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The genome of the Java medaka (*Oryzias javanicus*): Potential for its use in marine molecular ecotoxicology

Bo-Young Lee[a,1], Jun Chul Park[a,1], Min-Sub Kim[a], Beom-Soon Choi[b], Duck-Hyun Kim[a], Jong-Sung Lim[c], Seungshic Yum[d], Un-Ki Hwang[e], Gyoung Ju Nah[c,*], Jae-Seong Lee[a,*]

[a] Department of Biological Sciences, College of Science, Sungkyunkwan University, Suwon 16419, South Korea
[b] Phyzen Genomics Institute, Seoul 08787, South Korea
[c] NICEM, College of Agriculture and Life Sciences, Seoul National University, Seoul 08826, South Korea
[d] Risk Assessment Research Center, Korea Institute of Ocean Science and Technology (KIOST), Geoje 53201, South Korea
[e] Marine Ecological Risk Assessment Center, West Sea Fisheries Research Institute, National Institute of Fisheries Science, Incheon 46083, South Korea

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ABSTRACT

The Java medaka (*Oryzias javanicus*) is distributed in tropical brackish water and is considered as an ecotoxicological experimental organism for assessing diverse pollutions and global climate change effects in the ocean. In this study, we sequenced and assembled the genome of *O. javanicus* using the Oxford Nanopore technique and anchored the scaffolds to the 24 genetic linkage map of a sister species *Oryzias melastigma*. The assembled genome consisted of 773 scaffolds including 24 LG-based scaffolds, and the estimated genome length was 846.3 Mb (N50 = 19.3 Mb), containing 24,498 genes. As detoxification processes are crucial in aquatic organisms, antioxidant-related genes including glutathione *S*-transferases, superoxide dismutase, catalase, and glutathione peroxidase were identified in this study. In the genome of *O. javanicus*, a total of 21 *GST*s, 4 *SOD*s, 1 *CAT*, and 7 *GPx*s were identified and showed high similarities between sister species *O. melastigma* and *Oryzias latipes*. In addition, despite having 8 classes of cytosolic GSTs family, medaka showed no presence of GST pi and sigma classes, which are predominantly found in carp and salmon, but not in neoteleostei. This study adds another set to genome-library of *Oryzias* spp. and is a useful resource for better understanding of the molecular ecotoxicology.

1. Introduction

Anthropogenic pollutants and global climate changes directly or indirectly affect aquatic organisms (Eissa and Zaki, 2011). To date, fish have been widely used as model species in ecotoxicological studies and diverse species have been proposed as useful animals for evaluating pollutant toxicity (Little et al., 1990; Heath, 1995; Carlson et al., 1998; Scholz et al., 2000; Hollis et al., 2000; Pane et al., 2003; Imai et al., 2007; Arellano-Aguilar et al., 2009). Fish are large enough to observe the effect of environmental changes so they are widely used for experiments in ecotoxicological study (Norrgren, 2012). Indeed, medaka (*Oryzias* spp.) is a widely used species in ecotoxicology, ecophysiology, and many other fields as a model organism along with zebrafish (Kasahara et al., 2007; Naruse et al., 2004a, 2004b; Crollius and Weissenbach, 2005). Furthermore, the first genome assembly of the freshwater Japanese medaka *Oryzias latipes* (Kasahara et al., 2007) allowed comparative studies with other fish species that inhabit different environmental conditions.

The *Oryzias* is a genus of ricefish and currently 33 species are recognized in FishBase (https://www.fishbase.de/home.htm). Each *Oryzias* species has a specific pattern of geographic distribution from freshwater to brackish, which makes this fish a potentially important species for diversity, evolution, and ecology (Inoue and Takei, 2002). Molecular phylogenetic studies divided the *Oryzias* species into three major species groups: latipes (*O. latipes*, *Oryzias curvinotus*, and *Oryzias mekongensis*), javanicus (*Oryzias javanicus*, *Oryzias melastigma*, and *Oryzias minutillus*), and celebensis (*Oryzias marmoratus* and *Oryzias celebensis*) groups (Takehana et al., 2005). The Java medaka (*O. javanicus*) (Bleeker, 1854) inhabit the brackish waters of South East Asia (Raymond Jani Angel et al., 2019). Two brackish water living ricefishes, *O. javanicus* and *O. melastigma*, have emerged as a new model fish for ecotoxicology studies in marine environments (Kim et al.,

* Corresponding authors.
E-mail addresses: gnah.nicem@snu.ac.kr (G.J. Nah), jslee2@skku.edu (J.-S. Lee).
[1] These authors contributed equally to this work.

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2016a; Kim et al., 2016b; Koyama et al., 2008; Horie et al., 2018). Given that the species *O. javanicus* is highly tolerant against changing conditions, it is considered to be a proper experimental organism for emerging global climate change and its following effects, including hypoxia and ocean acidification in the sea water (Koyama et al., 2008; Rositasari et al., 2018; Yusof et al., 2012; Zhang et al., 2009).

As aquatic organisms constantly face abiotic and biotic challenges, defense systems in living organisms are crucial for their survival in such environment. In particular, to sustain healthy condition and prolong their longevity, organisms continuously consume and excrete various exogenous substances, most of which are considered harmful due to increasing anthropogenic activities in the last few decades (Bukola et al., 2015; Subhendu, 2000). Among the defense mechanisms, detoxification system, comprised of three phases, namely phase I, II, and III, is extremely important. In this study, the fully constructed genome-library of *O. javanicus* was used to identify glutathione *S*-transferase (GST) gene family belonging to phase II detoxification system, which primarily involves the conjugation of glutathione (GSH) with xenobiotics to increase hydrophilicity for efficient excretion. In addition to GST family, other potential antioxidant biomarkers including catalase (CAT) (Ozmen et al., 2004), superoxide dismutase (SOD) (Kohen and Nyska, 2002), and glutathione peroxidase (GPx) (Brigelius-Flohé, 1999; Cossu et al., 1997), which are largely involved in mediation of oxidative stress, have been identified.

To date, several studies on *O. javanicus* have been conducted, focusing on various genes related to oxidative-stress defense and reproduction in response to various chemicals (Table 1). For example, various xenobiotics including organic chemicals (Won et al., 2014; Yum et al., 2015), endocrine disruptors (Woo et al., 2011), and metals (Kim et al., 2016a; Kim et al., 2016b)-induced differential gene expressions were observed by microarray technique in *O. javanicus*. In addition, the effect of metal exposures have been analyzed through identification and classification of metallothionein and changes in expression levels (Woo et al., 2006), and further used for the development of the protein chip analysis of the Java medaka metallothionein to check the metal levels (Lee et al., 2018). However, these approaches for the ecotoxicology with the Java medaka are limited at the genes, microarray and transcriptome. To date, genome-wide analysis with regard to environmental stressors and pollutants is allowed to establish multiple bio-markers and multi-omics approach to the ecotoxicologial experiments (Zhang et al., 2009).

In this study, we sequenced the genome of *O. javanicus* using a third generation sequencing technology, nanopore (ONT), and anchoring the genome scaffolds to the linkage map of *O. melastigma* to assemble the scaffolds at the chromosome level. This reference genome will facilitate further research to understand molecular mechanisms' biological responses to environmental toxicants.

Table 2
Information of sequence data used for genome assembly and annotation in *Oryzias javanicus*.

Genomic sequences	
PromethION	
Total number of reads	9,069,415
Total number of bases (bp)	74,091,915,108
Min length (bp)	19
Max length (bp)	173,513
Average length (bp)	8169
N50 (bp)	16,368
1k no./1k length/%	8,068,741/73,481,941,897/99
5k no./5k length/%	4,167,760/62,775,214,690/85
10k no./10k length/%	2,398,462/50,213,498,737/68
20k no./20k length/%	992,738/30,126,801,958/41
Genome coverage	87.5×
NCBI accession number	**SRR10988328**
Illumina paired-end 500 bp	
Total number of raw reads	123,650,688
Total number of raw bases (bp)	31,0363,22,688
Total number of cleaned reads	121,610,242
Total number of cleaned bases (bp)	19,909,035,988
Genome coverage	23.5×
NCBI accession number	**SRR10988327**
Total genome coverage	111×
RNA-Seq	
GS-FLX	
Total number of raw reads	1,853,195
Total number of raw bases (bp)	563,835,036
Total number of cleaned reads	886,137
Total number of cleaned bases (bp)	167,554,620
NCBI accession no.	**SRR10992869**

Table 1
Molecular studies for ecotoxicology in the *Oryzias javanicus*.

