Genetic Diversity Analysis of Mulberry Silkworm (*Bombyx Mori* L.) Hybrids and Breeds in Azerbaijan Using RAPD Markers

Ayaz Mammadov[1*], *Saltanat* Aghayeva[1], *Guduret* Bakırov[3], *Mahpuba* Gulubeyova[4], and *Gular* Ismayılova[2]

[1]Department of Natural Sciences, Western Caspian University, 17A, Ahmad Rajabli st., AZE1072, Baku Azerbaijan
[2]Genetic Resources Institute of the Ministry of Science and Education, Baku, Azerbaijan
[3]Department of Silkworms Breeding, Sheki Regional Scientific Center of ANAS, Baku, Azerbaijan
[4]Department of Breeding and Nutrition of Agricultural Animals of the Azerbaijan State Agrarian University, Baku, Azerbaijan

Abstract. In order to study the genetic diversity of silkworms (Bombyx mori L.) breeding in Azerbaijan, the following breeds and hybrids were analyzed: SHZEM 4 x GE 143, Chingiz x Yagub, Yagub x Chingiz, GE 143, Yagub, SHZEM 4 belted, Chingiz, GE 143 x SHZEM 4 using RAPD molecular markers. For this purpose, silkworm larvae were taken for DNA extraction and nuclear genome DNA was isolated from various parts of the silkworm (silk gland, epidermis, etc.). Four RAPD primers (BGN 04, BGL 02, BGY 06, BGA 02) were used in the study, resulting in 200-1200 bp 34 dots representing fragments. The average number of amplicons for each primer was 6-11, and 82-100% of the amplicons were polymorphic. The study found that RAPD markers do not fully reflect the similarity between the local breed and hybrids.

Keywords: silkworm, breeds, hybrids, homozygote, hereditary.

1 Introduction

The silkworm is a domesticated insect that has been cultivated for over 5,000 years [3]. It is an experimental object with very superior properties. All reproductive cycles have a large number of hereditary characteristics. Body color, cocoon weight, pup weight, etc. Morphological features such as this are the traditionally used method for gender identification. It is clear that tolerance to biotic and abiotic factors is controlled by polygenes together with genetic markers and environmental influences [8]. Therefore, it is advisable to breed the breeds in a suitable geographical region in a climate where they are adapted [6]. Molecular markers, modern biological research tools, are invaluable tools in breeding strategies and identifying resistance genes. Natural resources - flora and fauna, especially

* Corresponding author: ayaz.mammadov@wcu.edu.az

insects and the plants that make up their food, give a unique image to nature [1, 10]. Natural silk is a product of Lepidopteran insects.

The average genetic distance from analyzed samples proved to be relatively high, which can be due to the fact that hybrids are from two different species and also to the distant origin of these species. With RAPDs it is possible to assess genetic similarity amongst a range of silkworm genotypes on the basis of band sharing analysis. In conclusion, RAPD analysis promises to become a valuable tool for analysis of genetic variation, estimating genetic distance among populations and generating molecular markers for economic traits of the silkworm. From the DNA analysis it is clear that the samples are different from each other, and each sample has individual genetic characters. The hybrids showed average values in Phylogenetic Tree. To achieve good reproducibility and strong signal in the RAPD assay, one of the most important parameters is the concentration of genomic DNA [5].

Among these, PCR-RAPD markers are a simple technique for assessing genetic diversity of organisms. Several workers have used PCR-RAPD markers successfully for estimation of genetic diversity and relatedness of the mulberry silkworm strains [2, 9, 7]. Genetic diversity and polymorphism among strains is evident through amplification with tested primers and promising parents for productive and disease resistant silkworm strains can be obtained [11].

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 lociwere detected. All of them (100%) were polymorphic. “Null” alleles in four lociwere 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 strainsas well as deviations from Hardy-Weinberg equilibrium in almost all analyzed loci, resultingof excess of homozygotes. The value of FSTwas 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 withsubclades. 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 [12].

