



## Isoenzyme Polymorphism of Silkworm *Bombyx mori* L., 1758 (Lepidoptera: Bombycidae) Breeds from Germplasm Resources of Bulgaria

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**Abstract:** Polyacrilamide gel electrophoresis (PAGE) was used to study the isoenzyme polymorphism 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.). Genetic variations within and among five breeds kept in Bulgaria were found out. A total of seven polymorphic loci with 19 alleles were established. The observed polymorphism demonstrated breeds specificity. Inter-breeds differences regarding the degree of polymorphism and heterozygosity were established, too. The degree of polymorphism ranged from 77.80% to 11.10%. Low levels of observed heterozygosity and deviations from Hardy-Weinberg equilibrium in the most of analysed loci were found. The dendrogram constructed with the values of genetic distance showed that Hebar 2 formed one main cluster, while the rest breeds studied formed the other one. The results of the present study complement the data on the genetic heterogeneity of breeds from the silkworm genetic resources of Bulgaria and could help the breeders in the process to identify suitable parents for the breeding programs for yield improvement.

**Key words:** *Bombyx mori*, isoenzyme variability, phylogenetic relationships

### Introduction

The mulberry silkworm *Bombyx mori* L., 1758 is one of the species of insects that are considered to be "economically significant" since it has contributed to a great extent to the cultural development of humanity. Genetic resources of the silkworm are of great importance not only for the breeding programs of this species, but also for the conduct of research in the field of genetics and molecular biology. Dur-

ing the long-term selection of this species, it has been working to improve the biological and technological traits such as fecundity, disease resistance, single cocoon weight, cocoon shell weight, filament length etc. To increase overall adaptability in the creation of new breeds and hybrids, it is important to find appropriate markers for the selection and use of genetically diverse material. The geographical diversity is important factor for genetic divergence (MUKHERJEE et al. 1999, PAL & MOORTHY 2011).

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The silkworm strains have different geographical origins, mainly the Japanese, Chinese, European and tropical strains, which have distinct traits.

More than 450 morphological, physiological and biochemical characters are described in the silkworm whose determinant genes are located in 27 of the total of 28 chromosome groups (BINDROO & MOORTHY 2014). Research on genetic diversity is critical to success in animal breeding and it provides information about the genetic divergence and could be used as a base for specific breeding objectives. Genetic diversity could be assessed among different individuals within the same breed (intra-breed) or among the breeds (inter-breeds) and it plays an important role in the breeding either to exploit the heterosis effect or to generate productive hybrids. Genetic diversity has been studied on the basis of different techniques utilizing morphological, physiological and biochemical characters (BINDROO & MOORTHY 2014). In general, cocoon colour and cocoon shape, larval marking and quantitative traits have been used for differentiation of silkworm genotypes and based on that, parents are being selected (BINDROO & MOORTHY 2014). However, recent advent of different molecular techniques led breeders to estimate genetic diversity on the basis of data generated by different markers, which provided a means of rapid analysis of germplasm and estimates of genetic diversity, which were often found to corroborate phenotypic data. These markers are mainly categorised as biochemical and molecular markers. Isoenzymes and allozymes have been used as biochemical markers for the study of genetically-determined polymorphism in *B. mori* and to analyse the degree of genetic heterogeneity of the populations (STAYKOVA 2008, STAYKOVA et al. 2012, 2015, BINDROO & MOORTHY 2014, MOORTHY & CHANDRAKANTH 2015, BUHROO et al. 2016). Application of isoenzymes and other molecular markers helps to estimate genetic diversity much more accurately than that of morphological traits. Electrophoresis identifies variation (alleles) at loci that codes for enzymes (isozymes or allozymes). One advantage of allozyme loci is that they are codominant and heterozygotes can be scored directly. Isoenzymes like esterase, acid phosphatase, alkaline phosphatase, amylase, phosphoglucomutase, aspartate aminotransferase, malate dehydrogenase and glucose-6-phosphate dehydrogenase have been used by various authors to study diversity in silkworm genotypes (SCHABALINA 1990, SOMASUNDARAM et al. 2004, MOORTHY et al. 2007, ASHOK KUMAR et al. 2011, PATNAIK et al. 2012, STAYKOVA et al. 2012, RONQUI et al. 2013).

