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DEVELOPMENT AND EVALUATION OF CROSS BREED HYBRIDS FOR *Bm*BDV RESISTANCE THROUGH MOLECULAR MARKER ASSISTED SELECTION

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Abstract– *Bombyx mori* bidensovirus (*Bm*BDVs) are one of the causative agents of Flacherie disease which causes densonucleosis in the *B.mori* silkworm. In *Bm*BDV, resistance or the non-susceptibility is governed by a single recessive gene called non-susceptibility to *Bm*BDV (*nsd-2*). The deletion in *nsd-2* gene is associated with the *Bm*BDV resistance and hence can be used as a marker for molecular marker assisted breeding program. In the present study, cross breeds were developed by screening 17 multivoltine breeds popular among the breeders to develop hybrids for the Northeast regions of India. Out of 17 multivoltine, three showed presence of *nsd-2* resistant allele in heterozygous/homozygous condition. Among the breeds which carry *nsd-2* resistant allele, Lamerin breed showed complete homozygosity for *nsd-2* resistant allele through marker assisted selection and showed complete resistance to BmBDV infection and was taken as female parent. Another productive bivoltine breed CSR2R resistant to BmBDV was developed through marker assisted backcrossing method was taken as a male parent. The control hybrids Lamerin, CSR2 as well as the BmBDV resistant LamerinR and CSR2R were further crossed to develop crossbreed and these hybrids were confirmed for *Bm*BDV resistance through bioassay and absence of viral genome through conventional PCR.The BmBDV resistant hybrids showed >90% (90%) survivability compared to control susceptible hybrid with 10.66% survival post infection with the *Bm*BDV. Further, the BmBDV resistant hybrids (LamerinR X CSR2R) also showed significant improvement in the pre cocoon and the post cocoon traits at par with the (LamerinXCSR2) and there was no deviation or compromise in the economic characters. Hence, the developed cross breeds with the disease resistant trait along with the improved qualitative and quantitative traits will enhance the productivity which can be successfully commercialized in Northeastern part of India and Lamerin can also be used as foundation cross in Multi x Bi hybrids (Crossbreeds).

INTRODUCTION

The mulberry silkworm *Bombyx mori* L. is an economically important insect species which produces silk cocoons that have been bred in captivity for around 5000 years and now have been domesticated completely (Ito *et al.,* 2021). It has great economic value and is used as a model for genetics and molecular biology research. *Bm*BDVs or *Bm*DNV-2 are one of the causative agents of Flacherie disease which causes densonucleosis in the *B.mori* silkworm. *Bm*BDV infection is tissue specific and it occurs only in the columnar cells of midgut epithelium of infected larvae. The typical symptoms of *Bm*BDV infection include diarrhea, anorexia, retarded growth and flaccidity.

The silkworm *B.mori* like any other insect is susceptible to various pathogens. Among all the known silkworm pathogens, viruses are one of the major devastating ones that cause immense

economic losses for the sericulture farmers (Watanabe, 2002). Hence, genetic resistance of a silkworm breed against diseases would be one of the crucial factors that would determine its commercial success. Most of the viral disease resistance in silkworms is exhibited through polygenic interactions, however, in case of *Bm*BDV, resistance or the non-susceptibility to *Bm*BDV is governed by a single recessive gene called non-susceptibility to *Bm*BDV (*nsd-2*). This major resistance gene, *nsd-2* was identified as a plasma membrane localized putative amino acid transporter that may function as a receptor for *Bm*BDV and may be utilized as a marker for developing silkworm breeds and hybrids resistant to *Bm*BDV infection (Ito *et al.,* 2008). The natural deletion occurring in the *nsd-2* gene disrupting gene function has contributed to the evolution of *Bm*DNV-2 resistant silkworm breeds.

Several studies carried out so far have shown that certain strains of *B. mori* are susceptible to *Bm*BDV, while, some are resistant (Ponnuvel *et al.,* 2011; Watanabe and Maeda, 1981). The crosses made between the resistant and the susceptible breeds showed that resistance was controlled by both dominant and recessive genes. *Nsd-2* gene reported against *Bm*BDV can be used as functional marker for screening and development of *Bm*BDV resistant silkworm breeds. Earlier in Japan, 154 *B. mori* races have been reported as resistant to *Bm*DNV-2 (Furuta, 1995). In India, 70 multivoltine and 28 productive bivoltine germplasm resources were screened for *Bm*DNV-2 resistant/susceptible Indian *B. mori* races (Ponnuvel *et al.,* 2011). Four multivoltine races revealed the presence of *Bm*DNV-2 resistant gene. Later, 49 productive bivoltine breeds were screened and identified 28 breeds carrying the *nsd-2* resistant allele (Gupta *et al.,* 2019). Further, they have showed selection of *nsd-2* resistant allele in homozygous condition, as the *nsd-2* locus is sufficient for the development of *Bm*BDV resistant productive breeds (Gupta *et al*., 2019).

