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Identification of three doublesex genes in the monogonont rotifer *Brachionus koreanus* and their transcriptional responses to environmental stressor-triggered population growth retardation

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ABSTRACT

Doublesex and Mab-3-related transcription factor (*Dmrt*) gene family members have rarely been identified or characterized in aquatic invertebrates. In this study, we identified and characterized three DMdomain-containing genes – *Dmrt11E*, *Dmrt93B*, and *Dmrt99B* – in the monogonont rotifer, *Brachionus koreanus*. DMdomains of the proteins encoded by the *B. koreanus Dmrt* (*Bk-Dmrt*) genes had high similarities to DM domains of other invertebrate species. To understand the potential effects of environmental stressors on the transcriptional expression of *Dmrt* genes in rotifers, we exposed *B. koreanus* to a wide range of UV-B radiation and different concentrations of benzo[*a*]pyrene (B[*a*]P) over different time courses. Transcript levels of all *Bk-Dmrt* genes decreased significantly in response to relatively high doses of UV-B irradiation, and were also downregulated in response to exposure to UV-B radiation over time. Transcript levels of all *Bk-Dmrt* genes were downregulated in response to B[*a*]P exposure for 24 h. This decrease in expression of all *Bk-Dmrt* genes was concomitant with the growth retardation induced by UV-B and B[*a*]P exposure. We concluded that both environmental stressors have detrimental effects on transcriptional regulation of all *Bk-Dmrt* genes, especially relatively high doses of these stressors, leading to growth retardation. However, further studies are required to better understand the potential role of *Dmrt* genes in environmental stressor-triggered growth retardation in the rotifer *B. koreanus*.

1. Introduction

Doublesex and Mab-3-related transcription factor (*Dmrt*) proteins contain a conserved zinc finger-likeDNA-binding region referred to as a doublesex/Mab-3DNA-binding motif (DM domain). The functions of these transcription factors have not been identified in most invertebrates, although Kopp (2012) suggested that members of the *Dmrt*-family of transcription factors are likely to be major players in sex-specific differentiation in invertebrates. In vertebrates, the protein encoded by the *DMRT* gene is involved in development and sex determination (Raymond et al., 2000; Hong et al., 2007). However, the roles of these transcription factors in invertebrates have not yet been investigated extensively. To date, three highly conserved *Dmrt* genes have been identified in both *Drosophila melanogaster* and *Caenorhabditis elegans* (Raymond et al., 1998; Yi and Zarkower, 1999). These three genes have highly similar DM domains and function as transcription factors to regulate sexual differentiation and yolk protein gene transcription. In the water flea *Daphnia magna*, Kato et al. (2008) identified three DM domain genes corresponding to *Dmrt11E*, *Dmrt93B*, and *Dmrt99B*of *D. melanogaster*, and they further cloned two additional sex mechanism-related*Doublesex* (*dsx*) genes (*DapmaDsx1* and *DapmaDsx2*) and characterized their function in the sex-determining pathway (Kato et al., 2011). Tresser et al. (2010) demonstrated that null mutation of the *Dmrt1* gene led to abnormalities in the development of anterior neural plate derivatives in the sea squirt *Ciona*. Naimi et al. (2009) isolated a *Dmrt*-like gene and analyzed its transcriptional expression during developmentin the oyster *Crassostrea gigas*. However, DMdomain-containing genes have not been identified in the *Rotifera* as yet, although rotifers have many potential advantages for population growth studies as they have a unique reproductive system and a distinctive developmental process.

Ultraviolet B (UV-B) radiation is ubiquitous and is an emerging environmental stressor in aquatic animals. UV-B radiation has diverse detrimental effects on DNA, cells, tissues, growth, behavior, and survival, while it actively causes oxidative stress to aquatic organisms via water

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column penetration to ecologically significant depths (Dahms and Lee, 2010). However, only a few studies have reported UV-B effects in terms of UV-B sensitivity. In sea urchin embryos, Bonaventura et al. (2005) reported that higher sensitivity to UV-B radiation is common in early developmental stages compared to later stages. Lu and Wu (2005) reported that the sperm of the sea urchin *Anthocidaris crassispina* is more susceptible to UVR damage than eggs, embryos, and larvae. Recently, Kim et al. (2011) reported that UV-B radiation inhibited growth and survival rate via oxidative stress-triggered modulation of DNAreplication- and DNArepair-related genes in the rotifer, *Brachionus koreanus*. We conducted transcript profiling of *Bk-Dmrt* genes in *B. koreanus* exposed to UV-B radiation to investigate the potential correlation between UV-Bradiation-induced detrimental effects such as growth retardation and *Dmrt* gene expression.

Benzo[*a*]pyrene (B[*a*]P) is a polycyclic aromatic hydrocarbon (PAH); PAHs are potent environmental pollutants. B[*a*]P is the most studied PAH due to its relatively high levels in the environment and substantial toxicity. It can also occur naturally and be transferred from surface water to seawater by anthropogenic activity. In our recent study of the rotifer *B. koreanus*, we found that B[*a*]P inhibited their population growth rate for 10 days, and induced expression of a molecular detoxification system and antioxidant defense mechanisms to repair cellular damage (Kim et al., 2013). Rotifers maintain their populations by asexual and/or sexual reproduction. However, reproduction can be affected by environmental conditions, resulting in modulation of population growth (Snell and Janssen, 1995; Dahms et al., 2011). Despite numerous reports of physiological changes in response to environmental change, molecular responses in *Rotifera* have rarely been studied. Here, we analyzed transcriptional changes of *Bk-Dmrt* genes in response to B[*a*]P to gain a better understanding of the molecular responses of these rotifers to environmental pollutants.

In this study, we identified and characterized DM domain genes in *Rotifera* for the first time. We also analyzed the transcriptional profiles of DM domain genes to understand molecular responses of rotifers to environmental stressor-triggered growth retardation. These results provide insight into potential correlations of *Dmrt* expression with sexual metabolism and growth in rotifers.

2. Materials and methods

2.1. Rotifers

Individuals of the monogonont rotifer species *B. koreanus* were collected at Uljin (36°58′43.01″N, 129°24′28.40″E) in South Korea (Suppl. Fig. 1). Single individuals were isolated, reared, and maintained in filtered artificial seawater (TetraMarine Salt Pro, Tetra™, Cincinnati, OH, USA) adjusted to 25 °C under a LD 12:12 h photoperiod with 15 practical salinity units (psu) of salinity. Green algae (*Chlorella* sp.) were used as a live diet (approximately 6×10^4 cells/mL). Species identification was confirmed by assessing morphological characteristics and by mitochondrial genome analysis (Hwang et al., 2013a,b). A relative short life cycle was observed in *B. koreanus* of approximately 24 h. We assume that the rotifer *B. koreanus* reproduces preferentially by parthenogenesis without a sexual cycle, as we have never observed any males or resting eggs, even under harsh environmental conditions.

2.2. Retrieval and annotation of DM domain genes from the monogonont rotifer, B. koreanus

To obtain DM domain gene information, we searched the *B. koreanus* genomic DNA database that was constructed previously (Lee et al., 2011) and recently updated with RNA Seq information. Assembled contigs coding for proteins obtained in this study were subjected to BLAST analysis against the GenBank non-redundant (NR; including all GenBank, EMBL, DDBJ, and PDB sequence except expressed sequence tags (ESTs), sequence tagged sites (STSs), genome survey sequences (GSSs), or high-throughput genome sequence (HTGSs)) amino acid sequence database. All DM genes were subjected to 5′- and 3′-Rapid Amplification of cDNA Ends (RACE) to obtain full-length coding sequences according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). A series of RACE reactions were performed with target primers (Table 1) under the following conditions: 94 °C/4 min; 40 cycles of 98 °C/25 s, 55 °C/30 s, 72 °C/60 s; and 72 °C/10 min. Final RT-PCR products were isolated from 1% agarose/TBE gels, cloned into pCR2.1 TAvectors (Invitrogen, Carlsbad, CA, USA), and sequenced with an ABI PRISM 3700 DNA analyzer (Bionics Co., Seoul, South Korea). All gene information from *B. koreanus* has been submitted to GenBank; accession numbers of each gene are appended in Table 1.

