

The complex evolution of the metazoan HSP70 gene family

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Abstract

The metazoan 70-kDa heat shock protein (HSP70) family contains several members localized in different subcellular compartments. The cytosolic members have been classified into inducible HSP70s and constitutive heat shock cognates (HSC70s), but their distinction and evolutionary relationship remain unclear because of occasional reports of “constitutive HSP70s” and the lack of cross-phylum comparisons. Here we provide novel insights into the evolution of these important molecular chaperones. Phylogenetic analyses of ~100 full-length HSP70s revealed an ancient duplication that gave rise to two lineages from which all metazoan cytosolic HSP70s descend. One lineage (A) contains a relatively small number of Lophotrochozoan and Ecdysozoan genes, none of which have been shown to be constitutively expressed (i.e., either inducible or unknown). The other lineage (B) included both inducible and constitutive genes from diverse phyla. Species-specific duplications are present in both lineages, and Lineage B contains well-supported phylum-specific clades for Rotifera, Nematoda, and Chordata. Some genes in Lineage B have likely independently acquired inducibility, which may explain the sporadic distribution of “HSP70” or “HSC70” in previous analyses. Consistent with the diversification history within each group, inducible members show lower purifying selection pressure compared to constitutive members. These results illustrate the evolutionary history of the HSP70 family, encouraging us to propose a new nomenclature: “HSP70 + subcellular localization + lineage + copy number in the organism + inducible or constitutive, if known.” e.g., HSP70cA1i for cytosolic Lineage A, copy 1, inducible.

Key words: heat shock cognates, molecular evolution, rotifer, stress inducibility, synonymous and nonsynonymous substitution.

Introduction

The 70-kDa heat shock protein (HSP70) family members play important roles in various cellular processes including heat shock response, folding of newly synthesized proteins, protein transport, and protein degradation. These apparently diverse functions are attributed to their chaperone activity (Daugaard et al. 2007; Hartl et al. 2011), by which they prevent the aggregation and misfolding of target proteins. Typically, HSP70 family members bind to denaturing or newly synthesized proteins by recognizing up to ten hydrophobic amino acid residues exposed to the protein surface because of the misfolding. The release of the HSP70 members, which is triggered by ATP hydrolysis, facilitates the proper folding of the target proteins (Zuiderweg et al. 2017). The HSP70 family contains organelle-specific members localized in cytosol, endoplasmic reticulum (ER), mitochondria, and chloroplasts (Miernyk 1997), and these organelle-specific types not only perform chaperoning functions in the organelles but are also known to contribute to protein transport across organelle membranes.

HSP70 family members are often upregulated upon various stresses that disrupt protein folding, such as heat treatment, exposure to toxic materials, ultraviolet irradiation, and pathogen attack (Sørensen et al. 2003; Baird et al. 2006). The upregulation is primarily regulated at the transcription level by transcription factors called heat shock factors (HSFs), particularly HSF1. Under unstressed conditions, HSF1 remains in a monomeric state. Various stresses lead to the formation of HSF1 trimers, which in turn bind to heat shock elements (HSEs) in the promoter region of HSP70 family member genes and promote their transcription. The HSF1-dependent transactivation system is generally conserved among eukaryotes, although species-specific differences in HSF1 function have been reported in many organisms such as the fruit fly *Drosophila melanogaster* (Jedlicka et al. 1997; Marchler and Wu 2001) and yeast *Saccharomyces cerevisiae* (Sorger and Pelham 1988). The expression of HSP70 family member is also regulated by many other mechanisms such as the unfolded protein response, chromatin modification, and other transcription factors depending on the type of stress and subcellular localization (De Nadal et al.

2011; Garbuz 2017). HSP70 family member genes have therefore been used as biomarkers of environmental stresses both in laboratory and field experiments (Sanders 1993; Ceyhun et al. 2010; Judge et al. 2011).

On the other hand, several cytosolic HSP70 family members show constitutive expression patterns. These proteins are involved in the folding of newly synthesized proteins and are traditionally called 70-kDa heat shock cognates (HSC70). However, the distinction between HSP70s and HSC70s remains unclear because of the occasional reports of “constitutive HSP70s,” especially from invertebrates (Jayasena et al. 1999; Piano et al. 2002; Liu et al. 2017). Stress-induced upregulation of HSC70 has also been reported in several animals including shrimps (Luan et al. 2010), snails (Zheng et al. 2012), and fish (Yabu et al. 2011). Furthermore, little is known for the evolutionary relationship between metazoan HSP70s and HSC70s because of the lack of cross-phylum comparisons. A previous phylogenetic analysis indicated that metazoan HSP70 family members can be classified into invertebrate HSP70s, vertebrate HSP70s, and HSC70s from both vertebrates and invertebrates (Kourtidis et al. 2006), but this study comprises only a few phyla. In-depth phylogenetic analysis on HSP70 family members with a broad sampling would offer further insight into the classification of this important group of molecular chaperones.

In this study, we sought to trace the evolutionary history of metazoan HSP70 family members by cross-phylum phylogenetic analyses with a particular attention to their stress inducibility. The specific hypothesis was that the cross-phylum analysis would provide the essential information to solve the evolutionary relationship between metazoan HSP70s and HSC70s. In order to add insights from an emerging model in evolutionary biology to the analyses, we first cloned two stress-inducible HSP70 family member genes from a monogonont rotifer, *Brachionus plicatilis*. The phylum Rotifera, composed of mostly microscopic aquatic animals with about 1,000 cells and a ciliated head structure, is part of the Gnathifera, a group of basal-branching phyla related to Lophotrochozoa, which contains Mollusca and Annelida. Together these groups form a sister-clade to Ecdysozoa, which contains the established invertebrate models *Caenorhabditis elegans* and *D.*

melanogaster (Struck et al. 2014; Fröbuis and Funch 2017). Genetic information from Gnathifera remains limited, making rotifers useful models in evolutionary studies. Our results added novel groups of stress-inducible HSP70, providing important insight into the evolutionary history of the metazoan HSP70 family.

Results

Molecular Characterization of Rotifer HSP70 Genes

A combination of 3' and 5' RACE identified two *B. plicatilis* HSP70 genes consisting of 2,121 and 2,248 bp, designated as HSP70cB1i and HSP70cB2i, respectively (see Discussion for the nomenclature). The nucleotide sequence of the open reading frames, as well as the absence of introns, were ascertained by single PCR experiments using cDNA or genomic DNA, respectively, followed by DNA sequencing. The deduced amino acid sequences of HSP70-1 and HSP70-2 genes were more than 99% identical to each other ([supplementary fig. S1](#)). Both contain the HSP70 protein family signatures, IDLGTTYS, IFDLGGGTFDVSIL, and IVLVGGSTRIPKVQK (Prosite motifs PS00297, PS00329, and PS01036, respectively), as well as the non-organelle stress protein motif RARFEEL found in several cytosolic HSP70s (Lo et al. 2004; Cottin et al. 2008; Simoncelli et al. 2010; Zheng et al. 2012). The major difference between *B. plicatilis* HSP70 genes was the number of GGMP repeats, which are involved in binding to the HSP70-HSP90-organizing protein (Hop), a cofactor of HSP70 (Demand et al. 1998). The nucleotide sequences of *B. plicatilis* HSP70cB1i and HSP70cB2i genes were registered into the DDBJ/EMBL/GenBank databases with accession numbers AB775784 and AB775785, respectively. The two HSP70 genes were also found in the *B. plicatilis* genomic sequence.

We next examined whether the mRNA levels of *B. plicatilis* HSP70s are increased by heat stress. Due to the high sequence identity between *B. plicatilis* HSP70 genes and difficulty in designing a TaqMan probe, we employed semi-quantitative RT-PCR to measure their respective mRNA levels. Primers were designed to amplify DNA fragments of 112 and 124 bp from

HSP70cB1i and HSP70cB2i cDNAs, respectively (fig. 1A), and the PCR products were separated using a polyacrylamide gel. The mRNA levels of HSP70cB1i and HSP70cB2i in heat-treated rotifers were 2.9 and 7.5 times higher, respectively, than those in control rotifers (fig. 1B and 1C). These results indicate that both genes encode heat-inducible HSP70.

Since the two *B. plicatilis* HSP70 genes showed a similar expression pattern in response to heat stress, we used quantitative real-time PCR primers that amplify both HSP70cB1i and HSP70cB2i genes in the subsequent time-course expression analyses (fig. 1D). The relative mRNA levels were increased by heat stress at 40°C for 10 min and reached at the maximum level 2 h after the heat stress. The difference in mRNA levels between the control and 2 h groups was statistically significant. The mRNA levels of *B. plicatilis* HSP70s remained twofold 8 h after the heat stress. We then performed *in situ* hybridization using DIG-labeled RNA probes (fig. 1E). The probe region contained only five base pair differences between the two HSP70 genes, and thus the RNA probes were likely to hybridize with both HSP70cB1i and HSP70cB2i mRNAs. The hybridization signals increased in all tissues of heat-treated rotifers. Altogether, these results indicate that the *B. plicatilis* HSP70cB1i and HSP70cB2i genes are heat-inducible and have similar expression profiles.

Phylogenetic Analysis of HSP70 Family Members

To assess the relationship of the *B. plicatilis* HSP70 genes with those of other rotifers and other metazoans, we constructed gene trees of more than 100 HSP70 family members, including members associated with the mitochondria and ER and many sequences known only from automated genome annotation as “HSP70.” To assess the robustness of the results we used alignments made by three different methods [Clustal-Omega (Sievers et al. 2011), M-Coffee (Wallace et al. 2006), and Expresso (Armougom et al. 2006)] to construct consensus trees with maximum-likelihood (RAxML) and Bayesian (MrBayes) approaches. The RAxML PROTGAMMAAUTO function found that the LG model of protein evolution (Le and Gascuel 2008) was optimal for all three alignments and this model was used to construct ML and Bayesian

consensus trees. The six trees were in general agreement for major nodes; [fig. 2](#) shows the Bayesian consensus tree of the Expresso alignment and support for major nodes from each approach is shown in [table 1](#). In all analyses HSP70 family members known to be mitochondria- and ER-specific formed two monophyletic groups (nodes 11 and 12 in [fig. 2](#)) in agreement with previous reports (Boorstein et al. 1994; Miernyk 1997; Nikolaidis and Nei 2004). All family members known to be cytosolic formed a third group (node 1; clades 1–6 in [fig. 2](#)), with yeast cytosolic HSP70s (clade 6) basal to all metazoan cytosolic HSP70s. The tree topology generally supported the species phylogeny. Within the metazoan cytosolic HSP70s (node 2; clades 1–5) there were two lineages: Lineage A was well supported and contained of genes from arthropods, molluscs, and rotifers (node 3; clade 1); Lineage B was more poorly resolved and represented by many diverse vertebrate and invertebrate phyla (node 4; clades 2–5). There are two groups of vertebrate cytosolic HSP70s (nodes 8 and 9), which form a monophyletic group (node 7; clade 5) in Bayesian and RAXML analyses of Expresso and Clustal alignments. Unexpectedly, we found two additional clusters of cytosolic HSP70 family members in all analyses: one composed of HSP70 genes from the phylum Rotifera (node 5; clade 2) and the other from the phylum Nematoda (node 6; clade 3). This suggests the evolution of two unique subgroups of HSP70, distinct from conventional vertebrate and invertebrate HSP70s. We also constructed phylogenetic trees mainly using HSP70 family members of known expression patterns (supplementary figs. S2 and S3). Both of the two HSP70 lineages as well as most of the nodes described above were identified, supporting the robustness of the tree shown in [fig. 2](#), although several clades were poorly resolved in supplementary figs. 2 and 3 that have limited number of samples.

The phylogenetic breadth of our analyses provided the opportunity to search for sequence motifs characteristic of different groups within the HSP70 family. We identified a single region near the N terminus (but after signal peptide motifs) that discriminates between metazoan mitochondrial, ER, and cytosolic HSP70s ([fig. 3](#)), which enables better discrimination for organelle-specific HSP70s together with the well-known C-terminal motifs for cytosolic (EEVD) and ER

(HDEL/KDEL) HSP70s (supplementary fig. S4). We also found the RARFEEL motif, previously described as possibly cytosolic-specific in conjunction with the EEVD motif (Cottin et al. 2008; Simoncelli et al. 2010; Zheng et al. 2012), in several invertebrate genes that are found with high confidence in the ER-associated node 12 (fig. 2; *B. ibericus* HSC70, *C. gigas* GRP, and *B. manjavacas* HSC71). This result suggests that the RARFEEL is not a reliable predictor of cytosol association. Indeed, this motif was identified by a comparison of few sequences (Lo et al. 2004) and appears to have no strong scientific support.

Stress-inducible invertebrate HSP70s are reported to have an extra serine residue in their ATPase domain (Kourtidis et al. 2006; Garbuz et al. 2011). In our analysis this insertion occurs only in Lineage A irrespective of the stress-inducibility, where it is present in all sequences except *B. ibericus* HSP70 and one of four *Lottia* HSP70s; in *Priapulius* HSP70-1 the serine has been replaced by alanine (fig. 4). These results suggest that the extra serine residue is a characteristic of this lineage and may be associated with the stress-inducibility of the ancestral HSP70, which will be tested by broader sampling focusing on this residue. The alignment result supports the idea that HSP70s from Nematoda and Rotifera evolved separately from the those in Annelida, Mollusca and Arthropoda, which have been generally recognized as "invertebrate HSP70s."

Synonymous and Nonsynonymous Substitution Rates

Lastly, we calculated the synonymous and nonsynonymous substitution rates of 34 representative cytosolic HSP70 member genes. We primarily selected genes with known expression patterns (stress-inducible or constitutive) from each clade, hypothesizing that constitutive HSP70 family members have been under stronger purifying selection compared to stress-inducible members. Only well-conserved regions were used for the calculation (supplementary fig. S5). We noted that *B. ibericus* HSP70 (GU574486) has an insertion at nucleotide position 403 and a nucleotide deletion at position 563, causing a frame shift. Deduced amino acid sequences encoded by nucleotides 403–563 were markedly different from those of other HSP70 family members

because of the frame shift, which resulted in the long genetic distance in phylogenetic trees (fig. 2). Because the frame shift greatly affects the synonymous and nonsynonymous substitution rates, this sequence was excluded from our analysis.

For the selected 34 sequences, we calculated synonymous and nonsynonymous substitution rates (Ks and Ka) for all combinations of representative cytosolic members of the HSP70 family from metazoans (fig. 5A). Synonymous substitution rates (Ks) were saturated in many combinations and only a limited number of combinations produced meaningful results (229 out of 465 combinations, supplementary material 1). The Ka/Ks values calculated for the 229 combinations were averaged for each clade (fig. 5B). Overall, Ka/Ks values were markedly lower than 1, indicating that cytosolic HSP70 family member genes have been under purifying selection. The purifying selection pressure appears to have been less in Lineage A (clade 1) than in Lineage B (clades 2-5). The effect size between these lineages ranged from 0.19 to 1.64 (fig. 6C). Since the effect size larger than 0.8 indicate large difference in multiple comparisons, these results support our hypothesis that constitutive HSP70 family members [clade 4 and clade 5 (node 8)] have been under stronger purifying selection.

Within Lineage B, the selection pressure was strongest in Nematode HSP70s (clade 3), invertebrate HSC70/HSP70s (clade 4), and vertebrate HSC70s (clade 5, node 8). In particular, vertebrate HSC70s (clade 5, node 8) and HSP70s (clade 5, node 9) showed significant difference in the Ka/Ks with a large effect size of 0.86 despite their close phylogenetic relationship. These results also support the above hypothesis. To confirm how much this finding depends on the sampling, we conducted the same analysis with a larger sample size, including most genes used in the phylogenetic analysis, and obtained consistent results (supplementary fig. S6).

Discussion

A previous phylogenetic analysis by Kourtidis et al. (2006) classified metazoan cytosolic HSP70s into three groups, invertebrate HSP70, vertebrate HSP70, and HSC70 including both

vertebrate and invertebrate orthologues using data from a few taxa (Kourtidis et al. 2006). The monophyletic origin of HSC70s was also proposed by Nikolaidis and Nei (2004) based on their analysis using *Drosophila* and nematode sequences (Nikolaidis and Nei 2004). Surprisingly, to our knowledge, there has been no significant update on the evolution of metazoan HSP70 family after these small-scale studies despite the continuing expansion of available genomic data. Here we show that there are at least two types of ancestral cytosolic HSP70 family member genes in all analyzed phyla of metazoans, one (node 3; clade 1) giving birth to Lineage A of invertebrate HSP70s, and a second (node 4; clade 5) to Lineage B of both vertebrate and invertebrate HSP70s and all HSC70 genes. This second lineage has further diversified within diverse phyla: Rotifera (node 5; clade 2), Nematoda (node 6; clade 3), and Chordata (node 7; clade 5) (fig. 2), which has not been identified in the previous reports. The tree topology is generally consistent with the species phylogeny, indicating that the effect of sequence contamination is very limited.

Inter-clade comparison of the synonymous and nonsynonymous substitution rates shows that while all HSP70 family members are under strong purifying selection, the pressure to conserve amino acid sequence varies across clades. The presence of purifying selection has been reported for mammalian (Hess et al. 2018), nematode (Nikolaidis and Nei 2004), and molluscan (Kourtidis et al. 2006) HSP70 genes, and our cross-phylum analysis was consistent with these reports. Most dramatically, the canonical HSC70 genes of vertebrates (node 8; clade 5) showed stronger purifying selection pressure than the HSP70 family members in node 9 (clade 5). This concept can be further generalized by thoroughly investigating the saturation rate between each HSP70 gene (Pollock and Larkin 2004). It is thus speculated that the role of HSC70 protein, the folding of newly synthesized proteins, is more essential for cell survival compared to the role of the HSP70 cognates (node 9; clade 5). In line with this speculation, cells deficient in the HSC70 gene are non-viable (Florin et al. 2004), whereas HSP70 knockout mice are mostly viable and fertile, although they are known to be sensitive to stress (Daugaard et al. 2007). Meanwhile, genetic distance between each clade is not uniform, and the gene conversion may also affect the accuracy of this calculation. In this regard our

data should be interpreted in a semiquantitative manner.

It is known that nematode, mammalian, and molluscan HSP70 families have experienced gene conversion events (Nikolaidis and Nei 2004; Kourtidis et al. 2006; Hess et al. 2018). In addition to the purifying selection, gene conversion also likely contributes to the highly conserved nature of the HSP70 family members found in this study. The tree topology might have been affected by the gene conversion in closely related paralogues and orthologues such as human HSPA1A and HSPA1B (Hess et al. 2018). However, gene conversion generally functions to conserve the sequence in a species, and thus the overall topology of the metazoan tree is unlikely to be significantly affected. Gene conversion rate should be about 10 times higher than the mutation rate to significantly affect the topological distance according to a genomic simulation study (Touchon et al. 2009), but the nucleotide identity between sequences is often lower than 70% in our phylogenetic analysis.

Given the presence of inducible and constitutively expressed genes in many clades of metazoan cytosolic HSP70, it is difficult to predict the ancestral state of this phenotype, and whether a gene is inducible or constitutive expression does not predict its relationship to other family members. Phylogenetic analyses, the novel signature sequences near the N terminus (fig. 4), and the extra serine residue (fig. 5) would provide better clues for the classification of HSP70 family members than their expression patterns under stress.

Based on the largest-ever cross-phylum phylogenetic analysis on HSP70 family, we propose a new nomenclature that reflects the phylogenetic relationship of the HSP70 family members (fig. 6). The proposed name of an HSP70 family member protein consists of “HSP70 + subcellular localization + lineage + copy number found in the organism + inducible or constitutive, if known.” For example, HSP70cA1i represents a cytosolic HSP70 in Lineage A, copy 1, inducible; and HSP70mA1 for mitochondrial lineage of unknown inducibility. Although the current study found only one lineage in mitochondrial and ER members, the use of “A” in these organelle-specific isoforms would be beneficial. Systematic lineage names such as HSP70cD and HSP70mB can be assigned for novel lineages that may be discovered in future. Gene names are represented by

lowercase italics of protein names (e.g., *hsp70cb2i* and *hsp70era1*).

In conclusion, the present study identified two novel HSP70 family members from a monogonont rotifer, an emerging model in evolutionary biology. The subsequent phylogenetic analyses illustrated the evolutionary history of the metazoan HSP70 family, in which stress inducibility does not reflect evolutionary history. The proposed nomenclature based on molecular evolution will help us understand the true nature of the HSP70 family.

Materials and Methods

Culture

We used the *B. plicatilis* Ishikawa strain (also called *Brachionus* sp. ISKW), originally isolated from a Japanese eel culture pond (Yoshinaga et al. 2004), for cDNA cloning, semi-quantitative RT-PCR, quantitative real-time PCR, and *in situ* hybridization. The cytochrome oxidase subunit I gene sequence has been registered for detailed identification of this strain (GenBank accession number LC422762). Rotifers were cultured at 25 °C by a standard protocol (Kaneko et al. 2016) using half-diluted Brujewicz artificial seawater and fed the algae *Chlorella regularis* (Nikkai Center, Tokyo, Japan) at a final concentration of approximately 7×10^6 cells/mL. See [supplementary material 2](#) for details of molecular characterization of rotifer HSP70 genes.

Phylogenetic Analysis

Metazoan HSP70 family genes were selected based on the following criteria: 1) genes from diverse metazoan phyla are included; 2) HSP70 genes with known expression patterns are prioritized; 3) both Lineage A and Lineage B genes from the same organism are included when possible; and 4) the number of genes from a single species is limited to ~5 because within-phylum gene duplications do not change the tree topology. Annotated and unannotated genome and transcriptome databases were used. A fasta file containing sequences used for the tree is provided as [supplementary material 3](#).

A multiple sequence alignment of HSP70 and HSC70 amino acid sequences was produced using Clustal Omega, Expresso, and M-Coffee with default parameters. Bayesian phylogenetic analysis was performed using MrBayes (v3.2.6) on the CIPRES scientific gateway v3.3 (Miller et al. 2010) with four chains each for 10^7 generations. Every 1000th trees were sampled, and the first 25% of samples were discarded as a burn-in. Bayesian consensus trees were visualized with the TreeView software v1.4.3.

Synonymous and Nonsynonymous Substitution Rates

R version 4.0.4 was used for the calculation of the synonymous and nonsynonymous substitution rates (the kaks function), Kruskal-Wallis test (kruskal.test function), and post-hoc test (pairwise.wilcox.test function) on the Macintosh platform. Heat maps were created in Microsoft Excel 2016 (Redmond, MA).

Author Contributions

Conceived and designed the experiments: TY, GK. Performed experiments and data analyses: EMY, FLJ, DMW, GK. Contributed reagents/materials/analysis tools: EH, DMW, GK. Wrote the paper: DMW, GK. Read and approved the final manuscript: EMY, TY, FLJ, EH, DMW, GK.

