ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 9 (1): 41-47, 2010
ISSN 1682-296X

The QTLs' Determination of Cocoon Shell Weight Trait in Mulberry Silkworm (*Bombyx mori* L.)

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Abstract: The silkworm is an important economical insect in sericultural field. Its major production-economic characteristics are polygenic. In this study, three F2 populations (second generation) derived from three cross between three pairs' parents of Lemon Khorasan (as maternal) and 107 (as paternal) lines. we contacted AFLP (amplified fragment length polymorphism) technique for mapping genetic factors or QTLs that effect on Cocoon Shell Weight (CSW) trait. Following it we used 20 selected primer combinations of *Pst*I/*Taq*I and DNAs were individually extracted to phenol-chloroform method. They digested by two restriction enzymes (*Taq*I and *Pst*I) and also produced DNA fragments amplified by appropriate adaptors separately. After transferring of DNAs samples on annealed 6% polyacrylamide gels and genotyping of individuals, the linkage maps of each population were drawed. The QTLs for cocoon shell weight trait in LRS = LRT>12.5 (LOD>2.71) threshold level based on permutation test (n = 1000) and using compound interval mapping methods were analyzed and detected 5, 1 and 1 QTLs that were localized on linkage groups 9, 11; 12 and 24 in each studied F2 populations, respectively. The QTLs had different gene effects from over dominance, dominance to partial dominance.

Key words: Silkworm, QTLs, linkage map, molecular markers, AFLP

INTRODUCTION

The mulberry silkworm *Bombyx mori* (Lipdopterae: Bombycidae) includes n = 28 chromosomes (Tazima, 2001). This insect is the most important economic organism, so that sericulture industry is depended on it, to produce natural silk (Nguu *et al*., 2005). Silk fibers are derived from the cocoon of silkworm *Bombyx mori*, which was domesticated from the wild progenitor *Bombyx mandarina* during 5000 years ago (Zhan *et al*., 2009). Presently there are about 3000 silkworm genotypes being maintained in Europe and Asia (Nagaraju, 2000). The genetic stocks consist of about 500 mutants that vary in different physical, physiological and biochemical traits (Koshy *et al*., 2008). Genomic studies on this insect lead us to different linkage maps based on morphological (Doira, 1992) and different molecular markers (Tan *et al*., 2001). Through the efforts of silkworm breeders over several thousands years, many silkworm strains have been collected. Until now, for breeding and achievement to commercial lines they used only crossbreeding (Zhan *et al*., 2009; Mirhoseini *et al*., 2004) by different selection strategies based on different traits amounts (Seidavi *et al*., 2008) or through selection index (Ghanipoor *et al*., 2006) or control of selection severity (ESCAP, 1993).

Many silkworm genotypes have the same phenotype inspite of unique genetic characteristics or the same genotypes can be having different phenotype under different ecological conditions. Such similarities and differences have caused problems for silkworm breeders (Nagaraju and Singh, 1997).

Using DNA markers in plants and animals breeding has created a new theme in agricultural section as molecular breeding (Rafalski and Tingey, 1993). Today, molecular breeding is considered as a major success in plant and animal species (Stuber *et al*., 1987; Williams, 2003). Many plant and animal breeding centers accepts such capacity for development and selection by markers (Lee, 1995). In this reason, we are observing different software analysis procedures that developing for all these places now (Kreasey, 1998).

Important economic traits in silkworm such as cocoon weight, cocoon shell weight, cocoon shell

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percentage, length and thickness of silk; reproduction and resistance are quantitative traits. Because of these traits affected on each one by many small genes with small effects and environmental conditions. Our information is not enough about the number of genes of these traits and their QTLs and products. The identification of these loci with an appropriate molecular markers that have such close conjunction with desired traits or characteristics are required to access the information, reducing choice risk and prevent to waste of fund and time (Nagaraju and Goldsmith, 2002).

