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Identification of a new nucleopolyhedrovirus from naturally-infected *Condylorrhiza vestigialis* (Guenée) (Lepidoptera: Crambidae) larvae on poplar plantations in South Brazil

Maria Elita B. Castro [a,*], Zilda Maria A. Ribeiro [a], Ana Cláudia B. Santos [a], Marlinda L. Souza [a], Edilene B. Machado [b], Nilton J. Sousa [c], Flávio Moscardi [d]

[a] *Embrapa Recursos Genéticos e Biotecnologia, Parque Estação Biológica W5 Norte Final, 70770-917 Brasília-DF, Brazil*
[b] *Swedish Match do Brasil S.A., Curitiba-PR, Brazil*
[c] *Universidade Federal do Paraná, UFPR, Curitiba-PR, Brazil*
[d] *Embrapa Soja, Londrina-PR, Brazil*

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ABSTRACT

A baculovirus was isolated from larvae of *Condylorrhiza vestigialis* (Guenée) (Lepidoptera: Crambidae), a pest of a forest species known as Poplar (family Salicaceae, genus: *Populus*) with high economic value. Electron microscopy analysis of the occlusion body obtained from diseased larvae showed polyhedra containing multiple nucleocapsids per envelope. This baculovirus was thus named *Condylorrhiza vestigialis multiple nucleopolyhedrovirus* (CoveMNPV) and characterized by its DNA restriction endonuclease pattern, polyhedral protein, viral protein synthesis, and infectivity in insect cell lines. Restriction endonuclease profiles of viral DNA digested with five restriction enzymes were obtained and the CoveMNPV genome size was estimated to be 81 ± 2.5 kbp. The isolation of the polyhedra (OBs) was done from the crude extract of infected larvae by ultracentrifugation through sucrose gradients. These viral particles were analyzed by denaturing polyacrylamide gel electrophoresis (SDS–PAGE), which showed a strong band with approximately 33 kDa, corresponding to the main protein of the occlusion bodies (polyhedrin). Also, a similar band was observed for CoveMNPV infected *Spodoptera frugiperda* cells (SF-21 AE) pulse-labeled with [^{35}S] methionine and fractionated by SDS–PAGE. Of the four insect cell lines tested for susceptibility to CoveMNPV infection, the SF-21 AE was the most susceptible with occlusion bodies produced in most of the inoculated cells. This is the first record of an NPV from *C. vestigialis*.

1. Introduction

The *Baculoviridae* is a family of insect specific viruses with a large, circular, covalently closed, double-stranded DNA molecule that ranges in size from 80–180 kilobases (Theilmann et al., 2005). Two genera, *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GV), have been recognized and distinguished by occlusion body morphology. Occlusion bodies (OBs), also called polyhedra, contain many virions and are designated as single (S) or multiple (M) based on the number of nucleocapsids (NC) packaged in a virion (Theilmann et al., 2005). Based on phylogenetic relationships within the family, the NPV have been subdivided into groups I and II (Zanotto et al., 1993; Herniou et al., 2003).

Baculoviruses have high potential to be used as biological control agents against insect pests, besides their wide use as gene expression vectors in biotechnology. Due to their naturally high specificity, they are regarded as safe for environment and vertebrates (including man), and some species have been shown to be very effective biopesticides (Moscardi, 1999; Souza et al., 2007).

In this context, the current study is focused on characterization of a baculovirus isolated from *Condylorrhiza vestigialis* (Guenée, 1854) (Lepidoptera: Crambidae) infected larvae found in a forest species known as Poplar. This tree (*Populus* sp, family Salicaceae) is currently cultivated in approximately 5,000 ha, to supply the match industry and the manufacture of splints and boxes, and more recently as a possible source of biofuels. Recently, a group of scientists representing 34 institutions around the world published a draft genome of one of the varieties, the black cottonwood tree *Populus trichocarpa*, as an interesting model forest species for comparative plant genomics (Tuskan et al., 2006). According to the research team, this is the first tree and the third plant genome to be sequenced, coming after the herbaceous annual *Arabidopsis* and rice. Of the 45,500 genes identified, 93 were associated with the production of cellulose and lignin.

