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Original article (Orijinal araştırma)

Characterization of a novel baculovirus isolate from *Malacosoma neustria* (Linnaeus, 1758) (Lepidoptera: Lasiocampidae) in Samsun and its pathogenicity in different hosts

Samsun'da *Malacosoma neustria* (Linnaeus, 1758) (Lepidoptera: Lasiocampidae)'dan yeni bir bakülovirüs izolatının ve farklı konukçularda patojenitesinin belirlenmesi

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Abstract

Malacosoma neustria (Linnaeus, 1758) (Lepidoptera: Lasiocampidae) causes economic losses in apple, pear, plum, willow, oak and other economically important trees. In this study, an *Alphabaculovirus* was isolated from the larval population of *M. neustria* from Samsun in the central Black Sea Region of Turkey between 2015 and 2016. Electron microscope analysis of occlusion bodies (OBs) obtained from dead larvae showed that the nucleocapsids of a new isolate (ManeNPV-T4) are multiply enveloped. The Kimura two-parameter analysis and the phylogenetic tree were performed based on concatenated nucleotide and amino acid sequences of the partial *lef-8, lef-9* and *polh* genes from ManeNPV-T4 isolate compared to those of other 51 baculoviruses. Insecticidal activity tests against third instar *M. neustria* larvae produced 48 to 100% mortalities. The LC_{50} of ManeNPV-T4 was 0.78 x 10^3 OBs/ml in *M. neustria*. Additionally, the isolate caused mortalities lower than 50% in *Spodoptera exigua* (Hübner, 1808) (Lepidoptera: Noctuidae), *Lymantria dispar* (Linnaeus, 1758) (Lepidoptera: Erebidae), *Hyphantria cunea* (Drury, 1773) (Lepidoptera: Arctiidae) and *Helicoverpa armigera* (Hübner, 1805) (Lepidoptera: Noctuidae) larvae. Consequently, the new nucleopolyhedrovirus is infectious on *M. neustria* larvae and other lepidopterans.

Keywords: Baculovirus, identification, *Malacosoma neustria*, mortality

Öz

Malacosoma neustria (Linnaeus, 1758) (Lepidoptera: Lasiocampidae), Avrupa, Asya ve Kuzey Afrika'ya dağılmış dünya çapında bir zararlıdır. Elma, armut, erik, söğüt ve meşede ekonomik kayıplara neden olur. Bu çalışmada, Türkiye'nin Orta Karadeniz Bölgesi'nde bulunan Samsun'dan 2015-2016 yıllarında toplanan *M. neustria* larvalarından bir *Alphabakülovirüs* izole edilmiştir. Ölü larvalardan elde edilen oklüzyon badilerin (OB) elektron mikroskobu analizi, çoklu nükleokapsidlere sahip yeni bir izolat (ManeNPV-T4) olduğunu gösterdi. Kimura iki-parametre analizi ve filogenetik ağaç, ManeNPV-T4 izolatından elde edilen kısmi *lef-8, lef-9* ve *polh* genlerinin birleştirilmiş nükleotit ve amino asit dizilerine dayanarak, diğer 51 bakülovirüsünki ile karşılaştırıldı. Üçüncü evre *M. neustria* larvalarına uygulanan insektisidal aktivite testleri sonucunda %48 ile %100 arasında ölüm gözlendi. ManeNPV-T4'ün *M. neustria* için LC_{50} değeri 0,78 x 10^3 OB/ml olarak belirlendi. Ek olarak, viral izolat *Spodoptera exigua* (Hübner, 1808) (Lepidoptera: Noctuidae), *Lymantria dispar* (Linnaeus, 1758) (Lepidoptera: Erebidae), *Hyphantria cunea* (Drury, 1773) (Lepidoptera: Arctiidae) ve *Helicoverpa armigera* (Hübner, 1805) (Lepidoptera: Noctuidae) larvaları üzerinde %50'den düşük ölüm oranı oluşturdu. Sonuç olarak, bu yeni nükleopolihedrovirüs *M. neustria* ve diğer lepidopter larvalarına karşı da enfektiviteye sahiptir.

Anahtar sözcükler: Bakülovirüs, tespit, *Malacosoma neustria*, ölüm oranı

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Introduction

The baculoviruses are a family (Baculoviridae) of large, enveloped viruses, with circular covalently closed double stranded DNA genomes ranging from 80 to 180 kbp in size (Rohrmann, 2013). Baculoviruses infect mainly insects from the orders Diptera, Hymenoptera and Lepidoptera (Slack & Arif, 2007). A major distinctive feature is that these viruses have occlusion bodies (OBs), major proteins that are produced by the virus genes (*polyhedrin* or *granulin*), assembled into a protective paracrystalline matrix around the enveloped virions. The family Baculoviridae consists of four genera: *Alpha*-, *Beta*-, *Gamma*- and *Deltabaculovirus*. While the lepidopterans are infected by *Alphabaculovirus* and *Betabaculovirus*, hymenopterans and dipterans are infected by *Gammabaculovirus* and *Deltabaculovirus*, respectively (Jehle et al., 2006).

