Detection of pebrine disease and Bombyx mori Nuclear Polyhedrosis Virus (BmNPV) in silkworm moths (Bombyx mori. L)

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Abstract. Silkworm is an insect that can produce silk fiber at any step of life, one of them in the moth phase. There are still not many researchers who have researched pebrine and BmNPV disease detection in the moth phase. This research purpose was to find out pebrine and BmNPV disease detection on moth phase. This research was conducted from January 2021 to March 2021 with samples obtained from the Center for Social Forestry and Environmental Partnership, South Sulawesi in Bili-Bili, Gowa district. The data processing was done at the Laboratory of Biotechnology and Tree Breeding, Faculty of Forestry, Universitas Hasanuddin, Makassar. Data analysis was done using the descriptive analysis method by looking at the primer amplification of Pebrine disease and BmNPV in the silkworm moth phase. The detection of pebrine disease ad BmNPV from the two primers showed that from 12 moths studied, none of the moths detected either pebrine disease or BmNPV disease.

1. Introduction
Silkworm (Bombyx mori L) is an insect-producing silk thread fiber at a stage of its life cycle. It metamorphoses from egg to larvae, larvae to pupae, then larvae to moths. The moths can be directly recognized by the dusty-like scale at their wings which will be attached on the fingers when humans touching it [1].

The moth undergoes several changes in its life to get into an adult form. Starting from eggs, larvae, pupae, and finally moths. This series of events is known as complete metamorphosis and occurs in approximately one month. The number of eggs per parent ranges from 400 to 500 eggs. In general, the eggs hatch after ten days of incubation. Silkworm larvae consist of five instars, the first instar to the third instar are called tiny caterpillars, and the fourth and fifth instars are called large caterpillars [1].

According to the Indonesian Silk Association (ASSIA) in Estetika (2018), Indonesia can only meet the domestic supply of silk thread by 5% of the total demand of 900 tons/year, while 95% is imported
from China. South Sulawesi is the largest silk-producing area in Indonesia, around 80% of Indonesia's total production. There are three central districts in South Sulawesi that have become the centers of natural silk production: the Enrekang, Soppeng, and Wajo districts. South Sulawesi is recorded as the largest producer of silkworm cocoons in Indonesia, reaching an average of 110,307 kg per year [2]. However, natural silk production conditions continue to decline due to the low levels of cocoons production. According to [3], the leading cause of the fall in natural silk production is viruses and bacteria that attack mulberry plants, resulting in decreased yarn production. Decreased production is caused by caterpillars that eat mulberry plants are attacked by viruses and bacteria, causing the caterpillars to die and failed to produce silk thread. Two types of serious diseases can attack silkworms and moths, namely pebrine disease caused by Nosema bombycis and Bombyx mori Nuclear Polyhedrosis Virus (BmNPV) or brasserie disease caused by a virus. Global natural silk has always been reported regarding the attack of the BmNPV virus disease or known as grasseria disease [4,5].

Research on early detection related to diseases caused by BmNPV and Pebrine is usually found only in the caterpillar and egg phases. However, it can also be found in the moth phase. Therefore, this research is necessary because the detection of pebrine disease and BmNPV in moths have never been conducted until now. This study is expected to provide information on whether the pebrine disease and BmNPV can be found from the egg or in moths directly.

2. Methods

Samples obtained from the BPSKL (Center for Social Forestry and Environmental Partnership) South Sulawesi region in Bili-Bili, Gowa district. The data processing was done at the Laboratory of Biotechnology and Tree Breeding, Faculty of Forestry, Universitas Hasanuddin, Makassar.

2.1. Tools and materials

The tools used were tubes, tips, pens/markers, tweezers, analytical balances, measuring cups, Vortex, water baths, microwave, mini centrifuge, micropipettes, PCR machine, Centrifuge machine, digital camera, agar molds, electrophoresis machine, gel doc, Enlemeyer Tube, The materials used were silkworm moth DNA (Bombyx mori. L), 2% agarose, alcohol, aquadest, ddH2O, 1 x TAE buffer, red gel, PCR mix Kapa, white tip, and PCR products.

2.2. DNA isolation

DNA isolation was carried out using the Plant mini Kit method. A sampling of silkworm moth (the belly of the moth with a total of 12 samples) was put into a tube, and then 200 ml of GT Buffer was added. After that, it was crushed, then added 20 micro proteinases and vortexed for 15 seconds. The following process was incubation at 60°C for ± 45 minutes (Vortex every 5 minutes), each sample that has been incubated was added with GBT Buffer 200 microns then vortexed and incubated again at 60°C for ± 20 minutes (Vortex every 5 minutes) then Centrifuged at 10,000 G for 3 minutes.