Stressors	Endpoints	Results summary	References
B[α]p	*CYP1A*	mRNA expression level of *CYP1A* was significantly increased with dose-dependent of B[α]P	Won et al. (2011)
	Microarray	*ChgH* expression was increased 4.37-fold in the male medaka indicating the occurrence of endocrine disrupting activity.	Won et al. (2014)
		Tumor suppressor gene *RARRE3* expression was decreased and *TNF-1* (which is related to the tumorigenesis) was increased	
		In detoxification, *CYP1A* was increased 5.33-fold, but expressions of *GST* and *G6PD* genes were decreased	
Heavy metal	Metallothionein	mRNA expression level of metallothionein was up-regulated in response to 6 different heavy metal exposure, especially at the liver	Woo et al. (2006); Lee et al. (2018)
		MT were highly detected by Protein-chip with high $CdCl_2$ concentration	
	CAT, *GST*, *SOD*, *CAT*, *GPx*, *CYP1A*	*GST*, *SOD*, and *CAT* genes expression levels were increased while *CYP1A* expression was decreased	Woo et al. (2009)
		Expression of *GPx* increased in response to Ag, Cd, Cu, and Zn exposure but decreased in Cr and Ni exposure.	
	Transcriptome	*Vtg1* expression were decreased while *Apo4*, related genes were expressed more with Cd	Kim et al. (2016a); Kim et al. (2016b)
		Transcriptome expression patterns were reversed with organic pollutants expression data	
17β-Estradiol	Oxidative stress and gene	In most tissues, mRNA expression of antioxidant genes were increased but patterns were slightly different	Woo et al. (2012)
		In the intestine, mRNA expression was decreased at all genes	
		GR and *UB* were the highest up-regulated genes in the liver while *GST* was highly expressed at the muscle	
	Transcriptome	Differentially expressed gene were identified and characterized with 24 h and 48 h exposure of E2	Woo et al. (2011)
		mRNA expression level of *apo* and *vtg1* genes were increased while *cyp1a*, *glud1*, and *transferrin* expressions were decreased	
4-Nonylphenol	Transcriptome	Energy metabolism, lipid metabolism, oxidative stress, detoxification, growth/differentiation, and the immune system were affected by 4-nonylphenol *Vtg1* and *ChgL* were increased both microarray and qRT-PCR experiments	Won et al. (2014); Yum et al. (2015)

Table 3
Information of anchoring *Oryzias javanicus* reference genome onto *Oryzias melastigma* genetic linkage map.

LG	Physical Length (bp)	No. of anchors	No. of scaffolds	No. of oriented scaffolds	Length of oriented scaffolds (bp)
Oj01	25,814,124	11	7	4	18,883,250
Oj02	16,610,604	8	7	1	4,213,677
Oj03	19,108,275	16	8	5	13,980,660
Oj04	25,880,235	16	10	7	22,438,292
Oj05	29,460,115	10	10	3	13,996,023
Oj06	28,870,867	20	11	10	28,130,519
Oj07	20,108,269	13	9	3	8,874,997
Oj08	15,744,308	10	3	3	15,744,308
Oj09	24,183,002	21	11	5	12,492,036
Oj10	22,904,608	16	13	5	13,985,792
Oj11	16,899,021	12	10	3	7,463,814
Oj12	21,192,871	16	7	3	15,734,022
Oj13	22,153,140	16	14	7	10,782,728
Oj14	27,044,193	19	15	7	18,337,951
Oj15	25,440,418	17	10	6	21,717,435
Oj16	26,621,043	17	11	6	19,066,879
Oj17	31,877,992	18	13	5	20,807,047
Oj18	13,079,881	9	8	3	6,705,506
Oj19	16,343,203	11	7	4	13,196,092
Oj20	19,923,579	14	6	2	13,661,645
Oj21	24,039,527	13	9	7	22,127,346
Oj22	19,309,036	9	5	5	19,309,036
Oj23	21,165,622	13	9	4	11,062,235
Oj24	25,566,155	10	7	3	18,516,070
Total	539,340,088	335	220	111	371,227,360

Table 4
Anchoring of the *Oryzias javanicus* assembly with *Oryzias melastigma* genetic linkage map.

Statistics	Value
Number of scaffolds	773
Length of scaffolds (bp)	846,333,395
N50 (bp)	19,309,436
Largest scaffold (bp)	31,879,192
Gap (%)	0.002
GC content (%)	38.89
No. of unanchored scaffolds	749
Length of unanchored scaffolds (bp)	306,973,707

2. Materials and methods

2.1. Fish culture

The fish used in this study was obtained from Korea Institute of Ocean Science and Technology (kindly provided by Dr. Seungshic Yum) and were reared in the aquarium facility in Sunkyunkwan University (Suwon, South Korea). The fish were maintained in artificial sea water (Tetra Marine Salt Pro, Tetra™, Cincinnati, OH, USA; 5.71 ± 0.19 mgO_2/L, 12 practical salt unit) at 26 °C with 12L:12D. All procedures of animal handling and experiments were approved by the Animal Welfare Ethical Committee and Animal Experimental Ethics Committee of Sungkyunkwan University (Suwon, South Korea).

Table 5
Completeness assessment of the anchored *Oryzias javanicus* assembly.

	Metazoa		Actinopterygii	
	%	No. of genes	%	No. of genes
Complete BUSCOs (C)	98.7	965	96.3	4415
Complete and single-copy BUSCOs (S)	94.9	928	93.7	4295
Complete and duplicated BUSCOs (D)	3.8	37	2.6	120
Fragmented BUSCOs (F)	0.4	4	2.1	98
Missing BUSCOs (M)	0.9	9	1.6	71
Total BUSCO groups searched		978		4584

Table 6
Gene annotation statistics for the assembled *Oryzias javanicus* genome.

Categories	Statistics
Number of genes	24,498
Total coding sequence length (bp)	31,926,183
Average gene length (bp)	7037
Largest gene length (bp)	112,429
Average CDS length (bp)	1303
Average intron length (bp)	945
GC content (%)	54.09

2.2. Genomic DNA extraction

To extract genomic DNA, muscle and liver of a single male Java medaka were homogenized with a sterile Teflon homogenizer in DNA extraction buffer (100 mM NaCl, 10 mM TrisCl, pH 8.0, 25 mM ethylenediaminetetraacetic acid [EDTA], 0.5% sodium dodecyl sulfate, 100 μg/mL proteinase K, and 1 μg/mL RNase). The homogenized sample was incubated in a heat block at 55 °C overnight and standard phenol/chloroform extraction was carried out. The genomic DNA was precipitated with 0.5 volumes of isopropanol and 0.2 volumes of 10 M ammonium acetate under centrifugation at 7500 × *g* for 10 min. The DNA pellet was washed with 70% ethanol and dissolved in TE buffer (10 mM TrisCl, pH 8.0, 1 mM EDTA) after air dry. The isolated DNA was quantified and qualified using a QIAexpert system (Qiagen, Hilden, Germany) and electrophoresis on a 0.8% agarose gel.

2.3. Construction of sequencing libraries and sequencing analysis

Whole genome sequencing of Java medaka was performed using PromethION from Oxford Nanopore technology (ONT, Oxford, UK). Nanopore library was constructed with the ligation sequencing 1D kit (SQK-LSK108). An Illumina Paired-End library (PE500) was constructed and sequencing was performed on HiSeq2500 platform (Illumina, San Diego, CA, USA) to correct errors in Nanopore sequences. Library construction and sequencing processes for both platforms were performed at the National Instrumentation Center for Environmental Management (NICEM; Seoul, South Korea) according to the manufacturer's instructions.

2.4. Genome assembly

De novo assembly was initially performed with Nanopore sequences using SMARTdenovo (https://github.com/ruanjue/smartdenovo). Nanopore sequences data was again mapped on the initial *de novo* assembly using Medaka (https://github.com/nanoporetech/medaka) to correct errors in nanopore sequences. Illumina reads were mapped to nanopore assembly to correct and polish the consensus sequences using Pilon (Walker et al., 2014) (https://github.com/broadinstitute/pilon/wiki). BUSCO version 3 (Simão et al., 2015) was used to investigate the completeness of the final assembly using metazoan and Actinopterygii database.