2.3. Amino acid comparison, phylogenetic analysis, and genomic structure analysis

Multiple alignments of rotifer DM domain genes were performed using ClustalX software (ver. 1.83) at the level of deduced amino acid sequences. Domains and motifs were identified through Pfam HMM searches (http://pfam.sanger.ac.uk) and MotifScan (http://myhits.isb-sib.ch/cgi-bin/motif_scan). To determine the phylogenetic placement of the identified rotifer DM domain genes, we aligned them with those of other species using ClustalX 1.83. Gaps and missing data in the matrix were excluded from the analysis. Generated data matrix was converted to nexus format, and the data matrix was analyzed with Mr. Bayes v3.1.2 software using the general time-reversible (GTR) model. The analysis was run for a total of 1,000,000 generations, and the sampling frequency was every 100 generations. After analysis, the first 10,000 generations were deleted as "burn-in", and a consensus tree was constructed and then visualized with the Tree View module of PHYLIP (Felsenstein, 1989).

2.4. UV-B irradiation

Acute and chronic mortality in response to exposure to UV-B irradiation was assessed as described in our previous study (Kim et al., 2011).

Table 1
Primers used in RACE and real-timeRT-PCR analyses.

Gene	Oligo name	Sequence (5 → 3′)	Remarks
Dmrt11E (JX908759)	5GSP1	GAGCCACCTGAGCAGCCATAACTC	5′-RACE
	5GSP2	GTTGCATTCGCGCCATCTACAATAC	
	3GSP1	GTAGAAATCACGGTGTCGTATCATG	3′-RACE
	3GSP2	GTATTGTAGATGGCGCGAATGCAAC	
	Real-time RT-F	GCATCATTAAATAGTCAGAAGACCG	Real-time RT-PCR
	Real-time RT-R	GCAACAATGAGTCTACTTGAGGCAGCA	Amplification
Dmrt93B (JX908760)	5GSP1	CATTACTCGCTGTCTCTCGGCAATC	5′-RACE
	5GSP2	CTTACAATGCCTTTTGTGTCCTTTC	
	3GSP1	CATTGTAAGTTCAAGGACTGTGTGTG	3′-RACE
	3GSP2	CCTGATTGCCGAGAGACAGCGAG	
	Real-time RT-F	CAATGAATGGTTCTAGCGGTTCTTTGGC	Real-time RT-PCR
	Real-time RT-R	GGGCTATCTGATTCTTGGAACGGTG	Amplification
Dmrt99B (′)	5GSP1	CATGACCCTCTGTCTCTCGGCAATC	5′-RACE
	5GSP2	GAGAGTGGATACGACGCCGTGGTTTC	
	3GSP1	GAAACCACGGCGTCGTATCCACTCTC	3′-RACE
	3GSP2	GATTGCCGAGAGACAGAGGGTCATG	
	Real-time RT-F	GCTGCATCATCTTCTCAATCAAGCTC	Real-time RT-PCR
	Real-time RT-R	CATCATAGCTGCCAGTCTCATTGTCG	Amplification
18S rRNA	Real-time RT-F	TCGGGCTGTCTCGTTCGTGATTC	Real-time RT-PCR
	Real-time RT-R	TGCCACAGTCGACAGTTGATAGG	Amplification

Abbreviations: GSP,gene-specific primer; F, forward; R, reverse.

The LD_{50} value was 24.6 kJ m^{-2} (95% CI (confidence interval): 23.8–25.4). Based on the mortality schedules, we measured the population growth rate of *B. koreanus* in response to different doses of UV-B radiation in this study. Rotifers were irradiated with UV-B using a UV-B lamp (wavelength range of 280 to 360 nm; G15T8E, Sankyo Denki, Japan) with a quartz cover (Taemin Science, Suwon, South Korea) to allow UV-B penetration and to prevent evaporation during UV-B exposure. Intensity of UV-B radiation was measured by a UVX radiometer (Model M007-043; loaded Mid Range UVX 300 nm Probe, Model M007-045, CON-TROL-CURE®, Chicago, IL, USA). To check growth retardation of *B. koreanus* after UV-B irradiation, we placed 10 neonates (less than 12-h-old) into the wells (working volume, 4 mL) of 6-well culture plates and irradiated them with UV-B under dark conditions. Subsequently, we counted the number of rotifers at 0, 2, 4, 6, 8, and 10 kJ m^{-2} over 10 days. During the experiment, we supplied an algal diet of the green algae *Chlorella* sp. (approximately 5×10^4 cells/mL) every 24 h.

To analyze the dose-dependent effect of UV-B radiation on transcript levels of the *Bk-Dmrt* genes, we placed approximately 7000 adult rotifers into a petri dish (working volume, 20 mL) and irradiated the rotifers with UV-B at 2, 4, 6, 8, and 10 kJ m^{-2} under dark conditions. Rotifers were harvested at 24 h after UV-B irradiation, and total RNA was extracted for further analysis. For time course experiments, rotifers were exposed to different doses of UV-B (2 and 10 kJ m^{-2}). Rotifers exposed to 2 kJ m^{-2} were sampled after 10 days, while rotifers exposed to 10 kJ m^{-2} were sampled after 24 h, as dead rotifers were observed at 3 days after exposure to 10 kJ m^{-2}.

2.5. B[a]P exposure and experimental design

B[*a*]P was purchased from Sigma (B1760; Sigma-Aldrich, Inc., St. Louis, MO, USA; purity >96%) and was dissolved in dimethylsulfoxide (DMSO, Sigma-Aldrich, Inc., St. Louis, MO, USA). The effect of each concentration of B[*a*]P on rotifer population growth rate was determined as described in our previous study (Kim et al., 2013). We could not measure the exact No Observed Effect Concentration (NOEC) and Lethal Concentration (LC) values of B[*a*]P for 24 h, as the rotifers did not show mortality at even 1 mg/L of B[*a*]P exposure due to minor precipitation at high concentrations over 2 mg/L in artificial seawater (15 psu). Therefore, we employed a wide range of B[*a*]P concentrations (1–100 μg/L) to measure the population growth rate of B[*a*]P-exposed *B. koreanus* in this study.

To examine the transcriptional response of the *Bk-Dmrt* genes to B[*a*]P exposure, we exposed rotifers to different concentrations of B[*a*]P (1, 10, and 100 μg/L) for 24 h. The control group was exposed to 0.01% DMSO. DMSO concentrations in the control and treatment groups were lower than 0.01%. Approximately 7000 rotifers were exposed to each concentration of B[*a*]P for 24 h. To analyze the transcriptional profile of *Bk-Dmrt* genes when population growth was inhibited, rotifers were sampled at 10 days after exposure to 100 μg/L B[*a*]P. During the experiment, we supplied an algal diet of *Chlorella* sp. (approximately 6×10^4 cells/mL) every 24 h.

2.6. Total RNA extraction and single-strand cDNA synthesis

Pooled whole rotifers (≈7000 for each experiment) were homogenized in three volumes of TRIZOL® reagent (Molecular Research Center, Inc., OH, USA) with a tissue grinder and stored at −80 °C until use. Total RNA was isolated from the tissues according to the manufacturer's instructions. Genomic DNA was removed using DNase I (Sigma-Aldrich, Inc., St. Louis, MO, USA). Quantity of total RNA was measured at 230, 260, and 280 nm using a spectrophotometer (Ultrospec 2100 pro, Amersham Bioscience, Freiburg, Germany). To check for genomic DNA contamination, we loaded total RNA on a 1% agarose gel that contained ethidium bromide (EtBr) and visualized the gel using a UV transilluminator (Wealtec Corp., Sparks, NV, USA). Furthermore, to verify total RNA quality, we loaded total RNA on a 1% formaldehyde/agarose gel stained with EtBr and checked the *18/28S* ribosomal RNA integrity and band ratio. Single-stranded cDNA was synthesized from total RNA using oligo$(dT)_{20}$ primers for reverse transcription (SuperScript™III RT kit, Invitrogen, Carlsbad, CA, USA).

