DOUBLE MOLECULAR MARKERS-ASSISTED BREEDING OF SILKWORM, 
*BOMBYX MORI*, RESISTANT TO THE VIRUS, BMNPV AND BMDNV-Z

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ABSTRACT

In order to improve the classic techniques of silkworm breeding and achieve the integration of good economic traits and disease resistance traits, a cross-breeding and molecular marker assisted breeding technology combined was developed in this experiment. The practical strain dingsong and the strain NB, highly resistant to the BmNPV found from the library of silkworm races and strains in China, were selected as the breeding material. Through cross-breeding and backcrossing, the disease resistant gene was introduced into the economic strain Jingsong. At the same time, the strain Quifeng, resistant to the BmDNV, was used as the donor material of resistance gene. In this experiment, a new strain resistant to the two virus, BmNPV and BmDNV-Z, was obtained through the method of virus administration and molecular marker assisted breeding technology.

Key words: Bombyx mori, Molecular markers, BmNPV, BmDNV-Z, Breed.

INTRODUCTION

China has a history of over 5,000 years in silkworm raising. At present, China is still the biggest silkworm raising country in the world. Sericulture is practiced in more than 10 provinces by over 30 million farmer households in China. Its output of silkworm cocoons, silk and silk fabrics accounts for over 70% of the world’s total. Apart from China, sericulture is also practiced in Japan, the former Soviet Union and Brazil etc. Silkworm viral diseases, mainly caused by nuclear polyhedrosis virus, cytoplasmic polyhedrosis virus and densovirus, are major diseases causing great loss in silkworm raising, among which the disease caused by nuclear polyhedrosis virus (NPV) is the most disastrous to sericulture.

Silkworm nuclear polyhedrosis virus (NPV) was the first virus discovered in the history of insect virology studies (Lu 1998). Silkworm resistance to NPV disease is a very important economic character. The world’s silkworm raising countries suffered great loss due to NPV infection. Many of the current research base that the silkworm resistance to NPV is mainly controlled by a pair of autosomal dominant major genes and sex chromosomes minor genes (Chen et al. 1996). Several silkworm genes associated with resistance to the NPV, such as RFP were (Hayashiya et al. 1976), serine protease (Nakazawa et al. 2004), NADPH Oxidoreductase (Ruchita et al. 2007). The disease caused by densoviruses occurs in silkworm raising frequently. Silkworm nonsusceptibility to DNV-1 in B. mori lies under the control of two independent genes nonsusceptibility to BmDNV-1 (nsd-1) (Watanabe 1981) and non-infection to DNV-1 (Nid-1) (Eguchi et al.1991), but the resistance mechanism of silkworm to DNV-1 is still unkown. Presently the mechanism of silkworm resistance to DNV-2 has been studied more clearly. It is controlled by a recessive nonsusceptibility gene, nsd-2. Japanese scholars’ research believe that the virus resistance is caused by a 6-kb deletion in the ORF of a gene encoding a 12-pass transmembrane protein, a member of an amino acid transporter family, and expressed only in midgut (Ito et al. 2008). In the past 50 years, a series of studies have been focused on the two

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disease characters, infection pathways and control methods, and on silkworm’s resistance to NPV, the resistance’s inheritance characters and breeding methods aiming to develop highly resistant strains. Significant progress has been made in these aspects (Meng, 1982; Chen et al., 1991 and 1996).

From the breeding point of view, an effective way to reduce the loss of sericulture will no doubt to cultivate a new variety, which is strong resistant to pathogenic. But at present no other than our lab find the only germplasm with high resistance to BmNPV in the world. The traditional hybridization breeding method relies on selection of the resistant individuals after virus administration. Although this method can effectively obtain a variety of resistant varieties, but the process takes a long period of time and the select accuracy is poor. Meanwhile, the select process can be influenced by environmental factors. In recent years, with the rapid development of molecular markers, especially the results of whole-genome shotgun sequencing reported by Southwest Agriculture University and Genome Research Department of Japan (Mita et al. 2004; Xia et al. 2004), and several finished silkworm linkage maps based on molecular markers, including randomly amplified polymorphic DNA (RAPD) (Promboon et al. 1995), simple sequence repeat (SSR) (Miao et al. 2005), amplified fragment length polymorphisms (AFLP) (Tan et al. 2001), and single nucleotide polymorphisms (SNPs) (Kadono-Okuda et al. 2002), enable the molecular marker assisted breeding technology more mature. Breeding through molecular markers can track fragments linked to the resistance accurately, and the breeding efficiency is greatly enhanced. In this study, we are the first use molecular marker assisted breeding technology to cultivate a new variety, which is resistant to two virus, BmNPV and BmDNV has been used for the first time in this experiment. This can provide high quality, efficient, and stable production of a new variety resistant to NPV and DNV for silk production.

