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The *hsp40* Gene Family in Japanese Flounder: Identification, Phylogenetic Relationships, Molecular Evolution Analysis, and Expression Patterns

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Heat shock proteins (hsps) are cellular chaperones that are involved in developmental stages and stress responses. Hsp40 is the major subfamily of hsps, but has not been fully characterized in Japanese flounder (*Paralichthys olivaceus*), especially their roles in immune response. In this study, a comprehensive identification and analysis of hsp40 in flounder is presented, including gene structures, evolutionary relationships, conserved domains, molecular evolution analysis, and expression patterns. Sequence features and phylogenetic analysis revealed that *hsp40* genes could be grouped into 40 distinct subfamilies and most of them (96%) in Japanese flounder possessed no less than two introns. Molecular evolution analysis indicated that the *hsp40* genes were conservative during evolution and were functional-constrained. Meanwhile, *hsp40* genes were found to express in different embryonic and larval stages and might play the role of sentinel in healthy organisms. Furthermore, *hsp40* genes' expression profiles after *Edwardsiella tarda* injection were determined in Japanese flounder without precedent, and 88% (44/50) of *hsp40* genes showed differential expression patterns after bacterial challenge. Our findings provide basic and useful resources for understanding the immune responsibilities of *hsp40* genes in flatfish.

Keywords: heat shock protein, Hsp40, Japanese flounder, *Edwardsiella tarda*, immune response

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INTRODUCTION

In fisheries, fish are often affected by a variety of stresses during growth and development, including abiotic and biotic stresses such as poor water quality, thermal stress, environmental pollution, osmotic pressure, as well as bacterial and viral infections, which may influence the balance between fish and the environment and cause the stress responses of fish (Chen et al., 2010; Roberts et al., 2010; Zhang et al., 2011; Eissa, 2014; Eissa et al., 2018; Lee et al., 2018; Wen et al., 2019; Xu Z. N. et al., 2019). Over the course of long-term evolution, fish proceed changes in reaction to a variety of stressors, which are named the "general adaptation syndrome" (GAS) (Pickering, 1998). "Cellular stress response" is one feature of GAS that has been little studied.

Heat shock proteins (hsps), first reported in *Drosophila* under heat stress, are a superfamily of stress proteins expressed ubiquitously in most species from bacteria to human beings (Ritossa, 1962; Whitley et al., 1999). Although first discovered in response to thermal stress, hsps were also

found to participate in a great number of stress conditions including radiation (UV), heavy metals, pesticides, hypoxia, oxygen radicals, anti-inflammatory drugs, malignant transformation, bacterial, and viral infection (Fuller et al., 1994; Sørensen et al., 2003; Gehrmann et al., 2004; Multhoff, 2006; Akira et al., 2008). Based on the molecular weight, sequence homology, and domain structures, the hsp superfamily can be classified into several subfamilies, including Hsp90, Hsp70/Hsp110, Hsp10/Hsp60, Hsp40, and Hsp20 (sHsp) families (Gething, 1997).

Hsp40 proteins (also referred to DnaJ proteins) constitute one of the largest subfamilies among the hsp superfamily. Each member of hsp40 proteins contains the J domain (JD), a 70-amino-acid domain with similarity to the initial 73 amino acids of the *Escherichia coli* hsp40 (Georgopoulos et al., 1980; Zylicz et al., 1985). The conserved JD is necessary for hsp40 to bind to hsp70 and regulate the ATPase activity of hsp70 proteins (Ohtsuka and Hata, 2000; Qiu et al., 2006; Li et al., 2009). Except for JD, members of *E. coli* hsp40 proteins typically have three other distinct regions: glycine/phenylalanine-rich region (G/F domain), cysteine-rich region (CRR domain), and variable C-terminal domain (CTD) (Bork et al., 1992; Hennessy et al., 2005; Qiu et al., 2006). Based on the homology of hsp40 proteins of *E. coli*, DnaJ proteins are divided into three categories: Type I DnaJ proteins (DnaJA) possess all four regions of DnaJ protein in *E. coli*; Type II hsp40 proteins (DnaJB) lack the CRR domain; and Type III hsp40 proteins (DnaJC) only possess the JD, which is not necessarily located at N-terminus of the protein (Cheetham and Caplan, 1998; Kampinga et al., 2009). The concept of type IV DnaJ protein family was raised, which owns a "J-like" domain (Walsh et al., 2004; Botha et al., 2007; Morahan et al., 2011) containing a wide range of mutations in a highly conserved histidine, proline, and aspartic acid–HPD motif located between helices II and III in the DnaJ domain (Tsai and Douglas, 1996; Mayer et al., 1999; Hennessy et al., 2000). Cooperating with hsp70, another molecular chaperone that couples the cycles of ATP binding, hydrolysis, and ADP release, the hsp40 proteins are involved in numerous cellular functions, including regulation of protein folding, translocation, and assembly (Cheetham and Caplan, 1998; Ohtsuka and Hata, 2000).

Except for the traditional functions, previous studies suggested that hsps may actually play important roles in immune reactions (Srivastava, 2002; Roberts et al., 2010). Hsps took part in humoral and cellular responses in innate immunity (Sung and MacRae, 2011) and also played a role as the danger signal *in vitro* to communicate innate immune responses by activating various cells (Chen et al., 1999; Kol et al., 2000; Singh-Jasuja et al., 2000). Besides, hsps can induce a variety of cytokines including interleukin-12, nitric oxide, tumor necrosis-a, interleukin-1β, and several chemokines (Basu et al., 2000; Lehner et al., 2000; Moré et al., 2001; Panjwani et al., 2002). Additionally, in adaptive immunity, hsps can also function as powerful danger signals and antigen carriers. For example, Hsp60, Hsp70, and Hsp90 act as ligands for a number of clusters of differentiation and cell-surface receptors (Ohashi et al., 2000; Basu et al., 2001; Vabulas et al., 2001; Habich et al., 2002). Furthermore, the increase of *hsp* gene expressions induced by stressors influences the immune resistance of aquatic animals (Wilhelm et al., 2005; Sung and MacRae, 2011). However, little is known about the participation of *hsp40* genes in the immune reactions of flatfish except the differential expression of three *hsp40* genes in the embryonic cells of Japanese flounder after virus infection (Dong et al., 2006).

Because of various advantages such as rapid growth rate and delicious taste, Japanese flounder is a high-value flatfish in Asian countries including China, Japan, and Korea (Fuji et al., 2006). Nevertheless, the development of industrial farming has caused the Japanese flounder's susceptibility to various pathogens like bacteria, parasites, and viruses, resulting in numerous diseases that are infectious and severe losses in aquaculture recently (Isshiki et al., 2001; Moustafa et al., 2010). Edwardsiellosis is a serious illness caused by *Edwardsiella tarda*, which has led to sizable economic losses all over the world in aquaculture (Hoshina, 1962; Meyer and Bullock, 1973; Yasunaga, 1982; Bang et al., 1992; Nougayrede et al., 1994). Japanese flounder that suffers from Edwardsiellosis often shows various symptoms including a swollen abdomen, pigmentation loss, dermal damages, and spiral movement; as a result, Edwardsiellosis has affected the flounder breeding industry tempestuously (Bang et al., 1992; Moon et al., 2014). Recently, immune responses in different organs of Japanese flounder after *E. tarda* affection have been reported (Takano et al., 2006; Taechavasonyoo et al., 2013; Li et al., 2014; Liu et al., 2017; Thanasaksiri et al., 2017).

Up to now, the systematic efforts to reveal the roles of *hsps* in economically important fish' immune reactions have not been completed. In order to avoid commercial losses caused by *E. tarda* in aquaculture, more in-depth comprehension about the involvement of *hsps* in the resistance to *E. tarda* is of great importance. Since the first discovery of *hsp40* in bacteria, thousands of *hsp40* genes have been identified in prokaryotes and eukaryotes (Yochem et al., 1978; Song et al., 2014; Chen T. et al., 2018; Huang et al., 2018; Xu Y. et al., 2019). Previous researches illustrated that the expression of several *hsp* subfamilies from Japanese flounder could be affected after the infection of several pathogens, e.g., *Streptococcus parauberis* (Dong et al., 2006; Chen et al., 2010; Sung and MacRae, 2011; Cha et al., 2013; Wei et al., 2013). Nevertheless, with respect to the roles of *hsp40* genes in the disease resistance in Japanese flounder, extensive understanding is lacking. In this study, we conducted the genome-wide characterization of 50 *hsp40* genes, including sequence information, selective pressures, and phylogenies. We also determined their expression profiles in embryonic and larval stages in *E. tarda*-infected and healthy tissues, to give early reference about the characteristics of *hsp40* genes in the immune reaction in Japanese flounder.

MATERIALS AND METHODS

Identification of *hsp40* Family Members in Flounder Genome

To identify the *hsp40* genes, the whole genome database of Japanese flounder (NCBI accession number MPLB00000000.1) was searched using available *hsp40* sequences from teleosts [zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), tilapia

(*Oreochromis niloticus*), channel catfish (*Ictalurus punetaus*), yellow catfish (*Tachysurus fulvidraco*), spotted gar (*Lepisosteus oculatus*), and fugu (*Takifugu rubripes*)] from the Heat Shock Protein Database Information Resource (Sinha et al., 2012). The *E*-value was set at 1e-10 to acquire as many candidate *hsp40* genes as possible. Redundant sequences were removed. Afterward, Pfam (El-Gebali et al., 2018) and SMART (Letunic and Bork, 2017) databases were applied to confirm the conserved *hsp40* domain, namely, DnaJ_CXXCXGXG (PF00684), DnaJ_C (PF01556), DnaJ (PF00226), and Pam16 (PF03656), and sequences without any *hsp40* domain were excluded. Newly identified *hsp40* genes of flounder were named following Zebrafish Nomenclature Guidelines and the Guidelines for the nomenclature of the human hsps (Kampinga et al., 2009), and the genes were named after the zebrafish orthologs whenever possible (**Table 1**).

Phylogenetic Tree Conduction of *hsp40* Gene Families

The amino acid sequences of hsps of seven teleosts above and Japanese flounder were used for phylogenetic analysis. The MEGA7 (Kumar et al., 2016) software was used to construct a phylogenetic tree, using parameters of the WAG model and the maximum-likelihood method. Further, we applied the Evolview (Zhang et al., 2012) to visualize the tree.

Sequence Structure and Motif Prediction

We utilized the MEME program (Bailey et al., 2009) to evaluate the motifs in the hsp40 sequences; the parameters were as follows: any number of repetitions, maximum of eight motifs, and an optimum motif width of 6–200 amino acid residues. Then, we used the Gene Structure Display Server (GSDS[1]) to identify exon–intron organizations of the *hsp40* genes from Japanese flounder. Then, TBtools software was applied to construct a diagrammatic sketch (Chen C. et al., 2018). Finally, the ProtParam Tool (Walker, 2005) predicted the biophysical properties of each hsp40 protein.

Molecular Evolution Analysis

To investigate the selective pressure of *hsp40* genes, the relative rates of non-synonymous substitutions (dN) and synonymous substitutions (dS) were used to represent the natural selective pressure of eight different teleosts above (Kryazhimskiy and Plotkin, 2008). According to previous research, a dN/dS ratio of greater than 1 forecasts positive selection and less than 1 forecasts negative selective pressure, whereas a ratio equal to 1 hints neutral selection (Nei and Gojobori, 1986). We applied the ClustalW for codon-based alignment and removed the terminator. Then, the single likelihood ancestor counting (SLAC) method (Kosakovsky Pond and Frost, 2005) in Datamonkey (Delport et al., 2010) was utilized to predict selective pressure on individual codons (sites) within the CDS of the *hsp40* genes. SLAC is good at exploring non-neutral evolution in over 50 sequences, using likelihood-based branch lengths, nucleotide and codon substitution parameters, and ancestral sequence reconstructions (Pond and Frost, 2005).

Subcellular Localization and Secondary Structure Analysis of hsp40 Proteins

The secondary structure of *hsp40* genes in Japanese flounder was predicted by SOPMA (Geourjon and Deleage, 1995) with the following parameters: output width, 70; number of conformational states, four (helix, sheet, turn, and coil); similarity threshold, 8; and window width, 17. The subcellular localization was performed using the amino acid sequences of hsp40 proteins by PSORT (protein subcellular localization prediction tool) (Nakai, 1999).

Expression Profiles in Developmental Stages, Challenged and Unchallenged Tissues

We analyzed the expression profiles of *hsp40* genes in unchallenged Japanese flounder tissues and *E. tarda*-infected Japanese flounder, namely, 11 tissues (heart, spleen, liver, kidney, intestines, muscle, brain, gill, stomach, testis, and ovary) and six embryonic and larval stages (stages 1–6) (NCBI accession number: SRX500343, PRJNA319595).

