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[Review Article]

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Prospects of Application of Linkage Disequilibrium Mapping for Crop Improvement in Wild Silkworm (*Antheraea mylitta* Drury)

Kunjupillai Vijayan, Ravindra Nath Singh* and Beera Saratchandra
Central Silk Board, BTM Layout, Madiwala, Bangalore-560068, Karnataka, India

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The wild silkworm, *Antheraea mylitta* Drury (Lepidoptera: Saturniidae) is a polyphagous silk producing insect that feeds on *Terminalia arjuna, T. tomentosa* and *Shorea robusta* and is distributed in the forest belts in different states of India. Phenotypically distinct populations of the *A. mylitta* are called "eco-race" or "ecotypes". Genetic improvement of this wild silkworm has not progressed much due to lack of adequate information on the factors that control the expression of most of the economically important traits. Considering the amazing technological advances taking place in molecular biology, it is envisaged that it is now possible to take greater control on these intractable traits if a combination of genetic, molecular and bioinformatics tools are used. Linkage disequilibrium (LD) mapping is one such approach that has extensively been used in both animal and plant system to identify quantitative trait loci (QTLs) for a number of economically important traits. LD mapping has a number of advantages over conventional biparental linkage mapping. Therefore, LD mapping is considered more efficient for gene discovery to meet the challenge of connecting sequence diversity with heritable phenotypic differences. However, care must be taken to avoid detection of spurious associations which may occur due to population structure and variety interrelationships. In this review, we discuss how LD mapping is suitable for the dissection of complex traits in wild silkworms (*Antheraea mylitta*).

Key words: *Antheraea mylitta*, Association mapping, Linkage disequilibrium, Tasar silkworm

*To whom the correspondence addressed
Central Silk Board, BTM Layout, Madiwala, Bangalore-560068, Karnataka, India
Tel:+91-80-26688704; Fax: +91-80-26681511;
E-mail: spilorns@yahoo.com

Introduction

Among the silkworms, the tropical tasar silkworm *Antheraea mylitta* Drury produces the largest cocoons (Akai, 2000). The silk obtained from these cocoons is used for making fabrics that is appreciated widely for its distinctive color and texture. Being a polyphagous insect, tasar silkworm feeds on a number of tree species such as *Shorea robusta, Terminalia arjuna*, *Terminalia tomentosa, Lagerstroemia indica, Zizyphus mauritiana*, *Madhuca indica*, *Hardwickia binata, Bauhinia variegate, Melastoma malabaricum,* and *Tectona grandis*. The distinct populations of this silkworm present in different forest patches are generally called "eco-race" or "ecotypes" (Gaur, 1992; Jolly *et al*., 1974), a term generally used for defining distinct races of a species genetically adapted to particular habitats. The fundamental phenotypic differences among these eco-races are their geographic distributions, voltinisms, host plant preferences, fecundity, larval durations, larval weights, shell weights and cocoon weights. Since most of these phenotypic traits are highly influenced by the environmental factors such as temperature, relative humidity and rainfall, considerable variations among the individuals of the same eco-race have also been noticed (Srivastava *et al*., 2000; 2002). For instance, the same eco-race may behave as trivoltine, bivoltine and univoltine depending on the altitude of the locality (Kar *et al*., 2000; Nayak and Dash, 1991). It is also believed, though scientifically not yet proved, that all these eco-races have evolved from a single population that had been distributed extensively across these forest stretches. At present 44 eco-races have been widely recognized (Table 1), of which Daba, Raily, Sukinda, Sarihan Modal, Bhandra and Andhra local have great economic importance (Srivastava *et al*., 2000; 2002). Some of the important eco-races along with their relevant characters are given in Table 2.

Genetic studies in wild silk moths (including tasar silk-

Table 1. Diversity of *Antheraea mylitta* Drury in relation to distribution, soil type, food plant and forest type

