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Full Length Research Paper

Isolating silkworm genomic DNA without liquid nitrogen suitable for marker studies

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Genomic DNA was isolated from posterior silk gland of silkworms, *Antheraea assama*. Absolute alcohol was used as tissue fixing solution instead of grinding in liquid nitrogen, which yielded high molecular weight DNA (>40 kb). Samples yielded similar amount of DNA when fixed in absolute alcohol (400 µg/g of silk gland tissue) and ground in liquid nitrogen (456 µg/g of silk gland tissue). RAPD profile of the isolated DNA revealed high degree of polymorphism. The silkworms were analysed using 50 random primers among which 36 polymorphic primers gave 309 amplicons. The average amplicons per primer found to be 8.58 and 94.82% amplicons were polymorphic. Cluster analysis based on Jaccard's similarity coefficients resulted in the formation of two main clusters with S9 on one cluster and the remaining strains on the other cluster. Jaccard's similarity coefficients ranged from 0.122 to 0.863 indicating a high level of genetic diversity within muga silkworm collection. Isolated DNA was also suitable for cloning and restriction enzyme digestion. This method does not require liquid nitrogen for fixation, grinding or storage at -80°C, making it advantageous over other common protocols.

Key words: Genomic, silkworms, muga, molecular breeding.

INTRODUCTION

Silkworm is a domesticated insect having been cultured for a period of over 5000 years (Goldsmith, 1995). 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 (Ashwath, 2000; Datta, 1984; Vijaya et al., 2006; Singh et al., 1997; Williams et al., 1990). It is well known that the resistance to biotic and abiotic constraints is governed by polygenes with complex inheritance patterns and with lot of environmental influences (Promboon et al., 1995). Therefore, it is required to produce genotypes for particular geographical environ-ment 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 (Murthy et al., 2006).

The North Eastern region of India is endowed with large natural wealth of both fauna and floral especially in case of sericigenous insects and their food plants which have given a unique outlook to the region (Chowdhury, 1983; Thangavelu, 1991). Natural silk is the product of a group of sericigenous lepidopteran insects. Muga silk is a wonderful gift of nature extracted from an insect species called *Antheraea assama.* It is unique to the North Eastern region of India particularly Assam and its neighbouring states. It is semi-domesticated in the sense that the worms, which crawl down at the end of their larval period are collected and are allowed to spin cocoons inside rearing house, however, some wild species are also found in isolated areas of North East India. Muga

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Table 1. DNA yield obtained from silkworm strains with different treatments.

Silkworm	λ260/ λ280	λ260/ λ230	DNA* concentration, µg/µl	DNA yield, µg/g tissue
A(Alcohol+Li. nitrogen)	1.94	2.07	0.274	548
B(Without alcohol&Li. nitrogen)	1.97	2.11	0.145	290
C(Li. nitrogen)	1.82	3.15	0.228	456
D(Alcohol)	1.82	2.13	0.200	400

*DNA diluted a thousand times to measure OD.

silkworms are raised primarily on two types of trees that is, som (*Persea bombycina* Kost) and soalu (*Litsea polyantha* Juss). Muga is a mono-race. The primary food plants of muga silkworms are som and soalu. Both the plants can be propagated through seeds. Muga silkworm is multivoltine in nature and 5 to 6 crops are raised in a year. Two are commercial crops, including Jethua (May to June) and Kotia (October to November) and remaining four are pre-seed (Jarua: December to January and Aherua: June to July) and seed crops (Chotua: February to March and Bhodia: July to August). North East India as a whole produced 101.5 MT of muga worth Rs. 35.52 crore in the year 2002 to 2003 with Assam's share of 96 MT of raw muga.

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. The cloning of important genes requires the extraction of large insert genomic DNA. Liquid nitrogen can be difficult to procure in remote locations; thus, a method not requiring its use would be helpful. We have developed a protocol in which tissues are fixed in alcohol before DNA extraction, making liquid nitrogen unnecessary. DNA isolated by this method is suitable for various molecular biology applications.

