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Population genetics of the sugarcane borer *Diatraea saccharalis* (Fabr.) (Lepidoptera: Crambidae)

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ABSTRACT. *Diatraea saccharalis* is the principal pest of sugarcane in Brazil and is found throughout the sugarcane crop. Information about its population genetics is scarce, but population genetic analysis is of particular importance as a basis for a successful pest control program. Pest control requires a constant evaluation of genetic variability so that appropriate strategies can be employed. In this study, the structure of *D. saccharalis* populations in sugarcane crops was analyzed with PCR-RAPD (Polymerase Chain Reaction-Random Amplified Polymorphic DNA) markers. Samples were collected from four sugarcane fields in ParanáState and one in São PauloState. Twelve PCR-RAPD primers were used. The analysis produced 216 fragments, with a 99.54% polymorphism rate. The Shannon index averaged 0.3797 (± 0.1729), and the average value of G_{ST} for the five populations was 0.0909. These results showed that the populations are differentiated. An analysis of molecular variance showed that 92% of the polymorphism occurs within populations. The high polymorphism rate within these populations shows that these insects have the potential to develop resistance to insecticides. The control and management of *D. saccharalis* should be conducted cautiously and integrated using chemical and biological control.

Keywords: molecular marker, polymorphism, PCR-RAPD, sugarcane pest.

Genética de populações da broca da cana-de-açúcar *Diatraea saccharalis* (Fabr.) (Lepidoptera: Crambidae)

RESUMO. *Diatraea saccharalis* são consideradas as principais pragas da cana-de-açúcar, estando distribuídas em todas as regiões canavieiras do Brasil. As informações sobre a genética de populações em *D. saccharalis* são raras e sua análise é muito importante para o sucesso de programas de controle da praga. O controle de praga necessita de uma constante avaliação da variabilidade genética para que estratégias adequadas sejam empregadas. A estrutura de populações de *D. saccharalis* presentes em culturas de cana-de-açúcar foi analisada por meio do marcador PCR-RAPD. As amostras foram coletadas em canaviais de quatro regiões do noroeste do estado do Paraná e uma do estado de São Paulo. Foram empregados 12 *primers* RAPD, os quais produziram 216 fragmentos, com 99,54% de polimorfismo. O índice de Shannon foi em média 0,3797 (± 0,1729) e o valor médio encontrado do G_{ST} nas cinco populações foi de 0,0909, mostrando que as populações estão diferenciadas. Análise molecular de variância mostrou que 92% do polimorfismo ocorre dentro das populações. O amplo polimorfismo intrapopulacional mostra que esses insetos têm potencial para desenvolver resistência a inseticidas. O controle e o manejo da *D. saccharalis* deve ser realizado com cautela e integrado, ou seja, controle químico e biológico.

Palavras-chave: marcador molecular, polimorfismo, PCR-RAPD, praga da cana-de-açúcar.

Introduction

Diatraea saccharalis (Lepidoptera: Crambidae) causes considerable losses in sugar and ethanol production in Brazil. If the intensity of infestation by *D. saccharalis* is equal to 1% of the production, the resulting losses would be 0.77% of the sugarcane weight, 0.25% of the sugar production and 0.20% of the ethanol production (CAMPOS; MACEDO, 2004).

The sugarcane borer is originally from the Western Hemisphere (CAPINERA, 2007). The pest is distributed between 30° north latitude and 30° south latitude. In Brazil, it occurs in localities that have sugarcane fields (GUEVARA; WIEND, 1980).

The larvae borer into the sugarcane stalks and cause damage to the plant (CAPINERA, 2007). If borers are present, the amount and purity of the juice that can be extracted from the cane is reduced, and the sucrose yield may be decreased 10 to 20% (CAPINERA, 2007).

The sugarcane borer also increases the susceptibility of the plant to disease. The plant diseases invade the cavities left by the larvae and by

stem decay. Red rot, caused by *Physalospora tucumanensis* fungi, is particularly common. These forms of indirect damage are considered the most important and have negative effects on the production of sugar and alcohol (BOTELHO; MACEDO, 2002).

In the Latin American countries, biological control is the most common approach to the management of *D. saccharalis* (WEIR et al., 2007). Parasitic insects, such as Hymenoptera, are used widely in the biological control of *D. saccharalis* (BOTELHO, 1992).

Ruvolo-Takasusuki et al. (2002) evaluated esterase polymorphisms in laboratory-reared *D. saccharalis* pupae. Eight esterase activity regions were detected in this study, but only EST-3 was polymorphic. The authors detected the presence of two high-heterozygozity alleles for this isoenzyme and concluded that EST-3 could be used as a molecular marker for this insect.

