ACTA ZOOLOGICA BULGARICA
Acta zool. bulg., Suppl. 4, 2012: 89-94

Genetic Variability in Silkworm (*Bombyx mori* L.) Strains with Different Origin

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Abstract: Genetic diversity within and among ten silkworm *Bombyx mori* strains was investigated using isoenzymes of four metabolic enzymes viz., phosphoglucomutase (PGM), malate dehydrogenase (MDH), acid phosphatase (ACP) and aspartataminotransferase (AST) by polyacrilamide gel electrophoresis (PAGE). Three of the studied enzyme systems – PGM, MDH and ACP manifested intra- and inter-strain polymorphism with three or five alleles. AST was monomorphic in all strains tested. The observed heterozygosity was found to range from 0.000 to 0.266. Allele frequencies of all loci were used to estimate Nei's (1972) genetic distance, which was found to range between 0.004 and 0.457 among the strains studied. A perusal of genetic diversity within and among strains indicated that 34.72% of the observed variation occurred among strains and the rest of the variation (65.28%) within strains. Genetic relatedness of ten strains revealed by UPGMA dendrogram, showed genetic grouping of strains in two clusters. Populations of silkworm strains Kinshu and E 29, and Asahi and Kinshu showed the highest percent of polymorphism and more number of alleles, respectively. Their rich genetic diversity needs to be exploited in conservation and breeding programme.

Key words: *Bombyx mori* L., isoenzymes, genetic variability, phylogeny

Introduction

Mulberry silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae), has long been used as a model system for basic research in microbiology, physiology, and genetics because of its economic importance in sericulture. Silkworm became a model organism after *Drosophila* because of its large size and the possibilities to keep a culture in laboratory condition. The well-developed genetic resources of this species include more than 4000 strains. There are more than 400 described mutants, which have been mapped to more than 200 loci in 28 linkage groups (Mita *et al*. 2004). These mutations and different strains help fundamental research. Assessment of heterozygosity in each silkworm race is important for efficient management and conservation of genetic resources (Graner *et al*. 2004). Application of isoenzymes and other molecular markers help estimate genetic diversity much more accurately than that of morphological traits. Information on these various markers and their application is important in understanding the molecular basis of differentiation between races and their phylogenetic relationships.

The genetic variability among the silkworm strains reared in Bulgaria has been estimated mainly on the base of some qualitative and quantitative traits (total larval duration, cocoon shape, cocoon colour, weight of single cocoon, weight of single shell, shell ratio, fecundity, etc). Isoenzymic polymorphism has been slightly studied (Shabalina 1990, Stoykova *et al*. 2003, Staykova, Grekov 2006, Staykova 2006, Staykova 2008). It has not been made phylogeny reconstruction of the strains.

The present study was carried to determine the degree of diversity and the existing relationships between ten strains belonging to the silkworm germplasm bank of Agricultural University of Plovdiv, using isozyme markers.

Material and Methods

Silkworm strains and rearing

Twelve strains of silkworm (offsprings of 2010/11 year) with different origin (Table 1) were obtained from Agricultural University in Plovdiv. All individuals were nourished at a standard regime of silkworm breeding. On the fifth day of the fifth instar, 80-87 larvae were selected randomly from each strain (Table 3).

Haemolymph and silk glands extraction and electrophoretic analysis

The larval haemolymph was taken with a transactional cut through one of the prolegs. To avoid the activity of prophenol oxidase followed by melanization of haemolymph, a small amount phenylthiourea was added to the samples as well as 0.8 M tris-phosphate buffer at pH 6.7. The silk glands were isolated through dissection, rinsed with distilled water, squashed with quartz sand in 0.8 M tris−phosphate buffer at pH 6.7, left for extraction for 18 h at 4 °C and centrifuged for 45 min at 5000 rpm. The spectrum of malate dehydrogenase (MDH) (EC 1.1.1.37) and acid phosphatase (ACP) (EC 3.1.3.2) from hemolymph and the spectra of phosphoglucomutase (PGM) (EC 5.4.2.2) and aspartate aminotransferase (AST) (EC 2.6.1.1) from silk glands of larvae were studied by means of 7.5% PAGE (Stoykova *et al*. 2003, Staykova *et al*. 2004). 10μL of each sample was applied into the gel. Method of Shaw, Prasad (1970) was used to visualize the malate dehydrogenase, methods of Spencer *et al*. (1964) and Schmidtke, Engel (1972) were used to visualize the phosphoglucomutase and the aspartate aminotransferase respectively. Acid phosphatase isoenzymes were visualized according to Staykova *et al.* (2010).

