



Research article

Diversity of gut microbes in freshwater and brackish water ricefish (*Oryzias minutillus* and *O. javanicus*) from Southern Thailand

Arin Ngamniyom^{a,*}, Thayat Sriyapai^a, Pichapak Sriyapai^b, Busaba Panyarachun^c

^a Major in Environment, Faculty of Environmental Culture and Eco-tourism, Srinakharinwirot University, Bangkok 10110, Thailand

^b Department of Microbiology, Faculty of Sciences, Srinakharinwirot University, Bangkok 10110, Thailand

^c Department of Anatomy, Faculty of Medicine, Srinakharinwirot University, Bangkok 10110, Thailand

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Abstract

Fish belonging to the genus *Oryzias*, commonly known as medaka or ricefish, are frequently used as a vertebrate model, especially the Japanese ricefish *O. latipes*. In Thailand, Thai ricefish (*O. minutillus*) are widely distributed throughout natural freshwater, whereas Javanese ricefish (*O. javanicus*) mainly inhabit brackish water along the coast of southern Thailand. To date, nothing has been published on the endosymbionts of *Oryzias* inhabiting different natural environments in Thailand. The current study used 16S rDNA sequencing to investigate communities of bacteria and photosynthetic unicellular organisms in the gastrointestinal tracts of Thai ricefish from fresh water and in Javanese ricefish from brackish mangrove swamps. The family Aeromonadaceae was identified as the most dominant phylum of the Proteobacteria in Thai ricefish, but not in Javanese ricefish. The cyanobacterium *Synechococcus* was more prevalent in the gut of Thai ricefish than Javanese ricefish. Among the family Vibrionaceae, the genus *Vibrio* was found only in Javanese ricefish. In contrast, the genus *Escherichia* was the predominant class of Gammaproteobacteria in all gut samples of both ricefish species. This study showed that gut microbiomes of Thai and Javanese ricefish each consists of a core group of bacteria and photosynthetic unicellular organisms. Furthermore, microbiota may be dependent on the ricefish species and the different levels of salinity in their environments.

Introduction

The fish genus *Oryzias* comprises several bony fish, including the Japanese ricefish or medaka (*O. latipes*), which is well known as an experimental aquatic animal model for several fields of biology (Takehana et al., 2005; Hirako et al., 2017; Hilgers and Schwarzer, 2019; Lu et al., 2019). In the *Oryzias* genus, more than 30 species are recognised as being distributed in South, Southeast and East Asia (Mokodongan et al., 2014). In Thailand, five species of *Oryzias*

have been reported: *O. minutillus*, *O. mekongensis*, *O. dancena*, *O. javanicus* and *O. songkhramensis* (Parenti, 2008; Magtoon, 2010). They are important species in trophic levels of aquatic ecology in Thailand (Ngamniyom et al., 2009). In the tropical environment of Thailand, the smallest sized *Oryzias* species is *O. minutillus* or Thai ricefish (also known as Thai medaka or dwarf medaka); it is widespread in shallow fresh water (Takata et al., 1993). In saline environments, *O. javanicus* (commonly known as Javanese ricefish or Java medaka) inhabits brackish or marine habitats near mangrove forests (Roberts, 1998; Parenti, 2008). Javanese ricefish are utilized

* Corresponding author.

E-mail address: arin@g.swu.ac.th (A. Ngamniyom)

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as a model organism for endocrinology, developmental biology, and physiology experiments (Inoue and Takei, 2003; Imai et al., 2005; Yusof et al., 2012; Takehana et al., 2020).

Many studies have reported on gut microbial diversity in teleosts, which is important to understanding endosymbiotic and pathogenic interactions in aquatic fish (Wong and Rawls, 2012; Lescak and Milligan-Myhre, 2017; Li et al., 2018). For several decades, 16S rRNA gene sequencing has been used as a powerful method to examine microorganism communities and the influence of various environmental factors on the diversity of these communities (Kessel et al., 2011). Specific examples in bony fish include a study by Carda-Diéguez et al. (2014) in which the microbes in the intestinal tracts of cultured sea bass (*Dicentrarchus labrax*) were sequenced, and a study by Roeselers et al. (2011) in which the gut microbiota in zebrafish were sequenced. Finally, Chang et al. (2019) showed the impact data of cadmium exposure on the intestinal bacterial community of common carp.

