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**Research Article**

# Prevalence and molecular characterization of viruses causing diseases in *Bombyx mori* L. (Lepidoptera: Bombycidae) from different climatic regions of India

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**ABSTRACT:** A number of viruses are known to cause the disease in mulberry silkworm, *Bombyx mori L.* and cause significant cocoon crop loss to the farmers. The study has been undertaken to assess the prevalence of silkworm viral diseases caused by *B. Mori* Nucleopolyhedrovirus (BmNPV), *B. mori* Infectious Flacherie Virus (BmIFV) and *B. mori* Densovirus1 (BmDNV1) through external symptoms and molecular characterization from different climatic regions of India. During the intensive exploratory surveys in 2017, silkworm larvae with typical symptoms of BmNPV, BmIFV and BmDNV1 were collected across seven provinces from North India [Kashmir (temperate), Jammu, Ghumarwin in Himachal Pradesh, Dehradun in Uttarakhand (subtropical) and South India [Karnataka, Andhra Pradesh and Tamil Nadu (tropical)]. Dissection of diseased specimens confirmed the presence of virus through anatomical changes viz, size, shape and colour of organs after disease attack. The viruses were isolated and identified through PCR amplification of highly conserved genes. The results reveal that BmNPV, BmIFV and BmDNV1 are evenly prevalent across India. The infection percentage of BmNPV, BmIFV and BmDNV1 in North India (11.41 ± 1.21, 7.81 ± 0.67 and 7.40 ± 0.61) was significantly higher than South India (1.61 ± 0.17, 1.52 ± 0.14 and 1.62 ± 0.14), respectively. The highest prevalence of these viruses was observed from subtropical followed by temperate and tropical climate. The knowledge of the prevalence of these viruses in India and their synergism with bacteria and influence of other possible factors is important for preventing cocoon crop losses caused by viral diseases in India.

**KEYWORDS:** *Bombyx mori*, BmNPV, BmIFV, BmDNV1, characterization, prevalence

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## INTRODUCTION

The mulberry silkworm, *Bombyx mori* Linneaus (Lepidoptera: Bombycidae) is an economically important insect domesticated for silk production. The major constraints for increased cocoon yield in sericulture are the occurrence of diseases such as grasserie, flacherie, muscardine and pebrine caused by virus, bacteria, fungi and microsporidia, respectively. Although there is no single factor responsible for these losses, viruses are considered as one of the most important contributors to this situation. A number of viruses, including nucleopolyhedrovirus (NPV; Group I double stranded DNA virus; Genus: Alphabaculovirus; Family: Baculoviridae), infectious flacherie virus (IFV; Group IV single stranded RNA virus;Genus: Iflavirus; Family: Iflaviridae) and densovirus (DNV; Group II single stranded DNA virus; Genus: Iteravirus; Family: Parvoviridae) are known to infect the *B. mori*. The disease caused by viruses is more severe than any other disease in silkworm and the larval populations frequently harbour viral infections, often at prevalences enough to cause disease epizootics. Because of the outbreak of these diseases, crop failures in silkworm rearing are common phenomenon (Gani *et al*., 2017). The silkworm crop losses due to diseases in India are about15-20% and out of this more than 50% is attributed to viral infection only (Reddy and Rao, 2009; Khurad *et al.,* 2006). This substantial economic loss can be stopped by preventing spread of the disease and by applying suitable control measures at the right time against the disease.

The identification of insect viruses is an important step in disease monitoring, control and in host-virus association under natural epizootic conditions. Polymerase Chain Reaction (PCR) with specific primers for highly conserved genes provides a powerful tool to identify lepidopteran-specific viruses and to study their diversity (Jehle *et al*., 2006). Since,

the presence of BmNPV is reported throughout sericulture practising areas of India but, the studies are still incipient. The BmIFV and BmDNV1 have been reported in silkworm larval populations in southern tropical part of the India only, but till now no such study has been reported from subtropical and temperate climates. Therefore, the logic behind this study was to assess and compare the presence of BmNPV, BmIFV and BmDNV1 in silkworm larval populations from different climatic conditions of India.

