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CLUSTER ANALYSIS THROUGH SDS-PAGE UTILIZING HAEMOLYMPH PROTEIN OF THE SILKWORM, *BOMBYX MORI*.

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ABSTRACT
Qualitative analysis of haemolymph protein was carried out through the SDS-PAGE utilizing eighteen silkworm breeds *viz.*, six bivoltines (C_{108}, Kalimpong-A, NB_4D_2, CSR_2, P_{31}, NB_{18}), six multivoltines (Pure Mysore, Nistari, C. nichi, MU_1, *npnd*, Hosa Mysore) and six mutants (zebra, ursa, knobbed, *pere*, lemon and *pre*). The haemolymph was extracted by following the standard procedure and the haemolymph of 2nd, 4th and 5th days of V instar larvae was subjected to 8% gel electrophoresis. Based on the documented protein banding patterns from overall profiles, a highest of 31 protein bands were recorded each in P_{31} race and MU_1 breed during 2nd and 4th day of V instar respectively. Whereas, a lowest of 12 protein bands were observed during 5th day of the V instar in the Nistari race with their different intensity and molecular weight. The calculated data revealed an average of 22 protein bands during 2nd day, 25 during 4th day and 20 during 5th day of V instar larval stage. The expression of protein banding patterns falls between 43.0 to 97.4 kDa molecular weight. Further, the different clusters constructed based on the protein profiles of eighteen genotypes have clearly established their genetic identity. Through the UPGMA dendrogram, it is possible to establish the genetic distance and relationship among the populations of selected races/breeds. The present findings from qualitative analysis have clearly showed genetic differences and phylogenetic relationships between the silkworm races/breeds, which will be one of the important yardsticks in selecting parents for silkworm breeding programmes.

KEYWORDS: SDS-PAGE, haemolymph, genetic differences, phylogenetic relationships races/ breeds/ mutants.

INTRODUCTION

The SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), is one of the powerful tool, which has been helping in understanding allelic variations through separation of protein bands based on their different intensity and molecular weight among the different population of plants, animals and insects, *etc*. The changes in haemolymph protein profiles can be observed through the SDS-PAGE, which is one of the most widely utilized procedures in contemporary biochemistry and molecular biology of insects. Among various classes of organic molecules within cells, it is the proteins that are without doubt the most variable in terms of size, structure and functions. Because proteins are the "working molecules" of the cells and these large and complex macromolecules are of interest to researchers working in many areas of contemporary biological research. Further, the electrophoresis offers much more precise indications about the features of hybridization than morphology of lepidopteron insect *parnassius* (Descimon and Geiger, 1988). The morphological markers were found to be insufficient to expose the genetic diversity between the morphological overlap species and the morphological identical accessions. The advent of the electrophoresis as an analytical tool provides an indirect method for genome probing by exposing structural variations in enzymes or other protein genome (Cook, 1984 and Gilliland, 1989). This electrophoretic marker technique offers significant advantages over protein variability as well as morphological methods of species/variety identification, since they are rapid and relatively cheap depending upon the range/type of the markers/techniques. However, Hubby and Lewontin (1966) explained the importance of protein polymorphism in *Drosophila melanogaster* in the light of speciation and evolutionary mechanism. Thus, the importance of the study for polymorphic proteins through SDS-PAGE added a new dimension in plant and animal breeding (Frankel and Brown, 1983). The protein polymorphism (genetic variability) and phylogenetic relationships have to be observed in silkworm *Bombyx mori* by their differential protein mobilities, intensity and molecular weight. Perusal of literature indicated that, haemolymph protein as one of the most extensively studied biochemical tools for understanding banding profiles and with wide overlapping substrate specificities and pattern of inhibition. Protein polymorphism occurs in numerous forms expressed by distinct gene loci that generally have a high degree of genetic variability (Takasusuki *et al.*, 2006). The importance of similar study relevant to animal and plant breeding (Frey *et al.*, 1983) and conservation of genetic resource (Zeng *et al*., 2003), genetic variability in mosquitoes (Pushpalatha and Vijayan, 1999) and in silkworms (Hegde and Krishnamurthy, 1980; Subramanya and Sreerama Reddy, 1982; Somasundaram *et al*., 2004; Doddaswamy and Subramanya, 2007 and Talebi, 2010) are clearly established. Keeping the above aspects in mind, the present research work is being undertaken utilizing genotypes of silkworm of *Bombyx mori* and aimed to assess the protein polymorphism during 2nd, 4th and 5th

