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Diversity analysis of *Bm*NPV Isolates Infecting Mulberry Silkworm (*Bombyx mori*) using Restriction Endonuclease Digestion Profiling

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ABSTRACT

Understanding the genetic diversity of *Bm*NPV isolates causing grasserie viral disease of mulberry silkworm *Bombyx mori* L. is essential for adoption of management strategies including biotechnological tools. The present study was aimed at the use of restriction fragment length polymorphism (RFLP) profiling for studying the molecular diversity analysis of six *Bm*NPV isolates collected from Devanahalli, Kolar, Shidlaghatta, Hosakote, Tumakuru, and Ramanagara areas in Karnataka, India (*Bm*NPV-Ko, *Bm*NPV-Ho, *Bm*NPV-SG, *Bm*NPV-DH, *Bm*NPV-TM, *Bm*NPV-Ram respectively). DNA was extracted from each of these isolates and subjected to digestion with different restriction enzymes *Eco*R1, *Bam*H1, *Sma*1, *Nco*1, and a combination of *Bam*H1+*Nco*1 and electrophoresed in 0.8% w/v agarose gel to visualize restriction enzyme profile. The analysis revealed that all the six *Bm*NPV isolates had similar *Nco*1 and *Sma*1 restriction patterns, although there was variation in low molecular weight fragments. The *Eco*R1 and *Bam*H1 restriction patterns were nearly the same for all the isolates except for the presence of an approximately 4kb and an additional 1.5kb polymorphic band only in *Bm*NPV-TM and *Bm*NPV-Ram isolates. *Bam*H1+*Nco*1 digestion of the DNA from each isolate yielded numerous fragments, which was different in *Bm*NPV-Ram isolate. Molecular diversity analysis can helps in understanding the evolution and phylogeny of the virus, enhance the knowledge on its pathogenicity and can help to develop and adopt suitable measures to combat and diagnose the disease to reduce crop loss and increase income generating ability of the farmer.

KEYWORDS: *Bombyx mori*, diversity, Grasserie, NPV, restriction digestion, silkworm, virus

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1. INTRODUCTION

The silkworm *Bombyx mori* L. has been utilized for the production of silk which is generally referred as "queen of fabrics" due to its luster, softness, colour, biodegradability, elegance, biocompatibility, strength and flexible properties and also used as powerful biological model system. Globally silk production is estimated was 1,59,648 mt (2018). China is lead producer of silk (1,20,000 mt / 75.16%) followed by India (35,261 MT/22.08%) (Anonymous, 2018). The silkworm *Bombyx mori* is highly susceptible to multiple diseases caused by protozoa, virus, bacteria and fungi pathogens. The disease caused by *Bombyx mori nuclear polyhedrosis virus* (*Bm* NPV) causes drastic damage in the cocoon yield affecting the income of silkworm growers. Grasserie disease causes more than 15 % loss in yield and accounts for 25–58% in total disease incidence (Sharma et al., 2014). The NPV infects various tissues, and multiplies in the nucleus forming inclusion bodies called polyhedra, which are occluded viral particles (Jiang et al., 2012).

*Bm*NPV is arthropod-specific, enveloped, rod-shaped virus belonging to the family Baculoviridae characterized by a circular, supercoiled and dsDNA-containing genome infecting lepidoptera group of insects (Kool et al., 1995; Gomi et al., 1999). *Bm*NPV is well characterized baculoviruses with a genome of 132kb in size (Palhan and Gopinathan, 1996) with a biphasic life cycle, two virion phenotypes are formed such as occlusion-derived virus (ODV) and budded virus (BV). ODV is involved in the lateral transmission between individuals whereas BV plays an important role in spreading the infection throughout the host (Wang et al., 2018).

Phylogenetic analysis of common ORFs of six strains of *Bm*NPV were studied in which the genome of the Indian strain consisted of 126,879 nucleotides, with a 40.4% of G+C. Variation in terms of survival rate, virulence and host range has been well documented among isolates of *Heliothis* NPVs (Bilimoria, 1983). Sub molar fragments have been reported by restriction digestion of a wild isolate of *AcNPV,* which resulted in the isolation of several variants of the particular isolate (Summers and Smith, 1978; Lee and Miller, 1978). Similarly, the RFLP profiles of MNPV genome from *A. californica, Tricoplusia ni*, *Rachiplusia ou* and *Galleria mellonella* has been well studied (Smith and Summers, 1979; 1980). Further, RFLP, was used to study the genetic variation within *Malacosoma californicum pluviale* NPV wild type populations (Cooper et al., 2003).

