

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

Maryam Talebi Haghighi\* and T.S. Jagadeesh Kumar

Department of Studies in Sericulture Science, Department of Studies in Biotechnology

University of Mysore, Manasagangothri, Mysore, India

\*Corresponding Author E-mail: [haghighi.1987@gmail.com](mailto:haghighi.1987@gmail.com)

Received: 10.08.2016 | Revised: 16.08.2016 | Accepted: 19.08.2016

### 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<sup>1,2</sup>. 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 polyvoltine (non-diapause)

breeds complete five to six generations per year and are adapted to tropical climatic conditions with low yield<sup>3</sup>. 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<sup>4,5</sup>.

**Cite this article:** Haghighi, M.T. and Kumar, T.S.J., Genetic Divergence and Voltinism's Expression in Silkworm: Revealed through DNA Barcoding, *Int. J. Pure App. Biosci.* 4(4): 255-263 (2016). doi: <http://dx.doi.org/10.18782/2320-7051.2352>

DNA markers are used to provide raw information, based on which an ecologist make estimates of gene flow between species<sup>6</sup>. 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<sup>7, 8</sup>. This method has been applied excellently in lepidopteran insects from different geographic regions for the genetic biodiversity analysis and species authentication<sup>9,10,11</sup>. The *mtCOI* gene can aid the resolution of diversity and discrimination of closely allied species of lepidopterans<sup>12</sup> and it is used widely as a marker, due to it has high inter-species nucleotide divergence than intra-species nucleotide divergence<sup>13</sup>. DNA bar-coding offers taxonomists the opportunity to increase the scale and success of biodiversity science<sup>14</sup>. For instance,<sup>15</sup> 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<sup>16</sup>. The study of insect ecology is also important to understand their evolution, and genetic diversification<sup>17</sup>.

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<sub>2</sub>, CSR<sub>4</sub>, and two polyvoltine namely, PM (Pure Mysore) and *C. nichii* 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. nichii* (polyvoltine breeds) reared five to six times per year show non-diapausing eggs, originated from tropical countries, unlike CSR<sub>2</sub> and CSR<sub>4</sub> (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 silkworm samples as standard protocol<sup>18</sup>. The primers sequences used for amplification of ~520-bp fragment of *COI* were forward 5'-TGATCAAATTTATAATAC-3' and reverse 5'-GTAAAATTTAAAATATAAAC-3'<sup>19, 20</sup>. 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<sup>21</sup>, 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<sup>22</sup>. The consensus sequences were blasted in National Center for Biotechnology Information (NCBI) for the nearest similar sequence matches<sup>23</sup> 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<sup>24</sup> and all codon positions were included. Pairwise genetic distances were calculated using the Kimura two-parameter model.

