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Source: Florida Entomologist, 85(1) : 186-190

Published By: Florida Entomological Society

URL: https://doi.org/10.1653/0015-4040(2002)085[0186:SIOSFL]2.0.CO;2

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STRAIN IDENTIFICATION OF *SPODOPTERA FRUGIPERDA* (LEPIDOPTERA: NOCTUIDAE) INSECTS AND CELL LINE: PCR-RFLP OF CYTOCHROME OXIDASE C SUBUNIT I GENE

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ABSTRACT

A simple method was developed to analyze the two morphologically indistinguishable host-associated strains of the fall armyworm (FAW), *Spodoptera frugiperda* (J. E. Smith). Total DNA extracted from the FAW corn and rice strains, as well as from the *S. frugiperda* cell line (SF9) was used to PCR amplify a 569 base pairs region of the mitochondrial gene cytochrome oxidase subunit I (COI). The amplified DNA from the *Spodoptera* corn strain and the SF9 cell line contained a restriction fragment length polymorphism (RFLP) marker, the MspI recognition site that was not present in the rice strain. This PCR-RFLP method does not require purification of mitochondrial DNA (mtDNA) or the use of radioactive isotopes, and differs from previous methods in that only a few nanograms of total DNA are needed to yield clear and accurate strain identification of individual insects.

Key Words: Fall armyworm, mitochondrial DNA, host strain discrimination, cytochrome oxidase I

RESUMEN

Un método simple ha sido desarrollado para analizar las dos cepas biológias, hospedero-dependiente, morfológicamente indistinguibles de *Spodoptera frugiperda* (J. E. Smith). El ADN total, extraído de insectos de *S. frugiperda* de las cepas de arroz y maíz, asi como también del cultivo de células SF9, fue utilizado para la amplificación, mediante el método de PCR. La región amplificada, de 569 pares de bases de la subunidad I del gen Citocromo Oxidase (COI), está localizada en el ADN mitocondrial. El ADN amplificado a partir de larvas y adultos de *S. frugiperda* de la cepa de maíz, contiene como marcador molecular, la secuencia reconocida por la enzima de restricción MspI, que no está presente en la cepa de arroz. Este método que combina la amplificación de ADN por PCR y subsecuente digestión enzimática (Polimorfismo de Longitud de Fragmento de Restricción-RFLP), no requiere la purifiación del ADN mitocondrial ni el uso de isótopos radioactivos. Se diferencia de otros métodos ya que sólo pocos nanogramos de ADN total son necesarios para obtener una identificación clara y precisa de insectos individuales.

The fall armyworm (FAW), *Spodoptera frugiperda* (Lepidoptera: Noctuidae) (J. E. Smith) is an agricultural pest that over-winters in Florida and causes costly damage to corn, rice, peanuts, cotton, soybeans, alfalfa and forage grasses (Knipling 1980, Pashley 1986, Lu & Adang 1996). There are at least two morphologically indistinguishable host-plant specific strains of FAW. One strain feeds on corn and sorghum (corn strain) and the other on rice and bermudagrass (rice strain).

Several studies have been focused on the behavioral and biochemical differences between these two FAW strains. They mate unidirectionally in the lab and at different times in the night, display differential responses to control agents and differ in development on plant hosts (Pashley et al. 1992, Pashley et al. 1985, Quisenberry & Whitford 1988, Pashley 1986). The two strains also diverge in allelic frequencies of several glycolytic enzymes, in migratory pathways, and in mitochondrial DNA (mtDNA) sequences (Pashley et al. 1985, Maas & Sanjur 1996). The existence of the FAW strains complicates the management of these pests as demonstrated in studies where bermudagrasses specifically bred for pest resistance showed differential resistance to the corn strain FAW (Quisenberry & Whitford 1988).

