UTTAR PRADESH JOURNAL OF ZOOLOGY

42(12): 42-48, 2021
ISSN: 0256-971X (P)

EARLY DETECTION OF SIGNIFICANT DISEASES IN EGGS AND LARVA OF SILKWORM WITH A MOLECULAR APPROACH: A CASE STUDY OF SOUTH SULAWESI

IKRAENI SAFITRI[1], SITTI NURAENI[1*] AND SITI HALIMAH LAREKENG[1]
[1]Faculty of Forestry, Universitas Hasanuddin, Makassar, South Sulawesi, Indonesia.

AUTHORS' CONTRIBUTIONS
This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

Editor(s):
(1) Dr. Pınar Oguzhan Yildiz, Ataturk University, Turkey.
Reviewers:
(1) Rachna Pande, ICAR- Central Institute for Cotton Research, India.
(2) Jalajakshi. S., Bangalore Central University, India.

Received: 20 March 2021
Accepted: 27 May 2021
Published: 02 June 2021

Original Research Article

ABSTRACT

Silkworm (*Bombyx mori* L.) is an insect in Ordo Lepidoptera which produces cocoons before becoming an adult insect. Silkworm disease that causes decreased in cocoon production is *Bombyx mori* Nucleapolyhidrosisvirus (*Bm*NPV) and pebrine. The Borcelina virus causes NPV disease, while pebrine caused by *Nosema bombycis* spores. This study aimed to identify NPV and *N. bombycis* in silkworms using a molecular approach. The materials used were 13 egg samples from strain C301 (Perum Perhutani, Soppeng District), strain PS01 (Puslitbang LHK, Bogor), strains S01, S02, S03, broodstock eggs, F1 hybrid (BPSKL Bili-Bili, Gowa District), and 14 larvae samples (strains C301, PS01, S01, S02, S03). The amplification results showed that during the egg phase of all samples were not detected with NPV while egg samples from strains S02 and S03 were detected with pebrine disease. While in the larval phase, NPV was detected in larvae PS01 4th instar (from Wajo District), 5th instar (from Soppeng District), and pebrine disease was detected in all larvae samples 3rd, 4th and 5th instars.

Keywords: *Nucleapolyhidrosis* Virus (NPV); *Neosema bombycis*; Silkworm.

1. INTRODUCTION

Natural silk is one of the commodities that can meet domestic and export needs, either in the form of cocoons, threads, or finished goods. Its activities have a strategic role such as 1) it can involve workers, including farmers; 2) open up business opportunities; 3) provide opportunities to develop the populist economy; 4) increase community income; 5) increase foreign exchange; and 6) opportunities in the service sector [1].

**Corresponding author: Email: nuraenisitti@gmail.com;*

The productivity of national natural silk in Indonesia has decreased after experiencing a disease epidemicwhich is caused by various viruses, bacteria, fungi, and microsporidia. However, at each cultivation stage, some diseases cause harvest failure of the cocoons, namely pebrine disease and *Nuclearpolyhedrosis* virus [2,3,4].

Pebrine disease is infected by *Nosema bombycis,* which had failed the sericulture industry in Europe, especially in France and Italy in the mid-19th century [5]. Pebrine disease first occurred in Indonesia around 1973 [6] until the end of 2010. Several cultivation centers have implemented a moratorium on the cultivation of silkworms, such as in Enrekang, Majalengka, and Bogor districts and some in West Java due to the high intensity of disease [6].

Examination of pebrine disease has only been carried out through female silkworms after laying the eggs. If *N. bombycis* spores are found in the parent, all laid eggs must be destroyed [7]. Meanwhile, controlling NPV disease is still challenging to do because not included in preventive control [8]. In tropical countries, NPV disease is a devastating disease, and the virus multiplies in the nucleus of infected cells [9]. Kaewwises et al. [10] stated that silkworms infected with NPV showed symptoms during the larval development stage and died without cocoon production. The conventional method is commonly used in seed-producing countries and was first applied during the pebrine epidemic in the 19th century [11]. With the molecular approach, detection can be performed on eggs after being placed. The use of polymerase chain reaction (PCR) molecular methods replaces a conventional method in early detection of silkworm disease, including pebrine and NPV with a higher degree of accuracy [12]. Despite the high cost, provision of material can be used in many samp, and has rapid results. A high degree of accuracy means it can be done in the egg phase and allows detection of low-level disease DNA [10].