The current study aimed to investigate the diversity of bacteria and photosynthetic unicellular organisms in the gastrointestinal tract of *O. minutillus* and *O. javanicus* collected from natural freshwater and brackish water environments, respectively, in southern Thailand. Molecular analysis was based on 16S rDNA sequences of the V1–V3 hypervariable region to identify bacteria including photosynthetic unicellular organisms using the BGISEQ-500 sequencing system.

Materials and Methods

Collection of ricefish

Freshwater natural ponds were sampled for Thai ricefish ($n = 4$) and brackish water near mangrove swamps was sampled for Javanese ricefish ($n = 4$) in Pattani province, southern Thailand (Fig. 1).

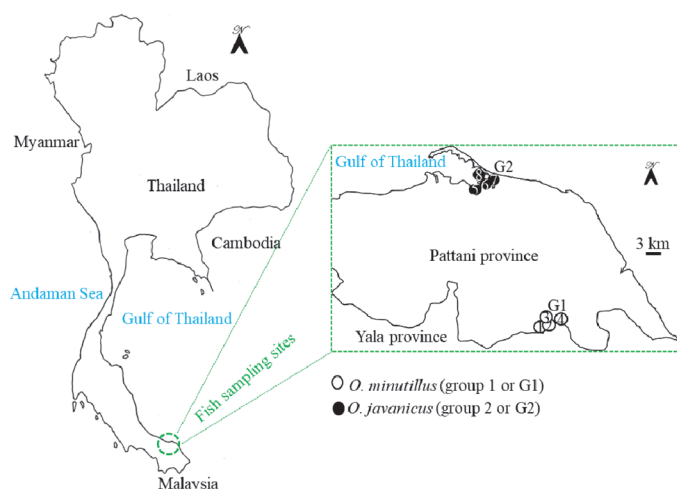


Fig. 1 Sampling sites of ricefish from natural aquatic environments in southern Thailand, where Thai ricefish (*Oryzias minutillus*) were collected from four freshwater sites (G1, open circles sites 1–4) and Javanese ricefish (*O. javanicus*) were collected from four brackish water coastal environmental sites (G2, filled circles, sites 5–8).

Group 1 (G1) consisted of Thai ricefish from sites 1–4, and group 2 (G2) consisted of Javanese ricefish from sites 5–8. The sampling sites were in one of several areas in southern Thailand where Javanese ricefish live abundantly (Termvidchakorn and Magtoon, 2008) and were not impacted by industrial or domestic activities (Kaewtubtim et al., 2017). Water conditions in each locality were measured using a Hach HQ40d portable multi-parameter meter (Hach; Loveland, CO, USA). The ricefish sampled were defined as healthy by observing their swimming locomotion and non-characterized diseases on the fish skin. Adult Thai and Javanese ricefish, 1.8–2.2 cm and 2.5–3.1 cm, respectively, were used in this study. The sex of each fish was distinguished as males or females based on the secondary sex characters of fins and genital papillae (Parenti, 2008). The ricefish were maintained in aquaria that had been aseptically cleaned with 250 mg/L of oxytetracycline (Sigma Aldrich; St Louis, MO, USA) and ultraviolet light and dried before filling with water from each fish collection site. No antibiotic, probiotic or prebiotic treatments were used.

Isolation of ricefish gut tissue

The ricefish were directly transferred to a mobile laboratory and euthanized using tricaine methanesulfonate solution (Sigma-Aldrich; St. Louis, MO, USA) prior to gut dissection. The fish skins were gently cleaned with 250 mg/L of oxytetracycline (Sigma Aldrich; St. Louis, MO, USA) (Campos et al., 2001), and then the whole guts (fore, mid, and hind guts) of the Thai or Javanese ricefish were immediately removed aseptically using a sterile scalpel blade. Four gut tissue samples (one each from two male and two female Thai ricefish and Javanese ricefish) were preserved separately in absolute ethanol at -20°C prior to nucleic acid isolation. It should be noted that there was no pooling of tissue samples or genomic DNA for molecular processes, following Anand et al. (2016).

The methods used for the sacrifice of fish were consistent with the Canadian Council on Animal Care Guidelines on the Care and Use of Fish in Research, Teaching and Testing, 2005 (<https://www.ccac.ca/Documents/Standards/Guidelines/Fish.pdf>) and the National and Institutional Guidelines for Animal Care and Use for Vertebrates from the Institute for Animals for Scientific Purpose Development (IAD) National Research Council of Thailand (NRCT). The Animal Care and Use Committee of Srinakharinwirot University granted ethics approval for all animal experiments in this study under approval license SWU-A-014-2563.