Differences in morphology, physiology and behavior in *D. saccharalis* from Brazil and Louisiana were analyzed by electrophoresis (PASHLEY et al., 1990). The Brazilian collection showed significant differences at all polymorphic loci, and one locus was diagnostic. The variations within the sugarcane borer populations analyzed showed interspecific differences, suggesting that *D. saccharalis* may represent two species.

Lange et al. (2004) used partial sequence data from two mitochondrial genes, COII and 16S, to construct a molecular phylogeny of Noctuidae and Pyraloidea based on 26 species from ten genera and six tribes. Of the three crambine genera examined, *Diatraea* was monophyletic and *Chilo* paraphyletic. *Eoreuma* was basal to the other two genera. These data provide a basis for the development of DNA-based diagnostics for rapidly identifying many species at any developmental stage.

Heideman et al. (2010) used the PCR-RAPD sex molecular marker GyakuU-13, which is specific for the W chromosome of *Bombyx mori*, to differentiate male and female *D. saccharalis*. An analysis of molecular markers showed only one fragment, of approximately 700 bp, that could be considered a female sex marker in this moth.

The use of a dominant molecular marker such as PCR-RAPD (Random Amplified Polymorphic DNA - Polymerase Chain Reaction) may contribute to the knowledge of the diversity and population genetics of these pests. Genetic diversity and gene flow within and among populations of pest species play an important role in resistance or susceptibility to insecticides. This information is necessary to define the management and control of these insects.

Population genetic studies of the sugarcane borer *D. saccharalis* have not previously been conducted.This study was performed to detect the occurrence of genetic polymorphisms and to analyze the relationships within and among populations with PCR-RAPD markers in *D. saccharalis* in sugarcane fields located in the states of Paraná and São Paulo, Brazil. These results will contribute to better management practices and pest control in sugarcane crops.

Material and methods

Materials

Samples of *D. saccharalis* larvae were collected in four regions of the states of Paraná and São Paulo. The samples were collected from three sugarcane producers (Tables 1 and 2). Samples were collected from random plots in the sugarcane fields. After collection, the larvae were sacrificed and placed on ice, taken to the laboratory and stored at -20°C for DNA isolation. The DNA was isolated as soon as possible after storage.

Table 1. Geographic distances in kilometers (km) between the localities sampled for *D. saccharalis*.

	Engenheiro Beltrão (PR)	Tapejara (PR)	Centenário do Sul (PR)	Itororó do Paranapanema (SP)
Paranacity (PR)	123	185	72	62
Engenheiro Beltrão (PR)		81	148	162
Tapejara (PR)			250	240
Centenário do Sul (PR)				43

Table 2. Identification of *D. saccharalis* populations, geographical coordinates, sampling locations and sample sizes.

Geographical Coordinates	Locality	State	Samples Number
22º55'48"S 52º09'03"W	Paranacity	Paraná	12
23°47'49" S 52°16'08" W	Engenheiro Beltrão	Paraná	12
23º43'59"S 52º52'24"W	Tapejara	Paraná	12
22°49'15"S 51°35'42" W	Centenário do Sul	Paraná	12
22º 36'35"S 51º 43'16" W	Itororó do Paranapanema	São Paulo	12

Isolation of genomic DNA

Genomic DNA was extracted with the modified protocol of Doyle and Doyle (1990). The samples were homogenized individually in 500 µL of buffer (0.1 M pH 8.0 Tris-HCl, 1.4 M NaCl, 2% CTAB and 0.02 M EDTA) and 5 µL of proteinase K (25 µg μL^{-1}), remaining in a water bath for 2 hours at 65°C. They were then centrifuged at 11,751 g^{-1} for 10 min. at room temperature. The supernatant was removed, and 500 µL of phenol: chloroform: isoamyl alcohol (25:24:1) was added. The resulting sample was then centrifuged at 11,751 g^{-1} for 10 min. at room temperature. After

this period, the supernatant was removed and transferred, and two parts of chloroform: isoamyl alcohol (24:1) were added consecutively. After the homogenization and centrifugation of the last phase of chloroform: isoamyl alcohol, the supernatant was transferred to a new tube and 250 µL of isopropanol added for DNA precipitation for 12 hours. After this period, the DNA was washed twice with cold 70% ethanol and left to dry at room temperature. The DNA was resuspended in 60 µL of TE buffer (0.01 M pH 8.0 Tris-HCl, 0.001 M EDTA) and stored at -20°C.

