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THESIS

ANALYSIS OF THE *DOUBLESEX* AND *VITELLOGENIN* GENES IN RICE MOTH, *Corcyra cephalonica* Stainton

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A Thesis Submitted in Partial Fulfillment of
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Mayura Veerana 2014: Analysis of the *doublesex* and *vitellogenin* Genes in Rice Moth, *Corcyra cephalonica* Stainton. Master of Science (Genetics), Major Field: Genetics, Department of Genetics. Thesis Advisor: Associate Professor Lertluk Ngernsiri, Ph.D. 161 pages.

Rice moth, *Corcyra cephalonica* Stainton, is one of the devastating stored insect pests. The *doublesex (Ccedsx)* and *vitellogenin (CceVg)* genes of *C. cephalonica* were investigated. Three *doublesex* (*dsx*) mRNAs, two from female (*CcedsxF1* and *CcedsxF2*) and one (*CcedsxM*) from male, were identified. The *CcedsxF1* and *CcedsxF2* contained the complete open reading frame (ORF) of 801 and 741 bp length, respectively. While the *CcedsxM* was the 831 bp partial ORF sequence. The nucleotide sequences of these *dsx* mRNAs were identical except the *CcedsxM* had no the nucleotide at the position 744-1018. The difference between *CcedsxF1* and *CcedsxF2* is the present of 15 bp sequence containing a stop codon in the *CcedsxF2*. The expression pattern of *Ccedsx* was very low in eggs and increased gradually to maximum at adult females and males. The *vitellogenin* gene of *C. cephalonica* was 6,721 bp length including a 60 bp 5'UTR, 5,382 bp six exons, 1238 bp five introns and 41 bp 3'UTR. The 5,382 bp open reading frame (ORF) encodes a predicted protein of 1,793 amino acids residues with 16 amino acid signal peptide and four conserved domains. CceVg contained three polyserine tracts, two located at the N-terminus and the other at the C- terminal, which has not been reported in Lepidopteran insect Vgs. The *CceVg* expression pattern was first detected in very low level at the early larval stage but disappeared in later stage of larvae and resumed in female at the early pupal stage. Interestingly, at adult stage, the highest expression was detected in females while the low expression level was detected in males. Injections of *CceVg* double strand RNA into early emerged females caused severe abnormal ovaries.

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Student's signature Thesis Advisor's signature

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Mayura Veerana

March, 2014

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LIST OF ABBREVIATIONS

A	=	Adenine
aa	=	amino acid
°C	=	degrees Celsius
%	=	Percent
BLAST	=	Basic Local Alignment Search Tool
Bp	=	base pair
C	=	Cytosine
C_T	=	Baseline-subtracted Threshold
cDNA	=	complementary DNA
DEPC	=	diethylpyrocarbonate
DNA	=	deoxyribonucleic acid
dNTP	=	deoxyriboNucleotide TriPhosphate
dsRNA	=	double-stranded RNA
esiRNA	=	endogenous siRNA
EST	=	Expressed Sequence Tag
EtBr	=	Ethidium Bromide
g	=	grams
G	=	Guanine
GI	=	Genbank ID
H	=	hour
IPTG	=	Isopropyl β-D-1-thiogalactopyranoside
kDa	=	Kilo Dalton
LB	=	Luria Bertani
M	=	Molar
mg	=	milligrams
ml	=	millilitres
mM	=	millimolar
mRNA	=	messenger RNA
µg	=	micrograms
miRNA	=	micro RNA

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LIST OF ABBREVIATIONS (Continued)

µL	=	microlitres
µM	=	micromolar
ng	=	nanogram
nt	=	nucleotide
PBS	=	Phosphate Buffered Saline
PCR	=	Polymerase Chain Reaction
*p*mol	=	picomole
q-RT-PCR	=	quantitative Real Time PCR
RACE	=	Rapid Amplification of cDNA Ends
RISC	=	*RNA-induced silencing complex*
RNA	=	ribonucleic acid
RNAi	=	RNA interference
rpm	=	revolutions per minute
RT	=	Reverse Transcription
SDS-PAGE	=	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
siRNA	=	short interfering RNA
SIT	=	sterile insect technique
ssRNA	=	Single-Stranded RNA
T	=	Thymine
TAE	=	Tris Acetate EDTA
Tm	=	Melting Temperature
U	=	units

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ANALYSIS OF THE *DOUBLESEX* AND *VITELLOGENIN* GENES IN RICE MOTH, *Corcyra cephalonica* Stainton

INTRODUCTION

The rice moth, *Corcyra cephalonica* (Stainton) is one of the major pests of stored cereals world. Its larval stages cause dramatically damage in many grain species such as rice, sorghum, maize, currants, gram, groundnuts, cocoa beans, peanuts, cotton seeds, linseeds, raisins, chocolates, army biscuits, nutmeg and milled products, resulting in loss of profit for farmers (Hutson, 1920; Rao, 1954; Kennard, 1965; Highlands, 1978). The use of chemical insecticides to eliminate insect pests including rice moth has still conducted, particularly in developing countries including Thailand. These chemical insecticides cause hazard to environment and human health. In recent years, the molecular mechanisms of sex determination have received special attention due to their potential use in sterile insect technique (SIT) programs for the control and elimination of insect pests.

The animal kingdom possesses a wealth of mechanisms in which gender is decided, all of which are represented among the insects. In most insects, they are monoseious, males produce sperm whereas female produce eggs. Sex is determined after fertilization to commit the embryo developing into either male or female. The sex determination genes have analysed in dipteran and nondipteran insects (Sanchez, 2008). The sex determination of *Drosophila melanogaster* (Diptera) is clearly discussed in genetic cascade. In *Drosophila melanogaster*, the flies with one X chromosome (XY) are male, and those with two X chromosomess (XX) are female. However, the sex of *D. melanogaster* is not specified by sex chromosome but by the ratio between the number of X chromosome and the number set of autosome. X-linked genes located on X chromosome act together to activate *Sex-lethal* (*Sxl*) in XX flies (Salz and Erickson, 2010). The *Sxl* gene produced SXL protein which regulates the splicing of *transformer* (*tra*) (Inoue *et al*., 1990; Bopp *et al*., 1991). The TRA protein together with TRA2 in turn promotes female-specific splicing of *doublesex*

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(*dsx*), getting DSX-F. While, in male flies, only TRA2 regulates mRNA *dsx* splicing, yielding DSX-M (Amrein *et al*., 1988; Nagoshi *et al*., 1988; Hoshijima *et al*., 1991; Inoue *et al*., 1992). The DSX-F and DSX-M then regulate the expression of downstream genes for somatic sex-specific functions (Burtis and Baker, 1989; Coschigano and Wensink, 1993; An and Wensink, 1995). *Vitellogenin* (Vg) gene is one of the target genes of DSX protein (Shukla and Nagaraju, 2010). It is a large precursor protein of egg yolk vitellin (Vn) that provides energy reserves for the embryonic development of oviparous vertebrates and invertebrates. In many insect species, Vgs are synthesized mainly in fat body cells at sex-, tissue-, and stage-specific manners. The product is secreted into the hemolymph and then sequestered by developing oocytes via receptor-mediated endocytosis (Raikhel and Dhadialla, 1992). After taken up by oocytes, Vgs are stored in cytoplasmic granules as Vns which provide raw materials for development of the embryos (Kunkel and Nordin, 1985).

Dsx gene is one of the potential candidate genes proposed to be used in sterile insect technique programs (Saccone *et al*., 2002) and control *vg* gene. Therefore, it may be useful to investigate *dsx* and *vg* gene in insects, particularly Lepidoptera which are a large number of pests worldwide. Thus in this study, the *dsx* and *vg* gene of rice moth had investigated.

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OBJECTIVES

1. To identify *doublesex* (*dsx*) and *vitellogenin* (*vg*) gene sequences of rice moth (*Corcyra cephalonica* Stainton.)

2. To study the *doublesex* RNA processing between rice moth male and female.

3. To study the function of *vitellogenin* gene using RNA interference technique.

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LITERATURE REVIEW

Rice moth (*Corcyra cephalonica* Stainton)

The rice moth, *Corcyra cephalonica* (Stainton) is a major pest of stored cereals and cereal commodities in Asia and South America as well as in other tropical and subtropical regions of the world. Its larval stages cause appreciable loss to rice, sorghum, maize, currants, gram, groundnuts, cocoa beans, peanuts, cotton seeds, linseeds, raisins, chocolates, army biscuits, nutmeg and milled products (Hutson, 1920; Rao, 1954; Kennard, 1965; Highlands,1978).

Scientific classification

Kingdom Animalia
Phylum Arthropoda
Class Insecta
Order Lepidoptera
Family Pyralidae
Genus *Corcyra*
Species *cephalonica*

The life cycle of rice moth

The life cycle of rice moth is considered one of the most advanced forms of metamorphosis in insects, called complete metamorphism or holometabola, the life cycle consists of 4 stages, egg, larva, pupa and adult (Figure 1).

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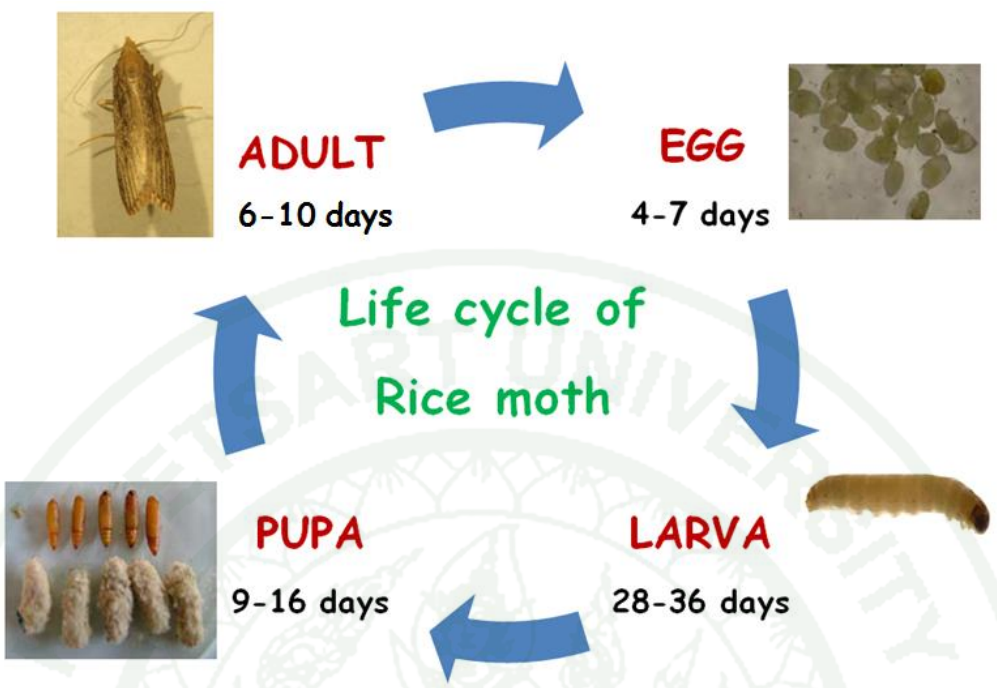

Figure 1 Life cycle of rice moth (*Corcyra cephalonica* Stainton).

Egg

Females lay eggs anywhere such as on the grains, among grains, on the containers or on any surface near the grains, either singly or in clusters. Egg is period of 4-7 days. The eggs are whitish, oval in shape, 0.5 mm long (Grist and Lever, 1969).

Larvae

Larvae are tiny and creamy-white color. They move actively and feed on broken grains and start spinning web to join grains. Full grown larvae are pale whitish in color, 15 mm long with short scattered hairs and no markings on body. Larval period is about 28-36 days depending on temperature.

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Pupae

Pupae develop inside an extremely tough, opaque whitish cocoon that is surrounded by webbed grains (Hinton, 1963). Pupae color are light brown, 7.5-8.5 mm long and a wing span of about 1.45-2.6 mm. Pupa periods is about 9-16 days but may extend to 40-50 days if the weather is cold.

Adult

After emerging from cocoons adult moths can start mating and lay egg immediately after emergence (Ayyar, 1934). Adults are light greyish-brown in color, 12 mm long and a wing span of about 15 mm, no any markings on the wings but veins are slightly darkened, a projected tuft of scales on head. Moths have short life around 6-10 days. One female can lay eggs up to 150-200 within a few days after emergence (Ayyar, 1934).

Sex chromosome

In insects, sex can be determined by various mechanisms: by Y (W) chromosomal factors, by autosomal factors, by the number of X chromosomes, by haploidy versus diploidy, or by infection of microorganisms (Ohbayashi *et al*, 2002). In *Drosophila melanogaster*, the first signal of sex differentiation is the ratio of X chromosomes to sets of autosomes (A). The X: A ratio, a balance mechanism in which X chromosomal gene products are titrated against autosomal gene products, governs sex determination. Although the sex-determining mechanism of *Drosophila melanogaster* is one of the best-understood pathways, the balance mechanism observed in *Drosophila* is not common in any other previously examined insects. For example, even in Diptera, an epistatic maleness factor found in the Y chromosomes of several species, such as the housefly *Musca domestica*, fruit fly *Ceratitis capitata*, *Lucilia cuprina* and *Bactorocera tryoni* determines sex by an epidtatic male-determining factor on the Y chromosome.

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In Lepidoptera, in contrast to most other animal groups, females are the heterogametic sex (ZW) while males are homogametic (ZZ) (Table 1). Sex chromosome systems with female heterogamety are referred to as WZ/ZZ and ZO/ZZ (alternatively designated WZ and Z0) depending on the presence or absence of a W chromosome (Traut *et al.*, 2007). Rice moth has 2n = 54 (Mohanty and Nayak, 1981).

Table 1 Lepidopteran karyotypes with identified sex chromosomes

Family	**Species**	**2n chromosome constitution**	
		Females	**males**
Arctiidae	*Lophocampa maculata*	33,Z	34,ZZ
	*Phragmatobia fuliginosa*b	57,W1W2Z	56,ZZ
Bombycidae	*Bombyx mori*	56,WZ	56,ZZ
Crambidae	*Ostrinia scapulalis*	62,WZ	62,ZZ
Gelechiidae	*Exoteleia dodecella*	23,Z	24,ZZ
	Exoteleia pinifoliella	21,Z	22,ZZ
	Phthorimaea operculella	58,WZ	58,ZZ
Lasiocampidae	*Trabala vishnu*	51,WZ1Z2	52,Z1Z1Z2Z2
Lymantriidae	*Lymantria dispar*	62,WZ	62,ZZ
	Orgyia antiqua	28,WZ	28,ZZ
	*Orgyia thyellina*c	22,WZ	22,ZZ
	*Orgyia thyellina*c	23,W1W2Z	22,ZZ
Micropterigidae	*Micropterix calthella*	57,Z	58,ZZ
Noctuidae	*Orthosia gracilis*	27,Z	28,ZZ
Pieridae	*Pieris brassicae*	30,WZ	30,ZZ
Psychidae	*Luffia lapidella*	61,Z	62,ZZ
	*Solenobia triquetrella*b	61,Z	62,ZZ
	Talaeporia tubulosa	59,Z	60,ZZ
Pyralidae	*Cadra cautella*	60,WZ	60,ZZ
	Ectomyelois ceratoniae	62,WZ	62,ZZ
	Ephestia kuehniella	60,WZ	60,ZZ
	Galleria mellonella	60,WZ	60,ZZ
	Witlesia murana	59,W1W2Z	58,ZZ
	Plodia interpunctella	60,WZ	60,ZZ
Saturniidae	*Antheraea yamamai*	62,WZ	62,ZZ
	Caligula japonica	61,Z	62,ZZ
	*Samia cynthia*c	27,Z	28,ZZ
	*Samia cynthia*c	26,WZ	26,ZZ
	*Samia cynthia*c	25,WZ1Z2	26,Z1Z1Z2Z2
	Saturnia pyri	60,WZ	60,ZZ

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Table 1 (Continued)

Family	Species	2n chromosome constitution	
		Females	**males**
Sphingidae	*Mimas tiliae*	58,WZ	58,ZZ
Tischeriidae	*Tischeria ekebladella*	46,WZ	46,ZZ
Tortricidae	*Bactra lacteana*	59,W1W2Z	58,ZZ
	Cydia pomonella	56,WZ	56,ZZ
Yponomeutidae	*Yponomeuta cagnagellus*	61,WZ1Z2	62,Z1Z1Z2Z2
	Yponomeuta rorellus	61,WZ1Z2	62,Z1Z1Z2Z2
	Yponomeuta gigas	61,WZ1Z2	62,Z1Z1Z2Z2
	Yponomeuta evonymellus	61,WZ1Z2	62,Z1Z1Z2Z2
	Yponomeuta malinellus	61,WZ1Z2	62,Z1Z1Z2Z2
	Yponomeuta padellus	61,WZ1Z2	62,Z1Z1Z2Z2

Source: Traut *et al.* (2007)

Sex determination genes

In most insects, sex determination is determined after fertilization and embryos develop into either male or female. The best study of sex-determination genes in insects came from the studies in *Drosophila melanogaster* (Diptera) (MacDougall *et al.*, 1995; Cline and Meyer, 1996; Schutt and Nothiger, 2000; Sanchez 2008).

Sex determination gene of *Drosophila melanogaster* (Diptera)

In *D. melanogaster*, sex determination is under the control of the gene *Sex lethal (Sxl)* (Cline, 1978; Penalva and Sánchez, 2003). The epistatic relationships between *Sxl* and the other sex determination genes *transformer (tra), transformer-2 (tra-2)* and *doublesex (dsx)* have revealed that a hierarchical interaction exists among them (Baker and Ridge, 1980). On the hierarchical cascade, sex-specific splicing of their primary transcripts controls the sex-specific splicing of the pre-mRNA of the downstream gene in the cascade during development (Figure 2).

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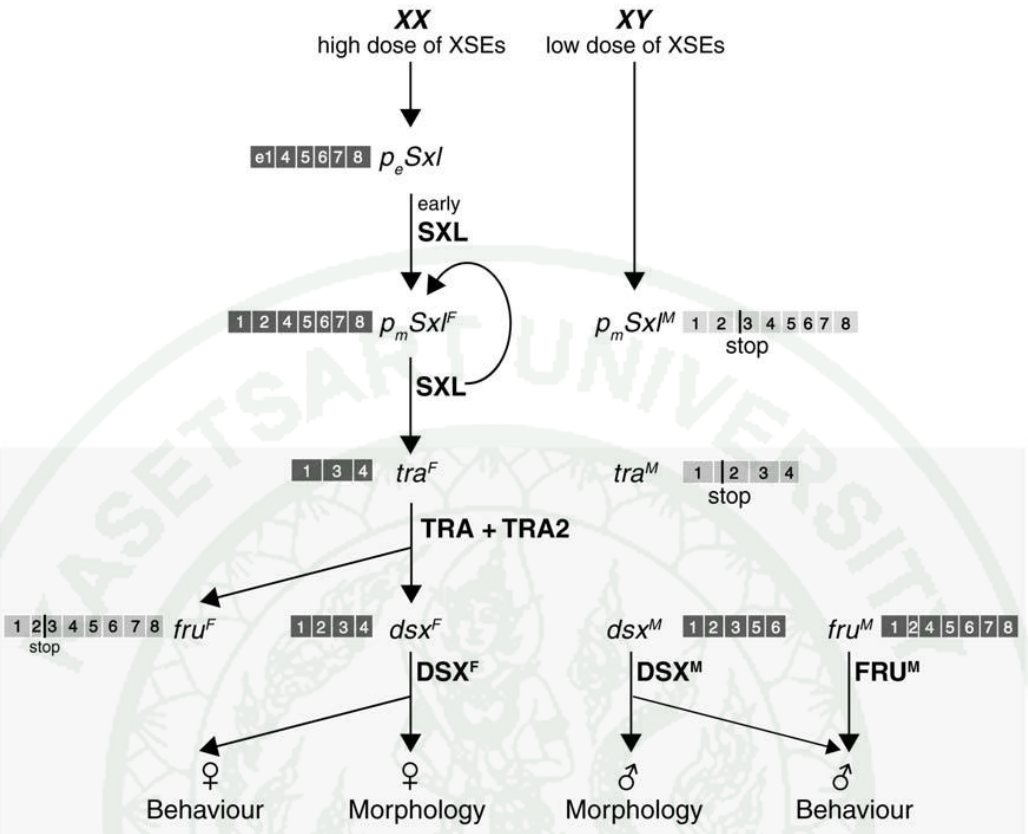

Figure 2 The sex determination cascade in *Drosophila melanogaster*.

Source: Eveline *et al*. (2010)

The process of sex determination in *Drosophila* is initiated by the signal under the control of the X:A ratio (Bridges and Anderson, 1925). *Sex-lethal* (*Sxl*), the top most gene of *Drosophila* sex determination cascade (Cline 1978, 1983; Penalva and Sanchez, 2003), contains two promoters: early and late promoters. The primary signal is based on the concentration of X-linked genes that activate. The early Sxl promoter is activated by the primary singnal produced by X-linked genes in high concentration of diploid XX individuals only at blastoderm stage. While in males X genes are present in single dose and they remain inactive (Erickson and Quintero, 2007). Once the activity of *Sxl* is established, the signal from X-linked is no longer needed (Sanchez and Nothiger, 1983; Bachiller and Sanchez, 1991). The *Sxl* late promoter (*Sxl-pm*) is active in both males and females but the transcript. Only in females, the

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Sxl transcript from the late promoter is spliced to remove the stop codon by the functional Sxl protein (Bell *et al.*, 1988; Bopp *et al.*, 1991). In males, the absence of the transcribed from late promoter had still have the stop codon resulting in the non-functional Sxl protein. An auto regulatory feedback loop ensures sustained production of functional Sxl protein throughout development only in females (Cline, 1984; Bell *et al*., 1991; Keyes *et al*., 1992; Penalva and Sanchez, 2003).

The functional Sxl protein in females regulates the splicing of its immediate downstream target gene *transformer* (*tra*) by removing the stretch encompassing the termination codon in the mature *tra* transcript, yielding the production of full length Tra protein. In the absence of Sxl protein in males *tra* splices in a default mode, leading to the selection of proximal 30 splice site including the stretch harbouring in frame stop codon resulting in truncated non-functional protein (Boggs *et al*., 1987; Belote *et al*.,1989; Valcarcel *et al*., 1993).

TRA protein in female interacts with the nonsex-specific transformer2 protein (TRA2) (Amrein *et al.*, 1988) and binds to the *dsx* transcript in the middle of exon 4, called the *dsx* repeat element (*dsx*RE). This *dsx*RE contains six copies of the 13 nucleotide sequence TC (T/A)(T/A)C(A/G)ATCAACA (Tian and Maniatis, 1993). Located between repeat element five and six of the *dsx*RE is a purine-rich enhancer element (PRE) which is required for the specific binding of TRA2 to the dsxRE (Lynch and Maniatis, 1995).The binding of TRA/TRA2 to the *dsx*RE and PRE sites retains exon four in the *dsx* pre-mRNA resulting in female-specific splicing of *dsx* at the bottom of the cascade (Hoshijima *et al*., 1991; Hedley and Maniatis, 1991; Ryner and Baker, 1991). The *dsx* premRNA sex-specifically splices to produce sex-specific transcripts which encode sex-specific proteins, DsxF in female and DsxM in male. (Figure2).

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Homologous of *doublesex* gene in other insects

Dsx in Diptera

Outside drosophilids, homologues of *dsx* have been found in many other dipterans, including *Megacelia scalaris* (Sievert *et al*., 1997; Kuhn *et al*., 2000), *Musca domestica* (Hediger *et al*., 2004), and *Anopheles gambiae* (Scali *et al*., 2005), in the tephritids *Bactrocera tryoni* (Shearman and Frommer 1998), *Bactrocera oleae* (Lagos *et al*., 2005), *Ceratitis capitata* (Saccone *et al*., 2008) and *A. obliqua* (Ruiz *et al*., 2005, 2007). The pre-mRNAs of all the dipteran *dsx* studied to date sex-specifically splice to produce sex-specific transcripts that encode putative male-specific and female-specific Dsx proteins having common N-terminal but different C-terminal regions. As in the case of *Drosophila dsx*, the *dsx* pre-mRNA of other dipterans also contain Tra-Tra2 binding sites (*dsx*RE elements) in the female-specific exon, suggesting a common mode of sex-specific splicing of *dsx* in dipterans; default splicing in males and Tra-mediated splicing of female-specific exon (Sanchez, 2008). An exception to the sex-specific splicing of *dsx* pre-mRNA among dipterans is seen only in *M. domestica*, where the male specific exon (exon m) of *M. domestica dsx* (*Mddsx*) lies within the female-specific intron (intron 3). A similar type of splicing is also found in the Australian sheep blowfly, *Lucilia cuprina* (Calliphoridae) (Concha *et al*., 2010)

Dsx in Hymenoptera

The pre-mRNA of *A. mellifera dsx* gene (*Amdsx*) splices to produce three types of transcripts (Cho *et al*., 2007): one male-specific (*Am-dsxm*), two female-specific (*Am-dsxf1* and *Am-dsxf2*), and one common to both male and female (*Am-dsxb*). ORFs of both the female-specific *Am-dsx* transcripts (*Am-dsxf1* and *Am-dsxf2*) are identical leading to the production of the same putative protein from both transcripts. In this way, *A. mellifera* is similar to dipteran insects where *dsx* has been characterized to produce one male and one female specific Dsx protein. Initial studies by Cho *et al*. (2007) suggested that the splicing pattern of *Am-dsx* shows similarity to

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that of *Drosophila dsx* as well as to that of *Bombyx dsx* (*Bmdsx*). Comparison of the female-specific transcript *Amdsxf1* with the male-specific transcript (*Am-dsxm*) indicates that the splicing pattern of *Am-dsx* is similar to that of *Bmdsx*; the female-specific exon is having a strong splice site thus leading to the female default splice form, and skipping of the female-specific exons occurs in males because of the presence of splicing suppressor. Also, as in the case of *Bmdsx*, there is no *dsx*RE or PRE in the female-specific exons and the 3'splice site preceding the female-specific exon is supposedly not weak (probably leading to the female splice form as the default form) (Cho *et al*., 2007). Similarity in splicing pattern of *Am-dsx* and *Drosophila dsx* is evident from the comparison of another female-specific transcript, *Am-dsxf2* with the male-specific transcript (*Am-dsxm*); the sex-specific splice forms differ from each other by the differential polyadenylation sites (Cho *et al*., 2007). Later, the knockdown studies by Hasselmann *et al*. (2008) discarded the possibility of any of the female splice form of *Am-dsx* to be the default form (and hence any similarity of *Am-dsx* with *Bmdsx*) since RNAi-induced knockdown of *fem* and *csd* alleles in separate experiments resulted into male-specific *Am-dsx* splice variant in females, whereas no change was observed in males. Unlike *Apis*, the haplodiploid wasp, *N. vitripennis* produces one male and one female-specific *dsx* (*Nvdsx*) splice form (Oliveira *et al*., 2009). Although *Nvdsx* clusters together with that of *Apis*, the DM domains of these two hymenopterans show a high degree of amino acid divergence (Oliveira *et al*., 2009). As mentioned above *Nvdsx* is sex-specifically spliced, in females, by a zygotically active *transformer* gene that is autoregulated by maternally provided *Nvtra* transcript (Verhulst *et al*., 2010). The *Nvdsx* in haploid embryos which are unable to activate the autoregulatory loop of *tra*, splices in a default male mode (Verhulst *et al*., 2010). Recently, putative binding sites for sex-specific splicing factors found in *Nasonia fruitless* and *doublesex* as well as *A. mellifera doublesex* transcripts have been identified, which suggest that similar factors in both hymenopteran species (*A. mellifera* and *N. vitripennis*) could be responsible for sex-specific splicing of both the genes (Bertossa *et al*., 2009), though a functional demonstration is required to confirm this point.

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Dsx in Lepidoptera

Among lepidopterans, *dsx* homologues have been characterized in *B. mori* (Ohbayashi *et al*., 2001), *A. assama* and *A. mylitta* (Shukla and Nagaraju 2010), *O. scapulalis* (Sugimoto *et al*., 2010) but the molecular mechanism involved in the sex-specific splicing of *dsx* pre-mRNA has been investigated only for *B. mori* (*Bmdsx*). In an earlier study, pre-mRNA of *Bmdsx* was shown to be sex-specifically spliced to generate one female (*Bmdsxf*) and one male (*Bmdsxm*) specific mRNA which is expressed in various tissues at larval, pupal and adult stages of the silkworm (Ohbayashi *et al*., 2001). Recently, one more female specific splice form of *Bmdsx* pre-mRNA has been characterized which differs from the *Bmdsxf* by the presence of additional stretch of 15 bp (GTACGGACTTTA ATA) after exon 3 as a result of alternative 5'splice site selection (Shukla and Nagaraju 2010). This novel splice form expresses in various tissues and at different developmental stages. *Bmdsx* gene consists of six exons; the female-specific *Bmdsx* transcripts (*Bmdsxf1* and *Bmdsxf2*) contain all the six exons out of which the first four exons constitute its ORF. In male-specific *Bmdsx* transcript (*Bmdsxm*), exons 3 and 4 are skipped and the resulting ORF includes exons 1, 2 and 5 (Suzuki *et al*., 2001) (Figure 3). Exon 5 encodes male-specific C-terminal amino acid sequence, while it is transcribed as a 3'-UTR in females. The ORF of *Bmdsxf2* differs from that of *Bmdsxf1* by the early occurrence of stop codon in the former resulting in the putative protein (BmDsxF2) which differs from BmDsxF1 at its extreme C-termini. The isoforms of the DSX protein (DSX-F and DSX-M) then regulate the expression of downstream genes for somatic sex-specific functions (Burtis and Baker, 1989; Coschigano and Wensink, 1993; An and Wensink, 1995). *Vitellogenin* (Vg) gene is one direct target gene of DSX protein (Shukla and Nagaraju, 2010).

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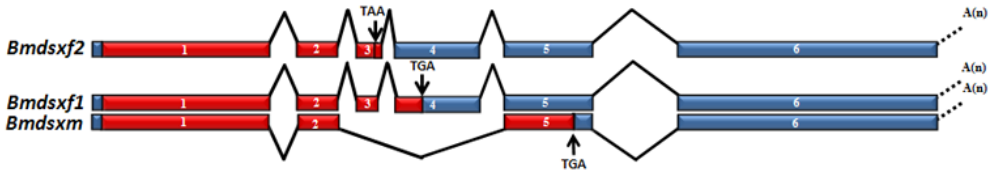

Figure 3 Pre-mRNA of *Bmdsx* splices to produce three products i.e., *Bmdsxf1*, *Bmdsxf2* and *Bmdsxm*. Boxes represent exons, whereas lines represent introns. Exons have been numbered. A small stretch of 15 nt (GTACGGACTTTAATA) after exon 3 (small box after exon 3) generated as a result of alternative 5'splicing, leads to the production of second female splice form i.e., *Bmdsxf2*.

Source: Shukla *et al*. (2010)

Vitellogenin **gene**

Vitellogenin (Vg), a major lipoprotein in many oviparous animals, is a precursor of major yolk protein vitellins (Vn). It has been shown that Vg is secreted by the liver in vertebrates and the intestine in nematodes. In vertebrates, Vg production is in control of estrogen receptor (ER) pathway. However, in invertebrates such as bivalves, vitellogenesis seems to be under the control of both estradiol (E2) and a neuropeptide (Osada *et al*., 2003). In vertebrates, Vg levels can remain high in the plasma for some time as Vg proteins degrade slowly (Denslow *et al*., 1999). Therefore, measurement of plasma Vg levels, especially in immature females and males, is considered a useful biomarker of exposure to estrogenic compounds (Hansen *et al*., 1998; Okoumassoun *et al*., 2002; Marin and Matozzo, 2004; Matozzo *et al*., 2008). In insects, the vitellogenesis generally proceeds from the biosynthesis of Vg, the processing in the fat body, the secretion of the processed Vg into the hemolymph, the selective uptake by competent oocytes and finally to the utilization of Vn by the developing embryo (Byrne *et al*., 1989; Wyatt, 1991; Raikhel and Dhadialla, 1992; Izumi *et al*., 1994; Hagedorn *et al*., 1998; Sappington and Raikhel, 1998; Giorgi *et al*., 1999). During these processes, Vg and Vn are modified through cleavage,

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glycosylation, lipidation and phosphorylation (Raikhel and Dhadialla, 1992; Sappington and Raikhel, 1998).

Vitellogenin gene in insect

Vitellogenin (Vg) is precursor of major yolk protein in insects (Wahli *et al*., 1981; Kunkel and Nordin, 1985; Sappington *et al*., 2002), and synthesized one or more large precursors (Della-Cioppa and Engelmann, 1987) in fat body and secreted into the hemolymph. It will then be up taken by vitellogenin receptors (VgRs) through receptor-mediated endocytosis (Raikhel and Dhadialla, 1992; Sappington and Raikhel, 1998; Snigirevskaya and Raikhel, 2005). Vgs played important roles in promoting growth and differentiation of oocytes and transporting metallic ions, lipid, thyroxine, vitamin A, carotenoid and riboflavin into oocyte. So far, Vgs have been sequenced from 25 insect species. Alignment of the Vgs sequences revealed that some conserved features such as cysteine residues, the GL/ICG and DGXR motifs, were found (Tufail and Takeda, 2008) at the carboxy terminal. Among the Lepidoptera insects, the Vgs have been identified from *Bombyx mori* (Yano *et al*.,1994a; Yano *et al*.,1994b), *Bombyx mandarina* (Meng *et al*., 2006b), *Actias selene* (Yin *et al*., 2007), *Antheraea pernyi* (Yokoyama *et al*.,1993; Liu *et al*., 2001, Zhu *et al*., 2010), *Antheraea yamamai* (Liu *et al*., 2001; Meng and Liu, 2006a), *Saturnia japonica* (Meng *et al*., 2008), *Samia cynthia ricini*, *Lymantria dispar* (Hiremath and Lehtoma, 1997) and *Philosamia cynthia ricini* (Liu *et al*., 2003). Researches on insect Vgs provide great theoretical and practical significances for utilization of beneficial insects and prevention of harmful insects (Brownes, 1986). The primary structures of many insect Vgs (Tufail and Takeda, 2008) and some advanced structure are known (Sharrock *et al*., 1992).

Structure of Vitellogenins

Vitellogenins are large (200-700 kDa) homologous phosphoglyco-lipoproteins that are often oligomeric in their native state, with monomers consisting of one to four subunits. In most insect, Vg monomer are compose two subunits (one

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of large subunit > 150 kDa and one of small subunit < 65 kDa) (Kunkel and Nordin, 1985; Raikhel and Dhadialla, 1992; Valle, 1993) derived from the cleavage of a single precursor in the fat body (Bose and Raikhel, 1988; Dhadialla and Raikhel, 1990; Heilmann *et al*., 1993; Kageyama *et al*., 1994; Yano *et al*., 1994; Hiremath and Lehtoma, 1997). Exceptions include the Vgs of higher Hymenoptera (suborder Apocrita) (Wheeler and Kawooya, 1990; Kageyama *et al*., 1994; Nose *et al*., 1997) and two species of whitefly (Homoptera) (Tu *et al*., 1997) which are not cleaved. There is evidence that some Orthopteran and sawfly Vgs consist of three or four subunits (Kunkel and Nordin, 1985; Della-Cioppa and Engelmann, 1987; Wyatt, 1988; Kim and Lee, 1994; Takadera *et al*., 1996). However, it is possible that some of the observed multiple subunits are an artifact of cleavage by proteases during sample preparation, and more experiments are needed to confirm these observations.

