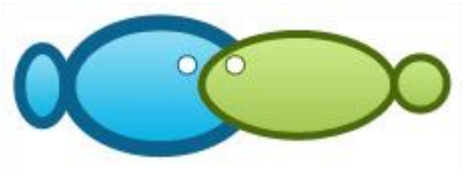

Genetic characteristics of ricefish from Lake Poso, Central Sulawesi, Indonesia

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Abstract. This study determined the genetic characteristics of ricefish from Lake Poso, Sulawesi Tengah-Indonesia based on cytochrome c oxidase subunit I (COI) gene marker. We collected and analyzed 2 tissue samples from Lake Poso, about 54 sequences from GenBank, and 100 sequences from Bold taxonID of ricefishes. The two tissue samples were extracted to obtain whole genome then amplified and sequenced to obtained COI nucleotides. The nucleotide frequencies were A = 23.8%, T/U = 29.8%, C = 26.8%, and G = 19.6%. The AT content (53.85%) in the COI region of ricefish was higher than that of GC (46.2%). We identified 217 codons from two sequences of our samples. Ninety-six percent of nucleotide changes were observed at the 3rd codon position of COI sequence, but the amino acid compositions translated by COI sequences of two samples stayed the same. We identified also 170 cut positions from two sequences of our samples. The phylogenetic tree estimated by Neighbour-joining method, Blastn and Boldsystem results, and nucleotide composition indicated that the ricefish is *Oryzias nebulosus*. Individuals from operational taxonomic units designated as same ricefish species, supporting morphological evidence for each of these being similar species. It is suggested that the COI barcoding can be used to identify ricefish species of *Oryzias*.
Key Words: COI, *Oryzias nebulosus*, ricefish, Lake Poso, Blastn.

Introduction. Lake Poso or Danau Poso is the third-deepest lake in Indonesia with a depth of 450 meters at its deepest points, two other lakes are Lake Matano in Sulawesi (590 m) and Lake Toba in Sumatera (529 m) (Lehmusluoto & Machbub 1997). Lake Poso is a tectonic lake located at 657 m above sea level in Sulawesi Tengah, Indonesia. Sulawesi is a particularly rich area of endemism for atherinomorph fishes. The lake is known to supply drinking water for domestic use and irrigation of farm lands, recreational activities and means of transportation. Fishing activities also take place in the river and the people depend to a large extent on their fish catches as one of the major sources of protein. The ancient lake has 32 km length, and 16 width. The lake spans across an area of 32,000 hectares and contains various fish, including the eel *Anguilla marmorata* (Haryani & Hehanussa 2000), a large number of endemic *Tylomelania* freshwater snails (von Rintelen & Glaubrecht 2010), several endemic *Caridina* shrimps (von Rintelen & Cai 2009) and Parathelphusid crabs-genera *Migmathelphusa, Parathelphusa* and *Sundathelphusa* (Chia & Ng 2006) and also 11 fish species that are endemic to the lake (Naruse et al 1993), notably buntingi ricefish.

Oryzias is a genus of ricefishes native in Lake Poso. These ricefishes are all highly threatened; in some case possibly already extinct (Kottelat 1990; Parenti & Soeroto 2004). One of the reasons for the drastic decline of the native fish is the introduction of some non-native fish species, particularly Mozambique tilapia (*Oreochromis mossambicus*) and common carp (*Cyprinus carpio*) (Parenti 2008). The *Oryzias* is a reference to the scientific name for rice, *Oryza* (Parenti 2008). According to Parenti (2008) some species of the genus are commonly used in science as a model organism. The *Oryzias* have an unusual reproductive behavior where the female facultatively carries the eggs in a cluster at the pelvic or anal fins for a period after they have been fertilized (Parenti 2008; Herder et al 2012). They are small, up to 8 cm (3.1 in) long, and most are relatively plain in colour (Froese & Pauly 2018). There are currently 33 recognized species in this genus (Froese & Pauly 2018). Of the 33 ricefishes species currently recognized, seven are endemic to Lake Poso (Parenti & Soeroto 2004), namely *Oryzias nebulosus* sp. nov., *Oryzias nigrimas* Kottelat 1990b, *Oryzias orthognathus* Kottelat 1990b, *Adrianichthys kruyti* Weber 1913, *Adrianichthys roseni* sp. nov., *Xenopoecilus oophorus* Kottelat 1990b, and *Xenopoecilus poptae* Weber & de Beaufort 1922.

Morphological characters have been used to describe variation among individuals and populations of the species and also have been useful in determining fish species, sex and larval stages. The characters used are meristic (countable) such as number of fin rays or vertebrae and morphometric (measurable) expressed as ratios of standard length or fork length (Parenti et al 2013; Marčeta 2016). Morphological characters have limitations for describing intraspecific genetic diversity as they are polygenic and expression can be modified by the environment. Their use in individual characterization studies has been superseded for the most part by the development of direct genetic methods. Hence, genetic or DNA-based techniques are being used for such purposes (Teletchea 2009; Wong 2011). In this technique, individual characteristics could be detected and could produce fine genetic markers (Ali et al 2004; Ahmed et al 2004; Theodorakis & Bickham 2004).

Here, we describe a genetic characteristic of ricefishes, ikan padi or buntingi (in Sulawesi vernacular) based on cytochrome oxidase subunit I (COI) gene. The COI gene, within the mitochondrial genome, is suitable for discriminating close relationship between the species across various animal phyla and it has been used for both marine and freshwater species (Hajibabaei et al 2005; Steinke et al 2005). The gene is widely used owing to their slowest mutation and lower substitution rates compared to other mitochondrial DNA genes (Castresana et al 1994). These results have prompted to screen the species diversity and process the identification of ricefishes species. *Oryzias asinua*, *O. wolasi*, and *O. woworae* are the first ricefishes reported in this genus (Parenti et al 2013), and will provide potential genetic resources for further evolutionary studies of the genus *Oryzias* and other relatives.

Material and Method

Specimen collection and DNA extraction. Fish samples were collected on April 2017 from three stations or sites at Lake Poso location in the Central Sulawesi (Figure 1). We were also collected about 54 nucleotide sequences and 100 sequences of ricefishes from GenBank (https://www.ncbi.nlm.nih.gov) and from Bold taxonID (http://www.boldsystems.org), respectively (Figure 2).