Bulgaria is one of the European countries, having long history and tradition in sericulture and silk industries development. The genetic resources of *B. mori* in Bulgaria are quite rich and have been included more than 250 breeds with different geographical origin (PETKOV et al. 2006). Some of them have been studied earlier to find out the isozyme variability and the genetic relationships on this base (STOYKOVA et al. 2003, STAYKOVA 2008, STAYKOVA et al. 2015). For many of the breeds, however, such information is missing.

The present study aimed to detect the genetic diversity in silkworm breeds from the germplasm resources of Bulgaria that were not studied in this aspect previously and to reveal the relationships between breeds using isoenzyme markers.

## Materials and Methods

Total of five breeds of silkworm *B. mori* with different geographical origin and phenotype characteristic were used in the present investigation (Table 1). The breeds were obtained from the Scientific Centre on Sericulture, Vratsa, Bulgaria. The larvae were reared following the standard procedure.

Totally, 187 larvae (male and female) on the fifth day of the fifth instar were studied. Larvae were selected randomly from each breed and were submitted to electrophoretic analysis of haemolymph, silk glands and midgut tissues (mean sample size per locus ranged from 34 to 40).

The tissue extracts were prepared according to STOYKOVA et al. (2003), STAYKOVA et al. (2004, 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 haemolymph; 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 analyse 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 described earlier (STAYKOVA 2008). The staining mixtures for the enzymatic activities tested were taken from BOYER (1961), SPENCER et al. (1964), EATON et al. (1966), SHAW & PRASAD (1970), SCHMIDTKE & ENGEL (1972) and STAYKOVA et al. (2010). 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

**Table 1.** Origin and phenotype characteristic of tested silkworm breeds

Name of breed	Origin	Main characteristic
Hebar 2	Bulgaria	Uni-bivoltine, created at SCS - Vratsa. The egg serosa colour is green, chorion colour is yellow and eggs are sticky. The larvae are white in colour and plain. The cocoons are white with oval shape.
Sh 4	China	Uni-bivoltine, introduced in 1990. The egg serosa colour is grey, chorion colour is white and eggs are sticky. The larvae are white in colour with markings. The cocoons are white in colour and elongated.
Tahvon 106	North Korea	Uni-bivoltine, introduced in 1974. The egg serosa colour is grey, chorion colour is white and eggs are sticky. The larvae are white in colour with markings or plain. The cocoons are white in colour with oval shape.
Tg	Italy	Univoltine, introduced in 1971. The egg serosa colour is grey, chorion colour is white and the eggs are sticky. The larvae are white in colour with markings and with translucent yellow haemolymph. The cocoons are cream in colour, elongated with low constriction.
AES 1-zebra	Spain	Univoltine, introduced in 2008. The egg serosa colour is green and grey, chorion colour is white or yellow and eggs are sticky. The larvae are with zebra markings. The cocoons are elongated and spindle with yellow colour.

( $H_o$ ) 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

### Isoenzyme polymorphism

By PAGE, we found isoenzyme variability among the studied breeds. Seven of the nine loci were polymorphic. A total of 19 alleles were detected. Results for genetic heterogeneity of the breeds are presented in Table 2 and 3. Figs. 1–6 show electrophoretic spectra of the individual enzymes.

**Nonspecific esterases** (EC 3.1.1.11). In Spanish breed AES1-zebra, we found a two-allele polymorphism of the locus Bes A with the presence of the "null" allele - Bes A<sub>0</sub> (Fig. 1) and the allele Bes A<sub>1</sub> (Table 2). The same locus (Bes A) was monomorphic for all other breeds and it was represented by Bes A<sub>1</sub> allele, which was fixed. HE (1995) also established a polymorphism with the presence of a "null" allele of the locus Bes A in some breeds and hybrids grown in China.

