MOLECULAR CHARACTERIZATION OF *Bm*NPV AND CLONING OF *Bm*NPV ANTIVIRAL ENCODING PROTEIN GENES SERINE PROTEASE AND LIPASE

NATESH, J.

PALB 5274

DEPARTMENT OF PLANT BIOTECHNOLOGY

UNIVERSITY OF AGRICULTURAL SCIENCES

BENGALURU – 560 065

2017

MOLECULAR CHARACTERIZATION OF *Bm*NPV AND CLONING OF *Bm*NPV ANTIVIRAL ENCODING PROTEIN GENES SERINE PROTEASE AND LIPASE

NATESH, J.

PALB 5274

Thesis submitted to the
UNIVERSITY OF AGRICULTURAL SCIENCES, BENGALURU
In partial fulfilment of the requirements
for the award of the Degree of

MASTER OF SCIENCE (Agriculture)

in

PLANT BIOTECHNOLOGY

BENGALURU JULY, 2017


Affectionately Dedicated to My
Grand Father
Sampangi Ramakrishnappa,
Parents, Brother, Teachers and
Friends

DEPARTMENT OF PLANT BIOTECHNOLOGY
UNIVERSITY OF AGRICULTURAL SCIENCES
GKVK, BENGALURU – 560 065

CERTIFICATE

This is to certify that the thesis entitled, **"MOLECULAR CHARACTERIZATION OF *Bm*NPV AND CLONING OF *Bm*NPV ANTIVIRAL ENCODING PROTEIN GENES SERINE PROTEASE AND LIPASE"** submitted in partial fulfilment of the requirement for the degree of **MASTER OF SCIENCE (AGRICULTURE)** in **PLANT BIOTECHNOLOGY** to the University of Agricultural Sciences, GKVK, Bengaluru, is a *bonafide* record of research work done by **Mr. NATESH, J., ID. No. PALB 5274** during the period of his study in this University, under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles.

Bengaluru
July, 2017

Dr. NAGESH, N
(Major Advisor)

APPROVED BY:

Chairperson : _____
(NAGESHA. N)

Members : 1. _____
(ANITHA PETER)

2. _____
(P. H. RAMANJINI GOWDA)

3. _____
(NATARAJA KARABA)

4. _____
(NAGESHA, S. N.)

ACKNOWLEDGEMENT

I would like to convey my sincere gratitude and thanks to all those who have been instrumental in the course of my studies at the ***University of Agricultural Sciences, Bengaluru****. It is not possible to mention the names of all the individuals who contributed to this piece of work but I fully recognize and appreciate your valuable contributions.*

My deep gratitude goes to my chair person ***Dr. Nagesha N,*** *for his guidance and encouragement during my entire postgraduate study. Mere any words from the vastness of literature fails to express my sense of gratitude to him, for his continued calm endurance shown throughout my research programme and compilation of this thesis, without which this work would not have seen the light of the day.*

I deem it a great privilege, in placing my esteem and deep sense of gratitude to ***Dr. Anitha peter,*** *Professor, Department of Plant biotechnology, for her parental care, impeccable guidance, genuine counseling and encouragement for the successful completion of this research work.*

I am also grateful to thanks to all my advisory committee ***Dr. Ramanjini Gowda. P. H****., Professor, Department of Plant Biotechnology,* ***Dr. Nataraja Karaba****, Professor, Department of Crop physiology,* ***Dr. Nagesh S. N.*** *Assistant Professor****,*** *Department of Plant Biotechnology, College of Agriculture, Hassan, for there valuable advises and guidelines throughout my research work.*

I am extremely thankful to ***Dr. D. Dayal Doss****, Professor and Head, Department Plant Biotechnology and all the staff members who have provided an excellent studying atmosphere during my Masters programme.*

I am thankful to the staff members of the Department of Plant Biotechnology ***Dr. H.E. Shashidhar, Dr. R. L. Ravikumar, Dr. Theertha Prasad, Dr. Harini Kumar K. M, Dr. S. Shyamalamma, Dr. Veena. S. Anil, and Dr. Kavitha*** *Professors for their valuable guidance and moral support during my stay in this Department.*

Words of encouragement, boundless love, unflagging inspiration, interest and selfless sacrifice of my parents I would not have been what I am

today. I owe all my success to them. The special persons in my life my Father ***Sri Jagadish T*** *Mother* ***Smt. Manjula R,*** *Brother****, Shivadarshan,*** *Sister* ***Archana,*** *without their love and blessings this study would have scarily accomplished. I dedicate this piece of work to them.*

I owe thanks from depth of my heart to my lab mates*, who were always stood and guided me and felt all were like a family of Brothers and Sisters, Swapnil, Kheta ram, Chaithrakka, Savithakka, Rohini akka, Meenashi akka, Bharath, and friends Ram and Jaanu* ***for their love, affection and constant encouragement throughout the study.***

I owe thanks from depth of my heart to my well-wishers, *Dr. Sham, Dr. Ningaraju, Manoj, Krupakka, Raghu, Sunil Subramanya, Avinash M, and Santhosh Xan* ***for their love, affection and constant encouragement throughout the study.***

I owe thanks from depth of my heart to my friends, *Chandrakala Ram, Vinayaka, Janani, Subbu, Reddy, Sampad, Anjali, Mouna, Monica, Amulya, Chitra, Jack, Lobo, Roopashree, Praveen, Krishna, Nagappa, Manu, Ashwin, Pranam, Hari, Nayana, Hemanth T N, Abhi S. N* ***and*** *juniors BJ, Sha, RNA, KN, Jalhalli, Suresh, Dube, Vijesh, Kavya, Sagar, Vinith, Rohith, Non staff- Shashank, Shivanna, Ananth, Prathima, Manjunath, Shivakumar* ***for their constant encouragement and co-operation throughout the study.***

I also owe thanks *to Mr. Thillai Chidabaram P., Dr. Leela Reddy and Dr. Selva kesavan who thought me cloning techniques at internship in MRC, Bangalore which helped me a lot in* ***understanding and completing my research work****.*

Last but not the least I thank all those who helped me directly or indirectly during period of my stay in this UAS, GKVK. Campus.

Bengaluru

July, 2017 **(Natesh, J.)**

MOLECULAR CHARACTERIZATION OF *Bm*NPV AND CLONING OF *Bm*NPV ANTIVIRAL ENCODING PROTEIN GENES, SERINE PROTEASE AND LIPASE

NATESH, J

ABSTRACT

Sericulture is an important agro based cottage industry in Karnataka and silkworm *Bombyx mori* L. is being commercially utilized for the production of silk. Silkworms are prone to many microbial infection which causes different diseases during domestication. Among these, *Bombyx mori* nuclear polyhedrosis virus (*Bm*NPV) causes Grasserie a viral disease in silkworm which is a major disease causing great economic loss to sericulture industry. The present investigation lays main emphasis on genetic diversity of six different *Bm*NPV isolates collected from different locations of Karnataka and molecular characterization of antiviral proteins. The genetic diversity of six different *Bm*NPV isolates were analyzed by restriction endonuclease digestion. The diversity dendogram was drawn based on polymorphic banding pattern in which Ramanagara isolate showed more variation followed by Tumakuru isolate and not much variation observed in the remaining isolates which were shown similar banding pattern for specific endonucleases digestion. Silkworm digestive juice contains number of different antiviral proteins among these lipase and serine protease were shown strong antiviral properties against *Bm*NPV. These genes were isolated from midgut of silkworm, amplified using gene specific primers. These genes were cloned into pGEMT cloning vector, sequence and identity was confirmed using NCBI BLAST analysis. Sequence variation for antiviral protein genes were analysed for the resistant breed Pure Mysore, Susceptible breed CSR2, and a hybrid Kolar Gold. Multiple sequence alignment of these genes showed variation in nucleotides sequence around 0.33 % to 1.33 % for lipases and 1.29 % to 4.4 % for serine proteases.

July, 2017

Department of Plant Biotechnology
UAS, GKVK, Bengaluru

NAGESHA, N
Major Advisor

ಬಿಎಮ್ಎನ್‌ಪಿವಿಯ ಆಣ್ವಿಕ ಗುಣಲಕ್ಷಣಗಳ ವಿವರಣೆ ಮತ್ತು ಬಿಎಮ್ಎನ್‌ಪಿವಿ ನಂಜಾಣು ವಿರೋಧಿ ಪ್ರೋಟಿನ್‌ಗಳಾದ ಸಿರೈನ್ ಪ್ರೋಟಿಯೆಸ್ ಮತ್ತು ಲಿಪೆಸ್‌ನ ಅನುವಂಶಿಕ ಧಾತುಗಳ ಕ್ಲೋನಿಂಗ್.

ನಟೇಶ್ ಜೆ

ಅಮೂರ್ತ

ರೇಷ್ಮೆ ಕೃಷಿ, ಕರ್ನಾಟಕದ ಪ್ರಮುಖ ಕೃಷಿ ಆಧಾರಿತ ಕುಟೀರ ಉದ್ಯಮವಾಗಿದೆ ಮತ್ತು ರೇಷ್ಮೆ ಹುಳು (ಬಾಂಬಿಕ್ಸ್ ಮೋರಿ)ವನ್ನು ರೇಷ್ಮೆ ಉತ್ಪಾದನೆಗಾಗಿ ವಾಣಿಜ್ಯ ಬೆಳೆಯಾಗಿ ಬಳಸಲಾಗುತ್ತಿದೆ. ರೇಷ್ಮೆ ಹುಳುಗಳ ಸಾಕಾಣಿಕೆಯ ಸಮಯದಲ್ಲಿ ವಿವಿಧ ರೋಗಗಳಿಗೆ ಕಾರಣವಾಗುವಂತಹ ವಿವಿಧ ಸೂಕ್ಷ್ಮ ಜೀವಿಗಳ ಸೋಂಕಿಗೆ ಒಳಗಾಗುತ್ತದೆ. ಇವುಗಳಲ್ಲಿ ಮುಖ್ಯವಾಗಿ ಬಾಂಬಿಕ್ಸ್ ಮೋರಿ ನ್ಯೂಕ್ಲಿಯರ್ ಪಾಲಿಹೆಡ್ರೋಸಿಸ್ ನಂಜಾಣು (ಬಿಎಮ್ಎನ್‌ಪಿವಿ), ಇದು ರೇಷ್ಮೆ ಹುಳುವಿನಲ್ಲಿ ಹಾಲುತೊಂಡೆ ರೋಗವನ್ನು ಉಂಟುಮಾಡುತ್ತದೆ. ಇದು ರೇಷ್ಮೆ ಕೃಷಿ ಉದ್ಯಮಕ್ಕೆ ಹೆಚ್ಚಿನ ಆರ್ಥಿಕ ನಷ್ಟವನ್ನು ಉಂಟುಮಾಡುವ ಪ್ರಮುಖ ನಂಜಾಣು ರೋಗವಾಗಿರುತ್ತದೆ. ಪ್ರಸ್ತುತ ಅಧ್ಯಯನದಲ್ಲಿ, ಕರ್ನಾಟಕದ ವಿವಿಧ ಸ್ಥಳಗಳಿಂದ ಆರು ಬಿಎಮ್ಎನ್‌ಪಿವಿ ಪ್ರತ್ಯೇಕ ಗಳನ್ನು ಸಂಗ್ರಹಿಸಲಾಗಿದೆ. ಆರು ವಿಭಿನ್ನ ಬಿಎಮ್ಎನ್‌ಪಿವಿ ಪ್ರತ್ಯೇಕತೆಗಳ ಅನುವಂಶಿಕ ವೈವಿಧ್ಯತೆಯನ್ನು ಪ್ರತಿಬಂಧಕ ಎಂಡೋನ್ಯೂಕ್ಲೀಯೇಸ್‌ಗಳಿಂದ ವಿಶ್ಲೇಷಿಸಲಾಗಿದೆ. ವೈವಿಧ್ಯತೆಯ ವಿಕಸನೀಯ ವೃಕ್ಷವನ್ನು ಬಹುರೂಪದ ಬ್ಯಾಂಡಿಗ್ ಮಾದರಿಯ ಆಧಾರದ ಮೇಲೆ ಚಿತ್ರಿಸಲಾಗಿದ್ದು, ಇದರಲ್ಲಿ ರಾಮನಗರದ ಪ್ರತ್ಯೇಕತೆ ಹೆಚ್ಚಿನ ವ್ಯತ್ಯಾಸವನ್ನು ತೋರಿದ್ದು, ತದ ನಂತರ ತುಮಕೂರು ಪ್ರತ್ಯೇಕತೆ ಇದೇ ಮಾದರಿಯ ವ್ಯತ್ಯಾಸವನ್ನು ತೋರಿಸಿದೆ. ಉಳಿದಿರುವ ಪ್ರತ್ಯೇಕತೆಗಳು, ಒಂದೇ ರೀತಿಯ ಬ್ಯಾಂಡಿಗ್ ಮಾದರಿಯನ್ನು ಹೊಂದಿರುತ್ತದೆ. ರೇಷ್ಮೆ ಹುಳುವಿನ ಜೀರ್ಣಕಾರಿ ರಸವು ಹಾಗೂ ಮಧ್ಯ ಅನ್ನನಾಳವು ವಿವಿಧ ಬಗೆಯ ನಂಜಾಣು ವಿರೋಧಿ ಪ್ರೋಟಿನ್‌ಗಳನ್ನು ಹೊಂದಿದ್ದು, ಇವುಗಳಲ್ಲಿ ಸಿರೈನ್ ಪ್ರೋಟಿಯೆಸ್ ಮತ್ತು ಲಿಪೆಸ್ ಪ್ರೋಟಿನ್‌ಗಳು ಹೆಚ್ಚಿನ ನಂಜಾಣು ವಿರೋಧಿ ಗುಣಲಕ್ಷಣವನ್ನು ಬಿಎಮ್ಎನ್‌ಪಿವಿ ವಿರುದ್ಧ ತೋರಿಸಿದೆ. ಈ ವಂಶಧಾತುವನ್ನು ರೇಷ್ಮೆಹುಳುವಿನ ಮಧ್ಯ ಅನ್ನನಾಳದಿಂದ ಬೇರ್ಪಡಿಸಿ ನಿರ್ದಿಷ್ಟ ಪ್ರೈಮರ್ಗಳನ್ನು ಬಳಸಿ ವರ್ಧಿಸಲಾಗಿದೆ. ಈ ವಂಶಧಾತುಗಳನ್ನು ಪಿಜಿಇಎಂಟಿ ಕ್ಲೋನಿಂಗ್ ವೆಕ್ಟರ್‌ನಲ್ಲಿ ಕ್ಲೋನ್ ಮಾಡಲಾಗಿದ್ದು, ಇದರ ಅನುಕ್ರಮ ಗುರುತನ್ನು ಎನ್‌ಸಿಬಿಐ ಬ್ಲಾಸ್ಟ್ ವಿಶ್ಲೇಷಣೆಯನ್ನು ಬಳಸಿ ದೃಢೀಕರಿಸಲಾಗಿದೆ. ಬಿಎಮ್ಎನ್‌ಪಿವಿ ನಂಜಾಣು ವಿರೋಧಿ ಪ್ರೋಟೀನ್ ವಂಶಧಾತುಗಳ ಅನುಕ್ರಮ ಗುರುತುಗಳಲ್ಲಿನ ವ್ಯತ್ಯಾಸವನ್ನು ತಿಳಿದುಕೊಳ್ಳಲು ಪ್ರತಿರೋಧಕ ತಳಿಯಾದ ಪ್ಯೂರ್ ಮೈಸೂರು, ಸೋಂಕಿಗೆ ಒಳಗಾಗುವ ತಳಿಯಾದ ಸಿಎಸ್‌ಆರ್2 ಮತ್ತು ಹೈಬ್ರಿಡ್ ಕೋಲಾರ್ ಗೋಲ್ಡ್‌ಗಳಿಂದ ವಂಶಧಾತುಗಳನ್ನು ಪ್ರತ್ಯೇಕಿಸಿ ವಿಶ್ಲೇಷಿಸಲಾಗಿದೆ. ಈ ಅನುವಂಶಿಕ ಧಾತುಗಳ ಅನುಕ್ರಮ ಜೋಡಣೆಯ ನ್ಯೂಕ್ಲಿಯೋಟೈಡ್ಸ್‌ಗಳ ಅನುಕ್ರಮದಲ್ಲಿ ಲಿಪೆಸ್ ಅನುವಂಶಿಕ ಧಾತುಗಳು ಶೇಕಡ ೦.೩೩ ರಿಂದ ೧.೩೩ ಮತ್ತು ಸಿರೈನ್ ಪ್ರೋಟಿಯೆಸ್ ಅನುವಂಶಿಕ ಧಾತುಗಳು ಶೇಕಡ ೧.೨೯ ರಿಂದ ೪.೪ ವರೆಗೆ ವ್ಯತ್ಯಾಸವನ್ನು ತೋರಿಸಿದೆ.

ಜುಲೈ, ೨೦೧೭

ಸಸ್ಯ ಜೈವಿಕ ತಂತ್ರಜ್ಞಾನ ವಿಭಾಗ
ಕೃಷಿ ವಿವಿ, ಜಿಕೆವಿಕೆ, ಬೆಂಗಳೂರು

ನಾಗೇಶ ಎನ್
(ಮುಖ್ಯ ಸಲಹೆಗಾರರು)

Molecular Characterization of *Bm*NPV and Cloning of *Bm*NPV Antiviral Encoding Protein Gene Serine Protease/ Lipase

NATESH, J. and NAGESHA, N.

Department of Plant Biotechnology, University of Agricultural Sciences (B), GKVK, Bengaluru-65

INTRODUCTION

❖The silkworm *Bombyx mori* has been commercially utilized for the production of silk, expression of recombinant proteins of pharmaceutical importance and a biological model system for studying the lepidopteron insect pests of Agriculture.

❖Nuclear polyhedrosis in silkworm is caused by *Bombyx mori* Nuclear polyhedrosis virus (*Bm*NPV), and this accounts for more than 15 % loss in yield and 25-58 % in total disease incidence.

❖The silkworm mid gut is an important immune organ which provides the first line of resistance against pathogens. The Antiviral proteins present in the mid gut includes *Bm*Serine protease (*Bm*SP), *Bm*lipase, etc., were reported to have antiviral activity against *Bm*NPV infection.

❖In the present investigation, isolation of antiviral genes *Bm*Serine Protease, *Bm*Lipase and characterization of these genes are focused.

OBJECTIVES

1. Collection of *Bombyx mori* Nuclear polyhedrosis virus isolates from different locations of Karnataka and molecular characterization of isolates using Restriction endonucleases (REN).
2. Cloning and characterization of the antiviral Serine protease/Lipase gene.
3. Screening of a few breeds of the silkworm *Bombyx mori L* for resistance to *Bombyx mori* nuclear polyhedrosis virus and sequence comparison of serine protease/lipase gene in these breeds.

MATERIALS & METHODS

Cloning and characterization of the antiviral genes Serine protease/Lipase from silkworm.

- Collection of Mulberry Silkworm and isolation RNA from midgut of silk worm using Nucleospin RNA isolation kit.
- c-DNA synthesis from Total RNA using TAKARA RT-PCR kit.
- Amplification of Serine protease and Lipase genes using sequence specific primers.

Serine protease forward primer

5' ATATCTAGAATGAAGGTCTTCGCAGCAGTACT 3'

Serine protease reverse primer

5' GAGCTCTTAAATTCTAGCCCGGATCCAGGAGT 3'

Lipase forward primer

5' ATTGAATTCATGCCTGATGGCGAGGGTGTT 3'

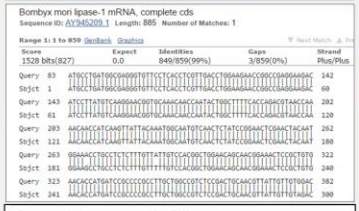
Lipase reverse primer

5' ATTGAGCTCTTAGAAAGGCCAACTGCT 3'

- Amplicons are integrated into T/A cloning vector pGMET and transformed to DH5α *E.coli* cells.
- Recombinant colonies was selected by blue white screening and by PCR using gene specific and M13 primers.
- Plasmid DNA was isolated from the recombinant colonies and confirmed by restriction digestion and sequencing.
- Further, sequences aligned with reference genome sequences and BLAST analysis was done for confirmation.

RESULTS

- ✓ Total RNA was isolated from midgut of silk worm (Fig. 1) and synthesised cDNA from RNA (Fig. 1).
- ✓ Amplification of Serine protease and Lipase gene was done using gene specific primers (Fig. 2).
- ✓ Integration of antiviral genes into pGMET vector (Fig. 3) by ligation (Fig. 4).
- ✓ Confirmed the recombinant colonies of by blue white screening (Fig. 5).
- ✓ Sequencing results were analysed through BLAST analysis. Lipase gene was showing 99 % identity and Serine Protease gene was showing 97 % identity with reference gene sequences.
- ✓ It showed that the cloned gene was similar to the reference gene sequence of Serine protease and Lipase genes (Fig. 6).
- ✓ Restriction profiling of recombinant colonies was done (Fig. 7).



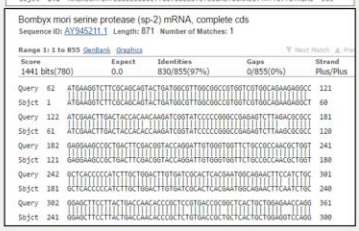


Fig.5 : Blast analysis of Lipase gene showing 99 % identity and Serine Protease showing identity of 97 % with reference gene sequences.

DISCUSSION

- ❖ From the BLAST result it has been shown that similarity of the antiviral genes with reference gene sequence of Lipase(AY945209.1) and Serine Protease(AY945211.1) (Cheng *et al.,* 2014)
- ❖ Variation within the gene sequence may be due to isolation of these genes from different breed of silk worm where as reference gene sequence from other breed.
- ❖ Comparison of these antiviral gene sequence with susceptible and resistant silkworm breed for *Bm*NPV may give variation at nucleotide level and can evaluate for resistance using Bioinformatics tools.

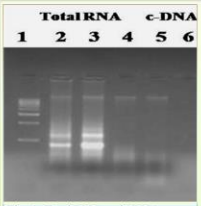


Fig. 1: Total RNA and cDNA
Lane 1: 1kb ladder
Lane 2 & Lane 3: Total RNA from silkworm
Lane 4 & Lane 5: cDNA from total RNA

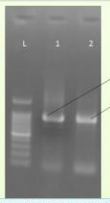


Fig. 2: PCR amplification of *Bm*SP and *Bm*Lipase gene Products
Lane 1:100bp Ladder
Lane 2: Lipase of size 895 bp
Lane 3:Serine protease of size 850 bp

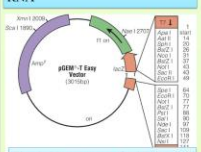


Fig. 3: Vector map of pGEMT

Fig. 4: Ligation Products
Lane 1 & Lane 4: 1KB and 100bp ladder
Lane 2 & Lane 3: SP/LP ligated product+ Plasmid +Amplified product

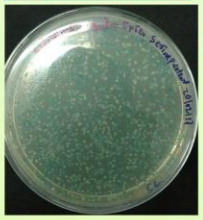
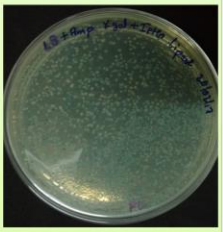


Fig.5 : Screening of transformants through White colonies (transformants) and Blue colonies (Non-transformants) of E- coli.



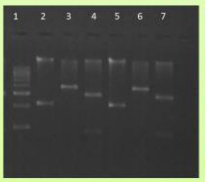


Fig.7: Restriction Digestion confirmation of recombinant E-coli colonies
Lane 1- 1kb ladder
Lane 2 & Lane 5: undigested plasmid of SP & LP.
Lane 3 & Lane 6: Single digested plasmid of SP & LP of 4KB
Lane 4 & Lane 7: Double digested product plasmid of SP & LP of 3KB & 1KB

SUMMARY

❖From the Mulberry silk worms, total RNA was isolated from the midgut intestinal fluids using Nucleospin RNA isolation kit. The cDNA synthesis was done using the cDNA synthesis kit (TAKARA Bio Inc).

❖Serine protease and Lipase genes were amplified using gene specific primers and cloned into cloning vector and transformed to *E. coli* and confirmed transformation.

❖Sequencing result shown antiviral genes were similar to reference gene sequences.

❖Further, antiviral genes will be transforming into mulberry using plant expression vector and will study protein expression of these genes in mulberry.

ADVISORY COMMITTEE

Chairperson: Dr. Nagesha N.
Members: Dr. Anitha Peter
Dr. Ramanjini Gowda P.H.
Dr. Nataraja Karaba
Dr. Nagesha S.N.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

PLATE No.	TITLE	BETWEEN PAGES
14	Agarose gel electrophoresis of confirmation of recombinant pGEM-T- *Bm*Lp gene and pGEM-T- *Bm*Sp gene construct with *Xba1 and SacI* restriction digestion.	53-54
15	Transformed *E. coli* DH5α cells with *Bombyx mori* lipase-pGMET vector isolated from CSR2 breed.	53-54
16	Transformed *E. coli* DH5α cells with *Bombyx mori* lipase-pGMET vector isolated from Pure Mysore breed	53-54
17	Transformed *E. coli* DH5α cells with *Bombyx mori* serine protease- pGME-T vector, isolated from CSR2 breed	53-54
18	Transformed *E. coli* DH5α cells with *Bombyx mori* serine protease- pGME-T vector, isolated from Pure Mysore breed.	53-54
19	Agarose gel electrophoresis of colony PCR for confirmation of recombinant clones CSR2 Lp- pGEMT.	53-54
20	Agarose gel electrophoresis of colony PCR for confirmation of recombinant clones PM Lp- pGEMT clones.	53-54
21	Agarose gel electrophoresis of colony PCR for confirmation of recombinant CSR 2 Sp- pGEMT clones.	53-54
22	Agarose gel electrophoresis of colony PCR for confirmation of recombinant PM Sp- pGEMT clones.	53-54
23	Agarose gel electrophoresis of confirmation of recombinant pGEMT- PmLp gene, pGEMT- PmSp, pGEMT- CSR2 Lp and pGEMT- CSR2 Sp gene construct with *BamH1*, and *Xba1* + *SacI* restriction digestion.	53-54

I INTRODUCTION

The silkworm *Bombyx mori* L. has been utilized for the production of silk. Silk is called as "queen of fabric" because of its luster, softness, color, biodegradability, elegance, biocompatibility, strength and flexible properties. Currently, silkworm is used as a powerful biological model system of lepidopteran insects. It is also a source of economic growth of our society as it generates employment for rural people. It has the unique ability to convert leaf protein into a silk protein. Globally silk production is estimated that 2, 02,072.83 MT in 2016 in which China and India contributed 1, 70,000 MT (84.13 %) and 28,523 M.T. (14.11 %) respectively (ISC, 2016).

The silkworm *Bombyx mori* is susceptible to various diseases caused by protozoa, virus, bacterial and fungal pathogens. Among these, viral diseases causing drastic loss in the cocoon yield there by reducing the income of silkworm growing farmers. Nuclear Polyhedrosis viral disease in silkworm is caused by *Bombyx mori* Nuclear Polyhedrosis Virus (*Bm*NPV), generally known as Grasserie, Jaundice, milky disease and hanging disease are synonyms for Nuclear Polyhedrosis (Lakshmi *et al*., 2014). Grasserie disease accounts for more than 15 % loss in yield and 25-58 % in total disease incidence (Sharma *et al*., 2014). *Bm*NPV is a member of the Baculoviridae family, which has a circular double-stranded DNA genome (Gomi *et al*., 1999). Palhan and Gopinathan (1996) reported that genome size of the *Bm*NPV was 132kb. The nuclear polyhedrosis virus (NPV) infects various tissues, and multiplies in the nucleus forming inclusion bodies called polyhedra, which occlude virus particles (Jiang *et al.*, 2012).

RFLP has been used extensively to study polymorphism of NPVs. Information on AFLP analysis of NPVs is very limited, however it has been used to study diversity of fungi and bacteria. The NPVs are highly polymorphic (Herniou *et al.,* 2003). It is therefore interesting to study the diversity of *Bm*NPV to develop an effective immunodiagnostic kit and also to study its evolution and phylogeny. With recent advances in DNA analysis technology, NPV genomes have been analyzed and their relationships to each other have been investigated using techniques like RFLP to distinguish and characterize closely related genotypic variants.

Silkworm diseases are and will continue to be a major challenge to tackle even with improved breeds and scientific rearing technology. A quest for alternative strategies is therefore imperative breeding resistant strain by traditional or transgenic method is an approach to silkworm disease control. Disinfection of rearing house, its surrounding and appliances with any recommended disinfectants, collection of diseased larvae carefully before rupturing of the skin and disposing properly are methods of managing diseases Traditional breeding methods have limitations such as enhancing pathogen resistance at the expense of the quality of economically important characteristics (Jiang *et al.*, 2012). Limitations of traditional breeding methods might be avoided by transgenic technology like over expression and RNAi which are two established gene regulation strategies. The problem remains to be solved which include further enhancing the traits against *Bm*NPV and determining if a single major silkworm gene is responsible for resistance to *Bm*NPV (Jiang and Xia, 2014).

The genetic analysis studies have been carried out for screening the resistant and susceptible strains of silkworm against viral infections. The various antiviral genes encoding proteins are expressed against viruses during the infection, some of which are *serine proteases*, *lipases*, red fluorescent proteins (RFP), *gloverrin 4, gloverin 3, lebocin, serpin 5, arylphorin, promoting protein, cathespin B and actin 3* etc. (Bao *et al*., 2009). Ponnuvel *et al.* (2003) investigated that, *Bmlipase*-1 has strong antiviral activity against *Bm*NPV, which is highly expressed in the anterior and middle portions of the silkworm midgut and *BmSP*-2, a hormonally regulated 24 kDa protein, also has antiviral effects against *Bm*NPV. *BmSP*-2 is expressed in the entire midgut of silkworm larvae (Nakazawa *et al*., 2004). Studies on resistant and susceptible breeds of silkworm were shown an increase of antiviral activity in resistant breeds.

Hence the present study was undertaken to understand the diversity of *Bm*NPV isolates and to screen few breeds of silkworm for its resistance against *Bm*NPV and characterization of antiviral protein genes *Bm Lipase* and *Bm serine protease* from the silkworm breeds. In this regard, the following objectives were designed to achieve the goal of developing molecular strategies for managing *Bm*NPV infection of the silkworm *Bombyx mori* L.

Objectives

1. Collection of *Bombyx mori* nuclear Polyhedrosis virus isolates from different locations of Karnataka and molecular characterization of isolates using restriction endonucleases (REN).
2. Cloning and characterization of the antiviral encoding genes *serine protease* and *lipase* gene.
3. Screening of a few breeds of the silkworm *Bombyx mori L* for resistance to *Bombyx mori* nuclear polyhedrosis virus and sequence comparison of *serine protease* and *lipase* genes from these breeds.

II REVIEW OF LITERATURE

Mulberry silkworm is a commercially grown cocoon crop which is susceptible to various diseases caused by virus, fungi and protozoa. Among these, viral infections are major causes of concern about crop failure. The present work is designed to explore the diversity studies of NPV collected from different cocoon growing areas of Karnataka and also the antiviral mechanism exhibited by the silkworms. A detailed review of literature as an overview pertaining to the silk worm *Bombyx mori* L., the *Bm*NPV affecting it, the use of molecular markers in understanding NPV diversity, antiviral mechanisms against *Bm*NPV along with the role of antiviral proteins gene *lipase* and *serine protease* are elaborated in this chapter.

2.1 Importance of Sericulture and Silk Production

Sericulture has been one of the main branches of agriculture in Asiatic countries and maintenance of silkworm races in scientific manner is called Sericulture (Watanabe, 2002). The domesticated silkworm, *Bombyx mori* has been reared by humans from the past thousands of years and used as a model insect system in studies of physiology, genetics, molecular biology and biotechnology of the lepidopterans. It is an economically important insect and has been exploited as a silk producer for over 5000 years. Nearly about 90% of silk fabric manufactured in the world is of mulberry silkworm cocoon. Demand on silk raw material in the world is increasing annually, and therefore sericulture, as such is faced with a task to elevate productivity of mulberry silkworm breeds and hybrids (Baramidze *et al.,* 2016.).

Silk, popularly known in the textile industry for its luster and mechanical properties, is produced by cultured silkworms. Silks are produced by members of the class Arachnida (over 30,000 species of spiders) and by several worms of the order Lepidoptera, which includes mites, butterflies and moths. Silks are fibrous proteins synthesized in specialized epithelial cells that line glands in these organisms. The silk of the mulberry silkworm *B.mori* is made up of fibroin fibers which are about 10–25 mm in diameter and consist of two proteins: a light chain (26 kDa) and heavy chain (390 kDa)

which are present in a 1:1 ratio and linked by a single disulfide bond (Veparia and Kaplan, 2007).

