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Genetic Analysis of Isoenzyme Polymorphism in Silkworm (*Bombyx mori* L.) (Lepidoptera: Bombycidae) Strains and Phylogenetic Relationships

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Abstract: This study was carried out in order to evaluate the polymorphism in the silkworm of thirty strains of various origins using the isoenzyme electrophoresis for detecting biochemical markers and to investigate the population genetics of those strains. The isozymes of nonspecific esterases (EST), malate dehydrogenase (MDH) and acid phosphatase (ACP) from the hemolymph, as well as phosphoglucomutase (PGM), hexokinase (HK) and aspartat aminotranspherase (AST) from the silk glands were studied by electrophoresis in 7.5% polyacrylamide gel. A total of 9 loci were detected, and 8 (89%) of them were polymorphic. Intra- and inter-strain polymorphism was observed. The mean number of alleles per polymorphic locus ranged from 1.0 to 2.2. The value of F_{st} (0.4556) showed that the strains are differentiated. The obtained dendrogram with the values of genetic distance separated two main clusters and many sub-clusters. The acquired results demonstrated that the hemolymph EST, ACP and MDH as well as PGM and HK from the silk glands are appropriate markers for examining the genetic diversity and differentiating the silkworm strains.

Keywords: Lepidoptera, mulberry silkworm, phylogeny

Introduction

The economic and scientific significance of mulberry silkworm *Bombyx mori* L. have made it a subject of intensive genetic studies since the last century (Miao *et al*. 2005). This silkworm has a large number of geographical races and inbred lines, which show substantial variation in a large number of quantitative traits (Reddy *et al*. 1999). Using the appropriate markers to differentiate the silkworm strains makes it possible to develop a classification, which is important for the species breeding with regard to increasing the adaptive potential and productivity (Mirhoseini, Gholami 2002). The identification of isoenzyme and molecular markers is important to the breeding and marker-assisted selection in the silkworm.

The genetic resources of the mulberry silkworm kept in Bulgaria include more than 250 strains of various origins (Petkov *et al*. 2006). In order to differentiate the strains, the studies so far have concerned mainly some qualitative and quantitative traits, such as, among others, the total larval duration, cocoon shape, cocoon colour, weight of single cocoon, weight of single shell, shell ratio and fecundity. The selection of parental strains for a breeding programme is usually based on those parameters. The isoenzymic polymorphism and phylogeny has been less studied (Shabalina 1990, Stoykova *et al*. 2003, Staykova, Grekov 2006, Staykova 2006, Staykova 2008, Staykova *et al*. 2010, Staykova *et al*. 2012).

The present study aimed to determine the degree of diversity and the existing relationships between 30 silkworm strains of various origins kept in Bulgaria by using isoenzyme markers.

Material and Methods

Thirty strains of silkworm of various origins (Table 1) were obtained from the germplasm bank of the Sericulture Experiment Station in Vratsa and the Agricultural University in Plovdiv. All individuals were nourished under a standard regime of silkworm breeding. On the fifth day of the fifth instar, from 80 to 87 larvae were selected randomly from each strain (Table 3) and were subjected to electrophoretic analysis of the hemolymph and silk glands.

The tissue extracts were prepared according to Stoykova *et al*. (2003, 2004, 2012). The 7.5% polyacrylamide gel electrophoresis (PAGE) (Daevis *et al*. 1964) was applied to six enzyme systems: nonspecific esterases (EST – EC 3.1.1), malate dehydrogenase (MDH – EC 1.1.1.37) and acid phosphatase (ACP – EC 3.1.3.2) – from the hemolymph; and phosphoglucomutase (PGM – EC 5.4.2.2), hexokinase (HK – EC 2.7.1.1) and aspartate aminotransferase (AST – EC 2.6.1.1) from the silk glands. The used buffers and histochemical staining for each enzyme system were according to Shaw, Prasad (1970), Staykova *et al.* (2010), Spencer *et al*. (1964), Eaton *et al.* (1966), and Schmidtke, Engel (1972).

After the establishment of the isozyme activity regions, the phenotypes of the discovered loci were recorded. The allele frequencies, mean number of alleles per locus, proportion of polymorphic loci, observed (H_o) and expected (H_e) heterozygosity, deviation from the Hardy-Weinberg equilibrium, Nei's genetic distance (D) (1972), and Wright's fixation index, F_{ST} (Wright 1965), were calculated using BIOSYS-1 (Swofford, Selander 1981). The phylogenetic tree was constructed on the basis of Nei's genetic distance (Nei 1972) by the UPGMA method (Sneath, Sokal 1973), using the PHYLIP (Felsenstein 1993) software package.

