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Current Trends in Silkworm Molecular Studies: A Review

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Abstract

Silkworm is an economically significant insect. It holds value in sericulture, protein synthesis,and as a model species for pest-destroying strategies. The construction of molecular maps and genome sequencing by utilizing Whole Genome Sequencing, Next Generation Sequencing, Third Generation Sequencing, and long reads from Pacbio, Bac, or Fosmid libraries has helped in studying its population genetics and developing it as a reference organism for lepidopteran order. Sequencing of mitochondrial DNA and cytochrome b gives insights into silkworm domestication and separation on the Phylogenetic tree of *Bombyx Mori* and *Bombyx Mandarina* species. The impact of various environmental stresses and coping mechanisms is being adopted by silkworms at various life stages. Humidity affects cocoon coloration, larvae growth, and the quality of produced cocoon. We have also reviewed genetic engineering tools available to induce double-strand breaks DSBs in the silkworm DNA sequence; highlighting the drawbacks of Zinc Finger Nucleases and Transcription Activator-like (TAL) Effector Nucleases (TALENs) and the advantages of utilizing the CRISPR tool for editing genes at various targeted sites. This review discusses molecular tools that facilitate genetic engineering in silkworm species and the status of silkworm molecular genetics research to enable the study of gene expression patterns in environmentally stressed conditions.

Keywords: **S**ilkworm, Model Species, Gene Editing, Molecular Biology, Genome Sequencing, CRISPR

Introduction

For past 5000 years, silkworm *Bombyx mori*, has been used to produce silk. It is now a wholly domesticated arthropod, that mostly relies on humans for sustenance and breeding. In developing countries, it holds economic significance as it can be cultured at a large scale and used as a silk producer for textile industry. It plays an essential role as a cultivable insect for recombinant protein synthesis congruent with the advancement of biotechnology.

B. mori is an ideal insect for Lepidopteran order, which encompasses several destructive agricultural insects. This genomic information will help in mitigating damages caused by pests on global staple diet and fiber production.

In 2004, Chinese (Kawamoto et al., 2019) and Japanese (Kawamoto et al., 2019) teams conducted independent whole-genome shotgun (WGS) sequencing projects for male *B. mori*. However, these datasets had limitations for constructing comprehensive scaffolds. In 2008, both groups decided to collaborate to integrate data sets and produced an assembly of its genome. In 2019, genome sequences of p50T silkworm strain were assembled utilizing PacBio long reads and BAC sequences (Kawamoto et al., 2019).

The silkworm *Bombyx mori*, has woven a bond with humanity, driving financial survival and cultural richness. Evolving from a symbol of art along Silk Road to a scientific model in nineteenth century, stands significant in genetics, genomics, sericulture, and biotechnology (Goldsmith et al, 2004).

In India's sericulture history, Pure Mysore and Nistari stand as the oldest silkworm races in the country. Pure Mysore is believed to trace its roots from China brought by Mysore King Tippu Sultan in 1875. Nistari is thought to have been introduced from China in West Bengal region.

Molecular biology studies on Mitochondria offer insights into domestication of silkworms. It aids in revealing distinct population dynamics and crucial role of cytochrome b in silkworm domestication.

Various species of moths within Bombycoidea superfamily produce range of silk fibers. This includes *B. mori* from Bombyidae family and wild moths from Saturniidae family, such as *Antheraea mylitta* (Indian tropical tasar silkworm), *A. pernyi* (Chinese oak tasar silkworm), *A. assama* (Indian golden silkworm), *A. yamamai* (Japanese oak silkworm), and *Philosamia cynthia ricini* (Indian castor silkworm). The production of silk from worms, particularly from *B. mori, A. pernyi, A. mylitta,* & *A. assama,* contributes significantly to promote economies of densely populated low and middle income countries.

1. Environmental stress and adaptation:

1.1 Temperature stress:

Insects are cold-blooded animals, which rely on environmental conditions to regulate their body temperatures. Thermal shock results in either elimination of species or causes natural selection of adaptive survival mechanisms in silkworms. The changes in worldwide climate affect growth, demographics and feeding habits of insects (Chakraborty & Dastidar, 2022). Global warming also poses a serious threat to growth and breeding of silk moth (Ismail et al., 2023). Elevated temperatures impact intestinal microbes of *B. mori* larvae, with females displaying more sensitivity to these changes. The alteration of biochemical processes of silkmoth's gut tissues under elevated temperatures can be associated with changes in composition of intestinal microbes. High temperatures may disrupt functioning of gastrointestinal proteins and transportation in gut, impacting digestion, nutrient absorption, and consequently overall maturation of silkworm insects. Additionally, exposure to high

temperatures causes morphological changes and permanent harm to midgut of silkworm larvae (Sun et al., 2022).

