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## Original Article

# cDNA cloning and expression pattern of two enolase genes from the Chinese oak silkworm, *Antheraea pernyi*

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In this study, two enolase genes were isolated and characterized from the Chinese oak silkworm, *Antheraea perny*, which were designated as *enolase I* and *II*, respectively. The *enolase I* cDNA sequence was 1712 bp with an open reading frame (ORF) of 1302 bp encoding 433 amino acids. The *enolase II* cDNA sequence was 1549 bp with an ORF of 1296 bp encoding 431 amino acids. The amino acid sequences of the two genes share several conserved features/sites of enolase. *Antheraea pernyi* enolase I shows 93%–97% sequence identity to enolases of lepidopterans available to date, 75%–82% identity to enolases of other invertebrates, 60%–72% identity to enolases of other organisms including vertebrates, plants, and fungi. *Antheraea pernyi* enolase II shows 84% identity to *Bombyx mori* enolase II, but 60% identity to *A. pernyi* enolase I. In the phylogenetic tree, enolase II sequences from *A. pernyi* and *B. mori* were clearly separated from the majority of enolase sequences of higher organisms including *A. pernyi* and *B. mori* enolase I sequences. By sequence comparisons and phylogenetic analysis, we suggest that enolase II from *A. pernyi* and *B. mori* may be a new member of the enolase superfamily. *Antheraea pernyi enolase I* mRNA was found in all tested tissues whereas *enolase II* mRNA was expressed specifically in the spermaries and ovaries, suggesting that the product of *enolase II* gene may be related to reproduction. The transcript abundance of *A. pernyi enolase I* gene was significantly down-regulated after cold shock and significantly up-regulated after heat shock, suggesting that *A. pernyi enolase I* gene may be inducible by temperature stress.

*Keywords*  *Antheraea pernyi*; enolase; cloning; expression pattern

## Introduction

Enolase (2-phospho-D-glycerate hydrolyase) [EC 4.2.1.11] is an essential enzyme catalyzing the conversion of 2-phosphoglycerate (2-PGE) to phosphoenolpyruvate (PEP), the only dehydration step in the glycolytic pathway [1]. The Embden-Meyerhof-Parnas pathway of glycolysis is the backbone of energy metabolism (ATP synthesis) in eukaryotes. Compared with other enzymes involved in glycolysis and gluconeogenesis, enolase is one of the most conservatively evolved glycolytic enzymes [2]. It is found in all organisms and belongs to a highly conserved gene family that allows for the evolutionary study of gene duplication in animals [3]. Eukaryotic enolases have been shown to be multifunctional proteins presenting a variety of activities besides their glycolytic activities [4], and play important roles in many biological and disease processes [5]. It was reported that enolase has the ability to bind to polynucleotides and cytoskeletal proteins [6], and functions like a heat shock protein [7]. Moreover, enolase is found to be identical to the heat shock protein HSP48 and, hence, is important in thermal tolerance and growth control of yeast [8]. Recently, it has been reported that enolase is involved in both temperature and salt stress tolerance in algae [9].

The enolase has been studied from a wide variety of organisms, from bacteria to mammals. In humans and other mammals there are three enolase isoenzymes; their cell types and developmental specificity have been demonstrated [10–12]. There are about 900,000 known insect species, three times as many as all other animal species

together, and thousands of new ones are described each year. In the past years, published articles are starting to shed light on the biology of insect enolase. Recently, it has been reported that *Aphidius ervi* enolase may be involved in the regulation of teratocyte-mediated degradation of host tissues and immune evasion [13]. However, knowledge on insect enolase remains extremely limited.

The Chinese oak silkworm, *Antheraea pernyi* (Lepidoptera: Saturniidae), is a well-known economic insect species. This insect is known to be domesticated in China around the 16th century [14], and it is commercially cultivated for wild silkworm silk production. The silk-worm, including larva, pupa and moth, is also used for a high-quality protein food [15]. Moreover, this species has become an excellent natural bioreactor for the production of recombinant proteins [16]. We have constructed a full-length cDNA library from *A. pernyi* pupa [17] and per-formed the expressed sequence tags (EST) sequencing to identify the functional genes of this species. Using this strategy, a lysophospholipase gene of this species has been cloned and characterized [18].

In this paper, two enolase genes from *A. pernyi* were isolated and characterized for the first time. We compared the deduced protein sequence with those of other organisms, and examined the expression patterns at various developmental stages and in different tissues in the fifth instar larvae. Finally, the effects of cold and heat shocks on the relative mRNA expression levels were determined. To our knowledge, this is the first report to describe the tissue-specific expression pattern of insect enolase. The results presented here would provide basic information for their functional analyses.

## Materials and Methods

### Insects and tissues

Larvae of *A. pernyi* strain *Shenhuang No. 1* used in this study were reared on oak trees (*Quercus liaotungensis*) in the field. Blood, fat body, midgut, silk glands, body wall, Malpighian tubules, spermaries, ovaries, brain and muscle were dissected from silkworm larvae at Day 10 of the fifth instar and immediately frozen in liquid nitrogen and then stored at $-80^{\circ}$C. Eggs at Day 5, fifth instar larvae, pupae and moths were also frozen in liquid nitrogen and stored at $-80^{\circ}$C for later use. To examine the effect of temperature stress on the mRNA expression of *A. pernyi* enolase gene, three stress conditions, including $46^{\circ}$C-heat shock treat-ment for 3 h, $26^{\circ}$C-control treatment for 3 h and $4^{\circ}$C-cold shock treatment for 3 h, were used. After treatment, fat body was immediately dissected from silkworm pupae to extract total RNA. Tissue samples from 18 silkworm pupae were pooled randomly into three groups for each treatment.

### Total RNA extraction, first-strand cDNA synthesis and RT-PCR

Total RNA was extracted from *A. pernyi* samples using RNAsimple Total RNA Extraction Kit (TIANGEN Biotech, Beijing, China) according to the manufacturer's instructions. DNase I was used to remove contaminating genomic DNA. The purity and quantity of extracted RNA were quantified by the ratio of $OD_{260}/OD_{280}$ with an ultra-violet spectrometer. Using 2 μg of total RNA per sample, the first-strand cDNA was generated with TIANScript cDNA Synthesize Kit (TIANGEN Biotech) following the manufacturer's instructions. RT-PCR amplification was carried out in a total reaction volume of 25 μl, containing cDNA sample, 20 pmol of each primer, 2 mM $MgCl_2$, 0.25 mM dNTP, $1\times$ buffer, and 2.5 units of Taq DNA polymerase (TIANGEN Biotech). PCRs were performed with the following cycles: initial denaturation at $95^{\circ}$C for 5 min; followed by 35 cycles of 1 min at $95^{\circ}$C, 30 s annealing at $55^{\circ}$C, 30–90 s extension at $72^{\circ}$C; and a final extension at $72^{\circ}$C for 10 min.

### Isolation of the *A. pernyi* enolase cDNAs

A full-length *A. pernyi* pupal cDNA library was con-structed in our laboratory [17], and the randomly selected positive clones were sequenced by the EST method. An EST representing the *enolase I* gene (GenBank accession no. GH335056) was first isolated. Then, the cDNA clone was used to complete the full-length cDNA sequence of the *enolase I* gene.

