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Research Article
Transcriptomic Profiles of Male Thai Ricefish (*Oryzias minutillus*) after Encountering Two Related Species of Males (*Oryzias latipes* or *Oryzias woworae*)

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Abstract

Background and Objective: The Thai ricefish (*Oryzias minutillus*) is the smallest *Oryzias* spp. and is important in the trophic structure of freshwater ecological systems. However, interactions with related species via gene expression profiles are unknown in this species. Here, this study reports on the first attempt to investigate the transcriptome profiles of male Thai ricefish induced by the males of two *Oryzias*. Japanese ricefish (*O. latipes*) and Daisy's ricefish (*O. woworae*, a remarkably colourful *Oryzias*) were used in the experiments. **Materials and Methods:** *Oryzias minutillus* was put in the presence of *O. latipes* (as group 1) or *O. woworae* (as group 2) for 7 days in aquaria divided by a transparent partition wall. Thai ricefish faced the same species as control group. Fish in each group were measured the distance between fish individuals of *O. minutillus* to *O. latipes* or *O. woworae*. One-way ANOVA with *post hoc* Tukey's test was used to analyse the significant differences among groups. *Oryzias minutillus* from groups 1 and 2 on day 7 were subjected to RNA-sequencing analysis via next-generation sequencing. **Results:** Long-distance encounters of fish appeared in group 2 on day 7, but there were no significant differences between fish distances. Among the differentially expressed genes, the up-and downregulated genes were more highly expressed in group 2 than in group 1. According to gene ontology term enrichment analysis, genes downregulated in the "locomotion" pathway were detected in group 1 but not in group 2. Conversely, downregulation of "pigmentation" and "reproductive process" was detected only in group 2. **Conclusion:** These results suggested that the different patterns of gene expression in *O. minutillus* may be affected by the presence of *O. latipes* and *O. woworae*.

Key words: Thai ricefish, RNA, gene expression, transcriptomic profiles, Daisy's ricefish (*O. woworae*)

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The biological relationships between species within ecosystems involve symbiotic interactions such as commensalism, amensalism and competition[1-3]. These interactions can influence the food web, biodiversity, adaptations, genetics and changes in relationships between species[4-7]. Moreover, interactions between species or single species via visually mediated individuals also affect behaviour and physiology[8-10]. For instance, Detto *et al.*[11] described visually mediated recognition of fiddler crabs (*Uca* spp.). Schläge *et al.*[12] provided a great model to estimate the interaction of concurrent movements between individuals of bank voles (*Myodes glareolus*). Furthermore, Hotta *et al.*[9] successfully reported the primary face-viewing characteristics of cichlid fish (*Neolamprologus brichardi*) that these fish observed on the faces of other fish. Thus, the above studies of animal relationships emphasize the understanding of biological interactions among different or the same species.

Teleost fishes have emerged as nonmammalian vertebrate models in several fields of scientific research, including genetics, environmental biology and ethology[9,13,14]. In addition, Nunes *et al.*[15] successfully used zebrafish (*Danio rerio*) as a powerful model to show the understanding of visual cues with social attraction to basic perceptual mechanisms. Similar to zebrafish, fish of the genus *Oryzias*, especially Japanese medaka or Japanese ricefish (*O. latipes*), are widely utilized as model organisms in many fields, such as physiology, neurology, endocrinology and molecular genetics, including animal behaviour[16-18]. Isoe *et al.*[19] used *O. latipes* to demonstrate visually mediated social behaviour in response to visual cues. Yokoi *et al.*[20] contributed to the understanding of mate-guarding behaviour for familiar mates in *O. latipes*. Recently, Audira *et al.*[21] compared the interspecies behavioural variability and biomarker expression among *O. latipes*, *O. dancena*, *O. woworae* and *O. sinensis* and their results suggested a need for phenomic studies in ricefish.

