

# Cloning and Expression Analysis of the Heat-Shock Protein 70 Gene in *Eogystia hippophaecolus* (Lepidoptera: Cossidae)

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## Abstract

*Eogystia hippophaecolus* is a major borer pest of sea buckthorn in China, damaging the environment and sea buckthorn industry. It is widely distributed in the Three-North region of China, and its larvae are resistant to low temperatures. Because heat-shock protein 70 can repair misfolded proteins under low temperatures, thus preventing cell damage and improving tolerance, we investigated the adaptation of *E. hippophaecolus* HSP70. We screened the transcriptome of *E. hippophaecolus* for HSP70 homologs, identified a full-length gene, and cloned to obtain the open reading frame, which was 1896 bp in length and encoded 631 amino acids, with a molecular weight of 69.7 kDa. The amino acid sequence contained three signature sequences of the HSP70 family and a C-terminal cytoplasmic character sequence. A phylogenetic tree demonstrated that *EhHSP70* has high homology with HSP70 genes from other insect species. We measure expression of the HSP70 gene across tissues when larvae of *E. hippophaecolus* were cold shocked. We also measured expression during cold shock recovery. *EhHSP70* expression varied across tissues at 25 °C, with the highest expression in the midgut. Compared with the control, the expression of *EhHSP70* in the cuticle was fourfold higher after a cold shock of –5 °C for 1 h. *EhHSP70* expression was also significantly increased during the 1-h period following recovery from cold shock, then decreased. In summary, cold shock promoted the expression of *EhHSP70*, which may play an important role in the adaptation mechanism of cold tolerance in *E. hippophaecolus* larvae.

**Key words:** *Eogystia hippophaecolus*, heat-shock protein 70, expression pattern, cold shock

The sea buckthorn (*Hippophae rhamnoides* L.) is a nitrogen-fixing dioecious deciduous shrub. It is found in northwestern Europe and in Asia, within northwestern China, the Altai Mountains, and the northern Himalayan Mountains (Rousi 1971, Mao et al. 2010). Sea buckthorn has an adaptable, well-developed root system, rapid growth, and resistance to drought, wind-blown sand, and saline soil. It can improve the environment because it plays important roles in water conservation, climate regulation, and soil and water conservation (Wang 1994). In addition, it is a valuable medicinal and edible plant resource (Shang et al. 2013).

*Eogystia hippophaecolus* (Hua et al. 1990) (Lepidoptera: Cossidae) is a moth and the major wood borer damaging sea buckthorn in China, and its population size has increased since 2001. They are currently distributed across the major sea buckthorn cultivation areas in the Inner Mongolia, Shaanxi, Shanxi, Liaoning, Gansu, Qinghai, and Ningxia provinces. They have caused great damage to the environment and the sea buckthorn industry in China (Luo et al. 2003, Zong et al. 2010). They mainly bore into sea buckthorn trunks

and roots, hollowing those structures and leading to the death of whole plants (Zong et al. 2005). According to an overwintering survey, the mature larvae do not die during winter. In fact, the mortality of early-instar larvae was <50%, and second- to third-instar larvae had a rate of only 10% (Zong et al. 2006). That leads to a large number of larvae that successfully overwinter, resulting in a large increase in population size in late spring, despite the fact that the larvae experience low temperatures during spring and winter. This survival is due to the long-term evolution of low-temperature adaptation strategies (Han et al. 1989), for example, by regulating the expression of heat-shock protein (HSP), which helps the insect resist the adverse effects of low temperatures (Matz et al. 1996, Sørensen et al. 2003). Many genes and their encoded protein products associate with tolerance to low temperatures in insects, including antifreeze proteins (Devries 1971), ice-nucleating protein (Maki et al. 1974), and the DCA (Goto 2000) and FST proteins (Goto 2001).

Heat-shock proteins, which are produced under high temperature and other environmental stresses, also play a crucial role in cold

tolerance (Ritossa 1964, Jing and Kang 2002). Heat-shock proteins can be divided into six categories according to their relative molecular weight, structure, and function: HSP100, HSP90, HSP70, HSP60, HSP40, and small molecular weight proteins (sHSPs, molecular weight between 12–43 kDa; Kim et al. 1998, Feder and Hofmann 1999, Sørensen et al. 2003). Among these categories, HSP70 is the most widely studied, and it is also one of the most highly conserved protein families (Lindquist 1986). Functional counterparts of members of the HSP70 family have been identified in an extremely wide range of animals and plants, ranging from primitive bacteria to humans. More than eight genes coding for HSP70 family members have so far been identified. The two major cytoplasmic isoforms are HSC70 and HSP70 (Massey et al. 2010). Initially, HSP70 was considered to be only induced by heat shock, namely, a brief exposure to a nonlethal temperature higher than that necessary for best growth and development (Tissières et al. 1974). Later, it was found that a variety of environmental stresses caused a similar up-regulation of HSP expression (Korsloot et al. 2004). In addition, HSP70 repairs misfolded proteins under low temperatures, increases cell viability, prevents cell damage, and enhances tolerance. It also acts as a chaperone, stabilizing and protecting other proteins (Moseley 1997, Rani et al. 2013). HSP70 genes are central components of the cellular network of molecular chaperones and folding catalysts. They assist in a large variety of protein-folding processes in the cell via the ability of their substrate-binding domains to associate with short hydrophobic peptide segments within substrate proteins (Mayer and Bukau 2005).

Because HSP70 genes are important in the cold tolerance of insects, we screened the *E. hippophaecolus* transcriptome obtained previously for HSP70 homologs. We obtained an open reading frame (ORF) of HSP70 using cloning. In addition, we studied its expression across the different tissues of *E. hippophaecolus* in response to cold shock and its expression during cold shock recovery using qRT-PCR. The results of this study lay the foundation for further analysis of the molecular mechanisms of cold resistance in *E. hippophaecolus*, provide a theoretical basis for the formulation of a better comprehensive control strategy, and provide a valuable reference for improving cold tolerance through transgenic technology.

## Materials and Methods

### Experimental Insects

The larvae of *E. hippophaecolus* were collected from a sea buckthorn forest in Jianping, Liaoning Province, during late October 2016. The larvae were stored in a bucket and kept in fresh sea buckthorn root. The soil of the sea buckthorn root was kept in the bottom of the bucket to retain humidity. The top of the bucket was sealed with a stainless steel net to prevent the larvae from escaping. The larvae were placed in a 25 °C incubator until further experiments.

