Expression Profiling of Growth Specific Genes Associated Silk Productivity in Bombyx Mori

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Abstract

Silkworm Bombyx mori exhibits wide variability and development attributes. The genetics of yield expression, shown to be of polygenic nature, is poorly studied in silkworm, to identify markers associated with 5 selected yield traits, with two RFLP-derived sequence –tagged site (STS) primers on the genomic DNA of silkworm germplasm stocks of different origin and diverse yield potential. The analysis led to the identification of two markers showing significant association with the yield traits. The markers could classify the stocks according to yield potential, irrespective of their origin. The relevance of the STS primers is discussed in the context of Appling multiple regression models for identifying markers associated with yield expression and stability for molecular breeding work in *B. mori* for yield improvement.

Introduction

Sericulture is an important rural agro-industrial practice in India and all the major commercial silks are produced in the country [1]. Sericulture, the practice of breeding silkworms for the production of raw silk, has been underway for at least 5000 years in China, from where it was spread to Korea and Japan and later to India and the west. The prestigious silk fiber is produced by the monophagous insect, the silkworm *Bombyx mori*, a Lepidopteron; whose sole food is mulberry. Silkworm is a holometabolous insect, which completes its life cycles of four different metamorphosing phases i.e., egg, larva, pupa and adult 50–55 days.

More than 4000 *Bombyx mori* strains are currently available throughout the world and these strains are maintained by continuous sibling mating. At Central Sericultural Germplasm Resources Centre (CSGRC), Hosur, Tamil Nadu, where it was undertaken around 450 strains of silkworm are maintained and conserved.

The gene pool available in the country can be broadly divided in to two groups, the low yielding stocks characterized by high adaptability to tropical conditions and the yielding stocks exhibiting regular diapause, but suffer from the low adaptability to the highly variable tropical agro-climatic conditions [2, 3] and are mainly developed from Japanese hybrids[4]. The proposed ‘gene tagged breeding’ to combine the best qualities of the temperate high yielding stocks with tropical hardy ones [5, 6].

The analyzed many cDNA libraries prepared from various tissues[7] and at different developmental stages that cover almost entire set of Bombyx genes, comprising 35,000 expressed sequence tags (EST) from 36 cDNA libraries. Expressed sequence Tags or ESTS are small pieces of DNA sequences that are generated by sequencing either one or both ends of an expressed gene. Once generated, they are useful in cloning specific genes of interest and mapping of functional genes in various related organisms. Such markers are obtained by partial sequencing of random clones. ESTs are popularly used in full genome sequencing and mapping program underway for a number of organisms and for identifying active genes thus helping in identification of diagnostic markers. EST is very simple tool for genome analysis. ESTs

provide researchers with a quick and inexpensive route for discovering new genes, for obtaining data on gene expression and regulation, and for constructing the genome maps. EST database was also used to develop simple sequence repeat (SSR) markers for various species.

To build a foundation for the complete genome analysis of the silkworm, *Bombyx mori*, EST database has been constructed [8] covering about 55% of all genes of silkworm. EST's (Expressed Sequence Tags) are small pieces of DNA sequence, usually 200–500 nucleotides long, that are generated by either one or both ends of a cDNA. ESTs are presently being used as markers for genome mapping and identification of expressed genes. ESTs libraries and databases have proven to be powerful tools for gene discovery, gene mapping, and for the analysis of quantitative traits. ESTs are generated by large-scale sequencing of randomly picked clones from cDNA libraries constructed from mRNA isolated at a particular development stage and/or tissue. Japanese scientists are actively involved in the genome analysis in the mulberry silkworm. It has a large collection of cDNA libraries prepared from various and different developmental stages to cover the entire set of silkworm's genes. It is also engaged in construction of BAC (Bacterial Artificial Chromosome) library and making BAC contigs based on DNA fingerprinting and EST markers anchored to linkage maps.

Vitellogenins, the precursors of major yolk proteins of oviparous animals, Vitellogenin of the silkworm, *Bombyx mori* ,is a tetramer with molecular weight of 440K,composed of each two molecules of non-identical subunits termed heavy chain and light chain [9].

The prothoracic gene of *Bombyx mori* is a polymorphic one. The gene for the prothoracicotropic hormone (Ptth) in Bombyx mori has been already cloned and characterized. Three alleles a,b,c were identified in the Ptth locus which encodes PTTH in the silkworm ,Bombyx mori. These three alleles can be easily diagnosed by using the PCR technique because their length of the 3rd and 4th introns varies depending on the genotype [10].

