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Analysis of Genetic Diversity and Relationships of Silkworm Varieties Using Molecular Marker

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Abstract: ***The genomic DNA of seventeen silkworm were amplified with OPA-02 primer revealed 45 RAPD bands out of which 90% is polymorphism and 10% is monomorphic silkworm is polygenic. Silkworm variety 9 was not shown any amplification of bands however the varieties 1 and 2 showed one band each some of the DNA fragments were strain specific and some could differentiate the multivoltine from the bivoltine strains or vice versa. Silkworm genetic resources that are being maintained in Hosur, Tamil Nadu, India, are yet to be adequately topped to develop elite varieties that are subjected to different agro-eco-climatic condition of the country like India. Molecular markers are known to provide unambiguous estimated of genetic variability of silkworm populations. Since they are independent confounding effects of environment, the genetic similarity among the seventeen silkworm varieties/strains and their genetic diversity and relationships were discussed. The amplification bands were very poor.***

Key words: Genetic diversity, Silkworm varieties, molecular marker, RAPD.

I. INTRODUCTION

A wide array of DNA marker techniques is available for genetic studies. All DNA markers reflect differences in DNA sequences. Typically, in a diploid organism, each individual can have one or two different states (all-eles) per character (locus). The choice of a particular genetic marker often depends upon the purpose of the study and is usually a trade-off between practicality and precision of genetic markers. One manifestation of this is the dichotomy between multilocus DNA techniques which include Random Amplified Polymorphic DNAs (RAPDs), Amplified Fragment Length Polymorphisms (AFLPs) and Inter-Simple Sequence Repeat PCR markers (ISSR-PCR) and single locus techniques which include single copy nuclear DNA regions and microsatellites. Multilocus methods result in simultaneous visualization of many dominant markers and are technically convenient, but have some marked weaknesses and limitations, including dominant inheritance - the DNA fragment can be scored only as present or absent, and in many cases substantial proportion of variation they detect can be non-heritable or not even derived from the target organism. By contrast, single locus markers are quite informative because they provide alleles whose zygosity status can be assayed easily. Mulberry silkworm *(Bomby mori* L.), the most important silk producing insect, exhibits wide diversity in morpho-biochemical and biometric characters. In general, silkworm strains from temperate countries like China, Japan and Korea complete two generations per year (bivoltines) by adopting egg diapauses. On the other hand, those from tropical countries like India, Bangladesh and tropical belts of China complete five to six generations, per year without undergoing diapauses, hence they are called polyvoltine silkworms. The polyvoltines are comparatively smaller in size but can with stand adverse climatic conditions such as higher humidity, temperature and to a certain extent exhibit tolerance to diseases. However, from the productivity point of view, bivoltines are preferred'. Since the bivoltines are prone to abiotic and biotic stresses prevailing in the tropical sericulture belt, attempts are being made to develop silkworm hybrids with productivity traits of bivoltines and tolerance of polyvoltines[2]. In order to achieve this goal, knowledge on the genetic makeup of silkworm strains is necessary. The present investigation is an attempt in this direction where bivoltines are compared with polyvoltines along with a few mutants derived

from bivoltines but with intermediate voltinism pattern (Table 1).Using RAPD markers, researchers have characterized silkworm strains[3,5]. However, in the present study the silkworm mutants, which are believed to have originated from bivoltines, but closer to polyvoltines in yield attributes, are also included to understand the genetic relatedness with either of the genotypes. The ISSR primers are core sequences of SSRs and reported to be widely spread over genome[6], provide more realistic genetic information in closely related genotypes like silkworm. In addition to RAPD and ISSR markers, RFLP-STS markers are also used, thus including both dominant and co-dominant markers for drawing precise conclusions on their genetic relatedness.

