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Screening and classification of mulberry silkworm, *Bombyx mori* based on thermotolerance

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Abstract

The tropical climate prevailing in India adversely affects temperate bivoltine silkworm rearing and causes crop loss especially during summer. Identification of high temperature tolerant bivoltine breeds by screening for thermotolerance in the silkworm, *Bombyx mori* (Lepidoptera: Bombycidae) is an essential prerequisite for the development of thermotolerant bivoltine breeds / hybrids. Therefore, in this study, 20 silkworm breeds were reared at different temperatures (25 ± 1°C,32 ± 1°C, 34 ± 1°C and 36 ± 1°C) for 6 h every day from 3rd d of 5th instar to till spinning. Significant differences ($p < 0.01$) were found among all the rearing traits over temperature. Based on pupation percentage, SK4C and BHR3 were identified as thermotolerant bivoltine breeds. Hierarchical clustering analysis based on rearing traits at tested temperatures grouped 20 silkworm breeds in four clusters which included one cluster each of susceptible and tolerant, and two clusters of moderately tolerant silkworm breeds. This suggests that clustering based on rearing data at high temperatures by using Euclidean distance can be an effective approach in classifying the silkworm breeds on their thermotolerance capacity. The identified breeds would be used for development of thermo tolerant bivoltine silkworm breeds / hybrids.

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Introduction

The silkworm, *Bombyx mori* L. has a long history of domestication as an economically important insect. Intensive and careful domestication over centuries has apparently deprived this commercial insect of the opportunity to acquire thermotolerance (Kumari *et al*., 2011). This vulnerability is more pronounced in bivoltine breeds compared to multivoltine breeds. Thus, among many factors responsible for poor performance of the bivoltine breeds under tropical conditions, the major one is lack of thermotolerance (Kumari *et al*., 2011). Many quantitative characters decline sharply at higher temperature (Krishnaswami *et al*., 1973). Therefore, high temperature is one of the main factors for bivoltine crop failure in the tropics (Kumar *et al*., 2001).

Sericulturists from the tropical countries like India relay on climatic conditions prevailing in the field and restrict themselves to rear silkworm breeds suitable for

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high temperature conditions. This prompts the commercial exploitation of multivoltine x bivoltine hybrids in India, as they are hardy and ably to survive and reproduce under fluctuating climatic conditions (Kumari *et al*., 2011). The quantity of silk produced by multivoltine x bivoltine hybrids meet the domestic needs but the quality of silk is low compared to bivoltine silk of international grade. High quality, internationally recognised silk production can be achieved only by rearing bivoltine breeds (Begum *et al*., 2001). To overcome this drawback, compatible bivoltine hybrids for rearing under tropical conditions were developed (Datta *et al*., 2001; Kumar *et al*., 2002, 2006; Lakshmi *et al*., 2011) and selected for rearing in field conditions. However, the attempts to spread bivoltine silkworm breeds / hybrids throughout the sericulture belt of India resulted in extensive crop loss, especially in the hot and humid climatic conditions of tropics (Chavadi *et al*., 2006). These productive hybrids were vulnerable to varied environmental conditions, as they originated from temperate regions (Kumari *et al*., 2011).

Performance of each organism is determined and affected by genotype, environment and interactions between them. Therefore, individuals with high genetic potential must be selected in order to improve the quantity and quality of silk production, besides improving environmental conditions (Nezhad *et al*., 2010). Knowledge of genetic diversity and genetic relationships between different pure lines can be a valuable factor to improve silkworm breeding strategies. The selection of divergent parents present in the breeding stocks forms the base for any breeding program (Vijayan *et al*., 2010). Genetic diversity in silkworm is generally measured based on the phenotypic traits that have been used for differentiation of silkworm genotypes because most of the traits are either qualitative or quantitative (Bindroo and Moorthy, 2014). However, molecular markers are other types to estimate genetic diversity, which can provide a rapid means of analysis of genetic diversity in germplasm (Bindroo and Moorthy, 2014). Anyhow, many important phenotypic traits in silkworms decline above 28°C (Krishnaswami *et al*., 1973). Therefore, the estimation of phenotypic changes at high temperature is considered of great importance for sustainable progress in bivoltine breeding (Kumari *et al*., 2011).

Euclidean or straight-line measure of distance is the most commonly used statistics for estimating genetic distance between individuals by morphological data (Mohammadis and Prasanna, 2003). Numerous studies represent the hierarchical clustering of different silkworm breeds based on their performances with respect to quantitative traits. The economically important quantitative traits like fecundity, larval weight, yield/ 10000 larvae by weight, yield/ 10000 by number, shell weight, cocoon weight, shell %, filament length, filament size, neatness, etc., have been traditionally used for characterization / diversity studies (Thangavelu *et al*., 2000). Applying D^2 (Mahalonobis' distance) statistics, Kumaresan *et al*. (2007) categorized 58 multivoltine silkworm genotypes traits into nine clusters using ten economic. Nezhad *et al*. (2010) classified all 51 pure lines of Iran silkworm germplasm based on larval traits into two distinct groups using Ward's method and UPGMA (Unweighted Pair-group Method using Arithmetic mean) method. Pal and Moorthy (2011) studied variability in larval and cocoon traits in 19 bivoltine silkworm breeds and reported their division into three distinct clusters. Such classification is also important for breeding work aimed at improving adaptability and other yield components. The aim of this study was to identify high temperature tolerant bivoltine breed and an attempt was also made to classify 20 silkworm breeds based on the rearing performance under high temperature conditions using Euclidean distance.