To analyze the expression levels of *hsp40* mRNA in Japanese flounder blood, gill, and kidney samples during *E. tarda* infection, we applied the RNA-seq data from previous research of our lab (Liu et al., 2017; Li et al., 2018a,b). The individuals for infection provided by the Yellow Sea Aquatic Product Co. Ltd. in China were approximately 1 year old with an average body length of 16.3 ± 1.5 cm (mean ± SD) and an average weight of 70.5 ± 7.9 g (mean ± SD). They were acclimatized in aerated seawater at 19°C for 7 days before injection. The *E. tarda* strain EIB202 was acquired from the Key Laboratory of Microbial Oceanography, Ocean University of China. It was a chloramphenicol, tetracycline, rifampicin, and streptomycin strain isolated from an outbreak in farmed turbot in Shandong province of China (Xiao et al., 2008; Wang et al., 2009). We did not detect pathogenic *E. tarda* from the Japanese flounder before the experiment utilizing primers based on the specific esaV gene of pathogenic pathogen (Tan et al., 2005). We incubated the *E. tarda* strain in Luria–Bertani (LB) medium to mid-logarithmic stage at 28°C and then harvested it by centrifugation and re-suspended it to a final concentration of 2×10^7 colony-forming units (CFU) ml^{-1} in Ringer's solution.

Before the formal injection, we performed two pre-experiments to confirm that the injections did make healthy individuals sick and even die. After we validated that *E. tarda* infections were virulent, the formal injections were performed. The Japanese flounder was randomly spilt into three groups: 60 individuals as bacteria-challenge experiment group (BCEG), 60 in the Ringer's solution control group (RSCG), and 10 as the blank control group (BCG). Japanese flounder in BCEG was injected intraperitoneally with 1 ml of the abovementioned pathogen suspension. Additionally, RSCG individuals were injected with the same dosage of Ringer's solution, and individuals in BCG have not been injected. Then, their kidney,

[1]http://gsds.cbi.pku.edu.cn/

TABLE 1 | Comparison of copy numbers of *hsp40* genes among selected teleosts genomes.

Species	*Ictalurus punetaus*	*Tachysurus fulvidraco*	*Danio rerio*	*Oryzias latipes*	*Takifugu rubripes*	*Oreochromis niloticus*	*Paralichthys olivaceus*	*Lepisosteus oculatus*	Total
dnaja1	3	2	1	2	1	1	2	2	14
dnaja2	2	2	2	2	2	2	1	1	14
dnaja3	2	1	3	2	2	2	1	1	14
dnajb1	3	3	2	3	3	3	2	2	21
dnajb3	1	1	1	1	1	1	1	1	8
dnajb4	1	1	1	1	1	1	1	0	7
dnajb5	2	2	2	2	3	2	2	1	16
dnajb6	2	2	1	2	2	2	2	1	14
dnajb9	2	1	2	2	2	3	2	2	16
dnajb11	2	1	1	0	1	1	1	0	7
dnajb12	2	2	2	2	2	2	2	1	15
dnajb14	1	1	1	1	1	1	0	1	7
dnajc1	1	1	1	1	1	1	0	0	6
dnajc2	1	1	1	1	1	1	1	0	7
dnajc3	2	2	2	2	2	2	2	1	15
dnajc4	1	1	1	1	1	1	1	0	7
dnajc5	4	4	4	4	5	4	2	2	29
dnajc6	1	1	2	1	1	1	1	1	9
dnajc7	0	1	1	2	2	2	2	0	10
dnajc8	0	1	1	1	1	1	1	1	7
dnajc9	1	1	1	1	1	1	1	1	8
dnajc10	1	1	1	1	1	1	1	1	8
dnajc11	0	2	2	2	2	2	2	1	13
dnajc12	1	1	1	1	1	1	1	1	8
dnajc14	0	1	1	1	1	1	1	0	6
dnajc15	1	1	2	1	0	1	1	1	8
dnajc16	2	2	2	2	2	2	2	0	14
dnajc17	0	1	1	1	1	1	1	1	7
dnajc18	1	1	1	1	1	2	1	1	9
dnajc19	2	2	1	1	1	1	1	1	10
dnajc21	1	1	1	1	1	1	1	0	7
dnajc22	1	1	1	1	1	1	1	1	8
dnajc24	1	1	1	1	1	3	1	1	10
dnajc25	1	1	1	1	1	1	1	1	8
dnajc27	1	0	1	1	1	1	1	0	6
dnajc30	1	1	2	2	2	2	2	0	12
dnajgak	0	1	2	1	1	1	1	1	8
dnajpam16	0	1	1	2	1	2	1	1	9
dnajsec63	1	1	1	1	1	1	1	1	8
hscb	1	1	1	1	1	1	1	1	8
Total	50	53	57	57	57	61	50	33	418

gill, and blood samples were extracted and stored in liquid nitrogen until RNA extraction. Three time points (0, 8, and 48 h) were chosen to sample collection at each group: BCG for 0 h (Bl-BC), BCEG for 8 h (Bl-8hE), RSCG for 8 h (Bl-8hC), BCEG for 48 h (Bl-48hE), and RSCG for 48 h (Bl-48hC). We have set the necessary biological duplication, and equal molar ratios of two individuals' RNA were pooled as one replicate for further study. Sequencing libraries were constructed using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (#E7530L, NEB, United States) following the manufacturer's recommendations, and index codes were added to attribute sequences to each sample. Raw reads were cleaned by removing adaptor sequences, low-quality sequences (Sanger base quality < 20), and reads with unknown nucleotides larger than 10%. The TopHat–Cufflinks–Cuffmerge–Cuffdiff pipeline was used to analyze the clean data by using default parameters, and then the parameter fragments per kilobase of transcript per million mapped reads (FPKM) was used to quantify the abundance of assembled transcripts (Trapnell et al., 2012; Ghosh and Chan, 2016; Li et al., 2018b). Thirty sequencing libraries from four groups were

constructed totally. The raw sequencing reads were submitted to Sequence Read Archive (SRA) in NCBI with accession numbers PRJNA359626, PRJNA359627, SRR5713071, SRR5713072, SRR5713073, SRR5713074, SRR5713075, SRR5713076, SRR5713077, SRR5713078, SRR5713079, and SRR5713080. Last, the expression levels in unchallenged (RSCG) and challenged groups (BCEG) were examined to find out further information about *hsp40* genes' differential expression in reaction to *E. tarda* injection (Liu et al., 2017; Li et al., 2018a,b). Then, we applied R package pheatmap to visualize the profiles in different tissues (Kolde, 2018).

RESULTS

Sequence Extraction From Japanese Flounder Genome

We applied 57 zebrafish *hsp40* sequences extracted from the Heat Shock Protein Database Information Resource as a query to search against the Japanese flounder genome. Afterward, SMART and Pfam databases were applied to validate the putative *hsp40* genes by the standard of hsp40 domains (PF00226, PF00684, PF01556, and PF03656). Sequences that were repetitive or lacked any hsp40 domains were deleted. As a result, 50 *hsp40* sequences (*dnaja1*, *dnaja1a*, *dnaja2*, *dnaja3*, *dnajb1*, *dnajb1a*, *dnajb3*, *dnajb4*, *dnajb5*, *dnajb5a*, *dnajb6*, *dnajb6a*, *dnajb9*, *dnajb9a*, *dnajb11*, *dnajb12*, *dnajb12a*, *dnajc2*, *dnajc3*, *dnajc3a*, *dnajc4*, *dnajc5aa*, *dnajc5ga*, *dnajc6*, *dnajc7*, *dnajc7a*, *dnajc8*, *dnajc9*, *dnajc10*, *dnajc11*, *dnajc11a*, *dnajc12*, *dnajc14*, *dnajc15*, *dnajc16*, *dnajc16a*, *dnajc17*, *dnajc18*, *dnajc19*, *dnajc21*, *dnajc22*, *dnajc24*, *dnajc25*, *dnajc27*, *dnajc30*, *dnajc30a*, *dnajgak*, *dnajpam16*, *dnajsec63*, and *hscb*) identified from the Japanese flounder genome were named based on the Guidelines for the nomenclature of the human hsps as well as the Zebrafish Nomenclature Guidelines (**Table 1**).

Phylogenetic Analysis

To evaluate the evolutionary history of the *hsp40* genes collected from eight teleosts above, a phylogenetic tree (maximum-likelihood method, WAG model) was constructed by MEGA7 (**Figure 1**). The 418 *hsp40* genes fell into 40 specific subfamilies: 14 *dnaja1*, 14 *dnaja2*, 14 *dnaja3*, 21 *dnajb1*, eight *dnajb3*, seven *dnajb4*, 16 *dnajb5*, 14 *dnajb6*, 16 *dnajb9*, seven *dnajb11*, 15 *dnajb12*, seven *dnajb14*, six *dnajc1*, seven *dnajc2*, 15 *dnajc3*, seven *dnajc4*, 29 *dnajc5*, nine *dnajc6*, 10 *dnajc7*, seven *dnajc8*, eight *dnajc9*, eight *dnajc10*, 13 *dnajc11*, eight *dnajc12*, six *dnajc14*, eight *dnajc15*, 14 *dnajc16*, seven *dnajc17*, nine *dnajc18*, 10 *dnajc19*, seven *dnajc21*, eight *dnajc22*, 10 *dnajc24*, eight *dnajc25*, six *dnajc27*, 12 *dnajc30*, eight *dnajgak*, nine *dnajpam16*, eight *dnajsec63*, and eight *hscb*. Each of the homologous *hsp40*

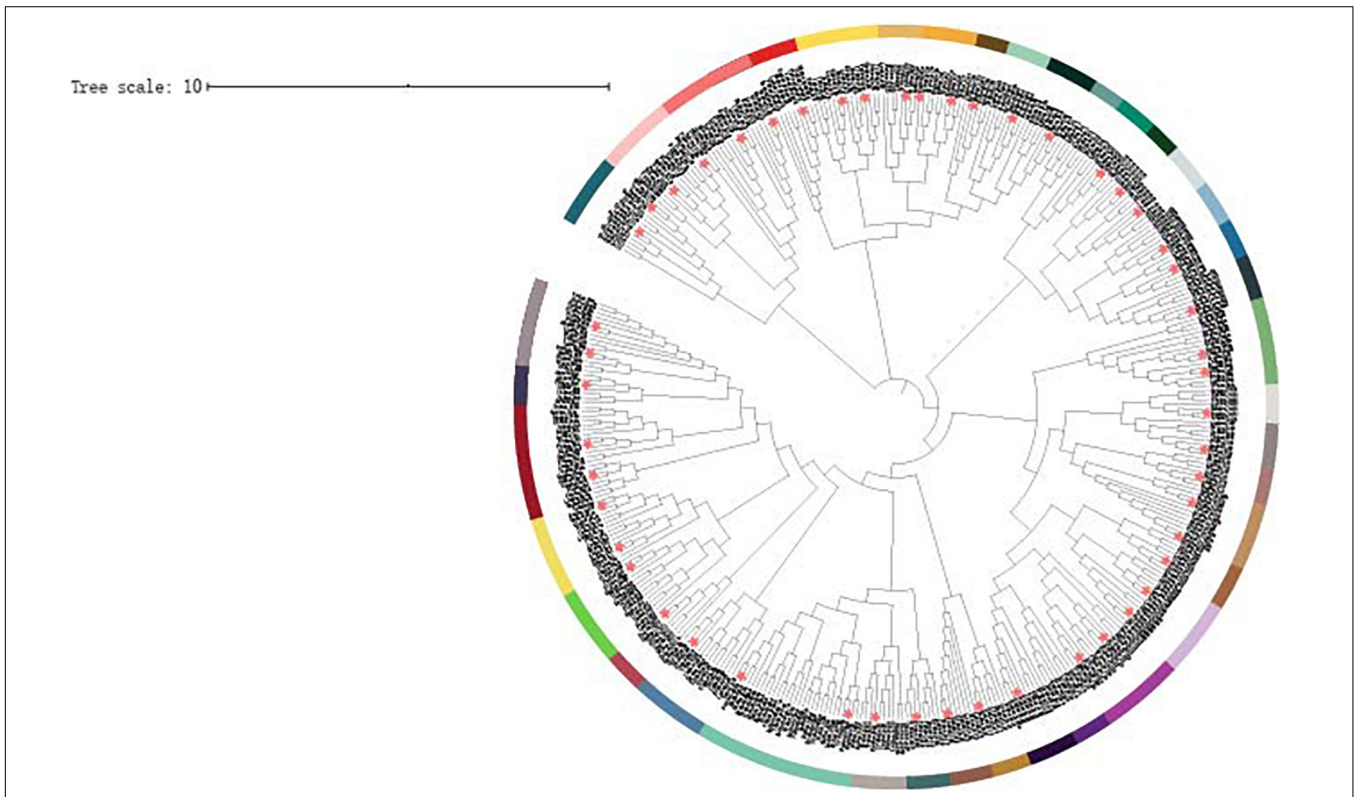

FIGURE 1 | Phylogenetic analysis of 418 *hsp40* genes constructed using ML method and WAG models. Different subfamilies were distinguished in different colors, and the Japanese flounder *hsp40* genes were asterisk labeled. Abbreviations: On, *Oreochromis niloticus*; Tr, *Takifugu rubripes*; Dr, *Danio rerio* Ol, *Oryzias latipes*; Ip, *Ictalurus punetaus*; Tf, *Tachysurus fulvidraco*; Lo, *Lepisosteus oculatus*.

genes from eight teleosts was divided into the same cluster. Furthermore, *hsp40* genes whose protein shows high similarity in structure share a close range in the phylogenetic tree.

