

1   **The complex evolution of the metazoan HSP70 gene family**

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17

18 Abstract

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

37

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

40

41 **Introduction**

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

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

67 2011; Garbuz 2017). HSP70 family member genes have therefore been used as biomarkers of  
68 environmental stresses both in laboratory and field experiments (Sanders 1993; Ceyhun et al. 2010;  
69 Judge et al. 2011).

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

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

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

97

## 98 **Results**

### 99 Molecular Characterization of Rotifer HSP70 Genes

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

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

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

123 Since the two *B. plicatilis* HSP70 genes showed a similar expression pattern in response to  
124 heat stress, we used quantitative real-time PCR primers that amplify both HSP70cB1i and  
125 HSP70cB2i genes in the subsequent time-course expression analyses (fig. 1D). The relative mRNA  
126 levels were increased by heat stress at 40°C for 10 min and reached at the maximum level 2 h after  
127 the heat stress. The difference in mRNA levels between the control and 2 h groups was statistically  
128 significant. The mRNA levels of *B. plicatilis* HSP70s remained twofold 8 h after the heat stress. We  
129 then performed *in situ* hybridization using DIG-labeled RNA probes (fig. 1E). The probe region  
130 contained only five base pair differences between the two HSP70 genes, and thus the RNA probes  
131 were likely to hybridize with both HSP70cB1i and HSP70cB2i mRNAs. The hybridization signals  
132 increased in all tissues of heat-treated rotifers. Altogether, these results indicate that the *B. plicatilis*  
133 HSP70cB1i and HSP70cB2i genes are heat-inducible and have similar expression profiles.  
134

### 135 Phylogenetic Analysis of HSP70 Family Members

136 To assess the relationship of the *B. plicatilis* HSP70 genes with those of other rotifers and  
137 other metazoans, we constructed gene trees of more than 100 HSP70 family members, including  
138 members associated with the mitochondria and ER and many sequences known only from  
139 automated genome annotation as “HSP70.” To assess the robustness of the results we used  
140 alignments made by three different methods [Clustal-Omega (Sievers et al. 2011), M-Coffee  
141 (Wallace et al. 2006), and Expresso (Armougom et al. 2006)] to construct consensus trees with  
142 maximum-likelihood (RAxML) and Bayesian (MrBayes) approaches. The RAxML  
143 PROTGAMMAAUTO function found that the LG model of protein evolution (Le and Gascuel  
144 2008) was optimal for all three alignments and this model was used to construct ML and Bayesian

145 consensus trees. The six trees were in general agreement for major nodes; [fig. 2](#) shows the Bayesian  
146 consensus tree of the Espresso alignment and support for major nodes from each approach is shown  
147 in [table 1](#). In all analyses HSP70 family members known to be mitochondria- and ER-specific  
148 formed two monophyletic groups (nodes 11 and 12 in [fig. 2](#)) in agreement with previous reports  
149 (Boorstein et al. 1994; Miernyk 1997; Nikolaidis and Nei 2004). All family members known to be  
150 cytosolic formed a third group (node 1; clades 1–6 in [fig. 2](#)), with yeast cytosolic HSP70s (clade 6)  
151 basal to all metazoan cytosolic HSP70s. The tree topology generally supported the species  
152 phylogeny. Within the metazoan cytosolic HSP70s (node 2; clades 1–5) there were two lineages:  
153 Lineage A was well supported and contained of genes from arthropods, molluscs, and rotifers (node  
154 3; clade 1); Lineage B was more poorly resolved and represented by many diverse vertebrate and  
155 invertebrate phyla (node 4; clades 2–5). There are two groups of vertebrate cytosolic HSP70s  
156 (nodes 8 and 9), which form a monophyletic group (node 7; clade 5) in Bayesian and RAxML  
157 analyses of Espresso and Clustal alignments. Unexpectedly, we found two additional clusters of  
158 cytosolic HSP70 family members in all analyses: one composed of HSP70 genes from the phylum  
159 Rotifera (node 5; clade 2) and the other from the phylum Nematoda (node 6; clade 3). This suggests  
160 the evolution of two unique subgroups of HSP70, distinct from conventional vertebrate and  
161 invertebrate HSP70s. We also constructed phylogenetic trees mainly using HSP70 family members  
162 of known expression patterns ([supplementary figs. S2 and S3](#)). Both of the two HSP70 lineages as  
163 well as most of the nodes described above were identified, supporting the robustness of the tree  
164 shown in [fig. 2](#), although several clades were poorly resolved in [supplementary figs. 2 and 3](#) that  
165 have limited number of samples.

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

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

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

187

#### 188 Synonymous and Nonsynonymous Substitution Rates

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

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

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

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

219

## 220 Discussion

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

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

235 Inter-clade comparison of the synonymous and nonsynonymous substitution rates shows that  
236 while all HSP70 family members are under strong purifying selection, the pressure to conserve  
237 amino acid sequence varies across clades. The presence of purifying selection has been reported for  
238 mammalian (Hess et al. 2018), nematode (Nikolaïdis and Nei 2004), and molluscan (Kourtidis et al.  
239 2006) HSP70 genes, and our cross-phylum analysis was consistent with these reports. Most  
240 dramatically, the canonical HSC70 genes of vertebrates (node 8; clade 5) showed stronger purifying  
241 selection pressure than the HSP70 family members in node 9 (clade 5). This concept can be further  
242 generalized by thoroughly investigating the saturation rate between each HSP70 gene (Pollock and  
243 Larkin 2004). It is thus speculated that the role of HSC70 protein, the folding of newly synthesized  
244 proteins, is more essential for cell survival compared to the role of the HSP70 cognates (node 9;  
245 clade 5). In line with this speculation, cells deficient in the HSC70 gene are non-viable (Florin et al.  
246 2004), whereas HSP70 knockout mice are mostly viable and fertile, although they are known to be  
247 sensitive to stress (Daugaard et al. 2007). Meanwhile, genetic distance between each clade is not  
248 uniform, and the gene conversion may also affect the accuracy of this calculation. In this regard our

249 data should be interpreted in a semiquantitative manner.

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

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

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

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

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

281

## 282 Materials and Methods

283 Culture

284 We used the *B. plicatilis* Ishikawa strain (also called *Brachionus* sp. ISKW), originally  
285 isolated from a Japanese eel culture pond (Yoshinaga et al. 2004), for cDNA cloning, semi-  
286 quantitative RT-PCR, quantitative real-time PCR, and *in situ* hybridization. The cytochrome oxidase  
287 subunit I gene sequence has been registered for detailed identification of this strain (GenBank  
288 accession number LC422762). Rotifers were cultured at 25 °C by a standard protocol (Kaneko et al.  
289 2016) using half-diluted Brujewicz artificial seawater and fed the algae *Chlorella regularis* (Nikkai  
290 Center, Tokyo, Japan) at a final concentration of approximately  $7 \times 10^6$  cells/mL. See  
291 [supplementary material 2](#) for details of molecular characterization of rotifer HSP70 genes.

292

293 Phylogenetic Analysis

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

301 A multiple sequence alignment of HSP70 and HSC70 amino acid sequences was produced  
302 using Clustal Omega, Expresso, and M-Coffee with default parameters. Bayesian phylogenetic  
303 analysis was performed using MrBayes (v3.2.6) on the CIPRES scientific gateway v3.3 (Miller et  
304 al. 2010) with four chains each for  $10^7$  generations. Every 1000th trees were sampled, and the first  
305 25% of samples were discarded as a burn-in. Bayesian consensus trees were visualized with the  
306 TreeView software v1.4.3.

307

#### 308 Synonymous and Nonsynonymous Substitution Rates

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

313

#### 314 Author Contributions

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

318

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324

#### 325 Figure Legends

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

327 reverse transcription-PCR. A representative result is shown out of three independent experiments.  
328 Location of primers rHSP70\_gapF and rHSP70\_gapR that amplify cDNA fragment of 112 and 124  
329 bp from HSP70cB1i and HSP70cB2i cDNAs, respectively (A). Polyacrylamide gel electrophoresis  
330 patterns of the RT-PCR product. Cycle numbers within a linear range of PCR amplification were  
331 determined to be 24 to 28 cycles for both cDNAs by preliminary experiments on the basis of signal  
332 intensities of amplified products by RT-PCR (B). Signal intensities of HSP70cB1i and HSP70cB2i  
333 genes standardized to those of  $\beta$ -actin (C). (D) Heat stress-induced expression of HSP70 quantified  
334 by real-time PCR. Beta-actin gene was used as the internal control. Bars represent standard errors  
335 from three replications. One-way ANOVA detected significant effects of heat treatment ( $F = 3.281$ ,  
336  $df = 5$ ,  $P = 0.0426$ ). Dunnett's multiple comparison was used to detect significant differences  
337 between the control and other groups. Difference between control and two hours group was  
338 statistically significant ( $t = 3.246$ ,  $P = 0.028$ ). (E) *In situ* hybridization for *B. plicatilis* HSP70  
339 genes. Rotifers were fixed 4 h after the heat treatment.

340

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

348

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

353 I14V, I15L, A16E, D17A, and Q18E.

354

355 **FIG. 5.** Comparison of the amino acid sequences of the HSP70 family members from invertebrates.

356 Nodes in the phylogenetic tree ([fig. 3](#)) are indicated on the right margin, and serine residues specific  
357 to node 3 are highlighted. Stress-inducible and non-inducible genes are shown in red and blue,  
358 respectively. No data on the stress inducibility is available for genes shown in black. The accession  
359 numbers and other information are summarized in [supplementary table S2](#).

