Genome-wide identification and characterization of heat shock protein family 70 provides insight into its divergent functions on immune response and development of *Paralichthys olivaceus*

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Flatfish undergo extreme morphological development and settle to a benthic in the adult stage, and are likely to be more susceptible to environmental stress. Heat shock proteins 70 (*hsp70*) are involved in embryonic development and stress response in metazoan animals. However, the evolutionary history and functions of *hsp70* in flatfish are poorly understood. Here, we identified 15 *hsp70* genes in the genome of Japanese flounder (*Paralichthys olivaceu s*), a flatfish endemic to northwestern Pacific Ocean. Gene structure and motifs of the Japanese flounder *hsp70* were conserved, and there were few structure variants compared to other fish species. We constructed a maximum likelihood tree to understand the evolutionary relationship of the *hsp70* genes among surveyed fish. Selection pressure analysis suggested that four genes, *hspa4l*, *hspa9*, *hspa13,* and *hyou1*, showed signs of positive selection . We then extracted transcriptome data on the Japanese flounder with *Edwardsiella tarda* to induce stress, and found that *hspa9*, *hspa12, hspa4l, hspa13,* and *hyou1* were highly expressed, likely toprotect cells from stress . Interestingly, expression patterns of *hsp70* genes were divergent in different developmental stages of the Japanese flounder. We observed that there was always one or more *hsp70* genes highly expressed in various stages of embryonic development of Japanese flounder, indicating that the *hsp70* genes were constitutive expression in Japanese flounder. Our study provides basic and useful re sources to better understand *hsp70* genes in flatfish .

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Abstract

Flatfish undergo extreme morphological development and settle to a benthic in the adult stage, and are likely to be more susceptible to environmental stress. Heat shock proteins 70 (*hsp70*) are involved in embryonic development and stress response in metazoan animals. However, the evolutionary history and functions of *hsp70* in flatfish are poorly understood. Here, we identified 15 *hsp70* genes in the genome of Japanese flounder (*Paralichthys olivaceus*), a flatfish endemic to northwestern Pacific Ocean. Gene structure and motifs of the Japanese flounder *hsp70* were conserved, and there were few structure variants compared to other fish species. We constructed a maximum likelihood tree to understand the evolutionary relationship of the *hsp70* genes among surveyed fish. Selection pressure analysis suggested that four genes, *hspa4l*, *hspa9*, *hspa13,* and *hyou1*, showed signs of positive selection. We then extracted transcriptome data on the Japanese flounder with *Edwardsiella tarda* to induce stress, and found that *hspa9*, *hspa12, hspa4l, hspa13,* and *hyou1* were highly expressed, likely to protect cells from stress. Interestingly, expression patterns of *hsp70* genes were divergent in different developmental stages of the Japanese flounder. We observed that there was always one or more *hsp70* genes highly expressed in various stages of embryonic development of Japanese flounder, indicating that the *hsp70* genes were constitutive expression in Japanese flounder. Our study provides basic and useful resources to better understand *hsp70* genes in flatfish.

Introduction

Heat shock proteins (HSPs) are a super family of proteins that are induced by physical, chemical and biological stressors in all living organisms from bacteria to humans (Kregel, 2002). They were first discovered as genes that are involved in heat-shock response in the fruit fly *Drosophila melanogaster* (Ritossa, 1962). Based on their roles and expression patterns, HSPs were categorized into two different types: constitutive heat shock proteins (HSCs) that are expressed constitutively, and inducible forms that are expressed in response to certain factors (Boone and Vijayan, 2002). HSCs are expressed early in organismal development and are involved in cellular activity, in contrast, inducible HSPs are involved in the response to harmful circumstances and protect the cell from stress (Angelidis et al., 1991; Whitley et al., 1999). HSPs have also been classified based on their protein molecular weight, where they are divided into HSP90 (83~110 KD), HSP70 (66~78 KD), HSP60 (58~65 KD) and other small molecular weight proteins (Morimoto et al., 1990).

Characterization of HSPs in a species genome will facilitate a better interpretation of how an organism responds to environmental stressors.

HSP70 are the most conserved HSPs across different species (Hunt and Morimoto, 1985; Mayer and Bukau, 2005). HSP70 proteins have a characteristic N-terminal ATPase domain, substrate binding domain and terminal domain (Schlesinger, 1990; Kiang and Tsokos, 1998), the N-terminal ATPase domain, and the substrate binding domain are often more conserved than the C-terminal domain (Munro and Pelham, 1987). Humans, bird, amphibian, zebrafish, catfish and medaka contain 17, 12, 19, 20, 16 and 15 *hsp70* genes, respectively (Song et al., 2015). In previous studies, it was shown that *hsp70* genes play fundamental roles as chaperones involved in maintaining cellular function, they can facilitate protein-folding, regulate kinetic partitioning, and reduce protein aggregation (Gething and Sambrook, 1992; Pratt and Toft, 1997; Parsell and Kowal, 1994; Morimoto et al., 1997; Pratt, 1993).

HSP70 is a well-known stress protein in the aquatic organisms, which is involved in stress response, including thermo tolerance as well as regulating the immune system (Gornati et al., 2004; Poltronieri et al., 2009; Bertotto et al., 2011; Wallin et al., 2002; Tsan and Gao 2009). For example, hyper-thermic treatment of *Penaeus monodon* increases *hsp70* expression and reduces the replication of gill associated virus (GAV) (Vega et al., 2006). In addition, upregulation of endogenous HSP70 in the *Artemia franciscana* (Kellogg) occurs simultaneously when shielding bacterial infection (Sung et al., 2009). Coho salmon infected with *Renibacteriumsal moninarum* shows higher *hsp70* expression in the liver and kidney comparing with uninfected one, highlighting the importance of *hsp70* genes in immune response of fish (Forsyth et al., 1997). Juvenile rainbow trout (*Oncorhynchus mykiss*) infected with *Vibrio anguillarum* has higher *hsp70* expression in hepatic and kidney tissues before showing clinical signs of disease (Paige and George, 2001). Therefore, *hsp70* are important for the immune response of aquatic species against diverse infections.

In addition to its role in cellular function, stress response and immunity, HSPs are also involved in the development of the embryo and extra-embryonic structures (Morange et al., 1984; Voss et al., 2000; Matwee et al., 2001; Louryan et al., 2002; Rupik et al., 2006). Many of HSPs exhibit complex spatial and temporal expression patterns during embryonic development (Krone et al., 1997). For example, mouse embryos treated with anti-HSP70 showed significant reduction in the progression of development (Neuer et al., 1998). Zebrafish have low and constitutive *hsp90a* expression during embryonic development, and these levels increase when the gastrula and later stage embryos exposed to heat (Krone and Sass, 1994). Moreover, *hsp47* shows higher expression in response to stress (Pearson et al., 1996), and is involved in the formation of embryonic tissues in fish through its interaction with procollagen (Krone et al., 1997). Therefore, HSPs play an important role during embryonic development in addition to their basic cellular functions.

