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Allele-specific expression of biochemical markers with special reference to silkworm voltinism

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Abstract
The activity level of esterase and acid phosphatase in bivoltine and polyvoltine silkworm strains by Native-PAGE was documented for investigation of genetic diversity and their voltinism expression. Furthermore, the quantitative amount of total protein of haemolymph was estimated with help of SDS-PAGE. The expression of seven α-esterases and five acid phosphatase specific alleles were observed. The dendrogram produced by UPGMA analysis, based on Dice's coefficient, clustered four races into two major groups which accurately segregated them according to their inheritance of voltinism. The genetic distance between strains ranged from 0.07 to 0.43. The research outcome showed that, the Pure Mysore strain exhibited higher allelic expressions of protein when compared to other strains. Despite exhibiting their unique breed, these biochemical markers represented voltinism-specificity interpreting the origin of each breed and phylogenetic relationships among them. Hence, isozyme fingerprinting technique is beneficial for revealing the genetic variability and polymorphism differentiation among closely related strains.

Keywords: Esterase, acid phosphatase, specific-alleles, voltinism expression, polymorphism

1. Introduction
The silkworm (*Bombyx mori*) is of great economic value from an industrial perspective. Knowing their genetic structure may provide improve the conservation of commercial lines [1, 2]. The silkworm's genotypes are largely categorized into bivoltine (egg-diapause) breeds completes two generations per year and are adjusted to temperate conditions with high yielding production and polyvolitine (non-diapause) breeds complete five to six generations per year and are adapted to tropical climatic conditions with low yield [3].

Using the appropriate markers to differentiate the silkworm strains makes it possible to develop strategies for classification, which is significant for the species breeding with regard to enhancing the adaptive potential and productivity [4]. Silkworm phylogenetic diversity is substantial for selection of functional parent and specific fingerprints. Application of isoenzymes and other molecular markers assist to estimate genetic diversity much more accurately than morphological traits [5]. Moreover, haemolymph proteins play an important role in insects for transport functions, as well as for their enzyme action. The synthesis and utilization of haemolymph proteins are controlled by genetic and hormonal factors [6]. In 2011, [7] reported that, the protein polymorphism gives a clue on the heterosis expression for selected traits and can be used as an index in silkworm breeding. The haemolymph composition of insects reflects the nature and degree of the protein metabolism may be for synthesis and breakdown of specific proteins which are due to differential racial characters [8].

We found that, little attention paid to genetic variability and voltinism expression of protein, esterase and acid phosphatase isozymes during the larval period in tissue and haemolymph of *B. mori*. Hence, present chapter aimed to investigate the existing polymorphism of acid phosphatase, esterase, to highlight the heterogeneity and mode of inheritance of these isozymes and to maintain the variability within and among population for conservation of genetic resources followed by detection of geographical relationship among different breeds and their uniqueness profiles. The data gathered from this study will be also beneficial during the breeding of new silkworm breeds with better commercial characters. The selected silkworm breeds reared in Mysore region had not yet been investigated for specific-alleles of gene/s linked to the expression of voltinism.

2. Materials and Methods

Four disease-free layings of two bivoltine strains of the silkworm *B. mori* namely, CSR_2, CSR_4, and two multivoltines namely, Pure Mysore and C. nichi, showing different in the expression of morphological traits originating from India, China and Japan were maintained from August 2015 to June 2016 at Germplasm Bank, Department of Studies in Sericulture Science, Manasagangothri in Mysuru, Karnataka, India (Table 1).

Table 1. Lineages of races, their origin and voltinism

Lineage	Origin	Voltinism
Line-1 (PM)	Indo-China	Multivoltine
Line-2 (C.nichi)	Japanese	Multivoltine
Line-3 (CSR_2)	CSR & TI, Mysore, India	Bivoltine
Line-4 (CSR4)	CSR & TI, Mysore, India	Bivoltine

2.1 Qualitative analysis of isozymes by – Native Poly Acrylamide Gel Electrophoresis

The qualitative analysis of esterases and acid phosphatase were carried out utilizing the selected tissue and haemolymph samples in Poly Acrylamide Gel Electrophoresis (PAGE) with the discontinuous buffer system following the procedure as described by [9]. The vertical slab gel apparatus (Chromous Biotech, India) was utilized.