2.7. Real-time reverse transcription-polymerase chain reaction (real-time RT-PCR)

Significant changes in transcript levels were analyzed using real-timeRT-PCR. Primers for each gene were designed after the exon/intron boundary was compared to genomic DNA using GENRUNNER software (Hastings Software, Inc., NY, USA) and confirmed by the Primer 3 program (Whitehead Institute/MIT Center for Genome Research). Optimized conditions were determined using the CFX96™ real-timeRT-PCR protocol described below (Bio-Rad, Hercules, CA, USA). For real-timeRT-PCR amplification, each reaction consisted of 1 μL of cDNA that was reverse transcribed from 2 μg of total RNA and 0.2 μM primer each of real-timeRT-F/R and the reference gene primer *18S rRNA*RT-F/R (Table 1). All real-timeRT-PCR reactions were carried out in an unskirted low 96-well clear plate (Bio-Rad, Hercules, CA, USA). Reaction conditions were 94 °C/4 min; 35 cycles of 94 °C/30 s, 55 °C/30 s, 72 °C/30 s; followed by 72 °C/10 min. SYBR® Green (Molecular Probe Inc., Invitrogen, Carlsbad, CA, USA) was used to detect specific amplified products. To confirm the amplification of specific products, cycling was continued under the following conditions to check the melting curve: 95 °C/60 s, 55 °C/60 s, and 80 cycles of 55 °C/10 s with a 0.5 °C increase per cycle. All PCR products were sequenced by Bionics (Seoul, South Korea). Amplification and detection of SYBRGreen-labeled products were performed using the CFX96 real-timePCR system (Bio-Rad, Hercules, CA, USA). Data from triplicate experiments were expressed relative to *18S rRNA* levels to normalize for any differences in reverse transcriptase efficiency. Fold-change in gene expression was determined by the $2^{-\Delta\Delta C}$t method (Livak and Schmittgen, 2001).

2.8. Statistical analysis

SPSS ver. 17.0 (SPSS Inc., Chicago IL, USA) was used for statistical analyses. Data are expressed as means ± S.D. Significant differences between observations of different groups were analyzed using Student's paired *t*-test and multiple-comparisonANOVA followed by Tukey's test. Any difference showing $P < 0.05$ was considered significant.

3. Results

3.1. Sequence analysis of rotifer Dmrt genes

This is the first report of the cloning and characterization of *Dmrt* genes in *Rotifera*. Full-length rotifer *Dmrt* genes were completely sequenced from cDNA (Suppl. Figs. 2, 3, and 4) and sequences were deposited in Genbank (Accession No. JX908759 for *Dmrt11E*, JX908760 for *Dmrt93B*, JX908761 for *Dmrt99B*).

Complete cDNA sequence of *Dmrt11E* was 1337 bp in length including 77 bp of 5′-untranslated region (UTR), 1179 bp of open reading frame (ORF), and 75 bp of 3′-UTR with a poly (A) tail (Suppl. Fig. 3). ORF encoded a polypeptide of 393 amino acids. The predicted molecular weight and theoretical isoelectric point (p*I*) of the Dmrt11E protein were calculated as 45.14 kDa and a theoretical p*I* of 9.06, respectively.

The cloned full-length*Dmrt93B* cDNA region was 1156 bp in length including 76 bp of 5′-UTR and 114 bp of 3′-UTR with a poly (A) tail (Suppl. Fig. 4). ORF was composed of 963 bp that translated into a putative polypeptide of 321 amino acid residues. *Dmrt93B* polypeptide has a putative molecular mass 35.96 kDa and a theoretical p*I* of 8.62.

Full-length*Dmrt99B* is shown in Suppl. Fig. 5. The 1408 bp full-length cDNA contained a region (1143 bp) coding for a 381 amino acid protein

with a molecular mass of 95.72 kDa and a p*I* of 5.01. Sequence analysis revealed an 81 bp 5′-UTR and 181 bp 3′-UTR with a poly (A) tail.

All *Dmrt* genes identified to date have DM domains except for the *Dmrt8* gene; mammalian DMRT8 has no DM domain (Volff et al., 2003; Veith et al., 2006). In vertebrates, Dmrt proteins can be divided into two superfamilies; those with a DM domain only and those with a DM and a conserved motif (DMA) outside the DM domain (Ottolenghi et al., 2002; Wang et al., 2012). Furthermore, these proteins have a short conserved motif (7 amino acid residues) downstream of the DM domain. Bk-Dmrt proteins contained the DM domain consensus amino acid sequence with conserved cysteine and histidine residues of the Dmrt family (Fig. 1A). A nuclear localization signal (NLS; KGHKR) and two zinc (Zn^{2+}) binding sites (site I and II) were observed in the DM domains of the gene we identified. However, there was low sequence identity between the DMA domain and short conserved motif of Bk-Dmrt93B and Bk-Dmrt99B compared to other Dmrt proteins (Fig. 1B).

3.2. Phylogenetic analysis

Phylogenetic analysis showed that the Bk-Dmrt proteins clustered with those of Dmrt proteins from other invertebrates (Fig. 2). Phylogenetic positions of the Bk-Dmrt proteins are congruent with existing knowledge within several invertebrates.

3.3. Transcript profiles of Bk-Dmrt genes after exposure to UV-B radiation

We checked the effect of UV-B radiation on growth over a period of 10 days. Compared to the control (0 kJ m^{-2}), a relatively low dose (2 kJ m^{-2}) resulted in growth retardation. Statistical differences ($P < 0.05$) between control and UV-B-exposed groups were observed from 3 days. Rotifers irradiated with 4 kJ m^{-2} showed growth recovery 7 to 8 days after UV-B radiation (Fig. 3A). Thus, the effects of UV-Bradiation on rotifer population growth was similar to that observed in our previous study (Kim et al., 2011), although the total number of rotifers counted each day was slightly different compared to our previous study. After UV-B radiation, threshold dose–response patterns for the *Bk-Dmrt* genes were observed from 4 to 10 kJ m^{-2} ($P < 0.05$) (Fig. 3B).

In a time course experiment, transcript levels of *Bk-Dmrt11E* were significantly downregulated 2 days after exposure to 2 kJ m^{-2}UV-B, but transcript levels recovered to the control level at 4 days (Fig. 4A). When *B. koreanus* was exposed to 10 kJ m^{-2} of UV-B radiation, *Bk-Dmrt11E* transcript levels decreased after 6 h. In the case of exposure to 2 kJ m^{-2}UV-B radiation, *Bk-Dmrt93B* transcript levels were downregulated from day 2 to 4, and then increased significantly at 5 days (Fig. 4B). Moreover, at 10 kJ m^{-2} of UV-B radiation, levels of *Bk-Dmrt93B* decreased significantly after 3 h. In rotifers exposed to 2 kJ m^{-2}UV-B radiation, transcript levels of *Bk-Dmrt99B* decreased