The integrity and quantification of DNA were evaluated in 0.8% agarose gel 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}), the DNA bands were visualized under UV light and the image was captured with the EDAS system (Kodak 1 D Image Analysis 3.5).

DNA Amplification

For the PCR-RAPD analysis, 80 random primers, developed by Operon Technologies Inc., Alameda, CA, USA, were tested, of which 12 were amplified (Table 3). The amplification reactions were performed in a Techne thermocycler according to the original protocol described by Williams et al. (1990) with minor modifications.

Table 3. Primer nucleotide sequences, fragment numbers and polymorphic fragments obtained per primer in PCR-RAPD analysis of *D. saccharalis*.

Primer	Nucleotide sequence	Fragments Number	Polymorphic fragments number
OPA- 01	5'-CAGGCCCTTC–3'	16	16
OPA- 02	5'-TGCCGAGCTG-3'	11	11
OPA -08	5'- GTGACGTAGG -3'	20	20
OPA- 11	5'- CAATCGCCGT -3'	21	21
OPB- 04	5' - GGACTGGAGT -3'	19	19
OPB- 08	5'- GTCCACACGG -3'	20	20
OPB- 10	5'- CTGCTGGGAC -3'	15	14
OPB- 18	5'- CCACAGCAGT- 3'	15	15
OPC- 11	5'- AAAGCTGCGG -3'	21	21
OPC- 19	5' - GTTGCCAGCC -3'	15	15
OPP- 10	5'- TCCCGCCTAC -3'	18	18
OPP- 17	5'- TGACCCGCCT -3'	25	25
Total		216	215

The reactions were performed in a final volume of 25 µL containing 0.2 µM of primers, 2 units of Taq-DNA polymerase (Invitrogen), 2.5 µL of 10X reaction buffer (Invtrogen), 0.1 mM of each dNTP and 2.5 to 3.0 mM $MgCl_2$.

The initial DNA denaturation was at 94°C for 5 minutes. This step was followed by 45 amplification cycles (94°C: 1 min.; 35°C: 1 min.; 72°C: 2 min.). After the cycles, a final extension of 7 min. was performed at 72°C.

The amplification products were prepared and separated on 1.5% agarose gel with 1X TBE buffer (89 mM Tris, 89 mM boric acid and 2 mM EDTA). After this period, the gels were stained with an ethidium bromide bath (0.5 mg mL^{-1}). The DNA fragments were visualized under UV light, and the image was captured with the EDAS system (Kodak 1 D Image Analysis 3.5).

A molecular weight marker DNA (1 Kb Kaddi - Gibco-BRL) was used to determine the size of the generated fragments.

Population genetic analysis

The molecular data analysis involved the interpretation of the obtained DNA fragments (bands). The individuals were compared within and among populations. The comparisons were based on the presence (1) or absence (0) of fragments of identical molecular size (located in the same place) for each individual analyzed.

The genetic variability within and among populations was determined by the percentage of polymorphic loci. The Shannon index (I) and G_{ST} values were calculated with Popgene 1.31 (YEH et al., 1999) software.

The similarity matrix was subjected to a UPGMA cluster analysis (Unweighted Pair-Group Method Using an Arithmetic Average) to construct a dendrogram. For this analysis, the NTSYS 1.7 (Numerical Taxonomy and Multivariate Analysis System) program was used (ROHLF, 1989). The analysis was based on the arithmetic complement of the Jaccard coefficient.

GenALEX 6 (PEAKALL; SMOUSE, 2006) software was used to estimate Nei's (1978) genetic distance and to perform an AMOVA (Analysis of Molecular Variance).

A tree of the genetic relationships among *D. saccharalis* populations based on Nei and Li/Dice was constructed. The percentage confidence level was obtained from a bootstrap analysis (1,000 replicates) with FreeTree software (HAMPL et al., 2001). The TreeView (PAGE, 1996) program was used to draw the tree.

Results and discussion

Tests were performed to identify the primers with the highest number of bands to verify the reliability of the RAPD results. The repeats showed the same banding pattern in all analyses. The consistency of the marker was also confirmed with

tests performed to evaluate the amounts of NaCl and magnesium used. This molecular marker is a simple technique for the estimation of genetic diversity. RAPD markers have been reported to be an efficient tool to differentiate geographically and genetically isolated populations (JAIN et al., 2010). The polymorphisms are observed in non-coding regions of the genome (VUCETICH et al., 2001).

Table 4 shows that 216 fragments were obtained, 215 polymorphic, accounting for 99.54% of the overall polymorphism. The sugarcane borer showed a high level of PCR-RAPD polymorphism. The OPP-17 primer had the highest number of fragments (25). The lowest number (11) was detected with the OPA-02 primer.