360

361 **FIG. 6.** Selection pressure on cytosolic HSP70 family member genes. (A) Nonsynonymous  
362 substitution rates (Ka values) between 34 HSP70 family member genes. Gene number 1 is the fruit  
363 fly HSP70 Bb (AF295957) gene. The substitution rate of fruit fly HSP70 Aa and Bb gene was  
364 00395526, which is shown as 0.00 in the figure. Accession numbers for other genes are indicated in  
365 the figure. Clades and nodes correspond to those in [fig. 2](#). (B) Bee swarm boxplots of Ka/Ks values  
366 for each clade. Only Ka/Ks values calculated from inter-cluster pairs were averaged. Statistical  
367 differences were calculated by Kruskal-Wallis test (chi-squared = 88.704, df = 5, p < 2.2e-16)  
368 followed by the non-parametric post-hoc tests (pairwise Wilcox test with P value adjustment by the  
369 Holm method). Clades sharing same letters are not significantly different at the 5% level of  
370 significance. One outlier in clade 3 (0.53) is not included in the plot (see supplementary material 1)  
371 although this value was used for all statistical analyses. (C) Effect sizes (Cohen's d) for all  
372 comparisons. Combinations where significant differences were found in the pairwise Wilcox test are  
373 shown in bold.

374

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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

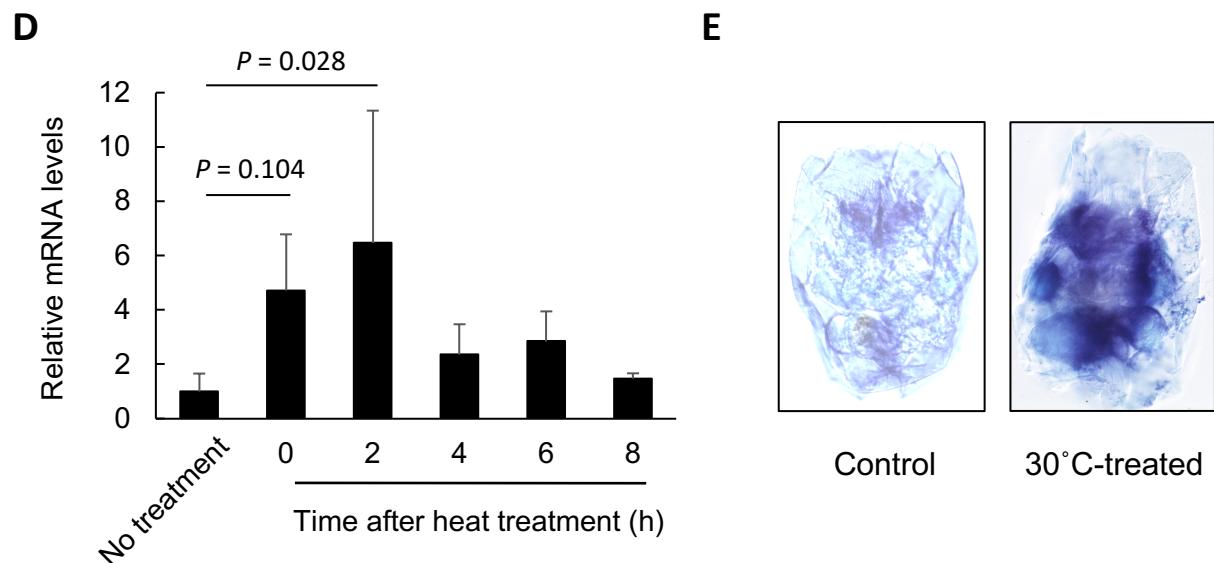
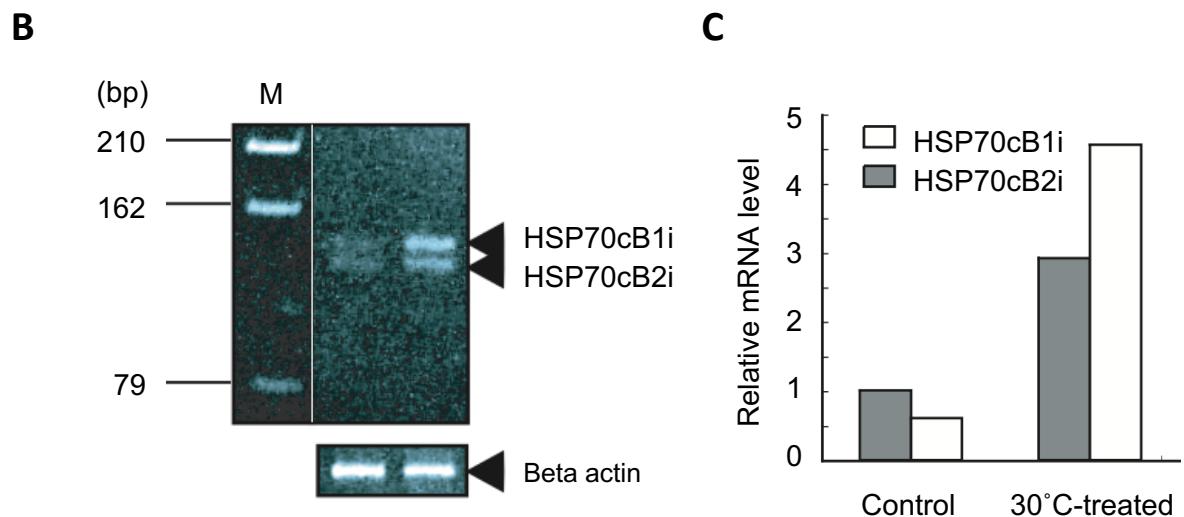
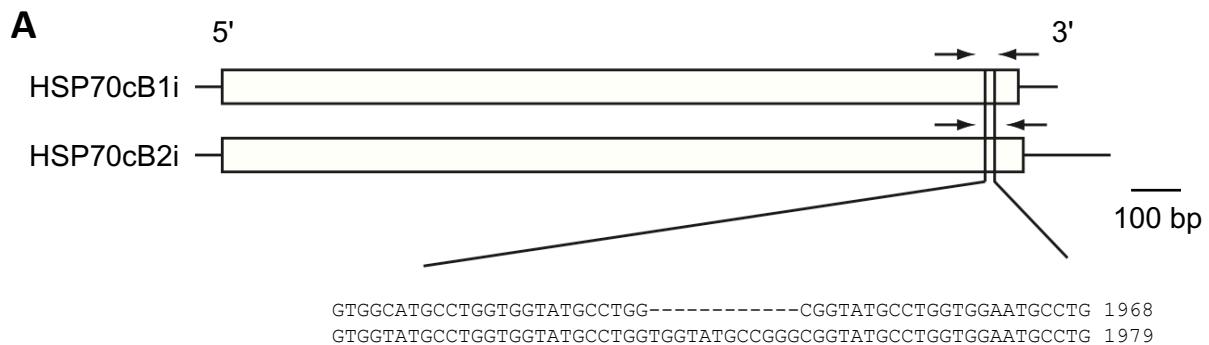
(a) Rotifer genes (node 5) is included in node 3 with 83% support

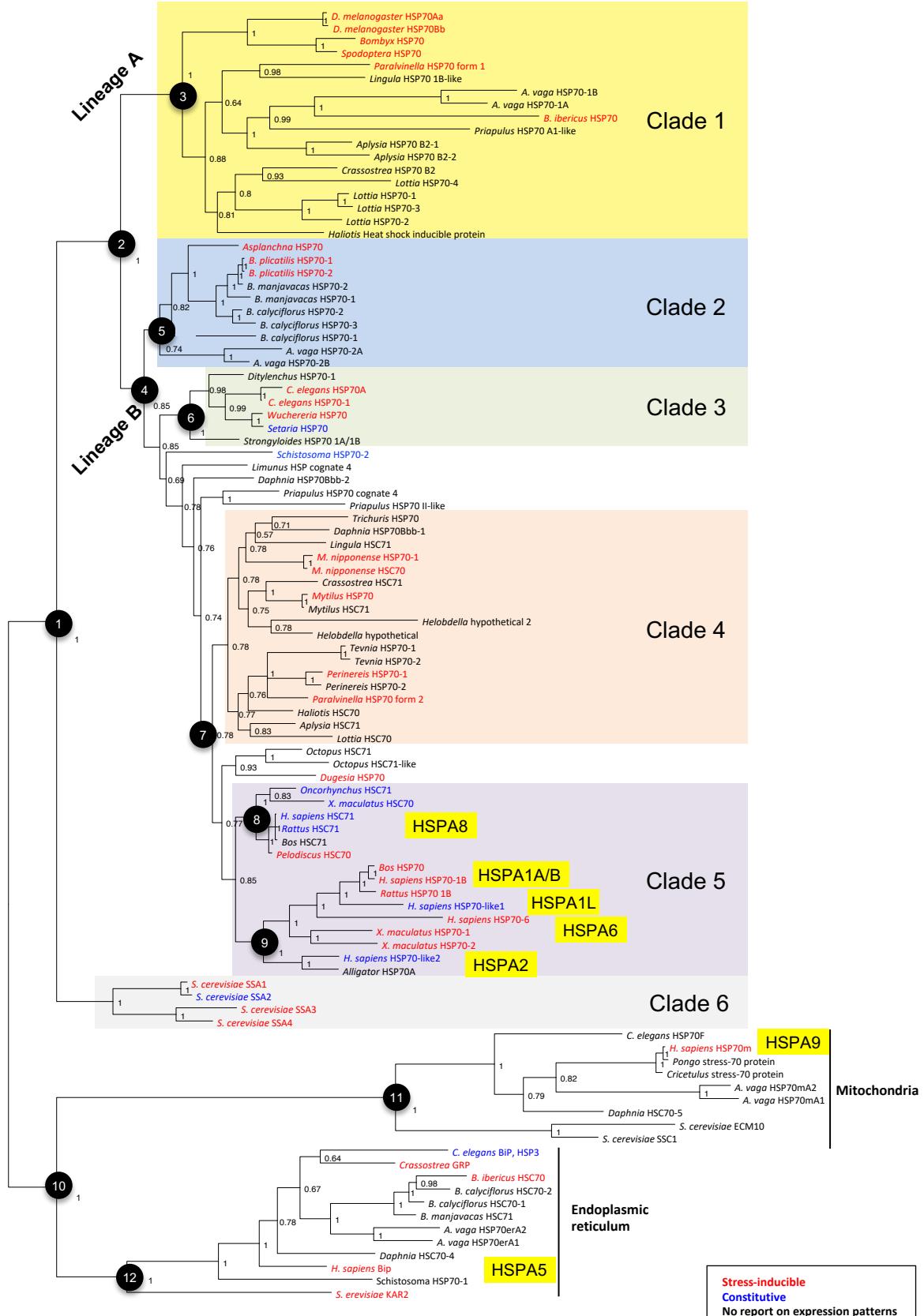
(b) Rotifer genes (node 5) sister group to node 3 with 80% support, other paraphyly

