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Genetic Analysis of Izoenzymes Polymorphisms in Silkworm (*Bombyx mori* L.) Strains

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ABSTRACT. This work carried out to evaluates the polymorhism in the silkworm of different lineages using the isoenzymes electrophoresis to detect biochemical markers and to investigate the genetics of populations for those lineages. They were used as samples individual extracts of silk glands of second day old larvas of the fifth instar, originating from seven Japanese lineages and eight pure Chinese lineages maintained by the Cocamar-Cooperativa Agroindustrial de Maringá. The isozymes acid phosphatase (ACP), alkaline phosphatase (AKP) and carbonic anhydrase (CA) they were submitted to the electrophoresis in starch gels 14%. The esterases (EST) were analyzed in polyacrylamide gels to 10% and stained with α and β-naphtyl acetate. The total of 21 loci was detected, and 04 (19.05%) they are polymorphic, *Est-11*, *Acp*, *Akp*, *Ca*. The fixation index (F_{is}) for the analyzed isozymes it was 0.0751, indicating excess of homozygotes. The value of F_{st} (0.6128) it shows that the lineages are well differentiated. The dendrogram obtained with the values of genetic distance didn't separate the Chinese and Japanese lineages analyzed totally. That preliminary evaluation of the lineages of *B. mori* shows that they present genetic material that it can be used in breeding programs that have the purpose of producing hybrid for silk production.

Keywords: silkworm, genetic polymorphism, isoenzymes.

Análise genética de polimorfismos bioquímicos em linhagens do bicho da seda (*Bombyx mori* L.)

RESUMO. Este trabalho teve como objetivo avaliar o polimorfismo em lagartas do bicho-da-seda de diferentes linhagens utilizando a eletroforese de isoenzimas para detectar marcadores bioquímicos e investigar a genética de populações para essas linhagens. Foram utilizados como amostras extratos individuais de glândulas sericígenas de lagartas do segundo dia da quinta idade, de sete linhagens japonesas e oito linhagens chinesas puras mantidas pela Cocamar-Cooperativa Agroindustrial de Maringá. As isozimas fosfatase ácida (ACP), fosfatase alcalina (AKP) e anidrase carbônica (CA) foram avaliadas por meio de eletroforese em géis de amido de milho a 14%. As esterases (EST) foram analisadas por meio de eletroforese vertical em géis de poliacrilamida a 10% e coloração com α e β-naftil acetato. Foram observados 21 locos, dentre os quais quatro (19.05%) são polimórficos, *Est-11, Acp*, *Akp*, *Ca*. O índice de fixação (F_{is}) para as isozimas analisadas foi 0.0751, indicando excesso de homozigotos. O valor de F_{st} (0.6128) permite sugerir que as linhagens estão bem diferenciadas. O dendograma obtido a partir dos valores de distância genética não separou totalmente as linhagens chinesas e japonesas analisadas. Essa avaliação preliminar das linhagens de *B. mori* mostra que elas apresentam material genético que pode ser utilizado em programas de cruzamentos que tenham a finalidade de produzir híbridos para produção de seda.

Palavras-chave: bicho-da-seda, polimorfismos genéticos, isoenzimas.

Introduction

The world production of silk is around 635 thousand tons, only the China produces 70%, following by India with 20% (ANN, 2008). Brazil participates in 3.2% of that production, arriving to 10.300 tons. Paraná State is the largest producing of Brazil silk, it was responsible for 89% of the harvest 2006/2007 (IAPAR, 2008).

The silkworm silk can be classified according to their geographical origin, such as Japanese, Chinese, Indian or European. This insect has morphological and physiological differences correlated with the production of silk because of its origin and, therefore, different breeds ent origins have been, used in crosses, called arrays, as a source of characters for selection in favor of sericulture (PORTO et al., 2004).

The silkworm has been used as model for genetic studies since the discovery of the Mendelian inheritance in the beginning of the last century, due

to its large size, easy of maintenance in the laboratory, and the economic importance (NAGARAJU, 2000). According to this author, *Bombyx mori* is, after *Drosophila melanogaster*, the second most important model insect in genetic studies. Over 400 mutations have been mapped in 28 linkage or chromosomes groups in successful genetic studies with the species (NAGARAJU, 2000). Besides, hundreds of geographical races and genetically improved strains are maintained in different countries where sericulture is in vogue. These races and strains differ not only in well characterized Mendelian traits but also in not so well-studied complex or quantitative traits such as body size, feeding duration, thermal tolerance, and disease resistance (NAGARAJU, 2000).