For the amplification of the 5′ end fragment of *A. pernyi enolase II* gene, the primer pair, LYQ170 (5′-CACAA AATGC CNATT AAG-3′) (N = A/G/C/T) and LYQ171 (5′-AGAAT GGTGA AATGA GGTAC-3′), was first designed and used in the RT-PCR reaction. The two sequences were obtained by aligning the sequences from the putative *Antheraea assama* enolase II (WildSilkbase accession no. Aats3556), *Samia cynthia ricini* enolase II (WildSilkbase accession no. Scfb_Ec6565) and *Bombyx mori* enolase II (SilkDB accession no. BGIBMGA00 2337-TA). Spermary cDNA sample was used as the tem-plate for RT-PCR amplification. The amplification products were analyzed on 1.0% agarose gels stained with ethidium bromide. The RT-PCR products were purified from the gel and sequenced.

Based on the sequence obtained, the 3′ rapid amplifica-tion of the cDNA ends (3′ RACE) of *A. pernyi enolase II* cDNA was performed with TIANScript cDNA Synthesize Kit (TIANGEN Biotech) following the manufacturer's instructions. The first-strand cDNA was synthesized from 2 μg of total RNA of spermaries using a cDNA synthesis primer AP (5′-ATCCT GACAC TATCG TACAC $(T)_{20}$-3′). Using primer LYQ187 (5′-ACGGA CAGTA AAAAG CTTGC-3′) synthesized based on the known sequence as

the forward primer and the Universal Amplification Primer (UAP, 5′-ATCCT GACAC TATCG TACAC-3′) as the reverse primer, 3′ RACE was performed. The PCR products were purified from the gel and sequenced.

## Sequence analysis

DNA sequences were assembled and edited to obtain a consensus sequence. DNASTAR software (DNASTAR Inc., Madison, USA) was used to identify the open reading frame (ORF), deduce amino acid sequence, and predict the isoelectric point and molecular weight of the deduced amino acid sequence. Blast search was performed at http://www.ncbi.nlm.nih.gov/blast.cgi. Conserved domains were predicted at http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi. The *in silico* gene expression analysis was employed at http://www.ncbi.nlm.nih.gov/Unigen/EST profileViewer based on the available EST resources and at http://silkworm.swu.edu.cn/silkdb based on the extensive microarray information.

## Phylogenetic analysis

Clustal X software [19] was used to align amino acid sequences. A phylogenetic tree was constructed by MEGA version 4 [20] using the neighbor-joining method [21]. A Poisson-corrected distance was used, and the statistical significance of group in the neighbor-joining tree was assessed by the bootstrap probability with 1000 replications. A maximum parsimony analysis was also used to construct the phylogenetic tree by MEGA version 4 [20].

## Semi-quantitative RT-PCR analysis

A previously isolated actin gene (GenBank accession no. GU073316) was used as the internal control for adjustment of template RNA quantity with the gene-specific primer pair LYQ85 (5′-CCAAA GGCCA ACAGA GAGAA GA-3′) and LYQ86 (5′-CAAGA ATGAG GGCTG GAAGA GA-3′), which generated a 468 bp fragment [22]. The cDNA samples were amplified by semi-quantitative RT-PCR method using the gene-specific primer pair LYQ116 (5′-TGCTG TTGGT GATGA GGGTG-3′) and LYQ117 (5′-CGATG CGTTT TGGGT TTGTT-3′) for *A. pernyi enolase I* gene, which generated a 374 bp fragment, and LYQ187 (5′-ACGGA CAGTA AAAAG CTTGC-3′) and LYQ171 (5′-AGAAT GGTGA AATGA GGTAC-3′) for *A. pernyi enolase II* gene, which generated a 347 bp fragment. PCRs were performed with the following cycles: initial denaturation at 95°C for 5 min; followed by 25 cycles of 1 min at 95°C, 30 s annealing at 55°C, 30 s extension at 72°C; and a final extension at 72°C for 10 min. The amplification products were analyzed on 1.0% agarose gels stained with ethidium bromide. To avoid sample DNA contamination, the negative RT-PCR control reactions were performed with total RNA as templates. To

confirm the specificity of RT-PCR amplification, the RT-PCR products were purified from the gel and sequenced. The RT-PCR experiments were performed three times. Statistical analysis was performed with SPSS v11.5. A two-tailed Student's test was used to determine the statistical difference between the groups and $P < 0.01$ was considered as significant.

# Results

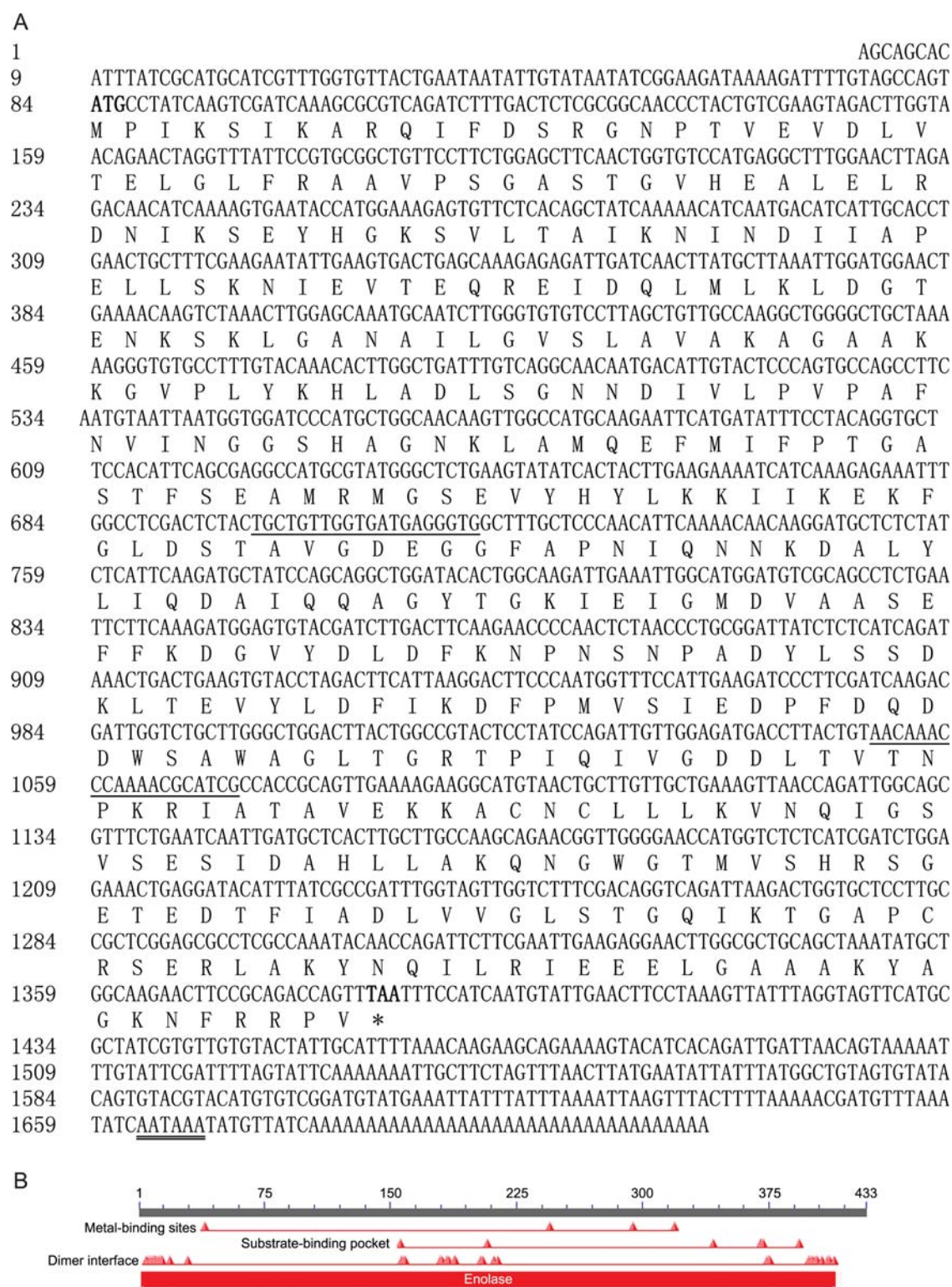## Sequence analysis of the *A. pernyi* enolase gene