Japanese flounder is endemic to the northwestern Pacific Ocean (Minami and Tanaka, 1992). It is the dominant flatfish species in the aquaculture industry because of its rapid growth rate, delicious taste and high nutritional value, becoming an economically important marine species in China, Korea and Japan (Fuji et al., 2006). The genome of Japanese flounder was recently completed (Shao et al., 2017), thereby facilitating the discovery of *hsp70* genes. Here, we identified and characterized the Japanese flounder *hsp70* family and determined whether these genes are involved in stress response to a pathogen, and embryonic development. Comparative genomics between the other closely related species offer a chance to understand the evolutionary relationship of *hsp70* and the selective pressures that affect evolution of these genes. Our study provides insights into the function of *hsp70* in embryonic development and disease defense in Japanese flounder, which may help future improvement of the Japanese flounder for aquaculture.

Materials & methods

Database mining and sequence extraction

A comprehensive search of the sequence database on the NCBI website and Ensemble website was carried out to identify *hsp70* orthologs among six different teleost fish, including: zebrafish, stickleback, medaka, tilapia, platyfish, and tetraodon. Protein sequences of all chosen species were collected, HSP70 proteins were selected from zebrafish according to the accession number, and HSP70 protein sequences from zebrafish were used as queries to search against the Japanese flounder gene set with an intermediate stringency of e-10. Redundant gene sequences were removed by setting the identity value and coverage of the alignment length to 70% and 60%, respectively. All remaining sequences were manually confirmed for the presence of known HSP70 domains using SMART (Schultz et al., 1998; 2000) to remove pseudogenes. When applying a similar method, *hsp70* gene sequences were retrieved from the gene set of other species, including stickleback, medaka, platyfish, tilapia, and tetraodon. The Zebrafish Nomenclature Guidelines were used as a benchmark to name *hsp70* genes in flounder. Furthermore, the isoelectric point (*pI*) of the HSP70 protein was determined using ExPASy (https://www.expasy.org/).

Phylogenetic analyses

To investigate the phylogenetic relationship of *hsp70* genes among the surveyed fish species, the sequences were processed as follows. Protein sequences were aligned using Guidance2 with MAFFT as the MSA algorithm and with 100 bootstrap repeats. Ambiguous sites were manually trimmed while aligning sequences. The multiple sequence alignment was used as input into MAGE7 (Kumar et al., 2016) to construct a phylogenetic tree. The phylogenetic relationships of *hsp70* genes of seven teleost fishes were constructed using the ML method in MEGA7 (Kumar et al., 2016). The maximum composite likelihood model was used in the ML analyses, and a total of

1000 bootstrap replicates were conducted for each calculation. Finally, Evolview was used to visualize the phylogenetic tree (Zhang et al., 2012).

Sequence structure analysis and motif prediction of *hsp70*

To analyze the gene structure of *hsp70* in the Japanese flounder, the GFF file was downloaded from NCBI. The annotation for *hsp70* genes was obtained from the GFF file, and the Gene Structure Display Server of Peking University (Hu et al., 2015) was used to display the intron and exon structure of all *hsp70* genes. To identify the motif of *hsp70* genes, a structural motifs search was conducted using the MEME (Machanick and Bailey, 2011) with the target motif number setting of 15.

Molecular evolution analysis

Protein sequences from each clade in the phylogenetic tree were retrieved and used for multiple sequence alignment with Guidance2 (Sela et al., 2015). Unreliable sites were trimmed in the multiple sequence alignment, and a tree was constructed using IQ-TREE (Nguyen et al., 2014). Codon alignment of protein sequences was converted by pal2nal (Suyama et al., 2006). Using these data, molecular evolution analysis was conducted to measure the selection pressure within each clade, and CODEML program from PAML (Yang, 1997; 2007) was used to estimate the ω value using the branch site model. The aim of the branch-site test was to identify episodic Darwinian selection along a prespecified branch in a phylogenetic tree that impacts only a few codons in the coding sequence of a gene. In this model, we detected genes under positive selection and the corresponding sites with nonsynonymous/synonymous ratio of ω>1 (Yang and Nielsen, 2002; Yang and Reis, 2011; Zhang et al., 2005).

Structure modeling

To better understand the protein structure of genes under positive selection in Japanese flounder, PHYRE2 (Kelley and Sternberg, 2009) was used to predict the protein structure and secondary structure using the default parameter. The sites under positive selection were marked by PyMol 2.0.

Immune response expression profile of *hsp70* genes against *Edwardsiella tarda* infection in Japanese flounder

The RNA-seq data was downloaded from Sequence Read Archive (SRA) database in NCBI with the following accession numbers: SRR5713071, SRR5713072, SRR5713073, SRR5713074, SRR5713075, SRR5713076, SRR5713077, SRR5713078, SRR5713079 and SRR5713080. These data represent Japanese flounder that was challenged with *E. tar* at 0 h, 8 h, and 48 h, as well as a control injected with Ringer's solution (Li et al., 2018). The data was trimmed and the quota

transcripts per million of each gene (TPM) was used to display the expression profile of *hsp70* genes.

Expression pattern of *hsp70* genes during embryonic development of Japanese flounder

The *hsp70* gene expression analysis was conducted during early stages of embryonic development and mature gonads of Japanese flounder. The family of Japanese flounder with the cross of normal female and male are produced and kept in separate units until collecting samples of sperm, oocyte, 4 cell stage, 32 cell stage, 128 cell stage, high blastula stage, low blastula stage, early gastrula stage, late gastrula stage, myomere stage, heart beat stage, and hatched larva stage. RNA-seq was conducted on all the above developmental samples (unpublished data). In addition, raw sequence data of ovary and testis was downloaded from NCBI (accession numbers SRR3509719 and SRR3525051). Gene expression levels were assessed using TPM, and then the R package pheatmap (Kolde et al., 2018) was used to illustrate the expression patterns at different developmental stages.

Results

Identification of *hsp70* superfamily genes

A total of 112 genes were retrieved from seven fish species (Japanese flounder, zebrafish, stickleback, medaka, tilapia, platyfish, and tetraodon), where the number of *hsp70* genes ranged from 9 to 21, depending on the species. There are 9 *hsp70* genes in tetraodon, while tilapia has 21 *hsp70* genes. Fifteen *hsp70* genes (*hspa1a, hspa4a, hspa12a, hsc70, hspa5, hspa9, hspa1b, hspa12b, hspa14, hspa13, hspa4l, hspa4b, hspa8a, hspa8b* and *hyou1*) were identified in the Japanese flounder (**Table1**). All the genes contained the necessary domains of *hsp70*. The length of the corresponding protein ranged from 442 to 1020 amino acids. The p*I* of different genes was variable, ranged from 4.97 to 8.17 (**Table1**).

