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Cloning and Characterization of the *HSP70* Gene, and Its Expression in Response to Diapauses and Thermal Stress in the Onion Maggot, *Delia antiqua*

Bin Chen[1,2,3,*], Takumi Kayukawa[1], Antónia Monteiro[2] and Yukio Ishikawa[1]

[1]Laboratory of Applied Entomology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo 113-8657, Japan
[2]Department of Ecology and Evolutionary Biology, Yale University, New Haven, CT 06520, USA
[3]The Key Laboratory of Animal Biology in Chongqing, College of Life Sciences, Chongqing Normal University, Shapingba, Chongqing 400047, P.R. China

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The cytosolic members of the HSP70 family of proteins play key roles in the molecular chaperone machinery of the cell. In the study we cloned and sequenced the full-length cDNA of *Delia antiqua HSP70* gene, which is 2461 bp long and encodes 643 a.a. with a calculated molecular mass of 70,787 Da. We investigated gene copies of cytosolic HSP70 members of 4 insect species with complete genome available, and found that they are quite variable with species. In order to characterize this protein we carried out an alignment and a phylogenetic analysis with 41 complete protein sequences from insects. The analysis divided the cytosolic members of the family into two classes, HSP70 and HSC70, distinguishable on the basis of 15 residues. HSP70 class members were slightly shorter in length and smaller in molecular mass relative to the HSC70 class members, and the conservative and functional regions in these sequences were documented. Mainly, we investigated the expression of *Delia antiqua HSP70* gene, in response to diapauses and thermal stresses. Both summer and winter diapauses elevated *HSP70* transcript levels. Cold-stress led to increased *HSP70* expression levels in summer- and winter-diapausing pupae, but heat-stress elevated the levels only in the winter-diapausing pupae. In all cases, the expression levels, after being elevated, gradually decreased with time. *HSP70* expression was low in non-diapausing pupae but was up-regulated following cold- and heat-stresses. Heat-stress gradually increased the mRNA level with time whereas cold-stress gradually decreased levels after an initial increase.

Keywords: Cloning, *Delia antiqua*, Diapause, Expression, Genome, *HSP70*, *HSC70*, Insect, Phylogeny, Thermal stress

*To whom correspondence should be addressed.
Tel: 86-23-65362652; Fax: 86-23-65362775
E-mail: c_bin@hotmail.com

Introduction

Heat shock genes are a subset of a larger group of genes coding for molecular chaperones (Sorensen *et al.*, 2003). In insects there are four heat-shock gene families: the small HSP (sHSP) family with molecular masses ranging from 20-30 kDa, the HSP60 family with molecular masses of approximately 60 kDa, the HSP70 family with molecular masses of approximately 70 kDa, and the HSP90 family with higher masses (Denlinger *et al.*, 2002). The HSP70 family is one of the most highly conserved gene families and its proteins are the most widely studied stress proteins (Gupta and Golding, 1993; Boorstein *et al.*, 1994). The members of the HSP70 family are nuclear genes, and their proteins have been localized to various cellular compartments, including the cytosol, mitochondria, chloroplast, and endoplasmic reticulum. Cytosolic members include the heat-inducible *HSP70* and the constitutively expressed heat shock cognate 70 (*HSC70*), both of which play key roles in the molecular chaperone machinery (Caplan, 2003; Sorensen *et al.*, 2003). Although the expression patterns of these two genes are quite different, their proteins share common structural features. These proteins consist of 2 domains: a 44-kDa N-terminal ATP-binding domain, and a 30-kDa C-terminal substrate-binding domain that bears the highly conserved EEVD terminal sequence, unique to the cytosolic forms of HSP70 family members (Dang and Lee, 1989; Kiang and Tsokos, 1998). Little is known about the evolutionary relatedness and characteristic difference between HSP70 and HSC70 proteins in insects, as earlier phylogenetic studies only involved one insect species, *Drosophila melanogaster* (Boorstein *et al.*, 1994; Gupta and Singh, 1994; Gupta *et al.*, 1994).

To survive seasonally reoccurring environmental stresses, such as cold, heat or dry seasons, most insects enter diapause (Rinrehart *et al.*, 2000). Initiated by environmental cues, diapause is characterized by developmental arrest, decreased

metabolism (Tauber *et al.*, 1986), and an increase in resistance to stresses (Adedokun and Denlinger, 1984). Diapausing individuals demonstrate striking differences in gene expression when compared with non-diapausing ones: i.e. most genes are silenced but a few are highly upregulated during diapause (Denlinger, 2002). Whereas some studies have found that *HSP70* is upregulated during insect diapause (Rinehart *et al.*, 2000; Yocum, 2001), others have not found the same pattern (Goto *et al.*, 1998; Tachibana *et al.*, 2005). This inconsistency needs to be elucidated with additional species. The onion maggot *Delia antiqua* can be easily induced in laboratory into both summer diapause (SD) and winter diapause (WD) occurring at a comparable developmental stage, the pupal stage (Ishikawa *et al.*, 2000; Chen *et al.*, 2005a, 2005b), thus is an ideal model species for diapause research.

In the present study, we clone the *HSP70* cDNA of the onion maggot *Delia antiqua*, investigate 4 insect species of genomes that have been completely sequenced, characterize HSP70 and HSC70 protein sequences of insects by bioinformatics analyses, and examine HSP70 expression in response to both summer and winter diapause as well as to thermal stress through diapause and in non-diapausing pupae.