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Figure Legends

FIG. 1. Molecular characterization of *Brachionus plicatilis* HSP70 genes. (A-C) Semi-quantitative

reverse transcription-PCR. A representative result is shown out of three independent experiments. Location of primers rHSP70_gapF and rHSP70_gapR that amplify cDNA fragment of 112 and 124 bp from HSP70cB1i and HSP70cB2i cDNAs, respectively (A). Polyacrylamide gel electrophoresis patterns of the RT-PCR product. Cycle numbers within a linear range of PCR amplification were determined to be 24 to 28 cycles for both cDNAs by preliminary experiments on the basis of signal intensities of amplified products by RT-PCR (B). Signal intensities of HSP70cB1i and HSP70cB2i genes standardized to those of β -actin (C). (D) Heat stress-induced expression of HSP70 quantified by real-time PCR. Beta-actin gene was used as the internal control. Bars represent standard errors from three replications. One-way ANOVA detected significant effects of heat treatment ($F = 3.281$, $df = 5$, $P = 0.0426$). Dunnett's multiple comparison was used to detect significant differences between the control and other groups. Difference between control and two hours group was statistically significant ($t = 3.246$, $P = 0.028$). (E) *In situ* hybridization for *B. plicatilis* HSP70 genes. Rotifers were fixed 4 h after the heat treatment.

FIG. 2. Bayesian consensus tree of HSP70 family members. Nodes discussed in the text are indicated. Numbers on the branches indicate the posterior probability support for each node. Stress-inducible and constitutive genes are shown in red and blue, respectively. No data on the stress inducibility is available for genes shown in black. Approved HGNC names of human HSP70 family members are shown in yellow boxes for reference. The DDBJ/EMBL/GenBank accession numbers and other information are summarized in [supplementary table S2](#). Clades 1 to 6 include only cytosolic members of the HSP70 family.

FIG. 3. Logo visualization of mitochondrial-, endoplasmic reticulum-, and cytosolic-specific sequence near the N terminus of the HSP70 family members. Compared to the cytosolic form, ER-specific forms have several conserved amino acid substitutions: Q8K, H9N, K11R, and T23I. Similarly, mitochondrial forms have Y1N, G5A, F7M, Q8E, H9G, G10K, K11T/Q, V12P/A, E13K,

I14V, I15L, A16E, D17A, and Q18E.

FIG. 5. Comparison of the amino acid sequences of the HSP70 family members from invertebrates. Nodes in the phylogenetic tree (fig. 3) are indicated on the right margin, and serine residues specific to node 3 are highlighted. Stress-inducible and non-inducible genes are shown in red and blue, respectively. No data on the stress inducibility is available for genes shown in black. The accession numbers and other information are summarized in [supplementary table S2](#).

FIG. 6. Selection pressure on cytosolic HSP70 family member genes. (A) Nonsynonymous substitution rates (Ka values) between 34 HSP70 family member genes. Gene number 1 is the fruit fly HSP70 Bb (AF295957) gene. The substitution rate of fruit fly HSP70 Aa and Bb gene was 0.00395526, which is shown as 0.00 in the figure. Accession numbers for other genes are indicated in the figure. Clades and nodes correspond to those in fig. 2. (B) Bee swarm boxplots of Ka/Ks values for each clade. Only Ka/Ks values calculated from inter-cluster pairs were averaged. Statistical differences were calculated by Kruskal-Wallis test ($\chi^2 = 88.704$, $df = 5$, $p < 2.2e-16$) followed by the non-parametric post-hoc tests (pairwise Wilcoxon test with P value adjustment by the Holm method). Clades sharing same letters are not significantly different at the 5% level of significance. One outlier in clade 3 (0.53) is not included in the plot (see supplementary material 1) although this value was used for all statistical analyses. (C) Effect sizes (Cohen's d) for all comparisons. Combinations where significant differences were found in the pairwise Wilcoxon test are shown in bold.

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 475

Table 1. Support for major nodes from each approach

		ClustalOmega		Expresso		M-Coffee	
		MrBayes	RAxML	MrBayes	RAxML	MrBayes	RAxML
1	Cytosolic	100	99	100	99	100	100
2	Metazoan	100	95	100	99	100	94
3	Lineage 1	(a)	99	100	99	100	100
4	Lineage 2	(b)	41	85	67	(b)	(c)
5	Rotifer	100	48	74	48	100	65
6	Nematode	94	93 (d,e)	100 (e)	96 (e)	100 (e)	86 (e)
7	Chordate		42	85	35		
8	HSC	100	91	100	86	100	31
9	HSP	100	88	100	83	100	87
10	Outgroup	100	99	100	99	100	100
11	Mitochondrial	100	100	100	97	100	100
12	ER	100	99	100	100	100	100

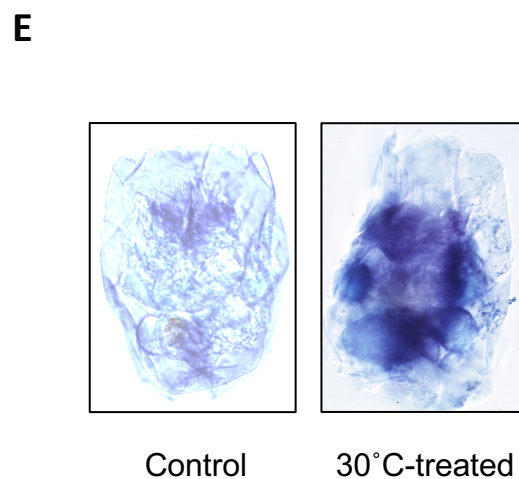
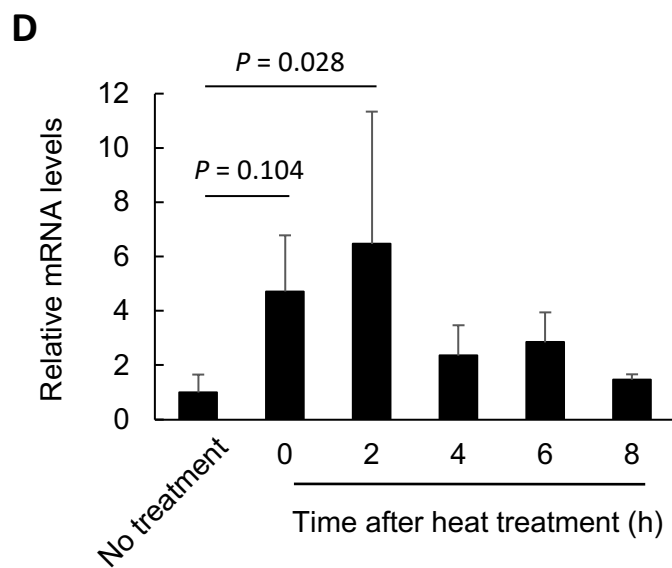
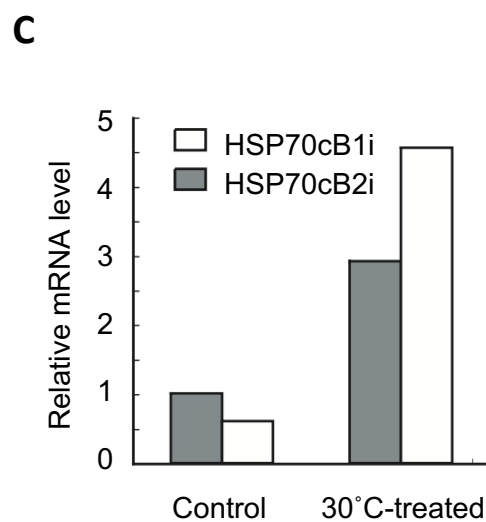
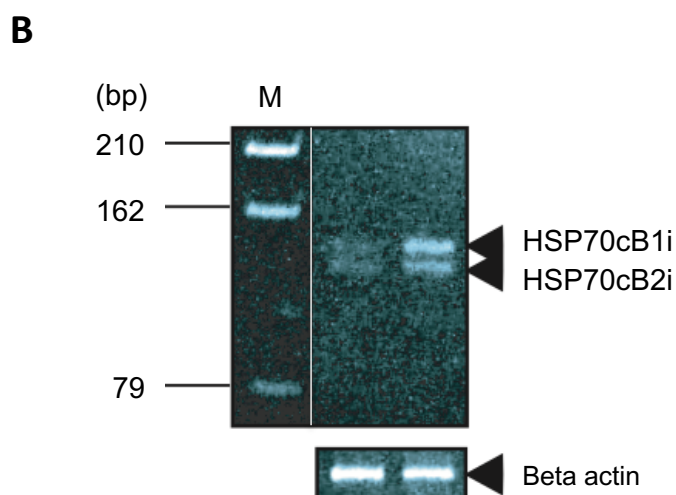
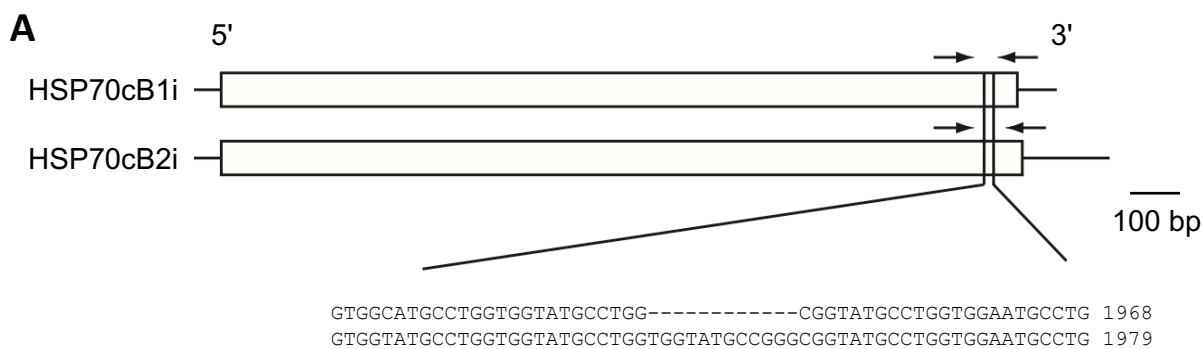
(a) Rotifer genes (node 5) is included in node 3 with 83% support

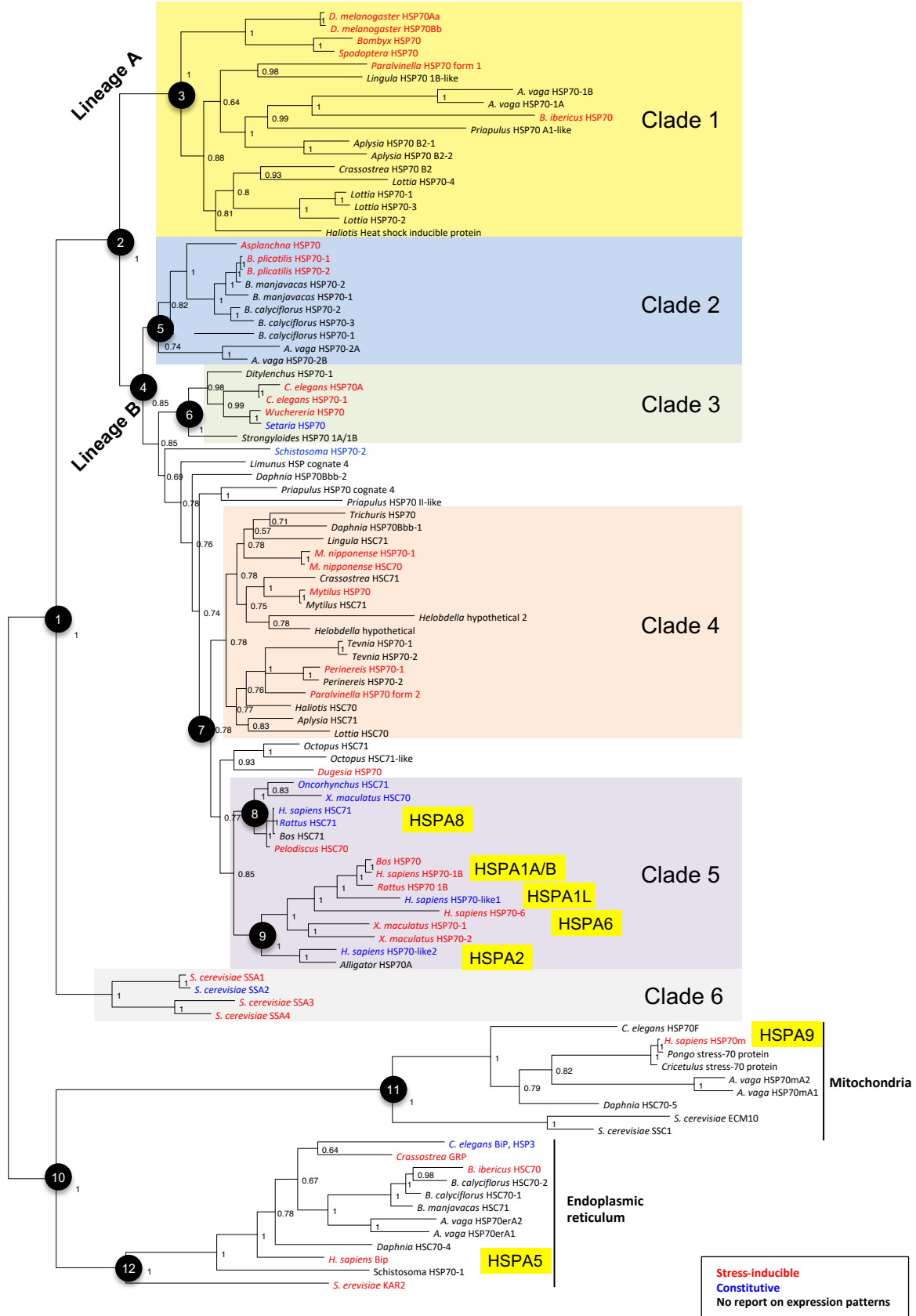
(b) Rotifer genes (node 5) sister group to node 3 with 80% support, other paraphyly

(c) Nematode genes (node 6) basal; rotifer genes sister group to node 3 with 46% support, other paraphyly

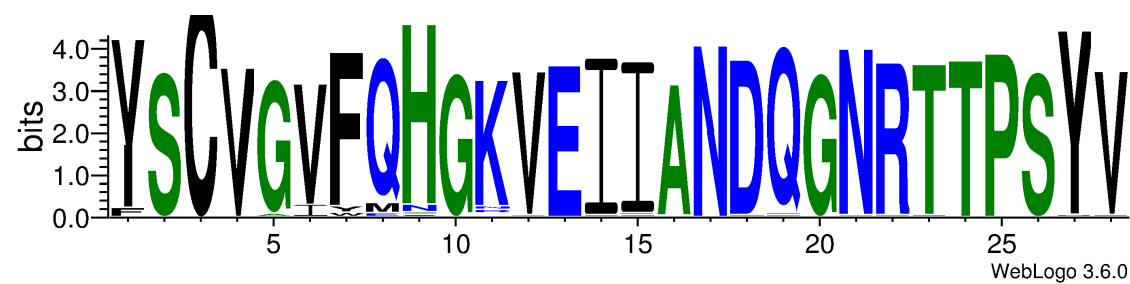
(d) 36% with Trichuris (immediate outgroup to node 6)

(e) Trichuris not in node 6

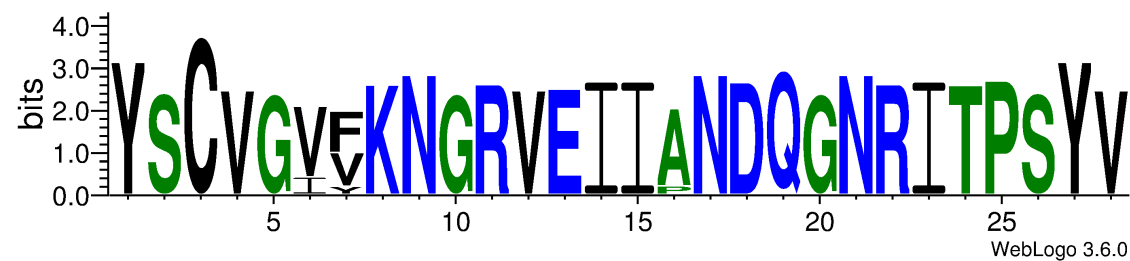




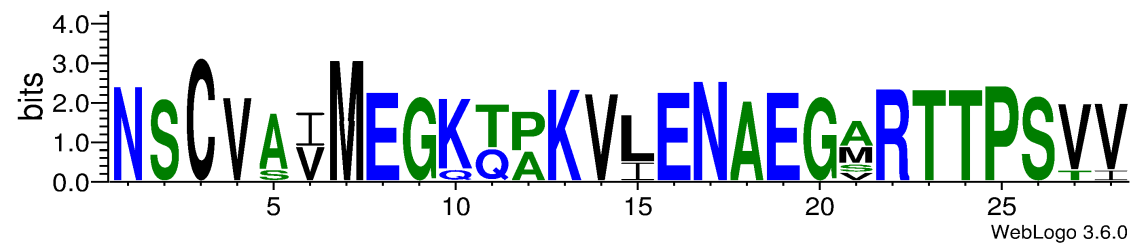
Cytosol



Endoplasmic reticulum



Mitochondrial



<i>D. melanogaster</i> HSP70A	KGERNVLIFDLGGGTFDVSILTIDE----GSLFEVRSTAGDT	224
<i>D. melanogaster</i> HSP70B	KGERNVLILDLGGGTFDVSILTIDE----GSLFEVRSTAGDT	224
<i>Bombyx</i> HSP70	KGERNVLIFDLGGGTFDVSILTIDE----GSLFEVKSTAGDT	224
<i>Spodoptera</i> HSP70	KGERNVLIFDLGGGTFDVSILTIDE----GSLFEVRATAGDT	224
<i>Paralvinella</i> HSP70-1	SGEKNVLIFDLGGGTFDVSILSIAD----GSLFEVKSTAGDT	229
<i>Lingula</i> HSP71	QGEKNVLIFDLGGGTFDVSILSIAD----GSLFEVRSTAGDT	228
<i>A. vaga</i> HSP70-1B	HRDQNILIYDLGGGTFDVSILTISGGDVSGSVFEVKATAGDT	238
<i>A. vaga</i> HSP70-1A	QRDQNILIYDLGGGTFDVSILTISGGDAGGSVFEVRSTAGDT	238
<i>B. ibericus</i> HSP70	KRRKNVLIYDLGGGTFDVSILSIND----G-LFEVLSTNGNT	224
<i>Priapulius</i> HSP70-1	AGERNVLVYDLGGGTFDVSILTIDE----GAMFEVRSTAGDT	226
<i>Aplysia</i> HSP70-1	KGEKHVLIFDLGGGTFDVSILAIIDE----GSIFEVKATAGDT	227
<i>Aplysia</i> HSP70-2	SGEKNVLIFDLGGGTFDVSILTIDE----GSMFEVKATAGDT	230
<i>Crassostrea</i> HSP70	SGEKNVLIFDLGGGTFDVSILTIDE----GSIFEVRSTAGDT	228
<i>Lottia</i> HSP70-4	DTEQHILIFDLGGGTFDVSILAIIDE----G-VFEVLATAGDT	227
<i>Lottia</i> HSP70-1	SGEKNVLIFDLGGGTFDVSILTIDE----GSLFEVKSTAGDT	225
<i>Lottia</i> HSP70-3	SGEKNVLIFDLGGGTFDVSILTIDE----GSLFEVKSTAGDT	226
<i>Lottia</i> HSP70-2	SGEKNVLIFDLGGGTFDVSILAIIDE----GSLFEVKSTAGDT	226
<i>Haliotis</i> HSP70	KGEKNVLIFDLGGGTFDVPVLTIDE----GSMFEVRSTAGDT	231
<i>B. plicatilis</i> HSP70-1	GGEKNVLIYDLGGGTFDVSILTIEE----G-IFEVKSTAGDT	226
<i>B. manjavacas</i> HSP70-1	AGEKNVLIYDLGGGTFDVSILTIED----G-IFEVKATAGDT	226
<i>A. vaga</i> HSP70-2A	SGERNVLIYDLGGGTFDVSILKIEE----G-IFEVKSTAGDT	184
<i>C. elegans</i> HSP70A	HGERNVLIYDLGGGTFDVSILTIED----G-IFEVKSTAGDT	227
<i>Wuchereria</i> HSP70	HGERNVLIYDLGGGTFDVSILTIED----G-IFEVKSTAGDT	226
<i>Setaria</i> HSP70	HGERNVLIYDLGGGTFDVSILTIED----G-IFEVKSTAGDT	226
<i>Strongyloides</i> HSP70	ATERNVLIYDLGGGTFDVSILTIED----G-IFEVKSTAGDT	227
<i>Schistosoma</i> HSP70-2	GGERNVLIYDLGGGTFDVSILTIED----G-IFEVKSTAGDT	224
<i>M. nipponense</i> HSP70-1	GGERNVLIYDLGGGTFDVSILTIED----D-IFEVKSTAGDT	226
<i>Mytilus</i> HSP70	GGERNVLIYDLGGGTFDVSILTIKD----G-IFEVKSTSGDT	227
<i>Tevnia</i> HSP70-1	GGEQNVLIYDLGGGTFDVSILTIED----G-IFEVKSTAGDT	226
<i>Perinereis</i> HSP70-1	GGERNVLIYDLGGGTFDVSILTIED----G-IFEVKSTAGDT	225
<i>Lottia</i> HSP70c	GGERNVLIYDLGGGTFDVSILTIED----G-IFEVKSTAGDT	226
<i>Dugesia</i> HSP70	GIERNVLIYDLGGGTFDVSILTIED----G-IFEVKSTAGDT	226
<i>Oncorhynchus</i> HSC71	GAERNVLIYDLGGGTFDVSILTIED----G-IFEVKSTAGDT	226
<i>H. sapiens</i> HSC71	GAERNVLIYDLGGGTFDVSILTIED----G-IFEVKSTAGDT	226
<i>Pelodiscus</i> HSC70	GAERNVLIYDLGGGTFDVSILTIED----G-IFEVKSTAGDT	226
<i>H. sapiens</i> HSP70-1B	KGERNVLIYDLGGGTFDVSILTIDD----G-IFEVKATAGDT	226
<i>H. sapiens</i> HSP70-likel	QGERHVLIFDLGGGTFDVSILTIDD----G-IFEVKATAGDT	228
<i>X. maculatus</i> HSP70-1	RGERNVLIYDLGGGTFDVSILTIED----G-IFEVKSTAGDT	228
<i>Alligator</i> HSP70A	AGEKNVLIYDLGGGTLDVSILTIED----G-IFEVKSTAGDT	229

