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LUANA WALRAVENS BERGAMO

GENÔMICA POPULACIONAL DA MOSCA-DA-BICHEIRA *COCHLIOMYIA HOMINIVORAX* (DIPTERA:CALLIPHORIDAE)

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Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do Título De Doutora em Genética e Biologia Molecular, na Área de Genética Animal e Evolução.

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RESUMO

A pecuária é uma importante atividade econômica da América do Sul que sofre perdas significativas devido à presença de parasitas. Um importante ectoparasita causador de miíase primária endêmico das Américas é a mosca-da-bicheira, *Cochliomyia hominivorax*. Essa espécie foi erradicada nos Estados Unidos e países continentais da América Central devido à implementação de uma estratégia de controle baseada na técnica do inseto estéril (SIT). No Brasil e demais países da América do Sul o planejamento de um programa de controle semelhante ao SIT seria uma alternativa promissora em relação ao uso exclusivo de inseticidas, que até o momento consiste no método estabelecido para combate da mosca-da-bicheira. Porém, a delimitação de regiões e escalas geográficas é um fator crucial para que o programa seja realizado com sucesso. Estudos que buscam investigar a estrutura genética das populações de *C. hominivorax* e os corredores de conexão entre os grupos identificados mostraram que o padrão de distribuição da espécie é complexo, sendo necessários estudos complementares. Estudos em escala microgeográfica em regiões estratégicas utilizando uma grande quantidade de marcadores se mostram interessantes nesse sentido. Adicionalmente aos marcadores microssatélites e sequências de DNA mitocondrial, é possível obter uma grande quantidade de marcadores moleculares SNPs (do inglês *single nucleotide polymorphism*) para várias amostras a partir da recente técnica de genotipagem-por-sequenciamento (GBS). Diante desse cenário, esta tese teve como objetivo geral investigar a estrutura genética de populações de *C. hominivorax* em escala microgeográfica e temporal, em localidades ao sul da sua atual distribuição, através do uso de diferentes marcadores moleculares, incluindo SNPs. Em escala espacial, considerando duas localidades da fronteira Brasil-Uruguai, não foi observada estrutura populacional. Já em escala temporal, considerando uma população do Uruguai, observou-se estrutura genética e sinais de flutuações populacionais. Esses resultados podem ser associados a diferentes fatores, incluindo disponibilidade de hospedeiros, uso de inseticidas e mudanças climáticas sazonais.

Palavras-chave: DNA mitocondrial, genômica populacional, genotipagem-por-sequenciamento, microssatélites, sequenciamento de nova geração (NGS), SNP, variação temporal.

ABSTRACT

Livestock production is an important economic activity in South America, but has been suffering significant losses due to the impact of parasites. The New World screwworm fly (NWS), *Cochliomyia hominivorax*, is an important ectoparasite and myiasis causing fly endemic from the Americas. This species was eradicated in United States of America and continental part from Central America due to the implementation of the Sterile Insect Technique (SIT). Planning a similar control program in Brazil and other South America countries could be an interesting alternative to the exclusive use of insecticides, which until now is the method established for combating the NWS fly. However, the delineation of regions and geographic scales is a crucial factor to the successful of the program. Studies that investigated the genetic structure of NWS fly populations and the conecction corridors between the identified groups indicated that the distribution pattern of the species is complex, being necessary complementary studies. Studies in microgeographic scale of strategic regions using numerous markers can be interesting in this sense. Additionally to microsatellite and mitochondrial DNA sequences it is possible to obtain high quantity of SNPs markers (Single Nucleotide Polymorphisms) for numerous samples with the recent technique Genotyping-By-Sequencing (GBS). Considering this scenario, the general objective of this thesis was to investigate the genetic structure of *C. hominivorax* populations on a microgeographic and temporal scale, in localities from the southern end of its current distribution, by using different molecular markers, including SNPs. In spatial scale, considering two localities in Brazil-Uruguay border, it was not observed population structure. However, in temporal scale considering one Uruguayan population, genetic structure and signals of population fluctuations were observed. These results can be associated to different factors, including host availability, insecticides use and sazonal climatic changes.

Keywords: genotyping-by-sequencing, microsatellite, mitochondrial DNA, next-generation sequencing (NGS), population genomics, SNP, temporal variation.

SUMÁRIO

INTRODUÇÃO GERAL

1. Pecuária e Economia

A pecuária é uma das principais atividades econômicas do Brasil e da América do Sul. Só no Brasil, os registros oficiais do setor de exportação de carne bovina no ano passado foi equivalente a US$ 6,28 bilhões (ABIEC 2017). Apesar disto, a presença de ecto e endoparasitas representa um importante obstáculo para a criação animal, uma vez que afeta diretamente a produção de carne, leite e a qualidade do couro, além de levar à mortalidade, redução da fertilidade, perda de peso e gastos com o tratamento dos animais infestados (Otranto and Stevens 2002, Vargas-Terán et al. 2005), culminando em consideráveis prejuízos econômicos. Embora não exista uma avaliação conclusiva e oficial sobre as perdas econômicas na produção animal causadas por infestações de ectoparasitas, no Brasil algumas estimativas podem chegar a US$ 6,9 bilhões anuais (Grisi, Leite, Martins, Barros, Andreotti, Cançado, León, Pereira, and Villela 2014).

2. A espécie *Cochliomyia hominivorax*

Neste cenário de perdas econômicas associadas à presença de ectoparasitas, destaca-se a espécie *Cochliomyia hominivorax* (Diptera: Calliphoridae) (Coquerel 1858), conhecida popularmente como mosca-da-bicheira, que é um importante ectoparasita obrigatório causador de miíases das Américas (Guimarães and Papavero 1999). Miíase é a infestação de animais vertebrados por dípteros que em alguma fase do seu desenvolvimento se alimentam de tecidos vivos ou mortos do hospedeiro (Zumpt 1965). As infestações de *C. hominivorax* são notórias principalmente em animais domésticos como gado bovino, ovino e suínos, além da ocorrência de infestações em animais silvestres, como jaguatirica (Pulgar H. et al. 2011), lobo-guará (Cansi et al. 2011), tamanduá e bicho-preguiça (Mastrangelo, 2012, comunicação pessoal).

O ciclo de vida completo da espécie *C. hominivorax* compreende aproximadamente três semanas. As fêmeas fecundadas depositam seus ovos na borda de feridas pré-existentes ou em orifícios do corpo, como olhos, ouvidos e narinas, de animais vertebrados de sangue quente. Após 12 a 24 horas as larvas eclodem e passam a se alimentar dos fluidos corporais e tecidos vivos do hospedeiro por aproximadamente 4 a 8 dias, passando por três estágios larvais, e caem no solo para se transformarem em pupas. O período de pupa dura em média 8 dias, mas pode chegar até dois meses dependendo da temperatura, seguido pela emergência dos adultos. Após um período de 5 a 10 dias, as fêmeas já estão prontas para serem fecundadas e realizar a

oviposição, fechando o ciclo de vida da espécie (Guimarães and Papavero 1999). Uma vez que que as fêmeas precisam encontrar hospedeiros vertebrados vivos para realizar a oviposição, a dispersão é um componente importante da história de vida desta espécie causadora de miíase (Krafsur et al. 1979; Thomas & Mangan 1989). Como estes sítios de oviposição não tem uma localização geográfica definida, a fêmea é o sexo com maior capacidade de dispersão. Mayer & Atzeni (1993), analisando dados de marcação-recaptura, mostraram que *C. hominivorax* dispersa por distâncias relativamente pequenas em ambientes favoráveis (fêmeas: 2,85km, machos: 1,25km, em média), aumentando a distância em aproximadamente 8 vezes em ambientes menos favoráveis (fêmeas: 22,07km, machos: 9,64km, em média), indicando que as distâncias de dispersão são dependentes das condições de habitat locais.

A distribuição original de *C. hominivorax* compreendia desde a região sul dos Estados Unidos da América até a Argentina e o Uruguai (Hall and Wall 1995, Guimarães and Papavero 1999). Porém a distribuição atual da espécie é neotropical, sendo sua ocorrência relatada em todos os países da América do Sul e países da América Central, como Cuba, República Dominicana, Haiti e Jamaica (Hall and Wall 1995). A redução da área ocupada por essa espécie ocorreu devido ao sucesso da implementação da técnica do inseto estéril (SIT-*Sterile Insect Technique*), iniciada na década de 1950 nos EUA (Knipling 1955, Wyss 2000). Essa técnica se baseia na liberação de moscas estéreis no campo, de modo que os machos estéreis irão competir com os machos selvagens pelo acasalamento com as fêmeas. Como as fêmeas são monógamas, ou seja, se acasalam uma única vez, a prole gerada pelo cruzamento com um macho estéril será inviável. Em grande escala, esses acasalamentos que não geram prole viável levam à redução populacional da espécie (Knipling 1955).

3. Implementação de programas de controle: fatores fundamentais

Apesar dos resultados positivos, alguns fatores são fundamentais para o sucesso da implementação de um programa baseado na SIT. Dentre eles, pode-se citar: (i) a necessidade de baixa densidade populacional previamente à liberação das moscas estéreis no campo (Knipling 1979), fator este fortemente dependente do uso de inseticidas químicos na primeira etapa do programa de controle (ver seção 3.1.); e (ii) a delimitação de regiões e escalas geográficas adequadas (Tabachnick and Black 1995), que podem ser determinadas a partir de estudos sobre a estrutura genética das populações de interesse (ver seção 3.2.).

3.1. Controle por inseticidas e resistência

Na América do Sul, o controle dos ectoparasitas é realizado através do uso de inseticidas químicos. O uso indiscriminado desses compostos tem levado ao aumento da frequência de indivíduos resistentes, tornando assim o controle através dos mesmos cada vez mais desafiador (Georghiou 1990, Feyereisen 1995). Os mecanismos moleculares de resistência a inseticidas envolvem predominantemente a alteração da sensibilidade do sítio alvo para que não seja inibido pelo inseticida ou a desintoxicação metabólica do produto químico antes que atinja o sítio alvo. Sozinhos ou em combinação, esses mecanismos conferem resistência a todas as classes de inseticidas disponíveis (Hemingway et al. 2004). Um exemplo de alteração da sensibilidade ao sitio alvo é a modificação do sítio de ligação da enzima acetilcolinesterase (AChE), impedindo que os inseticidas fosforilem uma serina no seu sítio alvo e inibam irreversivelmente a enzima, que tem função de hidrolisar o neurotransmissor acetilcolina após a sinapse química. Mecanismos de desintoxicação metabólica através de amplificação gênica, regulação transcricional e mutações em genes codificando as enzimas carboxilesterases, citocromo P450 monooxigenases (P450) e glutationa S-transferases (GST) têm sido identificados como mecanismos de resistência em diversas espécies (Li et al. 2007).

O controle de infestações causadas por *C. hominivorax* é feito majoritariamente com inseticidas da classe dos organofosforados (OP), que são divididos nos sub-grupos dietil-OPs e dimetil-OPs, e o principal mecanismo de resistência parece ocorrer através de alterações qualitativas no gene da carboxilesterase E3, denominado *ChαE7*, através de duas substituições de aminoácidos (Carvalho et al. 2006; Carvalho et al. 2010a), que resultam em uma habilidade para hidrolisar substratos organofosforados em detrimento dos substratos carboxilésteres (Newcomb et al. 1997a; Newcomb et al. 1997b; Campbell et al. 1998; Claudianos et al. 1999). A substituição de uma glicina para aspartato na posição 137 (G137D) da proteína E3 confere um largo espectro de resistência a OPs, principalmente dietil-OPs (Newcomb et al. 1997b). Já a substituição do aminoácido triptofano na posição 251 por uma leucina ou serina (W251L e W251S) tem sido associada a um baixo nível de resistência aos dietil-OPS e alto nível de resistência a outro grupo de inseticidas OPs, os dimetil-OPs, e pode estar relacionada/envolvida com resistência cruzada a inseticidas piretróides (Heidari et al. 2005). Essas mutações foram detectadas em uma ampla região do Brasil e Uruguai, apresentando frequências elevadas em diversas localidades (Carvalho et al. 2010b; Bergamo et al. 2015), contribuindo para a diminuição da eficiência dos inseticidas contendo compostos organofosforados, representados por 42 dos 59 produtos registrados junto ao Ministério da Agricultura Pecuária e Abastecimento (MAPA) (Sindan 2010). Dessa maneira, o monitoramento das mutações associadas à resistência

se mostra uma ferramenta de extrema importância, capaz de prever a eficiência da primeira etapa do programa de controle baseado na SIT. Um estudo sobre a frequência destas mutações em populações de *C. hominivorax* está sendo realizado em nosso laboratório (projeto de Iniciação Científica PIBIC/CNPq), incluindo amostras de 7 localidades do Maranhão, 9 localidades da Amazônia, 1 localidade do Mato Grosso do Sul, 10 localidades do Peru e uma análise temporal nos anos de 2015 e 2016 de uma localidade do Uruguai. O objetivo é obter um mapeamento das duas mutações do gene da carboxilesterase E3 (*ChaE7*) nestas localidades, uma vez que este é o principal mecanismo de resistência genética detectado pela aplicação de organofosforados para o controle desta espécie no Brasil e demais países da América do Sul.

3.2. Estudos populacionais

Como já citado acima, outro importante fator para o sucesso de um programa de controle baseado na SIT é a delimitação de regiões e escalas geográficas adequadas (Tabachnick and Black 1995) a partir de estudos sobre a estrutura genética das populações. No caso da espécie *C. hominivorax*, estudos vem sendo realizados há alguns anos com base em diversos marcadores moleculares (RFLP, RAPD, alozimas, PCR-RFLP, microssatélites, sequências de genes mitocondriais e nuclear) e têm dado embasamento para distintas hipóteses sobre a estrutura populacional e a distribuição da diversidade genética (Infante-Vargas and Azeredo-Espin 1995, Infante-Malachias et al. 1999, Lyra et al. 2005, 2009, Azeredo-Espin and Lessinger 2006, Torres and Azeredo-Espin 2009, Fresia et al. 2011, 2013, 2014, Mastrangelo et al. 2014, Bergamo et al. 2015).

Em escala continental, a distribuição atual da espécie se divide em quatro grupos regionais: (1) Cuba (CG); (2) República Dominicana (DRG); (3) Jamaica, Trinidad e Tobago, Colômbia, Equador e Venezuela, definido como norte da região amazônica (NAG-North Amazon Group) e (4) sul da região amazônica (SAG-South Amazon Group) (Fresia et al. 2011). O processo de expansão populacional da espécie parece ter se iniciado na região norte, tendo ocorrido uma primeira separação entre o grupo da América do Norte/Central e o grupo da América do Sul, após o Último Máximo Glacial, seguida de uma segunda separação entre os grupos regionais do Norte e do Sul da região Amazônica (NAG e SAG) entre o Pleistoceno e o Holoceno (Fresia et al. 2013). A região SAG apresenta baixa diferenciação populacional sem uma clara correlação geográfica (Fresia et al. 2011, Bergamo et al. 2015), provavelmente associado ao processo de expansão populacional (Fresia et al. 2013). Os grupos NAG e SAG não compartilham haplótipos mitocondriais, indicando o isolamento entre as regiões norte e sul da América do Sul (Fresia et al. 2013), provavelmente associado a uma barreira ao norte da

bacia Amazônica uma vez que o norte e o sul da bacia compartilham haplótipos apenas com populações do sul do Brasil (Mastrangelo et al. 2014). As populações da bacia Amazônica apresentam variáveis níveis de diferenciação e alta diversidade genética (Mastrangelo et al. 2014). Fortes corredores de conexão entre as regiões NAG e SAG foram preditos na região próxima ao Oceano Atlântico e também na região entre o Noroeste do Brasil e o Peru (Fresia et al. 2014), apesar das limitações metodológicas e ausência de amostragem nessas regiões.

Em escala regional dentro do grupo SAG, localidades do Uruguai foram estudadas e parecem se comportar como uma única população panmítica (Lyra et al. 2005) ou estão pouco estruturadas (Torres 2006).

Adicionalmente ao estudo espacial, o estudo temporal, em que populações são amostradas em períodos diferentes com o intuito de observar o comportamento temporal das frequências alélicas, também é importante para o melhor entendimento da dinâmica populacional da espécie. Segundo Lyra (2008), os eventos históricos e demográficos que influenciam a dinâmica de populações de *C. hominivorax* poderiam ser melhor investigados através de estudos microgeográficos e temporais.

Os estudos de genética de populações realizados na espécie *C. hominivorax* citados anteriormente obtiveram informações relevantes para a compreensão da dinâmica da espécie tanto em escala local quanto continental. Apesar disso, o padrão de distribuição da espécie é complexo e ainda não foi completamente compreendido. Estudos em escala microgeográfica em regiões estratégicas tanto do ponto de vista ecológico e evolutivo quanto de atividade pecuária, com diferentes tipos de marcadores e em maior quantidade, foram conduzidos nesta tese na tentativa de ajudar a esclarecer esse padrão e o entendimento da dinâmica populacional da espécie.

4. Genômica populacional

Uma área da genética que tem crescido consideravelmente nos últimos anos é a genômica populacional que, segundo Luikart et al. (2003), consiste no uso de vários locos ou regiões genômicas para entender os processos evolutivos que influenciam a variação genômica e populacional, como deriva genética, fluxo gênico, mutação e seleção natural. Para Luikart et al. (2003), uma grande quantidade de locos representativos do genoma deveria ser obtida de maneira simples, através de um único experimento, em um estudo de genômica populacional.

Com os avanços das técnicas de sequenciamento de nova geração (NGS – *next generation sequencing*) foi possível a elaboração de inúmeras estratégias para a obtenção de milhares a centenas de milhares de marcadores de uma grande porção do genoma de uma

maneira relativamente simples, como sugerido por Luikart et al. (2003), e de menor custo. Várias estratégias que descobrem os marcadores (*single nucleotide polymorphisms* - SNPs) e fazem a genotipagem dos indivíduos simultaneamente foram desenvolvidas (Davey et al. 2011), sem a necessidade de um genoma referência. Dentre elas estão (i) RNA-seq, (ii) captura de sequência (*Sequence capture*) e (iii) baseada em métodos que utilizam enzimas de restrição para reduzir o tamanho do genoma a ser amostrado (Davey et al. 2011). Uma revisão elaborada de cinco métodos dessa última estratégia foi feita por Davey et al. (2011), com destaque para o RAD-seq (*Restriction-site Associated DNA sequencing*) (Baird et al. 2008) e o GBS (G*enotyping-By-Sequencing*) (Elshire et al. 2011).

Os métodos RAD-seq e GBS descritos por Baird et al. (2008) e Elshire et al. (2011) possuem várias semelhanças, como por exemplo a digestão de múltiplas amostras de DNA genômico por uma enzima de restrição; a ligação de adaptadores com *barcodes* para a identificação dos indivíduos; a mistura de vários indivíduos em um único *pool* de amostras (*multiplexing*); a seleção ou redução dos fragmentos digeridos; e o sequenciamento por NGS do conjunto final de fragmentos (Davey et al. 2011). Basicamente, a diferença entre os dois métodos é que no GBS não há nenhum passo intermediário de seleção dos fragmentos (corte aleatório e seleção por tamanho dos fragmentos) como no RAD-seq. Porém, em anos posteriores à difusão destes métodos, novas variantes foram surgindo com pequenas alterações no protocolo original (e.g. Peterson et al. 2012 e Poland et al. 2012), de modo que os métodos RAD-seq e GBS convergiram e hoje esses termos são considerados equivalentes. Ao longo da tese, o termo GBS será utilizado para se referir a essas técnicas de maneira generalizada (e.g. GBS, RAD-seq, ddRAD-seq).

Apesar do potencial da técnica de GBS, poucos estudos com insetos as utilizaram até o momento, com objetivos variados. No caso da espécie *Timema cristinae*, um estudo obteve 86.130 SNPs para oito populações naturais, que foram capazes de detectar quais fatores estavam envolvidos na divergência genômica desse sistema em processo de especiação ecológica (Nosil et al. 2012). Nesta mesma espécie foram obtidos 186.576 SNPs com o objetivo de verificar mudanças nas frequências alélicas durante um experimento de associação inseto-hospedeiro e encontrou tanto mudanças devido à deriva genética quanto à seleção (Gompert et al. 2014). Em torno de 10.000 SNPs foram obtidos para as espécies de abelha *Bombus impatiens* e *Bombus pensylvanicus*, cujos índices de diversidade sugerem histórias demográficas um pouco divergentes das inferidas com base em microssatélites (Lozier 2014). Populações de *Aedes aegypti* do Rio de Janeiro foram analizadas com marcadores mitocondriais, microssatélites e 1496 SNPs, de modo que os marcadores nucleares revelaram baixa estrutura espacial enquanto

os marcadores mitocondriais revelaram alta estrutura (Rašić et al. 2015). Um estudo com a espécie de mariposa *Grapholita molesta* utilizou 1.226 SNPs e detectou estrutura genética populacional, indicando que esses marcadores são informativos para este tipo de estudo com insetos praga (Silva-Brandão et al. 2015). Outro estudo com inseto praga, a espécie de mariposa *Helicoverpa armigera*, utilizou aproximadamente 20.000 SNPs para estudar populações em vários continentes de sua ocorrência e foi capaz de aumentar a resolução da estrutura populacional em comparação com marcadores mitocondriais e detectar fluxo gênico (Anderson et al. 2016). Um estudo de estrutura populacional baseado em 15.123 SNPs foi conduzido para duas espécies da ordem Plecoptera, a espécie alada *Zelandoperla decorata* que ocorre em regiões de baixa altitude e a espécie não-alada *Zelandoperla fenestrata* que ocorre em regiões de alta altitude, e os resultados suportam a hipótese de que a perda das asas pode iniciar um processo de diversificação das populações (Dussex et al. 2016). Pavinato et al. (2017) desenvolveram um protocolo para obtenção de SNPs na espécie de broca da cana-de-açúcar *Diatraea saccharalis*. Uma baixa estruturação genética com base em 6.461 SNPs foi observada para populações de *Anopheles moucheti* dos Camarões (Fouet et al. 2017). Ragland et al. (2017) utilizaram 10,256 SNPs para investigar a associação entre a diapausa e o processo de diferenciação de raças na mosca *Rhagoletis pomonella*. Brunet et al. (2017) utilizaram 2.218 SNPs para investigar os padrões de diferenciação do complexo de espécies de mariposas *Choristoneura fumiferana*, *C. occidentalis* e *C. biennis* e rever a classificação em espécies e subespécies.

Nesta tese é apresentado o primeiro trabalho que implementou a estratégia de GBS para a espécie praga *C. hominivorax*. O objetivo deste trabalho foi realizar a padronização do protocolo de GBS e verificar o potencial da utilização dos SNPs em escala microgeográfica para esta espécie, com a perspectiva de futuramente poder ser implementado em estudos populacionais em escala regional e macrogeográfica.

OBJETIVO GERAL

A presente tese teve como objetivo central investigar a estrutura populacional e a dinâmica temporal de *C. hominivorax* em localidades ao sul da sua distribuição atual utilizando diferentes marcadores moleculares e, assim, complementar estudos prévios e fornecer dados para o estabelecimento de unidades de manejo necessárias para o planejamento de uma eficiente estratégia de controle.

OBJETIVOS ESPECÍFICOS

1. Investigar a variabilidade genética de *C. hominivorax* em uma região da fronteira entre o Brasil e o Uruguai, região em escala microgeográfica localizada ao sul da distribuição da espécie e onde foi realizado um projeto piloto de controle em 2009;
2. Investigar a variação temporal (intervalo de 1 ano) de uma população de *C. hominivorax* do Uruguai (Cañas, Cerro Largo);

APRESENTAÇÃO DA TESE

Esta tese está organizada em três capítulos, que são precedidos por uma seção denominada "Material e Métodos" em que é apresentada, de maneira geral, a metodologia empregada em cada um deles.

O Capítulo 1 se trata de um capítulo de livro em processo de publicação, em que é apresentada uma revisão bibliográfica completa dos estudos populacionais e filogeográficos de *C. hominivorax* na América do Sul e Caribe, definindo um cenário consenso atual para a distribuição da variabilidade genética da espécie.

O Capítulo 2 apresenta um estudo em escala local com amostras de *C. hominivorax* de duas localidades adjacentes da fronteira entre Brasil e Uruguai, amostragem essa que foi realizada em 2009 concomitantemente à execução de um projeto piloto de controle da espécie. A partir dos resultados obtidos, é discutida a escala geográfica mais adequada para se determinar unidades de manejo, aspecto importante para a elaboração de um programa de controle efetivo. Esse capítulo está submetido à publicação em revista indexada internacional.

O Capítulo 3 apresenta um estudo temporal de uma população de *C. hominivorax*, em que foram utilizados três marcadores moleculares diferentes (i.e. sequências de DNA mitocondrial, microssatélites e SNPs) com o intuito de detectar variações temporais nas frequências alélicas e flutuações nos tamanhos populacionais.

Ao final da tese, é apresentada uma discussão geral dos resultados obtidos nos três capítulos e uma análise preliminar, com amostras do Maranhão e Peru, baseada no cenário

obtido. Com isso, pretendeu-se enriquecer a discussão sobre a dinâmica populacional e auxiliar no delineamento da continuidade dos estudos populacionais da espécie.

MATERIAL E MÉTODOS

1. Organismos de estudo

Conforme já descrito anteriormente, a mosca-da-bicheira *Cochliomyia hominivorax* é um ectoparasita obrigatório causador de miíases, de modo que a fase larval se alimenta dos fluidos corporais de animais vertebrados (Guimarães and Papavero 1999). Dessa maneira, pelo fato das larvas não se deslocarem independentemente (o deslocamento pode ocorrer caso haja o transporte do hospedeiro), a coleta de larvas dessa espécie se torna mais simples do que a coleta da fase adulta (fotos de ambas as fases são apresentadas na Figura 1). Por conta disso, todas as amostras utilizadas no presente projeto eram de estágio larval, em diferentes fases.

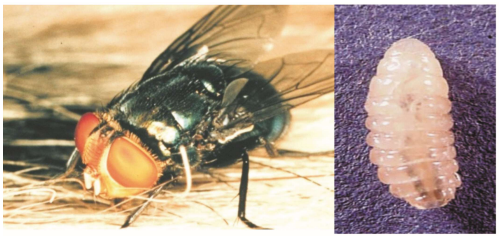

Figura 1. Adulto (esquerda) e larva (direita) de *Cochliomyia hominivorax*.

2. Desenho amostral

As coletas de *C. hominivorax* para os trabalhos apresentados nos capítulos 2 e 3 foram realizadas em localidades ao sul da distribuição atual da espécie: em duas localidades na fronteira entre Brasil e Uruguai e uma outra localidade no Uruguai (Figura 2), respectivamente, sendo esta última amostrada temporalmente com um intervalo de aproximadamente 1 ano.

Foram obtidas também amostras de diferentes localidades do estado do Maranhão, através de colaboração com o Prof. Dr. Livio Martins Costa-Júnior (Departamento de Patologia, Universidade Federal do Maranhão), e do Peru, em colaboração com Robin Manuel Gamarra Madueño (Servicio Nacional de Sanidad Agraria, SENASA, Peru) (Figura 2).

Figura 2. Pontos de coleta de *Cochliomyia hominivorax*. Os pontos indicados em vermelho são as localidades amostradas para os trabalhos dos capítulos 2 e 3 da tese. Os pontos em azul e verde são as localidades amostradas no Peru e Maranhão, respectivamente, cujos resultados preliminares são apresentados na "Discussão Geral".

