PhD THESIS

Assessing the potential of distinct *Bombyx mori* breeds for producing transgenic larvae in the Pharma-Farming breeding system

SUMMARY OF PhD THESIS

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INTRODUCTION

The silkworm, *Bombyx mori* (Lepidoptera: Bombycidae), is one of the most important insects from an economic point of view. This insect plays an essential role in silk production and is a textile industry's mainstay. Its potential in biological areas has been massively exploited since 2004 when its entire genome sequence was published (Mita et al., 2004; Moise et al., 2020). The genetic deepening of each species is the starting point for improving organisms in terms of qualitative and quantitative production (Pusta, 2018), in this case, silk production.

Among the most important contributions of *B. mori* in the scientific fields, its applications as a model organism and bioreactor are the most important. In terms of products derived from sericulture, we mention fibroin and sericin, the two major components of silk thread. Of the two proteins, fibroin exhibits a wide range of properties with significant impact on the biotechnological field, the most important being biodegradability, biocompatibility and robust mechanical strength. Molecularly, fibroin consists of two chains: fibroin heavy chain (FIBH) and fibroin light chain (Fib-l) (Matei et al., 1997; Mărghitaș et al., 2003).

In the context of Pharma-Farming, the sericulture industry and the area of molecular biology are the main components involved. The combination of the two areas represents an approach that aims to optimize the process of obtaining essential compounds for the pharmaceutical industry and the medical field (Scholar et al., 2022). Consolidating knowledge of an organism's genetics is the foundation for applying genetic engineering techniques to genome editing (Coșier, 2007).

In this thesis, the starting point was the application of the RAPD technique to confirm the genetic variation between breeds and hybrids of *B. mori*. The results of this analysis facilitated the selection of three breeds, namely Băneasa 1 (B1), Băneasa Yellow (GB) and JH3, for which specific bands were determined, allowing their identification but also highlighting genetic variability. Next, the three silkworm breeds were analyzed in terms of biological, technological and economic characteristics, outlining a holistic approach to the improvement and optimization of silk production, which is subsequently used in the textile industry as well as in fields such as medicine and pharmaceuticals.

Regarding recombinant protein production or fibroin extraction for biomaterial development, it is essential to identify the breeds that exhibit the best feasibility in terms of silk synthesis yield. In this direction, the pillar of research is the evaluation of the genes involved in silk synthesis, namely FIBH and Fib-l. In the present study, we performed the analysis of the *Fib-l* gene sequence, specifically exon 3, together with the related upstream intronic sequence. This approach represents an optimal strategy to comprehensively assess the role of the target gene in silk production and to identify sequence diversity among target breeds. Following the identification of mutations compared to the reference genome, the SilkBase database and the ChIP-seq mapping tool were used to assess in detail the impact of the determined mutations in the *Fib-l* sequence, specifically in the binding sequences of the BmHP1a protein belonging to the three *B. mori* breeds, on the binding potential of this protein, which plays a crucial role in regulating gene expression.

Moreover, the expression profile of genes involved in fibroin synthesis was analyzed; these genes exhibit a distinct pattern depending on the larval developmental stage. Moreover, the variability of the gene expression patterns among distinct *B. mori* breeds may serve as an indicator of genetic diversity, facilitating the identification of breeds and hybrids.

These types of research, on the expression profile and regulatory mechanism of the two genes, provide the foundation for the process of improving silk fiber characteristics through genetic engineering techniques. After an exhaustive evaluation of the genes involved in fibroin synthesis and identification of variability in order to create a guide sequence that serves the gene editing process, the first step was to optimize the microinjection protocol. This technique is crucial for the efficient delivery of the transgenic construct into *B. mori* eggs. Optimization of this method is essential to increase the efficiency and success rate of the experiments, allowing precise insertion of exogenous genetic material into the target genomic sequence of silkworms.

