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Genome-wide identification of 31 cytochrome P450 (*CYP*) genes in the freshwater rotifer *Brachionus calyciflorus* and analysis of their benzo[α]pyrene-induced expression patterns

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Abstract

While marine invertebrate cytochrome P450 (CYP) genes and their roles in detoxification mechanisms have been studied, little information is available regarding freshwater rotifer CYPs and their functions. Here, we used genomic sequences and RNA-seq databases to identify 31 *CYP* genes in the freshwater rotifer *Brachionus calyciflorus*. The 31 *Bc-CYP* genes with a few tandem duplications were clustered into CYP 2, 3, 4, mitochondrial, and 46 clans with two marine rotifers *B. plicatilis* and *B. koreanus*. To understand the molecular responses of these 31 *Bc-CYP* genes, we also examined their expression patterns in response to benzo[α]pyrene (B[α]P). Three *Bc-CYP* genes (*Bc-CYP3044B3*, *Bc-CYP3049B4*, *Bc-CYP3049B6*) were significantly upregulated ($P<0.05$) in response to B[α]P, suggesting that these *CYP* genes can be involved in detoxification in response to B[α]P exposure. These genes might be useful as biomarkers of B[α]P exposure in *B. calyciflorus*. Overall, our findings expand the repertoire of known CYPs and shed light on their potential roles in xenobiotic detoxification in rotifers.

Keywords: Freshwater rotifer, *Brachionus calyciflorus*, cytochrome P450s, benzo[α]pyrene

1. Introduction

Cytochrome P450s (CYPs) comprise a multigene superfamily belonging to the phase I detoxification system in diverse living organisms (e.g., bacteria, fungi, and animals) (Nelson et al., 1996; Werck-Reichhart and Feyereisen, 2000). CYPs have important roles in the metabolism of endogenous (e.g., steroid hormones, bile acids, and fatty acids) and exogenous (e.g., drugs, toxicants, and environmental pollutants) compounds (Nelson et al., 1993). Thus, CYPs are considered to be reliable biomarkers for monitoring environmental pollutants in aquatic animals (Flammarion et al., 2002; Mayon et al., 2006; Rhee et al., 2013; Kim et al., 2014). CYPs belonging to families 1-4 have crucial roles in xenobiotic detoxification in vertebrates and invertebrates (Baldwin et al., 2009). In particular, the CYP1 family (CYP1A, CYP1B, CYP1C, and CYP1D) has been well studied for its role in detoxification through the aryl hydrocarbon receptor (AhR)-CYP1 signaling pathway, which is involved in biotransformation of diverse xenobiotics such as benzo[α]pyrene (B[α]P) and polycyclic aromatic hydrocarbons (PAHs) in marine vertebrates (Goksøyr, 1995; Whitlock, 1999; Wang et al., 2006). For example, in the killifish *Fundulus heteroclitus* (Wang et al., 2006) and the marine medaka *Oryzias melastigma* (Kim et al., 2013), *CYP1A* has been shown to be significantly upregulated in response to B[α]P. However, the invertebrate AhR-CYP signaling pathway remains unclear, because no CYP1 family members have been identified in marine invertebrates (Han et al., 2017b). Rather than the CYP1 family, the CYP2 and CYP3 families were recently reported to be involved in detoxification in marine invertebrates such as the polychaete *Perinereis nuntia* (Won et al., 2013) and the copepods *Tigriopus japonicus* (Han et al., 2014) and *Paracyclopina nana* (Han et al., 2015). However, knowledge of CYP complements is limited in freshwater invertebrates except for the water flea *Daphnia pulex* compared to what is known about CYP complements in vertebrates and marine invertebrates (Nelson, 2009; Baldwin et al., 2009; Han et al., 2017b). Of note, CYPs have been identified in diverse organisms, and a large body of information has been uploaded into the CYP database (http://drnelson.uthsc.edu/cytochromeP450.html). Thus, genome-wide identification of *CYP* genes and analysis of their transcriptional profiles in freshwater invertebrates are important for better understanding AhR-CYP signaling pathways associated with detoxification mechanisms in aquatic invertebrates.