Statistical and clustering methodology

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). UPGMA dendrogram (Sneath, Sokal 1973) was constructed using PHYLIP software package (Felsenstein 1993).

Table 1. Tested strains of silkworm (*Bombyx mori* L.).

Strains	Origin
Asahi	Japan
Kinshu	Japan
Gergana 1	Bulgaria
Gergana 2	Bulgaria
Line 22 Pv	Uzbekistan
Ogosta 1	Bulgaria
Mziuri 1 Pv	Georgia
M-6	Azerbaijan
Almaz	Azerbaijan
E 29	Egypt

Results and Discussion

Three of the studied enzyme systems – phosphoglucomutase, malate dehydrogenase and acid phosphatase manifested intra- and inter-strain polymorphism (Table 2). The aspartate aminotransferase was monomorphic in all strains tested.

PGM from *B. mori* silk glands is under monogene control within polymorphism described (Staykova 2006, Staykova 2008). In the gene pool of the strains Gergana 1, Line 22 Pv, Ogosta 1, Mziuri 1 Pv, Almaz and E 29, the Pgm A locus was presented with three alleles – Pgm A_1, A_2 and A_3 (Table 2). In Kinshu strain gene pool we found alleles Pgm A_2 and A_3. For eight out of ten strains, the highest frequency belonged to Pgm A_2 allele, which was fixed in the gene pool of strains Asahi, Gergana 2 and M-6. Staykova (2008) described polymorphism of Pgm locus in Asahi with tree alleles and the highest frequency of Pgm A_2. The fixing of this allele in the gene pool of Asahi is probably resulting from the gene drift.

Kageyama *et al*. (1972) described two forms of MDH from silkworm eggs (sMDH and mMDH) with molecule weight 56kD. sMDH has been prevailed over mMDH. Marcato *et al*. (1990) reported for lacking of polymorphism by Mdh locus and other enzyme loci in some Italian strains, which demonstrated low level of the genetic diversity. Egorova,

Table 2. Allele frequencies in strains tested.

Locus	Strains									
	Asahi	**Kinshu**	**Gergana 1**	**Gergana 2**	**Line22 Pv**	**Ogosta 1**	**Mziuri1 Pv**	**M-6**	**Almaz**	**E29**
PgmA										
A_1	0.0	0.0	0.265	0.0	0.267	0.097	0.063	0.0	0.050	0.200
A_2	1.0	0.306	0.500	1.0	0.467	0.236	0.547	1.0	0.600	0.550
A_3	0.0	0.694	0.235	0.0	0.267	0.667	0.391	0.0	0.350	0.250
MdhA										
A_1	0.031	0.113	0.0	0.0	0.200	0.0	0.0	0.0	0.050	0.0
A_2	0.859	0.855	1.0	1.0	0.683	0.972	1.0	1.0	0.950	0.950
A_3	0.109	0.032	0.0	0.0	0.117	0.028	0.0	0.0	0.0	0.050
Bph A										
A	0.219	0.306	0.529	0.550	0.0	0.0	0.625	1.0	1.0	0.600
B	0.047	0.387	0.471	0.450	1.0	1.0	0.375	0.0	0.0	0.400
C	0.641	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
D	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0	0.094	0.306	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Ast A										
A	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

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

Strains	**Mean sample size per locus**	**Mean no. of alleles per locus**	**Percent polymorphic loci (P=0.99)**	**H_o**	**H_e**
Asahi	82.0±0.0	2.3±0.80	50.0	0. 102±0.062	0. 198±0.128
Kinshu	81.0±0.0	2.3 ±0.50	75.0	0. 266±0.130	0. 341±0.142
Gergana 1	84.0±0.0	1.8±0.50	50.0	0. 184±0.114	0. 285±0.167
Gergana 2	80.0±0.0	1.3±0.30	25.0	0. 108±0.108	0. 126±0.126
Line 22 Pv	87.0±0.0	2.0±0.60	50.0	0. 092±0.063	0. 285±0.168
Ogosta 1	86.0±0.0	1.8±0.50	50.0	0. 063±0.046	0. 138±0.120
Mziuri 1 Pv	82.0±0.0	1.8 ±0.50	50.0	0. 172±0.118	0. 257±0.149
M-6	80.0±0.0	1.0±0.00	0.0	0. 000±0.000	0. 000±0.000
Almaz	80.0±0.0	1.8±0.50	50.0	0. 125±0.095	0. 155±0.125
E 29	80.0±0.0	2.0±0.40	75.0	0. 217±0.099	0. 297±0.147

Nasirillaev (1993) described polymorphism on MDH of hemolymph in some Russian strains. In six out of ten studied strains with different origin, kept in Bulgaria we established polymorphism by Mdh locus, too (Table 2). In the gene pool of strains Asahi, Kinshu and Line 22 Pv we detected tree alleles (Mdh A_1, A_2 и A_3). In the gene pool of Ogosta 1 and E 29 we found Mdh A_2 and A_3 alleles and for Almaz – Mdh A_1 and A_2. Mdh A_2 allele was the most common for all tested strains with polymorphism by Mdh locus. The same allele was fixed in the gene pool of Gergana 1, Gergana 2, Mziuri 1 Pv and M-6.