Materials and Methods

Silkworm breeds and thermal treatment

Twenty silkworm breeds consisting of two multivoltine (Nistari and Cambodge) and eighteen bivoltine breeds (CSR2, CSR17, S5, D13, 5HT, 8HT, BHR2, BHR3, D6(P), SK3, SK4, D6(P)N, SK4C, B37, S38, NN6D, ATR16, ATR29) were used for the study. Multivoltine breeds were considered as reference to compare with the performance of bivoltine breeds. Silkworm rearing was conducted following the standard method under recommended temperature and humidity conditions till 2^{nd} d of 5^{th} instar as suggested by Krishnaswami *et al*. (1973). With regard to thermal exposure, the 3^{rd} d 5^{th} instar larvae were reared in a SERICATRON (Environment chamber with temperature and humidity control) at three different temperatures *viz*., 32 ± 1°C, 34 ± 1°C and 36 ± 1°C with an exposure duration of six h (10:00-16:00) per d. Fresh mulberry leaves were given twice a day and silkworm rearing was continued as suggested by Kato *et al*. (1989) and Kumar *et al*. (2001). The larvae reared at 25 ± 1°C under normal conditions were considered as control. The experiment was performed in triplicate for each temperature treatment with 100 larvae per replicate. The ripened larvae were mounted on plastic mountages. After 48 h of mounting, when the larvae formed hammock, the mountages were turned upside down. Cocoon harvesting was carried out on the 7th d of spinning. Cocoons were deflossed and the defective ones were sorted out. Assessment of all the breeds was carried out on the same day of development.

Rearing traits

During silkworm rearing, data on six quantitative traits were collected separately for control and each treatment batch (32 ± 1°C, 34 ± 1°C and 36 ± 1°C). The traits like pupation percentage (%), larval weight (g), cocoon yield for 10,000 larvae by weight (kg), cocoon weight (g), shell weight (g) and shell percent (%) were noted. The overall mean of each trait was calculated for each breed at all four temperatures. Pupation percentage was estimated as live pupa present inside the cocoon during metamorphosis of larva into pupa expressed as a percent. The percentage change in the performance of the breeds in treated

Table 1. Rearing performances of twenty silkworm breeds at 25 ± 1°C

Breed	Pupation percentage (%)	Larval wt. (g)	Cocoon yield / 10,000 by wt. (kg)	Cocoon wt. (g)	Shell wt. (g)	Shell percent (%)
ATR 16	89.24	39.305	12.83	1.575	0.307	19.511
ATR 29	92.13	37.1	13.765	1.618	0.329	20.338
B37	86.89	38.502	10.532	1.526	0.291	19.056
BHR 2	90.62	40.105	15.284	1.67	0.317	18.981
BHR 3	92.25	34.328	12.638	1.525	0.297	19.455
CSR2	92.76	46.41	15.498	1.76	0.401	22.797
CSR17	93.79	43.093	16.948	1.762	0.4	22.698
D13	90.46	42.448	15.22	1.715	0.367	21.399
D6(P)	87.81	36.815	14.087	1.618	0.333	20.584
D6(P)N	91.93	33.572	14.127	1.539	0.298	19.368
NN6D	92.91	36.143	12.279	1.557	0.297	19.054
S38	92.34	39.658	13.752	1.565	0.293	18.697
S5	92.40	44.107	15.757	1.727	0.404	23.379
SK3	89.92	33.262	14.536	1.64	0.34	20.717
SK4	90.95	36.953	13.82	1.572	0.307	19.504
SK4C	92.76	34.42	14.362	1.627	0.334	20.51
5HT	91.93	41.365	15.127	1.683	0.353	21
8HT	93.96	43.215	15.933	1.912	0.43	22.501
Nistari	94.95	23.28	12.689	1.182	0.168	14.255
Cambodge	94.19	25.657	9.641	1.23	0.179	14.544
Mean	91.66	37.487	13.941	1.600	0.322	19.917
Minimum	86.89	23.280	9.641	1.182	0.168	14.255
Maximum	94.95	46.410	16.948	1.912	0.430	23.379
Standard deviation	20.49	8.382	3.117	0.358	0.072	4.454

over control with respect to their pupation percentage was calculated. This genetic trait was considered as the measure of index of thermotolerance in silkworm. Percentage change / reduction in the pupation percentage was calculated as follows (Kumari *et al*., 2011)

Statistical analysis

Hierarchical clustering was done by using the rearing data of twenty silkworm breeds at four temperatures. Euclidean distance was calculated as a measure of genetic diversity by using the rearing parameters of the twenty silkworm breeds at 25 ± 1°C, 32 ± 1°C, 34 ± 1°C and 36 ± 1°C. Based on Euclidean distance dendrogram was constructed using Ward's method with a bootstrap value of 10000. Analysis of variance was performed on pupation percentage, larval weight, cocoon yield for 10,000 larvae by weight, cocoon weight, shell weight and shell percent of silkworm breeds using temperature as factor. All the analysis was done with SPSS software package.

