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The Characteristics of Cytochrome C Oxidase Gene Subunit I in Wild Silkmoth *Cricula trifenestrata* Helfer and Its Evaluation for Species Marker

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ABSTRAK

Penelitian bertujuan untuk mengkarakterisasi dan mendeteksi situs diagnostik dari parsial gen sitokrom C oksidase sub unit I (COI) ulat sutera liar *Cricula trifenestrata,* dan mengevaluasi gen tersebut sebagai penanda spesies. Sebanyak 15 larva *C. tifenestrata* dikoleksi dari Kabupaten Bogor, Purwakarta, dan Bantul. DNA genom diekstrak dari kelenjar sutera larva, kemudian diperbanyak dengan metode PCR dan disekuensi. Hasil sekuensi dikarakterisasi runutan nukleotida dan diprediksi kandungan asam aminonya. Hasilnya menunjukkan bahwa 595 runutan nukleotida pada ujung 5′ gen COI *C. tifenestrata* bersifat kekal pada level spesies, tetapi beragam pada level *family*. Nukleotida didominasi oleh basa timin dan adenin (± 70%). Terdapat 25 situs diagnostik bagi *C. tifenestrata* dan 4 situk diagnostik pada level genus. Seratus delapan puluh sembilan (189) asam amino yang disejajarkan, menunjukkan satu persen variasi antar spesies. Asam amino ke-107 (valin) dan asam amino ke-138 (treonin) merupakan asam amino diagnostik bagi *C. tifenestrata.* Berdasarkan runutan nukleotida dan asam aminonya, filogeni kekerabatan menunjukkan bahwa *C. tifenestrata* terdapat pada nodus yang sama dengan spesies *Antheraea,* sehingga *family* Saturniidae bersifat monofiletik.

Kata kunci: ulat sutera liar, C. trifenestrata, sitokrom oksidase sub unit I, nukleotida diagnostik, asam amino diagnostik

ABSTRACT

The study was conducted to assess the characteristics of partial gene of cytochrome C oxidase subunit I (COI) of wild silkmoth *Cricula trifenestrata,* and to detect the diagnostic sites from these gene for evaluation as species marker. A total of fifteen larvae of *C. tifenestrata* were collected from Bogor, Purwakarta, and Bantul Regencies. Genomic DNA was extracted from silk gland of individual larvae, then amplified by PCR method and sequenced. DNA sequencing was done to characterize their nucleotide and amino acid contents. The results showed that 595 nucleotides at the 5 'end of COI gene of *C. tifenestrata* was conserved at the species level, but varies at the family level. Nucleotide dominated by thymine and adenine bases (± 70%). There were 25 diagnostic sites for *C. tifenestrata,* and four diagnostic sites for genus level. One hundred eigthty nine (189) amino acids were alignment, and only one percent of the genes was varied among species. The 107th amino acid (valine) and 138th (threonine) were diagnostics amino acid for *C. tifenestrata*. Based on nucleotides and amino acids sequences, the phylogeny showed that *C. tifenestrata* lied on the same nodes with *Antheraea,* so the Saturniidae family is monophyletic.

Key words: wild silk moth, C. trifenestrata, cytohrome C oxidase subunit I, nucleotide diagnostic, amino acid diagnostic

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INTRODUCTION

The golden silkmoth, is one local name for *Cricula trifenestrata* Helfer; and the member of the Saturniidae family (superfamily Bombycoidea), in which include the giant silkmoths, royal moths, and emperor moths. This family is the largest and most spectacular of the Lepidoptera, with an estimated number of 1,300–1,500 species (Tuskes *et al*., 1996). The Saturniidae family, for example domesticated silkmoth (*Bombyx mori*), has a great attraction among the silkmoth family (Bombycidae). In addition, some species of the family are wild silkmoths which can also produce silk. *C. trifenestrata* was conspicuous by having perforated cocoon, which golden yellow color. Male wing span is 56-78 mm, while female wing span is 75-81 mm (Kakati & Chutia, 2009). This species is distributed in throughout Filipine, India and Indonesia, and there are many endemic species in Indonesia Island such as in Sumatera, Java, and Sulawesi (Nassig *et al.,* 2006). Study based on biology, ecology and genetics aspects of this insect is therefore necessary. As *C. trifenestrata* is a closely related to species of *Antheraea* and *B. mori*, the mitochondrial genome information of the species may provide fundamental information for future phylogenetic analyses and evolutionary biology.