3. Marcadores moleculares e análises

Nos dois manuscritos presentes nesta tese, foram utilizadas sequências de DNA mitocondrial e locos microssatélites, marcadores que já haviam sido previamente empregados com sucesso em outros trabalhos (Torres et al. 2004, Torres and Azeredo-Espin 2005, 2009, Fresia et al. 2011, Mastrangelo et al. 2014, Bergamo et al. 2015). No capítulo 3, em que é apresentado um estudo temporal de uma localidade do Uruguai, utilizamos ainda marcadores SNPs obtidos com o uso da técnica de GBS com duas enzimas de restrição (Poland et al. 2012). Esse é o primeiro trabalho a empregar o uso dessa técnica e dos SNPs para o estudo de *C. hominivorax*.

Em ambos os manuscritos apresentados nos capítulos 2 e 3, fizemos análises de diversidade, estrutura populacional e inferências demográficas. No capítulo 2 essas análises foram conduzidas em âmbito espacial, na tentativa de determinar a menor escala geográfica em que ocorre diferenciação populacional. Já no capítulo 3, o foco foi investigar a variação

temporal das frequências alélicas de uma população e, assim, tentar detectar sinais de mudanças no tamanho populacional e levantar hipóteses acerca dessas mudanças.

Na discussão geral do trabalho, são apresentados também os resultados preliminares de análises conduzidas para as amostras do Peru e Maranhão, utilizando sequências de mtDNA. Essas duas regiões são importantes por terem sido preditas como corredores de conexão entre as regiões NAG e SAG (Fresia et al. 2014) e pretendemos utilizar esses dados para complementar tal estudo.

CAPÍTULO 1

Phylogeography and insecticide resistance of the new world screwworm fly in South America and the Caribbean

(Capítulo em fase de publicação no livro "Area-wide Integrated Management: Development and Field Application" – J. Hendrichs, R. Pereira e M. J. B. Vreysen, Editora Springer)

BOOK CHAPTER:

Phylogeography and insecticide resistance of the new world screwworm fly in South America and the Caribbean

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SUMMARY

Insect pests have a widespread negative impact on livestock production, resulting in large economic losses. Monitoring and surveillance of pest species are fundamental to manage their populations and reduce the damage they inflict on livestock. In addition, resistance to pest control methods, such as the use of insecticides, is becoming an increasingly important issue. Inferring population structure, the phylogeographic pattern of pest species, and the connectivity among populations is key to understanding migration patterns, which can be used to delineate area-wide pest surveillance and management schemes such as the sterile insect technique (SIT). This review provides a summary of phylogeographic patterns of the New World screwworm (NWS) fly, Cochliomyia hominivorax Coquerel, a myiasis-causing fly that leads to significant losses in livestock production, based on molecular markers and the monitoring of insecticide resistance to improve its management. The species' current geographic distribution comprises most of the Neotropical region, having been eradicated in North and Central America after area-wide integration of the SIT with other methods. Introducing similar management programmes in South America and the Caribbean could be a strategic alternative to the permanent and exclusive use of insecticides, which has a negative environmental impact and is a growing challenge because of increasing resistance development in NWS. Such an area-wide approach requires NWS population delineation at regional and geographic scales, and the monitoring of mutations involved in insecticide resistance in natural populations.

Key Words: Carboxylesterase, insecticide resistance, management unit, microsatellites, mitochondrial DNA, population structure

1. INTRODUCTION

Successful eradication of the New World screwworm (NWS) fly Cochliomyia hominivorax Coquerel from North and Central America, using an area-wide integrated pest management (AW-IPM) (Klassen 2005) approach that included a sterile insect technique (SIT) component, has triggered discussions about its potential eradication in the Caribbean and South America (Vargas-Terán et al. 2005). However, the high livestock density and wildlife distribution in the NWS fly's current habitat area, with the geographical and environmental settings including large rainforests, wetlands, and huge grasslands, make area-wide management and eventual eradication a great challenge.

The efficient area-wide management of a pest requires the control of all its target populations in a delimited geographic region, requiring a minimum area sufficiently large to

guarantee that natural dispersion only occurs inside it (Klassen 2005). E. F. Knipling (1972) showed that the survival of a small remnant fraction of the population (i.e. 1% of the original population) is enough for it to recover to a density capable of causing economic damages in a few generations. In this sense, the delimitation of adequate target regions and geographic scales is extremely important as well the understanding of gene flow pattern among populations (Tabachnick and Black 1995). Several studies, reviewed below, have aimed to characterize the NWS fly population structure and infer gene flow patterns at different geographic scales, from local to continental, providing a basis for distinct hypotheses about the distribution of genetic variability and its possible effects on control strategies.

Another important requisite for the effective application of the SIT is a low density of the target field populations (Knipling 1979). Due to the relatively high density of NWS populations in some local situations (Krafsur et al. 1985), complementary actions need to be taken to ensure their reduction prior to the release of sterile insects. Wound and myiasis treatment, which relies on the application of insecticides (e.g. organophosphates and pyrethroids), is the standard method to reduce NWS fly populations in the first step of a management programme (reviewed in Mangan 2005; Vargas-Terán et al. 2005). However, chemical treatment will not succeed if populations are resistant to the used compounds. Thus, studies that aimed to discover the main genes involved in NWS fly insecticide resistance and monitor the frequencies of mutations in the genes associated with this resistance in natural populations are also reviewed here.

2. POPULATION GENETICS AND PHYLOGEOGRAPHY

Over the last three decades, technological advances in molecular biology have led to the introduction of many types of molecular markers to assay genetic variation. Accompanying these advances, the genetic variability and structure of NWS fly natural populations in South America and the Caribbean region have been extensively studied and characterized (see Table 1 for a summary).

2.1. NWS Population Genetic Studies from South America and Caribbean Region

Restriction fragment length polymorphism of mitochondrial DNA (mtDNA RFLP) was the first method used and a seasonal analysis of a single population from Brazil (Caraguatatuba, São Paulo) indicated a high genetic heterogeneity for some restriction sites over time, with seven haplotypes exclusively found during summer and fall (Azeredo-Espin 1993). A study of four other populations from the same state, São Paulo, showed 15 haplotypes,

with a small number of haplotypes widely distributed and a large number that appeared to be local (Infante-Vargas and Azeredo-Espin 1995). Similarly, Infante-Malachias et al. (1999) explored the nuclear genome with Random Amplified Polymorphic DNA (RAPD) markers, and detected moderate genetic differentiation among 6 populations from south-eastern Brazil and one from northern Argentina.

Subsequently, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis was used to characterize mtDNA variation in Caribbean, Central, and South American NWS fly populations (Taylor et al. 1996). Fourteen mtDNA haplotypes were observed among 18 flies, indicating high variability. These haplotypes, based on phenetic analysis, were divided into three discontinuous assemblages: "North and Central America", "South America", and "Jamaica". Notably, the Cuban sample seemed to be more closely related to Central American populations, while Dominican Republic samples were grouped with those from South America, suggesting a scenario of multiple origins of the NWS fly throughout the Caribbean.

Table 1. New world screwworm fly population genetic studies from South America and Caribbean region.

Reference	Region	Marker	Var.	F_{ST}
Azeredo-Espin (1993)	South-eastern BR	RFLP	-	-
Infante-Vargas and Azeredo-Espin (1995)	South-eastern BR	RFLP	H	-
Infante-Malachias et al. (1999)	Northern AR, South-eastern BR	RAPD	-	0.122
Taylor et al. (1996)	CB, CR, DR, JM, TT, Southern BR	PCR-RFLP	H	-
Lyra et al. (2005)	UY	PCR-RFLP	H	0.145*
Torres et al. (2007)	UY	SSR	M	0.031
Torres and Azeredo-Espin (2009)	CB, DR, JM, TT	SSR	M/H	0.157
Griffiths et al. (2009)	BR, JM, TT, UY	SSR	M/H	-
McDonagh et al. (2009)	BR, CB, CO, DR, EC, JM, PE, TT, US, UY, VE	mtDNA, Nuc	-	-
Lyra et al. (2009)	BR, CB, CO, DR, EC, JM, PY, TT, UY, VE	PCR-RFLP	L/H	0.130
Fresia et al. (2011)	AR and Lyra et al. (2009)	mtDNA	H	0.496
Fresia et al. (2013)	BL, CR, MX, US and Fresia et al. (2011)	mtDNA	-	0.155-0.718
Mastrangelo et al. (2014)	Amazon Basin BR	mtDNA, SSR	H	0.24(mtDNA) 0.099(SSR)
Fresia et al. (2014)	Fresia et al. (2011) and Mastrangelo et al. (2014)	mtDNA	-	-

Var., variability; F_{ST}, fixation index; SSR, microsatellites; mtDNA, mitochondrial DNA; Nuc, nuclear marker; H, high; M, moderate; L, low; AR, Argentina; BL, Belize; BR, Brazil; CO, Colombia; CB, Cuba; CR, Costa Rica; DR, Dominican Republic; EC, Ecuador; JM, Jamaica; MX, Mexico; PY, Paraguay; PE, Peru; TT, Trinidad and Tobago; UY, Uruguay; US, United States of America; VE, Venezuela. *Value not statistically significant.

The mtDNA variation was also investigated by PCR-RFLP in 7 populations from Uruguay (Lyra et al. 2005). High genetic variability and no evidence of subpopulation differentiation were observed, indicating the existence of a single panmictic population. This lack of differentiation was attributed to the absence of geographical and/or climatic barriers and to the fact that Uruguay is almost at the southern extreme of the species' distribution. These same populations from Uruguay were also investigated by Torres et al. (2007) using nuclear microsatellites. A moderate degree of polymorphism and an excess of observed homozygosity were found, which could have been caused by demographic changes in response to the decrease in temperature and humidity in the Uruguayan winter and/or persistent insecticide treatment. It is likely that the low population differentiation was caused by passive migration of larvae through the movement of infested animals, or by recent recolonization events.

Microsatellite markers were also used to investigate 10 populations from 4 Caribbean islands (Torres and Azeredo-Espin 2009) and, contrary to expectations, the level of genetic variability of some Caribbean populations was not lower than that of continental samples. In fact, moderate to high levels of genetic variability and a high level of population differentiation were found, even among populations within the same island.

Despite small sample sizes, an analysis of 9 populations from South America and the Caribbean islands found microsatellite differences between Trinidad and Jamaica, and in relation to the mainland (Griffiths et al. 2009). Population structure in mainland South America was more difficult to describe, but some weak signals of structure were detected, suggesting that population differentiation may exist between NWS flies from at least some areas.

McDonagh et al. (2009), utilizing the sequences of two mitochondrial (COI and 12S) and one nuclear (EF1α) gene investigated the phylogenetic relationship of NWS fly populations from the Caribbean, South America, and Texas ("historical" North American samples). This study found that NWS fly populations of the Caribbean islands were structured, and suggested a period of isolation and/or founder effects following colonization from South America. The data did not support a North American origin of the Cuban NWS population, as previously hypothesized by Taylor et al. (1996). The NWS samples from Texas were in a different lineage as compared with South American and Caribbean samples, indicating a possible north-south division.

Lyra et al. (2009) conducted the first study on a continental-scale that encompassed NWS fly populations covering its entire distribution area. Thirty-four populations from 10 South American and Caribbean countries were analysed using mitochondrial PCR-RFLP. Population structure with significant fixation indices and low variability were found in the

Caribbean, indicating that island populations have been evolving independently due to geographical isolation, but are connected by restricted gene flow. In contrast, mainland populations presented high genetic variability and low differentiation, with no correlation of genetic and geographic distances. The moderate and non-homogeneous level of genetic differentiation of the NWS fly in its current distribution area, as well as its high genetic variability, was described as being the product of several historical demographic processes.

In order to highlight and test the results obtained by Lyra et al. (2009), the same NWS fly samples and samples from four other populations were investigated using mtDNA sequences (Fresia et al. 2011). This study found that genetic diversity is distributed in four main groups of populations, corresponding to Cuba (CG), the Dominican Republic (DRG), and North and South Amazon regions (NAG and SAG, respectively). This phylogeographic structure of the NWS populations over its entire range was characterized by distinct historical events: (i) island colonization from mainland (a North American and/or Central American colonization was suggested for Cuba, whereas the other Caribbean islands were colonized from South America); (ii) recent separation of NAG and SAG probably associated to a barrier in the Amazon region resulting in separate populations in NAG and SAG; and (iii) population expansion that started approximately 20–25,000 years ago and that increased exponentially up to date. This expansion was probably linked with climatic oscillations in the late Pleistocene and resource availability. The population expansion probably caused the low divergence detected within SAG, erasing genetic and geographic correlations even among distant populations (maximum distance of 10,000 km).

In analysing mtDNA sequences from 60 populations (see Figure 1), a North to South colonization was proposed for the continental Americas (Fresia et al. 2013). According to the best population divergence model chosen by Approximate Bayesian Computation (ABC), a first split occurred between North/Central American and South American populations at the end of the Last Glacial Maximum. A second split occurred between the North and South Amazonian populations in the transition between the Pleistocene and Holocene eras. The NWS fly went through a population expansion during its dispersal toward its current geographic range, with the strongest signals in SAG. This work concluded that climatic oscillations only were not sufficient to explain the phylogeographic patterns observed, and human activity might have played a crucial role in shaping the current distribution of the NWS fly.

The most recent survey of genetic variability was conducted on under-explored NWS populations of the highly important region in Amazonia, in an attempt to better understand the NAG-SAG evolutionary relationships (Mastrangelo et al. 2014). Based on 3

mtDNA genes and 8 microsatellite loci, a high genetic diversity and differentiation was revealed among 9 populations. These Amazonian populations only share mtDNA haplotypes with SAG, suggesting that the NAG-SAG split is the result of a barrier in the north of the Amazon Basin rather than of the basin environment itself.

Finally, pairwise F_{ST} among South American NWS fly populations were mapped with a Geographical Information System (GIS) on a friction layer derived from the MaxEnt niche modelling in order to identify connection corridors between NAG and SAG (Fresia et al. 2014). Despite methodological limitations, it was possible to identify two strong connections between the populations of the NAG and SAG: one along the Atlantic Ocean passing through the Northwest of Brazil and the other passing through Peru. The main limitations for this approach are the sampling strategy based mainly on larvae, because it does not capture with precision the adults´ habitat, and the genetic distances estimation based only on mitochondrial DNA sequences.

2.2. Consensus Scenario and Main Conclusions

Synthesizing the results from the previous studies presented above, we established the distribution of genetic diversity and population structure of the NWS populations in the Caribbean and South America (Figure 1).

Caribbean populations are structured (Taylor et al. 1996; Griffiths et al. 2009; Lyra et al. 2009; McDonagh et al. 2009; Torres and Azeredo-Espin 2009; Fresia et al. 2011) and several events hypothetically resulted in their current distribution, such as Cuba having been originally colonized by North and/or Central American populations and the other Caribbean islands colonized by South American populations (Torres and Azeredo-Espin 2009; Fresia et al. 2011). However, the lack of congruence between nuclear (Torres and Azeredo-Espin 2009) and mtDNA (Lyra et al. 2009) genetic diversity in the Caribbean suggests a complex scenario of population structure.

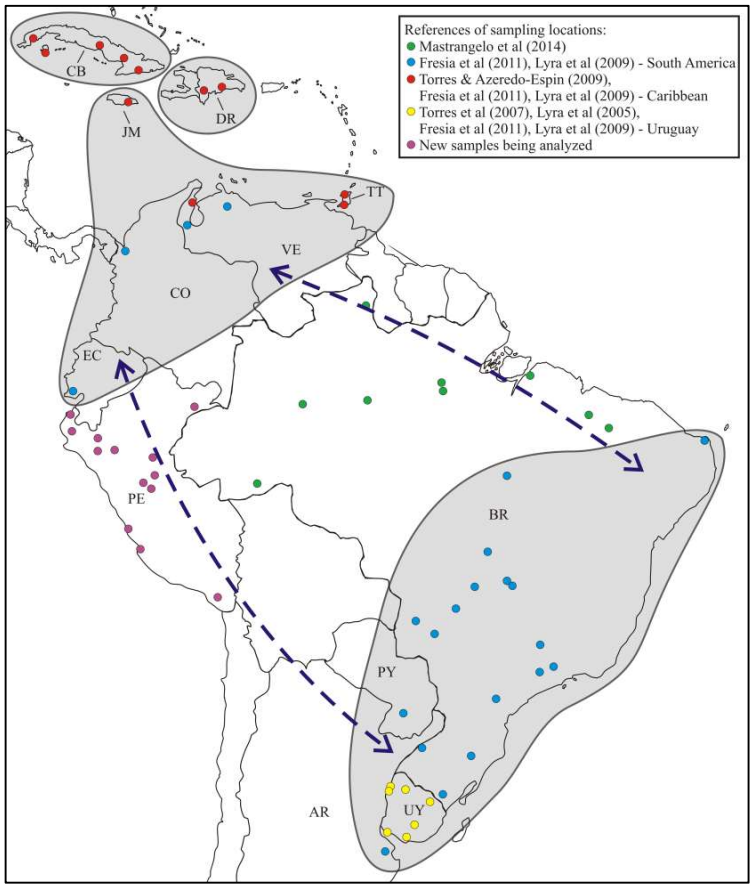

Figure 1. Consensus map showing sampled populations (coloured points), and current population structure scenario (the four main groups are highlighted in grey: Cuba, Dominican Republic, and North and South Amazon), and predicted connection corridors (dashed arrows) for NWS fly populations from the Caribbean and South America.

Unlike the Caribbean populations, South American patterns of genetic variability and structure are not completely clear, but, in general, populations present a high genetic variability and low differentiation with no correlation to geographic distance. There are two distinct genetic groups, NAG and SAG (Fresia et al. 2011), probably separated by a barrier in the north of the Amazon Basin (Mastrangelo et al. 2014) during the transition between the Pleistocene and Holocene eras (Fresia et al. 2013). Populations experienced an expansion during the North-South colonization, mainly SAG, which is probably the cause of its low genetic divergence. All these historical factors and climatic oscillations are important to explain the pattern observed in South America, but current factors may also be influencing it, such as livestock movement and human activity.

Results of NWS phylogeography and population genetics studies can be of relevance to the operation of SIT programmes (Krafsur 1985). However, the significant genetic differences found in these studies do not result in mating incompatibility. Strains from three

different locations in Brazil (i.e. Pará state, in the Amazon basin; Piauí state, in the northeast; and São Paulo state, in the southeast) were crossed and showed no significant differences in all biological parameters assessed and no evidence of hybrid dysgenesis (Mastrangelo et al. 2014). Similarly, the crossing between a Brazilian strain from Goiás state in central Brazil, and the Jamaica-06 wt-strain, which is currently being mass-reared in Panama, did not show any evidence of genetic incompatibility or hybrid dysgenesis (Mastrangelo et al. 2012). The absence of mating incompatibility indicates that sterile males from the Jamaica strain reared in Panama could be used in future SIT-based control programmes throughout Brazil and, possibly, South America.

Target management units still need to be determined within the NAG and SAG large geographic distribution area and a better understanding of the distribution of genetic variability in Amazonia is required before considering starting an AW-IPM programme against NWS in these regions. Regional-scale studies in South America were conducted only in Uruguay, a region that coincides with the southernmost distribution of the species, and different degrees of population polymorphism and structure were reported (Lyra et al. 2005, Torres et al. 2007). These differences can be associated with the distinct molecular markers used (Table 1), as they present different modes of inheritance (the effective size of mtDNA populations is one-quarter the size of nuclear DNA populations) and/or mtDNA can present a sex-biased gene flow among the populations.

2.3. Perspectives

In 2009, a study was carried out in a 100 x 60 km area situated at the Brazil-Uruguay border with samples collected during a pilot SIT project against the NWS (Pontes et al. 2009). The high genetic diversity and absence of population structure indicate that the target population limits are certainly larger than the pilot area, and consequently, the management unit should be larger than this pilot project.

Population analyses can be further refined through the use of new and more genetic markers. To reach this goal, we standardized a Genotyping-By-Sequencing (GBS) protocol for the species and the sequencing of the first library, which contains samples from one Uruguayan population, resulting in approximately 1,000 filtered single-nucleotide polymorphisms (SNP). Another library is being constructed with individuals from the same population that were sampled one year later. After generating these data, we aim to evaluate if the obtained SNPs will give an increased resolution for temporal population genetic analyses in comparison to other molecular markers (mtDNA and microsatellites).

Recently the evolutionary relationships and the phylogeographic structure of populations from the Northwest of Brazil and Peru (i.e. the predicted corridor connecting NAG and SAG (Fresia et al. 2014)) were investigated using samples of 13 NWS populations from Peru that were obtained with the assistance of the Servicio Nacional de Sanidad Agraria (SENASA)-Peru (Figure 1), and three mitochondrial regions (COI, COII, and CR) are being sequenced. Preliminary results suggest the presence of genetically distinct groups with some geographic isolation, high haplotype diversity, low nucleotide diversity, and significant negative values of Tajima's D and Fu's Fs, indicating population expansion.

3. INSECTICIDE RESISTANCE

3.1. Investigation of the Molecular Basis of Resistance Mechanisms

NWS fly management throughout South America is mostly carried out independently on each farm and the farmer decides on the used control strategy. Topical insecticide application on livestock is the most popular and effective suppression method, and two main classes of compounds are used, i.e. organophosphates (OPs) and pyrethroids, which can be applied separately or in combination (Coronado and Kowalski 2009; SINDAN 2010).

A decrease in carboxylesterase activity has been observed in OP-resistant strains of some arthropod species (Van Asperen and Oppenoorth 1959; Towsend and Busvine 1969; Hughes and Raftos 1985), that has resulted in the formulation of a mutant ali-esterase hypothesis. This suggests that a structural mutation in a carboxylesterase results in a reduced ability to hydrolyse aliphatic ester substrates, but also in an acquired ability to hydrolyse OP substrates (Claudianos et al. 1999).

In the Australian sheep blowfly, *Lucilia cuprina* (Wiedemann), which belongs to the same family Calliphoridae as the NWS fly, the *LcαE7* gene encodes the ali-esterase E3 isozyme. Biochemical assays with proteins produced by different *LcαE7* alleles showed that an amino acid substitution at position 137 (Gly137Asp) abolished the ali-esterase activity and increased diethyl-OP hydrolase activity, while a second amino acid substitution (Trp251Leu) increased dimethyl-OP hydrolase activity (Campbell et al. 1998). These two amino acid substitutions confer insecticide resistance because they are part of the active site of the enzyme (Newcomb et al. 1997, Campbell et al. 1998).

Based on these previous studies and in view that OPs are commonly used to suppress the NWS fly, the E3 gene in this species (*ChαE7*) was partially characterized (Carvalho et al. 2006, Carvalho et al. 2009). Mutations at the positions responsible for conferring OP resistance in *L. cuprina* (Gly137 and Trp251) were identified, but unlike with *L.*

cuprina, NWS fly samples with a mutation in the Trp251 residue showed the substitution of a tryptophan for a serine. It is suggested that this new substitution has the same effect of reducing esterase activity (Taşkin et al. 2004) and may also be involved in pyrethroid resistance and be the molecular basis of cross-resistance between OPs and pyrethroids (Heidari et al. 2005). The strong association between this mutation (Trp251Ser) and dimethyl-OP resistance was later confirmed (Carvalho et al. 2010a).

Population genetic analyses assessed the selective pressures that have shaped carboxylesterase E3 evolution in NWS (Bergamo et al. 2015) and found a negative association between the Gly137Asp and Trp251Ser mutations. Fay & Wu's H value was significantly negative for the exons in which these mutations occur, which suggests that the E3 gene has evolved under positive selection, which is indirect evidence of its role in insecticide resistance.

This association between carboxylesterase E3 mutations and insecticide resistance were not directly proven by bioassays. Only the study involving bioassays by Silva and Azeredo-Espin (2009) indicated a correlation between the Trp251Ser mutation and moderate resistance to the pyrethroid cypermethrin. However, the high conservation of mutations in this gene among dipteran species suggests that the same resistance mechanism could have evolved in the NWS fly. Moreover, mutation-mediated resistance conferred by the E3 gene appears to be the main resistance mechanism selected in this species.

Other mechanisms of insecticide resistance were also investigated for the NWS fly: point mutations in the sodium channel, known as "knockdown resistance" (kdr) (Silva and Azeredo-Espin 2009); point mutations in acetylcholinesterase (AChE) (Carvalho et al. 2010a, Silva et al. 2011); changes in the expression levels of glutathione S-transferases and cytochrome P450 monooxygenases (Carvalho et al. 2010a); and glutamate-gated chloride channels (Lopes et al. 2014). However, no evidence of their association to insecticide resistance was detected.

3.2. Field Monitoring of Mutations in the Carboxylesterase E3 Gene Associated with Organophosphate Insecticide Resistance in South America

In view of the mutations of the carboxylesterase E3 gene that were identified as an important insecticide resistance mechanism in the NWS fly, the characterization of this gene in natural populations of the species throughout its current geographic distribution area can be an important tool for area-wide monitoring of resistance to insecticides. This information can then be used to select and implement more effective pest management programmes.

The Trp251Ser and Gly137Asp mutations were screened in ten NWS fly populations from Brazil, Colombia, Cuba, Paraguay, Uruguay and Venezuela (Silva and

Azeredo-Espin 2009; Silva et al. 2011, respectively). Although sample size was small, with only one population from each country (except for Brazil), the Trp251Ser mutation was detected in all populations. In Brazil, allelic frequencies varied from 15.6% to 46.7%. In Cuba, the frequency was 16.7%. In Uruguay, where the use of pyrethroids seems to be common, the frequency was 28.1%, while the highest frequencies were found in Colombia and Venezuela (93.7% and 100%, respectively). The Gly137Asp mutation, however, was not detected in Colombia, Cuba, and Venezuela, although it was present in high frequencies in Brazilian and Uruguayan NWS populations.

The changes in the frequency of both mutations in three different regions of Uruguay in two years (2003 and 2009) were investigated by Carvalho et al. (2010b). The NWS populations of the three regions showed high frequencies of mutated alleles, but whereas the frequency of the Gly137Asp mutation was reduced in 2009 as compared with 2003, the frequency of the Trp251Ser mutation was significantly higher in 2009. This change is probably associated with the current intense use of pyrethroids and dimethyl-OP compounds for NWS fly control in Uruguay.

Analysis of the structure of 21 NWS populations in the SAG area showed three distinct population groups when considering the carboxylesterase E3 gene, with some differences related to both mutation frequencies (Bergamo et al. 2015). Resistant genotypes were observed in high frequencies in all sampled areas, but the frequency of the Trp251Ser and Gly137Asp mutations was higher at lower and higher latitudes.

There is a need for further resistance monitoring studies that would cover the largest possible area of the current distribution of the NWS fly, in addition to studies that would measure changes in temporal frequencies of mutations associated with insecticide resistance. However, the studies presented above clearly indicate that insecticide resistance is widespread throughout studied South American NWS populations.

3.3. Perspectives

Frequencies of both mutations of the E3 gene associated with OP resistance are being monitored in strategic regions of South America that have not been analysed before. The first region of interest is Amazonia, whose NWS populations showed, based on our preliminary results, a considerable frequency of mutant individuals (24% and 16% of the Gly137Asp and Trp251Ser mutations, respectively). The other important region that is currently being analysed for both E3 mutations is Peru, which is located along a putative connection corridor for the

species (Fresia et al. 2014) and consequently can be a key region for the spread of resistance mutations among populations.