Results and Discussion

Nine loci with a total of 27 alleles were observed in the analysed strains of six enzyme systems (Table 2). Four enzyme systems, i.e. nonspecific esterases, phosphoglucomutase, acid phosphatase and malate dehydrogenase, were polymorphic, with inter- and intra-strain polymorphism. The aspartate aminotransferase was monomorphic in all tested strains. The hexokinase manifested only inter-strain polymorphism.

Polygene control and polymorphism of nonspecific esterases in the hemoplymph of *Bombyx mori* L. were described earlier (Egorova *et al*. 1985, He 1995, Stoykova *et al*. 2003). Arai *et al*. (2000) reported that Bes B in the hemolymph is a carboxylesterase. In this study, we determined polymorphism on the loci Bes B (with three alleles), Bes D (with four alleles) and Bes E (with three alleles) in all tested strains (Table 2) with the following exceptions:

The locus Bes B was monomorphic in the strains Ukrainian 20 (with the allele Bes B_1 in the gene pool only), Vratza 40, Japanese 106, PS, China, and Jena (with the allele Bes B_2).

The locus Bes D demonstrated monomorphism in the strains Vratza 40, M-6, Asahi, J-90, Rg-90 (with the allele Bes D_1 in the gene pool only), Japanese 106, PS, China, E27 (with the allele Bes D_2 in the gene pool), and E28 strain (with the allele Bes D_3 only).

The locus Bes E was monomorphic in the strains Ogosta 1, M-6, Japanese 106, Asahi and MNB (with the allele Bes E_1 in the gene pool only), E10, Ukrainian 12, Ukrainian 20, China, Jena, E27, and

Table 1. Silkworm strains tested

Strain number	**Strains with white cocoon**	**Origin**
1	Vratza 1	BULGARIA
2	Vratza 37	
3	Vratza 40	
4	Belopol 1/18	
5	Belopol 2/21	
6	Gergana 1	
7	Gergana 2	
8	Ogosta 1	
9	Gindga 8	AZERBAIJAN
10	M-6	
11	TV	JAPAN
12	Japanese 106	
13	Asahi	
14	Kinshu	
15	E 10	EGYPT
16	J-90	ROMANIA
17	Alb Cislau 29	
18	Ukrainian 12	UKRAINE
19	Ukrainian 20	
20	Tashkent12	UZBEKISTAN
21	Syria 1	SYRIA
22	MNB	MADAGASCAR
	Strains with colored cocoon	
23	Rg-90	ROMANIA
24	PS	NORTH KOREA
25	China	CHINA
26	Jena	AUSTRIA
27	E 27	EGYPT
28	E 28	
29	E 29	
30	E 30	

E28 (with the "null" allele Bes E_0 in the gene pool).

The number of alleles as well as allele and genotype frequencies of the loci Bes B, D and E manifested the strain specificity.

In this study, we found polymorphism of the Bes A locus, which was not described before (Table 2). In the gene pool of the Egyptian strains E27 and E29, we found two alleles Bes A_1 and Bes A_2, while in the gene pool of the Romanian strain Alb Cislau 29 – the Bes A_1 and Bes A_0 alleles. The Bes A locus was monomorphic and presented by the allele Bes A_1 in the remaining strains. He (1995) described polymorphism with a "deletion" type esterase (BesAo) in some races and hybrids bred in China.

Three-allele polymorphism of the phosphoglucomutase locus in *B. mori* was described by Staykova (2006, 2008). In the gene pool of the tested strains Belopol 1/18, Belopol 2/21, Gergana 1, Ogosta 1, E 10, J-90, Tashkent 12, China, and E 29, the Pgm A locus was presented with all three alleles (Pgm A_1, A_2 and A_3 – Table 2). The allele Pgm A_1 was missing in Vratza 1, Vratza 37, Vratza 40, Gindga 8, TV, Kinshu, Ukrainian 20, Syria 1, Rg-90, Jena, E 27, and E 30. The allele Pgm A_3 was missing in Ukrainian 12, PS, and E 28. The allele Pgm A_2 was present in the gene pool of all tested strains. This allele was fixed in the gene pool of six of them: Gergana 2, M-6, Japanese 106, Asahi, Alb Cislau 29, and MNB (Table 2). Polymorphism of the Pgm locus in Asahi with three alleles and the highest frequency of the Pgm A_2 was described earlier by Staykova (2008). The fixing of this allele in the Asahi gene pool, as established in this study, was probably a result of gene drift.

