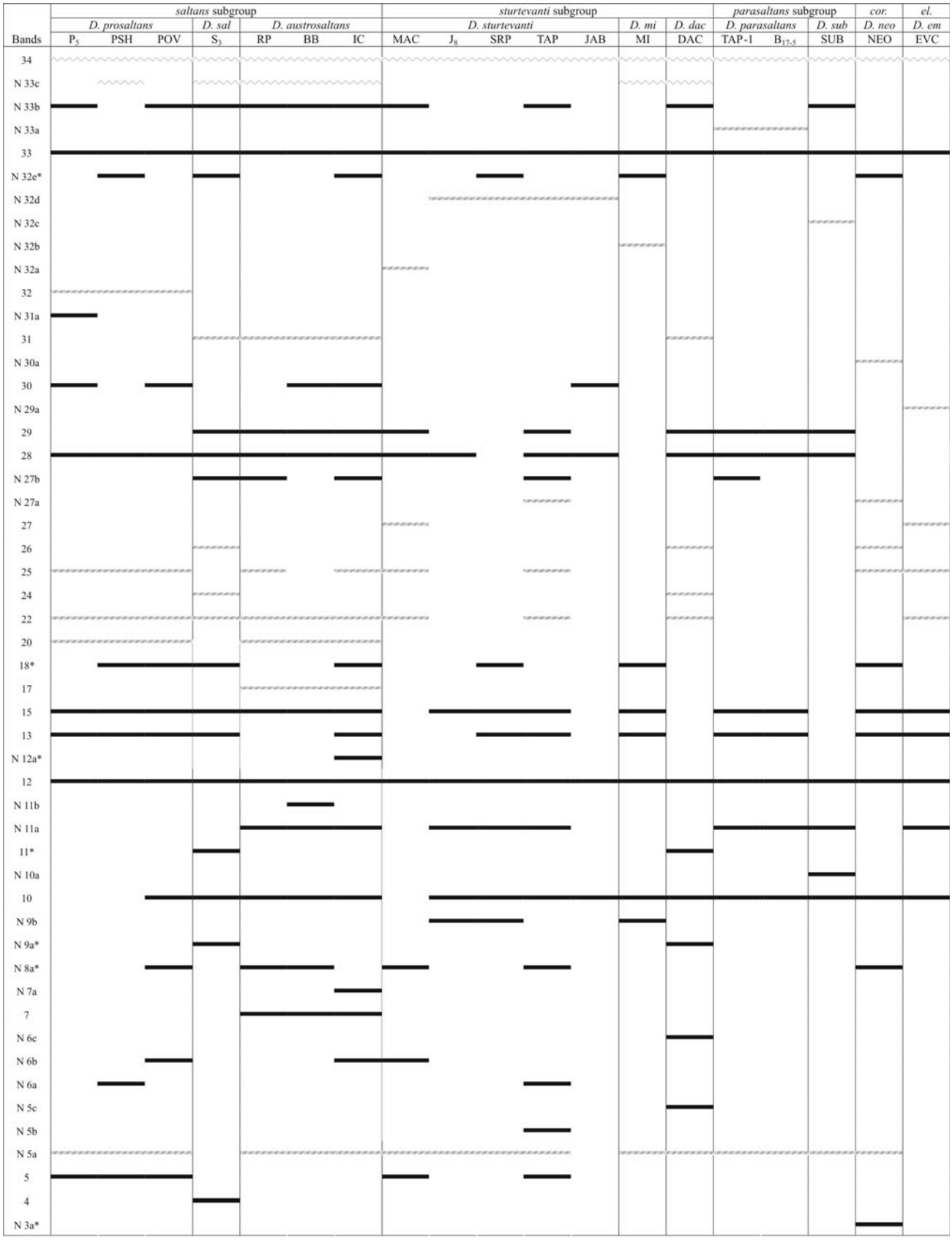

Figure 2. The bands are represented in the sequence of their location in the gels, numbered from less anodic ends. Bands substrate specificity: ▬ = α-esterase; ▨ = β-esterase; ∿ = α/β-esterase. * = male- especific esterase. *D. sal*= *D. saltans*; *D. mi*= *D. milleri*; *D. dac*= *D. dacunhai*; *D. sub*= *D. subsaltans*; *D. neo*= *D. neocordata*; *D. em*= *D. emarginata*; *cor.*= *cordata* subgroup; *el.* = *elliptica* subgroup.

Table 2. Number of α-esterases (α), β-esterases (β) and α/β-esterases (α/β) detected in the species and strains used.

			Bands			
subgroup	Species	Strains	α	β	α/β	TS
saltans	*D. prosaltans*	P_5	9	5	1	15
		PSH	9	5	2	16
		POV	12	5	1	18
						TSp 22
	D. saltans	S_3	14	4	2	20
						TSp 20
	D. austrosaltans	RP	11	6	2	19
		BB	12	5	2	19
		IC	17	6	2	25
						TSp 27
						TSg 36
sturtevanti	*D. sturtevanti*	MAC	8	5	1	14
		J_8	7	2	1	10
		SRP	9	2	1	12
		TAP	14	5	1	20
		JAB	5	1	1	7
						TSp 27
	D. milleri	MI	8	2	2	12
						TSp 12
	D. dacunhai	DAC	10	5	2	17
						TSp 17
						TSg 36
parasaltans	*D. parasaltans*	TAP-1	9	2	1	12
		$B_{17\text{-}5}$	8	2	1	11
						TSp 12
	D. subsaltans	SUB	8	2	1	11
						TSp 11
						TSg 15
cordata	*D. neocordata*	NEO	9	5	1	15
						TSp 15
						TSg 15
elliptica	*D. emarginata*	EVC	6	4	1	11
						TSp 11
						TSg 11

TS= total of esterases in the strains, TSp= total in the species; and TSg= total in the subgroup.

Table 3. The ten loci tentatively established, the esterase bands that were included in each of them, their biochemical classification and degree of inhibition by Malathion and eserine sulphate.