Breeding is an important tool utilized by many breeders has played a vital role in increasing the productivity in sericulture (Seshagiri and Raju, 2020). Cross breeds are produced by crossing multivoltine x bivoltine or vice-versa. In India, 70% of the mulberry raw silk production in India depends on multivoltine x bivoltine hybrids (CSB, Annual report, 2022). The raw silk production of crossbreed was substantially increased by 4.5% from 17,113 MT during 2020-21 to 17,877 MT during 2021-22 (CSB, Annual report, 2022). Crossbreed silk cocoons would continue to hold a place of importance because of its adaptation to the field constraints, economic/social compulsions and agro-climatic conditions. The general feeling in the Indian silk industry that lustre and stiffness of crossbreed silk is superior to bivoltine silk and is preferred over by the handloom sector (Dayananda *et al.,* 2016). The crossbreed production in Southern India is mainly by crossing females of Pure Mysore (PM) race with males of productive bivoltine breed, CSR2. Multivoltine breeds such as Pure Mysore, Nistari, Sarupat and Moria were reared on commercial scale in different parts of India especially in South, East and North Eastern states (Raghavendra Rao *et al.,* 2003).

Lamerin is a hibernating multivoltine silkworm breed which was reared in Northeast parts of India especially in Manipur region. It is a tetra moulter pure mulberry silkworm race with five larval ages. Lamerin race spins small, orange yellowcolored cocoons showing inferior economic traits and irregular shaped cocoons, but the silkworm breed is resistant and can survive inspite of infection by microsporidian Lb_{ms} (*Nosema bombycis*) for generations without causing much harm (Bhat and Nataraju, 2009; Bhat and Nataraju, 2005). The exploitation of hybrids and its usage was initiated in West Bengal and Jammu and Kashmir much later during 1956 and 1959, respectively (Thangavelu, 1997).

Later in 1960s cross breeding took place by crossing multivoltine Pure Mysore with exotic bivoltine breed such as J112, C108, J124 and NN6D and was found superior to pure breeds. During late 1960s and early 1970s Central Silk Board, India had developed new bivoltine breeds such as KA, NB4D2, NB7 and NB18 through systematic breeding programme(Datta, 1984) and utilized for the development of improved multivoltine x bivoltine hybrids namely PM xNB4D2 and PM x NB18, which ruled the silk industry of India for more than two decades covering major group of sericulture farmers (Seshagiri and Raju, 2020).These breeds were also used as male parent for preparation of F1 hybrids crossed with Pure Mysore and Nistari in South and Eastern Indian region, respectively (Raghavendra Rao *et al.,* 2003). The utilization of these cross breed combinations has bought quantum jump in cocoon production. During past few years a number of multi x bi hybrids was developed (Raghavendra Rao *et al.,* 2003).

Several studies were undertaken to evaluate and

identify silkworm hybrids to improve productivity through various breeding programs by the breeders (Singh *et al.*, 1990, 1998, 2001, 2004; Rao *et al.*, 2004; Seshagiri *et al.*, 2011; Seshagiri and Raju, 2016). Based on multiple trait evaluation studies, several cross breeds including MY1 x NB18, BL24 x NB4D2, BL23 x NB4D2, P2D1 x NB18 and PM x CSR2 were developed at Silkworm Breeding laboratory of CSRTI-Mysore and authorized by Central Silk Board, Bangalore for commercial exploitation. Presently >90% of crossbreed DFLs (Disease Free Layings) reared are from PM x CSR2 in South India. The existing crossbreeds have certain limitations like low cocoon shell, cocoon shell percentage and high renditta besides inferior silk quality (Dayananda *et al.*, 2016).