Bivoltine silkworms raised in tropical regions exhibit a degree of thermotolerance. Yet, extended contact to intense heat can prove lethal. In a study, fifth larval stage of bivoltine silkmoth was subjected to thermal stress at 40 ± 2 °C for an hour daily to assess alteration in intestinal microbiota. The results demonstrated increased temperature negatively impacted gut microbiota diversity in contrast to control group that was bred under ideal temperature (25 ± 3°C). Control group reported 458 species of microbiota whereas experimental group reported only 434 species. Exposure to heat shock results in a decrease in morphological measurements of silkworm larvae, and cocoons *(*Ismail et al., 2023*).*

High-temperature treatment during fifth larval stage affected the overall maturation of silkworms, with females being heavier than males. The exposure to elevated temperature displayed increased feeding habits and digestion in silkworm larvae, resulting in increased weight in contrast to control group. During recovery period, feeding and digestion of silkworms was reduced as compared to control group, leading to lower body weights. High temperature also resulted in higher mortalities of cocoons, vacuolation in midgut cells of both male and female silkworms indicating its negative impact on health and growth of silkworms. (Sun et al., 2022)

<u>Activation of molecular chaperone genes:</u>

To overcome thermal stress, silkworm body accommodates a protective mechanism by synthesizing proteome enriched with Heat shock proteins (HSPs). Heat shock proteins ensure survival under hostile conditions either by protecting against damage or by aiding in damage repair. HSPs act as molecular chaperones, assisting in proper folding of proteins and barring their clumping under heat strain (Chakraborty & Dastidar, 2022).

HSPs are conserved protein groups present in all insects. HSPs can be classified into tetrad groups HSP90, HSP70, HSP60, and sHSP, based on molar mass, sequence of amino acids in polypeptides, and functional roles (Liu et al., 2018). The activation of genes such as HSP70, HSP40, HSP20.8 in response to heat strain in silkworm *B. mori* was analyzed using reverse transcriptase-polymerase chain reaction (Velu et al., 2008).

Assessment of Hsp 70 and Hsp 40 gene activation in heat-tolerant (Nistari) & thermosusceptible (NB4D2) silkworm varieties showed differences in their gene activity levels, with Nistari showing more gene activity. Hsp 70 and Hsp 40 displayed higher levels of expression compared to small molecular chaperon genes Hsp 20.8 and Hsp 20.4. The expression of Hsp 70 surged amidst recovery phase, while Hsp 40, Hsp 20.8, and Hsp 20.4 genes displayed increased levels of expression during preliminary phase which steadily decreased throughout recovery phase. Tissue-specific expression of Hsp 70 varied among different tissues, with midgut and adipose tissues displaying more expression than epidermis and labial glands tissue. These findings provide insights into cellular shielding mechanisms from environmentally extreme conditions, particularly thermal assault (Velu et al., 2008).

Exposure of silk moth to elevated temperature also causes activation of BmTmC27 gene. BmTmC27 gene is engaged in transport of materials across plasma membrane. The altered expression of this gene may disrupt normal transport of materials in midgut, leading to homeostatic disturbances (Sun et al., 2022).

An empirical study was conducted to assess heat responsiveness of larvae of polyvoltine white nistari breed. Eggs, larvae (4th day of Vth instar), pupae, and adult silkworms were subjected to thermal conditions ranging between 18°C to 44°C for 3 successive days with each session of 90 minutes. During this study, it was observed that rising temperature to 44°C, induced appearance of a 72kDa protein in hemolymph of Vth instar larva. Cocoon and shell weight

increased considerably following heat stress, credited to the activation of Hsp72 during Vth larval phase. These findings suggest *B. mori* may be bred under high temperature to produce superior cocoons (Chakraborty & Dastidar, 2022).

Small heat shock proteins (sHSPs) are proteins engaged in various biological activities across insects, including diapause, metamorphosis, embryo formation, apoptosis, autophagy, and immune responses against pathogens. In *B. mori*, exposure to nucleopolyhedrovirus (NPV) triggers altered expression of several HSP genes, such as HSP 19.9, HSP 20.1, HSP 20.4, HSP 20.8, HSP 21.4, HSP 23.7, HSP 40, HSP 70, and HSP 90. Similarly, in *A. Pernyi*, NPV infection causes activation of sHSP20.8, sHSP21.4, and sHSP25.4 in various tissues like midgut, hemocytes, and fat body (Liu et al., 2018).

A wild silkmoth variety *A. Pernyim*, is economically reared in South Asian countries for silk generation, conventional pharmaceutical (specific to Chinese practices), and as a protein rich diet source (Liu et al., 2018).

It is recognized that synthesis of HSPs can be changed upon exposure to temperature stress. Many HSPs are involved in induction of immune response. Expression of ApsHSP21 (sHSP) responses was examined in *A. pernyi*, lymphoid tissues using qRT-PCR upon exposure to various stimulants including LPS, PGN, glucan, and NPV. Post LPS challenge, expression of ApsHSP21 increased and reached highest after 6 and 12 hours of injection respectively. Conversely, post PGN challenge, activation of ApsHSP21 reached highest point after 3 hours of injection and similarly, exposure to glucan challenge also resulted in peaking ApsHSP21 expression after 3 hours of injection. Following NPV challenge, expression of ApsHSP21 peaked after 36 h of injection. (Liu et al., 2018).