Loci	Bands	BC	Inhibition pattern	
			Malathion	Eserine
1	5a; 1 (?); 2 (?)	β, Ace	-	-
2	10; 9b; 9a; 9; 8a; 8; 7a; 7; 6c; 6b; 6a; 6; 5c, 5b; 5; 4; 3a (?); 3	α, CE	+ +	-
3	18; 15; 14; 13; 12a; 12; 11b; 11a; 11; 10a	α, Ace	-	-
4	21; 20; 19; 17; 16	β, CE	+ + +	-
5	27a; 27; 26; 25; 24; 23; 22	β, CE	+ + +	-
6	30; 29; 28; 27b	α, Ace	-	-
7	33a; 32d; 32c; 32b; 32a; 32; 31; 30a; 29a	β, CE	+ + +	-
7/8	33b; 32e; 31a (?)	α, CE	+ +	-
8	33	α, ChE	+ +	+ + +
9	34; 33c	α/β, ChE	+	+

+, + + and + + + = increasing degrees of inhibition; - = absence of inhibition; BC = Biochemical Classification: α= alpha-esterase, β= beta-esterase, α/β= alpha/beta-esterase; CE= carboxylesterase, Ace=acetylesterase, ChE= cholinesterase; ? = band not found in the treated gels; the underlined bands were previously described by Nascimento and Bicudo (2002), but they were not dectected in the present study.

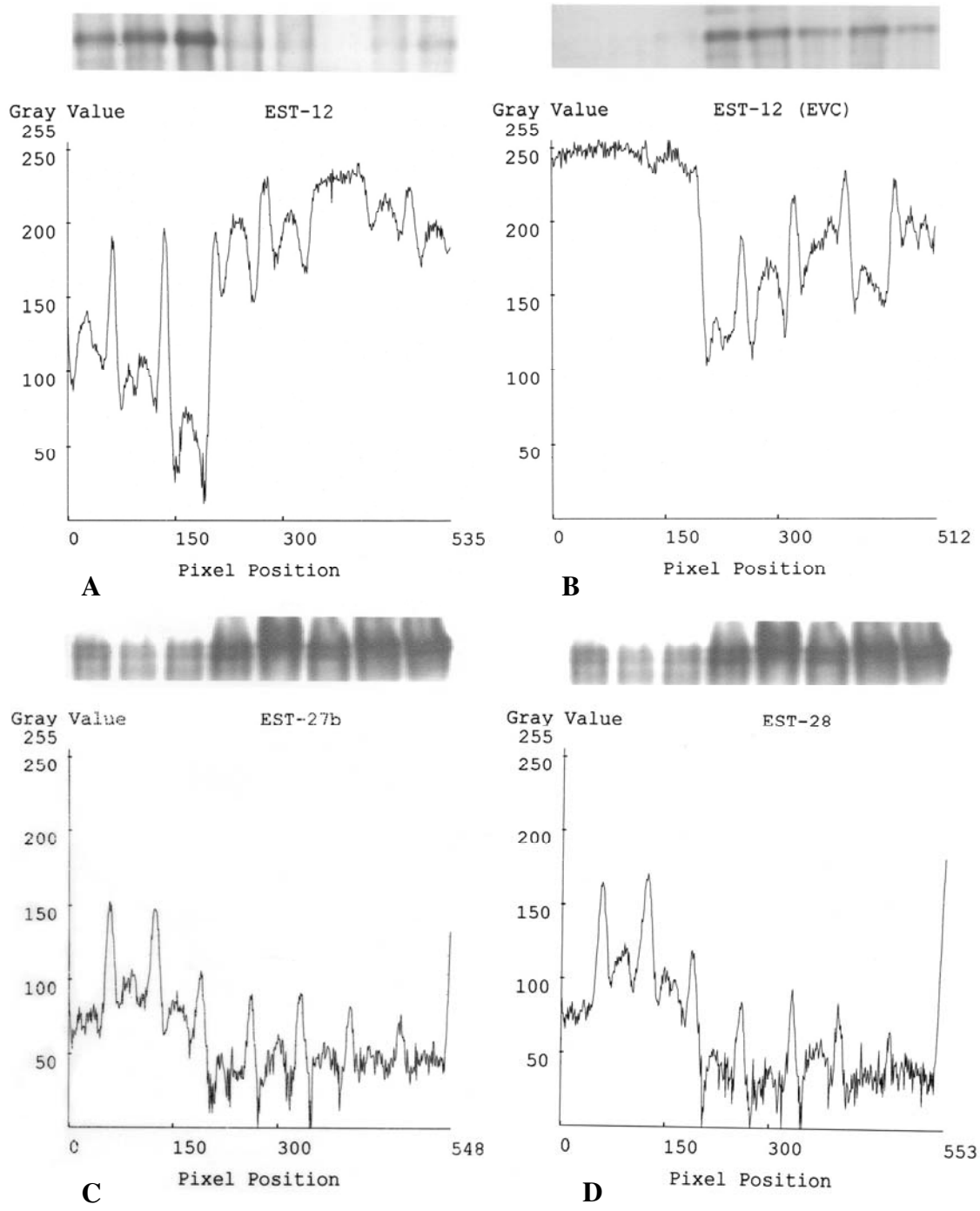

Figure 3. A-D. Profile graphs for comparisons of the activity degree of some esterase bands in males and females. In every case, three samples of males (left) and five samples of females (right) were used. (A) EST-12 band from *D. prosaltans* (strain P_5); (B) EST-12 band from *D. emarginata* (strain EVC); (C) EST-27b and (D) EST-28 bands from *D. austrosaltans* (strain IC). The strips of gel containing the band, located above the graphs were used for obtaining each graph along a horizontal line segment.

Table 4. Expression degree of the β- esterases from loci 5, 6 and 7 in the body parts (head, thorax and abdomen) of five species from the *saltans* group. - = no expression, +, + + and + + + = increasing degrees of esterase expression.

species	Body parts		
	Head	Thorax	Abdomen
D. prosaltans	+ +	+ + +	-
D. sturtevanti	+	+ + +	+ +
D. parasaltans	-	+ + +	+
D. subsaltans	-	+ + +	+
D. neocordata	+	+ + +	+

Table 5. Expression degree of the α-esterase bands in the body parts of species *D. prosaltans* (strain P_5), *D. sturtevanti* (strain JAB), *D. parasaltans* (TAP-1), *D. subsaltans* (SUB) and *D. neocordata* (NEO).

