ISSN 1009-3095 *Journal of Zhejiang University SCIENCE* V.3, No.3, P.348-354, July-Aug., 2002
http://www.periodicals.com.cn; http://www.zju.edu.cn/English

http://www.zjupress.com; http://lib.zju.edu.cn/eindex.htm; jzu_s@mail.hz.zj.cn

Analysis of bulked segregants to identify molecular markers linked with cocoon weight and cocoon shell weight in the silkworm *Bombyx mori* L

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Received Dec.29, 2001; revision accepted Feb.25, 2002

Abstract: Two silkworm strains viz, B20 Λ (high cocoon shell ratio) and C.Nichi (low cocoon shell ratio) were sib mated for 10 generations to determine the homozygosis. Both bulked segregant analysis (BSA) and near isogenic lines (NIL) studies were done to identify the RFLP markers closely linked to cocoon shell parameters. Three hundred and fifty-two random clones were identified as the low copy number sequence and used for identification of Restriction Fragment Length Polymorphic (RFLP) marker linked to cocoon weight and cocoon shell character. In the bulk segregant analysis, DNA from the parents (B20 Λ, C.Nichi), F_1 and F_2 progeny of high shell ratio (HSR) and low shell ratio (LSR) were screened for hybridization with the random clones. Polymorphic banding pattern achieved through southern hybridization with different probes indicated the probable correlation of polymorphism with high and low cocoon shell character which are possible landmarks in identifying the putative marker(s) for the cocoon shell character. Out of the 100 probes tried with parents, F_1, F_2 and their bulks, 10 probes were found to be closely linked to cocoon shell characters.

Key words: Restriction fragment length polymorphic (RFLP), Molecular marker, *Bombyx mori* L, Shell ratio, Bulked segregant analysis(BSA), Near isogenic lines
Document code: A **CLC number:** Q 819

INTRODUCTION

The indigenous multivoltine breeds, although seemingly disease tolerant and showing higher survival potential under tropical climate, are characterized by low silk yield and poor fiber quality, whereas bivoltine breeds of temperate region are endowed with high silk yield and improved yarn quality. The recombinational breeding programs involving these low and high yielding breeds by conventional methods, have so far, achieved limited success in improving the productivity of Indian breeds(Datta, 1998).

Over the past few years, directional breeding technique using DNA markers like RFLP, RAPD, etc., which are linked to specific traits is being widely explored and successfully used for the improvement of the target traits in crop plants and livestocks (Tanksley et al., 1989; Tanksley et al., 1995). In recent years, rapid progress had been made in the development of RFLP based QTL maps for important crop species like pepper and tomato (McCouch et al., 1988) maize (Halentjaris, 1987), lettuce (Landry et al., 1987), potato (Bomierhole et al., 1988). However, very little work has been done in animals in general and silkworm in particular to establish QTL linkage maps and to identify markers for the gene of interest.

Hence, the new strategy of molecular

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marker to facilitate investigations on silkworm was initiated with the objective of identifying DNA markers closely linked to important yield traits, such as cocoon weight and cocoon shell weight, which mainly determine the silk yield. The present work carried out to identify DNA markers for both low and high cocoon shell weight so that these markers could be used as molecular tags during breeding in the coming years.

MATERIALS AND METHODS

1. Selection of silkworm strains and development of inbred lines for parents

B. 20A with high shell ratio (HSR-24%) and C. Nichi with low shell ratio (LSR-11%) were selected as parents for the present study. Parental lines were maintained separately by full sib mating. At each generation the cocoons were assessed for HSR and LSR character in B. 20A and C. Nichi, respectively. Full sib mating was continued up to 10 generations and the homozygosity at the DNA level was confirmed by RAPD analysis. Bulked segregant analysis (BSA) was carried out to identify RFLP and RAPD markers closely linked to cocoon shell characters.

2. Population for bulked segregants

The bulked segregants were prepared and analyzed as described by Michelmore et al. (1991). The two parental strains, C. Nichi and B. 20A were used for obtaining bulked segregants. The former is non-diapausing strain and the latter is a diapausing strain; both exhibit high phenotypic diversity for complex characters such as size, growth rate, diapause, morphology, nutritional requirements, general vigor and cocoon properties suggesting that there exists considerable polymorphism at DNA level. Both these strains had undergone high degree of inbreeding and so, are relatively homozygous. A single C. Nichi female was mated to a single B. 20A male to produce F_1 offspring. The F_2 progeny were raised from a single pair mating of F_1 sibs and each of the 430 individuals was assessed for single cocoon weight traits.