2 Material and methods

8 different local mulberry silkworm breeds and hybrids from the Sheki Regional Scientific Center of ANAS, were characterized in this study. These are ShZEM 4 x GE 143 (S1), Chingiz x Yaqub (S2), Yaqub x Chingiz (S3), GE 143 (S4), Yaqub (S5), ShZEM 4 belted (S6), Chingiz (S7), It was GE 143 x ShZEM 4 (S8) The research work was carried out in the Department of Genetic Resources of Agricultural Animals of the Institute of Genetic Resources of the Ministry of Science and Education and the Laboratory of Genetics and Molecular Biology of the Western Caspian University.

2.1 RAPD primer

RAPD markers are PCR-based markers that determine genetic diversity. The main advantages of the RAPD marker technology are that it does not require any information about the genome, does not require DNA with a high quality indicator, and does not require any radioactive chemicals during its application. Currently, RAPD molecular markers are of great

importance in the study of genetic diversity - polymorphism. Based on the conducted scientific studies, it was determined that RAPD molecular markers are widely used in the study of the genetic diversity of mulberry silkworm breeds in countries where sericulture is developed.

RAPD markers were used in this study. In the conducted study, RAPD profile was performed with BGN 04, BGW 02, BGY 06, BGA 02 primers. Indicators such as primer sequence, number of amplified DNA fragments per primer and melting temperature of primers are given in table 1.

Table 1. List of RAPD primers, nucleotide sequence, fragment size and melting temperature.

№	Name of primers	Sequences (5'-3')	Size aof fragment, bp	Melting temperature (T.m)
1	BGN 04	GACCGACCCA	300-1200 bp	38.6^0C
2	BGW 02	ACCCCGCCAA	400-1150 bp	42.9^0C
3	BGY 06	AAGGCTCACC	450-1200 bp	34.1^0C
4	BGA 02	TGCCGAGCTG	200-1100 bp	40.7^0C

2.2 Genomic DNA extraction

Samples of each breed and hybrid of mulberry silkworm grown in the 5th age period were taken and DNA was extracted. A kit belonging to the EURx company was used for extraction. The tissue is prepared for extraction, first the solvent and then the denaturing enzymes are added. The collected tissue sample is crushed with a mortar and then liquid nitrogen is added to homogenize it. Then, the extraction steps were performed as described below. A tissue fragment (maximum 10 µg) is added to a 2 µl Eppendorf tube. 350 µl of lysed BT is added to it, then 20 µl of proteinase K is added and mixed. The samples are placed in a water bath at 56°C for 3-6 hours, stirring every 15 minutes. Then, 350 µl of Sol BT buffer is added to the samples and mixed several times. Samples are incubated at 70°C for 10 minutes. 180 µl of 96-100% ethyl alcohol or ethanol is added to each tube and mixed several times. It is centrifuged at 14000 rpm for 2 minutes. Then, 600 µl from each tube is added to spin columns and centrifuged at 12000 rpm for 1 minute. The sediment in the spin column is discarded. The rest of the samples are weighed and added to spin columns. Centrifuge again for 2 minutes at 12000 rpm. The liquid in the spin column is discarded. 500 µl of detergent BTX1 is added to the collection tube and centrifuged at 12000 rpm for 1 minute. The liquid in the spin column is discarded and 500 µl of detergent BTX 2 is added to it again and centrifuged at 12000 rpm for 2 minutes. The upper part of the spin columns is transferred to new collection tubes (1.5-2 µl), and 50 µl of solvent buffer heated at 70°C is added to it. Spin columns are incubated at room temperature for 5 minutes. Spin columns are centrifuged at 12000 rpm for 1 minute, DNA is transferred to tubes. The DNA smples are stored at -20°C.

2.3 PCR (polymerase chain reaction)

It was carried out in a Biorad T100 Thermal Cycler device, in 25 µl reaction mixture. The amount of eacrivs used for 1 sample - 2 µl of MgCl2, 2 µl of 10xPCR buffer, 0.5 µl of primer, 0.5 µl of dNTP, 0.25 µl of Taq DNA polymerase and 16.75 µl of distilled water were used. 4 primers were used during the reaction: BGN 04, BGL 02, BGY 06, BGA 02. The PCR program performed: during 1 cycle of the program consisting of 35 cycles, the reaction was initial denaturation at 95°C for 5 minutes, denaturation at 95°C for 30 seconds, 38°C at 1 minute (varies depending on the melting temperature of the primers) the step of joining the

primer to the chain (annealing), the chain extension reaction at 72°C for 2 minutes and the last extension reaction at 72°C for 5 minutes continues.

