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## Characterization of a new isolate of *Malacosoma neustria* nucleopolyhedrovirus (ManeNPV) from Turkey

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**Abstract:** The lackey moth, *Malacosoma neustria* (Linnaeus, 1758), a worldwide pest, causes extensive economic losses on particularly hazelnut, *Prunus*, *Quercus*, *Populus*, and *Salix* trees. In this study, a local nucleopolyhedrovirus (NPV) was isolated from the larvae of *M. neustria* in the northeast of Turkey. It was named ManeNPV-T2. Electron microscopic observations showed that the polyhedra of the new isolate contain several virions with multiple nucleocapsids packaged within a single viral envelope. Restriction endonuclease analysis of ManeNPV-T2 DNA indicated that it has a different restriction profile compared to the previous Turkish and Latvian isolates. The phylogenetic analysis of the amplified *polh* sequence of ManeNPV-T2 showed its relation to the other NPVs from *Malacosoma* species. Mortality values for third instar *M. neustria* larvae ranged from 48% to 100% according to the concentration of polyhedral occlusion bodies. Based on screening tests, the LD<sub>50</sub> value of the new isolate was calculated as  $1.34 \times 10^4$  occlusion bodies per larvae. Consequently, ManeNPV appears to be a promising microbial control agent for biocontrol of *M. neustria*.

**Key words:** *Malacosoma neustria*, nucleopolyhedrovirus, characterization, ManeNPV, insect virus

### 1. Introduction

Baculoviruses are enveloped viruses that have double-stranded, circular DNA genomes ranging in size from 80 to 180 kbp (1,2). Baculoviruses have been isolated from more than 600 insect species belonging to the orders of Lepidoptera, Hymenoptera, Diptera, Orthoptera, Coleoptera, Neuroptera, Thysanura, and Trichoptera (3). The most notable characteristic of baculoviruses is the occlusion body (OB). The occlusion body is a crystalline matrix composed of a protein called polyhedrin, which provides protection to the virions in the environment against proteolytic and chitinolytic enzymes in the decomposing larvae and spreads infection among insects (4,5). Baculoviruses have been taxonomically divided based on their OB morphology into nucleopolyhedroviruses (NPVs) and granuloviruses (GVs), forming polyhedra and granula, respectively (6). In the NPV, the OB ranges in size from 0.4 to 5 µm in diameter and contains several virions (7,8). The OBs of NPVs are most easily seen under light microscope due to their larger size and their light refractory polyhedral structure. The OBs of GV, called granula, appear as dark granules and are comparatively more difficult to resolve under light microscope. They are ovoid-shaped and about 500 nm long and 20 nm wide (8,9).

The family *Baculoviridae* is divided into 4 genera according to common biological and structural

characteristics and patterns of host associations: *Alphabaculovirus*, which includes lepidopteran-specific baculoviruses and is subdivided into Group I or Group II based on the phylogenetic analysis of the polyhedrin genes from different baculoviruses; *Betabaculovirus*, comprising lepidopteran-specific granuloviruses; *Gammabaculovirus*, which includes hymenopteran-specific baculoviruses; and finally *Deltabaculovirus*, which contains dipteran-specific baculoviruses (4,10,11).

*Malacosoma neustria* (Linnaeus, 1758) (Lepidoptera: Lasiocampidae), known as the European tent caterpillar, is an important defoliator of various fruit trees such as apple, pear, and plum; wild and ornamental trees and shrubs, including oak and rose species, oleaster, sea buckthorn, barberry, elm trees, willow, poplar, and aspen; and birch, particularly in eastern and central Turkey (12–14). The caterpillars first eat the buds and then leaves of the trees. During some years their population reaches such high numbers that they leave the trees completely leafless. Factors such as weather conditions and natural enemies including predators, parasitoids, and pathogenic microorganisms have historically been important regulatory elements in the population cycles of this insect.

The key microbial pathogens of this insect include NPVs (15). The presence of NPVs in *M. neustria* was reported by several authors (16–31). In 2009, Demir et al.

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(31) worked with a field isolate of ManeNPV from Turkey (ManeNPV-T2). They studied the in vitro replication properties of this virus in the Md203 cell line, derived from *Malacosoma disstria*. In another study (32), they demonstrated the susceptibility of various cell lines derived from different lepidopteran hosts to that field isolate. In the current study we report the morphological features, molecular characterization, and partial polyhedrin (*polh*) gene analyses of ManeNPV-T2 from Turkey (31), using electron microscopy and DNA restriction endonuclease profile and phylogenetic analysis. The virulence of the virus was also tested against *M. neustria* larvae.