The silk fiber protein is synthesized by silk gland cells and stored in the lumen of the silk glands, subsequently it is converted into silk fibers. When the silkworms secrete the liquid silk during spinning, it passes through the anterior gland and is expelled out through the spinneret opening. Quantity and nature of sericin are fundamental characteristics in conferring distinctive traits of the cocoon (Mondal *et al*., 2007).

2.2 Present Status of Silk Industry

Presently, natural silk is produced in 60 countries all over the world. It is practiced in tropical and sub-tropical countries due to its beneficial aspects. The present leading silk producing countries of the world are China, India, the Republic of Korea and a few states of Russia. In the past, production of silk was confined to China alone but gradually it spread to other parts of the world including India. Silk production was practiced from the prehistoric time in India and mulberry silk production has remained a major domestic industry. China and India contributed 1, 70,000 M.T. (84.13 %) and 28,523 M.T. (14.11 %) respectively of the global silk production 2,02,072.83 M.T. (ISC, 2016) amongst 60 sericulture countries in the world.

In India, the modern silk industry has grown to meet the domestic rather than export requirements and this is a fact of great importance for the industry. Presently in India, the industry provides employment opportunities to approximately 8.1 million people (ISC, 2016) and plays a key role in the upliftment of rural economy besides earning considerable foreign exchange.

India produces mainly four types of silk namely mulberry, tasar, eri and muga. Mulberry sericulture is mainly confined to the provinces Karnataka, Andhra Pradesh, Tamil Nadu, Jammu and Kashmir and West Bengal. Tasar silk is reared in Bihar, Madhya Pradesh, Maharashtra, Orissa, Andhra Pradesh and West Bengal. Golden Silk or Muga is produced in the states of Assam and other North Eastern States and the eri silk production is confined to northern Bihar and some regions of the north eastern

Himalayan regions. However, India holds the monopoly of muga production and marketing in the world.

India is a mega biodiversity country by virtue of having both tropical and temperate climatic conditions needed for sericulture. The mulberry silkworm *Bombyx mori* has been domesticated since more than 400 years, its adaptability to environmental conditions is different from that of wild insects and the rearing techniques differ to suit the requirements under different ecological conditions. In Karnataka, sericulture is practiced as multiple crop of 4-6 per year whereas in Jammu and Kashmir sericulture is practiced as a mono crop with single crop harvest per year (Deb, 2013).

India, with a total geographical area of 3.8 million square miles, 2.09 lakh hectare of land was reported to be under mulberry cultivation during the year 2015-16. It produces 1,51,787 MT of cocoon, and 20,478 MT of raw silk and total foreign exchange earnings from silk production was Rs. 2,495.99 Crores in the year 2015-16 (CSB, 2016). In the last two decades, mulberry acreage, cocoon and raw silk production has been remarkably increased by 223 %, 289 % and 556 % respectively (Deb, 2013).

2.3 Diseases of silkworm *Bombyx mori*

The outbreak of diseases in sericulture has hampered progress and inflicted heavy losses and hence the silk industry often faces a serious setbacks. The common major diseases of silkworm are caused by various pathogens viz., protozoan, viral, fungal and bacteria. In India, before the introduction of new techniques of silkworm rearing, one out of every three or more crops suffered due to one disease or other. However, with the advent of improved rearing technology, package of practices of mulberry cultivation and proper disease and pest management techniques, the cocoon production has increased in recent years inspite of this, the cocoon crop losses due to diseases in India accounts for more than 15 % loss in yield and 25-58 % in total disease incidence (Sharma *et al*., 2014). The most common source of pathogen for infection and stress over the disease during the rearing are the contaminated rearing trays and sheet papers (Ishikawa, 1958). Most of the damage to sericulture can be attributed directly to silkworm diseases, rather than to unfavorable weather conditions that lead to a poor harvest of mulberry leaves.

Therefore, prevention of silkworm diseases and breeding of silkworm varieties with high productivity are important problems in the commercial aspects of sericulture.

2.3.2 Grasserie disease in *Bombyx mori*

Among the silkworm diseases, which causes economic damages, viral diseases of silkworms are the most important. The viral disease of silkworm possesses a great threat to the silk industry that causes approximately 70 % loss of the crop every year. The viral diseases of the silkworm includes grasserie disease caused by nuclear polyhedrosis virus (NPV), cytoplasmic polyhedrosis virus (CPV), infectious flacherie caused by infectious flacherie virus (IFV), and densonucleosis caused by densonucleosis virus (Watanabe, 2002), kempu sappe or kenchu caused by kenchu virus (KV) a bidensovirus viral diseases of silkworm are of two types, inclusion and non-inclusion type.

The inclusion virus disease forms typical inclusion bodies which are nuclear polyhedrosis and cytoplasmic polyhedrosis that can be identified by ocular microscope. The non-inclusion type consists of infections like flacherie and densonucleosis that can be detected only by electron microscope (Vago and Atger, 1951). Grasserie disease in silkworm is caused by nuclear polyhedrosis and it is commonly known to the farmers by different vernacular/colloquial terms like, '*rasa*' in West Bengal, '*halu hula'* in Karnataka, '*polapurugu*' in Andhra Pradesh and '*palpoochhi*' in Tamil Nadu. The virus usually affects the late fourth instar larvae, but the damage may be initiated in the late third instar also. The infected larvae appear absolutely normal and feed as usual till they are close to death. About six or seven days after the infection the inter-segmental membranes are swollen and the worms appear to be under stress and exhibit restless behaviour. At this stage, various tissues like integument, fat bodies, alimentary systems, silk glands, pericardial cells are affected and the most important thing to observe is that the haemolymph turns turbid and milky due to multiplication of polyhedral bodies in multiple numbers (Chisti and Schaf, 1990). The integument loses its elasticity and become fragile and gets ruptured easily releasing the milky haemolymph. The onset of death from the time of swelling of intersegments is relatively rapid, usually ranging from a few hours to less than a day. The dead worms become soft and flabby. The nuclear

polyhedrosis virus multiplies in the nucleus of the infected cells of various tissues and gets crystallized on maturation into proteinaceous material forming polyhedra (Kobayashi *et al.,* 1981).

2.3.3 Symptomatology of *Bm*NPV disease

Diagnosis is one of the most important and one of the most complex branches of the study of disease incidence. It is fundamental to insect pathology, both basic and applied. The importance of diagnosis in insect pathology lies in the fact that one must know the nature of the disease and what ails or has killed an insect before the disease can be properly studied, controlled or suppressed.

The external symptoms of the *Bm*NPV disease of silkworm in the fourth and fifth instar larva, which are visible about a week after infection, includes the following (Fig. 1).

- ✓ Swelling of inter segmental areas
- ✓ Shining and yellowish body
- ✓ Hyperactivity
- ✓ Crawling around trays and hanging
- ✓ Rupturing of the skin
- ✓ White ooze filled with polyhedra
- ✓ Crawling around the periphery of the rearing tray
- ✓ Death of worms
- ✓ Rotting and secondary infections
- ✓ Nuclei of cells of various tissue contain polyhedral bodies

Epidermal cells and cells of all infected tissue become abnormal, If infection occurs in the early instars, the worms fail to spin the cocoons and die, whereas if the infection occurs at later stages, the worms could spin the cocoons but subsequently die inside producing melted cocoons, and the affected cocoons become unfit for reeling (Chitra *et al*., 1975; Gowda and Ramaiah 1976; Peter *et al*., 1999).

2.3.4 *Bm*NPV Disease Management

Several prophylactic management practices for Grasserie disease of silkworm have been evolved. Use of disinfectants like formalin and sodium hypochlorite, formalin, phenolic compounds, asiphor and bleaching powder, and sanitech are well documented for management of *Bm*NPV disease of silkworm.

Similarly, several bed disinfectants like Pafzol, Resham Keet Oushad (RKO) [captan- paraformaldehyde-benzoic acid and lime (1:1:2:96)] were found to be effective in reducing grasserie disease (Samson *et al,* 1998). Singh *et al*., (1999) reported that, alternate application of RKO and formalin chaff in sequence was more effective in prevention of *Bm*NPV under normal conditions. Vijeta is also being used as an effective disinfectant. Chemicals like bleaching powder, sodium hypochlorite, calcium hydroxide, trisodium monophosphate and oxidizing agents were used to suppress the occluded virus as they posed health hazards like dermal irritation, nausea and lachrymation. Some of the alkylating agents like formaldehyde were reported to be carcinogenic (Nataraju *et al.,* 2001). Chlorinating agents like bleaching powder is corrosive in nature and is unstable during storage. All these factors have necessitated identification of newer chemical disinfectants, and alternative strategies like Breeding of resistive silk worm breeds, enhancing of antiviral activity against NPV using transgenic approach are new arena for the management of the disease.

2.4.1 *Bombyx mori* nuclear polyhedrosis virus

Morphology of NPVs, either single or multiple envelopes do not appear to be crucial in taxonomy. For example, *Bombyx mori Sn*NPV (*Bm*SnNPV) and *Autographa californica* MnNPV (*Ac*MnNPV) are far more closely related to each other than they are to most other NPVs whether they are SnNPVs or MnNPVs (Hunter *et al*., 1998). The virus particle is composed of a protein shell that surrounds the nucleic acid. Within the capsid, the DNA is condensed into a nucleoprotein structure known as the core. The capsid plus the core are collectively referred to as the nucleocapsid.

A

B

C

D

(Deb, 2013)

Fig. 1: Symptoms exhibited by silkworm from Grasserie disease caused by BmNPV

A) Rupturing of the skin, shining and yellowish body

B) White ooze filled with polyhedra, swelling of inter segmental areas

C) Crawling around trays and hanging

D) Rotting and death of worms

Nucleocapsids contain a single molecule of circular super coiled double stranded DNA. The length of baculoviral DNA is between 80 and 200 kb. Nucleocapsids are made in the nucleus of infected cells and are subsequently enveloped by one of two processes. Nucleocapsid can bud through the plasma membrane of the infected cell, such budded virus particles are released into the extracellular fluid with a loosely fitting membrane envelope. Nucleocapsids may also acquire an envelope within the nucleus where they are produced. The envelope of virions embedded in 13 occlusion bodies (OBs) fits closely around the nucleocapsids, and additional nuclear material may be compressed between the membrane and the nucleocapsids during the occlusion process (Sugimori *et al*., 1990). Viral occlusion bodies are formed in the nucleus and comprise enveloped nucleocapsids embedded in a crystalline protein matrix. Polyhedral occlusion bodies of NPVs are known as polyhedra occlusion viruses or polyhedral inclusion bodies.

A characteristic feature of *Bm*NPV infection is the production of crystalline proteinaceous structures called occlusion bodies or polyhedra, in which several virions are embedded and thus protected from UV rays, desiccations, proteases and nucleases (Horton and Burand, 1993). *Bombyx mori* nuclear polyhedrosis virus (*Bm*NPV) infects the mulberry silkworm *Bombyx mori* and is of major economic concern to sericulture. This virus has also been exploited as a baculovirus expression vector and is next only to the most widely exploited *Autographa californica* multinucleocapsid nuclear polyhedrosis virus (*Ac*MNPV) in popularity (Acharya *et al*., 2002). It is a rod-shaped enveloped virion which contains a circular double-stranded DNA genome of 128.41 kb in size encoding 143 predicted open reading frames (ORFs) (Sehrawat *et al*., 2002).

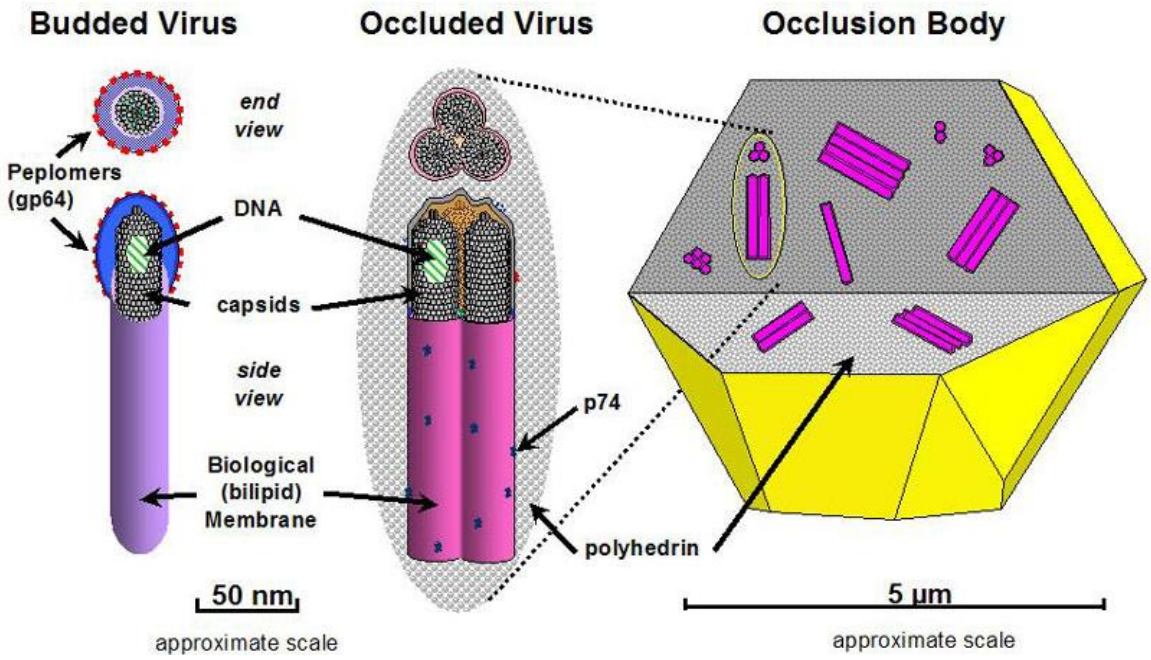
The nuclear polyhedrosis virus classified under the genus Nucleopolyhedrovirus, family *Baculoviridae*. The family *Baculoviridae* is classified by the basic characters as an enveloped, rod-shaped virion (approximately 50 × 250 micron) containing a circular double-stranded DNA genome ranging from 50 to 100 million daltons (Deb, 2013).

Baculoviridae comprises only two genera, nuclear polyhedrosis virus (NPVs) and granulosis virus (GVs). Baculovirus have only been found in over 600 species of

arthropod hosts. The majority of baculovirus hosts are within the order Lepidoptera (Hong *et al.*, 2000).

*Bm*NPV is a species affecting only the silkworm *Bombyx mori.* The virus particles require two phenotypically different but genetically identical virions (Fig. 2) to complete their life cycles, both forms have different role during pathogenesis (Engelhard and Volkman, 1995; Nakazawa *et al*., 2004). The virus particles are embedded in proteinaceous occlusions which are released into the larval midgut by the combined action of alkaline gut pH and proteases. Infection begins when the envelops of occlusion derived virus (ODV) fuse with the microvillar membrane of mature or differentiating columnar cells (Yao *et al*, 2006). Occluded viruses (OV) are present on plant surfaces (Miller, 1988; Maeda and Majima, 1990). They are involved in the horizontal transmission of virus. These are covered by a polyhedrin coat protein that helps to protect the embedded virions from inactivation during the interval of transmission between hosts. It helps in effective release of virions at primary site of infection in the midgut (Blissard and Rohrmann, 1990). Infected midgut cells produce virions primarily as single nucleocapsids through the basal plasma membrane. This budded virus (BV) thereby gains an envelope studded with GP-64, a viral encoded glycoprotein which is important for the infection of neighboring host cells and tissues (Monsma *etal*., 1996). Cells of the insect tracheal system are the important targets of BV, and their infection is critical for the rapid spread of the virus because they provide access to larval tissues surrounded by basal laminar barriers (Yao *et al*, 2006). By the end of the infection cycle, the body of the lepidopteron larvae is liquefied and transformed into millions of new occlusion bodies that spread into the surrounding environment.

Some tissues, however, escape infection. *Bm*NPV does not infect the silk gland cells of *B. mori* L., which constitute a major component of the larval body. The BVs enter the cell through receptor-mediated endocytosis. Following penetration of the plasma membrane, the nucleocapsid moves towards the cell nucleus by a process that requires actin micro- filaments. At the nucleus, the viral nucleocapsid is uncoated and the DNA gets released. The nucleus becomes enlarged and a distinct electron dense granular structure, called virogenic stroma is formed. This structure gets associated with nuclear



http://silkpathdb.swu.edu.cn/virus_bmnpv

Fig. 2: Structure of BmNPV virus representing two types of virions.

matrix and forms the site of nucleocapsid assembly. Viral transcription and replication take place in this region (Sriram, *et al*., 1997, Sehgal and Gopinathan, 1998).

During baculovirus infection, more than hundred viral genes are expressed in a cascade that can be divided into four phases, viz., immediate early, delayed early, late and very late (Krappa *et al*., 1992). Immediate early genes are required for infection, delayed early for replication of the virus and manipulation of the host, late genes for nucleocapsid synthesis and very late for polyhedrin synthesis (Lu *et al*., 1998). Expression of immediate early viral genes is driven by host cellular factors, while delayed-early genes are transcribed in the presence of cellular factors (Krappa and Mordorf, 1991).

2.4.2 Structure of polyhedra

The polyhedra of insect NPVs are generally dodecahedral, tetrahedral, cubical or irregular in shape whereas the polyhedra of *Bm*NPV are dodecahedral in shape. Mutants with irregular polyhedral inclusion bodies (PIBs) have also been reported. Variants with defects in the *de-novo* synthesis of envelope and formation of polyhedra devoid of virions and variants with no PIBs producing normal progeny are also found (Noguchi, 1991). Two polyhedron deficient mutants number 126 and 136 produced small-uncrystallized particles of polyhedrin in the nuclei, number 211 produced large number of variable sized polyhedra, number 220 produced large cuboidal polyhedra, and mutant number 24 did not produce polyhedrin as reported in a review (Peter *et al.*, 1999).

The polyhedra are roughly spherical and usually hexagonal in shape, measuring 164-211μm. In a study Palhan and Gopinathan. (1996), found that Bangalore isolate of *Bm*NPV (*Bm*NPV-BGL) measures 2μm in length and 0.5 μm in width whereas Hayakawa *et al.* (2000), have reported a size of 5-15μm.

The polyhedral proteins of *B. mori* L. and *Galleria mellonella* NPV obtained by dissolving inclusion bodies in acetic acid showed only one main component with molecular weight of about 29 kd. In *A. californica* only one polypeptide with a molecular

weight of 28-29 kd protein was observed in SDS-PAGE irrespective of the origin and the procedure applied (Maskos and Miltenburger, 1981).

2.4.3 *Bm*NPV genome

Palhan and Gopinathan. (1996), reported a size of 132kb for the genome of *Bm*NPV. The T_3 strain of the *Bm*NPV is 130kb nucleotides long, with a G+C content of 40% and 143 open reading frames (ORFs). The baculovirus was originally described when silkworm cultures were threatened by a disease indicated as jaundice (Yang and Zhang, 2012), and the isolation and chemical composition of the virus resulted in the new era of the baculovirus research. Baculoviruses contains large double-stranded circular DNA genomes of 80-180 kbp. The *Baculoviridae* family can be divided into four genera according to common biological and structural characteristics: *Alphabaculovirus*, which includes lepidopteran specific baculoviruses and is subdivided into Group I or Group II based on the type of fusogenic protein, *Betabaculovirus*, comprising lepidopteran-specific granuloviruses, *Gammabaculovirus*, which includes hymenopteran-specific baculoviruses, and *Deltabaculovirus*, which, to date, comprises only *Cuni*NPV (Miele *et al.*, 2011).

There are currently 54 complete baculovirus genomes deposited in GenBank (Yang and Zhang, 2012) reported. These include 37 *Alphabaculoviruses*, 13 *Betabaculoviruses*, 3 *Gammabaculoviruses*, and 1 *Deltabaculovirus*. Despite the diversity in gene content present in different baculovirus genomes, there are now 31 core genes conserved in all sequenced baculovirus genomes, the function of these genes can be divided into replication, transcription, packaging and assembly, cell cycle arrest, and oral infectivity. Most of the data comes from the *Ac*MNPV, as it is the model type for baculovirus and has been extensively studied. Four core genes have been identified as essential players in viral DNA replication those are *DNApol, Helicase, Lef-1* and *Lef-2*. Nearly half of the core genes are involved in the packaging (Table 1) and assembly of *Bm*NPV (Yang and Zhang, 2012).

Xue *et al*., (2012) reported that *Bm*NPV (T3 strain) genome has a size of 12, 843 bp with a G+C content of 40 % and encoding 142 predicated open reading frames (136 of

the ORFs encoding predicted proteins of over 60 amino acids). *Bm*NPV strain (S1) isolated from the wild silkworm (*Bombyx mandarina*) was also sequenced. The complete nucleotide sequence of the S1 strain was compared with the T3 strain. The S1 strain was 126,770 nucleotides long, with a G+C content of 40.23 %. The genome contained 133 potential ORFs encoding predicted proteins of over 60 amino acids. The *Bm*NPV genome was closely related to that of AcMNPV, sharing over 90 % similarity to about three-quarters of the genome of *Ac*MNPV and the relatedness of predicted amino acid sequences of corresponding ORFs between *Bm*NPV and *Ac*MNPV was about 90 % identical.

The first NPV genome to be sequenced was that of *Ac*NPV. Presently 12 NPVs have been sequenced viz., *A. californica* NPV (*Ac*MNPV), *B. mori* L. NPV (*Bm*NPV), *Epiphyas postvittana* NPV (*Eppo*NPV), *Orygyia pseudotsugata* NPV (*Op*MNPV), *Helicoverpa armigera* NPV (*Ha*SNPV), *Heliothus zea* NPV (*HzS*NPV), *Spodoptera litura* NPV (*Spli*NPV), *S. exigua* NPV (*Se*NPV), *Lymantria dispar* NPV (*Ld*NPV), *Cydia pomonella* NPV (*Cp*NPV), *Plutella xylostella* NPV (*Pn*NPV), *Xestiac-nigrum* NPV (*Xc*NPV) and *Culex nigripalpus* NPV (*Cun*iNPV). These NPVs have common 30 identified genes. A putative function can be assigned to 20 of these identified genes. They include genes that act at various stages of the baculovirus infection cycle and can be grouped into five functional categories: RNA transcription, DNA replication, structural proteins, auxillary proteins and proteins of unknown function (Herniou *et al.*, 2003).

2.5 Baculovirus phylogeny and evolution:

Grouping of baculoviruses based on comparison of individual gene sequences like the polyhedrin / granulin gene (pol h) and other genes such as DNA polymerase (dpo I), egt, gp 41, chitinase, cathepsin, lef 2 and gp 37 have led to conflicting conclusions as a result entire genomes can be used to reconstruct baculovirus phylogenetics (Herniou *et al.*, 2003).

There are two potential approaches in grouping these viruses: i) to analyze each gene separately and to derive a consensus from the resulting phylogenies or ii) to concatenate the genes and analyze them together. In principle, the second approach gives

Table 1: Functions of core genes of *Bm*NPV and their position

Name	ORF No. in *Bm*NPV	Localization	Function
Lef-1	6	nucleosome	DNA primase
DNApol	53	nucleosome	DNA polymerase
Helicase	78	nucleosome	DNA helicase
Lef-2	135	cell nuclei	Bind to both DNA and LEF- 1; activate late transcription
P47	31	nuclei	RNA polymerase subunit
Lef-8	39	Nuclei	RNA polymerase subunit
Lef-9	50	cell nuclei	RNA polymerase subunit
Lef-4	73	cell nuclei	RNA polymerase subunit
Lef-5	83	cell nuclei	initiation factor
VP1054	43	nucleosome	structural protein; nucleo- capsid assembly
Vlf-1	63	nucleosome	lambda integrase; nucleo- capsid assembly
GP41	66	tegument	ODV protein; BV production
ODV -NC42	56	capsid	ODV protein; Assemble nucleocapsid into ODV
VP91/P95	69	envelope	Capsid-associated protein
VP39	72	capsid	major capsid protein
P33	75	nonstructural	sulfhydryloxidase; BV/ODV associated protein
38K	82	nucleosome	Nucleocapsid assembly
P6.9	84	capsid	DNA binding
ODV-EC43	92	capsid	BV/ODV Protein
Alk-exo	110	nucleosome	5'-3'exonulcease
P49	118	capsid	Nucleocapsid assembly
ODV-E18	119	envelope	BV/ODV Protein; BV production
desmoplakin	54		BV production
ODV-EC27	120	tegument	ODV associated protein; cyclin
Pif-2	13	envelope	mediate binding in midgut
OEV-E28/19K/Pif4	79	envelope	primary oral infection
Pif-3	95	envelope	form a complex with PIF-1 and PIF-2
Pif-1	97	envelope	mediate binding in midgut
Pif-0/P74	115	envelope	mediate binding in midgut
Pif5/ODV-E56	124	envelope	primary oral infection

(Yang and Zhang, 2012)

better results because each gene contributes to the overall phylogenetic signal and a synergistic effect is produced by combination of all the signals (Herniou *et al.*, 2003).

2.5.1 Diversity studies of nuclear polyhedrosis viruses (NPVs) using molecular markers

Genotypic variation in baculovirus populations *Heliothis armigera* MNPV, *H. armigera* SNPV, *H. zea* SNPV, *Spodoptera litura* and *S. frugipeda* has been detected at several ecological scales, from between geographical regions (Gettig and McCarthy, 1982) to within virus isolates (Maeda and Majima, 1990). Differences in pathogenicity have also been demonstrated between geographical isolates and within single agricultural fields (Summers and Smith, 1978; Cooper *et al.,* 2003) and between genotypes derived from the same isolate by *in vitro* techniques (Stiles and Himmerich, 1998). Very recently baculovirus genotypes derived from a single isolate, have been studied using *in vitro* techniques and have been compared. Despite this, the evolutionary and ecological processes leading to the existence and persistence of genotypic variation in baculovirus populations have received little attention (Cory *et al.,* 1997). This level of variation has led scientists to understand the influence of various ecological and other factors underlying this polymorphism between strains using various molecular tools.

Several molecular markers are available to study the genetic diversity of organisms. These include Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Simple Sequence Repeats (SSRs), Sequence Tagged Sites (STS), Single Nucleotide Polymorphism (SNP), Amplified Fragment Length Polymorphism (AFLP) and many other markers. RFLP has been used extensively to study polymorphism of NPVs. Information on AFLP analysis of NPVs is very limited, however it has been used to study diversity of fungi and bacteria.

2.5.2 Restriction Endonucleases Analysis (REN) of NPVs

2.5.2.1 Insect NPVs

A restriction endonucleases analysis of three NPVs, viz., *Ac*NPV, *Op*NPV and *Hz*NPV using *EcoRI, SalI, BamHI, HindIII, HaeIII, HhaI* and *PstI* revealed characteristic

patterns, which could probably be used in their identification. These NPVs have been used as biopesticide formulations and can recombine with each other, 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).

Variation has been reported in the survival time among isolates of *Heliothis* NPVs. Variation among NPVs also has been documented in terms of virulence and host range (Bilimoria, 1983). Sub molar fragments have been detected in restriction digests of a wild isolate of AcNPV resulting in the isolation of several variants of the particular isolate (Summers and Smith, 1978; Lee and Miller, 1978). The genomes of MNPV from *A. californica, Tricoplusia ni, Rachiplusia ou* and *Gallaria mellonella* have similar restriction fragmentation profiles (Smith and Summers, 1979; 1980).

Cooper *et al.,* (2003) have studied the hierarchial spatial structure of genetically variable NPVs infecting cyclic population of western tent caterpillars *Malacosoma californicum pluviale* NPV (*Mc*plNPV) over a period of three years (1998-2000). The sampling was done in southwestern British Columbia and western Cypress. The extent of genotypic variation was examined using *Hind III*, the study showed that considerable genetic variation was present within the McplNPV wild type populations and that this variation is structured at different spatial scales: this was the first study to analyze baculovirus populations in the field in such detail. Fourteen dominant genetic variants were identified in 48 *Mc*plNPV isolates from individual host larvae examined across 3 years of declining and low density host populations.

Kumar *et al*., (2015), analyzed polyphagous insect pest *Spilarctia obliqua* in which they observed an epizootic caused by a Nucleopolyhedrovirus (NPV) in field populations of the insects. The NPV was purified and characterized. The isolated NPV was tetrahedral in shape and belonged to multicapsid NPV. Using REN profile of the *Spo*NPV genome with *Pst I, Xho I and Hind III* enzymes they showed a genome size of 99.1 ± 3.9 kbp of *Spo*NPV.

Avile *et al*., (2017), studied the four multiple nucleocapsid nucleopolyhedrovirus isolates recovered from infected larvae of beet armyworm *Spodoptera exigua* on crops in two different geographical regions of Mexico. Molecular characterization was compared with characterized *S. exigua* multiple nucleopolyhedrovirus (*Se*MNPV), isolates from the United States (*Se*US1 and *Se*US2) and Spain (*Se*SP2). Digestion with *Bgl II* resulted in a characteristic DNA profile for the Mexican isolates (SeSIN6, SeSIN8, SeSLP6, and SeSLP8), as indicated by the presence of the marker fragment (2.5 kb, absent in the corresponding profile of the reference isolates SeSP2 and SeUS2. Moreover, SeSP2-BglII isolate showed two *Bgl II* fragments of 1.5 and 2.4 kb that were absent in the Mexican isolates, whereas SeUS2-BglII presented a marker fragment of 9.5 kb that was absent in the Mexican isolates. When compared with the SeUS1 isolate the Mexican isolate *Bgl II* profiles presented a more similar pattern of bands, and a number of sub molar bands. Overall, Restriction endonuclease analysis of viral DNA confirmed that all Mexican isolates were contained a number of sub molar bands indicating that the isolates comprised genotypic variants in different proportions.

2.4.2.2.1 Molecular marker analysis of *Bm*NPVs

2.4.2.2.1 REN analysis of *Bm*NPVs

Palhan and Gopinathan (1996) constructed a genetic map of the *Bm*NPV (BGL), which was similar to *Bm*NPV T_3 isolate, but the *Eco*RI and *Hind III* digestions showed some polymorphic sites. This genetic map was then compared with the map of *Bm*NPV (Dharwad isolate). Restriction site polymorphism was observed between the two, especially for the Hind-III digest. A single base pair substitution or deletion in the recognition sequence of the restriction enzyme can lead to these changes and are commonly encountered in large virus samples isolated from infected animals.

Genetically distinct four isolates of *Bm*NPVs, from a sericultural Korean farm *Bm*NPV- K1, K2, K3 and K4 were purified and characterized by their DNA restriction pattern, virus replication, polyhedra production and gene structures. The *EcoRI* and *Sal I* fragments showed similar overall patterns with minor differences but distinguishable patterns in each isolate. The wild isolate is probably a mixture of four or more closely

related genotypic mutants. There was no significant difference in the virus replication pattern, yield of total polyhedra production and polyhedra morphology, but the yield of released polyhedra by *Bm*NPV- K1 in *Bm*5 cells was 2 to 5 times higher than that of other isolates (Hong *et al.*, 2000).

2.4.2.2 AFLP analysis

Peter *et al*., (2016) characterized five *Bm*NPV isolates by using Amplified fragment length polymorphism (AFLP). From four AFLP primer combinations a total of 235 bands were scored and 202 (86%) were polymorphic with an average of 50 polymorphic bands per primer combination was observed. Cluster analysis based on AFLP markers showed concordant relationships among the isolates in relation to geographical origin with high bootstrap values. The Survey of unique AFLP bands across five *Bm*NPV isolates revealed correlation between number of unique bands noticed for each isolate and their existing genetic relationships.

2.5.1 Insect immunity against pathogens

Management of *Bm*NPV by various methods such as usage of chemicals like alkylating agent like formaldehyde, Chlorinating agents, like bleaching powder is corrosive in nature and is unstable during storage (Nataraju *et al.,* 2001).. All these factors have necessitated identification of newer chemical disinfectants, and alternative strategies like Breeding of resistive silk worm breeds, enhancing of antiviral activity against NPV using transgenic approach are new arena for the management of the disease.

Insects are the earliest and most diverse taxon of animals accounting for more species than all other animals put together because of their high reproductive potential and varied niche. Though cellular reactions like phagocytosis, nodulation and encapsulation and humoral reaction through secretion of antibacterial proteins are known for fungus and bacterial invaders, little is known about insect defense against insect virus infection (Lavine and Strand, 2002).

Insect immunity plays an important role in the interaction between the host and pathogen as a part of survival strategy including physical blockades such as cuticle and

peritrophic matrix, epithelial barriers, protease cascades leading to coagulation and melanization, cellular responses such as phagocytosis and encapsulation and also the production of certain antimicrobial peptides (Yao *et al.,* 2006). The first step in any immune response is the recognition of an invading organism as foreign. Once such recognition has taken place, a protective response involving blood cells or soluble plasma proteins (Yu *et al.,* 2002) may be triggered.

Lepidopteron larvae resist baculovirus infection by selective apoptosis of infected cells from the midgut epithelial cells and by sloughing off infected cells from the midgut cells. Even then the virions can successfully enter the host body (Yao *et al,* 2006). The mechanism by which the insect resists viral infections include, recognition of infected cells and recruitment of immune cells to the infective foci or clearing of infected cells is poorly understood (Popham *et al*, 2004). Insects seemingly lack any adaptive immune responses that operate analogously to the well documented antibody or histocompatibility adaptive immune responses as in vertebrates (Hoffmann, 2003).

