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Geographic variation of sex pheromone and mitochondrial DNA in *Diatraea saccharalis* (Fab., 1794) (Lepidoptera: Crambidae)

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ABSTRACT

(9*Z*,11*E*)-hexadecadienal and (*Z*11)-hexadecenal, the main sex pheromone components of the sugarcane borer, *Diatraea saccharalis*, were identified and quantified from four Brazilian and one Colombian populations using GC-EAD, GC–MS and GC analyses. Three different ratios were observed, 9:1, 6:1, and 3:1. The pheromone concentration for the major component, (9*Z*,11*E*)-hexadecadienal, varied from 6.8 ng/gland to 21.9 ng/gland and from 1.7 ng/gland to 6.5 to the minor component, (*Z*11)-hexadecenal. The 25 *D. saccharalis* cytochrome oxidase II sequences that were analyzed showed low intra-specific variation and represented only 11 haplotypes, with the most frequent being the one represented by specimens from São Paulo, Paraná, and Pernambuco states. Specimens from Colombia showed the highest genetic divergence from the others haplotypes studied. Data on the genetic variability among specimens, more than their geographic proximity, were in agreement with data obtained from analyses of the pheromone extracts. Our data demonstrate a variation in pheromone composition and a covariation in haplotypes of the *D. saccharalis* populations studied.

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1. Introduction

Pheromone composition in moths can vary drastically, especially in those species with wide distribution ranges (Cardé and Haynes, 2004). This geographic dissimilarity may have a genetic basis due to founder effect, genetic drift, or even natural or sexual selection (Templeton, 1980; Rice, 1987). However, the evolutionary basis for such divergence is not clear.

There are reported examples of pheromone blend differences among and within populations of a single species. For instance, there are studies describing some differences in species from the family Crambidae (Sorenson et al., 1992; Huang et al., 1998; Kawazu et al., 2000; Kawazu et al., 2009), which encompass the *Diatraea*. The chemical composition of the pheromone blend, the ratio between the components, and the rate of pheromone emission are crucial to the male response.

Diatraea saccharalis occurs in several countries in the Americas, and is a pest of several grasses of economical importance, including sugarcane, *Saccharum officinarum* L. (Long and Hensley, 1972; Cueva et al., 1980). The simultaneous presence of all developmental stages throughout the year, as well as, the location of larval and pupal stages inside the plant make control of the sugarcane borer difficult (William et al., 1969; Gomez and Lastra, 1995). Two pheromone components for *D. saccharalis*, (9*Z*, 11*E*)-hexadecadienal (Svatoš et al., 2001) and (*Z*11)-hexadecenal (Kalinová et al., 2005), have been reported. Both components showed antennal activity by GC-EAD and elicited positive responses in wind tunnel bioassays at ratios of 10:1 respectively (Kalinová et al., 2005). However, these components exhibited low attractiveness in field experiments when compared with traps containing virgin females (Patrick and Hensley, 1970; Hammond and Hensley, 1971; Almeida and Arrigoni, 1989), suggesting that additional unidentified pheromone components are involved in male attraction. Despite the incomplete identification of the sex pheromone of *D. saccharalis*, it is still important to determine the appropriate ratio of two known pheromone components in order to maximize their attractiveness since deviations from the optimal blend can decrease male attraction.

Demonstration of geographic variation in pheromone composition may be helpful to develop efficient trapping schemes for accurately monitoring this economically important insect, as well as to shed light on whether this variation is an important contributor to population divergence and potential speciation. This information can also be complemented by determining the phylogenetic history of the populations showing pheromone composition variation. In Lepidoptera, DNA sequences of the

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protein cytochrome oxidase II have been used successfully for determining phylogenetic relationships of populations (Sperling and Hickey, 1994), possibly being useful for the phylogenetic reconstruction of populations of the sugarcane borer.

Therefore, our aim was to study the variation of the ratio of the main sex pheromone components of different populations of *D. saccharalis*, as well as to analyze the divergence of its mitochondrial cytochrome oxidase II (COII) sequences.

2. Material and methods

2.1. Sample collection for molecular analyses

Adult male and female specimens of *D. saccharalis* were collected in Carpina ($n = 3$), Pernambuco State (PE); Paranavaí ($n = 2$), Paraná State (PR); Piracicaba ($n = 3$), São Paulo State (SP); São José de Rio Claro ($n = 2$), Mato Grosso State (MT); and Santo Antônio de Goiás ($n = 3$), Goiás State (GO) in Brazil; Artigas Department ($n = 3$) in Uruguay; and Valle de Cauca Department ($n = 3$) in Colombia. Specimens from Goiás State were collected on rice (*Oryza sativa* L.), while all others were collected on sugarcane (*Saccharum* sp.). Insects were kept in 100% ethanol at −20 °C until analysis.