### Extraction of Total RNA and Synthesis of First-Strand cDNA

Total RNA was extracted from one larva using TRIzol reagent (number 15596018; Invitrogen) and the RNeasy Plus Mini Kit (number 74134; Qiagen, Hilden, Germany), and then stored at –80 °C. The concentration, purity, and quantity of the RNA were assessed using agarose gel electrophoresis and the NanoDrop 8000 (Thermo Scientific, Waltham, MA). First-strand cDNA synthesis was performed using the PrimeScript RT Reagent Kit (number RR047Q; TaKaRa, Dalian, China). The reverse transcription was performed in 20 µl of reaction mix using 1 µg of RNA template, 2 µl

of 5× gDNA Eraser Buffer, 1 µl of gDNA Eraser, 4 µl of 5× PrimeScript Buffer 2, 1 µl of PrimeScript RT Enzyme Mix I, 1 µl of RT Primer Mix, and RNase-free dH<sub>2</sub>O to 20 µl. The cycling condition was 15 min at 37 °C, and then 5 s at 85 °C. The product was used immediately or stored at –20 °C.

### Primer Design

The UniGene sequence obtained from the larval transcriptome data established previously in our laboratory was screened for HSP70 protein homologs. Then, the ORF region was predicted using ORF finder (<https://www.ncbi.nlm.nih.gov/orffinder>). The ORF had a length of 1896 bp and was considered to be full length because the lengths of insect HSP70 genes in the NCBI database are between 1872 and 2004 bp. The complete ORF sequence was named *EhHSP70*. Primers were designed to this full-length sequence using Primer Premier 5.0. The sequences of all primers used in this study are summarized in Table 1.

### Cloning of the Target Gene

The ORF of *EhHSP70* was cloned using its cDNA as a template for PCR. The PCR amplifications were performed in 25 µl of reaction mix using 2 µl of cDNA template, 12.5 µl of 2× PrimeSTAR Max Premix, 0.75 µl each of the forward and reverse primers, and 9 µl of ddH<sub>2</sub>O. It was cycled for 10 s at 98 °C, followed by 35 cycles of 10 s at 55 °C, 10 s at 72 °C, and 1 min at 72 °C.

The amplified PCR products were run on a 1.2% agarose gel to verify the presence of the product. Then, the purified PCR products were inserted into a pEASY-Blunt Simple Cloning Vector (number CB111-01; TransGen Biotech, Beijing, China). The ligated product was transformed into Trans1-T1 Phage Resistant Chemically Competent Cells (number CD501-01, TransGen Biotech). Then, the positive colonies were identified using blue and white selection and sequenced at RuiBiotech Co., Ltd. After alignment, the ORF sequence was obtained.

### Bioinformatics and Sequence Homology Analysis

The amino acid sequence of *EhHSP70* was translated using DNAMAN, and the physical and chemical properties were analyzed using ProtParam (<http://web.expasy.org/protparam/>). The amino acid sequence was analyzed for hydrophilicity, hydrophobicity, and transmembrane structures using ProtScale (<http://web.expasy.org/protscale/>). The subcellular localization was predicted using PSORT (<http://psort.hgc.jp/form.html/>). The presence of signal peptides was predicted using SignalP 4.1 (<http://www.cbs.dtu.dk/services/SignalP/>). The domains of the protein were predicted and analyzed using ScanProsite and SMART (<http://prosite.expasy.org/scanprosite/>; <http://smart.embl-heidelberg.de/smart/>). The sequence of the full-length cDNA of *EhHSP70* was used to search for homologs in GenBank using BLAST, available at the NCBI website (<http://www.ncbi.nlm.nih.gov/>). The Molecular Evolutionary Genetic Algorithm (MEGA7) was used to analyze phylogenetic relationships. A neighbor-joining (NJ) tree was constructed, and the reliability of the tree was tested using bootstrap analysis with 1,000 replications.

### Expression of HSP70 Across Tissues After Cold Shock

13th to 16th instar larvae were selected, washed with DEPC H<sub>2</sub>O, placed in culture dishes, cold shocked at –5 °C for 1 h, and recovered at 25 °C for 1 h. Four tissues (hemolymph, fat body, midgut, and cuticle) of the *E. hippophaecolus* larvae were obtained by dissection under an anatomical lens and frozen with liquid nitrogen immediately. The same instar of normal-feeding larvae at 25 °C was used as a control, and the experiments were repeated at least three

**Table 1.** Primer sequences used for full-length ORF cloning of *E. hippophaecolus* HSP70 and real-time quantitative PCR

Primer name	Primer sequence (5'-3')	Use of primers
EhHSP70-F1	ATGCCAGCTGTTGGTATCGATCTTG	Amplification of ORF
EhHSP70-R1	TTAGTCGACTTCCTCTACGGTGGTC	Amplification of ORF
EhHSP70-F2	TGTCAATCAACCCCTGACGAA	Amplification of target gene in qRT-PCR
EhHSP70-R2	AGCAGCACGTCTGGATTCT	Amplification of target gene in qRT-PCR
actin-F	CGACTTCGAACAGGAGATGG	Amplification of actin in RT-qPCR
actin-R	TCGTCTCATGAATGCCACAG	Amplification of actin in RT-qPCR

times. RNA was extracted according to method described in “Extraction of total RNA and synthesis of first-strand cDNA” and reverse transcribed into cDNA after passing quality inspection.

Four actin genes were identified and selected from the *E. hippophaecolus* antennal transcriptome and evaluated using Normfinder and GeNorm. The actin gene with the lowest M value (GeNorm) and stability value (Normfinder) was selected as the reference gene for qRT-PCR (Hu et al. 2016). The fluorescent chimeric dye SYBR Green I was used for qRT-PCR. The PCR reaction was carried out using the Bio-Rad CFX96 PCR System (Hercules, CA) and conducted in 12.5 µl of reaction mix using 6.25 µl of SYBR Premix Ex Taq II, 0.5 µl of each primer (10 mM), 1 µl of cDNA template, and 4.25 µl of dH<sub>2</sub>O. The qRT-PCR cycling parameters were 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. After this, melting curves were generated. The Ct value was recorded after the reaction was completed. The qRT-PCR reaction was performed using cDNA at different concentrations. HSP70 and actin were found to have the highest amplification efficiency using 100× diluted cDNA as a template. To assess reproducibility, each qRT-PCR reaction for each tissue was performed in three biological replicates and four technical replicates. The relative fold change in mRNA expression was determined by the change in ΔΔC<sub>t</sub>. Bio-Rad CFX Manager (version 3.1.1517.0823) was used to normalize expression based on the 2<sup>-ΔΔC<sub>t</sub></sup> method (Livak and Schmittgen 2001).

