UNIVERSIDADE ESTADUAL DE CAMPINAS
INSTITUTO DE BIOLOGIA

Fabricio José Biasotto Francischini

Morphological and molecular characterization of species of *Diatraea* ssp. (Lepidoptera: Crambidae) and elucidation of dispersal pattern in America continent

Caracterização morfológica e molecular de espécies de *Diatraea* ssp. (Lepidoptera: Crambidae) e elucidação dos padrões de dispersão no continente americano

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Orientadora: Profa. Dra. Maria Imaculada Zucchi
Coorientador: Dr. Tederson Galvan

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Para

Josy

Minha eterna esposa

Juntos recebemos o dom Da Graça!

Constantemente você me ensina a construir nossa família alicerçados em Jesus.

Onde, com o amor Dele soubemos enfrentar a vida

E saborear o presente precioso que Deus nos deu!

“Honre sua esposa, como herdeira do dom da graça”

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Ao meu super herói Noah e minha doce princesa Liz....

“Os filhos são herança do Senhor, uma recompensa que ELE dá....

....Como é feliz o homem que tem a sua aljava cheia deles”

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RESUMO

A broca-da-cana-de-açúcar ou broca-do-colmo-do-milho (*Diatraea* spp.) é um inseto polífago, considerada inseto-praga de culturas importantes como milho, sorgo e cana-de-açúcar. Prejuízos originados pelo ataque de espécies de *Diatraea* ssp. têm se constituído em um sério problema podendo acarretar perdas de 0,25% de açúcar, 0,20% de álcool e 0,77% de peso a cada 1% de infestação para a cultura da cana-de-açúcar e de até 21% na produção de milho. No Brasil, existem duas espécies principais desta praga: *Diatraea saccharalis* e *Diatraea flavipennella*. Atualmente existem poucas informações sobre a presença de outras espécies causando injúria nas culturas da cana-de-açúcar e do milho. Além disso, pouco se sabe sobre a diversidade e estrutura genética e os padrões de dispersão desta espécies. Desta forma, os objetivos do presente trabalho foram identificar espécies de *Diatraea* que acarretam danos e prejuízos as culturas, além de avaliar a estrutura genética de populações de *D. saccharalis* para realizar inferências sobre a dispersão da espécie no continente americano. A identificação das espécies foi realizada através de caracterização morfológica dos órgãos reprodutivos e do polimorfismo do gene mitocondrial citocromo c oxidase subunidade I (COI). Os padrões de estrutura e diversidade genética foram avaliados com marcadores microssatélites (SSR) e polimorfismos de nucleotídeos únicos (SNP). Basedo na análise de 95 indivíduos coletados nas principais regiões produtoras do território brasileiro é possível afirmar que *Diatraea saccharalis* e *Diatraea impersonatella* são as únicas espécies responsáveis pelos ataques observados nestas culturas. Os resultados obtidos utilizando marcadores microsatelites demosntraram baixa conexão genética entre as populações, revelando uma estruturação geográfica e preferência em relação ao hospedeiro em populações de *Diatraea saccharalis*. Estes resultados mostram claramente um processo de fragmentação entre as regiões produtoras e consequente interrupção do fluxo gênico e aumento da endogamia intrapopulacional. Utilizando marcadores SNPs obtidos por genotipagem por sequenciamento (GBS) foi possível sugerir com os resultados obtidos duas hipóteses sobre a dispersão de *Diatraea saccharalis* no continente Americano, ambas influenciadas pelas migrações humanas em dois momentos distintos da história. Os resultados obtidos com o presente projeto de pesquisa permitem refinar o entendimento sobre aspectos evolutivos de *Diatraea saccharalis* a fim de se desenvolver práticas de manejo populacional mais eficaz e sustentável. Os resultados fornecem também informações importantes sobre a relação de espécies de *Diatraea* que ocorrem nas culturas agrícolas.

Palavras Chave: Cana-de-açúcar, Broca-da-cana-de-açúcar e Marcadores Moleculares

ABSTRACT

The Sugarcane borer or Corn Stalk borer, *Diatraea* spp. is a polyphagous insect pest of many important crops such as corn, sorghum and sugarcane. Losses arising from the attack of of *Diatraea* spp. have constituted a serious problem which may cause loss of 0.25% for sugar, 0.20% for alohol, and 0.77% of body weight every 1% of infestation in sugar cane plantations and up to 21 % in corn production. In Brazil, there are two major species of this pest: *Diatraea saccharalis* and *Diatraea flavipennella*. Currently little information exists about the presence of other species causing injury to sugarcane and corn plantations. Moreover, little is known about the diversity and genetic structuring and dispersion patterns of *Diatraea* species. In this way, the objectives of the present work were to identify the species of *Diatraea* which cause damage in sugarcane and corn plantations in Brazil, as well as evaluating the genetic diversity and structuring of *D. saccharalis* populations to make inferences about the species' dispersion in the Americas. The species identification ws performed through morphological characterization of the reproductive organs and the polymorphism of the mitochondrial gene cytochrome oxidase subunit I (COI). The patterns of genetic diversity and structuring were evaluated with microsatellite markers (SSR) and single nucleotide polymorphisms (SNP). Based on the analysis of more than 100 individuals collected in the main sugarcane and corn producing regions of Brazil, *Diatraea saccharalis* and *Diatraea impersonatella* are the only species responsible for the observed attacks on these crops. The results obtained using microsatellite markers demonstrated a low connection among populations, revealing a geographic structuring and preference in relation to the hosts in populations of *Diatraea saccharalis*. These results clearly show a process of fragmentation among producing regions and consequent disruption of gene flow, which increases intrapopulation endogamy. Using SNP markers obtained with genotyping-by-sequencing (GBS) it was possible to suggest two hypothesis about the dispersion of *Diatraea saccharalis* in the American continent, both influenced by human migrations in two different historical moments. The results obtained with the present research project allow understanding the evolutionary aspects of *Diatraea saccharalis* in order to develop more effective and sustainable population management practices. The results also provide important information on the relationship of species of *Diatraea* occurring in the agricultural crops involved in this research.

Key words: Sugarcane, Sugarcane Borer and Molecular Markers.

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1. INTRODUÇÃO GERAL

Ao longo dos anos a população mundial irá crescer gradativamente, dos atuais 6,9 bilhões de habitantes para um total estimado de 9,2 bilhões em 2050, com crescimento concentrado quase que inteiramente em regiões menos desenvolvidas (UNITED NATIONS, 2009).

Se a tendência de urbanização e o crescimento de renda continuar em países em desenvolvimento, acarretará em um maior consumo e conseqüentemente aumento da procura de cereais para alimentação humana e principalmente para criação de gado. Essas mudanças na demanda irão conduzir a necessidade de acréscimos significativos na produção de alimentos e forragem. Com isso, o uso de *commodities* agrícolas, como o milho e a cana-de-açúcar, também continuará a crescer (ROSEGRANT, 2008).

Neste cenário, o Brasil tem se destacado pelo seu alto potencial agrícola, como um dos maiores países na produção de culturas como soja, arroz, feijão, cana-de-açúcar e milho.

Para a cultura do milho o Brasil é o terceiro maior produtor mundial, com uma área de cultivo estimada em 17.346,5 milhões de hectares. Onde foram produzidos 93.835,6 milhões de toneladas na safra 2016/2017 (CONAB, 2017). A média de produtividade nacional foi em torno de 5.409 kg/ha, apresentando um crescimento de 8% em relação à safra anterior (CONAB, 2017). O principal destino da safra são as indústrias de rações para animais e uma pequena porção da produção atende ao consumo na mesa dos brasileiros.

Enquanto que para a cultura da cana-de-açúcar o Brasil se destaca por ser o maior produtor, e por estar entre os primeiros do mundo na produção de açúcar e etanol. A área cultivada para a safra corrente está estimada em 8,84 milhoes de hectares, distribuídos em todos estados produtores. A previsão do total de cana esmagada na safra 2017/18 é de 647,6 milhoes de toneladas, sendo que para a produção prevista de açúcar é de 38,70 milhões de toneladas, e para a produção de etanol será de 26,45 bilhões de litros, redução de 4,9% em razão da preferência pela produção de açúcar (CONAB, 2017).

Apesar de todo o aumento de produtividade observado nos últimos anos a agricultura brasileira apresenta problemas e desafios, que vão desde a reforma agrária, a logística para escoamento da produção, mudanças climáticas e entre outros os problemas fitossanitários. Dentre todos os desafios para o crescimento da produção de alimentos, os problemas fitossanitários apresentam-se como um dos mais expressivos em todo o mundo, podendo causar perdas significativas em toda a cadeia produtiva, desde a produção no campo, armazenamento e

comercialização. Segundo Oerke (2006) anualmente estima-se perdas mundiais de produção agrícola em decorrência do ataque de pragas entre 7,9% e 15,1%, mesmo que o uso de medidas de controle seja adotado corretamente. No Brasil as pragas podem ser responsáveis por perdas na ordem de 14,7 bilhões de reais nas principais culturas (OLIVEIRA, 2014). Segundo dados do Departamento de Agricultura dos EUA são coletadas e classificadas anualmente cerca de cinco mil novas espécies de insetos em todo o mundo (GALLO, 2002; CAMARGO, 2007). Estima-se que atualmente são conhecidas 100 mil espécies de insetos consideradas pragas, ou seja, que prejudicam plantas, animais e o próprio homem (GALLO, 2002).

Como importante praga para a cultura do milho e cana-de-açúcar, destacam-se espécies do gênero *Diatraea* (DYAR & HEINRICH, 1927). Estes insetos são nativos do hemisfério ocidental, e ocorrem desde o sul dos Estados Unidos, às Antilhas, e da America Central até a Argentina (CAPINERA, 2001; LONG AND HENSLEY, 1972). Estas espécies são popularmente conhecidas como broca comum da cana-de-açúcar, broca pequena da cana-de-açúcar, broca da cana-de-açúcar, broca do colmo do milho, ou broca do colmo, devido ao seu hábito alimentar que se caracteriza pela penetração do seu estágio larval no colmo para alimentação e finalização do seu ciclo biológico. Ataques causados por espécies de *Diatraea* têm se constituído em um sério problema para várias gramíneas, entre estas milho e cana-de-açúcar (DYAR & HEINRICH, 1927). Em altas infestações, o ataque destes insetos pode causar perdas de até 21% na produção de milho (GITAHY, 2006) e em regiões canavieiras tem se observado elevados prejuízos econômicos. Diversos autores mostram que para cada 1% de intensidade de infestação da praga, ocorrem prejuízos de 0,25% de açúcar, 0,20% de álcool e 0,77% de peso de planta (GALLO et al., 2002; CAMPOS & MACEDO, 2004).

A fim de minimizar esses prejuízos, o manejo integrado da broca vem sendo realizado em regiões produtoras utilizando-se o controle químico e principalmente o controle biológico. Entretanto, o uso de agroquímicos possui eficiência reduzida devido a necessidade de ser utilizado antes da broca penetrar no colmo. Com isso, o uso do controle biológico apresenta grande importância no combate destas pragas. A utilização dos parasitóides *Cotesia flavipes* e *Trichogramma* spp. em associação reduzem em até 60% a intensidade de infestação por *Diatraea saccharalis* (GALLO et al., 2002). Além disso, a eliminação de restos culturais de plantas hospedeiras ajuda a reduzir a infestação na próxima safra. Os métodos de controles utilizados para

insetos-praga por muitas vezes são específicos e diferem em eficiência entre gêneros, espécies e indivíduos.

Referências quanto às espécies de *Diatraea* ocorrendo no Brasil são muito conflitantes. Há relatos da ocorrência de diversas espécies, dentre estas *D. myersi, D. incertella, D. brunnescens, D. ragonoti, D. amnemonella, D. amazônica, D. angustella, D. impersonatella, D. albicrinella*, *D. flavipennella* e *D. indiginella* em território brasileiro (CRUZ, 1976; FREITAS et al., 2006; LESLIE, 2007; CORTÉS, 2010). Entretanto, Gallo (1963) realizou coletas de larvas em diferentes regiões do estado de São Paulo e manteve-as em laboratório até a fase adulta. Durante o processo de identificação do material este autor encontrou somente exemplares de *Diatraea saccharalis*.

Cada espécie forma um grupo de indivíduos único na natureza que cruzam entre si e produzem descendentes férteis, sendo isolados reprodutivamente de outras espécies, e possuem comportamentos específicos, como agressividade, preferência por oviposição e resistência diferencial aos métodos de controles adotados entre outros (MAYR, 1963). Desta forma a constatação de espécie deve ser realizada cuidadosamente, através de estudos dos caracteres morfológicos internos e genéticos. Entre os parâmetros morfológicos a genitália, principalmente do macho, é utilizada para determinação de espécies e classificação de insetos (SCHILTHUIZEN, 2003; PEAIRS & SAUNDERS, 1980). Outras características como coloração da caixa encefálica, pigmentação das asas da mariposa e do corpo da larva podem ser usadas para agregar mais informações (BLESZYNSKI, 1969).

Para os caracteres genéticos, alterações na sequência de nucleotídeos, ou seja, a presença ou ausência de sequências especificas pode variar entre indivíduos, gerando assim polimorfismos. Diversas técnicas, como isoenzimas, RFLP, RAPD, AFLP, SNP e SSR têm sido utilizadas para identificação de espécies e diversidade genética dentro da ordem dos lepidópteros (MARTINELLI et al., 2007; PAVINATO et al., 2011). Estudos de genética de populações utilizando insetos-praga como modelo também permitem responder questionamentos de biologia evolutiva e aprimorar o entendimento sobre padrões que afetam a variabilidade genética natural. Tais estudos aumentam a compreensão das diferenciações genéticas associadas a distintas escalas geográficas, à planta hospedeira e à ecologia da hibridação interespecífica (VIA, 1990).

Além das técnicas citadas acima, o estudo do DNA mitocondrial, em particular a sequência do gene citocromo oxidase I (COI), tem sido extensivamente utilizado em estudos para caracterizar espécies e espécimes de animais, pois apresenta sequências nucleotídicas altamente conservadas

(PALUMBI, 1996; HEBERT et al., 2003) desta forma sendo possível construir relações filogenéticas entre as espécies. O sistema de identificação COI foi proposto como um processo para o diagnóstico universal de espécies, por fornecer uma solução confiável, econômica e acessível (HEBERT et al., 2003). Esse marcador foi denominado DNA barcode, pois sequências desse gene funcionam como código de barras através da possibilidades de combinações de nucleotídeos que estabelece códigos únicos permitindo que cada táxon seja identificado por apresentar uma sequência única de DNA barcode (HEBERT et al., 2003).

Outra possibilidade para o estudo de variabilidade e estrutura genética é o uso de sequências de repetição simples, ou microssatélites. Estas sequências são regiões de DNA repetitivo não codificantes compostas de um a seis nucleotídeos repetidos em "tandem" (FERREIRA & GRATTAPAGLIA, 1998; TOTH et al., 2000). São marcadores com alto poder discriminatório, pois possuem alta taxa de mutação, aumentando a variação das sequências microssatélites entre indivíduos diferentes. Atualmente, tem-se intensificado o interesse no uso dos marcadores SNPs (*Single Nucleotide Polymorphisms*) para estudos populacionais devido à quantidade de marcas geradas, ampla cobertura genômica e por permitir responder questões relacionadas aos processos de adaptação local (OUBORG et al., 2010). Os SNPs podem ser obtidos através de técnicas de sequenciamento de nova geração, como a tecnologia de genotipagem por sequenciamento (GBS), que possui custo bastante reduzido em comparação a genotipagem utilizando chips de DNA (ELSHIRE et al., 2011).

O estudo genético de *Diatraea saccharalis* permitirá refinar o entendimento sobre aspectos evolutivos destes insetos-praga. Avaliando características morfológicas e variabilidade genética será possível inferir sobre a composição do gênero *Diatraea*, ou seja, identificar a estrutura e diversidade populacional no Brasil, determinando os possíveis padrões de dispersão desta espécie.

1.1 Considerações gerais sobre a taxonomia de *Diatraea* spp.

A ordem Lepidoptera contém cerca de 150.000 espécies conhecidas e são facilmente separados de outros grupos de insetos por possuírem asas membranosas cobertas por escamas que se destacam facilmente. Esta ordem reúne todas as borboletas e as mariposas, sendo dividida em quatro subordens. Desta, a subordem Glossata é a mais importante, pois reúne 98% das espécies de lepidópteros conhecidas, sendo composta por 13 superfamílias que são consideradas pragas importantes em muitas culturas. Dentre estas superfamílias estão inseridas Castnioidea,

representada por *Telchin licus licus* (*Castnia licus licus*), Noctuoidea, que inclui *Spodoptera frugiperda*, *Helicoverpa zea* entre outras espécies consideradas pragas agrícolas e a superfamília Pyraloidae (GALLO, et. al. 2002).

A superfamília Pyraloidae é dividida em duas famílias, a família Crambidae e a Pyralidae, sendo que a característica utilizada para esta divisão é a diferença na estrutura auditiva chamada *praecinctorium*, que faz a junção entre as duas membranas timpânicas presente na família Crambidae e ausente em Pyralidae (KRISTENSEN, 1999).

A família Crambidae é composta por duas subfamílias, Pyraustinae e Crambinae. Sendo que a espécies do gênero *Chilo* e *Diatraea* fazem parte desta última subfamília. Esta subfamília pode ser dividida em razão do seu hábito larval em brocas e desfolhadores (BLESZYNSKI, 1969).

Membros do gênero *Diatraea* são muitos próximos de espécimes do gênero *Chilo*, formando um compacto e monofilético grupo sendo difícil separá-los, pois muitas espécies de *Chilo* possuem o ocelli pouco desenvolvidos ou ausentes com exemplares do gênero *Diatraea* (BLESZYNSKI, 1969). No velho mundo predomina os gêneros *Chilo* e *Sesamia*, já no novo mundo se destacam as espécies do gênero *Diatraea.*

1.2 Considerações gerais sobre espécies de *Diatraea* no Brasil

O primeiro relato de espécimes de *Diatraea* no Brasil foi realizado por Dyar em 1911, que observou sete espécies. O mesmo autor estudando o gênero identificou 32 espécies de *Diatraea*, sendo que 12 ocorriam no país (DYAR AND HEINRICH, 1927). Novas espécies foram inseridas ao gênero *Diatraea* por Box (1931), o que elevou de 32 para 48 espécies no continente americano, e conseqüentemente de 12 para 16 as espécies presentes no Brasil. Posteriormente em 1935, mais três novas espécies foram incluídas no gênero (BOX, 1935), sendo catalogada para o Brasil a espécie *Diatraea myersi*, citada na região do baixo Amazonas. Almeida e Souza (1936) indicaram a ocorrência de 10 espécies atacando cana-de-açúcar. Em 1948, Box apresentaram a classificação sistemática e as características do gênero com uma lista das espécies e suas distribuições geográficas. Desta forma, este autor se opôs a indicação de Dyar e Heinrich (1927) e do próprio Box em 1931, retirando da lista *Diatraea maronialis* como espécie presente na fauna brasileira.

No mesmo ano, Box (1948) estudando insetos coletados do Museu Nacional de Historia Natural de Paris descreveu uma nova espécie, *Diatraea ragonoti*, como proveniente de Petrópolis, Rio de Janeiro. Com a criação do gênero *Eodiatraea* Box (1953) transferiu quatro espécies que

estavam alocadas em *Diatraea*, duas desta, *D. ammemonella* e *D. amazônica*, relatadas no Brasil. Em 1955 foi criado o gênero *Crambidiatraea*, o qual recebeu quatro espécies de *Diatraea*, sendo *D. cayenella, D. strigipenella, D. castrensis e D. entreriana* (BOX & CAPPS, 1955). No ano de 1956 foram realizados novos registros sobre a distribuição de espécies de *Diatraea*. *D. flavipennella*, anteriormente citada apenas no estado do Paraná e de São Paulo, passou a ser referida também no estado do Rio de Janeiro. *D. angustella* foi relatada no Rio Grande do Sul. Enquanto que neste ano ocorreram os primeiros relatos de *D. impersonatella* atacando cana-de-açúcar no país (BOX, 1956).

