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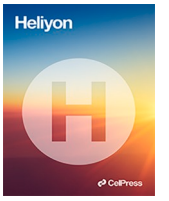

Research article

The analysis of inducible family members in the water flea *Daphnia magna* led to the identification of an uncharacterized lineage of heat shock protein 70

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ABSTRACT

To explore the function and evolutionary relationships of inducible heat shock protein 70 (Hsp70) in *Daphnia magna*, cDNAs of four Hsp70 family members (*DmaHsp70*, *DmaHsp70-2*, *DmaHsp70-12*, *DmaHsp70-14*) were cloned. While all DmaHsp70s possess three function domains, it is noteworthy that only DmaHsp70 ends with a "EEVD" motif. Phylogenetic analysis indicates that the Hsp70-12 lineage is distanced from the rest, and therefore it is an uncharacterized lineage of Hsp70. The differences in isoelectric point and 3-dimensional (3D) conformation of the N-terminal nucleotide binding domain (NBD) of DmaHsp70s further support the theory. DmaHsp70s exhibit varied motif distribution patterns and the logo sequences of motifs have diverse signature characteristics, indicating that different mechanisms are involved in the regulation of ATP binding and hydrolysis for the DmaHsp70s. Protein-protein network together with the predicted subcellular locations of DmaHsp70s suggest that they likely fulfill distinct roles in cells. The transcription of four DmaHsp70s were changed during the recovery stage after thermal stress or oxidative stress. But the expression pattern of them were dissimilar. Collectively, these results collectively elucidated the identification of a previously uncharacterizedHsp70 lineage in animal and extended our understanding of the Hsp70 family.

1. Introduction

The 70-kDa heat shock proteins (Hsp70s) are a family of the Hsp superfamily, a highly conserved group of proteins functioning as molecular chaperones in almost all the living species [1,2]. Of the superfamily, Hsp70s are one of the most well-characterized members for their involvement in a wide range of housekeeping processes in cells, including the folding of newly synthesized proteins, the translocation of polypeptides into mitochondria, chloroplasts and endoplasmic reticulum (ER), the disassembly of protein complexes and the regulation of protein activity [3–5]. Additionally, within cells, Hsp70 also prevents the aggregation and promotes the refolding of misfolded denatured proteins, solubilizes aggregated proteins and cooperates with cellular degradation machinery to clear aberrant proteins and protein aggregates [2,6,7]. Consequently, the primary function of Hsp70 is to safeguard cells against various proteotoxic stresses such as extreme temperature, heavy metal, toxic organic chemicals, abiotic stresses and so on [6,8–10].

Hsp70 contains two groups, the stress-inducible Hsp70 and the constitutive Hsp70 [11,12]. Traditionally, the former group is

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considered to be induced by environmental stimulation and returns to basal levels thereafter, while the latter group is reckoned to continuously express in cells to maintain correct protein folding without responding to environmental stress [13]. Nonertheless, all family members share a common structural framework, with a N-terminal nucleotide binding domain (NBD) followed by a substrate binding domain (SBD) and a variable region [14]. For eukaryotic species, Hsp70 generally ends with a conserved motif (Glu-Glu--Val-Asp; EEVD), which is functional in interacting with cofactors of Hsp70s [15,16]. In metazoan, Hsp70s are grouped into four lineages, cytosolic A, cytosolic B, endoplasmic reticulum (ER), and mitochondria, with *Drosophila* Hsp70s falling into these four groups [17]. A previous study further classified cytosolic Hsp70s into eight clades based on their phylogenetic relationships. And Hsp70s in different clades tend to have varied subcellular localization and probably different functions [18]. Up until now, 13 Hsp70 members were identified in Human being, with most of them distributed to the four lineages or eight clades, but there are some non-canonical Hsp70s including HspA12, HSPA13 and HspA14 that could not be classified into the traditional groups [18,19].

Given its upregulation in response to environmental challenges, Hsp70 is considered as a reliable stress indicator [14,20]. In arthropods that constantly facing harsh environments, Hsp70 is critical. Hsp70 protected the apple maggot *Rhagoletis pomonella* against high temperature and aids survival in the gall fly *Eurosta solidaginis* upon freezing [21,22]. Moreover, the synthesis of Hsp70 occurred in several *Drosophila* species during the recovery from heat or cold stress [23,24]. Other than in insects, Hsp70 synthesis was activated by the hypothermic, hyperthermic and oxidative stresses in shrimps and several amphipods 6, [25]. Notably, within a given arthropod species, more than one type of Hsp70 may exist, and the varied expression patterns of Hsp70s against environmental challenges are documented. For example, *Drosophila Hsp68*, *Hsp70Aa* are cold inducible, whereas *Hsc70-1* is not [26,27]. And *PmHsc70, PmHsp70a* and *PmHsp70b* are all inducible by heat shock of *Pieris melete* under summer diapause but *PmHsc70* is upregulated more significantly than the other two [28]. Such distinct responses of Hsp70s to stresses imply divergent roles within organisms.

The water flea *Daphnia magna* is a planktonic crustacean ubiquitously found in the fresh water environment [29]. It is a keystone species in the freshwater ecosystem and occupy an important position in the aquatic food chain. *D. magna* has long been used as a model crustacean mainly in ecological and toxicological studies [30–32]. Living mostly in small ponds and rivers, *D. magna* constantly encounters various environmental challenges [33]. Therefore, Hsp70s are critical to their survival in the unstable environment. Previous research identified two *hsp70s* from *D. magna* and demonstrated that both of them were functional against heat stress during embryo development [34]. However, with the *D. magna* genome available, more Hsp70s are to be found in the crustacean. Here, we established inducible Hsp70s in *D. magna* and further investigate their functions and evolutionary relationships.

In the current study, we identified and cloned four *Hsp70s* from *D. magna.* The transcription of *DmaHsp70*s increased upon heat shock, cold shock or oxidative stress despite the varied expression patterns. But phylogenetic analysis together with the differences in signature sequences indicated that *DmaHsp70-12* diverged from the rest three Hsp70s. Structural comparison and analysis of DmaHsp70s confirmed the theory that the Hsp70-12 subgroup was distanced from other Hsp70s. Together, the study of inducible Hsp70s in *D. magna* described the uncharacterized lineage of Hsp70-12 in the Hsp70 family and hinted a different origin of Hsp70-12.

Table 1
Primers and their applications in this study.

