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RESEARCH PAPER

# Genetic structure analysis of several *peroral infectivity factor* gene in S*podoptera litura* Nucleopolyhedrovirus

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## ABSTRACT

*Spodoptera litura* Nucleopolyhedrovirus (*Splt*NPV) is an entomopathogenic virus from the *Baculoviridae* family, currently under development as a biological control agent for cutworm *Spodoptera litura*. The NPV's ability to infect its pest host can be determined by expression of pif protein complex by *pif* gene. The research aims to acquire genetic character information of the *pif* gene of *Splt*NPV from Bogor. Amplification of the NPV gene was carried out using the specific primers to amplifies two types of *pif* gene. The PCR products were sequenced then the DNA sequences were analyzed with the BioEdit and BLAST programs. The PCR amplification results showed that the size of the sample DNA fragment was 900 bp, 1300 bp and 710 bp. Based on the sequence analysis results, *Splt*NPV isolates from Bogor are closely related to *Splt*NPV and *Spli*NPV isolates from China. The highest nucleotide homology values of *Pif*-1, *Pif*-2, and *Pif*-3 gene were 99.56%, 99.37% and 100%, respectively. Based on the results of phylogenetic analysis, *Hear*NPV isolates from Bogor belong to the same group as the NPVs that infect the species *Spodoptera litura* and closely related to NPV that infect *Spodoptera littoralis*. The amino acid sequence analysis showed the number of mutated sites in *pif-1*, *pif-2*, and *pif-3* in this study are 1, 3, and 0, respectively, which indicates that protein mutation in *Splt*NPV Bogor did not significantly alter the viral infection process.

**Key words:** *Baculoviridae*, entomovirus, infectivity factor, matrix identity, phylogeny

## INTRODUCTION

*Spodoptera litura* Nucleopolyhedrovirus (*Splt*NPV) is an entomopathogenic virus from the *Baculoviridae* family that has the potential to be developed as a biological control agent against *Spodoptera litura* larvae. NPV acts as a stomach poison in its target host (Cuartas-Otálora et al., 2019). Occlusion bodies (OBs) enter the larvae digestive system through the ingestion of contaminated polyhedra on the food surface (Harrison & Hoover K, 2012). Infected larvae typically exhibit symptoms such as reduced appetite, decreased mobility, a shiny body appearance, body swelling, and death, often with their bodies hanging from plant shoots or twigs (Claus et al., 2012). Larvae generally die within 3–5 days after infection occur (Ali et al., 2018).

The use of *Splt*NPV for controlling *S. litura* holds promise as an effective and environmentally safe biological control method. Each NPV species, including *Splt*NPV, is host-specific and does not negatively impact non-target insects such as pollinators, predators, and parasitoids (Rodgers, 1993). *Splt*NPV has been widely used for *S. litura* management worldwide and is beginning to be developed in Indonesia. In Taiwan and India, *Splt*NPV applications have successfully reduced *S. litura* infestationss in peanuts, cotton, and rice (Das & Durga, 1996; Moscardi et al., 2011). *Splt*NPV has also been commercially applied for *S. litura* control in China and Japan (Mitsuhashi, 2009; Sun, 2015). In Indonesia, a *Splt*NPV isolate from China has been developed as a biological agent against *S. litura,* but indigenous isolates are still studied at the laboratory scale (Bedjo, 2017).

The morphology and physiology of a virus are determined by the genetic information within its genome (Nayak, 2007). The *pif* gene (Per os infectivity factor) is one of the core genes in the NPV genome and plays a crucial role in the viral infection process in the host's small intestinal epithelial cells (Boogaard et al., 2018). The *pif* protein consists of multiple protein types, forming a complex that facilitates the entry of the nucleocapsid into epithelial cells. The ability of viral particles to enter host cells is a key factor in determining the pathogenicity and virulence of the virus.

Genetic studies of NPV in *S. litura* in Indonesia have been reported previously. The *lef-8* gene of

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*Splt*NPV was identified in Indonesia by Kusumah et al. (2023). Similarly, molecular characterization of NPV isolates from other lepidopteran pests has been conducted. For instance, Kusumah et al. (2017) analyzed the lef-8 gene of Hyposidra talaca NPV (HytaNPV) from tea plantations in Gunung Mas, Bogor, revealing a high nucleotide and amino acid homology (98% and 100%, respectively) with NPV isolates infecting the genus Helicoverpa from Brazil, Australia, Spain, and the Netherlands. Additionally, Kusumah et al. (2018) conducted a molecular characterization of Helicoverpa armigera NPV (HearNPV) in H. armigera larvae, demonstrating a close relationship with other HearNPV strains, with nucleotide sequence homology ranging from 98.4% to 99.4% and amino acid sequence homology ranging from 98.2% to 99.2%. This study aims to investigate one of the key genes involved in the primary infection process of SpltNPV, namely the pif genes. Comprehensive information on pif genes is essential for understanding the virulence factors of NPV, which serve as a reference for biological pest control applications.

## MATERIALS AND METHODS

**Research Site.** This research was conducted at the Insect Pathology Laboratory and the Integrated Laboratory of Plant Protection, Department of Plant Protection, Faculty of Agriculture, IPB University. Nucleopolyhedrovirus (NPV) strains were obtained from taro plants in the area around the IPB University campus.

**Viral Isolation.** The virus were extracted using a gradual centrifugation method with a refrigerated benchtop centrifuge, Sorvall Biofuge Fresco (Kendro Laboratory Products, USA) (Kusumah et al., 2022). Nucleopolyhedrovirus-infected larvae were crushed in 0.1% SDS buffer using a sterile mortar. A 1.5 mL of virus suspension was transferred into a 1.5 mL microtube and centrifuged for 1 min at 380 × g for 20 min. The resulting white-colored pellet was resuspended in sterile water until a final volume of 1.5 ml was reached, then homogenized using a vortex for 30 seconds. The suspension was centrifuged again for 1 min at 380 × g, and the supernatant was subjected to a second centrifugation at 2375 × g for 20 min. The supernatant was discarded, and the pellet was resuspended in sterile water to obtain a relatively pure virus suspension..