The present PhD thesis involves two distinct parts: " Current state of knowledge" and " Personal contribution". The first part consists of two chapters: "Ch. 1. Impact of sericulture in the life sciences" and "Ch. 2. Pharma-Farming: Concept and applicability". The second part of the thesis consists of eight separate chapters, namely "Chap. 3. Objectives pursued", "Chap. 4. Preliminary analysis of genetic variation between different *Bombyx mori* breeds", "Chap. 5. Comparative analysis of biological and economic parameters in three distinct silkworm breeds", "Chap. 6. Identification of particularities of the *Fib-l* gene level in three *Bombyx mori* breeds", "Chap. 7. Identification of the expression profile of genes involved in fibroin synthesis", "Chap. 8. Optimization of microinjection protocol to obtain transgenic larvae used in Pharma-Farming breeding systems", "Chap. 9. Conclusions and recommendations", "Chap. 10. Original aspects of the thesis". Within these chapters, there are 22 tables and 31 figures.

1. Sericulture's impact on life science domains

1.1. Sericulture: An overview

The domestication of one of the most economically important insects, the silkworm (*B. mori*), dates back over 5000 years. *B. mori* is an oligophagous insect with complete metamorphosis. The main food source of the *B. mori* silkworm is mulberry leaves, a nutritional preference that directly impacts its biological and economic parameters (Mărghitaș, 1995; Pașca, 2004; Montali et al., 2022).

Representing an insect with a significant impact on the economic sector, more than 4000 silkworm breeds and hybrids have been described (Matei et al., 2005). Regarding the genetics of *B. mori*, the genome of this species has been completely sequenced (Mita et al., 2004), which has revealed a lot of information on the evolution of the silkworm, and its biology, and has also provided a clear insight into silk production. The genome of *B. mori* comprises approximately 432 million bp, including approximately 14,623 genes (Mita et al., 2004; Dezmirean et al., 2010).

Silk is the main product of sericulture and plays an essential role as a raw material in the textile industry. Apart from the main product, a large-scale by-product chain with a wide range of applications is also obtained from sericulture (Baci et al., 2022).

1.2. Sericultural by-products

1.2.1. Fibroin

Silk is one of the most valued natural polymers, alongside cellulose and chitosan. Several insects produce this biopolymer, but the most explored is the silk produced by larvae of *B. mori*. It exhibits outstanding characteristics, including biocompatibility, versatility, and biodegradability. Based on these characteristics, silk has been used as a surgical suture since ancient times. The silk proteome contains two key proteins, namely fibroin and sericin (Baci et al., 2023).

Molecularly, fibroin involves two chains: the fibroin heavy chain (FIBH) and the fibroin light chain (Fib-l). Between the two chains, there is a disulfide link through which the two proteins are connected. In addition to FIBH and Fib-l, fibroin includes another protein, namely fibrohexamerin (P25). The *FIBH* contains several repetitive sequences that are involved in the development of antiparallel structures. On the other hand, the *Fib-l* contains non-repetitive sequences responsible for the chain's hydrophilicity and the fibroin's elasticity. The molar ratio of the three proteins is 6:6:1 (Lujerdean et al., 2022).

1.2.2. Sericin

Even though fibroin captures the attention of the scientific community, and sericin is removed by some thermochemical methods, studies have recently been described that highlight various uses of sericin. This research highlights the potential of this protein for various applications. For example, some studies have been published showing the therapeutic effects of sericin, including anti-inflammatory and antioxidant effects (Jian & Huan, 2024).

1.2.3. Mulberry

One of the richest plants in bioactive components is mulberry. A wide range of recent studies have highlighted the beneficial effects of different parts of mulberry on human health. It is well documented that this plant exhibits specific therapeutic effects, including its fruits, leaves or roots. As for the composition of mulberry, it includes a considerable content of carbohydrates, and proteins, but also lipids, vitamins, or minerals (Pașca, 2004; Baciu et al., 2023).

1.2.4. Chrysalis

The chrysalis of *B. mori* contains a significant proportion of nutrients, including a high protein and fat content, and its composition also includes minerals, vitamins or polyphenolic compounds. Protein accounts for 55.6% of the dry matter of the pupa, being the most abundant category of compounds. The active ingredients in silkworm pupae exhibit a wide range of pharmacological activity, showing significant therapeutic effects against some target diseases (Hăbeanu et al., 2023).

1.3. *Bombyx mori* as a model organsim

B. mori plays a crucial role in the life sciences and is one of the most widely used model organisms due to its unique characteristics. This insect has a short life cycle, with the larval stage lasting 25-30 days. Another important advantage is that it produces a large number of offspring.