Mulberry silkworm has various applications in pharmaceutical sector, agriculture, medicine and industry (Yamamoto *et al*., 2006). Cocoon weight and shell weight are the important or main traits evaluated for productivity in sericulture and these characters have been used for breeding more than half a century (Gaviria *et al*., 2006). The genomic study on silkworm has recently started by molecular markers so, that some researches done by sericultural researchers. Now, different linkage maps and important traits' QTL(s) have presented by using of various silkworm lines, segregated populations and molecular markers methods such as: AFLP (Tan *et al*., 2001; Lu *et al*., 2005), RFLP (Goldsmith, 1991), RAPD (Yuji 1998), SSR (Damodar *et al*., 1999; Miao *et al*., 2005), SNP (Yamamoto *et al*., 2006).

Amplified Fragment Length Polymorphism (AFLP) method is a Polymerase Chain Reaction (PCR) based technique that avoids the laborious steps involved in other methods such as: Restriction Fragment Length Polymorphism (RFLP) and generally shows a much higher level polymorphisms' production potency and informativeness (Mackill *et al*., 1996).

Silkworm breeding in this way is still in the early stages of their way because the formations of high density Linkage map will acquire difficultly. Such continuity plans will be included more genes loci (Sima *et al*., 2006). In this study, we tried for detection of cocoon shell weight trait QTLs' under composite interval mapping analysis.

MATERIALS AND METHODS

This study was conducted during April 2008 until September 2009. Linkage map creates a framework for anchoring morphological or other molecular markers and identifying Quantitative Trait Loci (QTL) for taxon diagnostic, geographically varying and economically important traits. This map also can be utilized to locate genes of interest and to develop DNA probes, SNPs, STS/expressed sequence tags or other codominant DNA markers with a wider range of applications (Wang and Porter, 2004).

For linkage map construction we must produce a mapping population then identifying the polymorphisms and doing Linkage analysis of markers (Collard *et al*., 2005).

Mapping population: For mapping population, selected parents must vary at one or more traits of interest. Therefore, segregation populations are included a mixture of parental and recombinant genotypes. The frequency of recombinant genotypes can be used to calculate recombination fractions which may be used to computation of the genetic distance between markers. The relative order and distance between markers can be determined by analyzing of markers segregation (Collard *et al*., 2005).

My study focused on F2 populations. In attention to this, three individuals of each pure-breeding families of two species Khorasan pink (Iranian local race that has a lower cocoon shell weight, as maternal) and P107 (breeded japonese line that has a higher cocoon shell weight, as paternal) were randomly crossed to originated three isolated F1 families. Then in each F1 family, we selected some individuals for crossing and producing F2 populations. Now we had three F2 populations with 33, 36 and 34 male individuals, respectively. Because genetic recombination occurs only in males in *B. Mori* and other lepidopteran insects therefore, this Lepidoptera-related phenomenon leads to the fact that marker loci derived from the female and male parents cannot be integrated into a single map (Tan *et al*., 2001). Therefore, we used male individuals only.

At the end of rearing and after harvesting cocoons, cocoon shell weight was recorded in each case by an electronic balance so that all phenotypic data obtained to two significant figures.

Identification of polymorphism: This is known as marker genotyping of the population (Collard *et al*., 2005). It is critical that sufficient polymorphism exists between parents in order to construct a linkage map (Young, 1994). For identifying of polymorphic markers, they must be screened across the entire mapping population, including the parents, F1 and F2 Progeny (Collard *et al*., 2005). But before that, genomic DNA was extracted individually from all parents, F1 and F2 populations from moth following the phenol/chloroform method as described by Suzuki *et al*. (1972) and as modified by Nagaraja and Nagaraju (1995). Then, all individuals were subjected to genotyping with AFLP markers according to Vos *et al*. (1995) with some modifications. Briefly, genomic DNA was double digested with *Pst*I (six base cutters) and *Taq*I (four base cutters)

Table 1: Adapters used in AFLP analysis

Name	Sequence
Adapters/*Pst*I	
*Pst*I top strand	5'-GACGTGACGGCCGTCATGCA-3'
*Pst*I bottom strand	3'-GCACTGCCGGCAGT-5'
***Taq*I/Adapters**	
*Taq*I top strand	5'-GACGATGAGTCCTGAG-3'
*Taq*I bottom strand	3'-TACTCAGGACTCGC-5'