4. CONCLUSIONS

Identification of isolated populations or groups of populations is very important to determine target management units for effective AW-IPM programmes of the NWS fly in its current geographic distribution area. Many insights on genetic variability, population structure, and even migration patterns have been obtained, but, except for the Caribbean islands, the identified mainland areas (NAG and SAG regions) are very large and have no identifiable barriers that limit NWS dispersion. The identification of restricted areas within NAG and SAG will be essential for the success of NWS area-wide programmes, both for managing the logistics of implementing the SIT and other suppression methods, and also for the economic implications.

Furthermore, monitoring the spread of insecticide resistance among NWS fly natural populations is equally important, as the effective use of insecticides will be necessary for population suppression activities as part of future area-wide management programmes that integrate the SIT. However, already the current resistance scenario represents a significant challenge.

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CAPÍTULO 2

High genetic diversity and no population structure of the New World Screwworm fly *Cochliomyia hominivorax* (Coquerel, 1858) (Diptera: Calliphoridae) on a microgeographic scale: implications for management units

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ORIGINAL ARTICLE:

High genetic diversity and no population structure of the New World Screwworm fly *Cochliomyia hominivorax* (Coquerel, 1858) (Diptera: Calliphoridae) on a microgeographic scale: implications for management units

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Abstract

The New World Screwworm (NWS) fly *Cochliomyia hominivorax* is an important livestock pest endemic to the Americas that has been eradicated from North and continental Central America with a control program based on the Sterile Insect Technique (SIT). The establishment of target management units is a strategic step in the implementation of new control programs, which can be achieved using genetic studies of natural populations. Previous studies of NWS fly populations were conducted on the continental scale and identified four main groups: two in South America and two in the Caribbean. However, studies within these groups are needed to determine which smaller geographic areas can be treated as management units. Here, we analyze the genetic variability distribution and the population demographic signals of the NWS fly in a 6,000 km^2 area located along the border of Brazil and Uruguay. This area was the subject of the first control pilot program conducted in South America. We studied eight microsatellite loci and sequences from two mitochondrial DNA regions in individuals sampled at 20 to 25 livestock breeding farms. We observed no population structure and found high genetic variability on the geographical scale sampled for both molecular markers. Our microsatellite data suggest that these populations are not in equilibrium, and demographic analyses based on mitochondrial data indicate population expansion. These results suggest that this geographic scale is not adequate for future NWS fly management in South America.

Key-Words: Control program, genetic diversity, local population scale, management unit, South America.

Introduction

Cochliomyia hominivorax (Coquerel, 1858) (Diptera: Calliphoridae), the New World screwworm (NWS) fly, is an obligatory ectoparasite endemic to the Americas that causes severe economic losses in the livestock industry (Guimarães et al. 1982, Guimarães and Papavero 1999). This myiasis-causing fly has a variety of warm-blooded vertebrate hosts, ranging from humans to wildlife, but is most evident in livestock. The life cycle of the species comprises approximately 21 days: gravid female flies lay their eggs in wounds or natural cavities of a host and the emerging larvae feeds on living tissue, causing injuries to the host and consequently declining the production and quality of milk and meat (Guimarães et al. 1982, Hall and Wall, 1995); after four to eight days, passing through three instars, larvae fall to the ground and pupate, with adults emerging after an average of 8 days (Guimarães et al. 1982).

Historically, the geographic distribution of NWS fly extended from the southern USA to Uruguay and Argentina (Hall and Wall 1995). This insect pest has been fought for over 60 years with Area-Wide Integrated Pest Management (AW-IPM) approaches involving the Sterile Insect Technique (SIT) (Wyss 2000, Klassen 2005, Klassen and Curtis 2005, Vargas-Terán et al. 2005) and the current distribution of the species includes some Caribbean islands and South America (Klassen and Curtis 2005). The SIT involves the release of sterile flies into field populations; sterile males mate with wild females, preventing their mating with wild males and, consequently, their reproduction (Robinson 2005). On a large scale, matings that do not generate viable offspring lead to population reduction and ultimately to the extinction of the species (Knipling 1955).

NWS fly control programs were first successfully conducted in the USA (1957 - 1966), followed by Mexico (1972-1991), Guatemala (1988-1994), Belize (1988-1994), El Salvador (1991-1995), Honduras (1991-1995), Nicaragua (1992-1999), Costa Rica (1995-2000) and Panama (1997-2000), where a permanent biological barrier has been maintained. In Caribbean islands, control programs were implemented in Curaçao (in 1954 and again in 1976, after a reintroduction), Puerto Rico (1975), the US Virgin Islands (1971-1972) and the British Virgin Islands (1971-1972) (see Vargas-Terán et al. 2005). However, this pest continues to represent a serious problem in Cuba, Dominican Republic, Haiti and Jamaica, despite the attempts to establish suppression and eradication programs (Vargas-Terán et al. 2005). In addition, the presence of the NWS fly in these regions facilitates possible reintroductions in the free areas.

According to Klassen (2005), control of all populations of a given pest within a delimited geographic area is necessary for its efficient management. Knipling (1972) showed

that the survival of a small fraction of a pest population is enough for the pest to recover and cause economic damage within a few generations. Therefore, establishing populations or groups of populations delimited geographically (i.e., management units) and estimating gene flow patterns are important tasks for the development of effective pest control strategies (Tabachnick and Black 1995). In this way, population genetic studies can provide important information for planning and evaluating the implementation of AW-IPM programs based on SIT (Krafsur 2005).

In recent years, population genetics studies have been conducted in current areas of NWS fly occurrence (i.e., Caribbean islands and South America) with the goals of analyzing the geographical distribution of genetic diversity and understanding the demographic history of the species. Currently, the species is known to be distributed among four main regional genetic groups: two in the Caribbean region (Cuba and the Dominican Republic), and two in South America (the North Amazon region and South Amazon region) (Lyra et al. 2009, Fresia et al. 2011).

Within the South Amazon region group, only two studies were conducted on a smaller, regional scale (Lyra et al. 2005, Torres et al. 2007). Both studies analyzed populations from Uruguay. Lyra et al. (2005) used mitochondrial DNA (mtDNA) markers and showed no population structure for the species, while Torres et al. (2007) used microsatellites markers and showed little but significant levels of subdivision between populations. The differences were attributed to the different inheritance patterns of the markers used, but the authors also suggested that there might be sex-biased gene flow among these populations and noted the need to study the species at different geographic scales (Torres et al. 2007).

Considering this scenario, conducting microgeographic (i.e., local scale) studies is important to improve our knowledge of NWS fly populations, thereby improving our understanding of the species' population dynamics and limits and helping to identify management units for control.

A NWS fly control pilot program was conducted between January and May 2009 at the Brazil-Uruguay border in an area of 100x60 kilometers encompassing the municipalities of Quaraí (Brazil) and Artigas (Uruguay). The aim of the project was to transfer the technology applied previously in other countries and to evaluate the viability of its implementation. Over the course of 13 weeks, sterile flies produced in Mexico were released and the characteristics of the flies and egg masses were quantified. After this period, approximately 21.5% of analyzed egg masses gave rise to unviable offspring, which was considered a promising result (Pontes et al. 2009).

Here, we investigated the genetic variability, population structure and demographic history of NWS flies in the small area where this pilot program was conducted, using both mitochondrial DNA sequences and microsatellites. We aimed to gain insight into neighborhood size and dimensions of the management units necessary for implementing an effective control program on this landscape.

Materials and Methods

Study area

The study area is located at the border between Brazil and Uruguay and comprises the municipalities of Artigas (Uruguay) and Quaraí (Brazil). The area comprises 100 km in length by 60 km in width (30 km within each country). Geographic locations of the sampling sites (i.e., livestock breeding farms) are indicated in Figure 1. Details about the sampling sites (names, ID codes and coordinates) are presented in Supplementary Table 1.

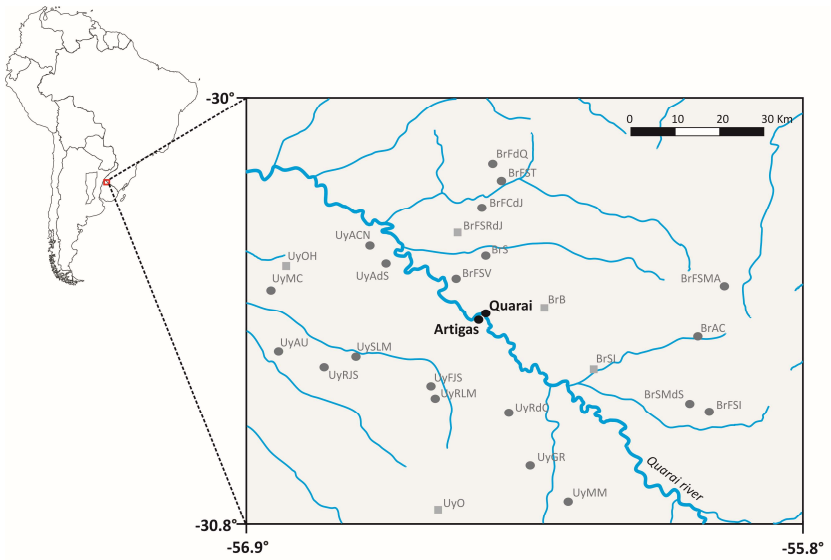

Figure 1. Sampling locations from the local scale study. The closest view of the area in the borders of Brazil and Uruguay shows the 25 NWS fly sampling sites. Sites represented by squares were only analyzed with mitochondrial data. In blue are the main rivers and streams found in the region.

Data collection

Larvae of the NWS fly were collected from January to March 2009, during the control pilot program at the Brazil-Uruguay border. Second and third instar larvae were collected in the wounds of sheep and cattle at 25 different farms (Figure 1) and fixed in 100% ethanol. Sampling at each farm was carried out on different animals, on only one wound per animal. Despite NWS fly larvae being morphologically similar to *Cochliomyia macellaria*

(Fabricius 1775), the last is a secondary screwworm that feed on putrefying meat and very rarely appears in wounds.

Only one larva per wound was used for the total genomic DNA extraction, which was conducted using a standard phenol:chloroform method adapted for microcentrifuges (Lyra et al. 2009). The extractions were stored at -20°C until further use.

We amplified and sequenced two mitochondrial fragments - the B domain of the control region (CR) (Lessinger and Azeredo-Espin 2000) and the cytochrome c oxidase subunit II gene (COII) - using primers and conditions described in Fresia et al. (2011). Purified PCR products were sequenced using a BigDye Terminator Cycle Sequencing Kit (version 3.0, Applied Biosystems, Foster City, CA, USA) in an ABI 3730 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA) at Centro de Biologia Molecular e Engenharia Genética, Universidade Estadual de Campinas (UNICAMP; Campinas, SP, Brazil).

Eight polymorphic microsatellite loci (Torres et al. 2004; Torres and Azeredo-Espin 2005 – CH01, CH05, CH09, CH12, CH14, CH15, CH24 and CH26) were independently amplified by PCR following the steps described in Torres et al. (2004) and Torres and Azeredo-Espin (2005). Reaction products were verified via 6% denaturing polyacrylamide gels stained with silver nitrate. Positive amplicons for each locus were multiplexed (4-plex) and sequenced using an ABI 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Mitochondrial haplotype reconstruction and microsatellite genotyping

The sequences of CR and COII were aligned independently using ClustalX software (Thompson 1997) and manually inspected in Mega 6 (Tamura et al. 2013). The COII fragment was translated to protein in Mega 6 (Tamura et al. 2013) to confirm the open reading frame. Individual sequences of both fragments (CR and COII) were collapsed into haplotypes with Fabox (Villesen 2007) and compared to those obtained in previous works (Fresia et al. 2011, Mastrangelo et al. 2014, Bergamo et al. 2015). Each insertion/deletion (*indel*) in the CR fragment was considered a single mutational step and recoded as a single position in the final alignment in Mega 6 (Tamura et al. 2013) before haplotype collapsing.

Microsatellite alleles were genotyped by size using the software GeneMarker 2.4.2 (SoftGenetics, LLC, PA, EUA) and were also checked manually. The presence and frequency of null alleles were estimated with the software Microchecker (Van Oosterhout et al. 2003), which also adjusted the allelic and genotypic frequencies.

Genetic diversity

The haplotype diversity ($\hat{H}$) and nucleotide diversity (π) were estimated with the software Arlequin 3.5 (Excoffier and Lischer 2010) for mitochondrial concatenated sequences.

The genetic variability of microsatellite loci was accessed through different parameters: the mean number of alleles per locus and allelic richness (AR) were calculated with MSA 4.05 (Dieringer and Schlotterer 2003), thereby allowing a comparison of the variability among the different localities independent of sample size. The observed (Ho) and expected (He) heterozygosities were obtained using the software GenAlex 6.5 (Peakall and Smouse 2012). Departures from Hardy-Weinberg equilibrium were tested for each microsatellite locus with the web version of Genepop (Raymond and Rousset 1995, Rousset 2008) considering the probability-test (i.e. the ‘exact HW test’ of Guo and Thompson 1992). The Markov chain (MC) algorithm was used to obtain a non-bias estimate of the exact P-value of this test, following the parameters of 1,000 series of 1,000 iterations with 1,000 steps of dememorization.

Haplotype network and population structure

Relationships between haplotypes generated with the concatenated sequences (CR-COII) were inferred with Haploviewer (Salzburger et al. 2011), using a maximum parsimony tree reconstructed using DNApars extension from Phylip 3.6 package (Felsenstein 2005).

A Bayesian clustering analysis implemented in Structure 2.3.3 (Pritchard et al. 2000) was performed for microsatellite data set only. This analysis considered the admixture model with correlated allele frequencies, 100,000 MCMC replications, and 10,000 burn-in, with K varying from 1 to 10 and with 10 iterations for each value of K. The number of genetic groups (K) that best fit the data was determined using the method of Evanno et al. (2005) with Structure Harvester 0.6.94 software (Earl & Von Holdt 2012). The results from the Structure runs for the best K value were summarized with Clumpp 1.1.2 software (Jakobsson and Rosenberg 2007) and graphically represented using Distruct 1.1 (Rosenberg 2004).

Demographic inferences

The demographic history of the species was inferred from mitochondrial data with Tajima’s D (Tajima 1989) and Fu’s Fs (Fu 1997) neutrality tests, which were calculated using Arlequin 3.5 (Excoffier and Lischer 2010). The statistical significance of Tajima’s D was obtained by two-tailed tests, while that of Fu’s Fs was determined by coalescence, with 1,000 replicates and a confidence interval of 95%.

Results

Mitochondrial diversity

The final alignment consisted of 431 bp of the CR fragment and 493 of the COII fragment.CR sequences were obtained for 218 individuals and collapsed into 69 haplotypes, of which 35 have not been reported previously (GenBank accession numbers: MG581836 to MG581870). COII sequences were obtained for 209 individuals and collapsed into 30 haplotypes, 18 of which were new compared to the studies indicated above (GenBank accession numbers: MG581818 to MG581835). The previously reported COII and CR haplotypes are well distributed geographically and have been reported in Uruguay, many regions of Brazil, Paraguay, Argentina, Venezuela, Colombia, Jamaica and Ecuador (Fresia et al. 2011, Fresia et al. 2013, Mastrangelo et al. 2014, Bergamo et al. 2015). Sequences from both fragments were concatenated for 201 individuals, resulting in 921 bp collapsed into 91 haplotypes to be used in the population analyses. Of the 91 haplotypes, named here as H1 to H91, 67 were unique and 24 were observed in more than one individual. The two most frequent haplotypes were found in 45 (22.4%) and 24 (11.9%) samples (Supplementary Table 2)

The mitochondrial data from our study area is characterized by high haplotype diversity ($\hat{H}$) and low nucleotide diversity (π) (Table 1). In each sampling site, $\hat{H}$ ranged from 0.50 to 1.00 (mean=0.919) and π ranged from 0.0005 to 0.0087 (mean=0.006) (Supplementary Table 2).

Table 1. Mitochondrial DNA diversity indices when considering all sampling sites together.

Sampling site	N	Nh	Haplotypes (no of individuals)	$\hat{H}$ (s.d.)	π (s.d.)
All	201	91	H1 (45), H2 (24), H3 to H5 (6), H6 (5), H7 (4), H8 to H11 (3), H12 to H24 (2), H25 to H91 (1)	0.9327 (0.0126)	0.005880 (0.003148)

N: number of individuals; Nh: number of haplotypes; $\hat{H}$: haplotype diversity; π: nucleotide diversity.

Nuclear genetic diversity

Genotyping of the eight microsatellite loci was performed for samples from 20 of the 25 sampling locations (Figure 1; locations UyOH, UyO, BrFSRdJ, BrB and BrSI were excluded due to low sampling ($N < 4$)). Samples that failed to amplify two or more loci were excluded from the analysis, resulting in a final dataset of 190 individuals.

Null alleles were detected and allelic frequencies were subsequently adjusted for almost all loci found in at least one sampling site, with the exception of the locus CH05 (Supplementary Table 3). Genetic diversity and population structure analyses were conducted

based on these adjusted allelic and genotypic frequencies. Allele frequencies for each locus at each sampling site are presented in Supplementary Table 4.

When considering all sampling sites together, most of the loci showed high genetic diversity (Table 2), with a mean observed heterozygosity of 0.58 and expected heterozygosity of 0.72. Significant departures from Hardy-Weinberg equilibrium were observed for 6 of 8 tests after Bonferroni correction ($p<0.006$).

Table 2. Genetic variability of microsatellite loci for all sampling sites together.

Sampling Site		Loci							
		CH01	**CH05**	**CH09**	**CH12**	**CH14**	**CH15**	**CH24**	**CH26**
All	**N**	178	187	173	188	162	133	175	180
(N=190)	**Na**	10	10	6	15	9	8	12	16
	Ho	0.494	0.679	0.341	0.707	0.463	0.511	0.583	0.867
	He	0.682**	0.675	0.528**	0.834**	0.650**	0.812**	0.730**	0.892*

N: sample size; Na: number of alleles per locus; Ho: observed heterozygosity; He: expected heterozygosity. Significant departures from Hardy-Weinberg equilibrium were indicated by asterisks in the value of He. * $p < 0.05$; ** $p < 0.005$

Population structure and Demographic inferences

The haplotype network from the concatenated sequences revealed a star-like topology, with two main haplotypes widespread in the region (H1 and H2) and connected to numerous less frequent haplotypes (Figure 2), illustrating the presence of at least two genetic groups. However, these genetic groups were not geographically structured, as illlutraded by the colors in the Figure 2.

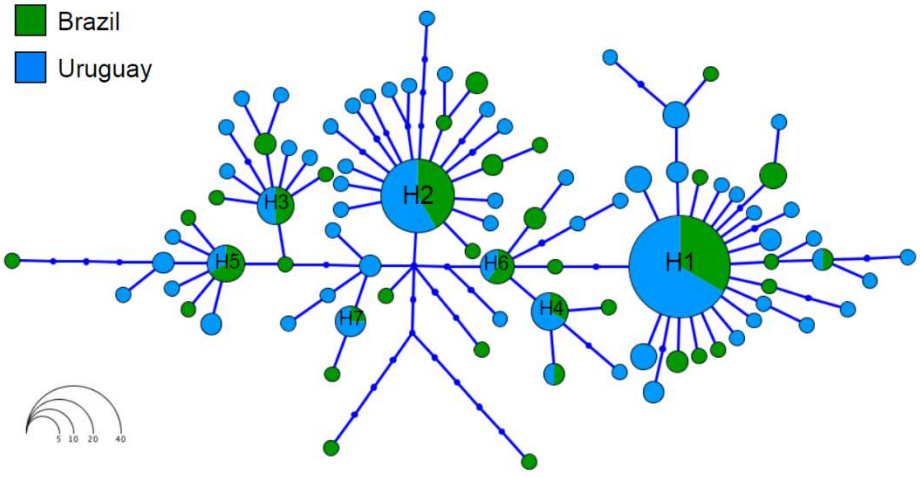

Figure 2. Mitochondrial DNA haplotype network of concatenated sequences (CR-COII). Circles sizes are proportional to the number of samples related to each haplotype and circle colors indicate the country of origin of the samples. Only most frequent haplotypes (N>4) are indicated by names. For details see Supplementary Table 2.

Evanno et al.'s (2005) method indicated K=3 as the best group scenario to Structure assignment tests with microsatellite data, which suggests the presence of 3 possible genetic groups (Supplementary Figure 1). However, plotting our results for this K value revealed no geographic structure between samples (Figure 3), indicating that they represent a unique panmictic population.

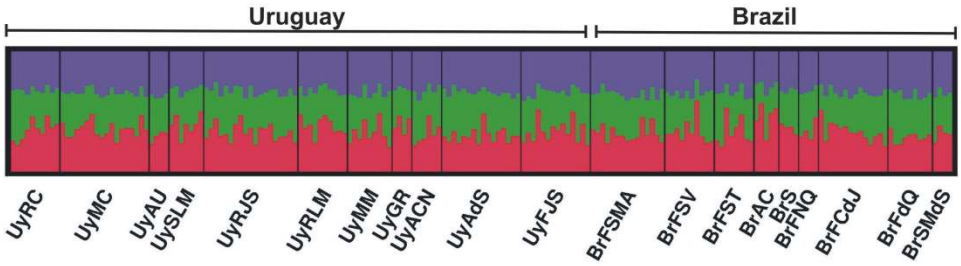

Figure 3. Microsatellite Structure analysis result for K=3. Each color represents a genetic group, defined by the analysis. Vertical lines represent individuals, which are colored proportionally to their probability of assignment to each genetic group.

Both neutrality tests resulted in significant negative values (Tajima´s D = -1.85346 (p-value=0.008) and Fu´s Fs = -25.05329 (p-value=0.000)), which are indicative of population expansion.

Discussion

Livestock production in Brazil and Uruguay is an important economic activity that suffers considerable annual losses due to the NWS fly (Vargas-Terán et al. 2005, Grisi et al. 2014). The present study is the first one to investigate the genetic variability and population structure of the NWS fly on a local scale at the border between Brazil and Uruguay in the Southern region of South America. This study provides first insights for evaluating whether this local scale could be considered a management unit for future control programs.

NWS fly samples from this local scale presented high genetic diversity and showed a signature of population expansion, but lacked geographic population structure. In the case of the mtDNA data, the haplotype ($\hat{H}$ = 0.919) and nucleotide (π = 0.00588) diversities observed are similar to the ones found in other NWS fly population studies, involving many countries of the current NWS fly distribution (i.e. Brazil Uruguay, Paraguay, Argentina, Ecuador, Colombia, Venezuela, Trinidad & Tobago, Jamaica, Dominican Republic and Cuba) (Fresia et al. 2011, Mastrangelo et al. 2014). Although the nucleotide diversity found here is not so low compared to other 23 invertebrate species (Goodall-Copestake et al. 2012), the haplotype and

nucleotide diversities indices patterns we report in the present work reflects in the star-like topology of the mtDNA haplotype network, which is typical in populations that have undergone recent population expansion (Avise 2000). This scenario of population expansion is corroborated by the neutrality tests results, as well as by the mismatch distribution (Supplementary Figure 2). Despite the presence of at least two major genetic groups in the haplotype network, no geographical population structure was detected (also corroborated by pairwise Φ_{ST} analysis - Supplementary Figure 3). Similarly, signals of population expansion and absence of geographic structure were observed in the South Amazon regional group (Lyra et al. 2005, 2009, Fresia et al. 2011), with expansion beginning approximately 20-25,000 years ago (Fresia et al. 2011).

For microsatellite loci, variability was moderate (Ho and He ranging from 0.341 to 0.867 and from 0.528 to 0.892, respectively) and Structure analysis indicates absence of population structure (also supported by pairwise F_{ST} analysis - Supplementary Figure 3). Torres et al. (2007), considering samples from seven geographic locations throughout Uruguay (i.e., on the regional scale), observed a moderate degree of polymorphism for these microsatellite loci (Ho ranging from 0.19 to 0.91 and He ranging from 0.37 to 0.87), as well as low but significant population structure ($F_{ST} = 0.031$, $p < 0.001$). When we considered all sampling sites to be a unique panmictic population for our microsatellite dataset, significant deviations from Hardy-Weinberg equilibrium were observed in almost all cases (Table 2), in which we observe excess of homozygotes (i.e. the observed heterozygosity is lower than expected). This pattern is not a result of linkage disequilibrium between loci (Supplementary Table 5) but could be the result of many distinct factors, either separately or in combination.

One such factor could be sampling effects (i.e. Wahlund effects), once our samples were collected from many distinct farms. However, we did not found structure among these farms (Supplementary Figure 3) and homozygote excess is also observed for some loci in some of these farms (Supplementary Table 6), suggesting that we could have sampled only few families in these locations, reflecting the mating system (i.e. inbreeding, distinct levels of reproductive fitness among individuals and/or sex ratio biases). Another factor that could also explain the pattern observed is the presence of null alleles. Null alleles are alleles at microsatellite loci that failed to be amplified by PCR due to mutations in primer annealing sites (Callen et al. 1993) and, consequently, they can lead to a misinterpretation of the number of heterozygotes at the population level. However, there is no direct relation between the loci with deviations in each locality and the presence of null alleles. Furthermore, fluctuations in population size between generations is an important factor that can be caused by climate

variation and/or natural selection mediated by insecticides (Bergamo et al. 2015). This last explanation of demographic changes is congruent with the one suggested by Torres et al. (2007) for the homozygote excess observed for these loci.

In the present study, the local scale includes the Southern distribution of the NWS fly species, which is characterized by cold autumns and severe winters followed by the warm months of spring and summer. This climatic regime can lead to seasonally fluctuating populations, with population retraction and expansion cycling according to the season, as proposed by Shpak et al. (2009). In this scenario, some individuals survive in small refuges during fall and winter; when spring arrives and the climate is more favorable, these initially small isolated demes expand, increasing gene flow, and after some time overlap, behaving similar to a unique population. Although we cannot directly test this with our data, the absence of population structure, high genetic diversity, deviations from Hardy-Weinberg equilibrium, and signs of recent population expansion collectively indicate that seasonal fluctuations in effective size are plausible. According to Vargas-Terán et al. (2005), NWS fly population size suffers variation during the year (which comprises approximately 17 generations, considering a generational time of 21 days) and fly populations are greater in size during the hot and humid season. Under this assumption, NWS fly populations in Uruguay would begin expanding in September-October and would reach their maximum size in February-March, the period coincident with the sampling effort detailed in this study. Testing this hypothesis of seasonal population fluctuation requires a specific experimental design (i.e. collecting samples from more localities in many periods, including all seasons of the year), additional molecular markers such as SNPs and conducting analyses of model selection that were capable to compare distinct demographic scenarios, such as Approximate Bayesian Computation methods (ABC) (Beaumont et al. 2002).

The results presented in this work indicate that the local scale should not be considered as a target management unit because no structure was found and there is likely to be gene flow with other locations at a larger scale. Based on this finding and the previous study of Torres et al. (2007), which detected a significant degree of population structure in Uruguay, the regional scale seems more appropriate than the local scale as a management unit for establishing a pest control program. However, further studies at other regional scales in South America should be conducted to determine whether this assumption holds for all continental distributions of the NWS fly.

