

Cloning and expression pattern of a heat shock cognate protein 70 gene in ticks (*Haemaphysalis flava*)

Lei Liu¹ · Tian-yin Cheng¹ · Ya Yang¹

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Abstract Ticks and tick-borne-diseases have serious public health implications, and screening feasible protein candidates for vaccines development is identified to be an effective alternative to control of tick infestations. In current study, we focused on cloning the full-length gene encoding a heat shock cognate protein 70 (Hsc70), a molecular chaperone of critical functional roles belonging to heat shock protein 70 (HSP70) family, in salivary glands of *Haemaphysalis flava*, namely *Hf-Hsc70*, and analyzing the expression of *Hf-Hsc70* in different life phases, organs and ambient temperatures. Rapid amplification of cDNA ends (RACE) was performed to amplify the 5' and 3' ends of *Hf-Hsc70*. The expression profiles of *Hf-Hsc70* were studied by semi-quantitative real-time PCR (RT-PCR). The full-length of *Hf-Hsc70* was 2363 bp, and contained an ORF of 1965 bp encoding a protein of 648 amino acids. The expression levels of *Hf-Hsc70* at different life phases were in the order of female larvae < female fully engorged nymphs < male adult ticks < female full engorged adult ticks < female half engorged adult ticks. The relative expression of *Hf-Hsc70* in salivary glands was steadily higher than that in midguts ($p < 0.05$) regardless of feeding status. A 3-h of heat stress did not significantly induce the up-regulation of *Hf-Hsc70* transcription. These results indicated that *Hf-Hsc70* was a constitutive form of HSP70 family, and its expression pattern in different life phases and organs suggested a possible role in blood feeding, which would further make Hsc70 a potential candidate for the development of vaccines against ticks.

Keywords Tick · *Hsc70* · heat shock protein · RACE · RT-PCR

Haemaphysalis flava, a kind of ticks belonging to the family of Ixodidae, is geographically ubiquitous in the Far East and many countries in Asia (Yan and Cheng 2015). In China, except for Tibet, Qinghai and Xinjiang, it can be found in the rest provinces, especially in southern and eastern China (Yan and Cheng 2015). *H. flava* is able to survive in vacuum condition under scanning electron microscopes, comparable to tardigrades (trivial name “water bear”), an ecdysozoa known for resistance to various extreme environments (Ishigaki et al., 2012). It attacks human, cattle, goats, dogs, giant pandas and hedgehogs etc., causing lesions, dermatitis, anemia and even death in hosts, and carries specific pathogenic microorganisms like encephalitis virus (Sungjin et al. 2010), *Francisella tularensis* (Ozawa et al., 1982), *Rickettsiae* (Noh et al., 2017), *Coxiella*, *Pseudomonas*, *Ehrlichia*, *Escherichia*, *Acinetobacter*, *Citrobacter*, *Cupriavidus*, *Staphylococcus* and *Wolbachia* (Duan and Cheng, 2016) etc. The extensive distribution, and vectors of a variety of pathogens, as well as a wide range of hosts, make it of specific importance for public health.

It is generally believed that the most promising way for control of tick infections is to develop vaccines against ticks. To screen possible candidates for vaccines development, we previously conducted the sialotranscriptome and midgut transcriptome of *H. flava* using next-generation sequencing (NGS) (Xu et al., 2015a, b). A bunch of genes involved in feeding and digestion were identified, like genes encoding cysteine protease, longipain, subolesin, calreticulin, metalloproteases, serine protease inhibitor, enolase and AV422 (Xu et al., 2015a, b) etc. Specifically, the expression patterns of SubA and Enolase, as well as possible functions, were further characterized in *H. flava* in our lab (Liu et al., 2016; Xu et al., 2016).

✉ Tian-yin Cheng
hn5368@163.com

¹ College of Veterinary Medicine, Hunan Collaborative Innovation Center for Safety Production of Livestock and Poultry, Hunan Agricultural University, Changsha 410128, China

HSP, cellular chaperones expressed both under normal physiological situations and stresses, are involved in the folding and transportation of the newly synthesized peptides. HSP can be divided into 6 major subsets based on their molecular weights, including HSP100, HSP83–90, HSP70, HSP60, HSP40 and small HSP family etc. (Wang et al., 2013) HSP70 are the most widely studied ones, and currently can be further divided into 4 members according to their localizations in cells, namely Hsp70, Hsc70, Hsp75 and glucose-regulated protein 78 (GRP78) (Bausero et al., 2005). The cytosolic localized HSP70 members includes Hsc70 and Hsp70, the former being constitutive expressed while the latter being heat-inducible (Daugaard et al., 2007; Kabani and Martineau, 2008). Accumulated evidences are supporting a role of HSP70 family in the differentiation, virulence adaption, resistance and protection from oxidative damage of parasites (Polla, 1991). We previously found that Hsc70 was a protein of high abundance using saliva and midguts content proteomes of *H. flava* (unpublished data). The DNA fractions encoding *Hsc70* (Contig2727) were also identified in libraries of salivary glands and midguts transcriptomes (Xu et al., 2015a, b).

So far the full-length and expression patterns of *Hsc70* have been studied in turtles, frogs, shrimps and other invertebrates etc., but not extensively in ticks. We speculated that *Hf-Hsc70* would also be conserved, as seen in other species, and its transcript levels would be varied in different organs and life phases, but would be stable under different ambient temperatures. In the present study, for the first time, the full length of *Hf-Hsc70* were cloned by RACE, and its expression patterns under different circumstances were determined by RT-PCR.

Materials and methods

Ticks

H. flava were collected, identified and cultured in Hunan Agricultural University, Changsha, China. Fasted *H. flava* (adult ticks) were inoculated onto 4 hedgehogs, 40 ticks (20 males, 20 females) each. *H. flava* then were harvested at half-engorged and full-engorged state, respectively. Procedures involving hedgehogs in the present study were approved and overseen by the Hunan Agricultural University Institutional Animal Care and Use Committee (No.43321503).