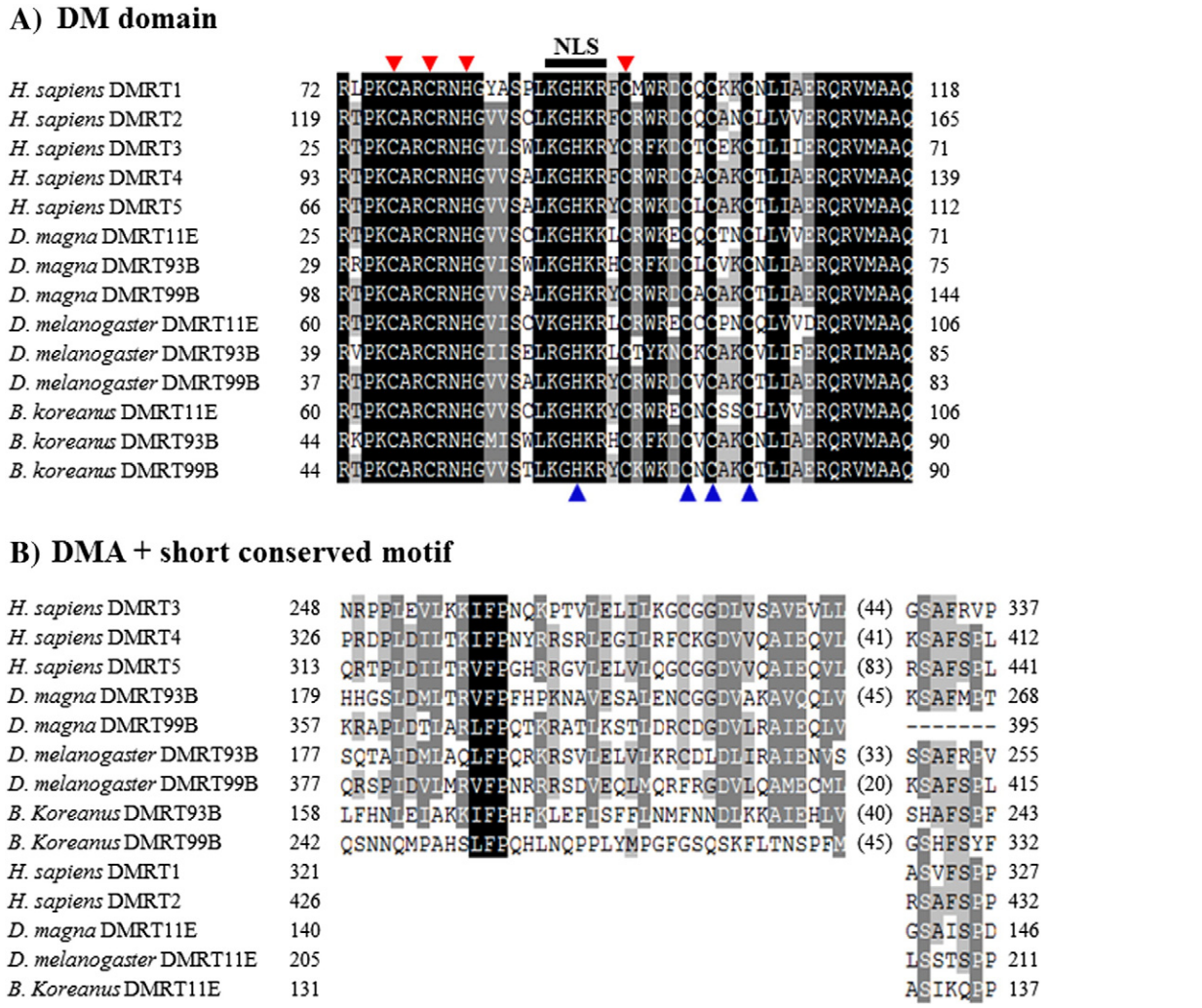

Fig. 1. Multiple alignments of the amino acid sequences of A) DM domains and B) DMAs with short conserved motifs of Bk-Dmrt proteins using Clustal X. The conserved nuclear localization signal (NLS) is indicated by the black shaded bar, and two conserved zinc (Zn^{2+}) binding sites are marked with red inverted triangles for site I (CCHC) and blue triangles for site II (HCCC).

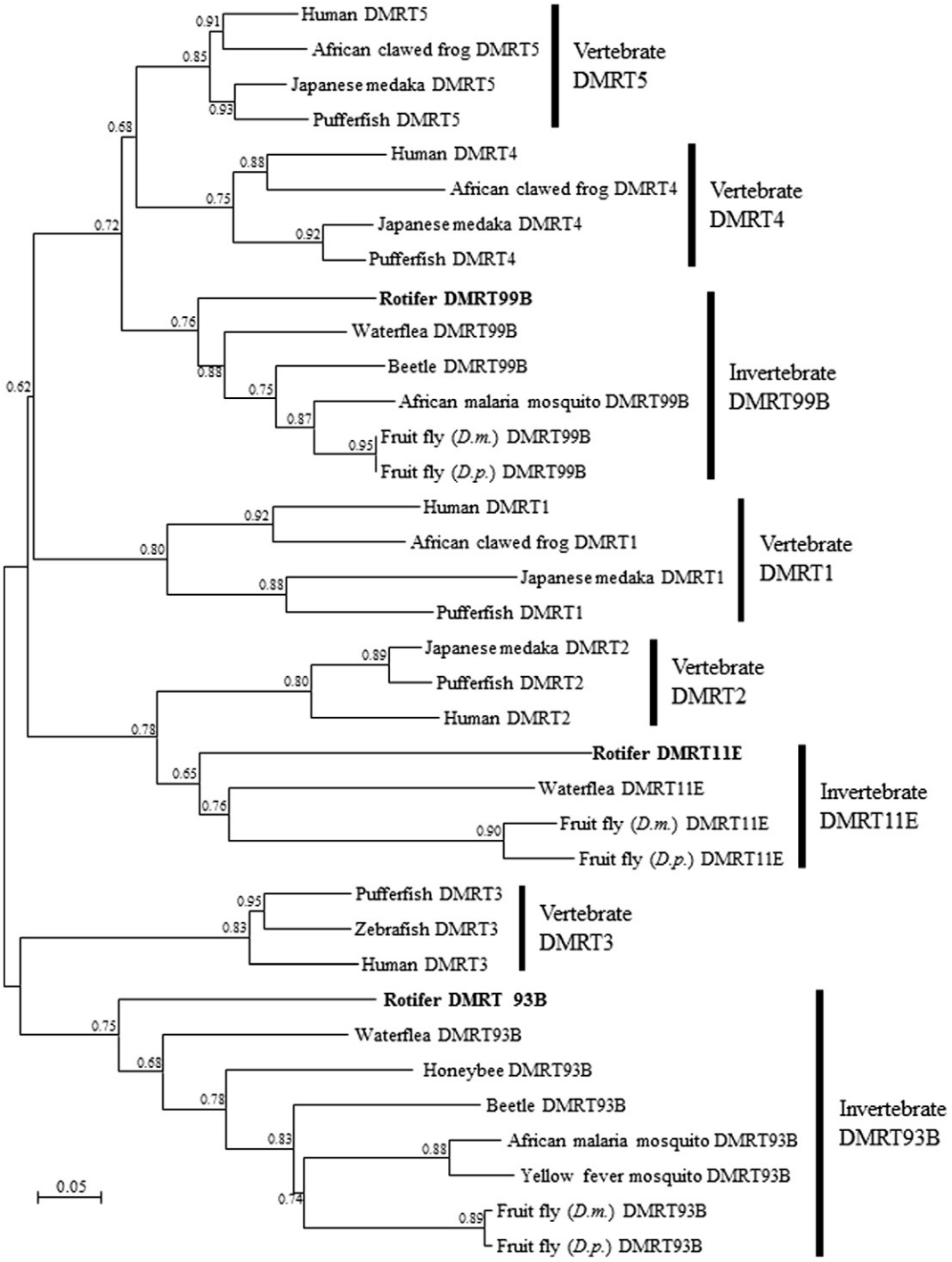

Fig. 2. Bayesian phylogenetic analysis of Bk-Dmrt proteins and Dmrt proteins of other species. Numbers at branch nodes represent posterior probability values. Abbreviations: *D. m.*, *Drosophila melanogaster*; *D. p.*, *Drosophila pseudoobscura*.

from days 2–4, but transcript levels of this gene in rotifers exposed to 10 kJ m^{-2} of UV-B radiation decreased time-dependently over 24 h (Fig. 4C).

3.4. Transcript profiles of Bk-Dmrt genes after exposure to B[a]P

We were not able to measure mortality in 24 h acute toxicity tests as more than 2 mg/L of B[*a*]P aggregated in artificial seawater (15 psu) (Kim et al., 2013). Also, in this study, exposure of rotifers to different concentrations of B[*a*]P (1, 10, and 100 μg/L) for 24 h did not result in mortality. However, 100 μg/L of B[*a*]P caused gradual growth retardation of *B. koreanus* over 10 days (Fig. 5A). Particularly, statistical differences ($P < 0.05$) between control and B[*a*]P-exposed groups were observed from 7 to 8 days in 10 μg/L of B[*a*]P exposure. Also, significant differences ($P < 0.05$) were analyzed in 100 μg/L of B[*a*]P-exposed rotifers from 4 to 10 days. Subsequently, transcript levels of *Bk-Dmrt* genes were investigated in B[*a*]P-exposed *B. koreanus* (Fig. 5B). At 24 h, all *Bk-Dmrt*-transcript levels in rotifers exposed to 100 μg/L B[*a*]P had decreased, but only transcript levels of the *Bk-Dmrt93B* gene were significantly decreased in response to a 24-h exposure to 10 μg/L B[*a*]P (Fig. 5B).