Electrophoresis

Fifteen µl of each extracted samples were subjected to electrophoresis on a 8 % polyacrylamide gel. The PAGE was carried out with transmiting initial voltage of 50 and followed by 100 V for 3 h until the tracing dye reached the bottom of the gel. The relative mobility (*R*f value) was calculated for each polypeptide from the formula: *R*f =distance of protein migration /distance of dye migration [10].

Esterase

Gels were stained for α-esterase activity of haemolymph. Gels were rinsed rapidly in an ice-cold distilled water and incubated in the following solution C in a rotary shaker at 37ºC under dark condition for 8 h or until the bands emerged. After the appearance of bands, the gels were scanned, analyzed and photographed with help of a gel scanner.

Acid phosphatase

The gels, soon after the removal, washed in a running ice-cold distilled water followed by the incubation in 100 ml of acetate buffer (0.125 M pH 5) consist of polyvinyl pyrolidone 500 mg, fast blue RR salt 100 mg, sodium alpha napthyl phosphate 100 mg, at 37ºC in rotary shaker in dark for 8 h or until the bands emerged. Then the gels were scanned analyzed and photographed with help of a gel scanner.

2.2 Qualitative analysis of total proteins by Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE)

The qualitative analysis of total proteins present in haemolymph was carried out according to [11] method in Sodium Dodecyl Sulphate - Poly Acrylamide Gel Electrophoresis (SDS-PAGE) with the discontinuous buffer system. The vertical slab gel apparatus (Chromous Biotech, India) was used. The cluster analysis was carried out using UPGMA method (Unweighted Pair Group Method Algorithm) developed by [12] with help of computer software Alphaview version 3.0 for SDS-PAGE.

3. Results

3.1 Esterase

The genetic versatility also existed in haemolymph α -esterases of the silkworm strains with help of α – naphthylacetate. The expression of seven α-esterases alleles, Est-a, b, c, d, e, f, and Est-g in selected silkworm lineages has been depicted in Fig. 1 and the relative mobility (*R*f) value of each esterase band was calculated (Table 2). The α -Est-a with *Rf* value of 0.21 and EST-f with *Rf* value 0.47 confirmed to be a race-specific esterase as they only appeared in CSR4 strain belong to India and the line Pure Mysore (an Indo-Chinese strain) respectively. Every larvae display bands α-Est-c (*Rf* value of 0.32), Est-d (*Rf* value of 0.39), and Est-e (*Rf* value of 0.43) stained by α -naphthylacetate. There was not presence of a voltinism-specific marker with help of haemolymph α-esterases of *B. mori*. However, it could express alleles of presenting race-specificity in two different strains.

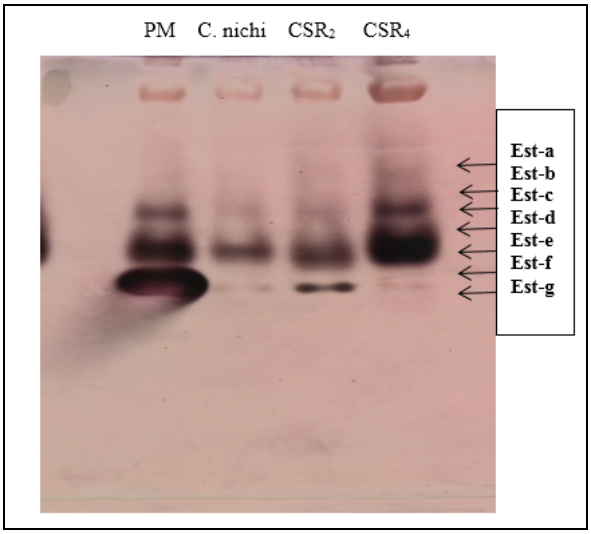

Fig 1: Vertical 8 % PAGE of α -esterase isozyme banding pattern of haemolymph of *B. mori* lines were identified using 2% α -naphthyalacetate as a substrate. Lane1-Pure Mysore, lane 2-C.nichi, lane 3-CSR_2, lane 4-CSR_4.