1.2 Humidity stress:

Silk production is a trait of around 98 % Lepidopteran species. Silk is produced internally but performs external functions, and responses to environmentally stressed conditions. Since silk moths are ectothermic, while spinning, surrounding conditions have an influence on both physiology and behavior of moths, resulting in impacts on characteristics of silk (Offord et al., 2016).

The interaction between heat and moisture significantly impacts optimal development of silkworms and quality of cocoon production. Humidity affects physiological processes of silkworms, with younger larvae demonstrating greater tolerance to high humidity compared to older ones. Under high humidity, young silkworms exhibit vigorous growth. The effect of climate on cocoon anatomy can be divided into two categories: cocoon shape (impacted by heat) and cocoon hue (impacted by moisture). The caterpillars of *B. Mori* synthesize a shielding cocoon before undergoing metamorphosis. This procedure entails weaving for numerous hours as caterpillar produces a seamless silk strand from its silk glands (Offord et al., 2016).

A study aimed to examine how environmental conditions impact spinning by analyzing physical characteristics of *B. Mori* cocoons, showed elevated temperatures prompted to produce longer, thinner cocoons, to regulate heat dissipation. This could help maintain an optimal temperature range for metamorphosis, given higher surface area to volume ratio of longer, thinner cocoons. Increasing relative humidity (RH) facilitated easier fiber peeling but reduced distinction between individual layers (Offord et al., 2016). Additionally, humidity affected cocoon coloration, with higher RH resulting in darker, tanned colors compared to cocoons spun under lower humidity (Table 1, Table 2).

Table 1: Range of Temperature and Relative Humidity and consequences on silkworm (Offord et al., 2016).

Temperature (ºC)	**Relative humidity (%)**	**Consequences**	**References**
25	65-75	Optimum range	(Offord et al., 2016)
25	85	Mortality of larvae increases	(Offord et al., 2016)
35	100	Lowest survival of larvae	(Offord et al., 2016)
15	10	90 percent larvae survived	(Offord et al., 2016)

Table 2: Range of Temperature and Relative Humidity Affecting Silkworm (Islam, 2018).

Temperature (ºC)	**Relative Humidity**	**Affects**	**References**
25 ºC	70% RH	Ideal for enhanced silk gland activity and increased weights of larvae and cocoon.	(Islam, 2018)
22-26º C	80-85% RH	Least larval mortality. Weight of cocoon and pupae is more	(Islam, 2018)
25°-28° C	60-75% RH	Suitable for development and adult emergence	(Islam, 2018)
28°-32°C	80-95% RH	Reduces developmental time, impact performance of silkworm lines negatively.	(Islam, 2018)
25°C	55 - 65% RH	lowered hatchability and pupation, higher larval mortality	(Islam, 2018)
°22 C	65±5% RH	good quality cocoon	(Islam, 2018)

For commercial silkworm rearing, most favorable conditions were found to be at 25-32°C and 75-80% RH. Temperatures exceeding 35°C hindered growth, with embryogenesis halting and eggs failing to hatch between 35°-38°C. Impact of temperature variations within the range of 25° to 32°C on *B. Mori's* maturation was notable, with shortened incubation, larval, and pupal periods observed. Overall, total maturation time of silkworm decreased from 25°C to 32°C,

indicating a significant inverse relationship between rearing temperature and immature developmental periods. (Islam, 2018).

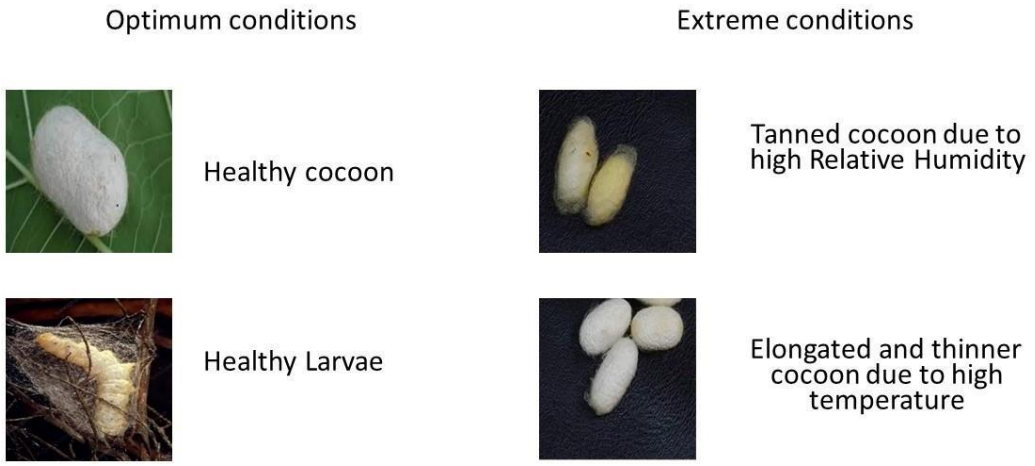

Figure 1: Impact on Cocoon of *B. Mori* of high thermal conditions and Humidity (Goetgheluck (Silkworm (Bombyx mori) spinning a silk cocoon) & Yellow and white silkworm cocoons (online image).

