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Genetic characterization of silkworm (*Bombyx mori* L.) strains (Lepidoptera: Bombicidae) with different geographical origin on the basis of isozyme markers

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ABSTRACT. The aim of the present study was to assess genetics variations within and among strains with different geographical origin, belonging to Bulgarian germplasm bank of mulberry silkworm (*Bombyx mori* L.) and to establish their relationships using isoenzyme markers. Polyacrilamide gel electrophoresis (PAGE) was used to study the isoenzymes of nonspecific esterases (EST), malate dehydrogenase (MDH) and acid phosphatase (ACP) from haemolymph, phosphoglucomutase (PGM) and hexokinase (HK) from silk glands and alkaline phosphatase (ALP) from midgut of mulberry silkworm (*Bombyx mori* L.). Variability was evident in all of these enzyme systems among twelve strains from Bulgarian germplasm resources of *B. mori*. Total of nine *loci* were detected. All of them (100%) were polymorphic. "Null" alleles in four *loci* were determined. Intra- and inter-strain polymorphism was obtained. The degree of polymorphism ranged from 0% to 77.80%. Low levels of observed heterozygosity in comparison with the expected one have been calculated in almost all of strains as well as deviations from Hardy-Weinberg equilibrium in almost all analyzed *loci*, resulting of excess of homozygotes. The value of F_{ST} was 0.4903. The dendrogram constructed with the values of genetic distance manifests that Romanian strain Cislau Tokay is formed one main clade while the rest strains studied (from Bulgaria, Japan, China, Vietnam, Spain, Syria and Egypt) are formed the other clade with subclades. The genetic data of the tested strains from different geographical regions, would be used for identifying suitable parents for breeding programs with a view to yield improvement in this species with economic significance.

Keywords: mulberry silkworm; genetic diversity.

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Introduction

Mulberry silkworm *Bombyx mori* (Linnaeus, 1758) is the model organism for Lepidoptera, including many races with different geographical origin and phenotypic characterization. More than 4000 univoltine, bivoltine and polyvoltine strains are maintained in the world germplasm of *B. mori* (Bindroo & Moorthy, 2014). The economic, cultural and scientific significance of this species have made it the subject of intensive studies. Genetics researches of silkworm have a great impact in improving sericulture. The traditional breeding activities, involving hybridizations between members of elite groups, are adding new varieties every year (Reddy, Abraham, & Nagaraju, 1999). A number of quantitative, qualitative and ethological traits are used for selection of parental strains for breeding programs, but many of silkworm varieties cannot be differentiated by the use of conventional characteristics, only. Isoenzymes have become a highly informative class of genetic markers for breeding programs and marker-assisted selection, as well as for conservation strategies of silkworm *Bombyx mori* L. Allozyme markers are powerful molecular tools that have facilitated the study of genetic diversity. Allozyme electrophoresis is a technique that has proved useful for determining the genetic variations within and among populations (Egorova, Naletova, & Nasirillaev, 1985; Eguchi, Takahama, Ikeda, & Horii, 1988; Marcato, Trevisan, Cappellozza, & Bisol, 1990; He, 1995; Staykova, 2008; Staykova et al., 2015).

Silk producing countries have collected number of silkworm strains suitable for a wide range of agroclimatic conditions (Bindroo & Moorthy, 2014). A large number of strains (more than 250) with

different origin compose Bulgarian germplasm resources of *Bombyx mori* (Petkov, Tzenov, Petkov, Natcheva, & Vasileva, 2006). They have been described mainly on the base of their morphological characteristics that are highly variable and environment dependent. Isoenzymic variations and phylogenetic relatedness among strains have been slightly studied (Shabalina, 1990; Staykova, 2008; Staykova et al., 2010; Staykova, Ivanova, Grekov, & Avramova, 2012; Staykova et al., 2015). Due to this fact the purpose of the present study was to assess genetics variations within and among twelve strains with different origin belonging to Bulgarian germplasm bank of silkworm and to establish their relationships using isoenzyme markers.

Material and methods

The silkworm resources used in the present investigation include a total of twelve strains with different geographical origin and phenotype characteristic (Table 1), which were obtained from the Sericulture Experiment Station germplasm bank in Vratza, Bulgaria. All individuals were nourished at a standard regime of silkworm breeding.