Recent investigations have suggested mitochondrial genome (mtDNA) as a tool for studying the taxonomy, and evolution of animal populations. The cytochrome oxidase subunit I (COI) gene is a part of mtDNA. This gene was chosen because of its central role in metabolism and its presence is in almost all *eukaryotes*. Additionally, the size and structure of COI gene has been well conserved in the animal groups analysed so far, a feature which makes it especially suitable for evolutionary studies (Lunt *et al*., 1996). The COI, however, is one of the most conserved mitochondrial protein-coding genes in animals (Mueller, 2006), and thus displays a better phylogenetic signal (Wilson, 2010). COI as a barcode marker has been used very successfully in many animal *taxa*, for examples: differentiated among family in Lepidoptera (Wilson, 2010), beetles (Funk *et al*., 1995), some insect pests (Toda & Murai, 2006) *Mermerodes hamona* moth (Hulrc *et al.*, 2007), mosquito (Cywinska *et al*., 2006), and *Thrips tabaci* (Karimi *et al.,* 2010).

Several cytochrome oxidase genes have been sequenced and characterized in silkmoth, *i.e*: *Bombyx mori* (Li *et al*., 2005; Arunkumar *et al*., 2006). Similar studies in wild silkmoth (Saturniidae family) have been done, especially in some species of Antheraea (Arunkumar *et al*., 2006; Mahendran *et al*., 2006), but has not been done in the Saturniidae of Indonesia. Study of *C. trifenestrata* is still minimally performed in the existing of taxonomy based on morphology. Molecular data is an appropriate alternative to complement the morphological data. Beside as the basic information, molecular data can be used as the basic study for selection, the business development of the utilization of wild silkworm, especially *C. trifenestrata*. Up to present, there are only limited data on the molecular characteristics of the partial coding genes in insect silk (Suriana *et al*., 2011). It is therefore, important to do more advanced research.

This study aimed to: (1) characterize, (2) detect the site of nucleotide and amino acid diagnostics COI gene for *C. trifenestrata*, and (3) examine the relationship of kinship among the silk-producing insects based on nucleotide and amino acid COI sequences. The results were described based on genetics backgrounds, so it would be the first molecular database for *C. trifenestrata* wich can be used as species marker.

MATERIALS AND METHODS

A total of fifteen larvae of the wild silkmoth *C. trifenestrata* were collected from Bogor and Purwakarta Regencies of West Java, and Bantul Regency of Central Java, Indonesia.

Genomic DNA Isolation

Genomic DNA was prepared from a pair of silk glands of final instar larvae of *C. trifenestrata* using a standard technique (Sambrook *et al*., 1989) that has been adjusted.

Electrophoresis

Genomic DNA was migrated on 1.2% agarose gel in 1xTBE solution using tools of submarine electrophoresis (Hoefer, USA). Ethidium bromide (0.5 ug/ml) was used for staining of the gel, and visualized under UV transiluminator (λ= 300 nm).

COI Gene Amplification and Sequencing

A region of COI gene derived from *C. trifenestrata* was amplified by using a Thermal Cycler Eppendorf Type 5332 machine. The primer used were forward 5'-TGATCA-AATTTATAATAC-3' and reverse 5'-GTAAAAT TAAAA-TATAAAC-3' (Mahendran *et al.,* 2006).

The program of PCR amplification was as follows 1 min at 94 ºC, followed by 35 cycles of 30 sec at 94 ºC, 45 sec at 40ºC, and 1 min at 72 ºC, with a subsequent 5 min for final extension at 72 ºC. Reagent components were 2 µl DNA template, 1.5 µl of each 10 picomol primer, 12.5 µl of buffer ready to mix, 0.5 ul MgCl2 50mM, and added double destiled H_2O up to 25 µl total volume. DNA sequencing was done by using Applied Biosystem 3730 XL DNA Analyzer.

Phylogenetic Construction

Characterization of *C. trifenestrata* COI gene was carried out using MEGA 4. Software (Tamura *et al*., 2007); sequence was alignmented to determine the position of these gene at COI gene data from GenBank. COI data for same species and genus were not available on the GenBank, therefore we used COI data for family level, i.e: six species of Antheraea (Saturniidae): *A. pernyi* (HQ264055), *A. mylitta* (AY 605 255) and *A. yamamai* (AF029067). *A. assama* (AY605249), *A. proiily* (AY960274), and *A. roilei* (AY960274). *Bombyx mori* (AF027953) sequence was used as outgroup. The final step phylogenetics tree was constructed by using the Neighborn

Joining method with 1000x boostrapped, and Kimura 2-parameter model (Nei & Kumar, 2003).

RESULTS AND DISCUSSION

General COI Partial Gene Features of *C. trifenestrata*

Amplification results of COI gene derived from *C. trifenestrata* was at long sequence of 595-602 base pairs (bp). The nucleotide sequence was aligned parallel to the nucleotide positions 138-740 in the COI gene sequences from GenBank data. This position was an area proposed as the animal barcode region (Hulrc *et al*., 2007). Sequence data are characterized along of 595 bp, encoding 198 amino acids. The position of these amino acids was aligned with 48th up to 245th of the amino acid *A. pernyi* as a reference. These sequences have been submitted to GenBank with the accession number of JF264728.

