Genetic Phylogeny and Diversity of some Romanian Silkworms Based on RAPD Technique

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Abstract
Randomly amplified polymorphic DNA (RAPD) markers were used in the present study to analyze genetic diversity and phylogenetic relationships among some race and hybrids of Romanian *Bombyx mori.* DNA from 8 hybrids and 1 race was amplified with 35 highly polymorphic RAPD primers, of which 21 markers generated polymorphic bands that were used to analyze genetic phylogeny and diversity. A total of 921 polymorphic bands were detected and UPGMA cluster analysis of Jaccard's genetic distance grouped silkworm strains on the basis of their origin, obtaining a dendogram reflecting their genetic relationship.

Keywords: hybrids, phylogenetic relationships, RAPD technique, silkworm (*Bombyx mori L.)*

1. Introduction

RAPD technique is also known as AP-PCR (Arbitrary Primers-PCR, [1] and DAF (DNA Amplified Fragments [2]. By this technique, DNA fragments are amplified by PCR, using short sequences of nucleotides, usually of 10 bp (base pairs) [3]**.** In this method, electrophoresis of a set of products amplified from different DNA regions randomly chosen by the primer produces a series of bands (pattern) of different intensity [4]**.**
The polymorphic pattern depends on the specific DNA and primer, primer length, the amplification and annealing conditions and the enzyme quality [5]**.** Although newer techniques like AFLP and SSR are preferred, due to their informativeness, RAPD is still a method of choice because its simplicity and low cost [6].
The present study is trying to estimate the average genetic distance, genetic similarity, phylogenetic relations among the samples of silkworms Bombyx mori from Romania by using RAPD technique.

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2. Materials and methods

Genomic DNA, silkworm eggs and larvae were obtained from SC. Sericarom SA. Bucuresti, Romania.
The biological material was: 1 race (S8) and 8 hybrids (S8 x Ac 29/T, Ac 29/T x S8, Ac x B1, B1 x Ac, Hesa1 x Svila 2, B1 x Svila 2, B1 x Hesa 2, Vratza 35 x Svila 2).
35 RAPD primers were used according to the working protocol of [7].
The RAPD analysis of genetic phylogeny and diversity had the following stages:
- DNA extraction from Bombyx mori;
- Quantity and purity determination;
- Amplification with RAPD primers (35);
- Samples electrophoresis;
- Agarose gel pictures;

Results interpretation.
DNA extraction from Bombyx mori: genomic DNA was isolated from the posterior silk glands of fifth instar larvae and eggs [8]. DNA extraction was performed using one extraction kit from

Promega (Promega Wizard DNA Purification Kit - Animal Tissue).

Determination of the Quantity and purity of the DNA: DNA concentration was measured spectrophotometrically with a NanoDrop Spectrophotomer ND-1000.

Amplification with RAPD primers (35): the amplification of genomic DNA was performed according to [3]. The amplification was carried out in a reaction volume of 25 µl, containing: H2O-11.3 µl, PVP- 2 µl, Buffer 5x-5 µl, $MgCl_2$ - 2.5 µl, dNTP-0.5ul, Primer- 0.5 µl, Go Taq Polymerase -0.2 µl, ADN- 3 µl, with the help of a PCR thermal cycler (BioRad, iCycler).

Reagents used: Master Mix [5x Green Taq Flexi Buffer (Promega), $MgCl_2$ - 25 mM (Promega), dNTP - 10 mM (Promega), Go Tag Polymerase - 5u/µl (Promega)], Primer - 10 pmol/µl, H_2O sterile.

Electrophoresis: The amplified products were separated according to molecular size on 2% agarose gels in TBE buffer 1x, with SYBR Safe, followed by visualization and photography of gels. The molecular marker used was 100 bp DNA Ladder (Fermentas). For the image capture a UVP system (BioSpectrum AC Imaging System) and BioChemi HR Camera, SYBR Gold 485-655nm filter were used. Data analysis Gel images were analyzed using TL120 software (Nonlinear Dynamics, Newcastle upon Tyne, UK). Amplified bands were scored present (1) or absent (0) and data entered into a binary matrix. The genetic distance between accessions was calculated using Jaccard's coefficient of similarity [9]. Cluster analysis was conducted with an UPGMA (Unweighted Pair Group Method with Arithmetic mean) algorithm using FreeTree software [10] and a dendrogram was constructed, using the TreeView software [11]. Its consistency was assessed using bootstrap method in 1000 repetitions. A synthetic outgroup, generated as an individual in whom all alleles were absent was used for dendrogram rooting.