MATERIALS AND METHODS

Silkworm collection

Eleven morphologically distinct strains of *A. assama*, collected from different regions of North and Eastern India from Assam and neighbouring states (Table 1), were used for this study. The silkworms collected were Mangaldoi (S1), Tura 1 (S2), Jorhat (S3), Tura 2 (S4), Tura 3 (S5), Tura 4 (S6), Tura 5 (S7), Lahing (S8), Mokokchung 1 (S9), Mokokchung 2 (S10) and Baghmara (S11).

Mangaldoi and Jorhat are in Assam, while Tura and Baghmara in Meghalaya and Lahing and Mokokchung are in Nagaland.

Genomic DNA isolation

Each individual larva at 5th instar was collected and DNA was isolated from the posterior silk gland (Suzuki et al., 1972; Thanananta et al., 1997; Nagaraja, 2002; Nagaraja and Nagaraju, 1995). The tissues were dipped in fixing solution, absolute alcohol to denature enzymes. The treated tissue material was handled as follows: submerge 100 mg of tissue in 500 µl of alcohol for 30 min; allow alcohol to evaporate. Grind the tissue with a mortar and pestle; transfer the homogenized tissue to prewarmed extraction buffer (100 mM Tris buffer, pH 8.0 containing 2.5% SDS, 1.4 M NaCl, 25 mM EDTA, pH 8.0 and 0.8% β-mercaptoethanol); incubate for 1 h at 65°C in a water bath, occasionally mixing by gentle swirling; remove from water bath. Add equal vol of chloroform-isoamylalcohol (24:1). Mix by inversion for 10 min; spin at 10,000 *g* for 10 min; transfer the aqueous phase to another tube; add 0.6 vol of chilled isopropanol to precipitate the DNA; centrifuge briefly to pellet the DNA; wash with 70% alcohol and then allowed to dry for 30 min for approximately 1 h; dissolve the dried DNA in $T_{10}E_1$ buffer (pH 8); add 2 µl of RNAse to 0.2 ml of crude DNA; mix thoroughly but gently and incubate at 37°C for 1 h; add equal vol of phenol: chloroform-isoamylalcohol (25:24:1). Mix thoroughly for 10 min; centrifuge at 10,000 *g* for 10 min; remove the upper phase (avoid the whitish interface layer) and add equal vol of chloroform-isoamylalcohol (24:1). Mix by inversion for 10 min; remove the upper phase and add 0.1 vol 3 M sodium acetate (pH 5.4/5.5) and 2.5 vol chilled absolute alcohol. Keep in -20°C for 15 min; centrifuge at 10,000 *g* for 15 min; wash the pellet with 70% alcohol. Dry overnight; redissolve the DNA in 200 ml $T_{10}E_1$ buffer; dilute the DNA 1000 times in $T_{10}E_1$ buffer and quantify spectrophotometrically (BioPhotometer, Eppendorf, Germany); observe purified DNA on 0.8% agarose gel after staining with ethidium bromide to ascertain its integrity.

The DNA isolated following the earlier mentioned method was compared with that of DNA extracted using liquid nitrogen and at the same time compared with that of DNA isolated without the use of any fixing solution and liquid nitrogen.

RAPD analysis

A set of 50 decamer RAPD primers was developed from Bangalore Genei, Bangalore, India. The PCR was performed in a 25 µl reaction mixture containing 2.5 µl buffer (10 X *Taq* DNA polymerase buffer containing 15 mM MgCl2, Bangalore Genei); 2.5 µM dNTPs (from 10-mM stock, Bangalore Genei), 20 pM primer (random decamer primer), 1 unit of *Taq* DNA polymerase (Bangalore Genei) and 30 ng of genomic DNA. Amplification was performed in a thermal cycler (Applied Biosystems, Germany) using the following program: 1 cycle of 94°C for 4 min, 40 cycles of 94°C for 1 min, 37°C for 1 min, 72°C for 2 min, followed by 72°C for 5 min. PCR products were resolved on 2% agarose gel, employing 100 bp ladder as a molecular weight standard, stained with ethidium bromide (0.5 µg ml^{-1}) and run in 1X TBE (100 mM Tris-HCl, pH 8.0, 83 mM Boric acid, 1 mM EDTA, pH 8.0) at 80 voltage. Gels were visualized with UV transilluminator (Consort, Belgium) and photographed. The DNA fragments amplified by a primer were scored as present (1) or absent (0) for the strain studied.

