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Short Communication

Genome survey report of Thai ricefish (*Oryzias minutillus*) (Actinopterygii: Beloniformes)

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Abstract

Thai ricefish (*Oryzias minutillus*) is the smallest species in the *Oryzias* genus, widely distributed throughout Thailand. They are important in the trophic level of food webs within freshwater ecosystems. However, knowledge about the molecular data of this fish is lacking. Therefore, in this study, we surveyed the genome data of Thai ricefish collected from Mae Hong Son Province, Northwestern Thailand. In the initial assembly, the total genome and mitochondrial genome of Thai ricefish were 824 Mb and 16,954 bp, respectively. The total sizes of contig and scaffold were 547.7 and 585.9 Mb, respectively. The total genome size per mitochondrial size was 0.049. Phylogenetic relationship of Thai ricefish and related species of *Oryzias* was constructed based on mitochondrial genome. The nucleotide similarity of all genes in the mitogenome of Thai ricefish was compared with the related species of *Oryzias* from nucleotide database of the National Center for Biotechnology Information. The results provide data that increase knowledge of molecular genetics and a basis for further work on fish in genus *Oryzias*.

Keywords: Thai medaka, northwestern Thailand, genome size, mitogenome

1. Introduction

More than 30 fish in genus *Oryzias* have been identified, mainly inhabiting East and Southeast Asia (Hilgers & Schwarzer, 2019). Particularly, *Oryzias latipes* (Japanese medaka), *O. javanicus* (Java medaka), and *O. dancena* (Indian medaka) provide aquatic animal models for many biological studies, such as those on molecular genetics, endocrinology, toxicology, and biochemistry (Inoue & Takei, 2003; Kirchmaier, Naruse, Wittbrodt & Loosli, 2015; Koyama *et al*., 2008). The final assembly of *O. latipes* and *O. javanicus* genomes were reported, providing us an understanding of the evolutionary biology of teleost fish (Kirchmaier, Naruse, Wittbrodt, Loosli, 2015; Takehana *et al*., 2020).

In Thailand, 5 species of *Oryzias* have been reported: *O. minutillus*, *O. javanicus*, *O. dancena*, *O. mekongensis*, and *O. songkhramensis* (Magtoon, 2010). *O. minutillus* are the smallest species in genus *Oryzias* and commonly called Thai ricefish or dwarf medaka. Thai ricefish are an important organism in the trophic levels of the food webs within freshwater ecosystems; they are mainly found in shallow ponds of natural freshwater in many regions in Thailand (Magtoon, Nadee, Higsdhitani, Takata, & Uwa, 1992). However, molecular data for this species are lacking. Therefore, we report the genome survey data of Thai ricefish collected from Mae Hong Son province, Northwestern Thailand.

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2. Materials and Methods

Thai ricefish were collected from the shallow ponds in Mae Hong Son province, Northwestern Thailand. Mae Hong Son has a complex pattern of high mountain ranges (Chumpu, Khamsemanan, & Nattee, 2019) isolated from other areas. As such, fish cannot easily migrate from this origin. Males were separated from females using the secondary sex characteristic of the anal fin (Ngamniyom, Magtoon, Nagahama, & Sasayama, 2009) and maintained in a tank containing freshwater with aquarium air pumps. Fish were acclimatized for 1 month in the following aquatic conditions: pH, 7.0–7.4; salinity, 0.05–0.08 ppt; temperature, 27–29 °C; and dissolved oxygen, 6.0–6.5 mg/L. For related species, *O.*

uwai (origin from Myanmar) were purchased from a fish pet shop and maintained in an aquarium under the same conditions as Thai ricefish. Mitogenome sequencing of *O. uwai* was conducted to add nucleotides to the molecular tree.

Total genomic DNA was extracted from 35 male Thai ricefish individuals (XY chromosome for male (Nagai, Takehana, Hamaguchi, & Sakaizumi, 2008)) using a QIAamp Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions, and stored at –20 °C. For quality and quantity of nucleic acids, DNA were electrophoresed on 1% agarose gel, and the concentrations were measured on NanoDrop 2000/2000c spectrophotometers (Thermo Fisher Scientific, MA, USA) and confirmed on a Qubit 3.0 Fluorometer (Thermo Fisher Scientific, MA, USA).