Results

Morphological variations

Table 2. Rearing performances of twenty silkworm breeds at 32 ± 1°C

Breed	Pupation percentage (%)	Larval wt. (g)	Cocoon yield / 10,000 by wt. (kg)	Cocoon wt. (g)	Shell wt. (g)	Shell percent (%)
ATR 16	66.667	32.573	9.225	1.351	0.226	16.734
ATR 29	69.333	33.743	9.022	1.294	0.245	18.977
B37	53.667	27.947	6.62	1.252	0.209	16.673
BHR 2	67.667	30.977	8.625	1.367	0.223	16.338
BHR 3	71.083	28.287	8.471	1.219	0.22	18.084
CSR2	32.333	38.28	5.098	1.577	0.337	21.352
CSR17	56.333	39.683	9.039	1.605	0.331	20.635
D13	59.667	34.433	8.408	1.473	0.289	19.612
D6(P)	49.697	32.84	6.645	1.332	0.25	18.759
D6(P)N	69.667	31.117	8.62	1.249	0.237	18.991
NN6D	72.727	28.903	8.588	1.368	0.223	16.294
S38	64	31.603	8.717	1.282	0.233	18.187
S5	70.667	40.087	10.217	1.564	0.322	20.57
SK3	69.231	31.27	9.81	1.414	0.28	19.773
SK4	54.016	31.038	6.832	1.312	0.224	17.047
SK4C	73	31.667	9.04	1.239	0.237	19.108
5HT	65.556	36.805	9.705	1.498	0.295	19.76
8HT	64	34.907	9.378	1.47	0.309	21.028
Nistari	86.333	21.697	9.336	1.081	0.142	13.14
Cambodge	83.7	23.087	9.185	1.097	0.143	12.992
Mean	64.967	32.047	8.529	1.352	0.249	18.203
Minimum	32.333	21.697	5.098	1.081	0.142	12.992
Maximum	86.333	40.087	10.217	1.605	0.337	21.352
Standard deviation	14.527	7.166	1.907	0.302	0.056	4.070

Eighteen bivoltine and two multivoltine breeds were considered for the study. Variations in the larval marking, cocoon shape and cocoon colour were observed in experimental breeds. Among multivoltines, Nistari and Cambodge spun yellow coloured and oval elongate cocoons. Nistari larvae are marked and Cambodge larvae are plain. Among bivoltines, ATR16, B37, CSR2, CSR17, 8HT, S5, NN6D, S38 and SK3 larvae were plain and spun oval shaped cocoons. Other bivoltines *viz*., ATR29, BHR2, BHR3, 5HT, D13, D6(P), D6(P)N, SK4 and SK4C larvae were marked and spun dumbbell shaped cocoons. All the bivoltines spun white coloured cocoons.

Overall performance of silkworm breeds under normal and high temperature treatments

Six important rearing traits *viz.,* pupation percentage (%), larval weight (g), cocoon yield, cocoon weight (g), shell weight (g) and shell percent (%) were analysed in twenty silkworm breeds by exposing them to temperatures at 32 ± 1°C, 34 ± 1°C and 36 ± 1°C (Treated) against 25°C ± 1°C (Control). Differences in the studied parameters over temperature suggested the response of each breed to high temperature. Generally the control larvae performed better than the treated. In order to know the breed that is performing

Table 3. Rearing performances of twenty silkworm breeds at 34 ± 1°C

Breed	Pupation percentage (%)	Larval wt. (g)	Cocoon yield / 10,000 by wt. (kg)	Cocoon wt. (g)	Shell wt. (g)	Shell percent (%)
ATR 16	36.959	28.317	4.716	1.291	0.223	17.3
ATR 29	45.045	28.743	5.429	1.223	0.207	16.915
B37	22.333	27.392	2.631	1.245	0.202	16.236
BHR 2	53.246	29.902	6.439	1.308	0.194	14.835
BHR 3	68.174	27.853	8.287	1.206	0.208	17.271
CSR2	30.473	36.117	4.229	1.394	0.267	19.124
CSR17	53.333	34.82	7.978	1.496	0.291	19.478
D13	38.931	32.03	5.383	1.406	0.268	19.044
D6(P)	40.271	27.975	4.731	1.269	0.221	17.44
D6(P)N	57.581	25.408	6.299	1.24	0.204	16.483
NN6D	50.15	27.743	5.862	1.217	0.187	15.37
S38	43.667	30.382	5.236	1.273	0.205	16.1
S5	58.476	34.503	8.583	1.445	0.272	18.855
SK3	33.675	30.437	4	1.375	0.261	19.014
SK4	50.833	29.902	6.065	1.301	0.203	15.588
SK4C	68	29.395	7.649	1.227	0.223	18.159
5HT	55.311	33.57	7.721	1.457	0.277	19.021
8HT	57.611	32.178	8.146	1.425	0.267	18.756
Nistari	85.333	21.407	9.104	1.04	0.133	12.803
Cambodge	80.387	20.313	8.313	1.034	0.132	12.716
Mean	51.489	29.419	6.340	1.294	0.222	17.025
Minimum	22.333	20.313	2.631	1.034	0.132	12.716
Maximum	85.333	36.117	9.104	1.496	0.291	19.478
Standard deviation	11.513	6.578	1.418	0.289	0.050	3.807