ACP is an important phosphatase which isozyme profiles are highly complex and its heterogeneity arises from multiple genetic loci. It is involved as mediator in the energy transfer. Yoshitake, Akiyama (1964) and Eguchi *et al*. (1988) described five isozyme forms of ACP from hemolymph (BPH – blood phosphatases) designated as A, B, C, D and 0, which were considered to be controlled by codominant

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

	Asahi	Kinshu	Gergana 1	Gergana 2	Line22 Pv	Ogosta 1	Mziuri1 Pv	M-6	Almaz	E29
Asahi	*****	0. 302	0. 198	0. 113	0. 362	0. 453	0. 192	0. 154	0. 226	0. 180
Kinshu		*****	0. 094	0. 198	0. 174	0. 096	0.069	0. 278	0. 163	0. 095
Gergana 1			*****	0. 056	0. 133	0. 139	0. 015	0. 113	0. 083	0. 004
Gergana 2				*****	0. 201	0. 269	0. 056	0. 053	0. 106	0. 045
Line22 Pv					*****	0. 057	0. 186	0. 457	0. 424	0. 161
Ogosta 1						*****	0. 158	0. 521	0. 396	0. 169
Mziuri1 Pv							*****	0. 086	0. 045	0. 007
M-6								*****	0. 037	0. 082
Almaz									*****	0. 056
E29										*****

alleles Bph A, Bph B, Bph C, Bph D and Bph 0, respectively. The 0 type has been symbolized because it does not show any active band. Among the strains included in this study, we found the presence of four alleles in the gene pool of Asahi strain – BphA, B, C and 0 (Table 1). We established three alleles in Kinshu – Bph A, B and 0. In the gene pool of strains Gergana 1, Gergana 2, Mziuri 1 Pv and E 29, only Bph A and B alleles were presented. Bph A allele was fixed in Azerbaijan strains M-6 and Almaz, and Bph B allele was fixed in Line 22 Pv and Ogosta. We found that Bph A allele was the most common for Gergana 1, Gergana 2, Mziuri 1 Pv, and E 29 strains. Bph B and C alleles were with the highest frequency for Kinshu and Asahi, respectively.

Polymorphism by AST for some Russian strains of *B. mori* L. has been reported by Zhukova *et al.* (1974) and Minina (1979). However, this locus was monomorphic for the ten strains included in this study.

In ten silkworm strains analysed using four enzyme loci the number of alleles calculated with BIOSYS-1 software package ranged from 1.0 (for M-6) to 2.3 (for Asahi and Kinshu) per locus (Table 3). The degree of polymorphism (according to the criterion 0.99) was highest for strains Kinshu and E 29 (75%), and lowest – for M-6 (0% – no polymorphic loci). The observed heterozygosity (H_o) varied from 0.000 (for M-6) to 0.266 (for Kinshu). With all analysed strains the expected heterozygosity (H_e) by polymorphic loci was higher than the observed one. There were significant deviations of genotype frequencies from Hardy-Weinberg expectations for some of the strains. Chi-Square (df: 1-6) tests showed that the deviations were generally in favour of homozygotes. The lower degree of observed heterozygosity and the higher degree of homozygotes proved the inbreeding effect.