Materials and Methods

Experimental insects and treatment. The non-diapausing (ND) colony of *D. antiqua* was reared on an artificial diet at 20°C with a 16L:8D photocycle and 50-70% relative humidity, as described by Ishikawa *et al* (1983). At the fourth day after pupariation (D4) pupae were subject to cold stress at −10°C with 0L:24D, or heat stress at 35°C with 16L:8D, both for 2, 4 or 6 days. To induce SD, larvae were maintained at 25°C with 16L:8D. Newly formed puparia were kept under the same conditions as the larvae until day 15 after pupariation (D15) and then transferred to 16°C and 16L:8D to trigger diapause termination (Ishikawa *et al.*, 2000). A few co-occurring non-diapausing pupae, which could be discriminated by direct observation through the semi-transparent pupal case (Nomura and Ishikawa, 2001), were eliminated at D8. D5 diapausing pupae were subject to cold stress at −15°C with 0L:24D, or heat stress at 35°C with 16L:8D, both for 2, 4 or 6 days. To induce WD, larvae were reared at 15°C with 12L:12D (Nomura and Ishikawa, 2000), and pupae were kept under this condition throughout. The pupae at D40 were treated for 5, 10 or 15 days either at −20°C with 0L:24D or 35°C with 16L:8D. Pupae collected at various stages were snap frozen in liquid nitrogen and stored at −80°C prior to RNA extraction.

cDNA synthesis, cloning and sequencing. mRNA was isolated from the pupae using a MicroPoly(A)Pure™ kit (Ambion). The first-strand cDNA was synthesized from the mRNA using the oligo(dT)-anchor primer from a 5'/3' RACE Kit (Roche Applied Science). After purification with a SUPREC™-02 kit (TaKaRa), a 185 bp of *HSP70* cDNA fragment was amplified by PCR from cDNA using degenerate primers (5'-GYBMTGACHAARGAYRA CAA-3' for forward; 5'-CKWCCYTTRTCRTTYTTDAT-3' for reverse), designed using consensus mRNA sequences of *HSP70* genes (in *sensu stricto*) from 17 insect species showed in Fig. 2. The 185 bp of fragment ranges bp 1518-1702 in the final *HSP70* cDNA sequenced in the present study (GenBank accession number DQ017057). The fragment was then purified with a PCR Purification Kit (Qiagen) and cloned into a pGEM-T vector (Promega). The *HSP70* fragment was amplified from positive clones by PCR with vector-specific primers and sequenced using BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) on an ABI PRIM 377HN Sequencer (Perkin-Elmer).

For 3' RACE (rapid amplification of cDNA ends), the cDNA was amplified with the oligo(dT)-anchor primer and a *HSP70*-specific forward primer (5'-ATGCTAATGGTATTCTCAAT-3', designed based on the cDNA fragment obtained earlier). The PCR product was reamplified using the anchor primer mentioned above and another gene-specific forward primer (5'-AGATGAGCACCGGCA ACGTT-3'). For 5' RACE, template cDNA was synthesized with a *HSP70*-specific reverse primer (5'-AACGTTGCCGGTGCTCATC T-3'). After purification, the 3' end of the cDNA was attached with a poly(A)-tail using dATP and terminal deoxynucleotidyl transferase. Then, the cDNA was amplified by PCR using the anchor primer and a gene-specific primer (5'-ATTGAGAATACCATTAGCAT-3'). The PCR product was amplified once more with the anchor primer and another gene-specific primer (5'-TAGGTTGTTGTCTTTGGTC A-3'). The second-round PCR products for both 3' RACE and 5' RACE were cloned and sequenced as described above. The initial *HSP70* cDNA fragment and cDNA ends derived from the 5' and 3' RACE were edited and assembled with BioEdit (Hall 1999).

Quantitative real-time PCR (Q-RT-PCR). Total RNA was isolated from the pupae using a RNeasy Midi/Maxi kit (Qiagen) and treated with RNase-Free DNase I (Qiagen). cDNA was reverse-transcribed from total RNA using random nanomers with a RNA PCR kit (TaKaRa) and purified with a SUPREC™-02 kit (TaKaRa). The reverse transcribed cDNA samples were used for real time-PCR, which was performed on an ABI PRISM 7700 thermal cycler (PE Biosystems). A *HSP70* cDNA fragment (189 bp) was amplified using gene-specific primers 5'-TGACCAAAGA CAACAACCTA-3' and 5'-GCGACAATCGACCTTTGTCA-3'. The 18S rRNA gene was chosen as a reference for normalizing the *HSP70* mRNA levels. Primers for 18S rRNA were the same as those used by Chen *et al.* (2005a), which amplified a 333 bp fragment. One cDNA sample, diluted to 1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}, was employed as an internal standard. Q-RT-PCR was performed in 20 µl reactions containing 1 µl of template cDNA or the standard, 1× QuantiTect SYBR Green PCR premix (Qiagen), and 0.3 µM of each primer. Thermal cycling conditions were: 95°C for 15 min, 45 cycles of 95°C for 15 s, 58°C for 30 s and 72°C for 30 s, then 40°C for 30 s and 95°C for 1 min with ramp time of 19'59". After Q-RT-PCR, the absence of unwanted by-products was confirmed by automated melting curve analysis and agarose gel electrophoresis of the PCR product. The relative molar amounts of *HSP70* and 18S rRNA transcripts were calculated based on crossing point analysis, using standard curves generated from the cDNA standards. *HSP70* mRNA levels were normalized with those of 18S rRNA in the same samples quantified in the same manner, and the final relative mRNA levels of *HSP70* were averages of five replicates. Regression analysis was carried out with the software Statistica (release 4.5, StatSoft, Inc).

