

Full Length Research Paper

Detection of homozygosity in near isogenic Lines of non-susceptible to Zhenjiang strain of densovirus in silkworm

Li Muwang^{1*}, Hou Chengxiang¹, Zhao Yunpo³, Xu Anying¹, Guo Xijie^{1,3} and Huang Yongping²

¹Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang 212018, P. R. China.

²Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences 200032, P. R. China.

³College of biotechnology, Jiangsu University of Science and Technology, Zhenjiang 212018, P. R. China.

Accepted 21 March, 2007

The near isogenic lines (NILs) of non-susceptible to DNV-Z were bred through backcrossing successively using *L10* as donor parent and *Js* as recurrent parent. The homozygosity of the NILs was detected using the SSR markers from the SSR linkage map. The results showed that the ratio of the linkage groups from the recurrent parent in the NILs increased rapidly along with the increase of the backcrossing generations. After 6 generations of backcrossing, it was selfed and was fed with DNV-Z. The linkage groups of 9 of the 5 BC₆ pairs, whose 1/4 offspring were non-susceptible to DNV-Z, were all replaced by the recurrent parent except the linkage group that held *nsd-Z*, and only part of one linkage group from the other one individual had not been replaced. According to the markers linked to *nsd-Z*, FI0316 had not been replaced in all of the 10 individuals, but crossover happened in 2 individuals between FI0568 and *nsd-Z*.

Key words: Silkworm, Non-susceptible to DNV-Z, Near Isogenic line, SSR marker, Homozygosity and Linkage group.

INTRODUCTION

Near isogenetic lines (NILs) mean a group of lines that are genetically identical except at one or a few loci. A NIL is developed by several backcrosses (more than six generations) between the recurrent and donor (which take the aim marker) parent. In each generation, the individuals which are similar to the recurrent parent except the aim gene should be selected and are backcrossed to the recurrent parent. Self-crossing is taken out after backcrossing for more than 6 generations, and the aim gene is homozygous. When the NIL is completed, it is genetically identical to the recurrent parent except the region close to the aim gene locus on the chromosome. Molecular markers linked to the aim gene can be identified using this NIL, and the molecular linkage map can be parallelism to the traditional mutant linkage map. Young et al. (1988) Identified DNA markers linked to the

Tm-2a gene in tomato using isogenic lines. Martin et al. (1991) used random markers and NILs to identify markers linked to a pseudomonas resistance gene in tomato. Abe et al. (1998, 2000) bred the NILs of *nsd-1* and *nsd-2*, then identified and mapped several RAPD markers linked to the *nsd-1* or *nsd-2* allele successively. Li et al. (2001) constructed the near-isogenic lines of *nsd-Z* and had identified RAPD markers linked to *nsd-Z*. Li et al. (2003) had constructed NILs for all 28 linkage groups by successive backcrossing method.

Bombyx mori densovirus (BmDNVs) belong to the genus Iteravirus of the family Parvoviridae (Lü, 1998). BmDNVs, which cause serious economic damage to sericulture, were discovered in 1976. They comprise several strains, such as Ina virus, Saku virus, Yamanashi virus and Zhenjiang (China) virus (DNV-Z). As distinguished by Watanabe et al. (1986), Ina virus belongs to the DNV-1 type, and Saku virus and Yamanashi virus belong to the DNV-2 type. Iwashita et al. (1983) discussed the chemical characteristics of DNV-Z, and Qian et al.

*Corresponding author. E-mail: cangege@pub.zj.jsinfo.net. Fax: 86-511-5622507. Tel: 86-511-5616575.