Rotifers are aquatic invertebrates that are distributed worldwide and play crucial roles as energy transmitters between producers and consumers in aquatic food webs. Rotifers in the genus *Brachionus* (e.g., *B. calyciflorus*, *B. koreanus*, and *B. plicatilis*) have been considered as suitable model species for ecology, gerontology, and ecotoxicology (Dahms et al., 2011;

Smith and Snell, 2014; Snell et al., 2015; Won et al., 2017) due to their many advantages (e.g., small size [≈ 150-200 μm], short generation cycle [≈24 h], high fecundity, and easy laboratory maintenance). Furthermore, extensive RNA-seq information from the rotifers *B. koreanus* and *B. plicatilis* has allowed a better understanding of physiological effects at the cellular and molecular levels in response to environmental stressors (e.g., gamma radiation, microplastics, and methylmercury [MeHg]) (Han et al., 2014; Jeong et al., 2016; Lee et al., 2017). Indeed, entire *CYP* genes have been identified, and the functions of these genes in detoxification mechanisms were uncovered using B[α]P-exposed *B. koreanus* (Kim et al., 2013b) and *B. plicatilis* (Kim et al., 2017). However, genome-wide identification of *CYP* genes and analysis of their expression patterns have not yet been performed in the freshwater rotifer *B. calyciflorus*.

In this study, we identified 31 *CYP* genes from genome sequences and RNA-seq databases of the freshwater rotifer *B. calyciflorus* and analyzed their expression patterns in response to B[α]P exposure. This is the first study to identify the entire *CYP* gene complement in a freshwater rotifer. Our results contribute to the understanding of molecular defense mechanisms involving CYP genes in response to environmental pollutants in freshwater rotifers.

2. Materials and methods

2.1. Culture and maintenance

Resting eggs of the monogonont freshwater rotifer *B. calyciflorus* were collected in Zwartenhoek, The Netherlands (52.0263N and 4.18355E) by Dr. Steven Declerck (Netherlands Institute of Ecology). A mass culture of a healthy resting egg strain was kindly provided by Prof. Atsushi Hagiwara (Nagasaki University, Japan). *B. calyciflorus* rotifers were reared and maintained at the aquarium facility of the Department of Biological Science, Sungkyunkwan University (Suwon, South Korea). Briefly, *B. calyciflorus* rotifers were cultured at 25°C in filtered freshwater with a light/dark ratio of 12L:12D. The rotifers were fed the green alga *Chlorella* sp. (approximately 6×10^4 cells/ml). Species identification of *B. calyciflorus* was verified by morphometric analysis and sequencing of the universal marker cytochrome oxidase 1 (*COI*) gene (Hwang et al., 2013; Mills et al., 2017).

2.2. Identification and nomenclature of CYP genes

Whole genome data were obtained by RNA-seq using an Illumina HiSeq 2000 platform (300 bp, 500 bp, and 800 bp paired-end libraries and 2 kb, 5 kb, and 10 kb mate-pair libraries). Sequencing was performed at the National Instrumentation Center for Environment Management (NICEM), Seoul National University in Seoul, South Korea. After sequencing, the raw sequenced reads were pre-processed using Trimmomatic (http://www.usadellab.org/cms/?page=trimmomatic). The pre-processed raw reads were assembled *de novo* using the Trinity software suite, and the coding sequences were extracted using Platanas assembler v1.2.4 (http://platanus.bio.titech.ac.jp) and HaploMerger 2 v20151124 (http://mosas.sysu.edu.cn/genome/download_softwares.php). Whole genome assembly yielded a total genome length of 129,636,934 bp (scaffold nos. 1,041; N50=787 kb) in *B. calyciflorus* (unpublished data).

To obtain the sequences of the *Bc-CYP* genes, all contigs in the transcriptome database of *B. calyciflorus* (unpublished data) were subjected to BLAST analysis (tBLASTn) against the non-redundant (NR) database at GenBank (http://www.ncbi.nlm.nih.gov/genome/seq/database.html). All acquired contigs were mapped to the *B. calyciflorus* genome to obtain the complete *CYP* gene sequences and to determine the structures of the *CYP* genes in the scaffolds. *CYP* gene annotation and nomenclature followed the criteria of the Cytochrome P450 Nomenclature Committee (Nelson, 2009). All *CYP* gene information was deposited at GenBank; the accession numbers of the *CYP* genes are provided in **Table S1**.

2.3. Phylogenetic analysis

To determine the phylogenetic positions of the 31 Bc-CYP genes, the deduced amino acid sequences of the *B. calyciflorus* CYP genes were aligned with those of other marine rotifer species using Geneious software (ver. 8.1.9; https://www.geneious.com) (Kearse et al., 2012) and the MUSCLE alignment algorithm (Robert, 2004). Representative *CYP* family genes from the marine rotifers *B. koreanus* (Bk; 25) and *B. plicatilis* (Bp; 28) were obtained from the GenBank database. According to the results of the model test, maximum likelihood phylogenetic analyses were performed with the LG + G + I model using MEGA software (ver. 7.0; Center for Evolutionary Medicine and Informatics, Tempe, AZ, USA).