The supernatant/solution was transferred to a new tube. Before re-centrifuged, 200-micron absolute ethanol was added, then vortex again to separate the supernatant and solid material, then the solution transferred into a 65 column with 2 ml tube and then centrifuged at 10,000 G for 4 minutes. The solution contained in the tube was discarded, then 400 micro W1 Buffer was added and then centrifuged at 10,000 G for 1 minute.

After that, the solution in the tube was discarded again, then added 600 Wash Buffer, then centrifuged at 10,000 G for 1 minute. The solution was discarded again and centrifuged at 10,000 G for 4 minutes; the 65 columns were transferred to a new 2 ml tube. In the final stage of isolation, the sample solution was added with 50 microns of elution buffer (the elution buffer must be heated before use) and then centrifuged again at 10,000 G for 1 minute, then put into the freezer before the primer or PCR testing.
2.3. Primer and PCR test
The next step was primer and PCR test. This stage was done by selecting 12 samples because there were only 12 temperature gradients on the primer engine. Each time the PCR reaction was made with various solutions consisting of 1µl of working DNA, 1µl of primer, PCR mix Kapa 6,25 µl, and 3µl of dH2O with a total reaction was 10 µl, then proceed to DNA amplification using a PCR machine. The stages of the PCR amplification process consist of three steps, as follows [6–8].

1. Denaturation step for separating two double strands of DNA for 15-60 seconds at a temperature of 90 – 95 ºC.
2. The annealing stage for binding or re-binding between the DNA template strands and the primers with complementary sequences for 30-60 seconds at a temperature of 50-60ºC.
3. The extension/elongation stage for DNA polymerase synthesis into a complementary strand was at a temperature of 72ºC. The enzyme reads the opposite sequence of the strand and elongates the primer by adding nucleotides in the DNA sequence; thus, the DNA is elongated. The whole process was repeated, so the number increases exponentially. The whole process is highly dependent on the temperature and time of each stage. The primers used can be seen in Table 1.

| Target Strains | Primer Name | Sequence | Number of Nucleotide | Type of Disease |
|----------------|-------------|----------|----------------------|----------------|
| Polh           | Polhefor    | 5’CGTGTA CGACAACAAGTACTACA3’ | 23              | BmNPV          |
|                | Polherev    | 5’AAAGTGA GTTTTGGTTTTTGCC3’ | 24              |                |
| Nb             | NBEF 35F    | 5’-TG GGC CGTG TTTGATAAAGAGTT-3’ | 21              | Pebrine        |
|                | NBEF957R    | 5’-AATT TAGCAACACAAGCCTTAT-3’ | 22              |                |

Source: Polh [9]; Nb [10].

2.4. Electrophoresis
At the silkworm stage, there was an electrophoresis stage where the process used agarose as a medium to detect the silkworm disease based on the temperature. Electrophoresis can be carried out after the silkworm samples have been isolated from DNA, given a buffer solution and extracted with the primer used, and then applied with a special tool (PCR machine).

3.6 g of agarose was weighed in the electrophoresis stage and then put into a 500 ml Erlenmeyer. Dissolved in 180 ml of TAE (Tris-acetate-EDTA) stirred until evenly distributed, then the solution was put in the microwave for 5 minutes. During the first 3 minutes was stirred and put back into the microwave for 2 minutes until dissolved. After that, the solution was removed and added with 1.5 micro red gel liquid, stir until evenly distributed, and then allowed to stand until warm. After the solution has cooled, it was poured into the agarose template container for 1 hour until the agarose hardens. After 1 hour, the comb was removed and then put into a tank containing 1 x TAE buffer solution. The PCR sample was put into the well/hole in the agarose. Then it was running at 120 volts for 60 minutes. After that, the agar was released from the electrophoresis template and then placed into the Geldoc to be documented.

2.5. Data Analysis
Data analysis was done using the descriptive analysis method by looking at the primer amplification of Pebrine disease and BmNPV in the silkworm moth phase.

3. Result and discussion
Electrophoresis is the final stage of the molecular analysis process. The results of electrophoresis will determine the amplified DNA from the primer detection result through the PCR machine. The primer detection stage using the DNA isolation method. DNA isolation is a crucial step and must be fulfilled in molecular studies. The DNA isolation stage started by selecting 12 DNA samples.
According to [11] in [12], the primer used for further analysis is a primer that produces clear, bright, and polymorphic bands. The primer selection stage is carried out by making a PCR reaction with several different primers under the same conditions. At the PCR stage, it can be seen that the primer selection results have optimum conditions and the level of band variation in each primer.