The tropical climatic conditions prevailing in West Bengal and Jharkhand regions are most unpredictable and beset with many problems such as fluctuation in temperature and humidity favouring pathogen infection. Hence,silkworm breeders focus on season specific, disease resistance breeds which can produce good quality and quantity silk throughout the year. Developing disease resistant cross breeds can help in producing quality cocoons. In that, *Bm*BDV infection is one of the major viral diseases which cause flacherie due to fluctuating temperature and humidity. Earlier reports indicated that screening of multivoltine and bivoltine breeds resistant to *Bm*BDV infection reveal some of the breeds to be resistant to *Bm*BDV virus even after inoculating high dosage of virus (Ponnuvel *et al.*, 2011; Gupta *et al.*, 2019, Murthy *et al.*, 2014). In earlier reports, Sarupat was the multivoltine breed which showed resistance to *Bm*BDV infection through *nsd-2* marker (Ponnuvel *et al.*, 2011). Further, CSR6R and CSR26R bivoltine breeds were screened and developed (Gupta *et al.*, 2019). CSR & TI, Berhampore had developed and authorized foundation cross Nistari x (SK6 x SK7), M6 (DP) C x (SK6 x SK7) and 12Y x BFC1 for hot climate conditions which was commercialized in North eastern parts of India (Dayananda *et al.*, 2016, CSB Annual report, 2022). But, there is no report on development of cross breeds which are resistant to *Bm*BDV infection. Therefore, there is need to develop *Bm*BDV resistant cross breeds.

MATERIALS AND METHODS

Screening and selection of silkworm breeds for *Bm*BDV resistance

The bivoltine and multivoltine silkworm breeds used in breeding programs in North eastern region were obtained from Central Sericultural Research & Training Institute (CSR&TI), Berhampore, West Bengal, India. In the present study 17 multivoltine breeds were screened for identification of *Bm*BDV resistance *nsd-2* gene. Along with these breeds, the earlier developed CSR2R bivoltine breed was utilized as a male parent for development of cross breeds. The moths of the parents that is used for the breeding were tested with PCR to identify the presence of *nsd-2* resistant allele.

Isolation of genomic DNA

Genomic DNA was isolated from midgut of moth using 2PK buffer (200 mM Tris Ph-8.0,25mM EDTA, 300mM NaCl, 2% SDS). The dissected midgut tissue was crushed with micro pestle and immediately 500 ml of 2PK buffer was added in 1.5 ml vial and the tissue was homogenized completely. The homogenized sample was centrifuged at 13000 rpm for 10 min to get a clear supernatant devoid of cell debris. The supernatant was transferred to a fresh vial and equal volumes of phenol:chloroform: isoamyl alcohol mixture (25:24:1) was added and centrifuged. The upper aqueous layer was collected into a fresh 1.5 ml vial and to it equal volumes of chloroform: isoamyl alcohol mixture was added and centrifuged. The aqueous layer was collected into a fresh 1.5 ml vial and equal volumes of chilled isopropanol was added and tubes were inverted several times to precipitate the genomic DNA and centrifuged to pellet the genomic DNA. The supernatant was discarded after the centrifugation and 700ul of 70% ethanol was added and centrifuged to remove the excess salt. The 70% alcohol in the tubes was discarded completely without leaving any traces of alcohol and air dried the pellet. All the centrifugations involved were carried out at 13,000 rpm for 5 min. The DNA pellet was resuspended in 50µl of autoclaved MiliQ water and the quality of DNA was analyzed on 1.2% agarose gel.

*Bm*BDV resistant *(nsd-2)* marker analysis and *Bm*BDV inoculum preparation

Based on earlier reports, two sets of primers were designed for the detection of *nsd-2* resistant and susceptible alleles (Gupta *et al.*, 2019). One for determining the resistant allele that had binding site within exon 4, while, the aa-trans1 reverse primer had binding site within exon 14. On the other hand the second set of primer was designed for

determining the susceptible allele, denoted as aa-trans3 forward primer that had binding site at exon 13 while the aa-trans 3 reverse primer targeted exon 14. The reverse primer sequence was identical for determining both resistant and susceptibility alleles (Table 1).

The PCR reaction conditions includes initial denaturation at 98 °C for 1 min, then 30 cycles of denaturation at 98 °C for 30s, annealing at 55 °C for 30s, extension at 72°C for 1 min and nal extension at 72 °C for 8 min. The amplied products were run on 1.2% agarose gel to identify resistant and susceptible alleles. The expected product size for *nsd-2* resistant allele was 1200bp and for *nsd-2* susceptible allele was 830bp. *Bm*BDV inoculum was prepared from fifth instar diseased larval samples showing typical acherie disease symptoms such as accid body, non-spinning worms and dark brown color collected from different sericulture farms for bioassay studies. The mortality rate with multiple infections was checked with specific primers of *N.bombycis*, *Bm*NPV and *Bm*BDV VD2 ORF1 infections with multiplex PCR (Table 1).