Voucher specimens were deposited in the Fisheries Laboratory of Tadulako University, Palu. All specimens were preserved in 96% ethanol. Tissue samples were dissected from the body muscle, and genomic DNA was extracted from the muscle tissue following the method described by Lopera-Barrero et al (2008) with some modification. Approximately, 0.3 g of each muscle tissue was crushed with a mortar and pestle in 500 µL extraction buffer (100 mM Tris, 8.5 mM EDTA, 500 mM NaCl) and transferred to a polypropylene microfuge tube. Then 20% SDS was added; the mixture was vortexed briefly and incubated in a gentle shaking water bath at 65°C for 10 min. After that 160 µL of 5 M potassium acetate was added after bringing the sample to room temperature, then vortexed and centrifuged at 10 000 *g* for 10 min. Supernatant was removed (about

500 µL) into another Eppendorf tube and 200 µL of cold isopropanol was added and mixed after which samples were kept on at -4°C for 15 min before centrifuging at 10 000 *g* for 10 min to sediment the DNA. Supernatant was decanted after centrifuging and pellets were washed with 500 µL of 70% ethanol and centrifuged at 10 000 *g* for 10 min. The ethanol was decanted and the DNA was air-dried at room temperature (37°C) and re-suspended in 50 µL TE (10mM Tris and 1mM EDTA) buffer and stored in the freezer prior to PCR analysis. The integrity of the DNA was verified by electrophoresis on a 1% agarose gel.

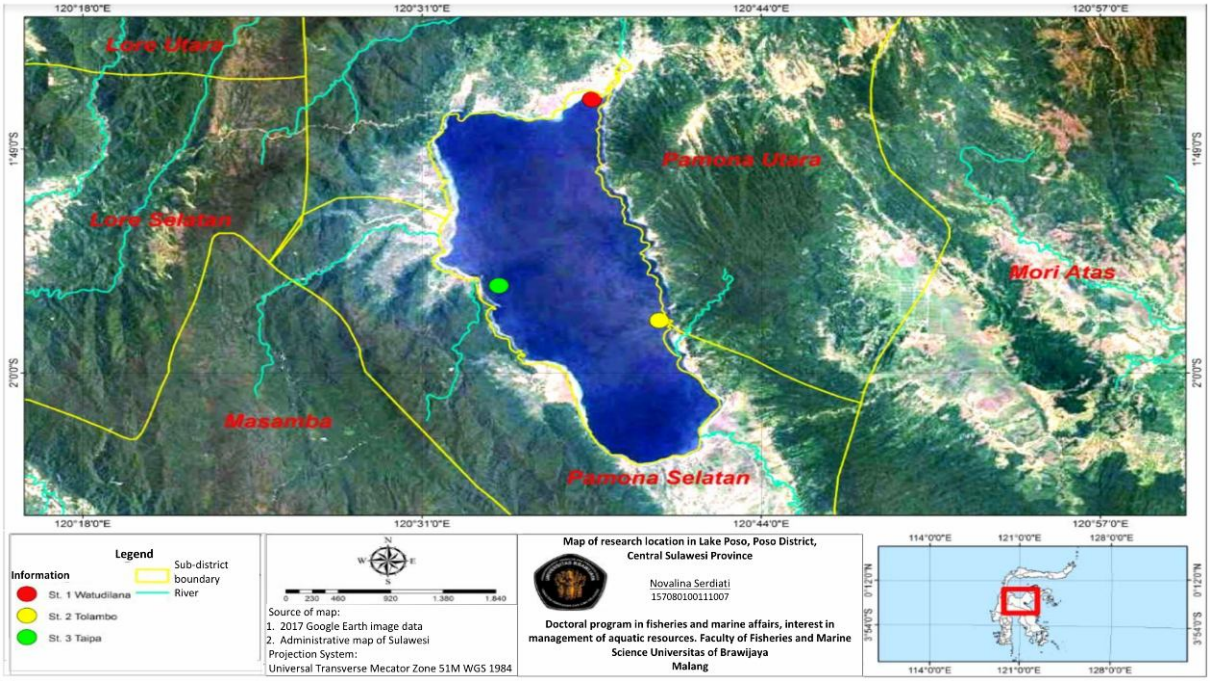

Figure 1. Map of Lake Poso (the sampling stations showed by yellow, green and red circles).

PCR and DNA sequencing. Fragments of cytochrome c oxidase subunit I (COX1, COI) gene were amplified using primer Fish BCL (Forward): 5'-TCAACYAATCAYAAAGATATYGGCAC-3' and primer Fish BCH (Reverse): 5'-ACTTCYGGGTGRCCRAARAATCA-3' (Baldwin et al 2008). PCR reactions were carried out using MastercyclerH Eppendorf gradient thermal cyclers (Brinkmann Instruments, Inc.). The reaction mixture of 18 µL ddH_2O, 2.5 µL fish BCL (10 µM), 2.5 µL fish BCH (10 µM), 25 µL Go Taq Green PCR mix (Promega) 2X was prepared for each plate. The mixture contained 50 µL of mixture and 2 µL DNA template.

PCR amplifications were performed with the following conditions: 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 40 s, and extension at 72°C for 1 min, with an initial denaturation at 94°C for 2 min and final extension at 72uC for 7 min. Amplified products were visualized in 1% agarose gel, and purified products were directly sequenced on an Applied Biosystems 3730 sequencer using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems, Inc.). Sequencing primers were the same as those listed above for PCR. All sequencing reactions were performed according to the manufacturer's instructions.

Data analyses. COI sequences were aligned and translated into amino acids with MEGA 6.06 software (Tamura et al 2013) in order to exclude sequencing errors. Obtained sequences were blasted (*basic local alignment search tools*) through NCBI and BOLD web-based systems. The hits with the highest query coverage and maximum identical values (> 98%) were chosen as reference sequences. Nucleotide composition, genetic distances, and codon usage were calculated in MEGA 6.06 software (Tamura et al 2013). Substitution pattern and rates were estimated under the Kimura (1980) 2-parameter model. These sequences, as well as 50 more (representing 22 species) downloaded from

GenBank, including two voucher samples, were added to the aligned sequences generated in this study for further phylogenetic analyses. Phylogenetic analyses were performed using neighbor-joining (NJ) methods based on K2P distances that was created using MEGA 6.06 software (Tamura et al 2013). The enzyme recognition sites of the sequence of COI are determined online on http://www.restrictionmapper.org.

Results. A data set (652 base pairs) of two COI sequences from Lake Poso, about 54 sequences from GenBank, and 100 sequences from Bold taxonID of ricefishes was obtained.

Species identification. DNA barcoding uses the 652 base pairs segment of the COI gene as the basis for an identification system for members of the animal kingdom showed at Figure 2.

