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Genome-wide identification and structural analysis of heat shock protein gene families in the marine rotifer *Brachionus* spp.: Potential application in molecular ecotoxicology

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ABSTRACT

Heat shock proteins (Hsp) are class of conserved and ubiquitous stress proteins present in all living organisms from primitive to higher level. Various studies have demonstrated multiple cellular functions of Hsp in living organisms as an important biomarker in response to abiotic and biotic stressors including temperature, salinity, pH, hypoxia, environmental pollutants, and pathogens. However, full understanding on the mechanism and pathway involved in the induction of Hsp still remains challenging, especially in aquatic invertebrates. In this study, the entire Hsp family and subfamily members in the marine rotifers *Brachionus* spp., one of the cosmopolitan ecotoxicological model organisms, have been genome-widely identified. In *Brachionus* spp. Hsp family was comprised of Hsp10, small hsp (sHsp), Hsp40, Hsp60, Hsp70/105, and Hsp90, with highest number of genes found within Hsp40 DnaJ homolog subfamily C members. Also, the differences in the orientation of the conserved motifs within Hsp family may have induced differences in transcriptional gene modulation in response to thermal stress in *Brachionus koreanus*. Overall, Hsp family-specific domains were highly conserved in all three *Brachionus* spp., relative to *Homo sapiens* and across other animal taxa and these findings will be helpful for future ecotoxicological studies focusing on Hsps.

1. Introduction

Heat shock proteins (Hsps), also referred to as heat stress proteins and/or molecular chaperones, are a family of highly conserved proteins across primitive prokaryotes to higher level eukaryotes (Srivastava, 2002). The very first discovery of Hsp was from observation of puffs in polytene chromosomes in the salivary glands of *Drosophila* larvae under high temperature (Ritossa, 1962), which was later validated by the synthesis of new set of proteins called Hsps or stress proteins (Tissiers et al., 1974). In general, these family of proteins are cosmopolitan in all living organisms and are produced in response to thermal stress as well as various stressful conditions including toxins, oxidative conditions, hypoxia, nutritional deprivation, and infection (Santoro, 2000; Srivastava, 2002; Senf, 2013; Mahmood et al., 2014; Wang et al., 2017; Johnston et al., 2018), thus making Hsp as an extremely important "moonlighting" protein family. To date, Hsp families are categorized based on their molecular weights: Hsp10, small Hsp (sHsp), Hsp40, Hsp60, Hsp70/105, and Hsp90 (Feder and Hofmann, 1999). Despite growing evidences and accumulating results on Hsps in response to various environmental pollutants and stressors (Table 1), limited information is available on genome-wide identification of the entire Hsp families in aquatic organisms.

Due to increasing use of commercially manufactured products and heavy industrialization, living organisms are constantly being challenged by various stressors (e.g., metals, xenobiotics, radiation), which ultimately lead to deleterious effects on cellular infrastructure as well as homeostasis imbalance (Gupta et al., 2010). Fortunately for organisms, such disturbance in homeostasis and cellular damages are significantly reduced by establishment of adaptive cellular stress response pathways, however, only if the stress is below the threshold which differs in species-specifically. Among the various stress response pathways available, the heat shock response is considered crucial stress-response pathways (Westerheide and Morimoto, 2005). Also, various types of chemicals such as pesticides, metals, polycyclic aromatic hydrocarbons, and industrial discharges are known to induce heat shock response. Aside from xenobiotic stressors, also biotic (e.g. predation) and abiotic

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Table 1
Summary of modulations in heat shock proteins (Hsps) of some species with Hsps described in terms of protein levels and gene expression from previous studies.

Hsp family	Organism	Stressor	Responses	Reference
Hsp10	*Chironomus riparius* (Harlequin fly)	Toxic material (Azadirachtin) 0.2, 0.3, 0.4, 0.5, 1 mg/L, for 3, 24 h	Significant increase of mRNA at 0.05, 1 mg/L at 3 h (~2 folds each), at entire concentration for 24 h (~4 folds each) compared to the control.	Lencioni et al., 2016
	Chironomus riparius (Harlequin fly)	Toxic material (Butyl benzyl phthalate) 0.001, 0.01, 0.1, 1 μg/L for 48 h	Significant decrease of mRNA at whole body in every concentration (~0.5 folds decrease) in fourth instar larvae	Herrero et al., 2015
	Brachionus koreanus (Monogonont rotifer)	Toxic material (Triclocarban) 25, 50, 100, 200 μg/L for 24 h	Significant increase of mRNA in 200 μg/L (5 folds), significant decrease of mRNA in 25, 50 μg/L (~0.5 folds)	Han et al., 2016
	Tigriopus japonicus (Harpacticoid copepod)	Toxic material (Cd, As, Zn) 5, 10, 50, 100 μg/L for 96 h	Significant decrease of mRNA at whole body (Cd at 50 μg/L, As, Zn at 100 μg/L) (0.5 folds each)	Kim et al., 2014
	Paracyclopina nana (Cycloid copepod)	UV-B irradiation (306 nm) 50 μW/cm^2 for 1,3,6,12, and 24 h	Significant increase of mRNA at whole body in 6 h (2 folds)	Won et al., 2015
Hsp20	*Tigriopus japonicus* (Intertidal copepod)	Heat and Cold shock (15, 35 °C), 25 °C Control 30, 60, 90, 120 min	Significant increase of mRNA at whole body in 30, 60 90 min (low temperature, 3 folds), and 30, 60, 90, 120 min (high temperature, 5 folds)	Han et al., 2018
	Tigriopus kingsejongensis (Antarctic copepod)	Cold shock (4 °C), 14 °C Control 30, 60, 90, 120 min	Significant increase of mRNA at whole body in 30 min at low temperature (~2 folds)	
	Brachionus sp. (Monogonont rotifer)	0.1 mM Hydrogen peroxide for 3, 6, 12, and 24 h	Significant increase of mRNA at 12 h, 24 h (2 folds, 3 folds each)	Rhee et al., 2011
		Heat and cold shock (15, 37 °C) 25 °C Control for 10, 20, 30, 60, and 90 min	Significant increase of mRNA at 60, 90 min (3 folds, 4 folds each) at high temperature, significant increase of mRNA at 20, 30, 60, 90 min (2, 2, 3, 4 folds each)	
	Crassostrea gigas (Pacific oyster)	Toxic material (Tributyltin, Diuron, Irgarol), 0.01, 0.1, 1 μg/L for 96 h	Significant increase of mRNA in gill by TBT (0.1, 1 μg/L), Diuron (1 μg/L), and Irgarol (0.01, 1 μg/L) exposure	Park et al., 2016
Hsp40	*Litopenaeus vannamei* (Pacific white shrimp)	Heat shock (37 °C), 28 °C Control, for 6 h	Significant increase of mRNA in hepatopancreas (5 folds), muscle (8 folds), and gill (4 folds)	Chen et al., 2018
		Low pH (pH 6.8), pH 8.2 Control for 6 h	Significant increase of mRNA in hepatopancreas (2 folds) and gill (3 folds)	
	Crassostrea gigas (Pacific oyster)	Toxic material (Tributyltin, Diuron, Irgarol) 0.01, 0.1, 1 μg/L for 96 h	Down-regulation of Hsp40 by TBT (0.1 and 1 μg/L)	Park et al., 2016
	Pinctada fucata martensii (Pearl oyster)	Heat and cold shock (17, 32 °C) 22 °C Control for 6 h, 1 day, and 3 days	Significant increase of mRNA in gill (more than 2 folds, maximum 15 folds in 1 day at high temperature)	Wang et al., 2019
	Chironomus riparius (Harlequin fly)	Toxic material (Butyl benzyl phthalate) 0.01 μg/L ~ 100 mg/L for 24 h	Significant decrease of mRNA at whole body in every concentration (0.5– 0.8 folds decrease) in fourth instar larvae	Herrero et al., 2015
	Tigriopus japonicus (Intertidal copepod)	Toxic material (As, Cu, Ag, Zn) 5, 10, 50, 100 μg/L for 96 h	Significant decrease of mRNA at whole body in 100 μg/L (every material, ~0.5 folds)	Kim et al., 2014
	Tigriopus japonicus (Intertidal copepod)	Heat and Cold shock (15, 35 °C), 25 °C Control for 30, 60, 90, and 120 min	Significant increase of mRNA at whole body in 60, 90, 120 min at high temperature (~5 folds)	Han et al., 2018
Hsp60	*Crassostrea gigas* (Pacific oyster)	Toxic material (Tributyltin, Diuron, Irgarol), 0.01, 0.1, 1 μg/L for 96 h	Significant increase of mRNA in gill by TBT (1 μg/L), Diuron (1 μg/L), and Irgarol (1 μg/L) exposure (3 folds each)	Park et al., 2016
	Macrophthalmus japonicus (Mud crab)	Toxic material (Di-2-ethylhexyl phthalate, DEHP) 1, 10, 30 μg/L for 1, 4, and 7 days	Significant increase of mRNA in gill in 1 μg/L for 1, 4 days (8, 3 folds each), in 10 μg/L for 1 day (4 folds)	Park et al., 2020b
			Significant decrease of mRNA in gill in 10, 30 μg/L for 7 days (0.5, 0.25 folds each)	
Hsp70	*Litopenaeus vannamei* (Pacific white shrimp)	Heat shock (37 °C), 28 °C Control for 6 h	Significant increase of mRNA in hepatopancreas (7 folds), muscle (8 folds), and gill (9 folds)	Chen et al., 2018
		Low and High pH (pH 6.8, 8.9) pH 8.2 Control for 6 h	Significant increase of mRNA in hepatopancreas (3 folds), muscle (3 folds), and gill (2 folds) at low pH	
			Significant increase of mRNA in gill (3 folds) at high pH	
	Tigriopus japonicus (Intertidal copepod)	Toxic material (Ag, Cd, Cu, As, Zn) 5, 10, 50, 100 μg/L for 96 h	Significant increase of mRNA at whole body (Ag at 50, 100 μg/L, Cd at 100 μg/L, As at 10, 50, 100 μg/L, Cu, Zn at every concentration) (5 folds each)	Kim et al., 2014
	Chironomus riparius (Harlequin fly)	Toxic material (Butyl benzyl phthalate) 1 - 100 mg/L for 24 h	Significant increase of mRNA at whole body in every concentration (1.5– 4.5 folds) in fourth instar larvae	Herrero et al., 2015
		Toxic material (Azadirachtin) 0.2, 0.3, 0.4, 0.5, 1 mg/L for 3 and 24 h	Significant increase of mRNA at 1 mg/L at whole body for 3 h (~2 folds), at 0.3, 0.4, 0.5, 1 mg/L for 24 h (~4 folds each, 16 folds for 1 mg/L)	Lencioni et al., 2016
Hsp90	*Sparus aurata* (Gilthead sea bream)	Food-deprivation (12*h*), reduced-oxygen (3.5 mg/L O_2, 1 h), 5.3 mg/L O_2 Control Heat shock (25 °C, 1 h), 20 °C Control	Induction of HSP90 protein at whole body (200– 250% for food-deprivation, reduced-oxygen, heat shock each) in 22 days larvae	Cara et al., 2005
	Crassostrea gigas (Pacific oyster)	Toxic material (Tributyltin, Diuron, Irgarol), 0.01, 0.1, 1 μg/L for 96 h	Significant increase of mRNA in gill by TBT (0.1, 1 μg/L), Diuron (0.1, 1 μg/L), and Irgarol (1 μg/L) (3 folds each)	Park et al., 2016
	Tigriopus japonicus (Intertidal copepod)	Toxic material (Cu, Zn) 5, 10, 50, 100 μg/L for 96 h	Significant increase of mRNA at whole body (Cu at every concentration, Zn at 100 μg/L) (5 folds each)	Kim et al., 2014
	Tigriopus kingsejongensis (Copepod)	Heat and Cold shock (24, 4 °C), 14 °C Control for 30, 60, 90, and 120 min	Significant increase of mRNA at whole body in 90, 120 min at high temperature (~5 folds)	Han et al., 2018
			Significant decrease of mRNA at whole body in 90, 120 min at low temperature (~0.5 folds)	