Selection for development of *Bm*BDV resistant multivoltine and bivoltine breeds and their hybrids

In the study, 17 multivoltine breeds were screened for the presence of of *Bm*BDV resistance *nsd-2*alleles either in heterozygous/homozygous conditions. Out of 17 breeds, 03 breeds showed the presence of nsd-2 resistnce alleles in homozygous/herterozyous condition, however, only Lamerin breed showed BmBDV resistance (+nsd-2/nsd-2) alleles in homozygous condition. The multivoltine breeds are indigenous and superior in their survival and hardiness but are relatively inferior in terms of qualitative traits, mostly the silk quality therefore the multivoltine parents are crossed with the bivoltine parents to develop cross breeds with improved survival and qualitative traits. In the northern and West Bengal regions, the cross breeds (Mutivoltine X Bivoltine crosses) are most popular because of its adaptability to the varied climatic conditions. Therefore to develop a hybrid (cross breed), the Lamerin breed was selected as a female parent and CSR2, a commercially exploited, productive bivoltine breed was used as a male parent for the breeding program. Female moths of Lamerin R (resistant to BmBDV) and male moths from CSR2R (Resistant to BmBDV) breed were crossed to prepare disease free laying (DFL) and individual moths were labelled and tested using *nsd-2* resistant and susceptible primers through conventional PCR for genotyping. Once the hybrids were developed, they were evaluated for the pre-cocoon and post cocoon parameters. The cross hybrid combination along with their parents was reared by following standard rearing techniques (Krishnaswami, 1973).

Bioassay studies to validate the BmBDV resistance in the hybrids

The bioassay studies wereconducted to check the resistance and susceptibility of selected silkworm breeds to *Bm*BDV infection. The newly hatched first instar larvae of breeds and cross breeds (hybrids) *i.e.*, Lamerin X CSR2 (susceptible, control) and LamerinR X CSR2R , BmBDV resistant hybrids were artificially inoculated with *Bm*BDV through two continuous feedings of mulberry leaves smeared with the 100 times diluted *Bm*BDV viral inoculum for *Bm*BDV infection. Subsequently, fresh mulberry leaves were fed to the silkworm till spinning. The uninfected and infected control for each breed was maintained separately. In each breed, the larvae

Table 1. List of primers used in screening and detection of *Bm*BDV, *Bm*NPV, *N.bombycis* infection in multiplex PCR,*nsd-2* resistant and susceptible allele

Sl. No.	Primer	Sequence (5′-3′)	Amplicon size(bp)	
1.	*nsd-2* resistant allele	Forward	TCTACGTGCTTTCATACTACGTATC	1200
		Reverse	TTCCTCACGTTTCTGAATTTCTCTTG	
2.	*nsd-2* susceptible allele	Forward	GGTAAGAGGTCCAACGCTGTTAAGT	830
		Reverse	TTCCTCACGTTTCTGAATTTCTCTTG	
3.	*Bm*BDVVD2 ORF1 segment	Forward	ACTCCTAGATGTGGTAATG	115
		Reverse	CTCCAAATCTGAATGGTAAA	
4.	*Bm* NPV	Forward	CGCGGATCCGGTACAAGGCCTTCGAAA	310
		Reverse	CCGTCCGGACCGGACCACATCCACCAATTCCAT	
5.	*Nosema bombycis*	Forward	AGACGAACAGCTCAGTAACTCTT	780
		Reverse	CGGCCATGCACCACTATCAT	

were maintained in triplicates with n=50. The mortality and survival percentage of larvae in control and infected samples were recorded. The total number of larvae in each breed that went for spinning cocoons was also recorded and the Effective Rate of Rearing (ERR %) was calculated. In addition to the disease resistance traits, it also important to evaluate the pre-cocoon and post cocoon parameters in the developed cross breed. Therefore, in the developed cross breed different economic characteristics such as fecundity, hatching%, larval weight, single cocoon weight, pupal weight, single shell weight (g) and shell ratio were recorded. Approximately 1.5 to 2.0 kg cocoons were randomly selected from the breed for post cocoon quality assessment.