Nucleotide Composition and Diagnostics Sites

Compared base N composition of each species is presented in Table 1. The base N composition of each species showed varies. There are trend that timine was higest base N, while guanine was lowest base N percentage for each species.

Based on Table 1, this base N composition was consisted of 38%-41.8% thymine (T), 27.6%-32.9% adenine (A), 15.8%-18.5% cytosine (C), and 13.3%-14.6% guanine (G). The dominance of T and A bases in each species were mainly found in the third codon, as has also been observed in other insect: *B. mori* (Arunkumar *et al*., 2006), *A. pernyi* (Liu *et al*., 2008), *A. yamamai* (Kim *et al*., 2009), and *Hyphantria cunea* (Liao *et al*., 2010), *Eriogyna pyretorum* (Jiang *et al*., 2009). A possible explanation for this differences that the base constraints on T+A content in this first and second codon positions are less relaxed than those in the third codon position is due to degenerated genetic code (Funk *et al*., 1995; Liu *et al*., 2008).

The alignments results of 595 bp COI gene among *C. trifenestrata* populations showed that genes were highly conserve the species level, but diverse with other silkmoth species, both in Saturniidae (the same family), and Bombycidae (different family). There were 454/595 (76.3%) conserved nucleotides, and 141/595 (23.7%) variable nucleotides. The variable nucleotides, consisted of 99/595 (16.6%) parsimony informative nucleotides and 42/595 (7%) singleton nucleotides (Table 2). Similar results were observed in other insect, for examples: a lower (1%) of intra-species nucleotide diversity based on the COI gene was also found by Li *et al.* (2009); Weagener *et al.* (2006); Cywinska *et al.* (2006). Nevertheless, at the level of genus, COI was successed to separate the beetle genus *Ophraella* and showed the diversity among species up to 24% (Funk *et al.* 1995), insect pests *Thrips tabaci tobacco* up to 4.8% (Toda & Murai, 2007), and mosquitoes (Culicidae) 0.2%-17.2% (Cywinska *et al*., 2006).

Table 1. Percentage base N composition of COI gene *C. trifenestrata* and GenBank data

Species	T	C	A	G
C. trifenestrata	40.7	18.5	27.5	13.3
A. pernyii	40.4	17.3	29.4	12.9
A. yamamai	41.8	15.9	28.9	13.4
A. mylitta	41.3	16.5	28.9	13.3
A. assama	40.2	17.1	29.2	13.4
A. proylei	40.7	17.0	29.2	13.1
A. roylii	41.0	16.5	29.2	13.3
B. mori	38.0	15.8	32.9	13.3

Composition of the COI nuleotida *C. trifenestrata* is relatively consistent with the nucleotide composition of the COI sequences found in other insects, particularly Lepidoptera. There is a tendency of dominance of thymine and adenine bases that caused by the use of bases such as triplet codons. The applicability of the triplet codons is associated with the availability of the corresponding tRNA and also the rate of gene expression. Addition of nucleotide composition related to the rate of base substitution. Transition substitution was larger than transversion substitution. This is consistent with the results of this study, and as found by Toda & Murai (2006) wich A ↔ G substitutions, was greater than the C ↔ T, synonymous mutations was larger than non-synonymous mutations. Due to the substitution it was found 5 diagnostic nucleotide sites that can be utilized as species marker for *C. trifenestrata.*

Nucleotide differences among species ranged from 14 up to 92 were presented in Table 3. From those nucleotide differences, there were 25 sites of diagnostic for the *C. trifenestrata* species, and four diagnostics sites for genus of silkmoth, *i.e* 30th, 174th, 325th, and 396th sites respectively. Diagnostic sites among species, genus and family of silkmoth based on 595 bp COI gene is presented in Table 4. The diagnostic site was used as a molecular marker species, family and other *taxa* because it showed diverse parameters. This study was shown many diagnostic sites for *C. trifenestrata*. However, the data are not available from GenBank for other *Cricula* species, so the comparison performed at the family level is rougher.

Amino Acid Composition and Diagnostics Sites

The COI gene of *C.trifenestrata* has 595 bp long, translated into 189 amino acids which are aligned with *A. pernyi* amino acid as a reference at the position of 48th up to 245th. The COI amino acids of *C. trifenestrata*, were conserved at the species level. Amino acid composition was dominated by leucine and isoleucine (up to 25% from 189 amino acid sequence), whereas other amino acids were present in smaller amounts (Table 5).

Contrary to the nucleotide, amino acid alignment results of *C. trifenestrata* with amino acid in the GenBank data, showed a little variability. There were 178/198 (90%) that conserved amino acids, the remainder was 20/198 (10%) variable amino acid. The varies amino acid among species and their sites is presented in Table 6.