(e.g. temperature) stress affect *Hsp* expression in zooplankton (Pijanowska and Kloc, 2004; Mikulski et al., 2009; Mikulski et al., 2011). Indeed, vast amount of studies in aquatic organisms have shown overexpression of Hsps in response to various stressors (Table 1). As anticipated, most of Hsp families (Hsp10, sHsp, Hsp40, Hsp60, Hsp70, and Hsp90) were differently modulated across various organisms, however, mostly up-regulated or overexpressed under stressful conditions. While various types of environmental xenobiotics have shown strong inducibility of Hsp synthesis in most reported cases, ubiquitous environmental pollutants and a potent procarcinogen and mutagen can possibly inhibit Hsp synthesis through the production of anti-Hsps (Wu and Tanguay, 2006), ultimately resulting in impaired cellular function

and defense. Thus, Hsps, involved in cellular protein homeostasis and repair (De Jong et al., 2008), are considered as a useful and potential biomarker tool for ecotoxicological studies and early detection of ecological risks in aquatic biota (Triebskorn et al., 2002; Yoshimi et al., 2002).

Among many aquatic model organisms, rotifers belonging to Lophophorata are one of the cosmopolitan species that are found in both fresh and marine environments (Segers, 2008; Mills et al., 2017). In fact, rotifers constitute an important part of zooplankton communities and have recently been widely applied in the field of ecology, evolution biology, and ecotoxicology (Harvell, 1990; Dahms et al., 2011; Gilbert, 2017). The monogonont rotifers are considered as suitable model organisms due to their morphological and ecophysiological characteristics including small body size (100 to 250 μm), ease of culture and maintenance, rapid developmental time to adult (~ 24 h), and high sensitivity in response to various toxic substances. Among the monogonont rotifers, *Brachionus* spp. (class Monogononta) play an important role in aquatic ecosystems and recently genome databases have been constructed for *Brachionus plicatilis* (http://rotifer.skku.edu:8080/Bp) (Han et al., 2019), *Brachionus koreanus* (http://rotifer.skku.edu:8080/Bk) (Park et al., 2020a), and *Brachionus rotundiformis* (http://rotifer.skku.edu:8080/Br) (Kang et al., 2020).

This study is the first study to report on the genome-wide identification of the entire Hsp families in aquatic invertebrate rotifer *Brachionus* spp. (*B. koreanus*, *B. plicatilis*, and *B. rotundiformis*). This study provides full characterization of the entire Hsps including genomic structure, conserved domains and motifs, and thermal-stress induced differential gene expression in the monogonont *B. koreanus*. Furthermore, the information provided on the rotifer Hsps will be helpful for a better understanding of mechanisms for the regulation of Hsps in various aquatic invertebrates under different stress conditions.

2. Materials and methods

2.1. Species and culture conditions

The monogonont rotifer *B. koreanus* was collected at Uljin (36°58′43.01″ N, 129°24′28.40″ E) in South Korea, while the two other rotifers *B. plicatilis* and *B. rotundiformis* were originally provided by Prof. Atsushi Hagiwara (Nagasaki University, Nagasaki, Japan) and their specific mitochondrial DNA gene cytochrome oxidase 1 (*CO1*) has been analyzed for further verification of species identification (Hwang et al., 2013; Hwang et al., 2014; Mills et al., 2017). These three different strains of rotifers were reared and maintained in filtered artificial seawater with 15 practical salinity units (psu) (TetraMarine Salt Pro, Tetra, Cincinnati, OH, USA) under a light:dark, 12:12 h photoperiod at 25 °C. The rotifers were daily fed with the green microalga *Tetraselmis suecica* (approximately 6×10^4 cells/mL). Rotifers have been maintained at the aquarium facility at the Department of Biological Sciences, Sungkyunkwan University (Suwon, South Korea).

2.2. Identification of heat shock protein families in three marine rotifers *Brachionus spp.*

Previously constructed genome databases of the three rotifer species (Han et al., 2019; Park et al., 2020b; Kang et al., 2020) have been employed for the computational analysis of Hsps in *Brachionus* spp. To obtain the entire available Hsps, *in-silico* screening of Hsps from various organisms including *Homo sapiens* was initially performed with accessible data obtained from NCBI (https://www.ncbi.nlm.nih.gov) and Ensembl (https://ensembl.org) as query sequences to search against whole genome and RNA-seq results of *Brachionus* spp. The screened Hsp candidates were subjected to BLAST analysis in the GenBank non-redundant (NR; including all GenBank, EMBL, DDBJ, and PDB) amino acid sequence database (http://blast.ncbi.nlm.nih.gov/). All acquired contigs were mapped to the genome for obtaining the complete sequence using Geneious (v.10.0.7; Biomatters Ltd., Auckland, New Zealand) (Kearse et al., 2012). Annotation and nomenclature of all Hsp genes were completed based on amino acid sequence similarities and phylogenetic analysis under the guidance of the recommendations from the HUGO Gene Nomenclature Committee, with an exception of small Hsp family, which has been annotated based on their molecular weights (kDa).

2.3. Phylogenetic analysis of the entire heat shock protein families

To better understand the presence and significance of the types of Hsp families identified from the three *Brachionus* spp. at the evolutionary scale, the protein sequences of the entire Hsps from *Brachionus* spp. were used to construct phylogenetic tree and compared with Hsps from other organisms; organisms used for identification and phylogenetic analysis are available in Supplementary file. The final phylogenetic tree was obtained and finalized after family-specific phylogenetic tree analysis using sequences retrieved from various organisms in NCBI database [sHSPs: honey bee *Apis mellifera*, mosquito *Anopheles gambiae*, silk moth *Bombyx mori*, fruit fly *Drosophila melanogaster*, shrimp *Penaeus vannamei*, brine shrimp *Artemia franciscana*, copepods *Eurytemora affinis*, *Tigriopus japonicus*, *Caligus rogercresseyii*, molluscs *Crassostrea virginica*, *Mytilus galloprovincialis*, *Mizuhopecten yessoensis*, *Haliotis discus hannai*, and human *Homo sapiens*] [Hsp40/DnaJ homolog subfamily A: arthropods *Agrilus planipennis*, *Anoplophora glabripennis*, *Aphis gossypii*, *Bactrocera dorsalis*, *Drosophila* spp., *Scaptodrosophila lebanonensis*, molluscs *Hyalella azteca*, *Octopus vulgaris*, *Pecten maximus*, African clawed frog *Xenopus laevis*, and human *H. sapiens*]. [Hsp40/DnaJ homolog subfamily B: arthropods *Acyrthosiphon pisum*, *Aedes aegypti*, *Apis dorsata*, *Apis mellifera*, *Bactrocera dorsalis*, *Bobus impatients*, *Bombyx mori*, *Caligus clemensi*, *Daphnia magna*, *Diaphorina citri*, *Drosophila* spp., *Eumeta japonica*, *E. affinis*, *Ixodes scapularis*, *Lepeophtherirus salmonis*, *Leptinotarsa decemplneata*, *Limulus polyphemus*, *Panaeus vannamei*, *Varroa destructor*, molluscs *Aplysia californica*, *Crassostrea gigas*, *Crassostrea virginica*, *Mizuhopecten yessoensis*, *Octopus vulgaris*, *Pomacea canaliculata*, and human *Homo sapiens*]. [Hsp40/DnaJ homolog subfamily C: *Drosophila grimshawi*, *I. scapularis*, *P. vannamei*, *C. virginica*, *M. yessoensis*, *O. vulgaris*, *Pecten maximus*, *P. canaliculata*, and *H. sapiens*]. [Hsp60/T-complex protein subunit: *Bombyx mori*, *C. gigas*, *Crassostrea glacialis*, *Culex quinquefasciatus*, *Tigiriopus japoncius*, *I. scapularis*, *Schistosoma japonicum*, *Danio rerio*, *Oryctolagus cuniculus*, and *H. sapiens*]. [Hsp70: *A. planipennis*, *A. franciscana*, *Cherax* spp., *D. magna*, *Eriocheir sinensis*, *E. affinis*, *Macrobrachium nipponense*, *Leptinotarsa decemlineata*, *H. azteca*, *Onthophagus taurus*, *Penaeus* spp., *Sus scrofa*, *Rattus norvegicus*, *C. gigas*, *M. yessoensis*, *O. vulgaris*, *A. californica*, *Haliotis fulgens*, *Biomphalaria glabrata*, and *H. sapiens*]. [Hsp90: *B. mori*, *A. franciscana*, *Chiromantes haematocheir*, *D. magna*, *E. sinensis*, *Eurytemora pacifica*, *Paracyclopina nana*, *P. vannamei*, *Portunus trituberculatus*, *Probambarus clarkia*, *Pseudodiaptomus annadalei*, *Tigriopus japonicus*, *Drosophila busckii*, *Operophtera brumata*, *Azumapecten farreri*, *Callistoctopus minor*, *Crassostrea ariakensis*, *C. gigas*, *M. yessoensis*, *M. galloprovincialis*, *O. vulgaris*, *Pinctada imbricate*, *P. canaliculata*, *Ruditapes philippinarum*, and *H. sapiens*].