Data collection and statistical analysis

Data are presented as mean of three independent experiments @ 50 larvae / experiment with standard deviation and standard error. Values were statistically analysed using the ANOVA test. The significant difference in all the parameters were indicated as i.e., * *p<.05*, ** *p<.001*, *** *p<.0001* compared to control using SPSS software version 23.

RESULTS

Development of *Bm*BDV resistant multivoltine and bivoltine breeds

To develop *Bm*BDV resistant homozygous lines, screening and selection of *nsd-2* resistant allele through marker assisted selection was employed. Out of the 17 multivoltine breeds that were screened, Lamerin, *C.nichi* and Sarupat showed *nsd-2* resistant allele in heterozygous/homozygous condition. In that, only Lamerin breed showed *nsd-2* resistant allele in homozygous condition with 100% resistant genotype, while the others exhibited a heterozygous genotype (Table 2 and Fig. 1). This Lamerin breed was selected as multivoltine parent for development of cross breed. For bivoltine selection, CSR2R breed developed was selected for crossing with multivoltine. Female moth of LamerinR and male moth from CSR2R breed were crossed and disease free laying (DFLs) were prepared. The individual moths were labelled and tested with PCR using *nsd-2* resistant and susceptible primers for genotyping. The results reveal, both male and female parents show *nsd-2* resistant allele in all DFLs (Fig. 2). This developed F1 hybrids were further evaluated for the pre-cocoon and post cocoon parameters.

Evaluation of BmBDV resistance and the economic traits of *Bm*BDV resistant cross breeds

In the bioassay studies, Lamerin breed did not show any symptoms of viral falcherie however, the growth and development was similar to the uninfected control. But, the visible symptoms of flacherie such as flaccidity, less intake of feed and

Table 2. *nsd-2* screening of 17 productive multivoltine races of North-eastern Indian region collected from CSR&TI, Berhampore

Sl. No	Breed/ Accession	Presence/ absence of *nsd-2* resistant allele	Frequency of different genotypes at nsd-2 locus (%)			Expected *Bm*BDV resistant individuals (%)
			+nsd-2/+nsd-2	*+nsd-2/**nsd-2***	***nsd-2/ nsd-2***	
1	M6 DPC	Absent	100	0	0	0
2	M12 W	Absent	100	0	0	0
3	Nistari 0017	Absent	100	0	0	0
4	Nistari 0018	Absent	100	0	0	0
5	Nistari 0019	Absent	100	0	0	0
6	Nistari Plain	Absent	100	0	0	0
7	Nistari (D)	Absent	100	0	0	0
8	Nistari (C)	Absent	100	0	0	0
9	Nistari Mark	Absent	100	0	0	0
10	Np1	Absent	100	0	0	0
11	12 Y	Absent	100	0	0	0
12	12y (Cr)	Absent	100	0	0	0
13	Mcon -4	Absent	100	0	0	0
14	Mcon-1	Absent	100	0	0	0
15	Sarupat	**Present**	0	24.5	85.5	85.5
16	C.nichi	**Present**	0	10.5	89.5	89.5
17	Lamerin	**Present**	0	0	100	100

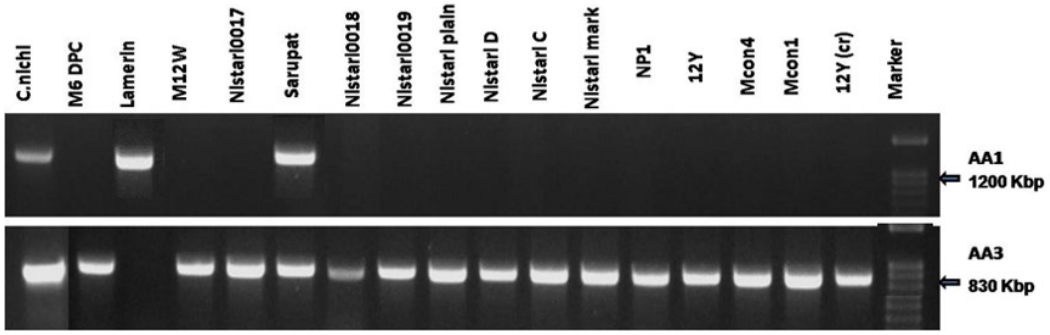


Fig. 1. Screening of productive multivoltine races from West Bengal region for the presence of *nsd-2* marker

