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Genetic Variability of the Silkworm, *Bombyx mori* (L., 1758) (Lepidoptera: Bombycidae) of Different Geographical Origin as Revealed by mtDNA Gene Segment Sequencing Analysis

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Abstract: Genetic variability and phylogenetic relationships in eight strains of the economically important silkworm *Bombyx mori* of different geographical origin and belonging to the Bulgarian germplasm were examined for the first time using 12S rDNA mtDNA gene segment sequencing analysis. We found 58 variable sites. Intra-strain variability was detected based on neighbour-joining, minimum evolution, maximum parsimony, UPGMA and PCA analyses. Eight haplotypes were revealed corresponding to the eight strains studied. PCA analysis distinguished Plovdiv 14, Plovdiv 18 and Maiak 5 strains from the rest. Maximum parsimony and UPGMA trees demonstrated that the strain Plovdiv 14 showed a large sequence divergence as compared to the other studied strains. Our results could be applied in future conservation strategies and breeding programs for *B. mori* in Bulgaria. 12S rDNA mtDNA gene segment sequencing analysis could be used with isoenzyme analysis for discriminating of strains of different geographical origin in relation to their involvement in breeding programs and preserving their original characteristics.

Key words: *Bombyx mori*, sequencing data, mtDNA, conservation, Bulgaria

Introduction

Silk moths are the best studied lepidopteran insects and most of them belong to two families, Bombycidae and Saturniidae. *Bombyx mori* (Linnaeus, 1758) (Lepidoptera: Bombycidae) is known to have many mutations in its genome that affect aspects such as its behaviour, morphology and development. Sericulture is flourishing economically in many countries, such as China, Japan, India, Russia, Korea, Bulgaria, and Iran. This is the reason why a number of silkworm breeds growing in these countries has been considered suitable for a wide range of climatic conditions. More than 4000 strains are maintained in the germplasm of *B. mori* (Bindroo & Moorthy 2014).

The appropriate markers used to study the silkworm strains can compose a classification, which is important for the species breeding regarding the increasing of its adaptive potential and productivity (Mirhoseini & Gholami 2002). Recently completed genome sequence of *B. mori* (Mita *et al*. 2004, Xia *et al*. 2004) provides useful information regarding this aspect. Mitochondrial DNA (mtDNA) has been widely employed in phylogenetic studies of animals because it evolves much more rapidly than nuclear DNA, resulting in the accumulation of differences between closely related species (Brown *et al*. 1979, Mindell *et al*. 1997, Moore 1995). Molecular phylogeny of silk moths was studied using gene rearrangements and sequences of mtDNA (Yukuhiro *et al*. 2002), mitochondrial cytochrome b gene (Li *et al*. 2005), 12S rRNA, 16S rRNA, and cytochrome

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oxidase I gene (Arunkumar *et al*. 2006). The analysis of the mitochondrial genome has showed that the studied local strains of *B. mori* are closer to Chinese *Bombyx mandarina* than to Japanese one. Genetic and evolutionary relationships of domestic and wild species of silkworms have been studied on the base of mtDNA sequences of cytochrome oxidase subunit-I gene by Lenin (2015). Their results revealed the genetic affinity and phylogenetic status of *B. mori* as compared to 13 wild species of silkworms, demonstrating the close genetic relationship between *B. mori* and *B. mandarina* as well. Chen *et al*. (2007) studied the structure of A-T- rich regions of mtDNA and evolution of several Chinese races of *B. mori*. Their results have showed that one of the local race – Gansu, formed one group by itself and its evolution has preceded the one of the other races in the group.

The Bulgarian germplasm includes more than 250 strains of mulberry silkworms *B. mori* of different origin (Petkov *et al*. 2006). The genetic variability among the silkworm strains reared in Bulgaria has been estimated mainly on the base of qualitative and quantitative traits (total larval duration, cocoon shape, cocoon colour, weight of single cocoon, weight of single shell, shell ratio, fecundity, etc.). Intra- and inter-strain genetic varyability of isoenzymes and phylogenetic relationships have been also studied (Shabalina 1990, Stoykova *et al*. 2003, Staykova & Grekov 2006, Staykova 2006, 2008, Staykova *et al*. 2010, 2015). There is no information about genetic variations of mtDNA in strains of Bulgarian germplasm resources and their evolution.

The aim of the present study was to determine the genetic variability and phylogenetic relationships in eight strains of mulberry silkworm originated in different geographical regions and belonging to the germplasm bank of Bulgaria, using mtDNA gene segment sequencing analysis.