Many of the studies with *B. mori* are related with molecular biology including the cloning of many silkworm genes as e.g., genes of the enzymes sorbitol dehydrogenase (NIIMI et al., 1996), trealase (SU et al., 1996); homeoproteins such as antennapedia (UENO et al., 1996 apud NAGARAJU, 2000); diapause hormone (SATO et al., 1993); sericin (MICHAILLE et al., 1990), heavy chain fibroin (MITA et al., 1994) and light chain fibroin (YAMAGUCHI et al., 1989).

Since 1995, different molecular markers such as RAPD (PROMBOON et al., 1995) AFLP (TAN et al., 2001) and microsatellites (MIAO et al., 2005) are being used to detect polymorphisms and construct linkage maps for silkworm.

Aside from the different molecular markers available nowadays, biochemical markers such as isoenzymes have generated a broad array of practical information for the identification of hybrid species and natural and cultivated populations of diverse live organisms. Compared with other markers, these analyses are relative simple, fast and cheap (TEIXEIRA et al., 2004).

Polymorphism of nonspecific esterases from pupal haemolymph was analyzed, as well as of phosphoglucomutase from different organs of larvae, pupae and imago, from eight introduced from Bulgaria were analysed by Staykova (2008).

The observation of genetic polymorphisms and molecular markers of those lineages can contribute with an improvement in the handling and consequently with the silk production in the northwest of Paraná State.

This study aimed to evaluate the genetic polymorphism in larvae of Japanese and Chinese silkworm (*B. mori* L.) lineages through isoenzymes, to detect molecular markers and investigate the population genetics of these strains.

Material and methods

Two-day-old larvae of the 5th instar of different *Bombyx mori* lineages were provided by ACESP (Associação dos Sericicultores de Nova Esperança e Regiões Sericícolas), Paraná, Brazil. Of the 15 lineages under study, seven were of Japanese and eight of Chinese origin (Table 1).

Table 1. *Bombyx mori* lineages and their origin.

Lineages	Origem	Lineages	Origem
Line1	Japonese	Line8	Chinese
Line2	Japonese	Line9	Japonese
Line3	Chinese	Line10	Chinese
Line4	Japonese	Line11	Japonese
Line5	Japonese	Line12	Chinese
Line6	Chinese	Line13	Japonese
Line7	Chinese	Line14	Chinese
		Line15	Chinese

The larvae were deep-frozen at –20ºC in duly closed and identified flasks for the laboratory analysis.

For the electrophoresis the silkworms were dissected and the silk glands removed under a stereomicroscope in a dissection dish.

Esterase (EST - EC 3.1.1.1)

Native polyacrylamide gels (10%, T= 30.8%; C = 2.6%) were used together with 5% stacking gels (T= 10.5%; C = 4.8%) (LAPENTA et al., 1995).

Electrophoresis was performed for 4.5 hours at 4ºC, at a constant voltage of 200 V. Running buffer was 0.1M Tris-glycine, pH 8.3.

The samples were homogenized in centrifuge tubes (1.5 mL) in 150 µL β-mercaptoethanol and 40 µL carbon tetrachloride with a glass stick and immediately centrifuged at 12000 xG for 15 minutes to 2ºC. Of the supernatant, 20 µL were used with 20 µL of 0.1 M Tris-HCl solution pH 8.8 containing 10% glycerol. This solution (15 µL) was then applied on the gel.

After the electrophoresis the gel was incubated for 30 min. in 50 mL sodium phosphate buffer solution at 0.1 M pH 6.2. Then the gel was incubated for ± 1 hour at 38°C in staining solution (50 mL phosphate buffer 0.1 M pH 6.2; 5 mL N-propanol; 0.06 g Fast Blue RR Salt; 0.03 g α-naphthyl acetate and 0.02 g β-naphthyl acetate) (LAPENTA et al., 1995).

Starch gel

Horizontal electrophoresis was carried out using the starch gel technique according Ruvolo-Takasusuki et al. (2002). After the electrophoretic running the starch gel was cut in three homologous parts, used for the staining of three enzyme systems (Acid phosphatase, alkaline phosphatase and carbonic anhydrase).

The buffer system used for the gel was Tris-HCl pH 7.5 0.02M and for the run Tris-HCl at 0.3 M pH 7.5.

Acid phosphatase (ACP – EC 3.1.3.2)

The acid phosphatase (ACP) activity was identified using 0.5 mL 1 M magnesium chloride, 0.05 M sodium acetate buffer pH 5.5, 0.04 g Fast Blue RR Salt and 3.5 mL 1% α- naphthyl phosphate.