2.2. DNA extraction

Total genomic DNA was extracted from thoracic muscle using the salt extraction protocol (Sunnucks and Hales, 1996) with proteinase-K digestion, and voucher specimens with genitalia were deposited in the Museu de Entomologia, Universidade de São Paulo (Piracicaba). The polymerase chain reaction (PCR) to amplify the COII DNA fragment was conducted using1 μl of DNA, 5 μl 5× PCR Buffer (10 mM Tris–HCl, 100 mM KCl, 15 mM $MgCl_2$, pH 8.0), 200 μM of each dNTP (Fermentas), 320 pM of each primer (A-298: 5′-ATRGGACATCAATGATATTGA-3′; 1-tLYS: 5′-GTAAGAGACCAG-TACTRG-3′), 0.125 μl Taq polymerase (5 U μl^{-1}) (Fermentas), plus sterile Milli-Q water bringing the total reaction volume to 25 μl. Thermal cycling was performed in an iCycler (Bio Rad) using the cycling conditions of initial denaturation at 94 °C for 1 min, then 35 cycles at 94 °C for 30 s; 50 °C for 60 s, 72 °C for 60 s, with a final extension at 72 °C for 1 min (Lange et al., 2004). The PCR products generated were resolved in 1.5% agarose gel electrophoresis in Tris–acetate/EDTA buffer (TAE), stained with 0.5 μl/ml of ethidium bromide, and visualized under a UV image capture and analysis system (Bio-Imaging Systems). PCR products were purified following precipitation with 80% ethanol (Davis et al., 1994) before sequencing.

2.3. Cloning and sequencing

The amplified PCR products were ligated into the pGEM-T Easy vector (Promega) according to the manufacturer's protocol. The ligation mixture was introduced into NEB5α competent *Escherichia coli* cells by electroporation. The transformed cells were spread on an LB agar plate containing ampicillin (50 μg/mL), 5-bromo-4-choloro-3-indolyl-β-D-galactoside (X-Gal) and isopropyl thiogalactoside (IPTG) under sterile conditions. Positive and negative controls were processed simultaneously. Screening of the colonies was performed by choosing white colonies in LB broth containing ampicillin (50 μg/mL). The plasmid DNA from the selected *E. coli* cells was extracted by the mini-prep plasmid isolation method (Sambrook and Russel, 2001). Insert size was checked by PCR amplification using the primers of the cloning kit, followed by agarose gel electrophoresis. Clones were sequenced bidirectionally by the Centro de Estudos do Genoma Humano (CEGH-USP) (http://genoma.ib.usp.br/).

2.4. Sequence alignment

Sequences were aligned using the computer program CLUSTAL X (Thompson et al., 1997), and edited by visual inspection with the help of the program BioEdit v.7.0.9.0 (Hall, 1999). To better understand the intra- and inter-specific variation of COII, published sequences by Lange et al. (2004) from *D. saccharalis* specimens originating from unknown localities in Brazil (GenBank accession no. AY320496); Jamaica (GenBank accession no. AY320498); Mexico (GenBank accession no. AY320499); Texas State, USA (GenBank accession no. AY320500); Florida State, USA (GenBank accession no. AY320497); and Venezuela (GenBank accession no. AY320501) were included in our alignment. COII sequences were also included for root the tree from the following related species: *Diatraea busckella* Dyar & Heinrich (GenBank accession no. AY320490), *Diatraea centrella* Möschler (GenBank accession no. AY320492), *Diatraea grandiosella* Dyar (GenBank accession no. AY320949), *Diatraea rosa* Heinrich (GenBank accession no. AY320495.1), and *Chilo sacchariphagus* Bojer (GenBank accession no. AY320483). To infer protein codon positions of the alignment, DNA sequences were translated into protein sequences minimizing stop codons using MacClade v.4.08 (Maddison and Maddison, 2005), according to the *Drosophila* mitochondrial DNA code. The alignment for phylogenetic analyses excluded the last two nucleotides, immediately after the final stop codon.

2.5. Haplotype network and phylogenetic analyses

A haplotype network was constructed using the method of Templeton et al. (1992) with the aid of TCS v.1.18 (Clement et al., 2000) treating gaps as missing data, and 95% connection limit.

