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First Record of a NPV Isolated from *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae) in Turkey and Its Molecular Identification According to the Partial *lef-8* Gene

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Abstract: *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae) is an important pest in Turkey and causes extensive economic losses in many cultivated crops. The viruses within the family Baculoviridae are specific pathogens of insects. The nucleopolyhedroviruses (NPVs), 1 of the 2 genera of this family, have been isolated from many insect orders, primarily from the lepidopterans. In our study, a local NPV was isolated from the larvae of *Spodoptera littoralis* fed in the cotton fields in southern Turkey (Mersin). For the molecular identification of the virus, polyhedra (occlusion bodies [OBs]) were isolated from insects and total DNA extraction was performed using a commercial kit from OBs. One of the genes involved in late gene expression found in the SpliNPV genome, *lef-8*, was partially amplified by PCR. Nucleotide sequence analysis of this region was performed by MWG (Ebersberg, Germany). According to the nucleotide sequences of partial *lef-8*, searches carried out with the updated GenBanks showed that the isolate was a *Spodoptera littoralis* NPV-B variant. This NPV was the fifth SpliNPV-B variant isolated worldwide and was named SpliNPV-TR1 isolate as it is the first record in Turkey of a NPV isolated from *Spodoptera littoralis*.

Key Words: Baculovirus, NPV, *Spodoptera littoralis*, *lef-8*, PCR, Nucleotide sequence analysis

Spodoptera littoralis (Boisd.) (Lepidoptera: Noctuidae)'ten İzole Edilen Bir NPV'nin Türkiye İçin İlk Kaydı ve Kısmi *Lef-8* Genine Göre Moleküler Tanımlaması

Özet: *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae) Türkiye'de önemli bir zararlı olup, bir çok üründe ciddi ekonomik kayıplara neden olmaktadır. Baculoviridae familyasında yer alan virüsler böceklerin spesifik patojenlerindendir. Nükleopolihedrosis virüsler (NPVs) bu familyada yer alan iki cinsten biri olup başta lepidopterler olmak üzere pek çok böcek takımından izole edilmiştir. Çalışmamızda, Türkiye'nin güney bölgelerindeki (Mersin) pamuk alanlarında beslenen *Spodoptera littoralis* larvalarından lokal bir NPV izole edilmiştir. Virüsün moleküler tanımlamasında polihedra'lar (occlusion body'ler [OB'ler]) böceklerden izole edilmiş ve toplam DNA ekstraksiyonu ticari bir kit kullanılarak OB'lerden gerçekleştirilmiştir. SpliNPV genomunda bulanan ve gecikmis gen ifadesinden sorumlu genlerden biri olan *lef-8* PCR'la kısmi olarak çoğaltılmıştır. Bu bölgenin nükleotid dizin analizleri MWG (Ebersberg, Almanya) tarafından gerçekleştirilmiştir. Kısmi *lef-8* geninin nükleotid dizinlerine göre, güncellenen gen bankalarındaki yapılan araştırmalar izolatın bir *Spodoptera littoralis* NPV-B varyantı olduğunu göstermiştir. Bu NPV tüm dünyada izole edilen beşinci SpliNPV-B varyantını oluşturmuş ve Türkiye için *Spodoptera littoralis*'ten izole edilen ilk kayıt olması sebebiyle SpliNPV-TR1 izolatı olarak adlandırılmıştır.

Anahtar Sözcükler: Bakulovirüs, NPV, *Spodoptera littoralis*, *lef-8*, PCR, Nükleotid sekans analizleri

Introduction

The family *Baculoviridae* contains viruses that are pathogens only for arthropods, mainly insects. Because baculoviruses are environmentally benign and do not replicate in vertebrates, they have been the subject of intensive study as possible alternatives or supplements to chemical insecticides (1). In the family *Baculoviridae*, 2 genera of viruses were recognized, the nucleopolyhedroviruses (NPVs) and the granuloviruses (GVs) (2). These entomopathogen viruses have a circular double stranded DNA genome ranging from 90 to 160 kb (3) contained within enveloped, rod-shaped virions. Their