Apoptosis or programmed cell death is one of the phenomena evolved by certain vertebrates and invertebrates lacking humoral immunity to function as antiviral defense mechanism (Narayan, 2004). This mechanism is a controlled biochemical pathway distinguishable from cell necrosis by characteristics that include cellular shrinkage, membrane blebbing, chromatin condensation, apoptotic body formation and fragmentation (Wyllie *et al*, 1980).

2.5.2 Proteins involved in antiviral mechanisms of silkworm against *Bm*NPV

The primary/major route of infection is through the food, there must be some antiviral mechanism/substances existing in the gut juice of the caterpillar. The presence of such antiviral substances was isolated and identified from the haemolymph and intestinal fluids of silkworm by some of the earlier workers (Funakoshi and Aizawa, 1989).

Hayashiya and Nishida (1968; 1976) and Hayashiya and Matsubara (1971) reported the red fluorescent proteins (RFPs) could inactivate the *Bm*NPV. RFP, is a

conjugated protein bearing a chromophore, its physiological activities revealed that it had antiviral effect against *Bm*NPV and Galleria NPV. It was observed that silkworm larvae reared continuously in the dark are more susceptible to peroral infection with NPV than those reared continuously in environment with light. The increased susceptibility may be due to absence or lower production of RFP in the larvae reared in the dark. Although the exact mechanism of the antiviral action of RFP is still unknown, it is believed that it destroys the nucleocapsid of NPV or blocks the multiplication of NPV or agglutinates the virus and is excreted along with faeces (Yao *et al*., 2006).

A number of studies have been conducted on insect resistance to NPV. *Bombyx mori serine protease*-2, *lipase*-1, and alkaline trypsin protein purified from the digestive juice of *B.mori* larvae showed strong antiviral activity to *Bm*NPV *in vitro* (Ponnuvel *et al*., 2003; Nakazawa *et al*., 2004; Ponnuvel *et al*., 2012). Soluble *B. mori* NADPH oxidoreductase (*Bm*Nox) isolated from silkworm larval gut juice was also confirmed to have antiviral activity to *Bm*NPV (Selot *et al*., 2007; Selot *et al*., 2010). Recently, techniques such as differential display, cDNA microarray assay, and two-dimensional gel electrophoresis (2-DE), have become routine to examine changes in gene expression. Using the fluorescent differential display (FDD) technique, *Bmsop2* and *Bms3a* were identified to be related to *Bm*NPV resistance in silkworm (Xu *et al*., 2005; Xu *et al*., 2008). A gene encoding arginine kinase in *B. mori* has been identified differentially expressed in the midgut of *B. mori* resistant and susceptible strains to *Bm*NPV by two-dimensional gel electrophoresis (Kang *et al*., 2011).

NPV infection alters cellular events such as the formation of actin cables (Roncarati and Knebel-Morsdorf, 1997) and the arrest of the host cell cycle at the G2/M transition phase (Axen *et al*., 1997). Furthermore, viral infection causes a global shutoff of host protein synthesis and transcription at 12–18 hrs after infection in the late phase of virus replication (Nobiron *et al*., 2003). However, this global shutoff mechanism is still unknown. In contrast, some particular genes were reported to escape the global shutoff. Quadt *et al*. (2002) reported that TATA binding protein dramatically increased during the late phase of infection. Genes encoding cytochrome oxidase I, III, ADP/ATP translocase, and the lepidopteran specific recognition protein, hemolin, are also believed to not be

affected by the global shutoff (Hirai *et al*., 2004). In addition, some genes such as heat shock protein (Hsp) 70 cognate (Hsc70) have been shown to be up-regulated in the early stage of NPV infection (Nobiron *et al*., 2003). Iwanaga *et al*. (2007) showed that the level of general mRNA content in infected-silkworm cells slightly increased 2–6 hours after *Bm*NPV infection, suggesting some genes may be differentially regulated during the early phase of viral infection. Moreover, they identified 7 up-regulated and 4 down-regulated genes in the early phase of infection by subtractive hybridization and Northern blot analysis (Iwanaga *et al*., 2007).

The genome-wide host gene expression profile in response to NPV infection has not yet been analyzed. Recently, a nearly complete genome sequence from *Bombyx mori* has been reported for the first lepidopteran insect which is available at http://kaikoblast.dna.affrc.go.jp/ and a silkworm cDNA database has also been generated (http://kaikocdna.dna.affrc.go.jp/). A 44K oligonucleotide microarray containing 34,631 oligonucleotide probes derived from the silkworm expression sequence tag (EST) database was available to analyze the global expression profile of the silkworm. Therefore, genome-wide analysis and post-genome studies using the silkworm have a great advantage over other lepidopteran insects (Sagisaka *et al.*, 2010).

Silkworm strains are identified to be highly resistant to *Bm*NPV. To explore the silkworm genes involved in this resistance, Lu *et al.* (2013) performed comparative real time PCR, ATPase assay, over-expression and sub-cellular localization experiments. It was found that when inoculated with *Bm*NPV both the expression and activity of V-ATPase were significantly up-regulated in the midgut columnar cells of *Bm*NPV-resistant strains (NB and BC8), the main sites for the first step of *Bm*NPV invasion, but not in those of a *Bm*NPV-susceptible strains. Furthermore, this up-regulation mainly took place during the first 24 hours post inoculation (hpi), the essential period required for establishment of virus infection, and then was down-regulated to normal levels. Transient over expression of V-ATPase c subunit in *Bm*NPV-infected silkworm cells could significantly inhibit *Bm*NPV proliferation. By this it was demonstrated clearly that V-ATPase is indeed involved in the defense response against *Bm*NPV. V-ATPase functions

as fast acidification of endosomes and/or lysosomes to render them competent for degradation of invading viruses.

26.5 kDa protein named *Bm*Nox in the gut fluid of Nistari strain of *Bombyx mori* was identified which possessed antiviral activity against *Bm*NPV *in-vitro*. Selot and Kumar. (2010) reported that, the characterization of the full-length gene encoding *Bm*NOX and the levels of expression of this gene in selected tissues of silkworm larvae from a *Bm*NPV susceptible and a *Bm*NPV resistant strain to the defense capability in *Bombyx mori* larvae challenged with *Bm*NPV. Nistari, a multivoltine strain of silkworm, expressed *Bm*NOX during all five larval stages, and were highly resistant to *Bm*NPV infection. In sharp contrast, CSR2, a Bivoltine strain, showed weaker expression of *Bm*NOX in the anterior midgut in larval life and was highly susceptible to *Bm*NPV infection. *Bm*Nox over-expressed in the silkworm gut fluid of disease resistant multivoltine races rendered them NPV resistant (Selot *et al*., 2007).

2.5.2.1 *Bombyx mori lipase* (*Bmlipase*) an antiviral protein

Yao *et al*, (2006) reported that *lipases* are likely to contribute immune defenses, conceivably acting directly against invading microorganisms. *Bmlipase-1*, a *lipase* purified from the digestive juice of *B. mori* larvae proved to have a strong antiviral activity against *Bm*NPV and showed 56% homology with *Drosophila melanogaster lipase* and 21% homology with human *lipase*. It has also been confirmed that the *Bmlipase*-1 gene is expressed only in the midgut tissue, but not in other tissues. When the fifth instar larvae of silkworm were orally inoculated with pre-treated *Bm*NPV-ODV (ODV incubated with *Bmlipase*); the larvae showed resistance to viral infection and successfully entered the pupal stage, thereby indicating the suppression of viral proliferation by midgut *Bmlipase*1 (Ponnuvel *et al*., 2003).

Bmlipase-1 gene regulation is unique as gene expression of this enzyme is not unregulated by viral infection but seems to be hormonally regulated, suggesting that the main role of the enzyme is food digestion. However, *Bmlipase*-1 is also involved in primary defense against viral infection to protect *B. mori* midgut epithelial cells from

ODV at the initial infection stage. The antiviral mechanisms of *Bmlipase*-1 which suppress *Bm*NPV replication remain unclear (Jiang and Xia, 2014).

It was reported that over expressing the *Bmlipase-1* gene in silkworm reduced the mortality of the transgenic line by approximately 33% compared to the non-transgenic line when the virus dose was 106 OB/larva (Jiang *et al,* 2012). Cheng *et al*, (2014) reported that relative expression level of *Bmlipase-1* in resistant strains increased significantly compared to the susceptible strain in the larval haemolymph and midgut. Du *et al*. (2012) identified four *lipase*s directly involved in PBAN (Pheromone biosynthesis activating neuropeptide) stimulated sex pheromone biosynthesis in *Bombyx mori* using the DGE and RNAi approaches, but there is no evidence demonstrating the relevance between PBAN and viral resistance in insects (Cheng *et al*., 2014). Expression of *Bmlipase-1* gene was found only in the midgut, but not in other tissues, nor is it activated by *Bm*NPV infection. In addition, the *Bmlipase*-1 gene was shown not to be expressed in the molting and wandering stages, indicating that the gene is hormonally regulated (Cheng *et al*, 2014).

2.5.2.2 *Bombyx mori serine protease* (*BmSP*) an antiviral protein

*Serine protease*s are among the group of proteins that regulate several invertebrate defense responses including haemolymph coagulation, antimicrobial peptide synthesis and melanization of pathogen surfaces (Gorman and Paskewitz, 2001). Nakazawa *et al*. (2004), showed that the presence of *serine protease* in the digestive juice of silkworm larvae has strong antiviral activity against *Bm*NPV. The molecular mass and partial N-terminal sequence was determined and cDNA showed 94% homology with *B. mori serine protease*.

The *serine protease* had an isoelectric point of pH 10–11 and the pH optimum for degradation activity of sucinyl-Leu-Val-Tyr was about 10 (Kotani *et al*., 1999). The pH optimum of *BmSP*-2 was also determined to be about 11 showing this enzyme is a highly basic protease. A sequence comparison of *BmSP*-2 with other proteases indicated that *BmSP*-2 is a mammalian-type *serine protease* with a catalytic triad composed of His 45, Asp 92, and Ser 186. A large number of Arg may contribute to the stability and function

of *BmSP*-2 by remaining charged at a high pH as suggested by Kotani *et al*. (1999). Nakazawa *et al*. (2004) isolated and studied pure *BmSP*-2, it showed that a larva contains approximately 0.58 µg *BmSP*-2. The LD50 of ODV was determined to be 12.0 ng per larva. The LD50 was also confirmed by a mortality of 100% in *B. mori* larvae infected with 22.5 ng ODV. The examination of effect of *BmSP*-2 concentration on a fixed amount of ODV (860 ng per larva) showed that ODV treated with more than 3.89 µg of *BmSP*-2/larva cannot propagate at all. These data suggested that the physiological concentration of *BmSP*-2 cannot protect against infection with 22.5 ng of ODV, although the same amount of ODV can be completely inactivated by 2.2 µg of *BmSP*-2. The results obtained by Nakazawa *et al*, (2004) indicated that *Bm-serine protease*-2 was a midgut-specific protease and could significantly reduce the infectivity of *Bm*NPV.

Li *et al*., 2017 reported that *Bm-SP*142 is a 35 kDa protease in the silkworm, but its exact functions remain unknown. Recombinant *Bm*NPV treated with purified *Bm-SP*142 effectively impaired its ability to infect BmN cells, and Bm-SP142 decreases the efficiency of *Bm*NPV and *Bm*BDV propagation in silkworms. Furthermore, overexpression of *Bm-SP142* in BmN cells inhibited viral propagation.

The haemolymph of mosquito, *Anopheles gambiae* also contains five *serine proteases*, which play an important role in insect immunity (Gorman and Paskewitz, 2001). Among them sp22D was found to be the largest protease with potential pathogen binding domains that is expressed in the midgut epithelium, fat body and haemocytes. sp14A, sp14D1 and sp14D2 are clip domain *serine proteases* that are similar to enzymes with presumed roles in melanization (Yao *et al*., 2006).

2.6 Resistance and susceptible breeds of silkworm

Different races of silkworm exhibit marked variation in their susceptibility to various diseases Watanabe. (1967). India has a rich silkworm germplasm with promising economic and breeding characters. The utilization of disease resistant varieties is the most economical and effective way in earning better cocoon yield. Larval susceptibility to viral disease greatly differed in various breeds/ hybrids. Researchers have reported that, interbreed/ strain differences were noticed in susceptibility to *Bm*NPV (Aratake, Y.,

1971). The resistance in silkworm breeds has physiological and genetic origins that are controlled by polygenes.

Preliminary screening of multivoltine and bivoltine hybrids for their comparative rearing performance (Tikoo *et al.,* 1971) and pure races (Krishnaswamy and Tikoo, 1971) was conducted. The differential responses of few breeds to the infections of silkworm pathogens have been recorded (Chinnaswamy and Devaiah, 1984). Pure Mysore and Nistari breeds have been observed to be less susceptible to *Bm*NPV (Baig, *et al.,* 1991). Baig *et. al.,* (1998) screened 21 races of silkworm including pure and hybrid races for their relative susceptibility to NPV under natural and induced conditions. There was also attempt to the induction of resistance to *Bm*NPV into susceptible silkworm breeds.

Baig *et al*., (1998) confirmed wide variation in resistance, both among the multivoltine and bivoltine breeds, on challenging with uniform dose of *Bm*NPV inoculum. Among the multivoltine breeds screened Sarupat, Nistari, Madagaskar, Pure Mysore, BL 37, BL 36, 96C, Moria, MAR, Mysore Princess and MR136 were comparatively more resistant than other breeds and differed from each other in resistance to nuclear polyhedrosis. Similarly among the bivoltine breeds, CSR19, S-1, 5- HT, D-13, 61N, D-7, CSR4, D-15, CSR2(SL), 8HT, CSR2, CSR-18 and JPN8, were comparatively more resistant than other breeds and differed from each other in resistance to nuclear polyhedrosis.

Asha and Bhaskar. (2011) studied that infection during fourth and fifth instar of silk worm caused more ET50 value for symptom expression and less mortality in both the instars of Pure Mysore breed. It was also noted that Pure Mysore breed took more time (7.67 and 8.33 days) for first symptom expression. Among hybrids, PMxCSR16 has recorded highest ET50 value (5.67 and 6.67 days). The susceptible hybrids viz., CSR2xCSR4 and CSR2xCSR16 have reported 100 percent total larval mortality indicating their high sensitiveness to the disease while the hybrids involving Pure Mysore multivoltine breed as maternal parent exhibited more ET50 value.

2.6.1 Differential expression of *Bm*NPV antiviral genes in silkworm resistant and susceptible breeds

In order to gain a better understanding of the mechanisms that underlie resistance to *BmNPV* in *B. mori* immune-related cells, Bao *et al*, (2010) investigated the infection profiles of *Bm*NPV in the larval fat body and haemocyte of the susceptible and resistant *B. mori* strains by directly injecting the BV virion into *B. mori* larval haemocoel. Results revealed that *Bm*NPV invaded the fat body and haemocyte of both strains and also noted slow viral proliferation in fat body and haemocyte of the resistant strain. Genes with potential antiviral activities were found by comparing *Bm*NPV responsive genes in the larval fat body of *B. mori* resistant (KN) and susceptible strain 306 by suppression subtractive hybridization (Diatchenko *et al.*, 1996).

Real-time quantitative PCR (qPCR) analysis confirmed that several genes were significantly upregulated following *Bm*NPV infection in the larval fat body and haemocyte of the resistant KN strain. Genes in the larval midgut of the resistant strain and a large number of additional *Bm*NPV-activated genes with oral route of entry, the midgut was the defensive barrier against *Bm*NPV infection, whereas fat body and haemocyte indirectly responded to viral infection. Comparison of *Bm*NPV responsive genes in immune-related cells of resistant and susceptible *B. mori* strains indicated potential candidate genes, which may have important functions in aspects of resistance against viral infection (Bao *et al*., 2010)**.**

Bao *et al*. (2010) constructed two complementary fat body cDNA libraries of resistant and susceptible breeds of silkworm against *Bm*NPV, and conferred that gene with high abundance in the R library but low abundance or absence from the S library. Of the 96 functional genes detected in the two libraries, transcripts for ribosomal protein and protein synthesis related genes showed the greatest abundance in both S and R libraries (25.7 and 17.9%, respectively), indicating that these genes are most likely commonly expressed in both *B. mori* strains. Transcripts for the genes encoding intermediate synthesis and catabolism enzymes as well as protein degradation and processing enzymes were highly abundant in the S library, comprising a total of 28.6% of the clones. In

contrast, some genes only comprised 8.3% in the R library, suggesting that these genes may not be highly expressed in *B. mori* resistant larvae. Immune-related gene transcripts, such as defense, stress response, proteases and protease inhibitor gene groups showed a relatively high abundance in the R library, comprising a total of 27.1% of the clones and suggested that immune related genes may have potential roles in *B. mori* antiviral infection mechanisms.

III MATERIAL AND METHODS

In the present study, molecular characterization of *Bm*NPV isolates collected from different locations of silkworm growing areas as done for diversity analysis. Isolation, cloning and sequence analysis of the antiviral encoding protein genes *serine protease*/ *lipase* was done. All the research activities pertaining to the "Molecular characterization of *Bm*NPV and cloning of *Bm*NPV antiviral encoding protein gene *serine protease*/ *lipase*" was carried out in the Department of Plant Biotechnology, University of Agricultural Sciences, GKVK, Bengaluru-560065 during the year 2016-2017. The materials and methods adopted for these studies are presented in this chapter.

3.1 Experimental materials

3.1.1 Biochemicals

The molecular biology bio-chemicals used in the study were of either molecular biology or analytical grade.

3.1.2 Chemicals

Ethidium bromide, PCR-primers, Isopropanol, DEPC-H_2O etc., were from Sigma Chemicals Co., St. Louis. DNA ladder and rulers, Chloroform, Phenol, Isoamyl alcohol, Agarose, Sodium carbonate, Sodium dodecyl sulphate, Sodium chloride, EDTA, Sucrose, *Taq polymerase*, *Taq* buffer, Nuclease free water, $MgCl_2$, dNTP, Ribonuclease inhibitor, SDS, β-Mercaptoethanol, Glycerol, Bromophenol blue, Ethanol were from Bangalore Genei or HiMedia..

3.1.3 Glass ware and plastic ware

All the glassware used in the study was from Borosil India Ltd. They were thoroughly washed and sterilized as per standard procedure. Micro-centrifuge tubes and micropipette tips were from Tarsons (India).

3.1.4 Other chemicals and consumables

Other chemicals of analytical grade were either from Glaxo Laboratories India Ltd. or E. Merck India Ltd.

3.1.5 Media and buffer

Composition of media, buffers and solutions used in this study are given in Appendix I, II and at appropriate places.

3.1.6 Equipment

- Refrigerated micro Centrifuge, MPW, Poland.
- Refrigerated high speed centrifuge, Sorvall RC5C, Dupont, USA.
- Single pan digital balance, Mettler, AK-100, Switzerland.
- Bio-Rad Power pack, Bio-Rad, India.
- Water bath, Heto, Denmark.
- Gel documentation system, Bio-Rad, India.
- Air displacement pipettes P20, P100 -Eppendorf, Germany.
- LG refrigerator, India, -20°C low freezer, Blue Star, India.
- -70°C ultralow freezer, Sanyo, India; Brunswick Scientific, USA.
- Microwave oven MX 2100, Microwin Electronic India Ltd.
- pH meter, ECIL India.
- Electronic Shaking incubator, Innova-4000, New Brunswick Scientific, USA.
- Horizontal laminutesar air flow, Klenzaids, India.
- Bio-Rad PCR Machine, Bio-Rad, India.
- Microfuge, Eppendorf, model 5415 C, Brinkman Inc, Germany.
- UV Transilluminutesator, Chouromous Biotech Pvt. Ltd., India.
- Ultra-centrifuge, Beckman coulter, USA.

3.2 Molecular Characterization of *Bm*NPV using Restriction endonuclease profiling

Isolation of *Bm*NPV viral DNA and molecular characterization of *Bm*NPV isolates collected from different localities experimental overview is depicted in Fig. 3.

3.2.1 DNA isolation from the *Bm*NPV strains

The viral DNA from *Bm*NPV strains of different localities of Karnataka (Kolar, Shidlaghatta, Devanahalli, Ramanagar, Hosakote and Tumakuru) was isolated by following a modified standard protocol (Govindan *et al*., 1998).

3.2.1.1 Preparation of polyhedra from infected silkworm

- Silkworms were collected based on symptoms which were typically infected with nuclear polyhedrosis virus.
- Haemolymph was collected from diseased silkworms by cutting the front pair of prolegs and the turbid milky haemolymph was stored in sterilized glass tube and subjected to refrigeration.
- After refrigeration, haemolymph was taken and diluted two fold by adding distilled water.
- The haemolymph was filtered through double layer cheese cloth, and the filtrate was subjected to centrifugation at 12,000 rpm for 15 minutes at 4˚C.
- The pellet was resuspended in half the quantity of original volume of sterile distilled water and centrifuged at 5,000 rpm for 15 minutes at 4˚C.
- Repeated cycles of centrifugation at 12,000 rpm followed by 5000 rpm were done for 15 minutes each to obtain milky whiteamorphous sediment of nuclear polyhedra and the polyhedral were stored in distilled water as suspension.

3.2.1.2 Purification of virions using sucrose density gradient centrifugation.

1. Different gradients of sucrose were prepared in Beckman centrifuge tubes in 5 ml layers of 60, 55, 50, 45, and 40 % (W/W) sucrose solution, and incubation of the gradients at 4˚C overnight.

2. Three ml of amorphous sediment of nuclear polyhedra was loaded on to the sucrose gradient, without disturbing the gradients and ultra-centrifuged at 32,000 rpm for 60 minutes at 4˚C, pellet was then collected and dissolved in 1.5 ml of distilled water.

3.2.1.3 Isolation of DNA from purified virions.

- 1.5ml of resuspended polyhedral pellet from ultra-centrifugation was taken and pelleted at 9000 rpm for 2 min. The pellet was resuspended in 200µl distilled water.
- 20µl of 0.5M EDTA and 2µl of proteinase K were added and incubated for 5 hour.
- Half the volume of sodium carbonate (0.1M) was added to this and incubated at 37° C for 15 minutes, 20µl of 10 % w/v SDS was added and incubated at 37°C for 15 minutes and then 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 to this and gently agitated for 2 minutes followed by centrifugation at 9000 rpm for 2 minutes 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 minutes followed by centrifugation at 9000 rpm for 2 minutes, 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 at 9000 rpm, supernatant was removed and pellet was allowed to dry, the pellet was then dissolved in nuclease free water and stored in a freezer.

3.2.1.4 Confirmation of viral DNA using gel electrophoresis:

Viral DNA isolated from the *Bm*NPV infected silk worm samples (5 µl) was mixed with 1 µl of loading dye and eletrophoresed in 0.8 % w/v gel in TAE buffer

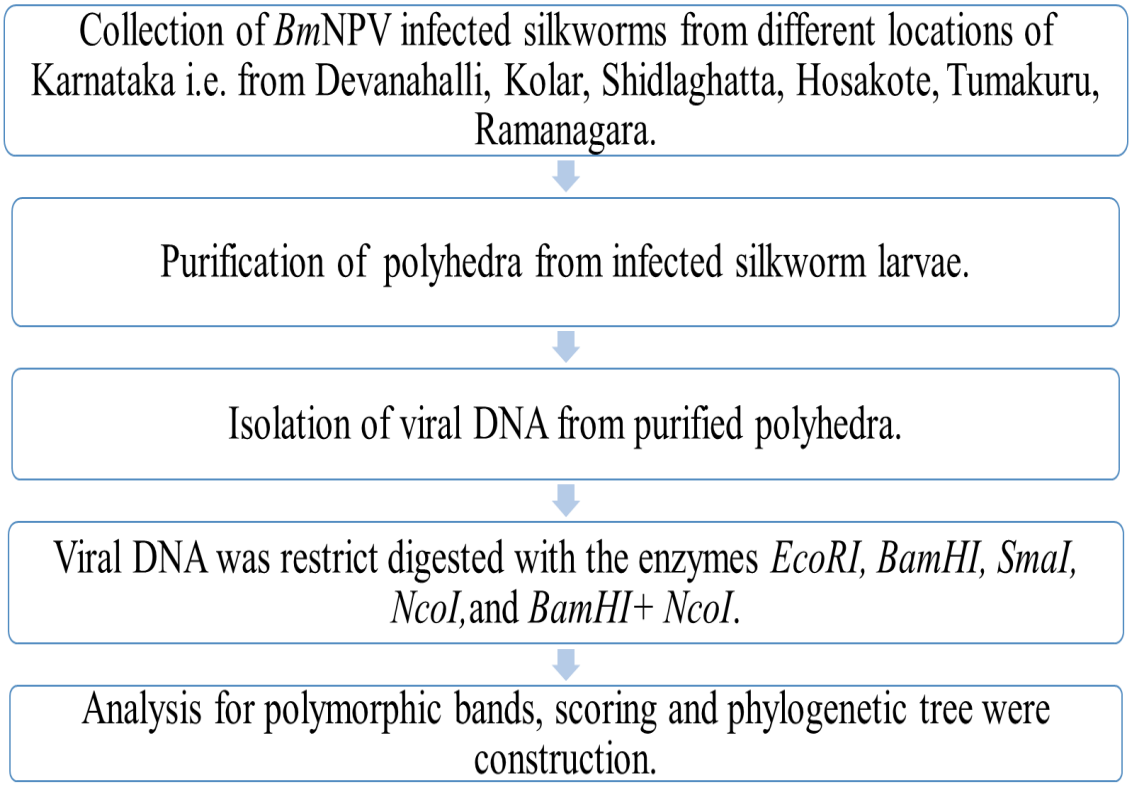


Fig. 3: Schematic representation of overall method followed for diversity analysis of *Bm*NPV by restriction endonuclease profiling.

(Appendix I) along with 1 Kb ladder and then run at 70 V for 1 hour 30 minutes. The DNA bands in the gel were visualized on a UV- transilluminator.

3.2.2 Restriction endonuclease profiling

Components	Quantity (µl)
*Bm*NPV DNA	2.0 µg
10X NEB Cut Smart Buffer	2.0 µl
Restriction enzyme HF (20units)	0.5 µl
Nuclease free water	10.5 µl
Total	20.0 µl

All the above components were mixed properly. The reaction mixture was incubated at 37°C for 3 hours and then inactivated at 60°C for 20 minutes. The restriction digested sample was then run on 0.8 % w/v agarose gel (Appendix 1) to visualize restriction enzyme profiling. Restriction enzymes used for the characterization were *EcoRI, SacI, BamHI, SmaI* and *BamHI+NcoI*.

3.2.3 Lethal dose 50 (LD50) study of silkworm

3.2.3.1 Rearing of Silkworm

For lethal dose 50 study hybrid Kolar Gold (Pure Mysore x CSR2) was used. Rearing of silkworm was conducted in the Department of Plant Biotechnology, UAS, GKVK, Bengaluru. The disease free chawki worms of 2nd instar, of the hybrid Kolar Gold (KG) were procured from Q3 Chawki center, Devanahalli. Silk worms were reared on disinfected plastic trays and were fed fresh mulberry leaves 3 times in a day as per the standard schedule. During first and second instars, silkworms are fed with chopped tender and succulent mulberry leaves and from third instar, 3-4 pieces of medium sized to large sized leaves were given to the silkworms. For LD-50 studies silkworms were divided into 9 treatments (1 control+ 8 treatment) and for each treatment 3 replication of silkworms containing 50 worms were separated.

3.2.3.2 Purification of polyhedra

Grasserie infected silkworm of fifth instar larvae were collected and haemolymph was taken into sterilized glass tubes from infected worms shortly before death by puncturing the first pair of prolegs with the help of surgical blade followed by gentle pressing. The turbid milky haemolymph was stored and then subjected to refrigeration for few days till the polyhedra settled at the bottom. Later, the haemolymph was filtered thourough 2 layers of muslin cloth. The filtrate was subjected to differential centrifugation at 12000 rpm for 15 minutes followed by 5000 rpm for 15 minutes. The pellet was resuspended in sterile distilled water to half of the original volume. Viral suspension centrifuged further at 12000 rpm for 15 minutes and at 5000 rpm for 15 minutes was repeated till milky whitish amorphous sediment of purified nuclear polyhedral suspension. The polyhedral inclusion bodies were suspended in distilled water and stored at 4˚C in the refrigerator.

3.2.3.3 Serial dilution of polyhedral suspension

From the original stock suspension of the polyhedral inclusion bodies, eight concentrations *viz*., 10^{0} to 10^{-7} were serially diluted in distilled water

3.2.3.4 Infectivity technique

The infection of *Bm*NPV to the silkworms was carried out per os. The worms were starved for 24 hrs and virus suspension was smeared on mulberry leaves which dried under shade and then cut to a size 50 cm^2 were prepared and washed in sterile water and surface sterilized by using 70 % ethanol (by cotton swab). The sterilized leaves were shade dried and such leaves were smeared evenly with virus suspension using non-adsorbent cotton. The leaves were shade dried and fed to the silkworm. Control batches were fed with surface sterilized mulberry leaves for the first feed. For subsequent feeding, inoculum free leaves were provided for both treated and untreated batches. Three replication were maintained for each treatment.

The treatment details were as follows

$T_1=10^{-0}$	$T_6= 10^{-5}$
$T_2=10^{-1}$	$T_7=10^{-6}$
$T_3=10^{-2}$	$T_8= 10^{-7}$
$T_4= 10^{-3}$	$T_9=$ Absolute control.
$T_5=10^{-4}$	

3.3 Cloning and characterization of the antiviral *serine protease* and *lipase* genes

The strategy used for this study is outlined in Fig. 4

3.3.1 Isolation of total RNA from silkworm midgut

Precautions: All materials used for RNA isolation such as pestle, mortar, scalpel, forceps, micro tips, water, surgical blade which were treated with DEPC water and autoclaved before use.

RNA isolation was done using NucleoSpin RNA isolation kit of catalogue number 740955.50 from Macherey- Nagel Company and the protocol used was as follows

- Silkworm of 5^{th} instar was used for RNA isolation. Using surgical blade, thoracic region of larvae was cut and from mid gut portion mid gut juice was collected.
- The collected sample of approximately 30 mg was homogenized by grinding in pestle and mortar.
- Sample was lysed by adding 350 µl Buffer RA1 and 3.5 µl ß-mercaptoethanol (ß-ME) to the sample pellet and vortexed vigorously.
- To reduce viscosity and clear the lysate by filtration thourough NucleoSpin® Filter (violet ring), the sample was applied and centrifuged for 1 minute at 11,000 rpm.

- The NucleoSpin® Filter was discarded and 350 µl ethanol (70 %) was added to the homogenized lysate and mixed by pipetting up and down (5 times).
- For each sample preparation, one NucleoSpin® RNA Column (light blue ring) was taken and placed in a collection tube. Lysate was pipetted up and down for 2–3 times and the lysate loaded to the column and centrifuged for 30 s at 11,000 rpm and the filtrate discarded.
- 350µl of MDB (Membrane Desalting Buffer) was added and centrifuged at 11,000 g for 1 minute to dry the membrane.
- DNase reaction mixture was prepared in a sterile 1.5 ml micro centrifuge tube: For each isolation, 10µl of reconstituted DNase was added to 90µl reaction buffer for DNase and mixed by flicking the tube. 95µl DNase reaction mixture was directly applied onto the center of the silica membrane of the column and incubated at room temperature for 15 minutes.
- Washing and drying of silica membrane
    - 1st wash- 200µl buffer RAW2 was added to the NucleoSpin® RNA Column. centrifuged for 30 seconds at 11,000 rpm, and the flow thourough was discarded.
    - 2nd wash- 600µl Buffer RA3 was added to the NucleoSpin® RNA Column. centrifuged for 30 seconds at 11,000 rpm, flow thourough discarded.
    - 3rd wash- added 250µl Buffer RA3 to the NucleoSpin® RNA Column. centrifuged for 2 minutes at 11,000 rpm to dry the membrane completely. Then column was placed in a nuclease free collection tube.
- The RNA was eluted by adding 60 µl RNase-free H_2O, and centrifuge at 11,000 rpm for 1 minute and the eluted RNA was stored at -20˚C for further use.

3.3.2 Synthesis of C-DNA from total RNA

PrimeScript™ 1st strand cDNA Synthesis Kit of catalogue number # 6110A from TAKARA was used and procedure was as follows.

