

Notas Científicas

Mitochondrial markers to distinguish *Spodoptera frugiperda* populations associated with corn and cotton crops

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Abstract – The objective of this work was to analyze the genetic variability of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) populations collected from corn and cotton crops in Brazil. The samples were analyzed by DNA markers. The dendrogram produced by the 20 RAPD primers evaluated showed a correlation between genetic profile and feeding behavior. The analysis of the mitochondrial *NDI* gene allowed identifying the insect populations in both crops, and, in corn, in several geographical regions. The presented strategy allows the identification of *Spodoptera frugiperda* populations associated with corn and cotton crops.

Index terms: *Gossypium hirsutum*, *Zea mays*, fall armyworm, haplotypes, *NDI* gene, RAPD.

Marcadores mitocondriais para diferenciar populações de *Spodoptera frugiperda* associadas às culturas de milho e algodão

Resumo – O objetivo deste trabalho foi avaliar a variabilidade genética de populações de *Spodoptera frugiperda* (Lepidoptera: Noctuidae) coletadas nas culturas de milho e algodão do Brasil. As amostras foram analisadas por marcadores de DNA. O dendrograma produzido pelos 20 iniciadores de RAPD avaliados revelou correlação entre o perfil genético e o comportamento de alimentação. A análise do gene *NDI* mitocondrial permitiu identificar populações do inseto em ambas as culturas, e, em milho, em várias regiões geográficas. A estratégia apresentada permite a identificação de populações de *Spodoptera frugiperda* associadas às culturas de milho e algodão.

Termos para indexação: *Gossypium hirsutum*, *Zea mays*, lagarta-do-cartucho, haplótipos, gene *NDI*, RAPD.

Spodoptera frugiperda (J.E. Smith, 1797) (Lepidoptera: Noctuidae), usually known as fall armyworm, has a polyphagous feeding behavior and is a serious pest of 80 plant species, including cotton (*Gossypium hirsutum* L.), corn (*Zea mays* L.), and soybean (*Glycine max* L.) (Martinelli et al., 2006; Barros et al., 2010).

Initial studies of *S. frugiperda* populations from North American and Caribbean countries showed the existence of two strains: the corn strain, associated with corn and sorghum (*Sorghum bicolor* L.); and the rice strain, found preferentially in rice (*Oryza sativa* L.) and turfgrassess. These strains differ in their host preference, physiology, behavior, and pesticide

susceptibility (Prowell et al., 2004). However, both host strains can only be reliably distinguished by biochemical and molecular markers, which remain the most accurate indicators of strain identity worldwide (Nagoshi & Meagher, 2008).

Busato et al. (2004) and Pinto et al. (2015) reported genetic variability among *S. frugiperda* populations associated with cotton and corn in Brazil, using amplified fragment length polymorphism (AFLP) and random amplified polymorphic DNA (RAPD) markers. Lewter et al. (2006) distinguished rice and corn strains of *S. frugiperda* by analyzing the polymorphisms in the mitochondrial cytochrome oxidase 1 (*COI*) gene. However, in the present study, a mitochondrial

marker based on the NADH dehydrogenase 1 (*NDI*) gene was adopted; this gene has been previously used for phylogenetic analyses of different lepidopteran species, including *S. frugiperda* (Pashley & Ke, 1992).

The objective of this work was to analyze the genetic variability of *S. frugiperda* populations collected from corn and cotton crops in Brazil.

Immature stages (second or third instar) of *S. frugiperda* were collected from different corn and cotton field crops in Brazil: corn crop in Planaltina, in the city of Brasília, DF; cotton crop in the municipality of Porto Alegre do Norte, in the state of Mato Grosso; and cotton crop in the municipality of Londrina, in the state of Paraná. All insects were collected and stored in 100% ethanol, at -20°C, until use. Other *S. frugiperda* mass-reared individuals were obtained from: Embrapa Recursos Genéticos e Biotecnologia, located in Brasília, DF, Brazil; Universidad Autónoma de México, located in Cuernavaca, Mexico; Corporación para Investigaciones Biológicas, located in Medellín, Colombia; and Universidad de Costa Rica, located in San José, Costa Rica. All these rearing strains were established with insects collected from corn crops in each country, and the insects were reared in artificial diet under laboratory conditions, at 28±2°C and 65±5% relative humidity, under a 12:12 (light-dark) photoperiod (Monnerat et al., 2006).