The biology and genetics of silkworm, *Bombyx mori, is* the most advanced of any lepidopteran species. Its rich repertoire of genetic resources and potential applications in sericulture and as a model for other Lepidoptera led to the initiation of genomics research. During the past decade much effort has been made in the areas of marker development, and molecular maps have been constructed in standard strains with the use of RFLPs, RAPDs, ISSRs, STSs, and microsatellites. The potential applications of molecular markers and linkage maps include stock identification, Marker Assisted Selection (MAS), identification of Quantitative Trait Loci (QTL), and, ultimately, positional cloning of visible mutations and QTL. To these ends, BAC libraries have been constructed and are being used to make large-scale physical maps, with markers based on ESTs as framework anchors. Altogether this work provides a foundation for identification of gene function, gene and chromosome evolution, and comparative genomics.

II. GENETIC MARKERS

In the past decade, several key advances in molecular biology have greatly advanced the impact of molecular genetics in biology. Most important have been: (i) development of the polymerase chain reaction (PCR), which amplifies specific stretches of DNA to usable concentrations, (ii) the application of evolutionarily conserved sequences as PCR primers, (iii) the advent of hyper variable microsatellite loci as genetic markers and (iv) the advent of routine DNA sequencing in biology laboratories. These innovations, coupled with the recent explosion of powerful analysis and relatively user-friendly computer programs, have accelerated the pace at which molecular genetic data can be used for various programmes. These developments led to the detection, analysis and exploitation of naturally occurring DNA sequence polymorphisms in eukaryotes and laid a foundation for contemporary genomics of several organisms. Polymorphic genetic markers have found potential applications in animal and plant improvement programmes as a means for varietal and parentage identification, construction of molecular linkage maps and evaluation of polymorphic genetic loci affecting quantitative economic traits.

Considering the genetic in formativeness offered by microsatellite loci, which are co-dominant, a microsatellite discovery programme in the silkworm genome was initiated[21]. Microsatellites, also known as simple sequence repeats (SSRs), are short stretches of nucleo-tides in which a motif of one to six bases is tandemly repeated, have emerged as ideal markers in eukaryotic genetics. They are ubiquitous in prokaryotic and eukaryotic genomes and are randomly distributed, both in protein coding and non-coding regions. The sequences in microsatellite motifs can differ in repeat number among individuals, providing a ready source of polymorphism. With the advent of the PCR, this property of microsatellite DNA was converted into highly versatile genetic markers[17,19]. PCR products of different lengths can be amplified using primers flanking the variable microsatellite region Due to the advantages offered by microsatellites they have been used to construct linkage maps. In addition to their clear utility for practical applications, information about the distribution and variability of microsatellite sequences in the genome of a given species can help elucidate its genetic history from the standpoint of evolution and artificial selection.

The perusal of the earlier literature indicates that most of the RAPD and ISSR molecular markers were carried out on different varieties silkworm. It is also also noted that earlier researchers have not investigated the molecular markers studies in some of the silkworm varieties. The investigation of RAPD is necessary to be used as a tool to investigate the genetic diversity and relationships of silkworm varieties. It is appropriate to evaluate their use in breeding purpose. Although sporadic reports on molecular markers in silkworm varieties (are mostly known from the earlier researchers) they have not examined the RAPD and ISSR in detail in other silkworm varieties regarding it is, therefore pertinent to investigate the above said parameters

In the present investigation, 17 silkworm varieties from Hosur germplasm Tamil nadu, was selected to carryout RAPD molecular markers to study genetic diversity and relationships silkworm varieties

III. MATERIALS AND METHODS

The present investigation was carriedout in the Department of 0m –Bioscience research center, 2nd stage, Indiranagar, Bangalore, and Horticulture, Biotechnology Centre, DNA Fingerprinting Laboratory, Govt of Karnataka, Hulimavu, Banerghata Road, Bangalore.Karnataka.India, during the year January-2006 to December-2007.The materials used and methods followed in this study are presented here.

