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

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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.

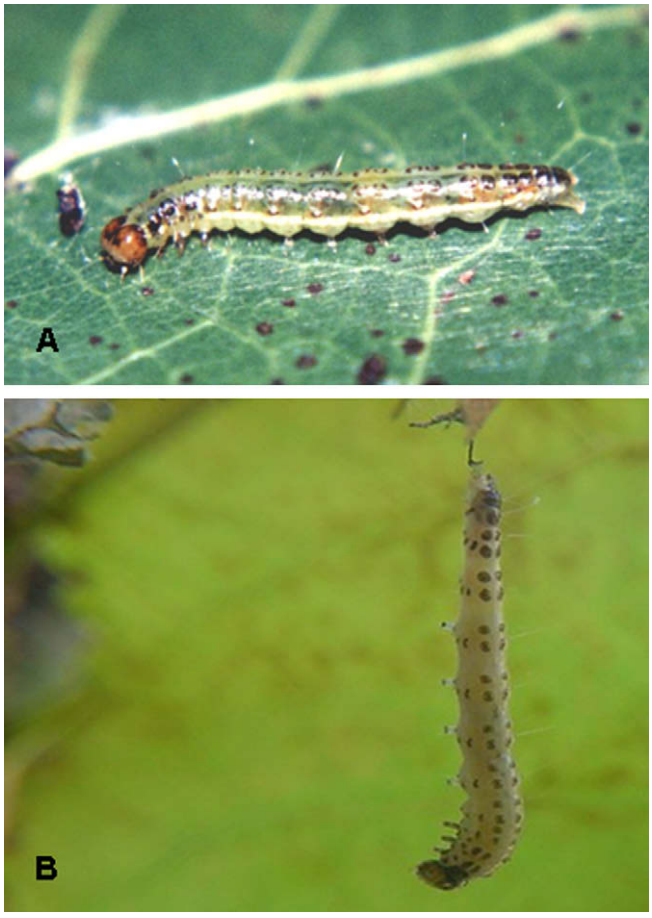


**Fig. 1.** Larvae of *Condylorrhiza vestigialis* (Guenée) (Lepidoptera:Crambidae). (A) Healthy larva; (B) virus diseased larva hanging from the top of poplar plant.

*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 $\lambda$-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

**Fig. 2.** Poplar (*Populus* sp.; Salicaceae) plantations, in São Mateus do Sul, PR, Brazil. At right, heavy defoliation caused by the lepidopterous pest *Condylorrhiza vestigialis*.

1.5% agarose gel. The electrophoresis was carried out at 30 V overnight (14–16 h) using TAE buffer (0.04 M Tris/acetate, 0.001 M EDTA, pH 8.0). The DNA fragments were visualized by staining with 0.5 μg/ml ethidium bromide and photographed under UV light. Two commercial 1 kb DNA ladders, containing fragments with sizes ranging from 500–10,000 bp (Sigma) and 250–10,000 bp (Promega), were used as standard for size determination.

### 2.6. Polyacrylamide gel electrophoresis (SDS–PAGE) of occlusion bodies (OBs)

Purified OB samples were disrupted by boiling for 5 min in loading buffer containing β-mercaptoethanol and electrophoresed through 15% sodium dodecyl sulphate–polyacrylamide gel according to the method of Laemmli (1970). Virus samples and molecular weight standard were run simultaneously for 18 h at constant voltage (40 V). Proteins were visualized by staining with solution containing 40% (v/v) methanol, 10% (v/v) acetic acid and 0.1% (w/v) *Coomassie Brilliant Blue R-250*, followed by destaining with 40% methanol and 10% glacial acetic acid.

### 2.7. Viral infection in vitro

Cells from each cell line were seeded at a density of $3 \times 10^5$ cells per well (6-well tissue culture plates) and incubated overnight at 27 °C, to allow their attachment. Subsequently, the cells were inoculated with hemolymph from CoveMNPV-infected larvae. The controls (mock-infected cells) received the same treatment, except that the inoculum did not contain any virus. After 1 h of adsorption, the inoculum was removed, the cells were washed and fresh medium was then added. The infected cells were incubated at 27 °C and examined at 24, 48, and 72 h post infection (h p.i.) by phase-contrast microscopy.