Exon–Intron Organizations and Motif Patterns

Gene structure and motif patterns of *hsp40* genes were analyzed to illustrate their similarities and differences and provide deeper comprehension about their evolutionary relationship (**Figure 2**). As for the 50 *hsp40* genes of Japanese flounder, two (*dnajc30* and *dnajc30a)* had no introns, belonging to the "no intron" group, whereas other genes had no less than two introns and were divided into the "multiple introns" group. We identified eight evolutionary-conserved motifs from flounder *hsp40* genes. Results indicated that *hsp40* genes had diverse motif patterns, and genes with close phylogenetic

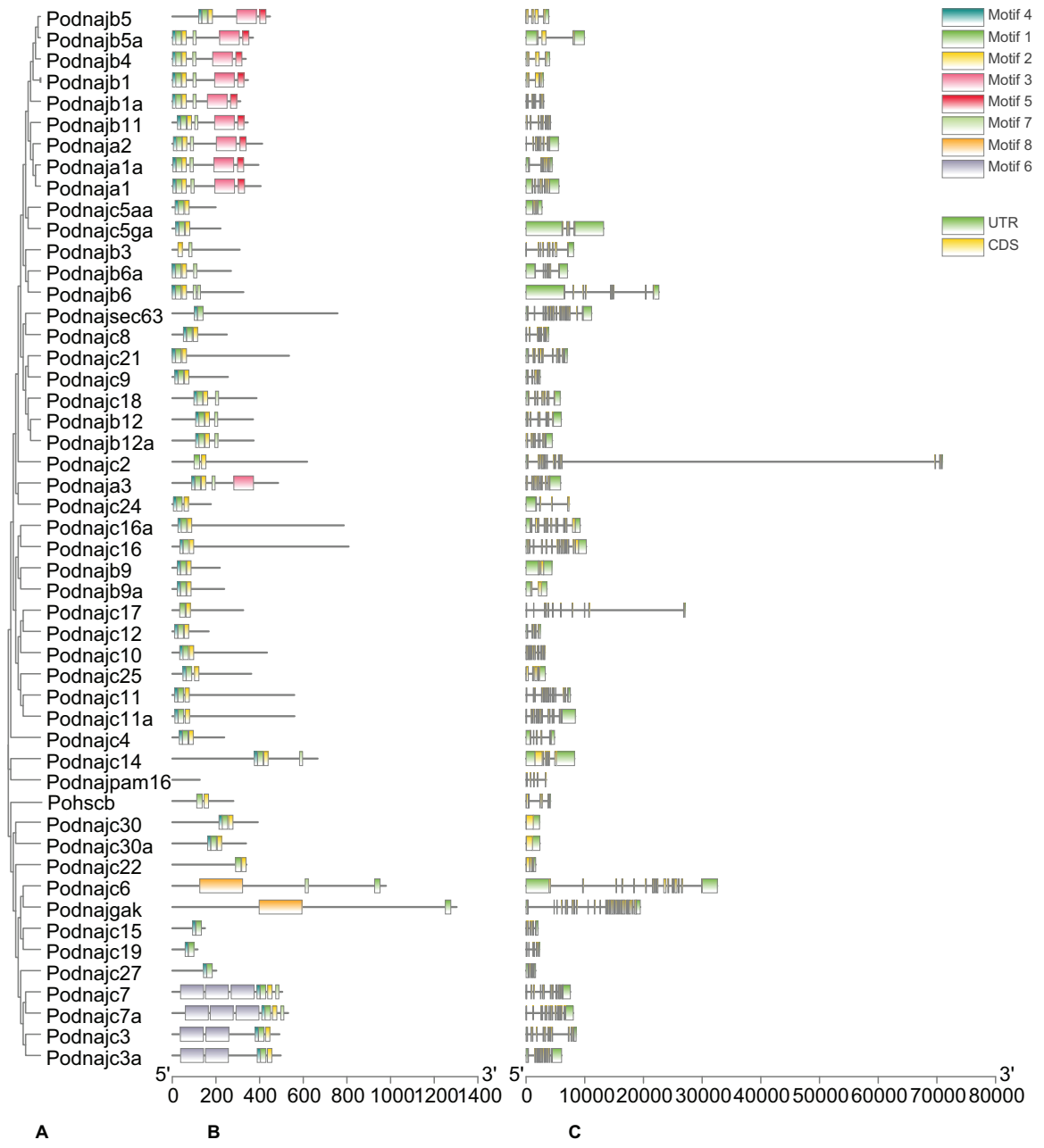

FIGURE 2 | Phylogenetic analysis, exon/intron organization, and motif analysis of *hsp40* genes. **(A)** Phylogenetic tree of 50 hsp40 proteins. The maximum-likelihood phylogenetic tree was constructed using amino acid sequences of 50 hsp40 proteins with MEGA7 software. **(B)** Distributions of conserved motifs in *hsp40* genes. Eight motifs were distinguished in different colored boxes. **(C)** Gene structure of *hsp40* genes. Yellow lines represent CDSs and green boxes with the same length represent UTRs. The length of exons can be inferred by the scale.

relationship had similar motif patterns. Additionally, the biophysical properties are listed in **Table 2**. The amino acid numbers of hsp40 proteins were with the scope of 116 (dnajc19) to 1303 (dnajgak); the molecular masses were between 12.48259 kDa (dnajc19) and 143.23479 kDa (dnajgak), and the pI values are confined from 4.89 (dnajc24) to 10.42 (dnajc30).

Molecular Evolution Analysis of the *hsp40* Genes

In order to get more information about the evolutionary history of *hsp40* gene interspecies, we used the synonymous rate to infer whether fixation of non-synonymous mutations is strengthened or weakened by selection pressure, and the coding sequences of the 50 *hsp40* genes were applied for calculating the dN/dS ratio (**Table 3**). In this study, the dN/dS ratio of all the 50 *hsp40* genes was less than 1, indicating pronounced negative selective pressure. We cannot find any positive selection sites of all the *hsp40* genes from Japanese flounder.

Subcellular Localization and Secondary Structure Analysis of hsp40 Proteins

Subcellular localization analysis showed that the *hsp40* genes of Japanese flounder were mainly expressed in the cytoplasm, nucleus, mitochondria, and endoplasmic reticulum (ER) (**Table 4**), and less expressed in peroxisome, cytoskeleton, and Golgi apparatus. In detail, most *hsp40* genes were expressed mainly in the nucleus, nine genes (*dnaja1a*, *dnaja2*, *dnajb1*, *dnajb1a*, *dnajb4*, *dnajb6*, *dnajc7*, *dnajc11*, and *dnajc27*) were mainly expressed in the cytoplasm, six genes (*dnaja3*, *dnajb9a*, *dnajc10*, *dnajc14*, *dnajc19*, and *dnajc25*) were expressed highly in mitochondria, and four genes (*dnajb11*, *dnajc16*, *dnajc16a*, and *dnajc22*) were mainly expressed in ER. The secondary structure of hsp40 proteins consisted of alpha helix, extended strand, beta turn, and random coil (**Table 4**). Among the 50 hsp40 proteins in Japanese flounder, danjb12 was alpha helix = random coil > extended strand > beta turn, dnajc9 was alpha helix > random coil > beta turn > extended strand, 19 proteins (dnajc2, dnajc3, dnajc3a, dnajc4, dnajc7, dnajc7a, dnajc8, dnajc10, dnajc15, dnajc17, dnajc19, dnajc21, dnajc22, dnajc24, dnajc25, dnajc27, dnajsec63, hscb, and dnajpam16) were alpha helix > random coil > extended strand > beta turn, and the other 29 proteins were random coil > alpha helix > extended strand > beta turn. In summary, the alpha helix and random coil were the main components of the hsp40 secondary structure.

Expression Profiles Analysis

The expression profiles of *hsp40* genes at embryo and larval developmental stages and tissues extracted from healthy individuals and after *E. tarda* injection were illustrated using our previous data. In order to visualize the expression profiles in detail, heat maps with the phylogenetic tree were constructed (**Figure 3**). Results showed that most *hsp40* genes participated in six embryonic and larval stages of Japanese flounder, while three of them (*dnajb1a*, *dnajb5*, and *dnajb12a*) were

TABLE 2 | Features of hsp40 proteins in Japanese flounder identified.

ID	NCBI accession number	Size (aa)	pI	Mw (kDa)
Podnaja2	XP_019934545.1	412	5.91	45.18112
Podnaja1a	XP_019935083.1	395	5.9	44.77599
Podnaja1	XP_019955997.1	405	8.33	45.92839
Podnajb4	XP_019963110.1	337	8.81	37.77469
Podnajb5	XP_019937162.1	448	9.48	50.09499
Podnajb1	XP_019940040.1	347	8.96	38.23021
Podnajb11	XP_019960126.1	346	5.16	39.48552
Podnajb5a	XP_019956370.1	370	8.91	41.8922
Podnajb1a	XP_019941143.1	312	8.67	35.15
Podnaja3	XP_019965729.1	485	9.32	52.23182
Podnajc5aa	XP_019962986.1	199	5.21	22.2869
Podnajb6a	XP_019967712.1	269	7.24	29.63975
Podnajb12	XP_019951913.1	370	8.93	42.16221
Podnajc18	XP_019941514.1	386	8.43	44.52194
Podnajc16a	XP_019944601.1	786	7.13	90.59462
Podnajc10	XP_019968568.1	434	5.98	49.65526
Podnajb12a	XP_019938503.1	373	9.1	42.75287
Podnajc5ga	XP_019965682.1	221	4.91	24.40564
Podnajb6	XP_019943374.1	326	8.35	36.09796
Podnajb9	XP_019934058.1	218	9.08	25.5084
Podnajc16	XP_019942216.1	808	7.21	93.06036
Podnajb9a	XP_019947865.1	238	7.4	27.77144
Podnajc21	XP_019938005.1	535	5.07	62.21628
Podnajc14	XP_019942158.1	666	6.74	74.7478
Podnajc11	XP_019963719.1	559	8.09	63.42023
Podnajc7	XP_019949855.1	504	6.28	57.59444
Podnajc7a	XP_019938771.1	531	6.72	60.31686
Podnajc11a	XP_019941868.1	560	6.98	63.12494
Podnajc9	XP_019952654.1	255	5.34	29.36504
Podnajc3	XP_019945309.1	491	6.64	56.04434
Podnajc3a	XP_019951274.1	497	5.58	56.891
Podnajc24	XP_019934316.1	177	4.89	20.54465
Podnajc4	XP_019965877.1	238	8.96	27.99151
Podnajc25	XP_019963320.1	362	8.76	43.16232
Podnajb3	XP_019945294.1	309	5.28	34.48404
Podnajc30	XP_019964323.1	392	10.42	44.95075
Podnajc27	XP_019967980.1	202	5.73	22.72074
Podnajc30a	XP_019940424.1	338	9	37.44297
Podnajc17	XP_019940273.1	325	8.94	36.936
Podnajsec63	XP_019947337.1	757	5.18	86.6702
Podnajc6	XP_019954137.1	979	7.33	104.25921
Podnajgak	XP_019954890.1	1303	5.69	143.23479
Podnajc12	XP_019945743.1	167	5.4	19.38179
Podnajc15	XP_019966627.1	150	9.8	16.22657
Podnajc19	XP_019959696.1	116	10.12	12.48259
Podnajc22	XP_019944261.1	340	8.79	38.01626
Pohscb	XP_019954647.1	280	5.87	31.79024
Podnajc8	XP_019958083.1	250	9.14	29.70666
Podnajpam16	XP_019963217.1	126	9.52	13.8867
Podnajc2	XP_019955430.1	618	6.8	71.15799

TABLE 3 | Selection pressure of *hsp40* genes (dN/dS) in teleosts.