Silk fibroin is secreted into the lumen of the posterior silk gland. Silk fibroin is composed of one heavy chain (≈ 350 KDa) and one light chain (25 KDa) [11, 12]. The identified a new mRNA species in the posterior silk gland encoding a silk protein of 25.197 KDa which was termed as P_{25}. P_{25} protein is produced in equimolar concentrations to major Fibroin [13, 14].

Therefore, in the present study, organization of yolk protein gene and PTTH gene genetic diversity were analyzed and their variations associated with productive traits in silkworm germplasm

Materials And Methods

Silkworm Races

A total 105 Silkworm accessions (30 MV and 75BV accessions) were screened using growth gene (PTTH) and Yolk protein gene primers. Silkworm strains from different origin, parentage and donor were chosen

from the silkworm stocks based on the yield diversity. Strains were maintained at Central Sericultural Germplasm Resources Centre, Hosur, Tamil Nadu. Data's were given in **table 1 & 2**.

Genomic DNA isolation

Genomic DNA was isolated from all the selected strains by standard phenol chloroform method (Nagaraja and Nagaraju, 1995). Extraction buffer (100Mm tris HCL, pH 8.0, 50Mm Nacl, 50,50Mm EDTA and 1 % SDS) and proteinase k (100 µg/ml) was added to the ground tissue and incubated at 37°c. Then DNA was extracted with phenol-chloroform-isoamylalcohol. DNA was dissolved in TE (10Mm Tris-HCL, 1Mm EDTA, pH 8.0). The genomic DNA was quantified in 0.8% Agarose gels and diluted to a uniform concentration (20ng/µL). PCR was carried out with two expressed sequence tag primers (EST) viz., PTTH and yolk protein gene and then PCR products were restricted with EcoRI restriction enzyme.

Primer Designing

The up and down gene-specific primers were designed for available gene sequence of yolk protein gene and PTTH gene using the software programme of primer3 [15]

PCR condition and analysis of amplified product

The amplification of genomic DNA was performed according to the amplification reaction was carried out in a 20µL reaction volume containing 20ng of template DNA, 10×PCR buffer (10Mm Tris–HCL, pH 8.3,1.5Mm Mgcl2 and 50MmKCL), 0.2µM primer, 100µM each of dATP, dCTP, dGTP and dTTP, and 3units of Taq DNA polymerase (genei) [16]. Amplification was performed in a MJ RESEARCH Peliteir thermal cycler (PTC 200 Controller). PCR schedule was 94°c for 2min, followed by 45 cycles of 94°c for 30 sec, 55°c for 30 sec and 72 °c for 2min and final extension was at 72 °c for 10 minutes. The amplified products were resolved on 1.5% Agarose gels in TBE buffer (89Mm Tris, 89Mm boric acid and 2Mm EDTA) and electrophoresis was carried out with a constant voltage. gels were stained with ethidium bromide(0.5 ug/ml) and analysis of restriction banding pattern were carried out using 1.5% Agarose gel, and photographed through gel documentation system. The presence of an amplified product was identified as 1 and the absence was designated as 0

Statistical Data Analysis

The SPSS/PC-11.5 (M.J.Norusis,SPSS Inc.,Chicago) package [17,18] was used for various statistical analyses. The PCR profiles were subjected to binary scoring (1 for presence and 0 for absence of DNA fragments).Clustering of 105 genotypes was done on genetic distance. Genetic similarity coefficients among twenty races were estimated from the binary data by Hierarchical cluster analysis using Ward method [19-21]. Two major groups are found in the cluster obtained.

Results And Discussion

A total 105 Silkworm accessions (30 MV and 75BV accessions) were screened using growth gene (PTTH) and Yolk protein gene primers. The DNA restriction patterns enzymes showed three band patterns which are designated as a, b and c. The presence and absence of these three types were entered in the ward's minimum variance test for all the 75 BV and 30 MV accessions. Three restriction banding pattern were observed among the screened silkworm genetic resources and their scoring revealed high, moderate and low fecundity and growth groups

The PCR product of PTTH and Yolk protein genes **(Figs. 1 & 2)** after digestion with restriction enzymes showed three band patterns which are designated as a, b and c. The presence and absence of these three types were entered in the ward's minimum variance test for all the 75 BV and 30 MV accessions (**Figs. 3 & 4**). Cluster groups were identified into high, moderate and low productive groups. The presence of allele / band a & b were seen among high productive group and a and c in the moderate and low productive groups (Tables 3 & 4). Three restriction banding pattern were observed among the screened silkworm genetic resources and their scoring revealed high, moderate and low fecundity and growth groups aa, ab banding types were found in Bivoltine high yielding groups and ac, bc found in high yielding Multivoltine groups.

