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Variation in esterases activity of different races and hybrids of *Bombyx mori* l. as a tool for genetic relationships analysis

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ABSTRACT

Variation in esterases activity in different silkworm races and hybrids were investigated to determine the usefulness of this marker for the assessment of genetic variation among them. A study on the haemolymph esterases pattern of different silkworm populations from different origins (two races and five hybrids) kept in Egypt was undertaken for enhancing the breeding program in the Sericulture Research Department (SRD).

The larval haemolymph showed esterolytic activity capable of hydrolysing α and β naphthyl acetate substrates. A total of 14 α and 10 β – esterase and 21 α and 24 β-esterase bands were detected during the fourth and the fifth larval instars, respectively.

By evaluating the degree of similarity among the tested populations, it was found that similarity of α esterase between Race 2, Hybrids 2 and 3 during the fourth larval instar was 50%. The similarity percentage was 40% between Race 2 and Hybrid 5 during the fifth larval instar. For β esterase, the similarity was 66% between Race 2 and both Hybrid 1 and Hybrid 4 during the fourth larval instar and was 66% between Race 2 and Hybrid 5 during the fifth larval instar.

It may be recommended to dispense Race 2 from the breeding program as it shows the highest degree of similarity with the other tested populations.

At the physiological level, esterases appeared to be a suitable marker to estimate the genetic variation among *Bombyx mori* races and hybrids.

Keywords : *Bombyx mori*, esterases and genetic variation

INTRODUCTION

Gel electrophoresis of proteins and isozymes are powerful tools to distinguish between genotypes (He, 1995). It has been studied in silkworm by Takeda *et al*., 1990 and 1993; Nagaoka *et al*., 1997 and Popov *et al*., 1999. Most of them have been made on the esterases (Krishnamurthy & Umakanth, 1997; Stoykova *et al*., 1998) as esterase exhibits a greater degree of polymorphism than other enzymes because they act on a class of molecules many of which come directly from external environment (Kojima *et al*., 1970). Most enzymes of this class hydrolyze endogenous substances and are important in intermediary metabolism (Sivakumarm and Maya, 1991).

A study on the variation in esterases activity of seven *Bombyx mori* populations was carried out to determine the usefulness of this marker for the assessment of genetic variation among them. The haemolymph esterases pattern of different silkworm populations from different origins (two races and five hybrids) kept in Egypt were determined to use as a tool of selection for enhancing the breeding program in the Sericulture Research Department (SRD).

It can be concluded that, studying the esterases pattern reflects the activity and the healthy of the living organism as

well as determining the similarity degrees and the genetic variation among the selected races and hybrids. The establishment of a suitable biochemical marker for analyzing the degree of genetic relationships between different populations is a tool for increasing the selection effectiveness as reported by Staykova & Grekov (2005) and may facilitate maintenance, conservation and cost effective management of plant and animal genetic resources (Bruford and Wyne, 1993). Thereby helps to eliminate duplicates and reduce the cost of germplasm maintenance and volume of work.

MATERIAL AND METHODS

Bioassay:

Silkworm eggs of two races and five hybrids were obtained from the Sericulture Research Department (SRD) of Plant Protection Research Institute:

380 a Chinese monovoltine race imported from Italy: coded as Race 1

Novi a European monovoltine race imported from Italy: coded as Race 2

ChJ a Chinese X Japanese hybrid: coded as Hybrid 1

DCh a Japanese X Chinese hybrid: coded as Hybrid 2

Tail a Thai hybrid: coded as Hybrid 3

TJ a Thai X Japanese hybrid: coded as Hybrid 4

TCh a Thai X Chinese hybrid: coded as Hybrid 5

The rearing technique was done following the standard methodology of rearing in SRD. Larvae were fed on leaves of Kokuzo-27 mulberry variety.

Esterase determination:

Polyacrylamide gel electrophoresis was done on a 1.0 mm vertical slap according to Turunen & Chippendale, (1977) at the Central Lap, Faculty of Education. The running gel (8%) was prepared in 1.5 M Tris buffer pH (8.8); the stacking gel (4%) was made with 0.5 M Tris buffer pH (6.8). The temperature of the gel was maintained at 7°C by refrigerating the gel chamber during a run. The gel was stained for esterolytic activity by incubation at 25°C in a solution of 100 mg α-naphthyl acetate in 2 ml acetone (as substrate) and 100 mg fast blue RR salt (as diazocoupler) in 200 ml of 0.1 M phosphate buffer, pH 6.5 (Sell *et al*., 1974). The α-naphthol, which was released on hydrolysis of the substrate, coupled with the dye salt to produce an insoluble pigment at the site of enzyme activity. β-naphthyl acetate also used as substrate. After incubation, the gel was distained in 7% acetic acid.