### 2.7 Expression of HSP70 During Cold Shock Recovery

9th to 12th instar larvae of *E. hippophaecolus* were placed in culture dishes and cold shocked at 0 °C for 10 h, and then quickly transferred into an incubator, where they were allowed to recover at 25 °C for 0.5, 1, 2, 4, and 8 h before freezing in liquid nitrogen. The same instar of normal-feeding larvae at 25 °C was used as a control, and the experiments were repeated at least three times. RNA was extracted using method “Extraction of total RNA and synthesis of first-strand cDNA,” reverse transcribed into cDNA after passing quality inspection, and stored at -20 °C for expression analysis. The qRT-PCR reaction was the same as in “Expression of HSP70 across tissues after cold shock.”

### Statistical Analysis

The differences in relative expression across tissues between -5 °C and 25 °C were compared using an independent-samples t-test. The relative expression at different recovery time points after cold shock was analyzed using one-way analysis of variance (ANOVA), and differences in relative expression were compared using Dunnett's multiple comparison. All data were analyzed using GraphPad 7.0.

## Results

### Cloning and Characterization of *EhHSP70*

The nucleotide sequence of the cloned *EhHSP70* gene (GenBank KY930331) in Fig. 1 was confirmed by sequencing, and it was also confirmed to belong to the HSP70 gene family using BLAST.

The ORF of *EhHSP70* contained 1896 bp, encoding 631 amino acids with a calculated mass of about 69.7 kDa and a theoretical isoelectric point (pI) of 5.47. The instability index was 37.50, which classifies this protein as stable, and its grand average of hydropathicity (GRAVY) was -0.484, making it a hydrophilic protein (Fig. 2). In addition, *EhHSP70* did not have a signal peptide. The heat shock domain is encoded by nucleotides 4–609 in the sequence.

Three highly conserved segments defining the HSP70 family in eukaryotes, IDLGTTYS, IFDLGGGTFDVSIL, and VVLVGGGSTRIPKVQS, were located at positions 6–13, 194–207, and 332–346, respectively. Two glycosylation sites, NLSI and NVSA, were located at positions 358–361 and 485–488, respectively. The nonorganellar consensus motif RARFEEL was at 297–303. The GGMP tetrapeptide and the cytoplasmic HSP70 carboxyl terminal region, EEVD, occupied the C-terminal. Together, this indicates that *EhHSP70* is a member of the cytoplasmic HSP family.

The deduced amino acid sequence of *EhHSP70* had a high degree of similarity with the HSP70 proteins of other insects, as assessed using ClustalX multiple sequence alignment and amino acid BLAST to seven other eukaryotic insects (Fig. 3). *EhHSP70* had the highest identity (92%) with HSP70s from *Antheraea pernyi* (ADI50267.1), *Antheraea yamamai* (BAD18974.1), and *Bombyx mori* (BAF69068.1). It also shared 91% and 90% identity to HSP70s from *Melitaea cinxia* (AGR84224.1) and *Spodoptera litura* (ADV03160.1), respectively.

A phylogenetic tree of 17 HSP70 sequences from Lepidopterous, Homoptera, and Hymenoptera insects is shown in Fig. 4. As expected, *EhHSP70* was clearly grouped into the Lepidoptera clade and clustered the *A. yamamai* and *A. pernyi* HSP70s in one group.

### Tissue-Specific Expression of *EhHSP70*

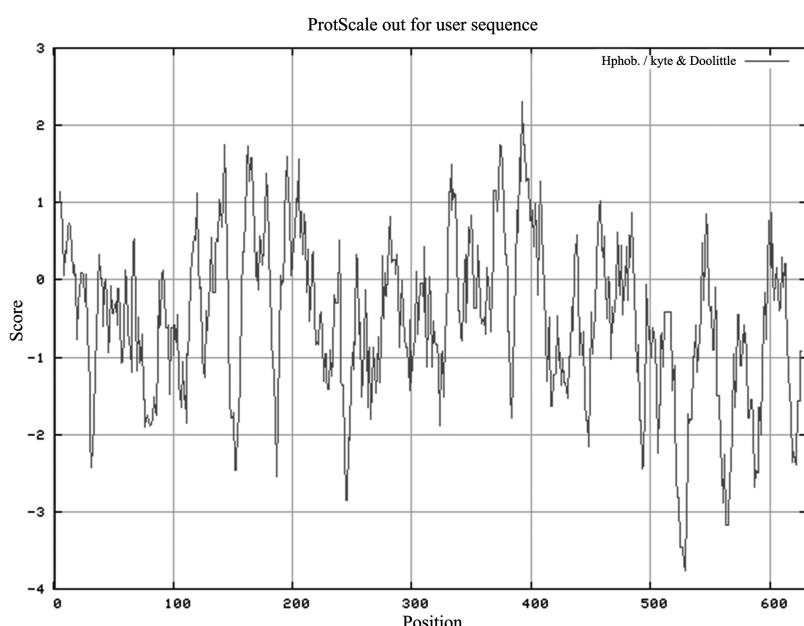
We measured the relative expression of *EhHSP70* across larval tissues with qRT-PCR, using the expression in the midgut of larvae at 25 °C as a reference. At 25 °C, expression in the cuticle, hemolymph, and fat body was relatively low, with the expression in the midgut higher than in the other three tissues (Fig. 5). Compared with the control larvae at 25 °C, the expression of *EhHSP70* in the cuticle was fourfold higher after cold shock at -5 °C for 1 h, and the difference was statistically significant ( $P=0.0288$ ). It was also much higher than the expression in the hemolymph, fat body, or midgut. Thus, cold shock significantly induced *EhHSP70* expression in the cuticle of *E. hippophaecolus*, suggesting that the cuticle is the most active tissue for cold shock responses.