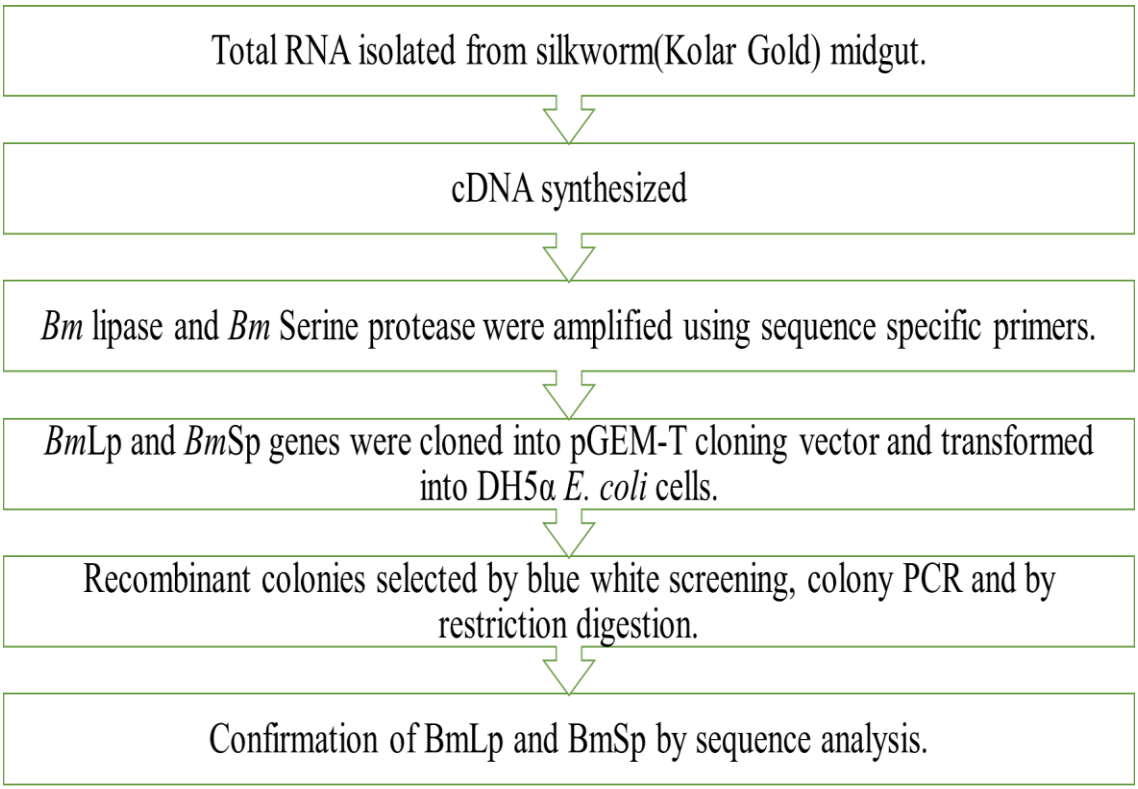


Fig. 4: Schematic representation of overall method followed for cloning and characterization of the antiviral protein genes serine protease and lipase.

1. The following mixture was prepared in a microtube.

Reagent	Volume (µl)
Oligo dT Primer (50 µM)	1
dNTP Mixture (10 mM each)	1
Template RNA	5
RNase Free dH2O	3
Total	10

2. The mixture was incubated at 65℃ for 5 minutes, and then cooled immediately on ice.
3. The next reaction mixture was prepared in a total volume of 20 µl as follows

Reagent	Volume
Template RNA Primer Mixture (from step 2)	10
PrimeScript Buffer	4
5X RNase Inhibitor (40 U/µl)	0.5 (20 units)
PrimeScriptRTase (200 U/µl)	1.0 (200 units)
RNase free H_20	4.5
Total	20

4. The reaction mixture was incubated at 42℃ for 30 - 60 minutes
5. The enzyme was inactivated by incubating at 95℃ for 5 minutes and stored at -20˚C.

3.3.3 Designing primer for *lipase* and *serine protease* gene

Bombyx mori lipase and *serine protease* gene sequence were downloaded from the NCBI nucleotide database of gene I. D AY945209.1 for *lipase* and AY945211.1 for *serine protease*, these sequences were aligned through Bio Edit (Version 7.0) sequence alignment software and two regions with maximum sequence homology were selected and designed.

Primers for *lipase* gene:

Forward Primer : 5'ATTGAATTCATGCCTGATGGCGAGGGTGTT 3'

Reverse Primer : 5' ATTGAGCTCTTAGAAAGGCCAACTGCT 3'

Primers for *serine protease*:

Forward Primer : 5'ATAGAATTCATGAAGGTCTTCGCAGCAGTACT 3'

Reverse Primer : 5' ATTGAGCTCTTAAATTCTAGCCCGGATCCAGGAGT 3'

Primers with the restriction sites of *XbaI* and *SacI* were added respectively for forward and reverse primers using Primer3 software.

3.3.4 Amplification of *lipase* and *serine protease* gene

The c-DNA synthesized from m-RNA which was isolated from silkworm midgut was subjected to polymerase chain reaction using *Bombyx mori lipase* and *serine protease* gene specific primers.

PCR reaction mixture

PCR reaction components	**Quantity (µl)**
Deionised nuclease free water	11.0
10X PCR buffer with 15 mM MgCl2	2.0
2 mM dNTPs	2.0
Forward primer(10 pmol/µl)	1.0
Reverse primer (10 pmol/µl)	1.0
*Taq*DNA polymerase(3 U/µl)	1.0
DNA template (50-60 ng)	2.0
Total	20

The PCR amplification of *lipase* and *serine protease* gene using gene specific primers was carried out in a thermal cycler (Bio-Rad) with the following temperature profile:-

Initial denaturation	94ºC for 5 minutes
Denaturation	94ºC for 45 seconds
Annealing	55ºC for 1 minute
Extension	72ºC for 1 minute 30 seconds
Final Extension	72ºC for 10 minutes
Hold	4ºC (∞)

3.3.5 Analysis of amplified gene product using agarose gel electrophoresis

The amplified PCR product of 20 µl was mixed with 4 µl of loading dye and electrophoresed in 1 % w/v agarose gel along with 100 bp ladder at 50 V for the initial 30 minutes and then at 70 V for 1 hour. The buffer used was 1X TAE pH 8 (Appendix I). After electrophoresis, the DNA bands were visualized on UV Transilluminator.

3.3.6 Development of recombinant *B. mori lipase* (*Bm-Lp*) and *B. mori serine protease* (*Bm- Sp*) construct for molecular characterization

PCR based cloning

Cloning of the amplified *Bm-Lp* and *Bm- Sp* was done in pGEM-T Easy cloning vector (Fig. 5) using InsT/A cloning kit. The amplified *Bm-Lp* and *Bm- Sp* genes were loaded in the gel and specific amplicons of 885 bp and 855 bp respectively were excised using a sharp sterile surgical blade on a low intensity UV Transilluminutesator and collected in sterile pre-weighed 2.0 ml micro centrifuge tubes. The Fermentas GeneJET gel extraction kit (#K0691) was used to elute the amplicons from the agarose block as described below:

- Equal weight by volume (1:1) of Solution Buffer was added to the gel slice and incubated at 60 degree for 10 minutes.
- The tube was mixed by inversion every few minutes to facilitate the melting process till a yellow colour was obtained.

- The solution was then poured to a fresh adsorption column and centrifuged at 13000 rpm for 1 minute. Flow-thourough was discarded.
- 700 µl washing buffer (WB) was added and centrifuged at 13000 rpm for 1 minute.
- The liquid in the collection tube was poured off into beaker and washing step was repeated again. Empty column spun again for 1 minute to remove the ethanol.
- The column was put into a fresh 1.5ml tube and 50µl elution buffer (EB) was added and spun at 13,000 rpm for 1 minute to elute the DNA and stored at -20˚ C.
- 5µl of the eluted sample was electrophoresed to confirm the gene.

3.3.7 Cloning of PCR product

The purified PCR fragments of about 885 bp and 855 bp were separately ligated to pGEMT Easy cloning vector (Fig. 5) (3015 bp) as described in InsTAclone™ PCR cloning kit, Fermentas, USA.

3.3.7.1 Ligation

An optimal molar ratio of ends of vector: insert (1:3) was computed and the components of ligation mix were added to 0.5 ml micro centrifuge tubes and incubated at 4°C overnight for ligation.

Components	**Volume (µl)**
pGEM-T Easy Cloning vector (50ng)	1.0
Purified PCR fragment (Approx. 150ng)	3.0
5x ligation buffer	6.0
T4 DNA ligase, 5U	1.0
Deionized water	18.0
Total	30.0

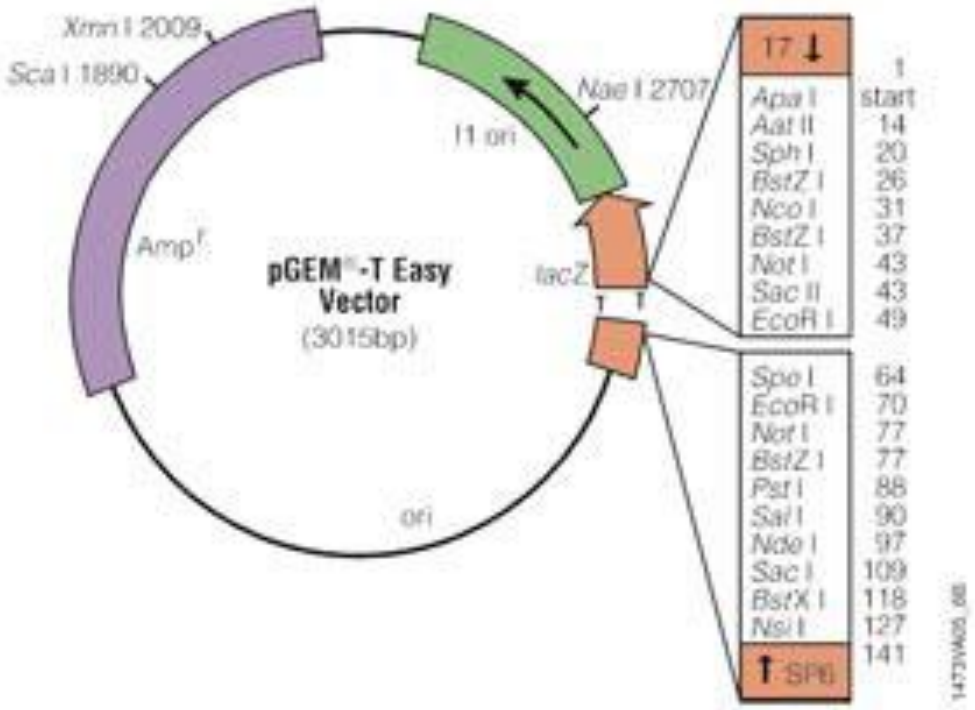


Fig. 5. Vector map of T/A cloning vector pGEM-T Easy vector.

3.3.7.2 Preparation of competent cells

1. The competent cells of *E.coli* strain α-DH5 were prepared following the protocol mentioned by Sambrook and Russell (2001) with minute modifications.
2. An isolated colony from *E.coli* α-DH5 plate was inoculated into 5 ml of Luria broth (Appendix III) and incubated at 37°C overnight at 200 rpm.
3. The next day, the culture was diluted to 1:100 using Luria broth *i.e.,* 0.5 ml culture was added to 50 ml Luria broth.
4. It was incubated for 2 – 3 hours till it attained an OD of 0.3 to 0.4 at 600 nm. The culture was chilled on ice for 30 minutes and 25 ml of culture was dispensed into two centrifuge tubes of capacity 50 ml.
5. The cells were pelleted at 6000 rpm for 10 minutes at 4˚C. The supernatant was discarded and the pellet was resuspended in 12.5 ml of ice-cold 0.1M calcium chloride.
6. The centrifuge tubes were again kept in ice for 10 minutes and later centrifuged at 6000 rpm for 10 minutes.
7. The pellet was dispensed in 1 ml of 0.1M $CaCl_2$ and to this 88µl of glycerol was added. About 200 µl of cells were distributed to each chilled micro centrifuge tubes and used immediately.

3.3.7.3 Transformation of *E. coli* α-DH5 cells

1. About 100 µl of freshly prepared competent cells were taken in chilled centrifuge tubes and 10 µl of ligation mixture (pGMET- *Bmlp* and pGMET- *BmSp*) was added and mixed gently.
2. The mixture was chilled on ice for 45 minutes and subjected to heat shock by shifting the chilled mixture to a pre heated 42°C water bath for exactly 90 seconds.
3. It was immediately transferred to ice to chill for 5 minutes. To this 800 µl of Luria broth (Appendix III) was added and incubated at 37°C at 200 rpm for 1 hour to

allow bacteria to recover and express the antibiotic marker encoded by the plasmid.

4. The culture was centrifuged at 13,000 rpm for one minute and about 700 µl of supernatant was discarded and the pellet was dissolved in remaining supernatant and spread on the agar plates with Ampicillin (50 mg/L), X-Gal and IPTG which was then incubated overnight at 37°C.
5. The recombinant clones were identified by blue- white screening.

3.3.8 Confirmation of clones

The confirmation for the presence of desired DNA fragment in cloning vector was done by PCR amplification using gene specific primers and by restriction analysis.

a) PCR confirmation of clones

For PCR confirmation of clones, the template DNA from plasmid was isolated following the alkaline lysis protocol of Brimbion and Dolly (1979).

Plasmid Isolation

A) Reagents (Appendix II)

B) Protocol for plasmid isolation

1) Single white colony from the transformed plate was inoculated and grown overnight in Luria Bertani (LB) broth (Himedia) at 37 °C.
2) Bacterial culture of 2 ml was micro-centrifuged at 6,000 rpm for 5 minutes at 4 °C and this step was repeated.
3) The pellet was resuspended in 200 µl of ice cold solution I and vortexed. RNase A (10 mg/ml) was added and vortexed.
4) Further 100 µl of solution II was added and mixed gently.
5) The mixture was then incubated for 5 minutes at room temperature.
6) To this mixture 150 µl solution III was added and incubated on ice for 5 minutes.

7) The bacterial lysate in the tube was centrifuged at 13,000 rpm for 15 minutes at 4 °C and supernatant was transferred to a fresh tube.

8) To the supernatant collected, 240 µl of isopropanol was added, mixed well and centrifuged at 13,000 rpm for 15 minutes at 4 °C.

9) Pellet was washed in 750 µl of 70 % (v/v) ethanol (10,000 rpm for 5 minutes at 4 °C) and pellet was air dried.

10) Plasmid was dissolved in 40 µl of 10 mM Tris-EDTA (TE) buffer (Appendix I) and stored at -20 °C.

Confirmation of the presence of cloned fragment was done by PCR amplification of clones with respective primer pairs.

b) Restriction analysis of recombinant clones

Restriction digestion of T/A cloning vector with *Bm Lp* and *BmSp* gene

Components	**Quantity (µl)**
T/A cloning vector+ *Bm Lp/Sp*	20
10X NEB Cut Smart Buffer	5
XbaI-HF (20units)	1.5
SacI-HF(20units)	1.5
Nuclease free water	22
Total	50

All the above components were mixed properly. The reaction mixture was incubated at 37°C for 3 hours and then inactivated at 60°C for 20 minutes. The restriction digested sample was then run on 0.8 % gel to confirm the 800 bp release from the recombinant clones.

3.3.9 Sequencing of cloned *lipase* and *serine protease* gene into T/A cloning vector

The strategy used for this study is outlined in Fig. 6

The *Bmlp* and *BmSp* genes cloned into pGEM-T Easy vector were sequenced using M13 forward and reverse primers at Chromous Biotech Pvt. Ltd., Bangalore. The sequence was analyzed using BLAST algorithm available at http://www.ncbi.nlm.nih.gov.

3.4 Screening of a few breeds of the silkworm *Bombyx mori L* for resistance to *Bm*NPV and sequence comparison of *serine protease* and *lipase* genes in these breeds.

3.4.1 Rearing of Silkworm

Rearing of silkworm Breeds *i.e*. Pure Mysore, CSR2, and their hybrid Kolar Gold (PM x CSR2) was conducted at Department of Plant Biotechnology, UAS, GKVK, Bengaluru for screening of resistance against *Bm*NPV and sequence characterization of *Bm*NPV antiviral proteins *Bm lipase* and *Bm serine protease*. The disease free chawki worms of 2nd instar, of the breeds CSR2, Pure Mysore (PM), and Kolar Gold (KG) was procured from Q3 Chawki center, Devanahalli. Silkworms were reared on plastic trays and were fed 3 times in a day, morning (9-10 A.M.), afternoon (1-2P.M.), and night (8-9 P.M.). During first and second instars, silkworms were fed with chopped tender and succulent mulberry leaves and from third instar, 3-4 pieces of medium sized to large sized leaves were fed to the worms.

3.4.1.1 Screening of PM, KG, and CSR2 for their resistance against *Bm*NPV

Silkworms of breeds Pure Mysore, CSR2, and their hybrid Kolar Gold (PM x CSR2) were taken in 3 replications of 30 worms each. LD-50 concentration of 10^{-2} was used for infection. Infectivity to silkworms was done as mentioned in 3.2.3.4 i.e. leaves were smeared with 10^{-2} dilution of *Bm*NPV and fed to each replications and for control each breed were fed with non-treated leaves.

3.4.2 Isolation of total RNA from Pure Mysore, Kolar Gold and CSR2 was done as mentioned in 3.3.1

3.3.3 Synthesis of c-DNA from Pure Mysore, Kolar Gold and CSR2 total RNA was done as mentioned in 3.3.2

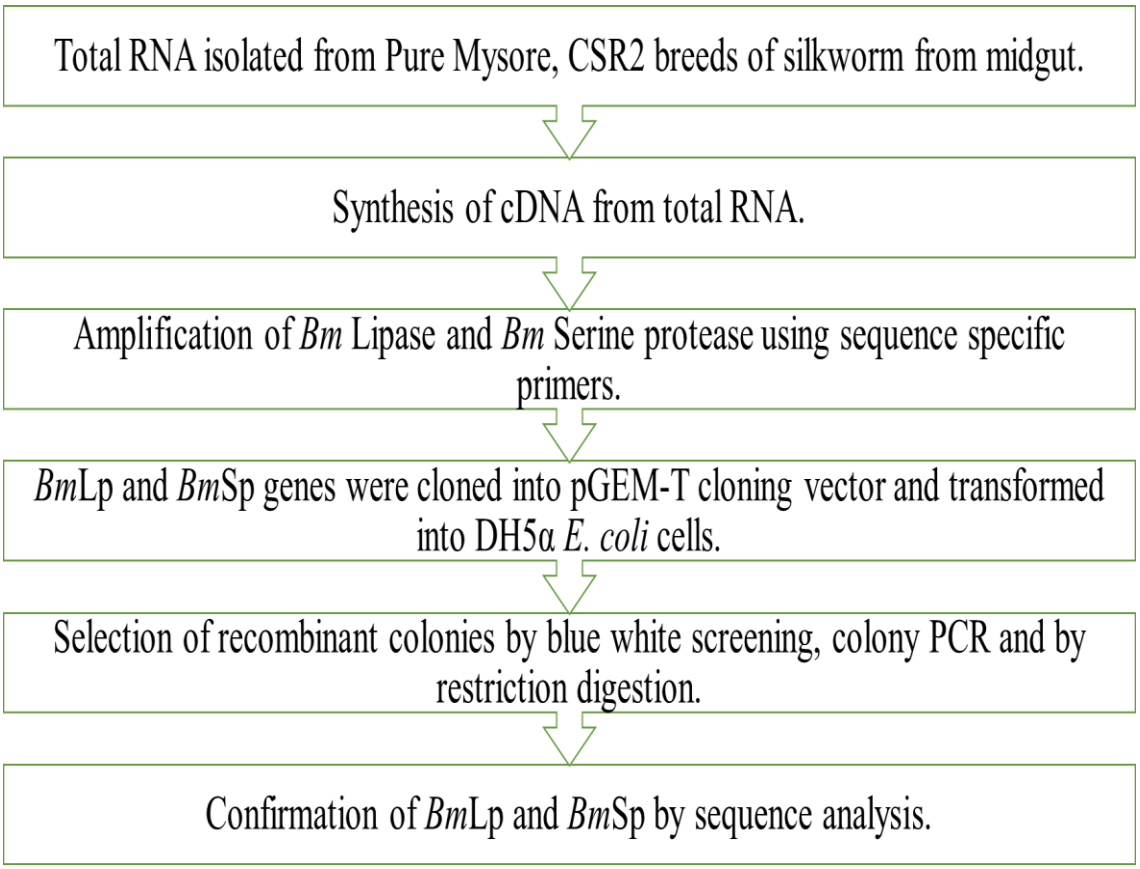


Fig. 6: Schematic representation of overall method followed for sequence comparison of serine protease/lipase genes isolated from Pure Mysore and Kolar Gold.

3.3.4 Amplification of *BmLp* and *BmSp* from Pure Mysore, Kolar Gold and CSR2 c-DNA using PCR was done as mentioned in 3.3.4

3.3.5 Integration of *BmLp* and *BmSP* intopGEMT cloning vector and transformation into DH5α E-coli was done as mentioned in3.3.6 and 3.3.7.

3.3.6 Confirmation of integration using colony PCR and restriction digestion was done as mentioned in 3.3.8

3.3.7 Sequencing of cloned *BmLp* and *BmSp* from PM, CSR2, and KG breeds gene was done as mentioned in 3.3.9.

3.3.8 Analysis of sequences from these breeds

After sequencing, obtained sequence results were confirmed using NCBI Blast. Using Mega 6 software multiple sequence alignment was done and analyzed for variations in nucleotide variations between the breeds of silkworm. Amino acid sequences from nucleotide sequence were predicted using EMBL- Transeq tool. Multiple amino acid sequence alignment of these genes were done using Clustlw tool. Secondary and tertiary structure of these predicted amino acid were done using phyre2 tool.

IV EXPERIMENTAL RESULTS

The present research work focuses on Molecular characterization of *Bm*NPV and cloning of *Bm*NPV antiviral encoding protein gene *serine protease*/ *lipase*.

4.1 Molecular characterization of *Bombyx mori* nuclear polyhedrosis (*Bm*NPV) isolates using restriction endonucleases (REN).

4.1.1 Purification and isolation of *Bm*NPV viral DNA

*Bm*NPV infected silkworms were collected from different regions of Southern Karnataka such as Devanahalli, Hosakote, Shidlagatta, Kolar, Ramanagaram, Tumakur. Haemolymph from *Bm*NPV infected worms was collected, purified by differential centrifugation and sucrose density gradient centrifugation and the presence of *Bm*NPV confirmed under 100 x compound microscope (Plate 1). Viral DNA was isolated from purified polyhedra and confirmed by gel electrophoresis (Plate 2).

4.1.2 Restriction endonuclease profiling of *Bm*NPV viral genome.

The isolated viral DNA was digested with *EcoRI, BamHI, SmaI, NcoI, and BamHI + NcoI.* Results obtained in banding pattern were scored and analyzed using NTYSYS software to obtain phylogenetic tree. Most of the banding pattern (plate 3-7) were same between isolates except that restriction digestion of viral genome with *BamH1* enzyme produced a polymorphic band of 4kb in Tumkuru and Ramanagara isolates and another band at 1.5 kb only in Ramanagara isolate. *EcoRI* digestion produced a band at 3.5 kb of Ramanagara isolate and digestion with *NcoI + BamHI* yielded a polymorphic band at 2 kb. Based on the scoring results a dendrogram was drawn (Fig. 7) using NTYSYS software which represented the relationship among six isolates of *Bm*NPV containing two large clusters, in one cluster Ramanagra isolate was present, in another cluster containing two sub clusters with one sub cluster containing Tumkuru isolate and the other Devanahalli, Shidlaghatta, Hosakote and Kolar isolates. Similarity indices between the isolates shown in Table 2.

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

	Kolar	Hosakote	Shidlghatta	Devanahalli	Tumakuru	Ramanagar
Kolar	1.000					
Hosakote	1.000	1.000				
Shidlghatta	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

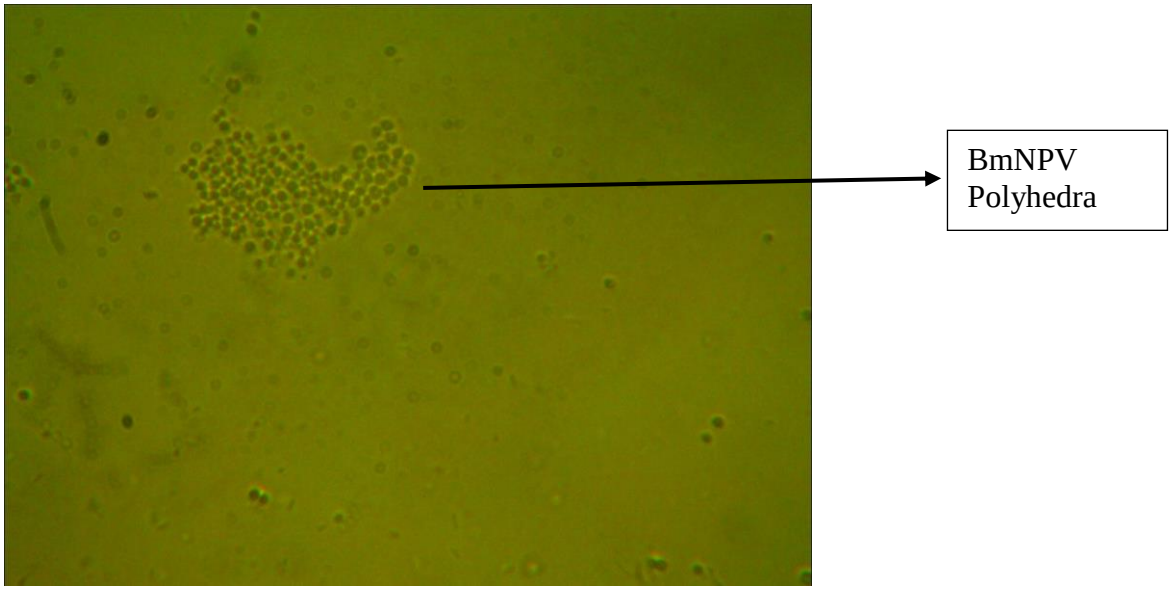


Plate 1: Confirmation of presence of polyhedra under 100x compound microscope

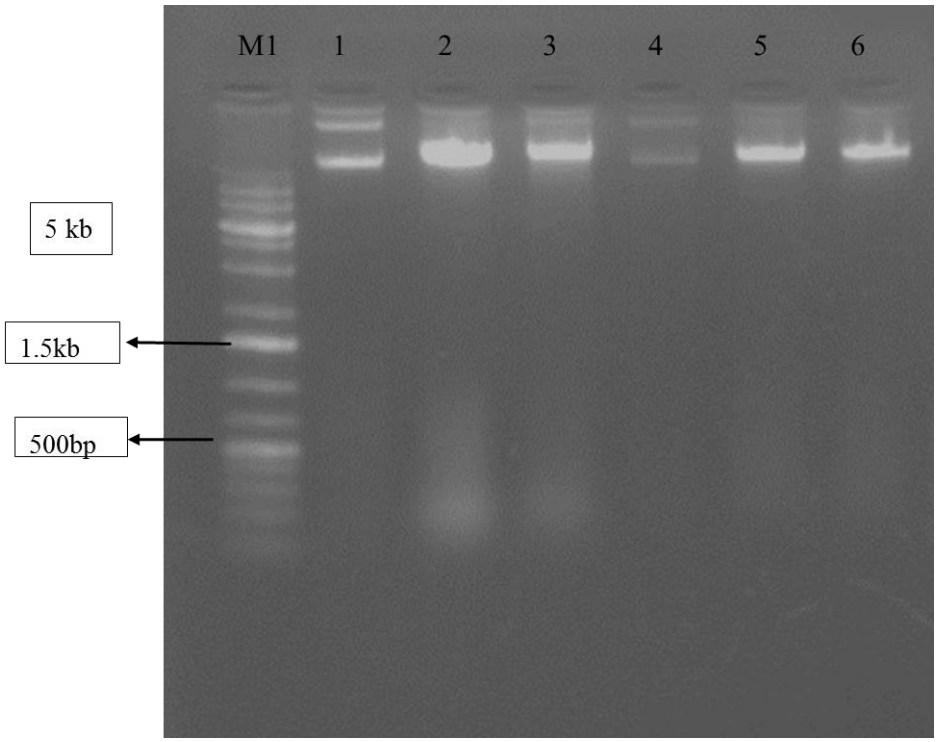


Plate 2: Agarose gel electrophoresis of viral DNA isolated from *Bm*NPV infected silkworms collected from different locations of Karnataka.

M 1- 1 Kb ladder (Thermoscientific)

Lane 1- Kolar isolate

Lane 2- Tumakur isolate

Lane 3- Shidlaghatta isolate

Lane 4- Devanahalli isolate

Lane 5- Hosakote isolate

Lane 6- Ramanagar isolate

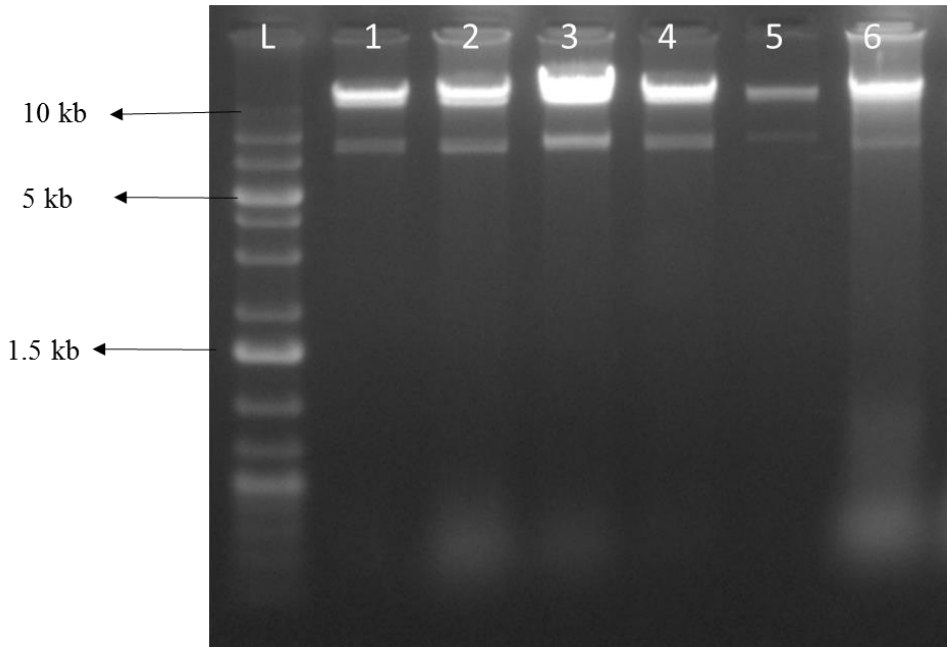


Plate 3: Agarose gel electrophoresis of restriction endonuclease profiling of *Bm*NPV isolates using *SmaI* enzyme.

M 1- 1 Kb ladder (Thermoscientific)

Lane 1- Kolar isolate

Lane 2- Tumakur isolate

Lane 3- Shidlaghatta isolate

Lane 4- Devanahalli isolate

Lane 5- Hosakote isolate

Lane 6- Ramanagar isolate

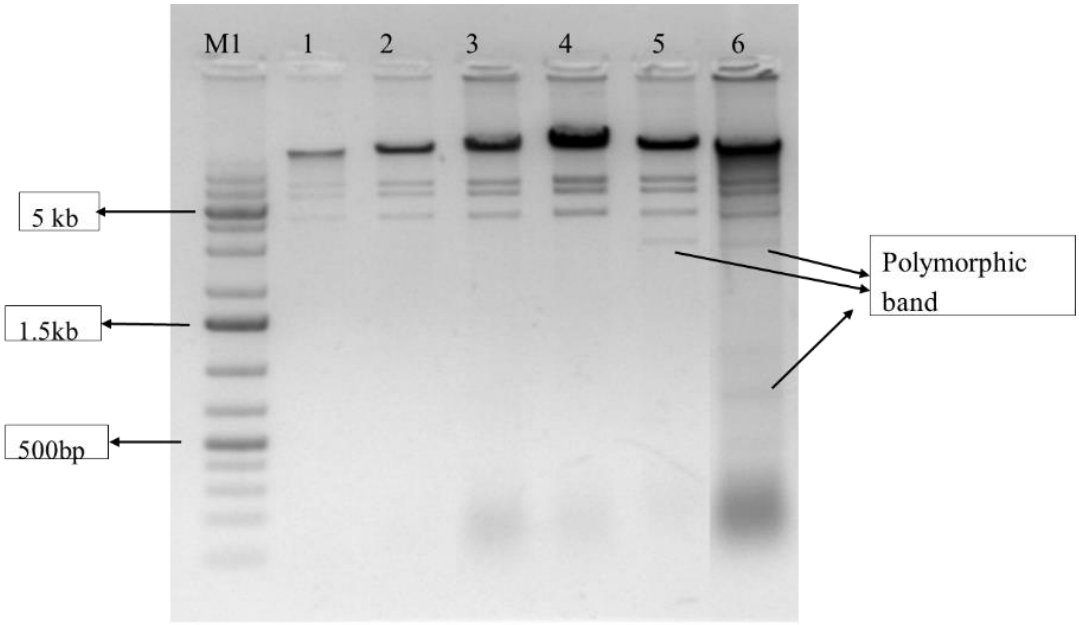


Plate 4: Agarose gel electrophoresis of restriction endonuclease profiling of *Bm*NPV isolates using *BamHI* enzyme.