IV. SILKWORM STRAINS

The 17 different silkworm strains **ANISH E2(M), SANISH E2(M)** , **SANISH-8** , **SANISH-17, SANISH-18(M), SANISH-18(P) ,SHEIKHI-I , SANIS H-30, SHEIKHI-II**, **TETRA HYBRID (P), SHINKI REYAKU, NB4D2, NB7, SH-6, SF-19 and JD-6SANISH** comprising of bivoltine and polyvoltine accessions silkworm stocks having divergent geographical origin were used in the study. These silkworms were maintained at Central Sericultural Germplasm Resources Centre, Hosur, Tamil Nadu, India (77.51°E, 12.45°N, 942m AMSL) [www.silkgermplasm.com], following the recommended rearing practices.

V. DNA Isolation

Genomic DNA was extracted from the silkworm moth using phenolchloroform method briefly; Silk moths were frozen first with liquid nitrogen and homogenized with cooled mortar and pestle. The powdered content was transferred to an Oakridge tube and 5

mL of the extraction buffer (100 *mM* Tris-HCl, 8.0,50 mM EDTA and 1% SDS) and 25 uL of proteinase K (100 ug/mL) were added before incubating it at 37°C for 2 h with occasional swirling. DNA was extracted twice with phenol-chloroform-isoamyi alcohol (24:24:1) and once with chloroform. The supernatant DNA was precipitated in ethanol in the presence of 3 *M* sodium acetate (pH 5.2), re-suspended in TE buffer (10 *mM* Tris-HCl, 1 *mM* EDTA, *pH* 8.0). The RNA contamination was removed by incubating with RNase A (100 ug/mL) at 37°C for 1 h. DNA was re-extracted with phenol-chloroform and precipitated with ethanol. The genomic DNA was quantified on 0.8% agarose gel and diluted to uniform concentrations (10 ng/ul) for PCR amplification.

VI. PCR Amplification of Genomic DNA

For RAPD, the amplification of genomic DNA was carried out in a PTC-200 Thermal-cycler (MJ Research Inc., Watertown, Massachusetts, USA) using 12 random decamer primers will be obtained from Operon Tecnhnologies, USA (OPW series) following the standard procedure Twenty-one ISSR (Inter simple sequence repeat) primers will be procured from the University of British Columbia, Vancouver, Canada (UBC set No. 9), will be tested for their efficacy in amplification of Silkworm DNA employed in this study. PCR amplification of the DNA was carried in the Thermal-Cycler, PTC 200[10].

VII. DNA Electrophoresis

Amplified DNA fragments were separated out on 1.2% agarose gel stained with ethidium bromide. Running buffer containing Tris-buffer, Acetic acid and EDTA (pH 8.0) was used for electrophoresis and for preparing gels. Wells were loaded with 25µl reaction volume and 5 µl of loading buffer (Sucrose, Bromophenol blue and Xylene cyanol) together. Electrophoresis was conducted at 45 volts for 3 hours and the gel photographed under UV light using gel dock system (Herolab).

VIII. STATISTICAL ANALYSIS.

Binary coding was used to score gel and each band of primer was scored of 6 species and 12 primers with 100 to 1000 base pairs Marker level pair wise squared Euclidean species was calculated and utilizing these distances, species were clustered following Wards method. The segregation of species was also assessed through principle component analysis, the Statistica version 5.0 a computer application was used to generate dendrogram using squared Euclidean distance and Wards method.

IX. RESULTS

The yield and quality of DNA extracted by following the procedure described earlier was 10-25 ng per µl for silkworm moth. The DNA obtained was amplifiable and of high quality. Spectrophotometer reading of 1.7-1.8 (260nm/280nm) confirmed the quality of DNA.DNA isolated from silkworm moth using 20 ml extraction buffer yielded good quality, high molecular weight DNA (above 50 kb). The quality of the DNA was also confirmed by gel electrophoresis.

In this study, 100 Operon random ten-base long, single stranded arbitrary primers (OPW -02, OPW-O4, OPW-05 OPW-06 OPW-011 OPW-12 OPW-13 OPW16 OPW17 OPW18 OPW19 and OPW-20 Table-1) were screened using the silkworm varieties, which on an average gave nine bands. The selected 12 arbitrary RAPD primers were used for the screening of the silkworm varieties, in which 20% monomorphic and 80% polymorphic and were considered for the precise calculation of genetic diversity.Twelvel RAPD primers revealed 45 markers of 32 which polymorphic. All the silkworm varieties grouped in to four groups and indicating RAPD bands lie in between the 1.0 kb to 2.0kb

The basic protocol described elsewhere for PCR was optimized for high quality amplification and intense repeatable banding patterns. However, a reduction in the amplification of fainter bands was noticed with large changes in template DNA concentrations, while too much DNA produced a smear effect, which emphasized the importance of quantification of the DNA for clear amplification.