Poucos anos depois foi publicada uma lista de espécies de *Diatraea* com ocorrência comprovada em cana-de-açúcar, sendo assinaladas para o Brasil apenas *D. saccharalis*, *D. impersonatella*, *D. albicrinella* e *D. flavipennella* (BOX, 1960).

Bleszynski e Collins (1962) publicaram um catálogo dos crambídeos do mundo, assinalando a ocorrência de 46 espécies de *Diatraea*, sendo sete presentes no Brasil. O mesmo autor publicou em 1966 uma revisão sobre o gênero, nesta considerando errônea a alocação de espécies de *Diatraea* realizada por Box (1960) e Box & Capps (1955) em outros gêneros. Desta forma, o autor concluiu pela sinonímia destes gêneros com *Diatraea*.

Dados publicados na ultima revisão do gênero descrevem como catalogadas cerca de 47 espécies de *Diatraea* ocorrendo em todo continente americano. Dez espécies de *Diatraea* (*Diatraea saccharalis, Diatraea considerata, Diatraea centrella, Diatraea dyari, Diatraea guatemalella, Diatraea indigenella, Diatraea magnifactella, Diatraea rosa, Diatraea tabernella* e *Diatraea veracruzana*) possuem importância econômica em cana-de-açúcar e milho (Bleszynski, 1969). Estas estão presentes por toda America do Sul e Central.

Bleszynski (1969) considera outras 13 espécies (*Diatraea albicrinella, Diatraea amnemonella, Diatraea amazônica, Diatraea busckella, Diatraea crambidoides, Diatraea flavipennella, Diatraea grandiosella, Diatraea impersonatella, Diatraea lineolata, Diatraea minimifacta, Diatraea muellerella, Diatraea pittieri* e *Diatraea rufescens*) como de pouco importância econômica ou encontrada ocasionalmente em cana-de-açúcar. No mesmo manuscrito, Bleszynski (1969) discorreu sobre outras 20 espécies de *Diatraea* identificadas por Dyar & Heinrich (1927) como sem importância ou sem referências recentes em cana-de-açúcar, são estas: *Diatraea angustella, Diatraea bellifactella, Diatraea canella, Diatraea continens, Diatraea cayennella, Diatraea evanescens, Diatraea fuscella, Diatraea gaga, Diatraea incomparella,*

Diatraea instructella, Diatraea maronialis, Diatraea moorella, Diatraea pallidostricta, Diatraea pedibarbata, Diatraea postlineella, Diatraea strigipennella, Diatraea schausella, Diatraea umbrialis, Diatraea venosalis e Diatraea zeacolella.

Cruz (1976) realizando coleta través de armadilhas luminosas instaladas em canaviais paulista identificou, pelo estudo da genitália masculina, dez espécies de *Diatraea*, entre elas: *Diatraea angustella*, *Diatraea bellifactella*, *Diatraea brunnescens*, *Diatraea castrensis, Diatraea cayenella, Diatraea flavipennella, Diatraea impersonatella, Diatraea pallidostricta, Diatraea saccharalis e Diatraea strigipennella*. Sendo que *Diatraea saccharalis* foi a única espécie encontrada em colmos de cana-de-açúcar.

Levantamentos recentes atestaram *Diatraea centrella, Diatraea tabernella, Diatraea flavipennella, Diatraea grandiosella* e *Diatraea indiginella* como importantes pragas de cana-de-açúcar e milho em Trinidade e Guiana, Costa Rica, Estado Unidos e Brasil (MAHADEO et al., 1976; VALVERDE et al., 1991; DASRAT et al., 1997; LESLIE, 2007; FREITAS et al., 2006).

1.3 Caracteres morfológicos para identificação das espécies de *Diatraea*

Membros da classe Insecta podem ser separados em ordens e posteriormente até espécies através de caracteres de identificação. A categoria básica sobre a qual se baseia toda classificação animal é a espécie. Sendo assim, espécie é uma população de animais de uma área, onde ocorre cruzamento e há produção de prole fértil (ODUM, 1988).

Nas classificações de espécies do gênero *Diatraea*, estes insetos são distribuídos conforme seus caracteres específicos, que podem estar relacionadas com as peças bucais, a forma e venação das asas, a estrutura da larva e da posição de cerdas primárias, cápsula cefálica e comprimento do ovipositor (Enciclopédia Britânica, 2011). Tais características são consideradas de importância secundárias, pois podem variar conforme o tamanho do corpo e influenciadas por fatores ambientais locais.

Atualmente para classificação e identificação na ordem lepidóptera são usadas comparações da anatomia dos órgãos reprodutivos, especificamente as genitálias de macho e fêmea, pois possuem características conservadas dentro de cada espécie (SCUDDER, 1971; PEAIRS e SAUNDERS, 1980; SCHILTHUIZEN, 2003). Os órgãos genitais são complexos e fornecem a base para a discriminação das espécies na maioria das famílias e também na identificação de família (POWELL, 2009).

O arranjo da genitália é importante no namoro e acasalamento e impedem cruzamentos interespecíficos e hibridação. Ou seja, a genitália do macho e fêmea de qualquer espécie é adaptada uma para o outro como chave e fechadura (HOSKINS, 2010).

1.4 Morfologia: Genitália do Macho

A estrutura da genitália masculina de lepidópteros está envolvida pelos segmentos abdominais IX-X (CRUZ, 1980). O 10° segmento abdominal consiste de uma porção dorsal denominada *uncus* e uma ventral, o *gnathos.* A forma do *uncus* é variável entre as espécies sendo caracterizado como espinho, gancho, liso, piloso, simples, bifurcado ou trífido, podendo estar reduzido e até mesmo ausente. O *gnathos* é formado por dois lobos fixos a margem posterior do *tegumen* e a base do *uncus*, estendendo-se ventralmente. Algumas vezes presos a base do *uncus* encontram-se processos pareados denominados *socii*, que são estruturas macias ou fracamente esclerotizadas, usualmente pilosas, podendo ser pequenas ou até mesmo pecioladas (OGATA et al., 1957). Enquanto que o 9° segmento abdominal é representado pelo *tegumen* dorsalmente e pelo *vinculum* ventralmente, formando um anel transverso, que serve como base para a união da genitália feminina (KLOTS, 1970). Articulados ao anel transverso estão presentes duas estruturas, com forma de asas, denominadas *valvae*, cuja função é auxiliar a cópula. Encerrando a porção posterior do abdômen esta presente o *aedeagus*, órgão reprodutivo do macho, através do qual secreta o esperma dos testículos durante a cópula (Figura 1.1).

A genitália masculina de espécies do gênero *Diatraea* possui caracteristicamente o *uncus* e o *gnathos* bem desenvolvidos. O *gnathos* é raramente bifucardo, enquanto que o *uncus* em algumas espécies é ampliado. O *tegumen* muitas vezes conta com uma extremidade forte. A *valvae* na sua maioria é aberta com a extremidade da base forte, sendo por vezes um processo sub-basal. Este gênero possui macho com *saccus* presente e *vinculum* estreito. O *aedoagus* possui uma armadura simples e as vezes com um esclerito.

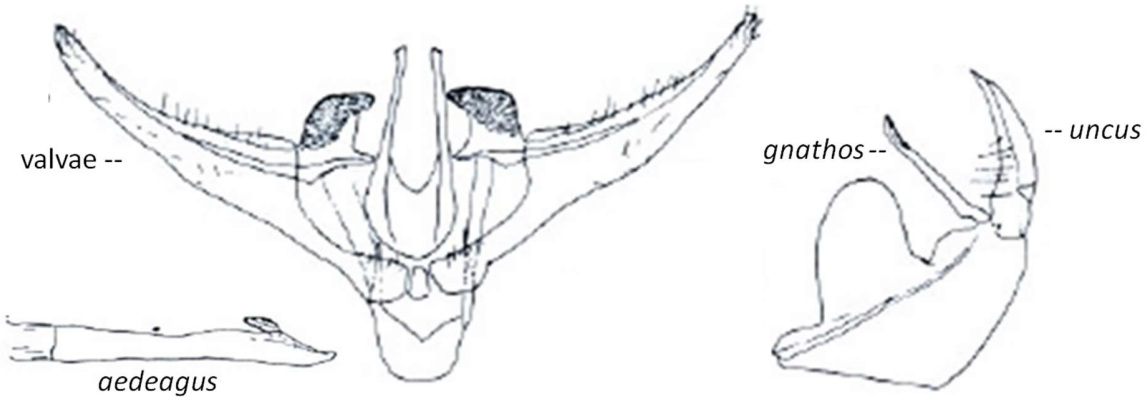

Figura1.1. Estrutura reprodutiva de um espécime macho característica da ordem lepidóptera

1.5 Caracteres genéticos para identificação das espécies de *Diatraea*

Atualmente, além das características morfológicas, outros caracteres são usados para classificar os organismos ao longo das linhas evolutivas e agregar valor a identificação, tais como a bioquímica, os fósseis e principalmente a biologia molecular. Com o advento de métodos utilizando a análise de DNA, o estudo da genitália e de outros caracteres externos se tornou apenas umas das técnicas utilizadas na taxonomia (HOSKINS, 2010). Desta forma a análise de dados moleculares apresenta-se como uma alternativa para ampliar o conhecimento sobre um determinado grupo de organismos (BROWN *et al*., 1999). Uma série de técnicas moleculares ou marcadores moleculares vêm sendo vastamente empregados na delimitação de espécies. As principais vantagens do uso dos marcadores moleculares baseados em DNA é que existe neutralidade em relação a efeitos fenotípicos (efeito epistático ou pleiotrópico mínimo ou nulo) e pode-se analisar um grande número de locos com alto nível de polimorfismos, desta forma com uma maior quantidade de informação genética por loco (FERREIRA & GRATTAPAGLIA, 1998).

O marcador genético mais utilizado para discriminação a nível de espécie inclui a variação na sequência do DNA mitocondrial, por este ser haplóide e fácil de amplificar em vários táxons. O sequenciamento pode ser facilmente obtido sem a clonagem e existe uma grande quantidade de cópias de DNA mitoncrial na célula (AVISE *et al*., 1987). Também apresenta uma alta taxa evolutiva permitindo o reconhecimento dos padrões de mudanças e o tempo dos eventos históricos recentes, ou seja, determinando relações filogenéticas, filogeografia, evolução molecular, dinâmica e estrutura de populações e vários aspectos biológicos. As seqüências são usadas para construir inter-relações entre as espécies, permitindo uma abordagem multidisciplinar que inclui a

taxonomia morfológica, molecular e a distribuição de dados é essencial para a compreensão da biodiversidade (BRAVO, 2008). No DNA mitocondrial a sequência parcial do gene da citocromo c oxidase subunidade I (COI), tem sido objeto de numerosos estudos funcionais para caracterizar espécies e espécimes de animais. O segmento COI que é comumente utilizado tem aproximadamente 658 pares de bases (pb), a partir da base 58 da extremidade 5' (Herbert et al., 2003). Este segmento de DNA Mitocondrial, definido como DNA Barcoding ou "código de barras do DNA" vem sendo utilizado desde 2004 como parte central de um sistema global de identificação pelo "Consortium for the Barcode of Life" (CBOL) (www.barcoding.si.edu).

Para estudos de dinâmica e estrutura de populações a metodologia mais utilizadas são os marcadores moleculares microssatélites (SSR), pois são ferramentas úteis na construção de mapas genéticos, fluxo gênico, análise de paternidade entre outros (CHASE et al., 1996; KALIA et al., 2011). Os marcadores microssatélites são altamente polimórficos e abundantes nos genomas de eucariotos. Suas características inerentes, tais como alto polimorfismo, genotipagem fácil e confiável e herança codominante, associado a métodos estatísticos poderosos como análises Bayesiana e métodos de máxima verossimilhança (LUIKART, 1999) tornaram-os amplamente utilizados pelos geneticistas de populações de insetos e ecologistas (BEADELL et al., 2010; PAVINATO et al. 2011). Dentre as aplicações de marcadores microssatélites em estudos ecológicos e evolutivos de insetos podemos destacar: estudos genéticos para obter informações sobre ecologia básica (ENDERSBY et al., 2007); sobre padrões de mudanças na composição genética de populações associada a planta hospedeira (CARLETTO et al., 2009); e pela existência de descontinuidades espaciais (ABILA et al., 2008) e temporais (FRANCK & TIMM, 2010) e processos de especiação simpátrica (SANTOS et al., 2010). Marcadores moleculares microssatélites já foram descritos para as principais insetos-praga agrícolas como *Ostrinia nubilalis* (BOURGUET et al., 2000; COATES et al., 2008), *Helicoverpa armigera* (SCOTT et al., 2006), e *Spodoptera frugiperda* (MARTINELLI et al., 2007).

Atualmente os custos de sequenciamento de DNA caíram vertiginosamente quando comparados à década passada, o que têm possibilitado um acelerado desenvolvimento e avanço de tecnologias de genotipagem em larga escala. Novas metodologias, baseadas em sequenciamento de nova geração (NGS), combinam a descoberta de polimorfismo e genotipagem em um único passo (BAIRD et al., 2008; HUANG et al., 2009; ELSHIRE et al., 2011). Uma nova metodologia denominada genotipagem por sequenciamento (*Genotyping by Sequencing* – GBS) possibilita

amostrar o polimorfismo de SNPs em regiões amplamente distribuídas ao longo do genoma utilizando, por exemplo, enzimas sensíveis a metilação, evitando dessa forma o sequenciamento massal de regiões repetitivas e complexas, que dificultam o processo de análise. Os macadores SNPs tem como base genética mutações em base única na sequência de DNA, ou inserções / deleções.

O estudo genético de espécies consideradas pragas agrícolas permite um melhor entendimento sobre a dinâmica populacional dessas espécies. Desta forma, podem ser identificandas relações evolutivas em resposta ao ambiente, que por sua vez podem ser utilizadas na proposição de mecanismos de controle onde a estrutura e diversidade genética, devem ser levadas em conta para a tomada de decisão.

2. OBJETIVO GERAL

O presente trabalho teve como objetivos identificar populações do gênero Diatraea no Brasil oriundas das culturas do milho e da cana-de-açúcar e estimar a diversidade e estrutura genética em populações de *Diatraea saccharalis*. Desta forma descrever o sistema dispersão da pragas no continente Americano para elucidar a história evolutiva desta espécie.

2.1 OBJETIVOS ESPECÍFICOS

1) Descrever as características morfológicas das estruturas reprodutivas de mariposas de *Diatraea saccaharalis*;

2) Gerar padrões para análise filogenética e de filogeografia através da divergência das seqüências do gene mitocondrial *Citocromo Oxidase* I (COI);

3) Determinar as sequências barcodes entre diferentes espécies utilizando o gene do *Citocromo Oxidase* subunidade I;

4) Análisar a variabilidade genética e estrutura populacional utilizando marcadores de marcadores microssatélites (SSR) e comparação da estrutura genética em escala local, regional e/ou associada à planta hospedeira além de investigar padrões de fluxo gênico em Diatraea;

5) Avaliar os padrões de dispersão no continente Americano através da diversidade e estrutura genética SNPs;

3. Capítulo 1 Morphological and Molecular Characterization of Brazilian Populations of *Diatraea saccharalis* (Lepidoptera: Crambidae) and the Evolutionary Relationship among Diatraea spp.

SUMÁRIO

Abstract

The sugarcane borer or corn stalk borer, *Diatraea spp.* (Lepidoptera: Crambidae) are a polyphagous insect pest of many important crops such as corn, sorghum and sugarcane. Losses arising from the attack of *Diatraea* species have been a serious problem, which may cause loss in sugarcane production around 0.25% in sugar, 0.20% in alcohol and 0.77% of body weight for every 1% infestation and up to 21% in corn production fields. In Brazil, the most commonly reported species are *Diatraea saccharalis* and *Diatraea impersonatella* (= *D. flavipennella)*. However, multiple other species of *Diatraea* have been identified in Brazil according to the literature. Currently, little information exists on the presence of the other species causing injury to sugarcane and corn. The objectives of this study were to improve the accuracy of species assignment, evaluate the population genetic structure, and address many of the outstanding questions of systematics and evolution of Brazilian *Diatraea*. To solve these main questions, classical taxonomic methods were used, focused on morphological characterization of the reproductive organs, especially the male genitalia. In addition, genetic studies were performed using simple sequence repeats (SSR) and the mitochondrial gene cytochrome oxidase I (COI). The data and findings from this research will refine the understanding of evolutionary aspects of insect pests in order to develop more effective and sustainable population management practices.

Key words: Sugarcane, Stemborer, COI, Microsatellites and DNA barcoding.

3.1 Introduction

Diatraea Guilding is a genus that is composed of a significant number of species, which contains some of the most important lepidopteran pests of crops, including corn, sorghum, and sugarcane (DYAR, 1927; LONG, 1972; ROE, 1981; RODRIGUEZ-DEL-BOSQUE, 1988). The species of this genus are restricted to the New World, being widely distributed throughout South America, Central America, the Caribbean, and the southern United States (BOX, 1931; BLESZYNSKI, 1969; LANGE, 2004, CORTES, 2010). Currently, in Brazil, all *Diatraea* species are popularly known as sugarcane or corn stalk borers. Because of the lack of distinction between species, borers discovered in agricultural fields are known as *Diatraea saccharalis* (FABRICIUS, 1794). Based on this principle, *D. saccharalis* has been reported to be distributed in sugarcane belts across Brazil (DINARDO-MIRANDA, 2008) whereas *Diatraea impersonatella* (WALKER, 1863) another important pest, has been reported only in some states, such as Espírito Santo, Rio de Janeiro, Minas Gerais, and in all the Northeastern states (MENDONÇA, 1996; FREITAS, 2006; NETO, 2014). These two species of *Diatraea* can be morphologically distinguished in the larvae phase. *D. saccharalis* larvae has a reddish brown head or dark. The body often dirty white with the pinacula golden brown being that the D2 on A1-7 tend to be on oval to rectangular. The anal shield usually pale (PASSOA, 2014). Larvae of *D. impersonatella* have a head capsule that is yellow or brownish. The body is cream or yellowish with mesothoracic and metathoracic extra pinacula oval with a median indentation. In addition, the anal crochets in a short arc. The mandible with five teeth (MENDONÇA, 1996; FREITAS, 2006; NETO, 2014).

In spite of these differences, farmers, students, and researchers often fail to separate these species correctly. The confusion is likely due to the variability of these characteristics between individuals according to the age of the larvae and environmental conditions (BOX, 1951; PASHLEY, 1990; RILEY, 2005; PASSOA, 2014) and the lack of comparative morphological studies across larvae and pupae to confirm the species level.

The incorrect identification may cause problems for the control of sugarcane borers, given that all actions are targeted towards *D. saccharalis*. Chemical control of populations is often not effective given the endophagous feeding habits of the insect and the constant availability of host

plants in the field throughout the year (CIRELLI, 2003). Appropriate biocontrol methods, in many cases, have a high degree of host specificity (NETO, 2014; GOMÉZ, 1995).