Gene	dN/dS	No. of positive sites	No. of negative sites
dnaja1	0.135	0	131
dnaja1a	0.11	0	71
dnaja2	0.0551	0	201
dnaja3	0.148	0	179
dnajb1	0.103	0	142
dnajb1a	0.117	0	140
dnajb3	0.259	0	82
dnajb4	0.0557	0	122
dnajb5	0.0911	0	109
dnajb5a	0.139	0	70
dnajb6	0.159	0	103
dnajb6a	0.142	0	51
dnajb9	0.135	0	77
dnajb9a	0.182	0	51
dnajb11	0.0798	0	86
dnajb12	0.14	0	123
dnajb12a	0.168	0	79
dnajc2	0.1	0	174
dnajc3	0.129	0	152
dnajc3a	0.102	0	114
dnajc4	0.0506	0	194
dnajc5aa	0.0864	0	29
dnajc5ga	0.118	0	16
dnajc6	0.127	0	272
dnajc7	0.0622	0	105
dnajc7a	0.109	0	26
dnajc8	0.0809	0	89
dnajc9	0.171	0	76
dnajc10	0.144	0	270
dnajc11	0.0624	0	224
dnajc11a	0.0642	0	136
dnajc12	0.227	0	40
dnajc14	0.21	0	97
dnajc15	0.137	0	30
dnajc16	0.141	0	213
dnajc16a	0.152	0	192
dnajc17	0.182	0	85
dnajc18	0.123	0	128
dnajc19	0.0812	0	44
dnajc21	0.198	0	105
dnajc22	0.212	0	52
dnajc24	0.251	0	35
dnajc25	0.128	0	99
dnajc27	0.0185	0	82
dnajc30	0.375	0	34
dnajc30a	0.379	0	18
Dnajgak	0.117	0	349
dnajpam16	0.0991	0	45
dnajsec63	0.065	0	280
Hscb	0.329	0	60

expressed only at a certain part of developmental stages. Furthermore, there were six genes (*dnaja2*, *dnajb3*, *dnajc3a*, *dnajc4*, *dnajc5aa*, and *dnajc19*) that showed high expression levels at all stages and 12 genes (*dnaja1*, *dnaja1a*, *dnaja3*, *dnajb6a*, *dnajb9*, *dnajb11*, *dnajb12*, *dnajc8*, *dnajc9*, *dnajc10*, *dnaj16*, and *dnajc21*) had relatively high expression levels at one or several developmental stages. Under normal conditions, all *hsp40* genes were expressed to maintain homeostasis, as a reserve in case of sudden pathogen invasion. The expression of *hsp40* genes in different tissues had various patterns, and some genes showed preferential expression in certain tissues. For instance, several *hsp40* genes were highly expressed in one specific tissue, but had low expression levels or not expressed in other tissues, namely, *dnajc22* in the heart, *dnajc3a* in the gill, *dnaja3* and *dnajc21* in the muscle, *dnajb11* in the stomach, *dnajb6* and *dnajc7* in the intestines, and *dnajb5*, *dnajb12a*, *dnajc5aa*, *dnajc6*, and *dnajc11a* in the brain. Additionally, we also found that *dnajb1a* had weak expression levels in 11 tissues, which required deeper studies.

To investigate the role of *hsp40* family members in response to *E. tarda*, we analyzed the expression levels of the 50 *hsp40* genes from three tissues and three time points after *E. tarda* infection from the RNA-seq data of previous research (**Figure 4**); the summary of the comparison of Ringer's solution and bacterial-challenge group, namely, log2(fold_change) and *p*-value, is shown in **Supplementary Table 1**. In total, 41 *hsp40* genes except *dnajb1a*, *dnajb4*, *dnajb5*, *dnajb6a*, *dnajb12a*, *dnajc5aa*, *dnajc6*, *dnajc22*, and *dnajc30* were significantly influenced by Ringer's solution and the differential expression showed a tissue- and gene-specific pattern. After the injection with Ringer's solution in the blood, the expression of eight genes (*dnaja1*, *dnajb5a*, *dnajc3*, *dnajc16a*, *dnajc18*, *dnajc24*, *hscb*, and *dnajsec63*) was significantly influenced at 8 h and the amount of 10 genes' transcripts (*dnaja3*, *dnajb9*, *dnajb9a*, *dnajc4*, *dnajc7*, *dnajc11a*, *dnajc12*, *dnajc15*, *dnajc18*, and *dnajc25*) was not influenced until 48 h, whereas that of eight genes (*dnaja1a*, *dnajb1*, *dnajb3*, *dnajb12*, *dnajc5ga*, *dnajc9*, *dnajc10*, and *dnajgak*) was influenced from 8 to 48 h. After Ringer's injection in the gill, gene expression of 11 genes (*dnaja2*, *dnajb9a*, *dnajc10*, *dnajc11*, *dnajc14*, *dnajc15*, *dnajc16*, *dnajc17*, *dnajc18*, *dnajc21*, and *dnajc30a*) was prominently influenced at 8 h whereas the expression of eight genes (*dnaja1a*, *dnajb1*, *dnajb9*, *dnajc2*, *dnajc12*, *dnajc19*, *dnajgak*, and *hscb*) was influenced at 48 h. In addition, gene expression of 13 genes (*dnaja3*, *dnajb3*, *dnajb6*, *dnajb11*, *dnajc3*, *dnajc3a*, *dnajc5ga*, *dnajc7a*, *dnajc8*, *dnajc25*, *dnajc27*, *dnajsec63*, and *dnajpam16*) was influenced by Ringer's solution throughout all the time points investigated. After the injection with Ringer's solution in the kidney, the expression of 11 genes (*dnaja2*, *dnajb9a*, *dnajc10*, *dnajc11*, *dnajc14*, *dnajc15*, *dnajc16*, *dnajc17*, *dnajc18*, *dnajc21*, and *dnajc30a*) was up- or downregulated at 8 h, whereas that of eight genes (*dnaja1a*, *dnajb1*, *dnajb9*, *dnajc2*, *dnajc12*, *dnajc19*, *dnajgak*, and *hscb*) was influenced at 48 h. The expression of 13 genes (*dnaja3*, *dnajb3*, *dnajb6*, *dnajb11*, *dnajc3*, *dnajc3a*, *dnajc5ga*, *dnajc7a*, *dnajc8*, *dnajc25*, *dnajc27*, *dnajsec63*, and *dnajpam16*) was influenced from 8 to 48 h.

TABLE 4 | Subcellular localization and secondary structure of hsp40 proteins.

Gene name	Alpha helix	Extended strand	Beta turn	Random coil	Subcellular localization
Podnaja2	106 (25.73%)	67 (16.26%)	40 (9.71%)	199 (48.3%)	Cytoplasmic
Podnaja1a	99 (25.06%)	74 (18.73%)	33 (8.35%)	189 (47.85%)	Cytoplasmic
Podnaja1	108 (26.67%)	74 (18.27%)	37 (9.14%)	186 (45.93%)	Nuclear
Podnajb4	79 (23.44%)	63 (18.69%)	27 (8.01%)	168 (49.85%)	Cytoplasmic
Podnajb5	110 (24.55%)	91 (20.31%)	30 (6.7%)	217 (48.44%)	Nuclear
Podnajb1	78 (22.48%)	65 (18.73%)	23 (6.63%)	181 (52.16%)	Cytoplasmic
Podnajb11	109 (31.5%)	64 (18.5%)	31 (8.96%)	142 (41.04%)	Endoplasmic reticulum
Podnajb5a	76 (20.54%)	65 (17.57%)	24 (6.49%)	205 (55.41%)	Nuclear
Podnajb1a	82 (26.28%)	59 (18.91%)	22 (7.05%)	149 (47.76%)	Cytoplasmic
Podnaja3	113 (23.3%)	87 (17.94%)	35 (7.22%)	250 (51.55%)	Mitochondrial
Podnajc5aa	63 (31.66%)	30 (15.08%)	10 (5.03%)	96 (48.24%)	Nuclear
Podnajb6a	59 (21.93%)	51 (18.96%)	31 (11.52%)	128 (47.58%)	Nuclear
Podnajb12	167 (45.14%)	29 (7.84%)	7 (1.89%)	167 (45.14%)	Nuclear
Podnajc18	174 (45.08%)	27 (6.99%)	9 (2.33%)	176 (45.6%)	Nuclear
Podnajc16a	306 (38.93%)	123 (15.65%)	26 (3.31%)	331 (42.11%)	Endoplasmic reticulum
Podnajc10	173 (39.86%)	86 (19.82%)	16 (3.69%)	159 (36.64%)	Mitochondrial
Podnajb12a	163 (43.7%)	28 (7.51%)	8 (2.14%)	174 (46.65%)	Nuclear
Podnajc5ga	72 (32.58%)	17 (7.69%)	7 (3.17%)	125 (56.56%)	Nuclear
Podnajb6	87 (26.69%)	55 (16.87%)	38 (11.66%)	146 (44.79%)	Cytoplasmic
Podnajb9	87 (39.91%)	25 (11.47%)	6 (2.75%)	100 (45.87%)	Nuclear
Podnajc16	304 (37.62%)	120 (14.85%)	23 (2.85%)	361 (44.68%)	Endoplasmic reticulum
Podnajb9a	98 (41.18%)	23 (9.66%)	6 (2.52%)	111 (46.64%)	Mitochondrial
Podnajc21	288 (53.83%)	25 (4.67%)	17 (3.18%)	205 (38.32%)	Nuclear
Podnajc14	276 (41.44%)	56 (8.41%)	42 (6.31%)	292 (43.84%)	Mitochondrial
Podnajc11	180 (32.2%)	131 (23.43%)	29 (5.19%)	219 (39.18%)	Cytoplasmic
Podnajc7	319 (63.29%)	29 (5.75%)	22 (4.37%)	134 (26.59%)	Cytoplasmic
Podnajc7a	322 (60.64%)	27 (5.08%)	24 (4.52%)	158 (29.76%)	Nuclear
Podnajc11a	177 (31.61%)	132 (23.57%)	27 (4.82%)	224 (40%)	Nuclear
Podnajc9	166 (65.1%)	9 (3.53%)	16 (6.27%)	64 (25.1%)	Nuclear
Podnajc3	327 (66.6%)	28 (5.7%)	21 (4.28%)	115 (23.42%)	Nuclear
Podnajc3a	344 (69.22%)	31 (6.24%)	21 (4.23%)	101 (20.32%)	Nuclear
Podnajc24	68 (38.42%)	36 (20.34%)	9 (5.08%)	64 (36.16%)	Nuclear
Podnajc4	130 (54.62%)	15 (6.3%)	2 (0.84%)	91 (38.24%)	Nuclear
Podnajc25	227 (62.71%)	32 (8.84%)	11 (3.04%)	92 (25.41%)	Mitochondrial
Podnajb3	109 (35.28%)	52 (16.83%)	23 (7.44%)	125 (40.45%)	Nuclear
Podnajc30	162 (41.33%)	49 (12.5%)	13 (3.32%)	168 (42.86%)	Nuclear
Podnajc27	98 (48.51%)	24 (11.88%)	11 (5.45%)	69 (34.16%)	Cytoplasmic
Podnajc30a	123 (36.39%)	32 (9.47%)	16 (4.73%)	167 (49.41%)	Nuclear
Podnajc17	199 (61.23%)	31 (9.54%)	11 (3.38%)	84 (25.85%)	Nuclear
Podnajsec63	327 (43.2%)	106 (14%)	23 (3.04%)	301 (39.76%)	Nuclear
Podnajc6	212 (21.65%)	140 (14.3%)	56 (5.72%)	571 (58.32%)	Nuclear
Podnajgak	394 (30.24%)	160 (12.28%)	65 (4.99%)	684 (52.49%)	Nuclear
Podnajc12	74 (44.31%)	11 (6.59%)	5 (2.99%)	77 (46.11%)	Nuclear
Podnajc15	75 (50%)	18 (12%)	11 (7.33%)	46 (30.67%)	Nuclear
Podnajc19	71 (61.21%)	10 (8.62%)	7 (6.03%)	28 (24.14%)	Mitochondrial
Podnajc22	171 (50.29%)	42 (12.35%)	17 (5%)	110 (32.35%)	Endoplasmic reticulum
Pohscb	135 (48.21%)	33 (11.79%)	6 (2.14%)	106 (37.86%)	Nuclear
Podnajc8	164 (65.6%)	16 (6.4%)	9 (3.6%)	61 (24.4%)	Nuclear
Podnajpam16	78 (61.9%)	14 (11.11%)	1 (0.79%)	33 (26.19%)	Nuclear
Podnajc2	392 (63.43%)	38 (6.15%)	14 (2.27%)	174 (28.16%)	Nuclear

DISCUSSION

The industrial farming of Japanese flounder was affected by *E. tarda* infection seriously (Egusa, 1976; Nakatsugawa, 1983; Miyazaki and Kaige, 1985; Isshiki et al., 2001; Moustafa et al., 2010; Park et al., 2012). Previous studies indicated that hsps participated in disease defense and resistance of teleosts, e.g., channel catfish (Xie et al., 2015; Song et al., 2016).