The prothoracic gene of *Bombyx mori* is a polymorphic one. The gene for the prothoracicotropic hormone in Bombyx mori has been already cloned and characterized Three alleles a,b,c were identified in the ptth locus which encodes PTTH in the silkworm ,Bombyx mori. These three alleles can be easily diagnosed by using the PCR technique because their length of the 3rd and 4th introns varies depending on the genotype [22, 23].

Conclusion

Express Sequence Tag site technique was employed as an important molecular tool to identify productive breeds among promising silkworm genetic resources. Identified productive races based on growth and yield specific genes can be used as high productive/robust breeds by the breeders for marker assisted selection breeding to evolve robust breed for field utilization and augmentation of silk productivity in the field.

Declarations

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Tables

Table1.**Allelic variations in PTTH & yolk protein gene primers in Bivoltine accessions**

ACC.NO	Races	PTTH allele	yolk allele	Fec	Hat%	WTG	VLD(h)	TLD(h)	SCW(g)	SSW(g)
BBE-0001	Alps jaunne	ab	aa	368	91.5	34	144	574	1.53	0.24
BBE-0002	Alps Yellow	aa	ab	436	91.4	34.9	172	602	1.63	0.28
BBE-0003	Cevenese Yellow	ab	ab	422	91.2	36.3	154	574	1.72	0.28
BBE-0004	Ascoli Yellow	ab	aa	410	94.6	35.7	148	582	1.56	0.25
BBE-0005	Meigitsu	aa	ab	403	92.5	41.9	147	577	1.77	0.32
BBEI-0006	B-36	ab	aa	486	88.2	41.3	170	597	1.73	0.31
BBE-0007	B-37	aa	aa	450	82.6	36.8	156	612	1.56	0.28
BBE-0008	B-40	ac	aa	425	89.4	35.7	144	578	1.55	0.26
BBE-0009	B-40	ab	aa	466	93.1	41.9	149	582	1.56	0.25
BBE-0010	J-112	aa	ab	467	96.2	41.3	142	568	1.66	0.28
BBE-0011	J122	ab	ab	418	82.2	36.8	154	582	1.91	0.36
BBE-0012	Yakwei	ab	aa	460	92.2	37.9	159	592	1.56	0.28
BBE-0013	Chaung Naung	aa	aa	453	96.5	36	146	576	1.6	0.29
BBE-0014	C-122	aa	aa	467	93.8	40.3	154	582	1.64	0.28
BBE-0015	C-108	aa	aa	394	93.4	46.6	155	584	1.54	0.26
BBEI-0016	C-110	aa	aa	449	96.8	35.6	158	588	1.69	0.3
BBE-0017	Chukwei	ab	aa	458	90.7	40	145	572	1.57	0.27
BBE-0018	Chinese Farmer	ab	bb	402	90.8	38.4	161	590	1.54	0.26
BBE-0019	Chinese Golden-70	ab	bb	440	87.2	38.1	142	572	1.53	0.24
BBE-0020	Chinese Golden-80	aa	bb	408	94.3	36.1	177	630	1.34	0.22

BBE-0021	Chinese Golden -90	ac	bb	425	92.8	27.7	138	573	1.67	0.28
BBE-0022	Haulak	ac	bb	427	78.4	41.8	148	582	1.46	0.26
BBE-0023	King Haung	ac	bb	376	89.9	35	162	592	1.65	0.28
BBEI-0024	Hauchi	ac	ab	458	94.2	40.5	152	588	1.79	0.31
BBE-0025	Nan Naung 6A	ac	ab	375	88.9	38.6	148	571	1.61	0.38
BBE-0026	Nan Naung 6D	bc	ab	382	88.1	32	142	577	1.39	0.24
BBE-0027	Chinese Yellow	bc	aa	365	93.8	37.5	152	575	1.72	0.28
BBE-0028	Azad	ab	aa	419	91.4	38	152	578	1.58	0.26
BBE-0029	AzerbaiJan	ab	ab	383	97	37.5	160	592	1.61	0.27
BBE-0030	Sanish E1(P)	ab	ab	395	91.1	33.9	160	604	1.47	0.27
BBE-0031	Sanish-E1(M)	ab	ab	412	95.2	39.4	163	600	1.7	0.28
BBE-0032	Sanish-E2(M)	ac	ab	529	95.1	38.5	156	587	1.7	0.32
BBE-0033	Sanish-8	ac	aa	464	91.8	34.5	172	610	1.47	0.27
BBE-0034	Sanish-17	ac	ab	455	90.2	32.8	158	604	1.47	0.25
BBE-0035	. Sanish-18(M)	bc	aa	390	91.4	39.1	162	596	1.63	0.29
BBE-0036	Sanish-18 (P)	ac	aa	495	94.3	37.7	168	601	1.57	0.3
BBE-0037	Sanish-30	ac	aa	445	95.7	39.1	146	579	1.61	0.28
BBE-0038	. Sanish-21	ac	ab	482	92.6	35.8	156	590	1.7	0.29
BBE-0039	Shekhi-1	ac	ab	485	89.5	37.8	152	584	1.56	0.28
BBE-0040	Sheikhi-11	ac	aa	450	86.5	39.2	167	598	1.71	0.32
BBE-0041	Tashkahashi-112	ac	aa	448	89.8	39.1	162	596	1.64	0.29