Alkaline phosphatase (AKP – EC 3.1.3.1)

For the visualization of alkaline phosphatase (AKP), a solution was used containing 0.5 mL 1 M magnesium chloride, 0.05M Tris-HCl buffer pH 7.5, 0.04 g Fast Blue RR Salt, and 3.5 mL 1% α-naphthyl phosphate.

Carbonic anhydrase (CA – EC 4.2.1.1)

Fluorescein diacetate (10 mg) was dissolved in 0.5 mL acetone and mixed in 100 mL sodium phosphate buffer 0.1 M, pH 6.5. Gel was soaked in that solution and incubated at 37°C for 40 minutes, and was observed under long wave ultraviolet light.

Analysis of population genetics

After the revelation of the isozyme activity regions, the phenotypes of the discovered loci were recorded. The gene frequencies as well as the measure of the genetic variability were directly estimated, to determine the deviations from the expected genotypic frequency according to the Hardy-Weinberg equilibrium. Furthermore the similarity coefficients and genetic distances for all analyzed strains were calculated, using software Popgene 1.31 (YEH et al., 1999) and Genepop 4.1.1 (RAYMOND; ROUSSET, 1995).

Results and discussion

In the analyses in four enzyme systems with 15 *B. mori* L. lineages, (eight Chinese and seven Japaneses lineages) 21 loci were observed. Four of these were polymorphic (19.05% polymorphism) with the polymorphic loci Est-11A (0.8974) and rarest Est-11B (0.1026), AcpA (0.300), AcpB (0.3917), AcpC (0.3083), AkpA (0.2500), AkpB (0.5333), AkpC (0.2167), CaA (0.4750), CaB (0.5250). Table 2 shows the frequencies of the loci for analyzed lineages.

The esterases are a widely distributed isozyme family that is found in bacteria as well as in human beings. Those isozymes present wide specificity to substrate, with a differential distribution in tissues and in the different development stages. In *Diatraea saccharalis* (Lepidoptera, Pyralidae) eight esterase loci were observed, of which EST-3 was polymorphic (RUVOLO-TAKASUSUKI et al., 2002).

Table 2. Allelic frequency of the polymorphic loci of *Bombyx mori* lineages.

	EST – 11		ACP			AKP			CA	
	A	B	A	B	C	A	B	C	A	B
1⋆	0.666	0.333	0.000	1.000	0.000	0.250	0.750	0.000	0.500	0.500
2⋆	---	---	0.000	0.250	0.750	0.000	0.000	1.000	1.000	0.000
3⋆	---	---	0.000	0.500	0.500	0.000	0.000	1.000	1.000	0.000
4⋆	0.250	0.750	0.500	0.000	0.500	1.000	0.000	0.000	0.500	0.500
5⋆	---	---	0.000	0.750	0.250	0.000	1.000	0.000	0.000	1.000
6⋆	1.000	0.000	0.500	0.500	0.000	1.000	---	0.000	0.000	1.000
7⋆	1.000	0.000	0.500	0.000	0.500	0.000	1.000	0.000	0.000	1.000
8⋆	1.000	0.000	0.750	0.250	0.000	0.000	1.000	0.000	0.875	0.125
9⋆	1.000	0.000	0.000	0.500	0.500	0.000	1.000	0.000	0.000	1.000
10⋆	1.000	0.000	0.750	0.250	0.000	0.000	1.000	0.000	0.750	0.250
11⋆	1.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000
12⋆	1.000	0.000	0.500	0.500	0.000	0.000	0.750	0.250	0.500	0.500
13⋆	---	---	0.000	0.250	0.750	0.250	0.250	0.500	1.000	0.000
14⋆	1.000	0.000	0.000	0.125	0.875	0.250	0.250	0.500	0.000	1.000
15⋆	1.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000

⋆lineages.

The mean heterozygosity observed for all loci was 0.0350, while the expected heterozygosity was 0.0929 (Table 3). These results indicate low heterozygosity for the analyzed loci and suggest that these lineages can be crossed with others for genetic improvement.

Table 3. Observed and expected heterozygosity of isoenzymes for 15 loci of *Bombyx mori* lineages.