Bioinformatics analyses. The *HSP70* family of protein sequences was retrieved by PSI-BLAST (Altschul *et al.*, 1997) searches against the Arthropoda protein database at NCBI (http://www.ncbi.nlm.nih.gov) using the HSP70Ba and HSC70-4 protein sequences of *D. melanogaster* (accession numbers NP_731716.1 and NP_524356.1, respectively) as queries. The search resulted in a list of similar sequences, which were added by the next round of PSI-BLAST iteration searches. The search continued until no new sequence with an alignment score above the default threshold was retrieved. The cytosolic HSP70 and HSC70 protein sequences were subsequently recognized from the retrieved collection through their characteristic EEVD C-terminus residues (Kiang and Tsokos, 1998; Gupta *et al.*, 1994) or via their high similarity with EEVD-ended sequences for incomplete sequences. The sequences were aligned via Clustal X alignments (Thompson *et al.*, 1997).

Four insect species of nuclear genomes that have been completely sequenced were investigated (Table 1). To localize the members of the HSP70 gene family within each genome, we used MapViewer at NCBI. A TBLASTN search against each genome assembly was applied using *D. melanogaster* HSP70Ba and HSC70-4 protein sequences mentioned above as queries, whereas a BLASTN search was performed with the nucleotide sequences of these two genes as queries. Subsequently, a 200 kb genome sequence flanking each hit, or close hits, was downloaded from the corresponding strand of the chromosome and analyzed as Chen *et al.* (2005c; 2006).

In order to identify biologically significant motifs and domains for each divergent protein sequence we used the program InterProScan (Zdobnov *et al.*, 2001) to search against InterPro, a database of protein domains and functional sites (http://www.ebi.ac.uk/interpro). The signal peptide was predicted using both neural network (NN) and hidden markov model (HMM) methods with the program SignalP v3.0 (Bendtsen *et al.*, 2004; Nielsen *et al.*, 1997). The protein alignment was used to explore HSP70- and HSC70-specific residues and in subsequent phylogenetic analysis. Sequence identity and molecular mass were calculated using BioEdit.

In order to understand the evolutionary relationships of cytosolic HSP70 copies in insects we performed a phylogenetic analysis using protein sequences with PAUP* v4.0b10 (Swofford, 2001). Maximum parsimony was used for phylogenetic inference. We performed an heuristic search employing step-wise addition with 200 random taxon addition sequence replicates and 10 best trees held at each step. All characters were given equal weight and gaps were treated as "missing". As endoplasmic reticulum (ER) HSP70 homologue is the sister of cytosolic HSP70 genes (Boorstein *et al.*, 1994), we used the ER HSP70 sequences of *Drosophila melanogaster* (NP_727564.1) and *Apis mellifera* (XP_393090.2) as a combined outgroup. The node support was assessed using 1000 bootstrap pseudo-replicates with full-heuristic algorithm.

Results

HSP70* cDNA in *Delia antique. Cloning and sequencing of the cDNA fragment amplified by the degenerate primers yielded a 285 bp expressed sequence tag. Based on the tag sequence, we designed 5 gene-specific primers and amplified both the 5' and 3' ends of its cDNA. The full-length *HSP70* cDNA of *Delia antiqua*, assembled from the tag sequence and both 5' and 3' ends, is 2461 bp long and contains a unique open reading frame (ORF) spanning nucleotides 183-2114. It has a polyA tail at its 3' end, and a polyadenylation signal (AATAAA) at nucleotides 2424-2729. The polypeptide deduced from the ORF comprises 643 a.a., with a calculated molecular mass of 70,787 Da and an estimated isoelectric point of 5.65. The deduced sequence was confirmed to belong cytosolic HSP70 gene via alignment with sequences of other insects, and is 85.0% identical to *D. melanogaster* cytosolic *HSP70Ba* (NP_731716.1). The cDNA and its deduced protein sequence were deposited in GenBank with accession numbers DQ017057 and AAY28732, respectively.

Protein sequence properties and phylogenetic relationships. We obtained 144 cytosolic protein sequences from insects (61 for *D. melanogaster* alone) from databases searches. From these, 85 belong to HSP70 and 59 belong to HSC70 protein classes. After removing redundant or unconfirmed, or very similar sequences in each species, 24 HSP70 (including *Delia antiqua* HSP70) and 17 HSC70 complete protein sequences were subject to subsequent protein sequence characterization and phylogenetics analysis.

Using InterProsite searches and primary literature we identified 3 signature motifs, 2 nuclear localization signals, an ATP-binding domain, a substrate binding domain, and α- and β-domain in the substrate binding domain of cytosolic HSP70 proteins from insects (Fig. 1). We recognized 15 a.a. residues that distinguish HSP70 from HSC70 proteins in our alignment. All 41 cytosolic HSP70 sequences terminate with the motif EEVD. No signal peptide, characteristic of a secreted protein, unlike that found for mitochondrial and ER localized proteins, was detected for these protein sequences. The length of HSP70 protein sequences range from 633 a.a. (*Manduca sexta*) to 645 a.a. (*Chironomus yoshimatsui*), making them slightly shorter than those of HSC70 that range from 649 a.a. (*Bombyx mori*) to 656 a.a. (*Cotesia rubecula*). The molecular masses of HSP70 proteins range from 69.5 kDa (*Man. sexta*) to 71.2 kDa (*Chi. yoshimatsui*), slightly smaller than those of HSC70 that ranges from 71.1 kDa (*Apis mellifera*) to 71.9 kDa (*Trichoplusia ni*). The length and mass differences are mainly due to two non-conservative regions recognized in the present study (a and b, Fig. 1), which are slightly shorter in HSP70 proteins.