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

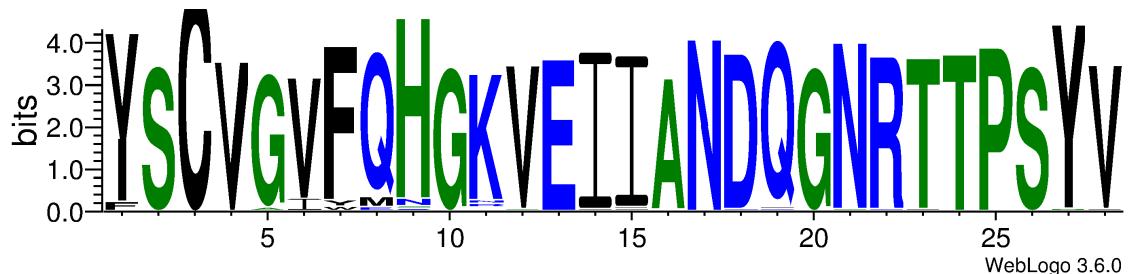
(d) 36% with Trichuris (immediate outgroup to node 6)

(e) Trichuris not in node 6

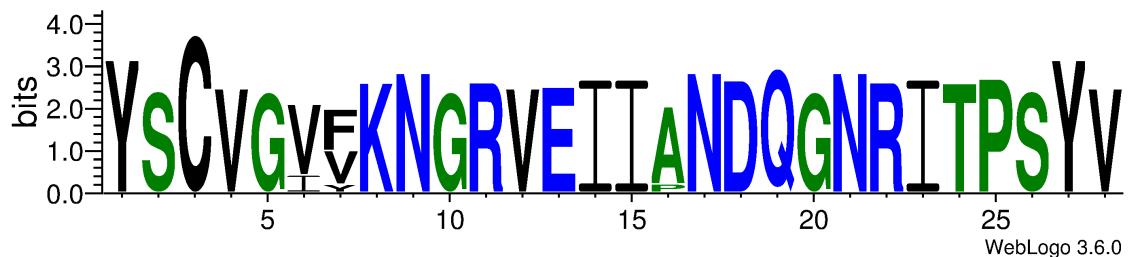




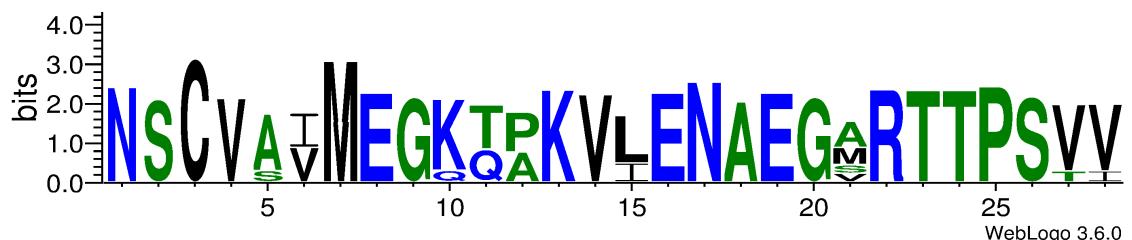
## Cytosol



## Endoplasmic reticulum



## Mitochondrial



<i>D. melanogaster</i> HSP70A	KGERNVLI <b>F</b> DLGGGTFDVSI <b>L</b> TIDE----G <b>S</b> LF <b>EVR</b> STAGDT	224
<i>D. melanogaster</i> HSP70B	KGERNVLI <b>I</b> DLGGGTFDVSI <b>L</b> TIDE----G <b>S</b> LF <b>EVR</b> STAGDT	224
<i>Bombyx</i> HSP70	KGERNVLI <b>F</b> DLGGGTFDVSI <b>L</b> TIDE----G <b>S</b> LF <b>EVK</b> STAGDT	224
<i>Spodoptera</i> HSP70	KGERNVLI <b>F</b> DLGGGTFDVSI <b>L</b> TIDE----G <b>S</b> LF <b>EVR</b> RATAGDT	224
<i>Paralvinella</i> HSP70-1	SGEKNVLI <b>F</b> DLGGGTFDVSI <b>L</b> SIAD----G <b>S</b> LF <b>EVK</b> STAGDT	229
<i>Lingula</i> HSP71	QGEKNVLI <b>F</b> DLGGGTFDVSI <b>L</b> SIAD----G <b>S</b> LF <b>EVR</b> STAGDT	228
<i>A. vaga</i> HSP70-1B	HRDQNILIYDLGGGTFDVSVLTISGGDVSG <b>S</b> VFEVKATA <b>G</b> DT	238
<i>A. vaga</i> HSP70-1A	QRDQNILIYDLGGGTFDVSI <b>L</b> TISGGDAGG <b>S</b> VFEVRSTAGDT	238
<i>B. ibericus</i> HSP70	KRRKNVLIYDLGGGTFDVSVLSIND----G <b>-L</b> FEVLSTNGNT	224
<i>Priapulus</i> HSP70-1	AGERNVLYD <b>L</b> GGGTFDVSVLTIDE----GAMFEVRSTAGDT	226
<i>Aplysia</i> HSP70-1	KGEKHVLIFDLGGGTFDVSVLAIDE----G <b>S</b> IFEVKATA <b>G</b> DT	227
<i>Aplysia</i> HSP70-2	SGEKNVLI <b>F</b> DLGGGTFDVSVLTIDE----G <b>S</b> MFEVKATA <b>G</b> DT	230
<i>Crassostrea</i> HSP70	SGEKNVLI <b>F</b> DLGGGTFDVSI <b>L</b> TIDE----G <b>S</b> IFEVRSTAGDT	228
<i>Lottia</i> HSP70-4	DTEQHILIFDLGGGTFDVSI <b>L</b> AIDE----G <b>-V</b> FEVLATAGDT	227
<i>Lottia</i> HSP70-1	SGEKNVLI <b>F</b> DLGGGTFDVSI <b>L</b> TIDE----G <b>S</b> LF <b>EVK</b> STAGDT	225
<i>Lottia</i> HSP70-3	SGEKNVLI <b>F</b> DLGGGTFDVSI <b>L</b> TIDE----G <b>S</b> LF <b>EVK</b> STAGDT	226
<i>Lottia</i> HSP70-2	SGEKNVLI <b>F</b> DLGGGTFDVSI <b>L</b> AIDE----G <b>S</b> LF <b>EVK</b> STAGDT	226
<i>Haliothis</i> HSP70	KGEKNVLI <b>F</b> DLGGGTFDV <b>P</b> VLTIDE----G <b>S</b> MFEVRSTAGDT	231
<i>B. plicatilis</i> HSP70-1	GGEKNILIFDLGGGTFDVSI <b>L</b> TIEE----G <b>-I</b> FEVKSTAGDT	226
<i>B. manjavacas</i> HSP70-1	AGEKNILIFDLGGGTFDVSI <b>L</b> TIED----G <b>-I</b> FEVKATA <b>G</b> DT	226
<i>A. vaga</i> HSP70-2A	SGERNILIFDLGGGTFDVSVLKIEE----G <b>-I</b> FEVKSTAGDT	184
<i>C. elegans</i> HSP70A	HGERNVLI <b>F</b> DLGGGTFDVSI <b>L</b> TIED----G <b>-I</b> FEVKSTAGDT	227
<i>Wuchereria</i> HSP70	HGERNVLI <b>F</b> DLGGGTFDVSI <b>L</b> TIED----G <b>-I</b> FEVKSTAGDT	226
<i>Setaria</i> HSP70	HGERNVLI <b>F</b> DLGGGTFDVSI <b>L</b> TIED----G <b>-I</b> FEVKSTAGDT	226
<i>Strongyloides</i> HSP70	ATERNVLI <b>F</b> DLGGGTFDVSVLTIED----G <b>-I</b> FEVKSTAGDT	227
<i>Schistosoma</i> HSP70-2	GGERNVLI <b>F</b> DLGGGTFDVSI <b>L</b> TIED----G <b>-I</b> FEVKSTAGDT	224
<i>M. nipponense</i> HSP70-1	GGERNVLI <b>F</b> DLGGGTFDVSI <b>L</b> TIED----D <b>-I</b> FEVKSTAGDT	226
<i>Mytilus</i> HSP70	GGERNVLI <b>F</b> DLGGGTFDVSI <b>L</b> TIKD----G <b>-I</b> FEVKSTSGDT	227
<i>Tenvia</i> HSP70-1	GGEQNVLIFDLGGGTFDVSI <b>L</b> TIED----G <b>-I</b> FEVKSTAGDT	226
<i>Perinereis</i> HSP70-1	GGERNVLI <b>F</b> DLGGGTFDVSI <b>L</b> TIED----G <b>-I</b> FEVKSTAGDT	225
<i>Lottia</i> HSP70c	GGERNVLI <b>F</b> DLGGGTFDVSI <b>L</b> TIED----G <b>-I</b> FEVKSTAGDT	226
<i>Dugesia</i> HSP70	GIERNVLI <b>F</b> DLGGGTFDVSI <b>L</b> TIED----G <b>-I</b> FEVKSTAGDT	226
<i>Oncorhynchus</i> HSC71	GAERNVLI <b>F</b> DLGGGTFDVSI <b>L</b> TIED----G <b>-I</b> FEVKSTAGDT	226
<i>H. sapiens</i> HSC71	GAERNVLI <b>F</b> DLGGGTFDVSI <b>L</b> TIED----G <b>-I</b> FEVKSTAGDT	226
<i>Pelodiscus</i> HSC70	KGERNVLI <b>F</b> DLGGGTFDVSI <b>L</b> TIDD----G <b>-I</b> FEVKATA <b>G</b> DT	226
<i>H. sapiens</i> HSP70-1B	QGERHVLIFDLGGGTFDVSI <b>L</b> TIDD----G <b>-I</b> FEVKATA <b>G</b> DT	228
<i>H. sapiens</i> HSP70-like1	RGERNVLI <b>F</b> DLGGGTFDVSI <b>L</b> TIED----G <b>-I</b> FEVKSTAGDT	228
<i>X. maculatus</i> HSP70-1	AGEKNVLI <b>F</b> DLGGGTLDVSI <b>L</b> TIED----G <b>-I</b> FEVKSTAGDT	229
<i>Alligator</i> HSP70A		

