



# Marker-assisted selection in breeding silkworm strains with high silk production and resistance to the densovirus

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**ABSTRACT.** In the silkworm (*Bombyx mori*), resistance to the Zhenjiang (China) strain of the densovirus (DNV-Z) is controlled by the recessive gene *nsd-Z* (non-susceptible to DNV-Z), which is linked to 7 simple-sequence repeat markers. Marker-assisted evaluation and selection of DNV-Z-resistant silkworms were used for predicting DNV-resistance in backcrossed animals. A silkworm race was bred using this method, and its economic characteristics were found to be similar to those of commercial silkworm races. These markers will therefore be useful for silkworm breeding

programs and in screening for densovirus resistance in segregating populations.

**Key words:** *Bombyx mori*; Densovirus; Non-susceptible gene; Marker-assisted selection

## INTRODUCTION

The *Bombyx* densovirus (BmDNV) causes flacherie disease in the silkworm (*Bombyx mori*). BmDNV multiplies only in the nuclei of the columnar cells of the larval midgut epithelium (Seki and Iwashita, 1983; Guo et al., 1985). DNV viruses possess a small, single-stranded, linear DNA genome, and are classified into the family Parvoviridae (Lü, 1998). The parvoviruses are one of the smallest viruses with genomic DNA, and they require factors from the host cell and/or helper viruses for efficient replication (Watanabe et al., 1986; Abe et al., 1987; Goldsmith et al., 2005).

BmDNVs comprise several strains, such as the Ina, Saku, and Yamanashi viruses. As classified by Watanabe et al. (1986), the Ina virus belongs to the DNV-1 type, while the Saku and Yamanashi viruses belong to the DNV-2 type. Iwashita and Chao (1983) described the chemical characteristics of DNV-Z, and Qian et al. (1985) demonstrated that it was similar to the Saku virus.

Non-susceptibility to DNV-1 is controlled by the recessive gene *nsd-1* and the dominant gene *Nid-1* control (Eguchi et al., 1991). The *nsd-2* and *nsd-Z* genes confer resistance to DNV-2 and DNV-Z, respectively (Hu et al., 1984; Abe et al., 1987; Qin and Yi, 1996). Abe et al. (1998, 2000) identified and mapped several random-amplified polymorphic DNA (RAPD) markers linked to the *nsd-1* and *nsd-2* genes. Ogoyi et al. (2003) mapped *nsd-2* using the restriction fragment length polymorphism method, and obtained 3 closely linked cDNA markers. Based on a map-based cloning analysis, Li et al. (2001a) identified a RAPD marker linked to *nsd-Z*. Furthermore, Ito et al. (2008) found that the virus resistant to BmDNV-2 was caused by a deletion in the open reading frame of an amino acid transporter.

Based on viral resistance characteristics, it has been proposed that homozygous *nsd-Z* silkworm strains can be used in breeding to prevent economic losses due to DNV-Z. The incorporation of the *nsd-Z* gene and the elimination of the susceptibility gene(s) during breeding programs could provide significant advantages for the production of commercial silkworm strains. Historically, DNV has been fed to silkworms during breeding to select for individuals resistant to DNV-1 or DNV-Z (Eguchi et al., 1998; Li et al., 2001b). More recently, marker-assisted selection (MAS) has been widely used in breeding programs (Yau et al., 2005; Steele et al., 2006), although, to date, it has not been applied in silkworm breeding.

Microsatellites, or simple-sequence repeats (SSRs), are short, tandemly repeated motifs of 1 to 6 bases found in all prokaryotic and eukaryotic genomes. SSRs are widely used in population genetics and linkage map construction because of their high levels of polymorphism and reproducibility (Tautz, 1989), and their genome-wide distribution. SSRs are inherited in a Mendelian fashion and show co-dominant alleles. Reddy et al. (1999) obtained 28 SSR markers and used 15 of them to estimate the relationships among 13 strains of silkworm. Miao et al. (2005) constructed a silkworm SSR linkage map.