Primers

5'-primers and 3'-primers for RACE were synthesized based on Contig2727 in the transcriptome library of salivary glands in *H. flava* (NCBI-GSE67247). Primers for RT-PCR were synthesized based on hypervariable regions of *Hf-Hsc70*. All primers were synthesized and sequenced by Sangon (Shanghai).

Total RNA extraction

Ticks were sterically observed under stereomicroscope after surfaces were cleaned, and sterilized by 75% ethanol. Ticks were dissected to isolate salivary glands and midguts. Dissected tick organs, as well as whole ticks were well grinded in liquid nitrogen, and were extracted for RNA by an EasyPure RNA Kit (Transgen Biotech, Beijing). The A260 to A280 ratio of RNA extracts was measured by a NanoDrop ND-2000 (Thermo Scientific Waltham, MA), and the RNA extracts was further quantified by an Agilent Bioanalyzer 2100 (Agilent, CA).

Rapid amplification of cDNA ends (RACE) for *Hf-Hsc70*

The 3' and 5' RACE were applied to Contig2727 to clone the full-length of *Hf-Hsc70* using a 3'-Full RACE Core Set with PrimeScript™ Rtase (Takara, Dalian) and a SMARTer™ RACE cDNA Amplification Kit (Takara, Dalian). Gene-specific primers (GSP) for 3'-RACE and 5'-RACE were listed in Table 1.

The PCR conditions and follow-up operations were similar to our previous study (Liu et al., 2016). First-strand cDNA was reversely transcribed from 1.5 μL of RNA using PrimeScript Reverse Transcriptase from the kit. For the 3' RACE, the first round of amplification was accomplished with the synthesized cDNA as templates, and 3'R-GSP1 and 3'R-outerP as primers through 22 cycles. The PCR programs were: initial denaturation at 94 °C for 3 min, denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 45 s, and a final extension at 72 °C for 10 min. A 1.0 μL of PCR products from the first amplification was used to perform the second round of amplification with 3'R-GSP2 and 3'R-innerP as primers through 32 cycles. The PCR programs were: initial denaturation at 94 °C for 3 min, denaturation at 94 °C for 30 s, annealing at 65 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 10 min.

The outer PCR for 5'-RACE was conducted with 5'R-GSP1 and 5'R-outerP as primers through 32 cycles. The

Table 1 Primers for RACE and RT-PCR

Primer	Sequence	Primer for
5'R-GSP ₁	5'-CACTTGCGTTCTCCCGTA-3'	5'-RACE
5'R-GSP ₂	5'-AAAGGTCTTAGTCTCGCCC-3'	5'-RACE
3'R-GSP ₁	5'-CCGTCTATCCAAGGAGGAGA-3'	3'-RACE
3'R-GSP ₂	5'-GCAGGAGACCATCAAGTGCTG-3'	3'-RACE
<i>Hf-Hsc70</i> F	5'-AGACTTCTACTCCACCATCACCC-3'	RT-PCR
<i>Hf-Hsc70</i> R	5'-CTTGTCATCTTGGCATCG-3'	RT-PCR
β-actinF	5'-AGATCTGGCACACACCTTC-3'	RT-PCR
β-actinR	5'-CTTCTCCCGTTAGCCTTG-3'	RT-PCR

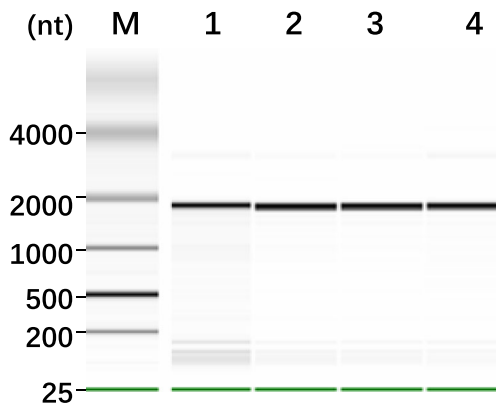


Fig. 1 Electrophoresis of total RNA from *H. flava* in different life phases. 1 the fasted adult ticks, 2 the larvae, 3 the female nymphs, 4 the female engorged adult ticks, M Markers

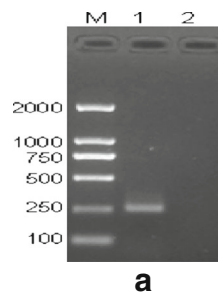
PCR programs were initial denaturation at 94 °C for 3 min, denaturation at 94 °C for 30 s, annealing at 64 °C for 30 s, extension at 70 °C for 30 s, and a final extension at 70 °C for 10 min. The inner PCR for 5'-RACE was conducted with 5'R-GSP2 and 5'R-innerP as primers through 32 cycles. PCR programs were the same to the outer PCR except annealing at 60 °C instead of 64 °C.

The full-length of *Hf-Hsc70* was obtained by reassembly of the 3' end and 5' end of PCR products and Contig2727. The encoding protein sequence and homology of Hsc70 was analyzed by software DNA (version 2.5.1). The protein structure and motifs were analyzed by InterPro scan (<http://www.ebi.ac.uk/interpro/search/sequence-search>). A phylogenetic tree of *Hf-Hsc70* was also generated using software MEGA 5.0 based on available mRNA sequences of typical selected species in Genbank.

Expression pattern of *Hf-Hsc70* in different organs, life phases and ambient temperatures

40 female adult *H. flava*, half engorged, were randomly divided into 5 groups. 10 ticks in control group were placed at room temperature (25 °C) for 3 h. Other 30 ticks were placed at 23 °C, 28 °C, 33 °C and 38 °C for 3 h ($n = 7$ or 8). Total RNA extracts from different organs and whole ticks in different life phases were achieved as described previously.

