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Isozymic variations in specific and nonspecific esterase and its thermostability in silkworm, *Bombyx mori* L.

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Abstract

Esterase isozymic variations were documented in the haemolymph of developed multivoltine and bivoltine silkworm breeds during unfavorable seed crop seasons of May – September using α- and β- napthylacetate separately to identify specific and nonspecific esterase having thermotolerant potentiality. Variations existed in the isozyme pattern with three bands (Est-2, 3 and 4) in pure Nistari race and other developed multivoltine and bivoltine breeds. Est-2 and Est-3 were non-specific esterases as they were observed when both α- and β-napthylacetate was used as substrates separately. Est- 4 band was observed only with α-napthylacetate as substrate and was therefore confirmed to be specific α-esterase band in the haemolymph of silkworm, *Bombyx mori* L. Zymograms showed that the non-specific esterase band (Est-3) with R_f of 0.43 and specific α-esterase band (Est-4) with R_f of 0.32 predominately withstood a temperature of 70 ± 2°C for a duration of 10 min and were confirmed as thermostable esterases in haemolymph of silkworm, *Bombyx mori* L. This also categorized the presence of thermostable esterases in developed multivoltine and bivoltine breeds of silkworm, even though the qualitative activity was more in the former than the latter. The qualitative presence of thermostable esterases and their activity could be adopted as an indicative biochemical marker in relation to thermotolerance in silkworm.

Key words

Isozyme variations, Esterases, Thermotolerance, Breed development, *Bombyx mori*

Introduction

Esterases (EST, 3.1.1.2) are ubiquitous in living organisms. Several esterases have been isolated from various tissues of microbes, plants and animals and investigated for their biochemical properties (Thomas *et al*., 1993; Shahjahan *et al*., 2008). They have been found widespread in insect species and function in the digestion of nutritional materials, detoxification of xenobiotics (Oakeshott *et al*., 1993; Shiotsuki and Kato, 1999; Amanullah *et al*., 2010), and in reproduction (Krishnamurthy and Umakanth, 1997).

Insect esterases are classified according to their reactions to different substrates. Use of α- or β-napthylacetate, a hydrolyzing substrate, for nonspecific esterases (α and β) have been reported

by several workers (Stoykova *et al*., 1998; Chattopadhyay *et al*., 2001). Some authors have used esterases as genetic markers for studying enzyme polymorphism in different insect species (Patnaik *et al*., 1994; Ivanova *et al*., 1994, 1995, 2007; Ivanova and Popov, 1996).

Polymorphism of *Bombyx mori* esterases in different tissues viz. haemolymph, silk glands, fat bodies, midgut, eggs and integuments have been observed by their differential mobility in agar gel electrophoresis (He, 1995; Stoykova *et al*., 2003). The allozyme technique, therefore, provides an opportunity to establish genetic relationships and is used to estimate genetic diversity. Eguchi *et al*. (1965) has described the types and inheritance of haemolymph esterase in silkworm stating the presence of four fundamental types of esterases controlled by co-dominant alleles Bes^A, Bes^B, Bes^C and Bes^O (blood esterase). Gamo (1978) linked these genes on 11th chromosome. Arai *et al*. (2000) purified and characterized major esterase Bes^B from the haemolymph of *B. mori*.

Most significantly, heat stable esterases were reported in midgut of silkworm (Liu et al., 1984). Wu and Hou (1993) studied the direct relationship between thermotolerance and heat stable esterase in larval body or midgut of bivoltine silkworm, *Bombyx mori* originating from temperate zones and suggested it as an indicator for studies on silkworm thermotolerance. Reports of esterase isozyme polymorphism in digestive juice and haemolymph of tropical multivoltine silkworm, *Bombyx mori* L. breed CB5 and its syngenic lines (CB5Lm^e-1, CB5Lm-2 and CB5Lm-5) using nonspecific substrates and presence of natural thermostable β-esterase 3 were documented by Chattopadhyay *et al*. (2001). Moorthy *et al*. (2007b) subsequently reported heat stable α-esterase-3 band is associated with thermotolerance capacity of some elite multivoltine lines.