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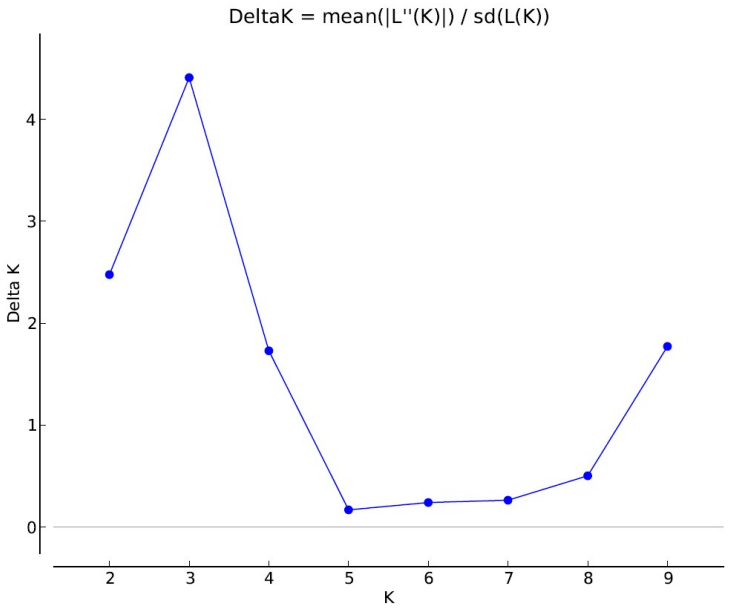
Supplementary Information

Supplementary Figure 1.



Supplementary figure 1. Result of the Evanno et al.'s (2005) method, obtained with Structure Harvester 0.6.94 software (Earl & Von Holdt 2012). This graph is indicating K=3 as the best group scenario to Structure assignment tests with microsatellite data.

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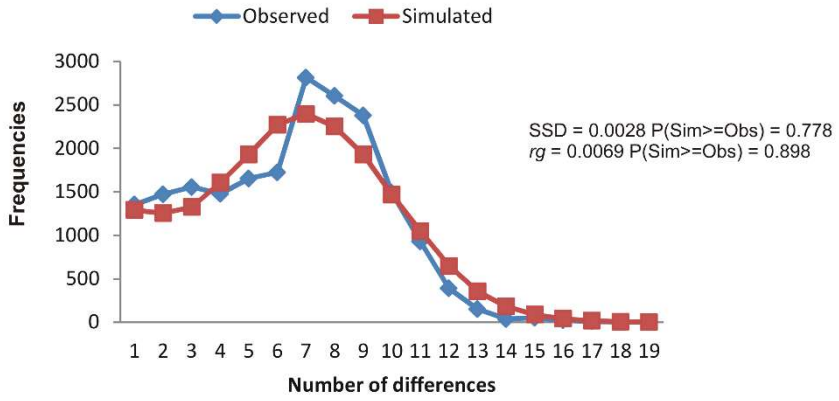
Supplementary Figure 2.



Supplementary figure 2. Mitochondrial DNA mismatch distribution analysis of the panmitic population. This analysis was performed using Arlequin 3.5 software (Excoffier and Lischer 2010). The sum of the square deviations (SSD) and the *Raggedness* index (*rg*) were used to measure the adjustment to a population expansion model, with significance determined by 10,000 permutations. The observed unimodal distribution of pairwise differences, with the observed distributions adjusted to the theoretical distributions in both tests of goodness-of-fit (SSD and *rg*), is indicative of recent population expansion (Excoffier et al. 1992, Rogers and Harpending 1992, Rogers et al. 1996, Schneider and Excoffier 1999).

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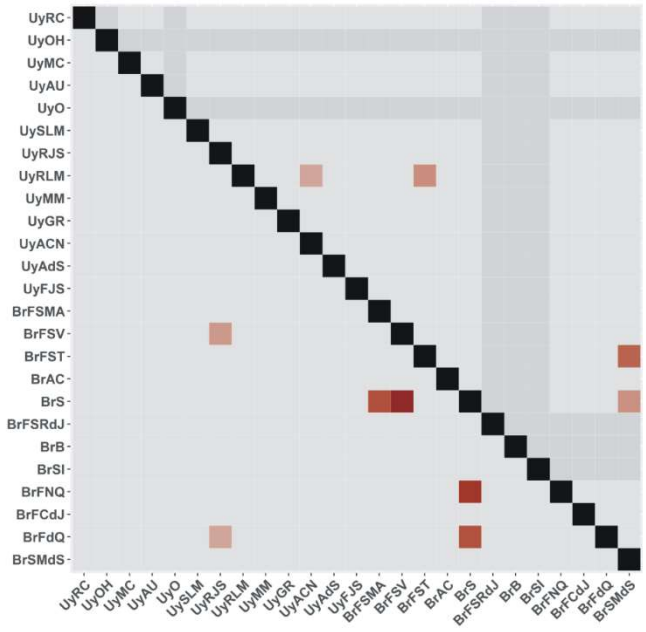
Supplementary Figure 3.



Supplementary figure 3. Pairwise F_{ST} matrix. Matrix of pairwise Φ_{ST} values for mitochondrial DNA (below diagonal) and pairwise F_{ST} values for microsatellites (above diagonal) between the sampling sites analyzed (Reynolds et al. 1983, Slatkin 1995) calculated using Arlequin 3.5 (Excoffier and Lischer 2010). The statistical significance of these analyses was accessed through 10,000 permutations. Light gray boxes indicate the comparisons that were not statistically significant. Gray boxes indicate the comparisons that were not conducted due to the absence of microsatellite data for those sampling sites. Red boxes indicate the F_{ST} values for the comparisons that were statistically significant ($p<0.05$). All significant values found can be related to sampling problems (most sites involved in those pairwise comparisons were undersampled). These results indicate the absence of geographic structure for both mitochondrial and microsatellite data.

References:

Excoffier, L., and H. E. L. Lischer. **2010**. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Mol. Ecol. Resour. 10: 564–7.

Reynolds, J., B. S. Weir, and C. C. Cockerham. **1983**. Estimation of the coancestry coefficient: basis for a short-term genetic distance. Genetics. 105: 767–79.

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Supplementary Table 1. Details about the 25 sampling sites.

Site number	Country	City	Location/Site	ID code	Latitude	Longitude
1	Uruguay	Artigas	Rincon del Catalan	UyRdC	30° 35' 13.37''	56° 24' 14.27''
2	Uruguay	Artigas	Olearraga Hnos.	UyOH	30° 17' 22.17''	56° 51' 29.10''
3	Uruguay	Artigas	Miguel Cuggeri	UyMC	30° 20' 23.46''	56° 53' 15.02''
4	Uruguay	Artigas	Alberto de Urgaray (de Vargas)	UyAU	30° 27' 52.88''	56° 52' 17.78''
5	Uruguay	Artigas	Odriozola	UyO	30° 46' 42.22''	56° 32' 58.88''
6	Uruguay	Artigas	Saul Leites de Moraes	UySLM	30° 28' 36.86''	56° 43' 02.66''
7	Uruguay	Artigas	Raul Jacquez Sarasua	UyRJS	30° 29' 41.47''	56° 46' 56.60''
8	Uruguay	Artigas	Richel Leites de Moraes	UyRLM	30° 33' 29.67''	56° 33' 22.29''
9	Uruguay	Artigas	Moema Medina / Green	UyMM	30° 46' 01.76''	56° 17' 03.47''
10	Uruguay	Artigas	Gustavo Riani / Macedo	UyGR	30° 41' 45.00''	56° 21' 44.81''
11	Uruguay	Artigas	Alfredo Castro Navarro	UyACN	30° 15' 00.19''	56° 41' 09.91''
12	Uruguay	Artigas	Auta Dos Santos	UyAdS	30° 17' 06.63''	56° 39' 09.81''
13	Uruguay	Artigas	Fund. Jaureche-Solari	UyFJS	30° 31' 55.57''	56° 33' 58.33''
14	Brasil	Quaraí	Estância Santa Maria Angélica (Suzana Albornoz)	BrFSMA	30º 20' 016"	55º 58' 052"
15	Brasil	Quaraí	Estância Santa Virgínia (Condomínio Santa Virgínia)	BrFSV	30º 18' 507"	56º 30' 576"
16	Brasil	Quaraí	Fazenda Santa Tereza	BrFST	30º 07' 05,8"	56° 25' 22,3''
17	Brasil	Quaraí	Agropecuária Caty (Adroaldo Bernardo Potter)	BrAC	30º 25' 515"	56º 01' 283"
18	Brasil	Quaraí	Sobrado (Agrop. Rio Bonito - José Delmar Pradella e Out.)	BrS	30º 16' 951"	56º 27' 822"
19	Brasil	Quaraí	Fazenda Santa Rita do Jarau	BrFSRdJ	30º 13' 10,0"	56º 30' 29,5"
20	Brasil	Quaraí	Fazenda Branquilho	BrB	30º 22' 30,7"	56° 20' 04,2''
21	Brasil	Quaraí	Fazenda Santa Isabel	BrSI	30º 29' 43,4"	56° 14' 07,7''
22	Brasil	Quaraí	Fazenda Nova Querencia	BrFNQ	30º 35' 19,9"	56° 00' 08,0''
23	Brasil	Quaraí	Fazenda Cerro do Jarau	BrFCdJ	30º 10' 16,1"	56° 27' 38,4''
24	Brasil	Quaraí	Fazenda da Quizilla	BrFdQ	30º 05' 09,6"	56º 26' 16,2"
25	Brasil	Stna do Livramento	São Miguel do Sarandy (Ricardo Brochado)	BrSMdS	30º 34' 925"	56º 02' 355"

Supplementary Table 2. Mitochondrial DNA diversity indices in each sampling site in Quaraí (Br) and Artigas (Uy). ID: sampling site identification; N: number of individuals; Nh: number of haplotypes; Ĥ: haplotype diversity; π: nucleotide diversity.

ID	N	Nh	Haplotypes (no of individuals)	Ĥ (s.d.)	π (s.d.)
BrAC	5	3	H1 (3), H6 (1), H37 (1)	0.7000 (0.2184)	0.004144 (0.002925)
BrB	1	1	H15 (1)	---	---
BrFCdJ	14	10	H1 (2), H2 (2), H5 (1), H6 (1), H8 (2), H12 (1), H13 (1), H17 (2), H18 (1), H42 (1)	0.9560 (0.0377)	0.005656 (0.003270)
BrFdQ	8	8	H2 (1), H5 (1), H8 (1), H15 (1), H43 (1), H44 (1), H45 (1), H46 (1)	1.0000 (0.0625)	0.008529 (0.005063)
BrFNQ	3	3	H5 (1), H16 (1), H41 (1)	1.0000 (0.2722)	0.009078 (0.007237)
BrFSMA	14	6	H1 (4), H2 (6), H12 (1), H13 (1), H25 (1), H26 (1)	0.7692 (0.0895)	0.005387 (0.003131)
BrFSRdJ	2	2	H7 (1), H14 (1)	1.0000 (0.5000)	0.008724 (0.009253)
BrFST	9	8	H1 (2), H3 (1), H5 (1), H6 (1), H33 (1), H34 (1), H35 (1), H36 (1)	0.9722 (0.0640)	0.005543 (0.003367)
BrFSV	9	9	H2 (1), H3 (1), H4 (1), H27 (1), H28 (1), H29 (1), H30 (1), H31 (1), H32 (1)	1.0000 (0.0524)	0.006089 (0.003662)
BrS	4	2	H1 (3), H14 (1)	0.5000 (0.2652)	0.000545 (0.000676)
BrSI	2	2	H3 (1), H4 (1)	1.0000 (0.5000)	0.007634 (0.008161)
BrSMdS	4	4	H1 (1), H38 (1), H39 (1), H40 (1)	1.0000 (0.1768)	0.007270 (0.005188)
UyACN	7	6	H1 (1), H2 (1), H10 (2), H11 (1), H70 (1), H71 (1)	0.9524 (0.0955)	0.006439 (0.004008)
UyAdS	17	13	H1 (4), H2 (1), H11 (2), H19 (1), H21 (1), H23 (1), H72 (1), H73 (1), H74 (1), H75 (1), H76 (1), H77 (1), H78 (1)	0.9485 (0.0435)	0.005501 (0.003145)
UyAU	4	4	H1 (1), H3 (1), H6 (1), H57 (1)	1.0000 (0.1768)	0.005271 (0.003876)
UyFJS	16	14	H1 (2), H2 (1), H24 (2), H79 (1), H80 (1), H81 (1), H82 (1), H83 (1), H84 (1), H85 (1), H88 (1), H89 (1), H90 (1), H91 (1)	0.9833 (0.0278)	0.006334 (0.003581)
UyGR	4	3	H1 (1), H2 (2), H23 (1)	0.8333 (0.2224)	0.006180 (0.004473)
UyMC	18	12	H1 (4), H2 (1), H3 (1), H4 (1), H5 (2), H6 (1), H7 (2), H9 (2), H18 (1), H54 (1), H55 (1), H56 (1)	0.9412 (0.0388)	0.006165 (0.003468)
UyMM	12	8	H1 (3), H2 (3), H4 (1), H67 (1), H68 (1), H69 (1), H86 (1), H87 (1)	0.9091 (0.0649)	0.005320 (0.003140)
UyO	1	1	H1 (1)	---	---
UyOH	3	3	H1 (1), H4 (1), H53 (1)	1.0000 (0.2722)	0.007270 (0.005886)
UyRdC	11	9	H1 (2), H2 (2), H19 (1), H47 (1), H48 (1), H49 (1), H50 (1), H51 (1), H52 (1)	0.9636 (0.0510)	0.006774 (0.003933)
UyRJS	16	11	H1 (5), H2 (2), H4 (1), H7 (1), H9 (1), H10 (1), H16 (1), H20 (1), H21 (1), H61 (1), H62 (1)	0.9083 (0.0633)	0.004898 (0.002851)
UyRLM	10	8	H1 (2), H3 (1), H20 (1), H22 (2), H63 (1), H64 (1), H65 (1), H66 (1)	0.9556 (0.0594)	0.006495 (0.003828)
UySLM	7	5	H1 (3), H2 (1), H58 (1), H59 (1), H60 (1)	0.8571 (0.1371)	0.007374 (0.004533)

Supplementary Table 3. Individual genotypes for all microsatellite loci. Loci adjusted for null loci in each sampling site are indicated in gray.

Individuals	CH01		CH05		CH09		CH12		CH14		CH15		CH24		CH26	
Site UyRC	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B
UyAr16	121	125	118	124	101	101	81	87	178	178	182	0	136	136	173	173
UyAr17	119	123	118	118	101	101	65	81	176	178	180	0	136	136	165	177
UyAr19	123	123	112	118	101	101	65	81	178	178	186	186	140	148	157	177
UyAr20	123	123	118	122	99	99	79	81	178	188	180	0	136	136	161	189
UyAr21	123	123	118	122	101	101	63	81	172	178	180	180	0	0	193	193
UyAr22	119	131	118	118	91	99	65	83	178	178	174	182	138	138	165	173
UyAr23	119	123	118	122	101	101	65	79	178	188	178	184	140	148	177	177
UyAr24	121	123	116	122	101	101	63	63	172	178	180	180	140	148	165	177
UyAr25	117	131	122	124	101	101	87	87	178	178	182	0	130	138	173	173
UyAr26	123	125	112	118	91	101	81	81	178	178	184	184	140	148	153	169
Site UyMC																
UyAr30	119	131	118	122	99	99	83	83	178	0	182	182	136	142	157	169
UyAr31	123	131	122	124	101	101	81	0	174	188	174	186	136	136	157	177
UyAr32	123	123	110	116	101	101	81	81	178	0	0	0	136	136	153	177
UyAr33	117	123	112	118	91	91	63	81	180	180	186	0	136	136	161	169
UyAr34	131	131	118	122	99	101	65	65	176	0	0	0	136	146	161	173
UyAr35	123	123	118	128	101	101	79	87	176	178	178	182	136	136	145	173
UyAr36	121	121	112	122	101	0	65	77	180	0	180	0	136	142	153	161
UyAr37	123	127	124	124	101	0	0	0	178	0	176	176	136	136	169	177
UyAr38	123	123	116	122	101	0	81	87	182	0	182	0	134	136	169	173
UyAr39	121	123	118	130	101	0	77	81	180	0	0	0	136	136	161	169
UyAr40	123	125	122	124	101	101	63	77	188	188	180	186	134	138	153	165
UyAr41	119	123	124	124	101	101	85	85	180	0	178	186	136	0	177	177
UyAr42	119	123	116	118	101	101	79	85	178	180	180	180	134	136	141	177
UyAr44	123	123	124	132	101	101	65	81	180	182	182	0	136	0	149	181
UyAr45	117	121	118	118	91	101	65	79	178	178	174	182	136	0	0	0
UyAr46	123	123	118	118	101	101	65	79	176	182	180	182	134	134	177	177
UyAr48	123	123	118	118	0	0	75	75	180	0	186	0	138	138	153	161
UyAr49	115	123	118	122	101	101	79	79	180	180	178	178	138	138	157	161

Individuals	CH01		CH05		CH09		CH12		CH14		CH15		CH24		CH26	
	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B
Site UyAU																
UyAr50	123	123	118	124	91	101	65	65	178	180	174	182	136	138	165	173
UyAr51	117	131	118	124	101	101	65	87	174	178	184	184	136	138	149	165
UyAr52	123	131	118	118	99	99	71	85	174	180	182	182	136	136	161	169
UyAr53	117	123	118	124	91	101	83	83	178	180	174	186	136	136	161	169
Site UySLM																
UyAr55	117	123	118	122	101	0	63	81	176	178	0	0	134	134	165	173
UyAr56	121	121	112	118	89	89	81	83	178	180	0	0	136	136	161	169
UyAr57	123	123	124	124	91	91	87	87	180	180	0	0	136	136	0	0
UyAr58	123	123	118	122	101	0	63	79	176	180	178	180	134	140	169	173
UyAr59	119	123	112	118	91	101	81	81	178	178	178	178	136	138	157	185
UyAr60	117	121	0	0	101	101	65	87	176	178	174	180	136	136	145	173
UyAr61	123	131	118	122	93	93	83	83	176	178	184	188	136	136	0	0
Site UyRJS																
UyAr62	119	123	0	0	101	101	63	81	178	0	182	0	136	136	165	181
UyAr63	131	145	118	124	101	101	81	81	178	0	186	0	138	138	169	173
UyAr64	123	123	118	122	91	91	89	89	178	178	182	186	142	142	165	189
UyAr65	123	123	118	122	101	101	83	85	178	178	182	182	134	140	169	173
UyAr66	125	125	118	118	101	101	81	87	176	176	186	186	136	142	165	181
UyAr67	123	123	124	130	91	101	81	81	180	0	182	186	136	138	157	173
UyAr68	123	123	118	124	101	101	67	87	176	176	186	0	136	138	145	153
UyAr70	121	123	118	118	91	101	71	87	0	0	182	0	138	148	153	161
UyAr71	117	117	118	130	101	101	65	65	178	180	176	176	134	140	169	177
UyAr72	123	125	118	122	91	101	83	89	176	180	186	186	136	136	161	165
UyAr73	123	0	118	118	91	91	65	65	178	180	178	186	136	136	169	177
UyAr74	121	121	118	124	99	101	79	85	178	178	180	188	136	138	169	185
UyAr75	119	123	118	118	99	101	79	81	178	180	174	180	136	136	169	189
UyAr76	123	0	110	118	101	101	81	87	174	180	180	182	136	142	157	181
UyAr77	121	125	118	118	101	101	81	81	178	178	182	186	136	136	0	0
UyAr78	123	0	122	124	101	101	81	87	178	178	180	182	134	138	161	169
UyAr79	123	123	118	122	91	91	65	77	178	0	184	184	136	136	169	173
UyAr80	121	121	118	124	99	101	67	81	178	0	182	186	136	138	165	181
UyAr81	123	0	118	118	91	91	65	87	180	0	180	186	136	138	177	177

Individuals	CH01		CH05		CH09		CH12		CH14		CH15		CH24		CH26	
	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B
Site UyRLM																
UyAr83	117	131	118	118	93	101	81	83	178	178	180	186	134	142	153	161
UyAr84	117	117	118	118	91	0	87	87	178	182	184	184	136	140	157	197
UyAr85	121	123	118	124	93	101	81	81	176	180	0	0	134	138	161	173
UyAr86	123	0	118	118	101	101	81	87	178	178	182	0	0	0	173	177
UyAr87	121	121	112	118	93	99	81	81	176	178	186	186	134	138	161	165
UyAr88	119	121	118	124	93	101	61	81	176	176	182	182	138	138	161	173
UyAr89	123	123	118	122	101	0	63	81	182	184	0	0	136	138	169	173
UyAr90	123	123	118	118	91	91	81	83	178	182	180	0	0	0	153	165
UyAr92	123	0	116	124	99	99	83	87	174	180	180	182	138	140	165	173
UyAr93	117	0	122	124	91	91	65	87	178	178	180	188	138	138	165	169
Site UyMM																
UyAr96	123	123	122	124	101	101	87	87	178	178	182	182	136	136	153	173
UyAr97	121	121	118	118	91	101	65	79	174	174	182	0	138	138	169	177
UyAr98	117	123	118	124	101	101	65	79	178	178	178	0	138	138	161	173
UyAr99	123	123	122	124	101	101	77	97	182	190	0	0	136	136	165	181
UyAr100	123	123	118	118	101	101	65	79	178	182	180	180	136	136	161	173
UyAr101	121	121	118	122	101	101	87	87	178	180	174	178	136	138	153	177
UyAr102	121	131	118	122	91	101	81	83	178	178	0	0	142	158	173	173
UyAr103	121	123	116	130	101	101	81	85	178	182	178	0	136	136	161	161
UyAr105	123	123	118	124	101	101	81	87	178	178	186	186	136	138	157	165
Site UyGR																
UyAr106	123	123	112	118	91	101	81	87	176	180	178	182	136	138	141	173
UyAr107	119	125	110	118	101	101	81	87	172	174	174	182	134	136	149	153
UyAr108	119	119	122	124	91	101	63	63	178	178	182	182	136	136	153	177
UyAr109	121	121	118	130	91	101	83	87	180	180	176	176	138	144	153	169
Site UyACN																
UyAr110	119	123	124	124	99	101	65	81	178	178	186	186	136	138	0	0
UyAr111	123	123	118	130	99	101	81	85	176	0	182	186	134	136	169	169
UyAr112	123	123	124	124	101	101	77	77	178	0	180	186	136	138	153	177
UyAr113	119	121	110	124	101	101	67	79	172	172	174	186	136	136	165	173
UyAr115	119	123	118	122	101	101	79	81	178	0	178	182	136	138	0	0
UyAr116	119	121	122	122	101	101	81	83	176	0	182	186	136	136	133	165

Individuals	CH01		CH05		CH09		CH12		CH14		CH15		CH24		CH26	
	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B
Site UyAds																
UyAr117	121	123	118	122	101	101	65	81	178	178	182	0	0	0	181	181
UyAr118	121	131	116	124	101	101	81	87	178	180	186	0	136	136	173	177
UyAr119	119	123	118	118	101	101	81	81	176	176	180	0	126	136	153	189
UyAr120	119	123	118	124	91	101	65	65	178	178	180	0	126	136	157	177
UyAr121	119	119	124	124	101	101	83	87	178	178	178	186	136	142	161	173
UyAr123	123	123	124	124	101	101	63	81	178	178	182	182	0	0	149	149
UyAr124	117	123	110	118	91	101	81	87	180	182	180	180	136	136	153	157
UyAr125	123	123	118	124	101	101	81	81	178	178	182	182	140	140	165	173
UyAr126	123	123	118	122	89	99	63	83	178	182	180	0	136	0	161	185
UyAr127	123	123	118	124	91	101	63	81	178	188	180	180	136	136	169	181
UyAr128	123	123	118	122	101	101	65	65	178	188	186	0	136	0	165	173
UyAr129	123	131	118	118	99	99	65	81	178	188	178	184	0	0	153	153
UyAr130	121	123	118	118	101	101	77	79	178	180	182	0	138	138	169	181
UyAr133	123	123	118	124	91	101	65	81	178	188	180	0	0	0	157	165
UyAr134	123	123	118	124	99	99	77	87	178	180	178	182	0	0	153	169
UyAr135	121	121	118	124	101	101	81	81	178	180	180	186	0	0	161	169
Site UyFJS																
UyAr138	123	123	118	118	99	99	65	85	178	178	182	182	136	136	169	173
UyAr139	117	123	118	122	101	101	79	87	182	182	176	186	138	140	173	177
UyAr140	123	123	118	122	101	101	65	87	178	180	178	182	134	138	157	173
UyAr141	121	123	118	118	89	89	63	81	174	176	180	188	136	140	161	161
UyAr142	121	123	118	122	91	101	81	83	176	178	178	180	136	136	161	177
UyAr143	123	123	124	124	101	0	65	81	178	178	174	180	138	142	173	177
UyAr144	123	123	118	122	89	99	65	83	178	178	0	0	134	136	0	0
UyAr146	123	127	118	124	91	101	65	65	178	178	180	182	138	140	161	193
UyAr147	121	121	122	124	101	0	65	87	174	178	182	182	134	134	181	193
UyAr152	117	119	118	118	101	101	65	87	182	188	174	182	136	136	157	161
UyAr154	123	123	118	134	101	0	83	87	176	178	182	188	124	138	161	173
UyAr155	119	123	122	124	91	101	65	81	176	178	186	186	136	138	165	181
UyAr156	117	123	118	122	99	99	83	97	178	180	182	182	136	138	0	0
UyAr157	123	123	118	124	101	101	79	87	178	178	186	186	136	136	0	0

Individuals	CH01		CH05		CH09		CH12		CH14		CH15		CH24		CH26	
	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B
Site BrFSMA																
BrQi01	123	123	118	118	101	101	83	87	174	178	182	188	136	136	149	185
BrQi02	123	123	118	124	91	101	63	81	178	0	188	188	136	138	169	177
BrQi03	119	119	122	122	89	89	65	81	178	178	186	0	126	138	165	173
BrQi04	121	123	118	124	101	0	65	65	178	0	182	0	136	136	165	173
BrQi05	123	125	118	124	101	101	67	87	178	178	186	188	134	138	161	169
BrQi06	127	127	122	124	101	0	65	65	180	180	186	186	136	138	161	165
BrQi07	123	131	122	124	101	101	65	87	178	178	180	0	138	138	169	189
BrQi08	123	123	118	118	99	101	87	87	180	180	180	0	136	140	165	169
BrQi09	123	131	118	118	101	0	65	79	178	178	182	186	138	138	157	157
BrQi10	121	131	124	130	99	101	81	87	178	178	180	186	138	138	169	173
BrQi11	117	123	110	118	99	99	61	83	178	188	178	186	134	134	165	169
BrQi12	123	123	116	118	99	99	87	87	178	0	184	184	134	138	161	165
BrQi13	121	123	122	124	93	101	67	81	178	178	182	182	138	144	169	169
BrQi14	117	123	118	118	99	101	65	87	178	178	180	180	0	0	149	161
BrQi84	123	123	118	118	101	101	65	81	176	178	186	0	136	142	165	177
Site BrFSV																
BrQi15	117	123	122	122	101	101	81	83	0	0	182	182	136	136	169	181
BrQi16	123	0	0	0	91	91	65	87	0	0	0	0	134	138	0	0
BrQi17	119	123	122	124	99	101	81	87	176	178	184	188	138	140	161	165
BrQi18	131	131	118	118	101	101	65	81	180	180	182	182	136	140	157	173
BrQi19	117	129	118	118	99	101	65	77	178	178	182	186	138	140	169	185
BrQi20	131	131	118	118	99	101	65	87	188	188	182	186	134	136	149	149
BrQi21	121	121	118	122	93	103	61	61	178	178	174	174	138	140	157	165
BrQi22	123	0	118	122	101	101	81	81	178	182	178	186	134	138	157	169
BrQi23	121	123	118	124	101	101	81	81	178	178	0	0	136	138	157	173
BrQi24	123	123	118	118	101	101	81	87	176	178	182	186	140	140	177	181