To evaluate changes in transcript levels of *Bk-Dmrt* genes according to exposure to different concentrations of B[*a*]P, we analyzed changes

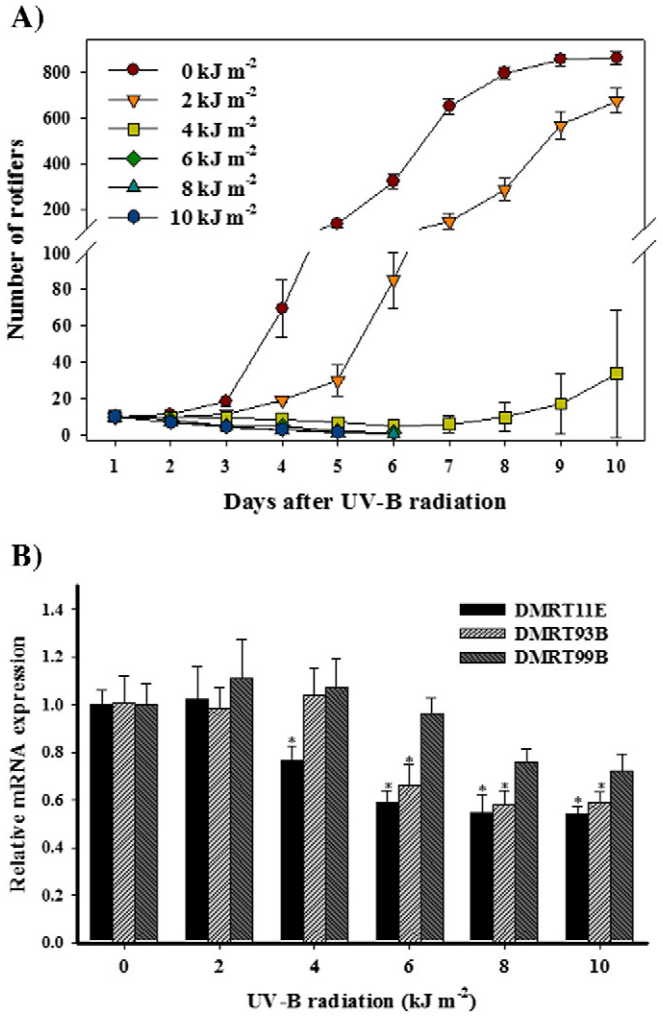

Fig. 3. A) Effect of UV-B radiation on *B. koreanus* growth rate for 10 days. Six *B. koreanus* groups were exposed to 0, 2, 4, 6, 8, and 10 kJ m^{-2} of UV-B radiation, respectively. Error bars indicate means ± S.D. B) Effects of exposure of the monogonont rotifer, *B. koreanus*, to different doses of UV-B radiation (0, 2, 4, 6, 8, 10 kJ m^{-2}) for 24 h on transcript levels of *Dmrt11E*, *Dmrt93B*, and *Dmrt99B*. Transcript changes are shown as relative to *18S rRNA*, which was used as the reference housekeeping gene. Data are means ± S.D. of three replicates. Asterisk (*) indicates $P < 0.05$.

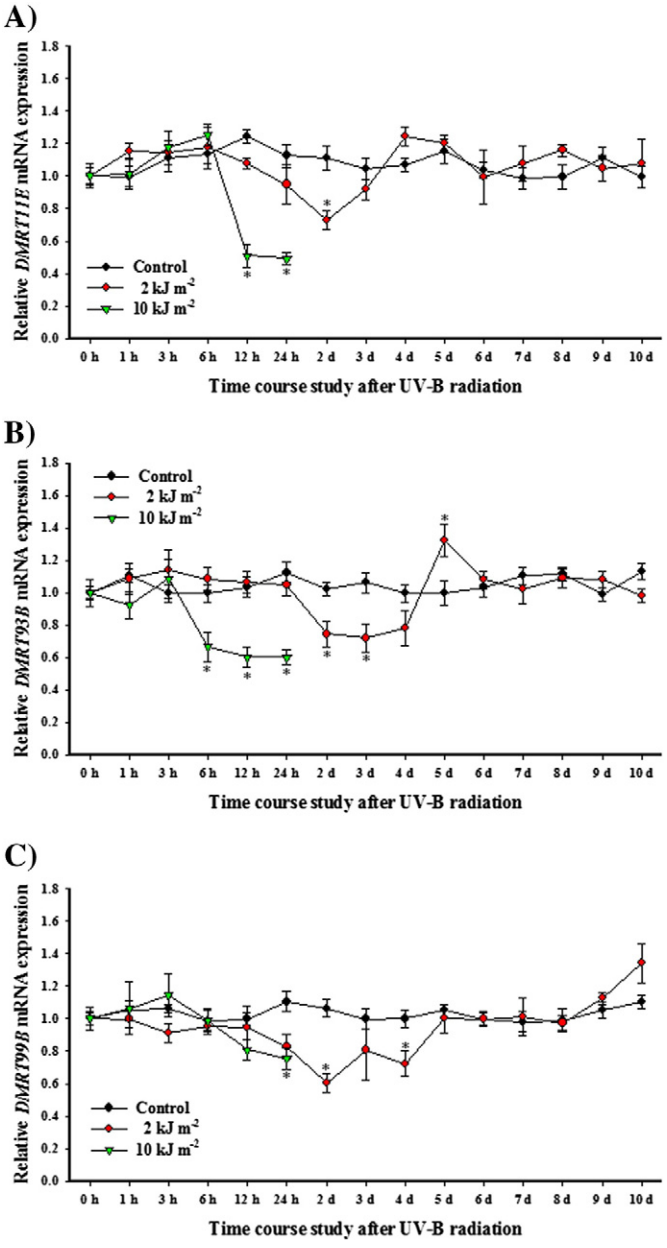

Fig. 4. Transcript profiles of (A) *Dmrt11E*, (B) *Dmrt93B*, and (C) *Dmrt99B* over 10 days after irradiation with 2 or 10 kJ m^{-2}UV-B. Transcript changes are shown as relative to *18S rRNA*, which was used as a reference housekeeping gene. Data are means ± S.D. of three replicates. Asterisk (*) indicates $P < 0.05$.

in transcript levels in rotifers exposed to 100 μg/L B[*a*]P over 10 days (Fig. 6). B[*a*]P exposure significantly decreased *Bk-Dmrt* transcript levels from 12 h to 3 days. Interestingly, transcript levels of both *Bk-Dmrt93B* and *Bk-Dmrt99B* increased at 5 days ($P < 0.05$), and reached control levels by the end of the experiment.

4. Discussion

Dmrt genes have not been identified in *Rotifera* prior to this report, even though rotifers have an interesting life cycle and a reproductive system that responds to ambient environmental conditions. Therefore, molecular characterization of essential genes that are involved in diverse physiological functions can provide a better understanding of invertebrates' growth, sexual maturation, and population maintenance. *Dmrt* or *Dmrt*-like genes have been cloned in several invertebrates, but annotation and characterization of *Dmrt* genes in diverse invertebrate taxa is difficult due to lack of genomic information. In this study, we identified three *Dmrt* genes in *B. koreanus* that correspond to *Dmrt11E*, *Dmrt93B*, and *Dmrt99B* of *D. magna* and *D. melanogaster*, and performed a series of in silico analyses such as amino acid translation, domain/motif identification and comparison, and phylogenetic analysis to characterize the *Bk-Dmrt* genes (Miller et al., 2003; Kato et al., 2008; Naimi et al., 2009; Wang et al., 2012). Structural characteristics of the sequences were consistent with them being *Dmrt* gene family members; translated amino acid sequences had a clearly identifiable DM domain, conserved cysteine/histidine residues for DNA-binding, a DMA, and short conserved motifs. Phylogenetic analysis revealed that the Bk-Dmrt proteins clustered with the Dmrt sequences of other invertebrates. We were not able to find other DMdomain-containing genes in the *B. koreanus* genome database and RNA-Seq database beyond the three we identified, suggesting that rotifers have only three *Dmrt* genes.