Among the analysed breeds, we found a Bes B polymorphism with three codominant alleles – Bes B<sub>1</sub>, B<sub>2</sub> and B<sub>3</sub> (Table 2, Fig. 1). In the gene pool of Sh 4, we have established the presence of all three alleles. In the gene pool of the breeds Tahvon 106, Tg and AES1-zebra, we found two of them - Bes B<sub>2</sub> and B<sub>3</sub>. In the gene pool of Hebar 2, the Bes B<sub>2</sub> allele was fixed. We have established breed specificity in the frequencies of the different alleles on the poly-

morphic Bes B locus. The Bes B<sub>1</sub> allele was represented only in the gene pool of the Sh 4 breed. The highest frequency of the Bes B<sub>2</sub> allele was found in the AES1-zebra and the lowest in the Sh 4. The Bes B<sub>3</sub> allele had the highest frequency at Sh 4 and the lowest at the AES1-zebra. The same allele was missing from the gene pool of the Hebar 2 breed.

We have established a four-allele polymorphism in the Bes D locus with the presence of a "null" allele (Bes D<sub>1</sub>, D<sub>2</sub>, D<sub>3</sub> and D<sub>0</sub>). All four alleles were represented in the gene pool of the Sh 4 breed. In the other breeds we found a two-allele polymorphism with the presence of Bes D<sub>1</sub> and D<sub>2</sub> alleles (Table 2). The highest frequency of the Bes D<sub>1</sub> allele was found in the Tahvon 106 and the lowest in the Sh 4. Bes D<sub>2</sub> showed the highest frequency in the gene pool of Tg and the lowest in the gene pool of the Sh 4.

Polymorphism with three alleles was found on the Bes E locus, including a "null" allele (Bes E<sub>1</sub>, E<sub>2</sub> and E<sub>0</sub>). The existence of "null" alleles in esterase loci has been reported by other authors (EGUCHI et al. 1965, SHABALINA 1990, HE 1995, STAYKOVA 2008). In the gene pool of the Tahvon 106, Tg and AES1-zebra breeds, we found the presence of all three alleles (Table 2). In the gene pool of Sh 4, we have established a two-allele polymorphism with the presence of Bes E<sub>2</sub> and E<sub>0</sub> alleles. In Hebar 2, the Bes E locus was monomorphic and the Bes E<sub>0</sub> allele was fixed. It should be noted that the "null" allele (Bes E<sub>0</sub>) had the highest frequency in the gene pools of all tested breeds (0.738 ÷ 1.0). The highest frequency of the Bes E<sub>1</sub> allele was found in the Tg breed and the Bes E<sub>2</sub> allele - in Tahvon 106.

**Phosphoglucomutase** (EC 5.4.2.2). The comparative analysis of the individuals from the tested breeds showed well expressed intra- and inter-breed

**Table 2.** Allele frequencies in breeds tested

Locus (alleles)	Breeds				
	Hebar 2	Sh 4	Tahvon 106	Tg	AES 1-zebra
Bes A					
A <sub>1</sub>	1.000	1.000	1.000	1.000	0.622
A <sub>0</sub>	0.000	0.000	0.000	0.000	0.378
Bes B					
B <sub>1</sub>	0.000	0.149	0.000	0.000	0.000
B <sub>2</sub>	1.000	0.135	0.346	0.500	0.514
B <sub>3</sub>	0.000	0.716	0.654	0.500	0.486
Bes D					
D <sub>1</sub>	0.632	0.108	0.808	0.538	0.581
D <sub>2</sub>	0.368	0.149	0.192	0.463	0.419
D <sub>3</sub>	0.000	0.527	0.000	0.000	0.000
D <sub>0</sub>	0.000	0.216	0.000	0.000	0.000
Bes E					
E <sub>1</sub>	0.000	0.000	0.077	0.200	0.135
E <sub>2</sub>	0.000	0.027	0.179	0.063	0.041
E <sub>0</sub>	1.000	0.973	0.744	0.738	0.824
Pgm					
A <sub>2</sub>	1.000	0.473	1.000	0.550	0.338
A <sub>3</sub>	0.000	0.527	0.000	0.450	0.662
Mdh					
A <sub>2</sub>	1.000	1.000	1.000	1.000	0.946
A <sub>3</sub>	0.000	0.000	0.000	0.000	0.054
Bph					
A	0.000	0.162	0.130	0.000	0.176
B	1.000	0.000	0.000	0.000	0.027
0	0.000	0.838	0.897	1.000	0.797
Alp					
A <sub>1</sub>	1.000	1.000	1.000	1.000	1.000
Hk					
A <sub>2</sub>	1.000	1.000	1.000	1.000	1.000

