Molecular characterization of *Spodoptera litura* Nucleopolyhedrovirus (*S*NPV) Labuhan Batu isolate

D P Sinambela¹, D Bakti¹, M C Tobing¹ and Y M Kusumah²

¹Agrotechnology Department, Agriculture Faculty, Universitas Sumatera Utara, Medan, Indonesia, 20155
²Plant Protection Department, Agriculture Faculty, Institut Pertanian Bogor, Bogor, Indonesia, 16680

Corresponding author email: dbakti0656@gmail.com

Abstract. *Spodoptera litura* F. is a new pest that potentially becomes a serious pest threatening palm oil industry. The control can be carried out using biological control agents *S. litura* Nucleopolyhedrovirus (*S*NPV). Molecular approach using polymerase chain reaction (PCR) has been used to characterize the *S*NPV isolate. The aim of this study was to determine the molecular characteristics of *S*NPV Labuhan Batu isolate based on DNA sequence analysis. The *NPV* isolate was collected from oil palm plantation Negeri Lama Estate Group, PT. Hari sawit Jaya, Asian Agri, Labuhan Batu. The *S*NPV DNA extraction was conducted using the DNA gSYNC kit. The amplification of *S*NPV DNA was done by specific primers with oligonucleotide sequences forward 5'-ACGTTACGATCTAGATCC-3' and reverse 5'-AACGTTAGACAGACATAT-3' with ±700bp of amplicon. Homology and phylogeny characteristics were used to determine the relationship of *S*NPV isolate from Labuhan Batu to NPV from other countries were reported in the GeneBank of National Center for Biotechnology Information (NCBI). The results showed that *S*NPV isolate from Labuhan Batu had the closest related to *S*NPV from Australia, *S*NPV from Canada and *S*NPV from China, with the similarity percentage 100%, 96% and 90% respectively. They belong to the same group as the NPVs that infected the *Spodoptera* genus.

Keywords: gen polymerase, molecular characterization, NPV, *Spodoptera litura*

1. Introduction

Oil palm is one of the plantation commodities that has an important role in the Indonesian economy. Oil palm can produce vegetable oil which is needed by the industrial sector for various purposes, such as cooking oil, industrial oil, and biodiesel fuel [1]. The number of derivative products that can be produced from oil palm commodities is the reason why oil palm plantation companies have survived since the early development of oil palm plantations in the 1980s until now, which generally have entered the second generation.

*Spodoptera litura* Fabricius. (Lepidoptera:Noctuidae) is a polyphagous pest that attacks food, horticulture, and estate crops. In addition to soybeans, these insects have also been found to attack tobacco, cotton, cabbage, and green beans [2]. In oil palm plantations this pest is a newcomer that appears in the second generation of young plants, however, the intensity of its attack in the field was recorded at 1.2 – 5.4% of the total 7,861 Ha area of young plants aged 1-2 years in 2017 (Source of data: Asian Agri).
As *S. litura* attacks in the field, there are several natural enemies whose presence is consistent over time and is found in several attack locations. One of the natural enemies of *S. litura* that has the potential to be developed as a biological control agent is *Spodoptera litura* Nucleopolyhedrovirus (*SlNPV*) which has high virulence against *S. litura* larvae. Samsudin [3] reported that two *SlNPV* isolates namely isolates B01 and B02 with a dose of $1.5 \times 10^{12}$ PIBs/ha can reduce the population of *S. litura* between 90-94% at 6 days after application. Besides, *SlNPV* symptoms were also found in the pupa and imago of *S. litura* [4]. The results of Bedjo's research [5] found that *Borrelinavirus litura* (Virus: *Borrelinaceae* *SlNPV* isolate JTM 97c had the same effectiveness as the insecticide lambda-cyhalothrin against *S. litura* larvae. According to Cory *et al* [6], *Baculovirus* isolates obtained from the same species in different geographic locations often show genetic variation and differences in virulence. Therefore, a more precise molecular identification of host-specific virulent strains is needed to ensure virulence stability in the field.

Polymerase Chain Reaction (PCR) is a detection method that can be used to identify Nucleopolyhedrovirus using primers for the polymerase gene and allow for the use of freely available software to determine the extent of similarity and the translated protein sequence from amplified sequences [7]. The aim of this study was to determine the molecular characteristics of *SlNPV* Labuhan Batu isolate based on DNA sequence analysis.

