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Genetic Divergence and Voltinism's Expression in Silkworm: Revealed through DNA Barcoding

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ABSTRACT

The phylogenetic relationships between bivoltine and polyvoltine silkworm B. mori strains were examined sequencing~520-bp of mitochondrial gene cytochrome c oxidase I (mtCOI). Multiple alignments of the sequences ascertained the probability of Transition/Transversion substitutions. The percentage of A+T content was higher than that of C+G region. Maximum likelihood (ML) analysis clustered four strains into two major groups and accurately segregated them according to their voltinism inheritance. The open reading frames of the gene were found exhibited the strain-specificity indicating their geographical origin. The protein coding sequence identified which commenced with 4-bp TTAG putative initiator codon. The outcome suggested the mtCOI gene has not only crucial task in learning the evolutionary evidences but it can also be characterized as a bar-coding marker for revealing voltinism's expression and genetic variation in the silkworm, discriminating their genotype based on COI gene nucleotide versatilities. The study was fruitful as we noticed prominent intra-specific versatility among B. mori species, indicating presence of allele/s regulating diapause development-linked to COI gene. We conclude with an insight into potential utility of DNA bar-coding technique for ecological classification and taxonomic investigation of insect's biodiversity.

Key words: *DNA bar-coding, Mitochondrial cytochrome c oxidase I gene, voltinism's expression, genetic variation*

INTRODUCTION

The silkworm (*B. mori*) is of great economic value from an industrial perspective. Knowing its genetic structure may provide improve the conservation of commercial lines[1,2]. The silkworm's genotypes are largely categorized to bivoltine (egg-diapause) breeds completes two generations per year and are adjusted to temperate conditions with high yield production and polyvolitine (non-diapause) breeds complete five to six generations per year and are adapted to tropical climatic conditions with low yield[3]. The genetic structures of these populations are under constant pressure from varying geographic conditions that induce and hence it is essential to develop a DNA marker system to study genetic diversity among varieties of the silkworm[4, 5].

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DNA markers are used to provide raw information, based on which an ecologist make estimates of gene flow between species [6]. Recent work has created a new taxonomic system, founded on *mtCOI*, called "DNA bar-coding" referring to the technique of sequencing a short fragment of the gene[7, 8]. This method has been applied excellently in lepidopteran insects from different geographic regions for the genetic biodiversity analysis and species authentication[9,10,11]. The *mtCOI* gene can aid the resolution of diversity and discrimination of closely allied species of lepidopterans [12] and it is used widely as a marker, due to it has high inter-species nucleotide divergence than intra-species nucleotide divergence[13]. DNA bar-coding offers taxonomists the opportunity to increase the scale and success of biodiversity science[14]. For instance,[15] characterized the nucleotide sequence variation in *mtCOI* gene among *B. mori* strains from Japanese, Chinese, and European. DNA barcoding techniques will be increasingly used by ecologists[16]. The study of insect ecology is also important to understand their evolution, and genetic diversification[17].

The present study deals with the utility of DNA bar-coding through *COI* sequencing of bivoltine and multivoltine *B. mori* strains based on their genome fingerprints to compare their voltinism's expression pattern and address their evolutionary issues, therefore an attempt was made to probe the genetic versatility and phylogenetic relationships of these commercially important insects at the intra-species level. The other intention was to identify and present the genetic marker to improve the marker-assisted selection for future breeding programmes, and to distinguish their geographical origin enhancing their inherent characters, thus leading to the production of better quality yield.

MATERIALS AND METHODS

Silkworm Strains collection

Four disease-free laying of the two bivoltine silkworm namely, CSR_2, CSR_4, and two polyvoltine namely, PM (Pure Mysore) and C. nichi originating from different locations were obtained from Germplasm Bank, Department of Studies in Sericulture Science, Mysuru. The fresh cocoons were stored frozen at -80°C. Pure Mysore is unique in that it takes more than 28 days to complete its larval life. The isolate PM and C. nichi (polyvoltine breeds) reared five to six times per year show non-diapausing eggs, originated from tropical countries, unlike CSR_2 and CSR_4 (bivoltine breeds) produce diapausing eggs that complete two generations per year and are adjusted to temperate conditions, however they show susceptibility to the pathogens during silkworm rearing (Table 1).