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genome replicates in the host cell nucleus and associates with capsid proteins to form rod-shaped nucleocapsids. After this they are enveloped in a lipoprotein membrane to form infectious virions. The virions are thus occluded in polyhedral occlusion bodies (OBs) called polyhedra and are protected against environmental conditions for years. The baculoviruses have 2 distinct phenotypes in their replicative cycle: the budded virus (BV) and the occlusion derived virus (ODV). BVs provide the dissemination of infection within insect tissues while the infections are spread by ODVs to other susceptible larvae. The initial signs of baculoviral infections are gradual changes in the color and luster of the integument with an increase in opaqueness, milkiness and glossiness (4). Infected larvae begin to appear swollen and the cuticle ruptures by liquefaction of the larval carcass at the end of baculovirus infection (5).

Although the virions present in both forms differ in some of their virus-derived proteins and in the composition of their viral membranes (6), they contain the same genome (7). Gene expression in the viruses has been extensively studied in the *Autographa californica* (Speyer) (Lepidoptera:Noctuidae) multiple nucleopolyhedrosis virus (AcMNPV) and 4 phases of expression were classified: immediate-early, delayed-early, late and very late (8). Considerable progress has been achieved in the identification and characterization of genes involved in late gene expression. In the AcMNPV genome, 19 genes are required for the transactivation of late gene expression such as *vp39* and *p6.9* genes and the very late polyhedrin and *p10* genes, including *ie-1*, *ie-2*, *lefs 1-12*, *dnapol*, *p143*, *p47*, *p35* and *39K* (9). These genes are referred to as late expression factor (*lef*) genes. Many of these *lefs* have been transcriptionally analyzed (10). A possible candidate for a viral gene involved in late gene expression is one of the late expression factor genes of AcMNPV, *lef-8*, which has been shown to be necessary for the efficient expression of late and very late gene promotors in a transient expression assay (11). A gene similar to *lef-8* of the AcMNPV was identified in the *S. littoralis* (*Spli*) MNPV and localized on the genomic map between 26.9 and 29 map units and is flanked by a chitinese gene and p47 gene (12).

The Egyptian cotton leafworm, *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae), is an important pest in Turkey, especially in the south agricultural fields and greenhouses, and it causes extensive economic losses in many cultivated crops. Furthermore, the insect is resistant to many commonly used chemical insecticides. Baculoviruses have great potential to be used as biological insecticides (13) and cause lethal epizootic diseases in their host-insect populations (14). Several hundred NPV isolates have been described from insects primarily of the order Lepidoptera (15). Different SpliNPV isolates have been isolated from *S. littoralis* to date. These isolates were designated as SpliMNPV-A, SpliMNPV-B (16) and SpliMNPV-C (17) according to their restriction-fragment profiles. The ability of SpliMNPV-B to successfully infect several *Spodoptera* species including *Spodoptera exigua* (Hübner), *Spodoptera exempta* (Walker), *Spodoptera frugiperda* (Smith) and *Spodoptera litura* (Fabricius) make it a suitable candidate for use as a microbial pest control agent (18). SpliNPV-B variants have been isolated from diseased *S. littoralis* larvae collected in Israel, Egypt and Morocco and from diseased *S. litura* larvae collected in Japan to date (16,17,19). In this study, we isolated a new local SpliNPV-B variant as the fifth sample isolated worldwide from the larvae of *S. littoralis* fed in cotton fields in southern Turkey (Mersin). We partially amplified the *lef-8* gene of our virus isolate by PCR and described it according to the nucleotide sequences of the partial *lef-8* gene.

Materials and Methods

Insect and insect rearing

S. littoralis larvae were reared on lettuce leaves in plastic cages (24 x 33 x 15 cm) at the Department of Plant Protection, Faculty of Agriculture, University of Ankara, as a continuously maintained culture that was first brought to the department in 2002. Lettuce leaves were surface sterilized with 1% NaOCl before being given to the larvae. By pupation, individual pupae were transferred into adult rearing cages with 15% sucrose solution. A 5-cm-wide and 20-cm-long paper towel strip was hung in the jar for the egg deposition. Eggs on the paper towel strip were transferred into a clean glass container for hatching. *S. littoralis* were reared under controlled conditions with 16:8 h (L:D) photoperiod, 27±0.5 °C temperature and 70% relative humidity.