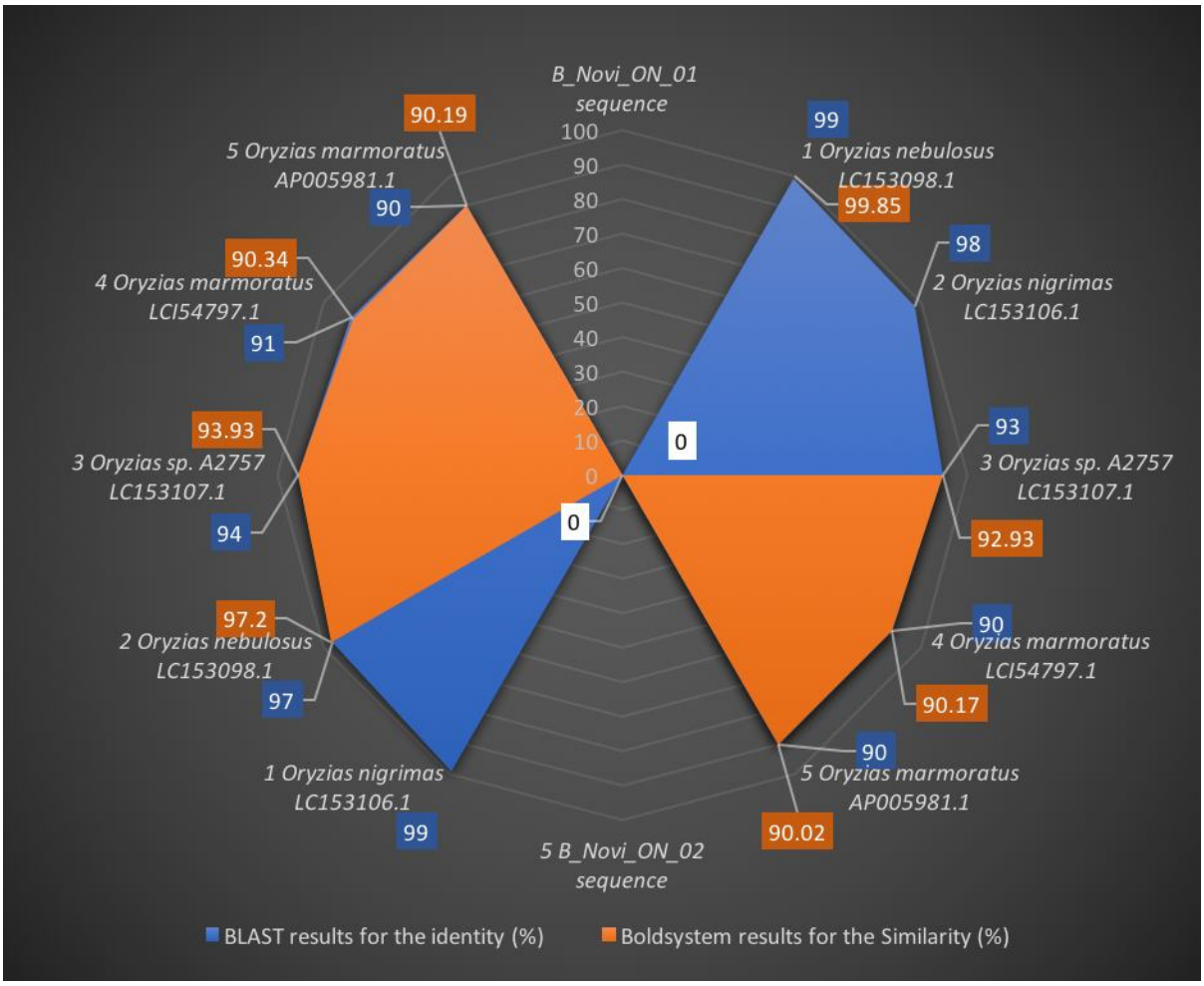

Figure 2. BLAST (Blastn) and Boldsystem results for DNA barcoding used the COI gene fragment of paddy fish from Lake Poso.

We see that these five database matches span almost the entire length of our query sequence. We also notice that the top five hits match to our sequence than the remaining both Boldsystem and Blastn hits. Figure 2 shows *O. nebulosus* and *O. nigrimas* as the top of reference for sample 1 (B_Novi_ON_01) and sample 2 (B_Novi_ON_02), respectively. Blastn tool identified COI sequences of two tissue samples as *O. nebulosus* and *O. nigrimas*. These samples were further compared with standard BOLD system of *O. nebulosus* and *O. nigrimas*. Based on further analysis it was determined that both samples came from one species, *O. nebulosis* (see nucleotide composition, phylogenetic tree and Bold taxonID tree). Morphological features of samples found matching with physical samples used in study.

Polymorphic sites and nucleotide composition. Nucleotide composition of COI gene of paddy fish in Lake Poso varied greatly, ranging from 40.8 to 54.8% of GC content. In all cases, GC content decreased from the first to the third codon position with mean values of 54.8%, 42.9% to 40.8% respectively (Figure 3). The pattern of variance (standard error) confirmed that the highest range in GC content was observed in the first codon position: the third position displayed the least variation.

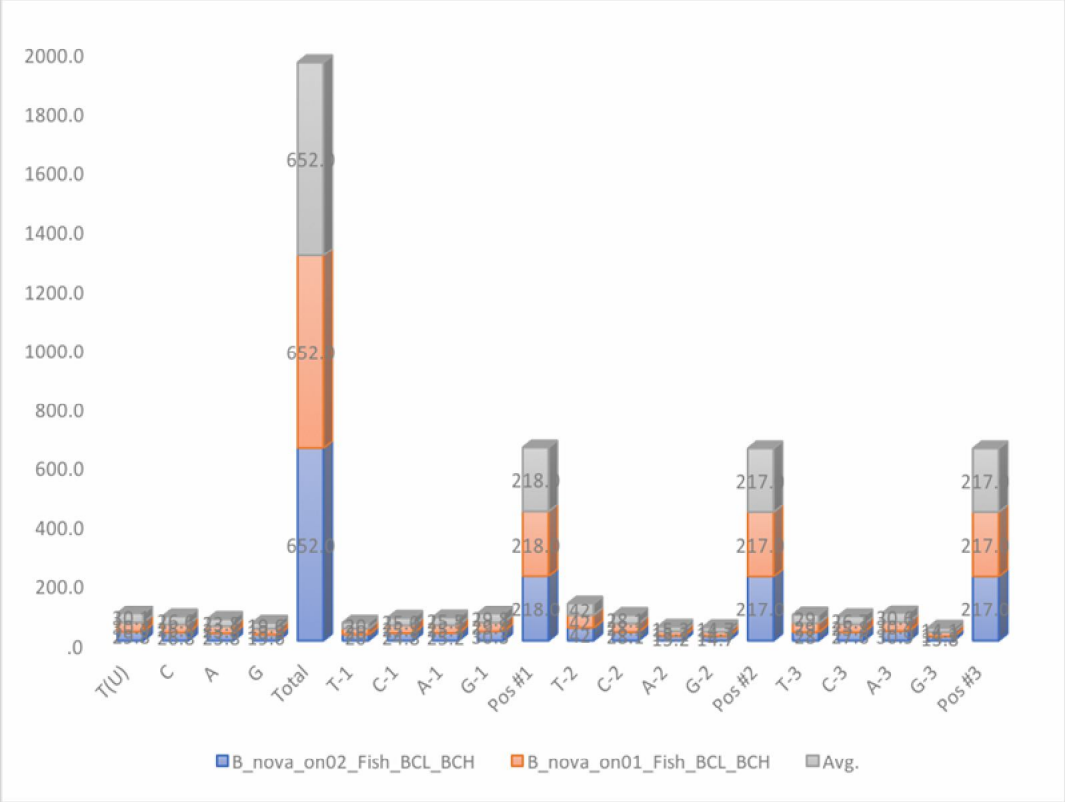

Figure 3. Nucleotide composition from the COI gene fragment of paddy fish from Lake Poso.