3

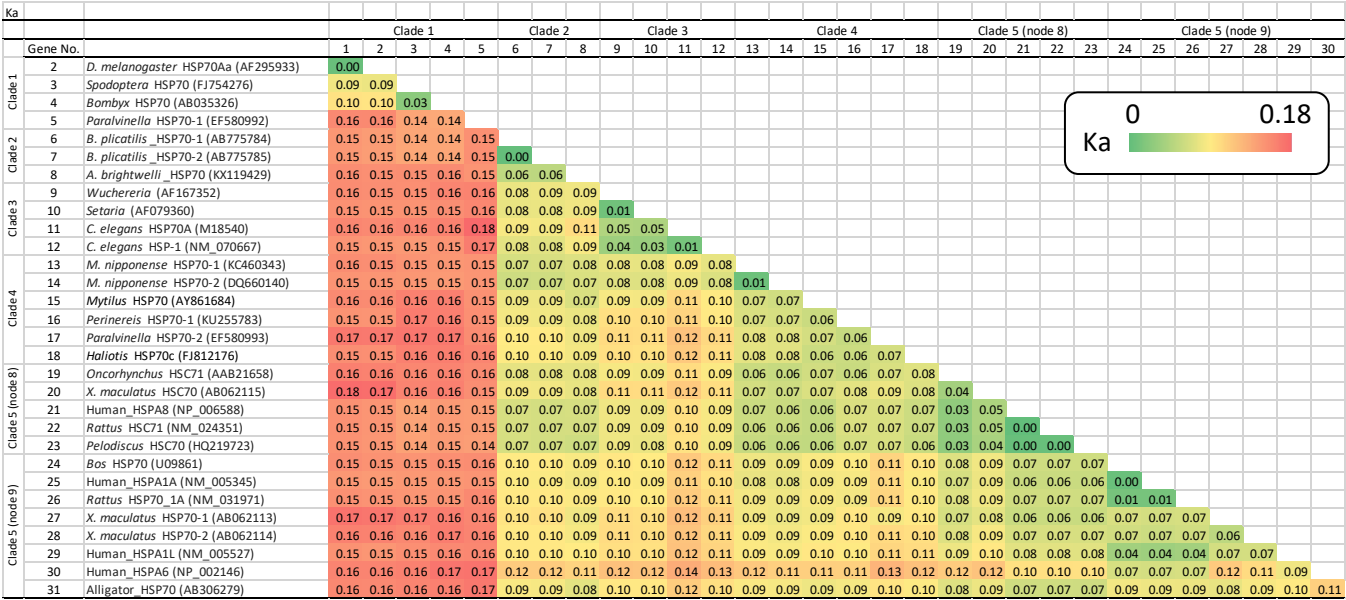
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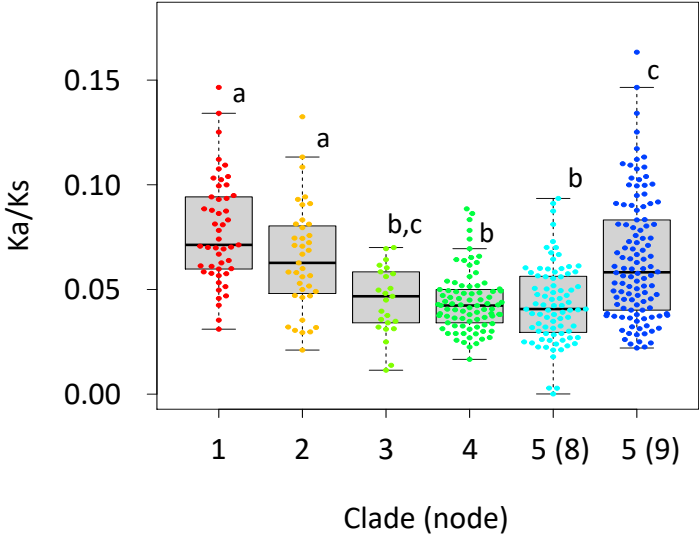
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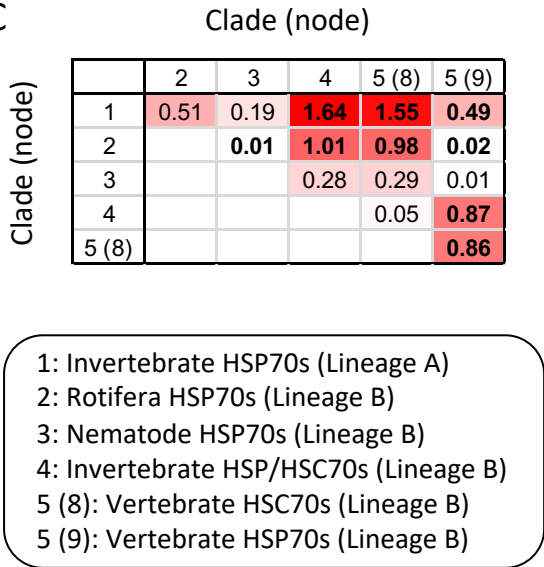
A



B



C



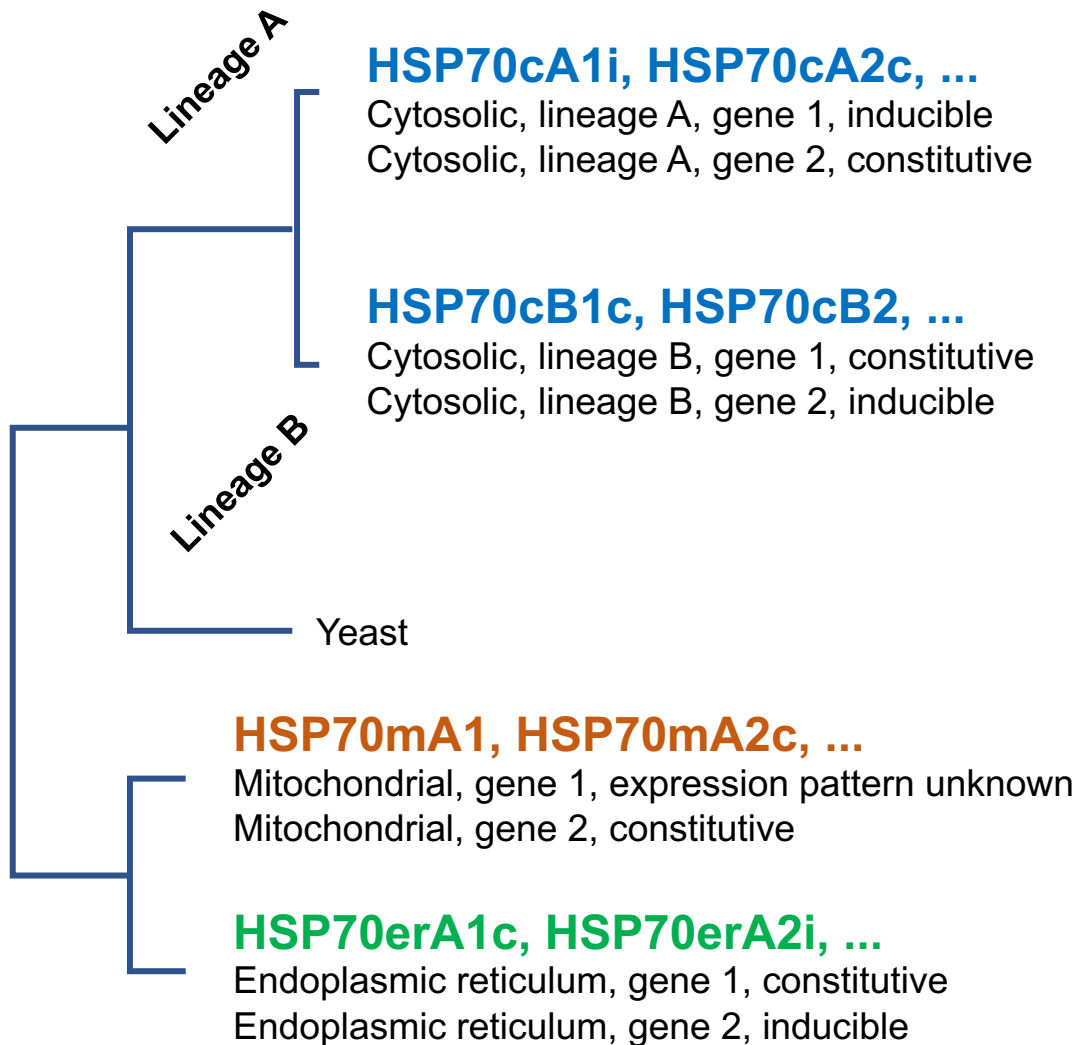


Table 1. Nucleotide sequences of primers used in this study

Primer name	Sequence (5'-3')	Nucleotide position	Gene
<i>cDNA cloning</i>			
rHSP70_5RACE1	AAAAGTCCTGGAGCAGCTT	1107-1125*	HSP70-1, 2
rHSP70_5RACE2	GAATCCTCGACAAAATGGTT	780-800*	HSP70-1, 2
rHSP70_5RACE3	CAGGTCAAAGATCAGGATGTT	645-665*	HSP70-1, 2
rHSP70_3RACE1	AGGCCAACATCGAAATAGA	901-919*	HSP70-1, 2
rHSP70_3RACE2	GACAAGTCAAGTGTGAACGAG	1044-1064*	HSP70-1, 2
rHSP70_full_f1	ACATACACCCAGACACACAA	1-20*	HSP70-1, 2
rHSP70_full_r1	GTCGACTTCATCAATGGTGG	2002-2021*	HSP70-1, 2
GeneRacer 5' RACE	CGACTGGAGCACGAGGACACTGA	-	-
GeneRacer 5' RACE Nested	GGACACTGACATGGACTGAAGGAGTA	-	-
Oligo-dT adapter	GGCCACGCGTCGACTAGTAC (T) ₁₇	-	-
AUAP	GGCCACGCGTCGACTAGTAC	-	-
<i>Semi-quantitative RT-PCR</i>			
rHSP70_gapF	CGATTATTACTCAGTTGTATCAG	1879-1901*	HSP70-1, 2
rHSP70_gapR	ATCAATGGTAGGTCCACTGC	1993-2012*	HSP70-1, 2
rActinF	CCTGCCATGTACGTAGCC	2-19	Actin
rActinR	ATCACTTGACCATCGGGC	340-357	Actin
<i>Quantitative real-time PCR</i>			
RT-hsp70-140F	AGTGGAATAATCGCCAACGA	140-160*	HSP70
RT-hsp70-208R	TCGGTGTCGGTGAACGCTA	190-208*	HSP70
RT-AC-306F	CGTCCAGTTCCTCGTTGGAA	306-325	Actin
RT-AC-373R	CTCGTTGCCAATGGTGATCA	354-373	Actin
<i>In situ hybridization</i>			
rHSP70_insituF	CCATTTACAAACACAAATATT	2026-2046*	HSP70-1, 2
rHSP70_insituR	GTCAAACAAATCTTTATC	2085-2103*	HSP70-1, 2

*Nucleotide positions in HSP70-1 gene

) family members used for the construction of phylogenetic trees

Organism and protein name	Proposed name	Nucleotide accession number	Protein ID	Tissue	Treatment	Quantification method	Expression	Reference for expression patterns
<i>D. melanogaster</i> HSP70Aa	HSP70cA1i	AF295933.1:486-2411	AAG26887	Whole body of larvae	Heat	-	Inducible	UniProtKB, ID P82910
<i>D. melanogaster</i> HSP70Bb	HSP70cA2i	AF295957.1:215-2140	AAG26911	Whole body of larvae	Heat	-	Inducible	UniProtKB, ID Q9BIS2
<i>Bombyx</i> HSP70	HSP70cA1i	AB035326.1:102-2024	BAF69068	Diapause egg	Heat	Real-time PCR	Inducible	Moribe et al., 2010
<i>Spodoptera</i> HSP70	HSP70cA1i	FJ754276.1:151-2154	ACN78407	Fat body	Heat and cold shock	Northern blot	Inducible	Xu et al., 2011
<i>Paralvinella</i> HSP70 form 1	HSP70cA1i	EF580992.1:1-1947	ABU63808	Posterior part of the body	Heat	Western blot	Inducible, but the data may contain other members	Cottin et al., 2008
<i>Lingula</i> HSP70 1B-like	HSP70cA1	XM_013527000.2:123-2033	XP_013382454	-	-	-	-	-
<i>A. vaga</i> HSP70-1B	HSP70cA2	-	GSADVT00016830001	-	-	-	-	-
<i>A. vaga</i> HSP70-1A	HSP70cA1	-	GSADVT00001811001	-	-	-	-	-
<i>B. ibericus</i> HSP70	HSP70cA1i	GU574486.1:1-1860	ADR79281	Whole body	Ultraviolet	Real-time PCR	Inducible	Kim et al., 2011
<i>Priapul</i> HSP70 A1-like	HSP70cA1	XM_014826376.1:219-2171	XP_014681862	-	-	-	-	-
<i>Aplysia</i> HSP70 B2-1	HSP70cA1	XM_005103777.2:352-2253	XP_005103834	-	-	-	-	-
<i>Aplysia</i> HSP70 B2-2	HSP70cA2	XM_005100297.2:191-2110	XP_005100354	-	-	-	-	-
<i>Crassostrea</i> HSP70 B2	HSP70cA1	JH818426.1:39359-41263	EKC30019	-	-	-	-	-
<i>Lottia</i> HSP70-4 (hypothetical protein LOTGIDRAFT_190284)	HSP70cA4	XM_009058212.1:120-2033	XP_009056460	-	-	-	-	-
<i>Lottia</i> HSP70-1 (hypothetical protein LOTGIDRAFT_181897)	HSP70cA1	XM_009053468.1:343-2253	XP_009051716	-	-	-	-	-
<i>Lottia</i> HSP70-3 (hypothetical protein LOTGIDRAFT_209056)	HSP70cA3	XM_009053469.1:216-2129	XP_009051717	-	-	-	-	-
<i>Lottia</i> HSP70-2 (hypothetical protein LOTGIDRAFT_198956)	HSP70cA2	XM_009047345.1:307-2226	XP_009045593	-	-	-	-	-
<i>Halotis</i> heat shock inducible protein 70	HSP70cA1	FJ812177.1:169-2079	ACO36048	-	-	-	-	-
<i>Asplanchna</i> HSP70	HSP70cB1i	KX119429.1:81-2009	ARU12813	Whole body of larvae	Oxidation stressors (Paraquat and juglone)	Real-time PCR	Inducible	Xu et al., 2017
<i>B. plicatilis</i> HSP70cB1i	HSP70cB1i	AB775784.1:66-2024	BAO23806	Whole body of larvae	Heat	Real-time PCR and in situ hybridization	Inducible	This study
<i>B. plicatilis</i> HSP70cB2i	HSP70cB2i	AB775785.1:65-2035	BAO23807	Whole body of larvae	Heat	Real-time PCR and in situ hybridization	Inducible	This study
<i>B. manjavacas</i> HSP70-1	HSP70cB1	-	BmTi01_isotig01884_1998_175_2172_f_0 BmTi01_isotig01885_1998_27_2_024_f_2	-	-	-	-	-
<i>B. manjavacas</i> HSP70-2	HSP70cB2	-	BmTi01_isotig03034_2025_165_2189_r_2	-	-	-	-	-
<i>B. calyciflorus</i> HSP70-2	HSP70cB2	-	NTPY01000792	-	-	-	-	-
<i>B. calyciflorus</i> HSP70-3	HSP70cB3	-	NTPY01000899	-	-	-	-	-
<i>B. calyciflorus</i> HSP70-1	HSP70cB1	-	NTPY01000620	-	-	-	-	-
<i>A. vaga</i> HSP70-2A	HSP70cB1	-	GSADVT00032221001	-	-	-	-	-
<i>A. vaga</i> HSP70-2B	HSP70cB2	-	GSADVT00050272001	-	-	-	-	-
<i>Diitylenchus</i> HSP70-1	HSP70cB1	HQ386232.1:49-1986	AEP19214	-	-	-	-	-
<i>C. elegans</i> HSP70A	HSP70cB1i	M18540.1:423-627,677-1464,1659-2339,2395-2643	AAA28078	Whole body	Heat	Northern blot	Inducible	Snutch et al., 1988
<i>C. elegans</i> HSP70-1	HSP70cB2i	NM_070667.4:1-1923	NP_503068	Whole body	-	-	Inducible	WormBase ID: WBGene00002005
<i>Wuchereria</i> HSP70	HSP70cB1i	AF167352.1:973-1069,1451-1555,1731-2092,2179-2302,2418-2571,2690-2948,3076-3297,3400-3747,3830-4016,4116-4195	AAF32254	Whole body	Heat	SDS-PAGE and blotting, ELISA	Inducible, but the data may contain other members	Ravi et al., 2004

<i>Setaria</i> HSP70	HSP70cB1c	AF079360.1:711-807,1003-1107,1212-1573,1654-1777,1864-2017,2103-2361,2450-2671,2772-3119,3198-3384,3499-3578	AAD13154	Whole body	Heat	Northern blot	Non-inducible	Jayasena et al., 1999
<i>Strongyloides</i> HSP70 1A/1B	HSP70cB1	XM_024648782.1:1-1935	XP_024502726	-	-	-	-	-
<i>Schistosoma</i> HSP70-2	HSP70cB1	L02415.1:533-2446	AAA29898	Whole body	Heat	Northern blot	Non-inducible	Neumann et al., 1993
<i>Limulus</i> HSP cognate 4	HSP70cB1	XM_013923214.2:160-2091	XP_013778668	-	-	-	-	-
<i>Daphnia</i> HSP70Bbb-2	HSP70cB2	LRGB01001774.1:337-903,963-1285,1342-1597,1655-1858,1928-2025,2086-2194,2252-2641	gijl1022765301 gb KZS10545.1	-	-	-	-	-
<i>Priapulus</i> HSPc	HSP70cB1	XM_014812613.1:143-2113	XP_014668099	-	-	-	-	-
<i>Priapulus</i> HSP70 II-like	HSP70cB2	XM_014817626.1:190-2052	XP_014673112	-	-	-	-	-
<i>Trichuris</i> HSP70	HSP70cB1	KL363195.1:c58762-57407,c57345-57028,c56967-56683	KFD56098	-	-	-	-	-
<i>Daphnia</i> HSP70Bbb-1	HSP70cB1	LRGB01002121.1:c532073-531866,c531808-531464,c531400-530010	gijl1022763482 gb KZS09073.1	-	-	-	-	-
<i>Lingula</i> HSP71c	HSP70cB1	XM_013525464.2:202-2139	XP_013380918	-	-	-	-	-
<i>M. nipponense</i> HSP70-1	HSP70cB1i	KC460343.1:253-2202	AGM50430	Gill	Heat	Real-time PCR	Inducible	Xiu et al., 2014
<i>M. nipponense</i> HSC70	HSP70cB2i	DQ660140.1:99-2048	ABG45886	Gill	Heat	Real-time PCR	Inducible	Xiu et al., 2014
<i>Crassostrea</i> HSP71c	HSP70cB1	AB122064.1:33-2012	BAD15287	-	-	-	-	-
<i>Mytilus</i> HSP70	HSP70cB1i	AY861684.1:71-2035	AAW52766	Whole body	Heat, heat-killed bacteria	Real-time PCR	Inducible	Cellura et al., 2006
<i>Mytilus</i> HSP71c	HSP70cB2	AJ783714.1:475-682,1087-1292,1752-1904,2462-3017,5566-5967,6815-7254	CAH04109	-	-	-	-	-
<i>Helobdella</i> hypothetical protein 2 HELRODRAFT	HSP70cB1	XM_009023904.1:1-1878	XP_009022152	-	-	-	-	-
<i>Helobdella</i> hypothetical protein HELRODRAFT	HSP70cB2	XM_009031197.1:188-2125	XP_009029445	-	-	-	-	-
<i>Tevnia</i> HSP70-1	HSP70cB1	FN860147.1:1-411,757-2100,2515-2709	CBM42052	-	-	-	-	-
<i>Tevnia</i> HSP70-2	HSP70cB2	FN860148.1:1-411,865-2208,2623-2817	CBM42053	-	-	-	-	-
<i>Perinereis</i> HSP70-1	HSP70cB1i	KU255783.1:49-2019	AND99892	Whole body	Cu ²⁺	Real-time PCR	Inducible	Zhang et al., 2016
<i>Perinereis</i> HSP70-2	HSP70cB2	HQ449186.1:89-2050	ADR66514	-	-	-	-	-
<i>Paralvinella</i> HSP70 form 2	HSP70cB1i	EF580993.1:1-1962	ABU63809	Posterior part of the body	Heat	Western blot	Inducible, but the data may contain other members	Cottin et al., 2008
<i>Haliois</i> HSC70	HSP70cB1	FJ812176.1:77-2032	ACO36047	-	-	-	-	-
<i>Aplysia</i> HSC71	HSP70cB1	XM_005097955.2:103-2067	XP_005098012	-	-	-	-	-
<i>Lottia</i> HSC70 (hypothetical protein LOTGIDRAFT_177837)	HSP70cB1	XM_009048116.1:1-1962	XP_009046364	-	-	-	-	-
<i>Octopus</i> HSC71	HSP70cB1	XM_014927588.1:112-2067	XP_014783074	-	-	-	-	-
<i>Octopus</i> HSP71-like	HSP70cB2	XM_014929288.1:100-2058	XP_014784774	-	-	-	-	-
<i>Dugesia</i> HSP70	HSP70cB1	EU380241.1:53-1999	ABY83101	Whole body	Heat, amputation and starvation	Semi-quantitative RT-PCR	Inducible	Ma et al., 2009
<i>Oncorhynchus</i> HSC71	HSP70cB1c	S85730.1:2774-2978,3222-3427,3528-3680,3904-4459,4686-4888,5108-5306,5536-5768,5965-6165	AAB21658	Gonad cell line	Heat	Northern blot	Non-inducible	Zafarullah et al., 1992
<i>X. maculatus</i> HSC70	HSP70cB1c	AB062115.1:135-2048	BAB72169	Muscle and liver	Heat	Northern blot	Non-inducible	Yamashita et al., 2004
<i>H. sapiens</i> HSC71 (HSPA8)	HSP70cB6c	NM_006597.6:79-2019	NP_006588	-	-	-	-	-
<i>Rattus</i> HSC71	HSP70cB1c	NM_024351.2:81-2021	NP_077327	Brain	Axonal synapsin	Two-color superresolution microscopy	Non-inducible	Ganguly et al., 2017
<i>Bos</i> HSC71	HSP70cB1	NM_174345.4:76-2028	P19120	-	-	-	-	-
<i>Pelodiscus</i> HSC70	HSP70cB1i	HQ219723.1:81-2021	ADO17794	Liver, lung, heart, and skeletal muscle	Heat	Western blot, Real-time PCR	Inducible	Li et al., 2012
<i>Bos</i> HSP70	HSP70cB2i	U09861.1:157-2082	AAA73914	Skeletal muscle	Bacteria	Two-dimensional gel electrophoresis	Inducible	Gutierrez and Guerriero, 1995