Polyserine domains

The most striking feature of vertebrate and some insect Vg primary structures are the presence of one to three domains containing long runs of serine residues. Three of the six sequenced insect Vgs contain polyserine domains: two in the silk moth and sawfly Vgs, They contain two polyserine domains and the mosquito Vg habor three domains. There is a region in the wasp Vg rich in serines (corresponding to the second polyserine domain of mosquito, silk moth, and sawfly) but they are not arranged as a long run. The serine runs arose through serial amplification of serine codons, and are located in regions that are evolving at a faster rate than most of the rest of the gene (Byrne *et al*., 1989; LaFleur *et al*., 1995; Chen *et al*., 1997).

The role of the polyserine domains is unknown, and their presence cannot yet be correlated with peculiarities in the life histories of the insects harboring them. Their significance probably lies in being good substrates for kinases. Vertebrate phosvitin is indeed heavily phosphorylated (Byrne *et al*., 1989), as is mosquito Vg (Dhadialla and Raikhel, 1990), but the phosphorylation sites in the latter have not been identified. Phosphoserine tracts represent extreme concentrations of negative

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charge (Goulas *et al*., 1996), which may promote solubility of the Vg (Gerber-Huber *et al*., 1987) or provide a region for chelating essential metal ions such as Ca^{2+} and Fe^{3+} (Nardelli *et al*., 1987; Taborsky, 1991). Dephosphorylation of Vg reduces its uptake by oocytes (Miller *et al*., 1982; Dhadialla *et al*., 1992), suggesting a role of phosphorylated residues in VgR recognition or maintenance of tertiary structure. *Bombyx mori* Vg is further phosphorylated during embryogenesis, possibly as a signal to initiate its proteolysis (Takahashi *et al*., 1992; Izumi *et al*., 1994).

Cleavage site of the VG precursor

Most of the insect Vgs for which the sequences are known are of the type that are cleaved once in the fat body to produce two subunits. The cleavage sites are immediately preceded by a motif, (R/K)X(R/K)R or RXXR, specifically recognized by the subtilisin-like proprotein endoproteases, convertases (Barr, 1991; Rouille´ *et al*., 1995). In the case of the wasp Vg which is not cleaved (Nose *et al*., 1997), the sequence at the presumed ancestral cleavage site has been mutated from RXRR to LYRR and is apparently no longer recognized by convertases. In addition to the recognition motif, each cleavage site is either immediately preceded or followed by a predicted b-turn, a requirement for optimal recognition by this family of endoproteases (Brakch *et al*., 1993). Indeed, a mosquito fat-body-specific convertase has been cloned and sequenced, and when coexpressed with mosquito Vg in an *in vitro* trancription/translation system, the convertase correctly cleaved the pro-Vg (Chen and Raikhel, 1996). The primary structure of this mosquito vitellogenin convertase (VC) is similar to that of human and *Drosophila* furins (Barr *et al*., 1991; Roebroek *et al*., 1991, 1992), and another *Drosophila* convertase, dKLIP-1 (Hayflick *et al*., 1992). Unlike the mosquito VC, however, the native substrates of the *Drosophila* convertases have not yet been identified. Interestingly, the putative cleavage site for mosquito VC auto-activation is a paired di-basic motif, RAKR211-RPKR215 (Chen and Raikhel, 1996). The probable cleavage site of *C. elegans* Vg6 and *Dolichorabditis* Vg (Winter *et al*., 1996) resemble those of insects, but *C. elegans* Vg5 has an RSRR motif in the same relative position and is not cleaved. The reason for its not being cleaved is not readily apparent from its primary sequence, but unlike

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the other sites, the nematode Vg5 cleavage motif is immediately flanked on both sides by probable b-turns rather than having a turn on one side only. Although the cleavage sites in insect and nematode Vgs are structurally similar, their location in the precursor is not strictly conserved. In *Anthonomus, Aedes, Bombyx,* and *Athalia*, the cleavage site is near the N-terminus of the precursor (Heilmann *et al*., 1993; Chen *et al*., 1994; Kageyama *et al*., 1994; Yano *et al*., 1994), but this site has been lost at least twice, once within the Lepidoptera e.g., *L. dispar* (Hiremath and Lehtoma, 1997), and once within the Hymenoptera e.g., *P. nipponica* (Nose *et al*., 1997). In the case of *L. dispar*, the Vg is still cleaved, but the cleavage site is located near the C-terminus (Hiremath and Lehtoma, 1997). In nematodes the site is nearer the center of the precursor (Winter *et al*., 1996). However, it is clear that the cleavage sites have not moved via exon shuffling, because multiple alignments indicates a one-to-one correspondence of domains along the length of the precursors (Chen *et al*., 1997). The common insect cleavage sites are in a region of relatively low conservation; an area not represented in vertebrate or nematode Vgs at all, and is flanked by polyserine domains. The putative nematode cleavage site (Winter *et al*., 1996) is also located in a region of low conservation (Chen *et al*., 1997). The *Lymantria* cleavage site is within a domain of comparatively high conservation, but its immediate vicinity appears to have undergone relatively rapid evolution (as suggested by low sequence conservation compared to other regions of the domain). Thus, the evolutionary loss or relocation of the Vg cleavage site is due to local modifications in the amino acid sequence, not to rearrangement of the gene.

RNA interference (RNAi)

RNA interference (RNAi) is a newly identified mechanism of post-transcriptional regulation of gene-expression which is highly conserved among higher eukaryotes (Carthew and Sontheimer, 2009; Berezikov, 2011). RNAi, initially coined by Fire *et al.*, (1998), is the sequence-specific gene silencing induced by double-stranded RNA. This phenomenon has generated an explosion of interest and enthusiasm by scientists, similar to that created by the entrance of the green fluorescent protein as a species-independent molecular reporter in the mid-1990s.

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Since its inception, RNAi has been induced in organisms ranging from trypanosomes (Ngo *et al.*, 1998) to nematodes (Fire *et al.*, 1998) to flies (Kennerdell and Carthew, 1998) to vertebrates (Wianny and Zericka-Goetz, 2000). Because of its specificity, efficiency, and cost-effectiveness, RNAi has drawn the bulk of attention away from previous antisense methods such as ribozymes and oligodeoxynucleotides (ODNs). Furthermore, technical expertise accumulated from previous approaches is now being applied to RNAi, thus rapidly advancing its application to the medicinal domain (Kim, 2003). Currently, three areas of human disease research have already strongly embraced the RNAi phenomenon: infectious disease, cancer, and dominantly inherited disorders (Kim, 2003; Wall and Shi, 2003). RNAi has also shown exceptional promise as a genome-wide screening tool (Kamath *et al*., 2003; Kiger *et al.*, 2003; Berns *et al.*, 2004; Paddison *et al.*, 2004) The level and complexity of gene regulation by RNAi appears to be much more intricate than originally anticipated, which has led to the opinion that this process is the principle means of fine tuning proteins levels in cells (Bartel, 2009).

RNAi in insects

To avoid confusion when talking about different mechanisms involved in RNAi, clear definitions should be determined. We would like to adopt the definitions proposed by Whangbo and Hunter (2008) to discuss RNAi and the uptake mechanisms of dsRNA in insects. RNAi can be divided in cell-autonomous and noncell-autonomous RNAi. As the name suggests, in the case of cell-autonomous RNAi, the silencing process is limited to the cell in which the dsRNA is introduced/expressed and encompasses the RNAi process within individual cells. The mechanism of cell-autonomous RNAi is best known: dsRNA is cleaved by an RNaseIII, often called Dicer, into 21-25 nt-long short interfering RNA duplexes (siRNA). These siRNAs are incorporated in the RNA induced silencing complex (RISC); after discarding the passenger strand, the RISC will bind to a homolog mRNA, cutting it and thereby hindering translation (Figrue 4).

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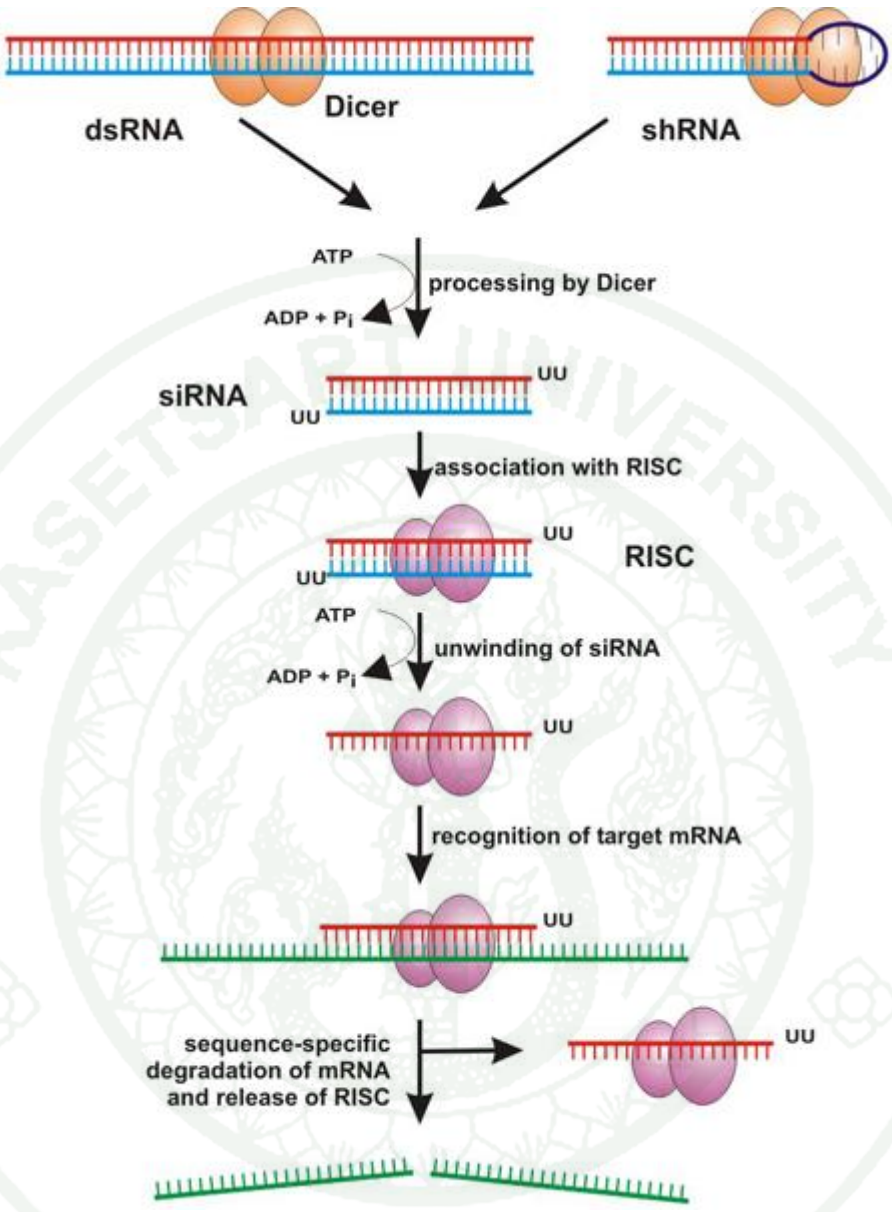

Figure 4 The RNAi process within individual cells.

Source: Rutz and Scheffold (2004)

In case of non-cell-autonomous RNAi, the interfering effect takes place in tissues/cells different from the location of application or production of the dsRNA. There are two different kinds of non-cell-autonomous RNAi: environmental RNAi and systemic RNAi (Figure 5). Environmental RNAi describes all processes in which dsRNA is taken up by a cell from the environment. Therefore, this process can also be

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observed in unicellular organisms. Systemic RNAi can only take place in multicellular organisms because it includes processes in which a silencing signal is transported from one cell to another or from one tissue type to another. In multicellular organisms environmental RNAi can be followed by systemic RNAi and non-cell-autonomous RNAi will always be followed by cell-autonomous RNAi. Research on non-cell-autonomous RNAi is mainly conducted on plants and the model organism *C. elegans*. More detailed information on environmental RNAi is reviewed by Whangbo and Hunter (2008) and on systemic RNAi by van Roessel and Brand (2004), Grishok (2005) and Jose and Hunter (2007). For the efficient application of RNAi in insect control, we have to focus on non-cell-autonomous RNAi. The insect will have to internalize the dsRNA of a target gene through feeding. In order to silence the target gene, this dsRNA must be taken up from the gut lumen into the gut cells, representing environmental RNAi. If the target gene is expressed in a tissue outside of the gut, the silencing signal will also have to spread via cells and tissues, which is systemic RNAi.

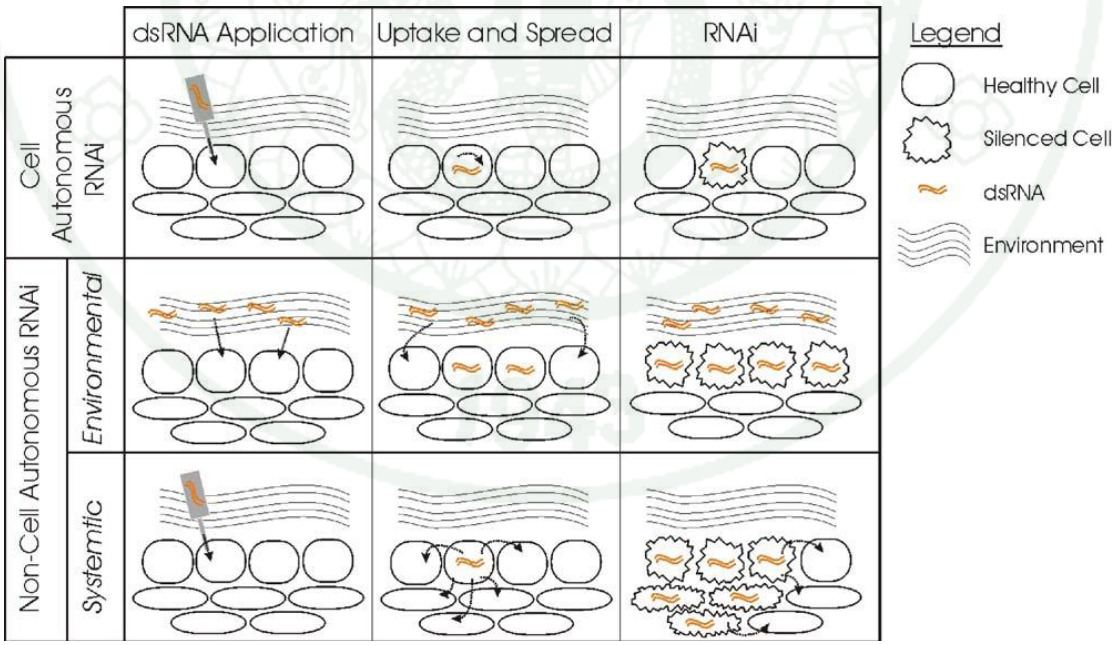

Figure 5 RNAi in cell-autonomous and noncell- autonomous.

Source: Huvenne and Smagghe (2010)

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Methodology of dsRNA uptake in insects

Methods of dsRNA uptake in insects can greatly vary and strongly influence the efficiency of gene silencing, thus their potential as insect pest control agent. It is important to note that since gene silencing is only limited to cells that are infected, the main challenge is the selection of the delivery system (Terenius *et al*., 2011). In both types, methods of delivery must be first defined, being effectively easier and better understood for cell-autonomous RNAi machinery (Siomi and Siomi, 2009). The main uptake (or delivery) methods include injection, soaking, feeding, transgenic technique, and viral infection. This section examines these various mechanisms and their effectiveness in delivering RNAi and gene silencing in various species.

Microinjection

Microinjection is the direct injection of dsRNA into the body of insects, has been one of the most effective delivery methods for systemic RNAi types. Short dsRNA have had the most success with this mechanism (Siomi and Siomi, 2009). In addition, the 5' end of the dsRNA can affect the effectiveness of RNAi; a phosphorylated 5' end exhibits better gene silencing rate than does a hydroxylated 5' end (Boutla *et al*., 2001). The major advantage of injecting dsRNA into the insect body is the high efficiency of inhibiting gene expression. There are however some limitations with micro-injection. First, the cost for in vitro synthesis and storage of dsRNA is relatively high, and the steps are complicated. In addition, injection pressure and the wound generated inevitably affect the insects. It has been shown that skin damage stimulates the immune response. In practice, this delivery method would have very limited application as pest control agent.

Soaking

Soaking *D. melanogaster* embryos in a dsRNA solution can inhibit gene expression, and its effectiveness is comparable to the injection method in that it requires a higher concentration of dsRNA (Eaton *et al*., 2002). Soaking *D.*

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melanogaster S2 cells in *CycE* and *ago* dsRNA solutions has been shown to effectively inhibit the expression of these two genes for cell cycle, thereby elevating levels of protein synthesis (March and Bentley, 2007). The soaking method is suitable only for certain insect cells and tissues as well as for specific insects of developmental stages that readily absorb dsRNA from the solution, and therefore, it is rarely used.

Feeding of artificial diet

Compared to other methods, dsRNA feeding is the most attractive primarily because it is convenient and easy to manipulate. Since it is a more natural method of introducing dsRNA into insect body, it causes less damage to the insect than microinjection (Chen *et al*., 2010). It is especially popular in very small insects that are more difficult to manipulate using microinjection. Early insect RNAi feeding studies were frustrating; for example, the injection of dsRNA effectively silenced the aminopeptidase gene *slapn*, which is expressed in the midgut of *Spodoptera littoralis*, but feeding with dsRNA did not achieve RNAi (Rajagopal *et al*., 2002). Fortunately, there are other studies showing that dsRNA feeding can be successful for RNAi studies in insects. Feeding dsRNA to *E. postvittana* larvae has been shown to inhibit the expression of the carboxylesterase gene *EposCXE1* in the larval midgut and also to inhibit the expression of the pheromone-binding protein *EposPBP1* in adult antennae (Turner *et al*., 2006). dsRNA feeding also inhibite the expression of the nitrophorin 2 (*NP2*) gene in the salivary gland of *Rhodnius prolixus*, leading to a shortened coagulation time of plasma (Araujo *et al*., 2006). dsRNA feeding has also been successful in many other insects, including insects of the orders Hemiptera, Coleoptera, and Lepidoptera (Baum *et al*., 2007; Mao *et al*., 2007).

The main challenge remains that there needs to be a greater amount of material for delivery as silencing has been shown to be incomplete (Chen *et al*., 2010). This phenomenon has been observed after ingestion of *CELL-1* dsRNA by the termite *Reticulitermes flavipes* (Zhou *et al*., 2008), *TPS* dsRNA in *N. lugens* nymphae (Chen *et al*. 2010), *Nitrophorin 2* dsRNA by *Rhodnius prolixus* (Araujoa *et al*., 2006). In addition, different species of insects have different sensitivities to RNAi molecules

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when delivered orally. For example, *Glossina morsitans* fed with dsRNA may effectively inhibit the expression of *TsetseEP* in the midgut, but cannot inhibit the expression of the transferrin gene *2A192* in fat bodies due to lack of transfer capacity between tissues (Walshe *et al*., 2009). The mechanisms associated with the transfer of gene expression through feeding delivery method still need further study. In addition, one method that may be better than direct feeding with dsRNA is the use of transgenic plants to produce dsRNA (Baum *et al*., 2007; Mao *et al*., 2007). The advantage of this method is the generation of continuous and stable dsRNA material. Genetically engineered dsRNA-producing yeast strains have also been developed to feed *D. melanogaster*, but gene silencing was not successful (Gura, 2000). However, dsRNA produced in bacteria is effective in *C. elegans* (Timmons and Fire, 1998). Therefore, the use of bacteria, especially insecticidal microorganisms, to produce dsRNA for insect RNAi needs further study.

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MATERIALS AND METHODS

Materials

1. Insect

Rice moth, *C. cephalonica* are kindly given by Postharvest and Products Processing Research and Development Office, Department of Agriculture, Ministry of Agriculture and Cooperatives. Then, we cultured at the Department of Genetics, Faculty of Science, Kasetsart University.

2. Bacterial strain

Escherichia coli strain DH5α

3. Type of vector

pGEM-T® easy vectors (Promega, USA)

4. Chemicals and Reagents

4.1 General Chemicals and Reagents

Absolute ethanol (C_2H_5OH) (Merck, Germany)
Acetic acid glacial (CH_3COOH) (BDH, UK)
Bromophenolblue (Merck, Germany)
Chloroform (Merck, Germany)
Glucose (Sigma, USA)
Glycerol (BDH, UK)
Hydrochloric acid (Merck, Germany)
Isoamyl alcohol (Merck, Germany)

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Isopropanol (Merck, Germany)

Nuclease-free water

Potassium chloride (Sigma, USA)

Sodium acetate (Merck, Germany)

Sodium chloride (Merck, Germany)

Sodium dodecyl sulfate (SDS) (Merck, Germany)

Sodium hydroxide (BDH, UK)

Xylene cyanol FF (Sigma, USA)

4.2 Chemicals for Bacterial Culture

Bacto agar (Difco, USA)

Bacto tryptone (Difco, USA)

Tryptone (Difco, USA)

Yeast extracts (Difco, USA)

4.3 Chemicals and Reagent for Molecular Cloning

Agarose (Sekem)

Ampicillin (Sigma, USA)

5-bromo-4-chloro-3-indoyl-ß-D-galactopyranoside (X-gal) (Sigma, USA)

Chloroform (Merck, Germany)

Dextran sulphate (Sigma, USA)

Diethyl pyrocarbohydrate (DEPC)

100 mM dATP, dCTP, dGTP, and dTTP (Thermo Scientific)

DNA marker: lampda DNA, 1 Kb plus DNA ladder and 100 bp ladder (Thermo Scientific)

Ethanol, absolute (Merck, Germany)

Ethidium bromide (EtBr) (Sigma, USA)

Ethylene diamine tetraacetic acid, disodium salt dihydrate (EDTA) (Merck, Germany)

GeneDireX PiNk Plus Prestained Protein Ladder (USA)

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Isopropyl-ß-D-thiogalactoside (IPTG) (Sigma, USA)

Phenol (Sigma, USA)

Tris-(hydroxy methyl)-aminomethane (Sigma, USA)

4.4 Reagent Kit for Molecular Cloning

RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) TRIzol reagent (Gibco BRL, USA)

Favorgen® Gel/PCR purification kit

FavorPrep™ Plasmid Extraction MiNi kit

GeneRacer™ Kit (Invitrogen)

SMARTer™ RACE cDNA Amplification Kit User Manual (Clontech)

FavorPrep™ Tissue Genomic DNA Extraction Mini Kit

4.5 Enzyme and Restriction enzymes

Advantage® 2 PCR Enzyme System (Clontech, USA)

Emerald Amp® PCR master Mix (Takara, japan)

Restriction enzymes *Eco*RI (New England BioLabs, UK)

T7 RNA polymerase (New England BioLabs, UK)

Taq DNA polymerase (recombinant) (Thermo Scientific)

ThermOne™ Real-Time Premix (SYBR Green) (RBCBioscience)

RNase-free DNase (Thermo Scientific)

RNase innibitor (New England BioLabs, UK)

5. Equipments for analysis

Autoclave: Model HA-300M

Autopipette: Pipetteman (Gilson, France)

Centrifuge, refrigerated centrifuge: Model BR 4i (JOUAN SA)

Centrifuge, microcentrifuge: Model Spectrafuge 16M

Electrophoresis unit: Submerged Agarose Gel Electrophoresis System

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Hamilton microsyringe (10 µl)

Incubator: Model IPR 150.XX2.C

Incubator shaker: Model IOC400.XX2.C (GALLENKAMP PLC)

Incubator water bath: Model INNOVA 3100 (New Brunswick Scientific)

Larminar flow: BH-120 (GelmanScience)

Magnetic stirrer

Microwave

Olympus stereomicroscope (Leica MX8)

Olympus DP72 digital camera

Olympus SZX16 for screening

pH meter

Thermal Cycler (Px2Thermal cycle: Thermo hybrid)

UV Visible spectrophotometer (Beckman)

Vortex mixer

Spectrophotometer

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Methods

1. Cloning and sequencing of *doublesex* gene in *C. cephalonica*

1.1 RNA Isolation

1.1.1 Total RNA isolation from whole body of *C. cephalonica* female and male

Total RNA was extracted from whole body of *C. cephalonica* using TRIzol reagent (Gibco BRL, USA). Fifty microgram of the whole body was homogenized by a pestle in 500 µl TRIzol reagent and incubated at room temperature for 5 min to dissociate nucleoprotein complex. The mixture was centrifuged at 12,000 rpm for 10 min at 4 °C. Supernatant was removed into a new microcentrifuge tube and 200 µl of chloroform was added into that tube, shake the tube vigorously by hand for 15 sec and then incubated for 15 min on ice and 3 min at room temperature for precipitation proteins. The mixture was centrifuged at 12,000 rpm at 4°C for 15 min. Following centrifugation, the mixture separated into a lower red phase, phenol-chloroform phase and a colorless upper phase. The upper aqueous phase was transferred to a new microcentrifuge tube. The RNA was precipitated by mixing with 200 µl of isopropanol. The sample was incubated at -20 °C for 1 hr. Total RNA was precipitated by centrifugation at 12,000 rpm at 4 °C for 10 min. After the supernatant was removed, the RNA pellet was washed twice with 75% (v/v) ethanol 500 µl and centrifuged at 7,500 rpm at 4 °C for 5 min. The washed RNA pellet was air-dried until ethanol evaporated completely and dissolved in RNase-free water (DEPC-treated water) 20 µl.

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1.1.2 Determination of RNA quality and concentration

1.1.2.1 Spectrophotometric determination

RNA concentration was estimated by UV spectrophotometer at the absorbance 260 nm (A260). An absorbance of 1.0 corresponds to 40 µg/ml of RNA.The RNA concentration of each sample was calculated in µg/ml by the following equations:

[RNA] = A260 x dilution factor x 40 µg/ml

Quality of the purified total RNA was determined by calculating the A260 /A280 ratio. The ratio between 1.8 and 2.0 indicated good quality of the RNA. The RNA was stored at -20 ºC. For long term storage, the total RNA was precipitated and kept in absolute ethanol at -80 ºC.

1.1.2.2 Agarose gel electrophoresis

Agarose was mixed with 1X Tris-acetate EDTA (TAE) buffer and heat in a microwave until complete solubilization. An appropriate comb was selected for forming the sample slot in the gel. The warm agarose (55 °C) was poured into the casting tray. After the gel setting completely, the comb was removed carefully. The gel is placed in an electrophoresis chamber containing TAE buffer.

The RNA sample was mixed with 6X gel-loading buffer and the sample mixture was loaded slowly into the slots of the submerged 1% agarose gel. The lid of the gel chamber was closed and the electrophoresis was carried out in 1X TAE running buffer. The RNA should migrate toward the positive anode at 100 volts and the bromophenol blue should migrate from the wells into the body of the gel within a few min. The gel was run until the bromophenol blue and xylene cyanol FF have migrated to an appropriate distance through the gel. After finishing, the gel was stained in 0.1 mg/ml ethidium bromide (EtBr) solution for 10 min and destained by

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submerging in an excessive amount of distilled water for 2 min. The nucleic acid bands were visualized under UV transilluminator.

1.2 Amplification and sequencing *doublesex* gene

1.2.1 Synthesis the first strand cDNA

First strand cDNA was synthesized with 1 µg of total RNA (from section 1.1.1) using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). The reaction mixture of 20 µl contained 3 µl of total RNA, 1 µl of oligo$(dT)_{18}$ primer, and add nuclease free water up to 10 µl. The solution reaction was mixed gently, centrifuged briefly and incubate at 65 ºC for 5 min. Chilled on ice, spun down and place the vial back on ice. The 4 µl of 5X Reaction Buffer, 1 µl of RiboLock RNase Inhibitor (20u/µl) 2 µl of 10 mM dNTP mix and 1 µl of RevertAid M-MuLV Reverse Transcriptase (200u/µl) were added into the solution mixture. The solution reaction was mixed and incubated at 42 ºC for 60 min and then incubated at 70 ºC for 5 min. First strand cDNA product can be directly used on PCR applications or stored at -20 ºC.

1.2.2 Oligonucleotide primers design

The *dsx* sequences of *Ostrinia scapulalis* and *Antheraea mylitta* between female and male available on the database (www.ncbi.nlm. nih.gov) were aligned using ClustalW program (http://www.ebi.ac.uk/Tools/clustalw2 /index.html). The primers were designed on the basis of consensus nucleotide sequences. The forward primer (DsxF1) and reverse primers (DsxR1, DsxR2) were showed in the table 2.

1.2.3 PCR amplification of *doublesex* gene

The *doublesex* gene of *C. cephalonica* was amplified by polymerase chain reaction (PCR) using the first strand cDNA as a template and used the

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EmeraldAmp® PCR Master Mix (Takara). The 50 µl reaction was added 1 µl of the first-stranded cDNA template, 25 µl of EmeraldAmp® PCR Master Mix, 1 µl of primer sense (DsxF1 10 pmol/ µl), 1 µl of primer antisense (DsxR1, DsxR2 10pmol/ µl) and added sterilized distilled water up to 50 µl. PCR procedure was performed as followed: preheated at 98 ºC for 3 min, followed by 35 cycles of denaturing at 94 ºC for 10 sec, annealing at 55 ºC for 30 sec and extension at 72 ºC for 1 min. The final extension was carried out at 72 ºC for 2 min.

1.2.4 Determination of PCR product quality

The PCR product was mixed with 6X gel-loading buffer and the sample mixture was loaded slowly into the slots of the submerged 1% agarose gel. The lid of the gel chamber was closed and the electrophoresis was carried out in 1X TAE running buffer. The PCR product should migrate toward the positive anode at 100 volts for 30 min and the bromophenol blue should migrate from the wells into the body of the gel within a few min. The gel was run until the bromophenol blue and xylene cyanol FF have migrated to an appropriate distance through the gel. After finishing, the gel was stained in 0.1 mg/ml ethidium bromide (EtBr) solution for 10 min and destained by submerging in an excessive amount of distilled water for 2 min. The nucleic acid bands were visualized under UV transilluminator.

1.2.5 Purification of DNA fragment from agarose gel

DNA fragment was purified from agarose gel by using the Favorgen® Gel/PCR purification kit. DNA fragment on agarose gel (from section 1.2.4) was cuted and transferred to a microcentrifuge tube. The 500 µl of FADF buffer was added to sample and mixed by vortexing. The sample was incubated at 55-60 ºC for 10-15 min or until the agarose gel was completely dissolved. During incubation, the solution was inverted every 2-3 min and cooled down the dissolved sample mixture at room temperature. The 800 µl of sample mixture was transfered into FADF column and centrifuged at 14,000 rpm at room temperature for 30 seconds. The FADF column was removed from the collection tube and discarded the

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liquid inside the tube. The FADF column was placed back into the tube and added 750 µl of wash buffer. The sample was centrifuged at 14,000 rpm for 30 seconds at room temperature. The flow-through was discarded. The FADF column was placed back into the tube and centrifuge for 3 min at 14,000 rpm to dry the column matrix. The dried column was transfered to a new microcentrifuge tube. The DNA fragment was eluted by adding 50 µl of Elution buffer and incubated for 2 min at room temperature. After incubation, the column was centrifuge at 14,000 rpm for 2 min. The concentration of purified DNA fragment was then determined using gel electrophoresis and stored at –20 ºC until use.

1.2.6 DNA ligation

The purified DNA fragment from section 1.2.5 was ligated into pGEM-T® easy vectors (Promega) (Figure 6) which possessed a single 3' deoxythimidine (T) overhanged at both ends. This vector allowed easy cloning of PCR product based on the fact that Bio-X-ACT Short DNA Polymerase used in PCR tend to add an additional nucleotide, usually a deoxyadenosine (A) to the 3'-end of each strand that it synthesized. Therefore, a double-stranded PCR product has a single adenosine nucleotide overhang. The molar ratio of the insert DNA to the vectors is 3:1. The amount of inserted DNA depended on the length of the inserted DNA fragment. The quantity of inserted DNA was estimated in ng by the following equation:

$$\text{x ng of insert DNA} = \frac{\text{(50 ng of pGEM-T vector)(y bp of insert DNA) x insert : vector Molar ratio}}{\text{3000 bp of pGEM-T vector}}$$

Where x was the amount of insert DNA of y bp to be ligated for 3:1 (insert: vector molar ratio). The 10 µl of each ligation reaction composed of inserted DNA, 1 µl of 5 µl of 2X T4 DNA ligase buffer, pGEM-T vector (50 ng), 1 µl of 3 unit/µl of T4 DNA ligase and sterile deionized water was added to the final volume of 10 µl. Each mixture was gently mixed by pipetting and then incubated at 16 ºC for 3-4 hr.

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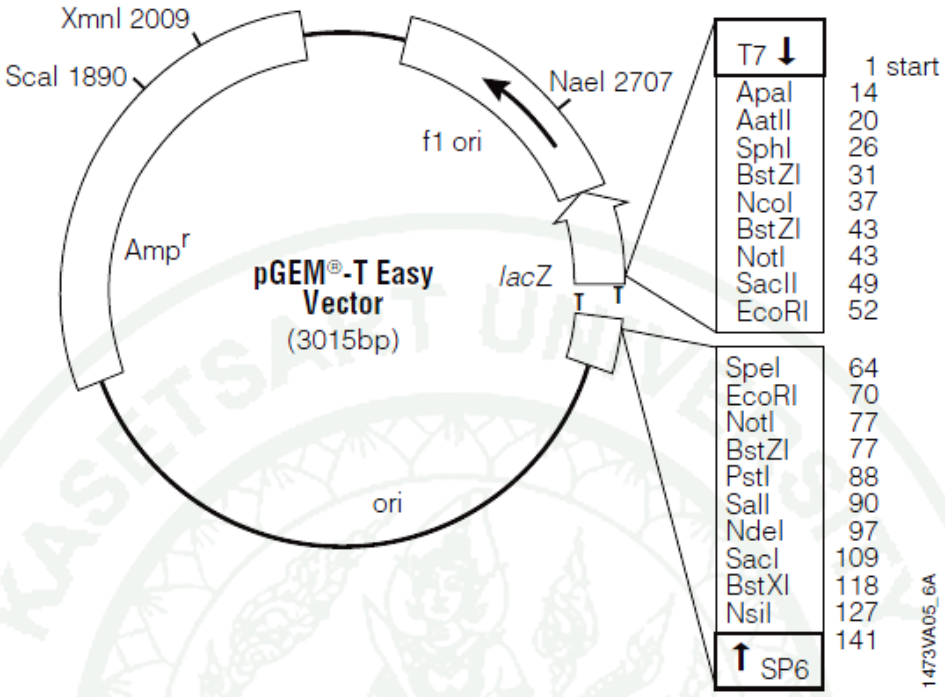

Figure 6 Diagram shows structure of pGEM-T® easy vectors.

Source: Promega (2010)

1.2.7 Transformation into *E.coli* using heat shock transformation

The 50 µl of the *E.coli* (DH5α) was mixed with 2 µl of ligation mixture in a tube, was placed on ice for 30 min. The mixed solution in the tube was heat-shocked in a water bath at exactly 42 ºC (do not shake) for 1 min and then placed immediately on ice for 2 min. The tube containing cells transformed with ligation reactions was added with 1,000 µl of LB broth and incubated at 37 ºC for 90 min with shaking at 250 rpm. After incubated, the tube was centrifuged at 8,000 rpm for 2 min. Supernatant about 800 µl was discarded. Before the transformed mixture was plated on LA ampicillin plates with 100 µl of 100 mM Isopropylthio- ß-D-galactoside (IPTG) and 20 µl of 20 mg/ml 5–Bromo-4-chloro-3-indoly-ß-D-galactosidase (X-Gal) were spreaded over the surface of an LA ampicillin plate. The plate was allowed to absorb at 37 ºC for 30 min prior to use and the transformed mixture were spreaded

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over the surface of an LA ampicillin plate. The plate was incubated at 37 ºC overnight (16-18 hr). Recombinant clones were analyzed by blue/white screening. The transformant colonies were counted and selected randomly to check the transformation of the inserted DNA by colony PCR.