Among the genera of ricefish, dwarf ricefish (*O. minutillus*) (commonly known as Thai medaka or Thai ricefish) are the smallest of this genus and widely inhabit natural freshwater environments throughout Thailand[22-24]. Unlike *O. latipes* and some *Oryzias* spp., *O. minutillus* is not frequently used in biological experiments and few studies on this species have been reported; examples of such studies include morphology, developmental biology, cytogenetics and molecular genetics[22-27]. Moreover, visual interactions among different species related to gene expression profiles may be unclear in this species. *Oryzias woworae* is a freshwater fish with a very colourful pattern native to Sulawesi, Indonesia[28,29]. Therefore, this study aimed to investigate the transcriptomic pattern of Thai ricefish induced by *O. latipes* or Daisy's ricefish (*O. woworae*) in separate aquaria.

MATERIALS AND METHODS

For adult fish, the shiro-medaka strain (white colouration of the body) of *O. latipes* and the wild-type *O. woworae* (2.5-3.5 cm in standard length) were purchased from the ornamental fish market in Bangkok, Thailand. The study was carried out from June, 2023 to October, 2023. Adult male Thai ricefish (1.6-2.0 cm in standard length) kept in a freshwater fish tank at the Faculty of Environmental Culture and Ecotourism, Srinakharinwirot University, were used in these experiments. To avoid the fish mating with each other, only adult males were used in the current study. Adult male individuals were clearly distinguished from female individuals by identifying secondary sex characteristics of the dorsal and anal fins[26,28]. The fish were acclimated to the aquaria containing freshwater for one week under the following conditions: 12 hrs light: 12 hrs dark, 27-29°C, pH 7.4-7.6 and 5-7 mg/L for dissolved oxygen. The fish were fed *ad libitum* with Kyorin Hikari food for medaka (Kyorin, Japan) two times per day and the freshwater was changed every day.

In the experimental groups, *O. minutillus* was placed in the presence of *O. latipes* in group 1 or placed in the presence of *O. woworae* in group 2 for 7 days in freshwater. The aquaria were divided into transparent partition walls between the ricefish species. The acrylic aquarium was 12 cm wide, 12 cm long and 15 cm tall and the aquarium acrylic thickness was 0.5 cm. Freshwater was added to the aquaria at a height of 12-13 cm. For each aquarium, one individual species of *O. minutillus* faced one individual fish of *O. latipes* or *O. woworae*. The species of *O. minutillus* faced with *O. minutillus* was used as the control group. The environments of the fish in the aquaria were the same as those described above. The fish were fed with Kyorin Hikari in the morning and evening at 8:00 am and 5:00 pm, respectively. Half the volume of aquarium water was gently replaced by fresh water on day 2 and 5. The experiments were conducted ten times.

Fish in the aquaria of each experiment were recorded by a VStarcam C22Q IP Camera (Shenzhen VStarcam Technology, Shenzhen City, Guangdong) and Canon 600D Digital SLR Camera (Canon, Japan) to measure the distance from *O. minutillus* to *O. latipes* or *O. woworae* in the morning and evening on day 1, 4 and 7. To compare the distances between individual fish, the distances from the edge of the lower jaw of *O. minutillus* to the edge of the lower jaw of

other Ricefish were measured (Fig. 1a). One-way ANOVA with *post hoc* Tukey's test ($p<0.05$) was used to determine the significant differences among groups.

Ten fish from each group were euthanized by treatment with tricaine methanesulfonate (ms-222) at 200 mg/L and transferred to RNAlater solutions for RNA stabilization and storage (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Ethical consideration: The animal research ethics of this study were approved by the Animal Care and Use Committee of Srinakharinwirot University (COA/AE-007-2565).

Total RNA was extracted from 10 male individuals for each experiment using the RNeasy Mini kit (Qiagen, Germany) with DNase treatment (Qiagen, Germany) according to the manufacturer's instructions. For RNA quality control, total RNA samples were subjected to gel electrophoresis on 0.75% agarose gels stained with SYBR Safe and viewed under a blue-light transilluminator. The RNA concentration, RNA integrity and fragment length were measured by using an Agilent 2100 Bioanalyzer (Agilent RNA 6000 Nano Kit).