## 2. Materials and methods

### 2.1. Insect and virus

*Malacosoma neustria* larvae were collected from both hazelnut and various fruit trees in the northeastern Black Sea Region of Turkey and brought to the laboratory where they were reared on appropriate foliage until they either died or pupated.

The ManeNPV used in this study was isolated from dead and field-collected *Malacosoma neustria* larvae from the northeast of Turkey by Demir et al. (31). After detecting the baculovirus infection under light microscope from dead insects, OBs were purified according to the procedure described by O'Reilly et al. (33). Viral propagation was performed in healthy *M. neustria* larvae in the laboratory. The larvae were placed in plastic dishes, fed with a few leaves contaminated with OBs isolated from the infected larvae, and maintained at 25 °C to develop infection. OBs from newly infected larvae were purified and stored at -20 °C.

### 2.2. Electron microscopy

A suspension of purified OBs was placed on a round coverslip and allowed to air dry. The coverslip was glued onto a 1.27-cm aluminum stub, sputter-coated with gold for 3 min, and examined with a JEOL JSM 6400 scanning electron microscope.

For transmission electron microscopy analysis, purified OBs were fixed in 2% glutaraldehyde and 0.1 M phosphate buffer (pH 7.2), and postfixed in 1% OsO<sub>4</sub>. OBs were then embedded in Epon-Araldite resin, and ultrathin sections were cut using glass knives on a Leica Reichert Ultracut S ultramicrotome stained with uranyl acetate and then examined on a transmission electron microscope (Zeiss EM900) at 80 kV (34).

### 2.3. Isolation of viral DNA and restriction enzyme analysis

Purified OBs were resuspended in 0.1 M Na<sub>2</sub>CO<sub>3</sub> and incubated at 37 °C for 30 min to dissolve the polyhedrin matrix and release the virions. Viral DNA was extracted from the polyhedra-derived virus particles according to the method described by Reed et al. (35). After phenol

chloroform extraction, viral DNA solution was dialyzed against 3 changes of 0.1X TE buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) at 4 °C for 24–48 h.

For restriction enzymes analyses, 5 µg of viral DNA was digested with *Bam*HI, *Kpn*I, *Pst*I, *Hind*III, and *Eco*RI restriction enzymes (Promega) at 37 °C for 3.5 h. Digested fragments were separated by electrophoresis in a 1% agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 15 mA for 18 h. The gel was stained with ethidium bromide and photographed on a UV transilluminator.

### 2.4. Amplification of *polh* gene and phylogenetic analysis

The ManeNPV *polh* gene was amplified from viral DNA by polymerase chain reaction (PCR). The degenerate primer set used in this study (F: 5'-TAY GTG TAY GAY AAC AAG T-3' and R: 5'-TTG TAR AAG TTY TCC CAG-3') was previously described by de Moraes and Maruniak (36). M13 (-20) forward and reverse primer sequences were added to the ends of the degenerate polyhedrin forward and reverse primers, respectively. The PCR reaction mixture was prepared as 25 µL containing 30–50 ng of viral DNA, 400 nM of each primer, 0.2 mM of each dNTP, 0.5 U of *Taq* DNA polymerase (Promega), 1.5 mM of MgCl<sub>2</sub>, and 2.5 µL of 10X reaction buffer (Promega). Reaction was carried out using the following parameters: after 5 min of denaturation step at 95 °C, 10 cycles of 60 s at 94 °C, 45 s at 45 °C, and 60 s at 72 °C, followed by 25 cycles of 45 s at 94 °C, 30 s at 50 °C, and 60 s at 72 °C. Amplification was completed with a final extension step of 5 min at 72 °C. The PCR product was first cloned into the pGEM-T Easy (Promega) vector and then sequenced.

To show the position of the ManeNPV-T2 isolate among the other *Malacosoma* NPVs in GenBank, we performed a phylogenetic analysis using the partial polyhedrin gene sequences of 10 other isolates belonging to *Malacosoma* species. Additionally, 2 polyhedrin gene sequences and a granulins sequence belonging to other baculoviruses were also used in order to infer the phylogenetic relationships within these isolates. Polyhedrin gene sequences were obtained from GenBank. A maximum parsimony phylogenetic tree was constructed based on the DNA sequence alignment using MEGA 5 software. The robustness of the phylogenetic tree was tested by bootstrap analysis with 1000 replicates.