Table 2: Preamplification primers used in AFLP analysis

Name		Sequence
P_{000}	*Pst*I	5'-GACGGCCGTCATGCAG-3'
T_{000}	*Taq*I	5'-GATGAGTCCTGAGCGA-3'

Table 3: Selective amplification primers (based on bolded selective nucleotide) used in AFLP analysis

Name	Sequence
***Pst*I**	
P_1	5/-GACGGCCGTCATGCAG**TA**
P_2	5/-GACGGCCGTCATGCAG**AT**
P_3	5/-GACGGCCGTCATGCAG**TC**
P_4	5/-GACGGCCGTCATGCAG**AC**
P_5	5/-GACGGCCGTCATGCAG**AAC**
P_6	5/-GACGGCCGTCATGCAG**AAG**
P_7	5/-GACGGCCGTCATGCAG**AGA**
P_8	5/-GACGGCCGTCATGCAG**ATG**
P_9	5/-GACGGCCGTCATGCAG**TAT**
***Taq*I**	
T_1	5/-GATGAGTCCTGAGCGA**TA**
T_2	5/-GATGAGTCCTGAGCGA**AT**
T_3	5/-GATGAGTCCTGAGCGA**TC**
T_4	5/-GATGAGTCCTGAGCGA**TG**
T_5	5/-GATGAGTCCTGAGCGA**AAT**
T_6	5/-GATGAGTCCTGAGCGA**ACA**
T_7	5/-GATGAGTCCTGAGCGA**AAG**
T_8	5/-GATGAGTCCTGAGCGA**AGC**
T_9	5/-GATGAGTCCTGAGCGA**TAC**

restriction enzymes. According to report of Tan *et al.* (2001), these enzymes can produce polymorphic DNA fragments in silkworm. The DNA fragments were ligated with *Pst*I and *Taq*I adapters, generating template amplification were designed on the basis of adaptor sequences and restriction site sequences. Selective nucleotide sequences were added to the 3'-end of each primer. The PCR amplification was conducted in two steps: a preselective amplification and a selective amplification. For selective amplification, a total of 81 primer combinations were screened. Among them, 20 primer pairs that produced fragments with clear dominance inheritance patterns and reproducibility were used for linkage analysis. The adaptors, preamplification primers and selective amplification primers used in AFLP analysis were as Table 1-3.

The 50 μL digestion reaction system contained 10 μL (50 ng μL^{-1}) extracted DNA, 0.5 μL (10 u μL^{-1}) of both restriction enzymes, 5 μL (10X) PCR buffer and 34 μL ddH_2O. Then this mixture was stored at 37°C in 17-18 h.

The 50 μL ligation reaction system contained 40 μL digested DNA, 1 μL (50PM μL^{-1}) *Taq*I adapter, 1 μL (5 PM μL^{-1}) *Pst*I adapter, 1 μL (1 u μL^{-1}) T4 DNA ligase, 1 μL (10X) PCR buffer and 5 μL ddH_2O.

The 25 μL PCR preamplification reaction system contained 2 μL of digested and ligated DNA diluted 1:5 V/V double distilled (ddH_2O), 1 μL (60 ng μL^{-1}) of both primers (*Pst*I-P_{000} and *Taq*I-T_{000}), 0.2 μL (5u μL^{-1}) *Taq* DNA polymerase, 2.5 μL^{-1} (2 mM) of dNTPs, 1 μL (50 mM) $MgCl_2$, 2.5 μL (10X) PCR buffer and 14.8 μL ddH_2o. The preamplification reaction conditions and steps employed were as described by Vos *et al.* (1995). After this step, we diluted preamplificated mixture 1:5 V/V with double distilled H_2O (dd H_2O).

Now, the 15 μL PCR selective amplification system contained 2 μL of product from the diluted preamplification reaction, 0.6 μL (60 ng) of both selective primers, 0.2 μL (5u μL^{-1}) of *Taq* DNA polymerase 1.5 μL (2 mM) of dNTPs, 0.6 μL (50 mM) $MgCl_2$ 1.5 μL (10X) PCR buffer and 8 μL ddH_2O. All amplification reactions were performed in touch gene model thermocycler.