**Viral Propagation.** Nucleopolyhedrovirus (NPV) propagation was carried out at the Insect Pathology Laboratory, Department of Plant Protection, IPB University. A 50 µL artificial diet, previously prepared based on Singh & Moore (1985), was smeared onto a 90 mL plastic cup. Then, 1 µL of virus suspension with a concentration of $10^7$ POBs/mL was added to the diet. Second instar-larvae were starved for 2 h before being fed the prepared diet until it was completely consumed. The larvae were then fed a virus-free artificial diet and maintained at 25 ºC with a 16-h photoperiod until death. Dead larvae exhibiting dark, soft, and watery bodies were collected and stored in a freezer at -20 ºC.

**DNA Extraction.** Viral DNA was extracted using a modified Cetyl-Trimethyl Ammonium Bromide (CTAB) method (Doyle & Doyle, 1987). A 0.1 g sample of NPV-infected larvae was placed into a 1.5 mL microtube and mixed with 400 µL of CTAB extraction buffer (2% (v/v) CTAB, 0.05 M Tris-HCl, 0.02 M EDTA, 1.4 M NaCl, 1% (v/v) β-mercaptoethanol, $ddH_2O$). The larvae were crushed with a micropestle and homogenized using a vortex for 30 s. The mixture was incubated at 60 ºC for 30 min. Then, 58 µL of chloroform and 2 µL of isoamyl alcohol were added, followed by vortex homogenization for 30 seconds. The mixture was centrifuged at 9500 × g for 3 min, and the supernatant was transferred into a new microtube. DNA precipitation was performed by adding an equal volume of isopropanol at -20 ºC, followed by centrifugation at 9500 g for 3 min. The supernatant was discarded, and the pellet was washed twice with 500 µL of 70% ethanol. The pellet was then air-dried at room temperature for 2 h. The dried pellet was resuspended in 50 µL TE buffer (0.01 M Tris-HCl, 0.001 M EDTA, $ddH_2O$) and stored at -20 ºC.

**DNA Amplification.** *Splt*NPV DNA was amplified using pre-designed primer (Table 1). Each amplification reaction contained 2 µL of *Splt*NPV DNA (±40 ng/µL), 12.5 µL of 2 × Taq® Plus PCR Mastermix (Tiangen, Beijing), 1 µL forward primer (10 µmol), 1 µL reverse primer (10 µmol), 4 µL of DNA template, and 6,5 µL of nuclease free-water. The amplification steps consisted of pre-denaturation at 94 ºC for 4 min, followed by 30 cycles of denaturation at 94 ºC for 30 s, annealing at 54 ºC for 30 s, elongation at 72 ºC for 60 s, and a final elongation at 72 ºC for 5 min. The final storage temperature of the amplification product was set to 4 ºC.

A 5 µL aliquot of the PCR product was mixed with 1 µL of *PegGREEN* gel stain (Peqlab, Hongkong) and subjected to electrophoresis in 1% agarose gel

under a 50 V current for 50 min. The gel was visualized using a UV-transilluminator.

**DNA Sequencing and Data Analysis.** The amplified DNA fragments were sequenced by 1st BASE Malaysia and sent via PT. Genetika Science Indonesia using the Sanger sequencing method. The sequences were trimmed before being compared with published gene sequences from the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tools (BLAST) program (Altschul et al., 1990). Selected nucleotide sequences were aligned using the ClustalW algorithm in BioEdit ver 7.2.6 (Hall 1999). The sequence alignments were analyzed using the Sequence Dermacation Tools v2.1. for homology analysis and the Molecular Evolutionery Genetic Analysis (MEGA) v11.0 software for phylogenetic analysis (Tamura et al., 2021). The phylogenetic tree was constructed using the Maximum Likelihood approach with 1000 bootstrap replications.

Amino acid structure analysis was performed by translating the nucleotide sequences using the Expasy translation tool (website.expasy.org/translate/) and aligning the resulting protein sequences using the ClustalW algorithm in BioEdit v7.2.6.

## RESULTS AND DISCUSSION

**Symptoms of NPV Infection in *Spodoptera litura*.** Infected larvae showed symptoms of decreased feeding activity on the fourth day after inoculation. The larvae died on the sixth day after inoculation, displaying symptoms of pale body color and hanging upside down on the side of the cup (Figure 1).

**DNA Amplification Using the PCR Method.** The amplification result showed DNA bands with varying amplicon sizes. DNA amplification of *pif*-1 (*pif-1a* and *pif-1b*) genes produced an amplification product of 900 bp, while the *pif-2* and *pif-3* genes produced an amplification product of 1300 and 700 bp, respectively (Figure 2).

**Homology and Phylogenetic Analysis Based on Nucleotides.** BLAST analysis revealed that the Bogor *Splt*NPV isolate had the highest similarity to *Splt*NPV isolates from China, specifically isolates KY and T0, as indicated by their homology values. The highest nucleotide homology values for the *Pif-1*, *Pif-2*, and *Pif-3* genes were 99.56%, 99.37%, and 100%, respectively (Table 2). Based on the identity matrix and phylogenetic analysis, the Bogor *Splt*NPV isolate clustered within the same group as NPV species infecting *Spodoptera litura* and *Spodoptera littoralis* (Figure 3, 4, 5).

According to King et al. (2012), a high homology value with a nucleotide sequence similarity of more than 89% suggests that the virus belongs to the same

Table 1. Pre-designed primer used for PCR of several pif genes from *Splt*NPV

| Target gene | | Primer sequence (5′-3′) | Tm (ºC) | Amplicon (bp) |
|---|---|---|---|---|
| *pif-1a* | F | CGGCTAAATACAACGCTTC | 51.5 | ± 900 |
| | R | GTTACATTCAAGCTGACCATC | 51.5 | |
| *pif-1b* | F | CCAAATTTGTACTGTTCGG | 49.1 | ± 900 |
| | R | CATCGACATGTACGAGATAA | 49.1 | |
| *pif-2* | F | CCAGCATGAACACGATTAG | 51.0 | ± 1300 |
| | R | GGTATTTGAATTTGGCCATTG | 50.5 | |
| *pif-3* | F | TGTACGACCGCTCTAGGATAAG | 55.5 | ± 700 |
| | R | GTCTCGCCAAACATCGAACA | 55.6 | |

Figure 1. NPV-infected *Spodoptera litura* larvae.

species. The analysis showed that each *pif* gene from *Splt*NPV Bogor had more than 89% homology with *Splt*NPV isolates from China and Japan. However, the homology percentages of *Splt*NPV Bogor were lower than 89% when compared with *Spli*NPV isolates from Egypt and Tunisia for *pif-1* and *pif-3*, but not for *pif-2* (Figures 6, 7, and 8). These results suggest that *pif-2* is a more conserved sequence than *pif-1* and *pif-3*.