The gels were scanned to calculate the relative mobility and concentration of identified bands using Gel-Pro Analyzer software (V.3).

Similarity Index:

The similarity index between all tested races and hybrids were calculated according to Nei & Li (1979) using the formula

$$S.I = \frac{2N}{Na + Nb}$$

S.I: Similarity Index

N: the number of common bands

Na and Nb: the number of bands in individuals (a) and (b).

RESULTS AND DISCUSSION

A- Electrophoretic analysis of esterases pattern:

Polyacrylamide gel electrophoresis has been widely used for separation of different enzymes which help in explanation of different biological processes that occur inside the living organism (Staykova & Grekov 2005). Esterases can often be separated into isozymes with different substrate specificities (Dauterman, 1985).

In the present study, the analysis of larval haemolymph α-esterase enzyme showed a total of 14 and 21 polypeptide

fractions with different densities during the fourth and fifth larval stages, respectively.

The fourth larval instar had 14 bands of esterolytic activity capable of hydrolysing α-naphthyl acetate. The bands distributed in 9 zones with relative fragmentations (Rf) ranging from 0.24 to 0.63 (Table 1- Fig 1). In the fifth larval instar 21 bands distributed in 14 zones with Rf ranging from 0.20 to 0.59 were detected (Table 2- Fig 1).

Table 1: α- esterase pattern in the haemolymph of the fourth and fifth larval instars.

Fourth instar

Lanes	**lane 1 Race 1**		**Lane 2 Hybrid 1**		**Lane 3 Hybrid 2**		**Lane 4 Race 2**		**Lane 5 Hybrid 3**		**Lane 6 Hybrid 4**		**Lane 7 Hybrid 5**	
Rows	**Rf**	**Am%**	**Rf**	Am%	Rf	Am%	**Rf**	**Am%**	**Rf**	**Am%**	**Rf**	**Am%**	**Rf**	**Am%**
R 1			0.24	18.48										
R 2							0.25	17.46					0.25	9.57
R 3											0.28	8.80		
R 4													0.39	16.24
R 5			0.42	19.05										
R 6							0.44	15.96						
R 7			0.61	62.47							0.61	91.20	0.61	74.19
R 8	0.62	100												
R 9					0.63	100	0.63	66.58	0.63	100				

Fifth instar

lanes	**Lane 8 Race 1**		**Lane 9 Hybrid 1**		**Lane 10 Hybrid 2**		**Lane 11 Race 2**		**Lane 12 Hybrid 3**		**Lane 13 Hybrid 4**		**Lane 14 Hybrid 5**	
Rows	**Rf**	**Am%**	**Rf**	**Am%**	**Rf**	**Am%**	**Rf**	**Am%**	**Rf**	**Am%**	**Rf**	**Am%**	**Rf**	**Am%**
R1									0.20	17.08				
R2			0.21	18.84										
R3	0.22	14.22			0.22	14.55								
R4							0.23	18.26			0.23	8.30	0.23	22.42
R5											0.39	14.36		
R6									0.40	30			0.40	38.70
R7	0.46	40									0.46	20.6		
R 8					0.48	31.93								
R 9											0.53	24.86		
R 10									0.55	52.92				
R 11							0.56	81.74						
R 12					0.57	53.52								
R 13			0.58	81.16							0.58	31.86		
R 14	0.59	45.78											0.59	38.88

Relative fragmentation (Rf) – Amount percentages (Am %)

Table 2: β- esterase pattern in the haemolymph of the fourth and fifth larval instars.

