Media Peternakan, April 2011, hlm. 23-29
EISSN 2087-4634
Terakreditasi B SK Dikti No: 43/DIKTI/Kep/2008

Versi *online*:
http://medpet.journal.ipb.ac.id/
DOI: 10.5398/medpet.2011.34.1.23

Characterization of Partial Coding Region Fibroin Gene on Wild Silkmoth *Cricula trifenestrata* Helfer (Lepidoptera: Saturniidae)

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(Received 5-12-2010; accepted 25-02-2011)

ABSTRACT

The study was conducted to characterize coding region of wild silkmoth *C. trifenestrata* partial fibroin gene, and detect these gene potential as molecular marker. A total of six larvae *C. trifenestrata* were collected from Bogor, Purwakarta and Bantul Regency. Genomic DNA was extracted from silk gland individual larvae, then amplified by PCR method and sequenced. DNA sequenced result was 986 nucleotide partial fibroin gene of *C. trifenestrata*, which are comprising complete coding region of first exon (42 nucleotide), an intron (113 nucleotide), and partial of second was exon (831 nucleotide). Only coding region was characterized. Results showed that first exon very conserved in *C. trifenestrata*. These gene consisted of 31%, thymine, 28% guanine, 21% cytosine, and 19% adenine. Cytosine and thymine (sites of 25th and 35th respectively) were marker for *C. trifenestrata* species. The first exon encoding 14 amino acids. Valine amino acid (12th site) was marker to the species *C. trifenestrata*. The partial second exon consisted of guanine (32.7%), alanine (26.5%), thymine (21%) and cytosine (19.7%). These region encoded 277 amino acids, which were dominated by the alanine (27.8%) and glycine (21.66%). Alanine formed polyalanine sequence with different motifs namely: AAAAAAASS, AAAAAAAAAAGSSG, AAAAAAAAAAAGSGTGFGGYDS, AAAAAAAAAGS SGRGGYDGVDGGYGSGSS, and AAAAAAAAAAAGSSGRGLGGYDGWVDDGYGSGSGS.

Key words: Wild silkmoth, C. trifenestrata, fibroin gene, nucleotide and amino acid marker, polyalanin sequence

INTRODUCTION

Indonesia is known as a country with mega diversity and mega center of biological resources. Recently many studies have been done on the basis of local genetic resources, for example in local duck (Muzani *et al*., 2005), sheep (Mansjoer *et al*., 2007) as well as Bali cattle (Zulkharnaim *et al*., 2010). Similar studies should be done, especially on Indonesia endemic animals, including pest and beneficial insects.

Silks play important roles in the lives of arthropods, for example, cocoon silks produced by Lepidopteran insects, silk egg stalk by Neuropteran insects, underwater silk prey capture nets by Trichopteran insects, and web building by spiders (Mondal *et al*., 2007). Silk has been analyzed in some detail in only three Lepidopteran families. In Saturniidae, the silk protein is relatively small (200- to 250-kDa). H-fibroin molecules consist of regularly alternating hydrophilic and hydrophobic regions (Hwang *et al*., 2001) seem to assemble in the elementary secretory units and in the filament polymer as homodimers (Tanaka & Mizuno, 2001).

The silk consisted of two components, sericin and fibroin. Sericin makes up the protein coating of the silk filament and it secreted into the lumen from cells of the middle division of the silk gland. Fibroin, the main part of the silk, it produced in the posterior region of the silk gland and contains three components: fibroin H chain, fibroin L chain, and fibrohexamarine/P25 (Zhou *et al*., 2000). Interestingly, the fibroin L chain and fibrohexamarine/P25 are not founded in *A. yamamai* and *A. mylitta* fibroin (Tanaka & Mizuno, 2001; Datta *et al*., 2001b). Most of Saturniidae *fibroin* protein consists of heavy chain dimer, which are formed by disulfide bound of two cysteine amino acid residues (Sezutsu & Yukuhiro, 2000; Sezutsu *et al*., 2010).

However no yet evident for all Saturniidae, expecially *Cricula* species. Fibroin gene in the various taxa

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vary in size, namely the Bombycoidae family (fibroin gene up to 80 009 bp in *B. mori*) while the Saturnidae family (*A. pernyi* have fibroin gene 9282 bp along Sezutsu & Yukuhiro, 2000). Although different in size, but there are similarities in the structure of this fibroin gene, which is composed of two exons and one intron (Zhou *et al*., 2000; Craigh & Riekel, 2002). Fibroin gene 5'flanking region (promoter), at bases -300 up to -20 base, in both families are homologous, as well as in first exon in both these families have a high homology. However, intron and the second exon in both families are very different (Zhou *et al*., 2000).