## MATERIALS AND METHODS

### Sampling and virus analysis

The study was carried out during 2017across seven provinces of India which include North India [Kashmir (temperate), Jammu, Ghumarwin in Himachal Pradesh, Dehradun in Uttarakhand (subtropical)] and South India [Mysore in Karnataka, Anantapur, Andhra Pradesh and Salem, Tamil Nadu (tropical)] (Table 1). The survey was made by selecting fifty sericulturists from five villages (@10 sericulturists per village) at each location. The *B. mori* larvae showing typical signs and symptoms of BmNPV, BmIFV and BmDNV1 infection during V instar (inspection time) were collected individually in 5 ml tubes labelled, transported to the laboratory and stored at -20°C until the isolation and purification of virus. The per cent infection was calculated by the following formula:

$$\text{Infection}(\%) = \frac{\text{Number of larvae infected}}{\text{Total number of larvae (healthy + infected)}} \times 100$$

Dissection of diseased specimens was done to study the anatomical changes: size, shape and colour of organs after disease attack. From the diseased samples the viruses vizBmNPV, BmIFV and BmDNV1 were purified and isolated as per the standard methods (Nataraju *et al*., 1994; Sivaprasad *et al*., 2003; O'Reilly *et al*., 1992; Palhan and Gopinathan, 1996; Luo *et al*., 2010; Vootla *et al*., 2013). Preliminary BmNPV identification was done by light microscopy by spreading smears from infected larvae thinly across a microscope slide followed by Giemsa staining and then examined through a phase-contrast microscope at 1000X magnification under oil immersion (Gupta *et al*., 2013). Molecular identification of BmNPV, BmIFV and BmDNV1isolates was done through PCR amplification of highly conserved genes. The *polh*gene of BmNPV and *RNA dependent RNA polymerase* of BmIFV were targeted for the molecular identification owing to their highly conserved nature. The BmDNV1 specific primers were designed from the nucleotide sequences of DNV 2&3 (Ravikumar *et al*., 2011). Only one virus was processed at a time to prevent any cross-contamination.

### Extraction of viral DNA/RNA and PCR amplification

The genomic DNA of individual isolates of BmNPV was extracted directly from the polyhedra stock suspension using the protocol given by Tang*et al*. (2017). The *polh*gene was amplified from the genomic DNA of the seven BmNPV isolates by optimizing the PCR using degenerate primer pairs as described by Lange *et al*.(2004). These samples represent isolates from 7 geographic locations including Kashmir, Jammu, Ghumarwin, Dehradun, Mysore, Anantapur and Salem as described previously. The extraction of genomic DNA of BmDNV1 isolates and its amplification using primers designed from the nucleotide sequences of DNV 2&3was done as per the methods described by Ravikumar *et al*. (2011). The extraction of RNA using the TriZol reagent (Invitrogen, Carlsbad, CA), cDNA synthesis and amplification of RNA dependent RNA polymerase gene of BmIFV was done as per the methods given by Vootla *et al*. (2013). The purified

**Table 1. Locations of collecting BmNPV, BmIFV and BmDNV1 infected silkworm larvae with their altitude, latitude, longitude, average annual temperature (°C) and average annual precipitation (mm)**

| S. No. | Collection area | Altitude (ft) | Latitude/Longitude | Average annual temperature (°C) | Average annual precipitation (mm) |
|---|---|---|---|---|---|
| 1 | Kashmir, J&K | 6070 | 34° 5' N/74° 47' E | 13.6 | 710.0 |
| 2 | Jammu, J&K | 1073 | 32° 43' N /74° 51' E | 24.2 | 76.2 |
| 3 | Ghumarwin, H.P. | 2133 | 31° 26' N /76° 42' E | 22.3 | 60.8 |
| 4 | Dehradun, Uttarakhand | 2142 | 30° 19' N/78° 1' E | 23.0 | 176.3 |
| 5 | Mysore, Karnataka | 2503 | 12° 18' N/76° 39' E | 25.0 | 85.7 |
| 6 | Anantapur, Andhra Pradesh | 1099 | 14° 41'N/77° 35'E | 28.0 | 53.3 |
| 7 | Salem, Tamil Nadu | 912 | 11° 39' N/78° 8' E | 30.0 | 58.1 |