### 2.5. Concentration–mortality response test

Concentration–mortality response tests were conducted with the new isolate on third instar *M. neustria* larvae. OB suspensions were prepared as 5 doses (10<sup>4</sup>, 10<sup>5</sup>, 10<sup>6</sup>, 10<sup>7</sup>, and 10<sup>8</sup> OB mL<sup>-1</sup>). Experiments were performed with 15 larvae per dose and were replicated 3 times for each dose. Larvae that had been starved for 12 h prior to the virus challenge were fed with natural foliage contaminated with OB suspensions. After 24 h, all larvae were fed with

fresh diets and incubated at 24 °C with an 8 h dark/16 h light photoperiod. The control larvae group was treated with only water. Mortality was assessed daily; dead larvae were removed and were checked for NPV infection under phase-contrast microscope, as determined by the presence of viral OBs. Mortality data were evaluated by using Abbott's formula (37) and the LD<sub>50</sub> value was calculated by probit analysis. Experiments were repeated 3 times.

### 3. Results

#### 3.1. Electron microscopy

The electron microscopy studies revealed typical baculovirus occlusion bodies (Figure 1). Scanning electron microscopy showed that the polyhedral inclusion bodies (PIBs) were irregularly shaped and ranged in size from 0.87 to 1.75 µm in diameter (Figure 1A). The transmission electron microscopy revealed that occlusion bodies were occupied by several virions with multiple nucleocapsids packaged within a single viral envelope (Figure 1B). The length of the rod-shaped nucleocapsids was approximately 260 nm and its width measured approximately 50 nm. Since there is another isolate of NPV from the European tent caterpillar from Turkey, we have designated this isolate as ManeMNPV-T2 to indicate its second isolation in Turkey.

#### 3.2. Restriction endonuclease analysis of ManeNPV

Restriction analysis of the ManeNPV-T2 DNA purified from the viral inclusion body yielded 20 *EcoRI* fragments, 12 *HindIII* fragments, 8 *PstI* fragments, 7 *KpnI* fragments, and 6 *BamHI* fragments. All restriction endonuclease

reactions resulted in different fragment profiles. The size of all restriction endonuclease fragments was observed clearly on 1% agarose gels (Figure 2).

#### 3.3. Polyhedrin gene phylogeny

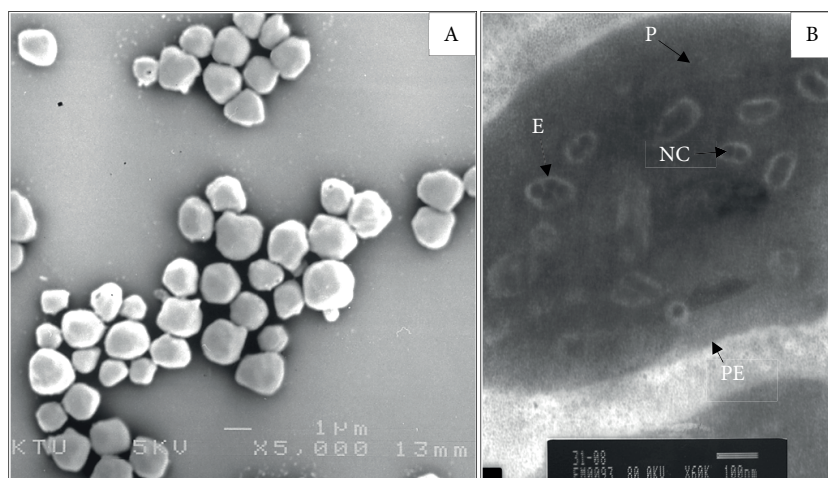
The purpose of this analysis was to show the taxonomic position of ManeNPV-T2 among the other *Malacosoma* NPVs. To this aim, the sequence of a nearly 500-nt fragment of ManeNPV-T2 polyhedrin gene was aligned with 10 polyhedrin genes from other *Malacosoma* NPVs, 2 polyhedrin genes from other baculoviruses, and a granulin gene from *Trichoplusia ni granulovirus* (GV). The phylogenetic analysis of the ManeNPV-T2 *polh* sequence showed its relation together with the NPVs from *Malacosoma* group (Figure 3).