M 1- 1 Kb ladder (Thermoscientific)

Lane 1- Kolar isolate

Lane 2- Hosakote isolate

Lane 3- Shidlaghatta isolate

Lane 4- Devanahalli isolate

Lane 5- Tumakur isolate

Lane 6- Ramanagar isolate

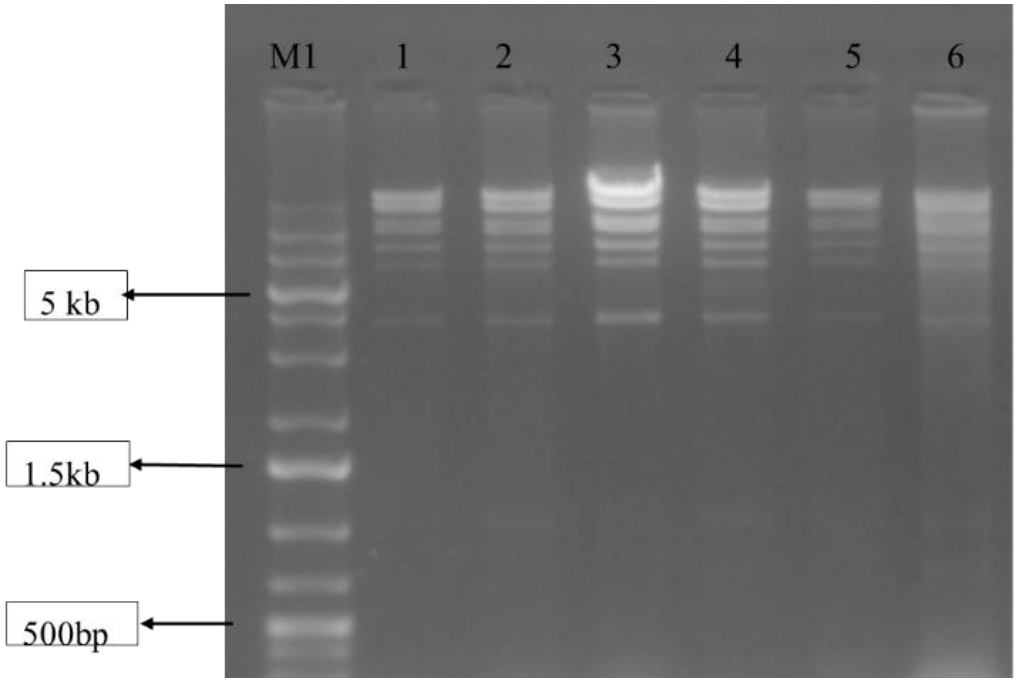


Plate 5: Agarose gel electrophoresis of restriction endonuclease profiling of *Bm*NPV isolates using *NcoI* enzyme.

M 1- 1 Kb ladder (Thermoscientific)

Lane 1- Kolar isolate

Lane 2- Hosakote isolate

Lane 3- Shidlaghatta isolate

Lane 4- Devanahalli isolate

Lane 5- Tumakur isolate

Lane 6- Ramanagar isolate

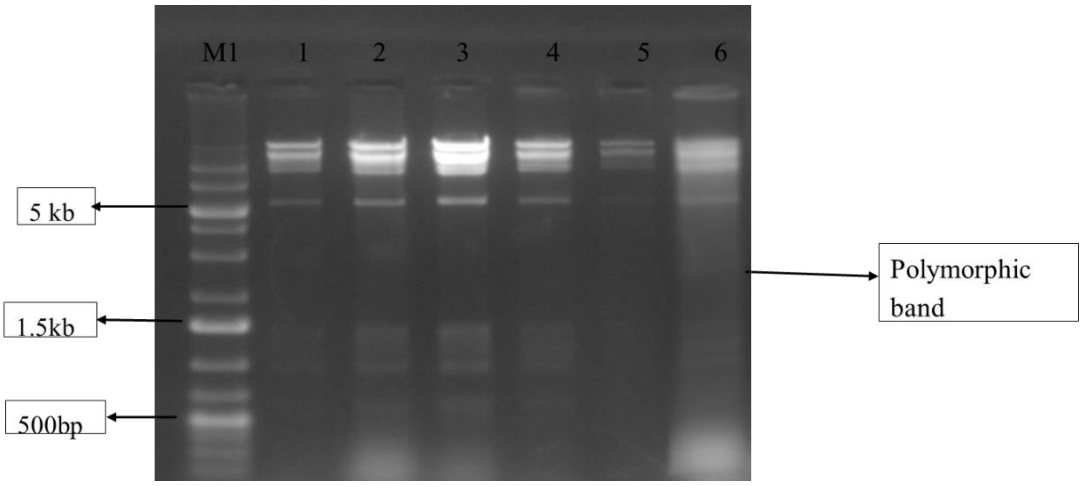


Plate 6: Agarose gel electrophoresis of restriction endonuclease profiling of *Bm*NPV isolates using *EcoRI* enzyme.

M 1- 1 Kb ladder (Thermoscientific)

Lane 1- Kolar isolate

Lane 2- Hosakote isolate

Lane 3- Shidlaghatta isolate

Lane 4- Devanahalli isolate

Lane 5- Tumakur isolate

Lane 6- Ramanagar isolate

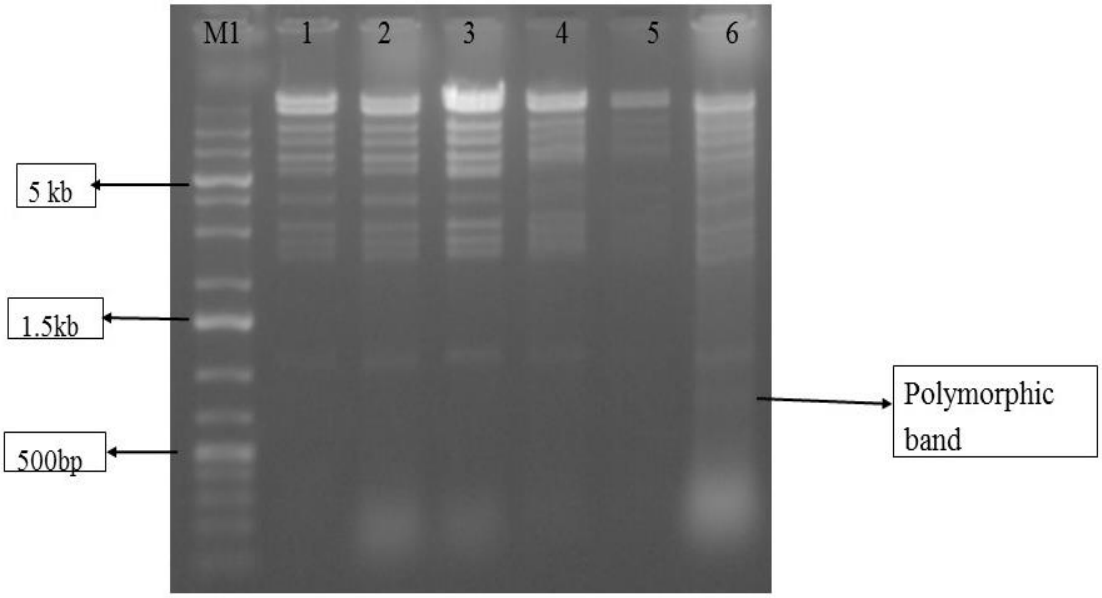


Plate 7: Agarose gel electrophoresis of restriction endonuclease profiling of *Bm*NPV isolates using *BamHI+NcoI* enzyme.

M 1- 1 Kb ladder (Thermoscientific)

Lane 1- Kolar isolate

Lane 2- Hosakote isolate

Lane 3- Shidlaghatta isolate

Lane 4- Devanahalli isolate

Lane 5- Tumakur isolate

Lane 6- Ramanagar isolate

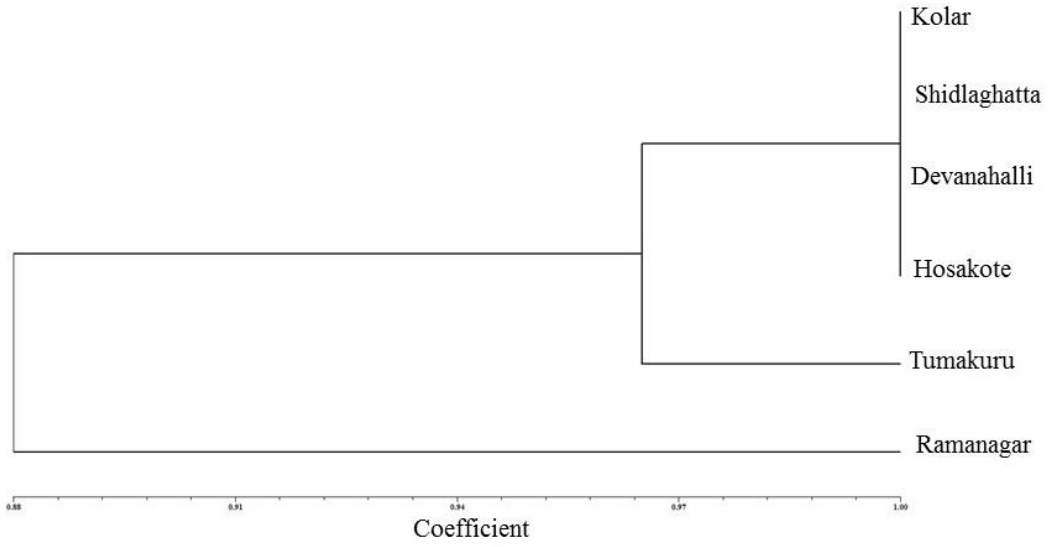


Fig. 7: Dendrogram representing relationship among six *Bm*NPV isolates

4.1.3 Lethal dose 50 study

Kolar Gold breed of silkworms were infected with 10^0 to 10^{-7} dilution of *Bm*NPV in three replications and a control after 3rd moult of silkworm. Initially for four to five days there were no symptoms of *Bm*NPV identified. After which specific symptoms of graserrie like crawling around the periphery of the rearing tray, swelling of inter segmental areas, shining and yellowish body, rupturing of the skin, white ooze filled with polyhedral were observed. After 6 hours of first symptom worms started to die, which were collected and separated to reduce secondary infection of *Bm*NPV. The mortality of worms were recorded on daily basis from each treatment and its replication. Maximum mortality was observed in stock (10^0) and minimum mortality was observed at 10^{-7}, 50% mortality was observed at 10^{-2} (Table 3). In control only 2% of mortality was observed, the dilution of 10^{-2} was taken as lethal dose 50 for further study (Fig. 8).

4.2 Isolation of *Bm*NPV antiviral *Bm*Lp and *BmSP* genes

4.2.1 Primer designing

The *BmLp* gene sequence (Ref. ID: AY945209.1) and *BmSP* gene (Ref. ID: AY945211.1) sequence was downloaded from NCBI nucleotide database and the primers were designed using software Primer3 such that the entire *BmLp* and *BmSp* gene were amplified.

4.2.2 PCR amplification of *BmLp* and *BmSP* genes.

Amplification of *BmLp* and *BmSP* genes from the cDNA using Taq DNA polymerase enzyme with gene specific primers revealed an amplicon size of 885 bp of *BmLp* and 855 bp of *BmSp* on 1% agarose gel which was eluted from the gel (Plate 8).

4.2.3 Cloning of *BmLp* and *BmSP* genes in T/A cloning vector

4.2.3.1 Ligation and transformation of *E.coli* DH5α

The amplified and eluted *BmLp* and *BmSP* genes and the vector pGEMT were ligated in the molar ratio 3:1 using T4 DNA ligase. The ligated product was transformed

Table 3: Lethal dose 50 study of *Bm*NPV on Kolar Gold breed of silkworm showing cumulative mortality at different dilution treatments.

Treatment	Replication			Cumulative mortality	Percent of Mortality
	1	2	3		
T1 (10^{-0})	39	36	35	110	73.33%
T2 (10^{-1})	34	25	29	88	58.67%
T3 (10^{-2})	21	24	30	76	50.67%
T4 (10^{-3})	18	20	13	51	34%
T5 (10^{-4})	10	13	11	34	22.67%
T6 (10^{-5})	10	10	11	31	20.67%
T7 (10^{-6})	7	3	5	15	10%
T8 (10^{-7})	5	3	6	14	9.3%
T9 (Absolute control)	3	0	0	3	2%

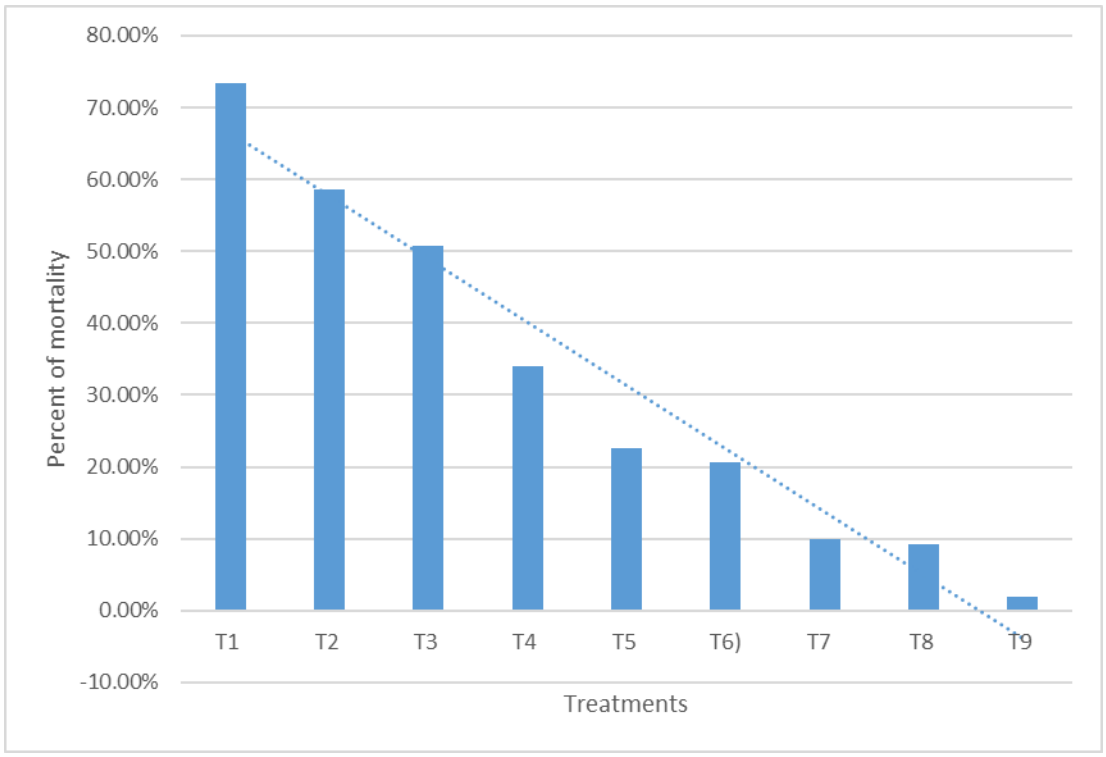


Fig. 8: Graph representing per cent cumulative larval mortality at different dilution treatments of *Bm*NPV

T1: 10^{-0} T4: 10^{-3} T7:10^{-6}

T2:10^{-2} T5: 10^{-4} T8: 10^{-7}

T3: 10^{-2} T6:10^{-5} T9: Absolute control

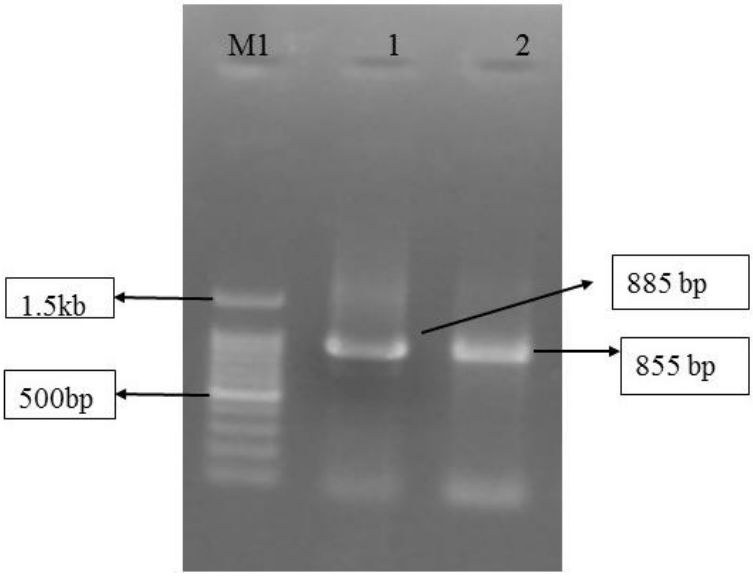


Plate 8: Agarose gel electrophoresis of amplification of *Bombyx mori* lipase and serine protease genes from the midgut of Kolar Gold breed of silkworm.

M1 : 100 bp DNA ladder (Genei)

Lane 1 : *Bombyx mori* lipase

Lane 2 : *Bombyx mori* serine protease

into *E.coli* DH5α cells by heat shock method. After incubation at 37˚C overnight, transformed colonies were observed (Plate 9 & 10).

4.2.4 PCR and restriction confirmation of recombinant pGEMT- *BmLp* and pGEMT- *BmSp* clones

Transformed colonies were selected based on blue white screening, white colonies were selected and by colony PCR transformed colonies were confirmed (Plate 11 & 12) and inoculated for plasmid isolation. The plasmid DNA from the transformed *E.coli* DH5α cell was cultured and the *BmLp* and *BmSP* genes in pGEMT plasmid was confirmed by plasmid restriction digestion using *Xba1* and *Sac1* and a fragment of 885 bp and 855 bp was found with vector backbone of 3kb fragment (Plate 13) and the integration of pGEMT- *BmLp* and pGEMT- *BmSp* was confirmed.

4.2.5 Sequencing of *BmLp* and *BmSp* gene

The plasmid DNA isolated from the transformed *E.coli* DH5α cells were sequenced using M13 primers. The sequences obtained from the automated sequencing was analyzed using NCBI-BLAST to find possible matches with the *BmLp* and *BmSp* genes reported globally and confirmed that isolated genes were *Bomyx mori lipase*s and *serine protease*s (Fig. 9 and 10).

4.3.1 Screening of a few breeds of silkworm *Bombyx mori L* for resistance to *BmNPV*

For this experiment Kolar Gold, Pure Mysore, CSR2 breeds of silkworms were chosen. Lethal dose 50 dilution 10^{-2} of *Bm*NPV was used to infect silkworms. In each breed three replications were made of thirty worms and infected after 3rd moult of silkworm. Larval mortality was observed based on symptoms and recorded mortality of worms. % of cumulative mortality of Kolar Gold breed was 48.89%, CSR2 breed was 65.56% and in Pure Mysore breed 35.56% of laval mortality were observed. (Table 4 and Fig. 11)

Table 4: Cumulative mortality of different breeds of silkworms at lethal dose 50 dilution of *Bm*NPV

Breeds	Replications			Control	Cumulative mortality	% of Mortality
	1	2	3			
Pure Mysore (PM)	13	9	10	1	32	35.56 %
CSR2	19	18	22	0	59	65.56%
Kolar Gold	17	13	14	4	44	48.89 %

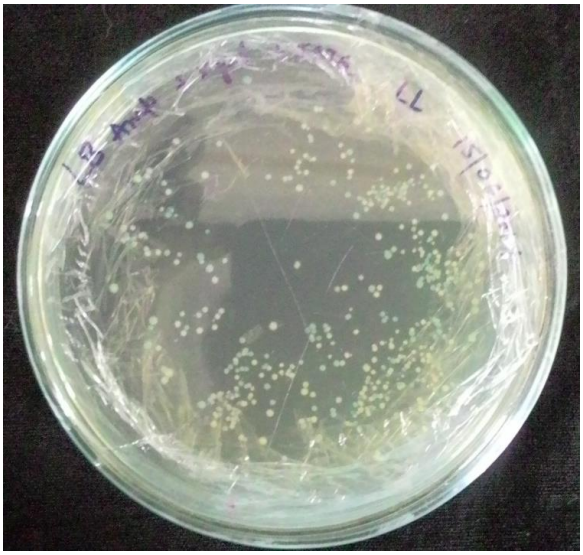


Plate 9: Transformed *E. coli* DH5α cells with *Bombyx mori* lipase- pGMET vector, selection by blue white screening.

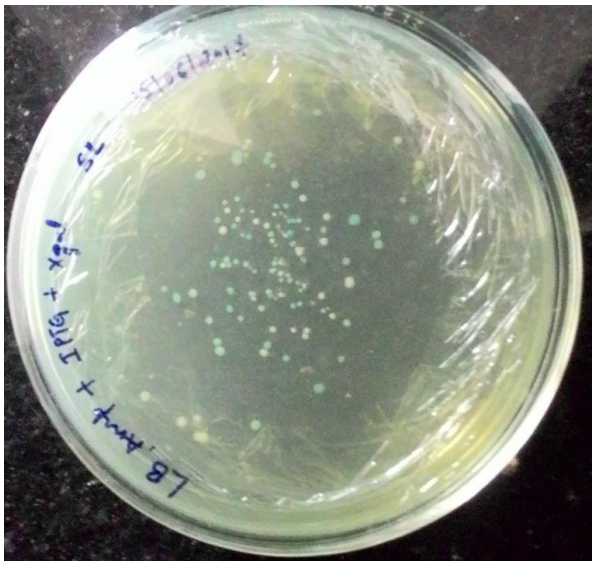


Plate 10: Transformed *E. coli* DH5α cells with *Bombyx mori* serine protease- pGMET vector, selection by blue white screening.

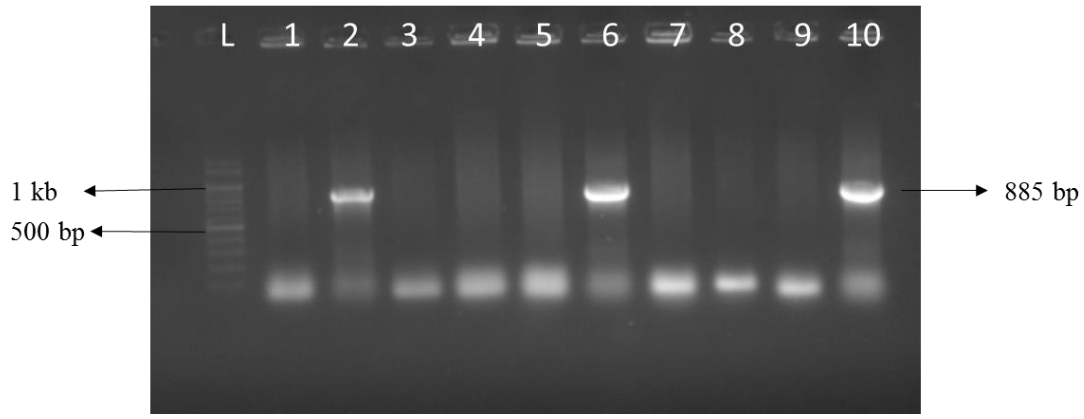


Plate 11: Agarose gel electrophoresis of colony PCR for confirmation of recombinant clones *Bm*Lp- pGEMT.

M1 : 100 bp DNA ladder (Genei)

Lane 1-10 : Clones in replicates

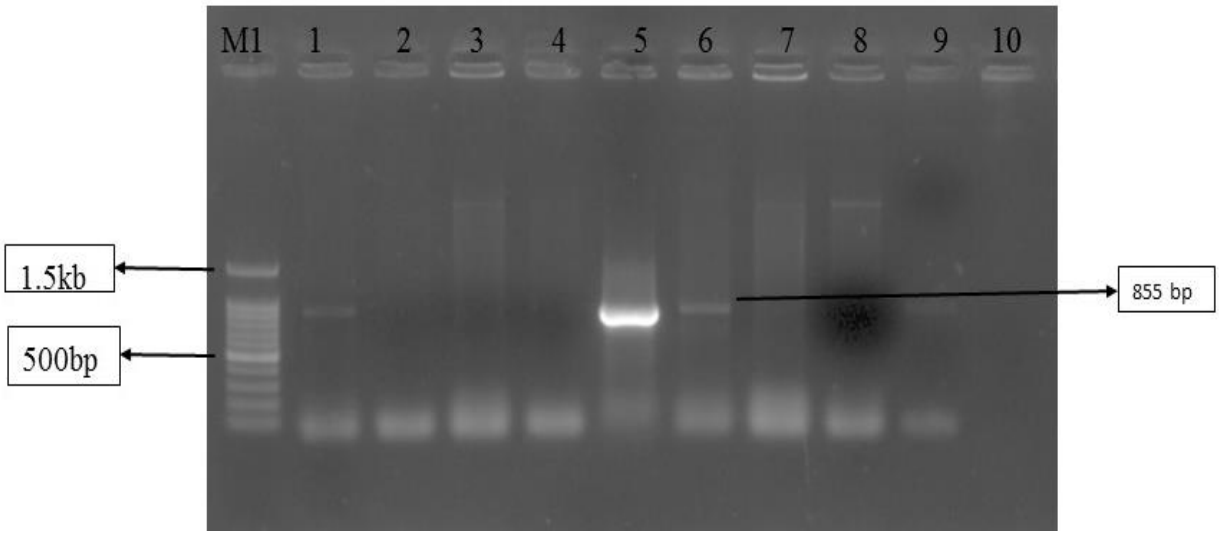


Plate 12: Agarose gel electrophoresis of colony PCR for confirmation of recombinant clones *Bm*Sp- pGEMT.

M1 : 100 bp DNA ladder (Genei)

Lane 1-10 : Clones in replicates

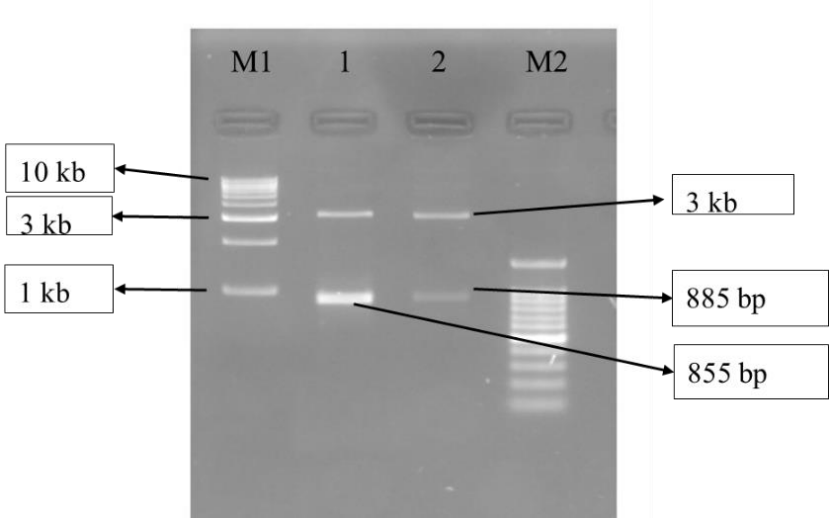


Plate 13: Agarose gel electrophoresis of confirmation of recombinant pGEM-T-*Bm*Lp gene and pGEM-T- *Bm*Sp gene construct with *Xba1 and SacI* restriction digestion.

M1 : 1Kb DNA ladder (Genei)

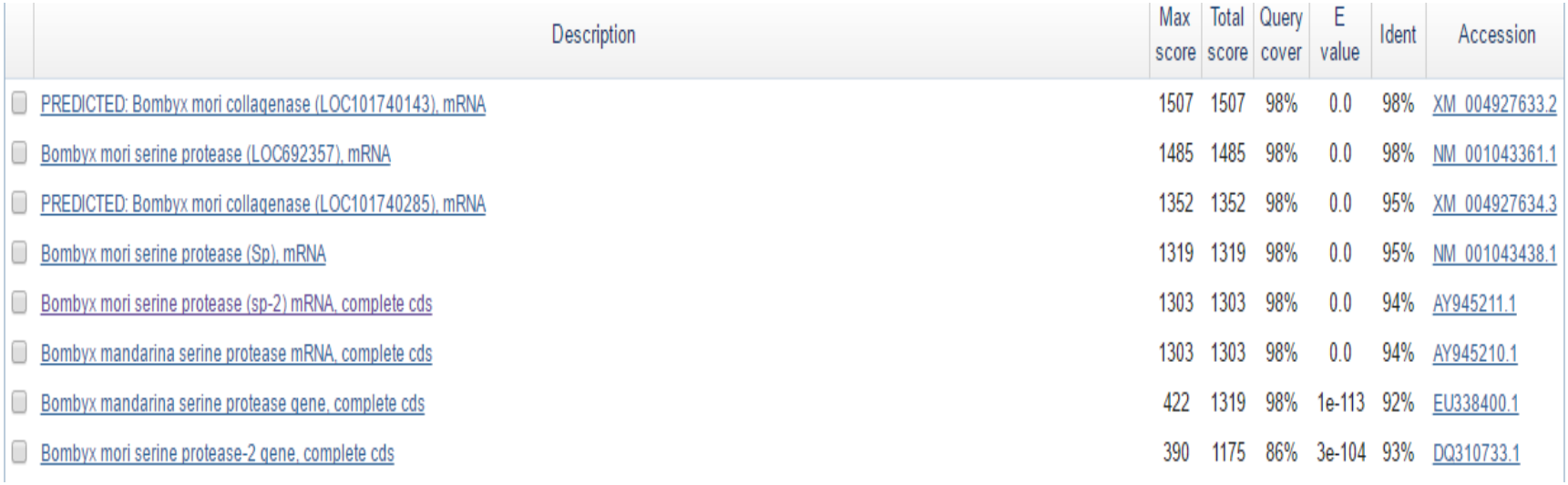
M2 : 100bp DNA ladder (Genei)

Lane 1 : Recombinant pGEMT- *Bm*Sp gene construct

Lane 2 : Recombinant pGEMT- *Bm*Lp gene construct

Description	Max score	Total score	Query cover	E value	Ident	Accession
PREDICTED: Bombyx mori lipase-1 (LOC692513), transcript variant X1, mRNA	1568	1568	91%	0.0	99%	XM_012695610.2
Bombyx mori mRNA, clone: fmqV52I07_K05239	1568	1568	91%	0.0	99%	AK378535.1
Bombyx mori mRNA, clone: fmqV44B09	1568	1568	91%	0.0	99%	AK378456.1
Samia cynthia ricini strain Banma lipase mRNA, complete cds	1568	1568	91%	0.0	99%	DQ149986.1
Bombyx mori lipase-1 (LOC692513), mRNA	1568	1568	91%	0.0	99%	NM_001043501.1
Bombyx mandarina lipase mRNA, complete cds	1554	1554	91%	0.0	98%	AY945212.1
Bombyx mori lipase-1 mRNA, complete cds	1541	1541	91%	0.0	98%	AY945209.1
Bombyx mandarina lipase gene, complete cds	719	1570	91%	0.0	99%	EU700252.1
Bombyx mori strain Qiufeng Baiyu lipase gene, complete cds	719	1579	91%	0.0	99%	DQ286554.1

Fig. 9: Sequencing result showing percent identity of *Bombyx mori* lipase gene isolated from Kolar Gold breed of silkworm with other *Bombyx mori* lipase genes using BLAST analysis.



Description	Max score	Total score	Query cover	E value	Ident	Accession
PREDICTED: Bombyx mori collagenase (LOC101740143), mRNA	1507	1507	98%	0.0	98%	XM_004927633.2
Bombyx mori serine protease (LOC692357), mRNA	1485	1485	98%	0.0	98%	NM_001043361.1
PREDICTED: Bombyx mori collagenase (LOC101740285), mRNA	1352	1352	98%	0.0	95%	XM_004927634.3
Bombyx mori serine protease (Sp), mRNA	1319	1319	98%	0.0	95%	NM_001043438.1
Bombyx mori serine protease (sp-2) mRNA, complete cds	1303	1303	98%	0.0	94%	AY945211.1
Bombyx mandarina serine protease mRNA, complete cds	1303	1303	98%	0.0	94%	AY945210.1
Bombyx mandarina serine protease gene, complete cds	422	1319	98%	1e-113	92%	EU338400.1
Bombyx mori serine protease-2 gene, complete cds	390	1175	86%	3e-104	93%	DQ310733.1

Fig. 10: Sequencing result showing percent identity of *Bombyx mori* serine protease gene isolated from Kolar Gold breed of silkworm with other *Bombyx mori* serine protease genes using BLAST analysis.