Table 6 showed that the valine (107th site) and threonine (138th site) are the diagnostic amino acid on *C. trifenestrata*. The diagnostics amino acid for *A. yamamai*

Table 2. The alignments result of 595 bp COI gene *C. trifenestrata* and with species from GenBank data

Species	Nucleotide sequences
*C.trifenestrata**	GATCAAATTTATAATACTATTGTAACAGCGCATGCTTTTATTATAATTTTTTTTATAGTAATACCT(66)
A.pernyi	..C.....C.....C.....T.....C.....T.....
A.yamamai	..C.....T.....T.....
A.mylitta	.....T.....T.....
A.assama	.....C.....T.....
A.proylei	.....C.....T.....C.....T.....
A.roylii	.....T.....C.....
B.mori	.....A.....T.....
C.trifenestrata	ATTATAATTGGAGGATTTGGTAATTGATTAATCCCCTTAATACTTGGAGCTCCTGATATAGCTTTC[132]
A.pernyi	.....A.....T..A.....T.A.....C.....
A.yamamai	.....A.....C....T..G.....T.A.....
A.mylitta	.....G....A.....T.A.....C.....
A.assama	.....A.....C..G....A.....T.A..T..C..C.....A...
A.proylei	..C.....A.....T..G.....T.A.....C.....
A.roylii	.....A.....T..A.....T.A.....
B.mori	.....A.....G.T..TC.T.....A.....A..A.....A...
C.trifenestrata	CCTCGAATAAATAATATAAGTTTTTGACTATTACCCCCTTCATTAGTACTTTTAATTTCCAGAAGT[198]
A.pernyi	..A.....C..T...ACC..A.....C.....A
A.yamamai	..A.....T.GC.C.....T...ACTT.A.....T.....A
A.mylitta	..C.....T.....T.....TC..ACT.....A.....A
A.assama	..C.....G...C.T.....T...ACT...C.....T.....A
A.proylei	..A.....C..T...ACC..A.....C.....A
A.roylii	..A.....C..T...ACT..A.....C.....A
B.mori	..A.....A.....CC.....C..CC.TA..T.A.....A.....A
C.trifenestrata	ATTGTTGAAAATGGAGCTGGAACAGGTTGAACGGTTTACCCCCCCCTTTCTTCCAATATTGCTCAC[264]
A.pernyi	.....A.....T..A.....A.....T..C.....A.....T
A.yamamai	.....A.....T..A.....A.....T.....A.....T
A.mylitta	.....T.....T..T..A.....A.....T..A..T..C.....T.....T
A.assama	.....A.....C.....T..A.....A.....T.....A.....A..A.....T
A.proylei	.....A.....T..A.....A.....T..C.....A.....T
A.roylii	.....A.....T..A.....A.....G..C.....T.....A..T
B.mori	.....A.....T..A.....A.....A.....A.....A..T.....C..A..T
C.trifenestrata	AGTGGAACTTCTGTTGATTTAGCTATTTTCTCCCTTCATCTTGCAGGAATTTCTTCAATTCTAGGC[330]
A.pernyi	G.A...T....A..A...C.T.....T.....T.....A.....T....A
A.yamamai	G.A..CT....A..A...C.T.....T..AT.A.....T.....T....A
A.mylitta	G.A..TT.C..A.....C.T.....T.....A.....T....G
A.assama	G.A..TT.A.....A..CC.....T..TT.A.....A..T...T....A
A.proylei	G.A...T....A..A...C.T.....T.....T.....A.....T....A
A.roylii	G.A...T....A..A...C.T.....T.....T.....A.....T....G
B.mori	..A....GA..C..A...C.T.....T..A..A...T.A.....T.....A.....A....A
C.trifenestrata	GCAATTAATTTTATTACAACTATTATTAACATACGAATAAATAACCTTTCCTTTGATCAAATACCA[396]
A.pernyi	.....T..A.....T.....TT.A..A.....T
A.yamamai	.....T..A.....T.....TT.A..A.....T
A.mylitta	..T.....T..A.....T.....TT.A..A.....T
A.assama	..T.....T..A.....T.....TT.A..A.....C.....T
A.proylei	.....T..A.....T.....TT.A..A.....T
A.roylii	.....T..A.....T.....TT.A..A.....T
B.mori	.....A..A.....T.....T.....TA.A..A.....T....C
C.trifenestrata	CTATTTGTTTGAGCTGTAGGAATTACTGCTTTCCTTTTACTTTTATCTCTTCCAGTTTTAGCTGGA[462]
A.pernyi	T.....C.....T.....A.....T.AC.T.....AT.A..T.....
A.yamamai	T.....C.....A..C..TT.AC.T.....AT.A..T.....
A.mylitta	T.....A..T.....C..A.....T.AC..TC.....A..A.....A.....A...
A.assama	.....G.....A..A..TT.AC.T.....T.A..T.....A...
A.proylei	T.....C.....T.....A.....T.AC.T.....AT.A..T.....
A.roylii	T.....C.....C.....A.....T.AC.T.....AT.A..T.....
B.mori	T.....A.....G.....A..A..TT.A...T.A.....A..A..T.....
C.trifenestrata	GCTATTACTATACTTTTAACCGACCGAAATTTAAATACTTCTTTTTTTGATCCAGCTGGAGGAGGT[528]
A.pernyi	.....A..T.....C.....T.....T.....A
A.yamamai	.....A...T.AC....A..T.....C.....T.....G..A
A.mylitta	.....A.....C.....C.....A
A.assama	.....A..T.....C.....C..T.....A
A.proylei	.....A..T.....C.....T.....T.....A
A.roylii	.....A..T.....C.....T.....T.....A
B.mori	.....A...T.A.....A..T.....C.....A..A.....T.....A
C.trifenestrata	GATCCTATCCTCTATCAACATTTATTTTGATTTTTTGGACACCCAGAAGTTTATATTTTAATTTTAC[595]
A.pernyi	.....A..TT.A..C.....T..T.....
A.yamamai	.....T..T.....T..T.....
A.mylitta	.....TT.A.....C..T..T.....
A.assama	.....A..T..T.....C..T.....
A.proylei	.....A..TT.A.....T..T.....
A.roylii	.....A..TT.A.....T..T.....
B.mori	..C..A..TT.A.....T..T.....