The PCR technique is a method used to detect pathogens in silkworms in the cultivation of farmers or egg producers, and to analyze disease transmission, as reported by Kawakami et al. [13], Hatakeyama and Hayakasa [12,14], Kaewwises et al. [10], Tekin et al. [15], Nuraeni [8] and Martínez-Zubiaur [16]. Detection for diseases such as NPV does not have a standard operating procedure; thus, this method can be performed using PCR techniques, which allow for more accurate results. Early detection research is essential thus, the farmers can carry out environmental sanitation and disinfection of larvae during silkworm rearing. This study aimed to focus on the early detection of two destructive diseases in silkworm by using a molecular approach.

2. RESEARCH METHODS

2.1 Sampling

The samples used for disease detection were eggs from producers and larvae that were being raised by farmers. Silkworm eggs used were strain C301 (Perum Perhutani, Soppeng District), strain PS01 (LHK Research and Development Center, Bogor), S01, S02, S03, broodstock, and F1 hybrid eggs (BPSKL Bili-Bili, Gowa District), silkworm larvae randomly collected at cultivation centers in Wajo District, Soppeng District, and Makassar City. The total samples collected were 13 eggs and 14 larvae samples (2^{nd} - 5^{th} instars).

2.2 DNA Isolation of Silkworm Eggs and Larvae

The DNA isolation method for the detection of NPV and *N. bombycis* pathogens was using the DNA extraction kit Genomic DNA Mini Plant (Geneaid) kit protocol with little modifications [17]. Egg samples (at least ten items) and caterpillars (head or tail) were ground by using a micro pestle in 200 µl GT buffer, and then 20 µl proteinase K was added and vortexed. Next, samples were incubated at 60^{o}C temperature for ± 45 minutes (vortexed every 5 minutes). GBT buffer (200 µl) were added and vortexed. Then, re-incubated in 60^{o}C for ± 20 minutes (vortexed every 5 minutes) and centrifuged 10,000 G for 3 minutes.

The supernatant was separated in a new tube, then added with 200 µl of absolute ethanol followed by the vortex. The supernatant was transferred to the gs column in a 2 ml tube then centrifuged at 10,000 G for 4 minutes. The solution in the tube was discarded, then 400 µl w1 buffer was added and centrifuged at 10,000 g for 1 minute. The solution was re-discarded, then 400 µl of wash buffer added and centrifuged at 10,000 g for 1 minute. Discard the solution, then centrifuged at 10,000 g for 4 minutes. Transfer the gs column to a new tube, then 50 µl of elution buffer (heated before use) was added, then centrifuged at 10,000 g for 1 minute.

2.3 DNA Dilution, PCR and Electrophoresis

The master DNA was diluted using the 20 µl method (1 µl of master DNA and 19 µl of ddH_2O). 5.4 g of agar was weighed and dissolved in 180 ml of TAE then heated using an oven for 5 minutes (stirred every 2 minutes), 1.5 µl of red gel solution added and put into an agarose molding container. After it became solid, 4 µl of the PCR product of each amplified

Table 1. PCR Primers for detection

Target Strains	Primer	Sequence	Amplicon
Polh	Polhefor (F)	CGTGTACGACAACAAGTACTACA3'	150 *bp*
	Polherev (R)	5'AAAGTGAGTTTTTGGTTTTTGCC3'	
Nb	NBEF 35F	5'-TGGCGCTGTTGATAAGAGATT-3	50 *bp*
	NBEF 957R	5'-AATTTAGCAACACAAGCCTTAT-3'	

Notes : Polh (NPV), Nb (N. bombycis)

sample was inserted into agarose well, ran on 120 V for 60 minutes.