1.2.8 Detection of DNA insertion by colony PCR

Colony PCR reactions were performed using the EmeraldAmp® PCR Master Mix (Takara) containing 25 µl of EmeraldAmp® PCR Master Mix, 1 µl of T7 primer 10 pmol/ µl, 1 µl of SP6 10pmol/µl, 27 µl of sterilized distilled water and pick up a single white colony into PCR reactions. Colony PCR procedure was performed as followed: preheated at 98 ºC for 3 min, followed by 35 cycles of denaturing at 94 ºC for 10 sec, annealing at 55 ºC for 30 sec and extension at 72 ºC for 1.30 min. The final extension was carried out at 72 ºC for 2 min.

1.2.9 Extraction of plasmid DNA by FavorPrep™ Plasmid Extraction MiNi kit

A single selected white colony of X-gal-IPTG-ampicillin plate was cultured in 3 ml of LB broth containing 50 µg/ml ampicillin and incubated at 37 ºC with shaking at 250 rpm overnight (12-16 hr). The culture was transferred into 1.5 ml microcentrifuge tube and centrifuged at 14,000 rpm for 1 min. The supernatant was discarded. The 200 µl of FADF1 buffer was added into the tube. The cell pellet was resuspended by vigorous pipeting. The 200 µl of FADF2 buffer was added and mix gently by inverting the tube 10 times. The tube was incubated at room temperature for a 2 min. The 300 µl of FADF3 buffer was added and mix gently by inverting the tube 10 times. The tube was centrifuged at 14,000 rpm at room temperature for 5 min. FADF Plasmid column was placed into a 2 ml collecting tube and transferred the supernatant into the column. The column was centrifuged at 14,000 rpm for 30 sec. The flow-through was discarded. The FADF Plasmid column was placed back into the 2 ml collecting tube. Added 400 µl of W1 buffer and centrifuged at 14,000 rpm at room temperature for 30 sec. The flow-through was discarded. The FADF Plasmid

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column was placed back into the 2 ml collecting tube and added 600 µl of wash buffer (with ethanol). The FADF Plasmid column was centrifuged at 14,000 rpm at room temperature for 30 sec. The flow-through was discarded and centrifuged again at 14,000 rpm at room temperature for 3 min. FADF Plasmid column was placed into a 1.5 ml microcentrifuge tube and added 50 µl of Elution buffer. The FADF Plasmid column was incubated at room temperature for 2 min. The FADF Plasmid column was centrifuged at 14,000 rpm at room temperature for 1 min. The Plasmid DNA was determined its concentration by spectrophotometer (nano drop) and stored at –20 ºC until use.

1.2.10 Detection of DNA insertion by restriction digestion

The digestion reaction of 10 µl contained 1 µl of the purified plasmid DNA of each clone, 1 µl of 10x *Eco*RI reaction buffer, 1 µl of *Eco*RI (New England Biolabs, UK), and sterile deionized water was added to the final volume. The digestion reaction was incubated at 37 ºC for 1 hr. The digestion reaction was detected by loading onto 1% agarose gel and electrophoresed at 100 volts for 30 min. The size of inserted DNA was compared with GeneRuler^{TM} DNA Ladder Mix (Thermo Scientific).

1.3 Cloning and sequencing cDNAs using 5'- Rapid Amplification of cDNA ends (5'- RACE)

1.3.1 Total RNA isolation from whole body of *C. cephalonica* female and male.

Total RNAs were extracted from whole body of *C. cephalonica* female and male using TRIzol reagent (Gibco BRL, USA). The 5'-RACE were performed to generate the full-length *doublesex* cDNAs using the GeneRacer^{TM} Kit (Invitrogen) according to manufacturer's instructions.

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1.3.2 Designing PCR Primers for 5'RACE

The primers were designed on the sequence of *Ccedsx* nucleotide sequences. The gene-specific primers used for 5' RACE (GSP1 and GSP2) were showed in the table 2.

1.3.3 Dephosphorylating RNA

The total RNAs or mRNAs were dephosphorylation with calf intestinal phosphatase (CIP) to remove the 5′ phosphates (Figure 7). The reaction was performed in a 1.5 ml sterile microcentrifuge tube using the reagents in the kit. The reaction mixture of 10 µl contained 1-5 µg of total RNA, 1 µl of 10X CIP Buffer, 1 µl of RNaseOut™ (40 U/µl), 1 µl of CIP (10 U/µl) and added DEPC water up to 10 µl. The reaction mixture was mix gently by pipeted, briefly vortexed and centrifuged to collect fluid. The reaction mixture was incubated at 50°C for 1 hour. After incubation, the reaction mixture was centrifuge briefly and place on ice. To precipitate RNA, the 90 µl of DEPC water and 100 µl of phenol: chloroform was added and vortex vigorously for 30 sec. The reaction mixture was centrifuged at maximum speed in a microcentrifuge for 5 minutes at room temperature and transfer aqueous (top) phase to a new microcentrifuge tube (~100 µl). The 2 µl of 10 mg/ml mussel glycogen, 10 µl of 3 M sodium acetate, pH 5.2, were added and mix well. The 220 µl of 95% ethanol was added to microcentrifuge tube, vortex briefly and incubated at -80°C for 20 min. After incubation, the solution was centrifuged at maximum speed in a microcentrifuge for 20 minutes at 4°C and removes the supernatant by pipet. The 500 µl of 70% ethanol was added, inverted several times, and vortex briefly. The solution was centrifuged at maximum speed in a microcentrifuge for 2 min at 4°C. The ethanol was removed and the remaining was air-dried the pellet for 1-2 min at room temperature. The pellet was resuspended in 7 µl of DEPC water.

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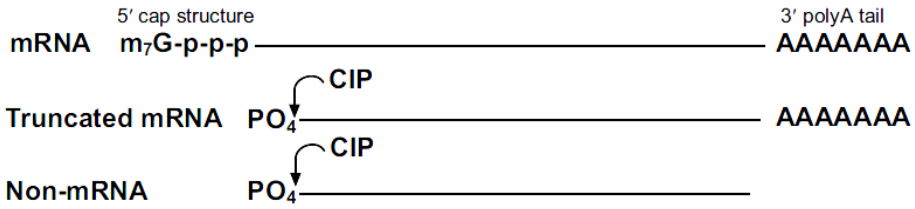

Figure 7 The process to remove the 5′ phosphates by treating total RNA or mRNA with calf intestinal phosphatase (CIP).

Source: Invitrogen (2004)

1.3.4 Removing the mRNA Cap Structure

After dephosphorylating and precipitating the RNA, the 5′ cap structure was removed from full-length mRNA (Figure 8). Decapping reaction was performed in a 1.5 ml sterile microcentrifuge tube using the reagents in the kit. The reaction mixture of 10 µl contained 7 µl of Dephosphorylated RNA, 1 µl of 10X TAP Buffer, 1 µl of RNaseOut™ (40 U/µl) and 1 µl of TAP (0.5 U/µl). The solution was mixed gently by pipeted, briefly vortexed and then centrifuged briefly to collect fluid. The solution was incubated at 37°C for 1 hour and then centrifuged briefly and place on ice. The 90 µl of DEPC water and 100 µl of phenol: chloroform were added in the solution and vortexed vigorously for 30 sec and then centrifuged at maximum speed for 5 min at room temperature. The aqueous (top) phase was transfered to a new microcentrifuge tube (~100 µl) and added 2 µl of 10 mg/ml mussel glycogen, 10 µl of 3 M sodium acetate, pH 5.2, and mixed well. The 220 µl of 95% ethanol was added and vortexed briefly before incubation at -80°C for 20 min. After incubated, the solution was centrifuged at maximum speed for 20 min at 4°C and removes the supernatant by pipet. The 500 µl of 70% ethanol was added, inverted several times, and vortex briefly. The solution was centrifuged at maximum speed for 2 minutes at 4°C. The ethanol was removed and the remaining was air-dried the pellet for 1-2 min at room temperature. The pellet was resuspended in 7 µl of DEPC water.

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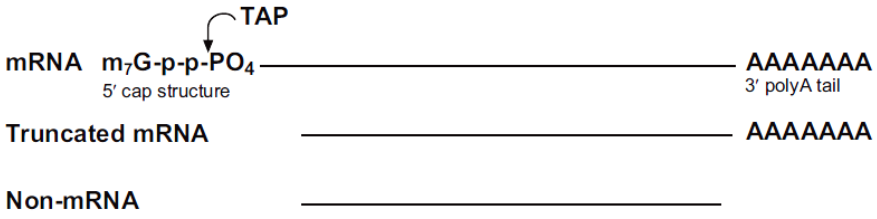

Figure 8 The process to remove the 5′ cap structure from intact, full-length mRNA by treating dephosphorylated RNA with tobacco acid pyrophosphatase (TAP).

Source: Invitrogen (2004)

1.3.5 Ligating the RNA Oligo to Decapped mRNA

The GeneRacer™ RNA Oligo was ligated to the 5′ end of the mRNA using T4 RNA ligase (Figure 9) and added the 7 µl of dephosphorylated, decapped RNA to the tube containing the pre-aliquoted, lyophilized GeneRacer™ RNA Oligo (0.25 µg). The solution was pipeted up and down several times to mixed and resuspend RNA Oligo and centrifuged briefly to collect the fluid in the bottom of the tube. The solution was incubated at 65°C for 5 minutes to relax the RNA secondary structure and placed on ice to chill (~2 minutes) and then centrifuged briefly. The 1 µl of 10X Ligase Buffer, 1 µl of 10 mM ATP, 1 µl of 10 RNaseOut™ (40 U/µl) and 1 µl of T4 RNA ligase (5 U/µl) were added to the tube, mixed gently by pipeted, and centrifuged briefly. The solution was incubation at 37°C for 1 hour and then centrifuged briefly and place on ice. The 90 µl of DEPC water and 100 µl of phenol: chloroform were added in the solution and vortexed vigorously for 30 sec and then centrifuged at maximum speed for 5 min at room temperature. The aqueous (top) phase was transfered to a new microcentrifuge tube (~100 µl) and added 2 µl of 10 mg/ml mussel glycogen, 10 µl of 3 M sodium acetate, pH 5.2, and mixed well. The 220 µl of 95% ethanol was added and vortexed briefly before incubation at -80°C for 20 min. After incubated, the solution was centrifuged at maximum speed for 20 min at 4°C and removes the supernatant by pipet. The 500 µl of 70% ethanol was added,

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inverted several times, and vortex briefly. The solution was centrifuged at maximum speed for 2 minutes at 4°C. The ethanol was removed and the remaining was air-dried the pellet for 1-2 min at room temperature. The pellet was resuspended in 10 µl of DEPC water.



Figure 9 GeneRacer™ RNA Oligo to ligated the 5′ end of the mRNA using T4 RNA ligase.

Source: Invitrogen (2004)

1.3.6 Reverse Transcribing mRNA

The ligated mRNA was reverse transcribed using SuperScript™ III RT and the GeneRacer™ OligodT Primer to create RACEready first-strand cDNA (Figure 10). The 1 µl of Primers, 1 µl of dNTP Mix and 1 µl of Steriled water were added to the 10 µl of ligated RNA and incubated at 65°C for 5 min to remove any RNA secondary structure. The solution was chilled on ice for at least 1 min and then centrifuged briefly. The 4 µl of 5X First Strand Buffer, 1 µl of 0.1 M DTT, 1 µl of RNaseOut™ (40 U/µl) and 1 µl of SuperScript™ III RT (200 U/µl) were added to the 13 µl ligated RNA and primer mixture and mixed well by pipeted gently up and down. The solution was centrifuged briefly and incubated at 50°C for 30-60 min. the RT reaction was inactivated at 70°C for 15 min and chilled on ice for 2 min and then centrifuged briefly at 15,000 rpm. The 1 µl of RNase H (2 U) was added to the reaction mix and incubated at 37°C for 20 min. The solution was centrifuged briefly and use immediately for amplification or store at -20°C.

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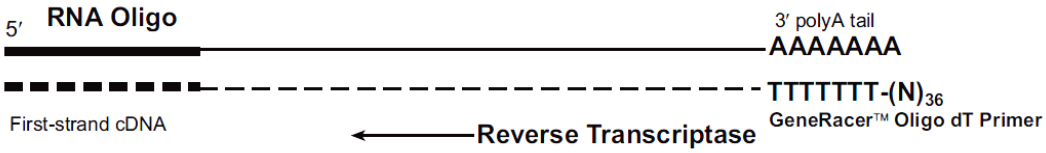

Figure 10 The pocess to Reverse transcription of the ligated mRNA using SuperScript™ III RT and the GeneRacer™ Oligo dT Primer.

Source: Invitrogen (2004)

1.3.7 Amplifying cDNA 5' Ends of *doublesex*

The first-strand cDNA was amplified using a reverse genespecific primer (Reverse GSP) and the GeneRacer™ 5′ Primer (Figure 11). The 5' RACE reaction was used the GeneRacer™ Kit (Invitrogen) according to manufacturer's instructions, using one adapter-specific and the other gene-specific primers. The gene-specific primers used for 5' RACE of *Ccedsx* were GSP1 and GSP2. First RACE-PCR was was performed as followed: preheated at 98 ºC for 3 min, followed by 35 cycles of denaturing at 94 ºC for 10 sec, annealing at 60 ºC for 30 sec and extension at 72 ºC for 2 min. The final extension was carried out at 72 ºC for 2 min. After that, nested RACE-PCR reaction was performed by using product of first RACE-PCR for template and nested RACE-PCR was performed as followed: preheated at 98 ºC for 3 min, followed by 5 cycles of denaturing at 94 ºC for 30 sec, annealing at 72 ºC for 2 min and 5 cycles of denaturing at 94 ºC for 30 sec, annealing at 70 ºC for 2 min and 22 cycles of denaturing at 94 ºC for 30 sec, annealing at 65 ºC for 30 sec, extension at 68 ºC for 2 min. The final extension was carried out at 68 ºC for 2 min. The PCR products of 5'- RACE fragments was detected by 1% agarose gel and purified from agarose gel using Favorgen® Gel/PCR purification kit. The PCR purified was cloned into pGEM-T®easy vector and transformed into *E coli* DH5α by heat shock transformation. The plasmid containing these fragments was extracted using FavorPrep™ Plasmid Extraction MiNi kit and sent to Macrogen, Korea for sequencing.

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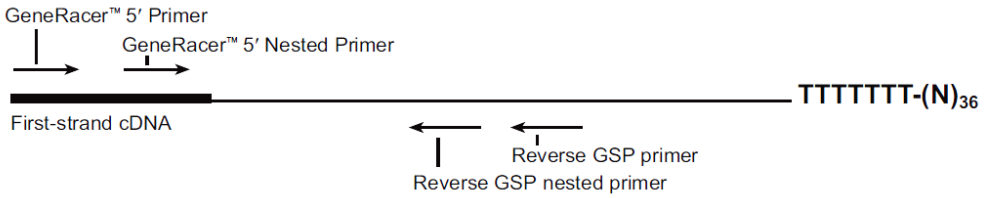

Figure 11 Diagram of the first-strand cDNA amplification using a reverse genespecific primer (Reverse GSP) and the GeneRacer™ 5′ Primer.

Source: Invitrogen (2004)

2. Detection the expression of *doublesex* gene by RT-PCR

2.1 RNA Isolation and the first strand cDNA synthesis

Total RNAs were extracted from eggs, larva, pupal and adult female and male of *C. cephalonica* using TRIzol reagent (Gibco BRL, USA). The total RNAs were used as a template to synthesize the first strand cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific).

2.2 Oligonucleotide primers design

The primers were designed using the sequence of *Ccedsx* nucleotide sequences. The primers for amplifiled *doublesex* gene were *Ccedsx*RT_F and *Ccedsx*RT_R and the primers for amplifiled *β-actin* gene were β-ActinF and β-ActinR showed in the table 2.

2.3 RT-PCR amplification of *Ccedsx*

The expression of *Ccedsx* were amplified by Reverse transcriptase polymerase chain reaction (RT-PCR), using the first strand cDNA (from section 2.1) as a template. The primers were *Ccedsx*RT_F and *Ccedsx*RT_R. The reaction of 50 µl

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was amplified using the *Taq* DNA Polymerase (recombinant) (Thermo Scientific) contained 1 µl of the first-stranded cDNA template, 5 µl of 10X *Taq* buffer, 5 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTP, 2 µl of primer (*Ccedsx*RT_F 10 pmol/ µl) and (*Ccedsx*RT_R 10pmol/ µl), 0.5 µl of *Taq* DNA polymerase and added sterilized distilled water up to 50 µl. The PCR reaction was performed as followed: preheated at 94 ºC for 3 min, followed by 30 cycles of denaturing at 94 ºC for 30 sec, annealing at 57 ºC for 30 sec and extension at 72 ºC for 30 sec. The final extension was carried out at 72 ºC for 10 min. PCR products of *Ccedsx* fragments was detected by 1% agarose gel.

3. Sequence alignment and phylogenetic tree reconstruction

Phylogenetic analysis of *C. cephalonica dsx* was performed using Neighbor-Joining and Maximum Likelihood analysis with amino acid sequences from the GenBank database. All the sequences were aligned with ClustalX (ver. 1.83) by the multiple alignment method. Gaps and missing data were completely excluded from the analysis. The generated data matrix was converted to mega format and analyzed with MEGA 5.03 program. The *dsx* sequences used for comparison were showed in appendix table A1**.**

4. Cloning and sequencing of *vitellogenin* gene in *C. cephalonica*

4.1 RNA Isolation and the first strand cDNA synthesis

Total RNA was extracted from adult female of *C. cephalonica* using TRIzol reagent (Gibco BRL, USA). The total RNA was used as a template to synthesize the first strand cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific).

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4.2 Oligonucleotide primers design

The *vg* sequences of *Antheraea pernyi*, *Antheraea yamamai*, *Bombyx mandarina*, *Bombyx mori*, *Saturnia japonica*, *Samia Cynthia pryeri* and *Samia ricini* available on the database (www.ncbi.nlm. nih.gov) were aligned using ClustalW program (http://www.ebi.ac.uk/Tools/clustalw2/ index.html). The three sets primers (Table 2) were designed on the basis of consensus these nucleotide sequences. The set one primer were CceVgF1 and CceVgR1, The set two primers were CceVgF2 and CceVgR2 and The set three primers were CceVgF3 and CceVgR3.

4.3 PCR amplification of *vitellogenin* gene from cDNA

The *vitellogenin* gene of *C. cephalonica* was amplified by polymerase chain reaction (PCR), using the first strand cDNA as a template and amplified with the three sets primers to get three DNA fragments. The reaction of fragment 1 were amplified using the *Taq* DNA Polymerase (recombinant) (Thermo Scientific) contained 1 µl of the first-stranded cDNA female template, 5 µl of 10X *Taq* buffer, 5 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTP, 2 µl of primers (CceVgF1 10 pmol/ µl) and (CceVgR1 10pmol/ µl), 0.5 µl of *Taq* DNA polymerase and added sterilized distilled water up to 50 µl. PCR procedure was performed as followed: preheated at 94 ºC for 3 min, followed by 35 cycles of denaturing at 94 ºC for 30 sec, annealing at 55 ºC for 30 sec and extension at 72 ºC for 1 min. The final extension was carried out at 72 ºC for 10 min.

The reaction of fragment 2 were amplified using the *Taq* DNA Polymerase (recombinant) (Thermo Scientific) contained 1 µl of the first-stranded cDNA female template, 5 µl of 10X *Taq* buffer, 5 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTP, 2 µl of primers (CceVgF2 10 pmol/ µl) and (CceVgR2 10pmol/ µl), 0.5 µl of *Taq* DNA polymerase and added sterilized distilled water up to 50 µl. PCR procedure was performed as followed: preheated at 94 ºC for 3 min, followed by 35 cycles of denaturing at 94 ºC for 30 sec, annealing at 43 ºC for 30 sec and extension at 72 ºC for 1 min. The final extension was carried out at 72 ºC for 10 min.

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The reaction of fragment 3 were amplified using the *Taq* DNA Polymerase (recombinant) (Thermo Scientific) contained 1 µl of the first-stranded cDNA female template, 5 µl of 10X *Taq* buffer, 5 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTP, 2 µl of primers (CceVgF3 10 pmol/ µl) and (CceVgR3 10pmol/ µl), 0.5 µl of *Taq* DNA polymerase and added sterilized distilled water up to 50 µl. PCR procedure was performed as followed: preheated at 94 ºC for 3 min, followed by 35 cycles of denaturing at 94 ºC for 30 sec, annealing at 57 ºC for 30 sec and extension at 72 ºC for 3 min. The final extension was carried out at 72 ºC for 10 min.

Three fragments of PCR products *vitellogenin* gene were detected by 1% agarose gel and purified from agarose gel using Favorgen® Gel/PCR purification kit. The PCR purified were cloned into pGEM-T®easy vector and transformed into *E coli* DH5α by heat shock transformation. The plasmids containing these fragments were extracted using FavorPrep™ Plasmid Extraction MiNi kit and sent to Macrogen, Korea for sequencing.

4.4 Cloning and sequencing cDNAs using 5’ and 3’ Rapid Amplification of cDNA Ends

The 5’ and 3’ RACE reactions were performed using the SMARTer™ RACE cDNA Amplification Kit User Manual (Clontech) according to manufacturer’s instructions, using one adapter-specific and the other gene-specific primers. The overview of full-length ds cDNA synthesis was showed in Figure 12.

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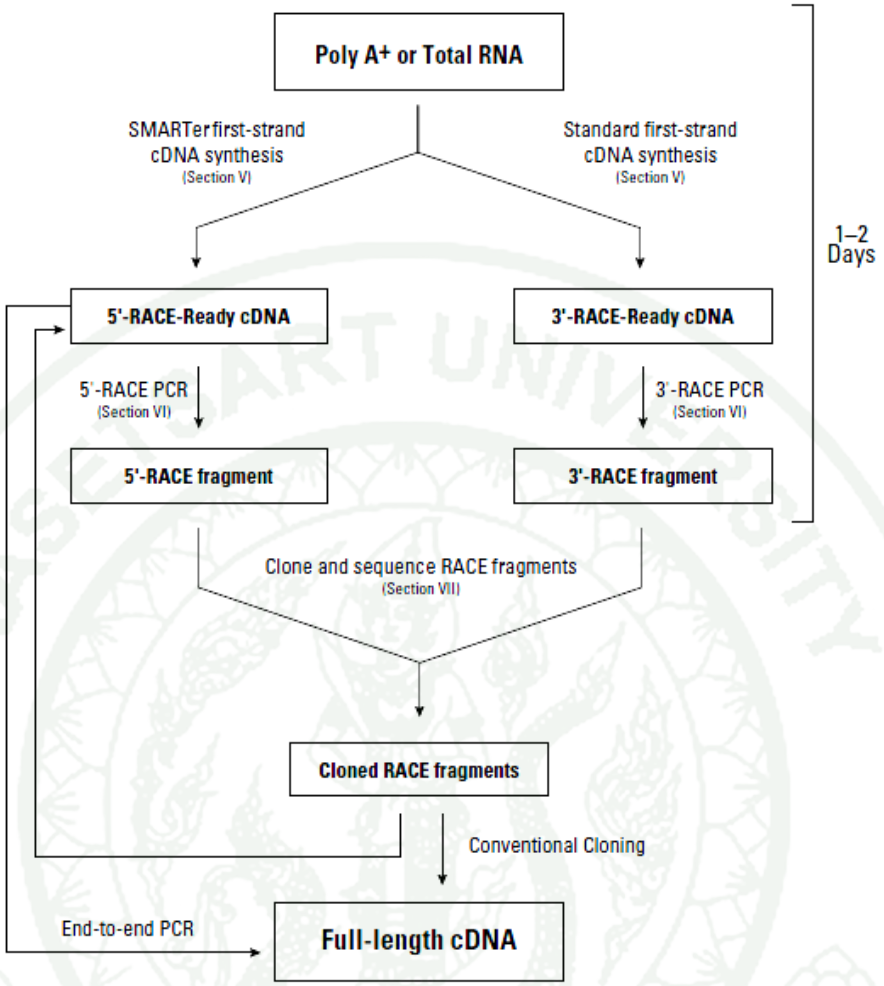

Figure 12 Overview of the SMARTer RACE procedure.

Source: Clontech (2012)

4.4.1 Designing PCR Primers for 5’ and 3’ RACE

The primers were designed using the sequence of *Ccedsx* nucleotide sequences. The gene-specific primers used for 5’ and 3’ RACE (5'RACE-VG and 3'RACE-VG) were showed in the table 2.

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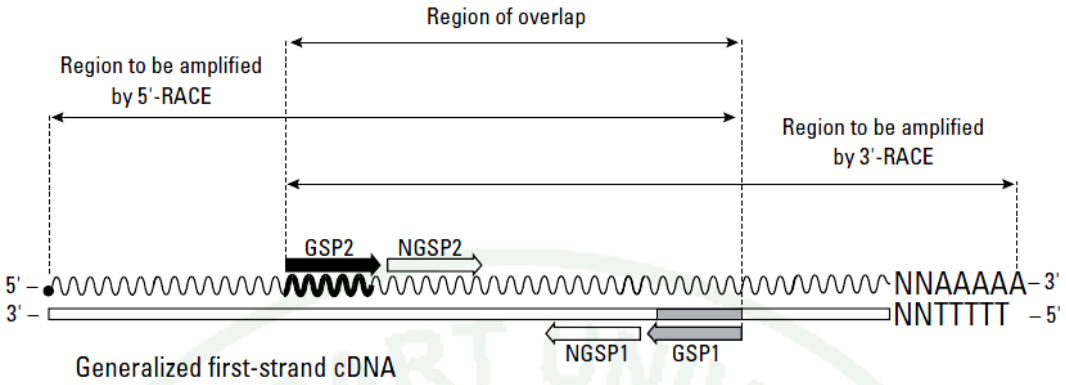

Figure 13 Schematic of the 5’ and 3’ RACE techniques.

Source: Clontech (2012)

4.4.2 Total RNA isolation from whole body of *C. cephalonica* female

Total RNA was extracted from whole body of adult female using TRIzol reagent (Gibco BRL, USA). The 5’and 3’ RACE were performed to generate the full-length *vitellogenin* cDNAs using the SMARTer^{TM} RACE cDNA Amplification Kit User Manual (Clontech).

4.4.3 First-Strand cDNA Synthesis of 5’-RACE

The Buffer Mix for 5’-RACE-Ready cDNA synthesis reactions was prepared by added 2.0 µl of 5X First-Strand Buffer, 1.0 µl of DTT (20 mM) and 1.0 µl dNTP Mix (10 mM), mixed the reagents and spin briefly in a microcentrifuge, then set aside at room temperature. The reaction of 5'-RACE -Ready cDNA was added 2.75 µl of total RNA and 1 µl 5'-CDS Primer A and mixed contents and spin the tubes briefly in a microcentrifuge. The solutions were incubated at 72°C for 3 min, cool the tubes at 42°C for 2 min and then spin the tubes briefly for 10 seconds at 14,000 g to collect the contents at the bottom. The 1 µl of the SMARTer IIA oligo, 4.0 µl of Buffer Mix, 0.25 µl of RNase Inhibitor (40 U/µl) and 1 µl of SMARTScribe Reverse Transcriptase (100 U) were added to reaction, mixed gently by pipeted, and then spin the tubes briefly to collect the contents at the bottom. The solution was incubated at

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42°C for 90 min by hot-lid thermal cycler and heated tubes at 70°C for 10 min and dilutes the first-strand reaction product with Tricine-EDTA Buffer.

4.4.4 First-Strand cDNA Synthesis of 3’-RACE

The Buffer Mix for 3’-RACE-Ready cDNA synthesis reactions was prepared by added 2.0 µl of 5X First-Strand Buffer, 1.0 µl of DTT (20 mM) and 1.0 µl dNTP Mix (10 mM), mixed the reagents and spin briefly in a microcentrifuge, then set aside at room temperature. The reaction of 3'-RACE -Ready cDNA was added 3.75 µl of total RNA and 1 µl 5'-CDS Primer A, mixed contents and spin the tubes briefly and incubated the tubes at 72°C for 3 min, then cool the tubes to 42°C for 2 min and then spin the tubes briefly for 10 seconds at 14,000 g to collect the contents at the bottom. The 4.0 µl of Buffer Mix, 0.25 µl of RNase Inhibitor (40 U/µl) and 1 µl of SMARTScribe Reverse Transcriptase (100 U) were added to reaction, mixed the tubes by gently pipeted and spin the tubes briefly to collect the contents at the bottom. The solution was incubated at 42°C for 90 min by hot-lid thermal cycler and heated tubes at 70°C for 10 min and dilutes the first-strand reaction product with Tricine-EDTA Buffer.

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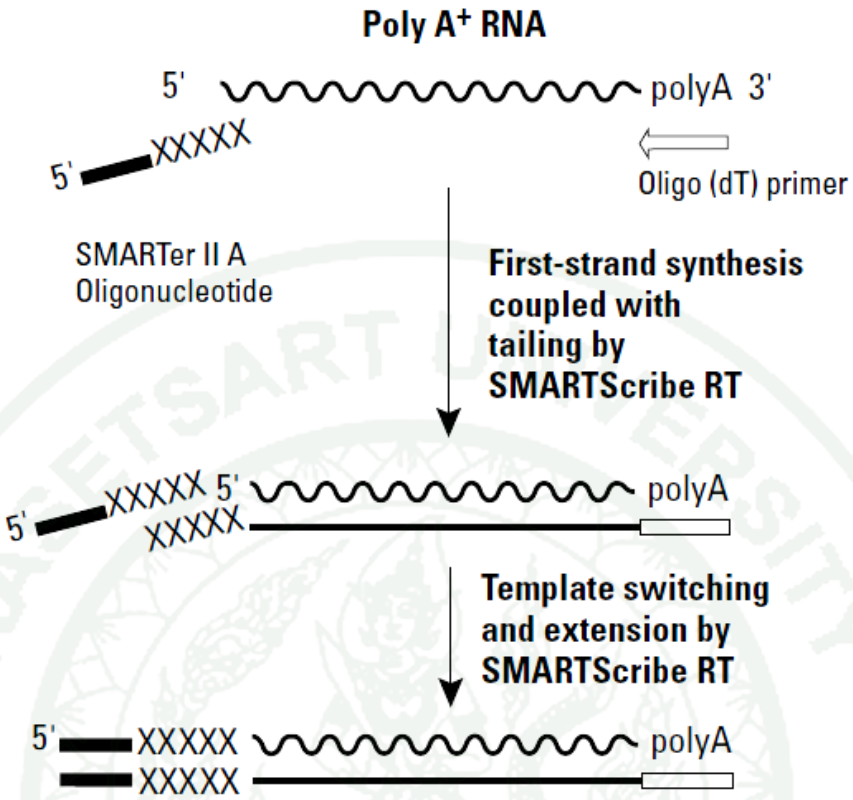

Figure 14 Mechanism of SMARTer cDNA synthesis.

Source: Clontech (2012)

4.4.5 Rapid amplification of 5’ ends of *CceVg*

The 5’ RACE reactions was performed using the SMARTer™ RACE cDNA Amplification Kit User Manual (Clontech) according to manufacturer’s instructions, using one adapter-specific and the other gene-specific primers. The gene-specific primers of 5’ RACE *CceVg* was 5’RACE-VG (Table 2). The reaction used the Advantage 2 Polymerase Mix (Clontech). The 5’ RACE-PCR was performed as followed: preheated at 94 ºC for 3 min, followed by 5 cycles of denaturing at 94 ºC for 30 sec, annealing at 72 ºC for 3 min, 5 cycles of denaturing at 94 ºC for 30 sec, annealing at 70 ºC for 30 sec, extension at 72 ºC for 3 min and 20 cycles of denaturing at 94 ºC for 30 sec, annealing at 68 ºC for 30 sec, extension at 72 ºC for 3 min . The final extension was carried out at 72 ºC for 10 min. The PCR product of 5’- RACE

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fragment was detected by 1% agarose gel and purified from agarose gel using Favorgen® Gel/PCR purification kit. The PCR purified was cloned into pGEM-T®easy vector and transformed into *E coli* DH5α by heat shock transformation. The plasmids containing DNA fragment was extracted using FavorPrep™ Plasmid Extraction MiNi kit and sent to Macrogen, Korea for sequencing.

4.4.6 Rapid amplification of 3' ends of *CceVg*

The 3' RACE reactions was performed using the SMARTer™ RACE cDNA Amplification Kit User Manual (Clontech) according to manufacturer's instructions, using one adapter-specific and the other gene-specific primers. The gene-specific of 3' RACE *CceVg* was 3'RACE-VG (Table 2). The reaction was used the Advantage 2 Polymerase Mix (Clontech). The 3' RACE-PCR was performed as followed: preheated at 94 ºC for 3 min, followed by 5 cycles of denaturing at 94 ºC for 30 sec, annealing at 72 ºC for 3 min, 5 cycles of denaturing at 94 ºC for 30 sec, annealing at 70 ºC for 30 sec, extension at 72 ºC for 3 min and 20 cycles of denaturing at 94 ºC for 30 sec, annealing at 68 ºC for 30 sec, extension at 72 ºC for 3 min . The final extension was carried out at 72 ºC for 10 min. The PCR products of 3'- RACE fragment was detected by 1% agarose gel and purified from agarose gel using Favorgen® Gel/PCR purification kit. The fragment was cloned into pGEM-T®easy vector and transformed into *E coli* DH5α by heat shock transformation. The plasmids containing DNA fragment was extracted using FavorPrep™ Plasmid Extraction MiNi kit and sent to Macrogen, Korea for sequencing.

5. DNA sequence analysis of *vitellogenin* gene in *C. cephalonica*

5.1 Genomic DNA extraction by FavorPrep™ Tissue Genomic DNA Extraction Mini Kit.

Genomic DNA of *C. cephalonica* was extraction using the FavorPrep™ Tissue Genomic DNA Extraction Mini Kit. The whole body was collected from adult female and transferred into a 1.5 ml microcentrifuqe tube. The 200 µl of FATG1

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Buffer was added, homogenized the sample with micropestle and then added 20 µl Proteinase K (10 mg/ml) to the sample mixture and mixed thoroughly by vortexed. The sample mixture was incubated at 60 °C for 60 min and vortexed every 10-15 min during incubation and spin down the tube to remove drops from inside of the lid. The 200 µl of FATG2 Buffer was added to the sample mixture, mixed thoroughly, vortexed and incubation at 70 °C for 10 min. The 200 µl of ethanol (96-100%) was added to the sample mixture, mixed thoroughly, vortexed and briefly spins the tube to remove drops from inside of the lid. FATG Mini Column was placed in a Collection Tube and transfered the sample mixture carefully to FATG Column and then centrifuged at 14,000 rpm for 1 min and discard the flow-through then placed FATG Column to a new Collection Tube. The 500 µl of W1 Buffer was added to FATG Column, centrifuged for 1 min and then discard the flow-through. The 750 µl of Wash Buffer was added to FATG Column and centrifuged for 1 min then discard the flow-through. The FATG Column was centrifuged again for 3 min to dry the column and placed FATG Column to a new microcentrifuge tube. The 50 µl of Elution Buffer was added to the membrane center of FATG Column, standed FATG Column for 3 min and then centrifuged for 2 min to elute total DNA.