Cloning cathepsin B gene

Cathepsin B gene specific primers were designed using DNAstar

Table 2. The nucleotide sequences of the primers, number of amplicons, fragment sizes, and number of polymorphic fragments scored using RAPD profiles of 11 muga silkworms, *A. assama* DNA in a PCR.

S/N	RAPD Primer	Nucleotide sequence (5'→3')	Number of amplicon	Fragment size range (bp)	Polymorphic fragment
1	BGA-01	CAGGCCCTTC	6	350 - >1000	6
2	BGA-02	TGCCGAGCTG	10	450 - >1000	10
3	BGA-05	AGGGGTCTTG	9	150 - >1000	9
4	BGA-16	AGGTGACCGT	9	400 - >1000	8
5	BGC-12	TGTCATCCCC	11	250 - >1000	11
6	BGD-19	GGGGTGACGA	7	400 - >1000	7
7	BGK-01	CATTCGAGCC	9	400 - >1000	9
8	BGK-19	CACAGGCGGA	8	300 - >1000	8
9	BGL-02	TGGGCGTCAA	12	200 - >1000	12
10	BGL-06	CAGGGAAGAG	9	200 - >1000	9
11	BGL-17	AGCCTGAGCC	17	400 - >1000	17
12	BGM- 20	AGGTCTTGGG	8	300 - >1000	8
13	BGN-03	CTGTTGCTAC	9	300 - >1000	9
14	BGN-04	GACCGACCCA	12	300 - >1000	11
15	BGN-05	ACTGAACGCC	9	150 - >1000	9
16	BGN-16	AAGCGACCTG	10	250 - >1000	10
17	BGW-01	CTCAGTGTCC	8	400 - >1000	7
18	BGW-02	ACCCCGCCAA	13	250 - >1000	12
19	BGW-03	GTCCGGAGTG	10	150 - >1000	10
20	BGW-04	CAGAAGCGGA	7	450 - >1000	7
21	BGX-06	ACGCCAGAGG	9	175 - >1000	9
22	BGY-02	CATCGCCGCA	5	350 - 900	5
23	BGY-03	ACAGCCTGCT	5	350 - 1000	4
24	BGY-04	GGCTGCAATG	8	375 - >1000	6
25	BGY-05	GGCTGCGACA	7	275 - >1000	4
26	BGY-06	AAGGCTCACC	10	350 - >1000	9
27	BGY-07	AGAGCCGTCA	4	280 - >1000	4
28	BGY-08	AGGCAGAGCA	9	300 - >1000	8
29	BGY-09	AGCAGCGCAC	7	250 - >1000	5
30	BGY-10	CAAACGTGGG	4	200 - 900	4
31	BGY-11	AGACGATGGG	5	250 - >1000	4
32	BGY-13	GGGTCTCGGT	7	300 - 800	7
33	BGY-14	GGTCGATCTG	6	250 - 1000	6
34	BGY-16	GGGCCAATGT	8	200 - >1000	7
35	BGY-17	GACGTGGTGA	10	400 - >1000	10
36	BGY-18	GTGGAGTCAG	12	200 - >1000	12
			309		293