Fig. 2 Amplification of *Hf-Hsc70* by 3'RACE and the 3' end sequence of *Hf-Hsc70*. (a) Amplification of *Hf-Hsc70* by 3'RACE (b) The 3' end sequence of *Hf-Hsc70*



cDNA synthesis was accomplished using 2 μ L total RNA according to the manual of PrimeScript™ RT reagent Kit with gDNA Eraser (Transgen Biotech, Beijing). A 20 μ L reaction system of RT-PCR with 2 μ L of cDNA templates was constructed by following the manual of SYBR® Premix Ex Taq™ (Takara, Dalian). The program for RT-PCR was as follows: initial denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 31 s, 95 °C for 15 s, and a final 60 °C for 1 min and 95 °C for 15 s. Expression levels of *Hf-Hsc70* was estimated by the $2^{-\Delta\Delta C_t}$ method. Normalized expression of *Hf-Hsc70* was compared among different organs, life phases and ambient temperatures using Student's t test.

Results

Total RNA extraction

The A_{260} to A_{280} ratios were 1.98 and 2.00 for full-engorged ticks by spectrophotometer, as shown in Fig. 1. It revealed that the extracted total RNA were of sound quality.

RACE for Contig2727

The amplified fragments with 3'R-GSP1, 3'R-outerP, 3'R-GSP2 and 3'R-innerP as primers were 250 bp in length (Fig. 2). After gene sequence alignment, it was found that 180 bp was lost from 3' end of Contig2727 (Fig. 4).

Similarly, the amplified fragments with 5'R-GSP1, 5'R-outerP, 5'R-GSP2 and 5'R-innerP as primers were 750 bp in length (Fig. 3). It was found that 562 bp was lost from 5' end of Contig2727 (Fig. 4).

After alignment, it was revealed that the full-length of *Hf-Hsc70* was 2363 bp, and contained an ORF of 1965 bp.

Sequence and phylogenetic analysis of full length of *Hf-Hsc70*

The full-length of *Hf-Hsc70* was 2363 bp, with an ORF at 1965 bp in length. The encoded protein was 648 aa in length, with molecular weight at 71.09 kDa and pI at 5.25.

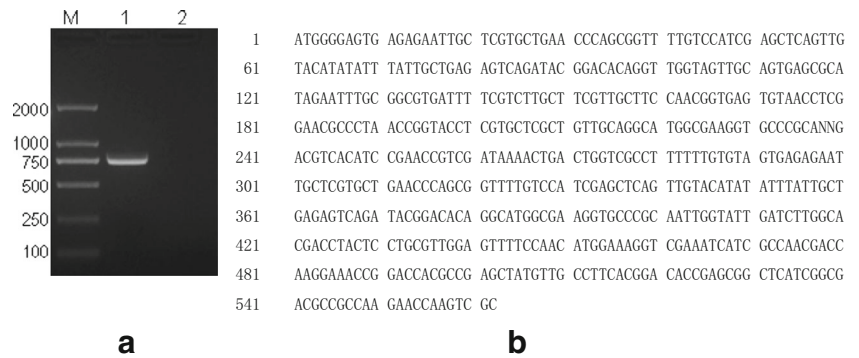
The complete amino acid sequence could be separated into three separate regions: ATPase domain (1 aa-385 aa), the

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1 GCAGATCTGC AACCCCATCA TCAGCAAGCT GTACCAGGCT GGAGGCATGC CTTCTGGTGG
61 ATTCCCAGGT GCCCGAGGAC CCGGTGGTGC TGGTGCAGCT CCTGGAGGAG GTGCCGGCAG
121 TGGGCCCA CA ATTGAAGAAG TGGATTAGAC AAGAGGAAAA AAAAAAAAAA AAAAAAAAAA

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Fig. 3 Amplification of *Hf-Hsc70* by 5'RACE and the 5' end sequence of *Hf-Hsc70*. (a) Amplification of *Hf-Hsc70* by 5'RACE (b) The 5' end sequence of *Hf-Hsc70*



peptide-binding domain (386 aa-543 aa) and C-terminal domain (538 aa-648 aa) (Fig. 5). Three signature sequences of HSP70 was detected, and that was IDLGTTYs (9 aa-16 aa), IFDLGGGTfdvSIL (197 aa-210 aa), and IvLvGGsTRIPkIqK

(334 aa-348 aa). It was also found that 2 tetrapeptide motifs (GGMP and GGFP), the putative bipartite nuclear location signal (KK and RRLRT), a potential non-organelle eukaryotic consensus motif (RARFEEL), the extreme C-terminal domain

Fig. 4 Comparison of Contig2727 from *H. flava* with Hsc70 from *I. scapularis*. The amino acid sequences in gray indicate detected peptide fractions using proteomics