The difference of fibroin gene is based on the number of nucleotide composition, i.e introns in *B. mori* consists of 970 bp (Zhou *et al*., 2000), but intron *A. yamamai* consists of 150 bp (Hwang *et al*., 2001), intron *A. mylitta* consists of 133 bp (Datta *et al*., 2001b). The second exon in *B. mori* consists of 15 750 bp, which encoded 5250 amino acid residues, while the second exon *A. yamamai* consists of 7923 bp, which encoded 2461 amino acid residues (Hwang *et al*., 2001) and *A. pernyi* second exon consists of only 7878 bp, which encoded 2626 amino acid residues (Sezutsu & Yukuhiro, 2000; Zhou *et al*., 2000). Differences in size and amino acid sequences of these genes may be affected on the physical properties of the fiber (Yonemura & Sehnal, 2006; Sehnal & Sutherland, 2008).

To date, all silk and fibroin gene studies of Lepidoptera have focused on *B. mory* (Bombycidae) (Zhou *et al*., 2000; Katsuhiko *et al*., 2007; Lefevre *et al*., 2007), *Antheraea* spesies and *Samia cynthia ricini* (Saturniidae) (Datta *et al*., 2001a, b; Sezutsu *et al*., 2009; Sezutsu & Yukuhiro, 2000; Sezutsu *et al*., 2010), *Araneus diadematus*, and *Nephila edulis* (Araneoidae), *Galleria mellonella,* and *Hepialus californicus* (Hepialidae) (Zurovec & Sehnal, 2002; Yonemura & Sehnal, 2006; Chen *et al*., 2006; Saravanan *et al*., 2006; Collin *et al*., 2010). No date for *C. trifenestrata*. *C. trifenestrata* is endemic wild silk moth in some islands in Indonesia, while it has silk fiber with the original color of golden yellow, so it is the potential as a natural source of silk fiber. Fibroins are important candidates for the production of transgenic silkworms that would secrete novel types of silk (Komija *et al*., 2007; Sezutsu *et al*., 2009; Kobayashi *et al*., 2009). Furthermore spesivicity of the silk protein has inspired people in both academia and industry to prepare silk-mimetic polymers and proteins by chemical and/or biotechnological means (Hardy & Scheibel, 2009). It is therefore essential to characterize fibroin genes in species that produce attractive silks as *C. trifenestarata*.

In this study, we describe fibroin gene especially partial coding region of *C. trifenestrata*. We compare the fibroin sequences to those known in other Lepidoptera. We then determine the nucleotide and amino acid composition, and detection nucleotide and amino acid maker for each species. In addition, by comparing the sequences of *C. trifenestrata* to other known fibroins, we shed light on the potential development of these insects as a source of natural silk fibers.

MATERIALS AND METHODS

Samples

A total six larvae of the wild silkmoth *C. trifenestrata* were collected from Bogor and Purwakarta Regency (West Java), and Bantul Regency (Central Java).

Genomic DNA Isolation and Purification

Genomic DNA was prepared from a pair of silk glands of final instar larvae of *C. trifenestrata* using a standard technique that has been adjusted (Sambrook *et al*., 1989; Duryadi, 1993).

Electrophoresis Results of Genomic DNA Purification

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

Primer Design

Primer for DNA amplification of *C. trifenestrata* fibroin gene was designed by using Primer3 software that available in http://frodo. wi. mit. edu. Primer designed based on *Antheraea pernyi* fibroin gene (AF083334). This primer consisted of: forward primer FFCt 5'-CATAACCATGAGAGTAATAGCC-3', and reverse primer RFCt 5'-CTGCTGAGTCTGATCCGTAA-3' (Figure 1).

Fibroin Gene Amplification

Amplification of *C. trifenestrata* fibroin gene use thermal cycler Eppendorf Type 5332 machine. The PCR amplification was performed using the following procedure: 1 min at 94 ^{o}C, followed by 35 cycles of 30 sec at 94 ^{o}C, 45 sec at 52 ^{o}C, and 1 min at 72 ^{o}C, with a subsequent 5 min final extension at 72 ^{o}C. Reagent components are: 2 ul DNA template, 1.5 ul of each 10 pmol primer, 12.5 ul of buffer ready to mix, 0.5 ul $MgCl_2$ 50mM, and added double distilled H_2O up to 25 ul volume.