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1.3 Air and light conditions:

Silkworms inhale oxygen through 18 spiracles on each side of their bodies and distribute further through trachea. In rearing room, excessive gases like carbon dioxide, formaldehyde, sulfur dioxide (SO2), and ammonia can cause silkworms to become sluggish and cease feeding. Safe gas limits for rearing are 1-2% for carbon dioxide, 1% for formaldehyde, 0.02% for SO2, and 0.1% for ammonia. Young silkworms are more susceptible to toxic gases. The presence of SO2 can negatively impact cocoon quality and decrease filament reliability. Low Ammonia level can render sericin insoluble during reeling.

2. Silkworm genetic engineering tools

Genetic engineering enables alteration of DNA sequences within living organisms. The approach of genetic engineering is based on causing double-strand breaks (DSBs) in DNA at targeted site, instructed by either customized mobile protein like zinc fingers (ZFs) or transcription activator-like effectors (TALEs) or short guide RNAs (gRNAs). DNA DSBs possibly can be fixed by two mechanisms. The one through non-homologous end-joining (NHEJ) method, which may lead to alteration of specific nucleotide by inserting or deleting sequences (knockout variation). The other is through homology-directed repair method which facilitates insertion of external DNA sequences at specific site through homologous recombination (HR), thereby generating a knock-in variation (Xu & O'Brochta, 2015).

2.1 Zinc finger nuclease editing tool.

It comprises set of tailored proteins, all constructed from three adjustable ZF motifs. These are designed to bind to a specific trinucleotide sequence and cleave DNA at site. Linked to these motifs is a single unit of nonspecific DNA endonuclease derived from Fok I protein. The tailored domains containing ZF establish site of protein interaction within genome, whereas paired Fok I enzyme serve as DNA cleavers to induce DSBs at desired site.

ZFNs can be applied in *Bombyx mori* to alter gene *BmBLOS2* and *white egg Bmwh3* , both of them possess latent amorphic mutations. Lately, ZFNs were utilized to interfere fibroin heavy chain (BmfibH) gene, which encodes for silk protein.

Producing inheritable constitutional variants with this tool proved to be ineffective and intricate. To engineer genes by utilizing ZFNs is a challenging and time-consuming endeavor due to difficulty in tailoring and constructing ZFs with efficiency. This was challenged by limited availability of recognized ZFs for a few codon and contextual impacts of a single ZFs within assembly (Xu & O'Brochta, 2015).

2.2 Transcription activator-like effector nucleases editing tool

TALE nucleases (TALENs) represent a potent, adaptable genetic engineering tool. Similar to ZFNs, TALENs comprise a set of customized proteins, each comprised of approximately 17 highly similar motifs, consisting of around 34 amino acids. Each of these motifs can competently associate to a single nucleotide and is connected to a single unit of nonspecific DNA restriction enzyme Fok I . Since each nucleotide ties to 43 amino acid motifs, one can tailor proteins which have potential to target nearly any genetic code within a genome.

Findings suggest TALENs is a preferable gene engineering method in *Bombyx mori* compared to ZFNs. As they can be tailored and constructed using a straightforward code to target specific DNA sequences. Additionally, it is utilized to simultaneously knockout more than two genes (Xu & O'Brochta, 2015).

Genome engineering of ku80 gene using TALEN:

Genetic engineering is a potential tool used to disrupt or modify desired genes in different organisms. Knockout efficiency of TALEN is high in contrast to editing using CRISPR-cas9 in *B.mori*. It forms a complex with Ku70 gene and binds to DNA ends and activates enzyme Kinase. Inactivating Ku80 could increase number of knock in events through homologous recombination. Incorporation of a donor vector into ku80 gene can be induced using TALEN mediated PITCH (Tsubota et al., 2017).

2.3 Gene engineering by utilizing CRISPR-cas9

The emerging cutting-edge technology for genetic engineering is clustered regularly interspersed short palindromic repeats (CRISPR)-associated 9 (Cas9). This tool is independent of tailoring site-specific transcription factors, unlike ZFN s and TALENs. It relies on a DNA nuclease that targets DNA sequences specified by a user-tailored gRNA. This makes it simpler, less time-consuming, and cost-effective. CRISPR utilizes RNA-DNA base pairing, which is

efficient and stable targeting mechanism than protein-DNA interaction employed by ZFNs and TALENs.

It is utilized to produce synonymous mutations, extensive chromosomal knockout mutations (approx. 3.5 kb), inversion mutations in a singular gene and to facilitate mutagenesis of maximum six genes concurrently in silkworm species *Bombyx mori*. Hence CRISPR/Cas9 is the potential tool for genome engineering (Xu & O'Brochta, 2015).

In 2013, first modification of *B. Mori* DNA sequences by utilizing CRISPR-CAS9 was documented (Wang et al., 2013). They focused on a fundamental gene BmBlos2, similar to human gene Blos2. They tailored two sgRNAs (23-bp) to create modifications, resulting in the inactivation of function of targeted gene. Each complex consisting of sgRNA and Cas9 restriction enzyme was injected in early embryonic stage before germinal disc formation (Baci et al., 2021).