Genomic analysis revealed that *D. melanogaster* has 8 HSP70 and 2 HSC70 class of genes (Table 1). Six out of 8 HSP70 genes (Aa = Ab, Bb = Bc, Ba and Bbb) are actually very similar with only 0-2 a.a. difference. The *HSC70-2* and *HSC70-3*, which were treated as HSC70 class of genes, are decided to be a gene of HSP70 class, and a gene functioning in endoplasmic reticulum (with terminus sequence KDEL) via sequence and phylogenetic analysis in the present study, respectively. Relatively less, *Anopheles gambiae* has only 1 HSP70 and 2 HSC70 class of genes, and *Tribolium castaneum* has 3 HSP70 and 1 HSC70 class of genes. Surprisingly, we did not find any HSP70 class of gene in *Apis mellifera* but 2 HSC70 genes,

Table 1. Cytosolic *HSP70* genes present on the complete genomes of 4 insect species, with *Delia antiqua HSP70* gene

Gene[a]	Accession number		Chromosomal location[b]	Exon no[c]	a.a. length	Mass (kDa)	Genome size (bp)
	mRNA	a.a.					
Delia antiqua							
HSP70	DQ017057	AAY28732	N/A	N/A	643	70.78	N/A
Drosophila melanogaster							
HSP68 [1]	NM_079750.3	NP_524474.1	3R- 95D11	1(1)	635	69.74	2228
HSP70Aa	NM_169441.1	NP_731651.1	3R- 87A2	1(1)	642	70.16	2630
HSP70Ab	NM_080059.2	NP_524798.2	3R+ 87A3	1(1)	642	70.16	2399
HSP70Ba	NM_169469.1	NP_731716.1	3R- 87B12	1(1)	641	70.18	2474
HSP70Bb	NM_080188.2	NP_524927.2	3R+ 87B14	1(1)	641	70.19	2591
HSP70Bbb	NM_176486.1	NP_788663.1	3R+ 87B14	1(1)	641	70.25	2591
HSP70Bc	NM_141952.1	NP_650209.1	3R+ 87B14-15	1(1)	641	70.19	2211
HSP70D (HSC70-2)[1]	NM_079615.2	NP_524339.1	3R+ 87D10	2(2)	633	69.72	2626
HSC70-1	NM_079339.2	NP_524063.1	3L+ 70C9	2(2)	641	70.68	3347
HSC70-4	NM_079632.4	NP_524356.1	3R+ 88E4	1(2)	651	71.13	3970
Anopheles gambaie							
HSP70-1[1,2]	ENSANGT00000001468.2	ENSANGT00000001468.2	2R-	3	641	70.73	9896
HSC70-1 (HSP70)[1,2]	AAAB01008948.1	EAA10375.2	2L-	1	561	61.36	1683
HSC70-2[1,3]	ENSANGT00000019887.2	ENSANGT00000019887.2	2R+	1	653	71.10	1962
Apis mellifera							
HSC70-1 (HSP70)[1]	XM_623127.1	XP_623130.1	LG15-	3(4)	640	70.38	3729
HSC70-2[1]	XM_392933.2	XP_392933.2	not mapped+	2(3)	658	71.77	4326
Tribolium castaneum							
HSP70-1[1]	newly-predicted	newly-predicted	LG7+	1	645	70.86	1938
HSP70-2[1]	newly-predicted	newly-predicted	LG7+	1	646	70.80	1941
HSP70-3[1]	newly-predicted	newly-predicted	not mapped-	2	689	75.99	6585
HSC70[1]	newly-predicted	newly-predicted	not mapped+	2	649	71.08	2868

[a][1]the gene names are given temporarily in the study with old name in parentheses.
[2]sequence with complete coding region (CDs) only, [3]sequence with incomplete CDs only, and all others are full-length of cDNA.
[b]+, forward strand, preceded by chromosomal name; –, reverse strand, followed by location in a given chromosome. N/A indicates unavailability of the chromosomal information.
[c]Total number of exons in CDs. The numbers in parentheses include exons in untranslated regions.

and the HSP70 gene earlier named for the species is decided to be a HSC70 gene in the present study. There are 1-3 exons in coding region with a genome size smaller than 10 kb throughout all these cytosolic HSP70 genes.

Maximum parsimony analysis of the sequence data resulted in the most parsimonious trees shown in Fig. 2 with tree length = 2049, consistency index = 0.605, homoplasy index = 0.395, retention index = 0.765 and rescaled consistency index = 0.463. Among the 729 a.a. characters used, 251 characters were constant, 121 were parsimony-uninformative and 357 were parsimony-informative. The complete ingroup, HSP70 and HSC70 are separately monophyletic with 100%, 99% and 95% bootstrap supports, respectively (Fig. 2). However, in both HSP70 and HSC70 clades, there is no >50% bootstrap value to support the relationships among different orders of insects. This is mainly due to high conservation of HSP70 sequences and different number of copies in different species.

Expression of HSP70 through diapauses. When onion maggot larvae were reared at 25°C and 16L:8D, 98% of the pupae enter summer diapause (SD) at 2.2 days after pupariation (Ishikawa *et al.*, 2000). Although the SD period is quite variable at 23°C and 16L:8D, lasting between 15-45 days, pupae complete SD and start postdiapause development soon after the temperature decreases to 16°C (Ishikawa *et al.*, 2000). The relative amounts of *HSP70* transcript, normalized to 18S rRNA levels, were very low in both prediapause (0.03-0.21 for D0.5, D1 and D2) and postdiapause pupae (0.55-0.12 for D18, D21 and D24) (Fig. 3). At the onset of SD (D3) the amount increased to 0.86; interestingly, three days after, the amount steeply rised to 4.53 (D6), 5.27 times higher than that for D3. Subsequently the amounts gradually decreased from 3.23 (D9), 2.41 (D12) to 2.04 (D15) with a slope rate of –0.276/day.