Phylogenetic analyses of unique haplotypes were conducted in order to understand their evolutionary history. Most parsimonious trees were found with a heuristic algorithm with 10,000 random addition sequences (RAS) and tree-bisection and reconnection (TBR) branch swapping using PAUP* 4.0 (Swofford, 2000), and treating gaps as missing data. A Bayesian consensus (post-10%-burnin) topology was calculated based on the results of four independent runs of four Markov chains for five million generations sampling every thousandth topology, and assuming flat priors using MrBayes v.3.1 (Ronquist and Huelsenbeck, 2003). The sequences were partitioned according to their codon position and each partition was analyzed independently with a different model of evolution. Models of molecular evolution were selected based on the scores of optimized maximum likelihood topologies calculated by PhyML v.3.0 (Guindon and Gascuel, 2003), using the Bayesian information criterion (BIC: Schwarz, 1978) with jModeltest v.0.1.1 (Posada, 2008). Models chosen for each codon position of COII amongst the 24 tested were HKY + I, F81, and HKY + G.

Clade support was given based on posterior Bayesian probabilities (Alfaro et al., 2003), and non-parametric bootstrap percentages based on parsimony heuristic searches (100 RAS with TBR) of topologies generated from 10,000 matrix pseudoreplicates (Felsenstein, 1981).

2.6. Insect rearing condition and pheromone extraction

Colonies of *D. saccharalis* from São Paulo and Paraná states were established in the Semiochemicals Laboratory at the Universidade Federal do Paraná under a L12:D12 photoperiod at 24 ± 2 °C and 70% r.h. conditions. Adults were kept in PVC tubes (10 cm × 20 cm) covered with a mesh on the upper part, lined inside with sulfite paper to allow oviposition. A 10% sucrose solution was provided daily. Egg masses were kept until larval eclosion in 10-cm diameter Petri dishes with cotton cloth soaked in water inside to maintain humidity. Larvae

were transferred to glass tubes (2.5 cm × 8 cm), and fed an artificial diet based on soybean, sucrose and wheat germ (Parra and Mishfeldt, 1992).

Biologically active compounds were obtained from pheromone glands of 2–4-day-old virgin females, between the 5th and 7th hour of the scotophase. Glands were excised with forceps, transferred to conical vials for extraction at room temperature (10 μl hexane/gland/20 min) (Berger, 1972), transferred to glass tubes, and stored at -20 °C until analyses.

Pheromone gland extracts from Mato Grosso and Pernambuco states were obtained from 2- to 4-day-old females from pupae provided by the Universidade Federal do Mato Grosso and Universidade Federal Rural de Pernambuco rearing laboratories, respectively. Larvae were fed with the same artificial diet used for populations from São Paulo and Paraná. Pheromone extracts of specimens from Colombia were obtained from females provided by the Centro de Investigación de la Caña de azúcar de Colombia (Cenicaña), reared on an artificial diet based on wheat germ and carrot (Lastra and Gómez, 2006). Gland extracts were prepared from 33 females from São Paulo, 35 from Paraná, 28 from Pernambuco, 37 from Mato Grosso, and 28 from Colombia.

2.7. Sex pheromone instrumental analyses

2.7.1. Gas chromatography with electroantennographic detection (GC-EAD)

Pheromone extracts of each population were analyzed by GC-EAD using a Shimadzu GC-2010 GC and a Syntech electroantennography system (Hilversum, The Netherlands). The GC was equipped with a DB-5 column (30 m × 0.25 mm i.d. and 0.25 μm film thickness; J&W Scientific Inc., USA). One μl of extract was injected using the splitless mode with the injector temperature at 250 °C. The column oven temperature was maintained at 100 °C for 1 min, then raised to 270 °C at a rate of 10 °C min^{-1}, and held at this temperature for 7 min. Helium was used as carrier gas at a column head pressure of 170 kPa. The column effluent was split 1:1, with one part going to the flame ionization detector (FID) with temperature of 270 °C, and the other through a heated transfer line into a humidified airstream (300 mL min^{-1}) directed at an antennal preparation of a male. The antennae were fixed between two stainless-steel electrodes using electrically conductive gel (Signa gel, Parker Labs., NJ). Ten antennae for each Brazilian population were tested. GC-EAD recordings were analyzed with Syntech GC-EAD32 software (version 4.6).

2.7.2. Gas chromatography–mass spectrometry (GC–MS)

Extracts of each population were analyzed by using a GC coupled with a Shimadzu QP-5050A MS equipped with a DB-5 capillary column as for GC-EAD (30 m x 0.25 mm id and 0.25 μm film thickness; J&W Scientific Inc., USA) in the electron impact mode at 70 eV. The injection was made in the splitless mode for 1 min at 250 °C. The column oven temperature program was: initial temperature at 50 °C (1 min); increasing 7 °C min^{-1} up to 250 °C and held for 10 min. The transfer line operated at 270 °C. Helium was used as the carrier gas. The pheromone components that repeatedly elicited antennal responses were identified by comparing retention times with synthetic references, and with mass spectra from libraries (NIST27 and NIST147), and verified with authentic standards.