DNA extraction and 16S rDNA sequencing

Genomic DNA was extracted from whole gut tissue using a DNeasy Blood & Tissue Kit (Qiagen; Hilden, Germany) according to the manufacturer's instructions. For polymerase chain reaction (PCR) amplification, paired primers for the 16S rDNA target (V1–V3 hypervariable region; Wang and Qian, 2009) were used with *Pfu* DNA polymerase MasterMix (Bioneer; Daejeon, South Korea). The bacteria and photosynthetic unicellular organisms were identified using the V1–V3 region of the 16S rRNA gene according to McAllister et al. (2011), Hanshew et al. (2013), Beckers et al. (2016), Parulekar et al. (2017) and García-López et al. (2020). The PCR conditions were 94°C for 3 min to

pre-denature, followed by 34 cycles at 94°C for 1 min to denature, 54°C for 35 s to anneal, 70°C for 2 min for extension and a final extension for 5 min. The 16S rDNA PCR products were analyzed using electrophoresis through a 1% agarose gel, staining with SYBR Safe DNA Gel Stain (Invitrogen; Carlsbad, CA, USA) and by observation under a blue light emitting diode transilluminator. DNA products (~490 bp) were purified from the agarose using a Gel Extraction Kit (Qiagen; Hilden, Germany). The DNA concentrations were quantified using a NanoDrop 2000/2000c and confirmed using Qubit 2.0 fluorometric quantitation (Thermo Fisher Scientific; Waltham, MA, USA). The qualified DNA was used to construct libraries according to Mak et al. (2017). Sequencing was performed using the BGISEQ-500 sequencer (Zhang, 2004).

The metagenomic datasets were deposited in the published databases of The National Center for Biotechnology Information. The Data Availability Statement (<https://www.ncbi.nlm.nih.gov/sra/PRJNA679409>) of sequences was under accession number PRJNA679409 for Bioproject, SAMN16833206-SAMN16833213 and SRX9530368-SRR13083675 for the Sequence Read Archive (SRA).

Data analysis

Operational taxonomic unit (OTU) analyses were clustered by scripts of software USEARCH (v7.0.1090) (Edgar et al., 2011) and Naïve Bafesian Classifier v2.2 for taxonomic ranks (Wang et al., 2007). Taxonomy to OTU of photosynthetic protists and microalgae sequences was referred as the taxonomic class level (Dalevi et al., 2007; Sayers et al., 2011; Ye et al., 2014; Mancini et al., 2018). In databases of species annotation, taxonomy classifications of bacteria and photosynthetic unicellular organism were set against Greengene (V201305) (DeSantis et al., 2006) and RDP (Release9 201203) (Cole et al., 2014). A consensus base was generated by the Fast Length Adjustment of SHort reads, v1.2.11 (Magoc and Salzberg, 2011). The collection of the relative abundance of taxonomic rank, heat map representation and phylogenetic imaging was performed using the software R (v3.1.1) (Zhang, 2004). Phylogenetic analysis was aligned using PyNAST and molecular trees were constructed using QIIME (v1.80) (Caporaso et al., 2010; Kuczynski et al., 2011). The information statistic index (Shannon diversity) was calculated according to the criteria of Hill et al. (2003). Alpha and beta diversities measurement followed the report of Tuomisto (2010). The Bray-Curtis diversity distance used the method of Bray and Curtis (1957). Statistical analysis using the unpaired t-test and Mann-Whitney U test were used to justify the water quality and the OTU differences of bacteria and photosynthetic unicellular organisms, respectively, between freshwater and brackish water fish at $p < 0.05$ using the SPSS Statistics v20 software package (IBM; Somers, NY, USA). Results were presented as the mean $\pm$ standard deviation (SD).

Results and Discussion

Water quality

The average water quality conditions of the Thai ricefish collection

site were 7.13 ± 0.24 for pH, 0.12 ± 0.06 parts per thousand (ppt) for salinity, $27.29 \pm 1.0^\circ\text{C}$ and 6.18 ± 0.58 mg/L for dissolved oxygen. In Javanese ricefish, the water quality was 7.32 ± 0.52 for pH, 4.11 ± 0.69 ppt for salinity, $29.9 \pm 0.79^\circ\text{C}$ and 7.1 ± 1.47 mg/L for dissolved oxygen. Salinity of the water of Java ricefish collection site was significantly ($p < 0.001$) higher than those of Thai ricefish whereas non-significant differences were observed for other water quality parameters between collection sites of the two species.