In Brazil, the defoliating caterpillar *C. vestigialis* is considered the main pest on poplar plantations in southern states, mainly in

* Corresponding author. Tel.: +55 61 3448 4691; fax: +55 61 3448 4673.
E-mail address: elita@cenargen.embrapa.br (M.E.B. Castro).

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Parana, since the 90's decade (Marques et al., 1995; Diodato and Pedrosa-Macedo, 1996). According to Diodato (1999), the *C. vestigialis* attack to *Populus* spp is always in patches causing a significant damage in the period of highest vegetative plant growth (December to March). In the larval stage, this pest is able to cause defoliation levels above 50% and up to 100% in two-year old plants, significantly affecting tree trunk diameter in subsequent years.

Currently, control of *C. vestigialis* larvae has mainly been made with the application of chemical insecticides of the pyrethroid group. Given the possibility for pests to develop resistance to this chemical group, researchers involved with this forest crop have investigated a new pest control alternative that lowers the environmental impact and other adverse effects from the use of these insecticides.

In this paper, the identification of a nucleopolyhedrovirus infecting *C. vestigialis* larvae is being reported for the first time based on morphological, biochemical and molecular data.

2. Materials and methods

2.1. Insects and cell lines

Original *C. vestigialis* infected larvae used in these studies were collected from poplar plantations located in São Mateus do Sul, Parana State, Brazil (Figs. 1 and 2). Initial larval examination and polyhedra detection were made at a Swedish Match laboratory in Curitiba, PR, using an optical microscope at 400x magnification. A purified OB stock was prepared in sufficient quantity and then used for all the steps of the experimental work reported herein.

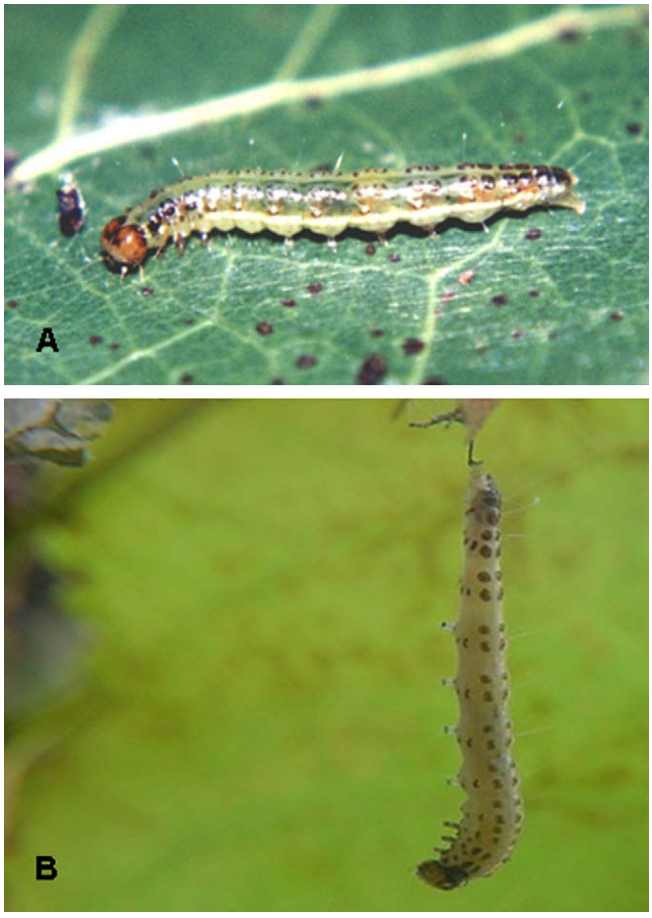

Fig. 1. Larvae of *Condylorrhiza vestigialis* (Guenée) (Lepidoptera:Crambidae). (A) Healthy larva; (B) virus diseased larva hanging from the top of poplar plant.