<i>H. sapiens</i> HSP70-1B (HSPA1B)	HSP70cB2i	NM_005346.5:214-2139	NP_005337	-	-	-	Inducible	Uniprot P0DMV8
<i>Rattus</i> HSP70	HSP70cB2i	NM_031971.2:1248-3173	NP_114177	Pancreas	Heat	Western blot	Inducible	Lee et al., 2018
<i>H. sapiens</i> HSP70-like1 (HSPA-1L)	HSP70cB3c	NM_005527.4:409-2334	NP_005518	HeLa cells	Heat	Northern blot	Non-inducible	Uniprot P34931, Milner and Campbell, 1990
<i>H. sapiens</i> HSP70-6 (HSPA6)	HSP70cB5i	NM_002155.5:120-2051	NP_002146	Neuronal cells	Aarimoclomol	Immunostaining, Western blot	Inducible	Deane and Brown, 2018, UniProt P17066
<i>X. maculatus</i> HSP70-1	HSP70cB1i	AB062113.1:83-1999	BAB72167	Muscle and liver	Heat	Northern blot	Inducible	Yamashita et al., 2004
<i>X. maculatus</i> HSP70-2	HSP70cB2i	AB062114.1:131-2050	BAB72168	Muscle and liver	Heat	Northern blot	Inducible	Yamashita et al., 2004
<i>H. sapiens</i> HSP70-like2 (HSPA2)	HSP70cB4c	NM_021979.3:383-2302	NP_068814	Lung carcinoma cell	Heat	Immunostaining, Western blot	Non-inducible	Ściegłńska et al., 2008
<i>Alligator</i> HSP70A	HSP70cB1	AB306279.1:43-1962	BAF94142	-	-	-	-	
<i>S. cerevisiae</i> SSA1	-	NC_001133.9:c141431-139503	YAL005C	-	Heat	-	Inducible	Boorstein et al., 1994
<i>S. cerevisiae</i> SSA2	-	NC_001144.5:c97485-95566	YLL024C	-	Heat	-	Non-inducible	Boorstein et al., 1994
<i>S. cerevisiae</i> SSA3	-	NC_001134.8:c86448-84499	YBL075C	-	Heat	-	Inducible	Boorstein et al., 1994
<i>S. cerevisiae</i> SSA4	-	NC_001137.3:364589-366517	YER103W	-	Heat	-	Inducible	Boorstein et al., 1994
<i>C. elegans</i> HSP70F	HSP70mA1	NM_071890.4:1-1974	NP_504291	-	-	-	-	-
<i>H. sapiens</i> HSP70m (HSPA9)	HSP70mA1i	NM_004134.7:84-2123	NP_004125	Cancer cells	Ovarian carcinoma	Immunostaining, Western blot	Inducible	Xu et al., 2019
<i>Pongo</i> stress-70 protein	HSP70mA1	NM_001133388.1:101-2140	NP_001126860	-	-	-	-	-
<i>Cricetulus</i> stress-70 protein	HSP70mA1	NM_001246829.1:1-2040	NP_001233758	-	-	-	-	-
<i>A. vago</i> HSP70mA2	HSP70mA2	-	GSADVT00049157001	-	-	-	-	-
<i>A. vago</i> HSP70mA1	HSP70mA1	-	GSADVT00003702001	-	-	-	-	-
<i>Daphnia</i> HSC70-5	HSP70mA1	LRGB01000716.1:c3467-3389,c3326-3203,c3078-2880,c2819-2228,c2144-1297	gij1022772237 gb KZS16423.1	-	-	-	-	-
<i>Scerevisiae</i> ECM10	-	NC_001137.3:94644-96578	YEL030W	-	-	-	-	-
<i>Scerevisiae</i> SSC1	-	NC_001142.9:c521602-519638	YJR045C	-	-	-	-	-
<i>C. elegans</i> BiP, heat shock protein 3	HSP70erA1c	M26604.1:442-628,675-1148,1387-2132,2237-2815	AAA28074	Whole body of larvae	Heat	Not mentioned	Non-inducible	Heschl and Baillie, 1989
<i>Crassostrea</i> GRP	HSP70erA1i	AB122065.1:108-2093	BAD15288	Gill and adductor muscle	Heat	Northern blot	Inducible	Yokoyama et al., 2006
<i>B. ibericus</i> HSC70	HSP70erA1i	GU574487.1:1-1962	ADR79282	Whole body	Ultraviolet	Real-time PCR	Inducible	Kim et al., 2011
<i>B. calyciflorus</i> HSC70-2	HSP70erA2	NTPY01000000	NTPY01001024	-	-	-	-	-
<i>B. calyciflorus</i> HSC70-1	HSP70erA1	NTPY01000000	NTPY01001024	-	-	-	-	-
<i>B. manjavacas</i> HSC71	HSP70erA1	-	1977_126_2102_r_0_1 BmTi01_isotig03139	-	-	-	-	-
<i>A. vago</i> HSP70erA2	HSP70erA2	-	GSADVT00030804001	-	-	-	-	-
<i>A. vago</i> HSP70erA1	HSP70erA1	-	GSADVT00008959001	-	-	-	-	-
<i>Daphnia</i> HSC70-4	HSP70erA1	LRGB01000024.1:1105780-1105910,1105969-1106200,1106260-1106476,1106539-1106876,1106942-1107467,1107528-1108063	gij1022777923 gb KZS21417.1	-	-	-	-	-
<i>H. sapiens</i> Bip	HSP70erA1i	NM_005347.5:218-2182	NP_005338	Umbilical vein endothelial cells	Thapsigargin (endoplasmic stress)	Western blot	Inducible	Uniprot P11021, Milner and Campbell, 1990
<i>Schistosoma</i> HSP70-1	HSP70erA1	XM_018794726.1:55-2001	XP_018649109	-	-	-	-	-
<i>S. cerevisiae</i> KAR2	-	NC_001142.9:381327-383375	YJL034W	-	Heat	-	Inducible	Boorstein et al., 1994

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Bp_HSP70-1 -----MSKGPVAVGIDLGTTTSCVGVFQHGKVEI IANDQG 34
Bp_HSP70-2 -----MSKGPVAVGIDLGTTTSCVGVFQHGKVEI IANDQG 34
Bi_HSC70 MNKLGLFLLLVLSSISYLNQKDEKKESYGTVIGIDLGTTTSCVGVVKNGRVEI IANDQG 60
Bi_HSP70 -----MSPAIGIDLGTTTSCVAVFENGKVEI IANDQG 32
Ab_HSP70 -----MAKTAIGIDLGTTTSCVGVFQHGKVEI IANDQG 33
Pr_HSP70 -----MARAIGIDLGTTTSCVGIFQHGKVEI IANDQG 32
                .:*****:***.:.:*:*****

Bp_HSP70-1 NRTTPSYVAFT-DTERLIGDAAKNQVAMNPNTVFDKRLIGRKFDLTVQADMKHWPFT 93
Bp_HSP70-2 NRTTPSYVAFT-DTERLIGDAAKNQVAMNPNTVFDKRLIGRKFDLTVQADMKHWPFT 93
Bi_HSC70 NRITPSYVAFTSDGERLIGDAAKNQLTSNPENTIFDAKRLIGREFKDSSVQGMKYWPFK 120
Bi_HSP70 NRTTPSYVAFT-DTERIIGDAAKAQVATNPNTVFDKRLIGRRFDEHTVQSDMKHWPFT 91
Ab_HSP70 NRTTPSYVAFT-DTERLIGDAAKNQVAMNPNTVFDKRLIGRRFDDSTVQSDMKHWPFT 92
Pr_HSP70 NRTTPSYVAFT-DSERLIGDAAKNQVAMNPNTIFDAKRLIGRKFDATVQADMKHWPFT 91
** ***** * **:***** *: * ***:*****.:.: **:***:***.

Bp_HSP70-1 VISDSGRPKIQVEFKGETKSFYPEEVSSMVLTKMKETA EAYLGKKVTD AVVTPPAYFNDS 153
Bp_HSP70-2 VISDSGRPKIQVEFKGETKSFYPEEVSSMVLTKMKETA EAYLGKKVTD AVVTPPAYFNDS 153
Bi_HSC70 VVDKNNKPHIKVKSADDEEKLFAAEVVSAMVLGKMKEIAEAYLGKPVTHAVVTPPAYFNDA 180
Bi_HSP70 LVNVNGKPKIQVEFKNSKTFSP E EISSMVLTKMKETAESYLRKKS KCSHHSTGLFQRF 151
Ab_HSP70 VVNEGKPKIQVEFKGEKKTFFPEEISSMVLTKMKETAEAYLGKKVND AVITVPAYFNDS 152
Pr_HSP70 VISEGGKPKIQVEYKNETKSFTPEEVSSMVLTKMKETAEAYLGKKISEAVITVPAYFNDS 151
.:. .:***:*. . * * *:***:*** ***:** * . . . *:

Bp_HSP70-1 QRQATKDAGAIAGLNVLR IINEPTAAAIAYGLDKKGGGEKNILIFDLGGGTFDVSILTIE 213
Bp_HSP70-2 QRQATKDAGAIAGLNVLR IINEPTAAAIAYGLDKKGGGEKNILIFDLGGGTFDVSILTIE 213
Bi_HSC70 QRQATKDAGTIAGMTVMRI IINEPTAAVYCLRLGQK-EGEKNILVFDLGGGTFDVSLLTID 239
Bi_HSP70 ATTSYQRCWINSRFKCATYYQRAYCCCSCLWLRKKFKRRKNVLIYDLGGGTFDVSVLSIN 211
Ab_HSP70 QRQATKDAGAIAGLNVLR IINEPTAAAIAYGLDKKVGGEKNVLIIFDLGGGTFDVSILTIE 212
Pr_HSP70 QRQATKDAGVIAGLNVLR IINEPTAAAIAYGLDKKVTGERNLIIFDLGGGTFDVSVLKIE 211
        : : . : :. :. . . * : * .:***:*****:***:

Bp_HSP70-1 EGIFEVKSTAGDTHLGGEDFDNRLVNHVFVEEFKRKNKKDITSNKRALRRLRTACERAKRT 273
Bp_HSP70-2 EGIFEVKSTAGDTHLGGEDFDNRLVNHVFVEEFKRKNKKDITSNKRALRRLRTACERAKRT 273
Bi_HSC70 NGVFEVVATNGDTHLGGEDFDNRVMEHF I KLFKKKTGKDIRKDNRAVQKLREVEKAKRT 299
Bi_HSP70 DGLFEVLSTNGNTHLGGEDFDNNLVNFFASEFRKHGKDL SRNQALRRLRTACERAKRT 271
Ab_HSP70 EGIFEVKSTAGDTHLGGEDFDNRLVNHVFVEEFKRKHKKDITGNKRAIRRLRTACERAKRT 272
Pr_HSP70 EGIFEVKSTAGDTHLGGEDFDNRMVSHFVQEFKRKNKDL SQNKRLRLRTACERAKRT 271
*:*** : * *:*****.:.:. * :*: ** : : : : : * :****

Bp_HSP70-1 LSSSAQANIEIDSLHEGVDFYTSITRARFEELCADLFRGTLDPVEKALRDAMDKSSVNE 333
Bp_HSP70-2 LSSSAQANIEIDSLHEGVDFYTSITRARFEELCADLFRGTLDPVEKALRDAMDKSSVNE 333
Bi_HSC70 LSTQFDTRIEIESFDGEDFSETFTRARFEELNMDLFRNTLKPQKVL EDAGLKKEIDE 359
Bi_HSP70 LSSSAEATIEVDSLYEGTDFNSKISRARFEELNMDLFRSTLHLVEQALKDARITKTKIDE 331
Ab_HSP70 LSSSAQANIEIDSLHEGVDFYTNITRARFEELNADLFRGCLDPVEKSLRD AKLDKAQIHE 332
Pr_HSP70 LSSSSQASIEIDSLHEGIDFYSTITRARFEELCADLFRSTLEPVEKALRDAMDKASIHE 331
**.: : :*:***:*** ** .:***** ****. * .*: * ** : : : : *

Bp_HSP70-1 IVLVGGSTRIPKVQKLLQDFFNGKELNKSINPDEAVAYGAAVQAA I LTGDKSEAVQD LLL 393
Bp_HSP70-2 IVLVGGSTRIPKVQKLLQDFFNGKELNKSINPDEAVAYGAAVQAA I LTGDKSEAVQD LLL 393
Bi_HSC70 IVLVGGSTRIPKIQQLVKEYFDGKEPSRGINPDEAVAYGAAVQGGVLSGEDVG--GEVVL 417
Bi_HSP70 IVLVGGSTRIPRIQKAFRGFFNGKQLNKSINPDEAVAYGAAVQAA I LSGDKSREIKDVL 391
Ab_HSP70 IVLVGGSTRIPKVQKLLQDFFNGKELNKSINPDEAVAYGAAVQAA I LTGDKSEAVQD LLL 392
Pr_HSP70 IVLVGGSTRIPKVQKLLQDFFNGKELNKSINPDEAVAYGAAVQAA I LTGDKSEEVKDVL 391
*****:***: .: :*:***: .:*****:*****.:***: . : : *

Bp_HSP70-1 LDVAPLSLGIETAGGVMTALIKRNTTIPTKQTQTFTTYADNQP GVLIQVYEGERAMTKDN 453
Bp_HSP70-2 LDVAPLSLGIETAGGVMTALIKRNTTIPTKQTQTFTTYADNQP GVLIQVYEGERAMTKDN 453
Bi_HSC70 LDVNPLTMGIETVGGVMTKLIPRNTVIPTKNP-KFSTASDNQPTVTIAVYEGERPMTKDN 476
Bi_HSP70 VDVAPLSLGIETAGGVMTNLINRNSRIPVKTSQIFTTYSDNQTA V SIRVFEGERALVQDN 451
Ab_HSP70 LDVAPLSLGIETAGGVMTSLIKRNTTIPTKQTQVFTTYSDNQP GVLIQVYEGERAMTKDN 452
Pr_HSP70 LDVAPLSLGIETAGGVMTPLIKRNTTIPTKQTQTFTTYSDNQP GVDIKVFEGERSMTRDN 451
: * * *:***:***** * * *: * * * * : * * * * * : : *

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Bp_HSP70-1  HLLGKFELSGIPAPRGVPQIEVTFDIDANGILNVSAADKSTGKTNKITITNDKGRLSKE  513
Bp_HSP70-2  HLLGKFELSGIPAPRGVPQIEVTFDIDANGILNVSAADKSTGKTNKITITNDKGRLSKE  513
Bi_HSC70    HLLGKFDLTGIPAPRGVPQIEVTFEIDANGILKVSADKGTGNKNNIVINNQNRLSPE  536
Bi_HSP70    HLLGNFIDLVGIPAPRGVPIQIDVTFDIDANGILSVSAKENSSGKSNNITIKNDKGRLSKE  511
Ab_HSP70    NLLGKFELTGIPAPRGVPQIEVTFDIDANGILNVSAADKSTGKQNKITITNDKGRLSKE  512
Pr_HSP70    HLLGNFELSGIPAPRGVPQIEVTFDIDANGILNVTAVDKSTGRENKITITNDKGRLSKD  511
:***:*:* *****:***:***:*****.*:* :.:*. *:.*.*:..*** :

Bp_HSP70-1  EIDRMVNEAEKYKKDDEEQRDKVAAKNSLESYCFNMKQTVED-EKLAAKISADDKKKILD  572
Bp_HSP70-2  DIDRMVNEAEKYKKDDEEQRDKVAAKNSLESYCFNMKQTVED-EKLAAKISADDKKKILD  572
Bi_HSC70    EIERMIKDAEKFADEDKKVKEKVEAKNELESYVYSLKNQLNDKEKLGKLSSEDEKETINS  596
Bi_HSP70    EIERMINEAERFRAEDDRQRDRIASKNKLETYIFAVKQALDD-AK---NLSSSDKKVCQE  567
Ab_HSP70    DIERMVNDAEKYKKDDEQQRERITAKNSLESYCFNMKQTIED-EKLAAKVSADEKKKILD  571
Pr_HSP70    QIEQMVAEAEKYKKEDEAQRDRISAKNSLESYCFNMKTSIND-DKIGAKISADDKAKITE  570
:***:*: :***: :*. :.: :*.***:* : :* :.* * :.* :.* .

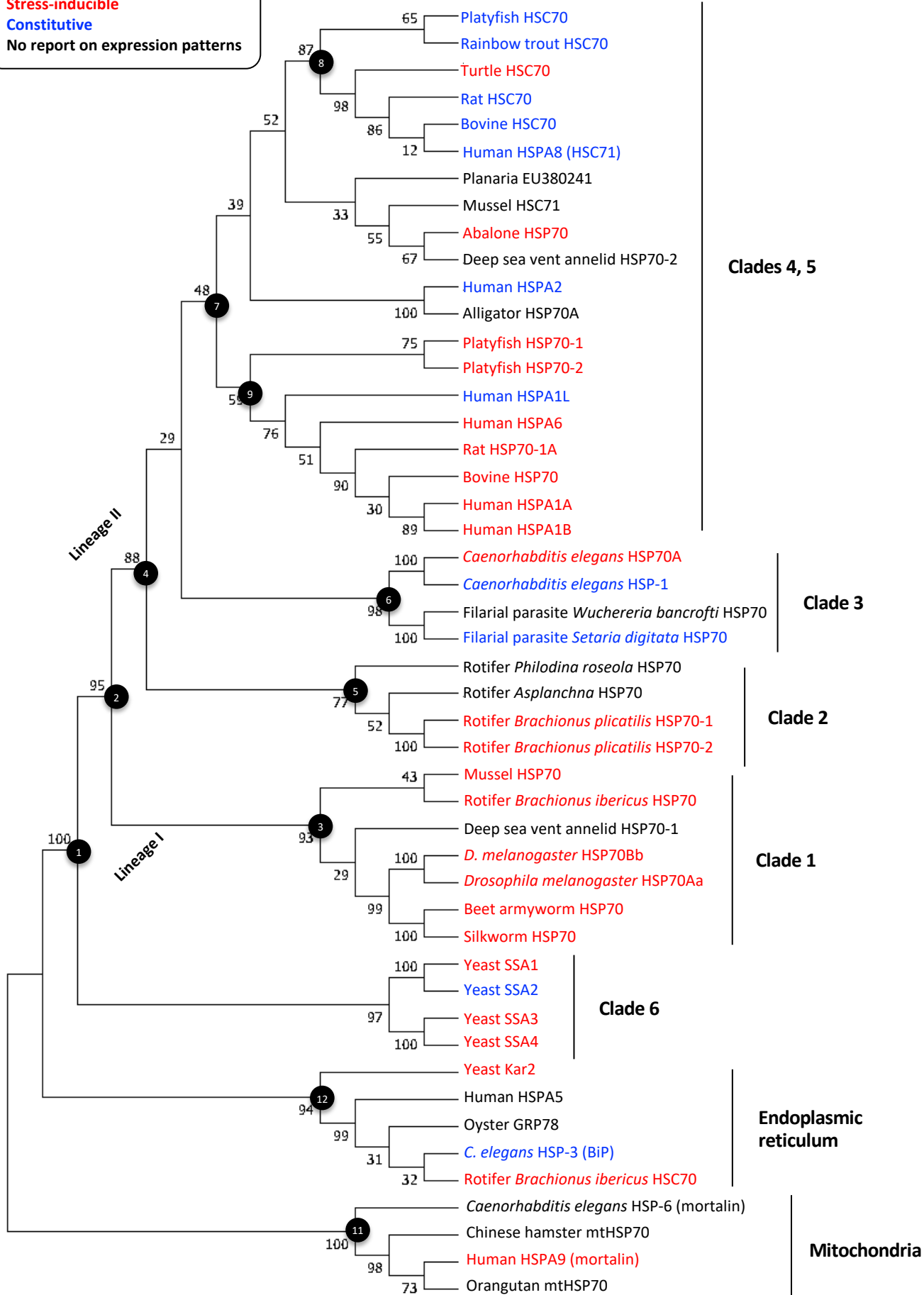
Bp_HSP70-1  ACEEALKWLDNSQTAEKDEFEHKMKEVEKICSPIITQLYQGAGGAPGGMPGGM----PGG  628
Bp_HSP70-2  ACEEALKWLDNSQTAEKDEFEHKMKEVEKICSPIITQLYQGAGGAPGGMPGGMPGGMPGG  632
Bi_HSC70    AVDEKIKWLESNSDAEVDDFKEQKKALEEIVNPIMTKLYQQNSENAGSSDSQTNDEL---  653
Bi_HSP70    ACRKELKWLEANQMADKDEFAFHYKELSRKCMPLMKKIHSGERNGPTVEEVE-----  619
Ab_HSP70    ACENALKWLDANQTAEKDEFEHKLKEIEKTCSPIITKLYQG--GAPGGMPGGMPGGAGA-  628
Pr_HSP70    TIESALKWMETNQLAEKDEFEHKLKEVEKICSPIMTKLYGGEAGGHAG-PGGHAGGA---  626
: . :***:*. *:* * : * .. *::*:

Bp_HSP70-1  MPGGMPGAGPESAGRSGPTIDEVD  652
Bp_HSP70-2  MPGGMPGAGPESAGRSGPTIDEVD  656
Bi_HSC70    -----  653
Bi_HSP70    -----  619
Ab_HSP70    --GGAS-----GAGPKIEEVD  642
Pr_HSP70    -PGGS-----KSGGKGPTIEEVD  643

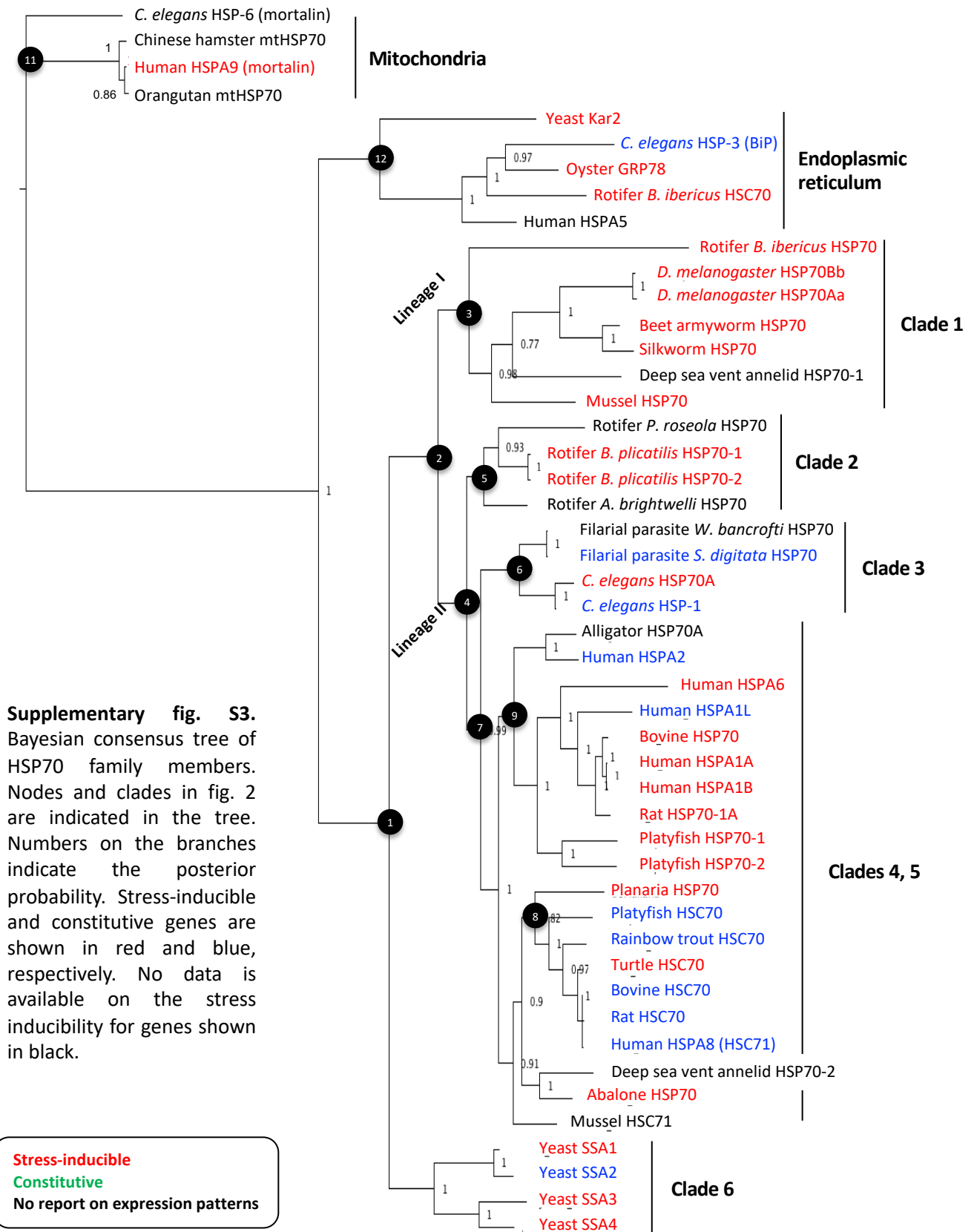
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Supplementary fig. S1. Comparison of the deduced amino acid sequences of cDNAs encoding HSP70-1 and HSP70-2 of *Brachionus plicatilis* with those of the HSP70 family members from other rotifers. Dashes denote gaps introduced to maximize homology. The HSP70 protein family signatures are meshed, and the EEVD motifs are boxed. The non-organelle consensus motifs and bipartite nuclear localization motifs are shown in blue and red letters, respectively. The numbers in the right margin of the sequences represent residues from the N-terminus. Bi, *B. ibericus* (ADR79281, ADR79282); Ab, *Asplanchna brightwelli* (ARU12813).

