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DNA Polymorphism and Genetic Variation among Bivoltine Silkworm (*Bombyx mori* L.) Genotypes Revealed by RAPD Markers

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Authors' contributions

This work was carried out in collaboration between all the authors. Author ZIB carried out laboratory work and contributed to the protocol (writing of the manuscript). Authors NAG and MAM conceptualized the study and contributed to the protocol. Author NAG contributed to the experimental design and performed the statistical analysis. Authors ZIB and MAM wrote the first draft of the manuscript and revised by authors NAG and ASK. All authors read and approved the final manuscript.

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ABSTRACT

Background: In breeding programs, the selection of parental lines based on their phenotypic traits along with genetic variation at their DNA level is the better option in directional breeding as specific DNA profiles would be useful in producing reliable estimates of genetic identity and diversity at their genomic levels. In this context, Random amplified polymorphic DNA (RAPD) marker approach was used to ascertain genetic diversity and relationship among 12 bivoltine silkworms (*Bombyx mori* L.) genotypes for the identification of diverse genotypes to be used as parents for the improvement of quantitative traits in silkworms.

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Results: RAPD analysis with 12 silkworm genotypes revealed high polymorphism of 88.88%. Ten RAPD primers utilized in this study generated PCR products in the range of 202-2876 *bp* across 12 silkworm genotypes. The Polymorphism information content (PIC) ranged from 0.295 to 0.493%. Based on Nei's similarity coefficients, the genetic distance among the studied silkworm genotypes ranged from 0.93 to 0.99. Of the pairwise combinations, CSR_{18} and SH_6, showed the highest similarity (0.99) whereas, SKAU-R-6 and DUN_6 showed the lowest similarity (0.93) among the genotypes. The dendrogram generated, using Unweighted pair group method using the arithmetic average (UPGMA) from this marker, grouped the 12 silkworm genotypes into five main clusters, which corroborates the differences in their phenotypic characters.

Conclusion: The study clearly indicated the potential use of RAPD markers in differentiating silkworm genotypes based on their characters. The information generated on genetically divergent genotypes identified in this study would be useful in future silkworm breeding programmes for modifying the yield potentials of silkworms so as to boost up bivoltine silk production in the interest of temperate sericulture industry.

Keywords: Bivoltine; DNA; genetic; marker; polymorphism; RAPD; silkworm; variation.

1. INTRODUCTION

Conventional breeding approaches employed during the last few decades have resulted in the development of many productive silkworms (*Bombyx mori* L.) breeds which have significantly contributed in increasing the yield potential of silkworms as well as silk production in India. Of late, a major thrust has been given to quality rather than quantity of silk produced. In this direction, efforts made during the 90's lead to the evolution of highly productive CSR bivoltine silkworm breeds with the potential to produce international grade silk. However, these new silkworm breeds continue to suffer badly under adverse climatic conditions of low/high temperature, humidity, poor leaf quality as well as inadequate rearing and management practices prevalent with the small and marginal farmers in the temperate region of Jammu and Kashmir. Unlike tropics, temperate sericulture being carried out under highly fluctuating environmental conditions and poor leaf quality urgently need evolution and development of broad-based silkworm breeds with higher genetic plasticity to buffer the adverse climatic conditions. In this context, it is pertinent to take note of the recent approaches of molecular biology and biotechnology which are now being successfully used in the improvement of crop plants and livestock. In India also, DNA profiles of silkworm genotypes have been carried out using Randomly Amplified Polymorphic DNA-RAPD markers [1-5], microsatellite or Simple Sequence Repeats-SSR markers [6-8] and Inter-Simple Sequence Repeat-ISSR markers [9-13], which have revealed distinct and unique DNA profile specific breeds of silkworms.