days of V instar larval developmental stage in eighteen races/breeds and mutant stocks of the silkworm, *Bombyx mori* having different genetic backgrounds. Further, through statistical analysis, dendrograms (cluster analysis) were formed by applying Unweight Pair Group with Arithmetic Mean (UPGMA).

MATERIALS AND METHODS

Parental seed cocoons of the above said eighteen races/breeds and mutants were collected from the Germplasm Bank of Department of Studies in Sericulture, Manasagangotri, Mysore and layings were prepared and reared by adopting the methods described by Tazima (1978) and Krishnaswami (1978) respectively. The haemolymph was extracted on 2nd 4th and 5th days of V instar larvae and subjected to qualitative analysis through sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) by following the method described by Laemmli (1970) with slight modification as suggested by Wenk and Fernandis (2007).

SDS Gel Preparation Procedure

The extracted haemolymph samples from silkworm were preserved in 4^{O}C. Subsequently, the 8% of Sodium Dodecyl Sulfate Polyacylamide Gel Electrophoresis (SDS-PAGE) method was followed to achieve a high resolution of protein banding profiles. Meanwhile, at the time of sample loading, the sample was dissolved by using sample buffer with 1:10 ratio (*viz.*, 1 *µl* of haemolymph sample and 10 *µl* of sample buffer). Subsequently, the mixed samples in Eppendorf tube were kept in a float of water bath at 95^{O}C for 3 minutes. The casting of gel was carried out by adding the stacking as well as separating gels by mixing different proportion of solutions as shown in below Table-1. The 8% SDS-Polyacrylamide gel was prepared and the 10 *µl* samples were loaded into each slot with bromophenol blue as the indicating dye.

TABLE 1: The proportion of stacking and separating solutions added for the gel casting experiment

Solutions	Sealing solution	Lower gel buffer (LGB)	Upper gel buffer (UGB)
LGB (separating gel)	0.531	5.0	-
UGB (stacking gel)	-	-	0.63
Acrylamide	0.869	5.3	0.83
Distilled water	0.923	9.3	3.40
APS	0.031	0.2	0.05
10% SDS	---	0.2	0.05
TEMED	0.010	0.012	0.005
Total amount of solution	2.364 ml	20 ml	4.965 ml

Electrophoresis running procedure

The 10 *µl* samples were loaded into each slot, which are then carefully layered with running (tray) buffer (0.05 M Tris, 0.384 M glycine buffer containing 10% SDS). The electrophoresis running was performed through dual gel electrophoresis units from starting current at 50 mA, and then shifted to 100 mA for 3 hours at room temperature until the dye front was 1cm from lower end of the gels. The gels were stained with staining solution (9% methonal, 2% acetic acid and 0.05% CBB R-250) overnight and de-stained in de-staining solution (9% methonal and 2% acetic acid) for about 8 hours until the protein bands appeared clearly. The staining and de-staining was conducted on shaker at the speed of 40 r/minute and the gels were scanned through Alpha Innotech (USA) Gel Documentation Image System. The qualitative analyses of profiles were carried out through different intensities/molecular weights and UPGMA phylogenetic trees (Dendrograms) were constructed based on the protein patterns of the selected eighteen races/strains of the silkworm *Bombyx mori.*