In the present study, two enolase genes were cloned from *A. pernyi*, and designated as *A. pernyi enolase I* and *II*, respectively. *Antheraea pernyi enolase I* gene was identified from the pupal cDNA library [17]. Based on the cDNA clone Appu0227, we isolated and sequenced the full-length cDNA of the *enolase I* gene. The cDNA sequence and deduced amino acid sequence of the *enolase I* gene is shown in **Fig. 1(A)**. The obtained 1712 bp cDNA sequence contains a 5′-untranslated region (UTR) of 83 bp with one TATA box (5′-TATAAT), a 3′ UTR of 292 bp with a polyadenylation signal sequence AATAAA at position 1663 and a poly (A) tail, and an ORF of 1302 bp encoding a polypeptide of 433 amino acids. The deduced amino acid sequence has a predicted molecular weight of 47.009 kDa and an isoelectric point of 5.4. Conserved domains prediction revealed that the amino acid sequence shares several conserved features/sites of enolase, such as the metal-binding sites, the substrate-binding pocket, and the dimer interface [**Fig. 1(B)**]. This cDNA sequence has been deposited in GenBank under accession no. GU289926.

The cDNA sequence of *A. pernyi enolase II* gene obtained was 1549 bp in length with an ORF of 1296 bp, a 3′ UTR of 227 bp and a poly (A) tail. However, no polyadenylation signal sequence AATAAA was found. The cDNA sequence and deduced amino acid sequence of the *enolase II* gene is shown in **Fig. 2(A)**. The *enolase II* gene encodes 431 amino acids with a predicted molecular weight of 46.976 kDa and an isoelectric point of 5.9. Conserved domains prediction also revealed that the amino acid sequence shares several conserved features/sites of enolase mentioned above [**Fig. 2(B)**]. This cDNA sequence has been deposited in GenBank under accession no. HM755879.

## Homologous alignment

The domesticated silkworm *Bombyx mori* is a major insect model for research and the first lepidopteran for which draft genome sequences became available in 2004 [23,24]. By search against *B. mori* genome at the SilkDB, an open-access database for *B. mori* genome biology [25], two enolase genes were identified with ID BGIBMGA
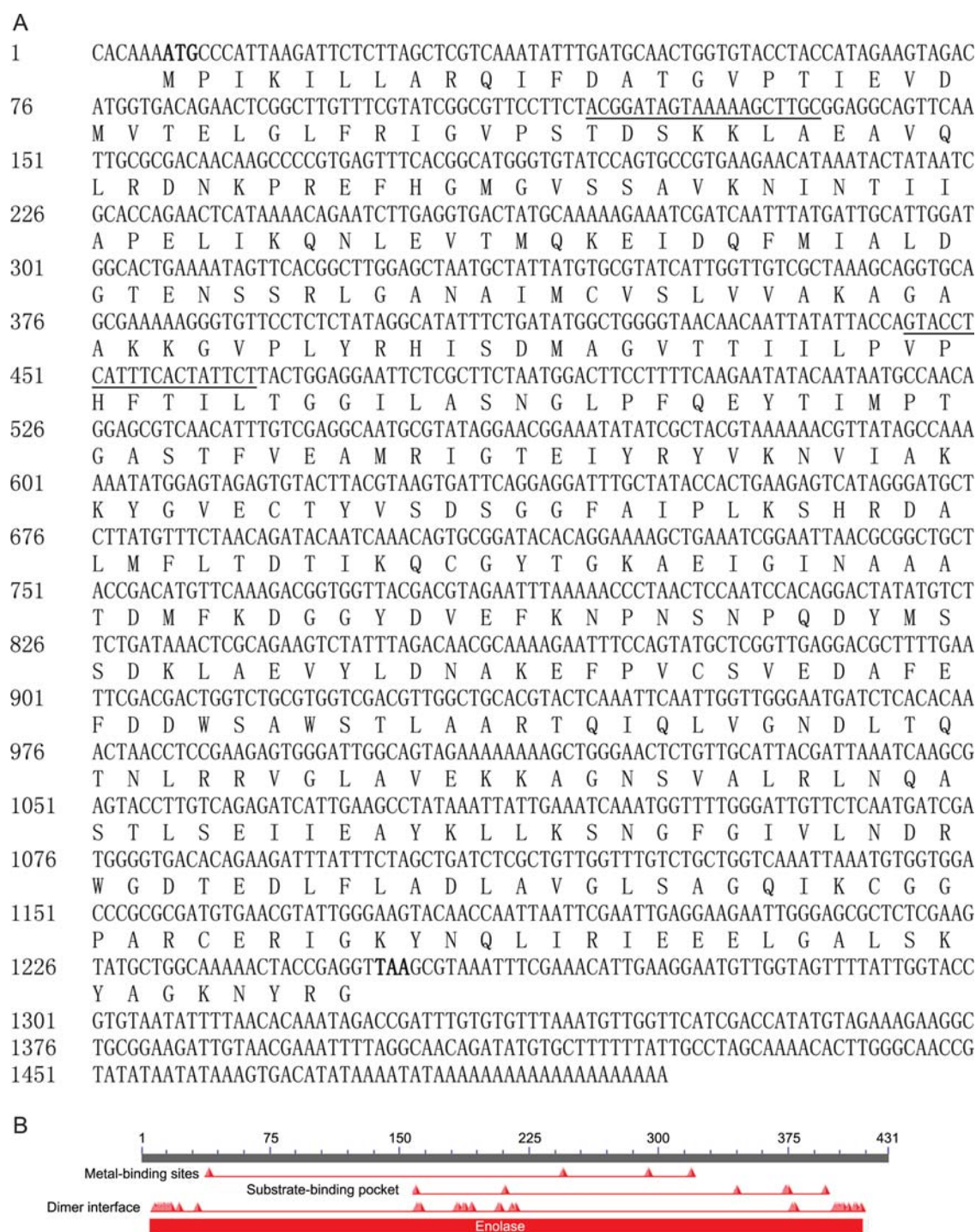
A

