

Cloning and Expression Analysis of the *Bombyx mori* α -amylase Gene (Amy) from the Indigenous Thai Silkworm Strain, Nanglai

Authors: Ngernyuang, Nipaporn, Kobayashi, Isao, Promboon, Amornrat, Ratanapo, Sunanta, Tamura, Toshiki, et al.

Source: Journal of Insect Science, 11(38) : 1-16

Published By: Entomological Society of America

URL: <https://doi.org/10.1673/031.011.0138>

BioOne Complete (complete.BioOne.org) is a full-text database of 200 subscribed and open-access titles in the biological, ecological, and environmental sciences published by nonprofit societies, associations, museums, institutions, and presses.

Your use of this PDF, the BioOne Complete website, and all posted and associated content indicates your acceptance of BioOne's Terms of Use, available at www.bioone.org/terms-of-use.

Usage of BioOne Complete content is strictly limited to personal, educational, and non - commercial use. Commercial inquiries or rights and permissions requests should be directed to the individual publisher as copyright holder.

BioOne sees sustainable scholarly publishing as an inherently collaborative enterprise connecting authors, nonprofit publishers, academic institutions, research libraries, and research funders in the common goal of maximizing access to critical research.



Cloning and expression analysis of the *Bombyx mori* α -amylase gene (*Amy*) from the indigenous Thai silkworm strain, Nanglai

Nipaporn Ngernnyuang^{1,4a}, Isao Kobayashi^{2,4b}, Amornrat Promboon^{3c}, Sunanta Ratanapo^{3d}, Toshiki Tamura^{2e} and Lertluk Ngernsiri^{1f*}

¹The Department of Genetics, Faculty of Science, Kasetsart University, Bangkok, Thailand

²The National Institute of Agrobiological Sciences, Tsukuba, Ibaraki 305-8634, Japan

³The Department of Biochemistry, Faculty of Science, Kasetsart University, Bangkok, Thailand

⁴These two authors contributed equally to this work.

Abstract

α -amylase (*Amy*) is a digestive enzyme that hydrolyzes starch into maltose and glucose. In *Bombyx mori* L. (Lepidoptera: Bombycidae), *Amy* is found in both digestive fluid and hemolymph. Here, the complete genomic sequence of the *Amy* gene encoding α -amylase from a local Thai silkworm, the Nanglai strain, was obtained. This gene was 7981 bp long with 9 exons. The full length *Amy* cDNA sequence was 1749 bp containing a 1503 bp open reading frame. The ORF encoded 500 amino acid residues. The deduced protein showed 81-54% identity to other insect α -amylases and more than 50% identity to mammalian enzymes. Southern blot analysis revealed that in the Nanglai strain *Amy* is a single-copy gene. RT-PCR showed that *Amy* was transcribed only in the foregut. Transgenic *B. mori* also showed that the *Amy* promoter activates expression of the transgene only in the foregut.

Keywords: *Amy*, *Bombyx mori*, GAL4-UAS, silkworm

Abbreviations: *Amy*, α -amylase gene; BmAMY, *Bombyx mori* α -amylase enzyme

Correspondence: ^a yingjung@hotmail.com, ^b isaori@affrc.go.jp, ^c fsciarp@ku.ac.th, ^d fscistr@ku.ac.th, ^e ttamura@affrc.go.jp, ^f khawlln@yahoo.com, *Corresponding author

Editor: Craig Coates was editor of this paper

Received: 23 February 2010, **Accepted:** 5 June 2010

Copyright : This is an open access paper. We use the Creative Commons Attribution 3.0 license that permits unrestricted use, provided that the paper is properly attributed.

ISSN: 1536-2442 | Vol. 11, Number 38

Cite this paper as:

Ngernnyuang N, Kobayashi I, Promboon A, Ratanapo S, Tamura T, Ngernsiri L. 2011. Cloning and expression analysis of the *Bombyx mori* α -amylase gene (*Amy*) from the indigenous Thai silkworm strain, Nanglai. *Journal of Insect Science* 11:38 available online: insectscience.org/11.38

Introduction

! "# \$ % & () * ; ! " < = " 4 & , ' . " = " 4 & , ' . - 2 % 3 1 - & () ? * + (* ' * \$) \$ @) 1 * - 0 * the glycoside hydrolase family 13, that catalyzes * 5 2) * 2 % 3 1 - & (+ (* - 0 * 5 2) * ! " ; < = ? * 4 & , - (+ 3 + , * & . 8 ' 4) (* + , * (5 ' 1 , 2 * ' . 3 * 1) & 5) 3 * , - \$ A - > . 3 ((Janecek 1997). ! "# \$ % & () (from many animals have been characterized both biochemically and molecularly. In the silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae), there are two sources of ! " ' \$ % & () (, the digestive fluid and the hemolymph (Yokoyama 1959). The activity of *B. mori* ! " ' \$ % & () (BmAMY) was studied in both polyvoltine and bivoltine races. The polyvoltine races have adapted to tropical climate zones and exhibit high survival rate and short rearing time, although their silk fibers are short (~500 – 700 meters) and of poor quality. Conversely, the bivoltine races have adapted to temperate zones and produce longer (1200–1500 meters) and higher quality silk fibers. However, the bivoltine races are weak and susceptible to diseases when reared in tropical zones (Murakami 1989). Interestingly, the activity of ! " ' \$ % & () in the digestive fluid of the polyvoltine races was higher than that of the bivoltine races, although the activity in their hemolymph did not differ. It was suggested that the increased enzyme activity of the polyvoltine races might be an adaptation to survive better in the tropical conditions (Abraham et al. 1992). Moreover, the activity of the ! " ' \$ % & () in digestive fluid was higher than that in the hemolymph (Abraham et al. 1992; Promboon et al. 1993). In Thai polyvoltine races, the ! " ' \$ % & () of the digestive fluid from the gut had an optimal pH of 9.8, whereas that of the hemolymph was 6.5 (Promboon et al. 1993). However, the function of ! " ' \$ % & () in hemolymph is still unclear and it has been

suggested that it may be involved in the degradation of fat body glycogen (Wyatt 1967).

The *Amy* genes have been studied in many animals such as insects (Grossman et al. 1997; Da Lage et al. 2003; Saltzmann et al. 2006), chicken (Benkel et al. 1997), shrimp (Moal et al. 2000), oyster (Sellos et al. 2003), pig (Darnis et al. 1999), and human (Horii et al. 1987). In *B. mori*, the truncated sequence of the *BmAmy* cDNA has been registered in the National Center for Biotechnology Information (NCBI) database and its gene expression in the salivary gland has been reported (Parthasarathy and Gopinathan 2005). Since the activity of ! " ' \$ % & () in the digestive fluid is different between the polyvoltine and bivoltine races, the complete sequence of the gene encoding BmAMY should be studied for further elucidation of the difference. In this study, the full-length cDNA sequence, the complete genomic sequence of *BmAmy* in a polyvoltine race of Thailand, Nanglai strain and the expression of this gene are reported and it was found that this gene was expressed only in the foregut at the larval stage. Using a transgenesis technique, it was shown that the *BmAmy* promoter was active in the foregut.

Materials and Methods

Insects

A local Thai silkworm, the Nanglai strain, was obtained from Phuttaisong Silkworm Research Station, Thailand, and the larvae were reared on mulberry leaves. The w1-pnd strain (Uchino et al. 2006) and the *UAS-GFP* line (Kobayashi et al. 2007) were obtained from the National Institute of Agrobiological Sciences, Tsukuba, Japan, and reared on an artificial diet (Nihonnosanko, Japan) at 25° C.