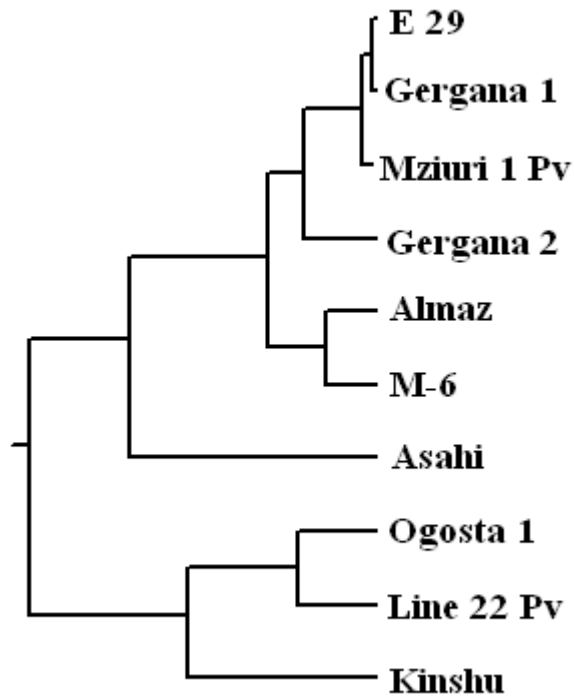

Fig. 1. UPGMA dendrogram

The estimated mean F_{ST} value was 0.3472 which shows that 34.72% of the overall genetic diversity observed was among strains, as opposed to 65.28% within strains. A higher degree of inter-strain variability was reported on the acid phosphatase (0.4708), and a lower one – on the malate dehydrogenase (0.1100).

The values of genetic distance (Nei 1972) were calculated using the allele frequencies and ranged

from 0.004 (between the strains Gergana 1 and E 29) to 0.457 (between strains Line 22 Pv and M-6) (Table 4).

In UPGMA dendrogram strains studied are grouped in two clades. Kinshu, Line 22 Pv and Ogosta 1 were clustered in the first clade and all other strains were clustered in the second one (Fig. 1).

Both biochemical and molecular markers have recently been employed to estimate the extent of genetic diversity present among various types of silkworm strains such as mono-, bi- and multivoltines kept in China, Japan, Korea, India, and several other countries (Xia *et al*.1998, Reddy *et al*. 1999a, b, Nagaraju *et al*. 2001, Li *et al*. 2005, Prasad *et al*. 2005, Dalirsefat *et al*. 2009). The results obtained in this study showed that the acid phosphatase and malate dehydrogenase of silkworm haemolymph, and phosphoglucomutase of silk glands are reliable genetic markers in order to determine the level of inter- and intra-strain diversity and strain differentiation. These polymorphic systems could be use in the breeding programs for increasing the productive and adaptive potential of the newly created strains and hybrids of mulberry silkworm *Bombyx mori* L.

References

Dalirsefat S., A. Meyer and S. Mirhoseini 2009. Comparison of similarity coefficients used for cluster analysis with amplified fragment length polymorphism markers in the silkworm, *Bombyx mori*. – *Journal of Insect Science*, **9**:71. Available online: http://insectscience.org/9.71.

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.

Graner A., K. Dehmer, T. Thiel and A. Borner 2004. Plant genetic resources: benefits and implications of using molecular markers. – In: de Vicente MC (Ed.): Issues in Genetic Resources, **11**: 26-32.

Kageyama T., S. Takahashi and E. Ohnishi 1972. Malate dehydrogenase in the eggs of the silkworm, *Bombyx mori*: purification and properties. – *Insect Biochemistry*, **2** (6): 186-196.

Li M., L. Shen, A. Xu, X. Miao, C. Hou, P. Sun, Y. Zang and Y.Huang 2005. Genetic diversity among the silkworm (*Bombyx mori* L., Lep., Bombicidae) germplasm revealed by microsatellites. – *Genome*, **48**: 802-810.

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*, **LXXIII** (2): 595-608. (In Italian, English summary).

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).

Mita K., M. Kasahara, S. Sasaki, Y. Nagayasu and T. Yamada 2004. The genome sequence of silkworm, *Bombyx mori*. – DNA *Researchers*, **11**: 27–35.

Nagaraju J., K. Reddy, G. Nagaraja and B. Sethuraman 2001. Comparision of multilocus RFLPs and PCR-based marker systems for genetic analysis of the silkworm *Bombyx mori*. – *Heredity*, **86**: 588-597.

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

Prasad M., M. Muthulakshmi and M. Madhu 2005. Survey and analysis of microsatellites in the silkworm, *Bombyx mori*: Frequency, distribution, mutation, marker potential and their conservation in heterologous species. – *Genetics,* **169** (1): 197-214.

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

Reddy K., J. Nagaraju and E. Abraham 1999b. Genetic characterization of the silkworm *Bombyx mori* by simple sequence repeat (SSR) anchored PCR. -*Heredity*, **83** (6): 681-687.

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.

Shmidtke 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.

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

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, Vratza, Bulgaria, 197-201.

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

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., 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., 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.

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.

Xia Q., Z. Zhou, C. Lu and Z. Xiang 1998. Molecular Phylogenetic study on the racial differentiation of *Bombyx mori* by random amplified polymorphic DNA (RAPD) markers. – *Acta Entomologica Sinica***, 41**: 32-40.

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).