BBE-0042	Gyandza	aa	aa	516	87.3	35.8	162	594	1.58	0.28
BBE-0043	Belkokona-11	ac	aa	485	93.5	37.8	168	604	1.72	0.29
BBI-0044	NB4D2	ac	aa	479	92.1	34.4	206	646	1.82	0.36
BBI-0045	SH-6	ac	aa	518	95.3	34.8	172	612	1.46	0.26
BBI-0046	YS-3	aa	bb	438	94.4	37.8	156	598	1.5	0.27
BBI-0047	SF-19	ac	aa	504	96	34.4	180	623	1.41	0.27
BBI-0048	JD6	ac	aa	475	95.4	33.9	164	596	1.53	0.27
BBE-0049	UKR-1	ac	bb	499	87.6	32.9	190	628	1.67	0.3
BBE-0050	UKR-2	cc	ab	483	91.6	36.4	185	621	1.79	0.36
BBE-0051	Merefa-6	aa	aa	376	94	35.8	158	596	1.63	0.33
BBI-0052	Jam-1	ac	aa	421	84.1	34.2	172	606	1.6	0.23
BBI-0053	Jam-2	ac	aa	426	86	32.5	166	596	1.56	0.25
BBI-0054	.Jam-11	ac	aa	360	85.8	33.6	158	590	1.46	0.24
BBI-0055	Jam-121	bc	ab	432	88.2	38.4	186	616	1.71	0.35
BBI-0056	Jam-21	ac	aa	407	89	30.9	160	606	1.55	0.27
BBI-0057	Jam-23	ac	ab	439	84.5	33.7	158	594	1.7	0.28
BBI-0058	. Jam-27	ac	aa	435	88.5	36.4	163	597	1.54	0.26
BBI-0059	Jam-103	aa	bb	417	92.6	38.1	152	586	1.53	0.26
BBI-0060	Jam-110	aa	aa	392	92	38.7	161	596	1.49	0.24
BBI-0061	Jam-118	ac	aa	430	82.4	30.6	159	588	1.75	0.31
BBI-0062	Jam-119	aa	aa	514	91.5	35.2	164	596	1.74	0.3
BBI-0063	Jam-122	ac	ab	411	89.7	30.6	206	648	1.54	0.27
BBI-0064	Jam-124	ac	aa	385	89.7	35.6	181	616	1.54	0.27
BBI-0065	Jam-125	aa	ab	433	95.8	34.2	166	604	1.69	0.3
BBI-0066	Pam-101	aa	ab	467	96.3	34.4	168	604	1.58	0.27
BBI-0067	Pam-102	ac	aa	508	89.3	36.3	145	577	1.65	0.25
BBI-0068	Pam-103	aa	aa	409	82.4	36.4	144	574	1.58	0.28
BBI-0069	Pam-104	aa	aa	430	92.5	35.9	168	598	1.62	0.28
BBI-0070	Pam-105	aa	aa	438	90.2	33.6	174	611	1.65	0.31
BBI-0071	Pam-106	ab	aa	430	88.2	37	169	602	1.6	0.29

BBI-0072	Pam-107	ac	aa	451	86.8	39.7	156	590	1.66	0.3
BBI-0073	Pam-108	ac	aa	392	85.8	38.4	189	610	1.54	0.29
BBI-0074	Pam-109	ac	aa	413	84.1	36.6	176	608	1.68	0.32
BBI-0075	Pam-110	ac	aa	412	88.1	35.1	162	594	1.58	0.3

Table. 2. **Allelic variations in PTTH & yolk protein gene primers in multivoltine accessions**