Malacosoma neustria (Linnaeus, 1758) (Lepidoptera: Lasiocampidae), also known as the European tent caterpillar, often cause serious damage to economically important fruit trees including apple, plum, hazelnut and pear; wild shrubs and ornamental trees including oleaster and oak, rose species, sea buckthorn, poplar, barberry, elm trees, willow and aspen, particularly in central and eastern Turkey (Ozbek & Calmasur, 2005; Ozbek & Coruh, 2010). The hatchlings migrate directly towards the new branches of the plant after emerging from egg clusters. The caterpillars feed on buds then on the upper epidermis and finally the parenchyma of the leaf tissue. In some years, the host plants are completely defoliated due to high numbers of this pest. Caterpillars are susceptible to parasitoids, predators and entomopathogens, which vary in their contributions to the larval mortality across regions.

As the European tent caterpillar larvae live together in a community, an infectious virus like a baculovirus may be effective for their control. Larvae emerging from tents may contact foliage contaminated with virus during feeding, and then infect other larvae upon returning to the tent. The use of baculovirus as a biological control agent has already been proven effective with agricultural pests (Granados, 1980).

The susceptibility of *Malacosoma* spp. to baculoviruses had been noted on numerous occasions (Jankevica et al., 2002; Progar et al., 2010). Moreover, the presence of baculoviruses in various *Malacosoma* spp. populations had been previously reported in Turkey (Yaman, 2003; Demir et al., 2013, 2014).

Host range is critical for determining the persistence of baculovirus isolates in an ecosystem, depending on the availability of primary and alternative hosts. Wider host ranges facilitate coevolution of baculoviruses and their hosts (Herniou et al., 2004). Likewise, broad host range is an important feature for developing effective commercial biocontrol agents (Brodeur, 2012).

In 2015, during field research in Samsun in the central Black Sea Region of Turkey, we observed baculovirus epizootics among *Malacosoma neustria* larvae populations. In this study, we aimed to characterize the biological properties of this baculovirus. We determined the morphological properties, molecular structure and phylogenetic position of new Malacosoma neustria nucleopolyhedrovirus isolate (ManeNPV-T4) from Turkey. Also, the pathogenicity of the virus was tested on various hosts including *Malacosoma neustria*, *Helicoverpa armigera* (Hübner, 1805) (Lepidoptera: Noctuidae)*, Hyphantria cunea* (Drury, 1773) (Lepidoptera: Arctiidae), *Lymantria dispar* (Linnaeus, 1758) (Lepidoptera: Erebidae) and *Spodoptera exigua* (Hübner, 1808) (Lepidoptera: Noctuidae) larvae.

Materials and Methods

Insect

Spodoptera exigua and *H. armigera* eggs used were obtained from Keyun Biocontrol Company, China. The eggs were allowed to hatch under laboratory conditions. After hatching, the neonate larvae were reared on a semisynthetic diet (Poitout & Bues 1974). *Malacosoma neustria*, *H. cunea* and *L. dispar* larvae were collected near Samsun and Trabzon in the Black Sea Region of Turkey. Collected larvae were fed with fresh leaves obtained from hazelnut (for *M. neustria*), mulberry (*H. cunea*) and oak (for *L. dispar*) trees till pupal stage in the laboratory. The pupae were then placed inside the oviposition chamber. After adult emergence, cotton soaked with 10% (w/v) sugar solution was provided for as a food source to increase the fecundity. Filter paper was placed inside adult emergence cage for egg laying. The eggs were then hatched and reared under laboratory conditions. Third instar larvae were used in the experiments described below. The eggs and the larvae were reared in an incubator at 65% RH, 25±1°C and 16:8 h L:D photoperiod.

Virus detection, isolation and propagation

Dead larvae of *M. neustria* were collected from various fruit trees in Samsun and brought to the microbiology laboratory of the Biology Department, Karadeniz Technical University, Trabzon during 2015 and 2016. These larvae were checked under phase-contrast microscope (Nikon Eclipse E600) for baculovirus infection. After detecting baculovirus infection, OBs were isolated using the procedure of Muñoz et al. (1997). These OBs were used for propagating the virus in healthy field collected *M. neustria* larvae. Subsequently, the larvae, fed with the leaf pieces (~30 mm^2) contaminated with OBs (10^6), were placed in Petri dishes. Insects were supplied with fresh leaves following consumption of the inoculum and maintained at 28°C until virus symptoms were observed. The OBs, observed under phase-contrast microscope, were reisolated from dead larvae and stored at -20°C. The isolate was designated as ManeNPV-T4.

Electron microscopy

The suspension of the purified OBs was transferred onto the coverslips and then air-dried. The sample was sputtered with gold for 3 min and examined with a Zeiss EVO LS10 scanning electron microscope. SmartSEM program was used for measuring the diameters of OBs (30 OBs/isolate). Purified OBs, fixed in 2% glutaraldehyde, 0.1 M phosphate buffer (pH 7.2) and post fixed in 1% OsO_4, analyzed using transmission electron microscope. For transmission electron microscopy (Zeis EM900), OB's were embedded in resin and ultra-thin sections stained with uranyl acetate were examined at 80 kV.