Diversity of bacteria and photosynthetic unicellular organisms at phylum-genus levels

At the class level, class the *Gammaproteobacteria* were the predominant phylum of Proteobacteria and the class *Bacilli* were the predominant phylum of *Firmicutes* in the gut of Thai ricefish from all four sites and in the gut of Javanese ricefish from site 5 (Figs. 2A and B). The class *Gammaproteobacteria* (phylum Proteobacteria), class *Cyanophyceae* (phylum Cyanobacteria), and class *Mollicutes* (phylum Tenericutes) predominated in Javanese ricefish from site 6 among other sites. The class *Cyanophyceae* also dominated in the gut of Javanese ricefish from site 7, whereas Javanese ricefish from site 8 were dominated by chloroplasts (eukaryote). In addition, *Euglenozoa* (a photosynthetic protist belonging to the chloroplasts) was found in the gut of freshwater fish from all sites. Members of the phylum *Spirochaetes* were detected in fish from sites 4, 5 and 8, but at low numbers (Figs. 2A and 2B).

At the order and family levels, the order *Enterobacteriales* and family *Enterobacteriaceae*, respectively, predominated in the gut of fish from sites 1–5 (Fig. 2). Members of the family *Synechococcaceae* (order *Synechococcales*) and unclassified microbes predominated in the gut of fish from site 6. The order *Synechococcales* and *Stramenopiles* (photosynthetic unicellular microbes) dominated in fish from site 7 and site 8, respectively. Members of the order *Enterobacteriales*, order *Synechococcales*, and order *Actinomycetales* were also detected in the gut of fish from site 8. Unclassified bacteria and photosynthetic unicellular organisms formed the largest group at the family level in the Javanese ricefish from site 8 (Figs. 2C and 2D).

At the genus level, *Escherichia* was the predominant microbe in the gut of Thai ricefish from all sites as well as in Javanese ricefish from site 5. *Mycoplasma* and *Vibrio* were also relatively abundant communities in the gut of fish from site 5. The genus *Synechococcus* was the most abundant in the gut of fish from site 7. In contrast, unclassified bacteria dominated the gut of fish from site 8. Furthermore, the genus *Escherichia* predominated the gut microbiota of fish from sites 1–5. Other species found in the gut of fish from site 4 included the genera *Aeromonas*, *Cetobacterium*, *Plesiomonas* and *Staphylococcus*, whereas the gut of fish from site 5 harbored the genera *Vibrio*, *Mycoplasma* and *Photobacterium*. Members of the genus *Vibrio* were detected in the gut of Javanese ricefish from all sites and this was the most abundant genus. Among the genera of photosynthetic unicellular microbes, communities of the genus *Acutodesmus* were found in fish from sites 4, 6 and 7, but not in fish from sites 1, 2, 3, 5 and 8 (Fig. 2E).