Cryptic host-strains of *S. frugiperda* have been previously distinguished through differences in protein composition, nuclear and mtDNA restriction fragment length polymorphism (RFLP) (Pashley et al. 1985, Lu et al. 1992, Adamczyk 1993, Lu & Adang 1996). The mtDNA genome is estimated to be 14.8 kb. Single restriction enzyme digestion with BstNI, HinfI or MspI differentiates corn and rice strain mtDNA (Adamczyk 1993, Lu & Adang 1996). McMichael & Prowell (1999) distinguished fall armyworm host strains using the amplified fragment-length polymorphic (AFLP) loci method. AFLP has the ability to detect many loci differences of the FAW strains as well as hybrids. They have been able to distinguish corn and rice FAW strains 92 to 97% of the time when compared to allozyme patterns and mtDNA profiles.

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The mitochondrial protein cytochrome oxidase c is a highly conserved electron transport protein coded by multiple genes containing regions that evolve at different rates (Lunt et al. 1996). Maas & Sanjur (1996) published partial cytochrome oxidase subunit I (COI) DNA sequences for *S. frugiperda* strains further substantiating mtDNA sequence divergence in the two strains. The study of host-plant strain variations in the mitochondrial genes that code for cytochrome oxidase c proteins may be of value in determining the evolution of toxin resistance and food preference in animals.

Many toxins are known to inhibit electron transport from cytochrome oxidase b to cytochrome c (Bolter & Chefurka 1990, Kamin & Masters 1968). These toxins include a number of drugs and pesticides and may include some plant allelochemicals. A factor important to the evolution of host races may be the development of resistance to host-plant allelochemicals (Yu 1987).

The mitochondrial DNA of Lepidoptera has been used in phylogenetic and population genetic studies (Weller & Pashley 1994). Universal primers have been designed to amplify the insect COI DNA region using the polymerase chain reaction (PCR) method (Kambhampeti & Smith 1995; Simon et al. 1994; Zhang & Hewitt 1996). However, PCR has not been used previously for strain identification of *S. frugiperda* larvae or cell cultures.

Sf9 cells are a subclone of IPLB-SF-21 cells (Vaughn et al. 1977) originally cultured from pupal *S. frugiperda* tissue used in expression vector systems (Luckow & Summers 1988a and 1988b, Oker-Blom & Summers 1989). These cells are also used to study viral infection for the development of highly specific biological pesticides (Danyluk & Maruniak 1987, Sieburth & Maruniak 1988). Different clones from the same cell line can present varying susceptibility to infection by baculoviruses (Volkman & Summers 1975, Maruniak et al. 1994). Cells cultured from either corn or rice strain may differentially respond to viruses, therefore, the strain identity of Sf9 could be useful information.

Cellular morphology in insect cells changes with cell division phase, media and even subtle culture variations; therefore, morphology is not a recommended method to characterize insect cell cultures (Granados & McKenna 1995). Isozyme analysis and restriction enzyme analysis of mtDNA with EcoRI and HindIII can distinguish Sf9 cells from other Lepidoptera cell lines and from contaminants (Danyluk & Maruniak 1987). However, this analysis of the total mitochondrial genome requires greater than 20 grams of larvae or 1×10^8 cells. In this study, a set of primers were designed to amplify a COI region containing a molecular marker to determine the plant-host strain identity of *S. frugiperda* insects and Sf9 cells.

Material and Methods

PCR primers

Nucleic acid sequences for *S. frugiperda* corn and rice strains COI partial genes were obtained from GenBank of the National Center for Biotechnology Information (Benson et al. 1998, Maas & Sanjur 1996). The Genetic Computer Group (GCG Wisconsin sequencing analysis package) (Devereaux et al. 1984) Program MapSort was used to create restriction enzyme maps of each strain sequence. A set of primers flanking the diagnostic molecular marker MspI in the COI gene was designed using the Oligonucleotide Selection Program (OSP) (Hillier & Green, 1991). The DNA Synthesis Core at the University of Florida synthesized the primer set JM-76 5'-GAGCTGAATTAGG(G/A)ACTCCAGG-3' and JM-77 5'-ATCACCTCC(A/T)CCTGCAGGATC-3'