The products of end step of AFLP method added with loading buffer (formamide 10 mL, Xylene cyanol FF 10 mg, Bromophenol blue 10 mg, 0.5 M EDTA pH 8.0/200 μL) then they were detected by silver staining after loaded and running on large denaturing polyacrylamide gel 6% in the vertical GT nucleic acid Sequencing cell-BioRad Sequi-Gen model PV3000 (Promega, Madison, WI) as described by Chalhoub *et al.* (1997) and gel images were scanned and saved as jpg files with GS-800 Calibrated Densitometer scanner device, related software and computer for genotyping by scoring as presence (1) or absence (0) of amplification bands and further analysis. The electrophoresis parameters were set to 75 watt, 50°C and a run time of 1.5-2.0 h.

Linkage analysis and QTL mapping: This step involves coding data for each DNA marker on each individual of segregation population (F2 offspring) that followed by using of computer programs (Collard *et al.*, 2005).

After that, gel images were scored by using a binary scoring system as 1 and 0 (presence and absence of bands, respectively) and these extracted informations inverted to codes D and B or C and A, according to SIS or TRANS dominant polymorphic markers in Mapmanager/QTX software manual (Manly *et al.*, 2001). Then the computer software QTL Cartographer ver 2.5 (Wang *et al.*, 2007) was used to determine QTL positions, the expected additive and dominance effects and phenotypic variance explained by individual QTL. An important and widely-used method is Composite Interval Mapping (CIM). The results of test statistic for Composite Interval Mapping=CIM (as profile of likely sites for a QTL

between adjacent linked markers) are presented using a logarithmic of odds=LOD score or Likelihood Ratio Statistic=LRS (Collard *et al*., 2005). It must be noticed that we used appropriate minimum threshold level of LOD=2.71 or LRS=LRT=12.5 for QTLs detection, based on permutation test (n=1000) (Churchill and Doerge, 1994).

RESULTS

AFLP markers screening: We selected 20 pair of primer combinations for segregation analysis on the F2 intercross populations on the basis of reproducibility and the extent of polymorphism. A total of were 930, 944 and 810 that gave us an average of 46.5, 47.2 and 40.5 bands per primer combinations for each F2 population, respectively. Only the polymorphic fragments that showed dominant segregation and could be scored unambiguously were used for construction of a linkage map. Following it we had a total of 142, 171 and 178 qualified polymorphic bands or there were 15.27, 18.11 and 21.97% polymorphism, respectively. It means that, on average, each primer combination generated 7.1, 8.55, 8.9 polymorphic fragments that conformed to a 3:1 segregation ratio with sizes ranging from 95-595 bp, which could be used for each linkage mapping, respectively. The most qualifying polymorphic bands in all three cross produced in relation to TP16 (12 polymorphic bands), TP01 (15 polymorphic bands) and TP10 (15 polymorphic bands) primer combination and also the lowest qualifying polymorphic bands produced in relation to TP04 (4 polymorphic bands), TP04 and TP12 (5 polymorphic bands) and TP04 (5 polymorphic bands) primer combination, respectively.

Linkage map Construction and its characteristics: The linkage maps obtained from the cross between three couples of two parental lines contained 119, 157, 160 AFLP polymorphic bands, respectively that were assigned to three linkage maps at the LOD threshold of 2.5. The 23, 14 and 18 AFLP markers could not be assigned to any linkage groups because conforming to the 3:1 segregation ratio weren't able to link. Each linkage maps were included 16, 18 and 24 linkage groups with total length of 2186.40 cM, 2582.50 cM, 2392.60 cM and the average length of a linkage group in each linkage maps were 136.65 cM, 143.47 cM and 99.69 cM. Meanwhile, number of markers/one linkage groups in each three linkage maps varied from 2 to 48, 2 to 30, 2 to 39 and the average distance between markers also was 18.37 cM, 16.45 cM and 14.95 cM, respectively.