Among the thirty strains included in the present study, we found polymorphism in five alleles on the acid phosphatase locus Bph (Bph A, Bph B, Bph C, Bph D and Bph 0 – Table 2). These results correspond with the results of Yoshitake, Akiyama (1964), Eguchi *et al*. (1988), and Staykova *et al*. (2010). We found (Table 2):

all five alleles in the gene pool of strains Vratza 37, Gandga 8 and Tashkent 12;

four alleles in Vratza 1, Vratza 40, TV, Asahi, E 10, J-90, Rg-90 and China (Bph A, B, C, 0); and in Ukrainian 12 (Bph A, C, D, 0), MNB and Alb Cislau 29 (Bph A, B, C, D);

tree alleles in Kinshu, Ukrainian 20, E 30 (Bph A, B, 0), and Jena (Bph A, B and D);

two alleles in Gergana 1, Gergana 2, Syria 1, PS and E 29 (Bph A, B), as well as in Belopol 1/18, Belopol 2/21, and E 27 (Bph A, 0).

The Bph A allele was fixed in the genebpool of strains M-6 and Japanese 106, while the Bph B allele in Ogosta 1, and Bph 0 in E28.

The allele frequencies on the acid phosphatase locus manifested strain specificity. The frequency of the Bph A allele was the highest for the PS strain, that of the Bph B allele – for Alb Cislau 29, of the Bph C allele – for Asahi, of the rare Bph D allele – for Jena, and of the Bph 0 – for the Egyptian E27 strain (Table 2).

We established tree-allele polymorphism on the Mdh locus in the Japanese Asahi and Kinshu strains (Mdh A_1, A_2 and A_3) (Table 2). Two of those alleles were presented in the gene pool of E30 (Mdh A_1 and A_2) and Ogosta 1 and E 29 (Mdh A_2 and A_3). The allele Mdh A_2 was the most common in all these strains and was fixed in the gene pool of all the remaining strains with exception of PS (Table 2). In the gene pool of PS, the Mdh A_3 allele was fixed. Our results correspond both with these of Marcato *et al*. (1990), who reported for lacking of polymorphism by the Mdh locus in some Italian strains, and Egorova, Nasirillaev (1993), who described polymorphism on the MDH of the hemolymph in some Russian strains.

We established inter-strain polymorphism of the hexokinase with Hk A_1 allele in the gene pool of Japanese 106, Jena and E 27, and with Hk A_2 – in the gene pool of the remaining strains (Table 2).

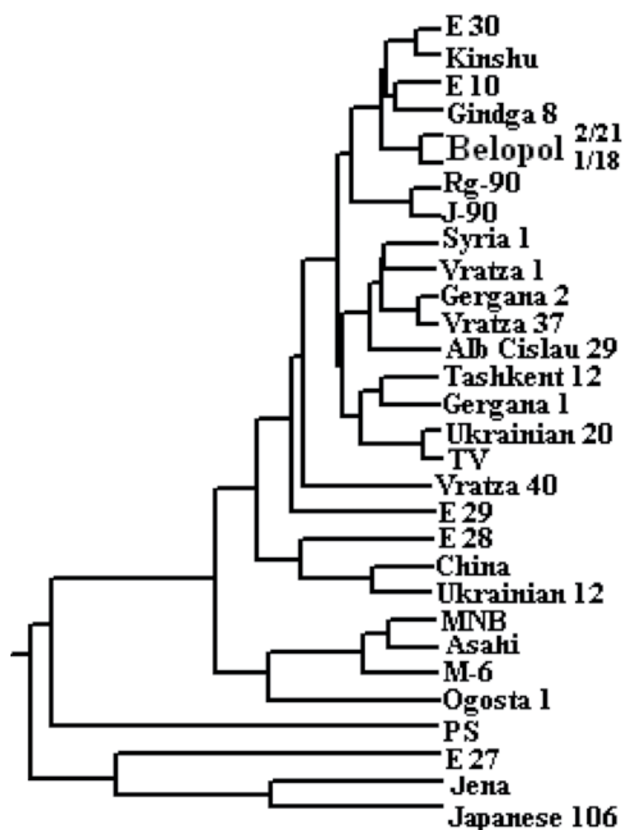

Fig. 1. UPGMA dendrogram constructed on the basis of genetic distance between studied strains of *Bombyx mori*.