Primer name	Primer sequences (5′–3′)	Tm (°C)	Objective
DmaHsp70AF	CGTCACCTCGGCAAATTTAC	55.40	Full-length amplification
DmaHsp70AR	CGAGCAAATTAGCTTAGGAGC	55.61	Full-length amplification
DmaHsp70-2 GSP1	AACGAACCCTGGTGATGCACGAACTG	62.75	1st round 5′-RACE
DmaHsp70-2 NGSP1	CGCAAGCTAAACGAGGACGATGCAAAGC	64.01	2nd round 5′-RACE
DmaHsp70-2 GSP2	GATTACGCCAAGCTTCGTTGTCACCGTAGGAA	64.78	1st round 3′RACE
DmaHsp70-2 NGSP2	GATTACGCCAAGCTTTCTTATGCCAATCA	59.62	2nd round 3′-RACE
DmaHsp70-12 GSP1	TTCGGTTTTCAGTTGGTGTCGTTTC	57.94	1st round 5′-RACE
DmaHsp70-12 NGSP1	CACTTGGTGAGGTAAACGATCAGAACC	61.12	2nd round 5′-RACE
DmaHsp70-12 GSP2	CGGTTCTGATCGTTTACCTCACCAAG	61.17	1st round 3′-RACE
DmaHsp70-12 NGSP2	TTAGGCAAACCAGGACCTCTGAAACC	61.17	2nd round 3′-RACE
DmaHsp70-14 GSP1	ATGCCTCCACGAACATTAACCAC	57.77	1st round 5′-RACE
DmaHsp70-14 NGSP1	TGGGCAGACAGATGTGGACTGGA	61.33	2nd round 5′-RACE
DmaHsp70-14 GSP2	TGCTGCTTGGTGTATCGCTGTG	59.54	1st round 3′-RACE
DmaHsp70-14 NGSP2	AGCGACGCTGGATGTCTCCGTGGT	64.69	2nd round 3′-RACE
UPM primer	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT*	72.00	1st round 5′-RACE
Short primer	CTAATACGACTCACTATAGGGC*	51.3	2nd round 5′-RACE
Outer primer	GCGAGCACAGAATTAATACGACT*	58.6	1st round 3′-RACE
Inner primer	CGCGGATCCGAATTAATACGACTCACTATAGG*	72.00	2nd round 3′-RACE
DmaHsp70 qF	CGGCATTTTGAACGTTACTG	56.90	qRT-PCR
DmaHsp70 qR	CTTAATGGCTTCACTGCACT	53.00	qRT-PCR
DmaHsp70-2 qF	CATTGGAATTGACCTTGGTAC	53.66	qRT-PCR
DmaHsp70-2 qR	ATCGACATCTTTGAGTTGACG	53.66	qRT-PCR
DmaHsp70-12 qF	ACACCAACTGAAAACCGAA	50.85	qRT-PCR
DmaHsp70-12 qR	TCCACAATCCACGACCATA	53.01	qRT-PCR
DmaHsp70-14 qF	CACAGGCTGGTCTGACTGC	59.48	qRT-PCR
DmaHsp70-14qR	ATCGTATCCCAAGGTTCTCC	55.40	qRT-PCR

*Commercial primers provided by manufacturer.

2. Materials and methods

2.1. Animal culture

D. magna was purchased online from Qingxiang Aquarium (Yantai, Shandong province) and parthenogenetically raised in the lab. Animals were maintained at a temperature of 22 ± 1 °C under natural sunlight in an aquarium and fed daily with an appropriate amount of algae puree. The remnants and exuvia were removed weekly and half of the water was exchanged every other day with newly prepared dechlorinated tap water. The studies and the use of animals were approved by the School of Marine Science and Engineering, Qingdao Agricultural University by following the national general standard.

2.2. RNA extraction, cDNA synthesis and cloning of D. magna Hsp70s

Total RNA was extracted from twenty *D. magna* heat-shocked at 35 °C for 20 min using FastPure Cell/Tissue Total RNA isolation Kit V2 Vazyme Cat (Vazyme Biotech, Nanjing, China). Reverse transcription was performed with 1.0 μg of total RNA as a template for first-strand cDNA synthesis using PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa Biotechnology (Beijing), Beijing, China).

Partial fragments of Hsp70s from the genomic library of *D. magna* were identified. The partial sequences were then amplified in a mixture with 2 μl of each specific primer (Table 1), 25 μl PCR mixtures (Vazyme Biotech), 19 μl H_2O, 2 μl first-strand cDNA as template respectively. The products were resolved on 1.2 % agarose gels and DNA bands of appropriate lengths were recycled using a FastPure Gel DNA Extraction Mini Kit (Vazyme Biotech) and sequenced by Ruibio Biotech (Qingdao, China).

To obtain full-length *D. magna Hsp70*s, rapid-amplification of cDNA ends (RACE) was employed. Gene-specific primers were designed based on the acquired partial sequences (Table 1). The 5′ and 3′ ends of *D. magna Hsp70*s were amplified with SMARTer RACE 5′/3′Kit (Vazyme Biotech) using nested PCR. Amplification products were resolved on agarose gels and DNA bands of the correct sizes were recovered using a FastPure Gel DNA Extraction Mini Kit (Vazyme Biotech), ligated into the pMD-19T vector (TaKaRa Biotechnology (Beijing)), and used to transform *Escherichia coli* competent cells (DH5α) (TransGen Biotech). Positive clones were selected, cultured and then sequenced by Ruibio Biotech. The *D. magna Hsp70*s acquired were named as *DmaHsp70*s, to avoid confusion with *Drosophila melanogaster Hsp70*s. All *DmaHsp70* sequences were uploaded to GenBank.

2.3. Sequence analysis

Open reading frames (ORF) of *DmaHsp70*s were identified by NCBI ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Identifications of the cDNA clones were determined by the BLAST search in GenBank (http://www.ncbi.nlm.nih.gov/BLAST). The molecular weights (Mw) and isoelectric point (pI) values were calculated by SMS2 server (http://www.detaibio.com/sms2/) and presented with Origin software package (version 9.8.0.200, Origin Lab, Massachusetts, USA). The subcellular localization of DmaHsp70s were predicted by WoLF PSORT online server (https://wolfpsort.hgc.jp). SMART server (SMART: Main page (embl.de)) was employed to identify the conserved domains of DmaHsp70s using default parameters. Characteristic sequences of DmaHsp70s were identified by Prosite (https://prosite.expasy.org/). The 3D structure of the DmaHsp70s were simulated by homology modeling (https://swissmodel.expasy.org/). PyMOL software (version 2.4, Schrödinger, New York, USA) was used to visualize the 3D structures. DmaHsp70s were aligned respectively with Hsp70s from other species in Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) using default setting and presented by the DNAMAN9 software package (Lynnon Corporation, Pointe-Claire, Quebec, Canada).

2.4. Phylogenetic analysis

Phylogenetic tree was constructed by MEGA 10.0 software (Mega Limited, Auckland, New Zealand) using the Maximum Likelihood method with 200 bootstrap replications under Poisson model. DmaHsp70s and 104 full-length Hsp70 amino-acid sequences from other animals including metazoan Hsp70s that used to mark the different lineages as well as three Hsp90s as an outgroup were aligned. The animals used and the corresponding Genbank IDs were listed in Supplementary Table S2.

2.5. Comparison of NBDs and motif identification

Three signature motif sequences in NBDs of DmaHsp70s were identified and visualized with the MEME program version 5.3.3 (https://meme-suite.org/meme/tools/meme) using default setting. Motif logos were computed by the MEME program. Multiple sequence alignments of the NBDs of DmaHsp70s were performed with Clustal Omega and presented by the DNAMAN9 software package.

The 3D structure of the NBDs of DmaHsp70s were simulated by homology modeling. PyMOL software was used to visualize the 3D structures and to perform the structural alignment. The Root-mean-square deviations (RMSD) between 3D structures were calculated by VMD software. The numbers of α-helixes and β-strands in the NBDs were calculate based on the simulated 3D structures.

2.6. Protein-protein interaction (PPI) network study

The PPI data of DmaHsp70s was obtained from the String server (version11.5, https://string-db.org). The size & brightness of nodes were drawn according to their degree values, and the size of edges were generated according to their combined scores. The Cytoscape

software (version3.9.1) was used to construct the interaction network maps.