3. Results and discussion

Amplification of silkworm genomic DNA with random decamer primers resulted in a series of discrete bands of different intensities. For each primer evaluated, a multiple band profile comprising of one to five major amplification products was observed.

- 21 of the 35 used primers have generated 921 polymorphic bands, which were used for the statistical interpretation of phylogenetic relations (Total Lab 120, UVP soft). Competition in the generation of PCR products has been postulated as important in determining amplification. Competition between priming sites for successful amplification may depend on the priming sequences and context [12-13], or degree of mismatching between the primer and template relative to other priming site in the genome as whole [14].

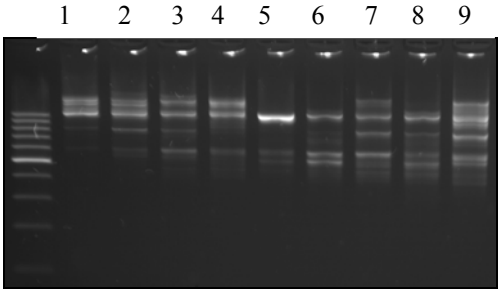

Figure 1. Amplicons obtained using OPAB 11 primer

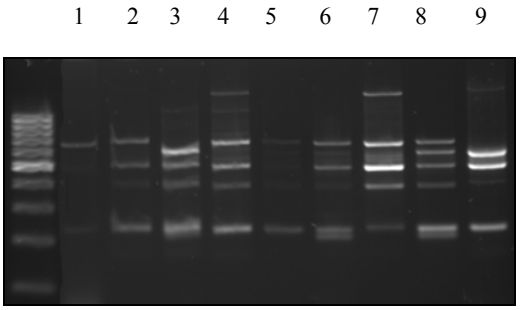

Figure 2. Amplicons obtained using OPD 16 primer

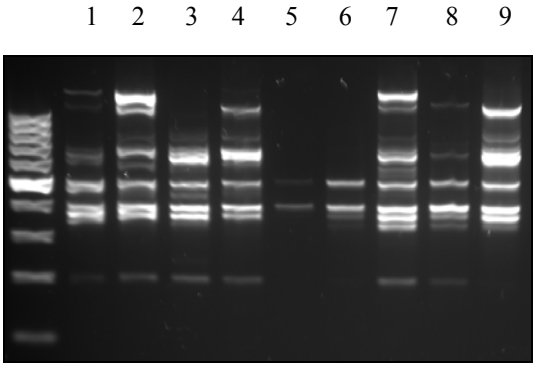

Figure 3. Amplicons obtained using OPE 02 primer

*1-S8 x Ac29/T
*2-Ac29/T x S8
*3- Acx B1
*4-B1 x Ac
*9-S8
*5-Hesa1 x Svila2
*6-B1 x Svila2
*7- B1 x Hesa2
*8-Vratza 35x Svila2

In Figure 4 is represented the dendrogram generated using TreeView software, based on the

genetic relationships between the analyzed silkworms, calculated using Jaccard's coefficient with FreeTree software.

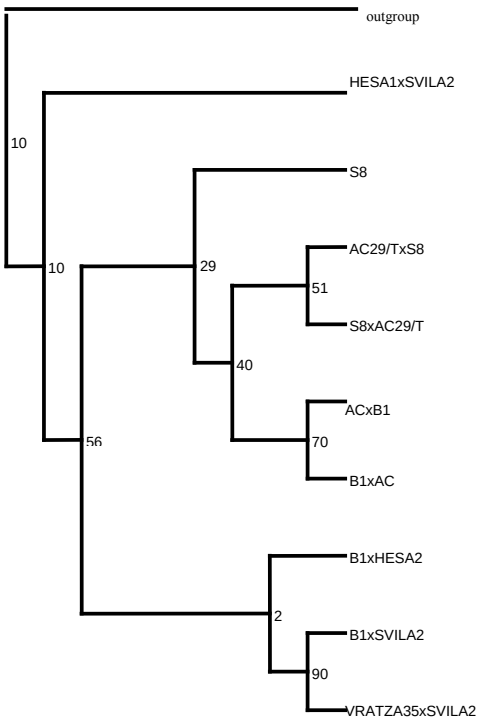

Figure 4. UPGMA dendrogram generated using Tree View [11] software, based on the genetic relationships between the analyzed *Bombyx mori* race and hybrids, calculated using Jaccard's coefficient with Free Tree [10] software.