Full-length *BmAmy* cDNA sequence

Total RNA was extracted from the heads to the third segments of 5th instar larvae of the Nanglai strain using TRIzol reagent (Gibco BRL, www.lifetech.com). First strand cDNA was synthesized using the Reverse Transcription System Kit (Promega, www.promega.com) according to the manufacturer’s instructions. The primers (Table 1) were designed based on the truncated sequence of the *BmAmy* gene (GenBank Accession No.U07847). First strand cDNA was used as a template to amplify the *BmAmy* cDNA with the primers amyF1 and amyR1 (Table 1) under the following conditions: 95° C for 3 min; 20 cycles at 94° C for 1 min; 60° C for 30 s; 70° C for 2 min; and 70° C for 10 min. The PCR product was cloned into the pGEMT Easy plasmid vector (Promega) and sequenced at Macrogen, Korea. To obtain the full-length *BmAmy* cDNA of Nanglai, both 5' and 3' Rapid Amplification of cDNA Ends (RACE) were conducted. The 3'-RACE was performed using the Marathon cDNA Amplification Kit (Clontech, www.clontech.com) and the gene-

specific primer, amyF1 (Table 1). Amplification condition included preheating 1min at 94° C, 10 cycles of denaturing at 94° C for 30 sec, annealing at 72° C for 4 min, extending at 70° C for 4 min and 20 cycles of denaturing at 94° C for 20 sec, annealing and extending at 68° C for 4 min. The 3'-RACE PCR fragment was cloned and sequenced. The 5'-RACE was carried out using the FirstChoice RLM-RACE Kit (Ambion, www.ambion.com) and the gene-specific primers outer reverse primer and inner reverse primer (Table 1). The PCR condition was preheated at 95° C for 2 min, followed by 35 cycles of denaturing at 95° C for 30 sec, annealing at 60° C for 30 sec, extending at 68 ° C for 1 min and final extending was carried out at 68° C for 5 min. The 5'-RACE PCR fragments were cloned and sequenced.

Sequencing the genomic *BmAmy* gene

To identify the structure of the Nanglai *BmAmy* gene, KAIKOBLAST (<http://kaikoblast.dna.affrc.go.jp/>) was used to find the scaffold corresponding to the Nanglai *Amy* cDNA in the database of the silkworm genome project. Four primer sets (GAMYF 1–

Table 1. Primers used for the amplification of Nanglai *BmAmy* cDNA and the complete genomic sequence.

Primer name	Sequence (5'--3')	Primer description	Amplified region	PCR product (bp)
amyF1	ATGTTTCGGTGACATCCTTCTACT	<i>Amy</i> cDNA, Forward primer for RT-PCR	cDNA at + 55 to + 1545	1491
amyR1	GTCAGCACCCCTATGGATAG	<i>Amy</i> cDNA Reverse primer for RT-PCR		
ORP	GGCCGTGCTTCCACCTGTACCAAC	outer reverse primer	5' UTR <i>Amy</i>	453
IRP	TTGCGGGACCAGATTACCAAATTCTCGT	inner reverse primer	5' UTR <i>Amy</i>	266
proF1	GCGCGCCCGGGTTTCATTTTATTACACG	Forward primer for promoter	Promoter region of <i>Amy</i>	2448
proR1	GAATTCCTAACAAGCGAAAGTTTGAAA	Reverse primer for promoter		
GAMYF1	CGGGTTTCATTTTATTACACGAT	Forward primer	Genomic <i>Amy</i> at 1 to 2434	2434
GAMYR1	CTAACAAAGCGAAAGTTTGAAA	Reverse primer		
GAMYF2	GTATTTTGGGCAGATCTCATGG	Forward primer	Genomic <i>Amy</i> at 946 to 3810	2865
GAMYR2	AAACCTGACGCCAACATTGT	Reverse primer		
GAMYF3	ATGTTTCGGTGACATCCTTCTACT	Forward primer	Genomic <i>Amy</i> at 2436 to 6546	4110
GAMYR3	GTCAGCACCCCTATGGATAG	Reverse primer		
GAMYF4	CCGGTGCTCGTCCCTACATC	Forward primer	Genomic <i>Amy</i> at 5051 to 7981	2931
GAMYR4	TTAACCCGGTTTCCAATCGTTAGC	Reverse primer		
RTF1	ACTGGCCTCATTGTGTCACTACTG	Forward primer for RT-PCR	<i>Amy</i> cDNA at + 506 to + 835	330
RTR1	TGTATTCGTTGCGACTGATAGCTT	Reverse primer for RT-PCR		
RTF2	CCTTCTACTTTCGGCCGTGACT	Forward primer for RT-PCR	<i>Amy</i> cDNA at +69 to + 453	385
RTR2	GGCCGTGCTTCCACCTGTACCA	Reverse primer for RT-PCR		
actinF	AGGCCAACAGAGAGAAGATGACCCAGAT	Forward primer for RT-PCR	<i>BmActin3</i> cDNA at 410 to 813	404
actinR	GACCTGACCGTCGGGAAGTTCGTA	Reverse primer for RT-PCR		

ORP, outer reverse primer; IRP, inner reverse primer

4 and GAMYR 1–4, Table 1) were designed for the amplification of the entire Nanglai *BmAmy* genomic region using the sequences of the scaffold and the Nanglai cDNA. Genomic DNA extracted from 1st instar larvae of the Nanglai strain was used as the template for amplification with each primer set. Four obtained PCR products were cloned and sequenced. These sequences were combined together to obtain the complete genomic *Amy* gene sequence.

Expression analysis using RT–PCR

Total RNAs were extracted with TRIzol reagent (Gibco BRL) from several tissues including the hemolymph, salivary gland, Malpighian tubule, silk gland, gut, and fat body of mid-5th instar larvae of the Nanglai strain. RT-PCR High-Plus kit (Toyobo, www.toyobobiologics.com) and three primer sets, set 1 (RTF1 and RTR1), set 2 (RTF2 and RTR2) and set 3 (amyF1 and amyR1) (Table 1) were used to perform reverse transcriptase polymerase chain reactions (RT-PCR) with the extracted total RNAs. The primers actinF and actinR (Table 1) were used for RT-PCR of the *B. mori* *Actin3* gene (Accession No. U49854.1) as control. With the primer set 1, 2 and the primers used to amplify *Actin3*, the PCRs were performed at 95° C for 2 min, followed by 25 cycles of denaturing at 95° C for 15 sec, annealing at 64° C for 1 min and extending at 60° C for 5 min. The PCR condition used for RT-PCR with the primer set 3 was the same condition used to amplify *BmAmy* cDNA. The PCR products were analyzed on 1% agarose gels.

Southern blotting

Southern blotting was performed using the Amersham gene images AlkPhos direct labeling detection system (Amersham, www.gelifesciences.com). Ten micrograms of the genomic DNA extracted from 5th instar

larvae of the Nanglai strain were digested with two restriction enzymes, *Bam*HI and *Eco*RI, at 37° C overnight and analyzed on a 0.8% agarose gel. The DNA fragments transferred from the gel were hybridized with the probe generated from the amplified product (1491 bp) of amyF1 and amyR1 (Table 1).

Construction of the *pBac[BmAmy-GAL4, 3xP3-DsRed]*

The 2448 bp promoter region of the *BmAmy* gene was amplified using Nanglai genomic DNA as a template with the primers proF1 and proR1 (Table 1). The DNA fragment was cloned into the pTA2 vector (Toyobo) and sequenced. The *Actin3* (*A3*) promoter from *pBac[A3-GAL4, 3xP3-DsRed2]* (Imamura et al. 2003) has been replaced by Nanglai *BmAmy* promoter to form the *pBac[BmAmy-GAL4, 3xP3-DsRed]* plasmid.

