Thais de França Patarro

Produtividade em espécies *de Drosophila* do subgrupo *saltans* (grupo *saltans*, subgênero Sophophora): efeitos da infecção por *Wolbachia* em linhagens normais e introgredidas

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Orientadora: Profª Drª Hermione Elly Melara de Campos Bicudo.

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DISSERTAÇÃO APRESENTADA PARA OBTENÇÃO DO TÍTULO DE DOUTOR

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"Tente uma, duas, três vezes e se possível tente a quarta, a quinta e quantas vezes for necessário. Só não desista nas primeiras tentativas, a persistência é amiga da conquista. Se você quer chegar aonde a maioria não chega, faça o que a maioria não faz."

Bill Gates

RESUMO

Mecanismos de isolamento reprodutivo são agentes que impedem ou diminuem a troca de genes entre duas espécies ou populações de uma mesma espécie que se encontram em processo de especiação. Esses mecanismos são compreendidos por uma serie de processos que atuam em diferentes níveis da reprodução, incluindo desde barreiras pré-zigóticas até barreiras pós-zigóticas. Nas ultimas décadas, muitos estudos têm indicado que a ausência de híbridos pode também ser promovida por interações entre microorganismos simbiontes e seus hospedeiros. Um dos principais simbiontes capazes de interagir com os insetos, levando a modificações do processo reprodutivo, são as alfaproteobactérias do gênero *Wolbachia*. No presente estudo, foram analisados os efeitos da infecção pela *Wolbachia* causados no parâmetro produtividade (número de descendentes), em intra e intercruzamentos de linhagens de *Drosophila. saltans*, *D. prosaltans* (espécies próximas pertencentes ao subgrupo *saltans*) e mais duas linhagens, obtidas por introgressão a partir de híbridos F1 do intercruzamento dessas duas espécies. Análises preliminares para detecção da infecção pela *Wolbachia* nas linhagens de *Drosophila* mostraram que cada uma das seis linhagens estava infectada com uma linhagem do simbionte. Os resultados quanto à produtividade foram obtidos de intra e intercruzamentos das linhagens nas condições infectadas e não infectadas A eliminação do simbionte foi realizada por tratamento com o antibiótico tetraciclina. O principal mecanismo resultante da interação simbionte-hospedeiro mencionado na literatura é chamado incompatibilidade citoplasmática (IC) e ocorre nos intercruzamentos de fêmeas não infectadas com machos infectados. Considera-se que nos machos infectados ocorrem alterações nos espermatozoides que somente os ovócitos de fêmeas infectadas podem reverter, reestabelecendo a produtividade. Os resultados obtidos, neste trabalho, sobre a infecção pela *Wolbachia* nas linhagens do grupo *saltans* de *Drosophila* foram variáveis. Em vários cruzamentos, as combinações que deveriam ser estéreis ou quase estéreis, se ocorresse o efeito IC, foram as mais produtivas ou uma das mais produtivas. Em outros casos, os intercruzamentos de ambos os pais infectados, esperados serem altamente férteis, em caso de IC, eram estéreis ou apresentavam baixa fertilidade. Considerando todos os cruzamentos analisados, os resultados foram indicativos de ausência do efeito IC. O principal resultado obtido foi alta produtividade das combinações heterogêneas de fêmeas infectadas com machos não infectados, mostrando que essa combinação é responsável pela manutenção e disseminação da infecção nas linhagens analisadas. De acordo com os resultados predominantes, no presente estudo a

eliminação da bactéria nos machos produziu efeitos benéficos, anulando a esterilidade dos cruzamentos. Os efeitos dos intra e intercruzamentos de *D. prosaltans* e *D. saltans* foram discutidos em relação à compatibilidade reprodutiva das linhagens e os efeitos da infecção pela *Wolbachia*. Também procuramos entender como as linhagens introgredidas diferenciaram-se uma da outra e das espécies parentais, levando em conta a constituição cromossômica para a qual evoluíram.

Palavras-chave: incompatibilidade citoplasmática, interação hospedeiro-simbionte, isolamento incipiente, introgressão, *fitness reprodutivo*

ABSTRACT

Reproductive isolation mechanisms are agents that prevent or decrease the exchange of genes between species or populations of the same species that are in process of speciation. These mechanisms are comprised of a series of processes operating at different levels of reproduction, ranging from pre-zygotic to post-zygotic barriers. In recent decades, studies have indicated that the absence of progeny can also be promoted by interactions of symbiont microorganisms and their hosts. Presently, the most known endosymbionts capable of interact with the insects, interfering in the reproductive process, are the alphaproteobacteria of the genus *Wolbachia*. In the present study, we investigated the *Wolbachia* effects on reproduction, focusing the parameter productivity (number of progeny) in crosses involving four strains of *Drosophila saltans* and *D. prosaltans* (close species belonging to the *saltans* group, Sophophora subgenus) and two introgressed strains started with F1 hybrids of these two species. Preliminary tests for screening *Wolbachia* showed that each of the six strains was infected with one strain of the symbiont. The results on productivity were obtained from intra and intercrosses of the strains in the conditions infected or uninfected. The elimination of *Wolbachia* was performed by treatment of the strains with the antibiotics tetracycline. The main mechanism resulting from the interaction symbiont-host described in the literature is called cytoplasmatic incompatibility (CI) and occurs in the intercrosses of uninfected females with infected males. It is considered that, in infected males, there are changes in the sperm that only the oocytes of infected females are able to correct, reestablishing the productivity. The present results on *Wolbachia* infection of the species from the *saltans* group were variable. In several crosses of the strains, the combinations that are sterile or almost sterile when CI effect occurs, were the most productive or one of the more productive combinations. In other cases, in which the CI-indicative combination is sterile or almost sterile, the intercrosses of both infected parents, expected to be highly fertile, were sterile or very low fertile. Considering all the crosses analyzed, the results are rather indicative of inexistence of CI effect in the strains used. The main result was high productivity of the heterogeneous combinations of infected females with uninfected males, indicating that this combination is responsible for the maintaining and spreading of the *Wolbachia* infection in the used strains. According with the prevalent results, the elimination of the bacteria in male parents produced beneficial effects, annulling the cross sterility. The effects of the intra and intercrosses of *D. prosaltans* and *D. saltans* were discussed in relation to the crossability of the strains and the

Wolbachia infection effects. We also tried to understand how the introgressed strains differentiated from each other and from their parental species, taking into account their chromosome constitution.

Keywords: cytoplasmic incompatibility, host-symbiont interaction, incipient isolation, introgression, reproductive fitness

SUMÁRIO

INTRODUÇÃO

Dobzhansky (1937) propôs a designação "mecanismos de isolamento" para todos os agentes que diminuem ou impedem a troca de genes entre populações. Dois tipos básicos de mecanismos de isolamento foram diferenciados: o geográfico ou espacial, e o reprodutivo (DOBZHANSKY, 1951).

Tendo em vista que os mecanismos de isolamento reprodutivo, atualmente também denominados barreiras reprodutivas, têm papel chave no processo de especiação, e que a especiação é o evento que promove o aumento da diversidade biológica, estudos que têm por objetivo a compreensão desses mecanismos são de extrema importância no que se refere ao entendimento da evolução dos organismos. Entretanto, mesmo tanto tempo depois da proposição de Dobzahnsky (1937), ainda não são suficientemente conhecidos os processos envolvidos na geração do isolamento reprodutivo, seus componentes e seus mecanismos genéticos.

Os mecanismos de isolamento reprodutivo incluem uma variedade de processos que podem atuar em cada estágio da reprodução, desde a corte, passando pelas interações entre espermatozóide e óvulo, até as características dos híbridos. Por isso, são divididos de acordo com seu nível de atuação em mecanismos de isolamento pré-zigóticos (que atuam antes do cruzamento) ou mecanismos de isolamento pós-zigóticos (que atuam após a formação do zigoto). A seleção sexual (aceitação do macho pela fêmea) é um mecanismo pré-zigótico enquanto a inviabilidade e a esterilidade dos híbridos são mecanismos pós-zigóticos (Mayr, 1963).

Especialmente nas últimas décadas, tem havido um interesse crescente na abordagem dos processos de isolamento reprodutivo (Matute e Coyne, 2009; Sweigart, 2010; Jennings et al., 2011; Nanda e Singh, 2011; Kim et al., 2012; Takahashi et al., 2012). Boa parte dessa abordagem tem sido realizada com diferentes espécies de *Drosophila*. Híbridos de espécies desse gênero consideradas completas ou nascentes podem ser obtidos em laboratório com maior ou menor dificuldade, dependendo das espécies e de sua origem geográfica. Isto facilita grandemente o estudo desses processos.

Segundo Dobzhansky (1937) e Muller (1942), o isolamento reprodutivo surge como subproduto das modificações genéticas que ocorrem e são fixadas nas populações. A presença desses genes pode então levar à produção de interações prejudiciais, nos híbridos dessas populações. Isto faz pensar que seus processos estão na dependência da ação de um número grande de genes. No modelo Dobzhansky-Muller é ainda esperado que a incompatibilidade dos híbridos seja poligênica e devida a genes com funções variadas (Tao et al. 2003). Considera-se,

porém, que pares de espécies antigas, isto é, que divergiram há um longo tempo podem levar à superestimativa do número de genes requerido para originar problemas nos híbridos (Orr e Irving, 2001).

Isolamento reprodutivo em *Drosophila*

Os primeiros estudos no que se refere à compreensão do isolamento reprodutivo foram realizados em espécies de *Drosophila* e publicados em meados da década de 40 por Dobzhansky e Patterson (Dobzhansky, 1944; Patterson e Dobzhansky, 1945). Esses estudos baseavam-se, em grande parte, na descoberta de espécies morfologicamente idênticas e geneticamente próximas, mas que demonstravam um forte isolamento reprodutivo na forma de híbridos inviáveis ou estéreis e discriminação sexual (Mallet, 2006). Posteriormente, foram realizados estudos envolvendo microorganismos, plantas e vertebrados (Ramsey et al., 2003; Levitan et al., 2004; Hofreiter et al., 2004; Liti et al., 2006; Amato et al., 2007). Contudo, os estudos envolvendo *Drosophila* lideram no que diz respeito à compreensão dos mecanismos ecológicos, comportamentais e genéticos do isolamento reprodutivo (Noor et al., 2001; Sawamura e Tomaru, 2002; Mallet, 2006; Laturney e Moehring, 2012; Nanda e Singh, 2012). Inclusive, o gênero em questão é responsável por propiciar a caracterização da grande maioria dos genes considerados responsáveis pelos processos de isolamento reprodutivo e especiação (Orr, 2005; Presgraves, 2010; Nosil e Schluter, 2011).

Os progressos acerca do entendimento desses processos são devidos, entre outros fatores, ao fato da *Drosophila* ser um organismo modelo para as ciências biológicas, utilizado há mais de cem anos, e por essa razão já ter gerado uma boa compreensão de seus aspectos biológicos.

Grupo *Saltans*: dados gerais sobre a constituição, distribuição geográfica e filogenia

O grupo *saltans* é um dos dez grupos que compõem o subgênero *Sophophora*, que por sua vez pertence ao gênero *Drosophila*. Atualmente, este subgênero é dividido em dois grandes clados: o do velho mundo, que inclui os grupos *melanogaster*, *montium*, *ananassae*, *fima* e *obscura*; e o neotropical, que contem os grupos *willistoni* e *saltans*, além do gênero *Lordiphosa* (considerado parafilético) e do grupo *ducani* composto apenas pela espécie *Hirtodrosophila duncani* (Throckmorton, 1975; Da Lage et al., 2007; Van der Linde et al., 2010). O décimo grupo chamado *populi*, inclui apenas duas espécies encontradas na região ártica, e ainda tem posição

indefinida dentro desses dois clados (Wheeler e Throckmorton, 1960; Vilela e Bächili, 2009). Juntos, esses dez grupos possuem aproximadamente 300 espécies conhecidas (Wheeler, 1986; Lemeunier et al., 1986)

O grupo *saltans* é subdividido em cinco subgrupos: (1) *saltans*, composto pelas espécies *D. saltans*, *D. lusaltans*, *D. prosaltans*, *D. nigrosaltans*, *D. septentriosaltans*, *D. austrosaltans e D. pseudodaltans*; (2) *parasaltans*; formado por *D. parasaltans*, *D. subsaltans* e *D. pulchela*; (3) *sturtevanti*, composto por *D. sturtevanti*, *D. milleri* e *D. rectangularis*, *D. magalhaesi* e *D. dacunhai*; (4) *elliptica*, que compreende as espécies *D. elliptica*, *D. emarginata*, *D. neoelliptica* e *D. neosaltans*; e (5) o subgrupo *cordata* composto pelas espécies *D. cordata* e *D. neocordata* (Magalhães, 1962; Mourão e Bicudo, 1967).