2. Material and methods
2.1. Collection of *SlNPV* isolate
*SlNPV* isolate was collected from *S. litura* larvae cadaver infected by NPV from oil palm plantation Negeri Lama Estate Group, PT. Hari sawit Jaya, Asian Agri. The visible late symptoms of *S. litura* larvae infected by NPV were larvae become inactive, the body swelled and slightly shiny, the cuticle color changed become browner and the larvae died. The visible further symptoms were the larvae cuticle being lysed so that becomes fragile and secrete a clear liquid, pinkish milky white and brownish milky white depending on the severity of the infection (Figure 1). The cadavers of the symptomatic *S. litura* larvae collected from the field were put in a vial glass and stored in a low-temperature freezer at -20°C to prevent spoilage or contamination from other microbes.

![Figure 1. The symptoms of NPV attack on S. litura larvae.](image)

2.2. Extraction of *SlNPV*’s DNA
DNA extraction of *Spodoptera litura* NPV Labuhan Batu was isolated using a gSYNC DNA extraction kit (Geneaid Biotech Ltd). The tissue dissociation process was carried out by inserting 50 mg of asymptomatic *S. litura* carcass sample into a 1.5 ml micro-tube, 200 μl of GST buffer and 20 μl of proteinase K added to the sample and then ground using a micropistil and then incubated in a water bath at 60°C for 2 hours. Every 10 minutes the microtube was inverted to speed up the lysis process until the lysate was clear. The cell lysis process was carried out by centrifugation of the lysate for 2 minutes at a speed of 14,000 rpm. The supernatant formed from the centrifugation process was taken and put into a new 1.5 ml microtube, then 200 μl of GSB buffer was added and incubated at room temperature for 5 minutes. The DNA binding process was carried out by adding 200 μl of ethanol absolute to the supernatant and then mixed using a vortex for 10 seconds. The supernatant precipitate that has not been destroyed by this process is crushed using a pipette tip. After being homogeneous and there was no precipitate, the supernatant solution was put into the GS column and centrifuged for
1 minute at a speed of 14.000 rpm. The new supernatant formed in this process was discarded and the GS column was transferred to a new collection tube. The washing process was carried out by adding 400 μl of W1 buffer into the GS column then centrifuged for 30 seconds at a speed of 14.000 rpm. The newly formed supernatant was discarded and the GS column was transferred to a new collection tube. A total of 600 μl of wash buffer was put into the GS column then centrifuged again at 14,000 rpm for 30 seconds. The newly formed supernatant was discarded, then centrifuged again at 14.000 rpm for 3 minutes to dry the column matrix. The elution process was carried out by transferring the GS column into a new 1.5 ml microtube. A total of 100 μl of elution buffer (which had previously been incubated at 60°C for 10 minutes), then placed in the middle of the column matrix. The GS column was incubated in an upright position for 3 minutes for complete absorption. After that, the GS column was centrifuged at 14.000 rpm for 30 seconds and the pure DNA was ready to be used as a template for the PCR reaction.

2.3. Amplification of SlNPV and visualization of amplification results
SlNPV DNA amplification was carried out using specific primers for the amplification polymerase gene with oligonucleotide sequences forward 5'- ACACG TTA CGA TCT AGA TCC -3' and reverse 5'- AAG CTA AGT AGA CAG ACA TAT -3' and DNA amplification targets ±700 bp [8]. In each reaction of SlNPV amplification were required 2 μl of DNA (±40 ng/μl), 12.5 μl of Taq polymerase, 1 μl of forward and reverse primers, and 8.5 μl of ddH2O. The steps carried out during amplification were pre-denaturation at 94°C for 4 minutes, denaturation at 94°C for 1 minute, annealing at 46°C for 1 minute, elongation at 72°C for 2 minutes, post-elongation at 72°C for 10 minutes and storage of the amplification results at 4°C for an indefinite period. The denaturation, annealing and elongation stages were repeated 30 times. The visualization of DNA bands was performed through electrophoresis. A total of 5 μl of the amplified DNA was inserted into a 2% agarose gel well which was immersed in Tris Borate EDTA (TBE) solution and then an electric current of 50 V was applied for 100 minutes. The gel resulting from the electrophoresis was immersed in an EtBr solution for 20 minutes. DNA visualization was performed using a UV-transilluminator.

2.4. Sequence analysis and phylogenetic tree construction
The amplified DNA fragments were sent to 1st BASE Laboratories, Malaysia for DNA sequencing. The nucleotide sequences obtained were compared with other NPV nucleotides published on the GenBank National Center for Biotechnology Information (NCBI) website using the basic local alignment search tools (BLAST) program. The nucleotide sequences selected were aligned with the Clustal W alignment program in Bioedit sequence alignment editor software version 7.1.3. The Phylogeny tree was constructed by using the Maximum Likelihood method and Tamura-Nei model [9] on the Molecular Evolutionary Genetics Analysis software version 10.0 (MEGA X) with 1000 x bootstraps.