The number and proportion of polymorphic fragments detected in the five analyzed populations of *D. saccharalis* are shown in Table 4. The highest proportion of polymorphic fragments (87.50%) was identified in the population from Paranacity, Paraná State, whereas the lowest proportion (75.46%) was identified in the population of Centenário do Sul, Paraná State.

Table 4. Number and percentage of polymorphic fragments and Shannon diversity index obtained from the PCR-RAPD analysis of five *D. saccharalis* populations.

Population	Number of polymorphic fragments	Polymorphic fragments (%)	Shannon index (I) (standard deviation)
Paranacity (PR)	189	87.50	0.3577 (± 0.2153)
Engenheiro Beltrão PR)	171	79.17	0.3536 (± 0.2355)
Tapejara (PR)	171	79.17	0.3368 (± 0.2328)
Centenário do Sul PR)	163	75.46	0.3222 (± 0.2402)
Itororó do Paranapanema (SP)	176	81.48	0.3130 (± 0.2108)
Total	216	99.54	0.3797 (± 0. 1729)

A similar result was obtained by Martinelli et al. (2006) in a study of genetic variability in 10 populations of *S. frugiperda* (Lepidoptera: Noctuidae) with PCR-RAPD markers, an average of 98% of the 208 fragments amplified were polymorphic. An analysis of *Sesamia nonagrioides* (Lepidoptera: Noctuidae) population genetics with PCR-RAPD showed that 78% of the fragments obtained were polymorphic (POZA et al., 2008).

Genetic diversity, estimated in *D. saccharalis* with the Shannon index, averaged 0.3797 (± 0.1729) in all populations (Table 4). A higher genetic diversity (35.77% ± 0.2153) was detected in the Paranacity population. The lowest genetic diversity was observed in the Itororó do Paranapanema (São Paulo State) population (31.30% ± 0.2108).

The G_{ST} value for the five populations was 0.0909, indicating that they were differentiated. AMOVA analysis showed that 92% of the variation obtained with the RAPD markers occurred within populations and 8% among populations. Moreover, the geographic distance between the sampled populations was variable (shortest distance – 43 km between Centenário do Sul and Itororó do Paranapanema; longest distance – 250 km between Centenário do Sul and Tapejara).

It is probable that the variation within populations prevented the separation of the populations based on the Jaccard coefficient (Figure 1).

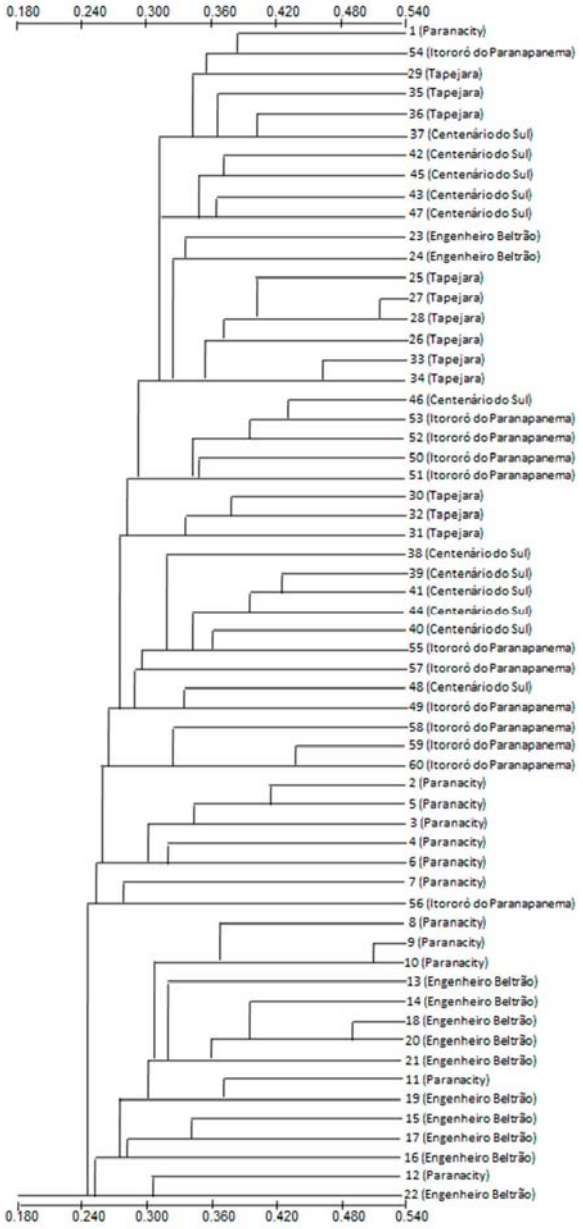

Figure 1. Dendrogram based on the arithmetic complement of the Jaccard coefficient, obtained by PCR-RAPD markers. Individuals 1-12 (Paranacity), 13-24 (Engenheiro Beltrão), 25-36 (Tapejara), 37-48 (Centenário do Sul), 49-60 (Itororó do Paranapanema). These specimens were clustered with the UPGMA method.