Insect and cell line sources

A confirmed rice strain *S. frugiperda* larva, collected in 1989 and freeze dried, was donated by Dr. D. Prowell of Louisiana State University, Baton Rouge, and was used as a standard for comparison to the unknown insects. Live, laboratory reared larvae of *S. frugiperda* were donated by Dr. E. Mitchell and Mr. F. Adams and *Plodia interpunctella* by Dr. D. Silhacek, of the USDA-ARS, Gainesville. Dr. J. Hamm, USDA-ARS, Tifton, Georgia donated live *S. frugiperda* larvae collected from corn, sorghum and bermudagrass. Adult *S. frugiperda, Apis mellifera* pupae and adult house flies (*Musca domestica*) were obtained from Drs. S. Yu, G. Hall and J. Butler, respectively, from the University of Florida. *Lymantria dispar* adult insects were obtained from Combined Scientific. Sf9 cells were maintained in tissue culture flasks (Corning) in TC-100 medium pH 6.2 containing 10% fetal bovine serum (Atlanta Biologicals) and 50 µg/ml gentamicin.

DNA isolation

Total nucleic acid (mtDNA, RNA and nuclear DNA) was isolated from *S. frugiperda* larvae and adults. Individual insects (whole larvae and only the thorax of adults) were homogenized in 0.5 ml STM (0.32 M sucrose, 50 mM Tris pH 7.25, 10 mM $MgCl_2$, 0.5% NP 40 detergent) and 0.1 ml of 0.5 M EDTA. The suspension was centrifuged at low speed for 4 min and the supernatant was removed. The pellet was resuspended in 0.5 ml STE (75 mM NaCl, 25 mM EDTA, 10 mM Tris pH 7.8) and 1% SDS. The homogenate was digested with 500 µg Proteinase K for 1 hour and extracted with phenol and chloroform. The DNA was ethanol precipitated, resuspended in 60 µl 10 mM Tris pH 8.0 and stored at -20°C (modified from Hall,

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1990). The SF9 DNA was extracted from 5×10^5 cells following the microextraction procedure detailed in Maruniak et al. (1999).

PCR-RFLP analysis

For PCR amplification, the total DNA extracted from *S. frugiperda* insects and SF9 cells were diluted (200× dilution factor for larvae and adult preparations and 100× for SF9 cells) in 10 mM Tris pH 8.0 prior to use as template in the PCR reactions. The reactions had a final volume of 100 ml and comprised approximately 10 to 20 ng of insect DNA template or 1 to 5 ng of SF9 DNA (4 µl of the diluted DNA), 40 pmoles of JM76 and JM 77 primers, 2.5 mM $MgCl_2$, 200 µM dNTP mix, 10.0 µl of 10× reaction buffer (10 mM Tris-HCl pH 8.8, 50 mM KCl, 0.1% Triton X-100) and two units of Taq DNA Polymerase (Gibco/BRL). DNA from other insects including *Lymantria dispar*, *Plodia interpunctella*, *Musca domestica* and *Apis mellifera* were used as negative controls to illustrate specificity of the primer set.

Temperature cycling was carried out by an MJ Research, Inc. thermal cycler starting with two min at 94°C and then 35 cycles of the following: one min at 94°C, 45 sec at 66°C, two min at 72°C. After the 35 cycles, the reactions were held at 72°C for 10 min. The PCR amplified DNA products were digested (5 to 7 ml) with MspI restriction endonuclease (New England Biolabs). Samples were incubated overnight at 37°C. The restriction enzyme profiles were visualized with ethidium bromide on a 2.0% Synergel/agarose (Diversified Biotech, Inc.) gel.