5.2 Oligonucleotide primers design

The primers were designed using the sequence of *CceVg* nucleotide sequences for amplifiled *vitellogenin* gene. The forward primer (GVG-F) and reverse primer (GVG-R) were showed in the table 2.

5.3 PCR amplification of *vitellogenin* gene from genomic DNA

The *vitellogenin* gene of *C. cephalonica* was amplified by polymerase chain reaction (PCR), using the genomic DNA of adult female (from section 5.1) as a template. The 50 µl was amplified using the High Fidelity PCR Enzyme Mix (Thermo Scientific) contained 1 µl of DNA female template, 5 µl of 10X High Fidelity PCR Buffer with 15 mM $MgCl_2$, 5 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTP, 2 µl of primers (GVG-F 10 pmol/ µl) and (GVG-R 1 10pmol/ µl), 0.5 µl of High Fidelity

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PCR Enzyme Mix (5 u/µl) and added sterilized distilled water up to 50 µl. The PCR procedure was performed as followed: preheated at 94 ºC for 2 min, followed by 35 cycles of denaturing at 94 ºC for 30 sec, annealing at 57 ºC for 30 sec and extension at 68 ºC for 8 min. The final extension was carried out at 68 ºC for 5 min. The PCR product was detected by 1% agarose gel and purified using Favorgen® Gel/PCR purification kit and sent to Macrogen, Korae for sequencing.

6. Multiple alignment and Phylogenetic tree analysis of *CceVg*

Phylogenetic analysis of *C. cephalonica vg* was performed using Neighbor-Joining and Maximum Likelihood analysis with amino acid sequences of 42 Vg sequences from 41 arthropod and non-arthropod species retrieved from the GenBank database. All the sequences were aligned with ClustalX (ver. 1.83) by the multiple alignment method. Gaps and missing data were completely excluded from the analysis. The generated data matrix was converted to mega format and analyzed with MEGA 5.03 program. The Vg sequences used for comparison were showed in appendix table A2**.**

7. Detection the expression of *vitellogenin* gene by RT-PCR

7.1 RNA Isolation and the first strand cDNA Synthesis

Total RNA were extracted eggs, early larva, late larva, early female pupae, early male pupae, late female pupae, late male pupae, adult female and adult male of *C. cephalonica* using TRIzol reagent (Gibco BRL, USA). The Total RNAs were used as template for synthesize the first strand cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific).

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7.2 Oligonucleotide primers design

The two set of primers were designed using the *CceVg* nucleotide sequences. The set one was VG-RTF1 and VG-RTR1. The set two was VG-RTF2 and VG-RTR2. The two sets of primers were showed in the table 2.

7.3 RT-PCR amplification of *CceVg*

The expression of *CceVg* were amplified by reverse transcriptase polymerase chain reaction (RT-PCR), using the first strand cDNA (from section 7.1) as a template and amplied by two sets of primers. The first RT-PCR was amplified by used VG-RTF1 and VG-RTR1 primers. The 50 µl of reaction was amplified by using the *Taq* DNA Polymerase (recombinant) (Thermo Scientific) contained 1 µl of the first-stranded cDNA template, 5 µl of 10X *Taq* buffer, 5 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTP, 2 µl of primer (VG-RTF1 10 pmol/ µl) and (VG-RTR1 10pmol/ µl), 0.5 µl of *Taq* DNA polymerase and added sterilized distilled water up to 50 µl. PCR procedure was performed as followed: preheated at 94 ºC for 3 min, followed by 30 cycles of denaturing at 94 ºC for 30 sec, annealing at 57 ºC for 30 sec and extension at 72 ºC for 30 sec. The final extension was carried out at 72 ºC for 10 min. The second RT-PCR was amplified by VG-RTF2 and VG-RTR2 primers. The 50 µl of reaction was amplified by using the *Taq* DNA Polymerase (recombinant) (Thermo Scientific) contained 1 µl of the first-stranded cDNA template, 5 µl of 10X *Taq* buffer, 5 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTP, 2 µl of primer (VG-RTF2 10 pmol/ µl) and (VG-RTR2 10pmol/ µl), 0.5 µl of *Taq* DNA polymerase and added sterilized distilled water up to 50 µl. The PCR procedure was performed as followed: preheated at 94 ºC for 3 min, followed by 30 cycles of denaturing at 94 ºC for 30 sec, annealing at 57 ºC for 30 sec and extension at 72 ºC for 30 sec. The final extension was carried out at 72 ºC for 10 min. PCR products of *CceVg* fragments was detected by 1% agarose gel.

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8. Study the function of *vitellogenin* gene by RNA interference

8.1 Design primer of dsVg and dsDsRed

The 19-25 nucleotide-long gene-specific primers were design from target gene (preferably > 200). The T7 RNA polymerase promoter sequence (5'-CCGGATCCTAATACGACTCACTATAGGGCG-3') was added to the 5' end of each gene-specific primer. The primers of *CceVg* were (dsVG-F and dsVG-R) and the primers of *DsRed* were (dsDsRed-F and dsDsRed-R) and showed in the table 2.

8.2 Amplification of *vitellogenin* and *DsRed* gene

The *vitellogenin* and *DsRed* gene were amplified by polymerase chain reaction (PCR), using the *Taq* DNA Polymerase (recombinant) (Thermo Scientific) contained 1 µl of the Plasmid DNA of *CceVg* and *DsRed* for templates, 5 µl of 10X *Taq* buffer, 5 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTP, 2 µl of primer (10 pmol/µl) and (10pmol/µl), 0.5 µl of *Taq* DNA polymerase and added sterilized distilled water up to 50 µl. PCR procedure was performed as followed: preheated at 94 ºC for 3 min, followed by 35 cycles of denaturing at 94 ºC for 30 sec, annealing at 50 ºC for 30 sec and extension at 72 ºC for 1 min. The final extension was carried out at 72 ºC for 10 min. PCR products was detected by 1% agarose gel and purified using Favorgen® Gel/PCR purification kit.

8.3 Double stranded RNA (dsRNA) synthesis

The 50 µl of dsRNA reaction was added 1 µg of PCR DNA template, 2 µl of NTPs (100 mM) (ATP,CTP,GTP,UTP), 5 µl of 10X buffer, 5 µl of T7 RNA polymerase (New England Biolabs, UK), RNase inhibitor, 1.25 µl (New England Biolabs, UK) and added sterilized distilled water up to 50 µl. The solution was incubated at 37º C for 2 hour. The solution was added 5 µl of RNase-free DNase for remove DNA template, incubatied at 37º C for 15 min and stop the reaction by heated at 65º C for 10 min. The 50 µl of phenol: 50 µl of chloroform was added, vortexed 20

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sec and then centrifuged at high speed for 2 min at room temperature.The supernatant was transfered into new tube and added 0.1 volumes of 3M sodium acetate, 2.5 volumes of 100% ethanol to the solution mixed well and incubated at -80º C for 20 min. The solution was centrifuged at high speed for 35 min at 4º C and discards the supernatant. The 500 µl of 70% ethanol was added, centrifuged at high speed for 10 min at 4º C. The supernatant was removed and dry the pellet for 5 min at room temperature. The 12 µl of TE buffer was added to RNA pellet and mixed well. The 1 µl of dsRNA was analyzed by 1% agarose gel electrophoresis to check the quality of dsRNA. The concentration of dsRNAs was checked using Nano Drop. The dsRNA solution was aliquoted to 5 tubes (2 µl of dsRNA, 8 µl of DEPC, 1 µl of 3M sodium acetate and 25 µl of 100% ethanol per tube) and keeped at -80º C. Before injection, dsRNA was centrifuged at high speed for 40 min at 4º C and discard the supernatant. The 500 µl of 70% ethanol was added to dsRNA pellet and mixed for wash the pellet and centrifuged dsRNA at high speed for 10 min at 4º C. The supernatant was removed and dried the pellet for 5 min at room temperature. The dsRNA was dissolved with dH_2O (1 µg/µl).

8.4 dsRNAs injection into *C. cephalonica*

The 1 µg of *Vg* and DsRed dsRNAs were injection into the second abdominal segment of adult female within 1 hour after emergence using a 10 µl Hamilton microsyringe. Two females were collected on the 1, 3 and 5 days post injection and kept at -80º C for analysis expression of *CceVg* gene. Three days after injection, the remaining four females were dissected and their ovaries were observed under an Olympus stereomicroscope and taken photographs with a DP72 digital camera of Olympus. *DsRed* dsRNAs was used as the control.

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8.5 Detection the expression level of *vitellogenin* gene by quantitative real-time RT-PCR (qPCR)

8.5.1 RNA Isolation and Synthesis the first strand cDNA

Total RNAs were extracted from adult females at injection by dsVg and dsDsRed (1, 3 and 5 days) using TRIzol reagent (Gibco BRL, USA). The total RNAs were used as template for synthesize the first strand cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific).

8.5.2 Oligonucleotide primers design

The primers were designed using the *vitellogenin* and *actin* sequences of *C. cephalonica* for quantitative real-time RT-PCR. The primer *vitellogenin* gene was RealT-VGF and RealT-VGR and primer *actin* gene was RealT-β-ActinF and RealT-β-ActinR and showed in the table 2.

8.5.3 Quantitative real-time RT-PCR

Quantitative real-time RT-PCR assays were performed using the Mastercycler® ep realplex (Eppendorf Bio Inc.). The reactions were performed using ThermOne™ Real-Time Premix (SYBR Green) (RBCBioscience). The 12.5 µl of reaction mixture contained 6.25 µl of SYBR Green, 0.25 µl of forward primer (10 pmol/ µl), 0.25 µl of reward primer (10 pmol/ µl), 2 µl of cDNA and added sterilized distilled water up to 12.5 µl. The cDNA was amplified one cycle of 94 °C for 3 min and 40 cycles of 94 °C for 30 sec, 57 °C for 30 sec and 72 °C for 30 sec, followed by melting curve analysis to detect-specific product amplification. Real-time PCR primers were as follows: RealT-VGF, RealT-VGR and RealT-β-ActinF, RealT-β-ActinR. Each sample were analyzed in duplicate and normalized to the β-actin expression of *C. cephalonica* as internal control. Real-time data were calculated the relative expression level by $2^{-\Delta\Delta C}{}_{T}$ method (Lival and Schmittgen, 2001).

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$$\text{Relative expression ratio} = 2^{-\Delta\Delta C_T}$$

$$\Delta\Delta C_T = (C_{T,\text{Target}} - C_{T,\text{Calibrator}})_{\text{Time x}} - (\text{Mean } C_{T,\text{Target}} - \text{Mean } C_{T,\text{Calibrator}})_{\text{Time 0}}$$

Time *x* is any time point and Time 0 represents the 1 x expression of the target gene normalized to *β-actin*. The mean C_T values for both the target and internal control genes were determined at time zero. The fold change in the target gene, normalized to *β-actin* and relative to the expression at time zero, was calculated for each sample using $2^{-\Delta\Delta C_T}$.

9. Identification Vg protein of *C. cephalonica* by SDS-PAGE

The 100 mg of fresh eggs, adult female and male were homogenized on ice with 200 ml of 20 mM Phosphate Buffer pH 7 (1M KH_2PO_4 and 1M K_2HPO_4). The homogenous solutions were then centrifuged at 10,000 rpm for 30 min at 4º C and transfered supernatant into new 1.5 microcentrifuge tube. The protein concentration of each sample was checked using Bradfrod method at 595 nm (Bradfrod, 1976). The 20 µg of each protein sample was boiled for 10 min with loading buffer then centrifuged briefly. The samples were separated by electrophoresis on 15% Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) at 150 V for 90 min. Following electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250 (45 ml methanol, 10 ml glacial acetic acid, 45 ml water, 0.25 g Coomassie Brilliant Blue R-250) overnight. The gels were destained using a mixture of 40% methanol and 10% acetic acid overnight, and then scanned using the Connon MG3100 series Printer (Cannon, Japan). To determine the relative MW of the putative Vn or Vg bands, MW standards (GeneDireX PiNk Plus Prestained Protein Ladder, USA) were run in a separate lane of each gel.

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Table 2 Primers in these experiments.

Primer name	Sequences primer 5'- 3'
DsxF1	AGTCGCATCGTGACCGCGCGCCG
DsxR1	CACRTACCGCGGCGAGCAGCACAT
DsxR2	GGTCGCGCCGCTACAAGTGCGC
GSP1	TACCGCCGGTTGCTGAGGAGGCA
GSP2	TACACAGTTGCGGTACTTGCAGTAGC
*Ccedsx*RT_F	GAACACGGCTGAACACGAGATCC
*Ccedsx*RT_R	GCTCCGGTCTATCCAATTCTACC
CceVgF1	ATTCAGGAAGATGGAAACTGA
CceVgR1	ACAACCTGAGCAGCTTCTTC
CceVgF2	ATGGGATGATATTCCGGGATGC
CceVgR2	GTCATTTCAGCCATTGCCTGCATC
CceVgF3	GAAGTGTTCGCTCCTTACTTAGAGGG
CceVgR3	TAATCGTAGCTGCGGTTGCTGAAGG
5'RACE-VG	CAAATGAGGGTGCACATGCACTGTGC
3'RACE-VG	GGAGCGCAGCAAGAAATTCGAGG
GVG-F	ACCATTGAACATCAGGTGTGGAAGG
GVG-R	CGCAGATGGTACTCTGTACTGAACG
VG-RTF1	TGCATCAGAATGGCGTCGTGAGC
VG-RTR1	CCTGCTCTTTGTCTTCTTCGCATG
VG-RTF2	CAACAAAGAGTACGTCGGTCC
VG-RTR2	CAATGCTGCGACTGGTTTGAGC
dsVG-F	CCGGATCCTAATACGACTCACTATAGGGCGGGTGTCG ATAATGTTCCCAGTAGC
dsVG-R	CCGGATCCTAATACGACTCACTATAGGGCGAGCGTAT CATAAACTGCGGTCAGC
dsDsRed	CCGGATCCTAATACGACTCACTATAGGGCGGTGCTTG TCAATGCGGTAAGTG
dsDsRed-R	CCGGATCCTAATACGACTCACTATAGGGCGCTCGAGA TCTGACAATGTTCAG

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Table 2 (Continued)

Primer name	Sequences primer 5’- 3’
RealT-VGF	GCATGTGCACCCTCATTTGTATGG
RealT-VGR	CAGACGACCGTCTTCGAGTTTGG
RealT-β-ActinF	GTGCCCATCTACGAGGGTTACG
RealT-β-ActinR	CTCGGCTGTGGTGGTGAATGAG
β-ActinF	GACGCCCCAGACATCAGGGT
β-ActinR	GTGATGACCTGACCGTCGGG

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RESULTS AND DISCUSSION

Results

1. Cloning and sequencing of *doublesex* gene in *C. cephalonica*

1.1 PCR Amplification

The total RNAs of adult female and male were used as a template to synthesis the first strand cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). Then, the 1st strand cDNA were used as a template for amplification *doublesex* cDNA by used two set primers (DsxF1, DsxR1) and (DsxF1, DsxR2). The result was showed PCR products of *doublesex* gene, approximately 849-1,221 bp long (Figure 15). There PCR products were purified from agarose gel and ligated into pGEM-T® easy vectors. The recombinant vectors were transformed into *E. coli* DH5α by heat shock transformation.

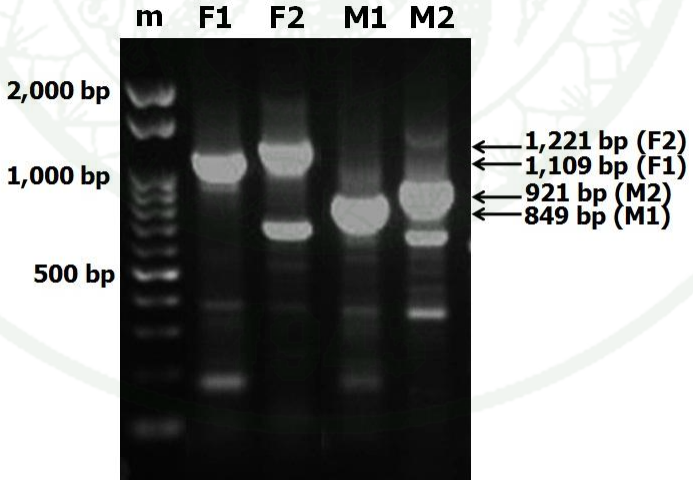

Figure 15 Agarose gel electrophoresis of PCR products of *doublesex* cDNA. Lane 1: DNA ladder, Lane 2: PCR products of female used primer DsxF1 and DsxR1, 3: PCR products of female used primer DsxF1 and DsxR2, Lane 4: PCR products of male used primer DsxF1 and DsxR1 and Lane 5: PCR products of male used primer DsxF1 and DsxR2.

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1.2 Detection of DNA Insertion by Restriction Digestion

The recombinant plasmids containing the *doublesex* cDNA were screened by the formation of blue/white colonies on LA ampicillin plate. Four white colonies were randomly selected for DNA plasmid isolation and analyzed by cutting with *Eco*RI to get four *doublesex* cDNA fragments (Figure 16). The vectors containing the cDNA fragment were sent to Macrogen, Korea for sequencing. The obtained sequences were aligned and the conserved region was used for design the specific primer for further experiment.

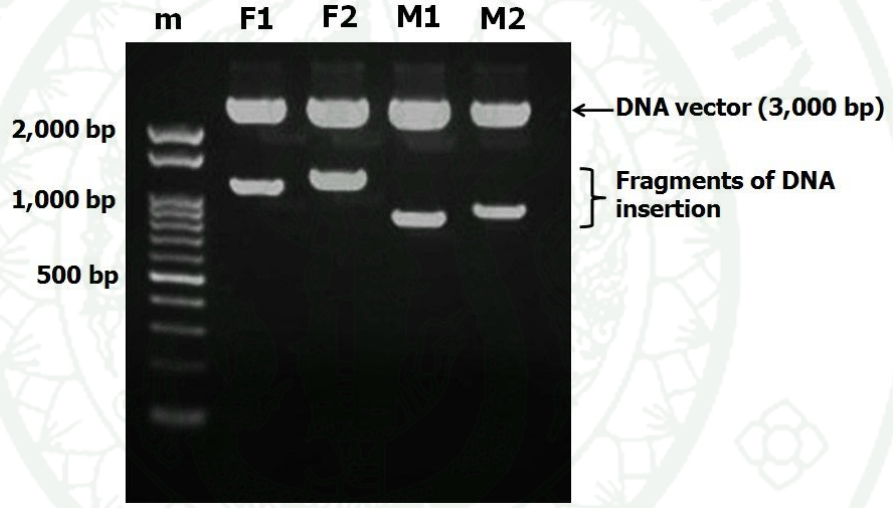

Figure 16 Agarose gel electrophoresis of recombinant vectors digested with *Eco*RI to select the expected inserted fragments of *doublesex* gene. Lane 1: DNA ladder and Lane 2-5: *Eco*RI digested recombinant plasmid clones.

1.3 Amplifying the 5’ ends of *doublesex* cDNA gene

The total RNAs of adult female and male were used as a template to synthesize the first strand cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). The first strand cDNA were then used as the template for amplification first RACE-PCR of 5’ end *doublesex* using primer GeneRacer™ 5′ Primer and GSP1. Then, the nested RACE-PCR reactions were performed using the

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product of first RACE-PCR as a template and the primer GeneRacer™ 5′ Primer and GSP2. The obtained PCR product, approximately 300 bp length (Figure 17), were purified and ligated into pGEM-T® easy vectors. The recombinant vectors were transformed into *E. coli* DH5α by heat shock transformation.

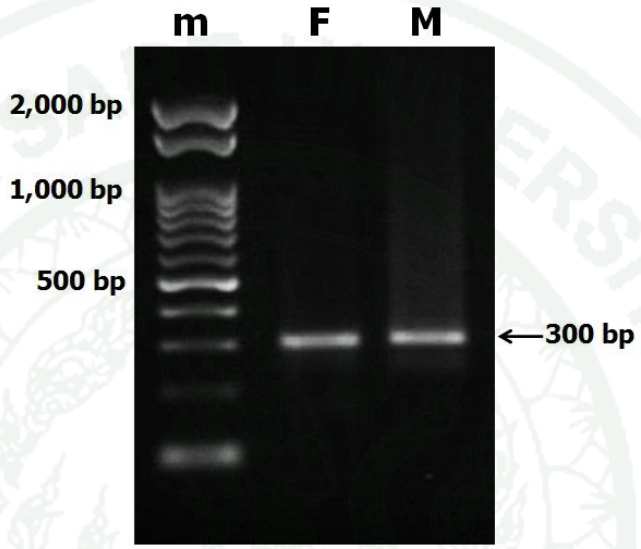

Figure 17 Agarose gel electrophoresis of PCR products 5’ Ends of *doublesex* cDNA. m: DNA ladder, F: PCR products of female and M: PCR products of male

1.4 Detection of DNA Insertion by Restriction Digestion

The recombinant plasmids containing the 5’ ends *doublesex* cDNA were screened by the formation of blue/white colonies on LA ampicillin plate. Two white colonies were randomly selected for DNA plasmid isolation and analyzed by cutting with *EcoR*I to get 5’ ends *doublesex* cDNA fragments (Figure 18). The vectors containing the cDNA fragment were sent to Macrogen, Korea for sequencing.

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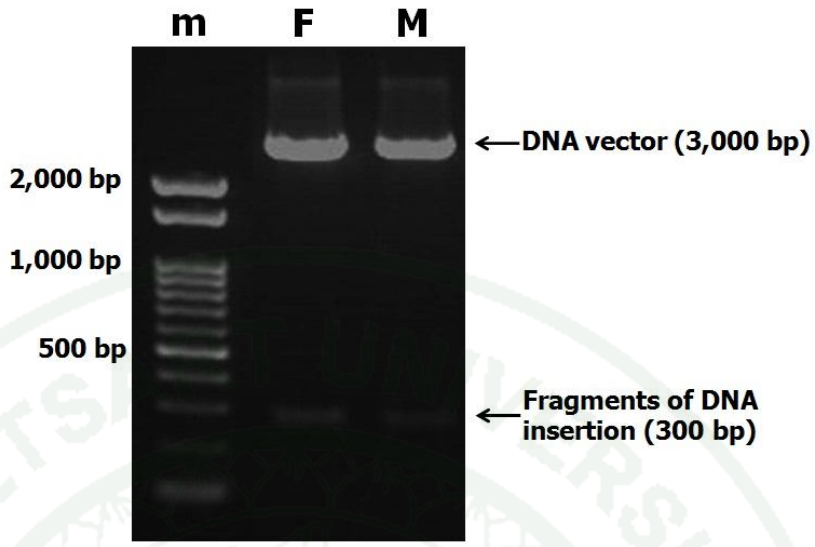

Figure 18 Agarose gel electrophoresis of recombinant vectors digested with *Eco*RI to select the expected inserted fragment of 5’ Ends *doublesex* gene. m: DNA ladder and F, M: *Eco*RI digested recombinant plasmid clones.

1.5 Nucleotide sequences of *doublesex* gene of *C. cephalonica* female and male

The obtained sequences were blast using the Blastn program of NCBI to confirm that the sequences were *dsx* gene. The 5’ ends *doublesex* cDNA sequences was 94 bp with to get long sequence. The 3’ ends *doublesex* cDNA was identified but unsuccessful. Therefore, in this study the partial nucleotide sequences of *doublesex* cDNA of *C. cephalonica* female and male were expected by assembly the nucleotide sequence of 5’RACE using CAP3 program. *Ccedsx* sequences contained three isoforms (one isoform in male and two isoforms in female). The results of RT-PCR indicated that major transcripts of *Ccedsx* mRNA was different between the males and females because 744-1018 nt of female cDNA was skipped over in the male transcript and produces two specific to female and one specific to male. The only difference between *CcedsxF1* and *CcedsxF2* were the present of 15 bp sequence (TACGGACTT TAATAG) in the *CcedsxF2*. From the results of a 5’RACE experiment, it was confirmed that the *Ccedsx* mRNAs of both sexes would share identical ATG start codons. These results indicate that the production of sex-specific transcripts of *Ccedsx*

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was due to alternative splicing of pre-mRNA originated from the same gene rather than transcription from two different genes (Figure 19). The putative *doublesex* cDNA sequence of *CcedsxF1* was 1,113 bp (Figure 20), *CcedsxF2* was 1,225 bp (Figure 21) and *CcedsxM* was 925 bp, (Figure 22).

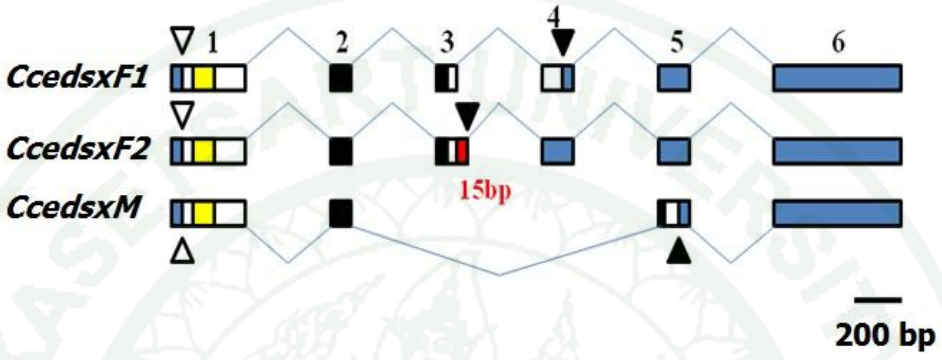

Figure 19 Sex-specific of *Ccedsx* transcript exon/intron boundaries of *Ccedsx*. Exons are shown as boxes, and exon numbers are indicated. Untranslated regions are shown as blue boxes, and coding regions as open boxes. DM domains are indicated as yellow boxes. OD2 domains are indicated as black boxes. An open arrowhead indicates the initiation codon, and a solid arrowhead indicates the stop codon.

```
5′  1 GAAAAGTCGCATCGTGACCGCGCGCCGTATCGCGTGTGCATCGCTCTGCT  50
   51 CGCGCTGGATTACCGCGAACACGGCTGAACACGAGATCCGAGCCATGGTT 100
  101 TCCATGGGCGCGTGGAGGCGCCGCGCGCCCGACGATTGTGAAGAACGCTC 150
  151 AGAGCCTGGTGCTTCTAGTTCGGCCGTGCCGCGAGCTCCACCCAACTGTG 200
  201 CCCGGTGCCGCAACCACCGTCTCAAGATCGAGCTGAAAGGCCACAAGCGC 250
  251 TACTGCAAGTACCGCAACTGTGTATGCGAGAAGTGCCAGTTGACTGCTGA 300
  301 TAGGCAGCGGGTGATGGCACTTCAGACGGCGCTGAGGCGTGCTCAGGCGC 350
  351 AGGACGAGGCGAGAGCGCGAGCCATTGAGTCCGGGCAGCCACCGAATGGG 400
  401 GTAGAATTGGATAGACCGGAGCCTCCTGCCGTGAAGATGCGAAGAAGTCC 450
  451 AGTGCCGCCGCCCCCTCCTCGCTCGATGGGCTCGGCTAGTTGCGATTCAG 500
  501 TGCCGGGCTCCCCCGGAGTATCCCCGTACGCGCCCCCTCCACCGCCGTCC 550
  551 GCGCCCCCGCCGCCGTCCATGCCGCCGCTGCTGCCTCCTCAGCAACCGGC 600
  601 GGTATCCCTTGAAACCCTAGTGGAGAATTGTCAGAAATTATTGGAGAAGT 650
  651 TCCACTATTCCTGGGAGATGATGCCTTTAGTCCTTGTCATTCTTAATTAC 700
  701 GCGGGAAGCGATTTGGAGGAGGCGTCGAAGAAAATTGACGAAGGGAAGAT 750
  751 GATCATCAACGAGTACGCCAGGAAGCACAACCTAAACATCTTCGACGGTG 800
  801 TCGAATTAAGGAACTCGACTCGCCAGAAAATGCTGCAAAGCGAAATTAAT 850
```

Figure 20 Partial nucleotide sequence of *CcedsxF*

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```
 851 AATATAAGTGGTGTACTATCATCATCGATGAAGTTGTTTTGCGAATGATA 900
 901 CTTTGTTTTACAAGTGCGTTGTTTTGTGTGGACGCATGCTGTGCGCCGCT 950
 951 GTGTGTTGCGAATCTCGTCGGACAATACTGTGCTTGTTACTCTGGACAAA 1000
1001 TAGCCCACTGGGTGGTCCACCAGTGGCGGCTGTACGAGCGCTCGCTGTGC 1050
1051 TCGCTGCTGGAGCTGCAGGCACGGAAGGGCACGTACTCGATGTGCTGCTC 1100
1101 GCCGCGGTACGTG 1113 3′
```

Figure 20 (Continued)

```
5′ 1 GAAAAGTCGCATCGTGACCGCGCGCCGTATCGCGTGTGCATCGCTCTGCT 50
  51 CGCGCTGGATTACCGCGAACACGGCTGAACACGAGATCCGAGCCATGGTT 100
 101 TCCATGGGCGCGTGGAGGCGCCGCGCGCCCGACGATTGTGAAGAACGCTC 150
 151 AGAGCCTGGTGCTTCTAGTTCGGCCGTGCCGCGAGCTCCACCCAACTGTG 200
 201 CCCGGTGCCGCAACCACCGTCTCAAGATCGAGCTGAAAGGCCACAAGCGC 250
 251 TACTGCAAGTACCGCAACTGTGTATGCGAGAAGTGCCAGTTGACTGCTGA 300
 301 TAGGCAGCGGGTGATGGCACTTCAGACGGCGCTGAGGCGTGCTCAGGCGC 350
 351 AGGACGAGGCGAGAGCGCGAGCCATTGAGTCCGGGCAGCCACCGAATGGG 400
 401 GTAGAATTGGATAGACCGGAGCCTCCTGCCGTGAAGATGCGAAGAAGTCC 450
 451 AGTGCCGCCGCCCCCTCCTCGCTCGATGGGCTCGGCTAGTTGCGATTCAG 500
 501 TGCCGGGCTCCCCCGGAGTATCCCCGTACGCGCCCCCTCCACCGCCGTCC 550
 551 GCGCCCCCGCCGCCGTCCATGCCGCCGCTGCTGCCTCCTCAGCAACCGGC 600
 601 GGTATCCCTTGAAACCCTAGTGGAGAATTGTCAGAAATTATTGGAGAAGT 650
 651 TCCACTATTCCTGGGAGATGATGCCTTTAGTCCTTGTCATTCTTAATTAC 700
 701 GCGGGAAGCGATTTGGAGGAGGCGTCGAAGAAAATTGACGAAGGGAAGAT 750
 751 GATCATCAACGAGTACGCCAGGAAGCACAACCTAAACATCTTCGACGGTG 800
 801 TCGAATTAAGGAACTCGACTCGCCAGTACGGACTTTAATAGAAAATGCTG 850
 851 CAAAGCGAAATTAATAATATAAGTGGTGTACTATCATCATCGATGAAGTT 900
 901 GTTTTGCGAATGATACTTTGTTTTACAAGTGCGTTGTTTTGTGTGGACGC 950
 951 ATGCTGTGCGCCGCTGTGTGTTGCGAATCTCGTCGGACAATACTGTGCTT 1000
1001 GTTACTCTGGACAAATAGCCCACTGGGTGGTCCACCAGTGGCGGCTGTAC 1050
1051 GAGCGCTCGCTGTGCTCGCTGCTGGAGCTGCAGGCACGGAAGGGCACGTA 1100
1101 CTCGATGTGCTGCTCGCCCCGGTACGTGCTGGCGCCGGAGTACGCGCCGC 1150
1151 ATCTGCTGCCGCTACCCCTCACCACCACCACGCAGCGGCCGTCGCCGCCC 1200
1201 CTCGCGCACTTGTAGCGGCGCGACC 1225 3′
```

Figure 21 Partial nucleotide sequence of *CcedsxF2*

```
5′ 1 GAAAAGTCGCATCGTGACCGCGCGCCGTATCGCGTGTGCATCGCTCTGCT 50
  51 CGCGCTGGATTACCGCGAACACGGCTGAACACGAGATCCGAGCCATGGTT 100
 101 TCCATGGGCGCGTGGAGGCGCCGCGCGCCCGACGATTGTGAAGAACGCTC 150
 151 AGAGCCTGGTGCTTCTAGTTCGGCCGTGCCGCGAGCTCCACCCAACTGTG 200
 201 CCCGGTGCCGCAACCACCGTCTCAAGATCGAGCTGAAAGGCCACAAGCGC 250
 251 TACTGCAAGTACCGCAACTGTGTATGCGAGAAGTGCCAGTTGACTGCTGA 300
 301 TAGGCAGCGGGTGATGGCACTTCAGACGGCGCTGAGGCGTGCTCAGGCGC 350
 351 AGGACGAGGCGAGAGCGCGAGCCATTGAGTCCGGGCAGCCACCGAATGGG 400
 401 GTAGAATTGGATAGACCGGAGCCTCCTGCCGTGAAGATGCGAAGAAGTCC 450
```

Figure 22 Partial nucleotide sequence of *CcedsxM*

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```
451 AGTGCCGCCGCCCCCTCCTCGCTCGATGGGCTCGGCTAGTTGCGATTCAG 500
501 TGCCGGGCTCCCCCGGAGTATCCCCGTACGCGCCCCCTCCACCGCCGTCC 550
551 GCGCCCCCGCCGCCGTCCATGCCGCCGCTGCTGCCTCCTCAGCAACCGGC 600
601 GGTATCCCTTGAAACCCTAGTGGAGAATTGTCAGAAATTATTGGAGAAGT 650
651 TCCACTATTCCTGGGAGATGATGCCTTTAGTCCTTGTCATTCTTAATTAC 700
701 GCGGGAAGCGATTTGGAGGAGGCGTCGAAGAAAATTGACGAAGCCCACTG 750
751 GGTGGTCCACCAGTGGCGGCTGTACGAGCGCTCGCTGTGCTCGCTGCTGG 800
801 AGCTGCAGGCACGGAAGGGCACGTACTCGATGTGCTGCTCGCCCCGGTAC 850
851 GTGCTGCTGCCGCTACCCCTCACCACCACCACGCAGCGGCCGTCGCCGCC 900
901 CCCGCGCACTTGTAGCGGCGCGACC 925 3′
```

Figure 22 (Continued)

1.6 Nucleotide and deduced amino acid sequence of *Ccedsx* female

CcedsxM was specifically found in males, and contained an 831-bp ORF encoding a protein of 277 amino acids. *CcedsxF1* and *CcedsxF2* were found in females, *CcedsxF1* contained an 801-bp ORF encoding a protein of 267 amino acids. *CcedsxF2* contained a 741-bp ORF encoding a protein of 247 amino acids. Both the female proteins (CceDSXF1 and CceDSXF2) differ from each other in 20 amino acid residues at C-terminus. (Figure 23)

```
CcedsxF1        ATGGTTTCCATGGGCGCGTGGAGGCGCCGCGCGCCCGACGATTGTGAAGAACGCTCAGAG 60
CcedsxF2        ATGGTTTCCATGGGCGCGTGGAGGCGCCGCGCGCCCGACGATTGTGAAGAACGCTCAGAG 60
                  M  V  S  M  G  A  W  R  R  R  A  P  D  D  C  E  E  R  S  E

CcedsxF1        CCTGGTGCTTCTAGTTCGGCCGTGCCGCGAGCTCCACCCAACTGTGCCCGGTGCCGCAAC 120
CcedsxF2        CCTGGTGCTTCTAGTTCGGCCGTGCCGCGAGCTCCACCCAACTGTGCCCGGTGCCGCAAC 120
                  P  G  A  S  S  S  A  V  P  R  A  P  P  N  C  A  R  C  R  N

CcedsxF1        CACCGTCTCAAGATCGAGCTGAAAGGCCACAAGCGCTACTGCAAGTACCGCAACTGTGTA 180
CcedsxF2        CACCGTCTCAAGATCGAGCTGAAAGGCCACAAGCGCTACTGCAAGTACCGCAACTGTGTA 180
                  H  R  L  K  I  E  L  K  G  H  K  R  Y  C  K  Y  R  N  C  V

CcedsxF1        TGCGAGAAGTGCCAGTTGACTGCTGATAGGCAGCGGGTGATGGCACTTCAGACGGCGCTG 240
CcedsxF2        TGCGAGAAGTGCCAGTTGACTGCTGATAGGCAGCGGGTGATGGCACTTCAGACGGCGCTG 240
                  C  E  K  C  Q  L  T  A  D  R  Q  R  V  M  A  L  Q  T  A  L

CcedsxF1        AGGCGTGCTCAGGCGCAGGACGAGGCGAGAGCGCGAGCCATTGAGTCCGGGCAGCCACCG 300
CcedsxF2        AGGCGTGCTCAGGCGCAGGACGAGGCGAGAGCGCGAGCCATTGAGTCCGGGCAGCCACCG 300
                  R  R  A  Q  A  Q  D  E  A  R  A  R  A  I  E  S  G  Q  P  P

CcedsxF1        AATGGGGTAGAATTGGATAGACCGGAGCCTCCTGCCGTGAAGATGCGAAGAAGTCCAGTG 360
CcedsxF2        AATGGGGTAGAATTGGATAGACCGGAGCCTCCTGCCGTGAAGATGCGAAGAAGTCCAGTG 360
                  N  G  V  E  L  D  R  P  E  P  P  A  V  K  M  R  R  S  P  V

CcedsxF1        CCGCCGCCCCCTCCTCGCTCGATGGGCTCGGCTAGTTGCGATTCAGTGCCGGGCTCCCCC 420
CcedsxF2        CCGCCGCCCCCTCCTCGCTCGATGGGCTCGGCTAGTTGCGATTCAGTGCCGGGCTCCCCC 420
                  P  P  P  P  P  R  S  M  G  S  A  S  C  D  S  V  P  G  S  P
```

Figure 23 Nucleotide and deduced amino acid sequence of *Ccedsx* female.

