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# Genomic DNA extracted from long preserved silkmoth samples suitable for genetic diversity studies

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**Abstract**
In the present study, preserved silkmoths served as samples for DNA extraction. The wings, legs and antennae of moths were cut with sterile scissors before preservation of the samples. The samples were placed in airtight cryovials snap frozen in liquid nitrogen and immediately stored at -80 $^0$C for further use. Genomic DNA was isolated after six months from the frozen silkmoth samples, crushed under liquid nitrogen which yielded high molecular weight DNA. RAPD profile of the isolated DNA revealed high degree of polymorphism. The silkworm strains were analyzed using 6 random primers out of which 5 polymorphic primers gave 61 amplicons. The average amplicons per primer found to be 10.16 and 73.02% amplicons were polymorphic. Cluster analysis based on Jaccard's similarity coefficients resulted in the formation of two main clusters with SKAU-R-1,CSR$_{18}$, SH$_6$ and NB$_4$D$_2$ in one cluster and the remaining two genotypes viz., CSR$_2$ and DUN$_{22}$ in other cluster. Jaccard's similarity coefficients ranged from 0.97 to 0.99 indicating considerable genetic diversity within bivoltine silkworm groups. Isolated genomic DNA from silkmoths was found suitable to undertake genetic diversity studies using molecular marker approach for identifying diverse genotypes for taking up breeding programs to push up bivoltine silk production

**Keywords:** DNA, diversity, extraction, marker, preserved, silkmoth, samples

## 1. Introduction
Silkworm is a domesticated insect having been cultured for a period of over 5000 years [1]. It possesses excellent characteristics as an experimental organism. There are numerous characters in all stages of silkworm that are heritable. The morphological characters like body colour, shell weight, cocoon weight, etc, has been traditionally used to identify a strain. Lack of assessing genetic diversity in the available germplasm, unavailability of modern tools to know the genomes at molecular level, environmental disturbances during the time of selection and phylogenetic control of various traits in silkworm have led to the poor selection of parents in breeding programs [2-6]. Therefore, it is required to produce genotypes for particular geographical environment by utilizing the races acclimatized to that location. New tools like molecular markers can be effectively applied with conventional breeding strategies and the genes for the resistance can be discovered [7]. In silkworm *Bombyx mori*, a great diversity has been reported to exist globally through which several breeds are evolved by selection or cross-breeding. These different genotypes display large differences in their qualitative and quantitative traits that ultimately control silk yield [8]. Many genotypes have same phenotype in spite of unique genetic characteristics or the same genotypes are having different phenotype under different ecological conditions. Such similarities and differences cause problems for silkworm breeders when choosing parental genotypes for cross breeding programmes [9]. These limitations call for harnessing the recent developments in molecular biology for rational utilization of silkworm genetic resources and such an attempt is bound to enhance our ability to gain deeper insights into the genome of this economic insect.
Molecular techniques provide an understanding of genetic identity and relationships among insects in terms of their evolution and genetic diversity. Molecular marker techniques have proven powerful in estimation of genetic diversity. Polymerase Chain Reaction (PCR) based multiple loci marker techniques which include Inter-Simple Sequence Repeat (ISSR) Randomly Amplified Polymorphic DNA (RAPD) and Simple Sequence Repeat (SSR) or microsatellites [10] are playing important role in crop improvement [11]. The isolation of intact, high-molecular-mass genomic DNA is essential for many molecular biology applications

including PCR, endonuclease restriction digestion, Southern blot analysis and genomic library construction [12]. Therefore the first and foremost step in undertaking any such molecular work is the extraction of quality DNA from the desired sample. Since we are dealing with insects (Silkmoth), the best source of insect DNA for molecular study is fresh/live insects. However, Insects frozen at -20˚C or -80˚C, dried, and preserved in 70% or 100% ethanol can be a substitute when collection of live insects is not possible due to costs, remoteness, transportation or time constraint [13]. The quality and amount of isolated DNA from preserved samples may be lower or degraded and may not be even appropriate for PCR. Therefore, the preservation technique is one of the crucial steps for the whole molecular process [14, 15]. In this context, the present study with RAPD-PCR technique was applied to determine the suitability of DNA isolated from the preserved Silkmoth samples at -80 $^0$C for assessment of genetic identity and relationship among silkworm *B. mori* L. genotypes.