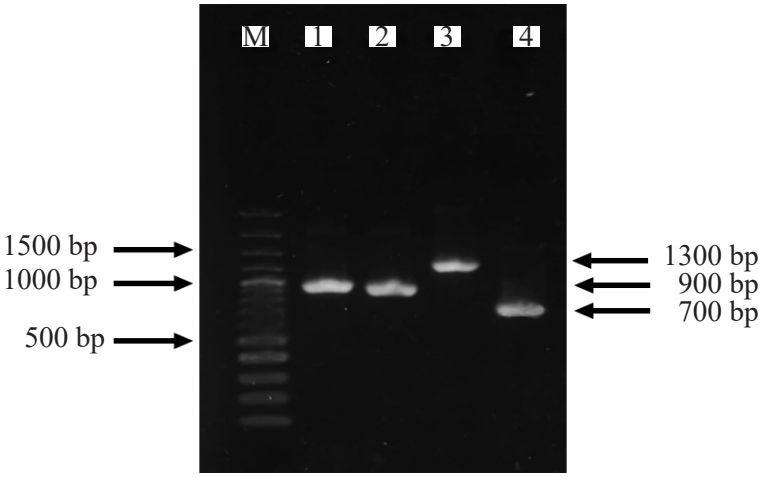


Figure 2. Visualization of *Splt*NPV pif gene amplification on 1% agarose gel using UV-transilluminator. M= marker 1 kb (Thermo Scientific, US); lane 2= *pif-1a* gene; lane 2= *pif-1b* gene; lane 4= *pif-2* gene; lane 5= *pif-3* gene.

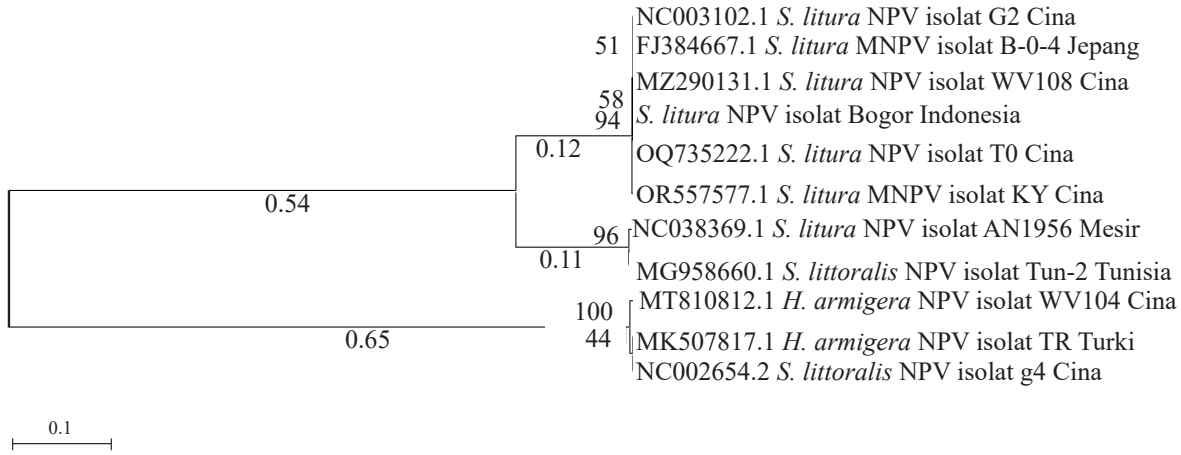


Figure 3. Phylogeny tree of *Splt*NPV *pif-1* gene nucleotide sequences based on Maximum-likelihood method with Kimura-2 approach (1000× bootstrap).

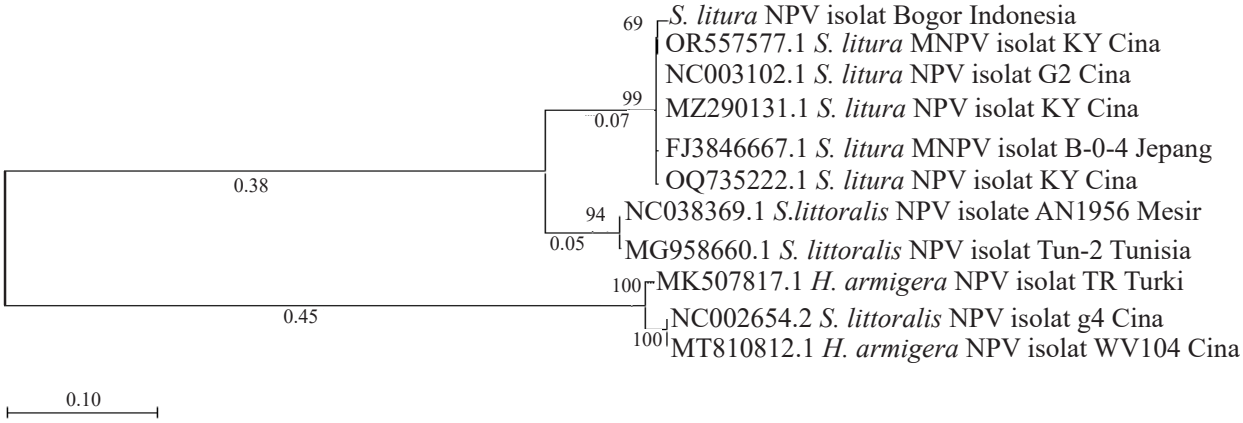


Figure 4. Phylogeny tree of *Splt*NPV *pif-2* gene nucleotide sequences based on Maximum-likelihood method with Kimura-2 approach (1000× bootstrap).