Chateigner et al. (2015) reported that the population polymorphisms are essential for functional diversity in model baculovirus *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV). The major factors required for the adaptation during virus-host interaction is genetic variation (Obbard and Dudas, 2014; Holmes, 2011). Categorization of different isolates of SfMNPV and ensuring their field efficacy polymorphism exhibited by the isolates proved to be very essential (Alletti et al., 2017).

Restriction digestion analysis helps in studying the polymorphisms among different organisms in which, RFLP was used extensively to study the polymorphism in NPVs (Herniou et al., 2003). The highly polymorphic nature of NPVs has led to study the diversity of *Bm*NPV. The RFLP technique was used to analyze the NPV genomes by using different restriction enzymes to differentiate the *Ac*NPV, *Orgyia pseudotsugata* nuclear polyhedrosis *virus* (*Op*NPV) and *Helicoverpa zea* nuclear polyhedrosis virus (*Hz*NPV) associated with different insect hosts. These NPVs wee used as bio-pesticide formulations in different combinations, without affecting the quality of the pesticides as there is a lot of specificity between the NPV strains and the host they infect (Miller and Dawes, 1978). Restriction digestion of PCR products of 25kbp region using different restriction enzymes showed genetic diversity among the 7 *Heliothis armigera* nuclear polyhedrosis virus (*Hear*NPV) isolates (Mehrvar et al., 2008). The *polyhedron* (*polh)* gene encoding structural protein of the occlusion bodies was used to study the diversity of the distinct isolates of *Hear*NPV by PCR analysis (Singh et al., 2019). The five *Bm*NPV isolates were characterized based on the amplified fragment length polymorphism pattern (AFLP) using four AFLP primer combinations (Peter et al., 2016). Diversity analysis of *Bm*NPV could help in studying its evolution and phylogeny and its diagnostics.

Hence, the present investigation is undertaken to study the diversity analysis of *Bm*NPV isolates procured from different silkworm growing areas of Karnataka using restriction endonuclease digestion profiling analysis.

2. MATERIALS AND METHODS

2.1. Collection of BmNPV isolates

The *Bm*NPV infected silkworms were collected from six different districts of Karnataka viz., Devanahalli (Latitude: 13° 14' 47.58" N; Longitude: 77° 42' 42.59" E), Kolar (Latitude: 13° 07' 48.00" N; Longitude: 78° 07' 48.00" E), Shidlaghatta (Latitude: 13° 23' 24.00" N; Longitude: 77° 51' 36.00" E), Hosakote (Latitude: 13° 04' 14.52" N; Longitude: 77° 47' 53.30" E), Tumakuru (Latitude: 13° 20' 28.90" N; Longitude: 77° 06' 7.92" E), Ramanagara (Latitude: 12° 42' 34.02" N; Longitude: 12° 42' 34.02" E) during the year 2016-2018. The silkworms were collected based on their symptoms caused by *Bm*NPV. The research was conducted at Department of Plant Biotechnology, College of Agriculture, UAS (B), GKVK, Bangalore-560065, Karnataka, India.

2.2. Preparation of polyhedra and purification of virions

A modified standard protocol was followed for the isolation of *Bm*NPV viral DNA (Govindan et al., 1998). The NPV infected silkworms were used for the collection of turbid milky haemolymph by cutting the front pair of prolegs and stored in sterilized glass tubes followed by immediate refrigeration. The haemolymph was then diluted two fold by adding distilled water and filtered through double layer cheese cloth and centrifuged at 12,000 RPM for 15 min at 4°C. After centrifugation, the pellet was resuspended in sterile distilled water and centrifuged at 5,000 RPM for 15 min at 4°C. Repeated cycles of centrifugation at 12,000 RPM and 5,000 RPM was done for 15 min each to get milky white amorphous sediment of nuclear polyhedra. The polyhedra were suspended in distilled water and stored. The different sucrose gradients layers of 60, 55, 50, 45, and 40% (W/W) sucrose solution were prepared in Beckman 5 ml centrifuge tubes and incubated at 4°C overnight. Further, three ml of amorphous sediment of nuclear polyhedra was loaded on to the sucrose gradient and ultra-centrifuged at 32,000 RPM for 60 min at 4°C. The pellet was collected and dissolved in 1.5 ml of distilled water.