2.7.3. Gas chromatography (GC)

GC analyses were conducted (same conditions as for GC-EAD) in order to quantify the identified compounds. The concentration and the blend ratio of pheromone components were determined based on the area of GC peaks, and analytical curves were calculated based on authentic standards. A series of saturated C10-C26 hydrocarbons was co-injected with analyzed samples to calculate the Kovats Index (KI) of electrophysiologically active compounds. These retention characteristics were subsequently used to compare the identity and antennal activity of authentic compounds with synthetic or purified standards. The concentration values obtained for the two pheromone components were based on analyses of three batches of extracts with approximately 30 pheromone glands dissected from calling females for each population. These results were submitted to one-way analysis of variance (ANOVA) and compared using Tukey's test ($p < 0.05$) using BioEstat 3.0 (Ayres et al., 2003).

2.7.4. Chemical standards

(9*Z*,11*E*)-hexadecadienal, (*Z*11)-hexadecenal, decane, dodecane, tetradecane, hexadecane, octadecane, nonadecane, eicosane, docosane, tetracosane, hexacosane were purchased from Aldrich Chemical Company (Milwaukee, Wisconsin, USA).

3. Results

Genetic divergences between COII sequences of *D. saccharalis* and related *Diatraea* species studied (Table 1) averaged 0.042 (dp = 0.047), while intra-specific divergence varied from 0.000 to 0.035 (average = 0.012; dp = 0.011). Out of the 25 sequences of *D. saccharalis* studied, only 11 were unique (=haplotypes). Within the seven geographical populations sequenced herein, only specimens from Goiás showed some genetic variation in their COII sequences (average = 0.004; dp = 0.002). All specimens from Brazilian states of Pernambuco, Paraná, and São Paulo were represented by a single identical haplotype, the same previously sequenced from an unknown locality in Brazil (Lange et al., 2004). This most frequent haplotype (Fig. 1) differed by one mutation from the haplotypes from Mexico, Uruguay, and a unique haplotype that includes two sequences from Mato Grosso and one from Goiás; and by two mutations from the Venezuelan haplotype. The haplotype shared by all Colombian specimens was the most divergent of all *D. saccharalis* specimens studied (average = 0.027; dp = 0.006), and differed from the most frequent one by 13 mutations, and from the haplotypes from Jamaica and Texas by 10 and 8 mutations, respectively (Fig. 1). From the 11 different DNA sequence haplotypes, only four showed variation in their amino acid sequence composition when compared to the most common haplotype: the Colombian population expressed valine instead of isoleucine at position 26, and methionine instead of valine at position 111; the Venezuelan population had tryptophan rather than serine at position 58 and phenylalanine instead of leucine at 87; one sample from Goias (GO1) had proline instead of serine at position 21; and GO2 switched from phenylalanine to leucine at position 85.

Parsimony and mixed-model Bayesian analysis of COII sequences supported the shared origin of *D. saccharalis* haplotypes and its sister relationship to the clade *D. busckella* + *D. rosa* (Fig. 2). However, the relationships amongst *D. saccharalis* haplotypes were poorly resolved, and good support in the Bayesian analysis was only found for the relationship of the Venezuelan haplotype as sister to the remaining haplotypes, a shared origin of the three Goiás haplotypes, and a clade containing the Colombian haplotype associated to the U.S. and Jamaican haplotypes (Fig. 2). On the other hand, these well-supported clades were not recovered in the parsimony analysis, which resulted in 26 most parsimonious trees (*L* = 138, CI = 0.75, RI = 0.75), all positioning the Colombian haplotype as sister to the remaining *D. saccharalis*.

Two EAD-active components were found in the sex pheromone gland extracts of the *D. saccharalis* females from São Paulo, Paraná, Pernambuco, and Mato Grosso (Fig. 3), and identified as the two