### Expression of *EhHSP70* During Recovery From Cold Shock

*Eogystia hippophaecolus* larvae were subjected to prolonged cold stress for 10 h at 0 °C and allowed to recover at 25 °C for up to 8 h. The relative expression level of *EhHSP70* was measured using qRT-PCR and compared with that of untreated larvae at the same period. *EhHSP70* expression increased from 0 to 1 h during cold shock recovery, reaching a peak of threefold higher expression at 1 h, but its

1       **atgccagctgttgttatcgatcttgataacataattcgctcgctggagttggcaacatggcaacgtggaaatcattgcgaacgtcag**  
 1        M P A V G **I D L G T T Y S C** V G V W Q H G N V E I I A N D Q  
 91        ggcaatcgtagcacccatcatacgtcgcccttacggacacggactcatcgccatgcggccaaaggaaaccaggatcgctttaacccc  
 31        G N R I T P S Y V A F T D T E R L I G D A A K N Q V A L N P  
 181        aacaacacgtgttcacgcaaaccgtactcggtcgaaatttgcacggccaaagatccagaagacatgaaacattggcccttaaag  
 61        N N T V F D A K R L I G R K F D D P K I Q Q D M K H W P F K  
 271        gtgatcaacgactggcaaaccgaaagattcaggctcgagttcaaaggcgaaacagaaagagggttgcgcctgaagaaatcagtagcatgg  
 91        V I N D C G K P K I Q V E F K G E T K R F A P E E I S S M V  
 361        ttgacaaagatgaaaggaaactcgccaggcgatctcggtacgtcgatgcggatcactgtgcggcttactcaacgactcg  
 121        L T K M K E T A E A Y L G T S V R D A T V P A Y F N D S  
 451        cagcgtcaggccactaaagacgcggccatcgccgggttgcacgtgttgcatacaacgagccccaggccggcgtagcgat  
 151        Q R Q A T K D A G A I A G L N V L R I I N E P T A A A L A Y  
 541        ggctcgacaaagaaccaaaggcgacgcacgttctcatcttcgacccctggcggtggcaccttcgacgtgtccatcttgcac  
 181        G L D K N L K G E R N V L **I F D L G G G T F D V S I L S I D**  
 631        gaagggttctgttgcagaatccacggcggtgatcgcacatctcgccggcgaggacttgcataacagattggtaatttttagcg  
 211        E G S T L F E V K S T A G D T H L G G E D F D N R L V N Y L A  
 721        gaggagtccaaacgcaactacaagaaagatttgcgtgatccgcgcgcgtcgccgtctacgcacccgcgcgtagcgtaaaacgc  
 241        D E F K R K Y K K D L C V N P R A L R R L R T A A E R A K R  
 811        acgcttcttcgagactgaggccacatagaatcgcgcctactcgagggtatcgcatttcacacgcgttgcgcgtcgcttc  
 271        T L S S S T E A T I E I D A L Y E G I D F Y T R L S R A R F  
 901        gaagaatttgcacggcgttgcgttgcgtactcgacccggcgagaaggctctgaaaggacgcgaaactcgataaaggtaaaatccac  
 301        E E L N A D L F R G T L E P V E K A L K D A K L D K G Q I H  
 991        gactgttcttgcggaggctcgacccgcattctcaaggccatgcgttgcacagatcttcgtgcacaaacttaatctgc  
 331        **D V V L V G G S T R I P K V Q S L L Q N F F C G K K L N L S**  
 1081        atcaaccctgacgaacggcggtatggactcgccgtacaggccgcattctgactggcgaaacgcactcgagaatccaacgcgtctg  
 361        **I N P D E A V A Y G A A V Q A A I L S G E S D S R I Q D V L**  
 1171        ctcgacgtggcgctcgcttcgcgcataaaacggccgtggcgcatgcacatcgacgcacgcgtcgatccatgc  
 391        L V D V A P L S L G I E T A G G V M T K I I E R N S K I P C  
 1261        aagcgtcgacgttgcacccgtactctgacaaaccagccgcgtcaccatccaatgttgcaggcgacgcgtgcacatgactaaggat  
 421        K Q S Q T F T T Y S D N Q P A V T I Q V Y E G E R A M T K D  
 1351        aacaacttgcgtggtaactgcgttgcatttgcggcattccaccggcactcggggggtgcggatgcacgttgcacatggacgt  
 451        N N L L G T F D L T G I P P A P R G V P K I D V T F D M D A  
 1441        aaggcatcttgcacggtaactgcgttgcggaaaggagaatagcgggttgcgtgatggaaatactatgtatcaag NcLtsS aggtgcgttgc  
 481        N G I L N V S A K E N S T G R S K N I V I K N D K G R L S Q  
 1531        gccggaaatcgatcgatgcgttgcggggccgacgcgtacaaaaggaggatgagaagcggcgtgtggccgcgcacaccatgc  
 511        A E I D R M L S E A R Y K E E D E K Q R Q R V A A R N Q F  
 1621        gaatcttataatcttcagcgtaaaacgacgttgcgttgcacgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgt  
 541        E S Y I F S V K Q A L D D A G S K L D E Q D K N R A R N E C  
 1711        gacaaaggcgctgcgttgcgttgcacacaacacgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgttgcgt  
 571        D E A L R W L D N N T L A E Q E E Y E H K L K E V Q R V C S  
 1801        cccatcatgacaaaggatgcacggcgccggggccggcatggccggccggccaccaacggccacaaacggaccaccgttagggaaat  
 601        P I M S K M H G A G A **G G M P G G H Q Q P N N G P T V E E V**  
 1891        gactaa  
 631        **D \***

**Fig. 1.** Nucleotide and deduced amino acid sequence of the *E. hippophaecolus* HSP70. Note: The initiation codon, atg, is shown in bold, and the termination codon, taa, is shown in bold and marked with an asterisk. The three signature sequences of the HSP70 family are marked by red boxes, the cytoplasmic HSP70 carboxyl terminal region is marked by a red background, the glycosylation sites are marked by a gray background, and the nonorganelle motif is underlined.