	D. prosaltans			*D. sturtevanti*			*D. parasaltans*			*D. subsaltans*			*D. neocordata*		
	H	Th	A	H	Th	A	H	Th	A	H	Th	A	H	Th	A
EST-5	+++	-	+++												
EST-12	-	+	+++	-	+	+++	-	+	+++						
EST-13	-	-	+++				-	-	+++						
EST-27b							+	+++	+++						
EST-28							+	+++	+++	+	++	++			
EST-29							+	+++	+++	+	++	++			
EST-30				-	+++	+									
EST-31a	-	+++	+++												
EST-33	+++	++	+	+++	++	+	+++	++	+	+++	++	+	+++	++	+

H=head; Th= thorax; A= abdomen

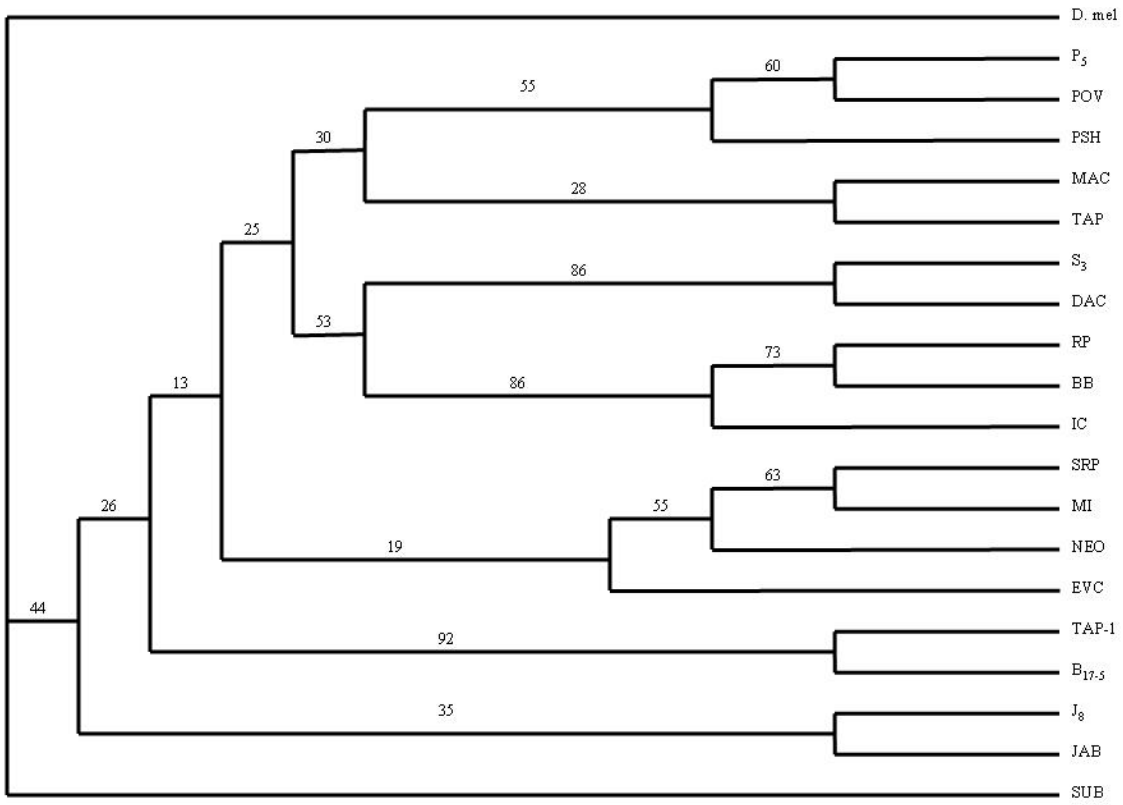

Figure 4. Bootstrap 50% majority-rule consensus tree, criterion distance, based on esterase band pattern of species in the *saltans* group. D. mel= *D. melanogaster* (outgroup); P_5, POV, PSH= *D. prosaltans*; S_3= *D. saltans*; RP, BB, IC= *D. austrosaltans*; MAC, TAP, J_8, JAB, SRP = *D. sturtevanti*; DAC= *D. dacunhai*; MI= *D. milleri*; TAP-1, $B_{17\text{-}5}$ = *D. parasaltans*; SUB= *D. subsaltans* ; NEO= *D. neocordata*; EVC= *D. emarginata*.