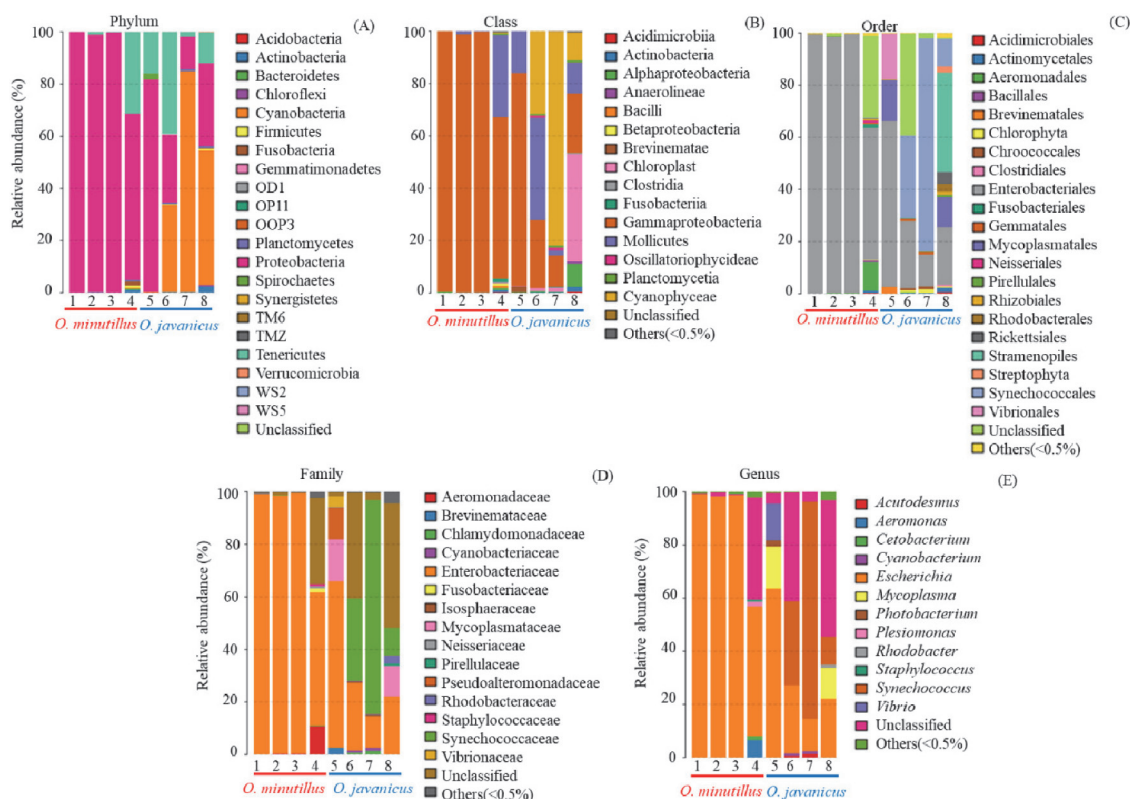


Fig. 2 Diversity of bacteria and photosynthetic unicellular organisms by percentage of relative abundance in guts of Thai ricefish (*Oryzias minutillus*) and Javanese ricefish (*O. javanicus*) from phylum to species level ($n = 4$ for both species)

Comparisons of relative abundance and similarity of bacteria and photosynthetic unicellular organisms among sites

The phylum-level heat map of relative abundance showed the similarity of all microorganisms in sites 1–3 and 5, and in sites 4 and 6–8 (Fig. 3). At the class and order levels, the relative abundance of gut microbes in fish from sites 1–3 and 5 was separated from that in fish from sites 4 and 6–8. At the family and genus level, the relative abundance of the microbial community in fish guts were clustered in fish from sites 1–3; sites 4, 6, and 7; and sites 5 and 8. In contrast, there was no clustering at the species level in the heat map of relative abundance and similarity (Figs. 3A–E).

The absolute abundances of two of the three dominant phyla (Proteobacteria and Tenericutes) in the Thai ricefish group were significantly higher than those in the Javanese ricefish group ($p < 0.05$; Fig. 4A). Conversely, the abundance of the phylum Tenericutes in the Javanese ricefish group was higher than in the Thai ricefish group but not significantly (Fig. 4A). There was no significant difference in the absolute abundance of the phylum Cyanobacteria between the Thai ricefish and Javanese ricefish groups (Fig. 4A). The Shannon diversity index was greater for the group of Javanese ricefish than the Thai ricefish group (Fig. 4B). The Bray-Curtis diversity distance of bacterial diversity and photosynthetic unicellular organisms in the ricefish gut was divided into two groups: group 1 consisted of bacteria and photosynthetic unicellular organisms from fish sites 1–5; and

group 2 consisted of bacteria and photosynthetic unicellular organisms from fish sites 5–8 (Fig. 4C).

According to Anand et al. (2016), the pooling of individual DNA samples prior to sequencing gives rise to poor accuracy of variant estimation, sequencing errors and false positive variants. Therefore, the current study did not pool DNA from individual fish gut samples prior to sequencing.