**Fig. 2.** Hydropathicity analysis of *E. hippophaecolus* HSP70.

EhHSP70	MPAAGIDLGTTYSBCVGWVQHGNVEIIANDQGNRTTPSYVAFTD	ERLIGDAAKNQVALNE	NNTVFDARE	LIGRKFDDKEIQCQDMKHWPFK	90	
SlHSP70	MPAAGIDLGTTYSBCVGWVQHGNVEIIANDQGNRTTPSYVAFTD	ERLIGDAAKNQVALNE	SNTVFDARE	LIGRKFDDKEIQCADMKHWPFK	90	
BmHSP70	MPAAGIDLGTTYSBCVGWVQHGNVEIIANDQGNRTTPSYVAFTD	ERLIGDAAKNQVALNE	NNTVFDARE	LIGRKFDDKEIQCQDMKHWPFK	90	
McHSP70	MPAAGIDLGTTYSBCVGWVQHGNVEIIANDQGNRTTPSYVAFTD	ERLIGDAAKNQVALNE	NNTVFDARE	LIGRKFDDKEIQCQDMKHWPFK	90	
AyHSP70	MPAAGIDLGTTYSBCVGWVQHGNVEIIANDQGNRTTPSYVAFTD	ERLIGDAAKNQVALNE	SNTVFDARE	LIGRKFDDKEIQCQDMKHWPFK	90	
ApHSP70	MPAAGIDLGTTYSBCVGWVQHGNVEIIANDQGNRTTPSYVAFTD	ERLIGDAAKNQVALNE	SNTVFDARE	LIGRKFDDKEIQCQDMKHWPFK	90	
HzHSP70	MPAAGIDLGTTYSBCVGWVQHGNVEIIANDQGNRTTPSYVAFTD	ERLIGDAAKNQVALNE	NNTVFDARE	LIGRKFDDKEIQCQDNQHWPFK	90	
HaHSP70	MPAAGIDLGTTYSBCVGWVQHGNVEIIANDQGNRTTPSYVAFTD	ERLIGDAAKNQVALNE	NNTVFDARE	LIGRKFDDKEIQCQDNQHWPFK	90	
Consensus	mpaagidlggttysbcvgwvqhgnveiiandqgnrttppsvaftd	erlighdaakqvalnp	ntvfdak	ligrkfddkeiqcqdmc	dm hwpfk	
EhHSP70	VINDCGKPKIQLEFFKGEIKPEFAPEEISSMVILTKMKGTAEEAYLG	SVRDAVITV	PAYFNDSQRQATKDAGAIAGLN	LRIINEPTAAALAY	180	
SlHSP70	VVSDCGKPKIQLEFFKGEIKPEFAPEEISSMVILTKMKGTAEEAYLG	ITV	DARVNFSQRQATKDAGAIAGLN	LRIINEPTAAALAY	180	
BmHSP70	VINDCGKPKIQLEFFKGEIKPEFAPEEISSMVILTKMKGTAEEAYLG	SVRDAVITV	PAYFNDSQRQATKDAGAIAGLN	LRIINEPTAAALAY	180	
McHSP70	VVSDCGKPKIQLEFFKGEIKPEFAPEEISSMVILTKMKGTAEEAYLG	SVRDAVITV	PAYFNDSQRQATKDAGAIAGLN	LRIINEPTAAALAY	180	
AyHSP70	VVNDCGKPKIQLEFFKGEAKPEFAPEEISSMVILTKMKGTAEEAYLG	ITV	DARVNFSQRQATKDAGAIAGLN	LRIINEPTAAALAY	180	
ApHSP70	VVNGCGKPKIQLEFFKGEAKPEFAPEEVSSMVILTKMKGTAEEAYLG	SVRDAVITV	PAYFNDSQRQATKDAGAIAGLN	LRIINEPTAAALAY	180	
HzHSP70	VINDCGKPKIQLEFFKGEIKPEFAPEEISSMVILTKMKGTAEEAYLG	SVRDAVITV	PAYFNDSQRQATKDAGAIAGLN	LRIINEPTAAALAY	180	
HaHSP70	VINDCGKPKIQLEFFKGEIKPEFAPEEISSMVILTKMKGTAEEAYLG	SVRDAVITV	PAYFNDSQRQATKDAGAIAGLN	LRIINEPTAAALAY	180	
Consensus	v d kpkia effkge k fapeevssmvltkmkgtaeaylg	vrdav	tvpayfnfsqrqatkdagaiagln	lriineptaaalay		
EhHSP70	GLDKNLGERNVLIFLDLGGGTFDVSI	SIDEGLSF	EVKSTAGDTHLGGEDFDNRVLNVLADEF	RKUKKDI	CVNERALRRRLTAAERAKR	270
SlHSP70	GLDKNLGERNVLIFLDLGGGTFDVSI	SIDEGLSF	EVKSTAGDTHLGGEDFDNRVLNVLADEF	RKUKKDI	MRRNERNALRRRLTAAERAKR	270
BmHSP70	GLDKNLGERNVLIFLDLGGGTFDVSI	SIDEGLSF	EVKSTAGDTHLGGEDFDNRVLNVLADEF	RKUKKDI	LRNSRALRRRLTAAERAKR	270
McHSP70	GLDKNLGERNVLIFLDLGGGTFDVSI	SIDEGLSF	EVKSTAGDTHLGGEDFDNRVLNVLADEF	RKUKKDI	LRNSRALRRRLTAAERAKR	270
AyHSP70	GLDKNLGERNVLIFLDLGGGTFDVSI	SIDEGLSF	EVKSTAGDTHLGGEDFDNRVLNVLADEF	RKUKKDI	LRNSRALRRRLTAAERAKR	270
ApHSP70	GLDKNLGERNVLIFLDLGGGTFDVSI	SIDEGLSF	EVKSTAGDTHLGGEDFDNRVLNVLADEF	RKUKKDI	LRNSRALRRRLTAAERAKR	270
HzHSP70	GLDKNLGERNVLIFLDLGGGTFDVSI	SIDEGLSF	EVKSTAGDTHLGGEDFDNRVLNVLADEF	RKUKKDI	LRNSRALRRRLTAAERAKR	270
HaHSP70	GLDKNLGERNVLIFLDLGGGTFDVSI	SIDEGLSF	EVKSTAGDTHLGGEDFDNRVLNVLADEF	RKUKKDI	LRNSRALRRRLTAAERAKR	270
Consensus	glodknlkgernvliifdlgggtfdvsi	ldegslef	ev tagdthlggedfdnrlvn la ef rk kkd n	ralrrrlrtaaerakr		
EhHSP70	TLSSTSSTEAEIIDLAEIGIDFYTRVSRARFEELNADLFRGTL	PEV	KALKDAKIDKQCIHDVVVLVGGSTRIPKVQ	SILLQNFFCGKKI	LS	360
SlHSP70	TLSSTSSTEAEIIDLAEIGIDFYTRVSRARFEELNADLFRGTL	PEV	KALKDAKIDKQCIHDVVVLVGGSTRIPKVQ	SILLQNFFCGKKI	LS	360
BmHSP70	TLSSTSSTEAEIIDLAEIGIDFYTRVSRARFEELNADLFRGTL	PEV	KALKDAKIDKQCIHDVVVLVGGSTRIPKVQ	TMLQNFFCGKKI	LS	360
McHSP70	TLSSTSSTEAEIIDLAEIGIDFYTRVSRARFEELNADLFRGTL	PEV	KALKDAKIDKQCIHDVVVLVGGSTRIPKVQ	SMLQNFFCGKKI	LS	360
AyHSP70	TLSSTSSTEAEIIDLAEIGIDFYTRVSRARFEELNADLFRGTL	PEV	KALKDAKIDKQCIHDVVVLVGGSTRIPKVQ	SILLQNFFCGKKI	LS	360