2.3. Isolation of DNA from purified virions

The re-suspended polyhedral pellet obtained from the ultra-centrifugation was pelleted by centrifugation at 9000 RPM for 2 min. The pellet was re-suspended in 200 µl distilled water. 20 µl of 0.5M EDTA and 2 ml of proteinase K were added and incubated for 5 hours. Half the volume of sodium carbonate (0.1M) was added and incubated at 37°C for 15 min, 20ml of 10% w/v SDS was added and incubated at 37°C for 15 min and centrifuged for 30 s at 6500 RPM, the supernatant was collected in a clean test tube. An equal volume of Tris-saturated phenol was added and gently agitated for 2 min followed by centrifugation at 9000 RPM for 2 min. The upper phase was removed carefully and transferred to a clean tube without disturbing the interface. An equal volume of 25:24:1 Tris-saturated-phenol: chloroform: isoamylalcohol was added and gently agitated for 2 min followed by centrifugation at 9000 RPM for 2 min; the upper phase was removed carefully and transferred to a clean tube without disturbing the interface. To the supernatant, 2.5 times absolute alcohol was added and incubated at -20°C overnight and then the pellet was collected after centrifugation at 9000 RPM. The supernatant was removed and pellet was dried and dissolved in nuclease free water and stored in a -20°C freezer. 5 µl of isolated *Bm*NPV viral DNA was mixed with 1 µl of loading dye and electrophoresed in 0.8% w/v gel in TAE buffer along with 1 Kb ladder and then run at 70 V for 1 hour 30 min. The DNA bands in the gel were visualized on a UV- Transilluminator.

2.4. Restriction endonuclease profiling

The purified *Bm*NPV DNA was used for restriction analysis using the different restriction enzymes. The reaction mixure consisted of *Bm*NPV DNA, 10x NEB cut smart buffer, different restriction enzymes viz., *Eco*R1, *Bam*H1, *Sma*1, *Nco*1, *and Bam*H1+*Nco*1 (20 units), and nuclease free water. The reaction mixture was incubated at 37°C for 3 h and then inactivated at 60°C for 20 min. The restriction digested sample was then run on 0.8% w/v agarose gel to visualize restriction enzyme profiling. The polymorphic bands were scored as presence (1) or absence (0) of bands. The analyses were performed using NTSYS-pc software, version 2.0 (Rolhf, 1990). The data matrix was used to calculate Jaccard's similarity coefficient (Sneath and Sokal 1973) which does not consider the joint absence of a marker as an indication of similarity. A dendrogram was constructed using the unweighted pair group method analysis (UPGMA). The frequencies of the RFLP fragments were estimated for each of the *Bm*NPV isolates infecting silkworm.

3. RESULTS AND DISCUSSION

3.1. Purification and isolation of BmNPV viral DNA

The haemolymph from *Bm*NPV infected worms collected from different regions of Southern Karnataka as purified by differential centrifugation and sucrose density gradient centrifugation and the presence of *Bm*NPV polyhedra's were confirmed under compound microscope (100×) (Plate 1). Viral DNA isolated from purified polyhedra was confirmed by gel electrophoresis (Figure 1).

3.2. Restriction endonuclease profiling of BmNPV viral genome

The total viral DNA isolated from the six *Bm*NPV isolates (*Bm*NPV-Ko, *Bm*NPV-Ho, *Bm*NPV-SG, *Bm*NPV-DH,

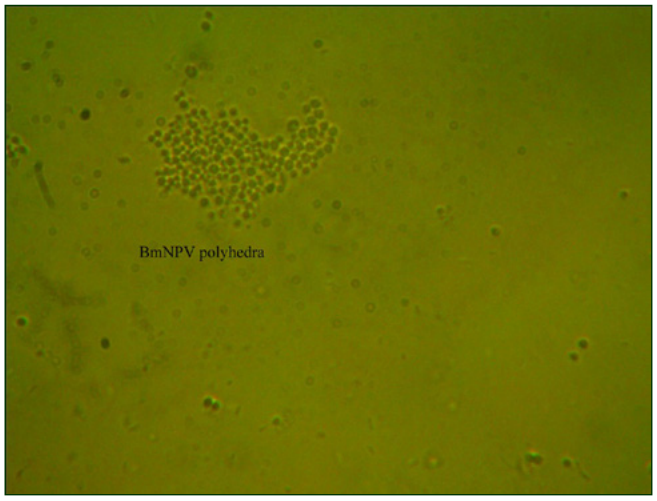

Plate 1: Confirmation of presence of *Bm*NPV polyhedra under 100× compound microscope