The Baculovirus genome contains approximately 895 open reading frames, 31 of which are core genes (Miele et al., 2011). *Per os* infectivity factor (pif) genes are among these core genes, expressed in the late phase of infection and playing a crucial role in oral viral infection in the host's digestive tract. The PIF protein facilitates the attachment of the occlusion body and the fusion of the nucleocapsid into the epithelial cells of the host midgut (Liao et al., 2013). The *pif* gene sequence is highly conserved and is also found in other virus groups, including nudiviruses and polydnaviruses (Burke et al., 2013). Several known *pif* genes in the baculovirus genome include *pif-0* (P74), *pif-1*, *pif-2*, *pif-3*, *pif-4*, *pif-5*, *pif-6*, *pif-7*, *pif-8*, and *pif-9* (Makalliwa et al., 2018).

Each *pif* genes encodes a protein that plays a distinct role in the viral infection process. P74 (*pif-0*) facilitates the attachment of viral particles to midgut epithelial cells (Haas-Stapleton et al., 2004). *Pif-1* and *Pif-2* are involved in binding the viral to the epithelial cell (Ohkawa et al., 2005). The *pif-3* protein does not directly participate in virus attachment but aids in viral translocation across the microvilli (Slack & Arif, 2006). A deletion in any of these genes can disrupt the viral entry process, reducing virulence without eliminating viral pathogenicity (Mu et al., 2014).

**Amino Acid Sequence Analysis.** This study suggests that *Splt*NPV from Bogor may represent a different strain compared to isolates from China and Japan. This conclusion is supported by differences in the number of cysteine residues and the presence of mutations.

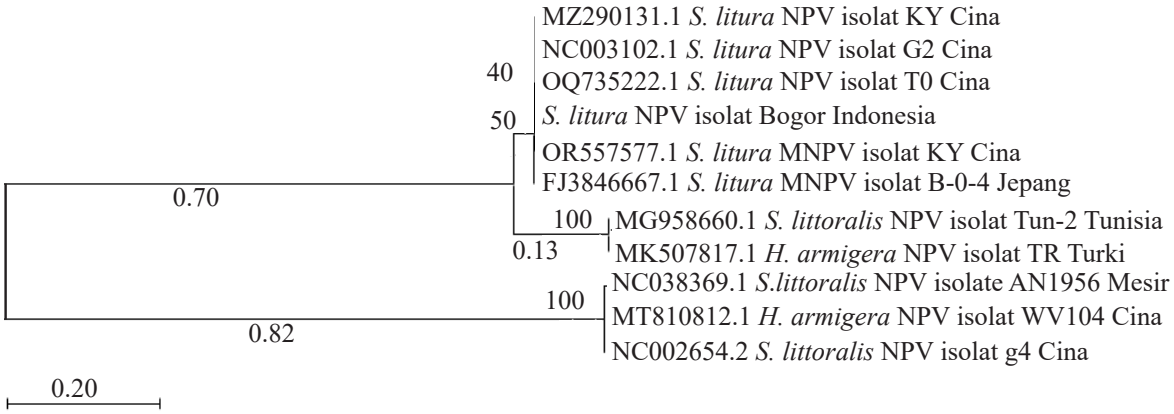


Figure 5. Phylogeny tree of *Splt*NPV *pif-3* gene nucleotide sequences based on Maximum-likelihood method with Kimura-2 approach (1000× bootstrap).

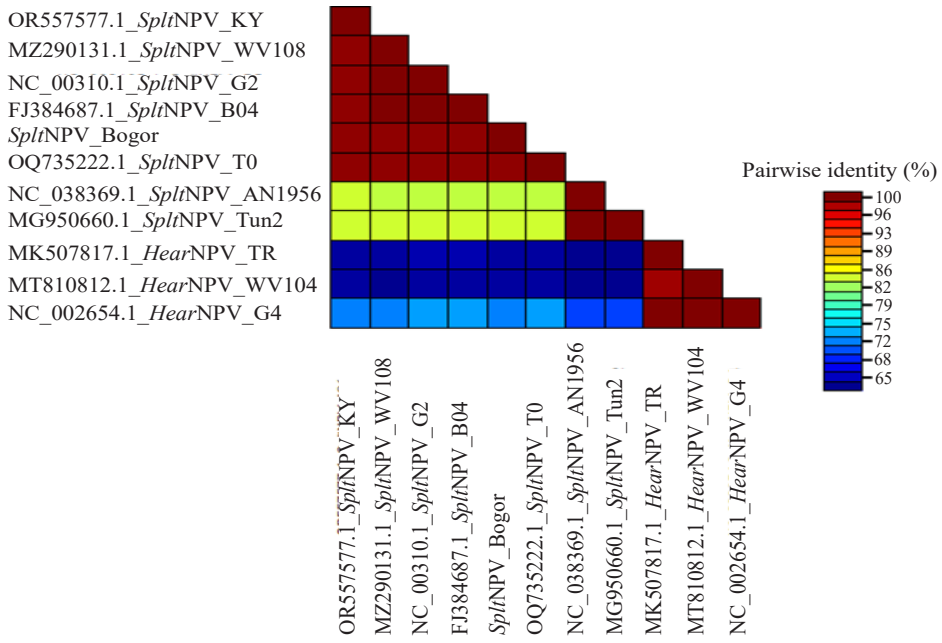


Figure 6. Homology of SpltNPV pif-1 gene nucleotide sequence based of sequence identity matrix. Matrix identity were generated with twelve NPV isolates obtained from NCBI (www.ncbi.nlm.nih.gov).