## 2. Materials and Methods

### 2.1 Experimental Material

The silkmoth samples from six bivoltine mulberry silkworm *B. mori* L. genotypes viz., SKAU-R-1, $CSR_2$, $CSR_{18}$, $NB_4D_2$, $SH_6$, and $DUN_{22}$ (Table-1) were obtained from the Germplasm Bank of Temperate Sericulture Research Institute (TSRI), Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir (SKUAST-K) Mirgund, Central Sericultural Germplasm Resources Centre (CSGRC) Hosour, Tamilnadu, India and Central Sericultural Research and Training Institute (CSR&TI) Central Silk Board (CSB) Pampore Srinagar Kashmir. The study was undertaken during the year 2012 and 2013. The silkworm rearing was carried out in the rearing laboratory of Temperate Sericulture Research Institute (TSRI), SKUAST-K Mirgund and the molecular studies were carried out at genomics laboratory, Division of Biotechnology, Faculty of Veterinary Sciences and Animal Husbandry, SKUAST-K, Shuhamma.

### 2.2 Sample collection

Silkmoths were collected in labeled perforated plastic boxes (Fig.1) and were carried to Genomics Laboratory, Division of Biotechnology, Faculty of Veterinary Sciences and Animal Husbandry, SKUAST-K, Shuhamma for conducting molecular studies.

### 2.3 Genomic DNA Extraction

Genomic DNA was extracted from the preserved silkmoth samples (Fig.2) of *B. mori* L., genotypes. The wings, legs and antennae of moths were cut with sterile scissors before extraction process. In each of the genotype/strain, 2-3 moths were frozen with liquid nitrogen and homogenized in pestle and mortar. The powdered content was transferred to fresh/autoclaved tubes containing DNA extraction buffer (50 mmol Tris–HCI/L, pH 8.0, 100 mmol NaCl/L, 20 mmol EDTA/L) having 100 µg/mL Proteinase K. After digestion with Proteinase K at 55 °C for 1 h, phenol/chloroform extraction was carried out and DNA was recovered by ethanol precipitation (Fig.3). Purified DNA was dissolved in 1X Tris–EDTA buffer (pH 8.0). DNA concentration was measured using spectrophotometer. Phenol-Chloroform method of DNA extraction [16] was followed for DNA extraction but [17] modified method was found suitable for DNA extraction in Silkmoth *B. mori* L., samples.

### 2.4 Spectrophotometric Estimation of Genomic DNA

The yield of DNA was measured using a UV-Spectrophotometer at 260 nm. The purity of DNA was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm. The DNA concentration was determined by the formula:

DNA concentration (µg/ml) = $OD_{260}$ × Dilution Factor × 50 µg/ml

The reading at 260 nm allowed calculating the concentration of DNA in the given sample. The ratio between 260nm/280nm i.e. $\lambda_1/\lambda_2$ provided the purity of DNA. Pure preparation of DNA had $OD_{260}/OD_{280}$ a value of 1.8-2.0.

### 2.5 Quality Check through Agarose Gel Electrophoresis

The quality of DNA was checked by Agarose gel electrophoresis. 1% Agarose gel was prepared for checking the quality of gDNA.

### 2.6 RAPD Marker Analysis

RAPD-PCR was performed in 25 µl of a reaction mixture containing 30 ng DNA, 2.5 µl of 10X Buffer, 2.0 µl of dNTP mix (10 mM), 2 mM MgCl2, 1.0 µl of primer (0.5 µM) and 1.0 U of Taq DNA polymerase (Genie). The DNA amplification reactions were performed in a thermal cycler (Applied Biosystems) and were carried out as follows: one cycle at 94°C for 5 min, 40 cycles each of 94°C for 1 min, 36°C for 1 min and 72°C for 2 min and a final extension for 10 min at 72°C. The RAPD products were analyzed by electrophoresis by resolved on 1.5% Agarose gel. The gel was prepared in 0.5X TAE buffer. Ethidium bromide was added at a concentration of 0.5µg/ µl. The gel was run at 90 volts and visualized under UV light and photographed using UV Photo Gel Documentation System (Bio-Rad, USA). The experiment was performed thrice and reproducibility of each marker was checked. Only consistent reproducible bands were scored and used for further analysis. RAPD data was analyzed by counting the banding pattern generated by each primer. The DNA fragments amplified by a primer were scored as present (1) or absent (0) for the genotype studied. POPGENE software program version 1.31 [18] was used to construct the dendrogram using Nei's genetic distance by Unweighed Pair Group Method with Arithmetic Mean (UPGMA) clustering method. Nei's similarity coefficients were used to calculate similarity between pairs of accessions [19].