Virus isolate, virus production and isolation of OBs

The original virus isolate was obtained from diseased *S. littoralis* larvae collected in cotton fields in Mersin, Turkey, in 2002. The larvae thought to show baculovirus

infection symptoms were brought to our laboratory and kept at –20 ºC until the isolation of OBs (polyhedra). Production of the isolate was performed in third instar *S. littoralis* and virus concentrations were quantified with an improved hemocytometer (Hausser Scientific, improved Neubauer hemocytometer, 0.100 mm deep) under a light microscope. Six counts per hemocytometer were measured to reduce counting errors. After the isolation of OBs from the insects, a stock virus suspension was prepared and a 3 x 10^6 OBs/ml concentration was diluted from the suspension. Thirty-two larvae were placed in individual 17-ml jelly cups and starved overnight. Three thousand OBs (1 µl from the concentration of 3 x 10^6 OBs/ml per disk) were given to larvae on 5 cm diameter lettuce leaf disks individually and thirty-two control larvae were inoculated with sterile water. The lettuce leaf disks were sterilized with 1% NaOCl, washed with distilled water 3 times and dried before being inoculated with the virus. Larvae that consumed the whole disk contaminated with virus were transferred to new cups and supplied with additional lettuce leaves daily. For the isolation of OBs, the larvae were treated with 0.1% SDS (1 ml per larva) for 1 night at 4 ºC and filtered through 5 layers of cheesecloth. OBs were pelleted by centrifugation at 3600 g for 10 min at room temperature in 50 ml centrifuge tubes. The pellet was resuspended in 0.5% SDS and centrifugation and resuspension were repeated with 0.3 M NaCl before final resuspension of the OBs in distilled water (modified from 20). The OBs were further purified according to sucrose gradient centrifugation using the method described by Cheng et al. (21).

Isolation of viral DNA

DNA extractions were performed using the DNeasy Tissue kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. Quantity of extracted virus DNA was estimated by the Ethidium Bromide Agarose Plate assay (22). The DNA yields were stored at –20 °C until used.

PCR conditions and electrophoresis of PCR product

To characterize the partial *lef-8* gene in the virus, this gene was amplified by polymerase chain reaction (PCR). PCR amplification of the partial lef-8 gene was carried out in a reaction volume of 48 µl for the isolate, containing 33 µl of sterile H_2O, 5 µl of 10x reaction buffer, 5 µl of dNTPs (1 mM) 1 µl of primer forward (200 µM), 1 µl of primer reverse (200 µM), and 1 µl of purified DNA (~ 50 ng). The degenerate primers used in the PCR were prL8-1 and prL8-2, which were developed by Lange et al. (23) and reported as *lef-8* specific (23). The amplification was accomplished with a DNA thermal cycler (Perkin Elmer Cetus). The samples were placed in the thermal cycler, which was preheated to 95 °C and incubated at 95 °C for 4 min, followed by 35 cycles of 95 °C for 2 min, 46 °C as annealing temperature for 2 min, and at 72 °C for 1 min. In the first step, before the annealing temperature, 0.2 µl of Taq polymerase and 1.8 µl of Taq polymerase dilution buffer were added to the tube. Thus, the final reaction volume was 50 µl. At the end of the PCR cycle, a final step of 2 min at 72 °C was included to ensure all of the final amplification products were full length. Agarose gel electrophoresis was carried out at the end of the PCR for verification using the followed method. In the electrophoresis of PCR yields, 7 ?l of PCR product and 7 µl of marker (100 bp ladder, GibcoBRL) were added separately to containers with loading buffer of 3 µl volume. The PCR product was analyzed by 1% agarose gel electrophoresis at 80 V for 1 h. After the PCR, the gel was stained with ethidium bromide and photographed under a UV light.

Sequencing of PCR product

The PCR product used was purified with the GFX PCR DNA and Gel Band Purification Kit (Amersham, Freiburg, Germany) before sequencing and DNA sequencing of partial *lef-8* was performed by MWG (Ebersberg, Germany). Homology searches were carried out with the updated GenBank EMBL, DDBJ and PDB sequences databases via the NCBI nucleotide database using the BLAST algorithm (24).