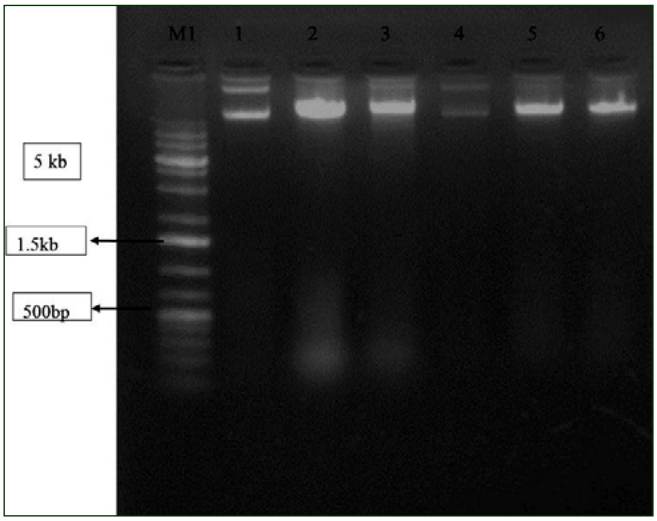

Figure 1: Ethidium bromide stained agarose gel showing viral DNA isolated from *Bm*NPV infected silkworms collected from different locations of Karnataka.
Lane M 1:1 Kb ladder; Lane 1: Kolar isolate; Lane 2: Tumakur isolate; Lane 3: Shidlaghatta isolate; Lane 4: Devanahalli isolate; Lane 5: Hosakote isolate; Lane 6: Ramanagar isolate

*Bm*NPV-TM, *Bm*NPV-Ram) were digested with different restriction enzymes which includes *Eco*R1, *Bam*H1, *Sma*1, *Nco*1, and *Bam*H1+*Nco*1 as they were previously used in similar studies (Hong et al., 2000) (Figure 2–6). The restriction profiles of each enzyme are shown in Table 1. All the six *Bm*NPV isolates had a quite similar *Nco*1 and *Sma*1 restriction patterns, although low molecular fragments showed a minor difference. Whereas *Eco*R1 and

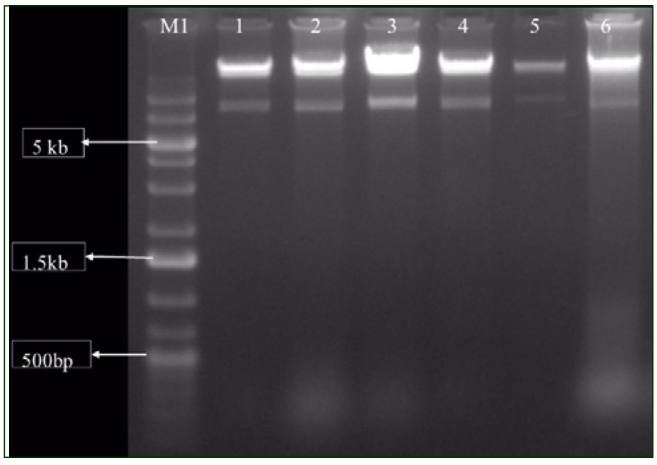

Figure 2: Ethidium bromide stained agarose gel showing restriction endonuclease profiling of *Bm*NPV isolates using *Sma*1 enzyme.
M 1: 1 Kb ladder; Lane 1: Kolar isolate; Lane 2: Tumakuru isolate; Lane 3: Shidlaghatta isolate; Lane 4: Devanahalli isolate; Lane 5: Hosakote isolate; Lane 6: Ramanagara isolate