2.7. Sub-lethal heat and cold treatments

In order to obtain the sub-lethal temperature of *D. magna*, a serial temperature treatment was performed. Six treatment temperatures were set for *D. magna* from 35 °C to 45 °C with a 2 °C interval in the Digital display constant temperature water bath (Changzhou Nuoji Instrument, Changzhou, Jiangsu, China). Ten adult animals were randomly picked and put into each treatment group. All treatments were maintained for 30 min and repeated at least three times. The number of dead animals were recorded. A standard curve was created based on the data acquired and a sub-lethal temperature of 39 °C for *D. magna* was calculated based on the standard curve (Supplementary Fig. S3). Sub-lethal temperature of cold treatment was determined by putting ten *D. magna* in a temperature set of water from 2 °C to 10 °C with a 2 °C interval in a biochemical incubator (Zhongxingweiye, Beijing, China) for 30 min. The experiment was repeated three times. It was shown that 4 °C is the lowest temperature to cause 50 % animals enter hardening without dying (Supplementary Fig. S3), and all the animals going through cold hardening could return to normal action within 5 min after putting into water of 23 °C.

To generate a sub-lethal heat shock on *D. magna*, twenty animals were placed in a 500 ml beaker with 200 ml water and incubated in a 39 °C water bath. For cold shock, the same amount of *D. magna* was incubated in 4 °C water bath. Animals incubated at room temperature were used as control. After 30 min of heat or cold treatment, the animals were left to recover at room temperature. The treated *D. magna* were collected separately at 0, 2, 4, 6, 8 h during recovery. Every treatment at each time point was repeated three times.

2.8. Hydrogen peroxide treatment

In order to obtain the sub-lethal concentration of hydrogen peroxide for *D. magna*, a primary treatment was performed. Treatment sets of five concentrations from 0.002 g/ml to 0.032 g/ml were used. Ten animals in a 500 ml beaker containing 200 ml water with specific concentration of hydrogen peroxide were considered as a trial. Each treatment concentration contained three trials. All treatments were maintained for 6 h, with half of the solution changed once every 2 h to maintain a relatively stable hydrogen peroxide concentration. The number of the dead animals were recorded. A standard curve was created based on the data acquired and a sub-lethal concentration of hydrogen peroxide for *D. magna* was calculated based on the standard curve (Supplementary Fig. S3).

D. magna was then treated with the sub-lethal concentration of hydrogen peroxide. Twenty animals were placed in a 500 ml beaker with 200 ml hydrogen peroxide solution at a concentration of 0.008 g/ml for 6 h. Same number of animals incubated in 200 ml dechlorinated tap water were used as control. Animals were collected separately at 0, 2, 4, 6, 8 h after treatments. Three trials were performed for each time points.

2.9. Quantification of DmaHsp70s by real time quantitative PCR

Real time quantitative PCR (RT-qPCR) was used to determine expression patterns of Hsp70s in response to temperature stresses. Primers used for the amplification were as listed in Table 1. *GAPDH* was chosen as a reference gene because the relatively stable expression during treatment [34]. Total RNA of all samples, including the animals treated by heat, cold or oxidative stress, was extracted using the method mentioned above and cDNAs were synthesized. Transcript levels of each gene were estimated from the Ct (cycle threshold) value, and normalized against the reference gene. The relative mRNA levels were calculated using the $2^{-\triangle\triangle CT}$ formula. All data were analyzed using a one-way ANOVA followed by Tukey's multiple range test for pairwise comparison ($p < 0.05$) and expressed as means ± SE. All statistics were carried out using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Characterization of DmaHsp70s

Using the RACE method and based on the partial sequences acquired from NCBI，cDNAs of four *Hsp70*s were cloned from *D. magna* and designated as *DmaHsp70*, *DmaHsp70-2*, *DmaHsp70-12* and *DmaHsp70-14* respectively. All sequences were uploaded to Genbank and the assigned accession numbers were as in Table 2. The putative isoelectric points (pI) of the DmaHsp70s are of 5.1–8.51 with DmaHsp70-12 the most alkaline and DmaHsp70-14 the most acidic (Table 2). The four DmaHsp70s have predicted molecular weights

Table 2
Characterization of identified DmaHsp70s.

Gene	ORF Length(bp)	Protein Sequence (aa)	Predicted subcellular location(s)	Mw (kDa)	pI	Accession No.
DmaHsp70	1947	648	Nucleus, Cytoplasm	70.96	5.10	OP597763
DmaHsp70-2	1842	613	Cytoplasm	68.06	7.98	OP597765
DmaHsp70-12	2010	669	Cytoplasm. Mitochondrion. Endoplasmic reticulum.	75.18	8.51	OP597766
DmaHsp70-14	1506	501	Cytoplasm	54.25	6.62	OP597767

(Mw) of 54.25 kDa–75.68 kDa, with DmaHsp70-14 has the smallest Mw and DmaHsp70-12 the largest (Table 2). Similar to the result in *D. magna*, the average pI of the Hsp70-12 subgroup is the highest and the Hsp70-14 the smallest. The average Mw of Hsp70-12 is the largest and the others are more or less the same (Fig. 1A and B).

The predicted subcellular localizations of the DmaHsp70s are as listed (Table 2). DmaHsp70 locates to nucleus, DmaHsp70-2 and DmaHsp70-14 locate to cytoplasm, and DmHsp70-12 is the only one with the potential to locate to ER and mitochondrial besides cytoplasm. The acquired DmaHsp70s all have NBD and SBD connected by linker, but only DmaHsp70 ends with "EEVD" motif (Supplementary Figs. S1A–D, Fig. 2A). The 3D configuration is simulated for the DmaHsp70s, with NBD as indicated (Fig. 2B–E). Signature sequences of Hsp70 were identified in the DmaHsp70s (Supplementary Figs. S1A–D). Three conserved sequences IDLGTTYS (residues 10–17), LIFDLGGGTFDVSIL (residues 197–211) and IVLVGGSTRIPKVQK (residues 335–349) are found in DmaHsp70. Similarly, three conserved sequences IDLGTTY (residues 50–56), LIFDLGGGTFDVS (residues 233–245) and IIVIGGSTRIPKIRK (residues 375–389) are identified in DmaHsp70-2. But in DmaHsp70-12, only two signature sequences IDFGTTYS (residues 133–140) and MVVDCGGGTVDIT (residues 375–387) are found. And in DmaHsp70-14, the two predicted signature sequences are LHVGRTNS (residues 7–14) and VIVCGGTAKMPRLQQ (residues 334–348). What's more, a GGAP motif was identified in DmaHsp70 but not other DmaHsp70s.

3.2. Phylogenetic relationships of DmaHsp70s

A phylogenetic tree was constructed with the Hsp70s from *D. magna* as well as other species using three arthropod Hsp90s as an outgroup. But because Hsp70-12 and Hsp70-14 are rarely investigated in arthropods, both Hsp70s from available species were collected to build the phylogenetic tree. The Hsp70 clades and lineages defined by Yu et al. [18] were applied in this study. Four DmaHsp70s are allocated to different lineages. DmaHsp70 and DmaHsp70-2 are cytosolic Hsp70s allocated to cytosolic B lineage (purple-colored shade) (Fig. 3). The result is in consistent with their subcellular localization. To be noticed is that DmaHsp70-12 and DmaHsp70-14 were not included in the traditional lineages. Especially DmaHsp70-12, together with other Hsp70-12s, formed a lineage distanced from the rest Hsp70s (Fig. 3). Alignment of Hsp70 groups respectively confirmed the conservatism of the Hsp70 groups (Supplementary Figs. S2A–D).