Of late, genome analysis of mulberry silkworm using molecular markers has already been initiated in India and also elsewhere. Linkage maps based on RAPDs, RFLPs and AFLPs have been constructed by Japanese and Chinese groups in silkworm. Today, silkworm genetic manipulation is possible due to the availability of more than 400 visible mutations, 3000 diverse silkworm strains, high-density molecular linkage and physical maps, complete whole genome sequence of 428 Mb, 16,000 expressed sequence tags (ESTs) and well established transgenic system. Silkworm has now become a model organism for understanding the biology to manipulate and reconstruct the genes to produce high-quality silk. Some of the significant findings include construction of early linkage maps, characterization of DNA markers, sex-linked markers, the establishment of stable germ line transformation, production of pharmaceutically important proteins including immune response proteins. Lepidoptera specific genes, horizontal gene transfer, and characterization of essential baculoviral genes have been established. The development and improvement in silkworm transgenesis protocol has opened new areas of application. The nucleo-polyhedrosis virus is also being exploited as a vector for introduction of foreign genes. Expression of marker proteins *viz*., luciferase and green fluorescent protein has been successfully achieved in cell lines and larval caterpillars of the silkworm (*B. mori*) employing recombinant BmNPV vector harboring reporter genes. In India, attempts have also been made to develop molecular markers for high cocoon shell weight as well as virus resistance markers and the characters are being established using divergent parents.

Modern Molecular biology tools and 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 [14] are playing important role in crop improvement [15]. The RAPD method generates PCR products by annealing to randomly distributed homologous target sites of the template DNA. This technique mostly generates dominant markers, although length polymorphisms caused by insertions/deletions can also occur at low frequencies. Because of its relative simplicity, RAPD technology is being extensively used in genetic analysis of various plant and animal species. RAPD detects both allelic and locus variation in a single assay and deals with insertions, deletions, point mutations which influence the base sequence of primer binding sites, allowing polymorphism to be detected [16]. These markers can be effectively utilized to unravel the genetic potential of silkworm strains maintained in the germplasm centers for further crossbreeding programmes to improve the quantitative traits in silkworms. The selection of parents based on phenotypic traits along with genetic variation (DNA profiles) may be the better option as phenotypic traits express variation due to their interaction with the environment. It is also believed that unique DNA profiles could provide a solution to the said problem as they are environmentally neutral. Such varietal DNA profiles would be useful in producing reliable estimates of genetic diversity for the selection of parents to develop elite hybrids [8]. Hence, an urgent need was felt to reorient our approach using molecular biology tools and techniques with available genetic stocks to supplement the conventional practices adopted in breeding programmes. In this context, the present study was aimed to assess the genetic identity and relationship among twelve potential bivoltine silkworm genotypes to identify divergent genotypes which could be used for future breeding programmes to push up productivity levels in temperate sericulture belt of India.

2. MATERIALS AND METHODS

2.1 Experimental Material

Twelve bivoltine mulberry silkworm genotypes namely; CSR_2, CSR_4, CSR_{18}, CSR_{19}, NB_4D_2, SH_6, SKAU-R-1, SKAU-R-6, SKUAST-28, SKUAST-31, DUN_6 and DUN_{22} formed the basis for this study. The disease-free laying's (DFL's) of these selected silkworm genotypes 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 2012-2013. The characteristic features of the silkworm genotypes used in the study are given in Table 1.

2.2 Silkworm Rearing

Silkworm rearing was carried out at Temperate Sericulture Research Institute (TSRI), Mirgund Sher-e- Kashmir University of Agricultural Sciences and Technology of Kashmir during the year 2012-2013, following the standard package of practices as outlined by [17].

2.3 Sample Collection

Live silkmoths formed the sample for DNA extraction and further studies. The sample live moths were collected in labeled perforated plastic boxes and were carried to Genomics Laboratory, Division of Biotechnology, Faculty of Veterinary Sciences and Animal Husbandry, SKUAST-K, Shuhamma for conducting molecular studies.

2.4 Genomic DNA Extraction

Genomic DNA was extracted from adult silk moths. The wings, legs and antennae of moths were cut with sterile scissors before extraction process. In each of the genotype, 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–HCl/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. Purified DNA was dissolved in 1X Tris–EDTA buffer (pH 8.0). DNA concentration was measured using spectrophotometer. The protocol of [18] was followed for DNA extraction with minor modifications as suggested by [4].