For the transcriptome library, mRNA enrichment was performed from the total RNA using oligo(dT)25 mRNA isolation beads at 200 ng or ribosomal depletion, which resulted in rRNA fragmentation at 250 bp. The N6 random primers were used for reverse transcription of RNA fragments to double-stranded cDNA. The cDNAs were synthesized from dNTPs and *E. coli* polymerase I and treated with RNase H. The synthesized cDNA was processed for end repair and adenylation. Adaptors were ligated to the ends of these 3' adenylated cDNA fragments, after which the ligation products were purified. Enrichment of the purified cDNA by PCR amplification. The cDNA library quantification was performed by using a Qubit 4.0 fluorometer with a dsDNA HS assay kit (Thermo Fisher Scientific, Delaware, USA). An Agilent 2100 Bioanalyzer was used to confirm the sizes of the inserts. The sequences were analysed by using a BGISEQ-500 platform (BGI-Shenzhen, China)[30].

Before downstream analysis, the sequencing reads composed of low-quality reads, reads with adaptor contamination and reads with a high content of unknown bases were removed using SOAPnuke (v1.5.2, BGI, China). The *de novo* transcriptome was assembled using Trinity v2.0.6, which consists of Inchworm, Chrysalis and Butterfly[31]. The TgicI v2.0.6 was used to cluster the transcripts[32]. The assembly result was mapped to the unigenes with Bowtie2 software v2.2.5 and the gene expression level was calculated with RSEM v1.2.12. The FPKM (fragments per kilobase million) method was used to calculate expression levels (FPKM$\leq$1, FPKM 1~10, FPKM$\geq$10)[33].

The NCBI BLAST v2.2.23 and Diamond v0.8.31 software was used to annotate the NT and NR (nonredundant protein sequences) databases, respectively. Moreover, Diamond v0.8.31 was used for SwissProt (UniProtKB/Swiss-Prot) and KOG (euKaryotic Orthologous Groups). The KEGG Automated Annotation Server (KAAS) (r140224) was used for Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. The HMMER 3.0 package with hmmscan was used to search against Pfam and for protein prediction. Gene ontology (GO) annotations from the NR and Pfam results were carried out using Blast2GO v2.5.0. The KEGG annotations were performed by KAAS. The KEGG enrichment was performed using GOSeq 1.10.0 and topGO 2.10.0 for GO and KOBAS v2.0.12 for KEGG. The candidate coding regions within the transcript sequences were identified by TransDecoder v3.0.1. Heatmaps and volcano plots generated with R software 4.0.4 were generated to visualize the differentially expressed genes.

RESULTS AND DISCUSSION

A comparison of the distances between *O. minutillus* and related species revealed no significant differences between *O. minutillus* and *O. latipes* or *O. woworae* ($p>0.05$) on day 1, 4 and 7 in either group. However, group 2, in which *O. minutillus* was put in the presence *O. woworae*, seemed to be biased towards long distances in the early morning on day 7 (Fig. 1b).

In preliminary data, approximately 13.51 Gb were generated. The assembly of all samples and the abundance of filtering yielded 75,652 unigenes. In addition, the total length was 83,747,669 bp and the average length was 1,107 bp. The percentages of unigenes that were annotated by alignment with 7 functional databases were 47,364 for NR (62.61%), 55,654 for NT (73.57%), 40,200 for Swissprot (53.14%), 35,156 for KOG (46.47%), 40,489 for KEGG (53.52%), 11,323 for GO (14.97%) and 36,109 for InterPro (47.73%).

For the FPKMs of the control group, group 1 and 2, the gene expression levels at FPKMs 1-10 were greater than those at FPKMs$\leq$1 and $\geq$10. The gene expression level at FPKM$\leq$1 was similar to the gene expression level at FPKM$\geq$10 for gene numbers. There were different FPKMs for gene numbers between group 1 and 2. Moreover, a low gene expression level at FPKM $\leq$1 predominated in group 2, but a moderate level (at FPKM 1-10) predominated in the control group (Fig. 2a). For predictions of transcription factor (TF) expression levels, the pattern distribution of TF expression