Phylogenetic analysis of *hsp70* in fish

We next conducted a phylogenetic analysis using 112 *hsp70* genes from seven teleost species (**Fig. 1**). In our analysis, *hsp70* genes were divided into eight sub-clades, which matched the known subfamilies of *hsp70* genes. However, we observed ambiguous separation between *hspa1 hsc70*, *hsp70* and *hspa8*. Not all the fish species had genes from each clade. For example, tetraodon did not contain *hspa14* and medaka did not contain *hyou1*. All the members of the flounder *hsp70* were split into distinct clades and were grouped with the corresponding genes from zebrafish and other fish.

Sequence structure analysis and motif prediction of *hsp70* gene family

In general, *hsp70* genes are variable in length, ranging from 1838 bp to 21276 bp (**Fig. 2**). They have diverse numbers of exons, for instance, *hspa1a* and *hspa1b* contained one exon, *hspa4a,*

hspa4b and hspa4l that belong to the same subfamily had 19 to 23 exons. Other genes within the same subfamily shared similar number of introns and exons. The gene structures of *hsp70* from the seven species included in this study are displayed in **Supplementary Figure 1**. The *hsp70* found in flounder had variable protein motif patterns (**Fig. 3**). Genes *hspa12a* and *hspa12b* contained three motifs, and *hspa1a* and *hspa1b* contained the maximum number of motif (15). The motif compositions of different *hsp70* genes are listed in **Supplementary Figure 2**.

Molecular evolution analysis

Though eight subclade can be found, since *hspa1, hsc70* , *hsp70* and *hspa8* clade shows ambiguous separation, which could not be used for positive selection analysis. We only used data from the other seven *hsp70* subclade genes in Japanese flounder to identify signatures of evolution.We identified four genes, *hspa4l*, *hspa9*, *hspa13* and *hyou1*, as having signatures of positive selection in the Japanese flounder, with $P < 0.05$. Among them, *hspa4l* and *hspa13* contained one positively selected site with posterior probabilities values > 0.95, while *hspa9* contained two positively selected sites. The sites were as follows: the Cys in the protein sequence of gene *hspa4l*, which is the 235th amino acid; the 582th and 587th amino acid Thr are in the protein of *hspa9* gene and His is the 337th amino acid in gene *hspa13*(**Supplementary Table1**).

Protein structure of genes under positive selection

Next, we generated three-dimensional protein structures of HSPA4L, HSPA9, HSPA13, and HYOU1 using PHYRE2. However, we were unable to predict the structure of HSPA9 and HYOU1. The site under positive selection in significant level is marked in the predicted proteins of HSPA4L and HSPA13 (**Fig. 4).** The predicted secondary structure of HSPA4L demonstrates that the Cys under positive selection is located in a α-helix, and the His under positive selection is located in a β-strand in HSPA13 (**Fig. 5**).

Immune response expression profile of *hsp70* genes against *Edwardsiella tarda* infection in Japanese flounder

To test the role of *hsp70* in response to an infection, we analyzed previously generated RNA-seq data of Japanese flounder blood from samples infected with *E. tar*. Overall, the *hsp70* genes showed diverse expression patterns after the *E. tar* infection. Expression levels of *hspa8b, hspa12b, hspa1a, hspa8a, hsc70* and *hspa1b* decreased after 48 hours of treatment with *E. tar*. Other genes, such as *hspa9, hspa12a*, *hspa4l*, *hspa13,* and *hyou1* showed increased level of expression after 48 hours' treatment. Only *hspa4a* had similar expression after 48 hours' treatment (**Fig. 6**). Expression of *hspa1b, hspa4a, hspa9, hspa12a, hspa4l, hspa13* and *hyou1* was dramatically changed in the samples injected with Ringer's solution after 8 h, but expression of

genes *hspa12a*, *hspa13* and *hyou1* returned to the original stage 48 h after injection with Ringer's solution.

Expression pattern in developmental stages of Japanese flounder

We next investigated the expression profile of *hsp70* genes in various developmental stages of the Japanese flounder. We observed significant differences in gene expression based on the developmental stage. Differential expression were observed between the oocyte and sperm, where most *hsp70* genes , like *hspa4l*, *hspa4a*, *hspa9*, *hsc70* and *hspa1a*, in the oocyte had higher expression level than the sperm. Comparing expression of *hsp70* in sperm and testis, some genes, including *hspa4l*, *hspa4a, hspa9*, *hspa13*, *hspa1b* and *hspa8a* had a higher expression level in the testis than sperm. Comparison of expression of ovary and oocyte, some genes, for instance, *hspa1b* and *hspa8a* showed higher expression in the ovary than oocyte, while other genes, for example, *hspa9*, *hsc70*, and *hspa1a* showed the opposite expression pattern. In early embryonic development, from oocyte to high blastula stage, *hspa9, hsc70*, *hspa1a*, *hspa4l* and *hspa4a* had high expression. Interestingly, the expression of these genes decreased from the low blastula stage to hatching stage. In contrast, expression of *hspa8b*, *hspa13, hspa4b* and *hspa8a* increased during the later developmental stages (**Fig. 7**).

Discussion

Studies on HSPs have mainly focused on model organisms such as zebrafish, mouse, and fruit flies (Rupik et al., 2011). With increasing genomic data available for other organisms, more in-depth studies can be carried out in a variety of species. Here, we identified and characterized HSPs at the genome level, then explored the evolution of HSPs and its divergent functions on the immune response and development stage of the Japanese flounder.

The *hsp70* family genes in Japanese flounder were divided into numbers of branches containing genes *hsc70*, *hspa1*, *hspa4*, *hspa5*, *hspa8*, *hspa9*, *hspa12*, *hspa13*, *hspa14,* and *hyou1*. The phylogenetic relationship and topology of *hsp70* were consistent with previous studies (Daugaard et al., 2007), indicating the confidence of the retrieved sequences in species that were included in the study. Most *hsp70* showed similar intron-exon boundary patterns, suggesting that these genes are highly conserved in fish. However, *hspa8a* (17) had doubled the number of exons in the flounder compared to other fish (8), and *hspa4l* from all the other species had about 19 exons, whereas the flounder had 23 exons. Interestingly, we found signatures of positive selection in *hspa4l*, further indicating the evolutionary difference of *hspa4l* between flounder and the other species.