Table 1. Origin and phenotype characteristic of tested silkworm strains

Name of strain	Origin	Main phenotype characteristic (Petkov et al., 2006)
VB 1	Bulgaria	Uni-bivoltine strain, created at SES - Vratza in 2001. The egg serosa color is gray, chorion color is white and eggs are sticky. The larvae are white with marking. The cocoons are white in color and elongated.
371		Uni-bivoltine strain, created at SES - Vratza in 1959. The egg serosa color is gray, chorion color is white and eggs are sticky. The larvae are white in color and plain with translucent yellow haemolymph. The cocoons are cream in color, elongated with low constriction
Cislau Tokay	Romania	Uni-bivoltine strain, introduced in 2003. The egg serosa color is green, chorion color is yellow and eggs are sticky. The larvae are white in color and plain. The cocoons are white with oval shape.
F – 13	Japan	Bivoltine strain, introduced in 1998. The egg serosa color is light brown, chorion color is white and eggs are sticky. The larvae are white in color with marking. The cocoons are small, white and elongated.
Par +		Uni-bivoltine strain, introduced in 1998. The egg serosa color is gray, chorion color is white and eggs are sticky. The larvae are white in color and plain with translucent yellow haemolymph. The cocoons are yellow in color and elongated.
Shandon		Three moulter strain, introduced in 1998. The egg serosa color is grey brown, chorion color is white and eggs are sticky. The larvae are white in color and plain. The cocoons are spindle with cream color.
Daizo		Poly - bivoltine strain, introduced in 1998.The egg serosa color is green brown, chorion color is yellow and eggs are sticky. The larvae are white in color with marking and with translucent haemolymph. The cocoons are green yellow in color and spindle.
Sh 3	China	Uni-bivoltine strain, introduced in 1990.The egg serosa color is gray or green, chorion color is white or yellow and the eggs are sticky. The larvae are white in color with marking or plain. The cocoons are white in color and oval-elongated in shape.
70-42	Vietnam	Uni-bivoltine strain, introduced in 1989.The egg serosa color is gray, chorion color is white or yellow and the eggs are sticky. The larvae are white in color with marking. The cocoons are white in color, elongated with low constriction.
AES 1 wh	Spain	Uni-bivoltine strain, introduced in 2008. The eggs serosa color is green and gray, chorion color is white or yellow and eggs are sticky. The larvae are plain and with zebra marking. The cocoons are elongated and spindle with yellow color.
Syria 2	Syria	Uni-bivoltine strain, introduced in 1991.The egg serosa color is green, chorion color is yellow and eggs are sticky. The larvae are white in color and plain. The cocoons are white in color with oval shape.
E 31	Egypt	Uni-bivoltine strain, introduced in 2001.The egg serosa color is gray, the chorion color is white the eggs are sticky. Larvae are white in color, with markings. The cocoons are white in color, elongated with low constriction.

Totally 477 larvae (male and female) on the fifth day of the fifth instar were studied. Larvae were selected randomly from each strain and were submitted to electrophoretic analysis of haemolymph, silk glands and midgut tissues (mean sample size per locus ranged from 33 to 41, Table 3).

The tissue extracts were prepared according to Stoykova, Popov, and Dimitrov (2003), Staykova, Grekov, and Panayotov (2004), Staykova et al. (2012) and Staykova (2013). The individual samples were studied by 7.5% polyacrilamide gel electrophoresis (PAGE) (Davis, 1964) for 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 hemolymph; hexokinase (HK, EC 2.7.1.1) - from silk glands and alkaline phosphatase (ALP, 3.1.3.1) – from midgut. The 6% PAGE was used to analyze phosphoglucomutase (PGM, EC 5.4.2.2) from silk glands. This concentration

of dividing gel was more appropriate for investigation of PGM polymorphism, as was pointed earlier (Staykova, 2008). The staining mixtures for the enzymatic activities tested were taken from Shaw and Prasad (1970), Staykova et al. (2010), Spencer, Hopkinson, and Harris (1964), Eaton, Brewer, and Tashia (1966), Schmidtke and Engel (1972) and Boyer (1961). All the reagents were high purity grade, provided by Sigma-Aldrich™.