This feature enables to induce mutations by gene editing at different target sites simultaneously. It was used to perform targeted mutation on insulin-like growth factor-like peptide (IGFLP), which has a crucial function in the development of genital disc. The lack of IGFLP causes reduced sized of ovaries and lesser egg count in contrast to its wild type. However, there was no impact on laid egg's size and its development indicating IGFLP hormone has no effect on fertility and ovarian development (Baci et al., 2021).

Researchers conducted a study to examine effects of BmFibH gene expression in *B. mori* embryonic cells. Deletion of BmFibH gene by sophisticated variant of cas9 nuclease (dcas9) causes significant changes like naked pupa or weak cocoons. Furthermore, deactivating this gene led to enhanced activities of other genes used in degradation processes, such as autophagy. The research also provides an understanding about the function of FibH protein in labial glands development.

To determine expression of silk genes from spider in *Bombyx Mori* to increase silk production, researchers effectively inserted spider silk gene in silkworm DNA sequence. This research provides a potential insight in utilizing CRISPR-Cas9 technology in to synthesize silk with improved mechanical characteristics on a large-scale production.

CRISPR-Cas9 technique was utilized to assess the role of miR-2 in mulberry silkworm. At initial stage, Gal4/UAS system was used to augment miR-2, which yielded deformities in adult appendages. In subsequent stage, CRISPR-Cas9 tool was utilized to delete two miR-2 targeted genes, BmFng and BmAwd which resulted in wing deformation. Both stages of research affirmed vital role of miR-2 in appendage development of silk moth (Baci et al., 2021).

Utility of CRISPR-Cas in Anti-BmNPV Treatment

In antiviral approaches, distinct versions of Cas9, Cas12, and Cas13 have shown positive results against BmNPV in *B. mori*. CRISPR-Cas has been applied in combating BmNPV by targeting genes engaged in replication and propagation of baculovirus such as immediate early-1 (ie-1) and me53. Furthermore, separate studies have utilized CRISPR-Cas9 to target other genes, including ie-0 and ie-2 (Baci et al., 2021).

To achieve simultaneous targeting of multiple genes in BmNPV genome, researchers created a transgenic line to target three genes in BmNPV genome ie-1, gp64, DNApoly. The transgenic line was hybridized with another transgenic line carrying FnCpf1 gene. This resulted in creation of an FnCpf1 x gNPVM binary hybrid expression system, which allowed for simultaneous expression of both genes. (Xuan et al., 2022)

New variants of CRISPR have been discovered, such as Cas12 (Cpf1), CasX, Cas13, Cas3, Cas14. These variants have different PAM requirements making them more efficient in gene engineering. Cpf1 is a newer type of CRISPR/Cas restriction enzyme, smaller than Cas9. It functions as an endoribonuclease to cleave DNA sequences and create double-strand breaks.

Unlike CRISPR/Cas9 system, Cpf1 processes crRNA (CRISPR RNA) and only needs a 23 bp crRNA scaffold to recognize its target, dispensing with the need for tracrRNA. Study showed that Cpf1 when targeted at same site as cas9, cpf1 can produce greater deletion of gene fragments than Cas9 thereby improving gene therapy in transgenic insects (Xuan et al., 2022). In an another research study, BmChit β-GlcNAcase gene was knocked out, facilitated by CRISPR- cas9 produced rough silk, indicating the role of this gene in determining silk fineness (Yu et al., 2023).

ReMOT control in *Bombyx mori* based on Cas9 gene engineering

In a study, researcher developed a new method called receptor mediated Transduction of Cargo (ReMOT) for gene editing in Sikworm. Instead of injecting gene editing tools into embryos, they injected them into female pupae. A peptide ligand (BmOTP) targeting oocytes was discovered, and Cas9 complex was injected in the haemocoel fluid of vitellogenin females. Gene engineering causes hereditary changes in silkworm pupae (Yu et al., 2023).

Individual injection is an easier and convenient method than embryo microinjection. It eradicates difficulty of dealing with tiny embryos and can facilitate genetic exploitation of other insects in lepidopteran order. ReMOT control tool has emerged as an alternative genetic engineering tool.

Modified ReMOT control tool exchanges ovary-targeting ligand from DmP2C to BmOTP. Original ReMOT control method used DmP2C, which was obtained from yolk protein of *D. melanogaster*, and has been proven effective for gene engineering in diverse insect species, including mosquitoes (Yu et al., 2023)

3. Challenges of genome editing techniques

These tools pose immediate challenges, especially in identifying variants without observable phenotypes. PCR and DNA sequencing tools are used to determine genotypes but comes with

limitations. However, modifying a dominant visible marker gene via HR could yield a variant lineage that is simpler to identify and sustain (Xu & O'Brochta, 2015).

