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COI gene-based identification and phylogenetic analysis of silkworm (*Bombyx mori*) from Changa Manga Forest, Pakistan

H. Alam*, M. Bilal*, N. Akhtar*, M. Yasmeen*, Q. A. Khan**, M. Hussain*

** University of Veterinary and Animal Sciences, Lahore, Pakistan*
*** Government College University, Lahore, Pakistan*

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Department of Wildlife and Ecology, University of Veterinary and Animal Sciences, Lahore, Pakistan.
Tel.: +92-303-487-34-40.
E-mail: 2022-phd-1065@uvas.edu.pk

Department of Zoology, Government College University, Lahore, Pakistan.
E-mail: qamarabbaskhan101@gmail.com

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The silkworm (*Bombyx mori*) is an important species in the sericulture industry, which contributes significantly to global silk production. However, identifying and distinguishing different strains of *B. mori* has been challenging, especially at the larval stages, where morphological similarities are prominent. This study aims to assess the genetic diversity and phylogenetic relationships of *B. mori* from the Changa Manga Forest, Punjab, Pakistan, by using mtDNA COI gene sequences. The DNA was extracted from thorax tissue and amplified using the COI gene's universal primers. The obtained DNA sequences were trimmed and analyzed using BioEdit. After trimming the ambiguous bases, the DNA sequences were submitted to Genbank and accession numbers were obtained. The phylogenetic analysis was performed through Neighbor-joining method using 100 bootstrap pseudo-replicates in MEGA X. The results showed that the *B. mori* strains present in Pakistan exhibited a high degree of genetic identity with Chinese strains. The mean genetic divergence of *B. mori* from *Bombyx mandarina* was found to be 0.016 ± 0.003. Furthermore, entropy plot analysis revealed both conserved and variable regions within the COI gene. The present study underscores the potential of the COI gene as a key marker for silkworm species identification and genetic characterization, contributing to the improvement of sericulture practices. The findings also have implications for biodiversity conservation and forensic applications in the sericulture industry. Future research with larger sample sizes across different regions will provide a more comprehensive understanding of the genetic diversity and strain characteristics of *B. mori*, which is crucial for enhancing silk production and sustainable sericulture practices.

Keywords: mtDNA; sericulture; Kasur; Pakistan; silk production; genetic diversity.

Introduction

Bombyx mori is a monophagous lepidopteran insect that exclusively feed on mulberry leaves. Silkworms play a significant role in the sericulture industry. The industry sector of sericulture comprises knitting, twisting, dyeing, printing and reeling (Wang et al., 2023). South and East Asia are the leading silk producing regions of the world as 92–94% of global production comes from China and India. South Korea, Japan, Vietnam, Thailand, Uzbekistan and Brazil produce the remaining 6–8% of silk (Sharma & Kapoor, 2020).

In Pakistan, sericulture was a thriving and profitable kind of entrepreneurship until the 1990s. The enormous potential of sericulture to absorb labor, particularly from women and young people, is what makes it significant. Overall, four types of silk have been reported so far and three varieties such as Eri, Tasar, and Muga silks included non-mulberry silk (Saikia et al., 2022). The cocoons of the mulberry silkworm (*B. mori*) which is raised in captivity, yield the most famous silk (Shahzadi et al. 2022). More than 3000 silkworm strains including those that produce different qualities and yields of silk are maintained worldwide. *B. mori* has played an essential role in the development of sericulture an industry that has been central to human civilization for thousands of years (Wang et al., 2023).

Beyond its economic significance, the silkworm has emerged as an important model organism for molecular and genetic studies. The domesticated insect *B. mori* has been the subject of extensive research, leading to significant advancements in our understanding of genetics, genomics, and molecular biology (Pavithra et al., 2024). Approximately, 180,000 lepidopteran species has been reported so far around the globe. The mtDNA based identification of *Bombyx mori* help in understanding its genetic structure, protein synthesis, gene regulation and the various mechanisms that control many physiological and developmental methods. However, the type of mulberry species affects the growth, development, immunity and survival rate of *B. mori* (Nguyen et al., 2024). The advancement in molecular biology helped us in understanding the genetic diversity of wild and domestic insects. mtDNA genes are mostly used to check the study genetic variation and phylogenetic relationships (Nagaraju, 2000).