Moreover, the level of attention received as an experimental model organism has increased significantly since the entire genome sequence was published (Matsumoto et al., 2021).

2. Pharma-Farming: concept and applicability

The concept of Pharma-Farming involves a holistic, innovative approach, combining the fields of animal husbandry and agriculture with the pharmaceutical industry. This concept promotes the use of animals or plants to their full potential by making full use of them. Farma-Farming also includes the optimization of practices in the two areas in order to maximize the yield of by-products of pharmaceutical interest, while aiming to minimize the environmental impact (Abiri et al., 2016).

2.1. Transgenic silkworms in the context of Pharma-Farming

The first experiment aimed at achieving transgenesis in silkworms was reported by Maeda et al. (1985). The authors first used nuclear polyhedrosis virus (BmNPV) as an expression system. After expression of the recombinant protein, i.e. human interferon α and its secretion into the hemolymph, it was observed that the exogenous protein is degraded by thiol proteases (Maeda et al., 1985). The process of obtaining recombinant proteins of interest using transgenic silkworms as a biotechnological platform is based on the silk synthesis system, implicitly the silk gland. Genes responsible for fibroin and sericin synthesis are involved in this process. To date, multiple expression systems based on the silk synthesis system have been described, each with both advantages and disadvantages. The choice of expression system is made according to the target protein and is the most important step for the production of recombinant proteins (Baci et al., 2022).

One of the most important advances in research fields has been the development of different genome editing tools. By using specific techniques for editing genetic material, genomic DNA belonging to a living organism can be subjected to guided modifications such as deletions, insertions, or sequence substitutions (Martin et al., 2024). In recent years, many such tools have been described in the field of genetic engineering, and among them, there are three prominent technologies, namely those based on programmable nucleases (e.g. TALEN), zinc-finger nucleases (ZFN) and nucleases associated with short interspaced palindromic repeats (CRISPR-Cas).

Proteins are complex molecules with a wide range of applications in various fields, playing a key role in the food, chemical, textile, agricultural, and cosmetic industries. In recent years, to meet the high demand for proteins with pharmaceutical applications, the scientific community has focused on developing feasible platforms for recombinant proteins. In this respect, research described in the literature has shown that the field of biotechnology applied to entomology shows the most promising results. In this direction, *B. mori* presents great potential as a bioreactor for the production of target proteins, since it presents certain advantages (Tatemastu, 2012).

3. Objectives pursued

The objectives pursued in this thesis mark the advancement of the sericulture field by providing a context on the application of innovative technologies to exploit silkworms in the life sciences. These objectives are the starting point for future research aimed at genomic editing of silkworms to obtain recombinant proteins or silk fibers with superior properties.

Five main objectives were considered in this thesis, namely "3.1. Evaluation of genetic variation between distinct *Bombyx mori* breeds", "3.2. Identification of economic and biological particularities in three distinct silkworm breeds" "3.3. Identification of particularities of the *Fib-l* gene in three *Bombyx mori* breeds", "3.4. Identification of the expression profile of genes involved in fibroin synthesis", "3.5. Optimisation of microinjection protocol to obtain transgenic larvae used in Pharma-Farming breeding systems".

4. Preliminary analysis of genetic variation between distinct *Bombyx mori* breeds

To achieve the first objective stated in this PhD thesis, the first study was carried out to identify genetic variations among 14 breeds and hybrids of *B. mori*. RAPD molecular markers described by Bajwa et al., (2017) were used, and these were associated with higher productivity in terms of silk production. The use of RAPD markers in applied genetic research in sericulture paves the way for the development of more accurate and efficient breeding strategies (Bajwa et al., 2017).