The isolated DNA was detected using the PCR technique. In the amplification process, a particular pair of pathogen detection primers were used. The primer sequences of this study are depicted in Table 1. PCR analysis for the identification of NPV pathogens by Martinez-Zubiaur et al. [16] is based on a polyhedrin (*polh*) gene using primers from various *nucleopolyhedrovirus* (NPV). Detection of *N. bombycis* pathogens refers to Hatakeyama and Hayakasa [14], who used NBEF 35F and NBEF 957R primers.

3. RESULTS AND DISCUSSION

3.1 Early Detection of Silkworm Seeds

The amplification results of egg samples tested for NPV and pebrine detection are presented in Table 2, and the agarose gel electrophoresis pattern of PCR products are in Figs. 1-2. The results of all egg samples amplification from producers were not detected (negative) with NPV pathogens. Several samples of silkworm eggs Strain S02 and S03 were detected with *N. bombycis*.

Visualization of electrophoresis result of silkworm egg samples from several producers (Fig. 1) showed NPV pathogen of PCR products at 150 bp, but all samples (numbers 1-13) were not detected with NPV. While Fig. 2 shows the visualization of PCR products of egg samples detected with *N. bombycis* at 50 bp (numbers 4, 10, 11).

The amplification results of larvae samples tested for NPV and *N. bombycis* detection are displayed in Table 3, and the electrophoresis pattern of PCR products are shown in Figs. 3-4. The results of amplification of samples of silkworm larvae strain PS01 (4^{th} instar), PS01 (5^{th} instar) from farmers' silkworm rearing were detected positive with NPV. Silkworm samples of all strains except PS01 (4^{th} instar) from the farmers' silkworm rearing were detected positive with *N. bombycis.*

Table 2. The amplification results of egg sample for NPV and *N. bombycis* pathogens detection in silkworms

Sample	Sample Origin	NPV	*N. bombycis*
C301 Eggs (1)	Perum Perhutani	-	-
PS01 Eggs (2)	Litbang LHK	-	-
S01 Eggs (3)	BPSKL	-	-
S02 Eggs (4)	BPSKL	-	+
S03 Eggs (5)	BPSKL	-	-
PS01 Eggs (6)	Litbang LHK	-	-
S01 Eggs (7)	BPSKL	-	-
Eggs (8)	Import	-	-
S01 Eggs (9)	BPSKL	-	-
S02 Eggs (10)	BPSKL	-	+
S03 Eggs (11)	BPSKL	-	+
Parent eggs (12)	BPSKL	-	-
F1 Hybrid Eggs (13)	BPSKL	-	-

Notes: (+): Positive, NPV and/or N. bombycis is detected, (-): Negative, no pathogens detected

Fig. 1. PCR products detected NPV in silkworm eggs in several strains. M. 50 bp marker; K. Positive Control; 1. strain C301 (producer); 2. Strain PS01 (producer); 3-5. Strains S01, S02, S03 (producer); 6. Strain PS01 (producer); 7. Strain S01 (producer); 8. Imported Eggs; 9-11. Strains S1, S2, S3; 12-13 (producer). Broodstock and F1 Hybrids (producers)