2.5. Anchoring genome assembly and genome annotation

Since the marine medaka (*O. melastigma*) and the Java medaka (*O. javanicus*) phylogenetically belong to the javanicus group, we used the linkage map of *O. melastigma* to anchor the reference genome of *O. javanicus* using Chromonomer v. 1.08 (http://catchenlab.life.illinois.edu/chromonomer/). The anchored genome assembly to the genetic map was re-assessed with BUSCO v.3.0 (Simão et al., 2015). The genome annotation of the final anchored assembly was performed by MAKER v.2.31.8 pipeline with manual curation (Holt and Yandell, 2011) using transcriptome data of *O. javanicus* (Table 2). The functional

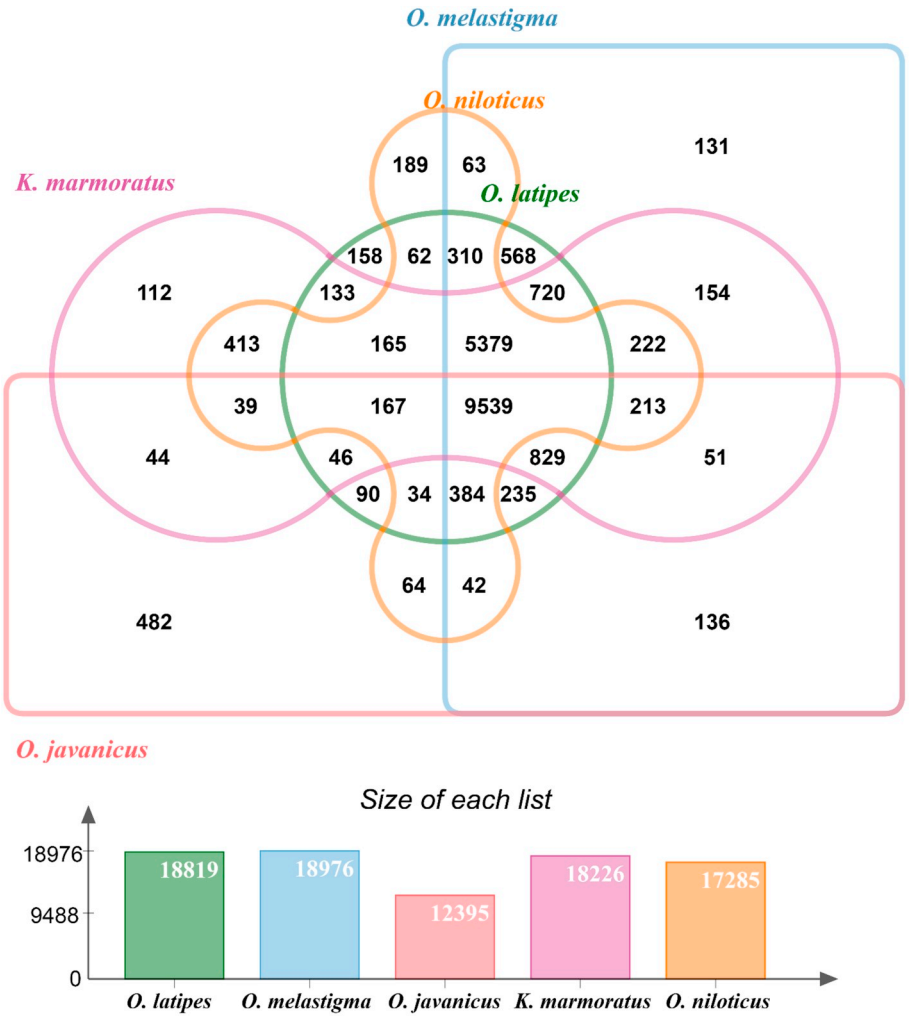


Fig. 1. Venn diagram of the orthologous cluster from five teleost fish including *Oreochromis niloticus*, *Kryptolebias marmoratus*, *Oryzias melastigma*, *Oryzias latipes*, and *Oryzias javanicus*.

annotation of the predicted genes was performed using Blast2GO_cli v1.1.5 (https://www.blast2go.com/) against the non-redundant (NR) database of *National Center for Biotechnology Information* (NCBI).

2.6. Orthologous comparison among teleost genomes

To compare the orthologous genes of *O. javanicus* with other teleost fish, OrthoVenn2 (Xu et al., 2019) was employed with the protein sequences of closely related teleost species, including *Kryptolebias marmoratus* (LWHD01000018.1), *Oreochromis niloticus* (GL831603.1), *O. latipes* (ASM223467v1), and *O. melastigma* (NVQA01000024.1).

2.7. Analysis of transposable elements in Oryzias genomes

The *de novo* repeat libraries from three *Oryzias* species were constructed using RepeatModeler version 1.0.10 (Smit and Hubley, 2014) and the unknown TEs were classified using TECLASS version 2.1.3 (Abrusan et al., 2009). To investigate the distribution of TE copies in *Oryzias* species, repeat regions of genomes were masked with the combined libraries of Repbase and the *de novo* Kimura distances were calculated by Repeat Landscape in RepeatMasker (Chalopin et al., 2015)

2.8. Identification and annotation of glutathione S-transferase, glutathione peroxidase, superoxide dismutase, and catalase in Oryzias javanicus

In this study, identification of antioxidant gene families including GSTs, GPx, SODs, and CAT in *O. javanicus* were performed by BLASTX searches to the NR database of NCBI and previously identified gene annotation from closely related sister species *O. melastigma* and *O. latipes* (ASM223467v1). To enhance the reliability and accuracy of the genes of interest, domain searches have been performed through InterproScan and PFAM searches to confirm their identity. Phylogenetic analysis was performed with RAxML V8.2.12 using maximum likelihood (LG + G + I: lnL = −24,747.17; bootstrapping 300) (Stamatakis, 2014). To obtain precise annotation of GSTs obtained from *in-silico* analysis, aquatic fish including Northern pike *Esox lucius* (Elu), zebrafish *Danio rerio* (Dre), killifish *Kryptolebias marmoratus* (Kma), tilapia *Oreochromis niloticus* (Oni), molly *Poecilia Formosa* (Pfo), puffer *Takifugu rubripes* (Tru), medaka *Oryzias melastigma* (Om), *Oryzias latipes* (Ol), eel *Monopterus albus* (Mal), killifish *Nothobranchius furzeri* (Nfu), platyfish *Xiphophorus couchianus* (Xco), carp *Cyprinus carpio* (Cca), rainbow trout *Oncorhynchus mykiss* (Omy), and salmon *Salmo salar* (Ssa) were used.

3. Results

3.1. Genome assembly in Oryzias javanicus

We obtained 74,091,915,108 bp sequences from PromethION with the mean read length of 8169.4 bp, after quality filtration (Q > 5) (Table 2). More than 120 million of Illumina reads were used to map to nanopore assembly for polishing (Table 2). Final genome assembly of *O. javanicus* consisted of 969 scaffolds with the N50 value of 2,264,148 bp,