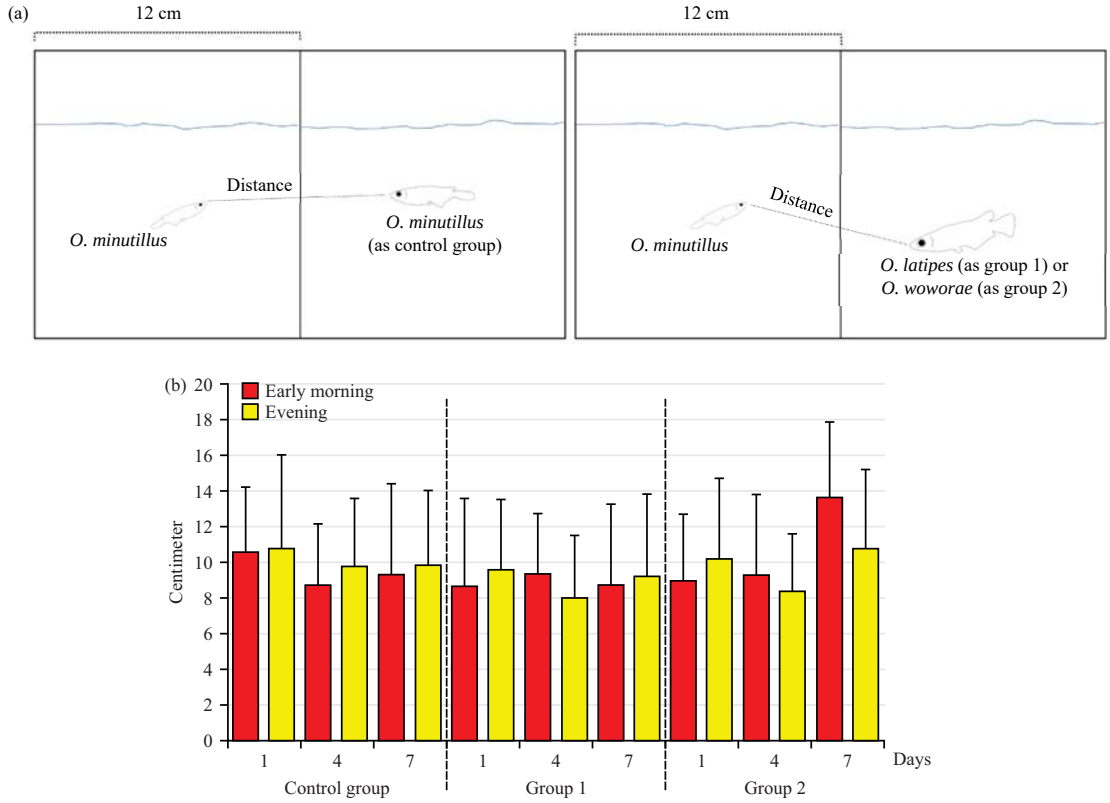

Fig. 1(a-b): Distances between individual fish were compared, (a) *O. minutillus* encountering *O. latipes* (group 1) or *O. woworae* (group 2). The *O. minutillus* faced to *O. minutillus* was obtained for control group and (b) Distance between the fish in control group, group 1 and those in group 2 at 1, 4 and 7 days