A filogenia do grupo tem sido discutida com base no estudo de diversos marcadores, incluindo marcadores morfológicos (Throckmorton e Magalhães, 1962; Throckmorton, 1975; Yassin, 2009; Souza et al., 2014), polimorfismo cromossômico (Bicudo, 1973a), isolamento reprodutivo (Bicudo, 1973a; Bicudo, 1973b), padrões de esterases (Nascimento & Bicudo, 2002; Nascimento e Bicudo 2006; Bernardo e Bicudo, 2009), análises moleculares (O'Grady et al., 1998; Rodriguez-Trelles et al., 1999) e elementos transponíveis (Clark et al., 1995; Clark e Kidwell, 1997; Silva e Kidwell, 2000; Castro e Carareto, 2004a; Castro e Carareto, 2004b).

Um dos estudos pioneiros, que reúne aspectos sobre a ecologia, filogenia e distribuição geográfica das espécies do gênero *Drosophila* foi realizado por Throckmorton (1975). Neste trabalho, o autor considerou que as espécies pertencentes ao subgênero *Sophophora* surgiram como parte de um amplo processo de radiação, incluindo também os gêneros *Chymomyza* e *Neotanygastrella* e que a radiação desse subgênero foi basal à radiação que deu origem ao gênero *Drosophila*. Estudos moleculares recentes apóiam a noção de que o subgênero *Sophophora* é basal dentro de *Drosophilinae* e relativamente distante do subgênero *Drosophila* (Remsen and DeSalle, 1998; Kwiatowski and Ayala, 1999; Remsen and O'Grady, 2002).

Com base nos padrões contemporâneos de distribuição e dados geológicos, Throckmorton (1975) propôs que o ancestral do subgrupo *saltans* foi originado na América do Norte tropical, onde os considerados subgrupos "primitivos" *cordata* e *elliptica* (Magalhães, 1962) são encontrados. Esse grupo ancestral teria colonizado o continente Sul-americano dando origem aos subgrupos *sturtevanti*, *saltans* e *parasaltans* (subgrupos considerados "derivados"), tendo se dispersado posteriormente para o norte através da região atualmente correspondente ao Istmo do Panamá. Há aproximadamente 4,5 milhões de anos, alguns membros do subgrupo *saltans*, como *D. saltans* e *D. prosaltans* teriam se dispersado de volta à América Central e à América do Norte.

Considera-se que os subgrupos *saltans* e *willistoni* compreendem um clado neotropical fortemente relacionado entre si, porém distinto dos outros táxons do subgênero *Sophophora* (Throckmorton, 1975). Todavia, dentro do subgrupo *saltans*, as relações entre espécies ainda são pouco compreendidas dado que estudos filogenéticos baseados em diferentes características mostram relações predominantemente incongruentes. É o que acontece na comparação de resultados obtidos em estudos sobre o isolamento reprodutivo, inversões cromossômicas e análise de DNA nuclear e mitocondrial (Bicudo, 1973a, 1973b; O'Grady et al., 1998). Essa incongruência tem sido atribuída à divergência recente das espécies do subgrupo (O'Grady et al., 1998).

Com relação à distribuição geográfica do grupo *saltans*, este se encontra predominantemente disperso ao longo da região Neotropical (Magalhães, 1962). Patterson e Stone (1952) mencionam que o subgrupo *elliptica* é encontrado exclusivamente na região Neoártica. Há ainda espécies como *D. lusaltans*, *D.milleri* e *D. pulchela*, incluídas respectivamente nos subgrupos saltans, sturtevanti e parasaltans que são encontradas apenas nas ilhas do Caribe. Todavia, outras espécies do grupo *saltans* podem ser encontradas tanto em território continental, quanto em insular, sendo *D. sturtevanti*, do subgrupo de mesmo nome, a espécie mais cosmopolita, ocupando quase que integralmente a amplitude de distribuição do grupo (Sturtevant, 1916; Duda, 1927; Sturtevant et al., 1942; Pavan, 1950; Magalhães, 1956; Spassky, 1957; Magalhães, 1962)

É interessante notar que não há relação entre os subgrupos e sua distribuição geográfica, uma vez que algumas espécies filogeneticamente próximas se encontram separadas por centenas de quilômetros de distancia, mostrando que possivelmente, essas espécies estiveram próximas em um período passado (Magalhães, 1962).

Em se tratando mais especificamente das espécies utilizadas no presente estudo (*D. saltans e D. prosaltans*), a distribuição geográfica de *D. saltans* compreende desde as regiões leste e sudoeste do México, passando pela Guatemala, Cuba, El Salvador e Costa Rica. *D. prosaltans*, por sua vez, tem ocorrência inicialmente na Costa Rica, seguindo ao sul pelos países Panamá, Trinidad e Colômbia. No Brasil, apresenta uma ampla distribuição desde o norte do país até o Rio Grande do Sul, chegando até o Paraguai (Magalhães, 1962; Bicudo, 1973a, 1973b; Drª. Beatriz Goñi, informação pessoal) (Figuras 1 e 2).

Figura 1. Distribuição geográfica de *Drosophila prosaltans* (Costa Rica ao Rio Grande do Sul, Paraguai e Uruguai).

Figura 2. Distribuição geográfica de *Drosophila saltans* (México a Costa Rica).

A seguir são apresentados alguns dados sobre o isolamento reprodutivo em espécies pertencentes aos subgrupos do grupo *saltans*.

Isolamento reprodutivo no grupo *saltans*

Subgrupo *saltans*

O isolamento reprodutivo entre espécies de diferentes subgrupos do grupo *saltans* é completo, isto é as espécies de diferentes subgrupos não se cruzam (dados não publicados de Hermione E. M. C. Bicudo, informação pessoal). Já o grau de isolamento entre espécies do mesmo subgrupo varia com o subgrupo e as espécies consideradas.

Bicudo (1973a) estudou o isolamento reprodutivo no subgrupo *saltans* utilizando sete espécies distribuídas desde o Haiti até o sudeste do Brasil. O isolamento reprodutivo observado nos cruzamentos entre as espécies ocorreu predominantemente em nível de inseminação e geralmente de forma não suficientemente eficaz para prevenir a produção de descendentes híbridos. O trabalho mostra que linhagens de espécies geograficamente isoladas como *D. saltans* e *D. prosaltans,* quando colocadas em contato, produzem, por vezes, descendentes híbridos férteis. Em contrapartida, linhagens dessas espécies, que apresentam distribuição sobreposta em áreas da Costa Rica possuem isolamento reprodutivo completo.

Ainda foi observado que alguns mecanismos de isolamento presentes em cruzamentos entre espécies já atuam em cruzamentos de indivíduos da mesma linhagem (intracruzamentos), demonstrando indícios do surgimento de variantes genéticas que estão levando à divergência dessas linhagens. Contudo, essas variantes ainda aparecem em baixa frequência intralinhagem, possivelmente por sofrerem o efeito da seleção natural. Por fim, Bicudo (1973a) propôs que a divergência entre as espécies de *D. saltans e D. prosaltans* ocorreu alopatricamente, tendo essas espécies, em algum momento, retornado à simpatria. A seleção natural, nesse momento, passou a favorecer os indivíduos incapazes de se reproduzir interespecíficamente, levando ao isolamento completo das espécies nessas áreas.

A compatibilidade biológica entre *D. prosaltans e D. saltans* permitiu a realização de um estudo de introgressão entre elas, em laboratório. Tadei e Bicudo (1981) analisaram a evolução cromossômica dessas duas espécies em dois tipos de populações experimentais, cada um com quatro réplicas, iniciados com moscas das duas espécies, em igual número e sexo (populações PS1 a PS4), ou iniciadas com híbridos F1, obtidos na direção de cruzamento fêmeas *D.*

prosaltans e machos *D. saltans* (populações H1 a H4). As quatro populações foram mantidas por transferência seriada, de acordo com o método de Buzzatii-Traverso (1955), com variações de acordo com Mourão e Ayala (1971).

O acompanhamento da evolução cromossômica foi realizado por cruzamentos de amostras das populações com a espécie parental *D. saltans*, sendo que a constituição cromossômica foi verificada pela análise, nas glândulas salivares, dos três pares de cromossomos que caracterizam essas espécies e que nessas glândulas são cromossomos gigantes. A presença de inversões interespecíficas e despareamentos, nos cromossomos dos híbridos, permitiu reconhecer a origem específica dos cromossomos ou de partes deles (Bicudo 1973b; Tadei e Bicudo, 1981). Foram realizadas quatro análises cromossômicas no período aproximado de três anos, as quais mostraram eliminação completa dos cromossomos de *D. prosaltans* nas populações PS, enquanto nas populações H, o comportamento diferiu duas a duas: as populações H1 e H4 eliminaram os cromossomos de *D. prosaltans* e as populações H2 e H3 mantiveram os cromossomos de *D. prosaltans* (X, II e III) e segmentos do genoma de *D. saltans*, incluindo os dois braços do cromossomo X , o braço IIL e vários segmentos do cromossomo III recombinados com segmentos do cromossomo III de *D. prosaltans*.

Vinte e um anos depois, Freschi (2002) confirmou a manutenção da constituição cromossômica das populações H2, H3 e H4, utilizando dados do polimorfismo de fragmentos de DNA gerado pela técnica de AFLP (Amplified Fragments Lengh Polymorphism). Os resultados mostraram maior proximidade entre H2 e H3, e destas com a espécie parental *D. prosaltans*. Por outro lado, H4 assemelhou-se à espécie parental *D. saltans*. Ao longo do tempo as populações foram mantidas por repiques semanais e depois por repiques mensais, sendo que as H2 e H4 ainda permanecem nos estoques do laboratório.

Bicudo (1978) avaliou a capacidade de intercruzamentos de linhagens de *D. prosaltans* provenientes de países da América Central e do Sul. Os resultados mostraram que as linhagens formam, em relação à sua distribuição geográfica e isolamento reprodutivo, três conjuntos distintos: o primeiro formado pelas linhagens da América Central, provenientes da Costa Rica e do Panamá; o segundo, formado por linhagens da Venezuela, Trinidad, Colômbia e Guiana, portanto, do norte da América do Sul; e o terceiro por linhagens do Brasil. A autora considerou estes três grupos semi-espécies de *D. prosaltans*, de forma semelhante ao que ocorre com *D. paulistorum* (Burla et al., 1949). Cruzamentos entre linhagens de diferentes semi-espécies mostraram algum grau de isolamento, sendo que os intercruzamentos que envolviam linhagens brasileiras com as dos outros conjuntos apresentaram isolamento mais acentuado, com algumas

combinações produzindo híbridos em uma única direção sendo os machos híbridos estéreis ou quase completamente estéreis e outras combinações produzindo híbridos nas duas direções, porém, numericamente escassos e completamente estéreis em uma das direções. A ausência de fêmeas inseminadas nos cruzamentos estéreis indicou que o isolamento sexual é também um mecanismo de isolamento atuando junto com a esterilidade dos híbridos. Cruzamentos entre linhagens da América Central e do Brasil mostraram maior discriminação sexual por parte das fêmeas brasileiras, sugerindo que as linhagens centro-americanas são derivadas das linhagens do sul e assim reforçando a hipótese de Throckmorton (1975). De acordo com Kaneshiro (1976) fêmeas de linhagens derivadas têm uma menor capacidade de discriminação sexual do que as fêmeas ancestrais, uma vez que as linhagens derivadas identificam elementos de corte que estão presentes tanto nas linhagens ancestrais como nas derivadas. Já as fêmeas ancestrais desconhecem os elementos recém-surgidos na linhagem derivada, o que lhes confere uma capacidade de discriminação dos machos mais acentuada.

Subgrupo *sturtevanti*

Cruzamentos por casal e em massa foram utilizados por Bicudo (1979) para compreender melhor os mecanismos de isolamento reprodutivo em três espécies do subgrupo sturtevanti: *D. magalhãesi*, *D. sturtevanti* e *D. milleri*. Os resultados mostraram graus variáveis de isolamento entre as combinações. *Drosophila magalhãesi* foi a espécie que apresentou maior isolamento em relação às outras espécies (*D. sturtevanti* e *D. milleri*). O trabalho mostrou que o isolamento entre as espécies ocorre predominantemente em nível de inseminação, sendo provavelmente devido ao comportamento de corte, já que os machos das três espécies apresentam genitálias morfologicamente muito semelhantes. Nos intercruzamentos que produziram alguns descendentes, os machos híbridos eram estéreis em uma direção dos cruzamentos, ou na única direção em que foram produzidos descendentes.

Hosaki-Kobayashi e Bicudo (1994) intercruzaram linhagens de laboratório e linhagens recém coletadas de *D. sturtevanti*. Os resultados mostraram uma alta produtividade em todos os tipos de cruzamentos, indicando que o isolamento geográfico entre as linhagens não resultou em uma diferenciação genética suficiente para causar isolamento reprodutivo entre elas, e que, apesar das condições laboratoriais propiciarem endogamia e deriva genética, houve preservação da variabilidade genética favorável a uma alta produtividade nas linhagens mantidas em laboratório.