Almost all the pupae enter winter diapause (WD) when

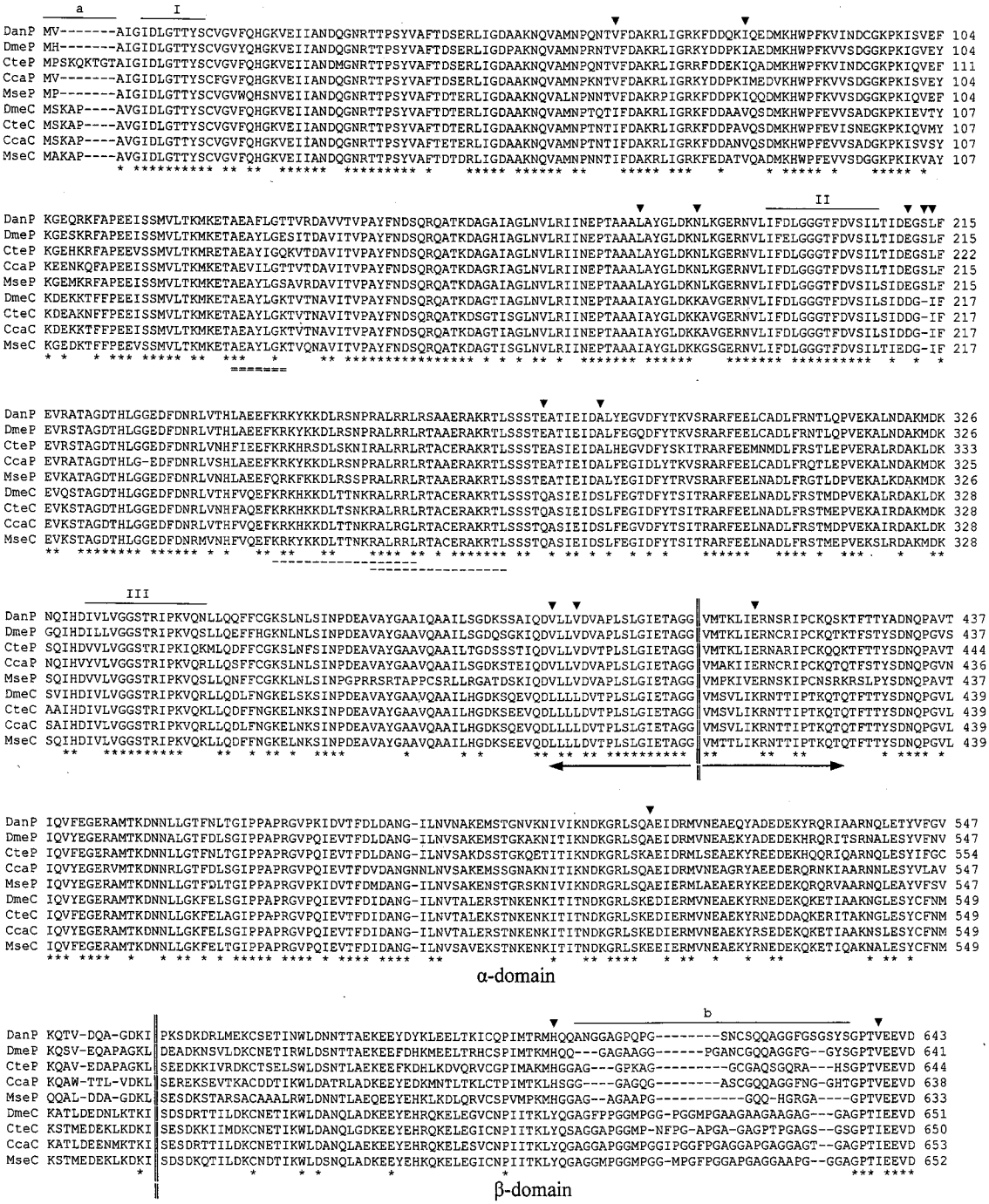

Fig. 1. The representative of the global alignment of cytosolic HSP70 (with P as suffix) and HSC70 (with C as suffix) amino acid sequences of insects, showing their difference, domains and functional regions. For comparison, completely conserved amino acid residues are marked below the alignment with " * ". A " - " corresponds to a gap and the last residue in each line is assigned a number. ▼ stands for distinguishable residues between HSP70 and HSC70. The conservative regions are indicated by: "I, II, III" for three HSP70 protein family signatures (document # PS00297 and PS00329 of database Prosite, and Liu *et al.* 2004); "a, b" for two non-conservative regions recognized in the present study; " = " for ATP/GTP binding site (Liu *et al.* 2004); " - " for nuclear localization signal (Liu *et al.* 2004), respectively. Backward "←" and forward "→" arrows separately indicate ATP-binding and substrate binding domains (Liu *et al.* 2004). The substrate binding domain is divided into α-domain and β-domain (Guy and Li, 1998), separated by "‖". The abbreviation for the sequence names used: HSP70: DanP-*Delia antique* (accession number: DQ017057.1), DmeP-*Drosophila melanogaster* HSP70Ba (NP_731716.1), CteP-*Chironomus tentans* (AAN85117.1), CcaP-*Ceratitis capitata* (CAA70153.1), MseP-*Mandura sexta* (AAO65964.1); HSC70: DmeC-*Dro. melanogaster* HSC70-4 (NP_524356.1), CteC-*Chi. tentans* (AAN14525.1), CcaC-*Cer. capitata* (AAC23392.1), and MseC - *Man. sexta* (Q9U639).