#### 3.4. Pathogenicity tests

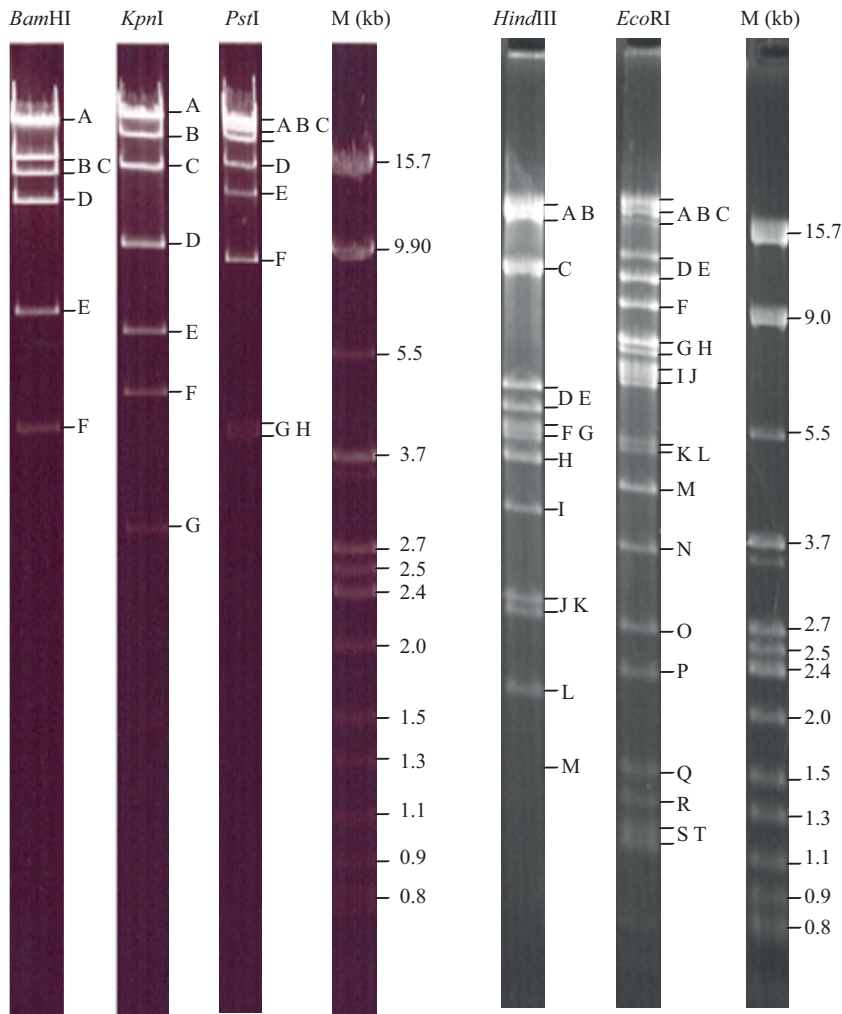
The insecticidal activity of ManeNPV-T2 was determined by bioassays using third instar *M. neustria* larvae. Doses of 10<sup>6</sup>, 10<sup>7</sup>, and 10<sup>8</sup> ManeNPV-T2 OBs caused 100% mortality. The mortality rate for 10<sup>5</sup> OBs was noted as 58%. The lowest mortality (48%) was obtained with 10<sup>4</sup> OBs (Figure 4). Larval deaths started at 4 days post infection (p.i.) with 10<sup>8</sup> OBs and reached 100% at 6 days p.i. Probit analysis was used to calculate the LD<sub>50</sub> value. The LD<sub>50</sub> of ManeNPV-T2 against 10 days after treatment was determined to be 1.34 × 10<sup>4</sup> OBs per larvae.

### 4. Discussion

We report here the characterization of a multicapsid NPV from *M. neustria* and its pathogenicity to the European tent caterpillar. The virus was isolated from diseased



**Figure 1.** Electron micrographs of purified PIBs from *Malacosoma neustria* nucleopolyhedrovirus. A) Scanning electron micrograph showing purified polyhedra. B) Transmission electron micrograph of section of purified polyhedron inclusion body. Details of a polyhedron showing multiple nucleocapsids surrounded by a single membrane. The polyhedron envelope (PE), the polyhedrin matrix (P), the virion envelope (E), and the rod-like nucleocapsid (NC) are indicated.