polymorphism of Pgm A locus with the presence of two codominant alleles – Pgm A2 and A3 (Table 2, Fig. 2). The Pgm A1 allele previously described by STAYKOVA (2008) in the other breeds was not found in any of the breeds included in this study. The Pgm A2 allele was present in the gene pool of all breeds tested and in Hebar 2 and Tahvon 106 this allele was fixed. In the gene pool of the Sh 4, Tg and AES1–zebra breeds, we also found the Pgm A3 allele.

**Malate dehydrogenase** (EC 1.1.1.37). Among the studied breeds, we found polymorphism with two alleles in the Mdh A locus (Mdh A<sub>2</sub> and A<sub>3</sub>) only in one of the tested breed – AES1–zebra (Table 2, Fig. 3). The remaining four breeds showed monomorphism at this locus with the presence of the Mdh

A<sub>2</sub> allele that was fixed. The allele Mdh A<sub>1</sub>, which was previously described in other breeds from the genetic resources of Bulgaria (STAYKOVA et al. 2012), was not found in the gene pool of any of the breeds studied here.

**Acid phosphatase** (EC 3.1.3.2). STAYKOVA et al. (2015) found that the study of haemolymph was most appropriate for analysing the intra- and inter-breed polymorphism of acid phosphatase in the mulberry silkworm. Among the breeds included in the present study, we obtained polymorphism and the presence of three alleles controlling the various allozyme forms of acid phosphatase from the haemolymph of larvae one of which was “null” (Bph A, B and 0) (Table 2, Fig. 4). In the gene pool of the Spanish breed AES 1-zebra, we recorded all three alleles - Bph A, B and 0 (Table 2). Two-allele polymorphism was observed in Sh 4 and Tahvon 106 (Bph A, Bph 0). In two of the five breeds tested (Hebar 2 and Tg), we observed monomorphism with respect to this locus, with the Bph B and Bph 0 alleles, respectively.

**Alkaline phosphatase** (EC 3.1.3.1). In the five breeds, we found monomorphism at the alkaline phosphatase (Alp A) locus with the presence of one allele in the gene pools of the breeds - Alp A<sub>1</sub> (Table 2, Fig. 5).

**Hexokinase** (EC 2.7.1.1). In the study of the hexokinase from the silk glands of the silkworm larvae among the tested breeds, we did not detect inter- and intra-breed polymorphism. We have established one from the two of Hk A alleles previously described by STAYKOVA et al. (2015). The obtained results showed that the allele Hk A<sub>2</sub> was fixed in the five breeds (Table 2, Fig. 6).

### Statistical analysis

In the silkworm breeds analysed using nine enzyme loci, the number of alleles per locus calculated with BIOSYS-1 software package, ranged from 1.1 (Hebar 2) to 2.0 (AES 1-zebra) (Table 3). Degree of polymorphism (according to the criterion 0.99) was the highest for strain AES 1-zebra (77.80%) and the lowest for Hebar 2 (11.10%). The heterozygosity (H<sub>o</sub>) by polymorphic loci observed varied from 0.029 (for Hebar 2) to 0.222 (for AES 1-zebra). The values of genetic distance (NEI 1972) were calculated using the allele frequencies (Table 4). The highest genetic distance value was found for breeds Hebar 2 and Sh 4 (0.310) and the lowest one for Tg and AES 1 – zebra (0.034). The tree drawn by UPGMA (SNEATH & SOKAL 1973), based on isoenzyme results, showed that Bulgarian breed Hebar 2 is formed one main clade, while the rest breeds studied are formed the other, which is differentiated in two subclades (Fig.