2.3. Expression of the 31 Bc-CYP genes in response to B[α]P

B[α]P was purchased from Sigma-Aldrich (Sigma Aldrich, Inc., St. Louis, MO, USA;

purity > 96%; Cat. no. B1760; molecular weight 252.31). B[α]P was dissolved in dimethylsulfoxide (DMSO) to prepare a concentrated stock solution. To determine the mRNA expression levels of the 31 *Bc-CYP* genes, approximately 300 rotifers were exposed to B[α]P (10 μg/L [3.96 nM] and 100 μg/L [39.6 nM]) for different lengths of time (0, 6, 12, and 24 h). These concentrations and time were chosen based on our previous studies of two marine rotifers *B. koreanus* (Kim et al., 2013b) and *B. plicatilis* (Kim et al., 2017).

Total RNA was isolated from each sample with TRIZOL® reagent (Invitrogen, Paisley, Scotland, UK) according to the manufacturer's instructions. The quantity and quality of the RNAs were assessed at 230, 260, and 280 nm using a spectrophotometer (QIAxpert, QIAGENGmbH, Hilden, Germany). To synthesize single-stranded cDNA for quantitative RT-PCR, total RNA (2 μg) and oligo$(dT)_{20}$ primers were used for reverse transcription (SuperScript™ II RT kit, Invitrogen, Carlsbad, CA, USA) under the following conditions: 65℃/5 min, 42℃/2 min, 42℃/50 min, and 72℃/15 min. The qRT-PCR thermocycling conditions were as follows: 95℃/4 min; 35 cycles of 95℃/30s, 58℃/30s, 72℃/30s; and 72℃/10 min. SYBR Green was used as a probe (Molecular Probe, Invitrogen), and the amplification reactions were performed in a CFX96™ real-time PCR system (Bio-Rad, Hercules, CA, USA). To confirm specific amplification of the desired products, melting curve cycles were run using the following conditions: 95℃/1 min; 55℃/1 min; and 80 cycles of 55℃/10s with a 0.5℃ increase per cycle using the real-time RT-PCR F or R primers **(Table S1)**. Three technical replicates were performed for each experiment. Fold-change in relative expression was calculated by the $2^{-\Delta\Delta C}$t method (Livak and Schmittgen, 2001).

2.4. Statistical analysis

SPSS® version 21 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. Data are expressed as mean values with standard errors (means ± SEs). Normal distributions and homogeneity of variances were assessed by Levene's test. Data were analyzed using one-way ANOVA, followed by Tukey's honest significant difference test (P<0.05).

3. Results and discussion

3.1. Annotation of the 31 Bc-CYP genes and phylogenetic analysis

Thirty-one full-length *CYP* genes were identified and annotated through *in silico* analysis

of the *B. calyciflorus* genome and RNA-seq assembly data. Gene synteny analysis of the 31 *CYP* genes was conducted by confirming the relative locations of the *CYP* genes in the scaffolds of the *B. calyciflorus* genome (**Fig. 1**). Of the 31 CYPs, four groups (*CYP3044B4* and *CYP3044B5*; *CYP3044B7* and *CYP4V38*; *CYP3045C11* and *CYP3047C13*; and *CYP3045C15* and *CYP3045C17*) were closely localized and showed tandem duplication (e.g., head-to-tail and head-to-head orientation) on the *B. calyciflorus* genome. This finding indicates that several CYP genes probably expanded through tandem duplication events during evolution of *B. calyciflorus*. Compared to the self-fertilizing fish *Kryptolebias marmoratus* (Rhee et al., 2017), rotifers generally have lower frequencies of transposable elements in the *B. caliciflorus* genome (unpublished data), resulting in fewer tandem duplications in CYP families. Previously, four tandem duplications were found among 52 *CYP* genes in the copepod *T. japonicus* (Han et al., 2017a). Thus, the fewer tandem duplications likely explain why the *B. caliciflorus* genome has fewer CYP genes than the copepod *T. japonicus* genome.

We found that the identified Bc-CYPs are divided into five distinct clans: clans 2, 3, 4, mitochondrial, and 46. These clans are found in two marine rotifers *B. koreanus* and *B. plicatilis* (**Fig. 2**). The 31 Bc-CYPs were classified into eight families and 40 subfamilies. Interestingly, seven Bc-CYPs (CYP3049A1, CYP3049B4, CYP3049B5, CYP3049B6, CYP3049E5, CYP3049E6, and CYP3049F2) belonging to clan 46 showed high sequence identity and similarity to their *B. koreanus* homologues (**Table S1**). The CYPs in clan 46 have been shown to function in cholesterol 24-hydroxylase metabolism in the vertebrate brain (Nebert et al., 2013). While the role of clan 46 CYPs is still unclear in invertebrates, they have been identified in marine invertebrates such as the rotifer *B. koreanus* (Kim et al., 2013b) and the annelid *Capitella teleta* (Dejong and Wilson, 2014). Thus, the identification of seven clan 46 CYPs in the *B. calyciflorus* genome could shed light on the mechanistic role of these CYPs by linking information from functional studies in vertebrates to invertebrates. However, further studies are needed to elucidate the function of the seven clan 46-CYPs in *B. calyciflorus*.