Individuals	CH01		CH05		CH09		CH12		CH14		CH15		CH24		CH26	
	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B
Site BrFST																
BrQi25	119	123	118	124	101	101	65	65	180	180	180	182	126	136	169	173
BrQi26	123	123	118	124	101	101	65	83	178	178	182	186	136	138	157	177
BrQi27	131	131	118	118	91	103	77	83	178	178	174	182	134	138	181	185
BrQi28	121	123	122	122	101	101	65	87	178	178	178	180	134	136	157	173
BrQi29	119	123	118	118	101	101	65	79	178	178	184	184	134	142	157	197
BrQi30	121	123	122	124	89	103	63	81	178	180	182	186	140	140	161	181
BrQi31	117	123	118	124	99	101	79	85	178	178	186	186	134	140	157	169
BrQi32	123	123	118	124	99	101	65	77	178	178	0	0	138	138	169	177
Site BrAC																
BrQi33	119	119	118	118	91	91	83	83	176	176	178	178	142	142	153	153
BrQi34	123	131	118	128	93	93	79	83	178	180	180	180	134	142	173	181
BrQi35	123	123	118	124	101	101	65	83	178	180	186	0	136	138	153	169
BrQi36	117	117	118	124	101	0	61	79	178	178	188	188	138	142	153	169
BrQi37	119	121	118	128	101	0	65	73	176	178	186	0	134	138	161	173
Site BrS																
BrQi42	123	131	112	122	91	101	63	79	178	178	182	182	138	138	157	161
BrQi43	123	123	110	122	91	101	79	81	188	188	182	182	138	140	149	169
BrQi44	121	121	118	118	101	101	81	81	176	188	182	182	138	138	165	185
BrQi45	123	123	118	118	93	101	81	81	178	178	184	186	136	136	169	181
Site BrFNQ																
BrQi54	117	131	118	124	101	101	81	81	178	180	182	182	138	140	177	193
BrQi55	121	121	118	124	99	101	81	87	178	180	182	182	136	142	161	161
BrQi56	123	123	118	124	101	101	83	83	178	178	182	182	136	136	161	165
BrQi57	123	123	118	118	93	101	67	83	178	178	180	180	140	142	157	177

Individuals	CH01		CH05		CH09		CH12		CH14		CH15		CH24		CH26	
	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B	Allele A	Allele B
Site BrFCdJ																
BrQi58	123	0	118	118	89	101	77	79	178	178	180	180	142	142	169	185
BrQi59	117	131	122	124	101	101	81	81	174	178	182	182	136	138	177	177
BrQi60	123	0	118	124	101	101	81	83	178	190	182	0	138	140	165	189
BrQi61	123	131	118	124	91	101	65	79	174	174	182	0	136	138	141	193
BrQi62	115	115	118	118	99	101	65	87	178	0	184	188	136	138	169	169
BrQi63	121	121	118	122	101	101	81	87	178	0	178	0	138	140	173	185
BrQi64	131	131	118	118	101	101	87	87	178	180	188	188	136	138	161	161
BrQi65	123	123	118	118	101	101	81	87	180	180	178	0	138	140	153	185
BrQi67	119	123	118	122	89	101	65	65	176	176	180	182	138	138	173	177
BrQi68	121	123	118	124	101	101	65	87	180	180	174	186	138	138	165	181
BrQi69	123	123	118	124	101	101	65	83	178	178	184	184	136	136	157	177
BrQi81	123	123	112	124	101	101	65	87	182	188	178	178	134	138	173	177
BrQi82	117	123	118	118	91	91	83	87	178	0	0	0	136	138	157	161
BrQi83	123	0	118	124	101	101	81	81	178	178	186	186	136	136	153	165
Site BrFdQ																
BrQi70	121	121	116	122	101	101	67	87	178	178	182	0	136	136	153	165
BrQi71	117	123	118	124	101	101	81	81	180	180	186	186	136	138	165	173
BrQi72	123	123	118	118	101	101	65	81	176	178	182	186	140	140	165	177
BrQi73	123	123	122	122	101	101	81	87	180	180	178	180	134	136	161	169
BrQi74	121	131	116	118	99	101	79	87	178	182	186	186	134	140	169	169
BrQi75	123	123	118	124	101	101	65	83	178	178	182	0	126	142	161	173
BrQi76	117	123	124	130	101	101	81	85	178	178	186	0	134	136	161	169
BrQi77	123	123	116	118	101	101	65	65	174	180	186	0	136	138	149	165
BrQi78	117	131	122	124	99	101	81	81	176	178	182	182	136	136	153	161
Site SMdS																
BrQi38	123	131	116	130	99	101	81	81	178	178	0	0	136	136	177	177
BrQi39	117	123	118	118	101	101	77	87	176	176	186	186	124	136	169	181
BrQi40	123	123	118	122	101	101	87	87	178	178	180	186	134	138	161	185
BrQi41	123	131	124	124	99	101	77	87	178	178	182	182	124	136	165	177

Supplementary Table 4. Allelic frequencies for all microsatellite loci in each sampling site.

Locus	Allele	UyRC	UyMC	UyAU	UySLM	UyRJS	UyRLM	UyMM	UyGR	UyACN	UyAdS	UyFJS	BrFSMA	BrFSV	BrFST	BrAC	BrS	BrFNQ	BrFCdJ	BrFdQ	BrSMdS
CH01	**115**	0.000	0.028	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.091	0.000	0.000
	117	0.050	0.056	0.250	0.143	0.067	0.214	0.056	0.000	0.000	0.031	0.107	0.067	0.125	0.063	0.200	0.000	0.125	0.091	0.167	0.125
	119	0.150	0.083	0.000	0.071	0.067	0.071	0.000	0.375	0.333	0.125	0.071	0.067	0.063	0.125	0.300	0.000	0.000	0.045	0.000	0.000
	121	0.100	0.111	0.000	0.214	0.200	0.286	0.333	0.250	0.167	0.156	0.143	0.100	0.188	0.125	0.100	0.250	0.250	0.136	0.167	0.000
	123	0.500	0.556	0.500	0.500	0.467	0.357	0.556	0.250	0.500	0.625	0.643	0.567	0.313	0.563	0.300	0.625	0.500	0.455	0.556	0.625
	125	0.100	0.028	0.000	0.000	0.133	0.000	0.000	0.125	0.000	0.000	0.000	0.033	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	127	0.000	0.028	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.036	0.067	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	129	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.063	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	131	0.100	0.111	0.250	0.071	0.033	0.071	0.056	0.000	0.000	0.063	0.000	0.100	0.250	0.125	0.100	0.125	0.125	0.182	0.111	0.250
	145	0.000	0.000	0.000	0.000	0.033	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
CH05	**110**	0.000	0.028	0.000	0.000	0.028	0.000	0.000	0.125	0.083	0.031	0.000	0.033	0.000	0.000	0.000	0.125	0.000	0.000	0.000	0.000
	112	0.100	0.056	0.000	0.167	0.000	0.050	0.000	0.125	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.125	0.000	0.036	0.000	0.000
	116	0.050	0.083	0.000	0.000	0.000	0.050	0.056	0.000	0.000	0.031	0.000	0.033	0.000	0.000	0.000	0.000	0.000	0.000	0.167	0.125
	118	0.500	0.361	0.625	0.417	0.611	0.600	0.444	0.375	0.167	0.500	0.500	0.500	0.611	0.500	0.600	0.500	0.625	0.607	0.333	0.375
	122	0.250	0.194	0.000	0.250	0.139	0.100	0.222	0.125	0.250	0.094	0.250	0.167	0.278	0.188	0.000	0.250	0.000	0.107	0.222	0.125
	124	0.100	0.194	0.375	0.167	0.167	0.200	0.222	0.125	0.417	0.344	0.214	0.233	0.111	0.313	0.200	0.000	0.375	0.250	0.222	0.250
	128	0.000	0.028	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.200	0.000	0.000	0.000	0.000	0.000
	130	0.000	0.028	0.000	0.000	0.056	0.000	0.056	0.125	0.083	0.000	0.000	0.033	0.000	0.000	0.000	0.000	0.000	0.000	0.056	0.125
	132	0.000	0.028	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	134	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.036	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Locus	Allele	UyRC	UyMC	UyAU	UySLM	UyRJS	UyRLM	UyMM	UyGR	UyACN	UyAdS	UyFJS	BrFSMA	BrFSV	BrFST	BrAC	BrS	BrFNQ	BrFCdJ	BrFdQ	BrSMdS
CH09	**89**	0.000	0.000	0.000	0.200	0.000	0.000	0.000	0.000	0.000	0.031	0.136	0.083	0.000	0.063	0.000	0.000	0.000	0.071	0.000	0.000
	91	0.100	0.115	0.250	0.300	0.289	0.250	0.111	0.375	0.000	0.125	0.136	0.042	0.100	0.063	0.333	0.250	0.000	0.107	0.000	0.000
	93	0.000	0.000	0.000	0.200	0.000	0.250	0.000	0.000	0.000	0.000	0.000	0.042	0.050	0.000	0.333	0.125	0.125	0.000	0.000	0.000
	99	0.150	0.115	0.250	0.000	0.079	0.188	0.000	0.000	0.167	0.156	0.227	0.292	0.150	0.125	0.000	0.000	0.125	0.036	0.111	0.250
	101	0.750	0.769	0.500	0.300	0.632	0.313	0.889	0.625	0.833	0.688	0.500	0.542	0.650	0.625	0.333	0.625	0.750	0.786	0.889	0.750
	103	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.050	0.125	0.000	0.000	0.000	0.000	0.000	0.000
CH12	**61**	0.000	0.000	0.000	0.000	0.000	0.050	0.000	0.000	0.000	0.000	0.000	0.033	0.100	0.000	0.100	0.000	0.000	0.000	0.000	0.000
	63	0.150	0.063	0.000	0.143	0.026	0.050	0.000	0.250	0.000	0.094	0.036	0.033	0.000	0.063	0.000	0.125	0.000	0.000	0.000	0.000
	65	0.200	0.188	0.375	0.071	0.158	0.050	0.167	0.000	0.083	0.219	0.321	0.300	0.200	0.375	0.200	0.000	0.000	0.250	0.222	0.000
	67	0.000	0.000	0.000	0.000	0.053	0.000	0.000	0.000	0.083	0.000	0.000	0.067	0.000	0.000	0.000	0.000	0.125	0.000	0.056	0.000
	71	0.000	0.000	0.125	0.000	0.026	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	73	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.100	0.000	0.000	0.000	0.000	0.000
	75	0.000	0.063	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	77	0.000	0.094	0.000	0.000	0.026	0.000	0.056	0.000	0.167	0.063	0.000	0.000	0.050	0.125	0.000	0.000	0.000	0.036	0.000	0.250
	79	0.100	0.188	0.000	0.071	0.053	0.000	0.167	0.000	0.167	0.031	0.071	0.033	0.000	0.125	0.200	0.250	0.000	0.071	0.056	0.000
	81	0.350	0.188	0.000	0.286	0.316	0.450	0.167	0.250	0.333	0.406	0.143	0.167	0.400	0.063	0.000	0.625	0.375	0.250	0.389	0.250
	83	0.050	0.063	0.250	0.214	0.053	0.150	0.056	0.125	0.083	0.063	0.143	0.067	0.050	0.125	0.400	0.000	0.375	0.107	0.056	0.000
	85	0.000	0.094	0.125	0.000	0.053	0.000	0.056	0.000	0.083	0.000	0.036	0.000	0.000	0.063	0.000	0.000	0.000	0.000	0.056	0.000
	87	0.150	0.063	0.125	0.214	0.158	0.250	0.278	0.375	0.000	0.125	0.214	0.300	0.200	0.063	0.000	0.000	0.125	0.286	0.167	0.500
	89	0.000	0.000	0.000	0.000	0.079	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	97	0.000	0.000	0.000	0.000	0.000	0.000	0.056	0.000	0.000	0.000	0.036	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Locus	Allele	UyRC	UyMC	UyAU	UySLM	UyRJS	UyRLM	UyMM	UyGR	UyACN	UyAdS	UyFJS	BrFSMA	BrFSV	BrFST	BrAC	BrS	BrFNQ	BrFCdJ	BrFdQ	BrSMdS
CH14	**172**	0.100	0.000	0.000	0.000	0.000	0.000	0.000	0.125	0.500	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	174	0.000	0.056	0.250	0.000	0.042	0.050	0.111	0.125	0.000	0.000	0.071	0.042	0.000	0.000	0.000	0.000	0.000	0.136	0.056	0.000
	176	0.050	0.111	0.000	0.286	0.208	0.200	0.000	0.125	0.000	0.063	0.143	0.042	0.125	0.000	0.300	0.125	0.000	0.091	0.111	0.250
	178	0.750	0.222	0.375	0.429	0.542	0.450	0.611	0.250	0.500	0.594	0.571	0.708	0.563	0.813	0.500	0.500	0.750	0.409	0.500	0.750
	180	0.000	0.333	0.375	0.286	0.208	0.100	0.056	0.375	0.000	0.156	0.071	0.167	0.125	0.188	0.200	0.000	0.250	0.227	0.278	0.000
	182	0.000	0.111	0.000	0.000	0.000	0.150	0.167	0.000	0.000	0.063	0.107	0.000	0.063	0.000	0.000	0.000	0.000	0.045	0.056	0.000
	184	0.000	0.000	0.000	0.000	0.000	0.050	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	188	0.100	0.167	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.125	0.036	0.042	0.125	0.000	0.000	0.375	0.000	0.045	0.000	0.000
	190	0.000	0.000	0.000	0.000	0.000	0.000	0.056	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.045	0.000	0.000
CH15	**174**	0.083	0.100	0.250	0.125	0.033	0.000	0.125	0.125	0.083	0.000	0.077	0.000	0.125	0.071	0.000	0.000	0.000	0.056	0.000	0.000
	176	0.000	0.100	0.000	0.000	0.067	0.000	0.000	0.250	0.000	0.000	0.038	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	178	0.083	0.200	0.000	0.375	0.033	0.000	0.125	0.125	0.083	0.188	0.077	0.050	0.063	0.071	0.333	0.000	0.000	0.111	0.100	0.000
	180	0.333	0.200	0.000	0.250	0.167	0.250	0.250	0.000	0.083	0.313	0.154	0.150	0.000	0.143	0.333	0.000	0.250	0.167	0.100	0.167
	182	0.083	0.250	0.375	0.000	0.267	0.250	0.250	0.500	0.250	0.313	0.385	0.200	0.438	0.286	0.000	0.750	0.750	0.167	0.300	0.333
	184	0.250	0.000	0.250	0.125	0.067	0.167	0.000	0.000	0.000	0.063	0.000	0.100	0.063	0.143	0.000	0.125	0.000	0.167	0.000	0.000
	186	0.167	0.150	0.125	0.000	0.333	0.250	0.250	0.000	0.500	0.125	0.192	0.300	0.250	0.286	0.000	0.125	0.000	0.167	0.500	0.500
	188	0.000	0.000	0.000	0.125	0.033	0.083	0.000	0.000	0.000	0.000	0.077	0.200	0.063	0.000	0.333	0.000	0.000	0.167	0.000	0.000

Locus	Allele	UyRC	UyMC	UyAU	UySLM	UyRJS	UyRLM	UyMM	UyGR	UyACN	UyAdS	UyFJS	BrFSMA	BrFSV	BrFST	BrAC	BrS	BrFNQ	BrFCdJ	BrFdQ	BrSMdS
CH24	**124**	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.036	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.250
	126	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.125	0.000	0.036	0.000	0.063	0.000	0.000	0.000	0.000	0.056	0.000
	130	0.056	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	134	0.000	0.167	0.000	0.214	0.079	0.188	0.000	0.125	0.083	0.000	0.143	0.143	0.150	0.250	0.200	0.000	0.000	0.036	0.167	0.125
	136	0.333	0.567	0.750	0.643	0.500	0.125	0.556	0.500	0.667	0.563	0.429	0.286	0.250	0.188	0.100	0.250	0.375	0.321	0.444	0.500
	138	0.167	0.167	0.250	0.071	0.237	0.500	0.333	0.250	0.250	0.125	0.250	0.429	0.300	0.250	0.300	0.625	0.125	0.464	0.111	0.125
	140	0.222	0.000	0.000	0.071	0.053	0.125	0.000	0.000	0.000	0.125	0.107	0.036	0.300	0.188	0.000	0.125	0.250	0.107	0.167	0.000
	142	0.000	0.067	0.000	0.000	0.105	0.063	0.056	0.000	0.000	0.063	0.036	0.036	0.000	0.063	0.400	0.000	0.250	0.071	0.056	0.000
	144	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.125	0.000	0.000	0.000	0.036	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	146	0.000	0.033	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	148	0.222	0.000	0.000	0.000	0.026	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	158	0.000	0.000	0.000	0.000	0.000	0.000	0.056	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Locus	Allele	UyRC	UyMC	UyAU	UySLM	UyRJS	UyRLM	UyMM	UyGR	UyACN	UyAdS	UyFJS	BrFSMA	BrFSV	BrFST	BrAC	BrS	BrFNQ	BrFCdJ	BrFdQ	BrSMdS
CH26	**133**	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.125	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	141	0.000	0.029	0.000	0.000	0.000	0.000	0.000	0.125	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.036	0.000	0.000
	145	0.000	0.029	0.000	0.100	0.028	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	149	0.000	0.029	0.125	0.000	0.000	0.000	0.000	0.125	0.000	0.063	0.000	0.067	0.111	0.000	0.000	0.125	0.000	0.000	0.056	0.000
	153	0.050	0.118	0.000	0.000	0.056	0.100	0.111	0.375	0.125	0.156	0.000	0.000	0.000	0.000	0.400	0.000	0.000	0.071	0.111	0.000
	157	0.050	0.088	0.000	0.100	0.056	0.050	0.056	0.000	0.000	0.094	0.091	0.067	0.222	0.250	0.000	0.125	0.125	0.071	0.000	0.000
	161	0.050	0.176	0.250	0.100	0.083	0.200	0.222	0.000	0.000	0.094	0.273	0.133	0.056	0.063	0.100	0.125	0.375	0.107	0.222	0.125
	165	0.150	0.029	0.250	0.100	0.139	0.200	0.111	0.000	0.250	0.094	0.045	0.233	0.111	0.000	0.000	0.125	0.125	0.107	0.222	0.125
	169	0.050	0.147	0.250	0.200	0.222	0.100	0.056	0.125	0.250	0.125	0.045	0.267	0.167	0.188	0.200	0.250	0.000	0.107	0.222	0.125
	173	0.250	0.088	0.125	0.300	0.111	0.250	0.278	0.125	0.125	0.125	0.227	0.100	0.111	0.125	0.200	0.000	0.000	0.107	0.111	0.000
	177	0.250	0.235	0.000	0.000	0.111	0.050	0.111	0.125	0.125	0.063	0.136	0.067	0.056	0.125	0.000	0.000	0.250	0.179	0.056	0.375
	181	0.000	0.029	0.000	0.000	0.111	0.000	0.056	0.000	0.000	0.125	0.091	0.000	0.111	0.125	0.100	0.125	0.000	0.036	0.000	0.125
	185	0.000	0.000	0.000	0.100	0.028	0.000	0.000	0.000	0.000	0.031	0.000	0.033	0.056	0.063	0.000	0.125	0.000	0.107	0.000	0.125
	189	0.050	0.000	0.000	0.000	0.056	0.000	0.000	0.000	0.000	0.031	0.000	0.033	0.000	0.000	0.000	0.000	0.000	0.036	0.000	0.000
	193	0.100	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.091	0.000	0.000	0.000	0.000	0.000	0.125	0.036	0.000	0.000
	197	0.000	0.000	0.000	0.000	0.000	0.050	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.063	0.000	0.000	0.000	0.000	0.000	0.000

Supplementary Table 5. P-values of linkage disequilibrium tests in each sampling site. Values with asterisk (*) are statistically significant ($p<0.05$) and "no info" refers to tables in which all rows or all columns marginal sums are 1.

Tests for detecting genotypic linkage disequilibrium (LD) found significant association between pairs of loci in only four cases for $p<0.05$. No LD was detected after Bonferroni correction ($p<0.006$).

Sampling site	Locus 1	Locus 2	P-value
UyRC	CH01	CH05	0.397937
UyRC	CH01	CH09	0.539799
UyRC	CH05	CH09	1
UyRC	CH01	CH12	1
UyRC	CH05	CH12	1
UyRC	CH09	CH12	0.467062
UyRC	CH01	CH14	1
UyRC	CH05	CH14	0.347444
UyRC	CH09	CH14	0.914851
UyRC	CH12	CH14	1
UyRC	CH01	CH15	1
UyRC	CH05	CH15	1
UyRC	CH09	CH15	0.397729
UyRC	CH12	CH15	No info
UyRC	CH14	CH15	0.265460
UyRC	CH01	CH24	1
UyRC	CH05	CH24	0.591762
UyRC	CH09	CH24	0.593057
UyRC	CH12	CH24	1
UyRC	CH14	CH24	1
UyRC	CH15	CH24	No info
UyRC	CH01	CH26	1
UyRC	CH05	CH26	1
UyRC	CH09	CH26	0.170982
UyRC	CH12	CH26	1
UyRC	CH14	CH26	0.485301
UyRC	CH15	CH26	No info
UyRC	CH24	CH26	1
UyMC	CH01	CH05	0.624197
UyMC	CH01	CH09	0.043063*
UyMC	CH05	CH09	0.981832
UyMC	CH01	CH12	1
UyMC	CH05	CH12	0.060059
UyMC	CH09	CH12	1
UyMC	CH01	CH14	1
UyMC	CH05	CH14	1
UyMC	CH09	CH14	1
UyMC	CH12	CH14	1

UyMC	CH01	CH15	No info
UyMC	CH05	CH15	No info
UyMC	CH09	CH15	No info
UyMC	CH12	CH15	No info
UyMC	CH14	CH15	No info
UyMC	CH01	CH24	0.852077
UyMC	CH05	CH24	1
UyMC	CH09	CH24	0.658691
UyMC	CH12	CH24	No info
UyMC	CH14	CH24	1
UyMC	CH15	CH24	No info
UyMC	CH01	CH26	1
UyMC	CH05	CH26	1
UyMC	CH09	CH26	0.553169
UyMC	CH12	CH26	No info
UyMC	CH14	CH26	No info
UyMC	CH15	CH26	No info
UyMC	CH24	CH26	0.321763
UyAU	CH01	CH05	No info
UyAU	CH01	CH09	No info
UyAU	CH05	CH09	0.500744
UyAU	CH01	CH12	No info
UyAU	CH05	CH12	No info
UyAU	CH09	CH12	No info
UyAU	CH01	CH14	No info
UyAU	CH05	CH14	0.499390
UyAU	CH09	CH14	0.166981
UyAU	CH12	CH14	No info
UyAU	CH01	CH15	No info
UyAU	CH05	CH15	No info
UyAU	CH09	CH15	No info
UyAU	CH12	CH15	No info
UyAU	CH14	CH15	No info
UyAU	CH01	CH24	No info
UyAU	CH05	CH24	1
UyAU	CH09	CH24	1
UyAU	CH12	CH24	No info
UyAU	CH14	CH24	1
UyAU	CH15	CH24	No info
UyAU	CH01	CH26	No info
UyAU	CH05	CH26	1
UyAU	CH09	CH26	1
UyAU	CH12	CH26	No info
UyAU	CH14	CH26	1
UyAU	CH15	CH26	No info
UyAU	CH24	CH26	0.332034

UySLM	CH01	CH05	1
UySLM	CH01	CH09	No info
UySLM	CH05	CH09	No info
UySLM	CH01	CH12	No info
UySLM	CH05	CH12	No info
UySLM	CH09	CH12	No info
UySLM	CH01	CH14	1
UySLM	CH05	CH14	0.266750
UySLM	CH09	CH14	No info
UySLM	CH12	CH14	No info
UySLM	CH01	CH15	No info
UySLM	CH05	CH15	No info
UySLM	CH09	CH15	No info
UySLM	CH12	CH15	No info
UySLM	CH14	CH15	No info
UySLM	CH01	CH24	1
UySLM	CH05	CH24	1
UySLM	CH09	CH24	No info
UySLM	CH12	CH24	No info
UySLM	CH14	CH24	0.629346
UySLM	CH15	CH24	No info
UySLM	CH01	CH26	No info
UySLM	CH05	CH26	No info
UySLM	CH09	CH26	No info
UySLM	CH12	CH26	No info
UySLM	CH14	CH26	No info
UySLM	CH15	CH26	No info
UySLM	CH24	CH26	No info
UyRJS	CH01	CH05	0.129088
UyRJS	CH01	CH09	0.483956
UyRJS	CH05	CH09	0.810620
UyRJS	CH01	CH12	1
UyRJS	CH05	CH12	1
UyRJS	CH09	CH12	0.126644
UyRJS	CH01	CH14	0.570304
UyRJS	CH05	CH14	0.665448
UyRJS	CH09	CH14	0.799082
UyRJS	CH12	CH14	0.649739
UyRJS	CH01	CH15	0.633948
UyRJS	CH05	CH15	1
UyRJS	CH09	CH15	0.943722
UyRJS	CH12	CH15	0.026999*
UyRJS	CH14	CH15	0.584803
UyRJS	CH01	CH24	0.156870
UyRJS	CH05	CH24	0.053747*
UyRJS	CH09	CH24	0.839681

UyRJS	CH12	CH24	0.739953
UyRJS	CH14	CH24	0.935649
UyRJS	CH15	CH24	0.836124
UyRJS	CH01	CH26	0.589642
UyRJS	CH05	CH26	0.610154
UyRJS	CH09	CH26	0.575934
UyRJS	CH12	CH26	0.192970
UyRJS	CH14	CH26	0.184464
UyRJS	CH15	CH26	1
UyRJS	CH24	CH26	1
UyRLM	CH01	CH05	1
UyRLM	CH01	CH09	No info
UyRLM	CH05	CH09	0.495058
UyRLM	CH01	CH12	1
UyRLM	CH05	CH12	0.307394
UyRLM	CH09	CH12	1
UyRLM	CH01	CH14	1
UyRLM	CH05	CH14	0.069594
UyRLM	CH09	CH14	1
UyRLM	CH12	CH14	1
UyRLM	CH01	CH15	No info
UyRLM	CH05	CH15	No info
UyRLM	CH09	CH15	No info
UyRLM	CH12	CH15	No info
UyRLM	CH14	CH15	No info
UyRLM	CH01	CH24	No info
UyRLM	CH05	CH24	1
UyRLM	CH09	CH24	1
UyRLM	CH12	CH24	0.075802
UyRLM	CH14	CH24	1
UyRLM	CH15	CH24	No info
UyRLM	CH01	CH26	1
UyRLM	CH05	CH26	0.168312
UyRLM	CH09	CH26	0.140712
UyRLM	CH12	CH26	1
UyRLM	CH14	CH26	1
UyRLM	CH15	CH26	No info
UyRLM	CH24	CH26	1
UyMM	CH01	CH05	0.647165
UyMM	CH01	CH09	0.278422
UyMM	CH05	CH09	1
UyMM	CH01	CH12	1
UyMM	CH05	CH12	0.391809
UyMM	CH09	CH12	0.832690
UyMM	CH01	CH14	0.842336
UyMM	CH05	CH14	0.646743