Results and Discussion

All *S. littoralis* larvae that were given 3000 OBs died from the baculovirus infection and showed typical baculovirus infection symptoms. Infected larvae showed pale swollen bodies and then the tissues liquefied. Infection of the epidermis caused the host to appear to melt. These symptoms were also reported by other researchers (4,5,25).

After the extraction of viral DNA, the quantity of viral DNA (~50 ng) estimated by the Ethidium Bromide Agarose Plate assay was found to be sufficient for the PCR. Following the DNA extraction, *lef-8* gene in the

virus genome was amplified partially by PCR. The PCR amplification product of the partial *lef-8* is shown in Figure 1. (In lanes 1,2 water was used as a control, in lanes 3-7 the PCR product is shown).

After the PCR, the amplified partial *lef-8* was analyzed according to the nucleotide sequences. Nucleotide sequences of the partial *lef-8* gene are shown in Figure 2. According to the nucleotide sequences, 714 nucleotides were aligned for the partial *lef-8* gene of the isolate.

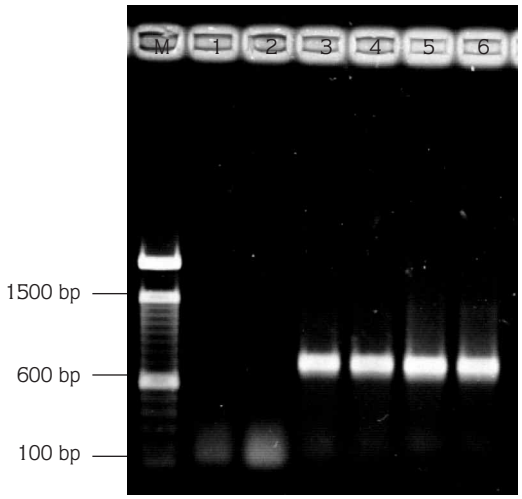

Figure 1. PCR amplification of the partial lef-8 gene coding region run at 1% agarose gel for 1 h at 80 V. (Marker (M): 100 bp ladder-GibcoBRL, Lanes 1-2: water control, Lanes 3-6: PCR product from virus)

The nucleotide sequence and open reading frames (ORFs) map of the *lef-8* gene of SpliMNPV were recently shown and the sequence analyses of the *lef-8* gene revealed an ORF of 2730 nucleotides (12), and the size of the amplification products obtained using prL8-1 and prL8-2 ranged from 681 to 771 nucleotides for several baculovirus isolates (23).

In Figure 3, the alignments of the partial *lef-8* gene of our isolate and the complete sequences of the *lef-8* gene of SpliMNPV described recently (12) are compared and the similarity is shown below.

BLAST analysis indicated that there is an identity between our isolate and the SpliNPV-B isolate E15 described by Faktor and Kamensky (12) recently, with an identity value of 99% (710/717) and an expect value of 0. Thus, our isolate was identified as a SpliNPV-B variant in the NCBI taxonomy database. A BLAST homology search revealed that SpliNPV nucleotide sequences showed high identities to homologues of other baculoviruses, such as *S. litura* nucleopolyhedrosis virus (SpltNPV) G2 strain, with the maximal identity reaching 89% and with a score of 658 bits (332) and an expect value of 0. Wang et al. (26) also found high identities based on *lef-8* amino acid sequences between SpliNPVs and SpltMNPV, with an identity value of 86%.

GTGGCGGGCAAAAAGTTTTTTCTGTGTCGAGACGTGCGACTTCCGAACGT
CGACTACGAGTCTATAGCGAAAAAATTTAAAGATTTGATTGAATCGAATC
TTATAGTGCGACGAGATGAGGACGAAAACGGCAGCGGCGGTGACGACGAC
GACGAATGGCTAATGATCGCGTTCAACAATAGACCTACGACGTTTTCGTG
CAAGCGAAAGCATCTAGTACGCATCGTGTACGAGTTTAAACGCAAACGGT
TTCCAGTCGAGATCAAACTGTCCAAAAGTATTCTATTTGTCAACCATCAC
GAAGGCATGATATGTATACGCAAGCAGGTTCGAATAAATGAAAAAGTAAA
CATTTCGGCGCTGCTCACTCCGTACGAGTACCACAACGTCGAGTCTATCA
TTGGCCAGATCGGAGCCCGGATCGTCGACGTCGATCATGTCTCTGCGCTA
ATGTCCAAGACGCTTCAGTACTATTACCGATCGCATTTGCACATTTTCGC
GACGATACCCGTGCCCAAACTGATCGTGTCCGTGACAAATTTGAAAAACG
CAATGCCCGTGATTGAATACGATGACGTCGAATGGAACGAAAACCGCGAC
ATGTTCATTAGAAACTTGCCGGTCGGCAATTCTGTCGTGGCGAGCCCCGG
TTCGGTGCACAACAACAAGATGATCAATCTGTGGACTCTGGTGCGAGACA
GCCGGCTCATGACC