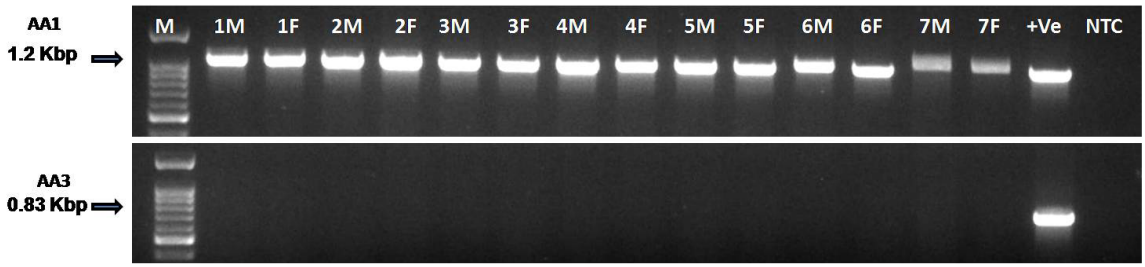


Fig. 2. The genotyping of the individual moths of LamerinR x CSR2R cross breed for *nsd-2* resistance and susceptible alleles; All DFLs resulting from the crossing moths show presence of *nsd-2* resistance allele and absence of susceptible allele indicating homozygous condition in both the male and female parents

failure to settle for molting were observed in CSR2 and control hybrid Lamerin x CSR2 10 days post infection compared to control (uninfected) and resistant breed (LamerinR X CSR2R). The infection was confirmed through PCR for the presence of viral genome in infected samples. However, LamerinR x CSR2R hybrid did not show any symptom of *Bm*BDV infection and conventional PCR also showed absence of viral genome from midgut tissue at different days after infection. Further, it was also observed that the mortality rate for *Bm*BDV infection was 90% in infected susceptible breedsand the hybrids, the infected resistant breeds showed >90% survivability and <10% mortality due to other infections such as *Bm*NPV and improper growth and development (Fig. 3). There was a significant difference in survival rate ($p<.0001$) between the infected resistant hybrids compared to the infected control batches (Fig. 4). The uninfected control and the resistant hybrids showed 91.66% survival rate.

The mean rearing performance data pertaining to nine economic traits of the cross breed along with their control are presented in Table 3. The harvested cocoons from both the susceptible and the resistant breeds were counted to compare the Effective Rate of Rearing (ERR %) after infection with *Bm*BDV at first larval instar. However, we found 0% ERR in the infected Lamerin x CSR2 hybrid having *nsd-2* susceptible allele as none of the larvae could survive till cocoon formation. However, > 88% (88.8%) ERR was observed in the infected LamerinR x CSR2R resistant hybrids, 89.33% in uninfected resistant hybrid, and 90.13% in the uninfected susceptible control hybrids indicating the efficiency of rearing.

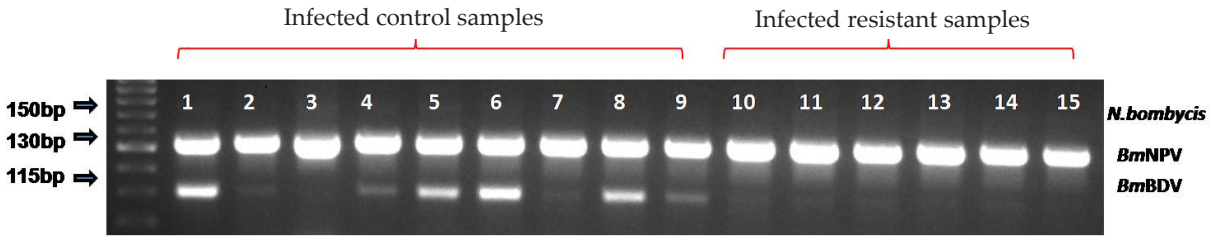


Fig. 3. Gel showing mortality rate in infected control and resistant with multiple infections with *Bm*NPV and *Bm*BDV with multiplex PCR.