Based on the findings reported in the current study, *E. coli* colonizes the intestine in teleosts, which are also commonly found in warm-blooded animals (Conway and Cohen, 2015). These coliform bacteria have been isolated from the digestive tract of freshwater fish, including the one-sided livebearer (*Jenynsia multidentata*), tetra (*Bryconamericus iheringi*) and the common carp (*Cyprinus carpio*) (Apun et al., 1999; Guzmán et al., 2004). In marine or brackish water fish, *Escherichia* populations have also been identified in grouper (*Epinephelus coioides*), black sea bass (*Centropristis striata*), great barracuda (*Sphyraena barracuda*) and blackchin tilapia (*Sarotherodon melanotheron*) (Givens et al., 2015; Abidemi-Iromini and Fofah, 2016). Furthermore, *Escherichia* populations have presented in the teleost guts due to contamination in aquatic environments (Givens et al., 2015; Cardozo et al., 2018). The results of the current study suggested that Gram-negative *Escherichia* may be a general bacterium in both these species of ricefish studied in Thailand, and it may be the predominant bacteria in Thai ricefish inhabiting fresh water. In addition, these results suggested that the predominant occurrence of

E. coli in fish guts might reflect the involvement of the bacterium in the trophic levels of the food web, the ambient environment or biocontamination. Izvekova et al. (2007) reported that members of the phylum Proteobacteria are dominant in the gastrointestinal tracts of marine and freshwater fish from different ecological systems, with the phylum diversity higher in freshwater fish than in marine fish. This was similar to the current data regarding both ricefish species. Therefore, in the current study, there may be a broad range of *Escherichia* in the gut of *Oryzias* inhabiting fresh or brackish water.

The phylum Spirochaetes and phylum Tenericutes have been detected in the guts of common dolphinfish (*Coryphaena hippurus*) and great barracuda (*Sphyraena barracuda*), respectively (Givens

et al., 2015), supporting the current finding of a microsymbiont Spirochaetes and Tenericutes in Javanese ricefish and Thai ricefish from site 4. Within the phylum Proteobacteria, a comparison between the class Gammaproteobacteria and the class Alphaproteobacteria communities indicated that the former had high community abundance of *Fundulus heteroclitus* in brackish water or saltwater and of *Lagodon rhomboides* in saltwater (Givens et al., 2015). In the current study, the predominant populations were of the class Gammaproteobacteria in the guts of each *Oryzias* species, with a larger community range in the Thai ricefish than in Javanese ricefish except in the fish gut from site 5. In contrast, the class Alphaproteobacteria in the Javanese ricefish was only high from site 8. In the current results at the genus level,