2.4 Agarose gel electrophoresis

Amplified DNA fragments were separated out on 1.5% agarose gel stained with ethidium bromide. Running buffer containing Tris-buffer, Acetic acid and EDTA (pH8.0) was used for electrophoresis and for preparing gels. Wells were loaded with 25µl reaction volume and 5µl of loading buffer (Sucrose, Bromophenol blue and Xylene cyanol) together. Electrophoresis was conducted at 75volts for 2 hours and the gel photographed under UV light using gel dock system (BioRAD).

2.4 Statistical analysis

Specific items were scored based on whether they were present (1) or absent (0) and a genetic diversity or distance (1-F) index was calculated. F quantity was calculated by Jaccard's similarity coefficient formula.
F=a/n-d
F- similarity index;
a is the number of items observed in both samples, the number of items not observed in both samples, and n is the number of total items.
Based on all the obtained data, the cluster analysis was built with the statistical program "Past for MS Windows".

3 Results and discussion

DNA extraction and result: silkworm larvae in the 5th age period were used for DNA extraction. Nuclear DNA was extracted by taking samples from different parts of the larva (silk gland, epidermis, etc.). The quantity (concentration) and quality (completeness of the DNA chain) of the resulting DNA molecule was checked, and the DNA extracted from the epidermis had higher quantitative and qualitative indicators. As a result of the procedure, a 17kb long DNA molecule was obtained.

Polymorphism indicators, genetic diversity coefficients, PIC, EMR, MRP, RP, MI indicators were calculated according to the RAPD primers used, and are listed in Table 4.2. A total of 200-1200 n.c. 32 fragments in length were amplified. Of the 32 observed synthesized dams, 30 (81%) were polymorphic dams. Each of the 4 primers used in the study showed high polymorphism, the minimum polymorphism (60%) was observed with the BGW 02 (TGGGCGTCAA) primer. The average number of items per primer was 6-11. The total polymorphism index was 81%. The highest number of fragments belongs to the BGN 04 primer, which fragments are amplified with the sequence "GACCGACCCA". Also, maximum polymorphism was observed in BGY 06 primer profile. 36 RAPD primers were studied on 11 breeds in India [4]. RAPD primers that we used were also used in the research. According to both studies, the matching indicators of the primers were compared. Thus, in the study conducted in India, the BGA 02 primer showed 100% polymorphism, and 10 of the 10 points synthesized during the PCR reaction were also polymorphic. The same primer showed 83% polymorphism in our research, 4 out of 6 synthesized points were polymorphic points. In another study, the polymorphism index of BGY 06 primer was 100%, while in our study it was calculated as 89%. The results of the comparison suggest that the primers we used in the study showed close but lower results compared to the results of the comparison study (Table 2.).

Table 2. Polymorphism indices and marker index with RAPD primers.

Primer	Sequence (5'-3')	Fragment lenth, bp	No. of total bands	No. of polymorphic bands	Polymorphism, %	Coefficient of genetic diversity	PIC	EMR	MRP	RP	MI
BGN 04	GACCGACCCA	300-1200	11	9	82	0.79	0.17	7.36	0.38	3.5	0.45
BGW 02	ACCCCGCCAA	400-1150	6	4	66	0.75	0.39	2.7	0.5	2	1.05
BGY 06	AAGGCTCACC	450-1200	9	8	89	0.88	0.38	7.1	0.59	4.75	2.69
BGA 02	TGCCGAGCTG	200-1100	6	5	83	0.79	0.43	4.2	0.55	2.75	1.81
Total			32	26	-	-	-	-	-	-	-
Average			8	6.5	81	0.80	0.34	5.34	0.51	3.25	1.5

Statistical parameters such as polymorphic information content (PIC), Effective multiplex ratio (EMR), marker index (MI), resolving power (RP) and average resolution MRP were studied in order to measure the usefulness of RAPD markers in the study of mulberry silkworm breeds and hybrids of the primers used during the research. Based on table 2, which shows the parameters, it was determined that the BGY 06 primer has the highest index (0.88) according to the Genetic Diversity Coefficient (GDI). Similarly, according to the RP and MRP parameters, the maximum indicator belongs to the BGY 06 primer.