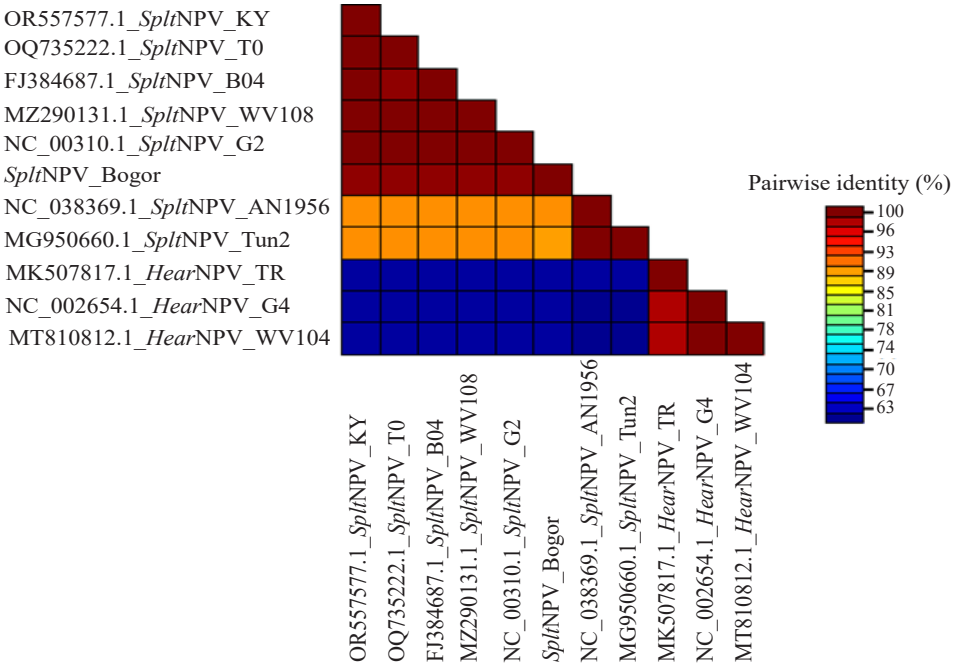


Figure 7. Homology of *Splt*NPV pif-2 gene nucleotide sequence based of sequence identity matrix. Matrix identity were generated with twelve NPV isolates obtained from NCBI (www.ncbi.nlm.nih.gov).

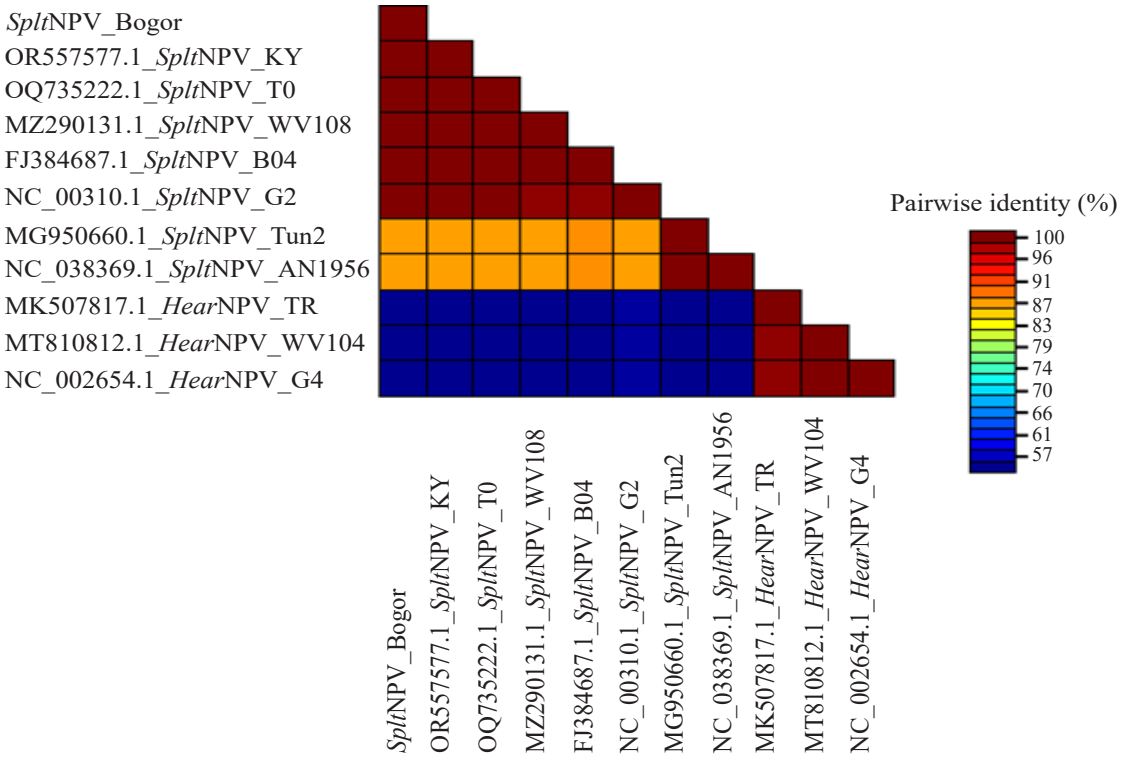


Figure 8. Homology of *Splt*NPV pif-3 gene nucleotide sequence based of sequence identity matrix. Matrix identity were generated with twelve NPV isolates obtained from NCBI (www.ncbi.nlm.nih.gov).

Based on amino acid alignment, the number of cysteine residues in *pif-1*, *pif-2*, *pif-3* from the Bogor *Splt*NPV isolate was 19, 14, and 13, respectively. *Pif-1* from *Splt*NPV Bogor had one additional cysteine residue at position 228 (Figure 9), which was found in *pif-2* or *pif-3* (Figure 10, Figure 11).

Mutation were observed in *pif-1* and *pif-2*. The *pif-1* sequence showed a single point mutation, where glycine (G) was replaced by cysteine (C) at position 228. The *pif-2* sequence contained three mutations:

Table 2. BLAST result of several *Pif* gene in *Splt*NPV Bogor isolate based on comparison with nucleotide data from NCBI (www.ncbi.nlm.nih.gov)

| Spesies | Accesion number | Country | Query cover (%) | | | Identity (%) | | |
|---|---|---|---|---|---|---|---|---|
| | | | Target gene | | | | | |
| | | | *Pif-1* | *Pif-2* | *Pif-3* | *Pif-1* | *Pif-2* | *Pif-3* |
| *S.litura* NPV isolate KY | OR557577.1 | China | 100 | 97 | 100 | 99.37 | 99.32 | 100 |
| *S.litura* NPV isolate T0 | OQ735222.1 | China | 100 | 97 | 100 | 99.56 | 99.07 | 100 |
| *S.litura* NPV isolate WV108 | MZ290131.1 | China | 100 | 97 | 100 | 99.49 | 99.24 | 99.83 |
| *S.litura* NPV isolate G2 | NC_003102.1 | China | 100 | 97 | 100 | 99.49 | 99.24 | 99.67 |
| *S.litura* NPV isolate B-0-4 | FJ384667.1 | Japan | 100 | 97 | 100 | 99.49 | 99.07 | 99.83 |
| *S.littoralis* NPV isolate AN1956 | NC_038369.1 | Egypt | 99 | 94 | 99 | 83.48 | 89.17 | 87.65 |
| *S.littoralis* NPV isolate Tun-2 | MG958660.1 | Tunisia | 99 | 94 | 99 | 83.61 | 89.08 | 87.65 |
| *H. armigera* NPV isolate TR | MK507817.1 | Turkey | 25 | 53 | 26 | 64.52 | 67.23 | 73.62 |
| *H. armigera* NPV isolate G4 | NC_002654.2 | China | 7 | 53 | 26 | 71.77 | 67.23 | 73.01 |
| *H. armigera* NPV isolate W104 | MT810812.1 | China | 25 | 53 | 26 | 64.69 | 66.72 | 73.01 |

histidine (H) to proline (P) at position 49, glutamic acid (E) to lysine (K) at position 383, and alanine (A) to arginine (R) at position 389 (Figure 10). However, no significant differences were observed in the N-terminal domain structures of *pif* genes from *Splt*NPV Bogor, China, Japan, Egypt, and Tunisia.