The deduced amino acid sequences of the Bc-CYPs showed high similarities to those of other rotifer species. Moreover, all CYP clans clustered well with those of the marine rotifers *B. koreanus* and *B. plicatilis*. This finding indicates that the CYPs of each clan are evolutionarily conserved among rotifer species, and that these CYPs might have important roles in xenobiotic detoxification in *B. calyciflorus*.

3.2. Amino acid alignments of the conserved domains of Bc-CYP genes

All the analyzed Bc-CYPs have typical conserved domains, including a K-helix domain (EXXR), a PERF domain (PXRX) (except for Bc-CYP3045C14 [clan 3]), and a cysteine heme-binding region (FXXGXRXCXG) (**Fig. 3**). Generally, the K-helix domain is essential for stabilizing the conserved core structure of CYPs (Sirim et al., 2010). The heme-binding domain is involved in the NADPH-dependent electron transport pathway and plays an important role in O^2-dependent hydroxylation reactions (Werck-Reichhart and Feyereisen 2000). These domains are highly conserved in CYPs in almost all organisms (e.g., bacteria, plants, and animals) (Nelson, 1999; Chapple, 1998; Otyepka et al., 2007; Saxena et al., 2013). For example, in the marine medaka *Orzias melastigama*, the heme-binding domain in the C-terminal region of CYP1A is conserved (Kim et al., 2013a). Also, in the marine ciliate *Euplotes crassus*, the K-helix, PERF, and heme-binding domains were found in all five CYPs (Yim et al., 2017). In the copepods *P. nana* (46 CYPs; Han et al., 2015) and *T. japonicus* (52 CYPs; Han et al., 2017a), heme-binding domains were also identified, indicating that CYPs are highly conserved and might have important roles across all living organisms in proton transfer during monooxygenation. Taken together, these data indicate that the general functions and classical CYP domains (e.g., K-helix, PERF, and heme-binding domains) are highly conserved in all living organisms, including rotifers.

3.3. Distribution of CYP clans in rotifers and comparison with the CYP clans in other invertebrates

The proportions of the CYP genetic clans differed in the freshwater rotifer *B. calyciflorus* compared to the proportions in other aquatic invertebrates (**Fig. 3**). Specifically, the 31 Bc-CYPs were composed of approximately 3% clan 2, 61% clan 3, 6.5% clan 4, 6.5% clan MT, and 23% clan 46 CYPs. Of note, clan 3 showed the highest proportion in *B. calyciflorus*. In the marine rotifers *B. koreanus* and *B. plicatilis*, the clan compositions of the 25 Bk-CYP and 28 Bp-CYP genes were similar (Kim et al., 2013b**;** Kim et al., 2017). However, in the cladoceran *D. pluex* and the copepods *T. japonicus* and *P. nana*, clan 2 had the highest proportion of all CYP clans, suggesting that each CYP clan has evolved lineage-specific CYP genes within each animal taxon. In addition, each CYP clan underwent evolutionary diversification to enable adaptation to different environmental stressors (Berenbaum, 2002). Thus, it is likely that the different CYP clan compositions between rotifers and other invertebrates are closely associated with their adaptations to xenobiotics and environmental stressors.