A existência de isolamento reprodutivo entre linhagens de *D. sturtevanti* provenientes do Brasil, Colômbia, Costa Rica e México foi analisada por Carareto (1994). As freqüências e as velocidades dos acasalamentos, assim como o grau de isolamento no que se refere à ocorrência de inseminação, fertilização e viabilidade zigótica precoce evidenciaram dificuldades assimétricas para o acasalamento, sugerindo o surgimento recente de isolamento comportamental entre essas linhagens.

Subgrupo *parasaltans*

No subgrupo *parasaltans*, o isolamento reprodutivo foi estudado nas duas espécies disponíveis, *D. parasaltans* (três linhagens) e *D. subsaltans* (uma linhagem) (Bicudo e Prioli, 1978). Os intercruzamentos das duas espécies mostraram isolamento completo, tanto em cruzamentos por casal como em cruzamentos em massa. Não houve produção de descendentes e nenhuma fêmea analisada estava inseminada. A maioria dos intercruzamentos das linhagens de *D. parasaltans* produziu descendentes em número inferior ao dos intracruzamentos, sendo que uma porcentagem das fêmeas dos casais de intercruzamentos que não produziram descendentes estava inseminada indicando a ocorrência de problemas pós-cópula.

Subgrupo *elliptica*

Bicudo e Prioli (1978) estudaram linhagens de *D. emarginata* provenientes do México e da Costa Rica. No que se refere à produtividade, os intercruzamentos foram tão bem sucedidos quanto os intracruzamentos. Os dados sugeriram isolamento incipiente apenas entre fêmeas do México e machos de Costa Rica, dado que, nessa direção, seus cruzamentos apresentaram menor fecundidade e fertilidade do que nos recíprocos.

Genes e mecanismos de isolamento

Muitos avanços já foram feitos em relação à compreensão de aspectos genéticos relacionados ao isolamento pré-zigótico. Entre eles estão a descoberta de interações entre regiões cromossômicas de machos híbridos de *D. persimilis* e *D. pseudoobscura* que levam a disfunções no processo de corte (Noor, 1997), a identificação de QTL's relacionados ao isolamento sexual *em D. yakuba* e *D. santomea* (Moehring et al., 2006), e a influencia do cariótipo na seleção

sexual (Nanda e Singh, 2011). Ainda nas espécies *D. santomea* e *D. yakuba*, Matute e Coyne (2009) descreveram uma nova barreira para a fecundação, a depleção do esperma mais rápida nos cruzamentos interespecificos do que nos intraespecificos.

Variações da expressão gênica afetando o isolamento pré-zigótico também já foram demonstradas. Bailey et al. (2011) mostraram que a superexpressão de genes relacionados à especiação nos tecidos nervoso e ovariano de fêmeas de *D. melanogaster* do Zimbábue potencializa sua preferência por machos que também apresentam superexpressão gênica nesses tecidos.

O isolamento comportamental é uma barreira reprodutiva que impede o acasalamento entre espécies devido a diferenças de comportamento de corte ou atração sexual (Bruker e Boderstein, 2012). É possível observar, entre os táxons, diversos mecanismos que levam ao isolamento comportamental. Alguns exemplos são a preferência por sons específicos emitidos durante a corte em sapos, grilos, e papa-moscas-monarcas (Ryan e Rand, 1993; Shaw, 2000; Uy et al, 2009); comportamentos de acasalamento em aranhas-lobo (Stratton e Uetz, 1986), padronização de cores em ciclídeos (Seehausen, 1997), forma do corpo em tentilhões (Ratciliffe e Grant 1983a, b), e feromônios em mariposas (Roelofs et al., 2002). Em *Drosophila* o som emitido pelo batimento das asas dos machos durante a côrte desempenha papel fundamental na discriminação sexual entre espécies (Hoikkala et al., 2000; Doi et al., 2001; Williams et al., 2001; Yamada et al., 2002). Da mesma forma, os feromônios estimulam os nervos olfativos e desencadeiam respostas comportamentais discriminatórias em machos e fêmeas (Marcillac et. al., 2005; Grillet et al., 2006; Kurtovic et al., 2007)

Além dos mecanismos citados, o isolamento comportamental pode ser promovido por microorganismos simbiontes. Os insetos são amplamente conhecidos por abrigarem em seus tecidos uma gama variável de espécies simbiontes (Bandi et al., 1994; Douglas et al., 1998; Dillon e Dillon, 2004; Moran et al., 2005; Pais et al., 2008; Teixeira et al., 2008). Evidencias teóricas e experimentais sugerem que interações a longo prazo entre simbionte e hospedeiro afetam o comportamento sexual de corte, podendo promover a especiação do hospedeiro (Miller et al., 2010).

Um exemplo de isolamento comportamental induzido por simbionte foi demonstrado por Sharon et al. (2010). Linhagens de *D. melanogaster*, geneticamente idênticas, que foram criadas em meios de cultura distintos (melaço e amido), adquiriram microbiotas diferentes, que por sua vez levaram a uma forte discriminação sexual entre elas.

Wolbachia e o isolamento reprodutivo

Um dos principais microorganismos capazes de interagir com os insetos são as proteobactérias do gênero *Wolbachia*. Esses microorganismos têm ampla distribuição entre os invertebrados. Estimativas recentes, baseadas em análises estatísticas estimam que 40% das espécies de artrópodes estão infectados com *Wolbachia* (Zug e Hammerstein, 2012). Esses microorganismos também foram encontrados em isopodos (Rousset et al., 1992), ácaros (Johanowicz e Hoy, 1995) e nematodas (Sironi et al., 1995).

A espécie tipo do gênero, *Wolbachia pipientis*, foi inicialmente descrita em *Culex pipiens* (Hertig e Wolbach, 1924). Com base nas sequencias ribossomais da porção 16S, somadas às informações de outras seqüências, as espécies do gênero *Wolbachia* foram categorizadas em oito supergrupos identificados de A a H (Casiraghi et al., 2005). Os supergrupos C e D são comumente encontrados em nematodas filarióides, enquanto os outros seis estão primordialmente presentes em artrópodes, sendo os grupos A e B os mais comuns (Werren et al., 2008).

Nas ultimas décadas, a *Wolbachia* tem chamado a atenção de pesquisadores pelo amplo espectro de efeitos causados em seus hospedeiros, que variam desde interações mutualísticas até sua capacidade de manipulação reprodutiva, além de potenciais aplicações no controle de doenças e pragas (Bourtzis, 2008). Particularmente em *Drosophila*, estes microoganismos foram relacionados a uma série de problemas relativos à fertilidade como incompatibilidade citoplasmática, partenogênese, feminização e morte em machos (Werren, 1997; Stouthamer et al., 1999; Stevens et al. 2001; Almeida e Carareto, 2002). Desses problemas, o mais estudado tem sido a incompatibilidade citoplasmática. Esta é resultante de alterações prejudiciais nos espermatozóides dos machos infectados que podem ser neutralizadas nas fêmeas também infectadas, mas que não são corrigidos se o cruzamento ocorre com fêmeas não infectadas (Yen e Barr, 1971; Werren et al., 1997)

Estudos recentes revelaram que a *Wolbachia* está associada à discriminação sexual em *D. paulistorum* (Miller et al., 2010) e redução da discriminação em vespas parasitóides *Nasonia* (Chafee et al., 2011). Miller et al. (2010) demonstraram que em *D. paulistorum*, a *Wolbachia* manipula o comportamento sexual acionando o isolamento pré-zigótico, ao fazer com que os indivíduos evitem seletivamente os parceiros em potencial, isto é, evitem parceiros que carregam variantes incompatíveis do simbionte. O estudo ainda revela que os endossimbiontes podem co-evoluir rapidamente com seus hospedeiros naturais e assim desempenham papel significativo na especiação de seus hospedeiros.

A atuação desses microorganismos é comprovada quanto à discriminação dos parceiros e facilmente "curada" expondo as moscas a um tratamento por antibióticos (Koukou et al., 2006; Gazla e Carracedo, 2011; Miller et al., 2010; Sharon et al., 2010). As populações tratadas podem ter sua capacidade de discriminação sexual restaurada através da reinfecção pelo simbionte através da sua inoculação. Acredita-se que a manipulação comportamental seja uma forma do endosimbionte aumentar sua capacidade de transmissão (Thomas et al., 2005).

Além de se alojar nos oocitos, a *Wolbachia* também é encontrada no cérebro de *Drosophila*, desde sua fase larval até o estágio adulto (Min e Benzer, 1997; Albertson et al., 2009). Albertson et al. (2013) examinaram três linhagens de *Wolbachia* em *D. melanogaster* e *D. simulans* e conseguiram demonstrar que essa bactéria se concentra preferencialmente na parte central do cérebro e, em concentrações menores, nos lobos ópticos. Nos neurônios da área central e do nervo ventral, a *Wolbachia* se aloja em maior concentração no corpo celular do que nos axônios. Além disso, o trabalho mostrou que a sua distribuição depende tanto da linhagem do microorganismo quanto da espécie hospedeira, e que está presente de forma tanto intra como extracelular nos cérebros de adultos. Apesar da *Wolbachia* ser um endossimbionte intracelular obrigatório, pois sua reprodução não acontece fora da célula (Rasgon et al., 2006), os autores atribuem esse fato a uma possível lise celular provocada por seus altos níveis intracelulares.

Contudo alguns trabalhos apontam alguns efeitos benéficos da relação mutualística entre os gêneros *Wolbachia* e *Drosophila*. Starr e Cline (2002) demonstraram que a *Wolbachia* pode reverter defeitos ovarianos provocados por alguns alelos mutantes, em *D. melanogaster*. Tendo em vista que a *Wolbachia* é selecionada de forma a estimular o desenvolvimento de ovos, o que permite a sua transferência para a próxima geração, mutações que afetam a ovogênese podem funcionar de forma compensatória na sua presença (Werren et al., 2008).

Teixeira et al. (2008) demonstraram que a infecção do microoorganismo *Wolbachia* em *D. melanogaster* pode funcionar como um agente protetor à infecção por vírus de RNA.

Todavia muitas perguntas permanecem em aberto acerca dos mecanismos usados por esses microorganismos para a manipulação comportamental de seus hospedeiros e de seu papel nos processos evolutivos, principalmente os que se referem à aceleração das taxas de especiação e aquisição de novos genes nas espécies hospedeiras (Werren et al., 2008).

OBJETIVOS

Objetivos gerais

Ampliar informações quanto aos mecanismos de isolamento reprodutivo que atuam nos intercruzamentos de *D. prosaltans* e *D. saltans*, analisando aspectos do processo sob influência da infecção com o simbionte *Wolbachia* e sob influência da introgressão gerada por intercruzamentos das duas espécies, em linhagens mantidas no laboratório do Departamento de Biologia desta Instituição desde 1977.

Objetivos específicos

1- Verificar a ocorrência de infecção pelo microorgnismo *Wolbachia* nas linhagens utilizadas.

2. Caracterizar as linhagens de *Wolbachia* que infectam cada linhagem de *Drosophila* analisada.

3- Comparar os resultados de intra e intercruzamentos das linhagens tratadas para depleção completa do simbionte e infectadas, considerando as linhagens das espécies originais e as produzidas por introgressão

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Os dados obtidos no presente trabalho foram reunidos em uma única publicação, escrita nos moldes da Revista Genética (The Netherlands), que se encontra na sequência.

Productivity in *Drosophila* species of the *saltans* subgroup (*saltans* group, Sophophora subgenus): effects of *Wolbachia* infection on normal and introgressed strains

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ABSTRACT

Reproductive isolation mechanisms are agents that prevent or decrease the exchange of genes between species or populations of the same species that are in process of speciation. These mechanisms are comprised of a series of processes operating at different levels of reproduction, ranging from pre-zygotic to post-zygotic barriers. In recent decades, studies have indicated that the absence of progeny can also be promoted by interactions of symbiont microorganisms and their hosts. Presently, the most known endosymbionts capable of interact with the insects, interfering in the reproductive process, are the alphaproteobacteria of the genus *Wolbachia*. In the present study, we investigated the *Wolbachia* effects on reproduction, focusing the parameter productivity (number of progeny) in crosses involving four strains of *Drosophila saltans* and *D. prosaltans* (close species belonging to the *saltans* group, Sophophora subgenus) and two introgressed strains started with F1 hybrids of these two species. Preliminary tests for screening *Wolbachia* showed that each of the six strains was infected with one strain of the symbiont. The results on productivity were obtained from intra and intercrosses of the strains in the conditions infected or uninfected. The elimination of *Wolbachia* was performed by treatment of the strains with the antibiotics tetracycline. The main mechanism resulting from the interaction symbiont-host described in the literature is called cytoplasmatic incompatibility (CI) and occurs in the intercrosses of uninfected females with infected males. It is considered that, in infected males, there are changes in the sperm that only the oocytes of infected females are able to correct, reestablishing the productivity. The present results on *Wolbachia* infection of the species from the *saltans* group were variable. In several crosses of the strains, the combinations that are sterile or almost sterile when CI effect occurs, were the most productive or one of the more productive combinations. In other cases, in which the CI-indicative combination is sterile or almost sterile, the intercrosses of both infected parents, expected to be highly fertile, were sterile or very low fertile. Considering all the crosses analyzed, the results are rather indicative of inexistence of CI effect in the strains used. The main result was high productivity of the heterogeneous combinations of infected females with uninfected males, indicating that this combination is responsible for the maintaining and spreading of the *Wolbachia* infection in the used strains. According with the prevalent results, the elimination of the bacteria in male parents produced beneficial effects, annulling the cross sterility. The effects of the intra and intercrosses of *D. prosaltans* and *D. saltans* were discussed in relation to the crossability of the strains and the *Wolbachia* infection effects. We also tried to understand how the introgressed strains

differentiated from each other and from their parental species, taking into account their chromosome constitution.