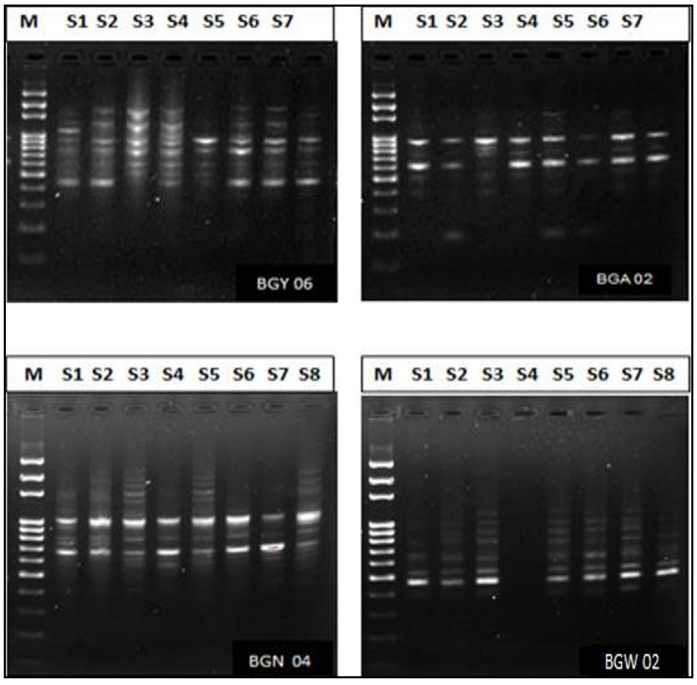

Fig. 1. Agarose gel visualization of results obtained with RAPD primers.
M= standard DNA marker, 100 bp DNA fragments (100 bp DNA ladder).

Cluster analysis was performed with "Past for MS Windows" software. Jaccard's similarity coefficient indicators between 0.300 and 0.975 were observed (Table 3). Maximum similarity was observed between samples S3 and S7, and minimum similarity was observed between samples S4 and S5. UPGMA cluster analysis results showing the relationship between 8 mulberry silkworm breeds and hybrids are shown in Figure 2.

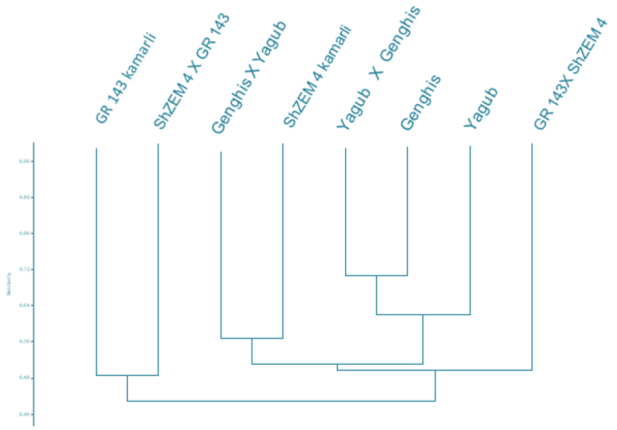

Fig. 2. Dendrogram of genetic relationship among 8 mulberry silkworm samples based on RAPD primers.

Table 3. Similarity and distance matrix of mulberry silkworm breed and hybrid.

Similarity and distance indices

	ShZEM-4_X	Chingiz_X_Y	Yaqub_X_Ch	GE-143_kem	Yaqub	ShZEM-4_ke	Chingiz	GE-143_X_S
ShZEM-4_X	1	0.425	0.46512	0.48649	0.525	0.44681	0.53659	0.38462
Chingiz_X_Y	0.425	1	0.56098	0.33333	0.40909	0.56818	0.45455	0.48649
Yaqub_X_Ch	0.46512	0.56098	1	0.37778	0.5814	0.5625	0.70732	0.525
GE-143_kem	0.48649	0.33333	0.37778	1	0.39535	0.42553	0.44186	0.39474
Yaqub	0.525	0.40909	0.5814	0.39535	1	0.55319	0.65854	0.43902
ShZEM-4_ke	0.44681	0.56818	0.5625	0.42553	0.55319	1	0.53061	0.46667
Chingiz	0.53659	0.45455	0.70732	0.44186	0.65854	0.53061	1	0.5641
GE-143_X_S	0.38462	0.48649	0.525	0.39474	0.43902	0.46667	0.5641	1