Table 4. Rearing performances of twenty silkworm breeds at 36 ± 1°C

Breed	Pupation percentage (%)	Larval wt. (g)	Cocoon yield / 10,000 by wt. (kg)	Cocoon wt. (g)	Shell wt. (g)	Shell percent (%)
ATR 16	19.167	20.937	2.213	1.236	0.189	15.265
ATR 29	40.366	24.67	4.315	1.129	0.186	16.454
B37	12.217	23.293	1.343	1.109	0.173	15.574
BHR 2	43.833	26.635	5.11	1.297	0.188	14.51
BHR 3	60.667	26.153	7.421	1.194	0.196	16.396
CSR2	12.497	32.738	1.585	1.308	0.239	18.245
CSR17	27.993	31.93	3.968	1.417	0.259	18.259
D13	18.537	20.298	2.271	1.258	0.227	18.063
D6(P)	28.13	26.548	3.419	1.216	0.203	16.731
D6(P)N	44.38	22.093	4.842	1.116	0.171	15.365
NN6D	44.149	21.22	4.764	1.17	0.178	15.188
S38	22.667	26.675	2.602	1.154	0.181	15.723
S5	36.414	32.78	5.096	1.391	0.253	18.208
SK3	33.208	28.922	3.743	1.335	0.246	18.402
SK4	38.532	27.085	4.532	1.295	0.197	15.235
SK4C	60.995	27.828	6.693	1.219	0.213	17.474
5HT	28.405	30.017	3.799	1.342	0.244	18.19
8HT	40.5	29.833	5.304	1.307	0.239	18.249
Nistari	80.667	20.672	8.39	1.021	0.123	12.015
Cambodge	75.833	19.072	7.826	0.965	0.114	11.777
Mean	38.388	25.970	4.462	1.224	0.201	16.266
Minimum	12.217	19.072	1.343	0.965	0.114	11.777
Maximum	80.667	32.780	8.390	1.417	0.259	18.402
Standard deviation	8.584	5.807	0.998	0.274	0.045	3.637

better in tested temperatures, the overall mean of particular trait of a breed at all temperatures (25°C ± 1°C, 32 ± 1°C, 34 ± 1°C and 36 ± 1°C) was considered. The performance of the breeds under normal and treated temperatures and their overall averages are presented in Tables 1-5. Among bivoltines, average pupation percentage (indicator of thermotolerance) was high in SK4C (73.34%) followed by BHR3 (73.05%) and low in CSR2 (42.02%) followed by B37 (43.53%) with 86.82% and 83.53% for Nistari and Cambodge, respectively, which were used as reference for thermotolerance. The rearing traits studied were low in multivoltines than bivoltines; therefore the comparison has been made only among bivoltines. The overall mean for weight of ten larvae was highest in CSR2 (38.39 g) and lowest in D6(P)N (28.05 g) followed by NN6D (28.5 g). Highest average cocoon yield per 10,000 larvae by weight was demonstrated in S5 (9.91 kg) followed by 8HT (9.69 kg) and lowest was noted in B37 (5.28 kg). The highest average cocoon weight and shell weight of 1.57 g and 0.32 g were observed in CSR17 and lowest of 1.28 g and 0.22 g in B37. Average shell percent was highest in CSR2 (20.38%) and lowest in BHR2 (16.17%) followed by NN6D (16.48%). Significant differences were noticed in all the studied rearing traits of silkworm breeds with regard to temperature (Table 6).

Table 5. Overall rearing performances of twenty silkworm breeds at four temperatures

Breed	Pupation %	Larval wt. (g)	Cocoon yield / 10,000 by wt. (kg)	Cocoon wt. (g)	Shell wt. (g)	Shell percent (%)
ATR16	53.01	30.28	7.25	1.36	0.236	17.20
ATR29	61.72	31.06	8.13	1.32	0.242	18.17
B37	43.53	29.28	5.28	1.28	0.219	16.88
BHR2	63.84	31.90	8.86	1.41	0.231	16.17
BHR3	73.05	29.16	9.20	1.29	0.230	17.80
CSR2	42.02	38.39	6.60	1.51	0.311	20.38
CSR17	57.86	37.38	9.48	1.57	0.320	20.27
D13	51.90	32.30	7.82	1.46	0.288	19.53
D6(P)	51.48	31.04	7.22	1.36	0.252	18.38
D6(P)N	65.89	28.05	8.47	1.29	0.228	17.55
NN6D	64.99	28.50	7.87	1.33	0.221	16.48
S38	55.67	32.08	7.58	1.32	0.228	17.18
S5	64.49	37.87	9.91	1.53	0.313	20.25
SK3	56.51	30.97	8.02	1.44	0.282	19.48
SK4	58.58	31.24	7.81	1.37	0.233	16.84
SK4C	73.34	30.83	9.44	1.33	0.252	18.81
5HT	60.30	35.44	9.09	1.50	0.292	19.49
8HT	64.02	35.03	9.69	1.53	0.311	20.13
Nistari	86.82	21.76	9.88	1.08	0.142	13.05
Cambodge	83.53	22.03	8.74	1.08	0.142	13.01

Table 6. ANOVA on rearing traits with temperature as a factor

Rearing trait	df	Mean square	F	P
Pupation percentage	3	10374.84	53.81	0.001
Larval weight	3	471.75	20.63	0.001
Cocoon yield /10000 larvae	3	336.32	110.91	0.001
Cocoon weight	3	0.54	27.09	0.001
Shell weight	3	0.06	20.49	0.001
Shell percent	3	50.58	10.60	0.001

Percentage reduction in pupation percentage of treated groups over control

The negative sign in the percent changes over control in treated groups indicate a reduction over control and the value near to zero suggests a better performance of the breed. At all the treated temperatures, among multivoltines, the highest reduction in pupation percentage was in Cambodge against Nistari (Table 7). At 32 ± 1°C, 34 ± 1°C and 36 ± 1°C reduction in pupation percentage in Cambodge was 11.14%, 14.66% and 19.5% over control, respectively. Similarly, reduction in pupation percentage in Nistari was 9.08%, 10.13% and 15.04% at 32 ± 1°C, 34 ± 1°C and 36 ± 1°C over control, respectively. Among bivoltines, highest reduction of 65.15% in pupation percentage was demonstrated in CSR2 under 32 ± 1°C and lowest reduction of 21.31% in SK4C. At 34 ± 1°C, B37 was highly affected by highest reduction in pupation percentage of 74% followed by CSR2 with 67.15%. At 36 ± 1°C, CSR2 had highest reduction in pupation percentage of 86.53% followed by B37 with 85.78%. BHR3 was more stable at 34 ± 1°C and 36 ± 1°C with lowest reduction in pupation percentage of 26.1% and 34.24%, respectively followed by SK4C with 26.7% and 35.76%, respectively (Table 7).