Historically there has been very little systematic research conducted on which species are infecting Brazilian corn and sugarcane fields. We aim to develop a comprehensive understanding of the relationships and diversity of *Diatraea* species in support of Brazilian agriculture. Other species of *Diatraea* have also been reported in the Brazilian territory. The first report of *Diatraea* in Brazil was by Dyar (1911), who described seven species. The same researcher, in 1927, reported 12 *Diatraea* species in Brazil (DYAR, 1927). Box (1931) revised the genus and increased the number of species in the Americas to 48, with 16 found in Brazil. Almeida and Souza (1936) reported the occurrence of 10 species attacking sugarcane in Brazil, however, Box (1960) narrowed that list down to three: *D. saccharalis*, *Diatraea albicrinella* (BOX), and *D. impersonatella*. Cruz (1976) published the last informative survey on the presence of *Diatraea* species within Brazil, reported 10 species in the country, and proposed that *D. saccharalis* was the sole pest in sugarcane stalks.

Box (1931) and Bleszynski (1969) performed very comprehensive studies on the genus *Diatraea* based on the morphology of the male and female genitalia. Expanding this study, Solis and Metz (2016), provided keys and illustrations of the male and female genitalia (including many primary types) for all known *Diatraea* species. Based on the variability of South American species, they synonymized the name *Diatraea flavipennella* (BOX, 1931) with *D. impersonatella*, and we follow the change herein.

The collection and observation of adult Lepidoptera is often preferred given the relative ease of identification. The internal morphological characterization of the anatomy of the reproductive organs, especially the male genitalia, has provided excellent results because they have important and conserved characteristics within species (SCUDDER, 1971; PEAIRS, 1980; SCHILTHUIZEN, 2003). The genitalia are complex, heavily sclerotized, and provide the basis for discrimination of many species and families (POWELL, 2009). The arrangement of the genitalia is important in courtship and mating feasibility, and can prevent attempted interspecific crosses and hybridization. The Lepidoptera male and female genitalia are adapted one to each other, like a lock and key mechanism (MIKKOLA, 1992; ARNQVIST, 1997).

In addition to morphological characterization, molecular analyses may be used to increase the knowledge of a particular group of organisms (BROWN, 1999). Several molecular techniques

have been widely used to delimit species, understanding the levels of population diversity, conducting phylogenetic analyses, and estimating gene flow among insect populations (SOSA-GÓMEZ, 2004; MARTINELLI, 2006; MARTINELLI, 2007; LEITE, 2014). For the identification of species, the most commonly used genetic marker is the polymorphism of sequence of cytochrome C oxidase subunit I (COI) gene. This relatively conserved gene has been useful for alpha level taxonomy because it is generally haploid, lacks introns, and has limited recombination (AVISE, 1987; BROWER, 1994; MORITZ, 1987; WILSON, 1985; HEBERT, 2003). Another useful molecular tool to study the diversity at the intraspecific level, are the microsatellite markers (SSRs). SSR markers are highly polymorphic and abundant in eukaryotic genomes, and they provide easy and reliable co-dominant genotyping (FERREIRA, 1998; GOLDSTEIN, 1999; TOTH, 2000).

Very little research on the genetic diversity and population structure of *Diatraea* species have been published in recent years. Some examples showed that Brazilian populations of *D. saccharalis* have a high level of polymorphism and genetic structure within the crop production regions (LOPES, 2014; SILVA-BRANDÃO, 2015). Studies using molecular markers have also shown the possible occurrence of more species than the single currently designated *D. saccharalis* (PASHLEY, 1990; JOYCE, 2014). Pashley et al. (1990) compared specimens collected in different countries and hosts, and found a cluster with populations from Louisiana and Mexico, and another cluster with Brazilian populations. Joyce et al. (2014) studied populations of *D. saccharalis* collected in the southern United States and identified two genetically distinct clusters using amplified fragment length polymorphism (AFLP) and COI sequencing. These authors suggested that Florida's *D. saccharalis* population could represent a distinct species. However, other studies suggest samples in South America form a separate cluster from those in Central America and the Southern United Stated of America (LANGE, 2004; JOYCE, 2016)

Discrepancies among studies may be associated with collection sites, individuals that were collected in light trap or in a host, development stage (adult or larvae) used for DNA analyses, and the lack of precision with species level identification. We addressed these disagreements with a sequence of methods that valid each other. This study aimed to fill a gap, building a comprehensive study of *Diatraea* specimens collected in sugarcane and corn plants in the same production regions of Brazil. Our objectives were (a) to improve the accuracy of species identification through morphological characterization and with the polymorphism of sequence of cytochrome C oxidase

subunit I (COI) gene (b) to establish a systematics and evolutionary comparison with *Diatraea* species and (c) to evaluate many of the outstanding questions about population structure with microsatellite (SSRs) fine-scale gene characterizations. In this study, we used these methods to assess the genetic variability of populations collected in the main crop production regions in Brazil. Furthermore, we used DNA barcodes of several species of *Diatraea* to evaluate the evolutionary relationships within this genus. The findings of this study may help to refine the understanding of evolutionary aspects of insect pests in order to develop more effective and sustainable population management practices. In addition, we adopt the use of *D. impersonatella* (WALKER, 1863) instead of *D. flavipennella* (BOX, 1931) as proposed by Solis and Metz (2016).

3.2 Materials and methods

3.2.1 Insect collections

During the Brazilian crop seasons of 2011–2012 and 2012–2013, 95 specimens of *Diatraea* were collected in the main corn and sugarcane production regions of Brazil. The larvae were collected in equidistant points inside the corn or sugarcane fields. In this study, we defined the term "population" as the city where collection was performed, and the host from where the insect was isolated. Thus, each population was defined as Piracicaba_Sugarcane, Piracicaba_Corn, Jaboticabal_Sugarcane, Morrinhos_Sugarcane, Morrinhos_Corn and Maceio_Sugarcane (Table 3.1).

Table 3.1 Populations collected and their geographic locations.

Species	Collection Location _Host	Latitude	Longitude	Number of Individuals	Female	Male
Diatraea impersonatella	Maceio_Sugarcane	-09° 39' 57"	-35° 44' 07"	13	11	2
Diatraea saccharalis	Jaboticabal_Sugarcane	-21° 15' 17"	-48° 19' 20'	24	11	13
	Morrinhos_Corn	-17° 43' 52"	-49° 05' 58"	8	3	5
	Morrinhos_Sugarcane	-17° 43' 52"	-49° 05' 58"	2	1	1
	Piracicaba_Corn	-22° 43' 31"	-47° 38' 57"	24	13	11

	Piracicaba_Sugarcane	-22° 51' 31"	-47° 77' 35"	24	9	15

Corn and sugarcane stalks showing the typical symptoms of attack by *Diatraea* borer were cut with the aid of a saw. The larvae found in each damaged plant were transferred to the laboratory, moved to Petri dishes containing artificial diet, identified, and kept separate. Each larva was assessed for the presence or absence of parasites. Larvae that were infected by parasites or other diseases were discarded. The specimens that passed visual screening were placed individually on Petri dishes with artificial diet and maintained at 27 ± 1 °C; 70% U.R. and photoperiod of 12 hours until they pupated. Then each individual pupa was transferred to cylindrical cages of 40 cm x 30 cm. The pupae were kept at 20 ± 1 °C with a 12-hour photoperiod until adults emerged. Moth emergence occurred within these cylindrical cages to allow the complete metamorphosis and for the moth to properly inflate their wings. All moths were transferred to individual micro centrifuge tubes and stored at – 80 °C.

The collections were carried out on private lands (with the permission of their owners), and no specific permits were required for these locations/activities because it did not involve endangered or protected species.

3.2.2 Morphological characters of the male and female genitalia

All moths that emerged were dissected and identified based on the morphological characteristics of the genitalia according to Robinson (1976). The technique consists of removing the abdomen using curved forceps that are gently pressed on the venter of the caudal end. Abdomens were placed in a 10% potassium hydroxide solution (KOH) and heated to boiling for 2-3 minutes. After this process, the genitalia were transferred to a Petri dish for cleaning in 50% ethanol. Scales were brushed from the abdomen and the genitalia was dissected with the aid of forceps and fine paint brushes under a binocular stereo microscope. Male specimens were dissected by gripping the anterior end of the abdomen while the sclerotized genitalia were gently pulled out of the posterior end. Females were dissected by cutting between abdominal segments VI and VII with the aid of forceps and iris scissors. The extracted genitalia were transferred to small polyethylene tubes (60 mm long by 5 mm diameter) containing glycerin or 85% lactic acid. Each tube contained an individual abdomen with a unique identifier to carefully maintain the

association of the genitalia with the specimen. The genitalia of each species were deposited as vouchers in the collection of the Illinois Natural History Survey at the Prairie Research Institute of the University of Illinois under the catalog codes INHS_814844 and INHS_814845. Photographs of representative dissections were taken with a Canon EOS 5D Mark II body and a MPE 65 mm 1x - 5x magnifying lens. The z-stacking camera setup was built by Visionary Digital and housed within the entomology collections at the Illinois Natural History Survey at the Prairie Research Institute of the University of Illinois. Photographs were first edited in Adobe Lightroom and then combined with the software package Zerene Stacker. The comparative analysis of male and female genitalia was performed following the description of the *Diatraea* morphology provided by Bleszynski (1969) and Solis & Metz (2016).

3.2.3 DNA extraction

The same specimens that were dissected were also used for DNA extractions. The male and female DNA was extracted from the thoracic tissues following the CTAB protocol described by Doyle and Doyle (1990) with slight modifications. The integrity and quantity of DNA were evaluated in 0.8 % agarose gels with 1x TAE buffer (TRIS, acetic acid, EDTA, pH 8.0). The amount of DNA present in each sample was estimated by comparison with known concentrations and graded standard DNA (λ phage). The gels were stained with an ethidium bromide bath (0.5 mg mL-1) and the DNA bands were visualized under UV light.

3.2.4 Analysis of the mitochondrial gene Cytochrome C Oxidase subunit I (COI)

3.2.4.1 Amplification, sequencing and alignment

A fragment of the COI mitochondrial gene was amplified by polymerase chain reaction (PCR) with the primers LCO 1490 (F) (5’ - GGT CAA CAA ATC ATA AAG ATA TTG G – 3’) and HCO 2198 (R) (5’ - TAA ACT TCA GGG TGA CCA AAA AAT CA – 3’) (FOLMER, 1994). The sequencing reactions were performed with the corresponding amplifying primers from both directions using a BigDye Terminator Cycle Sequencing Kit v.2.0 (Applied Biosystems, USA)

and the sequences obtained were processed by the 3730 / 3730xl Data Collection Software v3.0 (Applied Biosystems).

Multiple alignment of the sequences (Data available as Supporting Information: S1 Dataset) was done using the ClustalX Software (THOMPSON, 1997) with manual correction using Chromas 2.0 (http://www.technelysium.com.au/chromas.html).

Estimates of haplotype diversity were obtained with DnaSP5 (LIBRADO, 2009). The distribution of genetic diversity between and within populations and species were estimated by analysis of molecular variance (AMOVA) with Arlequin 3.5 (EXCOFFIER, 2010). Cluster analysis was performed with the neighbor joining (SAITOU, 1987) method using the MEGA4 (TAMURA, 2007).

The COI haplotypes found for *D. saccharalis* and *D. impersonatella* were also aligned with COI sequences from other *Diatraea* and related species available in GenBank (COI accession numbers: JQ888353, JQ888360, JQ888366, KJ657593, KM288999, KM289005, KP259615, KR070995, KR070998 and KR070999).

3.2.5 Analysis with nuclear microsatellite loci

3.2.5.1 Microsatellite genotyping

The microsatellite loci used in the study were provided by the Laboratory of Conservation Genetics and Genomics, Agribusiness Technological Development of São Paulo, Brazil and were developed by Pavinato (2013). Details and characteristics of the microsatellites are shown in S3.1 Table. Microsatellite amplification conditions and gel separation were performed according to Pavinato (2013).

3.2.5.2 Microsatellite data analysis

All individuals from *Diatraea* populations were genotyped with at least 10 highly polymorphic loci (Data available as Supporting Information: S2 Dataset). Genetic diversity and F statistics were estimated under a random model, in which the sampled populations were considered representative of the species and with a common evolutionary history. Allele frequencies, the number of alleles per locus (A), the observed heterozygosity (Ho) and expected (He) and Wright's F statistics (F_{IS}, F_{ST} and F_{IT}), assuming random model, were estimated using the *hierfstat* package (JEROME, 2015). Cluster analysis were based on the construction of dendrograms using Nei's genetic distance (NEI, 1978) and the UPGMA method, in the *poppr* package (KAMVAR, 2014). The stability of the clusters were tested, through 1,000 bootstraps resamples, also using the *poppr* package (KAMVAR, 2014). We applied the non-model based approach DAPC through the package adegenet (JOMBART, 2008)

3.3 Results and Discussion

3.3.1 Morphological description of *Diatraea* genitalia

For all 95 individuals collected and reared from sugarcane or corn we performed morphological analysis of the internal genitalia. Specimens were sorted by sex, totaling 47 males and 48 females (Table 3.1) following keys by Bleszynki (1969) and Solis and Metz (2016). We observed that 82 individuals showed little variation in the reproductive organs, independent of the site/city and host in which they were collected. These individuals were identified as *D. saccharalis* (Table 3.1). In addition, we observed that 13 individuals collected at Maceio, in Alagoas State, differed from the other 82 individuals. Using the above mentioned identification guides, we classified this group as *D. impersonatella*.

The results clearly demonstrate that all specimens of *Diatraea* collected randomly in sugarcane and corn plants were members of either *D. saccharalis* or *D. impersonatella*. While other *Diatraea* species are known to exist in Brazil, none was found in these sampling efforts. Interestingly, *D. impersonatella* was completely absent from fields of corn and sugarcane in São Paulo and Goiás state. However, *D. impersonatella* was the only species reared in the Northeast

region of Brazil. Freitas (2006) also noticed that *D. impersonatella* (89.80%) showed preponderance over *D. saccharalis* (10.20%) in some areas of the state of Alagoas.

3.3.2 Comparative morphology of the male genitalia

Forty-five male specimens were identified as *D. saccharalis* according to keys in Bleszynki (1969), Goyes (2008), and Solis and Metz (2016). The following important morphological characteristics are noted. The *vinculum* was smooth, U-shaped, and broadly rounded anteriorly. The *uncus* narrowed into a beak-like apex, and the *gnathos* were smooth except for approximately half of the dorsal surface that was densely covered with short teeth. The lateral lobe of the *tegumen* was rounded, as long as wide, and square in appearance. The basal costal lobe was present with the vertex slightly flattened. The apex of *juxta* arms were with a single point or rounded with a small, subapical tooth, but never bidentate (Fig 3.1, A and B).

Fig 3.1 *Diatraea saccharalis* male reproductive structures. (A) Posterior view of *D. saccharalis* male reproductive structures. (B) Lateral view of *D. saccharalis* male reproductive structures.

D. impersonatella is similar in appearance to *D. saccharalis* in general structure. The *uncus* and *gnathos* were beak-like, valva narrow, and the basal costal lobe pronounced as in *D. saccharalis*. Likewise, the lateral arms of *juxta* were slender and pointed. However, the basal costal lobe on the *valva* was elongated and narrow, less dentate, and not as darkened as the lobe of *D. saccharalis*. The lateral lobes of the *tegumen* were reduced and triangular in appearance (Fig 3.2, A and B). Males of *D. saccharalis* and *D. impersonatella* can easily be separated from each other, as well as from other members of the genus.

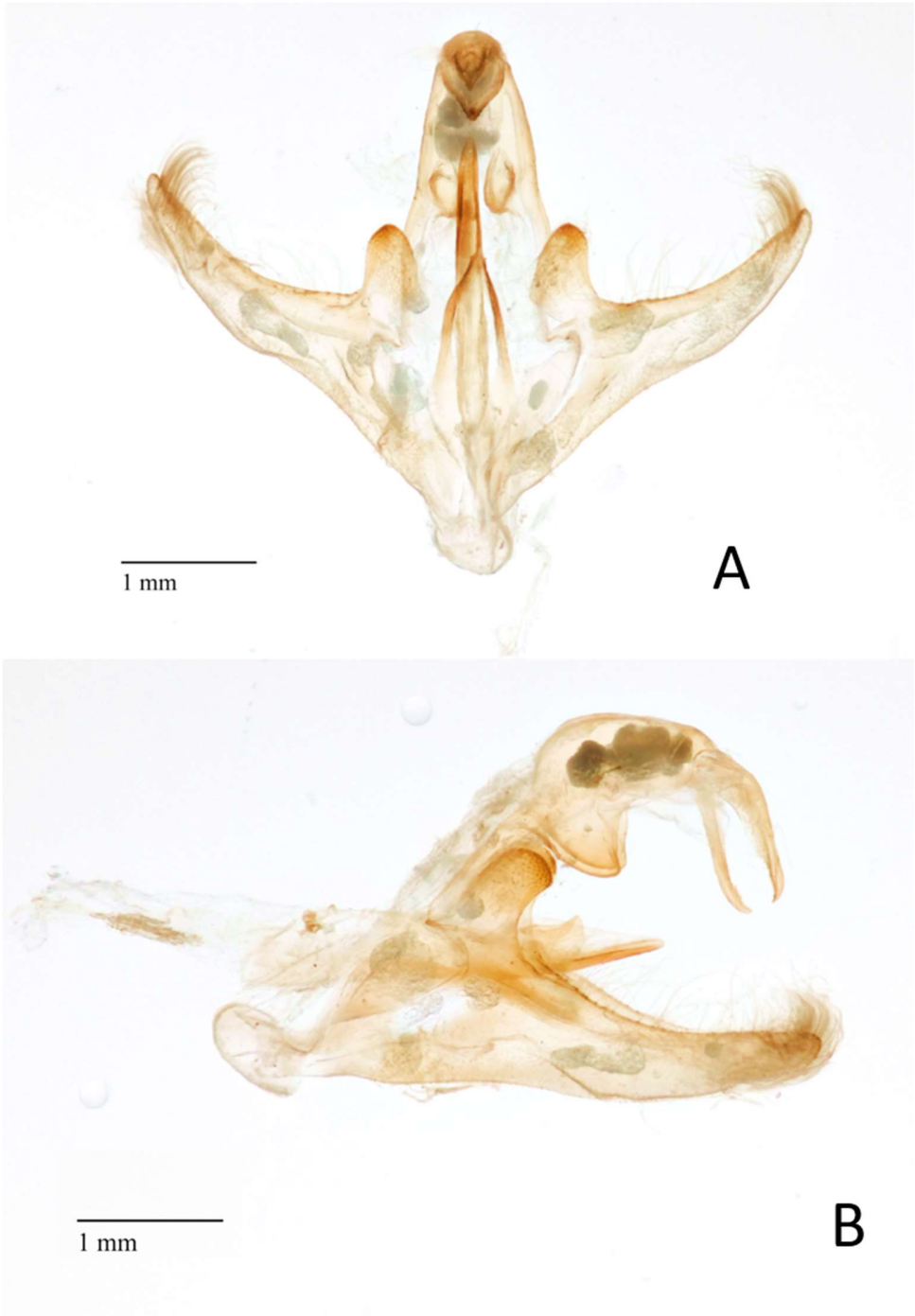

Fig 3.2. *Diatraea impersonatella* male reproductive structures. (A) Posterior view of *D. impersonatella* male reproductive structures. (B) Lateral view of *D. impersonatella* male reproductive structures.