Materials and Methods

Eight strains of mulberry silkworm *Bombyx mori* of different origin have been studied (Table 1). Larvae were reared using standard methods in the Agricultural University of Plovdiv, Bulgaria. During early stages (1st-3rd instar), the temperature was maintained about 25°-27°C, the air humidity was 85-90%, the premises were aired every three hours for 15 minutes. The larvae were fed on finely chopped mulberry leaves 3-4 times per day. Bedding was changed after each sleep cycle. The 4th-5th instar larvae were reared at a temperature of 20°-24°C, 65-75% air humidity, and the premises were aired every hour for 10 minutes. The larvae were given entire mulberry leaves 3-5 times a day. Bedding was changed every two days. The pupae samples for DNA sequencing analysis were placed alive in alcohol 95%, and stored in the laboratory at -20°C.

DNA Sequencing Analysis

Total DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's protocol after minor modifications. Fragment of 12S rDNA mtDNA gene segment was amplified using a PEQLAB Thermocycler (Primus 25 advanced). The primers used were 5-AAACTGGGATTAGATAC CCCACTAT-3 and 5-GAGGGTGACGGGCGGT-GTGT-3 (Palumbi *et al*. 1991).

The polymerase chain reaction (PCR) (Saiki *et al*. 1988) consisted of 2.4 units of *Taq* polymerase (Invitrogen), 5 μL of 10X reaction buffer (Invitrogen), 5 μL dNTPs mix (2 mM) (BioLabs), 5 μL $MgCl_2$ (5 mM) (Invitrogen), approximately 100 ng DNA, 0.5 μL of each primer (20mM) (BioLabs), and sterile water. PCR amplification conditions consisted of a four-minute denaturation step at 94°C followed by 35 cycles of 94°C for 1 min, annealing at 44°C for 1 min and extension at 72°C for 2 min. The PCR products were purified using the Nucleospin extract II kit (Macherey-Nagel) according to the supplier's protocol and were examined through agarose gel electrophoresis. Each sequence was determined via automated sequencing procedure provided by Macrogen Company (Seoul, Korea) using the same primers as in the amplification procedure. The sequences obtained have been deposited to Genbank with accession numbers KR707815- KR707822 (Table 2).

Data and Phylogenetic Analysis

Multiple-sequence alignments were done with CLUSTALW2 (Thompson *et al*. 1994). The computer-generated alignment was further adjusted manually. The authenticity of the produced mtDNA sequences was verified using BLAST program of NCBI.

Pairwise genetic distance, based on 12Sr DNA sequencing data, was estimated using MEGA v4.0.2 (Tamura *et al*. 2007) and the Kimura two-parameter model (Kimura 1980). Haplotypes were detected using DNA sp v. 5.10.00 package (Librado, Rozas 2009), as well as the number of variable sites, haplotype and nucleotide diversity.

Neighbor-joining (NJ), minimum evolution (ME), maximum parsimony (MP) and UPGMA analysis were performed using MEGA v4.0.2 (Tamura *et al.* 2007) package. Confidence in the nodes was evaluated by 1000 bootstrap replicates (Felsenstein 1985). Principal Component Analysis (PCA) was performed using GenAlEx 6.41 (Peakall, Smouse 2012).

Table 1. Strains of silkworm (*Bombyx mori* L.)

Name of strain	Origin
Plovdiv 18	Bulgaria
Plovdiv 14	Bulgaria
Maiak 5	Azerbaijan
China 23	China
Asahi	Japan
Almaz	Azerbaijan
Shova	Japan
Gindga 8	Azerbaijan

Table 2. Eight haplotypes revealed with the 12S rDNA gene segment sequencing analysis. Genbank accession numbers are also indicated

Haplotypes	Strain	Genbank Access. No
Haplotype 1	Plovdiv 18	KR707815
Haplotype 2	Plovdiv 14	KR707816
Haplotype 3	Maiak 5	KR707817
Haplotype 4	China 23	KR707818
Haplotype 5	Asahi	KR707819
Haplotype 6	Almaz	KR707820
Haplotype 7	Shova	KR707821
Haplotype 8	Gindga 8	KR707822

Results

The sequencing of 12S rDNA mtDNA gene segment produced an alignment of 342 bps and eight different haplotypes revealed (Table 2). The number of variable sites was 58 and the different nucleotides that are found in a specific position for the eight haplotypes are summarised in Table 3. The most frequent nucleotide was adenine (A), followed by thymine (T). Both of them (A+T) were around two time more as compared to cytosine (C) and guanine (G) (C+G). Moreover, there were very few cases in which the four nucleotides were present in the same site. In most cases there was only one different base, while the other seven haplotypes shared the same base at this position. For example, if at position 2, we found nucleotide A in the seven haplotypes, but only in the 8th haplotype there was the nucleotide G, demonstrating low variability in these silk worm populations.