3. Bulked segregant analysis (BSA)

Twelve individuals with a single cocoon weight of 0.843 − 1.031 gm and the same number of individuals with cocoon weight of 1.916 − 2.065 gm were polled separately to form low and high cocoon weight bulks respectively. Similarly twelve individuals with single cocoon shell weight of 0.099 − 0.129 gm and the same number of individuals with a single cocoon shell weight ranging from 0.239 − 0.269 gm were pooled separately from low and high cocoon shell weight bulks respectively. DNA from each individuals of each of the bulks was extracted. About 2μg DNA from each of the twelve individuals from both bulks were pooled. These bulked DNA samples were used to screen with random probes and primers to identify markers linked to cocoon weight and cocoon shell weight traits.

4. DNA isolation

Genomic DNA was isolated from posterior silk glands (PSG) of mid fifth instar larvae, pupae and moths (after egg laying). DNA was extracted according to Shi et al., 1995 with minor modification. Briefly a pair of PSG was disrupted with mortar and pestle into fine powder using liquid nitrogen. Ten ml of extraction buffer (100 mmol/L Tris-HCl (pH 8.0), 50 mmol/L EDTA) with protiense K(100 μg /ml) was added and incubated at 37℃, followed by addition of 1% SDS and incubation at 55℃ for 5 hour. Equal volume of Tris-saturated phenol was added to the tube; the contents were mixed by swirling for 5 − 10 minutes and centrifuging at 10 000 r/min for 15 min. The clear aqueous phase was transferred to a new centrifuge tube using wide mouthed pipette tips and after an equal volume of saturated phenol-chloroform - isoamyl alcohol (24:24:1) was added into the tube, it was spun at 10 000 r/min for 15 min. The aqueous phase was transferred to a new tube containing chloroform and spun at the same speed for 15 min; after which, the aqueous phase containing DNA solution was transferred to a new tube and the DNA was precipitated with 1/10 final volume of 3 mol/L sodium acetate (pH 5.2) and 2 volumes of absolute ethanol. The precipitated DNA was spooled out using a pipette tip and washed

once with 70% ethanol, air dried and resuspended in TE buffer (10 mmol/L Tris, 1mmol/L EDTA (pH 8.0)). RNase A (Amersham, UK) to a final concentration (100 μg/ml) was added to the TE buffer containing DNA and incubated at 37℃ for 2 hours.

5. Quantitation of genomic DNA

The quantity and purity of the extracted genomic DNA were checked spectrophotometrically at 260/280 nm absorbance as well as on 0.8% agarose minigel (Sambrook et al., 1989). The DNA quantity of each sample was estimated by comparing band intensity with uncut lambda DNA (Gibco, USA) controls of known concentration. The genomic DNA was diluted using TE buffer to a final concentration of 1 μg/μl for RFLP analysis and 0.1 μg/μl for RAPD analysis.

6. Southern blot preparation and hybridization

The genomic DNA from parent F_1, F_2 bulks (HSR/LSR) and BC progenies were extracted from pupae. After assessment, the DNA were digested with different restriction endonucleases and electrophoresed on 1% agarose gel. After processing the gel, the DNA was transferred to nylon membrane and hybridization was performed according to the standard protocols (Sambrook et al., 1989) using probes derived from partial genomic library constructed in pUC18 with pst1 digested DNA fragment (0.5 to 2.0 kb of C. Nichi genomic DNA).

RESULTS

1. Screening of genomic library for low copy probes

Seven hundred random clones from pst1 digested C. Nichi genomic library were screened through dot blot hybridization using alpha ^{32}p labeled genomic DNA of B.20 A as a probe. Based on the intensity of the signals developed, 352 clones were identified as low copy probes, which were used to study the polymorphism between parents and progenies (Fig. 1, Table 1).