Table 2. Allele frequencies in strains tested (strain's numbers correspond to the numbers from Table I)

Locus	Strain														
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Bes A															
A_1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
A_2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A_0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bes B															
B_1	.567	.183	0	.600	.256	.691	.200	0	.083	.283	.935	0	.078	.339	.150
B_2	0	.183	1.000	.325	.423	.147	.200	.528	.300	0	0	1.000	.203	.290	.550
B_3	.433	.633	0	.075	.321	.162	.600	.472	.617	.717	.065	0	.719	.371	.300
Bes D															
D_1	.350	.750	1.000	.138	.115	.824	.833	.556	.250	1.000	.306	0	1.000	.581	.383
D_2	.350	.250	0	.500	.654	0	.100	.444	.317	0	.694	1.000	0	.355	.417
D_3	.300	0	0	.363	.231	.176	.067	.000	.433	0	0	0	0	.065	.200
D_0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bes E															
E_1	.350	.167	.283	.112	.167	0	0	1.000	.283	1.000	.048	1.000	1.000	.258	0
E_2	.183	.000	.233	.112	.141	.338	.400	0	.017	0	.081	0	0	.194	0
E_0	.467	.833	.483	.775	.692	.662	.600	0	.700	0	.871	0	0	.548	1.000
Pgm A															
A_1	0	0	0	.038	.038	.265	0	.097	0	0	0	0	0	0	.333
A_2	.667	.900	.817	.438	.603	.500	1.000	.236	.750	1.000	.871	1.000	1.000	.306	.433
A_3	.333	.100	.183	.525	.359	.235	0	.667	.250	0	.129	0	0	.694	.233
Bph A															
A	.317	.618	.597	.225	.179	.529	.550	0	.333	1.000	.397	1.000	.219	.306	.464
B	.400	.206	.048	0	0	.471	.450	1.000	.152	0	.241	0	.047	.387	.196
C	.183	.074	.161	0	0	0	0	0	.076	0	.034	0	.641	0	.036
D	0	.015	0	0	0	0	0	0	.015	0	0	0	0	0	0
0	.100	.088	.194	.775	.821	0	0	0	.424	0	.328	0	.094	.306	.304
Ast A															
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Mdh A															
A_1	0	0	0	0	0	0	0	0	0	0	0	0	.031	.113	0
A_2	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.972	1.000	1.000	1.000	1.000	.859	.855	1.000
A_3	0	0	0	0	0	0	0	.028	0	0	0	0	.109	.032	0
Hk A															
A_1	0	0	0	0	0	0	0	0	0	0	0	1.000	0	0	0
A_2	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0	1.000	1.000	1.000

Table 2. Continued

Locus	Strain														
	16	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**
Bes A															
A_1	1.000	.700	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.162	1.000	.500	1.000
A_2	0	0	0	0	0	0	0	0	0	0	0	.837	0	.500	0
A_0	0	.300	0	0	0	0	0	0	0	0	0	0	0	0	0
Bes B															
B_1	0	.517	0	0	1.000	.467	.053	.371	0	0	0	0	0	0	.171
B_2	.417	.050	.333	0	.150	0	.429	.283	1.000	1.000	1.000	.575	.419	.317	.471
B_3	.583	.433	.667	0	.383	.200	.717	0	.617	0	0	.425	.581	.683	.357
Bes D															
D_1	1.000	.667	0	.551	.567	.487	.771	1.000	0	1.000	.559	0	0	.467	.529
D_2	0	0	.850	.449	.300	0	.229	0	1.000	1.000	.441	1.000	0	0	.471
D_3	0	.333	.150	0	.133	.513	0	0	0	0	0	0	1.000	0	0
D_0	0	0	0	0	0	0	0	0	0	0	0	0	0	.533	0
Bes E															
E_1	.150	.250	0	0	.333	.303	1.000	.317	.167	0	0	0	0	0	0
E_2	.083	0	0	0	0	.132	0	0	0	0	0	0	0	.367	.057
E_0	.767	.750	1.000	1.000	.667	.566	.000	.683	.833	1.000	1.000	1.000	1.000	.633	.943
Pgm A															
A_1	.300	0	.517	0	.617	0	0	0	.750	.583	0	0	.323	.200	0
A_2	.367	1.000	.483	.615	.300	.618	1.000	.733	.250	.250	.676	.425	.677	.550	.314
A_3	.333	0	0	.385	.083	.382	0	.267	0	.167	.324	.575	0	.250	.686
Bph A															
A	.333	.117	.100	.333	.446	.618	.100	.083	.719	.078	.603	.025	0	.600	.371
B	.183	.667	0	.321	.176	.382	.500	.067	.281	.313	.147	0	0	.400	.200
C	.050	.083	.033	0	.014	0	.271	.100	0	.047	0	0	0	0	0
D	0	.133	.017	0	.014	0	.129	0	0	0	.250	0	0	0	0
0	.433	0	.850	.346	.351	0	0	.750	0	.563	0	.975	1.000	0	.429
Ast A															
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Mdh A															
A_1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	.043
A_2	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.950	.957
A_3	0	0	0	0	0	0	0	0	1.000	0	0	0	0	.050	0
Hk A															
A_1	0	0	0	0	0	0	0	0	0	0	1.000	1.000	0	0	0
A_2	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0	0	1.000	1.000	1.000