From Figure 3, it is quite clear that nucleotide composition of two samples are a little different in terms of T and C nucleotide, which indicate that these two samples have different genetic makeup and belong to a single species. The estimated transition (A/C and C/T) and transversion (A/T; A/C; C/G; G/T) bias (R) is 5.12. The nucleotide frequencies were A = 23.8%, T/U = 29.8%, C = 26.8%, and G = 19.6%. The AT content (53.85%) in the COI region of paddy fish was higher than that of GC (46.2%). When COI gene fragment from our two samples are compared, they differ at about 17 in 652 bases sites. The two haplotypes are shown, along with their polymorphic sites in the sequences (Table 1).

Table 1

Polymorphic sites in the COI gene fragment sequences of paddy fish from Lake Poso

Sample	*Site*																		*Haplotype*
	0	*0*	*1*	*1*	*2*	*2*	*3*	*3*	*3*	*3*	*3*	*4*	*4*	*5*	*5*	*6*	*6*	
	6	*7*	*0*	*9*	*7*	*8*	*2*	*3*	*3*	*4*	*4*	*4*	*6*	*4*	*4*	*3*	*4*	
	9	*2*	*5*	*6*	*6*	*8*	*1*	*3*	*9*	*5*	*6*	*4*	*5*	*3*	*9*	*3*	*9*	
B_Novi_On_02	G	A	A	G	C	A	A	C	G	A	G	C	G	A	C	G	T	1
B_Novi_On_01	A	G	G	A	T	G	G	T	A	G	T	T	A	T	A	A	C	2
Mutation	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tv	Tv	Tr	Tr	

Notes: Tr = transition; Tv = transversion.

Both haplotypes differ in several polymorphic sites in 69, 72, 105, 196, 276, 288, 321, 333, 339, 345, 346, 444, 465, 543, 549, 633, 649, respectively caused by point mutation. Mutation mechanisms result either in transitions: purine-purine (A ⇔ G) or pyrimidine-pyrimidine (C ⇔ T) exchanges, or transversions: purine-pyrimidine or pyrimidine-purine (A⇔C, A⇔T) exchanges.

Codon usage and amino acid composition. We identified 217 codons from two sequences of our samples. Figure 4 shows codon usage of COI gene of paddy fish in Lake Poso. Relative synonymous codon usage is given in parentheses following the codon frequency.

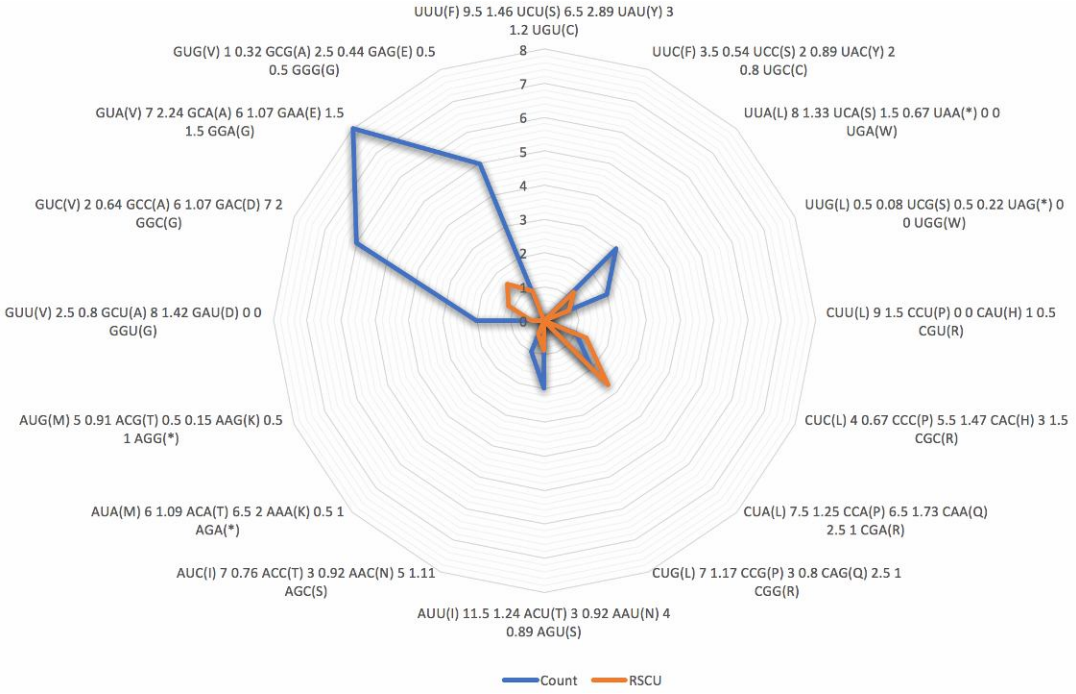

Figure 4. Codon usage and relative synonymous codon usage (RSCU) from COI gene fragment of paddy fish from Lake Poso. Symbols in parenthesis: A = Alanine, C = Cysteine, D = Aspartic acid, E = Glutamic acid, F = Phenylalanine, G = Glycine, H = Histidine, I = Isoleucine, K = Lysine, L = Leucine, M = Methionine, N = Asparagine, P = Proline, Q = Glutamine, R = Arginine, S = Serine, T = Threonine, V = Valine, W = Tryptophan, Y = Tyrosine, * = stop codon.

There are 64 sense codons that translated into 19 different amino acids (without Cysteine). Individual amino acids are encoded by up to six different codons but within codon families some are used more frequently than others. The Figure 4 has shown the top scores in count for AUU codon as Isoleucine (11.5), UUU codon as Phenylalanine (9.5), and CUU codon as Leucine (9) and even with relative synonymous codon usage. We did not found some codons such as UGU and UGC for Cysteine (C), CCU for Proline (P), CGU and CGG for Arginine (R), GAU for Asparagine (D), and AAA codon for Lysine (K). In addition, there are not the stop codons as AGA and AGG and we also discovered AUA codon responsible for start codon and Methionine (6) in mitochondria of paddy fish.

Cut positions and site length. The 652 base pairs fragment of the COI gene from paddy fish of Lake Poso can be recognized and cleaved by restriction enzyme. The Figure 5 provides the recognition sequences of COI of samples for some of the most frequently used restriction endonucleases.