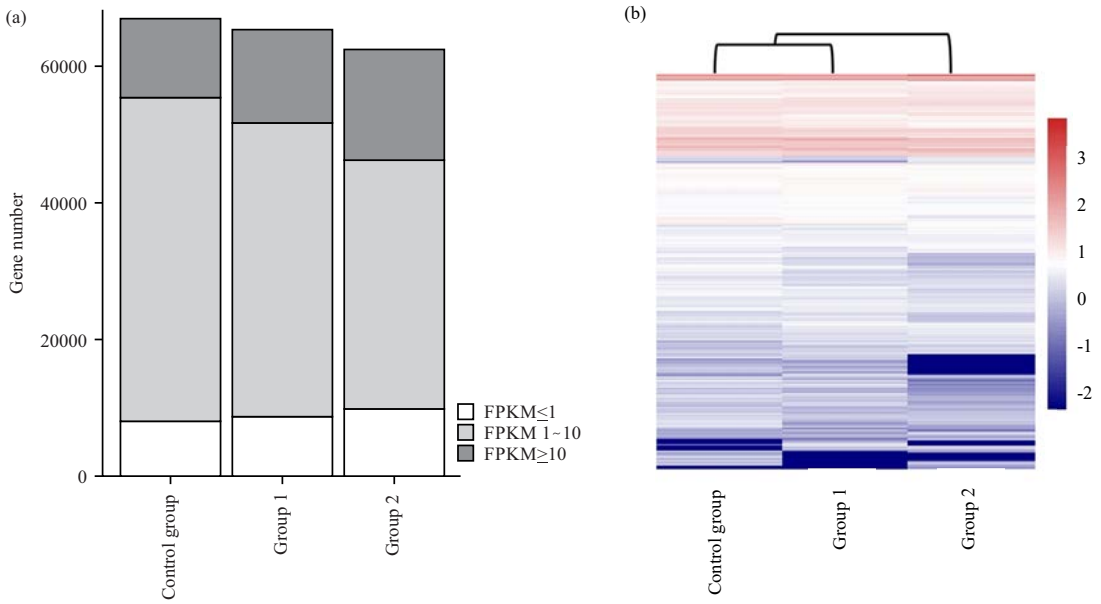

Fig. 2(a-b): Plot of the distribution of unigene expression levels in fragments per kilobase million, (a) FPKM≤1 (low expression level), FPKM 1~10 (medium expression level) and FPKM≥10 (high expression level) and (b) Distribution of a transcription factor expression level by heatmap

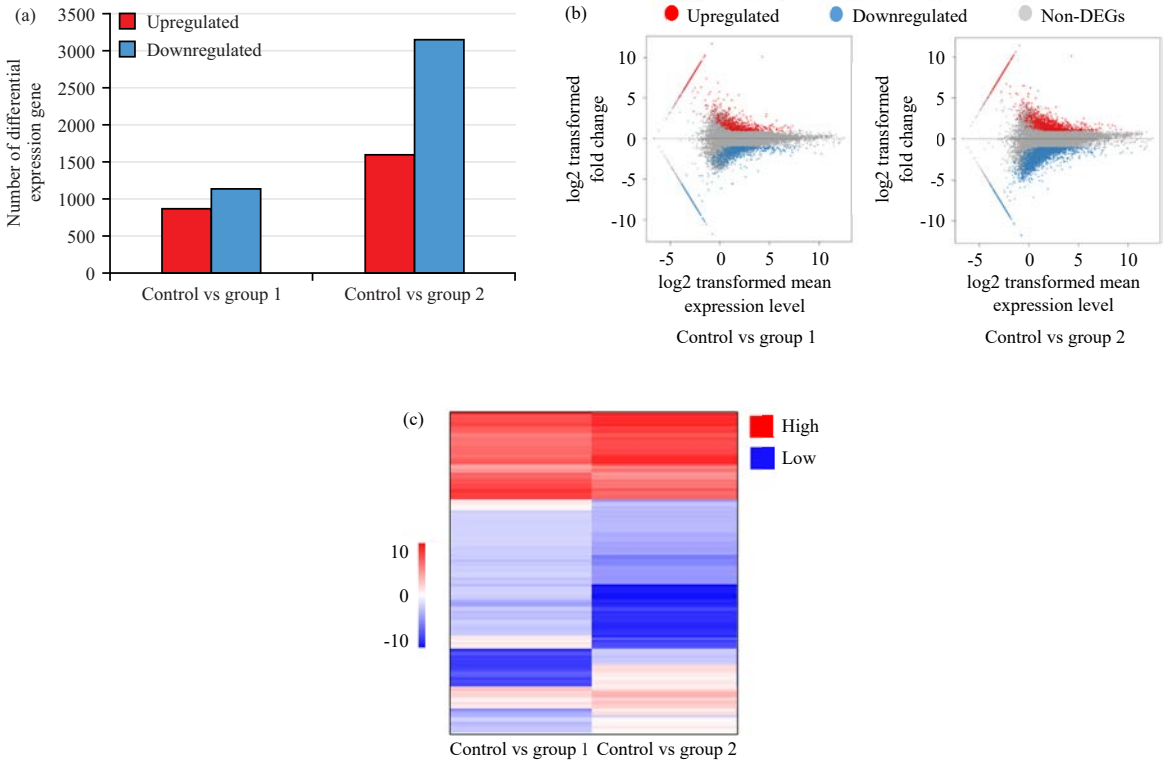

Fig. 3(a-c): DEGs (differentially expressed genes) for group 1 (*O. minutillus* to *O. latipes*) or group 2 (*O. minutillus* to *O. woworae*) compared with control group (*O. minutillus* to *O. minutillus*) (control vs group 1 or 2), (a) Number of DEGs, (b) MA plots and (c) Heatmap plots

levels in the control group was similar to TF levels in group 1. The zf-C_2H_2 and Homeobox were high levels of TF distributions. In contrast, lysosomal transcription factor, chorion-specific transcription factor, transcription factor nuclear factor YA and YB were low levels (Fig. 2b).