INTRODUCTION

Dobzhansky (1937) proposed the name "isolating mechanisms" for all agents that decrease or prevent the exchange of genes between populations. Two basic groups of isolating mechanisms were differentiated - geographic or spatial and reproductive, each of them including several different types (Dobzhansky 1951, Mayr 1963). Despite the recognized great importance of these mechanisms as they are at the basis of new species origin, and consequently the extensive number of studies that have focused on them over time, their knowledge is still far from enough. Relatively to basic knowledge, involving genes producing reproductive isolation and how they interact with other genes and with the environment, although still requires deeper information (Dobzhansky 1937, Orr 1987, Sawamura et al. 2004, Reed and Markow 2004, Sweigart 2010, Moran and Fontdevila 2014). The discovery of new elements that can influence these processes raises new approaches that are also used in order to clarify the obscure points.

In the sequence of events occurring during reproduction, the isolation barriers may act as early as at the courtship behavior, for example when interspecific differentiation changes components of this process, preventing that males and females recognize each other for crossing (Bruker and Boderstein 2012, Hoikkala et al. 2000, Doi et al. 2001, Williams et al. 2001, Yamada et al. 2002) or when pheromone modifications impair recognition between partners (Coyne et al. 1994, Marcillac et al. 2005, Grillet et al. 2006, Kurtovic et al. 2007). However, when isolation barriers don't work at the first level and interspecific crosses occur, the barriers still may act at subsequent levels of the reproduction process, blocking the formation of viable hybrids or producing hybrids that in most cases are sterile (Dobzhansky 1937, Bicudo 1973a, Heikkinen and Lume 1991, Matute and Coyne 2009).

Species from the *saltans* group have been used in several studies focusing on isolation process. Some of the first studies on this subject were performed with *Drosophila* species from this group and published in the middle 40's (Dobzhansky 1944, Dobzhansky and Patterson 1945). The *saltans* group is among the ten groups included in the *Sophophora* subgenus, which in turn belongs to the genus *Drosophila*. The *saltans* and the *willistoni* groups are the two neotropical clades from the *Sophophora* subgenus (Throckmorton 1975, Da Lage et al. 2007). This group

includes five subgroups (*saltans, sturtevanti, parasaltans, elliptica* and *cordata*), with a total of 21 species (Magalhães 1962, Mourão et al. 1965). In the group, isolation studies using species from the *saltans* subgroup predominate. The seven species that the subgroup includes (*D. prosaltans, D. saltans, D. lusaltans, D. septentriosaltans, D. austrosaltans, D. nigrosaltans and D. pseudosaltans*) showed a variable capacity of interbreeding, yielding hybrids in some combinations (Bicudo 1973a).

The reproductive isolation was also studied in intercrosses of 18 *D. prosaltans* strains from different geographic origins (Bicudo 1978). The results showed three different patterns of incipient isolation, differentiating strains from Central America, from South America at North of Amazon River and Brazilian strains at South of Amazon River. The similarity of these results to data obtained previously in *D. paulistorum* (Dobzhansky and Spassky 1959, Dobzhansky and Powell 1975) lead the author to the hypothesis that *D. prosaltans* could be formed by three groups of species in *status nascendi*, name coined for *D. paulistorum* by Dobzansky and Spassky (1959).

Drosophila prosaltans and *D. saltans* were also studied as to their capacity of introgression by analysis of their ability to maintain, in the same individual, a mixture of chromosomes of both species (Tadei and Bicudo 1981). In laboratory, populations started with F1 hybrids of both species have preserved a combination of parts of both genomes.

In the present study we used four strains of *D. prosaltans* and *D. saltans* and two introgressed strains aiming to observe how they face *Wolbachia* infection relatively to the parameter productivity. *Wolbachia* is a genus of microorganisms (alphaproteobacteria) widely prevalent in invertebrates. Due mainly to the large involvement of *Wolbachia* with host biology and its wide distribution in arthropods, Warren et al. (2008) called this symbiont "master manipulators of invertebrate biology".Particularly in *Drosophila*, this symbiont have been related to a number of reproductive interferences, such as cytoplasmic incompatibility, parthenogenesis, feminization and male killing (Werren 1997, Stouthamer et al. 1999, Stevens et al. 2001, Cordeaux et al. 2011). Evolutionary consequences of the *Wolbachia* infection have also been pointed out in several studies, suggesting for example that the infection by this endosymbiont might be important in the reinforcement of isolation when incipient species come into secondary contact (Charlat et al. 2003, Jaenike et al. 2006, Versace et al. 2014, Choi and Aquadro 2014). These observations and a rich literature on the subject point to the importance of including this approach in studies of the *saltans* group.

In this study, the interference of *Wolbachia* in the yielding of progeny in *D. prosaltans* and *D. saltans* strains was observed in intraspecific and interspecific crosses. The use of the introgressed strains was considered as to the possibility of revealing how the mixture of genotypes of both species responds to the infection in comparison with the species that originated them.

MATERIALS AND METHODS

Species and strains used in the experiments

Strains of *Drosophila saltans* and *D. prosaltans* plus two introgressed strains resulting from both species, maintained in our laboratory for a long time (about 37 years), were used in the experiments (Table 1). The strains and experiments were kept at 20° C ± 1° C, in a constant temperature room, located at the Department of Biology, IBILCE-UNESP.

Characteristics of the hybrid strains

Tadei and Bicudo (1981) started four laboratory populations using F1 hybrids of intercrosses between females *D. prosaltans* and males *D. saltans*. This direction of intercrosses yields F1 fertile females and males (Bicudo 1973a). After maintaining the populations for some months, chromosomal analyses in the larval salivary glands showed that they differed along time. Two were formed by flies bearing exclusively the chromosomes of *D. saltans*, that is, they apparently eliminated completely the chromosomes of *D. prosaltans* (these populations were named H1 and H4), while the other two, in addition to the chromosomes of *D. prosaltans*, segments of *D. saltans* genome maintained the X chromosome, the IIL chromosome recombined with the IIR of *D. prosaltans* and several types of recombinant chromosomes III with a mixture of parts of both species (populations H2 and H3).

This chromosome constitution remained the same over time. Its detection was made observing regions of asynapsis and interspecific inversions already known from previous study of both species (Bicudo 1973b). Figures 1 and 2 show prints of

Table 1. Code, laboratory reference and geographic origin of species, strains and introgressed populations used.

Species	Strain		
	Code	Reference	Origin
D. prosaltans	P1	-	Cachoeira dos Monteiros, Bahia, Brasil
	P2	-	Taquarebó, Uruguai
D. saltans	S1	H180.40 (S1)	San Jose, Costa Rica
	S3	H1911.5 (S3)	Guatemala, Guatemala
Introgressed populations	H2	H2536.9 (P7) plus H336.24 (P6)	Eldorado, Rio Grande do Sul, Brasil;
		X	Belém, Pará, Brasil
	H4	H1401.4 (S6)	Huichiuayan, México

Figure 1. Pairing figures depicting inversions in the polytene chromosomes X, II and III of *Drosophila prosaltans* (P) x *D. saltans* (S) hybrids used for recognizing the specific origin of the chromosome segments. The arrow points asynapsis of the distal region of the chromosome III (Bicudo 1973b).

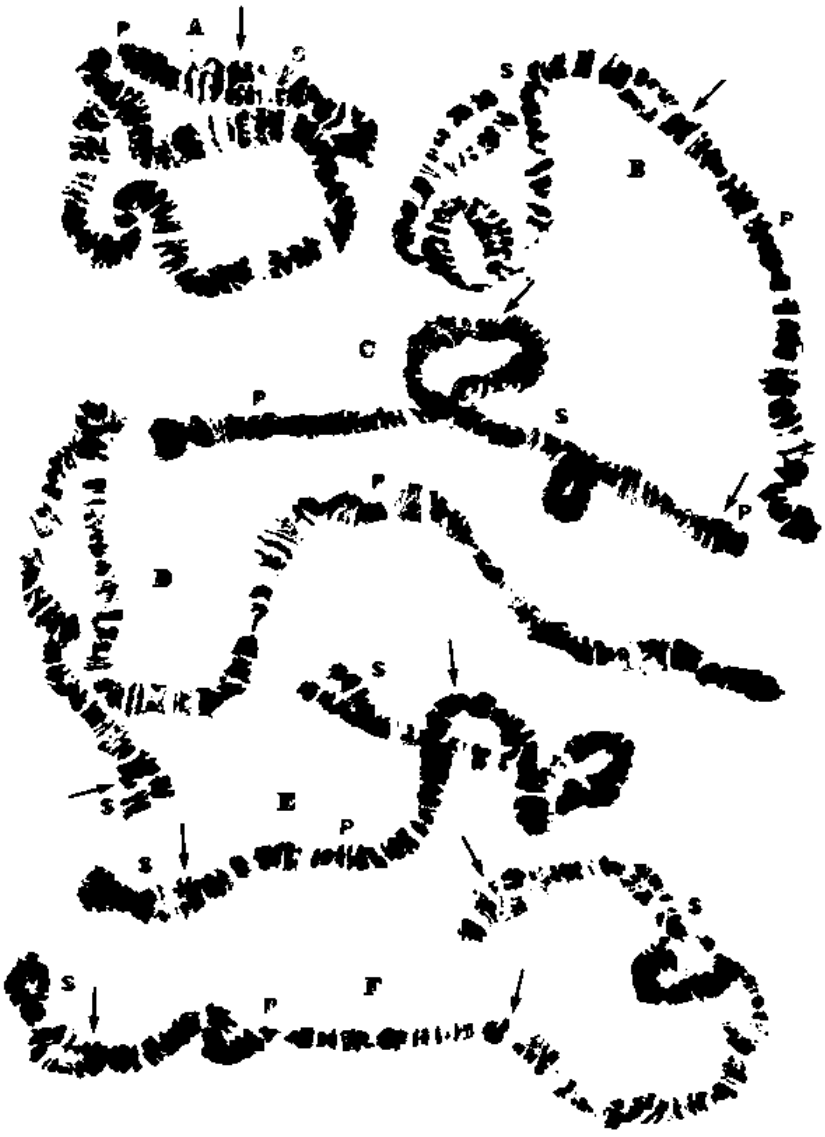

Figure 2. Pairing figures of the six subtypes of recombinant chromosomes III observed in the polytene chromosomes of flies from crosses of the introgressed populations H2 and H3 with *D. prosaltans*. P = *D. prosaltans*, S = *D. saltans*. The arrows indicate boundaries of segments of different specific origin. (Tadei and Bicudo 1981).

hybrids of both species, where the interspecific inversions and the regions of asynapsis are observed in the three pairs of chromosomes that characterize these species (two metacentric chromosomes - X and II; and one acrocentric chromosome -III). Figure 3 shows several types of chromosome III with recombination of segments from both species, found by Tadei and Bicudo (1981) in H2 and H3 populations.

Preparation of the intra and interspecific mass crosses

In order to study the productivity, intra and interspecific mass crosses were prepared with five virgin couples aged 7 to 9 days. The crosses were maintained in vials (tubes) containing banana-agar culture medium and transferred once to new flasks containing freshly prepared culture medium. Interspecific crosses were prepared in both directions. The productivity (number of progeny) of each cross was counted separately for males and females till the last fly emerged. F1 progeny obtained in each vial was put together in order to observe if they could produce F2. In vials that didn't produce F1 offspring, the parental females were dissected and their reproductive organs (spermatecae and seminal spiracle) were analyzed with a microscope in order to verify the occurrence of insemination.

Preparation of aposymbiotic strains (treatment of strains with antibiotics)

The term aposymbiotic is used when a host species naturally does not contain its symbiont or due to elimination. In this study, we used strains as they were originally in the stocks and the same strains treated with antibiotics for eliminating the symbionts. With this aim, the six strains were maintained during three generations in culture medium treated with tetracycline (Sigma - Aldrich) 0.03 %, in order to make them free of the α-proteobacteria symbiont *Wolbachia pipientis*. To prepare the medium, a stock solution of tetracycline diluted in 98% ethanol was used at 30mg/ml concentration and kept at -20°C. After three generations, the flies were maintained for other three generations in culture without tetracycline, in order to exclude possible remains of the antibiotic that could interfere with the results.