Table 1
Uncorrected distance matrix of *Diatraea saccharalis* specimens and related species. Specimens 12 through 30 sequenced in this study collected in CO: Colombia; GO: Goiás, Brazil; MT: Mato Grosso, Brazil; PE: Pernambuco, Brazil; PR: Paraná, Brazil; SP: São Paulo, Brazil; and UR: Uruguay.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	*C. sacchariphagus*	–														
2	*D. grandiosella*	0.107	–													
3	*D. centrella*	0.107	0.101	–												
4	*D. busckella*	0.129	0.082	0.096	–											
5	*D. rosa*	0.129	0.093	0.101	0.036	–										
6	Brazil	0.141	0.118	0.099	0.102	0.096	–									
7	Jamaica	0.146	0.112	0.107	0.110	0.101	0.024	–								
8	Mexico	0.138	0.115	0.096	0.099	0.093	0.003	0.027	–							
9	Florida	0.141	0.112	0.096	0.102	0.096	0.011	0.014	0.014	–						
10	Texas	0.146	0.112	0.101	0.104	0.093	0.019	0.011	0.022	0.008	–					
11	Venezuela	0.138	0.112	0.099	0.096	0.091	0.005	0.030	0.008	0.016	0.024	–				
12	CO1	0.135	0.112	0.098	0.096	0.087	0.030	0.027	0.032	0.024	0.022	0.035	–			
13	CO2	0.135	0.112	0.098	0.096	0.087	0.030	0.027	0.032	0.024	0.022	0.035	0.000	–		
14	CO3	0.135	0.112	0.098	0.096	0.087	0.030	0.027	0.032	0.024	0.022	0.035	0.000	0.000	–	
15	GO1	0.146	0.120	0.103	0.104	0.095	0.005	0.024	0.008	0.016	0.019	0.011	0.029	0.029	0.029	–
16	GO2	0.146	0.120	0.103	0.104	0.095	0.005	0.024	0.008	0.016	0.019	0.011	0.029	0.029	0.029	0.005
17	GO3	0.143	0.117	0.101	0.101	0.093	0.003	0.022	0.005	0.013	0.016	0.008	0.026	0.026	0.026	0.002
18	MT2	0.143	0.117	0.101	0.101	0.093	0.003	0.022	0.005	0.013	0.016	0.008	0.026	0.026	0.026	0.002
19	MT3	0.143	0.117	0.101	0.101	0.093	0.003	0.022	0.005	0.013	0.016	0.008	0.026	0.026	0.026	0.002
20	PE1	0.140	0.117	0.098	0.101	0.095	0.000	0.025	0.003	0.011	0.019	0.006	0.029	0.029	0.029	0.005
21	PE2	0.140	0.117	0.098	0.101	0.095	0.000	0.025	0.003	0.011	0.019	0.006	0.029	0.029	0.029	0.005
22	PE7	0.140	0.117	0.098	0.101	0.095	0.000	0.025	0.003	0.011	0.019	0.006	0.029	0.029	0.029	0.005
23	PR1	0.140	0.117	0.098	0.101	0.095	0.000	0.025	0.003	0.011	0.019	0.006	0.029	0.029	0.029	0.005
24	PR2	0.140	0.117	0.098	0.101	0.095	0.000	0.025	0.003	0.011	0.019	0.006	0.029	0.029	0.029	0.005
25	SP1	0.140	0.117	0.098	0.101	0.095	0.000	0.025	0.003	0.011	0.019	0.006	0.029	0.029	0.029	0.005
26	SP2	0.140	0.117	0.098	0.101	0.095	0.000	0.025	0.003	0.011	0.019	0.006	0.029	0.029	0.029	0.005
27	SP3	0.140	0.117	0.098	0.101	0.095	0.000	0.025	0.003	0.011	0.019	0.006	0.029	0.029	0.029	0.005
28	UR1	0.143	0.115	0.101	0.098	0.093	0.003	0.027	0.005	0.013	0.022	0.008	0.031	0.031	0.031	0.007
29	UR2	0.143	0.115	0.101	0.098	0.093	0.003	0.027	0.005	0.013	0.022	0.008	0.031	0.031	0.031	0.007
30	UR3	0.143	0.114	0.101	0.098	0.092	0.003	0.027	0.005	0.013	0.022	0.008	0.032	0.032	0.032	0.007

		16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	*C. sacchariphagus*															
2	*D. grandiosella*															
3	*D. centrella*															
4	*D. busckella*															
5	*D. rosa*															
6	Brazil															
7	Jamaica															
8	Mexico															
9	Florida															
10	Texas															
11	Venezuela															
12	CO1															
13	CO2															
14	CO3															
15	GO1															
16	GO2	–														
17	GO3	0.002	–													
18	MT2	0.002	0.000	–												
19	MT3	0.002	0.000	0.000	–											
20	PE1	0.005	0.002	0.002	0.002	–										
21	PE2	0.005	0.002	0.002	0.002	0.000	–									
22	PE7	0.005	0.002	0.002	0.002	0.000	0.000	–								
23	PR1	0.005	0.002	0.002	0.002	0.000	0.000	0.000	–							
24	PR2	0.005	0.002	0.002	0.002	0.000	0.000	0.000	0.000	–						
25	SP1	0.005	0.002	0.002	0.002	0.000	0.000	0.000	0.000	0.000	–					
26	SP2	0.005	0.002	0.002	0.002	0.000	0.000	0.000	0.000	0.000	0.000	–				
27	SP3	0.005	0.002	0.002	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	–			
28	UR1	0.007	0.005	0.005	0.005	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	–		
29	UR2	0.007	0.005	0.005	0.005	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.000	–	
30	UR3	0.007	0.005	0.005	0.005	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.000	0.000	–

main sex pheromone components, (9*Z*,11*E*)-hexadecadienal and (*Z*11)-hexadecenal. Besides the main components, additional ones eliciting antennal responses were also observed in some extracts as minor constituents.