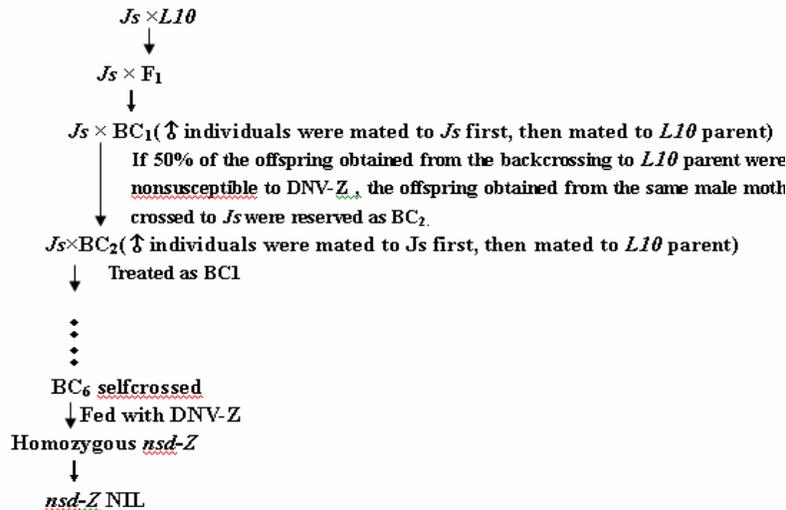


Figure 1. The flow chart of construction the NIL of *nsd-Z*.

(1985) demonstrated that it is similar to Saku virus. BmDNVs multiply only in the nuclei of the columnar cells of the larval midgut epithelium (Guo et al., 1985; Seki and Iwashita, 1983). Certain silkworm strains are highly susceptible, while other strains are non-susceptible (completely resistant) to DNV-1, DNV-2 or DNV-Z even though they are fed a high concentration of virus inocula. It was reported that a recessive gene, *nsd-1*, and another dominant one, *Nid-1*, control the non-susceptibility to DNV-1 severally (Watanabe and Maeda, 1981; Eguchi et al., 1991). *nsd-2*, a recessive gene conferring resistance to DNV-2, was shown to be different from *nsd-1* (Abe et al., 2000). Hu et al. (1984) demonstrated that a third recessive gene, *nsd-Z*, controls the non-susceptibility to DNV-Z. RAPD markers linked to *nsd-Z* had been screened (Li et al., 2001) and the molecular linkage map of *nsd-Z* by SSR markers had been constructed (Li et al., 2006). Two SSR markers, F10316 and F10568, were the nearest markers on its both sides; they were 4.4cM and 11.7cM from *nsd-Z* respectively.

In this research, NILs of *nsd-Z* was bred by backcrossing to recurrent parent successively. SSR markers were selected to detect the homozygosity in the NILs to inspect the replacement of the linkage groups of the donor parent.

MATERIALS AND METHODS

Silkworm strains

The donor parent, *L10* strain (*nsd-Z/nsd-Z*), non-susceptible to DNV-Z, and the recurrent parent, *Js* strain ($+^{nsd-Z}/+^{nsd-Z}$), susceptible to DNV-Z, were maintained by single pair mating in SRI-CAAS.

Virus inoculation and serological diagnosis

Dried silkworm midgut that contained DNV-Z was ground in distilled water until it turned into a dense solution. The solution was filtered

through gauze and centrifuged at 3500 rpm for 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 0.5% solutions of tissue. The bioassays were conducted at 25°C. DNV-Z was fed to newly hatched larvae for the first instar, followed by rearing on uncontaminated fresh mulberry leaves since the second instar. Normally, silkworms that are susceptible to DNV-Z develop very slowly and cannot survive through the third instar. All of the infected silkworms were checked for DNV by PCR methods (Hou et al., 2005).

Construction of near allele lines

To construct a near allele line homozygous for the *nsd-Z* in a *Js* background, for the crossing over experiments, a female of *Js* was crossed with a male of *L10*, then females of *Js* were mated to (*Js* × *L10*) F1 males or other backcross generations (6 in all) to eliminate the chromosomes of *L10* except the region close to the *nsd-Z* locus. Because of its being recessive, the *nsd-Z* allele would be lost if the methods of selection were not efficient during the backcrossing. Therefore, in order to ensure that the *nsd-Z* allele would not be lost, we used a single male moth, which carried the *nsd-Z* allele, to mate successively with two females of different races from the BC1 generation. In other words, the male moth was first mated to a female moth of *Js*, and then successively mated to another female moth of *L10*. The eggs from the two females were reared separately, and the offspring of backcrossing to *L10* were fed with DNV-Z to test for the presence of *nsd-Z*. If all of those individuals died, all of its offspring (including those crossed to *Js*) were discarded. On the contrary, if 50% of the offspring obtained from the backcross to *L10* survived, the offspring obtained from the same male moth crossed to *Js* were reared. Selfing was adopted in the B6 generation (Figure 1).