It is known that Thai ricefish has XY sex-determination system (Nagai, Takehana, Hamaguchi, & Sakaizumi, 2008). Therefore, the genomic materials were obtained from males according to the report of Takenaka *et al*., (2020). Total genomic DNA was extracted from 35 male Thai ricefish individuals using a QIAamp Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions, and stored at –20 °C. Random genomic fragments were constructed to a paired-end library with an insert size of 500 bp for producing contigs for *de novo* sequencing by filling gaps. Sequencing was performed using BGISEQ500 (Kozarewa &Turner, 2012). After cluster preparation and sequencing, the intermediate result was filtered to obtain high quality reads. Clean reads were obtained with a read length of 150 bp for K-mer analysis and heterozygous simulation. We obtained 17-mers, and genome size was calculated by K-mer number/peak depth (Huang *et al*., 2019). *Platanus* software v1.2.4 was used for assembly of the *de novo* sequence (Kajitani *et al*., 2014), and GC depth distribution was analyzed using SOAPaligner (Gu, Fang, & Xu, 2013). Gene predictions of Thai ricefish were similarity matched to the nucleotide databases of teleost fish from The National Center for Biotechnology Information (NCBI) by using the Basic Local Alignment Search Tool (BLAST).

For the mitochondrial genome, the tissue from three caudal fins of Thai ricefish from each individual was used as biological replicates. One individual of *O. uwai* was also used in this molecular analysis. Total DNA was isolated by QIAamp Mini Kit (Qiagen, Germany). DNA products were amplified by PCR with LongAmp *tag* polymerase (BioLabs, MA, USA), using two 2 primer pairs: 5 '-GCCACACCCCC AAGGGAACTCAGC-3 ' and 5 '-AGTAGGGRYTTTCCTG TTTCCGG-3 ' G, (~16,600 bp) and 5 '-GTTACCCACCAWG CCGAGCRTTC, and CCGCGGYGGCTGGCACGAG-3 ' (~1200 bp). Primer designs were applied from sequences of *O. melastigma* (Hwang, Kim, Au & Lee, 2012) and *O. javanicus* (Setiamarga *et al*., 2009). Thermal cycles consisted of an initial denaturation at 94 °C for 4 min; 34 cycles of denaturation for 30 s at 94 °C, annealing for 35 s at 60 °C, and extension for 17 min 30 s (~16,600 bp) and for 2 min 30 s (~1200 bp) at 70 °C; and a final extension for 10 min at 70 °C. PCR products were extracted from gels by using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). An Ion Plus Fragment Library Kit with single-end and 137 and 130 bp average read lengths was used. Library size was checked using LabChip GX Touch 24 Nucleic Acid Analyzer with DNA High Sensitivity Reagent Kit. IontTorrent sequencing platform PI V3 was preferred for mitochondrial DNA sequencing (Zhou *et al*., 2016). Mitogenome assembly was processed using MIRA v3.4.1.1 and MITObim v1.8 programs, and we manually checked the structures by referring to the mitogenome sequences of *Oryzias* from the National Center for Biotechnology Information (NCBI). Nucleotide data were deposited in GenBank with accession numbers MN832874 and MT127789, and BioSample accession SAMN14331746. Other sequences of *Oryzias* used in this study were retrieved from NCBI. Sequences were aligned and trimmed using BioEdit 7.2 software. A molecular tree with neighbor joining, maximum likelihood, and UPGMA was built using MEGA 6 (1000 bootstraps) with >80% of bootstrap support. Based on phylogenetic analysis of 3 Thai ricefish in this study, the same species from different biogeography and the closest relative species were selected for comparison of the mitochondrial genes. Regarding the phylogenetic results, three individuals of Thai ricefish, O. uwai, and the Thai ricefish in Setiamarga *et al*., (2009) were selected to compare the mitochondrial genes. Thai ricefish reported by Setiamarga *et al*., (2009) were used as representing the same species with a biogeographic difference and *O. uwai* as the closest relative species to Thai ricefish. Gene abbreviations were designated according to NCBI: cytochrome c oxidase subunit (CO), cytochrome b (CYTB), NADH dehydrogenase (ND), adenosine triphosphate (ATP) synthase and displacement loop (D-loop), or control region.