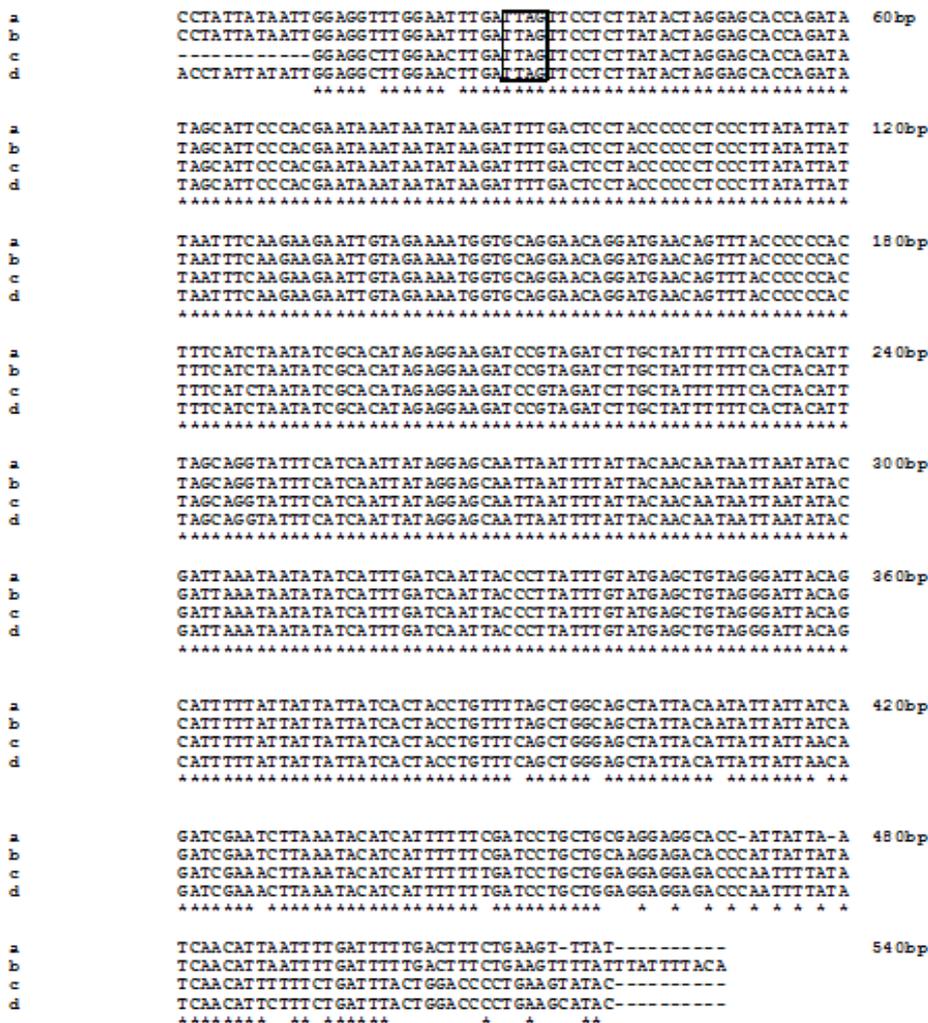


Fig. 1: Comparison of the nucleotide by multiple alignment of 520-bp 3' end fragment of *COI* gene sequences. Asterisks (\*) indicates nucleotide common to all species, and (-) indicates lack of sequence information; C. nichii (a), Pure Mysore (b), CSR<sub>4</sub> (c) and CSR<sub>2</sub> (d).

**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<sup>25</sup>. 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<sub>2</sub> and CSR<sub>4</sub> showed lowest genetic distance of 0.002 whereas highest distance of 0.058 was observed between *C. nichii* and CSR<sub>4</sub> 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. nichii* 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<sub>2</sub> strain commence “ACCTATTATA” with the ORF1 from base 352 to 483, and CSR<sub>4</sub> 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 *mtDNA* 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. nichii* 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).

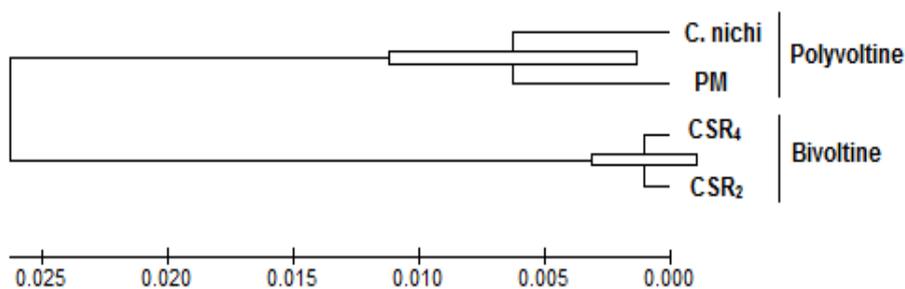
**a)**  
 ORF1 in reading frame 2 extends from base 83 to 190:  
 YKIL TPTPLPYIINFKKNCRKW CRNRMNSLPPTFI\*  
 ORF2 in reading frame 2 extends from base 323 to 418:  
 SITLICMS CRDYSIFIIHITTCFSWELLQYY\*  
 ORF1 in reading frame 3 extends from base 165 to 344:  
 TVYPPLS SNIHRGRSVDLAIFSLHL AGIS SIIGAINFITTIINIRLNNISFDQL PL FV\*  
 ORF2 in reading frame 3 extends from base 345 to 449:  
 AVGITAFLLLLSLPVL AGSY YNIINRS KL KYIIF\*  
**b)**  
 ORF1 in reading frame 1 extends from base 82 to 189:  
 YKIL TPTPLPYIINFKKNCRKW CRNRMNSLPPTFI\*  
 ORF2 in reading frame 1 extends from base 322 to 489:  
 SITLICMS CRDYSIFIIHITTCFSWS Y YNIINR.SNLNTSFFDPAE EETHFISTLF\*  
 ORF1 in reading frame 2 extends from base 164 to 343:  
 TVYPPLS SNIHRGRSVDLAIFSLHL AGIS SIIGAINFITTIINIRLNNISFDQL PL FV\*  
**c)**  
 ORF1 in reading frame 1 extends from base 382 to 498:  
 LELLHIINRS KLKYIIFL IL LEE ETQFYINIFYDFLDS\*  
 ORF1 in reading frame 2 extends from base 71 to 178:  
 YKIL TPTPLPYIINFKKNCRKW CRNRMNSLPPTFI\*  
 ORF2 in reading frame 2 extends from base 311 to 436:  
 SITLICMS CRDYSIFIIHITTCFSWS Y Y ILL TDRNLNTSFF\*  
 ORF1 in reading frame 3 extends from base 153 to 332:  
 TVYPPLS SNIHRGRSVDLAIFSLHL AGIS SIIGAINFITTIINIRLNNISFDQL PL FV\*  
**d)**  
 ORF1 in reading frame 1 extends from base 352 to 483:  
 GLQHFY Y YHYLFS WS Y YNIITDRNLNTSFFDPAGGGDPILYQH\*  
 ORF1 in reading frame 2 extends from base 83 to 190:  
 YKIL TPTPLPYIINFKKNCRKW CRNRMNSLPPTFI\*  
 ORF2 in reading frame 2 extends from base 323 to 430:  
 SITLICMS CRDYSIFIIHITTCLAGAITILLQIET\*  
 ORF1 in reading frame 3 on the direct strand extends from base 165 to 344:  
 TVYPPLS SNIHRGRSVDLAIFSLHL AGIS SIIGAINFITTIINIRLNNISFDQL PL FV\*