DNA extraction

DNA samples were extracted from the moth. The moth was ground with a mechanical homogenizer in a micro-centrifuge tube and suspended in DNA extraction buffer [50 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 20 mM EDTA] that contained 150 µg/ml proteinase K. After digestion with proteinase K at 50°C for 8 - 10 h, phenol-chloroform extraction was carried out and the DNA was recovered

Table 1. The achievement of the parents and backcrossing generations.

Race/generatinn	Rearing season	Duration of 5 th instar (D:H)	Duration of feeding period (D:H)	Cocoon weight (g)	Cocoon shell weight (g)	Cocoon shell ratio (%)	Ratio of cocoon mortality (%)	Larva-pupa survival rate (%)
F ₁	2003Au	6:18	21:06	1.283	0.227	17.69	4.32	91.33
BC ₁	2004Sp	7:00	22:12	1.591	0.362	22.75	3.87	96.00
BC ₂	2004Su	7:12	23:06	1.741	0.411	23.61	3.45	95.67
BC ₃	2004Au	8:00	23:18	1.882	0.452	24.02	2.23	96.86
BC ₄	2004LAu	7:12	23:00	1.688	0.401	23.76	4.67	91.23
BC ₅	2005Sp	8:12	25:00	1.822	0.441	24.20	4.32	94.88
BC ₆	2005Su	7:18	24:00	1.791	0.428	23.90	8.55	82.89
BC ₆ F ₂	2005Au	8:00	24:18	1.876	0.463	24.68	6.75	89.73
BC ₆ F ₃	2005LAu	8:12	25:00	1.968	0.491	24.95	2.67	96.59
<i>Js</i>	2005LAu	8:00	24:18	1.861	0.458	24.61	7.26	88.06
<i>Js</i>	2005Sp	8:12	25:00	1.931	0.483	25.01	2.88	95.41
<i>L10</i>	2005Sp	4:00	17:08	0.630	0.070	11.11	2.90	97.00

*D (Day; H) Hour; Sp (Spring; Su) Summer; Au (Autumn; Lau) Late autumn.

by isopropanol precipitation. The purified DNA was dissolved in 0.1 × TE buffer (pH 8.0). The DNA concentration was measured spectrophotometrically (BioPhotometer, Eppendorf) and the samples were diluted to a concentration of 10 ng/μl for use in the PCR analysis.

Methods for detection the homozygosity of the NIL

Two to four polymorphic SSR markers were selected from the constructed SSR linkage map (Miao et al., 2005). In each backcrossing generation, 3 male moths which had been demonstrated to harbor *nsd-Z* gene and had been selected to generate next generation were detected for their genotype of these SSR markers.

PCR and fragment analyses

The PCR reactions were performed using the Flexigene Cycler (Techne, England). The following PCR conditions were used for the micro-satellite loci: (1) 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, fourteen-step touchdown decreasing by 0.5°C at each step to 56°C (40 s), and 72°C for 1 min; (2) the conditions for the last 24 cycles were 94°C for 40 s, 56°C for 40 s, and 72°C for 1 min, followed by (3) a final elongation step of 10 min, with extension at 72°C. The PCR was performed in a final volume of 15 μl that contained 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM of each dNTP, 0.2 μM of each primer, 20 ng of genomic DNA, 0.5 U Taq polymerase, and distilled deionized water. The PCR products at about 0.5 ng (0.01 pM) per sample were analyzed in an ABI377 DNA sequencer (ABI PRISM) 2.5.