The silkworm, *Bombyx mori* L., is a poikilothermic insect, with its optimal temperature ranging from 20 - 30 ºC. The tropical multivoltine breeds exhibit greater survivability and adaptability with significant tolerance towards disease but produce inferior quality silk. Rearing of bivoltines, producing superior quality silk in tropical and subtropical zones is limited due to their low adaptive potential and increased susceptibility to pathogens (Chattopadhyay and Chatterjee, 1990). Also generation of multi x bi hybrid layings production during autumn (Nov-Dec commercial crop season) becomes a constraint due to lack of bivoltine male cocoons during preceding seed crop season (Sept-Oct) due to high temperature (above 33°C) and high humidity (~90%) (Moorthy *et al*., 2007a). It was therefore imperative to address the need to qualitatively assess the potential multivoltine and bivoltine breeds of silkworm, *Bombyx mori* L. and explore the uniqueness in esterase isozyme variation with special reference to thermotolerance.

Materials and Methods

Silkworms and collection of haemolymph : Multivoltine and bivoltine silkworm breeds having potentiality towards fluctuating tropical conditions with good yield potential were selected from the germplasm bank of Central Sericultural Research and Training Institute, Berhampore, West Bengal. Multivoltine breeds (viz., M6DP(C), M6M81, M12(W), CB5, M.Con.1 and M.Con.4) and pure strain Nistari were reared at 27-32°C and 60-75% RH in unfavorable tropical temperatures during May-July of the year. Developed bivoltine breeds selected (viz., NB_4D_2, D6(P)S, D6(P)N, MC4(O), YB, SK4(C), SK6, SK7, B.Con.1 and B.Con.4) were reared in unfavorable seed crop season of September.

Haemolymph were collected from Vth instar-5th day larva by puncturing the prolegs with a sterilized needle and samples (approximately 0.5 ml) were collected in chilled microfuge tubes with 1 mg phenylthiourea to avoid the activity of prophenol oxidase leading to melanization of haemolymph. The haemolymph was centrifuged at 750 g for 10 min to remove the haemocytes. The supernatant were collected in microfuge tubes after centrifugation and stored at -20°C.

Polyacrylamide Gel Electrophoresis (PAGE) : 8% gel was prepared using 8 ml of acrylamide: Bisacrylamide (30:0.8), 7.5 ml Tris-separating buffer, 0.6 ml of Ammonium persulphate (AMPS) as initiator, 0.018 ml of tetra ethyl methyl ethylene diamine (TEMED) as catalyst and 13.90 ml of distilled water. All chemicals were purchased from E-Merck India Ltd., Mumbai.

Ten microlitre of each sample were electrophoresed under non-denaturing conditions on a 8% polyacrylamide gel following Pharmacia Laboratory techniques using dual vertical gel electrophoresis system (Omega) attached to thermo-controlled water bath (Pharmacia LKB – MultiTemp II). The PAGE was carried out under 110 V constant voltage for 3-4 hr at 4°C until the tracing dye reached the bottom of the gel.

Esterase isozyme patterns were studied by staining the gels in the presence of α and β napthylacetate as substrates following basic techniques described by Johnson and Deniston (1964) and Simms (1965). Following electrophoresis, the gels were immersed in 0.5 M boric acid (pH 4.1) for 30 min at 4°C. Gels were then rinsed rapidly in two changes of ice-cold distilled water and placed separately in trays with substrate (2% α or β-napthylacetate in acetone), 40 mg of fast blue BB salt and 100 ml of 0.2 M sodium phosphate buffer A (pH 9.2) and B (pH 4.3). Gels were incubated under dark conditions at 30°C for 4-8 hr. Gel documentation was performed with the GDS-7600-UVP white/UV transilluminator. The relative mobility (R_f value) was calculated (Shi and Jackowski, 1998) for each polypeptide using the formula: R_f = distance of protein migration/ distance of dye migration.

Normal gels of haemolymph samples after electrophoresis were incubated at 70 ± 2°C in a water bath for 10 min prior to the addition of α and β napthylacetate as substrates. Those esterases maintaining activity were regarded Heat stable esterases (HsEST).

