

Molecular evaluation of bivoltine, polyvoltine and mutant silkworm (*Bombyx mori* L.) with RAPD, ISSR and RFLP-STS markers

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Mulberry silkworm (*Bombyx mori* L.), the most important silk producing insect, exhibits wide diversity in morphological and biometric characters. Characterization of vast genetic resources based on the morphological and quantitative traits is not solely dependable as the phenotypic traits are influenced by environment. In order to study genetic relatedness of the selected and varied genotypes, six each of the bivoltine, polyvoltine silkworm accessions and mutant stocks, were studied with RAPD, ISSR and RFLP-STS markers. Twelve RAPD primers generated 172 markers of which 161 were polymorphic, generating 93.60% polymorphism. Pair-wise genetic divergence varied from 0.209 between Mysore Princess and Rong Diazo to 0.588 between Boropolu and TMS-35. The ISSR primers generated 156 markers of which 132 were polymorphic thus generating 84.62% polymorphism. The pair-wise genetic diversity among the genotypes varied from 0.189 between Rong Diazo and BL-23 to 0.438 between MU-10 and TMS-35. Similarly, 10 RFLP-STS primers produced a total of 69 bands, out of which 53 were polymorphic thus realizing 75.6% polymorphism. Genetic distance varied from 0.242 between Nistari-M and BL-23 to 0.730 between Fengshong and TMS-17. On clustering with UPGMA and principal component analysis (PCA), RAPD and ISSR markers clearly discriminated the bivoltines and multivoltines and a multivoltine Tamil Nadu white occupied the positions among bivoltines, since it has bivoltine parentage. Boropolu, an original land race from North East India, and Feng shong, a Chinese silkworm strain, also showed a closer genetic relationship.

Keywords: *Bombyx mori*, silkworm, genetic characterization, ISSR, RAPD, RFLP-STS, bivoltine, polyvoltine

Introduction

Mulberry silkworm (*Bombyx 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 diapause. 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 diapause, hence they are called polyvoltine silkworms. The polyvoltines are comparatively smaller in size but can 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 preferred¹. 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 polyvoltines². In order to achieve this goal, knowledge on the genetic make up 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 strains³⁻⁵. 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 genome⁶, 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.

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Table 1—Name, origin and quantitative traits of 18 silkworm accessions studied

No.	Accession No.	Name	ORIGIN	TLD (h)	VLD (h)	LWT (g)	CWT (g)	SWT (g)	SR (%)	Life cycle/ year
1	BBI-0140	Boropolu	India	585	151	37.26	1.700	0.298	17.462	Two
2	BBE-0174	Feng shong	China	620	177	37.437	1.686	0.339	20.189	Two
3	BBE-0196	R7042	Vietnam	609	170	35.498	1.662	0.320	19.288	Two
4	BBE-0211	European P	France	632	182	39.098	1.655	0.336	20.384	Two
5	BBE-0224	JZH (MO)	Brazil	628	182	38.914	1.714	0.356	20.857	Two
6	BBE-0232	NB1	Japan	626	180	40.089	1.752	0.331	19.014	Two
7	BMI-0004	TN white	India	558	147	24.585	1.163	0.173	14.973	Six
8	BMI-0007	M. Princess	India	551	143	27.068	1.257	0.191	15.383	Six
9	BME-0012	Rong Diazo	China	574	169	26.294	1.368	0.191	14.104	Six
10	BMI-0018	Nistari M	India	550	143	21.071	1.039	0.143	13.920	Six
11	BMI-0058	BL-23	India	558	147	29.182	1.32	0.219	16.671	Six
12	BMI-0062	MU-10	India	553	137	27.061	1.239	0.198	16.108	Six
13	BBE-0320	TMS-02	Japan	553	146	21.788	1.004	0.158	15.940	Four
14	BBE-0321	TMS-17	Japan	559	133	19.264	1.941	0.130	14.124	Four
15	BBE-0322	TMS-31	Japan	557	150	17.717	0.896	0.133	14.984	Four
16	BBE-0308	TMS-32	Japan	562	130	15.332	0.829	0.103	12.466	Four
17	BBE-0309	TMS-33	Japan	540	128	22.385	0.992	0.134	13.634	Four
18	BBE-0310	TMS-35	Japan	580	147	18.659	1.054	0.130	12.346	Four

Note: Nos1-6 are bivoltines, 7-12 are polyvoltines and 13-16 are mutants.