DNA Extraction, PCR amplification and sequencing

Genomic DNA was isolated from silkmoth samples as standard protocol [18]. The primers sequences used for amplification of ~520-bp fragment of *COI* were forward 5′-TGATCAAATTTATAATAC-3′ and reverse 5′-GTAAAATTAAAATATAAAC-3′ [19, 20]. PCR was carried out in 50 µl of reaction mixture. The PCR schedule was 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 47°C for 45 s, 72°C for 2 min and a final extension of 10 min at 72°C. The ~ 520-bp DNA fragment PCR products were checked by electrophoresis on a 1.0% agarose gel, and purified using the Nucleo-pore PCR Clean-Up Gel Extraction Kit (Genetix, Molecular Devices, Hampshire). Sequencing of *COI* PCR product (50 ng) was carried out after PCR elution of pure DNA from the gel with forward and reverse primers. The cycling conditions were: 96 °C for 1 min, followed by 25 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4min. Samples were precipitated and washed with 70% ethanol to clean up the reactions. DNA sequencing was performed following the dideoxynucleotide chain termination method [21], using an automated ABI 3730 sequencer. Products were labeled using the Big Dye Terminator V.3.1 Cycle Sequencing Kit (Applied Biosystems, Inc).

Sequence alignment and Phylogenetic analysis

The *COI* sequence analysis was done for molecular authentication of species of *B. mori*.

The DNA sequences were aligned using ClustalW (Fig. 1) and phylogenetic analysis performed using MEGA 5.2 [22]. The consensus sequences were blasted in National Center for Biotechnology Information (NCBI) for the nearest similar sequence matches [23] and submitted to GenBank. The open reading frames (ORF) were identified using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The *mtCOI* genes and C+G, A+ T-rich regions were found by aligning the nucleotide sequences with homologous regions of full-length insect mitochondrial sequences using ClustalW. Bootstrap values were included to test the reliability of inferred trees [24] and all codon positions were included. Pairwise genetic distances were calculated using the Kimura two-parameter model.