3.4. Expression of the 31 Bc-CYP genes in response to B[α]P

Most of the *Bc-CYP* genes were differentially regulated in response to 10 μg/L and 100 μg/L B[α]P for different lengths of time (0, 6, 12, and 24 h). In particular, the transcript levels of four *CYP* genes (*CYP3044B3*, *CYP30445* [Clan 3] and *CYP3049B4*, *CYP3049B6* [Clan 46]) were highly upregulated in B[α]P-exposed *B. calyciflorus* (**Fig. 4**). In vertebrates, members of the CYP1 family have been used as biomarkers for monitoring of environmental toxicants through the PAH-AhR-CYP1A signaling pathway (Whitlock, 1999; Wang et al., 2006; Kim et al., 2013a). However, in invertebrates, this signaling pathway has not been studied, since the CYP1 family has not been identified in the genome. However, rather than CYP1, clan 2 and clan 3 CYPs are viewed as potential biomarkers for detection of environmental pollutants via the AhR-mediated CYP signaling pathway in marine invertebrates. Indeed, some CYPs or even entire CYP complements have been identified in a number of marine invertebrates (e.g., the mussel *Mytilus californianus*, the oyster *Crassostrea gigas*, and the copepods *P. nana* and *T. japonicus*) whose gene expression patterns change in response to environmental toxicants (Zanette et al., 2009; Han et al., 2014; Han et al., 2017a). For example, in the rotifer *B. koreanus*, *CYP3042A1* and *CYP3043A1* (clan 2) were significantly upregulated in response to B[α]P exposure (Kim et al., 2013b). In addition, in the rotifer *B. plicatilis*, *CYP3047B1* (clan MT) and *CYP3045C3-2* (clan 3) were highly up-regulated in response to B[α]P (Kim et al., 2017). Moreover, in the copepods *T. japonicus* and *P. nana*, some CYPs (*Tj-CYP3024A2*, *Tj-CYP3024A3*, *Tj-CYP3027C2*, *Pn-CYP3027F1*, and *Pn-CYP3027F2*) belonging to clan 3 were shown to be significantly upregulated in response to the water-accommodated fraction (WAF) of crude oil (Han et al., 2014; Han et al., 2015). Furthermore, in B[α]P-exposed *T. japonicus* (Kim et al., 2015) and WAF-exposed *P. nana* (Han et al., 2015), *AhR* and aryl hydrocarbon receptor nuclear translocator (ARNT) genes were shown to be up-regulated, suggesting that CYPs (clan 2 and clan 3) have an important role in detoxification of xenobiotics through the PAH-AhR-CYP signaling pathway in invertebrates. In addition, two *Bc-CYP* genes (*CYP3049B4*, *CYP3049B6* [Clan 46]) were shown to be up-regulated in response to B[α]P. Previously, in *B. koreanus*, *CYP3047A1* (clan MT) and *CYP3045C1* (clan 3) were shown to be significantly upregulated in response to WAF (Won et al., 2016). Also, expression of *CYP3049D1* and *CYP3049* (clan 46) and *CYP3027* (clan MT) was shown to be induced in chlorpyrifos-exposed *B. koreanus* (Kim et al., 2016), suggesting that CYPs might be involved in xenobiotic detoxification. However, different CYPs appear to show species-specific responses to a given toxicant. Thus,

knowledge of the expression patterns of these 31 *Bc-CYP* genes enhances our understanding of the biotransformation and detoxification mechanisms of CYPs in response to B[α]P in *B. calyciflorus*.

Overall, our findings contribute to our understanding of CYP evolution in rotifers and shed light on their potential role in detoxification and/or biotransformation mechanisms in the freshwater rotifer *B. calyciflorus*.

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Figure legends

Fig. 1. Gene synteny analysis of the 31 CYPs identified in *B. calyciflorus*. Genes are represented by colored arrows in the scaffolds. Each gene locus is presented in proportion to the length of the scaffold. Positions that are too close to represent with colored arrows are indicated by vertical bars.