UyMM	CH09	CH14	0.608564
UyMM	CH12	CH14	1
UyMM	CH01	CH15	No info
UyMM	CH05	CH15	No info
UyMM	CH09	CH15	No info
UyMM	CH12	CH15	No info
UyMM	CH14	CH15	No info
UyMM	CH01	CH24	0.186985
UyMM	CH05	CH24	0.669765
UyMM	CH09	CH24	0.168705
UyMM	CH12	CH24	0.682855
UyMM	CH14	CH24	0.912952
UyMM	CH15	CH24	No info
UyMM	CH01	CH26	1
UyMM	CH05	CH26	1
UyMM	CH09	CH26	0.616214
UyMM	CH12	CH26	0.111232
UyMM	CH14	CH26	1
UyMM	CH15	CH26	No info
UyMM	CH24	CH26	1
UyGR	CH01	CH05	No info
UyGR	CH01	CH09	No info
UyGR	CH05	CH09	No info
UyGR	CH01	CH12	No info
UyGR	CH05	CH12	No info
UyGR	CH09	CH12	1
UyGR	CH01	CH14	No info
UyGR	CH05	CH14	No info
UyGR	CH09	CH14	No info
UyGR	CH12	CH14	No info
UyGR	CH01	CH15	No info
UyGR	CH05	CH15	No info
UyGR	CH09	CH15	No info
UyGR	CH12	CH15	No info
UyGR	CH14	CH15	No info
UyGR	CH01	CH24	No info
UyGR	CH05	CH24	No info
UyGR	CH09	CH24	No info
UyGR	CH12	CH24	No info
UyGR	CH14	CH24	No info
UyGR	CH15	CH24	No info
UyGR	CH01	CH26	No info
UyGR	CH05	CH26	No info
UyGR	CH09	CH26	No info
UyGR	CH12	CH26	No info
UyGR	CH14	CH26	No info

UyGR	CH15	CH26	No info
UyGR	CH24	CH26	No info
UyACN	CH01	CH05	1
UyACN	CH01	CH09	1
UyACN	CH05	CH09	1
UyACN	CH01	CH12	No info
UyACN	CH05	CH12	No info
UyACN	CH09	CH12	No info
UyACN	CH01	CH14	No info
UyACN	CH05	CH14	No info
UyACN	CH09	CH14	No info
UyACN	CH12	CH14	No info
UyACN	CH01	CH15	1
UyACN	CH05	CH15	1
UyACN	CH09	CH15	1
UyACN	CH12	CH15	No info
UyACN	CH14	CH15	No info
UyACN	CH01	CH24	0.198894
UyACN	CH05	CH24	0.265781
UyACN	CH09	CH24	0.602005
UyACN	CH12	CH24	No info
UyACN	CH14	CH24	No info
UyACN	CH15	CH24	1
UyACN	CH01	CH26	No info
UyACN	CH05	CH26	No info
UyACN	CH09	CH26	No info
UyACN	CH12	CH26	No info
UyACN	CH14	CH26	No info
UyACN	CH15	CH26	No info
UyACN	CH24	CH26	No info
UyAdS	CH01	CH05	0.140213
UyAdS	CH01	CH09	0.918156
UyAdS	CH05	CH09	0.455463
UyAdS	CH01	CH12	0.914354
UyAdS	CH05	CH12	0.883446
UyAdS	CH09	CH12	0.674513
UyAdS	CH01	CH14	0.655444
UyAdS	CH05	CH14	0.446053
UyAdS	CH09	CH14	0.217075
UyAdS	CH12	CH14	0.868312
UyAdS	CH01	CH15	0.423092
UyAdS	CH05	CH15	1
UyAdS	CH09	CH15	0.078419
UyAdS	CH12	CH15	1
UyAdS	CH14	CH15	0.324171
UyAdS	CH01	CH24	0.276018

UyAdS	CH05	CH24	1
UyAdS	CH09	CH24	1
UyAdS	CH12	CH24	0.266323
UyAdS	CH14	CH24	1
UyAdS	CH15	CH24	0.168231
UyAdS	CH01	CH26	0.371763
UyAdS	CH05	CH26	1
UyAdS	CH09	CH26	0.604355
UyAdS	CH12	CH26	1
UyAdS	CH14	CH26	1
UyAdS	CH15	CH26	No info
UyAdS	CH24	CH26	1
UyFJS	CH01	CH05	0.825223
UyFJS	CH01	CH09	0.863553
UyFJS	CH05	CH09	0.989917
UyFJS	CH01	CH12	1
UyFJS	CH05	CH12	1
UyFJS	CH09	CH12	0.029234*
UyFJS	CH01	CH14	0.095042
UyFJS	CH05	CH14	0.704620
UyFJS	CH09	CH14	0.862297
UyFJS	CH12	CH14	1
UyFJS	CH01	CH15	1
UyFJS	CH05	CH15	1
UyFJS	CH09	CH15	0.401177
UyFJS	CH12	CH15	1
UyFJS	CH14	CH15	1
UyFJS	CH01	CH24	0.851104
UyFJS	CH05	CH24	0.814850
UyFJS	CH09	CH24	0.862474
UyFJS	CH12	CH24	1
UyFJS	CH14	CH24	0.763650
UyFJS	CH15	CH24	1
UyFJS	CH01	CH26	1
UyFJS	CH05	CH26	1
UyFJS	CH09	CH26	No info
UyFJS	CH12	CH26	1
UyFJS	CH14	CH26	1
UyFJS	CH15	CH26	1
UyFJS	CH24	CH26	1
BrFSMA	CH01	CH05	0.298125
BrFSMA	CH01	CH09	0.906224
BrFSMA	CH05	CH09	0.295066
BrFSMA	CH01	CH12	0.425332
BrFSMA	CH05	CH12	1
BrFSMA	CH09	CH12	1

BrFSMA	CH01	CH14	0.909803
BrFSMA	CH05	CH14	0.967398
BrFSMA	CH09	CH14	0.857708
BrFSMA	CH12	CH14	0.574533
BrFSMA	CH01	CH15	No info
BrFSMA	CH05	CH15	No info
BrFSMA	CH09	CH15	No info
BrFSMA	CH12	CH15	No info
BrFSMA	CH14	CH15	No info
BrFSMA	CH01	CH24	0.603554
BrFSMA	CH05	CH24	1
BrFSMA	CH09	CH24	1
BrFSMA	CH12	CH24	1
BrFSMA	CH14	CH24	0.124582
BrFSMA	CH15	CH24	No info
BrFSMA	CH01	CH26	1
BrFSMA	CH05	CH26	1
BrFSMA	CH09	CH26	1
BrFSMA	CH12	CH26	1
BrFSMA	CH14	CH26	1
BrFSMA	CH15	CH26	No info
BrFSMA	CH24	CH26	1
BrFSV	CH01	CH05	0.214105
BrFSV	CH01	CH09	1
BrFSV	CH05	CH09	0.809272
BrFSV	CH01	CH12	1
BrFSV	CH05	CH12	1
BrFSV	CH09	CH12	0.669617
BrFSV	CH01	CH14	1
BrFSV	CH05	CH14	1
BrFSV	CH09	CH14	1
BrFSV	CH12	CH14	0.265025
BrFSV	CH01	CH15	1
BrFSV	CH05	CH15	0.070837
BrFSV	CH09	CH15	0.290510
BrFSV	CH12	CH15	1
BrFSV	CH14	CH15	1
BrFSV	CH01	CH24	1
BrFSV	CH05	CH24	1
BrFSV	CH09	CH24	0.795485
BrFSV	CH12	CH24	1
BrFSV	CH14	CH24	0.390386
BrFSV	CH15	CH24	1
BrFSV	CH01	CH26	1
BrFSV	CH05	CH26	1
BrFSV	CH09	CH26	0.362807

BrFSV	CH12	CH26	1
BrFSV	CH14	CH26	1
BrFSV	CH15	CH26	No info
BrFSV	CH24	CH26	1
BrFST	CH01	CH05	0.627096
BrFST	CH01	CH09	0.621101
BrFST	CH05	CH09	0.489238
BrFST	CH01	CH12	No info
BrFST	CH05	CH12	No info
BrFST	CH09	CH12	No info
BrFST	CH01	CH14	1
BrFST	CH05	CH14	0.501115
BrFST	CH09	CH14	0.502312
BrFST	CH12	CH14	No info
BrFST	CH01	CH15	1
BrFST	CH05	CH15	1
BrFST	CH09	CH15	1
BrFST	CH12	CH15	No info
BrFST	CH14	CH15	1
BrFST	CH01	CH24	No info
BrFST	CH05	CH24	No info
BrFST	CH09	CH24	No info
BrFST	CH12	CH24	No info
BrFST	CH14	CH24	No info
BrFST	CH15	CH24	No info
BrFST	CH01	CH26	No info
BrFST	CH05	CH26	No info
BrFST	CH09	CH26	No info
BrFST	CH12	CH26	No info
BrFST	CH14	CH26	No info
BrFST	CH15	CH26	No info
BrFST	CH24	CH26	No info
BrAC	CH01	CH05	No info
BrAC	CH01	CH09	No info
BrAC	CH05	CH09	No info
BrAC	CH01	CH12	No info
BrAC	CH05	CH12	No info
BrAC	CH09	CH12	No info
BrAC	CH01	CH14	No info
BrAC	CH05	CH14	1
BrAC	CH09	CH14	No info
BrAC	CH12	CH14	No info
BrAC	CH01	CH15	No info
BrAC	CH05	CH15	No info
BrAC	CH09	CH15	No info
BrAC	CH12	CH15	No info

BrAC	CH14	CH15	No info
BrAC	CH01	CH24	No info
BrAC	CH05	CH24	No info
BrAC	CH09	CH24	No info
BrAC	CH12	CH24	No info
BrAC	CH14	CH24	No info
BrAC	CH15	CH24	No info
BrAC	CH01	CH26	No info
BrAC	CH05	CH26	0.199768
BrAC	CH09	CH26	No info
BrAC	CH12	CH26	No info
BrAC	CH14	CH26	1
BrAC	CH15	CH26	No info
BrAC	CH24	CH26	No info
BrS	CH01	CH05	1
BrS	CH01	CH09	1
BrS	CH05	CH09	1
BrS	CH01	CH12	1
BrS	CH05	CH12	0.167633
BrS	CH09	CH12	1
BrS	CH01	CH14	1
BrS	CH05	CH14	1
BrS	CH09	CH14	1
BrS	CH12	CH14	1
BrS	CH01	CH15	1
BrS	CH05	CH15	1
BrS	CH09	CH15	0.500952
BrS	CH12	CH15	1
BrS	CH14	CH15	1
BrS	CH01	CH24	1
BrS	CH05	CH24	1
BrS	CH09	CH24	1
BrS	CH12	CH24	1
BrS	CH14	CH24	1
BrS	CH15	CH24	0.501960
BrS	CH01	CH26	No info
BrS	CH05	CH26	No info
BrS	CH09	CH26	No info
BrS	CH12	CH26	No info
BrS	CH14	CH26	No info
BrS	CH15	CH26	No info
BrS	CH24	CH26	No info
BrFNQ	CH01	CH05	1
BrFNQ	CH01	CH09	1
BrFNQ	CH05	CH09	0.500864
BrFNQ	CH01	CH12	No info

BrFNQ	CH05	CH12	No info
BrFNQ	CH09	CH12	No info
BrFNQ	CH01	CH14	0.331868
BrFNQ	CH05	CH14	1
BrFNQ	CH09	CH14	1
BrFNQ	CH12	CH14	No info
BrFNQ	CH01	CH15	1
BrFNQ	CH05	CH15	0.249360
BrFNQ	CH09	CH15	0.498755
BrFNQ	CH12	CH15	No info
BrFNQ	CH14	CH15	1
BrFNQ	CH01	CH24	No info
BrFNQ	CH05	CH24	No info
BrFNQ	CH09	CH24	No info
BrFNQ	CH12	CH24	No info
BrFNQ	CH14	CH24	No info
BrFNQ	CH15	CH24	No info
BrFNQ	CH01	CH26	No info
BrFNQ	CH05	CH26	No info
BrFNQ	CH09	CH26	No info
BrFNQ	CH12	CH26	No info
BrFNQ	CH14	CH26	No info
BrFNQ	CH15	CH26	No info
BrFNQ	CH24	CH26	No info
BrFCdJ	CH01	CH05	1
BrFCdJ	CH01	CH09	0.207156
BrFCdJ	CH05	CH09	0.855065
BrFCdJ	CH01	CH12	1
BrFCdJ	CH05	CH12	1
BrFCdJ	CH09	CH12	0.185830
BrFCdJ	CH01	CH14	1
BrFCdJ	CH05	CH14	0.704109
BrFCdJ	CH09	CH14	0.657483
BrFCdJ	CH12	CH14	1
BrFCdJ	CH01	CH15	No info
BrFCdJ	CH05	CH15	No info
BrFCdJ	CH09	CH15	No info
BrFCdJ	CH12	CH15	No info
BrFCdJ	CH14	CH15	No info
BrFCdJ	CH01	CH24	1
BrFCdJ	CH05	CH24	0.454716
BrFCdJ	CH09	CH24	0.742058
BrFCdJ	CH12	CH24	0.613176
BrFCdJ	CH14	CH24	0.388474
BrFCdJ	CH15	CH24	No info
BrFCdJ	CH01	CH26	1

BrFCdJ	CH05	CH26	1
BrFCdJ	CH09	CH26	1
BrFCdJ	CH12	CH26	1
BrFCdJ	CH14	CH26	1
BrFCdJ	CH15	CH26	No info
BrFCdJ	CH24	CH26	1
BrFdQ	CH01	CH05	1
BrFdQ	CH01	CH09	0.111237
BrFdQ	CH05	CH09	0.889153
BrFdQ	CH01	CH12	1
BrFdQ	CH05	CH12	1
BrFdQ	CH09	CH12	1
BrFdQ	CH01	CH14	1
BrFdQ	CH05	CH14	1
BrFdQ	CH09	CH14	0.304824
BrFdQ	CH12	CH14	1
BrFdQ	CH01	CH15	1
BrFdQ	CH05	CH15	No info
BrFdQ	CH09	CH15	1
BrFdQ	CH12	CH15	1
BrFdQ	CH14	CH15	1
BrFdQ	CH01	CH24	1
BrFdQ	CH05	CH24	1
BrFdQ	CH09	CH24	0.669425
BrFdQ	CH12	CH24	1
BrFdQ	CH14	CH24	1
BrFdQ	CH15	CH24	No info
BrFdQ	CH01	CH26	1
BrFdQ	CH05	CH26	1
BrFdQ	CH09	CH26	0.610800
BrFdQ	CH12	CH26	1
BrFdQ	CH14	CH26	1
BrFdQ	CH15	CH26	No info
BrFdQ	CH24	CH26	0.081715
BrSMdS	CH01	CH05	No info
BrSMdS	CH01	CH09	0.333088
BrSMdS	CH05	CH09	No info
BrSMdS	CH01	CH12	1
BrSMdS	CH05	CH12	No info
BrSMdS	CH09	CH12	1
BrSMdS	CH01	CH14	0.502132
BrSMdS	CH05	CH14	No info
BrSMdS	CH09	CH14	1
BrSMdS	CH12	CH14	1
BrSMdS	CH01	CH15	No info
BrSMdS	CH05	CH15	No info

BrSMdS	CH09	CH15	No info
BrSMdS	CH12	CH15	No info
BrSMdS	CH14	CH15	No info
BrSMdS	CH01	CH24	1
BrSMdS	CH05	CH24	No info
BrSMdS	CH09	CH24	1
BrSMdS	CH12	CH24	0.164786
BrSMdS	CH14	CH24	1
BrSMdS	CH15	CH24	No info
BrSMdS	CH01	CH26	No info
BrSMdS	CH05	CH26	No info
BrSMdS	CH09	CH26	No info
BrSMdS	CH12	CH26	No info
BrSMdS	CH14	CH26	No info
BrSMdS	CH15	CH26	No info
BrSMdS	CH24	CH26	No info

Supplementary Table 6. Genetic variability of microsatellite loci in each sampling site. N: Sample size; Na: Number of alleles per locus; AR: Allelic richness; Ho: observed heterozygosity; He: expected heterozygosity. Significant departures from Hardy-Weinberg equilibrium were indicated by asterisks in the value of He. * $p < 0.05$; ** $p < 0.005$

Sampling Site		Loci							
		CH01	CH05	CH09	CH12	CH14	CH15	CH24	CH26
UyRC	N	10	10	10	10	10	6	9	10
(N=10)	Na (AR)	6 (4.164)	5 (3.654)	3 (2.460)	6 (4.554)	4 (2.705)	6 (3.920)	5 (4.149)	9 (5.357)
	Ho	0.700	0.800	0.200	0.700	0.500	0.333	0.556	0.600
	He	0.695	0.665	0.405*	0.780	0.415	0.778**	0.759**	0.830*
UyMC	N	18	18	13	16	9	10	15	17
(N=18)	Na (AR)	8 (3.912)	9 (4.530)	3 (2.241)	9 (5.452)	6 (4.235)	6 (4.011)	5 (3.226)	11 (5.491)
	Ho	0.556	0.722	0.154	0.625	0.556	0.600	0.400	0.882
	He	0.654	0.781	0.382*	0.861	0.784	0.815	0.618*	0.858
UyAU	N	4	4	4	4	4	4	4	4
(N=4)	Na (AR)	3 (3)	2 (2)	3 (3)	5 (5)	3 (3)	4 (3.678)	2 (2)	5 (5)
	Ho	0.750	0.750	0.500	0.500	1.000	0.500	0.500	1.000
	He	0.625	0.469	0.625	0.750	0.656	0.719	0.375	0.781
UySLM	N	7	6	5	7	7	4	7	5
(N=7)	Na (AR)	5 (3.923)	4 (3.800)	4 (3.800)	6 (4.853)	3 (2.970)	5 (4.214)	4 (3.088)	7 (5.978)
	Ho	0.571	0.833	0.200	0.571	0.714	0.750	0.286	1.000
	He	0.673	0.708	0.740*	0.796	0.653	0.750	0.531	0.820
UyRJS	N	15	18	19	19	12	15	19	18
(N=19)	Na (AR)	7 (3.818)	5 (3.168)	3 (2.473)	11 (5.213)	4 (2.998)	8 (3.673)	6 (3.650)	11 (5.779)
	Ho	0.400	0.667	0.316	0.684	0.417	0.667	0.579	0.944
	He	0.713**	0.576	0.511*	0.831*	0.618*	0.778	0.673*	0.877
UyRLM	N	7	10	8	10	10	6	8	10
(N=10)	Na (AR)	5 (3.833)	5 (3.350)	4 (3.743)	6 (3.955)	6 (4.156)	5 (3.827)	5 (3.933)	8 (5.250)
	Ho	0.429	0.600	0.500	0.700	0.600	0.500	0.750	1.000
	He	0.735	0.585	0.742*	0.705	0.720	0.778	0.680	0.830
UyMM	N	9	9	9	9	9	4	9	9
(N=9)	Na (AR)	4 (2.877)	5 (3.751)	2 (1.706)	8 (5.307)	5 (3.448)	5 (4.061)	4 (2.877)	8 (5.353)
	Ho	0.333	0.778	0.222	0.778	0.444	0.250	0.333	0.778
	He	0.574	0.698	0.198	0.827**	0.580	0.781	0.574*	0.827
UyGR	N	4	4	4	4	4	4	4	4
(N=4)	Na (AR)	4 (4)	6 (6)	2 (2)	4 (4)	5 (5)	4 (3.464)	4 (4)	6 (6)
	Ho	0.250	1.000	0.750	0.750	0.500	0.500	0.750	1.000
	He	0.719*	0.781	0.469	0.719	0.750	0.656	0.656	0.781
UyACN	N	6	6	6	6	2	6	6	4
(N=6)	Na (AR)	3 (2.907)	5 (4.224)	2 (1.909)	7 (5.483)	3 (3)	5 (3.408)	3 (2.648)	6 (6)
	Ho	0.667	0.500	0.333	0.833	0.000	0.833	0.667	0.750
	He	0.611	0.722	0.278	0.806	0.500	0.667	0.486	0.813
UyAdS	N	16	16	16	16	16	8	8	16
(N=16)	Na (AR)	5 (3.187)	5 (3.071)	4 (2.743)	7 (4.323)	5 (3.380)	5 (3.430)	5 (3.562)	11 (6.065)
	Ho	0.438	0.688	0.313	0.688	0.625	0.500	0.375	0.813
	He	0.564	0.621	0.486**	0.754	0.600	0.750*	0.633	0.893

UyFJS	**N**	14	14	11	14	14	13	14	11
(N=14)	**Na (AR)**	5 (3.198)	4 (3.116)	4 (3.292)	8 (4.754)	6 (3.696)	7 (3.882)	6 (3.917)	8 (5.193)
	Ho	0.500	0.714	0.364	0.929	0.571	0.615	0.643	0.909
	He	0.548	0.640	0.661**	0.801	0.630	0.772	0.719	0.826
BrFSMA	**N**	15	15	12	15	12	10	14	15
(N=15)	**Na (AR)**	7 (3.915)	6 (3.530)	5 (3.048)	8 (4.484)	5 (2.668)	6 (3.856)	7 (3.862)	9 (5.156)
	Ho	0.533	0.600	0.417	0.733	0.250	0.500	0.571	0.867
	He	0.644	0.664	0.611	0.780	0.465*	0.795	0.709	0.831
BrFSV	**N**	8	9	10	10	8	8	10	9
(N=10)	**Na (AR)**	6 (4.375)	3 (2.676)	5 (3.260)	6 (4.244)	5 (3.800)	6 (3.624)	4 (3.708)	9 (5.941)
	Ho	0.500	0.444	0.400	0.700	0.375	0.625	0.800	0.889
	He	0.781*	0.537	0.540*	0.745	0.633	0.719*	0.735	0.864
BrFST	**N**	8	8	8	8	8	7	8	8
(N=8)	**Na (AR)**	5 (3.800)	3 (2.887)	5 (3.533)	8 (5.296)	2 (1.900)	6 (4.102)	6 (4.723)	8 (5.661)
	Ho	0.625	0.625	0.500	0.875	0.125	0.714	0.750	1.000
	He	0.633	0.617	0.570	0.797	0.305	0.786	0.797	0.844
BrAC	**N**	5	5	3	5	5	3	5	5
(N=5)	**Na (AR)**	5 (4.578)	3 (2.955)	3 (3)	5 (4.555)	3 (2.978)	4 (3.857)	4 (3.778)	5 (4.555)
	Ho	0.400	0.800	0.000	0.800	0.600	0.000	0.800	0.800
	He	0.760*	0.560	0.667	0.740	0.620	0.667	0.700	0.740
BrS	**N**	4	4	4	4	4	4	4	4
(N=4)	**Na (AR)**	3 (3)	4 (4)	3 (3)	3 (3)	3 (3)	3 (2.5)	3 (3)	7 (7)
	Ho	0.250	0.500	0.750	0.500	0.250	0.250	0.250	1.000
	He	0.531	0.656	0.531	0.531	0.594	0.406	0.531	0.844
BrFNQ	**N**	4	4	4	4	4	4	4	4
(N=4)	**Na (AR)**	4 (4)	2 (2)	3 (3)	4 (4)	2 (2)	2 (1.964)	4 (4)	5 (5)
	Ho	0.250	0.750	0.500	0.500	0.500	0.000	0.750	0.750
	He	0.656*	0.469	0.406	0.688	0.375	0.375	0.719	0.750
BrFCdJ	**N**	11	14	14	14	11	9	14	14
(N=14)	**Na (AR)**	6 (3.929)	4 (2.872)	4 (2.435)	6 (4.264)	7 (4.093)	7 (4.403)	5 (3.409)	12 (6.240)
	Ho	0.455	0.643	0.286	0.714	0.364	0.333	0.643	0.786
	He	0.723	0.556	0.365	0.776	0.748**	0.846**	0.663	0.895
BrFdQ	**N**	9	9	9	9	9	5	9	9
(N=9)	**Na (AR)**	4 (3.412)	5 (4.149)	2 (1.706)	7 (4.558)	5 (3.565)	4 (2.827)	6 (4.300)	7 (5.095)
	Ho	0.444	0.778	0.222	0.667	0.444	0.400	0.667	0.889
	He	0.623	0.759	0.198	0.759	0.654*	0.640	0.728	0.821
BrSMdS	**N**	4	4	4	4	4	3	4	4
(N=4)	**Na (AR)**	3 (3)	5 (5)	2 (2)	3 (3)	2 (2)	3 (3)	4 (4)	6 (6)
	Ho	0.750	0.500	0.500	0.500	0.000	0.333	0.750	0.750
	He	0.531	0.750	0.375	0.625	0.375	0.611	0.656	0.781

CAPÍTULO 3

Temporal genetic diversity of a New World screwworm fly population inferred by the use of mitochondrial DNA, microsatellites and single-nucleotide polymorphisms

(Manuscrito em preparação)

ORIGINAL ARTICLE:

Temporal genetic diversity of a New World screwworm fly population inferred by the use of mitochondrial DNA, microsatellites and single-nucleotide polymorphisms

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Abstract

The New World screwworm fly (NWS), *Cochliomyia hominivorax,* is an economically important ectoparasite which is currently distributed in South America and Caribbean. The successful eradication of this species in USA, Mexico and continental Central America involved the implementation of a program based on sterile insect technique (SIT). To implement a similar control program in its current area of occurrence, it is first necessary to understand the NWS fly population structure and the species dynamics. Spatial genetic structure of the NWS fly has been previously reported, however, to date, no study has investigated temporal changes in the genetic composition of the species. In the present study, temporal population genetic structure was accessed by 3 mitochondrial DNA sequences, 8 microsatellite markers and 828 SNPs for a time scale of approximately 1 year. Temporal change in genetic composition was observed for all three markers, and the patterns observed can possibly be associated to host availability, insecticide use and climatological changes in this southern region. These results provided new clues and perspectives on the population genetic structure of the NWS fly, which could have significant implications for the planning and implementation of management programs.

Key-words: climatological changes, effective population size, genetic variability, genotyping-by-sequencing, temporal population structure.

Introduction

The New World screwworm (NWS) fly, *Cochliomyia hominivorax* (Coquerel, 1858) (Diptera: Calliphoridae), is an ectoparasite species that causes myiasis in warm-blooded vertebrates and, consequently, leads to negative impacts in livestock breeding (Guimarães et al. 1982). The current distribution of this species is Neotropical, but it originally extended from the Southern USA to Argentina (Wyss 2000). The reduction in the territory occupied by this species involved management programs based on the sterile insect technique (SIT) (Knipling 1955, reviewed in Vargas-Terán et al. 2005).