The *Pif* proteins *(pif-0, pif-1*, *pif-2*, *pif-3*, *pif-4*) form a protein complex crucial for the fusion process and determining viral host specificity (Ohkawa et al., 2005). The ability of the virus to infect its host is influenced by the stability of this protein complex. A more stable complex resists proteolytic degradation by factors such as high protease concentrations and the alkaline pH of the midgut environment (Boogaard et al., 2017). Proteases in the midgut can inhibit the attachment of the pif protein complex, thereby affecting viral infectivity (Horton & Burand, 1993).

The stability of *pif* protein complexes is closely associated with the number of cysteine residues and the structure of the inner nuclear membrane sorting motif (INM-SM) in the N-terminal domain. *Pif* proteins are cysteine-rich, which indicates by high-level of cysteine in every type of pif protein. In amino acid sequence, high concentration of cysteines is needed to form disulfide bonds that facilitates protein complex formation and assists protein folding process (Wang et al., 2017). The INM-SM sequence consists of 18 hydrophobic amino acids with one or more positively charged amino acids at the end (Boogaard et al., 2018). According to Braunagel et al. (2004), the N-terminal domain is essential for binding to the plasma membrane and translocating to the inner nuclear membrane.

Based on nucleotide identity differences and mutations in *pif-1* and *pif-2*, the Bogor *Splt*NPV isolate may represent a new strain. PIF proteins, particularly *pif-1*, *pif-2*, and *pif-3*, are highly conserved, as indicated by their percentage of amino acid constant sites (PAACS). In *Bombyx mori* nucleopolyhedrovirus, a model species, PAACS values for *pif-1*, *pif-2*, and *pif-3* range from 97% to 98% (Xu et al., 2013). Mutations in conserved viral proteins suggest strain differences within the same species (Doud et al., 2015).

The study of viral protein structures, which primarily act as factors of infection, is essential for understanding the physiology and pathogenicity potential of the virus within its host. A mutation in one or more amino acids in this region could potentially affect the structure of the pif protein, which may lead to changes in its function. According to Boogaard et al. (2020), mutations could impact the *pif* protein's ability to bind to the host cell and its proteolytic resistance. Based on this study, the mutations that occurred in the amino acids were not significant to the conserved regions, as evidenced by the number of mutations found in the sequence. According to a Baculovirus genetic variation study, the maximum number of mutations in *pif-1*, *pif-2*, and *pif-3* are 26, 8, and 6 sites, respectively (Xu et al., 2013). The number of mutated sites in *pif-1*, *pif-2*, and *pif-3* in this study are 1, 3, and 0, respectively. This information indicates that protein mutation in *Splt*NPV Bogor did not significantly alter the viral infection process.

Understanding the structure of infection-related viral proteins is crucial for evaluating the potential of NPV as a biological control agent. Low pathogenicity is observed in NPV isolates lacking