Transgenesis and screening of *B. mori*

To generate transgenic silkworms with the *pBac[BmAmy-GAL4, 3xP3-DsRed]* construct, the *pBac[BmAmy-GAL4, 3xP3-DsRed]* was co-injected with the helper plasmid *pHA3PIG* into pre-blastoderm embryos of the w1-pnd strain as described previously (Tamura et al. 2000). After injection, the G1 embryos were allowed to develop at 25° C and reared on an artificial diet (Nihonnosanko, Japan). Transgenic silkworms were screened from the G1 embryos using a fluorescence stereomicroscope (Leica, www.leica.com) equipped with the filter sets to detect DsRed expression in their stemmata. The expression of *DsRed* was controlled by the activity of the artificial promoter, 3xP3.

Results

Sequencing of the full length *BmAmy* cDNA of the Nanglai strain

A truncated sequence of *BmAmy* cDNA has been registered in GenBank (Accession No. U078470). According to this information, RT-PCR was performed to obtain a partial *BmAmy* cDNA sequence using primers designed from the database sequence and total RNA from the Nanglai strain. To identify the full-length Nanglai *BmAmy* cDNA, 5' and 3' rapid amplifications of cDNA ends (RACE) were carried out. The full-length Nanglai *BmAmy* cDNA sequence (Accession No. GQ274006) was 1749 bp long and contained a 1503 bp ORF (Figure 1). The 54 bp long 5' untranslated region was followed by the start codon ATG at position +55 to +57. The termination codon TAA was at position +1555 to +1557 and was followed by a 192 bp 3' untranslated region containing a polyadenylation site (AATAAA) at position +1724 to +1729 (Figure 1). The *BmAmy* open reading frame (ORF) sequence was aligned with the 738 bp ORF of the database sequence. Although the *BmAmy* ORF sequence alignment showed 99 % identity with the previous data (Accession No. U078470), the ORF sequence had three more extra regions, at positions +378 to +674 (297 bp in length), positions +1068 to +1077 (10 bp in length) and positions +1100 to +1557 (99 bp in length). The *BmAmy* cDNA sequence encoded for a deduced protein with 500 amino acid residues. The protein contained a signal peptide spanning amino acid residues 1-16 that would be cleaved off, leaving a mature protein of 484 amino acids. The theoretical molecular weight and predicted isoelectric point of the mature BmAMY protein were 55 kDa and 8.24, respectively, when calculated using the compute pI/Mw tool of ExPASy web (<http://www.expasy.ch/tools/pi-tool.html>).

Genomic structure of *BmAmy* gene

Genomic DNA of the Nanglai strain was amplified with four primer sets. The PCR fragments were sequenced and combined together, revealing the 7981 bp Nanglai *BmAmy* genomic DNA sequence (Accession No. GQ274006), including 5'- and 3'-flanking regions, exons, and introns (Figure 2). The Nanglai *BmAmy* gene consisted of nine exons separated by eight introns. The first exon, the smallest, was 53 bp long and the largest exon, the sixth exon, was 509 bp long. The other exons varied in size from 99 to 201 bp. The intron size varied considerably, from 133 bp for the fourth intron to 1048 bp for the second intron. The translation start codon ATG was found in the second exon and the stop codon TAA was located in the ninth exon. The 5'-end non-coding region was 1429 bp long upstream of the transcription start site (+1). A putative consensus TATA (TATAA) box and a putative GC box were located from nucleotide position -29 to -21 and from -388 to -379 upstream of the transcription start site, respectively. Southern blot analysis was performed to estimate the copy number of the *BmAmy* gene in the Nanglai genome, using the cDNA as a probe. Genomic DNA digested with *Bam*HI showed a single fragment of 5.1 kb, whereas genomic DNA digested with *Eco*RI showed two fragments of 4.5 kb and 3.7 kb (data not shown). It is suggested that the *BmAmy* is a single-copy gene in Nanglai.

Analysis of Nanglai BmAMY

Multiple alignment of the deduced amino acid sequence of Nanglai BmAMY with known sequences of ! " \$ % & () (from other species was performed to identify identity percentage (Table 2) and conserved regions (Figure 3). Two aspartic acid residues (D209 and D311) and a glutamic acid (E246) residue present in the Nanglai BmAMY protein are conserved in the active sites for catalytic activity of most ! " \$ % & () * protein sequences. In addition, three

Table 2. Pair-wise ClustalW analysis using the deduced amino acid sequences of Nanglai BmAMY compared with AMYs from other known species that are available in the NCBI database.

Species	Accession number	Identity (%)	Amino acids
Bombyx mori from database	U078470	99	245
Diatraea saccharalis	AAP92665.1	79	500
Ostrinia nubilalis	AAP92665	77	497
Spodoptera frugiperda	AF280891	81	505
Drosophila simulans	BBA04586	60	494
Anopheles gambia	AAA29357	54	511
Tenebrio molitor	IJAEA	61	471
Tribolium castaneum	NP001107848	59	490
Litopenaeus vannamei	AA54524	58	512
Crassotrea gigas	AF320688	54	520
Sus scrofa	AF064742	55	511
Homo sapiens	NM000699	53	511

conserved histidine residues involved in substrate recognition were present at His117, His213, and His310 (Qian et al. 1994). Four amino acid residues, asparagine (N116), arginine (R170), aspartic acid (D179), and histidine (H213) comprised a putative calcium-binding site. Moreover, three amino acid residues, arginine (R207), asparagine (N309), and glutamine (Q347) involved in chloride binding were found and were needed for full catalytic activity. The latter amino acid residue, Q347, was not found in the !"\$ %& () * proteins of other animals, and instead, an arginine (R) residue was present at this site (Strobl et al. 1997; 1998). Twelve cysteine residues were found at positions 44, 102, 155, 164, 165, 172, 380, 386, 422, 445, 452, and 464 (Figure 3). Eight of these 12 cysteine residues (positions 44, 102, 155, 172, 380, 386, 452, and 464) were conserved in all animal !"\$ %& () * proteins and formed four disulfide bridges (Janacek 1993). The two cysteine residues at positions 422 and 445 may form another disulfide bridge, as was suggested for *Penaeus vannamei* and *Aedes aegypti*, which also have two cysteines at the same positions (Grossman and James 1993; Van Wormhoudt and Sellos 1996). The remaining pair at position 164 and 165 has been found only in some species of Lepidoptera but not in other animals. The

different number of disulfide bridges may be related to differences in enzyme activity (Da Lage et al. 1996). Finally, the seven conserved motifs found in all animal !"\$ %& () * proteins were also present in Nanglai BmAMY (Figure 3).

A phylogenetic tree of !"\$ %& () * was constructed with the deduced amino acid sequences from some animal species using *MEGA* version 3.1 (Kumar et al. 2004). The tree clearly showed four clades of the orders Lepidoptera, Diptera, Coleoptera and of the non-insects (Mollusca, Shrimp and Mammalia) (Figure 4). As expected, the tree grouped *B. mori*, Nanglai strain, into the Lepidoptera clade. Therefore, the !"\$ %& () * amino acid sequence can be applicable for the classification of insect order.