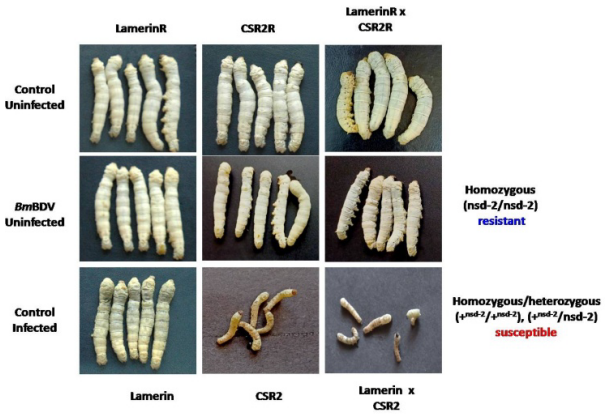
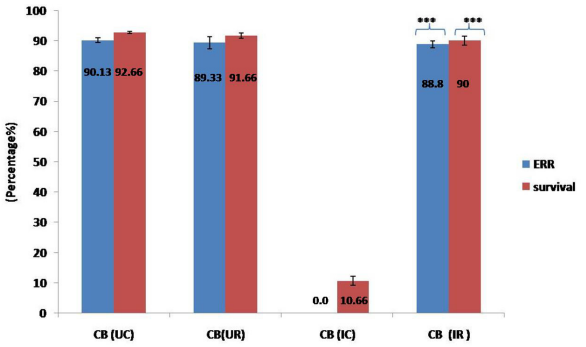


Fig. 4. Bioassay studies validate the *Bm*BDV resistance in marker assisted selected parents and Lamerin x CSR2 cross breeds.



*(UC)- uninfected control, (UR)- uninfected resistant, (IC)-Infected control, (IR)-Infected resistant

Fig. 5. Graphical representation of economic parameters of ERR and survivality of the cross breeds in resistant and control batches through two way ANOVA. The significant difference in all the parameters were indicated as * i.e., *p<.05*, ** *p<.001*, ****p<.0001*

The pre-cocoon and the post cocoon traits were compared between the resistant and control uninfected hybrids, which revealed no significant differences in pre cocoon sand post cocoon parameters and were at par with the control and therewas no deviation or compromise in the economic characters in the resistant lines (Table 3 and Fig. 5).

DISCUSSION

Majority of Indian Sericulture Industry is crossbreed oriented and contributes to more than 70% of mulberry raw silk production and more than ninety percent of the silk produced mainly by cross breeds (multivoltine x bivoltine) (Sheshagiri *et al.*, 2012) (CSB, Annual report, 2022). In general, silkworm races of Indian origin are poor in silk yield ascompared to Chinese and / or Japanese breeds (Thiagarajan *et al.*, 1993). It has been observed that silk productivity of crosses is better than that of parents (Hirobe, 1961; Kobayashi *et al.*, 1968). In India, the main challenge for the silkworm breeders is to develop suitable crossbreed to produce quality raw silk. Accordingly Central Silk Board and its institutes have developed and introduced several hybrids for improving the crossbreed cocoon productivity and silk quality (Narayanaswamy *et al.*, 2002; Dandin *et al.*, 2007, CSR and TI, Berhampore Brochure, 2014, 2015, 2020). Due to its hardness and lusture, cross breeds silk is superior to that of bivoltine silk and is preferred over by the handloom sector (Dayananda *et al.*, 2016). Selection of potential hybrid combination is one of the pre-requisites to the success of crossbreeds under the given environment conditions. However, most of the breeds are prone to diseases and pathogen infection due to fluctuation of climatic conditions. Thus, development of disease resistant crossbreeds is necessary for improvement of sericulture in different parts of India.

Table 3. Economic parameters and characteristics of parental and cross breeds

Sl. No.	Parameters	Lamerin	CSR2	LamerinR	CSR2R	Lamerin x CSR2	LamerinR x CSR2R
1.	Fecundity (no.)	332±11.13	457±10.58	333.33±7.63	438.66±8.50	404±2.64	408±21.70
2.	Hatching %	94.33±2.51	95.33±2.08	94.66±2.08	95.66±0.57	92.33±2.08	97.33±0.57
3.	Larval weight (V instar)	3.13±0.07	3.47±0.26	3.13±0.01	3.44±0.08	3.26±0.06	3.27±0.08
4.	Single cocoon wt. (g)	1±0.75	1.52±0.19	1.02±0.17	1.51±0.15	1.11±0.11	1.10±0.15
5.	Pupal weight (g)	0.87±0.07	1.22±0.18	0.88±0.15	1.22±0.14	0.92±0.10	0.92±0.14
6.	Single cocoon shell wt.(g)	0.12±0.09	0.29±0.04	0.12±0.02	0.29±0.02	0.17±0.01	0.17±0.02
7.	Cocoon shell ratio (%)	12.19±1.08	19.11±2.27	12.26±0.58	19.36±1.58	15.91±1.46	15.80±1.75
8.	ERR (%)	94.86±1.00	93	95.63±1.10	93.5	90.13±1.34	88.8±1.91
9.	Survival % (*Bm*BDV)	97.33±1.15	25	98±1	94.5	10.66±2.51	90±2.64(***)