New favorable genetic variants sweep population, which is called positive seletion. (Wagner, 2007; Darwin, 1912). Genes involved in metabolism, stress response and reproduction tend to be

under positive selection (Oliver, et al., 2010; Koester et al., 2013). Among the 15 *hsp70* identified in Japanese flounder, we found signatures of positive selection in four genes, *hspa4l*, *hspa9*, *hspa13* and *hyou1,* using the branch site model in PAML. Genes under positive selection tend to express less than genes subject to neutral or purifying selection, which tend to be expressed in specific tissues or conditions (Hodgins et al., 2016). Purifying and neutral selection tend to affect variants that are deleterious for the organism, and positive selection tend to affect variants that provide an adaptive advantage to the animal (Rocha and Eduardo,
2006). Interestingly, *hyou1* was not expressed at any of the developmental stages.This finding was consistent with previous studies that indicated that genes under positive selection have low expression levels, and are often involved in stress and metabolism-related activities.

The functions of *hsp70* were determined by their cellular location, and intracellular *hsp70* genes protect the cell from stress, while extracellular *hsp70* genes are involved in the immune system (De Maio et al., 2014). For example, *hsp70* can be the cross-presenters of immunogenic peptides in MHC antigens or stimulators that induce innate immune responses (Pockley et al., 2008; Asea et al., 2000). *Aeromonas hydrophila* challenged with *Labeorohita* showed up-regulation of *apg2*, *hsp90*, *grp78*, *grp75,* and *hsc70*, however, *hsp70* was down-regulated upon infection (Das et al., 2015). Here, we used RNA-seq data of the Japanese flounder injecting with *E. tarda* or Ringer's solution, and we found similar expression patterns as previously published studies (Li et al., 2018). However, *hsc70* expression was decreased in Japanese flounder 48 h after injection with *E. tarda*, which was opposite from the expression pattern of *A. hydrophila*, suggesting a species-specific expression pattern of this gene. Interestingly, some genes were up-regulated shortly after injection with Ringer's solution, and returned to the original expression levels after 48 h. However, samples injected with *E. tarda* maintained differences in gene expression even after 48 h. Such divergent expression pattern suggests that some *hsp70* genes are involved in the response to *E. tarda* infection.

Recent studies demonstrated that heat shock proteins play an important role in the sperm–egg recognition and embryonic development (Li and Winuthayanon, 2017; Luft and Dix, 1999). In mouse, *hsp70* is constitutively expressed from the two-cell to blastocyst stages (Hahnel et al., 1986). In this study, from the four-cell stage to high blastula stage, five genes, including *hspa4l*, *hspa4a*, *hspa9*, *hsc70* and *hspa1a*, were highly expressed and then ceased expression in later stages, besides these five genes also shows highly expression in the oocyte cell. A reasonable conclusion of such similar expression pattern between the oocyte cell and early stage of embryonic development is an initial, constitutive burst of *hsp70* expression after boosting the zygotic genome from four cell stage to high blastula stage. From the low blastula stage other genes, *hspa8b*, began to be expressed at a high level, then *hspa13* and *hspa8a,* and *hspa4b* showed highly expresssion in chronological order. Overall, from the beginning of embryonic development to sexual maturation stage, different *hsp70* gene is highly expressed in various developmental stages,

besides, there is always one or more *hsp70* gene expressed in the high-level during all the stages of embryonic development. This kind of expression pattern during the whole embryonic development thus has been proved that *hsp70* genes were constitutive expression in the embryonic development of Japanese flounder.

Conclusions

HSP70 constitutes an important group of proteins that respond to stress. The *hsp70* in the Japanese flounder are divided into eight clades, similar as in other species. Structure analysis of *hsp70* showed that these genes were highly conserved among different species. Four genes were found under positive selection. Genes *hspa9, hspa12a, hspa4l, hspa13,* and *hyou1* were highly expressed in flounders challenged by *E. tarda*, suggesting that these *hsp70* were induced to protect cells from stress. Expression analysis during the developmental stages indicated that *hsp70* were involved in embryonic development of the Japanese flounder in a temporal manner. In conclusion, *hsp70* play important roles in both immune response and embryonic development of the Japanese flounder.

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Author Contributons

C.S. and Z.D. conducted the research; X.H., J.H. and K.L. collected the samples; K.L., C.S., X.L., Q.W. and Z.D. analyzed the data; K.L. and C.S. participated in manuscript writing and revisions. All authors have reviewed and approved the manuscript.

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Table 1:
Summary of *hsp70* genes in the Japanese flounder genome.
p*I* indicates the protein isoelectric point

Supplemental Table 1.
Summary of statistics for detecting selection criteria using the Branch site model.
NP: number of estimated parameters.
LnL: log likelihood score.
LRT $P < 0.5$ indicates the positive selection gene.
Positively selective site > 0.95 indicates positive selection site (significance level: *,0.05; **,0.01).

Figure 1. Phylogenetic tree of *hsp70* from flounder, medaka, tilapia, zebrafish, platyfish, tetraodon, and stickleback.
The color in the background indicates the branch of sub-family and corresponds to the sub-family names marked in the same color as the circle beyond. The hsp70 genes from flounder are marked with a red star.

Figure 2. Intron-exon structure of *hsp70* genes in flounder.
The phylogenetic tree on the left panel was generated using MEGA7 with the Neighbor-joining (NJ) method and 1000 bootstrap replicates. The right of the panel shows exon and intron structure of *hsp70*, where the orange rectangles represent exons, black polylines indicate introns, orange and black line indicates scale.

Figure 3. Schematic representation of conserved motifs in HSP70 proteins.
Each colored box represents a motif and boxes in the same color indicate the same motif.

Figure 4. Multiple alignments of positively selected sites in *hspa4l*(A) and *hspa13*(B).
The amino acid residue in the red square represents the positively selective site. The secondary structure was predicted by PHYER2, and α-helixes were indicated in yellow and β-sheets were indicated in blue. The number on the top indicates the position of the amino acid residue in the protein.

Figure 5. The 3D-structural models of HSPA4L (A) and HSPA13 (B).
The amino acid under positive selection in HSPA4L is indicated in black (Cys 235) and located in an α-helix. The site under positive selection in HSPA13 is indicated in orange(His 337) and located in a β-sheet.

Figure 6. Expression patterns *hsp70* in Japanese flounder.

Each column represents a time point, and each row represents a gene. The relative expression level is indicated by the color bar on the top right. 0h represents the blank control group at the beginning of the experiment, C 8h, and C 48h indicates Ringer's solution control group, whereas E 8h and 48 h indicate a bacteria-challenged experimental group.