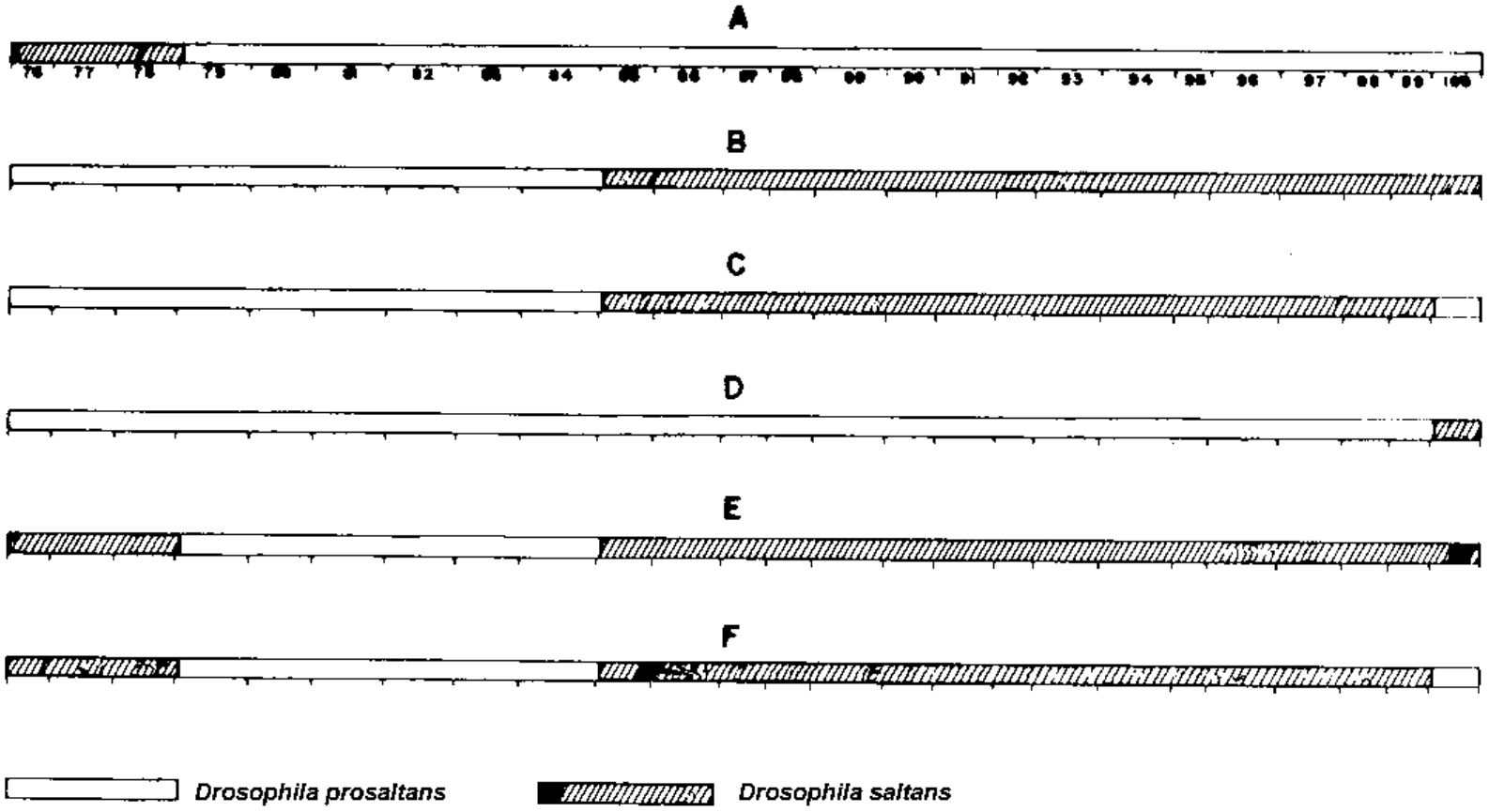

Figure 3. Diagram of 6 subtypes of polytene recombinant chromosomes III detected in the introgressed populations H2 and H3 (Tadei e Bicudo 1981).

Screening of *Wolbachia* strains

DNA extraction

DNA extractions were conducted according to Dellaporta et al. (1983) with modifications. Samples containing 10 flies were homogenized in 160µl of solution I (10 mM Tris, 60 mM NaCl, 5% sucrose, 10 mM EDTA, pH 7.8). 200µl of solution II (300mM Tris, 1.25% SDS, 5% sucrose, 10 mM EDTA, pH 8.0) were added to each sample, mixed and incubated in water bath at 65°C for 30 minutes. Then, 60µl of potassium acetate 3M (pH 5) were added to the samples and cooled at -20°C for 20 minutes. After centrifuging at 13000 rpm for 20 minutes, the supernatants were transferred to new tubes containing 400µl of isopropanol were incubated at room temperature for 5 minutes. Pellets were obtained after applying 500 µl 70% ethanol, centrifuging samples at 13000rpm for 10 minutes and air drying. The DNA was resuspended in 100 µl ultra-pure water and maintained at -20 ° C until use.

Amplification of DNA sequences

Specific primers were used for amplifications of the locus *Wsp* (*Wolbachia* Surface Protein) (Jeyaprakash et al. 2000), insertion sequences of the transposon IS5 in two loci (IS5 - WD0516/7 and IS5-WD1310), and *in tandem* sequences VNTR-105 and VNTR-141 (Riegler et al. 2005). All primer sequences are listed in Table 2. The reactions were prepared according to specific protocols for each primer. *Wsp*-PCR mix *r*eactions were prepared as described in Miller et al. (2010) and amplifications according to Jeyaprakash et al. (2000). *IS5*-PCR was conducted as described in Iturbe-Ormaetxe et al. (2005). *VNTR*–PCR was performed as described in Miller et al. (2010).

Identification of the amplification products

The amplified samples were subjected to electrophoresis using 8% polyacrylamide gel (30% bisacrylamide, TBE 10X Glycerin 10% ammonium persulfate and TEMED). 10µl of each sample were subjected to electrophoresis at 90 volts for 3 hours in the presence of 1X TBE buffer solution (Tris, boric acid and 0.5M EDTA pH 8.0). The fragments were visualized after fixation (10% ethanol, 0.75% Glacial Acetic Acid), and staining of the gel

Table 2. Primer sequences used in the screenings of *Wolbachia* in the *Drosophila* strains.

Primer	Sequence
Wsp-F	5-TGGTCCAATAAGTGATGAAGAAACTAGCTA-3
Wsp-R	5-AAAAATTAAACGCTACTCCAGCTTCTGCAC-3
IS5-WD0516/7-foward	5-CCATCAAGGTCTCTTTCA -3
IS5-WD0516/7-reverse	5-TGCAAGGAAAACTAAACCAG-3
IS5-WD1310-foward	5-AGGAGAACTGGTCTACGC-3
IS5-WD1310-reverse	5-TGTTGCTGAGCTTTGCT-3
VNTR-105-foward	5-GCAATTGAAAATGTGGTGCC-3
VNTR-105-reverse	5-ATGACACCTTACTTAACCGTC-3
VNTR-141-foward	5-GGAGTATTATTGATATGCG-3
VNTR-141-reverse	5 –GACTAAAGGTTAGTTGCAT-3

with silver nitrate followed by development (sodium hydroxide and formaldehyde). The gels were air dried and preserved wrapped in cellophane film (Ceron et al. 1992).

Characterization of the *Wolbachia* strain in the *Drosophila* strains

DNA sequencing was conducted in Centro de Estudos do Genoma Humano, USP, São Paulo. Amplified fragments were excised from the gels and purified using the QIAEX II Gel Extraction Kit (Qiagen). The sequencing reactions were made using the BigDye® Terminator v3.1 Cycle Sequencing Kit (4337456 code). The runnings were made in capillaries of 36 cm using the POP7 polymer. The sequences were analyzed by the Sequencing Analysis 5.3.1 software using the Caller Base KB.

The sequence alignments were made with the software Bioedit (Hall 1999) and MUSCLE method (Edgar 2004), followed by manual alignments. Segments ambiguous relatively to the consensus sequences were discarded.

The strain sequences were compared with reference sequences obtained in NCBI database and submitted to similarity analysis using MatGat software (Capanella et al. 2003). Reference for sequences obtained from *primers* IS5 WD1310 and IS5 WD0516/7 were not available. Therefore the comparisons were made exclusively among the sequences obtained in the experiments.

Statistics

Productivity data was submitted to Kolmogorov-smirnov test for normality verification. Student's t test was used for pairwise comparison of females, males and total progeny, in each group of heterogeneous and homogeneous combinations (Biostat 2009 – Pimentel and Smith 1986)

Chi-square test was used to compare the total productivity within each group and pairwise comparisons were made for crosses with productivity different from zero, for males, females, and their sum (total) (Minitab - Ryan et al. 1994).

RESULTS

Figures 4 and 5 show, in gels, the results of the molecular assays performed to detect the presence of *Wolbachia* in the six *Drosophila* strains. The amplification products were obtained from five loci (WSP, VNTR-105, VNTR-141, IS5-WD1310, IS5-WD0516/7). All strains proved to be infected. The production of a single fragment in each of the tests indicated that all of them are infected by a single strain of *Wolbachia*.

In Table 3 are data on F1 productivity obtained in mass intracrosses of untreated (infected) strains and strains treated for elimination of *Wolbachia* (uninfected). These crosses involved *D. saltans* (strains S1 and S3), *D. prosaltans* (strains P1 and P2) and the introgressed strains (H2 and H4). Crosses in which both parents were uninfected or infected we named homogenous combinations and crosses in which one parent was uninfected and the other infected we named heterogeneous combinations.

All comparisons of productivity data using Student's t test showed no significant results. Chi-square tests were then applied, showing highly significant results for all comparisons. Because the results of both tests were not consistent with the differences observed in the light of numbers, we concluded that they don't have the sensitivity level required to analyze small numbers as we obtained in the present study. Tables A to C, containing the results of the statistical analysis are included as supplementary material.

The analysis of the data made in the light of the numbers showed that, among the homogenous intracrosses of infected strains (N), S1 X S1, P1X P1 and H2 X H2 yielded progeny, being the number of progeny in H2 X H2 nine times that in the other fertile intracrosses. Among the homogeneous intracrosses of the same strains after treatment (T), only H4 X H4 didn't yield progeny but, among the three that produced progeny, the productivity in H2 X H2 was about 20% lower than that in P1 X P1 and about 44% lower than in P2 X P2 (Table 3).

The intrastrain crosses in heterogeneous combinations gave different results, depending on the strain and the direction of crosses (Table 3). For P1, in infected females X uninfected males (N F X T M) and P2 (also N F X T M) the number of progeny was greater than in the reciprocal combination, but the productivity difference was greater in P1 than in P2. In H2, both heterogeneous combinations didn't produce progeny while, in H4, both were fertile but six times more fertile in the direction T F X N M.

Data on F1 productivity of interstrain crosses in homogeneous and heterogeneous combinations are included in Table 4. In order to facilitate comparisons, the Table is arranged

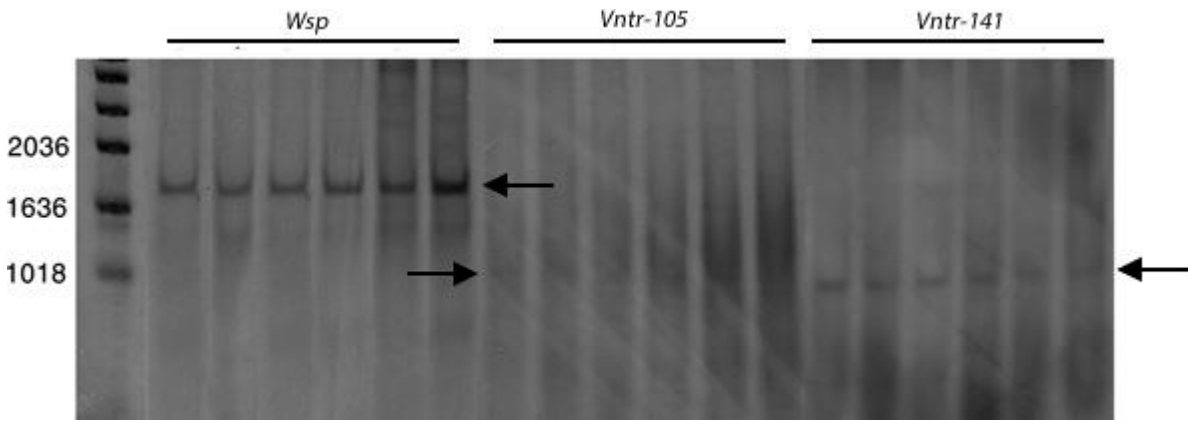

Figure 4. DNA fragments obtained by PCR using the primers *Wsp*, *VNTR-141* and *VNTR-105* (arrows). Samples from left to right: H2, H4, P1, P2, S3, S1