All animal experiments were conducted under the National and Institutional Guidelines for the Animal Care and Use for vertebrates by the Institute for Animals for Scientific Purpose Development (IAD) National Research Council of Thailand (NRCT). The license was provided by Animal Care and Use Committee of Srinakharinwirot University (SWU-A-001_2563).

3. Results

As an overview of the genomic analysis, 38.94 Gb of raw data of sequencing were produced, and 35.47 Gb of data were used with a depth/(x) of 43 after low quality reads filtering. Sequencing depth data were expected to be 50.67-fold. Total data were maintained for 17-mer analysis (Figure 1) with a 35 peak depth of 17 mer distribution, a 28,833,070,078 total K-mer count, and 236,448,838 used reads. The genome size of Thai ricefish was estimated to be 824 Mb and the heterozygous rate is 0.22%. The repeat rate is 52.56%. For an initial assembly, the contig showed 1611 bp for N50 with 89,784 contig number and 183 bp of N90 with 492,053 contig number. Total size was 547,715,854 bp. Total numbers of ≥100 bp and ≥2 kb are 893,082 and 64,117, respectively. In the scaffold, N50 is 4663 bp with a scaffold number of 32,369. N90 is 190 bp with a 272,068 scaffold number. Total size is 585,878,698 bp. Total numbers of ≥100 bp and ≥2 kb are 693,534 and 74,756, respectively (Tables 1 and 2).

Gene predictions in Thai ricefish were similarity based calls with reference to the nucleotide databases of teleost fish from NCBI. Examples of the genes include galanin receptor 1, collagen type XXVII alpha 1 chain, stAR-related lipid transfer protein 13-like, growth arrest specific 2, non-LTR retrotransposon, attractin, transmembrane protein 198-B, hyaluronan and proteoglycan link protein 4, histone deacetylase 4-like, cartilage acidic protein 1-like, G2/M

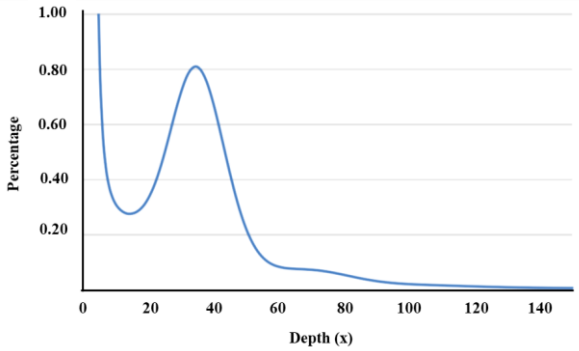

Figure 1. 17-mer depth distribution with percentage of Thai ricefish genome

Table 1. Contig and scaffold summary of Thai ricefish

Contig	
Total size	547,715,854 bp
N50 length	1,611 bp
N90 length	183 bp
Total Number(>=100bp)	893,082
Total Number(>=2kb)	64,117
Longest	55,729 bp
Scaffold	
Total size	585,878,698 bp
N50 length	4,663 bp
N90 length	190 bp
Total Number(>=100bp)	693,534
Total Number(>=2kb)	74,756
Longest	79,970 bp