3.3.3 Comparative morphology of the female genitalia

Females of both *D. saccharalis* and *D. impersonatella* have *papillae anales* separated anteroventrally, parallel and flattened, with longer setae of the outer margin (Figs 3.3 and 3.4). Ventral lobes of the *anales* were slightly swollen. The anterior *apophysis* nearly twice as long as the posterior *apophysis*, slightly curved, and marginally tapering anteriorially. The sternite VIII were with broad, transverse, indentations obstructing the *ostium bursae*. The *corpus bursae* were membranous, lacking any crenulations, and the signa was absent. Moths of *D. saccharalis* had an anterodorsal lobe off the *corpus bursae*, although it varied in size and shape. Careful dissection of the female was required to ascertain the true shape of the *bursae*, which can be easily ruptured or crushed. Posterior projections of the lamella *antevaginalis* were irregular and triangular, wrinkled and densley setose. *Ductus seminalis* originated at the posterior end of the *corpus bursae*. In specimens of *D. saccharalis* from this study, the anteroventral swelling of the *papillae anales* were more pronounced than in *D. impersonatella*. Females of these species cannot reliably be separated by genitalia, and this study lacked sufficient numbers of specimens to ascertain regional variation. Solis and Metz (2016) further described variation across the range of the *D. impersonatella* group, and the problems with determining species based on female genitalia.

Fig 3.3. Female reproductive structures of *D. saccharalis*.

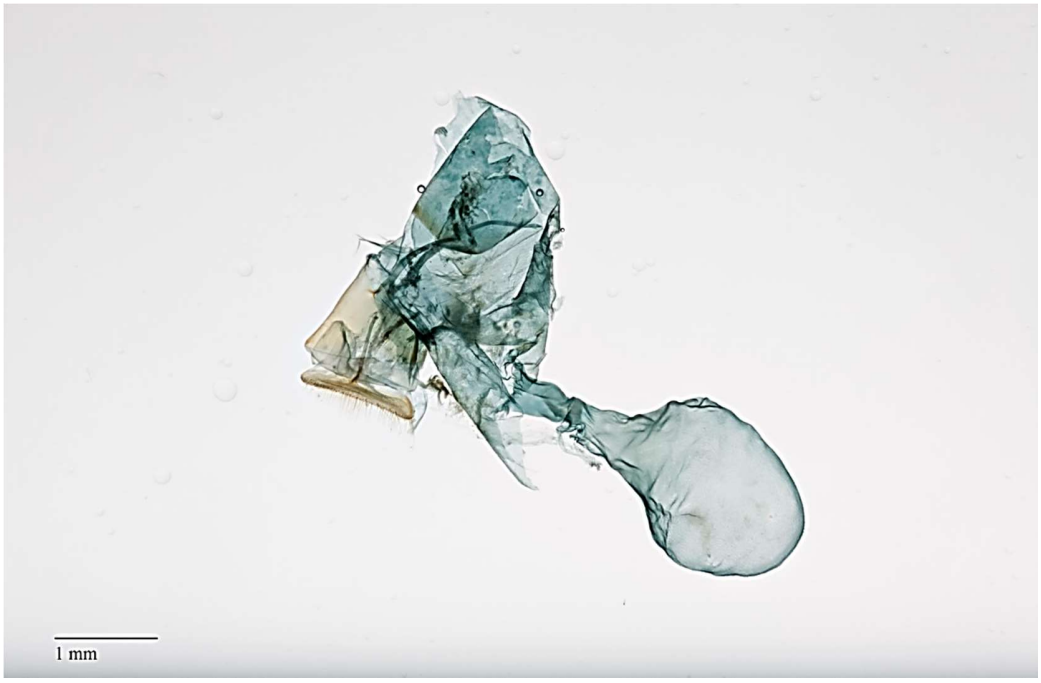

Fig 3.4. Female reproductive structures of *D. impersonatella*.

3.3.4 Analysis of COI mitochondrial gene

In addition to classification by taxonomy, we also classified the 95 individuals collected based on DNA polymorphism of sequence of cytochrome C oxidase subunit I (COI) gene. The COI sequence polymorphisms allowed the estimation of the genetic relationships among individuals. Aligned sequences of DNA consisted of 666 base pairs for the COI mitochondrial gene for both species. The average frequency of A, C, G and T was 37.8%, 15%, 16.1% and 31.1%, respectively. The strong AT bias (68.9%) is typical of insect mitochondrial genomes (MORITZ, 1987; HARRISON, 1989). Neighbor-joining clustering of the COI sequences using Jukes & Cantor distance (1969) produced two well-defined groups that perfectly matched the taxonomic findings: one with the 82 individuals identified as *D. saccharalis*, while the other one with the 13 individuals of *D. impersonatella* separated by a node with 100% of consistency (Fig 3.5). This result suggests that *D. saccharalis* and *D. impersonatella* clearly differ from each other. This grouping pattern was expected, because the COI is an efficient method for separation and confirmation of species (HEBERT, 2003; AVISE, 2000).

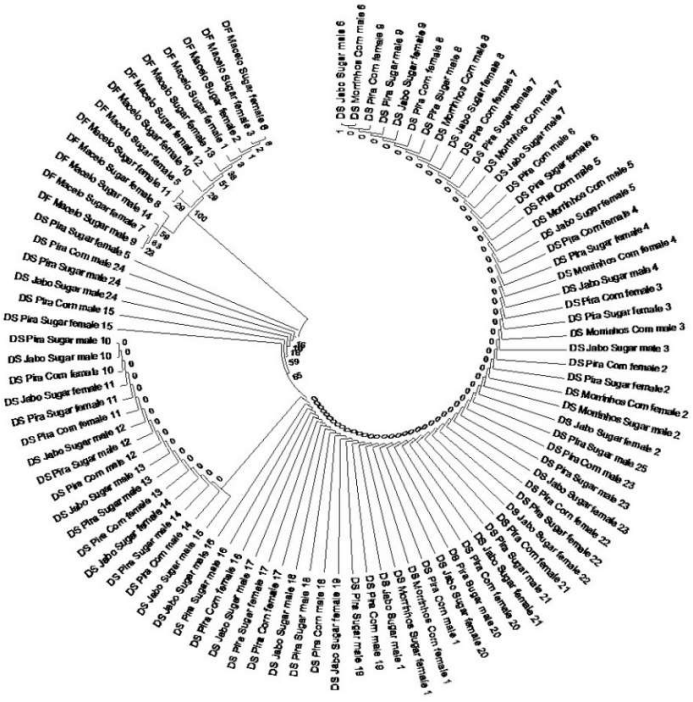

Fig 3.5. Relationships among individuals of *D. saccharalis* and *D. impersonatella* from the COI gene.

A total of 10 haplotypes were obtained from the COI sequencing, showing consistency with previous results (Table 2). Six of the haplotypes were present in *D. impersonatella* specimens and four were present in *D. saccharalis* specimens (Fig 3.6). The *D. impersonatella* population had the highest haplotype diversity (Table 3.2 and Fig 3.6).

Table 3.2. Genetic characterization of populations based on mitochondrial COI barcode sequence analysis.

Species	Number of Individuals	Number of Haplotypes	Haplotype Diversity	Nucleotide Diversity	Tajima's D test (p value)	Fu's Fs test (p value)
Diatraea impersonatella	13	6	0.769	0.00316	-0.25752 (=0.437)	-1.00562 (=0.246)
Diatraea saccharalis	82	4	0.14	0.00032	-1.73486 (=0.008)	- 2.52261 (=0.0016)

All individuals of this species were collected in a unique location, and had sugarcane as their host. Haplotypes H3 and H6 were the most frequently found in this species (6/13) and (3/13), respectively. Four haplotypes (H1, H2, H4 and H5) occurred in only one individual. In addition, *D. impersonatella* showed high haplotype diversity (0.769) and low nucleotide diversity (0.00316) (Table 3.2) indicating only a small difference between haplotypes.

Although haplotype diversity was high, low nucleotide diversity values indicate the existence of few segregating sites across different haplotypes. The combination of high haplotype diversity and low nucleotide diversity, as observed in our data, may be a signature of a rapid demographic expansion from a small effective population size (AVISE, 2000). Recently, there has been an inversion in the prevalence of the *Diatraea* species that attack sugarcane in Alagoas state. During the 1970s, *D. saccharalis* was the predominant species (RISCO, 1975). Currently, Freitas et al. (2006) observed 10.20% of *D. saccharalis* and 89.80% of *D. impersonatella* in Alagoas, indicating the recent expansion of species in this region.

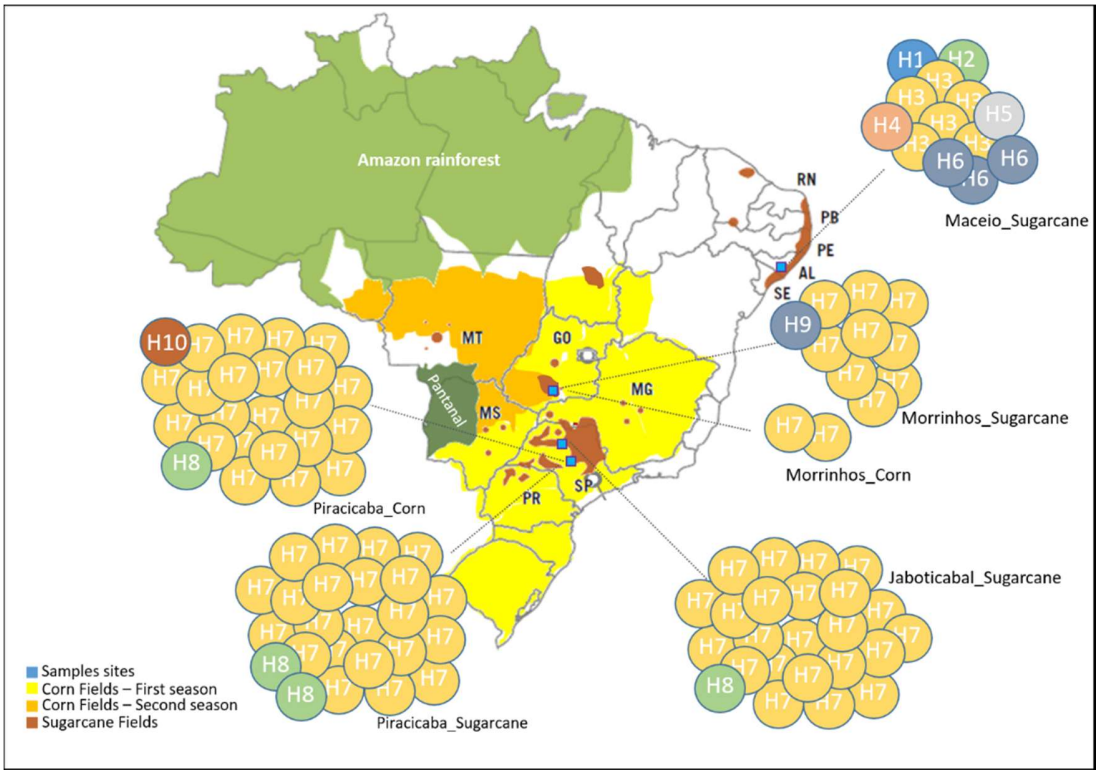

Fig 3.6. Geographic distributions of COI haplotypes of *D. saccharalis* and *D. impersonatella*.

Each colored circle represents the haplotypes identified in a given population. The number within circles denote the COI haplotypes identified in each population. The descriptions refer to the sampled locations and crops.

D. saccharalis haplotype diversity and nucleotide diversity were 0.14 and 0.00032, respectively (Table 3.2), and the population genetic diversity ranged from 0.083 to 0.2 and nucleotide diversity ranged from 0.0003 to 0.00042. In terms of population differentiation, the spatial distribution of haplotypes demonstrates that the *D. saccharalis* populations show relatively little divergence, and shared the most common haplotype, H7 (78/82) that was represented in all collection sites. The second most common haplotype was H8 (4/82), being that one individual was collected in Jaboticabal in the sugarcane crop and three in Piracicaba, one was from corn and two from sugarcane. The H9 and H10 haplotypes were represented by unique specimens, and both were collected in corn fields from Morrinhos and Piracicaba. Low genetic variation in the COI sequences was detected in *D. saccharalis*. The main production regions where the insects were

collected and where they shared the same haplotypes, were located within the approximately 200 km between Jaboticabal and Piracicaba, the 500 km between Jaboticabal to Morrinhos, and the 650 km between Piracicaba to Morrinhos. Collection sites located in the Piracicaba region had a distance of approximately 30 km between each location, and shared the same haplotypes in different hosts. In Morrinhos city the specimens were collected on the same farm, but from different hosts. Thus, the spatial distribution of haplotypes revealed no major groupings of *D. saccharalis* haplotypes according to either host plant or geographical location. Each haplotype, when present in two or more individuals, had a wide geographic distribution.

When genetic structure has been influenced by rapid range expansion, the Tajima's D value is expected to be negative, indicating an excess of rare nucleotide variants compared to the expected under a neutral model of evolution (TAJIMA, 1989). In this study, the Tajima's D values were negative for both species, -1.734 (p-value < 0.01) for *D. saccharalis*, and -0.25752 (p-value =0.437) for *D. impersonatella*. These results show that the mutations found in the *D. saccharalis* sequences probably occurred due a genetic drift, not selective pressure. While for *D. impersonatella*, the negative Tajima's D was not statistically significant, and therefore the hypothesis of neutral evolution was rejected for this species. The results of Fu's FS test, which is based on the distribution of haplotypes, also had negative values for all *D. saccharalis* populations, confirming an excess of rare haplotypes over what would be expected under neutrality. Just as for Tajima's D, Fu's FS was not significant for *D. impersonatella*.

An analysis of molecular variance (AMOVA) was performed to verify how the genetic variability was distributed among and within the two species collected. Consistent with the other analyses presented here, the AMOVA also shows the separation of the samples into two species of *Diatraea*. The AMOVA results showed a high percentage of variation between species 99.21% (F_{ST} = 0.99). Moreover, low genetic variations were observed among populations within species and within species, -0.02% and 0.81%, respectively (Table 3.3). An AMOVA with only *D. saccharalis* samples revealed that the largest percentage of variation occurred within populations, with 100%, suggesting that the variation was distributed randomly between sites (S3.2 Table).

Table 3.3. Hierarchical analysis of molecular variance (AMOVA) for population genetic structure of *D. saccharalis* and *D. impersonatella* based on the variation of the mitochondrial COI gene. D.f. = degrees of freedom.

Hierarchical levels	d.f.	Sum of Squares	Variance components	Variance (%)	Fixation Indices	p-value
Three-hierarchical-levels						
Among species	1	639.475	28.48853	99.21	F_{ST} = 0.99215	< 0.001
Among populations within species	2	0.184	-0.00612	-0.02	F_{CT} = -0.02714	0.4531
Within species	91	21.078	0.23163	0.81	F_{SC} = 0.991913	< 0.001
Total	94	660.737	28.71404			

The high-level divergence between *D. saccharalis* and *D. impersonatella* is also evident by the F_{ST} estimates among their populations. The pairwise F_{ST} estimates between *D. saccharalis* populations ranged from -0.33172 to 0.05546. The highest pairwise F_{ST} estimate was observed between populations from different hosts, Jaboticabal_Sugarcane and Morrinhos_Corn (F_{ST}= 0.05546), however this genetic divergence was not significant (S3.3 Table).

The relationship between these two species of *Diatraea* has always been unclear, and there is little historical knowledge about demographic expansion, especially for *D. impersonatella*. Myers (MYERS, 1932 and 1935) conducted a fascinating global search for primitive habitats and original host-plants of *Diatraea* species. He concluded that *D. saccharalis* co-evolved with riparian aquatic vegetation, and that the probable center of origin was between the delta region of the Orinoco River, Venezuela, and the lower Amazon River, Brazil. Taking into account that *D. flavipennella* is considered a synonym of *D. impersonatella* (BOX, 1931; BLESZYNSKI, 1969; SOLIS, 2016) the species was recorded as an original member of a true savannah and riparian vegetation (MYERS, 1935). During the colonization of Amazon basin these two species shared the same host, *Paspalum fasciculatum* (Poaceae), and was often found in association with insect damage in that region. The expansion of *Diatraea* species to other parts of Brazil is unknown. We can speculate that these pests were introduced into Brazil in the 16th century or later as stalks of

sugarcane varieties or native plants from the Amazon basin, were transported throughout the region by colonists.

Dissimilarities among the species sequences indicate genetic divergence as the result of molecular evolution during the course of time (PATWARDHAN, 2014). In analyzes of the COI sequence of these two species, combining with sequence data from other *Diatraea* species (obtained from GenBank and BOLD) showed that these species cluster together. Other clusters contained species originating from other localities and hosts (Fig 3.7). COI sequences were not available from the public databases for *D. impersonatella* or *D. flavipennella* to add to that analysis.

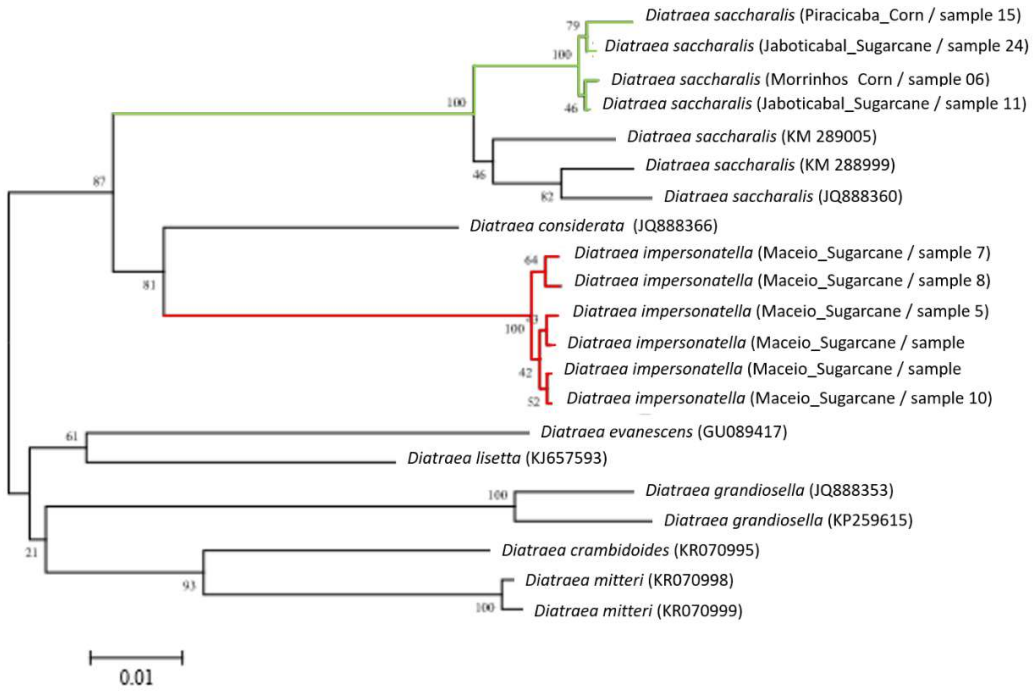

Fig 3.7. Neighbor joining dendrogram showing the relationships among haplotypes of the COI gene for *Diatraea* species. Individuals collected as part of this study are indicated with colored branches (Green: *D. saccharalis* and Red: *D. impersonatella*). Individuals showed in black were obtained from GenBank and BOLD databases. Confidence level values were based on 1000 bootstrapping.

Studies with mitochondrial DNA COI and COII sequences of *D. saccharalis* populations, including overseas populations, have shown a divergence between individuals and thus a geographic population structure (LANGE, 2004; CORTES, 2010; SILVA-BRANDÃO, 2015; JOYCE, 2014). It is clear in the results of these researchers that there exists a divergence between populations from South America to those collected in the southern USA. These studies, with different molecular markers, were able to identify clusters that can be associated with several introductions of this borer into each country (MYERS, 1932 AND 1935; BOX, 1950; HOLLOWAY, 1928). There have been few studies to determine the population structure of *D. saccharalis* in Brazil. Cortes et al. (2010) analyzed sequence variation in the barcode region of the COII gene and found low intra-specific variation among samples collected, with 98% of individuals sharing a common haplotype similar to that also found in all samples collected from in São Paulo, Paraná and Pernambuco state. Noteworthy that the most frequent haplotype observed by Cortes (2010) had the same sequence previously reported by Lange et al. (2004) in a Brazilian population.