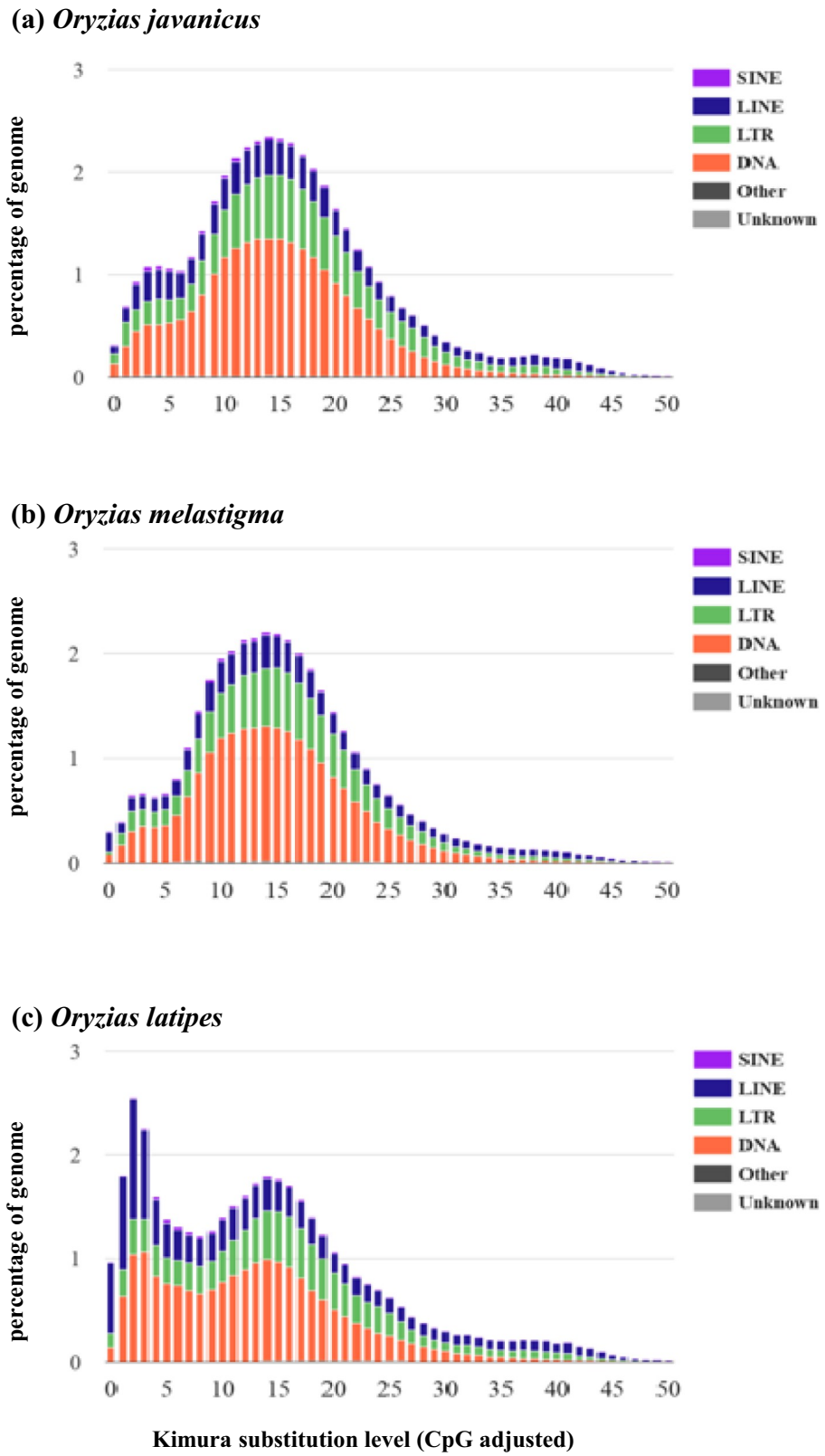


Fig. 2. Distribution of transposable elements based on Kimura distance-based copy divergence analysis in three *Oryzias* species.

which made the genome length to 846.3 Mb (Suppl. Table S1). Benchmarking universal single-copy orthologs (BUSCO) values assessed that the *O. javanicus* represented the 98.6% of metazoan genes (Suppl. Table S1). The data sets supporting the results in this article are available in the NCBI under the accession numbers **PRJNA603867**.

3.2. Anchoring of de novo assembly onto the Oryzias melastigma linkage map

Since the *O. melastigma* and *O. javanicus* belong to the javanicus group of *Oryzias*, we used the genetic linkage map of *O. melastigma* for

Table 7
Identification of glutathione *S*-transferases and antioxidant genes (superoxide dismutase, catalase, glutathione peroxidase) in *Oryzias javanicus*.

	Oryzias melastigma (Kim et al., 2018)	*Oryzias javanicus* (in this study)	*Oryzias latipes*
Genome size	**779.4 Mb**	**846 Mb**	**746.7 Mb**
GST			
Cytosolic			
Alpha	1	1	1
GDAP	2	2	2
Mu	2	2	2
Omega	2	2	2
Rho	5	5	6
Theta	2	2	2
Zeta	1	1	1
PTGES2	1	1	1
Mitochondrial			
Kappa	1	1	1
MAPEG			
Microsomal	4	4	4
Total	21	21	22
Antioxidant related			
MnSOD	1	1	1
Cu/ZnSOD	3	3	3
GPx	7	7	7
Catalase	1	1	1
Total	12	12	12

anchoring *O. javanicus* genome assembly (Suppl. Fig. S1). A total of 335 *O. melastigma* genetic markers were used for the anchors and 220 scaffolds were aligned to the linkage map (Suppl. File 1; Table 3). A total of 111 anchored scaffolds were oriented, making the length of oriented scaffolds 371.2 Mb (Table 3). After anchoring, *O. javanicus* assembly consisted of 773 scaffolds with N50 value of 19.3 Mb (Table 4), which represented 98.7% and 96.3% of genes in Metazoa and Actinopterygii, respectively (Table 5).

3.3. Annotation of Oryzias javanicus genome

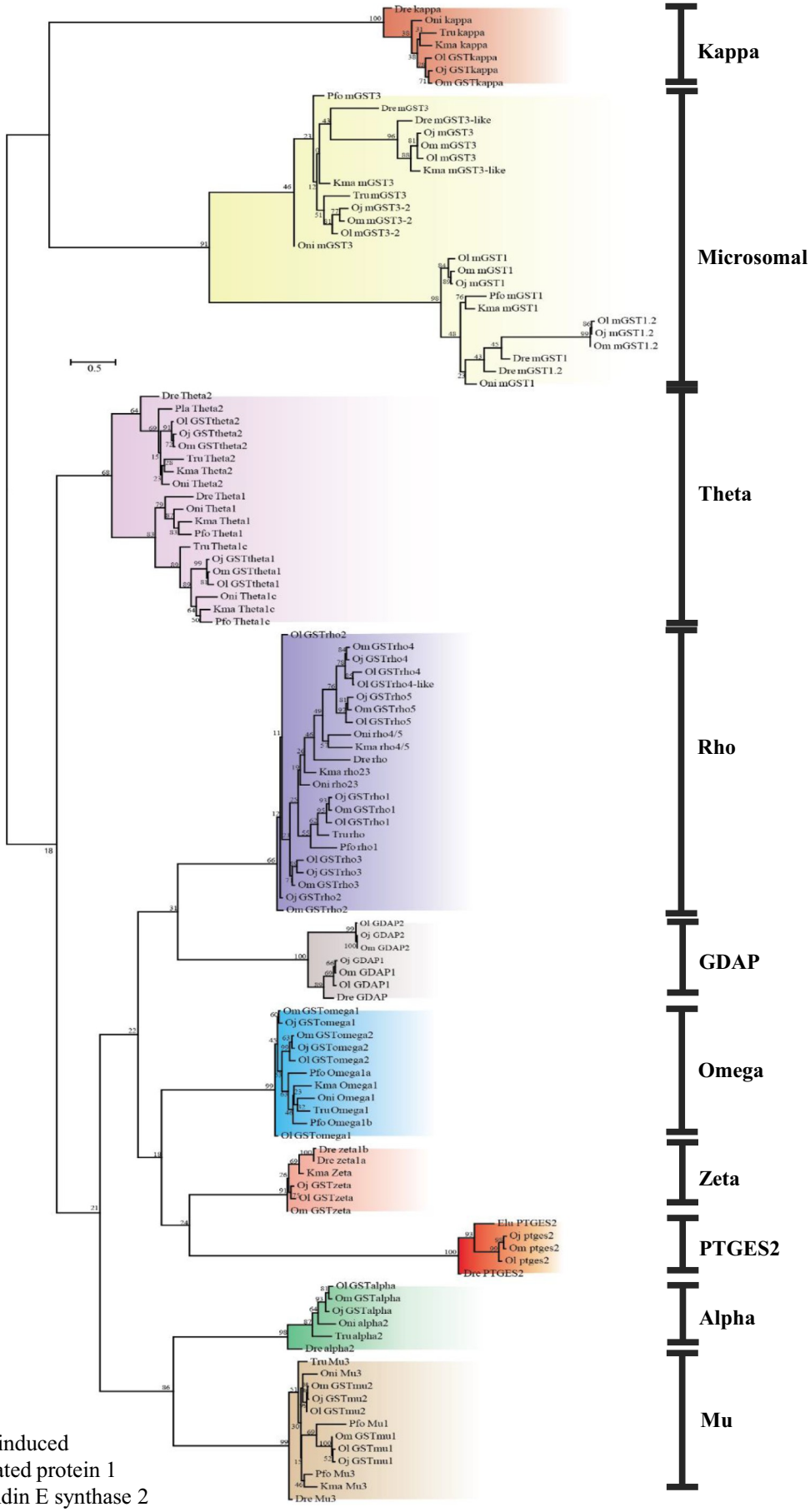
Genome annotation of *O. javanicus* found 24,498 genes based on *O. javanicus* RNA-seq evidence data with protein data of closely related species (Tables 2 and 6). Among the annotated gene sequence, 17,934 (73%) sequences were functionally annotated (Suppl. File 2) and can be accessed through the JBrowse (http://rotifer.skku.edu:8080/Oj).