DNA sequencing

DNA sequencing done with DNA sequencing service providers companies.

DNA Alignment and Characterization of *C. trifenestrata* Fibroin Gene

The sequence alignment was carried out using ClustalW available at Mega 4.0 software (Tamura *et al*., 2007). The genes *C. trifenestrata* fibroin were determined by comparing them with homologous regions in *A. per-*

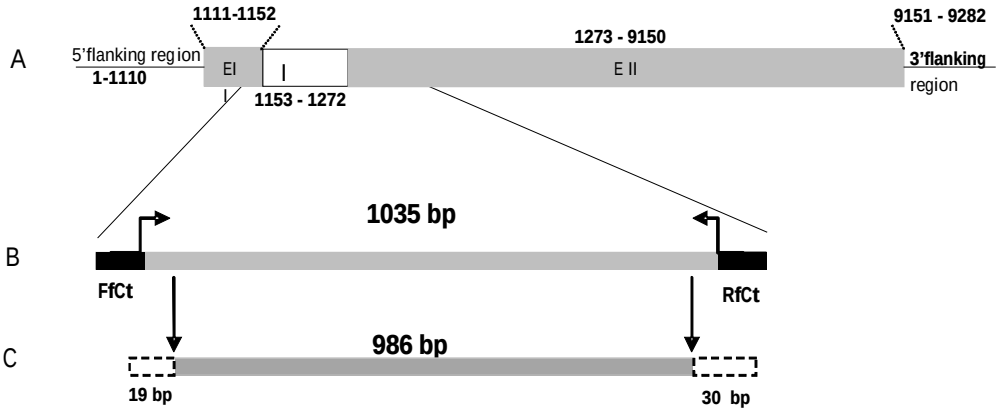

Figure 1. Scheme of the complete sequence fibroin gene *A. pernyi* (9282 bp). These gene consisting of: 5'flanking region (1110 bp), EI (first exon 42 bp), intron 120 bp, and EII (second exon), 7878 bp, 132 bp region 3'flanking (AF083334), which was used as templates for designing primers fibroin gene of *C. trifenestrata* (A). The position of primer RfCt and FfCt on fibroin gene fragment *A. pernyi*. The fibroin gene of *C. trifenestrata* was amplified over 1035 bp by these primers (B), and capable to read 986 bp long (C).

nyi fibroin gene. The results of alignment divided into first exon, an intron, and second exon. Only first and second exon that were characterized. These fragments aligned again with known in other Lepidoptera, and then determine the nucleotide type and their composition, amino acid type and their composition, number of different nucleotide and amino acid, and detection diagnostic marker for each species.

RESULTS AND DISCUSSION

The sequenced results of *C. trifenestrata* fibroin gene from of Bogor, Puwarkarta, and Bantul Regency are the DNA fragments 984-1035 nucleotides (nt) long. After multiple alignment, and cutting no matching sequence, the nucleotide consist of 986 nt, which are composed of 42 nt first exon, 113 nt intron and 831 nt partial of second exon. Only coding region (873 nt) was characterized. These sequence have been submitted to GenBank with accession number JF264729, and we will be release after this article was published.

Conserved Nature of First Exon Coding Region Fibroin Gene of *C. trifenestrata*

The coding region of first exon *C. trifenestrata* fibroin gene (Ct-fibroin) was determined with the initiation codon ATG. This region was highly conserved at spesies level, did not reveal any variation within population of *C. trifenestrata* (Table 1). Similar results were found in *B. mori*. *B. mori* fibroin cds consists of 16 788 nucleotides. Of these region, the 5 'end (+1 up to +1449), are identical at 99% of intra species. One percent variation may result from polymorphism between different strains (Zhou *et al*., 2000). Besides that, Collin *et al*. (2010) was aligned H-fibroin of *H.californicus* and *did* not reveal any variation intra those population. The fibroin protein is tipycal structural protein, and the main protein in silk fiber, that are spesies spesific (Zurovec & Sehnal 2002; Mondal *et al*., 2007). Hwang *et al*. (2001) have analyzed and aligned 155 nucleotides that includes first exon and the 5 ' end second exon in *A. yamamai* and also compare them with *A. pernyi*, founded a 95% similarity in these

Table 1. The first exon nucleotide fibroin gene of *C. trifenestrata*, *Antheraea*, and *B. mori*