Fourth instar

Lanes	lane 1 Race 1		Lane 2 Hybrid 1		Lane 3 Hybrid 2		Lane 4 Race 2		Lane 5 Hybrid 3		Lane 6 Hybrid 4		Lane 7 Hybrid 5	
Rows	**Rf**	**Am%**	**Rf**	Am%	Rf	Am%	**Rf**	**Am%**	**Rf**	**Am%**	**Rf**	**Am%**	**Rf**	**Am%**
R 1													0.22	7.49
R 2							0.46	36.96					0.46	46.69
R 3	0.49	100											0.49	45.82
R 4			0.51	100			0.51	63.04			0.51	100		
R 5					0.52	100								
R 6									0.53	100				

Fifth instar

lanes	Lane 8 Race 1		Lane 9 Hybrid 1		Lane 10 hybrid 2		Lane 11 Race 2		Lane 12 Hybrid 3		Lane 13 Hybrid 4		Lane 14 Hybrid 5	
Rows	**Rf**	**Am%**	**Rf**	**Am%**	**Rf**	**Am%**	**Rf**	**Am%**	**Rf**	**Am%**	**Rf**	**Am%**	**Rf**	**Am%**
R1					0.16	10.15								
R2	0.20	15.68	0.20	11.76										
R3									0.21	9.97				
R4					0.22	8.97								
R5							0.23	9.49			0.23	10.84	0.23	19.32
R6											0.34	16.62		
R7													0.35	35.80
R 8					0.39	12.25					0.39	17.57		
R 9	0.40	40.55												
R 10			0.42	52										
R 11									0.43	39.17				
R 12					0.45	30.87	0.45	44.34						
R 13	0.48	43.77	0.48	36.24										
R 14					0.49	37.76			0.49	50.86				
R 15							0.50	46.17					0.50	44.88
R 16											0.51	54.97		

Relative fragmentation (Rf) – Amount percentages (Am %)

Race 1, Hybrid 2 and Hybrid 3 had one band during the fourth larval instar with Rf 0.62, 0.63 and 0.63, respectively. Three bands during the fifth larval instar with Rf values ranged from 0.22 to 0.59, however, no common bands were detected among them.

Hybrid 4 showed the highest enzyme activity. The number of bands increased from two bands with Rf 0.28 and 0.61 in the fourth instars to five bands with Rf values ranged from 0.23 to 0.58 in the fifth larval instars.

Hybrid 5 showed three bands in the fourth and in the fifth larval instars with different amount percentages. In the fourth larval instar, the Rf values were 0.25, 0.39 and 0.61 with amount percentages 9.57, 16.24 and 74.19, respectively. While in the fifth larval instar, the Rf values were 0.23, 0.40 and 0.59 with amount percentages 22.42, 38.70 and 38.88, respectively.

On the other hand, both Race 2 and Hybrid 1 showed the same trend, the enzyme activity was decreased from three bands with Rf values ranged from 0.24 to

0.63 during the fourth larval instar to two bands with Rf values ranged from 0.21 to 0.58 during the fifth larval instar.

The Common bands were detected during the fourth larval instar between: Hybrid 2, Race 2 and Hybrid 3 with Rf value 0.63 and amounts 100%. Hybrid 1, Hybrid 4 and Hybrid 5 with Rf value 0.61 and amounts 62.47, 91.20 and 74.19%, respectively.

The detected common bands during the fifth larval instar were between Race 1 and Hybrid 2 with Rf value 0.22 and amounts 14.22 and 14.55%. Race 2, Hybrid 4 and Hybrid 5 with Rf value 0.23 and amounts 18.26, 8.30 and 22.42%, respectively. Hybrid 3 and Hybrid 5 with Rf value 0.40 and amounts 30 and 38.70%, respectively. Race 1 and Hybrid 4 with Rf value 0.46 and amounts 40 and 20.6%, respectively. Hybrid 1 and Hybrid 4 with Rf value 0.58 with amounts 81.16 and 31.86%, respectively. Race 1 and Hybrid 5 with Rf value 0.59 and amounts 45.78 and 38.88%, respectively.

The differentiation in densities could be attributed to the insect development and expression of resistance which concerned with the changes of the insect physiological states as esterase patterns are related to the degree of resistance and attributed to genetic variations within and among populations. (Owusu *et al*., 1996).

Larval haemolymph of the selected races and hybrids of the fourth larval instar had 10 bands of esterolytic activity capable of hydrolysing β-naphthyl acetate. The bands distributed in 6 zones with relative fragmentations (Rf) ranging from 0.22 to 0.53. In the fifth larval instar; 24 bands distributed in 16 zones with Rf ranging from 0.16 to 0.51 were detected (Table 2-Fig. 2).