RESULTS

The characters of the NIL

All of the individuals in these NILs expressed the same characters as *Js* except the aim gene, *nsd-Z*. The egg color, larva color, larva body form, cocoon shape, and other characters such as economic characters were all

same as *Js*. Table 1 showed the economic characters of the NIL and the donor parent and recurrent parent.

The detection results of each backcrossing generation

The 3 male moths which had been demonstrated to harbor *nsd-Z* and had been selected to generation next generation were detected their genotype of the SSR markers. Only 5 pairs of BC₆ moths whose 1/4 offspring were nonsusceptible to DNV-Z were detected for the genotype too. The results showed that linkage groups from the recurrent parent in the NILs increased rapidly along with the increase of the backcrossing generations (Figure 2). The average linkage groups which had been replaced by *Js* were 12, 19.3, 22.7, 24.3 and 26.7 in BC₁, BC₂, BC₃, BC₄ and BC₆ generation respectively. The linkage groups of 9 of the 5 BC₆ pairs, whose 1/4 offspring were non-susceptible to DNV-Z, were all replaced by the recurrent parent except the linkage group that held *nsd-Z*, and only part of one linkage group from the other one individual had not been replaced, as one marker showed the genotype of *L10*, while another showed the genotype of *Js*. According to the markers linked to *nsd-Z*, FI0316 had not been replaced in all of the 5 pair moths, the genotype of FI0568 showed *Js* type in 2 individuals, indicating crossover happened between FI0568 and *nsd-Z* (Figure 2) in them.

DISCUSSION

NIL populations have been used extensively in genetic studies due to the advantages derived from their homozygosity. NILs were important material to locate and clone new genes, in a NIL population, the phenotypic variation

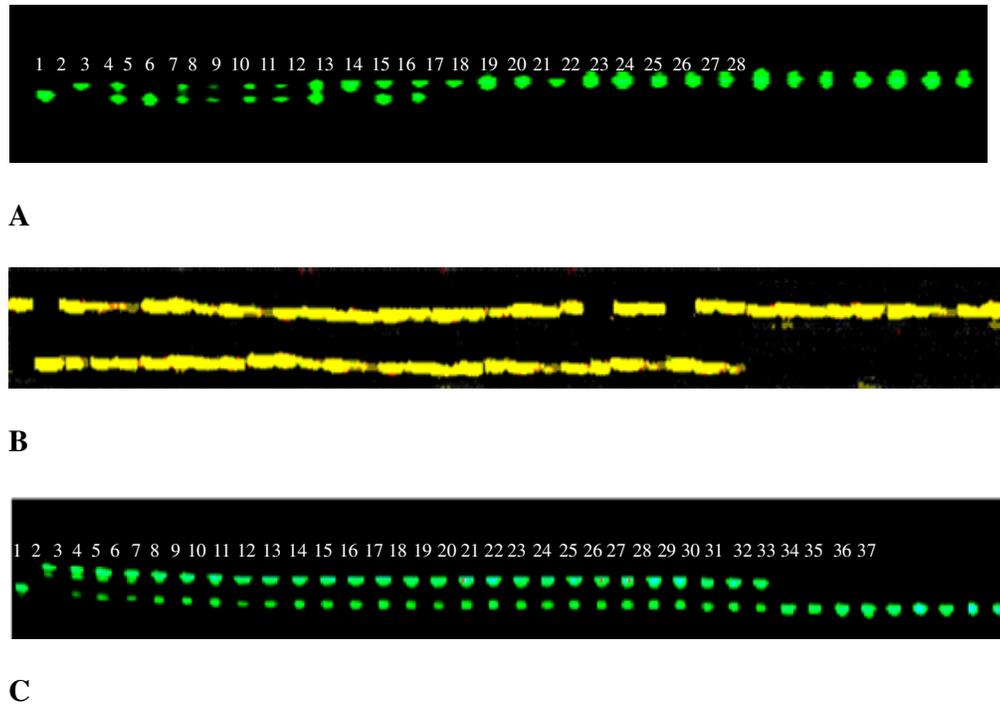


Figure 2. Profile of part of the SSR markers in the NIL. A, S1124; B, FI0568; and C, FI0316. 1. L10; 2. Js; 3. F₁; 4-6. BC₁; 7-9. BC₂; 10-12. BC₃; 13-15. BC₄; 16-18. BC₅; 19-28. BC₆; 29-37. Nonsusceptible individuals of BC₆F₂.

observed between pairs of lines can be assigned directly to the distinct genomic regions introgressed in another similar genetic background. Depending on the desired resolution one can minimize the number of lines by analyzing lines carrying large introgressions or even chromosome substitution strains (Nadeau et al., 2000).