3.4. Comparison of Oryzias spp. genomes

Genome annotation of *O. javanicus* found 24,498 genes based on *O. javanicus* RNA-seq evidence data with protein data of closely related species (Tables 2 and 6). OrthoVenn2 found 18,226 clusters in *K. marmoratus*, 17,285 in *O. niloticus*, 18,819 in *O. latipes*, 18,976 in *O. melastigma*, and 12,395 in *O. javanicus* (Fig. 1). Five teleost species shared 9539 clusters in common and GO enrichment analysis showed that RNA-mediated transposition (GO:003219) was significant (Suppl. Table S2a). OrthoVenn2 found that 235 clusters were specific to *Oryzias* fish (Fig. 1) and GO enrichment test of those gene clusters showed that hormone activity (GO:005179) was the most significant GO term ($p = 5.28 \times 10^{-5}$), followed by recycling endosome, (GO:0055037), DNA-mediated transposition (GO:0006313), immune response (GO:0006955), and sensory perception of chemical stimulus

Table 8
Genomic structure information of glutathione *S*-transferases and antioxidant families in *Oryzias* spp.

Genes	*Oryzias melastigma* (Kim et al., 2018)			*Oryzias javanicus* (in this study)			*Oryzias latipes*		
	ORF (bp)	Exon	Chr	ORF (bp)	Exon	Chr	ORF (bp)	Exon	Chr
GST alpha	675	6	15	675	6	15	672	6	15
GST omega1	720	6	15	720	6	15	720	6	15
GST omega2	720	6	15	720	6	15	720	6	15
GST rho1	675	5		684	6	11	684	6	11
GST rho2	678	6	11	681	6	11	684	6	11
GST rho3	681	6	11	681	6	11	684	6	11
GST rho4	672	4	11	672	6	11	672	6	11
GST rho4-like							672	6	11
GST rho5	672	6	11	672	6	11	672	6	11
GDAP1	1122	6	20	1086	6	20	1086	6	80
GDAP2	1101	6	7	1101	6	123	1101	6	7
GST mu1	660	8	7	660	8	68	660	8	7
GST mu2	660	10	7	660	8	68	660	8	7
GST theta1	723	5		723	5	134	723	5	9
GST theta2	687	5	9	687	5	130	684	5	9
GST zeta	651	9	22	663	9	22	663	9	22
PTGES2	1152	7	7	1122	7	134	1119	7	9
GST kappa	687	7	16	687	7	16	687	7	16
mGST1	456	3	22	456	3	22	456	3	22
mGST1.2	441	3	12	441	5	12	441	3	12
mGST3-2	459	5	17	459	5	249	459	5	17
mGST3	423	4	22	423	4	507	423	4	22
GPx1-1	567	2	5	567	2	5	567	2	5
GPx1-2	576	2	5	576	2	5			
GPx2	576	2	22	576	2	22	624	2	22
GPx3	666	5	10	666	5	10	672	5	10
Gpx6	546	5	19	543	5	19	546	5	19
GPx7-1	558	3	4	558	3	4	558	3	4
GPx7-2							558	3	4
GPx8	633	3	9	633	4	9	630	3	9
CAT	1584	13	6	1584	13	6	1584	13	6
Cu/ZnSOD1	465	5	14	465	8	14	465	5	14
Cu/ZnSOD2	633	2	1	618	5	1	636	1	1
MnSOD	678	5	24	678	6	24	681	5	24



GDAP: ganglioside-induced differentiated-associated protein 1
PTGES2: prostaglandin E synthase 2

(caption on next page)

Fig. 3. Phylogenetic tree analysis of aquatic fish glutathione *S*-transferases using maximum likelihood model (LG + G + I: lnL = −24,747.17; bootstrapping 300) using RAxML program. Each class belonging to a different subfamily (cytosolic, mitochondrial, and microsomal) is represented in a different color scheme as indicated in a color box. Species included in this analysis are as follows: Northern pike *Esox lucius* (Elu), zebrafish *Danio rerio* (Dre), killifish *Kryptolebias marmoratus* (Kma), tilapia *Oreochromis niloticus* (Oni), molly *Poecilia Formosa* (Pfo), puffer *Takifugu rubripes* (Tru), medaka *Oryzias melastigma* (Om), *Oryzias latipes* (Ol), eel *Monopterus albus* (Mal), killifish *Nothobranchius furzeri* (Nfu), platyfish *Xiphophorus couchianus* (Xco), carp *Cyprinus carpio* (Cca), rainbow trout *Oncorhynchus mykiss* (Omy), and salmon *Salmo salar* (Ssa).

(GO:0007606) (Suppl. Table S2b).

We investigated and compared the transposable elements (TEs) among three *Oryzias* genomes. TEs occurred in 46.4% of the genome in *O. javanicus*, 41.0% in *O. melastigma*, and 40.3% *O. latipes* (Suppl. Table S3). Of TEs, all three species predominantly contained DNA transposons (46.3% in *O. javanicus*, 48.4% in *O. melastigma*, and 43.9% in *O. latipes*), followed by LTR (23.6% in *O. javanicus*, 23.1% in *O. melastigma*, and 23.1% in *O. latipes*) and LINE (17.2% in *O. javanicus*, 15.8% in *O. melastigma*, and 26.4% in *O. latipes*). Kimura distance–based copy analysis showed that the two *Oryzias* species in the javanicus group had similar divergent patterns with one transposition burst (peak in the graph), while *O. latipes* showed more recent activity with two transposition bursts (Fig. 2).

3.5. Comparative analysis of genomic structures of glutathione S-transferases and antioxidant gene families in Oryzias javanicus, Oryzias melastigma, and Oryzias latipes (Oryzias spp.)

In-silico and phylogenetic analysis of GSTs and antioxidant gene families using the whole genome-library combined with RNA-seq data, have revealed a total of 21 GSTs, 7 GPxs, 4 SODs, and one CAT in *O. javanicus* (Tables 7 and 8). In comparison to other closely related sister species *O. melastigma* and *O. latipes*, the number of GSTs and genes belonging to antioxidant families (*i.e.*, CuZnSODs, MnSOD, GPx, and CAT) conformed one to one orthologous relationship (Tables 7 and 8). In detail, among the identified 7 GST classes, namely alpha, ganglioside-induced differentiation-associated protein 1 (GDAP), mu, omega, rho, theta, and zeta, the most dominant class with highest number of duplicates was found within the rho class, following the microsomal GST classes in *O. javanicus*, which were similar to those of closely related sister species *O. melastigma* and *O. latipes* (Figs. 3 and 4). Among the GST classes identified in this study, a unique feature demonstrated among the three *Oryzias* spp. is extra duplicated genes of GST rho4-like in *O. latipes*. In addition, genomic structural features such as the overall lengths of ORF region and exon in different classes of GSTs and antioxidant gene families showed similar homology among the three *Oryzias* spp. (Table 8).

Synteny analysis of tandemly duplicated GSTs, *GST omega*, *rho*, and *mu* showed high conservation of neighboring genes (Fig. 5). These three classes are considered tandemly duplicated GSTs, as they were duplicated in a short span within the same chromosomes (data not shown). The distance between the two *GST omega* genes (*GSTo1* and o2) in the three *Oryzias* spp. (*O. melastigma*, *O. javanicus*, and *O. latipes*) was quite different, ranging from 10.9, 11.4, and 14.7 kb, respectively (Fig. 5A). Similarly, *GST mu* genes in three *Oryzias* spp. also showed identical neighboring genes with small differences in the distances between the duplicated regions. However, synteny analysis of GST rho regions showed differences in the neighboring genes with large disparity in distances within the duplicated GST rho genes. In addition, *O. latipes* contained two extra rho genes (rho-like) in the region, resulting in a total spanning length over 144 kb.

Antioxidant gene families including *GPx*, *CAT*, and *SOD*s have been identified in *O. javanicus* and compared to two closely related *Oryzias* spp. A total of 7 *GPx*s have been identified, one *CAT*, and 4 *SOD*s (2 *Cu/ZnSOD*s, *MnSOD*, and chaperone-*Cu/ZnSOD*) in *O. javanicus* (Figs. 6 and 7). Comparative analysis with the other two *Oryzias* spp. showed one to one orthologous relationship within CAT and SODs, while an extra duplicated gene of *GPx1* have been identified in *O. melastigma* and *O. javanicus* and *GPx7* in *O. latipes*, contributing to the identical total number of duplicated genes in three *Oryzias* spp.

4. Discussion

4.1. Oryzias javanicus genome assembly and comparison among Oryzias genomes

We developed the reference genome of Java medaka (*O. javanicus*) using a long read-based nanopore sequencing technology and the linkage map of *O. melastigma*, a sister species in javanicus group of the genus *Oryzias*. A total genome size of *O. javanicus* was 846.3 Mb with 87× coverage of nanopore reads. Compared with *O. melastigma* reference genome, which was assembled with only 150× coverage of Illumina data (Kim et al., 2018), nanopore sequencing technology appeared to be very efficient for whole genome assembly, and anchoring scaffolds to the genetic map was useful to increase the contiguity of assembly to chromosome level.