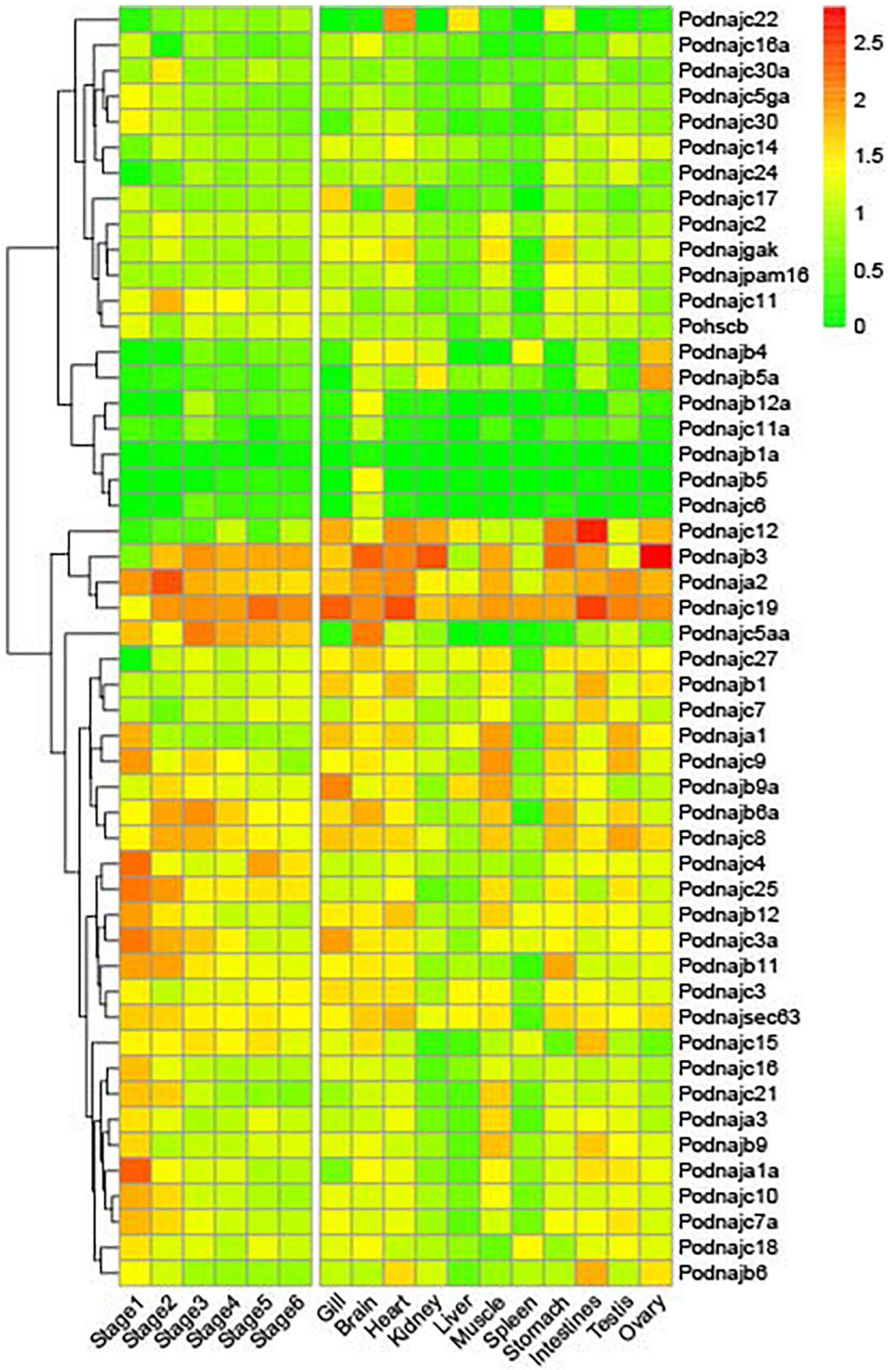

FIGURE 3 | Expression patterns of *hsp40* genes in 11 tissues and six embryonic and larval stages in healthy individuals. Each cell in the heat map corresponds to an expression level and the numbers in cell are FPKM values. Six stages of embryonic and larval development: stage 1 (from two cells to morula), stage 2 (from early gastrula to late somites), stage 3 (from hatching stage to 2 days after hatching), stage 4 (before metamorphosis), stage 5 (metamorphosis stages 1–2), and stage 6 (metamorphosis stages 3–5).

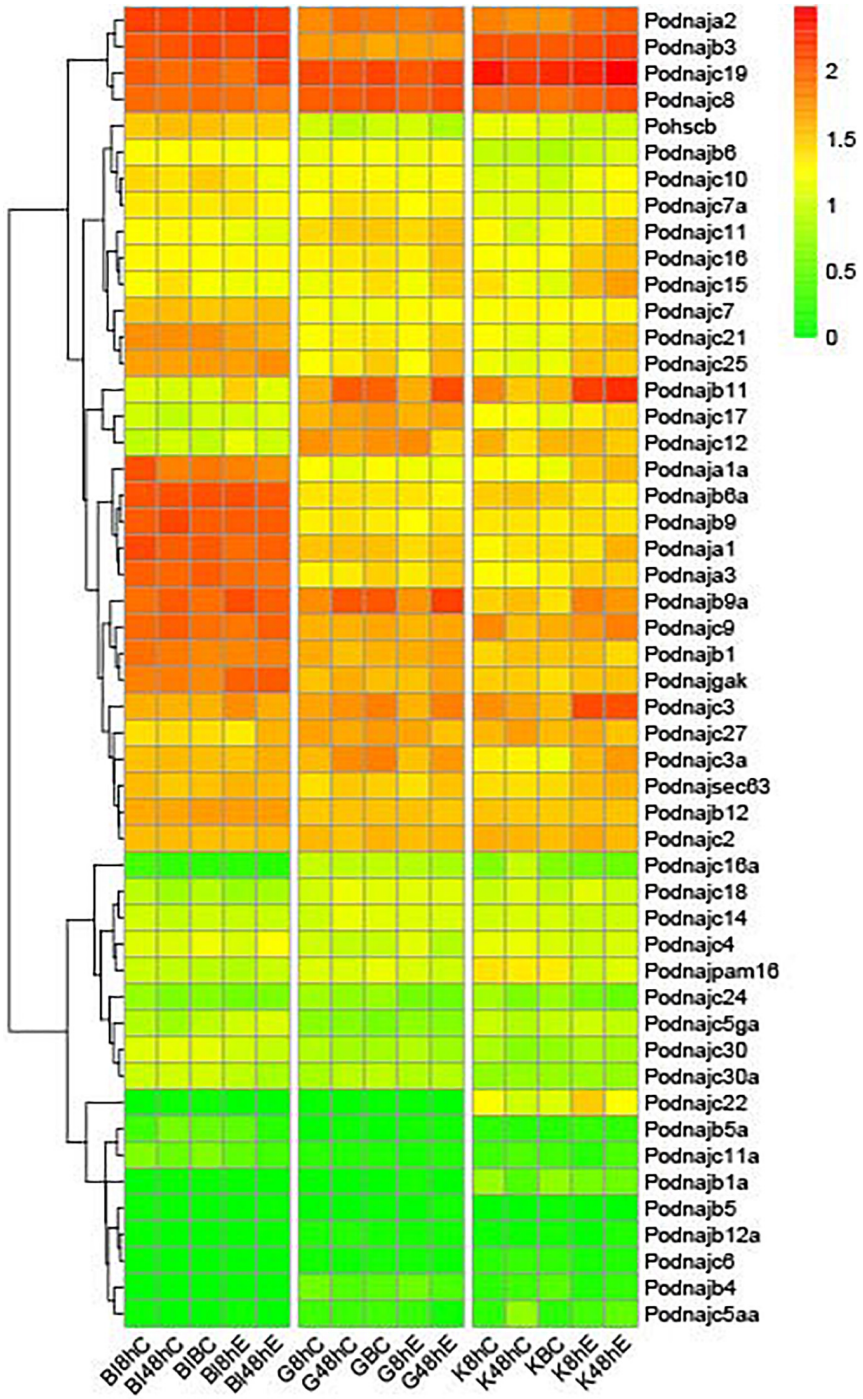

FIGURE 4 | Expression profiles of *hsp40* genes after *E. tarda* injection. Each row represents a gene, and each column represents a time point. The relative expression level is indicated by the color bar on the top right. G represents gill, K represents kidney, and Bl represents blood. 0 h represents the blank control group at the beginning of the experiment, C 8 h and C 48 h indicate Ringer's solution control group, whereas E 8 h and E 48 h indicate *E. tarda*-challenged experimental group, and BC represents blank control.

However, despite these observations, a deeper understanding of the molecular mechanism of Japanese flounder immune responses is required. In this study, we identified 61 *hsp40* genes from tilapia, 57 from medaka, 57 from fugu, 57 from zebrafish, 53 from yellow catfish, 50 from channel catfish, 50 from Japanese flounder, and 33 from spotted gar, indicating a relatively similar *hsp* gene number. In order to elucidate the evolution history of hsp40 proteins, we conducted a maximum-likelihood

phylogenetic gene tree of eight teleosts above. As shown in **Figure 1**, each of the *hsp40* genes was divided into 40 subfamilies (*dnaja1*, *dnaja2*, *dnaja3*, *dnajb1*, *dnajb3*, *dnajb4*, *dnajb5*, *dnajb6*, *dnajb9*, *dnajb11*, *dnajb12*, *dnajb14*, *dnajc1*, *dnajc2*, *dnajc3*, *dnajc4*, *dnajc5*, *dnajc6*, *dnajc7*, *dnajc8*, *dnajc9*, *dnajc10*, *dnajc11*, *dnajc12*, *dnajc14*, *dnajc15*, *dnajc16*, *dnajc17*, *dnajc18*, *dnajc19*, *dnajc21*, *dnajc22*, *dnajc24*, *dnajc25*, *dnajc27*, *dnajc30*, *dnajgak*, *dnajpam16*, *dnajsec63*, and *hscb*) with no obvious extension between species, and Japanese flounder harbored most genes, indicating a high evolutionary conservativeness. Although we conducted complete searches with all Japanese flounder genomic resources available, the *dnajb14* and *dnajc1* have not been found in the Japanese flounder genome. Together with selective pressure analysis, dN/dS analysis of all *hsp40* genes experienced pronounced negative selection, indicating that there were no non-synonymous nucleotide changes at that codon. There existed three genes (*dnajc30*, *dnajc30a*, and *hscb*) whose dN/dS ratio was more than 0.3, which is greater than that of others, hinting that they may experience a higher evolutionary dynamic. Previous studies have found that the evolution of new genes was usually accompanied by changes that occurred in both their sequence and structure, while mutation is the original condition in the evolution of genes. Furthermore, positive Darwinian selection may be another important power forcing new genes' evolution. In addition, natural selection pressure might affect particular sites but not the whole gene, namely, site-specific selection (Koester et al., 2012). These results revealed that the *hsp40* genes between teleosts above were evolutionary-constrained and they were function-conserved; therefore, negative selection might account for the loss of genes in the Japanese flounder genome. In addition, according to the subcellular localization analysis, we assume that most *hsp40* genes function in the nucleus of cells because of their expression preference. As for the structural characteristics of hsp40 proteins, the results above could predict their potential roles and ultimately provide their sequence–structure–function relationships by binding and acting with other proteins.

During embryogenesis, there exist vigorous cell proliferation and differentiation as well as gene expression and protein synthesis, the intra- and extracellular environments experience steady changes, and cells are extremely sensitive to external stimuli (Haanen and Vermes, 1996). Thus, the change and function of *hsps* may be more vital (Walsh et al., 1997; Neuer et al., 1999). *Hsp* genes are regarded as chaperones in morphologic development of cells and organisms and are believed to be related to normal and abnormal development of embryo (Neuer et al., 1999; Brown et al., 2007). Nevertheless, among the *hsp* superfamily, compared to the abundant researches about *hsp20* and *hsp10/60* genes with respect to embryonic and larval development, there is a lack of systemic studies about the role of *hsp40* genes in normal development of embryo and larva (Mao and Shelden, 2006; Elicker and Hutson, 2007; Xu et al., 2011; Middleton and Shelden, 2013; Wang et al., 2017). As for the model species, *hsp40* gene was reported to participate in the embryonic development of mouse forelimbs (Zhu et al., 2010). With regard to marine animals, we also found reports about the roles of *hsp40* genes in sea urchin cilia regeneration during embryogenesis (Casano et al., 2003). Furthermore, previous research also indicated that *hsp40* genes were upregulated after pathogen injection in Japanese flounder embryonic cells (FECs) (Dong et al., 2006). Herein, results indicated that *hsp40* genes had different expression profiles during different embryonic and larval stages and six of them (*dnaja2*, *dnajb3*, *dnajc3a*, *dnajc4*, *dnajc5aa*, and *dnajc19*) had high expression levels at all developmental stages, which hints that *hsp40* genes may be involved in the development of Japanese flounder embryo and larva.