**MATERIALS AND METHODS**

**Silkworm strains:** Through our investigation to the NPV resistance of 340 species, from the library of silkworm races and strains in China, a Chinese local variety was found with high ability to resist NPV, whose median lethal concentration is 1000 times higher than sensitive species. After eight generation of system segration and filter of high virus concentration, a high resistance specie was obtained, named NB (RR). The sensitive specie was named 306 (rr). Near isogenic lines BC8, constructed by eight generation of hybridization and backcrossing, was used as a molecular marker validation material.

Through investigation to the DNV resistance of 220 species, from the library of silkworm races and strains in China, the silkworm strain Quifeng (nsd-Z /nsd-Z) highly resistant to DNV and the silkworm strain Huaba35 (+nsd-Z / +nsd-Z) highly susceptible to DNV was selected as material. Current production variety Jingsong (rr, +nsd-Z / +nsd-Z) was selected as breed parents for improving the resistance to virus.

**Selection and validation of molecular marker:**

Genome DNA was extracted by the method of Bender et al. (Bender et al. 1983). Molecular marker of NPV resistance (R<sub>npv</sub>) was selected refer to the SCAR sequence (AY380833) published on the GenBank, then a pair of specific primer (anti-NPV-F: 5′-GCTACGACCCAGACCTGACTC-3′ anti-NPV-R 5′-GCCTGGCAGCTTAATG TAACA-3′) was synthesized; Molecular marker of DNV susceptibility (S<sub>dnv</sub>) linked to the sensitive gene was selected refer to the reference (Liu et al. 2008), then a pair of specific primer (sus-DNV-F: 5′-CCGACGGAATGATGG AATCG-3′ sus-DNV-R 5′-CCGACGGAATGATGG AATCG-3′) was synthesized according to the marker’s sequence.

**FIG. 1:** Amplification result of primer anti-NPV-F and anti-NPV-R against the populations of NB, 306 and near isogenic lines BC<sub>8</sub>. Lane 1: Molecular marker; Lane 2: NB; Lane 3: BC<sub>8</sub>; Lane 4: 306.
To validate the molecular marker of NPV resistance ($R_{NPV}$) obtained by our lab before and the molecular marker of DNV susceptibility ($S_{DNV}$) from the Japanese researcher's paper (Ito et al. 2008), firstly the resistant strains, sensitive strains and near isogenic lines were used as material to administrate virus, then selected 60 resistant individuals and sensitive individuals, respectively. After this, genome DNA extracted from 30 individuals mixed was used to carry out the experiment of colony validation, meanwhile, the remained 30 individuals' DNA was extracted respectively to individual validation.

**PCR amplification:** Total reaction volume was 20μl that contained 10× Taq polymerase buffer 2.0μl, 200μM dNTPs, 2mM Mg²⁺, 1.0 μl primer, 1U Taq polymerase and 20-30ng template DNA. The amplification procedure was as follows: 30 reaction cycles plus a final extension reaction at 72°C for 10 minutes. For the molecular marker $R_{NPV}$, each reaction cycle consisted of 94°C 45s, 56°C 45s, and 72°C 70s; while for the molecular marker $S_{DNV}$, each reaction cycle consisted of 94°C 45s, 60°C 45s, and 72°C 60s. PCR products were analyzed with electrophoresis on 1.2% agarose gels. The gels were stained with ethidium bromide and were consequently photographed and analyzed by GelDoc-2000 gel image analyzing system (Bio-Rad).

**Hybrid breeding method:** The theories of the resistance breeding trial design based on the literature Yao et al. (2003). According to the dominant inheritance of silkworm resistant to BmNPV, the female Jingsong and male NB were used as hybrid breeding material, then the offspring has been always selected to backcross with the female cyclical parent Jingsong. Virus administration
chose the concentration of $4.8 \times 10^7$ NPV polyhedra/ml. After using a syringe inject quantitative 5µl into every silkworm through the mouth, the life individuals with good economic traits remained. The moth district obtained from the districts which is not separated after virus administraton, then it was meted with silkworm Qiufeng resistant to DNV. Because of the gene resistant to DNV was a recessive single gene inheritance, when hybrid in the first generation, we did not feed with virus, then obtained the resistant individuals after $F_1$ selving with virus administration. After completion of all of the above, three generation of selving to get the resistant homozygotes are still needed.