A representative of the PCR amplification product of the 17 silkworm varieties is as shown in fig.1 which yielded sufficient polymorphisms to distinguish between the silkworm varieties by Ward's method of analysis with Squared Euclidean distance gave dendrogram. Based on the number of bands all the 17 silkworm varieties were grouped in three clusters **(Fig. 2).** The dendrogram revealed a maximum similarity between the silkworm varieties. Every single silkworm accessions /hybrid could be identified using these selected twelve primers.

Analysis of diversity based on PCR fragments amounted to saturating the genome. The analysis of 17 silkworm varieties revealed that diversity is moderate to high and has shown even differences also.The maximum polymorphism between silkworm varieties could reveal significant correlation.

X. DISCUSSION

The data regarding RAPD molecular marker on selected 17 silkworm varieties showed considerable variability. The larval duration was in the different ranges and final instar duration varied from from 128-181 h. The larval, cocoon and shell weight were showing considerable variation between silkworm varieries. The squared Euclidean distances was calculated using the data on 17 silkworm varieties with 12 Primers (OPW -02, OPW-O4, OPW-05 OPW-06 OPW-011 OPW-12 OPW-13 OPW16 OPW17 OPW18 OPW19 and OPW-20 Table-1) Highest distance was observed between JD and Sanish E2 and Sanish E2and SH-6. The lowest distance was recorded between Sheikhi-1and. Sheikhi-2

The distance within silkworm varieties was least (2.84 units) and highest (4.6 units) and intermediate distance between (3.82 units). However, this was in line with the results obtained in Wards genetic diversity calculations. The dendrogram (Fig.2) showed four distinct clusters namely Cluster1Sanish E2a,SanishE2b, Sanish8 and Cluster 2 included Sanish17, Sanish18, ,

Sanish30,Cluster3 Sheikhi-i Sheikhi-ii tetra hybrid (p) kyorieshimpaku (p) kyorieshimpaku (m)and shinki reyaku (m0) and cluster4 included NB4D2 Sf-19 Sh-6 and Jd-6 From the pattern of clustering, it was pertinent that RAPD technique was efficient in segregating silkworm varieties into different four clusters. More significantly, the clustering had been largely successful in retaining the relationship between Silkworm varieties as proosed by Chikkaswamy In the light of current study at varietal level, it can be seen in the clustering pattern that the series were clearly distinguished in silkworm varieties. The association of the varieties observed in the present study was similar to the pattern observed by Awasthi et.al, (2008) and Nagaraj and Nagaraj (1995).and Chatterjee and Pradeep (2003).