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1                                                                      AGCAGCAC
9      ATTTATCGCATGCATCGTTTGGTGTTACTGAATAATATTGTATAATATCGGAAGATAAAAGATTTTGTAGCCAGT
84     ATGCCTATCAAGTCGATCAAAGCGCGTCAGATCTTTGACTCTCGCGGCAACCCTACTGTCGAAGTAGACTTGGTA
       M  P  I  K  S  I  K  A  R  Q  I  F  D  S  R  G  N  P  T  V  E  V  D  L  V
159    ACAGAACTAGGTTTATTCCGTGCGGCTGTTCCTTCTGGAGCTTCAACTGGTGTCCATGAGGCTTTGGAACTTAGA
       T  E  L  G  L  F  R  A  A  V  P  S  G  A  S  T  G  V  H  E  A  L  E  L  R
234    GACAACATCAAAAGTGAATACCATGGAAAGAGTGTTCTCACAGCTATCAAAAACATCAATGACATCATTGCACCT
       D  N  I  K  S  E  Y  H  G  K  S  V  L  T  A  I  K  N  I  N  D  I  I  A  P
309    GAACTGCTTTCGAAGAATATTGAAGTGACTGAGCAAAGAGAGATTGATCAACTTATGCTTAAATTGGATGGAACT
       E  L  L  S  K  N  I  E  V  T  E  Q  R  E  I  D  Q  L  M  L  K  L  D  G  T
384    GAAAACAAGTCTAAACTTGGAGCAAATGCAATCTTGGGTGTGTCCTTAGCTGTTGCCAAGGCTGGGGCTGCTAAA
       E  N  K  S  K  L  G  A  N  A  I  L  G  V  S  L  A  V  A  K  A  G  A  A  K
459    AAGGGTGTGCCTTTGTACAAACACTTGGCTGATTTGTCAGGCAACAATGACATTGTACTCCCAGTGCCAGCCTTC
       K  G  V  P  L  Y  K  H  L  A  D  L  S  G  N  N  D  I  V  L  P  V  P  A  F
534    AATGTAATTAATGGTGGATCCCATGCTGGCAACAAGTTGGCCATGCAAGAATTCATGATATTTCCTACAGGTGCT
       N  V  I  N  G  G  S  H  A  G  N  K  L  A  M  Q  E  F  M  I  F  P  T  G  A
609    TCCACATTCAGCGAGGCCATGCGTATGGGCTCTGAAGTATATCACTACTTGAAGAAAATCATCAAAGAGAAATTT
       S  T  F  S  E  A  M  R  M  G  S  E  V  Y  H  Y  L  K  K  I  I  K  E  K  F
684    GGCCTCGACTCTACTGCTGTTGGTGATGAGGGTGGCTTTGCTCCCAACATTCAAAACAACAAGGATGCTCTCTAT
       G  L  D  S  T  A  V  G  D  E  G  G  F  A  P  N  I  Q  N  N  K  D  A  L  Y
759    CTCATTCAAGATGCTATCCAGCAGGCTGGATACACTGGCAAGATTGAAATTGGCATGGATGTCGCAGCCTCTGAA
       L  I  Q  D  A  I  Q  Q  A  G  Y  T  G  K  I  E  I  G  M  D  V  A  A  S  E
834    TTCTTCAAAGATGGAGTGTACGATCTTGACTTCAAGAACCCCAACTCTAACCCTGCGGATTATCTCTCATCAGAT
       F  F  K  D  G  V  Y  D  L  D  F  K  N  P  N  S  N  P  A  D  Y  L  S  S  D
909    AAACTGACTGAAGTGTACCTAGACTTCATTAAGGACTTCCCAATGGTTTCCATTGAAGATCCCTTCGATCAAGAC
       K  L  T  E  V  Y  L  D  F  I  K  D  F  P  M  V  S  I  E  D  P  F  D  Q  D
984    GATTGGTCTGCTTGGGCTGGACTTACTGGCCGTACTCCTATCCAGATTGTTGGAGATGACCTTACTGTAACAAAC
       D  W  S  A  W  A  G  L  T  G  R  T  P  I  Q  I  V  G  D  D  L  T  V  T  N
1059   CCAAAACGCATCGCCACCGCAGTTGAAAAGAAGGCATGTAACTGCTTGTTGCTGAAAGTTAACCAGATTGGCAGC
       P  K  R  I  A  T  A  V  E  K  K  A  C  N  C  L  L  L  K  V  N  Q  I  G  S
1134   GTTTCTGAATCAATTGATGCTCACTTGCTTGCCAAGCAGAACGGTTGGGGAACCATGGTCTCTCATCGATCTGGA
       V  S  E  S  I  D  A  H  L  L  A  K  Q  N  G  W  G  T  M  V  S  H  R  S  G
1209   GAAACTGAGGATACATTTATCGCCGATTTGGTAGTTGGTCTTTCGACAGGTCAGATTAAGACTGGTGCTCCTTGC
       E  T  E  D  T  F  I  A  D  L  V  V  G  L  S  T  G  Q  I  K  T  G  A  P  C
1284   CGCTCGGAGCGCCTCGCCAAATACAACCAGATTCTTCGAATTGAAGAGGAACTTGGCGCTGCAGCTAAATATGCT
       R  S  E  R  L  A  K  Y  N  Q  I  L  R  I  E  E  E  L  G  A  A  A  K  Y  A
1359   GGCAAGAACTTCCGCAGACCAGTTTAATTTCCATCAATGTATTGAACTTCCTAAAGTTATTTAGGTAGTTCATGC
       G  K  N  F  R  R  P  V  *
1434   GCTATCGTGTTGTGTACTATTGCATTTTAAACAAGAAGCAGAAAAGTACATCACAGATTGATTAACAGTAAAAAT
1509   TTGTATTCGATTTTAGTATTCAAAAAAAATTGCTTCTAGTTTAACTTATGAATATTATTTATGGCTGTAGTGTATA
1584   CAGTGTACGTACATGTGTCGGATGTATGAAATTATTTATTTAAAATTAAGTTTACTTTTAAAAACGATGTTTAAA
1659   TATCAATAAATATGTTATCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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B



**Figure 1  The complete nucleotide and deduced amino acid sequence of *Antheraea pernyi enolase I* gene**   (A) cDNA sequence and deduced amino acid sequence of *enolase I* gene. The amino acid residues are represented by one-letter symbol. The initiation codon ATG is bolded and the termination codon TAA is bolded and marked with an asterisk. The polyadenylation signals AATAAA are double underlined. The gene-specific primer sequences used in the semi-quantitative RT-PCR experiment are underlined. The cDNA sequence was deposited in GenBank under accession no. GU289926. (B) Conserved domains of *A. pernyi* enolase I determined by http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi/.

005493-PA (ABL73887 in GenBank) and BGIBMGA002337-PA, which were also named as *B. mori enolase I* and *II*, respectively. Both of them had EST evidence. In this study, two enolase genes were also isolated from *A. pernyi*. By searching in GenBank and available database, many complete sequences of the *enolase I*