3.3. NBDs of DmaHsp70s have different numbers of conserved motifs

To better understand the biological functions of DmaHsp70s, the conserved protein domain structure was constructed and analyzed. Typical Hsp70 has NBD, SBD and the variable domain. Alignment of the amino acid sequences of NBDs from four DmaHsp70s was carried out using Clustal Omega and the motif sequences were boxed. DmaHsp70 and DmaHsp70-2 has all three signature motifs of a typical Hsp70, while DmaHsp70-14 has two and DmaHsp70-12 has only one in NBD (Fig. 4A and B). Sequence logos generated with MEME show that Box1 of Hsp70 has a higher conservativity than the other two Hsp70 groups. Nevertheless, Box1 of all three Hsp70 groups has similar V[x]AN[xx]G[x]R[x]TP logo. In the second box or the middle box, Hsp70 and Hsp70-2 exhibit very similar pattern, with six conserved lysine (K) and three glutamic acid (E) as well as a few other conserved amino acid residues. As for box3, Hsp70 and Hsp70-12 groups have the same logo pattern. Hsp70-12 shares a V[x]TVPA with the first two Hsp70 groups. And all four Hsp70s have a conserved EP[x]AA logo (Fig. 4C).

3.4. 3-D structure of NBD in DmaHsp70-12 is different from in the other three DmaHsp70s

The protein domains with critical roles in organisms tend to have conserved primary and tertiary structures [35]. Therefore, to investigate the characteristics of different Hsp70s, 3D structure of NBD was obtained and the conserved motifs were marked with different colors (Fig. 5A–D). NBD of the DmaHsp70s contains 12–14 α-helixes and 13–16 β-strands (Fig. 5F), with no huge difference among the four DmaHsp70s. But further 3D alignment of NBDs of the four Hsp70s acquired shows that DmaHsp70, 70-2, 70-14 are

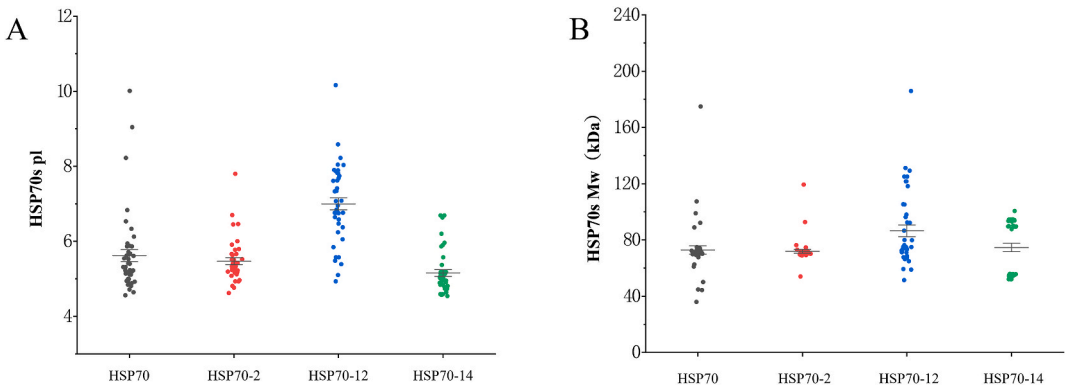


Fig. 1. The pI (A), Mw (B) of four type of Hsp70s from different organisms.

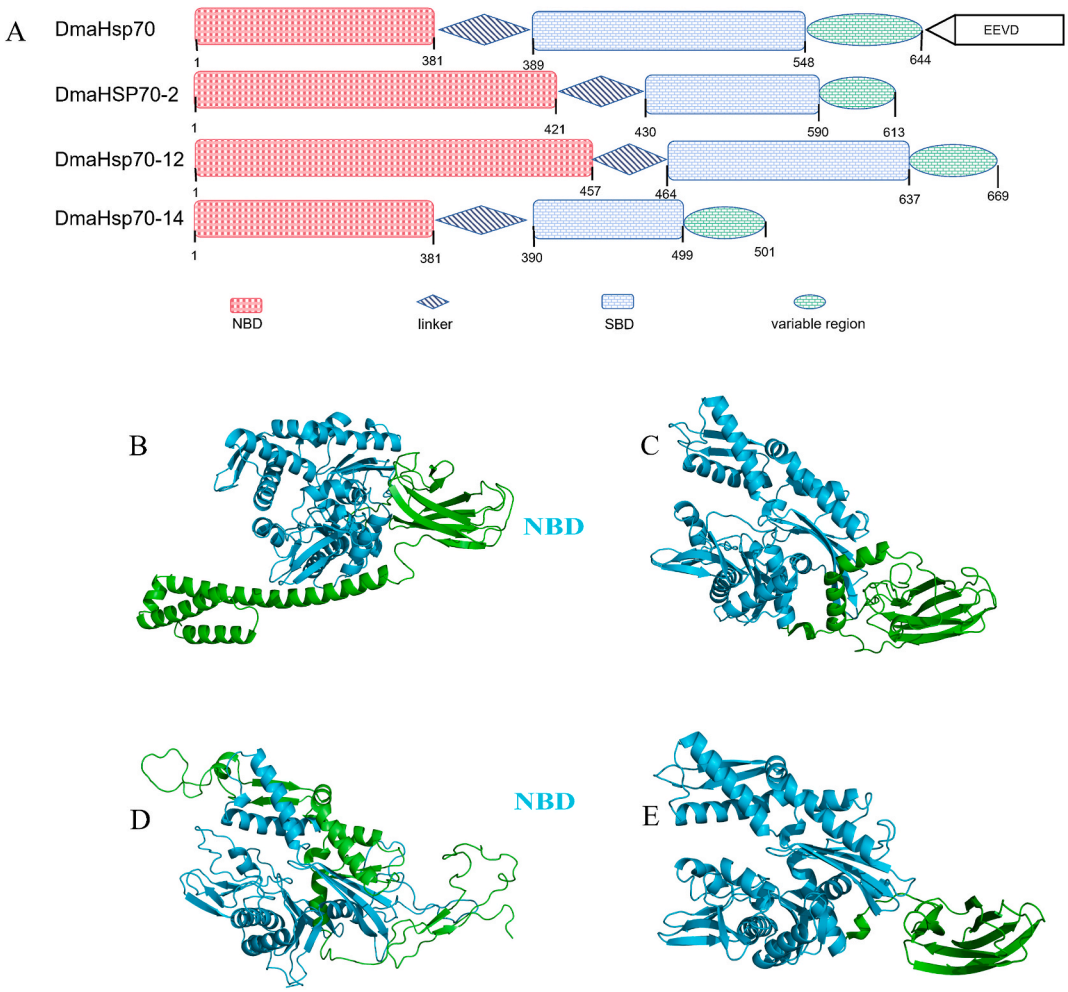


Fig. 2. Structural analysis of DmaHsp70 proteins. (A) Schematic representation of the domain organization of DmaHsp70s. Different colors and shapes represent different domains & regions. (B–E) The 3D model of DmaHsp70s simulated and marked NBDs (Blue color). DmaHsp70 (B), DmaHsp70-2 (C), DmaHsp70-12 (D), DmaHsp70-14 (E). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

similar in spatial conformation, with a RMSD ranging from 0.169 to 0.51 by pair-to-pair comparison (Fig. 5E–G). But the spatial conformation of DmaHsp70-12 is different from the other three, with a RMSD value ranging from 6.833 to 8.172 compared to the rest DmaHsp70s individually (Fig. 5E–G).