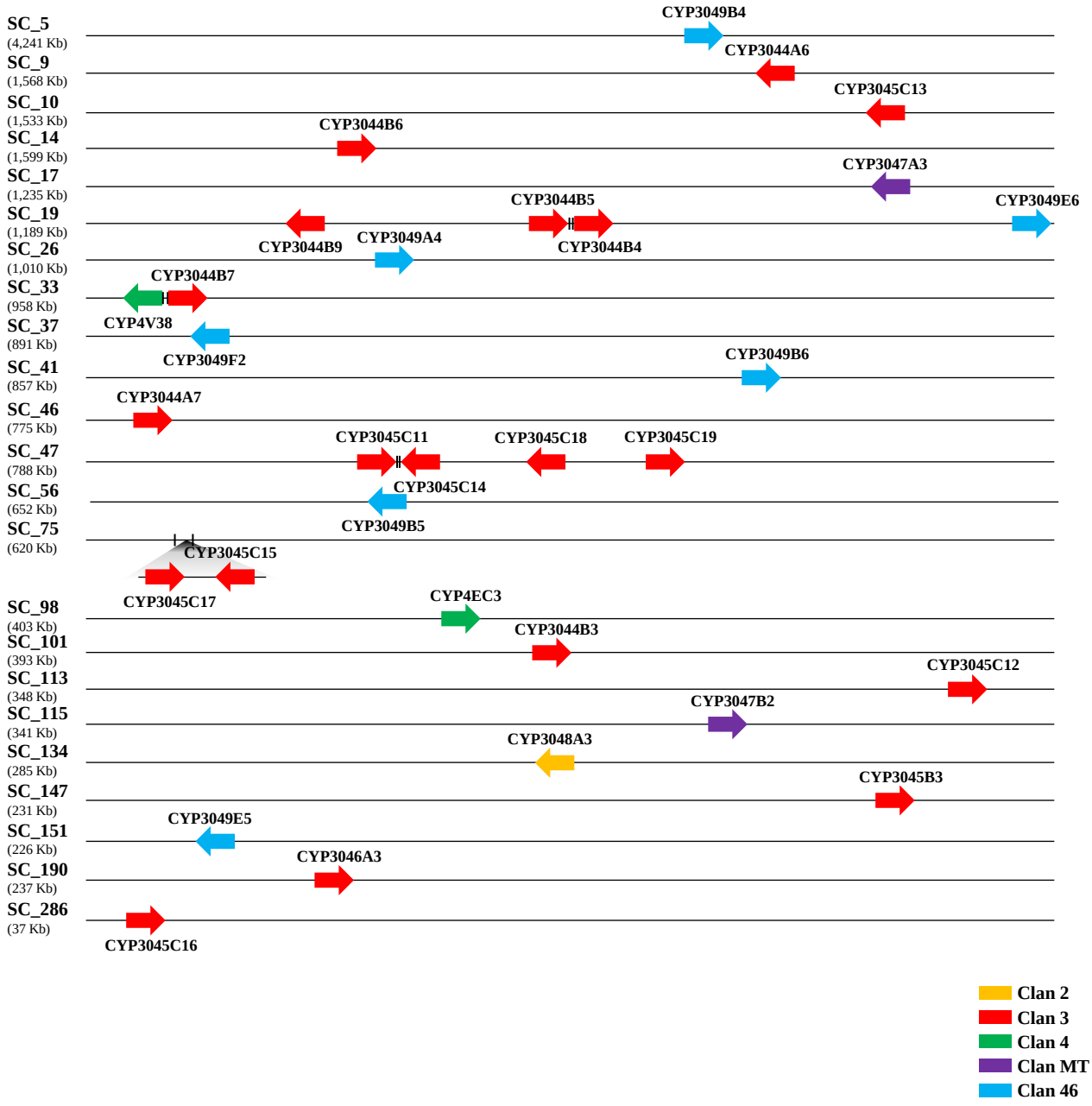
Fig. 2. Phylogenetic analysis of the 31 Bc-CYPs and comparison to the CYPs of two marine rotifers, *B. koreanus* (Bk; 25) and *B. plicatilis* (Bp; 28), using a maximum likelihood model.

Fig. 3. Multiple alignments of the deduced amino acid sequences of the conserved heme-binding domain of the 31 Bc-CYPs. The conserved heme-iron ligand signature motif (FXXGXRXCXG) is designated with a box.

Fig. 4. Distribution of each CYP clan in *B. calyciflorus* and comparison of the clans with the CYP clans in other animals.

Fig. 5. Expression patterns of the 31 CYPs in response to 10 and 100 μg/L B[α]P for different lengths of time (0, 6, 12, and 24 h). Expression profiles are presented by a heat map. Blue and red indicate downregulation and upregulation, respectively, of mRNA expression compared with control level (white).

Fig. 1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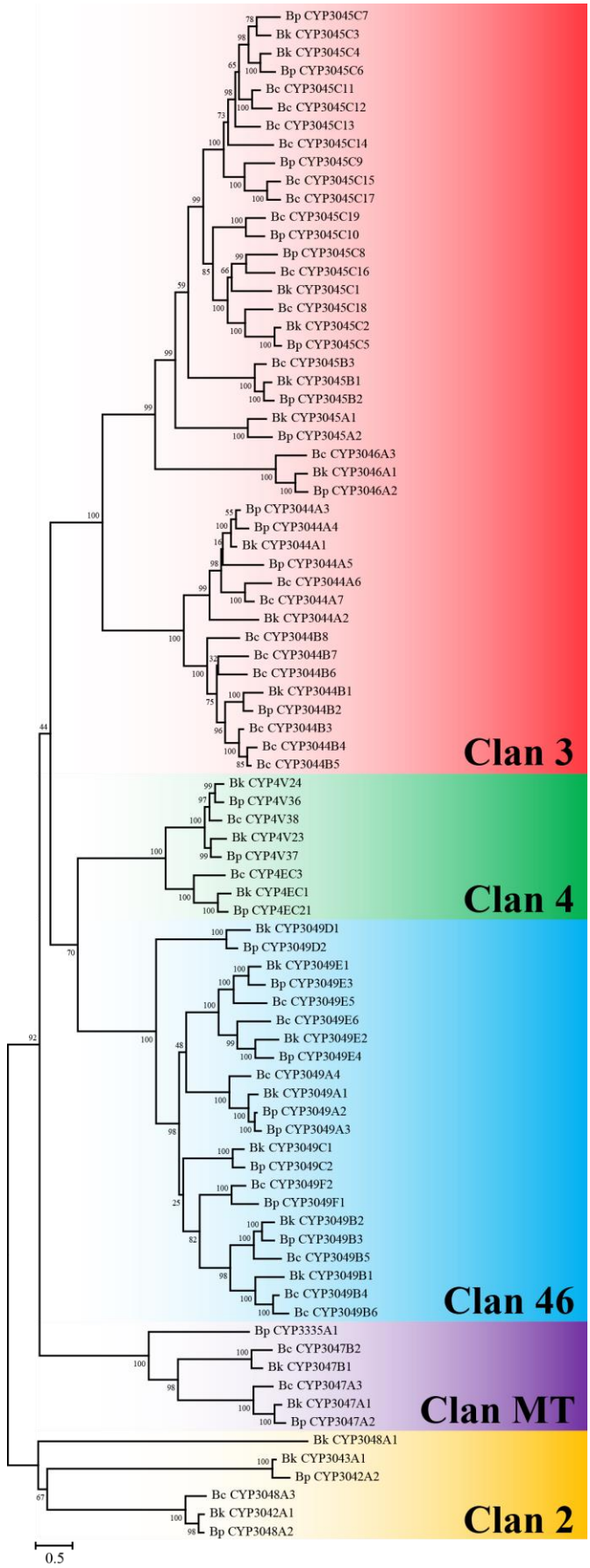
Clan 3
Clan 4
Clan MT
Clan 46