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1     CACAAAATGCCCATTAAGATTCTCTTAGCTCGTCAAATATTTGATGCAACTGGTGTACCTACCATAGAAGTAGAC
         M  P  I  K  I  L  L  A  R  Q  I  F  D  A  T  G  V  P  T  I  E  V  D
76    ATGGTGACAGAACTCGGCTTGTTTCGTATCGGCGTTCCTTCTACGGATAGTAAAAAGCTTGCGGAGGCAGTTCAA
         M  V  T  E  L  G  L  F  R  I  G  V  P  S  T  D  S  K  K  L  A  E  A  V  Q
151   TTGCGCGACAACAAGCCCCGTGAGTTTCACGGCATGGGTGTATCCAGTGCCGTGAAGAACATAAATACTATAATC
         L  R  D  N  K  P  R  E  F  H  G  M  G  V  S  S  A  V  K  N  I  N  T  I  I
226   GCACCAGAACTCATAAAACAGAATCTTGAGGTGACTATGCAAAAAGAAATCGATCAATTTATGATTGCATTGGAT
         A  P  E  L  I  K  Q  N  L  E  V  T  M  Q  K  E  I  D  Q  F  M  I  A  L  D
301   GGCACTGAAAATAGTTCACGGCTTGGAGCTAATGCTATTATGTGCGTATCATTGGTTGTCGCTAAAGCAGGTGCA
         G  T  E  N  S  S  R  L  G  A  N  A  I  M  C  V  S  L  V  V  A  K  A  G  A
376   GCGAAAAAGGGTGTTCCTCTCTATAGGCATATTTCTGATATGGCTGGGGTAACAACAATTATATTACCAGTACCT
         A  K  K  G  V  P  L  Y  R  H  I  S  D  M  A  G  V  T  T  I  I  L  P  V  P
451   CATTTCACTATTCTTACTGGAGGAATTCTCGCTTCTAATGGACTTCCTTTTCAAGAATATACAATAATGCCAACA
         H  F  T  I  L  T  G  G  I  L  A  S  N  G  L  P  F  Q  E  Y  T  I  M  P  T
526   GGAGCGTCAACATTTGTCGAGGCAATGCGTATAGGAACGGAAATATATCGCTACGTAAAAAACGTTATAGCCAAA
         G  A  S  T  F  V  E  A  M  R  I  G  T  E  I  Y  R  Y  V  K  N  V  I  A  K
601   AAATATGGAGTAGAGTGTACTTACGTAAGTGATTCAGGAGGATTTGCTATACCACTGAAGAGTCATAGGGATGCT
         K  Y  G  V  E  C  T  Y  V  S  D  S  G  G  F  A  I  P  L  K  S  H  R  D  A
676   CTTATGTTTCTAACAGATACAATCAAACAGTGCGGATACACAGGAAAAGCTGAAATCGGAATTAACGCGGCTGCT
         L  M  F  L  T  D  T  I  K  Q  C  G  Y  T  G  K  A  E  I  G  I  N  A  A  A
751   ACCGACATGTTCAAAGACGGTGGTTACGACGTAGAATTTAAAAACCCTAACTCCAATCCACAGGACTATATGTCT
         T  D  M  F  K  D  G  G  Y  D  V  E  F  K  N  P  N  S  N  P  Q  D  Y  M  S
826   TCTGATAAACTCGCAGAAGTCTATTTAGACAACGCAAAAGAATTTCCAGTATGCTCGGTTGAGGACGCTTTTGAA
         S  D  K  L  A  E  V  Y  L  D  N  A  K  E  F  P  V  C  S  V  E  D  A  F  E
901   TTCGACGACTGGTCTGCGTGGTCGACGTTGGCTGCACGTACTCAAATTCAATTGGTTGGGAATGATCTCACACAA
         F  D  D  W  S  A  W  S  T  L  A  A  R  T  Q  I  Q  L  V  G  N  D  L  T  Q
976   ACTAACCTCCGAAGAGTGGGATTGGCAGTAGAAAAAAAAGCTGGGAACTCTGTTGCATTACGATTAAATCAAGCG
         T  N  L  R  R  V  G  L  A  V  E  K  K  A  G  N  S  V  A  L  R  L  N  Q  A
1051  AGTACCTTGTCAGAGATCATTGAAGCCTATAAATTATTGAAATCAAATGGTTTTGGGATTGTTCTCAATGATCGA
         S  T  L  S  E  I  I  E  A  Y  K  L  L  K  S  N  G  F  G  I  V  L  N  D  R
1076  TGGGGTGACACAGAAGATTTATTTCTAGCTGATCTCGCTGTTGGTTTGTCTGCTGGTCAAATTAAATGTGGTGGA
         W  G  D  T  E  D  L  F  L  A  D  L  A  V  G  L  S  A  G  Q  I  K  C  G  G
1151  CCCGCGCGATGTGAACGTATTGGGAAGTACAACCAATTAATTCGAATTGAGGAAGAATTGGGAGCGCTCTCGAAG
         P  A  R  C  E  R  I  G  K  Y  N  Q  L  I  R  I  E  E  E  L  G  A  L  S  K
1226  TATGCTGGCAAAAACTACCGAGGTTAAGCGTAAATTTCGAAACATTGAAGGAATGTTGGTAGTTTTATTGGTACC
         Y  A  G  K  N  Y  R  G
1301  GTGTAATATTTTAACACAAATAGACCGATTTGTGTGTTTAAATGTTGGTTCATCGACCATATGTAGAAAGAAGGC
1376  TGCGGAAGATTGTAACGAAATTTTAGGCAACAGATATGTGCTTTTTTATTGCCTAGCAAAACACTTGGGCAACCG
1451  TATATAATATAAAGTGACATATAAAATATAAAAAAAAAAAAAAAAAAAAA
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B



**Figure 2 The nucleotide and deduced amino acid sequence of *Antheraea pernyi* enolase II gene** (A) cDNA sequence and deduced amino acid sequence of *enolase II* gene. The amino acid residues are represented by one-letter symbol. The initiation codon ATG is bolded and the termination codon TAA is bolded and marked with an asterisk. The gene-specific primer sequences used in the semi-quantitative RT-PCR experiment are underlined. The cDNA sequence was deposited in GenBank under accession no. HM755879. (B) Conserved domains of *A. pernyi* enolase II.

genes have been published, while only two complete sequences of the *enolase II* genes from *A. pernyi* and *B. mori* are available till now. The amino acid sequences of the four enolase proteins from *A. pernyi* and *B. mori* were aligned and compared (**Fig. 3**). *Antheraea pernyi* enolase I shows 93% identity to *B. mori* enolase I (ABL73887), and

*A. pernyi* enolase II shows 84% identity to *B. mori* enolase II (BGIBMGA002337-PA in SilkDB). However, *A. pernyi* enolase I exhibits only 60% identity to *A. pernyi* enolase II and *B. mori* enolase I exhibits only 59% identity to *B. mori* enolase II. This result showed that *A. pernyi enolase II* gene was related to *B. mori enolase II* gene,
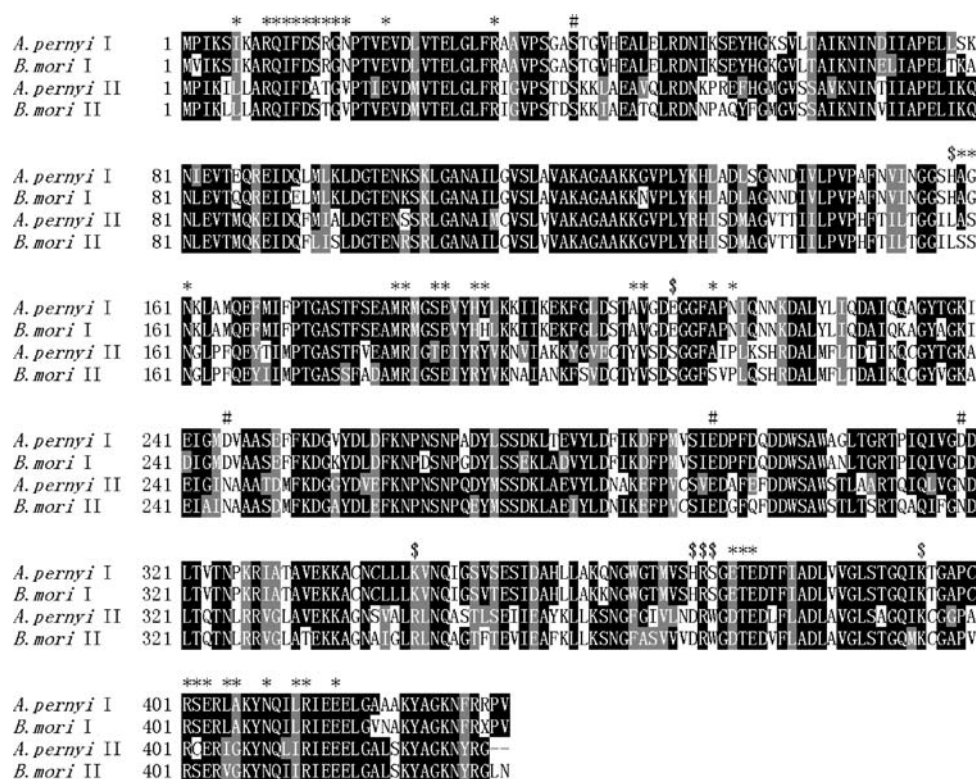
**Figure 3 Sequence alignment of enolase from *Antheraea pernyi* and *Bombyx mori*** *A. pernyi* enolase I shows 93% identity to *B. mori* enolase I (ABL73887), and *A. pernyi* enolase II shows 84% identity to *B. mori* enolase II (BGIBMGA002337-PA in SilkDB). However, *A. pernyi* enolase I exhibits only 60% identity to *A. pernyi* enolase II, and *B. mori* enolase I exhibits only 59% identity to *B. mori* enolase II. The alignment was generated using Clustal X, together with the Boxshade server (http://www.ch.embnet.org/software/BOX_form.html). The sign hash shows the position of the metal-binding site, sign dollar shows the position of the substrate-binding pocket, and sign asterisk shows the position of the dimer interface. Identical amino acids are highlighted in black and positive amino acids are highlighted in gray.

rather than *A. pernyi enolase I* gene, suggesting that the *enolase II* genes from *A. pernyi* and *B. mori* may be new members of the enolase superfamily.