(9*Z*,11*E*)-hexadecadienal and the (*Z*11)-hexadecenal were quantified after extraction from virgin female insect glands (Fig. 4). The concentration of the major component, (9*Z*,11*E*)-hexadecadienal, varied from 6.8 ng/gland for the Colombian population to 21.9 ng/gland for insects from Pernambuco. The major component concentrations for insects from São Paulo, Paraná, and Pernambuco were similar (Fig. 4). Also, the concentrations of the major component obtained from Mato Grosso, São Paulo, and Paraná populations were similar, while the Colombian population represented a third group, which is statistically different from all others (Fig. 4). A different pattern was observed when the minor component, (*Z*11)-hexadecenal, was evaluated. The minor component concentrations ranged from 1.7 ng/gland for the Colombian to 6.5 ng/gland for the Mato Grosso populations.

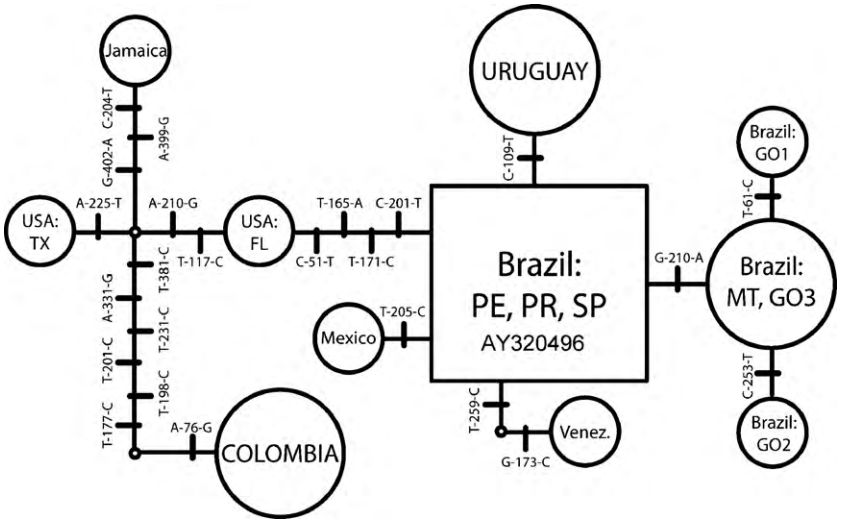

Fig. 1. Haplotype network based on 25 sequences of cytochrome oxidase II of *D. saccharalis* specimens from Colombia: Valle del Cauca; Uruguay: Artigas; and Brazil: Goiás (GO); Mato Grosso (MT); Paraná (PR); Pernambuco (PE); and São Paulo (SP). Additional sequences from Lange et al. (2004) included from Brazil (same as most common haplotype); Jamaica; Mexico; USA: Florida and Texas; and Venezuela. Most common haplotype represented as large square with $n = 9$, larger circle with $n = 3$, and small circle $n = 1$.