After the revelation of the isozyme activity regions, the phenotypes of the discovered *loci* were recorded. 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) (Nei, 1972), and Wright's fixation index, F_{ST} (Wright, 1965) were calculated using BIOSYS-1 (Swofford & Selander, 1981). Dendrogram was constructed using Nei's (1972) genetic distance, by UPGMA (Sneath & Sokal, 1973) method using the PHYLIP (Felsenstein, 1993) software package.

Results

A total of nine izoenzymes of nonspecific esterases, tree allozymes of phosphoglucomutase, malate dehydrogenase and acid phosphatase, and two allozymes of alkaline phosphatase and hexokinase were detected by PAGE electrophoresis.

The six enzyme systems recorded a total of nine polymorphic *loci* with 26 heterogeneous alleles (Table 2). Nonspecific esterases, phosphoglucomutase, acid phosphatase and malate dehydrogenase manifested inter- and intra-strain polymorphism. Alkaline phosphatase was polymorphic in AES 1 wh and the hexokinase - in F-13 strain, only.

Table 2. Allele frequencies in strains tested

Locus (allele)	Strain											
	VB 1	371	Cislau Tokay	F-13	Par+	Shandon	Daizo	Sh 3	70-42	AES 1 wh	Syria 2	E 31
Bes A												
A_1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.697	1.000	1.000
A_0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.303	0.000	0.000
Bes B												
B_1	0.446	0.000	0.250	0.203	0.000	0.329	0.000	0.134	0.576	0.000	0.378	0.545
B_2	0.554	0.000	0.039	0.176	1.000	0.671	1.000	0.476	0.424	0.645	0.000	0.091
B_3	0.000	1.000	0.711	0.622	0.000	0.000	0.000	0.390	0.000	0.355	0.622	0.364
Bes D												
D_1	0.000	0.618	0.000	0.000	0.000	0.000	0.000	0.463	0.576	0.158	0.851	0.515
D_2	0.514	0.000	1.000	0.000	1.000	0.000	0.000	0.000	0.424	0.842	0.000	0.485
D_3	0.486	0.382	0.000	0.676	0.000	1.000	0.000	0.537	0.000	0.000	0.149	0.000
D_0	0.000	0.000	0.000	0.324	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000
Bes E												
E_1	0.162	0.000	0.579	0.000	0.000	0.543	0.000	0.000	0.106	0.092	0.149	0.000
E_2	0.149	0.000	0.132	0.351	0.000	0.000	1.000	0.000	0.076	0.092	0.189	0.152
E_0	0.689	1.000	0.289	0.649	1.000	0.457	0.000	1.000	0.818	0.816	0.622	0.848
Pgm A												
A_1	0.027	0.149	0.000	0.000	0.467	0.000	1.000	0.000	0.258	0.000	0.000	0.000
A_2	0.446	0.479	0.436	1.000	0.533	1.000	0.000	0.549	0.576	0.474	0.419	0.439
A_3	0.527	0.372	0.564	0.000	0.000	0.000	0.000	0.451	0.167	0.526	0.581	0.561
Mdh A												
A_1	0.000	0.026	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
A_2	1.000	0.474	0.361	0.500	0.143	1.000	1.000	1.000	1.000	1.000	1.000	1.000
A_3	0.000	0.500	0.639	0.500	0.857	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Bph A												
A	0.000	0.961	0.473	0.639	0.653	1.000	1.000	0.341	0.424	0.197	0.081	0.091
B	0.324	0.039	0.000	0.361	0.347	0.000	0.000	0.000	0.000	0.039	0.000	0.333
C	0.135	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
D	0.000	0.000	0.527	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.576
0	0.541	0.000	0.000	0.000	0.000	0.000	0.000	0.659	0.576	0.763	0.919	0.000
Alp A												
A_1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.566	1.000	1.000
A_2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.434	0.000	0.000
Hk A												
A_1	0.000	0.000	0.000	0.178	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000
A_2	1.000	1.000	1.000	0.822	1.000	0.000	1.000	1.000	1.000	1.000	1.000	1.000