We observed that the environmental stressors of UV-B radiation and B[*a*]P exposure significantly retarded growth and reproduction in *B. koreanus*, consistent with our previous studies (Kim et al., 2011, 2013). These results are also consistent with the previously-reportedeffects of UV-B radiation and B[*a*]P treatment on the growth and physiology of aquatic animals (Lawrence and Poulter, 2001a,b;

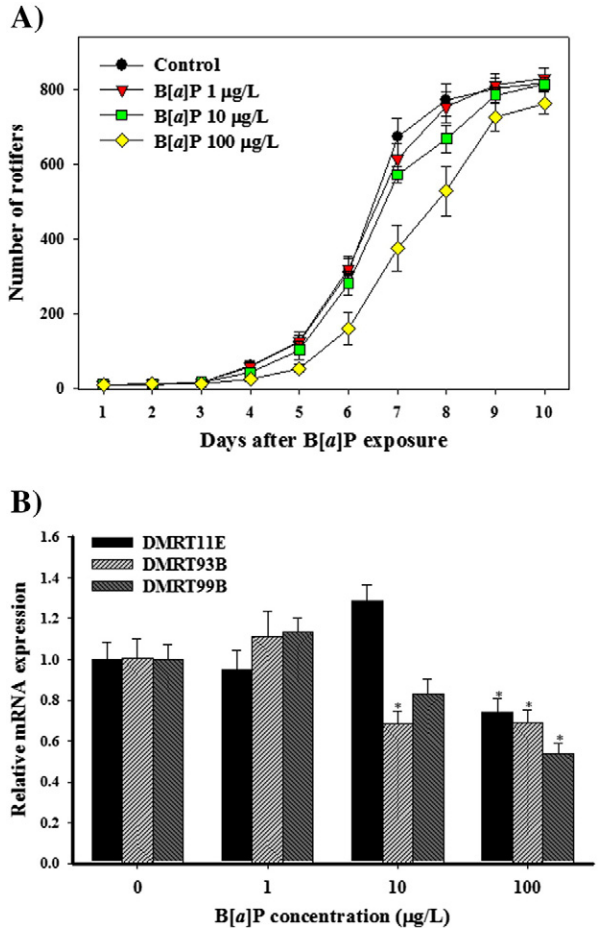

Fig. 5. A) Effect of B[*a*]P on *B. koreanus* growth rate for 10 days. Four *B. koreanus* groups were exposed to 0, 1, 10, and 100 μg/L B[*a*]P, respectively. Error bars indicate means ± S.D. B) Effects of exposure of rotifers to 0, 1, 10, or 100 μg/L B[*a*]P for 24 h on transcript levels of *Dmrt11E*, *Dmrt93B*, and *Dmrt99B*. Changes in transcript levels are shown as relative to *18S rRNA*, which was used as a reference housekeeping gene. Data are means ± S.D. of three replicates. Asterisk (*) indicates $P < 0.05$.

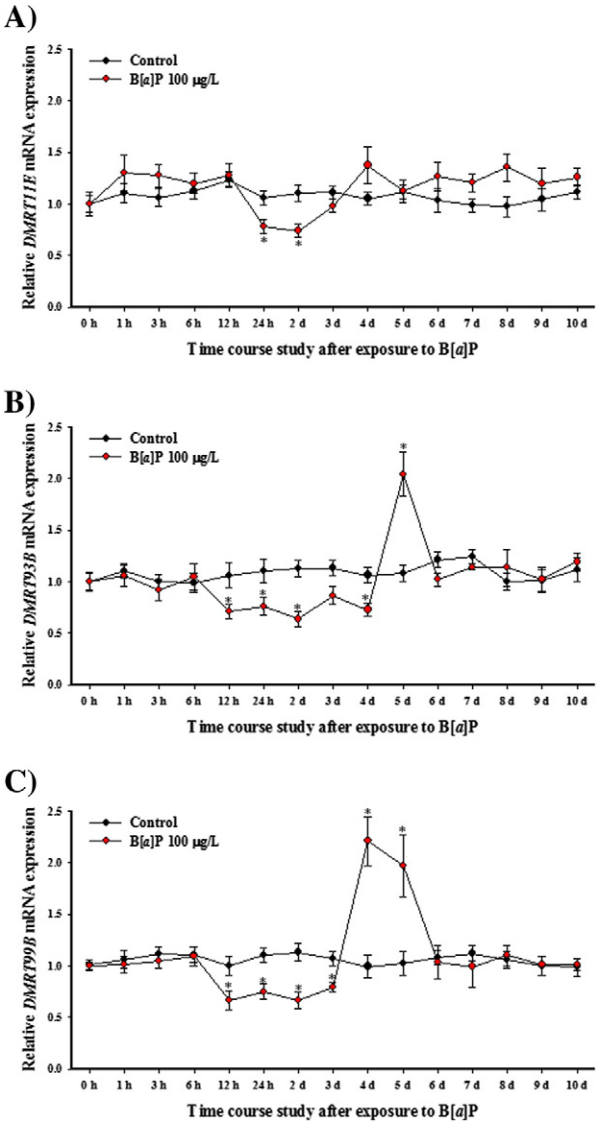

Fig. 6. Transcript profiles of (A) *Dmrt11E*, (B) *Dmrt93B*, and (C) *Dmrt99B* over 10 days after exposure to 100 μg/L B[*a*]P. Transcript changes are shown relative to *18S rRNA*, which was used as a reference housekeeping gene. Data are means ± S.D. of three replicates. Asterisk (*) indicates $P < 0.05$.

Lesser et al., 2004; Choy et al., 2007; Häder et al., 2007). Physiological activities can be directly or indirectly affected by environmental stressors, as an organism might consume metabolic energy due to increased endogenous metabolism within the limited total energy budget, even in animals that have a complex life-cycle like rotifers (Ramirez Llodra, 2002; Sebens, 2002; Nespolo et al., 2009). In the rotifer *B. koreanus*, several gene batteries involved in DNA replication, DNA repair processes, and molecular chaperoning were significantly modulated by exposure to sub-lethal doses of UV-B radiation, resulting in intracellular oxidative stress induction and growth retardation (Kim et al., 2011). In a previous study, we suggested that retardation of both growth and reproductive events were directly affected by B[*a*]P exposure, as rotifers spend their endogenous metabolic energy for detoxification activity by inducing the entire defensome (Kim et al., 2013). Diverse intra- and/or extracellular metabolic processes other than those mentioned above are likely modulated by environmental changes in rotifers. To expand our knowledge of the molecular effects of such environmental modulators, we analyzed transcriptional responses of *Dmrt* genes in *B. koreanus*, as Dmrt proteins are known to have diverse functions in physiology and reproductive events in organisms.