Spesies ↓	Nucleotide sites													
	123	456	789	111 012	111 345	111 678	122 901	222 234	222 567	223 890	333 123	333 456	333 789	444 012
C. trifenestrata (B1, B2, P1, P2, J1, J2)	ATG	AGA	GTA	ACA	GCC	TTC	GTC	ATC	CTG	TGC	TGC	GTT	TTG	CAG
A. yamamai	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	T . .	. . .	. . .	. C .	. . .	. . .
A. pernyi	. . .	. . .	. . .	T . .	. . .	. . .	. . .	. . .	T . .	. . .	. . .	. C .	. . .	. . .
A. mylitta	. . .	. . .	. . .	T . .	. . .	. . .	. . .	. . .	T . .	. . .	. . .	. C .	. . .	. . .
B. mori	. . .	. . .	. . C	. A .	. A .	T . .	. . .	. . .	T . .	. . .	. . .	. C .	C . .	. . .

↓ reading direction; (.) identical nucleotide; A= adenine; T= thymine; C= cytosine; G= guanine; marker nucleotide for *C. trifenestrata* are written in bold letters.

sequence. Furthermore, fibroin gene *A. yamamai* has rightly expressed in the posterior silk gland of *B. mori,* indicate that the promoter function and splicing machinery are well conserved between *A. yamamai, B. mori and other Lepidoptera insect* (Zhou *et al*., 2000; Fangzhou *et al*., 2002; Kobayashi *et al*., 2009; Sezutsu *et al*., 2009). There is homology of fibroin gene in those species, namely base -300 to -20, including the TATA box (Zhou *et al*., 2000; Sezutsu & Yukuhiro, 2000).

Multiple alignment of 42 nucleotidaes first exon fibroin gene *C. trifenestrata* and other genera, showed 83.3% (35/42) nucleotides that are conserved and 16.7% (7 / 42) nucleotides that are vary between them. Variable nucleotides are composed of 7.2% (3/42) parsimony informative and 9.5% (4/42) nucleotides that are singleton. Several nucleotides that are diagnostic, namely: nucleotides 9th, 13th, 18th, and 35th are diagnostic for *B. mori* (Bombycidae), which are different from the Saturniidae. None the diagnostic nucleotides for the *Antheraea* genera. However, there are two diagnostic nucleotide for *C. trifenestrata,* which are different from *Antheraea* and *B. mori*, namely: cytosine (25th site) and thymine (35th site). These nucleotide are marker for that species (Table 1).

Fourteen amino acid at the fisrt exon Ct-fibroin encoding 14 amino acid residues were also highly conserved at species level. Thirteen of these amino acid, which were identical for *C. trifenestrata* and *A. yamamai* fibroin heavy chain and 12 with *A. pernyi* and *A. mylitta.* Only 11 amino acid identical to *B. mori* fibroin heavy chain. Note that two cysteine residues were conserved in this region in all four Saturniidae fibroins and *B. mori* also (Table 2). The high conserved nucleotide (amino acid) of fibroin gene was founded in most Saturniidae (Hwang *et al*., 2001; Kobayashi *et al*., 2009; Sezutsu & Yukuhiro, 2000; Sezutsu *et al*., 2009; Sezutsu *et al*., 2010).

From the standpoint of evolution, the amino acid from first exon indicates the existence of specificity between the families. The amino acid threonine (T) was spesific for the Saturniidae family, while the amino acid lysine (K) and T were spesific in the Bombycidae family. When compared between *C. trifenestrata* and *Antheraea* and *B. mori*, there has been reduction in the number of amino acid alanine (A) in *C. trifenestrata*, followed by the addition of the amino acid valine (V). Thus suspected that the divergence begins fibroin gene of *B. mori* to *A. pernyi* and *A. myliita*, then to *A. yamamai* and last was *C. trifenestrata*. Furthermore, the valin amino acid (12th) was molecular marker for *C. trifenestrata* first exon.

Only A Small Range of Sequences Was Conserved in The Partial Second Exon

The results of multiple alignments 910 nucleotide second exon between *C. trifenestrata* with *A. pernyi, A. mylitta, A. yamamai* and *B. mori*, showed 35% (314/910) nucleotide were conserved and 62% (560/910) nucleotide were variable. The number of nucleotides that differ between species and the nucleotide sequence of different positions presented in Table 3. Nucleotide composition of fibroin gene second exon *C. trifenestrata* consisted of 32.7% guanine (G), 26.5% adenine (A), 21.1% thymine (T) and 19.7% cytosine (C). The content of G and A bases were higher than the other bases caused by the use of both these bases as part of a triplet codon for the amino