DNA was extracted from insect samples according to the procedure in Agusti et al. (1999). For RAPD molecular analysis, approximately ten immature stages (second or third instar) of *S. frugiperda* were collected from different corn and cotton field crops. The RAPD reactions were done following the procedures described by Monnerat et al. (2006), using the primers: OPA-01, OPA-02, OPA-03, OPA-04, OPA-05, OPA-07, OPA-10, OPA-11, OPA-13, OPA-14, OPA-15, OPA-17, OPA-20, OPE-01, OPE-03, OPE-07, OPE-08, OPE-14, OPE-15, OPR-01, OPR-02, OPR-03, OPR-04, OPR-05, OPR-07, OPR-10, and OPR-14; however, since seven (OPA-05, OPA-15, OPA-17, OPA-20, OPE-01, OPE-15, and OPR-05) of these primers did not generate useful PCR products, only 20 were analyzed. This experiment was repeated three times using randomly collected individuals.

For the execution of the mitochondrial analysis, approximately ten immature stages (second or third instar) of *S. frugiperda* were collected from different corn and cotton field crops. The polymerase chain

reaction (PCR) amplification of the *NDI* gene fragment was done in a 30-µL reaction containing 1X buffer (GE Healthcare, Buckinghamshire, UK), 0.17 mmol L⁻¹ dNTP (Invitrogen, ThermoFischer Scientific, Waltham, MA, USA), 0.5 µmol L⁻¹ of each primer (*NDI*-F: 5'CCC TCT TCT ATA CTC AAT AT3' and *NDI*-R: 5'CTT TAT TGG AGC GTA AGG TTT3'), and 0.15 U µL⁻¹ *Taq* DNA polymerase (GE Healthcare, Buckinghamshire, UK). These primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA, USA). The reaction was performed in a PTC-100 thermal cycler (MJ Research, Inc., Watertown, MA, USA) programmed for an initial denaturation step at 94°C for 3 min, followed by 35 cycles of denaturation at 92°C for 1 min, annealing temperature at 48°C for 1 min, amplification at 72°C for 1 min, and final amplification at 72°C for 5 min. The PCR reaction products were separated by 1.5% agarose gel electrophoresis for 3 hours, at 160 V, then stained for 20 min with 5.0 µg mL⁻¹ ethidium bromide and visualized in a photograph, using the Eagle Eye II Still Video System (Stratagene, Agilent Technologies, Santa Clara, CA, USA), under ultraviolet light.

The obtained PCR products (100 ng) were digested with the *Xba*I restriction enzyme, at 37°C, for 16 hours in a 20-µL reaction containing 1X One-Phor-All Buffer (GE Healthcare, Buckinghamshire, UK) and 10 U *Xba*I (Invitrogen, ThermoFischer Scientific, Waltham, MA, USA). This experiment was also repeated three times using randomly collected individuals.

The genetic variability of *S. frugiperda* populations found in corn and cotton crops in Brazil was first assessed by RAPD analysis. The 20 primers evaluated produced a total of 752±11 RAPD loci. The dendrogram performed with these RAPD products showed that Brazilian *S. frugiperda* populations can be grouped into two main groups: A, containing *S. frugiperda* populations from corn crops; and B, containing all insect populations collected from cotton crops. These data were validated by clustering insects collected from cornfields in Mexico with those isolated from cornfields in Brazil (Figure 1). It is important to note that the genetic similarity among these two main groups was quite low, only 5%. A similar result was observed between the Brazilian and Mexican corn populations (group C), which presented 6% genetic similarity (Figure 1).