The translated amino acids of Hsps from the three *Brachionus* spp. were first subjected to multiple sequence alignments using MAFFT and ClustalW algorithm for different Hsp families (e.g., Hsp10, sHsps, Hsp40/DnaJ homolog subfamilies, Hsp60/chaperonin 60, Hsp70/105, and Hsp90s). The aligned sequences were analyzed by maximum likelihood method with the parameter setting LG + G + I + F to generate the best fit phylogenetic tree using MEGA software ver.7.0 (Center for evolutionary Medicine and Informatics, Temple, AZ, USA) (Kumar et al., 2016). Each Hsp-family specific phylogenetic analysis was primarily performed using neighbor-joining method then finalized with maximum likelihood with bootstrapping value of 1000.

2.4. Bioinformatics analysis of heat shock protein family (Hsp) domains, conserved motifs, subcellular localization, and three-dimensional structure analysis of Hsps in Brachionus spp.

To identify whether the identified Hsps contain the putative conserved domains corresponding to each Hsp family (i.e., Hsp10, sHsp, Hsp40/DnaJ, Hsp60/TCP, Hsp70/105, and Hsp90s), structural domains were predicted with the SMART, PfamA, Phobius, and SuperFamily applications within the Geneious program (Geneious ver.10.2.3). To enhance the accuracy and reliability of the domains found, NCBI conserved domain database was used to validate the preliminary search results. Canonical motif consensus sequences reported from previous studies (Jungprung et al., 2019) have been used to analyze the presence of consensus motif in the identified Hsps from *Brachionus* spp. In addition, species-specific distribution of the conserved motif searches were performed using Motif-based sequence analysis tools (MEME) (Bailey et al., 2009) under discriminative mode to search against Hsps from *H. sapiens*, with parameter setting as follows: maximum length of the conserved motif, 8; minimum length, 3; number of motifs, 6.

To predict the subcellular localization of the entire Hsps, Hsps of *B. koreanus* was analyzed by WoLF PSORT (http://www.genscript.com/wolf-psort.html).

To show three-dimensional structure of Hsps in *B. koreanus*, structural analysis was performed using the identified Hsps and submitted to Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) to obtain the best fit 3D-structures.

2.5. RNA-seq analysis of transcriptomic changes in Brachionus koreanus under thermal stress

To investigate whether the identified *Hsps* in *B. koreanus* were responsive to thermal stresses, RNA-seq analysis was performed under 15 and 20 °C for 24 h, compared to the control 25 °C. The protocols for these experiments are provided in Supplementary information.

3. Results

3.1. Phylogenetic and in-silico synteny analysis of the entire heat shock protein families in Brachionus spp.

Based on previously reported genome databases of the three rotifers *B. plicatilis* (Han et al., 2019), *B. koreanus* (Park et al., 2020a), and *B. rotundiformis* (Kang et al., 2020), *in-silico* analyses of Hsps in the three *Brachionus* spp. have revealed a total of 206 *Hsp* genes (68, 69, and 69 Hsps distributed among *B. koreanus*, *B. plicatilis*, and *B. rotundiformis*, respectively) (Fig. 1 and Suppl. Fig. 1a–h). Specifically, 1 (*Hsp10*), 10 (*sHsps*), 32 (*Hsp40*s), 9 (*Hsp60*s), 13 (*Hsp70/105* s), and 4 (*Hsp90*s) genes were found in *B. koreanus*, while higher number of s*Hsp* (10 VS. 13) were found in *B. plicatilis* and *B. rotundiformis*, respectively. In addition, less number of Hsp40, specifically DnaJ homolog subfamily B, were identified in *B. plicatilis* compared to *B. koreanus* and *B. rotundiformis* (Table 2). Overall, one-to-one orthologous relationship has been shown in Hsp10, Hsp40 (DnaJ homolog subfamily C), Hsp60, and Hsp90 families in *Brachionus* spp. Within each Hsp family, few Hsp families (Hsp40/DnaJ, Hsp60s, and Hsp70s) were further divided into smaller subgroups with largest group of homolog members present within Hsp40/DnaJ homolog family in the *Brachionus* spp. As shown in the phylogenetic tree, DnaJ A, B, and C groups have been diversified into different forms, DnaJA1-3, DnaJB2/3/6/7/8, 4, 9, 11, 12/14, and 13, DnaJC with the largest number of C members (a total of 17) present (DnaJC1-5, 7-13, 16, 17, 21, 22, and 27). In addition, Hsp60 and its evolutionary homolog T-complex protein subunits were further classified into alpha, beta, delta, epsilon, eta, gamma, theta, and zeta in *Brachionus* spp.

Synteny analysis of the entire Hsps was conducted by confirming the relative localization of the *Hsps* in the *Brachionus* spp. genome (Fig. 2). In detail, a total of 67 *Hsps* of *B. koreanus* and 69 *Hsps* of *B. plicatilis* and *B. rotundiformis* were mapped onto 32, 45, and 21 scaffolds in *B. koreanus*, *B. plicatilis*, and *B. rotundiformis*, respectively. (Fig. 2A, B, and C). Synteny analysis revealed unique features in the location of *Hsps* in species-specifically manner. In *B. koreanus*, *DnaJA2* likes, *DnaJB2/3/6/7/8* likes were highly duplicated within the same scaffolds (SC2 and SC19), while in *Hsps* in *B. plicatilis* were highly dispersed throughout scaffolds, and *Hsps* in *B. rotundiformis* were highly clustered, with highly duplicated *DnaJB2/3/6/7/8* like genes within relatively short span. In addition, synteny analysis showed similar localization of specific *Hsps* (*sHsp*, *DnaJC10*, *13*, and *Hsp70* [*HYOU1*]) within the same scaffolds (Bk SC5 and Bp SC9) and *Hsp68* kDa and *Hsp70 14-likes* were localized in close proximity to *DnaJC2* and *DnaJC27*, respectively, in the three *Brachionus* spp. (Bk SC48, Bp SC95, and Br SC) (Bk SC30, Bp SC67, and Br SC31). Moreover, Hsp10 and Hsp60 in all three *Brachionus* spp., showed close localization in either head to head or tail to tail orientation.

3.2. Computational genomic structural analysis of heat shock proteins in Brachionus spp.

Genomic structure of the genome-widely identified *Hsps* in *Brachionus* spp. showed high conservation of the genomic structures including the number of exons, overall lengths, and the direction of the genes (Table 2) within each subfamily. In general the overall length of the entire Hsp families ranged from the shortest Hsp10 (306 bp) to the longest Hsp40 family DnaJ homolog subfamily C13 (approximately 6 k to 7 k bp). In particular Hsp10, Hsp60, and Hsp70s found in the three *Brachionus* spp. shared identical number of exons and overall lengths, with few exceptions in Hsp70 family members. Comparative analyses of open reading frames and coding sequence lengths resulted in family-specific variation in intron lengths. For example, sHsps, which in general, only have single or two introns, comprised of short introns in three *Brachionus* spp., while Hsp families such as Hsp40/DnaJC members have quite long intron lengths. In this study, intronless *Hsps* in the three *Brachionus* spp., were *Hsp10*, *Hsp40/DnaJA1*, *DnaJB2/3/6/7/8-like 1* and *2*, *DnaJB9*, *DnaJC27*, *Hsp70-BIP*, *HSC70* (like 1, 2, and 3), and *Hsp90 alpha1* and *2*. Also, the direction of gene has also been analyzed and showed no distinctive pattern among *Hsps*, in either species and Hsp family specific manner.

3.3. In-silico domain and multiple sequence alignment analysis of heat shock protein families in Brachionus spp.

To demonstrate conserved domains of Hsp across various organisms including in aquatic invertebrates, domain analyses using CDD and InterproScan were employed. In a total, the entire Hsps in the three *Brachionus* spp. were largely divided into 6 families: Hsp10/chaperonin10, sHsp/α-crystallin domain, Hsp40/DnaJ, Hsp60/chaperonin 60, Hsp70/105, and Hsp90. Each Hsp family contained distinct domains (Suppl. Fig. 2) similar to that of *H. sapiens*. Hsp20 or α-crystallin domain (ACD) containing protein family of *Brachionus* spp., clearly featured tripartite architecture possessing ACD consisting of approximately 80 aa in lengths, flanked by N- and C-terminal extensions (Suppl. Fig. 2a). Unlike other canonical domains of a-crystallin or small Hsps, rotifer sHsps showed the presence of multiple ACD domains.