Variable sites of the sequences obtained are presented in Fig. 1.

The average pairwise genetic distance was 0.068 (Kimura 2-parameter, Kimura 1980). The haplotype diversity Hd was 1.00, and the nucleotide diversity per site (pi) was 0.06318. The highest divergence value (0.149) was observed between the strains Plovdiv 14 and Plovdiv 18 in which there were the most polymorphic sites.

Table 3. Analysis of the 58 variable sites that were detected among the eight haplotypes and the different nucleotides per position

	Position (bp)	A	G	C	T		Position (bp)	A	G	C	T
1	1	3	1	0	4	**30**	103	0	0	2	6
2	7	7	1	0	0	**31**	104	1	7	0	0
3	8	5	1	0	2	**32**	141	7	0	1	0
4	9	1	0	0	7	**33**	144	0	0	1	7
5	12	6	2	0	0	**34**	153	1	0	0	7
6	16	7	1	0	0	**35**	159	7	0	0	1
7	17	1	0	7	0	**36**	162	6	2	0	0
8	18	7	0	1	0	**37**	164	6	0	2	0
9	19	1	0	0	7	**38**	169	2	0	0	6
10	20	0	0	1	7	**39**	172	6	0	2	0
11	21	0	0	1	7	**40**	174	1	0	7	0
12	26	3	0	5	0	**41**	203	3	0	0	5
13	27	0	1	7	0	**42**	214	0	6	2	0
14	34	5	1	1	1	**43**	231	7	1	0	0
15	37	1	1	0	6	**44**	241	0	0	6	2
16	38	7	1	0	0	**45**	242	0	0	2	6
17	40	2	0	1	5	**46**	258	1	0	0	7
18	42	0	0	3	5	47	275	6	0	0	2
19	43	1	0	2	5	**48**	296	7	1	0	0
20	54	6	0	2	0	**49**	297	0	0	7	1
21	58	5	1	2	0	**50**	298	0	0	1	7
22	60	2	0	0	6	**51**	305	0	5	3	0
23	66	7	1	0	0	**52**	306	0	0	3	5
24	67	7	1	0	0	**53**	307	7	0	0	1
25	76	0	2	0	6	**54**	309	0	0	4	4
26	78	7	0	1	0	**55**	310	7	0	1	0
27	80	0	2	6	0	**56**	312	0	0	1	7
28	99	7	0	0	1	**57**	316	0	0	2	6
29	102	1	0	7	0	**58**	319	6	1	1	0
Total								**180**	**40**	**95**	**149**

The trees drawn as a result of NJ, ME and MP analyses, based on sequencing results, exhibited the same topology. For this reason only the MP tree is presented here (Fig. 2).

According to this topology, Plovdiv 14 was in separate clade while the other studied strains were grouped together. The suggested topology was supported by the high bootstrap values.

UPGMA tree showed also a similar topology with two clades, with Plovdiv 14 in one main clade, while the studied other strains formed the other with two subclades (Fig. 3). One of the subclades included strains Almaz and Shova, and the second one includes Maiak 5, Plovdiv 18, Asahi, Gindga 8 and China 23.

The PCA discriminated strains Plovdiv 14, Plovdiv 18 and Maiak 5 from the other ones (Fig. 4).