ApHSP70	TLSSTSSTEAEIIDLAEIGIDFYTRVSRARFEELNADLFRGTL	PEV	KALKDAKIDKQCIHDVVVLVGGSTRIPKVQ	SILLQNFFCGKKI	LS	360
HzHSP70	TLSSTSSTEAEIIDLAEIGIDFYTRVSRARFEELNADLFRGTL	PEV	KALKDAKIDKQCIHDVVVLVGGSTRIPKVQ	SILLQNFFCGKKI	LS	360
HaHSP70	TLSSTSSTEAEIIDLAEIGIDFYTRVSRARFEELNADLFRGTL	PEV	KALKDAKIDKQCIHDVVVLVGGSTRIPKVQ	SILLQNFFCGKKI	LS	360
Consensus	tlssstea ieidal egidfytr srafeeln dlfrgtpk	levekalkdk	dk ihdvvvlvggstripk q lqnnffcgkkls	is		
EhHSP70	INPDEAVVYGAAVQAAILSGESDSEI	IQDVLVLDVAP	LSLGIETAGGVMTKIDERN	KIPCKQSQTFTTY	SDNQPAVTIQVYEGERA	450
SlHSP70	INPDEAVVYGAAVQAAILSGESDSEI	IQDVLVLDVAP	LSLGIETAGGVMTKIDERN	KIPCKQSQTFTTY	SDNQPAVTIQVYEGERA	450
BmHSP70	INPDEAVVYGAAVQAAILSGESDSEI	IQDVLVLDVAP	LSLGIETAGGVMTKIDERN	KIPCKQSQTFTTY	SDNQPAVTIQVYEGERA	450
McHSP70	INPDEAVVYGAAVQAAILSGESDSEI	IQDVLVLDVAP	LSLGIETAGGVMTKIDERN	KIPCKQSQTFTTY	SDNQPAVTIQVYEGERA	450
AyHSP70	INPDEAVVYGAAVQAAILSGESDSEI	IQDVLVLDVAP	LSLGIETAGGVMTKIDERN	KIPCKQSQTFTTY	SDNQPAVTIQVYEGERA	450
ApHSP70	INPDEAVVYGAAVQAAILSGESDSEI	IQDVLVLDVAP	LSLGIETAGGVMTKIDERN	KIPCKQSQTFTTY	SDNQPAVTIQVYEGERA	450
HzHSP70	INPDEAVVYGAAVQAAILSGESDSEI	IQDVLVLDVAP	LSLGIETAGGVMTKIDERN	KIPCKQSQTFTTY	SDNQPAVTIQVYEGERA	450
HaHSP70	INPDEAVVYGAAVQAAILSGESDSEI	IQDVLVLDVAP	LSLGIETAGGVMTKIDERN	KIPCKQSQTFTTY	SDNQPAVTIQVYEGERA	450
Consensus	inpdeava vgaavqaailsgesdse	s iqddvlvdvap	lslgietaggvmtki	ern kipckqsqtftty dnqavptiqyegera	tkd	
EhHSP70	NNLLGFEDLTGIPFAPRGVPKIDVTFD	DANGILNVSAKENSTGRSKNIVIKNDKGRLSQ	EIDRMLSE	APRKYKEDDEKQRVRVAPRNQF	540	
SlHSP70	NNLLGFEDLTGIPFAPRGVPKIDVTFD	DANGILNVSAKENSTGRSKNIVIKNDKGRLSQ	EIDRMLSE	APRKYKEDDEKQRVRVAPRNQF	540	
BmHSP70	NNLLGFEDLTGIPFAPRGVPKIDVTFD	DANGILNVSAKENSTGRSKNIVIKNDKGRLSQ	EIDRMLSE	APRKYKEDDEKQRVRVAPRNQF	540	
McHSP70	NNLLGFEDLTGIPFAPRGVPKIDVTFD	DANGILNVSAKENSTGRSKNIVIKNDKGRLSQ	EIDRMLSE	APRKYKEDDEKQRVRVAPRNQF	540	
AyHSP70	NNLLGFEDLTGIPFAPRGVPKIDVTFD	DANGILNVSAKENSTGRSKNIVIKNDKGRLSQ	EIDRMLSE	APRKYKEDDEKQRVRVAPRNQF	540	
ApHSP70	NNLLGFEDLTGIPFAPRGVPKIDVTFD	DANGILNVSAKENSTGRSKNIVIKNDKGRLSQ	EIDRMLSE	APRKYKEDDEKQRVRVAPRNQF	540	
HzHSP70	NNLLGFEDLTGIPFAPRGVPKIDVTFD	DANGILNVSAKENSTGRSKNIVIKNDKGRLSQ	EIDRMLSE	APRKYKEDDEKQRVRVAPRNQF	540	
HaHSP70	NNLLGFEDLTGIPFAPRGVPKIDVTFD	DANGILNVSAKENSTGRSKNIVIKNDKGRLSQ	EIDRMLSE	APRKYKEDDEKQRVRVAPRNQF	540	
Consensus	nnllgf dltgippaprgvpkidytfda	gilnvsakenstgrsknivikndkgrlsq	ei rml eae yk de q rv rnq			
EhHSP70	BSYIFSVKQALDAGSKLDECDAL	WLDNNNTLAQ	EEYEYEHK1REVQRVQSEIMSKMH	.....	AC...AGMPGGHQ	619
SlHSP70	BSYIFSVKQALDAGSKLDECDAL	WLDNNNTLAQ	EEYEYEHK1REVQRVQSEIMSKMH	GAGAQNAADMPEGGMSECPGGY	630	
BmHSP70	BSYIFSVKQALDAGSKLDECDAL	WLDNNNTLAQ	DEMEYEHK1LWDVQRVQSEIMSKMH	....APGGMPGGMCPGGYQ	627	
McHSP70	BSYIFSVKQALDAGSKLSEEDRN	WLDNNNTLAQ	DEMEYEHK1LWDVQRVQSEIMSKMH	....AGGMPEGGMCPGGMP	627	
AyHSP70	BSYIFSVKQALDAGSKLTDKSTAR	WLDNNNTLAQ	DEMEYEHK1LWDVQRVQSEIMSKMH	....GGAAAGTTPGCAFGY	623	
ApHSP70	BSYIFSVKQALDAGSKLTDKSTAR	WLDNNNTLAQ	DEMEYEHK1LWDVQRVQSEIMSKMH	....GGAAAGTTPGCAFGY	623	
HzHSP70	BSYIFSVKQALDAGSKLTDKSTAR	WLDNNNTLAQ	DEMEYEHK1LWDVQRVQSEIMSKMH	....AGADAGAGQQYR	621	
HaHSP70	BSYIFSVKQALDAGSKLTDKSTAR	WLDNNNTLAQ	DEMEYEHK1LWDVQRVQSEIMSKMH	....AGADAGAGQQYR	621	
Consensus	e y fsv qald ag k dk a cd al wldnnntla	ey hkl qr sp m kmhg	g g			
EhHSP70	Q.P..NNNGPTVEEV				630	
SlHSP70	GNQNGPTVEEV				642	
BmHSP70	QARSIDGPTVEEV				639	
McHSP70	RAQGSIDGPTVEEV				639	
AyHSP70	PK..IDGPTVEEV				633	
ApHSP70	PK..IDGPTVEEV				633	
HzHSP70	QQAHSGPTVEEV				633	
HaHSP70	QQAHSGPTVEEV				633	
Consensus	gpt eev					