Restriction analysis

Viral DNA isolation was performed according to the method of Reed et al. (2003). The quantity and quality of the isolated DNA were determined spectrophotometrically (260/280 nm). For restriction enzyme analyses, 5 µg of DNA, was digested with *Hind*III (New England Biolabs, Ipswich, MA, USA) at 37°C for 5 h. Digestion reaction were electrophoresed in a 0.8% agarose gel containing ethidium bromide in TAE buffer (1 mM EDTA and 40 mM Tris-acetate at pH 8.0) at 25 V for 15 h. Fragments were displayed under UV light.

PCR amplification and phylogenetic analysis

Genes, *lef-8*, *lef-9* and *polh,* of ManeNPV-T4 were amplified using the degenerate primers of De Moraes and Maruniak (1997), Herniou et al. (2003) and Jehle et al. (2006). PCR reactions were performed as previously described (Demir et al., 2014). Amplified fragments were cloned into a pJET1.2 (Thermo Fisher Scientific, Waltham, MA, USA) vector and subsequently sequenced by Macrogen Inc. (Amsterdam, The Netherlands). These sequences were searched for similarity with those of the other baculovirus genes and submitted to National Center for Biotechnology Information under accession numbers, MH809425, MN218195 and MN218194 for*, lef-8*, *lef-9* and *polh* genes, respectively.

Nucleotide distance matrices between ManeNPV-T4 and other baculoviruses were determined for concatenated partial *lef-8*, *lef-9* and *polh,* gene sequences using the Kimura 2-parameter (K2P) analysis (Wenmann et al., 2018). Additionally, a phylogenetic tree was built based on the concatenated amino acid sequences of these three genes using MEGA7. Bootstrap analysis was used for testing the robustness of the phylogenetic tree. Phthorimaea operculella granulovirus, Spodoptera frugiperda granulovirus, Mythimna unipuncta granulovirus, Mocis latipes granulovirus and Pseudaletia unipuncta granulovirus were used as out-groups in the phylogenetic analysis.

Pathogenicity experiments

The pathogenicity of ManeNPV-T4 was tested in five potential hosts (*S. exigua, H. armigera, H. cunea, L. dispar* and *M. neustria*) at five OB concentrations (10^3, 10^4, 10^5, 10^6 and 10^7 OBs/ml). Thirty third-instar larvae were used per concentration and all treatments were repeated three times. The larvae were fasted for 12 h and then fed with leaves inoculated with 100 µl of the different OB concentrations. After consumption of the inoculated leaves, fresh diets were added and incubated at 28°C under a 16:8 h L:D photoperiod. Water treated leaves were used for feeding the control group larvae. Mortality was checked daily for 14 d. At the end of the experiment, dead larvae were collected and checked for the presence of OBs under phase-contrast microscope for NPV infection. The mortality levels varied for all host over the 14 d period. Mortality data were evaluated by using the Schneider-Orelli formula (Püntener, 1981) and the LC_{50} values necessary for *M. neustria* host were calculated by probit analysis using IBM SPSS statistics 23 software.

Results

Electron microscopy

The electron micrograph study revealed typical baculovirus OBs. Scanning electron microscopy results showed irregularly shaped OBs that were measured as 1.94 ± 0.23 µm in diameter (Figure 1A). Transmission electron micrograph of the ManeNPV-T4 OB isolated from infected larvae is occupied by the virion envelope with many virions occluded in the occlusion body (Figure 1B). Nucleocapsid sizes were 224.07 nm long by 46.29 nm wide.

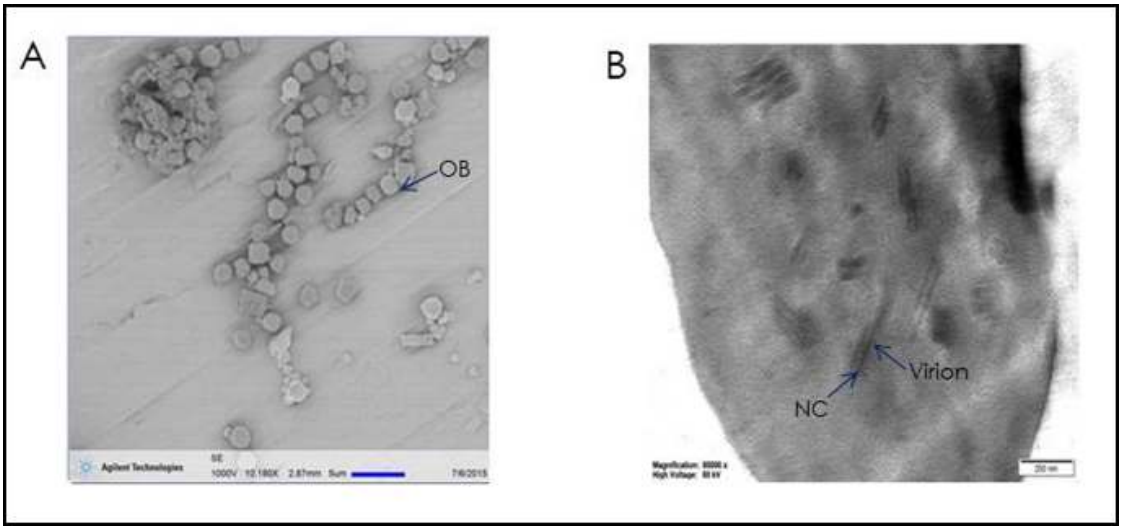

Figure 1. The electron micrographs of ManeNPV-T4: A) Scanning electron microscopy; B) Transmission electron micrographs. Virions are seen as dark rods and points in OBs; OB: occlusion bodies, NC: nucleocapsid.