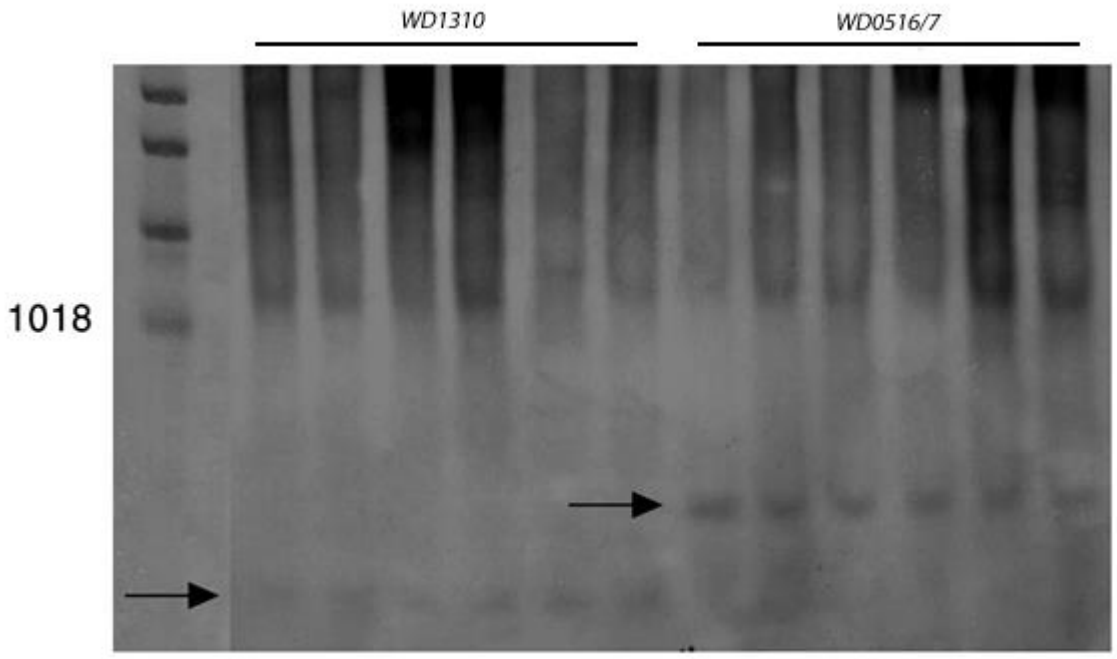

Figure 5. DNA fragments obtained by PCR using the *IS5* primers (arrows). Samples from left to right: H2, H4, P1, P2, S3, S1.

Table 3. Number of F1 males (M), females (F) and their sum (T) (productivity) obtained in intrastrain crosses of *Drosophila saltans* (strains S1 and S3), *D. prosaltans* (P1 and P2) and introgressed strains (H2 and H4), before treatment (N) and after treatment (T) to eliminate the *Wolbachia* infection.

	Crosses			Productivity		
	F		M	F	M	T
N x N	S1	x	S1	4	1	5
	S3	x	S3	0	0	0
	P1	x	P1	4	1	5
	P2	x	P2	0	0	0
	H2	x	H2	29	16	45
	H4	x	H4	0	0	0
T x T	P1	x	P1	17	17	34
	P2	x	P2	29	14	43
	H2	x	H2	17	7	24
	H4	x	H4	0	0	0
N x T	P1	x	P1	21	15	36
	P2	x	P2	13	12	25
	H2	x	H2	0	0	0
	H4	x	H4	5	2	7
T x N	P1	x	P1	2	3	5
	P2	x	P2	9	6	15
	H2	x	H2	0	0	0
	H4	x	H4	18	23	41

Table 4. Number of F1 males (M), females (F) and their sum (T) (productivity) obtained in interstrain crosses of *Drosophila saltans* (strains S1 and S3), *D. prosaltans* (strains P1 and P2) and introgressed strains (H2 and H4), before treatment to eliminate the *Wolbachia* infection (N) and after treatment (T).

Crosses			Productivity			Crosses			Productivity			Crosses			Productivity		
F		M	F	M	T	F		M	F	M	T	F		M	F	M	T
NS1	x	NS3	9	4	13	NS1	x	NH2	0	0	0	NH4	x	NP1	20	20	40
NS3	x	NS1	0	0	0	NS1	x	TH2	17	19	36	NH4	x	TP1	5	7	12
						NH2	x	NS1	0	0	0	TH4	x	NP1	24	16	40
						TH2	x	NS1	34	21	55	TH4	x	TP1	0	0	0
NP1	x	NP2	0	0	0	NS1	x	NH4	0	0	0	NP2	x	NH2	0	0	0
NP1	x	TP2	0	0	0	NH4	x	NS1	0	0	0	NP2	x	TH2	19	10	29
TP1	x	NP2	0	1	1	TH4	x	NS1	0	0	0	TP2	x	NH2	0	0	0
TP1	x	TP2	0	0	0	NS1	x	TH4	0	0	0	TP2	x	TH2	0	0	0
NP2	x	NP1	3	2	5	NS3	x	NH2	0	0	0	NH2	x	NP2	0	0	0
NP2	x	TP1	17	15	32	NS3	x	TH2	0	0	0	NH2	x	TP2	0	0	0
TP2	x	NP1	7	14	21	NH2	x	NS3	0	0	0	TH2	x	NP2	54	31	85
TP2	x	TP1	13	19	32	TH2	x	NS3	0	0	0	TH2	x	TP2	12	9	21
NS1	x	NP1	0	0	0	NS3	x	NH4	0	0	0	NP2	x	NH4	6	6	12
NP1	x	NS1	0	0	0	NH4	x	NS3	0	0	0	NP2	x	TH4	27	23	50
TP1	x	NS1	0	0	0	TH4	x	NS3	0	0	0	TP2	x	NH4	29	17	46
NS1	x	TP1	0	0	0	NS3	x	TH4	0	0	0	TP2	x	TH4	0	0	0
NS1	x	NP2	0	0	0	NP1	x	NH2	0	0	0	NH4	x	NP2	17	11	28
NP2	x	NS1	0	0	0	NP1	x	TH2	0	0	0	NH4	x	TP2	30	26	56
TP2	x	NS1	0	0	0	TP1	x	NH2	0	0	0	TH4	x	NP2	0	0	0
NS1	x	TP2	0	0	0	TP1	x	TH2	0	0	0	TH4	x	TP2	0	0	0
NS3	x	TP1	0	0	0	NH2	x	NP1	0	0	0	NH2	x	NH4	29	23	52
TP1	x	NS3	0	0	0	NH2	x	TP1	0	0	0	NH2	x	TH4	14	16	30
NS3	x	NP1	12	18	30	TH2	x	NP1	0	0	0	TH2	x	NH4	0	0	0
NP1	x	NS3	0	0	0	TH2	x	TP1	0	0	0	TH2	x	TH4	0	0	0
NS3	x	NP2	0	0	0	NP1	x	NH4	0	0	0	NH4	x	NH2	0	0	0
NS3	x	TP2	6	7	13	NP1	x	TH4	0	0	0	NH4	x	TH2	6	16	22
NP2	x	NS3	0	0	0	TP1	x	NH4	0	0	0	TH4	x	NH2	17	26	43
TP2	x	NS3	0	0	0	TP1	x	TH4	10	5	15	TH4	x	TH2	0	0	0

so that the different types of combinations, involving the same strains, are presented together, forming sets of four combinations. Homogeneous intercrosses of strains from the same species were performed for *D. saltans*, involving S1 and S3 that yielded progeny in the direction of the untreated combination S1F X S3M but not in the reciprocal crosses (only these two combinations were available for analysis of the productivity, involving these two strains of *D. saltans*). In *D. prosaltans*, the direction T P1F X N P2M yielded a single male. However, in the reciprocal crosses (P2 F X P1 M), the homogeneous and heterogenous combinations yielded hybrids that were in the homogeneous combination of untreated strains from about 4 to six times numerically lower.

Considering the intercrosses of *D. prosaltans* with *D. saltans* strains (S1 X P1, S1 X P2, S3 X P1 and S3 X P2) descendants were obtained only in two combinations: N S3F X N P1M and N S3 F X T P2M. The productivity of the first type of cross was about the double of the second.

In the intercrosses of *D. saltans* or *D. prosaltans* strains with the introgressed strains H2 and H4, all the combinations involving S1 X H4, S3 X H2 and S3 X H4 were unproductive. However, the intercrosses S1 X H2 yielded hybrids in the two heterogeneous combinations: N S1F X T H2M and T H2F X N S1M, with higher productivity in the second combination.

The intercrosses of *D. prosaltans* strain P1 with H2 were unproductive in both directions and all combinations. Differently, P1 X H4 yielded hybrids in the homogeneous treated combination P1F X H4M and in three of the four combinations involving H4F X P1M. The unproductive combination H4F X P1M was the homogeneous treated. The homogeneous untreated H4F X P1M and the heterogeneous T H4F X N P1M yielded more than three times the number of progeny obtained in the reciprocal heterogeneous combination (N H4F X T P1M).

The intercrosses P2F X H2M produced progeny only in the heterogeneous combination N P2F X T H2M while, in the reciprocal direction of crosses (H2F X P2M), the homogeneous treated combination and the heterogeneous T H2F X N P2M yielded progeny. This last combination produced the highest number of progeny obtained in the present study (85).

The intercrosses P2F X H4M yielded F1 progeny in the two heterogeneous combinations and in the homogeneous untreated combination, but the number of descendants in the two heterogeneous was about four times that in the homogeneous one. In the reciprocal crosses H4F X P2M, the homogeneous untreated combination and the heterogeneous

combination involving N H4F X T P2M were productive, but the first combination produced half the number of descendants obtained in the second.

Finally the intercrosses involving H2 X H4 showed two productive combinations in each direction of crosses. In H2F X H4M they were the homogeneous untreated combination and the heterogeneous N H2F X T H4M, this last yielding some less than half the number of the descendants of the first. In the reciprocal crosses the two heterogeneous combinations were productive; in N H4F X T H2M, the number of progeny was half the number of heterogeneous T H4F X N H2M.

A total of 100 types of crosses were performed in the present study. Thirty five of those that didn't yield progeny had the parental females analyzed as to the presence of spermatozoa in the spermatecae and seminal receptacles. Ten among 79 females analyzed were inseminated. The results were mentioned in the discussion when considered relevant.

DISCUSSION

In the present study we intended to add new data to the already accumulated on reproduction of *Drosophila* species in the *saltans* group. We studied the effects of *Wolbachia pipientis* infection, which has been widely associated with this process, in insects and other organisms. In *Drosophila* species, this symbiont, which is transmitted predominantly transovarially, has been extensively studied, considering biological and evolutionary effects.

We analyzed productivity in intra and intercrosses of two strains of the species *D. prosaltans* and *D. saltans* and two strains that started in the laboratory with F1 progeny of *D. prosaltans* females and *D. saltans* males and over time stabilized at different chromosome constitutions. We've chosen these two introgressed strains to observe how they respond to *Wolbachia* infection comparatively to the species that originated them.

Molecular analyses for the presence of *Wolbachia* infecting the six strains used in the present study were positive for all of them. The molecular reactions yielded a single DNA fragment per strain, indicating that each host is infected by one single bacteria strain. However, the analysis of DNA sequences for detecting which *Wolbachia* strain is present in each *Drosophila* strain didn't give clear results and will be repeated.

The treatment of the strains with the antibiotic tetracycline allowed to have them for analysis in the conditions infected (non-treated) and uninfected (treated, free of the *Wolbachia*). The use of this process has been considered secure in relation to possible influence of the antibiotics on the results. Specifically, previous studies tested the effect of

tetracycline treatment, confirming its efficiency for *Wolbachia* elimination and showing no significant effect on hosts fitness (Poinsot and Merçot 1997, Dedeine et al. 2001, Grenier et al. 2002, Fry et al. 2004).

The effect of *Wolbachia* infection on reproductive biology considered more widespread is the incompatibility egg-sperm . It is due to the interaction of the symbiont with the host reproductive tissues. This effect is named cytoplasmic incompatibility (CI) and is characterized by strong embryo mortality when uninfected females are crossed with infected males. It is considered that, in infected males, the sperm is modified but the changes may be corrected in the infected females. This favors the production of infected flies in intercrosses between infected females and males, favoring vertical transmission of the maternally inherited endosymbiont and *Wolbachia* establishment in wild populations. Uninfected females, however, are unable to do the correction of sperm, causing cross infertility (review in Cordaux et al. 2011).

In addition to the reproductive tissue, *Wolbachia* infects somatic tissues, including hemolymph, muscle, midgut, malphigian tubules, nervous tissue, fat body and wings, but the effects of the somatic infections have been less focused in the literature (McGraw and O'Neill 2004).