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```
CcedsxF1        GGAGTATCCCCGTACGCGCCCCCTCCACCGCCGTCCGCGCCCCCGCCGCCGTCCATGCCG 480
CcedsxF2        GGAGTATCCCCGTACGCGCCCCCTCCACCGCCGTCCGCGCCCCCGCCGCCGTCCATGCCG 480
                  G  V  S  P  Y  A  P  P  P  P  P  S  A  P  P  P  P  S  M  P

CcedsxF1        CCGCTGCTGCCTCCTCAGCAACCGGCGGTATCCCTTGAAACCCTAGTGGAGAATTGTCAG 540
CcedsxF2        CCGCTGCTGCCTCCTCAGCAACCGGCGGTATCCCTTGAAACCCTAGTGGAGAATTGTCAG 540
                  P  L  L  P  P  Q  Q  P  A  V  S  L  E  T  L  V  E  N  C  Q

CcedsxF1        AAATTATTGGAGAAGTTCCACTATTCCTGGGAGATGATGCCTTTAGTCCTTGTCATTCTT 600
CcedsxF2        AAATTATTGGAGAAGTTCCACTATTCCTGGGAGATGATGCCTTTAGTCCTTGTCATTCTT 600
                  K  L  L  E  K  F  H  Y  S  W  E  M  M  P  L  V  L  V  I  L

CcedsxF1        AATTACGCGGGAAGCGATTTGGAGGAGGCGTCGAAGAAAATTGACGAAGGGAAGATGATC 660
CcedsxF2        AATTACGCGGGAAGCGATTTGGAGGAGGCGTCGAAGAAAATTGACGAAGGGAAGATGATC 660
                  N  Y  A  G  S  D  L  E  E  A  S  K  K  I  D  E  G  K  M  I

CcedsxF1        ATCAACGAGTACGCCAGGAAGCACAACCTAAACATCTTCGACGGTGTCGAATTAAGGAAC 720
CcedsxF2        ATCAACGAGTACGCCAGGAAGCACAACCTAAACATCTTCGACGGTGTCGAATTAAGGAAC 720
                  I  N  E  Y  A  R  K  H  N  L  N  I  F  D  G  V  E  L  R  N

CcedsxF1        TCGACTCGCCAG---------------AAAATGCTGCAAAGCGAAATTAATAATATAAGT 765
CcedsxF2        TCGACTCGCCAGTACGGACTTTAATAGAAAATGCTGCAAAGCGAAATTAATAATATAAGT 780
                  S  T  R  Q  Y  G  L        K  M  L  Q  S  E  I  N  N  I  S

CcedsxF1        GGTGTACTATCATCATCGATGAAGTTGTTTTGCGAATGA 804
CcedsxF2        GGTGTACTATCATCATCGATGAAGTTGTTTTGCGAATGA 819
                  G  V  L  S  S  S  M  K  L  F  C  E
```

Figure 23 (Continued)

1.5 *Ccedsx* was transcribed into sex-specific mRNA isoforms as a result of alternative splicing

The two female-specific *Ccedsx* transcripts were encoded two female-specific putative proteins (CceDSXF1 and CceDSXF2) whereas the male specific transcript encoded male-specific putative protein CceDSXM. The putative DSX proteins of CceDSXF1, CceDSXF2 and CceDSXM were of 267, 247 and 277 amino acid, respectively. The deduced amino acid (aa) sequences of CceDSX proteins was encoded by sex-specific *Ccedsx* transcripts can be divided into three parts: the region common to both the sexes (aa 1-216), the female-specific regions (aa 217-267 of CceDSXF1 and aa 217-247 of CceDSXF2), and the male-specific region (aa number 217-277). The female-specific (CceDSXF1 and CceDSXF2) and male-specific (CceDSXM) proteins were similar at their N-termini but differ at their C-termini, starting from the C-terminal region of DBD/OD2 domains (Figure 24).

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Common region

```
AmyDSX    MVSVGAWRRRAPDDCEERSDPGASSSAVPRAPPNCARCRNHRLKVELKGHKRYCKYRYCN 60
AaDSX     MVSVGAWRRRAPDDCEERSDPGASSSAVPRAPPNCARCRNHRLKVELKGHKRYCKYRYCN 60
CceDSX    MVSMGAWRRRAPDDCEERSEPGASSSAVPRAPPNCARCRNHRLKIELKGHKRYCKYRNCV 60
OsDSX     MVSVGAWRRRAPDDCEERSEPGTSSSGVPRAPPNCARCRNHRLKVELKGHKRYCKYRYCT 60
BmDSX     MVSMGSWKRRVPDDCEERSEPGASSSGVPRAPPNCARCRNHRLKIELKGHKRYCKYQHCT 60
          ***:*:*:**.********:**:***.*****************:***********: *

AmyDSX    CEKCRLTADRQRVMALQTALRRAQAQDEARARAKETGGHLPGVELDRPEPPIVKAPRSPV 120
AaDSX     CEKCRLTADRQRVMALQTALRRAQAQDEARARAKETGGHLPGVELDRPEPPIVKAPRSPV 120
CceDSX    CEKCQLTADRQRVMALQTALRRAQAQDEARARAIESGQPPNGVELDRPEPPAVKMRRSP- 119
OsDSX     CEKCRLTADRQRVMALQTALRRAQAQDEARARSMESGIHPTGVELERPEPPVVKAPRSPV 120
BmDSX     CEKCRLTADRQRVMAKQTAIRRAQAQDEARARALELGIQPPGMELDRPVPPVVKAPRSPM 120
          ****:********** ***:************: * *    *:**:** ** **  ***

AmyDSX    VPPPPPRSLGSASCDSVPGSPGVSPYAPPPPS----VPPLQAMAPLMPPQQPAVSLETLV 176
AaDSX     VPPPPPRSLGSASCDSVPGSPGVSPYAPPPPS----VPPLQAMPPLMPPQQPAVSLETLV 176
CceDSX    VPPPPPRSMGSASCDSVPGSPGVSPYAPPPPP---SAPPPPSMPPLLPPQQPAVSLETLV 176
OsDSX     VPPPPPRSLGSASCDSVPGSPGVSPYAPPPPPPSSSAPPPPNMPPLLPPQQPAVSLETLV 180
BmDSX     IPPSAPRSLGSASCDSVPGSPGVSPYAPPPSV-----PPPPTMPPLIPTPQPPVPSETLV 175
          :**..***:**********************.      **   *.**:*. **.*. ****

AmyDSXF   ENCHKLLEKFHYSWEMMPLVLVILNYAGSDLEEASRKIDE 216
AaDSXF    ENCHKLLEKFHYSWEMMPLVLVILNYAGSDLEEASRKIDE 216
CceDSXF1  ENCQKLLEKFHYSWEMMPLVLVILNYAGSDLEEASKKIDE 216
CceDSXF2  ENCQKLLEKFHYSWEMMPLVLVILNYAGSDLEEASKKIDE 216
OsDSXF    ENCHKLLEKFHYSWEMMPLVLVILNYAGSDLDEASRKIDE 220
BmDSXF    ENCHRLLEKFHYSWEMMPLVLVIMNYARSDLDEASRKIYE 215
          ***::*******************:*** ***:***:** *
```

Female-specific region

```
AmyDSXF   GKMIINEYARKHNLNVFDGLELRNSTRQKML--EINNISGVLSSSMKLFCE 265
AaDSXF    GKMIINEYARKHNLNVFDGLELRNSTRQKML--EINNISGVLSSSMKLFCE 265
CceDSXF1  GKMIINEYARKHNLNIFDGLELRNSTRQKMLQSEINNISGVLSSSMKLFCE 267
CceDSXF2  GKMIINEYARKHNLNIFDGVELRNSTRQYGL---------------------- 247
OsDSXF    GKMIINEYARKHNLNIFDGVELRNSTRQKMLQSEINNISGVLSSSMKLFCE 271
BmDSXF    GKMIVDEYARKHNLNVFDGLELRNSTRQKML--EINNISGVLSSSMKLFCE 264
          ****::*********:***:********  *
```

Male-specific region

```
AmyDSXM   AHCVVHQWRLYERSLYS-LLELQARKG-TYAMCCSPRYVLAPEYAPHLLPIPLTTQRASP 274
AaDSXM    AHWVVHQWRLYEHSLCS-LLKLQAQKG-TYAMCCSPRYVLAPEYAPHLLPIPLTTQRASP 274
CceDSXM   AHWVVHQWRLYERSLCS-LLELQARKG-TYSMCCSPRYVLLP------LPLTTTTQRPSP 268
OsDSXM    AHWVVHQWRLYERSLCS-LLELQARKGSSYSMCCSPRYVLAPEYAPHILPLPLTTQRPSP 279
BmDSXM    GYWMMHQWRLQQYSLCYGALELSARKD-VAALCCLRDTCWRP-----------RSRRVWC 263
          .: ::***** : **        : :    ::**      *            * .

AmyDSXM   PPAHL---- 279
AaDSXM    PPAHL---- 279
CceDSXM   PPRTCSGAT 277
OsDSXM    PPAHL---- 284
BmDSXM    PSS------ 266
          *.
```

Figure 24 Comparison amino acid between the CceDSX with AmyDSX, AaDSX, BmDSX and OsDSX sequences. Identical amino acids are indicated by '*', and conservative substitutions by ':' or '.'. The DM domain is boxed in Yellow. Oligomerization domain 2 (OD2) is boxed in Green.

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1.6 Conservation of exon 4 in *CcedsxF*

The 4th exon of *doublesex* gene in females Lepidoptera insects contains the cis-acting element (PESS-Putative Exonic Splicing Silencer) that is responsible for the sex-specific splicing of *doublesex* pre-mRNA and skipping of exon 3 and exon 4 in *doublesex* of male splice form. On analyzing the nucleotide sequence of *CcedsxF* transcripts, the nucleotide sequence of 4th exon of *CcedsxF* is relatively well conserved in females Lepidoptera insects. The PESS sequence of *CcedsxF* is completely conserved (Figure 25). This sequence is presented in all the female splice forms but not in the male splice variants.

```
Amydsx          GAAAATGCTG------GAAATTAATAATATAAGTGGTGTACTGTCGTCATCGATGAAGTT 54
Aadsx           GAAAATGCTG------GAAATTAATAATATAAGTGGTGTACTGTCGTCATCGATGAAGTT 54
Bmdsx           GAAAATGCTG------GAAATTAATAATATAAGTGGTGTACTGTCTTCGTCAATGAAGTT 54
Osdsx           GAAAATGCTGCAAAGCGAAATTAATAATATAAGTGGTGTACTATCATCGTCGATGAAGTT 60
Ccedsx          GAAAATGCTGCAAAGCGAAATTAATAATATAAGTGGTGTACTATCATCATCGATGAAGTT 60
                **********     ************************* ** ** ** ********

Amydsx          ATTTTGCGAATGATACTTTGTTTTACGAGTGCCGTGGTTTTTGTGGACACATGCTGTGCG 114
Aadsx           ATTTTGCGAATGATACTTTGTTTTACGAGTGCCGTGGTTTTTGTGGACACATGCTGTGCG 114
Bmdsx           ATTTTGCGAATGATACTTAGTTTTACAAGTGCCGTGGTGTGTGTTGACACTTGCTGTGCG 114
Osdsx           GTTTTGCGAATGATACTTAGTTTTACCAGTG---TGTGTTGTGAGGACGCGTGCTGTGCG 117
Ccedsx          GTTTTGCGAATGATACTTTGTTTTACAAGTGCGTTGTTTTGTGTGGACGCATGCTGTGCG 120
                 ***************** ******* ****    **   * **  *** * *********

Amydsx          TTGCTCTGTGTTGCGAATTTCAACGGACAATTGTGT---TGTCGCTTCACTGGACATTTA 171
Aadsx           ATGCTCTGTGTTGCGAATTTCAACGGACAATTGTGT---TGTCGCTTCACTGGACATTTA 171
Bmdsx           ATGCTGTG-----CGAATTTCAACGGAAATATTTGT---TGTCGTAACATTGGATCTATG 166
Osdsx           TCGCTGTGTGTTGCGAA-CTCA--GAAGAATTGTGAACGGCTAGCTACAAAGGACTTTTA 174
Ccedsx          CCGCTGTGTGTTGCGAATCTCGTCGGACAATACTGT---GCTTGTTACTCTGGACAAATA 177
                   *** **     ****  **   * * *    **      * *   *   ***    *

Amydsx          G 172
Aadsx           G 172
Bmdsx           G 167
Osdsx           G 175
Ccedsx          G 178
                *
```

Figure 25 ClustalW alignment of 4th exon in *CcedsxF* with Lepidoptera insects. The yellow background is the cis-acting element responsible for the skipping of *Ccedsx* exon 3 and exon 4 in males.

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2. Detection the expression of *doublesex* gene by RT-PCR

We performed reverse transcription polymerase chain reaction (RT-PCR) to determine the expression patterns of *Ccedsx* at the different developmental stage. Total RNAs were isolated from the eggs, larva, pupal and adults of *C. cephalonica*. PCR primers were designed to amplify a 356 bp fragment of *Ccedsx*. The PCR products of *Ccedsx* was found in all developmental stages and continued to increase every stages to reach its maximum at adult females and males, and then started to decline (Figure 26).

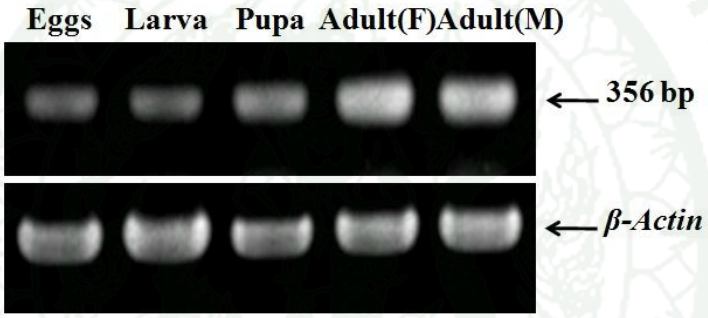

Figure 26 Agarose gel electrophoresis of *doublesex* gene expression at different stages of development, eggs, larva, pupal and adults of *C. cephalonica*. *β-actin* was used as a control.

3. Phylogenetic analysis of doublesex in *C. cephalonica* and other insects

Phylogenetic analysis of *C. cephalonica dsx* was performed using Neighbor-Joining and Maximum Likelihood analysis with amino acid sequences from the GenBank database. All the sequences were aligned with ClustalX (ver. 1.83) by the multiple alignment method. Gaps and missing data were completely excluded from the analysis. The generated data matrix was converted to mega format and analyzed with MEGA 5.03 program. The phylogenetic relationships among the DSX proteins of the Lepidoptera insects showed in figure 27 and 28. Phylogenetic analysis of *dsx* genes were performed using Neighbor-Joining (Figure 27) and Maximum Likelihood (Figure 28) analysis with amino acid sequences from the GenBank database. The

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phylogenetic tree of Neighbor-Joining and Maximum Likelihood analyze shows the relationships of each familys in order Lepidoptera. Pyralidae are more closely related to the Saturniidae than to Erebidae and Bombycidae and the results show very similar in both methods.

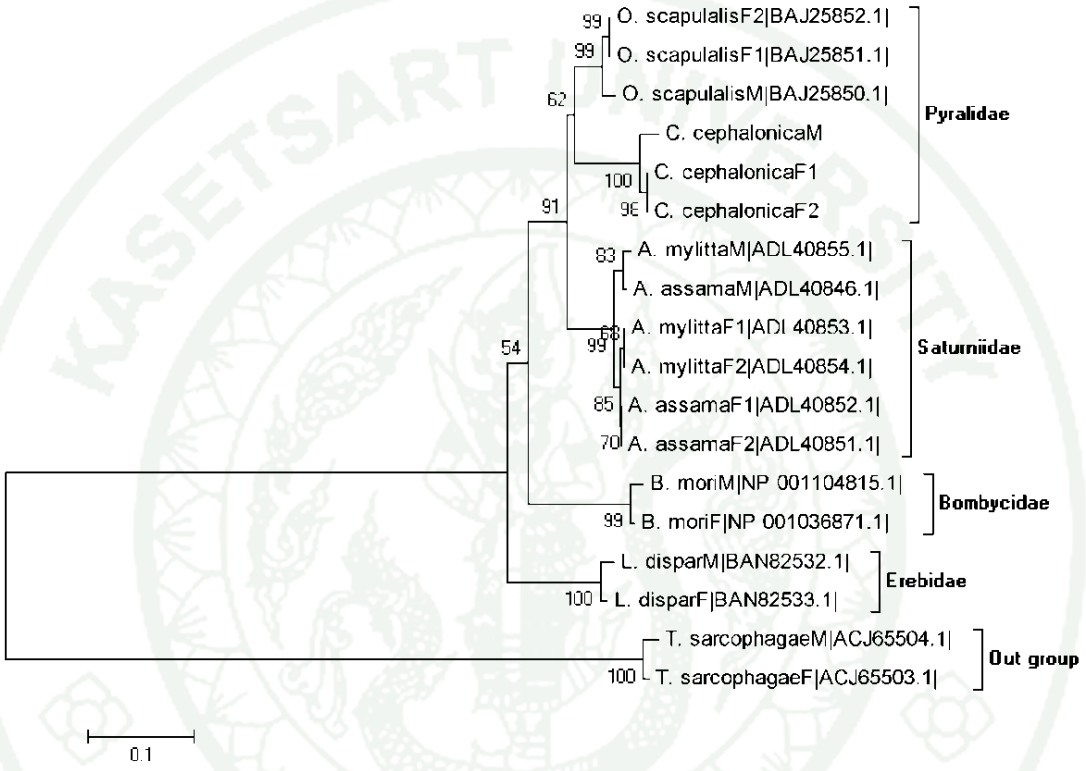

Figure 27 Phylogenetic tree constructed by using amino acid seguences of DSX proteins of some insects using MEGA 5.03 program, Neighbor-Joining method.

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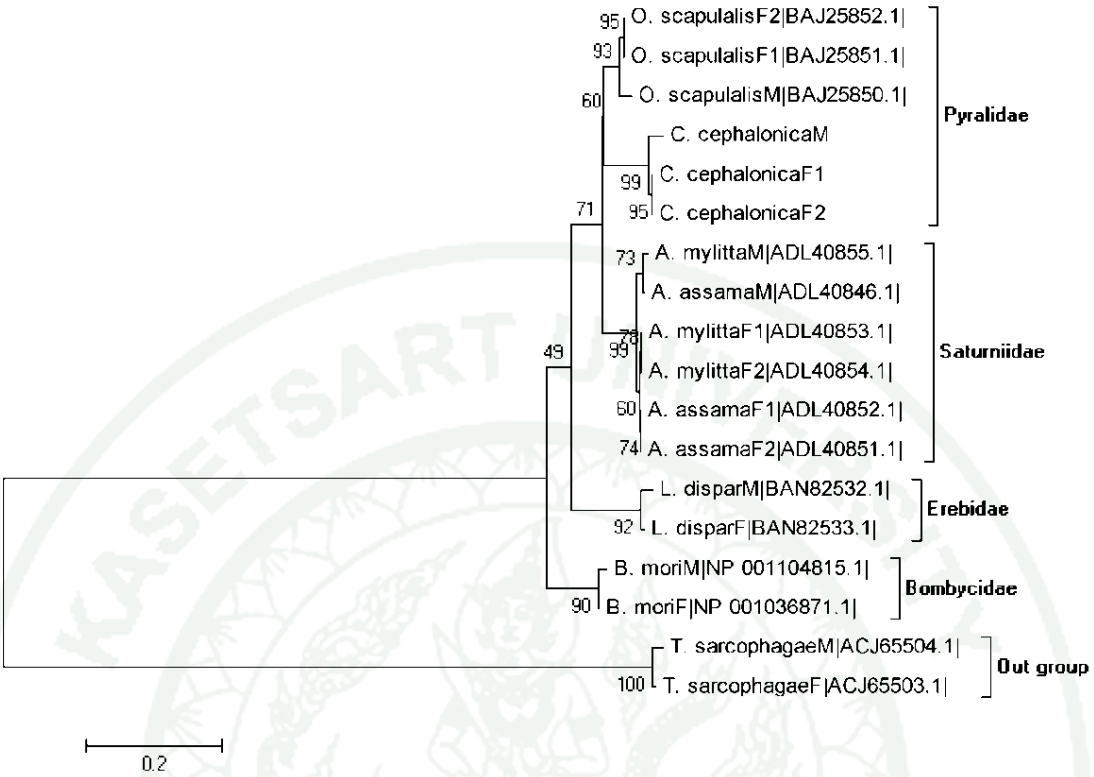

Figure 28 Phylogenetic tree constructed by using amino acid seguences of DSX proteins of some insects using MEGA 5.03 program, Maximum Likelihood method.

4. Cloning and sequencing of *vitellogenin* gene in *C. cephalonica*

4.1 PCR Amplification

The total RNA of adult female was used as a template to synthesis the first strand cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). Then, the first strand cDNA was used as a template for amplification three fragments of *vitellogenin* cDNAs by three primer sets, CceVgF1 and CceVgR1 for fragment 1(figure 29, A), CceVgF2 and CceVgR2 for fragment 2 (Figure 29, B) and CceVgF3 and CceVgR3 for fragment 3 (Figure 29, C). The obtained three PCR products were 1,086, 695 and 2,483 bp long, respectively. The three PCR products were purified and

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ligated into pGEM-T® easy vectors. Then, the three recombinant vectors were transformed into *E. coli* DH5α by heat shock transformation.

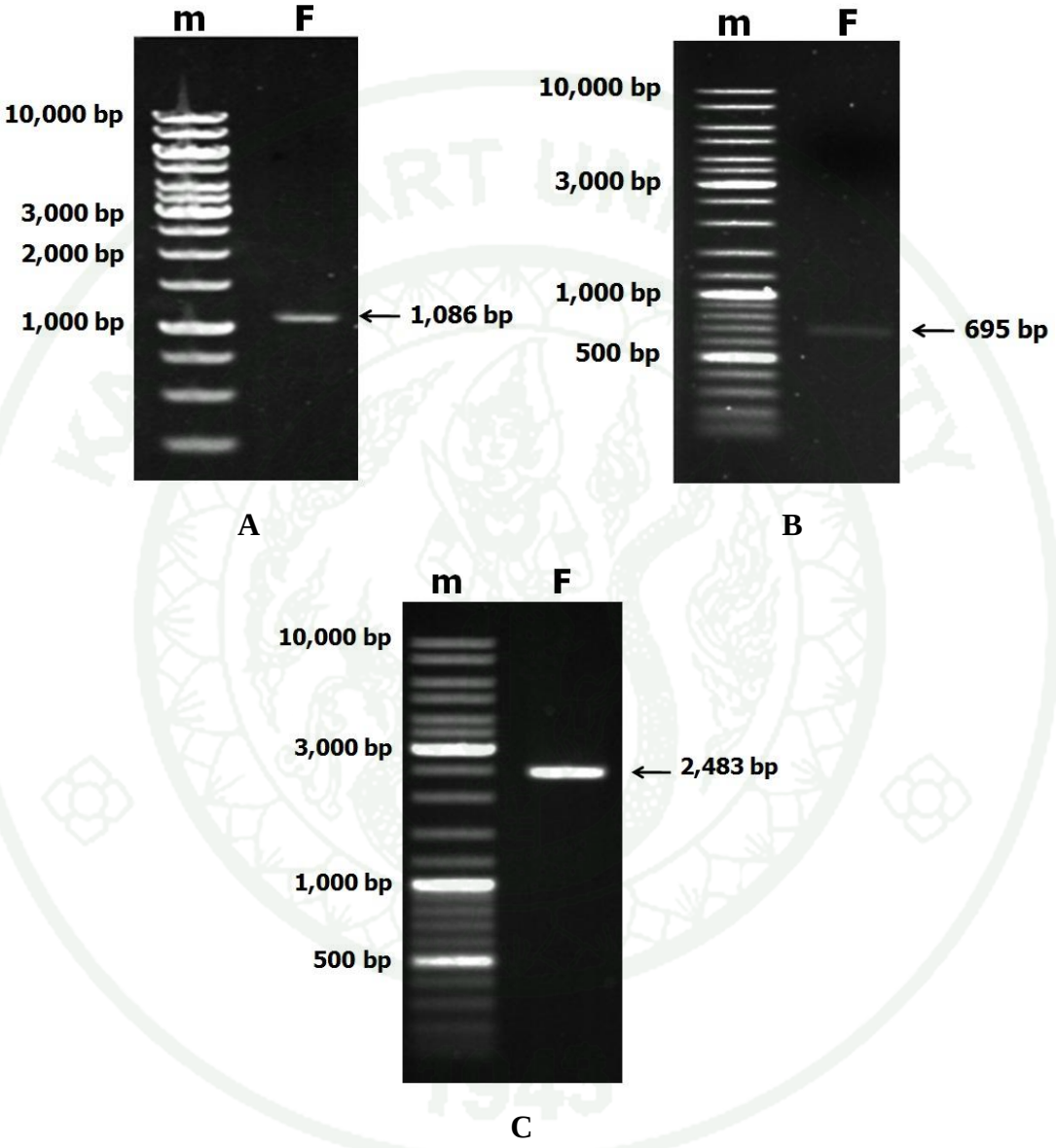

Figure 29 Agarose gel electrophoresis of the PCR product amplified from the female *vitellogenin* cDNA. A: PCR product of fragment 1, B: PCR product of fragment 2 and C: PCR product of fragment 3. m: DNA ladder and F: PCR products of *vitellogenin* gene from female**.**

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4.2 Detection of DNA Insertion by Restriction Digestion

The recombinant plasmids containing the *vitellogenin* cDNA were screened by the formation of blue/white colonies on LA ampicillin plate. Three white colonies were randomly selected for DNA plasmid isolation and analyzed by cutting with *Eco*RI to get *vitellogenin* cDNA fragments (Figure 30). The vectors containing the cDNA fragment were sent to Macrogen, Korea for sequencing.

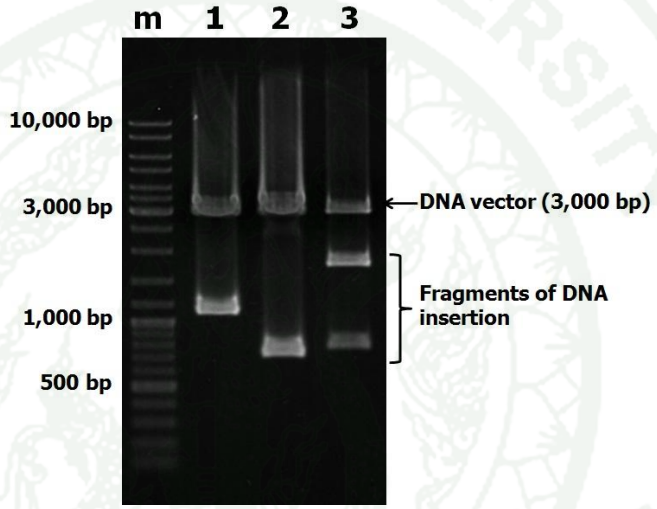

Figure 30 Agarose gel electrophoresis of recombinant vectors digested with *Eco*RI to select the expected inserted fragment of *vitellogenin* gene. Lane 1: DNA ladder and Lane 2-4: *Eco*RI digested recombinant plasmid clones.

4.3 Rapid amplification of the 5'ends of *CceVg*

The 5' RACE reactions was performed using the SMARTer^{TM} RACE cDNA Amplification Kit User Manual (Clontech) according to manufacturer's instructions, using one adapter-specific and the other gene-specific primers. The gene-specific primers used for 5' RACE of *CceVg* was 5'RACE-VG**.** The size of PCR product was 883 bp long (Figure 31) and purified PCR product from agarose gel and ligated into pGEM-T® easy vectors. Then, the recombinant vectors were transformed into *E. coli* DH5α by heat shock transformation.

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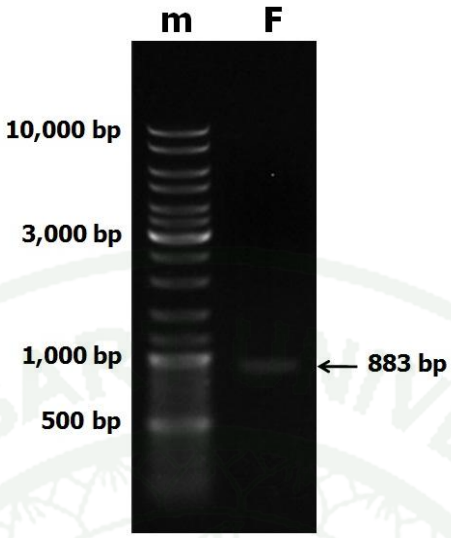

Figure 31 Agarose gel electrophoresis of the PCR product amplified from the female 5’ ends *vitellogenin* cDNA. m: DNA ladder, F: PCR products of 5’ ends *vitellogenin* gene

4.4 Detection of DNA Insertion by Restriction Digestion

The recombinant plasmids containing the 5’ ends *vitellogenin* cDNA fragment were screened by the formation of blue/white colonies on LA ampicillin plate. One white colony were randomly selected for DNA plasmid isolation and analyzed by cutting with *EcoR*I to get 5’ ends *vitellogenin* cDNA fragment (Figure 32). The vectors containing the DNA fragment were sent to Macrogen, Korea for sequencing.

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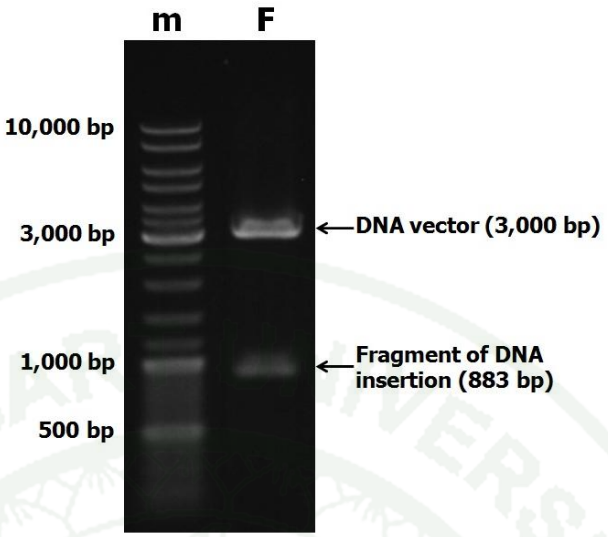

Figure 32 Agarose gel electrophoresis of recombinant vectors digested with *Eco*RI to select the expected inserted fragment of 5' ends *vitellogenin* gene. m: DNA ladder and F: *Eco*RI digested recombinant plasmid clones.

4.5 Rapid amplification of 3'ends of *CceVg*

To identify the 3' UTR of *CceVg* gene, the 3' RACE reactions was performed using the SMARTer™ RACE cDNA Amplification Kit User Manual (Clontech) according to manufacturer's instructions, using one adapter-specific and the other gene-specific primers. The gene-specific primers used for 3' RACE of *CceVg* was 3'RACE-VG**.** The size of PCR product was 1536 bp long (Figure 33). The PCR product was purified and ligated into pGEM-T® easy vectors. Then, the recombinant vectors were transformed into *E. coli* DH5α by heat shock transformation.

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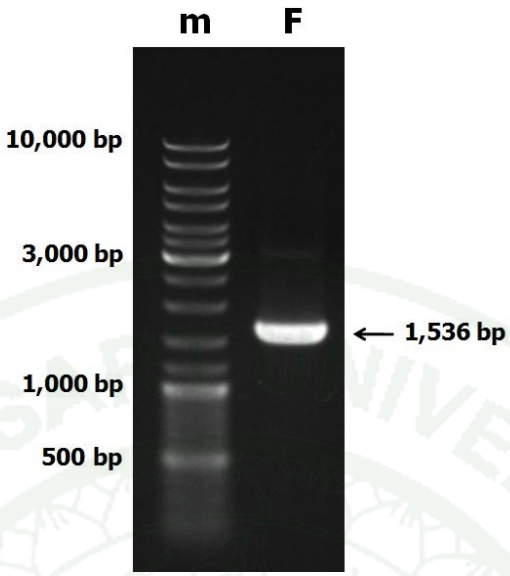

Figure 33 Agarose gel electrophoresis of the PCR product amplified from the female 3’ ends *vitellogenin* cDNA. m: DNA ladder, F: PCR products of 3’ ends *vitellogenin* gene

4.6 Detection of DNA Insertion by Restriction Digestion

The recombinant plasmids containing the 3’ ends *vitellogenin* cDNA fragment were screened by the formation of blue/white colonies on LA ampicillin plate. One white colony were randomly selected for DNA plasmid isolation and analyzed by cutting with *Eco*RI to get 3’ ends *vitellogenin* cDNA fragment (Figure 34). The vectors containing the DNA fragment were sent to Macrogen, Korea for sequencing

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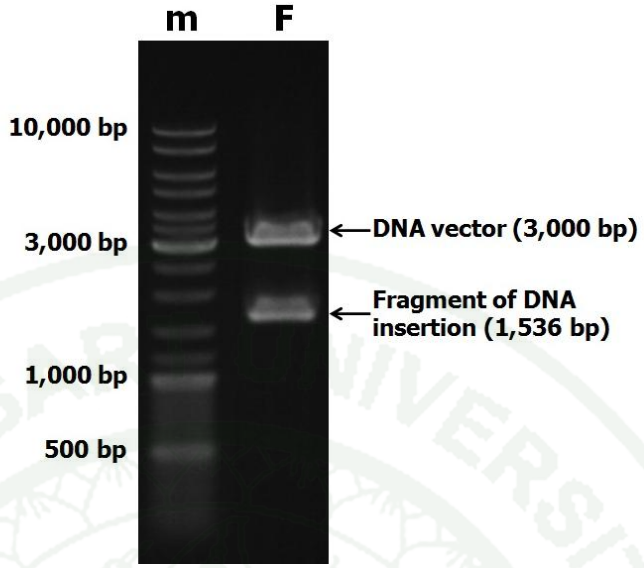

Figure 34 Agarose gel electrophoresis of recombinant vectors digested with *Eco*RI to select the expected inserted fragment of 3' ends *vitellogenin* gene. m: DNA ladder and F: *Eco*RI digested recombinant plasmid clones.

4.7 Complete Nucleotide and deduce amino acid sequences of *vitellogenin* gene of *C. cephalonica* from cDNA analysis

The sequences to determine the precise structures of *CceVg*, cloned cDNAs were sequenced by Macrogen Company. Nucleotides sequence of *vitellogenin* cDNA deduces amino acid sequences by ORFfinder. Complete nucleotide sequence of *vitellogenin* cDNA gene of *C. cephalonica* (*CceVg*) was obtained by assembly the nucleotide sequence of 5'RACE, the three fragments and 3'RACE using CAP3 program. Complete *CceVg* sequences was 5,483 bp long, consists of a 60 bp 5′-untranslated region (UTR), an 5382 bp open reading frame and a 41-nucleotide 3′-UTR. The ORF encoded a deduced CceVg protein of 1793 amino acid. The analysis of the primary protein revealed that the first 16 amino acids correspond to a putative signal peptide [as analysed with the SignalP-4.1 program (http://www.cbs.dtu.dk/services/SignalP/)] (Figure 35). The predicted molecular weight of the mature protein was 202.46 kDa with an isoelectric point (pI) of 9.09 by ExPASy. The National Center for Biotechnology Information (NCBI) Conserved Domain Database (CDD)

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search (http: //www.ncbi.nlm.nih.gov/Structure/cdd/cdd. shtml) predicted two vitellogenin-N domains (also known as the lipoprotein N-terminal regions) at the amino-terminus of the CceVg (amino acid positions: 27–350 and 420–737, respectively). Third domains, the domain of unknow function (DUF1943) (amino acid position: 769–1052). Finaly domain, the von Willebrand factor type D (VWD) domain, was identified at the C-terminus (amino acid position: 1453–1621). In addition, three polyserine regions, five RXXR consensus cleavage sites (RHIR, RVRR, RLCR, REDR and RPER), the GL/ICG motif, DGXR motif and the cysteine residues conserved at the C-terminus were identified (Figure 35). The sequence of DGXR motif in CceVg was DGQR (amino acid position: 1585–1588), while the sequence of the GI/LCG motif of all Lepidopteran Vgs including CceVg was GICG (amino acid position: 1602–1605). Furthermore, two putative glycosylation sites (NNT and NDT) were found in CceVg, but only the NNT site may be effectively glycosylated predicted by the NetNGlyc 1.0 program (http://www.cbs.dtu.dk/services/NetNGlyc/) and 127 potential phosphorylation residues were found in the CceVg sequence by including 81 serine (S), 17 Threonine (T) and 29 Tyrosine (Y). The interesting feature of vitellogenin protein is the presence of three polyserine tract in CceVg. Two of polyserine tract (8 and 12 serine residues) flanking the conserved cleavage site, RVRR, were at the N-terminal region and the third tract (12 serine residues) was at the C-terminal.

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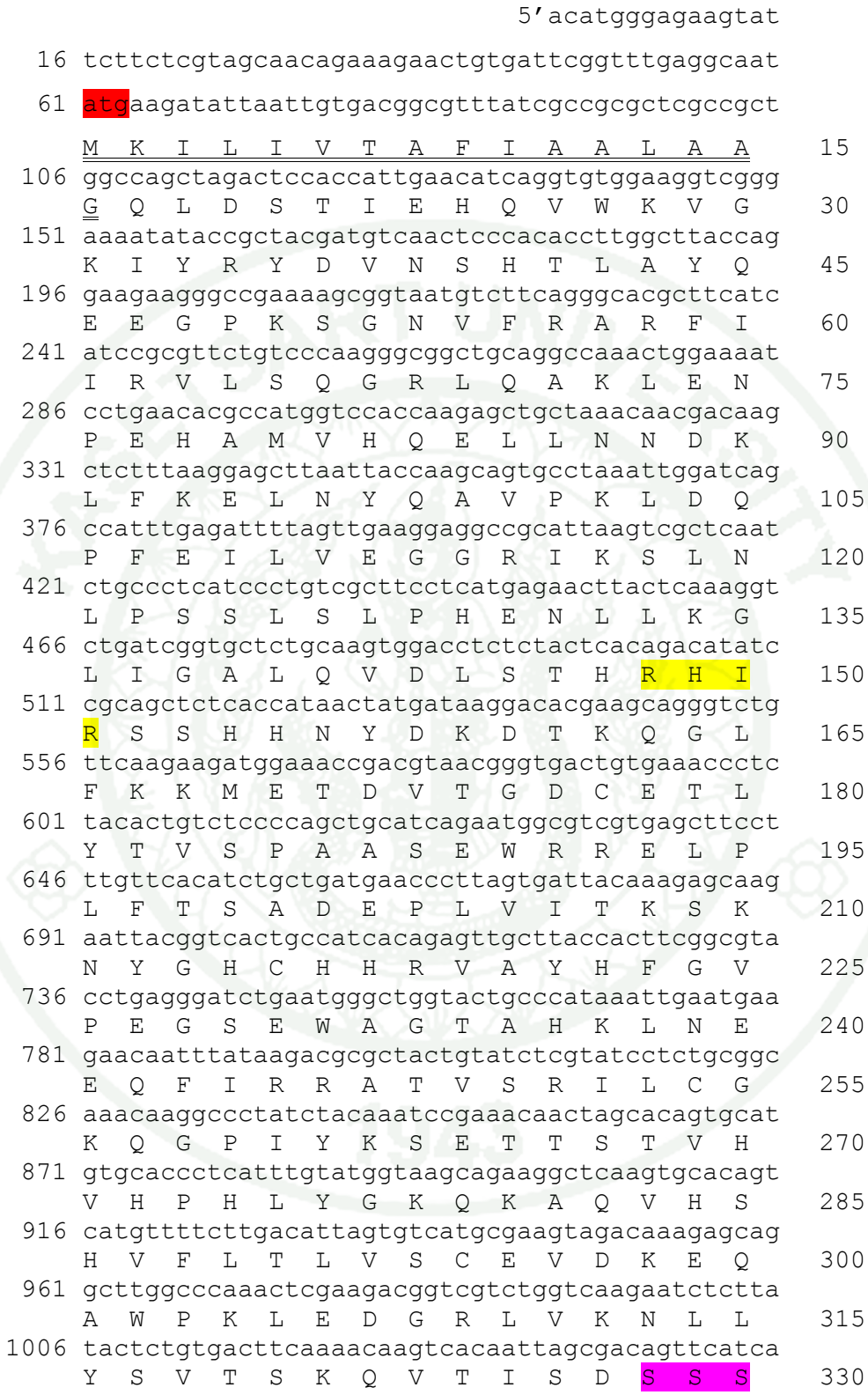

Figure 35 Complete nucleotide sequence and deduced amino acid sequence of the *vitellogenin* cDNA of *C. cephalonica.*

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```
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2086 caccaagttcgctctgttctattcagtatcttgaccaactcagca
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```

Figure 35 (Continued)

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Figure 35 (Continued)

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Figure 35 (Continued)

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4471 atcctgagcaaatcttggcacgtcgtgatgctagacagcaggcca
     I  L  S  K  S  W  H  V  V  M  L  D  S  R  P    1485
4516 gaacgcaatgacaaactggtagtcctcgctagaaagccgaatgag
     E  R  N  D  K  L  V  V  L  A  R  K  P  N  E    1500
4561 aaacaacaggaaatatacatttcttacgtgtccaagaacagcggc
     K  Q  Q  E  I  Y  I  S  Y  V  S  K  N  S  G    1515
4606 ttgcatctagaggtagaaatccaaccaactcgtgctgattctggc
     L  H  L  E  V  E  I  Q  P  T  R  A  D  S  G    1530
4651 aaatactccatgaaagttaacaccaatgccaagaagatctccgaa
     K  Y  S  M  K  V  N  T  N  A  K  K  I  S  E    1545
4696 ggcgacctaaccatgtactgggatgaggtcaacgaaatacctctc
     G  D  L  T  M  Y  W  D  E  V  N  E  I  P  L    1560
4741 ttccagtactacaccaattccgatggagtcttagttttggatatc
     F  Q  Y  Y  T  N  S  D  G  V  L  V  L  D  I    1575
4786 aagaaagatcgtctacgtgcaatgtacgatggtcaaaggattgtt
     K  K  D  R  L  R  A  M  Y  D  G  Q  R  I  V    1590
4831 gtcttatcaaaggaagaccgtagcaagacccgaggcatttgcgga
     V  L  S  K  E  D  R  S  K  T  R  G  I  C  G    1605
4876 ctcatgtctggatcaccccgtcatgattacctaaccccttacggc
     L  M  S  G  S  P  R  H  D  Y  L  T  P  Y  G    1620
4921 atggtcgacttgcccgagcattatggagccgctttctctctactc
     M  V  D  L  P  E  H  Y  G  A  A  F  S  L  L    1635
4966 aatgaagacgctgatcctaaaaccaaagaattgcttgaggaggcc
     N  E  D  A  D  P  K  T  K  E  L  L  E  E  A    1650
5011 aaggaagccgcataccagcccagaaaccagcccactttaatattg
     K  E  A  A  Y  Q  P  R  N  Q  P  T  L  I  L    1665
5056 aggtctaatggtgaatggaagaaggccatgtactccagttcatca
     R  S  N  G  E  W  K  K  A  M  Y  S  S  S  S    1680
5101 tcgtcttcttcgtcatcatcgtctgaggagaactatggatcgcag
     S  S  S  S  S  S  S  S  E  E  N  Y  G  S  Q    1695
5146 aacgtgtacagatcaaggaattacttgaaacaacgtggtgaatgc
     N  V  Y  R  S  R  N  Y  L  K  Q  R  G  E  C    1710
5191 caagttaaaagacaggtgcaatattacgagaaccatggcgagatc
     Q  V  K  R  Q  V  Q  Y  Y  E  N  H  G  E  I    1725
5236 tgcatcaccatcacgccattggagtcttgccagtcccactgcaat
     C  I  T  I  T  P  L  E  S  C  Q  S  H  C  N    1740
5281 gctgaaggttatcagattcaaccagtccaagtcacttgcagaccg
     A  E  G  Y  Q  I  Q  P  V  Q  V  T  C  R  P    1755
5326 aaaatagaccagcaattcatggctttcagggatgagattaggcga
     K  I  D  Q  Q  F  M  A  F  R  D  E  I  R  R    1770
5371 ggtgggaaccctaaggtgaccggtgtccctaaaatcgttcagtac
     G  G  N  P  K  V  T  G  V  P  K  I  V  Q  Y    1785
5416 agagtaccatctgcgtgcaaagtatagataaattataaataaaaa
     R  V  P  S  A  C  K  V  *                      1793
5461 aaaaaaaaaaaaaaaaaaaaa 5484 3′
```