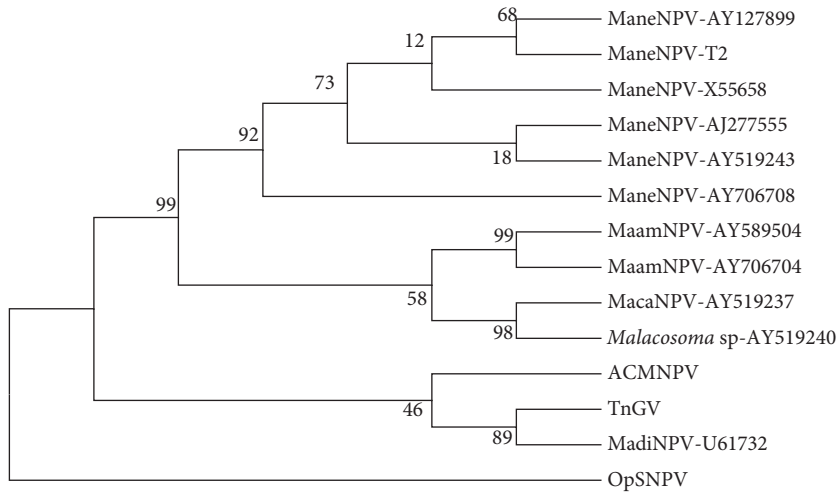


**Figure 2.** Restriction profile of ManeNPV-T2 genomic DNA. Restriction endonucleases: *Bam*HI, *Kpn*I, *Pst*I, *Hind*III, and *Eco*RI. Restriction fragments were separated by electrophoresis on a 1% agarose gel at 20 V. M: Lambda DNA's *Eco*RI/*Bam*HI/*Hind*III fragments (restriction fragments were run on two different gels).

*M. neustria* larvae from the northeast of Turkey. In this study, electron microscopic observations confirmed that this isolate was a multicapsid NPV (Figure 1B). Since baculoviruses are identified and named according to the insect host species from which they were first isolated, the viral isolate was designated as *M. neustria* NPV (ManeNPV). In order to differentiate our isolate from the previous Turkish isolate (30), this isolate has been further named as ManeNPV-T2. The diameter of the PIBs of ManeNPV-T2 measured between 0.87 and 1.75  $\mu\text{m}$ . When we compared the PIB diameter of ManeNPV-T2 with the PIB diameters of other ManeNPVs in the literature, it was obvious that the PIB diameter of ManeNPV-T2 was smaller than those of the previous Turkish isolate, the Polish isolate (38), and the isolate used by Ponsen et al. (39), which have PIB diameters of 0.76–3.85  $\mu\text{m}$ , 1–3.5

$\mu\text{m}$ , and 0.9–2.0  $\mu\text{m}$ , respectively. However, it is larger than the Latvian isolate's (29) PIB diameter, which is between 0.85 and 1.4  $\mu\text{m}$ .

The nucleocapsid sizes of different ManeNPV isolates in the literature were also compared to the nucleocapsid size of ManeNPV-T2, which has a nucleocapsid size of 250 nm in length and 50 nm in width. This comparison showed that ManeNPV-T2 has a smaller nucleocapsid size than the Polish isolate, the Latvian isolate, and the isolate used by Bergold (40), which have nucleocapsid lengths and widths of 310  $\times$  50, 360  $\times$  80, and 315–324  $\times$  40–46 nm, respectively. However, nucleocapsid lengths and widths of the previous Turkish isolate and the isolate used by Ponsen et al. (39) were 240  $\times$  35 and 250  $\times$  25 nm, respectively, which are smaller than the nucleocapsid size of ManeNPV-T2. In the cross-sections of polyhedra,

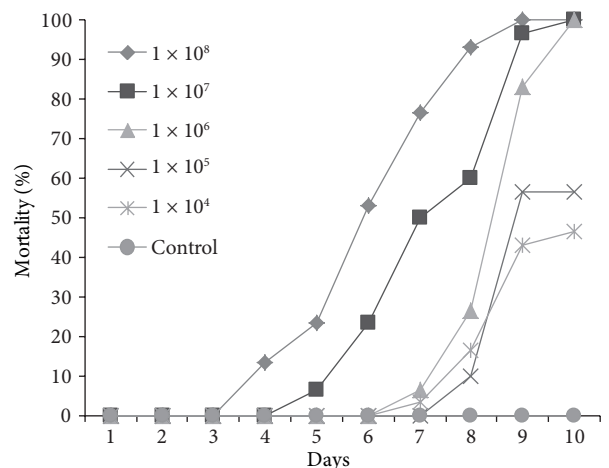


**Figure 3.** Phylogeny of NPV of *Malacosoma* species. The tree was obtained by maximum parsimony analysis of partial *polh* DNA sequence data. The numbers above branches indicate bootstrap scores. AcMNPV (*Autographa californica multiple nucleopolyhedrovirus*), OpSNPV (*Orgyia pseudotsugata single nucleopolyhedrovirus*), and TnGV (*Trichoplusia ni granulovirus*) were used as outgroups. ManeNPV: *Malacosoma neustria* NPV, MaamNPV: *Malacosoma americanum* NPV, MacaNPV: *Malacosoma californicum* NPV, MadiNPV: *Malacosoma disstria* NPV. The robustness of the phylogenetic tree was tested by bootstrap analysis of 1000 replicates.