RESULTS

The electrophoresed haemolymph protein of 2nd, 4th and 5th day of V instar larvae's banding patterns were subjected to Alpha Innotech Gel Documentation system for the statistical analysis and obtained results is shown in Table-2. It is evident from the pattern that, a maximum of 31 differentially expressed protein bands with their differential intensity was observed in P_{31} race and a least of 24 bands in C_{108} race. However, in the races/breed of CSR_2, KA, NB_4D_2 and NB_{18}, the protein bands recorded were 30, 28, 26 and 25 respectively. On the other hand, based on the protein pattern of six multivoltine breeds (Fig. 1, a & b), it is evident from the profiles that highest number of protein bands of 27 were recorded in Nistari race, whereas a lowest of 16 protein bands were observed in Hosa Mysore race and remaining MU_1, C.nichi, *npnd* and Pure Mysore races/breed recorded differentially expressed moderate protein bands of 24, 23, 22 and 19 respectively. Among six mutant stocks, the zebra mutant recorded a maximum number of 20 protein bands and a minimum of 17 different intensity bands were expressed in lemon mutant during 2nd day of V instar. Based on the gel documentation pattern, among six bivoltines (Table-2), a highest of 31 protein bands were recorded in P_{31} race, whereas a lowest of 26 protein bands were observed in the C_{108} race and KA and CSR_2 have expressed equal number of 30 each protein bands whereas, NB_{18} (29) and NB_4D_2 (27) revealed moderate number of protein bands with different intensity and molecular weight during 4th day of V instar larval developmental stage. On the other hand, protein profile of six multivoltine breeds during said day and instar, clearly revealed a minimum of 24 differentially expressed protein bands in C.nichi race; whereas a maximum of 31 bands were observed in MU_1 breed while Pure Mysore (28), Nistari (28), *npnd* (27) and Hosa Mysore (26) races/breed have expressed different intensity and molecular weight protein bands, which falls between 43.0 to 97.4 kDa. Further, among six mutant stocks ((Figure-2 a & b and Table-2), it is evident from the result that, a highest number of 25 differentially expressed protein bands were visible in *pere* mutant, a lowest number of 16 protein bands were recorded in zebra mutant and remaining 22, 21, 19 and 19 protein bands were recorded in knobbed, *pre*, lemon and ursa mutant stocks

with different intensity and molecular weight respectively during 4th day of V instar. Furthermore, the electrophoresed protein patterns of V instar 5th day of six bivoltines (Fig. 3 a & b), six multivoltines and six mutant stocks (Table-2) have clear shown that, a highest of 28 (P_{31}), 30 (Pure Mysore) and 20 (knobbed & pere) protein bands were revealed among six each of bivoltines, multivoltines and mutants respectively and a lowest of 18 (CSR_2), 12 (Nistari) and 14 (zebra) protein bands were expressed among six each of bivoltines, multivoltines and mutants respectively. Among the four bivoltines, NB_4D_2 and NB_{18} are exhibited 20 and 21 differentially expressed bands, whereas C_{108} and KA revealed 20 and 19 bands respectively. The protein bands numbering 26 (MU_1), 20 (C.nichi), 20 (*npnd*) and 19 (Hosa Mysore) with their different intensity were revealed in six multivoltines and protein bands numbering 17, 17 and 16 lemon, *pre* and ursa were recorded in six mutants with their different intensity and molecular weights respectively.

Further, it is interesting to note that, the average number of electrophoresed protein bands (Table-2) from the overall eighteen races/breeds and mutants during 2nd, 4th and 5th days of V instar larval developmental stage indicated 22.83 protein bands during 2nd day, 25.50 at 4th day and 19.88 at 5th day of V instar larval developmental stage. Further, the same table showed protein bands ranging from 16 to 31 with different intensity and molecular weights during 2nd day and from 16 to 31 differentially expressed protein bands during 4th day and from 14 to 30 protein bands during 5th day of V instar larval developmental stage.