The start of genomic technologies has transformed *B. mori* research. The complete sequencing of the *B. mori* genome has provided a comprehensive map of the genetic analysis. Mitochondrial DNA has become a central tool in animal phylogenetic studies due to its faster rate of evolution compared to nuclear DNA, leading to more rapid identification of closely related species (Mollah et al., 2024). In 1947, sericulture was first established in Taxila. Then it spread to forested areas where there were plenty of mulberry plantations (Pavithra et al., 2024). Changa Manga, Chichawatni, Daphor, Kamlia, Jouhrabad, Bhagat, Khanewal, and Kundian are some of these locations. *B. mori* is thought to be domesticated from the wild *B. mandarina*, about 5,000 to 10,000 years ago (Abdoli et al., 2022).

The COI gene is a standard marker in DNA barcoding because of its high mutation rate, which allows researchers to identify closely related species. The 16S rRNA gene is another mtDNA marker and it is highly conserved across species. Together, both genes offer a robust foundation for the molecular identification of *B. mori* and its strains (Alcudia-Catalma et al., 2021). Recently the entire genome for *B. mori* has been reported, which has provided a crucial molecular genetic resource for investigating a wide array of biological questions. Silkworm strains can be differentiated by various morphological and physiological traits, such as origin, voltinism, moltinism, and cocoon features. However, distinguishing *B. mori* strains can be challenging when the larval stages exhibit similar morphological features (Ashraf & Qamar, 2023). The circular, double-stranded DNA molecule that makes up the mitochondrial genome of metazoans is distinguished by its tiny size, rapid nucleotide mutation rate, and rare recombination. Moreover, compared to nuclear DNA, it is simpler to work with in laboratory settings (Kim et al., 1998). Molecular identification of *B. mori* holds significance far beyond taxonomy and phylogenetic analysis.

The genetic composition of silkworm strains directly impacts the quality and production of cocoons for silk. Many *B. mori* strains have been cross bred for better silk production, fiber quality, disease resistance and adaptability to different environmental conditions. mtDNA genes are used for exact species identification and strains of *B. mori*. These methods are helpful in making selective breeding programs more successful (Xia et al., 2014). mtDNA based identification of *B. mori* can be helpful in sericulture by confirming the availability of better quality *B. mori* strains (Meng et al., 2017). Sericulture has significant potential in small scale cottage industry and has substantial economic importance. The sustainability of sericulture is mostly vulnerable due to many factors such as *B. mori* strain, silkworm health, rearing conditions and environmental factors. The present study was therefore planned to identify different *B. mori* strains using mtDNA COI gene sequences.

Materials and methods

Changa Manga Forest, located in the district Kasur districts, Punjab, Pakistan has historically been a significant center for sericulture due to its extensive mulberry plantations. Established in 1866, the forest was initially planted with *Morus alba* (white mulberry) to support the silk industry by providing a sustainable source of silkworm feed. Over time, the forest expanded to include hybrid mulberry varieties, enhancing the availability and quality of mulberry leaves for silkworm cultivation. The forest's infrastructure facilitated the development of sericulture, with local farmers and communities engaging in silkworm rearing for silk production. However, in recent decades, the industry has faced challenges due to factors such as high silkworm mortality rates, low-quality mulberry trees, and competition from imported silk. Despite these setbacks, Changa Manga remains a notable example of integrating forestry and agriculture, offering valuable insights into sustainable practices and the potential for revitalizing sericulture in the region.

A total of 5 specimens of *B. mori* were collected from the sericulture unit of Changa Manga Forest. Each specimen was tagged with a specific voucher number. Specimens were euthanized and preserved in 75% ethanol for molecular characterization. The samples were brought to the Postgraduate Lab, Department of Wildlife and Ecology, UVAS, Ravi Campus for further analysis (Wang et al., 2023).

Total genomic DNA was extracted from thorax tissue of silkworm using salt extraction and Phenol Chloroform method (Ali et al., 2024). The quality of DNA samples was checked through gel electrophoresis using 1.2% agarose gel (Fig. 1) and quantified using Nanodrop one (Table 1).