In this study, five RAPD markers were analyzed to identify DNA polymorphisms and assess genetic variation among target breeds and hybrids of *B. mori*. Out of the total of five markers analyzed, three resulted in visible bands based on DNA amplification, namely Ga-12, CT-9 and Ca-9. PCR product amplification resulted in a variable number of bands. A total of 29 bands were obtained using these primers, indicating a polymorphism of 27.14%. Using the GA-12 genetic marker, a level of genetic variation of 21.42% was found, and the highest degree of polymorphism was identified using the CA-9 marker, i.e. 60%. In contrast, the use of the CT-9 marker did not reveal the presence of variable sequences. Specific bands were also obtained, and following their identification, we selected two breeds for further experiments. Another breed was selected for further experiments on the basis that band amplification was confirmed for each marker, namely the B1 breed. For the JH3 breed, specific traits were identified at two genetic markers, GA-12 and CA-9. Marker GA-12 showed two bands, one of which was specific to JH3 and the other common to another breed. For the GB breed, both markers, GA-12 and CA-9, produced amplifications, with the observation that GA-12 resulted in a specific band, while CA-9 generated a sequence of the same size, but also associated with another breed. The B1 breed was also included in the study, revealing bands for all three RAPD markers used: GA-12, CT-9, and CA-9, but without producing specific bands.

5. Comparative analysis of biological and economic parameters in three distinct silkworm breeds

The second study of this research involved the evaluation of silkworm breeds in terms of biological, technological, and economic characteristics. By combining this study with the identification of genetic variability using molecular biology techniques, a holistic approach to improving and optimizing silk production is outlined. The analysis of variability in all its dimensions, biological, economic, and genetic, is a pillar in achieving silk production that meets the industry's requirements in terms of quality.

In this study, we evaluated the biological and economic parameters of three silkworm (*B. mori*) breeds, namely B1 and GB, native Romanian breeds, and JH3, a breed of Japanese origin. Ten individuals from each breed were randomly selected and a series of parameters such as egg incubation period (days), larval length (mm), larval weight (g), and larval stage duration (days) were determined. In the last stage of the larval stage, the weight of the silk gland (g) was evaluated. After cocoon formation, another series of measurements were analyzed, namely the length of the transverse axis of the silkworm cocoon (mm), the length of the longitudinal axis of the cocoon (mm), the weight of the pupa (g), the weight of the cocoon (g), the number of eggs laid (no.)

The determined parameters were subjected to statistical analysis. In order to adjust the statistical analyses according to the distribution of the data, their normality was determined using a set of tests, such as the Kolmogorov-Smirnov and Shapiro-Wilk tests. At the same time, graphics in the form of histograms and quantile-quantile (Q-Q) plots were used in combination with the normality test. The results obtained indicated that the data corresponding to the majority of the measurements show a normal distribution, which justified the choice of performing parametric statistical tests. For three of the measured parameters, there were no statistically significant differences between the breeds evaluated in this study, namely 'Cocoon transversal axis, 'Chrysalis weight', and Cocoon weight'.

6. Identification of the particularities of the *Fib-l* gene in three *Bombyx mori* breeds

This study focused on identifying the sequence variability of genes involved in silk fiber synthesis, representing the direction with the greatest potential to improve not only silk yarn quality but also quantity. Using the reference genome, *B. mori* - breed p50T, available on NCBI, the design of the primers was performed using the reference sequences for chromosome 14 (NC_051371.1) and chromosome 25 (NC_051382.1). During the *in silico* design process, specialized databases for *B. mori*, such as SilkBase, were also consulted. A range of bioinformatics analyses was used for primer design through the use of online programs - BLAST, Primer 3, and the Ubuntu Linux 20.04 operating system. Specificity examination of the primers obtained was performed using the BLAST tool on the NCBI platform.

The amplicons resulting from the PCR reaction were sequenced, specifically, the products related to the *Fib-l* gene having a size of 390 bp. Any differences from the reference sequence were considered mutations. Regarding the mutations identified in exon 3 of the *Fib-l*

gene, a particularity is highlighted in the JH3 breed, where two synonymous mutations were identified: g.9,689,073A>G (G98G) and g.9,689,145A>G (D74D), the other breeds, showing no mutations compared to the reference sequence available in the NCBI database.

On the other hand, in the intronic region upstream of exon 3, mutations were identified in each evaluated breed. The B1 breed is distinguished by the presence of four mutations in this region, all of which are homozygous, which is a specific feature of this breed, in contrast to the other breeds that have heterozygous mutations. The g.9,689,398 locus was affected by mutations within each breed, while the g.9,689,403 and g.9,689,412 loci were mutated in B1 and JH3, but not GB. A specific feature of the Japanese-origin breed that distinguishes it from the Romanian-origin breeds is the mutation identified at locus g.9,689,269, and this breed also has a small insertion at the exon 3 border. Furthermore, the JH3 breed has a particular intronic mutation in the heterozygous state, symbolized by g.9,689,272T>G/A, which instead of T has one of the two alternative nucleotides, G or A).