Figure 2. Nucleotide sequences of the partial *lef-8* gene (714 nucleotides).

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Query: 1    gtggcgggcaaaaagttttttctgtgtcgagacgtgcgacttccgaacgtcgactacgag 60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1295 gtggcgggcaaaaagttttttctgtgtcgagacgtgcgacttccgaacgtcgactacgag 1354

Query: 61   tctatagcgaaaaaatttaaagatttgattgaatcgaatcttatagtgcgacgagatgag 120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1355 tctatagcgaaaaaatttaaagatttgattgaatcgaatcttatagtgcgacgagatgag 1414

Query: 121  gacgaaaacggcagcggcggtgacgacgacg---acgaatggctaatgatcgcgttcaac 177
            ||||||||||||||||||||||||||||||   ||||||||||||||||||||||||||
Sbjct: 1415 gacgaaaacggcagcggcggtgacgacgacgacgacgaatggctaatgatcgcgttcaac 1474

Query: 178  aatagacctacgacgttttcgtgcaagcgaaagcatctagtacgcatcgtgtacgagttt 237
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1475 aatagacctacgacgttttcgtgcaagcgaaagcatctagtacgcatcgtgtacgagttt 1534

Query: 238  aaacgcaaacggtttccagtcgagatcaaactgtccaaaagtattctatttgtcaaccat 297
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||
Sbjct: 1535 aaacgcaaacggtttccagtcgagatcaaactgtccaaaagtattctatttgtcaatcat 1594

Query: 298  cacgaaggcatgatatgtatacgcaagcaggttcgaataaatgaaaaagtaaacatttcg 357
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1595 cacgaaggcatgatatgtatacgcaagcaggttcgaataaatgaaaaagtaaacatttcg 1654

Query: 358  gcgctgctcactccgtacgagtaccacaacgtcgagtctatcattggccagatcggagcc 417
            |||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||
Sbjct: 1655 gcgctgctcactccgtacgagtaccacaacgtcgaatctatcattggccagatcggagcc 1714

Query: 418  cggatcgtcgacgtcgatcatgtctctgcgctaatgtccaagacgcttcagtactattac 477
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1715 cggatcgtcgacgtcgatcatgtctctgcgctaatgtccaagacgcttcagtactattac 1774

Query: 478  cgatcgcatttgcacattttcgcgacgatacccgtgcccaaactgatcgtgtccgtgaca 537
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1775 cgatcgcatttgcacattttcgcgacgatacccgtgcccaaactgatcgtgtccgtgaca 1834

Query: 538  aatttgaaaaacgcaatgcccgtgattgaatacgatgacgtcgaatggaacgaaaaccgc 597
            |||||||||||||||||||||||| || ||||||||||||||||||||||||||||||||
Sbjct: 1835 aatttgaaaaacgcaatgcccgtgatcgagtacgatgacgtcgaatggaacgaaaaccgc 1894

Query: 598  gacatgttcattagaaacttgccggtcggcaattctgtcgtggcgagccccggttcggtg 657
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1895 gacatgttcattagaaacttgccggtcggcaattctgtcgtggcgagccccggttcggtg 1954

Query: 658  cacaacaacaagatgatcaatctgtggactctggtgcgagacagccggctcatgacc 714
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1955 cacaacaacaagatgatcaatctgtggactctggtgcgagacagccggctcatgacc 2011
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Figure 3. Similarity between the sequence alignments of the partial *lef-8* gene from our isolate and nucleotide sequences of the *lef-8* gene region described by Faktor and Kamensky (12) from SpliMNPV. (Score = 1362 bits (687), Expect = 0.0 Identities = 710/717 (99%), Gaps = 3/717 (0%) Strand = Plus / Plus, NCBI Blast, Nucleic Acids Res. 25:3389-3402.)