Fig. 2: Coding sequences of the *mtCOI* showing the ORFs found on the direct strand in four strains of silkworm *B. mori*; *C. nichii* (a), Pure Mysore (b), CSR<sub>4</sub> (c) and CSR<sub>2</sub> (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*<sub>2</sub>, *CSR*<sub>4</sub> are closely related since they belong to same voltinism. Isolate PM along with cluster *C. nichi* forms a main cluster and isolates *CSR*<sub>2</sub> along with cluster *CSR*<sub>4</sub> 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
<i>C. nichi</i>	Japanese	Multivoltine	AB649188.1	68.78	31.22
<i>CSR</i> <sub>2</sub>	CSR & TI, Mysore, India	Bivoltine	GQ423217.1	68.04	31.96
<i>CSR</i> <sub>4</sub>	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	<b>10.42</b>
T	8.00	-	<b>5.88</b>	3.73
C	8.00	<b>11.93</b>	-	3.73
G	<b>22.39</b>	8.68	4.28	-

Note: Rates of different transitional substitutions are shown in bold and those of transversional substitutions are 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	<i>C. nichi</i>	PM	CSR <sub>4</sub>	CSR <sub>2</sub>
<i>C. nichi</i>	-	0.005	0.014	0.013
PM	0.013	-	0.012	0.012
CSR <sub>4</sub>	0.058	0.045	-	0.002
CSR <sub>2</sub>	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
<i>C. nichi</i>	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 <sub>4</sub>	43	22.6	19.3	15.8	35	12.9	45.3	6.5	30	17.4	34.7	21.1
CSR <sub>2</sub>	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<sup>26</sup>. 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<sup>27</sup>. Thus, understanding and preserving biodiversity is one of the most important global challenges that biologists are facing<sup>28</sup>. DNA sequences allow for the identification of genetic diversity and unusual patterns of genetic variability<sup>29</sup>. 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<sup>30</sup>. 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<sup>31</sup>. The *COI* gene also starts at a CGA codon for arginine, as found in other lepidopteran insects<sup>32, 33</sup>. The non-canonical putative sites has been designated as the *COI* start codon in silkmoth

and discussed extensively in lepidopteran<sup>34</sup>. 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 ORF1 elongated 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. nichii* 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<sup>35</sup>.

### Acknowledgements

The authors are thankful to Chairman, Department of Studies in Sericulture Science, University of Mysore, Manasagangothri for providing all possible facilities and support during my work carried out.