Many population genetic studies have been conducted in recent years in order to better understand the dynamic and distribution of NWS fly (reviewed in Bergamo et al, in press), with the aim to give basis for future implementation of similar control programs in its current area of occurrence. In South America, two genetic groups were identified: the North Amazon region (NAG) and the South Amazon region (SAG) (Lyra et al. 2009, Fresia et al. 2011). Inside SAG, studies diverged about the smaller scale in which genetic differentiation can be detected (Lyra

et al. 2005, Torres et al. 2007, Bergamo et al. 2015, Bergamo et al, submitted), what can be due to the distinct molecular markers used (sequences of mitochondrial DNA, microsatellites and sequences of carboxylesterase E3). More recently, the use of new and more abundant molecular markers, such as SNPs, has the promise to enrich population genetic studies, increasing the resolution of population structure and consequently allowing establishing boundaries and management units.

Currently, population genomics is one area of genetics that is considerably growing, accompanying the development of next-generation sequencing (NGS) technologies, and have the potential to improve population studies (Luikart et al. 2003). Many distinct approaches to obtain hundreds to thousands molecular markers over the genome were developed (Davey et al. 2011, Andrews et al. 2016). One of them is genotyping-by-sequencing (GBS) (Elshire et al. 2011, Poland et al. 2012), which is based on restriction enzymes to reduce the genome representation and is capable of obtaining a huge amount of single nucleotide polymorphisms (SNPs) for a lot of individuals. Despite of this, relatively few studies of population structure applying this or a similar approach were conducted, to date, for non-model insects (e.g., Nosil et al. 2012, Lozier 2014, Rašić et al. 2015, Silva-Brandão et al. 2015, Anderson et al. 2016, Dussex et al. 2016, Fouet et al. 2017 and others reviewed in Wachi et al, 2018).

Additionally, temporal studies, in which populations are sampled at different periods with the aim to observe the temporal changes of allelic frequencies, are also important to give insights about the species' population dynamics and demography. Effective population size (*Ne*), one important demographic parameter that is generally difficult to estimate, can be more robustly estimated with temporal sampling and population genomic data (i.e. GBS/RAD-Seq data) (Habel et al. 2014, Nunziata and Weisrock 2017).

In the present study, we standardized the two-enzyme GBS protocol (Poland et al. 2012) for NWS fly and used the SNPs markers, together with mtDNA sequences and microsatellites, to investigate temporal changes in the NWS fly population from an Uruguayan sampling locality. Our study is the first to implement the GBS approach for this pest species, allowing it future implementation in spatial NWS fly population studies.

Most studies that aimed to infer demographic dynamics did this based on historical timescales (i.e. thousands of generations) and not on an ecological timescale (i.e. tens of generations) (Nunziata et al. 2017). Understanding demographic changes at an ecological timescale is particularly important for NWS fly populations in its southern distribution (e.g. Uruguay), where the species is impacted by climate and environmental changes. We consider that there are two possible hypothesis for NWS fly populations dynamics in this region based

on climate changes: (i) during winter, characterized by cold temperatures and lower precipitation (i.e. unfavorable conditions), the species migrates to a northern region and, when conditions became favorable again (i.e., spring and summer, with warm temperatures and higher precipitation), it recolonizes the region again; or (ii) during winter, the population experience a bottleneck, with some individuals being maintained in refugees. When the climatic conditions became favorable, these individuals generate offspring and the population expands, giving rise to a cyclic phenomenon of population size changes based on annual seasons (Shpak et al. 2009). Besides climatic conditions, host availability and insecticide use are important factors that influence the NWS fly populations. These other two factors are also relevant for Uruguay, which ranks as one of the main cattle-raising countries in South America, mainly sheep.

Material and Methods

Sampling information. NWS fly samples were collected from wounds and natural cavities of sheep and cattle from the municipality of Cañas (Cerro Largo, Uruguay) (Figure 1) in two distinct periods: February/March 2015 and March 2016, here named as sample 1 and 2, respectively.

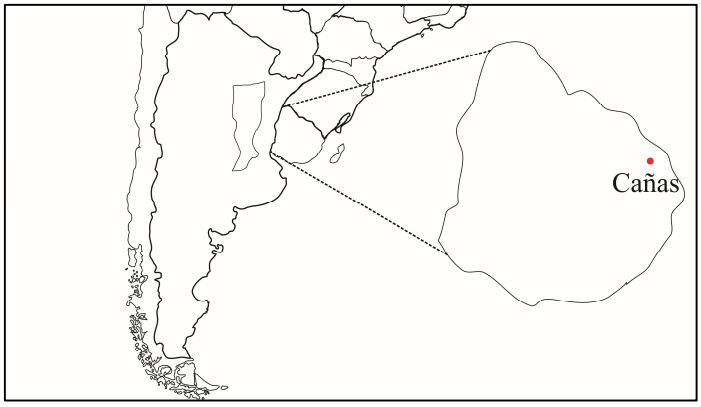

Figure 1. Schematic map showing the municipality of Cañas, Cerro Largo, Uruguay (32° 21' 21.96" S, 53° 49' 45.12"), where NWS fly samples were collected in two consecutive years (2015 and 2016).

Data collection. Total genomic DNA was extracted from one to three larvae per wound using the CTAB method (Doyle and Doyle 1987), with an additional step of RNAse treatment. DNA was eluted in 50µl AE buffer and stored at -20°C. The extractions were quantified by fluorescence using the Qubit DNA quantification system (Invitrogen, Carlsbad, CA, USA) and their quality and purity were verified using the NanoDrop UV spectrophotometer (Techno Scientific, Wilmington, DE, USA). The DNA amount per sampled was normalized to 20ng/µl.

Good quality DNA were obtained for 32 and 31 samples from sample 1 (2015) and sample 2 (2016), respectively. For these good DNA extractions, three distinct types of molecular markers were obtained: mitochondrial DNA, microsatellites and SNPs.

Three mitochondrial DNA fragments - the B domain of the control region (CR) (Lessinger and Azeredo-Espin 2000), the cytochrome c oxidase subunit I gene (COI) and the cytochrome c oxidase subunit II gene (COII) - were amplified according to the procedures described in Fresia et al. (2011). Purified PCR products were sequenced using a BigDye Terminator Cycle Sequencing Kit (version 3.0, Applied Biosystems, Foster City, CA, USA) in an ABI 3730 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA) at Life Sciences Core Facility (LaCTAD) from State University of Campinas (UNICAMP).

Eight polymorphic microsatellite loci (Torres et al. 2004; Torres & Azeredo-Espin 2005) were amplified according to the procedures described in Torres et al. (2004) and Torres & Azeredo-Espin (2005). PCR products were verified via 6% denaturing polyacrylamide gels stained with silver nitrate and the positive amplicons were multiplexed (4-plex) for subsequent sequencing in an ABI 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

SNPs were obtained using the Genotyping-By-Sequencing (GBS) approach. Two libraries were constructed with the individuals from samples 1 and 2, respectively, using standard protocols (Elshire et al. 2011, Poland et al. 2012) with minor modifications. DNA was double-digested with two high fidelity restriction enzymes, PstI (New England Biolabs, Ipswich, MA, EUA) and MspI (New England Biolabs, Ipswich, MA, EUA). Barcoded adapters were ligated on the rare cut site (i.e. PstI cut site CTGCAG) for each individual separately, and simultaneously the common adapter was ligated on the common cut site (i.e. MspI cut site CCGG) for all individuals. Individuals were pooled by year in two data sets (i.e. sample 1, with 32 individuals from 2015, and sample 2, with 31 individuals from 2016), which were amplified by multiplexed PCRs using standard forward primer A and modified reverse primer C (Sonah et al. 2013). PCR products were purified with Agencourt Ampure XP beads (Beckman-Coulter, Inc., Brea, CA, USA) and DNA amount was estimated with a NanoDrop UV spectrophotometer (Techno Scientific, Wilmington, DE, USA). The quality of the library was accessed with the evaluation of fragment sizes and the presence of adapter dimers in an Agilent BioAnalyzer 2100 at Life Sciences Core Facility (LaCTAD) from State University of Campinas (UNICAMP). The two libraries were sequenced separately in an Illumina NextSeq 500 (Illumina, Inc. San Diego, CA, USA) using 150bp single-end reads, at the IBTEC - UNESP (Botucatu, Brazil).

Mitochondrial haplotype reconstruction. CR, COI and COII sequences were independently aligned using ClustalX software (Thompson 1997) and manually inspected in Mega 6 (Tamura et al. 2013). COI and COII fragments were translated to protein in Mega 6 (Tamura et al. 2013) to confirm the open reading frame. Each insertion/deletion (*indel*) in the CR fragment was considered a single mutational step and recoded as a single position in the final alignment in Mega 6 (Tamura et al. 2013). Individual sequences of the three fragments were collapsed into haplotypes with Fabox (http://users-birc.au.dk/biopv/php/fabox/). Sequences of the three fragments for each individual were concatenated in a unique haplotype for further analyses.

Microsatellite genotyping. Microsatellite alleles were genotyped by size using the software GeneMarker 2.4.2 (SoftGenetics, LLC, PA, EUA) and manually checked. The presence and frequency of null alleles were estimated with the software Microchecker (Van Oosterhout et al. 2003), which also adjusted the allelic and genotypic frequencies.

SNP calling and outlier analysis. Stacks 1.48 *de novo* pipeline was used to demultiplex and perform the SNP calling without a reference genome (Catchen et al. 2011, 2013). The *process_radtags* from the pipeline was first used for raw data demultiplexing by barcode type, considering the parameters that remove low quality reads and reads with uncalled bases. The reads were trimmed to remove adaptors, restriction enzyme recognition sites and to consider only the initial 70bp (to keep most part of the reads in our dataset). After this processing, we excluded 5 and 6 individuals from samples 1 and 2, respectively (resulting in 27 and 25 remaining individuals, respectively), due to their low number of reads (less than 1% from the total number of reads in the sequenced flowcell). Considering this demultiplexed and cleaned dataset, the *ustacks* function was used to assemble *de novo* loci present in each individual, and calling SNPs (we considered 3 reads as a minimum stack depth of coverage and 2 mismatches as the distance allowed between stacks). Then, the data from each individual from both samples (i.e. samplings from 2015 and 2016) were merged into a catalog of loci using *cstacks* (distance allowed between catalog loci = 2), which contains all loci and alleles in the populations considered. Subsequently, each individual was matched against the catalog with *sstacks*. Finally, *populations* was executed for additional filtering, considering that (i) the locus must be present in both samples, (ii) only the first SNP of each locus was considered, (iii) the locus must be present in at least 75% of individuals in the samples, (iv) the minimum minor allele frequency to process a nucleotide site at a locus was 0.10, and (v) the minimum stack depth required for individuals at a locus was 3.

Additional filtering of data included pruning loci under Hardy-Weinberg disequilibrium and loci under selection in both samples. Hardy-Weinberg tests were conducted in the web version of Genepop (Raymond and Rousset 1995, Rousset 2008) considering a p-value of 0.05. Loci under selection were detected with Lositan (Antao et al. 2008), which calculates the F_{ST} values from each locus and compares them with the neutral distribution to detect outliers (i.e. loci under selective pressure) (Beaumont and Nichols 1996). The program was run three times to lower bias, as suggested by Antao et al (2008): (i) the first run used all loci to calculate a neutral mean FST, 50,000 simulations, 99% confidence interval, infinite alleles mutation model and false discovery rate of 0.1%; (ii) the second run was conducted with the same parameters from the first one, except that only the resulted neutral loci from the first run was considered to recalculate the mean neutral FST; (iii) the third run considered all loci again and the neutral FST calculated in the second run. Outlier loci in this last run were inferred as under selection and consequently excluded from the dataset for further population analyses. We conducted file conversions using PGDSpider v.2.0.5.1 (Lischer and Excoffier 2012) for downstream population genetic analyses.

Genetic diversity and population differentiation between years. We conducted genetic diversity and population structure analyses for each type of molecular marker in order to detect temporal changes in NWS fly population from Cañas in an interval of approximately one year.

Mitochondrial DNA haplotype diversity ($\hat{H}$), nucleotide diversity (π) and overall genetic structure (nonhierarchical AMOVA) were estimated with the software Arlequin 3.5 (Excoffier and Lischer 2010), with statistical significance obtained with 10,000 permutations. A parsimonious haplotype network was constructed with PopART 1.7 (Leigh and Bryant 2015) using TCS with 95% connection limit (Clement et al. 2002) to recover relationships between concatenated sequences.

The genetic variability of microsatellite loci was accessed through allelic richness (AR) with MSA 4.05 (Dieringer and Schlotterer 2003), observed (Ho) and expected (He) heterozygosities with the software GenAlex 6.5 (Peakall and Smouse 2012), and estimates of FIS with the web version of Genepop (Raymond and Rousset 1995, Rousset 2008). Each locus was tested for Hardy-Weinberg equilibrium with the web version of Genepop (Raymond and Rousset 1995, Rousset 2008), considering the Markov chain model from Guo and Thompson (1992) with the following parameters: 1,000 series, 1,000 iterations, 1,000 steps of "dememorization". Linkage disequilibrium between pairs of loci inside each sample was investigated using the web version of Genepop (Raymond and Rousset 1995, Rousset 2008).

Genetic structure was estimated by nonhierarchical AMOVA with the software Arlequin 3.5 (Excoffier and Lischer 2010), using 10,000 permutations to access statistical significance, and by a Bayesian clustering approach with the software Structure 2.3.3 (Pritchard et al. 2000). The parameters for Structure analysis were admixture model with correlated allele frequencies, 100,000 MCMC replications, 10,000 burn-in, K varying from one to five and 10 iterations for each K value. Evanno et al. (2005) method was used to determine the best K value with Structure Harvester 0.6.94 software (Earl & Von Holdt 2012). Results from this best K value were summarized with Clumpp 1.1.2 software (Jakobsson and Rosenberg 2007) and represented visually with Distruct 1.1 (Rosenberg 2004).

Filtered neutral SNPs were used to investigate nucleotide diversity, with observed heterozygosity (Ho), expected heterozygosity (He) and Wright´s F-statistic (FIS) being estimated by *populations* in Stacks pipeline. Genetic differentiation between the samples was estimated by nonhierarchical AMOVA with the software Arlequin 3.5 (Excoffier and Lischer 2010) and with the Bayesian approach implemented in Structure 2.3.3 (Pritchard et al. 2000), with the same parameters and procedures described for microsatellite data.

Demographic inferences. The demographic history of the species was inferred from mitochondrial data with Tajima's D (Tajima 1989) and Fu's Fs (Fu 1997) neutrality tests and mismatch distribution analysis. Tajima's D (Tajima 1989) and Fu's Fs (Fu 1997) were calculated using Arlequin 3.5 (Excoffier and Lischer 2010). For both summary statistics, values near zero are indicative of population size stability, negative values are indicative of recent population expansion, and positive values are indicative of population bottlenecks (Tajima 1989, Fu 1997). Mismatch distribution analysis was performed using Arlequin 3.5 software (Excoffier and Lischer 2010), with 10,000 permutations for statistical significance. The *Raggedness* index (*rg*) was used to measure the adjustment to a population expansion model.

For both microsatellite and SNP data, two-sample effective population size (*Ne*) estimates were calculated using the Jorde and Ryman (2007) method in NeEstimator 2.01 software (Do et al. 2014). This analysis considered a minimum allele frequency (MAF) cutoff of 0.01, 0.02 and 0.05 and 18 generations between both samples (NWS fly generation time is approximately 21 days in laboratory conditions, which corresponds to almost 18 generations in a period of 1 year).

Results

Diversity. Concatenated mtDNA sequences resulted in 21 haplotypes for both samples, with only two haplotypes shared between them (named here as H1 and H2) (Table 1). This most common haplotype H1 is the most represented in both samples, and became considerably more frequent in sample 2. Samples 1 and 2 showed high and moderate haplotype diversity for mtDNA, respectively, and low nucleotide diversity (Table 1).

Table 1. Mitochondrial DNA genetic diversity. N: number of individuals; Nh: number of haplotypes; $\hat{H}$: haplotype diversity; π: nucleotide diversity.

Sample	N	Nh	Haplotypes (nº of individuals)	$\hat{H}$ (s.d.)	π (s.d.)
1	32	19	H1 (8), H2 (4), H3 (2), H4 (2), H5 (2), H6 (3), H8 (2), H9 to H17 (1)	0.9375 (0.0286)	0.004530 (0.002372)
2	31	8	H1 (24), H2 (1), H7 (2), H18 to H21 (1)	0.4538 (0.1109)	0.001858 (0.001064)

Microsatellite loci showed a moderate to high degree of variability, with Ho ranging from 0.355 to 0.833 and He from 0.361 to 0.852 (Table 2). In sample 1, no locus showed significant deviations from Hardy-Weinberg equilibrium after Bonferroni correction ($p<0.006$). Otherwise, 4 from the 8 loci significantly deviated from Hardy-Weinberg equilibrium in sample 2 after correction ($p<0.006$), with CH15 and CH26 exhibiting excess of homozygotes (i.e. positive F_{IS}) and CH05 and CH14 exhibiting excess of heterozygotes (i.e. negative F_{IS}).

Table 2. Microsatellite genetic diversity for each locus in each NWS fly sample. N: sample size; Na: number of alleles per locus; AR: allelic richness; Ho: observed heterozygosity; He: expected heterozygosity; F_{IS}: inbreeding coefficient. Significant departures from Hardy-Weinberg equilibrium were indicated by an asterisk (*) in the value of He ($p < 0.006$).

Sample		Loci							
		CH01	CH05	CH09	CH12	CH14	CH15	CH24	CH26
Sample 1 (N=32)	N	23	32	31	30	24	21	21	32
	Na (AR)	7 (7)	5 (4.97)	5 (5)	11 (11)	4 (4)	8 (7.98)	6 (6)	11 (10.75)
	Ho	0.522	0.656	0.355	0.833	0.375	0.667	0.714	0.781
	He	0.693	0.665	0.361	0.804	0.533	0.811	0.787	0.815
	F_{IS}	0.2677	0.0291	0.0337	-0.0197	0.3157	0.2011	0.1163	0.0572
Sample 2 (N=31)	N	31	31	31	31	31	19	31	30
	Na (AR)	6 (5.99)	6 (6)	5 (5)	7 (6.97)	5 (4.90)	5 (5)	4 (4)	9 (9)
	Ho	0.548	0.645	0.613	0.710	0.645	0.579	0.613	0.833
	He	0.677	0.583*	0.624	0.729	0.616*	0.778*	0.670	0.852*
	F_{IS}	0.2056	-0.0909	0.0339	0.0435	-0.0318	0.2813	0.1009	0.0391

Tests for detecting genotypic linkage disequilibrium (LD) found significant association between pairs of loci only in sample 2, with 12 cases for $p<0.05$ and 8 cases after Bonferroni correction ($p<0.006$) (Table 3).

Table 3. Pairwise linkage disequilibrium between microsatellite loci. Statistically significant differences are indicated by asterisks: * $p<0.05$, ** $p<0.006$.

		P-value	
Locus 1	**Locus 2**	**Pop 1**	**Pop 2**
CH01	CH05	0.586370	0.586170
CH01	CH09	0.160350	0.034380*
CH05	CH09	0.454380	0.838580
CH01	CH12	1.000000	0.000000**
CH05	CH12	0.886270	0.124870
CH09	CH12	0.770180	0.000360**
CH01	CH14	0.220510	0.000000**
CH05	CH14	0.638530	0.119450
CH09	CH14	0.251030	0.536360
CH12	CH14	0.987650	0.002780**
CH01	CH15	0.374290	0.010970*
CH05	CH15	0.899110	0.542880
CH09	CH15	0.963080	0.259410
CH12	CH15	1.000000	0.246800
CH14	CH15	0.810280	0.327000
CH01	CH24	1.000000	0.029460*
CH05	CH24	0.659180	0.383390
CH09	CH24	0.843900	0.053430
CH12	CH24	0.543510	0.089310
CH14	CH24	1.000000	0.220070
CH15	CH24	0.368190	0.421300
CH01	CH26	0.175640	0.002990**
CH05	CH26	0.959870	0.007710*
CH09	CH26	0.864760	0.000000**
CH12	CH26	1.000000	0.000000**
CH14	CH26	0.623260	0.187100
CH15	CH26	0.401790	0.578220
CH24	CH26	0.742220	0.000000**

The SNP calling pipeline from Stacks, including *populations* filtering, recovered 1137 SNPs. Hardy-Weinberg tests recovered 52 loci in disequilibrium and Lositan indicated 257 outlier loci (i.e. putatively under selection), which were pruned from our data. After all these filtering steps, our data matrix for downstream analyses was represented by 828 loci.

Mean expected heterozygosity for both samples was similar, being somewhat lower in sample 2, and was little higher than the mean observed heterozygosities, culminating in small

positive F_{IS} estimates (Table 2). In addition to the 52 loci with deviations from Hardy-Weinberg equilibrium in both samples (that were previously excluded), 116 and 94 loci showed deviations in samples 1 and 2, respectively (data not shown).

Table 3. SNPs mean genetic diversity. N: number of individuals analyzed, Ho: observed heterozygosity; He: expected heterozygosity; F_{IS}: inbreeding coefficient.

Sample	N	Ho	He	FIS
1	27	0.1113	0.1317	0.0981
2	25	0.1044	0.1141	0.0498

Population differentiation. Haplotype network from mtDNA concatenated sequences shows that samples 1 and 2 present distinct haplotype distributions, sharing the common haplotype H1 (Figure 2).

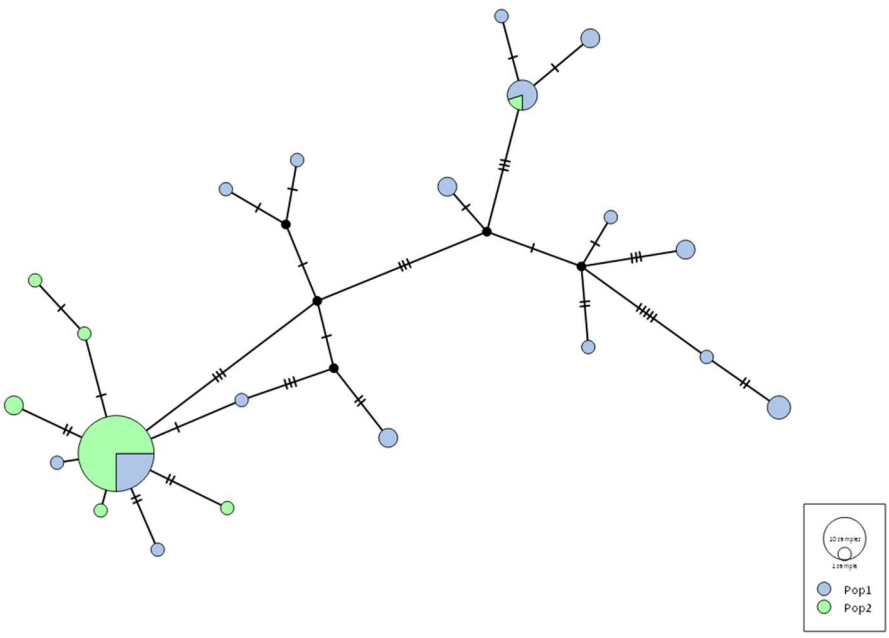

Figure 2. Haplotype network from mtDNA concatenated sequences. Circles sizes are proportional to the number of sequences representing the haplotype. Circle colors differentiate the sequences from each sample: sample 1 is represented in blue and sample 2 in green.

AMOVA result for mtDNA data was statistically significant (F_{ST} = 0.27537, p = 0.0000), indicating changes from one year to another. For microsatellite and SNP data, AMOVA results also indicated that both samples were genetically differentiated (F_{ST} = 0.08385, p = 0.0000 for microsatellites and F_{ST} = 0.01444, $p < 0.05$ for SNPs).

Bayesian inferences of population structure with Structure for microsatellite and SNP data indicate population differentiation over time, revealing the presence of 2 clusters for microsatellites and 3 for SNPs (Figure 3).

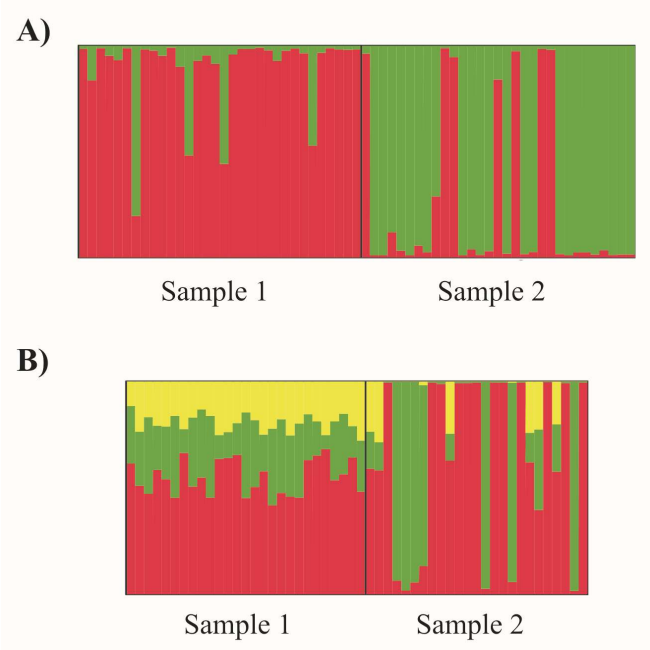

Figure 3. Structure results for microsatellite and SNP data. A) Structure inference for microsatellites - 2 clusters; B) Structure inference for SNPs - 3 clusters. Each color represents a genetic group and each vertical line represent an individual. Each individual is colored proportionally to their probability of assignment to each genetic group.

Demographic inferences. Neutrality tests for mtDNA were only statistically significant in one case: Tajima´s D for sample 2 (Table 4). The negative value of Tajima´s D is indicative of population expansion.

Table 4. Neutrality tests for mtDNA and their respective p-values.

	Tajima´s D	p-value	Fu´s FS	p-value
Sample 1	-0.32833	0.416	-2.77043	0.16600
Sample 2	-2.38060	0.000*	1.10613	0.72700

Since only sample 2 revealed some signal of population size change, the mismatch distribution analysis was conducted with this sample alone. This analysis resulted in a not so clear distribution (i.e., a definitely unimodal or multimodal distribution) and a non-significant *Raggedness* index (rg = 0.2978, P = 1.00).

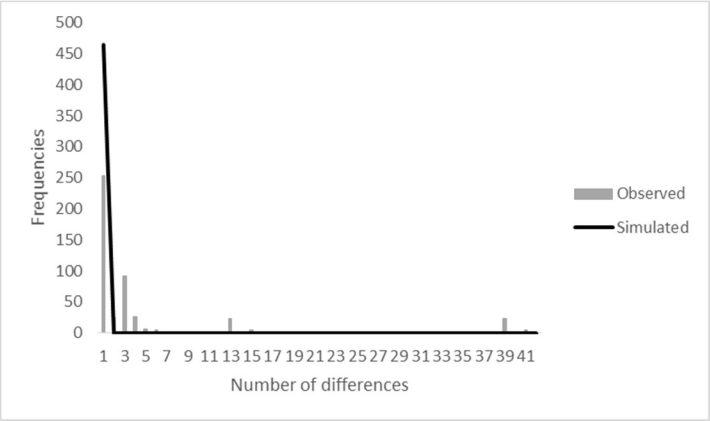

Figure 4. Mismatch distribution analysis for mtDNA sequences from sample 2.

Effective population size (*Ne*) estimates based on microsatellite and SNP data diverged about 3.5 times: microsatellites indicated a *Ne* of approximately 70 individuals, while SNPs estimated around 250 individuals (Table 5).