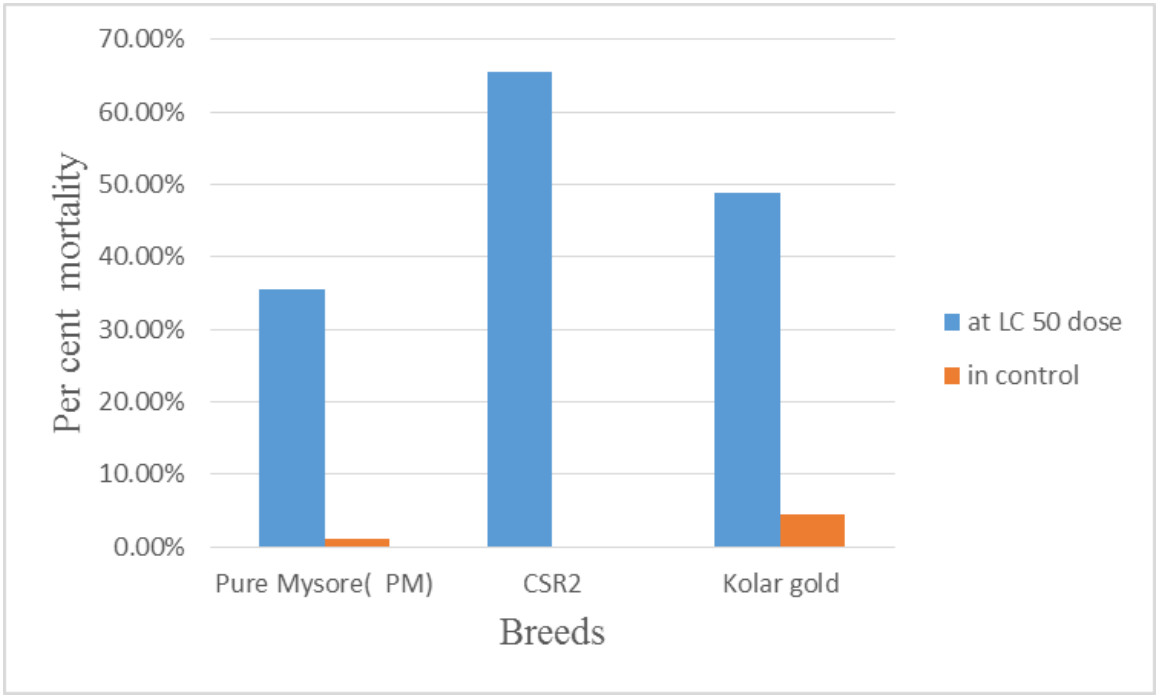


Fig. 11: Cumulative larval mortality of different breeds of silkworm at lethal dose 50 of *Bm*NPV.

4.3.2 Sequence comparison of *serine protease* and *lipase* genes in Kolar Gold, CSR2, and Pure Mysore breeds.

4.3.2.1 PCR amplification of *lipase* and *serine protease* genes from PM and CSR2 breeds

Amplification of l*ipase* and s*erine protease* genes from the cDNA using Taq DNA polymerase enzyme with gene specific primers revealed an amplicon size of 885 bp of *Lipase*s and 855 bp of *Serine protease*s on 1% agarose gel which was eluted from the gel (Plate 14).

4.3.2.2 Cloning of *lipase* and *serine protease* genes from PM and CSR2 breeds in T/A cloning vector and its confirmation

4.3.2.2.1 Ligation and transformation of *E.coli* DH5*α*

The amplified and eluted *lipase* and *serine protease* genes and the vector pGEMT were ligated in the molar ratio 3:1 using T4 DNA ligase. The ligated product was transformed into *E.coli* DH5α cells by heat shock method. After incubation at 37˚C overnight, transformed colonies were observed (Plate 15, 16, 17 and 18). Transformed colonies were selected based on blue white screening, white colonies were selected and by confirmed by colony PCR (Plate 19, 20, 21 and 22) and inoculated IN LB media for plasmid isolation. The plasmid DNA from the transformed *E.coli* DH5α cell was cultured and the *lipase* and *serine protease* genes in pGEMT plasmid was confirmed by plasmid restriction digestion using *Xba1+ Sac1* enzymes the of 885 bp and 855 bp of *lipase* and *serine protease* were found to be released along with vector backbone of 3kb fragment (Plate 23) by the integration of *lipase*s and *serine protease*s into pGEM-T vector was confirmed.

4.2.5 Sequencing of *lipase*s and *serine protease* genes in these breeds.

The plasmid DNA isolated from the transformed *E.coli* DH5α cells were sequenced using M13 primers. The sequences obtained from the automated sequencing was analyzed using NCBI-BLAST to find possible matches with the *BmLp* and *BmSp* genes reported globally and it was confirmed that the isolated genes were *Bomyx mori*

*lipase*s and *serine protease*s (Fig. 12, 13, 14 and 15). Using MEGA 4 software multiple sequence alignment was done a variation within nucleotides (Fig. 16 and 17) was observed in *serine protease* from Pure Mysore differed from that of Kolar Gold by 3.16 %, while variation between CSR2 and Kolar Gold was differed by 1.29 % and that between Pure Mysore and CSR2 *serine protease* was 4.4 % of variation. In *Bomyx mori lipase*, variation in nucleotides between CSR2 and Kolar Gold was 1.13 %, between Kolar Gold and Pure Mysore was 1.01 %, and between CSR2 and PM was 1.33 %.

Multiple amino acid sequence alignment (Fig. 18 and 19) shown variations within amino acid sequences were observed, in serine proteases PM differed from KG by 7.04 %, while variations between CSR2 and KG was differed by 1.76 % and between PM and CSR2 was 5.98 %. In lipase amino acid sequences, variations between KG and CSR2 0.34 %, between KG and PM was 2.04 % and between CSR2 and PM was 2.38 % were observed.

Using phyre2 tool secondary and tertiary structure of lipase and serine protease were predicted (Fig. 20, 21, 22, 23, 24 and 25). Minute variations were observed in helical and sheet structures between secondary structures of proteins. Predicted catalytic site for lipase found amino acid sequence of FLDH and for serine proteases HDGGG which were similar in sequence and no variations were observed in all breeds.

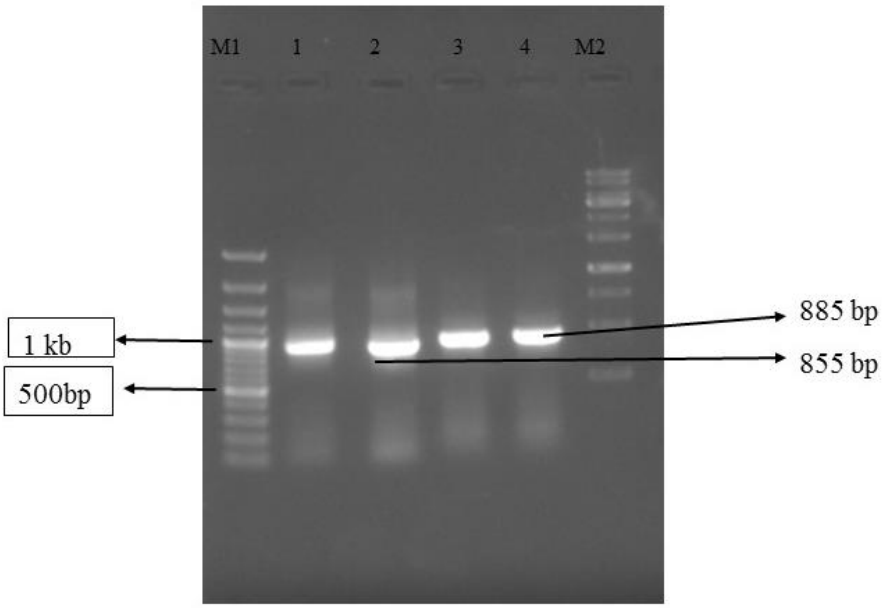


Plate 14: Agarose gel electrophoresis of confirmation of recombinant pGEM-T-*Bm*Lp gene and pGEM-T- *Bm*Sp gene construct with *Xba1 and SacI* restriction digestion.

M1 : 100 bp DNA ladder (Thermoscientific)

Lane 1 : Pure Mysore serine protease

Lane 2 : CSR2 serine protease

Lane 3 : Pure Mysore lipase

Lane 4 : CSR2 lipase

M2: 1 kb DNA ladder (Thermoscientific)

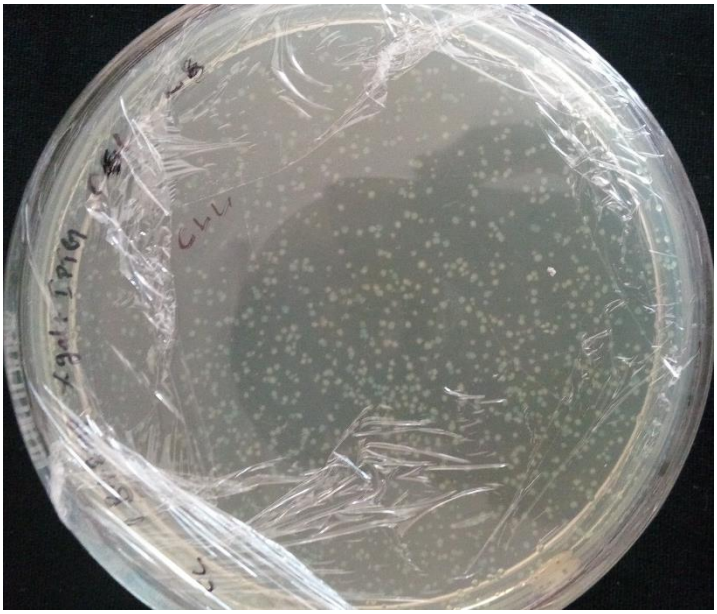


Plate 15: Transformed *E. coli* DH5α cells with *Bombyx mori* lipase- pGMET vector isolated from CSR2 breed.

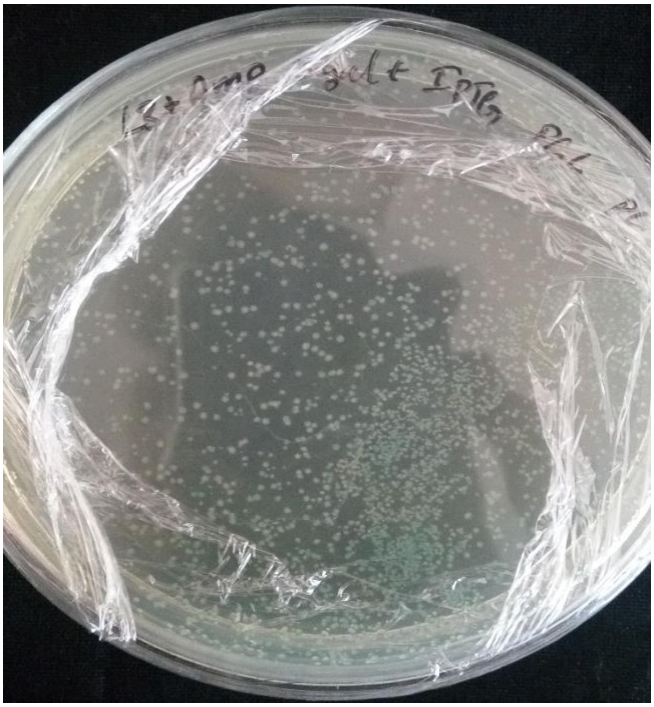


Plate 16: Transformed *E. coli* DH5α cells with *Bombyx mori* lipase- pGME-T vector isolated from Pure Mysore breed.

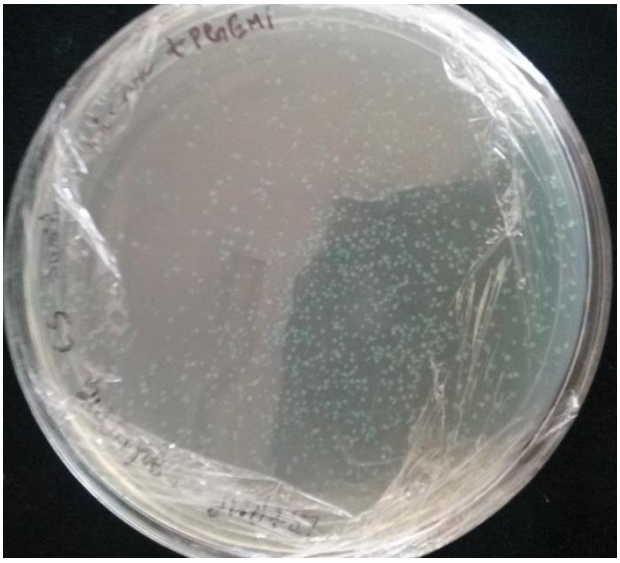


Plate 17: Transformed *E. coli* DH5α cells with *Bombyx mori* serine protease-pGMET vector, isolated from CSR2 breed.

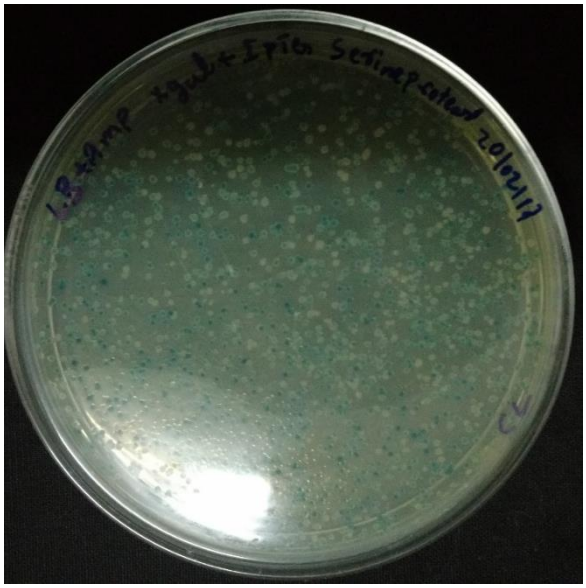


Plate 18: Transformed *E. coli* DH5α cells with *Bombyx mori* serine protease-pGMET vector, isolated from Pure Mysore breed.

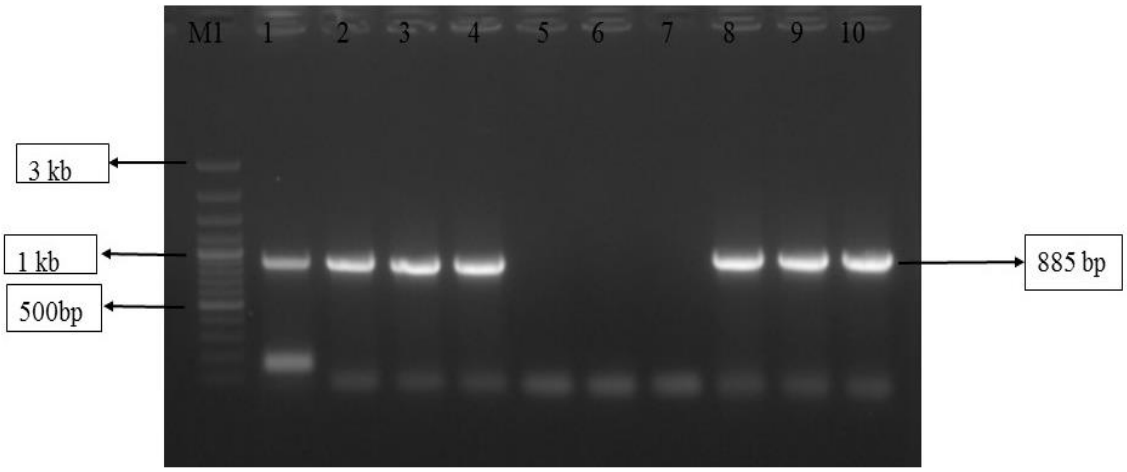


Plate 19: Agarose gel electrophoresis of colony PCR for confirmation of recombinant clones CSR2 Lp- pGEMT.

M1 : 100 bp DNA ladder (Thermoscientific)

Lane 1-10 : Clones in replicates

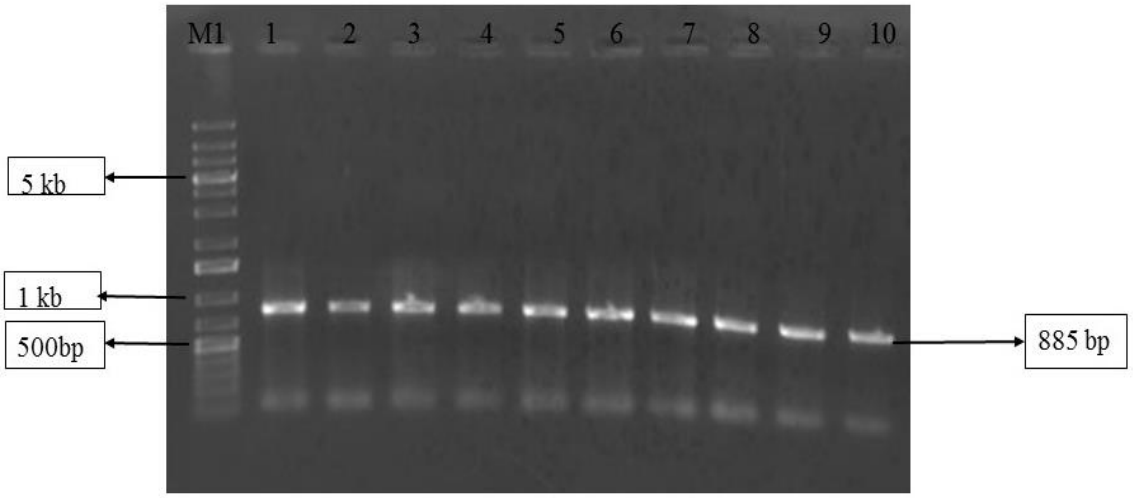


Plate 20: Agarose gel electrophoresis of colony PCR for confirmation of recombinant clones PM Lp- pGEMT clones.

M1 : 1 kb DNA ladder (GCC biotech)

Lane 1-10 : Clones in replicates

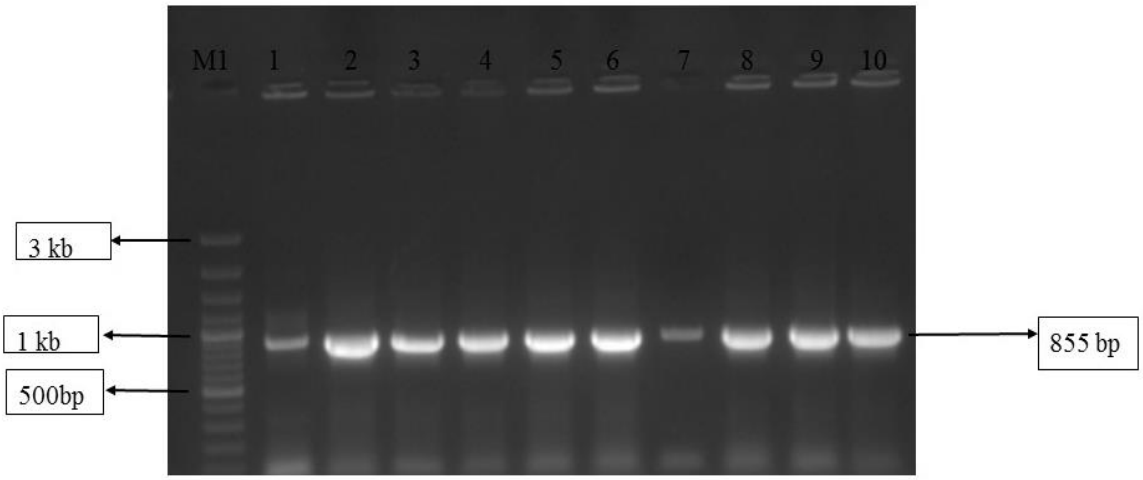


Plate 21: Agarose gel electrophoresis of colony PCR for confirmation of recombinant CSR 2 Sp- pGEMT clones.

M1 : 100 bp DNA ladder (Thermoscientific)

Lane 1-10 : Clones in replicates

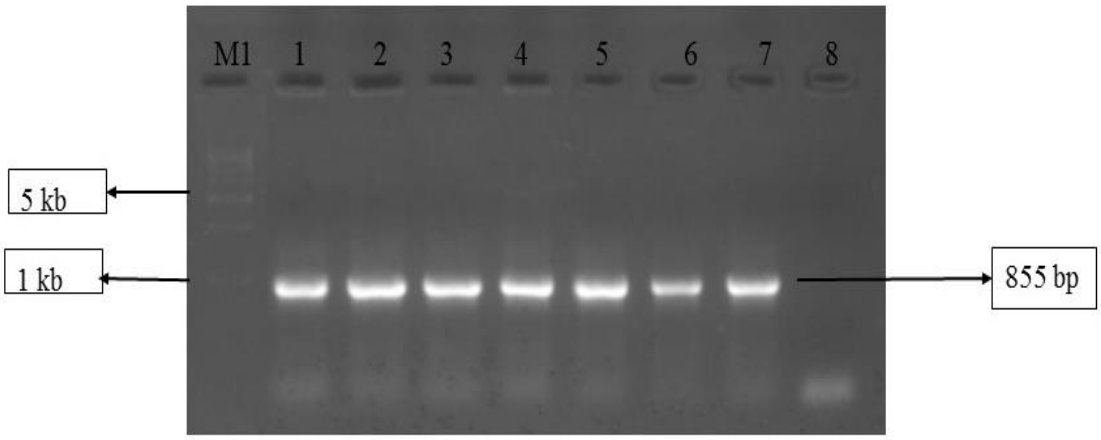


Plate 22: Agarose gel electrophoresis of colony PCR for confirmation of recombinant PM Sp- pGEMT clones.

M1 : 1kb DNA ladder (Genei)

Lane 1- 8 : Clones in replicates

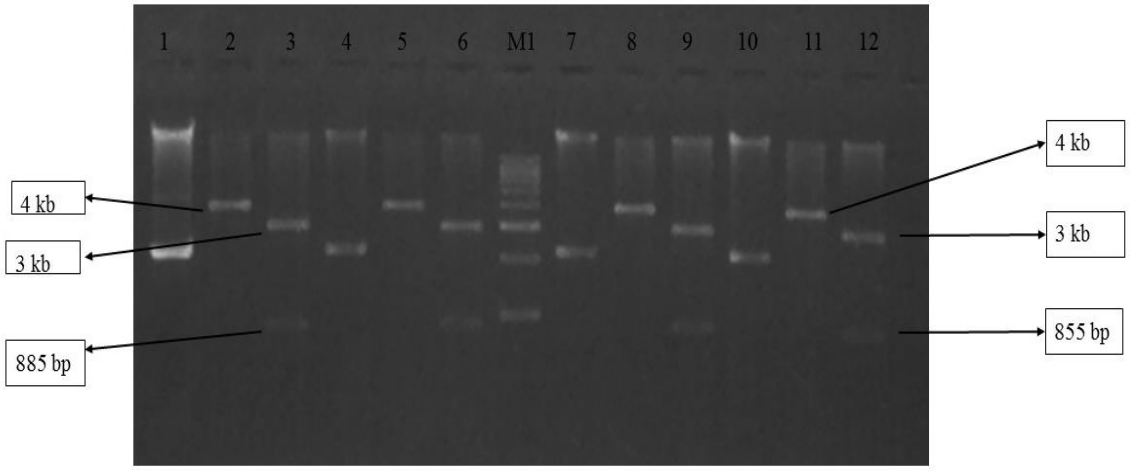


Plate 23: Agarose gel electrophoresis of confirmation of recombinant pGEMT-PmLp gene, pGEMT- PmSp, pGEMT- CSR2 Lp and pGEMT- CSR2 Sp gene construct with *BamH1*, and *Xba1 + SacI* restriction digestion.

M1: 1 kb DNA ladder (Genei)

Lane 1, 4, 7, 10: Undigested plasmid

Lane 2, 5, 8, 11: Single digested plasmid with size of 4 kb

Lane 3, 6, 9, 12: Double digested plasmid with size of 3 kb and 1 kb

Description	Max score	Total score	Query cover	E value	Ident	Accession
PREDICTED: Bombyx mori lipase-1 (LOC692513), transcript variant X1, mRNA	1555	1555	99%	0.0	99%	XM_012695610.2
Bombyx mori mRNA, clone: fmqV52I07_K05239	1555	1555	99%	0.0	99%	AK378535.1
Bombyx mori mRNA, clone: fmqV44B09	1555	1555	99%	0.0	99%	AK378456.1
Samia cynthia ricini strain Banma lipase mRNA, complete cds	1555	1555	99%	0.0	99%	DQ149986.1
Bombyx mori lipase-1 (LOC692513), mRNA	1555	1555	99%	0.0	99%	NM_001043501.1
Bombyx mandarina lipase mRNA, complete cds	1544	1544	99%	0.0	99%	AY945212.1
Bombyx mori lipase-1 mRNA, complete cds	1528	1528	99%	0.0	99%	AY945209.1
Bombyx mandarina lipase gene, complete cds	736	1530	97%	0.0	99%	EU700252.1
Bombyx mori strain Qiufeng Baiyu lipase gene, complete cds	736	1536	97%	0.0	99%	DQ286554.1
PREDICTED: Papilio xuthus pancreatic triacylglycerol lipase-like (LOC106114615), mRNA	198	198	65%	2e-46	73%	XM_013307884.1

Fig. 12: Sequencing result showing percent identity of Pure Mysore lipase gene with other reference sequences using BLAST analysis.

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
PREDICTED: Bombyx mori lipase-1 (LOC692513), transcript variant X1, mRNA	1574	1574	100%	0.0	99%	XM_012695610.2
Bombyx mori mRNA, clone: fmqV52I07_K05239	1574	1574	100%	0.0	99%	AK378535.1
Bombyx mori mRNA, clone: fmqV44B09	1574	1574	100%	0.0	99%	AK378456.1
Samia cynthia ricini strain Banma lipase mRNA, complete cds	1574	1574	100%	0.0	99%	DQ149986.1
Bombyx mori lipase-1 (LOC692513), mRNA	1574	1574	100%	0.0	99%	NM_001043501.1
Bombyx mandarina lipase mRNA, complete cds	1563	1563	100%	0.0	99%	AY945212.1
Bombyx mori lipase-1 mRNA, complete cds	1557	1557	100%	0.0	99%	AY945209.1
Bombyx mori strain Qiufeng Baiyu lipase gene, complete cds	736	1560	97%	0.0	99%	DQ286554.1
Bombyx mandarina lipase gene, complete cds	725	1543	97%	0.0	99%	EU700252.1

Fig. 13: Sequencing result showing percent identity of CSR2 lipase gene with other reference sequences using BLAST analysis.

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bombyx mori serine protease (Sp), mRNA	1458	1458	99%	0.0	97%	NM_001043438.1
Bombyx mori serine protease (sp-2) mRNA, complete cds	1441	1441	99%	0.0	97%	AY945211.1
Bombyx mandarina serine protease mRNA, complete cds	1380	1380	99%	0.0	96%	AY945210.1
PREDICTED: Bombyx mori collagenase (LOC101740143), mRNA	1352	1352	99%	0.0	95%	XM_004927633.2
PREDICTED: Bombyx mori collagenase (LOC101740285), mRNA	1352	1352	99%	0.0	95%	XM_004927634.3
Bombyx mori serine protease (LOC692357), mRNA	1330	1330	99%	0.0	95%	NM_001043361.1
Bombyx mandarina serine protease gene, complete cds	477	1387	99%	2e-130	95%	EU338400.1
Bombyx mori serine protease-2 gene, complete cds	401	1453	99%	1e-107	94%	DQ310733.1

Fig. 14: Sequencing result showing percent identity of PM serine protease gene with other reference sequences using BLAST analysis.

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bombyx mori serine protease (LOC692357), mRNA	1552	1552	98%	0.0	99%	NM_001043361.1
PREDICTED: Bombyx mori collagenase (LOC101740143), mRNA	1546	1546	98%	0.0	99%	XM_004927633.2
PREDICTED: Bombyx mori collagenase (LOC101740285), mRNA	1358	1358	98%	0.0	95%	XM_004927634.3
Bombyx mori serine protease (Sp), mRNA	1314	1314	98%	0.0	94%	NM_001043438.1
Bombyx mori serine protease (sp-2) mRNA, complete cds	1297	1297	98%	0.0	94%	AY945211.1
Bombyx mandarina serine protease mRNA, complete cds	1286	1286	98%	0.0	94%	AY945210.1
PREDICTED: Bombyx mori collagenase-like (LOC101738638), partial mRNA	444	444	27%	2e-120	99%	XM_012697054.1
Bombyx mandarina serine protease gene, complete cds	422	1302	98%	1e-113	92%	EU338400.1
Bombyx mori serine protease-2 gene, complete cds	396	1169	86%	6e-106	94%	DQ310733.1

Fig. 15: Sequencing result showing percent identity of CSR2 serine protease gene with other reference sequences using BLAST analysis.

```
#CSR2SP ATG AAG GTC TTC GCA GCA GTA CTG ATG GCG TTG GCG GCC GTG GTC GTG GCA GAA GAG CCC ATC GAA
#KGSP   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... G.. ... ...
#PMSP   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... G.. ... ...

#CSR2SP ATC AAG ATC GGT ATC CCC CGG GCC GAG AGT CTT AGA CGC GCC GAG GAA GCC GCT GAC TTC GAC GGT
#KGSP   .A. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#PMSP   .A. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

#CSR2SP GGT GGT TCT GCC GCC AAC GCT GGT GCT CAC CCC CAT CTT GCT GGA CTT GTG ATC GCA CTG ACG AAT
#KGSP   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..C ... ...
#PMSP   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..C ... ...

#CSR2SP ATC TGC GGA GCT TCC TTA CTG ACC AAC ACC CGC TCC GTG ACC GCC GCT CAT TGC TGG AGG ACC AGG
#KGSP   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..G ... ..C ... ... ..A ... ...
#PMSP   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..G ... ..C ... ... ..A ... ...

#CSR2SP CGT CAG TTC ACC CTC GCT CTT GGC ACA GCT AAC ATC TTC TCC GGA GGC ACC A-G GGT CAC CAC CTC
#KGSP   ... ... ... ... ... ... T.. ..T ... ... ... ... ... ... ..C ... ... .A. ... ... ... ...
#PMSP   ... ... ... ... ... ... T.. ..T ... ... ... ... ... ... ..C ... ... .-. ... ... ... ...

#CSR2SP GCA CGG CAG CTA CAA CAT GGA CAC CCT CCA CAA CGA CGT CGC CAT CAT CAA CCA CAA CCA TGT TGG
#KGSP   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#PMSP   ... ... ... ... ... ... .A. ..A ... .A. ... ... ... ... ... ... ... ... ... ... ... ...

#CSR2SP CAT CCA GCG CAT CAA CCT AGC CAG TGG AAG CAA CAA CTT TGC TGG TAC TTG GGC CTG GGC TGC CGG
#KGSP   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#PMSP   ... ... ... ... ... ... ... ... C.. ... ... ... ... ... ... ... ... ... ... ... ... ...


#CSR2SP CTC CGA TGC TGC TTC GGG AGC CAA CAA CCA ACA AAA ACG CCA AGT GAG CCT CCA GGT CAT TAC CAA
#KGSP   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#PMSP   T.. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... C.. ... T.. ... ... ... T..

#CSR2SP CCG CAC GTT TGG AAA CAA TGT GAT CAT TGC CTC CAC CCT CTG TGT TGA CGG CTC TAA CGG TCG CAG
#KGSP   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#PMSP   ... G.. T.. ... ... ..C .C. ... ... ..G ... ... ... ... ... ... ... ... ... ... ... ...

#CSR2SP AGA CTC CGG CGG CCC CTC TCA CCA TCG GCA GCG GCG GAA GCC GTC AAC TGA TCG GTA TCA CAT CGT
#KGSP   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#PMSP   ... ... ... ... ... -.. ... ... ... ... ... ... ..G ... ... .G. ... ... ... ... ... ...

#CSR2SP AAG GTT GCC CAG AGA GGC CAC CCT GCC GGC TTC GCC AGA GTC ACA TCC TTC AAC TCC TGT ATC CGG
#KGSP   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#PMSP   ... .C. ... -.. ... ... TT. ..C ... .C. ... ... ... ... ... ..T ..T ... ... ... ... ...

#CSR2SP AGC TCA ATA ATC ACT A
#KGSP   ... ... ... ... ... .
#PMSP   --- --- --- --- --- -
```

Fig. 16: Multiple sequence alignment of serine proteases isolated from PM, CSR2, KG breeds of silkworm.