The analysis conducted showed monomorphic and fixed allele of Bes A locus in all tested strains (Bes A_1 allele), except for AES 1wh (Table 2). In the gene pool of this strain introduced from Spain we found polymorphism with "null" allele - Bes A_o. Homozygous Bes A_o/A_o specimens did not show any bands in the BES A zone of the gel plate. For the Bes B locus three alleles were recorded (Bes B_1, B_2 and B_3). Bes B_2 allele was fixed in the gene pool of Par+ and Daizo, whereas Bes B_3 was fixed in the gene pool of 371 strain (Table 2). In Cislau Tokay, F-13, Sh 3 and E31 we obtained all three alleles of the Bes B locus. Polymorphism with two alleles we recorded in VB1, Shandon and 70-42 (Bes B_1 and B_2 were presented in the gene pools), in Syria 2 (Bes B_1 and B_3) and in AES 1 wh (Bes B_2 and B_3). Among the strains with polymorphism on Bes B locus, the allele Bes B_1 showed the highest frequency in 70-42, Bes B_2 – in Shandon and Bes B_3 – in Cislau Tokay. Polymorphism with four alleles we found on the Bes D locus (Tab. 2). Bes D_1 and D_2 alleles were presented in the gene pool of 70-42, AES 1wh and E31, Bes D_1 and D_3 – in 371, Sh3 and Syria2, Bes D_2 and D_3 – in VB1, Bes D_3 and D_o – in F-13. Bes D_2 allele was fixed in the gene pool of Cislau Tokay and Par+ and Bes D_3 – in Shandon. For Daizo we obtained Bes D_o alele, only. Homozygous Bes D_o/D_o specimens did not show any bands in the corresponding esterase zone BES D. Frequency of the allele Bes D_1 was the highest in Syria 2, of Bes D_2 – in AES 1wh and of Bes D_3 – in F-13 (except for strains with fixing Bes D alleles). Three allele polymorphism were found in Bes E locus (Bes E_1, E_2 and E_o alleles) in VB1, Cislau Tokay, 70-42, AES 1wh and Syria 2. Two of these alleles we obtained in Shandon (Bes E_1 and E_o), F-13 and E31 (Bes E_2 and E_o). All tested individuals from Daizo were homozygous of Bes E_2 allele, whereas the individuals from 371, Par+ and Sh3 were homozygous of Bes E_o allele. This "null" allele demonstrated the highest frequency in all polymorphic strains tested, except for Cislau Tokay and Shandon (Table 2).

The electrophoretic separation of allozyme forms of phosphoglucomutase from silk glands exhibited variations with three Pgm alleles - Pgm A_1, A_2 and A_3 in VB1, 371 and 70-42 strains. Pgm A_1 and A_2 were presented in the gene pool of Par+ and Pgm A_2 and A_3 –in Cislau Tokay, Sh3, AES 1wh, Syria 2 and E31 (Table 2). Pgm A_1 allele had the highest frequency in Par+, Pgm A_2 – in 70-42 and Pgm A_3 – in Syria 2. In Daizo we obtained Pgm A_1 allele, while in F-13 and Shandon strains - Pgm A_2 allele, only.

For the Mdh locus, three alleles were recorded in strain 371 (Mdh A_1, A_2 and A_3). Two of them (Mdh A_2 and A_3) were found in the gene pool of Cislau Tokay, F-13 and Par+ (Table 2). Mdh A_3 was the most common allele among these four strains with polymorphism of the Mdh locus. Mdh A_2 allele was fixed in the gene pools of the rest eight strains.

Electrophoresis of acid phosphatase allozymes from hemolymph showed total of five alleles in tested strains (Bph A, B, C, D and 0) (Table 2). The allele symbolized as Bph 0 was "null" allele. Three of the Bph alleles we found in AES 1wh (Bph A, B and 0), E31 (Bph A, B and D), VB1 (Bph B, C and 0). Two alleles were recorded in 371, F-13, Par+ (Bph A and B), Cislau Tokay (Bph A and D), Sh3, 70-42 and Syria 2 (Bph A and 0). Bph A allele was fixed in Shandon and Daizo strains. The Bph 0 allele was the most common allele in five of the tested strains with extremely high frequency in Syria 2. Bph A was the most expressed allele in three of strains with extremely high frequency in 371. Bph D was more frequently allele in two of the strains – Cislau Tokai and E31.