Fig. 2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CYP isoform
K-helix
PERF
Heme-binding
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Clan 3
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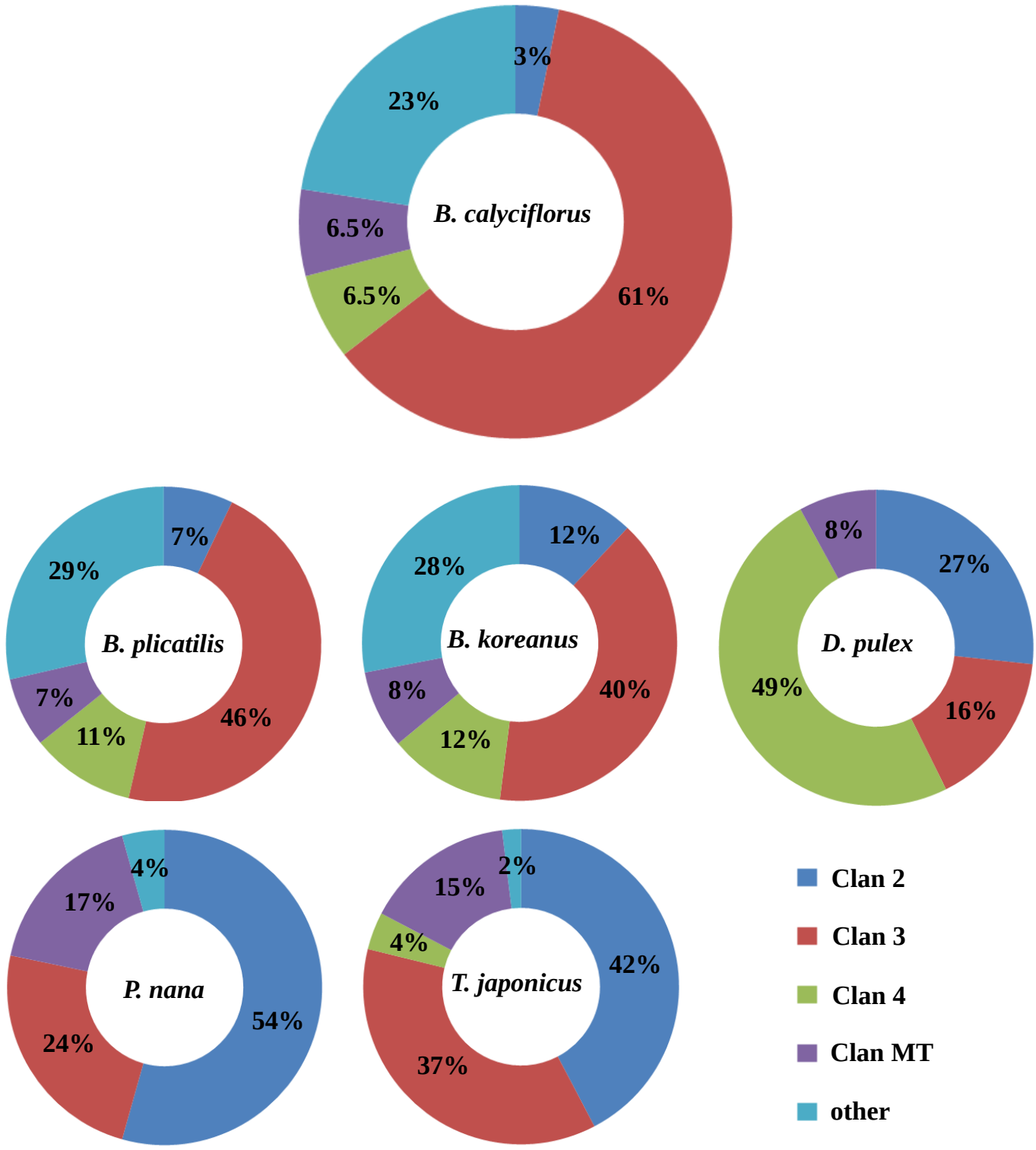