Sl. No	Ecorace	Collection site	Predominant food plant	Soil type	Forest type
1	Daba	Singhbhum (Jharkhand)	Terminalia arjuna T. Tomentosa	Red loamy	Tropical moist deciduous
2	Sarihan	Santhal pargana (Jharkhand)	Terminalia arjuna T. tomentosa	Bed loamy	Tropical moist deciduous
3	Munga	Santhal Pargana (Jharkhand)	Shorea robusta	Red loamy	Tropical moist deciduous
4	Modia	Dhanbad (Jharkhand)	Shorea robusta	Red loamy	Tropical moist deciduous
5	Laria	Peterbar, Hazairbagh (Jharkhand)	Shorea robusta	Red loamy	Tropical moist deciduous
6	Lodhma	Ranchi (Jharkhand)	Shorea robusta	Red loamy	Tropical moist deciduous
7	Palma	Ranchi (Jharkhand)	Shorea robusta	Red loamy	Tropical moist deciduous
8	Japia	Palamau (Jharkhand)	Shorea robusta	Red loamy	Tropical moist deciduous
9	Kowa	Palamanu (Jharkhand)	Shorea robusta	Red loamy	Tropical moist deciduous
10	Barharwa	Simdega (Jharkhand)	Shorea robusta	Red loamy	Tropical moist deciduous
11	Modai	Keonjhar (Orissa)	Shorea robusta	Red loamy	Tropical moist deciduous
12	Nalia	Sundergarh (Orissa)	Shorea robusta	Red loamy	Tropical moist deciduous
13	Sukinda	Sukindagarh (Orissa)	Terminalia arjuna T. Tomentosa	Red loamy	Tropical moist deciduous
14	Boadh	Phulbani (Orisssa)	Terminalia arjuna T. Tomentosa	Red loamy	Tropical moist deciduous
15	Simlipal	Phulbani (Orissa)	Terminalia arjuna T. Tomentosa	Red loamy	Tropical moist deciduous
16	Omarkote	Kalahandi (Orissa)	Terminalia arjuna T. Tomentosa	Red loamy	Tropical moist deciduous
17	Sikly	Khairpali (Orissa) Beramkela (Chhattisgarh)	Shorea robusta, Anogeissus latifolia	Red loamy	Tropical dry deciduous
18	Raily	Bastar (Chhattisgarh)	Shorea robusta	Sandy red	Tropical dry deciduous
19	Kurudh	Kurudh (Chhattisgarh)	Terminalia tomentosa	Red loamy	Tropical moist deciduous
20	Multai	Multai (Madhya Pradesh)	Terminalia arjuna T. Tomentosa	Red loamy	Tropical moist deciduous
21	Mandalla	Mandalla (Chattisgarh)	Shorea roubusta	Red loamy	Tropical moist deciduous
22	Jhabua	Jhabua (Madhya Pradesh)	Shorea robusta	Red loamy	Tropical moist deciduous
23	Bhopalpantnam	Bhopalpantnam (Chhattisgarh)	Shorea robusta	Red loamy	Tropical moist deciduous
24	Pipral	Pipral (Madhya Pradesh)	Shorea robusta	Red loamy	Tropical moist deciduous
25	Seoni	Seoni (Madhya Pradesh)	Shorea robusta	Red loamy	Tropical moist deciduous
26	Janghbhir	Bastar (Chhattisgarh)	Shorea robusta	Red loamy	Tropical moist deciduous
27	Korbi	Korba (Chhattisgarh)	Shorea robusta Terminalia sps	Red loamy	Tropical moist deciduous
28	Tira	Purulia (West Bengal)	Lagerstroemia parviflora. speciosa	Red loamy	Tropical moist deciduous
29	Bankura	Bankura (West Bengal)	Lagerstroemia parviflora.	Red loamy	Tropical moist deciduous
30	Dadar & Nagar Haveli	Dadar & Nagar Haveli (Union Territory)	Terminalia parviflora	Red loamy	Tropical deciduous
31	Shiwalika	Batote (J&K) Palampur (H.P)	Zizyphus jujube	Forest hill & Alluvial	Mountain sub-tropical
32	Bhandara	Bhandara (Maharashtra)	Terminallia arjuna. T. tomentosa	Black clayey	Tropical dry deciduous
33	Andhra local	Adilabad, Karimnagar (Andhra Pradesh)	Terminalia arjuna T. tomentosa	Black clayey	Tropical dry deciduous

Table 1. Continued

Sl. No	Ecorace	Collection site	Predominant food plant	Soil type	Forest type
34	Monga	Deoria (Uttar Pradesh)	Ternminalia arjuna, T. tomentosa zizyphus jujube	Red loamy	Tropical dry deciduous
35	Mirzapur	Mizapur (Uttar Pradesh)	Zizyphus jujube	Red loamy	Tropical dry deciduous
36	Sultanpur	Sultanpur (Uttar Pradesh)	Terminalia arjuna T. tomentosa	Red loamy	Tropical dry deciduous
37	Tesera	Sahabad (Rajasthan)	Zizyphus jujube	Red loamy	Tropical dry deciduous
38	Nowgong	Nowgong (Assam)	Zizyphus jujube	Red loamy	Tropical dry deciduous
39	NE1, 95	Boko (Assam)	Zizyphyus jujube	Red loamy	Tropical dry deciduous
40	NE2, 95	Mendipathar, Resubelpara (Meghalaya)	Zizyphus jujube, Careya aroborea	Laterite	Tropical wet ever green
41	Jiribam	Jiribam (Manipur)	Zizyphus jujuba	Red loamy	Tropical wet ever green
42	NG, 94	Dimapur (Nagaland)	Zizyphus jujuba	Red loamy	Tropical wet ever green
43	Belgaum	Belgaum (Karnataka)	Hardwickia binata	Laterite	Tropical wet ever green
44	KEo_2	Moorkhanparamoba (Kerala)	Ancardium occidentale	Laterite	Tropical moist evergreen