(***) indicates significant *p value* at (*p <0.0001*) of survival % in the resistant crossbreed hybrids

In the present study, 17 multivoltine breeds were screened from West Bengal region of India through marker assisted selection among the monly Lamerin, a multivoltine breed showed complete resistance to *Bm*BDV infection due to the presence of *nsd-2* resistant alleles in homozygous condition. In earlier studies, it is reported that Lamerin breed is the only breed which survives with associated microsporidian (Lb_{ms}) infection from generations (Bhat and Nataraju, 2005). During the study it was observed that the cocoon characters of Lamerin are inferior compared to the bivoltine breeds but the breed was hardy and was able to survive under unfavorable conditions (Bhat and Nataraju, 2009). To develop cross breed resistant to *Bm*BDV the male and female moths of Lamerin breed were labelled after crossing and tested through conventional PCR for the presence of *nsd-2* marker in homozygous condition in both the parents. From the results it was found that in all the DFLs the *nsd-2* alleles were in homozygous condition, which was also confirmed byresistance to *Bm*BDV infection shown by the lamerin breed in our bioassay studies. Hence, our results reveal Lamerin breed is resistant to *Bm*BDV virus even though inoculated with high concentration of *Bm*BDV inoculum.

In other experiment, CSR2R bivoltine breed which was developed through backcross method (data not shown) was taken as bivoltine parent for development of cross breed. The female moth of LamerinR was crossed with male moth of CSR2R and DFLs were prepared. Both the male and female moths of individual DFLs were tested for the presence of *nsd-2* marker through conventional PCR. The PCR results reveal presence of nsd-2 alleles in homozygous condition indicating complete resistance to *Bm*BDV. To confirm this, first instar larva of F1 hybrid was collected and inoculated with *Bm*BDV virus inoculum and checked for the survival rate in triplicates. After 10 days post infection these resistant breeds were compared with control. In this, LamerinR x CSR2R hybrid did not show any symptoms of *Bm*BDV infection and conventional PCR also showed absence of viral genome from midgut tissue at different days after infection.

Further, the mortality rate for *Bm*BDV infection was 90% with 10.66% survival rate in infected control breeds while, the resistant breeds showed >90% survivability and <10% mortality due to other infections such as *Bm*NPV and improper growth and development. The infected susceptible breeds showed multiple infections with *Bm*BDV and *Bm*NPV. There was no significant difference in the growth and development of the larvae and cocoons between the infected resistant breeds and the uninfected control batches. Similar results were reported by Dayananda *et al.*, 2016 with high survival (>90%) coupled with higher cocoon weight recorded in the newly identified improved crossbreeds *viz.*, NDV6 x CSR51 and L14 x CSR50 (Dayananda *et al.*, 2016). Later, 12(Y) x BFC1 was identified as promising hybrid and compared with Nistari x SK6 x SK7 crossbreed showing high survival rate of 94-96% with improved economic parameters for NE region (CSR & TI, Berhampore, 2020). We also observed zero ERR% in infected susceptible control and 88.8% in infected resistant breeds and 89.33% in uninfected resistant breeds. Further, there was an significant improvement in the pre cocoon and the post cocoon characters of LamerinR X CSR2R cross breed that was at par with the control.

In conclusion, in the present study, *nsd-2* marker was utilized for development of LamerinR multivoltine resistant breed and LamerinR x CSR2R crossbreed for *Bm*BDV resistance without compromising economic characters of the control crossbreeds. Hence, the developed cross breeds of LamerinR x CSR2R will improve productivity due to its disease resistant trait particularly against *Bm*BDV resistance which can be successfully commercialized in Northeastern part of India. In this context, the multivoltine Lamerin breed can be used as parents for preparation of foundation crosses of ruling hybrids for Northeast India which could be reared successfully in all the seasons to fulfill the demand of bivoltine seed cocoons for preparation of Multi x Bi hybrids.

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Conflict of Interests

The authors have no relevant financial or non-financial interests to disclose; therefore the authors declare no conflict of interests.

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