Restriction endonuclease analysis of viral DNA

Restriction endonuclease (RE) analysis of the ManeNPV-T4 DNA, purified from viral OBs, yielded 15 *Hind*III fragments. These fragments were named alphabetically. *Hind*III digestion of the completely sequenced ManeNPV-T2 genome (Gencer et al., 2018) was done with Benchling Life Sciences program and yielded 20 different bands (Figure 2). The sizes of the fragments were estimated according to *Hind*III digested λ DNA and 1 kb markers (Table 1). The ManeNPV-T4 complete genome was estimated to be 119.7 kbp consistent with the *Hind*III restriction profile.

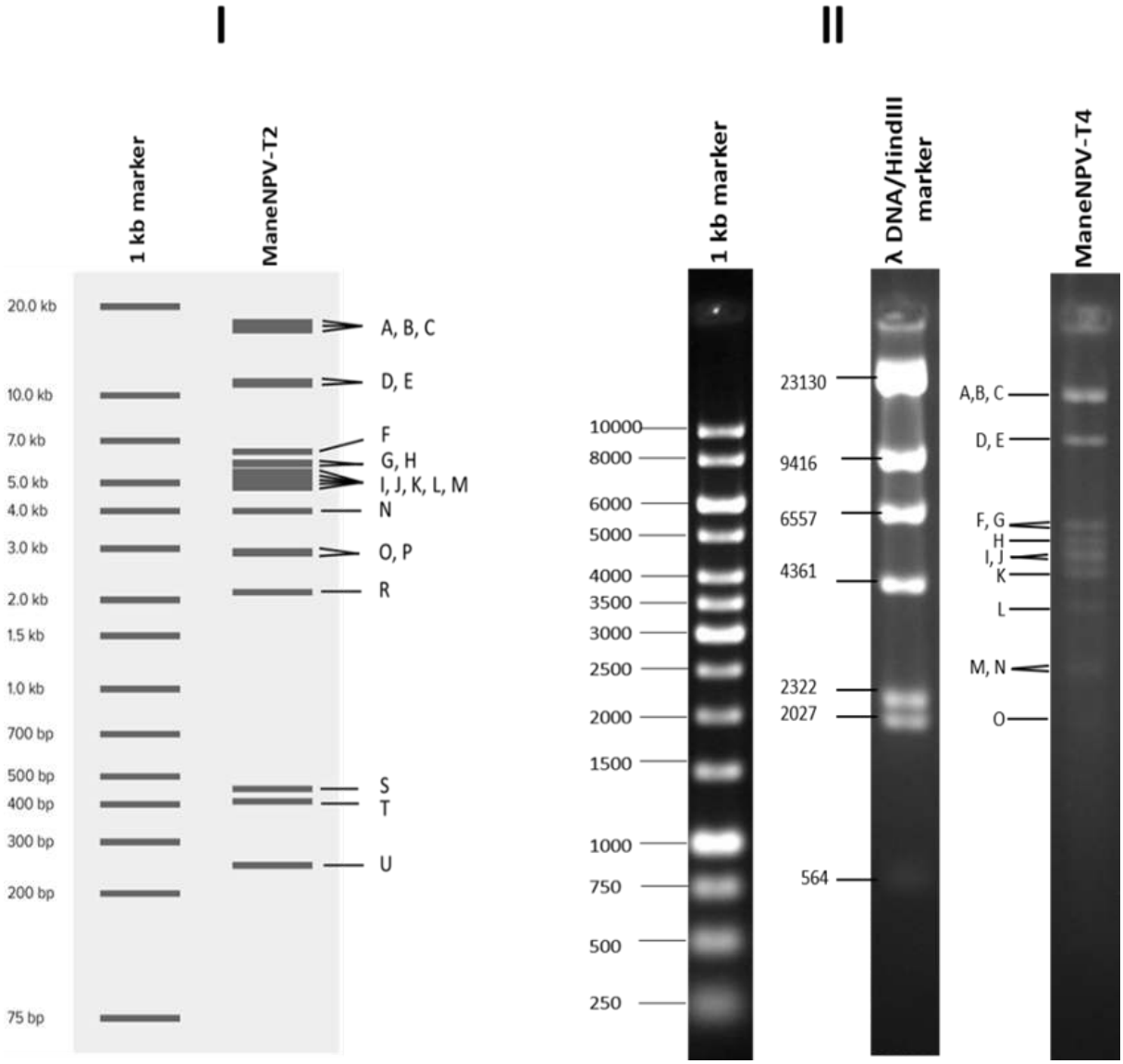

Figure 2. Restriction endonuclease mapping of ManeNPV-T4 DNA with *Hind*III. Fragments generated by *Hind*III enzyme are indicated by the letters near columns. I) ManeNPV-T2 digested with *Hind*III in silico. 1 kb marker: GeneRuler 1 kb Plus DNA Ladder, Thermo Scientific. II) ManeNPV-T4 DNA digested with *Hind*III in vitro. 1 kb marker: GeneRuler 1 kb DNA Ladder, Thermo Scientific. λ DNA/*Hind*III marker, Bio Basic.