In the rotifer *Brachionus* spp., three Hsp40 subfamilies DnaJ A, B, and C have been identified through *in-silico* analysis. The first type DnaJ homolog subfamily A contains typical DnaJ N-terminal domain (Suppl. Fig. 2b), with its signature motif histidine, proline, and aspartate (HPD). In addition to the canonical domain regions, DnaJ A also shows the presence of both glycine/phenylalanine-rich regions and cysteine-rich zinc finger motif (CXXCXGXG) (Suppl. Figs. 2b-1 and 3b). One key features of DnaJ was high composition of amino acids with hydrophobic side chains (e.g., A, D, L, V, Y). The second type DnaJ homolog subfamily B showed presence of typical DnaJ N-terminal and peptide

Fig. 1. Phylogenetic tree analysis of the entire heat shock protein (Hsp) families obtained from *in silico* analysis of genome databases of *Brachionus koreanus*, *Brachionus plicatilis*, and *Brachionus rotundiformis* using maximum likelihood model (LG + G + I + F). Each Hsp family is indicated by the colors shown in the color legend box below.

Table 2
Genomic structure analysis of the entire heat shock proteins in *Brachionus* spp.

Family	*Brachionus koreanus*					*Brachionus plicatilis*					*Brachionus rotundiformis*				
	Gene	Exon	Str	CDS (bp)	ORF (bp)	Gene	Exon	Str	CDS (bp)	ORF (bp)	Gene	Exon	Str	CDS (bp)	ORF (bp)
Hsp10	*Hsp10*	1	R	306	306	*Hsp10*	1	F	306	306	*Hsp10*	1	R	306	306
Hsp20/small Hsp	*sHSP27.6 kDa*	1	F	738	738	*sHSP 16.3 kDa*	1	R	432	432	*sHSP 25.9 kDa*	2	R	678	730
	sHSP 28,0.3 kDa	2	F	735	784	*sHSP 24.6 kDa*	1	F	648	648	*sHSP 27.4 kDa*	1	F	732	732
	sHSP 29.1 kDa	1	F	759	759	*sHSP 25.4 kDa*	1	F	732	732	*sHSP 29.1 kDa*	1	F	759	759
	sHSP 31.8 kDa	1	F	825	825	*sHSP 26.32 kDa*	2	R	684	735	*sHSP 30.8 kDa*	1	F	810	810
	sHSP 31.9 kDa	1	F	825	816	*sHSP 31.08 kDa*	2	F	804	857	*sHSP 31 kDa*	1	F	804	807
	sHSP 34.1 kDa	1	R	912	861	*sHSP 31.09 kDa*	2	F	804	854	*sHSP 31.1 kDa*	2	F	837	855
	sHSP 34.9 kDa	2	R	961	963	*sHSP 31.4 kDa*	1	R	813	813	*sHSP 31.2 kDa*	2	R	816	885
	sHSP 38.6 kDa	1	R	990	990	*sHSP 31.7 kDa*	1	R	825	825	*sHSP 31.9 kDa*	1	R	807	816
	sHSP 53.4 kDa	2	F	1419	1468	*sHSP 31.9 kDa*	1	F	816	816	*sHSP 32.6 kDa*	2	R	834	881
	sHSP 58 kDa	2	F	1566	1626	*sHSP 32.5 kDa*	2	R	834	887	*sHSP 34.5 kDa*	1	R	882	882
						sHSP 34.8 kDa	1	F	894	894	*sHSP 35.2 kDa*	2	R	915	967
						sHSP 38.8 kDa	1	R	990	990	*sHSP 38.6 kDa*	1	F	990	990
						sHSP 53.9 kDa	2	F	1446	1521	*sHSP 57.5 kDa*	2	R	1521	1634
						sHSP 55.7 kDa	2	R	1509	1646					
Hsp40/DnaJ	*A1*	1	R	1200	1200	*A1*	1	R	1200	1200	*A1*	1	R	1200	1200
	A2-like 1	5	R	1224	1425	*A2-like 1*	5	F	1224	1424	*A2-like 1*	5	R	1071	1286
	A2-like 1–2	3	R	1224	1699										
	A2-like 3	2	F	1317	1369	*A2-like 3*	2	R	1308	1561	*A2-like 3*	2	R	1317	1369
	A3	3	R	1425	1677	*A3*	3	R	1425	1685	*A3*	3	R	1425	1676
	B2/3/6/7/8 like-1	1	F	696	696	*B2/3/6/7/8 like-1*	1	F	702	702	*B2/3/6/7/8 like-1*	1	F	639	639
	B2/3/6/7/8 like-2	1	R	657	657	*B2/3/6/7/8 like-2*	1	R	663	663	*B2/3/6/7/8 like-2*	1	R	678	678
	B2/3/6/7/8 like-3	2	F	822	892	*B2/3/6/7/8 like-3*	2	R	771	2006	*B2/3/6/7/8 like-3*	2	F	825	881
	B4-like	2	R	1023	1074	*B4-like*	2	R	1023	1134	*B4-like*	2	F	1020	1077
	B9	1	R	534	534						*B9*	1	R	540	540
	B9-like	4	F	597	764	*B9-like*	4	F	597	749	*B9-like*	4	F	570	732
	B11-like	4	F	1071	1238	*B11-like*	4	R	1080	1240	*B11-like*	4	F	1080	1266
	B12/14-like	4	F	1104	2235	*B12/14-like*	4	R	1098	4130	*B12/14-like*	4	F	1092	1600
	B13-like	4	F	978	1998	*B13-like*	3	R	1014	1111	*B13-like*	3	R	1014	1126
	C1	5	R	1260	2708	*C1*	5	R	1257	5790	*C1*	5	F	1332	2726
	C2	2	R	1869	4812	*C2*	2	R	1845	3678	*C2*	2	R	1842	3061
	C3	6	R	1911	3827	*C3*	4	R	1479	3371	*C3*	4	R	1473	1656
	C4	6	R	729	1778	*C4*	6	R	729	1024	*C4*	6	F	732	998
	C5	4	F	645	1002	*C5*	3	F	609	1030	*C5*	3	R	609	969
	C7	6	F	1485	1849	*C7*	6	R	1497	1846	*C7*	6	F	1500	1787
	C8	3	R	948	1582	*C8*	2	R	1053	1138	*C8*	3	F	1017	2686
	C9	2	R	1029	1078	*C9*	2	F	1047	1111	*C9*	2	R	1050	1100
	C10	12	R	2523	4198	*C10*	12	F	2535	4017	*C10*	15	F	4722	7684
	C11	9	R	1740	2177	*C11*	9	F	1740	4901	*C11*	9	F	1740	2174
	C12		Unable to map			*C12*	4	F	456	4587	*C12*	4	R	459	1728
	C13	6	F	6876	8454	*C13*	4	R	6630	8575	*C13*	7	R	7008	7746
	C16	7	F	2535	2845	*C16*	8	F	2475	2839	*C16*	7	R	2454	2922
	C17	1	R	906	906	*C17*	1	R	909	909	*C17*	1	R	894	894
	C21	8	R	1515	3144	*C21*	7	R	1515	2392	*C21*	8	F	1527	1905
	C22	3	F	1143	1249	*C22*	3	R	1068	1164	*C22*	3	F	1068	1167
	C27	1	R	795	795	*C27*	1	R	795	795	*C27*	1	F	795	795
Hsp60/Chaperonin	*HSP60*	2	F	1746	1800	*HSP60*	2	R	1746	1807	*HSP60*	2	F	1737	1794
	TCP-1 alpha	4	F	1647	1812	*TCP-1 alpha*	4	R	1647	1865	*TCP-1 alpha*	4	F	1647	1820
	TCP-1 beta	2	R	1596	1653	*TCP-1 beta*	2	R	1596	1675	*TCP-1 beta*	2	R	1596	1651
	TCP-1 delta	2	R	1620	1680	*TCP-1 delta*	2	F	1644	1702	*TCP-1 delta*	1	F	1641	1641
	TCP-1 epsilon	2	F	1635	1713	*TCP-1 epsilon*	2	F	1635	1839	*TCP-1 epsilon*	2	F	1635	1759
	TCP-1 eta	2	R	1506	1562	*TCP-1 eta*	2	R	1506	1577	*TCP-1 eta*	2	F	1506	1552
	TCP-1 gamma	3	R	1674	1844	*TCP-1 gamma*	3	F	1674	1891	*TCP-1 gamma*	3	R	1674	1805
	TCP-1 theta	8	F	1635	2037	*TCP-1 theta*	8	R	1635	2050	*TCP-1 theta*	8	F	1635	2003
	TCP-1 zeta	2	R	1596	1652	*TCP-1 zeta*	2	F	1596	1648	*TCP-1 zeta*	2	R	1596	1651

(*continued on next page*)

Table 2 (*continued*)

Family	*Brachionus koreanus*					*Brachionus plicatilis*					*Brachionus rotundiformis*				
	Gene	Exon	Str	CDS (bp)	ORF (bp)	Gene	Exon	Str	CDS (bp)	ORF (bp)	Gene	Exon	Str	CDS (bp)	ORF (bp)
Hsp70	*ER BIP1a-like*	1	F	1971	1971	*ER BIP1a-like*	1	F	1971	1971	*ER BIP1a-like*	1	R	1971	1971
	ER BIP1b-like	1	R	1977	1977	*ER BIP1b-like*	1	R	1971	1977	*ER BIP1b-like*	1	R	1977	1977
	ER BIP1b-like2	1	F	1977	1977										
	Grp75	2	F	1980	2035	*Grp75*	2	F	2001	2069	*Grp75*	2	F	1980	2032
	HSC70 like 1	1	F	1947	1947	*HSC70 like 1*	1	R	1959	1959	*HSC70 like 1*	1	F	1947	1947
	HSC70 like 2	1	R	1911	1911	*HSC70 like 2–1*	1	F	1920	1920	*HSC70 like 1–2*	1	F	1947	1947
						HSC70 like 2–2	1	F	1677	1677	*HSC70 like 2*	1	R	1920	1920
	HSC70 like 3	1	F	1860	1860	*HSC70 like 3*	1	R	1860	1860	*HSC70 like 3*	1	F	1860	1860
	HSC70 like 4	4	R	2262	3564	*HSC70 like 4*	3	F	1902	2021	*HSC70 like 4*	3	R	1902	2015
	HSC70 like 5	2	R	2160	5275										
	HSP70 4 L-like	6	F	2856	4486	*HSP70 4 L-like*	4	R	2502	5246	*HSP70 4 L-like*	5	F	2418	3261
	HSP 68 kDa-like	5	F	2727	5760	*HSP 68 kDa-like*	5	F	2778	8313	*HSP70 68 kDa-like*	5	F	2739	3327
	HSP70 14-like	7	F	1482	1992	*HSP70 14-like*	7	F	1434	1863	*HSP70 14-like*	7	F	1488	1805
	HYOU1	16	F	2853	3656	*HYOU1*	16	R	2820	3638	*HYOU1*	15	R	2721	3508
Hsp90	*HSP90alpha 1*	1	R	2163	2163	*HSP90alpha 1*	1	F	2172	2172	*HSP90alpha 1*	1	F	2163	2160
	HSP90alpha 2	1	R	2163	2163	*HSP90alpha 2*	1	R	2169	2169	*HSP90alpha 2*	1	F	2163	2169
	HSP90B1 Grp94	3	F	2412	2595	*HSP90B1 Grp94*	3	R	2427	4686	*HSP90B1 Grp94*	3	R	2412	2564
	TRAP1	5	F	2031	2276	*TRAP1*	5	F	2061	2269	*TRAP1*	5	R	2031	2270