Figure 7. Expression profiles of *hspa4l*, *hsp4a*, *hspa9*, *hsc70*, *hspa1a*, *hspa8b*, *hspa13*, *hspa4b*, *hsp8a* and *hspa1b* during the life cycle of the Japanese flounder.

The panel is split into three parts by the three bars on the top, from left to right represents the germ cells, embryonic development stages, and mature gonads. The detailed stages are oocyte, sperm cell, 4 cell stage, 32 cell stage, 128 cell stage, high blastula, low blastula, early gastrula, late gastrula, myomere stage, heart beat stage, hatch stage, testis, and ovary stage. The relative expression level is indicated by the color bar on the top right.

Supplemental Figure 1. Intron-exon structure of *hsp70* genes in studied fish including medaka, platyfish, stickleback, tetraodon, tilapia, and zebrafish.

The orange rectangles represent exons and black lines indicate introns, black polylines indicate introns, orange, and the black line indicates the scale.

Supplemental Figure 2. Motif composition of HSP70 in flounder.

The motif corresponds to Figure 3 of the body page. The same number inside the legend of Figure 3 and this figure indicates the same motif.

Figure 1

Phylogenetic tree of *hsp70* from flounder, medaka, tilapia, zebrafish, platyfish, tetraodon, and stickleback.

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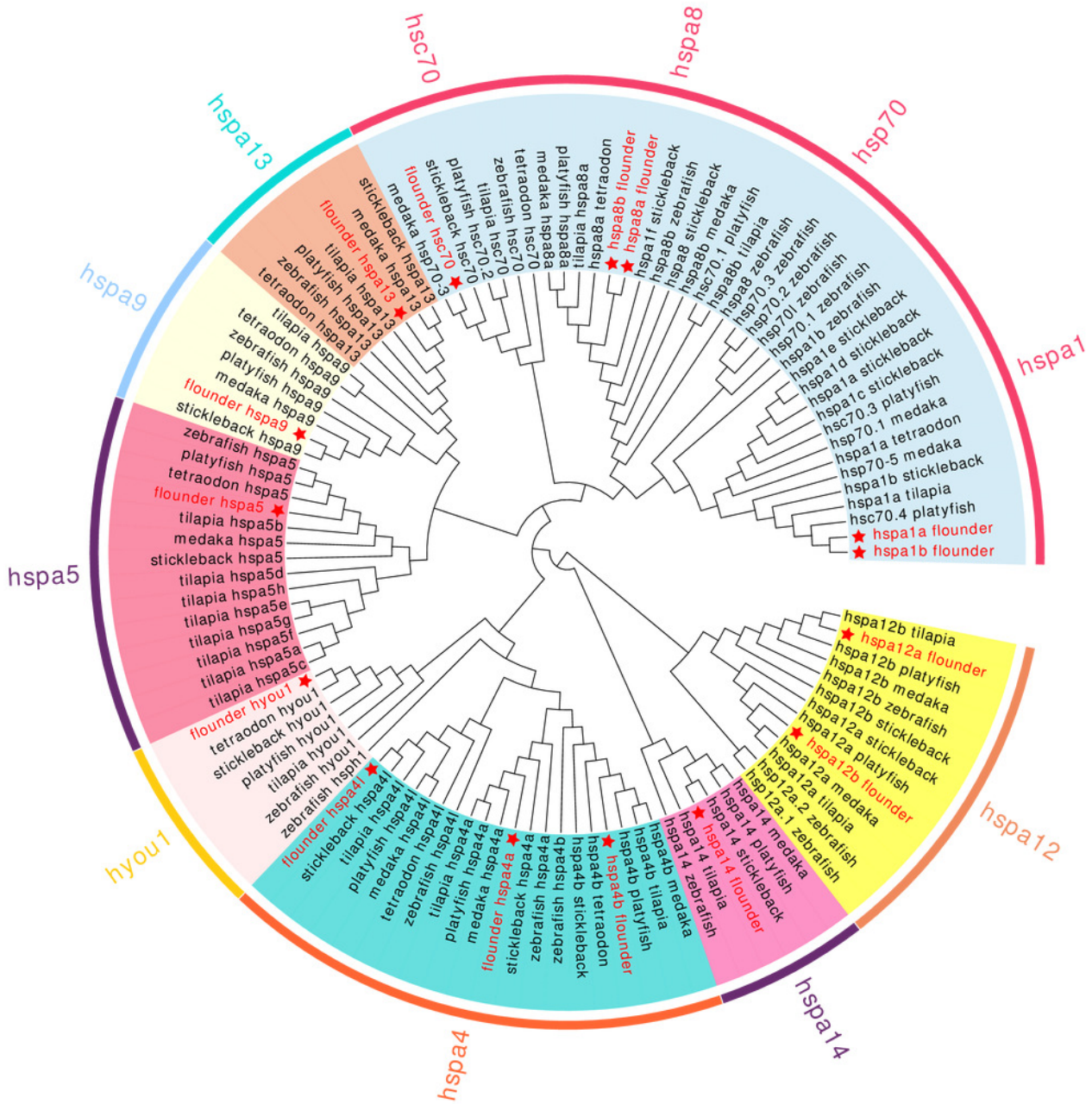
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hsp70
hspa1
hspa12
hspa14
hspa4
hyou1
hspa5
hspa9
hspa13
hspa8a tetraodon
hspa8b flounder
hspa8a flounder
hspa1f stickleback
hspa8b zebrafish
hspa8b stickleback
hsc70.1 medaka
hspa8b tilapia
hspa8 zebrafish
hsp70.3 zebrafish
hsp70.2 zebrafish
hsp70l zebrafish
hsp70.1 zebrafish
hspa1b zebrafish
hspa1e stickleback
hspa1d stickleback
hspa1a stickleback
hspa1c stickleback
hsc70.3 platyfish
hsp70.1 medaka
hspa1a tetraodon
hsp70-5 medaka
hspa1b stickleback
hspa1a tilapia
hsc70.4 platyfish
hspa1a flounder
hspa1b flounder
hspa12b tilapia
hspa12a flounder
hspa12b platyfish
hspa12b medaka
hspa12b zebrafish
hspa12a stickleback
hspa12a platyfish
hspa12b flounder
hspa12a medaka
hspa12a tilapia
hsp12a.2 zebrafish
hsp12a.1 zebrafish
hspa14 medaka
hspa14 platyfish
hspa14 stickleback
hspa14 tilapia
hspa14 flounder
hspa14 zebrafish
hspa4b medaka
hspa4b tilapia
hspa4b platyfish
hspa4b flounder
hspa4b tetraodon
hspa4b stickleback
zebrafish hspa4b
zebrafish hspa4a
stickleback hspa4a
flounder hspa4a
medaka hspa4a
platyfish hspa4a
tilapia hspa4a
zebrafish hspa4l
tetraodon hspa4l
medaka hspa4l
platyfish hspa4l
tilapia hspa4l
stickleback hspa4l
flounder hspa4l
zebrafish hsph1
zebrafish hyou1
tilapia hyou1
platyfish hyou1
stickleback hyou1
tetraodon hyou1
flounder hyou1
tilapia hspa5c
tilapia hspa5a
tilapia hspa5f
tilapia hspa5g
tilapia hspa5e
tilapia hspa5h
tilapia hspa5d
stickleback hspa5
medaka hspa5
tilapia hspa5b
flounder hspa5
tetraodon hspa5
platyfish hspa5
zebrafish hspa5
stickleback hspa9
flounder hspa9
medaka hspa9
platyfish hspa9
zebrafish hspa9
tetraodon hspa9
tilapia hspa9
tetraodon hspa13
zebrafish hspa13
platyfish hspa13
flounder hspa13
tilapia hspa13
medaka hspa13
stickleback hspa13
flounder hsc70
stickleback hsc70
platyfish hsc70.2
tilapia hsc70
zebrafish hsc70
tetraodon hsc70
medaka hspa8a
platyfish hspa8a
tilapia hspa8a