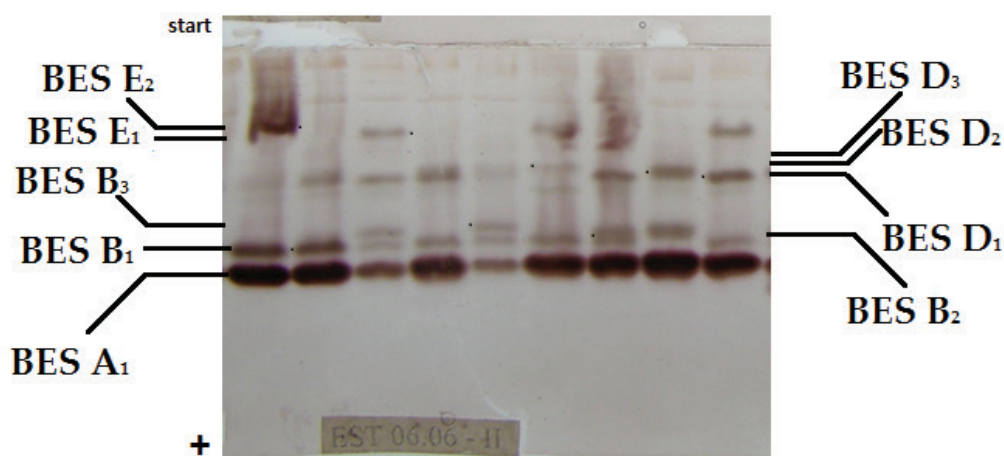


Fig. 1. Spectrum of non-specific esterases from the haemolymph of mulberry silkworm *B. mori* – larvae 5<sup>th</sup> instar.

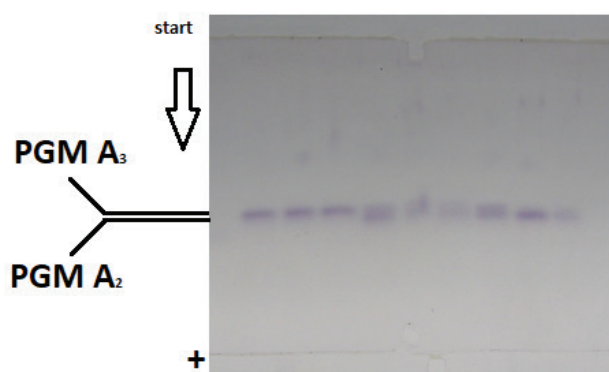


Fig. 2. Spectrum of phosphoglucosmutase from the silk glands of mulberry silkworm *B. mori* – larvae 5<sup>th</sup> instar.

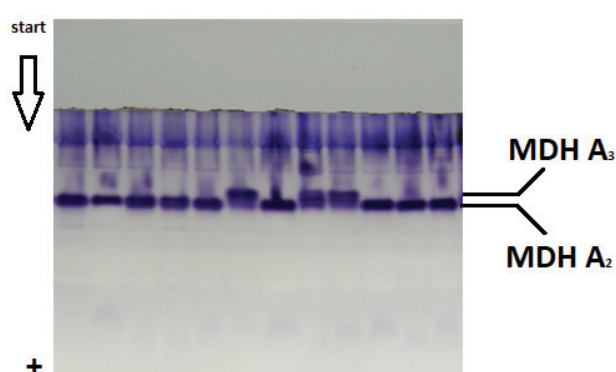


Fig. 3. Spectrum of malate dehydrogenase from the haemolymph of mulberry silkworm *B. mori* – larvae 5<sup>th</sup> instar.

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)	$H_o$	$H_e$
Hebar 2	34.0±0.0	1.1±0.1	11.1	0.029±0.029	0.052±0.052
Sh 4	37.0±0.0	1.9±0.4	55.6	0.144±0.057	0.215±0.087
Tahvon 106	39.0±0.0	1.6±0.2	44.4	0.097±0.042	0.153±0.065
Tg	40.0±0.0	1.6±0.2	44.4	0.119±0.063	0.214±0.085
AES 1- zebra	37.0±0.0	2.0±0.2	77.8	0.222±0.058	0.297±0.070

7). One of the subclade includes Sh 4 and the second one includes three others breeds (Tahvon 106, Tg and AES1–zebra). Tg and AES1–zebra breeds were grouped together.