Phylogenetic relationships (Cluster analysis)

Phylogenetic trees (Dendrograms) were constructed based on the protein patterns of six bivoltine races/breed (Fig.4), six multivoltine races/breed (Fig.5) and six mutant stocks (Fig.6) utilizing cluster analysis UPGMA statistical package to establish the genetic distance and relationships among the eighteen races/breeds. The Dendrogram (Fig.4) revealed that, NB_4D_2 and NB_{18} races were genetically close related and falling in one group with their genetic similarity co-efficient of 0.07, whereas KA and CSR_2 races falling grouped under another cluster with their genetic similarity co-efficient of 0.24 and C_{108} and P_{31} races falling in another cluster interlinked to each other with their genetic similarity co-efficient of 0.00. Hence, three group of clusters obtained from the dendrogram representation of the six bivoltine races has clearly established their genetic identity and branches in the UPGMA dendrogram. The banding profile of six multivoltine breed (Fig. 5) was also subjected for the construction of phylogenetic tree as mentioned above and obtained results clearly indicated that, all the six races were clustered together with a genetic similarity co-efficient ranging from 0.00 to 0.33. Moreover, it is evident from the UPGMA that, Pure Mysore and Nistari breeds falling under a major cluster with their genetic relationship similarity co-efficient of 0.33. Further, MU_1 breed is genetically inter-related with the Pure Mysore and Nistari breed with its similarity co-efficient of 0.22 and the remaining three races namely, Hosa Mysore, C.nichi, and *npnd* are inter-related with first and second clusters with their genetic similarity co-efficient of 0.00. Furthermore, third phylogenetic tree consisting of six mutants (Fig.6) was constructed based on the electrophoretic protein profile. It is perceptible from the tree that the entire six mutant stocks falling under three major clusters and their genetic similarity co-efficient ranges from 0.05 to 0.43. The first major cluster consists of *pere* and *pre* mutants with their genetic similarity co-efficient of 0.05 and second and third major clusters consists of four mutants (ursa, zebra, knobbed and lemon) with genetic similarity co-efficient of 0.43.

TABLE 2: Haemolymph protein banding pattern (numbers) in eighteen races/breeds/mutants of the *Bombyx mori*.

Races/breeds/mutants	Total number of bands during V instar		
Bivoltines	2nd day	4th day	5th day
C_{108}	24	26	20
KA	28	30	19
NB_4D_2	26	27	21
CSR_2	30	30	18
P_{31}	31	31	28
NB_{18}	25	29	21
Multivoltines			
Pure Mysore	19	28	30
Nistari	27	28	12
C.nichi	23	24	20
MU_1	24	31	26
npnd	22	27	20
Hosa Mysore	16	26	19
Mutants			
zebra	21	16	14
ursa	20	19	16
knobbed	19	22	20
pere	20	25	20
lemon	17	19	17
pre	19	21	17
Average	22.83	25.50	19.88
Range	16-31	16-31	12-30

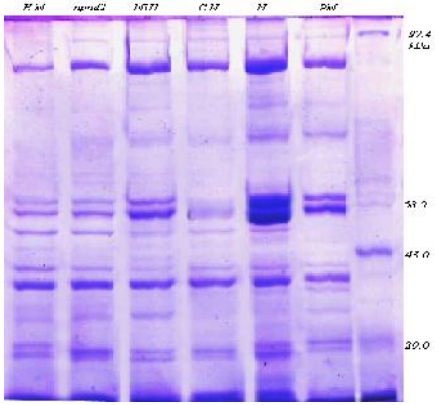

Fig. 1

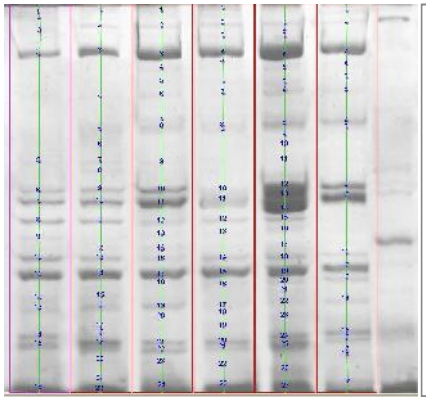

Fig.1 (a)

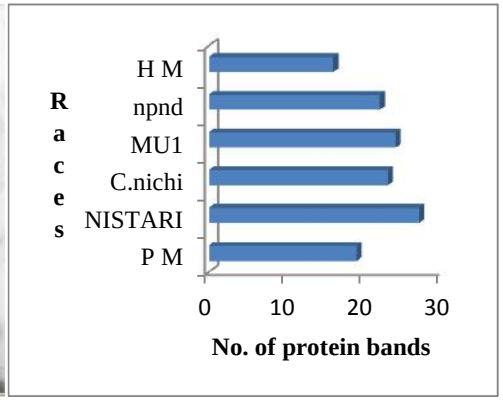

Fig. 1 (b)