Note: *There were 15 sequences, only one showed here because which were monomorphic. Numbers in parentheses indicate sites number. (.) indicate conserved nucleotides (identical nucleotides) among species. Italic letters indicate parsimony informative nucleotides, and bolled letters indicate singleton nucleotides for each species.

Table 3. The difference of number and percentage of COI nucleotide on *C. trifenestrata* compared with GenBank data

Species	1	2	3	4	5	6	7	8
1	-							
2	74(12,4)							
3	79(13,3)	39(6,6)						
4	73(12,3)	47(7,9)	56(9,4)					
5	82(13,8)	55(9,2)	46(7,7)	55(9,2)				
6	72(12,1)	55(9,2)	38(6,4)	46(7,7)	54(9,0)			
7	69(12,0)	15(2,5)	37(6,2)	44(7,3)	53(8,9)	14(2,3)		
8	92(15,3)	69(11,6)	69(11,6)	83(14,0)	80(13,4)	67(11,3)	66(11,0)	-

Note: 1= *C. trifenestrata*, 2= *A. pernyi*, 3= *A. yamamai*, 4:5= *A. assama*, 6= *A. proylei*, 7= *A. roylii*, 8= *B. mori.* Numbers in parentheses indicate percentage of nucleotides difference.

Table 4. Diagnostic sites among species, genus and family of silkmoth based on 595 bp COI gene

Species	Nucloetide sites				
	1111 30177 02148	12222 92356 85127	23333 92356 45010	33333 77789 56816	44555 48234 73870
C. trifenestrata	**G**CT**A**G	**TTGCT**	**C**C**C**CC	CCTC**A**	**TCTCC**
A. mylitta	TAATA	AAAAA	TTAAT	TTAAT	AAATA
A. yamamai	TGATA	AAAAA	TTAAT	TTAAT	AAATT
A. pernyi	TAATA	AAAAA	TTGAT	TTAAT	AAATA
A. proylei	TAATA	AAAAA	TTAAT	TTAAT	AAATT
A. assama	TGATA	AAAAA	TTAAT	TTAAT	AAATA
A. proiily	TAATA	AAAAA	TTGAT	TTAAT	AAATA
B. mori	ATA**C**A	AAAAA	T**A**AAT	TTAA**C**	AAATA

Note: G= guanine, A= adenine, C= cytosine, T= timine. Arrow shows the direction readings. Bolled letters indicate diagnostic sites for *C. trifenestrata* and underlined letters indicate diagnostic sites for each genus.

Table 5. Percentage 189 amino acid sequence on *C. trifenestrata* compared with GenBank data

Species	Ala	Cys	Asp	Glu	Phe	Gly	His	Ile*	Lys	Leu*	Met	Asn	Pro	Gln	Arg	Ser	Trh	Val	Trp	Tyr
C. trifenestrata	7.07	0.00	3.53	1.01	8.08	8.08	2.53	11.11	0.00	14.14	6.06	6.06	6.57	1.52	1.52	7.58	5.56	5.05	2.53	2.00
A. pernyi	7.07	0.00	3.53	1.01	8.08	8.59	2.53	11.11	0.00	14.14	6.06	6.06	6.57	1.52	1.52	7.58	5.56	4.52	2.53	2.02
A. yamamai	7.07	0.00	3.05	1.02	8.12	8.63	2.54	11.17	0.00	14.21	6.09	6.60	6.60	1.61	1.52	7.58	5.56	4.06	2.54	2.03
A. mylitta	7.07	0.00	3.54	1.01	8.08	8.59	2.53	10.61	0.00	13.64	6.06	6.06	6.57	1.52	1.52	8.04	5.56	5.05	2.53	2.02
A. assama	7.07	0.00	3.54	1.01	8.08	8.55	2.53	10.61	0.00	14.14	6.06	6.06	6.57	1.52	1.52	7.58	5.56	5.05	2.53	2.02
A. proylei	7.07	0.00	3.54	1.01	8.08	8.55	2.53	11.11	0.00	14.14	6.06	6.06	6.57	1.52	1.52	7.58	5.56	4.55	2.53	2.02
A. roylii	7.07	0.00	3.54	1.01	8.08	8.55	2.53	11.11	0.00	14.14	6.06	6.06	6.57	1.52	1.52	7.58	5.56	4.55	2.53	2.02
B. mori	6.57	0.00	4.50	1.01	7.07	7.58	2.53	10.61	0.00	14.14	7.07	5.56	6.57	1.52	1.52	9.09	4.55	5.05	2.53	2.53