### 2.8. Protein synthesis labeling assay

IPLB-SF-21AE cells were seeded at a density of $3 \times 10^5$ per well (6-well tissue culture plates) and inoculated with supernatant of CoveMNPV-infected cells. The control cells did not contain any virus. After an adsorption period of 1 h at 27 °C (zero h p.i.) the virus inoculum was removed and cells were washed and incubated in complete culture medium (TNMFH) for 48 and 72 h p.i., at 27 °C. At 30 min before the completion of each time period, the medium was replaced by phosphate-buffered saline (PBS, pH 6.2), and the incubation was continued (starvation period). The medium was removed and the cells were labeled with 50 μCi of [$^{35}$S] methionine in PBS for 1 h (pulse). Mock-infected cells were labeled and harvested at the 24 h time point. After labeling, the cells were collected by centrifugation for 30 s, rinsed with PBS and frozen at −20 °C. Sample disruption and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) were carried out according to Laemmli (1970). To visualize the labeled proteins, the gel was treated with a fluorographic reagent (Amplify; Amersham) for 30 min, dried and exposed to Kodak T-MAT film at −80 °C.

## 3. Results and discussion

### 3.1. Morphological data

Phase-contrast microscopy of the suspension material from diseased larvae revealed the presence of numerous occlusion bodies (OBs), which were highly refractive (Fig. 3A). Electron microscopy analyses revealed that these particles are polyhedral-shaped OBs, containing multiple-embedded, membrane-bound, and rod-shaped nucleocapsids (Fig. 3B, C). These observations suggested that the causal agent of the *C. vestigialis* disease was a *multiple nucleopolyhedrovirus* (CoveMNPV). The OB possessed the outer envelope

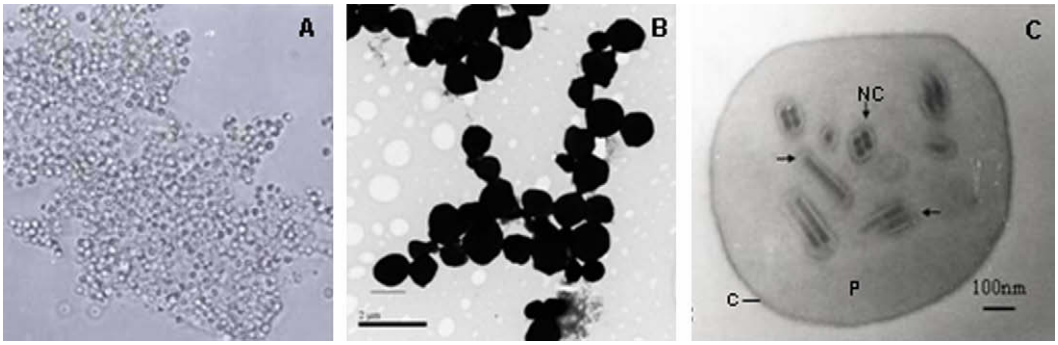


**Fig. 3.** Occlusion bodies purified from diseased *Condylorrhiza vestigialis* larvae. (A) Phase-contrast microscopy presenting numerous virus particles (1,000X). (B) Leaf-dip preparations showing multiple polyhedrical particles. *Scale bar* = 2.0 μm. (C) Transmission electron micrograph showing the paracrystalline structure of the occlusion body; cross and longitudinal sections of virions (*arrows*) containing multiple nucleocapsids (NC). P- polyhedrin protein. C- polyhedron calyx. *Scale bar* = 100 ηm.

(the polyhedron envelope of NPV) and the crystalline protein lattice surrounding the bundles of virions that is a characteristic feature of most baculoviruses (Theilmann et al., 2005).