### REFERENCES

1. Kim, K. Y., Kang, P. D., Lee, K. G., Oh, H. K., Kim, M. J., Kim, K. H. and Kim, I., Microsatellite analysis of the silkworm strains (*Bombyx mori*): high variability and potential markers for strain identification, *Genes & Genomics.*, **32**: 532-543 (2010).
2. Furdui, E. M., Mărghitaş, L. A., Dezmirean, D. S., Paşca, I., Pop, I. F., Erler, S. and Schlüns, E. A., Genetic characterization of *Bombyx mori* (Lepidoptera: bombycidae) breeding and hybrid lines with different geographic origins, *J. Insect Sci.*, **14**: 211 (2014).
3. Awasthi, A. K., Kar, P. K., Srivastava, P. P., Rawat, N., Vijayan, K., Pradeep, A. R. and Urs, S. R., Molecular evaluation of bivoltine, polyvoltine and mutant silkworm (*Bombyx mori* L.) with RAPD, ISSR and RFLP-STS markers, *Indian J. Biotechnol.*, **7**: 188-194 (2008).
4. Pradeep, A. R., Awasthi, A. K., Singh, C. K., Anuradha, H. J., Rao, C. G. P. and Vijayaprakash, N. B. Genetic evaluation of eri silkworm *Samia cynthia ricini*: ISSR loci specific to high and low altitude regimes and quantitative attributes, *J. Appl Genet.*, **52**: 345-353 (2011).
5. Chatterjee, S. N. and Tanushree, T., Molecular profiling of silkworm biodiversity in India: an overview. *Russ J. Genet.*, **40**: 1339-1347 (2004).

6. Behura, S. K., Molecular marker systems in insects: current trends and future avenues. *Mol Ecol*, **15**: 3087-3113 (2006).
7. Hebert, P. D., Ratnasingham, S. and de Waard, J. R., Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society of London B: Biol Sci*, **270**: S96-S99 (2003).
8. Wilson, J. J., DNA barcodes for insects. *DNA barcodes: methods and protocols*, 17-46 (2012).
9. Gaikwad, S. S., Ghate, H. V., Ghaskadbi, S. S., Patole, M. S. and Shouche, Y. S., DNA barcoding of nymphalid butterflies (Nymphalidae: Lepidoptera) from Western Ghats of India, *Mol Biol Rep*, **39**: 2375-2383 (2012).
10. Liu, Q. N., Chai, X. Y., Bian, D. D., Ge, B. M., Zhou, C. L. and Tang, B. P., The complete mitochondrial genome of fall armyworm *Spodoptera frugiperda* (Lepidoptera: Noctuidae). *Genes & Genomics*, **38**: 205-216 (2016).
11. Jalali, S. K., Ojha, R. and Venkatesan, T., DNA Barcoding for Identification of Agriculturally Important Insects. In *New Horizons in Insect Science: Towards Sustainable Pest Management*, (Springer India) 13-23 (2015).
12. Ramírez-Ríos, V., Franco-Sierra, N. D., Alvarez, J. C., Saldamando-Benjumea, C. I. and Villanueva-Mejía, D. F., Mitochondrial genome characterization of *Tecia solanivora* (Lepidoptera: Gelechiidae) and its phylogenetic relationship with other lepidopteran insects, *Gene*, **581**: 107-116 (2016).
13. Hebert, P. D., Cywinska, A. and Ball, S. L., Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London B: Biol Sci*, **270**: 313-321 (2003).
14. Hebert P D, & Gregory T R, The promise of DNA barcoding for taxonomy. *Syst Biol*, **54**: 852-859 (2005).
15. Yukuhiro, K., Sezutsu, H., Tamura, T., Kosegawa, E. and Kiuchi, M., Nucleotide sequence variation in mitochondrial *COI* gene among 147 silkworm (*Bombyx mori*) strains from Japanese, Chinese, European and moltinism classes, *Genes & genetic systems*, **86**: 315-323 (2011).
16. Valentini, A., Pompanon, F. and Taberlet, P., DNA barcoding for ecologists. *Trends in Ecology & Evolution*, **24**: 110-117 (2009).
17. Speight, M. R., Hunter, M. D. and Watt, A. D., *Ecology of insects: concepts and applications*. Blackwell Science Ltd, (1999).
18. Kar, P. K., Vijayan, K., Mohandas, T. P., Nair, C. V., Saratchandra, B. and Thangavelu, K. Genetic variability and genetic structure of wild and semi-domestic populations of tasar silkworm (*Antheraea mylitta*) ecorace Daba as revealed through ISSR markers, *Genetica*, **125**: 173-183 (2005).
19. Hwang, J. S., Lee, J. S., Goo, T. W., Yun, E. Y., Sohn, H. R., Kim, H. R. and Kwon, O. Y., Molecular genetic relationships between Bombycidae and Saturniidae based on the mitochondria DNA encoding of large and small rRNA. *Genetic analysis: biomolecular engineering*, **15**: 223-228 (1999).
20. Hwang, J. S., Lee, J. S., Goo, T. W., Kang, H. A., Sohn, H. R., Kim, H. R. and Kwon, O. Y., The comparative molecular study between Bombycidae and Saturniidae based on mtDNA RFLP and cytochrome oxidase I gene sequences: Implication for molecular evolution, *Zeitschrift für Naturforschung*, **C54**: 587-594 (1999).
21. Sanger, F., Nicklen, S. and Coulson, A. R., DNA sequencing with chain-terminating inhibitors. *Proc Nat Acad Sci*, **74**: 5463-5467 (1977).
22. Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S., MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods, *Mol Biol Evol*, **28**: 2731-2739 (2011).
23. Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J., Basic