it was visible that virions from ManeNPV-T2 contained more nucleocapsids per virion. Since ManeNPV-T2 showed different PIB and nucleocapsid sizes than the other ManeNPVs in the literature and contained more nucleocapsids, we classified it as a new Turkish isolate of ManeNPV.

For further characterization of ManeNPV-T2, restriction endonuclease (RE) analysis of viral DNA was performed. RE analysis is an important technique for comparing the different geographical isolates of the same virus (41). RE analysis of ManeNPV DNAs from 2 isolates (the previous Turkish and Ukrainian isolates) have been previously published (28,30). Viral DNA from the Turkish isolate of ManeNPV was digested with *Bam*HI, *Eco*RI, and *Hind*III restriction enzymes and the Ukrainian isolate with *Bam*HI, *Kpn*I, and *Pst*I enzymes. In this study, ManeNPV-T2 DNA was digested with *Bam*HI, *Kpn*I, *Pst*I, *Hind*III, and *Eco*RI enzymes, and the resultant RE profiles were compared with the other ManeNPV viral DNA restriction profiles. *Bam*HI, *Kpn*I, and *Pst*I restriction profiles of our isolate were different from the Ukrainian isolate's RE profile. While RE analysis of our isolate yielded 6 *Bam*HI, 7 *Kpn*I, and 8 *Pst*I fragments, the Ukrainian isolate, with the same enzymes, gave 8, 9, and 9 fragments, respectively. These data indicate that the Ukrainian isolate has more bands than our isolate and they are clearly different from each other. Additionally, *Hind*III and *Eco*RI restriction profiles of our isolate showed differences with

the previous Turkish isolate's restriction profile. According to the RE profiles, it is clearly seen that ManeNPV-T2 has more bands than the previous Turkish isolate. When we evaluate all of these restriction profile differences, we can clearly say that ManeNPV-T2 is a new isolate of ManeNPV. The difference in RE profiles indicated that ManeNPV is probably a mixture of more than one genotype, which is a generally common situation for baculoviruses (42).



**Figure 4.** The mortality rate in each dose on third instar *M. neustria* larvae infected by ManeNPV-T2. The bioassay showed that the incubation period of the disease was 10 day after infection with different doses of ManeNPV-T2. Mortality data were corrected according to Abbott's formula (37).

A phylogenetic analysis was performed using *polh* sequences of NPVs infecting the genus *Malacosoma* in the literature and the partial *polh* sequence of ManeNPV-T2. According to the maximum parsimony analysis, the resultant polyhedrin tree clustered ManeNPV-T2 together with other 5 *Malacosoma neustria* NPV species in GenBank. While 2 *Malacosoma americanum* NPV species clustered together, *Malacosoma californicum* and a *Malacosoma* sp. NPV species clustered together at that tree. However, *Malacosoma disstria* clustered at a farther position than the other *Malacosoma* species. From the tree, it is also seen that *Malacosoma* species are closer to the Group I NPVs than the Group II NPVs.

The biological activity of ManeNPV was characterized by bioassays. The mortality rate on *Malacosoma neustria* larvae reached 100% at  $10^6$ ,  $10^7$ , and  $10^8$  PIB/mL concentrations. The activity was noted as 70% when the concentration was reduced to  $10^5$  PIB/mL. According to the lowest concentration that has 100% mortality

on *Malacosoma neustria* larvae, the  $7 \times 10^4$  PIB/mL concentration is enough to provided 100% activity per larvae. The virulence obtained in our study is higher than that of the ManeNPV Latvian isolate, as Jankevica and Zarins found that  $10^5$  polyhedra per larvae were required for 100% mortality with the Latvian isolate (43). As a result, it is possible to conclude that since ManeNPV-T2 showed high virulence, it can be used as a source of viral insecticide to control populations of *M. neustria*.

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