Previous studies have shown the roles of *hsp40* genes in the reactions of a large number of stressors of aquatic animals; however, they emphasized on the abiotic stresses such as thermal, acidity/alkalinity, and salinity challenges (Chen T. et al., 2018; Huang et al., 2018; Xu Y. et al., 2019). As for the immune response, *hsp40* genes were found to be significantly regulated after *Edwardsiella ictaluri* and *Flavobacterium columnare* challenges in channel catfish (Song et al., 2014). Interestingly, in this study, we also found that a large percentage of *hsp40* family members, with a number of 44 genes (88%), were significantly influenced after pathogen injection. Though the mechanisms behind are ambiguous, *hsp40* genes were dramatically regulated after *E. tarda* challenge. These results indicate that *hsp40* genes participated in pathogen reactions and disease resistance against pathogens. In detail, the expression patterns showed a tissue-dependent feature after *E. tarda* infection: 35 genes were up- or downregulated in kidney, whereas 34 genes were regulated in blood while 31 were regulated in gill. In summary, 44 out of 50 *hsp40* genes participated in *E. tarda* defense reactions. After *E. tarda* infection in the blood, expression of three genes (*dnaja1a*, *dnajc11a*, and *dnajc21*) decreased from 8 to 48 h, whereas that of seven genes (*dnaja2*, *dnajb3*, *dnajb12*, *dnajc3*, *dnajc5ga*, *dnajsec63*, and *dnajgak*) was upregulated significantly. Gene expression of *dnajc19* and *dnajpam16* decreased at 8 h and then rose at 48 h, and gene expression of *dnajb5a* and *dnajc7a* was upregulated at 8 h but downregulated at 48 h. Gene expression of *dnajb9a*, *dnajb11*, and *dnajc11* was upregulated at 8 h but returned to the original level at 48 h, whereas that of nine genes (*dnaja1*, *dnaja3*, *dnajb1*, *dnajb6*, *dnajc9*, *dnajc10*, *dnajc16a*, *dnajc18*, and *dnajc24*) was downregulated at 8 h and kept in a normal standard at 48 h. In addition, of the 50 *hsp40* genes, nine did not show significant differential regulation until 48 h, among them, five (*dnajc3a*, *dnajc4*, *dnajc17*, *dnajc25*, and *dnajc27*) were upregulated at 48 h, whereas three genes (*dnajc15*, *dnajc30*, and *dnajc30a*) were downregulated at 48 h. After *E. tarda* injection in the gill, expression of *dnaja2* was dramatically increased from 8 to 48 h, whereas that of *dnajc24* decreased significantly after injection. The expression of *dnajc14* increased at 8 h and followed by a drop at 48 h. Gene expression of *dnajc19* dropped at 8 h and rose to a high level at 48 h. Besides, the expression of 19 *hsp40* genes (*dnaja1a*, *dnajb1a*, *dnajb3*, *dnajb4*, *dnajb5*, *dnajb5a*, *dnajb12a*, *dnajc4*, *dnajc5aa*, *dnajc5ga*, *dnajc6*, *dnajc9*, *dnajc10*, *dnajc11a*, *dnajc17*, *dnajc22*, *dnajc30*, *dnajc30a*, and *hscb*) was stable throughout all time period. Interestingly, *dnajb12* and *dnajc18* showed a different response pattern, which was upregulated at 8 h but returned

to the original level at 48 h, while that of *dnaja1* and *dnajb9* was downregulated at 8 h but restored to a normal level at 48 h. Additionally, gene expression of 16 genes (*dnaja3*, *dnajb1*, *dnajb6*, *dnajb9a*, *dnajb11*, *dnajc2*, *dnajc3*, *dnajc7*, *dnajc8*, *dnajc11*, *dnajc15*, *dnajc16*, *dnajc21*, *dnajc25*, *dnajsec63*, and *dnajgak*) was kept in a normal standard and showed a significant increase at 48 h, and the gene expression of seven genes (*dnajb6a*, *dnajc3a*, *dnajc7a*, *dnajc12*, *dnajc16a*, *dnajc27*, and *dnajpam16*) was kept normal and decreased prominently at 48 h. Besides, after *E. tarda* injection in the kidney, the gene expression of 20 genes (*dnaja1*, *dnaja1a*, *dnaja2*, *dnaja3*, *dnajb3*, *dnajb9a*, *dnajb11*, *dnajc3*, *dnajc3a*, *dnajc5ga*, *dnajc8*, *dnajc10*, *dnajc11*, *dnajc15*, *dnajc16*, *dnajc21*, *dnajc22*, *dnajc25*, *dnajcsec63*, and *dnajgak*) was prominently elevated after administration, and the expression of *dnajb6a*, *dnajpam16*, and *hscb* was downregulated from 8 to 48 h. The expression of *dnajb1* and *dnajc27* was elevated at 8 h and followed by a decrease at 48 h. Gene expression of six genes (*dnajb6*, *dnajc12*, *dnajc17*, *dnajc19*, and *dnajc30*) was kept in a normal standard and showed a significant increase at 48 h, whereas that of *dnajc16a* was kept normal but decreased at 48 h. Moreover, the expression of *dnajb9*, *dnajc7*, and *dnajc18* was upregulated at 8 h, whereas the original level was restored at 48 h, and gene expression of *dnajc24* was downregulated but restored to the normal standard at 48 h. Notably, 15 genes (*dnajb1a*, *dnajb4*, *dnajb5*, *dnajb5a*, *dnajb12*, *dnajb12a*, *dnajc2*, *dnajc4*, *dnajc5aa*, *dnajc6*, *dnajc7a*, *dnajc9*, *dnajc11a*, *dnajc14*, and *dnajc30a*) did not exhibit up- or downregulation from 8 to 48 h. In a word, results showed that a large percentage of *hsp40* gene in gill, blood, and kidney samples were likely to be involved in reaction to *E. tarda* injection, with the exception of *dnajb1a*, *dnajb4*, *dnajb5*, *dnajb12a*, *dnajc5aa*, and *dnajc6*. Interestingly, there existed a tissue-specific response pattern in these 44 regulated *hsp40* genes; namely, most *hsp40* genes had different reaction profiles in three tissues examined except *dnaja2*, which was upregulated in gill, kidney, and blood tissues from 8 to 48 h. In addition, there were still 14 *hsp40* genes (*dnaja1*, *dnajb3*, *dnajb6*, *dnajc3*, *dnajc5ga*, *dnajc16a*, *dnajc17*, *dnajc18*, *dnajc19*, *dnajc24*, *dnajc25*, *dnajc27*, *dnajgak*, and *dnajsec63*) that showed a similar response pattern in two of three particular tissues, among them, *dnaja1*, *dnajc19*, and *dnajc25* had the same patterns in blood and gill, while *dnajb3*, *dnajc3*, *dnajc5ga*, *dnajc17*, *dnajsec63*, and *dnajgak* had the same profiles in blood and kidney, whereas the remaining five genes had the same expression patterns in gill and kidney. Furthermore, the 44 regulated *hsp40* genes had different response efficiency: *dnajc2*, *dnajc4*, *dnajc17*, *dnajc30*, and *dnajc30a* did not show an up- or downregulation until 48 h after *E. tarda* injection, while the other 39 genes respond rapidly at 8 h after injection. Results above hint that five genes (*dnajc2*, *dnajc4*, *dnajc17*, *dnajc30*, and *dnajc30a*) showed a lower speed in regulating *E. tarda* challenge in flounder.

In total, 44 out of 50 *hsp40* genes seem to show a response in reaction to *E. tarda* administrations. The regulatory mechanism behind has not yet been fully validated nevertheless. Therefore, more in-depth research is needed to elucidate the mechanisms of differential expression and to verify the characteristics of *hsp40* genes in immune defenses. In summary, a full set of 50 *hsp40* genes derived from the Japanese flounder genome were identified and characterized in this study. As the largest subfamily of *hsp* superfamily that is involved in many vital physiological processes, *hsp40* genes were divided into 40 subfamilies in phylogenetic analysis, and selective pressure analysis indicated that *hsp40* genes experienced pronounced purifying selection. Additionally, we investigated the expression levels of the *hsp40* genes in *E. tarda*-injected and unchallenged organisms. In healthy individuals, *hsp40* genes played the sentinel role. In challenged individuals, 44 *hsp40* genes were up- or downregulated after *E. tarda* injection, hinting that they might play a role as a portion of the disease response while some of differential expression genes may participate in disease defense against *E. tarda*. These findings give elementary reference for in-depth validation of the characteristics of *hsp40* genes in the process of immune reactions and molecular evolutionary history in Japanese flounder.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of the Ocean University of China and the China Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training.

AUTHOR CONTRIBUTIONS

WY contributed to conceptualization, methodology, software, writing—original draft, writing—review and editing, and visualization. YQ contributed to software and resources. JQ contributed to software. XL contributed to formal analysis. QZ contributed to funding acquisition. XW contributed to conceptualization, methodology, project administration, and funding acquisition. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2020.596534/full#supplementary-material

REFERENCES

Akira, S., Bauer, S., and Hartmann, G. (2008). *Toll Like Receptors (TLRs) and Innate Immunity*. Berlin: Springer.

Bailey, T. L., Boden, M., Buske, F. A., Frith, M., Grant, C. E., Clementi, L., et al. (2009). MEME SUITE: tools for motif discovery and searching. *Nucleic Acids Res.* 37, W202–W208.

Bang, J.-D., Chun, S.-K., Park, S.-I., and Choi, Y.-J. (1992). Studies on the biochemical and serological characteristics of *Edwardsiella tarda* isolated from cultured flounder, *Paralichthys olivaceus*. *J. Fish Pathol.* 5, 29–35.

Basu, S., Binder, R. J., Ramalingam, T., and Srivastava, P. K. (2001). CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70, and calreticulin. *Immunity* 14, 303–313. doi: 10.1016/s1074-7613(01)00111-x

Basu, S., Binder, R. J., Suto, R., Anderson, K. M., and Srivastava, P. K. (2000). Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a partial maturation signal to dendritic cells and activate the NF-κB pathway. *Int. Immunol.* 12, 1539–1546. doi: 10.1093/intimm/12.11.1539

Bork, P., Sander, C., Valencia, A., and Bukau, B. (1992). A module of the DnaJ heat shock proteins found in malaria parasites. *Trends Biochem. Sci.* 17:129. doi: 10.1016/0968-0004(92)90319-5

Botha, M., Pesce, E., and Blatch, G. L. (2007). The Hsp40 proteins of Plasmodium falciparum and other apicomplexa: regulating chaperone power in the parasite and the host. *Intern. J. Biochem. Cell Biol.* 39, 1781–1803. doi: 10.1016/j.biocel.2007.02.011

Brown, D. D., Christine, K. S., Showell, C., and Conlon, F. L. (2007). Small heat shock protein Hsp27 is required for proper heart tube formation. *Genesis* 45, 667–678. doi: 10.1002/dvg.20340

Casano, C., Gianguzza, F., Roccheri, M. C., Giorgi, R. D., Maenza, L., and Ragusa, M. A. (2003). Hsp40 is involved in cilia regeneration in sea urchin embryos. *J. Histochem. Cytochem.* 51, 1581–1587. doi: 10.1177/002215540305101202

Cha, I. S., Kwon, J., Park, S. B., Jang, H. B., Nho, S. W., Kim, Y. K., et al. (2013). Heat shock protein profiles on the protein and gene expression levels in olive flounder kidney infected with *Streptococcus parauberis*. *Fish Shellf. Immunol.* 34, 1455–1462. doi: 10.1016/j.fsi.2013.03.355

Cheetham, M. E., and Caplan, A. J. (1998). Structure, function and evolution of DnaJ: conservation and adaptation of chaperone function. *Cell Stress Chaperon.* 3:28. doi: 10.1379/1466-1268(1998)003<0028:sfaeod>2.3.co;2

Chen, C., Xia, R., Chen, H., and He, Y. (2018). TBtools, a toolkit for biologists integrating various biological data handling tools with a user-friendly interface. *bioRxiv* [Preprint], doi: 10.1101/289660

Chen, T., Lin, T., Li, H., Lu, T., Li, J., Huang, W., et al. (2018). Heat shock protein 40 (HSP40) in Pacific white shrimp (*Litopenaeus vannamei*): molecular cloning, tissue distribution and ontogeny, response to temperature, acidity/alkalinity and salinity stresses, and potential role in ovarian development. *Front. Physiol.* 9:1784. doi: 10.3389/fphys.2018.01784

Chen, W., Syldath, U., Bellmann, K., Burkart, V., and Kolb, H. (1999). Human 60-kDa heat-shock protein: a danger signal to the innate immune system. *J. Immunol.* 162, 3212–3219.