- local alignment search tool. *J Mol Biol*, **215**: 403-410 (1990).
24. Felsenstein, J., Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, **39**: 783-791 (1985).
25. Tamura, K. and Nei, M., Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees, *Mol Biol Evol*, **10**: 512-526 (1993).
26. Lu, C., Yu, H. S. and Xiang, Z. H., Molecular systematic studies on Chinese mandarina silkworm (*Bombyx mandarina* M.) and domestic silkworm (*Bombyx mori* L.). *Agricultural Sciences in China*, **1**: 349-358 (2002).
27. Van Dyke, F., (Genetic Diversity–Understanding Conservation at Genetic Levels. *Conservation Biology: Foundations, Concepts, Applications*, 153-184 (2008).
28. Vijayan, K., Anuradha, H. J., Nair, C. V., Pradeep, A. R., Awasthi, A. K., Saratchandra, B. and Urs, S. R., Genetic diversity and differentiation among populations of the Indian eri silkworm, *Samia cynthia ricini*, revealed by ISSR markers, *J. Ins Sci.*, **6**: 30 (2006).
29. Meier, R., Shiyang, K., Vaidya, G. and Ng, P. K., DNA barcoding and taxonomy in Diptera: a tale of high intraspecific variability and low identification success, *Syst Biol*, **55**: 715-728 (2006).
30. Singh, M., Lakra, W. S., Bahuguna, S. N. and Kartavtsev, Y. P., Cytochrome b gene sequence divergence of seven sisorid species of catfish genus *Glyptothorax* (*siluriformes, sisoridae*) from India, *Mol Biol Rep*, **39**: 4275-4282 (2012).
31. Yukuhiro, K., Sezutsu, H., Itoh, M., Shimizu, K. and Banno, Y., Significant levels of sequence divergence and gene rearrangements have occurred between the mitochondrial genomes of the wild mulberry silkworm, *Bombyx mandarina*, and its close relative, the domesticated silkworm, *Bombyx mori*. *Mol Biol Evol.*, **19**: 1385-1389 (2002).
32. Kim, S. R., Kim, M. I, Hong, M. Y., Kim, K. Y., Kang P. D., Hwang, J. S. and Kim, I., The complete mitogenome sequence of the Japanese oak silkworm, *Antheraea yamamai* (Lepidoptera: Saturniidae), *Mol Biol Rep*, **36**: 1871-1880 (2009).
33. Liu, Q. N., Zhu, B. J., Dai, L. S., Wei, G. Q. and Liu, C. L., The complete mitochondrial genome of the wild silkworm moth, *Actias selene*, *Gene*, **505**: 291-299 (2012).
34. Hong, M. Y., Lee, E. M., Jo, Y. H., Park, H. C., Kim, S. R., Hwang, J. S. and Kim, I., Complete nucleotide sequence and organization of the mitogenome of the silk moth *Caligula boisduvalii* (Lepidoptera: Saturniidae) and comparison with other lepidopteran insects, *Gene*, **413** (2008) 49-57.
35. Maryam, T. H. and Kumar, T. S. J., Genetic divergence and allelic-specificity in relation to expression of voltinism in silkworm using ISSR and RAPD fingerprinting, *Russ J Genet*, **52**: 11-12 (2016).