FIGURES 1- (a & b): Haemolymph protein profile of V instar 2nd day of six multivoltines (Fig.1) with analyzed profile (Fig. 1 a) & number of bands (Fig. 1 b). M = Marker protein, 1 = PM, 2 = Nistari, 3 = C.nichi, 4 = MU_1, 5 = *npnd* and 6 = HM

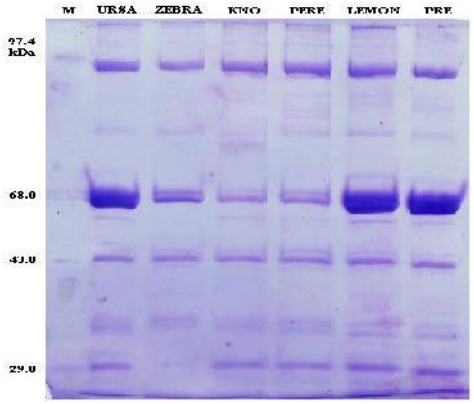

Fig. 2

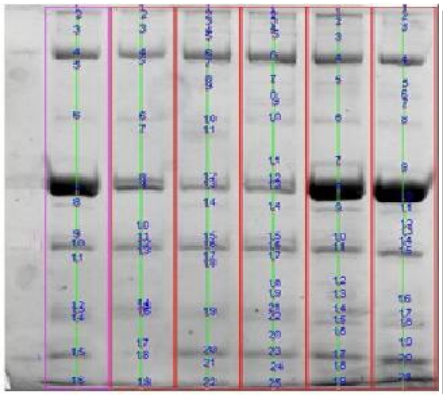

Fig. 2 (a)

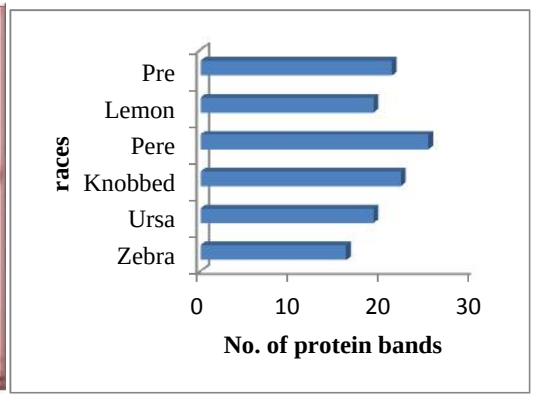

Fig. 2 (b)

FIGURES 2-(a & b): Haemolymph protein profile of V instar 4th day of six mutants (Fig.2) with analyzed profile (Fig.2 a) & number of bands (Fig. 2 b). M = Marker protein, 1 = zebra, 2 = ursa, 3 = knobbed, 4 = pere, 5 = lemon and 6 = *pre*

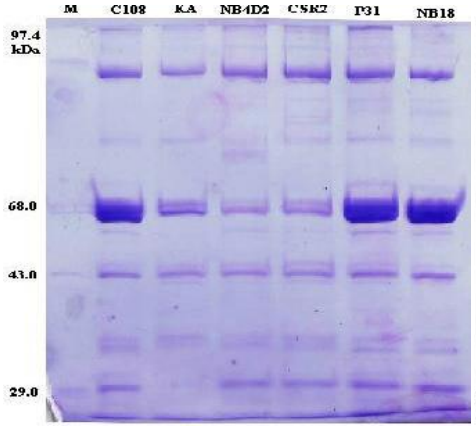

Fig. 3

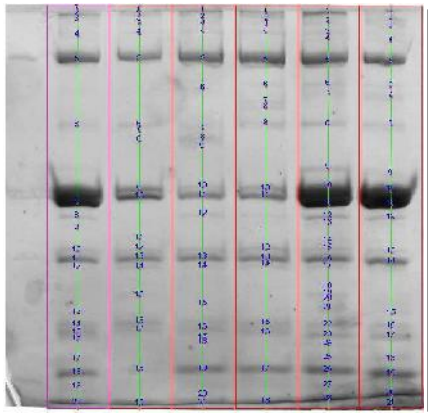

Fig. 3 (a)