## 3. Results

RAPD-PCR technique was applied to determine if the quality of DNA template obtained from the preserved Silkmoth *B. mori* L., samples was sufficient to produce multiple bands for genetic fingerprinting analysis. The result showed the pattern of random DNA fragments generated by the RAPD reaction was sufficient for undertaking diversity studies (Fig.4). Six RAPD primers were used for screening with six representative silkworm genotypes (Table-1). The reproducibility of each marker was checked at least three times and consistent results were obtained each time. In the present study, DNA amplification with RAPD primers showed that different primers generated different numbers and length of amplification products (Table-2). The electrophoretic patterns of the primers are shown in Fig. 4. Amplification products yielded a total of 61 scorable bands out of which 48 (73.02%) were polymorphic with an average of 8.0 bands per primer. The number of bands produced by each primer varied from 6 to 15 with an average of 10.16 bands per primer. The highest number of bands (15) was

obtained with primer UBC-782, while the lowest number (6) was obtained with primers UBC-769. The polymorphism percentage ranged from 75 to 100% with highest percentage of polymorphism (100%) generated by UBC-782 while as lowest percentage of polymorphism (75.00%) generated by UBC-783. Six RAPD primers generated PCR products in the range of 116-2876 bp generated by UBC-767 and UBC-782, respectively (Table-2). The Polymorphism information content (PIC) ranged from 0.353 (UBC-782) to 0.493 (UBC-767) with a mean PIC value of 0.322 per loci (Table-2). All the ten RAPD primers used in the study produced unambiguous markers and revealed considerable polymorphism among the silkworm genotypes studied. The genetic distance based on Nei's similarity coefficients among silkworm genotypes ranged from 0.97 to 0.99 with an average similarity coefficient of 0.98. Of the pair wise combinations, $CSR_{18}$ and $SH_6$, showed the highest similarity (0.99), whereas the lowest similarity (0.97) was observed between SKAU-R-1 and $CSR_2$ (Table-3, Fig. 5). Based on similarity coefficients of RAPD markers, the UPGMA cluster analysis separated the genotypes into two main clusters on the dendrogram. The first cluster included genotypes namely SKAU-R-1, $CSR_{18}$, $SH_6$, and $NB_4D_2$. Cluster-I was further divided into sub clusters with $CSR_{18}$ and $SH_6$ under sub cluster, which gathered at a highest similarity coefficient of 0.99. Cluster-II contained $CSR_2$, and $DUN_{22}$ with which grouped at a similarity coefficient of 0.98 (Fig. 5).

**Table 1:** Characteristic features of different silkworm genotypes under study

| Genotype | Parental Source | Voltinism | Larval pattern | Cocoon colour | Cocoon shape | Origin/ Evolution | Source |
|---|---|---|---|---|---|---|---|
| SKAU-R-1 | Shunrei × Shogetsu | Bivoltine | Marked | White | Constricted | TSRI, SKUAST-Kashmir-Mirgund | Silkworm Germplasm Bank, TSRI, SKUAST-K, Mirgund |
| $CSR_2$ | Shunrei × Shogetsu | | Plain bluish | Bright white | Oval | CSR&TI, Mysore-India | Silkworm Germplasm Bank, CSGRC, Hosur-Tamilnadu, India |
| $CSR_{18}$ | B201× BCS12 | | Plain & marked | Creamish white | Oval | | |
| $NB_4D_2$ | (Kokko × Seihaku) × (N124×C124) | | Plain faint bluish | White | Elongated, constricted | | |
| $SH_6$ | Shogetsu × Hoshu | | Moderately marked | White | Oval | RSRS, Majira, Dehradun- India | |
| $DUN_{22}$ | (KS × $NB_4D_2$) (AT × $NB_4D_2$) | | Marked | White | Oval | CSR&TI, Pampore-Kashmir | Silkworm Germplasm Bank, CSR&TI,CSB-Pampore |