Consequently, similar nucleotide alignments of *lef-8* between SpliNPV and SpltMNPV-G2 demonstrated that SpliNPVs were closely related to SpltNPVs. High identities between these isolates suggested that *lef-8* was well conserved in baculovirus genomes and an ideal candidate gene for molecular identification. The primers prL8-1 and prL8-2 have been used to amplify partial *lef-8* sequences of more than 100 different lepidopteran baculovirus isolates (23) and *lef-8* was identified in all sequenced baculoviruses (27); thus these also make it an ideal gene. The good conservation of *lef-8* in baculoviruses was also reported according to the sizes of *lef-8* and the high

identity values detected between some of the NPVs (26,27). Furthermore, *lef-8* was detected as the most conserved *lef* homologue with *lef-9* in the SpltMNPV genome according to the AcMNPV genome (28). In addition, the position of the *lef-8* gene in the genome was also comparable to that of other NPVs (26).

SpliNPV variants have been isolated from the Mediterranean region except for the Japanese isolate to date. This is of course related to the existence of *S. littoralis* in this region. The insect is highly migratory and commonly found in the Mediterranean region. Thus, sublethally infected adults might transport the virus from infected fields to other areas. In fact SpliNPV-B variants were isolated in Morocco, Egypt and Israel and lastly in Turkey by our record in the Mediterranean region to date. These locations are not far away for migrating infected adults and the isolate thus seems to spread in this region from the north coast of Africa to the eastern Mediterranean. Consequently SpliNPV-B variants may be distributed in other parts of the Mediterranean region. SpliNPV-A variants were also reported in Israel as equally distributed with SpliNPV-B (16), but it would not be wrong to describe SpliNPV-B as the most widely distributed SpliNPV variant in the Mediterranean region.

Molecular methods based on PCR and DNA sequences of PCR products can greatly reduce the amount of time needed for identifying unknown baculovirus isolates. The PCR is reported to be a highly sensitive technique that amplifies target DNA sequences, and viral DNA obtained from infected larvae is a good source of DNA for PCR amplification (13). The sensitivity of the PCR for baculovirus amplification has been also reported (29). Nevertheless a single target gene might in many cases be sufficient for a quick identification of an uncharacterized baculovirus isolate (23) but specifically the *lef-8* gene was suggested as particularly useful to meet targets for PCR amplification (30). Thus, Lange et al. (23) reported that the application of degenerate PCR combined with molecular phylogeny provides an excellent method for fast and reliable baculovirus identification and needs only tiny traces of a sample even if the virus cannot be recovered. In addition, virus DNA used for PCR amplification was obtained by complete DNA isolation of virus-infected larvae, indicating that insect DNA as a background neither disturbed the reaction nor resulted in false-positive amplification products (23). Lange and Jehle also pointed out that control experiments in which isolated genomic DNA of several uninfected insect larvae was used never resulted in a positive signal (Lange and Jehle, unpublished). Thus, possible insect DNA remaining after isolation from OBs did not constitute a false-positive amplification in the PCR due to the specificity of primers only to *lef-8* of the virus in our study.

In conclusion, we identified the virus isolate by nucleotide sequences of the partial *lef-8* gene following the PCR procedure with primers capable of amplifying the *lef-8* gene partially. According to the nucleotide sequences of partial *lef-8* and the homology searches carried out in the gene banks, it is proved that the isolate is a SpliNPV-B variant. Because baculoviruses are identified and named according to the insect host species from which they were isolated (23,31), this NPV was named SpliNPV-TR1 due to its being the first record for Turkey. This NPV also constituted the fifth SpliNPV-B variant isolated worldwide as a Turkish strain. As a result of this study, the procedure based on PCR and nucleotide sequences of *lef-8* gene is an easy and reliable way for identifying baculovirus isolates, and an important alternative candidate to chemical insecticides was identified for Turkey. We are currently investigating the restriction endonuclease (REN) analysis of viral DNA. Biological activities based on lethal doses of the isolate and survival times of infected *S. littoralis* larvae will be the subject of our next publication.

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