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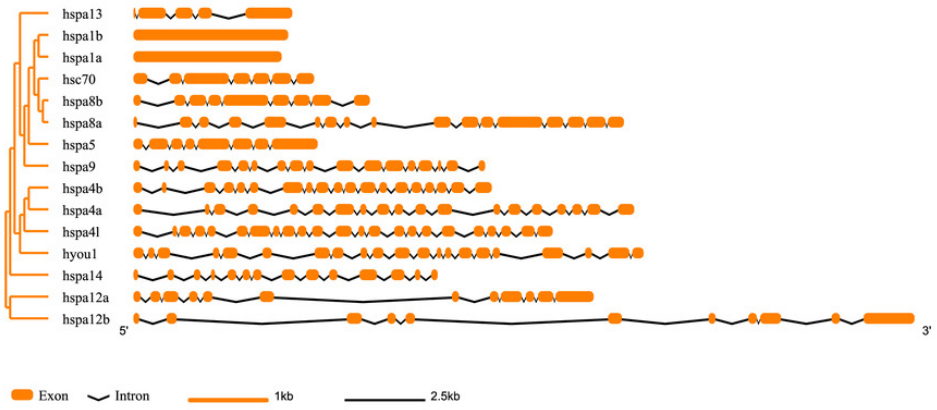
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hsc70
hspa8b
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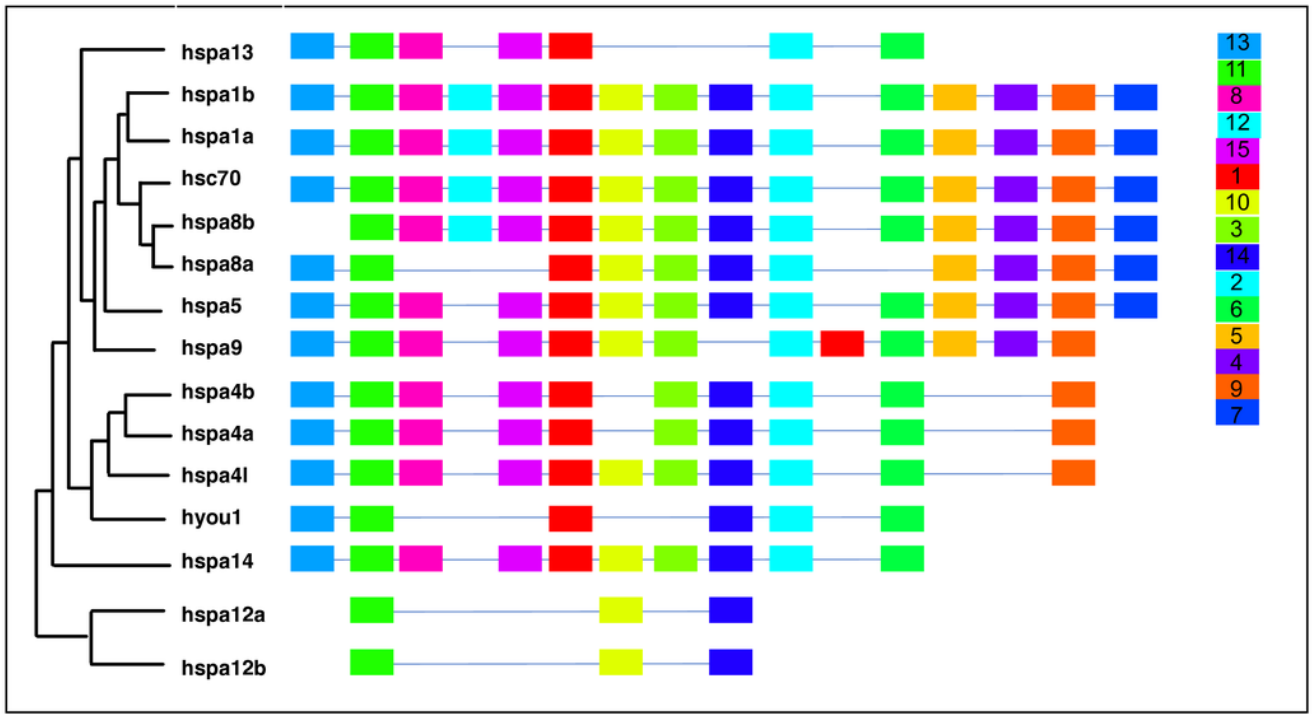
hspa5
hspa9
hspa4b
hspa4a
hspa4l
hyou1
hspa14
hspa12a
hspa12b
5'
3'
Exon
Intron
1kb
2.5kb

Figure 3

Schematic representation of conserved motifs in HSP70 proteins.

Each colored box represents a motif and boxes in the same color indicate the same motif.