Table 2. Amino acid the first exon fibroin gene of *C. trifenestrata*, *Antheraea*, and *B. mori*

Species	Amino acid sites														
	↓									1	1	**1**	1	1
	1	2	3	4	5	6	7	8	9	0	1	**2**	3	4
C. trifenestrata (B1, B2, P1, P2, J1, J2)	M	R	V	T	A	F	V	I	L	C	C	**V**	L	Q
A. yamamai	.	.	.	.	.	.	.	.	.	.	.	A	.	.
A. pernyi	.	.	.	I	.	.	.	.	.	.	.	A	.	.
A. mylitta	.	.	.	I	.	.	.	.	.	.	.	A	.	.
B. mori	.	.	.	K	T	.	.	.	.	.	.	A	.	.

↓ reading direction; (.) identical amino acid; V= valine; K= lysine; T= threonine; A= alanine; F= phenilalanine; I= isoleucine; L= leuicine; C= cysteine; Q= glutamine; amino acid marker for *C. trifenestrata* was written in bold letters.

Table 3. Number of different nucleotide second exon partial fibroin gene of *C. trifenestrata*, *Antheraea*, and *B. mori*

Species	1	2	3	4	5
1. *C. trifenestrata*	-				
2. *A. yamamai*	233	-			
3. *A. pernyi*	221	81	-		
4. *A. mylitta*	232	90	74	-	
5. *B. mori*	238	391	386	381	-

Table 4. Amino acid sequence second exon partial fibroin gene of *C. trifenestrata*, *Antheraea*, and *B. mori*

Species	Amino acid sequence sites				
	111	1111333344	4444444455	5555555566	66666666777
↓	1234567234	5678678901	2345678901	2345678901	23456789012
C. trifenestrata (B1, B2, P1, P2, J1, J2)	YATAKNIGRN	EYVGNNGELY	QRYSTHKQYE	RNAGVSPNLS	GDERLVRTIVV
A. yamamai	....N.LHHD	...D.H.Q.V	E.FT.R.H..	...ATR.H..	.N....E...L
A. pernyi	......LHHD	...D.H.Q.V	E.FT.R.HF.	...ATR.H..	.N....E...L
A. mylitta	.......HHD	...D.HSQ.V	E.FT.R.H..	...ATR.H..	.N....E...L
B. mori	AY.NA..FDE	D.F.AS.AVI	EEQI.T.KMQ	.KNKNHGI.G	KN.KMIK.F.I
			111111111	1111111111	11111111111
	7777777888	8888888999	9000000000	111111111	12222222222
	3456789012	3456789012	3012345678	9012345678	90123456789
C. trifenestrata (B1, B2, P1, P2, J1, J2)	EEDPTGHERI	FEEDDIIKRV	GAAAASS**AAA**	**AAAASS**AAA	IIVERDFGSRR
A. yamamai	....Y...D.	Y...VV.N..	PG.SS.A...	..AS.....	.....QASHGA
A. pernyi	....Y...D.	Y...VV.N..	PG.SS.A...	..AS.....	.....QASHGA
A. mylitta	....Y...D.	Y...VV.N..	PG.SS.A...	..AS.....	.T...QASHGA
B. mori	TT.SD.N.S.	V...VLM.TL	STV.Q.YV..	DAGAYSQSGP	YVSNSGYSTHQ
	1111111111	1111111111	1111111111	1111111111	11111111111
	3333333333	4444444444	5555555555	6666777778	88999999999
	123456789	123456789	123456789	123567890	12012345678
C. trifenestrata (B1, B2, P1, P2, J1, J2)	GHAAGSASAA	ASAAAAAAAS	AAGSSGRGLG	GYDGSVDGGY	GSGSGS**AAAAA**
A. yamamai	.G...A.AG.	.AGSS.RGG.	GFYETHDSYS	S.GS.GA..A	.G.Y..DS...
A. pernyi	.G...A.AG.	.AGSS.RGGG	GFYETHDSYS	S.GS.GA..V	.G.Y..DS...
A. mylitta	.G...A.AG.	.ASSSVRGGG	GFYETHDSYS	S.GS.GA..R	.H.Y..DS...
B. mori	.YTSDFSTS.	.VG.G.G.GA	....GAGAGA	..GAASGA.A	.A.A.AG.GYG
	1222222222	2222222222	2222222222	2222222222	22222222222
	9000000000	1111111122	2222222333	3333333444	44444456666
	9012345678	1234578901	2345789012	3456789013	45678904567
C. trifenestrata (B1, B2, P1, P2, J1, J2)	**AAAAAGSSGR**	**GGYDGVDGGY**	**GSGSS**AAAAA	AAAAAAAGSG	TGFGGYDS**AAA**
A. yamamai	.....AAAAG	S.AG.G....		.......RRV	GHDRAAG....
A. pernyi	.....AAASG	A.GS.GY...	..D.A.....	.........A	G.S...GA...
A. mylitta	.....AAAAA	S.AG.GH...	..D.A.....	.......AG.	A.GR.DGG...
B. mori	TG.G..AGAG	A.AGAAGA..	.A.AAG.G.G	YG.G.G..AA	GYGA.AGG.G.
	2222222222	2222222222	2222222222	2333333333	333333333
	6677777777	7788888888	8999999999	9000000000	011111111
	8901234567	8901234678	9012345678	9012345678	90124567
C. trifenestrata (B1, B2, P1, P2, J1, J2)	**AAAAAAAAAG**	**SSGRGLGGYD**	**GWVDDGYGSG**	**SGS**AAAAAAA	AAAAGSSG
A. yamamai	.........A	.GAG.S..GY	..G.G....D	.AA.....	....SGA.
A. pernyi	.........A	..AG.S..GY	..G.G....D	.AA.....	....SGA.
A. mylitta	.........A	.GAG.S..GY	.RG.G....D	.AA.....	...GSGA.
B. mori	G.GYG..SGA	GA.A.Y..VG	SGAAS.A.A.	A.AGS..GSG	.G.G.TGA