Tissue specificity of the BmAmy gene

To elucidate the tissue specificity of *BmAmy* gene, RT-PCRs were performed using total RNAs extracted from several tissues of the 5th instar larvae Nanglai strain at the 4th to 5th day. Three sets of primers, RTF1 and RTR1; RTF2 and RTR2; and amyF1 and amyR1, were used to amplify three different regions of the Nanglai *BmAmy* gene and three DNA fragments, 330 bp, 385 bp, and 1491bp, were obtained respectively. The results showed that the expression of *BmAmy* was found only in the gut (Figure 5A and B). Then, the gut was separated into three parts, the foregut, midgut, and hindgut, and the RT-PCRs were repeated. The result showed that gene expression was limited to the foregut (Figure 5D).

To investigate the promoter activity of *BmAmy* gene, a vector of the *GAL4* gene with the 5'-flanking region of the *Amy* gene was constructed (Figure 6A), and transgenic silkworm with the *pBac[BmAmy-GAL4, 3xP3-DsRed]* construct was generated (Table 3).

Table 3. Transgenesis and screening of silkworms containing pBac[BmAmy-Gal4, 3xP3-DsRed] vector

Number of injected eggs	Number of hatching eggs	Number of embryos with DsRed expression
542	344	4

The transgenic silkworm was crossed with the *UAS-EGFP* line and the expression of EGFP in the progeny was observed. It was found that the EGFP was expressed only in the progeny’s foregut (Figure 6B). Therefore, the result was in agreement with those obtained from the RT-PCR experiment and it is likely that the *BmAmy* gene isolated in this study is expressed only in the foregut and that BmAMY is secreted into the digestive fluid.

Discussion

The full-length *BmAmy* cDNA and its genomic structure in the Thai polyvoltine silkworm, Nanglai strain, have been successfully identified. This is the first report of the complete nucleotide sequence of the *BmAmy* gene in *B. mori*. The genomic sequence of *BmAmy* is approximately 7.9 kb long and contains nine exons, which is quite large compared with that of other insects, including *Drosophila melanogaster* (2.5 kb; Boer and Hickey 1987), *Aedes aegypti* (3.2 kb; Grossman et al. 1997), *Spodoptera frugiperda* (5.0 kb; Da Lage et al. 2002), *Ceratitis capitata* (2.3 kb. Da Lage et al. 2002), *Tribolium castaneum* (5.1 kb; Abukashawa et al. unpublished). The number of introns within the *Amy* genes of insects varies considerably. In the genus *Drosophila*, five of 146 species contain genes with no introns and the others have only one intron (Da Large et al. 1996). The *Amy* genes of *Apis mellifera* (Ohashi et al. 1999) and *Ceratitis capitata* (Da Lage et al. 2002) contain only two introns and that of *Tribolium castaneum* (Abukashawa et al. unpublished) has three introns. Conversely, the *Amy* genes of Lepidoptera species generally contain a large

number of introns (Sellos and Wormhoudt 2002). For example, *Spodoptera frugiperda* has six introns and *Ostrinia nubilalis* has seven introns (unpublished data from NCBI). The *BmAmy* gene obtained in this study has eight introns.

In *B. mori*, BmAMY is present in the digestive fluid and hemolymph (Yokoyama 1959; Promboon et al. 1993). The molecular weight of the enzyme purified from the digestive fluid of polyvoltine strains has been reported to be 55 kDa (Kanakatsu 1978). The molecular weight of the protein estimated from the gene sequence obtained in this study is 55 kDa. Therefore, it is likely that the *BmAmy* gene isolated in this study encodes the BmAMY enzyme found in digestive fluid. However, the molecular weight of BmAMY in hemolymph has not been reported. A comparison of the deduced amino acid sequence of the BmAMY with those of other insects reveal that the amino acid sequence of the BmAMY is 78, 79, and 81% identical to that of three species of Lepidoptera: *Ostrinia nubilalis* (AAA03715.1; Foster et al. unpublished), *Diatraea saccharalis* (AAP92665.1; Guerra et al. unpublished), and *Spodoptera frugiperda* (AF280891_1; Da Lage et al. 2002), respectively. In addition, it is more than 50% identical to that of other animals such as human, pig, oyster, shrimp, fruit fly, and mosquito. This homology clearly indicates that these proteins are of the same enzyme family. Moreover, when compared with BmAMY proteins of other animals, the amino acid sequence of BmAMY from Nanglai strain is highly conserved at the active sites and calcium binding sites.

The *BmAmy* encodes a chloride-dependent BmAMY that requires chloride for full activity (Janacek 1997; Qian et al. 2005). The three amino acid residues involved in the chloride

binding of most animal α -amylase (α -amylase) include two arginine (R) residues and an asparagine (N) residue. Conversely, *BmAmy* differs from those of other animals with the exception of Lepidoptera, in that one of the arginines is replaced with glutamine (Q347). The amino acid sequences of the chloride-binding sites in Lepidoptera are identical (Strobl et al. 1997). The seven conserved motifs that are commonly found in other animal α -amylase (α -amylase) are also present in the deduced BmAMY protein isolated from Nanglai strain (Svensson 1988; Janacek 1997).

In insects, *Amy* is expressed in different tissues. For example, in *Drosophila ananassae* (Da Lage et al. 2003) the *Amy* gene is expressed in the gut. Similarly, *Amy* is expressed in the crop and midgut of *Lutzomyia longipalpis* (Hill and Orchard 2005). In *Blattella germanica*, the tergal glands are specialized exocrine glands that secrete α -amylase (α -amylase) (Saltzman et al. 2006). In *Ae. aegypti*, *Amy1* is expressed only in salivary glands (Grossman and James 1993). In *B. mori*, whole-mount *in situ* hybridization reveals that *Amy* is expressed in the salivary glands (Parthasarathy and Gopinathan 2005). However, our results indicate that the *BmAmy* is expressed mainly in the foregut of a polyvoltine race, and the promoter activity of the gene supports this finding. Therefore, it is likely that the *BmAmy* gene isolated from Nanglai, a polyvoltine race, is expressed mainly in the gut and that the product is released to the digestive fluid. Two loci of *Amy* gene, *Amy-d* and *Amy-h*, have been reported to exist in *B. mori* (Matsumura 1933). Both loci are closely located on the chromosome 8. Null mutant of *Amy-d* is lacking amylase activity in the digestive juice (Kanekastu 1972) while in the null mutant of *Amy-h*, the α -amylase (α -amylase) activity in the hemolymph is very weak (Tanaka et al. 1976).

In the digestive juice, there are 5 isoforms of α -amylase (α -amylase) and all these isoforms have the same molecular weight (Asakawa and Hamano 1989). Therefore, it is likely that α -amylase (α -amylase) in the digestive juice is encoded by a single gene. Our results consistently indicate that the *BmAmy* gene identified in this study is present as a single copy in the Nanglai strain. Moreover, by searching the genomic database of *B. mori* for *Amy* homologs, no *Amy* homolog was found. This suggests that the *Amy* identified in this study may be that of *Amy-d* locus. However, if it is true that no *Amy* homolog exists and BmAMY is encoded by a single locus, the question arises why the α -amylase (α -amylase) activity in the digestive fluid is different from that in the hemolymph. At present, it is postulated that the *BmAmy* gene encodes the native proteins in the gut, and the enzyme is released into hemolymph and digestive fluid. In the different environments of these regions, the enzyme may be subjected to different post-translational modifications that are responsible for the differences in the activity of the enzyme from the two sources.