Race 1, Race 2, Hybrid 1, 2, 3 and Hybrid 4 showed increase in enzyme activity from the fourth to the fifth larval instars. Race 1, Hybrid 1 and Hybrid 3 had one band in the fourth larval instar with Rf values 0.49, 0.51 and 0.53, respectively. The activity increased in the fifth larval instar and showed 3 bands with Rf values ranged from 0.20 to 0.49. Race 2 showed slight increase, two bands in the fourth larval instar with Rf 0.46 and 0.51 increased to three bands with Rf values 0.23, 0.45 and 0.50 in the fifth larval instar**.** Hybrid 2 showed the highest increment, it recorded one band in the fourth larval instar with Rf 0.52 increased to five bands in the fifth larval instars with Rf values 0.16, 0.22, 0.39, 0.45 and 0.49. While Hybrid 5 showed only three bands during both fourth and five instars with Rf values ranged from 0.22 to 0.50.

The Common bands were detected during the fourth larval instar between: Race 1 and Hybrid 5 at 0.49 with 100% and 45.82 % activities, respectively. Hybrid 1, Race 2 and Hybrid 3 at Rf 0.51 with 100, 63.04 and 100% activities, respectively.

For the fifth larval instar the common bands were detected between, Race 1and Hybrid 1at Rf 0.20 with 15.68 and 11.76% activities, respectively. Race 2, Hybrid 4 and Hybrid 5 at Rf 0.23 with 9.49, 10.84 and 19.32%, respectively. Hybrid 2 and Hybrid 4 at Rf 0.39 with 12.25 and 17.57%, respectively. Hybrid 2 and Race 2 at Rf 0.45 and 30.87 and 44.34% activities, respectively. Race 1 and Hybrid 1 at Rf 0.48 with 43.77 and 36.24 % activities, respectively. Hybrid 2 and Hybrid 3 at Rf 0.49 with 37.76 and 50.86% activities, respectively. Race 2 and Hybrid 5 at Rf 0.50 with 46.17 and 44.88% activities, respectively.

Differences in esterase synthesis among stages are probably due to regulatory mechanisms acting in agreement with the requirements of a variable number of processes in which esterases are involved. Because of the larval stage is the most active in developmental processes and shows very intense intake of food and very high mobility, the variation in general esterase activity within samples is thought to be the result of age structure dynamics within the population. These features may need the increment of esterase production at that stage as suggested by Lima-Gatelani *et al*., 2004. According to Staykova *et al*., (2004), there is a correlation between the

number and activity of enzyme forms during the development of mulberry silkworm which was ascertained in the present study. As the larval haemolymph showed stage specific expression of α- and β- esterases with different amounts and Rf values.

The obtained results clearly clarified that esterases of *B. mori* haemolymph showed greatest specific activity toward α-naphthyl acetate and β-naphthyl acetate substrates. These results are in agreement with El-Bermawy, (2000) on *Bombyx mori* and *Spodoptera littoralis*.

The studied races and hybrids had common bands with the same Rf values, the enzyme activities or the enzyme amounts differed among them, which reflect the physiological conditions and processes. Thus, the similarities among theses races and hybrids can be estimated.

B- Similarity Index (S.I):

Estimating the similarity index (S.I) for α- esterase activity between the different populations during the fourth larval instar clarified that, the most similar populations were Hybrid 2 and Hybrid 3, as the S.I was 100%. Followed by Race 2, which recorded 50% similarity with both Hybrid 2 and Hybrid 3 (Table 3).

During the fifth larval instar, α-esterase activity was not exceeding 40% between tested populations. This percentage was recorded between Race 2 and Hybrid 5 (Table 4). It was obvious that, Race 2 was somewhat similar to the tested hybrids, (Hybrid 2, 3 and 5) during fourth and fifth larval stages.

Table 3: The Similarity Index (S.I) between tested samples of the fourth instar α – esterase

	S.I						
Lanes	**1**	**2**	**3**	**4**	**5**	**6**	**7**
1	/////	0	0	0	0	0	0
2		/////	0	0	0	0.4	0.34
3			/////	0.5	**1**	0	0
4				/////	0.5	0	0.34
5					/////	0	0
6						/////	0.4
7							/////

Lane 1: Race 1 Lane 2: Hybrid 1
Lane 3: Hybrid 2 Lane 4: Race 2
Lane 5: Hybrid 3 Lane 6: Hybrid 4
Lane 7: Hybrid 5

Table 4: The Similarity Index (S.I) between tested samples of the fifth instar. α- esteras

	S.I							
Lanes	**8**	**9**	**10**	**11**	**12**	**13**	**14**
8	/////	0	0.34	0	0	0.25	0.34
9		/////	0	0	0	0.29	0
10			/////	0	0	0	0
11				/////	0	0.29	**0.4**
12					/////	0	0.34
13						/////	0.25
14							/////

Lane 8: Race 1 Lane 9: Hybrid 1
Lane 10: Hybrid 2 Lane 11: Race 2
Lane 12: Hybrid 3 Lane 13: Hybrid 4 Lane 14: Hybrid 5

For β esterase activity during the fourth larval instar, Hybrid 1 recorded the highest similarity percentages, as it was 100% with Hybrid 4 and 66% with Race 2. Followed by Race 2 which similar to Hybrid 4 by 66% and Hybrid 5 by 40 % (Table 5).