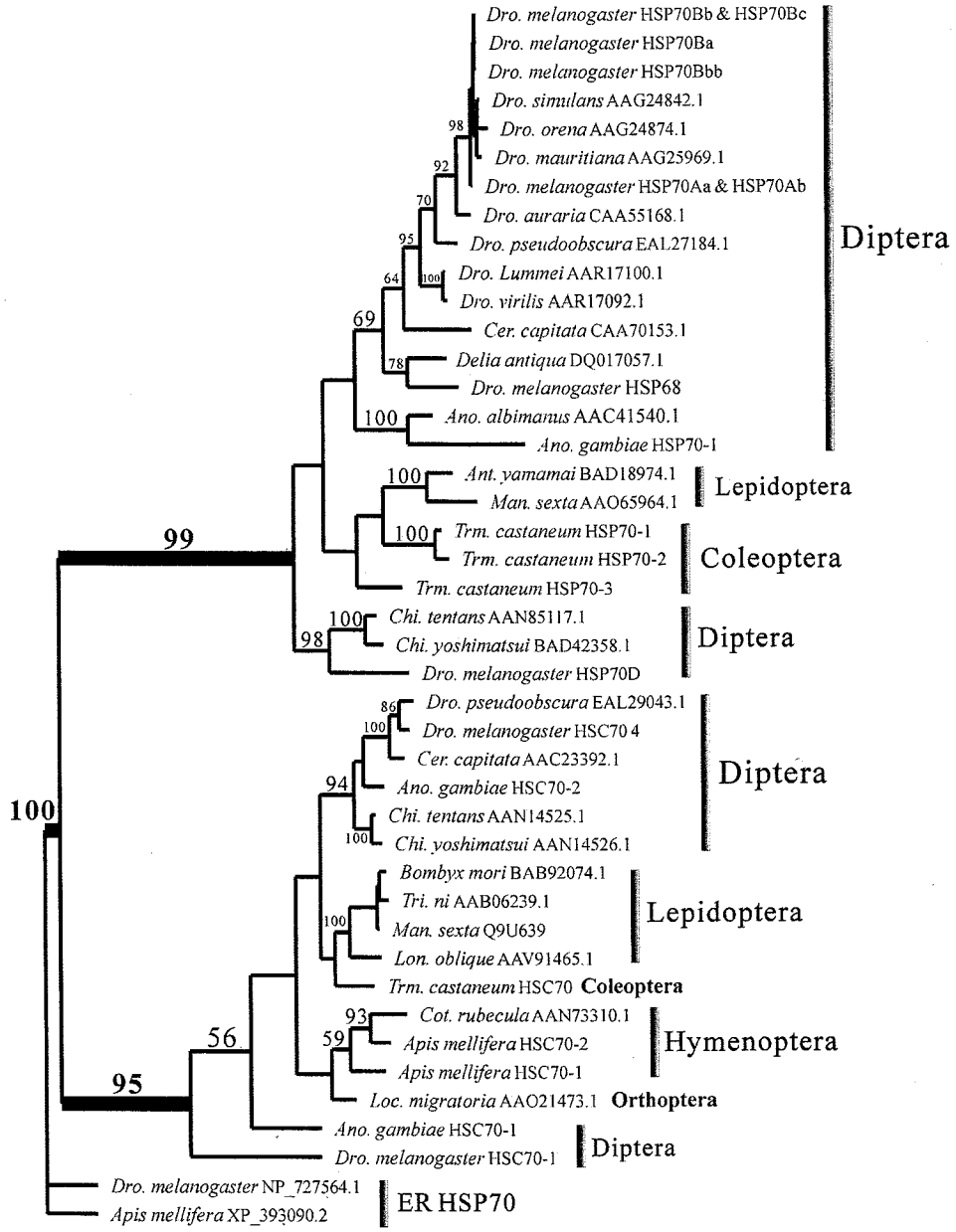

Fig. 2. The most parsimonious tree (2049 steps, see Results for parameter values) inferred from cytosolic HSP70 protein sequences and rooted with endoplasmic reticulum HSP70 homologues. Bootstrap percentages of 1000 replicates are shown above the branches where they exceed 50%. The abbreviations of genera: *Ano-Anopheles*, *Ant-Antheraea*, *Cer- Ceratitis*, *Chi-Chironomus*, *Cot-Cotesia*, *Dro-Drosophila*, *Loc-Locusta*, *Lon-Lonomia*, *Man-Manduca*, *Trm-Tribolium* and *Tri-Trichoplusia.*

larvae are maintained at 15°C and 12L:12D (Nomura and Ishikawa, 2000). WD starts on day 4.1 after pupariation and lasts for about 99 days with a postdiapause period of 23.5 days (Nomura, 2001). In a similar pattern to that observed under SD, the relative mRNA levels in the prediapause stages (D2, D3 and D4) were relatively low (0.15, 0.31 and 0.68), respectively, but increased to 2.52 in the early WD stage (D29; Fig. 3). Then the expression level gradually decreased from 1.97 (D54), to 1.29 (D79), to 0.88 (D105), with a slope rate of –0.022/day (Fig 3). The transcript amounts in three postdiapause stages, 0.49 (D112), 0.3 (D119) and 0.10 (D126) are comparable to the levels found in prediapause stages. The transcript amounts in prediapsuse and postdiapause pupae in SD and WD are similarly low compared to those in ND (between 0.14 at D2 and 0.45 at D6, Fig. 3).