TLD—Total larval duration, VLD- V^{th} instar larval duration, LWT- Larval weight,

CWT- Cocoon weight, SWT- Shell weight, SR- Ratio of shell weight to the cocoon (%)

Materials and Methods

Silkworm Strains

Eighteen silkworm strains comprising six each of bivoltine and polyvoltine accessions and equal number of mutant silkworm stocks having divergent geographical origin (Table 1) 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 practices⁷.

DNA Isolation

Genomic DNA was extracted from the silkworm moth using phenol:chloroform method⁸. 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, pH 8.0, 50 mM EDTA and 1% SDS) and 25 μ L of proteinase K (100 μ g/mL) were added before incubating it at 37°C for 2 h with occasional swirling. DNA was extracted twice with phenol-chloroform-isoamyl 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 μ g/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/ μ L) for PCR amplification.

PCR Amplification of Genomic DNA

For RAPD, the amplification of genomic DNA was carried out in a PTC-200 Thermal-cycler⁹ (MJ Research Inc., Watertown, Massachusetts, USA) using 12 random decamer primers (Table 2) obtained from Operon Technologies, USA (OPW series) following the standard procedure⁵.

Twenty-one ISSR (Inter simple sequence repeat) primers (Table 3) procured from the University of British Columbia, Vancouver, Canada (UBC set No. 9), were 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 200¹⁰.

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	14	13	92.86
2	OPW-04	CAGAAGCGGA	22	22	100.00
3	OPW-05	GGCGGATAAG	17	14	82.35
4	OPW-06	AGGCCCGATG	21	19	90.48
5	OPW-11	CTGATGCGTG	24	24	100.00
6	OPW-12	TGGGCAGAAG	8	6	75.00
7	OPW-13	CACAGCGACA	13	13	100.00
8	OPW-16	CAGCCTACCA	10	10	100.00
9	OPW-17	GTCCTGGGTT	11	10	90.91
10	OPW-18	TTCAGGGCAC	9	8	88.89
11	OPW-19	CAAAGCGCTC	12	12	100.00
12	OPW-20	TGTGGCAGCA	11	10	90.91
Total /Avg.			172	161	93.60

Table 3—Primers, and percentage of polymorphism realized with the ISSR primers

No.	Primer, UBC	Sequence	No. of total bands	No. of polymorphic bands	% Polymorphism
1	807	AGAGAGAGAGAGAGAGT	8	8	100.00
2	813	CTCTCTCTCTCTCTT	7	6	85.71
3	814	CTCTCTCTCTCTCTA	9	4	44.44
4	826	ACACACACACACACACC	10	8	80.00
5	827	ACACACACACACACAG	8	7	87.50
6	830	TGTGTGTGTGTGTGTGG	3	2	66.67
7	835	AGAGAGAGAGAGAGAGYC	17	17	100.00
8	836	AGAGAGAGAGAGAGAGYA	1	0	0.00
9	841	GAGAGAGAGAGAGAGAYC	6	5	83.33
10	842	GAGAGAGAGAGAGAGAYG	6	6	100.00
11	843	CTCTCTCTCTCTCTCRA	3	2	66.67
12	844	CTCTCTCTCTCTCTCRC	5	5	100.00
13	845	CTCTCTCTCTCTCTCRG	4	4	100.00
14	857	ACACACACACACACACYG	5	4	80.00
15	861	ACCACCACCACCACCACC	4	3	75.00
16	862	AGCAGCAGCAGCAGCAGC	9	6	66.67
17	864	ATGATGATGATGATGATG	11	11	100.00
18	873	GACAGACAGACAGACA	17	17	100.00
19	884	HBHGAGAGAGAGAGAGA	6	5	83.33
20	885	BHBGAGAGAGAGAGAGA	7	6	85.71
21	886	VDVCTCTCTCTCTCTCT	10	6	60.00
Total /Avg.			156	132	84.62

Ten RFLP-STS primers (Table 4) developed from silkworm were used for the study and PCR reaction was carried out as described earlier¹¹.