Regarding the differentially expressed genes (the control vs group 1 or 2), the number of upregulated gene was lower than the number of downregulated genes for group 1 and 2. In addition, the numbers of up- and downregulated genes were greater in group 2 than in group 1 (Fig. 3a). For the mean expression level of the genes whose expression increased, upregulated genes, downregulated genes and non-DEGs (differentially expressed genes) were more obvious and dominant in group 2 than in group 1 (Fig. 3b). Furthermore, the heatmap shows the DEGs of both groups. Group 1 showed a high level of clustering of DEGs that was similar to that of group 2. However, a lower level of clustering was predominant in group 2 than in group 1 (Fig. 3c).

In group 1 (the control vs group 1), the top five high levels of gene expression were (1) Class I histocompatibility antigen, F10 alpha chain-like, (2) Myosin regulatory light chain 2, skeletal muscle isoform, (3) Mitoferrin-2, (4) Hydroperoxide isomerase ALOXE3-like and (5) cdc42-interacting protein 4 homologue. The top five low levels were (1) E3 ubiquitin-protein ligase TRIM21-like, (2) Polypeptide *N*-acetylgalactosaminyltransferase 6-like, (3) Eukaryotic peptide chain release factor GTP-binding subunit ERF3A, (4) Pyruvate dehydrogenase (acetyl-transferring) kinase isozyme 2, mitochondrial-like and (5) CXADR-like membrane protein. In group 2 (the control vs group (2), the top five high levels were (1) Regulator complex protein LAMTOR3, (2) Exocyst complex component 1 isoform X5, (3) Splicing Factor 3B subunit 5, (4) Dual specificity protein phosphatase 5 and (5) Complement component C7 isoform X3. The top five low levels were composed of (1) E3 ubiquitin-protein ligase TRIM21-like, (2) Junctophilin-1, (3) Spartin-like isoform X1, (4) Matrix-remodelling-associated protein 7 isoform X3 and (5) Myomesin-1-like isoform X1.

For the GO (gene ontology) classification of the upregulated and downregulated DEGs, the downregulated "locomotion" gene was detected only in group 1 compared with the control group. For most of the GO terms, the number of downregulated genes was greater than that of

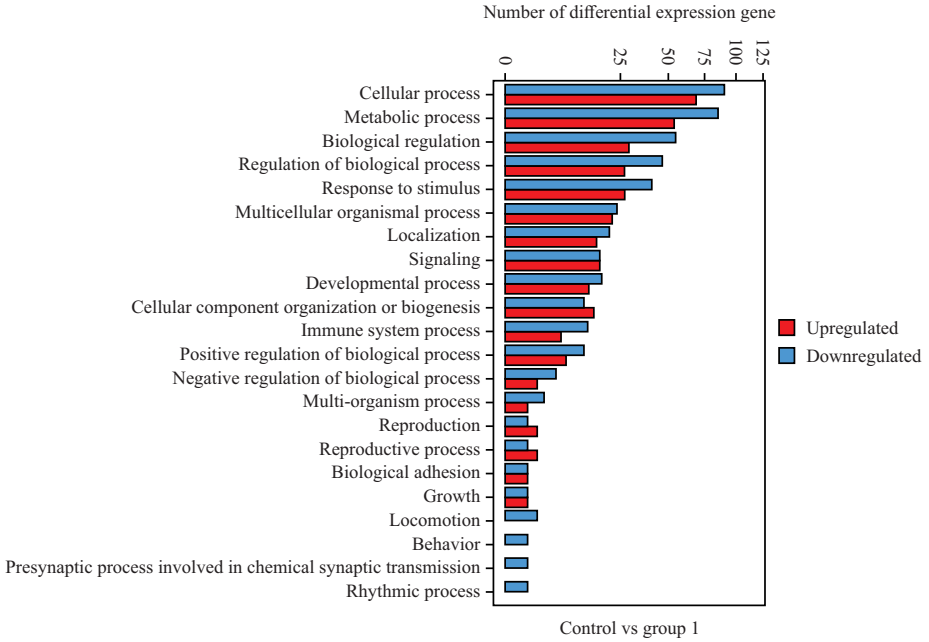

Fig. 4: Gene ontology (GO) classification of upregulated and downregulated genes between group 1 (*O. minutillus* to *O. latipes*) and control group (*O. minutillus* to *O. minutillus*)