DNA Sequencing

The PCR product (30 ng) of some samples were sequenced using 2 µl BigDye Terminator Sequencing Ready Reaction Kit (Perkin-Elmer Corporation), 3.2 pmol of either JM76 or JM77 primers and 2 ml of the ICBR (Interdisciplinary center for Biotechnology Research, UF) sequencing reaction mix. The samples were sequenced in a Thermocycler machine (MJ Research) as recommended in the BigDye kit and sent to the ICBR to be electrophoresed. The electropherograms were analyzed with the Sequencher (Gene Codes Corporation) program.

RESULTS

The analysis of the *S. frugiperda* corn and rice strain COI sequences from GenBank (Maas & Sanjur 1996) revealed that they are 98% homologous. Within the differences was found an MspI recognition site (CCGG) in the corn strain sequence, not present in the rice strain (Fig. 1). A set of primers, JM 76 and JM 77 was designed flanking this region and was able to amplify a 569 base pairs (bp) product from all the *Spodoptera* sources: larvae**,** adult and cell line. In Fig. 2 only three representatives of uncut *Spodoptera* samples are shown. The primers did not amplify any DNA from the other insect species that were tested (data not shown).

The MspI digestion of the PCR product obtained from the *Spodoptera* corn strain resulted in two fragments of 497 and 72 bp in length. The rice strain Spodoptera PCR product was not digested by MspI (Fig. 2).

The analysis of the *Spodoptera* larvae obtained from colonies and field collected is presented in Fig. 2. The USDA (Gainesville) colony larvae, the larvae collected on corn and sorghum fields, and the Sf9 cells corresponded with the expected fragment sizes for the corn strain *Spodoptera*. The PCR-RFLP profiles of the larva collected on bermudagrass and the confirmed rice strain larva corresponded with the expected profile for the rice strain *S. frugiperda*.

The strain identity of some *S. frugiperda* COI PCR products, characterized as rice and corn strains by MspI digestion, were further confirmed by DNA sequencing using the JM76 and JM77 primers (data not shown) and compared to *S. frugiperda* sequences in GenBank (Maas & Sanjur 1996). The sequences obtained verified the nucleotide differences expected between the two *Spodoptera* strains (Fig. 1).

DISCUSSION

The COI PCR-RFLP diagnostic test is quick and easy because PCR products can be amplified from total DNA extracts with no need of having purified mitochondrial DNA. The primers used in this study and reaction conditions work with both *S. frugiperda* insects and cell culture, as well as frozen and fresh specimens. MspI digestion analysis established strain identity. These PCR primers may have further use to confirm the strain of additional insect sources.

Analysis of total mtDNA restriction enzyme profiles by Lu & Adang (1996) substantiates that the strains differ in their MspI profiles. Probing with purified mtDNA labeled with ^{32}P is necessary to reveal the complete MspI mtDNA profiles (Adamczyk 1993; Lu & Adang 1996). Their method of strain determination requires several hundred nanograms of mtDNA. Our PCR-RFLP analysis required only a few nanograms of total DNA, used no isotope and confirmed that one of the distinguishing corn strain MspI sites described by Lu & Adang (1996) is located in the highly conserved COI gene. Although McMichael & Prowell (1999) are able to distinguish FAW strains, the AFLP technique they used required 1 µg of DNA and radioactive isotopes.

Danyluk & Maruniak (1987) characterized IPLB-SF-21AE cell cultures (parental cells of Sf9

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          *         *         *         *         *         *         *         *
1   CGAAAATGACTTTATTCAACAAATCATAAAGATATTGGAACATTATATTTTATTTTTGGAATTTGAGCAGGGATAGTAGG
    ..........................................................................A.......

81  TACTTCTTTAAGTTTATTAATTCGAGCTGAATTAGGGACTCCAGGATCTTTAATTGGAGATGATCAAATTTATAATACTA
    ....................................A.....................................

161 TTGTAACAGCTCATGCTTTTATTATAATTTTTTTTATAGTTATACCTATTATAATTGGAGGATTTGGAAATTGACTTGTA
    ..........C.....................................A.....................................

241 CCTTTAATATTAGGAGCCCCTGATATAGCTTTCCCACGTATAAATAATATAAGTTTTTGACTTTTACCCCCATCTTTAAC
    ................T.....................................................................