Li et al. (2005a,b, 2006) investigated the genetic diversity among silkworm germ-plasms using 26 SSR markers, and characterized 7 SSR markers linked to *nsd-Z*. In this study, we used these 7 SSR markers as tools to select silkworms that were not susceptible to DNV-Z, in order to breed non-susceptible silkworm races using an MAS approach.

## MATERIAL AND METHODS

### Silkworm strains

The L10 silkworm strain (*nsd-Z/nsd-Z*) is non-susceptible to DNV-Z, but its economic characteristics are unfavorable. The Jingsong strain (*+<sup>nsd-Z</sup>/<sup>nsd-Z</sup>*), which is susceptible to DNV-Z, is a productive commercial strain widely used in Chinese sericulture. These strains were maintained at the Sericulture Research Institute, Chinese Academy of Agriculture Sciences.

### DNA extraction and polymerase chain reaction (PCR)

DNA samples were extracted from whole moths and from the midgut of unhealthy larvae. The moth body (or midgut) was ground with a mechanical homogenizer in a microcentrifuge tube, and suspended in DNA extraction buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 20 mM EDTA) containing 150 µg/mL proteinase K. After digestion with proteinase K (50°C for 8-10 h), a phenol-chloroform extraction was performed, and the DNA was precipitated out with isopropanol. Purified DNA was dissolved in 0.1X TE buffer, pH 8.0. The DNA concentration was measured by spectrophotometry (BioPhotometer, Eppendorf), and the samples were diluted to a concentration of 10 ng/µL for use in PCR analysis.

The PCRs were performed using a Flexigene Cyclor (Techne, UK). The following PCR conditions were used for the microsatellite loci: i) 95°C for 3 min, 63°C for 40 s, and 72°C for 1 min, followed by 14 cycles of 94°C for 45 s, then a 14-step touchdown, decreasing by 0.5°C at each step to 56°C (40 s), and 72°C for 1 min; ii) conditions for the last 24 cycles were 94°C for 40 s, 56°C for 40 s, and 72°C for 1 min; and iii) a final elongation step of 10 min at 72°C. The PCR was performed in a final volume of 15 µL, containing 10 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP, 0.2 µM of each primer, 20 ng genomic DNA, 0.5 U Taq polymerase (Takara), and distilled, deionized water. PCR products at about 0.5 ng (0.01 pM) per sample were analyzed using an ABI377 DNA sequencer (ABI PRISM).

### Strategy for MAS

MAS was adopted to breed a new silkworm strain that is not susceptible to DNV-Z, based on co-dominant SSR markers linked to *nsd-Z*. For the crossing-over experiments, a female of the Jingsong strain was crossed with a male of the L10 strain, and then Jingsong females were mated to (Jingsong x L10) F<sub>1</sub>, or other backcross (BC) generation (6 in total), males in order to evaluate their economic characteristics. In each generation, 2 SSR markers (F10316 and F10568, located on either side of *nsd-Z*; Li et al., 2006) were

used to detect the genotype of male moths after they mated with Jingsong females. Amplification of both markers in 1 individual implied that the segments of the 2 chromatids from the 2 markers were highly likely to have come from both the L10 and the Jingsong strains, and this individual would therefore likely possess the *nsd-Z* gene. Otherwise, Jingsong-type homozygous animals were discarded, because if both chromatids were from the Jingsong parent, the animal would likely not possess the *nsd-Z* gene. Self-mating within a generation was carried out in the BC<sub>6</sub> generation, and these offspring were fed DNV-Z. Individuals with good economic characteristics that were not susceptible to DNV-Z were selected and self-mated to generate the next generation (BC<sub>6</sub>F<sub>3</sub>). Thus, a new silkworm race with good economic characteristics and no susceptibility to DNV-Z was bred.