Determined QTLs: Using the Composite Interval Mapping (CIM) method, we tried to define QTL(s) present in three populations of F2 individuals' link it. In this relationship, the CIM statistic test detected us 5, 1 and 1 QTLs in each populations that were placed on 9, 11, 12 and 24 linkage groups (or chromosomes) and the percentage of phenotypic variation explained by a QTL (R^2) for cocoon shell weight trait were 5.5-43, 70.4 and 29.6% in each studied segregation F2 populations, respectively (Table 4). These QTLs are designated as csw[x/y], that csw is cocoon shell weight, x is the linkage group and y is the AFLP marker closest to QTL (e.g: csw1/15).

According to Table 4 and h values (Stuber *et al*., 1987) in it, more detected QTLs in three F2 populations (3 No.) have underdominant or recessive effects (h<0) but the two QTLs have partial dominant effects (h>0.21-0.80) and remainder detected QTLs (2 No.) have dominant (h = 0.81-1.20) or over dominant effects on expression of cocoon shell weight trait (h>1.20).

Table 4: The determined QTLs of cocoon shell weight trait in three F2 populations of mulberry silkworm by Composite Interval Mapping (CIM) statistic test method at minimum threshold LRS (LRT) >12.5

Trait name	Linkage group	Nearest marker of QTL*	Adjacent markers to QTL	QTL	Likelihood Ratio Test (LRT)	Additive effect (H1:a)	Dominant effect (H2:d)	h**	R^2
First F2 population									
Cocoon shell weight (CSW)	9	28	TP15/120-TP08/285	*csw*9/28	15.08	0.0169	-0.0180	-1.065	0.204
	9	30	TP11/131-TP02/238	*csw*9/30	21.93	0.0419	-0.0690	-1.647	0.348
	9	39	TP07/170-TP05/320	*csw*9/39	12.56	0.0121	-0.0293	-2.421	0.055
	11	2	TP20/630-TP16/350	*csw*11/2	12.93	0.0421	0.0242	0.575	0.430
	11	3	TP16/350-TP16/162	*csw*11/3	12.72	0.0392	0.0263	0.671	0.427
Second F2 population									
Cocoon shell weight (CSW)	12	9	TP20/630-TP16/495	*csw*12/9	18.19	0.0474	0.0411	0.867	0.704
Third F2 population									
Cocoon shell weight (CSW)	24	22	TP08/205-TP06/314	*csw* 24/22	30.99	-0.0253	-0.0499	1.972	0.296

*The nearest place marker to QTL desired controller from the beginning side of linkage group. **: h is equal to the ratio dominance/additive effects. So if h<0, it show that QTL has underdominance or recessive effect on trait, if h = 0-0.20, it means that it has additive effect, if h = 0.21-0.80, it has partial dominance effect, if h = 0.81-1.20, it also has dominance effect and finally, if h>1.20, it means that QTL has over dominant effect on trait (Stuber *et al*., 1987)

DISCUSSION

Obtained results differ from previous studies, since, our varieties were unique and we conducted this experiment on these varieties firstly. Meanwhile our protocol was novel and interested method and we have minimum bias in our obtained results. Cocoon weight and cocoon shell weight are the main traits evaluated for productivity in sericulture and have been used for more than half a century shell weight gives a better measure of raw silk, but cannot be determined in commercial cultures because it requires damaging the cocoon. DNA markers closely linked to a characteristic of interest could be used to select and understand the genetics of productivity trait (Gaviria *et al*., 2006).

AFLP markers are excellences to applied variety of agronomically important organisms breeding because they have been widely employed to construct linkage maps and to map genes in them (Tan *et al*., 2001).

There is no absolute value for the number of DNA markers required for a genetic map, since the number of markers varies with the number and length of chromosomes in the organism. For detection of QTLs, a relatively sparse framework (or skeletal or scaffold) map consisting of evenly spaced markers is adequate and preliminary genetic mapping studies generally contain between 100 and 200 markers. However, this depends on the genome size of the species; more markers are required for mapping in species with large genomes. The power of detecting a QTL was virtually the same for a marker spacing of 10 cM as for an infinite number of markers and only slightly decreased for marker spacing of 20 or even 50 cM (Collard *et al*., 2005).