3. Result and discussion
3.1. Visualization of amplification results
The DNA bands visualization result of SlNPV Labuhan Batu isolates showed that amplification of SlNPV DNA using specific primers designed from SlNPV polymerase gene sequence was able to amplify the SlNPV DNA from Labuhan Batu isolate with a DNA band base fragment size of about ±700 bp (Figure 2). The specific primer was able to amplify the target gene with the size of the appropriate amplicon of SlNPV which has also been reported by previous researchers [8].
The virus genetic analysis method was carried out using the PCR technique, where the primers play a role in initiating the DNA molecules amplification. According to Aris et al [10], the presence of the PCR primer will amplify the target gene throughout the reaction. PCR analysis using specific primers is the best way for pathogen detection because it can determine the presence of pathogen target genes quickly.

3.2. *SlNPV* DNA fragments sequencing

Sequencing of DNA fragments which were resulted from *SlNPV* amplification was carried out by 1st BASE Malaysia through intermediary of PT. Genetika Science. The *SlNPV* DNA fragments sequencing results were obtained in chromatogram format (Figure 3). The chromatogram of the *SlNPV* DNA fragments sequence showed good results, which were indicated by little background noise found. The forward and reverse fragments obtained were processed and aligned using the Bioedit program to obtain the complete *SlNPV* sequence (Figure 4).

Figure 2. Visualization results of *SlNPV* DNA using DNA polymerase primers.

Figure 3. The results of sequencing *SlNPV* DNA fragments in the form of chromatograms

Figure 4. The results of sequencing forward and reverse *SlNPV* fragments using contig program.
3.3. *SlNPV* sequence relationship comparation analysis

The complete DNA fragments sequence of *SlNPV* Labuhan Batu was then analyzed by comparing it one to one to other virus DNA fragments sequences that had been reported on the GenBank National Center for Biotechnology Information (NCBI) website using the basic local alignment search tools (BLAST) program. The relationship comparison analysis result showed that *SlNPV* Labuhan Batu isolate was similar to five different NPV isolate from different countries and two of them showed identically similar with similarity percentage above 95% which were higher than another isolate (Table 1). However, the other three viruses isolate still have a high similarity relationship to *SlNPV* Labuhan Batu with a similarity percentage above 85%. One species has an identical similarity to others if it has more than 73% percentage of similarity [11].

**Table 1.** Results of BLAST on *polymerase gene* samples from *S.litura* NPV Labuhan Batu, Indonesia

| Species | Origin country | Access code | Max/Total score | Query cover (%) | Ident (%) |
|---------|----------------|-------------|-----------------|-----------------|-----------|
| *Spodoptera litura* NPV strain G2 | China | AF325155.1 | 1304/1304 | 100 | 99.4 |
| *Spodoptera litura* NPV DNA polymerase (dpol) gene | Australian | AF068187.1 | 1271/1271 | 100 | 98.8 |
| *Spodoptera littoralis* NPV DNA polymerase (pol) gene | Canadian | AF215639.1 | 905/905 | 100 | 89.2 |
| *Spodoptera littoralis* NPV isolate SpliNPV-Tun2 | Germany | MG958660.1 | 900/900 | 100 | 89.2 |
| *Spodoptera littoralis* NPV isolate AN1956 | USA | NC_038369.1 | 883/883 | 100 | 88.8 |

*SlNPV* Labuhan Batu isolates had the highest similarity with three *SlNPV* isolates, they were *SlNPV* isolate from China (Access No.: AF325155.1), *SlNPV* isolate from Australia (Access No.: AF068187.1) and *SlNPV* isolates from Canada (Access No.: AF215639.1), with 99.4%, 98.6% and 89.4% similarity percentage respectively, 1304/1304, 1271/1271 and 905/905 max/total score respectively and 100% query cover percentage respectively. In addition, *SlNPV* Labuhan Batu isolates also had high similarities with *SlNPV* isolate from Germany (Access No.: MG958660.1) and *SlNPV* isolates from USA (Access No.: NC_038369.1) with 89.2% and 88.8% similarity percentage respectively (Table 1).

The developed bio informative tools and application of latest genomic data facilitated the identification of viruses because of good primer designing. Ahmad [12], use molecular techniques to characterize native *S. litura* associated NPVs that were found identical to SpliNPVs from other countries, where LC<sub>50</sub> values against 2<sup>nd</sup>-5<sup>th</sup> instars with LT<sub>50</sub> values of this NPV isolate (SltNPV-FSD15) indicated a significant effective control against *S. litura*. Besides, Laarif et al [13] indicated that amino acid and nucleotide sequences of *S. littoralis* (Tun-SNPV) nucleopolyhedrovirus strain was almost identical with different GenBank deposited sequences of NPVs [14, 15].