DNA was amplified using primers set mentioned in Table 2. PCR amplification was done in 25 µL reaction mixture. 7 µL double distilled water, 1 µL forward primer (25 mM), 1 µL reverse primer (25 mM), 12 µL PCR master mix and 4 µL of DNA template were mixed in a 0.2 mL PCR tube. The following steps were performed for the amplification, 4 minutes of denaturing at 93 ºC followed by 35 cycles for 35 seconds at 93 ºC, primer annealing for 30 seconds at 48–56 ºC and elongation for 1 minute at 72 ºC and final 10 minutes at 72 ºC and infinity hold at 4 ºC (Ali et al., 2024). PCR products were checked on 1.2% agarose gels. All the DNA samples were Sanger sequenced in both directions using 3730XL DNA Analyzer from Korea.

The obtained DNA sequences were checked and trimmed in Bioedit 7.8. The newly obtained DNA sequence were aligned in Clustal X2 (Ali et al., 2024). The DNA sequences were subjected to BLAST analysis at NCBI. The closely related DNA sequences from the GenBank were downloaded and used in the Neighbor-joining (NJ) tree analysis using 100 bootstrap replicates in MEGA X. Genetic variations within and between silkworms' strains were calculated using p-distance in MEGA X (Ali et al., 2020).

Table 1
Quantification of DNA samples using NanoDrop One

Voucher specimens	Nucleic acid, ng/µL	A260/A280	A260/A230
BMUVAS101	410.04	1.643	0.602
BMUVAS102	478.50	1.618	0.934
BMUVAS103	368.15	1.366	0.534

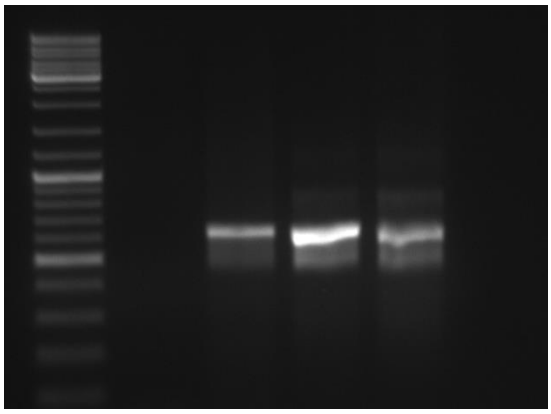

Fig. 1. Successfully extracted DNA samples of silkworm

Table 2
List of primers used during the present study

Gene	Primer	Primer sequence	Product size	References
COI	Univ1-F	5'-GTTGTAAAACGACGGCCA-3'	710–1280 bp	Simon et al. (1994), Niehuis et al. (2006)
	Univ1-R	5'-CAGGAAACAGCTATGA-3'		
	Univ2-F	5'-GGTTCGAATCCCTCTCTCTC-3'		
	Univ2-R	5'-GGTCGTGACCAAGAAACCAC-3'		
	Slkg-F	5'-TCCGGTCAAACATGAGGAAA-3'		
	Slkg-R	5'-CTGAGGTCATCCACTTCTTCG-3'		
	TY-J-1460	5'-TACAATTTATCGCCTAAACTTCAGCC-3'		
	Cl-N-2191	5'-CCCGGTAAAATTAAAATATAAACTTC-3'		

Results

The present one-year study was conducted at the sericulture unit, Changa Manga Forest and Department of Wildlife and Ecology, University of Veterinary and Animal Sciences, Ravi Campus from January–December 2024.

Silkworm eggs were incubated at 25 °C and 75–80% humidity for 10 days to hatch. Newly hatched larvae were shifted from Petri plates to trays and chopped mulberry leaves were provided for the silkworm larvae (Fig. 2). Three specimens of silkworms were collected and preserved in 75% ethanol for molecular characterization. All the preserved specimens were tagged with specific numbers.

The COI gene of all the three specimens was successfully amplified during the present study (Fig. 3). The obtained DNA sequences were first processed to ensure high quality DNA reads for further analysis using BioEdit. DNA sequences with low quality and ambiguous bases were excluded from further analysis. Any sequence with ambiguous or low-quality peaks, especially in the terminal regions or within homopolymer stretches, was excluded from the analysis. Table 3 summarizes the successfully amplified DNA and their GenBank accession numbers. After trimming ambiguous bases, the obtained COI fragments of *B. mori* were 810 bp. The COI fragments aligned with DNA sequences retrieved from GenBank comprised 790 bp.