Table 2: The relative mobility (*R*f) value of each α- esterase band of haemolymph of *B. mori* strains. Lane1-Pure Mysore, lane 2-C.nichi, lane 3-CSR_2, lane 4-CSR_4 using α-naphthylacetate separately as a substrate.

α-Esterase bands	Lane 1	Lane 2	Lane 3	Lane 4
EST-a	-	-	-	0.21
EST-b	0.28	-	-	0.28
EST-c	0.32	0.32	0.32	0.32
EST-d	0.39	0.39	0.39	0.39
EST-e	0.43	0.43	0.43	0.43
EST-f	0.47	-	-	-
EST-g	0.54	0.54	0.54	0.54

3.2 Acid phosphatase

The polymorphism among silkworm breeds existed in relation to isozyme of five acid phosphatase alleles. Five isozyme forms of acid phosphatase have been found in the various strains investigated. These our results correspond with the results of [13]. The acid phosphatase isozymes were designated as A, B, C, D and E (Fig. 2) and the relative mobility (*R*f) value of each acid phosphatase allele was calculated (Table 3). The Bph A with *Rf* value of 0.42 appeared in C.nichi and CSR_4 and could not discriminate the strain voltinism. The nonspecific Bph B (*Rf* value of 0.44) and Bph C (*Rf* value of

0.55) existed in all silkworm strains. The Bph D with *Rf* values 0.62 presented in PM, and C. nichi and confirmed as a multivoltine specific allele. The Bph E with *Rf* values 0.70 observed in CSR_2 and CSR_4 which made it a unique marker for bivoltine strains. There was not presence of a race-specific marker with help of acid phosphatase electrophoresis however it could differentiate bivoltine strains from polyvoltine.

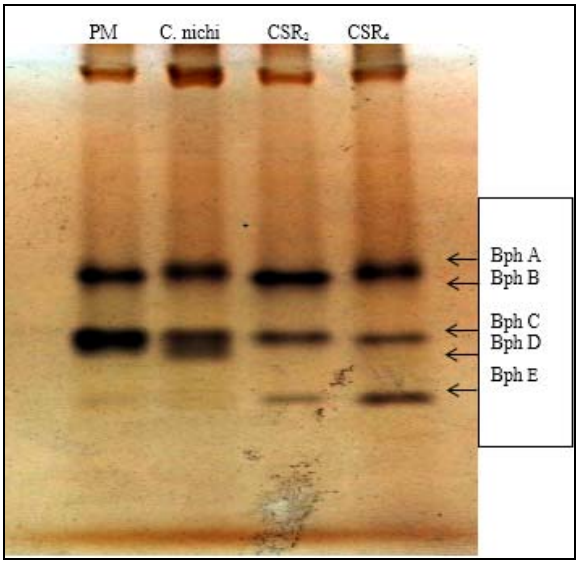

Fig 2: Vertical 8 % PAGE of acid phosphatase isozyme pattern of haemolymph of B. mori lines. Lane1-Pure Mysore, lane 2-C.nichi, lane 3-CSR_2, lane 4-CSR_4; the bands Bph A, Bph B, Bph C, Bph D, and Bph E.

Table 3: The relative mobility (*R*f) value of each acid phosphatase band of haemolymph of *B. mori* breeds. Lane1-Pure Mysore, lane 2-C.nichi, lane 3-CSR_2, lane 4-CSR_4.