## Discussion

Among the various enzymes, esterases, amylase, acid and alkaline phosphatase are much more thoroughly studied among insects, as they are involved in significant metabolic processes, in the differentiation of gonads, the functioning of the silk glands, etc.

(YOSHITAKE & AKIYAMA 1964, EGUCHI et al. 1965). Esterases are widespread isozymes, which show broad specificity to the substrate and demonstrate a tissue specific and stage specific expression in the course of ontogenesis (RONQUI et al. 2013, BUHROO et al. 2016). In insects, nonspecific esterases show a high degree of polymorphism and genetic variations (BUHROO et al. 2016). KASMAEI & MAHESHA (2012) by means of regression analysis establish that the activity of esterases of the haemolymph, from the midgut and from the fat body, positively correlates with some selectively valuable traits such as length

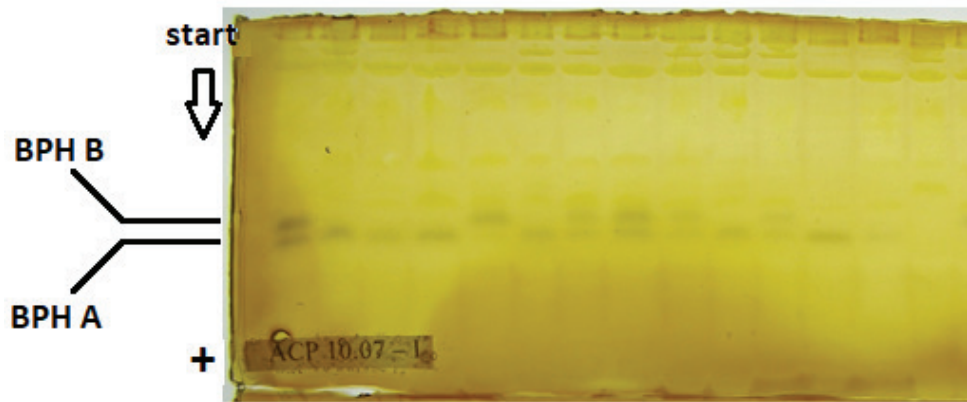


Fig. 4. Spectrum of acid phosphatase from the haemolymph of mulberry silkworm *B. mori* – larvae 5<sup>th</sup> instar.

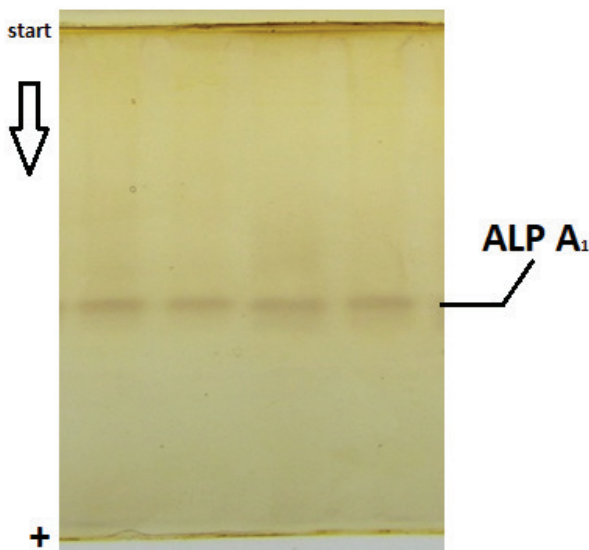


Fig. 5. Spectrum of alkaline phosphatase from the gut of mulberry silkworm *B. mori* – larvae 5<sup>th</sup> instar.

and thickness of the silk thread, duration of the larval stage, cocoon weight, weight of the silk thread and fertility.

EGOROVA & NASIRILLAEV (1993), HE (1995), STOYKOVA et al. (2003), STAYKOVA (2008), STAYKOVA et al. (2015) found that the most appropriate for analyzing the intra- and inter-breed polymorphism of silkworm nonspecific esterases, has been the haemolymph.