Table 1. Characteristic features of different silkworm genotypes used in the study

Genotype	Voltinism	Parental source	Larval pattern	Cocoon colour	Cocoon shape	Origin/ Evolution	Source
SKAU-R-1	Bivoltine	Shunrei × Shogetsu	Marked	White	Constricted	TSRI, SKUAST-Kashmir- Mirgund	Silkworm Germplasm Bank, TSRI, SKUAST-K, Mirgund
SKAU-R-6		Shogetsu × Hoshu	Plain	White	Slightly oval		
SKUAST-28		Evolved Under Broad Based Germplasm Complex, Comprising 10 Breeds With Marked Larvae	Marked	White	Short Dumbell		
SKUAST-31		Evolved Under Broad Based Germplasm Complex, Comprising 10 Breeds With Plain Larvae	Marked	White	Oval		
CSR_2		Shunrei × Shogetsu	Plain bluish	Bright white	Oval	CSR&TI, Mysore-India	Silkworm Germplasm Bank, CSGRC, Hosur-Tamilnadu, India
CSR_4		(BN18×BCS25) × NB_4D_2	Plain bluish	Bright white	Dumbell		
CSR_{18}		B201× BCS12	Plain & marked	Creamish white	Oval		
CSR_{19}		B201× BCS12	Plain & marked	Creamish white	Dumbell		
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_6		CC1 × NN6D	Plain	White	Oval	CSR&TI, Pampore-Kashmir	Silkworm Germplasm Bank, CSR&TI,CSB-Pampore
DUN_{22}		(KS × NB_4D_2) (AT × NB_4D_2)	Marked	White	Oval		

2.5 Amplification and Separation of DNA

A total of 17 RAPD primers from University of British Columbia, Biotechnology Laboratory [2,4] were screened initially out of which 09 RAPD markers produced amplifiable products with genomic DNA of 12 silkworm genotypes used in the present study. The list of RAPD primers which produced robust reproducible bands are presented in Table 2 along with their representative sequences. 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 $MgCl_2$, 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.

2.6 Detection and Visualization of RAPD Products

The RAPD products were analyzed by electrophoresis resolved on 1.5% Agarose gel. The gel was prepared in 0.5X TAE Buffer. Ethidium bromide was added at a concentration of 0.5 µg/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.

2.7 Data Analysis

RAPD data was analyzed by counting the banding pattern generated by each primer. All the reproducible bands amplified by the primers were counted starting from the bottom of the lanes and scored as '1' for presence and '0' for absence and this binary matrix was used for analysis. POPGENE software program version 1.31 [19] 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 the similarity between pairs of accessions [20].

3. RESULTS

3.1 RAPD Primer Analysis

Seventeen RAPD primers were used for initial screening with 12 representative silkworm genotypes. Out of seventeen, only 9 primers produced amplifiable polymorphic patterns (Table 2). The reproducibility of each marker was checked at least three times and consistent results were obtained each time. The amplification products yielded a total of 117 scorable bands out of which 104 (88.88%) were polymorphic with an average of 10.4 per primer. Strain-specific bands (indicated with arrows) were also obtained in the present study with RAPD markers. The number of bands produced by each primer varied from 6 to 23 with an average of 12 bands per primer. The highest number of bands (23) were obtained with primer UBC-778, while the lowest number (6) were

Table 2. RAPD Primers with their base sequences and degree of polymorphism in 12 silkworm genotypes

Primer	Primer Sequence	No. of Alleles	Allele size range (bp)*	Polymorphic Alleles	Polymorphism (%)	PIC
UBC760	5′- CCTTCCCTCC-3′	12	612-1528	12	100	0.303
UBC762	5′- GTGTGGTGGG-3′	11	229-890	11	100	0.322
UBC764	5′- CTCTCCTCCC-3′	7	336-1588	0	0.00	0.00
UBC767	5′- ACCCACCACC-3′	13	116-1912	12	92.30	0.493
UBC769	5′- GGGTGGTGGG-3′	6	488-1294	5	83.33	0.368
UBC778	5′- CCACACCACA-3′	23	212-1936	23	100	0.295
UBC782	5′- GGGAAGAAGG-3′	15	348-2876	15	100	0.353
UBC783	5′- GGTGGGTTGT-3′	12	202-2788	9	75.00	0.365
UBC785	5′- CACCCAACCA-3′	10	378-1854	10	100	0.356
UBC789	5′- GGAAGGGAGA-3′	8	730-1788	7	87.50	0.374
Total		**117**		**104**		
Average		**11.7**		**10.4**	**88.88**	**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*