<i>I. sc</i> Hsc70	1	MAKVPAIGIDLGTYSYCVGVFQHGKVEI IANDQGNRTTPSYVAFTDTERLIGDAAKNQVA
Contig2727	1
<i>I. sc</i> Hsc70	61	MNPNTVFDKRLIGRRFDDPAVQSDMKHWPFDVSDGGKPKIQVEYKGETKTFPPEEIS
Contig2727	1	MNPNTVFDKRLIGRRFDDPAVQSDMKHWPFEVSDGGKPKIQVEYKGETKTFPPEEIS
<i>I. sc</i> Hsc70	121	SMVLTKMKEIAEAYLGKTVTNAVVTVPAYFNDSQRQATKDAGT IAGLNVLR I INEPTAAA
Contig2727	61	SMVLTKMKEIAEAYLGKTVTNAVVTVPAYFNDSQRQATKDAGT IAGLNVLR I INEPTAAA
<i>I. sc</i> Hsc70	181	IAYGLDKRGTGERNVLI FDLGGGTFDVS ILTI EDG IFEVKSTAGDTHLGGEDFDNRMVNH
Contig2727	121	IAYGLDKKGTGERNVLI FDLGGGTFDVS ILTI EDG IFEVKSTAGDTHLGGEDFDNRMVNH
<i>I. sc</i> Hsc70	241	FVQEFKRKHKKDLTVNKRRLRRTACERAKRRLSSSTQASIEIDSLFEGVDFYSTITRA
Contig2727	181	FVQEFKRKHKKDLTVNKRRLRRTACERAKRRLSSSTQASIEIDSLFEGVDFYSTITRA
<i>I. sc</i> Hsc70	301	RFEELNADLFRSTLEPVEKALRDAKLDKSKVHDI VL VGGSTRIPKI QKLLQDFNGKELN
Contig2727	241	RFEELNADLFRSTLEPVEKALRDAKMDKSKVHDI VL VGGSTRIPKI QKLLQDFNGKELN
<i>I. sc</i> Hsc70	361	KSINPDEAVAYGAAVQAAILIGDKSEQVDLLLDVTPLSLGIETAGGVMTVL I KRNTTI
Contig2727	301	KSINPDEAVAYGAAVQAAILIGDKSEQVDLLLDVTPLSLGIETAGGVMTVL I KRNTTI
<i>I. sc</i> Hsc70	421	PTRQTQFTTYSNQPGLIQVFEGERAMTKDNNLLGKFELTGIPPAPRGVPQIEVTFDI
Contig2727	361	PTRQTQFTTYSNQPGLIQVFEGERAMTKDNNLLGKFELTGIPPAPRGVPQIEVTFDI
<i>I. sc</i> Hsc70	481	DANGILNVSVDKSTGKENKITITNDKGRLSKEEIERMVKDAEKYKDEDDKQKTRITAKN
Contig2727	421	DANGILNVSVDKSTGKENKITITNDKGRLSKEEIERMVKDAEKYKDEDDKQKTRISAKN
<i>I. sc</i> Hsc70	541	ALESYSFNKSTVEDEKLDKLSDDRKKILEKVEETIKWLDTNQLADKEEYEHRRQKELE
Contig2727	481	ALESYSFNKSTVEDEKLDKLSSEDRQKILEKVQETIKWLDNSQLAEKVEEYEHRRQKELE
<i>I. sc</i> Hsc70	601	QVCNPIITKLYQDGGMPAGGFPAGAGPGGAGAAPGAGAGSGPTIEEVD
Contig2727	541	QI.....

Fig. 5 The predicted structure of Hsc70 encoded by *Hf-Hsc70*. The protein structure and motifs were analyzed by InterPro scan (<http://www.ebi.ac.uk/interpro/search/sequence-search>). The characteristic HSP70 family signatures are underlined; the putative ATP/GTP binding site, localization signals, glycosylation domains and GGXP motifs are shown in gray

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451 CATGGGAAAGGTGCCGCAATGGTATGTGCTTGGCAAGCACTACTCTCGTGGAGTTTCCAAATCGAAAGTGCAGAAATCATGG
[1] M A K V P A I G I D L G T T Y S C V G V F Q H G K V E I I A
HSP70 Family Signature 1
541 CAAAGCAAGAAAGCCGACCGCAAGCTAGTGGCTTCCAGGACACGAGGCGCTCATGGCGACGCGCCAAAGCAACCAAGTGG
[31] N D Q G N R T T P S Y V A F T D T E R L I G D A A K N Q V A
681 CATGAACCTAACAAACAGTGTTCGACGCTAAGCGCGCTGATCGGGGCAAGCGCTTGTGACCCGCGCTTCAGTCGGACATGAAGCACTG
[61] M H P N T V F D A K R L I G R R F D D P A V Q S D M K H W
721 GCGCTTGAAGTGTCAAGCAAGCGCGCAAGCAAGATTAAGTGAAGTCAAGGCGAGACTAAGACTTTCCGGAAAGATCTC
[91] P F E V V S D G G K P K I Q V E Y K G E T K T F P F E I S
811 GTCGATGTACTCATCAAGATGAAGGATGCGGCAAGCGTACCTCGGCAAGCAAGCGTCAAGCGCGTGTCCAGCGTGTCCAGCGTACT
[121] S M V L I K M K E I A A R A Y I G K K T V V N A V V T V P A Y F
ATP/GTP Binding site motifA(P-loop)
901 TAAAGACTCTCAACCTCAGGCCAACAAAGAGCGGTAACATTGCCCGCTGAACGTGCTCCGATCATCAATAGCCAGCCCGGCTGC
[151] N D S Q R Q A T K D A G T I A G L N V L R I I N E P T A A A
991 TATCGCTAAGCGCTGGACAAAGGGAACGCGAGAACGCAATGTGCTCATCTCGACTTGGGGCGGTACCTTCGATGTTCCATTTT
[181] I A Y G L D K K G T G E R N V L I F D L G G G T F D V S I L
HSP70 Family Signature 2
1081 GACCATTGAGGACGGCATTTCGAAAGTCAAGTCCACGGCGGCAACCCATCTTTGGTGGCGAGACTTGCACACCGATGTGAAOCA
[211] T I E D G I F E V K S T A G D T H L G D E D F D N R M V N H
1171 CTTCGTCAGAGTTCAAGCGAAGCAAGAAAGCACTACCGTGAACAAAGGGCGCGTGGCAGCACTGAGAACTGCGTGGAGCGGGG
[241] F V Q E F K R K H K L D L T V N K R A L K R L R T A C E R A
Bipartite nuclear location signal
1261 CAAAGCGACCTCTCTTCGAGCACAGCGCAAGTTCGAGTTCGAGTTCGAGTTCGAGTTCGAGTTCGAGTTCGAGTTCGAGTTCGAGTTC
[271] K R T L S S S T Q A S I E I D S L F E G V D F Y S T I T A
1351 CGCCTTGAAGAGCTGAATCGCGACTCTTCGACGCAAGCGTGTGGAGAAAGGCTCCTCCGATGCGAAGATGCGAAGATGCGAAGATGCGA
[301] N A D L F R S T L E P V E K A L R D A K M D K S Q
Cytosolic localization signal
1441 GGTGACGACATTGTGCTGTGGCGGCTCGACCCGTATCCCAAGATTGAGAAGCTCTGCAAGACTCTCTCAATGGCAAGGACGTAAG
[331] V H D I V L V G G S T R I P K I Q K L L Q D K S I E Q V Q D
HSP70 Family Signature 3
1531 CAAATCCATCAACCCGACGAGGCTGTGGCTACGCGCTGCACTCAAGCTGCGATCCTCATTCGCGCAAGTCCGAGCAAGTCCGAAATGCAG
[361] K S I N P D E A V A Y G A A V Q A A I L I G D K S I E Q V Q D
Glycosylation domain ATP Binding domain SBD
1621 CCTCGCTGCTGCTGAAGTCACTCCCTCTCTTGGCATTGAGACCGCGCGGGGTGATGACCGTGTGATCAAGCGCAACACCAAT
[391] L L L L D V T P L S L G I E T A G G V M T V L I K R N T T I
1711 TCCGACGCGCAGACCCAGCTTCCACCACTACTGACACCAAGCGCGGTGCTGATCCAGGTGTCGAGGCGCAAGCGCGGATGAC
[421] P T R Q T Q T F T T Y S D N Q P G V L I Q V F E G R A M T
1801 CAAAGCAAAACCTGCTCGGAAAGTTCGAGTGAAGGCAATTCCTCCGCGCGCGGGTGAAGTCCCGCAATGAGGTCACTTTGACAT
[451] K D N N L L G K P E L T G I P P A P R G V P Q I E V T F D I
1891 TGATGCCAAGCGCTTCAAACTGTCTCGGTGCAAGAGCAAGCGCGCAAGCAAGATCCATCAGCAAGCAAGCGCGCTCT
[481] D A N G I L N V D K S T G K E N K I T I T N D K G R L
Glycosylation domain
1981 ATCCAGGAGGAGATTGAGCGGATGAGAGGATGCCGAGAAGTCAAGGACGAGGATGCAAGCAAGGACGAGGATCTCCCGCAAGAA
[511] S K E E I E R M V K D A E K Y K D E D D K Q K T R I S A K N
2071 TGCCGTGGAAGCTACTCGTCAATGTCAAGTCCACTGTGGAGGATGAAAGKAAAGCAAGATTCGCGAAGATGCGCAAGAAAT
[541] A L E S Y S F N V K S T V E D E K L K D K I S E E D R Q K I
SBD C-domain
2161 CTTGGAGAGGTGCAAGGAGACCAATCAAGTGGCTGGACTGGAACCAAGCTTGCAGAGAAAGAAAGATGAGCACCGCAAGAAAGTGG
[571] L E K V Q E T I K W L D S N Q L A E K E E Y E H R Q K E L E
2251 GCGATCTGCAACCCATCATCAGCAAGCTGACCGGCTGGAAGCATGCTTCTGGTATTCCCAAGTCCAGGAGGAGACCGCGTGTGC
[601] Q I C N P I I S K L Y Q A G G M P S G G F P F G A G G P G G A
GGXP motif GGXP motif
2341 TGGTGCAGCTCTGGAGAGGTGCCGCGCAAGTGGGCCCAATTCGAAAGTGGATTA
[631] G A P F G G A G S S G F P T I I E E V D *
Cytosolic localization signal