Figure 35 (Continued)

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The figure 35 shows nucleotides are numbered on the left of each line from the top. Amino acids are numbered on the right each line from the translation initiation methionine. The position of start and stop are red boxed. Consensus RXXR sequences for possible cleavage sites are yellow boxed and polyserine residues are pink boxed. The sequence with a consensus DGQR and GICG are shown with Blue boxed. Possible glycosylation sites are shown with Green boxed. A consensus polyadenylation signal is double underlined.

5. Identification the structure of *vitellogenin* gene in *C. cephalonica*

5.1 PCR amplification of *vitellogenin* gene from genomic DNA

Genomic DNA was extractrd from *C. cephalonica*. The *vitellogenin* gene of *C. cephalonica* was amplified by polymerase chain reaction (PCR), the genomic DNA of female was used as a template for amplification the *CceVg* gene using the High Fidelity PCR Enzyme Mix (Thermo Scientific) and Primer GVG-F and GVG-R. The obtained PCR product was 6,548 bp (Figure 36) and was purified and sent to Macrogen, Korea for sequencing.

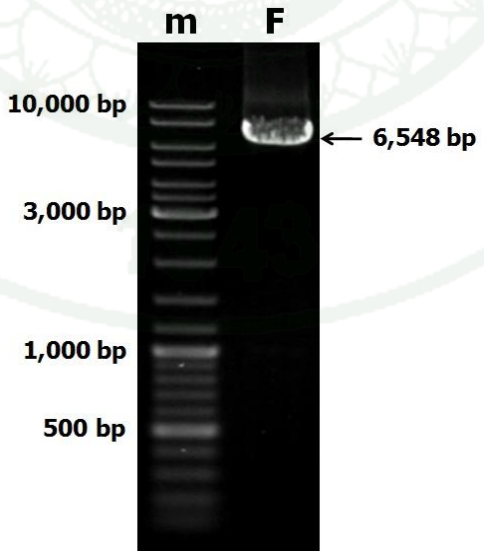

Figure 36 Agarose gel electrophoresis of the PCR product amplified from the female *vitellogenin* genomic DNA.

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5.2 Complete Nucleotide sequences of *vitellogenin* gene of *C. cephalonica* from DNA analysis

Complete nucleotide sequence of *vitellogenin* genomic DNA of *C. cephalonica* female was assembly the nucleotide sequence of 5'RACE and 3'RACE. The complete *CceVg* sequence was 6,721 bp long, included a 60 bp 5'- flanking region, 41bp 3'- flanking region and six exons (5382 bp) and five introns (1238 bp). The six exon are exon1 (2249 bp), exon2 (214 bp), exon3 (1003 bp), exon4 (885 bp), exon5 (178 bp) and exon6 (853 bp). The five intron are intron1 (238 bp), intron2 (123 bp), intron3 (242bp), intron4 (257 bp) and intron5 (378 bp). The start codon ATG (at position 61-63) was at the beginning of exon 1. The stop codon TAG was in the exon 6 at position 6678-6680 and the polyadenylation site (AATAAA) was at position 6691-6696 (Figure 37, 38). The sequence was submitted into Gene bank (accession no KJ540279).

```
5′  1 ACATGGGAGAAGTATTCTTCTCGTAGCAACAGAAAGAACTGTGATTCGGT  50
 51 TTGAGGCAATATGAAGATATTAATTGTGACGGCGTTTATCGCCGCGCTCG 100
101 CCGCTGGCCAGCTAGACTCCACCATTGAACATCAGGTGTGGAAGGTCGGG 150
151 AAAATATACCGCTACGATGTCAACTCCCACACCTTGGCTTACCAGGAAGA 200
201 AGGGCCGAAAAGCGGTAATGTCTTCAGGGCACGCTTCATCATCCGCGTTC 250
251 TGTCCCAAGGGCGGCTGCAGGCCAAACTGGAAAATCCTGAACACGCCATG 300
301 GTCCACCAAGAGCTGCTAAACAACGACAAGCTCTTTAAGGAACTTAATTA 350
351 CCAAGCAGTGCCTAAATTGGATCAGCCATTTGAGATTTTAGTTGAAGGAG 400
401 GCCGCATTAAGTCGCTCAATCTGCCCTCATCCCTGTCGCTTCCTCATGAG 450
451 AACTTACTCAAAGGTCTGATCGGTGCTCTGCAAGTGGACCTCTCTACTCA 500
501 CAGACATATCCGCAGCTCTCACCATAACTATGATAAGGACACGAAGCAGG 550
551 GTCTGTTCAAGAAGATGGAAACCGACGTAACGGGTGACTGTGAAACCCTC 600
601 TACACTGTCTCCCCAGCTGCATCAGAATGGCGTCGTGAGCTTCCTTTGTT 650
651 CACATCTGCTGATGAACCCTTAGTGATTACAAAGAGCAAGAATTACGGTC 700
701 ACTGCCATCACAGAGTTGCTTACCACTTCGGCGTACCTGAGGGATCTGAA 750
751 TGGGCTGGTACTGCCCATAAATTGAATGAAGAACAATTTATAAGACGCGC 800
801 TACTGTATCTCGTATCCTCTGCGGCAAACAAGGCCCTATCTACAAATCCG 850
851 AAACAACTAGCACAGTGCATGTGCACCCTCATTTGTATGGTAAGCAGAAG 900
901 GCTCAAGTGCACAGTCATGTTTTCTTGACATTAGTGTCATGCGAAGTAGA 950
951 CAAAGAGCAGGCTTGGCCCAAACTCGAAGACGGTCGTCTGGTCAAGAATC 1000
1001 TCTTATACTCTGTGACTTCAAAACAAGTCACAATTAGCGACAGTTCATCA 1050
1051 TCGTCTTCATCGTCAGAATCGAATGAAAATATAAACGATCAAATTGAAAA 1100
1101 TATTCAACAAAGAGTACGTCGGTCCGCAAAGAAGATTGTAAGCATTAACA 1150
```

Figure 37 Complete DNA sequence of *C. cephalonica vitellogenin* gene. The position of start and stop are red boxed and introns are pink boxed.

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1151 AGGTTGTCTTAAAGCAAAACGATGAAGCAGCTAGTTCCTCAAGTTCATCT 1200
1201 AGCTCATCCGCATCTTCTAGCTCCAGCGAATCAGCATCTTTCATGAACGA 1250
1251 TGACATACCAAAAAATAATGAGCCCGCTTATGCTGCTTTATATATGACTT 1300
1301 CCCATTCTCGCGGTGACAAGAAACAAAATCCGATGAATGCACAGAAACTT 1350
1351 ATTCAAGACATTGCTCAGCAGCTACAAAATCCTAACAATATGCCTAAAGC 1400
1401 CGATGCTTTGTCGAAATTCAACATCCTTGTTCGCATCTTAGCCTCAATGA 1450
1451 GTACTGAACAACTTGCTCAAACCAGTCGCAGCATTGAAGTTGGCAAATCA 1500
1501 TCCAACAACCACGTAAAATCAGATATGTGGATGATATTCCGGGATGCAGT 1550
1551 AGCCCAAGCAGGCACTCTTCCTGCCTTCCAACTGATCAAAACTTGGATTA 1600
1601 AAACTAATAAGATTGTAGAAGAGGAAGCAGCACAGGTGATAGCTGTACTT 1650
1651 GCTGGTACTCTGCGGTATCCCACCAAAGATCTTATGTTACAGTTCTTCGA 1700
1701 TCTCGCAATGAGTGCTGAAGTAAGAGAACAGAAATATCTTAATAACACCG 1750
1751 CTTTGATTATCGCAACTAGATTCATTAATAAAGGACAAGTGAACAACGAC 1800
1801 ACCGCTCATAATTTCTACCCAACTTATATGTATGGCCGTCTTTGTCGCCG 1850
1851 TCACGATCAATTTGTAACCGAGAAAGTCCTTCCACGTCTTTCTCAAGAAT 1900
1901 TGAAACAACTTATCCAGCAAGAGGATACTAATAAAGCACAGGTCTTTGTG 1950
1951 AGAGCGATTGGTAACTTGGGGCATCGTTCCGTCTTGGAAGTGTTCGCTCC 2000
2001 TTACTTAGAGGGCAAAATTGCAGTGACGAAGTTCCTTCGTCGTGAAATGG 2050
2051 TCAACAGTTTAGATGTTCTGGCATATCAAAGAGATCACCAAGTTCGCTCT 2100
2101 GTTCTATTCAGTATCTTGACCAACTCAGCAGAAGCATACGAAATAAGGGT 2150
2151 TGCTGCTATCAATAACATTATGACTGCCAAGCCAACGACTCCAATGATGA 2200
2201 TGGCGATGGCCCAAATGACCCAGCATGATCCTAGCATCCACGTTCGTGCT 2250
2251 TCACTCAAATCTGCGATTGAAACCGCTTCCAATTTGAAGAACCCTCGCTA 2300
2301 TCTGGATCTGTAAGTACTATTTGTTTCATAATGTAGAATACAATATTTAC 2350
2351 TTGAAATGTTAATTGGACTATAGGATTGTCGGTAAAGTTTCGTTTAGTTC 2400
2401 TTGAAATTCCAAAAGTAGGTATTATTATATTTTTATAACCACCTATTTAT 2450
2451 TTTAATATTTTATTATATTTATTAATAGGTTTTCCTTCTTTCTTGGATTG 2500
2501 TATTATTATATTATAAGTAAGTATTCATGGTTAACTTATTCTTACAGTGC 2550
2551 AAGAACAGCTAAAGCTGCTAAATGGATGGTGACCAAGGAAGATTTCGGAA 2600
2601 AACAATACTCCACCAAATGCTACGAAGACTTCCATAATAATCAATATGAA 2650
2651 AAGAGTATCTGGAAGGAAGTCACATCTATTGGAAGCCAAGATAGTCTTCT 2700
2701 ACCGAAATTCTTAAAATACTCTCTGAAGAGCAGATCCAGAGGCTGGAATA 2750
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2801 AACTATCTGAGGCAAGTACAAATAATCTTGACTAAACCGATTTTTTACCA 2850
2851 CGTTCAACGAACATATATTCTTTTCTTTTTACAGATATCTGCTTCAGTCT 2900
2901 CCAGCGTTAGTCAGTTTATCGATTTGTACAAGTACCTCACAAGTCAAATG 2950
2951 CACAGGAATCAACAGAACCAGCAAGAACCTAATCATAAGTTCACAGCTGA 3000
3001 CAACGTCGCAGAAATGTTGAACATTAAGCGTGAAGATCGCCAGCCCCTTG 3050
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3101 AGTGAATCCGATATTAAGCAACTGCCTAAATTCATGTCGGAGTACTTCTC 3150
3151 TGATGTCAACAAAGGCATTGAGAGGCATTACACCAAGATGTTCAACCAGG 3200
3201 CTCAGGTGTCGATAATGTTCCCAGTAGCTATGGGTGTACCGTTCATCTAC 3250
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3301 GGTTCAACAACCATCACAGGAGAACAGACATCACTCGGTCTCTGTCGATA 3350
3351 AAGAGGTGTACTTCACGTACGCAAGAAATCTTGATGGTAGCGTAGGATTC 3400
3401 TTGGATACTCTTTCTAATCAATATGCTTCTACTGGTGTAGTGAACAAGTT 3450
3451 GCAAGTGAGCATCCCTCTGAAGTTACACATTGGTATAAATTCCGGAGAAG 3500
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3551 CACTATAGCGTGTGGCCATATACATCTAACCAGAAGAAAGACAGTTTGAC 3600
3601 ACCGATCTCCCAAGATCCTACCACTAAGGTTATTAACCGTCAATTCAAAA 3650
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Figure 37 (Continued)

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3651 CATCAACCATTGACACGAAGTTCGGGCAATCCGTCGGCATGTTGTTCCAA 3700
3701 CTACAAGGACACTCCTACTCCTTAGACTACAAAAACTTTGGGGACATGCT 3750
3751 CTTAGAGGCATCACGAAATAAATTTTTGGATATTTACTCAGTTCTTTCCC 3800
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3851 AGCCAAAACAAGCGTGTAAAGCTGACCGCAGTTTATGGTGAGTACTTTCA 3900
3901 AATGCTAAATCGACAAAAGATATATAATAACGAACCATCATTATCATGTA 3950
3951 ATTAGTCCGCTGCATTGCCAACCTAGAATGGGAAATGGGGGTTGCGGAGA 4000
4001 AGATTAAAGAATTGGACGATTTGGACTATCAGCCTATTTTCGAACGAAAT 4050
4051 TCAGTATATCAAATTCAAAAATTTTATGAAAGTGTGTGTAAACTGCTTCA 4100
4101 TATTCATAGTATAAATGTTATTTTTACAGATACGCTCTACAATCAAAAGC 4150
4151 GAGACACAAAACAAGAGTATACTGCGACGAATGTCAAAGACGTTAAACCA 4200
4201 AACAGCGAAGATAGACGTCAAGAGTTGGTTAATCGTGTTGTGCCAGAAAT 4250
4251 AAACATAGCCACAGTCAAAGTACTCGACTTAAGCGCTACCTTTGAGGGAC 4300
4301 CACAACAAGTGGAATTCGTCTATACATCGGCCATGGCAAACAGTCCGGTT 4350
4351 GACCTGAAGATTCATTACGCTTTCTTTGTCGGTAAAAACTCTGAGCAACA 4400
4401 TGGAAACAGTCAACTTAATGGCATTGGAATGATTTTGAAACCACAAATGT 4450
4451 CATTCTTAAACTTCCTCGAAGCACTCAAGAAAGAAATGAAGACAGTATTT 4500
4501 GCTTTCGATGTCAATTTCGGTAAAAACAATGGAAACATTAATGTCCAAGG 4550
4551 TTTTTTGGAGCGCAGCAAGAAATTCGAGGAATTACTCCAGAACCACCCCT 4600
4601 TAGCCAAGATTTGCAAGGAACAAATGAGCCAAAACAATTATTATCAAGAA 4650
4651 GCTTGCCACAAAATGATAGTGTTAGCTCATGCTCCTGATTTCCTGAAGGC 4700
4701 TGCAGTCACATACAAGGAAGTTACTCCTTACGCTAAAGCAATAATGAGCC 4750
4751 AGGTTTACAGAATGCTGGAATACTTGGGTTACTTGCATTCCGAATCTAAC 4800
4801 CTAATGAAGGTAACTCCTGATGGTAAACTAGAGGTTGCCGCTCAGATTTC 4850
4851 GTACTGGCTACAGTCTATGAATTTGGTCATAAACTCAAGAATGGGTGAAC 4900
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4951 ATTTACTCACCTTTCAAAGCTAGTCAACGCGTGAAGAACTATTATACTAG 5000
5001 TCACCAATATCTACGTAAGTAATCATTATTTCAAATTTTCCCATATGCCT 5050
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5101 CTGAATTGAAATTTTGTTTAAATAAATAAGGAAACTTTTGGTGATCTTGA 5150
5151 ATTTACATTTCATCCTGTAAGAAAATGGTATAATAAATTTAAGTTCCATC 5200
5201 AAGTACCGACAATATACAATAACTTTATAATGATATATTTACTTAGAAAA 5250
5251 ATATTATTTTTTCTTTAACAGCTTATTGCTCTGTTGACACGAAGGCAGTG 5300
5301 AAAACATTCAGTAACCGCGCTTACGAATACATCCTGAGCAAATCTTGGCA 5350
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5501 TTGTCAAGAGACTATTAGGCCTTCCCCAGTGCTTTGGACAAATTAAAAAC 5550
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5651 ATAAACGTCTTCATAATTCGAAGATCACATAGCTAAAACTGTCCGTCCTG 5700
5701 ACTGCTATTGCGACTGCTGATTTCTAATTCATATAGTTAAAAAAAATCAA 5750
5751 GAAATATCGTGGATGGAAAAGGAAGGAATTTGTTAAAACTTTCCTGTATA 5800
5801 ATAATGTTATTTCTTTTTTTTTATTAGGTCCAAGAACAGCGGCTTGCATC 5850
5851 TAGAGGTAGAAATCCAACCAACTCGTGCTGATTCTGGCAAATACTCCATG 5900
5901 AAAGTTAACACCAATGCCAAGAAGATCTCCGAAGGCGACCTAACCATGTA 5950
5951 CTGGGATGAGGTCAACGAAATACCTCTCTTCCAGTACTACACCAATTCCG 6000
6001 ATGGAGTCTTAGTTTTGGATATCAAGAAAGATCGTCTACGTGCAATGTAC 6050
6051 GATGGTCAAAGGATTGTTGTCTTATCAAAGGAAGACCGTAGCAAGACCCG 6100
6101 AGGCATTTGCGGACTCATGTCTGGATCACCCCGTCATGATTACCTAACCC 6150
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Figure 37 (Continued)