3

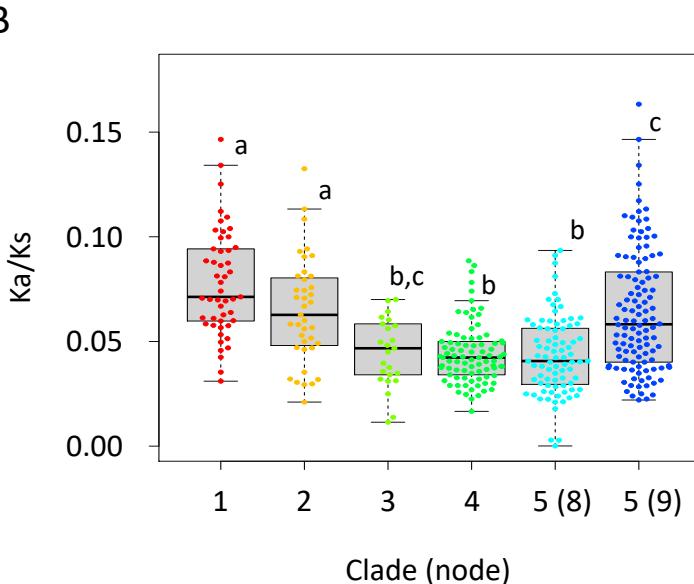
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A



	2	3	4	5 (8)	5 (9)
1	0.51	0.19	1.64	1.55	0.49
2		0.01	1.01	0.98	0.02
3			0.28	0.29	0.01
4				0.05	0.87
5 (8)					0.86

- 1: Invertebrate HSP70s (Lineage A)
  - 2: Rotifera HSP70s (Lineage B)
  - 3: Nematode HSP70s (Lineage B)
  - 4: Invertebrate HSP/HSC70s (Lineage B)
  - 5 (8): Vertebrate HSC70s (Lineage B)
  - 5 (9): Vertebrate HSP70s (Lineage B)

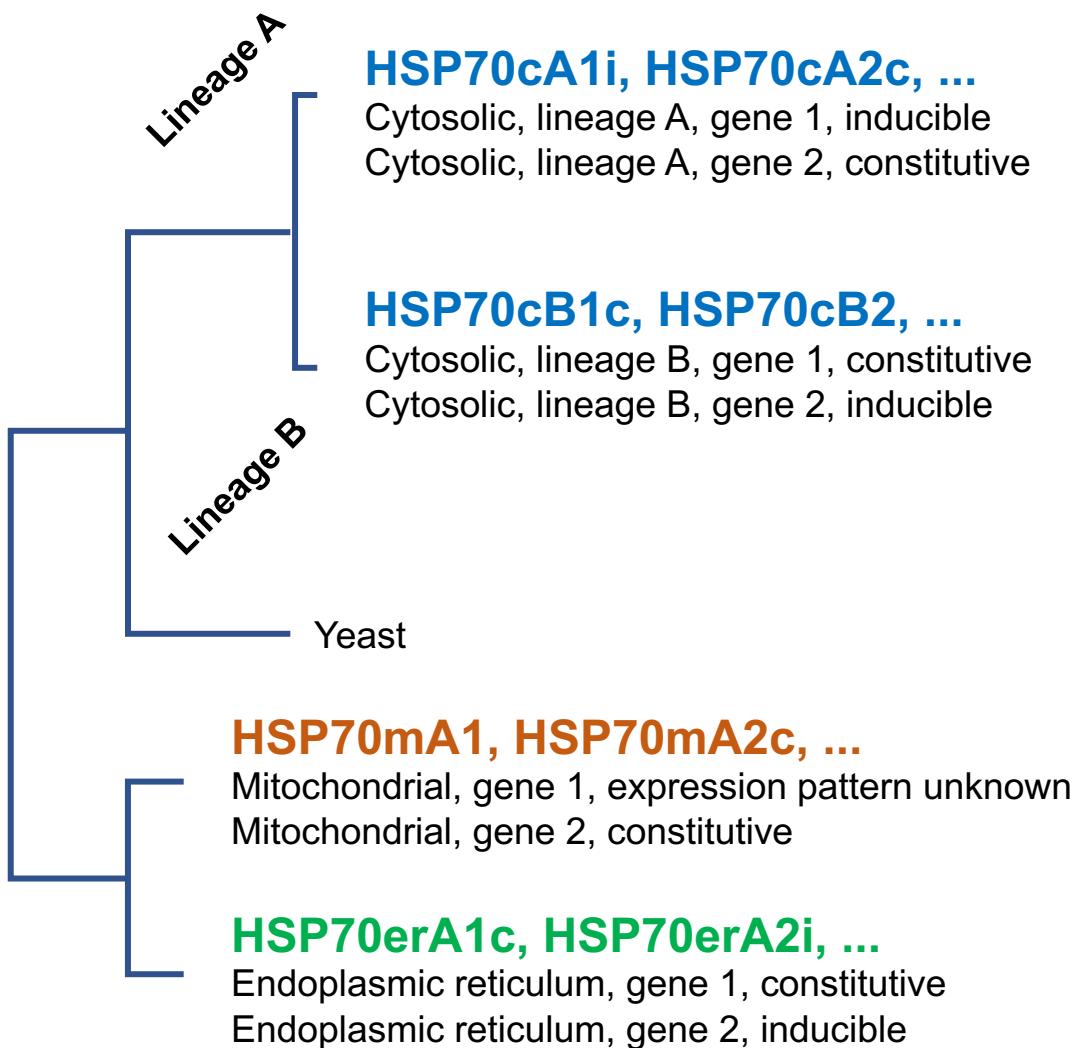


Table 1. Nucleotide sequences of primers used in this study

Primer name	Sequence (5'-3')	Nucleotide position	Gene
<i>cDNA cloning</i>			
rHSP70_5RACE1	AAAAGTCCTGGAGCAGCTT	1107-1125*	HSP70-1, 2
rHSP70_5RACE2	GAACTCCTGACAAAATGGTT	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) <sub>17</sub>	-	-
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	AGTGGAAATAATGCCAACGA	140-160*	HSP70
RT-hsp70-208R	TCGGTGTGGTGAACGCTA	190-208*	HSP70
RT-AC-306F	CGTCCAGTTCTCGTTGGAA	306-325	Actin
RT-AC-373R	CTCGTTGCCAATGGTGATCA	354-373	Actin
<i>In situ hybridization</i>			
rHSP70_insituF	CCATTACAAACACAAATATT	2026-2046*	HSP70-1, 2
rHSP70_insituR	GTCAAACAAATCTTTATC	2085-2103*	HSP70-1, 2

\*Nucleotide positions in HSP70-1 gene

0 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	-	GSADVT0001811001	-	-	-	-	-
<i>B. ibericus</i> HSP70	HSP70cA1i	GU574486.1:1-1860	ADR79281	Whole body	Ultraviolet	Real-time PCR	Inducible	Kim et al., 2011
<i>Priapulus</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>Haliotis</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_2024_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>Ditylenchus</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	1285,1342-1597,1655-1858,1928-2025,2086-2194,2252-2641	gi 1022765301 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	gi 1022763482 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>Tenvia</i> HSP70-1	HSP70cB1	FN860147.1:1-411,757-2100,2515-2709	CBM42052	-	-	-	-	-
<i>Tenvia</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 <sup>2+</sup>	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>Haliotis</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 Guerrero, 1995