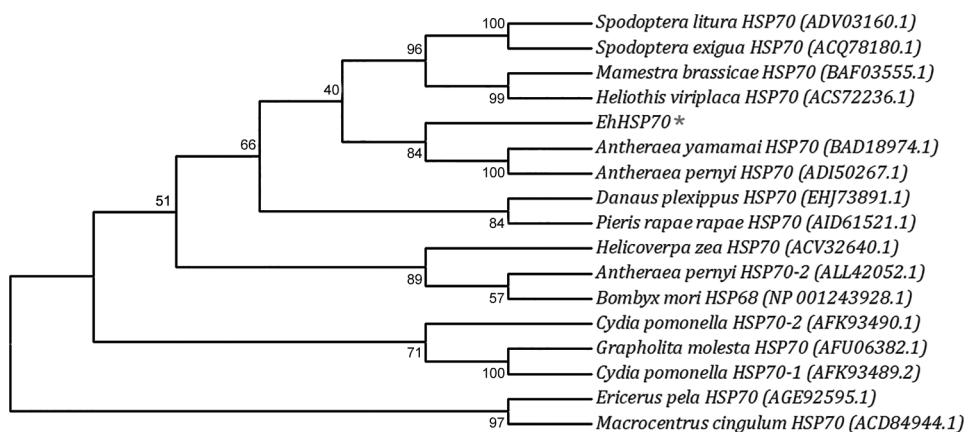
**Fig. 3.** Amino acid sequence comparison of HSP70 homologs in *E. hippophaecolus* and other insects. Note: Presented in the order of EhHSP70, *Spodoptera litura* (ADV03160.1), *Bombyx mori* (BAF69068.1), *Melitaea cinxia* (AGR84224.1), *Antheraea yamamai* (BAD18974.1), *Antheraea pernyi* (ADI50267.1), *Helicoverpa zea* (ACV32640.1), and *Helicoverpa armigera* (ADP37711.1).

expression decreased from 1 to 8 h (Fig. 6). The expression of EhHSP70 was relatively low in control larvae and larvae allowed to recover for 8 h. In addition, there was a statistically significant difference between the treated and untreated samples at 1 h (ANOVA;  $F_{(5, 12)} = 3.44$ ,  $P = 0.0369$ ), but no difference at the other recovery time points. The results indicate that HSP70 may play an important role in the recovery process after cold shock.

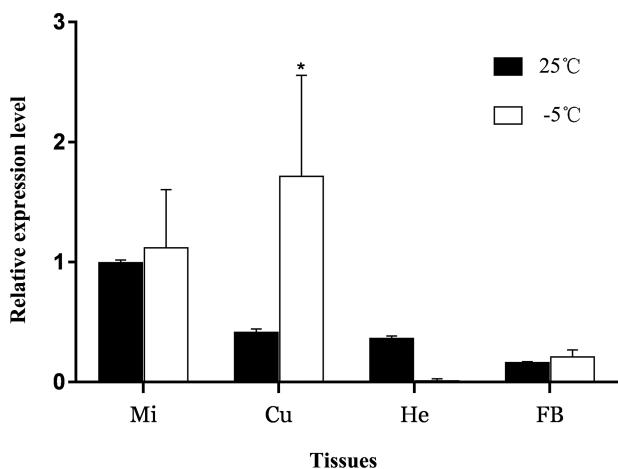
## Discussion

### Cloning and Characterization of EhHSP70

In this study, the transcriptome of *E. hippophaecolus* was screened for HSP70 homologs, and a full-length gene was identified. Primers were designed to clone the full-length ORF of HSP70 from cDNA, and we named the ORF EhHSP70. Sequence analysis found regions of the ORF that were highly conserved, including three HSP70



**Fig. 4.** Neighbor-joining phylogenetic tree showing *E. hippophaecolus* HSP70 alongside the other insects' HSP70s.

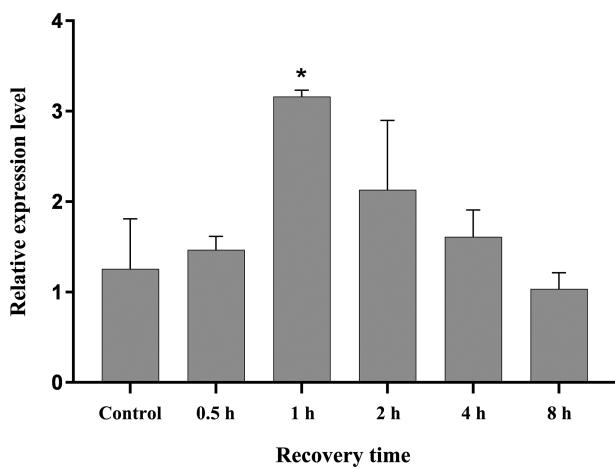


**Fig. 5.** Relative expression level of *EhHSP70* in different tissues of *E. hippophaecolus* larvae. **Note:** Mi: midgut, Cu: cuticle, He: hemolymph, FB: fat body. Actin was used as the reference gene to normalize target gene expression. Data in the figure are mean  $\pm$  SE, and the symbol (\*) indicates that the mean is significantly different from that of the untreated tissue ( $P < 0.05$ ).