Results and Discussion

Potential multivoltine (viz. Nistari, M6DP(C), M6M81, M12W, CB5, M.Con.1 & M. Con.4) and bivoltine breeds (viz. NB_4D_2, YB, MC4(O), SK6, SK7, D6(P)S, D6(P)N, SK4C, B. Con.1 & B.Con.4) of silkworm, *Bombyx mori* L. were selected from the central germplasm of institute for the present study with the objective of quality assessment during adverse seed crop seasons for utilization in breeding programme in developing superior breeds and identification of productive hybrids. The multivoltine breeds like M12(W), M6DP(C), M6M81 and bivoltine breed, YB were developed through hybridization and selection (Ghosh *et al*., 2001). Adopting similar techniques, bivoltine breeds like SK6 and SK7 were developed (Das *et al*., 2000). Breeds like M.Con.1, M.Con.4 (Multivoltine) and B.Con.1, B.Con.4 (Bivoltine) were developed through congenic breeding approach (Chattopadhyay *et al*., 2001).

The esterase spectra of different silkworm (*B. mori* L.) tissues and organs have been studied in series of investigations but were limited to races originating from temperate regions (Azivov, 1989; Stoykova *et al*., 1998). Earlier reports also revealed that the substrates utilized were either α- or β-napthylacetate or mixture of both as nonspecific substrate (Tavares *et al*., 1998).

The β-esterase isozyme pattern in haemolymph of selected multivoltine breeds showed two bands designated β-Est-2 and 3 (Fig. 1, lane 1-7) with R_f values of 0.52 and 0.43 suggesting homogeneity in the band presence. The α-esterase isozyme pattern in haemolymph of selected multivoltine breeds showed three conspicuous bands, designated α-Est-2, 3 and 4 (Fig. 3, lane 1-7) with R_f values of 0.52, 0.43 and 0.32. Although homogenous distribution of non-specific Est-2 and 3 was noticed in all the selected breeds, specific α-Est 4 was not noticed in breed CB5. The significant findings were the presence of α-napthylacetate specific band such as α-Est 4 in M6DP(C), M.Con.1, M12W, M6M81, M.Con.4 and Nistari breeds of silkworm, *Bombyx mori* L. α- and β-esterase bands, Est-2 and 3 in haemolymph were nonspecific as there were no differences in R_f values of each band in haemolymph of α- and β-esterase (Table 1). The results suggested substrate specificity of haemolymph esterases in *B. mori*. Moorthy *et al*. (2007c), earlier reported the conspicuous presence of five and eight bands in the fat body and midgut tissues of pure Nistari strain exposed to various temperature regimes for eight hours, with characteristic change in expression of Est-1 and Est-3 depending on exposing temperatures. Similar studies in haemolymph of pure multivoltine breeds like Nistari, NK4 and Cambodge showed the presence of three bands (Moorthy *et al*., 2007b). Jing *et al*. (2005), summarized the induction of Est-4 and Est-5 and depression of Est-2 in midgut of *Bombyx mori* L. ingested with Jatropherol-I from *Jatropha curcus* seeds.

The esterase bands showed intensity variation among the potential multivoltine breeds of *Bombyx mori* L. An understanding of intensity variations showed that β-Est 2 and 3 were deeply stained in case of M6DP(C) and faintly stained in M12W and CB5 breeds (Table 3). Moderately stained β-esterase bands were noticed in pure Nistari and developed M.Con1. Further, the response of the seven multivoltine silkworm strains to heat treatment at 70±2°C for 10 min, suggested the presence of thermostable nonspecific Est 3 in the haemolymph of the tropical strains (Fig. 2). Thermostable Est 3 was prominently present in M6DP(C), M6M81, M.Con.4 and Nistari as observed with the normal gel. Non- specific Est 2 lost its activity when the normal gel was heated to 70±2°C for 10 min before addition of β-napthylacetate as substrate. This esterase pattern proves and justifies the inherent tolerance capacity of these breeds to high temperature conditions. The results are in conformity with the findings of thermostable nonspecific β-Est-3 (R_f value of 0.53) in the haemolymph of tropical strain CB5, capable of withstanding a temperature of 80±1°C (Chattopadhyay *et al*., 2001).

An understanding of intensity variations in α-esterase bands (Fig. 3, Table 2) showed the prominent presence of specific α-Est 4 in M.Con.4 with moderate staining in M6DP(C) M.Con.1 and M12W

Table- 1: The relative mobility (R_f) value of each α- and β-esterase band in haemolymph of multivoltine and bivoltine breeds of *B. mori* using α- and β-napthylacetate as substrate. (Results of triplicate experiments are shown).