phase-specific E3 ubiquitin-protein ligase, HERV-H LTR-associating 1, microtubule-associated protein, pleckstrin homology domain-containing family A member 1, 5'-AMP-activated protein kinase subunit gamma-1, tyrosine-protein phosphatase F, suppressor of cancer cell invasion, bromodomain containing 2 gene, zinc finger protein 385C, PHD finger protein 20, rho GTPase-activating protein 39, solute carrier family 12 member 2, Na-K-Cl cotransporter 1 alpha, nebulin-like, E3 ubiquitin-protein ligase TRIM39, dual specificity protein phosphatase 14, neogenin 1, SRY-box containing transcription factor 3, ankyrin 3, sorting nexin 5, rhombotin 1, D-tyrosyl-tRNA deacylase 1, GRAM domain-containing protein 4, oligodendrocyte-myelin glycoprotein, vacuolar protein sorting 13 homolog B, clathrin adaptor protein 2, ADP-ribosylation factor 1, SH3-containing GRB2-like protein 3-interacting protein 1, sec1 family domain containing 2, nuclear factor 7 ovary-like, syncytin B, kingsleyae general transcription factor II-I repeat domain-containing protein 2, proteasome beta 9 subunit, ras-related protein Rab-14, ABL proto-oncogene 1, immunoglobulin superfamily member 3, isocitrate dehydrogenase, mannose receptor C type 2, glypican 1, OTU deubiquitinase 7B, type-4 ice-structuring protein LS-12, MAP/microtubule affinity-regulating kinase 4, sepiapterin reductase, indoleamine 2,3-dioxygenase 2, nidogen and EGF like domains 1, neuroligin 4, calcium uptake protein 3, teneurin transmembrane protein 4, EvC ciliary complex subunit 2, proteoglycan 4, cytoplasmic linker associated protein 2, inhibitor of nuclear factor kappa B kinase subunit gamma, skeletal muscle actin, pre-mRNA processing factor 6, FERM domain-containing protein 5, megakaryocyte-associated tyrosine kinase, C-terminal binding protein 1, complexin 2, phosphodiesterase 2A, cornichon family AMPA receptor auxiliary protein 2, voltage-dependent L-type calcium channel subunit alpha-1D, RNA binding motif protein 15, putative ZDHHC-type palmitoyltransferase 6, low-density lipoprotein receptor-related protein 8, desumoylating isopeptidase 1, myocyte-specific enhancer factor 2C, angiopoietin 2, LHFPL tetraspan subfamily member 4 protein, suppression of tumorigenicity 14, neural proliferation differentiation and control protein 1, ephrin A5b, G protein-coupled receptor 1, glutamate ionotropic receptor kainate type subunit 2, troponin T, tubulin beta-4B chain, NRDE 2, apoptosis regulator BAX, YTH domain-containing family protein 1, TOX high mobility group box family member 2, Lck interacting transmembrane adaptor 1, tumor necrosis factor alpha-induced protein 2, DCC netrin 1 receptor, clarin 1, relaxin family peptide receptor 2, tousled like kinase 2, macoilin, tetraspanin, nucleoporin, START domain-containing protein, calcineurin like EF-hand protein 1, stanniocalcin, raftlin, matrix metallopeptidase, alkaline ceramidase 3, gastrula zinc finger protein, aquaporin, and progestin receptor epsilon.

In mitogenome analysis, the nucleotides consist of 16,954 bp for the three Thai ricefish. The whole genome per mitochondrial genome is 0.049 for Thai ricefish (in this study), 0.054 for *O. javanicus*, 0.046 for *O. melastigma*, 0.048 for *O. latipes*, 0.102 for *Danio rerio*, and 0.025 for *Fugu*

Table 2. Whole genome, mitogenome and whole genome per mitogenome of ricefish

Species	Size		Whole genome:mitogenome	Reference
	Whole genome	Mitogenome		
O. minutillus	824 (Mb)	16,954 (bp)	0.049	In this study
O. uwai	nd	16,958	-	In this study
O. minutillus	nd	16,953	-	Setiamarga *et al.* 2009
O. javanicus	809.7	16,892	0.048	Takehana *et al.* 2019; Setiamarga *et al.* 2009
O. melastigma	779.4	16,864	0.046	Kim *et al.* 2018; Hwang *et al.* 2012
O. latipes	~800	16,715	0.048	Takeda, 2008; Ichikawa *et al.* 2007
Danio rerio	~1,700	16,596	0.102	Wixon, 2000; Broughton *et al.* 2001
Fugu rubripes	414	16,447	0.025	Neafsey & Palumbi, 2003; Elmerot *et al.* 2002