Table 7. Reduction in pupation percentage in silkworm breeds at high temperatures over control

Breeds	Reduction in 32°C	Reduction in 34°C	Reduction in 36°C
ATR 16	-25.30	-58.59	-78.52
ATR 29	-24.75	-51.11	-56.19
B37	-37.52	-74.00	-85.78
BHR 2	-25.33	-41.25	-51.63
BHR 3	-22.95	-26.10	-34.24
CSR2	-65.15	-67.15	-86.53
CSR17	-39.94	-43.14	-70.15
D13	-34.05	-56.97	-79.51
D6(P)	-43.40	-54.14	-67.97
D6(P)N	-24.22	-37.37	-51.73
NN6D	-21.73	-46.03	-52.49
S5	-23.52	-36.71	-60.59
S38	-30.70	-52.71	-75.45
SK 3	-23.01	-62.55	-63.07
SK 4	-40.61	-44.11	-57.64
SK4C	-21.31	-26.70	-35.76
8HT	-31.89	-38.69	-56.90
5HT	-28.69	-39.84	-69.10
Nistari	-9.08	-10.13	-15.04
Cambodge	-11.14	-14.66	-19.5

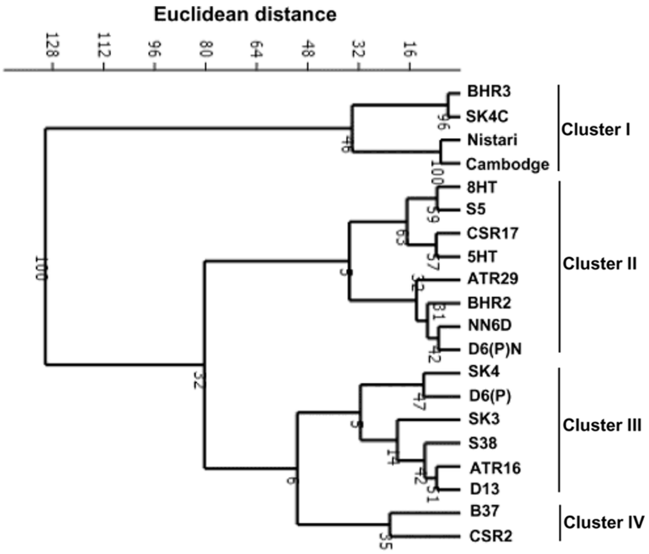

Fig. 1. Phylogenetic relationship of silkworm breeds based on six rearing traits at four different temperature regimes.

Cluster analysis

Pairwise Euclidean distance was estimated between silkworm breeds based on their rearing traits under normal and treated temperatures. Using Euclidean distance as measure of diversity the dendrogram was constructed by Ward's method. Twenty silkworm breeds were broadly divided into four clusters. Cluster I contained SK4C, BHR3, Nistari and Cambodge, which were thermo tolerant with pupation percentage of >70%. Cluster II was a large cluster with 8 silkworm breeds *viz*., 8HT, S5, CSR17, 5HT, ATR29, BHR2, NN6D and D6(P)N with pupation percentage of more than 60% and less than 70% except CSR17, which has a pupation percentage of 57.86%. Cluster III had 6 silkworm breeds namely, SK4, D6(P), SK3, S38, ATR16 and D13, and the pupation percentage of the members ranged between 51.48 and 58.58%. Cluster IV had 2 silkworm breeds namely, B37 and CSR2 with lowest pupation percentage of 43.53% and 42.02%, respectively (Fig. 1).

Genetic distance

The genetic distance ranged between 5.49 (between SK4C and BHR3) and 109.84 (between Nistari and CSR2) with an average of 36.74. In cluster I, four silkworm breeds were classified with an average genetic distance of 29.8 with 37.51 being highest between Nistari and SK4C and 5.49 being lowest between SK4C and BHR3. Similarly, in cluster II, eight silkworm breeds were divided with an average genetic distance of 17.63 with 30.92 being highest between CSR17 and NN6D and 9.78 being lowest between D6(P)N and NN6D. Silkworm breeds in cluster III were differentiated with an average genetic distance of 19.14 ranging from 10.58 between ATR16 and D13 to 28.21 between ATR16 and SK4. The two silkworm breeds, CSR2 and B37 formed cluster IV, which were distanced by a genetic distance of 31.38 (Supplementary material 1).

Discussion

On increase in the rearing temperature above 25°C, all the breeds showed a decline in rearing parameters which is similar to that reported by Kumar *et al*. (2002). The decline in the rearing parameters with increase in temperature was probably due to the low feeding activity of the silkworms during high temperature.

As a fact, high temperature reduces the moisture content in the leaf through evaporation thereby drying the leaf sooner and therefore not enabling to feed by silkworms (Kumar *et al*., 2012). Water is an essential requirement for metabolic activity and optimum growth of silkworm. At higher temperature, evapo-transpiration at body surface and respiratory epithelium of tracheal system significantly increases. The problem of water balance in silkworm at ambient temperature is further complicated by poor moisture content of the leaf, which finally affects the growth and productivity of silkworm (Rahmathulla, 2003). Moreover, heat stress affects biologically important molecules like DNA, RNA, lipids and halts normal protein synthesis mechanism. It even causes unfolding of the typical folded functional cellular proteins making it non-functional (Feder, 1996). All these changes tend to increase the vulnerability of the silkworm to other biological processes resulting in the decline in its performance (Kumari *et al*., 2011).