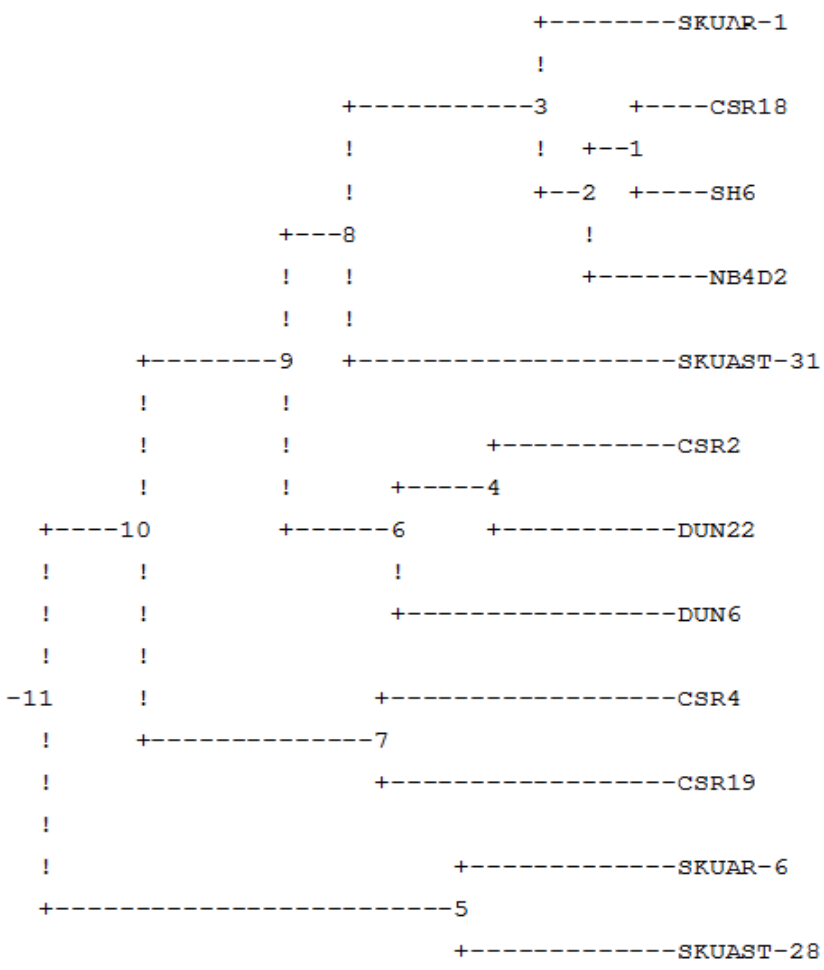


Fig. 1. Dendrogram of 12 silkworm genotypes realized from the Nei's similarity matrix derived from RAPD markers using UPGMA analysis

obtained with primers UBC-769. The highest percentage of polymorphism 100% was generated by UBC-760, UBC-762, UBC-778, UBC-782 and UBC-785 while as the lowest percentage of polymorphism 75.00% was generated by UBC-783. Ten RAPD primers generated PCR products in the range of 202-2876 bp generated by UBC-783 and UBC-782, respectively (Table 2, Fig. 1)

The Polymorphism information content (PIC) ranged from 0.295 to 0.493 with a mean PIC value of 0.322 per loci. The lowest (0.295) and highest (0.493) PIC values were recorded for UBC-778 and UBC-767, respectively (Table 2). All the ten RAPD primers used in the study produced unambiguous markers and revealed considerable polymorphism among the silkworm genotypes studied.

3.2 Genetic Diversity and Relationship among the Silkworm Genotypes

RAPD primers applied to twelve silkworm genotypes for DNA amplification showed that different primers generated different numbers and length of DNA amplification products as shown in Table 2. The electrophoretic patterns of the primers are shown in Fig. 2.

The genetic distance based on Nei's similarity coefficients among silkworm genotypes ranged from 0.93 to 0.99 with an average similarity coefficient of 0.96. Of the pairwise combinations, CSR_{18} and SH_6, showed the highest similarity (0.99), whereas the lowest similarity (0.93) was observed between SKAU-R-6 and DUN_6. Hence, the most distantly related genotypes in the dendrogram as revealed by RAPD markers (Table 3, Fig. 1).