Table 3. Mean number of alleles per locus, proportion of polymorphic loci, observed (H_o) and expected heterozygosity (H_e)

Strain	Mean sample size per locus	Mean no. of alleles per locus	Percent Polymorphic loci (P=0.99)	H_o	H_e
Vratza 1	82.0±0.0	2.0±0.40	55.6	0. 204±0.074	0. 330±0.108
Vratza 37	84.0±0.0	2.0 ±0.40	55.6	0. 113±0.049	0. 218±0.079
Vratza 40	84.0±0.0	1.7±0.40	33.3	0. 091±0.056	0. 170±0.090
Belopo1/18	87.0±0.0	2.0±0.30	55.6	0. 167±0.062	0. 268±0.089
Belopo2/21	87.0±0.0	2.0±0.30	55.6	0. 134±0.056	0. 274±0.092
Gergana 1	82.0±0.0	1.8±0.30	55.6	0. 124±0.056	0. 263±0.088
Gergana 2	80.0±0.0	1.7 ±0.30	44.4	0. 111±0.054	0. 206±0.085
Ogosta 1	80.0±0.0	1.6±0.20	33.3	0. 096±0.045	0. 173±0.082
Gandga 8	80.0±0.0	2.2±0.50	55.6	0. 140±0.048	0. 300±0.100
M 6	80.0±0.0	1.1±0.10	11.1	0. 033±0.033	0. 046±0.046
TV	80.0±0.0	1.9±0.40	55.6	0. 082±0.041	0. 190±0.080
Japanese 106	84.0±0.0	1.0±0.0	0	0±0	0±0
Asahi	82.0±0.0	1.8±0.40	33.3	0. 063±0.033	0. 137±0.073
Kinshu	81.0±0.0	2.2±0.30	66.7	0. 240±0.075	0. 354±0.098
E 10	80.0±0.0	2.0±0.40	44.4	0. 175±0.076	0. 285±0.113
J-90	80.0±0.0	1.9±0.40	44.4	0. 122±0.061	0. 248±0.102
Alb Cislau 29	80.0±0.0	1.9±0.40	55.6	0. 222±0.073	0. 260±0.084
Ukrainian 12	80.0±0.0	1.7±0.3	44.4	0.93±0.43	0.166±0.073
Ukrainian 20	85.0±0.0	1.4±0.20	33.3	0. 123±0.063	0. 184±0.094
Tashkent 12	82.0±0.0	2.2±0.50	55.6	0. 187±0.071	0. 316±0.102
Syria 1	87.0±0.0	1.7±0.20	55.6	0. 161±0.067	0. 238±0.087
MNB	83.0±0.0	1.7±0.40	33.3	0. 156±0.085	0. 185±0.097
RG-90	80.0±0.0	1.7±0.30	44.4	0. 107±0.044	0. 186±0.074
PS	85.0±0.0	1.3±0.20	33.3	0. 097±0.053	0. 119±0.061
China	82.0±0.0	1.6±0.40	22.2	0. 064±0.043	0. 130±0.086
Jena	86.0±0.0	1.4±0.20	33.3	0. 141±0.084	0. 167±0.084
E 27	87.0±0.0	1.4±0.20	33.3	0. 069±0.037	0. 146±0.072
E 28	80.0±0.0	1.2±0.10	22.2	0. 072±0.049	0. 104±0.069
E 29	80.0±0.0	1.9±0.20	77.8	0. 219±0.061	0. 346±0.080
E 30	82.0±0.0	1.9±0.30	55.6	0. 162±0.066	0. 268±0.094

These results confirmed the results published earlier (Staykova, Ivanova 2011).

Polymorphism by the aspartate aminotranspherase for some Russian strains of *B. mori* L. was reported by Zhukova *et al.* (1974) and Minina (1979). However, this locus was monomorphic for the strains included in the present study (Table 2).

In the silkworm strains analysed by using four enzyme loci, the number of alleles calculated with the BIOSYS-1 software package ranged from 1.0 (in Japanese 106) to 2.2 (in Gindga 8, Kinshu and Tashkent 12) (Table 3). The degree of polymorphism (according to the criterion 0.99) was the highest for strain E 29 (77.80%), and the lowest – for Japanese 106 (0% – no polymorphic loci). The expected heterozygosity (H_e) by polymorphic loci was higher than the observed one (H_o) in all tested strains (Table 3). The values of the genotype frequencies were calculated by the BIOSYS-1 and showed statistically significant deviations from the Hardy-Weinberg equilibrium, as confirmed by Chi-square test ($P < 0.01$) for the most loci in the 30 strains. This fact, in addition to the deficit of heterozygotes proved the inbreeding effect. The observed heterozygosity (H_o) varied from 0.000 (in Japanese 106) to 0.240 (in Kinshu).