Abiotic and biotic factors have a considerable impact on the success of sericulture industry and thus they are of great importance (Kumar *et al*., 2012). Among the abiotic factors, temperature plays a key role in growth and productivity of the silkworm, as it is a poikilothermic insect (Benchamin and Jolly, 1986). It is a well known fact that the late age silkworms prefer relatively lower temperature than young age and fluctuation of temperature during different stages of larval development is more favourable for growth and development of larvae than constant temperature. There is ample literature stating that good quality cocoons are produced within a temperature range of 22-27°C and above these levels the cocoon quality becomes poorer (Krishnaswami *et al*., 1973) indicating that studies on cocoon traits is basis for appropriate selection for required environment. The effect of high temperature in terms of cocoon crop depends on several factors that operate within and outside the body of the silkworm.

It is a difficult task to assess thermotolerance trait in silkworms as it is governed by genetic and environmental factors (Kumar *et al*., 2012). The effect of temperature higher than 30°C on silkworm larvae was reported earlier by Shirota (1992). Tazima and Ohnuma (1995) further confirmed the genetical nature of thermotolerance by selection based on pupation rate of silkworms reared under high temperature conditions in 5th instar. The experiments conducted by Kato *et al*. (1989) showed that the thermotolerance character in silkworm is heritable. Therefore, it is important to understand the performance of the breeds under a set of different high temperatures. In this study thermotolerance in twenty silkworm breeds was assessed under three high temperatures *viz*. 32 ± 1°C, 34 ± 1°C and 36 ± 1°C.

One of the objectives of the silkworm breeder is to recommend stable breeds to the farmers for rearing under different environmental conditions. Silkworm breeds reared over a series of environments exhibiting less variation are considered stable. According to Allard and Bradshaw (1964), performance of the breed itself in a given environment indicates its superiority. As the majority of the economically important genetic traits of silkworms are qualitative in nature and phenotypic expression is greatly influenced by environmental factors such as temperature, relative humidity, light and nutrition (Pillai and Krishnaswami, 1980, Ramesha *et al*., 2010; Zhang *et al*., 2002), it is essential to measure the degree of phenotypic difference of the economical traits under varied environmental conditions to understand the genetic steadiness. In the present study, the phenotypic expressions of six traits of economic importance were studied in twenty silkworm breeds exposed to different temperatures *viz*. 32 ± 1°C, 34 ± 1°C and 36 ± 1°C. The variations observed in the phenotypic manifestation for the traits measured can be attributed to the genetic constitution of the breeds and their degree of expression to the conditions to which they are exposed during their rearing. Such variations in the manifestation of phenotypic traits of the breeds studied can be ascribed to the influence of environmental conditions. Variable gene frequencies at different loci make them to respond differently. The results of the present study are in line with the findings of Kumar *et al*. (2012).

The multivoltine breeds were more tolerant to high temperature than the bivoltines. It is well established that multivoltine breeds originated and reared in tropical countries are tolerate slightly higher temperature, as are cross breeds that have been evolved for a tropical climate (Ramesha *et al*., 2009). In the present investigation, the productive bivoltine breeds like CSR2, CSR17 and SK3 showed low pupation percentage at high temperatures. This was also noticed by Kumar *et al*. (2002) and Kato *et al*. (1989) by observing higher survival in the hybrids than the pure races under high temperature conditions. The bivoltine breeds exposed to high temperature revealed a drastic reduction in the pupation rate and cocoon traits suggesting that bivoltines are more prone to high temperature.

In this study, the highest overall means of larval weight and shell percent were noticed in CSR2, while, the highest overall mean of cocoon and shell weights were observed in

CSR17. Highest cocoon yield per 10,000 larvae by weight was demonstrated in S5. The current study observed SK4C (73.34%) and BHR3 (73.05%) as two bivoltine breeds tolerant to high temperatures as they showed highest pupation percentage at all the temperatures tested. These breeds not only performed well under different high temperatures but also were stable with low percentage reduction in pupation rate over their controls indicating their ability to withstand high temperatures.

Classification of silkworm breeds based on six rearing parameters at high temperatures

In the current study, the genetic relationship between 20 silkworm breeds was determined by using rearing data of silkworm breeds at four different temperatures. Based on the data Euclidean distance was estimated and dendrogram was constructed using Euclidean distance and Ward's method. The lowest genetic distance was 5.49 between SK4C and BHR3. These two breeds had the highest pupation percentage at each high temperature as well as overall temperatures tested. They were developed by screening each generation at high temperature conditions and selection criteria for further generation was based on heat-tolerant traits (Moorthy *et al*., 2007). The highest genetic distance was 109.84 between Nistari and CSR2 that had contrasting characters with respect to cocoon traits and thermotolerance as revealed by this study. Nistari and CSR2 had highest and lowest pupation percentage at four tested temperatures, respectively, which probably might be the factor that separated Nistari and CSR2 with high genetic distance.