No report on expression patterns



Supplementary fig. S2. Maximum likelihood tree of HSP70 family members. A bootstrap consensus tree was constructed by the maximum likelihood method using the LG + G model. The bootstrap values from a 1000-replicate analysis are given at the nodes in percentage. Nodes and clades in fig. 2 are indicated in the tree. Stress-inducible and constitutive genes are shown in red and blue, respectively. No data on the stress inducibility is available for genes shown in black.



Supplementary fig. S3. Bayesian consensus tree of HSP70 family members. Nodes and clades in fig. 2 are indicated in the tree. Numbers on the branches indicate the posterior probability. Stress-inducible and constitutive genes are shown in red and blue, respectively. No data is available on the stress inducibility for genes shown in black.

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Aplysia_HSP70-2 -----MTKGMKTPAIGIDL 14
Haliotis_HSP70 -----MAPKGKSPAIGIDL 14
Lottia_HSP70-4 -----MAPKGPAIGIDL 12
Bplicatilis_HSP70-1 -----MSGGPAVGIDL 11
Avaga_HSP70erA -----MKIFTALSLIALTTITFFSSASEDAKKDIGTVIGIDL 37
Bibericus_HSC70 -----MNKLGFLLLVLSSISYLNQKDEKESYGTIVIGIDL 37
BcHSC70-2 -----GTVIGIDL 8
Celegans_HSP70F MLSARS-----FLSSARTIA---RSSLSARSLSDKPKGHVIGIDL 38
Daphnia_HSP70c5-1 ----- 0
Pongo_HSP70m MISASRAVAARLVGAAASRGPTAARYQDGWNGLSHEAFRIVSRRDYASEAIKGAUVGIDL 60
Hsapiens_HSP70m MISASRAAAARLVGAAASRGPTAARHQDSWNGLSHEAFRLVSRDYASEAIKGAUVGIDL 60

Aplysia_HSP70-2 GTTYSYSCVGFQHGKVEIIANDQGNRTTPSYVAFT-DTERLVGDAAKNQAAALNPSNTIFDA 73
Haliotis_HSP70 GTTYSYSCVGVFQHGQVEIIANDQGNRTTPSYVAFT-DTERLIGDAAKNQVALNPQNTVFDA 73
Lottia_HSP70-4 GTTYSYSCVGVFQHGAVEIIANDQGNRTTPSYVAFT-DSERLIGDSAKNQVALNPNTIFDA 71
Bplicatilis_HSP70-1 GTTYSYSCVGVFQHGKVEIIANDQGNRTTPSYVAFT-DTERLIGDAAKNQVAMNPNTVFDA 70
Avaga_HSP70erA GTTYSYSCVGFKNGRVEIIANDQGNRTTPSYVAFTAEGERLIGDAAKNQLTSPNPNTVFDA 97
Bibericus_HSC70 GTTYSYSCVGVKNGRVEIIANDQGNRTTPSYVAFTSDGERLIGDAAKNQLTSPNPNTIFDA 97
BcHSC70-2 GTTYSYSCVGVKNGRVEIIANDQGNRTTPSYVAFTSEGERLIGDAAKNQLTSPNPNTIFDA 68
Celegans_HSP70F GTTNSCVSIMEGKTPKVIENAEGVRTPSTVAFTADGERLVGAPAKRQAVTNSSANTLFAT 98
Daphnia_HSP70c5-1 -----MEGQTPKVIENAEGSRTPTSVIAFTKDGERLAGMPAKRQAVTNQONTLYAT 51
Pongo_HSP70m GTTNSCVAVMEGKQAKVLENAEGARTTPSVVAFTADGERLVGMPAKRQAVTNPNNTFYAT 120
Hsapiens_HSP70m GTTNSCVAVMEGKQAKVLENAEGARTTPSVVAFTADGERLVGMPAKRQAVTNPNNTFYAT 120
      .:      :: * : * * * : * * : * * * * * * . * * * . :

Aplysia_HSP70-2 KRLIGRKFTDKSVQSDMKHWPFFQVAE-VDSRPKIEAEYKGERKLFAPEEVSSMVLTKMKE 132
Haliotis_HSP70 KRLIGRQFDDSAVQKDMKHWPFRVNVSSGSKPKLQVEYKGEKKTFAPEEISSMVLNKMKE 133
Lottia_HSP70-4 KRLIGRKFDATIQSDMKHWPFFKVVK-DGDKPKLQAEFKNELKTFSPPEEVSAMVLTKMKE 130
Bplicatilis_HSP70-1 KRLIGRKFDLTVQADMKHWPFTVIS-DSGRPKIQVEFKGETKSFYPEEVSSMVLTKMKE 129
Avaga_HSP70erA KRLIGREFSDSSVQQDMKHFPFKVVE-KNSKPAIQISTGKEQKLFTPEEISAMVLGKMRE 156
Bibericus_HSC70 KRLIGREFKDSSVQGDMAKYWPFKVVD-KNNKPHIKVKSADEEKLFAAEVVSAMVLGKMKE 156
BcHSC70-2 KRLIGREFSDPSVQGDMAKYWPFKVVE-KNKKPYIKVKSQDQDKLFAPEEISAMVLGKMKE 127
Celegans_HSP70F KRLIGRRYEDPEVQKDLKVVPYKIVKASNGDAWVE---AQGKVYSPSQVGAFVLMKMKE 154
Daphnia_HSP70c5-1 KRLIGRKFEDEPVKKDMKTVSYKIVRASNGDAWVE---AQGKLYSPSQIGAFVLTMMKE 107
Pongo_HSP70m KRLIGRRYDDPEVQDKINVPFKIVRASNGDAWVE---AHGKLYSPSQIGAFVLMKMKE 176
Hsapiens_HSP70m KRLIGRRYDDPEVQDKINVPFKIVRASNGDAWVE---AHGKLYSPSQIGAFVLMKMKE 176
*****.: * ::*: * : : . : : . * : .: : : * * *: *

Aplysia_HSP70-2 TAEAYLGQKVTEAVITVPAYFNDSQRQATKDAGAIAGLNLVRMINEPTAAALAYGLDKGQ 192
Haliotis_HSP70 TAEAYLGQQVTDVAITVPAYFNDSQRQATKDAGAIAGLNLVRIINEPTAAALAYGLDKNL 193
Lottia_HSP70-4 TAEAYLGENVTVNAVITVPAYFTDSQRSATKDAGVIAGLNLVRIINEPTAAALAYGLDKKT 190
Bplicatilis_HSP70-1 TAEAYLGKKVTDVAVTVPAYFNDSQRQATKDAGAIAGLNLVRIINEPTAAAIAYGLDKKG 189
Avaga_HSP70erA IAEAYLGKVTTHAVVTVPAYFNDSQRQATKDAGTISGLNLVRIINEPTAAAIAYGLDKK- 215
Bibericus_HSC70 IAEAYLGKVPVTHAVVTVPAYFNDAQRQATKDAGTIAGMTVMRIINEPTAAVYCLRLGQK- 215
BcHSC70-2 IAEAYLGKNVTHAVVTVPAYFNDAQRQATKDAGTISGMTVMRIINEPTAAAIAYGLDKK- 186
Celegans_HSP70F TAESYLGTTVNNNAVTVPAYFNDSQRQATKDAGQISGLNLVRVINEPTAAALAYGLDKD- 213
Daphnia_HSP70c5-1 TAEAYLGTPVKNNAVTVPAYFNDSQRQATKDAGQISGLNLVRVINEPTAAALAYGLDKS- 166
Pongo_HSP70m TAENYLGHTAKNAVITVPAYFNDSQRQATKDAGQISGLNLVRVINEPTAAALAYGLDKS- 235
Hsapiens_HSP70m TAENYLGHTAKNAVITVPAYFNDSQRQATKDAGQISGLNLVRVINEPTAAALAYGLDKS- 235
      * * * . . *:*****.*:*.***** *:*.*:*.*****. . : :

Aplysia_HSP70-2 SGEKNVLIFDLGGGTFDVSULTIDEGSMFEVKATAGDTHLGGEDFDNRLVSHFLQEFKRK 252
Haliotis_HSP70 KGEKNVLIFDLGGGTFDVPVLTIDEGSMFEVRSSTAGDTHLGGEDFDNRLVEHFLQEFQRK 253
Lottia_HSP70-4 DTEQHILIFDLGGGTFDVSILAIEDG-VFEVLATAGDTHLGGEDFDNRMVNYFTQEFKRK 249
Bplicatilis_HSP70-1 GGEKNILIFDLGGGTFDVSILTIIEG-IFEVKSTAGDTHLGGEDFDNRLVNHVFEFFKRK 248
Avaga_HSP70erA EGEKNILVFDLGGGTFDVSLLTIDNG-VFEVVATNGDTHLGGEDFDQRMVEHFILKFKK 274
Bibericus_HSC70 EGEKNILVFDLGGGTFDVSLLTIDNG-VFEVVATNGDTHLGGEDFDNRMVEHFILKFKK 274
BcHSC70-2 EGEKNILVFDLGGGTFDVSLLTIDNG-VFEVVATNGDTHLGGEDFDNRMVEHFILKFKK 245
Celegans_HSP70F AGDKIIAVYDLGGGTFDVSILEIQKG-VFEVKSTNGDTFLGGEDFDHALVHHVGEFFKE 272
Daphnia_HSP70c5-1 -DDKI IAVYDLGGGTFDVSILEIQKG-VFEVKSTNGDTFLGGEDFDNALVNLVNFVRK 224
Pongo_HSP70m -EDKVIAYYDLGGGTFDISILEIQKG-VFEVKSTNGDTFLGGEDFDQALLRHIVKEFKRE 293
Hsapiens_HSP70m -EDKVIAYYDLGGGTFDISILEIQKG-VFEVKSTNGDTFLGGEDFDQALLRHIVKEFKRE 293
      : : : :*****: : * : * : * * : * * * : * * : * : .

Aplysia_HSP70-2 HNKDISKNARATRLRTACERAKRTLSSSSSEASIEIDSLFE---GMDYYTKISRARFEE 308
Haliotis_HSP70 TRKDISNTRAMRLRLHTACERAKRTLSSSTEASIEIDSLYE---GVDFYSKISRARFEE 309
Lottia_HSP70-4 FGKDLSSSRALRLRTACERAKRTLSSSAEANIEIDALFE---GVDFYSKITRARFEE 305
Bplicatilis_HSP70-1 NKKDITSNKRALRLRLRTACERAKRTLSSSAQANIEIDSLHE---GVDFYTSITRARFEE 304
Avaga_HSP70erA TGKDVRKDHRAVQKLRRVEKAKRTLSSQHQTKEIESFFD---NEDFSETLTRARFEE 330
Bibericus_HSC70 TGKDIRKDNRAVQKLRRVEKAKRTLSTQFDTRIEIESFFD---GEDFSETFTRARFEE 330

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BcHSC70-2	TGKDIRKDNRAVQKLRRVEKAKRTLSTQFDTRIEIESFFD----	SEDFSETFTRASF	301
Celegans_HSP70F	QGVDLTKDPQAMQRLREAAEKACELSSSTQTQDINLPYITMDQSGPKHLNKLTRAKFEQ		332
Daphnia_HSP70c5-1	QGLDITKDPAMQRVKEAAEKAKIELSSMQTDINLPYLTMDSSGPKHMLKMSRSKLES		284
Pongo_HSP70m	TGVDLTAKDNMALQVRREAAEKACELSSSVQTDINLPYLTMDSSGPKHLNMLKSRAQFEG		353
Hsapiens_HSP70m	TGVDLTAKDNMALQVRREAAEKACELSSSVQTDINLPYLTMDSSGPKHLNMLKTRAQFEG		353
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Aplysia_HSP70-2	LCADLFRSTLEPVESALRDAKLKDKGKIDEVVLVGGSTRIPKIQKLLSDFNKGKELNKSIN		368
Haliotis_HSP70	LCSDLFRSTLEPVKALRDAKLKDKSCIHDVVLVGGSTRIPKIQKLLQNFNMGKDLNKSIN		369
Lottia_HSP70-4	LCADLFRNTMVPVEKALRDAKLKDKSKIDEVVLVGGSTRIPKIQKLLKDFNMGKELNKSIN		365
Bplicatilis_HSP70-1	LCADLFRGTLDPVEKALRDAKMDKSSVNEIVLVGGSTRIPKVQKLLQDFNKGKELNKSIN		364
Avaga_HSP70erA	LNMDLFRSTMKPVQKVLDEADMKSDIAEVVLVGGSTRIPKVQQLVKDFFDQKEPSRGIN		390
Bibericus_HSC70	LNMDLFRNTLKPQKVLDEADGLKKEIDEIVLVGGSTRIPKIQQLVKEYFDGKEPSRGIN		390
BcHSC70-2	LNIELFRNTLKPQKVLDEADGLKKTIDEIVLVGGSTRIPKVQQLVKEFFNKGKEPSRGIN		361
Celegans_HSP70F	IVGDLIKRTIEPCRKALHDAEVKSSQIADVLLVGGMSRMPKVQATVQEIF-GKVPSKAVN		391
Daphnia_HSP70c5-1	LVGDLIKRTVGPQKALDKAEVSKNDIGDVLVGGMTRMPKVQETVKDIF-GRVPSKAVN		343
Pongo_HSP70m	IVTDLIRRTIAPCQKAMQDAEVSKSDIGEVILVGGMTRMPKVQQTQVQDLF-GRAPSKAVN		412
Hsapiens_HSP70m	IVTDLIRRTIAPCQKAMQDAEVSKSDIGEVILVGGMTRMPKVQQTQVQDLF-GRAPSKAVN		412
	: :*: : * * ..:.* .. : :*:** :*:** * : : : * : : : *		
Aplysia_HSP70-2	PDEAVAYGAAVQAAVLTDGKSEAIRDVLLVDVAPLSLGIETAGGVMTSLIKRGSTIPAKT		428
Haliotis_HSP70	PDEAVAYGAAVQAAVLSDSSDAIKDVLVDVAPLSLGIETAGGVMTKLIDRNTRIPTKA		429
Lottia_HSP70-4	PDEAVAYGAAVQAAVLIGDKSEKIKDVLVDVAPLSLGIETAGGVMTRIERNTRIPTKH		425
Bplicatilis_HSP70-1	PDEAVAYGAAVQAAVLTDGKSEAVDQLLLLDVAPLSLGIETAGGVMTALIKRNTIPTKQ		424
Avaga_HSP70erA	PDEAVAYGAAVQAGVLSGEEN--TGDIVLLDVNPLTMGIETVGGVMTKII PRNTVIPTKK		448
Bibericus_HSC70	PDEAVAYGAAVQGGVLSGEDV--GGEVLLDVNPLTMGIETVGGVMTKLI PRNTVIPTKN		448
BcHSC70-2	PDEAVAYGAAVQGGVLSGEDV--GGDVLLDVNPLTMGIETVGGMMTKLISRNTVIPTKK		419
Celegans_HSP70F	PDEAVAMGAAIQGAVLAGD---VTDVLLLDVTPLSLGIETLGGIMTKLIRNTIPTKK		447
Daphnia_HSP70c5-1	PDEAVAVGAAIQGGVLSGG---VTDILLLDVTPLSLGIETLGGVFTKLIQRNTIPTKK		399
Pongo_HSP70m	PDEAVAIGAAIQGGVLAGD---VTDVLLLDVTPLSLGIETLGGVFTKLINRNTIPTKK		468
Hsapiens_HSP70m	PDEAVAIGAAIQGGVLAGD---VTDVLLLDVTPLSLGIETLGGVFTKLINRNTIPTKK		468
	***** ***:..* * :*:** ***:** ***:** * : : * : : ** *		
Aplysia_HSP70-2	SQVFTTYSDNQPGVDIQVFEGERAMTKDNNLLGKQFSLGIPAPRGVPQIEVTFDIDANG		488
Haliotis_HSP70	SQIFTTYSDNQPGVSIQVFEGERALTKDNNILGKFELTGIPAPRGVPQIEVTFNIDANG		489
Lottia_HSP70-4	SQIFTTYSDNQPGVSIQVYEGERTMTKDNHMPGNFELSGIPAPRGVPQIEVTFIDANG		485
Bplicatilis_HSP70-1	TQTFTTYADNQPGVLIQVYEGERAMTKDNNLLGKFELSGIPAPRGVPQIEVTFIDANG		484
Avaga_HSP70erA	SQVFSTAAENQPSVTIQVFEGERPMTKDNHVLGKFDLTGIPAPRGVPQIEVTFEIDVNG		508
Bibericus_HSC70	-PKFSTASDNQPTVTIAVYEGERPMTKDNHLLGKFDLTGIPAPRGVPQIEVTFEIDANG		507
BcHSC70-2	SQVFSTAADNQPSVTIAVYEGERPMTKDNHLLGKFDLTGIPAPRGVPQIEVTFEIDVNG		479
Celegans_HSP70F	SQVFSTAADGQTQVQIKVFQGEREMATSNKLLGQFSLVGIPAPRGVPQIEVTFEIDANG		507
Daphnia_HSP70c5-1	SQVFSTAADGQTQVEIKVYQGEREMAADNKILGQFSLGIPAPRGVPQIEVTFEIDANG		459
Pongo_HSP70m	SQVFSTAADGQTQVEIKVCQGEREMAGDNKLLGQFTLIGIPAPRGVPQIEVTFEIDANG		528
Hsapiens_HSP70m	SQVFSTAADGQTQVEIKVCQGEREMAGDNKLLGQFTLIGIPAPRGVPQIEVTFEIDANG		528
	*: * : : * * * * : * : : : * : * * : : * : : * : : * : : *		
Aplysia_HSP70-2	ILNVTAQDKSTGKSSHITIKNERGRLSQAEIDRMLADADKYKDEDEKQREVRVSARNQLET		548
Haliotis_HSP70	ILNVSADVSTGKSNVNTISDDQSRLSKADIDRMVSEAEYKEDEKHKERIAARNHLEN		549
Lottia_HSP70-4	ILNVLAEDKSTGKSNKITITNDKGRLSSEEIEKLQVQAEKYKDEDEKQKRKIIARNQLES		545
Bplicatilis_HSP70-1	ILNVSADKSTGKTNKITITNDKGRLSKEEIDRMVNEAEKYKDDQQRDKVAAKNSLES		544
Avaga_HSP70erA	ILKVTAEDKGTGNKNNIVINSNTNRLSPDEIDRMKIDSEKFADEDKKVKERVDAKNELES		568
Bibericus_HSC70	ILKVSADKGTGNKNNIVINNQNRLSPPEIERMIKDAEKFADEDKKVKEKVEAKNELES		567
BcHSC70-2	ILKVTAEDKGTGNKNNIVINNQNRLSPPEIEKMIKDAEKFADEDKKVKEKVEAKNELES		539
Celegans_HSP70F	IVNVSARDRGTKGEQQIVIQS-SGGLSKDQIENMIKEAEKNAEDAKRKELVEVINQAE		566
Daphnia_HSP70c5-1	IVHVSARDKGTGKEQQIVIQS-SGGLSKDEIENMVRNAEQFAKEDQVKRDRVEAVNHAEG		518
Pongo_HSP70m	IVHVSARDKGTGREQQIVIQS-SGGLSKDDIENMVKNAEKYAEEDRRKKERVEAVNMAEG		587
Hsapiens_HSP70m	IVHVSARDKGTGREQQIVIQS-SGGLSKDDIENMVKNAEKYAEEDRRKKERVEAVNMAEG		587
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Aplysia_HSP70-2	YIFHVQKQAV-DA--AGDKLPSPDKDAVLKACNDSLSWLDNNSLADKEEVEDRLKELQKTT		605
Haliotis_HSP70	YVFSVRASV-DE--MGSQEDVDKQTVSKACEETLRWLDNNSLAEKEEFEQQYKQLEKLC		606
Lottia_HSP70-4	YVFNQKQVL-DNG-GADKICDSKQETVQCCDETIQWLDNNSLAEKDEYEHRLDEVQKVC		603
Bplicatilis_HSP70-1	YCFNMKQTV-EDEKLAAKISADKKKILDACEEALKWLDNSQTAEKDEFEHKMKEVEKIC		603
Avaga_HSP70erA	YAYSLKTQLNDKEKLGKLSDDKETIEKAVEEQIKWLESNQGAEVDELKEHKQLLEEIV		628
Bibericus_HSC70	YVYSLKNQLNDKEKLGKLSDEKETINSADVDEKIKWLESNSDAEVDDFKEQKALEEIV		627
BcHSC70-2	YVYSLKNQLSDKEKLGKLSSEEDKEKIQSSVEEKIKWLESNSNAEVEEFKDQKKELEEIV		599
Celegans_HSP70F	IIHDTAEKMT---EFADQLPKDCEALRTKIADTKKILDNKDNETPEAIKEACNTLQQQS		623
Daphnia_HSP70c5-1	IVHDTESKME---EFKEQLPAEECSKLEQISAVRDMVLNKSDEKTPPEIKKTTNDLQQAS		575
Pongo_HSP70m	IIHDTETKME---EFKDQLPADECNKLEKIEISKMRELLARKDSETGENIRQAASSLQQAS		644
Hsapiens_HSP70m	IIHDTETKME---EFKDQLPADECNKLEKIEISKMRELLARKDSETGENIRQAASSLQQAS		644
	. . : : : : *	: : . . : : :	

Aplysia_HSP70-2	SSVMAKLHTQGQSQGQSS-----QGSSGPHGPSV EE MD	639
Haliotis_HSP70	SPVMSKLNKGCQ--QGGSSKGPTV EE MD	636
Lottia_HSP70-4	SPIMTKLHGEANGQSTD-PTG-----RSGGNQGPTV EE ID	637
Bplicatilis_HSP70-1	SPIITQLYQGAGGAPGGMPGGMPGGMPGAGPESAGRSIPT DE VD	652
Avaga_HSP70erA	TPIMTKLYGQGGAGAGAPEDVPPPPHGHDD DS L	663
Bibericus_HSC70	NPIMTKLYQQNSENAGSSDSQT NDEL	653
BcHSC70-2	NPIMSKLY Q SG	611
Celegans_HSP70F	LKLFEEAAYKNMAAKNSGGDAQEAKT---AEE-----PKKEQN-	657
Daphnia_HSP70c5-1	LKLFEMAYKKMAADDRESSGGSTNSGDSTGST-----DKKEDKQ	613
Pongo_HSP70m	LKLFEMAYKKMASEREGSGSGTGEQ---KE-----DQKEEKQ	679
Hsapiens_HSP70m	LKLFEMAYKKMASEREGSGSGTGEQ---KE-----DQKEEKQ	679
	: : :	

Supplementary fig. S4. Alignment of several HSP70 family members with variations in the cytosolic (EEVD), endoplasmic reticulum (ER; HDEL/KDEL), and mitochondrial (PEAEYEEAKK) motifs. The three motifs are shown in bold. Distinctive amino acid residues in the newly identified motifs are shown in red (cytosolic), blue (ER), and green (mitochondrial). The discrimination of organelle-specific HSP70 family members becomes more evident by using multiple motifs.