No data (-)

rubripes (data used from NCBI) (Table 2). For mitochondrial structures, Thai ricefish contain the D-loop (control region), 12S rRNA, 16S rRNA, 22 tRNAs, and 12 coding genes. The most frequently used base was adenine (4,952-4,972 bp) followed by thymine (4,964-4,966 bp), cytosine (4,091-4,094 bp) and guanine (2,924-2,929, bp). In molecular based phylogenetic tree, Thai ricefish is closely related to *O. uwai* with 16,958 bp more so than to other species of *Oryzias* from NCBI databases and exactly separated from *Danio rerio* as outgroup (Figure 2). In this study, it was found in monophyletic clade that 3 samples of Mae Hong Son population exhibited differences to Thai ricefish by Setiamarga *et al*. (2009). In mitogenomic genes, only the D-loop and ND2 showed a difference in nucleotides for all comparisons between ricefish samples. However, COI was found to be the most different between the Mae Hong Son population and Thai ricefish from a different locality (97.3%-97.07%). CYTB of the Mae Hong Son samples was also diverged from another Thai ricefish (98.77%-98.95%). All genes of the Mae Hong Son clearly differed from *O. uwai* (Table 3 and Figure 3).

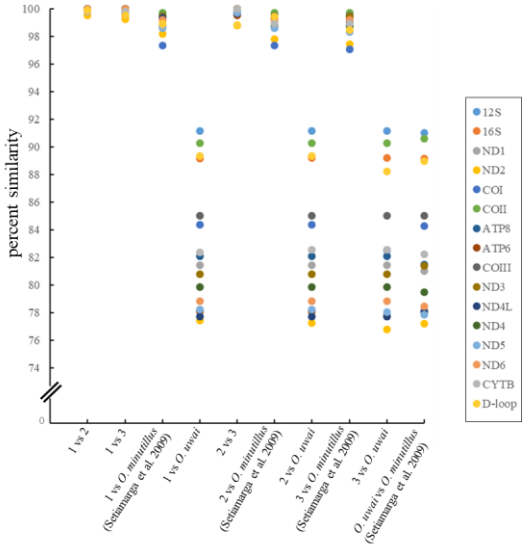

Figure 3. Comparison of percent similar identity between Thai ricefish, *O. uwai* and Thai ricefish from different region. Thai ricefish sample 1 (1), Thai ricefish sample 2 (2) and Thai ricefish sample 3 (3)

4. Discussion

Oryzias genomes are complete in three species: *O. javanicus*, *O. melastigma*, *O. latipes* (Takehana *et al*. 2019; Kim *et al*. 2018; Takeda, 2008). These fish are usually used as non-mammalian model organisms (Koyama *et al*., 2008; Inoue & Takei, 2003; Hilgers & Schwarzer 2019). Thai ricefish is the smallest among ricefish species (Nagai, Takehana, Hamaguchi, & Sakaizumi, 2008). In this study, we

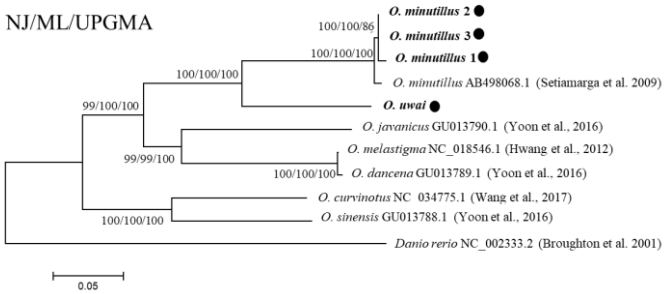

Figure 2. Phylogenetic tree of neighbor joining (NJ), maximum likelihood (ML) and unweighted pair group method with arithmetic mean (UPGMA) within ricefish species based on mitochondrial sequences

Table 3. Comparison of similar identity (%) of genes, tRNA, mRNA and control region in the mitochondria of Thai ricefish and *O. uwai*