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6151 CTTACGGCATGGTCGACTTGCCCGAGCATTATGGAGCCGCTTTCTCTCTA 6200
6201 CTCAATGAAGACGCTGATCCTAAAACCAAAGAATTGCTTGAGGAGGCCAA 6250
6251 GGAAGCCGCATACCAGCCCAGAAACCAGCCCACTTTAATATTGAGGTCTA 6300
6301 ATGGTGAATGGAAGAAGGCCATGTACTCCAGTTCATCATCGTCTTCTTCG 6350
6351 TCATCATCGTCTGAGGAGAACTATGGATCGCAGAACGTGTACAGATCAAG 6400
6401 GAATTACTTGAAACAACGTGGTGAATGCCAAGTTAAAAGACAGGTGCAAT 6450
6451 ATTACGAGAACCATGGCGAGATCTGCATCACCATCACGCCATTGGAGTCT 6500
6501 TGCCAGTCCCACTGCAATGCTGAAGGTTATCAGATTCAACCAGTCCAAGT 6550
6551 CACTTGCAGACCGAAAATAGACCAGCAATTCATGGCTTTCAGGGATGAGA 6600
6601 TTAGGCGAGGTGGGAACCCTAAGGTGACCGGTGTCCCTAAAATCGTTCAG 6650
6651 TACAGAGTACCATCTGCGTGCAAAGTATAGATAAATTATAAATAAAAAAA 6700
6701 AAAAAAAAAAAAAAAAAAAA 6721 3′
```

Figure 37 (Continued)

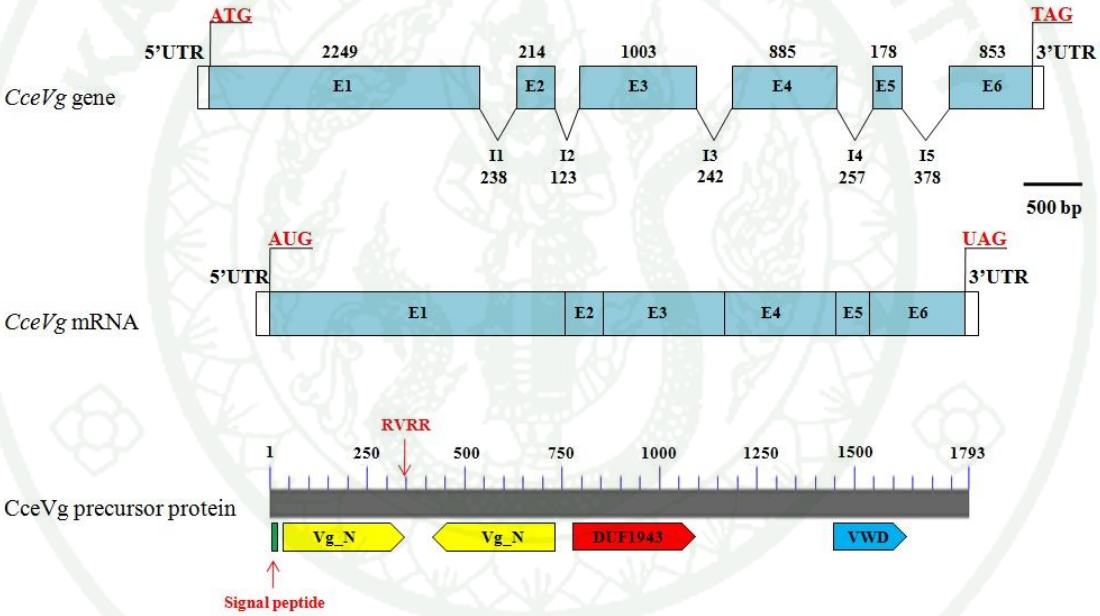

Figure 38 Diagram of the complete *CceVg* gene, structure mRNA and protein. Exons are indicated by blue, the black line between them mean introns.

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6. Multiple alignment and Phylogenetic tree analysis of CceVg

To clarify the evolutionary relationship of CceVg by used a neighbor-joining and Maximum Likelihood methods. Multiple aligment was performed using deduced amino acid sequence of CceVg and that of other 41 species (40 species of arthropod and one species of vertebrate, *Gallus gallus,* which was used as an out group. All the sequences were aligned with ClustalW (ver. 1.83) by the multiple alignment method. The generated data matrix was converted to mega format and analyzed with MEGA 5.03 program. As expected, the result showed that CceVg was grouped with all Lepidopteran Vgs and closed to the Vg of *Cnaphalocrocis medinalis*. In addition, insect Vgs were separated into two major clades when analyzed by Neighbor-Joining method. One clade consists of only all Lepidopteran Vgs, whereas all the remaining insect Vgs were grouped together in the other clade. The topology tree obtained from Maximum Likelihood method was differs from Neighbor-Joining method. From Maximum Likelihood method, insect Vgs were separated into three major clades. First clade consists of Lepidopteran, Dipteran, Dictyopteran, and Hemipteran Vgs, whereas the remaining clades separated between Coleopteran and Hymenopteran Vgs (Figure 39, 40).

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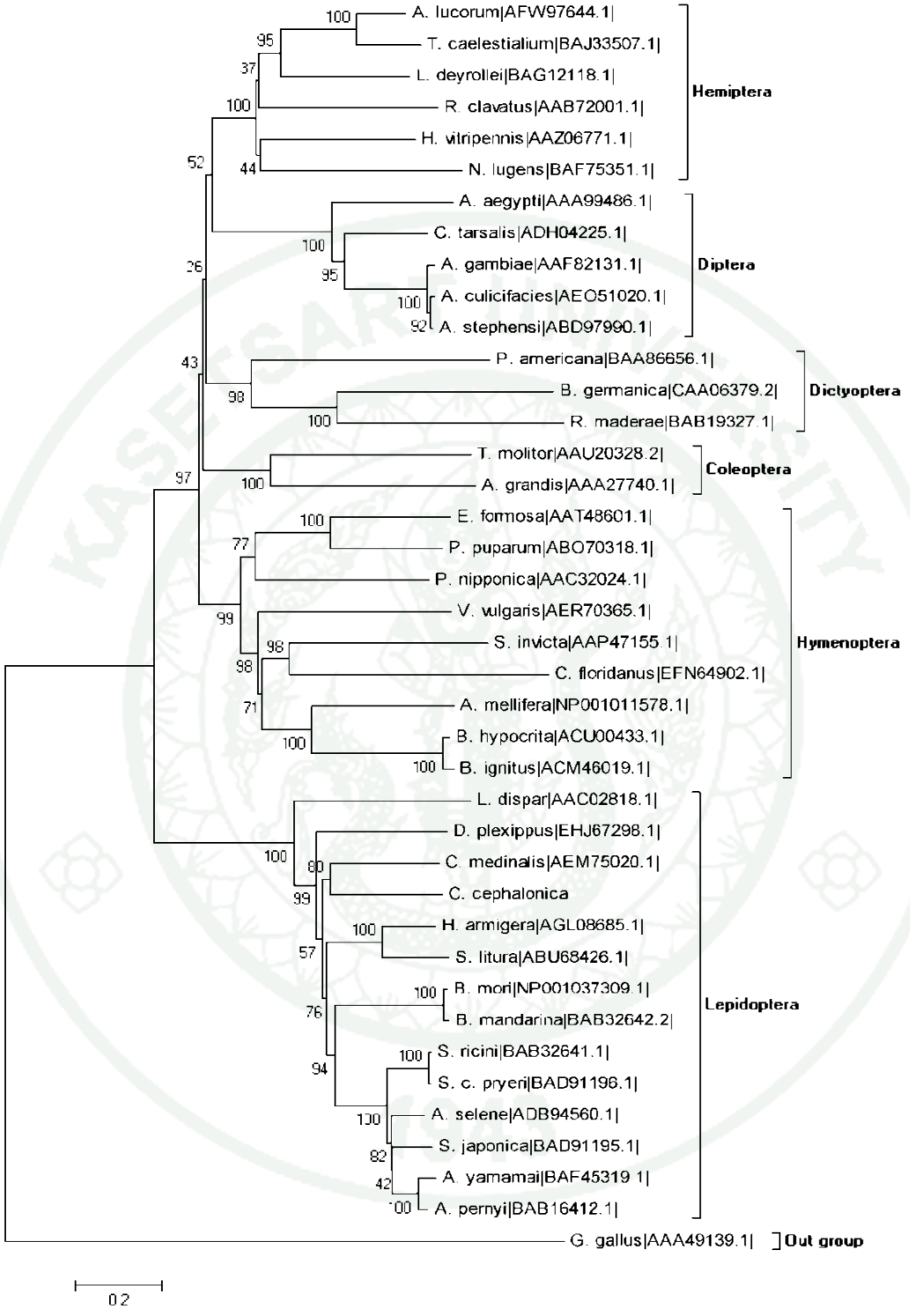

Figure 39 Phylogenetic tree constructed by using amino acid seguences of VG proteins of some insects using MEGA 5.03 program, Neighbor-Joining method

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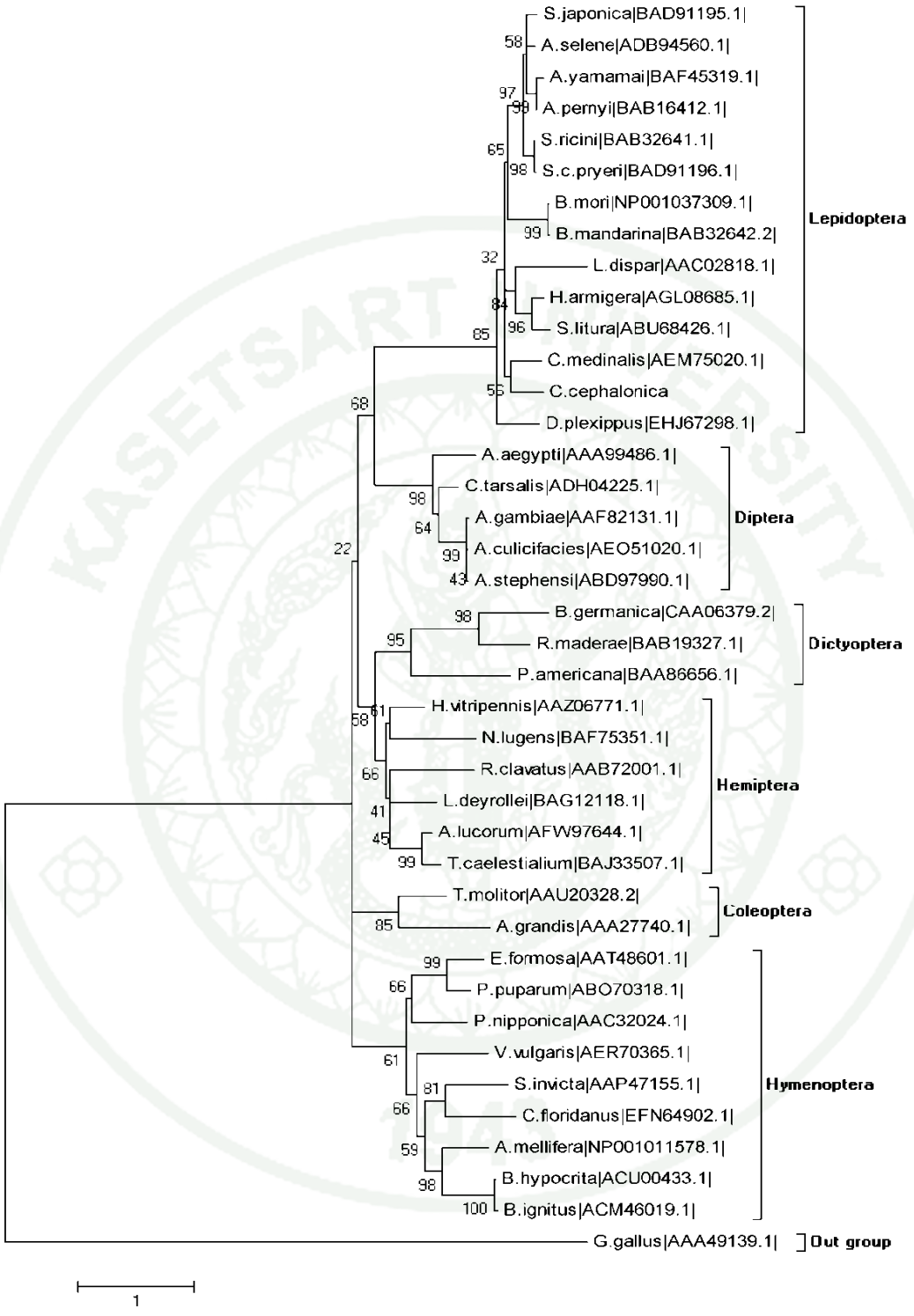

Figure 40 Phylogenetic tree constructed by using amino acid seguences of VG proteins of some insects using MEGA 5.03 program, Maximum Likelihood method

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7. Detection the expression of *vitellogenin* gene by RT-PCR

Reverse transcription polymerase chain reaction (RT-PCR) was performed to determine the expression patterns of *CceVg* at the all developmental stage. Total RNAs were isolated from the eggs, early larva, late larva, early female/male pupae, and late female/male pupae, adult of both female and male of *C. cephalonica*. The total RNAs were reversed to be cDNA which were used as template for RT-PCR with two primers sets. The product of first RT-PCR used VG-RTF1 and VG-RTR1 primer to amplify a 344 bp fragment of *CceVg*. The product of second RT-PCR used VG-RTF2 and VG-RTR2 primer to amplify a 382 bp fragment of *CceVg.* The results showed that *CceVg* gene was not expressed in the egg stage and began to express at low level in early larva stage. The expression of *CceVg* was not detected in late larva stage. Then, the *CceVg* gene resumed to expression at low level again in early pupa stage and substantial increase in late female pupa but not in late male pupa. The *CceVg* gene in female continues to increase to reach its maximum in adult females. Interestingly, the *CceVg* gene was found in adult male at low level (Figure 41).

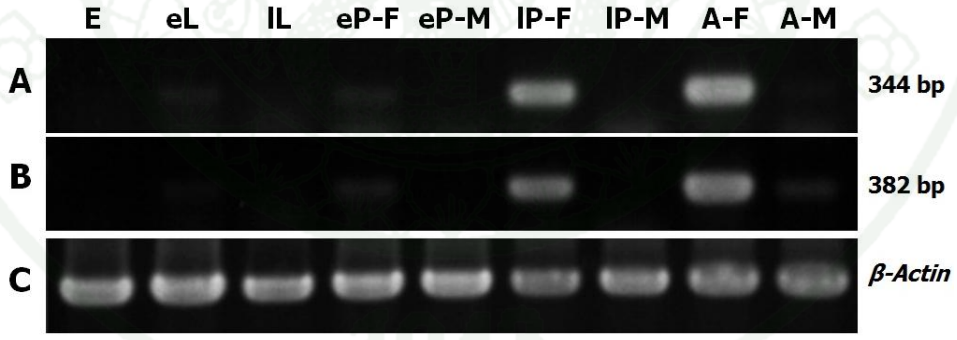

Figure 41 Agarose gel electrophoresis of *C. cephalonica vitellogenin* gene expression at the different developmental stage. A: amplification using VG-RTF1 and VG-RTR1 primer. B: amplification using VG-RTF2 and VG-RTR2 primer. C: *β-actin* was used as a control. (E = eggs, eL = early larva, lL = late larva, eP-F = early female pupae, eP-M = early male pupae, lP-F = late female pupae, lP-M = late male pupae, A-F = adult female and A-M = adult male)

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8. Study the function of *vitellogenin* gene by RNA interference

8.1 Effect of dsRNA injected on the newly adult females

In this study, the expression of *CceVg* gene in early emerged females was disturbed by RNAi experiment. The *vitellogenin* gene knockdown by RNAi was associated with a significant effect on the ovary development. The 1µg dsRNAs was injected into the second abdominal of newly emergent females. The ten females were injected with *CceVg* dsRNA and the other females were injected with *DsRed* dsRNA that was used as a control. After injection for three days, four injected females were dissected and their ovaries were observed. The results showed that the ovaries of female injected with *CceVg* dsRNA were seversly deformed, while the ovaries of females injected with *DsRed* dsRNA showed relatively normal to that of uninjected female (Figure 42). The remaining injected females of each group, two females were collected on the first, third and fifth days after injected and keep at -80°C for real-time PCR analysis.

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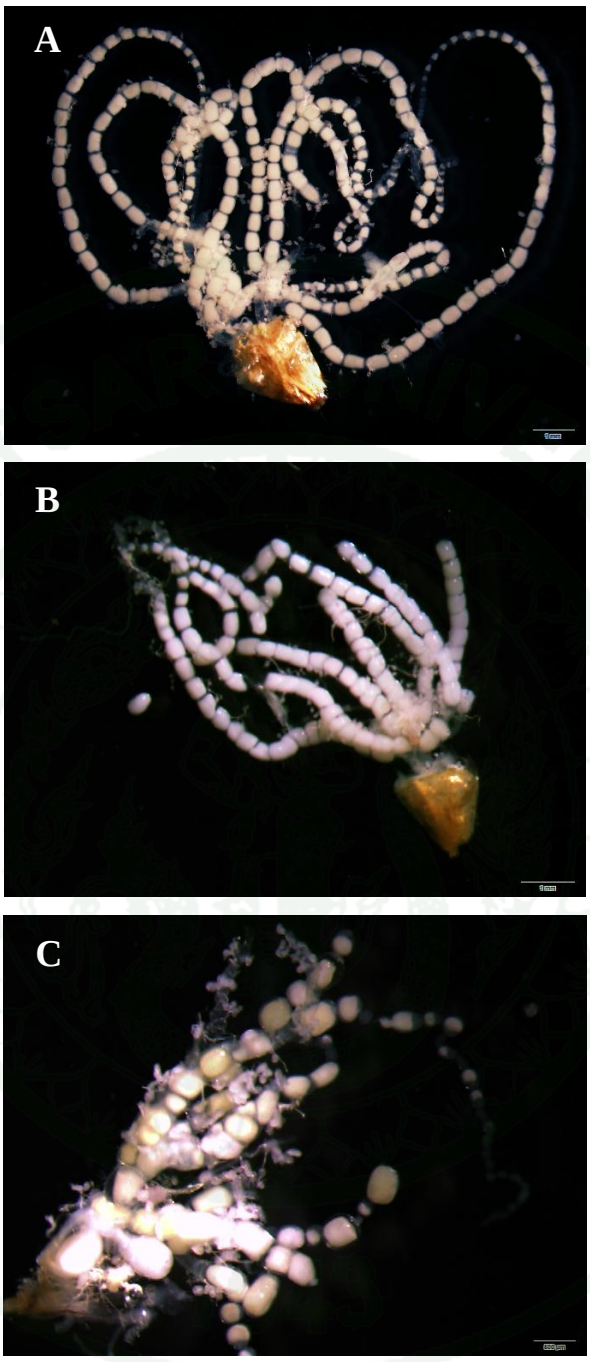

Figure 42 Effect of *CceVg* dsRNAs on the ovary development. (A) Ovary of adult female uninjected. (B) Ovary of adult female injected with *DsRed* dsRNA. (C) Ovary of adult female injected with *CceVg* dsRNA.

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8.2 Detection the expression level of *vitellogenin* gene by quantitative real-time PCR (qPCR)

Real-time PCR a highly sensitive tool used to detect small changes in targeted gene expression. To determine the effect of *CceVg* dsRNA on the level of *CceVg* transcripts, total RNAs were extracted from one female of each experiment days (1, 3 and 5 days) for performing real-time PCR. The results showed the level of *CceVg* mRNA was very low on the first day after injected with *CceVg* dsRNA and then increase in the similar level on the third and fifth days after injection. While the level of *CceVg* mRNA extracted from female injected with *DsRed* dsRNA showed the same level on the first, third and fifth days after injected and higher than the level mRNA injected with *CceVg* dsRNA (Figure 43).

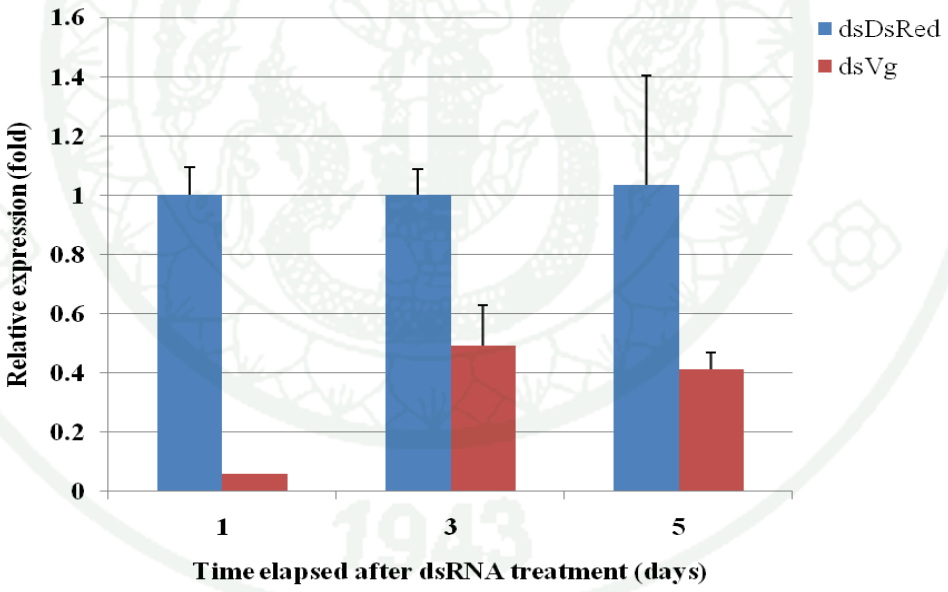

Figure 43 Relative vitellogenin expression in the adult female after dsRNA injection for 1, 3 and 5 days.

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9. Identification of *C. cephalonica* Vitellin protein by SDS-PAGE

To identily *C. cephalonica* yolk protein, the proteins were extracted from adult females, males and of eggs and run on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The result showed that two bands (~42 and ~170 kDa) were found in females and eggs extraction but were absent in that of males. Based on the deduced protein translated from the nucleotide sequence of *Ccevg* gene, the protein was cleaved into two subunits, one small and the other large. The molecular weights of the small and large were 38.46 and 164.01 kDa, respectively. These two subunits were corresponding well with 42 kDa and 170 kDa, identified by 15% SDS-PAGE (Figure 44).

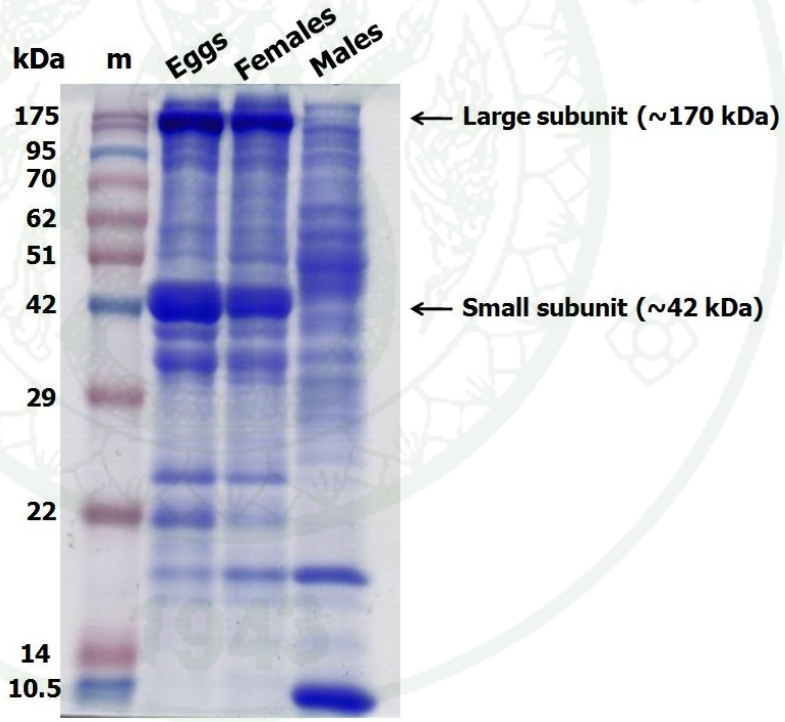

Figure 44 The 15% SDS-PAGE of Vitellin protein of *C. cephalonica*. Lane 1: Protein Ladder, Lane 2: protein from eggs, Lane 3: protein from adult females and Lane 4: protein from adult males.

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Discussion

1. Cloning and sequencing of *doublesex* gene in *C. cephalonica*

In this study, three *doublesex* (*dsx*) mRNA transcriptions were identified from rice moth (*C. cephalonica*). Two *dsx* mRNA (*CcedsxF1* and *CcedsxF2*) were isolated from female and one from male (*CcedsxM*). The lengths of *CcedsxF1, CcedsxF2* and *CcedsxM* were 1,113, 1,225 and 925 bp, respectively. The *CcedsxF1*and *CcedsxF2* harboured the complete open reading frame (ORF) of 801 and 741 bp length, respectively. The 801 and 741 bp ORFs encoded the deduced protein of 267 and 247 amino acid residues, respectively. Whereas, *CcedsxM* was an 831 bp partial ORF sequence that encoded a 277 amino acid deduced protein. Multiple alignment of these three mRNAs sequences revealed that thier nucleotide sequences were identical except the nucleotide at the position 744-1018 was absent in the male *dsx* mRNA. In Lepidopteran insects, their female produced more than one isoform of *dsx* mRNA whereas males possesses only one form such as *Bombyx mori dsx* (*Bmdsx*) (Shukla *et al*., 2011), *A. mylitta dsx* (*Amdsx)* (Shukla and Nagaraju, 2010) and *Ostrinia scapulalis dsx* (*Osdsx*) gene (Sugimoto *et al*., 2010). However, in *Anthearea assama*, the female produces six isoforms and one isoform found in male (Shukla and Nagaraju, 2010). Like most Lepidopteran insects, the *C. cephalonica* female produced two isoforms and male produced only one isoform.

Moreover, the multiple alignment of female insect *dsx* sequence also exhibited two interesting regions. One was the Putative Exonic Splicing Silence (PESS) sequence, responsible for sex-specific splicing. The sequences were completely conserved in all *dsx* female isoforms including *Bmdsx* (Suzuki *et al.*, 2008), *Aadsx* and *Amydsx* (Shukla and Nagaraju, 2010), *Osdsx* (Sugimoto *et al*., 2010) and the two *Ccedsx* isoforms isolated in this study. The other interesting region was the 15 bp stretch containing a stop codon. The difference of *dsx* female isoforms was the result of one isoform contains the stretch while the other had none, such as in *Bombyx mori* (Shukla *et al*., 2011) and *Anthearea mylitta* (Shukla and Nagaraju, 2010). In this

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study, the *CcedsxF2* contained the 15 bp stretch whereas the *CcedsxF1* did not. After translated, CceDSXF1 and CceDSXF2 were obtained. Existence of two female-specific DSX proteins in moths such as *A. assama*, *A. mylitta*, *B. mor*i and *C. cephalonica* has raises a possibility that both are required to exert effect on their downstream genes involved in sexual differentiation (Shukla and Nagaraju, 2010).

The amino acid sequences of doublesex proteions (DSXs) in all insects contain two conserved domains, an DNA binding domain (DM/OD1 domain) and oligomerization domain (OD2 domain) (Erdman and Burtis 1993). The CceDSX is also contained these two domains. The sequence of the DM/OD1 domain is highly conserved in both male and female DSXs. In *C. cephalonica*, the difference between male and female DSXs was at their C-terminal of OD2 domains since in CceDSXM, its OD2 domain sequences was longer than that of the CceDSXFs. As mentioned above, the gene *dsx* is subject to strong purifying selection to preserve the mechanism of action of DSX proteins, indicating the important function that *dsx* has in controlling the sexual development of insects (Ruiz *et al*, 2007).

2. Cloning and sequencing of *vitellogenin* gene in *C. cephalonica*

The complete nucleotide sequence of *C. cephalonica vitellogenin* was 6,721 bp long, including a 60 bp 5'UTR, 41bp UTR, six exons (5382 bp) and five introns (1238 bp). The start codon ATG (at position 61-63) was at the beginning of exon 1. The stop codon TAG was in the exon 6 at position 6678-6680 and the polyadenylation site (AATAAA) was at position 6691-6696. The lengths of the six exons were 2249, 214, 1003, 885,178, 853 bp, respectively, five introns were 238, 123, 242, 257, and 378 bp, respectively. The total length of exons was 5,382 bp that approximately 80% of the gene. The primary structure of *Ccevg* is highly homologous to other insect *vg* (Chen *et al*., 1997; Liu *et al*., 2001), but the DNA sequence is different from each other as far as the number and length of exons and introns are concerned. Comparison of the *Ccevg* gene with *vg* genes from twelve insect species having complete gene sequences available on the NCBI database showed that the structures of insect *vg* genes could be devided into three type (1, 2 and 3) based on the number of

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exon/intron. Type 1 is *vg* gene containing six exons and five introns such as *Vg* genes of *C. cephalonica*, *Antheraea pernyi* and *Actias selene*. While the type 2 composes of *vg* genes containing seven exons and six introns are found in *Antheraea yamamai* (Liu *et al*., 2001), *Bombyx mori* (Yano *et al*., 1994a; Yano *et al*., 1994b), *Lymantria dispar* (Hiremath and Lehtoma, 1997), *Anthonomus grandis* (Trewitt *et al*., 1992) and *Encarsia formosa* (Donnell, 2004). The last type contains only three exons and two introns such as the *vg* genes of all mosquitoes such as *Aedes aegypti* (Romans *et al*., 1995), *Culex tarsalis* (Chen *et al*., 2010) and *Toxorhynchites amboinensis* (accession no. GU361974). Even the numbers of exons and introns of mosquito *vg* genes are lower than that of the other two types but the lengths of their coding sequence genes are a bit longer than those of the insect *Vg* genes. The interesting feature of these insect *Vg* genes is the length of the first exons. In type 2 and 3 *Vg* gene, their first exons are very short, just only 31 bp length except *Encarsia formosa Vg* gene having the 34 bp first exon. While the first exons of type 2 *Vg* genes are about 70 folds longer than that of type 2 and 3 such as 2,249 bp in *CceVg*, 2,258 bp in *Antheraea pernyi Vg*, and 2,165 bp in *Actias selene Vg*. Although, the numbers and the lengths of exons and introns of these insect *Vg* genes are varied, the lengths of their coding sequences are similar and encode primary proteins with the similarity in sizes about 200 kDa.

The analysis of the primary protein revealed that the first 16 amino acids correspond to a putative signal peptide (SignalP-4.1 program). The predicted molecular weight of the mature protein was 202.46 kDa with an isoelectric point (pI) of 9.09 by ExPASy. The National Center for Biotechnology Information (NCBI) Conserved Domain Database (CDD) search predicted two vitellogenin-N domains (also known as the lipoprotein N-terminal regions) at the amino terminus of the CceVg. These domains are implicated in lipid transfer activity (Smolenaars *et al*., 2007). Third domains, the domain of unknow function (DUF1943). Finally domain, the von Willebrand factor type D (VWD) domain, was identified at the C-terminus. In addition, three polyserine regions, five RXXR consensus cleavage sites, the GL/ICG motif, and the cysteine residues conserved at the C-terminus were identified. Furthermore, two putative glycosylation sites (NXS/T) and 127 potential

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phosphorylation residues were found in the CceVg sequence. The presence of these modified residues in CceVg indicates that this molecule is likely to be highly phosphorylated. The phosphate moieties have a negative charge and may play a role in receptor binding as proposed by Sappington *et al*. (1996).

The molecular weight of primary CceVg protein is in the range of all insect Vgs. The exception is the yolk protein of higher Diptera that the molecular weight of their proteins are quite smaller, approximately 44-50 kDa (Tufail and Takeda, 2008). For example, the yolk protein precursor 1 (YP1) of *Drosophila melanogaster* was 48.7 kDa (Hovemann *et al*., 1981) and the vitellogenin 1 and vitellogenin 2 of *Ceratitis capitata* are 49 and 46 kDa, respectively (Rina and Mintzas, 1988). In most holometabolous insects, the molecular weight of their Vg primary precursors are approximately 200 kDa which are cleaved at the RXRR site into two polypeptides, one is small (49-60 kDa) and the other is large (140-190 kDa) (Tufail and Takeda, 2008). For example, the *Spodoptera litura* Vg precursor has 198.73 kDa which was cleaved into two subunits, 55.91 and 141.31 kDa (Shu *et al*., 2009) and the 210 kDa *A. yamamai* Vg precursor was cleaved into 184 and 42 kDa (Yokoyama *et al*., 1993). Even if the deduced CceVg contains five putative cleavage recognition sites (RHIR, RVRR, RLCR, REPR and RPER), the 202.46 kDa CceVg precursor peptide should be cleaved at the RVRR site, yielding two subunits, small and large subunits. The small subunit with predicted 38.46 kDa derived from the amino acid sequences between the N-terminal excluded the signal peptide to the cleavage site, RVRR, and the large subunit with 164.01 kDa was calculate from the first amino acid after the RVRR to the end of C-terminal sequences. The predicted molecular weights of two subunits were consistent with those of most insect Vgs.

The most unique feature that makes CceVg differed from other Lepidopteran Vgs is the presence of three polyserine tracts. In most insect Vgs including CceVg and almost all Lepidopteran Vgs, two polyserine tracts are conserved at N-terminal (Tufail and Takeda, 2008), except *Lymantria dispar* Vg has no any polyserine tract (Hiremath and Lehtoma, 1997). The presence of polyserine tract at C-terminal of

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CceVg is quite unique since other known lepidopterans Vgs have no polyserine tract at their C-terminal. The polyserine tract at the C-terminal is often found in the Vgs of hemimetabolous insect such as mosquito, cockroach and brown planthopper (Romans *et al*., 1995, Tufail *et al*., 2010). In addition, in the between the two polyserine tracts, a conserved cleavage site, RXRR, was located except *Antheraea pernyi* Vg and *Saturnia japonica* Vg had no the putative cleavage site flanked by two polyserine tracts (Liu *et al*., 2001, Meng *et al*., 2008). The polyserine regions were also suggested that they tend to be good sites for phosphorylation (Tufail and Takeda, 2008). Moreover, the role of the polyserine tracts was suggested that they might be phosphorylated and involved in the interaction between Vg and its receptor on the ovary membrane since the removal of phosphate caused the reduction in affinity between the Vg and its receptor (Dhadialla *et al*., 1992, Hirai *et al*., 1998). In CceVg, there are 127 putative phosphorylation sites including all serine residues of the three polyserine tracts so this study could only support the suggaestion that the polyserine region is appropriate sites for phosphorylation.

The deduced amino acid sequence of CceVg was aligned with that of some other insect Vgs available in the NCBI database using clustalW version 2.0.10. The result showed that sequence identities between the CceVg and other Lepidopteran Vgs were 51-56%, except 45.8% with *L. dispar* Vg and between the CceVg and Vgs other insect orders were about 20-30%. The interesting conserved motifs of all insect Vgs are GL/ICG and DGXR at the C-terminus. The GL/ICG motifs of all Lepidoptera Vgs including CceVg were GICG, while other insect Vgs possess GLCG. For the DGXR motif, its amino acid sequence quite varied. The sequence of DGXR motif in CceVg was DGQR which was the most often sequence found in insect Vgs. In addition, the DGXR motif is located 17 residues upstream of the GICG motif as present in all Lepidopteran Vgs, 18 residues in Hemipteran and Hymenopteran Vgs, and 19 residues in Coleopteran Vgs. Anoter interesting conserved sequence at the C-terminal is the number of cystein residue. CceVg contained 16 residues, 10 cystein residues, two residues being at the N-terminal and the remaining eigtht located at C-terminal are found in all Lepidopteran Vgs. The number of conserved cystein residues

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counted from the DGXR motif until the C-terminus of insect Vg s could vary from 6-10 such as in 10 in all Hymenoptera (Li *et al*., 2010, Havukainen *et al*., 2011). 9 in giant water bug (Nagaba *et al*., 2011), 8 in parasitoid wasp (Donnell, 2004) and all Lepidopteran Vgs including CceVg, and 7 in *A. grandis* Vg. In CceVg and all other Lepidopteran Vgs, their conserved GL/ICG domains were GLCG and 10 cystein residues. In vertebrate the GL/ICG motif and the conserve cysteins were suggested that they were necessary for the oligomerization Vitellins (Mayadas and Wagner, 1992, Mouchel *et al*., 1996), so these conserved sequences might do the same duty in insects.

Phylogenetic tree was performed using the deduced amino acid sequence of Vgs from *C. cephalonica* and some others insects available in the NCBI database. The vertebrate Vg from *Gallus gallus* was used as an out group. As expected, the result showed that CceVg was grouped with all Lepidopteran Vgs and closed to the Vg of *Cnaphalocrocis medinalis*. In addition, insect Vgs were separated into two major clades when analyzed by Neighbor-Joining method. One clade consists of only all Lepidopteran Vgs, whereas all the remaining insect Vgs were grouped together in the other clade. The topology of the tree obtained in this study was consistent with previous reports (Donnell, 2004, Meng *et al*., 2008, Tufail *et al*., 2010). Why the Lepidopteran Vgs differed from that of other insects. Multiple alignment of the C-terminal sequences of some insect Vgs available in the NCBI database showed some interesting regions that make them differ from other insect Vgs. One should be the conserved motifs GI/LCG in which all Lepidopteran Vgs possesses the GICG motif whereas all other insect harbor the GLCG motif. On the other hand, the topology tree obtained from Maximum Likelihood method was different from Neighbor-Joining method. From Maximum Likelihood method, insect Vgs were separated into three major clades. First clade consists of Lepidopteran, Dipteran, Dictyopteran, and Hemipteran Vgs, the second and the third clades are Coleopteran and Hymenopteran Vgs, respectively.