Table 3: Genome editing techniques and targeted genes

Genome editing technology	**Knock in or knock out technology**	**Targeted Gene name**	**Gene function**	**Reference**
Zinc finger nucleases (ZFN)	Insertion causing mutation and knock out	BmBLOS2	Epidermal color marker gene	Takasu et al., 2010
Zinc finger nucleases (ZFN)	knock-out	Bmfib-H gene	Encodes silk protein	Ma et al., 2014
TALENs	Knock in PITCH system)	BmBLOS2		Nakade et al., 2014
TALENs	Knock in PITCH system)	Ku80	Plays role in non-homologous end joining NHEJ process	Tsubota et al., 2017
TALENs	insertion	Gene of sericin	Silk production	Li et al., 2022
TALENs	Knock in system	fibroin heavy chain (*Fib-H*) gene	Protein production	Takasu et al., 2023
Cas9-sgRNA system	Knock in or insertion	BmBLOS2	Synthesis of uric acid molecules in larval cuticle.	Wang et al., 2013
CRISPR -Cas9	Knock out	Yellow-y	Melanin pigment formation	Tomihara et al., 2022
CRISPR -Cas9	Knockout	BmCPG25	Epidermis (outer layer of integument)	Wang et al., 2022
ReMOT control CRISPR-Cas9 Receptor-Mediated Ovary Transduction of Cargo (ReMOT) control technique	Knock out	BmBLOS2 gene	Synthesis of uric acid molecules in larval cuticle.	Yu et al., 2023
CRISPR -Cas9	Knockout	BmHR38	Nuclear receptor gene which plays role in regulating metamorphosis through ecdysone signaling pathway	Xu et al., 2024

4. Silkworm genomics and molecular biology

Substantial achievements have been made in comprehending silkworm including study of demographic history, uncovering genome sequence, genome mapping, developing diverse bioinformatics techniques (25). However, naturally occurring species of bombyx genus, Theophila and Ocinara can be found in Islands of Andaman, the Himalayan region of India and China and in the south east Asian countries like Jawam Sumatram Borneo etc. It is hypothesized domesticated silkworm, *Bombyx mori L*., originated from *B. mandarina* in China and subsequently distributed worldwide. (Muniraju & Mundkur, 2018).

Silk moths are divided in 2 families—Bombycidae and Saturniidae. Saterniidae contains around 1861 species, with 162 genera, and is the largest family in Lepidopteran order. In family Saturniidae, limited number of species, comprising *Antheraea yamamai* (Japanese oak wild silk moth) can be used for silk production. The Evolutionary history of silkworm reveals that family Saturniidae shares ancestry with Bombycidae family which includes *Bombyx mori*. It is estimated *B. mori* diverted from *A. yamamai* around is 84 million years ago (Kim et al., 2017).

This study presents first genome sequence of *Antheraea yamamai*, generating a total of 147 gigabase pairs with 210-fold coverage. The compiled genome size of *A. yamamai* was found 656 megabases (Mb) and consists of 3675 scaffolds with an N50 length of 739 kilobases (Kb) and a 34.07 GC ratio. It is assessed that compiled genome assembly is 96.7% complete. Through utilization of three distinct gene prediction techniques and manual curation, a total of 15,481 genes were identified within *A. yamamai* genome. The unique characteristics of *A. yamamai* silk, called tensan silk, have facilitated its application in diverse research domains, including biotechnology and medical science (Kim et al., 2017).

4.1 New genome assembly of silkworm

The study presents a new genome evaluation of *B. mori* p50T variety aimed at improving accuracy and completeness of its genome information. The genome assembly released in 2008 by International Silkworm Genome Consortium, had limitations such as unsequenced chromosome, inaccurate assembled sequence, and errors in gene prediction, hindering its application in advanced technologies like silkworm breeding and biotechnology (Kawamoto et al., 2019).

New genome assembly utilized deep sequencing of short and long reads, resulting in an assembly size of 460.3 Mb with minimal lacunae. N50 values for scaffolds and contigs were 16.8 Mb and 12.2 Mb, respectively, indicating high contiguity. Also generated new high-quality gene models based on improved genome assembly, utilizing mRNA and protein sequence data (Kawamoto et al., 2019).

To enhance accuracy of silkworm genome, researchers employed PacBio long-read and Illumina short-read sequencing technologies, achieving about 80× coverage for PacBio reads and 60× coverage for Illumina reads. Lacunae/voids in assembly were filled by utilizing sequences from BAC and Fosmid replicas (Kawamoto et al., 2019).

The study conducted a comparative analysis of mapping coverage for RNA-sequence including piRNA-sequence reads across both updated and previous genome assemblies of *B. mori* to assess improvements achieved with new assembly. Eight different tissues from epidermis, early embryo, fat body, brain, mid gut, internal genitalia, central and anterior labial gland were obtained for RNA sequence reads (Kawamoto et al., 2019).

The findings indicate updated genome assembly has wider mapping coverage and includes transcriptionally active regions of RNA of *B.mori* genome (Kawamoto et al., 2019). piRNA reads are involved in silencing repeat sequences that originate from transposable elements

(TEs). The mapping of piRNA reads showed a wider coverage than old assembly. It revealed that there was a decrease in count of piRNA clumps in new sequence however, aggregate length, mean length, median length, and N50 of these clusters in new sequence has multiplied significantly. Additionally, sets of piRNA aggregates were identified in voids between scaffolds of old DNA sequence, demonstrating repeat regions are precisely sequenced in the updated *B. mori* sequence utilizing long-read sequencing. (Kawamoto et al., 2019).