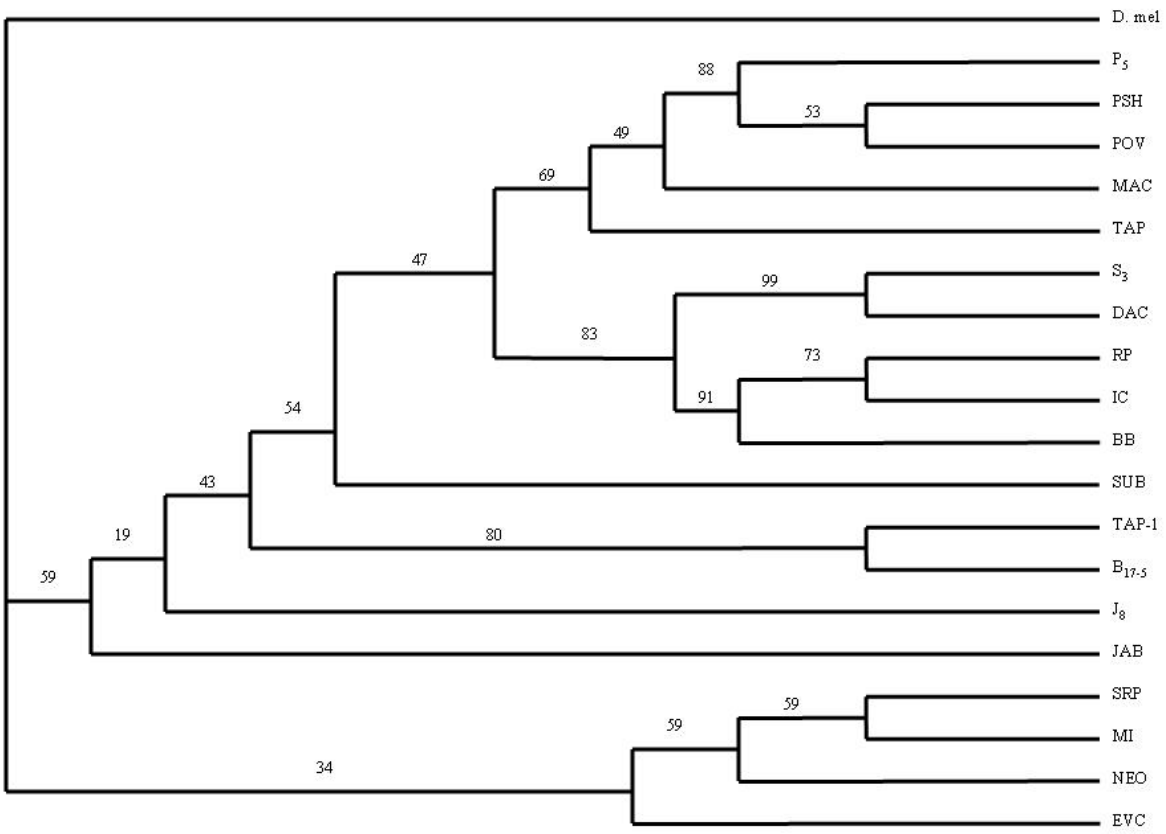

Figure 5. Bootstrap 50% majority-rule consensus tree, criterion parsimony, based on esterase band pattern of species in the *saltans* group. D. mel= *D. melanogaster* (outgroup); P_5, POV, PSH= *D. prosaltans*; S_3= *D. saltans*; RP, BB, IC= *D. austrosaltans*; MAC, TAP, J_8, JAB, SRP = *D. sturtevanti*; DAC= *D. dacunhai*; MI= *D. milleri*; TAP-1, $B_{17\text{-}5}$ = *D. parasaltans*; SUB= *D. subsaltans* ; NEO= *D. neocordata*; EVC= *D. emarginata*.

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IV. ARTIGO 2

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Esterase patterns used as markers for identification of *Drosophila* species from the *saltans* group

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Short Title: Identification of *Drosophila* species by esterases

Resumo

Neste trabalho, são descritos padrões de esterases detectados em espécies de *Drosophila*, do grupo *saltans*, úteis como marcadores para a sua identificação. Estas observações foram derivadas de um estudo anterior envolvendo 19 linhagens de 10 espécies dos cinco subgrupos do grupo *saltans* utilizando eletroforese em géis de poliacrilamida. Os marcadores estão localizados em uma região próxima a mais anódica do gel, a qual é rica em β-esterases, carboxilesterases, na maioria destas espécies. Esta região inclui um tipo de corrida β e algumas bandas α e β-esterásicas bem definidas. A presença de bandas específicas, tão bem como sua localização, o comprimento e a forma da corrida β são variáveis, mas característicos de diferentes espécies ou subgrupos. Quatro padrões foram basicamente reconhecidos. Cada um deles exibiu 100% de expressão nas moscas do taxon que elas caracterizam. Isto permite considerá-los bons marcadores. São também apresentados alguns comentários quanto à evolução do grupo *saltans* baseados na variabilidade do padrão da corrida β.

Abstract

In this paper, esterase patterns detected in *Drosophila* species from the *saltans* group, which are useful as markers for species identification are described. These observations were derived of previous study involving 19 strains of 10 species from the five subgroups of the *saltans* group, using electrophoresis in poliacrylamide gels. The markers are located in a region close to the more anodic part of the gel, which is rich in β-esterases, carboxylesterases in most of those species. This region includes a kind of β-spreading and some well defined α- and β-esterase bands. The presence of specific bands as well as the location, the length and the shape of the β-spreading are variable but characteristic of different species or subgroups. Four patterns were basically recognized. Each of them exhibited 100% expression in the flies from the taxon which they characterize. This allows considering them good markers. Some comments on evolution of the *saltans* group based on the variability of the β-spreading pattern are also presented.

Introduction

For about a hundred years, *Drosophila* species have been used in studies aiming to gain understanding of genetic processes. During this time *Drosophila* has been an important tool in the knowledge of a large range of different problems, from those involved in the answer to general questions about the laws governing heredity to those concerning molecular basis of gene function and its control. Its use has also been extremely useful in the disclosure of aspects concerning the evolutionary process. *Drosophila melanogaster* predominated in the mentioned studies, but several other *Drosophila* species have also been used as a source of information.

The identification of *Drosophila* species, which is essential for their use, has been usually made on the basis of morphological characteristics included in classification keys and illustrations. However, sometimes, these tools are not enough to recognize a species with certainty, mainly in the case of sibling species. Because of this, additional markers may be useful. Alternative markers for species identification have been developed in several organisms. Most of them are specific DNA sequences obtained by RAPD (Random Amplified Polymorphic DNA), such as STS (sequence tagged site), CAPS (cleaved amplified polymorphic sequence) and SCARs (sequence-characterized amplified region) (Li et al., 2002; Randig, Carneiro & Sereno, 2004; Barik, Senapati & Aparajita, 2006) or ITS (internal transcribed spacer) sequences from RNA (Vargas et al., 2005) or microsatelite markers –SSR (short sequence repeat) (Bianchi, Sansavini & Fachinello, 2004). Species-marker bands in native and SDS-PAGE gel electrophoresis of soluble proteins were described in fish by Hasnain et al. (2005).