2. Detection of polymorphism in bulked segregant analysis

Genomic DNA from the parents, C. Nichi and B.20A were hybridized with 352 random genomic low copy probes, the hybridized and amplification products that revealed polymorphism between the parents were identified (Fig. 2). Out of 352 random probes, 14 probes were informative and further used to

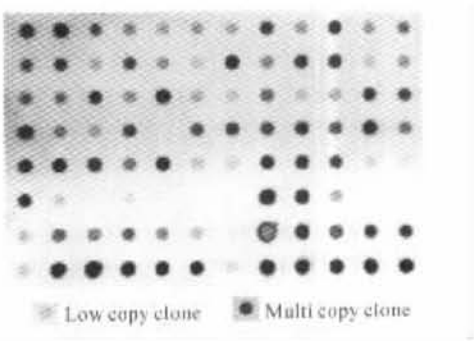

Fig. 1 Dot blotted recombinant clones are hybridized with DIG labeled genomic DNA

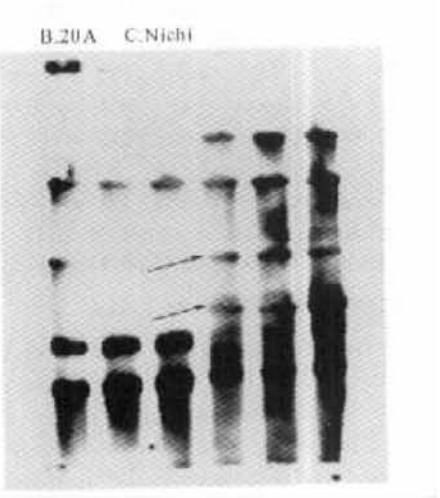

Fig. 2 Southernblot showing race specific polymorphism when EcoR1 digested DNA hybridized with radio labeled probe S-102

screen the DNA bulks with 'high' and 'low' cocoon weight and cocoon shell weight. Bulked segregant analysis is a fast way for identifying markers linked to specific genes; involves analysis of progenies segregating in the F_2 population in order to find the gene of interest; and overcomes several problems encountered with NILs. There is minimal chance that regions unlinked to this target region will differ in the bulked samples of many individuals. In bulked segregant analysis, all

polymorphic loci in the segregating population will segregate and can be mapped.

Bulked segregant analysis is being successfully used in a number of crop plants and livestock for analysis of genetically complex traits by screening bulks of individuals of a quantitative trait controlled by a few major genes (QTLs). Comparison of bulks of extreme individuals can rapidly identify markers linked to QTL Concepts of BSA analysis shown in Fig. 3 (Michelmore et al., 1991).

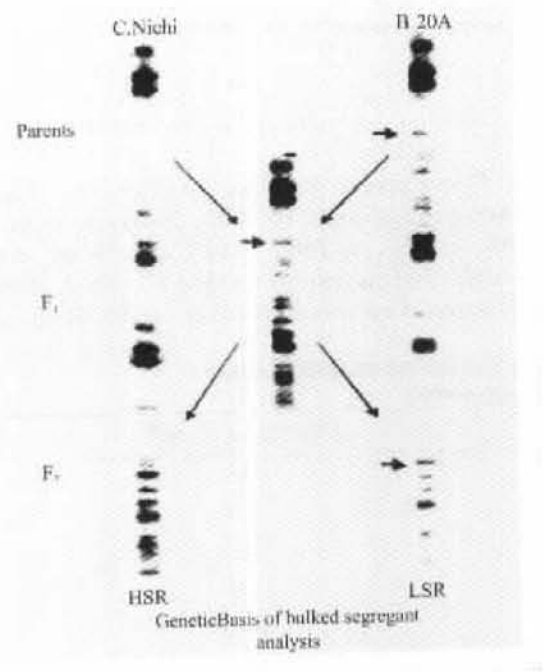

Fig. 3 The concept of bulked segregant analysis

3. RFLP and RAPD assay to screen bulk segregants

Prior to RFLP studies RAPD analysis of the DNA of parents, F_1 and F_2 bulks (High and Low) were also carried out which showed distinct polymorphic banding pattern when OPA-1, OPA-2, OPA-11 and OPA-13 primers were used; 6.7 kb as well as 2kb regions showed polymorphic bands between parents. When OPA-13 primer was used, it was found that 2.2 kb fragment was segregating in the high bulks progenies.