Source: https://www.timeanddate.com/weather/india

DNA pellet was dissolved in 0.1X TE buffer, pH 8.0 and stored at -20 °C until use. The purity and concentration of the isolated genomic DNA sample was estimated by using Nanodrop (Thermo Fisher). After amplification, 10µl of PCR product was electrophoresed at 70V for 1 h on 2.5% (w/v) agarose gel stained with ethidium bromide (0.2 µg/ml) in 1X TBE and viewed on Ultra Lum Gel documentation system. The 1kb DNA marker was used to estimate the size of the amplimers.

**Statistical analysis**

The mean prevalence of BmNPV, BmIFV and BmDNV1 from temperate, subtropical and tropical regions of India at each location was worked out using One-way Analysis of Variance (ANOVA) with Duncon's test. The comparison of BmNPV, BmIFV and BmDNV1prevalence between the locations was performed by Chi-square test using SPSS 16.0 software. Differences were considered statistically significant at $p<0.05$.

## RESULTS AND DISCUSSION

The results revealed that the BmNPV, BmIFV and BmDNV1 are widely distributed in all the climatic regions of India viz. temperate, subtropical and tropical with varying prevalences. The BmNPV larvae had milky white haemolymph, the DNV infected larvae were having pale yellow alimentary canal and the IFV infected larvae with dissolved alimentary canal. A microscopic examination through Giemsa staining of the tissue smears and haemolymph of the BmNPV infected larvae collected from all the locations revealed the presence of polyhedra as observed under a phase contrast microscope at X1000 magnification. The isolation and purification of the BmNPV, BmIFV and BmDNV1 from field collected larval samples with typical signs and symptoms was followed by viral DNA extraction and later the PCR method was used for their identification. The extracted viral DNV from different isolates of BmNPV, BmIFV and BmDNV1 was amplified successfully using gene specific primers and as expected the band size of the amplimers was 500, 214 and 391bp, respectively (Fig. 1).

The BmNPV was found as the most serious and dominant viral pathogen with the maximum prevalence of 17.32% in Ghumarwin, Himachal Pradesh and the lowest prevalence of 0.26% in Salem, Tamil Nadu, the differences being significant between the locations ($\chi^2$=17.21, df=6, $p$=0.032). The highest BmIFV prevalence of 9.19% was observed at Ghumarwin, Himachal Pradesh and the lowest 0.73% in Salem, Tamil Nadu with significant differences between the locations ($\chi^2$= 16.69, df=6, $p$=0.027). Significant differences ($\chi^2$=11.87, df=6, $p$=0.022) were also observed in the prevalence of BmDNV1

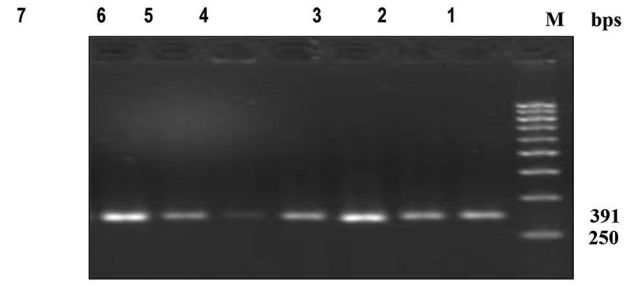


**Fig. 1. PCR amplified genomic DNA of BmDNV1 isolates using primers designed from the nucleotide sequences of DNV 2 & 3. Lane M: DNA molecular weight marker (1 kb), Lanes 1-7: amplified PCR product of 1=Kashmir;2=Jammu; 3=Dehradun; 4=Ghumarwin; 5=Mysore; 6=Anantapur; 7=Salem.**