Drosophila species from the *saltans* group have been the subject of many studies in genetics and evolution focusing aspects such as chromosome polymorphism, reproductive isolation, esterase patterns, transposable elements and phylogenetic relationships (Throckmorton & Magalhães, 1962; Bicudo, 1973a, 1973b; Clark et al., 1995; O'Grady, Clark & Kidwell, 1998; Rodriguez-Trelles, Tarrío & Ayala, 1999a, b; Nascimento & Bicudo, 2002, 2006; O'Grady & Kidwell, 2002; Castro & Carareto, 2004). Basic information on identification of *Drosophila* species included in this group were presented by Magalhães & Bjornberg (1957) and Magalhães (1962), including morphological descriptions of the external characteristics of the flies, descriptions of the male genitalia and a classification key.

Magalhães (1962) divided the *saltans* group into five subgroups, including 19 species distributed among them as follows: seven species in the *saltans* subgroup (*D. saltans*, *D. lusaltans*, *D. prosaltans*, *D. nigrosaltans*, *D. septentriosaltans*, *D. austrosaltans* and *D. pseudosaltans)*; three species in the *sturtevanti* subgroup (*D. sturtevanti*, *D. milleri* and *D. rectangularis*,); three species in the *parasaltans* subgroup (*D. parasaltans*, *D. subsaltans* and *D. pulchella*); four species in the *elliptica* subgroup (*D. elliptica*, *D. emarginata*, *D. neoelliptica* and *D. neosaltans*); and two species in the *cordata* subgroup (*D. cordata* and *D. neocordata*). Two more species were later added to the *sturtevanti* subgroup: *D. dacunhai* and *D. magalhaesi* (Mourão & Bicudo, 1967).

Despite the morphological descriptions of the flies present in the literature, sometimes the identification of *Drosophila* species from the *saltans* group is not easy, frequently requiring practice in the recognition of the genitalia pieces. Reevaluating the esterase patterns of species in the group described by Bernardo, Langeani & Bicudo (chapter 1 in this thesis), we detected features which are specific of a taxon. These features, which are being described in the present paper, may be useful in the case of doubt involving identification. The fact that they are present in 100% flies of the taxon reinforces their applicability.

Material and Methods

The esterase patterns of 19 strains from 10 species included in the five subgroups of the *saltans* group, previously described by Bernardo, Langeani & Bicudo (this thesis) were the source of the data used in this study. In that study, flies were maintained in laboratory in culture medium of corn meal-agar, at the temperature of 20°C ± 1,0°C. (Table 1). The electrophoresis procedure involved the use of two 10 days old flies of the same sex for preparing each sample. The flies were homogenized at 0°C, in 25 µl of the buffer solution (1.5 M Tris HCl, pH 8.8 plus 10% glycerol). Ten µl of the homogenate were used in each sample.

Esterase patterns were analyzed in polyacrylamide gels, using a separating 12% gel and a 4% stacking gel (Laemmli, 1970). After the sample application, the gels were subjected to electrophoresis for 4 h at ~25°C, using a running buffer (0.1 M Tris -glycine at pH 8.3) and a constant 200V.

The esterase bands were identified in gels by their color produced using α- and β-naphtil acetates as substrates. The process followed basically the technique described by Johnson et al (1966) and Steiner & Johnson (1973), involving preincubation (45 min) at room

temperature (~25°C) in 50 ml 0.1 M sodium phosphate, pH 6.2, followed by a staining reaction in the dark (1h) with a solution containing 20 mg of α-and 15 mg de β- naphtil acetate dissolved in 1 mL of acetone, used as substrates, 60 mg of Fast Blue RR and 5 mL of N-propanol in 50 mL of sodium phosphate solution. After this treatment, when the α- naphtil acetate is preferentially hydrolyzed, the bands in the gels become black and are named α-esterases. When the β- naphtil acetate is preferentially hydrolyzed, the bands in the gels become red and are named β-esterases.

The gels were distained (24-48 h) in a solution containing 250 mL of ethyl alcohol, 100 mL of acetic acid and 650 mL of distilled water. The gels were air dried at room temperature, following the technique of Ceron, Santos & Bicudo (1992). The biochemical characterization of the esterases involved the use of the inhibitors organophosphate Malathion (0.7 mM) and eserine sulfate (0,1 mM) (Holmes & Masters, 1967). In these tests the gels were preincubated for 1 h in 50 mL of 0.1 M aqueous sodium phosphate solution containing one of the inhibitors and then treated with the staining solution also containing the inhibitor. Using these tests, the esterases mentioned in this paper could be classified as carboxylesterases (those inhibited only_by Malathion) and acethylesterases (those which are not affected by those inhibitors) (Oakeshott et al., 1993).

Results and Discussion

As shown in Figure 1, the esterase markers for identification of *Drosophila* species from the *saltans* group are observed in a β-esterase carboxylesterase region located close to the more anodic part of the gel. The main marker is a kind of β-spreading (thus, stained red), which is variable in length and shape in different species or subgroups. In some cases this β-spreading includes well defined bands, which are also helpful in the recognition.

Figure 1A shows gels of strains from *D. prosaltans* (P_5, POV and PSH), *D. saltans* (S_3) and *D. autrosaltans* (RP and BB). The three species, which are included in the *saltans* subgroup, are considered sibling species. They show long β-esterase spreading, ending, in the upper limit, in a well defined β-esterase band, which is named EST-32 in *D. prosaltans* and EST-31 in *D. austrosaltans* and *D. saltans*. In this Figure, the esterase pattern of *D. austrosaltans* is shown in gels subjected or not to treatment with Malathion. In the treated gels, the β-spreading disappears (this is the reason why it was classified as carboxylesterase), but the α-esterase, acethylesterase bands that are in the region remain untouched (they are the

esterase bands EST-27b, EST-28 and EST 29). Among the three analyzed species from the *saltans* subgroup, *D. austrosaltans* is the easier to recognize using esterase patterns, due to the presence of these α-esterase bands at about the middle of the β-esterase spreading. *Drosophila prosaltans* and *D. saltans* may also be differentiated from each other because the *D. saltans* β-spreading upper limit starts slightly farther from the EST-33 band, and the lower limit ends after the lower limit of *D. prosaltans*. However, both species must be available for comparison. *Drosophila prosaltans* also has the EST-28 band but in low frequency, while in *D. austrosaltans* the α-esterase bands exhibit high frequency.