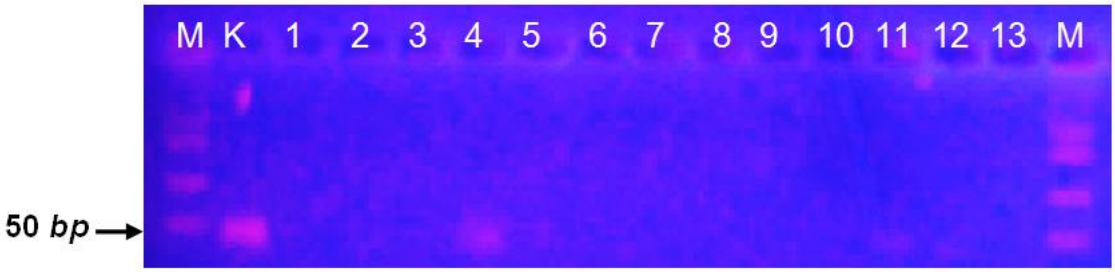

Fig. 2. PCR products detected *N. bombycis* in silkworm eggs in several strains. M. 50 bp marker; K. Positive Control; 1. Strain C301 (producer); 2. Strain PS01 (producer); 3-5. Strains S01, S02, S03 (producer); 6. Strain PS01 (producer); 7. Strain S01 (producer); 8. Imported Eggs; 9-11. Strains S1, S2, S3; 12-13 (producer). Broodstock and F1 Hybrids (producer)

Table 3. The amplification results of larvae sample for NPV and *N. bombycis* pathogens detection in silkworms at farmers' silkworm rearing

Sample	Sample Origin	NPV Detection	*N. bombycis* Detection
C301 larvae – 2nd instar (1)	Makassar	-	+
PS01 larvae – 4th instar (2)	Wajo	-	+
PS01 larvae – 4th instar (3)	Makassar	+	+
PS01 larvae - 5th instar (4)	Wajo	-	+
PS01 larvae - 5th instar (5)	Makassar	-	+
PS01 larvae - 3rd instar (6)	Wajo	-	+
PS01 larvae - 4th instar (7)	Wajo	-	-
S01 larvae - 3rd instar (8)	Wajo	-	+
S02 larvae - 3rd instar (9)	Wajo	-	+
S02 larvae - 3rd instar (10)	Makassar	-	+
S02 larvae - 2nd instar (11)	Wajo	-	+
S03 larvae - 3rd instar (12)	Wajo	-	+
S01 larvae - 5th instar (13)	Makassar	-	+
PS01 larvae - 5th instar (14)	Soppeng	+	+

Notes: (+): Positive, NPV and/or N. bombycis is detected, (-): Negative, no pathogens detected

The PCR amplification results for NPV detection of silkworm larvae samples PS01 4th instar and PS01 5th instar (Fig. 3) showed positive results (numbers 3, 14) at 150 bp. While the amplification result of *N. bombycis* detection (Fig. 4) in all strains of farmers' silkworms rearing showed positive results except PS01 (4th instar) at 50 bp.

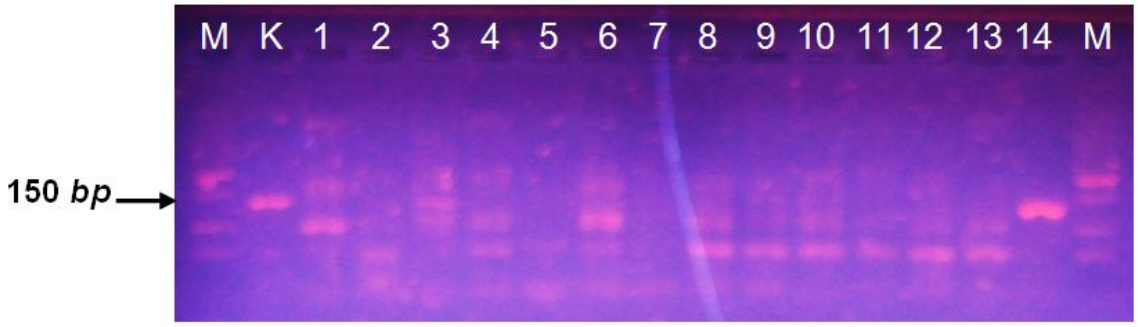

Fig. 3. PCR products detected NPV in silkworm larvae in several strains. M. 50 bp marker; K. Positive Control (purified NPV); 1. C301 larvae 2nd instar; 2-3. PS01 larvae 4th instar; 4-5. PS01 larvae 5th instar; 6. PS01 larvae 3rd instar; 7. PS01 larvae 4th instar; 8. S01 larvae 3rd instar; 9-10. S02 larvae 3rd instar; 11. S02 larvae 2nd instar; 12. S03 larvae 3rd instar; 13. S01 larvae 5th instar; 14. PS01 larvae 5th instar