primer select software which are described in detail in Table 5. The PCR was performed in a 20 µl reaction mixture containing 2 µl buffer (10 X *Taq* DNA polymerase buffer containing 15 mM MgCl2, Bangalore Genei,); 2 µM dNTPs (from 10-mM stock, Bangalore Genei), 10 pM of each primer, 0.3 unit of *Taq* DNA polymerase (Bangalore Genei) and 50 ng of silkworm genomic DNA. Amplification was performed in a thermal cycler (Applied Biosystems, Germany) using the following program: 1 cycle of pre-denaturation at 94°C for 1 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 30 s, extension at 72°C for 30 s, followed by final extension at 72°C for 7 min. PCR products were resolved on 1% agarose gel, employing 100 bp ladder as a molecular weight standard. PCR amplified products were gel eluted using gel extraction kit (Hi Media) and cloned into pGEMT easy vector (Promega) in 3:1 molar ratio and chemically transformed into chemically competent DH10β cells. Plasmids were extracted using SDS alkaline lysis method and sequenced using M13F and M13R primers in 3130 XL Genetic Analyzer (Applied Biosystems). The sequences obtained were analyzed using NCBI Blast for sequence homology.

Statistical analysis

Specific bands were scored as present (1) or absent (0) for each

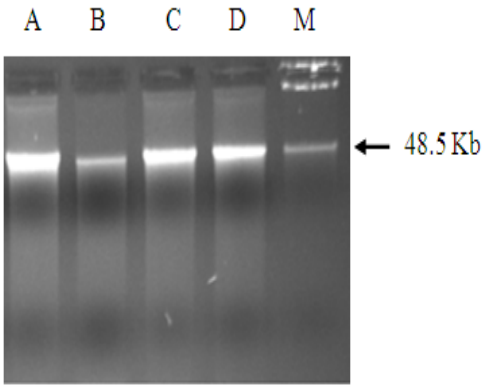

Figure 1. Genomic DNA isolated from silkworm strains. [A: sample was fixed with absolute alcohol and ground in liquid nitrogen; B: without the use of any fixing solution and liquid nitrogen; C: sample was not fixed with absolute alcohol and ground in liquid nitrogen; D: sample was fixed with absolute alcohol and without the use of liquid nitrogen; M: uncut λDNA (Bangalore genei, 48502 bp, 100 ng)].

band and index of genetic similarity or distance (1-F) was calculated. F value was calculated by Jaccard's similarity coefficient formula:

$$F = \frac{a}{n-d}$$

Where, F is the similarity index; a is the number of positive matches that is, the presence of bands in both samples; d is the number of positive matches that is, absence of bands in both samples and n is the the total sample size including both the number of matches and unmatched.

Based on the data, hierarchial cluster analysis was done using unweighted pair group method arithmetic mean (UPGMA) and dendogram was prepared using a statistical software package "SPSS for MS windows release 10.0".

Restriction analysis of genomic DNA

DNA was restricted by *Eco*R I (Bangalore Genei) using 1.5 U/µg of DNA. The reaction mixture was incubated at 37°C overnight. Digested DNA was separated on 0.8% agarose gel, stained with ethidium bromide and observed under UV light.

RESULTS AND DISCUSSION

DNA extraction and yield

The procedure yielded high molecular weight DNA (>40 Kb, Figure 1). The $\lambda_{260}/\lambda_{280}$ ratio was greater than 1.8 and the $\lambda_{260}/\lambda_{230}$ ratio was greater than 2.0 (Table 1), indicating the purity of extracted DNA (Sambrook et al., 1989; Henry, 1997). DNA yield ranged from 290 to 548 µg/g of silk gland tissue. The $\lambda_{260/\lambda280}$ ratio ranged from 1.82 to 1.97 and the $\lambda_{260/\lambda230}$ ratio ranged from 2.07 to 3.15, indicating good quality DNA by the method employed. Alcohol-fixed samples and ground with liquid nitrogen produced good quality DNA (548 µg/g of silk gland tissue). Samples without the use of any fixing solution and liquid nitrogen produced minimum DNA yield (290 µg/g of silk gland tissue). Samples yielded similar amount of DNA when fixed in absolute alcohol (400 µg/g of silk gland tissue) and ground in liquid nitrogen (456 µg/g of silk gland tissue). But liquid nitrogen can be difficult to procure in remote locations; thus, this method of DNA isolation not requiring its use would be helpful to researchers.