### Virus inoculation and diagnosis

Dried silkworm midgut containing DNV-Z was ground in distilled water until it turned into a dense solution. The solution was filtered through gauze and centrifuged (3500 rpm, 20 min). After adding an equal volume of 7% acetic acid to the supernatant, the solution was incubated at 25°C for 40 min, followed by adjustment to pH 7.0 and progressive dilution to a 0.5% tissue solution. Bioassays were conducted at 25°C. DNV-Z was fed to newly hatched larvae of the BC<sub>6</sub>F<sub>3</sub> generation for the first instar, followed by rearing on uncontaminated fresh mulberry leaves from the second instar onward. Normally, silkworms susceptible to DNV-Z develop very slowly and do not survive through the third instar. After the third instar, all sick silkworms were checked for DNV-Z, using a PCR-based method, to verify whether they were infected (Hou et al., 2005).

## RESULTS

### DNV-Z resistance and MAS of populations

In each BC generation, 12 male moths with good economic characteristics were selected to determine their genotypes with respect to the 2 co-dominant markers (Table 1). Approximately 4 to 6 moths were heterozygous for these 2 markers in each generation, and were selected as parents of the next generation (Table 2). A total of 24 pairs from the BC<sub>6</sub> generation, which demonstrated good economic characteristics, were mated to produce the BC<sub>6</sub>F<sub>2</sub> generation. Parents of 5 pairs were heterozygous at the 2 SSR loci, and their offspring were reared with DNV-Z-contaminated mulberry leaves to select for homozygous non-susceptible to DNV-Z individuals. The individuals selected to produce the BC<sub>6</sub>F<sub>3</sub> generation were also assessed with regard to these 2 SSR markers; only 1 male showed heterozygosity at the F10568 locus, and the others showed L10-type homozygosity at both loci. The BC<sub>6</sub>F<sub>3</sub> population was again fed DNV-Z, and proved to be non-susceptible to DNV-Z. Figure 1 shows the genotypes at the F10316 locus of all of the backcrossed male parents and of the non-susceptible individuals selected for rearing in each generation. Figure 2 shows the genotype at the F10316 locus of randomly selected BC<sub>6</sub>F<sub>2</sub> individuals. The ratio of the Jingsong type, heterozygotes, and the L10 type was consistently found to be 1:2:1.

**Table 1.** The co-dominant markers used in the marker-assisted selection.

Marker symbol	F Primer sequence (5'-3')	R Primer sequence (5'-3')
FI0316	GCGATAAGACCGCCTATTGAAC	GTGTATTAGGCACGAGAACTGACG
FI0568	TCGTCTACACTTGCGGGTT	TGTTTCGTCAAGTCTGCTCGGT
S2511	TCCAATGTTTCCTAACTAATTCGTGTA	CAAATTAGATCATGGCATATCGAAA

**Table 2.** Genotype of backcrossing individuals at the 2 SSR loci.

Backcrossing generation	FI0316		FI0568		Number of heterozygotes at both loci
	Number of heterozygotes	Number of homozygotes	Number of heterozygotes	Number of homozygotes	
BC <sub>1</sub>	6	6 (Js type)	5	7 (Js type)	5
BC <sub>2</sub>	5	7 (Js type)	6	6 (Js type)	5
BC <sub>3</sub>	4	8 (Js type)	5	7 (Js type)	4
BC <sub>4</sub>	7	5 (Js type)	7	5 (Js type)	7
BC <sub>5</sub>	6	6 (Js type)	5	7 (Js type)	5
BC <sub>6</sub>	11♀ 12♂	13 (Js type) 12 (Js type)	13♀ 12♂	11 (Js type) 12 (Js type)	11♀ 12♂ 5♀♂
BC <sub>6</sub> F <sub>2</sub>	0	24 (L10 type)	1	23 (L10 type)	0

\*Js = Jingsong.