Polymorphism of the Alph locus with two alleles (Alph $A_1$and A_2) was recorded in AES 1wh strain, only (Table 2). All the rest strains were monomorphic on this locus with fixed Alph A_1 allele.

Among the twelve strains included in the present study, we found polymorphism with two alleles on the hexokinase (Hk A) locus. Hk A_1 and A_2 alleles were recorded simultaneously in F-13, only. Hk A_1 allele was fixed in Shandon, whereas Hk A_2 allele was fixed in all the rest ten strains.

The number of alleles, allele and genotype frequencies of the *loci* of nonspecific esterases, phosphoglucomutase, malate dehydrogenase, acid phosphatase, alkaline phosphatase and hexokinase tested manifested strain specificity.

In the silkworm strains analysed using nine enzyme *loci*, the number of alleles per locus calculated with BIOSYS-1 software package, ranged from 1.0 (Daizo) to 2.0 (AES 1wh) (Table 3). The degree of polymorphism (according to the criterion 0.99) was the highest for strain AES 1wh (77.80%), and the lowest – for Daizo (0% - no polymorphic *loci*). The heterozygosity (H_o) by polymorphic *loci* observed varied from 0.000 (for Daizo) to 0.280 (for Cislau Tokay). The expected heterozygosity (H_e) was higher than the observed one (H_o) in all strains tested except Cislau Tokay, in which the value of H_o exceeded the value of H_e insignificantly (Table 3). Significant differences ($P < 0.05$) in genotype frequencies were seen at the most *loci* in strains studied. Chi-Square test (DF=1÷3) showed that the deviations from the Hardy-Weinberg equilibrium were in result of excess of homozygotes and deficiency of heterozygotes (Table 3).

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
VB 1	38.7.0±1.1	1.8±0.30	55.6	0. 204±0.074	0. 330±0.108
371	39.9±1.4	1.7 ±0.30	44.4	0. 169±0.079	0. 189±0.089
Cislau Tokay	38.7.0±1.1	1.8±0.30	55.6	0. 280±0.097	0. 275±0.088
F - 13	36.6±2.3	1.8±0.20	66.7	0. 191±0.063	0. 304±0.079
Par +	38.0±1.5	1.3±0.20	33.3	0. 096±0.034	0. 135±0.071
Shandon	36.6±1.5	1.2±0.10	22.2	0. 048±0.048	0. 106±0.070
Daizo	37.0±0.0	1.0 ±0.0	0.0	0. 000±0.000	0. 000±0.000
Sh 3	41.0±0.0	1.6±0.20	44.4	0. 157±0.066	0. 230±0.092
70-42	33.0±0.0	1.8±0.30	55.6	0. 246±0.092	0. 266±0.087
AES 1 wh	38.0±0.0	2.0±0.20	77.8	0. 164±0.048	0. 319±0.066
Syria 2	37.0±0.0	1.7±0.20	55.6	0. 135±0.052	0. 210±0.077
E 31	33.0±0.0	1.8±0.30	55.6	0. 195±0.078	0. 266±0.089

Summarized results of F-stastistics are presented in (Table 4). The mean F_{ST} value over all loci, which is associated with the level of inter-strain differentiation, was 0.4903 and shows that 49.03% of the overall genetic diversity observed was among strains. 50.97% of genetic variations were within strains. The higher level of genetic diversity among strains we found for the Hk A locus (0.8604) and the lower one – for the Bes A locus (0.2839). For three loci (Hk A, Bes D and Mdh A) we established a level of intra-strain differentiation over than 50%. The mean value of F_{IT} coefficient (0.6298) shows that there was a deficit of heterozygotes in the tested strains and correlates with the obtained lower level of heterozygosity observed compared to the expected one and with deviations from the Hardy-Weinberg equilibrium as well. This fact proved the inbreeding effect.