Statistical threshold is the most important in QTL analysis because the numbers of detected QTLs could be different when using different thresholds in QTL analysis (Lin *et al*., 1998). Most thresholds employed in published QTL analysis have been between LOD 2 and 3. In this study, our goal was to identify all possible putative QTLs, given that the F2 linkage map constructed is not saturated (Okogbenin *et al*., 2008), we used a threshold of LRT>12.5 for QTL mapping based on permutation test (n = 1000). Also, we applied three F2 populations here. Because these kind populations have non-stable status in comparison together, however they have been originated from same parental lines. But, its main advantage will be detected more QTLs.

Negative and positive h values of the QTLs show that the recessive, partial dominant, dominant and over dominant alleles controlled cocoon shell weight trait of mulberry silkworm and the alleles of identified QTLs which affected on cocoon shell weight were transmitted to progenies from both parents.

In total, the QTLs in each F2 populations which could be mapped, accounted for 146.4, 70.4 and 29.6% of phenotypic variance explained, respectively. More phenotypic variance explained in first F2 population can be in relation to additive effect of some QTLs interaction or appearance error for more distance (>20 cM) between two adjacent markers in a linkage group.

The remaining variation which could not be explained by the QTL model in other F2 populations may be due to undetected QTLs with too small effects not resolvable by this experiment, interaction between these QTLs and interaction of individual F2 genotypes with environment in the experiment (Okogbenin *et al*., 2008).

Some identified QTLs were to small effects because they are dependent upon interaction with other loci. It must be notice, for more detecting of QTLs with small effects we will need more number of progeny. An individual QTL is generally described as major or minor. This definition is based on the proportion of the phenotypic variation explained by a QTL (R^2 value), major QTLs will account for a relatively large amount (e.g. >10%) and minor QTLs will usually account for <10%. Also it is may be that sometime, major QTLs refer to QTLs that are stable across environments whereas minor QTLs may refer to QTLs that may be environmentally sensitive (Li *et al*., 2001; Lindhout, 2002).

There are many factors that influence the detection of QTLs segregating in a population (Asins, 2002). The main ones are genetic properties of QTLs that control traits, environmental effects, population size and experimental error. Genetic properties of QTLs controlling traits include the magnitude of the effect of individual QTLs and the distance between linked QTLs. Only QTLs with sufficiently large phenotypic effects will be detected and QTLs that are closely-linked (approximately 20 cM or less) will usually be detected as a single QTL in typical population sizes (<500) (Tanksley, 1993).

Therefore, on the base of QTLs' tables (Table 4) we can conclude that content genomes (amount and combination) in each F2 generation families have differences. It may be due to several factors: First, Lemon Khorasan and 107 parents are two distinct silkworm lines. The former is Iranian bivoltine and latter is from Japanese bivoltine races. Second, the crossing overs act as a variation apparatus in here as others animals. Third, a large fraction of the silkworm genome consists of families of transposable elements and the distribution of these elements has varied between silkworm strains (Tan *et al*., 2001).

In recent years some studies were done in order to identify and characterization of QTLs and genes controlling of economical traits of silkworm (Tan *et al*., 2001; Lu *et al*., 2005; Sima *et al*., 2006; Li *et al*., 2006).

The analysis of cocoon weight QTLs with these cocoon shell weight QTLs, suggest that there is not QTLs with pleoitropic effects or different QTLs affecting on these traits tend to be clustered together into closely linked groups (Okogbenin *et al*., 2008).

CONCLUSIONS

The identified QTLs in this study may be different to prior results because used lines, kind of populations, markers, molecular methods and some other conditions (such as experimental errors: mistakes in marker genotyping and errors in phenotypic evaluation) were non-equal. Ultimately these results can be used in marker assisted selection or gene transmission in breeding programs after confirming of these QTLs by other mapping populations and other molecular marker methods.

Of course ultimate perspective in this study were the reduction of breeding process expenditure and facility of attainment to new lines of silkworm. These can not realize until we extend this activity with complementary researches, such as cloning genes and transduction them by different methods.

ACKNOWLEDGMENTS

This study was prepared from MSc thesis of Alireza Bizhannia at Islamic Azad University, Science and Research Branch, Tehran-Iran. The authors also acknowledge the kind advice of Dr. Alireza Seidavi and Mr. Payam Potki for valuable comments and assistances.

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