### 3.2. Restriction endonuclease analysis of viral DNA

Genomic DNA restriction analyses were made with the aim to obtain useful evidences for identifying the virus and estimating the size of its genome. The CoveMNPV DNA was digested with five restriction endonucleases and analyzed by agarose gel electrophoresis (Fig. 4). This analysis yielded distinct fragments for each restriction enzyme: 8*Bam*HI fragments, 14*Bst*EII fragments, 7*Eco*RI fragments, 18*Hin*dIII fragments and 14*Pst*I fragments. These numbers represent the minimal number of cleavage sites on the genome for these enzymes. Fragments smaller than about 1,000 bases were not detected on the 1.5% agarose gel. From the sum of the fragment sizes relative to the 1 kb DNA ladder, the CoveMNPV genome was estimated to be 81 ± 2.5 kbp.

This prediction is within the range from 80–180 kbp for baculovirus genomes (Theilmann et al., 2005), and places the CoveMNPV very close to the smallest baculovirus genomes published so far. Of the genome sequences of 48 baculoviruses currently available in NCBI databases, the *Neodiprion lecontei* NPV with 81.755 kbp (Lauzon et al., 2004), *Neodiprion abietis* NPV with 84.264 kbp (Duffy et al., 2006), and *Neodiprion sertifer* NPV with 86.462 kbp (Garcia-Maruniak et al., 2004) have the smallest genomes. However, given that the estimated genome sizes of the sequenced lepidopteran baculoviruses are all above 100 kbp, further sequencing studies are required to determine CoveMNPV genome size more accurately.

Restriction profiles of CoveMNPV were distinct from those of other well-known NPVs, such as AcMNPV (Smith and Summers, 1978, 1979; Miller and Dawes, 1979), AgMNPV (Johnson and Maruniak, 1989), CfMNPV (Arif et al., 1984), OpMNPV (Chen et al., 1988), LdMNPV (McClintock and Dougherty, 1988), SfMNPV (Maruniak et al., 1984), SeMNPV (Brown et al., 1984), and some others described in a publication on DNA homology among 18 baculoviruses (Smith and Summers, 1982).

### 3.3. Analysis of viral structural proteins by SDS-PAGE

The protein components of the occlusion bodies were analyzed by denaturing polyacrylamide gel electrophoresis (SDS–PAGE) and sizes were estimated using molecular weight markers (Sigma) (Fig. 5). The protein profile showed, specifically, a single major protein band with an estimated molecular weight of 33 kDa. This is a characteristic of polyhedrin proteins, which are the most abundant components of the occlusion bodies of nucleopolyhedroviruses (NPV) (Theilmann et al., 2005).

### 3.4. Specificity of the baculovirus CoveMNPV to different insect cell lines and protein synthesis in CoveMNPV-infected SF-21AE cells

Considering that no cell lines from its natural host is currently available for the newly identified baculovirus (CoveMNPV) and