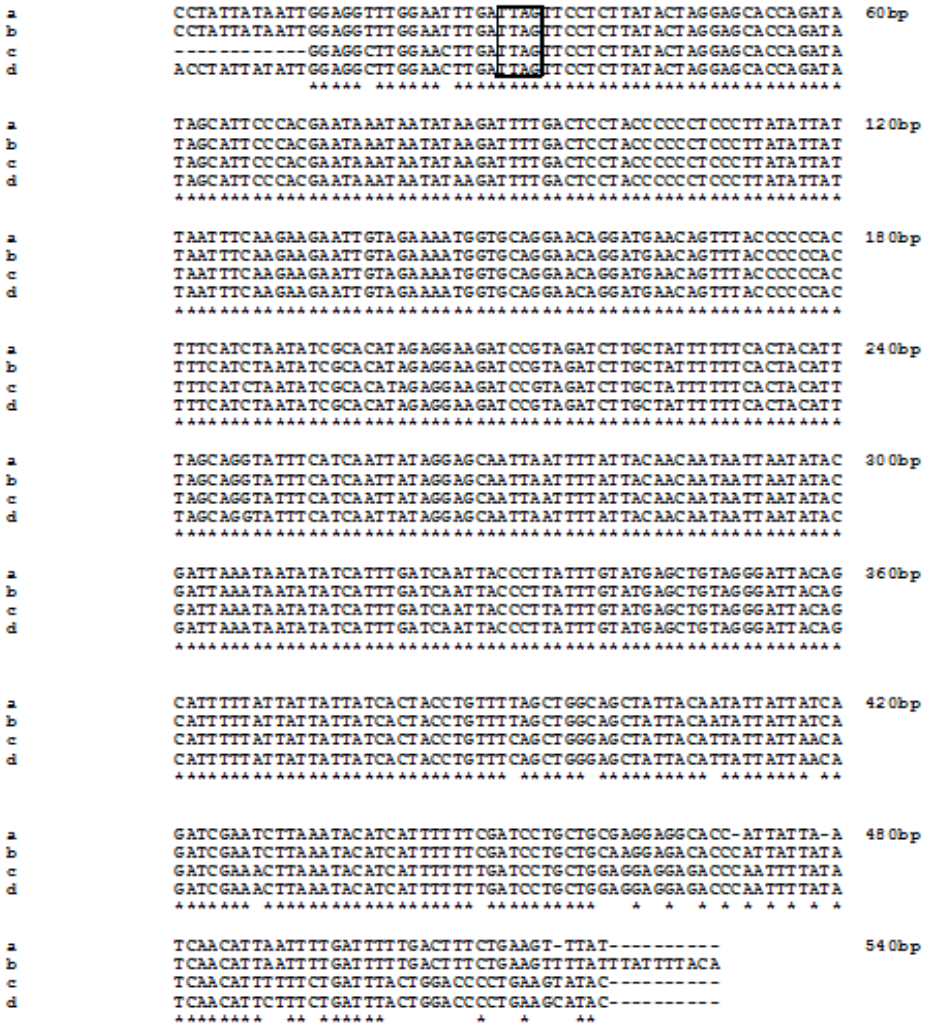

Fig. 1: Comparison of the nucleotide by multiple alignment of 520-bp 3′end fragment of *COI* gene sequences. Asterisks (*) indicates nucleotide common to all species, and (-) indicates lack of sequence information; C. nichi (a), Pure Mysore (b), CSR_4 (c) and CSR_2 (d).

RESULTS

Nucleotide sequence analysis

By sequencing the PCR products, the four 5´ - end sequences of *COI* (520 bp) were obtained. The sequences of *COI* gene have been submitted to GenBank and their accession numbers are listed in Table 1. All of these 4 sequences were analyzed for sequence divergence and phylogenetic relationships. The 5´ - end sequences of *COI* commenced with 4-bp TTAG initiator codon, and the percentage of A+T content was higher than

that of G+C (Table 1). Multiple alignments of the sequences revealed the probability of Transition/Transversion substitution rates under the[25]. We detected the highest rate of transitional substitution occurred at G-A, and the lowest was at T-C (Table 2). The NCBI database showed a homology of 99% with the *B. mori* species sequenced. In addition, the sequence comparison showed the intra-species nucleotide divergences of *B. mori* species, and the average of genetic distances were 0.037. The distance between silkworm species ranged from 0.002 to 0.058. The strains CSR_2 and CSR_4 showed lowest genetic distance of 0.002 whereas highest distance of 0.058 was observed between C. nichi and CSR_4 species (Table 3).

Coding sequence divergence

The sequence alignments revealed the open reading frames (ORF) of *mtCOI* gene in four breeds of domesticated silkworm. In polyvoltine species the coding sequence of C. nichi strain starting with "CCTATTATAA" shows the ORF1 was found on the direct strand extends from base 83 to 190 and PM strain starting "CCTATTATAA" the first ORF elongates from base 82 to 189, but in bivoltine strains the coding sequence of CSR_2 strain commence "ACCTATTATA" with the ORF1 from base 352 to 483, and CSR_4 strain begins with "GGAGGATTGG" and the ORF1 extends from base 382 to 498 (Fig. 2). The concatenated amino acid fragment of the *COI* gene from the *mt*DNA were aligned with MEGA 5.2 to compare the sequences as the amino acid data sets commonly provide better supports in phylogenetic relationships. Furthermore, A+T contents are highly represented in all three codon positions of each *COI* sequence. No ORF was found in reading frame 3 of PM strain and in reading frame 1 of C. nichi strain. The outcome also shows bivoltine species exhibited slightly higher expression of C+G ratio in the protein-coding gene compared to polyvoltine strains of *B. mori* (Table 4).

a)
ORF1 in reading frame 2 extends from base 83 to 190:
YKILTPTPLPYIINFKKNCRKWCRNRMNSLPPTFI*
ORF2 in reading frame 2 extends from base 323 to 418:
SITLICMSCRDYSIFIIIITTCFSWELLQYY*
ORF1 in reading frame 3 extends from base 165 to 344:
TVYPPLSSNIAHRGRSVDLAIFSLHLAGISSIIGAINFITTIINIRLNNISFDQLPLFV*
ORF2 in reading frame 3 extends from base 345 to 449:
AVGITAFLLLLSLPVLAGSYYNIINRSKLKYIIF*
b)
ORF1 in reading frame 1 extends from base 82 to 189:
YKILTPTPLPYIINFKKNCRKWCRNRMNSLPPTFI*
ORF2 in reading frame 1 extends from base 322 to 489:
SITLICMSCRDYSIFIIIITTCFSWSYYNIINRSNLNTSFFDPAEEETHFISTLF*
ORF1 in reading frame 2 extends from base 164 to 343:
TVYPPLSSNIAHRGRSVDLAIFSLHLAGISSIIGAINFITTIINIRLNNISFDQLPLFV*
c)
ORF1 in reading frame 1 extends from base 382 to 498:
LELLHIINRSKLKYIIFLILLEEETQFYINIYFDFLDS*
ORF1 in reading frame 2 extends from base 71 to 178:
YKILTPTPLPYIINFKKNCRKWCRNRMNSLPPTFI*
ORF2 in reading frame 2 extends from base 311 to 436:
SITLICMSCRDYSIFIIIITTCFSWSYYILLTDRNLNTSFF*
ORF1 in reading frame 3 extends from base 153 to 332:
TVYPPLSSNIAHRGRSVDLAIFSLHLAGISSIIGAINFITTIINIRLNNISFDQLPLFV*
d)
ORF1 in reading frame 1 extends from base 352 to 483:
GLQHFYYYYHYLFSWSYYNIITDRNLNTSFFDPAGGGDPILYQH*
ORF1 in reading frame 2 extends from base 83 to 190:
YKILTPTPLPYIINFKKNCRKWCRNRMNSLPPTFI*
ORF2 in reading frame 2 extends from base 323 to 430:
SITLICMSCRDYSIFIIIITTCLAGAITILLQIET*
ORF1 in reading frame 3 on the direct strand extends from base 165 to 344:
TVYPPLSSNIAHRGRSVDLAIFSLHLAGISSIIGAINFITTIINIRLNNISFDQLPLFV*