**Table 2. Type of Primer and Number of Amplified DNA Bands.**

| Target strains | Primer    | Sequence (5’- 3’)                                      | Annealing Temperature (°C) | Remarks                                      |
|---------------|-----------|--------------------------------------------------------|----------------------------|----------------------------------------------|
| NPV           | Polhefor  | 5’CGTGTACGACAACAAAGTACTACA3’                           | 54.7                       | Bright, clear, and monomorphic bands         |
|               | Polherev  | 5’AAAGTGAGTTTTTGGTTTTTGCC3’                            |                            |                                              |
|               | NBEF 35F  | 5’-TGGCGCTGTGATAAGAGATT-3’                             | 52.2                       | Bright, clear, and monomorphic bands         |
| Nb            | NBEF957R  | 5’-AATTTAGCAACACAAGCCTTAT-3’                           |                            |                                              |

Primer selection greatly influences the resulting band polymorphism because each primer has its attachment site in the number of base pairs and bands. The selection has been carried out using 12

![Figure 1](image1.png)

**Figure 1.** Electrophoresis of POLHE primer PCR Amplification Results (Remarks M = marker and 1-12 = Moth Sample Code).

DNA samples on moths with two types of primers. The primers with the number and size can be seen in Table 2. The primer selection in Table 2 used two primers, where the Polhefor and Polherev primers with a temperature gradient of 54.7°C that almost the same temperature as the research by [9], which was 55 °C, while the NBEF35F and NBEF95 7R primers used a temperature gradient of 52.2°C.

Figure 1 is the electrophoresis of PCR amplification results from POLHEFOR with 5’CGTGTACGACAACAAAGTACTACA-3’ sequence and POLHEREV with 5’AAAGTGAGTTTTTGGTTTTTGCC-3’ sequence, which was amplified and produced 12 clear, bright, and monomorphic bands, however not the target gene position.

Primer selection is needed to support the success of the amplification process and can produce the desired amplification product. At the molecular stage, the electrophoresis result shown in Figure 6 indicates that primer types were not suitable for detecting BmNPV disease. Therefore the amplification result obtained was monomorphic.

The results of this primer selection can show that the use of primers developed from silkworm moths does not mean that the amplification process will consistently produce polymorphic, clear, and bright bands and produces monomorphic bands.
Figure 2 above is electrophoresis of PCR amplification results from NBF 35F with 5’ TGGCGCTGTTGATAAAGAGATT-3’ and NBEF95 7R with 5’AATTTAGCAACACAAGCCTTAT-3’ sequences which is amplified and produced 19 clear, bright, and polymorphic bands; thus, primer can be used for analysis. Pebrine disease varies depending on the strain, stage of development, and environmental conditions. If the virus enters the insect's body, the period of infection and death varies and depends on factors, especially larval age, temperature, virulence, virus concentration, and host nutrition [13]. In moths, pebrine disease is more easily marked directly, such as by the late release of the moth from the cocoon, incomplete moth wings, dull brown moth. If the moth has detected pebrine or BmNPV pathogens, the eggs produced by the moth will contain the pathogen and cause death at the third instar larval stage [14].

Figure 2 produces a polymorphic band below 50 bp, which might be caused by too high of an annealing temperature, causing the primer attachment to be non-specific or not located in the target area [15,16]. Good band visualization is obtained from perfect amplification results and proper electrophoresis and staining processes [17]. According to [18,19] that if the primer is not attached to the DNA template perfectly, it can be caused by the inaccurate concentration of PCR components. In addition, the quality of the DNA template also affects. The presence of polyphenols and other secondary metabolites such as tannins can reduce DNA purity and inhibit primer attachment. The results using the molecular method in the 12 samples, AB-1, AB-2, AB-6, A23-1, A23-2, A23-6, A23-10, A24-1, A24-8, A24 -5 and A24-10 were negative, which means that no pebrine and BmNPV were found. Thus it can be said that in this phase, pebrine and BmNPV disease were not detected. According to [9], it was reported that N.Bombycis spread more frequently and could be easily determined in the silkworm egg stage.

4. Conclusion
The detection of pebrine disease and BmNPV from the two primers showed that from 12 moths studied, none of the moths detected either pebrine disease or BmNPV disease.

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