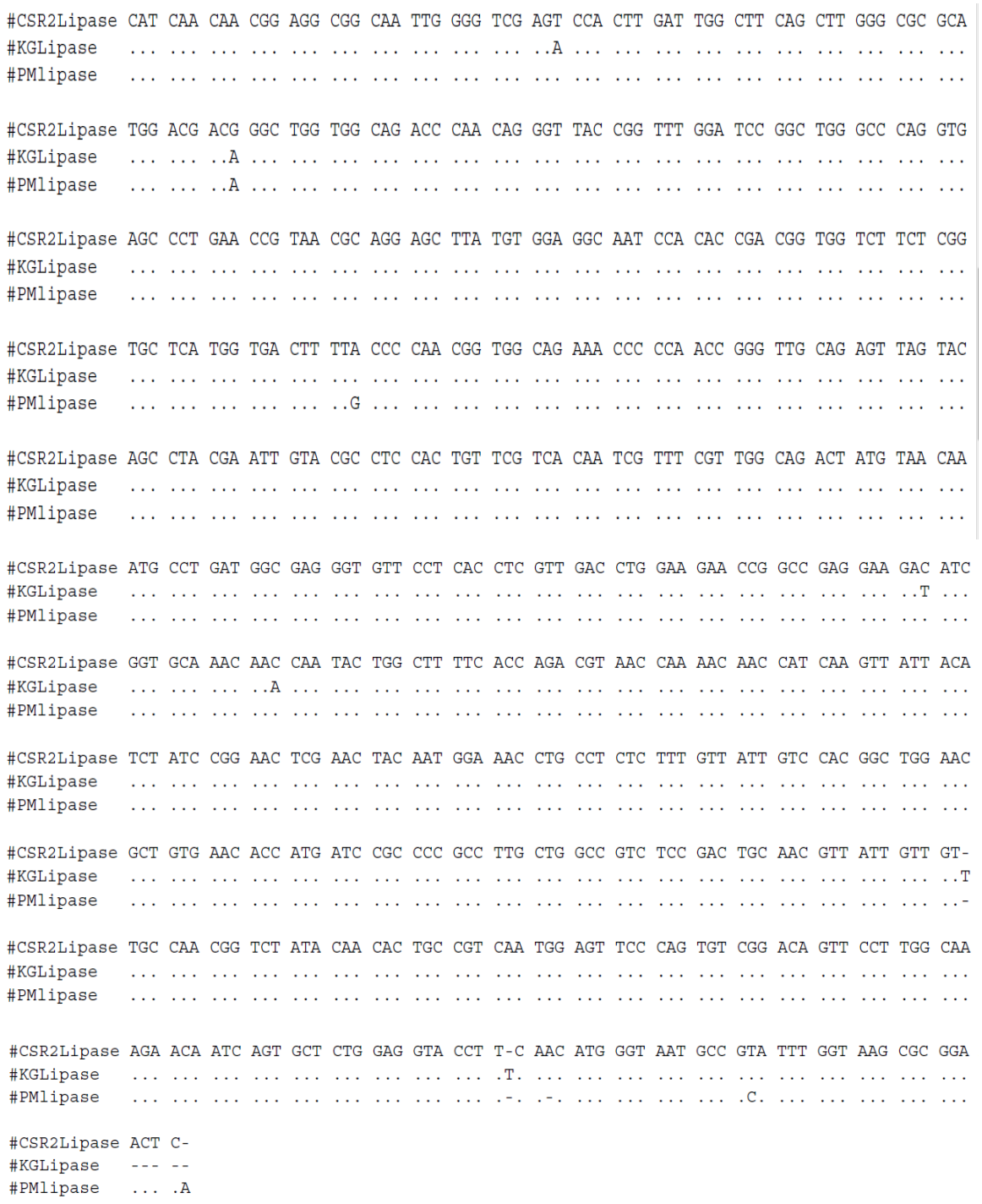


Fig. 17: Multiple sequence alignment of lipase isolated from PM, CSR2, KG breeds of silkworm.

Fig. 18: Multiple amino acid sequence alignment of predicted lipase isolated from PM, CSR2, and KG breeds of silkworm.

Fig. 19: Multiple amino acid sequence alignment of predicted serine protease isolated from PM, CSR2, and KG breeds of silkworm.

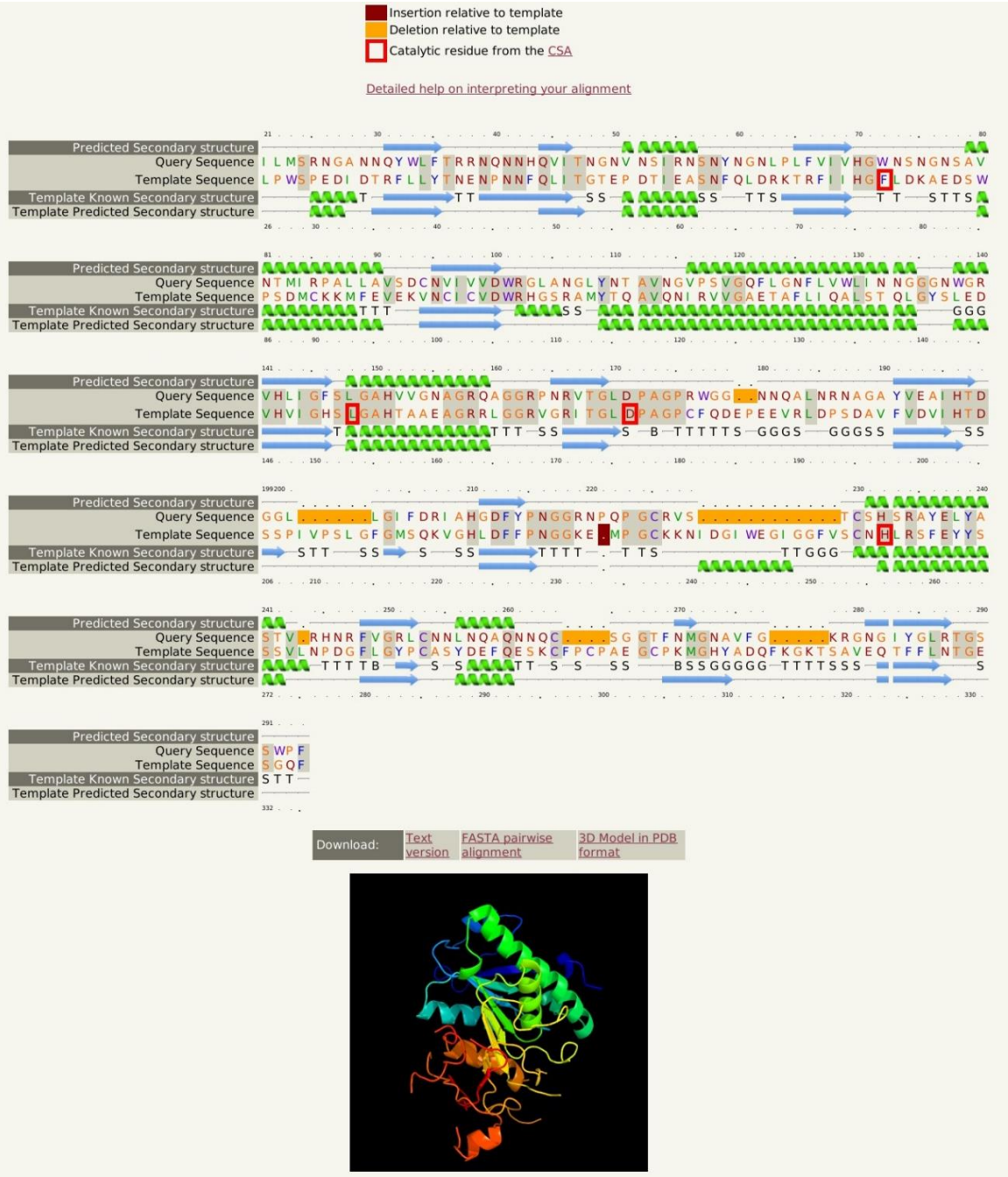


Fig. 20: Predicted secondary and tertiary structure of KG lipase using phyre 2.

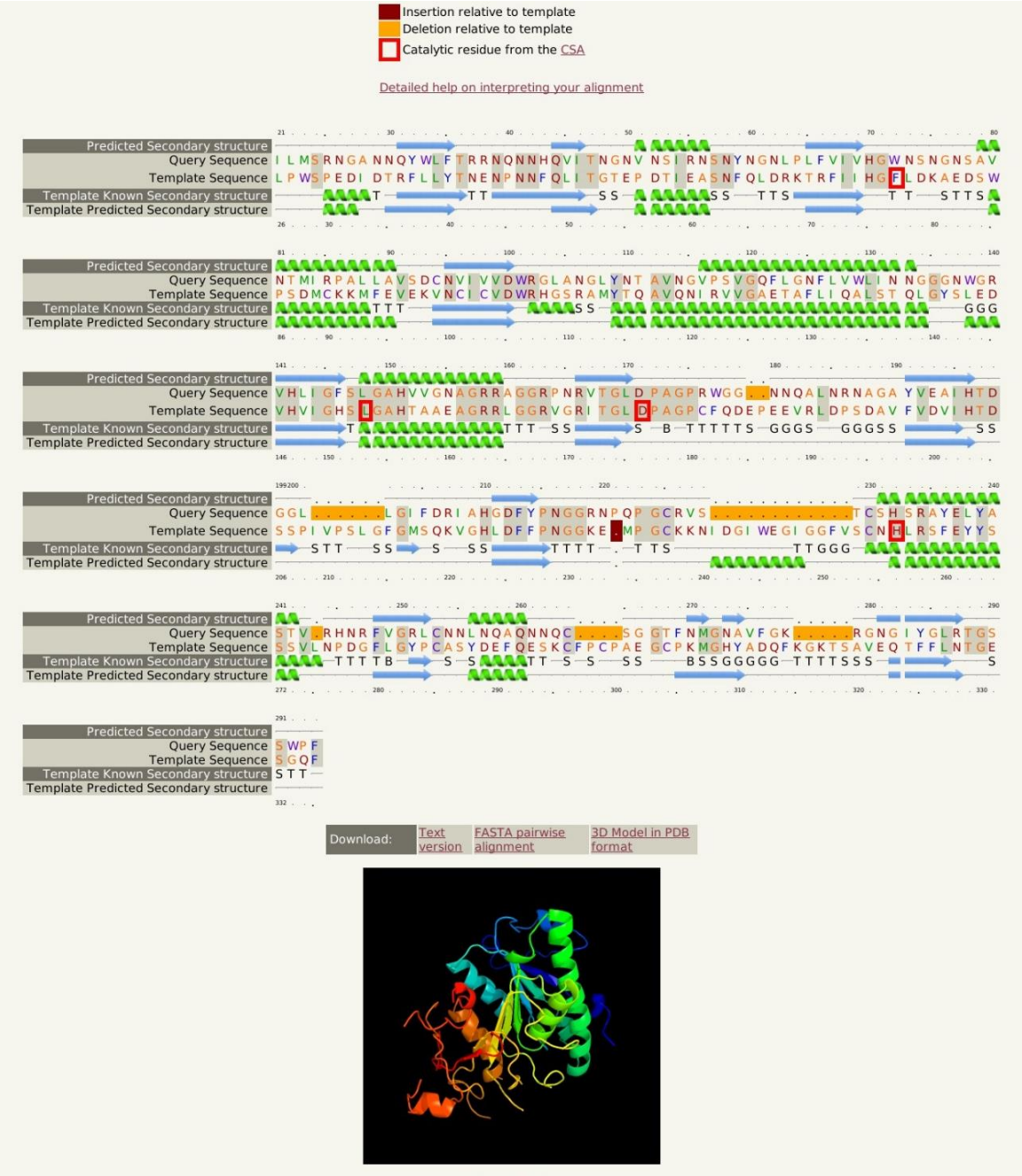


Fig. 21: Predicted secondary and tertiary structure of CSR2 lipase using phyre 2

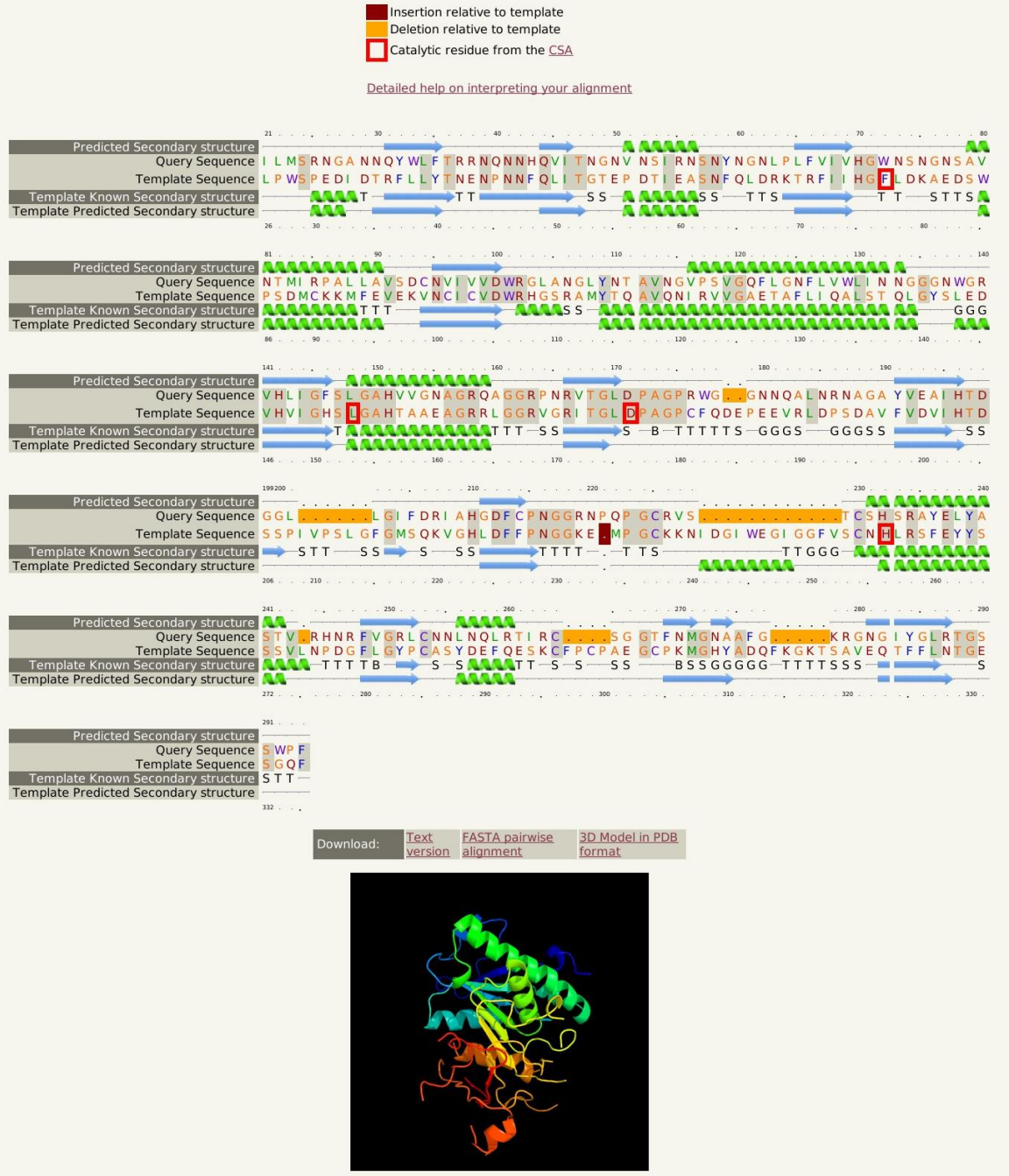


Fig. 22: Predicted secondary and tertiary structure of Pure Mysore lipase using phyre 2.

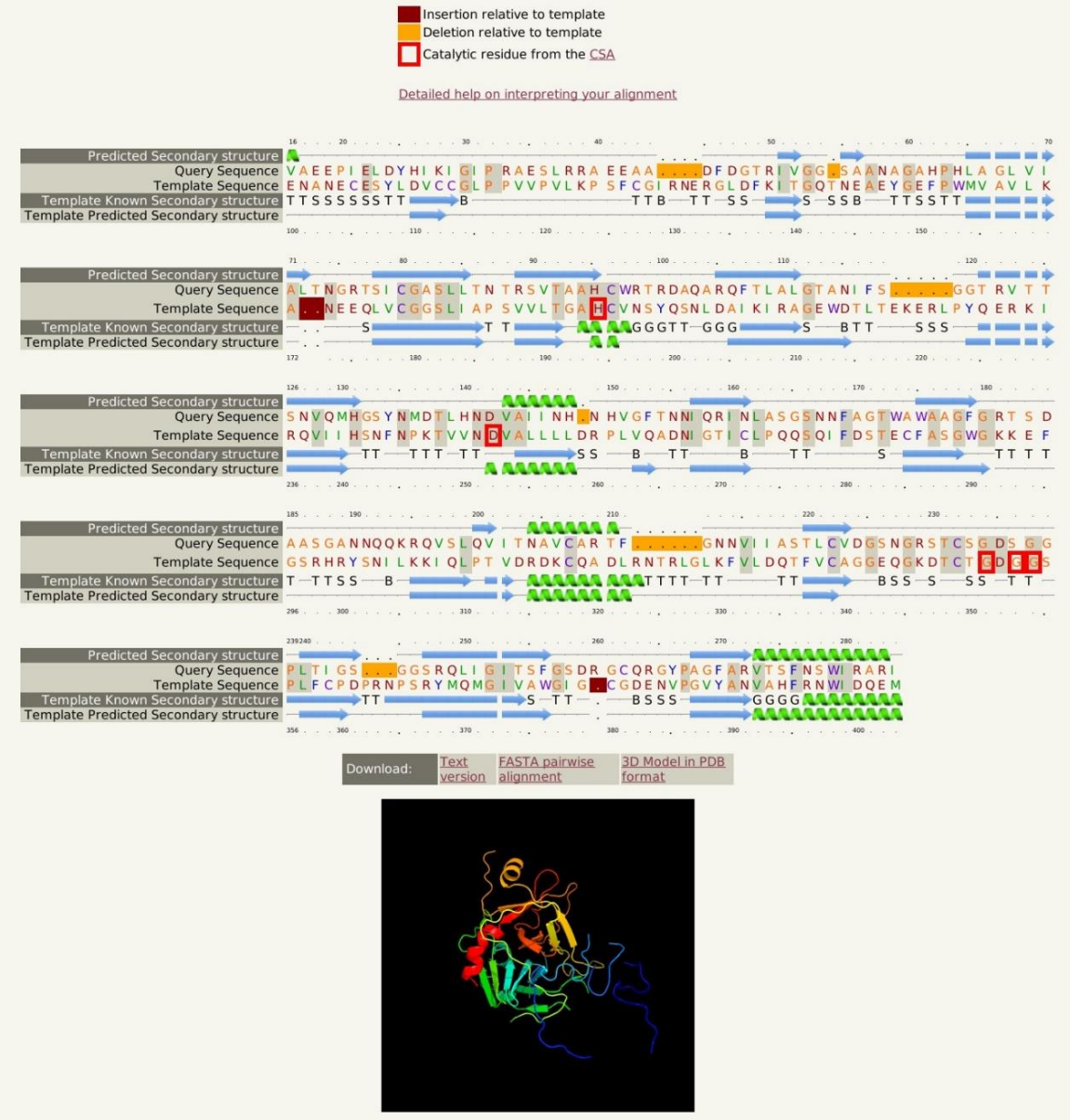


Fig. 23: Predicted secondary and tertiary structure of KG serine protease using phyre 2.

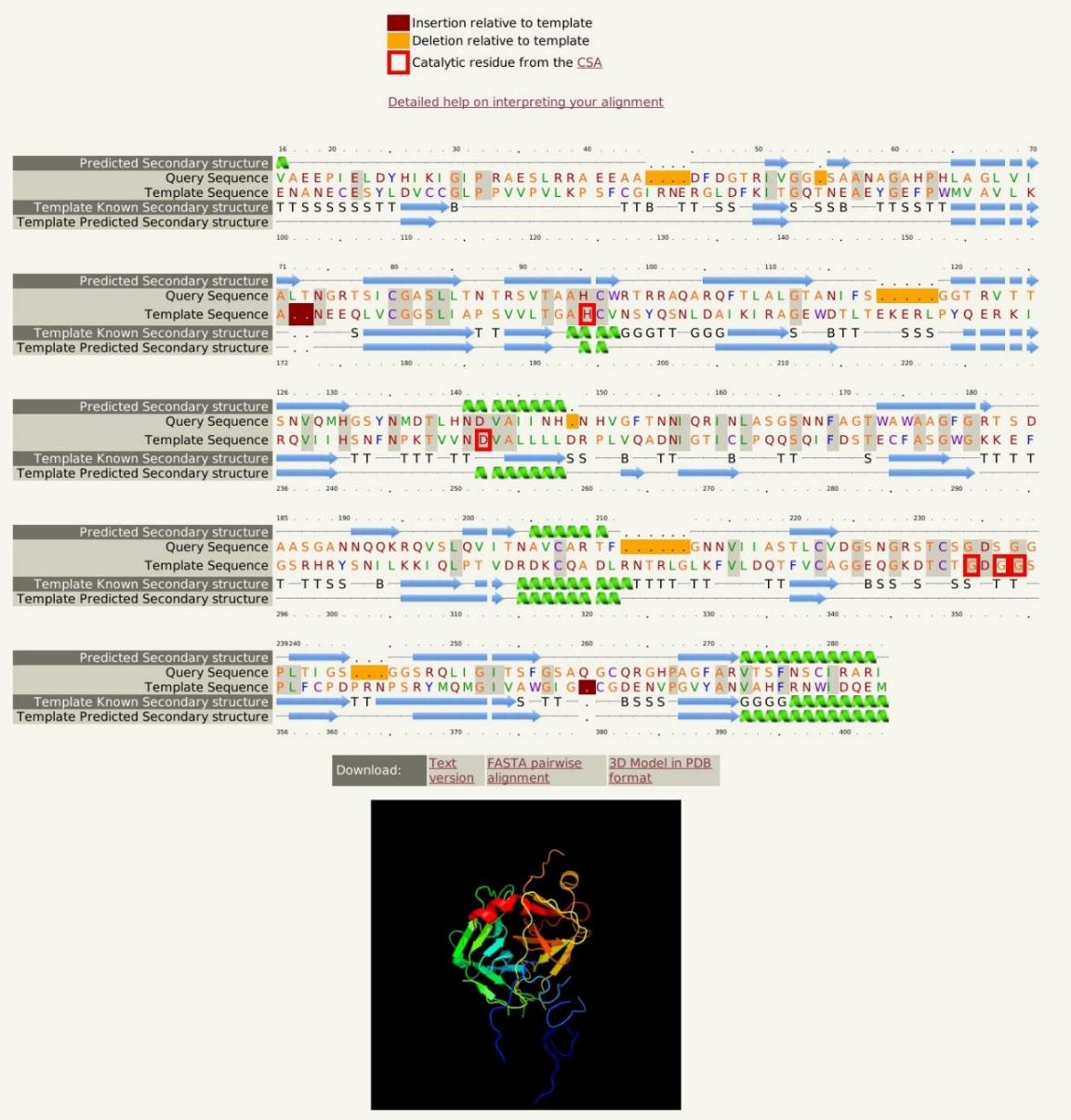


Fig. 24: Predicted secondary and tertiary structure of CSR2 serine protease using phyre 2

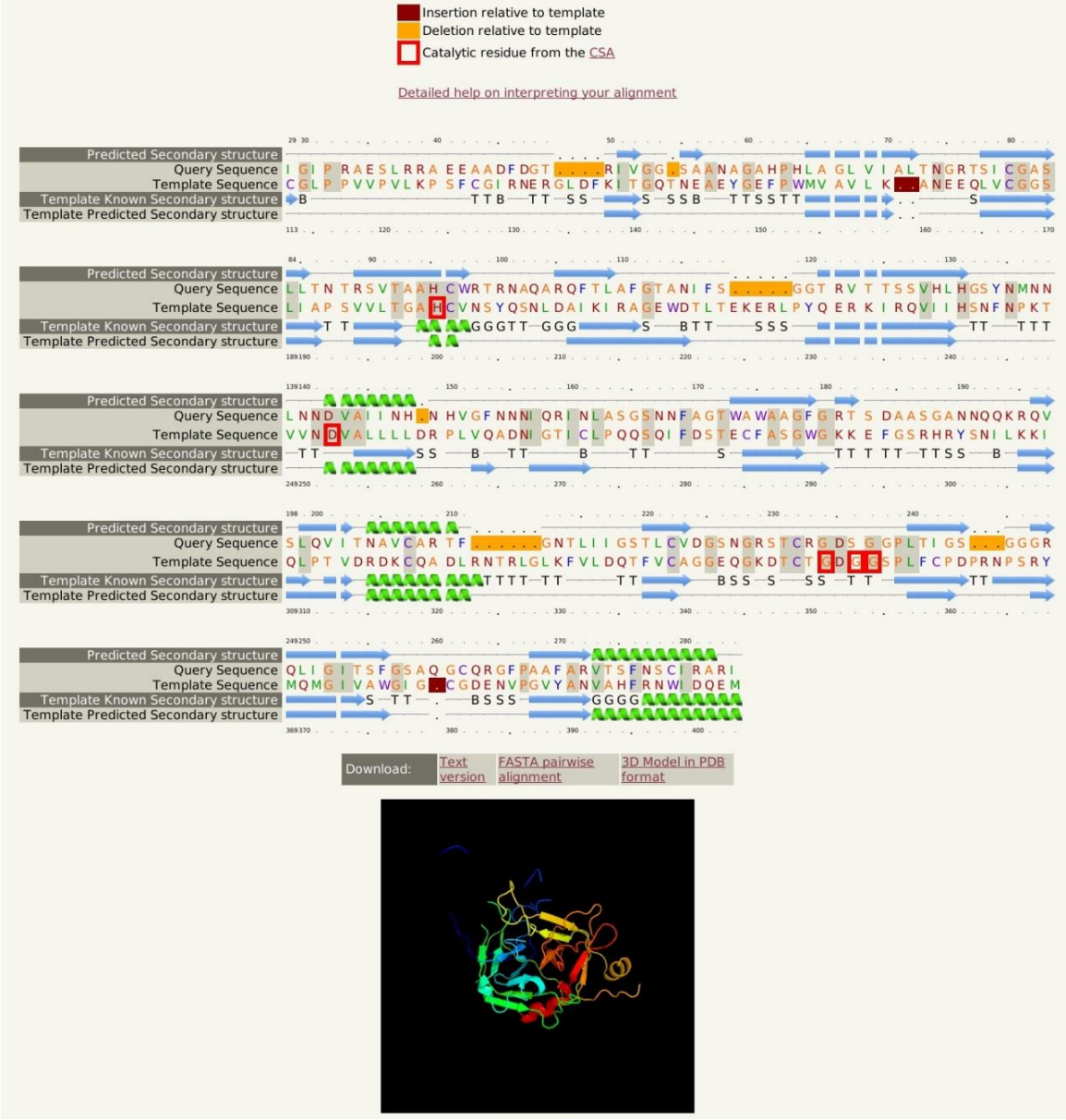


Fig. 25: Predicted secondary and tertiary structure of CSR2 serine protease using phyre 2.

V DISCUSSION

The *B. mori* nuclear polyhedrosis virus (*Bm*NPV) is one of the most harmful pathogens in the silk industry as it causes substantial damage in the production of silk (Tamura *et al*., 2000). Management of *Bm*NPV by traditional breeding methods have limitations such as enhancing pathogen resistance at the expense of the quality of economically important characteristics. It can be improved by transgenic technology such as overexpression of an endogenous or exogenous antiviral gene, RNA interference of the *Bm*NPV gene, or regulation of the immune pathway to inhibit *Bm*NPV at different stages of infection (Jiang and Xia, 2014).

The results of the present investigation have revealed interesting facts regarding diversity studies in *Bm*NPV using restriction endonuclease profiling and the findings regarding sequence comparision of antiviral protein genes from resistant and susceptible breeds are discussed in this section.

5.1 Molecular characterization of *Bombyx mori* nuclear polyhedrosis (*Bm*NPV) isolates using restriction endonucleases (REN).

In the present investigation infected *Bm*NPV isolates were collected from different locations of Karnataka, i.e. Devanahalli, Shidlaghatta, Kolar, Hosakote, Tumakuru and Ramanagar from these isolates polyhedra were purified and viral DNA was isolated. Restriction fragment analysis was done using the enzymes *BamHI, EcoRI, SmaI, NcoI,* and *BamHI+NcoI,* similar banding pattern was observed in all isolates but also sub molar variation in banding pattern was observed in Ramanagar and Tumakuru isolates. Based on banding pattern dendogram showed that Ramanagara isolate was separated in a single large cluster whereas there were two sub clusters formed, Tumakuru isolate was in one of these sub clusters and the remaining isolates were in the other.

Similar study was done by Peter *et al*. (2016), used AFLP marker to characterize the *Bm*NPV ioslates which were collected from different locations of Karnataka. Results obtained were showed Mandya isolate was more polymorphic. Phylogenetic analysis shown Mandya isolate separated in a large cluster and remaining isolate Kolar, Melur

divivided into a sub cluster and Hebbal, Devanahalli, isolates were grouped in other sub cluster.

Palhan and Gopinathan (1996), who found distinct differences for restriction enzyme *Hind III* for the Bangalore (BLR) and Dharwad (DHR) isolates of *Bm*NPV in Karnataka. Hong *et al*., (2000) also used restriction endonuclease analysis of viral DNA which has been used to distinguish and characterize closely related genotypic variants and species and observed *Bm*NPV isolates from wild stocks were closely related but still slightly different in banding pattern.

Similar differences in fragmentation profile of different insect NPVs have been noticed. Difference in fragmentation profile for MNPV and SNPV infecting *Heliothis* and *Orygyia pseudotsugata* has 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. 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 pathogenicity of virus. (Williams and Payne, 1984; Bilimoria, 1983; Burgess, 1977; Smith and Summers, 1979).

The reasons for these variations could be due to the insertion of host DNA into the viral genome, duplication of virus sequences, insertions, deletions and point mutations in the viral DNA. Thus molecular markers in viruses can be used to understand biodiversity of the viruses, 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.

The present study has shown genotypic variation between isolates for particular restriction enzymes. Further analysis of this variation can enhance the knowledge pathogenicity of *Bm*NPV can help with suitable measures to combat and diagnose this disease.

5.2 Cloning and characterization of the antiviral *serine protease* and *lipase* genes.

The total RNA was isolated from the midgut of silkworm, cDNA was synthesized from total RNA, using sequence specific primers of *lipase*s and *serine protease*s, antiviral genes were amplified and an amplified product of 885 bp for *lipase* and 855 bp for *serine protease* was obtained, which were cloned into pGMET cloning vector and transformed into DH5α *E. coli* cells. Recombinant clones were confirmed by colony PCR and restriction digestion and by sequencing. BLAST analysis shown that sequenced *lipase* gene showing 99 % of identity with other reference sequences whereas *serine protease* shown 94-96 % of identity.

Ponnuvel *et al.* (2003) isolated *Bmlipase* gene from silkworm and has shown its homology with other *lipases* and suggested that *lipases* act as physiological barriers against *Bm*NPV at the initial site of viral infection. Jiang *et al.* (2012) reported that overexpression of *lipase* decreased larval mortality against *Bm*NPV in silkworms.

Nakazawa *et al*. (2004), showed that the cDNA showed 94% homology with *B. mori serine protease* in the digestive juice of silkworm larvae which had strong antiviral activity against *Bm*NPV. He also studied concentrations of *serine protease*s at which mortality of silkworm were reduced. These studies have reported the antiviral activity of *lipase* and *serine protease* against *Bm*NPV, its characterization can enhance the knowledge of these enzymes.

5.3 Screening of a few breeds of the silkworm *Bombyx mori* L. for resistance to *Bombyx mori* nuclear polyhedrosis virus and sequence comparison of *serine protease* and *lipase* genes in these breeds.

5.3.1 LC_{50} studies for breed resistance

The lethal dose 50 concentration was determined by treating purified polyhedra at different concentrations on Kolar Gold breed of silkworm, and based on % of mortality and survivability lethal dose 50 concentration of 10^{-2} was determined. For screening of resistance against *Bm*NPV, Pure Mysore, Kolar Gold, and CSR2 breeds of silkworms were infected with 10^{-2} dilution of polyhedra and analyzed for mortality. Results shown

that maximum 65.56 percent of mortality was observed in CSR2 breed, minimum mortality 35.56 percent was observed in Pure Mysore, in Kolar Gold it was 48.89 percent.

Asha and Bhaskar, (2011) found similar observations in which multivoltine breed i.e. Pure Mysore showed minimum mortality, and the bivoltine breeds such as CSR2 recorded maximum mortality was observed.

It can thus be infer that Pure Mysore is a resistant breed for *Bm*NPV, and analyzing and characterizing antiviral properties in this breed can combat *Bm*NPV.

5.3.2 Sequence comparison of *serine protease* and *lipase* genes in Kolar Gold, Pure Mysore, and CSR2 breeds.

The total RNA was isolated from the midgut of silkworm from KG, PM and CSR2 breeds, cDNA was synthesized from total RNA, using sequence specific primers of *lipases* and *serine protease*s antiviral genes were amplified and obtained amplified product of 885 bp for *lipase*s and 855 bp for *serine protease*ss which were cloned into pGMET cloning vector and transformed into DH5α *E. coli* cells. Recombinant clones were confirmed by colony PCR and restriction digestion and by sequencing. BLAST analysis shown that the sequenced *lipase* gene i.e. CSR2 *lipase* and PM *lipase* shown 99 percent identity, and CSR2 *SP* and PM *SP* shown 94-96 percent with other reference sequences. Multiple sequence alignment was done to analyze sequence variation between breeds and it recorded that *serine protease* variations between Pure Mysore with Kolar Gold was 3.16% of variation, between CSR2 and Kolar Gold 1.29 and between Pure Mysore and Kolar Gold it was 4.4 percent. In *Bomyx mori lipase*, variation between CSR2 and Kolar Gold were 1.13 percent, between Kolar Gold and Pure Mysore 1.01%, and between CSR2 and PM a variation of 0.33% was noticed. These results probably indicates single nucleotide variations.