**Table 2:** Randomly Amplified Polymorphic DNA (RAPD) Primer sequences and degree of polymorphism

| Primer | Primer Sequence | No. of Alleles | Allele size range (bp)* | Polymorphic Alleles | Polymorphism (%) | PIC |
|---|---|---|---|---|---|---|
| UBC-767 | 5′- ACCCACCACC-3′ | 13 | 116-1912 | 12 | 92.30 | 0.493 |
| UBC-769 | 5′- GGGTGGTGGG-3′ | 6 | 488-1294 | 5 | 83.33 | 0.368 |
| UBC-782 | 5′- GGGAAGAAGG-3′ | 15 | 348-2876 | 15 | 100 | 0.353 |
| UBC-783 | 5′- GGTGGGTTGT-3′ | 12 | 202-2788 | 9 | 75.00 | 0.365 |
| UBC-764 | 5′- CTCTCCTCCC-3′ | 7 | 336-1588 | 0 | 0.00 | 0.00 |
| UBC-789 | 5′- GGAAGGGAGA-3′ | 8 | 730-1788 | 7 | 87.50 | 0.374 |
| Total | | 61 | | 48 | | |
| Average | | 10.16 | | 8.00 | 73.02 | 0.322 |

Single letter abbreviations for mixed base positions: R = (A, G); Y = (C, T) Abbreviations: A= Adenine, G= Guanine, C= Cytosine, T= Thymine; *bp= base pairs

**Table 3:** Genetic identity among silkworm genotypes based on Nei's similarity coefficient using RAPD markers

| Genotype | SKAU-R-1 | $CSR_2$ | $CSR_{18}$ | $NB_4D_2$ | $SH_6$ | $DUN_{22}$ |
|---|---|---|---|---|---|---|
| SKAU-R-1 | **** | 0.9763 | 0.9908 | 0.9905 | 0.9915 | 0.9780 |
| $CSR_2$ | | **** | 0.9853 | 0.9859 | 0.9869 | 0.9883 |
| $CSR_{18}$ | | | **** | 0.9915 | 0.9952 | 0.9791 |
| $NB_4D_2$ | | | | **** | 0.9922 | 0.9787 |
| $SH_6$ | | | | | **** | 0.9838 |
| $DUN_{22}$ | | | | | | **** |

**Fig 1:** Silkmoth samples of *B. mori* collected for genomic studies

**Fig 2:** Silkmoth samples preserved under Cryo-vials at -80 $^{0}$C

**Fig 3:** DNA samples extracted from preserved silkmoth genotypes

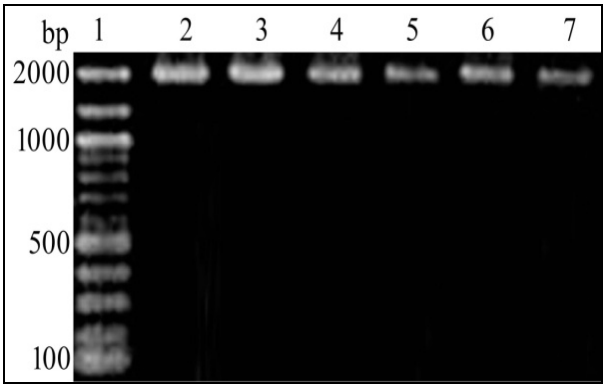


**Fig 4:** DNA profile from 6 silkworm genotypes resolved on 1.5% agarose gel.

Numbers represent 6 bivoltine silkworm genotypes.

| Lane 1= Molecular Weight Marker | | |
|---|---|---|
| Lane 2 = SKAU-R-1 | Lane 3= CSR2 | Lane 4 = CSR18 |
| Lane 5 = NB4D2 | Lane 6 = SH6 | Lane 7 = DUN22 |