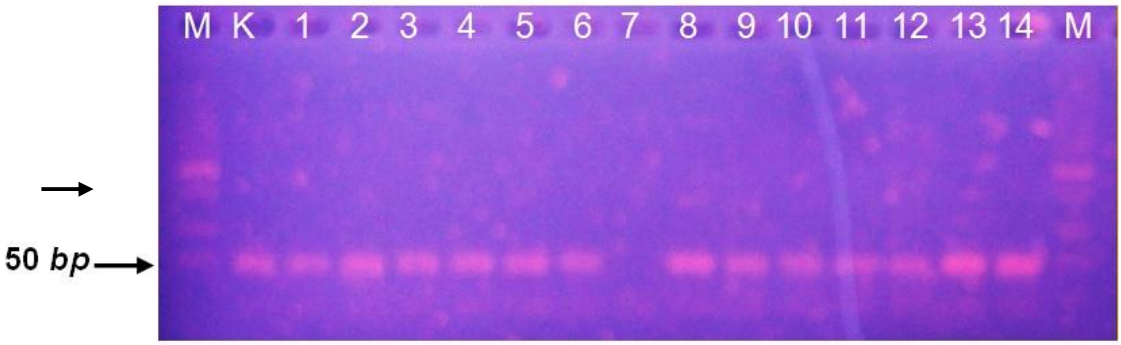

Fig. 4. The PCR product detected *N. bombycis* pathogen in silkworm larvae in several strains. M. 50 bp marker; K. Positive Control; 1. C301 larvae 2nd instar; 2-3. PS01 larvae 4th instar; 4-5. PS01 larvae 5th instar; 6. PS01 larvae 3rd instar; 7. PS01 larvae 4th instar; 8. S01 larvae 3rd instar; 9-10. S02 larvae 3rd instar; 11. S02 larvae 2nd instar; 12. S03 larvae 3rd instar; 13. S01 larvae 5th instar; 14. PS01 larvae 5th instar

4. DISCUSSION AND CONCLUSION

Silkworms are essential for the production of silk threads. Pathological and genetic studies of the disease have been conducted significantly and extensively. Viruses and bacteria cause death in silkworms and reduce cocoon yields caused by pebrine disease and *Bombyx mori* Nucleopolyhedrovirus (*Bm*NPV) pathogens. Early detection is part of efforts to control diseases transmitted by seeds, although they still use conventional methods but have shown promising results [4].

The PCR technique is a method used to detect pathogens in silkworms in the cultivation of farmers or egg producers, and to analyze disease transmission, as reported by Kawakami et al. [13], Hatakeyama and Hayakasa [12,14], Kaewwises et al. [10], Tekin et al. [15], Nuraeni [8] and Martínez-Zubiaur [16]. Another technique that was developed is the multiplex PCR performed by Hatakeyama and Hayakasa [14] to detect different pathogens. Early detection of silkworm pathogens helps research and extension centers to ensure that caterpillars are free from pathogens before being supplied to farmers [8].

Identification of *Bm*NPV that affecting silkworm larvae is vital to prevent NPV disease. The visualization of the gel is visible on the PCR band products. It provides evidence that PCR is a competent tool for detecting NPV in silkworms [9]. In the visualization, silkworms gel infected with *Bm*NPV at 150 bp using polh primer and molecularly detected NPV in larvae samples from Makassar 4th instar and 5th instar rearing in Soppeng. Similar results were also found in several places such as Thailand [10], Soppeng and Enrekang [18], Soppeng [8], Cuba [16], India [19].

The NPV pathogen is the Borrelina virus, which attacks outer skin cells (epidermis), silk glands, and blood cells, then to the cell nucleus [20]. The development of diseases in silkworms is mainly due to stressful conditions *viz*., variations in temperature, humidity, poor ventilation, nutritional deficiencies [16], and ineffective disinfectants in the rearing facilities, and tools used [19].