3.5. The PPI networks of DmaHsp70s

In order to understand the different functions of the DmaHsp70s, PPI networks were constructed based on Hsp70 analogs of other crustaceans. A total of 34 nodes are presented in the network (Fig. 6). The combined network shows that there are several key proteins inside. The protein to be noticed is a constitutively expressed Hsp, HSC4, that interacts with 22 nodes, with a degree value of 22. What's more, there are 26 other nodes with degree values greater than or equal to 10. Among them, Nup133, Nup85 and WRRD-contain interact with 19 nodes and Nup37 with 18 nodes (Fig. 6A–E). DmaHsp70, DmaHsp70-12 and DmaHsp70-14 are predicted to interact with Heat shock factor (HSF), a transcriptional factor for HSPs. To be noticed, DmaHsp70-12 interacts with several Dynein subunits or chains and DmaHsp70-14 interacts with various nuclear pore complex proteins, with the interaction not shared by other DmaHsp70s (Fig. 6D and E).

3.6. Expression analysis of DmaHsp70s upon temperature stresses

To clarify the role of *DmaHsp70s* upon temperature stress, the mRNA transcription levels were measured in *D. magna* incubated in a non-lethal heat shock temperature of 39 °C and a non-lethal cold shock temperature of 4 °C. All DmaHsp70s were upregulated in

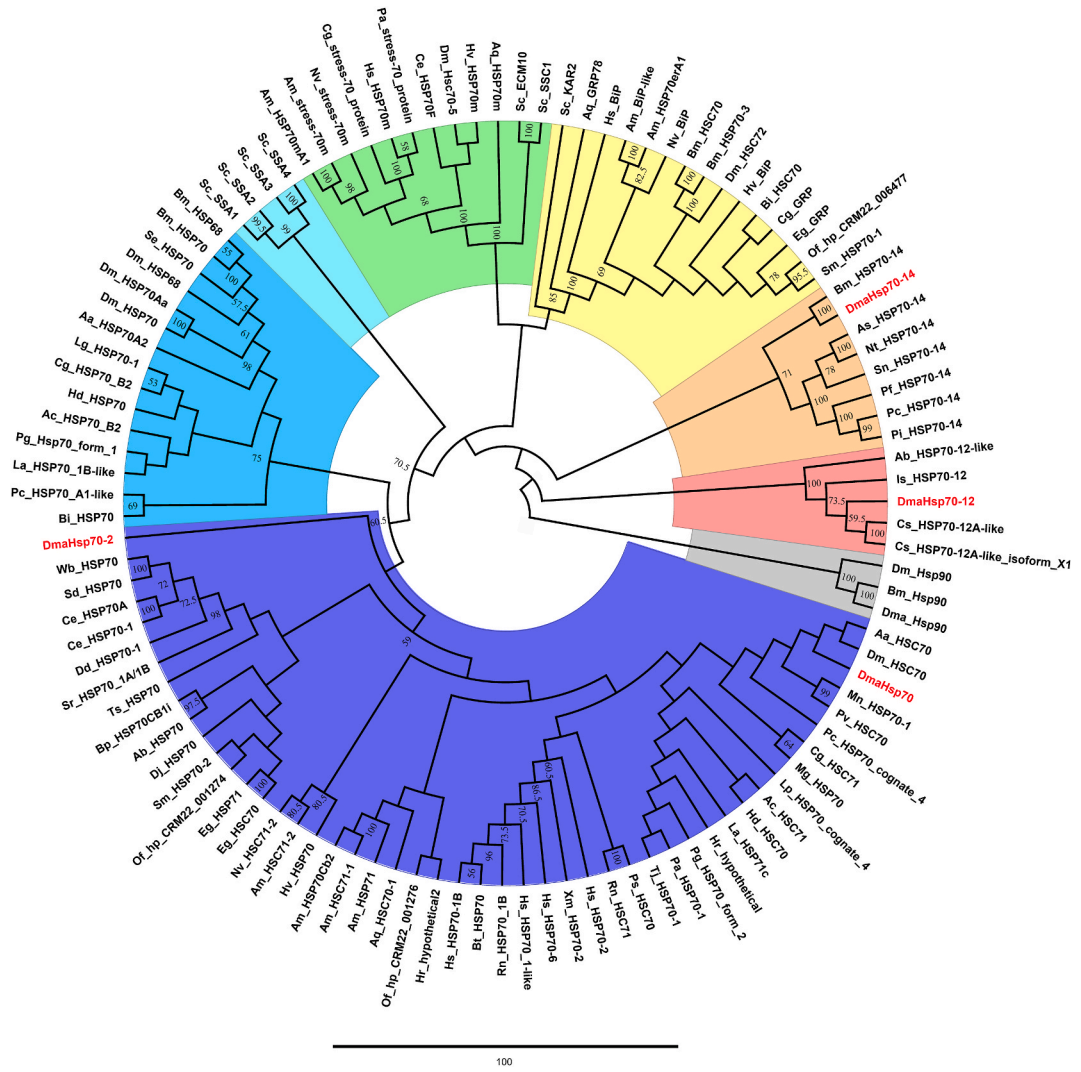


Fig. 3. Phylogenetic classification of DmaHsp70s. The Hsp70s sequences collected from NCBI were taken as input, and the maximum likelihood (ML) method with 200 bootstrap replicates was employed to construct phylogenetic tree. Bootstrap value higher than 50 was marked. The tree was divided into seven major clusters. Rainbow colors were used to mark different lineages. Hsp90 (gray), Hsp70-12 lineage (red). Hsp70-14 lineage (orange). ER lineage (yellow). Mitochondria lineage (green). Yeast specific lineage (light green). Cytosolic Hsp70 lineage A (blue). Cytosolic Hsp70 lineage B (Purple). The name and information of the proteins used were abbreviated in the figure and the full information was in Supplementary Table S2. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

D. magna immediately after heat treatment but not cold treatment. But DmaHsp70s increased at different time points during the recovery stage at room temperature (Fig. 7A and B).

The four *DmaHsp70*s exhibited different expression patterns after hyperthermic treatment. The expression of *DmaHsp70*, *DmaHsp70-2*, *DmaHsp70-12* significantly increased ($p < 0.05$) at 0, 4 h during recovery after heat treatment and then return to a slightly higher but nearly normal level compared to the non-treatment group. While *DmaHsp70-14* increased but not significant ($p > 0.05$) at 0, 2 h during recovery after heat treatment. To be noticed, the expression of *DmaHsp70-14* suddenly dropped to a lower level at 4, 6, 8 h during recovery after the initial increase (Fig. 7A).

During the recovery after cold shock, the expression of *DmaHsp70*s also changed, although not as significant as after heat stress. Still, each *DmaHsp70* exhibited unique expression pattern. The expression of *DmaHsp70* increased after the treatment, but only significantly at 4, 6 h during recovery ($p < 0.05$) and then returned to normal at 8 h during recovery. The expression of *DmaHsp70-2* and *DmaHsp70-14* both decreased at 2 h during recovery, then increased at 4, 6 h and return to a nearly normal level at 8 h during recovery. But the increase or decrease of the two *DmaHsp70*s are not significant ($p > 0.05$). The amount of *DmaHsp70-12* mRNA increased immediately at the beginning of the recovery stage, peaked at 4 h, and still with a significantly higher level at 8 h during recovery (Fig. 7B).