Fig. 2: Coding sequences of the *mtCOI* showing the ORFs found on the direct strandin four strains of silkworm *B. mori*; C. nichi (a), Pure Mysore (b), CSR_4 (c) and CSR_2 (d).

Phylogenetic relationships

The maximum likelihood (ML) tree consisted of two major groups based on mitochondrial *COI* gene sequences of 4 strains of the *B. mori* and accurately segregated the silkworm strains according to their voltinism's expression. The dendrogram made monophyletic clades of single species and suggested that out of four isolates PM, C. nichi and CSR_2, CSR_4 are closely related since they belong to same voltinism. Isolate PM along with cluster C. nichi forms a main cluster and isolates CSR_2 along with cluster CSR_4 forms another main cluster indicating close phylogenetic relationship and early evolutionary relevance from a common ancestor (Fig. 3). Furthermore, the transition/transversion rate ratios were calculated between four species of *B. mori* for the estimation of the pattern of nucleotide substitution. This difference allows easy species discrimination based on *COI* sequence data, however the null hypothesis of equal evolutionary rate throughout the tree was not rejected at a 5% significance level (P = 0.104) and hence there was no real differences between four silkworms at congeneric intra-species level.

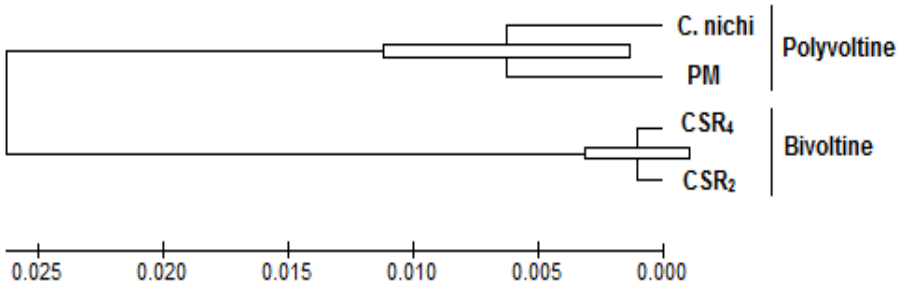

Fig. 3: Maximum Likelihood tree based on *COI* segment sequences illustrating the phylogenetic relationships among four *B. mori*, strains.

Table 1: Brief information on the strains of *B. mori*

Species name	Origin	Voltinism	GenBank accession No.	A+T %	C+G %
PM	Indo-China	Multivoltine	GQ423230.1	69.35	30.65
C. nichi	Japanese	Multivoltine	AB649188.1	68.78	31.22
CSR_2	CSR & TI, Mysore, India	Bivoltine	GQ423217.1	68.04	31.96
CSR_4	CSR & TI, Mysore, India	Bivoltine	AB649184.1	68.09	31.91

Table 2: Maximum likelihood estimate of substitution rates matrix

	A	T	C	G
A	-	8.68	4.28	**10.42**
T	8.00	-	**5.88**	3.73
C	8.00	**11.93**	-	3.73
G	**22.39**	8.68	4.28	-

Note: Rates of different transitional substitutions are shown in bold and those of transversionsal substitutionsare shown in italics.