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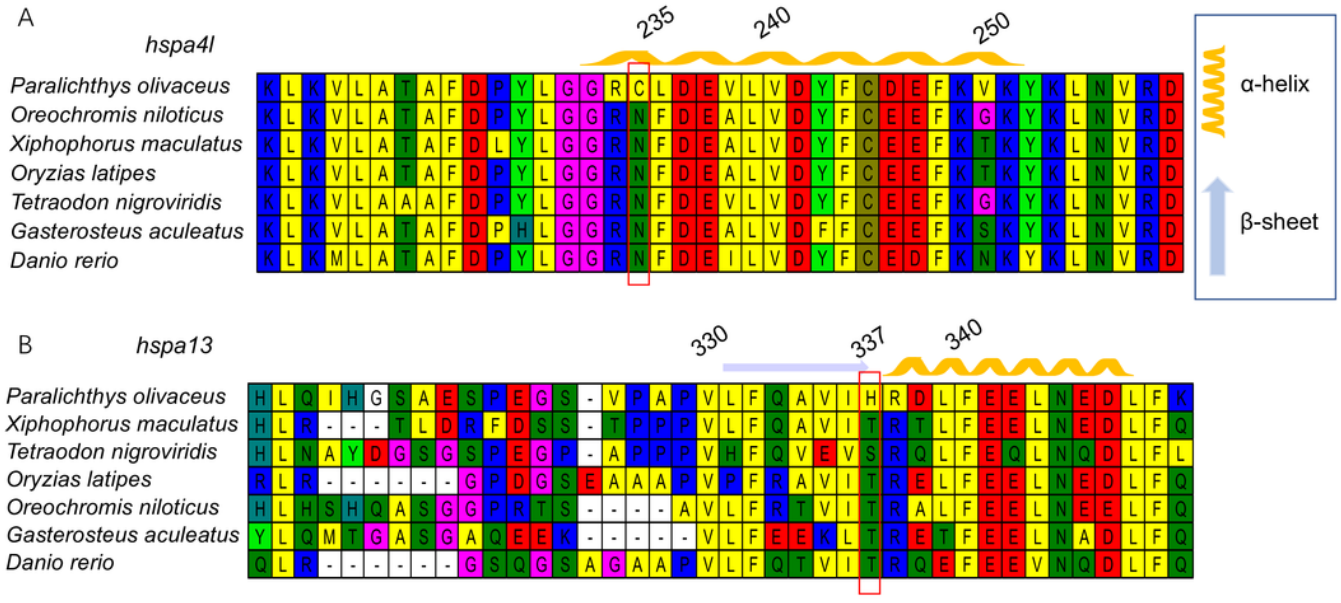

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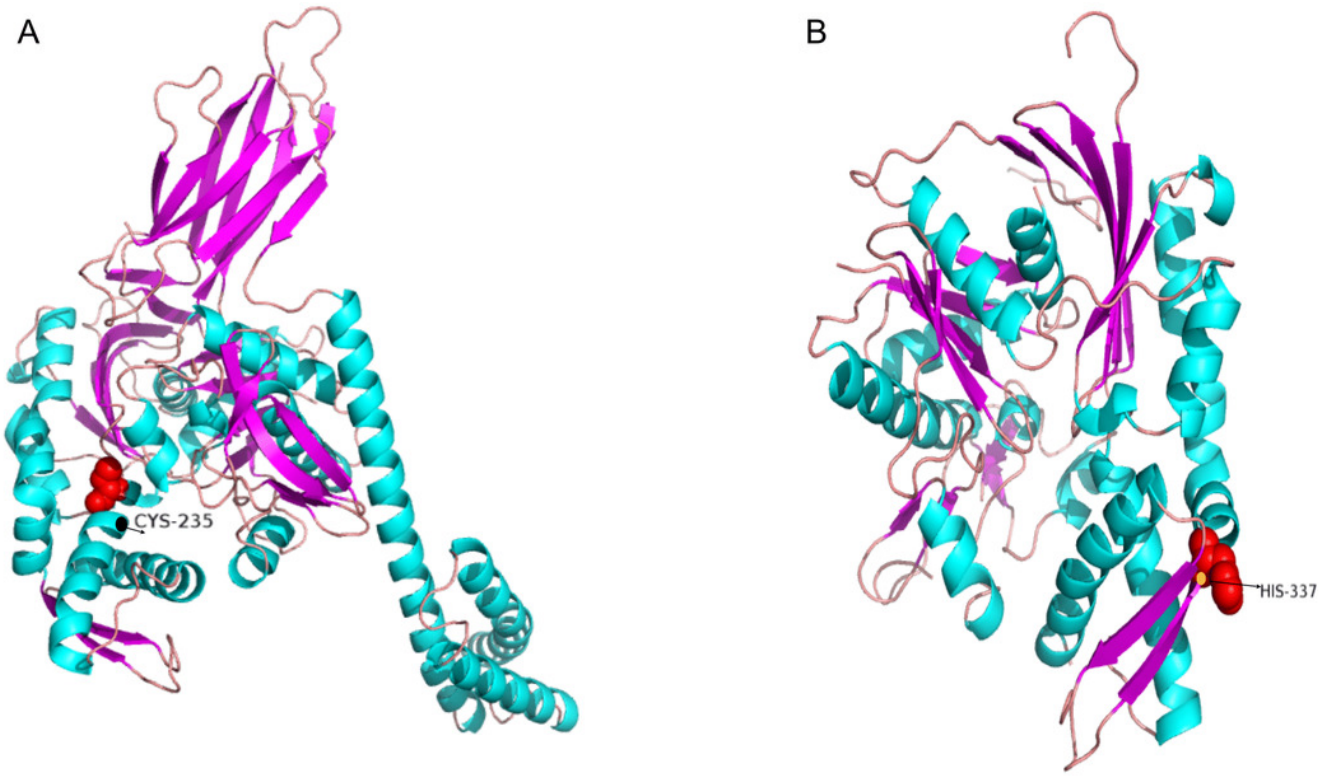

Figure 6

Expression patterns *hsp70* in Japanese flounder

Each column represents a time point, and each row represents a gene. The relative expression level is indicated by the color bar on the top right. 0h represents the blank control group at the beginning of the experiment, C 8h, and C 48h indicates Ringer's solution control group, whereas E 8h and 48 h indicate a bacteria-challenged experimental group.