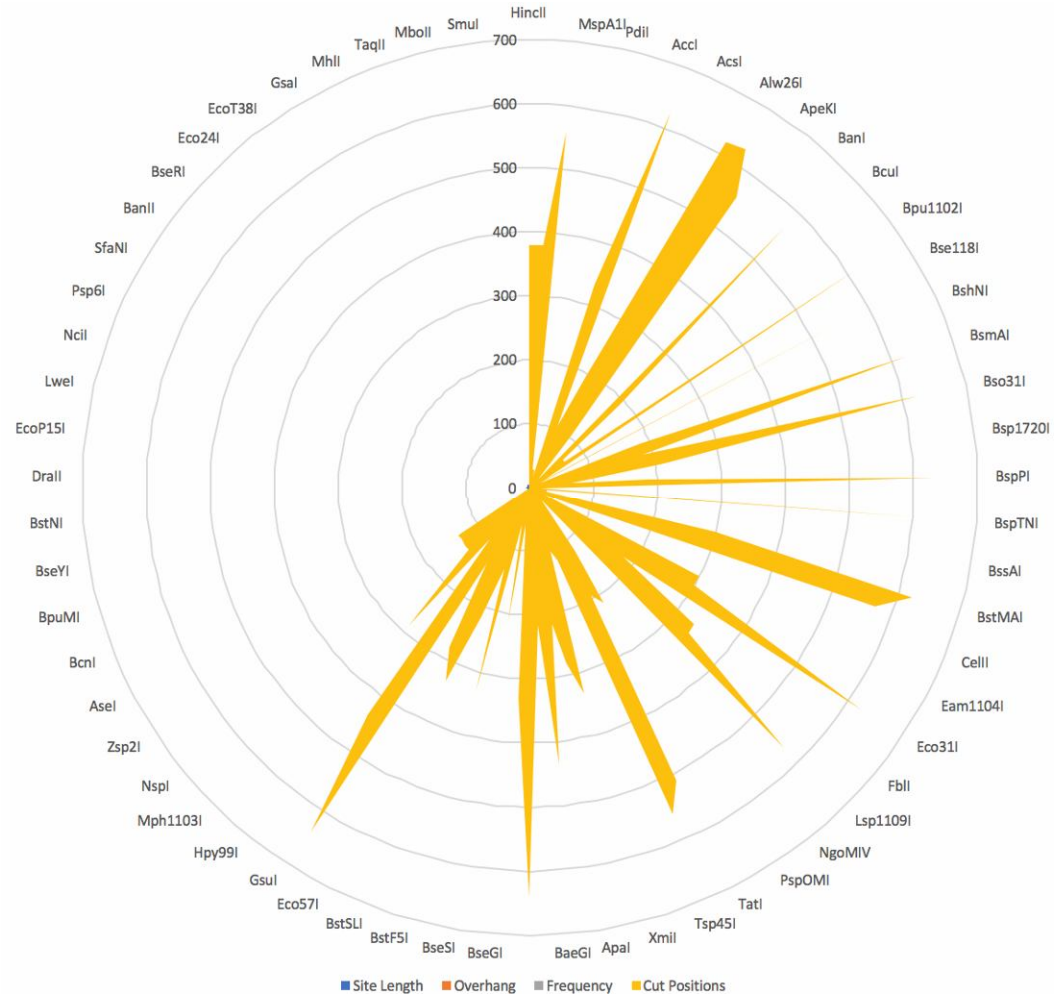

Figure 5. Recognition sequences for restriction endonucleases from COI gene fragment of paddy fish from Lake Poso.

We found 170 cut positions from two sequences of our samples with vary site length, range 5-6 nucleotides. More than a hundred restriction enzymes have been identified from COI fragments of *O. nebulosus* sequences from Lake Poso. Those all restriction enzymes are type II, which cleave DNA within their recognition sequences. These enzymes recognize particular base sequences (recognition sequences or recognition sites) in their target DNA and cleave that DNA at defined positions in the COI gene fragment of paddy fish from Lake Poso. We found there are many cut positions in this gene fragment (Table 2).

Phylogenetic position. We investigated the phylogenetic and Bold taxonID Tree position of our samples based on COI sequence data. Figure 6 shows the phylogenetic tree constructed by COI gene sequences of ricefish species using neighbor-joining algorithm with distance scale.


Oryzias_marmoratus LC154797.1_
Oryzias_marmoratus AP005981.1_
Oryzias_sp. LC153753.1_
Oryzias_matanensis LC153099.1_
Oryzias_sarasinorum LC154798.1_
Oryzias_sarasinorum AB370891.1
Oryzias_celebensis LC153105.1_
Oryzias_celebensis AB498070.1_
Oryzias_sp. LC153107.1_
B_nova_on02_Fish_BCL_BCH
Oryzias_nebulosus LC153098.1_
B_nova_on01_Fish_BCL_BCH
Sample 1
Sample 2
Oryzias_sinensis LC153109.1_
Oryzias_sinensis HQ536422.1_
Oryzias_sinensis HQ536428.1_
Oryzias_sinensis HQ536427.1_
Oryzias_sinensis HQ536426.1_
Oryzias_sinensis HQ536425.1_
Oryzias_sinensis HQ536424.1_
Oryzias_sinensis HQ536423.1_
Oryzias_setnai LC153752.1_
Oryzias_sp. LC153772.1_
Oryzias_pectoralis LC153100.1_
Oryzias_pectoralis KX987838.1_
A
Oryzias_sp. KC456403.1_
Oryzias_sp. KC456402.1_
Oryzias_sp. KC456404.1_
Oryzias_sp. KC456405.1_
Oryzias_javanicus MF170950.1_
Oryzias_javanicus LC153108.1_
Oryzias_javanicus KU692697.1_
Oryzias_javanicus KU692696.1_
Oryzias_javanicus KU692695.1_
Oryzias_latipes LC154799.1_
Oryzias_latipes AB498066.1_
Oryzias_latipes LC154800.1_
Oryzias_latipes AP004421.1_
Oryzias_latipes AB498065.1_
Oryzias_curvinotus LC153104.1_
Oryzias_curvinotus NC_034775.1_
Oryzias_curvinotus KY364884.1_
Oryzias_luzonensis NC_012979.1_
Oryzias_luzonensis AB498064.1_
Oryzias_minutillus NC_012975.1_
Oryzias_minutillus LC153115.1_
Oryzias_minutillus AB498068.1_
Oryzias_sp. LC153103.1_
Oryzias_dancena NC_012976.1_
Oryzias_dancena AB498069.1_
Oryzias_sp. LC153102.1_
Oryzias_melastigma NC_018546.1_
Oryzias_melastigma LC153114.1_
Oryzias_melastigma JQ713914.1_
Oryzias_dancena LC153101.1_
Oryzias_javanicus NC_012981.1_
Oryzias_javanicus DI374225.1.
Oryzias_javanicus AB498067.1_
0.1