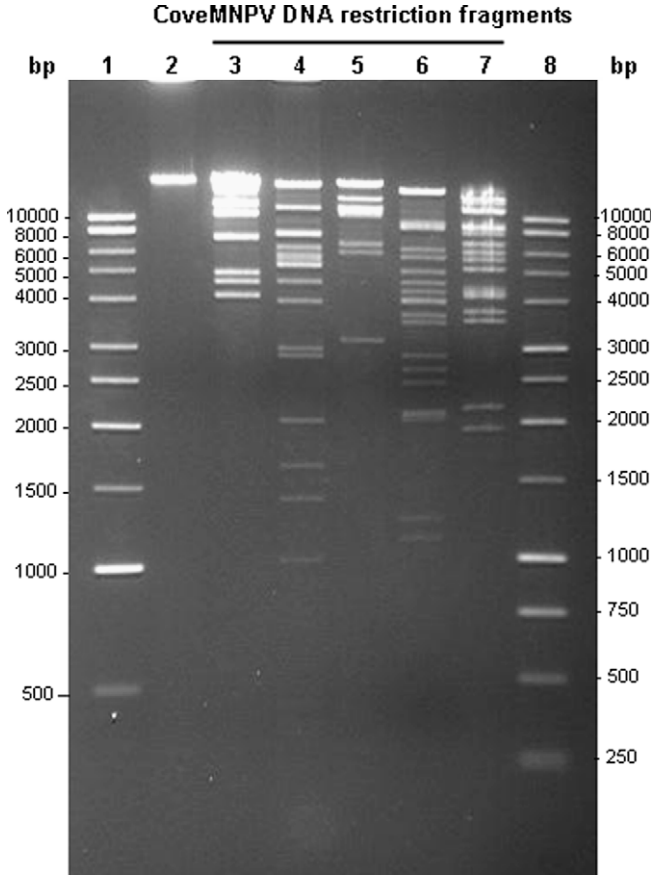


**Fig. 4.** Restriction endonuclease cleavage patterns of *Condylorrhiza vestigialis* MNPV DNA. Electrophoresis in 1.5% agarose gel stained with ethidium bromide. *Lanes 1* and *8* are molecular size markers in basepairs (bp): 1 kb DNA ladder (Sigma) and 1 kb DNA ladder (Promega), respectively. *Lane 2*: non-digested DNA (control); lane 3: *Bam*HI-restricted DNA; lane 4: *Bgl*II-restricted DNA; lane 5: *Eco*RI–restricted DNA; line 6: *Hin*dIII–restricted DNA; lane 7: *Pst*I-restricted DNA. Submolar bands were not apparent but may be present at a lower concentration.

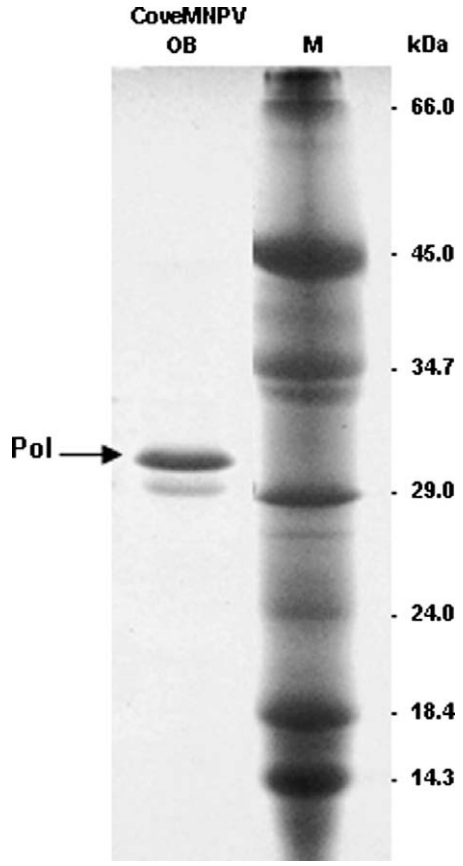


**Fig. 5.** Structural protein analysis of *Condylorrhiza vestigialis* MNPV. Purified occlusion bodies in 15% denaturing polyacrylamide gel stained with *Coomassie Blue*. A band of approximately 33 kDa, corresponding the polyhedrin, is shown in the first lane (arrow). Molecular mass (M) is expressed in kilodaltons on the right.

that the identification of an alternative host should be useful in biocontrol research, four lepidopteran cell lines were tested for their susceptibility to this baculovirus. CoveMNPV infectivity was tested in SF-21AE, Tn-5B1–4, UFL-AG-286 and BM-5 cells, the morphological alterations were observed by phase-contrast microscopy (Fig. 6), and protein synthesis was analysed for the most susceptible cell line to the virus (Fig. 7).

The SF-21AE and UFL-AG-286 lines were permissive to CoveMNPV exhibiting cellular rounding, nuclear hypertrophy and polyhedra formation. While the SF-21AE cell line was the most susceptible to CoveMNPV, presenting higher infection level than all others lines tested, the UFL-AG-286 presented very low polyhedra production and cell lysis. Tn-5B1–4 and BM-5 cells were not productive and underwent lysis after incubation with CoveMNPV, the effect being more prominent in the later. The result that SF-21AE cell line is susceptible to CoveMNPV infection is significant in that there are currently no cell lines from its natural host (*C. vestigialis*).