Table 1. Fragments and total genome size of ManeNPV-T4 isolate digested with *Hind*III enzyme

Fragment	ManeNPV-T4
A	17.7
B	17.0
C	16.7
D	11.1
E	11.0
F	6.4
G	5.9
H	5.8
I	5.5
J	5.4
K	5.2
L	4.0
M	3.0
N	2.9
O	2.1
Total (kbp)	119.7

Phylogenetic analysis

K2P analysis was performed to define baculovirus species based on nucleotide sequence distances (Jehle et al., 2006). In this study, we used K2P analysis to address the position of ManeNPV-T4 among the species from *Alphabaculovirus* Groups I and II, and *Betabaculoviruses*.

According to K2P analysis, two viruses are considered to be the same species if the nucleotide locus distances value is less than 0.015 and are considered as different virus species if the distance is greater than 0.050 (Jehle et al., 2006). Based on concatenated nucleotide sequences of the partial*, lef-8*, *lef-9* and *polh,* genes, the nucleotide locus distance between the previous Turkish isolate (ManeNPV-T2) and ManeNPV-T4 is found as 0.053 which indicates that these two viruses are different species according to Jehle et al. (2006) (Figure 3). However, the amino acid sequence identities of each three marker genes of both ManeNPV-T2 and ManeNPV-T4 was 100%. The nucleotide locus distance between two viruses may be high because of the different nucleotides, coding the same amino acids. Additionally, in a study performed with concatenated 38 baculovirus core gene sequences, it was reported that baculoviruses that have nucleotide distances between 0.021 and 0.072 can be classified as same species (Wenmann et al., 2018). Thus, ManeNPV-T4 can be thought as same species of *M. neustria* NPVs in Turkey.

The phylogenetic tree has been constructed based on the concatenated POLH, LEF-8 and LEF-9 amino acid sequences of baculoviruses from *Alphabaculovirus* groups I and II, and *Betabaculoviruses*. The resulted tree demonstrated that ManeNPV-T4 is closely related with ManeNPV-T2 (Figure 4).