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(SGPTIEEVD) motif and 2 glycosylation sites (NKSI and NVSA) were included in the protein structure (Fig. 5). Based on those structural characteristics, it could be concluded that the protein encoded was actually Hsc70.

As shown by both Blast P and gene alignment, Hsc70 was highly homologous with the identity of the protein in vertebrates like Pisces, Amphibian, Reptilian, Aves and mammals over 89.9%, and it was also highly homologous in invertebrates like Crustacea, Arachnoidea, and insect, with the identity over 90.33%. Notably, the identity among *H. flava*, *Ixodes scapularis* and *Ixodes ricinus* was over 99.02% (Fig. 6). It could be concluded that *Hf-Hsc70* was an evolutionarily conserved gene.

Expression patterns of *Hf-Hsc70* in different life phases, organs and ambient temperatures

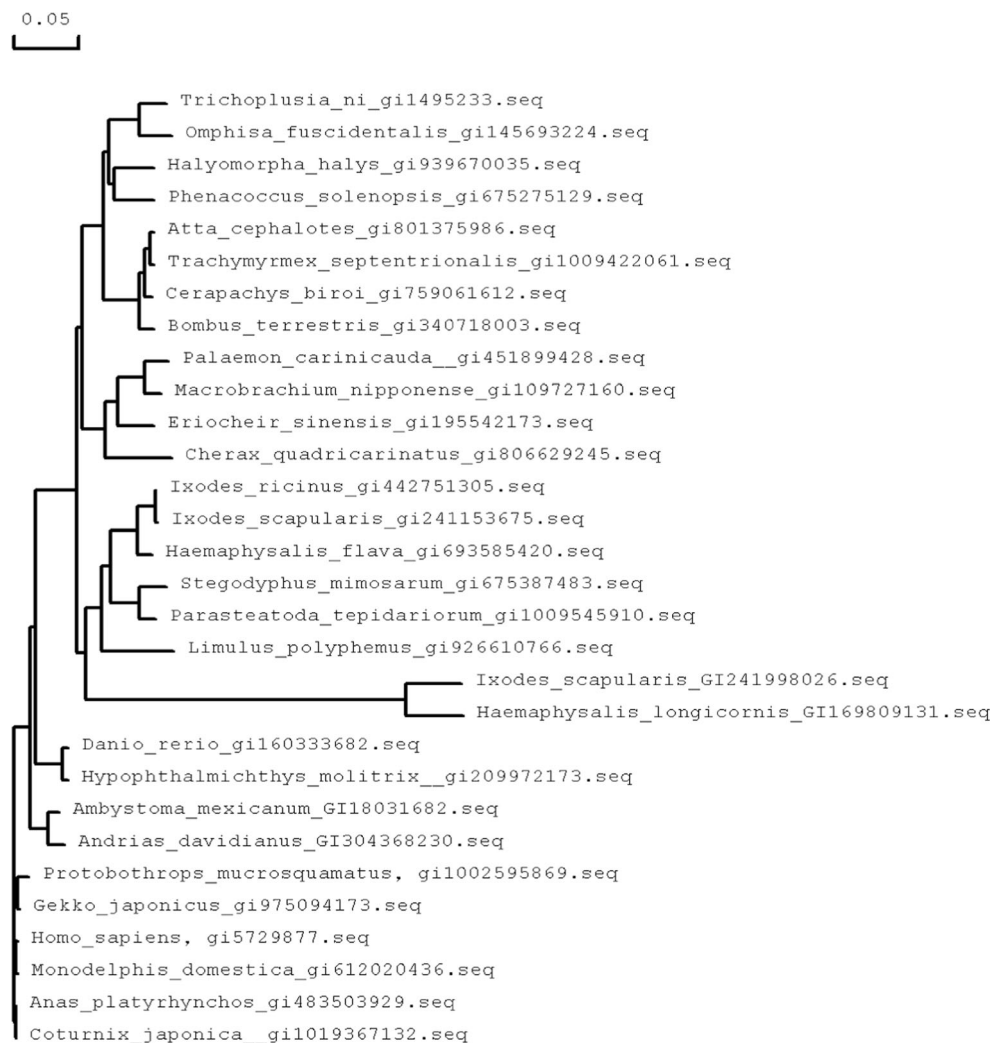
Expression levels of *Hf-Hsc70* in different organs were in the order of salivary glands of the female half-engorged > midguts of the female half-engorged > salivary glands of the female full-engorged > midguts of the female full-engorged. Specifically, the relative expression level of *Hf-Hsc70* in salivary glands was steadily higher than that in midguts ($p < 0.05$) regardless of feeding status (Fig. 7 A). Expression patterns of *Hf-Hsc70* in different life stages were in the order of female half engorged adult ticks > female full engorged adult ticks > male adult ticks >