Esterase bands	α-esterase		β-esterase	
	Multivoltine	Bivoltine	Multivoltine	Bivoltine
Est-1	-	-	-	-
Est-2$	0.52	0.52	0.52	0.52
Est-3*$	0.43	0.43	0.43	0.43
Est-4*#	0.32	0.32	-	-
Est-5	-	-	-	

*Heat stable; #Specific esterase. Values are mean of triplicate $Non-specific esterase

Table- 2: Electrophoretic banding pattern showing the intensity variation of esterase isozymes in haemolymph of multivoltine breeds of *B. mori* scored using α- napthylacetate as substrate. Lane 1 – M6DP(C), Lane 2 – M.Con.1, Lane 3 – M12W, Lane 4 – M6M81, Lane 5 – M.Con.4, Lane 6 – Nistari, Lane 7 – CB5 '+', '++', '+++', - denotes faintly, medium and deeply stained and absent in substrate respectively.

Esterase bands	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7
Est-2	+++	+++	+	+	+	+++	++
Est-3	+	+	+	+	++	+	+
Est-4	+	++	++	+	+++	+	-

Table-3: Electrophoretic banding pattern showing the intensity variation of esterase isozymes in haemolymph of multivoltine breeds of *B. mori* scored using β- napthylacetate as substrate. Lane 1 – Lane 1 – M6M81, Lane 2 – Nistari, Lane 3 – M12W, Lane 4 – M6DP(C), Lane 5 – M.Con.1, Lane 6 – M.Con.4, Lane 7 – CB5 '+', '++', '+++', - denotes faintly, medium and deeply stained and absent in substrate respectively.

Esterase bands	**Lane 1**	**Lane 2**	**Lane 3**	**Lane 4**	**Lane 5**	**Lane 6**	**Lane 7**
Est-2	+++	++	+	+++	++	+++	+
Est-3	++	++	+	+++	++	++	+

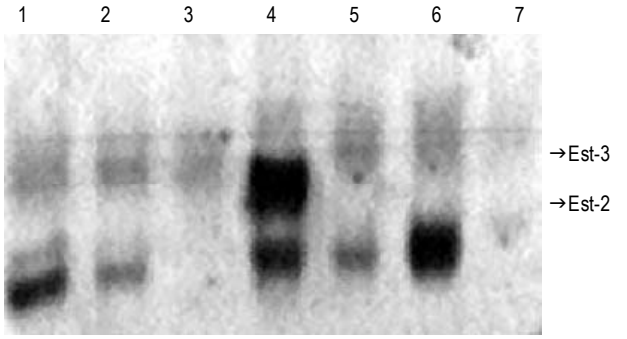

Fig. 1 : Vertical 8% PAGE of β-esterase isozyme pattern in haemolymph of multivoltine breeds of *B. mori*. Lane 1 – M6M81, Lane 2 – Nistari, Lane 3 – M12W, Lane 4 – M6DP(C), Lane 5 – M.Con.1, Lane 6 – M.Con.4, Lane 7 – CB5; Est – 2 and 3 are non-specific using 2% β-napthylacetate as a substrate. Results of triplicate experiments are shown.

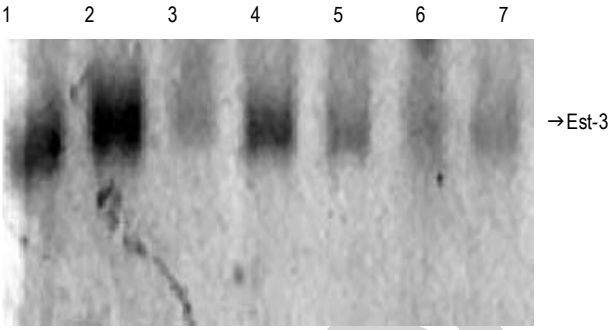

Fig. 2 : Vertical 8% PAGE of heat stable β-esterase isozyme pattern in haemolymph of multivoltine breeds of *B. mori*. Lane 1 - M6DP(C), Lane 2 - M6M81, Lane 3 - CB5, Lane 4 – M. Con.4, Lane 5 – Nistari, Lane 6 – M.Con.1, Lane 7 – M12W; Est-3 was detected when normal gel of haemolymph were incubated at 70 ± 2°C for 10 min prior to addition of 2% β-napthylacetate as a substrate. Results of triplicate experiments are shown.