Table 4. F-statistics for all polymorphic *loci* studied.

Locus	F_{IS}	F_{ST}	F_{IT}
Bes A	0.8130	0.2839	0.8661
Bes B	0.2213	0.4235	0.5510
Bes D	0.3445	0.6049	0.7410
Bes E	0.4627	0.4166	0.6865
Pgm A	0.2654	0.3459	0.5196
Mdh A	0.2271	0.5845	0.6788
Bph	0.0971	0.4817	0.5320
Alp A	0.3037	0.4121	0.5907
Hk A	0.0878	0.8604	0.8726
Mean	0.2737	0.4903	0.6298

The values of genetic distance (Nei, 1972) were calculated using the allele frequencies and ranged from 0.072 (between the strains Sh3 and Syria 2) to 0.651 (between strains Cislau Tokai and Daizo).

The tree drawn by UPGMA (Sneath & Sokal, 1973), based on isoenzyme results, showed that Romanian strain Cislau Tokay is formed one main clade, while the rest strains studied are formed the other, which is differentiated in two subclades (Figure 1). One of the subclade includes two Japanese strains Shandon and Par+ and the second one includes all others. Tree strains (AES 1 wh, Syria 2 and E31) were grouped to form one subclade and six strains (Sh3, F-13, 371, VB1, 70-42 and Daizo) were grouped to form another subclade. Five of them F-13, 371, VB1, 70-42 and Daizo strains were grouped together.

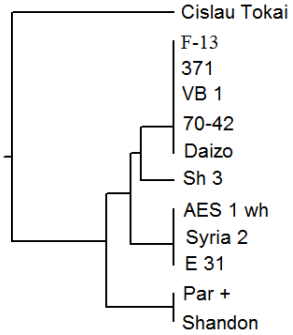

Figure.1. UPGMA dendrogram.

Discussion

Any attempt to improve silkworm breeds needs knowledge of their genetic variability and relationships. Silkworm breeding is the selection of superior genotypes and/or phenotypes over a period of time (Bindroo & Moorthy, 2014). The selection of best genotypes depends on a number of characters and diversity among them (Moorthy & Chandrakanth, 2015). Genetic variability can be analyzed by different methods and markers. According to Bindroo and Moorthy (2014) application of isoenzymes and other molecular markers helps to estimate genetic diversity much more accurately than that of morphological traits. Isoenzyme analysis appears to be useful in studing of intra- and inter-strain polymorphism of mulberry silkworm and determining the level of genetic changeability and genetic relationships (Egorova & Nasirillaev, 1993; Staykova, 2008; Staykova et al., 2010; Staykova et al., 2012; Staykova et al., 2015). Isozymes like esterase, acid phosphatase, alkaline phosphatase, malate dehydrogenase, phosphoglucomutase have been used by various researchers to study diversity in silkworm genotypes (Bindroo & Moorthy, 2014). Among them nonspecific esterases were most preferred because of its polygene control and polymorphic expression. The results in this study described indicate that esterase isozyme markers used could be successfully applied to examine the genetic variability in mulberry silkworm *Bombyx mori*. A total of twelve alleles of four esterase *loci* were recorded. Among strains tested "null" alleles of three esterase *loci* (Bes A_o, Bes D_o and Bes E_o) were found. Polymorphism with "null" allele of the Bes A and Bes E locus was described earlier in other strains of Bulgarian germplasm resourses of silkworm by Staykova et al. (2015). He (1995) also described polymorphism with a "deletion" type esterase (Bes A_o) in the corresponding zone of electrophoretic spectra in some races and hybrids in China. Polymorphism with five alleles was determined of the acid phosphatase. One of them was found as "null" type. Four electrophoretic variants (A, B, C, D) of hemolymph acid phosphatase in silkworm and a "null" mutant (0) controlled by total of five codominant alleles found Yoshitake and Akiyama (1964) and Eguchi et al. (1988). By means of linkage analysis Fujimori, Shimizu, and Enokijima (1984) located these alleles at 18.2 map unit on the chromosome 23 from silkworm karyotype. Polymorphism with five codominant alleles of the Bph locus was described earlier by Staykova et al. (2010) and Staykova et al. (2015) in other strains with different geographical origin, belonging to Bulgarian germplasm bank of silkworm. In this study polymorphism with three or two alleles of the phosphoglucomutase and malate dehydrogenase, and alkaline phosphatase and hexokinase *loci*, respectively was recorded. These results add new information about genetic variability of silkworm germplasm resources in Bulgaria (Staykova, 2008; Staykova et al., 2015). Some of the alleles of the polymorphic *loci* demonstrated strain specificity. For instance Bes A_o and Alp A_2 alleles were presented only in AES 1 wh strain, Mdh A_1 was presented in strain 371 and Hk A1 was presented in F-13, only.

