In this study, two enolase genes were isolated and characterized from the Chinese oak silkworm, *Antheraea pernyi*, 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 spermataries 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
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 silkworm, 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 performed 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 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.

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 OD260/OD280 with an ultraviolet 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 MgCl2, 0.25 mM dNTP, 1× buffer, and 2.5 units of Taq DNA polymerase (TIANGEN Biotech). PCRs were performed with the following cycles: initial denaturation at 95°C for 5 min; followed by 35 cycles of 1 min at 95°C, 30 s annealing at 55°C, 30–90 s extension at 72°C; and a final extension at 72°C for 10 min.

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

A full-length *A. pernyi* pupal cDNA library was constructed 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’-CACAATATATGGTATGTATTCTT-3’)(N = A/G/C/T) and LYQ171 (5’-AGATGGTGAAATGAAGTAC3’), 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. BGIBMGA002377-TA). Spermary cDNA sample was used as the template for RT-PCR amplification. The amplification products were analyzed on 1.0% agarose gels stained with etidium bromide. The RT-PCR products were purified from the gel and sequenced.

Based on the sequence obtained, the 3’ rapid amplification 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’-ATCTGGTCGACTGATTAC-3’). Using primer LYQ187 (5’-ACGGACAGTAAGAAGCTTGCG-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 LQY85 (5’-CCACA GGCCA ACAGA GAGAA GA-3’) and LQY86 (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 LQY116 (5’-TGCTG TTGGT GATGA GGGTG-3’) and LQY117 (5’-CGATG CGTTT TGGGT TTGTT-3’) for *A. pernyi enolase I* gene, which generated a 374 bp fragment, and LQY187 (5’-ACGGA CAGTA AAAAG CTTGC-3’) and LQY171 (5’-AGAAT GTGTA AATGA GTTAC-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
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*
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.
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* (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. Archaeabacteria enolase protein sequences from *Archaeoglobus fulgidus* (AAB90112), *Haloarcula marismortui* (P29201) and *Pyrococcus abyssi* (CA49458) were used as outgroups. It has been reported that the three representative archaeabacterial enolase sequences were not closely related to the majority of enolase sequences [3].
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 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

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**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_001414), 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.
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
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?

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 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, Veson’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.

**Funding**

This work was supported by the grants from the National Basic Research Program of China (2005CB121000), the National Natural Science Foundation of China (31072082), the National Modern Agriculture Industry Technology System Construction Project (Silkworm and Mulberry), and the Scientific Research Project for High School of the Educational Department of Liaoning Province (2008643).

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