binding domain, G/F-rich regions with HPD motif, however few members showed absence of G/F-rich regions (DnaJB12/14-like). However, lack of cysteine-rich zing finger motif was observed in DnaJ type B of *Brachionus* spp., compared to DnaJ A family (Suppl. Figs. 2b-2 and 3c). The last type identified in the *Brachionus* spp., showed no presence of conserved motif except for DnaJ domain with HPD motif. One of the interesting features of DnaJ type C was the presence of tetratricopeptide domain, which was present only in DnaJ type C member 3 and 7 of *Brachionus* spp. (Suppl. Figs. 2b-3 and 3d).

Hsp60 and its homologs T-complex protein 1 subunit in *Brachionus* spp. showed much more complex domain structures compared to other smaller Hsp families (Suppl. Figs. 2c and 3e) with close association with Hsp10. Overall structures of Hsp60 family (i.e., Hsp60 and T-complex protein 1 subunit members) contained three large domains: equatorial ATP-binding, an intermediate hinge domain, and an apical domain. One of the key signature motif of Hsp60 mitochondrial was identified (AAVEEGIVPGGG) which was conserved across the three *Brachionus* spp.

Hsp70/105 family in *Brachionus* spp. consisted of four domains; ATPase domain, middle domain with protease sensitive sites, peptide binding domain, and G/P rich C-terminal containing EEVD motif which is known to assist in co-chaperone binding (Suppl. Figs. 2d and 3f). Due to different homologs present within Hsp70 family, homolog-specific signature motifs were identified. Heat shock cognate 70 (Hsc70-likes) showed three signature motif I (IDLGTTF/YS), motif II (LIFDLGGGTF-DVSIL), and motif III (I[*V*]VLVGGST RIPKVQK). Immunoglobulin heavy chain binding protein (BIP), a HSC70-3 homolog, had one unique signal peptide for secretion into ER at N-terminus MKILVLLSLLAVAFA, ER retention tetrapeptide KDEL at C-terminus, and multiple conserved signature motifs IDLGTTYS, VYDLGGGTFDI(*V*)SIL. Also, ATP/GTP binding motif AEAYLEKK was only observed specifically in BIP1alpha. HSC70-5 or glucose-regulated protein 75 (Grp75) also contained multiple signature motifs in the three *Brachionus* spp., IDLGTTNS, VYDL-GGGTFDI(*V*)SIL, and ILLVGGVTRMPKVQ. Hypoxia up-regulated protein 1 (HYOU1) contained signal peptide for secretion to ER IILVGGNTRMPAVQA, which is slightly different from that of BIP motif.

Hsp90 family, similar to Hsp70/105 family, is comprised of multiple homologous families, resulting in different domain structures (Suppl. Figs. 2e and 3g). Hsp90 family, in general, consisted of three domains ATPase domain/Dna topoisomerase II/histidine kinase region, ribosomal protein domain, and C-terminal. Furthermore, differences in the signature motifs were identified. Grp94 showed KKIL (KKXX) ER retention motif, rather than the canonical KDEL. Hsp90 alpha and beta contained C-terminal MEEVD, while TRAP1 lacked both MEEVD, KDEL motifs. Thus, based on the multiple sequence alignment, the only MEEVD motif containing Hsp90 homologs are indeed the cytosolic Hsp90, alpha and beta isoforms of Hsp90s in *Brachionus* spp., while the Grp94 with KDEL-like motif for ER retention are ER homolog, and lastly the TRAP1 lacking all of the signature motifs found in other Hsp90s, is indeed the mitochondrial homolog of Hsp90 family.

3.4. Recognition of conserved sequences in heat shock proteins (Hsp40, Hsp60, Hsp70, and Hsp90s) across the three Brachionus spp.

To observe Hsp family-specific sequence conservation, motif analysis of Hsps in the three *Brachionus* spp. was conducted using MEME suite, relative to those of Hsps from *H. sapiens* (Suppl. Fig. 4). Overall, each Hsp family showed high conservation of sequences despite differences in sub-members but also few exceptional cases were observed in each Hsp family. For example, both DnaJA1 and DnaJA3 showed no presence of T/RK/QNM/LA/V/DY/L/F P/FL/MKV/M motif compared to DnaJA2 members. In addition, within Hsp70 family, only a single motif region was identified in Hsp70 4 L and HYOU1, compared to other Hsp70 subfamilies. Among Hsp90 families, TRAP1 only shared three specific consensus motif sequences among other members of Hsp90 subfamilies. More importantly, however, differences in the order of conserved motif sequences were shown in Hsp families. As shown in DnaJ subfamiliy A members, the conserved motifs found were different from the order of motif locations (Suppl. Fig. 4b). Also, the conserved consensus motifs found within Hsp60 families showed the highest variations in the order of motif locations compared to highly conserved Hsp families such as Hsp70s and Hsp90s.

3.5. Analysis of subcellular localization and 3-D structural prediction of heat shock proteins of Brachionus koreanus

Subcellular localization of the genome-widely identified *Hsps* in *B. koreanus* has been predicted using WoLF PSORT protein localization predictor (Suppl. Table 1). Based on the prediction algorithm, differences in the subcellular localization of each Hsp family have been

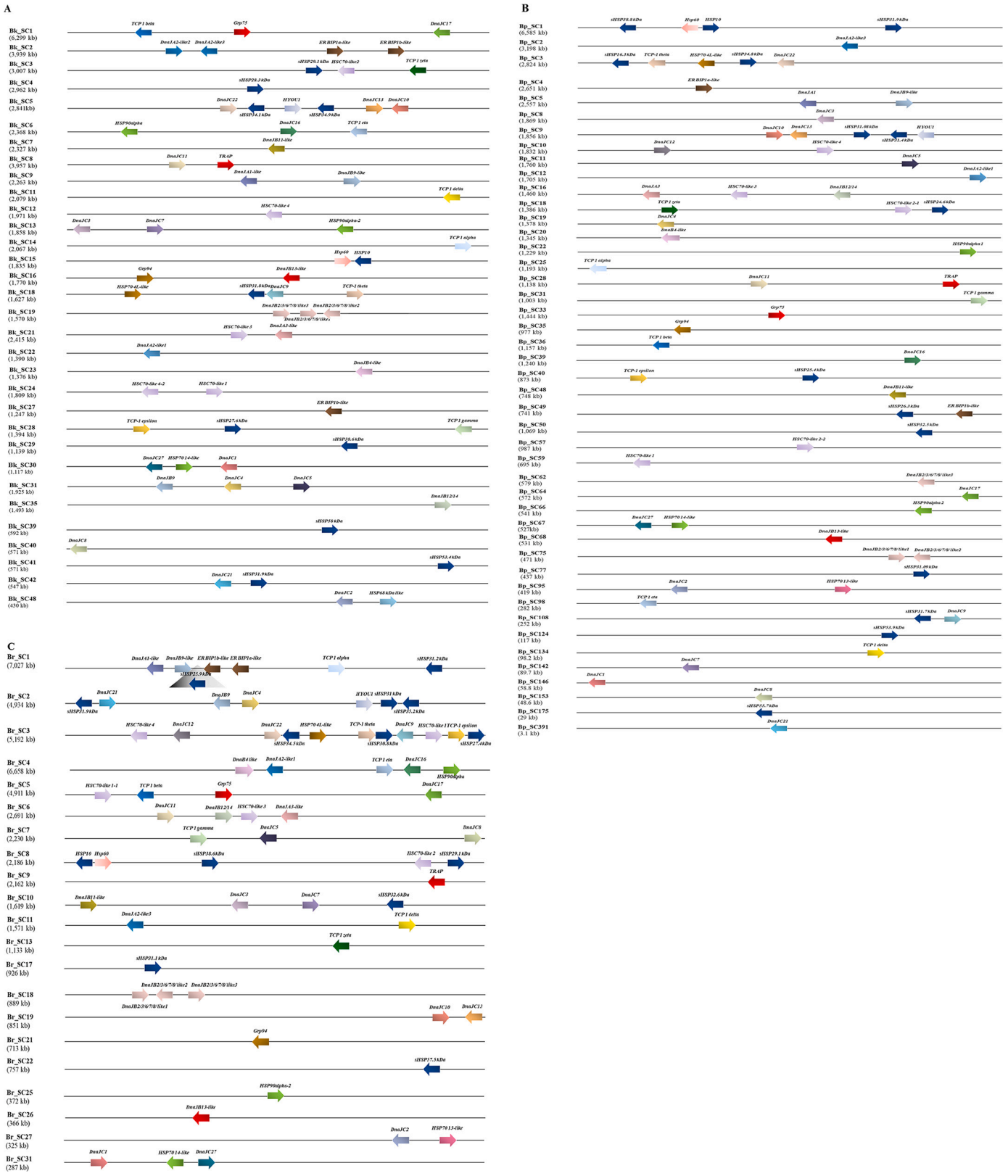

Fig. 2. Localization of gene analysis of the entire heat shock proteins identified in A) *Brachionus koreanus* B) *Brachionus plicatilis*, and C) *Brachionus rotundiformis*. Genes are represented by specific colored arrows, indicating the direction of the reads in each scaffold. Each gene locus is presented in proportion to the length of the scaffold. Clustered regions are represented are indicated by a set of vertical bars.