Next, the SilkBase database and the ChIP-seq mapping tool were used to assess in detail the impact of the mutations determined in the *Fib-l* sequence, specifically in the binding sequences of the BmHP1a protein, belonging to the three *B. mori* breeds. The ChIP-seq mapping tool uses the BLAST algorithm to align the collection of ChIP-seq reads generated for BmHP1a to a selected query sequence, thus providing estimates of the frequency and location of reads within the same query. The results indicated that the determined SNPs may influence the number and positioning of putative BmHP1a binding sites.

7. Determination of the expression profile of genes encoding fibroin

This study aimed to assess the expression profile of *Fib-l* and *FIBH* genes, which is a crucial step in understanding genetic diversity among silkworm breeds. On the 5th day of the last larval stage, the posterior part of the silk gland was extracted from nine individuals, which were randomly selected from the three breeds. The qRT-PCR technique was used to determine the relative gene expression of the two genes responsible for fibroin synthesis, *Fib-l* and *FIBH*, respectively, in the posterior part of the silk gland. Three technical replicates were performed for each gene. Analysis of qRT-PCR data was performed using the Livak method and the R script. For visualization of qRT-PCR data (mean ± standard error or SE), GraphPad Prism version 8.4.2 software was used.

In order to evaluate the raw data generated from the qRT-PCR reaction, the $2^{-\Delta\Delta Ct}$ method, or the Ct comparative approach, was used. This is a procedure used in molecular biology experiments to estimate gene expression variation. It calculates the difference in the expression level of a gene by comparing the number of cycles required to reach a significant level of DNA detection between samples. The *Fib-l* gene was overexpressed ($p < 10^{-4}$) in B1 individuals compared to JH3 (FC = 3.552) and GB (FC = 2.706). In contrast, JH3 showed statistically significant inhibition of *Fib-l* gene expression levels compared to GB (FC = 0.895).

For the *FIBH* gene, a mirror expression profile was generated with a significant increase in gene expression levels in B1 compared to JH3 (FC = 1.141) and a lower gene expression level

compared to GB individuals (FC = 0.688). As for the comparison of *FIBH* gene expression between JH3 and GB, there was an overexpression but not statistically significant.

A comparison of *Fib-l* and *FIBH* gene expression levels was also performed for each studied breed. In the case of the B1 breed, a significantly higher level of *Fib-l* gene expression was observed compared to *FIBH* (FC = 9.267; $p < 10^{-7}$). Also, in the case of the GB breed, a statistically significant overexpression of the *Fib-l* gene compared to *FIBH* was recorded (FC = 1.709). On the other hand, in the JH3 breed, no statistically significant overexpression was identified in terms of *Fib-l* and *FIBH* expression levels.

8. Optimization of microinjection protocol for obtaining transgenic larvae used in the Pharma-Farming breeding system

With regard to the delivery of exogenous material to obtain transgenic *B. mori*, it was described a protocol that shows superior results in terms of larval survival and genetic editing success. The application of the microinjection technique in sericulture is one of the most important directions for obtaining transgenic silkworms to obtain recombinant proteins, study gene function, and improve silk yarn characteristics.

In the first phase of the study, eggs were collected immediately after laying, i.e. within one hour, which was the first experimental batch. In the second phase of the study, eggs laid within four to six hours were collected to assess the impact of injection time on viability. This variable was analyzed in order to maximise both viability and the rate of genetic transformation following injection. In order to streamline the process of obtaining transgenic larvae, we adopted an injection technology that allows for the delivery of exogenous genetic material accurately into primordial germ cells.

To begin with, control of the initial mating process, placement of individuals at low temperatures, but also egg collection, provide the correct basis for subsequent experiments and contribute significantly to their success. In this context, the process of selecting eggs that show buoyancy for subsequent injection is an essential step in ensuring the quality of the biological material.