Random amplification of polymorphic DNA

Only 36 of the 50 RAPD primers yielded good amplification and hence were chosen for fingerprinting the 11 muga silkworm strains. RAPD profile generated with primers BGA-02, BGA-16, BGL-06, BGY-04, BGY-16 and BGY-17 are depicted in Figure 2. The total number of DNA fragments amplified and the number of polymorphic bands from each variety with individual primers are shown in Tables 2 and 3. All the 11 strains were quite distinct in their RAPD profiles. A total of 309 scorable fragments ranging from 150 to >1000 bp were generated by 36 RAPD primers. Out of this, 293 were polymorphic (94.82%). Percentage of polymorphism detected with each primer was as high as 100% in 24 primers (BGA-01, 02, 05, BGC-12, BGD-19, BGK-01, 19, BGL-02, 06, 17, BGM-20, BGN-03, 05, 16, BGW-03, 04, BGX-06 and BGY-02, 07, 10, 13, 14, 17, 18) to 57.14% detected by BGY-05. The number of bands per primer ranged from 4 (BGY-07 and BGY-10) to 13 (BGW-02) with an average of 8.58 scorable bands per primer. Cluster analysis (UPGMA) was generated by computing polymorphic as well as monomorphic markers to con-struct the dendogram (Figure 3). Jaccard's similarity coefficients ranged from 0.122 to 0.863 (Table 4). Maximum similarity was noticed between S2 and S3 strains and the minimum similarity was observed between S4 and S9. Associations among the 11 varieties revealed by UPGMA cluster analysis based on RAPD profile are presented in Figure 2. All the strains could be splited into two major clusters, at 50% similarity level, with S9 on one cluster and with the remaining strains on the other cluster. At 60% similarity level, strains S10 and S11 combine to form another cluster II and strains S2, S3, S1, S5, S6, S7, S8 and S4 combined to form cluster III, making three clusters along with cluster I. At 80% similarity level, all the strains were divided into 5 major clusters. The strains S9, S11 and S10, S4, S8 and S7 making four clusters: cluster I, II, III and IV, respectively and the remaining strains S2, S3, S1, S5 and S6 making the cluster V. Cluster V of 80% similarity level sub groups into 2 clusters at higher