In addition to the enolase I sequences from *A. pernyi* and *B. mori*, other 28 representative enolase sequences from different organisms were used to calculate the sequence identity. All these enolase sequences were from fungi (3), plant (2), vertebrate (3), and invertebrate (22) (**Fig. 4** and data not shown; see **Fig. 5** for details). The putative *Antheraea polyphemus* enolase (ABW39163) was included although it was a partial sequence [26]. The putative complete amino acid sequence of the enolase gene from *Spodoptera frugiperda* not available at GenBank was downloaded from ButterflyBase, an open-access Genomic Database for Lepidoptera [27]. The enolase sequences from other organisms were retrieved from GenBank or EMBL. Protein sequence alignment revealed that *A. pernyi* enolase I had the highest identity (97%) to that of *A. polyphemus*, and the lowest identity (60%) to those of *Schizosaccharomyces pombe* [28] and *Magnaporthe grisea* [29]. *Antheraea pernyi* enolase I also revealed 93% sequence identity to the enolase of *S. frugiperda*, 75%–82% identity to other invertebrates, 70%–72% identity to

vertebrates, 66% identity to plants, and 60%–64% identity to fungi. More than 60% amino acid sequence identity among these enolase proteins suggests that the enolases are highly conserved throughout evolution [3]. In **Fig. 4**, the sequence is shown aligned with various enolases from *A. polyphemus*, *B. mori*, *Drosophila melanogaster* [30], *Homo sapiens* [12], *Arabidopsis thaliana* [31], and *Saccharomyces cerevisiae* [32]. Since lepidopteran enolases including *A. pernyi* enolase I share a low degree of similarity with those of other invertebrates including insects, we consider them to be new members of the insect enolase family.

### Phylogenetic analysis
A total of 30 representative enolase protein sequences from different organisms including *A. pernyi* and *B. mori* were used to reconstruct the phylogenetic relationship. Archaebacteria enolase protein sequences from *Archaeoglobus fulgidus* (AAB90112), *Haloarcula marismortui* (P29201) and *Pyrococcus abyssi* (CAB49458) were used as outgroups. It has been reported that the three representative archaebacterial enolase sequences were not closely related to the majority of enolase sequences [3].
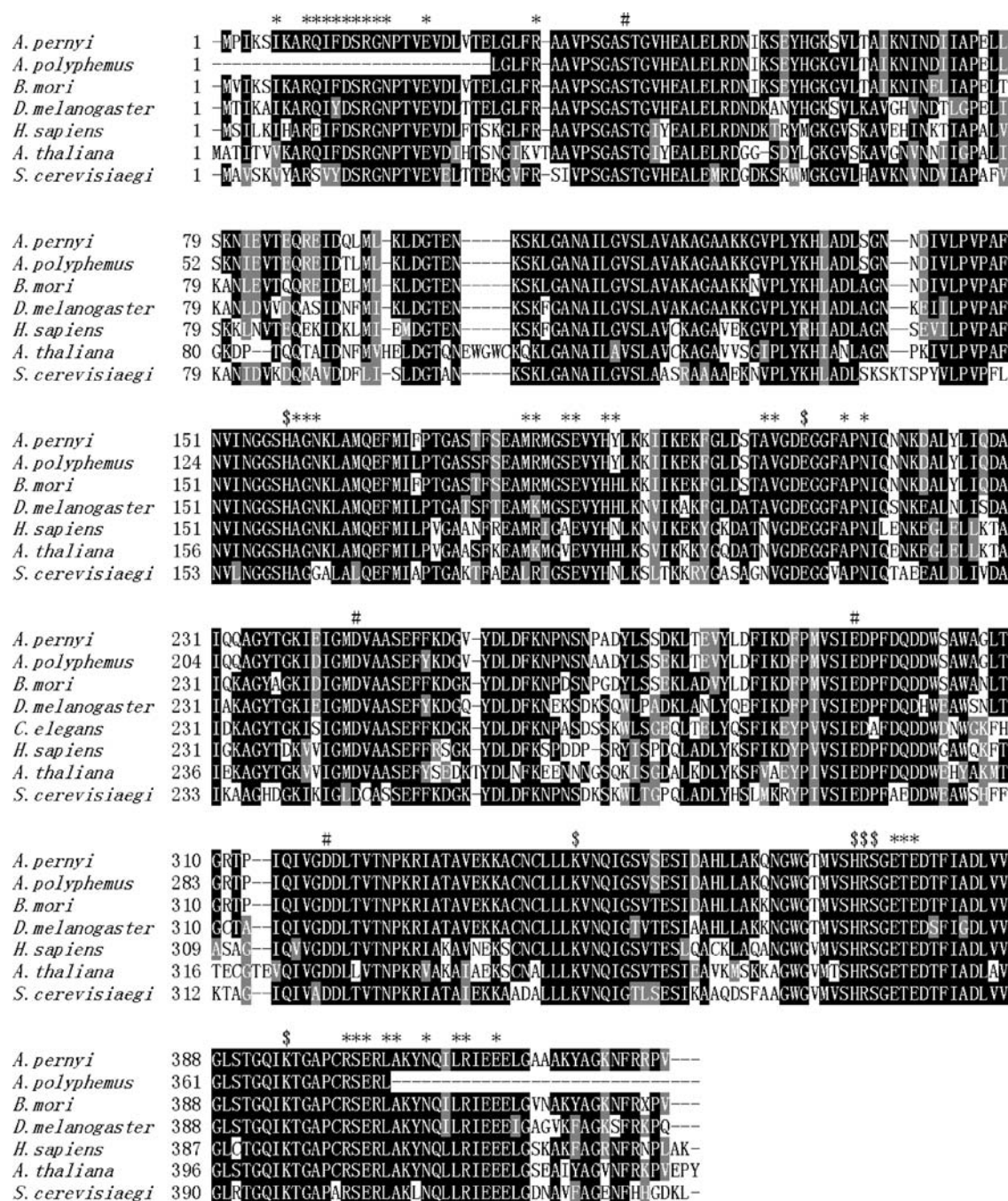
**Figure 4 Sequence alignment of enolase I proteins from *Antheraea pernyi* and other organisms** *A. pernyi* enolase I shows 97% identity to the enolase of *Antheraea polyphemus* (ABW39163), 93% to *Bombyx mori* (ABL73887), 80% to *Drosophila melanogaster* (ABH06829), 72% to *Homo sapiens* (NP_001419), 66% to *Arabidopsis thaliana* (P25696) and 64% to *Saccharomyces cerevisiae* (NP_011770). The sign hash (#) shows the position of the metal-binding site, sign dollar ($) shows the position of the substrate-binding pocket and sign asterisk (*) shows the position of the dimer interface. The alignment was generated using Clustal X, together with the Boxshade server.