4 Conclusion

Biodiversity of local silkworm (Bombyx mori L.) breeds and hybrids was studied for the first time in Azerbaijan based on RAPD (random amplified polymorphic DNA) molecular markers. Four RAPD primers (BGN 04, BGY 06, BGA 02, BGW 02) and four ISSR primers (UBC 807, UBC 857, UBC 813, UBC 827) were used in the experiment. The results showed that RAPD primers produced 32 measurable bands with fragment sizes ranging from 200 to 1200 bp. The average number of amplicons for each primer ranged from 6 to 11, and on average 81% of them were polymorphic. The same indicators for ISSR primers were slightly different. Thus, the total number of synthesized amplicons was 30 with fragment size from 400 to 1300 bp. All results were compared with primers, and other marker indices such as PIC, MI, EMR, MRP, RP and diversity index were calculated for each marker.

References

1. S.N.Chowdhury (1983). Eri silk industry. Directorate of Seric. Weaving, Government of Assam, India. pp. 1-23.
2. D. Eroğlu and S. Ç. Arica (2009). Molecular genetic analysis of three Turkish local silkworm breeds (Bursa Beyazı, Alaca and Hatay Sarısı) by RAPD-PCR Method. J. Appl. Biol. Sci. 3(2): 17-20.
3. M.R. Goldsmith (1995). Genetics of the silkworm. In: Mol. Model Systems in the Lepidoptera. Goldsmith MR & Wilkins AS Eds., Cambridge Univ. Press, London. pp. 21-76.
4. H.R.Hingh et al., Isolating silkworm genomic DNA without liquid nitrogen suitable for marker studies, African Journal of Biotechnology, 2011, v. 10, pp: 11365-11372.
5. H.B.Mahesha, F.G. Kasmaei and Thejaswini P.H. RAPD Analysis of Genetic Variability among Silkworm Breeds. Int. J. Pure App. Biosci. 5 (6): 1708-1718 (2017).
6. Murthy BCK, Prakash BM, Puttaraju HP (2006). Fingerprinting of nondispausing silkworm, Bombyx mori, using random arbitrary primers. Cytologia 71(4): 331-335.
7. Moorthy, S.M., N. Chandrakanth, A.S.K. Rao, V. Kumar, and B.B. Bindroo (2013). Genetic Diversity Analysis using RAPD Marker in some Silkworm Breeds of Bombyx mori L. Ann. Biol. Res. 4(12): 82-88.
8. Promboon A, Shimada T, Fujiwara H, Kobayashi M (1995). Linkage map of random amplified polymorphic DNAs (RAPDs) in the silkworm, Bombyx mori. Gen. Res. 66: 1-7.
9. Reddy, K.D., E.G. Abraham, and J. Nagaraju (1999). Microsatellites in the Silkworm, Bombyx mori: Abundance, Polymorphism, and Strain Characterization. Genome 42: 1057-1065.
10. Thangavelu K (1991). Wild sericigenous insects of India: A need for conservation. In: Wild Silkmoth 1991. Inter. Society for wild silkmoths: Jpn. pp. 71-77.
11. G. A. Bajwa, N. Ahmed, S. H. Shah and M. Adnan. Genetic diversity analysis of mulberry silkworm (bombyx mori) strains using rapd markers. The Journal of Animal & Plant Sciences, 27(2): 2017, Page: 575-581 ISSN: 1018-7081.
12. T.A. Staykova, P.I.Tzenov, Y.B. Vasileva, S.D. Aziz, E.N. Ivanova, I.I. Stoyanov, P.L.Vasileva, T.P. Popova. Genetic characterization of silkworm (Bombyx mori L.) strains (Lepidoptera: Bombicidae) with different geographical origin on the basis of isozyme markers. Acta Scientiarum. Biological Sciences, vol. 42, 2020.