Polylocus control of haemolymph esterases of *B. mori* was reported by other authors in previous studies (EGOROVA et al. 1985, SHABALINA 1990, HE 1995, STOYKOVA et al. 2003, STAYKOVA 2008, STAYKOVA et al. 2015). In the present study, we confirmed this fact. Appropriate markers for the analysis of genetically determined polymorphism in the breeds from genetic resources of Bulgaria are the loci Bes A, Bes B, Bes D and Bes E. We have established breed specificity in the expression of esterase loci.

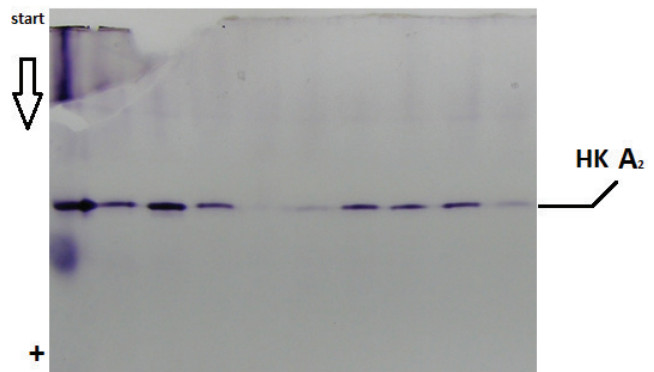


Fig. 6. Spectrum of hexokinase from the silk glands of mulberry silkworm *B. mori* – larvae 5<sup>th</sup> instar.

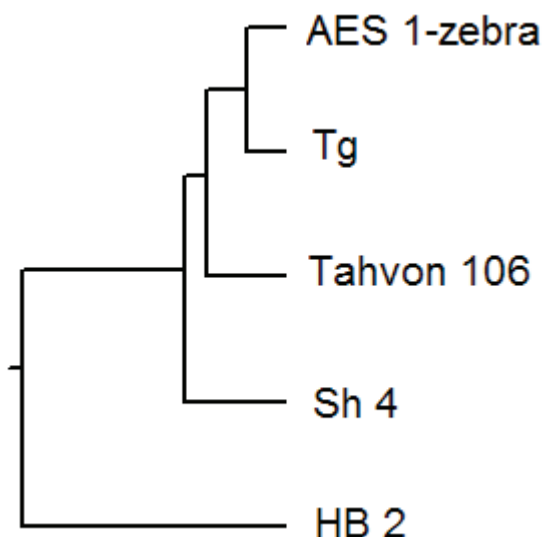


Fig. 7. UPGMA dendrogram (SNEATH & SOKAL 1973).

The phosphoglucomutase polymorphism found gives us reason to summarise that this enzyme is a very appropriate marker for analysing intra-breed heterogeneity and for inter-breed comparisons. This could be used in the course of selection as the increased expression of economically important

**Table 4.** Nei's (1972) genetic distance (above diagonal) based on isoenzymes.

Strains	Hebar 2	Sh 4	Tahvon 106	Tg	AES 1-zebra
Hebar 2	*****	0.310	0.191	0.212	0.241
Sh 4		*****	0.109	0.071	0.093
Tahvon 106			*****	0.044	0.099
Tg				*****	0.034
AES 1-zebra					*****

features (biological and technological) can be expected in the offspring of genetically different parents (KONICHEVA et al. 1975, EGOROVA et al. 1985, BINDROO & MOORTHY 2014).

EGOROVA & NASIRILLAEV (1993) reported the presence of malate dehydrogenase polymorphism in the *B. mori* haemolymph. Our results confirm the polymorphism of Mdh locus among the studied breeds from Bulgarian germplasm resources and differ from those of MARCATO et al. (1990) who have examined Italian breeds. Dissimilar results obtained by other authors, as well as the differences between the breeds studied, are probably related to the different geographical origin of the breeds and the "founder" effect.