In *Drosophila*, different degrees of CI have been associated with *Wolbachia* infection, in different species. As described by Fry et al. (2004), CI strength was variable in four strains of *D. melanogaster* from North America and Africa, a result that the authors considered conflicting with the generality of strong CI described before, in the same species. In *D. simulans*, natural populations from Australia didn't cause detectable harmful effects to the hosts, suggesting that in them the *Wolbachia* infection behaves like a neutral variant (Hoffmann et al. 1996). *D. suzukii* and *D. subpulchrella* also didn't manifest the CI effect in the intercrosses uninfected females X infected males, suggesting positive fitness effects (Hamm et al. 2014). The latter authors also looked for sex-ratio distortion in the analyzed species but also failed to find this effect. *D. auraria* and *D. sechellia* exhibited egg mortality denoting CI of about 60% (Bourtziz et al. 1996). Matings between females of *D. subquinaria* and males of *D. recens* resulted in high levels of offspring mortality due to interspecific cytoplasmic incompatibility caused by *Wolbachia* infection of *D. recens* (Jaenike et al. 2006).

The results of the present study showed that, in general, the effects of *Wolbachia* on productivity of the *Drosophila saltans* subgroup strains studied were variable, being similar for strains of the same species, but differing in other crosses, showing frequently asymmetric effects according with the combination and the incipient isolation of the strains.

In *D. prosaltans*, the two analyzed strains showed very similar *Wolbachia* effects in intracrosses, suggesting that the elimination of the symbiont was beneficial for productivity since the homogeneous matings of uninfected parents and the heterogeneous of infected females with uninfected males were the most productive. The heterogeneous combination expected to induce cytoplasmic incompatibility (TF X NM) also showed some productivity in P1 and P2, higher in P2. Apparently, *Wolbachia* infection in *D. prosaltans* affects males more strongly than females. Fry and Rand (2002) explained similar results observed in *D. melanogaster* by the existence of increased costs of reproduction in infected males.

The effect of *Wobachia* in intracrosses of S1 and S3 *D. saltans* strains was analyzed in both directions of the crosses, involving only the homogeneous infected combinations .The results were similar to the obtained for *D. prosaltans* strains, characterized by low number or absence of progeny.

Of course, the infected strains of *D. prosaltans* and *D. saltans* used are not really sterile because in this case they could be maintained in laboratory. What happens is that in the conditions of the experiments they have greater difficulty to mate than the other combinations. This also may explain why these strains are not so easy to maintain in laboratory.

Considering the intercrosses involving the six strains (Table 4), in this study 20 sets of four intercrosses were prepared, including homogeneous and heterogeneous combinations of infected and uninfected flies, in reciprocal crosses. Among the 20 sets, 12 produced progeny. In five of the 12 sets, the crosses involving uninfected females X infected males were the most productive or one of the more productive, indicating absence of CI impact. In addition, in eight of the 12 sets, the opposite direction (infected F X uninfected M) was the most fertile (in one case both heterogeneous directions had high productivity), a result already mentioned in the analysis of intracrosses. The results also showed that, in crosses that might indicate CI effect, the productivity of the other combinations frequently was not concordant. Following is a more detailed analysis of these results.

The interstrain crosses of the *D. prosaltans* showed asymmetrical results, yielding progeny only in the direction P2F X P1M (a single fly was obtained in the reciprocal crosses). The effects of infection in this case were very similar to the obtained for intrastrain crosses, reinforcing that the treatment was beneficial for productivity. The sterility of the reciprocal interstrain crosses (P1FX P2M) was probably due to incipient isolation of genetic origin. As mentioned, Bicudo (1978) observed degrees of incipient isolation among *D. prosaltans* geographic strains, manifested predominantly in one cross direction. The strains used in the present study weren't studied at that time since they were collected much later, but several

intercrosses of strains with different origin showed a low productivity or even sterility in one cross direction.

Productivity in the interspecific crosses of *D. prosaltans* strains (P1 and P2) with *D. saltans* strains (S1, S3) differed, depending on the strains that were intercrossed and the intercross direction. Descendants were not yielded in the interspecific crosses involving S1 with the two *D. prosaltans* strains, but they were produced in crosses of P1 or P2 with S3. The analysis of reproductive organs in parental females of crosses S1 X P1 or P2, performed, in the present study, showed absence of insemination, reinforcing previous observations that the isolation barriers among different species of the *saltans* subgroup act preferentially early in the reproductive process and that the degree of reproductive isolation vary for different strains (Bicudo, 1973a).

The fecundity of the intercrosses S3 X P1 or P2 was low, occurring in a single combination in both intercrosses. In both cases, the direction of the productive crosses involved S3 F and P1 or P2 M, but the productive combination differed: homogeneous infected (N) parents or infected F X uninfected M, respectively for P1 and P2. The analysis of female organs in the combination N S3F X T P1M, that didn't yield progeny, showed that four of the five females were inseminated, the absence of progeny being due to pre-zygotic barriers acting after mating in the isolation of *D. prosaltans* and *D. saltans* strains used. The results showed absence of CI.

The reproductive behavior of the introgressed strains showed that they differ from each other and from the parental species. This was expected, although both introgressed strains started with F1 hybrids of interspecific crosses between *D. prosaltans* females and *D. saltans* males, they evolved differently over time, as shown by the differences detected at the chromosome level. As mentioned, H2 has a chromosomal mixture of the two species while H4 apparently eliminated the entire *D. prosaltans* genome (Tadei and Bicudo 1981). However, we have to consider that this is a superficial analysis since the hybrids showed interspecific chromosome recombination; thus genes of one species may remain in the chromosomes of the other, but this cannot be detected by chromosome pairing analysis.

According to Baack and Rieseberg (2007) the introgressed organisms may furnish a new understanding of the basis of hybrid vigor. It is already known that hybridization can cause genomic changes occurring very rapidly and that among them are chromosomal rearrangements and differences in gene expression. The genomic changes may lead to new phenotypes that are beneficial for fertility.

The divergence between H2 and H4 relatively to the effect of *Wolbachia* infection was already indicated by the results of intrastrain crosses (Table 3). In H2, both homogeneous

combinations, uninfected or infected, yielded progeny, although more numerous when both parents were infected. This might suggest CI effect. However the sterility observed in both heterogeneous combinations may indicate harmfull effect originating intrastrain isolation. In turn H4, in the same conditions of homogeneous combinations, didn't yield progeny. The best result in terms of productivity for H4 X H4 was obtained when females were uninfected and males were infected, suggesting absence of CI effect.

The *Wolbachia* effects on the intercrosses H2 X H4 reinforced the idea of difference of the two strains. When females were H2, the combinations homogeneous infected and the heterogeneous infected females X uninfected males were productive. The reciprocal intercrosses (females H4 X males H2) were also productive in this last combination, but the heterogeneous reciprocal combination (uninfected females X infected males) was more productive and the homogeneous infected combination didn't yielded progeny. In H2 F X H4 M but not in the reciprocal crosses we may suppose the existence of CI effect.

The introgressed strains also differed from each other when compared with *D. prosaltans* and *D. saltans*. In intercrosses of H2 and H4 with *D. saltans* and *D. prosaltans* strains, H4 showed greater compatibility with the two *D. prosaltans* strains. Nine combinations of H4 with P1 and P2 yielded progeny whereas only three of H2 were productive and exclusively in crosses with P2. On the basis of the chromosome constitution, this result could be considered unexpected, since H2 has great part of the *D. prosaltans* genome that seems completely eliminated in H4. In addition, H2 produced hybrids exclusively in crosses with S1 but not with S3, while H4 didn't yield hybrids with S1 or S3. The intercrosses of H4 F X P2 M are the only that might show some CI effects. As to the introgressed strains, we think that the data at this time are not enough for other conclusions than recognizing the complexity of their behavior and the interesting pattern of divergence they show with each other and with *D. prosaltans* and *D. saltans*.

The extensive literature available on *Wolbachia* infection shows that the analysis of the effects in this endosymbiosis is not a simple task. As mentioned, the interaction of *Wolbachia* with hosts, including *Drosophila*, originates a complexity of interactive mechanisms that involve genetic processes of both organisms, resulting frequently in a variable interference in the fitness of the hosts. Besides, the efficiency of these mechanisms is under control of other elements. The environmental factors temperature and nutrition and the bacteria density were shown to influence cytoplasmic incompatibility (Clancy and Hoffmann 1998, Ponton et al. 2015). Also the specific *Wolbachia* strain that infects the host may be important. As much as possible in laboratory, the first two factors were controlled in the present tests, but the infection index needs special methods of analysis that were not part of

the present study. However, on the basis of the present results that represent a first view of the interaction between *Wolbachia* and species of the *D. saltans* subgroup we understand that the cytoplasmic incompatibility effect as defined in the literature is absent in the relation host-symbiont here analyzed.

The observation of a predominance of high productivity in the combination between infected females and uninfected males indicates that this is the main process selected to ensure the maintenance of infection in the analyzed species and may be related to the already mentioned fitness benefits caused by elimination of the symbiont in males.

The potential evolutionary aspects of the relationships between endosymbionts and their hosts have been intensively discussed in the literature, approaching different aspects of the speciation process. The study of this interaction originated the evolutionary concept known as the hologenome theory of evolution (Zilb-Rosemberg and Rosemberg, 2008; Serga and Kozeretskaya 2014). According to this theory, the hologenome (the host organism and its microbiota) forms a selection unity. In this sense, symbionts like *Wolbachia* may play a central role in the adaptation of their hosts since they are highly widespread in nature and developed special mechanisms for manipulation of the sexual reproduction. The potential of *Wolbachia* for causing evolutionary changes in the hosts begins with the opportunity to contribute to host functions or even with gene transference to the host nucleus (Charlat et al.2003).The main mechanism of change caused by *Wolbachia* in the host reproduction, which produces the cytoplasmic incompatibility effect and ensures the survival and spread of the symbiont, might be a first step for speciation, originating an incipient mechanism of sexual isolation (Gazla and Carracedo 2011).

Considering that the CI effect that occurs within species may also be present in matings between species, *Wolbachia* infection could be at the origin of asymmetrical hybrid fitness causing incipient isolation (Jaenike et al. 2006). An example involving this possibility in different species was described for *D. recens* (naturally infected) and *D. subquinaria* (naturally uninfected) that mated in the direction *D. recens* females and *D. subquinaria* males are sterile due to behavioral isolation, but the reciprocal crosses produce few viable progeny. Curing *D. recens* of their *Wolbachia* infection by antibiotic treatment, the production of viable hybrids via this route was restored, indicating that hybrid progeny production is greatly reduced by CI. Thus, behavioral isolation and *Wolbachia*-induced CI act together promoting asymmetrical isolation between these species (Shoemaker et al. 1999).

Has *Wolbachia* infection played a role in the evolution of the *saltans* group? The potential exists but we think that this has not happened in the strains analyzed because CI was not evidenced in them. In addition, this subject, in the literature, is still rather speculative than

proved and also involves a great amount of cumulative knowledge on the interaction of the specific host and the symbiont.

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CONCLUSÕES

1- Os testes para a triagem da *Wolbachia* em *Drosophila* mostraram que cada uma das seis linhagens analisadas estava infectada por uma única linhagem de *Wolbachia.*

2- O tratamento com Tetraciclina para a eliminação da *Wolbachia* permitiu obter linhagens aposimbióticas, isto é, livres do simbionte.

3- Os resultados referentes à produtividade mostraram que os intracruzamentos de linhagens infectadas e aposimbióticas em *D. prosaltans* e *D. saltans* comportaram-se da mesma forma, revelando ausência de incompatibilidade citoplasmática uma vez que os cruzamentos esperados serem estéreis foram produtivos e vice-versa.

4- Intercruzamentos de diferentes linhagens da mesma espécie (*D. prosaltans* ou *D. saltans*) concordaram com os resultados obtidos intralinhagem, isto é, revelaram ausência de incompatibilidade citoplasmática.

5- Intercruzamentos de linhagens de *D. prosaltans* com linhagens de *D. saltans* mostraram resultados de produtividade assimétricos, resultantes tanto de isolamento reprodutivo, já previamente demonstrado, como assimétricos em relação à produtividade das combinações de moscas com ou sem infecção.

6- Com relação à incompatibilidade citoplasmática, os intercruzamentos foram também indicativos de sua ausência nas linhagens de *D. prosaltans* e *D. saltans* analisadas.

7- A comparação das linhagens introgredidas H2 e H4, iniciadas com híbridos F1 das duas espécies, e que evoluíram cariotípicamente de forma diferente, mostrou que diferem entre si e em relação às linhagens parentais com relação ao parâmetro analisado.

8- Nos intracruzamentos bem como nos intercruzamentos, os resultados de produtividade das linhagens introgredidas diferiram, sendo que a linhagem H4 mostrou maior compatibilidade com *D. prosaltans* do que com *D. saltans*, resultado inesperado tendo em vista que esta linhagem evoluiu para a eliminação dos cromossomos da primeira espécie.

9- A linhagem introgredida H2, que manteve cromossomos de *D. prosaltans* e partes do cariótipo de *D. saltans*, demonstrou maior compatibilidade com as linhagens de *D. saltans* do que com *D. prosaltans*.