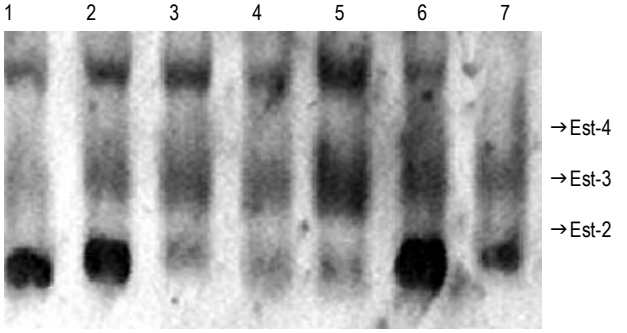

Fig. 3 : Vertical 8% PAGE of α-esterase isozyme pattern in haemolymph of multivoltine breeds of *B. mori*. Lane 1 – M6DP(C), Lane 2 – M.Con.1, Lane 3 – M12W, Lane 4 – M6M81, Lane 5 – M.Con.4, Lane 6 – Nistari, Lane 7 – CB5; Est – 2 and 3 are non-specific and Est – 4 are specific using 2% α-napthylacetate as a substrate. Results of triplicate experiments are shown.

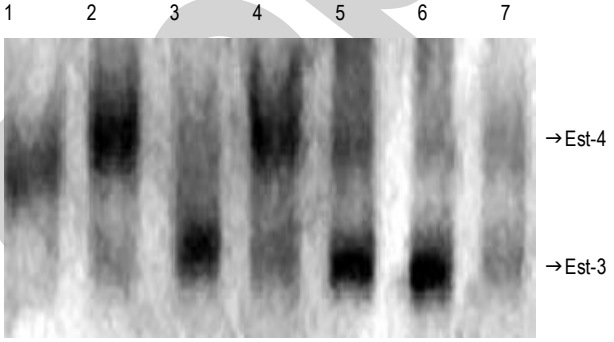

Fig. 4 : Vertical 8% PAGE of heat stable α-esterase isozyme pattern in haemolymph of multivoltine breeds of *B. mori*. Lane 1 - M6DP(C), Lane 2 - M6M81, Lane 3 - CB5, Lane 4 – M. Con.4, Lane 5 – Nistari, Lane 6 – M.Con.1, Lane 7 – M12W; Est-3 and 4 bands are detected when normal gel of haemolymph were incubated at 70 ± 2°C for 10 min prior to addition of 2% α-napthylacetate as a substrate. Results of triplicate experiments are shown.

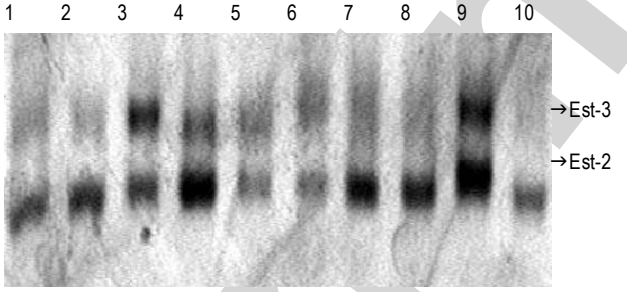

Fig. 5 : Vertical 8% PAGE of β-esterase isozyme pattern in haemolymph of bivoltine breeds of *B. mori*. Lane 1 – MC4(O), Lane 2 – SK7, Lane 3 – B.Con.4, Lane 4 – B.Con.1, Lane 5 – D6(P)S, Lane 6 – SK6, Lane 7 – NB_4D_2, Lane 8 – D6(P)N, Lane 9 – YB, Lane 10 – SK4(C); Est – 2 and 3 are non-specific using 2% β-napthylacetate as a substrate. Results of triplicate experiments are shown.

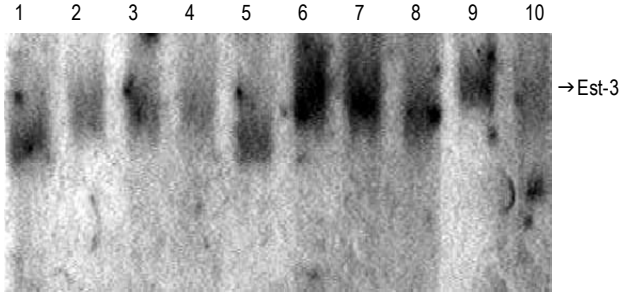

Fig. 6 : Vertical 8% PAGE of heat stable β-esterase isozyme pattern in haemolymph of bivoltine breeds of *B. mori*. Lane 1 – MC4(O), Lane 2 – SK7, Lane 3 – B.Con.4, Lane 4 – B.Con.1, Lane 5 – D6(P)S, Lane 6 – SK6, Lane 7 – NB_4D_2, Lane 8 – D6(P)N, Lane 9 – YB, Lane 10 – SK4(C); Est-3 was detected when nor mal gel of haemolymph were incubated at 70 ± 2°C for 10 min prior to addition of 2% β-napthylacetate as a substrate. Results of triplicate experiments are shown.