Fig. 4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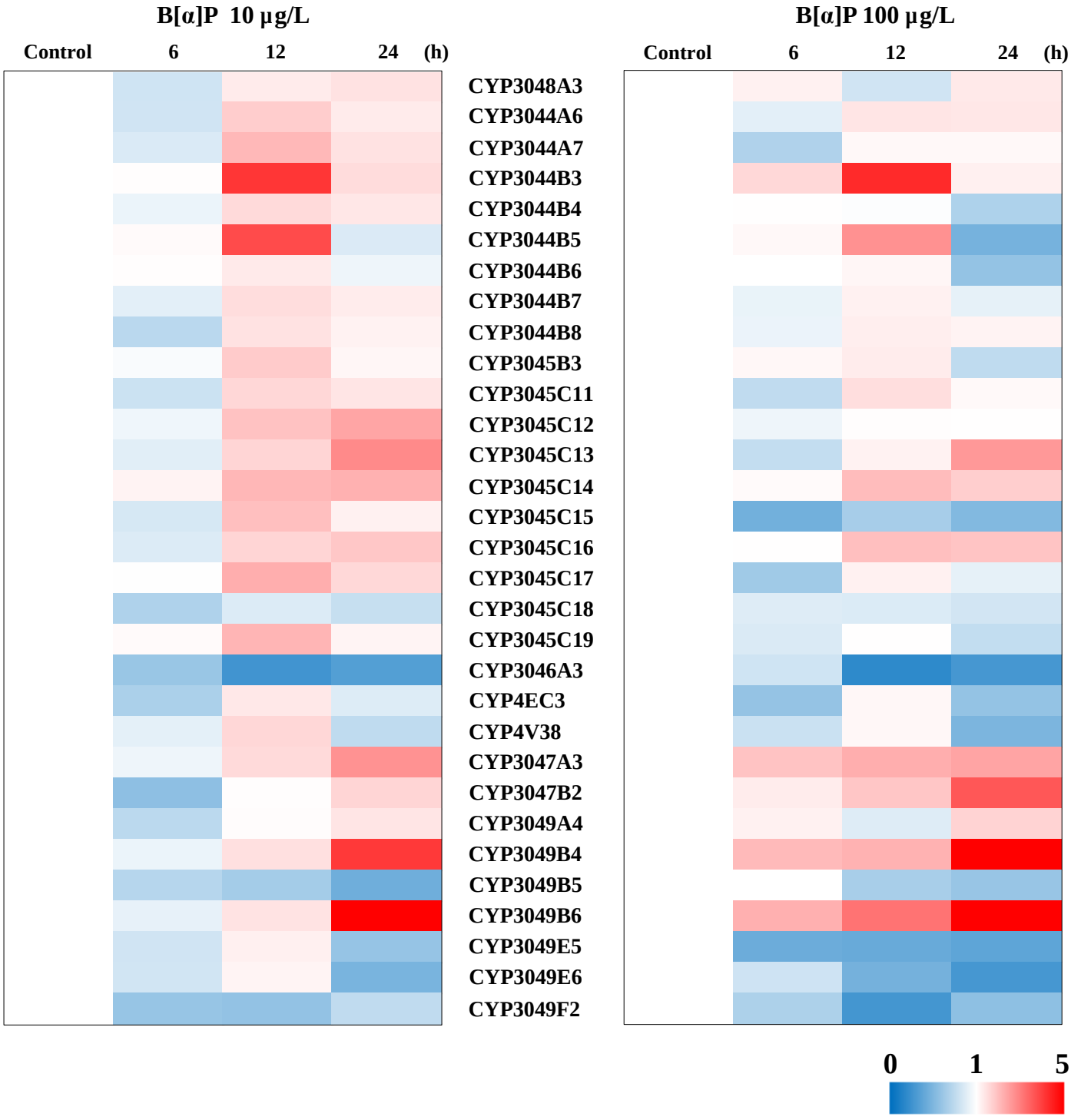
Clan 3
Clan 4
Clan MT
other

Fig. 5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