321 TTTATTAATTTCTAGTAGCATTGTAGAAAATGGAGCAGGAACTGGATGAACAGTTTACCCCCCCCTCTCCTCTAATATTG
    ..........................................................................

401 CTCATGGTGGTAGTTCAGTAGATTTAGCTATTTTCTCACTTCATTTAGCTGGAATTTCATCTATTTTAGGAGCTATTAAC
    ..........................................................................

481 TTTATTACTACTATTATTAATATACGATTAAATAATTTATCATTTGATCAAATACCTTTATTTATTTGAGCTGTAGGTAT
    ........C.....................................................................

561 TACTGCATTCTTATTATTATTATCTTTACCTGTTTTAGCCGGAGCTATTACTATATTACTTACTGATCGAAATTTAAATA
    ...C.....T.............................T.................................C.....

641 CATCATTTTTCGATCCTGCAGGTGGAGGTGATCCTATTCTTTA 683
    ......................A.....................
```

Fig. 1. Comparison of *S. frugiperda* sequences from corn (GenBank accession number U72974) and rice (accession number U72977) strain cytochrome oxidase subunit I gene regions. The corn strain (upper sequence) contains a MspI cut site (*CCGG*) not found for the rice strain (lower sequence). Primer locations are *underlined* and the direction of the primers is shown by the arrows (JM76 -> and JM 77 <-). Dotted lines represent identical sequences between the two strains and any base difference is indicated in its location. Asterisks at the top of the figure mark the distance between 10 bases.

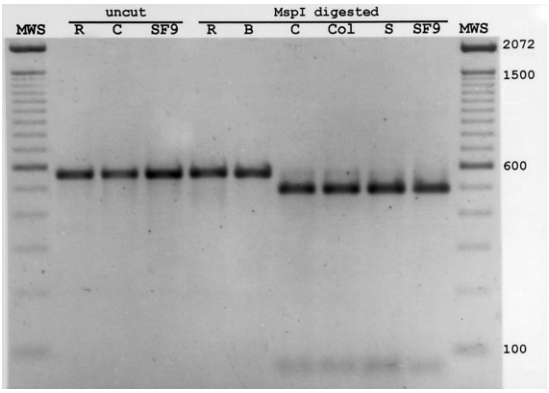


Fig. 2. PCR amplified DNA of COI mitochondrial region of *Spodoptera frugiperda* larvae and cell line. The size of the amplified DNA was the same (569 bp) independent of the FAW strain (uncut DNA). MspI restriction digestion of the PCR products distinguished DNA extracts from *S. frugiperda* corn and rice strains. MspI digested PCR products from corn (C), colony reared (Col) and sorghum larvae (S) as well as Sf9 cells (SF9) into expected sizes (497 and 72 bp) for *S. frugiperda* corn strain. However it did not digest PCR products from rice (R) or bermudagrass (B) larvae. The molecular weight standard (MWS) used was the 100-bp ladder (Gibco, BRL).

clonal cells) through isozyme analysis and autoradiography of ^{32}P labeled EcoRI restriction endonuclease profiles. Our non-radioactive analysis also characterized the *S. frugiperda* but concluded further that Sf9 was originally cultured from the corn strain.

This PCR-RFLP analysis of COI has been used to determine the isotype of several individual *S. frugiperda* larvae collected from corn, bermudagrass and sorghum. Strain identification of larvae is helpful in developing effective and specific pest management plans to control these insects. Field trapped *S. frugiperda* moths from various geographical locations could also be assessed by this method. Seasonal studies to investigate the population genetics of the *S. frugiperda* in which a large number of these individual insects are analyzed can exploit this mtDNA marker for distinguishing the cryptic strains.

ACKNOWLEDGMENTS

We thank Dr. James Nation for his help with this manuscript and the scientists mentioned in the text for the specimen donations. Florida Agricultural Experiment Station Journal Series No. R08045.

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