The observation regarding the necessity of using an agent with adhesive properties, contrary to the protocol described by Tamura et al., (2007), underlines the importance of adapting and optimizing the methodology according to the silkworm breeds used. The particularities of each breed can significantly influence the genetic transformation processes, in this case, the physical and chemical properties of the egg chorion resulted in low adhesion to the glass slide surface.

9. Conclusions and recomandations

The identification of specific bands using the GA-12 and CA-9 markers allowed the distinction and selection for further experiments of two breeds with potentially superior characteristics, namely JH3 and GB, highlighting the genetic diversity within the *B. mori* breeds.

Additionally, the B1 breed was selected for further studies as it showed bands following the use of the three RAPD markers GA-12, CT-9 and CA-9 without generating specific bands.

The study on the evaluation of biological and technological parameters of silkworms, focusing on B1, GB and JH3, highlights the need for a holistic approach combining careful genetic selection of superior performing breeds and optimization of diet and growth conditions. The use of advanced statistical tests allowed the comparison of breeds on specific parameters, highlighting the crucial role of genetic diversity and selection in improving the economic traits of silkworms. This research contributes to the advancement of the silkworm field, emphasizing the importance of in-depth analysis of biological, technological and economic parameters to maximize silk production and promote sustainability.

As for the third study, in the B1 breed, the absence of mutations in exon 3, combined with the presence of four homozygous mutations in the upstream intronic sequence, corresponds to the superior traits observed in silk production. This correlation highlights the link between the genetic stability of certain regions of the gene and high economic performance. JH3, on the other hand, shows mutations in both exon 3 and the adjacent intronic sequence. Although these mutations are unlikely to directly influence cis-regulatory sequences, they may impact gene expression, suggesting a possible impact on the quality and quantity of silk produced. Comparative analysis of regions containing binding sites for the BmHP1a protein provides an in-depth analysis of how genetic variation influences gene expression. Identifying the presence of mutations in these crucial regions highlights how *Fib-l* gene expression is regulated, directly affecting *B. mori* silk production and quality.

The use of qRT-PCR technology allowed a detailed assessment of the expression of the two genes involved in silk fiber production, providing insight into the differences between the three breeds analyzed. The analysis revealed significant differences in the expression profile of the *FIBH* gene compared to the *Fib-l* gene, with a focus on the B1 breed. These results indicate that the gene expression profile can serve as an indicator for silk fiber production yield *in B. mori*.

In the latter study, we encountered specific challenges, such as the lack of adherence of eggs to microscope slides, and the critical impact of exogenous injection timing on embryo survival rates. These impediments highlight the importance of adapting the standard protocol according to genetic variability to improve both the hatching rate and efficiency of genetic transformation. By adjusting standard methods, we can not only improve experimental efficiency but also provide a stronger basis for advancing sericulture research.

10. Original aspects of the thesis

This thesis establishes a solid foundation for the biotechnological exploitation of the sericulture field, proposing innovative directions for *B. mori* applicability. The main original aspect of the thesis is the focus of the experiments on Romanian breeds of *B. mori*, a subject that has not been extensively explored in the literature. The focus on local breeds provides insight into the local genotype, offering opportunities for economic growth in the Romanian sector.

This orientation offers a new perspective in applied genetic research in sericulture, highlighting the particularities of the examined breeds in the context of Pharma-Farming, underlining the applicability of silkworms in the biotechnological sphere. The contribution of

this thesis stands out in the field of sericulture by corroborating this area with molecular biology, in an attempt to correlate the identified genetic differences with the biological and economic parameters of the target breeds. In order to analyze the *Fib-l* gene, we used specific sequencing techniques, a methodology that facilitated an exhaustive comparison of specific gene sequences between the breeds studied. Results obtained from sequencing and qRT-PCR analysis were correlated with phenotypic observations on *B. mori* individuals and economic aspects.

This approach aimed to elucidate the links between genetic variability and larval phenotypic characteristics and their influence on the quality of silk produced. This topic of research offers promising prospects for obtaining transgenic larvae for the production of recombinant proteins. The use of advanced techniques in biotechnology allows the genetic manipulation of silkworms in order to obtain proteins with a broad spectrum of applicability in the pharmaceutical industry and other fields.

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