In the above breeding process, molecular marker assisted seleletion was used to verify the accuracy inoculated choice, meanwhile, the economic traits was considered to reservation.
RESULTS AND DISCUSSION

Linkage analysis of molecular marker R<sub>756</sub>: The primers anti-NPV-F and anti-NPV-R of molecular R<sub>756</sub> linked to the resistance gene amplified in the NB (resistant species), 306 (susceptible species) and near isogenic lines BC<sub>3</sub> (resistant species) populations and individuals (Fig. 1-2). The results showed that there was a specific band of approximately 700bp existed in the NB and near isogenic lines BC<sub>3</sub>, meanwhile, a specific band of approximately 1600bp existed in the 306 and near isogenic lines BC<sub>3</sub>. Sequencing results showed the specific band about 700bp existed in the NB was 736 bp, which was the same as the band existed in the near isogenic lines BC<sub>3</sub>. Moreover, the sequencing result of the band, approximately 1600bp existed in the 306, has the length of 1628bp, which was the same as the 1628bp shown in the near isogenic lines BC<sub>3</sub>. This not only showed that the molecular marker R<sub>756</sub> linked to the major dominant resistant gene, but also suggested that NILs locus was heterozygous.

Linkage analysis of molecular marker S<sub>851</sub>: The primers sus-DNV-F and sus-DNV-R of molecular S851 linked to the DNV susceptible gene amplified in the Qiu Feng (resistant strain), Huaba<sub>95</sub> (susceptible strain) populations and individuals (Fig. 3-4). A specific band of approximately 800bp was found in Huaba<sub>95</sub> while there was not any band existed in Qiu Feng. The sequencing result indicated it has the length of 851bp. This result showed that the reliability and hereditary stability of marker S<sub>851</sub>.

The new germplasm construction of the silkworm resistant to BmNPV and BmDNV-Z: Based on the phenomenon that the female silkworm did not occur the chromosome exchange in meiosis (Sturtevant 1915; Tanaka 1913), female NB and male Jingsong hybrid to obtain F<sub>1</sub> generation, then it backcross with the recurrent parent Jingsong until the sixth generation to get the BC<sub>6</sub> were used its genotype was about 98.5% similarity to the parent Jingsong (Yao 2003). After finished the above process, the male silkworm in BC<sub>6</sub> and female Qiu Feng, resistant to BmDNV, was selected to hybrid into BC<sub>6</sub>-F<sub>1</sub>, the next generation was assist-detected used the molecular marker R<sub>756</sub> and S<sub>851</sub>. Then selfing two generations could get the new germplasm resistant to BmNPV and BmDNV-Z (Fig. 5).

The assist-detection of molecular markers in the breeding process: To ensure the resistant gene can effectively inherit to the next generation, live silkworms after virus administrated were used to sampling inspection (Fig. 6). There were a few individuals mixed in the resistant population. This would inevitably interfere with the experiment. So in order to accurately conduct of breeding disease resistant individuals in the breeding process,
detection through the method of molecular marker was performed every two generations.

**Double molecular markers’ identification of the new variety:** After gained the dual-resistant variety, molecular markers R756 and S374 were used to ensure whether the BC2 F4 genotype were pure on the two alleles, NPV and DNV, through sampling 50 individuals re-identification. The result was shown in the Figure 7, from which we could clearly observe that the silkworm obtained were pure and the genotype were RR, nsd-Z / nsd-Z.

The genotype Rr and rr of BC1 segregants each accounted for 50%, while the Rr genotype of BC2 only accounted for 25%. If continuous backcross for several generations without virus filtration like this, the resistant gene would soon be lost. In addition, the individuals after DNA extraction were likely to die. Therefore, to ensure the accurate transmission of resistant gene and taking into account ease of detection, molecular markers-assisted selection was performed in each two generations. If the individuals, has the resistant marker, existed in the any moth district, then it remained for following breeding material. In this experiment, the modified backcross breeding method was used, so the economic traits were focused in the earlier generations. After obtained the heterozygote silkworm (Rr) with good economic characters, we performed selfing two generations to get the resistant homozygotes (RR). The silkworm resistant to BmDNV was controlled by a single recessive gene, so the generation of F1 did not food with virus. Only after obtained the F2 use molecular marker to perform selection using DNV administration was of course, after obtained the heterozygote silkworm (nsd-Z/ +, nsd-2), selfing was performed to purify the resistant gene to BmDNV.

The molecular marker S374 was selected according to the sequence of the BmDNV-2 resistant gene reported by Japanese scholars. The whole genomic sequencing of BmDNV-Z was finished previously by our lab. Through the comparison with the genome of BmDNV-2, The two genome sequence has the 97.7% similarity were found (Wang et al. 2007). In addition, the validation of resistant gene also was consistent. In a word, usage of the resistance gene as a molecular marker is reliable.

The strain NB, highly resistant to BmNPV, belongs to the Chinese local varieties. It has the problem of poor economic traits, so the resistant improvement of the offspring often brings other economic problems of poor characters. Moreover, if it was used as the only resistant material to improve the Chinese local varieties and the Japanese varieties synchronously, it will affect the heterosis.

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**REFERENCES**