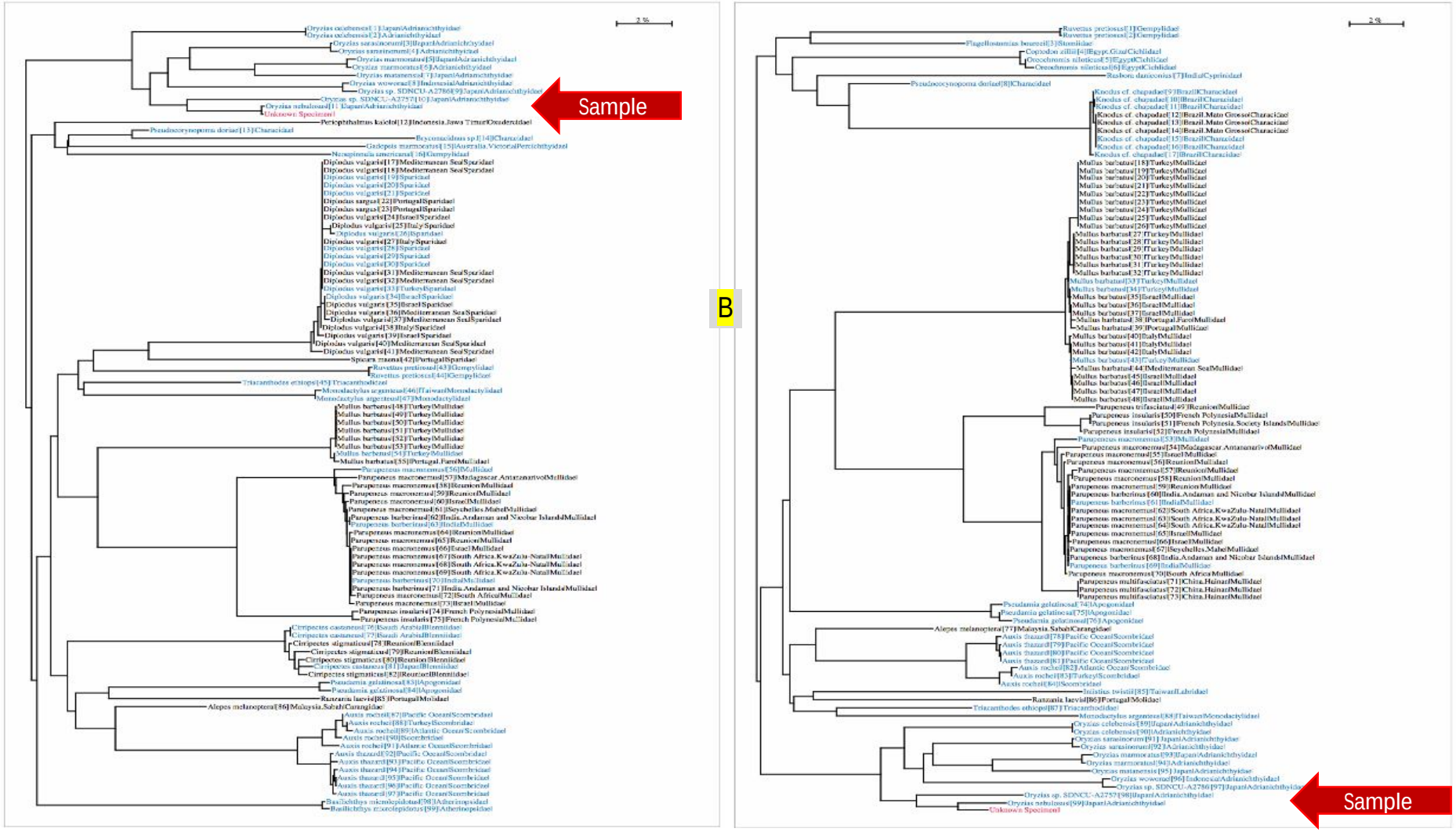

Figure 6. Phylogenetic tree (A) and Bold taxonID Tree (B). Those figures show the position sequence of sample in the tree. In total there are 54 sequences and two our samples (B_Novi_ON_01and B_Novi_ON_02)(A); and 100 sequence count, 29 species count, 17 genus count, 14 family count, and 1 unidentified (red color) as our samples (B).

In Figure 6 (A and B), it can be clearly seen that sample both B_Novi_ON_01and B_Novi_ON_02 formed a similar clade since they are genetically the same and evolved from the same ancestor. They also formed a same clade with paddy fish *O. nebulosus*. The tree shows a significant phylogenetic relationship among the studied several paddyfish species. In the present study, B_Novi_ON_01and B_Novi_ON_02 were clustered in a single clade with *O. nebulosus*. In addition, *O. nebulosus* was tightly clustered with *O. nebulosus* from Japan.

Discussion. DNA sequence analysis has been used to assist species identification of ricefishes. Species identification using the mtDNA COI gene is able to differentiate two ricefishes samples tested as *O. nebulosus*. *O. nebulosus* shares with *O. nigrimas* a unique sexual dichromatism in preserved specimens (Parenti & Soeroto 2004). Various methods and approaches have been used in fish species identification. Traditional and official methods used in fish species identification are based chiefly on external morphological features or various relative measurements of body parts (Strauss & Bond 1990), gill rakers counting (Iffat 2002), otoliths (Granadeiro & Silva 2000), the separation and characterization of specific proteins (Teletchea 2009). Yet, in some cases both morphological and protein features are of limited value for identification and differentiation purposes (Teletchea 2009). These difficulties explained why researchers have attempted to develop new methods for identifying fish species without relying chiefly on morphological features.

DNA-based identification methods offer an analytically powerful addition or even an alternative. According to Lefébure et al (2006) COI as a barcoding tool helps to identify an organism based on DNA sequence variability and assignment to a certain species. The DNA barcode sequences also can be used as a DNA taxonomy tool to perform prediction and classification of potentially new species (da Silva et al 2011). Da Silva et al (2011) stated the impacts of DNA barcoding is extended well beyond biodiversity science. This method has been broadly used in species identification and biodiversity studies (Bingpeng et al 2018), for taxonomy (Hebert et al 2003), species delimitation (Liu et al 2017), population and phylogeographic analyses, egg and larvae detection, and industrial applications (Ward 2009). Ferri et al (2009) stated that DNA barcoding will be useful not only in wildlife enforcement but also in biosecurity, food authentication, investigation against poaching or illegal trade of endangered species, and criminal casework. The COI gene is a commonly applied marker for identification studies in various animals, such as in Antartic ocean's animals (Grant et al 2010); marine crustacea (Radulovici et al 2009), marine metazoa (Bucklin et al 2011). The COI gene sequences was also effective for classifying and identifying vertebrates and invertebrates, and has been widely used in various biological groups (Weigt et al 2012; Dona et al 2015; Nzelu et al 2015; Staffen et al 2017). Similar results were obtained in a barcoding study on fish species (Ward et al 2005, 2008a, 2008b, 2009; Hubert et al 2008; Steinke et al 2009; Lakra et al 2011; Liu et al 2013).

In the entire sequences, the pair nucleotide frequencies provide the proper indication about diversity in the genetic makeup of two samples. The nucleotide pair frequencies provide the precise evidence about highest % identical sites and lowest rate of transversional pairs in the group. The AT content in the COI region of ricefish was higher than that of GC, which was similar with other species of Cypriniformes. Simon et al (1994) have reported AT rich nucleotide base compositions in COI gene of many organisms.