Genome annotation of *O. javanicus* identified 24,498 genes, which was very close to the number of genes in the latest genome of *O. melastigma* (Lee et al., 2019). GO analysis of orthologous genes specific to three medaka fish represented that the DNA-mediated transposition (GO:0006313) was most enriched among the biological process terms (Suppl. Table S2b), which was supported by the TE contents the *Oryzias* genomes. TE contents in *Oryzias* species followed the typical TE divergence pattern in fish genomes (Suppl. Table S3), with the most abundant being DNA transposon and LINEs and the least abundant being SINEs (Shao et al., 2019). Although five teleost fish predominantly contained DNA transposons, *Oryzias* species had about two times higher DNA transposons (Suppl. Table S3) (Conte et al., 2017; Rhee et al., 2017). Among the *Oryzias* species, TEs in *O. latipes* showed a different divergent pattern from those in the two species of the javanicus group with higher percentage of LINEs, which suggested that LINE seemed to be involved in the recent activity in the *O. latipes* genome. In this study, overall TE levels of three *Oryzias* species showed a positive relationship with genome sizes (Suppl. Table S3). TEs was present in 46.4% of the *O. javanicus* (846 Mb), 41.0% of *O. melastigma* (778 Mb), and 40.3% of *O. latipes* genome (746 Mb) (Suppl. Table S3). In fish, researches have supported the positive relationship between TE levels and genome size since the TEs function as a driving force in genome size (Gao et al., 2016; Shao et al., 2019).

4.2. Identification of antioxidant gene families in Oryzias javanicus

Glutathione *S*-transferase superfamily is an important detoxification enzyme superfamily which mediates phase II detoxification system, carrying primary function of glutathione (GSH) conjugation of various endogenous and exogenous compounds (Bašica et al., 2019) and other biological functions, including biosynthesis of biomolecules such as leukotrienes, prostaglandins, and isomerization of steroids (Hayes and Pulford, 1995; Sheehan et al., 2001; Sau et al., 2010), emphasizing their importance in cell signaling pathways (Glisic et al., 2015). Moreover, as GSTs are widely recognized as important cellular detoxification enzymes, their activity and expression have been frequently used as biomarker of various xenobiotic exposures (Table 1).

In *O. javanicus*, the number of identified GSTs and antioxidant families were in agreement to those found in *O. latipes* (ASM223467v1). In contrast to cytosolic GST classes including alpha, mu, pi, omega,

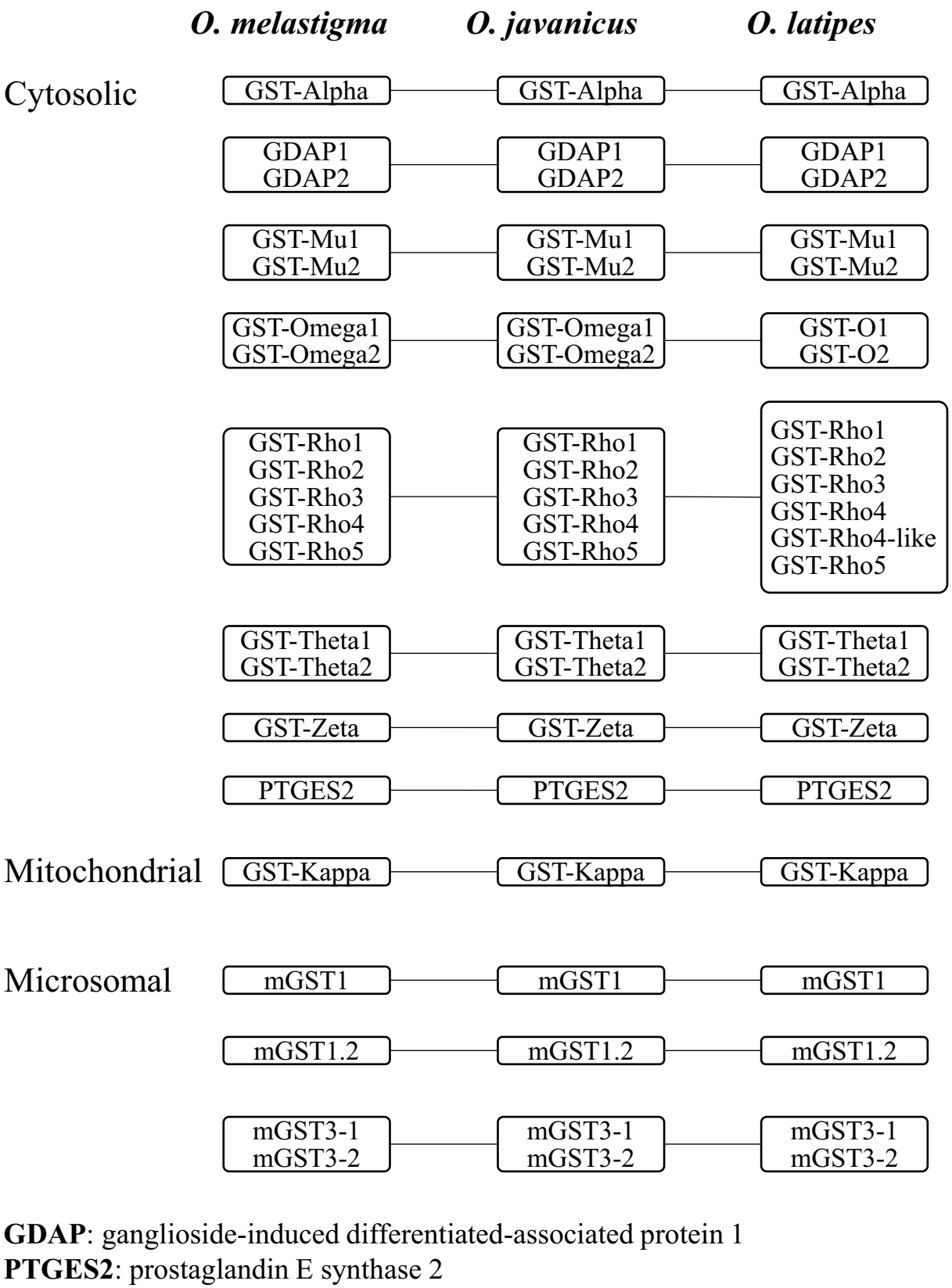
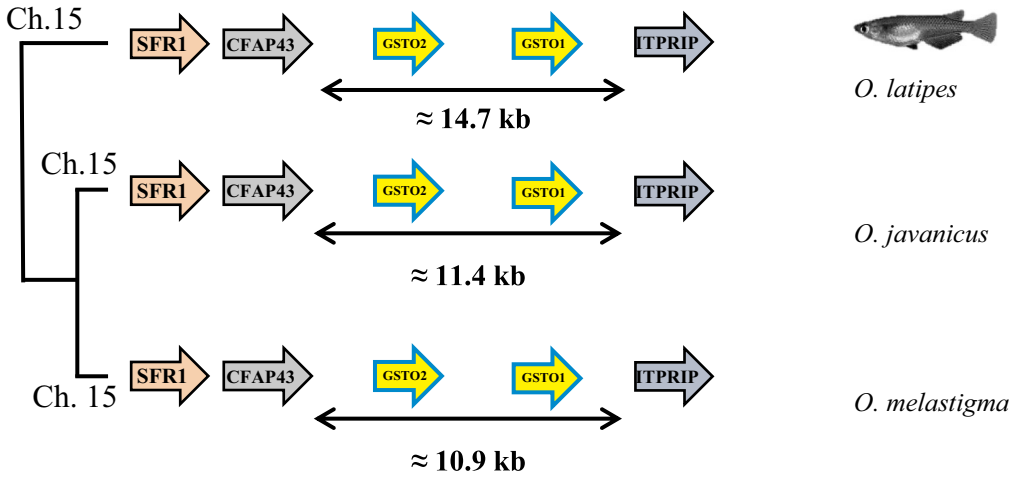