In contrast to this observations, Silva-Brandão et al. (2015), in a study using COI sequences combined with nad6 sequences, were able to distinguish a high level of genetic structure among samples of *D. saccharalis* collected in Brazilian corn and sugarcane fields, obtaining data with high values of F_{ST} and haplotype diversity, but low nucleotide diversity. Interestingly, the same haplotype was found at high frequency (68 of 125 specimens; 54.4%), possibly the consequence of using two molecular makers instead of one. The length of COI and *nad6* sequences used in that study were long, at 1,429 and 497 bases, respectively. The majority of studies involving COI sequences have used a short barcode section of mitochondrial DNA, the first approximate 650 bases of the 5'-end of this gene (HEBERT, 2003; ELIAS-GUTIERREZ, 2008; ROCK, 2008). Consistent with our work, the most frequent haplotype detected by Silva-Brandão et al. (2015) was identical to the most frequent haplotype in the present study. Furthermore, our findings showed no structured genetic variability in populations of *D. saccharalis,* and the haplotype diversity was lower than expected, in contrast to published records for this species (LANGE, 2004; SILVA-BRANDÃO, 2015; JOYCE, 2016).

Contrasting results may have emerged due to different factors, such as sampling and the methods employed. For example, light traps may attract insects with different hosts, which may

increase the diversity sampled. Other important point is the definition of development stage (moth or larvae) used for DNA extraction. Even though the use of larvae is allowed by International Barcode of Life project (iBOL), bacteria present in the larvae body may be a source of contaminant DNA. Thus, sampling of moths is an advantage with respect to the quantity and quality of DNA isolated (SHERE-KHARWAR, 2012; SHERE-KHARWAR, 2013). Moreover, another important aspect is the precise taxonomic identification to support molecular analyses of the species.

Based on these premises and on examination of almost 100 individuals we can show that low values of haplotype and nucleotide diversity and the higher frequency of the same haplotype is a realistic scenario. The homogeneity observed in COI among widely dispersed and geographically isolated Brazilian populations of *D. saccharalis* can be explained as a consequence of low mutation rates and/or stochastic processes that resulted in severe bottleneck of the population sizes, as described by negative values of Tajima's D. According to Myers (1932; 1935), the original hosts of *D. saccharalis* are aquatic and semi-aquatic grasses in the Orinoco Delta River. Therefore, we may speculate that when sugarcane was introduced in the New World by the sixteenth century, *D. saccharalis* migrated, colonized, and adapted to the new hosts and environments. The bottleneck signal observed suggest that during the colonization a relatively small number of individuals founded the new populations (ELTON, 1958; DEBACH, 1991). This demographic bottleneck could also be suggested by the genotype and haplotype composition, which indicated a reduced genetic diversity in Brazilian populations of *D. saccharalis* (NEI, 1975; TEMPLETON, 1980; BARTON, 1984; HARTL, 2007). Recently, various studies reported on species that have low mtDNA variation and no population structure (BROWER, 1991; CHAPCO, 1992; ZEHNDER, 1992; BOGDANOWICZ, 1993; MARTIN, 1990; PASHLEY, 1989). DNA identification will not work unless the variations are much less within a species than between species (LIPSCOMB, 2003). According to Hebert et al. (2003), the mean interspecific genetic divergence should be at least 10 times higher than the average intraspecific genetic distance in order to define the presence of species complexes. High levels of COI sequence variation within species could complicate efforts to use COI to differentiate between species (AVISE, 2000).

In this study, we showed that the analysis of polymorphism of oxidase cytochrome C oxidase subunit I (COI) mitochondrial gene is a powerful and accurate species discriminator for *D. saccharalis* and *D. impersonatella*. In relation to intraspecific studies within *D. saccharalis,*

COI analysis did not show genetic structure for populations sampled in this study. The use of combined morphological and molecular approaches, including both mtDNA and a nuclear DNA, is proposed to evaluate relationships that persist uncertain.

3.3.5 DNA polymorphism analysis with nuclear microsatellite loci

To investigate the genetic variation in populations of *D. saccharalis*, we performed microsatellite loci characterization on 80 of the 82 individuals collected (Table 3.1) with eleven microsatellite loci (S3.1 Table). Two individuals from Morrinhos_Sugarcane were removed of the study because they did not fit within the criteria for assignment. In the analysis of *D. saccharalis* populations using eleven microsatellite loci, we observed that the total number of alleles was 51, ranging from 2 to 7 alleles per locus. The loci Dsc1, Dsc2, Dsc10 and Dsc20 showed the smallest diversity, while the loci Dsc 03, Dsc9, Dsc11 and Dsc20 showed the highest (S3.4 Table). The average observed heterozygosity per loci was 0.42, ranging from 0.084 to 0.885, while the average expected heterozygosity was 0.49, ranging from 0.122 to 0.632. The average coefficient of inbreeding (intrapopulation fixation index) per loci was 0.14, ranging from -0.413 to 0.472 (S3.4 Table).

The average number of alleles found was 35.8, ranging from 30 to 40 alleles. The average allelic richness (Ar) per population was 29.83 and ranged from 32.4 to 25.19. The population Piracicaba_Sugarcane showed the highest value of allelic richness. The average values of observed and expected heterozygosity per population were 0.42 and 0.46, respectively. The highest heterozygosity was observed in Morrinhos_Corn (0.44), and the lowest in Jaboticabal_Sugarcane (0.4). For the expected heterozygosity, the highest value was 0.51 for Morrinhos_Corn, and the lowest was 0.39 for Piracicaba_Corn. The expected heterozygosity values were higher than observed heterozygosities for all populations, except for Piracicaba_Corn. The average coefficient of inbreeding was 0.10, ranging from -0.074 to 0.179. The estimate values of F_{IS} was positive for the populations from Jaboticabal_Sugarcane (0.1788), Morrinhos_Corn (0.1429), and Piracicaba_Sugarcane (0.1323), reflecting an excess of homozygotes in these populations, while Piracicaba_Corn was the only population with negative value of F_{IS}. These differences among the observed and expected heterozygosities can be attributed to non-random mating among the

individuals and inbreeding within populations with positive values of F_{IS}. While the negative inbreeding coefficient observed in Piracicaba_Corn suggests that these processes are not occurring in this populations (Table 3.4).

Table 3.4. Genetic diversity estimates for each population of *D. saccharalis* based on eleven microsatellite loci.

Population	n	N_A	PA	AR	H_E	H_o	F_{IS}
Jaboticabal_Sugarcane	24	40.0	3	30.93	0.480	0.400	0.179
Morrinhos_Corn	8	34.0	6	30.79	0.510	0.440	0.143
Piracicaba_Sugarcane	24	39.0	3	32.4	0.490	0.420	0.132
Piracicaba_Corn	24	30.0	0	25.19	0.390	0.420	-0.074
Average	-	35.8	-	-	0.46	0.42	0.10

This is the Table 3.4 legend.

n -Number of individuals, N_A - Number of alleles, PA - Private Alleles, AR - Allelic Richness, H_E - Expected heterozygosity, H_O - Observed heterozygosity, F_{IS} - coefficient of inbreeding.

We identified 12 private alleles, 50% were found in Morrinhos_Corn, and the Piracicaba_Corn population had no private alleles. The molecular marker, which identified the largest number of private alleles, was Dsc11, with 5 alleles distributed in Morrinhos_Corn, Jaboticabal_Sugarcane and Piracicaba_Sugarcane populations (S3.5 Table).

The values of pairwise F_{ST} indicated genetic differentiation among populations, and ranged from 0.0835 to 0.1812 (S3.6 Table). We observed that Jaboticabal_Sugarcane and Piracicaba_Corn were the most divergent populations (0.1812), while Morrinhos_Corn and

Piracicaba_Corn, with the same host, were the less divergent (0.0835). Using the UPGMA method (unweighted pair-group method with arithmetic mean) (SNEATH, 1973) a dendrogram based on Nei's standard distances (NEI, 1978) was generated. In this analysis, we observed the existence of well-defined groups, indicating the existence of genetic structure among hosts (Fig 3.8), suggesting that the populations of *D. saccahralis* collected in sugarcane and corn are not closely genetically related. Additionally, the clusters observed in the dendrogram are also in accordance with the sites of collection, which can be an indication of geographic structure.

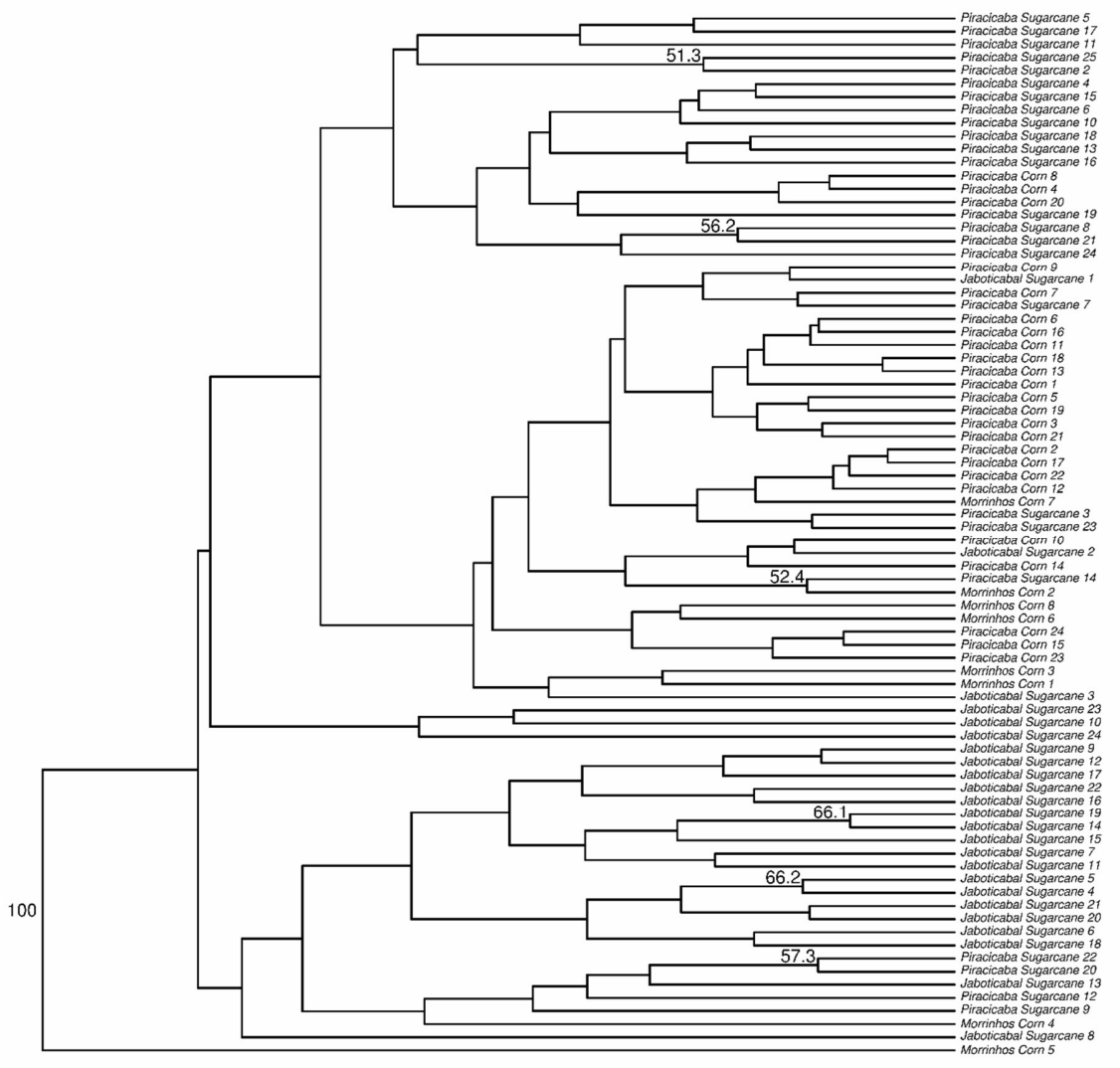

Fig 3.8. Dendrogram showing genetic relationships among 82 individuals of *D. saccharalis*.

Neighbor-joining tree based on the pairwise genetic distances between individuals estimated by the logarithm of the proportions of shared alleles, and 1000 bootstrap repetitions.

In the DAPC, 72% of the total genetic variation was captured by components of PCA and these were used as input to capture two DA functions. This analysis separated the samples in four major clusters that correspond to both geographic locations and hosts (Fig 3.9). Especially in Piracicaba the separation by host can be clearly visualized. The DAPC agreed the pairwise differentiation (F_{ST}) estimates among sampling sites.

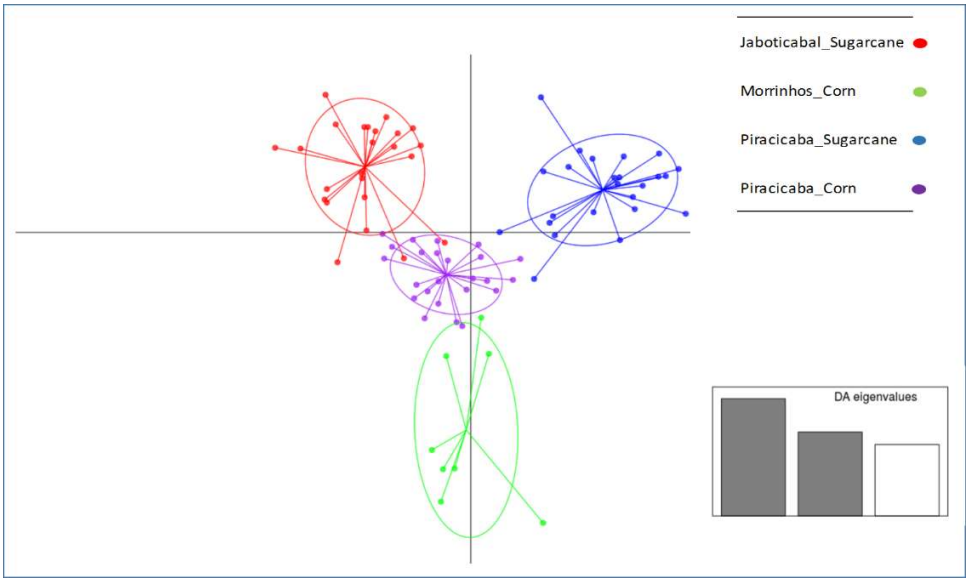

Fig 3.9. DAPC showing habitat profiling of individuals across four *D. saccharalis* populations, using the microsatellite data. Variation represented in x = 42% and in y = 30%.

In Brazil, sugarcane and corn are cultivated in a large diversity of environments, which differ in soil conditions, climate, availability and susceptibility of the variety and management control system. It is characteristic of inconstant environments to exhibit a significant loss and fragmentation of natural ecosystems (GEPTS, 2004).

Furthermore, these diverse cultivation ecosystems provide habitats for a wide range of pests. Insects demonstrate a high ability of local adaptation and acceleration of the evolutionary process (TISCHENDORF, 2003; VIALATTE, 2005). The fragmentation process can lead to

reduced effective insect population sizes and an increase in the mating between relatives. Especially in phytophagous insects, the process of fragmentation drives the reduction of gene flow and increases host plant specialization (STIREMAN, 2005). Our data show an overall deficit of heterozygotes, and a significant genetic differentiation among populations. According to Avise et al. (1987), the dispersion capacity, the geographic barriers and other related process may also affect the population structure of a given species. The agricultural production systems in Brazil may have considerably influenced *D. saccharalis* populations. The development of new varieties of sugarcane allowed the cultivation in a system ranging from 12 to 18 months. This sequential production system permits *D. saccharalis* to have host plants yearlong and limits the necessity of migration. In corn production systems, the introduction of Bt technology in the mid-1990s created an agricultural system less dependent on insecticides, but selectively eliminates certain species from the insect populations. Additionally, the Bt technology might provide the primary ecological opportunities needed for the first host shift and encourage adaptation to digest novel plant defensive proteins (SIMPSON, 1949; SIMPSON 1953; MITTER, 1991; SCHLUTER, 2000; YODER, 2010). Another important point to consider is related to the capacity of dispersal of this moth. The dispersal behavior of insects is the major factor that can influence gene flow among their populations (PETERSON, 1998). Results of mark–recapture studies suggest that dispersal rates in *D. saccharalis* are low, apparently as result of home-range behavior. Over 45% of the adults were recaptured around 50 meters of the release site. Extended dispersal was observed when the moths followed the wind, which increased their dispersal in around 800 meters (HAYWARD, 1943; CAIXETA, 2010).

Limited flight ability, genotype–environment interactions, and host year-availability affect the relationship of *D. saccharalis* with the major hosts and suggest that the current genetic divergence and inbreeding result from limited gene flow and natural barriers. Evidence of fragmentation influence on genetic diversity was found in other studies with *D. saccharalis* populations (PAVINATO, 2014; NASCIMENTO, 2015). Pavinato (2004) observed divergence and limited gene flow among populations of *D. saccharalis* collected in corn and sugarcane, just as the results found in our study. However, Nascimento (2015) reported low levels of structuring among populations of *D. saccharalis* in sugarcane. This lack of divergence observed between populations collected in sugarcane may be related with the proximity of samples sites, since all populations belonged to the same state in Brazil.

Another important factor for the population divergence observed in our study may involve different selective pressures across environments. In addition, low rates of gene flow between populations increase the likelihood of host-associated genomic differentiation. We found strong evidence of host-associated genetic divergence across the range of *D. saccharalis*, such as the DAPC and pairwise F_{ST} results. Pavinato (2014) support the hypothesis of ecological divergence between *D. sacharalis* populations from corn and sugarcane. Subpopulations of the same host (sugarcane or corn) tend to be more similar to each other (PAVINATO, 2014).

In conclusion, microsatellite loci were polymorphic and highly informative, allowing the study of genetic variability of *D. saccharalis* collected in Brazil, which suggested the presence of genetic groups correspondent with their geographic sampling locations. It is possible that these populations have evolved some degree of adaptation to local environmental conditions.

3.4 Conclusions

We conducted a systematic study of *D. saccharalis* and *D. impersonatella* that included characteristics from genitalia anatomy, mtDNA, and nuclear molecular markers. Our results clarified some outstanding questions about *Diatraea* populations in the Brazilian territory. In this study, we observed, through taxonomic methods and COI sequencing, that *D. saccharalis* and *D. impersonatella* are the two species responsible for attacking sugarcane and corn in Brazilian crop fields. Sequencing of COI revealed to be an accurate species discriminator for this genus. Microsatellite analyses revealed host-plant preference in populations of *D. saccharalis*. Moreover, genetic structure showed little connection among populations. There are preliminary indications that low interactions can relate to the fragmentation process between crop productions regions and may affect gene flow. In summary, we have demonstrated that microsatellite polymorphisms provide a valuable tool for population genetic analysis of *D. saccharalis*. Additionally, we strongly recommend the adoption of the name *D. impersonatella* as a nomenclatural change from *D. flavipennella* as proposed in Solis and Metz (2016). Our findings directly affect the adoption of control actions. The establishment of a functional program that will ensure the design and implementation of sustainable pest management strategies needs to take into account the genetic structure and local characteristics of *Diatraea* populations.