Gels of strains from species included in the *sturtevanti* subgroup are shown in Figure 1B. The β-spreadings in the three analyzed species from this subgroup (*D. milleri*, *D. dacunhai* and *D. sturtevanti*) are similar to those of species from the *saltans* subgroup: they are long and their upper limit is a well-defined β-esterase band. The level of the upper limit of the spreading varies among the species and also among strains of *D. sturtevanti* as shown in this Figure. The spreading in *D. dacunhai* (DAC) is the one which starts farthest from EST-33 and extends longer in the less anodic direction of the gel than in the other species from the same subgroup. In addition, the esterase band located in the upper limit of the spreading is the less evident. *Drosophila milleri* (MI) has a strongly stained band in the upper limit of the spreading, but the spreading is weakly stained when compared to the other species. Two strains of *D. sturtevanti* (MAC and J_8) are in Figure 1B, showing different morphological patterns for the β-spreading: in J_8, it starts closer to the EST-33 band and ends before in comparison with MAC. Because *D. sturtevanti* shows the greatest distribution area among species from the *saltans* group, covering almost the entire distribution area of the group, from Mexico to the South of Brazil, this variation may reflect the different degrees of differentiation characterizing its strains.

The β-spreading of species from the other three subgroups from the *saltans* group (*elliptica, cordata* and *parasaltans*) are shown in Figure 1C. This Figure includes gels with samples of a strain of *D. emarginata* (*elliptica* subgroup; strain EVC), a strain of *D. neocordata* (*cordata* subgroup-strain NEO), a strain of *D. subsaltans* (SUB) and two strains of *D. parasaltans* (TAP-1 and $B_{17\text{-}5}$) (both species from the *parasaltans* subgroup). The most differentiated species as to the β-esterase spreading region are included in these subgroups. *Drosophila emarginata, D. neocordata* and *D. subsaltans* have in common a concave upper limit in the β-spreading. However, the spreading differs clearly among the three species by the length and by the position in the gel. As shown in Figure 1C, the β-spreading location is less anodic for EVC and NEO, while in SUB it is more anodic (that is, it is closer to the EST-33

band). The mean length of the spreading was measured in the gels, starting in the center of the concave limit and extending to the lower limit, in 86 samples from EVC, 88 from NEO and 114 from SUB. The results showed the mean values 0.84 cm, 1.31 cm and 1.0 cm for the three species, respectively.

Drosophila parasaltans also from the *parasaltans* subgroup has the most differentiated pattern. In the two analyzed strains of this species, the β-spreading is absent, but a new β-esterase carboxylesterase band, expressed in high degree, with 100% frequency and exclusive, is present. It is located in a more anodic position, after EST-33 and is named EST-33a.

In short, among the three analyzed species from the *saltans* subgroup, which are sibling and very difficult to differentiate morphologically even analyzing the male genitalia, the pattern of the β-spreading region allows differentiating *D. austrosaltans* from both *D. prosaltans* and *D. saltans*, but it is less favorable for differentiation between the two last species. In the sibling species of the *sturtevanti* subgroup, the β-spreading region may be useful to recognize *D. dacunhai* and *D. milleri*, but may be confusing in the recognition of *D. sturtevanti* due to the pattern variation of its strains. However, *D. sturtevanti* and *D. milleri* are easily differentiated morphologically by the presence of an opaque, more or less round area in each side of the sixth tergite in *D. sturtevanti* females. *Drosophila milleri* females also show the opaque area in the same position, but it is more rectangular than in *D. sturtevanti*. This morphological feature of *D. sturtevanti* and *D. milleri* was described by Magalhães (1962). *Drosophila dacunhai* also has a rectangular opaque area (Mourão & Bicudo, 1967), which is more difficult to differentiate from that in the other species. Species from the *saltans* and *sturtevanti* subgroups are easily differentiated morphologically from each other due to the presence of a characteristic pattern of longitudinal stripes in the mesonotum, the presence of the opaque are in the sixt tergite of females and the frequently greater size of the flies in the *sturtevanti* subgroup. For species from the *elliptica*, *cordata* and *parasaltans* subgroups, the β-spreading region is a very helpful marker, allowing differentiating them from each other and from the species in the other subgroups. It is also very interesting that *D. parasaltans* and *D. subsaltans*, both included in the *parasaltans* subgroup by Magalhães (1962) show very different patterns in that region.

Considering the degree of morphological similarity of the patterns of β-spreading region detected in this study, four basic patterns can be recognized; one involving species from the *saltans* and *sturtevanti* subgroups (long β-spreading region, with a well defined band at the upper limit), other involving the species from the *elliptica* and *cordata* subgroups (short

spreading region with concave upper limit, located far from the EST-33 band, in less anodic position); a third pattern shown by *D. subsaltans*, included in the *parasaltans* subgroup (short β-spreading region, located in a more anodic position, close to the EST-33 esterase band) and a fourth pattern shown by *D. parasaltans*, also from the *parasaltans* subgroup (characterized by the absence of the β-spreading and presence of a single specific band classified as β-esterase carboxylesterase too) (figure 2).

Bernardo, Langeani & Bicudo (chapter 1 in this thesis) carried out a study on the expression of the esterases in body parts of the flies from the *saltans* group: head, thorax and abdomen. Because the expression pattern of the β-esterases in analysis in this paper was basically the same in every species (highest expression in the thorax), we may suppose that, in spite of the molecular changes which altered the migration pattern in the gel, those β-esterases preserved its function in the process of species divergence. Might those changes reflect the evolutionary divergence of the species? If yes, what subgroup is at the base of the evolutionary history of the group, the one with the band which migrates less in the gel (the different migration degrees are due to the protein molecular weight and electric charge), the *parasaltans* subgroup, or those with the longest β-spreading, *saltans* and *sturtevanti* subgroups. The pattern difference detected between *D. subsaltans* and *D. parasaltans*, both presently included in the *parasaltans* subgroup could indicate their separation in two different subgroups?