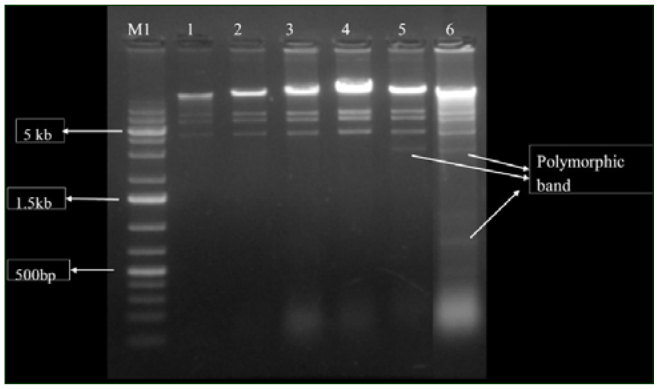

Figure 3: Ethidium bromide stained agarose gel showing restriction endonuclease profiling of *Bm*NPV isolates using *Bam*H1 enzyme.
M 1: 1 Kb ladder; Lane 1: Kolar isolate; Lane 2: Hosakote isolate; Lane 3: Shidlaghatta isolate; Lane 4: Devanahalli isolate; Lane 5: Tumakuru isolate; Lane 6: Ramanagar isolate

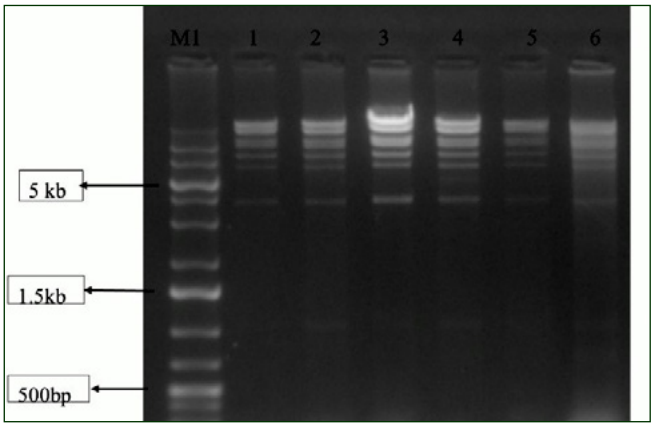

Figure 4: Ethidium bromide stained agarose gel showing restriction endonuclease profiling of *Bm*NPV isolates using *Nco*1 enzyme.
M1:1 Kb ladder; Lane 1: Kolar isolate; Lane 2: Hosakote isolate; Lane 3: Shidlaghatta isolate; Lane 4: Devanahalli isolate; Lane 5: Tumakuru isolate; Lane 6: Ramanagara isolate

*Bam*H1 restriction patterns were nearly the same for all the six isolates except for the presence of an approximately of 4kb band and an additional 1.5kb polymorphic band observed only in *Bm*NPV-TM and *Bm*NPV-Ram isolates. Similarly, *Bam*H1+*Nco*1 digestion of the DNAs from each isolate yielded numerous fragments, and *Bam*H1+*Nco*1 patterns were different in *Bm*NPV-Ram isolate. Based on the scores, a dendrogram was drawn (Figure 7) using XLSTAT software which represented the relationship among six isolates of *Bm*NPV containing two large clusters. *Bm*NPV-Ram isolate was present in one cluster, while the other cluster contained two sub-clusters one with *Bm*NPV-TM isolate and the other with *Bm*NPV-Ko,

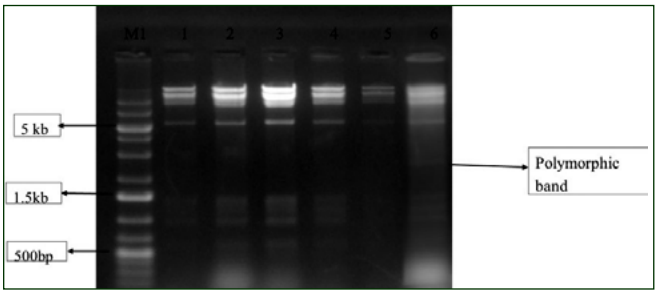

Figure 5: Ethidium bromide stained agarose gel showing restriction endonuclease profiling of *Bm*NPV isolates using *Eco*R1 enzyme.
M1:1 Kb ladder; Lane 1: Kolar isolate; Lane 2: Hosakote isolate; Lane 3: Shidlaghatta isolate; Lane 4: Devanahalli isolate; Lane 5: Tumakuru isolate; Lane 6: Ramanagara isolate