ACC.NO	Races	PTTH allele	yolk allele	Fecundity	Hat%	WTG	VLD(h)	TLD(h)	SCW(g)	SSW(g)
BMI-0001	Sarupat	ac	bc	405	85.7	25.21	149	569	1.16	0.17
BMI-0003	Moria	ab	bc	388	86	26.85	145	564	1.13	0.16
BMI-0004	T.white	ac	bc	429	86.8	26.41	147	566	1.21	0.19
BMI0008	Kolar gold	ac	bc	477	87.6	27.85	134	553	1.26	0.19
BME-0012	R. Daizo	ac	bc	433	88.6	29.27	168	572	1.41	0.21
BMEI-0013	G.Plain	ac	cc	417	90.3	24.32	135	558	1.13	0.17
BMI-0015	Raj	ac	bb	383	91	21.06	166	578	1.06	0.15
BMI-0016	G	ac	aa	432	90.4	25.37	149	571	1.22	0.18
BMI-0017	Nistari	ac	aa	381	82.3	20.91	143	557	1.02	0.18
BMI-0018	Nistari (M)	ac	aa	392	90.1	19.64	142	559	0.94	0.13
BMI-0019	Nistari (p)	aa	bb	382	88.8	20.58	134	549	0.97	0.14
BMI-0041	Wai-4	aa	bb	381	85.8	23.53	136	555	1.07	0.15
BMI-0042	MY-23	aa	bb	457	91.6	27.99	141	559	1.26	0.18
BMI-0043	MW13	aa	bb	470	88.6	28.96	138	558	1.33	0.2
BMI-0044	MHMP(W)	aa	bb	407	88	29.27	143	562	1.31	0.19
BMI-0045	MHMP(Y)	aa	bb	479	90.9	31.5	151	568	1.42	0.22
BME-0046	P4D3	aa	bb	461	92.9	27.85	153	571	1.34	0.21
BME-0047	NISTIDI (Y)	aa	bb	426	90.3	23.77	143	558	1.12	0.15
BME-0048	NISTIDI (W)	aa	bb	408	91	23.07	141	557	1.04	0.14
BME-0049	NK4	aa	bb	321	87.2	21.88	151	566	1.03	0.14
BME-	Cambodg	ac	ab	315	83.2	22.19	139	556	0.99	0.14

0050										
BME-0052	Daizo	ac	ab	393	87.7	21.76	162	564	0.96	0.13
BMI-0053	LMP	ac	ab	390	91.7	23.33	147	562	1.07	0.15
BMI-0054	DMR	ac	ab	410	90.8	24.13	138	549	1.06	0.16
BMI-0055	LMO	ab	ab	439	90.9	25.68	147	566	1.19	0.17
BMI-0056	MY1(SL)	bc	ab	492	89	23.98	153	581	1.18	0.18
BMI-0057	PM(SL)	ac	ab	477	91.9	26.61	144	563	1.23	0.18
BMI-0058	BL23	ac	bb	491	92.9	30.74	151	559	1.4	0.23
BMI-0059	B24	ac	ab	472	82.5	29.07	148	562	1.4	0.23
BMI-0060	MU303	ac	ab	522	91.2	27.87	157	574	1.34	0.2

Table3.Cluster groups of Bivoltine silkworm accessions based on EST gene primers

Acc. Nos.	No. of acc.	Group	Alleles	Number of qualified parameters
BBE-0001, BBE-0003, BBE-0004, BBE-0006,BBE-0011, BBE-0012, BBE-0013, BBE-0014,BBE-0016, BBE-0017, BBE-0018, BBE-0019, BBE-0026, BBE-0028, BBE-0029, BBE-0030,BBE-0031, BBE-0038, BBE-0041, BBE-0042,BBI-0044, BBI-0047, BBI-0048, BBE-0051, BBI-0052, BBI-0054, BBI-0058, BBI-0061, BBI-0062, BBI-0064, BBI-0065, BBI-0066, BBI-0067, BBI-0068, BBI-0069, BBI-0070	36	High	aa, ab	Fecundity, Larval growth, cocoon weight, Shell weight and shell ratio
BBE-0007, BBE-0008, BBE-0009, BBE-0020, BBE-0021, BBE-0023, BBE-0027 , BBE-0032, BBE-0033, BBE-0034, BBE-0035, BBE-0036, BBE-0037, BBE-0040, BBE-0043, BBI-0053, BBI-0063, BBI-0071, BBI-0072, BBI-0073, BBI-0074, BBI-0075	22	Moderate	aa, ac	Fecundity, Larval growth, cocoon weight, Shell weight and shell ratio
BBE-0002, BBI-0005, BBE-0010, BBE-0015, BBE-0022, BBE-0024, BBE-0025, BBE-0039, BBI-0045, BBI-0046, BBE-0049, BBE-0050, BBI-0055, BBI-0056 ,BBI-0057, BBI-0059, BBI-0060	17	Low	aa, ac	Fecundity, Larval growth, cocoon weight, shell weight and shell ratio