XI. Differential Polymorphism of DNA

The observed differences among 17 silkworm varieties could be ascribed to the fluctuating micro and macro climatic conditions of habitat. Ward's analysis of RAPD data also reveals that all silkworm varieties belonging to the state of Karnataka are genetically closer. The greater sensitivity of RAPD obtained in the result to species diversity may be derived from rapid evolution of non-coding, repetitive D N A sequence detected by R A P D s. This hypothesis has been corroborated from (Plomion et al. 1995). Akagi et al (1996), Akagi, et al. (1997), Anand, . and Murthy, (1968) , Anand, . and Rawat, (1984), Arunachalam and Bandyopadhyay (1984) Arunachalam, Bandyopadhyay, Nigam, and Gibbons, (1984), Ashoka, and Govindan, (1990a), Ashoka, and Govindan, (1990b), On comparison of 17 silkworm varieties in, it can be observed that though they differ largely in their, phenotypic expression, but the genetic difference as observed from DNA fingerprints was found to be low. The genetic variation as detected by RAPD analysis opens up the avenue for the proper identification and selection of the species/varieties that could be used for varietal identification and planning for future breeding programme.RAPD analysis revealed a high degree of genetic diversity among the species/varieties, examined in the study, which can contribute to the silkworm crop involvement. RAPD analysis can also be used for detecting gene flow between species. Furthermore, this technique is less restricting than other molecular technique RFLP (Restricted Fragment Length Polymorphism), as no hybridization and no use of radioisotopes is required, and it is therefore more convenient for use in research centers in developing countries.Thus our results demonstrate that DNA content and RAPD markers provide an effective tool for detection and evaluation of genetic variation existing among 17 silkworm varieties of the species *Bombyx mori.* Seventeen diverse strains of Silkworm (*Bombyx mori*) were analysed using the sequence repeat polymerase chain reaction for RAPD molecular marker. The result presented in the present study demonstrated the utility of using RAPD markers to characterize genetic diversity among 10 silkworm varieties. Differential polymorphism was noted in 17 silkworm varieties showing variation in percentage of polymorphic bands from 20% to 90% using 12 Primers (OPW - 02, OPW-O4, OPW-05 OPW-06 OPW-011 OPW-12 OPW-13 OPW16 OPW17 OPW18 OPW19 and OPW-20 Table-1) (. The observed high proportion of polymorphic loci reveals profound variation among the silkworm varieties. Significant genetic variations by RAPD have also been reported in other species leve Awasthi et.al, (2008), Nagaraj and Nagaraj (1995).and Chatterjee and Pradeep (2003). Basavaraja, et al (2001), Benchamin, et al (1988), Bhandari, et al (1997), Bhargava et al (1995), Buteler and LaBaonte (1999), Chandel and joshi (1983), Chatterjee, and Datta (1992), Chatterjee et al (1990), Chatterjee et al (1993), Chattopadhyay et al (1995), Chatterjee (1993), Das et al (1987), Das et al (1994), Data and Ashwath (2000), Data (1984), Data and Basavaraja, (1994) and Data and Pershad, (1988) Wide genetic distances determined by Euclidean Wards genetic distance reveals relatively high genetic variation among 17 silkworm varieties. The considerable polymorphism detected in the present day also illustrated genetic diversity among silkworm varieties of the same origin as reported among other varieties of Silkworm (by Srivastava(2005). However the variety 9 has not shown any amplification of RAPD bands, in varieties 1 and 2 were shown one band each

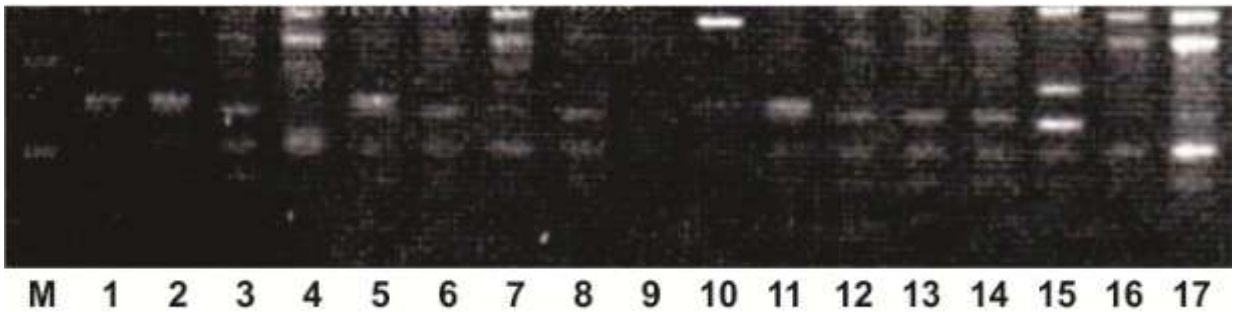

Figure-1: RAPD Profiles of Genomic DNA of 10 Silkworm Accessions generated with random primers OPW-02