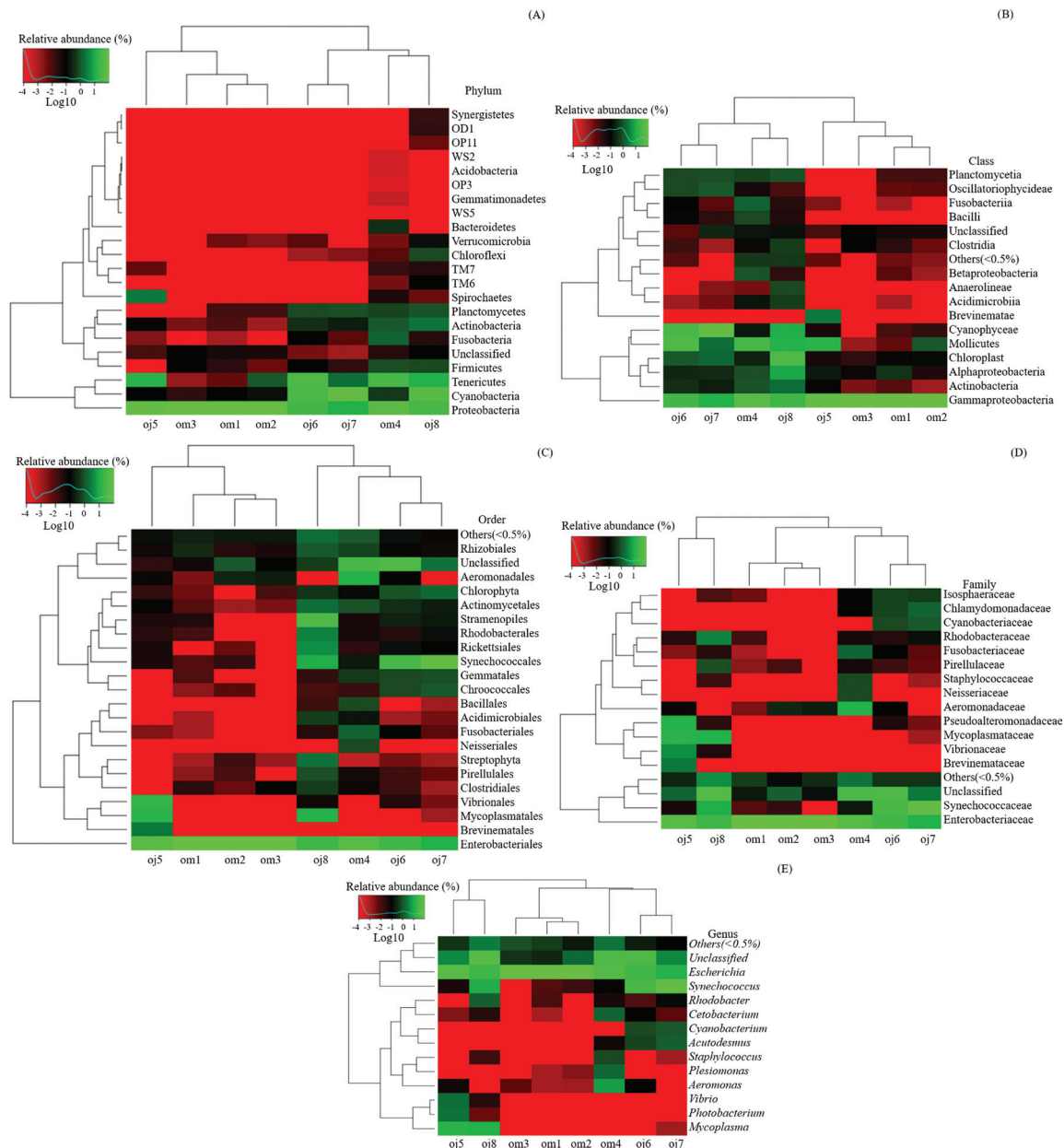


Fig. 3 Heat map representation of bacteria and photosynthetic unicellular organisms by percentage of relative abundance in guts of Thai ricefish (*Oryzias minutillus*) and Javanese ricefish (*O. javanicus*) from phylum to species level, where om = *Oryzias minutillus*, oj = *O. javanicus* and number following om or oj refers to fish sampling sites shown in Fig. 1

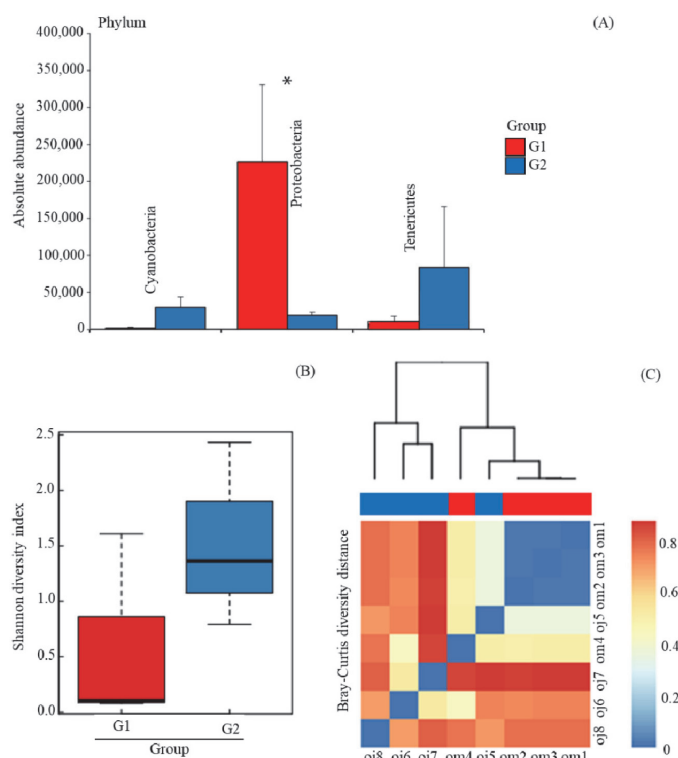


Fig. 4 (A) absolute abundance (mean $\pm$ SD) of Cyanobacteria, Proteobacteria, and Tenericutes in the gut of grouped Thai ricefish (*Oryzias minutillus*, G1, $n = 4$) and grouped Javanese ricefish (*O. javanicus*, G2, $n = 4$); (B) Shannon diversity index of bacteria and photosynthetic unicellular organisms of grouped Thai ricefish (G1, $n = 4$) and grouped Javanese ricefish (G2, $n = 4$); (C). Bray-Curtis diversity distance of gut microbial diversity in individual ricefish, where asterisk indicates a significant ($p < 0.05$) difference between G1 and G2 based on Mann-Whitney U test, om = *Oryzias minutillus*, oj = *O. javanicus* and number following om or oj refers to fish sampling sites shown in Fig. 1