female fully engorged nymphs > female larvae (Fig. 7 B). *Hsc70* expression was highest at 33 °C, and lowest at 38 °C, but not significantly different (Fig. 7 C).

Discussion

Our previous data from saliva and midgut content proteome in *H. flava* by LC/MS/MS revealed that 8 peptide fragments, namely ARFEELNADLFR, DAGTIAGLNVLR, IINEPTAAAIAYGLDK, SINPDEAVYGA AVQAAILGDK, STAGDTHLGGEDFDNR, TTPSYVAFTDTER, WLDTNQLADKEEYHR and VEIANDQGNR, were labeled as HSP70 unique peptide fragments of *I. scapularis* with total 69 specific peptides detected (unpublished data). 16 unigenes were annotated as *Hsp70* fragments of *I. scapularis* by searching in libraries of salivary glands and midguts transcriptomes in *H. flava* (GSE67247). Among them, contig2727 was 1629 bp in length, and showed an 86.40% identity with Hsp70 of *I. scapularis* (XM_002407088.1) and a 97.42% identity of Hsc70 (GI:241,153,675), but lacking VEIANDQGNR in amino terminal and VCNPIITKLYQDGGMPAGGFPAGAGPAGGAGAAPGAGA-GSGPTIEEVD in carboxyl terminal. We concluded that contig2727 was an encoding gene fraction of Hsc70, but was lack of the 3' end and 5' end.

Fig. 6 Phylogenetic analysis of *Hsc70* genes from representative species. A phylogenetic tree of *Hsc70* was constructed by software MEGA 5.0 based on available mRNA sequences of typical species in Genbank. The accession numbers of *Hsc70* sequences from multiple species are shown after their names



Of the 6 HSP families, HSP70 had the most members, and all members showed different bioactivity and different expression patterns (Bolhassani and Rafati, 2008). In the current study, full length of *Hf-Hsc70* was cloned by 3'-RACE and 5'-RACE based on the contig2727. It was found that the sequence obtained from RACE had 3 signature sequences of HSP70 family in the putative amino acids sequences, 2 characteristic motifs of Hsc70 (GGMP, SGPTIEEVD). Hence we claimed the sequence to be *Hsc70* (Bairoch, 1991; Rensing and Maier, 1994).