demonstrated in family specific manner. Chaperonin 10 (Hsp10) and majority of sHsps were localized in mitochondria or nucleus, whereas Hsp40 subfamilies were localized in a distinctive manner, where majority of DnaJA and DnaJB were localized in cytoplasm and nucleus but few exceptional cases have been observed among different Hsp families. Based on the computational analysis, DnaJA3 is highly localized in mitochondria and DnaJB9-like, DnaJC1, DnaJC5, and DnaJC10 are highly localized in extracellular matrix, whereas DnaJC4 and 7 are

Hsp10/Chaperonin 10

	20°C	15°C
HSP10	1.240321885	1.763184366

Hsp40/DnaJ homolog subfamily

		20°C	15°C
DnaJ A	*DnaJA1-like*	1.078296001	1.208397259
	DnaJA2-like 1	1.344023324	1.451895044
	DnaJA2-like1-2	1.387290012	1.286365412
	DnaJA2-like 3	1.294492425	1.290244938
	DnaJA3-like	1.293673825	1.227510157
DnaJ B	*DnaJB2/3/6/7/8/ like1*	1.105555556	1.39962963
	DnaJB2/3/6/7/8/ like2	0.74801061	0.895335986
	DnaJB2/3/6/7/8/ like3a	1.691770679	1.592764967
	DnaJB2/3/6/7/8/-like3b	0.872179126	0.840267983
	DnaJB4-like	1.268082448	1.261086821
	DnaJB9	0.189944134	1.279329609
	DnaJB9-like	0.946120482	0.949783133
	DnaJB11	1.191117764	1.25
	DnaJB12/14-like	1.036996287	1.204511071
	DnaJB13-like	0.906998679	0.731843048
DnaJ C	*DnaJC1*	1.093374153	0.958889132
	DnaJC2	1.15483871	2.3
	DnaJC3	1.275621335	1.127059481
	DnaJC4	1.267817618	1.358565737
	DnaJC5	1.017331023	0.83044483
	DnaJC7	0.963489028	1.077483383
	DnaJC8	0.9655706	0.867988395
	DnaJC9	0.984674823	0.939085641
	DnaJC10	0.696491745	0.49660967
	DnaJC11	1.134146341	1.06039489
	DnaJC13	1.211072664	0.869204152
	DnaJC16	0.828735034	0.745965643
	DnaJC17	1.092376682	1.357847534
	DnaJC21	0.826032541	0.826658323
	DnaC22	0.700583279	0.464031108
	DnaJC27	0.973972603	0.902739726

0 1 2

DEG analysis of Hsps in response to thermal stress

	20°C		15°C
DnaJB2/3/6/7/8/ like3	1.691770679	*DnaJC2*	2.3
HSP60 mito	1.556926528	*TCP1 beta*	2.003883495
TCP1 beta	1.877669903	*TCP1 delta*	1.9155785
TCP1 delta	2.358335864	*TCP1 eta*	2.436828166
TCP1 epsilon	1.585448144		
TCP1 eta	2.253154697		
TCP1 zeta	1.549357602		
HSC70-like 2	0.518313591		
HSC70-like 4-2	1.732799574		
Grp94	2.362910382		

0 1 2

Small Hsp

	20°C	15°C
sHSP 29.1	0.811672106	0.420349395
sHSP 31.8	0.740556962	1.264
sHSP 34.1	0.884350133	1.005229254
sHSP 28.3	0.913043478	0.550724638
sHSP34.9	0.846036585	0.990091463
sHSP58	0.842964053	0.649613544
sHSP31.9	0.944576363	0.916991751
sHSP27.6	0.924134036	0.608339608
sHSP53.4	0.876503373	0.900117336
sHSP38.6	0.922962382	0.983777429

Hsp60/T-complex subunit

	20°C	15°C
HSP60	1.556926528	1.311833988
TCP1 alpha	1.459960581	1.518723994
TCP1 beta	1.877669903	2.003883495
TCP1 delta	2.358335864	1.9155785
TCP1 epsilon	1.585448144	1.47161508
TCP1 eta	2.253154697	2.436828166
TCP1 gamma	1.044247788	0.789118322
TCP1 theta	1.407892994	1.440206595
TCP1 zeta	1.549357602	1.392505353

Hsp70

	20°C	15°C
ER BIP1a-like	1.259283327	1.199034534
ER-BIP1b-like	0.783595642	0.61440678
ER BIP1b-like	0.80737872	0.655320016
Grp75	1.045784398	0.77160317
HSC70-like 1	1.266201735	1.180756364
HSC70-like 2	0.518313591	0.721321696
HSC70-like 3	0.609848485	0.386363636
HSC70-like 4	0.693181818	1.5
HSC70-like 4-2	1.732799574	1.604820215
HSP704L-like	1.082715633	0.931940701
HSP70 68 kDa	0.613636364	0.764772727
HSP70 14-like	1.034679335	1.182897862
HYOU1	1.053591366	0.789728322

Hsp90

	20°C	15°C
HSP90 alpha 1	1.474635348	1.192271263
HSP90 alpha 2	0.547248127	0.492911433
Grp94	2.362910382	1.709849157
TRAP1	0.956957691	0.778674493

0 1 2

(caption on next page)

Fig. 3. RNA-seq analysis of the entire heat shock proteins in thermal-stress exposed *Brachionus koreanus*. *B. koreanus* was exposed to 15 °C and 20 °C for 24 h, compared to the control (25 °C). The relative expression levels are represented by heat map (red: up-regulation, blue: down-regulation, white: control) relative to the control.

highly localized in ER and Golgi apparatus, respectively. Hsp60 is highly localized in mitochondria, while its homologs were highly localized in cytoplasm/mitochondria. Hsp70/105 and Hsp90s showed different localization.

To exemplify the genome-widely identified Hsps through *in-silico* analysis, secondary structural analyses of Hsps of *B. koreanus* have been performed through computational 3-dimensional modeling (Suppl. Fig. 5). The secondary structure of the majority Hsps encoded by *B. koreanus* was mainly comprised of alpha helix, which accounted for 10 to 72%, whereas beta strand accounted for 0 to 52%. Information is provided in Suppl. Table 2.

3.6. RNA-seq analysis of heat shock proteins of Brachionus koreanus under thermal stress

To validate the inducibility of *Hsp*s identified *in-silico*, *B. koreanus* was exposed to different temperature conditions (20 and 15 °C) compared to the control (25 °C) at 24 h (Fig. 3). Most of the *Hsp*s were differently modulated, while significant modulations in transcription levels were observed in response to different temperatures. Under minor temperature reduction (20 °C vs. 25 °C), *DnaJB2/3/6/7/8/like 3a* and *Hsp60*, *TCP1 beta*, *delta*, *epsilon*, *eta*, and *zeta*, and *HSC70 like2*, *4-2*, and *Grp94* were significantly up-regulated ($P < 0.05$), while only *DnaJC2* and *TCP1 beta*, *delta*, and *eta* were significantly up-regulated ($P < 0.05$) under dramatic temperature reduction (15 °C vs. 25 °C). Differentially expressed genes (DEGs) analyses have shown that most *Hsp60*s were up-regulated to moderately low temperature (20 °C), while under low temperature (15 °C), dnaJC2 and Hsp60 member of TCP1 beta, delta, and eta were significantly up-regulated compared to the control.

4. Discussion

Brachionus spp., as a cosmolitan aquatic invertebrate, are distributed ubiquitously and have been widely used in aquaculture industry (Dhont et al., 2013) and ecotoxicology model species due to their rapid population growth and sensitivity and responsiveness to various environmental pollutants (Dahms et al., 2011). Due to their ubiquitous occupancy in the aquatic environment and species-specific feeding behavior as filter feeders (Arndt, 1993), they are likely to be heavily exposed to various environmental pollutants. Furthermore, due to constant increase in environmental pollution, unexpected changes in microbial ecosystem and virioplankton community are inevitable, which could cause deleterious effects on higher trophic organisms. Thus, it is important to revisit and reinvestigate one of the widely distributed yet, highly conserved stress proteins. In this study, we characterized a total of 209 *Hsp* genes were identified in three *Brachionus* spp., specifically 70, 70, and 69 *Hsp*s were distributed in *B. koreanus*, *B. plicatilis*, and *B. rotundiformis*, respectively.

Hsp10 is a mitochondrial-resident protein, which functions in protein assembly and disassembly and folding by co-chaperoning with mitochondrial Hsp60 (Jia et al., 2011). Besides its protein-structurally related functions, solitary function of Hsp10 also includes protection of cells against stresses caused by infection and inflammation (Cappello, 2003; Johnson et al., 2005), which has been strongly suggested by its interchangeable cellular locations either in cytosol or extracellular space. Moreover, such unrestricted localization of Hsp10 has been demonstrated by its expression in cytosol, cell surface, peripheral blood, which is closely associated with various immunomodulatory activities and cellular regulation (Cappello et al., 2005; Shamaei-Tousi et al., 2007). Though no functional studies are shown in this study based on genomic structure analysis of Hsp10 and mitochondrial Hsp60 genes, their localization within the same scaffold (i.e., Bk SC15, Bp SC1, and Br SC8) could possibly suggest the significance of co-chaperoning function of Hsp10 with Hsp60 in parallel with human Hsp10 and Hsp60 (Jia et al., 2011), however, the only head to head structures were shown in *B. koreanus*, while head to head on opposite directions were shown in *B. plicatilis* and *B. rotundiformis*. Since there is no presence of N-terminal signal peptide for secretion in Hsp10 sequences, its release into extracellular space is likely to be performed by non-canonical ER-Golgi-independent pathway, and further investigations on this translocations are required to fully understand the mechanisms how Hsp10s are highly found both in mitochondria and extracellular space.