Fig. 2. Silkworm (*Bombyx mori*) specimens during experimental period

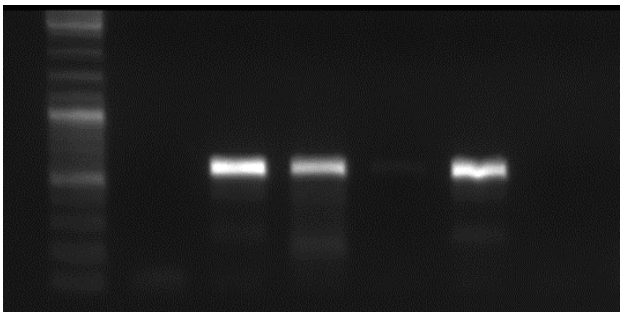

Fig. 3. Gel electrophoresis showing the PCR amplification of COI gene

Table 3
List of successfully amplified DNA of *Bombyx mori* and the GenBank accession numbers of voucher specimens

Family	Species	Voucher number	GenBank Accession Number
Bombycidae	*Bombyx mori*	BMUVAS101	PX097185.1
		BMUVAS102	PX097184.1
		BMUVAS103	PX097186.1

Table 4 summarizes the DNA sequence identity matrix of silkworm (*B. mori*) based on p-distance. The newly obtained DNA sequences of silkworm (*B. mori*) were subjected to BLAST analysis at NCBI. All the DNA sequences have shown clear species identification and related DNA sequences of *B. mori*'s COI gene were downloaded for further analysis. The overall, mean genetic divergence of *B. mori* with *B. mandarina* was 0.016 ± 0.003 calculated using MEGA X.

Phylogenetic analysis was conducted using the Neighbor-Joining (NJ) method based on p-distance using MEGA X. The phylogenetic tree constructed from the COI gene sequences indicated that the DNA of the silkworms used during the present study showed a high degree of genetic identity to Chinese strains. Figure 4 shows the Neighbor-Joining (NJ) tree of *B. mori* with closely related wild species.

The variability of the COI gene sequences of different silkworm samples was subjected to entropy plot analysis. Entropy plot analysis is critical for identifying different populations or studying evolutionary patterns in the silkworm strains. The entropy plot was generated from the aligned DNA sequences using Bioedit software. The entropy plot is a graphical representation of DNA sequence variability at each nucleotide position in a multiple DNA sequences alignment. The entropy plot value at each position redirects the variability at that particular nucleotide site across the aligned DNA sequences. High entropy values indicate greater sequence diversity and polymorphisms at a specific position, whereas lower entropy values means conserved positions with minimum variation. The entropy plot (Fig. 5) reveals regions of both high and low entropy along the COI gene sequence. The x-axis corresponds to the nucleotide positions in the alignment, ranging from position 1 to the final nucleotide position 790. The y-axis represents the entropy values with higher values indicating greater variability.

The sharp, tall red bars between positions 580 and 700, and again near the end of the sequence around position 740 to 790, indicate regions of high sequence variability, suggesting potential polymorphisms or mutations. The variable positions were successfully distinguishes different populations of silkworms.

The most of the region in COI gene sequences showed low entropy values. These positions show low variability and are represented by short bars indicating highly conserved regions in the aligned sequences. These conserved regions suggested that the COI can be used as stable markers for species differentiations. The conserved regions are typically less prone to mutations, which makes them reliable for molecular identification. The entropy plot analysis confirms that the COI gene in silkworm (*B. mori*) contains conserved and variable regions. The COI gene sequences can be used in molecular studies such as species identification, genetic diversity within populations and phylogenetic analysis.

Table 4
The DNA sequence identity matrix of silkworm (*Bombyx mori*) with closely related species based on p-distance