Theoretically, if there were n genes difference between recurrent parent and donor parent, after m times backcrossing, the proportion of homozygotes will account for $[1-(1/2)^m]^n$ (Xu and Zhu, 1994). In this research, the velocity of homozygosity was accord with it but was a little lower than the theoretic value. During the backcrossing, the genotypes of the SSR markers were not same in all of the detected individuals in one generation; their genotypes were stochastic but accorded with Mendel's law.

SSRs are widely used in population genetics and linkage map construction because of their high levels of polymorphism and reproducibility (Tautz, 1989), as well as their genome-wide distribution. SSRs are inherited in a Mendelian fashion and show co-dominant alleles, and had been widely used in constructing linkage map, studying genetic diversity, gene mapping, map-based cloning and marker-assisted selection (Miao et al., 2005; Li et al., 2005; Li et al., 2006; Ren et al., 2005; Danson et al., 2006). In this research, there was no crossover had been detected between *nsd-Z* and the nearest marker (FI0316, 4.4cM), but crossover had been detected between *nsd-Z*

and another marker, FI0568, which located on the other side of *nsd-Z*, indicated that marker assisted selection could be actualized to breed new silkworm races nonsusceptible to DNV-Z.

ACKNOWLEDGEMENTS

This project was supported by the National Basic Research (973) Program (grant No. 2005CB121000), National Natural Science Foundation of China (grant No. 30400327), High Technology Research Program of Jiangsu Province (No. BG2005302) and the Research Program of the Chinese Academy of Agricultural Sciences (2004 - 30).

REFERENCES

- Abe H, Harada T, Kanehara M, Shimada T, Ohbayashi F, Oshiki T (1998). Genetic mapping of RAPD markers linked to the denonucleosis refractoriness gene, *nsd-1*, in the silkworm, *Bombyx mori*. *Genes Genet. Syst.* 73(4): 237-242.
- Abe H, Sugasaki T, Kanehara M, Shimada T, Gomi SJ, Ohbayashi F, Oshiki T (2000). Identification and genetic mapping of RAPD markers linked to the denonucleosis refractoriness gene, *nsd-2*, in the silkworm, *Bombyx mori*. *Genes Genet. Syst.* 75(2): 93-96.
- Danson JW, Mbogori M, Kimani M, Lagat M, Kuria A, Diallo A (2006). Marker assisted introgression of opaque2 gene into herbicide resistant elite maize inbred lines. *Afr. J. Biotechnol.* 5(24): 2417-2422.
- Eguchi R, Ninaki O, Nara W (1991). Genetical analysis on the nonsusceptibility to denonucleosis virus in the silkworm, *Bombyx mori*, *J. Sericol. Sci. Jpn* 60(5): 384-389.