The DNA of parents, F_1 and F_2 bulks (High and Low) were screened for RFLPs with different probe-enzyme combinations. Among the different enzymes viz., *Hae*III, *Hind*III, *Hinf*I, *EcoR*I, *EcoR*V, *Sau*III and *Alu*I tried we found that *EcoR*V yielded better polymorphic pattern when used with different probes. Many mapping studies on plants have shown moderate to low levels of restriction fragment length polymorphism with both cDNA and genomic clones (Tanksley et al., 1989). In the case of *B. Mori*, genomic DNA clones showed sufficient levels of polymorphism for our RFLP studies. In general, we observed highest levels of polymorphism when probes were used with the restriction enzyme, *EcoR*V (Figs. 4, 5& 6), which is a hexacutter in the region of 6.6 kb size and below that up to 500 bp. To obtain more number of markers related to co-

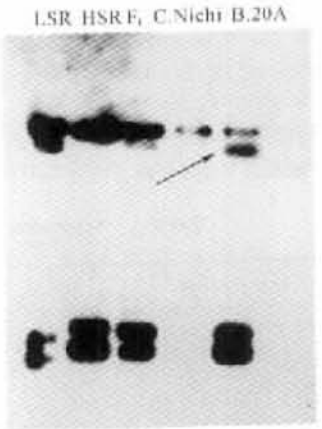

Fig. 4 Southern Hybridization of B. 20 A, C. Nichi, F_1 along with their two bulks with probe S-182

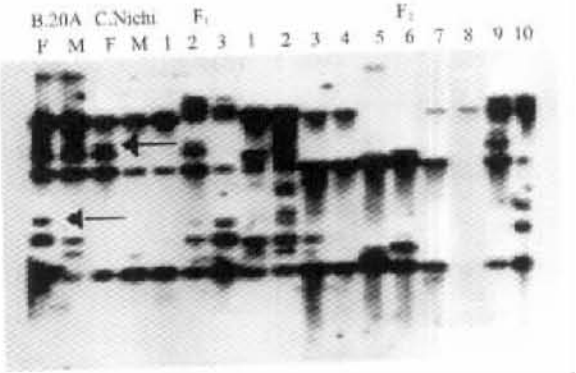

Fig. 5 RELP Pattern observed on autoradiograph of Eco-RV digested DNA of parents, F_1 & F_2 progeny probed with S-501

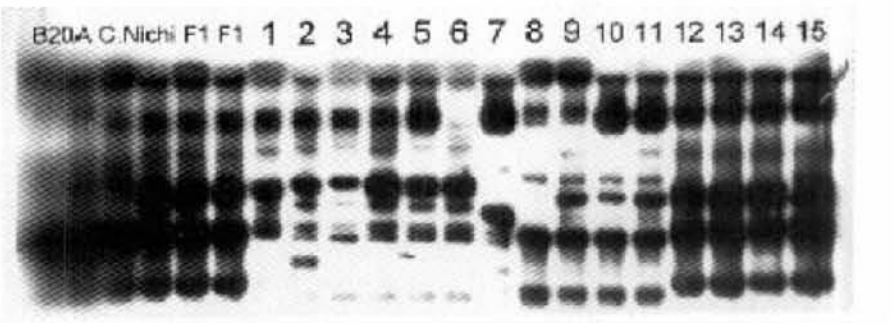

Fig. 6 RFLP Pattern observed on autoradiograph of Eco-RV digested DNA of parents, F_1 & F_2 progeny probed with S-96

coon shell character, we screened 100 random clones ranging from 0.5 to 2 kb in size. About 14% of the random clones produced intense smeared hybridized pattern, characteristic of highly repetitive DNA. Twelve of them produced very weak signal perhaps as a result of complex intron structure or numerous internal restriction sites in them (Laundry et al., 1987).

Polymorphic banding pattern was found when probes such as S-90, S-96, S-182, S-350, S-501, S-566, S-117 and S-352 were used for hybridization (Table 2) which clearly indicated correlation of bands with high and

Table 1 Screening of genomic library through dot blothybridization for selecting low copy probes

Copy number	Number of clones
Low copy	352
Medium copy	229
High copy	158
Clones without insert	103
Clones with single insert	402
Clones with double insert	214
Clones with multiple insert	221

Table 2 Hybridization signals observed in the digested DNA of parents, F_1 and F_2 bulks with low copy probes.