Our results correspond to data by YOSHITAKE & AKIYAMA (1964) and EGUCHI et al. (1988), which have described allozyme polymorphism of the acid phosphatase from the haemolymph of *B. mori*. They had found that the allozyme forms were controlled by five codominant alleles of the same gene, one of the alleles being "null" (Bph A, B, C, D, 0). We observed three of them (Bph A, B and 0) and the differences in the frequency of individual alleles between various breeds. The Bph C and Bph D alleles found earlier in other breeds from the genetic resources of Bulgaria (STAYKOVA et al. 2012) were not found among the studied breeds. The established differences in the composition of the gene pool of the breeds studied are important because they provide valuable information in the selection of parental breeds for hybridization. When crossing breeds with different phenotype on acid phosphatase, a high heterosis effect among hybrids has been found. On this basis, the multiple forms of acid phosphatase could be used as a test for the heterogeneous selection of parental breeds in order to obtain high heterosis offspring. MOORTHY et al. (2007) have observed a high degree of variability in acid phosphatases in silkworm breeds with different origin. HAGHIGHI & KUMAR (2017) have found that the "null" variant of acid phosphatase can be

used as a unique marker because it enables by electrophoresis to analyse the degree of voltinism. In the group of breeds we examined, this allele was present in the gene pool of four of breeds.

The monomorphism at the alkaline phosphatase described in this study corresponds to the lack of polymorphism on the locus encoding the soluble form of alkaline phosphatase which was found by TAKEDA et al. (1990) in *B. mori*. MIAO (2002) established that the activity of alkaline phosphatase varied among the breeds. He found a positive correlation between the activity of this enzyme and the quality of the cocoon.

STAYKOVA et al. (2015) described polymorphism of hexokinase among some breeds kept in Bulgaria. The allele Hk A<sub>1</sub> who was found by them did not occur in any of the breeds studied here, which we assume is the result of different origins and breeding activities.

Based on established isoenzyme polymorphism, we analysed the genotypic structure of the studied breeds and the level of heterozygosity. The expected heterozygosity ( $H_e$ ) was higher than the observed one ( $H_o$ ) in all strains tested. Significant differences ( $P < 0.05$ ) in genotype frequencies were seen at the most loci in strains studied. Chi-Square test ( $DF=1 \div 6$ ) showed that the deviations from the Hardy-Weinberg equilibrium were in result of excess of homozygotes and deficiency of heterozygotes. These results demonstrate the effects of inbreeding. Similar effects were found earlier by STAYKOVA et al. (2015).

The mean  $F_{ST}$  value over all loci, which is associated with the level of inter-breed differentiation, was 0.3103 and showed that 31.03% of the overall genetic diversity observed was among strains, while 68.97% of genetic variations were within breeds. The higher level of genetic diversity among breeds we found for the Bph A locus (0.6344). In the other loci, we observed a lower degree of inter-breed genetic variability: Bes A (0.3281), Bes B (0.2641), Bes D (0.2018), Pgm A (0.3389) and Bes E (0.0838). The lower level of genetic diversity among breeds we found for the Mdh A (0.0438) locus.

The differences between the studied breeds with respect to the genetically determined isoenzyme polymorphism showed that the highest level of genetic similarity was found between breeds Tg and AES 1-zebra which are originated from Italy and Spain. The Bulgarian breed Hebar 2 was genetically distant from all other breeds and could be used in various breeding programs.

## Conclusion

In the present study, we found a total of 7 polymorphic loci with 19 alleles. For the AES 1-zebra breed we found variability in all loci, whereas in Hebar 2 only at one of them. The observed polymorphism demonstrated breed specificity. The Bes A<sub>0</sub> allele was specific to the AES 1-zebra gene pool, the Bes D<sub>3</sub> and Bes D<sub>0</sub> alleles were specific for Sh 4 and Mdh A<sub>3</sub> was specific only for AES 1-zebra.

We also observed inter-breed differences regarding the degree of polymorphism and heterozygosity. The lowest values for both parameters were found in the Bulgarian breed Hebar 2 and the highest for the Spanish breed AES 1-zebra. The breeds were found to form two distinct clusters indicating the presence of distinct genetic diversity among them. Hebar 2 is formed one main cluster, while the rest breeds studied are formed the other one.

The present study complements the data on the genetic heterogeneity of breeds from the genetic resources of Bulgaria. The results described could help the breeders in the process to identify suitable parents for breeding programs and to recommend crossing the certain breeds from different clusters for yield improvement.

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