Cluster analysis revealed that SK4C and BHR3 (>70%) breeds were grouped together with the multivoltines (>80%), which had highest pupation percentage at all the tested high temperatures including overall means. Similarly, CSR2 (42.02%) and B37 (43.53%) showed an overall mean of lowest pupation percentage and high percentage reduction in pupation rate over control. Thus, CSR2 and B37 form a cluster. The clustering pattern and genetic distance suggests that the breeds were primary grouped based on their ability of thermotolerance epresented by pupation percentage. However, in cluster II, the silkworm breeds had pupation percentage between 60-70% except for CSR17, which had a pupation percentage of 57.86%, typical for cluster III (i.e. 50-60%). This clearly indicates the role of rearing traits other than pupation percentage in classification of silkworm breeds because CSR17 though had a pupation percentage of cluster III members; it shared very nearer values for other rearing traits with the members of cluster II. Therefore, it was grouped in cluster II.

In this study, the breeds reared in Southern India (S38 and D13) clustered with the breeds reared in Northern India (SK4, D6(P), SK3 and ATR16) to form cluster III. According to Chatterjee and Datta (1992) for the silkworm, domestication has played a major role in genetic diversification. As sericultural regions of the world have different climatic conditions, physiological diversification has also been influenced by agro-climatic factors. Thus, given geographic isolation and limited cultural exchange, some breeds of South India and North India inclusive in cluster III may have acquired similar genetics due to similar selection pressures. The above results revealed that the inclusion of silkworm breeds of the same region in different clusters clearly indicate the presence of considerable genetic diversity among the population used in this study. All these cases indicate that clustering on the basis of estimates of phenotype does not always reflect geographical distance, as has also been pointed out by researchers working on the clustering of plant materials (Harlan, 1971). Likewise Spagnoletti and Qualset (1987) pointed out that geographical position does not correspond with the phenotypic grouping for the origin of spike characteristics in Duram wheat. Kumaresan *et al*. (2007) reported the inclusion of silkworm genotypes of same origin in different clusters by applying D^2 (Mahalonobis' distance) statistics on 10 economical traits of 58 polyvoltine strains. In contrast, Nezhad *et al*. (2010) reported that 51 pure lines of Iran silkworm germplasm were clustered into different groups according to the geographic areas that were initially observed by using data on larval traits. However, in this study, clustering on the basis of rearing parameters at high temperature conditions separated most tolerant and most susceptible silkworm breeds in distinct clusters but failed to separate the breeds with moderate thermotolerance into distinct clusters. This is evident from the breed CSR17 (57.86%), which was grouped in cluster II but had a pupation percentage of members of cluster III (60-70%). This indicates that more rearing and economically important traits of silkworm have to be assessed at different high temperatures to classify silkworm breeds with moderate thermotolerance into distinct clusters.

Current study reveals a negative relationship between the tested temperature and the rearing parameters of silkworm breeds. SK4C and BHR3 with high pupation percentage at three different high temperatures and less percentage reduction in pupation percentage over controls, were identified as high temperature tolerant and stable bivoltine silkworm breeds over a range of 32 to 36°C. So far in silkworm, phenotypic studies to identify high temperature tolerant bivoltine breeds were carried out with a single high temperature treatment. In the present report, the high temperature tolerant bivoltine breeds were identified based on their rearing performance at three treated temperatures, which a breeder can rely on. Therefore, these bivoltine breeds can be recommended as potential breeding material for the development of thermotolerant silkworm breeds/ hybrids. Furthermore, this study also suggests that Euclidean distance estimated on the basis of rearing data of silkworm breeds under high temperature conditions can be an effective approach in classifying the silkworm breeds based on their capacity of thermotolerance. Based on the Euclidean distance 20 silkworm breeds were divided into 4 clusters, which represented one each of thermotolerant, thermo susceptible and two clusters of moderately tolerant silkworm breeds. The utilization of breeds from different clusters for breeding will increase heterosis. These results will enhance the knowledge of the silkworm breeders in developing thermotolerant breeds or hybrids with maximum heterosis.

References

Allard RB, Bradshaw AO (1964) Implications of genotype environment interaction in applied plant breeding. Crop Science 4, 503-506.

Begum AN, Basavaraja HK, Rekha M, Ahsan MM, Datta RK (2001) Breeding resource material for the evolution of thermo-tolerant breeds. Int J Ind Entomol 2 (2), 111-117.

Benchamin KV, Jolly MS (1986) Principles of silkworm rearing; in: Proceedings of seminar on problems and prospects of sericulture. Mahalingam S (eds.), pp. 63-106, Vellore, India.

Bindroo BB, Moorthy SM (2014) Genetic divergence, implication of diversity, and conservation of silkworm, *Bombyx mori*. International Journal of Biodiversity Article ID 564850, 15 pages.

Chatterjee SN, Datta RK (1992) Hierarchical clustering of 54 races and strain of mulberry silkworm, *Bombyx mori*: Significance of biochemical parameters. Theor Applications Genet 85, 394-402.

Chavadi VB, Aparna HS, Manjunatha HB (2006) Impact of heat shock on heat shock proteins expression, biological and commercial traits of *Bombyx mori*. Insect Science 13, 243-250.

Datta RK, Basavaraja HK, Mal Reddy N, Nirmal Kumar S, Suresh Kumar N, Ramesh Babu, Ahsan M, Jayaswal KP (2001) Breeding of new productive bivoltine hybrid, CSR12 x CSR6 of silkworm, *Bombyx mori* L. Int J Ind Entomol 3, 127-133.

Feder ME (1996) Ecological and evolutionary physiology of stress proteins and the stress response: the *Drosophila melanogaster* model; in: Animals and temperature: Phenotypic and evolutionary adaptation to temperature. Johnston IA, Bennett AF (eds.), pp. 79-102 Cambridge University Press.

Harlan JR (1971) Agricultural origins: centres and non-centers. Science 174, 468-474.