Table 3. Genetic distance among 12 silkworm genotypes based on Nei's similarity coefficient using RAPD markers

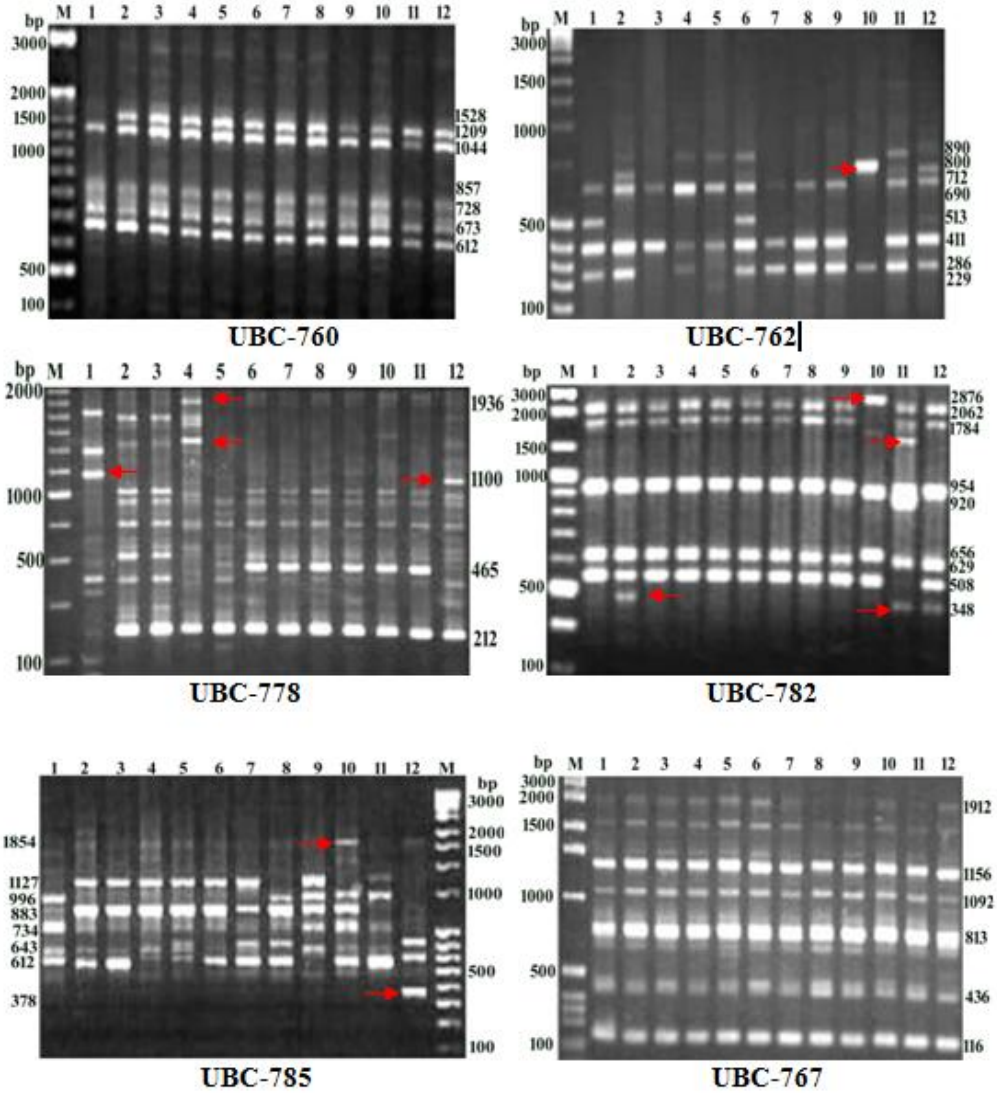
Genotype	SKAU-R-1	SKAU-R-6	SKUAST-28	SKUAST-31	CSR_2	CSR_4	CSR_{18}	CSR_{19}	NB_4D_2	SH_6	DUN_6	DUN_{22}
SKAU-R-1	****	0.9757	0.9748	0.9741	0.9763	0.9650	0.9908	0.9681	0.9905	0.9915	0.9663	0.9780
SKAU-R-6		****	0.9864	0.9705	0.9401	0.9573	0.9689	0.9517	0.9571	0.9608	0.9325	0.9596
SKUAST-28			****	0.9838	0.9573	0.9527	0.9792	0.9607	0.9667	0.9731	0.9446	0.9736
SKUAST-31				****	0.9770	0.9673	0.9896	0.9718	0.9748	0.9804	0.9592	0.9754
CSR_2					****	0.9588	0.9853	0.9692	0.9859	0.9869	0.9815	0.9883
CSR_4						****	0.9621	0.9816	0.9758	0.9579	0.9602	0.9594
CSR_{18}							****	0.9801	0.9915	0.9952	0.9664	0.9791
CSR_{19}								****	0.9893	0.9750	0.9618	0.9605
NB_4D_2									****	0.9922	0.9775	0.9787
SH_6										****	0.9696	0.9755
DUN_6											****	0 .9838
DUN_{22}												****