Acknowledgements

We thank Dr. Keiro Uchino, Mr. Koji Hashimoto, and Mr. Kaoru Nakamura for making the transgenic silkworms and rearing silkworms and Dr. Nora Facestii for critical reading of manuscript. This work was supported by a grant from the Insect Technology Project of the Ministry of Agriculture and Forestry; the Kasetsart University Research and Development Institute (KURDI); a Bilateral Research Cooperation (BRC) scholarship from the Faculty of Science, Kasetsart University; the student exchange program of the Transgenic Silkworm Research Center, National Institute of Agrobiological Science (NIAS); and Kasetsart University Graduate School grants.

References

- Abraham EG, Nagaraju J, Datta RK. 1992. Biochemical studies of amylases in the silkworm, *Bombyx mori* L., comparative analysis in diapausing and nondiapausing strains. *Insect Biochemistry and Molecular Biology* 22: 867-873.
- Asakawa H, Hamano K. 1989. Polymorphism of digestive amylase isozyme in the silkworm, *Bombyx mori*. *The Journal of Sericultural Science of Japan* 58: 322-326.
- Benkel BF, Nguyen T, Ahluwalia N, Benkel K, Hickey DA. 1997. Cloning and expression of a chicken *alpha*-amylase gene. *Gene* 192: 261-270.
- Boer PA, Hickey DA. 1987. The *alpha*-amylase gene in *Drosophila melanogaster*: nucleotide sequence, gene structure and expression motifs. *Nucleic Acids Research* 14: 8399-8411.
- Da Lage JL, Klarenberg A, Cariou M-L. 1996. Variation in sex, stage and tissue-specific expression of the amylase genes in *Drosophila ananassae*. *Heredity* 76: 9-18.
- Da Lage JL, Van Wormhoudt A, Cariou M-L. 2002. Diversity and Evolution of the *alpha*-amylase genes in animals. *Biologia, Bratislava* 57: 181-189.
- Da Lage JL, Maisonhaute C, Maczkowiak F, Cariou ML. 2003. A Nested Alpha-Amylase Gene in *Drosophila ananassae*. *Journal of Molecular Evolution* 57: 355-362.
- Darnis S, Juge N, Guo X-J, Marchis-Mouren G, Puigserver A, Chaix J-C. 1999. B- α , γ & β 1*, γ & β 4* . 3 β 1+ β ' 1 β *(51>, 5>1) * . ' β 8 (+(*-0*
- Grossman GL, James AA. 1993. The salivary glands of the vector mosquito, *Aedes aegypti*, express a novel member of the amylase gene family. *Insect Molecular Biology* 1: 223-232.
- Grossman GL, Campos Y, Severson DW, James AA. 1997. Evidence for two distinct members of the amylase gene family in the yellow fever mosquito, *Aedes aegypti*. *Insect Biochemistry and Molecular Biology* 27: 769-781.
- Hill SR, Orchard I. 2005. In vitro analysis of the digestive enzymes amylase and α -glucosidase in the midguts of α -glucosidase in the midguts of *Locusta migratoria* L. in response to myosuppressin, SchistoFLRFamide. *Journal of Insect Physiology* 51: 1-9.
- Horii A, Emi M, Tomita N, Nishide T, Ogawa M, Mori T, Matsubara K. 1987. Primary structure of human pancreatic *alpha*-amylase gene: its comparison with human salivary *alpha*-amylase gene. *Gene* 60: 57-64.
- Imamura M, Nakai J, Inoue S, Quan GX, Kanda T, Tamura T. 2003. Targeted gene expression using the Gal4/UAS system in the silkworm *Bombyx mori*. *Genetics* 165: 1329-1340.
- Janecek S. 1993. Sequence similarities in (α/β) 8-barrel enzymes reveals by observed regions of α -amylase. *FEBS* 316: 23-26.
- Janecek S. 1997. α -amylase family: molecular biology and evolution. *Progress in Biophysics and Molecular Biology* 67: 67-97.

- Kanekatsu R. 1972. Amylase in the digestive juice of silkworm larvae, *Bombyx mori*. *The Journal of Sericultural Science of Japan* 41: 445-451.
- Kanakatsu R. 1978. Studies on further properties for an alkaline amylase in the digestive juice of silkworm, *Bombyx mori*. *Journal of the Faculty of Textile Science and Technology* 76: 1-21.
- Kobayashi I, Uchino K, Sezutsu H, Iizuka T, Tamura T. 2007. Development of a new piggyBac vector for generating transgenic silkworms using the *kynurenine 3-mono oxygenase* gene. *Journal of Insect Biotechnology and Sericology* 76: 145-148.
- Kumar S, Tamura K, Nei M. 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Bioinformatics* 5: 150-163.
- Matsumura S. 1933. Genetics of four types of amylases in the silkworm, *Bombyx mori*. *The Journal of Sericultural Science of Japan* 4: 168-170.
- Moal J, Daniel JY, Sellos D, Wormhoudt AV, Samain JF. 2000. Amylase mRNA expression in *Crassostrea gigas* during feeding cycles. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology* 170: 21-26.
- Murakami A. 1989. Genetic studies on tropical races of silkworm (*Bombyx mori*), with special reference to cross breeding strategy between tropical and temperate races. 2. Multivoltine silkworms in Japan and their origin. *Journal of the Association for Research in Otolaryngology* 23: 123-127.
- Ohashi K, Natori S, Kubo T. 1999. Expression of amylase and glucose oxidase in the hypopharyngeal gland with an age-dependent role change of the worker honeybee (*Apis mellifera* L.). *European Journal of Biochemistry* 265: 127-133.
- Parthasarathy R, Gopinathan KP. 2005. Comparative analysis of the development of the mandibular salivary glands and the labial silk glands in the mulberry silkworm, *Bombyx mori*. *Gene Expression Patterns* 5: 323-339.
- Promboon A, Engkakul A, Ngernsiri L, Saksoong P. 1993. Amylase of the polyvoltine silkworm (*Bombyx mori*): variation of activity in the Thai local race. *Sericologia* 33: 603-609.
- Qian M, Haser R, Buisson G, Duee E, Payan F. 1994. The active center of a mammalian alpha-amylase. Structure of the complex of a pancreatic alpha-amylase with a carbohydrate inhibitor refined to 2.2 Å resolution. *Biochemistry* 33: 6284-6294.
- Qian M, Ajandouz EH, Payan F, Nahoum V. 2005. Molecular basis of the effects of chloride ion on the acid-base catalyst in the mechanism of pancreatic α -Amylase. *Biochemistry* 44: 3194-3201.
- Saltzmann KD, Saltzmann KA, Neal JJ, Scharf ME, Bennett GW. 2006. Characterization of BGTG-1, a tergal gland-secreted alpha-amylase, from the German cockroach, *Blattella germanica* (L.). *Insect Molecular Biology* 15: 425-433.
- Sellos DY, Van Wormhoudt A. 2002. Structure of the α -amylase genes in crustaceans and molluscs: evolution of the exon/intron organization. *Biologia, Bratislava* 57 (Suppl. 11): 191-196.

Sellos DY, Moal J, Degremont L, Huvet A, Daniel J-Y, Nicoulaud S, Boudry P, Samain J-F, Wormhoudt AV. 2003. Structure of amylase genes in populations of pacific cupped oyster (*Crassostrea gigas*): tissue expression and allelic polymorphism. *Marine Biotechnology* 5: 360-372.

Strobl S, Gomis-Ruth FX, Maskos K, Frank G, Huber R, Glockshuber R. 1997. The α -amylase from the yellow meal worm: complete primary structure, crystallization and preliminary X-ray analysis. *FEBS Letters* 409: 109-114.

Strobl S, Maskos K, Betz M, Wiegand G, Huber R, Gomis-Ruth FX, Glockshuber. 1998. Crystal structure of yellow meal worm α -amylase at 1.64 Å resolution. *Journal of Molecular Biology* 278: 617-628.