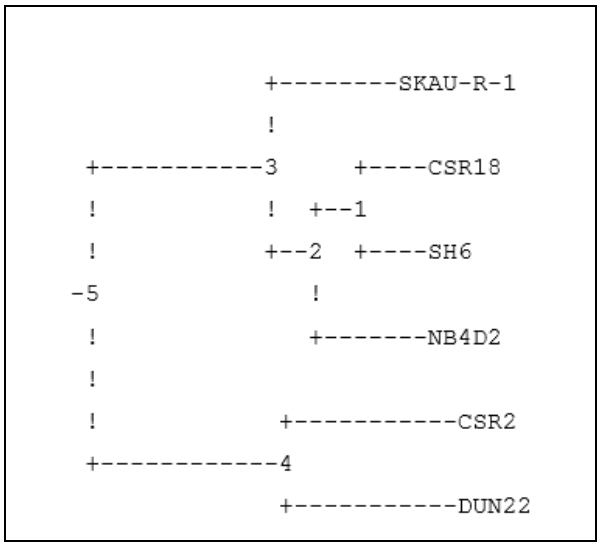


**Fig 5:** Dendrogram of 6 silkworm genotypes realized from the Nei's similarity matrix derived from RAPD markers using UPGMA analysis.

## 4. Discussion

DNA isolated from the long preserved Silkmoth samples was found intact and suitable for diversity studies. The DNA obtained without damage or shearing helped the random primer annealing for DNA fragment amplification. The present study with RAPD markers revealed considerable polymorphism among silkworm genotypes. The results of the current study are in contrast with the findings of [20] who have reported 82% polymorphism across ten silkworm stocks utilizing different RAPD markers. [21] reported 93.00% polymorphism with 12 different RAPD markers among silkworm stocks. Similar kind of polymorphism 90.00% was observed by [22] using same primers while evaluating genetic diversity among silkworm stocks. The Polymorphism information content (PIC) obtained in the present study is in conformity with the findings of [17] who have reported considerable polymorphism among silkworm genotypes using these markers. This kind of polymorphism was also reported by [23,24] utilizing two races of silkworm *B. mori* L. Similar results among closely related species are also reported in *Antheraea mylittae* [25] using different primers. The present findings also corrobates with the findings of [22, 26] in *B. mori* L. through RAPD-PCR. The genetic identity based on Nei's similarity coefficients among silkworm genotypes revealed that $CSR_{18}$ and $SH_6$ are the most closest genotypes as revealed by highest similarity values obtained for these genotypes in this study whereas, SKAU-R-1 and $CSR_2$ were found to be distantly related genotypes in the present study involving lowest similarity index values obtained for these genotypes. The lowest similarity coefficients obtained with RAPD markers among the genotypes reflects their genetic distinctness and depicts that the genotypes have not undergone much divergence which could the reason of low genetic distance observed between these genotypes. The present findings are in agreement with the findings of [27] who have obtained highest (0.99) pair wise genetic divergence between SK3, SK4 and SK4C and reported that this may be the reason for the least genetic distance among them. From the pattern of clustering, it is pertinent that RAPD technique is efficient in segregating silkworm varieties into different clusters. More significantly, the clustering had been largely successful in retaining the relationship between silkworm genotypes. 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 genotypes. The association of the genotypes observed in the present study was similar to the pattern observed by [21, 24, 9, 28]. Present study has revealed considerable polymorphism among the silkworm genotypes which illustrated their genetic diversity as also reported among other varieties of silkworm by [4, 22, 29, 30] using these stable markers.

## 5. Conclusion

Different preservation techniques are used to protect and preserve DNA samples from degradation. However, the quantity and quality of extracted genomic DNA from collections varies and may yield little or no DNA for PCR amplification. In the present study, adult silk moths were collected and preserved carefully at -80 $^0$C for a period of six months. The DNA extracted from these long preserved Silkmoth samples yielded good amplifiable DNA bands of varying size and revealed considerable polymorphism among the genotypes studied hence suitable for undertaking genetic diversity studies using PCR technique. Further, the genetic variation revealed by RAPD marker analysis opens up the avenue for the proper identification and selection of divergent silkworm genotypes that could be used in planning future breeding programmes in modifying the yield potentials of silkworms.

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