4.1. Hsp20/small heat shock proteins

sHsp family with molecular mass from 10 to 30 kD (also known as Hsp20 family), are relatively small mass stress-proteins, named according to their molecular masses. Due to lack of structural specificity of this particular Hsp family, sHsps are highly recognized by the presence of α-crystallin domain which is approximately 80 to 100 amino acids in length (Dejong et al., 1998; Denlinger et al., 2001; Kappé et al., 2010). The significance of this domain is the formation of a dimeric building block which assembles into oligomers through interactions in the sequence-variable N-terminal domain and the C-terminal end (Laganowsky et al., 2010; Braun et al., 2011; Jehle et al., 2011) and subsequent packing of these dimers is what defines the overall structure of sHsp oligomers (Hochberg and Benesch, 2015), resulting in a large diversity in both size and symmetry. Thus, in agreement, presence of ACDs in sHsps of *Brachionus* spp. may have resulted in a large disparity even among sHsp family with molecular size ranging from 16 to 58 kDa, which is relatively larger compared to other sHsps of other organisms. Despite a low degree of conservation compared to other Hsp families, sHsps play important roles in both stress-responses such as temperatures, UV irradiation, metals, and toxicants (Reineke, 2005; Waters et al., 2008) as well as in canonical biological processes including cell growth, apoptosis, differentiation, diapause, and lifespan in small invertebrates (Arrigo, 1998; Morrow et al., 2004; Gkouvitsas et al., 2008). Also, this particular Hsp family has shown its crucial functional role in protein aggregation and unfolding, demonstrated by various pathological conditions related to abnormal protein structures, including prion disease, cystic fibrosis, cataracts, or neurodegenerative disease (Johnston et al., 1998; Derham and Harding, 1999; Koyama and Goldman, 1999; Radford and Dobson, 1999). However, limited studies have provided full identification of the entire sHsp and some functional roles in aquatic invertebrates. For example, in Ascidian *Ciona savignyi*, three *sHsp*s have been identified and showed gene modulation in response to different temperatures and salinity (Huang et al., 2018). Also, Hsp27 has been characterized in the midge *Chironomus riparius* and different stressors including temperatures and environmental pollutants (tributyltin, nonylphenol, triclosan, bisphenol, and cadmium) induced increase in mRNA expression (Martínez-Paz et al., 2014). However, as sHsp family is comprised of multi-genes, genome-wide identification of sHsp family would be helpful for future environmental ecotoxicology.

In general, however, sHsps feature tripartite architecture composed of conserved ACD flanked by N-terminal region and C-terminal extensions (Haslbeck and Vierling, 2015). In contrast to the canonical architecture of sHsps, *Brachionus* spp. showed non-canonical structure having more than one ACD domain. In fact, some sHsps in *Brachionus* spp. contained p23-like domain, in addition to ACD domain with highly conserved specific amino acid sequences including valine, isoleucine, and leucine, which were highly homologous to that of humans. The

conservation of these hydrophobic side chains is likely to contribute to the hydrophobicity that affects the potential of sHsps to successfully bind aggregation-prone denaturing proteins (Posner et al., 2012) in an ATP independent manner and subsequent refolding by ATP-dependent chaperones such as Hsp60, 70, and 90s (Van Montfort et al., 2001). Yet, despite the presence of highly conserved ACD domain, due to low sequence similarities in the N-terminal and C-terminal extensions across different species (Basha et al., 2012), sHsps in *Brachionus* spp. indeed show high sequence variation and evolutionary divergence. Moreover, it has been shown that there is a considerable differences in tissue localization and modulations in *sHsps* expression (Vos et al., 2008; Mymrikov et al., 2011), suggesting that it is worthy to identify and further investigate highly varied sHsp in aquatic invertebrates for early detection in response to environmental pollutants.

Assembly of oligomers of sHsp or ACD through dimeric formation, which is altered by different factors, such as C-terminal motif (Poulain et al., 2010), is important for a dynamic subunit exchange for their role in chaperone function (Delbecq et al., 2015). Also, unusual member of sHsps having two ACDs, have also been found in a parasitic flatworm *Taenia saginata* (Stamler et al., 2005), which is an evolutionarily close species to rotifers. Thus, multiple ACD and/or p23-like domains (another monomeric ACD) present in *Brachionus* spp., can form higher-order structures and may possibly perform chaperoning function more efficiently.

4.2. Hsp40/DnaJ homolog subfamily members

Among Hsp families, Hsp40 family is structurally and functionally diverse group of members, consisting of three types (type I, II, and III) based on the degree of conservation of domains with those of *Escherichia coli* (Cheetham and Caplan, 1998; Pradeep et al., 2009). In particular, each sub-group contains a unique domain structure known as J-domain, consisting of 70-amino acids (Georgopoulos et al., 1980; Zylicz et al., 1985) residues with specifically highly conserved tripeptide motif comprised of histidine, proline, and aspartic acid (HPD) (Verma et al., 2017), responsible for binding of the ATPase domain of Hsp70 (Greene et al., 1998). In the three *Brachionus* spp., all DnaJ homologs (i.e., DnaJ homolog subfamily A, B, and C), showed highly conserved domain structures to that of *H. sapiens* and their genomic structures such as number of exons and the overall ORF lengths were homologous in subfamily specific manner. The highly conserved domain is crucial for the binding of Hsp40 to Hsp70 (Kelley, 1998), and the formation of the complex is very important for regulating ATP hydrolysis, involved in various housekeeping and stress-related functions (Fan et al., 2003). In detail, the classification of J-proteins is based on the presence of specific conserved regions. Type A, also known as DnaJA subfamily, consists of four distinct domains; N-terminal J-domain, glycine/phenylalanine (G/F)-rich region, four repeats of the CxxCxGxG-type zinc-finger domain, and a C-terminal domain. In the three *Brachionus* spp., not all DnaJA members contained G/F regions, which are associated with the specificity and modulation of the conformation of substrates for Hsp70 binding (Fan et al., 2003; Lopez et al., 2003). The next type B J-proteins consisted of domains similar to those of Type A, but lacked the zinc-finger domain in *Brachionus* spp. The last type C J-proteins are known to be the most diverse group as they are characterized only by J-domains that are located in non-specific manner (Qiu et al., 2006; Luo et al., 2019). Furthermore, some studies have suggested that C-type DNAJ proteins may not function as molecular chaperones due to their incapability to bind to non-native polypeptides (Qiu et al., 2006) and possibly linked to the lack of conserved domain compared to the other subfamilies. Also, among the DnaJ C subfamily members of the three *Brachionus* spp., only member 3 and 7 possessed tetratricopeptide (TPR-repeat protein domains consisting of ~34 residues), with tandem repeats of hydrophobic residues including alanine, methionine, tyrosine, and valine. TPR motif was originally identified in yeasts (Hirano et al., 1990), with minimally conserved regions, yet known to occur in a various types of proteins in both prokaryotes and eukaryotes (Cerveny et al., 2013) that participate in cellular functions. However, interesting to note here, is that not many proteins which contain TPR motifs interact with Hsps (Ballinger et al., 1999), and the significance of the presence of this particular motif could be linked to the potential of Hsp40s to interact with Hsp70s as a co-chaperone for ATPase activity. However, further studies are required to verify such mechanism.

Taken together, three DnaJ subfamilies (A, B, and C) identified in the three *Brachionus* spp., all showed highly conserved HPD motif in N-terminal, with high similarity to that of *H. sapiens*. As aforementioned, the type A dnaJ subfamily is most likely to mediate chaperone function by recognizing subsets of client conformers, facilitate binding to Hsp70, and provide molecular flexibility to assist in the transfer of the substrate from Hsp40 to Hsp70, attributing the presence of G/F-rich region (Ahmad et al., 2011; Stein et al., 2014) and a zinc-binding domain which has two functionally dissimilar zinc-binding sites potentially involved in substrate interaction (Linke et al., 2003; Tiwari et al., 2013), yet, further studies are required to understand post-translational modifications to validate whether these presence of multiple genes participate in the formation of protein-protein interaction.

4.3. Hsp10 co-chaperone with Hsp60/T-complex protein 1 subunits

In this study, a single Hsp10 and Hsp60 of mitochondria was identified in each *Brachionus* spp., and 8 different classes of T-complex protein 1 subunits were identified in each species, namely TCP1 alpha, beta, delta, epsilon, eta, gamma, theta, and zeta. Hsp 60 family of eukaryotes, the homolog of GroEL in *E. coli* (Hendrix, 1979), also referred to as chaperone60, was originally proposed to describe a class of protein that are ubiquitously found in organelles of living organisms with highly conserved sequence similarities (Hemmingsen et al., 1988; Gupta, 1995). To date, there are two known types of chaperonins, namely chaperonin 10 and 60, classified by their molecular mass represented in kDa which form a folding cage to produce a protein-editing machinery (GroES/GroEL complex) (Hayer-Hartl et al., 2015) that facilitate proper folding and assembly of mitochondrial-imported proteins (Hansen et al., 2003; Magen et al., 2008), yet, the regulatory mechanism of the system still remains in ambiguity in aquatic invertebrates. Despite their high degree of sequence similarities and function in intracellular folding and assembly of various polypeptides (Ellis and van der Vies, 1991) across various organisms and organelles, no close homologs of Hsp60 have been found in eukaryotic cell cytosol. However, another family of proteins known as T-complex polypeptide 1 has been proposed, which are distantly related to Hsp60s but share high sequence similarity and carry similar functions to that of Hsp60s (Gupta, 1990). In addition, most of Hsp60s have been preferentially found in mitochondria and the cytoplasm (Zügel and Kaufmann, 1999) and thus, considered cytosolic chaperone family (Trent et al., 1991; Ellis, 1992; Horwich and Willison, 1993). As site-specifically expressed, mitochondrial Hsp60 identified in *Brachionus* spp. were highly homologous to that of *H. sapiens*, as demonstrated by high sequence homology in the conserved domains. Besides the function in protein assembly and folding, Hsp60s have also gained their importance as major antigens due to high sequence similarities to that of microbial pathogens, inducing strong humoral and cellular immune responses against various infections (Zügel and Kaufmann, 1999). For example, the responsiveness of Hsp molecules to antibodies have been suggested by the involvement of GroE complex in the synthesis of bacterial cell wall (McLennan and Masters, 1998), which is suggestive of the conservation of this particular Hsp family members across all living organisms including prokaryotes and eukaryotes. In addition, canonical structural features of Hsp60s were observed including GGM amino acid sequences at the C-terminal end, however functional significance of the presence of GGM repeat still remains unknown (Sanchez et al., 1999).