Statistical Analysis

The presence of an amplified product was identified as "1" and the absence was designated as "0". The similarity coefficients were estimated with NTSYS-pc using the qualitative data option. Dendrograms from the above matrices were made using unweighted pair group method with arithmetic

averages (UPGMA) employing SAHN (sequential, agglomerative, hierarchical and nested clustering) routine from NTSYS-PC v.1.8 Program (Applied Biostatistics, Setauket, NY). The principal component analysis (PCA) was also done with NTSYS-pc.

Results and Discussion

Variability in Quantitative Traits

The quantitative data (Table 1) on the selected silkworms showed considerable variability. Total larval duration was in the range of 540-632 h and the

Table 4—List of RFLP-STs primers, their sequence, annealing temperature and polymorphism exhibited

No.	Primers	Sequence of the primers 5'-3'	Annealing temp. (°C)	No. of total bands	No. of polymorphic bands	% Polymorphism
1.	299	F CCTCCTTCGTCCATTTCG R GACCTTATAGAGCGGCAACG	58	10	10	100.0
2.	477	F TGGTACCCACATAAGCAG R CTTTAAGCCTCGACGACTC	49	7	6	85.7
3.	610	F TTGCTCCACTCACTATGG R CGCGTTTAGGCTTGCTCT	53	6	5	83.3
4.	633	F AAAAGTCTCGGACAGCATAT R GCTAGAAATAAGCCCTTACGT	55	8	4	50.0
5.	728	F CTTTCGTTTCGGGAAGATGTC R CGTAGCTTGCTGTTTCCTTC	60	5	3	60.0
6.	743	F TAGCCACGTCTGGATCTTCA R TGCGTCACTTTGGACTCAAC	62	3	2	66.6
7.	746	F AGCGTTGTGTGTAATCC R TAACCATGTGCGCCTCCT	50	10	7	70.0
8.	807	F AAGATGTGTGGCGTCGTG R GGTTGGCTCACTATAACG	57	10	8	80.0
9.	832	F GAATGCAGGGACACGTAAGC R GTCGAGGATGAGGAAGACCA	64	5	5	100.0
10.	963	F GACCGAAGAAATGGAGCAAG R GCACTGAAGGTGCCAAGTC	60	5	3	60.0
Total/Average				69	53	75.6

final instar duration varied from 128-182 h. The larval, cocoon and shell weights were in the range of 15.332-40.089 g, 0.829-1.752 g and 0.103-0.356 g, respectively. The shell-cocoon ratio varied from 12.346% to 20.857%.

Diversity Revealed by RAPD Markers

Twelve RAPD primers generated 172 markers of which 161 were polymorphic, generating 93.60% polymorphism. Primers OPW-04, OPW-11, OPW-13, OPW-16 and OPW-19 generated highest polymorphism (100%, Table 2). The profile of amplified PCR products for the primer OPW-16 is presented in Fig. 1a. Pair-wise genetic divergence varied from 0.209 between Mysore Princess and Rong Diazo to 0.588 between Boropolu and TMS-35. The dendrogram realized from RAPD matrix grouped 18 silkworms into four groups along with three silkworms as isolates. All the bivoltine silkworms were grouped into a single cluster but in the polyvoltines, Tamil Nadu White separated from others and figured as an isolate in between bivoltines and polyvoltines (Fig. 2a). In the case of mutants, there were two groups and two isolates showing greater diversity among them. Principal component analysis PCA showed that the bivoltine silkworms distributed across Y-axis of the two dimensional figure at the right hand side of the X-axis (Fig. 3a).