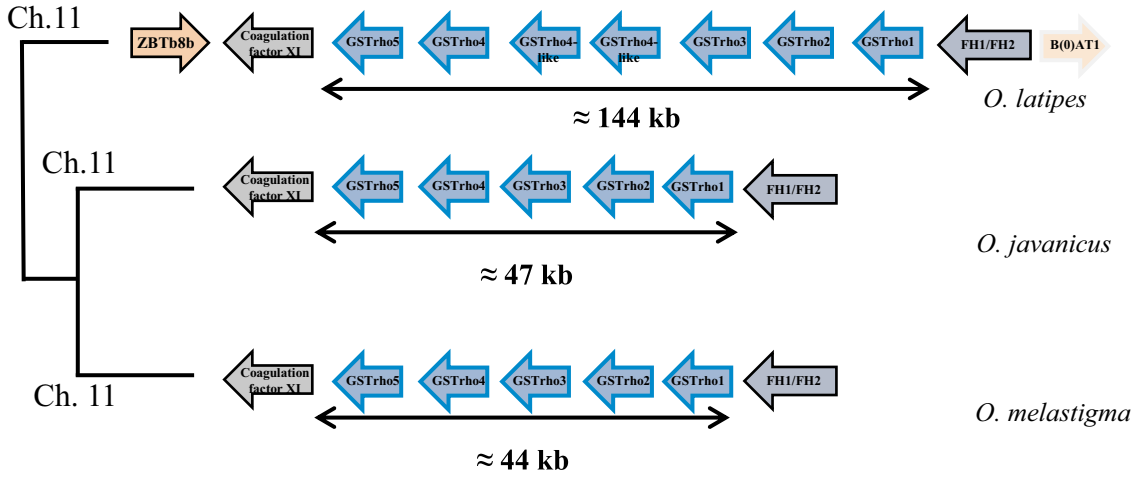
Fig. 4. Orthologous relationship between three *Oryzias* spp. (*Oryzias melastigma*, *Oryzias javanicus*, and *Oryzias latipes*) of the entire glutathione *S*-transferases identified from genome library.

theta, and zeta (Hayes and Pulford, 1995) which are highly found in both vertebrates and invertebrates, GST rho (Glisic et al., 2015) which also belongs to cytosolic family, has been identified, whereas pi class GST found in the zebrafish *D. rerio* (Glisic et al., 2015) was absent in *Oryzias* spp. Indeed, cytosolic GST rho class has been reported as the class which is only found in teleosts and cephalochordate or amphioxus

A) Synteny structure of *GST omega* genes



B) Synteny structure of *GST rho* genes



C) Synteny structure of *GST mu* genes

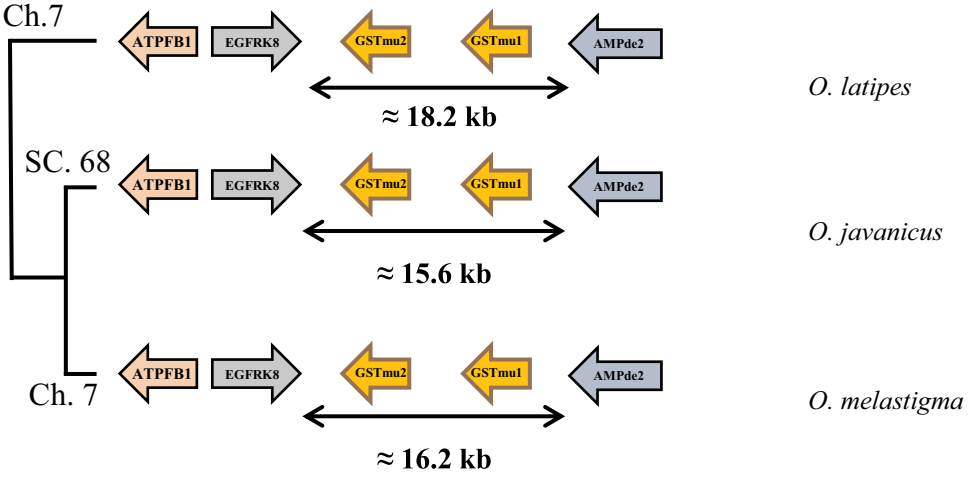


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Fig. 5. Synteny structural analysis of tandemly duplicated GSTs (A) *GST omega*, (B) *GST rho*, and (C) *GST mu* in *Oryzias javanicus* Sfr1: Swi5 dependent recombination DNA repair protein 1, CFAP43: cilia and flagella associated protein 43, ITPRIP: inositol 1,4,5-trisphosphate receptor interacting protein, B(0)AT1: sodium-dependent neutral amino acid transporter B(0)AT1, ZBTb8b: zinc finger and BTB domain containing 8B, ATPFB1: mitochondrial ATP synthase F0 complex subunit B1, EGFRK8: epidermal growth factor receptor kinase substrate 8-like protein 3, AMPde2: AMP deaminase 2 isoform X4.

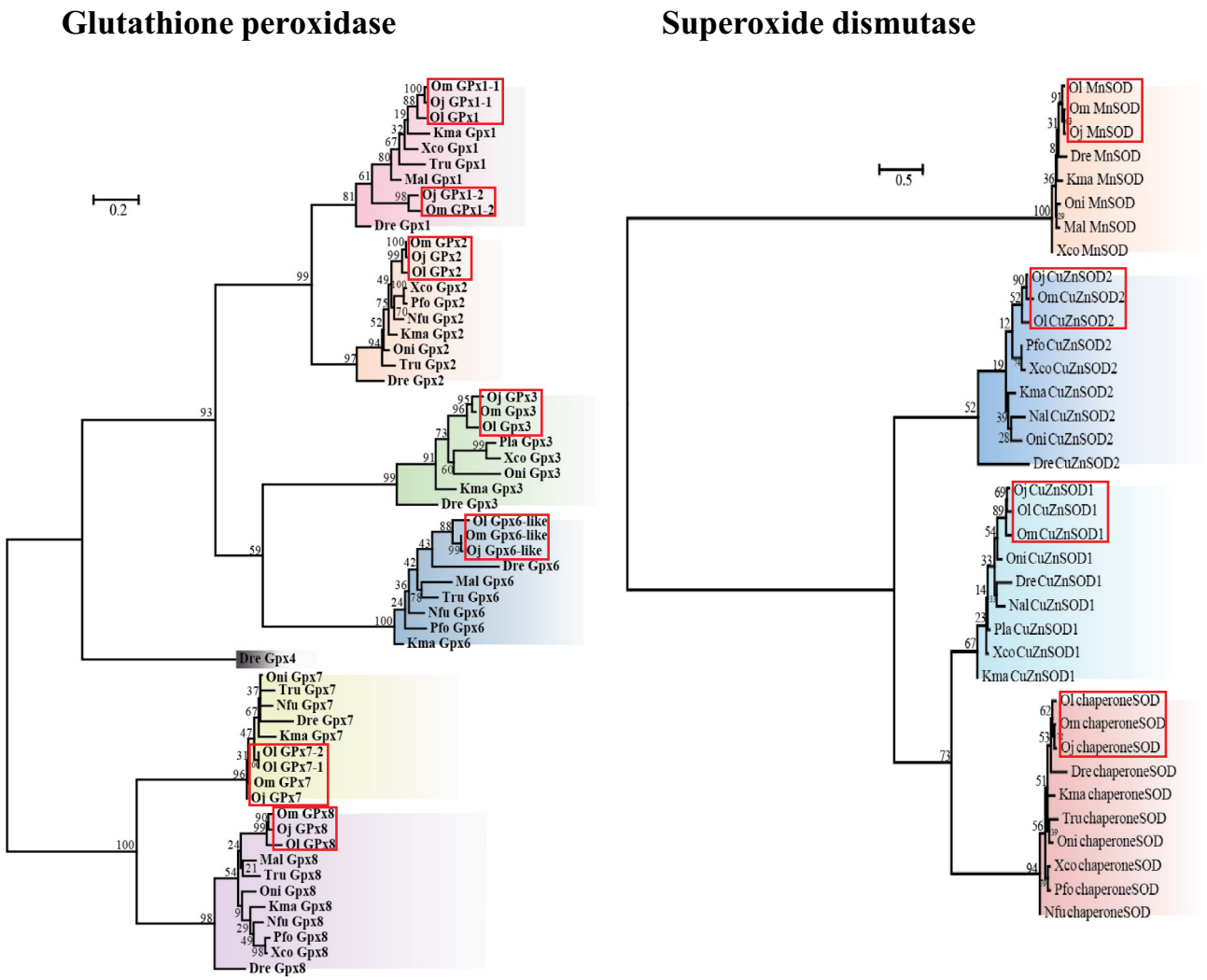

Fig. 6. Phylogenetic tree analysis of glutathione peroxidase and superoxide dismutase genes obtained from *in-silico* analysis of the genome library of the three *Oryzias* spp. (*Oryzias melastigma*, *Oryzias javanicus*, and *Oryzias latipes*) and compared to other fish species, using maximum likelihood (LG + G + I: lnL −6377; bootstrapping 100 and LG + G + I: lnL = −4087; bootstrapping 100, for glutathione peroxidase and superoxide dismutase, respectively). Species used in this study are as follows: Northern pike *Esox lucius* (Elu), zebrafish *Danio rerio* (Dre), killifish *Kryptolebias marmoratus* (Kma), tilapia *Oreochromis niloticus* (Oni), molly *Poecilia Formosa* (Pfo), puffer *Takifugu rubripes* (Tru), medaka *Oryzias melastigma* (Om), *Oryzias latipes* (Ol), eel *Monopterus albus* (Mal), killifish *Nothobranchius furzeri* (Nfu), platyfish *Xiphophorus couchianus* (Xco), carp *Cyprinus carpio* (Cca), rainbow trout *Oncorhynchus mykiss* (Omy), salmon *Salmo salar* (Ssa), and sailfin molly *Poecilia latipinna* (Pla).