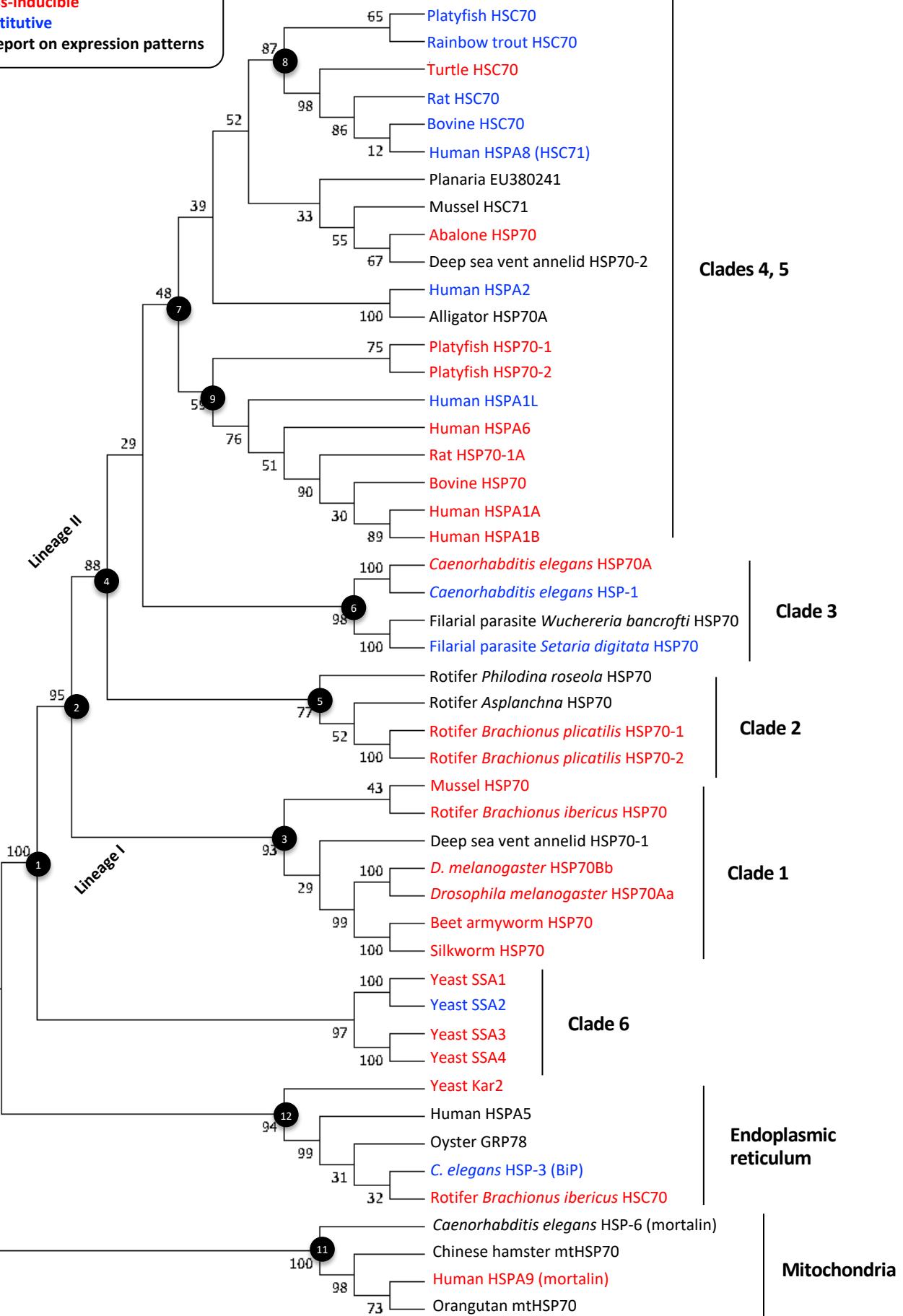
<i>H. sapiens</i> HSP70-1B (HSPA1B)	HSP70cB2i	NM_005346.5:214-2139	NP_005337	-	-	-	Inducible	Uniprot P0DMV8
<i>Rattus</i> HSP70	HSP70cB2i	NM_031971.2:1248-3173	NP_114177	Pancreas	Heat	Western blot	Inducible	Lee et al., 2018
<i>H. sapiens</i> HSP70-like1 (HSPA1L)	HSP70cB3c	NM_005527.4:409-2334	NP_005518	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	Aarimoclonol	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	Ścieglińska 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:c364589-366517	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		NM_001246829.1:1-2040	NP_001233758	-	-	-	-	
<i>A. vaga</i> HSP70mA2	HSP70mA2	-	GSADVT00049157001	-	-	-	-	-
<i>A. vaga</i> HSP70mA1	HSP70mA1	-	GSADVT00003702001	-	-	-	-	-
<i>Daphnia</i> HSC70-5	HSP70mA1	LRGB01000716.1:c3467-3389,c3326-3203,c3078-2880,c2819-2228,c2144-1297	gi 1022772237 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. vaga</i> HSP70erA2	HSP70erA2	-	GSADVT00030804001	-	-	-	-	-
<i>A. vaga</i> HSP70erA1	HSP70erA1	-	GSADVT00008959001	-	-	-	-	-
<i>Daphnia</i> HSC70-4	HSP70erA1	LRGB01000024.1:1105780-1105910,1105969-1106200,1106260-1106476,1106539-1106876,1106942-1107467,1107528-1108063	gi 1022777923 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



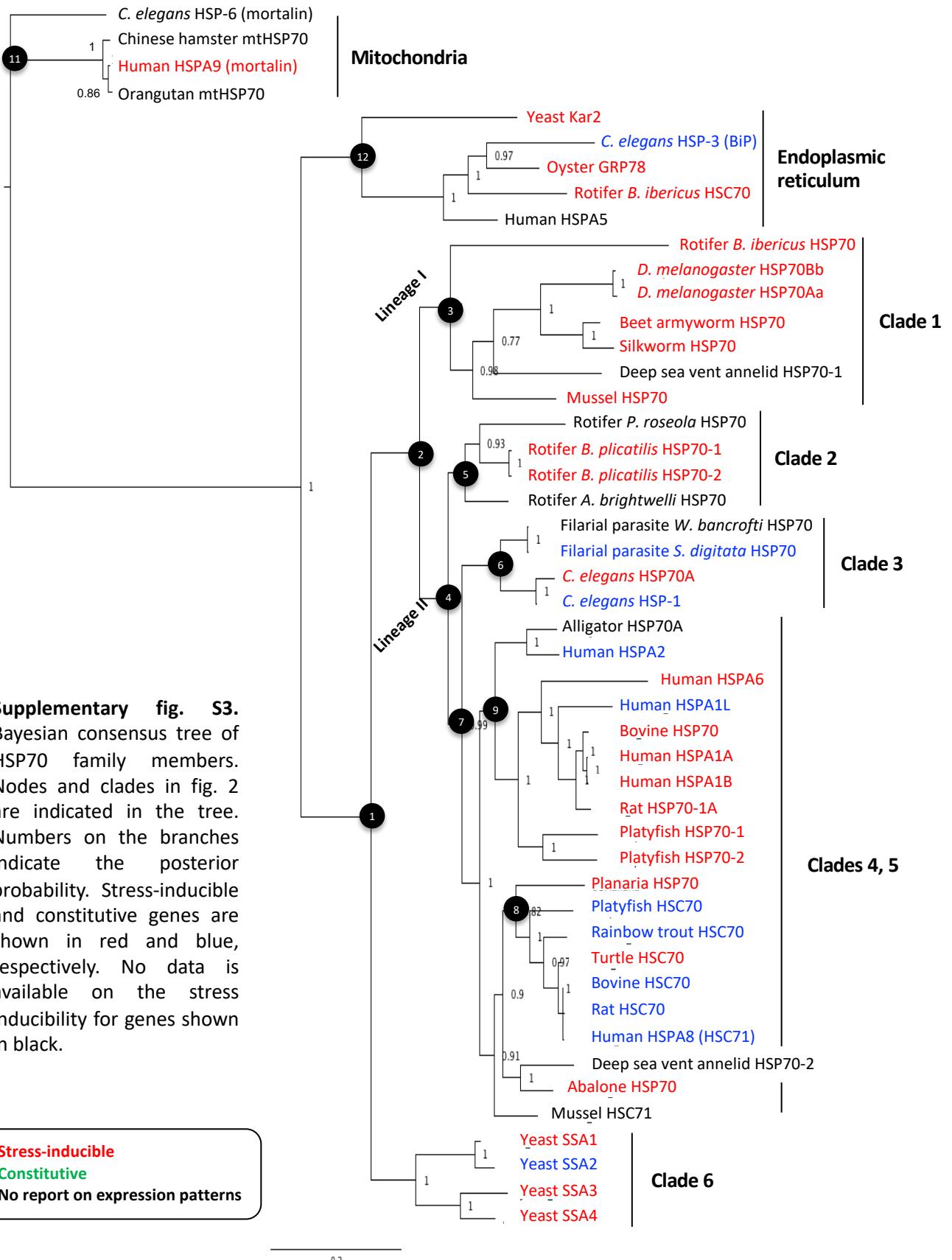
Bp_HSP70-1	HLLGKFELSGIPPAPRGVPQIEVTFDIDANGILNVSAADKSTGKTNKITITNDKGRLSKE	513
Bp_HSP70-2	HLLGKFELSGIPPAPRGVPQIEVTFDIDANGILNVSAADKSTGKTNKITITNDKGRLSKE	513
Bi_HSC70	HLLGKFDLTGIPPAPRGVPQIEVTFEIDANGILKVSAEDKGTGNKNNIVINNNQNRLSPE	536
Bi_HSP70	HLLGNFDLVGIPPAPRGIPQIDVTFDIDANGILSVSAKENSSGKSNNITIKNDKGRLSKE	511
Ab_HSP70	NLLGKFELTGIPPAPRGVPQIEVTFDIDANGILNVSAACDKSTGKQNKITITNDKGRLSKE	512
Pr_HSP70	HLLGNFELSGIPPAPRGVPQIEVTFDIDANGILNVTAVDKSTGRENKITITNDKGRLSKD	511
	:****:*** ****:*****:*****.*:*. :*:*. *:*. *. :**** :	
Bp_HSP70-1	EIDRMVNEAEKYKKDDEQRDKVAAKNSLESYCFNMKQTVED-EKLAAKISADDKKKILD	572
Bp_HSP70-2	DIDRMVNEAEKYKKDDEQRDKVAAKNSLESYCFNMKQTVED-EKLAAKISADDKKKILD	572
Bi_HSC70	EIERMIKDAEKFADEDKVKKEAKVEAKNELEYSYVSLKNQLNDKEKLGNLSEDEKETINS	596
Bi_HSP70	EIERMINEAERFRAEDDRQRDRIASKNKLETYIFAVKQALDD-AK---NLSSSDKKVCQE	567
Ab_HSP70	DIERMVNDAEKYKKDDEQQRERITAKNSLESYCFNMKQTIED-EKLAAKVSADEKKKILD	571
Pr_HSP70	QIEQMVAEAEKYKKDEAQDRRISAKNSLESYCFNMKTSIND-DKIGAKISADDKAKITE	570
	:*:*. :*:*. :*. :*: :**.*:*. :*: * :* * :*: .:*	
Bp_HSP70-1	ACEEALKWLDSNQTAEKDEFEHKMKEVEKICSPIITQLYQGAGGA <u>PGGMPGGM</u> ---PGG	628
Bp_HSP70-2	ACEEALKWLDSNQTAEKDEFEHKMKEVEKICSPIITQLYQGAGGA <u>PGGMPGGM</u> <u>PGGMPGG</u>	632
Bi_HSC70	AVDEKIKWL <u>ESNSDAEVDDFKEQKKALEEIVNPIMTKLYQQNSENA<u>GSSDSQTNDEL</u></u>	653
Bi_HSP70	ACRKELKWLEANQ <u>MADKDEFAFHYKELSRKCMPLMKKIHSGERNGPTVEEVE</u> -----	619
Ab_HSP70	ACENALKWL <u>DANQTAEKDEFEHKLKEIEKTCSPITKLYQG--GAPGGMPGGMPGGAGA-</u>	628
Pr_HSP70	TIESALKWMETN <u>QLAEKDEFEHKLKEVEKICSPIMTKLYGGAEAGGHAG-PGGHAGGA</u> ---	626
	: . :*:*. *: *:*. : * :.. *:***:	
Bp_HSP70-1	MPGGMPGAGPESAGRSGPTIDE <u>EV</u> D	652
Bp_HSP70-2	MPGGMPGAGPESAGRSGPTIDE <u>EV</u> D	656
Bi_HSC70	-----	653
Bi_HSP70	-----	619
Ab_HSP70	--GGAS-----GAGPKIE <u>EV</u> D	642
Pr_HSP70	-PGGS-----KSGGKGPTIE <u>EV</u> D	643