Kato M, Nagayasu K, Ninagi O, Hara W, Watanabe A (1989) Studies on resistance of the silkworm, *Bombyx mori* L. for high temperature. Proceedings of the 6th International Congress of SABRAO (II), India, 953-956.

Krishnaswami S, Narasimhanna MN, Surayanarayana SK, Kumararaj S (1973) Manual on sericulture - Silkworm rearing, UN Food and Agriculture Organisation, Rome.

Kumar NS, Basavaraja HK, Joge PG, Mal Reddy N, Kalpana GV, Dandin SB (2006) Development of new robust bivoltine hybrid (CSR46 x CSR47) of *Bombyx mori* L for tropics. Indian J Seric 45(1), 21-29.

Kumar NS, Basavaraja HK, Kishor Kumar CM, Mal Reddy N, Datta RK (2002) On the breeding of CSR18 x CSR19 a robust hybrid of silkworm, *Bombyx mori* L for tropics. Int J Indus Entomol 5(2), 153-162.

Kumar NS, Lakshmi H, Saha AK, Bindroo BB, Longkumer N (2012) Evaluation of bivoltine silkworm breeds of *Bombyx mori* L. under West Bengal conditions. Universal Journal of Environmental Research and Technology 2(5), 393-401.

Kumar NS, Yamamoto T, Basavaraja HK, Datta RK (2001) Studies on the effect of high temperature on F1 hybrids between polyvoltines and bivoltine silkworm races of *Bombyx mori* L. Int J Ind Entomol 2, 123-127.

Kumaresan P, Sinha RK, Raje Urs S (2007) An analysis of genetic variation and divergence in Indian tropical polyvoltine silkworm *(Bombyx mori* L.) genotypes. Caspian J Env Sci 5 (1), 11-17.

Kumari SS, Subbarao SV, Misra S, Murty US (2011) Screening strains of the mulberry silkworm, *Bombyx mori,* for thermotolerance. J of Insect Science 11, 116.

Lakshmi H, Chandrashekharaiah, Ramesh Babu M, Raju PJ, Saha AK, Bajpai AK (2011) HTO5 x HTP5, The new bivoltine silkworm (*Bombyx mori* L.) hybrid with thermo-tolerance for tropical areas. International Journal of Plant, Animal and Environmental Sciences 1(2), 88-104.

Mohammadis A, Prasanna BM (2003). Analysis of genetic diversity in crop plants. Crop Science 43, 1235-1248.

Moorthy SM, Das SK, Kar NB, Mandal K, Bajpai AK (2007) Breeding of bivoltine silkworm breeds suitable for tropics and identification of multi x bi silkworm hybrid for commercial exploitation in Eastern India. In perspectives of Cytology and Genetics 13, 215-227.

Nezhad SM, Mirhosseini SZ, Gharahveysi S, Mavvajpour M, Seidavi AR, Naserani M (2010) Genetic diversity and classification of 51 strains of silkworm *Bombyx mori* (*Lepidoptera: Bombycidae*) germplasm based on larval phenotypic data using Ward's and UPGMA methods. African Journal of Biotechnology 9(39), 6594-6600.

Pal NB, Moorthy SM (2011) Assessment of variability in larval and cocoon traits in some genotypes of bivoltine silkworm, *Bombyx mori* L. International Journal of Research in Biological Sciences 1(4), 59-65.

Pillai VS, Krishnaswami S (1980) Effect of high temperature on the survival rate, cocoon quality and fecundity of *Bombyx mori* L. In: Sericulture Symposium and Seminar, pp. 141-148, Tamil Nadu Agriculture University, Coimbatore, India.

Rahmathulla VK (2012) Management of climatic factors for successful silkworm (*Bombyx mori* L.) crop and higher silk production: A review. Psyche 2012, Article ID 121234, 12 pages.

Ramesha C, Anuradha CM, Lakshmi H, Sugnana Kumari S, Seshagiri SV, Goel AK, Suresh Kumar C (2010) Nutrigenetic traits analysis for identification of nutritionally efficient silkworm germplasm breeds. Biotechnology 9, 131-140.

Ramesha C, Seshagiri SV, Rao CGP (2009) Evaluation and identification of superior polyvoltine crossbreeds of mulberry silkworm, *Bombyx mori* L. Journal of Entomology 6, 179-188.

Shirota T (1992) Selection of healthy silkworm strain through high temperature rearing of fifth instar larvae. Reports of the Silk Science Research Institute 40, 33-40.

Spagnoletti Zeuli PL, Qualset CO (1987) Geographic diversity for quantitative spike characters in a world collection of durum wheat. Crop Science 27, 235-241.

Tazima Y, Ohnuma A (1995) Preliminary experiments on the breeding procedure for synthesizing a high temperature resistant commercial strain of the silkworm, *Bombyx mori* L. Silk Science Research Institute Japan 43, 1-16.

Thangavelu K, Sinha RK, Mahadevamurthy TS, Radha Krishnan S, Kumaresan P, Mohan B, Rayaradder FR, Sekar S (2000) Catalogue on silkworm (*Bombyx mori* L.) germplasm, CSGRC, Hosur, India.

Vijayan K, Nair CV, Raje Urs S (2010) Assessment of genetic diversity in the tropical mulberry Silkworm (*Bombyx mori* L.) with mtDNA-SSCP and SSR marker. Emir J Food Agric 22 (2), 71-83.

Zhang YH, Xu AY, Wei YD, Li MW, Hou CX, Zhang GZ (2002) Studies on feeding habits of silkworm germplasm resources for artificial diet without mulberry. Acta Sericologica Sinica 28, 333-336.