The dendrogram generated by UPGMA analysis based on isoenzyme polymorphism resolved twelve strains into two main clusters: one comprising strain Cislau Tokay and the other the rest strains. Classification of silkworm genotypes is very important in breeding programs, because commercial rearing of this species is based on hybrids crossed from pure lines (Moorthy & Chandrakanth, 2015). Isoenzyme and allozyme markers utilized in this study resulted in differentiating of some strains with different geographical origin in the same subclades and also strains with common origin in different subclades. According to Etebari, Mirhoseini, and Matindoost (2005) and Chandrakanth, Anusha, and Moorthy (2014) this may be due to the changes in biological and developmental process and strains' adaptation to environmental conditions. In our opinion genetically determined biochemical variances among tested strains from Bulgarian germplasm resources could be in result of founder effect provoked through introducing, also.

Almost all tested strains (eleven from twelve) had mean heterozygote deficiencies compared with Hardy-Weinberg expectation, which corresponds with high average value of F_{IT} coefficient. According to Bindroo and Moorthy (2014), reduction in genetic diversity in silkworm, might be mainly due to domestication, breeding systems, selection, genetic drift and inbreeding. The low level of heterozygosity among tested strains observed in this study, probably results from low effective number of reproductive individuals, which provokes genetic drift, selection process and inbreeding effect. According to Lande (1994) and Whitlock (2002), the effects of inbreeding can accumulate over many generations. Li et al. (2010) pointed out that the low level of mt sequence polymorphism in the domesticated varieties of *B. mori* is most likely caused by inbreeding or population bottlenecking (by Bindroo & Moorthy, 2014). In silkworm germplasm maintenance centers, at every cycle only 40-60 cocoons are selected from each strain from the next generation (Bindroo

& Moorthy, 2014). This size of population is small and may be led to bottleneck effect and to change the allelic frequencies. Bottleneck may restrict genetic diversity and breeding flexibility. On the other hand artificial selection for target characteristics has been widely utilized in the silkworm breeding programs (Bindroo & Moorthy, 2014). Breeders use the best genotypes available and select superior progeny, which narrows the gene pool variability to only those alleles that are available from the elite parent's genotypes and therefore tends to decrease the genetic variation of the population (Brown, 1988; by Bindroo & Moorthy, 2014). Systematic selection of parents' results in reduced genetic variation among their offspring. According to Bindroo and Moorthy (2014), to broaden the genetic diversity of silkworm, exotic elite strains were required to be introduced from various countries. Maintaining proper population size can prevent the loss of genetic variability due to genetic drift (Guzman & Lamkey, 2000). Genetic diversity is very important for selection of suitable parents required for successful development of improved varieties and hybrids of silkworm that have high adaptive potential lines (Bindroo & Moorthy, 2014). The results obtained in this study make use of breeding programs. Genetic variability, based on isoenzymes, could be also used during breeding of disease resistant silkworm strains (Mahesha & Thejaswini, 2014).

Conclusion

The present study is a step towards analyzing the genetic diversity in order to identify suitable parents for breeding programs with a view to cross different genotypes for yield improvement. The enzymes used in this investigation are further able to characterize genetic variability of silkworm strains and their relationships. Haemolymph esterases, acid phosphatase and malate dehydrogenase, as well as phosphoglucomutase and hexokinase from silk glands are appropriate markers for examination the genetic diversity and differentiating the silkworm strains. Allozyme data depicted substantiate amount of diversity among strains tested and hence, they can be utilized as parents in breeding programs.

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