Thermal stress response. Cold-stressing (–15°C in complete darkness) the SD pupae led to increased HSP70 expression levels from 3.30 (D5) to 4.06 (D7); however, heat-stressing (35°C and 16L:8D) the SD pupae from D5 did not significantly influence the expression level in the first two days (Fig. 3). After D7, the mRNA levels in cold- and heat-stressed pupae

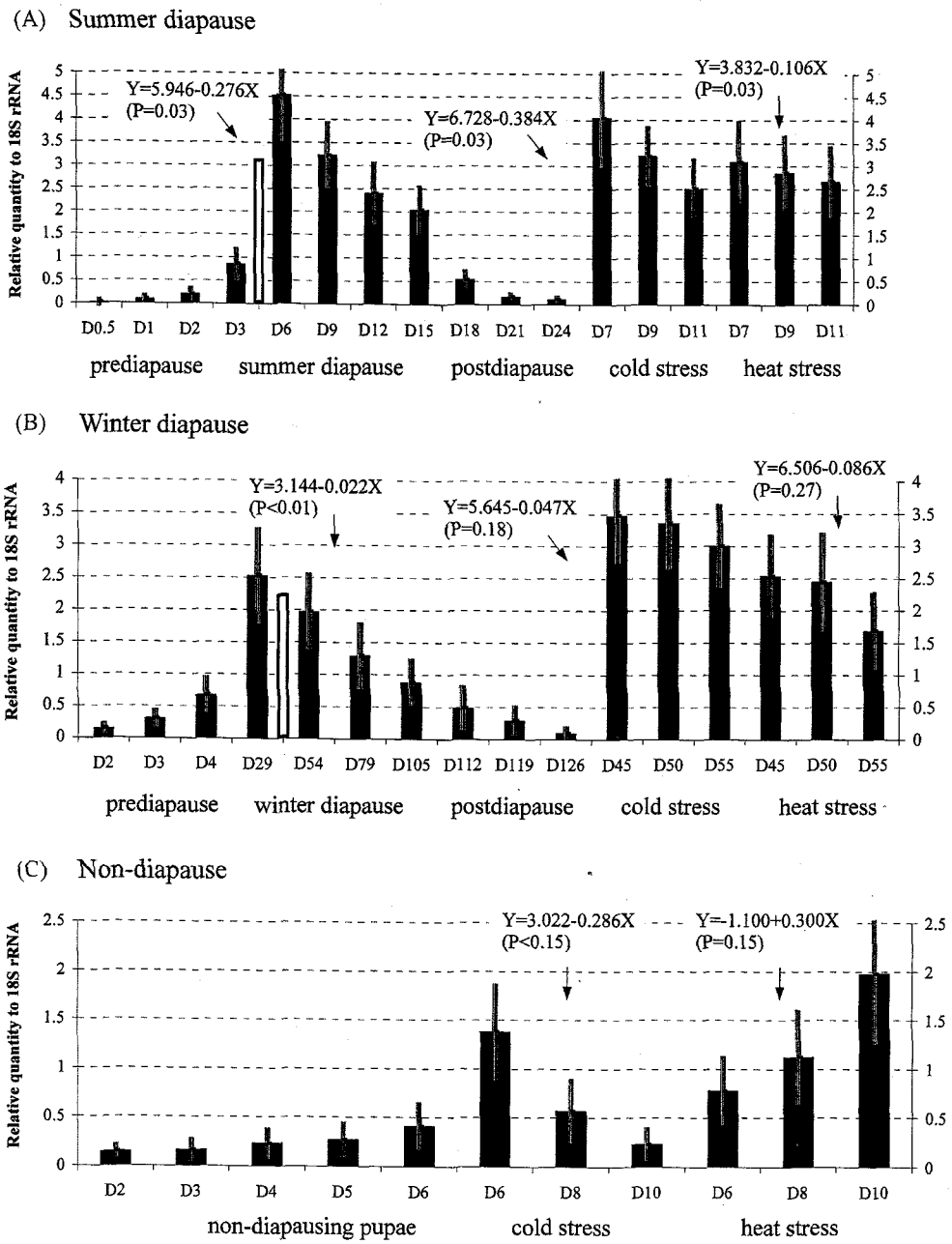

Fig. 3. Q-RT-PCR analysis of *Delia antiqua HSP70* mRNA levels in SD (A), WD (B), ND (C) and under cold- or heat-stress. The relative quantities indicate the levels of *HSP70* transcript normalized to the internal standard 18S rRNA. The shaded bars indicate the standard deviation of five repeats. The unfilled bars indicate the D5 pupae in SD and D40 pupae in WD, which were subject to heat- and cold stresses. Regression equations between the quantity (Y) and the day (X) are shown above each corresponding treatment group with the *P*-value in parentheses.

gradually decreased with treatment duration, consistent with the pattern of unstressed SD pupae. The slope rates of HSP70 decrease for cold- (–0.384/day) and heat-stressed pupae (–0.106/day) were 1.4 times faster and 0.6 time slower than that for unstressed pupae (–0.276/day), respectively (Fig. 3).

Cold-stressing (–20°C in complete darkness) and heat-stressing (35°C and 16L:8D) the WD pupae increased Cold-stressing (–10°C and 0L:24D) and heat-stressing (35°C and 16L:8D) ND pupae led to 3 and 2 fold increases in *HSP70* expression levels, respectively, from D4 toD6. After the D6 stage, mRNA levels in cold-stressed pupae gradually decreased with treatment duration with a slope rate of –0.286/day, whereas those in heat-stressed pupae gradually increased with a slope rate of 0.300/day.

Discussion

In the present study, we cloned the cDNA of *Delia antiqua* cytosolic *HSP70* gene, which is 2461 bp long and encodes a peptide of 643 a.a. with molecular mass of 70,787 Da. The protein sequence deduced from the cDNA is most similar to *D. melanogaster* HSP68 gene with 85.2% identity. We investigated complete nuclear genomes of 4 insect species, and found the cytosolic HSP70 gene copy number changes with different species. All 41 complete HSP70 sequences investigated terminate with EEVD, which supports the claim by Kiang and Tsokos (1998) and Gupta *et al.* (1994) that considered this motif the signature feature of cytosolic HSP70 proteins across all organisms. These sequences are consistently 633-656 a.a. long with molecular masses ranging from 69.5-71.9 kDa, and carrying no signal peptide. Three additional HSP70 family signatures, as well as an ATP/GTP binding site, 2 nuclear localization signals, an ATP-binding domain, and a substrate binding domain (identified from InterProScan searches; Guy and Li, 1998; Liu *et al.*, 2004) are all conserved in insect cytosolic members of HSP70 family proteins. In this study we additionally recognized two highly-variable regions located at the beginning and at the end of all cytosolic HSP70 protein sequences.