↓ reading direction; (.) amino acid identical; amino acid abbreviation refert to Table 2; sequences with different polyalanine motif written in bold and underlined.

acid alanine has the highest frequency among 3 triplet codons for amino acid alanine, i.e (GCA, GCT, GCG, GCC, with a frequency of consecutive 49, 17, 16, 1 times respectively). This result indicated that the composition of the nucleotide was similar, but their size was heterogeneous, such as the second exon of other Lepidoptera (Craigh & Riekel, 2002; Sezutsu *et al*., 2010)

Many Repetitif Alanine Amino Acid with Different Motif

The results of multiple alignments 317 amino acid second exon partial fibroin gen of *C. trifenestrata* and other genera showed that only 63/317 (20%) amino acids that were conserved inter genera, and the remaining 223/317 (70%) amino acid that were variable. The amino acids that differ between species presented in Table 4. From Table 4 showed that the amino acid composition has a specific pattern. There is a repetition of the amino acid alanine in sequence starting with the repetition of two up to 12 amino acid alanine (Polyalanin). This Polyalanin followed by amino acids serine and glycine, as well as other amino acids (Table 4). The composition of amino acids after polyalanin a repetition consists of the amino acid glycine (G) and serine (S). The amino acid arrangement like that, was similar with amino acid sequence of second exon fibroin of Saturniidae family (Datta *et al*., 2001b; Sezutsu *et al*., 2010). There are four type of polyalanine sequence motif in second exon fibroin *A. pernyi* (Sezutsu & Yukuhiro, 2000; Sezutsu, *et al*., 2009), while repetitive GAGASG pattern was founded in *B. mori* (Zhou *et al*., 2000). There is a relationship between the composition of nucleotides sequence, amino acids, and the nature of each silk fiber produced by each species, so there is high variability between species. Craigh & Riekel (2002) explained that these variability due to dynamic evolution of fibroin gene.

As described above, the first exon relatively short than the second exon, while these gene very conserved and the second exon very variable. This case indicates strong functional constraint on them, duplication and subsequent rearrangement of sequences with multiple motifs infers that the rearrangement rate is effectively large relative to the magnitude of negative natural selection. Variation in arrangement of motifs might be selectively neutral or adaptive (Sezutsu & Yukuhiro, 2000; Sezutsu *et al*., 2009).

CONCLUSION

The nucleotide of the first exon fibroin gene of *C. trifenestrata* was 42 nucleotide long, which are conserved in that species. Composition of this nucleotide was dominated by thymine and guanine (over 70% of total base). Cytosine and thymine (site of 25th and 35th respectively) were nucleotide marker for *C. trifenestrata* species. These exon encoded 14 amino acid residues, which was conservated too. Valine was marker to the species *C. trifenestrata*. Nucleotide composition of partial second exon was dominated by guanine and alanine. These exon encodes 277 amino acids that are dominated by the alanine (27.8%) and glycine (21.66%). Alanine formed polyalanine sequence with different motifs.

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