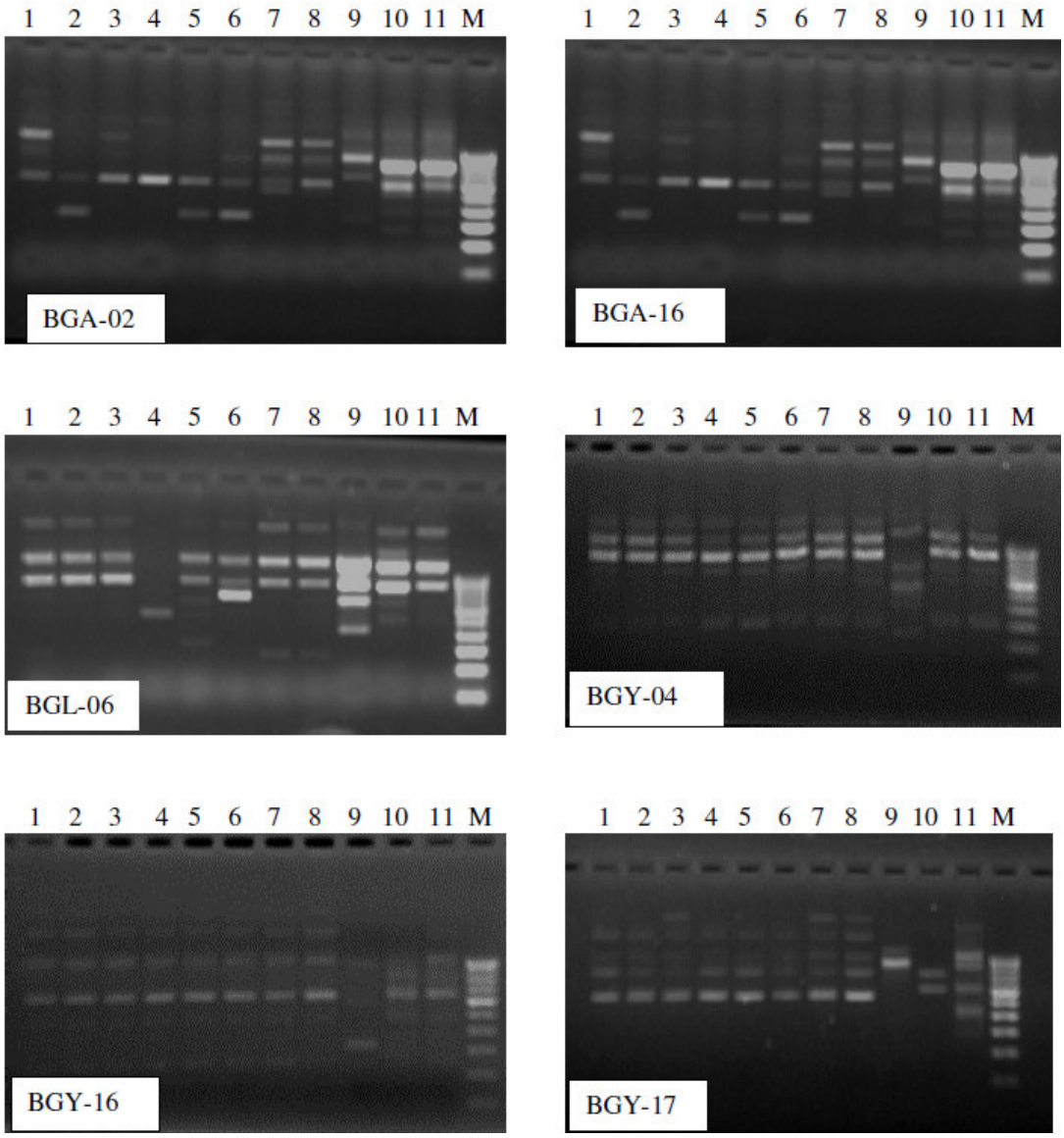

Figure 2. RAPD profile of muga silkworm *A. assama* genotypes obtained with primers BGA-02, BGA-16, BGL-06, BGY-04, BGY-16, BGY-17. Serial number of the genotypes is as given in the Table 1. M = Standard DNA marker, 100 bp DNA ladder.

Table 3. Summary of PCR amplification fragments from 11 muga silkworm strains *A. assama.*

Total number of amplicon	Total number of polymorphic band	Average fragment size range (bp)	Polymorphism (%)	Average amplicon per primer
309	293	150 - >1000	94.82	8.58

similarity levels: three domesticated strains (S1, S2, and S3) collected from Assam (Mangaldoi and Jorhat) and Meghalaya (Tura) combined to form one cluster and the other cluster comprised of two wild strains (S5 and S6).

Since RAPDs are random selection of DNA sequence, it was apparent in this study that RAPD technique was sensitive enough to detect differences between strains of muga silkworm in which differentiation is not always possible morphologically. In this study, 94.82% polymorphism demonstrated the potential of the method in evaluating genetic diversity within the germplasm. Wide range of similarity (0.122 to 0.863) indicated high genetic diversity in muga silkworm varieties. Cluster analysis of RAPD data using UPGMA revealed that three high-