The unweighted pair group method with arithmetic mean (UPGMA) was used to generate a cluster analysis (Free Tree Program, [10] using Jaccard's coefficient of similarity and visualized with the software Tree View [11] which plotted as a dendrogram (figure 4). The dendrogram generated by UPGMA analysis based on 35 RAPD primers grouped silkworm strains on the basis of their origin, obtaining a dendrogram of genetic relationship. The values presented near the dendrogram nodes represent bootstrap analysis results on tree made in 1000 repeats. The UPGMA cluster analysis separated the silkworms into 3 main groups, one consisting of hybrids Hesa 1 x Svila 2, which formed a separate group, reinforced by a very high bootstrap value, equal to 100. The second group consisted of S8 race, S8 hybrids and AC hybrids. The third group consisted of B1 and Svila hybrids.

The separate clustering of Hesa 1 x Svila 2 hybrids can be due to the presence of non-parental bands generated by RAPD markers, described by different authors [15-16-17-18-19]. The cause of this phenomenon can be explained by more hypotheses: the formation of heteroduplex molecules between the alleles, mutations or recombinations at the primer binding site or inside the amplified fragments, competition for primer binding sites or somatic rearrangements in perennial plants [20]. The samples AC x B1 and B1 x AC clustered together (bootstrap value 70), as well as the samples B1 x Svila2 and Vratza 35 x Svila2 (bootstrap value 90) and also the samples AC28/TxS8 and S8xAC29/T (bootstrap value 51). The calculated genetic distance between the analyzed samples, using Jaccard's coefficient is shown in table 1.

Table 1. The calculated genetic distance between the analyzed samples

Race/ Hybrid	VRATZA35 xSVILA2	B1xSVI LA2	HESA1x SVILA2	S8xAC29/T	B1xHES A2	ACxB1	S8	AC29 /TxS8	B1x AC
Vratza35 X Svila2									
B1 x Svila2	0.557								
Hesa1 x Svila2	0.689	0.481							
S8 x Ac29/T	0.639	0.479	0.553						
B1 x Hesa2	0.444	0.438	0.532	0.527					
ACxB1	0.582	0.365	0.352	0.503	0.436				
S8	0.609	**0.302**	0.494	0.434	0.440	0.376			
Ac29/T x S8	0.393	0.538	0.591	0.599	0.452	0.516	0.523		
B1 x Ac	0.590	0.664	0.672	**0.753**	0.652	0.687	0.708	0.495	

- The highest genetic distance (0.753) was registered between S8 x AC 29/T and B1xAC;
and the smallest (0.302) between AC x B1 and B1 x Svila.
- The average genetic distance among individuals was 0.53.

4. Conclusions

DNA extractions from eggs and glands from 5th instars silkworms were performed. The technique using liquid nitrogen resulted to be more efficient with a high purity of the DNA coming from glands samples.
Out of the 35 used primers, 21 have generated polymorphic bands like: OPA 05, OPA 17, OPAB 11, OPAB 18, OPB 10, OPB 18, OPC 04, OPC 08, OPD 16, OPD 20, OPE 02, OPE 14, OPF 02, OPF 13, OPF 16, OPF 20, OPH 20, OPH 12, OPH15, OPO 16, OPP 18, which are part of the mathematical-statistic interpretation of the phylogenetic relations.
Primers that have not generated polymorphic bands and did not allow the identification of relevant distances were: OPA 03, OPC 02 and OPB 09.
It can be observed, due to the dendrograme, the clustering of the analyzed samples into three main groups, the first containing the Hesa 1 x Svila 2 hybrid, that is removed from the other groups, observation sustained by a very high bootstrap value equal to 100.
The analyzed samples were clustered following the UPGMA analysis of Jaccard's coefficient distance matrix into three main groups, according to their origin.
The separate clustering of Hesa 1 x Svila 2 hybrids is due to the presence of non-parental bands generated by RAPD markers.
The cause of this phenomenon can be explained by more hypotheses: the formation of heteroduplex molecules between the alleles, mutations or recombinations at the primer binding site or inside the amplified fragments, competition for primer binding sites.
The average genetic distance between the samples was 0.53, similar results was obtained by [21].
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.

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