4.2 Development of novel gene models

Lepidopteran insects, including insect pests, have developed resistance to insecticides. These resistant pests exhibit an increased abundance of detoxification-related genes, including cytochrome P450, GST, COE, and ABC transporter. Gene families associated with detoxification in resistant insect pests display highly duplicated gene clusters, contributing to their heightened resistance to insecticides. Assessment of gene duplication levels in insects of lepidopteran order related to insecticide tolerance is frequently conducted through synteny analysis with *B. mori* (Kawamoto et al., 2019).

Next Generation sequencing has been used to re- sequence more than hundred genetic varieties of silkworm. Technical constraints in earlier research may have led to omission of many trait-associated sites, with structural variants (SVs) remaining largely unexplored (Tong et al., 2022)

To gain insights into genomic content of species, researchers have started focusing on a pan-genome approach. This approach involves utilizing third-generation sequencing (TGS) technologies to construct high-resolution pan-genome datasets. However, team has deeply re-sequenced 1078 silkworm varieties and generated long-read sequencing to overcome these limitations (Tong et al., 2022).

4.3 Recognition of genetic markers associated with commercially significant traits

Selective breeding in silkworms has focused on commercially valuable traits such as production or silk fineness. Only a few causative genes and loci of economically significant traits have been identified. Exploiting comprehensive pan-genome, study examined genes and variations would be useful for reproducing select traits.

The quantity of silk produced is decided by number and endoreduplication of labial gland cells. During breeding process, a significant selection signal was found in BmE2F1 gene. BmE2F1 gene has four updates- related SVs, comprising a knockout and three knock in within its sequence motifs and noncoding DNA. The frequency of these four SVs is increased in improved breed of silkmoths. Knockout of BmE2F1 using CRISPR-cas9 technology decreases count of cells in labial glands by 7.68% and silk production by 22%. On the other hand, genetically modified amplification of BmE2F1 gene, surges count of cells in labial glands by 23% and silk production by 16%. Therefore, it can be indicated that BmE2F1 gene contributes its importance in determining number of labial glands cells, which, in turn, affects silk yield (Tong et al., 2022).

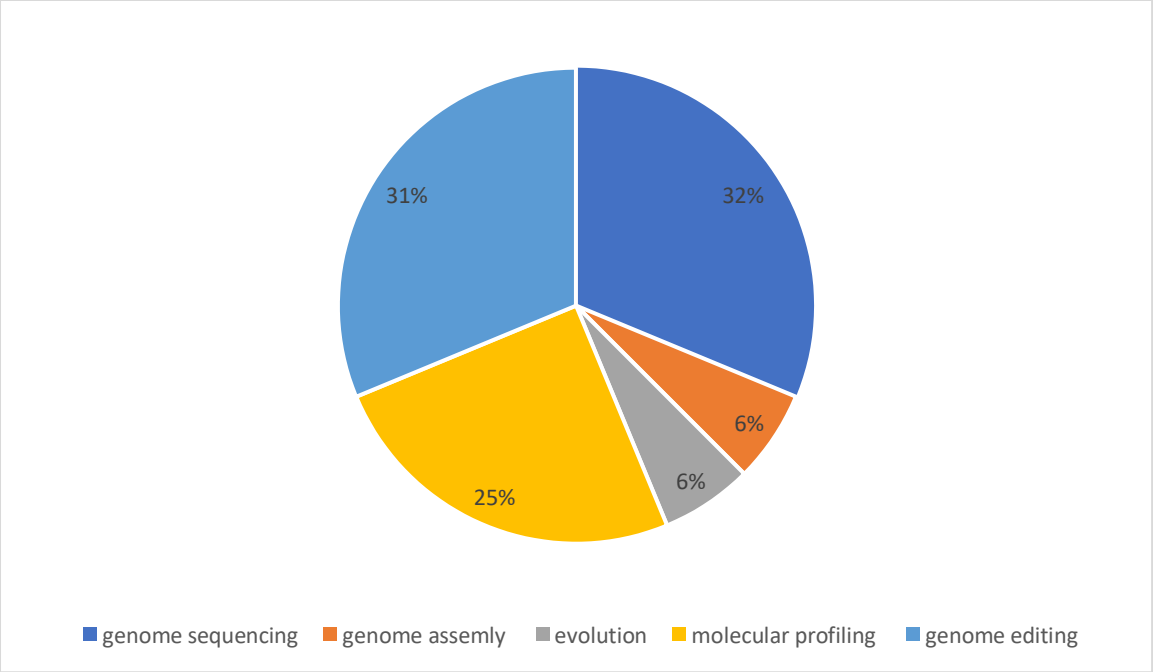
A seminal study was conducted to find out the gene responsible for fineness of silk. Researchers did RNA sequencing of labial glands in four breeds, two superior quality silk breeds (Suxiu, Chunfeng) and two rough silk quality breeds (Xiafang, Qiubai). It was found that BmChit β-GlcNAcase gene was expressed abundantly in fine silk quality silkworm breed. Knocking out of BmChit β-GlcNAcase gene, facilitated by CRISPR- cas9 produces rough silk, indicating the role of this gene in determining silk fineness. (Tong et al., 2022)