Table 5. *Ne* estimates from microsatellite (SSR) and SNP data, based on Jorde and Ryman (2007) method.

		Lowest allele frequency used			
		0.05	**0.02**	**0.01**	**0+**
Harmonic Mean Sample Size		27.1	27	27.2	27.5
SSR	**Fs**	0.17722	0.17167	0.17052	0.17003
	F´	0.13531	0.13013	0.12905	0.12859
	Ne	66.5	69.2	69.7	70.0
	(95% CI, parametric)	(39.5 - 100.5)	(43.9 - 100.1)	(45.1 - 99.6)	(46.5 - 98.2)
SNP	**Fs**	0.07969	0.08043	0.07811	0.07739
	F´	0.03631	0.03710	0.03469	0.03435
	Ne	247.9	242.6	259.5	262.0
	(95% CI, parametric)	(211.4 - 287.2)	(211.2 - 276.1)	(233.3 - 287.0)	(237.3 - 287.8)

Discussion

The three types of molecular markers indicated temporal genetic structure and gave some insights about the NWS fly demographic dynamic in a location from the temperate region, which is the southern region occupied by the species.

The mtDNA haplotype H1 is the most represented in both sampled years, becoming more frequent in sample 2. Sample 1 is more diverse for mtDNA, once it has more than twice as many haplotypes as sample 2, which is reflected in the higher haplotype diversity index ($\hat{H}$). The patterns of loss of rare alleles (i.e. haplotypes) and increasing in the number of individuals presenting the common haplotype from samples 1 to 2 are typical from a population that have

experienced a bottleneck followed by an expansion. Genetic structure was recovered by AMOVA analysis, which indicated a moderate to high degree of population structure between the two samples based on F_{ST} values. Comparatively, F_{ST} values inferred from mtDNA sequences were considerably higher than estimates for microsatellites or SNPs. This is expected due to the fourfold lower effective population size of mtDNA loci compared to nuclear markers (Larsson et al. 2009, Mesnick et al. 2011) and/or the population dynamics of the species, with females presenting higher dispersion capability than males (Mangan and Thomas 1989, Thomas and Mangan 1989).

Population size changes were detected only for sample 2 according to mtDNA neutrality tests and mismatch distribution, suggesting that sample 1 was stable. The negative Tajima´s D found for sample 2 means an excess of low frequency polymorphisms relative to expectation, indicating population size expansion after a bottleneck. This implies in a scenario in which sample 1 was stable and, for some reason, suffered a bottleneck in subsequent generations. After a period of this bottleneck, the population started expanding, and these signals of population size changes are present in sample 2, which was probably still expanding until reaching its maximum and population stability. MtDNA mismatch distribution is usually multimodal for populations at demographic equilibrium and unimodal for populations that have passed through a recent demographic expansion or through a range expansion with high levels of migration among neighboring demes (Excoffier et al. 1992, Rogers and Harpending 1992, Rogers et al. 1996, Schneider and Excoffier 1999). We are not able to discriminate undoubtedly the shape of the mismatch distribution for sample 2, but the non-significant *Raggedness* index indicates that we do not have any support for a stable population and we cannot reject the null hypothesis of population expansion.

Microsatellite loci from sample 1 showed no deviations for Hardy-Weinberg equilibrium and no linkage disequilibrium, indicating stability in population size, similar to mtDNA data. Differently, the distorted distribution of allele frequencies (i.e., half microsatellite loci showed deviations for Hardy-Weinberg) and increase in linkage disequilibrium in sample 2 could be due to populations bottlenecks or founder effects (Hartl and Clark 2007). It is notable that some loci presented less alleles from sample 1 to sample 2. This change in allelic diversity is a better parameter for bottleneck detection than heterozygosity, which is generally insensitive to these type of demographic change (Habel et al. 2014). A decline in genetic diversity, as observed here, and a shift in genetic composition can indicate a population bottleneck (Tison et al. 2015).

Additionally to these differences in genetic diversity, samples 1 and 2 were significantly low differentiated as indicated by AMOVA and Structure results, presenting a genetic profile change from one year to the other, with few individuals from one sample being more genetically related to the other.

The genotyping-by-sequencing (GBS) and similar techniques such as RAD-seq (e.g. Baird et al. 2008, Elshire et al. 2011, Peterson et al. 2012, Poland et al. 2012) radically changed the state of the art of the population genomics, allowing the obtainment of many informative genetic markers from the genome of any species, simultaneously for several individuals, for a relatively low cost (Davey et al. 2011). The huge amount of genetic information obtained with GBS is capable of estimating genetic variation and structure in many distinct situations. However, to date, few temporal population studies were conducted with markers obtained by GBS. Nunziata et al. (2017) studied the utility of genetically based demographic inference on salamander species with documented population declines and expansions in a time series over 37 years, which are known to be correlated with changes in wetland hydro-period. For this, they used ddRAD data from two temporally sampled populations and used coalescent-based demographic inference. Their results suggest that genome-based demographic inference has utility on an ecological scale (<100 years), but researches should also be cognizant that these methods have many difficulties and may not work in all systems and evolutionary scenarios.

Our work found that SNP data variability do not changed drastically from one year to the other: a similar number of loci presented deviations from Hardy-Weinberg equilibrium and mean observed and expected heterozygosity were similar. SNPs are bi-allelic and, then, it is expected to be less variable compared to microsatellites (Habel et al. 2014). Besides that, population structure analysis with SNPs indicate a very low, but significant, degree of differentiation between populations.

Some studies indicated genomic SNPs as good markers for improving resolution in population analyses compared to less informative markers (e.g. Reitzel et al. 2013). For insects, studies reported sufficient power for genomic SNPs to detect spatial population structure. Low significant levels of spatial population structure were reported for *Aedes aegypti* samples from Rio de Janeiro for nuclear markers (i.e. microsatellites and SNPs), while mtDNA variation revealed high spatial structure (Rašić et al. 2015). Population structure, with high FST value (0.19109), was observed for Brazilian populations of the insect pest *Grapholita molesta* based on 1,266 SNPs (Silva-Brandão et al. 2015). Anderson et al. (2016), working with the insect pest *Helicoverpa armigera*, reported improved resolution in population structure and gene flow using SNPs in relation to mtDNA data.

Our *Ne* estimates of approximately 70 (range 39.5 to 100.5) and 250 (ranging from 211.2 to 287.8) individuals for microsatellite and SNP loci, respectively, are based only in one sampling location in two temporal points and, then, any comparisons to other estimates should be made with reservations. Estimates of *Ne* for *Aedes aegypti* across 17 geographic localities and time points resulted in values slightly different from the ones we found, considering the same method of Jorde and Ryman (2007): for microsatellite loci, estimated *Ne* averaged 303.3, ranging from 25.0 to 1181.0, while for SNPs the average was 166.0, ranging from 22.9 to 549.2 (Saarman et al. 2017). According to the authors, these *Ne* values, which are not so different from the ones found in our study, are lower than estimates available for other insects.

In relation to the discrepancy between our *Ne* estimates based on microsatellite and SNP data, SNPs are considered superior to microsatellites for elucidating historical demography, once it represents an unbiased sampling of genomic variation (Brumfield et al. 2003). Therefore, we can consider that the estimated *Ne* of approximately 250 individuals can be more realistic for our NWS fly population. But this need to be considered with caution, once accurately estimation of population demographic changes in a contemporary time scale (20 generations) is dependent on the number of samples and the targeted number of GBS (or RADseq) loci (i.e. very large SNP data sets, with more than 25,000 SNPs, are necessary to detect population declines) (Nunziata and Weisrock 2017).

According to Whitlock (1992), population sizes of many species are dependent on many factors which can be variable through time, including the availability of resources, ecological processes (e.g. predation), migration rates, unusual events (e.g. floods), and extinction and recolonization processes related to temporarily favorable conditions. In the case of the NWS fly, host availability and insecticide use is likely to be important factors affecting population size, since females seek for wounds and natural cavities to do oviposition (Guimarães and Papavero 1999) and the control of this insect pest in South America countries is made by applying chemical insecticides without any regulation. Another important factor for this species, in its southern distribution, are climatic conditions (Vargas-Terán, Hofmann and Tweddle 2005). From this, we hypothesize that NWS fly populations migrate to northern regions during unfavorable conditions and recolonizes the southern in favorable ones, or are capable of maintaining few individuals (i.e. only a subset of the original diversity) in refugees during winter, characterizing a population bottleneck, followed by population expansion when springs start.

In conclusion, this is the first study to investigate the temporal genetic variation of a population of NWS fly with neutral markers and is the first study to utilize SNP markers for

this species, a widely dispersed and highly mobile insect pest from which we have little a priori indication of geographic population structure. All three types of molecular markers applied in the present work indicated significant temporal changes for the Uruguayan population analyzed, which we attributed mainly to population size fluctuations associated to climatological changes in the southern distribution of the species (but host availability and insectide use are also important). Testing and discriminating among distinct demographic hypothesis, including both hypotheses raised above (i.e. extinction-recolonization or maintenance of few individuals in refugees), and getting more robust *Ne* estimates involves the necessity of obtaining SNP data from more temporal points, in distinct seasons and climatic conditions, and more geographic sites, besides applying coalescent-based demographic analysis (e.g. ABC). Robust demographic inferences may be an important tool for population monitoring (Nunziata et al. 2017) and for the design of control programs, which is the ultimate goal in the case of the NWS fly in its current distribution.

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DISCUSSÃO GERAL

A presente tese se propôs a realizar estudos populacionais que contribuíssem para um melhor entendimento da dinâmica populacional da espécie *C. hominivorax*, uma importante praga agropecuária, e, assim, fornecer dados para o planejamento e posterior implementação de adequadas estratégias de controle.

A revisão de todos os estudos populacionais e filogeográficos realizados para *C. hominivorax* na América do Sul e Caribe apresentada no Capítulo 1 denota um cenário em que a América do Sul apresenta dois grandes grupos genéticos (denominados NAG e SAG), nos quais apenas dois estudos foram conduzidos em uma escala menor e regional (Lyra et al. 2005, Torres et al. 2007). Estes estudos, que utilizaram diferentes marcadores genéticos, resultaram em diferentes graus de variabilidade e estrutura genética populacional, levantando diferentes hipóteses acerca dos processos envolvidos.

Diante disso, nos capítulos 2 e 3 foram apresentados dois estudos em escala local/microgeográfica no Uruguai, cujos resultados contribuem para o estabelecimento de unidades de manejo e para o entendimento da dinâmica populacional de *C. hominivorax* na região ao sul da sua atual distribuição.

Com base em marcadores microssatélites e mtDNA, não foi observado nenhum sinal de estrutura geográfica populacional, indicando assim que, nessa escala local, as populações de *C. hominivorax* se comportam como populações panmíticas. Dessa maneira, podemos concluir que escalas regionais são mais adequadas para serem determinadas unidades de manejo em programas de controle. De maneira geral, grande parte das espécies de insetos praga apresentam padrões semelhantes ao de *C. hominivorax*, com baixa resolução da estrutura populacional (e.g. Anderson et al. 2016).

Apesar da ausência de estrutura espacial, foi possível detectar estrutura temporal para uma população do Uruguai em um intervalo de 1 ano, utilizando marcadores microssatélites, mtDNA e SNPs. A estruturação foi mais clara para o mtDNA, o que pode ser associado ao menor tamanho efetivo desse genoma em relação ao nuclear (Larsson et al. 2009, Mesnick et al. 2011), mas também foi significativa para os outros dois marcadores nucleares.

Este é o primeiro trabalho a utilizar a técnica de GBS para a espécie *C. hominivorax*. Mesmo envolvendo o estudo de uma única localidade geográfica em apenas dois períodos distintos, a quantidade de SNPs obtidas (i.e. 828 SNPs) foi similar à de outros estudos com mais localidades (e.g. Silva-Brandão et al. 2015), indicando o potencial de uso desta técnica em outros estudos populacionais para esta espécie. Adicionalmente, os 257 SNPs outliers (i.e.

possivelmente sob seleção) que foram excluídos do conjunto de dados utilizados neste estudo temporal serão investigados quanto à região genômica onde ocorrem para detectar quais genes estão sob seleção nesta espécie e, possivelmente, associá-los às suas funções (e.g. resistência a inseticidas).

Os distintos padrões de variabilidade genética encontrados em cada um dos dois períodos amostrados, assim como os sinais demográficos do estudo local na fronteira entre Brasil e Uruguai, sugerem a ocorrência de flutuações populacionais. Flutuações populacionais recentes podem ocorrer devido a inúmeros fatores. Em *C. hominivorax*, a presença de diferentes hospedeiros e/ou locais de intensa atividade pecuária associados à frequente utilização de inseticidas para o controle são fatores importantes que devem influenciar na dinâmica populacional desta espécie ao longo de toda sua área de ocorrência. No caso do Uruguai, ambos os fatores são altamente relevantes, por se tratar de um país com tradição em atividade pecuária, principalmente ovinos. Adicionalmente, as condições climáticas (temperatura, pluviosidade e umidade) podem ser citadas como outro fator relevante (Hightower et al. 1966, Stuart et al. 1995, Gutierrez and Ponti 2014), principalmente na região ao sul da distribuição da espécie, como é o caso do presente estudo. Diante disso, propomos duas hipóteses distintas para explicar as flutuações populacionais de *C. hominivorax* nessa região, ambas baseadas nas mudanças do clima: (i) "hipótese de extinção-recolonização", em que as populações migrariam para uma região mais ao norte em períodos de clima desfavorável (frio e seco), e retornariam à regiões mais ao sul quando as condições voltam a ser tornar favoráveis; (ii) "hipótese dos refúgios", em que o tamanho da população reduz drasticamente nos períodos desfavoráveis, com alguns poucos indivíduos sendo mantidos em refúgios; estes indivíduos rem anescentes gerariam nova prole e reestabeleceriam as populações nos períodos favoráveis. Essas duas hipóteses se baseiam em mudanças cíclicas nas condições climáticas, de modo que a segunda hipótese se baseia diretamente na hipótese elaborada por Shpak et al. (2009). Entretanto, para discernir entre ambas hipóteses, é necessário obter amostragem de mais períodos, em diferentes estações do ano, e provenientes de mais localidades.

Os resultados apresentados nesta tese sugerem que estudos em escala regional tem o potencial de elucidar os padrões populacionais da mosca-da-bicheira e, assim, permitir o estabelecimento de unidades geográficas distintas.

Estabelecendo alicerces para futuros estudos em escala regional

Neste sentido e baseado nos resultados obtidos, foram iniciadas no nosso laboratório análises de amostras de *C. hominivorax* do Maranhão e Peru, regiões estratégicas

subamostradas até o momento. Essas duas regiões são importantes por terem sido preditas como corredores de conexão entre populações do NAG e SAG (Fresia et al 2014), além de apresentarem intensa atividade pecuária. Adicionalmente, o Maranhão é um importante centro de recebimento de gado proveniente de todo o Brasil para exportação de gado em pé para diferentes países, como Turquia e Arábia Saudita (Livio Martins Costa-Júnior, comunicação pessoal).

Foram obtidas sequências de DNA mitocondrial (i.e. CR, COI e COII) para 100 amostras do Maranhão e 93 amostras do Peru, provenientes de 7 e 10 localidades geográficas distintas, respectivamente (Figura 2, página 18). Analisando a rede de haplótipos das sequências concantenadas dessas amostras (Figura 3) podemos observar que as duas regiões (i.e. Maranhão e Peru) se distinguem por apresentarem cada qual um conjunto de haplótipos bem diferentes. Porém, através das conexões dos haplótipos, nenhum tipo de estruturação geográfica fica evidente.

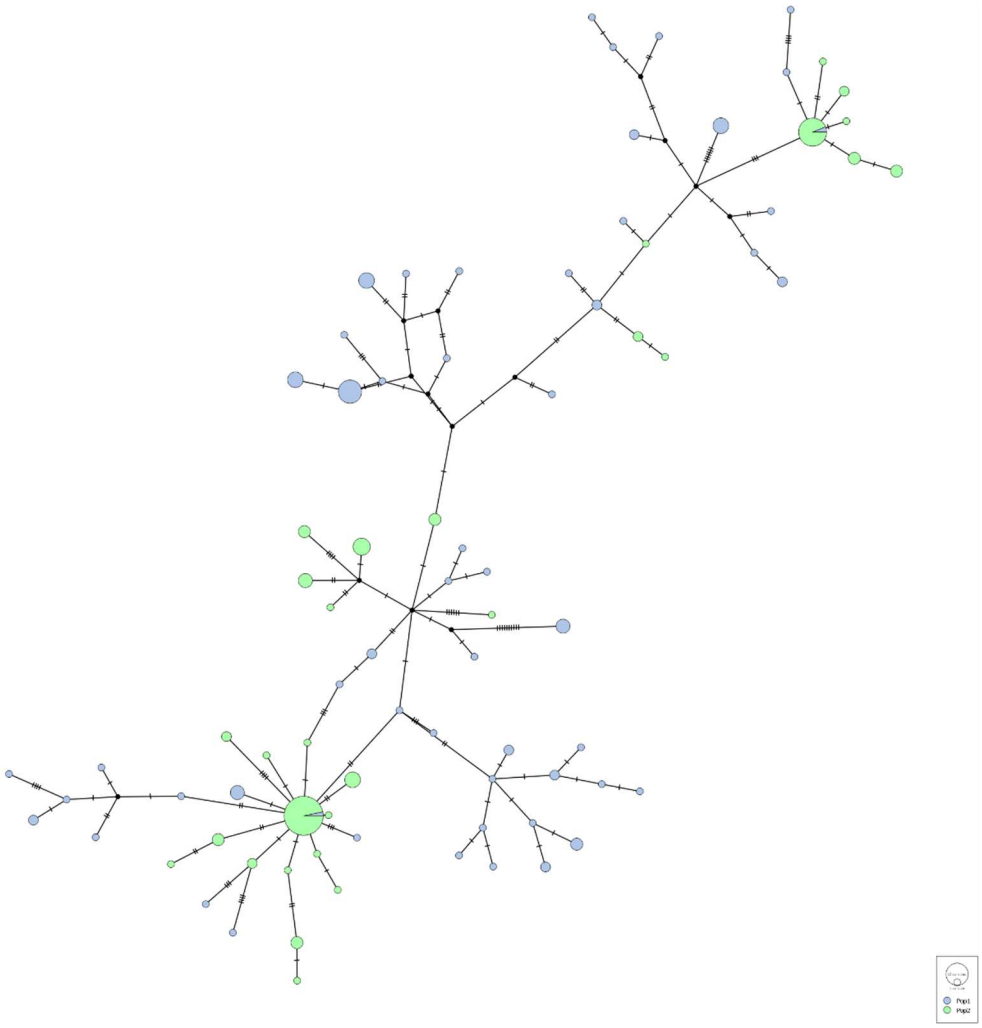

Figura 3. Rede de haplótipos das sequências mitocondriais concatenadas (CR-COI-COII) das amostras do Peru (em verde) e Maranhão (em azul).

As regiões amostradas apresentam altos índices de diversidade haplotípica (Ĥ) e baixos índices de diversidade nucleotídica (π) (Tabela 1), com exceção das localidades com baixa amostragem (i.e. N < 4), padrão esse observado em todos os estudos populacionais conduzidos para a mosca-da-bicheira até o momento, incluindo os trabalhos microgeográficos apresentados nos capítulos 2 e 3 desta tese.

Tabela 1. Diversidade genética das localidades amostradas no Maranhão e Peru. ID: identificação, Nh: número de haplótipos, Ĥ: diversidade haplotípica, π: diversidade nucleotídica.

	ID	Localidade	Nh	Haplótipos (no. indivíduos)	Ĥ	π
Maranhão	A	Serrano do Maranhão	3	h13(3)	0,0000 + - 0,0000	0,000000 + - 0,000000
	B	Sta Luzia Paruá	14	h1(8), h2(1), h7(2) h84(1), h85(1), h86(1)	0,9121 + - 0,0486	0,002262 + - 0,001327
	C	Humberto de Campos	3	h1(1), h27(2)	0,6667 + - 0,3143	0,003291 + - 0,002664
	D	São Luís	11	h1(1), h9(4), h19(3), h27(2), h83(1)	0,8182 + - 0,0826	0,004662 + - 0,002617
	E	Açailândia	20	h1(4), h2(2), h15(3), h17(1), h28(2), h78(1), h79(1), h81(1), h82(1), h88(1), h89(1), h90(1), h92(1)	0,9632 + - 0,0282	0,003471 + - 0,001900
	F	Fortuna	28	h1(12), h7(3), h14(3), h16(3) h29(3), h30(1), h76(1), h77(1), h80(1)	0,9127 + - 0,0264	0,002365 + - 0,001326
	G	Pirapemas	21	h2(11), h4(3), h17(2), h18(3), h87(1), h91(1)	0,9000 + - 0,0324	0,003450 + - 0,001885
Peru	H	Amazonas	8	h3(3), h5(5)	0,5357 + - 0,1232	0,004755 + - 0,002781
	I	Cajamarca	6	h32(1), h34(1), h35(1), h58(1), h59(1), h69(1)	1,0000 + - 0,0962	0,005851 + - 0,003572
	J	Huánuco	10	h20(1), h21(2), h36(1), h37(1), h38(1), h65(1), h66(1), h67(1), h68(1)	0,9778 + - 0,0540	0,004547 + - 0,002585
	K	Ica	11	h3(5), h6(5), h75(1)	0,6364 + - 0,0895	0,000538 + - 0,000431
	L	Lima	10	h3(3), h22(2), h31(1), h39(1), h40(1), h73(1), h74(1)	0,9111 + - 0,0773	0,004734 + - 0,002684
	M	Moquega	2	h41(1), h55(1)	1,0000 + - 0,5000	0,001480 + - 0,001709
	N	Piura	18	h8(4), h10(3), h23(2), h24(2), h42(1), h43(1), h44(1), h45(1), h62(1), h63(1), h64(1)	0,9281 + - 0,0401	0,004335 + - 0,002346
	O	Ucayali	13	h20(1), h25(1), h46(1), h47(1), h48(1), h49(1), h50(1), h51(1), h52(1), h70(1), h71(1), h72(1)	1,0000 + - 0,0340	0,004167 + - 0,002336
	P	Tumbes	2	h26(2)	0,0000 + - 0,0000	0,000000 + - 0,000000
	Q	Loreto	13	h11(3), h12(3), h33(1), h53(1), h54(1), h56(1), h57(1), h60(1), h61(1)	0,9231 + - 0,0572	0,005116 + - 0,002806

A AMOVA hierárquica revelou estrutura genética entre as amostras. Esta análise atribuiu 14,71% da variância total a comparações entre Peru e Maranhão (FCT = 0,14712, p = 0,0003), 23,10% a comparações entre as localidades dentro de cada uma dessas duas regiões (FSC = 0,27079, p = 0,0000) e 62,19% a variações dentro das localidades. O FST par-a-par para as localidades analisadas são apresentados na Tabela 2. Das 136 comparações entre as regiões, 102 foram significativas (p<0,05). Os maiores valores de FST não necessariamente foram observados entre localidades do Peru e Maranhão, embora ocorra em vários casos, indicando que não há uma completa correlação entre as distâncias genéticas e geográficas.

Tabela 2. FST par-a-par entre as localidades amostradas no Maranhão (A a G, em cinza claro) e no Peru (H a Q, em cinza escuro). Em asterisco (*) estão indicadas as comparações estatisticamente significativas (p<0,05).

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
A	0,00																
B	0,13	0,00															
C	0,57	0,34	0,00														
D	0,30	0,23*	0,07	0,00													
E	0,08	-0,01	0,18*	0,15*	0,00												
F	0,11	-0,02	0,30*	0,27*	0,02	0,00											
G	0,49*	0,37*	0,31*	0,08	0,27*	0,41*	0,00										
H	0,51*	0,50*	0,37*	0,34*	0,43*	0,53*	0,42*	0,00									
I	0,32*	0,33*	0,21	0,21*	0,26*	0,37*	0,35*	0,28*	0,00								
J	0,47*	0,42*	0,25*	0,17*	0,33*	0,46*	0,22*	0,25*	0,13*	0,00							
K	0,93*	0,75*	0,84*	0,61*	0,64*	0,71*	0,65*	0,58*	0,63*	0,57*	0,00						
L	0,36*	0,34*	0,17	0,18*	0,27*	0,35*	0,31*	0,23*	0,12	0,09*	0,40*	0,00					
M	0,93	0,67*	0,65	0,44*	0,55*	0,66*	0,57*	0,40*	0,35	0,40*	0,66*	0,24	0,00				
N	0,31*	0,31*	0,33*	0,30*	0,29*	0,33*	0,41*	0,39*	0,17*	0,30*	0,47*	0,16*	0,36*	0,00			
O	0,30*	0,25*	0,21*	0,14*	0,18*	0,28*	0,25*	0,29*	0,08	0,08	0,55*	0,06	0,39*	0,14*	0,00		
P	1,00	0,54*	0,61	0,32	0,39*	0,52*	0,46*	0,45	0,17	0,34*	0,91*	0,25	0,87	0,06	0,16	0,00	
Q	0,25	0,28*	0,28*	0,28*	0,26*	0,31*	0,40*	0,39*	0,14	0,29*	0,58*	0,20*	0,42	0,16*	0,14*	0,11	0,00

Os resultados preliminares aqui apresentados mostram que foi possível detectar estrutura genética populacional, e até certo ponto geográfica, tanto no Maranhão quanto no Peru. Estes dados serão utilizados, juntamente com todas as sequências mitocondriais já geradas pelo nosso grupo de pesquisa em trabalhos anteriores, para conduzir um estudo filogeográfico mais abrangente da espécie, na tentativa de corroborar os corredores de conexão preditos por Fresia et al (2014).

CONSIDERAÇÕES FINAIS

1) Nossos resultados sugerem que escala regionais possivelmente são as mais adequadas para o estabelecimento de unidades de manejo para a espécie *C. hominivorax*.

2) Mudanças temporais nas frequências alélicas em uma localidade do Uruguai indicam flutuações populacionais, possivelmente associadas a inúmeros fatores, como presença de hospedeiros, uso de inseticidas e flutuações climáticas sazonais.

3) Duas hipóteses acerca das flutuações populacionais devido a mudanças climáticas sazonais foram levantadas nesta tese: (i) hipótese de extinção-recolonização e (ii) hipótese dos refúgios.

4) A padronização da técnica de GBS para a espécie *C. hominivorax* permitiu a obtenção de uma boa quantidade de marcadores SNPs, que auxiliaram na investigação temporal de uma localidade do Uruguai. Esses marcadores tem ainda o potencial de refinar estudos populacionais geográficos.

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ANEXOS

ANEXO 1 – Declaração de Bioética e Segurança

UNICAMP

CBMEG

CIDADE UNIVERSITÁRIA "ZEFERINO VAZ",
17 DE MAIO DE 2018.

CIBIO: 01/2018

IDENTIFICAÇÃO

Doutorado: Luana Walravens Bergamo – CPG – GBM – UNICAMP.

PROJETO

Genômica populacional da mosca-da-bicheira *Cochliomyia hominivorax (Diptera: Calliphoridae).*

PARECER

Projeto aprovado pela CIBio / CBMEG sob número 01/2015 – Genômica da mosca-da-bicheira *Cochliomyia hominivorax (Diptera: Calliphoridae*): análise estrutural e funcional.

Coordenador: Profa. Dra. Ana Maria Lima de Azeredo-Espin.

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Campinas, 14 de Maio de 2018

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