	Sequences	Japanese wild silk moth, *Bombyx mandarina*						*Bombyx mori* from Changa Manga			Chinese wild silkworm *Bombyx mandarina*		
		AB 737935.1	AB 737902.1	AB 737942.1	AB 737943.1	AB 737939.1	AB 737944.1	PX 097185.1	PX 097186.1	PX 097184.1	GQ 423241.1	GQ 423239.1	GQ 423244.1
Japanese wild silk moth, *Bombyx mandarina*	AB 737935.1	ID	–	–	–	–	–	–	–	–	–	–	–
	AB 737902.1	0.997	ID	–	–	–	–	–	–	–	–	–	–
	AB 737942.1	0.997	0.997	ID	–	–	–	–	–	–	–	–	–
	AB 737943.1	0.997	0.997	0.997	ID	–	–	–	–	–	–	–	–
	AB 737939.1	0.995	0.995	0.995	0.995	ID	–	–	–	–	–	–	–
	AB 737944.1	0.995	0.995	0.995	0.995	0.994	ID	–	–	–	–	–	–
Bombyx mori from Changa Manga	PX 097185.1	0.791	0.791	0.791	0.792	0.792	0.792	ID	–	–	–	–	–
	PX 097186.1	0.791	0.791	0.791	0.792	0.792	0.792	1	ID	–	–	–	–
	PX 097184.1	0.791	0.791	0.791	0.792	0.792	0.792	1	1	ID	–	–	–
wild silk-worm *Bombyx mandarina*	GQ 423241.1	0.792	0.792	0.792	0.793	0.793	0.793	0.991	0.991	0.991	ID	–	–
	GQ 423239.1	0.791	0.791	0.791	0.792	0.792	0.792	0.99	0.99	0.99	0.998	ID	–
	GQ 423244.1	0.793	0.793	0.793	0.794	0.794	0.794	0.993	0.993	0.993	0.998	0.997	ID

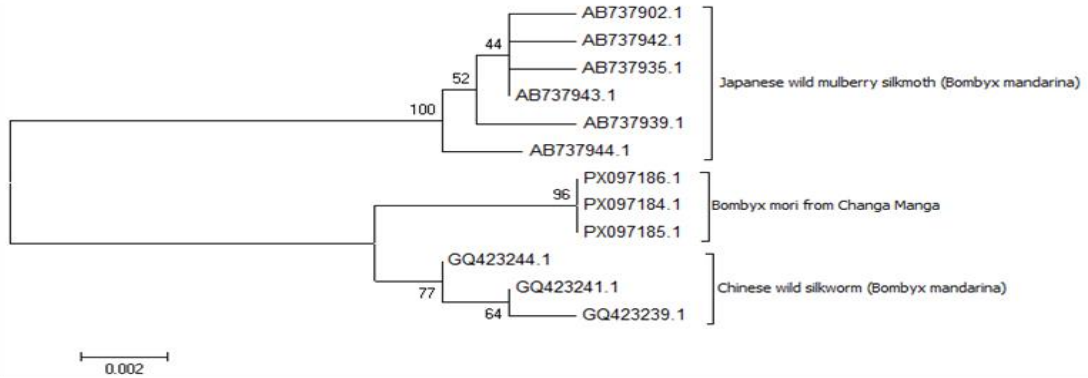

Fig. 4. NJ tree using COI gene sequence of silkworms (*Bombyx mori*) based on p-distance