10- De modo geral os resultados obtidos não mostraram o efeito da incompatibilidade citoplasmática nas linhagens analisadas.

11- As linhagens introgredidas não se comportaram da forma esperada pela sua constituição cromossômica, sugerindo, talvez, a troca de material genético por recombinação, não detectável pela análise cariotípica.

Anexo (Supplemental Material)

Table A. T test for productivity of intra and inter crossings of S1 and S3 (*D. saltans*), P1 and P2 (*D. prosaltans*) and introgressed strains H2 and H4. Comparisons were made pairwise within each group of crosses between treated and untreated flies to *Wolbachia* infection. $P \leq 0.05$.

	Crosses					t value			p value			
	N	S1	x	N	S1	2.774			0.891			
	N	S3	x	N	S3							
A	N	P1	x	N	P1	AB	3.250	BC	3.277	AB	0.918	BC	0.918
B	N	P1	x	T	P1	AC	0.000	BD	0.158	AC	0.000	BD	0.118
C	T	P1	x	N	P1	AD	3.338	CD	3.371	AD	0.922	CD	0.922
D	T	P1	x	T	P1								
A	N	P2	x	N	P2	AB	3.990	BC	1.348	AB	0.943	BC	0.726
B	N	P2	x	T	P2	AC	3.780	BD	1.282	AC	0.937	BD	0.691
C	T	P2	x	N	P2	AD	3.424			AD	0.924		
D	T	P2	x	T	P2	CD	7.582			CD	1.000		
A	N	H2	x	N	H2	AB	3.577	BC	-	AB	0.930	BC	-
B	N	H2	x	T	H2	AC	3.577	BD	3.244	AC	0.930	BD	0.917
C	T	H2	x	N	H2	AD	1.439			AD	0.744		
D	T	H2	x	T	H2	CD	3.244			CD	0.917		
A	N	H4	x	N	H4	AB	3.212	AD	-	AB	0.915	AD	-
B	N	H4	x	T	H4	AC	3.914	BD	3.212	AC	0.940	BD	0.915
C	T	H4	x	N	H4	BC	3.542			BC	0.999		
D	T	H4	x	T	H4	CD	5.011			CD	1.000		
	N	S1	x	N	S3	3.329			0.920			
	N	S3	x	N	S1							
A	N	S1	x	N	P1	AB	-	AD	-	AB	-	AD	-
B	N	P1	x	N	S1	AC		BD		AC		BD	
C	T	P1	x	N	S1	BC				BC			
D	N	S1	x	T	P1	CD				CD			
A	N	S1	x	N	P2	AD	-		-	AD	-		-
B	N	P2	x	N	S1	CD				CD			
C	T	P2	x	N	S1	AB		BC		AB		BC	
D	N	S1	x	T	P2	AC		BD		AC		BD	
A	N	S1	x	N	H2	AB	3.982			AB	0.942		
B	N	S1	x	T	H2	AC	-	CD	3.702	AC	-	CD	0.934
C	N	H2	x	N	S1	AD	3.702			AD	0.934		
D	T	H2	x	N	S1	BC	5.368	BD	1.891	BC	1.000	BD	0.804
A	N	S1	x	N	H4	AB	-	BC	-	AB	-	BC	-

B	N	H4	x	N	S1	AC		BD		AC		BD	
C	T	H4	x	N	S1	AD		CD		AD		CD	
D	N	S1	x	T	H4								
A	N	S3	x	T	P1	AB	-	BC	3.651	AB	-	BC	0.999
B	T	P1	x	N	S3	AC	3.651	BD	-	AC	0.999	BD	-
C	N	S3	x	N	P1	AD	-	CD	3.651	AD	-	CD	0.999
D	N	P1	x	N	S3								
A	N	S3	x	N	P2	AB	3.965	BC	3.965	AB	0.942	BC	0.942
B	N	S3	x	T	P2	AC	-	BD	3.965	AC	-	BD	0.942
C	N	P2	x	N	S3	AD	-	CD	-	AD	-	CD	-
D	T	P2	x	N	S3								
A	N	S3	x	N	H2	AB	-	BC	-	AB	-	BC	-
B	N	S3	x	T	H2	AC	-	BD	-	AC	-	BD	-
C	N	H2	x	N	S3	AD	-	CD	-	AD	-	CD	-
D	T	H2	x	N	S3								
A	N	S3	x	N	H4	AB	-	BC	-	AB	-	BC	-
B	N	H4	x	N	S3	AC	-	BD	-	AC	-	BD	-
C	T	H4	x	N	S3	AD	-	CD	-	AD	-	CD	-
D	N	S3	x	T	H4								
A	N	P1	x	N	P2	AB	-	BC	2.000	AB	-	BC	0.816
B	N	P1	x	T	P2	AC	2.000	BD	-	AC	0.816	BD	-
C	T	P1	x	N	P2	AD	-	CD	2.000	AD	-	CD	0.816
D	T	P1	x	T	P2								
A	N	P2	x	N	P1	AB	5.003	BC	2.322	AB	1.000	BC	0.876
B	N	P2	x	T	P1	AC	2.657	BD	0.699	AC	0.884	BD	0.450
C	T	P2	x	N	P1	AD	3.229	CD	1.321	AD	0.916	CD	0.808
D	T	P2	x	T	P1								
A	N	P1	x	N	H2	AB	-	BC	-	AB	-	BC	-
B	N	P1	x	T	H2	AC	-	BD	-	AC	-	BD	-
C	T	P1	x	N	H2	AD	-	CD	-	AD	-	CD	-
D	T	P1	x	N	H2								
A	N	H2	x	N	P1	AB	-	BC	-	AB	-	BC	-
B	N	H2	x	T	P1	AC	-	BD	-	AC	-	BD	-
C	T	H2	x	N	P1	AD	-	CD	-	AD	-	CD	-
D	T	H2	x	T	P1								
A	N	P1	x	N	H4	AB	-	BC	-	AB	-	BC	-
B	N	P1	x	T	H4	AC	-	BD	3.464	AC	-	BD	0.926
C	T	P1	x	N	H4	AD	3.464	CD	3.464	AD	0.926	CD	0.926
D	T	P1	x	T	H4								

A	N	H4	x	N	P1	AB	3.997	BC	5.868	AB	1.000	BC	1.000
B	N	H4	x	T	P1			BD	2.474			BD	0.969
C	T	H4	x	N	P1	AC	3.170	CD	9.487	AC	0.990	CD	1.000
D	T	H4	x	T	P1	AD	6.325			AD	1.000		
A	N	P2	x	N	H2	AB	10.232	BC	10.232	AB	1.000	BC	1.000
B	N	P2	x	T	H2	AC	-	BD	10.232	AC	-	BD	1.000
C	T	P2	x	N	H2	AD	-	CD	-	AD	-	CD	-
D	T	P2	x	T	H2								
A	N	H2	x	N	P2	AB	-	BC	3.622	AB	-	BC	0.932
B	N	H2	x	T	P2	AC	3.622	BD	3.883	AC	0.932	BD	0.940
C	T	H2	x	N	P2	AD	3.883	CD	10.786	AD	0.940	CD	1.000
D	T	H2	x	T	P2								
A	N	P2	x	N	H4	AB	5.699	BC	0.487	AB	1.000	BC	0.373
B	N	P2	x	T	H4	AC	2.872	BD	8.301	AC	0.898	BD	1.000
C	T	P2	x	N	H4	AD	3.464	CD	11.570	AD	0.995	CD	1.000
D	T	P2	x	T	H4								
A	N	H4	x	N	P2	AB	3.211	BC	8.635	AB	0.998	BC	1.000
B	N	H4	x	T	P2	AC	8.178	BD	8.635	AC	1.000	BD	1.000
C	T	H4	x	N	P2	AD	8.178	CD	-	AD	1.000	CD	-
D	T	H4	x	T	P2								
A	N	H2	x	N	H4	AB	3.468	BC	4.793	AB	0.999	BC	1.000
B	N	H2	x	T	H4	AC	9.092	BD	4.793	AC	1.000	BD	1.000
C	T	H2	x	N	H4	AD	9.092	CD	-	AD	1.000	CD	-
D	T	H2	x	T	H4								
A	N	H4	x	N	H2	AB	3.143	BC	2.103	AB	0.912	BC	0.960
B	N	H4	x	T	H2	AC	3.761	BD	1.759	AC	3.761	BD	0.907
C	T	H4	x	N	H2	AD	-	CD	4.288	AD	-	CD	1.000
D	T	H4	x	T	H2								

Table B. Values of p and Chi-square of the tests made for total productivity in each group.

Crosses	N	DF	Chi-square	P value
P1 x P1	80	3	45.100	0.000
P2 x P2	83	3	47.072	0.000
H2 X H2	69	3	81.783	0.000
H4 X H4	48	3	96.167	0.000
S1 X H2/H2 X S1	91	3	98.934	0.000
S3 X P1/P1 X S3	30	3	90.000	0.000
S3 X P2/P2 X S3	13	3	39.000	0.000
P2 X P1	90	3	21.733	0.000
P1 X H4	15	3	45.000	0.000
H4 X P1	92	3	53.391	0.000
P2 X H2	29	3	87.000	0.000
H2 X P2	106	3	183.283	0.000
P2 X H4	108	3	68.296	0.000
H4 X P2	84	3	102.667	0.000
H2 X H4	82	3	93.805	0.000
H4 X H2	65	3	78.569	0.000

Table C. Values of p and Chi-square of the pairwise comparisons for productivity of males, females and total in crosses within a group.

		Cmparisons					
		NP1xNP1 x NP1xTP1	NP1xNP1 x TP1xNP1	NP1xNP1 x TP1xTP1	NP1xTP1 x TP1xNP1	NP1xTP1 x TP1xTP1	TP1xNP1 x TP1xTP1
F	Chi-square	21.951	3.000	18.308	20.195	18.460	16.564
	P value	0.041	0.333	0.093	0.032	0.115	0.011
M	Chi-square	14.049	2.000	15.692	15.805	15.540	17.436
	P value	0.064	0.500	0.109	0.041	0.137	0.011
T	Chi-square	36.000	5.000	34.000	36.000	34.000	34.000
	P value	0.000	0.000	0.000	0.000	0.000	0.000
		NP2xNP1 x TP2xNP1	NP2xNP1 x NP2xTP1	NP2xNP1 x TP2xTP1	NP2xTP1 x TP2xNP1	NP2xTP1 x TP2xTP1	NH2xNH2 x TH2xTH2
F	Chi-square	8.077	17.297	13.838	14.490	15.000	30.000
	P value	0.144	0.005	0.051	0.435	0.267	0.033
M	Chi-square	12.923	14.703	18.162	17.510	17.000	15.000
	P value	0.090	0.006	0.039	0.360	0.235	0.067
T	Chi-square	21.000	32.000	32.000	32.000	32.000	45.000
	P value	0.000	0.000	0.000	0.000	0.000	0.000
		NP2xTP2 X TP2xNP2	NP2xTP2 X TP2xTP2	TP2xNP2 X TP2xTP2	NH4xNP1 X NH4xTP1	NH4xNP1 X TH4xNP1	NH4xTP1 X TH4xNP1
F	Chi-square	13.750	15.440	28.170	19.230	22.000	22.310
	P value	0.041	0.386	0.024	0.031	0.182	0.128
M	Chi-square	11.250	9.560	14.830	20.770	18.000	17.690

	P value	0.050	0.623	0.046	0.028	0.222	0.162
T	Chi-square	25.000	25.000	43.000	40.000	40.000	40.000
	P value	0.000	0.000	0.000	0.000	0.000	0.000
		NP2xNH4 X NP2xTH4	NP2xNH4 X TP2xNH4	NP2xTH4 X TP2xNH4	NH4xTH4 X TH4xNH4	NS1xTH2 X TH2xNS1	TH2xNP2 X TH2xTP2
F	Chi-square	26.610	27.760	29.170	19.646	30.820	52.920
	P value	0.006	0.056	0.161	0.138	0.327	0.022
M	Chi-square	23.390	18.240	20.830	21.354	24.180	32.080
	P value	0.006	0.084	0.225	0.127	0.417	0.036
T	Chi-square	50.000	46.000	50.000	41.000	55.000	85.000
	P value	0.000	0.000	0.000	0.000	0.000	0.000
		NH4xNP2 X NH4xTP2	TH2xNP2 X TH2xTP2	NH4xNP2 X NH4xTP2	NH2xNH4 X NH2xTH4	NH4xTH2 X TH4xNH2	
F	Chi-square	31.330	52.920	31.330	27.270	15.220	
	P value	0.057	0.022	0.057	0.110	0.209	
M	Chi-square	24.670	32.080	24.670	24.730	27.780	
	P value	0.072	0.036	0.072	0.121	0.115	
T	Chi-square	56.000	85.000	56.000	52.000	43.000	
	P value	0.000	0.000	0.000	0.000	0.000	