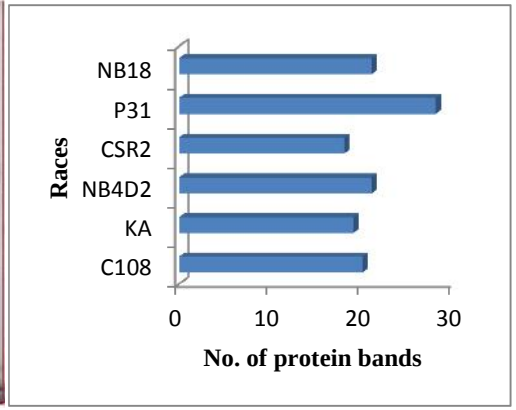

Fig. 3 (b)

FIGURES 3-(a& b): Haemolymph protein profile of V instar 5th day of six bivoltines (Fig.3) with analyzed profile (Fig.3 a) & number of bands (Fig. 3 b). M = Marker protein, 1 = C_{108}, 2 = KA, 3 = NB_4D_2, 4 = CSR_2, 5 = P_{31} and 6 = NB_{18}

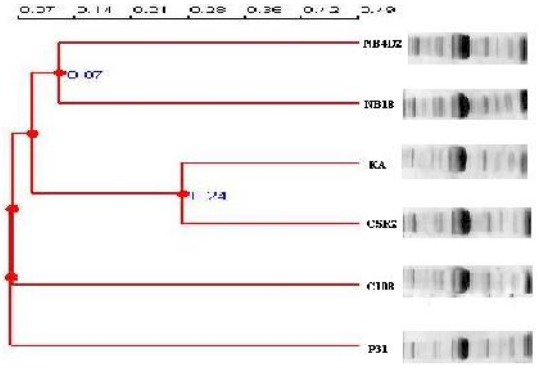

Fig. 4

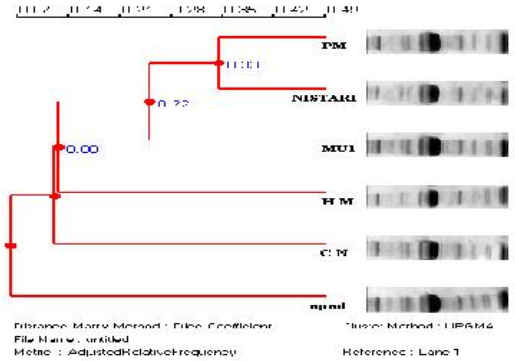

Fig. 5

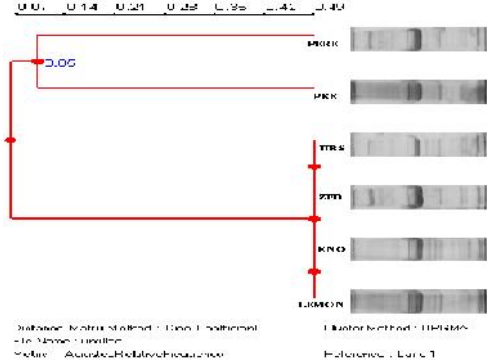

Fig. 6

FIGURES 4-6: Dendrograms showing cluster relationships of six bivoltines (Fig.4), six multivoltines (Fig.5) and six mutant stocks (Fig.6) of the silkworm, *Bombyx mori.*