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#Bombyx_mori-strain_Plovdiv_18 TAA TTA ATT AAA ATT ACA TTT AAA TCC AAT TTC AAT TAA TTT TTA AAA ATA ACC TCC AAA TAA ATA
#Bombyx_mori-strain_Plovdiv_14 ... ... GG. ..G ... GAC ACC ... .AG ... ... C.. A.. C.C C.. ... ... ..A ... G.T ... ..G
#Bombyx_mori-strain_Maiak_5    ... ... .A. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Bombyx_mori-strain_China_23   A.. ... .A. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..A ... ..T ... ...
#Bombyx_mori-strain_Asahi      A.. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..A ... ..T ... ...
#Bombyx_mori-strain_Almaz      ... ... .AA ... ... ... ... ... .A. ... ... T.. G.. A.C A.. ... ... ..A ... C.T ... ...
#Bombyx_mori-strain_Shova      G.. ... .A. ..G ... ... ... ... .A. ... ... G.. .G. A.C C.. ... ... ..A ... C.T ... ...
#Bombyx_mori-strain_Gindga_8   A.. ... .A. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..A ... ..T ... ...
#Bombyx_mori-strain_Plovdiv_18 TGC ATT ATA TTC TTA ATT ATA ATC TGC ATC TTG ATC TGA TTT AAC TTT TAA TAT AAA AAT TAA AAT
#Bombyx_mori-strain_Plovdiv_14 .C. ... ... ... ... ... ..T ..A CA. ... ... ... ... ... ... ... ... ... ... ... ... ...
#Bombyx_mori-strain_Maiak_5    .C. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..C ..C
#Bombyx_mori-strain_China_23   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Bombyx_mori-strain_Asahi      .C. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Bombyx_mori-strain_Almaz      .C. ... ... ... ... ... ... ... C.. ... ... ... ... ... ... ... ... ... ... ... ... ...
#Bombyx_mori-strain_Shova      .C. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Bombyx_mori-strain_Gindga_8   .C. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Bombyx_mori-strain_Plovdiv_18 TTA AAA TAT TTT TTT CAC AAC GAT ATA CAA AAT TAA ATA AAT TAA GTA AAT TTA TTC GTG GAA TAT
#Bombyx_mori-strain_Plovdiv_14 ..T ..G .C. ... ... A.. ... ... ... ... ... ... ... ... ... .A. ... ... ... C.. ... ...
#Bombyx_mori-strain_Maiak_5    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... C.. ... ...
#Bombyx_mori-strain_China_23   ... ... ... ... ... A.. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Bombyx_mori-strain_Asahi      ... ... ... ... ... A.. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Bombyx_mori-strain_Almaz      ... ... .C. ... A.. A.A ... ... ... ... ... ... ... ... ... .A. ... ... ... ... ... ...
#Bombyx_mori-strain_Shova      ... ..G ... ... A.. A.. ... ... ... ... ... ... ... ... ... .A. ... ... ... ... ... ...
#Bombyx_mori-strain_Gindga_8   ... ... ... ... ... A.. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Bombyx_mori-strain_Plovdiv_18 AGA TTC TTC TAA ATA AAC TAA AAT ACC GCC AAA TTA TTT AAG TTT TTA TAA ATA ATT ATA TAC TAT
#Bombyx_mori-strain_Plovdiv_14 ... ... CC. ... ... ... ... ..A ... ... ... ... ... .T. ... ... ... ... ... ... ... ...
#Bombyx_mori-strain_Maiak_5    ... ... CC. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Bombyx_mori-strain_China_23   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Bombyx_mori-strain_Asahi      ... ... C.. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... C..
#Bombyx_mori-strain_Almaz      ... ... C.. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .GT ...
#Bombyx_mori-strain_Shova      ... ... C.. ... ... ... ... ... ... ... ... ... ... .T. ... ... ... ... ... ... ... ...
#Bombyx_mori-strain_Gindga_8   ... ... C.. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Bombyx_mori-strain_Plovdiv_18 AAT TTA AAT TTT TAA TAG TGG GGT ATC TAA
#Bombyx_mori-strain_Plovdiv_14 ... ... G.. ... ... ... ... ... ... ...
#Bombyx_mori-strain_Maiak_5    ... C.. ... ... ... ... ... ... ... ...
#Bombyx_mori-strain_China_23   ... ... ... ... ... ... ... ... ... ...
#Bombyx_mori-strain_Asahi      ... ... C.. ... ... ... ... ... ... ...
#Bombyx_mori-strain_Almaz      ... ... ... ... ... ... ... ... ... ...
#Bombyx_mori-strain_Shova      ... C.. ... ... ... ... ... ... ... ...
#Bombyx_mori-strain_Gindga_8   ... ... ... ... ... ... ... ... ... ...
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Fig. 1. The variable sites of the sequences obtained (exported data from MEGA)