4. Capítulo 2 Utilizing genotyping-by-sequencing to elucidate *Diatraea saccharalis* (Lepidoptera: Crambidae) Dispersion in America continent and the Human-mediated migration dependence

Sumário

Abstract

The sugarcane borer, *Diatraea saccharalis*, is the main lepidopteran pest species attacking sugarcane and maize production fields in the Americas, with this pest being widely distributed throughout South America, Central America, the Caribbean, and the southern United States. However, its rate of spread is much higher than expected from an insect with low taxes of movement and home-range behavior more suggestive of a species inclined to live in family groups and not showing a necessity to search for new environments. The main objective of this study was to investigate this intriguing scenario through genetic structure and diversity of *D. saccharalis* populations in the Americas based on single nucleotide polymorphisms identified with genotyping-by-sequencing. Based on the results, we discuss possible hypotheses that may explain the migratory pathways from the probable center of origin in the Orinoco Delta River, Venezuela. Our findings showed a clear accordance between genetic structure and the geographical distributions of the sampled populations. The clustering analyses revealed three major distinct groups, one of them composed of Brazilian populations, another one represented only by the Argentine populations, and a third group which comprises populations from El Salvador and the United States. We suggest that human-mediated migration was most likely the responsible vectors for the vast spread of *Diatraea saccharalis* throughout the Americas in two distinct movements. Maize and sugarcane were the main host associations in the system, with maize most likely being the main vehicle of the first movement, and sugarcane the probable main vehicle of the second dissemination. Spread patterns that we suggest, show long-distance dispersal events with high genetic variability among countries. This study on targeted insect dispersal gives a better understanding of the past *D. saccharalis* movements, and can guide improved pest management techniques.

Key words: Sugarcane, stemborer, insect migration, genotyping-by-sequencing and population genetics

4.1 Introduction

Some insect populations have a predisposition for migration that allows them to escape a deteriorating habitat, and to colonize new areas or to seek temporary shelter such as overwintering sites (DINGLE, 1972). As a species invades a new environment, the selection pressures imposed by host-plants or environmental changes can promote rapid adaptive evolution in natural insect populations (CHEN, 2010; KIRK, 2013). The genetics of adaptation is fundamentally about gene substitution within populations over time. The alleles favored by natural selection replace other alleles and at the same time change traits within populations (BLACK, 2001). Advantageous alleles can be selected across generations to maximize fitness in the local environment by natural selection (KAWECKI and EBERT, 2004; AITKEN et al., 2008). An ample genome exploration of polymorphism and divergence can provide novel conceptions on the process of insect adaptive evolution. When individuals with certain variants of the trait survive and reproduce more than others, less successful variants, the population evolves. In addition, these adaptive divergences can initiate the speciation process by forming genomic islands of divergence (FEDER et al. 2012). The understanding of these genomic islands of divergence can help elucidating the dispersion pattern in a specific population of insect that share a recent common ancestral colonizer. The mechanism of dispersion in insects can be roughly divided into three broad categories:

(a) Migratory behavior and phenotypic characters that involves the condition of the flight muscles, the oviposition period and timing, the fecundity, the energy uptake and wing polymorphism (PANOS, 2005);

(b) Human-mediated migration (ABBO et al., 2006; ROULLIER et al., 2013) that is the result of three major modes of evolution (CHEN, 2016). First, according to Chen (2015), the insect herbivores previously adapted to the crop wild ancestor can shift onto the domesticated crop. Second, insect herbivores can shift from their native host plants onto introduced crops (BUSH, 1969; SHIRAL and MORIMOTO, 1999; CALCAGNO et al., 2007). Third, human-aided migration supports the invasion of insect herbivores to new geographic regions (GRAPPUTO et al., 2005; GUILLEMAUD et al., 2015);

(c) Natural forces, when the insect is accidental and passively transported by a large portion of air, such as by strong winds or tornados, or by a large body of flowing water, as in a river or

ocean (MYERS, 1935; AHMED et al., 2009; REYNOLDS and REYNOLDS, 2009; KERDELHUE, 2014).

Understanding how insects travel might assist in the prediction of new pest invasions, or deepen the understanding of past insect evolution (SAKAI, 2001; LOCKWOOD, 2007). Likely trajectories of introduced populations can estimate the evolutionary patterns by invoking various population dynamics and local ecological characteristics. Recently, substantial advancement has been made in the understanding patterns of insect migration (KIM and SAPPINGTON, 2004; MILLER et al., 2009), mainly with respect to certain moths (HOLLAND, 2006; KIM et al., 2009, 2011). Analysis of the spread of crop pests must consider where pests are currently found, where they could occur and how they are spreading (BEBBER, 2014). In this context, information about dispersion across the Americas of the important pest of sugarcane and maize, *Diatraea saccharalis*, remains unclear. According to the fascinating work of Myers (1935), the center of origin and original habitat of *D. saccharalis* seems to be the floating grasses (*Paspalum* spp. and *Panicum* spp.) along the banks of the Orinoco Delta River in Venezuela. Currently, *D. saccharalis* is widely distributed throughout South America, Central America, the Caribbean, and the southern United States (BOX, 1931; BLESZYNSKI, 1969; LANGE et al., 2004; CORTÉS et al., 2010). This widespread dispersal is surprising when considering that *D. saccharalis* has low rates of movement, and a home-range behavior or site fidelity suggestive of a more stagnant, family group lifestyle. Moreover, in mark-recapture studies, over 45% of the adults were recaptured around 50 meters of the release site (HAYWARD, 1943; CAIXETA, 2010). Extended dispersal was observed when the moths followed the wind, which increased their dispersal in around 800 meters. *D. saccharalis* shows a complex dispersion history, given this dichotomy between the insect behavior and the available dispersion information.

The study of insect dispersion becomes excessively complicated and difficult to validate when attempting to link patterns and mechanisms, and to account for multiple dispersal vectors (NATHAN, 2007). Moreover, generally it requires a combination of techniques to gain a comprehensive understanding of movement across different spatial scales (REYNOLDS et al., 2006). Genetic analysis of introduced populations is a promising strategy to discern the microevolutionary and ecological adaptations that underlie the ability of an insect pest to invade agro-ecosystems (CHU, 2014). This kind of information on dispersal capacity and dispersal

patterns that can be obtained from population genetic analyses would be difficult or impossible to acquire in other ways (MA, 2012; LLEWELLYN, 2003). Next-generation sequencing (NGS) technologies makes it possible to address these complex sets of information and questions of evolution in detail (TOEWS, 2015). The main objective of this study was to investigate the genetic structure and diversity of populations of *D. saccharalis* in the Americas using single nucleotide polymorphisms (SNPs) obtained through the genotyping-by-sequencing (GBS) approach (ELSHIRE, 2011). GBS generates hundreds to thousands of SNP markers providing ideal genetic data to study the evolutionary forces shaping the insect genomes and capture genetic variation that is specific to a given population (BLACK, 2001). This technology has been used to generate SNPs in several other organisms, including plants (ELSHIRE et al., 2011; POLAND et al., 2012), animals (DONATO, 2013; GORJANC, 2015) and insects (NOSIL, 2012; SILVA-BRANDÃO, 2015).

Population genetic approaches have been previously used study *D. saccharalis*. These studies have detected genetic differentiation and population structure in samples collected in several regions of North and South America (LANGE, 2004; CORTÉS et al., 2010; JOYCE, 2014). However, these studies relied on a limited number of genetic markers and therefore could not directly reveal the dispersion patterns of the *D. saccharalis*. In the presented work, we describe the analysis of SNP allele frequency differences among *D. saccharalis* populations, which provides a powerful approach to investigate the demographic history and identify in the genome signatures of selection, giving a framework for understanding the evolutionary patterns of introduced invasive populations of *D. saccharalis* in the Americas.

4.2 Materials and Methods

4.2.1 Insect collections

We collected 250 specimens of *Diatraea* in maize and sugarcane production regions in Brazil, Argentina, El Salvador and United States in the crop seasons 2011–2012 and 2012–2013 (Table 4.1).

The larvae were collected randomly in equidistant points inside the corn or sugarcane fields. Corn and sugarcane stalks showing the typical symptoms of attack by *Diatraea* borer, were cut with the aid of a saw. The larvae found in each damaged plant were transferred to the laboratory, moved to Petri dishes containing artificial diet, identified, and kept separate. Each larva was assessed for the presence or absence of parasites. Larvae that were infected by parasites or other diseases were discarded. The specimens that passed screening were placed individually on Petri dishes with artificial diet and maintained at 27 ± 1°C; 70% U.R. and photoperiod of 12 hours until they pupated. Then each individual pupa was transferred to cylindrical cages of 40 cm x 30 cm. The pupae were kept at 20 ± 1°C with a 12-hour photoperiod until adults emerged. Moth emergence occurred within these cylindrical cages to allow the complete metamorphosis and for the moth to properly inflate their wings. All moths were transferred into micro centrifuge tubes and stored at - 80 C.

The collections were carried out on private lands (with the permission of their owners), and no specific permits were required for these locations/activities because it did not involve endangered or protected species.

Table 4.1 - Populations collected and their geographic locations.

Code	Population (Country)	Population (State)	Population (City)	Host	Latitude	Longitude	Number of Individuals
LaCocha_Co	Argentina	Tucumán	La Cocha	Corn	-27.7680453	-65.5841519	10
LaCruz_Su	Argentina	Tucumán	La Cruz	Sugarcane	-29.1783205	-56.6378331	5
Jujuy_Su	Argentina	Jujuy	Jujuy	Sugarcane	-24.1843397	-65.302177	7
Perga_Co	Argentina	Buenos Aires	Pergamino	Corn	-33.8912831	-60.5745999	2
Qui_Co	Argentina	San Luis	Quines	Corn	-32.2337053	-65.8055325	4
Adam_Su	Brasil	São Paulo	Adamantina	Sugarcane	-21.717868	-51.0152294	1
Araras_Su	Brasil	São Paulo	Araras	Sugarcane	-22.3604911	-47.3798391	2
Goias_TBD	Brasil	Goiás	Goiás	TBD	-16.6868824	-49.2647885	4
Inac_Co	Brasil	Goiás	Inaciolândia	Corn	-18.4873555	-49.9892163	1
Jabo_Su	Brasil	São Paulo	Jaboticabal	Sugarcane	-21.2525138	-48.3256762	52
Minas_TBD	Brasil	Minas Gerais	Minas Gerais	TBD	-19.9465885	-43.9698479	1
Morr_Co	Brasil	Goiás	Morrinhos	Corn	-17.734945	-49.1208516	4
Morr_Su	Brasil	Goiás	Morrinhos	Sugarcane	-17.734945	-49.1208516	1
MS_TBD	Brasil	Mato Grosso do Sul	Mato Grosso do Sul	TBD	-17.7342695	-49.1193721	9
MT_TBD	Brasil	Mato Grosso	Mato Grosso	TBD	-15.5889647	-56.0814921	5
PAf_Su	Brasil	Tocantins	Pedro Afonso	Sugarcane	-8.9707508	-48.1733686	3
Parana_TBD	Brasil	Paraná	Paraná	TBD	-25.4244287	-49.2653819	3
Pira_Co	Brasil	São Paulo	Piracicaba	Corn	-22.7342864	-47.6480644	25
Pira_Su	Brasil	São Paulo	Piracicaba	Sugarcane	-22.7342864	-47.6480644	22
Rib_Su	Brasil	São Paulo	Ribeirão Preto	Sugarcane	-21.1704008	-47.8103238	4
Rondo_Co	Brasil	Mato Grosso	Rondonópolis	Corn	-16.4654757	-54.6387229	3

SHG_Su	Brasil	Goiás	Santa Helena de Goiás	Sugarcane	-17.8119748	50.5981252	4
SP_TBD	Brasil	São Paulo	São Paulo	TBD	-23.5505199	-46.6333094	18
Uber_Co	Brasil	Minas Gerais	Uberlândia	Corn	-18.8936275	-48.221351	1
ElNilo_Su	El Salvador	El Nilo	El Nilo	Sugarcane	NA	NA	4
ElPais_Su	El Salvador	El Paisnal	El Paisnal	Sugarcane	13.9755122	-89.217002	2
FLA_Su	USA	Florida	Unknown	Sugarcane	NA	NA	5
BGlade_Su	USA	Florida	Belle Glade	Sugarcane	26.6845104	-80.6675577	9
LA_Su	USA	Louisiana	Unknown	Sugarcane	NA	NA	4
Louis_Su	USA	Louisiana	Louisiana	Sugarcane	30.9842977	-91.9623327	15
Beaum_Su	USA	Texas	Beaumont	Sugarcane	30.080174	-94.1265562	13
Wesl_Su	USA	Texas	Weslaco	Sugarcane	26.1595194	-97.9908366	7

TBD = To be determined (information not available).

2

4.2.2 DNA Extraction

The DNA was extracted from the adult moth tissues following the CTAB protocol described by Doyle and Doyle (1990) with slight modifications. The integrity and quantification of DNA were evaluated in 0.8% agarose gels with 1X TAE buffer (Tris, acetic acid, EDTA, pH 8.0). The amount of DNA present in each sample was estimated by comparison with known concentrations of a DNA standard (λ phage). The gels were stained with an ethidium bromide bath (0.5 mg mL^{-1}), and DNA bands were visualized under UV light and the image was captured.

4.2.3 Genotyping-by-Sequencing

GBS libraries were produced as described by (POLAND et al. 2012). The restriction enzymes *Pst*I (New England Biolabs, Whitby, ON, Canada) and *Msp*I (New England Biolabs) were used to digest the DNA and reduce genome complexity. Barcoded *Pst*I adapters, and a common non-barcoded *Msp*I adapter, were ligated to the digested DNA and amplified by PCR to create a sequencing library. The resulting library was single-end sequenced to 100 bases on a single lane using the Illumina HiSeq 4000 sequencing kit v1 (Illumina, Inc., San Diego, CA, USA), and the fastq files were demultiplexed with the bcl2fastq v2.17.1.14 (Illumina) by the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana-Champaign producing nearly 404 million raw reads. The pipeline UNEAK, a multi-sample, SNP-calling approach developed for analyzing GBS data from species without reference genomes (LU et al. 2013), was used to analyze the first 64 bases (beginning with the *Pst*I restriction site) to call SNPs. TASSEL 5 (GLAUBITZ et al. 2014) was used to filter SNP data such that each marker must be present in at least 70% of the individuals within each population. With this filtering option, we identified 1,331 SNPs for the 250 individuals.

4.2.4 Population Genomics Analyses

File conversion to other population genetics programs were made using PGDSpider v. 2.1.0.3 (LISCHER AND EXCOFFIER, 2012). To identify candidate loci that may have been under selection during the range expansion of *D. saccharalis* in the Americas, the LOSITAN program (ANTAO et al., 2008), which employs the FDIST2 algorithm (BEAUMONT and NICHOLS, 1996), was used to detect SNPs that are F_{ST} outliers. This method evaluates the relationship between the expected distribution of F_{ST} and heterozygosity assuming an island model of

migration. In LOSITAN analysis, 100,000 simulations were run by using the stepwise mutation model with the option of neutral mean $F_{ST.}$ Markers that presented F_{ST} higher than the 95% confidence interval were considered candidates for divergent selection, and markers that presented F_{ST} lower than the 95% confidence interval were considered candidates for balancing selection. Genetic diversity and F statistics were estimated under a random model, in which the sampled populations were considered representative of the species and with a common evolutionary history. Allele frequencies, the number of alleles per locus (A), allelic richness (A_R) the observed heterozygosity (H_O) and expected (H_E) and the inbreeding coefficient (F_{IS}), assuming random model, were estimated using the diveRsity package (KEENAN et al., 2103). Cluster analysis were based on the construction of dendrograms using Nei's genetic distance (NEI, 1978) and the neighbor-joining method, in the poppr package (KAMVAR et al., 2014). The stability of the clusters was tested through 1,000 bootstrap resamples. We investigated the genetic structure employing the non-model based approach DAPC through the package adegenet (JOMBART, 2008). The hierarchical distribution of genomic diversity within and among groups of populations was investigated with Analyses of Molecular Variance (AMOVA) using the poppr package (KAMVAR et al., 2014).

4.3 Results and Discussion

4.3.1 GBS samples and sequencing

Sequencing data returned 403,918,781 sequence reads. From the initial 250 samples, we identified 1331 SNPs to use for the population analysis.

4.3.2 Detection of outlier SNPs

The LOSITAN analysis showed 125 loci possibly under positive selection, while 270 were putatively under balancing selection (Figure 4.1). This analysis has the purpose to detect genetic variation putatively driven by selective forces, because a shared demographic history should result in similar F_{ST}/H_E values for all loci but those that deviate may be candidates for selection (BEAUMONT, 2005). The genomic changes associated with the responses of organisms to their biotic and abiotic environment can increase the levels of divergence compared to a neutral model. The detection of loci putatively under selection may indicate that environmental factors are potentially responsible for adaptive divergence among *D. saccharalis* populations. However

further studies are needed to fully elucidate if the genomic regions where these outlier SNPs are located are truly influenced by selection, as well as what would be the major environmental forces responsible for such adaptive variation.

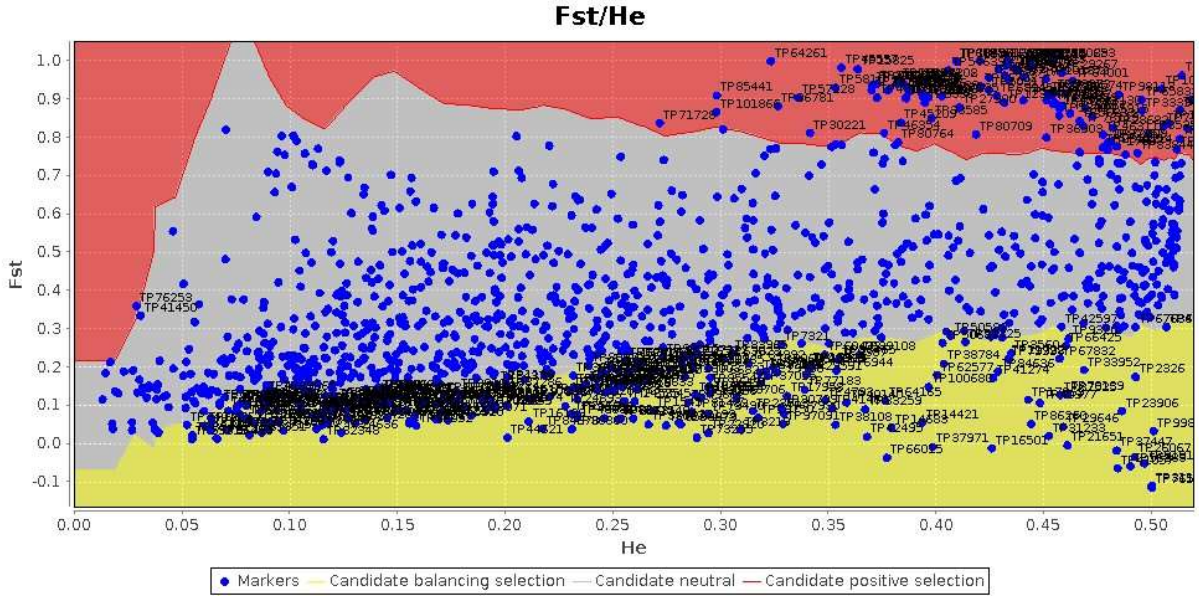

Figure 4.1– Loci distribution using the FST approach implemented in Lositan. Loci plotted in the red area are candidate for being under positive selection while loci plotted in the yellow area are candidate for being under balancing selection.