The final alignment resulted in 463 amino acid sites for the 33 enolase sequences including gaps. Of these sites, 42 were conserved, 410 were variable, and 323 were informative for parsimony. A neighbor-joining tree was constructed using amino acid sequences and a poisson-corrected distance (Fig. 5). It was well defined in the phylogenetic tree with 100% bootstrap confidence that enolase II sequences from *A. pernyi* and *B. mori* was not closely related to the

majority of enolase sequences. This result further suggested that enolase II from *A. pernyi* and *B. mori* was a new member of the enolase superfamily. The cluster-containing enolase II sequences from *A. pernyi* and *B. mori* is designated here as 'enolase II'.

The cluster with 75% bootstrap confidence containing the majority of enolase sequences from fungi (3), plant (2), vertebrate (3), and invertebrate (20) including *A. pernyi* and
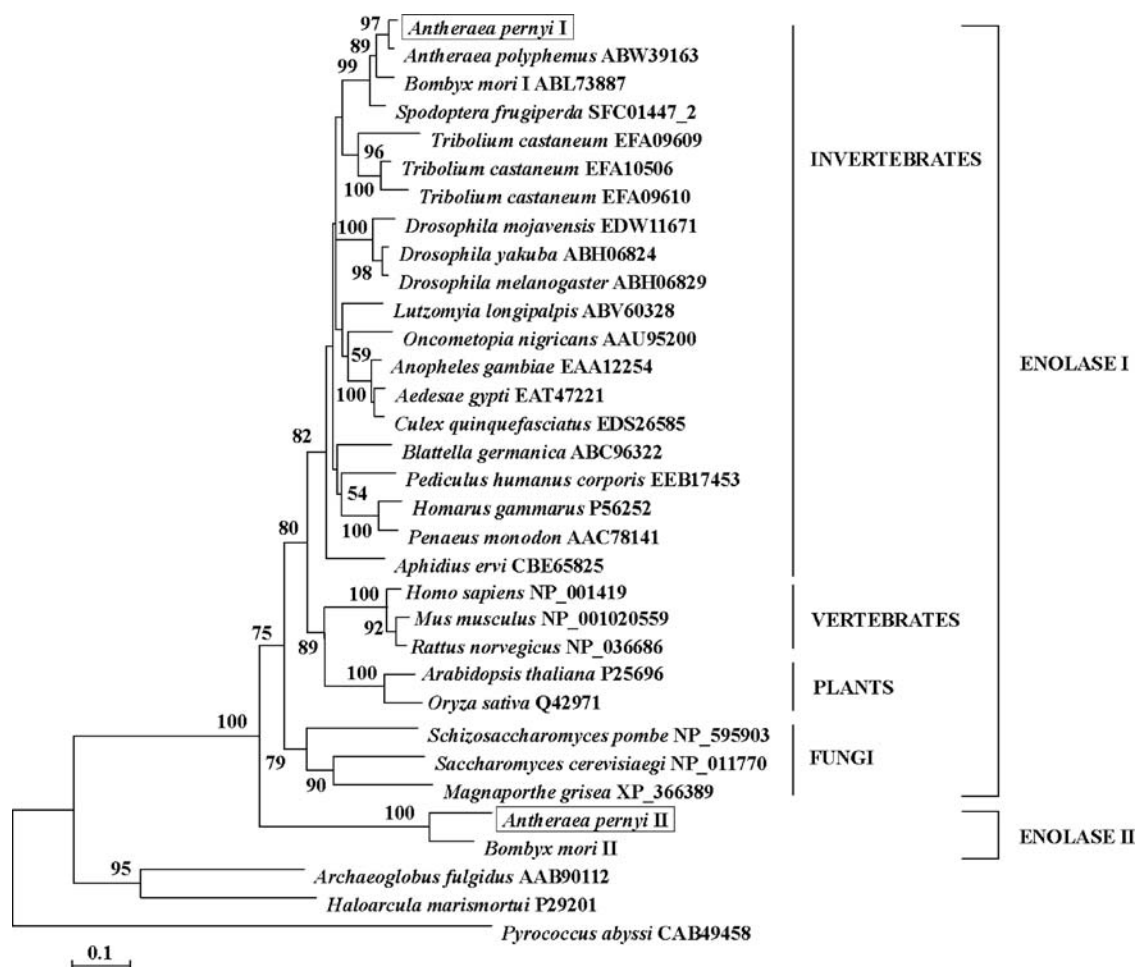
**Figure 5  Phylogenetic tree based on the amino acid sequence comparisons of enolases from various organisms including *Antheraea pernyi***   The topology was tested using bootstrap analyses (1000 replicates). Numbers at nodes are bootstrap *P*-values (>50%). Public database accession numbers of enolase proteins are shown following the names of organisms. The neighbor-joining tree was constructed by MEGA version 4 based on a poisson-corrected distance.

*B. mori* enolase I sequences is designated as 'enolase I'. In the phylogenetic tree of enolase I, the used enolase sequences were well divided into four groups corresponding to bacteria, plant, vertebrate, and invertebrate groups, which were consistent with the previously reported division [3]. Within the invertebrate group, four lepidopteran enolase sequences from *A. pernyi*, *A. polyphemus*, *S. frugiperda* and *B. mori*, were clustered into a monophyletic subgroup, which further supported that lepidopteran enolase I might be a new member of the insect enolase family. Similar results were obtained with the maximum parsimony method.

### Expression patterns of *A. pernyi enolase I* at different developmental stages and in various tissues
We employed the semi-quantitative RT-PCR to detect and quantify the expression levels of *A. pernyi* enolase genes during different developmental stages and tissue distributions in the fifth instar larvae. We used a constitutively expressed actin gene (GU073316) as the internal control [22]. The negative control exhibited no products (data not

shown). By sequencing, we confirmed that the positive RT-PCR products were amplified from the enolase gene sequence. The *enolase I* gene of *A. pernyi* was expressed during four developmental stages including egg, larva, pupa and adult [**Fig. 6**(A)].

*Antheraea pernyi enolase I* mRNA was found to be present in all tissues tested for the fifth instar larvae, including blood, midgut, silk glands, Malpighian tubules, spermaries, ovaries, brain, muscle, fat body and body wall; however, *A. pernyi enolase II* mRNA was expressed specifically in the spermaries and ovaries [**Fig. 6**(B)].

### Expression levels of *A. pernyi enolase I* under temperature stress
In order to examine the influence of temperature stress on *enolase I* gene expression, total RNA extracted from fat body of *A. pernyi* pupae was used as template to detect the levels of *enolase I* transcript after exposure to low- and high-temperature treatment. By semi-quantitative RT-PCR analysis, we found that the mRNA expression level of
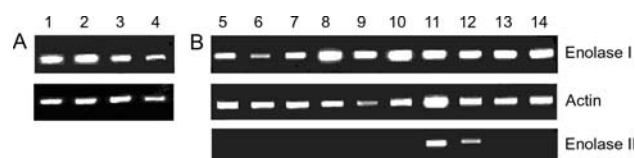
**Figure 6 Expression patterns of two *Antheraea pernyi* enolase genes** (A) Expressions in the four developmental stages: 1–4 represent egg, larva, pupa and moth, respectively. (B) Expressions in various tissues of the fifth instar larvae; 5–14 represent blood, fat body, midgut, silk glands, body wall, Malpighian tubules, spermaries, ovaries, brain and muscle, respectively. Expression patterns were analyzed by RT-PCR using gene-specific primer pair for *A. pernyi enolase I* or *II* gene. The *actin* gene was used as an internal standard.

*A. pernyi enolase I* gene was significantly down-regulated after cold shock (4°C for 3 h) and significantly up-regulated after heat shock (46°C for 3 h), compared with the control (26°C for 3 h) [**Fig. 7** (**A, B**)].