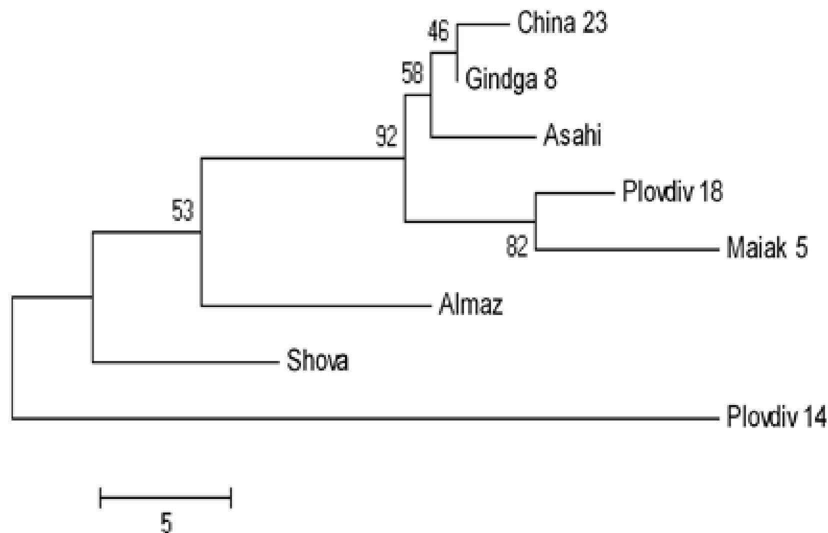

Fig. 2. Maximum Parsimony Tree based on the sequencing data obtained from 12S rDNA gene segment. Plovdiv 14 strain is in a separate clade. The numbers of bootstraps are indicated on the clades

Discussion

The domestication history of silkworm started around 5000 years ago. Races of *B. mori* were exported from China to other countries around 1500-2000 years ago (Li *et al*. 2005). The germplasm resources of silkworm in Bulgaria represent a large number of strains and genotypes that was also shown by the number of haplotypes revealed in the present study. Some of the strains have been imported from other countries and geographical regions, and the rest have been created in Bulgaria from initial populations with foreign origin. Biodiversity conservation programs are typical in silkworm breeding ac-

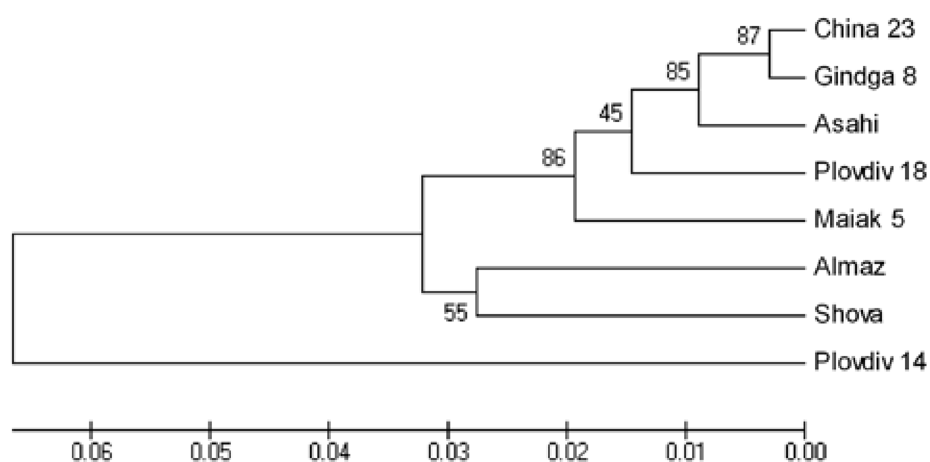

Fig. 3. UPGMA Tree based on the sequencing data obtained from 12S rDNA gene segment. Plovdiv 14 strain is in a separate clade. The numbers of bootstraps are indicated on the clades