4.3.3 Genomic diversity

The genomic diversity estimates for each *D. saccharalis* population are summarized in Table 4.2. The number of alleles ranged from 1,277 in El Pais_Sugarcane to 2,333 in Jaboticabal_Sugarcane. The total of number alleles considering the group of populations by country was 27,511 for Brazil, 8,517 for Argentina, 5,938 for the United States of America and 2,715 for El Salvador. The mean allelic richness (A_R) was 1.12 and ranged from 0.89 (El Pais_Sugarcane) to 1.41 (Jaboticabal_Sugarcane). The average allelic richness of Argentina, Brazil, El Salvador and the United States of America was 1.19, 1.08, 1.01 and 0.95, respectively. The population from Jaboticabal_Sugarcane showed the highest value of allelic richness for all populations. This high allelic richness can be related with a greater adaptive potential to future environmental changes (FISHER, 1930; WAGNER, 2008; CABALLERO, 2013). The average values of observed and expected heterozygosities for the populations were 0.12 and 0.16, respectively. The Jaboticabal_Sugarcane population showed the highest observed and expected heterozygosities, 0.24 for both estimates. While the lowest observed and expected heterozygosities were 0.03 and 0.08, respectively, for the Belle Glade_Sugarcane population. The expected heterozygosity values were higher than the observed heterozygosity for all populations, with the exceptions of Jaboticabal_Sugarcane, Araras_Sugarcane and Piracicaba_Corn, which were in Hardy-Weinberg equilibrium. Particularly great differences between observed and expected heterozygosities were observed in the case of populations Quilmes_Corn and El Pais_Sugarcane. The mean inbreeding coefficient (F_{IS}) across populations was 0.27, ranging from -0.009 (Piracicaba_Corn) to 0.727 (El Pais_Sugarcane). It has been shown that a decrease in the observed heterozygosity can lead to a decrease in the average fitness of individuals (REED, 2003; SZULKIN, 2010). Biological aspects of *D. Saccharalis* may explain these results. This insect is a preferential colonizer of annual plants, and the colonization is made primarily by few genotypes, which increases the chances of inbreeding (De BARRO, 2005). Mating among relatives will thus result in the heterozygote deficit observed within *D. saccharalis* populations.

Table 4.2 - Genomic diversity estimates based on 1,331 SNP loci for each *D. saccharalis* population.

Population Code	Number of Individuals	A	A_R	H_O	H_E	f_{IS}
LaCocha_Co	10	1859	1.13	0.1	0.13	0.244
LaCruz_Su	5	1721	1.1	0.1	0.12	0.159
Jujuy_Su	7	1866	1.13	0.11	0.14	0.222
Perga_Co	2	1519	1.05	0.12	0.14	0.183
Qui_Co	4	1552	0.98	0.07	0.13	0.475
Araras_Su	2	1723	1.14	0.16	0.16	0.02
Goias_TBD	4	1856	1.1	0.12	0.17	0.324
Jabo_Su	52	2333	1.41	0.24	0.24	0.006
Morr_Co	4	1910	1.16	0.13	0.17	0.243
MS_TBD	9	2129	1.23	0.14	0.2	0.306
MT_TBD	5	1818	1.12	0.11	0.15	0.225
PAf_Su	3	1751	1.12	0.11	0.15	0.253
Parana_TBD	3	1847	1.16	0.14	0.16	0.175
Pira_Co	25	2118	1.32	0.19	0.19	-0.009
Pira_Su	22	2296	1.38	0.22	0.23	0.046
Rib_Su	4	1916	1.11	0.14	0.18	0.246
Rondo_Co	3	1634	1.03	0.11	0.16	0.347
SHG_Su	4	1916	1.15	0.15	0.19	0.236
SP_TBD	18	2264	1.21	0.13	0.23	0.423
ElNilo_Su	4	1438	1.01	0.05	0.07	0.326
ElPais_Su	2	1277	0.89	0.04	0.13	0.727
BGlade_Su	9	1384	0.95	0.03	0.08	0.629
Louis_Su	15	1526	1.03	0.06	0.09	0.33
Beaum_Su	13	1556	1.07	0.08	0.09	0.149
Wesl_Su	7	1472	1	0.06	0.09	0.361

Number of individuals, A - Number of alleles, A_R - Allelic Richness, H_O - Observed heterozygosity, H_E - Expected heterozygosity, f_{IS} - Inbreeding coefficient

4.3.4 Genetic Structure of *Diatraea saccharalis* populations

The values of pairwise F_{ST} obtained between populations were highly variable (Table 4.3). The most divergent populations were San Luis – Louisiana (F_{ST} = 0.444), followed by Louisiana – Buenos Aires (F_{ST} = 0.418) and San Luis - El Nilo (F_{ST} = 0.411). In contrast, São Paulo state – Paraná state (F_{ST} = 0.003) and São Paulo state – Minas Gerais state (F_{ST} = 0.005) were the less divergent comparisons. These lower divergences may be explained by the geographical proximity among the states of São Paulo, Paraná state and Minas Gerais. These results may also indicate that these populations share a most recent common ancestor than the others do. Overall, the South America populations were genetically divergent from the Central and North America populations, as can be visualized in the heatmap of pairwise F_{ST} values (Figure 4.2).

Table 3 - Pairwise values of FST *Diatraea saccharalis* populations.

	Buenos Aires	El Nilo	El Pais	Florida	Goiás	Jujuy	Louisiana	Minas Gerais	Mato Grosso do Sul	Mato Grosso	Paraná	San Luis	São Paulo	Texas	Tocantins	Tucuman
Buenos Aires																
El Nilo	0.381															
El Paisnal	0.216	0.014														
Florida	0.329	0.180	0.146													
Goiás	0.050	0.204	0.177	0.242												
Jujuy	0.043	0.280	0.241	0.270	0.075											
Louisiana	0.418	0.089	0.056	0.261	0.304	0.348										
Minas Gerais	0.049	0.337	0.173	0.339	0.015	0.100	0.406									
Mato Grosso do Sul	0.048	0.231	0.201	0.260	0.033	0.066	0.323	0.028								
Mato Grosso	0.054	0.240	0.205	0.256	0.036	0.064	0.327	0.059	0.041							
Paraná	0.068	0.296	0.217	0.328	0.019	0.094	0.392	0.031	0.034	0.048						
SanLuis	0.034	0.411	0.334	0.349	0.067	0.052	0.444	0.119	0.065	0.068	0.107					
São Paulo	0.026	0.134	0.124	0.160	0.014	0.037	0.210	0.005	0.018	0.021	0.003	0.033				
Texas	0.399	0.067	0.037	0.233	0.298	0.336	0.015	0.396	0.316	0.317	0.376	0.419	0.211			
Tocantins	0.060	0.297	0.234	0.310	0.025	0.075	0.384	0.038	0.030	0.029	0.039	0.101	0.012	0.367		
Tucuman	0.039	0.272	0.237	0.268	0.114	0.044	0.350	0.129	0.100	0.095	0.129	0.047	0.063	0.337	0.106	0.000

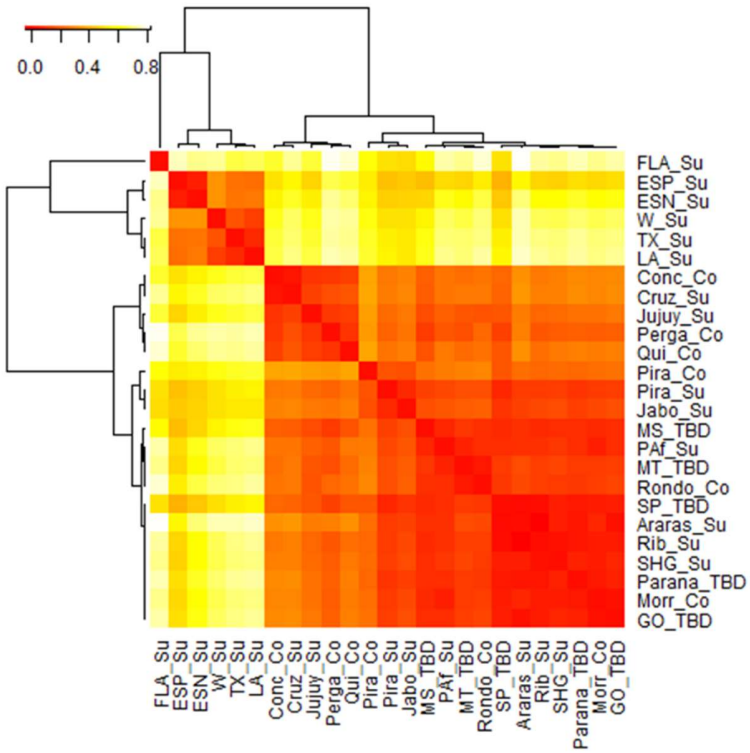

Figure 4.2 - Heatmap of pairwise F_{ST} values *Diatraea saccharalis* populations based on 1331 SNP loci. Dendrograms were plotted using unweighted-pair-group method with arithmetic mean (UPGMA). Degree of divergence is indicate by colors from red (low F_{ST} values, little genetic divergence) to light yellow (high F_{ST} values, strong genetic divergence).

The neighbor-joining dendrogram based on Nei's genetic distance (1978) showed three well-defined groups. The first group includes populations collected in Brazil, and it is closely related to the second group, which includes all populations from Argentina. The third group was comprised of populations from the USA and El Salvador (Figure 4.3). In general, we observed that the populations were clustered according to their geographic proximity. The group formed by Brazilian populations showed three large sub-clusters. That the first one included populations from the states of Goiás and Minas Gerais. The second sub-cluster was formed by populations from the states of São Paulo and Paraná, while the third group was formed by populations from the states of Mato Grosso do Sul, Mato Grosso and Tocantins. We also observed sub-clusters within the group of Argentine populations. The populations collected in La Cocha and La Cruz are both located in the Tucumán Province in northern Argentina, while the populations Pergamino and

Quilmes are both from Buenos Aires. In addition, within the third major group (populations from United States and El Salvador) we observed a clear separation between populations from Lousiana and Texas to populations collected in Florida. The population genetic structure reflects interactions among species with regard to their long-term evolutionary history, mutation and recombination, genetic drift, reproductive system, gene flow, and natural selection (SLAKIN, 1987; SCHAAL, 1998). The dendrogram corroborates pairwise F_{ST} estimates, and clearly indicates that populations from the United States are very divergent from the populations sampled in Argentina and Brazil.

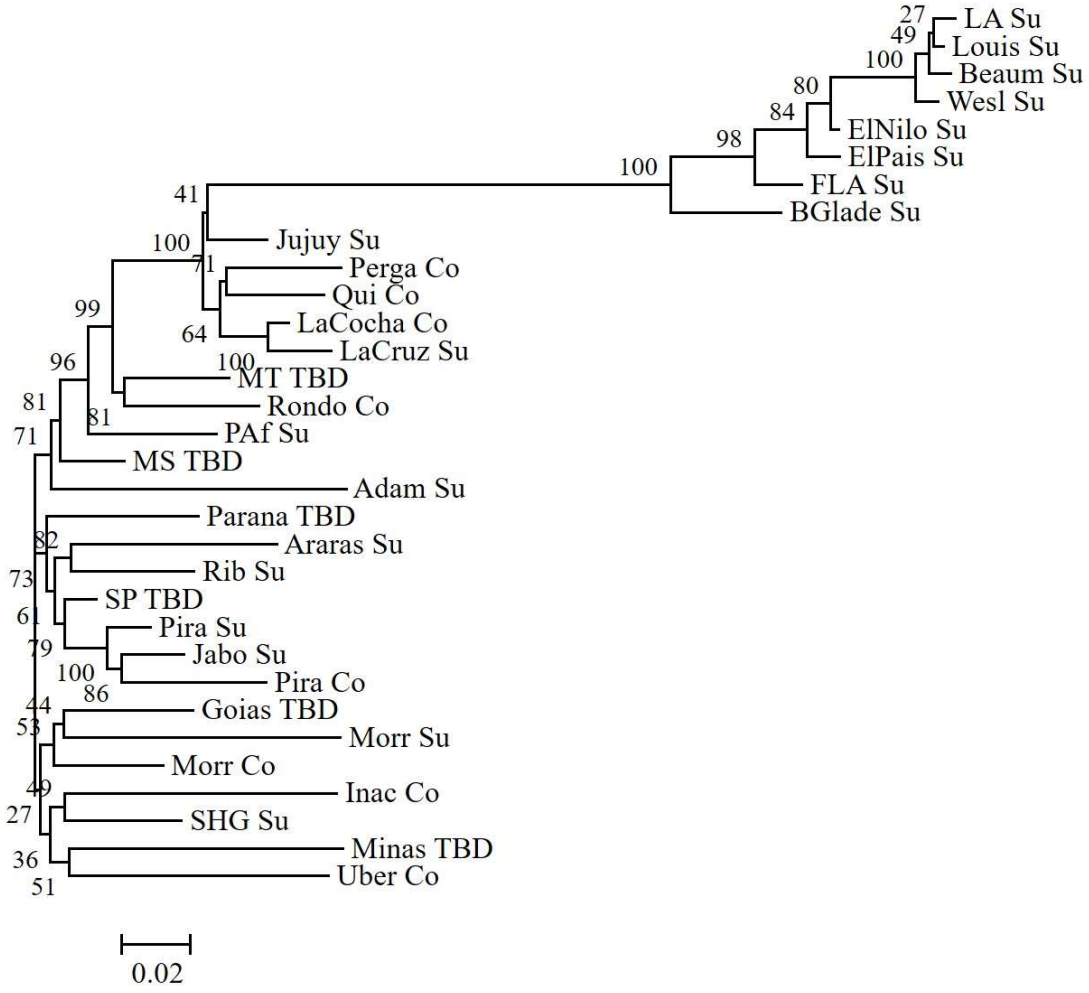

Figure 4.3 - Neighbor joining dendrogram showing the relationships among *Diatraea saccharalis* populations based on Nei's genetic distance (1978). Distances were estimated based on the variation of 1,331 SNP loci, and confidence of nodes were based on 1,000 bootstraps.

Discriminant analysis of principal components (DAPC) separated the *D. saccharalis* populations into four clusters (Figure 4.4). The results of DAPC are consistent with the Neighbor-joining cluster, and show a good concordance between genetic clusters and the countries of origin of each population. The individuals from Argentina and Brazil, showed a narrow genetic diversity. We can observe that the most individuals of these countries are closely to each other. The DAPC results also showed that the individuals from Florida were more distant in relation to the other samples collected in the United States (Louisiana and Texas states). This finding indicates that *D. sacharallis* populations are highly geographically structured in the United States, and that they might have been established from two different sources, which agrees with Joyce et al. (2014).

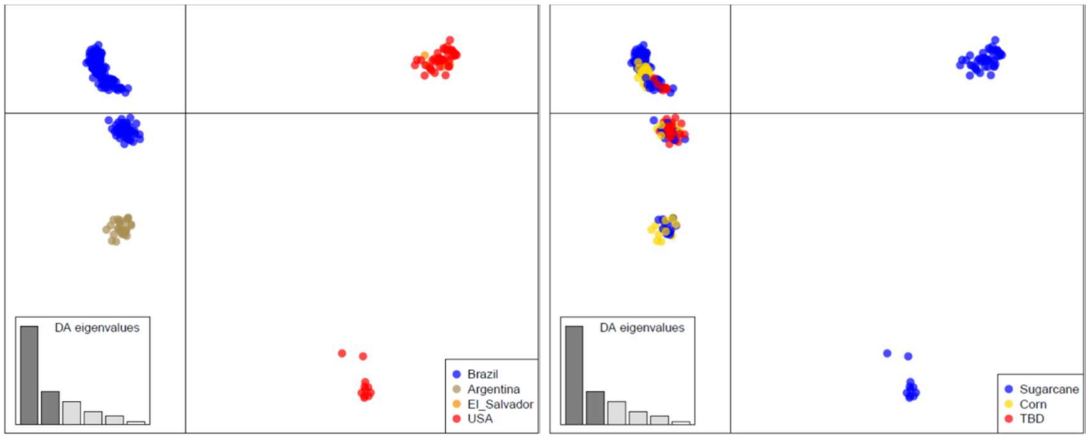

Figure 4.4 - Scatterplots from discriminant analysis of principal components (DAPC) based on 1,331 SNP loci showing the dispersion of 250 *Diatraea saccharalis* individuals across the first two principal components. Individuals (dots) are colored according to (A) their country of origin; and (B) the host crop in which they were collected. Variation represented in x = 44.4% and in y = 15%.

The higher level of divergence observed in *D. saccharalis* population collect in different countries in the American continent may result from evolutionary forces that act in several parts of the genome. Understanding the genomic alterations associated with the responses of the organisms to environmental changes and host adaptation can help clarify the process of colonization in a new habitat. The genetic structure observed among *D. saccharalis* populations appears to be primarily correlated with its geographical distribution across countries.

However, a very different picture emerges when evaluating the genetic structure of *D. saccharalis* among its main hosts, maize and sugarcane. We observed no genetic structure among

populations from these two crops (Figure 4.4). When different environments are geographically separated, the relative performance of populations on different hosts is not an issue, because populations can simply adapt independently to each environment when there is no migration (VIA AND LANDE, 1985). This might not be the case for *D. saccharalis* populations, since considerable overlap among populations from different hosts is observed in the DAPC (Figure 4.4).

The analyses of molecular variance (AMOVAs) revealed that most of the genetic variation is observed among populations of *D. saccharalis* from different countries (φst= 0.525, Table 4.4). In accordance with DAPC results, the AMOVA also revealed that a much smaller variation is observed among *D. saccharalis* populations from different hosts (φst= 0.122).

Table 4.4 - Analyses of Molecular Variance (AMOVAs) for different groups of *D. saccharalis* populations, based on the variation of 1,331 SNP loci and host-plant species and within geographic regions. φ statistics are indices of the amount of differentiation among populations, similar to Wright's F statistics. d.f. = degrees of freedom.

Source of Variation	d.f	sum of squares	mean squares	Sigma	% of variation	*P*
Among countries	3	39716.87	13238.96	329.12	52.59	φst= 0.525 p-value < 0.001
Within countries	192	56972.76	296.73	296.73	47.41	
Total	195	96689.62	495.84	625.86		
Between hosts	1	5052.01	5052.01	66.05	12.27	φst= 0.122 p-value < 0.001
Within hosts	194	91637.61	472.36	472.36	87.73	
Total	195	96689.63	495.84	538.41		
Among countries	3	39716.87	13238.96	290.58	46.51	φst= 0.549 p-value < 0.001

Between host within countries	2	3518.63	1759.32	52.79	8.45	φsc = 0.157 p-value < 0.001
Within hosts	190	53454.13	281.34	281.34	45.04	
Total	195	96689.62	495.84	624.71		

In sum, based on our results, we hypothesize that habitat heterogeneity and gene flow restrictions probably lead to genetic differentiation among populations of *D. saccharalis* during its early evolutionary history, which may have promoted adaptation to various environmental conditions. The populations develop into distinct genotypes due to differences in the demands driven by the environmental conditions. The *D. saccharalis* showed to be a successful invasive species that colonized several habitats with different environment conditions across the Americas. Multiple introductions of populations during a long period are important for the colonization success (ROMAN 2006; FACON et al. 2008), and possible multiple human-mediated passive transports could also enhanced demographic growth and increasing genetic diversity in previoulsy established isolated populations. Interestingly, *D. saccharalis* has a weak ability of dispersal, which was reflected in the high genetic differentiation among most of the populations included in our study. The lack of records of *D. saccharalis* dispersal in countries or neighboring areas close to where it currently occurs must be considered an uncertainty. For this, we analyzed all the information and results in the form of possibilities. In this historical analysis, it was not possible to compare the interval time of records between several introductions. Regardless of this lack of records, we propose two possibilities to explain the migration patterns of *D. saccharalis* in the Americas.