Table 3: Kimura two parameter distance (K2P) genetic distance (below diagonal) and standard error (above diagonal) of the genetic distance using *mtCOI* gene

Name of strain	*C. nichi*	PM	CSR_4	CSR_2
C. nichi	-	0.005	0.014	0.013
PM	0.013	-	0.012	0.012
CSR_4	0.058	0.045	-	0.002
CSR_2	0.056	0.047	0.002	-

Table 4: Codon usage in the protein-coding gene

Species	First position				Second position				Third position			
	T	C	A	G	T	C	A	G	T	C	A	G
C. nichi	41	21.6	20.9	14.7	38	11.5	44.7	4.8	28	16.6	33.2	20.8
PM	40.8	21.2	21	14.8	39	11.4	43.4	5.3	28	16.7	33.1	20.6
CSR_4	43	22.6	19.3	15.8	35	12.9	45.3	6.5	30	17.4	34.7	21.1
CSR_2	43	22.7	19.3	16.3	35	13.7	45.2	6.5	30	17.6	34.5	21.3

DISCUSSION

B. mori Genetic variation and ecological origin

The silkworm *B. mori* probably originates from *mandarina* silkworm with many ecological types including monovoltinism, bivoltinism and multivoltinism[26]. Preserving high level of heritable variation helps to retain a population's current reproductive fitness and its capacity to adapt to global environmental change over the long term[27]. Thus, understanding and preserving biodiversity is one of the most important global challenges that biologists are facing[28]. DNA sequences allow for the identification of genetic diversity and unusual patterns of genetic variability[29]. The difference between intra-species and congeneric interspecies levels of genetic diversity is an indicator of the barcoding value of a gene sequence for species discrimination[30]. An accurate analysis with a bar-coding marker like highly conserved *COI* sequence can offer better taxonomic insights among lepidopteran insects as well as closely related species in order to conserve their precious genome resources. The outcome of study was fruitful as we observed considerable intra-specific nucleotide and amino acid variations among *B. mori* species with different origin. On the other hand, there was certain intra-species nucleotides sequence divergence of *COI* gene between bivoltine and polyvoltine silkworms which discriminated and classified *B. mori* species based on distinct expression pattern and the genetic diversity. The present forward and reverse *COI* primers demonstrated the phylogenetic relationships among different ecotypes and it would also be suitable to examine the regulation of diapause mechanism in insect population. Besides, this research has ascertained the strain-specificity in the *B. mori*, indicating their genetic differentiation and geographical origin.

Protein-coding genes

The initiation codon of *COI* translation in *Bombyx* species was ambiguous, but may have occurred by the 4-bp putative initiation codon TTAG, as previously reported[31]. The *COI* gene also starts at a CGA codon for arginine, as found in other lepidopteran insects[32, 33]. The non-canonical putative sites has been designated as the *COI* start codon in silkmoth

and discussed extensively in lepidopteran[34]. In this investigation the canonical ATN initiator of this gene was absent in the start regions but it commenced with tetranucleotide, TTAG start codon as the initial site of transcription (Fig. 1). The *COI* sequence analysis showed intra-specific versatility within congeneric species. Because most coding genes are highly conserved, they have been strongly used to estimate phylogenetic relationships at higher taxonomic levels. In addition, the sequence alignments revealed the open reading frames (ORF) of *mtCOI* gene in four breeds of domesticated silkworm which separated the bivoltine and polyvoltine species based on coding sequences and reading frames of gene. The first ORF of *COI* gene extended from base 82 to 189 codes protein which could be responsible in multivoltine races and the ORF1elongated from base 352 to 498 codes protein that is most probably activated in bivoltine strains. However, no ORF was found in reading frame 3 of PM strain which may be due to its particular Indo-Chinese origin, but there was presence of ORF in third reading frame of other *B. mori* strains that code the same protein. There wasn't any ORF in reading frame 1 of C. nichi strain originating from Japan either.

CONCLUSION

Development of DNA barcoding techniques have extremely advanced our conception of genetic makeup of insects and molecular sequencing based techniques like *COI* coupled with PCR have become devices of choice for mapping genetic divergence between or within related species, population genetics. We have presented for the first time the utility of DNA bar-coding through *COI* sequencing for the ecological classification of silkworm species based on their genome fingerprints and voltinism's expression. Likewise, the genetic differences may provide applicable data for improvement of high yielding, disease resistant silkworm strains by hybridization of selected bivoltine and polyvoltine parents to assemble the desirable inherited traits such as productivity and temperature tolerance at an early stage of the breeding programme with genetic recombination techniques as well as construction of suitable construction of a single nucleotide polymorphism linkage map for positional cloning in silkworm[35].

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