Spodoptera frugiperda IPLB-SF-21AE (Vaughn et al., 1977), *Trichoplusia ni* BTI-Tn-5B1–4 (Granados et al., 1994), *Anticarsia gemmatalis* UFL-AG-286 (Sieburth and Maruniak, 1988), and *Bombyx mori* BM-5 (Grace, 1967) cell lines were maintained at 27 °C in medium supplemented with 10% heat-inactivated fetal bovine serum. The culture medium used was TNMFH (Grace's insect medium supplemented with lactalbumin hydrolysate and yeastolate), except for UFL-AG-286 cells, which were cultivated in TC-100 (Gibco-BRL). These insect cell lines were used for viral infectivity tests.

2.2. Purification of viral occlusion bodies (OBs)

Viral OBs were purified according to procedures described by Maruniak (1986). Briefly, viral OBs from homogenized larvae were purified by centrifugation on continuous 40–65% (w/w) sucrose density gradient at 100,000 *g* for 40 min at 4 °C (Sorvall AH 627 rotor). The band containing the virions was removed from the gradient with a Pasteur pipette, diluted to 3 times its original volume with TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and centrifuged again at 14,000 *g* for 30 min at 4 °C. The OBs were resuspended in sterile distilled water, quantified using a counting chamber (Haemocytometer) and stored at −20 °C. The OBs purified were either analyzed by microscopic studies and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) or used for viral DNA extraction.

2.3. Light and transmission electron microscopy

A drop of purified OB suspension was mounted on a glass slide with a coverslip and examined under an Axiovert 135 M Zeiss light microscope (OM). For electron microscopy (TEM), the pellets of purified OB were fixed in 2.5% glutaraldehyde in 0.02 M cacodylate buffer (pH 7.2) for 2 h, and then post-fixed in 1% osmium tetroxide in the same buffer for 1 h. The fixed samples were dehydrated by immersion in 0.5% uranyl acetate and washed in a graded series of ethanol, and then embedded in Spurr's resin (Spurr, 1969). Ultrathin sections were prepared on a Reichert OMU3 Ultramicrotome (Reichert Supernova Ultramicrotome), stained with 2% uranyl acetate followed by Reynolds lead citrate (Reynolds, 1963), and observed in a JEOL 1011 transmission electron microscope at 80 kV.

2.4. Extraction of viral DNA

Purified occlusion bodies were disrupted with a diluted alkaline solution (0.3 M sodium carbonate, 0.5 M NaCl, and 0.03 M EDTA, pH 10.5) at 37 °C for 30 min. Following the dissolution of the OB, the undissolved material was removed by low-speed centrifugation (1,500 g for 5 min), and the supernatant was treated with 1% SDS and 0.5 mg/ml proteinase K at 37 °C overnight. Viral DNA was extracted first with TE buffer-satured phenol, then with phenol/chloroform/isoamyl alcohol (25:24:1), and lastly with chloroform/isoamyl alcohol (24:1). The aqueous phase containing the viral DNA was precipitated by adding 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of absolute ethanol and then pelleting by centrifugation at 14,000 g for 15 min. The resulting DNA pellets were washed with 70% ethanol, dried, and then resuspended in TE buffer. The DNA concentration was estimated relative to a dilution series from the λ-DNA standard of known concentration in an agarose gel, stained with ethidium bromide.

2.5. Digestion of viral DNA with restriction endonucleases (REN)

For REN analysis, 3 μg viral DNA was digested with *Bam*HI, *Bst*EII, *Eco*RI, *Hin*dIII, *Pst*I separately in appropriate reaction buffers for 5 h at 37 °C. Equal amounts of each reaction was loaded on a