**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).

**Stress-inducible**  
**Constitutive**  
**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.

**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.

Aplysia_HSP70-2	-----	MTKGMKTPAIGIDL	14
Haliothis_HSP70	-----	MAPKGKSPAIGIDL	14
Lottia_HSP70-4	-----	MAPKGPAIGIDL	12
Bplacatilis_HSP70-1	-----	MSKGPAVGIDL	11
Avaga_HSP70eraA	MKIFTALSLIALTTIFTFSSASEDAKKDIGTVIGIDL		37
Bibericus_HSC70	MNKLGLFLLLVLSISSYLNQAKDEKKESYGTVIGIDL		37
BcHSC70-2	-----	GTVIGIDL	8
Celegans_HSP70F	MLSARS-----	FLSSARTIA--RSSLMSARSLSDKPKGHVIGIDL	38
Daphnia_HSP70c5-1	-----		0
Pongo_HSP70m	MISASRAVAARLVGAAASRGPTAACRYQDGWNGLSHEAFRIVSRRDYASEAIKGAVVGIDL		60
Hsapiens_HSP70m	MISASAAAARLVGAAASRGPTAACRHQDSWNGLSHEAFRLVSRRDYASEAIKGAVVGIDL		60

Aplysia_HSP70-2	GTTYS <del>CVGIFQHGKVEIIANDQGNRT</del> TPSYVAFT-DTERLVGDAAKNQAALNPNSNTIFDA	73
Haliotis_HSP70	GTTYS <del>CVGVFQNGQEIIANDQGNRT</del> TPSYVAFT-DTERLIGDAAKNQVALNPQNTVFD	73
Lottia_HSP70-4	GTTFSC <del>CVGVFQH</del> AVEIIANDQGNR <del>T</del> TPSYVAFT-DSERLIGDASAKNQVALNPNSNTIFDA	71
Bplacatilis_HSP70-1	GTTYS <del>CVGVFQHGKVEIIANDQGNRT</del> TPSYVAFT-DTERLIGDAAKNQVAMNPNNTVFD	70
Avaga_HSP70erA	GTTYS <del>CVGIFKNGRVEIIANDQGNR</del> TPSYVAFTAEGERLIGDAAKNQLTSNPENTVFD	97
Bibericus_HSC70	GTTYS <del>CVGVVKNGRVEIIANDQGNR</del> TPSYVAFTSDGERLIGDAAKNQLTSNPENTIFDA	97
BcHSC70-2	GTTYS <del>CVGVVKNGRVEIIANDQGNR</del> TPSYVAFTSEGERLIGDAAKNQLTSNPENTIFDA	68
Celegans_HSP70F	GTT <del>NSCVSIMEGKTPKVIENAEGVR</del> TPSTVAFATADGERLVGAPAKRQAVTN <b>SANTLFAT</b>	98
Daphnia_HSP70c5-1	----- <del>MEGKTPKVIENAEGSR</del> TPSVIAFKTGDGERLAGMPAKRQAVTN <b>AQNTLYAT</b>	51
Pongo_HSP70m	GTT <del>NSCAVMEGKQAKVLENAEGART</del> TPSVAFTADGERLVGMPAKRQAVTN <b>PNNTFYAT</b>	120
Hsapiens_HSP70m	GTT <del>NSCAVMEGKQAKVLENAEGART</del> TPSVAFTADGERLVGMPAKRQAVTN <b>PNNTFYAT</b>	120

Aplysia_HSP70-2	TAAEAYLGQKVTEAVITVPAYFNDSQRQATKDAGAIAGLNLVRMINEPTAAALAYGLDKQ	192
Haliothis_HSP70	TAAEAYLGQQVTDAVITVPAYFNDSQRQATKDAGAIAGLNLVRIIINEPTAAALAYGLDKNL	193
Lottia_HSP70-4	TAAEAYLGENVTNAIVTVPAYFDTSQRQATKDAGVIAGLNLVRIIINEPTAAALAYGLDKKT	190
Bplacatilis_HSP70-1	TAAEAYLGKVKTDAAVTVPAYFNDSQRQATKDAGAIAGLNLVRIIINEPTAAAIAYGLDKKG	189
Avaga_HSP70erA	TAAEAYLGKKVTHAAVTVPAYFNDAQRQATKDAGTIISGMNLVRIIINEPTAAAIAYGLDKK-	215
Bibericus_HSC70	IAEAYLGKPVTHAAVTVPAYFNDAQRQATKDAGTIAGMTVMRIIINEPTAAVYCLRLGQK-	215
BcHSC70-2	IAEAYLGKNTVTHAAVTVPAYFNDAQRQATKDAGTIISGMVMRIIINEPTAAAIAYGLDKK-	186
Celegans_HSP70F	TAESYLGTTVNNAAVTVPAYFNDSQRQATKDAGQISGMLNLVRVINEPTAAALAYGLDKD-	213
Daphnia_HSP70c5-1	TAAEAYLGTPVKNAVAVTVPAYFNDSQRQATKDAGQISGMLNLVRVINEPTAAALAYGMDKS-	166
Pongo_HSP70m	TAENYLGHATAKNAVITVPAYFNDSQRQATKDAGQISGMLNLVRVINEPTAAALAYGLDKS-	235
Hsapiens_HSP70m	TAENYLGHATAKNAVITVPAYFNDSQRQATKDAGQISGMLNLVRVINEPTAAALAYGLDKS-	235

Aplysia_HSP70-2	SGEKNVLIFDLGGGTFDVSVLTIDEGSMFEVKATAGDTHLGGEDFDNRVLVSHFLQEFKRK	252
Haliothis_HSP70	KGEKNVLIFDLGGGTFDVPLTIDEGSMFEVVRSTAGDTHLGGEDFDNRLVEHFLQEFQRK	253
Lottia_HSP70-4	DTEQHILIFDLGGGTFDVSILAIEDG-VFEVVLATAGDTHLGGEDFDNRMVNYFTQEKFKRK	249
Bplacatilis_HSP70-1	GGEKNILIFDLGGGTFDVSILTIIEG-IFEVKSTAGDTHLGGEDFDNRLVNHFVEEFKRK	248
Avaga_HSP70erA	EGEKNILVFDLGGGTFDVSLLTIDNG-VFEVVATNGDTHLGGEDFDQRMVMEHFIKLFKKK	274
Bibericus_HSC70	EGEKNILVFDLGGGTFDVSLLTIDNG-VFEVVATNGDTHLGGEDFDNRVMHEFIKLFKKK	274
BcHSC70-2	EGEKNILVFDLGGGTFDVSLLTIDNG-VFEVVATNGDTHLGGEDFDNRVMHEFIKLFKKK	245
Celegans_HSP70F	AGDKIIAVYDLGGGTFDVSILEI1QKG-VFEVKSTNGDTFLGGEDFDHALVHHLVGEFKKE	272
Daphnia_HSP70c5-1	-DDKIIIAVYDLGGGTFDISILEI1QKG-VFEVKSTNGDTFLGGEDFDNALVNLFVNEFKRD	224
Pongo_HSP70m	-EDKVIAVYDLGGGTFDISILEI1QKG-VFEVKSTNGDTFLGGEDFDQALLRHIVKEFKRE	293
Hsapiens_HSP70m	-EDKVIAVYDLGGGTFDISILEI1QKG-VFEVKSTNGDTFLGGEDFDQALLRHIVKEFKRE	293
	: : : : : * : : * : * : * : * : * : * : * : * : * : : . : * : :	

Aplysia_HSP70-2	HNKDISKNARATRRLRTACERAKRTLSSSEASIEIDSLFE---	GMDYTKISRARFEE	308
Haliotis_HSP70	TRKDISNNTRAMRRLRHTACERAKRTLSSSTEASIEIDSLYE---	GVDFYSKISRARFEE	309
Lottia_HSP70-4	FGKDLSKSSRALRRLRTACERAKRTLSSSAEANIEIDALFE---	GFDFYSKITRARFED	305
Bplacatilis_HSP70-1	NKKDITSNKRALRRLRTACERAKRTLSSAQANIEIDSLHE---	GVDFTSITRARFEE	304
Avaga_HSP70erA	TGKDVKRKDHRAVQKLRREVEKAKRTLSSQHOTKIEIESFFD---	NEDFSETLTRAKFE	330
Bibericus_HSC70	TGKDIRKDNRRAVQKLRREVEKAKRTLSTQFDTRIEIESFFD---	GEDFSETFTRARFEE	330