**Figure 1.** Genotype of the male of each backcrossing generation after detection for the next generation and non-susceptible individuals of the BC<sub>6</sub>F<sub>2</sub> generation at the FI0316 locus. Lane 1 = L10; lane 2 = (Jingsong x L10) F<sub>1</sub>; lane 3 = Jingsong; lanes 4 and 5 = BC<sub>1</sub>; lanes 6 and 7 = BC<sub>2</sub>; lanes 8 and 9 = BC<sub>3</sub>; lanes 10 and 11 = BC<sub>4</sub>; lanes 12 and 13 = BC<sub>5</sub>; lanes 14-17 = BC<sub>6</sub>; lanes 18-21 = susceptible individuals of the BC<sub>6</sub>F<sub>2</sub> generation; lanes 22-28 = non-susceptible individuals of the BC<sub>6</sub>F<sub>2</sub> generation.



**Figure 2.** Genotypes of randomly selected individuals of the BC<sub>6</sub>F<sub>2</sub> generation at the FI0316 locus. Lane 1 = L10; lane 2 = Jingsong; lane 3 = (Jingsong x L10) F<sub>1</sub>; lanes 4-23 = randomly selected individuals of the BC<sub>6</sub>F<sub>2</sub> generation.

### Economic characteristics of each generation

Using the L10 strain as the resistant parent, the Jingsong strain was used as a recurrent parent to improve the new silkworm strain's economic characteristics, and co-dominant SSR markers were used to ensure that the *nsd-Z* gene would not be lost during backcrossing. As shown in Table 3, the economic characteristics of the new strain were similar to those of the parent Jingsong strain.

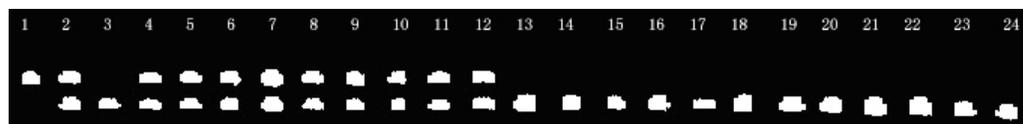
**Table 3.** Economic characteristics of the parents and the backcross generations.

Strain/generation	Year	Duration of 5th larvae (day:hour)	Duration of feeding period (day:hour)	Cocoon weight (g)	Cocoon shell weight (g)	Ratio of cocoon shell (%)	Ratio of cocoon mortality (%)	Larva-pupa survival rate (%)
F <sub>1</sub>	2006 Au	6:18	21:06	1.283	0.227	17.69	4.32	91.33
BC <sub>1</sub>	2007 Sp	7:00	22:12	1.591	0.362	22.75	3.87	96.00
BC <sub>2</sub>	2007 Au	7:12	23:06	1.741	0.411	23.61	3.45	95.67
BC <sub>3</sub>	2008 Sp	8:00	23:18	1.882	0.452	24.02	2.23	96.86
BC <sub>4</sub>	2008 Au	7:12	23:00	1.688	0.401	23.76	4.67	91.23
BC <sub>5</sub>	2009 Sp	8:12	25:00	1.822	0.441	24.20	4.32	94.88
BC <sub>6</sub>	2009 Au	7:18	24:00	1.791	0.428	23.90	8.55	82.89
BC <sub>6</sub> F <sub>2</sub>	2010 Sp	8:00	24:18	1.876	0.463	24.68	6.75	89.73
BC <sub>6</sub> F <sub>3</sub>	2010 Au	8:12	25:00	1.968	0.491	24.95	2.67	96.59
Jingsong	2010 Au	8:00	24:18	1.861	0.458	24.61	7.26	88.06
BC <sub>6</sub> F <sub>4</sub>	2012 Sp	8:06	24:18	1.931	0.483	25.01	2.88	95.41
Jingsong	2012 Sp	8:12	25:00	2.052	0.511	24.90	2.88	95.41
L10	2012 Sp	4:00	17:08	0.630	0.070	11.11	2.90	97.00

Sp = spring; Au = autumn.