3.3 Cluster Analysis

Based on similarity coefficients of RAPD markers, the UPGMA cluster analysis separated the genotypes into five main clusters on the dendrogram (Fig. 1). The first cluster included genotypes namely SKAU-R-1, CSR_{18}, SH_6, and NB_4D_2 with CSR_{18} and SH_6 under sub cluster, which gathered at the highest similarity coefficient of 0.99. Cluster II contained CSR_2, DUN_6 and DUN_{22} with CSR_2 and DUN_{22} under sub-cluster which grouped at a similarity coefficient of 0.98. Cluster III included two genotypes *viz*; CSR_4 and CSR_{19} which gathered at a similarity coefficient of 0.98 in the dendrogram. In cluster IV, two genotypes namely, SKAU-R-6 and SKUAST-28 were grouped together at a similarity coefficient value of 0.98. The genotype SKUAST-31 was placed separately in cluster V of the dendrogram generated by RAPD markers (Fig. 1).

4. DISCUSSION

The conventional breeding approaches employed over a long period of time to evolve high yielding silkworm races could not succeed due to natural biological limitations of the silkworm. As stated earlier, traditional landraces have got genetic plasticity to adjust to adverse climatic conditions, but their silk yield is poor, whereas temperate bivoltine silkworm breeds produce good quality silk, but at the same time these breeds are vulnerable to diseases. It is



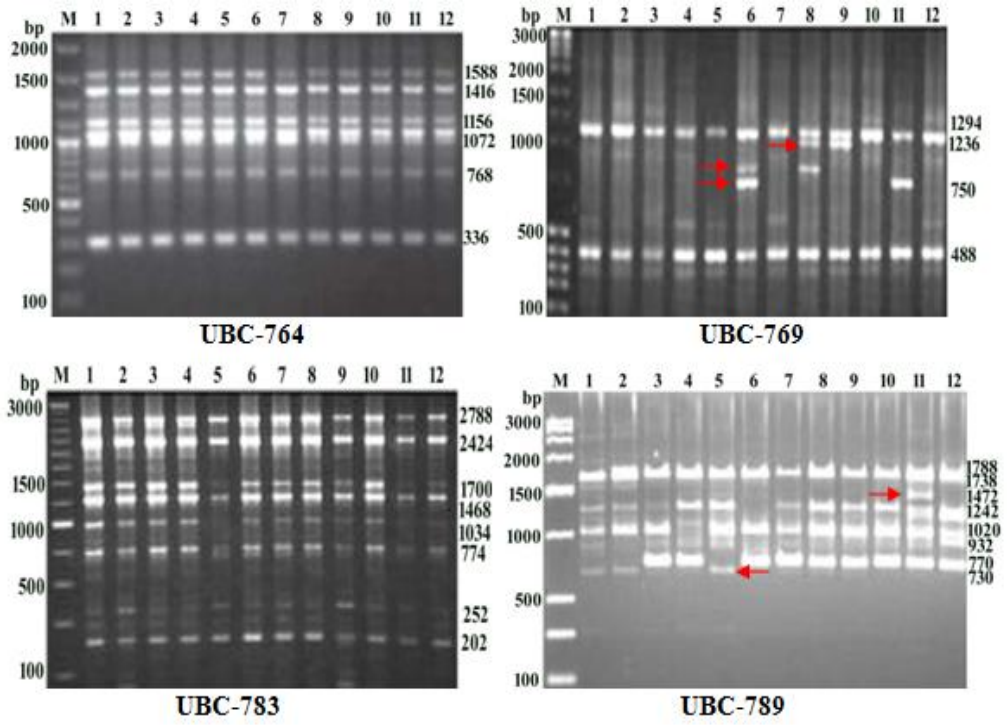


Fig. 2. DNA Profile of RAPD markers from 12 silkworm genotypes resolved on 1.5 % Agarose gel.