3.4. Nucleotide Homology of *SlNPV* Labuhan Batu

In addition to sequence Relationship Comparison Analysis, kinship analysis based on nucleotide homology is also carried out. Nucleotide homology analysis was used to compare the relationship between the six isolates that had similarities based on sequence relationship comparison analysis. The result showed that four isolates of them namely *SlNPV* isolate from Labuhan Batu (3051411 WR), *SlNPV* isolate from Australia (Access No.: AF068187.1), *SlNPV* from Canada (Access: AF215639.1), and *SlNPV* from China (Access No.: AF325155.1) had a closest relationship each other which can be seen from the value of homology percentage between each of them (Table 2).
Table 2. Nucleotide homology percentage of SlNPV isolate from Labuhan Batu, pol gene, with data from GenBank

|   | 1   | 2   | 3   | 4   | 5   | 6   |
|---|-----|-----|-----|-----|-----|-----|
| 1 | ID  |     |     |     |     |     |
| 2 | 0.904 | ID  |     |     |     |     |
| 3 | 1.256 | 1.505 | ID  |     |     |     |
| 4 | 0.838 | 0.153 | 1.427 | ID  |     |     |
| 5 | 0.965 | 1.626 | 1.625 | 1.605 | ID  |     |
| 6 | 0.835 | 0.154 | 1.425 | 0.005 | 1.611 |     |

Note. 1. SlNPV from Labuhan Batu, 2. SlNPV from China, 3. SlNPV from Australia, 4. SliNPV from Germany, 5. SlNPV from Canada, 6. SlNPV from USA.

The value of homology percentage between SlNPV isolate from Labuhan Batu (3051411 WR), SlNPV isolate from Australia (Access No.: AF068187.1), SlNPV from Canada (No. Access: AF215639.1), and SlNPV from China (Access No.: AF325155.1) were 100%, 96% and 90% respectively. The other two isolates showed the value of homology percentage about 0.5-100% (Table 2). According to Fauquet et al [16], if a virus species has a nucleotide sequence homology level of more than 72%, it is the same virus species. So that SlNPV isolates from Labuhan Batu (3051411 WR), SlNPV isolate from Australia (Access No.: AF068187.1), SlNPV from Canada (No. Access: AF215639.1) and SlNPV from China (Access No.: AF325155.1) can be put together in the same group that has the closest related to SlNPV Labuhan Batu.

3.5. Phylogenetic tree construction of SlNPV Labuhan Batu

The results of phylogenetic analysis based on the nucleotide sequence of the polymerase gene using the Maximum Likelihood method showed that SlNPV Labuhan Batu was closest related to SlNPV from Australia, SlNPV from Canada and SlNPV from China so can be put in the same group. SlNPV Labuhan Batu was outgroup with SliNPV from Germany and SlNPV from USA (Figure 5).

![Phylogenetic tree](image)

Figure 5. The phylogenetic tree of NPV nucleotide sequence was processed using MEGA 10 software based on the polymerase gene with the neighbor-joining method with 1000x bootstraps.
It means that SlNPV from Labuhan Batu, Indonesia, SiNPV from Australia, SlNPV from Canada, and SiNPV from China come from the same ancestor. In addition, DNA polymerase is one of the conserved genes that can be used to identify a species of NPV. According to Rohrmann [17], the pol/gran gene locus is one of the most conserved genes of lepidopteran-specific baculoviruses and was also the first baculovirus gene that was used for phylogenetic studies. Furthermore, Lange et al [18], state that for simple molecular identification and combined phylogenetic analyses, polh/gran is still very useful, especially because a great number of polh/gran gene sequences are available. The results showed that molecular methods based on DNA extraction and PCR amplification can significantly reduce the amount of time needed to identify unknown baculovirus isolate. According to De Moraes et al [19], PCR is a very sensitive technique that amplifies the target DNA sequence, and virus DNA obtained from infected larvae is a good source of DNA for PCR amplification.

4. Conclusion

The SlNPV from Labuhan Batu, Indonesia, SiNPV from Australia, SlNPV from Canada and SiNPV from China come from the same ancestor and they belong to the same group as the NPVs that infected the Spodoptera genus. Molecular methods based on DNA extraction and PCR amplification using DNA polymerase genes can be used to identify an unknown baculovirus isolate.

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