Acid phosphatase Bands	Lane 1	Lane 2	Lane 3	Lane 4
Bph A	-	0.42	-	0.42
Bph B	0.44	0.44	0.44	0.44
Bph C	0.55	0.55	0.55	0.55
Bph D	0.62	0.62	-	-
Bph E	-	-	0.70	0.70

3.3 Total protein profile pattern

The genetic versatility also existed in haemolymph protein of silkworm strains with help of SDS-PAGE. The expression of 34 alleles in selected silkworm lineages has been depicted in Fig. 3. Total protein analysis generated 34 different bands of which 50% were found to be polymorphic and were designated as Haemolymph Protein Band1 (Hpb1)…to Hpb34, in four strains of the silkworm and the polymorphism existed among them. The bands Hpb-1, -2, -5, -6, -7, -9, -11, and Hpb-12 appeared only in Pure Mysore and Hpb-4 marker with *Rf* value 0.14 was presented in C. nichi which confirmed to be as breed-specific markers. The markers Hpb-17,-26,-27, and Hpb-31 were presented in Pure Mysore, and C. nichi that made them multivoltine specific alleles. The bands Hpb-8,-25,-28, and Hpb-30 observed in CSR_2 and CSR_4 which made them unique markers for bivoltine strains. A maximum 24 bands of different molecular weight were appeared in Pure Mysore and a minimum 21 bands in both bivoltine strains.

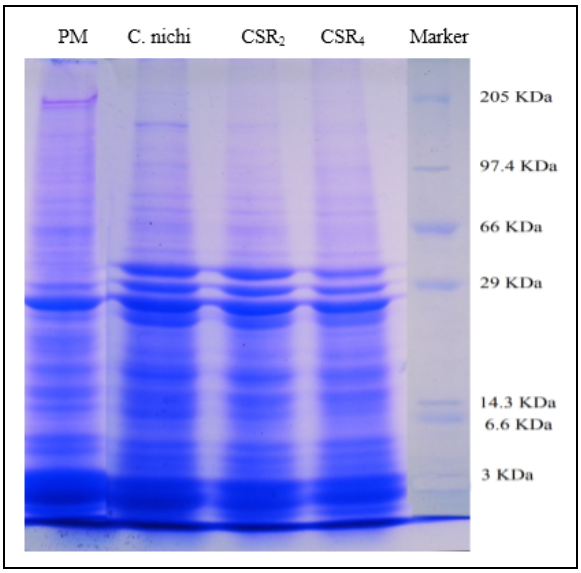

Fig 3: Vertical 8 % SDS-PAGE analysis of haemolymph protein pattern in *B. mori* lines. Lane1-Pure Mysore, lane 2-C.nichi, lane 3-CSR_2, lane 4-CSR_4. M- Molecular Weight Marker.

The results have clearly indicated, the differentially expressed protein banding pattern in the four *B. mori,* races with highest number of 25 bands were observed in Pure Mysore strain (Fig. 4).

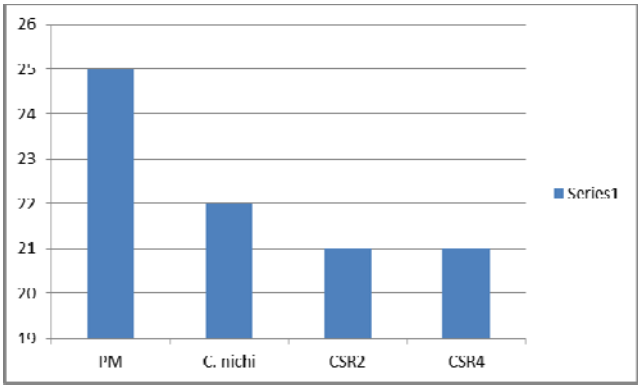

Fig 4: Graphical demonstraion of haemolymph protein bands in the four silkworm strains.

The banding pattern, which is measured with the help of transilluminator Alpha innotech (USA) Gel Documentation System along with marker proteins represented differential expression of proteins among the four breeds by UPGMA dendrogram, the protein fractions were classified into two groups, I, II (Fig. 5). The dendrogram based on *B, mori* haemolymph protein pattern suggests that, the strains PM, C. nichi and CSR_2, CSR_4 are closely related since they belong to similar voltinism. The genetic distance between strains ranged from 0.07 to 0.43.