Aplysia_HSP70-2	SSVMAKLHTQCQSQQGQGSS-----	QGSSGPHGPSV <b>EEMD</b>	639
Haliotis_HSP70	SPVMSKLHNKGCGQQ-----	QQGGSSKGPTV <b>EEMD</b>	636
Lottia_HSP70-4	SPIMTKLHGEANGSTD-PTG-----	RSGGNQGPTV <b>EEID</b>	637
Bplacatilis_HSP70-1	SPIITQLYQGAGGAPGGMPGGMPGGMPGGMPGAGPESAGRSGPT <b>IDEV</b> D	652	
Avaga_HSP70erA	TPIMTKLYGQGGAGAGAGPEDVPPPPHGH <b>DDSL</b> -----	663	
Bibericus_HSC70	NPIMTKLYQQNSENAGSSDSQT <b>NDEL</b> -----	653	
BcHSC70-2	NPIMSKLY <b>QQSG</b> -----	611	
Celegans_HSP70F	LKLFEAAAYKNMAAKNSGGDAQEAKT---AEE-----PKKEQN-	657	
Daphnia_HSP70c5-1	LKLFEMAYKKMAADRESSGGSTNSGDSTGST-----DKKEDKQ	613	
Pongo_HSP70m	LKLFEMAYKKMASEREGSGSSGTGEQ---KE-----DQKEEKQ	679	
Hsapiens_HSP70m	LKLFEMAYKKMASEREGSGSSGTGEQ---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

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-----ATGAGCAAAGGCCCGCTGTAGGCATCGACTTGGCACACGACTCTCGCGTG 54  
-----ATGAGCAAAGGCCCGCTGTAGGCATCGACTTGGCACACGACTCTCGCGTG 54  
-----ATGGCCAAAACAGCGATCGGTATTGATTGGCACACATATTCTCGTGTG 51  
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---ATGTCGGCAAGGGCTCGCATCGACCTGGCACCCACTACTCTCGCGTG 57

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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  
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M\_nipponense\_DQ660140  
Mytilus\_HSP70\_AY861684  
Perinereis\_KU255783  
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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  
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

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Drosophila\_Aa\_AF295933  
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Paralvinella\_EF580992  
B\_plicatilis\_HSP70-1\_AB775784  
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A\_brightwelli\_HSP70\_KX119429  
Wuchereria\_AF167352  
Setaria\_AF079360  
C\_elegans\_HSP70A\_M18540  
C\_elegans\_HSP-1\_NM\_070667  
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*Perinereis* KU255783  
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*Haliotis* FJ812176  
*Oncorhynchus* HSC71\_AAB21658  
*X\_maculatus* HSC70\_AB062115  
*Human* HSPA8\_HSC71\_NP\_006588  
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*Bos* HSP70\_U09861  
*Human* HSPA1A\_NM\_005345  
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*Human* HSPA1L\_NM\_005527  
*Human* HSPA6\_NP\_002146  
*Alligator* HSP70\_AB306279

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*Paralvinella\_EF580992*  
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*B\_plicatilis\_HSP70-2\_AB775785*  
*A\_brightwelli\_HSP70\_KX119429*  
*Wuchereria\_AF167352*  
*Setaria\_AF079360*  
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*C\_elegans\_HSP-1\_NM\_070667*  
*M\_nipponense\_KC460343*  
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*Haliotis\_FJ812176*  
*Oncorhynchus\_HSC71\_AAB21658*  
*X\_maculatus\_HSC70\_AB062115*  
*Human\_HSPA8\_HSC71\_NP\_006588*  
*Rattus\_HSC71\_NM\_024351*  
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*Human\_HSPA1A\_NM\_005345*  
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*X\_maculatus\_HSP70-2\_AB062114*  
*Human\_HSPA1I\_NM\_005527*  
*Human\_HSPA6\_NP\_002146*  
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Drosophila\_Bb\_AF295957  
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Spodoptera\_FJ754276  
Bombyx\_AB035326  
Paralvinella\_EF580992  
B\_plicatilis\_HSP70-1\_AB775784  
B\_plicatilis\_HSP70-2\_AB775785  
A\_brightwelli\_HSP70\_KX119429  
Wuchereria\_AF167352  
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M\_nipponense\_DQ660140  
Mytilus\_HSP70\_AY861684  
Perinereis\_KU255783  
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Halothis\_FJ812176  
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X\_macularatus\_HSC70\_AB062115  
Human\_HSPA8\_HSC71\_NP\_006588  
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Pelodiscus\_HSC70\_HQ219723  
Bos\_HSP70\_U09861  
Human\_HSPA1A\_NM\_005345  
Rattus\_HSP70\_1A\_NM\_031971

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X\_maculatus\_HSP70-1\_AB062113  
X\_maculatus\_HSP70-2\_AB062114  
Human\_HSPA1L\_NM\_005527  
Human\_HSPA6\_NP\_002146  
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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  
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C\_elegans\_HSP70A\_M18540  
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M\_nipponense\_KC460343  
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Mytilus\_HSP70\_AY861684  
Perinereis\_KU255783  
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Haliotis\_FJ812176  
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X\_maculatus\_HSC70\_AB062115  
Human\_HSPA8\_HSC71\_NP\_006588  
Rattus\_HSC71\_NM\_024351  
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Bos\_HSP70\_U09861  
Human\_HSPA1A\_NM\_005345  
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X\_maculatus\_HSP70-1\_AB062113  
X\_maculatus\_HSP70-2\_AB062114  
Human\_HSPA1L\_NM\_005527  
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Drosophila\_BB\_AF295937  
Drosophila\_Aa\_AF295933  
Spodoptera\_FJ754276  
Bombyx\_AB035326  
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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  
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*Setaria* AF079360  
*C. elegans* HSP70A M18540  
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*M. nipponense* KC460343  
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*Perinereis* KU255783  
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*Oncorhynchus* HSC71 AAB21658  
*X. maculatus* HSC70\_AB062115  
*Human* HSPA8 HSC71\_NP\_006588  
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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  
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Human\_HSPA1L\_NM\_005527  
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Bombyx\_AB035326  
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Setaria\_AF079360  
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M\_nipponense\_KC460343  
M\_nipponense\_DQ660140  
Mytilus\_HSP70\_AY861684  
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Haliotis\_FJ812176  
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Bos\_HSP70\_U09861  
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X\_maculatus\_HSP70-1\_AB062113  
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Drosophila\_Bb\_AF295957  
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Drosophila\_Aa\_AF295933  
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Bombyx\_AB035326  
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B\_plicatilis\_HSP70-2\_AB775785  
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Setaria\_AF079360  
C\_elegans\_HSP70A\_M18540  
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Mytilus\_HSP70\_AY861684  
Perinereis\_KU255783  
Paralvinella\_HSP70-2\_EF580993  
Haliothis\_FJ812176  
Oncorhynchus\_HSC71\_AAB21658  
X\_maculatus\_HSC70\_AB062115  
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Spodoptera\_FJ754276  
Bombyx\_AB035326  
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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  
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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  
C\_elegans\_HSP-1\_NM\_070667  
M\_nipponense\_KC460343  
M\_nipponense\_DQ660140  
Mytilus\_HSP70\_AX861684  
Perinereis\_KU255783  
Paralvinella\_HSP70-2\_EF580993  
Haliotis\_FJ812176  
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X\_maculatus\_HSC70\_AB062115  
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X\_maculatus\_HSP70-1\_AB062113  
X\_maculatus\_HSP70-2\_AB062114  
Human\_HSPA1L\_NM\_005527  
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Bombyx\_AB035326  
Paralvinella\_EF580992  
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A\_brightwelli\_HSP70\_KX119429  
Wuchereria\_AF167352  
Setaria\_AF079360  
C\_elegans\_HSP70A\_M18540  
C\_elegans\_HSP-1\_NM\_070667  
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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  
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X\_maculatus\_HSP70-1\_AB062113  
X\_maculatus\_HSP70-2\_AB062114  
Human\_HSPA1L\_NM\_005527  
Human\_HSPA6\_NP\_002146  
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Spodoptera\_FJ754276  
Bombyx\_AB035326  
Paralvinella\_EF580992  
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Wuchereria\_AF167352  
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Mytilus\_HSP70\_AY861684  
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Halictis\_FJ812176  
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X\_maculatus\_HSC70\_AB062115  
Human\_HSPA8\_HSC71\_NP\_006588  
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Drosophila\_Bb\_AF295957  
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Perinereis\_KU255783  
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Halictis\_FJ812176  
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 GGTGCTCCAGGGCTTGGGGCTTCGGGGCCAGGGCGCCCAAGGGAGGC---TCTGGGTG 1899  
 GCGGCAGGAAGCTCCAGCAGTCAAGGTGGAGGAGGCCACT-----AG 1890  
 AGGCCCACTGGGAGCTGCAGAGAGCAGGAGGCCACA-----GCCAG 1893  
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 GCCCCGCCGGCGGGCCCGTCCCGCTCCGCCGGCT-----CCGGC 1893

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  
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 Oncorhynchus\_HSC71\_AAB21658  
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 Human\_HSPA1A\_NM\_005345  
 Rattus\_HSP70\_1A\_NM\_031971  
 X\_maculatus\_HSP70-1\_AB062113  
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 Human\_HSPA6\_NP\_002146  
 Alligator\_HSP70\_AB306279