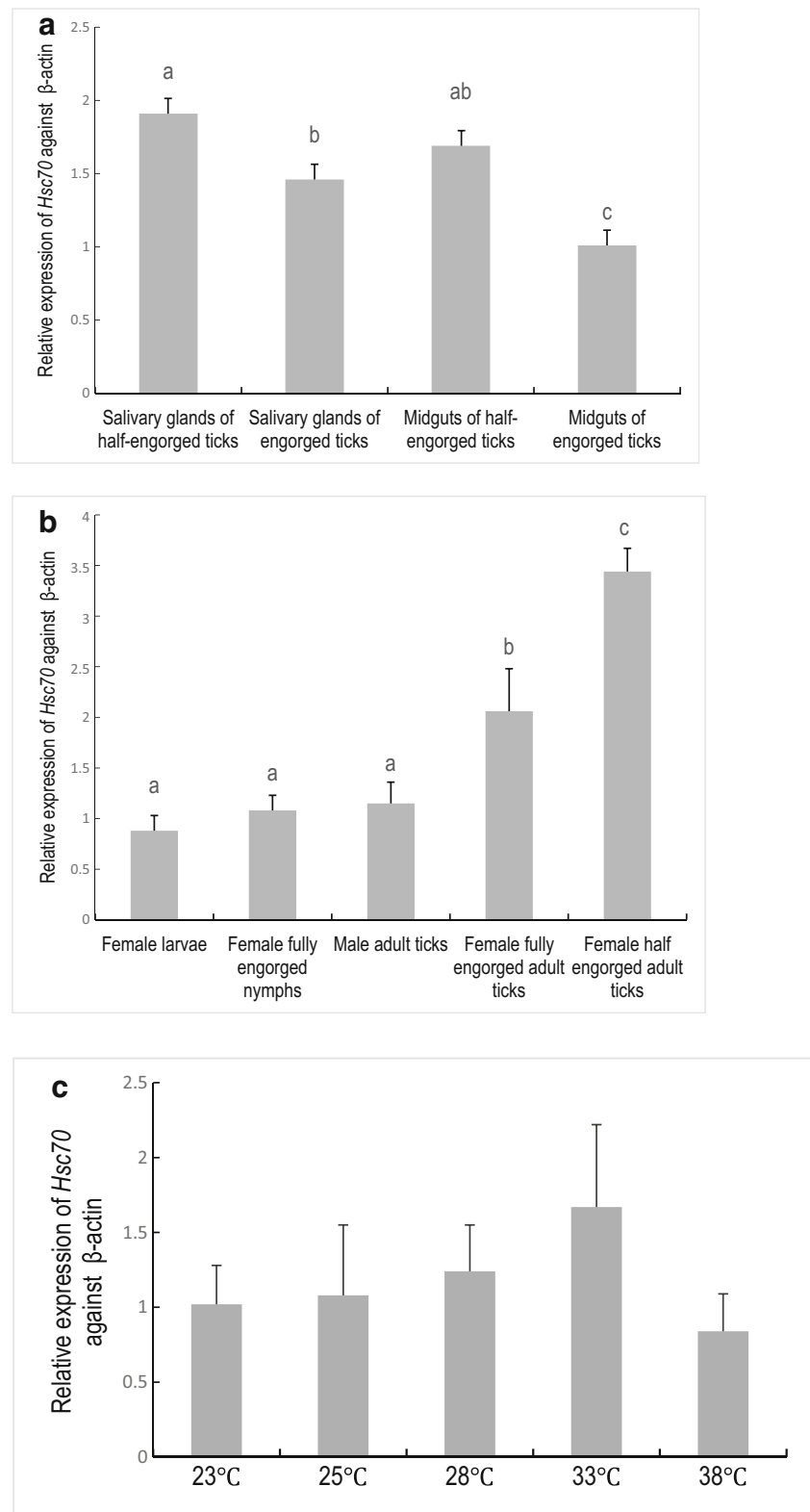
The phylogenetic analysis further indicated clearly the gene cloned was a constitutive HSP70 form other than a stress inducible form. *Hf-Hsc70* showed close relationships with *Hsc70* sequences of other typical selected vertebrates and invertebrates, especially with that of *I. scapularis* (GI:241,153,675) and *I. ricinus* (GI:442,751,305). However, *Hf-Hsc70* was not clustered with the sequence of *H. longicornis* (GI:169,809,131) to form a distinct clade. Only an identity of 64.6% was found between those two sequences (Tian et al., 2011). The author claimed that the

Hsp70, as well as that in *I. scapularis* both belonged to ER-localized HSP70 proteins (Tian et al., 2011), not *Hsc70*.

Our data revealed that *Hf-Hsc70* transcription was not significantly up-regulated after 3-h heat stress, indicating the protein encoded was not inducible by the 3-h heat stress. The observation was consistent with the data in frogs (Simoncelli et al., 2010), pleurodeles (Delelis-Fanien et al., 1997) and zebrafish etc. However, there were also data showing that *Hsc70* in some invertebrates and vertebrates, like moths (Sonoda et al., 2006), clams (Wu et al., 2014), shrimps (Lo et al., 2004), endoparasitoids (Wang et al., 2008) and turbot (Wang et al., 2013) etc., had an up-regulation pattern upon heat stress. One explanation for this was that possibly *Hsc70* was only account for the acquisition of tolerance to the beginning of heat stress, and other members of HSP70 would be induced and play a part in the long term heat exposure, like seen in *Pteromalus puparum* and *Drosophila melanogaster* (Li et al., 2012; Wang et al., 2008).

Expression pattern of *Hf-Hsc70* in different life phases showed that its expression was associated positively with the

Fig. 7 The expression of *Hf-Hsc70* in different organs (A), life phases (B) and ambient temperatures (C) by RT-PCR. *Hf-Hsc70* expression in different organs, life phases and ambient temperatures was compared by Student's t-test. Data not sharing a common letter indicated there was a significant difference ($p < 0.05$)



amount of blood ticks sucked. Salivary glands and midguts were both capable to express Hsc70 and secreted it into saliva and midgut contents as confirmed by transcriptomics and proteomics. Those observations suggested that Hsc70 was likely

to play a role in blood digestion, more likely, to play a part in anticoagulant or facilitation of the uptake of blood by somatic cells of ticks. Hsc70 depletion by RNAi knockdown in *Rhodnius prolixus* was lethal 40 d after dsRNA injection,

and those challenged insects died even earlier within 14–20 d after blood-feeding (Paim et al., 2016). It was suggested that Hsc70 knockdown bugs had impaired signaling pathways involved in blood processing and digestion. This observation was consistent with our assumptions. *Hsc70* was expressed in platelets, and was involved in the regulation of platelet adhesion by binding to protein phosphatase 1, and thus was functional in blood coagulation (Polanowska-Grabowska and Gear, 2000; Polanowska-Grabowska et al., 1997). However, more detailed molecular mechanism of Hsc70 on blood coagulation/anticoagulation awaits further investigations. Thus screening of peptides antigens based on the variable region of Hsc70 would be of significance to develop vaccines for the control of tick infestations. However, those assumption needs to be confirmed in further.

In conclusion, the current study cloned the full length of *Hf-Hsc70* and analyze its expression patterns under different circumstances. *Hf-Hsc70* was a conserved gene and belonging to a constitutive form of HSP70. Expression patterns of *Hf-Hsc70* in different life phases and organs suggested a possible role in blood feeding. Further work is needed to explore the functional roles of Hsc70 in the blood feeding process of ticks and make it a competent candidate for the development of vaccines against ticks.