Anyway a tentative hypothesis on the evolutionary history of the *saltans* group using this migration pattern could suggest the sequence but not the direction of subgroups divergence. A more detailed biochemical and molecular study will be certainly helpful.

The *saltans* subgroup has already been considered the most derivative in the *saltans* group in some phylogeny studies based on other characteristics (Throckmorton & Magalhães, 1962; Rodriguez-Trelles, Tarrío & Ayala, 1999b), with the *sturtevanti* subgroup, as alternative possibilities (Castro & Carareto, 2004) or with the *parasaltans* subgroup (O´Grady, Clark & Kidwell, 1998). The location of the *parasaltans* subgroup (*D. parasaltans)* at the base of the evolutionary process of the group was indicated by Rodriguez-Trelles, Tarrío & Ayala (1999b). The results shown in the two phylogenetic trees generated in the study of Bernardo, Langeani & Bicudo (first paper of this thesis), using the complete pattern of esterases in the *saltans* group, agree with those which indicated the *saltans* subgroup as the most recent, and the tree originated by neighbor-joining and criterion distance agree with the results obtained by Rodriguez-Trelles, Tarrío & Ayala (1999b), suggesting the *parasaltans* subgroup as the first derived. However, the high degree of incongruence among

different phylogenetic studies of species in the *saltans* group shows that this is a question which had still not been resolved.

Table 1. Subgroup, species and origin of the strains used.

Subgroup	Species	Strains	Geographical origin
saltans	*D. prosaltans*	P_5	Bucaramanga- Colombia
		PSH	Engo Schmidt (SP)-Brazil
		POV	Onda Verde (SP)- Brazil
	D. saltans	S_3	Guatemala- Guatemala
	D. austrosaltans	RP	S. J. do Rio Preto (SP)- Brazil
		BB	Barra Bonita (SP)- Brazil
		IC	Icém (SP)- Brazil
sturtevanti	*D. sturtevanti*	MAC	Macapá (AM)- Brazil
		J_8	Brotas (SP)- Brazil
		SRP	S. J. do Rio Preto (SP)- Brazil
		TAP	Tapuruquara (AM)- Brazil
		JAB	Jaboticabal (SP)- Brazil
	D. milleri	MI	El Yunque- Porto Rico
	D. dacunhai	DAC	Petionville- Haiti
parasaltans	*D. parasaltans*	TAP-1	Tapuruquara (AM)- Brazil
		$B_{17\text{-}5}$	Belém (PA)- Brazil
	D. subsaltans	SUB	Belém (PA)- Brazil
cordata	*D. neocordata*	NEO	Montes Claros (MG)- Brazil
elliptica	*D. emarginata*	EVC	Huatusco (Vera Cruz)- México