and absence in CB5 breeds of *Bombyx mori*. The results also established the novelty of specific α-Est 4 in combination with nonspecific Est-3 as thermostable esterase in multivoltine breeds of silkworm (Fig. 4).

The β-esterase isozyme pattern in haemolymph of selected bivoltine breeds showed two bands designated β-Est-2 and 3 (Fig. 5, lane 1-10) with R_f values of 0.52 and 0.43 suggesting homogeneity in the band presence. Est-2 and 3 are non-specific esterases as they were stained with both α- and β-napthylacetate separately. An understanding of intensity variations showed that β-Est 2 and β-Est 3 was deeply stained in case of B.Con.4, NB_4D_2, D6(P)N & YB and B.Con.1 and YB respectively (Table 5). The

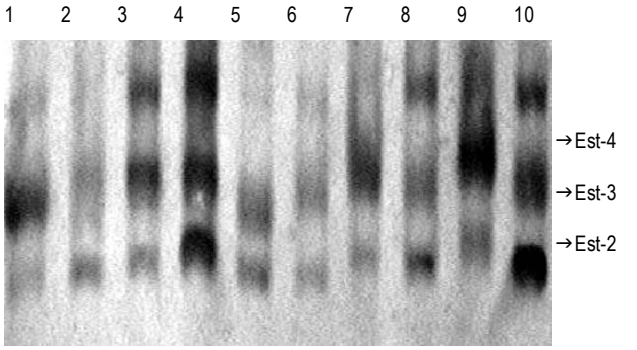

Fig. 7 : Vertical 8% PAGE of α-esterase isozyme pattern in haemolymph of bivoltine breeds of *B. mori*. Lane 1 MC4(O), Lane 2 – SK7, Lane 3 – B.Con.4, Lane 4 – B.Con.1, Lane 5 – D6(P)S, Lane 6 – SK6, Lane 7 – NB_4D_2, Lane 8 – D6(P)N, Lane 9 – YB, Lane 10 – SK4(C) ; Est – 2 and 3 are non-specific and Est – 4 is specific using 2% α-napthylacetate as a substrate. Results of triplicate experiments are shown.

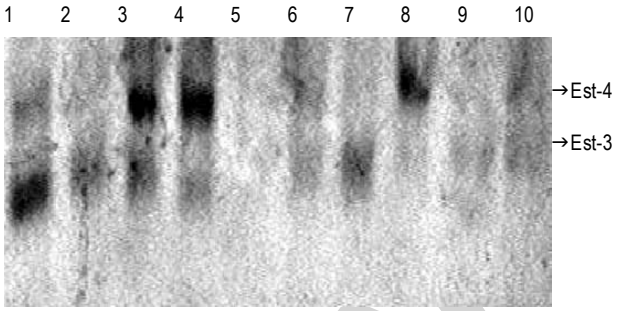

Fig. 8 : Vertical 8% PAGE of heat stable α-esterase isozyme pattern in haemolymph of bivoltine breeds of *B. mori*. Lane 1 MC4(O), Lane 2 – SK7, Lane 3 – B.Con.4, Lane 4 – B.Con.1, Lane 5 – D6(P)S, Lane 6 – SK6, Lane 7 – NB_4D_2, Lane 8 – D6(P)N, Lane 9 – YB, Lane 10 – SK4(C); Est-3 and 4 bands are detected when normal gel of haemolymph were incubated at 70 ± 2°C for 10 min prior to addition of 2% α-napthylacetate as a substrate.