Note: *The amino acids were dominant.

was asparagine (141th site), glutamine (169th site), and alanine (198th site). The diagnostics amino acid for *A. pernyi* was asparagine (226th sites), while the diagnostics amino acid for *B. mori* was methionine (107th site), methionine (156th site), methionine (165th site), leucine (170th site), methionine (173th site), leucine (178th site), serine (186th site), aspartic acid (187th site), tyrocine (188th site), serine (189th site), isoleucine (190th site), aspartic acid (211th site), and isoleucine (234th site).

The amino acid 107th was diagnostic on genus level (*Cricula, Antheraea,* and *Bombyx*). While at the family level (Saturniidae and Bombycidae), there were 12 amino acids, those are diagnostics of 156th, 165^{th,} 170^{th,} 173th, 178^{th,} 186^{th,} 187^{th,} 188^{th,} 189^{th,} 190^{th,} 211th, and 234th

sites. This means that only one amino acid sites that can be used as genus marker, and more than one amino acid that can be used as family marker.

Thus COI amino acids showed a low variation in species and genus level, compared to the family level. A possible explanation for this fact is that the amino acids function in the metabolism is universal, including the COI amino acids. Therefore, not surprising if there are small variation between species or genus. On the other hand, if there are large amino acid variations among species or genus means that there are large variations in metabolism (Lunt *et al.*, 1996).

The difference and percentage of amino acid among species were compared and presented in Table 7. *A. assama* and *A. roylii* has same amino acid. While another species has varies amino acid.

Table 7 showed that the amino acid differences were 0 up to 16 amino acids. *C. trifenestrata* and *A. assama* and *A. roylii* have smallest difference in their amino acid, while *B. mori* and *A. yamamai* has the biggest difference in their amino acid. *A. assama* and *A. roylii* has same amino acid. Logical explanation for this fact is the amino acid variation is a reflection of nucleotide variation, although this variation not showed a positive correlation. That mean amino acid varies because of nucleotide varies that derived from of transverse substitution. No change amino acid because of transition nucleotide substitution. Similar result was reported by Funk *et al.* (1995) which explained that the rate of nucleotide or amino acid substitution were varied in COI sequence based on each species.

Genetic Distance and Phylogenetic Relationships

Genetic distances among species were compared by Neighbor-Joining method, Kimura 2-parameter (K2P) model for nucleotide sequence and p-distance model for amino acid sequences (Table 8). The Table 8 showed that *C. trifenestrata* has the smallest genetic distance with *A. mylitta, A. proyley* and *A. roylii,* and *A. pernyii*, *A. assama* and last *B.mori*. This suggests that the smaller of genetic distance means greater than the similarities among species.

Based on genetic distances (Table 8), both of the topology of silkmoth phylogeny based on 595 bp COI is presented in Figure 1 and silkmoth phylogeny based on 189 amino acid COI is presented in Figure 2. *C. trifenestrata* lied on the same node with *Antheraea* species i.e: *A. mylitta, A. yamamai, A. assama, A. roylii, A. pernyi, and A. proylei*, while *B. mori* lied on the outermost node, as the out of group (Figure 1). This means that Saturniidae was philogenetics monophyletic. *C. trifenestrata* position was relatively consistent with filogenetic relationships based on nucleotide sequences,

Table 6. The varies amino acid and their sites among species

Species	Amino acid sites																				
	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	2
	8	0	3	3	4	5	6	6	7	7	7	8	8	8	8	9	9	1	2	3
	0	7	6	8	1	6	5	9	0	3	8	6	7	8	9	0	8	1	6	4
C. trifenestrata	I	**V**	S	**T**	D	L	I	R	M	L	M	G	I	T	A	F	V	N	I	F
A. mylitta	V	T	G	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A. yamamai	.	T	G	S	**N**	.	.	**Q**	.	.	.	.	.	.	.	.	**A**	.	.	.
A. pernyi	.	T	G	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	**N**	.
A. proylei	.	T	G	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A. assama	V	T	G	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A. proilly	.	T	G	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B. mori	V	**M**	.	S	.	**M**	**M**	.	**L**	**M**	**L**	**S**	**D**	**Y**	**S**	**I**	.	**D**	.	**I**

Note: V= valine, T= threonine, G= glycine, S= serine, N= asparagine, Q= glutamine, A= alanine, M= methionine, L= leucine, D= aspartic acid, Y= tyrosine, I= isoleucine. Bolled letters indicate diagnostic sites for each species.