Table 2. Commercial characters of different eco-races of *Antheraea mylitta*

Race	Volti-nism	Single cocoon weight (g)	Single shell weight (g)	Filamnet length (m)	Den ier	Silk recovery (g) from 1000 cocoon	No. of cocoons for 1 Kg silk
Daba	Bi	12~14	1.45~3.0	850~1200	10	750~1000	1000~1280
Raily	Uni / Bi	15~18	3.0~4.5	1200~1500	10	1500~1600	650~800
Sukinda	Tri	12~14	1~1.45	700~900	10	750~1000	900~1150
Sarihan	Tri	8~10	0.7~0.8	500~625	8	440~500	2280~2350
Bhandara	Tri	8.5~9.5	0.8~0.9	575~700	7	450~500	2220~2250
Andhra local	Bi	8.0~9	0.9~1.0	600~650	8	550~600	1750~1800
Modal	Bi	15~18	3~4	1200~1500	10	1800~1900	650~900
Laria	Bi	7~9	0.8~1.0	550~650	7	550~650	1900~1975

worm) have not been progressed as well as that in mulberry silkworm *Bombyx mori* L. Therefore, the true potential of these silkworms could not be exploited. Recently, a few attempts have been made to characterize these "eco-races" using morphological, biochemical and molecular markers which have provided some insights (Chatterjee *et al*., 2004; Kar *et al*., 2005; Mahendran *et al*., 2006; Saha and Kundu 2006; Srivastava *et al*., 2001; Vijayan *et al*., 2005). However, most of these studies have limited to understanding the differences between two or three eco-races or between populations of the same eco-races.

Genetic improvements of these ecoraces through conventional breeding have not met with much success because of the complexity of the traits and the lack of information on the factors controlling the expression of these traits. Most of the economically important traits like cocoon weight, silk yield etc. do not seem to follow the classical Mendelian inheritance (Lander and Schork, 1994), They are mostly of quantitative in nature, hence may be under the influence of a number of genes with minor effects. Phenotypic expression of these quantitative traits is the result of interaction among multiple quantitative trait loci (QTLs) (DNA segments that contribute to the expression of that trait), and between QTLs and environmental factors (Namkoong *et al*., 1988). Therefore, in order to tackle these quantitative traits, information on the number of QTLs involved in the expression of each of these traits and their interactions among themselves and with others need to be elucidated. Although QTL mapping has been in wide use for nearly two decades, it has not been progressed much in tasar silkworm. One of the reasons for this lack of progress on QTL mapping in tasar silkworm is that they are still in the wilderness and have high heterozygosity. Therefore, efforts to develop inbreeds through conventional breeding methods have also been under constraint from inbreeding depression and other related genetic bottle necks. Since QTL mapping is

an essential step to facilitate marker assisted breeding in tasar silkworm, attempts have been made in this article to review the current QTL mapping strategies and their possible applications in Tasar silkworm.

The two important strategies that have been used for QTL mapping in both plants and animals are the linkage analysis and the association mapping.

Linkage mapping

The traditional way of linkage mapping is to construct a genetic map based on data from experimental populations, which are raised through systematic breeding between two selected parents. Populations such as F2, back cross (BC), double haploid (DH), recombinant inbred lines (RIL) and near isogenic line (NIL) are used for investigating the segregation of traits of interest in different environmental conditions. Genotyping of the parents and segregating populations are done with DNA markers that are able to differentiate both parents and the segregating populations. Once the genotypic data are available, linkage maps are constructed using specific software. However, the success of linkage mapping is largely decided by the genetic variations that are present in the mapping population, the size of the mapping population and the number of marker loci used for analysis. The major disadvantage of biparental linkage mapping is the low resolution resulting from the few meiotic events occurring during the experimental mating. Besides in tasar silkworm, lack of inbreeds also poses serious problems in QTL analysis.