Numbers represent 12 bivoltine silkworm genotypes. Red arrow indicates strain specific bands

Lane M = Molecular Weight Marker

Lane 1 = SKAU-R-1	*Lane 5 = CSR2*	*Lane 9 = NB4D2*
Lane 2 = SKAU-R-6	*Lane 6 = CSR4*	*Lane 10 = SH6*
Lane 3 = SKUAST-28	*Lane 7 =CSR18*	*Lane 11 = DUN*
Lane 4 = SKUAST-31	*Lane 8 =CSR19*	*Lane 12 = DUN22*

essential to make genetic enhancement of the breeds by characterization of the breeds for specific traits using latest DNA based molecular biology tools. The DNA profiling has been carried out in silkworms for the conservation of bio-molecular genetics, identifying markers for traits, identification of genetic diversity and variation *etc.* The most popular or widely used techniques used with relevant to silkworms are RFLP, RAPD, ISSR, SSR etc. Several researchers [1-3, 21-29] have made foundational investigations on genome fingerprinting; genetic diversity and phylogenetic analysis of silkworms and have separated the silkworms genotypes based on their origin, evolution and voltinism using these DNA-based molecular approaches. In the present study, RAPD markers revealed considerable polymorphism among the silkworm genotypes. Ten RAPD primers applied to twelve genotypes of silkworm for DNA amplification showed that different primers generated different number and length of DNA amplification products. Some strain specific bands were also obtained in the present studies which reveal uniqueness among these strains from the others at their DNA levels. The level of polymorphism in the genotypes was evaluated from the number of alleles obtained and PIC value. A total of 117 alleles were identified across the 12 genotypes, out of which 104 were polymorphic revealing 88.88% polymorphism (Table 2). The results are in contrast with the findings of [30] who have reported 82.00% polymorphism across ten silkworm stocks utilizing different RAPD markers. [29] has reported 93.00% polymorphism with 12 different RAPD markers among silkworm stocks. Similar kind of polymorphism 90.00% was observed by [27] using same primers while evaluating genetic diversity among silkworm stocks. The Polymorphism information content (PIC) ranged from 0.295 to 0.493 with a mean PIC value of 0.248 per loci. The lowest (0.295)

and highest (0.493) PIC values were recorded for UBC-778 and UBC-767, respectively. The results are in agreement with the findings of [4] who have reported considerable polymorphism among silkworm genotypes using these markers. This kind of higher polymorphism was also reported by [1] utilizing two races of the silkworm. Higher polymorphism among closely related species is also reported in *Antheraea mylittae* [31] using different primers. The present findings also corrobates with the findings of [27,32,33] in silkworm through RAPD-PCR technique.

The genetic identity based on Nei's similarity coefficients among silkworm genotypes ranged from 0.93 to 0.99 with an average similarity coefficient of 0.96. Of the pairwise combinations, CSR_{18} and SH_6, showed the highest similarity (0.99), whereas the lowest similarity (0.93) was observed between SKAU-R-6 and DUN_6. The highest similarity coefficients obtained with RAPD markers among the genotypes depicts that the genotypes have not undergone much divergence, this could be attributed to the low genetic distance between the genotypes. The present findings are in agreement with the findings of [15] who have obtained highest (0.99) pairwise 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 varieties. The association of the genotypes observed in the present study was similar to the pattern observed by [1,3,29].

Significant genetic variations by RAPD have also been reported by [1,3,29,34] reported that wide genetic distances reveal relatively high genetic variation among silkworm genotypes. The considerable polymorphism detected in the present study also illustrated genetic diversity among silkworm genotypes as reported among other varieties of silkworm by [27,35]. Using RAPDs [36,37] separated 13 silkworm strains in two groups, six with diapause and seven without diapause and sex-specific bands were also identified.

5. CONCLUSION

Present investigation on genetic diversity in some potential bivoltine silkworm genotypes using RAPD markers clearly indicated the potential use of these markers in differentiating the silkworm genotypes based on their characters. The divergent parents identified in this study are expected to manifest high heterosis and hence, these genotypes from different clusters could be used for further breeding programs in modifying the yield potentials of the silkworms in the interest of temperate sericulture industry. The information generated in the present study is also useful in providing effective and efficient measures for germplasm conservation and utilization of silkworm breeds for maximizing productivity in temperate sericulture.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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