Table 4.Cluster groups of Multivoltine silkworm accessions based on EST gene primers

Acc.Nos.	No. of acc.	Group	Alleles	Number of qualified parameters
BMI-0001, BMI-0007, BME-0015, BMI-0019, BMI-0041, BMI-0042, BMI-0043, BME-0044, BMI-0045 , BME-0046, BME-0047, BME-0048, BME-0049, BMI-0061, BMI-0062, BMI-0065, BMI-0074	17	High	ac, bc	Fecundity, Larval growth, cocoon weight, Shell weight and shell ratio.
BMI-0016, BMI-0018, BME-0050, BME-0052, BMI-0053, BMI-0054, BMI-0055, BMI-0056, BMI-0057	9	Moderate	aa, ab	Fecundity, Larval growth, cocoon weight, Shell weight and shell ratio.
BMI-0017, BMI-0058, BMI-0059, BMI-0060	4	Low	ac, aa	Fecundity, Larval growth, cocoon weight, Shell weight and shell ratio.

Figures

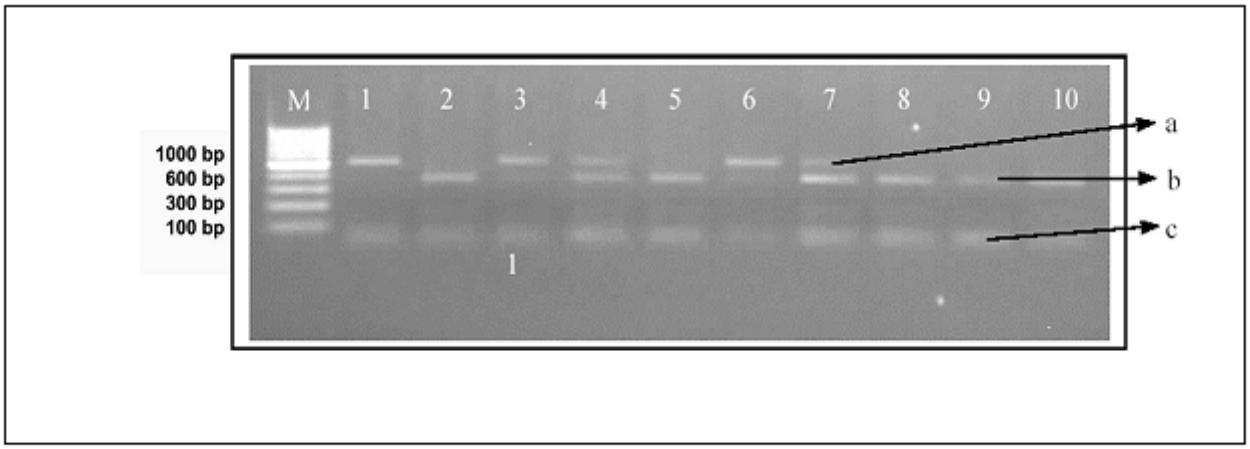
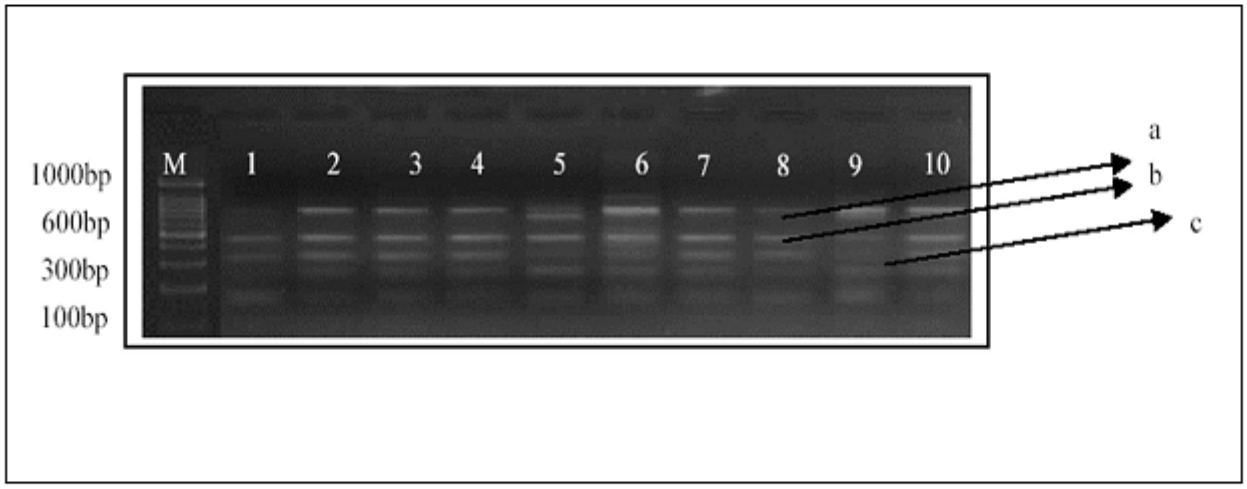