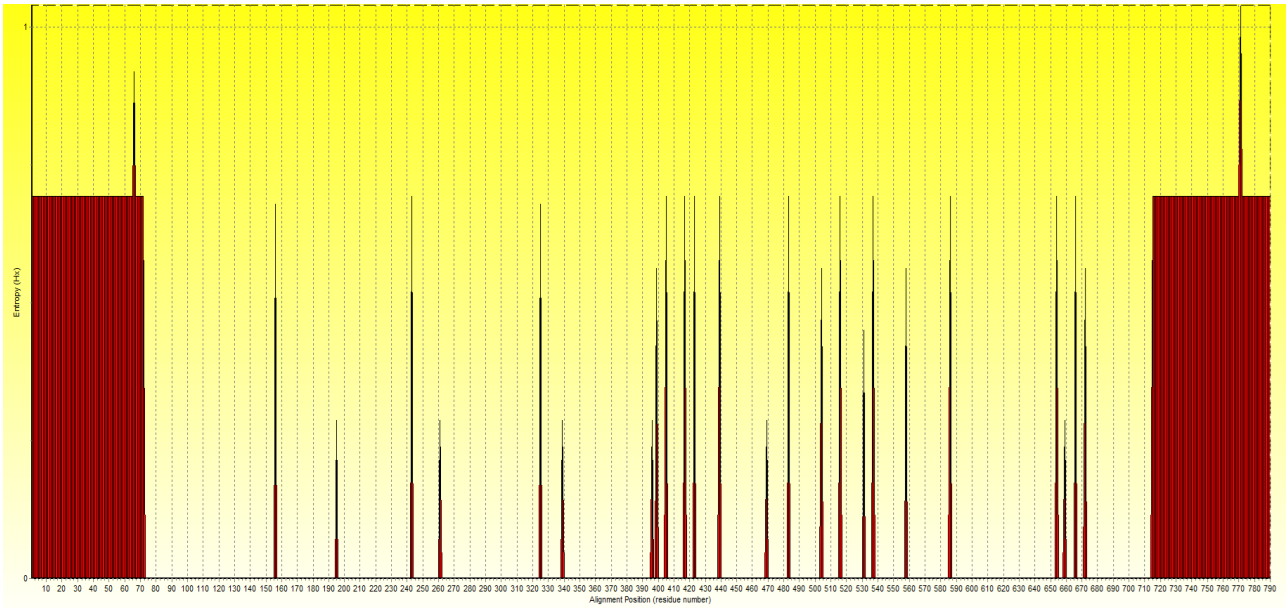

Fig. 5. Entropy plot of the COI gene sequences alignment from silkworm (*Bombyx mori*)

Discussion

Approximately 1.5 million species of a total estimated 5–100 million species existing on the globe have been described (Li et al., 2018). The molecular identification of *B. mori* strains has become a vital area of research as it is critical for sericulture. Recent studies have shown that researchers used various molecular techniques such as mitochondrial DNA and nuclear DNA markers to report the genetic variations between *B. mori* strains. One of the most diverse orders of insects is the order Lepidoptera, which includes butterflies and moths. Worldwide, 174,250 moth species have been reported so far, belonging to 126 families. Environmental factors can significantly influence the physical characteristics of many *B. mori* strains and making their identification challenging (Park et al., 2022). Moreover, solely relying on physical traits is not sufficient to identify cryptic species. The primary objective of the present study was to assess the genetic variation within *Bombyx mori* populations using the COI gene sequences. COI is a widely used marker for species identification and phylogenetic relationships (Kim et al., 2020).

During the present study, the DNA extraction was done through salt extraction method and phenol-chloroform technique. Both these techniques have been applied in studies of *B. mori*. Both the DNA extraction methods yielded high quality of DNA which is better for PCR amplification (Zhang et al., 2022). The quality of the DNA was

verified using gel electrophoresis where distinct bands with little to no degradation were observed. The findings of the current study are in line with (Park et al., 2022), who used salt extraction DNA methods and yielded high quality DNA for PCR amplification. The amplified DNA ranged in size from 710 to 720 bp. These findings were further confirmed by the studies of Kim et al. (2019) and Arunkumar et al. (2006), who reported same DNA band size for COI gene.

Silkworm is a monophagous insect that intensively feeds on mulberry leaves and plays a dominant role in the sericulture industry. Growing food plants, raising silkworms, and producing silk is known as sericulture. This is an agro-based industry. The two sectors of sericulture are agriculture and industry. Growing food plants for silkworms and raising them to lay eggs and cocoons are both part of the farming industry. The industry sector includes knitting, twisting, dyeing, printing, finishing, and reeling (Sharma & Kapoor, 2020).

In many nations where labor is in excess, manufacturing costs are low, and people are open to embracing new technologies, the silk industry has the potential to significantly boost GDP (Rain et al., 2019). For better sericulture practices, it is therefore crucial to choose and utilize suitable silkworm strains based on economic features and rearing performance in various climatic circumstances. After Nigeria, Ethiopia is the second most populous nation in Africa. There is a nationwide trend towards rising unemployment rates. The sericulture industry provides a greater business to the country's people (Saikia et al., 2019).

One of the main goals of this study was to assess the genetic variation of *B. mori* populations using the COI gene. The genetic variation observed in this study (0.023 ± 0.013) is within the expected range for intraspecific variation in silkworms. This level of genetic divergence is consistent with previous reports in the literature, such as Huang et al. (2006), who also found similar levels of genetic variation within silkworm populations across different regions in China. This study's genetic divergence value suggests that the silkworm samples used here are part of a relatively homogenous population, with minimal genetic differentiation, which could be attributed to the relatively controlled breeding and domestication of *B. mori* for sericulture purposes.