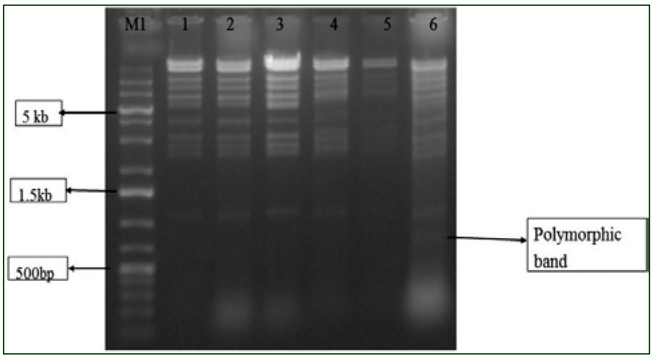

Figure 6: Ethidium bromide stained agarose gel showing restriction endonuclease profiling of *Bm*NPV isolates using *Bam*H1+Nco1 enzyme.
M1:1 Kb ladder; Lane 1: Kolar isolate; Lane 2: Hosakote isolate; Lane 3: Shidlaghatta isolate; Lane 4: Devanahalli isolate; Lane 5: Tumakuru isolate; Lane 6: Ramanagara isolate

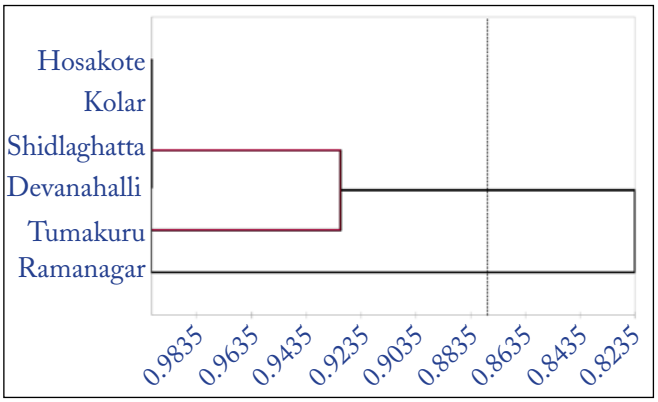

Figure 7: Dendrogram representing relationship among six *Bm*NPV isolates

*Bm*NPV-Ho, *Bm*NPV-SG and *Bm*NPV-DH isolates. Similarity indices of the values between the six isolates are shown in Table 2. The RFLP profile analysis of viral DNA has been used to distinguish and characterize closely related genotypic variants and species (Hong et al., 2000). In the present study, six *Bm*NPV isolates (*Bm*NPV-Ko, *Bm*NPV-Ho, *Bm*NPV-SG, *Bm*NPV-DH, *Bm*NPV-TM, *Bm*NPV-Ram) isolated from *B. mori* are closely related but slightly different with respect to *Eco*R1 and *Bam*H1 enzyme profiles. These variations may be due to insertion of host DNA into the viral genome or deletions / point mutations in the viral DNA.

AFLP markers were used to understand the diversityof *Bm*NPV isolates collected from different locations of Karnataka (Peter et al., 2016). Palhan and Gopinathan (1996) found distinct differences for restriction enzyme *Hind* III profile for Bangalore (BLR) and Dharwad (DHR) isolates of *Bm*NPV in Karnataka. Hong et al. (2000) also

Table 1: Restriction digestion banding pattern of *Bm*NPV DNA (*Bm*NPV-Ko, *Bm*NPV-Ho, *Bm*NPV-SG, *Bm*NPV-DH, *Bm*NPV-TM, *Bm*NPV-Ram) with different restriction enzymes

Restriction enzymes	Band Size	*Bm*NPV-Ko	*Bm*NPV-Ho	*Bm*NPV-SG	*Bm*NPV-DH	*Bm*NPV-TM	*Bm*NPV-RAM
*Bam*H1	>10kb	1	1	1	1	1	1
	9kb	1	1	1	1	1	1
	8kb	1	1	1	1	1	1
	5.8kb	1	1	1	1	1	1
	4kb	0	0	0	0	1	1
	1.5kb	0	0	0	0	0	1
*Eco*R1	11kb	1	1	1	1	1	1
	9.5kb	1	1	1	1	1	1
	9kb	1	1	1	1	1	1
	6.3kb	1	1	1	1	1	1
	3.5kb	0	0	0	0	0	1

Table 1: Continue...