(Glisic et al., 2015), playing a role in conjugation of 4-hydroxynonenal (Schlenk et al., 2008), which is a reactive aldehyde produced upon oxidative stress (Espinoza et al., 2013), suggesting a possible role of teleost specific GST rho class in defense against xenobiotic-induced oxidative stress. Moreover, the unique role of GST rho class in olfactory function has been reported as suggested by its activation during conjugation of various substrates (1-chloro-2,4-dinitrobenzene, 2-hydroxyethyl disulfide, and cumene hydroperoxide) and GSH-mediated demethylation of the organophosphate pesticide methyl parathion (Espinoza et al., 2013), a potent inhibitor of olfactory function in salmonids (Syvanen et al., 1996; Wang et al., 1991). One of the unique features demonstrated by *Oryzias* spp. was the absence of GST pi and sigma classes, which have been reported to be present in duplicate (GST pi) in zebrafish through teleost-specific genome duplication (TSD) (Meyer and Schartl, 1999; Glisic et al., 2015). In terms of evolutionary path of *GST* gene family, PTGES2, GST kappa, and microsomal GSTs are not considered true *GST* genes (Nebert and Vasiliou, 2004), however, due to their sequence similarity, phylogenetic analysis of GSTs in fish has shown that PTGES2 is closely associated with zeta class GST, possibly suggesting the difference in the function of PTGES2 in *Oryzias* spp. to that of other vertebrates.

Genomic structural analysis of the entire GSTs identified in *O. javanicus* in comparison to two closely related species *O. melastigma* and *O. latipes* has shown high conservation genomic structures in terms of the overall gene lengths and number of exons. In addition, synteny analysis of GSTs has revealed massive gene expansion through tandem duplication of certain GST classes, namely GST omega, rho, and mu, localized in chromosome 15, 11, and 7, respectively. In particular, the unique teleost-specific GST rho class resulted in 5 genes through duplication whereas omega and mu resulted in two genes, respectively.

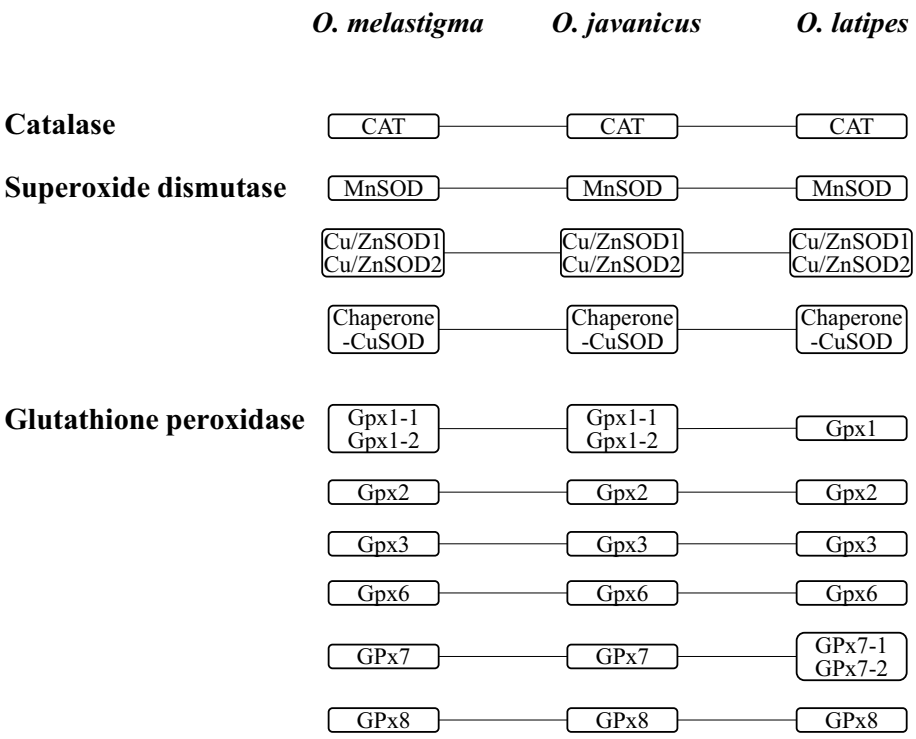

Fig. 7. Orthologous relationship between three *Oryzias* spp. (*Oryzias melastigma*, *Oryzias javanicus*, and *Oryzias latipes*) of the entire catalase, superoxide dismutase, and glutathione peroxidases identified from genome library.

Genomic structures of GST rho class in *O. javanicus* showed a total of 6 exons, almost identical to those found in *O. melastigma* and *O. latipes*, which are consistent to those found in amphioxus *Branchiostoma belcheri* (Fan et al., 2007). The significance of gene duplication is the generation of the genetic diversity, partly playing important roles in adaptability of a species to environmental changes (Ohno, 1970), suggesting that massive duplication of GSTs in *O. javanicus* may play important roles in unfavorable habitat condition, as the aquatic environment is constantly exposed to various xenobiotics; however, it is hard to assume that the degree of detoxification potential is positively correlated to the number of *GST* duplicates.

Consistent with the identified number of *GST*s, genome-wide based searches of antioxidant-related genes including *GPx*s, *CAT*, and *SOD* (*i.e.*, copper zinc *SOD* [*Cu/ZnSOD*] and manganese *SOD* [*MnSOD*]) in *O. javanicus* were identical to those identified in other *Oryzias* spp., conferring their close phylogeny relationship, showing one to one orthologous relationship. Interestingly, additional co-factor for the recruitment of copper for Cu/ZnSOD(Valentine and Gralla, 1997; Culotta et al., 1999), namely copper chaperone SOD (CCS), was also identified in all three *Oryzias* spp., possibly suggesting the importance of this specific gene through the evolutionary conservation of specific functions shared among the same genus and even further, across various animal taxa. Indeed, CCS is critical in cellular function which plays in sequestering and delivering of copper to target proteins, despite restricted intracellular free copper (Rae et al., 1999; Wong et al., 2000). However, further studies are required to elucidate a specific function of CCS in medaka and other marine vertebrates. In this study, the most significant finding in *O. javanicus* in comparison to closely related species *O. melastigma* and *O. latipes* is the conservation of chromosome segments between zebrafish and human, which has been verified by the genomic analysis of antioxidant-family genes shown in this study that parallels to those proposed by several researchers (Postlethwait et al., 1998, 2000; Barbazuk et al., 2000; Naruse et al., 2004a, 2004b).

In this study, we have fully established the whole genome library of Java medaka *Oryzias javanicus*. To demonstrate applications of the genome-library in ecotoxicological studies, gene families belonging to phase II of detoxification processes (glutathione S-transferases) and other important antioxidant gene families (glutathione peroxidase, catalase, and superoxide dismutase) were identified and high conservation of these gene families were observed among other *Oryzias* spp. In summary, the whole genomic dataset will be helpful for future studies of environmental ecotoxicology and for understanding of species-specific differences in sensitivity to various environmental stressors.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2020.111118.

CRediT authorship contribution statement

Bo-Young Lee:Investigation, Data curation, Visualization, Writing - original draft, Writing - review & editing, Funding acquisition.**Jun Chul Park**:Investigation, Data curation, Visualization, Writing - original draft, Writing - review & editing.**Min-Sub Kim**:Investigation, Data curation.**Beom-Soon Choi**:Investigation, Data curation.**Duck-Hyun Kim**:Investigation, Data curation.**Jong-Sung Lim**:Investigation, Data curation.**Seungshic Yum**:Visualization, Writing - original draft, Writing - review & editing.**Un-Ki Hwang**:Visualization, Writing - original draft, Writing - review & editing, Funding acquisition.**Jae-Seong Lee**:Conceptualization, Supervision, Visualization, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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