Phylogenetic analysis, performed using the Neighbor-Joining (NJ) and Maximum Likelihood (ML) methods, revealed a high degree of genetic identity between the silkworms from this study and Chinese strains (Kim et al., 2019). The NJ and ML trees (Fig. 4.4 and 4.5) show that the silkworm samples used in this study cluster closely with those from Chinese strains, suggesting that the silkworm population under study shares a common ancestry with the strains found in China. These findings are consistent with those of Li et al. (2019), who reported a similar genetic relationship between silkworm populations from China and neighboring regions. Additionally, studies by Li et al. (2005) and Zhang et al. (2022) have demonstrated the genetic closeness of silkworm populations from different parts of Asia, including China, Korea, and Japan, further corroborating the results of the current study.

The high genetic identity of silkworm populations observed in this study may also be explained by the widespread use of selective breeding programs in the sericulture industry, which could have reduced the overall genetic diversity of domesticated silkworm strains. This phenomenon has been discussed in several studies, including those by Hu et al. (2010), who observed a decrease in genetic diversity among domesticated silkworms due to selective breeding practices (Li et al., 2005). Entropy plot analysis was used to assess the variability and conservation of the COI gene sequences across the silkworm samples. The entropy plot revealed both high and low entropy regions, providing valuable insights into the genetic structure of the silkworm population (Zhang et al., 2022). High entropy was observed at positions between 580 and 700 bp, indicating significant variability across the sequences at these positions. This variability suggests the presence of polymorphisms or mutations in these regions, which could be useful for distinguishing between different genetic variants within the silkworm population. Similar findings were reported by Li et al. (2016), who also observed high entropy in specific regions of the COI gene, which were proposed as potential markers for studying genetic differentiation in silkworm populations. On the other hand, low entropy was observed in the regions between positions 10 and 570 bp, indicating that these regions are highly conserved across the silkworm population. The conserved nature of these regions suggests that they are functionally important and may serve as stable markers for species identification. Studies by Hu et al. (2010) and Zhang et al. (2022) have also highlighted the importance of conserved regions in the COI gene for accurate species identification in silkworms and other species. The presence of both highly variable and highly conserved regions within the COI gene further underscores the utility of this gene as a molecular marker for both species identification and genetic diversity studies.

Recently many researchers have used mtDNA genes, especially the COI gene to examine the genetic variations and phylogenetic relationships of *B. mori*. Zhang et al. (2022) used COI gene sequences to evaluate the genetic diversity of *B. mori* strains in China. Likewise, Kim et al. (2022) used COI gene sequences to study the genetic diversity of *B. mori* from China and Japan. The data revealed that both countries has genetic variations and have conserved regions of COI gene. These studies and our study concluded that the COI gene can be used as an effective molecular marker in species identification and to check the genetic diversity (Vimala et al., 2019). Additionally, the phylogenetic relationships using Neighbor Joining and Maximum likelihood methods are mostly used around the globe. Liu et al., (2013) made phylogenetic relationship trees of *B. mori* using COI gene sequences.

Furthermore, the identification and phylogenetic relationship of animals has used many DNA markers such as ND1, ND2 16S rRNA and mtDNA COI. The combination of nuclear and mitochondrial genes has also been used by others. However, there is availability of DNA barcodes but the accessibility of short sequences for the DNA based identification is deficient (Nneji et al., 2020). The primers set used by Ashraf & Qamar (2023) showed the same results for amplifying DNA sequences of species and made it potentially useful for providing shorter DNA sequences. The size of the sequence used in study was about 29 bp shorter than the 658-bp standard DNA barcode. On basis of the short DNA barcode sequence, there were three major groups from the phylogenetic relationship.

Conclusion

The current research gives valuable information regarding genetic diversity and phylogenetic relationship of domesticated silkworms (*B. mori*). The findings revealed that the mtDNA genes such as COI gene can be used as molecular markers for species identification and genetic variations. The high percentage of genetic identity between silkworm populations from different regions suggests that there is limited genetic differentiation. This could be due to the domestication and selective breeding of silkworms. Overall, the present study highlights the importance of the COI gene for its potential use in biodiversity conservation, breeding programs and forensic applications in sericulture. The identification of both variable and conserved regions within the COI gene provides further prospects for studying genetic diversity and developing new molecular markers for silkworms. Future studies with large sample sizes across various regions will provide more accurate information regarding different silkworm strains currently being used in sericulture.

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