Growing evidences on Hsp60s have demonstrated that these groups

of protein families play extremely important role in cellular homeostasis, which could possibly linked to relatively high expression levels in the normal cell (Gupta and Knowlton, 2002). Moreover, studies on stress responses of Hsp60s in response to various abiotic and biotic factors including trace metals, organic pollutants, temperature shifts, hypoxia/anoxia, and UV-radiation have been reported, indicating their importance in understanding molecular responses and evolutionary adaptation to environmental stressors (Sanders, 1990; Schlesinger, 1990; Sanders et al., 1991; Nepple and Bachofen, 1997; Gupta and Knowlton, 2002), ultimately establishing as important biomarkers for various stressors (Sanders, 1990; Bradely, 1993). For example, modulation of *Hsp60* expression levels has been demonstrated by the sea anemone *Anemonia viridis* in response to temperature changes following a seasonal variation (Choresh et al., 2001), suggesting higher *Hsp60* expression in response to increasing temperature is linked to higher protein damage. Indeed, Hsp60 has showed its potential as one of the major Hsp to environmental stressors (Tsan and Gao, 2004; Li et al., 2011), as demonstrated by modulations in temperature-dependent modulations in ATPase activity, which is essential for folding of denatured protein and stress responses (Gupta et al., 2008). Interestingly, in this study, RNA-seq analysis of temperature shift-exposed *B. koreanus* showed modulations of the entire *Hsps*, with significant up-regulation of Hsp60 family members. Indeed, differentially expressed gene analysis validated that mitochondrial *Hsp60*, *TCP1 beta*, *delta*, *epsilon*, *eta*, and *zeta* were significantly up-regulated in response to temperature shift (20 °C), while *TCP1 beta*, *delta*, and *eta* were significantly up-regulated in response to dramatic temperature shift (15 °C), compared to the control (25 °C) at 24 h exposure. Differences in transcriptional regulation of *Hsp* genes, particularly in Hsp60 family members could be linked to deviations in the orientation/order of locations of the conserved motifs within Hsp60s, which ultimately affect gene regulation (Westholme et al., 2008) However, limited studies have addressed the full genome-wide identification of Hsp60s in aquatic organisms and the information gained from this study will be helpful for future studies on evolutionary molecular ecotoxicology.

4.4. Hsp70

Organisms' ability to survive and thrive under environmental variation highly relies on adaptation and homeostatic maintenance. Another group of heat shock protein family is Hsp70, comprising the most conserved Hsps among Hsp families across different species (Hunt and Morimoto, 1985; Mayer and Bukau, 2005). Similar to other Hsp or chaperones, Hsp70s are also considered housekeeping protein family attributing to their assistance in a wide range of cellular functions, with majority functioning in protein folding and control of the regulatory proteins (Toft, 1999; Hartl and Hayer-Hartl, 2002; Ryan and Pfanner, 2002; Young et al., 2003). Specifically, three different activities are involved in the function of Hsp70s in folding of non-native proteins: prevention of aggregation, promotion of folding, and solubilization and refolding of aggregated proteins (Mayer and Bukau, 2005). In addition to folding function, Hsp70s assist in subcellular transport of proteins and vesicles (Pratt and Toft, 2003), shift between formation/dissociation (Young et al., 2003), protein degradation (Chiang et al., 1989; Bercovich et al., 1997). Furthermore, as this class of protein family is highly conserved across animal taxa, Hsp70s have been extensively implicated in a various field of studies including pathology, neurodegeneration, and immunology. In particular, extensive studies have been reported in humans as higher number of genes (13 Hsp70s) is present with differences in expression level, subcellular location, and amino acid constitution (Radons, 2016). Despite high number of Hsp70 genes in human, Hsp70 family, in general, is divided into four groups, namely Hsp70 (Liu and Cao, 2018), heat shock cognate 70 (Hsc70) (Wu et al., 2008), glucose-regulated protein 78 (Grp78)/immunoglobulin heavy-chain binding protein (BIP)/Hsc3 (Li et al., 2018), and glucose-regulated protein 75 (Grp75) (Daugaard et al., 2007), and each subfamily is expressed by different environmental stressors including heat, cold, salinity, pH, and metal. Among these four subfamilies, Grp75 has been known not to be induced in response to stressors, however, has been predominantly expressed in the mitochondria (Daugaard et al., 2007). In this study, *in-silico* analysis of the entire genome resulted in Grp78/BIP, Grp75, Hsc70, Hsp70, and hypoxia up-regulated protein 1 (HYOU1), with highest duplicates identified within Hsc70s. In addition, HYOU1 was comprised of the highest number of exons, 16 from *B. koreanus* and *B. plicatilis*, and 15 from *B. rotundiformis*. Previous studies have shown highly conserved structure of Hsp70 subfamilies. For example, Hsc70 has three signature motifs including IDLGTTYS, LIFDLGGGTFDVSIL, and IVLVGGSTRIPKVQK (Jungprung et al., 2019). Computational analysis of Hsp70 subfamilies have indeed demonstrated the presence of conserved motifs that are found in other invertebrates, suggesting possible functional role of Hsp70s in *Brachionus* spp., however, the conserved motifs within the rotifers showed minor differences in the amino acid features (i.e., hydrophobic, charged, polarity). Thus, to understand the significance of differences in AA residues and deviations within family members, functional studies are required to better understand the consequences of changes in residues.

4.5. Hsp90

The members of Hsp90 families are highly conserved and universal which are, similar to other chaperones, promotes the folding of synthesized or incorrectly folded proteins to avert aggregation (Hoter et al., 2018). Despite their ubiquitous presence, they are cellular-specifically expressed, where Hsp90 alpha and beta are located in the cytoplasm, Grp94 is localized in the ER, and tumor necrosis factor receptor-associated protein 1 (TRAP1) in the mitochondria (Hoter et al., 2018). As this particular Hsp family is highly conserved across various animal taxa with an exception of archaea (Chen et al., 2006), these four members of Hsp90 were also present in the rotifer *Brachionus* spp. with highly conserved number of exons among the three *Brachionus* spp., however, the direction of the strand were different in a species-specific manner. As suggested by numerous studies, key features that define Hsp90 protein family are (NKEIFLRELISN[S/A]SDALDKIR, LGTIA[K/R]SGT, IGQFGVGFYSA [Y/F]LVA[E/D], IKLYVRRVFI, and GVVDS[E/D]DLPLN[I/V]SRE) along with the consensus sequence (MEEVD) at the C-terminus (Zhao et al., 2011). Indeed, these conserved motifs were all present in the three *Brachionus* spp., Hsp90 family members however, some of the known key features of member-specific motifs were different compared to model species. For example, Grp94s of the *Brachionus* spp., lacked KDEL signal motif required for ER retention signal, but instead, has shown substitution of AA from KDEL to KKIL, the canonical dilysine (KK) motif, which has been suggested to confer ER localization in other organisms including plants (Gao et al., 2014). Also, another Hsp90 member, TRAP1 which is supposedly the mitochondrial resident of Hsp90 which is signified by lack of MEEVD and the charger linker domain found within the cytosolic homolog (Masgras et al., 2017), TRAP1 identified in *Brachionus* spp. showed absence of these signature motifs which indeed confirms its identity as mitochondrial Hsp90 member. Interestingly, however, TRAP1 of *Brachionus* spp. showed presence of KDEL motif in the NTD, which should only be found in ER or Golgi-related Hsps, and thus these genes required further qualification of the full sequences.

4.6. Global comparative analysis

As being one of the cosmopolitan species across diverse aquatic and limnoterrestial habitats (Wallace and Smith, 2009), rotifers could be an excellent model species to understand how they tolerate against constantly occurring natural perturbations, and further anticipate the aquatic food web status (Smith et al., 2012). In addition, as rotifers in the natural environment experience thermal fluctuations, particularly in shallow and temporary water bodies (Denekamp et al., 2009; Dupuis

and Hann, 2009), it is worthy to investigate the presence of the entire Hsp family genome-widely. In this study, the majority of Hsp families identified among the three rotifers were highly similar to one another, with an exception of small *Hsps*. The highest number of *sHsps* have been identified in *B. plicatilis*, which indeed has the largest genome size (106.9 Mb) (Han et al., 2019) compared to *B. koreanus* (85.7 Mb) and *B. rotundiformis* (58 Mb) (Kang et al., 2020). Moreover, unlike *B. koreanus*, *B. plicatilis* and *B. rotundiformis* both sustain the survival of their population through diapause and resting eggs (Gilbert, 2007) under unfavorable ambient conditions, and studies have shown that sHsps are likely to function in defensive role against stressful conditions (MacRae, 2010) during diapauses and resting eggs. Moreover, previous studies have shown that *B. plicatilis*, in general has shown, decreased susceptibility to environmental stressor or xenobiotic-induced oxidative stress compared to *B. koreanus* and *B. rotundiformis*, in terms of life cycle parameters including life span, reproduction under higher toxic concentrations no-observed effective concentration, implying that higher number of *sHsps* is possibly linked to increased tolerance against oxidative stress (Mayer et al., 2012). In addition, synteny analyses have shown relatively clustered organization of *Hsps* in *B. rotundiformis* compared to *B. koreanus* and *B. plicatilis*.

Taken together, this study provides the very first genome-widely identified Hsp families in one of the widely used ecotoxicological model aquatic invertebrates *Brachionus* spp. *In-silico* analysis have shown highly conserved domains within each Hsp family members, yet minor differences have been demonstrated. This study will provide a new set of dataset of aquatic invertebrate Hsps for a better understanding of the significance of amino acid residue differences and consequent functional role changes in species-specific manner.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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