Drosophila_Bb_AF295957
Drosophila_Aa_AF295933
Spodoptera_FJ754276
Bombyx_AB035326
Paralvinella_EF580992
B_plicatilis_HSP70-1_AB775784
B_plicatilis_HSP70-2_AB775785
A_brightwelli_HSP70_KX119429
Wuchereria_AF167352
Setaria_AF079360
C_elegans_HSP70A_M18540
C_elegans_HSP-1_NM_070667
M_nipponense_KC460343
M_nipponense_DQ660140
Mytilus_HSP70_AY861684
Perinereis_KU255783
Paralvinella_HSP70-2_EF580993
Haliotis_FJ812176
Oncorhynchus_HSC71_AAB21658
X_maculatus_HSC70_AB062115
Human_HSPA8_HSC71_NP_006588
Rattus_HSC71_NM_024351
Pelodiscus_HSC70_HQ219723
Bos_HSP70_U09861
Human_HSPA1A_NM_005345
Rattus_HSP70_1A_NM_031971
X_maculatus_HSP70-1_AB062113
X_maculatus_HSP70-2_AB062114
Human_HSPA1L_NM_005527
Human_HSPA6_NP_002146
Alligator_HSP70_AB306279

Drosophila_Bb_AF295957
Drosophila_Aa_AF295933
Spodoptera_FJ754276
Bombyx_AB035326
Paralvinella_EF580992
B_plicatilis_HSP70-1_AB775784
B_plicatilis_HSP70-2_AB775785
A_brightwelli_HSP70_KX119429
Wuchereria_AF167352
Setaria_AF079360
C_elegans_HSP70A_M18540
C_elegans_HSP-1_NM_070667
M_nipponense_KC460343

M_nipponense_DQ660140
Mytilus_HSP70_AY861684
Perinereis_KU255783
Paralvinella_HSP70-2_EF580993
Haliotis_FJ812176
Oncorhynchus_HSC71_AAB21658
X_maculatus_HSC70_AB062115
Human_HSPA8_HSC71_NP_006588
Rattus_HSC71_NM_024351
Pelodiscus_HSC70_HQ219723
Bos_HSP70_U09861
Human_HSPA1A_NM_005345
Rattus_HSP70_1A_NM_031971
X_maculatus_HSP70-1_AB062113
X_maculatus_HSP70-2_AB062114
Human_HSPA1L_NM_005527
Human_HSPA6_NP_002146
Alligator_HSP70_AB306279

CCATCATATGTTGCTTTACCGACACAGAGCGTCTTATGGTGATGCTGCCAAGAACCAG 174
CCAAGCTATGTCGCCCTTACAGACTCCGAGCTCCATGGAGATGCGCCAAGAACCAG 177
CCAGTTATGTCGCCCTTACAGACTCCGAGCTCCATGGAGATGCGCCAAGAACCAG 171
CCAAGTTATGTCGCCCTTACCGATTCCGAACGCTATTATGGAGATGGAGCCAAGAACCAG 174
CCCAGCTATGTTGCCCTTACCGACACAGAGCGTCTCATGGTGACGCTGCAAAGAACCAG 174
CCAAGCTACGTTGCCCTTCACTGACTCTGAGAGGCTCATCGGTGATGCTGCCAAGAATCAG 174
CCCAGTTATGTCGCCCTTACTGACACAGAGAGGCTGATTGGAGATGCAGCCAAGAACCAG 174
CCAAGCTATGTCGCCCTTACCGACTGAACGCTGATCGGTGATGCGCCAAGAATCAA 174
CCGAGCTATGTTGCTTTACCGACACAGAACGATTAATGGGGATGCGGCCAAGAATCAG 174
CCAAGTTACGTTGCCCTTACAGATACAGAGAGGTTGATCGGTGATGCTGCCAAGAACCAG 174
CCCAGCTACGTGGCCTTACCGATACCGAGCGGCTCATCGGGATGCGGCCAAGAACCAG 174
CCCAGCTACGTGGCCTTACCGACACCGAGCGGCTCATCGGGATGCGGCCAAGAACCAG 174
CCCAGCTACGTGGCCTTACCGACACCGAGAGGCTGATCGGGACGCGGCCAAGAACCAG 180
CCCAGCTACGTGGCCTTACCGACACCGAGCGGCTCATCGGGATGCGGCCAAGAACCAG 180
CCCAGCTACGTGGCCTTACCGACACCGAGCGGCTGGTGGGGACGCGGCCAAGAGCCAG 180
CCCAGCTACGTGGCCTTACCGACACCGAGCGGCTCATCGGCACGCGGCCAAGAACCAG 177
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Drosophila_Bb_AF295957
Drosophila_Aa_AF295933
Spodoptera_FJ754276
Bombyx_AB035326
Paralvinella_EF580992
B_plicatilis_HSP70-1_AB775784
B_plicatilis_HSP70-2_AB775785
A_brightwelli_HSP70_KX119429
Wuchereria_AF167352
Setaria_AF079360
C_elegans_HSP70A_M18540
C_elegans_HSP-1_NM_070667
M_nipponense_KC460343
M_nipponense_DQ660140
Mytilus_HSP70_AY861684
Perinereis_KU255783
Paralvinella_HSP70-2_EF580993
Haliotis_FJ812176
Oncorhynchus_HSC71_AAB21658
X_maculatus_HSC70_AB062115
Human_HSPA8_HSC71_NP_006588
Rattus_HSC71_NM_024351
Pelodiscus_HSC70_HQ219723
Bos_HSP70_U09861
Human_HSPA1A_NM_005345
Rattus_HSP70_1A_NM_031971
X_maculatus_HSP70-1_AB062113
X_maculatus_HSP70-2_AB062114
Human_HSPA1L_NM_005527
Human_HSPA6_NP_002146
Alligator_HSP70_AB306279

GTGGCCATGAACCCAGAAACACAGTGTGTTGACGCCAAGCGACTCATCGGCCGAAATAC 225
GTGGCCATGAACCCAGAAACACAGTGTGTTGACGCCAAGCGACTCATCGGCCGAAATAC 225
GTCGCCCTCAACCCCAACAACTGTGTTGACGCCAAGCGACTCATCGGAAGGAAATC 225
GTGCGCTGAACCCCAACAACTGTGTTGACGCCAAGCGGCTCATCGGCCAAGGAAATC 225
GTGGCGCTGAATCCGAGCAACACGCTATTGATGCGAAGAGGCTGATTGGTCGAAGATTT 240
GTGGCCATGAACCCCAATTAACACGGTGTGTCGACGCCAAGCGCTCATCGGCCGCAAGTTC 234
GTGGCCATGAACCCCAATTAACACGGTGTGTCGACGCCAAGCGCTCATCGGCCGCAAGTTC 234
GTGCGCTGAACCCCAACAACTGTGTTGACGCCAAGCGCTCATCGGCCAAGGAAATC 231
GTTGCAATGAATCCGACAACTGTGTTGACGCTAAACGACTGATTGGTCGTAAGTTT 231
GTTGCGATGAATCCGACAACTGTGTTGCGATGCGAAGAGTATGATCGGCCGTAAGTTT 231
GTTGCCATGAACCCACATAAAGTGTGTTGCGATGCGAAGAGTATGATTGGACGCAAGTTC 234
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GTGGCGCTGAACCCCAAGTCAACACGCTGTTGACGCCAAGCGGCTGATTGGCCGCAAGTTC 234
GTGGCGCTGAACCCCAAGTCAACACGCTGTTGACGCCAAGCGGCTGATCGGCCGCAAGTTC 234
GTTGCCATGAACCCCAAGTCAACACGCTGTTGATGCGAAGAGGCTCATGGTAAGAGGTTT 240
GTGGCCATGAACCCCAAGTCAACACGCTGTTGACGCCAAGAGGCTGATCGGCCGCAAGTTC 240
GTAGCAATGAATCCCAAGTCAACACGCTGTTGATGCTAAACGCTGATCGGCCGCAAGTTC 240
GCGGCCCTGAACCCCAAGTCAACACGCTGTTGATGCGAAGCGGCTGATCGGCCGCAAGTTC 240
GTGGCCATGAACCCCAAGTCAACACGCTGTTGACGCCAAGCGGCTCATCGGCCGCAAGTTC 237
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Drosophila_Bb_AF295957
Drosophila_Aa_AF295933
Spodoptera_FJ754276
Bombyx_AB035326
Paralvinella_EF580992
B_plicatilis_HSP70-1_AB775784
B_plicatilis_HSP70-2_AB775785
A_brightwelli_HSP70_KX119429
Wuchereria_AF167352
Setaria_AF079360
C_elegans_HSP70A_M18540
C_elegans_HSP-1_NM_070667
M_nipponense_KC460343
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Mytilus_HSP70_AY861684
Perinereis_KU255783
Paralvinella_HSP70-2_EF580993
Haliotis_FJ812176
Oncorhynchus_HSC71_AAB21658
X_maculatus_HSC70_AB062115
Human_HSPA8_HSC71_NP_006588
Rattus_HSC71_NM_024351
Pelodiscus_HSC70_HQ219723
Bos_HSP70_U09861
Human_HSPA1A_NM_005345
Rattus_HSP70_1A_NM_031971

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GACGACCCCAAGATCGCAGGACATGAAGCACTGGCCTTTCAAAGTTGTAAGCGACG-- 283
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GACGACCTGACCGTCCAGGCGGACATGAAGCACTGGCCTTTCAAAGTTGTAAGCGACG-- 292
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GACGACCTCACTGTCAGTCCGACATGAAGCACTGGCCTTTCAAAGTTGTAAGCGACG-- 289
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GACGATCCGACGATTCAGTCTGACATGAAGCACTGGCCATTTCAAAGTTGTAAGCGATG 294
GACGATCCGACGATTCAGTCTGACATGAAGCACTGGCCATTTCAAAGTTGTAAGCGATG 294
GAAGATCATGTAGTGCAGTCCGACATGAACATTGGCCTTTACAGTCATTAATGACA-- 292
GAAGATCATGTAGTGCAGTCCGACATGAACATTGGCCTTTACAGTCATTAATGACA-- 292
GATGACGCTACAGTCAATCAGACATGAAGCACTGGCCTTTCACTGTTGTCAATGATG-- 295
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GGCGACCCGTTGTCAGTCCGACATGAAGCACTGGCCCTTTCCAGGTGATCAACGACG-- 292

X_maculatus_HSP70-1_AB062113 GATGACCTGGTGGTCCAGTCTGACATGAACTCTGGCCGTTCAAGGTGATCAACGACA-- 298
X_maculatus_HSP70-2_AB062114 GAAGAGCCAGTGGTGCAGGCCGACATGAAGCACTGGCCCTTCAGGTGCTTTTCAGAGC-- 298
Human_HSPA1L_NM_005527 AATGATCCTGTTGTACAGCAGATATGAACTTTGGCCCTTTCAAGTGATTAATGAAG-- 298
Human_HSPA6_NP_002146 GCGGACACACCGGTGCAGTCCGACATGAAGCACTGGCCCTTCGCGGTGGTGAAGCAGG-- 298
Alligator_HSP70_AB306279 GACGACCCACGGTGCAGGCCGACATGAAGCACTGGCCCTTCACGTAAGTGTCCGAGG-- 295
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Drosophila_Bb_AF295957 -GCGGAAAGCCCCAAGATCGGGGTGGAGTATAAGGGTGAGTCCAAGAGATTTGCTCCCCGAG 342
Drosophila_Aa_AF295933 -GCGGAAAGCCCCAAGATCGGGGTGGAGTATAAGGGTGAGTCCAAGAGATTTGCCCCGAG 342
Spodoptera_FJ754276 -GTGGCAAACCGAAGATCCAAGTGGAGTTCAAGGGTGAAACGAAACGGTTCGCGCCGAG 342
Bombyx_AB035326 -GCGGCAAACCGAAAATACAGATCGAGTTCAAAGGTGAGACGAAACGATTTGCGCCAGAA 342
Paralvinella_EF580992 -GTGGAAAGCCAAAGATTGACGTGAGATGAAATACAAAGTGAGAGGAAACGCTTTTACTCCAGAA 357
B_plicatilis_HSP70-1_AB775784 -GCGGCCGGCCCCAAATCCAGGTGAGTTCAAAGGCGAGACCAAGAGCTTCTACCCGAA 351
B_plicatilis_HSP70-2_AB775785 -GCGGCCGGCCCCAAATCCAGGTGAGTTCAAAGGCGAGACCAAGAGCTTCTACCCGAA 351
A_brightwelli_HSP70_KX119429 -GCGGAAACCAAAATCCAAGTTCGAGTTCAAAGGCGAGAAAAAGACCTTCTTCCCTGAA 348
Wuchereria_AFL67352 GGTGTAAACCTAAAGTTCGAGTTGAAATACAAAGGGTGAGACAAAGACCTTTTACTCCAGAA 351
Setaria_AF079360 GGAGGCAAGCCAAAAGTTCAAGTGGAGTATAAGGGGGAGACGAAACCTTTCACGCCCGGA 351
C_elegans_HSP70A_M18540 GGAGCTAAGCCAAAGGTCCAAGTTGAGTACAAAGGAGAGAACAGATCTTCACTCCAGAA 354
C_elegans_HSP-1_NM_070667 GGAGCTAAGCCAAAGGTCCAAGTTGAGTACAAAGGAGAGAACAGATCTTCACTCCAGAA 354
M_nipponense_KC460343 -GCACGAAGCCCAAAATTCAGTTGATTACAAAGGAGAGACCAAAACCTTTTCCAGAA 351
M_nipponense_DQ660140 -GCACGAAGCCCAAAATTCAGTTGATTACAAAGGAGAGACCAAAACCTTTTCCAGAA 351
Mytilus_HSP70_AY861684 -CCTCCAAACCTAAAGTTACGGTAGACTACAAAGGAGAGAACAAAAACATTTTCCCTGAG 354
Perinereis_KU255783 -GTGGCAAACCAAGATCTCTGTTGATTACAAAGGGGAGAGAAATCATTTTACCAGAA 348
Paralvinella_HSP70-2_EF580993 -ATGGCAAGCCCAAAAGTTCGAGTTGAGTACAAAGGGGAGTTGAAAGACCTTCTATCCGAA 351
Haliotis_FJ812176 -GAGGCAAACCAAGATCCAAGTAAATACAAAGATGAACCAAAACCTTCTACCTGAA 351
Oncorhynchus_HSC71_AAB21658 -CTACTCGGCCTAAGCTCCAAGTTGAAATACAAAGGAGAGACTAAGTCCTTCTACCCAGAA 351
X_maculatus_HSC70_AB062115 -GCTCGAGGCCCAAGGTGAAAGTGGAGTACAAAGGGGAGACGAAGACCTTCTACCCAGAG 351
Human_HSPA8_HSC71_NP_006588 -CTGGCAAGCCCAAAAGTTCGAGTTGAAATACAAAGGGGAGAGACCAAAACCTTTTCCAGAG 351
Rattus_HSC71_NM_024351 -CAGGCAGGCCCAAGGTCCAAGTTCGAAATACAAAGGGGAGACCAAAAGTTTCTATCCTGAG 351
Pelodiscus_HSC70_HQ219723 -CTGGCAGGCCCAAGGTTCGAGTTGAGTACAAAGGGGAGACCAAGAGCTTCTATCCAGAG 351
Bos_HSP70_U09861 -GAGACAAGCCTAAGGTGCAGGTGAGTACAAAGGGGAGACCAAGGCGTTCTACCCGAG 351
Human_HSPA1A_NM_005345 -GAGACAAGCCCAAGGTGCAGGTGAGTACAAAGGGGAGACCAAGGCGATTCTACCCGAG 351
Rattus_HSP70_1A_NM_031971 -GCGACAAGCCCAAGGTGCAGGTGAACTACAAAGGGGAGAGAACCGGTCTGTTCTACCCGAG 351
X_maculatus_HSP70-1_AB062113 -ACGGAAGGCCCAAAAGTTCGAGTTGAAATATAAGGAGAGAAATTAAGACATTTCTGCTGAA 357
X_maculatus_HSP70-2_AB062114 -GAGGCAGGCCCAAAATTCAGGTGGAGTACAAAGGGGAGAGAACAAAGCCTTCTTCCCTGAG 357
Human_HSPA1L_NM_005527 -GAGGCAGGCCCAAAAGTTCGAGTTGAAATATAAGGAGAGAAATTAAGACATTTCTGCTGAA 357
Human_HSPA6_NP_002146 -GCGGCAAGCCCAAGGTGCGCGTATGCTACCGCGGGGAGGACAAAGACGTTCTACCCGAG 357
Alligator_HSP70_AB306279 -GCGGCAAGCCCAAGGTGCAGGTGGAGTATAAGGGCGAGGCCAAGACCTTCTTCCCCGAA 354
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Drosophila_Bb_AF295957 GAGATCAGTTCGATGGTCTGACCAAGATGAAGGAGACGGCGGAGGCGTATCTGGGCGAG 402
Drosophila_Aa_AF295933 GAGATCAGTTCGATGGTCTGACCAAGATGAAGGAGACGGCGGAGGCGTATCTGGGCGAG 402
Spodoptera_FJ754276 GAGATCAGCAGCATGGTCTGACGAAGATGAAGGAGACGGCGGAGGCGTATCTGGGCGAG 402
Bombyx_AB035326 GAAATGATCAGCATGGTCTGACCAAAATGAAGGAGACGGCGGAGGCGTATCTGGGCGAG 402
Paralvinella_EF580992 GAAATCAGCTCGATGATACTTACCAAGATGAAGGAGACGGCGGAGGCGTATCTGGGCGAG 417
B_plicatilis_HSP70-1_AB775784 GAAGTCTCATCCATGGTACTGACCAAAATGAAGGAGACGGCGGAGGCGTATCTGGGCGAG 411
B_plicatilis_HSP70-2_AB775785 GAAGTCTCATCCATGGTACTGACCAAAATGAAGGAGACGGCGGAGGCGTATCTGGGCGAG 411
A_brightwelli_HSP70_KX119429 GAAATCTCATCCATGGTCTTAATTAAGATGAAGGAGATCGCGAGGCTTATTTGGGAAAG 408
Wuchereria_AFL67352 GAGATTTTCGTCGATGGTCTTGGTTAAATGAAGGAGACGGCTGAAGCTTTTCTGGGTCAT 411
Setaria_AF079360 GAAATTTCTTCAATGGTCTTAGTTAAATGAAGGAGACAGCTGAGGCTATTTCTGGTCA 411
C_elegans_HSP70A_M18540 GAGATCTCCTCAATGGTCTGCTGAAGATGAAGGAGCTGCCGAGGCTTTCTTGAACCG 414
C_elegans_HSP-1_NM_070667 GAGATCTCCTCAATGGTCTTGGTGAAGATGAAGGAGCTGCCGAGGCTTTCTTGAACCG 414
M_nipponense_KC460343 GAGATCTCCTCAATGGTCTTATTAAGATGAAGGAGCTGCAGAGGCTTTTGGGTTGGT 411
M_nipponense_DQ660140 GAGATCTCCTCAATGGTCTTATTAAGATGAAGGAGCTGCAGAGGCTTTTGGGTTGGT 411
Mytilus_HSP70_AY861684 GAAATTTTCATCAATGGTCTGGTAAATGAAGGAGCTGCAGAGGCTATCTAGGAAAG 414
Perinereis_KU255783 GAGATCTCCTCATGTTGTTGTTAAGATGAAGGAGCTGCAGAGGCTTACATCGGCAAG 408
Paralvinella_HSP70-2_EF580993 GAAATTTCTCCTCAATGGTCTTGGTAAATGAAGGAGCTGCAGAGGCTTATCTGGGAAAG 411
Haliotis_FJ812176 GAGATTTCTCCTCAATGGTCTTGGTAAATGAAGGAGCTGCAGAGGCTTATCTGGGAAAG 411
Oncorhynchus_HSC71_AAB21658 GAAATTTTCATCTATGGTCTGCTGAAGATGAAGGAGATGCTGAGGCTACCTTGGGAAA 411
X_maculatus_HSC70_AB062115 GAGATCTCCTCATGGTCTGCTGAAGATGAAGGAGATGCTGAGGCTACCTTGGCAAG 411
Human_HSPA8_HSC71_NP_006588 GAGGTGTCTTCTCATGGTCTGCTGAAGATGAAGGAGATGCTGAGGCTACCTTGGGAAAG 411
Rattus_HSC71_NM_024351 GAAGTGTCTTCAATGGTCTGACCAAAATGAAGGAGATGCTGAGGCTACCTTGGGAAAG 411
Pelodiscus_HSC70_HQ219723 GAAATATCTTCCATGGTCTTGGTAAATGAAGGAGATGCTGAGGCTACCTTGGGAAAG 411
Bos_HSP70_U09861 GAGATCTCGTCAATGGTCTGACCAAGATGAAGGAGATGCTGAGGCTACCTTGGGCCAC 411
Human_HSPA1A_NM_005345 GAGATCTCGTCCATGGTCTGACCAAGATGAAGGAGATGCTGAGGCTACCTTGGGCCAC 411
Rattus_HSP70_1A_NM_031971 GAGATCTCGTCCATGGTCTGACCAAGATGAAGGAGATGCTGAGGCTACCTTGGGCCAC 411
X_maculatus_HSP70-1_AB062113 GAGATTTCTCCTCATGGTCTGTTAAATGAAGGAGATGCTGAGGCTTTCTGGGACAG 417
X_maculatus_HSP70-2_AB062114 GAGATCTCCTCATGGTCTGTTAAATGAAGGAGATGCTGAGGCTTTCTGGGACAG 417
Human_HSPA1L_NM_005527 GAAATCTCTCCTCATGGTCTGTTAAATGAAGGAGATGCTGAGGCTTTCTGGGACAG 417
Human_HSPA6_NP_002146 GAGATCTCCTCATGGTCTGTTAAATGAAGGAGATGCTGAGGCTTTCTGGGACAG 417
Alligator_HSP70_AB306279 GAGATCAGTCCATGGTCTGACTAAGATGAAGGAGATGCTGAGGCTACCTTGGGCGC 414
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Drosophila_Aa_AF295933 AGCATCACGAGCGAGTCATCACAGTTCCAGCTTACTTCAACGACTCCAGCGCCAGGCT 462
Spodoptera_FJ754276 ACAGTACGCGAGCGAGTCATCACAGTCCGCGCTACTTCAACGACTCTCAGCGCCAGGCT 462
Bombyx_AB035326 ACAGTCCGCGAGTCGAGTCATCACAGTTCCGCGCTACTTCAACGACTCCAGCGCCAGGCT 462
Paralvinella_EF580992 AAAGTGTGTAGATGCCGTCAACTGTCCCGCTTACTTCAACGACTCTCAGAGACAAGCT 477
B_plicatilis_HSP70-1_AB775784 AAGGTCACGGACGAGTGGTGACCGTCCCGCTTACTTCAACGACTCAGCGCCAGCG 471