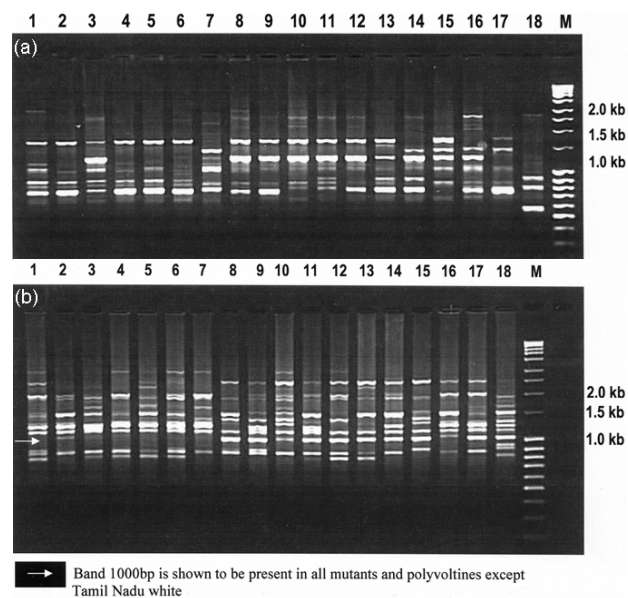


Fig. 1 (a-b)—a. RAPD profiles of genomic DNA of 18 silkworm accessions (as shown in Table 1) generated with random primer OPW-16 run on agarose gel and stained with ethidium bromide (Band 1350_{bp} is present in all mutants and polyvoltines and absent in all bivoltines, except R7042); b. Band profiles of the 18 silkworm accessions of *B. mori* (as shown in Table 1); genomic DNA amplified with ISSR primer UBC-873 and resolved on 2.0% agarose gel stained with ethidium bromide.

The silkworm strain Boropolu showed greater

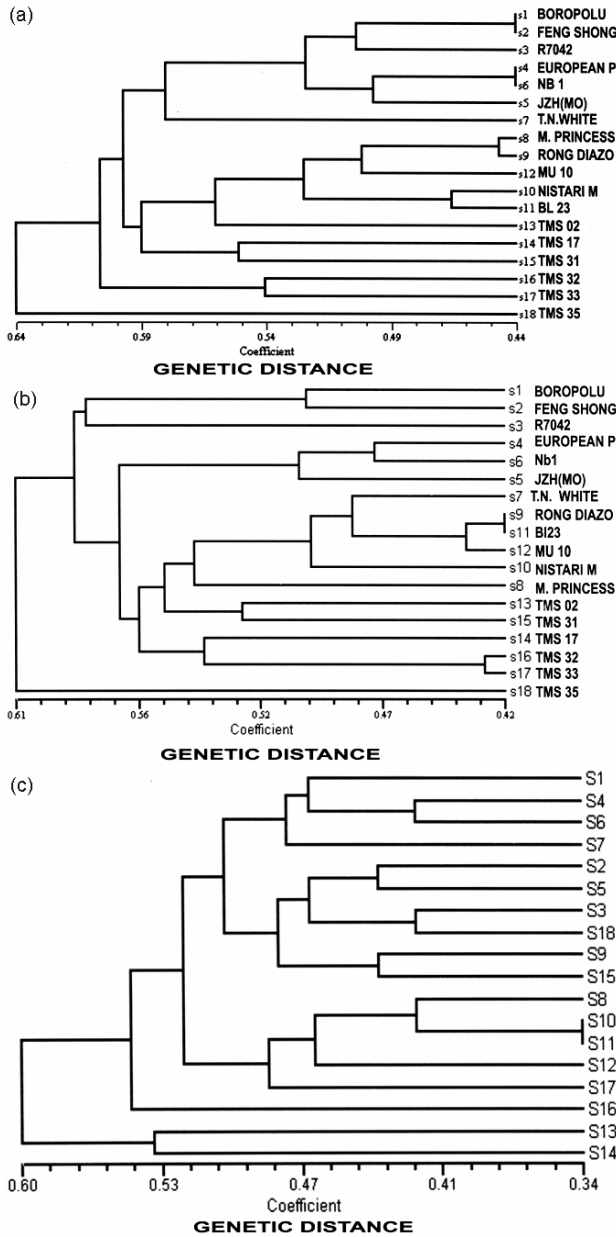


Fig. 2 (a-c)—Dendrograms based on RAPD markers (a), ISSR markers (b), and RFLP-STS markers using Nei and Li genetic distance on NTSYS-pc.

distance from others while Feng shong occupied a position in between Boropolu and other bivoltine silkworms. The polyvoltines and mutants were distributed widely and Tamil Nadu White showed more closeness towards the bivoltine groups.