The binary data generated by the RAPD markers from all *S. frugiperda* populations was subjected to the analysis of molecular variance (Ampova). When compared, the groups from cotton and corn crops showed the following sources of genetic variability: 2.8% among populations, 75.7% within each population, and 21.8% among populations inside each group of cotton or corn ($F_{ST} = 0.25$). These data indicated that RAPD analysis could be useful to distinguish insect populations collected from corn and cotton fields.

Martinelli et al. (2006), based on RAPD analysis using ten different primers, did not report genetic variation between *S. frugiperda* populations isolated from corn and cotton crops in Brazil. The authors found 206 loci, and that 15% of molecular variability occurred among

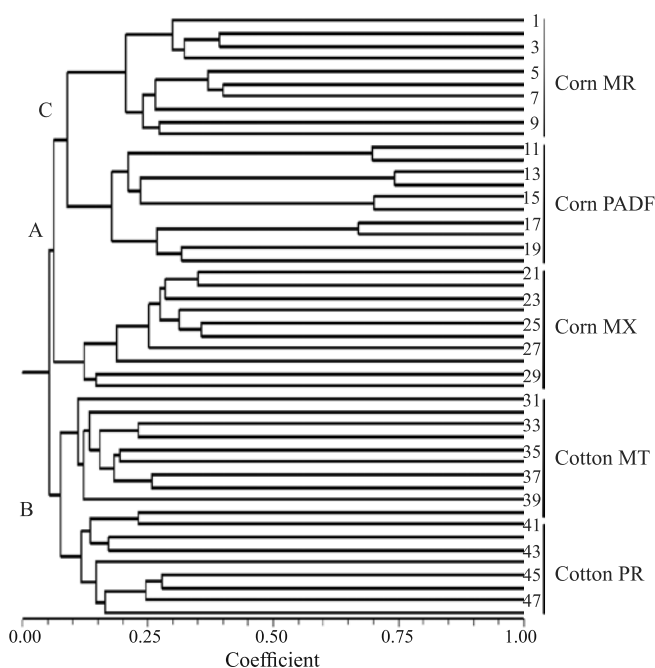


Figure 1. Genetic similarity between the five *Spodoptera frugiperda* populations, collected from corn (*Zea mays*) and cotton (*Gossypium hirsutum*) crops. Corn MR, mass-reared insects from corn, from the city of Brasília, DF, Brazil; Corn PADF, insects from corn, from Planaltina, Brasília, DF, Brazil; Corn MX, insects from corn, from the city of Cuernavaca, Mexico; Cotton MT, insects from cotton, from the municipality of Porto Alegre do Norte, in the state of Mato Grosso, Brazil; and Cotton PR, insects from cotton, from the municipality of Londrina, in the state of Paraná, Brazil. Numbers correspond to each individual larva.

populations and 85% within populations; however, mitochondrial markers were not analyzed. In present study, to validate the obtained results, these additional genetic markers were used, since they can distinguish among highly-related populations, such as those of *S. frugiperda* in cotton and corn. A total of 20 primers were used to distinguish 750 loci, which could explain the differences between both studies.

The sequence of the mitochondrial *NDI* gene from *S. frugiperda* was used to design primers that would amplify a 560-bp fragment of the *NDI* gene sequence. With these primers, individual insects of *S. frugiperda* collected from Brazilian corn and cotton fields were analyzed. The amplification of the *NDI* gene from insect samples collected in different Latin American regions was also assessed. Regarding the analysis of Brazilian populations, the expected 560-bp PCR product was generated only in insects from cornfields. These data indicate that insects associated with corn could be distinguished from those associated with cotton, in which a PCR amplification product was not observed. Furthermore, the data obtained show that *S. frugiperda* larvae from cornfields in Colombia, Costa Rica, and Mexico also amplified a DNA fragment of 560 bp.