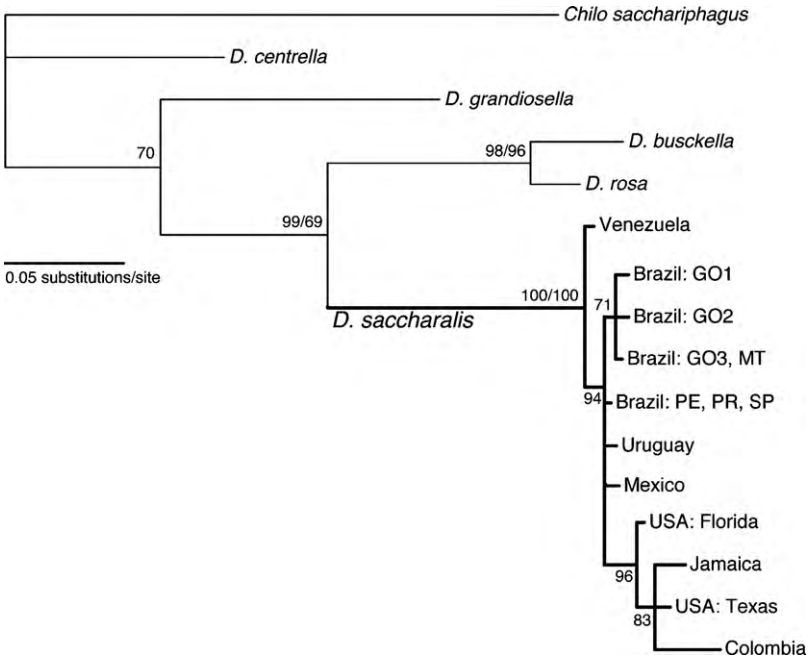

Fig. 2. Mixed-model Bayesian consensus phylogram resulting from the analysis of COII haplotypes of *D. saccharalis*. Sequences were partitioned according to codon position and model parameters were estimated independently based on HKY + I, F81, and HKY + G, respectively. Percentages associated to each clade refer to Bayesian posterior probabilities/parsimony bootstrap when over 50%.

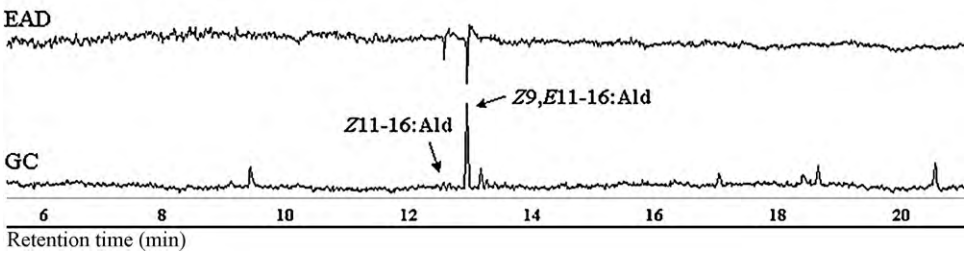

Fig. 3. Simultaneously recorded GC-FID and EAD responses using male *D. saccharalis* antenna stimulated by the extract obtained from the glands of 32 virgin females (2- or 3-day-old).

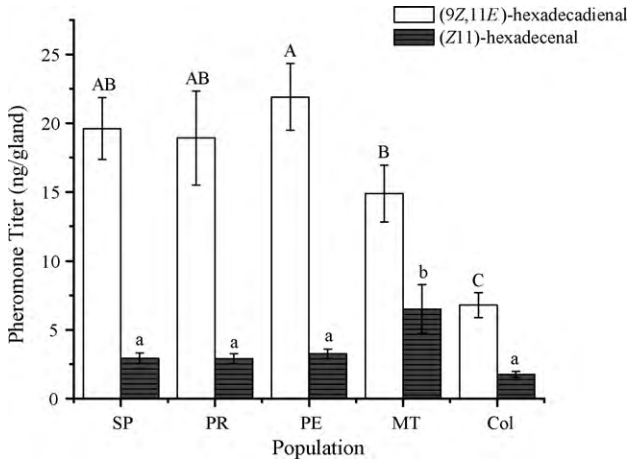

Fig. 4. Average concentration of the two main sex pheromone components, 9*Z*,11*E*-hexadecadienal, and (*Z*11)-hexadecenal, extracted from glands of 2- or 3-day-old *D. saccharalis* virgin females of different geographic origin. SP: São Paulo ($N = 33$); PR: Paraná ($N = 35$); PE: Pernambuco ($n = 28$); MT: Mato Grosso ($N = 37$); Col: Colombia ($n = 28$). Bars with different letters indicate significant differences (Tukey's test, $p < 0.05$).

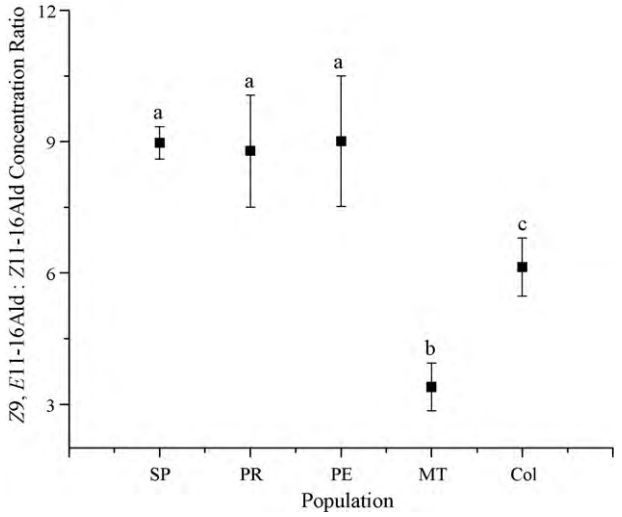

Fig. 5. Concentration ratios for the two sex pheromone components, 9*Z*,11*E*-hexadecadienal and (*Z*11)-hexadecenal, extracted from glands of 2- or 3-day-old virgin *D. saccharalis* females of different geographic origin. SP: São Paulo ($N = 33$); PR: Paraná ($N = 35$); PE: Pernambuco ($n = 28$); MT: Mato Grosso ($N = 37$); Col: Colombia ($n = 28$). Different letters indicate significant differences (Tukey's test, $p < 0.05$).