Fig.1: Restriction pattern profiles of Yolk protein gene among selected Bivoltine silkworm races

Figure 1

Restriction pattern profiles of Yolk protein gene among selected Bivoltine silworm races



Fig; 2 Restriction pattern profiles of PTTH gene among selected Bivoltine silkworm races

Figure 2

Restriction pattern profiles of PTTH gene among selected Bivoltine silworm races

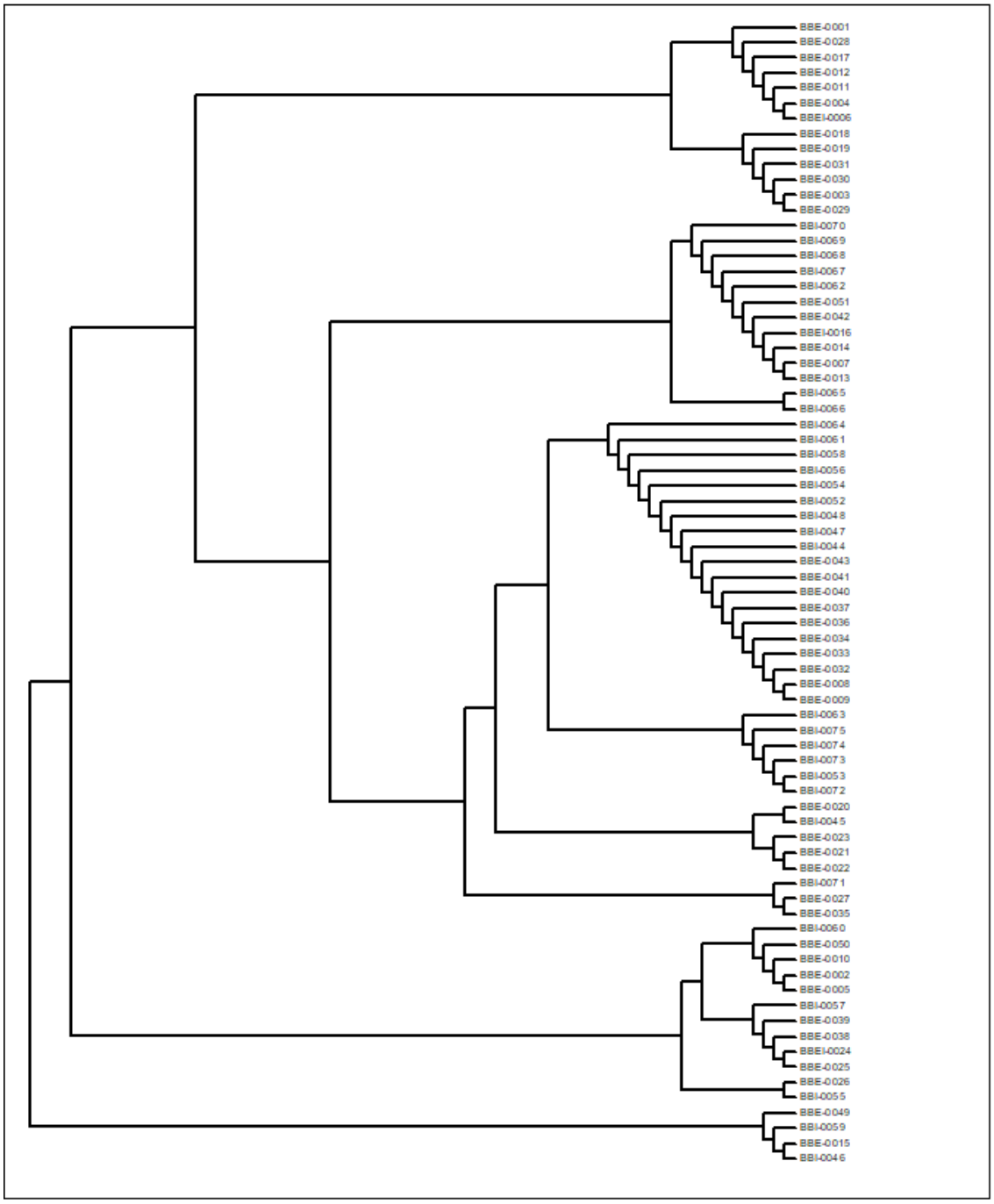


Fig 3 . Dendrogram – BV based on EST gene primers

Figure 3

Dendrogram - BV based on EST gene primers

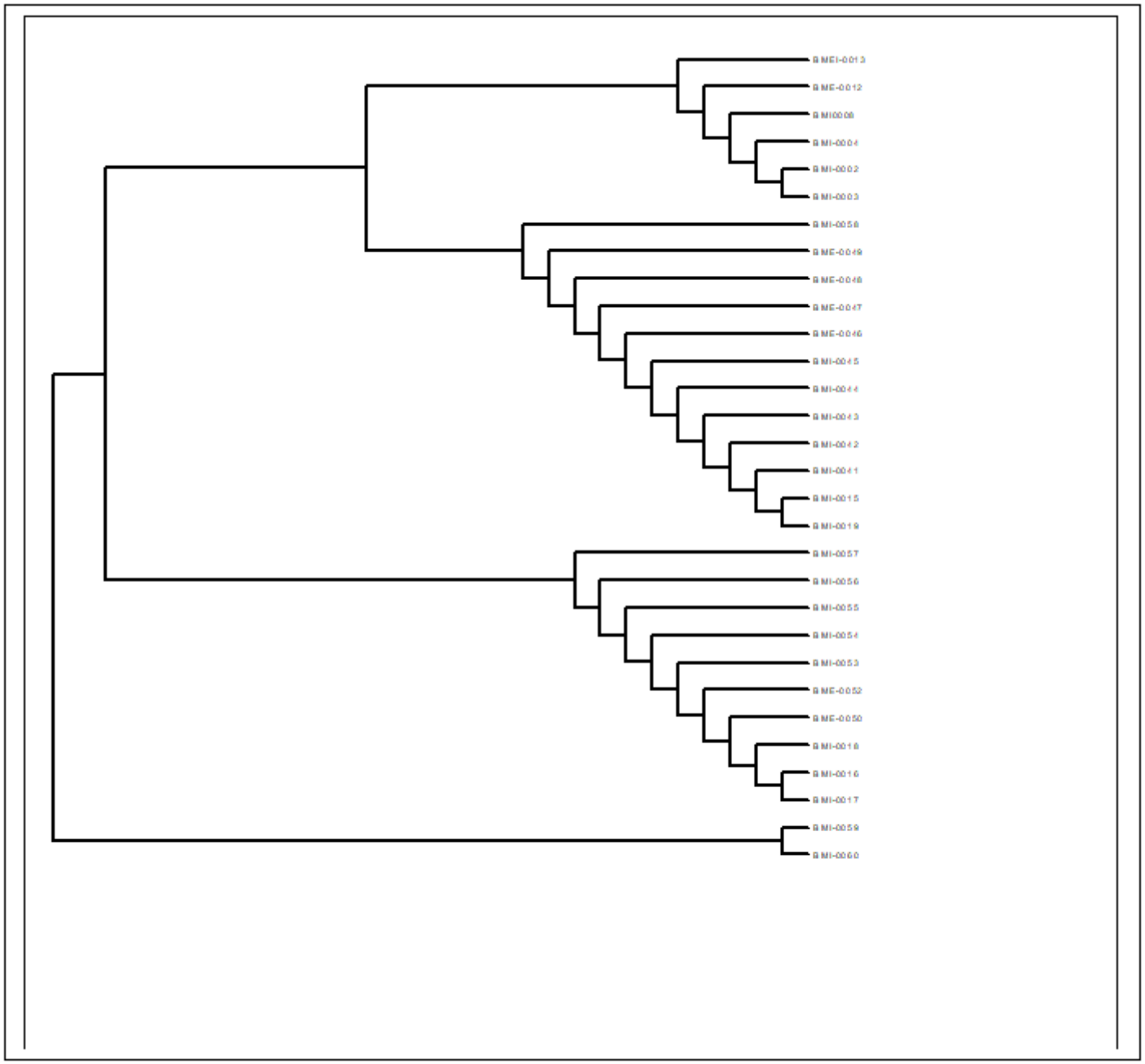


Fig 4. **Dendrogram – MV based on EST gene primers**

Figure 4

Dendrogram - MV based on EST gene primers