Svennson B. 1988. Regional distant sequence homology between amylase, α -glucosidase and transglucanases. *FEBS Letters* 236: 72-76.

Tamura T, Thibert C, Royer C, Kanda T, Abraham E, Kamba M, Komoto N, Thomas LL, Mauchamp B, Chavancy G, Shirk P, Fraser M, Prudhomme JC, Couble P. 2000. Germline transformation of the silkworm *Bombyx mori* L. using a piggyBac transposon-derived vector. *Nature Biotechnology* 18: 81-84.

Tanaka Y, Kusano T, Kobara R, Kawai T. 1976. An electrophoretic study on haemolymph amylase in the silkworm, *Bombyx mori* L. *The Journal of Sericultural Science of Japan* 45: 533-536.

Uchino K, Imamura M, Sezutsu H, Kobayashi I, Kojima K, Kanda T, Tamura T. 2006.

Evaluating promoter sequences for trapping an enhancer activity in the silkworm *Bombyx mori*. *Journal of Insect Biotechnology and Sericulture* 75: 89-97.

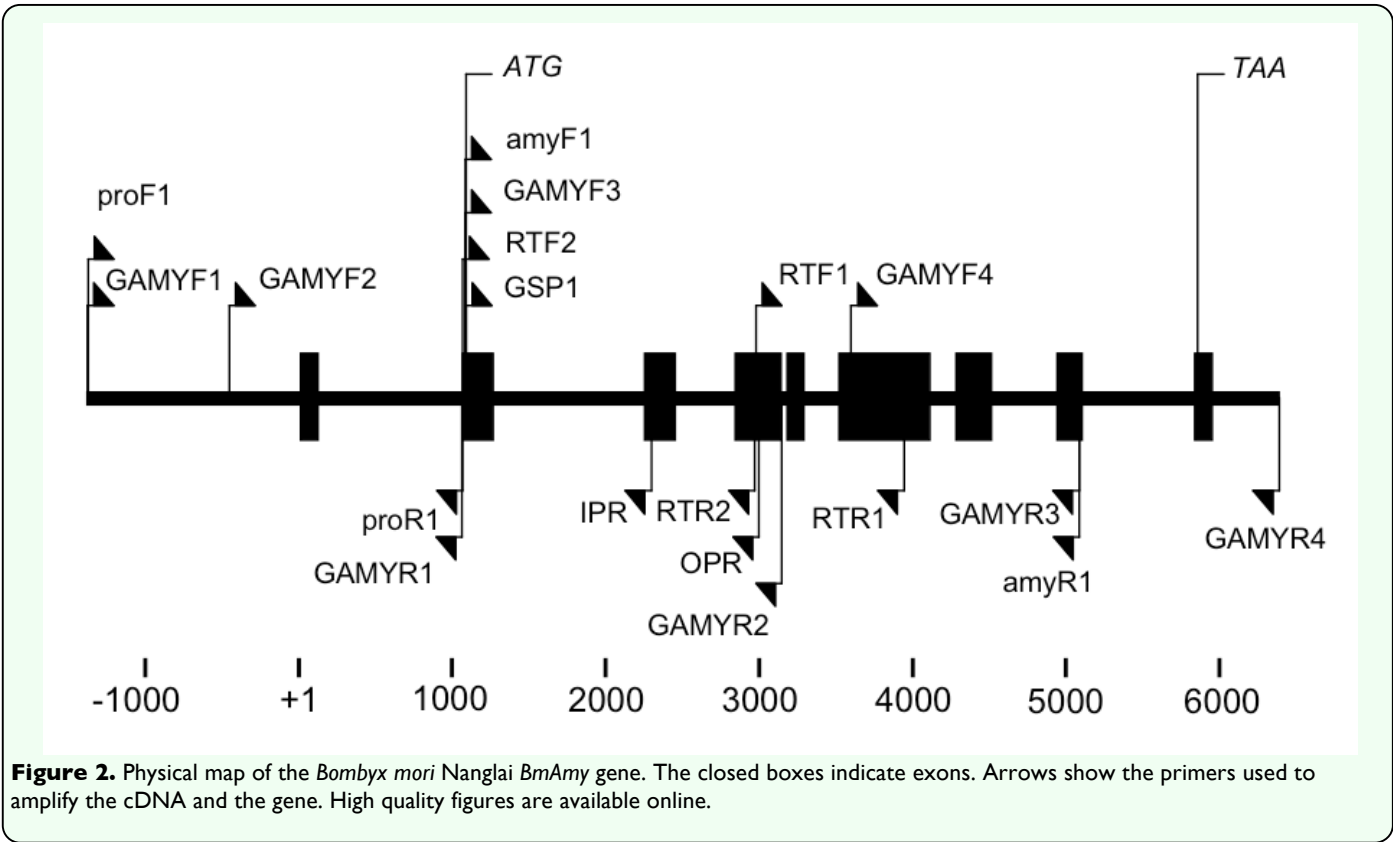
Van Wormhoudt A, Sellos DY. 1996. Cloning and sequencing analysis of three amylase cDNAs in the shrimp *Penaeus vannamei* (Crustacea decapoda): evolutionary aspects. *Journal of Molecular Evolution* 42: 543-551.

Wyatt GR. 1967. The biochemistry of sugars and polysaccharides in insects. *Advance in Insect Physiology* 4: 287-360

Yokoyama T. 1959. *Silkworm Genetics Illustrated*. Japanese society for promotion of science. Ueno Park, Tokyo.