α-esterase isozyme pattern in haemolymph of selected bivoltine breeds showed three conspicuous bands, designated α-Est-2, 3 and 4 (Fig. 7, lane 1-10) with R_f values of 0.52, 0.43 and 0.32. Although homogenous distribution of non-specific Est-2 and 3 was noticed in all the selected breeds, α-Est 4 was absent in breeds D6(P)S and YB. The significant findings were the presence of substrate specific band such as α-Est 4 in MC4(O), SK7, B.Con.4, B.Con.1, SK6, NB_4D_2, D6(P)N and SK4(C) breeds of silkworm, *Bombyx mori* L.

The prominent presence of specific α-Est 4 in B.Con.4, B.Con.1 and SK4(C) with moderate staining in D6(P)N and absence in D6(P)S and YB were observed among bivoltine strains of silkworm, *Bombyx mori* L. (Table 4). The novel presence of specific α-Est 4 in combination with nonspecific Est-3 as heat-stable bands (Fig. 8, lane 1-10) were noticed in bivoltine breeds but with reduction in induction when incubated at 70±2°C for 10 min in comparison with the normal gel. Most of the potential bivoltine breeds were developed through introgression of multivoltine character for survivability with B.Con.4 and B.Con.1 representing congenic lines of bivoltine D6P and JPN crossed with M6DP(C) and CB5 as donor multivoltine syngenic parents respectively. This was studied in relation to the presence of esterase heterogeneity, syngenic lines development and thermostable β-esterase band Est-3 in haemolymph of tropical silkworm, *Bombyx mori* L. (Chattopadhyay *et al*., 2001). The observation documented polymorphism in the esterase spectra with two non-specific β-esterase bands (Est-2 and Est-3) and five α-esterase bands (Est-1 to Est-5) in the haemolymph.

Similarly, Moorthy *et al*. (2007a) introduced Est-3 with Rm value of 0.247 into bivoltine SK4 from robust multivoltine parent, Cambodge to develop bivoltine SK4(C). Ashwath and Morrison (2001) used digestive amylase, as a surrogate marker in developing high survival bivoltine using multivoltine as donor. Polymorphism in relation to non-specific esterases in pupal haemolymph with polylocus control have been substantiated in eight introduced breeds of silkworm in Bulgaria by Staykova (2008).Wu and Hou (1993) studied heat-stable esterase (Est-5) activity in midgut of temperate silkworm strains after consecutive heat selection for 7 generations and suggested the strengthening of activity by rearing under high temperatures. Results therefore indicated a close relationship between heat-stable esterase and thermotolerance.

Table- 4: Electrophoretic banding pattern showing the intensity variation of esterase isozymes in haemolymph of bivoltine breeds of *B. mori* scored using α- napthylacetate as substrate. Lane 1 MC4(O), Lane 2 – SK7, Lane 3 – B.Con.4, Lane 4 – B.Con.1, Lane 5 – D6(P)S, Lane 6 – SK6, Lane 7 – NB_4D_2, Lane 8 – D6(P)N, Lane 9 – YB, Lane 10 – SK4(C) '+', '++', '+++', - denotes faintly, medium and deeply stained and absent in substrate respectively.

Esterase bands	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9	Lane 10
Est-2	++	++	+++	+++	++	++	++	++	++	+++
Est-3	+++	++	+++	+++	++	++	++	++	+++	+++
Est-4	+	+	+++	+++	-	+	+	++	-	+++

Table- 5: Electrophoretic banding pattern showing the intensity variation of esterase isozymes in haemolymph of bivoltine breeds of *B. mori* scored using β- napthylacetate as substrate. Lane 1 MC4(O), Lane 2 – SK7, Lane 3 – B.Con.4, Lane 4 – B.Con.1, Lane 5 – D6(P)S, Lane 6 – SK6, Lane 7 – NB_4D_2, Lane 8 – D6(P)N, Lane 9 – YB, Lane 10 – SK4(C) '+', '++', '+++', - denotes faintly, medium and deeply stained and absent in substrate respectively.

Esterase bands	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9	Lane 10
Est-2	++	++	++	+++	+	+	+++	+++	+++	++
Est-3	+	+	+++	++	+	+	+	+	+++	+

The thermostability of specific α-Est 4 has been reported for the first time in both multivoltine and bivoltine breeds, *Bombyx mori* . and would therefore help us to confer its relation to thermotolerance and some aspects of physiological performance or fitness. The characterization of these candidate thermostable esterases will enlighten its suitability as markers towards heat resistance and could be explored in the future breeding practices.

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