between the locations under study with the highest prevalence of 9.23% in Jammu and the lowest 0.70% in Salem, Tamil Nadu (Fig. 2). In general, the northern part of India (11.37, 7.81 and 7.34) had a significantly higher infection percentage of BmNPV (z=-3.838, $p$<0.05), BmIFV (z=-1.97, $p$<0.05) and BmDNV1 (z=-1.782, $p$<0.05) than southern part of India (1.62, 1.59 and 1.54), respectively. The highest prevalence of these viruses was observed from subtropical (12.71, 8.79, 7.5) followed by temperate (7.33, 4.89 and 6.85) and tropical climate (1.62, 1.59 and 1.54). The overall average infection percentage of BmNPV, BmIFV and BmDNV1 in silkworm larvae was 7.19, 5.14, 4.85 %, respectively irrespective of location during 2017 in India (Fig. 2). Illahi and Nataraju (2007) reported that the prevalence of BmNPV ranged from 13.85-26.03% in Jammu and Kashmir. Reddy and Rao (2009) reported 7.0-10.5 and 4.5-8.0% incidence of grasserie disease in Karnataka during 2004-05 and 2005-06, respectively. The variation in disease prevalence between the locations and years is evident as virus prevalence is multifactorial, being influenced by several factors including environmental, nutritional, microbial and genetic and their interactions. The presence of high viral loads in rearing area and the mismanagement such as high temperature and humidity and their sudden fluctuation, improper disinfection, lack of hygiene, ventilation and overcrowded conditions are observed to contribute to the high prevalence of the viral diseases. Savanurmath *et al*. (1992) also reported that fluctuations between day and night temperature and relative humidity prevailing in rearing house were the important causes for the occurrence of flacherie and grasserie diseases in silkworm larvae.

## CONCLUSION

The silkworm viruses viz. BmNPV, BmIFV and BmDNV1 are evenly prevalent in silkworm larval

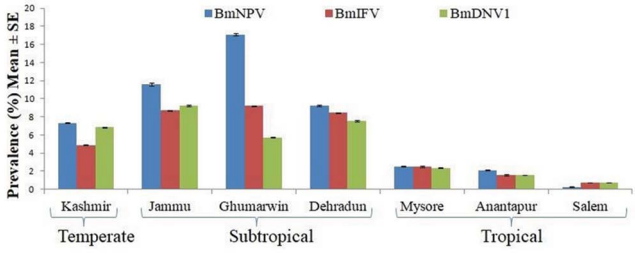


**Fig. 2. Prevalence of BmNPV, BmIFV and BmDNV1 from different climatic regions of India during 2017.**

populations in different climatic regions of India. A number of measures have been suggested for the prevention and control of silkworm viral diseases, but still the measures are ineffective and the full understanding of the genetic and host factors is not clear. The knowledge of the prevalence of these viruses in the region and their relation with the bacteria and other possible influencing factors is important for preventing cocoon crop losses in sericulture. Further studies are necessary to comprehensively analyze the host genes response to viral infection and its functional analysis to prevent virus replication and its transmission.

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## REFERENCES

Gani M, Chouhan S, Babulal, Gupta RK, Khan G, Kumar NB, Saini P, Ghosh MK. 2017. *Bombyx mori* nucleopolyhedrovirus (BmNPV): its impact on silkworm rearing and management strategies. *J Biol Control* **31**(3): 119–122. https://doi.org/10.18311/jbc/2017/16269

Gupta RK, Gani M, Jasrotia P, Srivastava K. 2013. Development of the predator *Eocanthecona furcellata* on different proportions of nucleopolyhedrovirus infected *Spodoptera litura* larvae and potential for predator dissemination of virus in the field. *BioControl* **58**(4): 543–552. https://doi.org/10.1007/s10526-013-9515-1

Illahi I, Nataraju B. 2007. Prevalence of nuclear polyhedrosis in mulberry silkworm, *Bombyx mori* L. in Jammu and Kashmir. *Indian J Seric*. **46**(1): 43–4