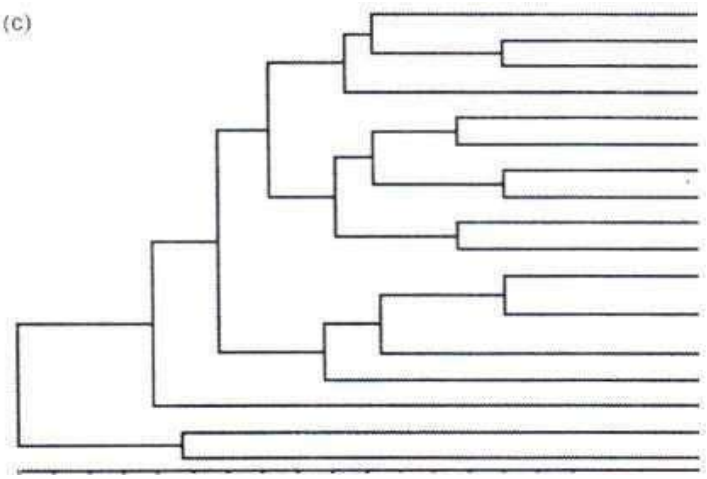

Figure-2: Dendrogram based on RAPD marker using Eucledian distance computer version 0.5

SANISH-30,
SHEIKHI-I,
SHEIKHI-II,
TETRAHYBRID(P),
KYORIESHIMPAKU (P),
KYORIESHIMPAKU (M),
SHINKI REYAKU (M),
SH-6,
SF-19 and JD-6SANISH
ANISH E2(M)
NB4D2,
SANISH E2(M)
SANISH-8
ANISH E2(M) SANISH E2(M)
SANISH-8 SANISH-17 SANISH-18(M) SANISH-18(P) SHEIKHI-I
SANIS H-30 SHEIKHI-II and **TETRA HYBRID (P)**
SANISH-17
SANISH-18(M)
SANISH-18(P)
SANIS H-3

Table 1 – Name, origin and quantitative traits of 1 silkworm accessions studied

No.	TLD (h)	VLD (h)	LWT (g)	CWT (g)	SWT (g)	SR (%)
1	585	151	37.26	1.700	0.298	17
2	620	177	37.437	1.686	0.339	20.189
3	609	170	35.498	1.662	0.320	19.288
4	632	182	39.098	1.655	0.336	20.384
5	628	182	38.914	1.714	0.356	20.857
6	626	180	40.089	1.752	0.331	19.014
7	558	147	24.585	1.163	0.173	14.973
8	551	143	27.068	1.257	0.191	15.383

9	574	169	26.294	1.368	0.191	14.104
10	550	143	21.071	1.039	0.143	13.920
11	585	151	37.26	1.700	0.298	17
12	620	177	37.437	1.686	0.339	20.189
13	609	170	35.498	1.662	0.320	19.288
14	632	182	39.098	1.655	0.336	20.384
15	628	182	38.914	1.714	0.356	20.857
16	626	180	40.089	1.752	0.331	19.014
17	558	147	24.585	1.163	0.173	14.973

Table 2 – Primers and percentage of polymorphism realized with the RAPD primers

No.	Primer (Operon)	Sequence	No. of total bands	No. of polymorphic bands	% polymorphism
1	OPW-02	ACCCCGCCAA	4	00	00
2	OPW-04	CAGAAGCGGA	4	00	.00
3	OPW-05	GGCGGATAAG	4	1	90.00
4	OPW-06	AGGCCCGATG	4	1	90.48
5	OPW-11	CTGATGCGTG	4	1	90.00
6	OPW-12	TGGGCAGAAG	4	1	90.00
7	OPW-13	CACAGCGACA	4	00	00.00
8	OPW-16	CAGCCTACCA	4	1	90.00
9	OPW-17	GTCCTGGGTT	3	00	00.91
10	OPW-18	TTCAGGGCAC	4	00	00.00
11	OPW-19	CAAAGCGCTC	4	00	00.00
12	OPW-20	TGTGGCAGCA	2	00	00.00
Total/Avg			45	5	95.00

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