Figure 2 shows that the values obtained from the bootstrap method with 1,000 repetitions were significant at the nodes representing Itororó do Paranapanema, Engenheiro Beltrão and Centenário do Sul. The populations of Paranacity and Tapejara showed no significant bootstrap values (Figure 2).

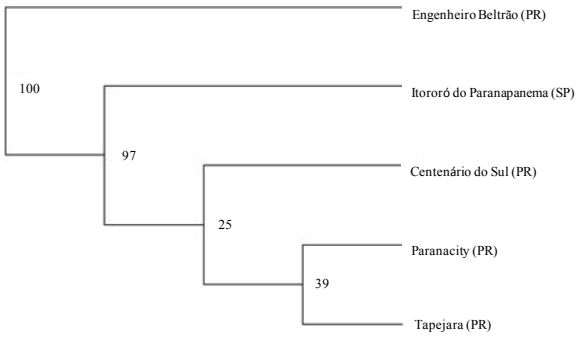

Figure 2. UPGMA dendrogram based on RAPD amplification patterns obtained for *D. saccharalis*. Bootstrap results are shown to the right of the nodes.

Nei's distance values among the five populations are presented in Table 5. The greatest similarity was observed between Centenário do Sul and Itororó do Paranapanema, most likely due to the small geographical distance (43 km) between them.

Table 5. Values of genetic identity and Nei's (1978) genetic distance for five *D. saccharalis* populations.

Locality	Paranacity	Engenheiro Beltrão	Tapejara	Centenário do Sul	Itororó do Paranapanema
Paranacity	---	0.932	0.927	0.923	0.935
Engenheiro Beltrão	0.071	---	0.911	0.916	0.915
Tapejara	0.075	0.094	---	0.926	0.936
Centenário do Sul	0.080	0.087	0.076	---	0.949
Itororó do Paranapanena	0.067	0.089	0. 067	0.052	---

*The values of Nei's genetic identity are shown above the diagonal, and Nei's genetic distances are shown below the diagonal.

It is probable that crosses between adult moths from different locations can produce insecticide resistance in two ways. First, populations that were not resistant to an insecticide would become resistant due to gene exchange through breeding among populations. Second, cross-resistance to insecticides can occur, i.e., if individuals from a population resistant to one type of insecticide bred with individuals from a population resistant to other insecticides, the descendants of the crosses would be resistant to both types of pesticides. This type of occurrence was described in a study by Saleem et al. (2008) of resistance to organochlorines, organophosphates, carbamates and pyrethroids in *Spodoptera litura* (Lepidoptera: Noctuidae) in Pakistan. The authors proposed that the detected resistance to insecticides may be due to the migration of the insects within areas in which resistance to different insecticides had previously been described.

In the cane fields analyzed in northwestern Paraná State and in Itororó do Paranapanema (located southwest of São Paulo State), migration and mixing have only a small probability because the populations are genetically differentiated and isolated from one other. However, the management of the sugarcane borer requires careful attention. First, the extensive intrapopulation polymorphism of the borer populations favors the occurrence of individuals with resistance to the agrochemicals used in the control of the pest. Second, the possible occurrence of gene flow between populations favors the emergence of cross-resistance.

Integrated control programs for sugarcane borers require more extensive supervision. It is necessary to analyze the *D. saccharalis* polymorphisms present in sugarcane crops to perform a more careful evaluation of the control programs that can be used and of the time to release *C. flavipes* for biological control of the pest. It would be important for sugar factories to work together to prevent the application of different insecticides. In this way, it may be possible to prevent cross-resistance based on the high degree of polymorphism of the pest, and it may also be possible to avoid breeding between populations from different locations.

Conclusion

High percentage of RAPD polymorphisms and the similarity between the populations of *D. saccharalis* evaluated showed that gene flow can occur within and between populations making possible increased resistance to pesticides used on sugarcane crops. This, ist becomes important to develop new strategies for managing this pest, among them, a periodic assessment of genetic polymorphism to make it difficult the occurrence of resistance to chemical and integrated control of insects.

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