AACTATTTTTGTACGGTACATTGGGGCGAGCAGCAAGAAATAT
-366 GAATAGTAAAATTAAAAATAACAAATTTAATAAAATCAATTAGTAGGATTATTTAAGC
-306 AAGAAGTTAACATTCCAACGCTGTGAAAAGCCATTTTTTTTTTAAAAACAGTTTCCTTCAA
-246 ATAACCGCTTTCAAACACAAATGATTTACAAATGAATCATTGGCTGAAATAATGTGT
-186 TTTAGGTGTGAAAATTTGATTTTTATCGTACAAAATCATCTTGGATACGGAGCGCCGAAT
-126 ATTTATCAATGTTTTTTTGAACTTTTATCGAACGATTATGTTTCATTATGTCGATAAATTT
-66 CGTGTATCGCAACACAGACCGAGATTTTATCTTCAAGTATATATAAATTGGGTACGGGAG
+1
-6 GCTGTTATCAGATTTATTTAATTATATCACACGACAGGAGGTCCAGTATATTAGATAAGG
+55 ATGTTTCGGTACATCCTTCTACTTTTCGGCCGTGACTCTGGCGTTGGCCTATAAAAACCTT
1 M F R Y I L L L S A V T L A L A Y K N P
+115 CACTATGCATCGGGTCGCACCACCATGGTACATCTCTTCGAATGGAAGTGGGATGACATC
21 H Y A S G R T T M V H L F E W K W D D I
+175 GCTGCTGAATGCGAAAGGTTCTTGGACCCCGAGGATTGGTGGTATTTCAGGTTTCGCCA
41 A A E C E R F L G P R G F G G I Q V S P
+235 CCAAACGAGAATTTGGTAATCTGGTCCCGCAACCGTCTTGGTGGGAGCGCTATCAACCA
61 P N E N L V I W S R N R P W W E R Y Q P
+295 ATCTCTACCGTCTAGTAACAAGATCTGGAATGAAATCAATTTTCGAATATGGTGGCT
81 I S Y R L V T R S G N E N Q F S N M V R
+355 CGCTGCAACAATGTTGGCGTCAGGATTTATGTGGACGCCATCATCAACCACATGACTGGA
101 R C N N V G V R I Y V D A I I N H M T G
+415 ACTTGGAAATGAGAATGTTGGTACAGGTGGAAGCAGGCCAATTTTCGAAACTGGCACTAT
121 T W N E N V G T G G S T A N F E N W H Y
+475 CCTGCTGTTCCCTACGGCAGGAATGACTTCAACTGGCCTCATTGTGTCATTACTGGGAGC
141 P A V P Y G R N D F N W P H C V I T G S
+535 GACTACAACGCTGCTCCCTGACAGAGTGGCAACTGTGAATTGTCTGGTCTGAAAGACTTG
161 D Y N C C P D R V R N C E L S G L K D L
+595 AACCAGGCTCTGATTATGTTAGACAGCAGATTCTAAATTATATGAACCGTCTTATTGAC
181 N Q G S D Y V R Q Q I L N Y M N R L I D
+655 ATGGGTGTTGCTGGTTTCAGAATTGATGCTGCAAAGCACATGTGGCCTCACGATTTGCGA
201 M G V A G F R I D A A K H M W P H D L R
+715 GTGATCTACGATCGTCTCCGCAATTTAAATACCGCCACGGTTTCCCATCCGGTGTCTGT
221 V I Y D R L R N L N T A H G F P S G A R
+775 CCCTACATCTACCAAGAAGTCATCGACCTCGGTGGTGAAGCTATCAGTCGCAACGAATAC
241 P Y I Y Q E V I D L G G E A S R N E Y T
+835 ACTCCACTTGCTGCCGTTACTGAGTTCAGATTTGGACTGGAACCTCAGTCAAGCTTTCCAA
261 P L A A I V T E F R F G L E L S Q A F Q
+895 AGAAGAAACAGCTTAGATGGTTGTTAACTGGGGTCCACAGTGGGGCCTTCTCGCTTCT
281 R R N Q L R W L V N W G P Q W G L L A S
+955 GGAGACGCTTTGACTTTTCATTGACAACACGACAACAGAGAGGTATGGCGCTGGTGGGA
301 G D A L T F I D N H D N Q R G H G A G G
+1015 AACATCTTGACATACAAGCAATCCCGACAATAACAAGGGCGCTATCGCTTTTCATGTTGGCA
321 N I L T Y K Q S R Q Y K G A I A F M L A
+1175 CATCCTTATGGCTACCCCTCAATTGATGAGTAGTTTCGCCTTCACGGACACCGAAGCTGGA
341 H P Y G Y P Q L M S S F A F T D T E A G
+1135 CCTCCAATGAACAGCAGGGGTGATATCACCTCTCTACTATCAACGCTGATAATTCTTGC
361 P P M N S R G D I T S P T I N A D N S C
+1195 GGCAATGGTTGGATATGCGAGCACCGTTGGCGTCAGATCCACAACATGGTTGTCTTCAGG
381 G N G W I C E H R W R Q I H N M V V F R
+1255 AACACCGCTGGTAACGGTGCCCTTACCAACTGGTGGGACAACGGTAGCAACAGATTGCT
401 N T A G N G A L T T N W W D N G S N Q I A
+1315 TTCTGCCGCGGCAACCGAGGCTTTCATTGCTTTCAACAACGATGCATGGGACATGGACCAG
421 F C R G N Q A F I A F N N D A W D M D Q
+1375 ACTCTTCAGACTTGCTCTCCCGCCGGAACATACTGCGATATTATTTCCGGCGCCAGGTCT
441 T L Q T C L P A G T Y C D I I S G A R S
+1435 GGTAACCGCTGCACTGGAATCTATTGTAGTAGGACGATGGTTCGCGCTCTTATCATC
461 G N R C T G K S I V V G S D G R A L I I
+1495 CACCGCAGCAACGAGTATGACATGATGGTTGCTATCCATAGGGGTGCTGACTCGAGATTG
481 H R S N E Y D M M V A I H R G A D S R L
+1555 TAAACGGGAACCGTACTGGCAATATTTGAAGACTGGAATCTGAAAATTGCAATAT
501 *
+1615 TAATTGTGAAATACATTTATGTCTTTCTTATAATAATGTTTTAATTTTATGATTAAAGCG
+1675 ATTTTCTTATCAAGGTTATTGCTTTCTAAGATTAGAAATAATAAGATTAAATAATACAT
+1735 AATATAAAAAAAAAA

Figure 1. Nucleotide sequence of the *Bombyx mori* Nanglai *BmAmy* cDNA , the 5' non-coding region and deduced amino acid sequences. The numbers with a plus sign indicate the nucleotide position from the transcription initiation site (+1). The amino acid sequence is numbered sequentially from the first amino acid, the methionine. The putative TATA box (TATATATAA) is bolded and underlined. The putative GC box is double underlined. The proposed signal peptide is underlined. The stop codon and consensus polyadenylated signal sites are in italics and underlined, respectively. High quality figures are available online.



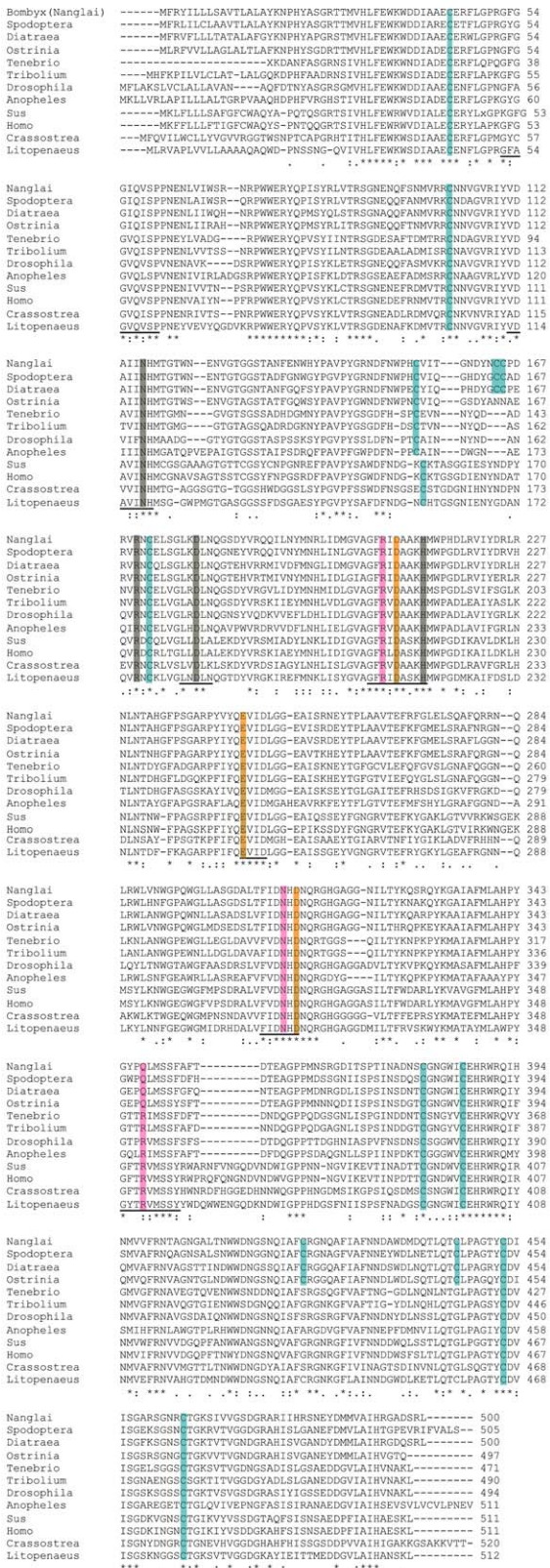


Figure 3. Multiple alignment of *Bombyx mori* α-amylase amino acid sequences using ClustalW version 1.81. The amino acid sequences of α-amylase were aligned to show maximum sequence identity. The seven conserved sequence regions common to all animal α-amylases are underlined. Active site residues (D209, E260, D311) are shaded in yellow. Calcium binding residues (N116, R170, D179, H213) are shaded in gray. The pink shade indicates chloride binding residues (R207, N309, Q347). Cysteine residues are shaded in blue. All amino acid sequences used for alignment were obtained from NCBI and their accession numbers are the same as in Table 2. High quality figures are available online.