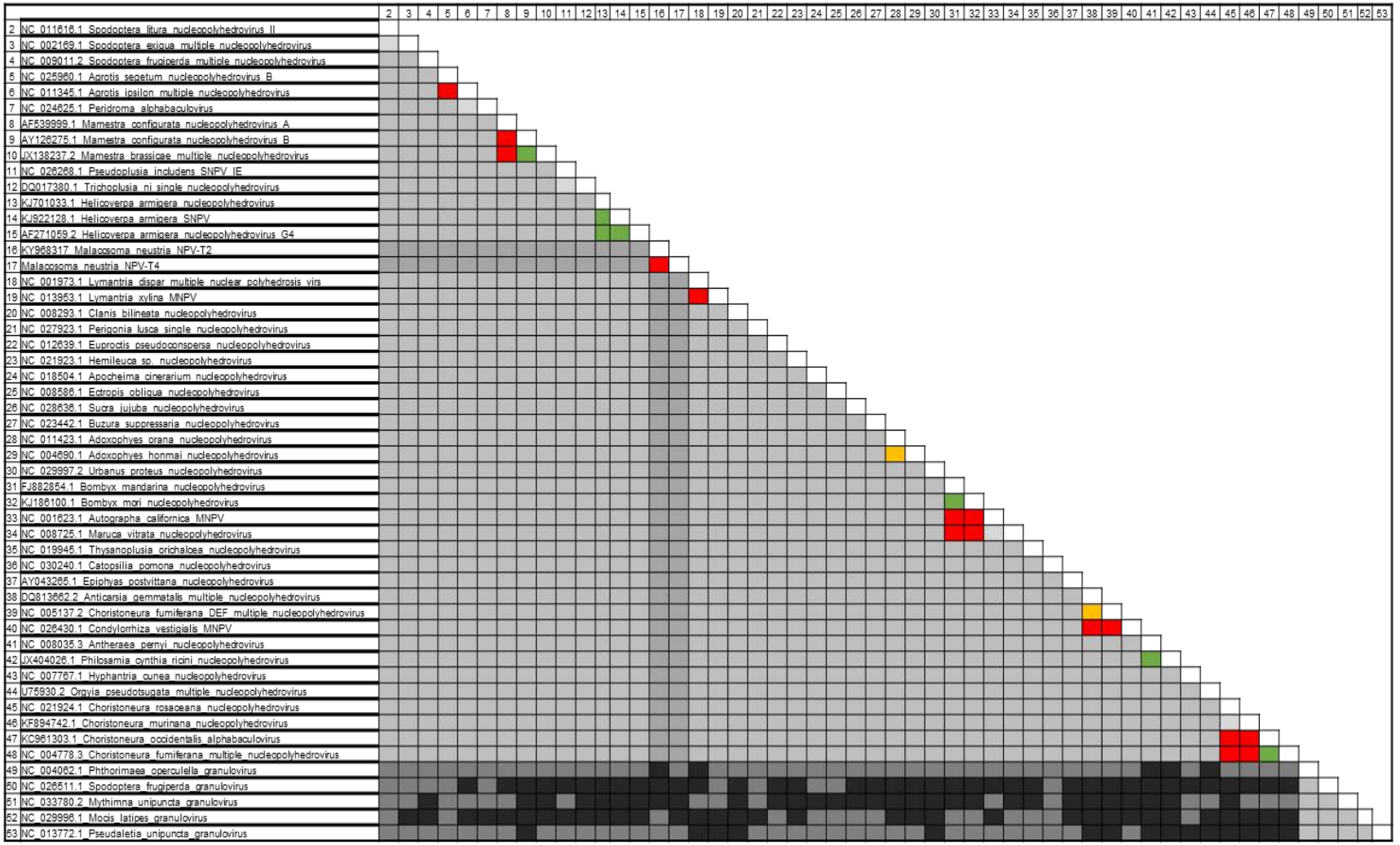

Figure 3. Pairwise distances of the nucleotide sequences of concatenated lef-8, lef-9 and polh fragments of baculoviruses. Kimura two-parameter thresholds were applied to indicate different colors as 0.000-0.015 green, 0.015-0.021 yellow, 0.021-0.05 orange, 0.05-0.072 red, 0.072 (light gray) >1 (black).