To investigate whether the CoveMNPV infection detected in SF-21AE cells would be productive or not, radiolabeling experiments were carried out to examine the viral protein synthesis in this system. SF-21AE cells infected with CoveMNPV and labeled with [$^{35}$S] methionine showed different polypeptide synthesis patterns from that observed in the control cells (Fig. 7). At late times post infection an intensive band of around 33 kDa was identified that corresponded to the size of polyhedrin proteins from other baculoviruses (Maruniak, 1986). This observation is consistent with the presence of polyhedra noted in infected SF-21AE cells (light microscopy) and the results obtained from viral structural protein analysis (SDS–PAGE).

In summary, our findings clearly show that the disease detected in *C. vestigialis* larvae was caused by a baculovirus, which was identified and designated here as *Condylorrhiza vestigialis multiple nucleopolyhedrovirus* (CoveMNPV). Therefore, this virus was named according to the insect host species from which it was first isolated. This baculovirus is being considered an interesting candidate for use in biological and integrated control programs that target *C. vestigialis*. Data demonstrate that this virus is highly virulent to third-instar *C. vestigialis* larvae in laboratory and field trials (Machado, 2006). Tests to evaluate the larval mortality using a virus suspension around $10^7$ and $10^8$ OBs/ml, resulted in average mortality rates from 80% to 100%, after 7–9 days of infection.

A method based on comparative sequence analysis of highly conserved genes has been successfully applied for identification of lepidopteran-specific baculoviruses (Lange et al., 2004). To better investigate the taxonomic and phylogenetic status of CoveMNPV, a procedure similar to this method, but based on the sequences of individual genes, was used in subsequent studies carried out in our laboratory. The *p74* gene encodes a protein that is highly conserved among all sequenced baculoviruses and is essential for *per os* infectivity. Thus, the *p74* gene from CoveMNPV was identified and sequenced (*GenBank/EMBL accession number EU919397*), and deduced amino acid and nucleotide sequences were compared with