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References

- Bairoch A (1991) PROSITE: a dictionary of sites and patterns in proteins. *Nucleic Acids Res* 19(Suppl):2241
- Bausero MA, Gastpar R, Multhoff G, Asea A (2005) Alternative mechanism by which IFN-gamma enhances tumor recognition: active release of heat shock protein 72. *J Immunol* 175:2900–2912
- Bolhassani A, Rafati S (2008) Heat-shock proteins as powerful weapons in vaccine development. *Expert Rev Vaccines* 7:1185
- Daugaard M, Rohde M, Jäättelä M (2007) The heat shock protein 70 family: highly homologous proteins with overlapping and distinct functions. *FEBS Lett* 581:3702
- Delelis-Fanien C, Penrad-Mobayed M, Angelier N (1997) Molecular cloning of a cDNA encoding the amphibian *Pleurodeles waltl* 70-kDa heat-shock cognate protein. *Biochem Biophys Res Commun* 238:159–164
- Duan D, Cheng TY (2016) Determination of the microbial community features of *Haemaphysalis flava* in different developmental stages by high-throughput sequencing. *J Basic Microbiol* 57:302–308
- Ishigaki Y, Nakamura Y, Oikawa Y et al (2012) Observation of live ticks (*Haemaphysalis flava*) by scanning electron microscopy under high vacuum pressure. *PLoS One* 7:e32676. doi:10.1371/journal.pone.0032676
- Kabani M, Martineau CN (2008) Multiple Hsp70 isoforms in the eukaryotic cytosol: mere redundancy or functional specificity? *Curr Genomics* 9:338–248
- Li XL, Kang Y, Zhang XY, Zhu BL, Fang WH (2012) Identification of a heat shock cognate protein 70 gene in Chinese soft-shell turtle (*Pelodiscus sinensis*) and its expression profiles under thermal stress. *J Zhejiang Univ Sci B* 13:465–477
- Liu L, Cheng TY, Yan F (2016) Expression pattern of subA in different tissues and blood-feeding status in *Haemaphysalis flava*. *Exp Appl Acarol* 70:511–512
- Lo WY, Liu KF, Liao IC, Song YL (2004) Cloning and molecular characterization of heat shock cognate 70 from tiger shrimp (*Penaeus monodon*). *Cell Stress Chaperones* 9:332
- Noh Y, Lee YS, Kim HC et al. (2017) Molecular detection of Rickettsia species in ticks collected from the southwestern provinces of the Republic of Korea *Parasite Vector* 10:20 doi:10.1186/s13071-016-1955-x
- Ozawa A, Yamaguchi N, Hayakawa K, Matsuo I, Niizuma K, Ohkido M (1982) [a case of tick bite (*Haemaphysalis flava*)—consideration of tularemia infection through tick bite] *Nihon Hifuka Gakkai Zasshi the Japanese. J Dermatol* 92:1415–1421
- Paim RM, Araujo RN, Leis M, Sant'anna MR, Gontijo NF, Lazzari CR, Pereira MH (2016) Functional evaluation of heat shock proteins 70 (HSP70/HSC70) on *Rhodnius prolixus* (Hemiptera, Reduviidae) physiological responses associated with feeding and starvation. *Insect Biochem Molec* 77:10–20. doi:10.1016/j.ibmb.2016.07.011
- Polanowska-Grabowska R, Gear AR (2000) Heat-shock proteins and platelet function. *Platelets* 11:6–22
- Polanowska-Grabowska R, Simon CG Jr, Falchetto R, Shabanowitz J, Hunt DF, Gear AR (1997) Platelet adhesion to collagen under flow causes dissociation of a phosphoprotein complex of heat-shock proteins and protein phosphatase 1. *Blood* 90:1516–1526
- Polla BS (1991) Heat shock proteins in host-parasite interactions. *Immunol Today* 12:A38–A41
- Rensing SA, Maier UG (1994) Phylogenetic analysis of the stress-70 protein family. *J Mol Evol* 39:80–86
- Simoncelli F, Morosi L, Di RI, Pascolini R, Fagotti A (2010) Molecular characterization and expression of a heat-shock cognate 70 (Hsc70) and a heat-shock protein 70 (Hsp70) cDNAs in *Rana* (Pelophylax) lessonae embryos. *Comp Biochem Phys A* 156:552–560
- Sonoda S, Ashfaq M, Tsumuki H (2006) Cloning and nucleotide sequencing of three heat shock protein genes (hsp90, hsc70, and hsp19.5) from the diamondback moth, *Plutella xylostella* (L.) and their expression in relation to developmental stage and temperature arch. *Insect Biochem* 62:80–90
- Sungjin K, Kang JG, Suyeon K et al (2010) Prevalence of tick-borne encephalitis virus in ticks from southern Korea. *J Vet Sci* 11:197–203
- Tian Z, Liu G, Zhang L et al (2011) Identification of the heat shock protein 70 (HLHsp70) in *Haemaphysalis longicornis*. *Vet Parasitol* 181:282
- Wang H, Dong SZ, Li K, Hu C, Ye GY (2008) A heat shock cognate 70 gene in the endoparasitoid, *Pteromalus puparum*, and its expression in relation to thermal stress. *BMB Rep* 41:388
- Wang TT, Wang N, Liao XL, Meng L, Liu Y, Chen SL (2013) Cloning, molecular characterization and expression analysis of heat shock cognate 70 (Hsc70) cDNA from turbot (*Scophthalmus maximus*). *Fish Physiol Biochem* 39:1377–1386
- Wu X, Tan J, Cai M, Liu X (2014) Molecular cloning, characterization, and expression analysis of a heat shock protein (HSP) 70 gene from *Paphia undulata* *Gene* 543:275–285

- Xu XL, Cheng TY, Yang H, Liao ZH (2015a) De novo assembly and analysis of midgut transcriptome of *Haemaphysalis flava* and identification of genes involved in blood digestion, feeding and defending from pathogens. *Infect Genet Evol* 38:62–72
- Xu XL, Cheng TY, Yang H, Yan F, Yang Y (2015b) De novo sequencing, assembly and analysis of salivary gland transcriptome of *Haemaphysalis flava* and identification of sialoprotein genes. *Infect Genet Evol* 32:135–142
- Xu XL, Cheng TY, Yang H (2016) Enolase, a plasminogen receptor isolated from salivary gland transcriptome of the ixodid tick *Haemaphysalis flava*. *Parasitol Res* 115:1955–1964
- Yan F, Cheng TY (2015) Morphological and molecular identification of *Haemaphysalis flava*. *Chinese J Vet Sci* 35:912–916