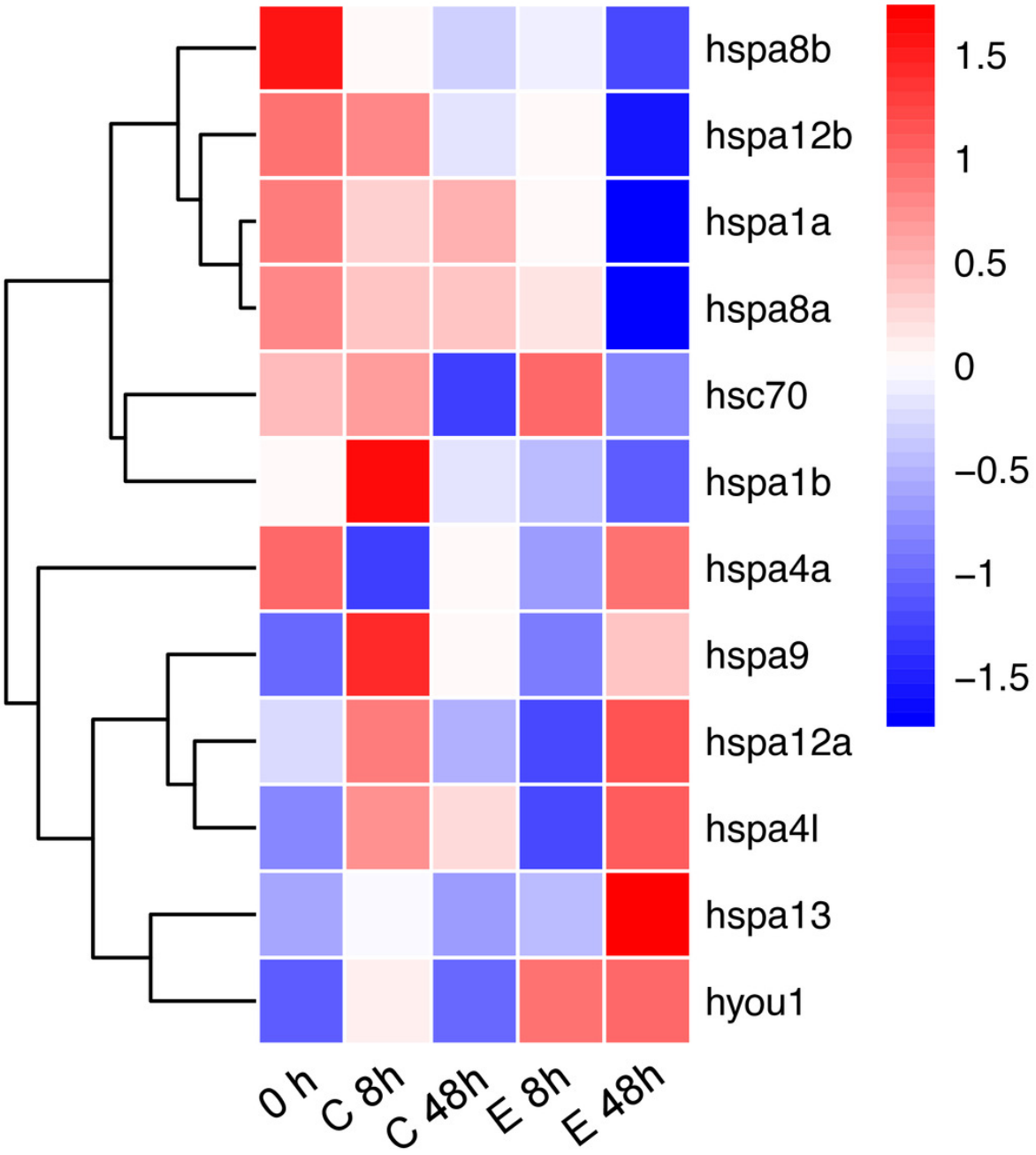
hspa8b
hspa12b
hspa1a
hspa8a
hsc70
hspa1b
hspa4a
hspa9
hspa12a
hspa4l
hspa13
hyou1
0 h
C 8h
C 48h
E 8h
E 48h
1.5
1
0.5
0
−0.5
−1
−1.5

Figure 7

Expression profiles of *hspa4l*, *hsp4a*, *hspa9*, *hsc70*, *hspa1a*, *hspa8b*, *hspa13*, *hspa4b*, *hsp8a* and *hspa1b* during the life cycle of the Japanese flounder.

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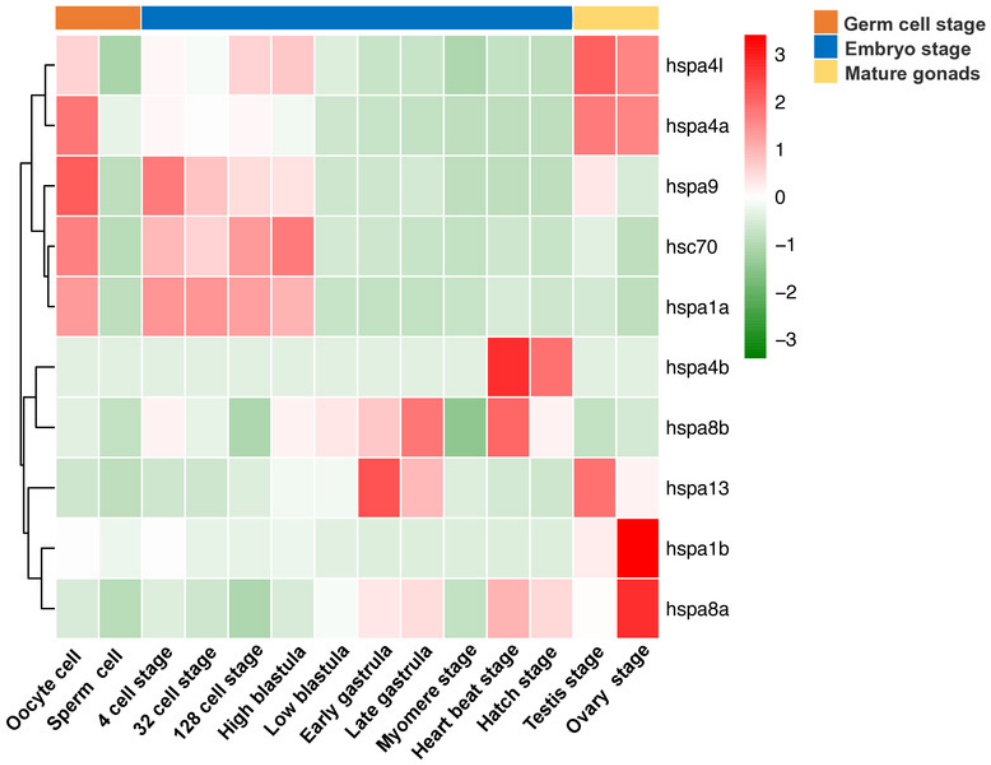

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p*I* indicates the protein isoelectric point

Table 1:

Summary of *hsp70* genes in the Japanese flounder genome

Name	Accession number	Length(a.a.)	p*I*
hspa1a	GS_000036	613	5.31
hspa4a	GS_001017	834	5.13
hspa12a	GS_001337	673	8.17
hsc70	GS_003073	578	5.08
hspa5	GS_008939	654	4.97
hspa9	GS_009710	716	6.23
hspa1b	GS_010306	640	5.42
hspa12b	GS_013272	655	7.30
hspa14	GS_015536	506	5.96
hspa13	GS_016130	442	5.50
hspa4l	GS_017566	1005	5.25
hspa4b	GS_018207	835	4.98
hspa8a	GS_021370	1020	6.47
hspa8b	GS_021371	659	5.32
hyou1	GS_021375	970	5.12

p*I* indicates the protein isoelectric point

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