NPV causes disease symptoms during the later stages of larval development and dies before pupating, resulting in economic losses. The treatment of NPV disease is by preventing disease transmission, but the most effective solution for disease control is to detect the virus as early as possible to stop transmission in the cultivation unit. Disease detection that is slow and less accurate will cause NPV pathogens to spread seasonally [10].

Pebrine disease is caused by *N. bombycis* spores that belong to the genus microsporidia, which causes death in the larval and pupal stages [20]. This disease can be observed at all stages of silkworm development [15]. Hatakeyama and Hayakasa [12] stated that the infection of pathogens can be through the mouth during the feeding period of mulberry leaves and through fertilization by moths with *N. bombycis* spores; thus, pathogen is present in the silkworm parent and the eggs will contain pebrine disease. The amplification results in the egg phase of the strains S02 and S03 from the producer (Table 1), and larvae samples from farmers' rearing detected the *N. bombycis* pathogen. In line with Nuraeni [8] findings, the presence of pebrine pathogens and NPV in 5th instar larvae originating from the farmers' silkworm rearing in Soppeng, which was taken in 2014. It shows that the cultivation site is challenging to avoid pathogens even though the seeds or eggs used are free of pathogens [8]. The PCR technique for detecting *N. bombycis* in silkworm seedlings was also reported by Hatakeyama and Hayakasa [12] Pan et al. [21], and the larval phase was reported by Nuraeni [8]. For the effective control of pebrine disease, outbreaks should be detected at an early stage and persistent infections should also be identified to prevent further transmittance [22].

In this study, the use of molecular methods was able to detect NPV and *N. bombycis* pathogens in silkworms from several producer samples as well as from farmers' rearing. This method has more benefits than conventional methods because high sensitivity strengthens target DNA and PCR amplification allows detection of viral DNA at low levels and faster [10, 23]. Therefore, the PCR technique can be an alternative and has the potential to detect NPV and *N. bombycis* pathogens at all stages of silkworm development. Microscopic examination method remains the conventional detection method for screening of microsporidia in sericulture. Molecular diagnosis tools have an advantage over microscopic detection as they are more specific, sensitive and aid in early detection [22].

ACKNOWLEDGEMENT

We would like to thank gratefully acknowledge to the South Sulawesi research and Development office Grant 2020 for the silkworm material.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Dinas Kehutanan Provinsi Sulawesi Selatan. Fasilitasi Penelitian Persuteraan Alam Di Sulawesi Selatan Tahun 2011 : Tantangan Komprehensif Persuteraan Alam di Sulawesi Selatan. Dinas kehutanan Provinsi Sulawesi Selatan dan Institute for Social and Political Economic Issue; 2011.
2. Etebari K, Bizhannia AR, Sorati R, Matindoost L. Biochemical changes in haemolymph of silkworm larvae due to pyriproxyfen residue. Pesticide Biochemistry and Physiology. 2007 May 1;88(1):14-9.
3. Hussain A, Buhroo AA. An alternative method to secure pebrine free silkworm eggs. International Society for Zoological Research (ISZR). International Journal of Entomology. 2011;2(1):40-2.
4. Nuraeni S. Gaps in the thread: Disease, production, and opportunity in the failing silk industry of South Sulawesi. Forest and Society. 2017 Nov 27;1:110-20.
5. Chakrabarty S, Manna B, Saha AK, Bindoroo BB, Trivedy K. Pebrine disease (*Neosema bombycis* N.) in *Bombyx mori* L.: The research trends. Sericologia. 2016;56:1-17.
6. Nuraeni S. Gaps in the thread: Disease, production, and opportunity in the failing silk industry of South Sulawesi. Forest and Society. 2017;1:110-120.
7. Mishra SA. Diseases of silk warm (*Bombyx mori*) and protocol for treatment. International Journal of Zoology Studies. 2017;2(6):116-118.