- Guo XJ, Qian YJ, Hu XF, Wang HL (1985). Studies on the infection site by the densovirus in the diseased silkworm (*Bombyx mori*) in China. *Acta Sericologica Sinica*, 11(2): 93-98.
- Hou CX, Li MW, Gui ZZ, Xu AY, Guo XJ (2005). PCR-based detection of densovirus infection in silkworm (*Bombyx mori* L.). *Int. J. Indust. Entomol.* 11(2): 135-138.
- Hu XF, Qian YJ, Wang HL (1984). Studies on infectivity of silkworm densovirus to some silkworm races. *Acta Sericologica Sinica*, 10(2): 87-90.
- Iwashita Y, Chao YS (1983). The development of a densovirus isolated from silkworm larvae, *Bombyx mori*, of China, *Acta Sericologica Sinica*, 9(4): 219-222.
- Li MW, Guo QH, Hou CX, Miao XX, Xu AY, Guo XJ, Huang YP (2006). Linkage and mapping analyses of the densovirus non-susceptible gene *nsd-Z* in the silkworm *Bombyx mori* using SSR markers. *Genome*, 49(4): 397-402.
- Li MW, Shen L, Xu AY, Miao XX, Hou CX, Sun PJ, Zhang YH, Huang YP. (2005) Genetic diversity among the silkworm (*Bombyx mori* L., Lep., Bombycidae) germplasm revealed by microsatellites. *Genome* 48: 802-810.
- Li MW, Xu AY, Hou CX, Zhang YH, Huang JT, Guo XJ (2003). Breeding of near isogenic lines of silkworm (*Bombyx mori* L.). *Int. J. Indust. Entomol.* 6: 207-210.
- Li MW, Yao Q, Hou CX, Lu C, Chen KP (2001). Studies on RAPD markers linked to the densovirus refractoriness gene, *nsd-Z* in silkworm, *Bombyx mori* L., *Sericologia*, 41(3): 409-415.
- Lü HS (1998). *Molecular biology of insect viruses*. Beijing: China agricultural sciencetech press. pp. 381-400.
- Martin GB, Williams JGK (1991). Rapid identification of markers linked to a *Pseudomonas* resistance gene in tomato by using random primers and near-isogenic lines. *Proc. Natl. Acad. Sci. USA*, 88: 2336-2340.
- Miao XX, Xub SJ, Li MH, Li MW, Huang JH, Dai FY, Marino SW, Mills DR, Zeng P, Mita K, Jia SH, Zhang Y, Liu WB, Xiang H, Guo QH, Xu AY, Kong XY, Lin HX, Shi YZ, Lu G, Zhang X, Huang W, Yasukochi Y, Sugasaki T, Shimada T, Nagaraju J, Xiang ZH, Wang SY, Goldsmith MR, Lu C, Zhao GP, Huang YP (2005). Simple Sequence Repeat-Based Consensus Linkage Map of *Bombyx mori*. *Proc Natl Acad Sci USA*. 102: 16303-16308.
- Nadeau JH, Singer JB, Matin A, Lander ES (2000) Analysing complex genetic traits with chromosome substitution strains. *Nat. Genet.* 24: 221-225.
- Qian YJ, Guo XJ, Hu XF, Watanabe H (1985). The serological relationship of *Bombyx mori* densovirus from China and Japan. *Acta Sericologica Sinica*, 11(4): 241-242.
- Ren ZH, Gao JP, Li LG, Cai XL, Huang W, Chao DY, Zhu MZ, Wang ZY, Luan S, Lin HX (2005). A rice quantitative trait locus for salt tolerance encodes a sodium transporter. *Nat. Genet.* 37: 1141-1146.
- Seki H, Iwashita Y (1983). Histopathological features and pathogenicity of a densovirus of the silkworm, *Bombyx mori*, isolated from sericultural farms in Yamanashi prefecture. *J. Sericol. Sci. Jpn.* 52(3): 400-405.
- Tautz D (1989). Hypervariability of simple sequences as a general source for polymorphic DNA markers. *Nucleic Acids Res.* 17: 6463-6471.
- Watanabe H, Kawase S, Shimizu T, Seki H (1986). Difference in serological characteristics of densovirus in the silkworm *Bombyx mori*. *J. Sericol. Sci. Jpn.* 55(1): 75-76.
- Watanabe H, Maeda S (1981). Genetically determined nonsusceptibility of the silkworm, *Bombyx mori* to infection with a densovirus. *J. Invertebr. Pathol.* 38: 370-373.
- Xu YB, Zhu LH (1994) *Molecular quantitative genetics*. Beijing: China agricultural press, Beijing, pp. 98-107.
- Young ND, Zamir D, Ganai MW, Tankley SD (1988). Use of isogenic lines and Simultaneous probing to identify DNA markers tightly linked to the *Tm2a* gene in tomato, *Genetics*, 120: 579-585.