Table 7. The difference of number and percentage of 189 amino acid sequence on *C. trifenestrata* compared with GenBank data

Species	1	2	3	4	5	6	7	8
1	-							
2	5(2,5)							
3	6(3,0)	5(2,5)						
4	4(2,2)	3(1,5)	4(2,0)					
5	3(1,5)	2(1,0)	3(1,5)	1(0,5)				
6	4(2,0)	1(0,5)	4(2,0)	2(1,0)	1(0,5)			
7	3(1,5)	2(1,0)	3(1,5)	1(0,5)	0	1(0,5)		
8	13(6,6)	13(6,6)	16(8,0)	14(7,0)	13(6,6)	12(6,0)	13(6,6) 12	-

Note: 1= *C. trifenestrata*, 2= *A. pernyi*, 3= *A. yamamai*, 4= *A. mylitta*, 5= *A. assama*, 6= *A. proylei*, 7= *A. roylii*, 8= *B. mori*. Numbers in parentheses indicate percentage of amino acid difference.

Table 8. Genetic distances between *C. trifenestrata* with species from GenBank data, based on 595 bp COI gene at below diagonal and amino acid sequence at above diagonal

Species	1	2	3	4	5	6	7	8
1	-	0.019	0.019	0.019	0.025	0.025	0.031	0.082
2	0.14		0.000	0.000	0.006	0.006	0.013	0.082
3	0.15	0.07		0.000	0.006	0.006	0.013	0.082
4	0.13	0.08	0.10		0.006	0.006	0.013	0.082
5	0.15	0.10	0.08	0.10		0.000	0.006	0.075
6	0.13	0.01	0.07	0.08	0.10		0.006	0.075
7	0.13	0.03	0.07	0.08	0.10	0.02		0.082
8	0.17	0.13	0.13	0.15	0.15	0.12	0.12	-

Note: 1= *C. trifenestrata*, 2= *A. pernyi*, 3= *A. yamamai*, 4= *A. mylitta*, 5= *A. assama*, 6= *A. proylei*, 7= *A. roylii*, 8= *B. mori.*