## Discussion

In the past years, more attention has been paid to the biology of insect enolase. Of the complete insect enolase sequences available to date, only a few belong to the lepidopteran including *B. mori* and *S. frugiperda*, although Lepidoptera is the second largest insect order and includes both harmful and beneficial agricultural insects. In this study, we for the first time isolated and characterized two enolase genes from *A. pernyi*, which were designated as *A. pernyi enolase I* and *II*, respectively. Sequence analysis indicated that *A. pernyi* enolase I protein showed more than 75% identity to the enolases of different invertebrates, and that *A. pernyi* enolase II showed 84% identity to *B. mori* enolase II. Bioinformatics analyses revealed that the amino acid sequences of the two genes shared several conserved features/sites of enolase, such as the metal-binding sites, the substrate-binding pocket, and the dimer interface. These results suggested that the two genes isolated from *A. pernyi* were the enolase genes.

The crucial amino acid residues for enolase activity have been defined in *S. cerevisiae* as Glu 211, Lys 345, and His 373 [33]. These three amino acid residues are part of conserved regions and His 373 is present within an enolase 'fingerprint' motif 371 [SHRSGETED]379 [34], which is highly conserved in all known enolases. The corresponding amino acid residues in *A. pernyi* enolase I are Glu 210, Lys 344, and His 372, as found in other known enolases, which suggests that the product of this gene was functional. However, the corresponding amino acid residues in *A. pernyi* enolase II were Ser 210, Arg 344, and Asp 372 (**Fig. 4**). The alteration of these three crucial amino acid residues was also observed in *B. mori* enolase II. Does this finding imply that enolase II is not a functional enolase?
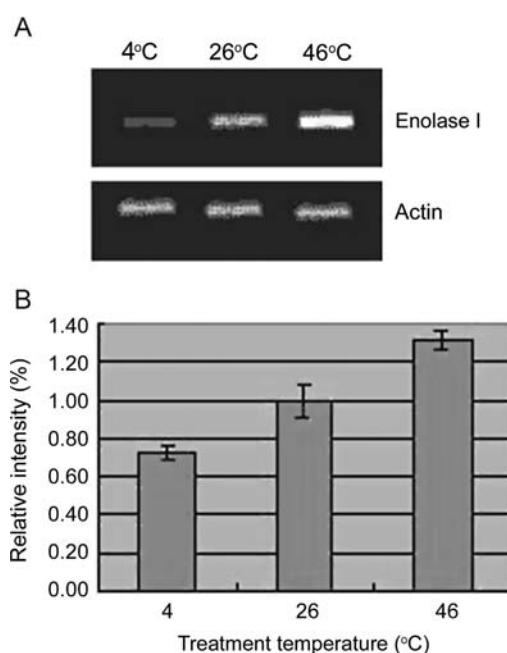


**Figure 7 Semi-quantitative expression analysis of *Antheraea pernyi enolase I* in response to temperature stress, including heat shock (46°C), control (26°C) and cold shock (4°C)** (A) The electrophoretic results. (B) The relative intensity. The *actin* gene was used as control.

To address this question, more studies including enzymatic studies of the expressed gene product need to be performed in the future.

Until now, only two complete sequences of *enolase II* genes from *A. pernyi* and *B. mori* characterized in this study are available. By searching the EST resources at the WildSilkbase, a BLAST searchable catalog of ESTs generated from three wild silkmoths, *A. assama*, *S. cynthia ricini* and *A. mylitta* [35], the putative *enolase II* gene sequences were also found from *A. assama* (accession nos Aats3556 and Aaov1664) and *S. cynthia ricini* (accession no. Scfb_Ec6565). Sequence alignment revealed that the protein sequence of *A. pernyi enolase II* gene shows 96% and 93% identities to those of *A. assama* and *S. cynthia ricini enolase II* genes, respectively (data not shown). This finding suggests that *enolase II* gene will be isolated from a wide variety of organisms, especially from silkworm species.

Amino acid sequence alignment of the four enolases from *A. pernyi* and *B. mori* revealed that the identity degrees between enolase I (93%) or II (84%) from different species were obviously larger than those between enolase I and II (about 60%) from the same species. Phylogenetic analysis also revealed a well-defined tree with 100% bootstrap confidence in which enolase II sequences from *A. pernyi* and *B. mori* was clearly separated from the majority of enolase sequences from fungi, plant, vertebrate, and invertebrate including *A. pernyi* and *B. mori* enolase I sequences. These results suggested that

enolase II from *A. pernyi* and *B. mori* may be a new member of the enolase superfamily.

Horizontal gene transfer can explain many of the complex patterns seen in gene and gene–family relationships involving the three domains of life [36,37]. However, another explanation that should be considered in some cases is a simple lack of phylogenetic resolution caused by the inescapable limitation of protein length [3]. The fact that both genes (*enolase I* and *enolase II*) have been sequenced from the same species (*A. pernyi* and *B. mori*) suggests that gene duplication, rather than horizontal transfer, is involved, although the latter cannot be ruled out [3]. This finding here supports the interpretation that an early gene duplication event, prior to the last common ancestor of higher organisms, leads to *enolase I* and *enolase II*. If this interpretation is correct, then *enolase II* was subsequently lost in the majority of higher organisms.

*Bombyx mori* is a major lepidopteran insect model for research. Large-scale EST resource and extensive microarray information for *B. mori* are available at GenBank and SilkDB. The *in silico* gene expression analysis based on the available EST resources showed that *B. mori enolase I* gene is expressed during four developmental stages, and is also expressed in eye, Verson's gland, imaginal disks, maxilla, and pheromone glands not analyzed in this study. Expression profiles based on the genome-wide microarray data at the SilkDB indicated that *B. mori enolase I* gene is widely expressed in the tissues including blood, head, Malpighian tubules, spermaries, ovaries, fat body, body wall, and silk glands; however, *enolase II* gene is expressed specifically in the spermaries and ovaries. In this study, semi-quantitative RT-PCR analysis revealed that *A. pernyi enolase I* gene was expressed during four developmental stages (egg, larva, pupa, and moth) and in all tissues tested (blood, midgut, silk glands, Malpighian tubules, spermaries, ovaries, brain, muscle, fat body and body wall), and that the *enolase II* gene was expressed specifically in the spermaries and ovaries. These results agreed well with the expression patterns of *B. mori enolase I* and *II* genes based on the EST resources and the genome-wide microarray data mentioned above.

In this study, we found that the *enolase II* mRNA from *A. pernyi* and *B. mori* were expressed specifically in the spermaries and ovaries. Spermary is an organ or a gland in which male gametes are formed, especially in invertebrates including *A. pernyi*. Ovaries are the female reproductive system of invertebrates that employ sexual reproduction, including *A. pernyi*. The result suggests that the *enolase II* genes from *A. pernyi* and *B. mori* are related to their reproduction.

It has been reported that yeast enolase was identical to HSP48 and was involved in thermal tolerance [8]. In ice plant *Mesembryanthemum crystallinum*, the enolase gene

transcripts increased in abundance in response to low and high temperature; however, no increase in enolase levels was observed [38]. Recently, it has been observed that, in alga *Dunaliella salina*, there was no significant change under low temperature treatment, but a gradual increase was observed under heat stress in the enolase protein level [10]. The preliminary results presented in this study by semi-quantitative RT-PCR analysis indicated that *A. pernyi enolase I* gene was cold- and heat-shock inducible. Meanwhile, some researchers put forward an opinion that the enolase genes appeared to be temperature-responsive to some extent, rather than inducible. To address this question, further investigations should be performed by increasing the sampling points with northern blot analyses of transcript abundance or quantitative RT-PCR.

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