Association mapping or Linkage disequilibrium (LD) mapping

Association mapping is a new method of identifying QTLs based on significant allele-frequency differences between individuals with the phenotype of interest ("cases") and controls (Farnir *et al*., 2000; Pritchard *et al*., 2000a). It has several advantages over the traditional biparental mapping strategy. Important among them are (1) Provides better mapping resolution by exploiting higher number of meiotic recombination. (2) It uses natural populations or germplasm, therefore, it reduces the research time taken for developing expensive and tedious biparental populations that are essential for classical linkage mapping. (3) LD mapping uses information from the whole population, hence, a broader genetic variation in a more representative genetic back ground is available (Kraakman *et al*., 2006; Nordbog and Tavare, 2002). A number of different methods are available for estimating LD in a population, but the most frequently used LD measures are D or D' [$(D = P_{AB} - P_A P_B = P_{AB}P_{ab} - P_{Ab}P_{aB})$, where P_A and P_B are allele frequencies], and r^2 (Hartl and Clarke, 1989; Hill, 1974). The D' is the standardized disequilibrium coefficient, which mainly measures recombinational history and is therefore useful to assess the probability of historical recombination in a given population. This statistic may be affected by sample size (with smaller size samples demonstrating larger D'_m values) and allele frequencies (Mohlke *et al*., 2001) and therefore may not be appropriate for comparison of LD between studies that use different sample sizes and markers with differing numbers of alleles and allele frequencies. The r^2 is essentially the correlation between the alleles at two loci and it summarizes both recombinational and mutational history (Hill, 1974). The r^2 values we use are the square of the Pearson correlation coefficients, and are related to D by $r^2 = D^2/[p_A(1-p_A)p_B(1-p_B)]$. The r^2 value of equal to 0.1 (10%) or above is considered as significant threshold for the rough estimate of LD to reveal association between pairs of loci. There are two common ways to summarize and visualize the LD across regions. LD decay plots are used to visualize the rate at which LD declines with genetic or physical distance while disequilibrium matrix is used for visualizing the liner arrangement of LD between polytrophic sites within a gene or loci along the chromosome.

Strategies of association mapping

The classical approach for LD mapping is the case-control strategy wherein populations are divided into a case, which is population with desired trait one lacking it is the control. The Pearson chi-square test or Fisher's exact test or Yates continuity correction is used for comparing allele frequencies to detect association between a phenotype in question and the neutral molecular markers. In case-control studies, sampling can be effectively targeted to maximize power. However, case-control method often suffers from the serious disadvantage of being affected severely by the population stratification which results in identification of "spurious associations" (Lander and Schork, 1994). Such association happens when the subpopulations show differences in the phenotype in question. A number of efforts have recently been made to eliminate this confounding effect of population stratification. Important among them is the structured association method developed by Pritchard *et al*., (2000b). This method employs Bayesian approaches to search for closely related clusters and uses the clustering matrix (Q) to correct the false association between marker and the trait. This method is implemented in the program STRUCTURE, which is available from http://www.stats.ox.ac.uk/~pritch/home/html.

Genotyping strategies

The two types of genotyping strategies used for LD mapping are candidate gene analysis and genome wide asso-

ciation analysis. Both candidate gene and whole genome scan analyses require large number of polymorphic markers. Thus, it is often necessary to conduct a preliminary survey on the nuclear diversity in the genomes. Similarly, due to the huge requirement of densely populated polymorphic markers for genome wide scan, in most of the crops candidate-gene analysis is the easiest way to start association mapping.

Candidate gene analysis

Candidate-gene association mapping relates polymorphisms in selected candidate genes that have purported roles in controlling phenotypic variation for specific traits (Zhu *et a*l., 2008). In this approach the critical factor is the selection of the candidate gene for the association mapping. Candidate genes are usually selected based on prior knowledge from mutational analysis, biochemical pathway, or linkage analysis of the trait of interest (Zhu *et al*., 2008). In general, candidate gene selection is easy for simple biochemical pathways but for complex traits the whole genome could potentially serve as a candidate gene (Yu and Buckler, 2006). One of the easy, reliable, and rational methods of candidate gene selection is the utilization of expressed sequence tag (EST) data bases of the crop. Once the candidate gene is identified, a chromosome segment of at most a few megabases that has strong association with the phenotype is identified. Candidate gene association mapping is, in fact, a hypothesis driven approach to dissect complex traits. In this method, relevant candidate genes are selected and ranked based on the evaluation of available results from genetic, biochemical, or physiological studies (Mackay and Powell, 2007). Genetic markers with very high frequency like SNPs, which are potentially in LD with causative polymorphism, are ideal for candidate gene mapping. The rate of LD decay for a specific candidate gene locus dictates the number of markers per unit length needed to identify significant associations. Since, SNPs present in the coding regions often cause phenotypic variation; they should be given top priority while selecting markers for candidate gene mapping.