Photobacterium and *Vibrio* were identified in the guts of Javanese ricefish, similar to reports for various marine fish (Labella et al., 2010; Parris et al., 2016; Halpern and Izhaki, 2017). In the family Mycoplasmataceae, *Mycoplasma* spp. were also isolated or detected in the intestines of marine fish (Llewellyn et al., 2016). In the Javanese ricefish, the intestinal bacteria were similar to genus *Mycoplasma*, which interestingly consists of mollicute bacteria that are a species-specific host to felid bloods (Kewish et al., 2004). It was suggested that the genus *Mycoplasma* in the whole gut tissues of the Javanese ricefish from sites 5 and 8 may be genetically related to the genera *Mycoplasma*, *Vibrio* and *Photobacterium* that were abundant only in the gut of the Javanese ricefish. The microbial communities harbored in the gut of the Javanese ricefish might have been related to the saline environment of the fish habitat. The diversity of gut microbes in the Thai ricefish appear to be different from that of the Javanese ricefish.

It is well known that microeukaryotes and the phylum “Cyanobacteria” are important primary food sources at the trophic level for fish (García et al., 2017; Fujibayashi et al., 2018). Friedland et al. (2005) reported that class Euglenophyceae (photosynthetic protists) was present in the esophagus of Atlantic menhaden (*Brevoortia*

tyrannus). Ye et al. (2014) reported that the phyla Streptophyta, Chlorophyta and Cyanobacteria were detected in the fore-gut and hind-gut of silver carp (*Hypophthalmichthys molitrix*) and gizzard shad (*Dorosoma cepedianum*). Therefore, the natural food sources of Thai or Javanese ricefish from Thailand may be the phylum Cyanobacteria based on the digestive tract sampling of the two ricefish.

Considering the salinity level, the genus *Vibrio* was detected in the gut tract of the Javanese ricefish group and not the Thai ricefish group. These results corresponded to the ecology of the genus *Vibrio* in saline water (Pérez et al., 2004; Eiler et al., 2007; Wang et al., 2020). This suggested that the genus *Vibrio* may contain specific core bacteria in the digestive tract of Javanese ricefish inhabiting saline aquatics. In the class Gammaproteobacteria, the genus *Escherichia* was predominant in the gut of the Thai ricefish living in freshwater, but low in the gut of the Javanese ricefish from brackish water. Enteric bacteria such as *E. coli* suffer from environmental stress upon exposure to seawater (Rozen and Belkin, 2001) and Korajkic et al. (2013) reported that the survival rates of *E. coli* declined under the influence of a saline environment. These studies supported the current of why the community of *Escherichia* sp. was low in the gastrointestinal tract of brackish ricefish. Therefore, the different abundance levels of this coliform bacterium between the two ricefish species might depend on the salt level in their aquatic environments. The unicellular cyanobacterium genus *Synechococcus* was found in the fish gut of brackish ricefish. Members of this genus *Synechococcus* are important primary producers that are widespread throughout the marine environment, including brackish environments (Kim et al., 2018), although they are also found in freshwater (Ruber et al., 2016). This cyanobacterium may be a core bacterium for the trophic consumption of Java ricefish living in a brackish ecology. The bacterial diversity results mentioned above may indicate that bacterial communities in Thai or Java ricefish depend on the different salinity levels in their aquatic ecologies.

This study was the first to report the gut microbiota of two species of *Oryzias* from fresh and brackish waters of Thailand. The collected data revealed the same abundance of representative populations of bacteria and photosynthetic unicellular organisms in the guts of the two ricefish species studied and described the communities of bacteria and photosynthetic unicellular organisms that were specific to each of the two ricefish species. The diversity profiles of the bacteria and photosynthetic unicellular organisms identified in the guts of the two ricefish could be species related to or associated with the different environmental habitats or both. Furthermore, understanding the gut microbiota of ricefish may be helpful in understanding the zoonotic transmission of bacterial diseases in the trophic levels of an ecosystem.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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