	12S	16S	ND1	ND2	COI	COII	ATP8	ATP6	COIII	ND3	ND4L	ND4	ND5	ND6	CYTB	D-loop
1 vs 2	100	99.94	100	99.52	100	100	100	100	100	100	100	100	100	100	99.82	99.92
1 vs 3	100	99.94	100	99.24	99.68	100	100	99.55	100	100	99.66	100	99.73	100	99.82	99.54
1 vs *O. minutillus* (Setiamarga *et al.* 2009)	99.6	99.4	99.28	98.19	97.33	99.71	99.4	99.41	99.49	99.43	99.33	98.76	98.59	99.23	98.77	98.93
1 vs *O. uwai*	91.2	89.14	81.42	77.42	84.39	90.3	82.1	78.07	85.04	80.8	77.74	79.84	78.25	78.83	82.38	89.35
2 vs 3	100	100	100	98.77	99.68	100	100	99.55	100	100	99.66	100	99.73	100	100	98.85
2 vs *O. minutillus* (Setiamarga *et al.* 2009)	99.6	99.46	99.28	97.81	97.33	99.71	99.4	99.41	99.49	99.43	99.33	98.76	98.59	99.23	98.95	99.46
2 vs *O. uwai*	91.2	89.19	81.42	77.27	84.39	90.3	82.1	78.07	85.04	80.8	77.74	79.84	78.25	78.83	82.56	89.34
3 vs O. minutillus (Setiamarga *et al.* 2009)	99.6	99.46	99.28	97.43	97.07	99.71	99.4	98.96	99.49	99.43	98.99	98.76	98.32	99.23	98.95	98.47
3 vs *O. uwai*	91.2	89.19	81.42	76.79	82.51	90.3	82.1	77.78	85.04	80.8	77.74	79.84	78.06	78.83	82.56	88.22
O. minutillus (Setiamarga *et al.* 2009) vs *O. uwai*	91	89.14	81.01	77.22	84.26	90.59	81.5	78.07	85.04	81.38	78.1	79.47	77.87	78.45	82.21	88.97

Thai ricefish sample 1 (1), Thai ricefish sample 2 (2) and Thai ricefish sample 3 (3)

found the whole genome size of Thai ricefish is larger than those of *O. melastigma* and *O. latipes*. The genome compositions of Thai ricefish are also different from *O. javanicus*, *O. melastigma*, and *O. latipes*. These results may provide important data to increase our molecular knowledge of *Oryzias* for screening selected genes and comparing with other animal models for further work. Genome size may not be associated with and may not depend on the total or standard length in ricefish species. For the whole genome size divided by mitogenome, the values of Thai ricefish, *O. uwai* in this study, and other ricefish differed from zebrafish and pufferfish (Neafsey & Palumbi, 2003; Wixon, 2000), respectively. This suggests the value of the whole genome per mitogenome might be unique within *Oryzias*. Both whole genome and mitogenome sizes in *Oryzias* may be about 700-900 Mb and 16,700-16,900 bp, respectively. The phylogenetic tree based on mitochondrial sequences showed that Thai ricefish is the most closely related with *O. Uwai*, more so than with *O. javanicus* and *O. melastigma*. Thai ricefish is similar in morphology to *O. uwai* but Thai ricefish have no a black pigment line on a pelvic fin (Uwa & Parenti 1988). The results suggest that the molecular phylogenetic tree based on mitogenome is congruent with this morphology. Cytochrome c oxidase subunit I (COI) is a potential molecular tool in the DNA barcode to study phylogenetic evolution for higher taxa and other fish populations (Dalziel, Moyes, Fredriksson, & Lougheed, 2006). Cytochrome b (CYTB) was also used for investigating the population diversity of wild ricefish in Japan (Takehana, Nagai, Matsuda, Tsuchiya, & Sakaizumi, 2003). We found few diversities in D-loop and ND2 among Thai ricefish. Therefore, D-loop and ND2 may provide an alternative DNA barcode for studying geographic variation in Thai ricefish population.

Our results provide new knowledge of the molecular biology of Thai ricefish and support the understanding of the evolution and geographic diversity of freshwater ricefish.

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