The next step was PCR-restriction fragment length polymorphism (RFLP) analysis of the *NDI* gene fragment using the restriction enzyme *XbaI*. The obtained results show two different patterns among the *S. frugiperda* samples. The *NDI* fragment in populations from Colombia and Costa Rica revealed two digestion fragments of 180 and 380 bp (Figure 2). However, the sample originated from Mexico was not digested by *XbaI*, indicating absence of this restriction site, which was confirmed by DNA sequencing. These results show that *NDI* polymorphism could be detected among the *Spodoptera* samples collected in these regions.

Using PCR-RFLP of the *COI* gene, Vélez-Arango et al. (2008) described two biotypes of *S. frugiperda* from corn and rice in Colombia. These authors also suggested a possible hybrid between these two biotypes. Lewter & Szalanski (2007) used PCR-RFLP of the *COI* mitochondrial DNA region, with the restriction enzymes *DraI*, *AluI*, and *NlaIII*, to distinguish biotypes of *S. frugiperda*, i.e., corn 1,2,3 haplotypes AAA, rice 1,2 haplotypes AAB, and rice 3 haplotype BAB.

Therefore, these two studies were able to distinguish corn and rice biotypes.

In this regard, the mitochondrial *NDI* gene marker was shown to be useful to distinguish *S. frugiperda* populations found in corn and cotton. Samples collected from corn crops in Colombia and Costa Rica could be differentiated from those isolated from corn in Mexico. These variations among geographically isolated *S. frugiperda* indicate the presence of factors that affect the genetic variability and selection of biotypes in the two analyzed crops. As recommended by Sosa-Gomez et al. (2004), in order to validate these results, it will be necessary to make ecological observations or characterizations of, for example, the insect's flying behavior, dispersion ability, and resistance to chemical or biological products. Despite this, the mitochondrial *NDI* gene showed high potential to discriminate these two biotypes of *S. frugiperda*, being a quick method to identify these pests.

Monnerat et al. (2006) also observed diversity of *S. frugiperda* insects isolated from cornfields associated with different geographical origins. These authors showed that there is a correlation among data on insect variability and susceptibility to different Cry toxins produced by *Bacillus thuringiensis*. Therefore, strain identification of specific lepidopteran larvae could help in developing effective pest management strategies to control these insects.

The *NDI* PCR-RFLP diagnostic test reported here is quick and easy to perform, since PCR products could be amplified from total *S. frugiperda* DNA extracts with no need to purify mitochondrial DNA. The primers used in the present study and the reaction

conditions established were useful for *S. frugiperda* samples maintained in cold 100% ethanol. In addition, the molecular patterns of RAPD and PCR-RFLP could be assessed to study the genetic variability in the corn strain and to analyze its distribution into cotton fields in the future.

In conclusion, the effectiveness of the mitochondrial marker *NDI* to identify different *S. frugiperda* populations from corn crops in Brazil was validated using insect populations from other geographical regions, including Mexico, Colombia, and Costa Rica.

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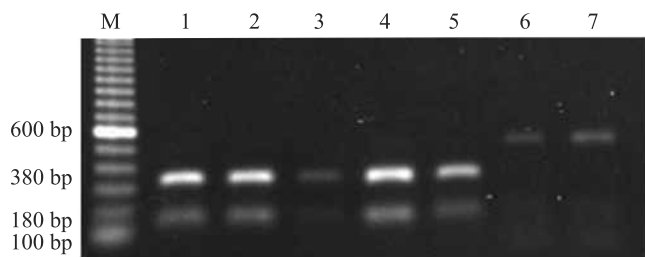


Figure 2. Pattern of PCR-RFLP analysis of the mitochondrial NADH-dehydrogenase 1 (*NDI*) gene, using the *Xba*I restriction enzyme. Lanes 1–3, insects from the city of Medellin, Colombia; lanes 4–5, insects from the city of San Jose, Costa Rica; and lanes 6–7, insects from the city of Cuernavaca, Mexico.

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