4.4 Silk production

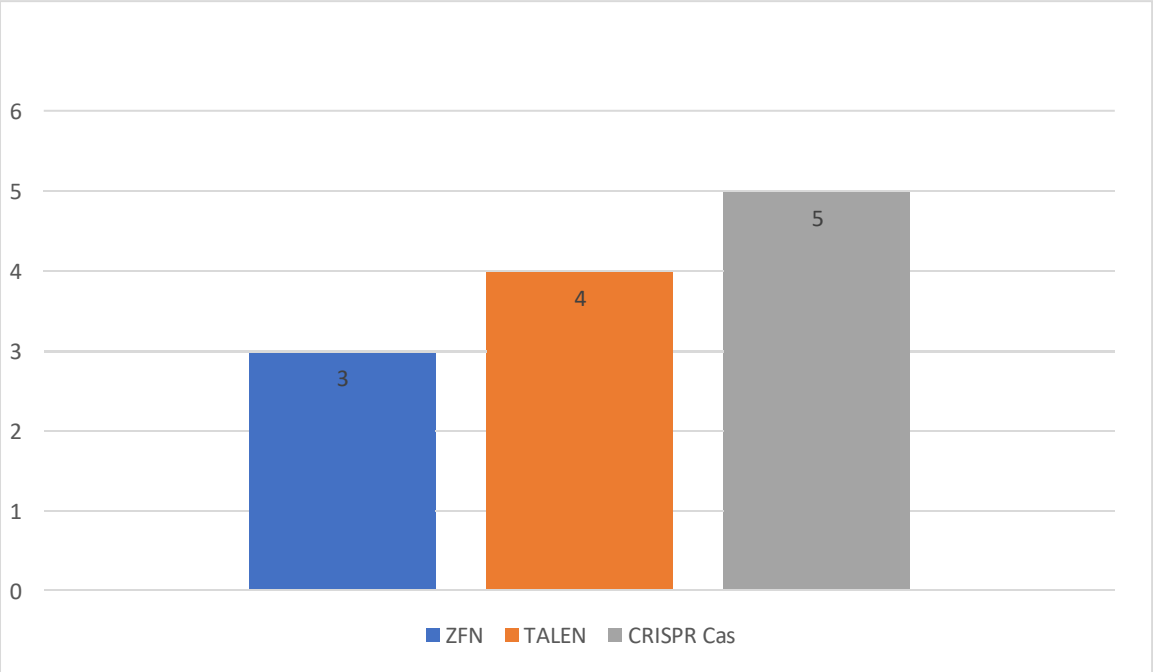
Silk comprises fibroin and sericin polypeptide polymers which are produced and discharged in abundance in hind and central region of labial glands throughout fifth larval stage. These

proteins traverse lumen into anterior labial gland and then extracted via spinnerets to produce silk fiber in cocoon.

Fibroin is composed of fibroin-H, fibroin-L, and fibrohexamerin of molar proportion 6–6–1. Fibroin-H is composed of three amino acids, Gly, Ala and Ser. Sericin is composed of Ser amino acid. Silk gland-specific tRNAs for glycine (tRNA-Gly1 and tRNA-Gly2), alanine (tRNA-Ala1), and serine (tRNA-Ser2 and tRNA-Ser4) play key roles in silk protein synthesis. Fibrohexamerin gene (Bmfhx) is expressed in hind labial glands for silk fibroin assembly. Ser1 located within a 2 Mb region on chromosome 11 is expressed in central and hind parts of middle silk gland (MSG), contributing to sericin synthesis. Silkworm tRNA genes for glycine, alanine, and serine are significantly higher compared to other amino acids, reflecting requirement for adapted tRNA synthesis during silk protein translation. Duplication events and shared promoters in tRNA gene clusters indicate an amplification strategy to meet high demand for specific tRNAs during silk protein synthesis (International Silkworm Genome, 2008).



Graph 1: Trends in study of silkworm in reviewed papers



Graph 2: Genome editing technologies reviewed

Discussion and conclusions

Molecular tools have provided a new way to comprehend biodiversity of nature. *B. mori* is a reference species in Lepidoptera, accuracy and precision of its genome information are crucial for the advancement of comparative genomics study and understanding of insect diversity. However, existing genome data is inadequate, with unsequenced chromosome regions, inaccurate sequence assembly, and errors in gene prediction.

Through mitochondrial genome sequencing, phylogeny of Bombycidae has been unveiled, highlighting recent divergence of domesticated silkworms from their Chinese wild counterparts. Looking ahead, exploration of silkworm–baculovirus dynamics provides future research endeavours, promising advancements in pest management, host immunity, and bioreactor optimization.

The isolation and sequencing of specific markers for different species have expanded our knowledge of silkworm biodiversity and offer avenues for leveraging identified alleles in future molecular breeding initiatives.

By delving into silkworm genomics, key genes and structural variations associated with economically significant traits can be identified. For example, BmE2F1 gene influences silk yield and BmChit β-GlcNAcase gene regulates silk fineness. Enhanced gene models and broader transcriptional coverage provides a robust foundation for research and applications in diverse fields, marking a significant advancement in understanding and utilizing silkworm genomics.

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