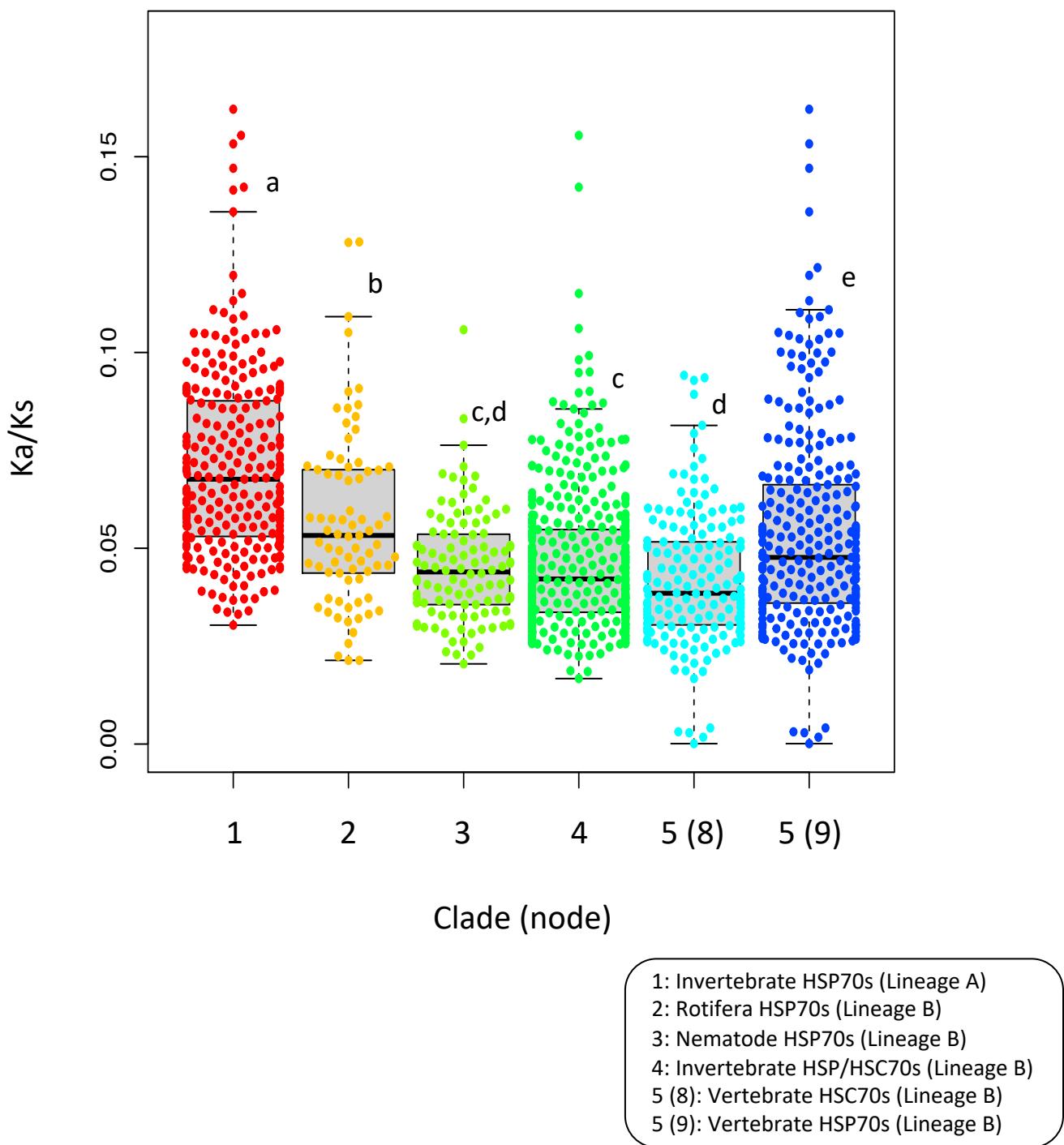
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 CAAGCGAGGAGTGTGGACCCACAGTGGAGGAAGTCGACTAA----- 1923  
 TATGGAAGT----- 1914  
 GGTGGAATG---CCTGGTGCCTGGACCTGAGAGTGC----- 1922  
 GGTGGAATG---CCTGGTGCCTGGACCTGAGAGC----- 1934  
 GGTGGCGCC---TCCGGAGCCGGA----- 1905  
 ACGGGAGGTGGACC----- 1916  
 GGTGGCAGTGGACC----- 1916  
 -----TGGAGGACC----- 1901  
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 GCTCCTG---GT-----GG----- 1913  
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 GGTGCTGGCGGGCAGCTGG-----TTC----- 1925  
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 ----- 1897  
 -----GTGGTGCCTC----- 1910  
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 GGC----- 1902  
 GGC----- 1902  
 GGG----- 1902  
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 GGG----- 1896  
 GGC----- 1902  
 GGC----- 1908  
 GGC----- 1896

Drosophila\_Bb\_AF295957  
 Drosophila\_Aa\_AF295933  
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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  
 Perinereis\_KU255783  
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----- 1926  
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 -----CCTAAAATCGAAGAGGGTGCACTAA----- 1929  
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 -----AACATCGAGGAGGTGCACTAA----- 1923  
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 -----TGGAAAGTGGTGGACCAACATTGAAGAGGTGCACTAA----- 1965  
 -----CCCAGGTGGTGGCCCCACAAATCGAAGAGGTGCACTAA----- 1971  
 -----CGGTGGCAGTGGACCCACCATTAAGGAGGTTGATTA----- 1963

Haliothis_FJ812176	CAGCTCCGGAGGCCACCATCGAGGAGGTTGATTA-	1956
Oncorhynchus_HSC71_AAB21658	-----ATCTGGACCAACCATTGAGGAAGTCGACTAA-	1956
X_maculatus_HSC70_AB062115	-----TAGAGGAGGTCGACTAA-	1914
Human_HSPA8_HSC71_NP_006588	-----CTCAGGGCCCACCATTAAGAGGAGGTTGATTA-	1941
Rattus_HSC71_NM_024351	-----TTCAGGGCCCCACCATTAAGAGGAGGTCGATTA-	1941
Pelodiscus_HSC70_HQ219723	-----ATCTGGACCAACCATTGAGGAAGTAGATTA-	1941
Bos_HSP70_U09861	-----CCCACCATTGAGGAGGTGGATTAG-	1926
Human_HSPA1A_NM_005345	-----CCCACCATTGAGGAGGTAGATTAG-	1926
Rattus_HSP70_1A_NM_031971	-----CCCACCATCGAGGAGGTGGATTAG-	1926
X_maculatus_HSP70-1_AB062113	-----CCCACTGTCGAGGAGGTCGACTAA-	1917
X_maculatus_HSP70-2_AB062114	-----CCCACTGTGGAGGAGGTGGACTGA-	1920
Human_HSPA1L_NM_005527	-----CCCACATTGAAGAAGTAGATTA-	1926
Human_HSPA6_NP_002146	-----CCCATCATTGAGGAGGTTGATTGA-	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 ( $\chi^2 = 200.45$ ,  $df = 5$ ,  $p\text{-value} < 2.2e-16$ ) 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 although this value was used for all statistical analyses.

Ka	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	D. melanogaster HSP70Aa (AF295933)	0.00																													
	3	Spodoptera HSP70 (FJ754276)	0.09	0.09																												
Clade 2	4	Bombyx HSP70 (AB035326)	0.10	0.10	0.03																											
	5	Paralvinella HSP70-1 (EF580992)	0.16	0.16	0.14	0.14																										
Clade 3	6	B. plicatilis_HSP70-1 (AB775784)	0.15	0.15	0.14	0.14	0.15			0.00																						
	7	B. plicatilis_HSP70-2 (AB775785)	0.15	0.15	0.14	0.14	0.15																									
Clade 4	8	A. brightwelli_HSP70 (KX119429)	0.16	0.15	0.15	0.16	0.15			0.06	0.06																					
	9	Wuchereria (AF167352)	0.16	0.15	0.15	0.16	0.16			0.08	0.09	0.09																				
Clade 5 (node 8)	10	Setaria (AF079360)	0.15	0.15	0.15	0.15	0.16			0.08	0.08	0.09	0.01																			
	11	C. elegans HSP70A (M18540)	0.16	0.16	0.16	0.16	0.18			0.09	0.09	0.11	0.05	0.05																		
Clade 5 (node 9)	12	C. elegans 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	M. nipponense 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	0.09	0.08															
Clade 4	14	M. nipponense 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	0.08	0.01														
	15	Mytilus 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	0.07													
Clade 5 (node 8)	16	Perinereis 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	0.06	0.06	0.07										
	17	Paralvinella 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	0.06	0.07										
Clade 5 (node 9)	18	Haliotis 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	0.08	0.06	0.06	0.07										
	19	Oncorhynchus 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	0.06	0.07	0.06	0.07	0.08									
Clade 4	20	X. maculatus 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	0.07	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	0.00							
Clade 5 (node 8)	22	Rattus 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	Pelodiscus 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	Bos 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	0.06	0.00			
Clade 4	26	Rattus 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	0.07	0.01	0.01	0.01	
	27	X. maculatus 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	0.09	0.10	0.07	0.08	0.06	0.06	0.07	0.07	0.07	0.07		
Clade 5 (node 8)	28	X. maculatus 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	0.09	0.10	0.11	0.10	0.08	0.09	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	0.09	0.10	0.10	0.12	0.11	0.09	0.09	0.10	0.11	0.11	0.09	0.10	0.08	0.08	0.08	0.04	0.04	0.04	0.04	0.07	0.07
Clade 5 (node 9)	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	0.13	0.12	0.12	0.12	0.10	0.10	0.07	0.07	0.07	0.12	0.11	0.09	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	0.08	0.09	0.10

Ka/Ks

## 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  $6 \times 10^4$  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 MgCl<sub>2</sub>, 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-μL reaction mixture contained approximately 1 μg of 1st strand cDNA, 10 pmol of rHSP70\_gapF and rHSP70\_gapR, 2 μL 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 μL reaction mixture containing approximately 1 μg of the genomic DNA of *B. plicatilis*, 10 pmol each of the HSP70\_insituf and HSP70\_insitur primers, 2 μL of 10 × 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.

Hothorn T, Bretz F, Westfall P. 2008. Simultaneous inference in general parametric models. *Biom J* 50:346-363.

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