DISCUSSION

The Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) is one of the powerful tools, which has helped in understanding allelic variations through separation of protein bands among different genotypes. Thus, the importance of the study of polymorphic proteins through SDS-PAGE added a new dimension in plant and animal breeding programmes (Frankel and Brown, 1983). Hence, keeping the above said concept in mind, this technique SDS-PAGE) has been utilized to understand the genetic differences and phylogenetic relationships. Based on the electrophoretic protein patterns and molecular weights, it is possible to arbitrarily classify the bands into three fractions namely major, intermediary and minor. All the three groups were observed in the range of 29.0 kDa to 97.4 kDa, wherein higher molecular weight bands falls under Group-I and lower molecular weight under Group-III. It is evident from the results that (Figs. 1 - 3), the variability of protein bands distinct in the middle of the gel and bands in the cathode side were common to all the profiles. On the other hand, the total number of protein bands obtained from the experiment was recorded during said different days revealed that, an highest of 31 differentially expressed protein bands observed in P_{31} race and a lowest of 16 bands recorded in Hosa Mysore during 2nd day. In case of 4th day a maximum of 31 bands obtained in both P_{31} race and MU_1 breed and a minimum of 16 bands were expressed in zebra mutant. Further, at 5th day a higher number of 30 bands were recorded in Pure Mysore, whereas lowest of 12 protein bands were observed in Nistari race. In a similar study utilizing SDS-PAGE, Dunbar, (1987) has observed that, the variations in the protein fractions are due to post-translational modifications. Further, the reports of Noel *et al.* (1979) and Seeberg *et al.* (1984) demonstrated that, the protein fractions noticed through SDS-PAGE may be due to changes in the sequence of single amino acid. The differential intensity and number of protein bands observed in the 2nd, 4th and 5th day larvae, supports the findings of Suzuki and Suzuki (1974), who demonstrated that the 5th day V instar larval stage is helpful for the protein assay either for quantification or qualitative estimation because of high transcription mechanism in this stage, which is ready for synthesizing the silk proteins. Moreover,the genetic variability observed in the present result on the electrophoretic protein patterns of *Drosophila willistoni* (Ayala *et al.*, 1974), in mosquitoes by Pushpalatha and Vijayan, (1999). Similarly, in lepidopteron silkworms, the polymorphic esterases groups are reported by Hegde and Krishnamurthy (1980); Sreerama Reddy and Subramanya, (1982) and Somasundaram *et al*., (2004).

However, understanding of the genetic diversity and population genetic structure is not only important for the conservation of many species of plants and animals but is also essential for maintenance of genetic diversity within the populations (Millar and Westfall, 1992). Similarly, haemolymph protein profiles in different multivoltine and bivoltine races of silkworm have been demonstrated by Somasundaram, *et al.* (2005) and Koundinya *et al.* (2007) among Indian races. Thus, in the present investigation utilizing silkworm haemolymph protein, it is important to note that, the banding pattern clearly varies between the races and exhibiting genetic differences. Such genetic variations are observed in the silkworm haemlymph protein profiles by Gamo, (1982); Doddaswamy and Subramanya, (2007) and Talebi, (2010). Similarly, Lakshmikumari, (1995) and Lakshmikumari *et al.,* (1997) has revealed that, the variability of haemolymph banding patterns due to the effect of the radiation. Further, the effect of CPV (Mahesha, 1997), a few mutagens (Sen *et al.,* 1999) and EMS (Mahesha *et al.,* 2000) on variable protein banding pattern is well documented. The results through UPGMA analysis (Figs. 4, 5 & 6) showed that, different genetic similarity co-efficient existing among the six bivoltines (Fig.4) where, NB_4D_2, NB_{18} and KA, CSR_2 and C_{108}, P_{31} races belong to cluster I, II & II with their similarity co-efficient of 0.07, 0.24 & 0.00 respectively. Further, six multivoltines (Fig.5) Pure Mysore and Nistari belongs to one cluster and remaining races clustered in other two respective clusters with their genetic similarity of 0.33, 0.22 and 0.00 respectively. Ultimately, six mutants (Fig.6), pere and *pre* mutants belong to a single cluster and remaining mutants in other two clusters with similarity co-efficient of 0.05 and 0.43 respectively. It is important that, the results of the present findings clearly established the genetic distances and differences, in addition to exhibiting phylogenetic relationships between eighteen genotypes of silkworm ***Bombyx mori***. Thus, results of the above research findings will be successfully utilized as an important yardstick in the silkworm genetics and breeding programmes**..**

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