	EST – 11		ACP		AKP		CA		Mean	Mean
	HO	HE	HO	HE	HO	HE	HO	HE	HO	HE
1⋆	0.6667	0.5333	0.0000	0.0000	0.5000	0.4286	1.0000	0.5714	0.5417	0.3833
2⋆	0.0000	0.0000	0.0000	0.4286	0.0000	0.0000	0.0000	0.0000	0.0000	0.1429
3⋆	0.0000	0.0000	0.5000	0.5714	0.0000	0.0000	0.0000	0.0000	0.1667	0.1905
4⋆	0.5000	0.4286	0.5000	0.5714	0.0000	0.0000	1.0000	0.5714	0.5000	0.3929
5⋆	0.0000	0.0000	0.0000	0.4286	0.0000	0.0000	0.0000	0.0000	0.0000	0.1429
6⋆	0.0000	0.0000	0.0000	0.5714	0.0000	0.0000	0.0000	0.0000	0.0000	0.1429
7⋆	0.0000	0.0000	1.0000	0.5714	0.0000	0.0000	0.0000	0.0000	0.2500	0.1429
8⋆	0.0000	0.0000	0.5000	0.4286	0.0000	0.0000	0.2500	0.2500	0.1875	0.1696
9⋆	0.0000	0.0000	0.0000	0.5714	0.0000	0.0000	0.0000	0.0000	0.0000	0.1429
10⋆	0.0000	0.0000	0.0000	0.4286	0.0000	0.0000	0.0000	0.4286	0.0000	0.2143
11⋆	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
12⋆	0.0000	0.0000	1.0000	0.5714	0.5000	0.4286	1.0000	0.5714	0.6250	0.3929
13⋆	0.0000	0.0000	0.5000	0.4286	0.5000	0.7143	0.0000	0.0000	0.3333	0.3810
14⋆	0.0000	0.0000	0.2500	0.2500	0.5000	0.7143	0.0000	0.0000	0.1875	0.2411
15⋆	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000

⋆lineages.

The fixation index (F_{is}) for the analyzed isozymes was 0.0751, indicating homozygous excess and that there is probably no inbreeding in the lineage (Table 4). The high F_{st} value (0.6128) allows the conclusion that the lineages are differentiated (Table 4).

Table 4. Values of the Fixation index (F_{is}) and differentiation degree (F_{st}) of *Bombyx mori* strains.

Locus	F_{is}	F_{st}	F_{it}
Est-11	- 0.2655	0.5857	0.4757
Acp	0.3014	0.4075	0.5861
Akp	0.1429	0.7569	0.7916
Ca	- 0.4444	0.7140	0.5868
Mean	0.0751	0.6128	0.6419

Silkworm hybrids are obtained by single crosses of two silkworm parent races, normally Japanese crossed with Chinese races, to obtain the so-called direct hybrids (KRISHNASWAMI et al., 1979). Another form of hybrid production is the mating of two F_1 hybrids produced by different combinations of Japanese and Chinese *B. mori* races, in other words, double cross hybrids or tetra-parental hybrids (NAGARAJU, 2002). Shows the Japanese and Chinese strains with lowest variability, which are considered pure and are therefore indicated for the development of hybrids with promising results regarding the performance in the silk production.

The values obtained by the genetic distance of Nei (1978) show that the strains are distinct. The results showed that the strain 5 and strain 15 are identical and can be considered a single lineage.

The dendrogram based on the distance of Nei (1978) by the UPGMA method (Figure 1) shows that the Japanese and Chinese strains could not be totally separated by the isoenzyme system analysis. The results indicate that in spite of the genetic distance and differentiation among the lineages, they cannot be separate just with the isozymes alleles.

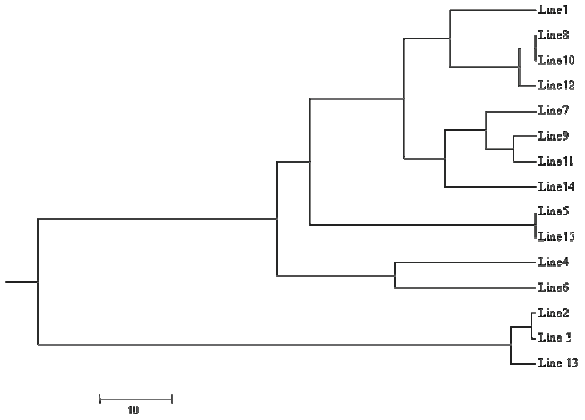

Figure 1. Dendrogram of genetic distance constructed by the UPGMA method based on Nei (1978) between Chinese and Japanese *Bombyx mori* lineages.