In a study by Silva *et al*., (2016) on tomato leaf miner *Tuta absoluta,* a major pest of tomato which is primarily controlled by the insecticide spinosad which targets the nicotinic acetylcholine receptor (nAChR). In leaf miners resistant and susceptibleto the

insecticides, cloning and sequencing of the nAChR α6 subunit from *T. absoluta*, the spinosad target-site revealed a single nucleotide change in exon 9 of the α6 subunit of the resistant strain, resulting in the replacement of the glycine (G) residue at position 275 with glutamic acid (E) and inferred that a nucleotide variation can lead to resistance and susceptibility.

Polymorphisms (SNPs) in the *Bm lipase* and *Bm serine protease* genes will be interesting to understand. These can create particular changes in the structure of these enzymes in the resistant and susceptible breeds, mainly in the binding sites of these enzymes to *Bm*NPV and thus inactivating it which could be of potential use.

VI SUMMARY

The results pertaining to the molecular characterization of nuclear polyhedrosis virus infecting *Bombyx mori* L. (*Bm*NPV) using restriction endonucleases, screening of few breeds for resistance against *Bm*NPV and cloning and characterization of *Bm*NPV antiviral encoding genes, *lipase* and *serine protease*s from Kolar Gold, Pure Mysore and CSR2 breeds are summarized in this section.

In the present study *Bm*NPV infected isolates were collected from different locations of Karnataka. Polyhedra were purified and viral DNA were isolated from these NPVs. Using restriction endonucleases banding pattern as analyzed for genotypic variation and it was found that Ramanagara isolate showed more variation for the specific endonucleases and followed by Tumakuru isolate, the remaining isolates were similar in banding pattern.

Screening of PM, KG, and CSR2 breeds for resistance revealed that PM was resistant and CSR2 was susceptible.

Cloning and characterization of antiviral protein genes from these breeds was done by isolating total RNA, synthesizing cDNA and amplification of the genes i.e. *lipase* and *serine protease* using sequence specific primers. Amplicon of size 885 bp and 855 bp corresponding to these genes was found , which were integrated into pGEMT cloning vector and transformed to *E. coli* DH5α cells, and confirmed for integration, sequencing analysis using BLAST tool, revealed that the *lipase* genes shown 99 % identity, whereas *serine protease*s revealed 96 % with other reference sequences. Multiple sequence alignment of these genes showed variation in nucleotides between breeds, between *lipases* it shown 0.33 % - 1.13 % variation and between *serine protease*s it was 1.29 % to 4.4 %.

Future line of work

- Characterization of antiviral protein genes, at protein levels in resistant and susceptible breeds can enhance the knowledge about antiviral activity.
- *Serine protease* activity in inhibition of ODV's has been revealed in earlier studies too, hence expression of these antiviral proteins in mulberry plants can increase the antiviral protein content and can resist against *Bm*NPV.

VII REFERENCES

ACHARYA, A., SRIRAM, S., SEHRAWAT, S., RAHMAN, M., SEHGAL, D. AND GOPINATHAN, K. P., 2002, *Bombyx mori* nucleopolyhedrosis virus: Molecular biology and biotechnological applications for large scale synthesis of recombinant proteins. *Curr. Sci.,* **83**: 455-465.

ARATAKE, Y., 1971, Strain difference of the silkworm *Bombyx mori* L. in the resistance to nuclear polyhedrosis. *J. Seric. Sci. Jpn.,* **42** (3): 230-238.

ASHA, M. H. AND BHASKAR, R. N., 2011, Silkworm breeds and their hybrids of *Bombyx mori* L. to *Bm*NPV stress. *Asian J. Bio. Sci.,* **9**(2): 220-223.

AVILE, N. Z., MURILLO, R., LASA, R., PINEDA, S., FIGUEROA, J. I., PATI, A. B., CORRALES, J. L. AND MARTINEZ, M. J. A., 2017, Genetic and biological characterization of four nucleopolyhedro virus isolates collected in Mexico for the control of *Spodoptera exigua* (Lepidoptera: Noctuidae). *J. Eco. Ento.*, **17**:1-11.

AXEN, A., CARLSSON, A., ENGSTROM, A. AND BENNICH, H., 1997, Gloverin, an anti bacterial protein from the immune haemolymph of *Hyalophora* pupae. *Eur. J. Biochem.,* **247**: 614-619.

BAIG, M., SHARMA, S. D., BALAVENKATASUBBAIAH, M., SAMSON, M. V. AND SASIDHARAN, T. Q., 1998, Relative susceptibility of different race of silkworm, *Bombyx mori* L. to nuclear polyhedrosis under natural and induced condition. *Sericologia*, **31**(3): 417-425.

BAIG, M., SHARMA, S. D., BALAVENKATESUBBAIAH, M., SAMSON, M. V., SASIDHARAN, T. O. AND NOAMANI, M. K. R., 1991, Relative susceptibility of different races of silkworm, *Bombyx mori* L. to nuclear polyhedrons under natural and induced conditions. *Sericologia,* **31**: 417-420.

BAO, Y. Y., LV, Z. Y., LIU, Z. B., XUE, J., XU, Y. P. AND ZHANG, C. X., 2010, Comparative analysis of *Bombyx mori* nucleopolyhedro virus responsive genes in fat body and haemocyte of *B. mori* resistant and susceptible strains. *Insect Mol. Bio*., **19**: 347-358.

BAO, Y. Y., TANG, X. D., LV, Z. Y., WANG, X. Y., TIAN, C. H., XU, Y. P. AND ZHANG, C. X., 2009, Gene expression profiling of resistant and susceptible *Bombyx mori* strains reveals nucleopolyhedro virus-associated variations in host gene transcript levels. *Genom.,* **94**: 138-145.

BARAMIDZE, N., KHUTSISHVILI, M., TSKARUASHVILI, Z. AND GUJABIDZE, I., 2016, Comparative study of Georgian breeds of mulberry silkworm according to the main biotechnological characteristics. *Ann. Agrar. Sci*., **14**(2): 56 -60

BILIMORIA, S. L., 1983, Genomic divergence among single-nucleocapsid nuclear polyhedrosis viruses of plusiine hosts. *Virol*., **127**: 15-23.

BLISSARD, G. W. AND ROHRMANN, G. F., 1990, Baculovirus diversity and molecular biology. *Annu. Rev. Entomol.,* **35**: 127-155.

BURGESS, S., 1977, Molecular weights of lepidopteran baculoviruses DNAs: derivation by electron microscopy. *J. Gen. Virol.,* **37**: 501-510.

CHENG, Y., WANG, X., DU, C., GAO, J. AND XU, J., 2014, Expression analysis of several antiviral related genes to *Bm*NPV in different resistant strains of silkworm, *Bombyx mori. J. Insect. Sci*., **14**: 1-9.

CHINNASWAMY, K. P. AND DEVAIAH, M. C., 1984, Susceptibility of different races of silkworm, *Bombyx mori* L. to aspergillosis caused by *Aspergillus tamarii* Kita. *Sericologia,* **24**: 513-517.

CHISTI, M. Z. AND SCHAF, K. A., 1990, Studies on the nuclear polyhedral disease of silkworm *B. mori* L. in Jammu and Kashmir state of India. *Indian J. Seric.*, **29**: 155-157.

CHITRA, C., KARNATH, N. G. K. AND VASANTHARAJAN, V. N., 1975, Diseases of the mulberry silkworm, *Bombyx mori* L. *J. Sci. Ind. Res.*, **34**: 386-401.

COOPER, D., CORY, J. S. AND MYERS, J. H., 2003, Hierarchical spatial structure of genetically variable nucleopolyhedro viruses infecting cyclic populations of western tent caterpillars. *Mol. Eco.,* **12**: 881- 890.

CORY, J. S., HAILS, R. S. AND SAIT, S. M., 1997, Baculovirus ecology. In: *The Baculoviruses* (L.K. Miller, ed.), New York: Plenum Press. pp. 301–339.

DEB, S., 2013, Studies on nuclear polyhedrosis (grasserie disease) of silkworm, *Bombyx mori* L. in West Bengal and its management. Thesis, Department of Zoology, The University of Burdwan, Burdwan – 713104, West Bengal, India, p. 34.

DIATCHENKO, L., LAU, Y. F. C., CAMPBELL, A. P., CHENCHIK, A., MOQADAM, F. AND HUANG, B., 1996, Suppression subtractive hybridization: A method for generating differentially regulatedor tissue-specific cDNA probes and libraries. *Proc. Natl. Acad. Sci. USA.*, **93**: 6025–6030.

DU, M. F., YIN, X. M., ZHANG, S. D., ZHU, B., SONG, Q. S. AND AN, S. H., 2012, Identification of *lipases* involved in PBAN stimulated pheromone production in *Bombyx mori* using the DGE and RNAi approaches. *PLoS ONE*, **7**: 2.

ENGELHARD, E. K. AND VOLKMAN, L. E., 1995, Developmental resistance in fourth instar *Trichoplusia ni* orally inoculated with *Autographa californica* M. nuclear polyhedrosis virus. *Virol*., **209**(2): 384-389.

FUNAKOSHI, M. AND AIZAWA, K., 1989, Antiviral substance in the silkworm gut juice against a nuclear polyhedrosis virus of the silkworm. *J. Invertebr. Pathol*., **54**(2): 151-155.

GETTIG, R. G. AND MC-CARTHY, W. J., 1982, Genotypic variation among wild isolates of *Heliothis* spp. nuclear polyhedrosis viruses from different geographic regions. *Virol.,* **117**: 245-252.

GOMI, S., MAJIMA, K. AND MAEDA, S., 1999, Sequence analysis of the genome of *Bombyx mori* nucleopolyhedro virus. *J. Gen. Virol.*, **80**: 1323–1337.

GORMAN, M. J. AND PASKEWITZ, S. M., 2001, *Serine protease*s as mediators of mosquito immune responses. *Insect. Biol. Mol. Biol*., **31**(3): 257-262.

GOVINDAN, R., NARAYANASWAMY, K. C. AND DEVAIAH, M. C., 1998, Principles of silkworm pathology, In: Seri scientific publishers, Bangalore, pp. 65

GOWDA, V. T. AND RAMAIAH, T. K., 1976, Effect of induced polyhedrosis on haemolymph transminases in the larvae of the silkworm *Bombyx mori* L. (Lep., Bombycidae). *J. Invertebr. Pathol.,* **28**: 271-272.

HAYAKAWA, T., ROHRMANN, F. G. AND HASIMOTO, Y., 2000, Pattern of genome organization and content in lepidopteran Baculovirus. *Virol.,* **278**: 1-12.

HAYASHIYA, K. AND MATSUBARA, F., 1971, Comparative experiments with the silkworm larvae reared on mulberry leaves and artificial diets: Comparison of antiviral activities in the digestive juice of larvae reared on natural and artificial diets. *Bull Fac. Text. Sci.,* **6**: 87-100.

HAYASHIYA, K. AND NISHIDA, J., 1968, Inactivation of nuclear polyhedrosis virus in the digestive of larvae reared on natural and artificial diets. *J. Appl. Entomol. Zool.,* **12**: 189-193.

HAYASHIYA, K. AND NISHIDA, J., 1976, The mechanism of formationof the red fluorescent protein in the digestive juice of silkworm larvae the formation of chlorophyllidaea. *J. Appl. Entomol. Zool*., **20**: 37-43.

HERNIOU, E. A., OLSZEWSKI, J. A., CORY, J. S. AND O'REILLY, D. R., 2003, The genome sequence and evolution of baculoviruses. *Annu. Rev. Entomol.*, **48**: 211-234.

HIRAI, M., TERENIUS, O., LI, W. AND FAYE, I., 2004, Baculovirus and dsRNA induce hemolin, but no antibacterial activity, in *Antheraea pernyi*. *Insect Mol. Biol*., **13**: 399-405.

HOFFMANN, J. A., 2003, The immune response in *Drosophila*. *Nature*, **426**(6962): 33-38.

HONG, H. K., WOO, S. D., CHOI, J. Y., LEE, H. K., KIM, M. H., JE, Y. H. AND KANG, S. K., 2000, Characterization of four isolates of *Bombyx mori* nucleopolyhedro virus. *Arch. Virol.*, **145**: 2351-2361.

HORTON, H. M. AND BURAND, J. P., 1993, Saturable attachment sites for polyhedron-derived baculovirus on insect cells and evidence for entryvia direct membrane fusion. *J. Virol.*, **67**: 1860-1868.

http://inserco.org/en/india

http://kaikocdna.dna.affrc.go.jp

http://www.csb.gov.in/assets/Uploads/rti-files/item-06-b01.pdf

HUNTER, F. R., ENTWISTLE, P. E., EVANS, H. F. AND CROOK, N. E., 1998, Insect viruses and pest management. Wiley and Sons, Chichester, pp. 48-62.

ISHIKAWA, S., 1958, Respiratory enzymes in the midgut of the cytoplasmic polyhedrosis infected silkworm *B. mori* L. *J. Sericult. Sci*., **27**: 99-103.

IWANAGA, M., SHIMADA, T., KOBAYASHI, T. AND KANG, W. K., 2007, Identification of differentially expressed host genes in *Bombyx mori* nucleopolyhedrovirus infected cells by using subtractive hybridization. *Appl. Entomol. Zool*., **42**: 151-159.

JIANG, L. AND XIA, Q., 2014, The progress and future of enhancing antiviral capacity by transgenic technology in the silkworm *Bombyx mori*. *Insect Biochem. Mol. Bio*., **48**: 1-7.

JIANG, L., CHENG, T. C., ZHAO, P., YANG, Q., WANG, G. H., JIN, S. K., LIN, P., XIAO, Y. AND XIA, Q. Y., 2012, Resistance to *Bm*NPV via overexpression of an exogenous gene controlled by an inducible promoter and enhancer in transgenic silkworm, *Bombyx mori*. *PLoS ONE*, **7**(8): 1-9.

KANG, L. Q., SHI, H. F., LIU, X. Y., ZHANG, C. Y. , YAO, Q., WANG, Y., CHANG, C., SHI, J., CAO, J., KONG, J., AND CHEN, K. P., 2011, Arginine kinase is highly expressed in a resistant strain of silkworm (*Bombyx mori*, Lepidoptera): Implication of its role in resistance to *Bombyx mori* nucleopolyhedrovirus. *Comparative Biochemistry and Physiology*, Part B, **158**: 230-234.

KOBAYASHI, M. AND KAWASE, S., 1981, Quantitative analysis of the metabolism on the isolated pupal abdomens of the silkworm *B. mori* L. infected with nuclear polyhedrosis virus. *J. Invert. Pathol.,* **38**: 4-11.

KOTANI, E., NIWA, T., TOKIZANE, M., SUGA, K., SUGIMURA, Y., ODA, K., MORI, M. AND FURUSAWA, T., 1999, Cloning and sequence of a cDNA for a highly basic protease from the digestive juice of the silkworm, *Bombyx mori*. *Insect Mol. Biol*., **8**: 299-304.

KRAPPA, R. AND MORSDORF, D. D., 1991, Identification of the very early transcribed Baculovirus gene *PE-38*. *J. Virol.,* **65**: 805-812.

KRAPPA, R., BEHN, A. K., JANNEL, R., DOERFLER, W. AND KNEBEL. M. D., 1992, Differential factor binding at the promoter of early Baculovirus gene *PE38* during viral infection: GATA motif is recognized by an insect protein. *J. Virol.*, **66**: 3494-3563.

KRISHNASWAMI, S. AND TIKOO, B. L., 1971, A comparative study of performance of pure races currently under rearing in Mysore state. *Ind. J. Seric.*, **10**(1): 66-71.

KUMAR, C. M. S., JACOB, T. K., DEVASAHAYAM, S., SILVA, S. D., JINSHA, J. AND RAJNA, S., 2015, Occurrence and characterization of a tetrahedral nucleopolyhedrovirus from *Spilarctia obliqua* (Walker). *J. Invert. Pathol.,* **132**: 135-141.

LAKSHMI, V. L., SUJATHAMMA, P. AND SIVAPRASAD, V., 2014, Evaluation and selection of potential parents tolerant to *Bombyx mori* nuclear polyhedrosis virus (*Bm*NPV) for development of disease resistant hybrids. *Intl. J. Plt. Ani. Envi. Sci.*, **4**: 332-336.

LAVINE, M. D. AND STRAND, M. R., 2002, Insect hemocytes and their role in immunity. *Insec. Biochem. Mol. Biol.*, **32**(10):1295-1309

LEE, H. H. AND MILLER, L. K., 1978, Isolation of genotypic variants of *Autographa californica* nuclear polyhedrosis virus. *J. Virol.*, **27**: 754-767.

LI, G., ZHOU, Q., QIU, L., YAO, Q., CHEN, K., TANG, Q, AND HU, Z, 2017, Serine protease *Bm-SP142* was differentially expressed in resistant and susceptible *Bombyx mori* strains, involving in the defence response to viral infection. *PLoS One*, e0175518

LU, M., SWEVERS, L. AND IATROU, K., 1998, The *p95* gene of *Bombyx mori* L. nuclear polyhedrosis virus; Temporal expression and functional properties. *Virol.*, **72**: 4789-4797.

LU, P., XIA, H., GAO, L., PAN, Y., WANG, Y., CHENG, X., LU, H., LIN, F., CHEN, L., YAO, Q., LIU, X., TANG, Q. AND CHEN, K., 2013, V-ATPase is involved in silkworm defense response against *Bombyx mori* nucleopolyhedrovirus. *PLoS ONE*, **8**(6): e64962.

MAEDA, S. AND MAJIMA, K., 1990, Molecular cloning and physical mapping of the genome of *Bombyx mori* L. nuclear polyhedrosis virus. *J. Gen. Virol.,* **71**: 1851-1855.

MASKOS, B. C. AND MILTENBURGER, G. H., 1981, SDS-PAGE comparative studies on the polyhedral and viral polypeptides of the nuclear polyhedrosis viruses of *Mamestra brassicae, Autographa californica* and *Lymantria dispar*. *J. Invertebr. Pathol.,* **37**: 174-180.

MIELE, S. A., GARAVAGLIA, M. J., BELAICH, M. N. AND GHIRINGHELLI, P. D., 2011, Baculovirus: Molecular insights on their diversity and conservation. *Int. J. Evol. Biol*., 379-424.

MILLER, L. K. AND DAWES, K. P., 1978, Restriction endonuclease analysis for the identification of baculovirus pesticides. *Appl. Environ. Microbiol.,* **35**: 411-421.

MILLER, L. K., 1988, Baculovirus as gene expression vectors. *Annu. Rev. Microbiol.,* **42**: 177-199.

MONDAL, M., TRIVEDY, K. AND KUMAR, S. N., 2007, The silk proteins, sericin and fibroin in silkworm, *Bombyx mori* L. - A review. *Caspian J. Env. Sci*., **5(**2): 63-76.

MONSMA, S. A., OOMENS, A. G. AND BLISSARD, G. W., 1996, The GP64 envelope fusion protein is as essential baculovirus protein required for cell-to-cell transmission of infection. *J. Virol*., **70**(7): 4607-4616.

NAKAZAWA, H., TSUNEISHI, E., PONNUVEL, K. M., FURUKAWA, S., ASAOKA, A., TANAKA, H., ISHIBASHI, J. AND YAMAKAWA, M., 2004, Antiviral activity of a *serine protease* from the digestive juice of *Bombyx mori* larvae against nucleopolyhedrovirus. *Virol*., **321**: 154-162.

NARAYANAN, K., 2004, Insect defence: Its impact on microbial control of insect pests. *Curr. Sci*., **86**(6): 800-814.

NATARAJU, B., BALAVENKATASUBBIAH, M., SELVAKUMAR, T. AND DATTA, R. K., 2001, Stabilised chlorine dioxide- an ideal disinfectant for sericulture. *Indian Silk*, **40**: 11-14.

NOBIRON, I., O'REILLY, D. R. AND OLSZEWSKI, J. A., 2003, *Autographa californica* nucleopolyhedrovirus infection of *Spodoptera frugiperda* cells: A global analysis of host generegulation during infection, using a differential display approach. *J. Gen. Virol.,* **84**: 3029–3039.

NOGUCHI, Y., 1991, Electron microscopic investigations on the multiplication of the variants of *Bombyx mori* L. nuclear polyhedrosis virus. *J. Seric. Sci. Jpn.,* **60**: 302-309.

PALHAN, V. B. AND GOPINATHAN K. P., 1996, Characterization of a local isolate of *Bombyx mori* nuclear polyhedrosis virus. *Curr. Sci.,* **70**: 147-153.

PETER, A., FATIMA, S. AND DEVAIAH, M. C., 1999, The viral, bacterial and protozoan diseases of the silkworm, *Bombyx mori.* In: Advances in Mulberry Sericulture. (Devaiah, M. C., Narayanaswamy, K. C. and Maribashetty, V. G. Eds.) CVG Publications, Bangalore, pp. 378-448.

PETER, A., PATIL, P., SHAMPRASAD, P., CHANDRAMOHAN, J., SHANTALA, J., VENKATESH, S. C. AND GOWDA, K. S. M., 2016, Molecular diversity of *Bombyx mori* nuclear polyhedrosis virus (*Bm*NPV) isolates using AFLP markers. *Indian J. Eco.,* **43** (1): 119-123.

PONNUVEL, K. M., NAKAZAWA, H., FURUKAWA, S., ASAOKA, A., ISHIBASHI, J., TANAKA, H. AND YAMAKAWA, M., 2003, A *lipase* isolated from the silkworm *Bombyx mori* shows antiviral activity against nucleopolyhedro virus. *J. Virol*., **77**(19): 10725 - 10729.

PONNUVEL, K. M., NITHYA, K., SIRIGINEEDI, S., AWASTHI, A. K. AND YAMAKAWA, M., 2012, *In vitro* antiviral activity of an alkaline trypsin from the digestive juice of *Bombyx mori* larvae against nucleopolyhedro virus. *Arch. Insect Biochem. Physi.,* **81**: 90-104.

POPHAM, H. J. R., SHELBY, K. S., BRANDT, S. L. AND COUDRON, T. A., 2004, Potent virucidal activity in larval *Heliothis virescens* plasma against *Helicoverpa zea* single capsid nucleopolyhedro virus. *J. Gen. Virol*., **85**: 2255-2261.

QUADT, I., MAINZ, D., MANS, R., KREMER, A. AND KNEBEL-MORSDORF, D., 2002, Baculovirus infection raises the level of TATA-binding protein that colocalizes with viral DNA replication sites. *J. Virol.*, **76**: 11123-11127.

ROHRMANN, G. F. AND BEAUDREAU, G. S., 1977, Characterization of DNA from polyhedral inclusion bodies of the nucleopolyhedrosis single-rod virus pathogenic for *Orgyia pseudotsugata*. *Virol.*, **83**: 474-478.

RONCARATI, R. AND KNEBEL-MORSDORF, D., 1997, Identification of the early actin rearrangement-inducing factor gene, *arif-1*, from *Autographa californica* multicapsid nuclear polyhedrosis virus. *J. Virol.*, **71**: 7933-7941.

SAGISAKA, A., FUJITA, K., NAKAMURA, Y., ISHIBASHI, J., NODA, H., IMANISHI, S., MITA, K., YAMAKAWA, M. AND TANAKA, H., 2010, Genome-wide analysis of host gene expression in the silkworm cells infected with *Bombyx mori* nucleopolyhedro virus. *Virus Res*., **147**: 166-175.

SAMBROOK, J. AND RUSSEL, D. W., 2001, Molecular Cloning: A laboratory Manual, 3rd edn. Cold Spring Harbor, N. Y.: Cold Spring Harbor Laboratory Press. ISBN 0-87969-576-5.

SAMSON, M. V., SINGH, R. N. AND SASIDARAN, T. D., 1998, Resham Jyothi a wide spectrum bed disinfectant. *Ind. Silk*, **38**: 9-11.

SEHGAL, D. AND GOPINATHAN, K. P., 1998, Recombinant *Bombyx mori* nucleopolyhedro virus harboring green fluorescent protein. *BioTechniques,* **25**: 997-1006.

SEHRAWAT, S., SRINIVASAN, N. AND GOPINATHAN, K. P, 2002, Functional characterization and structural modelling of late gene expression factor 4 from *Bombyx mori* nucleopolyhedro virus. *Biochem. J*., **368**: 159-169

SELOT, R., KUMAR, V., SEKHAR, S. C. AND KUMAR, P. G., 2010, Molecular characterization and expression analysis of *Bm*NOX in two strains of *Bombyx mori* with contrasting viral resistance phenotype. *Arch. Insect Biochem. Physiol.*, **73**: 163-175.

SELOT, R., KUMAR, V., SHUKLA, S., CHANDRAKUNTAL, K., BRAHMARAJU, M., DANDIN, S. B., LALORAYA, M. AND KUMAR, P. G., 2007, Identification of a soluble NADPH oxidoreductase (*Bm*NOX) with antiviral activites in the gut juice of *Bombyx mori*. *Biosci. Biotechnol. Biochem.,* **71**: 200-205.

SHARMA, R., GANIE, N. A., RUFAIE Z. H., KHAN, I. L. AND PARKASH, J., 2014, Screening of various bivoltine hybrids of silkworm *Bombyx mori* L. for disease susceptibility during monsoon season in Uttar Pradesh, *Intl. J. Adv. Biotech. Res.*, **4**(3): 352-356.

SILVA, W. M., BERGER, M., BASS, C., WILLIAMSON, M., MOURA, D. M. N., RIBEIRO, L. M. S. AND SIQUEIRA, H. A. A., 2016, A mutation (G275E) of the nicotinic acetylcholine receptor α6 subunit is associated with high levels of resistance to spinosyns in Tuta absoluta (Meyrick) (Lepidoptera: Gelechiidae). *Pest. Biochem, Physiol.*, **131**: 1–8

SINGH, K. K., SAVANURMATH, C. J. AND GOKHALE, S. B., 1999, Efficacy of some bed disinfectants against nuclear polyhedrosis virus of silkworm. *Proc. Natl. Sem. Tropl. Seric.,* UAS, Bangalore, pp. 233-234.

SMITH, G. E. AND SUMMERS, M. D., 1980, Restriction map of *Rachiplusia ou* and *Autographa californica* baculovirus recombinants. *J. Virol.,* **33**: 311-319.

SMITH, G. E. AND SUMMERS, M., 1979, Restriction maps of five *Autographa californica* MNPV variants, *Trichoplusia ni* MNPV, and *Galleria mellonella* MNPV DNAs with endonucleases *Sma*I, *Kpn*I, *Bam*HI, *Sac*I, *Xho*I and *Eco*RI. *J. Virol.,* **30**: 828-838.

SRIRAM, S., PALHAN, V. B. AND GOPINATHAN, K. P., 1997, Heterologous promoter recognition leading to high-level expression of cloned foreign genes in *Bombyx mori* cell lines and larvae. *Gene,* **190**: 181-189.

STILES, B. AND HIMMERICH, S., 1998, *Autographa californica* NPV isolates: Restriction endonuclease analysis and comparative biological activity. *J. Invertebr. Pathol.,* **72**: 174-177.

SUGIMORI, H., NAGAMANE, T. AND KOBAYASHI, M., 1990, Analysis of structural polypeptides of *B. mori* L. (Lepidoptera: Bombycidae), nuclear polyhedrosis virus. *App. Entomol. Zool*., **25**(1): 67-77.

SUMMERS, M. D. AND SMITH, G. E., 1978, Baculovirus structural polypeptides. *Virol.,* **84**: 390-402.

TAMURA, T., THIBERT, C., ROYER, C., KANDA, T., ABRAHAM, E., KAMBA, M., KOMOTO, N., THOMAS, J. L., MAUCHAMP, B. AND CHAVANCY, G., 2000, Germline transformation of silkworm using a piggyback transposon derived vector. *Nat. Biotech.,* **18**(1): 81-84.

TIKOO, B. L., KAPLIA, M. L. AND KRISHNASWAMI, S., 1971, Large-scale trial on the comparative performance of multivoltine x bivoltine hybrid of mulberry silkworm in Mysore state. *Indian J. Sericul.,* **10**(1): 57-65.

VAGO, C. AND ATGER, P., 1951, Multiplication massive des virus' D insects pendant la mue numphale. *Entomophaga,* **6**: 53-56.

VEPARIA, C. AND KAPLAN, D. L., 2007, Silk as a biomaterial. *Prog. Polym. Sci.*, **32**: 991-1007.

WATANABE, H., 1967, Development of resistance in the silkworm *Bombyx mori*, to peroral infection of a cycloplasmic polyhedrosis virus. *J. Invert. Pathol.*, **9**: 472-479.

WATANABE, H., 2002, Genetic resistance of the silkworm, *Bombyx mori* to viral diseases. *Curr. Sci.*, **83**(4): 439-446.

WILLIAMS, C. F. AND PAYNE, C. C., 1984, The susceptibility of *Heliothis armigera* larvae to three nuclear polyhedrosis viruses. *Annu. Appl. Biol.*, **104**: 405-412.

WYLLIE, A. H., KERR, J. F. R. AND CURRIE, A. R., 1980, Cell death: The significance of apoptosis. *Int. Rev. Cytol.*, **68**: 251-306.

XU, I. P., CHEN, K. P., LIU, M. H., YAO, Q., GAO, G. T. AND ZHAO, Y., 2008, Identification and characterization of *Bms3a* in *Bombyx mori* L. *African Journal of Biotechnology*, **7**: 3424-3430.

XU, I. P., CHEN, K. P., YAO, Q., LIU, M. H., GAO, G. T. AND ZHAO, Y., 2005, Identification and characterization of an NPV infection-related gene *Bmsop2* in *Bombyx mori* L. *Journal of Applied Entomology*, **129**: 425-431.

XUE, J., QIAO, N., ZHANG, W., CHENG, R. L., ZHANG, X. Q., BAO, Y. Y., XU, Y. P., GU, L. Z., HAN, J. D. J. AND ZHANGA, C. X., 2012, Dynamic Interactions between *Bombyx mori n*ucleopolyhedrovirus and its host cells revealed by transcriptome analysis. *J. Virol.*, **86**: 7345-7359.

YANG, N. V. AND ZHANG, C. X., 2012, Advances on *Bm*NPV functional genomics. *J. Biotechnol. Biomaterial*, **9**: 1-6.

YAO, H., FENG, X. W. AND GOKULAMMA K., 2006, Antiviral activity in the mulberry silkworm, *Bombyx mori* L. *J. Zhejiang Univ. Sci. A.*, **7**: 350-356.

YAO, H., HE, F., GUO, A., CAO, C., LU, X. AND WU, X., 2006, Gene analysis of an antiviral protein SP-2 from Chinese wild silkworm, *Bombyx mandarina* Moore. and its bioactivity assay. *Sci. China C. Life Sci.*, **51**: 879-884

APPENDIX I

50X TAE buffer (pH 8.0)

- Tris Buffer 242 grams
- Glacial acetic acid 57.1 ml
- EDTA 93 grams

Volume was made upto 1000 ml using distilled water

- Working concentration 1X TAE (2ml of 50X TAE/100 ml)

Loading dye (6X)

- 0.25 % Bromophenol blue – 0.25 g
- 0.25 % Xylene Cyanol FF
- 30 % Glycerol

Ethidium Bromide

- Ethidium bromide – 10 mg
- Double distilled water – 1 ml
- Note: Wrap the container with dark paper.

TE buffer

- 1M Tris-Cl – 10 ml
- 0.5M EDTA – 2 ml

Mix the solutions with distilled H_2O and make up the volume to 1000 ml.

Preparation of 0.8 % w/v Agarose gel

Weigh 0.8 gm of agarose, add in 100 ml of 1X TAE buffer and melt agarose using micro wave oven, after completion of melting kept for cooling, then add 4 µl of ethidium bromide, mix properly and pour on gel casting tray with required gel comb and allow for solidification.

APPENDIX II

Reagents for Plasmid Isolation

❖ **Extraction / Lysis buffer**

1) Alkaline lysis solution I

- 50 mM Glucose
- 25 mMTris-HCl (pH- 8.0)
- 10 mM EDTA (pH- 8.0)

2) Alkaline lysis solution II (freshly prepared)

- 0.2 N NaOH and
- 1 % SDS (w/v)

3) Alkaline lysis solution III

- 5 M Potassium acetate 60 ml
- Glacial acetic acid 11.5 ml
- Distilled water 28.5 ml

(Solution I and III were autoclaved and stored at 4 °C)

APPENDIX III

Media used in the experiments

I. LB media

Bactotryptone- 10 g/l

Bacto yeast extract- 5 g/l

Sodium chloride – 10 g/l

pH adjusted to 7.0

For Solid media Bacto agar of 1.5 % was included

IPTG

Dissolve 2 g IPTG in 8 ml water make up volume to 10 ml, filter sterilize, aliquot, store at –20°C

X-gal solution (2% w/v)

Dissolve X-gal at concentration of 20 mg/ml in water