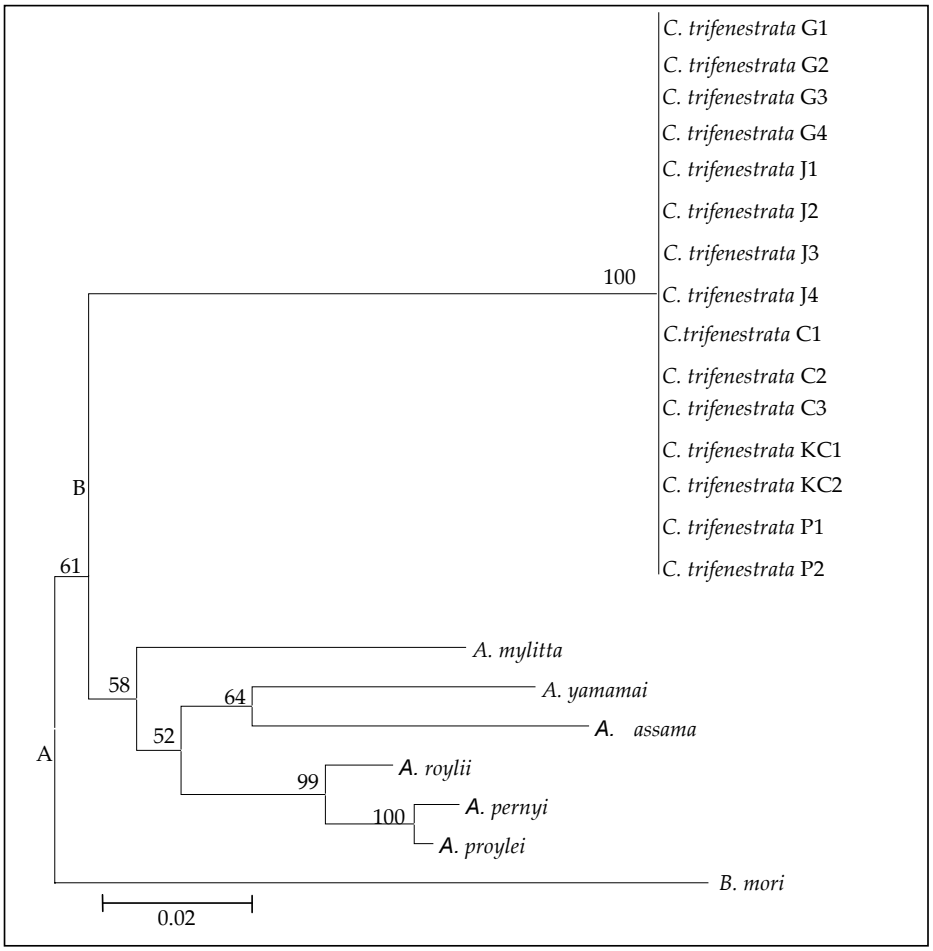

Figure 1. Silkmoth phylogeny based on 595 bp COI with Neighbor-Joining method, 1000x bootstrapped, and Kimura 2-parameter model, node for Bombycidae (A), node for Saturniidae (B).

while *A. mylitta* changed position with *A. assama* (Figure 2). These fenomenom clearly caused of changed amino acid among species that was compared.

The use of different families as outgroup when constructing phylogenies sometimes leads to differences in tree topology (Funk *et al*., 1995), but this did not occur in *C. trifenestrata*. Phylogenetic construction using Antheraea as fellow family level (Saturniidae) and *B. mori* (Bombycidae) as different families did not changed tree topology based on both in nucleotide and amino acid sequence. This means that the phylogenetic signal by COI is still robust. Similar results were also reported by Mahendran *et al.* (2006) which indicate that the phyilogenetic tree based on COI is more robust than phylogenetic tree based on 16SRNA sequence.

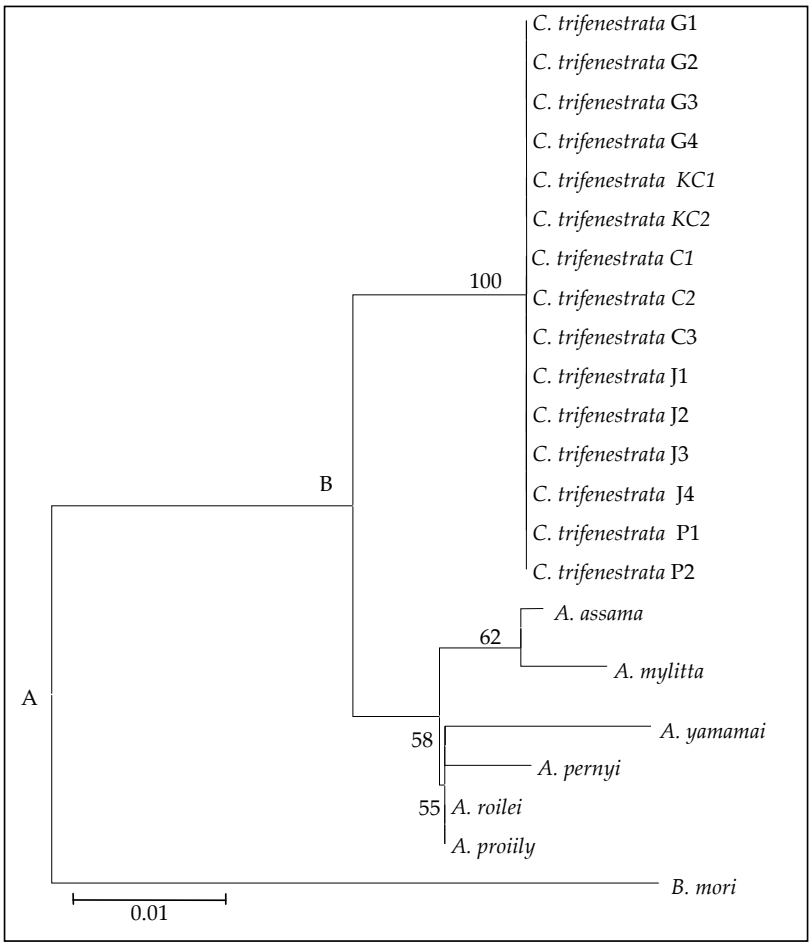

Figure 2. Silkmoth phylogeny based on 189 amino acid COI with Neighbor-Joining method, 1000x bootstrapped, and p-distance model, node for Bombycidae (A), node for Saturniidae (B).

CONCLUSION

This study successfully characterized the 595 nucleotides of COI gene of *C. trifenestrata*. Nucleotides were dominated by thymine (T) and adenine (A) bases (± 70%). Nucleotides of 595 bp of COI gene *C. trifenestrata*s encoded 189 amino acids. Two amino acids were obtained as species marker for *C. trifenestrata*, *i.e* Valine amino acid 107th and threonine amino acids 138th. *C. trifenestrata* lied at the same node with *Antherasea* spescies, while *B. mori* was at outhermost node. The family of Saturniidae is monophyletic.

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