upregulated genes, but this was not the case for the GO terms"signalling", "cellular component organization or biogenesis", "reproduction" and "reproductive process". However, there was little difference between "biological adhesion" and "growth". The DEGs among the upregulated genes were not related to "locomotion", "behaviour", "presynaptic process involved in chemical synaptic transmission" or "rhythmic process" (Fig. 4).

In contrast, "pigmentation" and "reproductive process" were downregulated only in group 2 compared with control. For all the GO terms, the number of downregulated genes was greater than that of upregulated genes. The DEGs among the upregulated genes were not related to "behaviour", "pigmentation", "reproduction", "reproductive process", "presynaptic process involved in chemical synaptic transmission" or "rhythmic process" (Fig. 5).

Many studies have reported inter- or intraspecific species relationships regarding behavioural patterns and biomarker expression[21,34-37]. Audira *et al.*[21] provided important data regarding the different behaviours of four species of ricefish in which each species exhibited behavioural differences. In this study of Thai ricefish, the distance between *O. minutillus* and *O. woworae* seemed to be greatest after 7 days. The results may help to support the design of time periods for further in-depth studies of interspecific distances. However, the behavioural performance associated with distance and body colour between different species is unclear since no significant differences were found. Therefore, these results suggest that the distance between *O. minutillus* and *O. latipes* or *O. woworae* may not be related to the distance between them.

In addition, transcription factor plays an important role in controlling transcription by binding to a specific DNA sequence[38]. This result in the present study suggests that the pattern of TF distribution levels of *O. minutillus* is closely related between Thai ricefish to Thai ricefish and Thai ricefish faced to Japanese ricefish. However, it is far to discuss that TF distribution levels may involve the body color of fish.

The number of differentially expressed genes was high for both *O. minutillus* and *O. woworae*. Furthermore, the number of genes with no differential gene expression was large when *O. minutillus* was confronted with *O. woworae*. Differential gene expression underlies gene expression patterns during cellular or tissue differentiation, suggesting differential transcription expression and progression of genes[39-41]. These results may reveal the transcriptional patterns, the core gene expression patterns and the differences in the expression of genes for male Thai ricefish encountering two males of related species of ricefish.

The genes with the greatest upregulation in *O. minutillus* to *O. latipes* were class I histocompatibility antigen, F10 alpha chain-like, myosin regulatory light chain 2, skeletal muscle isoform and mitoferrin-2. Vij *et al.*[42] predicted that the class I histocompatibility antigen F10 alpha chain-like in Asian sea bass (*Lates calcarifer*) is involved in the immune response.