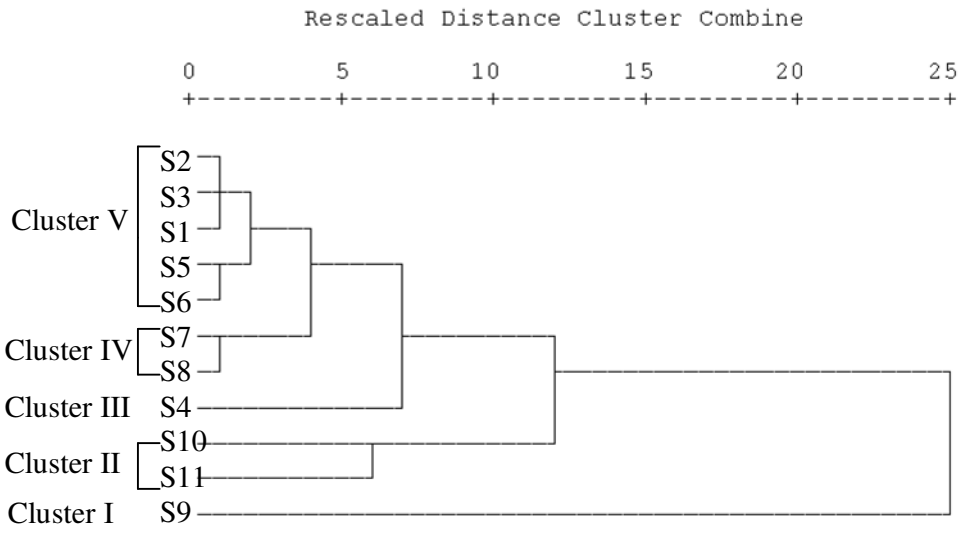

Figure 3. Dendrogram illustrating genetic relationships among the eleven muga silkworm strains *A. assama*. The dendrogram was obtained using the unweighted pair group method with arithmetic average (UPGMA) using the Jaccard's similarity coefficient derived from 36 RAPD primers data.

Table 4. Similarity matrix for Jaccard's coefficient based on RAPD banding pattern for 11 muga silkworm strains *A. assama*.

Parameter	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
S1		0.838	0.832	0.654	0.807	0.813	0.816	0.792	0.130	0.538	0.538
S2			0.863	0.667	0.799	0.842	0.784	0.728	0.127	0.500	0.546
S3				0.707	0.781	0.837	0.827	0.779	0.137	0.528	0.568
S4					0.667	0.673	0.667	0.616	0.122	0.475	0.475
S5						0.860	0.736	0.705	0.141	0.550	0.535
S6							0.809	0.753	0.150	0.547	0.555
S7								0.846	0.138	0.559	0.575
S8									0.129	0.607	0.591
S9										0.159	0.163
S10											0.710
S11											

Table 5. Gene, primer, primer sequences and expected product length from PCR.

Gene	Accession	Primer pair	Amplicon size (bp)
Antheraea assama cathepsin B	EU126818.1	ACB1F: 5' CCGGACGTAACTTTCCGATA 3' ACBR: 5' ACAACCGAGACCGCAAATAG 3'	332

yielding strains S9, S10 and S11 were genetically distinct from the remaining strains. In the dendogram, it was interesting to find that at higher similarity levels, there was clear demarcation between the wild and domesticated muga silkworm strains. The domesticated strains S2, S3 and S1 making one cluster, the other domesticcated strain S9 making the other cluster and the wild strains were grouped in five different clusters. The muga silkworms are susceptible to many dreaded bacterial diseases (Choudhury et al., 2002; Sharma et al., 2005) and many environmental stresses. The molecular studies for the reasons behind the diseases and stress factors will help to understand the genetics of resistance in muga silkworms and such studies on prevailing indigenous races will throw light on complex genotype x environment (GXE) interactions (Murthy et al., 2006). For sustenance of the silk industry such studies are very important and their utilization in silkworm breeding is inevitable for the