Changes in UV radiation (UVR) affect the zooplankton community of the aquatic ecosystem in many ways (Häder et al., 2007; Dahms and Lee, 2010). However, little is known about the effects of UV radiation on rotifers. UVR may have detrimental effects on rotifers, which may influence the entire aquatic ecosystem at all levels of the community, as rotifers play a crucial role in aquatic food chains. Of planktonic species, rotifers may be affected by acute UVR damage as they are showing less vertical migration than other species. In *B. koreanus*, UV-B radiation induced growth retardation and decreased transcript levels of *Dmrt* genes. Interestingly, rotifers exposed to 2 kJ m^{-2}UV-B produced lower transcript levels of *Dmrt* genes for 2 to 4 days, suggesting that UV-B-induced growth retardation was potentially correlated with downregulated transcription of *Bk-Dmrt* genes as their mRNA levels were not significantly modulated in the control group for 10 days. Indeed, UV-B doses higher than 10 kJ m^{-2} induced significant mortality, leading to a significant decrease in transcript levels of *Bk-Dmrt* genes after 24 h. Taken together, these results suggest that UV-B radiation is a negative transcriptional regulator of *Dmrt* genes in *B. koreanus*, and also suggest that processes that involve Dmrt in rotifers are direct or indirect targets of UV-B. The function(s) of *Bk-Dmrt* genes remains to be elucidated. In most vertebrates and several invertebrates, *Dmrt* genes have been suggested to have the conserved role of controlling sex differentiation and determination (Volff et al., 2003). However, other physiological roles such as animal development, embryogenesis, organogenesis, and differentiation of sexual organs have been suggested in various animal species (Hong et al., 2007; Naimi et al., 2009;

Tresser et al., 2010). In fact, population abundance and reproduction ability of aquatic invertebrate species such as *Asplanchna girodi* (Grad et al., 2001), *Chydorus sphaericus* (Cabrera et al., 1997), *Daphnia pulicaria* (Grad et al., 2001), *Tigriopus californicus*(Kane and Pomory, 2001), and several rotifer species (Sawada and Enesco, 1984; Persaud and Williamson, 2005) were significantly inhibited by UVR. Furthermore, in the water flea *D. magna*, embryonic transcript levels of *Dmrt11E*, *Dmrt93B*, and *Dmrt99B* were increased at different developmental points after ovulation, suggesting that *Dmrt* genes may be involved in invertebrate reproduction (Kato et al., 2011). We demonstrated that a decrease in *Bk-Dmrt* transcript levels was correlated with growth retardation and a decrease in reproductive rate in *B. koreanus*, even though the exact roles of the Dmrt proteins in rotifers have yet to be defined. Because *Dmrt* genes are known to have sex-related roles in other organisms, decreased *Bk-Dmrt* transcript levels may play a role in environmentally-induced asexual/sexual switches in *Rotifera*. Previously, Chalker-Scott (1995)-reported a differentially modulated sex ratio in different developmental stages of the copepod *Tigriopus californicus* in response to UV-B irradiation, suggesting that UV-B radiation may influence sex-related mechanisms in aquatic organisms. We are currently studying Dmrt-related sexual reproduction in rotifer species in response to UV-B radiation, as populations of *B. koreanus* are maintained by parthenogenesis.

Although no differences in mortality or growth rate were observed among groups of rotifers exposed to different concentrations of B[*a*]P for 2 days, we observed transcriptional differences in levels of *Bk-Dmrt* genes in response to B[*a*]P in 24 h. Upon exposure to high levels of B[*a*]P (100 μg/L B[*a*]P), transcript levels of all *Bk-Dmrt* genes significantly decreased, while *Bk-Dmrt99B* was slightly downregulated upon exposure to 10 μg/L B[*a*]P. A time course experiment of B[*a*]P exposure demonstrated that transcript levels of *Bk-Dmrt* genes were strongly modulated by B[*a*]P. In aquatic invertebrates such as the amphipods *Gammarus duebeni* and *Chaetogammarus marinus* (Lawrence and Poulter, 2001a,b) and the Pacific oyster *C. gigas* (Choy et al., 2007), B[*a*]P treatment has been reported to retard embryogenesis, impair swimming activity, and inhibit growth. Based on these results, we interpreted the effects of B[*a*]P on *Bk-Dmrt* transcript regulation as follows. First, B[*a*]P might be reproductively toxic, although it remains to be determined whether B[*a*]P has a detrimental effect on reproductive events in aquatic invertebrates. In zebrafish, waterborne B[*a*]P exposure modulated reproduction and altered transcriptional levels of several genes (*20β-HSD*, *CYP19A2*, and *vitellogenin*) that are important in regulating reproduction (Hoffmann and Oris, 2006). Furthermore, B[*a*]P treatment impaired the reproductive activity of male and female zebrafish with a reduction in egg production (Alsop et al., 2007). Therefore, B[*a*]P may have adverse physiological and reproductive effects in rotifer species via modulation of *Dmrt* genes. Second, growth retardation and transcriptional regulation of *Dmrt* genes could be affected indirectly by B[*a*]P treatment, as increased detoxification activity could trigger growth retardation and modulate physiological parameters through metabolic energy consumption. Rotifers may modulate their energy metabolism to balance detoxification costs and endogenous physiological conditions through regulation of transcription and translation of defense gene batteries. In fact, a series of detoxification mechanisms involved in *CYP*-mediated biotransformation were acutely induced in response to exposure of *B. koreanus* to B[*a*]P for 24 h (Kim et al., 2013). Moreover, transcriptional induction of two *Dmrt* genes (*Dmrt93B* and *Dmrt99B*) corresponded to a gradual increase in population growth in rotifers exposed to B[*a*]P after 4 days. Dmrt proteins may play a potential role in the growth and reproduction of rotifers, although further studies are necessary to uncover the molecular mechanism of *Bk-Dmrt* gene regulation by diverse environmental stressors in rotifers.

In conclusion, our results together with those of previous studies indicate that changes in environmental conditions, particularly those triggered by UV-B radiation and B[*a*]P treatment, affect the population growth of the monogonont rotifer *B. koreanus* and are associated with detoxification and molecular responses (Kim et al., 2011, 2013). Although slight discrepancies in correlation between transcriptional increase of *Bk-Dmrt* genes and population growth were observed at both exposures (*Dmrt93B* at 5 days post-exposure to UV or to B[*a*]P; *Dmrt99B* at 4 and 5 days post-exposure to B[*a*]P), transcript levels of *Bk-Dmrt* genes were strongly affected by these environmental stressors, and therefore the potential roles of *Bk-Dmrt* genes in environmental stressor-triggered growth retardation should be investigated. In particular, monogonont rotifers have a single gonad, and analysis of expression and functional characterization of *Dmrt* genes in rotifer will be interesting as *Dmrt* genes are specifically expressed in the developing gonads of almost all animals (reviewed by Kopp, 2012). In addition, analysis of Dmrt-mediated molecular and physiological events will be helpful infurthering our understanding of the community and health status of aquatic ecosystems, as rotifers are a food resource for consumers in aquatic food webs.

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References

Alsop, D., Brown, S., Van Der Kraak, G., 2007. The effects of copper and benzo[*a*]pyrene on retinoids and reproduction in zebrafish. Aquat. Toxicol. 82, 281–295.

Bonaventura, R., Poma, V., Costa, C., Matranga, V., 2005. UV-B radiation prevents skeleton growth and stimulates the expression of stress markers in sea urchin embryos. Biochem. Biophys. Res. Commun. 328, 150–157.

Cabrera, S., Lopez, M., Tartarotti, B., 1997. Phytoplankton and zooplankton response to ultraviolet radiation in a high-altitude Andean lake: short- versus long-term effects. J. Plankton Res. 19, 1565–1582.

Chalker-Scott, L., 1995. Survival and sex-ratios of the intertidal copepod, *Tigriopus californicus*, following ultraviolet-B (290–320 nm) radiation exposure. Mar. Biol. 123, 799–804.

Choy, E.J., Jo, Q., Moon, H.B., Kang, C.K., Kang, J.C., 2007. Time-course uptake and elimination of benzo(α)pyrene and its damage to reproduction and ensuing reproductive outputs of Pacific oyster, *Crassostrea gigas*. Mar. Biol. 151, 157–165.

Dahms, H.-U., Lee, J.-S., 2010. UV radiation in marine ectotherms: molecular effects and responses. Aquat. Toxicol. 97, 3–14.

Dahms, H.-U., Hagiwara, A., Lee, J.-S., 2011. Ecotoxicology, ecophysiology, and mechanistic studies with rotifers. Aquat. Toxicol. 101, 1–12.

Felsenstein, J., 1989. PHYLIP — Phylogeny Inference Package (Version 3.2). Cladistics 5, 164–166.

Grad, G., Williamson, C.E., Karapelou, D.M., 2001. Zooplankton survival and reproduction responses to damaging UV radiation: a test of reciprocity and photoenzymatic repair. Limnol. Oceanogr. 46, 584–591.