Diversity Revealed by ISSR Markers

The ISSR primers generated 156 markers of which 132 were polymorphic thus generating 84.62% polymorphism. Highest polymorphism was recorded for primers UBC-807, 835, 842, 844, 845, 864 and

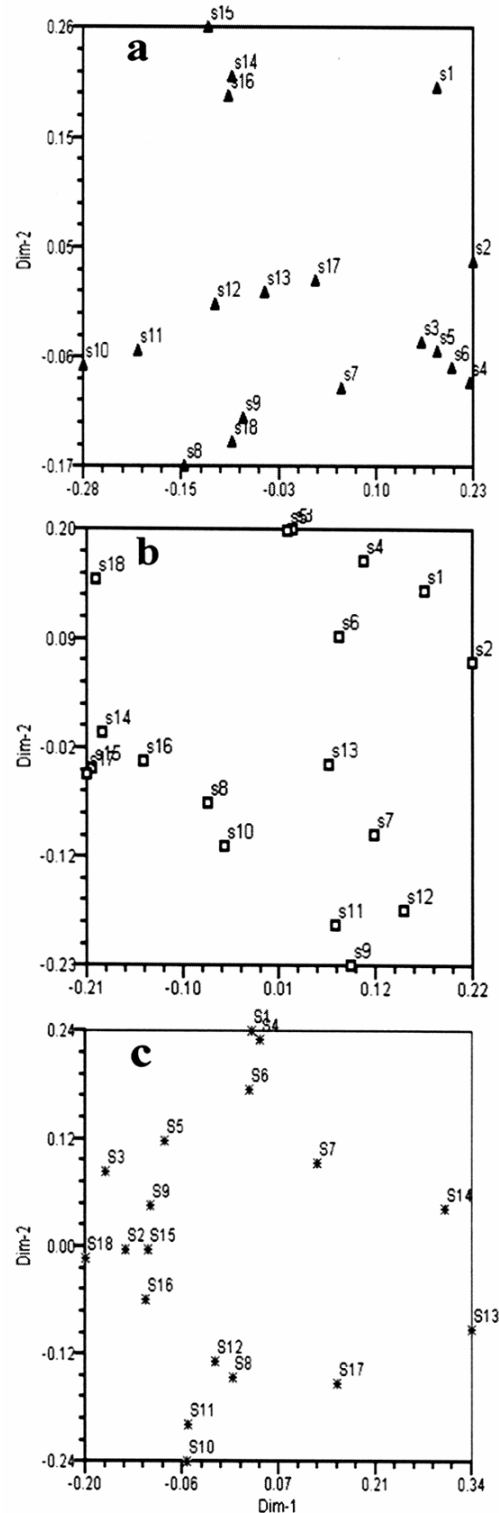


Fig. 3—(PCA) plots based on a. RAPD, b. ISSR and c. RFLP-STS markers.

873 (Table 3). The profile of PCR products for the primer UBC-873 is presented in Fig. 1b. The pairwise genetic diversity among the genotypes varied

from 0.189 between Rong Diazo and BL-23 to 0.438 between MU-10 and TMS-35. Dendrogram realized from the matrix clustered the silkworm strains according to their voltinism. Six bivoltines grouped into two clusters and one isolate (R-7042, a Vietnamese strain). Similarly, the polyvoltines grouped themselves into one major group comprising five silkworms and one isolate (Mysore Princess). Regarding the mutants, two distinct groups and an isolate could be discernible (Fig. 2b). In this case also TMS-35 stood as an isolate as observed during RAPD analysis. When PCA was applied, the bivoltines showed a confined assembly at the right top corner of the figure while the rest of the silkworms showed wide distribution across the PCA plot (Fig. 3b).