8. Nuraeni S. Kajian Epidemiologi Penyakit Pebrin dan NVP pada Beberapa Galur dan Sumber Bibit Ulatsutera dengan Menggunakan Metode Deteksi Molekuler [Disertasi]. Universitas Hasanuddin. Makassar; 2016.
9. Joshi RP. dan Raja A. Polymerase chain reaction based detection of Grasserie virus, BmNPV in Silkworm, *Bombyx mori*. Biotechnological Communication. 2016;9(3): 471-474.
10. Kaewwises M, Chaeychomsri S, S. Chowpongpang dan Attathom T. Identification of the Polyhedrin Gene of Thai *Bombyx mori* nucleopolyhedrovirus. Science Asia. 2006;32:421-427.
11. Bhat SA, Bashir dan I, Kamili AS. Microsporidiosis of silkworm, *Bombyx mori* L. (Lepidoptera-Bombycidae): A review. African Journal of Agricultural Research. 2009;4(13): 1519-1523.
12. Hatakeyama, Y. dan Hayakasa S. A new method of pebrine inspection of silkworm egg using multiprimer PCR. Journal of Invertebrate Pathology. 2003;82:148-151.
13. Kawakami Y, Inoue T, Uchida Y, Hatakeyama Y, Iwano dan H, Ishihara R. Specific amplification of DNA from reference strains of Noserma bombycis. Journal of Sericultural Science of Japan. 1995;64 (2):165-172.
14. Hatakeyama Y, Hayakasa S. Specific amplification of microsporidian DNA fragments using multiprimer PCR. JARQ. 2002; 36(2):97-102.
15. Tekin OK, Sezer D, Soganci dan K, Tunca RI. Molecular and microscopic detection of microsporidia in some silkworm (*Bombyx mori* L.) populations in Turkey. Türk Tarım ve Doğa Bilimleri Dergisi. 2014;1(4):450-453.
16. Martínez-Zubiaur Y, Abreu MP, Hernández MDCP, Sihler W, Falcão R, Ribeiro dan BM, Souza MLD. First record of a *Bombyx mori* Nucleopolyhedrovirus (BmNPV) isolate from Cuba. International Journal of Current Research. 2016;8(8):35766-35770.
17. Syahri YF, Rauf M, Paembonan SA, Larekeng, SH, Cahyaningsih YF. RAPD Amplification on Cocoa (*Theobroma cacao* L.) from East Kolaka, Southeast Sulawesi Province. IOP Conference Series: Earth and Environmental Science. 2019;270(1). DOI:https://doi.org/10.1088/1755-1315/270/1/012052
18. Nuraeni S, M. Nuraedah dan D. Sanusi. Respon Empat Ras Ulat Sutera Terhadap Bombyx mori Nuclear Polyhedrosis Virus (BmNPV). Prosiding Seminar Nasional Mikrobiologi Kesehatan dan Lingkungan. ISBN 978-602-72245-0-6; 2015.
19. Khyade VB, dan Mullis KB. Polymerase chain reaction for detection of grasserie virus, BmNPV in the fifth instar larvae of silkworm, *Bombyx mori* (L) (Race: PM x CSR2). International Journal of Scientific Research Chemistri (IJSRCH). 2018;3(5): 2456-8457.
20. Balai Persuteraan Alam Sulawesi Selatan. Laporan: Pengamatan Hama dan Penyakit Tanaman Murbei dan Ulat Sutera Tahun 2012. Sulawesi Selatan; 2012.
21. Pan ZH, Gong CL, Zheng XJ, Guo R. dan Shen WD. Application of pebrine detection by PCR infected *Bombyx mori* eggs. Advanced Materials Research. 2011;175-176:8-12.
22. Roy G, Mandal K, dan Ravikumar G. PCR-based detection of microsprodia in silkworms using non-conventional RNA polymerase primers. Biosci. Biotech. Res. Comm. 2017; 10(4):676-679 .
23. Larekeng SH, Restu M, Arif A, Cahyaningsih YF, Mukti J. A genetic approach to study mating system on Jabon Merah (*Anthocephalus macrophyllus* Roxb.) from three different provenances in South Sulawesi. IOP Conference Series: Earth and Environmental Science. 2019;235(1). DOI:https://doi.org/10.1088/1755-1315/235/1/012049