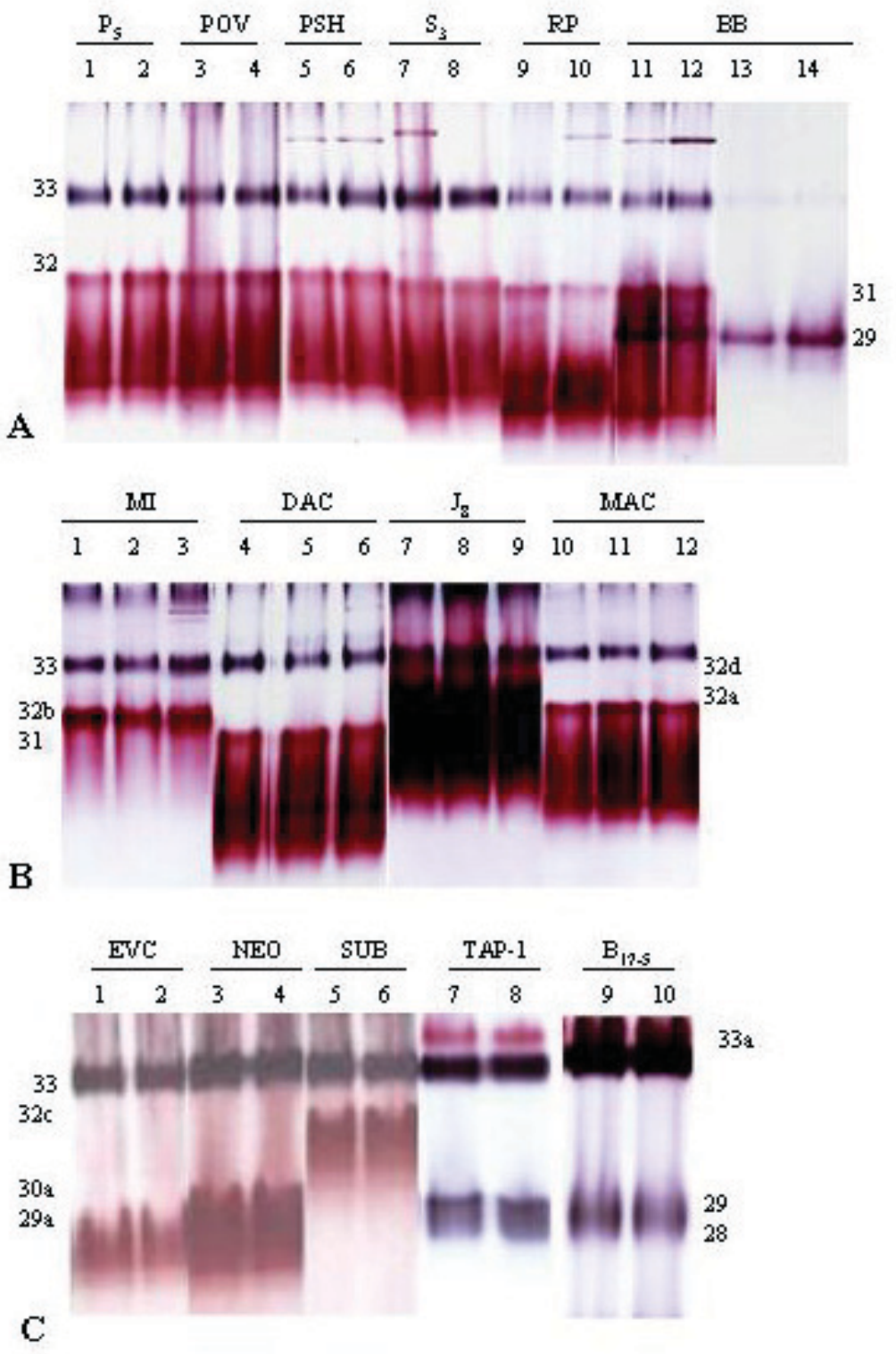

Figure 1. A-C. Morphology of the β- esterases and α- esterases mentioned in this study, as observed in the polyacrylamide gels. A. *saltans* subgroup: 1-6 = *D. prosaltans,* 7,8= *D. saltans*; 9-14= *D. austrosaltans* (13-14= gel treated with Malathion). B. *sturtevanti* subgroup: 1-3= *D. milleri*; 4-6= *D. dacunhai*; 7-12= *D. sturtevanti*. C. *elliptica*, *cordata* and *parasaltans* subgroups: 1,2= *D. emarginata*; 3,4= *D. neocordata*; 5,6= *D. subsaltans*; 7-10= *D. parasaltans*.

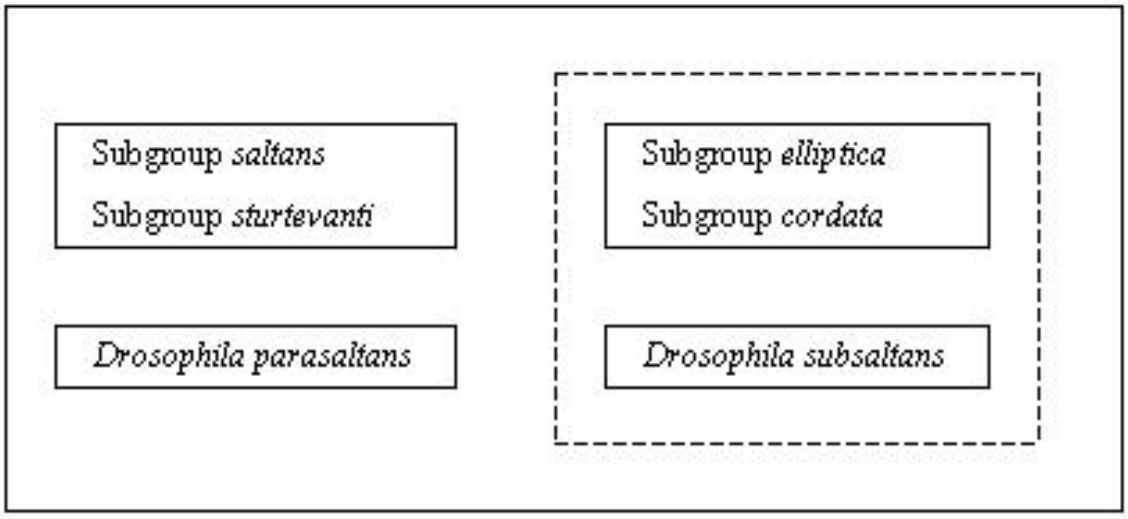

Figure 2. Diagram showing the proximity of the subgroups of the *saltans* group, based on the morphology of β- esterases useful as markers. The dasked line suggests a greather similarity between *elliptica* + *cordata* subgroups with *subsaltans* subgroups.

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V. CONCLUSÕES

V. Conclusões

Os resultados obtidos no presente trabalho, o qual utilizou dez espécies do grupo *saltans* de *Drosophila*, destacam que a análise dos padrões de esterases pela técnica de eletroforese em gel de poliacrilamida é uma importante ferramenta para investigar diferenças entre espécies crípticas, auxiliando tanto em estudos filogenéticos como de reconhecimento das referidas espécies. A partir dessas premissas, este estudo possibilitou concluir que:

1. Cinqüenta e uma bandas foram detectadas em machos e fêmeas adultos, sendo que vinte e oito são novas. Estas foram classificadas conforme a afinidade ao substrato em 31 α-esterases, 18 β-esterases e duas α/β- esterases.

2. Com base nos padrões de inibição promovidos pelo Malathion e sulfato de eserina, 34 bandas foram classificadas como carboxilesterases, 14 como acetilesterases e três como colinesterases.

3. Conforme a posição da banda no gel, especificidade ao substrato e padrões de inibição, este estudo estabeleceu tentativamente que as bandas esterásicas estariam distribuídas em 10 loci, ampliando estudos anteriores que sugeriram nove loci.

4. Todos os subgrupos mostraram bandas exclusivas, sendo nove no subgrupo *saltans*, sete no subgrupo *sturtevanti*, três no subgrupo *parasaltans*, duas no subgrupo *cordata* e uma em *elliptica*.

5. Sete bandas α-esterásicas, quatro carboxilesterases e três acetilesterases, foram encontradas exclusivamente em machos, podendo ser consideradas macho- específicas.

6. Variação quanto à espessura e intensidade de coloração das bandas, indicativa de atividade diferencial das enzimas a que elas correspondem, foi observada entre os sexos.

7. Estudos anteriores descreveram a banda EST-34 com sendo a única banda na região mais anódica do gel. Neste trabalho, foi detectada uma segunda banda, também α/β- esterase, colinesterase, muito próxima da primeira, nomeada EST-33c.

8. O padrão de esterases observados nas partes do corpo mostrou que o grau de expressão das β- esterases foi alto no tórax, enquanto as α- esterases expressaram-se predominantemente no abdome e tórax.

9. Foram propostas duas hipóteses filogenéticas com base na presença e ausência de bandas esterásicas entre as linhagens dos referidos subgrupos do grupo *saltans*, permitindo comparações com filogenias anteriores baseadas em caracteres morfológicos, bioquímicos e moleculares.

10. Com base nas variações morfológicas da região β- esterásica presente na região mais anódica do gel, foi possível definir marcadores para identificação de espécies do grupo estudado e também sugerir proximidades filogenéticas entre elas e entre os subgrupos.

VI. REFERÊNCIAS

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