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Identification of Diatraea spp (Lepidoptera: Crambidae) based on Cytochrome oxidase II and SSCP V.1

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Abstract

Diatraea spp. (Lepidoptera: Crambidae) are a group of insects considered as an agriculture pest in many economically relevant crops such as sugarcane, sorghum, corn and rice. Currently, identification is based on the male genitalia. However, the availability of specimens collected from field and subjectivity on the character recognition can seriously hamper species identification, and therefore result in inadequate pest management. We described a protocol based on a fragment of the *cytochrome oxidase subunit* II (CO II) mitochondrial gene and Single-Strand Conformation Polymorphism (SSCP) methodology to properly recognizing each species and in consequence is proposed as an effective tool in *Diatraea* species identification.

Guidelines

1. Simon C, Frati F, Beckenback A, Crespi B, Liu H, Flook P. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved PCR primers. Ann Entomol Soc Am. 1994; 87: 651-701.
2. Lange CL, Scott KD, Graham GC, Sallam MN, Allsopp PG. Sugarcane moth borers (Lepidoptera: Noctuidae and Pyraloidea): phylogenetics constructed using COII and 16S mitochondrial partial gene sequences. Bull Entomol Res. 2004; *94*(5): 457-464
3. Palacio-Cortés AM., Zarbin PH, Takiya DM, Bento JM, Guidolin AS, Consoli FL. Geographic variation of sex pheromone and mitochondrial DNA in *Diatraea saccharalis* (Fab., 1794) (Lepidoptera: Crambidae). J Insect Physiol. 2010; 56: 1624-1630.

Materials

MATERIALS

Acrylamide **P212121**

bind silane **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**17-1330-01

Troubleshooting

Safety warnings

Acrylamide solution may cause cancer, heritable genetic damage, possible risk of impaired fertility. It is toxic if swallowed, and may causeserious damage to health by prolonged exposure through inhalation, in contact with skin and if swallowed Harmful by inhalation and in contact with skin. Irritating to eyes and skin and may cause sensitisation by skin contact

1 Dissection, DNA extraction and COII fragment amplification:

Abdomens from insects must be dissected and maintained in ethanol at 70 % v/v in sterile distilled water to DNA extractions. DNA of insects must be obtained from complete abdomen by using commercial kit (DNeasy Blood & Tissue Kit, QIAGEN) and quantified by spectrophotometry (Nanodrop 1000, Thermo-Fisher).

COII amplification: In vitro amplifications of CO II fragments must be carried out by the Polymerase Chain Reaction method (PCR) in standard conditions (final volume 25 µL) using Taq polymerase (PROMEGA), 50 ng of template and the primers previously described and used in Diatraea: A-298 (5′- ATTGGACATCAATGATATTGA-3′) and B-tLYS (5′ GTTTAAGAGACCAGTACTTG-3′) (Simon et al. 1994; Lange et al. 2004, Palacio et al. 2010). Thermal cycling was performed using the following conditions: 1 cycle at 95 °C for 3 min; 34 cycles at 95 °C for 10 s; 53 °C for 45 s, and 72 °C for 30 s; and one cycle at 72 °C for 5 min. Aliquots of amplification products were resolved in 1% w/v agarose gel electrophoresis and later stained with SYBR-Safe (Invitrogen). Amplicons will be used for SSCP methodology.

Single-Strand Conformation Polymorphism (SSCP)

2 **Preparing electrophoresis equipment**
For this protocol the Biorad Sequi-Gen® GT (38 × 50 cm) was used. The detailed instructions for assembly are in
http://www.cmu.edu.cn/cmu/upl_files/327/2007111485016635.pdf
To prepare Sequi-gen glass plate (IPC) and outer plates follow the Biorad manual instructions. Additionally, after cleaning, add silicone and shine the IPC (rub until silicone residue is completely removed). The outer plate must be prepared with bind silane (See: Reagent Preparation) and after cleaned two times with ethanol.
Note: Tips and paper towels used for the treatment of outer plate should be discarded immediately after use avoiding contamination of IPC.
Assembly the glass plate sandwich and slowly inject the polyacrylamide solution (6%, 49:1) with a 100 mL syringe avoiding bubbles formation. To prepare polyacrylamide solution and electrophoresis buffers see Reagent Preparation.

3 **Preparing sample and running electrophoresis**
After PCR of COII fragment, prepare the sample by mixing 9 µL of loading buffer (95% v/v Formamide, 20mM EDTA, 0.05% w/v bromophenol blue and 0.05% w/v Xylene-cyanol, in distilled water),) with 1 µL of PCR product. Denature for 5 minutes at 95 °C and immediately after cool on ice for 3 minutes. Add approximately 1.3 µL of PCR product mix into the wells. Place the electrodes caps and connect to the power supply. Apply 600 volts and run for 8 hours.

4 **Staining the gel (See Reagent Preparation).**

Stain gel using and adapted silver nitrate method. Briefly, gels fix for 3 min (10% v/v ethanol, 1% v/v acetic acid in distilled water), oxide for 3 min (1.5 % v/v nitric acid in distilled water), stain for 20 min (0.1% w/v silver nitrate, 0.045 % v/v formaldehyde in distilled water), reveal for approximately 5 min (3% w/v $NaCO_3$, 0.02 % v/v formaldehyde in distilled water), and stop reaction by adding acetic acid solution (5 % v/v in distilled water).

5 **REAGENT PREPARATION**

Bind silane Solution:

Absolute ethanol	95%
Acetic acid glacial	5 %
Bind-Silane	0.13%
Apply 1mL to outer glass	

Acrylamide bisacrylamide Stock (49:1; 40%)

Acrylamide	39.2 g
Bisacrylamide	0.8 g
Distilled water to 100 mL	

6% polyacrylamide solution (49:1)

Acrylamide bisacrylamide stock	15 mL
10X TBE Solution	10 mL
Distilled water	75 m
Distilled water to 100 mL	

Immediately before use add Ammonium Persulfate (10%) 850 ul and TEMED108 ul

TBE buffer (10X)

Tris base	53.85 g
Boric acid	27.51 g
0.5M EDTA (pH: 8.0)	20 mL
Add water to 500 mL	

STAINING POLYACRYLAMIDE GELS

FIXATION SOLUTION

Absolute ethanol	150 mL
Acetic acid glacial	15 mL
Distilled water	1335 mL
Add water to 1500 mL	

Leave for three minutes. Wash with distilled water for three minutes.

OXIDATION SOLUTION

Nitric acid	22.5 ml
Distilled water	1477.5 ml

Add water to 1500 ml

Leave for three minutes. Wash with distilled water for three minutes.

NITRATE SOLUTION

Silver nitrate	1.5 g
Formaldehyde	1.8 mL

Add distilled water to 1500 mL

Leave for twenty minutes. Wash with distilled water for ten seconds.

REVEALED SOLUTION

Sodium carbonate	30 g
Formaldehyde	540 ul

Add distilled water to 1000 mL

Leave until the gel reveals.

STOP SOLUTION

Acetic acid glacial	75 mL
Distilled water	1425 mL

Add distilled water to 1500 mL

Leave for four minutes. Wash with distilled water for four minutes.