Genome wide association analysis

Genome wide association mapping or gene scan surveys genetic variation in the whole genome to locate genes or narrow regions that have significant statistical connections to various complex traits. The discovery of these genes and regions offers the potential to increase understanding of biological process controlling manifestation of these complex traits (Pan *et al*., 2009). To conduct a genome wide association analysis, enormous number of densely distributed markers is necessary. Therefore, whole genome scan is usually carried out using the most frequent genetic variants available in the genome, i.e., single nucleotide polymorphisms (SNPs). With the low cost genotyping technologies, it is now possible to collect extensive data on SNPs present in the genome of organisms.

Marker suitability

In general, the most preferred markers for association studies are SSRs and SNPs. Dominant markers such as RAPD, ISSR and AFLP are not suitable for association mapping due to the difficulties in interpreting the allelic-trait association. The major limitation of dominant markers is their limited statistical power as compared to that of the co-dominant markers. Nonetheless, RAPD, RFLP, AFLP markers can be used to achieve genome-wide coverage for characterizing the genetic composition of the individuals. This information is essential for assigning individuals to respective populations. This can prevent spurious association of markers resulting from population structure and consanguinity (Pritchard and Rosenberg, 1999). In this context, it is important to know that CDFD, Hyderabad, India has already generated 57,113 ESTs and 87 SSR primers for wild silkworms (Arunkumar *et al*., 2008, 2009). These genomic resources will be of much use for the association studies of this much valuable insect species.

Prospects of linkage disequilibrium mapping in tasar silkworms

Linkage disequilibrium is a powerful tool in gene mapping for a population that has derived from a relatively small number of founders and the expansion of the population has occurred by growth rather than by immigration. A population originating from a relatively small number of founding individuals is exposed to recombinations over many generations to create the larger set of recombined haplotypes of present day population. Association mapping exploits this recombination over a great many generations and does not require to collect the pedigree information. Since most of the ecoraces of *A, mylitta* currently available must have originated from a few founder individual of a single population through geographic isolation and subsequent adaptive evolution. Genetically isolated populations offer many advantages for association mapping. The founder effect and high degree of inbreeding in small populations result in an increased incidence of expression of rare recessive characters, which will be very hard to find in large heterogeneous populations (Peltonen, 2000). A relevant hypothesis says that in isolated populations fewer influencing genes behind a given traits will be encountered than in populations of more heterogenous origin (Lander and Schork, 1994). Several association mapping studies in polygenic

characters like diseases have been successfully carried out in isolated populations (Ginns *et al*., 1996). Recent studies with molecular markers like RAPD, ISSR (Chatterjee *et al.*, 2004; Kar *et al*., 2005; Mohandas et al., 2004; Saha and Kundu, 2006; Vijayan *et al*., 2005), RFLP (Mahendran *et al*., 2006) have shown genetic considerable variations among different ecoraces. These inter-ecoracial genetic diversity clearly suggest that association mapping is well suited to tasar silkworm. For analyzing the marker-phenotypic trait associations, case-control methods can be easily adopted in tasar silkworms as the ecorace with the given phenotypic trait under investigation can be considered as the "case" and a related but phenotypically distinct ecorace can be considered as the "control". However, there are also several cases where intermixing of ecoraces have been observed. In such cases the admixture of populations may pose problems for the application LD mapping. Nonetheless, with the help of the statistical methods developed by Pitchard *et al*., (2000a,b) and implemented in the program *structure,* it is possible to identify population structures and to assign the sampled individuals to putative "unstructured" subpopulations which do not exhibit association between unlinked markers.

Although LD has not been utilized in tasar silkworms, LD has been extensively used in many organisms such as human (*Homo sapience*), cattle (*Bos taurus*), fruit flies (*Drosophyila melanogaster*) (Flint-Garcia *et al*., 2003), and in a number of plants (Gupta *et al.* 2005). The results of these studies clearly suggest that LD mapping is a powerful strategy to identify QTLs for important traits without generating mapping populations. Similarly, LD mapping can also be used for population genetic and genetic diversity analysis of different ecoraces. Since the neutral theory of evolution states that majority of polymorphism observed within and among species are selectively neutral (Tajima, 1989), it is possible to investigate population dynamics such as selection, migration and demographic history with the neutral markers. It is also possible to infer demographic history of a population from the pattern of DNA polymorphism, if data from a number of independent loci are used and it is assumed that the demographic history affects the entire genome in the same way (Gupta *et al*., 2005).

Thus, the information generated through association studies will be of much use not only in breeding programs but also in formulating appropriate conservation strategies for this very valuable insect species.

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