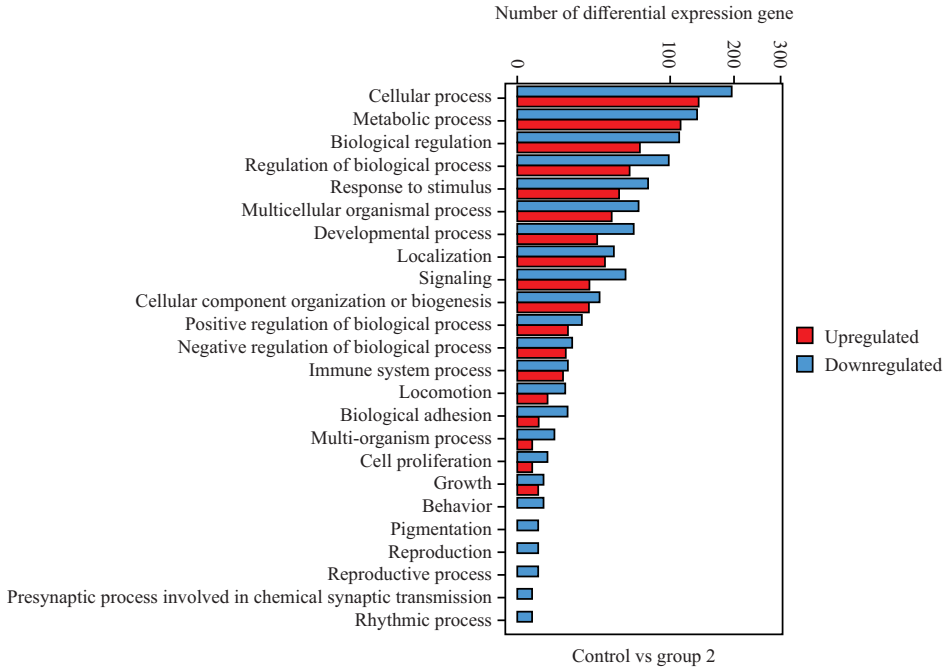

Fig. 5: Gene ontology (GO) classification of upregulated and downregulated genes between group 2 (*O. minutillus* to *O. woworae*) and control group (*O. minutillus* to *O. minutillus*)

Myosin regulatory light chain 2 functions as a regulatory light chain of myosin[43]. In addition, mitoferrin-2 is known as an iron importer for haem and Fe/S clusters in mitochondria[44]. Among the downregulated genes, the E3 ubiquitin-protein ligase TRIM21-like, polypeptide *N*-acetylgalactosaminyltransferase 6 like and eukaryotic peptide chain release factor GTP-binding subunit ERF3A were found. The E3 ubiquitin-protein ligase TRIM21 is a major autoantigen in the innate immune system[45]. The polypeptide *N*-acetylgalactosaminyltransferase 6 like responds to glycosylation in animal cells[46] and the eukaryotic peptide chain release factor GTP-binding subunit ERF3A may be involved in translational regulation of termination[47]. These results from the male Thai ricefish in group 1 predicted that the main genes with differential expression may be involved in immune processes or signalling regulation.

Regarding the genes whose expression was most upregulated in *O. minutillus* to *O. woworae*, the ragulator complex protein LAMTOR3, exocyst complex component 1 and splicing Factor 3B subunit 5 were detected. The ragulator complex protein LAMTOR3 plays a role in promoting cell growth[48] and the exocyst complex is an important component for secretory function[49]. Splicing Factor 3B subunit 5 is essential for mRNA splicing processes[50]. In contrast, the downregulated genes were the E3 ubiquitin-protein ligases TRIM21-like and Junctophilin-1 and the spartin-like isoform X1. Junctophilin-1 plays a role in intracellular calcium release channels[51]. Moreover, spartin has been proposed to regulate endosomal trafficking[52]. In this study, the E3 ubiquitin-protein ligase TRIM21 might be downregulated in different species. In addition, this result also suggested that there were genes whose expression differed between the Thai ricefish in group 1 and 2.

According to the GO classification, no DEGs related to pigmentation were detected when *O. minutillus* encountered *O. latipes*. Only downregulation was revealed when *O. minutillus* encountered *O. woworae*. The GO term "pigmentation" is a biological process that has been used to screen gene profiles such as cellular pigmentation, developmental pigmentation and pigment accumulation[53]. This study thus suggested that in pigmentation, differential gene expression may be related to fish body colour. Furthermore, upregulation, downregulation and no differential gene expression were detected between the fish in the experimental groups. The gene expression patterns in *O. minutillus* may visually influence those of the two related species.

CONCLUSION

To the present knowledge, it reports the first investigation in the transcriptome of male Thai ricefish compared to the males of Japanese ricefish or daisy's ricefish. Results showed

that the pattern of differential gene expression in Thai ricefish may be affected by the encounter of *O. latipes* and *O. woworae*. Furthermore, this study may contribute and increase the important data on transcriptomic profile for fish interactions of genus *Oryzias*.

SIGNIFICANCE STATEMENT

Distance encounters of *Oryzias* in this study were not significant differences between fish distances. The differentially expression genes, the up- and downregulation were higher in group 2 than in group 1. Downregulated genes in the "locomotion" pathway were detected in group 1 but not in group 2. In contrast, downregulated genes "pigmentation" and "reproductive process" were detected only in group 2. These results are hoped to supply the basic information on transcriptomic data for fish interactions for further work.

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