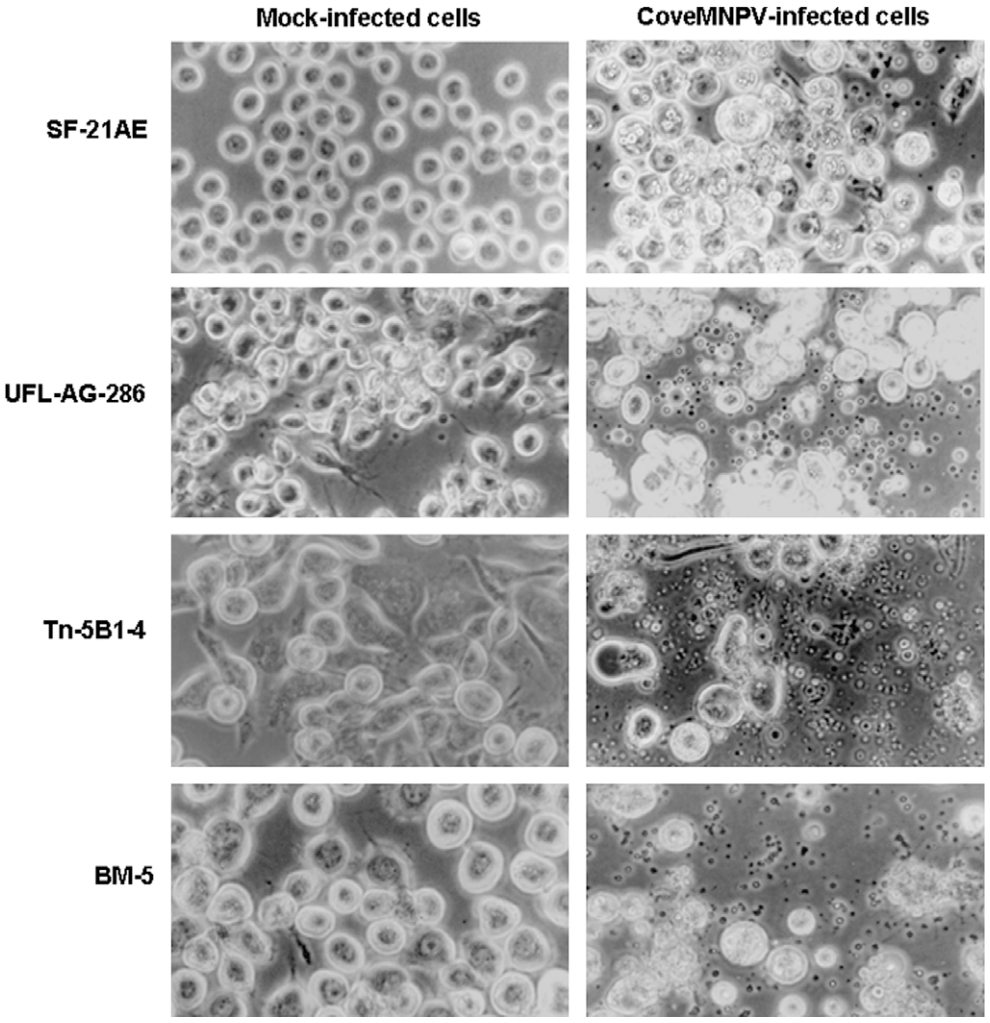


**Fig. 6.** Phase-contrast micrographs of different insect cell lines. Mock-infected cells (left) and CoveMNPV-infected cells at 72 h p.i. (right). 320X. Occlusion bodies are visualized in the CoveMNPV-infected SF-21AE cells.

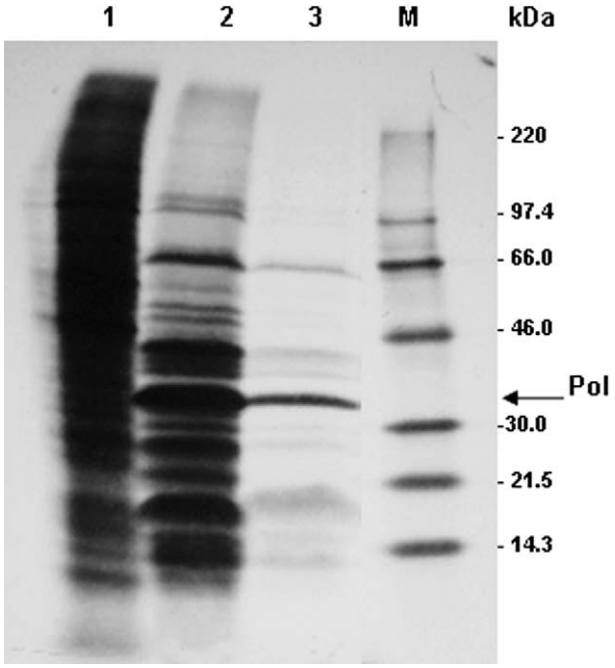


**Fig. 7.** CoveMNPV protein synthesis in SF-21AE cells. Autoradiogram of cells pulse-labelled with [$^{35}$S] methionine for 1 h: lane 1- mock-infected cells, lane 2- cells at 48 h p.i., lane 3- cells at 72 h p.i., M- molecular weight marker - Rainbow [$^{14}$C] methylated protein (Amersham) expressed in kilodaltons (14300–220000). A dominant protein band at ~33 kDa that corresponds to the expected size of polyhedrin is shown in the lanes 2 and 3 (arrow).

other baculoviral P74 sequences to estimate their phylogenetic relationships. These data showed that CoveMNPV differs from all other sequenced baculoviruses (Almeida, 2008).

The results obtained so far combined with the findings presented here provided consistent data to support that the virus CoveMNPV belongs to the lepidopteran NPV Group I, and that this species differs from the baculoviruses described to date. On this basis, we reported here that CoveMNPV was found for the first time in *C. vestigialis* and that this virus might be a distinct baculovirus species.

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