family signatures. The presence of EEVD at the *E. hippophaecolus* HSP70 C-terminal indicates that *EhHSP70* is a member of the cytosolic HSP family (Gupta, 1995). In addition, EEVD is believed to bind many synergistic chaperones containing a TPR motif, which is a structural motif consisting of a degenerate 34-amino acid sequence found in a wide variety of proteins such as Hop/Stil, Hip, Fkbps, PP5, Sgt, and Cyp40 (Pearl and Prodromou 2006, Allan and Ratajczak 2011, Gitau et al. 2012).

Aligning the *EhHSP70* nucleotide sequence with that of other species showed that it had the highest identity with HSP70 from Lepidoptera insects such as *A. pernyi*, *A. yamamai*, *S. litura*, and *B. mori*. This also confirmed that the obtained gene belongs to the HSP70 gene family.

Because HSP70 is highly conserved, it is widely used for the phylogenetic analysis of species. Phylogenetic analysis found that *EhHSP70* and HSP70 from other Lepidoptera insects clustered together with a high degree of similarity. However, because little is known about cold resistance genes and no HSP sequence information is in GenBank from other Cossidae insects, the *E. hippophaecolus* HSP70 could only be clustered with HSP70 from *A. yamamai* and *A. pernyi*, the clustering of which indicated that *EhHSP70* was closest to *AyHSP70* and *ApHSP70*, consistent with the homology between species.



**Fig. 6.** Relative expression level of *EhHSP70* during recovery from cold stress. **Note:** Larvae were treated at 0°C for 10 h and allowed to recover at 25°C for 0.5, 1, 2, 4, and 8 h. qRT-PCR experiments were carried out with the primers listed in Table 1. The experiment was performed in triplicate (mean  $\pm$  SE). All treated larvae were compared with the larvae after 8 h of recovery. The symbol (\*) indicates when a value is significantly different from the control ( $P < 0.05$ ).

#### Tissue-Specific Expression of *EhHSP70*

This study revealed the tissue-specific expression pattern of *EhHSP70*, which had a specific pattern even at 25°C in untreated larvae. The expression of the *EhHSP70* gene was most active in the cuticle after cold shock, which significantly increased expression. This expression pattern is similar to that of *Sarcophaga crassipalpis* (Rinehart et al. 2000) and *Lucilia sericata* (Tachibana et al. 2005). The increased expression might be because the insects' cuticle is the first line of defense against the external environment. The expression regulation function of the insect cuticle protein gene (ICPG) may take the lead in coordinating the response, resulting in tissue-specific expression when the insects encounter dry stress, temperature stress, and other environmental conditions (Liu et al. 2010). In addition, the higher expression of *EhHSP70* in the cuticle indicates that it is involved in important physiological processes that rapidly respond when the larvae feel environmental stress. The cuticle quickly and efficiently transcribes the HSP gene to prevent cold shock from damaging the organism, reflecting the deeper protective role of the insects' cuticle.

#### Expression of *EhHSP70* During Recovery

This study revealed a specific expression pattern of *EhHSP70* during recovery, in which expression increased then decreased.

The upregulation of HSP might be triggered by the accumulation of various freezing injuries (Petersen et al. 1990, Sejerkilde et al. 2003) and heat stress during recovery (Burton et al. 1988). Indeed, HSP70 plays an important role in repairing injuries caused by cold stress (Koštál and Tollarová-Borovanská 2009). The expression of many genes, proteins, or cold-tolerant substances might have changed (Colinet et al. 2007, Lalouette et al. 2007, Clark and Worland 2008) and participated in the process of recovery after cold shock, though the expression pattern indicated that *EhHSP70*, specifically, was involved in the reaction to cold shock and the recovery afterwards. Its expression, which increased then decreased, was similar to that of *Drosophila melanogaster* (Colinet et al. 2010), *Drosophila triauraria* (Goto and Kimura 1998), and *Polypedilum vanderplanki* (Gusev et al. 2011). In addition, the HSPs of *Apis cerana cerana* (Liu et al. 2014), Mediterranean fruit fly (Kokolakis et al. 2009), and *Chironomus riparius* (Martín-Folgar et al. 2015) have a similar expression pattern during recovery after heat shock. This confirms that the recovery after cold or heat shock induces the expression of HSP70 in a pattern of upregulation followed by down-regulation. The expression will likely be higher after heat shock than after cold shock, and the changes more direct, as HSP70 is delayed during cold shock (Colinet et al. 2010). Differences between cold and heat shock responses might be due to differential activation of the various heat shock factor (HSF) isoforms (Fujikake et al. 2005) or incomplete activation of HSFs under cold stress, as observed under mild heat stress (Park et al. 2005).

The maximum expression of *EhHSP70* was only attained after 1 h of recovery and underwent a 0.5-h delay. The reason for this delay is unknown, but it may reflect the strong repression of metabolic activity at low temperatures (Colinet et al. 2010). The expression waned to that of untreated larvae after 8 h of recovery. The expression level of HSPs in each species and population is a balance between benefits and costs, and overexpression of HSPs may negatively impact growth, development rate, and fertility (Sørensen et al. 2003). A pivotal trait of the heat shock response is its suppression following restoration of normal environmental conditions (Parsell and Lindquist 1993), reflected in our results.

In this study, the ORF of the *EhHSP70* gene was obtained for the first time using molecular cloning technology. To further investigate the cold-tolerance genes of *E. hippophaecolus*, we performed bioinformatics and phylogenetic analyses, which is of great significance for studying the expression and function of the *EhHSP70* gene in the future. In addition, this study provides an overview of the expression of the *E. hippophaecolus* HSP gene in response to low temperatures and provides the experimental and theoretical basis for the study of cold-tolerance adaptation through *EhHSP70*. With an increasing number of studies on the evolution, function, and mechanism of stress response genes in insects, this study will help us understand the adaptability of insects to diverse environments (Li et al. 2009). Comparing the stress response of *E. hippophaecolus* with that of other model insects will provide useful insights into insect biology.

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