The mean F_{ST} value over all loci was 0.4556, which showed that 45.56% of the overall genetic diversity observed was between strains, as opposed to 54.44% within strains. We found the highest level of genetic diversity between strains for the hexokinase (1.000) and the lowest level – for the phosphoglucomutase (0.3002). In three loci we established a

Table 4. Nei's (1972) genetic distance (below diagonal) based on isoenzymes (strain's numbers correspond to the numbers from Table I)

Strain	30	29	28	27	26	25	24	23	22	21	20	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1
30	****																													
29	.165	****																												
28	.207	.315	****																											
27	.374	.538	.473	****																										
26	.238	.387	.468	.295	****																									
25	.124	.338	.228	.348	.289	****																								
24	.346	.485	.541	.750	.452	.198	****																							
23	.102	.188	.176	.512	.364	.297	.606	****																						
22	.283	.301	.483	.871	.437	.402	.579	.178	****																					
21	.141	.116	.209	.697	.403	.395	.559	.125	.212	****																				
20	.097	.175	.192	.570	.354	.176	.345	.118	.189	.124	****																			
19	.092	.259	.291	.529	.340	.239	.473	.188	.254	.215	.099	****																		
18	.122	.276	.105	.321	.389	.081	.324	.173	.403	.245	.121	.215	****																	
17	.196	.177	.249	.718	.401	.378	.617	.144	.133	.111	.142	.125	.292	****																
16	.062	.130	.202	.545	.295	.225	.450	.034	.212	.105	.068	.167	.172	.154	****															
15	.034	.145	.136	.418	.215	.081	.257	.123	.259	.130	.054	.104	.079	.157	.066	****														
14	.030	.160	.258	.499	.315	.200	.398	.102	.178	.101	.082	.090	.190	.151	.069	.076	****													
13	.330	.298	.435	.960	.545	.569	.701	.131	.063	.182	.225	.371	.424	.194	.187	.316	.233	****												
12	.521	.624	.733	.473	.202	.456	.499	.611	.346	.551	.501	.616	.547	.635	.606	.451	.508	.461	****											
11	.124	.264	.277	.506	.345	.218	.422	.212	.228	.219	.108	.020	.174	.128	.212	.109	.123	.343	.483	****										
10	.320	.251	.492	1.026	.508	.641	.692	.179	.111	.135	.187	.319	.464	.204	.199	.298	.225	.071	.382	.290	****									
9	.074	.152	.089	.441	.304	.186	.409	.077	.179	.057	.087	.143	.092	.112	.091	.053	.079	.188	.416	.122	.194	****								
8	.231	.308	.511	.754	.503	.334	.519	.253	.122	.216	.236	.355	.404	.265	.224	.273	.128	.219	.459	.378	.272	.228	****							
7	.141	.101	.276	.705	.310	.342	.501	.105	.155	.072	.125	.151	.255	.070	.089	.116	.115	.167	.505	.140	.134	.093	.276	****						
6	.120	.147	.303	.764	.333	.308	.473	.165	.193	.120	.071	.070	.295	.089	.096	.100	.080	.266	.618	.103	.205	.143	.275	.062	****					
5	.062	.246	.109	.322	.320	.103	.370	.121	.259	.188	.110	.122	.054	.219	.139	.065	.087	.319	.425	.099	.336	.046	.287	.190	.199	****				
4	.066	.285	.133	.375	.344	.139	.415	.166	.300	.213	.107	.073	.110	.220	.164	.077	.082	.395	.517	.078	.384	.080	.331	.227	.157	. 023	****			
3	.140	.193	.320	.673	.220	.263	.414	.123	.144	.207	.172	.232	.323	.203	.092	.122	.143	.174	.366	.242	.181	.150	.271	.108	.137	.194	.223	****		
2	.089	.112	.235	.573	.265	.273	.429	.073	.158	.063	.084	.114	.179	.079	.064	.070	.093	.151	.444	.106	.116	.055	.270	.027	.082	.137	.171	.106	****	
1	.109	.169	.229	.603	.379	.274	.479	.140	.118	.067	.078	.078	.200	.072	.135	.105	.054	.171	.457	.068	.161	.055	.174	.080	.069	.105	.099	.190	.071	****

level of intra-strain differentiation more than 50% – Mdh A (F_{ST}=0.7276), Bes A (F_{ST}=0.6474) and Bes E (F_{ST}=0.5003).