Jehle JA, Lange M, Wang H, Hu Z, Wang Y, Hauschild, R. 2006. Molecular identification and phylogenetic analysis of baculoviruses from Lepidoptera. *Virology* **346**: 180–193. doi: PMid: 16313938 https://doi.org/10.1016/j.virol.2005.10.032

Khurad AM, Kanginakudru S, Qureshi SO, Rathod MK, Rai MM. 2006. A new *Bombyx mori* larval ovarian cell line highly susceptible to nucleopolyhedrovirus. *J Invertebr Pathol.* **92**: 59–65. PMid: 16713602 https://doi.org/10.1016/j.jip.2006.03.005

Lange M, Wang H, Zhihong H, Jehle JA. 2004. Towards a molecular identification and classification system of lepidopteran-specific baculoviruses. *Virology* **325**(1): 36–47. PMid: 15231384 https://doi.org/10.1016/j.virol.2004.04.023

Luo Y, Nartker S, Miller H, Hochhalter D, Wiederoder M, Wiederoder S, Setterington E, Drzal LT, Alocilja EC. 2010. Surface functionalization of electrospun nanofibers for detecting *Escherichia coli* O157:H7 and BVDV cells in a direct–charge transfer biosensor. *Biosens Bioelectron*. **26**: 1612–1617. doi: 10.1016/j.bios.2010.08.028 PMid: 20833013

Nataraju B, Sivaprasad V, Datta RK, Gupta SK, Shamim M. 1994. Colloidal textile dye-based dipstick immunoassay for the detection of nuclear polyhedrosis virus (BmNPV) of silkworm, *Bombyx mori* L. *J Invertebr Pathol.* **63**(2): 135–139. https://doi.org/10.1006/jipa.1994.1026

O'Reilly DR, Miller LK, Luckow VA. 1992. *Baculovirus expression vectors: A laboratory manual*. W. H. Freeman & Co., New York. https://doi.org/10.1016/0092-8674(93)90288-2

Palhan VB, Gopinathan KP. 1996. Characterization of a local isolate of *Bombyx mori* nuclear polyhedrosis virus. *Curr Sci*. 70(2): 147–153.https://www.jstor.org/stable/24096983

Ravikumar G, Urs SR, Prakash NV, Rao CGP, Vardhana KV. 2011. Development of a multiplex polymerase chain reaction for the simultaneous detection of microsporidians, nucleopolyhedrovirus, and densovirus infecting silkworms. *J Invertebr Pathol.* **107**(3): 193–197. PMid: 21570404 https://doi.org/10.1016/j.jip.2011.04.009

Reddy BK, Rao JVK. 2009. Seasonal occurrence and control of silkworm diseases, grasserie, flacherie and muscardine and insect pest, Uzi fly in Andhra Pradesh, India. *Int J Indus Entomol*. **18**(2): 57–61.

Savanurmath CJ, Basavarajappa S, Hinchigeri SB, Ingalhalli SS, Singh KK, Sanakal RD. 1992. Relative incidence of silkworm viral disease in agroclimatic zones of northern Karnataka, India. National conference on mulberry sericulture research, CSR & TI, Mysore, Dec. p. 123.

Sivaprasad V. Chandrasekharaiah C. Misra S. Kumar KPK. Rao YUM. 2003. Screening of silkworm breeds for tolerance to *Bombyx mori* nuclear polyhedrosis virus (BmNPV). *Int J Indus Entomol*. **7**: 87–91.

Tang FB, Zhanga Y, Shaoa Y, Zhua F, Huanga P, Baia X. 2017. Isolation and identification of a new *Bombyx mori* nucleopolyhedrovirus strain isolated from Yunnan, China. *Science Asia* **43**: 26–32. https://doi.org/10.2306/scienceasia1513-1874.2017.43.026

Vootla SK, Lu XM, Kari N, Gadwala M, Lu Q. 2013. Rapid detection of infectious flacherie virus of the silkworm, *Bombyx mori*, using RT-PCR and nested PCR. *J Insect Sci*. **13**(1): 120. PMid: 24785655 https://doi.org/10.1673/031.013.12001