Häder, D.P., Kumar, H.D., Smith, R.C., Worrest, R.C., 2007. Effects of solar UV radiation on aquatic ecosystems and interactions with climate change. Photochem. Photobiol. Sci. 6, 267–285.

Hoffmann, J.L., Oris, J.T., 2006. Altered gene expression: a mechanism for reproductive toxicity in zebrafish exposed to benzo[*a*]pyrene. Aquat. Toxicol. 78, 332–340.

Hong, C.-S., Park, B.-Y., Saint-Jeannet, J.-P., 2007. The function of *Dmrt* genes in vertebrate development: it is not just about sex. Dev. Biol. 310, 1–9.

Hwang, D.-S., Dahms, H.-U., Park, H.G., Lee, J.-S., 2013a. A new intertidal *Brachionus* and intrageneric phylogenetic relationship among *Brachionus* as revealed by allometry and *CO1-ITS1* gene analysis. Zool. Stud. 52, 13.

Hwang, D.-S., Suga, K., Sakakura, Y., Park, H.G., Hagiwara, A., Rhee, J.-S., Lee, J.-S., 2013b. Complete mitochondrial genome of the monogonont rotifer, *Brachionus koreanus* (*Rotifera*, Brachionidae). Mitochondrial DNA 25, 29–30.

Kane, K., Pomory, C.M., 2001. The effects of UV-B radiation on the reproduction and mortality of *Tigriopus californicus* (Copepoda: Harpacticoida). Hydrobiologia 444, 213–215.

Kato, Y., Kobayashi, K., Oda, S., Colbourn, J.K., Tatarazako, N., Watanabe, H., Iguchi, T., 2008. Molecular cloning and sexually dimorphic expression of DM-domain genes in *Daphnia magna*. Genomics 91, 94–101.

Kato, Y., Kobayashi, K., Oda, S., Colbourn, J.K., Tatarazako, N., Watanabe, H., Iguchi, T., 2011. Environmental sex determination in the branchiopod crustacean *Daphnia magna*: deep conservation of a doublesex gene in the sex-determining pathway. PLoS Genet. 7, e1001345.

Kim, R.-O., Rhee, J.-S., Won, E.-J., Lee, K.-W., Kang, C.-M., Lee, Y.-M., Lee, J.-S., 2011. Ultraviolet B retards growth, induces oxidative stress, and modulates DNArepair-related gene and heat shock protein gene expression in the monogonont rotifer, *Brachionus* sp. Aquat. Toxicol. 101, 529–539.

Kim, R.-O., Kim, B.-M., Jeong, C.-B., Nelson, D.R., Rhee, J.-S., Lee, J.-S., 2013. Expression pattern of entire cytochrome P450 genes and response of defensomes in the benzo [*a*]pyrene-exposed monogonont rotifer *Brachionus koreanus*. Environ. Sci. Technol. 47, 13804–13812.

Kopp, A., 2012. *Dmrt* genes in the development and evolution of sexual dimorphism. Trends Genet. 28, 175–184.

Lawrence, A.J., Poulter, C., 2001a. Impact of copper, pentachlorophenol and benzo[α]pyrene on the swimming efficiency and embryogenesis of the amphipod *Chaetogammarus marinus*. Mar. Ecol. Prog. Ser. 223, 213–223.

Lawrence, A.J., Poulter, C., 2001b. Impact of copper, pentachlorophenol and benzo[α]pyrene on the swimming efficiency and embryogenesis of the amphipod *Chaetogammarus marinus*. Mar. Ecol. Prog. Ser. 223, 213–223.

Lee, J.-S., Rhee, J.-S., Kim, R.-O., Choi, B.-S., Park, H.G., Park, G.S., Hagiwara, A., Choi, I.Y., 2011. Genomic DNA information (680 Mb) and molecular insights to the monogonont rotifer *Brachionus ibericus*. Hydrobiologia 662, 65–75.

Lesser, M.P., Lamare, M.D., Barker, M.F., 2004. Transmission of ultraviolet radiation through the Antarctic annual sea ice and its biological effects on sea urchin embryos. Limnol. Oceanogr. 49, 1957–1963.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real time quantitative PCR and the $2^{-\Delta\Delta C}_{t}$ method. Methods 25, 402–408.

Lu, X.Y., Wu, R.S.S., 2005. UV induces reactive oxygen species, damages sperm, and impairs fertilisation in the sea urchin *Anthocidaris crassispina*. Mar. Biol. 148, 51–57.

Miller, S.W., Hayward, D.C., Bunch, T.A., Miller, D.J., Ball, E.E., Bardwell, V.J., Zarkower, D., Brower, D.L., 2003. A DM domain protein from a coral, *Acropora millepora*, homologous to proteins important for sex determination. Evol. Dev. 5, 251–258.

Naimi, A., Martinez, A.S., Specq, M.L., Mrac, A., Diss, B., Mathieu, M., Sourdaine, P., 2009. Identification and expression of a factor of the DM family in the oyster *Crassostrea gigas*. Comp. Biochem. Physiol. A 152, 189–196.

Nespolo, R.F., Halkett, F., Figueroa, C.C., Plantegenest, M., Simon, J.C., 2009. Evolution of trade-offs between sexual and asexual phases and the role of reproductive plasticity in the genetic architecture of aphid life histories. Evolution 63, 2402–2412.

Ottolenghi, C., Fellous, M., Barbieri, M., McElreavey, K., 2002. Novel paralogy relations among human chromosomes support a link between the phylogeny of doublesex related genes and the evolution of sex determination. Genomics 79, 333–343.

Persaud, A.D., Williamson, C.E., 2005. Ultraviolet and temperature effects on planktonic rotifers and crustaceans in northern temperate lakes. Freshw. Biol. 50, 467–476.

Ramirez Llodra, E., 2002. Fecundity and life-history strategies in marine invertebrates. Adv. Mar. Biol. 43, 87–170.

Raymond, C.S., Shamu, C.E., Shen, M.M., Seifert, K.J., Hirsch, B., Hodgkin, J., Zarkower, D., 1998. Evidence for evolutionary conservation of sex-determining genes. Nature 391, 691–695.

Raymond, C.S., Murphy, M.W., O'Sullivan, M.G., Bardwell, V.J., Zarkower, D., 2000. Dmrt1, a gene related to worm and fly sexual regulators, is required for mammalian testis differentiation. Genes Dev. 14, 2587–2595.

Sawada, M., Enesco, H.E., 1984. Effects of UV radiation on the lifespan of the rotifer *Asplanchna brightwelli*. Exp. Gerontol. 19, 289–296.

Sebens, K.P., 2002. Energetic constraints, size gradients, and size limits in benthic marine invertebrates. Integr. Comp. Biol. 42, 853–861.

Snell, T.W., Janssen, C., 1995. Rotifers in ecotoxicology: a review. Hydrobiologia 313/314, 231–247.

Tresser, J., Chiba, S., Veeman, M., El-Nachef, D., Newman-Smith, E., Horie, T., Tsuda, M., Smith, W.C., 2010. Doublesex/mab3 related-1 (*dmrt1*) is essential for development of anterior neural plate derivatives in *Ciona*. Development 137, 2197–2203.

Veith, M., Klattig, J., Dettai, A., Schmidt, C., Englert, C., Volff, J.N., 2006. Male-biased expression of X-chromosomalDMdomain-less*Dmrt8* genes in the mouse. Genomics 88, 185–195.

Volff, J.N., Zarkower, D., Bardwell, V.J., Schartl, M., 2003. Evolutionary dynamics of the DM domain gene family in metazoans. J. Mol. Evol. 57, S241–S249.

Wang, F., Yu, Y., Ji, D., Li, H., 2012. The *DMRT* gene family in amphioxus. J. Biomol. Struct. Dyn. 30, 191–200.

Yi, W., Zarkower, D., 1999. Similarity of DNA binding and transcriptional regulation by *Caenorhabditis elegans*MAB-3 and *Drosophila melanogaster*DSX suggests conservation of sex determining mechanisms. Development 126, 873–881.