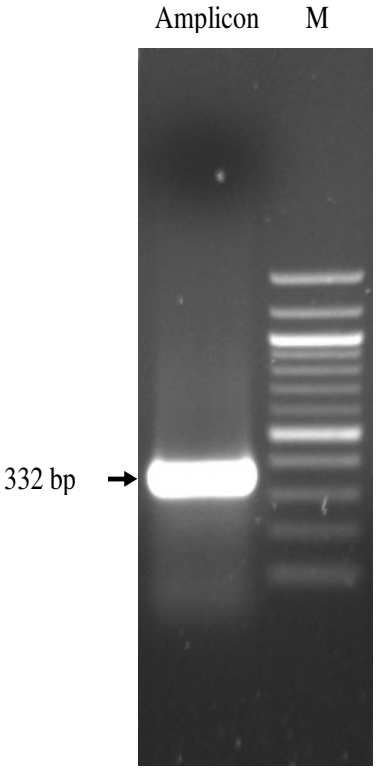

Figure 4. PCR amplification of partial sequence of cathepsin B gene using gene specific primers [M: 100 bp ladder].

development of region specific races. Such studies are very much necessary to develop a farmer preferred; resistant variety which will thrive very well at the prevailing local conditions of Indian villages particularly North Eastern region. Morphologically, all the strains are green in larval colour except the strain S8 which is blue in colour but it did not show any marked genetic divergence from other strains. It was also found that the progenies of S8 are not always blue in colour favoring the Mendel's law of segregation. Morphologically similar individuals were also genetically dissimilar and grouped into different clusters.

Modern tools like molecular markers offer a wide range of applications in silk breeding programs. India being a country with diverse environmental conditions, the local races is rich reservoirs of many resistant genes and molecular markers are inevitable tools to study inheritance of such complex traits. The amplification products resulting from the RAPD assay vary between strains and hence, can be used as genetic markers as well as to construct linkage maps. Considering the diversity, the genotypes belonging to different groups will constitute promising parents for hybridization in silk improvement program. Thus, this study along with the analysis of their rearing performance was essential to understand the genetic relationship among the 11 varieties for use in muga silkworm breeding program.

Cloning of cathepsin B gene

To examine the integrity of isolated DNA from silkworm and to check the presence of cathepsin B gene, PCR analysis was performed with gene specific primer to amplify partial sequence of the gene. The primer produced 332 bp fragment and the amplicon after sequencing was analysed using NCBI BLAST which showed 100% homology with cathepsin B gene of *A. assama* (Figure 4).

Restriction analysis of genomic DNA

Genomic DNA from all 11 silkworm species was restricted by EcoRI (2 unit of enzyme/µg of DNA, kept overnight at 37°C). The restricted DNA produced good patterns on 0.8% agarose gel, indicating complete digestion of DNA samples (Figure 5).

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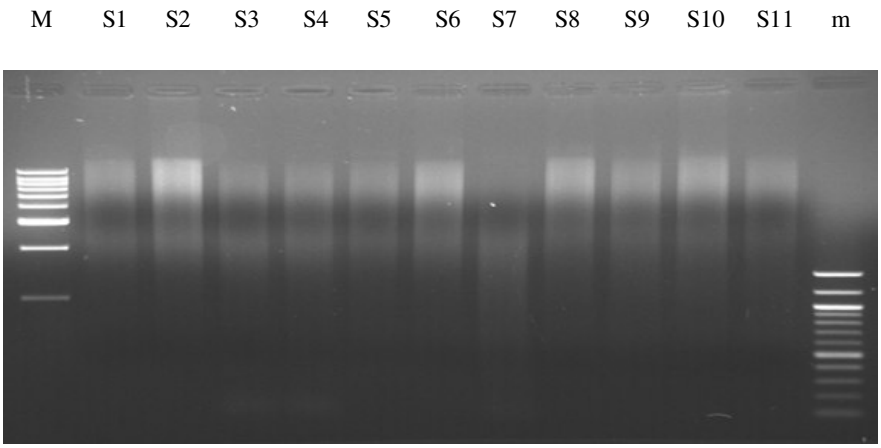

Figure 5. EcoRI digestion of genomic DNA isolated from 11 muga silkworm strains. M, 1 kb ladder; m, 100 bp ladder.

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