### Homozygosity in male moths of each backcrossed generation

Two SSR markers that showed polymorphism between Jingsong and L10 were selected from each linkage group of the SSR linkage map (Miao et al., 2005) to detect homozygosity of the backcrossed males throughout the breeding procedure. An average of 12 linkage groups were homozygous for Jingsong in the BC<sub>1</sub> generation, and an average of 19 linkage groups were homozygous for Jingsong in the BC<sub>2</sub> generation. By the BC<sub>3</sub> and BC<sub>4</sub> generations, the number of homozygous linkage groups increased to 23 and 25, respectively (Figure 3). Only 1 marker of 10 individuals was found to be heterozygous in the BC<sub>6</sub> generation, while the others were all homozygous, except those linked to *nsd-Z* (data not shown).



**Figure 3.** Genotype of the male of each backcrossing generation at the S2511 locus. Lane 1 = L10; lane 2 = (Jingsong x L10) F<sub>1</sub>; lane 3 = Jingsong; lanes 4-6 = BC<sub>1</sub>; lanes 7-9 = BC<sub>2</sub>; lanes 10-12 = BC<sub>3</sub>; lanes 13-15 = BC<sub>4</sub>; lanes 16-18 = BC<sub>5</sub>; lanes 19-24 = BC<sub>6</sub>.

### DISCUSSION

During sericulture, disinfection is used to eliminate pathogens. Several different chemical agents are generally used, many of which are harmful to both humans and the environment. Nonetheless, pathogens cannot be completely eliminated from rearing houses, and silkworm diseases occur frequently, especially in rural areas. Densonucleosis is common in rural areas, especially in the autumn, and the *nsd-Z* allele controls non-susceptibility to DNV-Z. The best way to control the spread of such diseases is to breed silkworm varieties that are resistant to pathogens.

It is possible to breed commercial silkworm strains that are not susceptible to DNV-Z,

and such strains have been bred using crossing methods (Eguchi et al., 1998; Li et al, 2001b). For example, the virus was fed to silkworm larvae throughout classical crossing procedures to ensure that the resistance gene would not be lost during backcrossing. Although it is relatively easy to make *nsd-Z* homozygous as a recessive gene, a good breeding design is necessary to ensure that the gene will not be lost. Furthermore, special care is necessary when feeding the virus to silkworms; i.e., using special rooms and appropriate tools to avoid infecting other silkworms, and rearing under sterilized conditions.

MAS has been widely used in crop breeding, along with developments in molecular biology, although it has not yet been widely applied in silkworm breeding. We screened for SSR markers linked to *nsd-Z*, and adopted the nearest 2 to select for silkworms that possess the *nsd-Z* gene. A new silkworm strain that was non-susceptible to DNV-Z was bred after 6 generations of backcrossing, and its economic characteristics were similar to its recurrent (Jingsong) parent.

The key to successful MAS is to select a tightly linked marker. The best option would be the gene itself or part of it, because this would prevent recombination during backcrossing, thus avoiding errors in selection. In our screening, the nearest SSR markers were 4.4 cM away from the *nsd-Z* gene, which suggested a high probability of losing *nsd-Z* during backcrossing if only 1 marker were used. Thus, we used 2 markers, located on either side of the *nsd-Z* gene. In a previous study, no double-recombination occurred among these 3 loci in 190 individuals. Therefore, we decided to rear offspring of fathers who expressed heterozygosity at both loci. We did observe recombination during backcrossing with these 2 markers, however (Table 3), and these individuals were discarded. Fortunately, there was not an important economic characteristic locus (except *nsd-Z*) located between these 2 loci. Otherwise, it would have been difficult to simultaneously improve economic characteristics while selecting for resistance by backcrossing.

In our study, individuals were detected after eclosion, and mated to females to produce the next generation. The silkworm body is small, and could easily be fatally damaged if tissues or blood are sampled for genotype detection. In the future, we would like to develop a method that allows detection of the larval genotype without the need to sample tissues. With such a method, it would only be necessary to rear a small group of individuals, thereby saving costs and improving efficiency.

In conclusion, we used SSR markers as tools to select for silkworms that are not susceptible to DNV-Z. A new non-susceptible silkworm strain was bred after 6 generations of backcrossing, and its economic characteristics were similar to those of its recurrent parent.

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