These concentrations are statically similar for insects from São Paulo, Paraná, Pernambuco, and Colombia, but significantly higher for those from Mato Grosso (Fig. 4).

Average ratios of the two main *D. saccharalis* sex pheromone components for the populations from São Paulo (9.0:1), Paraná (8.7:1), and Pernambuco (9.0:1) were similar (Fig. 5). The ratio for the population from Mato Grosso was 3.4:1, and for the Colombian population the average ratio was 6.1:1. The component ratios for Mato Grosso and Colombia are statistically different when compared to each other, and also significantly different from the ratios exhibited for São Paulo, Paraná, and Pernambuco populations (Fig. 5).

4. Discussion

The present study focused on the amounts and ratios of pheromone components for *D. saccharalis* geographic populations, and their relation to the mitochondrial sequence variability. The components, (9*Z*,11*E*)-hexadecadienal and (*Z*11)-hexadecenal, were detected in all extracts from Brazilian and Colombian populations. The average pheromone ratios obtained from pheromone glands of females from São Paulo, Paraná, and Pernambuco were similar to those reported by Batista-Pereira et al. (2002) and Kalinová et al. (2005) for populations from São Paulo, Rio de Janeiro, and Alagoas states. However, the analyses of the pheromone gland extracts of the populations studied revealed differences regarding the ratios and the amounts of pheromone compounds. Interestingly, these differences are in exact agreement with haplotype groupings found when studying COII intra-specific variation, but not geographical proximity. For example, although Pernambuco and Paraná states are distant from each other (ca. 3000 km), these populations still share the same haplotype and exhibit significantly similar pheromone ratios, while populations separated by about 1700 km, such as those from Mato Grosso and São Paulo states, show different haplotypes and significantly different pheromone ratios. Unfortunately, the few females collected from Goias did not allow us to gather pheromone data, but it would be interesting to carry out complementary study in the pheromone variation of this population, since it was the only collection site where multiple haplotypes were found.

If we take into account the three different pheromone ratios found and the different haplotypes from Colombia, Mato Grosso, and the most frequent one that include samples from São Paulo, Paraná, and Pernambuco, we suggest that *D. saccharalis* exhibits monomorphic pheromone variation. This variation occurs when different populations of the same species use the same compounds, but in different amounts and ratios (Löfstedt, 1990). For example, the pheromone blend of *Ostrinia nubilalis* (Hübner) (Lepidoptera:Pyralidae) consists of Z11–14:AC and *E*11–14:Ac components. Males of this specie present preferential response for the ratio 97:3 and 3:97 for North American and European populations, respectively (Klun and Cooperators, 1975). This variation was also reported for other lepidopterans, such as *Agrotis segetum* (Arn et al., 1983; Hansson et al., 1990), *Hemileuca eglanterina* (McElfresh and Millar, 2001), *Agrotis ipsilon* (Gemeno et al., 2000), and *Choristoneura rosaceana* (El-Sayed et al., 2003), among others.

When separate pheromone component amounts are compared to identified haplotypes in this analysis, no clear pattern was observed. For instance, the Colombian population showed the highest genetic divergence (3%) from the most common haplotype and a significantly different concentration of the major component in the sex pheromone. On the other hand, it showed a similar minor component concentration. The minor component concentration was only statistically different for the Mato Grosso population, which was actually very similar genetically (0.3%) to the most common haplotype. The pheromone concentration may be influenced by aspects such as adult size, temporal variation in pheromone production, and variable selection associate with the process of laboratory colonization as discussed by El-Sayed et al. (2003).

The mechanism by which divergence in sexual communication systems occurs, as well as the relevance that this may have on speciation, are a controversial subject of evolutionary biology (Lambert et al., 1987; Löfstedt, 1991, 1993; Linn and Roelofs, 1995; White et al., 1995). However, the pheromone blend produced by females of the various populations, and the patterns of detection and response by males, are congruent because mismatch of male and female reproductive signals and behaviors would constitute a constraint to reproductive success (McElfresh and Millar, 2001). Since the sex pheromone blend is important in eliciting male attraction, our data demonstrate a variation in pheromone composition and a covariation in haplotypes of *D. saccharalis* populations. Future trapping experiments

with different pheromone component ratios could be carried out with the different populations to evaluate the biologically significant of male response to ratios of the two pheromone components studied.

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