Table 5: The Similarity Index (S.I) between tested samples of the fourth instar

β- esterase

	S.I						
Lanes	**1**	**2**	**3**	**4**	**5**	**6**	**7**
1	/////	0	0	0	0	0	0.5
2		/////	0	0.66	0	**1**	0
3			/////	0	0	0	0
4				/////	0	0.66	0.4
5					/////	0	0
6						/////	0
7							/////

Lane 1: Race 1 Lane 2: Hybrid 1
Lane 3: Hybrid 2 Lane 4: Race 2
Lane 5: Hybrid 3 Lane 6: Hybrid 4
Lane 7: Hybrid 5

During the fifth larval instar the similarity percentage did not exceed 66%. Race 2 recorded 66% similarity with Hybrid 5 and 29% with Hybrid 4. Also between Race 1 and Hybrid 1 (S.I = 66%) (Table 6).

Table 6: The Similarity Index (S.I) between tested samples of the fifth instar

β – esterase

	S.I							
Lanes	**8**	**9**	**10**	**11**	**12**	**13**	**14**
8	/////	0.66	0	0	0	0	0
9		/////	0	0	**0**	0	0
10			/////	0.25	0.25	0.23	0
11				/////	0	0.29	0.66
12					/////	0	0
13						/////	0.23
14							/////

Lane 8: Race 1 Lane 9: Hybrid 1
Lane 10: Hybrid 2 Lane 11: Race 2
Lane 12: Hybrid 3 Lane 13: Hybrid 4
Lane 14: Hybrid 5

It may be concluded that, as the larvae aged the enzyme activity differed among the selected races and hybrids.The differentiation among tested samples may be related to age – structure dynamics within populations and to geographical distances between samples selected. These results agreed with Pruett *et al.*, (2001). It seemed to direct their biochemical processes to minimize the effect of toxic materials found in environment as suggested by El-Bermawy (2004). It can be recommended to include Race 1 and the other tested hybrids and dispense Race 2 from the breeding program, to manage costs and labor intense as this race showed the highest similarity with the other tested populations at α and β enzyme activities levels.