Sl. No.	Probe No.	Base pair (kb)	B.20A	C. Nichi	F_1	F_2 Bulk	
						HSR	LSR
1	S-90	1.8	+	−	+	+	−
2	S-96	1.6	+	−	+	+	−
3	S-119	1.2	+	−	+	+	−
4	S-350	1.6	+	−	+	+	−
5	S-501	1.7	+	−	+	+	−
6	S-566	0.5	+	−	+	+	−
7	S-117	0.4	−	+	+	−	+
8	S-188	1.1	−	+	+	−	+

low cocoon shell progenies segregating in the F_2. Out of these 8 probes, the first six revealed identical hybridization signals in the HSR parent, B. 20A and HSR bulk in the F_2; and at the same time, their absence in C. Nichi and LSR bulk. This indicates the possibility of these markers were closely linked to high shell ratio character. While the probe numbers S-117 and S-352 showed similar banding patterns in the LSR parent, C. Nichi, and the LSR bulk in the F_2 showed likely association of these markers with low shell character.

DISCUSSION

The present study examined the application of bulked segregant analysis to identify RAPD and RFLP markers linked to cocoon weight and cocoon shell weight characters in silkworm. The success of DNA pooling strategies for tagging economic traits of interest (ETLs) based solely upon phenotypic information is dependent on the magnitude of the phenotypic effect of individuals ETLs, the population size sampled and the influence of non-genetic factors on the phenotype. The present work used phenotypic extreme distribution analysis for making DNA bulks of high and low cocoon weight and cocoon shell characters. The phenotypic extreme distribution analysis has great advantage in terms of saving of time and resources in analyzing DNA markers. On the other hand, for the same number of individuals assayed for molecular markers in total population analysis versus extreme distribution analysis, the statistical power of detecting ETLs will be greater for the latter. However, extreme phenotypic distribution analysis has some disadvantages (I) more segregating F_2 individuals must be analyzed for the quantitative phenotype to collect enough individuals with extremes phenotypic distribution. In many instances, this is not a problem. However in some situations, the time and cost of characterizing the larger population phenotypically outweighs the advantages and in the case of some organisms (mostly animal species), it is difficult to raise the large number of populations required: these disadvantages can be overcome in the case of silkworm, because F_2 population size is in the range of 400-500 individuals; of which are enough number of individuals with extreme phenotypic distributions. Besides, the time required for raising F_2 population and cost of characterizing the population of silkworm phenotypically, unlike large animals, is much cheaper. (II) The extreme distribution analysis is more efficient at detecting linkage between marker loci and ETLs of interest; on the other hand it is less efficient in determining ETL effects as extreme phenotypic distribution individuals tend to have large number of either all positive or all negative ETLs, depending on which extremes they represent in the segregating population.

To tag quantitative traits using phenotype based DNA bulks, several recommended precautions including the use of cross in which extreme variation is observed, the use of large population, and the use of homozygous population i. e. , F_2 or BC derived lines for phenotypic values to make DNA bulks. ETLs with very large effect on phenotype can be quickly identified, if sufficiently large populations are studied. Two experimental aspects of DNA pooling strategies are that the DNA of each individual must be equally represented in DNA pools; and that the experiment must include measures for independent verification of putative new markers. Equal DNA concentration may be ensured by simply extracting the DNA from each member of the pool individually and quantitating. Of particular concern is that the non- radioactive based assays such as DIG and ECL, are less sensitive compared to ^{32}p assay.

Bulked segregant analysis overcomes several problems inherent in using near-isogenic lines (NILs) to identify molecular markers linked to a particular trait. There are minimal chances that the pooled samples of many individuals from a genomic region unlinked to the target region will differ from those of the target region. In contrast, near isogenic lines require many backcrosses to develop and is time consuming to generate. On the other hand,

bulked segregants can be made immediately for any locus or genomic region once the segregating population has been constructed. In silkworm, many of the characters such as resistance to *BmNPV*, larval span, fecundity, body weight, and filament length are introgressed from the donor parent to the recurrent parent through repeated backcrossing. Even after successive backcrosses many of the undesirable traits of the parent still persist in the inbred line. In such circumstances, molecular tags linked to the traits of interest could be precisely used to synthesize silkworm strains with desirable traits. In order to examine the feasibility of tagging DNA markers to the QTLs of interest, we raised F_2 population from a cross of two divergent parents. The differences in the low and high phenotypic extremes for cocoon and cocoon shell weight were almost two fold. The study could wait to benefit from the efforts on integration of RFLP, RAPD, ISSR and microsatellite that would lead to the construction of high density linkage map. Such a map would be ideal for searching markers linked to the QTLs with minimal major effects on the bulks.

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