Chen, Y.-M., Kuo, C.-E., Wang, T.-Y., Shie, P.-S., Wang, W.-C., Huang, S.-L., et al. (2010). Cloning of an orange-spotted grouper *Epinephelus coioides* heat shock protein 90AB (HSP90AB) and characterization of its expression in response to nodavirus. *Fish Shellf. Immunol.* 28, 895–904. doi: 10.1016/j.fsi.2010.02.004

Delport, W., Poon, A. F., Frost, S. D., and Kosakovsky Pond, S. L. (2010). Datamonkey 2010: a suite of phylogenetic analysis tools for evolutionary biology. *Bioinformatics* 26, 2455–2457. doi: 10.1093/bioinformatics/btq429

Dong, C.-W., Zhang, Y.-B., Zhang, Q.-Y., and Gui, J.-F. (2006). Differential expression of three *Paralichthys olivaceus* Hsp40 genes in responses to virus infection and heat shock. *Fish Shellf. Immunol.* 21, 146–158. doi: 10.1016/j.fsi.2005.11.002

Egusa, S. (1976). Some bacterial diseases of freshwater fishes in Japan. *Fish Pathol.* 10, 103–114. doi: 10.3147/jsfp.10.103

Eissa, N. (2014). Probiotic effect on molecular antioxidant profiles in yellow perch, *Perca flavescens*. *Glob. Jo. Fish. Aquac. Res.* 1, 16–29.

Eissa, N., Wang, H.-P., Yao, H., and Abou-ElGheit, E. (2018). Mixed Bacillus species enhance the innate immune response and stress tolerance in yellow perch subjected to hypoxia and air-exposure stress. *Sci. Rep.* 8, 1–10.

El-Gebali, S., Mistry, J., Bateman, A., Eddy, S. R., Luciani, A., Potter, S. C., et al. (2018). The Pfam protein families database in 2019. *Nucleic Acids Res.* 47, D427–D432.

Elicker, K. S., and Hutson, L. D. (2007). Genome-wide analysis and expression profiling of the small heat shock proteins in zebrafish. *Gene* 403, 60–69. doi: 10.1016/j.gene.2007.08.003

Fuji, K., Kobayashi, K., Hasegawa, O., Coimbra, M. R. M., Sakamoto, T., and Okamoto, N. (2006). Identification of a single major genetic locus controlling the resistance to lymphocystis disease in Japanese flounder (*Paralichthys olivaceus*). *Aquaculture* 254, 203–210. doi: 10.1016/j.aquaculture.2005.11.024

Fuller, K., Issels, R., Slosman, D., Guillet, J.-G., Soussi, T., and Polla, B. (1994). Cancer and the heat shock response. *Eur. J. Cancer* 30, 1884–1891.

Gehrmann, M., Brunner, M., Pfister, K., Reichle, A., Kremmer, E., and Multhoff, G. (2004). Differential up-regulation of cytosolic and membrane-bound heat shock protein 70 in tumor cells by anti-inflammatory drugs. *Clin. Cancer Res.* 10, 3354–3364. doi: 10.1158/1078-0432.ccr-03-0382

Georgopoulos, C. P., Lundquist-Heil, A., Yochem, J., and Feiss, M. (1980). Identification of the *E. coli* dnaJ gene product. *Mol. Gen. Genet. MGG* 178, 583–588. doi: 10.1007/bf00337864

Geourjon, C., and Deleage, G. (1995). SOPMA: significant improvements in protein secondary structure prediction by consensus prediction from multiple alignments. *Bioinformatics* 11, 681–684. doi: 10.1093/bioinformatics/11.6.681

Gething, M.-J. (1997). *Guidebook to Molecular Chaperones and Protein-Folding Catalysts*. Oxford: OUP Oxford.

Ghosh, S., and Chan, C.-K. K. (2016). "Analysis of RNA-Seq data using TopHat and Cufflinks, Plant Bioinformatics," in *Plant Bioinformatics. Methods in Molecular Biology*, Vol. 1374, ed. D. Edwards (New York, NY: Humana Press), 339–361. doi: 10.1007/978-1-4939-3167-5_18

Haanen, C., and Vermes, I. (1996). Apoptosis: programmed cell death in fetal development. *Eur. J. Obstetr. Gynecol. Reprod. Biol.* 64, 129–133. doi: 10.1016/0301-2115(95)02261-9

Habich, C., Baumgart, K., Kolb, H., and Burkart, V. (2002). The receptor for heat shock protein 60 on macrophages is saturable, specific, and distinct from receptors for other heat shock proteins. *J. Immunol.* 168, 569–576. doi: 10.4049/jimmunol.168.2.569

Hennessy, F., Cheetham, M. E., Dirr, H. W., and Blatch, G. L. (2000). Analysis of the levels of conservation of the J domain among the various types of DnaJ-like proteins. *Cell Stress Chaperon.* 5, 347–358. doi: 10.1379/1466-1268(2000)005<0347:aotloc>2.0.co;2

Hennessy, F., Nicoll, W. S., Zimmermann, R., Cheetham, M. E., and Blatch, G. L. (2005). Not all J domains are created equal: implications for the specificity of Hsp40-Hsp70 interactions. *Protein Sci.* 14, 1697–1709. doi: 10.1110/ps.051406805

Hoshina, T. (1962). On a new bacterium, *Paracolobactrum anguillimortiferum* n. sp. *Bull. Jpn. Soc. Sci. Fish.* 28, 162–164. doi: 10.2331/suisan.28.162

Huang, X., Li, S., Gao, Y., and Zhan, A. (2018). Genome-wide identification, characterization and expression analyses of heat shock protein-related genes in a highly invasive ascidian *Ciona savignyi*. *Front. Physiol.* 9:1043. doi: 10.3389/fphys.2018.01043

Isshiki, T., Nishizawa, T., Kobayashi, T., Nagano, T., and Miyazaki, T. (2001). An outbreak of VHSV (viral hemorrhagic septicemia virus) infection in farmed Japanese flounder *Paralichthys olivaceus* in Japan. *Dis. Aquat. Organ.* 47, 87–99. doi: 10.3354/dao047087

Kampinga, H. H., Hageman, J., Vos, M. J., Kubota, H., Tanguay, R. M., Bruford, E. A., et al. (2009). Guidelines for the nomenclature of the human heat shock proteins. *Cell Stress Chaperon.* 14, 105–111.

Koester, J. A., Swanson, W. J., and Armbrust, E. V. (2012). Positive selection within a diatom species acts on putative protein interactions and transcriptional regulation. *Mol. Biol. Evol.* 30, 422–434. doi: 10.1093/molbev/mss242

Kol, A., Lichtman, A. H., Finberg, R. W., Libby, P., and Kurt-Jones, E. A. (2000). Cutting edge: heat shock protein (HSP) 60 activates the innate immune response: CD14 is an essential receptor for HSP60 activation of mononuclear cells. *J. Immunol.* 164, 13–17. doi: 10.4049/jimmunol.164.1.13

Kolde, R. (2018). *Pheatmap: Pretty Heatmaps, R Packag. Version 1.0. 10.*

Kosakovsky Pond, S. L., and Frost, S. D. (2005). Not so different after all: a comparison of methods for detecting amino acid sites under selection. *Mol. Biol. Evol.* 22, 1208–1222. doi: 10.1093/molbev/msi105

Kryazhimskiy, S., and Plotkin, J. B. (2008). The population genetics of dN/dS. *PLoS Genet.* 4:e1000304. doi: 10.1371/journal.pgen.1000304

Kumar, S., Stecher, G., and Tamura, K. (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33, 1870–1874. doi: 10.1093/molbev/msw054

Lee, H. B., Kim, D. S., Gil, H. W., and Park, I.-S. (2018). Pysiological responses of diploid and triploid far eastern catfish, *Silurus asotus* to water temperature stress. *Dev. Reprod.* 22:165. doi: 10.12717/dr.2018.22.2.165

Lehner, T., Bergmeier, L. A., Wang, Y., Tao, L., Sing, M., Spallek, R., et al. (2000). Heat shock proteins generate β-chemokines which function as innate adjuvants enhancing adaptive immunity. *Eur. J. Immunol.* 30, 594–603. doi: 10.1002/1521-4141(200002)30:2<594::aid-immu594>3.0.co;2-1

Letunic, I., and Bork, P. (2017). 20 years of the SMART protein domain annotation resource. *Nucleic Acids Res.* 46, D493–D496.

Li, J., Qian, X., and Sha, B. (2009). Heat shock protein 40: structural studies and their functional implications. *Protein Pept. Lett.* 16, 606–612. doi: 10.2174/092986609788490159

Li, S., Li, X., Gen, X., Chen, Y., Wei, J., and Sun, J. (2014). Identification and characterization of lipopolysaccharide-induced TNF-alpha factor gene from Japanese flounder *Paralichthys olivaceus*. *Vet. Immunol. Immunopathol.* 157, 182–189. doi: 10.1016/j.vetimm.2013.11.006

Li, Z., Liu, X., Cheng, J., He, Y., Wang, X., Wang, Z., et al. (2018a). Transcriptome profiling provides gene resources for understanding gill immune responses in Japanese flounder (*Paralichthys olivaceus*) challenged with *Edwardsiella tarda*. *Fish Shellf. Immunol.* 72, 593–603. doi: 10.1016/j.fsi.2017.11.041

Li, Z., Liu, X., Liu, J., Zhang, K., Yu, H., He, Y., et al. (2018b). Transcriptome profiling based on protein-protein interaction networks provides a core set of genes for understanding blood immune response mechanisms against *Edwardsiella tarda* infection in Japanese flounder (*Paralichthys olivaceus*). *Dev. Compar. Immunol.* 78, 100–113. doi: 10.1016/j.dci.2017.09.013

Liu, X., Li, Z., Wu, W., Liu, Y., Liu, J., He, Y., et al. (2017). Sequencing-based network analysis provides a core set of gene resource for understanding kidney immune response against *Edwardsiella tarda* infection in Japanese flounder. *Fish Shellf. Immunol.* 67, 643–654. doi: 10.1016/j.fsi.2017.06.051

Mao, L., and Shelden, E. (2006). Developmentally regulated gene expression of the small heat shock protein Hsp27 in zebrafish embryos. *Gene Expr. Patter.* 6, 127–133. doi: 10.1016/j.modgep.2005.07.002

Mayer, M. P., Laufen, T., Paal, K., Mccarty, J. S., and Bukau, B. (1999). Investigation of the interaction between DnaK and DnaJ by surface plasmon resonance spectroscopy. *J. Mol. Biol.* 289, 1131–1144. doi: 10.1006/jmbi.1999.2844

Meyer, F., and Bullock, G. (1973). *Edwardsiella tarda*, a new pathogen of channel catfish (*Ictalurus punctatus*). *Appl. Microbiol.* 25:155. doi: 10.1128/aem.25.1.155-156.1973

Middleton, R. C., and Shelden, E. A. (2013). Small heat shock protein HSPB1 regulates growth of embryonic zebrafish craniofacial muscles. *Exp. Cell Res.* 319, 860–874. doi: 10.1016/j.yexcr.2013.01.002

Miyazaki, T., and Kaige, N. (1985). Comparative histopathology of edwardsiellosis in fishes. *Fish Pathol.* 20, 219–227. doi: 10.3147/jsfp.20.219

Moon, J. Y., Hong, Y.-K., Kong, H. J., Kim, D.-G., Kim, Y.-O., Kim, W.-J., et al. (2014). A cDNA microarray analysis to identify genes involved in the acute-phase response pathway of the olive flounder after infection with *Edwardsiella tarda*. *Vet. Immunol. Immunopathol.* 161, 49–56. doi: 10.1016/j.vetimm.2014.07.002

Morahan, B. J., Strobel, C., Hasan, U., Czesny, B., Mantel, P., Marti, M., et al. (2011). functional analysis of the exported Type IV HSP40 protein PfGECO in *Plasmodium falciparum* gametocytes. *Eukaryot. Cell* 10, 1492–1503. doi: 10.1128/ec.05155-11

Moré, S. H., Breloer, M., and von Bonin, A. (2001). Eukaryotic heat shock proteins as molecular links in innate and adaptive immune responses: Hsp60-mediated activation of cytotoxic T cells. *Int. Immunol.* 13, 1121–1127. doi: 10.1093/intimm/13.9.1121

Moustafa, E. M. M., Naota, M., Morita, T., Tange, N., and Shimada, A. (2010). Pathological study on the scuticociliatosis affecting farmed Japanese flounder (*Paralichthys olivaceus*) in Japan. *J. Vet. Med. Sci.* 72, 1359–1362. doi: 10.1292/jvms.10-0026

Multhoff, G. (2006). "Heat shock proteins in immunity," in *Molecular Chaperones in Health and Disease. Handbook of Experimental Pharmacology*, Vol. 172, eds K. Starke and M. Gaestel (Berlin: Springer), 279–304.