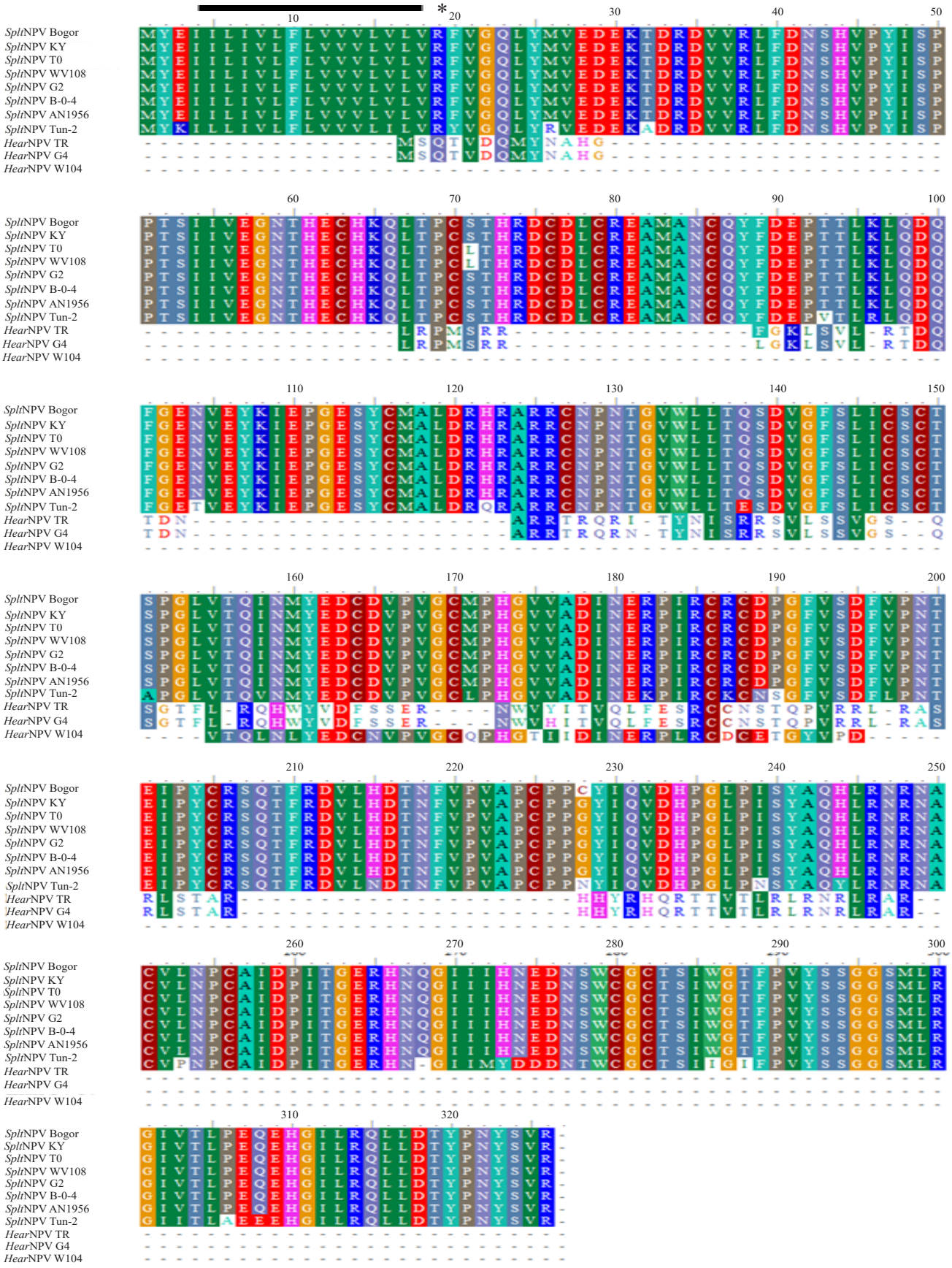


Figure 9. Amino acid sequence alignment of *Splt*NPV *pif-1* gene. Sequences with bold line (-) indicates hydrophobic region in N-terminal domain; sequences with asterisk (*) indicates the positively charged amino acid.

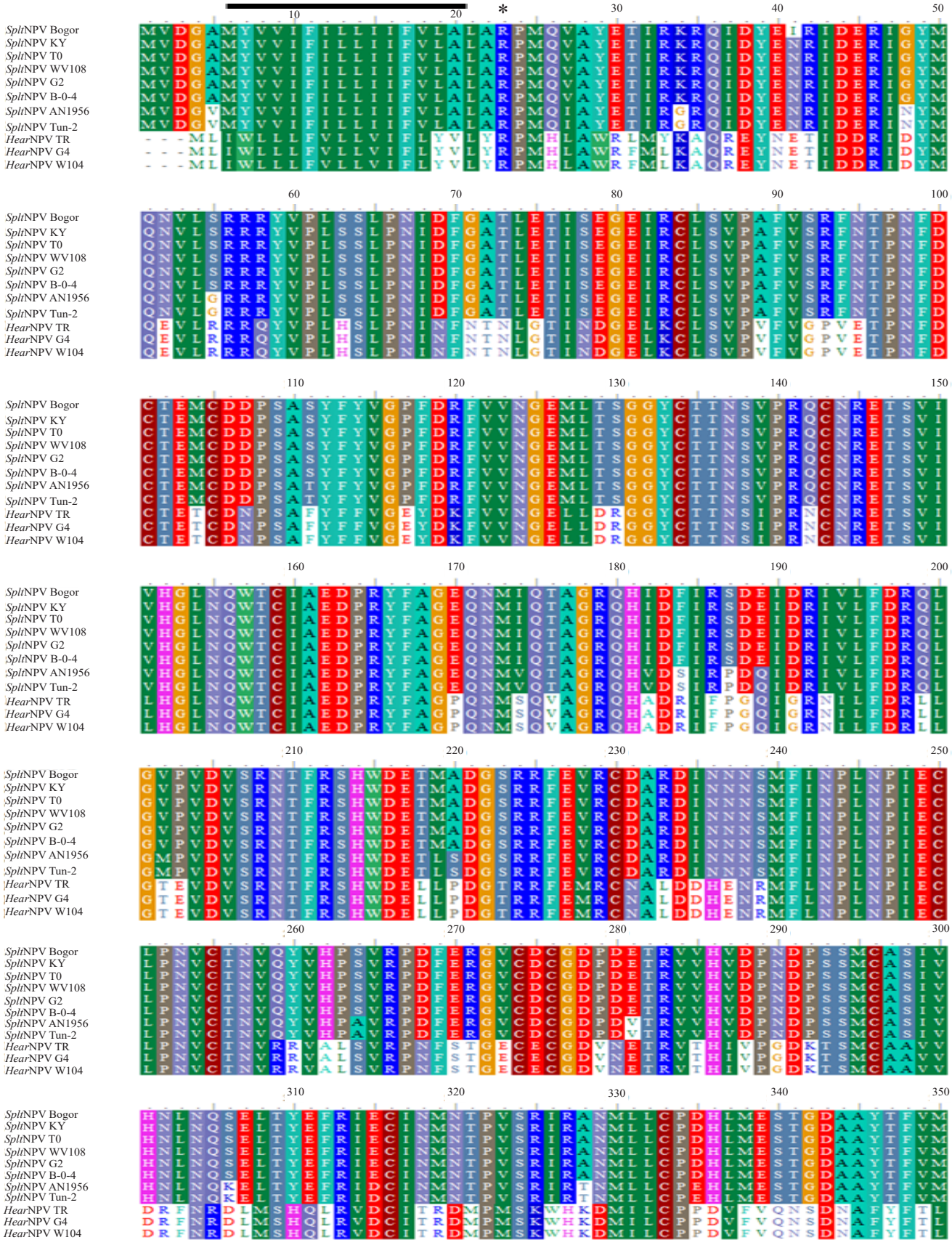


Figure 10. Amino acid sequence alignment of *Splt*NPV *pif-2* gene. Sequences with bold line (-) indicates hydrophobic region in N-terminal domain; sequences with asterisk (*) indicates the positively charged amino acid.