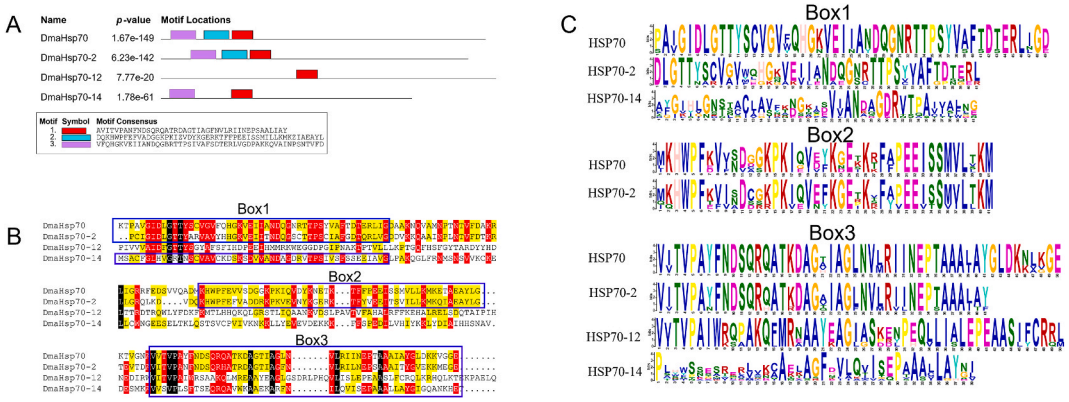


Fig. 4. Structural analysis of NBDs in DmaHsp70s. (A) Motif locations of four DmaHsp70s. Different motifs are represented by different colors. (B) Sequence alignment of NBDs in DmaHsp70s. Sequence in black squares are box 1–3 respectively. (C) Sequence logos of box 1–3 in NBDs of four different Hsp70 groups. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.7. Expression analysis of DmaHsp70s upon oxidative stresses

The amount of *DmaHsp70* mRNA increased prominently at 0 h after treatment and then started to decrease until 4 h after treatment, followed by an increased expression. During the recovery stage, the expression of *DmaHsp70-2* increased suddenly at 4 h after treatment and then returned to normal at 8 h after treatment. *DmaHsp70-12* had a similar expression pattern as *DmaHsp70-2*, only with a smaller magnitude. Of the four *DmaHsp70*s, only *DmaHsp70-14* showed no significant change ($p > 0.05$) during the recovery after an increase at the beginning (Fig. 7C).

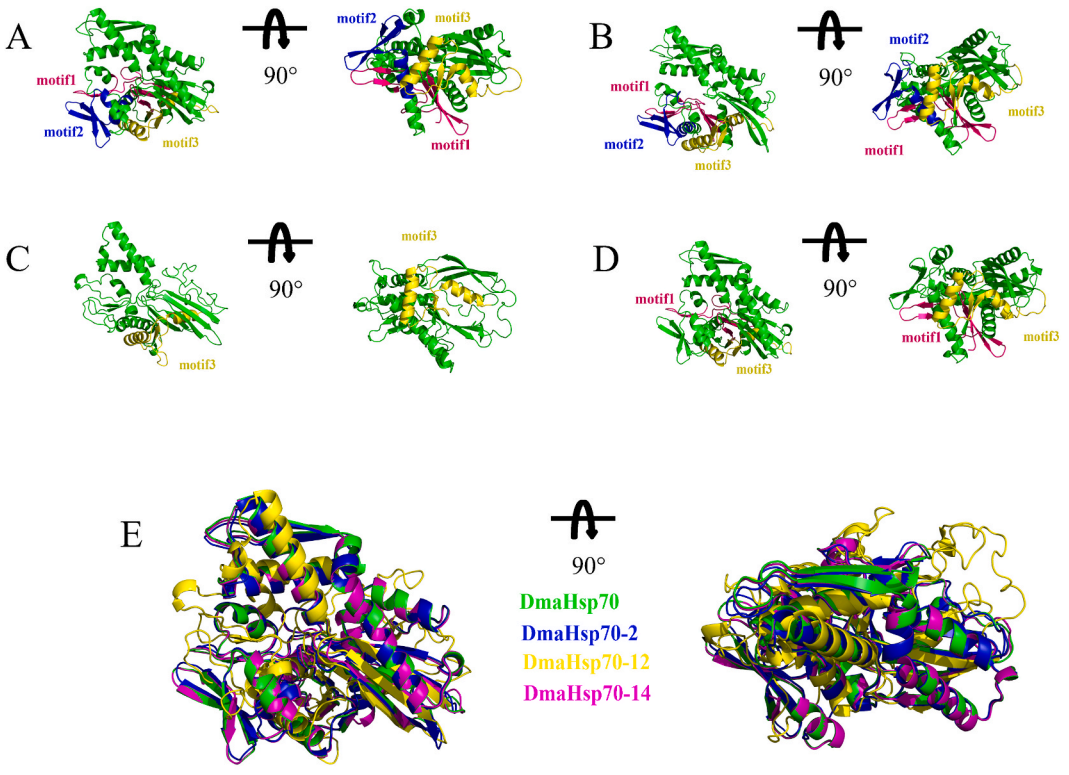
4. Discussion

In species ranging from bacteria to mammals, Hsp70s are widely found [1,14]. Nevertheless, the functions and especially evolutionary relationships of different Hsp70s in crustaceans are still obscure. In our present study, utilizing *D. magna* as a model species, four distinct Hsp70s were obtained and characterized. Despite variations in their molecular weights, by searching against GenBank database coupled with subsequent bioinformatical analysis, all of them were confirmed as Hsp70s with three functional domains. But the DmaHsp70s identified have different amounts of characteristic Hsp70 motifs (Fig. 4A). Furthermore, comparison of the logo sequences of characteristic motifs revealed high conservation between Hsp70 and Hsp70-2 group (Fig. 4C), indicating that these two groups are more closely related than the Hsp70-12 and Hsp70-14 groups. The result aligns with the phylogenetic analysis. Certain signature sequences and motifs were employed to discriminate Hsp70s from different lineages [11], yet the sequences are not applicable in analyzing non-canonical Hsp70s. To be noticed, only DmaHsp70 terminates with a "EEVD" motif (Supplementary Figs. S1A–D). "EEVD" motif binds to tetratricopeptide repeat domain co-chaperones as well as class B J-domain proteins containing a structurally distinct domain [15,36,37]. The absence of the motif in DmaHsp70-2, DmaHsp70-12 and DmaHsp70-14 implies alternative modes of interaction with co-chaperones. Considering that the three DmaHsp70s without C-terminal "EEVD" motif have different amino acid residues at the carboxylic end, it is plausible that their mechanisms of interaction with co-chaperones are diverse.

pI determines the interactions between proteins and is instrumental in predicting protein isoforms [38]. Notably, the average pI of Hsp70-12 is higher than the rest Hsp70 groups (Fig. 1B). A pI value higher than seven makes Hsp70-12s more of alkalinous proteins, instead of acidic ones. With a higher pI, Hsp70-12 probably interacts with different group of proteins and possibly in a different way comparing to other Hsp70s.

Genomes across a spectrum of organisms, from bacteria to humans, encode multiple Hsp70s. In humans, thirteen Hsp70 homologues were expressed in distinct cellular compartments (cytosol, nucleus, ER and mitochondria) according to cellular needs [14]. Thirteen Hsp70s were identified in *Drosophila* [17], and nine in the silkworm *Bombyx mori* [39], but the search for Hsp70s is still ongoing in many other species. Despite the chaperone activities in common, Hsp70s show a certain degree of specialization in their locations, substrates recognition and interactions with co-chaperones [40,41], facilitating their categorization within the family.