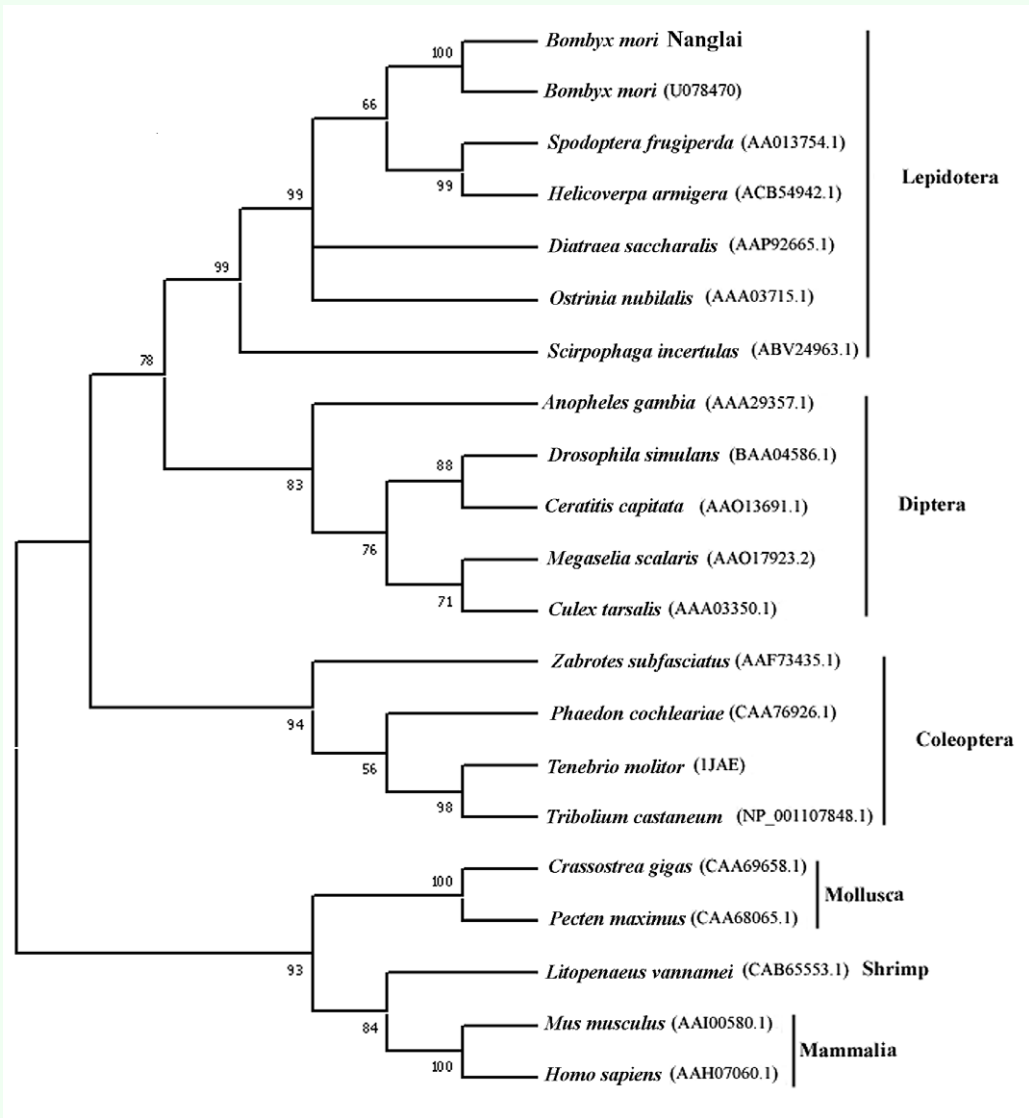


Figure 4. Phylogenetic analysis of *Bombyx mori* Nanglai BmAMY with α -amylases of some other species. The tree based on their amino acid sequences was constructed by MEGA 3.1. The numbers at the nodes are the percentage of 1000 bootstrap re-samplings. The accession number of each species is shown in parentheses. High quality figures are available online.

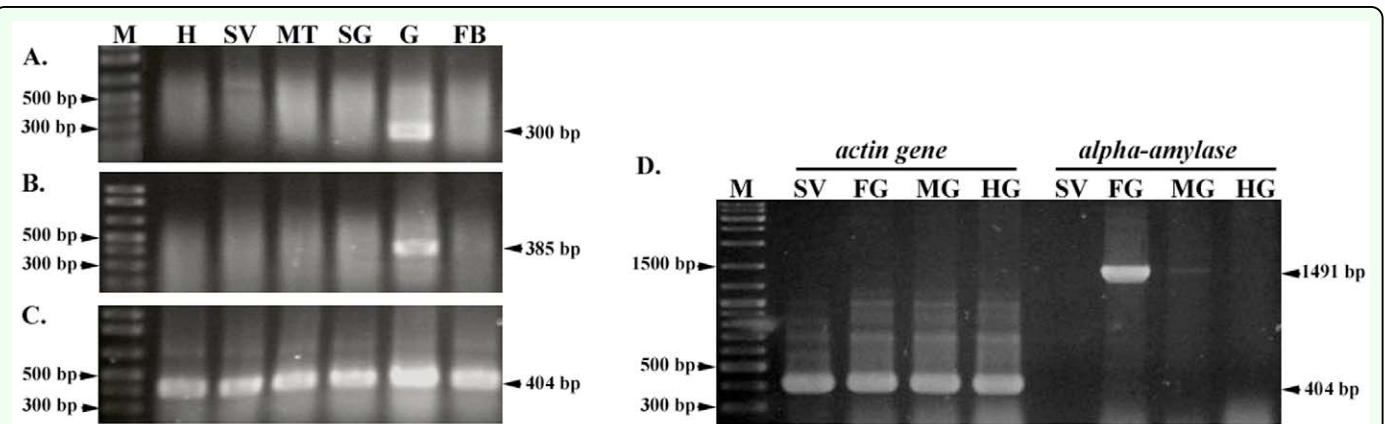


Figure 5. Tissue-specific expression of the *Bombyx mori* Nanglai BmAmy gene was analyzed using RT-PCR with primer sets 1 (A), 2 (B), and 3 (D). RT-PCR of the *Bombyx mori* actin3 gene is shown in C and D. Total RNAs extracted from the hemolymph (H), salivary gland (SV), Malpighian tube (MT), silk gland (SG), gut (G), fat body (FB), foregut (FG), midgut (MG), and hindgut (HG) of the Nanglai strain were used as templates for RT-PCRs. Lane M shows 100-bp and 1-kb DNA ladders as size markers. High quality figures are available online.