B_plicatilis_HSP70-2_AB775785
A_brightwelli_HSP70_KX119429
Wuchereria_AF167352
Setaria_AF079360
C_elegans_HSP70A_M18540
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M_nipponense_DQ660140
Mytilus_HSP70_AY861684
Perinereis_KU255783
Paralvinella_HSP70-2_EF580993
Haliotis_FJ812176
Oncorhynchus_HSC71_AAB21658
X_maculatus_HSC70_AB062115
Human_HSPA8_HSC71_NP_006588
Rattus_HSC71_NM_024351
Pelodiscus_HSC70_HQ219723
Bos_HSP70_U09861
Human_HSPA1A_NM_005345
Rattus_HSP70_1A_NM_031971
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Human_HSPA6_NP_002146
Alligator_HSP70_AB306279

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Drosophila_Aa_AF295933
Spodoptera_FJ754276
Bombyx_AB035326
Paralvinella_EF580992
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B_plicatilis_HSP70-2_AB775785
A_brightwelli_HSP70_KX119429
Wuchereria_AF167352
Setaria_AF079360
C_elegans_HSP70A_M18540
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M_nipponense_KC460343
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Mytilus_HSP70_AY861684
Perinereis_KU255783
Paralvinella_HSP70-2_EF580993
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Oncorhynchus_HSC71_AAB21658
X_maculatus_HSC70_AB062115
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Pelodiscus_HSC70_HQ219723
Bos_HSP70_U09861
Human_HSPA1A_NM_005345
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X_maculatus_HSP70-1_AB062113
X_maculatus_HSP70-2_AB062114
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Human_HSPA6_NP_002146
Alligator_HSP70_AB306279

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Drosophila_Bb_AF295957
Drosophila_Aa_AF295933
Spodoptera_FJ754276
Bombyx_AB035326
Paralvinella_EF580992
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B_plicatilis_HSP70-2_AB775785
A_brightwelli_HSP70_KX119429
Wuchereria_AF167352
Setaria_AF079360
C_elegans_HSP70A_M18540
C_elegans_HSP-1_NM_070667
M_nipponense_KC460343
M_nipponense_DQ660140
Mytilus_HSP70_AY861684
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Drosophila_Bb_AF295957
Drosophila_Aa_AF295933
Spodoptera_FJ754276
Bombyx_AB035326
Paralvinella_EF580992
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Wuchereria_AFL67352
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Perinereis_KU255783
Paralvinella_HSP70-2_EF580993
Haliotis_FJ812176
Oncorhynchus_HSC71_AAB21658
X_maculatus_HSC70_AB062115
Human_HSPA8_HSC71_NP_006588
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Pelodiscus_HSC70_HQ219723
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X_maculatus_HSP70-1_AB062113
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Human_HSPA1L_NM_005527
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Paralvinella_EF580992
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B_plicatilis_HSP70-2_AB775785
A_brightwelli_HSP70_KX119429
Wuchereria_AFL67352
Setaria_AF079360
C_elegans_HSP70A_M18540
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Mytilus_HSP70_AY861684
Perinereis_KU255783
Paralvinella_HSP70-2_EF580993
Haliotis_FJ812176
Oncorhynchus_HSC71_AAB21658
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Paralvinella_EF580992
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B_plicatilis_HSP70-2_AB775785
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Perinereis_KU255783	TCCTCCAGCACCAAGCCAGTATTGAAATTGATTCTTGTATGAAGGTGTCGACTTCTAC 879
Paralvinella_HSP70-2_EF580993	TCCAGCAGCACACAGGCAAGCATTGAAATAGATTCCCTCTATGAGGGCATAGACTATTAC 882
Haliotis_FJ812176	TCCTCCAGCACCCAGGCCAGCATAGAGATCGACTCCCTGTTTGAAGGAGTAGATTACTAC 882
Oncorhynchus_HSC71_AAB21658	TCCTCCAGCACCCAGGCCAGCATCGAGATCGACTCTTTGTACGAGGAATCGACTTCTAC 882
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Human_HSPA8_HSC71_NP_006588	TCTTCCAGCACCCAGGCCAGTATTGAGATCGATTCTCTCTATGAAGGAATCGACTTCTAT 882
Rattus_HSC71_NM_024351	TCCTCCAGCACCCAGGCCAGTATTGAGATTGATTCTCTCTATGAGGGAAATGACTTCTAC 882
Pelodiscus_HSC70_HQ219723	TCTTCTAGCACTCAGGCCAGTATTGAAATTGACTCGCTCTATGAGGGCATTGATTTTTAC 882
Bos_HSP70_U09861	TCGTCCAGCACCCAGGCCAGCCTGGAGATCGACTCCCTGTTTTCGAGGGCATCGACTTCTAC 882
Human_HSPA1A_NM_005345	TCGTCCAGCACCCAGGCCAGCCTGGAGATCGACTCCCTGTTTTCGAGGGCATCGACTTCTAC 882
Rattus_HSP70_1A_NM_031971	TCGTCCAGCACCCAGGCCAGCCTGGAGATCGACTCTCTGTTTCGAGGGCATCGACTTCTAC 882
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X_maculatus_HSP70-2_AB062114	TCCTCCAGCTCCAGGCCAGCCTGGAGATCGACTCTCTGTTTCGAGGGATTGAGCTTGTAC 888
Human_HSPA1L_NM_005527	TCGTCCAGCACCCAGGCCAACCAGAAATTGATTACCTTTATGAAGGCATTGACTTCTAT 888
Human_HSPA6_NP_002146	TCCTCCAGCACCCAGGCCACCCTGGAGATAGACTCCCTGTTTCGAGGGCGTGGACTTCTAC 888
Alligator_HSP70_AB306279	AGCTCCTCCAGCAGGCCAGCATCGAGATCGACTCGCTGTTTCGAGGGCATCGACTTCTAC 891
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Drosophila_Aa_AF295933	ACCAAAGTGAGCCGCGCCAGGTTTGAGGAGCTGTGCGCGGACCTCTTCCGCAACACCCCTG 936
Spodoptera_FJ754276	ACTTCGCTGTCCGCGCGCCAGTTCGAGGAGCTCAACGCTGACCTGTTCCGAGCCACCCCTC 936
Bombyx_AB035326	ACGCGAGTCTCCGCGCCCGCTTCGAGGAACCTGAACGCGGACCTGTTCCAGGGGAACCTCTG 936
Paralvinella_EF580992	ACAACATATTACCAGAGCCAGATTGGAAGAGCTCTGCTCCGATCTGTTTCAGGTCAACTTTG 951
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B_plicatilis_HSP70-2_AB775785	ACGAGCATCACAGAGCTCGCTTTGAAGAGCTGTGCGCCGATTGTTTCGCGGCACCCCTC 942
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M_nipponense_KC460343	ACTTCTATTACTCGTGCCAGGTTTGAGGAACCTTGTGGTGACTTGTTCGCTGGCACCCCTT 942
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Perinereis_KU255783	ACAACCATCACAGGGGCTCGTTTTGAGGAGCTGTGTGCCGATCTCTTCGAGGCACCTCTC 939
Paralvinella_HSP70-2_EF580993	ACAACATCACTAGAGCTAGATTGAGGAGCTGAATGCTGACCTATTCCGTGTGTACGCTG 942
Haliotis_FJ812176	ACCAGCATCACTAGAGCCAGGTTTGAGGAGCTCAATGCTGATCTTTCCGTGGCACACTA 942
Oncorhynchus_HSC71_AAB21658	ACCTCCATCACAGGGGCTCGTTTTGAGGAGCTCAATGCAGACCTTTTCCGCGCACCCCTT 942
X_maculatus_HSC70_AB062115	ACGTCCATCACAGGGGCGCGCTTTGAGGAGCTGAACGCGGACCTGTTCCGCGGAACGCTG 942
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Rattus_HSC71_NM_024351	ACCTCCATTACCCGTGCTCGATTGAGGAGTTGAATGCTGACCTGTTCCGTGGCACACTG 942
Pelodiscus_HSC70_HQ219723	ACCTCAATTACAGAGCTCGTTTTGAGGAATTAAATGCTGATCTGTTCCGTGGCATCTG 942
Bos_HSP70_U09861	ACGTCCATCACAGGGGCGCGCTTCGAGGAGCTGTGCTCCGACCTGTTCCGAGCACCCCTA 942
Human_HSPA1A_NM_005345	ACGTCCATCACAGGGGCGAGGTTTCGAGGAGCTGTGCTCCGACCTGTTCCGAAGCACCCCTG 942
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Human_HSPA6_NP_002146	ACGTCCATCACTCGTGCCCGCTTTGAGGAACCTGTGCTCAGACCTCTTCCGAGCACCCCTG 948
Alligator_HSP70_AB306279	ACGTCCATCACGCGGGCGCGCTTCGAGGAGCTGAACGCCGACCTGTTCCGCGGCACGCTG 951
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Drosophila_Aa_AF295933	CAGCCTGTGGAGAAGGCCCTCAACGATGCCAAGATGGATAAGGGTCAGATCCACGACATC 996
Spodoptera_FJ754276	GAGCCGGTTCGAGAAAGGCTTCAGAGACGCAAGATGGACAAGAGCCAGATCCACGACGTC 996
Bombyx_AB035326	GAACCCGTTCGAGAAGGCACTCAAGGATGCTAAACTCGACAAGAGTCAGATCCACGACGTC 996
Paralvinella_EF580992	GAGCCAGTAGAGAAAGCTCTCCGAGATGCCAAGATGGACAAGTCCAAGATCGACGAAGTG 1011
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A_brightwelli_HSP70_KX119429	GACCCAGTTCGAGAAATCTTTGAGAGACGCTAAACTCGACAAGCCAGATCCATGAAATT 999
Wuchereria_AFL67352	GATCCAGTTGAGAAAGCACTCCGTGATGCTAAGATGGACAAGGCCAAGTCCATGACATT 1002
Setaria_AF079360	GATCCAGTTGAGAAAGCATTCGCTGATGCCAAGATGGATAAGGCACAAGTTCATGATATT 1002
C_elegans_HSP70A_M18540	GACCCAGTTCGAGAAAGTCTCTCCGTGACGCCAAGATGGACAAGGCAAGTTCATGACATC 1005
C_elegans_HSP-1_NM_070667	GACCCAGTTCGAGAAAGTCTCTCCGTGACGCCAAGATGGACAAGGCAAGTTCATGACATC 1005
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M_nipponense_DQ660140	GAGCCAGTTGAGAAAGTCTCTGAGAGATGCAAAAATGGACAAGGCCAAATCTATGACATT 1002
Mytilus_HSP70_AY861684	GAACCAAGTTCGAGAAAGCTCTGAGCGATGCCAAACTAGACAAGGCTGCTGCTCATGAAATT 1005
Perinereis_KU255783	GAGCCAGTAGAGAAGTCCATCCGTGATGCCAAGATGGGAAAAGATGCCATCCACGACATC 999
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Haliotis_FJ812176	GAGCCAGTGGAGAAGGCTTTACGTGATGCCAAGGCAGACAAGTCTCCATCCATGACATA 1002
Oncorhynchus_HSC71_AAB21658	GACCCAGTGGAGAAATCCCTCCGCGACGCCAAGATGGACAAGGCCAGGTACACGACATC 1002
X_maculatus_HSC70_AB062115	GAGCCCGTGGAGAAGTCTCTCCGAGACGCCAAGATGGACAAGGCTCAGATCCACGAGATT 1002
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Rattus_HSC71_NM_024351	GACCCGTGTAGAGAAGGCCCTTCGAGATGCCAAACTAGACAAGTTCACAGATCCATGATATT 1002
Pelodiscus_HSC70_HQ219723	GATCCTGTGGAGAAGGCCCTTCGAGATGCCAAACTAGACAAGTTCACAGATCCATGACATT 1002
Bos_HSP70_U09861	GAGCCCGTGGAGAAGGCGCTACGCGACGCCAAGCTGGACAAGGCGCAGATCCACGACCTG 1002
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Haliotis_FJ812176
Oncorhynchus_HSC71_AAB21658
X_maculatus_HSC70_AB062115
Human_HSPA8_HSC71_NP_006588
Rattus_HSC71_NM_024351
Pelodiscus_HSC70_HQ219723
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Drosophila_Aa_AF295933
Spodoptera_FJ754276
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Paralvinella_EF580992
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Haliotis_FJ812176
Oncorhynchus_HSC71_AAB21658
X_maculatus_HSC70_AB062115
Human_HSPA8_HSC71_NP_006588
Rattus_HSC71_NM_024351
Pelodiscus_HSC70_HQ219723
Bos_HSP70_U09861

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Rattus_HSP70_1A_NM_031971
X_maculatus_HSP70-1_AB062113
X_maculatus_HSP70-2_AB062114
Human_HSPA1L_NM_005527
Human_HSPA6_NP_002146
Alligator_HSP70_AB306279

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Spodoptera_FJ754276
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B_plicatilis_HSP70-2_AB775785
A_brightwelli_HSP70_KX119429
Wuchereria_AFL67352
Setaria_AF079360
C_elegans_HSP70A_M18540
C_elegans_HSP-1_NM_070667
M_nipponense_KC460343
M_nipponense_DQ660140
Mytilus_HSP70_AY861684
Perinereis_KU255783
Paralvinella_HSP70-2_EF580993
Haliotis_FJ812176
Oncorhynchus_HSC71_AAB21658
X_maculatus_HSC70_AB062115
Human_HSPA8_HSC71_NP_006588
Rattus_HSC71_NM_024351
Pelodiscus_HSC70_HQ219723
Bos_HSP70_U09861
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Rattus_HSP70_1A_NM_031971
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Human_HSPA1L_NM_005527
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Alligator_HSP70_AB306279

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Drosophila_Bb_AF295957
Drosophila_Aa_AF295933
Spodoptera_FJ754276
Bombyx_AB035326
Paralvinella_EF580992
B_plicatilis_HSP70-1_AB775784
B_plicatilis_HSP70-2_AB775785
A_brightwelli_HSP70_KX119429
Wuchereria_AFL67352
Setaria_AF079360
C_elegans_HSP70A_M18540
C_elegans_HSP-1_NM_070667
M_nipponense_KC460343
M_nipponense_DQ660140
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Haliotis_FJ812176
Oncorhynchus_HSC71_AAB21658
X_maculatus_HSC70_AB062115
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Human_HSPA6_NP_002146
Alligator_HSP70_AB306279

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Drosophila_Aa_AF295933
Spodoptera_FJ754276
Bombyx_AB035326

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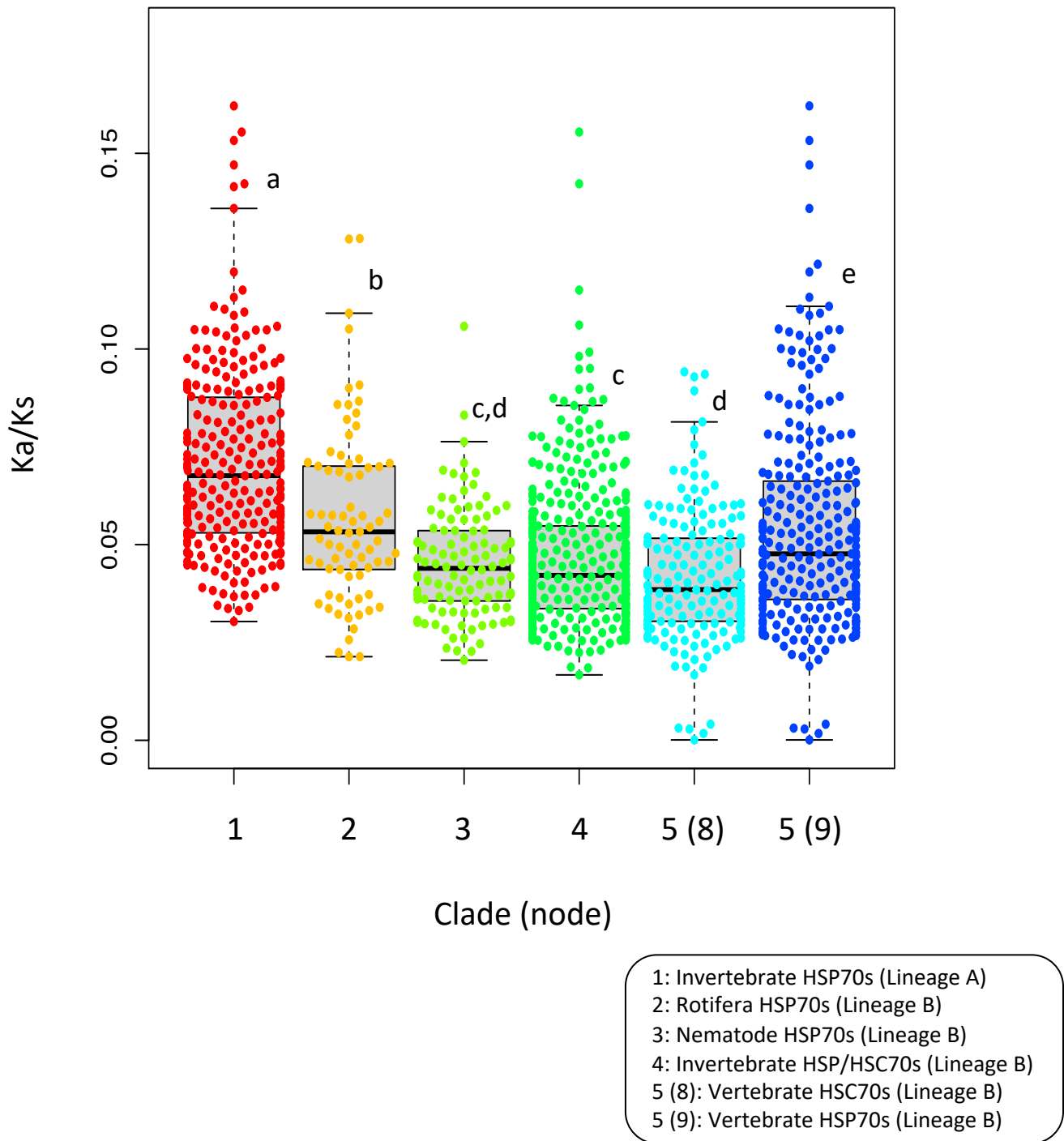
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B_plicatilis_HSP70-1_AB775784 GGTGGTGC GCCTGGTGGCATGCCTGGTGGTATGC-----CTGGCGGTATGCCT 1890
B_plicatilis_HSP70-2_AB775785 GGTGGTGC GCCTGGTGGTATGCCTGGTGGTATGCCGGGCGGTATGCCT 1902
A_brightwelli_HSP70_KX119429 GCGCCGGGCGGTATGCCGGGTGGTATGCCGGGCGGTGCTGGTGC-----T 1884
Wuchereria_AF167352 GGTGGTATGCCTGGAGGATGCCGGGTGGCATGCCTAGTGGAGCTCCAGGTGCTGGTTCA 1902
Setaria_AF079360 GGTGGTATGCCCGGAGGAATGCCTGGTGGTATGCCTGGTGGAGCTCCAGGTGGAGGTTTCG 1902
C_elegans_HSP70A_M18540 GGAGGAGCCCCACCAGGTGCTGCTCCAGGAGGAGCCGCCGAGGAGC----- 1892
C_elegans_HSP-1_NM_070667 GGAGGAGCCCCACCAGGTGGTGGTCCAGGAGGAGCCGCCGAGGAGC----- 1892
M_nipponense_KC460343 GGTGGTGTCTCTCCAGGTGGTATGCCAGGAGGTTTCCAGGTGCCCCAGGGGCTGGTGT 1902
M_nipponense_DQ660140 GGTGGTGTCTCTCCAGGTGGTATGCCAGGAGGTTTCCAGGTGCCCCAGGGGCTGGTGT 1902
Mytilus_HSP70_AY861684 GGTGGAGCCCCAGGTGGTGGTATGCCAAACTTTGGTGGAGCTGGTGGAGCCCCAGGAGGT 1905
Perinereis_KU255783 GCGGCCCTGCTGGTGGAAATGCCCGGTGGAATGCCCGGTGGAATGCCCGGAGGCTTCCCA 1899
Paralvinella_HSP70-2_EF580993 GGTGCTCCACCAGGCAGTATGCCAGGTGGCATGCCCGGTGGTATGCCAGGAGCGCTGGT 1902
Haliothis_FJ812176 GCGCGGTGCTGGTGGTATGCCCAACTTCAACCCAGGTGCTGCTGGTGTGGCGGTGCAGGA 1902
Oncorhynchus_HSC71_AAB21658 GGTGGGATGCCCGGCGGTATGCCTGAGGGCATGGCTGGCGGATTCCCTGGAGCTGGTGGT 1902
X_maculatus_HSC70_AB062115 GGAGGATGCCCTAGAGGTGGTGGTCCAGGAGAGGAGGCGGCTCGGGCCCCACCA---- 1897
Human_HSPA8_HSC71_NP_006588 GGAGGCATGCCAGGAGGAATGCCTGGGGGATTTCTGGTGGTGGAGCTCCTCCCTCTG-- 1900
Rattus_HSC71_NM_024351 GGTGGCATGCCTGGAGGAATGCCTGGTGGCTTCCCTGGTGGAGGAGCTCCTCCATCTG-- 1900
Pelodiscus_HSC70_HQ219723 GGAGGAATGCCTGGTGGCATGCCTGGGGGATTTCCCTGGAGGTGGAGCTGCCCCATCTG-- 1900
Bos_HSP70_U09861 GCGGCCCTGCTGGTGGTGGTGGGCTTGGGGCTCAGGGCCCTAAAGGGGGC---TCTGGGTCT 1899
Human_HSPA1A_NM_005345 GGTGGTCCCGGGCTGGGGGCTTCGGGGCTCAGGGTCCCAAGGAGGG---TCTGGGTCA 1899
Rattus_HSP70_1A_NM_031971 GGTGCTCCCGGGGCTGGGGGCTTCGGGGCCAGGCGCCCAAGGAGGC---TCTGGGTCTG 1899
X_maculatus_HSP70-1_AB062113 GCGGCAGGAAGCTCCAGCAGTCAGGCTGGAGGAGCCACTC-----AG 1890
X_maculatus_HSP70-2_AB062114 AGGCCCACTGGGAGGTGCAGAGAGCAGGCAGGAGGCCACA-----GCCAG 1893
Human_HSPA1L_NM_005527 TGCCTGGGCTGCTGCGGAACAGGGTATGTGCTG-----GAAGG---CCTGCCACA 1899
Human_HSPA6_NP_002146 GGTGTCCCTGGGGGCGAGCAGTTGTGGCACTCAAGCCCGCCAGGGGGAC---CCCAGCACC 1905
Alligator_HSP70_AB306279 GCCCCGCCGGCGGCCCGGTCCCGCGTCCCGCGGT-----CCGGC 1893