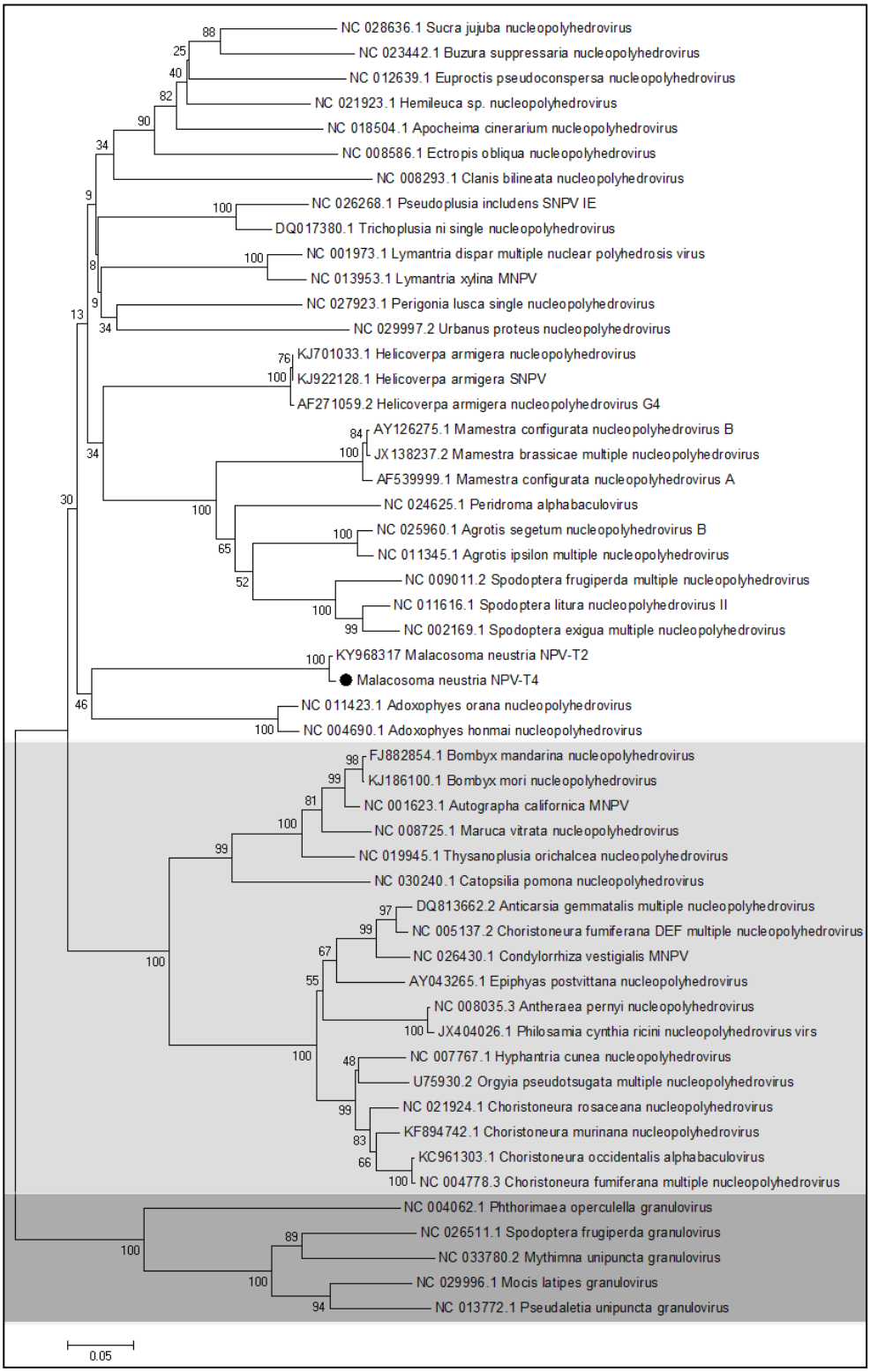

Figure 4. A phylogenetic tree (neighbor joining) according to the amino acid sequences of concatenated partial *lef-8*, *lef-9* and *polh*, genes. Bootstrap scores were showed with numbers on branches. Black dot indicates the location of the ManeNPV-T4. White, gray and dark gray areas show *Alphabaculovirus* group II, *Alphabaculovirus* group I and *Betabaculoviruses*, respectively.

Pathogenicity

The infectivity of the ManeNPV-T4 isolate was determined in *M. neustria* and four other lepidopteran hosts. Thirty third-instar larvae were used from each host at five virus concentrations, and tests were performed three times. Mortalities were assessed daily for 14 d. Mortality of *M. neustria*, ranged from 28 to 100%. The mortality of *M. neustria* larvae reached 100% with 10^7 OBs/ml within 14 d, whereas, this concentration caused 28, 30, 36 and 42% mortality of *H. armigera, H. cunea, S. exigua* and *L. dispar* larvae, respectively (Figure 5). The LC_{50} of ManeNPV-T4 in *M. neustria* was 0.78 x 10^3 (slope±se = 0.448±0.481; df = 3; X2 = 0.903). Since mortality of the hosts, other than *M. neustria*, did not exceed 50%, the LC_{50} for these hosts could not be calculated.

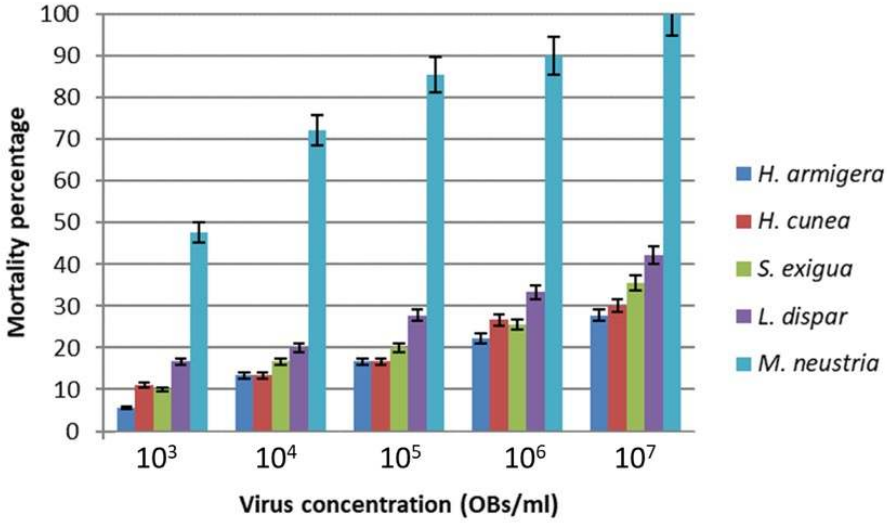

Figure 5. Pathogenicity of the ManeNPV-T4 isolate between 10^3-10^7 OBs/ml concentrations on third instar *Helicoverpa armigera*, *Hyphantria cunea*, *Spodoptera exigua*, *Lymantria dispar*, *Malacosoma neustria* larva 14 d after infection. Mortality data were corrected with Schneider-Orelli's formula (Püntener, 1981). Different lowercase letters represent statistically significant differences between mortalities according to LSD multiple comparison test (P < 0.05). Concentrations were evaluated each other. Bars show standard error.

Discussion

In recent years, baculoviruses have been used as biopesticide due to the negative effects of chemicals on environment and other non-target organisms. However, geographical baculovirus variants have different effects on local populations in laboratory and field conditions (Haase et al., 2015). In current study, we obtained a new isolate from *M. neustria* near Samsun, Turkey, and characterized its biological properties. Additionally, the pathogenicity of this isolate was determined in five species of lepidopteran insect larvae. This new isolate was designated ManeNPV-T4 to indicate its difference from previous Turkish isolates (Yaman, 2003; Demir et al., 2013, 2014). Scanning electron microscopy observations showed that the OBs of ManeNPV-T4 are irregularly shaped and about 1.5**-**2.17 µm in diameter. The OB dimension of ManeNPV-T4 was compared to those of other ManeNPVs, and ManeNPV-T4 OBs was found to be larger than the Latvian isolates (Jankevica et al., 1998), which have OBs of 0.85**-**1.4 µm, and also larger than previous Turkish isolates, which have OBs of 0.87-1.75 µm (Demir et al., 2013) and 1.0-2.1 µm (Demir et al., 2014). However, ManeNPV-T4 OBs are smaller than the other Turkish ManeNPV isolates (Yaman, 2003), Polish isolate (Lipa et al., 1968) and the isolate used by Ponsen et al. (1964) which have OB of 0.76-3.85, 0.9**-**2.0 and 1**-**3.5 µm, respectively.