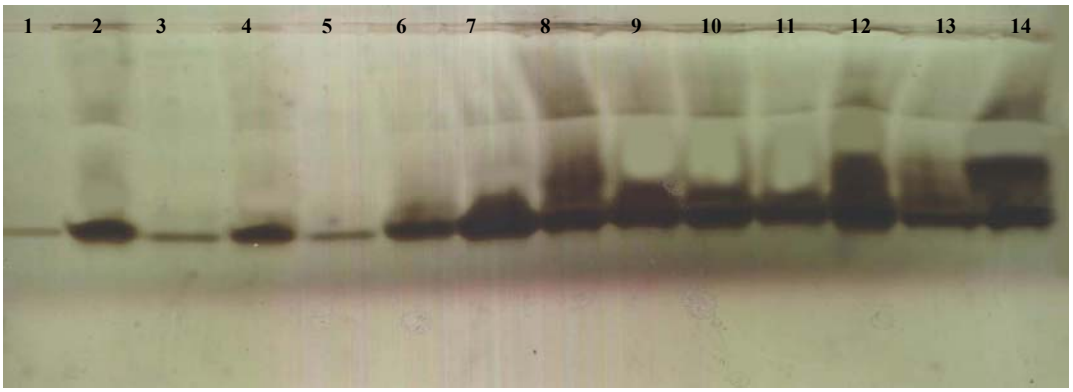

Fig 1

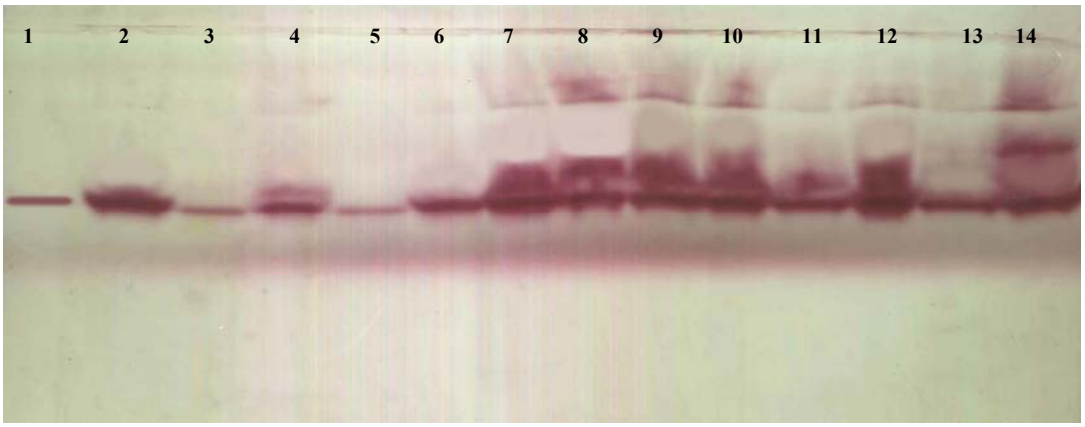

Fig 2

Esterases pattern of *Bombyx mori* L. haemolymph in the fourth and fifth larval instars using α-naphthyl acetate as substrate (1) and β-naphthyl acetate as substrate (2) for all tested races and hybrids.

Lane 1: Race 1 Lane 2: Hybrid 1 Lane 3: Hybrid 2 Lane 4: Race 2 Lane 5: Hybrid 3 Lane 6: Hybrid 4 Lane 7: Hybrid 5

Lane 8: Race 1 Lane 9: Hybrid 1 Lane 10: Hybrid 2 Lane 11: Race 2 Lane 12: Hybrid 3 Lane 13: Hybrid 4 Lane 14: Hybrid 5

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ARABIC SUMMARY

دراسة التباين فى نشاط إنزيم الإستيراز فى سلالات وهجن مختلفه من بومبكس مورای إل وإستخدامها كأداه لتحليل التنوع الجيني بينهم

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تم دررراسه الإختلاف فى نشاط إنزيم الإستيراز فى سلالات وهجن دودة الحرير المختلفه وذلك لتحديد فائدتها كدلاله لتقييم الإختلافات الجينيه بينهم.

لذلك قد تم دراسة شكل إنزيم الإستيراز فى هيموليمف مجموعات مختلفه من ديدان الحرير (٢ سلاله ٥ هجن) ذات منشأ مختلف والتى تحفظ فى مصر بغرض تحسين برنامج التربيه والإنتخاب لقسم بحوث الحرير.

وقد أظهر هيوليمف اليرقات نشاط إنزيمي قادر على تحليل مادتى ألفا وبيتا أسيتات. وقد تم الكشف عن ١٤ حزمه ألفا و١٠ حزم بيتا إستيرز و ٢١ حزمه ألفا و ٢٤ حزمه بيتا خلال العمرين الرابع والخامس على التوالى.

عن طريق تقدير درجة التشابه بين المجموعات المختلفه تحت الدراسه فقد وجد ان التشابه فى إنزيم ألفا إستيرز بين سلاله ٢ والهجين ٢ والهجين ٣ خلال العمر الرابع كانت ٥٠ % و نسبه التشابه كانت ٤٠ % بين السلاله ٢ والهجين ٥ خلال العمر الخامس.

أما بالنسبه للأنزيم بيتا إستيراز قد كانت نسبة التشابه ٦٦% بين السلاله ٢ وكل من هجين ١ و هجين ٤ خلال العمر الرابع و ٦٦% بين السلاله ٢ والهجين ٥ خلال العمر الخامس.

وبذلك يمكن التوصيه بإستبعاد السلاله ٢ من برنامج التربيه والإنتخاب حيث أظهرت أعلى درجه من التشابه مع المجموعات المختبره.

ويمكن إستنتاج ان نمط إنزيم الإستيراز يعكس نشاط الحشره ودرجة الإختلاف الجيني بين المجموعات المختلفه. وعلى المستوى الفسيولوجى فقد أظهر إنزيم الإستيراز انه دلاله مناسبه لتقدير الإختلافات الجينيه بين سلالات و هجن دوده الحرير التوتيه.