Restriction enzymes	Band Size	*Bm*NPV-Ko	*Bm*NPV-Ho	*Bm*NPV-SG	*Bm*NPV-DH	*Bm*NPV-TM	*Bm*NPV-RAM
*Sma*1	>10kb	1	1	1	1	1	1
	8kb	1	1	1	1	1	1
*Nco*1	>10kb	1	1	1	1	1	1
	10kb	1	1	1	1	1	1
	9kb	1	1	1	1	1	1
	7.8kb	1	1	1	1	1	1
	7kb	1	1	1	1	1	1
	5kb	1	1	1	1	1	1
*Bam*H1+*Nco*1	>10kb	1	1	1	1	1	1
	10kb	1	1	1	1	1	1
	9kb	1	1	1	1	1	1
	8kb	1	1	1	1	1	1
	7.5kb	1	1	1	1	1	1
	7kb	1	1	1	1	1	1
	5kb	1	1	1	1	1	1
	4.3kb	1	1	1	1	1	1
	4kb	1	1	1	1	1	1
	3.8kb	1	1	1	1	1	1
	2.2kb	1	1	1	1	1	1
	2kb	0	0	0	0	0	1

Table 2: Similarity indices of six *Bm*NPV isolates collected from different locations of Karnataka

	Kolar	Hosakote	Shidlaghatta	Devanahalli	Tumakuru	Ramanagara
Kolar	1.000					
Hosakote	1.000	1.000				
Shidlaghatta	1.000	1.000	1.000			
Devanahalli	1.000	1.000	1.000	1.000		
Tumakuru	0.964	0.964	0.964	0.964	1.000	
Ramanagar	0.871	0.871	0.871	0.871	0.871	1.000

used restriction endonuclease analysis of *BmNPV* viral DNA which has been used to distinguish and characterize closely related genotypic variants and observed that *Bm*NPV isolates from wild stocks were closely related with slightly different banding pattern. Similar differences in RFLP profile for multiple peptide occlusion nuclear polyhedrosis virus (MNPV) and single peptide occlusion nuclear polyhedrosis virus (SNPV) infecting *Heliothis* and *Orygyia pseudotsugata* respectively have been reported (Rohrmann and Beaudreau, 1977) also for *Tricoplusia ni* (Smith and Summers, 1979). These minor genotypic variations serve as genetic markers for the particular isolate. *Hear*NPV isolates of Spain and Portugal showed genetic variations based on their restriction fragment profile (Figueiredo et al., 1999). Restriction endonuclease analysis of *Spodoptera litura* NPV and *S. littoralis* NPV from Japan, Vietnam, Malaysia, India, and Egypt (Takatsuka et al., 2003) also revealed similar findings.

Genomic diversity acts a major determinant of viral evolutionary dynamics and virulence (Geoghegan and Holmes, 2018), particularly large DNA viruses (Renner and Szpara 2017). Mason et al. (2021) characterized the genetic diversity analysis of SfMNPV populations and showed that coding regions with higher genetic diversity are associated with oral infectivity or unknown functions.

Thus variation in baculoviruses has been observed between geographical regions, within virus isolates, within single agricultural fields, between different polyhedra in a single host and polymorphism has also been detected in baculovirus genotypes, derived from a single isolate. Variation has also been noticed in the pathogenicity of the virus (Williams and Payne, 1984; Bilimoria, 1983; Burgess, 1977; Smith and Summers, 1979). Thus molecular markers can be used for better understanding the biodiversity of the *Bm*NPV, their evolution patterns and recombination. It could also help in developing better immunodiagnostic kits for the identification of *Bm*NPV and better management of the disease.

4. CONCLUSION

Six *Bm*NPV isolates (*Bm*NPV-Ko, *Bm*NPV-Ho, *Bm*NPV-SG, *Bm*NPV-DH, *Bm*NPV-TM, *Bm*NPV-Ram) isolated from mulberry silkworm *B. mori* from different locations were found to be closely related but slightly different with respect to *Eco*R1 and *Bam*H1 enzyme profiles. These variations may be due to insertion of host DNA into the viral genome or deletions / point mutations in the viral DNA.

5. FUTURE RESEARCH

Further analysis of these genetic variation will lead to enhance the knowledge on the pathogenicity of *Bm*NPV and developing suitable measures to combat and diagnose the disease. These genetic diversity of *Bm*NPV isolates will be further validated by studying the expression of antiviral protein genes in silkworm from the respective locations.

6. ACKNOWLEDGEMENT

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