Drosophila_Bb_AF295957 GG-----CTACTCTGGACCCACAGTCGAGGAGGTTCGACTAA----- 1926
Drosophila_Aa_AF295933 GG-----CTACTCTGGACCCACAGTCGAGGAGGTTCGACTAA----- 1926
Spodoptera_FJ754276 GGAGGAATGCCGGGCGGAATGCCTGGTGGCATGCCCGCGGTATGCCAGGAGGAATGCCT 1950
Bombyx_AB035326 CAAGCGAGGAGTGATGGACCCACAGTTGAGGAAGTCGACTAA----- 1923
Paralvinella_EF580992 TATGGAAGT----- 1914
B_plicatilis_HSP70-1_AB775784 GGTGGAATG---CCTGGTGTGGACCTGAGAGTGC----- 1922
B_plicatilis_HSP70-2_AB775785 GGTGGAATG---CCTGGTGTGGACCTGAGAGTGC----- 1934
A_brightwelli_HSP70_KX119429 GGTGGCGCC---TCCGGAGCCGGA----- 1905
Wuchereria_AF167352 ACGGGAGGTGGACC----- 1916
Setaria_AF079360 GGTGGCAGTGGACC----- 1916
C_elegans_HSP70A_M18540 -----TGGAGGACC----- 1901
C_elegans_HSP-1_NM_070667 -----TGGAGGACC----- 1901
M_nipponense_KC460343 GCTCCTG-----GT-----GG----- 1913
M_nipponense_DQ660140 GCTCCTG-----GT-----GG----- 1913
Mytilus_HSP70_AY861684 GCACCTGGAG---CA-----GGAGGCACCTGG----- 1928
Perinereis_KU255783 GGTGGCGTGTCTCCCGGTGGAGACTCCGGTGTGTC----- 1934
Paralvinella_HSP70-2_EF580993 GGTGCTGGCGGGGCGAGCTGG-----TTC----- 1925
Haliothis_FJ812176 GGAGCTC---AGACAGG-----CGG----- 1919
Oncorhynchus_HSC71_AAB21658 GCTGCTCCTGGAGGTGGTGGATC----- 1925
X_maculatus_HSC70_AB062115 ----- 1897
Human_HSPA8_HSC71_NP_006588 -----GTGGTGTTC----- 1910
Rattus_HSC71_NM_024351 -----GTGGTGTTC----- 1910
Pelodiscus_HSC70_HQ219723 -----GAGGGGCTC----- 1910
Bos_HSP70_U09861 GGC----- 1902
Human_HSPA1A_NM_005345 GGC----- 1902
Rattus_HSP70_1A_NM_031971 GGG----- 1902
X_maculatus_HSP70-1_AB062113 GGT----- 1893
X_maculatus_HSP70-2_AB062114 GGG----- 1896
Human_HSPA1L_NM_005527 GGC----- 1902
Human_HSPA6_NP_002146 GGC----- 1908
Alligator_HSP70_AB306279 GGC----- 1896

Drosophila_Bb_AF295957 ----- 1926
Drosophila_Aa_AF295933 ----- 1926
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Bombyx_AB035326 ----- 1923
Paralvinella_EF580992 -----CAACAAGGACCAACAGTGGAAGAGGTGGATTAA- 1947
B_plicatilis_HSP70-1_AB775784 -----GGGACGAGTGGACCTACCATTGATGAGGTCGATTAA- 1959
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Wuchereria_AF167352 -----AACAATTGAAGAAGTTGATTAG- 1938
Setaria_AF079360 -----AACAATTGAAGAAGTTGATTAG- 1938
C_elegans_HSP70A_M18540 -----AACGATCGAGGAGGTTCGACTAA- 1923
C_elegans_HSP-1_NM_070667 -----AACGATCGAGGAGGTTCGACTAA- 1923
M_nipponense_KC460343 -----TGGTTTCATCAGGCCCCACAATTGAAGAGGTTCGATTAA- 1950
M_nipponense_DQ660140 -----TGGTTTCATCAGGCCCCACAATTGAAGAGGTTCGATTAA- 1950
Mytilus_HSP70_AY861684 -----TGGAAGTGGTGGACCAACCATTGAAGAGGTTCGATTAA- 1965
Perinereis_KU255783 -----CCCAGGTGGTGGCCCCACAATCGAAGAGGTTCGACTAA- 1971
Paralvinella_HSP70-2_EF580993 -----CGGTGGCAGTGGACCAACCATTGAGGAGGTTCGATTAA- 1963

Haliotis_FJ812176	-----CAGCTCCGGAGGCCCCACCATCGAGGAGGTTGATTAA-	1956
Oncorhynchus_HSC71_AAB21658	-----ATCTGGACCAACCATTGAGGAAGTCGACTAA-	1956
X_maculatus_HSC70_AB062115	-----TAGAGGAGGTCGACTAA-	1914
Human_HSPA8_HSC71_NP_006588	-----CTCAGGGCCCCACCATGAAGAGGTTGATTAA-	1941
Rattus_HSC71_NM_024351	-----TTCAGGCCCCACCATGAAGAGGTCGATTAA-	1941
Pelodiscus_HSC70_HQ219723	-----ATCTGGACCAACCATTGAGGAAGTAGATTAA-	1941
Bos_HSP70_U09861	-----CCCACCATTGAGGAGGTGGATTAG-	1926
Human_HSPA1A_NM_005345	-----CCCACCATTGAGGAGGTAGATTAG-	1926
Rattus_HSP70_1A_NM_031971	-----CCCACCATCGAGGAGGTGGATTAG-	1926
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X_maculatus_HSP70-2_AB062114	-----CCCACTGTGGAGGAGGTGGACTGA-	1920
Human_HSPA1L_NM_005527	-----CCCACAATTGAAGAAGTAGATTAA-	1926
Human_HSPA6_NP_002146	-----CCCATCATGAGGAGGTTGATTGA-	1932
Alligator_HSP70_AB306279	-----CCCACCATCGAGGAGGTGGACTAG-	1920

Supplementary fig. S5. Comparison of HSP70 nucleotide sequences used for the calculation of synonymous and nonsynonymous substitution rates. Regions shown in bold were used for the calculation.



Supplementary fig. S6. Bee swarm boxplots of Ka/Ks values for each clade. Only Ka/Ks values calculated from inter-cluster pairs were averaged. Statistical differences were calculated by Kruskal-Wallis test (chi-squared = 200.45, df = 5, p-value < 2.2e-16) followed by the non-parametric post-hoc tests (pairwise Wilcox test with P value adjustment by the Holm method). Clades sharing same letters are not significantly different at the 5% level of significance. One outlier in clade 3 (0.53) is not included in the plot although this value was used for all statistical analyses.

1a			Clade 1				Clade 2				Clade 3				Clade 4				Clade 5 (node 8)				Clade 5 (node 9)									
Gene No.			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Clade 1	2	<i>D. melanogaster</i> HSP70Aa (AF295933)	0.00																													
	3	<i>Spodoptera</i> HSP70 (FJ754276)	0.09	0.09																												
	4	<i>Bombyx</i> HSP70 (AB035326)	0.10	0.10	0.03																											
	5	<i>Paralvinella</i> HSP70-1 (EF580992)	0.16	0.16	0.14	0.14																										
Clade 2	6	<i>B. plicatilis</i> _HSP70-1 (AB775784)	0.15	0.15	0.14	0.14	0.15																									
	7	<i>B. plicatilis</i> _HSP70-2 (AB775785)	0.15	0.15	0.14	0.14	0.15	0.00																								
Clade 3	8	<i>A. brightwelli</i> _HSP70 (KX119429)	0.16	0.15	0.15	0.16	0.15	0.06	0.06																							
	9	<i>Wuchereria</i> (AF167352)	0.16	0.15	0.15	0.16	0.16	0.08	0.09	0.09																						
	10	<i>Setaria</i> (AF079360)	0.15	0.15	0.15	0.15	0.16	0.08	0.08	0.09	0.01																					
	11	<i>C. elegans</i> HSP70A (M18540)	0.16	0.16	0.16	0.16	0.18	0.09	0.09	0.11	0.05	0.05																				
Clade 4	12	<i>C. elegans</i> HSP-1 (NM_070667)	0.15	0.15	0.15	0.15	0.17	0.08	0.08	0.09	0.04	0.03	0.01																			
	13	<i>M. nipponense</i> HSP70-1 (KC460343)	0.16	0.15	0.15	0.15	0.15	0.07	0.07	0.08	0.08	0.08	0.09	0.08																		
	14	<i>M. nipponense</i> HSP70-2 (DQ660140)	0.15	0.15	0.15	0.15	0.15	0.07	0.07	0.07	0.08	0.08	0.09	0.08	0.01																	
	15	<i>Mytilus</i> HSP70 (AY861684)	0.16	0.16	0.16	0.16	0.15	0.09	0.09	0.07	0.09	0.09	0.11	0.10	0.07	0.07																
Clade 5 (node 8)	16	<i>Perinereis</i> HSP70-1 (KU255783)	0.15	0.15	0.17	0.16	0.15	0.09	0.09	0.08	0.10	0.10	0.11	0.10	0.07	0.07	0.06															
	17	<i>Paralvinella</i> HSP70-2 (EF580993)	0.17	0.17	0.17	0.17	0.16	0.10	0.10	0.09	0.11	0.11	0.12	0.11	0.08	0.08	0.07	0.06														
	18	<i>Haliotis</i> HSP70c (FJ812176)	0.15	0.15	0.16	0.16	0.16	0.10	0.10	0.09	0.10	0.10	0.12	0.11	0.08	0.08	0.06	0.06	0.07													
	19	<i>Oncorhynchus</i> HSC71 (AAB21658)	0.16	0.16	0.16	0.16	0.16	0.08	0.08	0.08	0.09	0.09	0.11	0.09	0.06	0.06	0.07	0.06	0.07	0.08												
Clade 5 (node 9)	20	<i>X. maculatus</i> HSC70 (AB062115)	0.18	0.17	0.16	0.16	0.15	0.09	0.09	0.08	0.11	0.11	0.12	0.11	0.07	0.07	0.07	0.08	0.09	0.08	0.04											
	21	Human_HSPA8 (NP_006588)	0.15	0.15	0.14	0.15	0.15	0.07	0.07	0.07	0.09	0.09	0.10	0.09	0.07	0.06	0.06	0.07	0.07	0.07	0.03	0.05										
	22	<i>Rattus</i> HSC71 (NM_024351)	0.15	0.15	0.14	0.15	0.15	0.07	0.07	0.07	0.09	0.09	0.10	0.09	0.06	0.06	0.06	0.07	0.07	0.07	0.03	0.05	0.00									
	23	<i>Pelodiscus</i> HSC70 (HQ219723)	0.15	0.15	0.14	0.15	0.14	0.07	0.07	0.07	0.09	0.08	0.10	0.09	0.06	0.06	0.06	0.07	0.07	0.06	0.03	0.04	0.00	0.00								
Clade 5 (node 9)	24	<i>Bos</i> HSP70 (U09861)	0.15	0.15	0.15	0.15	0.16	0.10	0.10	0.09	0.10	0.10	0.12	0.11	0.09	0.09	0.09	0.10	0.11	0.10	0.08	0.09	0.07	0.07	0.07							
	25	Human_HSPA1A (NM_005345)	0.15	0.15	0.15	0.15	0.15	0.10	0.09	0.09	0.10	0.09	0.11	0.10	0.08	0.08	0.09	0.09	0.11	0.10	0.07	0.09	0.06	0.06	0.06	0.00						
	26	<i>Rattus</i> HSP70_1A (NM_031971)	0.15	0.15	0.15	0.15	0.16	0.10	0.10	0.09	0.10	0.10	0.12	0.11	0.09	0.09	0.09	0.09	0.11	0.10	0.08	0.09	0.07	0.07	0.07	0.01	0.01					
	27	<i>X. maculatus</i> HSP70-1 (AB062113)	0.17	0.17	0.17	0.16	0.16	0.10	0.10	0.09	0.11	0.10	0.12	0.11	0.09	0.09	0.09	0.10	0.10	0.10	0.07	0.08	0.06	0.06	0.06	0.06	0.07	0.07	0.07	0.07		
Clade 5 (node 9)	28	<i>X. maculatus</i> HSP70-2 (AB062114)	0.16	0.16	0.16	0.17	0.16	0.10	0.10	0.09	0.11	0.10	0.12	0.11	0.09	0.09	0.09	0.10	0.11	0.10	0.08	0.09	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.06		
	29	Human_HSPA1L (NM_005527)	0.15	0.15	0.15	0.16	0.16	0.10	0.10	0.10	0.10	0.12	0.12	0.11	0.09	0.09	0.10	0.10	0.11	0.11	0.09	0.10	0.08	0.08	0.08	0.04	0.04	0.04	0.07	0.07		
	30	Human_HSPA6 (NP_002146)	0.16	0.16	0.16	0.17	0.17	0.12	0.12	0.11	0.12	0.12	0.14	0.13	0.12	0.11	0.11	0.11	0.13	0.12	0.12	0.12	0.10	0.10	0.10	0.07	0.07	0.07	0.12	0.11	0.09	
	31	Alligator_HSP70 (AB306279)	0.16	0.16	0.16	0.16	0.17	0.09	0.09	0.08	0.10	0.10	0.12	0.10	0.09	0.09	0.09	0.09	0.10	0.10	0.08	0.09	0.07	0.07	0.07	0.09	0.09	0.09	0.08	0.09	0.10	0.11

[illegible]

Supplementary material 2

Heat Treatment

For semi-quantitative RT-PCR, rotifers were batch-cultured in 400 mL at 25°C. In the exponential growth phase, approximately 6×10^4 individuals were randomly divided into two groups and concentrated to about 10 mL by filtration through 20 µm mesh filters. One group (heat-treated group) was transferred to culture medium at 30°C for 10 min, collected with a 20 µm mesh filter, and stored in ISOGEN (Nippon Gene, Tokyo, Japan) at -80°C. The control group was prepared by the same procedure at 25°C.

For quantitative real-time PCR, 8 rotifers bearing 2 or 3 eggs were selected from a batch culture population and inoculated into a well of a 12-well plate (Nunc, Rochester, NY) containing 1 mL of culture medium at 25°C. The 12-well plate was put on a water bath at 40°C for 10 min, and then transferred to a water bath at 25°C. Rotifers were sampled before heat treatment (control), just after heat treatment (time 0), and 2, 3, 4, 6, and 8 h after heat treatment ($n = 3$, each group contained 8 individuals). Rotifer samples were stored in 500 µL ISOGEN (Nippon Gene) and stored at -80°C until use. Rotifers used for *in situ* hybridization were prepared by the same method except the heat shock temperature was 30°C.

cDNA Cloning of HSP70

5' RACE for the partial sequence of *B. plicatilis* HSP70 gene [AB076052; (Kaneko et al. 2002)] was carried out using a GeneRacer kit (Invitrogen, Carlsbad, CA) with gene-specific primers rHSP70_5RACE1, rHSP70_5RACE2, and rHSP70_5RACE3 ([supplementary table S1](#)). Total RNA was extracted from a rotifer population in an exponential growth phase and used for 1st strand cDNA synthesis using the rHSP70_5RACE1 primer and the GeneRacer kit (Invitrogen). First round PCR was performed at 94°C for 3 min followed by 30 cycles of 94°C for 30 s, 55°C for 30 s and 72 °C for 1 min. The final extension step was performed at 72°C for 5 min. The 20 µL-reaction mixture contained about 1 µg of the 1st strand cDNA, 2 µL of Ex *Taq* buffer (Takara, Shiga, Japan), 1 U of Ex *Taq* DNA polymerase, 4 nmol of dNTP mixture, 10 pmol of rHSP70_5RACE2 primer and GeneRacer 5' primer (Table 1). The amplified products were diluted 10-fold with sterile distilled water and used as the template for the nested PCR with rHSP70_5RACE3 and GeneRacer 5' nested primers ([supplementary table S1](#)). The PCR conditions were same as the first round PCR except for the template and primers. The amplified products were subcloned into pGEM-T vector (Promega, Madison, WI) and sequenced as reported previously (Yoon et al. 2008).

3' RACE for rotifer HSP70 gene was carried out as follows. An aliquot of first strand cDNA was synthesized from rotifers in an exponential phase using an oligo-dT adapter primer ([supplementary table S1](#)). PCR was performed with the first strand cDNA as a template and with rHSP70_3RACE1 and AUAP primers. PCR was carried out with 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 2 min. The final extension step was performed at 72°C for 5 min. The 20 µl of reaction mixture contained 5 pmol of forward and reverse primers, approximately 1 µg of first strand cDNA, 4 nmol of dNTP mixture, 2 µL of 10 × PCR buffer [100 mM Tris-HCl, pH8.3, 500 mM KCl, 15 mM MgCl₂, 0.01% (w/v) gelatin] and 0.2 U of Ex *Taq* DNA polymerase (Takara). Nested PCR was performed under the same conditions using the PCR product diluted 100-fold and the rHSP70_3RACE2 primer. The amplified products were subcloned into pGEM-T vector (Promega) and sequenced as described above. The nucleotide sequence of the open reading frame was confirmed by a single PCR using rHSP70_full_f1 and rHSP70_full_r1 primers

([supplementary table S1](#)). Conditions of the single PCR were same as described in 5' RACE.

Semi-Quantitative RT-PCR

RNA extraction and 1st strand cDNA synthesis were performed as described above. Primers rHSP70_gapF and rHSP70_gapR were designed to amplify the region encoding the GGMP repeat of *B. plicatilis* HSP70cB1i and HSP70cB2i genes ([supplementary table S1](#)). The β -actin gene was used as the internal control with specific primers rActinF and rActinR reported previously (Kaneko et al. 2005). PCR was carried out at 94°C for 3 min followed by 20 – 40 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 30 s. A 20- μ L reaction mixture contained approximately 1 μ g of 1st strand cDNA, 10 pmol of rHSP70_gapF and rHSP70_gapR, 2 μ L of Ampli Taq buffer (Applied Biosystems, Foster City, CA), 4 nmol of dNTP mixture, and 0.2 U of Ampli Taq DNA polymerase (Applied Biosystems). The PCR products were separated with an 8% acrylamide gel and visualized by ethidium bromide staining.

Quantitative Real-Time PCR

Rotifers were collected 0, 2, 4, 6, and 8 h after the heat treatment, and stored in ISOGEN (Takara) at -80°C until use (three replicates for each time point, one replicate contained eight individuals). Rotifers collected before the heat treatment were used as the control. Quantitative real-time PCR was performed as reported previously (Kaneko et al. 2011). Primers for the β -actin gene were described previously (Kaneko et al. 2005); primers for HSP70 genes were designed using the Primer Express software ver. 2 (Applied Biosystems) ([supplementary table S1](#)). R version 3.5.1. and the multcomp package (Hothorn et al. 2008) was used for one-way analysis of variance (ANOVA) followed by the Dunnett's test on the Macintosh platform.

In situ Hybridization

Rotifers were fixed in PBS containing 4% paraformaldehyde 4 h after the heat treatment. *In situ* hybridization was performed as reported previously (Kaneko et al. 2011), using DIG-labeled RNA probes synthesized as follows. Primers HSP70_insituF and HSP70_insituR were designed to amplify the DNA fragment of 2026 - 2103 nt of *B. plicatilis* HSP70cB1i gene ([supplementary table S1](#)). PCR was performed in a 20 μ L reaction mixture containing approximately 1 μ g of the genomic DNA of *B. plicatilis*, 10 pmol each of the HSP70_insituF and HSP70_insituR primers, 2 μ L of 10 $\times$ PCR buffer, 4 nmol dNTP mixture, and 0.2 U of Ex Taq DNA polymerase (Takara). The amplification was carried out with the initial denaturation at 94°C for 3 min and 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1.5 min, followed by the final extension step at 72°C for 5 min. The amplified products were subcloned into pGEM-T easy vector (Promega). DIG-labeled RNA probes were synthesized with a DIG RNA labeling kit (Roche) according to the manufacturer's instructions.

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