The values of the genetic distance (Nei 1972) were calculated using the allele frequencies and ranged from 0.020 (between strains TV and Ukrainian 20) to 1.026 (between strains E 27 and M-6) (Table 4).

In the UPGMA (Sneath, Sokal 1973) dendrogram, the strains studied are grouped in two main clades (Fig. 1). Strains E 27, Japanese 106 and Jena were clustered in one clade, while the others were clustered in the second one with many sub-clusters. The obtained results showed that the analysed strains of various origins are genetically differentiated.

Conclusions

The results of this study provide new information concerning the genetic enzyme polymorphism in *Bombyx mori* strains originated from different countries. The hexokinase, malate dehydrogenase and nonspecific esterases from the mulberry silkworm hemolymph are suitable markers to analyse the inter-strain polymorphism and strain differentiation. Phosphoglucomutase from the silk glands and acid phosphatase from the hemolymph are better suited for investigating the intra-strain polymorphism and establishing the degree of intra-strain genetic variability.

The allozyme variability of the studied loci would be useful for the selection of parents in the development of elite hybrids. Strains E 27, Japanese 106 and Jena may be used in future breeding programs as donor strains since they are genetically distant from all others.

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References

Arai H., T. Okido, H. Fujii and H. Doira 2000. Purification and characterization of a major esterase BesB from hemolymph of the silkworm, Bombyx mori. – *Journal of Sericultural Science of Japan*, **69** (2): 121-130.

Daevis B. 1964. Disc electrophoresis. Methods and application to human serum proteins. – *Ann. New York Acad. Sci*., **121** (4): 404-427.

Eaton, G. M., G. J. Brewer and R. E. Tashian 1966. Hexokinase isozyme patterns of human erythrocytes and leucocytes. – *Nature*, **212** (5065): 944-946.

Egorova, T, E. Naletova and Y. Nasirillaev 1985. Polymorphic system of silkworm haemolymph esterases as a criterion to make programs for parental specimens crossing. – *Biochemistry of Insects – Moskow*: 54-62. (In Russian).

Egorova T., Y. Nasirillaev 1993. Polymorphic enzymes of silkworm and their using in breeding. – *State committee of science and technics of republic Uzbekistan,* GFNTI: 120 p. (In Russian)

Eguchi M., Y. Takahama, M. Ikeda and S. Horii 1988. A novel variant of acid phosphatase isozyme from hemolymph of the silkworm, *Bombyx mori*. – *Japan Journal of Genetics*, **63** (2): 149-157.

Felsenstein, J. 1993 PHYLIP (Phylogeny Inference Package). Version 3.5C Distributed by the author. Dept. of Genetics, Univ. of Washington, Seattle, W.A.

He, J. L. 1995. Studies on the inheritance of a deletion type of the blood esterase isoenzymes in the silkworm, *Bombyx mori* L. – *Sericologia*, **35**: 17-24.

Marcato S., M. Trevisan, L. Cappellozza and P. Bisol 1990. Variabilita di sistemi gene-enzima, in razze di Bombyx mori (Lepidoptera, Bombycidae) utilizzate per la produzione di seta. – *Estratto da Redia*, **73** (2): 595-608. (in Italian, English summary).

Miao X. X., S. J. Xu, M. H. Li, M. W. Li, J. H. Huang, F. Y. Dai, S. W. Marino, D. R. Mills, P. Zeng, K. Mita, S. H. Jia, Y. Zhang, W. B. Liu, H. Xiang, Q. H. Guo, A. Y. Xu, Kong X. Y., H. X. Lin, Y. Z. Shi, G. Lu, X. Zhang, W. Huang, Y. Yasukochi, T. Sugasaki, T. Shimada, J. Nagaraju, Z. H. Xiang, S. Y. Wang, M. R. Goldsmith, C. Lu, G. P. Zhao and Y. P. Huang 2005. Simple sequence repeat-based consensus linkage mao of the silkworm genome. – *Proceedings of the National Academy Sciences USA*, **102**: 16303-16308.

Minina N. 1979. Form dynamics of some ferments of nitrogen exchange in the ontogenesis process of silkworm. – *A collection of works of V.I. Lenin National Pedagogy Institute of Moscow*, **21**: 4-15. (In Russian).

Mirhoseini S. Z., M. R. Gholami 2002. Study on some economic characters of single, three-way and double crosshybrids obtained from four Iranian silkworm varieties. – *XIXth Congress of The International Sericultural Commission Proceedings, Thailand*, pp.190-194.

Nei M. 1972. Genetic distance between populations. – *American Naturalist,* **106**: 283-292.