Eight Hsp70s were identified in the yeast *Saccharomyces cerevisiae*, and they were further divided into four groups based on their subcellular localization and proposed evolutionary relationship [42]. Metazoan Hsp70s were classified into three groups by a previous phylogenetic analysis, and they are vertebrate Hsp70s, Hsc70s from both vertebrates and invertebrates, and invertebrate Hsp70s [43]. A recent research has expanded the classification, delineating the Hsp70 superfamily into eight clades and revealing that all metazoan cytosolic Hsp70s descended from two lineages that occurred due to an ancient duplication event [18]. Of the thirteen Hsp70s identified in human being, some were located into certain lineages, while others are not thoroughly investigated [18,19,44]. Human Hsp70s have various roles inside cells. For example, HspA5 are functional in cancer and even during the invasion of SARS-Cov-2, while HspA6 is involved in the tumorigenesis and tumor progression [44,45]. The cloned DmaHsp70s were assigned to different clades and/or lineages. However, DmaHsp70-12 could not be assigned to the traditional lineages. Therefore, it is proposed here that an uncharacterized



F

Proteins	Helix	Strand
DmaHsp70（A）	13	16
DmaHsp70-2（B）	12	15
DmaHsp70-12（C）	14	14
DmaHsp70-14（D）	13	13

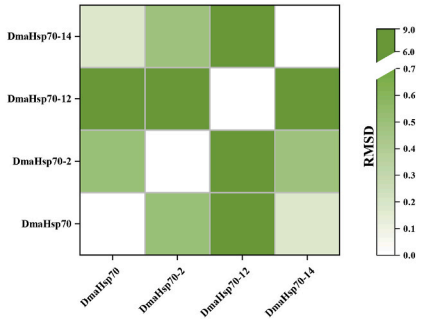


Fig. 5. The 3D model analysis of NBDs of DmaHsp70s. (A–D) 3D structures of NBD of DmaHsp70 (A), DmaHsp70-2 (B), DmaHsp70-12 (C) and DmaHsp70-14 (D) respectively. Different motif sequences are marked with different color. (E) Structural alignments of NBDs of DmaHsp70s. (F) Information of the structural components of NBDs of DmaHsp70s. (G) RMSD matrix of NBDs of DmaHsp70s. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

lineage of Hsp70 is identified. Meanwhile, the functional processes of the DmaHsp70s are yet to be determined.

NBD is essential in the function of Hsp70 since it governs substrate binding affinity through ATP binding and hydrolysis [46,47]. Therefore, it possesses high conservatism among species. Discrepancies in the 3-D structures of DmaHsp70s NBD suggest differential interactions with ATP and co-chaperones. In addition, variations in the abundance and sequence logos of the three characteristic motifs hint different regulatory mechanisms governing chaperone activity. The nucleotide-binding activity of NBD is realized through the rotation of lobe I and lobe II [48,49]. The function of the two NBD lobes and their linker depends on conserved motifs essential for ATP hydrolysis [50]. The lack of certain motif means that ATP hydrolysis regulation in DmaHsp70-12 differs from other DmaHsp70s.

Different Hsp70s have diverse roles in cells. For instance, in diapausing adults of the Colorado potato beetle, *Leptinotarsa decemlineata*, one of the two copies of *hsp70*, *hsp70A*, showed very low levels of up-regulation during diapause, while the other, *hsp70B*, was undetectable [51]. During development, different Hsp70s were employed in the formation of wings and the occurrence of eyes in *Drosophila* [52,53]. Based on the proposed PPI network, DmaHsp70-12 is possibly functional as a chaperone in the formation of Dynein complex. Dyneins are molecular motors composed of several subunits. Based on microtubule, Dyneins are essential in cellular processes including transportation of cytoplasmic proteins, mitosis and maintenance of cell organelles [54,55]. Consequently,

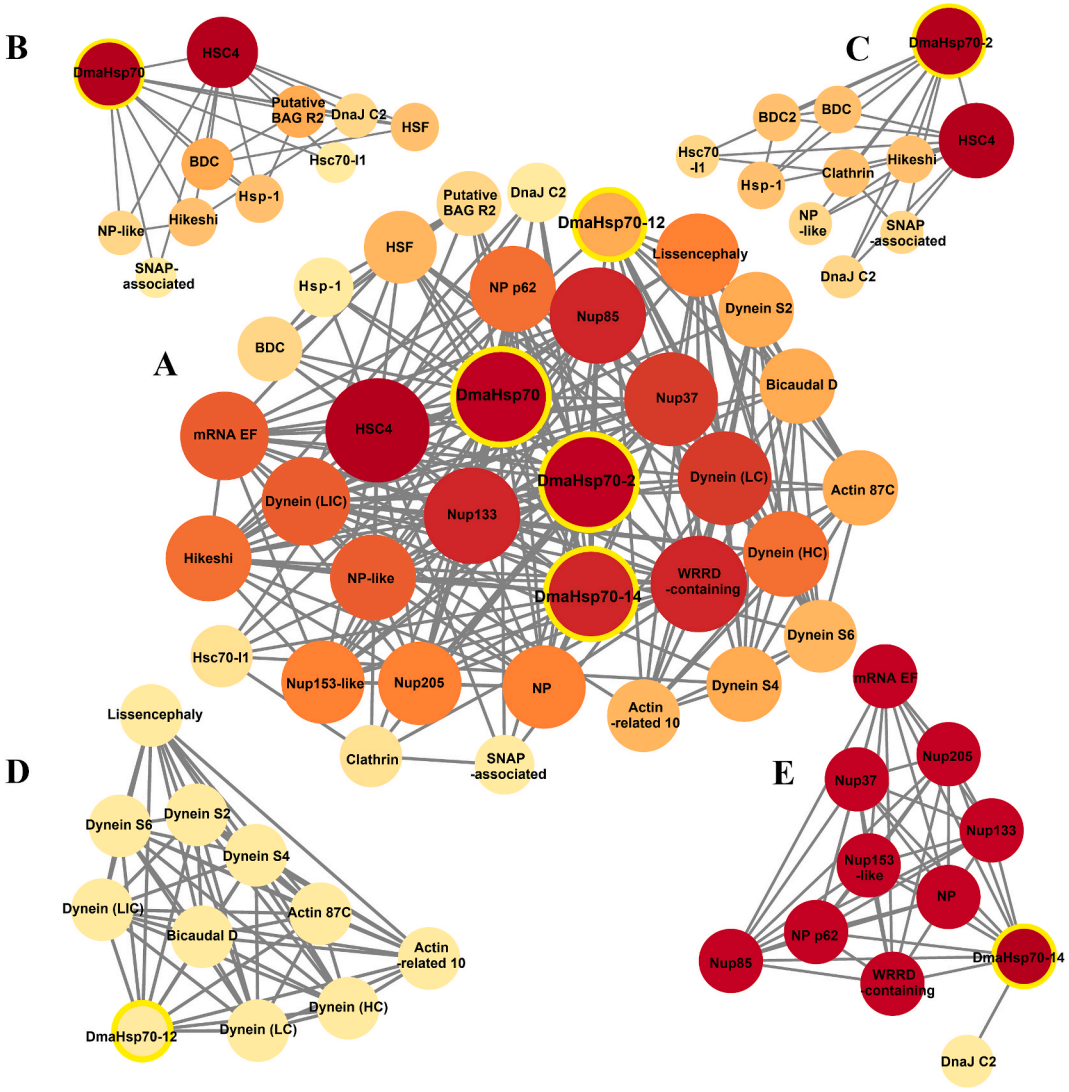


Fig. 6. The PPI networks of DmaHsp70s. (A) The interaction network of four DmaHsp70s. (B–E) The subnetworks of DmaHsp70 (B), DmaHsp70-2 (C), DmaHsp70-12 (D) and DmaHsp70-14 (E). Degree values of nodes were represented by the size & brightness, and combined scores were used to determine the size of edges. Full names of the proteins involved were listed in Table S3.