Almost all transitions and transversions occurred on the third codon positions and transitions are always higher than transversions indicating the nucleotide substitutions are yet unsaturated. This data suggested that COI markers are effective parameters for phylogenetic analyses of ricefish. According to Gold & Karel (1988) the %GC values among the species (15 species of teleost fishes) ranged from 37.3 to 41.6; compositional heterogeneity values were relatively uniform and ranged from 8.8 to 13.5; asymmetry values were generally low.

The COI gene fragment of *O. nebulosus* was 652 bp long. Its gene fragment content conforms to the vertebrate consensus, by containing the highly conserved set of 635 bp and variable sites of 17 bp, that are essential in mitochondrion respiration and cythcrome oxidase subunit I (COI) production. Resource polymorphism is widespread over several taxa including fish, amphibians, and birds (Smith & Skúlason 1996). According to Smith & Skúlason (1996) and Schluter (2000), resource polymorphism may be induced by phenotypic plasticity, genetic difference, or a combination of both. The mechanisms that generate and maintain the polymorphisms provide insights into the role of natural selection driving phenotypic, behavioral, and life-history diversification, which ultimately lead to speciation.

Fragment of COI gene of samples indicating that they represent fragments of functional mitochondrial genes (Bensasson et al 2001). The genetic code is redundant, with 64 sense codons translated into 19 different amino acids (without Cysteine, symbol Cys or C). Individual amino acids are encoded by up to six different codons but within codon families some are used more frequently than others. According to Brandis & Hughes (2016), this phenomenon is referred to as synonymous codon usage (SCU) bias. Williford & Demuth (2012) stated that SCU correlate with expression level in prokaryotes, as well as in unicellular and multicellular eukaryotes such as paddy fish. The strong positive correlation between codon usage bias and gene expression levels is attributed to selection for translational efficiency (Brandis & Hughes 2016).

The increase in SCU bias with gene expression levels has been observed in organisms from all domains of life including yeast (Ghaemmaghami et al 2003), human (Comeron 2004), nematode (Cutter et al 2006), flowering plant (Qiu et al 2011). According to Suzuki et al (2008) SCU varies both between organisms and among genes within a genome, and arises due to differences in G+C content, replication strand skew, or gene expression levels. We identified 19 amino acids from two sequences of our samples. Amino acid composition and their changes with expression levels have been also documented in a number of prokaryotes, *Escherichia coli* and *Bacillus subtilis* (Akashi & Gojobori 2002) and eukaryotes, human (Urrutia & Hurst 2003), nematode (Cutter et al 2006), yeast (Raiford et al 2008).

The restriction enzyme type II cuts DNA at varies the sequence of paddy fish from Lake Poso. The type II restriction systems typically contain individual restriction enzymes and modification enzymes encoded by separate genes. Type II restriction endonucleases recognize specific DNA sequences and cleave DNA at specified locations within or adjacent to their recognition sites to produce 5-phosphates and 3-hydroxyls (Halford 2001; Pingoud & Jeltsch 2001; Roberts et al 2003). Restriction sites, recognition sequence, site length of COI gene fragment from paddy fish of Lake Poso that recognized by restriction enzymes are showed in Table 2.

Table 2

Restriction sites (cut positions), recognition sequence, site length of COI gene fragment from paddy fish for the restriction enzyme type II

Cut positions	*Recognition sequence*	*Site length*	*Overhang*	*Frequency*	*Enzyme*
380	GTYRAC	6	blunt	1	HincII
380	GTYRAC	6	blunt	1	HindII
561	CMGCKG	6	blunt	1	MspA1I
29	GCCGGC	6	blunt	1	NaeI
29	GCCGGC	6	blunt	1	PdiI
18	GGYRCC	6	five_prime	1	AccB1I
334	GTMKAC	6	five_prime	1	AccI
629	GGATC	5	five_prime	1	AclWI
106	RAATTY	6	five_prime	1	AcsI
194	ACTAGT	6	five_prime	1	AhlI
622	GTCTC	5	five_prime	1	Alw26I
629	GGATC	5	five_prime	1	AlwI
558	GCWGC	5	five_prime	1	ApeKI
106	RAATTY	6	five_prime	1	ApoI
18	GGYRCC	6	five_prime	1	BanI

570	GCAGC	5	five_prime	1	BbvI
194	ACTAGT	6	five_prime	1	BcuI
67	GCTNAGC	6	five_prime	1	BlpI
67	GCTNAGC	6	five_prime	1	Bpu1102I
622	GGTCTC	6	five_prime	1	BsaI
27	RCCGGY	6	five_prime	1	Bse118I
570	GCAGC	5	five_prime	1	BseXI
18	GGYRCC	6	five_prime	1	BshNI
183	GGGAC	5	five_prime	1	BslFI
183	GGGAC	5	five_prime	1	BsmFI
622	GTCTC	5	five_prime	1	BsmAI
622	GGTCTC	6	five_prime	1	Bso31I
214	GGGCCC	6	five_prime	1	Bsp120I
67	GCTNAGC	6	five_prime	1	Bsp1720I
183	GGGAC	5	five_prime	1	BspLU11III
629	GGATC	5	five_prime	1	BspPI
18	GGYRCC	6	five_prime	1	BspT107I
622	GGTCTC	6	five_prime	1	BspTNI
27	RCCGGY	6	five_prime	1	BsrFI
27	RCCGGY	6	five_prime	1	BssAI
299	CTCTTC	6	five_prime	1	Bst6I
622	GTCTC	5	five_prime	1	BstMAI
570	GCAGC	5	five_prime	1	BstV1I
67	GCTNAGC	6	five_prime	1	CelII
27	RCCGGY	6	five_prime	1	Cfr10I
299	CTCTTC	6	five_prime	1	Eam1104I
299	CTCTTC	6	five_prime	1	EarI
622	GGTCTC	6	five_prime	1	Eco31I
183	GGGAC	5	five_prime	1	FaqI
334	GTMKAC	6	five_prime	1	FblI
337	GGATG	5	five_prime	1	FokI
570	GCAGC	5	five_prime	1	Lsp1109I
27	GCCGGC	6	five_prime	1	MroNI
27	GCCGGC	6	five_prime	1	NgoMIV
122	GTSAC	5	five_prime	1	NmuCI
214	GGGCCC	6	five_prime	1	PspOMI
194	ACTAGT	6	five_prime	1	SpeI
512	WGTACW	6	five_prime	1	TatI
558	GCWGC	5	five_prime	1	TseI
122	GTSAC	5	five_prime	1	Tsp45I
106	RAATTY	6	five_prime	1	XapI
334	GTMKAC	6	five_prime	1	XmiI
280	CTGAAG	6	three_prime	1	AcuI
218	GGGCCC	6	three_prime	1	ApaI
437	GGTGA	5	three_prime	1	AsuHPI
218	GKGCMC	6	three_prime	1	BaeGI
640	CTGGAG	6	three_prime	1	BpmI
330	GGATG	5	three_prime	1	BseGI
58	CTCAG	5	three_prime	1	BseMII
218	GKGCMC	6	three_prime	1	BseSI
59	CTCAG	5	three_prime	1	BspCNI
330	GGATG	5	three_prime	1	BstF5I
135	RCATGY	6	three_prime	1	BstNSI
218	GKGCMC	6	three_prime	1	BstSLI
330	GGATG	5	three_prime	1	BtsCI
280	CTGAAG	6	three_prime	1	Eco57I
137	ATGCAT	6	three_prime	1	EcoT22I
640	CTGGAG	6	three_prime	1	GsuI
437	GGTGA	5	three_prime	1	HphI
102	CGWCG	5	three_prime	1	Hpy99I
288	CCTTC	5	three_prime	1	HpyAV
137	ATGCAT	6	three_prime	1	Mph1103I
137	ATGCAT	6	three_prime	1	NsiI