Nakai, K. (1999). PSORT: a program for detecting the sorting signals of proteins and predicting their subcellular localization. *Trends Biochem. Sci.* 24, 34–35. doi: 10.1016/s0968-0004(98)01336-x

Nakatsugawa, T. (1983). *Edwardsiella tarda* isolated from cultured young flounder. *Fish. Pathol.* 18, 99–101.

Nei, M., and Gojobori, T. (1986). Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. *Mol. Biol. Evol.* 3, 418–426.

Neuer, A., Spandorfer, S., Giraldo, P., Jeremias, J., Dieterle, S., Korneeva, I., et al. (1999). Heat shock protein expression during gametogenesis and embryogenesis. *Infect. Dis. Obstet. Gynecol.* 7, 10–16. doi: 10.1155/s1064744999000034

Nougayrede, P., Vuillaume, A., Vigneulle, M., Faivre, B., Luengo, S., and Delprat, J. (1994). First isolation of *Edwardsiella tarda* from diseased turbot (*Scophthalmus maximus*) reared in a sea farm in the Bay of Biscay. *Bull. Eur. Assoc. Fish Pathol.* 14, 128–129.

Ohashi, K., Burkart, V., Flohé, S., and Kolb, H. (2000). Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. *J. Immunol.* 164, 558–561. doi: 10.4049/jimmunol.164.2.558

Ohtsuka, K., and Hata, M. (2000). Mammalian HSP40/DNAJ homologs: cloning of novel cDNAs and a proposal for their classification and nomenclature. *Cell Stress Chaperon.* 5:98. doi: 10.1379/1466-1268(2000)005<0098:mhdhco>2.0.co;2

Panjwani, N. N., Popova, L., and Srivastava, P. K. (2002). Heat shock proteins gp96 and hsp70 activate the release of nitric oxide by APCs. *J. Immunol.* 168, 2997–3003. doi: 10.4049/jimmunol.168.6.2997

Park, S. B., Aoki, T., and Jung, T. S. (2012). Pathogenesis of and strategies for preventing *Edwardsiella tarda* infection in fish. *Vet. Res.* 43:67. doi: 10.1186/1297-9716-43-67

Pickering, A. D. (1998). "Stress responses of farmed fish," in *Biology of Farmed Fish*, eds K. D. Black and A. D. Pickering (Sheffield: Sheffield Academic Press), 222–255.

Pond, S. L. K., and Frost, S. D. (2005). Datamonkey: rapid detection of selective pressure on individual sites of codon alignments. *Bioinformatics* 21, 2531–2533. doi: 10.1093/bioinformatics/bti320

Qiu, X.-B., Shao, Y.-M., Miao, S., and Wang, L. (2006). The diversity of the DnaJ/Hsp40 family, the crucial partners for Hsp70 chaperones. *Cell. Mol. Life Sci. CMLS* 63, 2560–2570. doi: 10.1007/s00018-006-6192-6

Ritossa, F. M. (1962). A new puffing pattern induced by heat shock and DNP in *Drosophila*. *Experientia* 18, 515–523.

Roberts, R., Agius, C., Saliba, C., Bossier, P., and Sung, Y. (2010). Heat shock proteins (chaperones) in fish and shellfish and their potential role in relation to fish health: a review. *J. Fish. Dis.* 33, 789–801. doi: 10.1111/j.1365-2761.2010.01183.x

Singh-Jasuja, H., Hilf, N., Scherer, H. U., Arnold-Schild, D., Rammensee, H.-G., Toes, R. E., et al. (2000). The heat shock protein gp96: a receptor-targeted cross-priming carrier and activator of dendritic cells. *Cell Stress Chaperon.* 5:462. doi: 10.1379/1466-1268(2000)005<0462:thspga>2.0.co;2

Sinha, D., Veedin Rajan, V. B., Esthaki, V. K., and D'Silva, P. (2012). HSPIR: a manually annotated heat shock protein information resource. *Bioinformatics* 28, 2853–2855. doi: 10.1093/bioinformatics/bts520

Song, L., Li, C., Xie, Y., Liu, S., Zhang, J., Yao, J., et al. (2016). Genome-wide identification of Hsp70 genes in channel catfish and their regulated expression after bacterial infection. *Fish Shellf. Immunol.* 49, 154–162. doi: 10.1016/j.fsi.2015.12.009

Song, L., Zhang, J., Li, C., Yao, J., Jiang, C., Li, Y., et al. (2014). Genome-wide identification of Hsp40 genes in channel catfish and their regulated expression after bacterial infection. *PLoS One* 9:e115752. doi: 10.1371/journal.pone.0115752

Sørensen, J. G., Kristensen, T. N., and Loeschcke, V. (2003). The evolutionary and ecological role of heat shock proteins. *Ecol. Lett.* 6, 1025–1037. doi: 10.1046/j.1461-0248.2003.00528.x

Srivastava, P. (2002). Roles of heat-shock proteins in innate and adaptive immunity. *Nat. Rev. Immunol.* 2, 185–194. doi: 10.1038/nri749

Sung, Y., and MacRae, T. (2011). Heat shock proteins and disease control in aquatic organisms. *J. Aquac. Res. Dev.* S2:006.

Taechavasonyoo, A., Kondo, H., Nozaki, R., Suzuki, Y., and Hirono, I. (2013). Identification of novel interleukin 1 beta family genes in Japanese flounder

Paralichthys olivaceus. *Fish Shellf. Immunol.* 34, 393–396. doi: 10.1016/j.fsi.2012.10.001

Takano, T., Kondo, H., Hirono, I., Saito-Taki, T., Endo, M., and Aoki, T. (2006). Identification and characterization of a myeloid differentiation factor 88 (MyD88) cDNA and gene in Japanese flounder, *Paralichthys olivaceus*. *Dev. Comparat. Immunol.* 30, 807–816. doi: 10.1016/j.dci.2005.11.003

Tan, Y., Zheng, J., Tung, S., Rosenshine, I., and Leung, K. (2005). Role of type III secretion in *Edwardsiella tarda* virulence. *Microbiology* 151, 2301–2313. doi: 10.1099/mic.0.28005-0

Thanasaksiri, K., Hirono, I., and Kondo, H. (2017). Molecular cloning and expression analysis of NOD-like receptor 5 in Japanese flounder (*Paralichthys olivaceus*) after injection with two different formalin-killed pathogenic bacteria and poly (I: C). *Dev. Comparat. Immunol.* 67, 481–484. doi: 10.1016/j.dci.2016.08.017

Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D. R., et al. (2012). Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat. Protoc.* 7, 562–578. doi: 10.1038/nprot.2012.016

Tsai, J., and Douglas, M. G. (1996). A Conserved HPD sequence of the J-domain is necessary for YDJ1 stimulation of Hsp70 ATPase activity at a site distinct from substrate binding. *J. Biol. Chem.* 271, 9347–9354. doi: 10.1074/jbc.271.16.9347

Vabulas, R. M., Ahmad-Nejad, P., da Costa, C., Miethke, T., Kirschning, C. J., Häcker, H., et al. (2001). Endocytosed HSP60s use toll-like receptor 2 (TLR2) and TLR4 to activate the toll/interleukin-1 receptor signaling pathway in innate immune cells. *J. Biol. Chem.* 276, 31332–31339. doi: 10.1074/jbc.m103217200

Walker, J. M. (2005). *The Proteomics Protocols Handbook*. Berlin: Springer.

Walsh, D., Li, Z., Wu, Y., and Nagata, K. (1997). Heat shock and the role of the HSPs during neural plate induction in early mammalian CNS and brain development. *Cell. Mol. Life Sci. CMLS* 53, 198–211. doi: 10.1007/pl00000592

Walsh, P., Bursać, D., Law, Y. C., Cyr, D., and Lithgow, T. (2004). The J-protein family: modulating protein assembly, disassembly and translocation. *EMBO Rep.* 5, 567–571. doi: 10.1038/sj.embor.7400172

Wang, P., Xuac, P., Zhouac, L., Zengac, S., and Liac, G. (2017). Molecular cloning, characterization, and expression analysis of HSP60 in mandarin fish *Siniperca chuatsi*. *Isra. J. Aquac. Bamidgeh* 69:13.

Wang, Q., Yang, M., Xiao, J., Wu, H., Wang, X., Lv, Y., et al. (2009). Genome sequence of the versatile fish pathogen *Edwardsiella tarda* provides insights into its adaptation to broad host ranges and intracellular niches. *PLoS One* 4:e7646. doi: 10.1371/journal.pone.0007646

Wei, T., Gao, Y., Wang, R., and Xu, T. (2013). A heat shock protein 90 β isoform involved in immune response to bacteria challenge and heat shock from *Miichthys miiuy*. *Fish Shellf. Immunol.* 35, 429–437. doi: 10.1016/j.fsi.2013.04.045

Wen, X., Hu, Y., Zhang, X., Wei, X., Wang, T., and Yin, S. (2019). Integrated application of multi-omics provides insights into cold stress responses in pufferfish *Takifugu fasciatus*. *BMC Genom.* 20:563. doi: 10.1186/s12864-019-5915-7

Whitley, D., Goldberg, S. P., and Jordan, W. D. (1999). Heat shock proteins: a review of the molecular chaperones. *J. Vasc. Surg.* 29, 748–751.

Wilhelm, V., Soza, C., Martínez, R., Rosemblatt, M., Burzio, L. O., and Valenzuela, P. D. (2005). Production and immune response of recombinant Hsp60 and Hsp70 from the salmon pathogen *Piscirickettsia salmonis*. *Biol. Res.* 38, 69–82.

Xiao, J., Wang, Q., Liu, Q., Wang, X., Liu, H., and Zhang, Y. (2008). Isolation and identification of fish pathogen *Edwardsiella tarda* from mariculture in China. *Aquac. Res.* 40, 13–17.

Xie, Y., Song, L., Weng, Z., Liu, S., and Liu, Z. (2015). Hsp90, Hsp60 and sHsp families of heat shock protein genes in channel catfish and their expression after bacterial infections. *Fish Shellf. Immunol.* 44, 642–651. doi: 10.1016/j.fsi.2015.03.027

Xu, X.-Y., Shen, Y.-B., Fu, J.-J., Liu, F., Guo, S.-Z., Yang, X.-M., et al. (2011). Molecular cloning, characterization and expression patterns of HSP60 in the grass carp (*Ctenopharyngodon idella*). *Fish Shellf. Immunol.* 31, 864–870. doi: 10.1016/j.fsi.2011.07.028

Xu, Y., Zheng, G., Liu, G., Yang, Q., and Yu, X. (2019). Molecular cloning, characterization of *Pomacea canaliculata* HSP40 and its expression analysis under temperature change. *J. Therm. Biol.* 81, 59–65. doi: 10.1016/j.jtherbio.2019.02.006

Xu, Z.-N., Zheng, G.-D., Wu, C.-B., Jiang, X.-Y., and Zou, S.-M. (2019). Identification of proteins differentially expressed in the gills of grass carp (*Ctenopharyngodon idella*) after hypoxic stress by two-dimensional gel electrophoresis analysis. *Fish Physiol. Biochem.* 45, 743–752. doi: 10.1007/s10695-018-0599-5

Yasunaga, N. (1982). Characteristics of fish pathogen *Edwardsiella* isolated from several species of cultured marine fishes. *Bull. Nagasaki Pref. Instit. Fish.* 8, 57–65.

Yochem, J., Uchida, H., Sunshine, M., Saito, H., Georgopoulos, C., and Feiss, M. (1978). Genetic analysis of two genes, dnaJ and dnaK, necessary for *Escherichia coli* and bacteriophage lambda DNA replication. *Mol. Gen. Genet. MGG* 164, 9–14. doi: 10.1007/bf00267593

Zhang, H., Gao, S., Lercher, M. J., Hu, S., and Chen, W.-H. (2012). EvolView, an online tool for visualizing, annotating and managing phylogenetic trees. *Nucleic Acids Res.* 40, W569–W572.

Zhang, X., Dai, L., Wu, Z., Jian, J., and Lu, Y. (2011). Expression pattern of heat shock protein 90 gene of humphead snapper *Lutjanus sanguineus* during pathogenic *Vibrio harveyi* stress. *J. Fish Biol.* 79, 178–193. doi: 10.1111/j.1095-8649.2011.03012.x

Zhu, Y., Zhu, J., Wan, X., Zhu, Y., and Zhang, T. (2010). Gene expression of sHsps, Hsp40 and Hsp60 families in normal and abnormal embryonic development of mouse forelimbs. *Toxicol. Lett.* 193, 242–251. doi: 10.1016/j.toxlet.2010.01.016

Zylicz, M., Yamamoto, T., McKittrick, N., Sell, S., and Georgopoulos, C. (1985). Purification and properties of the dnaJ replication protein of *Escherichia coli*. *J. Biol. Chem.* 260, 7591–7598.

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