DmaHsp70–12 may be instrumental in protein transportation and related process within cells. Another DmaHsp70, DmaHsp70-14 is probably involved in the transportation of mRNA into cytoplasm because it interacts with nuclear pore complex and mRNA export factor.

The primary function of Hsp70s in organisms is to cope with thermal stresses. In previous researches, the expression of *Hsp70* was shown to be strongly upregulated by extremely high and low temperatures in *Drosophila melanogaster*, the endoparasitoid *Cotesia chilonis*, and the serpentine leafminer *Liriomyza trifolii* [28,56–58]. Additionally, Hsp70 synthesis increased during recovery from cold in *Drosophila* species and the adult linden bug, *Pyrrhocoris apterus* [23,24,59]. In crustaceans, Hsp70 increased upon non-lethal heat or cold stress in the brine shrimp *Artemia franciscana* [9,60,61]. Hsp70 was also prominently induced in the hepatopancreas and gills of the shrimp *Fenneropenaeus chinensis* upon heat stress [62], and the shrimp *Litopenaeus vannamei* upon a brief cold shock at 13 °C [63]. However, not all Hsp70s necessarily respond to thermal stress. For example, *Mj*Hsp70 of the kuruma shrimp *Marsupenaeus japonicus* and *Lv*Hsp70 of *L. vannamei* exhibited no significant increase after heat stress [64,65]. Oxidative stress could be the a by-effect of heat or cold shock. It also leads to the synthesis of Hsp70 in certain cases [63]. Exposure to oxidative stress for 6 h strongly induced the synthesis of *Hsp7*0 mRNA in the Antarctica midge, *Belgica Antarctica* [66], probably because Hsp70 has a protective role against oxidative stress. The phenomenon that Hsp70 reduced the damage to quail spleen by oxidative stress further supports the point [67]. To elucidate the functions of DmaHsp70s, their expression during the recovery from different stresses was examined. Upon heat or cold shock or oxidative stress, most DmaHsp70s were upregulated during recovery and returned to a relatively lower level after. The reaction of DmHsp70s came out with no surprise since the function of Hsp70s is to protect proteins from denaturation and refold

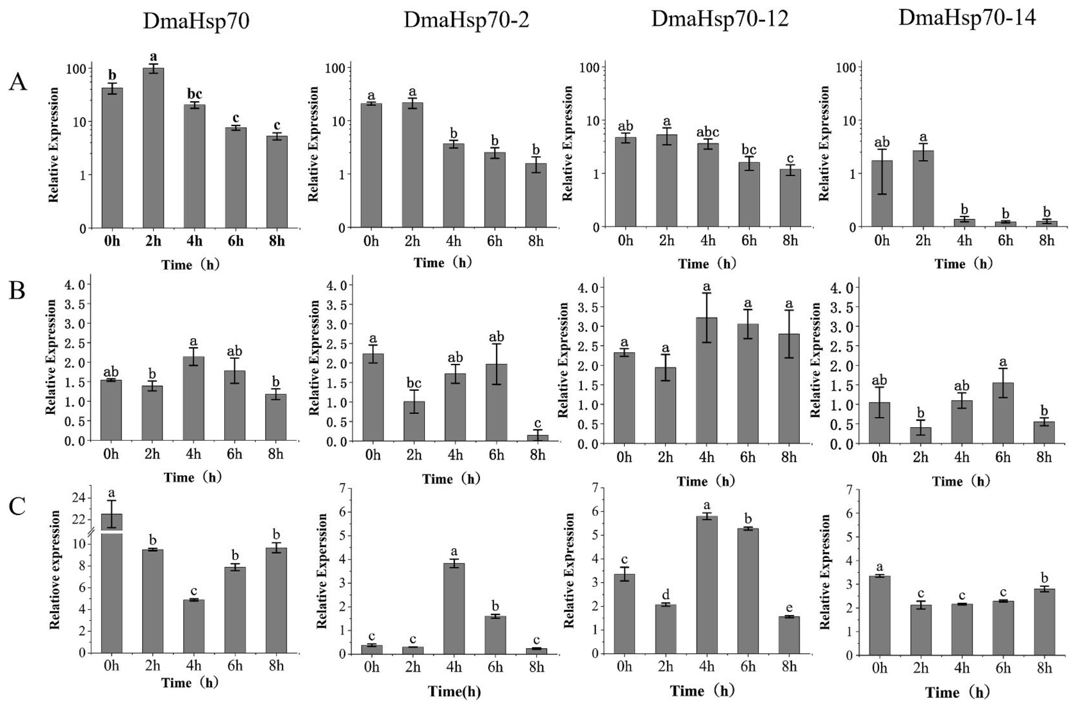


Fig. 7. Effects of sub-lethal heat and cold stresses on the expression of DmaHsp70s. (A) Relative expression of DmaHsp70s after sub-lethal heat shock. (B) Relative expression of DmaHsp70s after sub-lethal cold shock. (C) Relative expression of DmaHsp70s after sub-lethal oxidative stimulation. Every value (means ± SD) was calculated based on three independent data. Different letters indicated that there were significant differences ($p < 0.05$) between the amount of certain DmaHsp70 at two time points, while same letters meant that there were no significant differences ($p > 0.05$).

misfolded proteins. The results are in accordance with previous researches.

Four DmaHsp70s functioning in heat or cold shock or oxidative stresses may appear redundant for the creature. Yet similar results are recurrent in numerous researches. For example, both Hsp68 and Hsp70Aa are cold inducible in *Drosophila* [26,27]. The question of why do animals need multiple Hsp70s to cope with environmental stress is still to be answered. A theory here is that Hsp70s protect different client proteins given the variety of their domain structure. Combining the different functional location of the Hsp70s, they probably interact with distinct protein groups within different cell organelles.

5. Conclusion

Here, we identified four inducible Hsp70s in water flea and examined their structural characteristics, phylogenetic relationships and expression profiles upon distinct stresses. It was revealed that the four DmaHsp70s were distributed in different lineages. Especially that DmaHsp70-12, together with Hsp70-12s from other species, formed a lineage which has not been described before. DmaHsp70-12 has a different conformational structure of NBD comparing to the other three Hsp70s. The NBD of DmaHsp70-12 has only one traditional motif, less than in other DmaHsp70s. And sequence signatures of the motifs vary between Hsp70s. Analysis of transcription during the recovery after stresses indicated that all DmaHsp70s had certain roles in thermal and oxidative challenges. These findings will facilitate the understanding of the evolution and function of the Hsp70 family. Due to the lack of enough investigation, future works are needed on the functional mechanism of Hsp70s, especially Hsp70-12.

CRediT authorship contribution statement

Xiangyang Wu: Writing – original draft, Formal analysis, Data curation, Conceptualization. **Zhiwei Zhang:** Writing – original draft, Formal analysis, Data curation. **Wenfeng Cui:** Data curation. **Linfei Han:** Data curation. **Zijie Liu:** Data curation. **Xiaojun Song:** Writing – review & editing, Supervision. **Jiabo Tan:** Writing – original draft, Supervision, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e30288.

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