The transmission electron microscopy results demonstrated that ManeNPV-T4 has multinucleocapsids in its OBs. The nucleocapsid size of ManeNPV-T4, 224 × 46 nm, was compared with

the nucleocapsid sizes of other ManeNPV isolates. The nucleocapsid size of ManeNPV-T4 was similar to the Turkish isolates, ManeNPV (Yaman, 2003), ManeNPV-T2 and ManeNPV-T3, which have nucleocapsid sizes of 240 × 35, 250 × 50 and 194.5 × 40 nm, respectively. However, the Polish isolate, the Latvian isolate and the isolate used by Bergold (1953) being 310 × 50, 360 × 80 and 315-324 × 40-46 nm, respectively, had larger nucleocapsids than ManeNPV-T4.

The RE profile of the genome has been used to examine geographical variation within a single virus (Murillo et al., 2001). For further characterization, ManeNPV-T4 genome was digested with *Hind*III and the profile was compared with other Turkish isolates (Demir et al., 2013). The RE analysis of the new isolate showed that it is different to the other Turkish isolates. While the genome of another Turkish isolate (ManeNPV-T2) yielded 20 different fragments with *Hind*III enzyme in silico, ManeNPV-T4 genome yielded 15 fragments for the same enzyme. The pattern of digested viral genomes showed similarities among major fragments of both isolates. Restriction endonuclease digestion profiles are used in genome size calculation. The size of the ManeNPV-T4 genome was estimated at 119 kbp (Table 1). This size is compatible with the completely sequenced ManeNPV-T2 genome (130 kbp) (Gencer et al., 2018) and the range for the baculovirus genomes (80-180 kb) (Rohrmann, 2013). The profile obtained with RE analysis supported the microscopic observations and we can conclude that ManeNPV-T4 is a new M. neustria nucleopolyhedrovirus isolate from Turkey.

The genes*, lef-8, lef-9* and *polh/gran***,** and 38 core genes nucleotide sequences are used to identify similarities between baculovirus species using K2P (Jehle et al., 2006; Wennmann et al., 2018). This method does not necessarily prove whether viruses are different, but rather indicates similarity of virus species. According to the K2P results, ManeNPV-T4 and ManeNPV-T2 are similar species, but different isolates. However, more information is required to prove this outcome according to the distance parameters. The morphological and molecular characterization of these isolates showed that they are quite similar rand but different from other baculoviruses **(**Figure 3).

Concatenated partial POLH, LEF-8 and LEF-9 amino acid sequences of ManeNPV-T4 were used to construct a phylogenetic tree. This tree showed that Malacosoma NPVs cluster together as other NPVs, such as Lymantria or Spodoptera NPVs. ManeNPV-T4 isolate clustered with ManeNPV-T2 and these two isolates close to Adoxophyes NPV isolates. Although this phylogenetic tree, demonstrated that ManeNPV-T4 and ManeNPV-T2 are almost the same species with high similarity, these two isolates have different nucleotide sequences. This nucleotide diversification might be the result of geographical differences.

Bioassays were used to determine the pathogenicity of ManeNPV-T4 in different lepidopteran insect pests. The mortality with 10^7 OBs/ml reached 100% in *M. neustria* larvae after 14 d. Whereas 10^6 OBs/ml of ManeNPV-T2 was enough to cause 100% mortality on *M. neustria* larvae after 10 d (Demir et al., 2013). Furthermore, in another study, 10^6 OBs/ml concentration of ManeNPV-T3 caused 100% mortality in *M. neustria* larvae within 10 d (Demir et al., 2014). Additionally, ManeNPV-T4, at 10^7 OBs/mln, caused mortality between 28 and 42% in *H. armigera, H. cunea, S. exigua* and *L. dispar* larvae within 14 d. *Hyphantria cunea* is the most resistant to these baculovirus isolates and *M. neustria* is most susceptible of the species tested. It is important to note that the larvae used in bioassay were collected from different locations of Turkey, not from the same place as the isolate. These data indicate that ManeNPV-T4 caused different mortality tin *M. neustria* and other lepidopteran pests used in this study and has the potential to be used for improving the biological control programs.

As expected, ManeNPV-T4 caused high mortality in its source host. However, its pathogenicity was lower than that of other Turkish isolates, ManeNPV-T2 and ManeNPV-T3. Therefore, it is concluded that ManeNPV-T4 has relatively large OBs with less nucleocapsids compared to other isolates indicating that, different geographic isolates can have different microscopic properties, nucleotide sequences and pathogenicity.

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