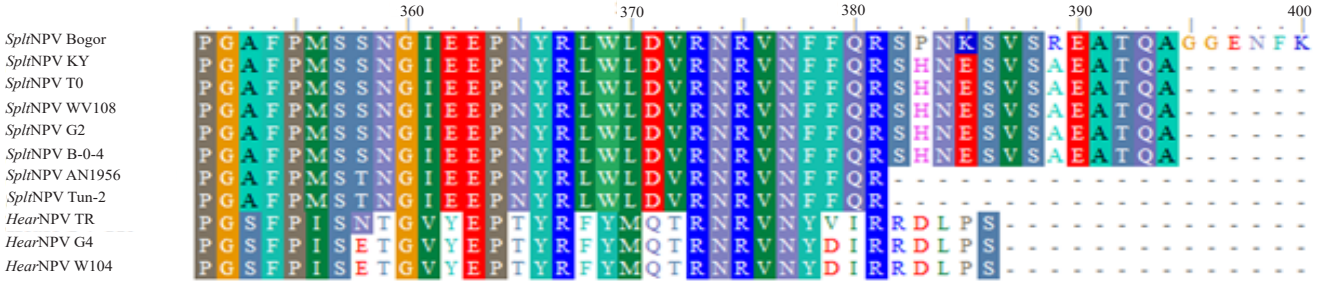


Figure 10. Continued. Amino acid sequence alignment of *Splt*NPV pif-2 gene. Sequences with bold line (-) indicates hydrophobic region in N-terminal domain; sequences with asterisk (*) indicates the positively charged amino acid.

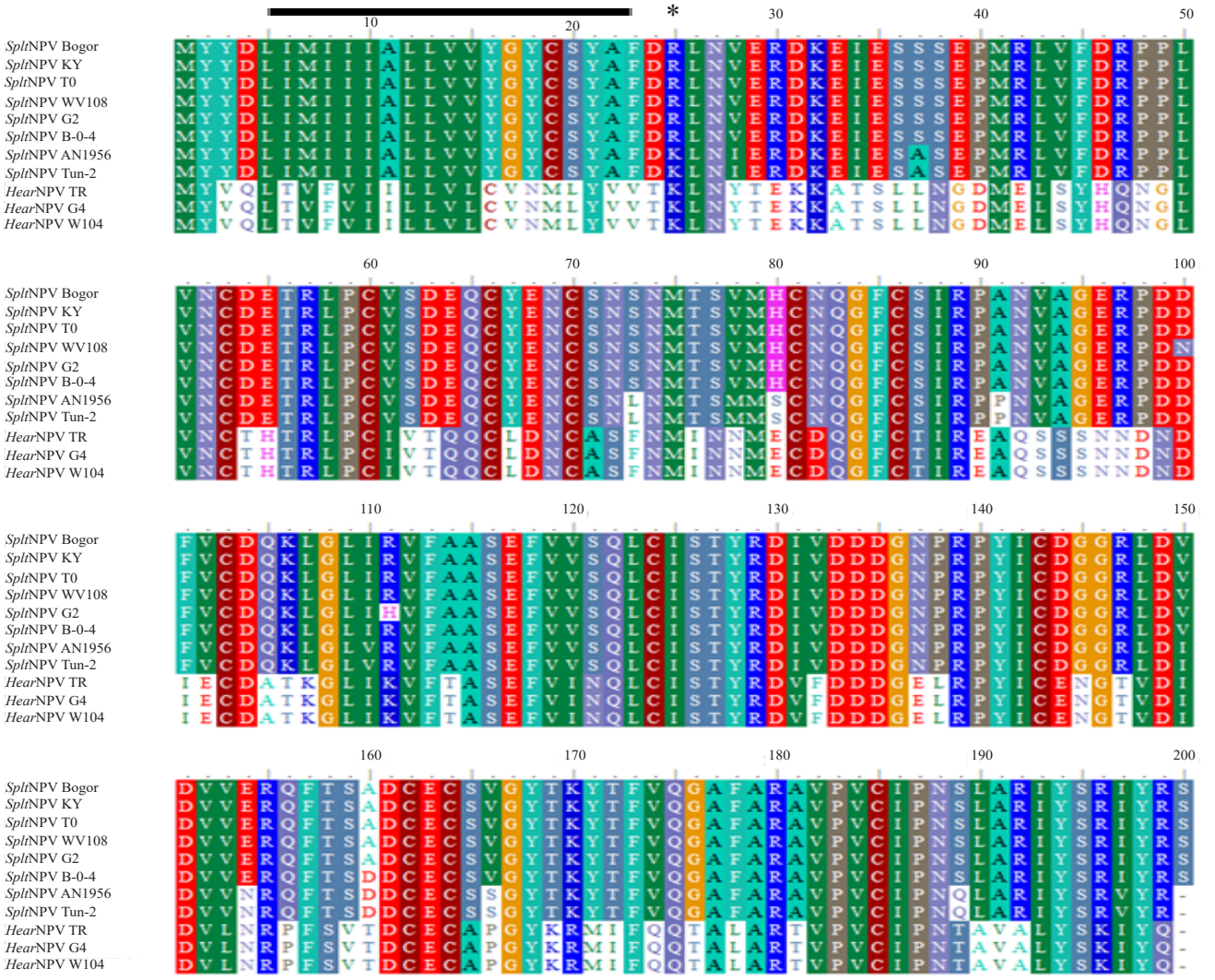


Figure 11. Amino acid sequence alignment of SpltNPV *pif-3* gene. Sequences with bold line (-) indicates hydrophobic region in N-terminal domain; sequences with asterisk (*) indicates the positively charged amino acid.

*pif* gene expression. Deletion of *pif* genes, especially *pif-1* and *pif-2*, prevents viral entry into host midgut cells, impairing the oral infection pathway (Mu et al., 2014). While low *pif-1* expression does not affect pathogenicity, it influences viral virulence (Wang et al., 2017).

## CONCLUSION

The result of BLAST analysis showed that the Bogor *Splt*NPV isolate had the highest similarity to the *Splt*NPV isolates from China, namely isolates KY and T0. Based on identity matrix and phylogeny analysis,

Bogor *Splt*NPV isolate is in the same group with NPV species that infect Spodoptera litura and Spodoptera littoralis. Pif-1 from *Splt*NPV Bogor have one additional cysteine amino acids compared to SpltNPV and *Splt*NPV isolates from NCBI. N-terminal domain in pif-1, pif-2, and pif-3 from *Splt*NPV Bogor shows no difference to pif genes from *Splt*NPV and *Splt*NPV from NCBI.

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This research was funded independently.

## AUTHORS' CONTRIBUTIONS

MC considered and planned the experiment, sampling, infecting larvae, carried out the isolation of DNA Spodoptera litura NPV, performed molecular work and analysis data sequencing. MC, YMK, and KHM interpreting sequence data. MC, YMK, KHM prepared the manuscript. All the authors have read and approved the final manuscript.

## COMPETING INTEREST

We confirm that we have no competing interests to declare regarding the publication of our work.

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