Diversity Revealed by RFLP-STs Markers

Ten RFLP-STs primers produced a total of 69 DNA bands, out of which 53 were polymorphic thus realizing 75.6% polymorphism (Table 4). Polymorphism ranged from 50% in the case of primer 633 to 100% in the case of primers 299 and 832. The PCR product of primer set 728 was only a single monomorphic band with the template DNA of 18 silkworms. This band, upon digestion with the restriction enzyme *Hind*III produced 5 DNA fragments of which 3 were polymorphic. These fragments were also included in the 69 fragments mentioned above. Digestion of the PCR products generated with four other primers with restriction enzymes was not informative as the products appeared to be monomorphic (Table 2). Genetic distance varied from 0.242 between Nistari-M and BL-23 to 0.730 between Feng shong and TMS-17. Unlike the dendrograms realized from RAPD and ISSR markers, the grouping from RFLP-STs markers did not show clear distinction for voltinism. The dendrogram grouped the silkworms into four groups and one isolate (Fig. 2c). The first group comprised three bivoltines along with a polyvoltine (TN White), the second group comprised six silkworm strains with different voltinism and origin. The third group comprised only polyvoltines except one mutant. The mutants, TMS-2 and TMS-17, were grouped together as the outermost group in the dendrogram showing their highest genetic distance from the rest. The mutant, TMS-32 stood as an isolate just behind the outermost group. No distinct grouping pattern was observed among the silkworms in the PCA plot and their distribution was intermingled (Fig. 3c).

The results of the present study clearly demonstrate

that using molecular markers, the genetic distance is more or less in conformity with the phenotypic character voltinism, and parentage. It is also seen that non-specific primers like RAPD and ISSR have generated more polymorphism than the silkworm specific primer like RFLP-STs. The higher polymorphism with microsatellite primers like ISSR is quite understandable as microsatellite regions harbor more variability than the rest of the genome due to higher mutation rate resulting from slippage of DNA polymerase activity and failure to repair mismatches, deletion and insertion of repeats¹². Similarly, RAPD can also generate more polymorphism than the specific primers due to the random annealing of the primers across the genome. However, RFLP-STs has the added advantage of being more reliable, repeatable and specific than the ISSR and RAPD primers. Being derived from the same genome, specificity of RFLP-STs primers is high, thus these primers are more useful in characterizing silkworm genome. Using RFLP-STs, markers associated with yield attributing traits in silkworm have been identified¹¹.

In the greater genetic diversity observed among the polyvoltines, Tamil Nadu White needs a special mention as this strain was developed through systematic breeding between a polyvoltine (Pure Mysore) and a bivoltine (J122) strain¹³. But the behaviour of this silkworm is similar to that of the polyvoltine and it would be difficult to distinguish it from the true polyvoltines. But the molecular analyses could decipher its bivoltine lineage and separated it from the rest. Similarly, Boropolu, an original land race from North East India, and Feng shong, a Chinese silkworm strain, also showed a closer genetic relationship between them but showed higher genetic distance from other strains. From the origin of these silkworm strains, it could be seen that both of them originated from regions, which have almost similar geographic features.

The higher genetic distance between the mutants and the original bivoltines is another point of interest. Most of the mutants though originated from bivoltines, later started behaving like polyvoltines and complete 3-4 life cycles in a year. Thus, the genetic make up of the mutants appear to be in the transitional stage between the two cycles of bivoltines and 5-6 cycles per year of polyvoltines. Interestingly, the molecular analyses also revealed an intermediate position of mutants with a skew towards the

polyvoltines.

DNA Markers

PCR amplification of silkworm genomic DNA with OPW-16 and UBC-873 demonstrated unique DNA fragments for polyvoltines and mutants (Figs 1 and 2). In case of UBC-873, the band of size 1000 bp was present in all the mutants and polyvoltines except Tamil Nadu White. The inheritance of these bands in the F₂ showed Mendelian pattern (data not shown).

The primer systems like RAPD, ISSR and RFLP-STS have once again proved to be ideal for studying genetic relatedness among the closely related population of *B. mori* strains. Even the race like Tamil Nadu White, which produces white cocoons but behaves like multivoltine could show close proximity to its old parentage i.e. bivoltine. Similarly, North East Indian race could show close proximity with Chinese race thus proving geographical proximity. The study also confirms genetic transition of the mutants which are mutated from bivoltine races and presently behave like multivoltines. Accordingly they occupied intermediate positions in genetic analyses too thus confirming their transition stage. Thus specific primers like RFLP-STS and non-specific primers like RAPD and ISSR could be effectively used to decipher genetic relatedness issues in close bred and mutant populations of silkworm.

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