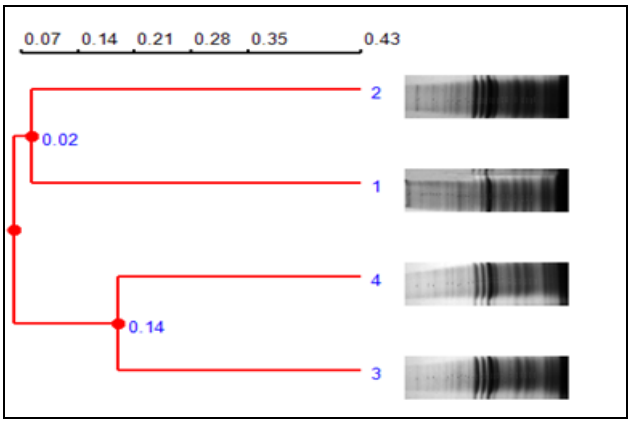

Fig 5: Dendrogram illustrating the phylogenetic relationship among four silkworm strains based on UPGMA cluster analysis of Dice's genetic distance**,** where (1) Pure Mysore, (2) C. nichi, (3) CSR_2 and (4) CSR_4.

4. Discussion

As the global scenario in terms of present trend is moving towards augmentation of plant and animal breeding with help of molecular markers (Isozyme/protein) techniques therefore fingerprinting of genetic versatility in the organisms can prove this object for improvement of desirable traits and elucidation of the evolutionary relationships. Among the different isoenzymes analyzed namely esterase was most preferred because of its diverse substrate specificity and polymorphic expression followed by acid phosphatase [14, 15, 13]. One of esterases in silkworm eggs named 'esterase A' is closely related to diapause character of the insect. The effect of this esterase on the yolk cells of diapause eggs was examined in order to discover the mechanism of diapause termination in silkworm eggs [16]. Moreover, in 2012, [17] evaluated the polymorphism of different silkworm using the isoenzymes electrophoresis to detect biochemical markers and indicated that in spite of the differentiation among the lineages, they could not be separated just with help of the isozymes alleles. Furthermore, [18] revealed the esterase isozyme polymorphism of specific and nonspecific esterase, syngenic lines development and natural occurrence of a thermostable esterase in the tropical silkworm, *B. mori* using α- and β-naphthylacetate as substrate where the races or breeds originated either from temperate or tropical regions. Besides, SDS-PAGE is a technique widely used in biochemistry, forensics, genetics and molecular biology to separate proteins according to their electrophoretic mobility or a function of length of polypeptide chain or molecular weight [19]. In 2010, [20] investigated silkworm hemolymph from different developmental stages was by two-dimensional electrophoresis and mass spectrometry to understand the intricate developmental mechanisms of metamorphosis. They identified 34 proteins involved in metamorphosis, programmed cell death, food digestion, metabolism, and nutrient storage and transport.

5. Conclusion

The results obtained in the present study provides new information concerning genetic variations among geographically isolated silkworm breeds by analyzing of different bio-chemichal markers with refer to heterozygosity to comprehend the significant role of any specific protein and isoenzyme specific allele/s of gene/s linked to polygenic systems. The genetic versatility existed in haemolymph esterase of the silkworm strains with help of α – naphthylacetate. The α -Est-a and EST-f approved to be a breed-specific esterase for CSR_4 and PM strains respectively. Furthermore, five forms of acid phosphatase alleles have been found in the all strains investigated. The Bph D presented in PM, and C. nichi and confirmed as a multivoltine specific allele. The Bph E observed in CSR_2 and CSR_4 breeds which made it a unique marker for bivoltine strains.

The study also shows the capacity of esterase and acid phosphatase revealing genetic variability and expression of voltinism in silkworm at strains. Moreover, the present investigation on haemolymph protein banding pattern through SDS-PAGE technique utilizing two voltine groups of the silkworm strains has been undertaken in order to study the function of any specific protein marker related to embryonic diapause development and assess the genetic variability at intra-species level.

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