Petkov, N., P. Tzenov, Z. Petkov, Y. Natcheva and Y. Vasileva 2006 Silkworm, Bombyx mori L. germplasm resources in Bulgaria. *National Centre for Agrarian Sciences – Sofia, Sericiltural Experiment Station – Vratza*, 264.

Reddy K., E. Abraham and J. Nagaraju 1999. Microsatellites in the silkworm *Bombyx mori*: Abundance, polymorphism, and strain characterisation. – *Genome,* **42** (6): 1057-1065.

Schmidtke J., W. Engel 1972. Duplication of the gene loci coding forthe supernatant aspartate aminotransferase by polyploidization in the fish family Cyprinidae. – *Experientia*, **28** (8): 976-978.

Shabalina A. 1990. Esterase genetic polymorphism in haemolymph of larvae *Bombyx mori* L. – *Comptes rendus de l'Academie bulgare des Sciences*, **43** (9), 105-108.

Shaw C., R. Prasad 1970. Starch gel electrophoresis of enzymes – a compilation of Recipes. – *Biochemistry Genetics*, **4** (2): 297-320.

Sneath P. H. A., R. R. Sokal 1973. Numerical Taxonomy – The principle and practice of numerical classification. W.H. Freeman and Co., San Francisco, 573 p.

Spencer N., D. Hopkinson and H. Harris 1964. Phosphogluco-

mutase polymorphism in man. – *Nature*, **204**: 742-745.

Staykova T., D. Grekov and M. Panayotov 2004. Electrophoretic analysis of nonspecific esterases in silkworm (*Bombyx mori* L.) female genital organs and eggs. – *International Journal of Industrial Entomology*, **9** (1): 59-63.

Staykova T. 2006. Genetic control of phosphoglucomutase in mulberry silkworm (*Bombyx mori* L.). – *International Jubilee Scientific Conference "Problems of maintenance and utilization of mulberry and silkworm genetic resources",* September 25-29, 2006, Vratsa, Bulgaria, 197-201.

Staykova T., D. Grekov 2006. Stage specificity and polymorphism of haemolymph esterases in races and hybrids of silkworm (*Bombyx mori* L.) kept in Bulgaria. – *Proceedings of the International workshop on silk handcrafts cottage industris and silk enterprises development in Africa, Europe, Central Asia and the Near East & Second executive meeting of Black, Caspian seas and Central Asia Silk Assosiation (BACSA),* Bursa, Turkey, 6-10 March, 2006, 667-674.

Staykova T. 2008. Genetically-determined polymorphism of nonspecific esterases and phosphoglucomutase in eight introduced breeds of the silkworm, *Bombyx mori*, raised in Bulgaria. 8 pp. – *Journal of Insect Science,* 8:18, available online: insectscience.org/8.18.

Staykova T., E. Ivanova, P. Tzenov, Y. Vasileva, D. Arkova-Pantaleeva and Z. Petkov 2010. Acid phosphatase as a marker for differentiation of silkworm (*Bombyx mori*) strains. – *Biotechnology & Biotechnological Equipment*, **24** (2): 379-384.

Staykova T., E. Ivanova 2011. Concerning genetic variability and usable isozyme markers for characterization of *A. mellifera* L. populations and *B. mori* L. breeds in Bulgaria. – *Advances in Bulgarian Science*, 20-28.

Staykova T., E. Ivanova, D. Grekov and K. Avramova. 2012. Genetic Variability in silkworm (Bombyx mori L.) strains with different origin. – *Acta Zoologica Bulgarica*, Suppl. **4**, 87-92.

Stoykova T., P. Popov and B. Dimitrov 2003. Electrophoretic analysis of non-specific haemolymph esterases during silkworm (*Bombyx mori* L.) ontogenesis. – *Sericologia*, **43** (2): 153-162.

Swofford D. L., R. B. Selander 1981. BIOSYS-1: A computer program for the analysis of allelic variation in genetics Rel. 1.0 Department of Genetics and Development University of Illinois at Urbana-Champaign, Urbana, Illinois 60801, USA.

Wright S. 1965. The Interpretation of population structure by F-Statistics with special regard to systems of mating. – *Evolution*, **19** (3): 395-420.

Yoshitake N., M. Akiyama 1964. Genetical studies on the acid phosphatase in the blood of the silkworm, *Bombyx mori* L. – *Japan Journal of Genetics*, **39**: 26-30.

Zhukova N., S. Klunova and Y. Filipovic 1974. Study on some ferments of the aminoacids exchange in silkworm tissues by the method of the enzyme electrophoresis in polyacrylamide gel. – *A collection of works of V.I. Lenin National Pedagogy Institute of Moscow*, **17**: 56-68. (In Russian).

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