The probable center of origin of *D. saccharalis* is pointed to be the region including the delta of the Orinoco River, Venezuela, extending to the lower Amazon River (MYERS, 1932; 1935). Both basins (Orinoco and Amazon) cover an area of 8,380,000 square kilometers (Figure 5a). According to Myers (1932), the dispersion of *D. saccharalis* across this region was by bored aquatic grasses that float in the water. This pattern reflects the original range expansion out of Venezuela through Brazil with an initial expansion between the Orinoco and Amazon River basins.

In addition, our results suggest two complex demographic dispersion histories of *D. saccharalis* that may have been influenced by human-mediated movements of sugarcane and corn (Figure 4.5). The first hypothesis involves the Early occupation of men in America, followed by the establishment of several civilizations (DILLEHAY, 1999; FRASER, 2014). These new America residents originally rely in hunting and gathering, but began building a more complex social and cultural systems in the areas where agriculture was developed, between 4.100 and 4.300 years ago (BRONK, 2001; REIMER, 2004). Noteworthy, approximately 13.000 years ago, humans began to domesticate plants and animals (DIAMOND, 2002) and there is evidence of corn cultivation possibly as early as 9.000 years ago (MATSUOKA, 2002). These new cultivation systems associated with early American human societies may have been responsible for corn dispersal from Mesoamerica to North and South America and the Caribbean following different routes (BEDOYA, 2017). Possibly, *D. saccharalis* dispersion to areas beyond the center of origin accompanied corn dispersal in the pre-Columbian America.

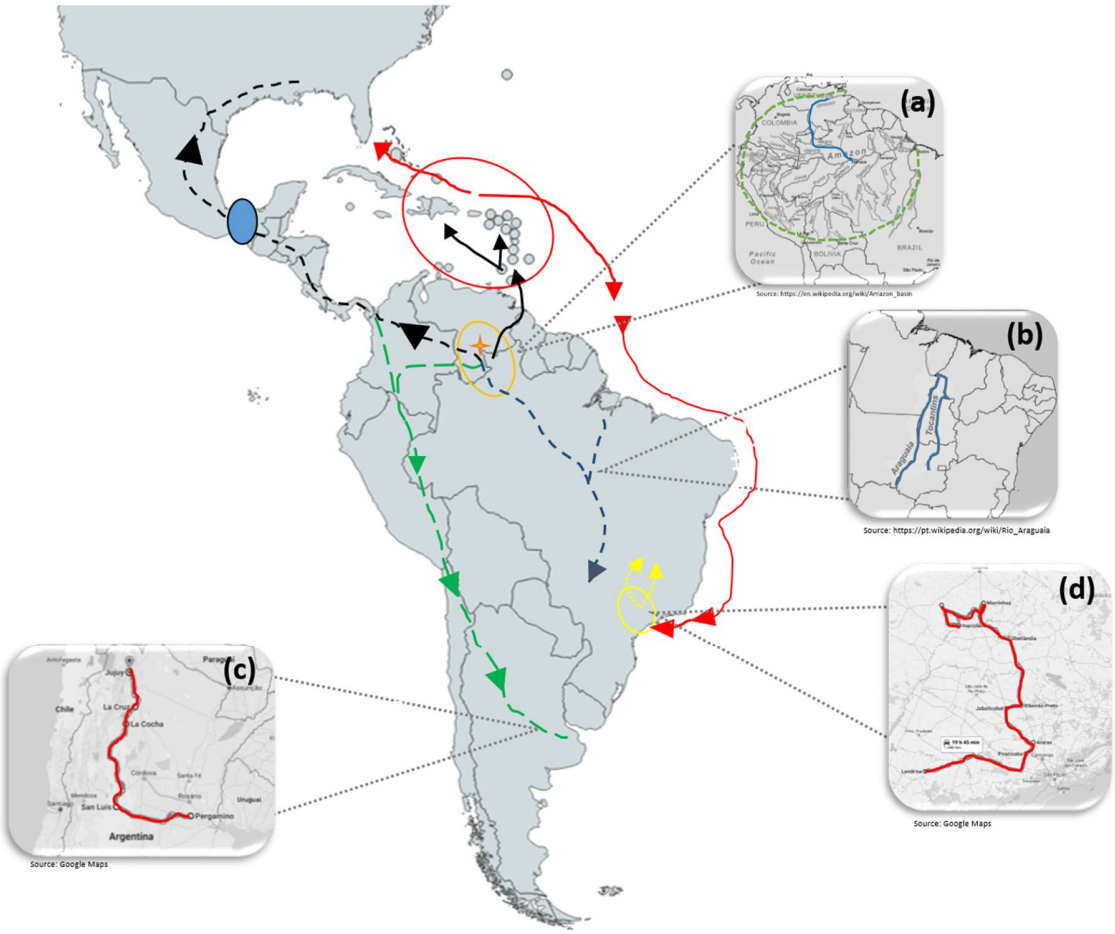

Figure 4.5 - Suggested *D. saccharalis* dispersion routes from its center of origin in the Orinoco Basin, Venezuela, based on historic information about corn dispersals and sugarcane introduction, and the genetic relationships observed in this study. Orange circle and (a) represent the *D. saccharalis* center of origin, in (a) The Orinoco River route in blue. Black arrows show the earliest *D. saccharalis* dispersion to Caribbean region by floating bored aquatic grasses. Blue dashed arrows and (b) show *D. saccharalis* dispersion from the Orinoco/Amazon Basin by Early Men migration route following the Tocantins and Araguaia rivers. Dashed green arrows and (c) represents the likely corn dispersal routes via the Andean region in Pre-Columbian period. Black dashed arrows indicate spread of *D. saccharalis* influenced by Human movement from Orinoco Basin to Central America and southwestern United State. Red arrows indicate dispersal of *D. saccharalis* influenced by the Columbian Exchange period from Caribbean region to Florida and to São Paulo State. Yellow circle, arrows and (d) shows the internal *D. saccharalis* dispersion route from São Paulo State to the Brazilian Cerrado. The blue circle represents the center of origin of corn in southern Mexico.

Archaeological evidence demonstrates that corn dispersals occurred through South America lowlands and the Andean region (FREITAS, 2013; BRACCO, 2016; BEDOYA, 2017). It is possible that the closer relationships among the populations from the states of Mato Grosso do Sul, Mato Grosso and Tocantins (Figure 4.3) may be associated with the Early American Men migrations to South America lowlands. This route of human migration group may have been dependent of transportation along rivers (RAMIREZ, 1961), probably following the Araraguaia River or/and the Tocantins River (Figure 4.5b). Both rivers are connected to the Amazon and Orinoco Basins. The closer relationships among *D. saccharalis* populations from Argentina may be associated with corn dispersals through the Andean region (Figure 4.5d). In North America (Figure 4.5), historical data traced corn dispersal from its center of origin in Mesoamerica towards Central America, northern Mexico and into the southwestern US, and finally into the northern US and Canada (BELLWOOD, 1997; VIGOUROUX, 2008). These routes of corn dispersal may have influenced the relationships among *Diatraea saccharalis* populations from the United States, since populations from Louisiana and Texas were in the same cluster and are closely related populations from Central America (El Salvador).

The second hypothesis we propose is that *D. saccharalis* dispersal across America is directly related with commercial movement of plants during the Columbian Exchange period in the 15th and 16th centuries. The Caribbean region was as crossing point, in the beginning of European colonization of the America, and served to exchange people and food between the Old World and the New World (GOODMAN, 1988). This vivacious and enthusiastic new market was the propitious environment to introduction of new disease and pests that affect the human native population and their native (corn) or introduced (sugarcane) crops. We can speculate that the exchange of damaged material from the center of origin in Venezuela to the Caribbean islands promoted the spread of *D. saccharalis* by sea routes, northwards to the United States of America, specifically Florida, as well as southwards, to Brazil, specifically the São Paulo state region. In both cases, it seems much probable that the sugarcane may have been infested from the corn.

While in Brazilian territory, we observed two groups, one group that is concentrated in São Paulo and Paraná states. Probably related with the first sugarcane plants that arrived in the captaincies of São Vicente, currently São Paulo state, from Caribbean routes. The second group, which contains populations from Goiás and Minas Gerais states, can be associated with dispersal

of bored material from São Paulo state (Figure 4.5d). Thus, it is possible that these populations share a common ancestor, given the low values of pairwise F_{ST} among them. The transportation of plants across states seems to be the most important pathway for *D. saccharalis* dispersion in Brazil countryside. Due to its high commercial value during the last decade, sugarcane had a considerable agricultural expansion in areas of the Brazilian Cerrado. This relatively recent introduction of *D. saccharalis* followed by its population expansion may be the cause of the low allelic richness observed in populations from the Central Brazil than that of populations collected in the states of São Paulo and Paraná.

4.4 Conclusions

Our study is the first one to apply the Genotyping-by-Sequence in *D. saccharalis* populations from the Americas. The results showed that the methodology allowed the detection and characterization of the genetic diversity among insect populations, showing effectiveness for the study of population structure. Recent advances in population genomics have made it possible to detect previously unidentified structure, obtain more accurate estimates of demographic parameters, and explore adaptive divergence, potentially revolutionizing the way genetic data are used to manage wild populations.

We hypothesize that human actions was the responsible to spread *Diatraea saccharalis* populations thought the America continent in several introductions. In the earliest *Diatraea saccharalis* dispersion, corn remains an important diffusion pathway. While sugarcane was a vehicle for dissemination in a second historical moment. The main hypothesis for the introduction of these insect from the Venezuela to others countries through contained corn and sugarcane bored plants.

5. DISCUSSION

In this study, we observed host-plant preference and genetic structure revealed by COI, Microsatellite loci and Genotyping-by-sequence. These molecular markers were polymorphic and highly informative, allowing the study of genetic variability of *D. saccharalis* populations. Our results suggested the presence of genetic groups correspondent with their geographic sampling locations. Moreover, that low interactions is relate to the fragmentation process between crop productions regions and consequently affect gene flow.

The actual sugarcane and corn production system is characterized by inconstant environments, which differ in soil conditions, climate, availability and susceptibility of the variety and management control system. We observed that these diverse cultivation ecosystems provide a habitat that influenced directlly the local adaptation and acceleration of the evolutionary process in *D. saccharalis*. It is clear that the current genetic divergence observed between *D. saccharalis* populations is related with inbreeding process result from these limited gene flow and natural barriers. The transfer of genes between these populations decreasingly in the past years, for this reason creating separated population. *D. saccharalis* is considered an invasive species that colonize a new environment and has been influenced by nature forces.The selection pressures imposed by local environmental changes promote rapid adaptive evolution in this specie that can be related with a populations adaptation over time.

Understanding the genetic background of *Diatraea* populations, it is important tool prior to a large investment in large-scale efforts aimed at controlling the borer. The inability to detect or improper detection of differences between populations can lead to drastic and costly consequences in pest management. The establishment of a functional program that will ensure the design and implementation of sustainable pest management strategies needs to take into account the genetic structure and local characteristics of *Diatraea* populations.

6. CONCLUSIONS

6.1 The results clearly demonstrate that all specimens of Diatraea spp. collected in corn and sugarcane crops in Brazilian territory that exhibiting characteristic damages are members of the species *Diatraea saccharalis* or *Diatraea impersonatella*.

6.2 Analysis of the cytochrome oxidase I gene of mitochondrial DNA is a precise and discriminant tool for species identification, especially in relation to the identification of Diatraea saccharalis and Diatraea impersonatella.

6.3 Through the estimates of genetic diversity, we can observe the presence of well-defined groups, indicating the existence of genetic structuring among the hosts. Suggesting that populations of Diatraea saccahralis collected in maize and sugarcane crops are not genetically related.

6.4 Parameters of genetic diversity and fixation index show the grouping in agreement with the collection sites, which indicates a geographical structuring due to fragmentation processes and low gene flow.

6.5 Diatraea saccharalis shows two possible dispersal routes in the American continent influenced by human migration actions in two different moments of history.

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8. ANEXOS

3. Capítulo 1 Morphological and Molecular Characterization of Brazilian Populations of *Diatraea saccharalis* (Lepidoptera: Crambidae) and the Evolutionary Relationship among Diatraea spp

S3.1 Table. Characteristics of the 11 microsatellite loci from *Diatraea saccharalis*.

Locus	GeneBank accession	Primer nucleotide sequence (5'-3')	Repeat motif	Ta (°C)	Size range (bp)
Dsc1	GF111048	F CGAGGCTATATTTGCGTGTG R GATGATGGAGTTGGAAGGTGA	(TG)10	56	180-192
Dsc2	GF111061	F GCGGTGCCTCTTTGTCATA R TTGACCAACTACTGCAAGACG	(CA)19	60	188-230
Dsc3	GF111049	F CCATCAAGCTCCTTCTAAGAGAC R CCTTGCTCAGTTACCATTCG	(AC)11	56	250-274
Dsc5	GF111050	F TCTTGCCTTTGCTCTTGAAA R GCGGGGTCAGCTAGTTATTC	(TG)19	60	146-190
Dsc7	GF111051	F TGTCGAGCTACTCCATGCTT R TGAGACTGAACACTGGCAAGA	(ATG)6	60	214-250
Dsc9	GF111052	F AACCTTCGATGAGCTACTGC R TGTGGTGATTTGTTTGCTTG	(TG)16	56	160-182
Dsc10	GF111060	F GGTCCGCGTTTGTTATTGTT R TCAAGTGCTCCTTAAAACACGA	(GT)7	56	270-280
Dsc11	GF111990	F ATACGGCTTCATTCGCTTC R GGTTTCGCACTCATCACG	(GT)10	54	220-228
Dsc13	GF111053	F CGTGGACTAACCCATAGAAGAT R GGTTTAGCAGAACTTGGCATA	(GT)18	54	220-270
Dsc19	GF111058	F CACACACGAACACACACGA R ATGGTTGGGTCTTTCCTTTT	(CA)10	60	160-170
Dsc20	GF111059	F TTGGCAGAGTTGTGGGTAAC R ACAGCAGCATCATCAGAAGG	(AG)8	54	222-230

F = forward primer sequences; R = reverse primer sequences; Ta = annealing temperature;

S3.2 Table. Hierarchical analysis of molecular variance (AMOVA) for population genetic structure of *Diatraea saccharalis* with a mithocondrial (COI) region marker.

Hierarchical levels	d.f.	Sum of Squares	Variance components	Variance (%)	Fixation Indices	P valor
Two-hierarchical-levels						
Among Populations	2	0.184	-0.00067	-0.63	-0.00625	0.45974
Within Populations	79	8.463	0.10712	100.63		
Total	81	8.646	0.10645			
Two-hierarchical-levels						
Among Host	1	0.076	-0.00079	-0.74	-0.00742	0.6998
Within Host	80	8.57	0.10712	100.74		
Total	81	8.646	0.10634			
Three-hierarchical-levels						
Among Populations	2	0.184	0.00271	2.54	F_{SC} = -0.046	1
Among host within populations	2	0.088	-0.00478	-4.48	F_{CT} = 0.02537	0.3993
Within Host	77	8.375	0.10877	101.94	F_{ST} = -0.0194	0.7271
Total	81	8.646	0.10669			

S3.3 Table. Estimates of pairwise Fst (lower diagonal) and p-values (upper diagonal) among populations of *Diatraea saccharalis* and *Diatraea impersonatella*, based on the variation of the mitochondrial COI gene.

	Jaboticabal_Sugarcane	Morrinhos_Corn	Morrinhos_Sugarcane	Piracicaba_Corn	Piracicaba_Sugarcane	Maceio_Sugarcane
Jaboticabal_Sugarcane		0.436	1.000	1.000	1.000	0.000
Morrinhos_Corn	**0.05546**		1.000	0.448	0.300	0.000
Morrinhos_Sugarcane	**-0.33172**	**-0.31765**		1.000	1.000	0.008
Piracicaba_Corn	**-0.01366**	**-0.00878**	**-0.31555**		1.000	0.000
Piracicaba_Sugarcane	**-0.02813**	**0.04**	**-0.29095**	**-0.02391**		0
Maceio_Sugarcane	**0.98657**	**0.97568**	**0.96682**	**0.9829**	**0.9857**	

S3.4 Table. Genetic variability and coefficient of inbreeding for microsatellite loci evaluated in *Diatraea saccharalis* populations.

Locus	**A**	**Ho ± SE**	**He ± SE**	**F_{IS} ± SE**
Dsc1	3	0.3125 ± 0.027	0.54775 ± 0.036	0.42275 ± 0.060
Dsc2	3	0.60025 ± 0.078	0.50575 ± 0.051	-0.20625 ± 0.161
Dsc3	6	0.3645 ± 0.104	0.59025 ± 0.077	0.3675 ± 0.184
Dsc5	4	0.3915 ± 0.101	0.632 ± 0.005	0.37825 ± 0.162
Dsc7	4	0.47925 ± 0.100	0.504 ± 0.026	0.01375 ± 0.245
Dsc9	6	0.28575 ± 0.121	0.51675 ± 0.109	0.472 ± 0.156
Dsc10	3	0.34975 ± 0.110	0.467 ± 0.095	0.34225 ± 0.152
Dsc11	7	0.27 ± 0.160	0.22675 ± 0.134	-0.1885 ± 0.013
Dsc13	6	0.602 ± 0.088	0.6325 ± 0.081	0.05225 ± 0.056
Dsc19	7	0.88525 ± 0.057	0.625 ± 0.026	-0.4135 ± 0.045
Dsc20	2	0.084 ± 0.068	0.12275 ± 0.071	0.3455 ± 0.309
Average	**4.64**	**0.42**	**0.49**	**0.14**

S3.5 Table. Nuclear microsatellite private alleles observed in four populations of *Diatraea saccharalis*.

Population	Locus	Allele	Frequency
Jaboticabal_Sugarcane	Dsc11	210	0.1363
	Dsc11	254	0.0227
	Dsc11	256	0.0454
Morrinhos_Corn	Dsc3	282	0.4375
	Dsc9	188	0.1428
	Dsc11	194	0.1250
	Dsc11	220	0.0625
	Dsc13	314	0.1875
	Dsc19	203	0.1250
Piracicaba_Sugarcane	Dsc2	228	0.2391
	Dsc19	169	0.0208
	Dsc19	175	0.0416

S3.6 Table. Estimates of pairwise Fst among the *Diatraea saccharalis* populations based on the variation of eleven microsatellite loci.

	Jaboticabal_Sugarcane	Morrinhos_Corn	Piracicaba_Sugarcane	Piracicaba_Corn
Jaboticabal_Sugarcane	0			
Morrinhos_Corn	0.1415	0		
Piracicaba_Sugarcane	0.1578	0.1046	0	
Piracicaba_Corn	0.1812	0.0835	0.1533	0

Declaração

As cópias de artigos de minha autoria ou de minha co-autoria, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, que constam da minha Dissertação/Tese de Mestrado/Doutorado, intitulada **Morphological and molecular characterization of Diatraea species (Fabricius, 1794) (Lepidoptera: Crambidae) and the elucidation of dispersal pattern in America continent**, não infringem os dispositivos da Lei n.° 9.610/98, nem o direito autoral de qualquer editora.

Campinas, 16 de outubro de 2017

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DECLARAÇÃO

Em observância ao **§5º do Artigo 1º da Informação CCPG-UNICAMP/001/15**, referente a Bioética e Biossegurança, declaro que o conteúdo de minha Tese de Doutorado, intitulada "***Morphological and molecular characterization of Diatraea species (Fabricius, 1794) (Lepidoptera: Crambidae) and the elucidation of dispersal pattern in America continent***", desenvolvida no Programa de Pós-Graduação em Genética e Biologia Molecular do Instituto de Biologia da Unicamp, não versa sobre pesquisa envolvendo seres humanos, animais ou temas afetos a Biossegurança.

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