This is the first comparative study of insect HSP70 and HSC70 proteins. In previous studies (Lo *et al.*, 2004; Boorstein *et al.*, 1994; Gupta *et al.*, 1994; and Gupta and Singn, 1994), only one insect species, *Dro. melanogaster*, was involved. Our phylogenetic analysis clearly differentiates cytosolic HSP70 proteins into two classes, HSP70 and HSC70. Fifteen characteristic residues can reliably distinguish the two classes of protein sequences in insects. In addition, the length of HSP70 sequences (633-645 a.a.) is shorter than that of HSC70 (649-656 a.a.), and the molecular masses of HSP70 (69.5-71.2 kDa) is smaller than that of HSC70 (71.1-71.9 kDa). The differences are mainly due to two non-conservative regions recognized in the present study (a and b, Fig. 1). We also found that the cytosolic HSP70 sequences are very conserved with 74.6-98.7% sequence identity in HSP70 class and 83.4-97.8% identity in HSC70 class. They do not seem good marker genes for insect phylogeny analysis due to their high conservation and variable gene copies.

We designed the real-time PCR primers from the *Delia antiqua HSP70* sequence in its gene specific region determined by *D. melanogaster* HSP68 and its most homologous copies. Therefore, the expression really come from the *Delia antiqua HSP70* gene cloned if there is more than 1 copy of cytosolic HSP70 class of gene in the species. In *Delia antiqua* we found that *HSP70* is upregulated during WD, and that the transcript level declines to prediapause levels when diapause is terminated. These results are comparable to those found for *HSP70* in *Sar. crassipalpis* WD (Rinehart *et al.*, 2000). The expression pattern of *HSP70* in *Delia antiqua* is comparable to that of some other genes we investigated in *Delia antiqua*, *DaTrypsin*, *TCP-1*, and *HSP90*, which are all upregulated in response to WD (Chen *et al.*, 2005a; Kayukawa *et al.*, 2005; Chen *et al.*, 2005b). In contrast, *HSP70* is not upregulated as a function of diapause in *Dro. triauraria* and *Lucilia sericata* (Goto *et al.*, 1998; Tachibana *et al.*, 2005). *HSP70* is slightly upregulated in the diapausing adults of Colorado potato beetle *Leptinotarsa decemlineata* (Yocum, 2001), and is only expressed in the diapausing pharate first instar larva of the gypsy moth *Lymantria dispar* after exposure to low temperature (Yocum *et al.*, 1991). Variation among species is also found in the expression of other heat stress genes, *HSP23* and *HSP90*. These genes are not regulated in *Dro. triauraria* and *Luc. sericata* during WD (Goto and Kimura, 2004; Tachibana *et al.*, 2005), whereas *HSP23* is highly upregulated and *HSP90* is down-regulated in *Sar. crassipalpis* (Yocum *et al.*, 1998; Rinehart and Denlinger, 2000). These results indicate that gene expression in response to WD diapause is species-specific.

This is the first study on *HSP70* expression in response to SD, and, thus, generalities relative to this gene's pattern of expression will only be possible once SD is further investigated in other species. In *Delia antiqua* under SD, *HSP70* transcript levels rise in the first three days and then gradually decrease with time. This pattern is not mimicked by *HSP90* transcripts where levels increase sharply in the first few days and then gradually increase with time (Chen *et al.*, 2005b), or by *DaTrypsin* transcript levels that rise in the first few days and then remain at similar levels until SD termination (Chen *et al.*, 2005a).

HSP70 transcripts are usually undetectable or constitutively expressed at low levels under normal (non-diapausing) conditions, but transcription is induced by heat and cold stresss in a variety of insect species (Goto *et al.*, 1998; Rinehart *et al.*, 2000; Tachibana *et al.*, 2005). Our results are consistent with these earlier reports. In contrast to the results obtained for other species (Rinehart *et al.*, 2000; Yocum *et al.*, 1991; Denlinger *et al.*, 1992), we showed that during diapause, heat stress significantly rised *HSP70* expression in *Delia. antiqua*. However, as in two of the previous studies (Yocum *et al.*, 1991; Denlinger *et al.*, 1992), cold stress did not obviously affect *HSP70* expression within a short period after the treatment (2 days for SD, and 5 days for WD). After this period, and despite the continuation of the temperature shock, the expression levels gradually decreased in both cold and heat stress treatments for both SD and WD.

To date, *HSP70* is the better-studied HSP gene regarding its response to various stresses (Sorensen *et al.*, 2003). HSP70 has been found to be up-regulated in response to high larval rearing density (Sorensen and Loeschcke, 2001), high inbreeding coefficient (Kristensen *et al.*, 2002), desiccation (Hayward *et al.*, 2004), and nutrient deprivation (Salvucci *et al.*, 2000). In addition, increased levels of HSP70 following heat-stress were found to correlate with increased longevity in transgenic *Dro. melanogaster* lines carrying extra copies of the gene (Tatar *et al.*, 1997). A single study looked at the expression of both *HSP70* and *HSC70* genes following

temperature stresses. While in non-diapausing pupae of the flesh fly, *Sar. crassipalpis*, heat-stresses only elevated the levels of the inducible *HSP70*, cold stresses up-regulated both the inducible and the constitutive *HSC70* (Rinehart *et al.*, 2000). It appears that our current knowledge of the function of these proteins in insects is still quite incomplete. Future comparative and functional studies should attempt to target both *HSP70* and *HSC70* genes. Like the earlier studies mentioned above, the expression experiments in the present study also only targeted the inducible *HSP70* gene.

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