The matrices that compose these strains are maintained by crosses that may reduce the genetic variability. It would therefore be important to continuously evaluate the polymorphism degree of the strains, to avoid a marked increase of homozygosis. This would result in the expression of deleterious genes that can cause high matrix mortality.

Several studies have been developed with molecular markers in silkworm to detect the polymorphism degree, develop linkage maps for genes related to silk production and to map economic relevant genes.

The first linkage map by the Amplified Fragment Length Polymorphisms (AFLP) marker in *B. mori* was developed by Tan et al. (2001). For the AFLP mapping 47 progenies of a backcross population were genotyped. The authors found a total of 1248 (60.7%) polymorphisms detected with 35 primer combinations with adaptor *PstI/TaqI*. Since each one of the primer combinations generated an average of 35.7 polymorphic markers, a total of 44% of the polymorphic markers was consistent with the segregation ratio of 1:1 at a significance level of P = 0.05. The total AFLP map length was 6512 cM. The genetic distances between two neighboring markers varied from 0.2 to 47 cM, with a mean of 18.2 cM. The *od* linked gene to the sex was located between the markers P1T3B40 and P3T3B27 at the end of group 3, indicating that the linkage group AFLP 3 was on the Z chromosome (sexual).

The domesticated silkworm, *B. mori*, is a herbivorous insect and a model organism for Lepidoptera. Chinese and Japanese scientists made great efforts to accomplish the sequencing project of the whole silkworm genome, which offers *Bombyx* researchers an opportunity to identify peptides using proteomic method (MITA et al., 2004).

The mtDNA of some insects has been completely sequenced, including the mtDNA-arrays of four races of *B. mori*: (1) Xiafang (LU et al., 2001), (2) Backokjam (LEE et al., 2002) (3) C108 (YUKUHIRO et al., 2002), (4) Aojuku (ITOH et al., 2002). These sequences were used to determine the average size of mtDNA from *B. mori* in 15,650 bp.

Prasad et al. (2005) evaluated the microsatellite frequency and distribution in 21.76-Mb random genome sequences, 0.67-Mb in BAC sequences of the Z chromosome, and 6.3-Mb EST ("Expressed Sequence Tags") sequences of *B. mori*. In this study microsatellites were estimated in 0.31% of the silkworm genome.

In 13 *B. mori* strains analyzed by Prasad et al. (2005), 23 polymorphic microsatellite loci were found with variation of 2 to 14 alleles, with a mean heterozygosity value of 0.54. Only 36 (18.2%) of 198 microsatellite loci were polymorphic between the two divergent silkworm populations and 10 (5%) loci had no alleles. The map based on these polymorphic markers presented eight linkage groups. The microsatellite loci were most conserved in the closest ancestor, *B. mandarina*, followed by the wild silkworm, Antheraea assama.

The analysis of 15 microsatellite loci of 13 *B. mori* strains revealed 3 to 17 alleles, with a heterozygosity between 0.66 and 0.90 (REDDY

et al., 1999). These microsatellites enabled the authors to obtain alleles for strains with and lineages without diapause.

Chatterjee and Pradeep (2003) identified molecular markers associated with silkworm growth and yield parameters. RAPD profiles were generated with 7 UBC primers for 14 silkworm lineages originated from China, Japan, India, and Russia and statistically analyzed. By multiple regressions analysis a significant association of 45 markers was detected with the larval development, the growth indices and the four relevant cocoon yield parameters for silk production.

Miao et al. (2005) constructed a dense linkage map of *B. mori* using 518 Simple Sequence Repeat (SSR) sequences or microsatellites, as part of an international genome program. Six representative strains and 2500 (CA)n and (CT)n microsatellite markers were used in the study.

These markers revealed a polymorphism of 17 - 24%, indicating the high homozygosity degree resulting from the long-standing history of inbreeding of silkworm. Those studies with molecular markers indicate that the genome of the silkworm is being studied broadly, for the development of better lineages of those insects.

The analyzed isozimas showed that the 15 Japanese and Chinese lineages maintained by ACESP-Brazil are genetically differentiated, possess a high polymorphism degree and can be used for production of hybrid and to do part of a future improvement program for those lineages.

Besides the improvement the isozymes may contribute to the understanding of the expression of functional genes, in different tissues or organs and in different phases of the development in *B. mori*.

Conclusion

Through the analysis of isoenzyme found an excess of homozygotes, results showed that the line 5 and line 15 are identical and can be considered a single lineage.

The genotypes of the study strains can be used in future studies of genetic improvement to develop superior hybrids for silk production.

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