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The expression patterns of *CceVg* were determined at the different developmental stage. *CceVg* gene was not expressed in the egg stage and began to express at low level in early larva stage. The expression of *CceVg* was not detected in late larva stage. Then, the *CceVg* gene resumed to expression at low level again in early pupa stage and substantial increase in late female pupa but not in late male pupa. The *CceVg* gene in female continues to increase to peak in adult females. Interestingly, the *CceVg* gene was found in adult male at low level. The expression of *Vg* gene in male insects was reported in some insect species, particulary in the social insects of Hymenoptera. In *Apis mellifera* worker larvae, and in terms of mRNA, *Vg* expression was detected at high levels early in development (second, third, and fourth larval instars), then expression decreased in fifth (last) larval instar and in prepupae, became practically undetectable during the early pupae, and increased again in late pupae (Guidugli *et al*., 2005), showing levels similar to those reported in young adults by Piulachs *et al*. (2003). Insect *Vgs* may be involved in the transport of sugars, lipids, phosphates, vitamins, and hormones (Chen *et al*., 1997; Sappington and Raikhel, 1998). In addition, *Vg* in *A. mellifera* has been implicated in metabolic functions like the synthesis of brood food, transport of zinc, longevity, and immune system (Amdam and Omholt, 2002; Amdam *et al*., 2003, 2004). These possible may explain the function of *Vg* in *C. cephalonica* male.

In insect, vitellogenin protein, a major precursor yolk protein, is mainly produced in fat bodies and released into hemolymp. The protein is then circulated to developing oocytes which take up the protein by receptor-mediated endocytosis (Raikhel and Dhadialla, 1992, sappington and Raikhel, 1998). If the production of the protein is decreased, the number and size of eggs might be smaller or lower than normal. In this study, the expression of *CceVg* gene in early emerged females was disturbed by RNAi experiment and the morphology/number of eggs were checked. Early emerged females were injected with 1 µg/µl double strand RNAs of *CceVg* into about upper abdominal segment. After injected for three days, the injected females were dissected. The ovaries of females injected with *CceVg* dsRNAs were deformed from mild to severe, while the ovaries of females injected with *DsRed* dsRNA

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showed relatively normal in both morphology and number of eggs. Expresion of *CceVg* checked by real-time PCR showed the level of *CceVg* mRNA was very low on the first day after injected with *CceVg* dsRNA and then increase in the similar level on the third and fifth days after injection. While the level of *CceVg* mRNA extracted from female injected with *DsRed* dsRNA showed the same level on the first, third and fifth days after injected and higher than the level mRNA injected with *CceVg* dsRNA.

The CceVgs were identified by comparing the polypeptide profiles of adult female and male whole body homogenates and of egg extracts. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) revealed two Vgs polypeptides of ~170 and ~42 kDa corresponding to the two Vns polypeptides, whereas these polypeptides were absent in the male sample. As estimated from previously reports, the primary peptides of most Lepidopteran Vgs were cleaved behind the RXRR cleavage recognition site (Tafail and Takeda, 2008). The CceVg precursor polypeptide is most probably cleaved following RVRR cleavage signal, yielding two polypeptides, one short and one long. The putative molecular weights predicted based on the amino acid sequences of small (38.46 kDa) and large (164.01 kDa) subunits correspond well with the molecular weights of the two protein bands, approximately 170 kDa and 42 kDa, identified by 15% SDS-PAGE. To verify whether the approximately 170 and 42 kDa were the cleaved products of the CceVg primary peptide at the RVRR site, the N-terminal sequences of the both polypeptides were identified. Unfortunately, the N-terminal amino acid sequences of these two proteins using chemical determining were unsuccessful. The obtained three amino acid sequences from the large polypeptide was SAV but the expected sequence should be SAK, whereas the expected sequence of the short polypeptide was QLD but that obtained from chemical determining was SVA which did not match any CceVg amino acid sequence. It is likely that the N-terminus of the proteins may be modified.

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CONCLUSION

1. The *doublesex* gene of *C. cephalonica* was three *doublesex* (*dsx*) mRNAs, two from female (*CcedsxF1* and *CcedsxF2*) and one (*CcedsxM*) from male. The *CcedsxF1* and *CcedsxF2* contained the complete open reading frame (ORF) of 801 and 741 bp long, respectively. While the *CcedsxM* was the 831 bp partial ORF sequence. The sequences of these *dsx* mRNAs were identical except the *CcedsxM* had no the nucleotide at the position 744-1018. In addition, the difference between *CcedsxF1* and *CcedsxF2* was the present of 15 bp sequence containing a stop codon in the *CcedsxF2* and two *CcedsxFs* was found the Putative Exonic Splicing Silencer (PESS) region responsible for the sex-specific splicing differing between male and female *dsx* pre-mRNA. The expression of *Ccedsx* was found all development stages by expression very low in eggs and increases every stage to reach its maximum at adult females and males.

2. The *vitellogenin* gene of *C. cephalonica* was 6,721 bp long including a 60 bp 5'UTR, a 5,382 bp six exons, a 1238 bp five introns and a 41 bp 3'UTR. The 5,382 bp open reading frame (ORF) encodes a predicted protein of 1,793 amino acids residues with 16 amino acid signal peptide and four conserved domains (two vitellogenin-N domains, DUF1943 and von Willebrand factor type D). In addition, the CceVg was found highly conserved sequences at the C-terminal such as DGXR, GI/ILC and cystein residues. Interestingly, CceVg contains three polyserine tracts. Two polyserine tracts were located at the N-terminus and one was at the C- terminal, has not been reported in Lepidopteran insect Vgs. The *CceVg* expression was first detected in very low level at the early larval stage but disappeared in later stage larvae and resumed expression in early pupal stage of female. At adult stage, the highest expression was detected in females while the low level expression was detected in males. In addition, *C. cephalonica* vitellins protein was identified by the 15% SDS-PAGE. Two putative protein bands (~42 and ~170 kDa) were found in females and eggs extraction but were absent in that of males. Based on the deduced protein translated from the nucleotide sequence of *CceVg* gene, the protein was cleaved into

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two subunits, one small and the other large. The molecular weights of the small and large were 38.46 and 164.01 kDa, respectively. These two subunits were corresponding well with 42 kDa and 170 kDa.

3. The function of *vitellogenin* gene in *C. cephalonica* was investigated by RNA interference technique. *CceVg* double strand RNA was injected into early emerged females and *DsRed* double strand RNA was used for a control. Vitellogenin protein, a major precursor yolk protein and circulated to developing oocytes. If the production of vitellogenin protein is decreased, the number and size of eggs might be smaller or lower than normal.

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APPENDICES

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Appendix A

Data for phylogenetic tree analysis

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Appendix Table A1 The doublesex protein sequences for phylogenetics analysis.

Order	Family	Species	Amino acid	GenBank Accession
Lepidoptera	Bombycidae	*Bombyx mori* (*Bmdsx*F)	264	NP 001036871.1
	Bombycidae	*Bombyx mori* (*Bmdsx*M)	266	NP 001104815.1
	Erebidae	*Lymantria dispar (Lddsx*F*)*	264	BAN82533.1
	Erebidae	*Lymantria dispar (Lddsx*M*)*	275	BAN82532.1
	Pyralidae	*Corcyra cephalonica* (*Ccedsx*F1)	267	this study
	Pyralidae	*Corcyra cephalonica* (*Ccedsx*F2)	247	this study
	Pyralidae	*Corcyra cephalonica* (*Ccedsx*M)	277	this study
	Pyralidae	*Ostrinia scapulalis* (*Osdsx*F1)	271	BAJ25851.1
	Pyralidae	*Ostrinia scapulalis* (*Osdsx*F2)	271	BAJ25852.1
	Pyralidae	*Ostrinia scapulalis* (*Osdsx*M)	284	BAJ25850.1
	Saturniidae	*Antheraea assama* (*Aadsx*F1)	265	ADL40852.1
	Saturniidae	*Antheraea assama* (*Aadsx*F2)	247	ADL40851.1
	Saturniidae	*Antheraea assama* (*Aadsx*M)	279	ADL40846.1
	Saturniidae	*Antheraea mylitta* (*Amydsx*F1)	265	ADL40853.1
	Saturniidae	*Antheraea mylitta* (*Amydsx*F2)	247	ADL40851.1
	Saturniidae	*Antheraea mylitta* (*Amydsx*M)	279	ADL40855.1

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Appendix Table A1 (Continued)

Order	Family	Species	Amino acid	GenBank Accession
Hymenoptera	Pteromalidae	*Trichomalopsis sarcophagae* (*Tsdsx*F)	235	ACJ65503.1
	Pteromalidae	*Trichomalopsis sarcophagae* (*Tsdsx*M)	223	ACJ65504.1

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Appendix Table A2 The vitellogenin protein sequences for phylogenetics analysis.

Order	Family	Species	Amino acid	GenBank Accession
Coleoptera	Curculionidae	*Anthonomus grandis*	1,790	AAA27740.1
	Tenebrionidae	*Tenebrio molitor*	1,821	AAU20328.2
Dictyoptera	Blaberidae	*Rhyparobia maderae*	1,913	BAB19327.1
	Blattidae	*Periplaneta Americana*	1,896	BAA86656.1
	Cicadellidae	*Blattella germanica*	1,862	CAA06379.2
Diptera	Culicidae	*Aedes aegypti*	2,148	AAA99486.1
	Culicidae	*Anopheles culicifacies*	2,052	AEO51020.1
	Culicidae	*Anopheles gambiae*	2,051	AAF82131.1
	Culicidae	*Culex tarsalis*	2,119	ADH04225.1
Hemiptera	Alydidae	*Riptortus clavatus*	1,876	AAB72001.1
	Belostomatidae	*Lethocerus deyrollei*	1,895	BAG12118.1
	Cicadellidae	*Homalodisca vitripennis*	1,890	AAZ06771.1
	Delphacidae	*Nilaparvata lugens*	2,063	BAF75351.1
	Miridae	*Apolygus lucorum*	1,995	AFW97644.1
	Miridae	*Trigonotylus caelestialium*	1,976	BAJ33507.1
Hymenoptera	Apidae	*Apis mellifera*	1,770	NP_001011578.1
	Apidae	*Bombus hypocrita*	1,772	ACU00433.1

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Appendix Table A2. (Continued)

Order	Family	Species	Amino acid	GenBank Accession
Hymenoptera	Apidae	*Bombus ignitus*	1,773	ACM46019.1
	Aphelinidae	*Encarsia formosa*	1,814	AAT48601.1
	Apocrita	*Pimpla nipponica*	1,807	AAC32024.1
	Formicidae	*Camponotus floridanus*	1,820	EFN64902.1
	Formicidae	*Harpegnathos saltator*	2,105	EFN86099.1
	Formicidae	*Solenopsis invicta*	1,641	AAP47155.1
	Leucospidae	*Pteromalus puparum*	1,803	ABO70318.1
	Tenthredinidae	*Athalia rosae*	1,872	BAA22791.1
	Vespidae	*Vespula vulgaris*	1,756	AER70365.1
Lepidoptera	Bombycidae	*Bombyx mandarina*	1,780	BAB32642.2
	Bombycidae	*Bombyx mori*	1,782	NP_001037309.1
	Crambidae	*Cnaphalocrocis medinalis*	1,789	AEM75020.1
	Erebidae	*Lymantria dispar*	1,747	AAC02818.1
	Noctuidae	*Helicoverpa armigera*	1,756	AGL08685.1
	Noctuidae	*Spodoptera litura*	1,748	ABU68426.1
	Nymphalidae	*Danaus plexippus*	1,763	EHJ67298.1
	Pyralidae	*Corcyra cephalonica*	1,793	this study

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Appendix Table A2 (Continued)

Order	Family	Species	Amino acid	GenBank Accession
Lepidoptera	Saturniidae	*Actias selene*	1,774	ADB94560.1
	Saturniidae	*Antheraea pernyi*	1,778	BAB16412.1
	Saturniidae	*Antheraea yamamai*	1,778	BAF45319.1
	Saturniidae	*Samia cynthia pryeri*	1,779	BAD91196.1
	Saturniidae	*Samia ricini*	1,779	BAB32641.1
	Saturniidae	*Saturnia japonica*	1,776	BAD91195.1
Galliformes	Phasianidae	*Gallus gallus*	1,852	AAA49139.1

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Appendix B

Comparison of *vitellogenin* gene from various insects

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Appendix Table B1 Comparison of *vitellogenin* gene from various insects.

Types		**Type 1**			**Type 2**					**Type 3**		
species		*C. cephalonica*	*A. pernyi*	*A. selene*	*B. mori*	*A. yamamai*	*L. dispar*	*A. grandis*	*E. formosa*	*A. aegypti*	*C. tarsalis*	*T. amboinensis*
Accession no.		KJ540279	EF683091	GU361974	D30733	AB247378	U90756	M72980	AY553878	L41842	GU017909	AY691326
Numbers and length (bp) of exons and introns	Exon 1	2249	2258	2246	31	31	31	31	34	31	31	31
	Intron 1	238	84	107	1588	4590	1667	2056	91	70	63	57
	Exon 2	214	205	205	2242	2227	2143	2003	725	5214	5061	4869
	Intron 2	123	78	84	91	77	254	85	85	57	61	58
	Exon 3	1003	982	982	208	205	214	197	894	1202	1262	1199
	Intron 3	242	410	626	81	77	781	57	84	-	-	-
	Exon 4	885	879	879	985	982	970	303	1077	-	-	-
	Intron 4	257	1055	706	88	423	374	103	69	-	-	-
	Exon 5	178	184	184	879	879	870	170	1710	-	-	-
	Intron 5	378	795	230	740	896	3016	59	72	-	-	-
	Exon 6	853	829	829	184	184	202	1692	446	-	-	-
	Intron 6	-	-	-	286	806	297	87	230	-	-	-
	Exon 7	-	-	-	820	829	814	977	558	-	-	-
Total exon length		5382 (80.0%)	5337 (65.5%)	5325 (72.6%)	5349 (55.3%)	5337 (40.2%)	5244 (32.5%)	5373 (32.6%)	5445 (81.8%)	6447 (73.4%)	6354 (65.6%)	6099 (71.2%)
Total intron length		1238 (18.4%)	2422 (29.7%)	1753 (23.9%)	2874 (29.7%)	6869 (51.8%)	6389 (39.6%)	2447 (24.4%)	631 (9.4%)	127 (1.4%)	124 (1.3%)	115 (1.3%)
Gene length		6721	8142	7329	9664	13260	16133	10017	6654	8780	9676	8565
No. of amino acid		1793	1778	1774	1782	1778	1747	1790	1814	2148	2117	2032
Molecular weight		202.46	201.6	201.3	203.05	201.49	198.18	205.85	204.57	250.25	242.43	233.41

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Appendix C

Data of real-time PCR analysis

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Document information

Software:	realplex 2.0	
File Name:	EPPENDORF\4607\rttrue	
Printed by:	EPPENDORF	
Created:	Nov/07/2013 12:33	
Serial No. Thermo Module:	5345 7363	
Serial No. realplex Module.:	630200043	
Acquisition Start Time:	EPPENDORF	Nov/07/2013 13:12
Acquisition End Time:	EPPENDORF	Nov/07/2013 15:21
Last updated:	EPPENDORF	Nov/07/2013 15:24
Background:	tube bioactive	Jan/12/2006 12:04
Color Calibration:	SYBR	Dec/14/2005 13:31
Evaluations for Dye SYBR:		
Evaluation 1:	Quantification	Nov/14/2013 14:50
Evaluation 2:	+/- Assay (PCR Data)	Nov/14/2013 14:51
Evaluation 3:	Gene Identification (PCR Data)	Nov/14/2013 14:51
Evaluation 4:	Melting Curve	Nov/14/2013 14:48
Comment:		

Appendix Figure C1 Data of real-time PCR analysis.

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Plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	DsRed1 1: IPC	DsRed1 1: IPC	DsRed1 1: IPC	vg1 1: IPC	vg1 1: IPC	vg1 1: IPC						
B	DsRed1 1: HKG	DsRed1 1: HKG	DsRed1 1: HKG	vg1 1: HKG	vg1 1: HKG	vg1 1: HKG						
C	DsRed3 1: IPC	DsRed3 1: IPC	DsRed3 1: IPC	vg3 1: IPC	vg3 1: IPC	vg3 1: IPC						
D	DsRed3 1: HKG	DsRed3 1: HKG	DsRed3 1: HKG	vg3 1: HKG	vg3 1: HKG	vg3 1: HKG						
E	DsRed5 1: IPC	DsRed5 1: IPC	DsRed5 1: IPC	vg5 1: IPC	vg5 1: IPC	vg5 1: IPC						
F	DsRed5 1: HKG	DsRed5 1: HKG	DsRed5 1: HKG	vg5 1: HKG	vg5 1: HKG	vg5 1: HKG						
G												
H												

Appendix Figure C1 (Continued)

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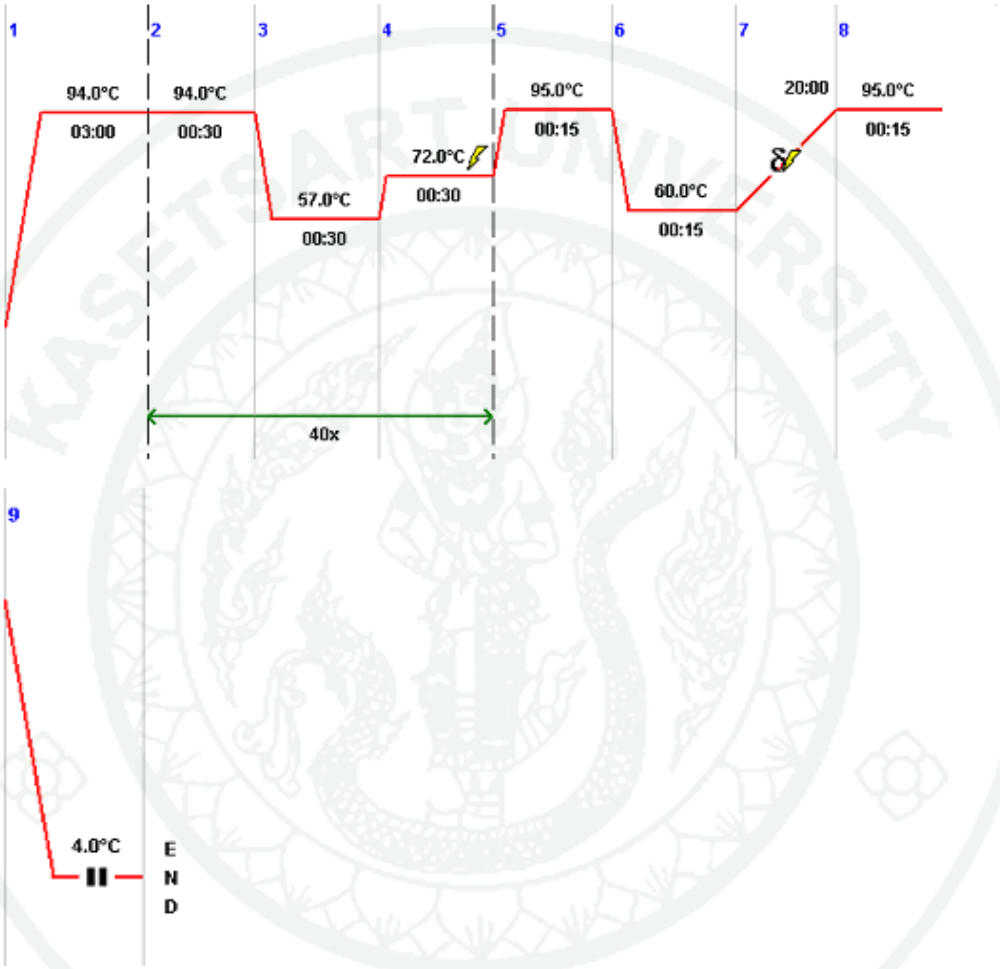
PCR Program



Appendix Figure C1 (Continued)

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Program Header

Lid Temp	105 °C	TSP Heated Lid	Yes
Control	Sim. Tube	Switch off lid at low block temp	Yes
Impulse	Yes	Simulate Mastercycler gradient	No

Appendix Figure C1 (Continued)

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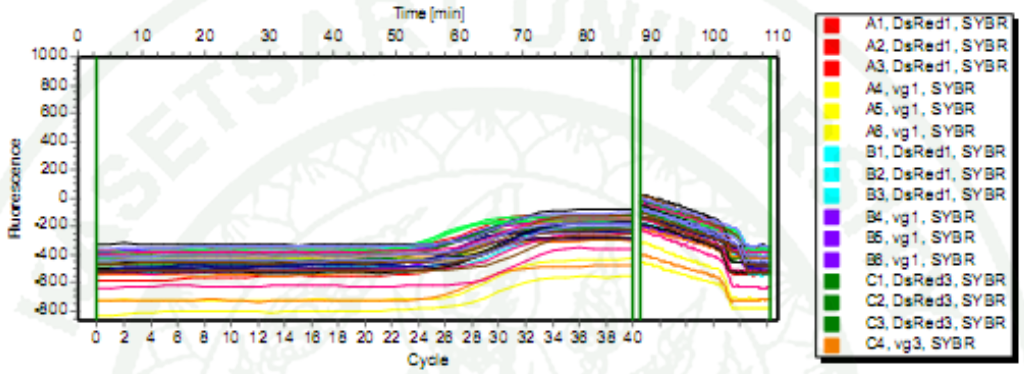
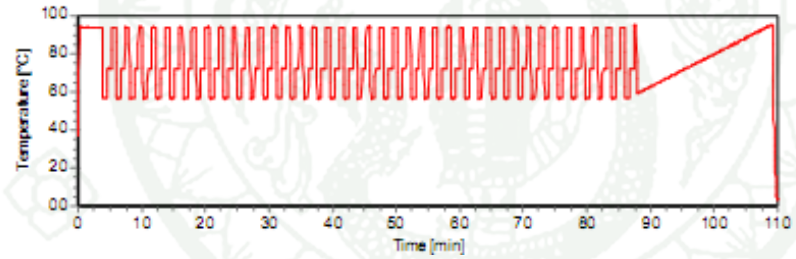
Raw Data SYBR

Fluorescence Profile



Temperature Profile



Appendix Figure C1 (Continued)

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Quantification SYBR

Pos	Name	Ct SYBR	Ct Mean SYBR	Ct Dev. SYBR	Amount SYBR	IPC SYBR	Target SYBR
A1	DsRed1	25.32	24.84	0.48	-	Positive	vg
A2	DsRed1	24.85	24.84	0.48	-	Positive	vg
A3	DsRed1	24.35	24.84	0.48	-	Positive	vg
A4	vg1	25.87	26.71	1.22	-	Positive	vg
A5	vg1	28.11	26.71	1.22	-	Positive	vg
A6	vg1	26.16	26.71	1.22	-	Positive	vg
B1	DsRed1	26.23	25.76	0.53	-		act
B2	DsRed1	25.19	25.76	0.53	-		act
B3	DsRed1	25.92	25.76	0.53	-		act
B4	vg1	24.49	23.79	0.63	-		act
B5	vg1	23.26	23.79	0.63	-		act
B6	vg1	23.60	23.79	0.63	-		act
C1	DsRed3	25.30	24.59	1.17	-	Positive	vg
C2	DsRed3	25.24	24.59	1.17	-	Positive	vg
C3	DsRed3	23.24	24.59	1.17	-	Positive	vg
C4	vg3	26.78	25.44	1.25	-	Positive	vg
C5	vg3	25.25	25.44	1.25	-	Positive	vg
C6	vg3	24.29	25.44	1.25	-	Positive	vg
D1	DsRed3	26.39	24.63	1.53	-		act
D2	DsRed3	23.86	24.63	1.53	-		act
D3	DsRed3	23.62	24.63	1.53	-		act
D4	vg3	22.96	22.45	0.48	-		act
D5	vg3	22.39	22.45	0.48	-		act
D6	vg3	22.00	22.45	0.48	-		act
E1	DsRed5	27.95	27.26	0.63	-	Positive	vg
E2	DsRed5	27.12	27.26	0.63	-	Positive	vg
E3	DsRed5	26.71	27.26	0.63	-	Positive	vg
E4	vg5	26.94	26.64	0.86	-	Positive	vg
E5	vg5	27.32	26.64	0.86	-	Positive	vg
E6	vg5	25.66	26.64	0.86	-	Positive	vg
F1	DsRed5	28.63	27.97	0.60	-		act
F2	DsRed5	27.47	27.97	0.60	-		act
F3	DsRed5	27.80	27.97	0.60	-		act
F4	vg5	26.51	27.10	0.92	-		act
F5	vg5	26.62	27.10	0.92	-		act
F6	vg5	28.17	27.10	0.92	-		act

Appendix Figure C1 (Continued)

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Melting Curve SYBR

Pos	Name	No. Tm SYBR	Tm x (°C) SYBR	Tm y (°C) SYBR	Mean SYBR	Dev. SYBR
A1	DsRad1	1	83.9		83.8	0.1
A2	DsRad1	1	83.8		83.8	0.1
A3	DsRad1	1	83.7		83.8	0.1
A4	vg1	1	83.8		83.7	0.1
A5	vg1	1	83.7		83.7	0.1
A6	vg1	1	83.5		83.7	0.1
B1	DsRad1	1	88.4		88.1	0.3
B2	DsRad1	1	88.0		88.1	0.3
B3	DsRad1	1	87.8		88.1	0.3
B4	vg1	1	87.8		87.5	0.3
B5	vg1	1	87.5		87.5	0.3
B6	vg1	1	87.2		87.5	0.3
C1	DsRad3	1	83.8		83.5	0.3
C2	DsRad3	1	83.4		83.5	0.3
C3	DsRad3	1	83.3		83.5	0.3
C4	vg3	1	83.2		83.1	0.1
C5	vg3	1	83.2		83.1	0.1
C6	vg3	1	83.0		83.1	0.1
D1	DsRad3	1	88.0		87.7	0.4
D2	DsRad3	1	87.8		87.7	0.4
D3	DsRad3	1	87.2		87.7	0.4
D4	vg3	1	87.2		87.1	0.1
D5	vg3	1	87.1		87.1	0.1
D6	vg3	1	87.0		87.1	0.1
E1	DsRad5	1	83.8		83.5	0.3
E2	DsRad5	1	83.4		83.5	0.3
E3	DsRad5	1	83.3		83.5	0.3
E4	vg5	1	83.0		82.9	0.2
E5	vg5	1	83.0		82.9	0.2
E6	vg5	1	82.7		82.9	0.2
F1	DsRad5	1	87.9		87.6	0.2
F2	DsRad5	1	87.6		87.6	0.2
F3	DsRad5	1	87.5		87.6	0.2
F4	vg5	1	87.4		87.2	0.2
F5	vg5	1	87.2		87.2	0.2
F6	vg5	1	87.1		87.2	0.2

Appendix Figure C1 (Continued)

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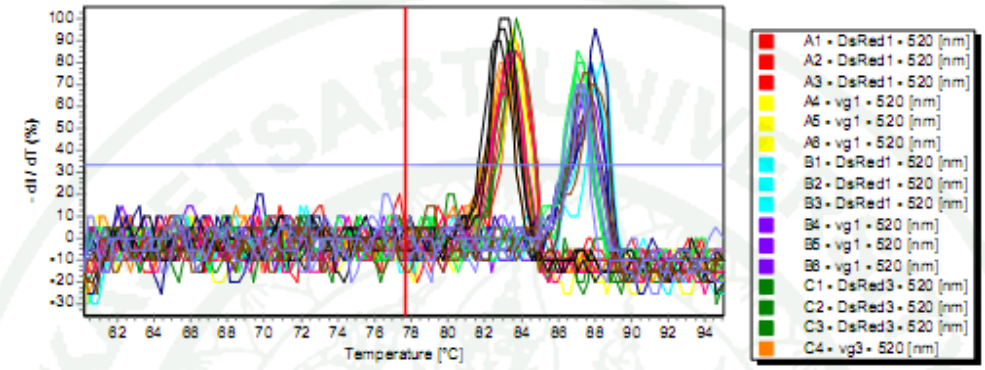
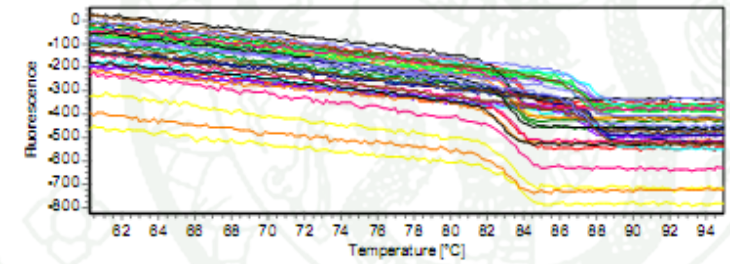
eppendorf MondoTech Nov/14/2013

Pos	Name	No. Tm SYBR	Tm x (°C) SYBR	Tm y (°C) SYBR	Mean SYBR	Dev. SYBR

Melting curve



Threshold 33%



Appendix Figure C1 (Continued)

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Appendix D

Structural comparison of the C-terminal part of Vgs

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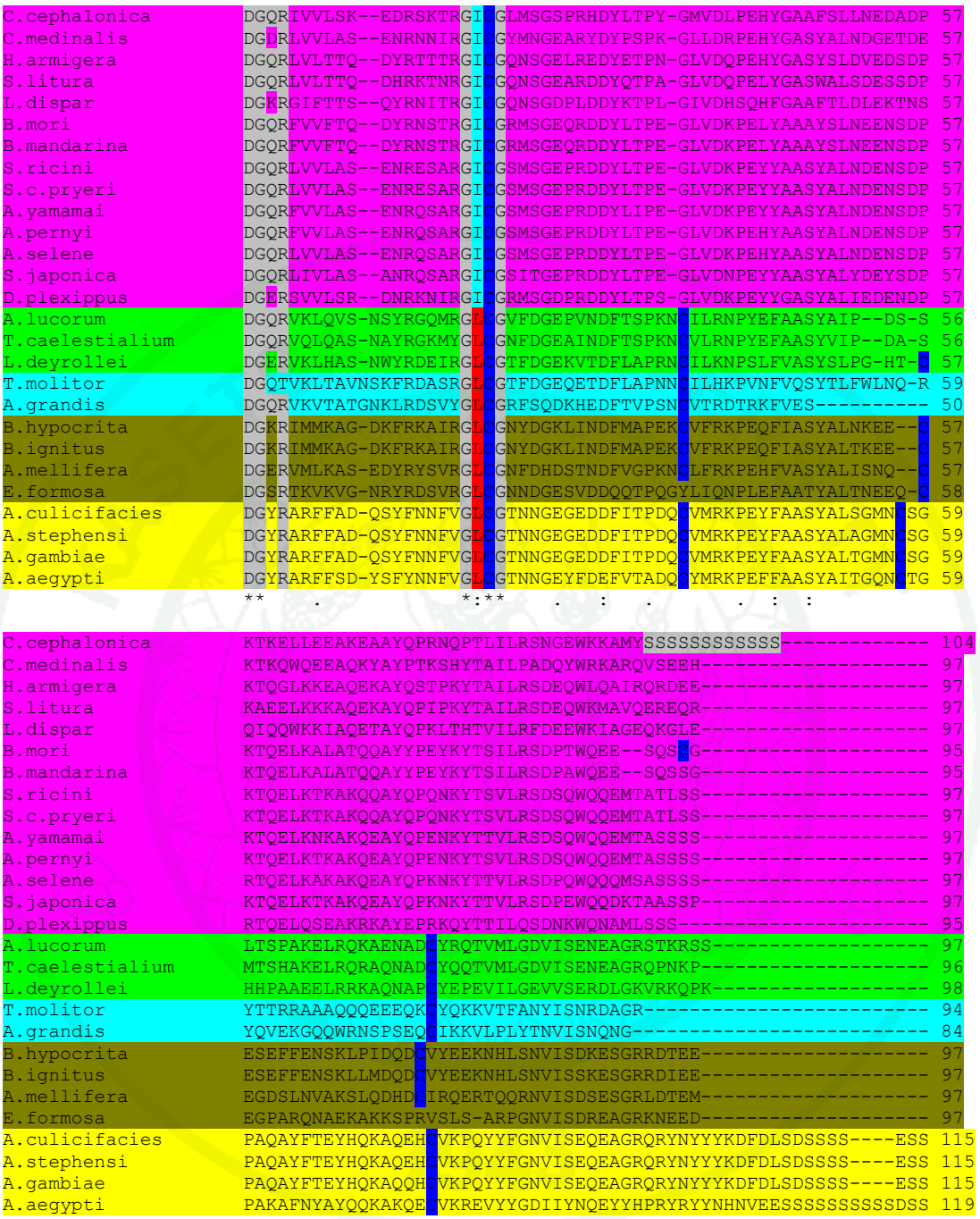

Appendix Figure D1 Structural comparison of the C-terminal part of Vgs.

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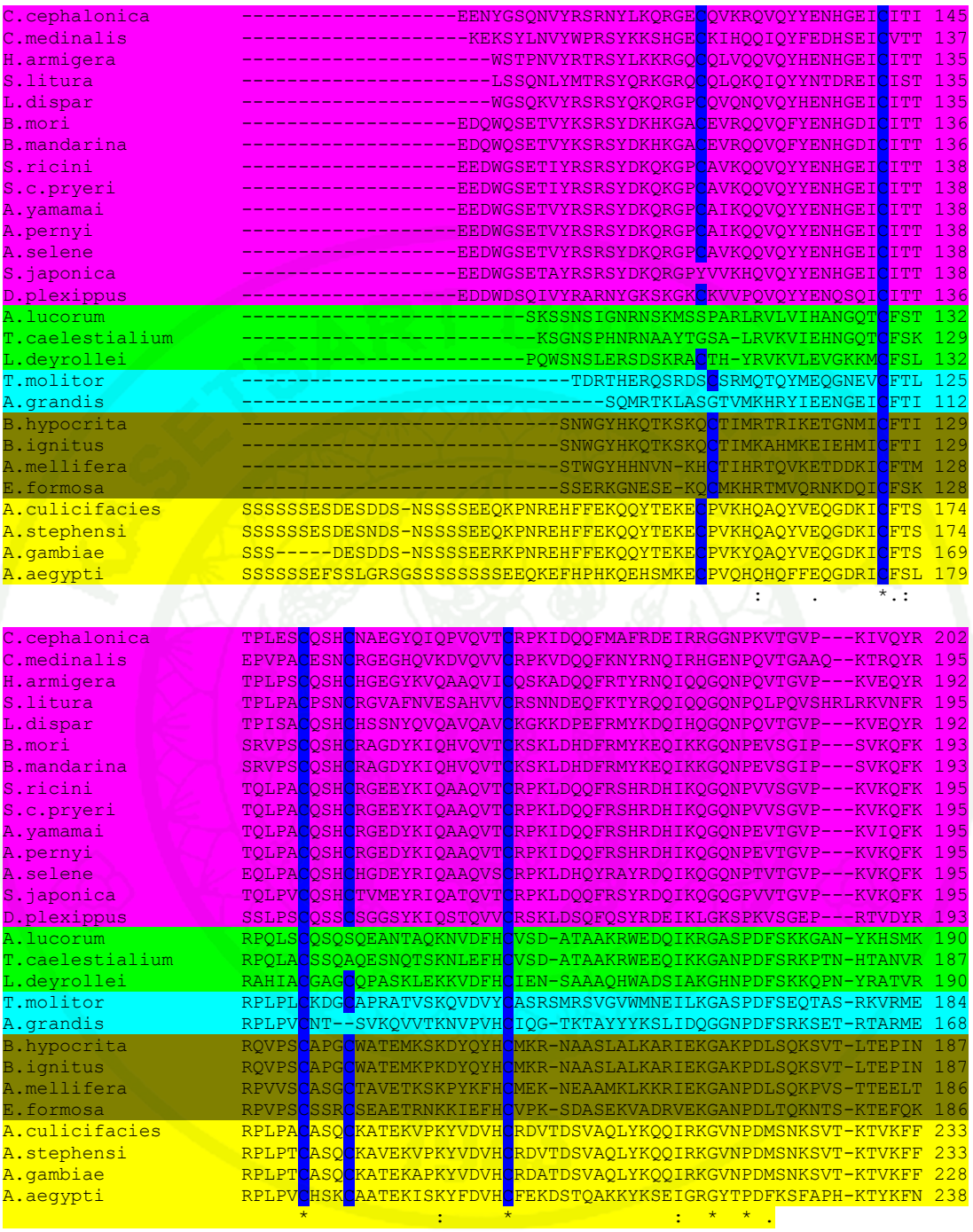

Appendix Figure D1 (Continued)

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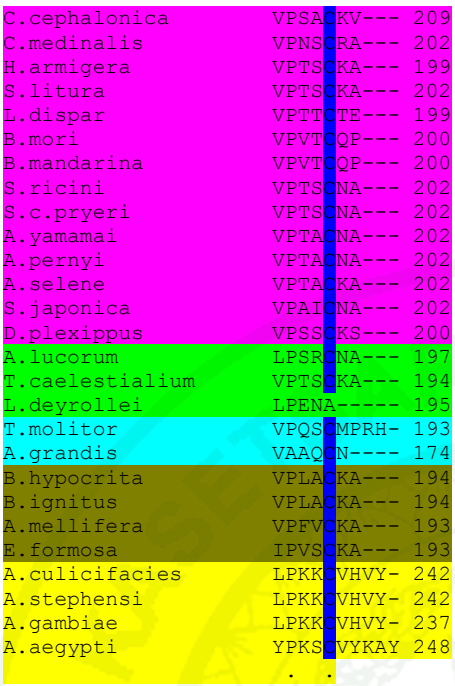

Appendix Figure D1 (Continued)

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Appendix E

Reagent preparation

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General reagent

0.5 M EDTA, pH 8.0

EDTA	18.61 g

The chemical was dissolved in distilled water, adjusted to pH 8.0 with 1 M NaOH. Distilled water was added to the final volume 100 ml. The solution was sterilized by autoclaving for 15 minutes at 121 ºC, 15 lb/square inches.

TE, pH 8.0

1 M Tris-HCl, pH 8.0	1.0 ml
1 mM EDTA, pH 8.0	0.2 ml

The mixture was mixed thoroughly and adjusted to the final volume of 100 ml with distilled water.

3.0 M Sodium acetate, pH 5.2

Sodium acetate	24.2 g

The chemical was dissolved in distilled water, adjusted to pH 5.2 with glacial acetic acid. The final volume was adjusted to 100 ml with distilled water.

10 % SDS

Sodium dodecyl sulfate	10 g
Distilled water was added to final volume	90 ml

The chemical was dissolved at 68 ºC and adjusted to pH 7.2 by adding 2-3 drops of concentrated HCl. The solution was adjusted to the volume 100 ml.

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Reagent for molecular cloning

50X TAE buffer (stock)

Tris	240.2 g
Glacial acetic acid	57.0 ml
0.5 M EDTA (pH 8.0)	100.0 ml

The chemicals were dissolved in distilled water, and adjust the final volume to 1 liter with distilled water. (Working solution is 1 X)

Chloroform: Isoamyl alcohol (24: 1, v/v)

Chloroform (Merck)	24.0 ml
Isoamyl alcohol	1.0 ml

Both reagents were mixed together and stored in a dark bottle at room temperature.

1.0 M IPTG

Isopropylthio-ß-D-galactoside	2.38 g
Distilled water	100 ml

The solution was sterilized by filtration through a 0.2 µm filter and dispensed the solution into 1 ml aliquot tube and stored at –20 ºC.

X-gal

5-bormo-4-chloro-3-indolyl-ß-D-galactoside	100 mg

The chemical was dissolved in 2 ml of dimethyl-formamide. The solution was stored in a tube covered with aluminum foil and stored at -20ºC.

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Ethidium bromide (10 mg/ml)

Ethidium bromide	1.0 g
Distilled water	10.0 ml

The solution was stored in a dark bottle at room temperature.

6X gel-loading dye buffer

0.25 % bromophenol blue
0.25 % xylene cyanol FF
30 % glycerol in water

The chemicals were dissolved and adjusted the final volume with distilled.

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Media for bacterial culture

Luria-Bertani medium (LB medium per liter)

Tryptone (Difco)	10 g
Yeast extract (Difco)	5.0 g
NaCl	10 g

Adjust pH to 7.0 with NaOH. Then the solution was adjusted to the final volume of 1000 ml with distilled water and sterilized by autoclaving.

LA plates with ampicillin

Fifteen grams of agar was added to 1 l of LB medium then the media was sterilized by autoclaving. The medium was allowed to cool to 50ºC before adding ampicillin to a final concentration of 100 µg/ml. The medium (30-35 ml) was poured into 85 mm petri dishes. The agar was allowed to harden. Agar plates were stored at 4ºC for up to 1 month or room temperature for up to 1 week.

LA plates with ampicillin / IPTG / X-gal

The LB with ampicillin was prepared then supplement with 0.5 mM IPTG and 80 µg/ml X-Gal and pour the plates. Alternatively, 100 µl of 100 mM IPTG and 20 µl of 50 mg/ml X-gal may be spread over the surface of an LA ampicillin plate and allowed to absorb for 30 minutes at 37ºC prior to use.

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