135	RCATGY	6	three_prime	1	NspI
135	RCATGY	6	three_prime	1	XceI
137	ATGCAT	6	three_prime	1	Zsp2I
21, 403	CCWGG	5	five_prime	2	AjnI
436, 457	ATTAAT	6	five_prime	2	AseI
82, 615	CCSGG	5	five_prime	2	AsuC2I
82, 615	CCSGG	5	five_prime	2	BcnI
196, 381	GCATC	5	five_prime	2	BmsI
82, 615	CCSGG	5	five_prime	2	BpuMI
23, 405	CCWGG	5	five_prime	2	BseBI
309, 470	CCCAGC	6	five_prime	2	BseYI
23, 405	CCWGG	5	five_prime	2	Bst2UI
23, 405	CCWGG	5	five_prime	2	BstNI
23, 405	CCWGG	5	five_prime	2	BstOI
214, 215	RGGNCCY	6	five_prime	2	DraII
214, 215	RGGNCCY	6	five_prime	2	EcoO109I
495, 548	CAGCAG	6	five_prime	2	EcoP15I
21, 403	CCWGG	5	five_prime	2	EcoRII
196, 381	GCATC	5	five_prime	2	LweI
23, 405	CCWGG	5	five_prime	2	MvaI
82, 615	CCSGG	5	five_prime	2	NciI
436, 457	ATTAAT	6	five_prime	2	PshBI
21, 403	CCWGG	5	five_prime	2	Psp6I
21, 403	CCWGG	5	five_prime	2	PspGI
196, 381	GCATC	5	five_prime	2	SfaNI
436, 457	ATTAAT	6	five_prime	2	VspI
87, 218	GRGCYC	6	three_prime	2	BanII
57, 91	TGANNNNNNTCA	6	three_prime	2	BdaI
637, 640	GAGGAG	6	three_prime	2	BseRI
87, 218	GDGCHC	6	three_prime	2	Bsp1286I
87, 218	GRGCYC	6	three_prime	2	Eco24I
280, 640	CTGRAG	6	three_prime	2	Eco57MI
87, 218	GRGCYC	6	three_prime	2	EcoT38I
87, 218	GRGCYC	6	three_prime	2	FriOI
313, 474	CCCAGC	6	three_prime	2	GsaI
315, 349	GAYNNNNNRTC	6	three_prime	2	HaeIV
87, 218	GDGCHC	6	three_prime	2	MhlI
87, 218	GDGCHC	6	three_prime	2	SduI
575, 601	GACCGA	6	three_prime	2	TaqII
341, 477	ATGAA	5	three_prime	2	TspDTI
273, 286, 409	GAAGA	5	three_prime	3	MboII
308, 341, 346, 554	CCCGC	5	five_prime	4	FauI
308, 341, 346, 554	CCCGC	5	five_prime	4	SmuI
316, 348, 619, 651	GAYNNNNNVTC	6	three_prime	4	Hin4I

These observations indicate that many type II restriction enzymes are indeed evolutionary related. Analyses of the sequences in greater detail suggest that bacteria may have obtained genes encoding these enzymes from other species by horizontal gene transfer, the passing between species of pieces of DNA (such as plasmids) that provide a selective advantage in a particular environment. In this study, the restriction enzymes recognize specific sequences 5 to 6 base pairs long in range 1-4 cut positions. These restriction sites are typically pallindromic - they read identically on both strands. The fragment lengths produced in the digestion reactions can be used to determine the species *O. nebulosus* from which the DNA sample was prepared, using the restriction fragment length polymorphism (RFLP) pattern matching software containing a database of experimentally-derived RFLP patterns from commercially relevant fish species (Formosa et al 2010).

The data obtained in this present work, indicate hundreds restriction enzymes that can be used to evaluate variation in COI gene fragment of mtDNA of *O. nebulosus* and serve as markers for population analyses. This practice can promote contact between the two subspecies and lead to gene flow and development of secondary hybrid populations. The development of reliable genetic markers will be of significant benefit in studies where the identification of maternal origin in commercial populations or of subspecies in natural populations is required.

Ricefishes are taxonomically classified into the Adrianichthyidae family, an indigenous family in Asia, composed of four genera, namely *Oryzias* with 20 species, *Adrianichthys* with two species, *Horaichthys* with one species and *Xenopoecilus* with three species (Nelson 2006). Most of the paddy fish distribution area is recorded in Sulawesi including Central Sulawesi. Paddy fish species in Central Sulawesi spread over Lake Lindu and Lake Poso. The endemic species of Lake Poso are *Adrianichthys kruyti*, *A. oophorus*, *A. poptae*, *A. roseni*, *O. nigrimas*, *O. orthognathus* and *O. nebulosus* (Parenti et al 2013).

The phylogenetic trees were clearly established among the species, under one node similar species were clustered while under the separate nodes dissimilar species were clustered. Ward et al (2005), stated based on the molecular and traditional methods of species identification and phylogenetic relationship are mostly concordant. Lievens et al (2001) stated also the intraspecific homogeneity level and interspecific heterogenenity level of species identification was judged by the molecular method. Zou et al (2011) demonstrated the effectiveness of the monophyly-based method for species identification in different taxonomic levels use COI as the ideal gene for barcoding. The monophyly-based method on a phylogenetic tree, can be used to flag species.

Conclusions. This study has determined that both samples of ricefish came from one species, *O. nebulosus*. Nucleotide composition of COI gene of ricefish in Lake Poso varied greatly, ranging from 40.8 to 54.8% of GC content. The COI gene fragment of ricefish differ at about 17 in 652 bases sites in two haplotypes. We identified 217 codons and found 170 cut positions from two sequences of our samples with varied site length, range 5-6 nucleotides. In the phylogenetic tree, both samples formed a similar clade since they are genetically the same and evolved from the same ancestor. They also formed a same clade with ricefish *O. nebulosus*.

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