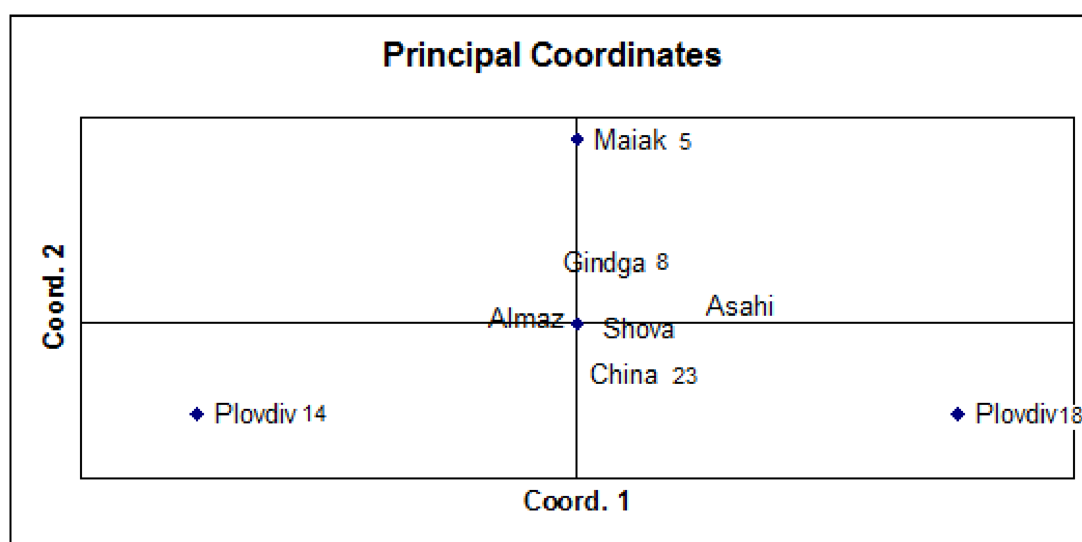

Fig. 4. PCA based on the sequencing data obtained from 12S rDNA gene segment. Plovdiv 14, Plovdiv 18 and Maiak 5 strains are distinguished from the rest of the studied strains

tivities during the recent years. The main purpose of the silkworm breeding efforts is to keep the original characteristics of the strains.

The eight silkworm strains studied have a wide geographical distribution in Asia, Japan and Europe. Two of them – Plovdiv 14 and Plovdiv 18 have been created in Bulgaria from an initial population named Plovdiv 10. Strain Plovdiv 14 is older. It was sertified in 1996, while Plovdiv 18 was sertified in 2008. The other six strains were imported from Azerbaijan (Almaz, Gindja 8 and Maiak 5), Japan (Asahi and Shova), and China (China 23). The eight haplotypes corresponded to these eight strains, demonstrating intra-strain variability, which was probably due to the different geographical origin and the lack of the gene flow among strains. Isozyme polymorphisms and diversity among the imported strains have been also described by Staykova (2008).

Plovdiv 14 showed a large sequence divergence in correlation the rest of the studied strains (Figs. 2, 3) that means that its evolution maybe was earlier than the others and the strain has different genetic characteristics of the 12S rDNA mtDNA probably due to the long-term breeding activity. PCA analysis discriminated strains Plovdiv 14, Plovdiv 18 and Maiak 5 from the rest, which confirmed the different origin of the strains and the absence or extremely low gene flow between them.

The highest divergence value was observed between Plovdiv 14 and Plovdiv 18. This fact is very interesting, because it shows that these two strains from Bulgaria have evolved from genetically distant sub-groups within the limits of the same initial hybrid population (Plovdiv 10) many ears ago.

On the basis of the obtained results the studied strains of mulberry silkworm stocks in Bulgaria,

showed genetic variability in terms of 12S rDNA mtDNA gene segment. Inter-race variability in the structure of A+T-rich regions of mitochondrial DNA of twelve local silkworm races, belonging to the germplasm of China and their phylogeny, have been reported by Chen *et al.* (2007), too. Genetic diversity among introduced strains Almaz, Gindja 8, Maiak 5, Asahi, Shova and China 23, that were included in this study, were confirmed through isoenzyme analysis of nonspecific esterases and phosphoglucomutase, expressed in the different allele composition of gene fund, different frequencies of alleles, different levels of heterozygosity and polymorphism (Staykova 2008). We used 12S rDNA mtDNA gene segment for the first time aiming to discriminate the different silkworm strains in the germplasm resources of Bulgaria. This molecular marker should be considered along with other biochemical markers, such as nonspecific esterases, phosphoglucomutase, acid phosphatase, malatedehydrogenase, etc., which were determined in previous studies (Stoikova *et* al. 1998, Stoykova *et al*. 2003, Staykova *et al*. 2010, 2015), in order to combine more factors that would be useful in future conservation strategies and breeding programs. Isoenzyme analysis is an accurate and efficient method not only for studying the inter-strain and intra-strain polymorphism of the mulberry silkworm, but also for determining the level of genetic changeability and phylogenetic relationships, as has been ascertained already (Staykova 2008, Staykova *et al*. 2010, 2015). Therefore, 12S rDNA mtDNA gene segment sequencing analysis described in the present study could be also use for discriminating of strains with different geographical origin in relation to their involving in breeding programs and preserving their original characteristics.

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