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DNA BARCODING USING COI GENE SEQUENCES OF WILD BETTA FIGHTING FISH FROM INDONESIA: PHYLOGENY, STATUS AND DIVERSITY

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ABSTRACT

The wild betta fish is a potential ornamental fish export commodity normally caught by traders or hobbyists in the wild. However, the population of wild betta in nature has declined and become a threat for their sustainability. This research was conducted to analyze the genetic diversity, phylogenetic relationships, and molecular identification through DNA COI gene sequence of Indonesian wild betta fish. A total of 92 wild betta fish specimens were collected in this study. Amplification of COI genes was carried out using Fish F1, Fish R1, Fish F2, and Fish R2 primers. The genetic diversity and phylogenetic relationships were analyzed using MEGA version 5 software program. Species identification of the specimen was conducted using BLAST program with 98-100% similarity value of the DNA sequences to indicate the same species. Phylogenetic tree construction showed seven monophyletic clades and showed that *Betta smaragdina* was the ancestral species of genus Betta in Indonesian waters. Genetic distance among species ranged from 0.02 to 0.30, whereas intra-species genetic distance ranged from 0 to 6.54.

Keywords: Wild Betta; Indonesian Waters; DNA Barcoding; Ornamental Fish

INTRODUCTION

Fighting fish is a common name for the fish of genus *Betta* (Tan & Ng, 2005a). The mapping of species shows that betta fish can be found in all over Southeast Asia, including Cambodia, Indonesia, Laos, Malaysia, Singapore, Thailand, and Vietnam (Witte & Schmidt, 1992; Tan & Ng, 2005ab; Schindler & Linke, 2013). Betta fish is classified into two major groups based on its caring for fertilized eggs and newly hatched fry. The first group is characterized by the male fish builds a bubble as a temporary shelter on the water surface to place the fertilized eggs, termed bubblenester. Moreover, the second group is distinguished by the male protects the fertilized eggs and the hatchlings in its mouth until release or called mouthbrooder (Panijpan *et al*., 2014).

As ornamental fish, there are several selling point characters of betta fish demanded by consumers in the market, such as fin shape, color brightness, and fighting ability (fighting fishes). The male fish from bubblenester group is normally found to have brighter color compared to the male fish from mouthbrooder group, except for species like Betta *macrostoma* and Betta *coccina*. Furthermore, it is easier to identify betta fish from bubblenester group for its fin shape as in *Betta splendens, Betta imbellis, Betta smaragdina, Betta mahachaiensis, Betta coccina,* and *Betta livida* (Panijpan *et al*., 2014).

Out of 75 betta fish species registered in fishbase.org and ITIS.gov, more than two-thirds of them inhabit Indonesian waters, particularly in Kalimantan and Sumatra Islands (Tan & Ng, 2005a). The high diversity of betta fish is due to its varied habitats, such as extreme waters with a pH=3, e.g. peatlands. Moreover, limited migration distance and unconnected habitat are other factors causing high diversity and endemicity level of the genus *Betta* (Kottelat & Ng, 1994).

Current identification of betta fish species is mostly based on morphological characters, including color patterns and appearances. Eventually, identification based on color characters often has limitations since preserved fish do not show colors, similar things occur when the maintenance environment is not suitable, causing the absence of color appearance in betta fish. The appearance of color in betta fish is strongly influenced by several factors, such as fish maturity, gonad maturity or reproductive cycle, and geographical conditions (Tan & Ng, 2005ab).

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Taxonomic revision of betta fish species has been conducted by Tan & Ng (2005) who revised the taxonomy of 23 betta fish species originated from Southeast Asian waters. Schindler & Schmidt (2006) reclassified six betta fish species from Thailand and Kowasupat *et al*. (2012) also performed species revision through molecular approach. Betta fish from mouthbrooder group has the most member, yet identification of 35 members of mouthbrooder group only 3 species clearly identified based on fin shape and color, namely *B. chanoides, B. unimaculata*, and *B. macrostoma* (Panijpan *et al*., 2014), while the other 32 species were hard to identify through morphological character. The mouthbrooder group normally has dull or grey color if disturbed or experiencing physiological change, such as replacement of fish maintenance tank or other stresses. Several morphological characters of the mouthbrooder group often result in confusion, including body color that quickly becomes dark, causing most fish in this species to have the same color as well as faded or invisible elongated and transverse bars in the fish body that also leads to obstacles in identification.

In addition, it is difficult to distinguish among species on the bubblenester group through morphological approach also, thus species identification became a major problem in the areas where this group is mostly found, such as in Thailand (Kowasupat *et al*., 2014). Therefore, after 2012, betta fish identification in Thailland was done through molecular approach, namely DNA barcoding using *COI* gene on species of *B. splendens, B. smaragdina, B. imbellis, B. mahachaiensis,* and *B. siamorientalis* (Panijpan *et al*., 2014). Ever since DNA barcoding succeeded to identify many fish species (Ward *et al.,* 2009; Zemlak *et al.,* 2009; Pereira *et al.,* 2013;), re-revision of existing fish species continues to be done.

Data and information on betta fish existence in Indonesia and other countries are still limited (Monvises *et al*., 2009). On the other hand, high capture rate of betta fish in the wild and conversion of habitat function into settlement and plantation threaten betta fish sustainability in nature (Chan, 2015). This study aimed to analyze genetic diversity and phylogenetic relationship of betta fish species in Indonesia based on *COI* gene.

MATERIALS AND METHODS

Specimens of betta fish used in this study were obtained from direct catch, collectors, and exporters of ornamental fish. A total of 98 wild betta specimens were collected from Kalimantan and Sumatra (a list of the specimens used and the location of capture was presented in Appendix 1). Moreover, interviews related to sampling location and determination of trade name for fish was also performed. For three months, a total of 92 betta fish samples were collected, consisting of 24 tradable species. Samples of collection were transported to the Research Institute of Ornamental Fish Culture for molecular analysis. DNA was collected from caudal fin tissue by cutting the tip of the caudal fin fish and placing it into a tube containing alcohol 70%. Alcohol replacement was applied for long-term analysis of tissue.

Extraction and Amplification of DNA

Total DNA (genome) was isolated from caudal fin tissue. DNA was extracted through spin-column method referred to the protocol, according to the manufacturer recommendation, is the following: approximately 25 mg of caudal fin tissue was incubated with lysis buffer and Proteinase K at 60^{o}C for 1 h or until lysis was complete, and then incubated with the second lysis buffer at 70^{o}C. The next steps were binding DNA with ethanol and separating DNA from undesirable material using a GD column. The last step was washing the DNA twice with different wash buffers. The genomic DNA was eluted by nuclease-free water. The quantity of DNA was checked by electrophoresis. Extracted DNA was migrated on 1.2% agarose gel in solution of 1xTAE using SYBR safe DNA gel stain. To observe the quality of extracted DNA, the DNA was visualized using blue light transilluminator (λ = 250 nm). Total DNA extracted was further used as a DNA template for amplification process through the process of Polymerase Chain Reaction (PCR). Primers used in the PCR process are listed in Table 1.

Table 1. List of primers used in this study

Primer	Sequence	Gene	PCR Product	Reference
Fish F1	TCA-ACC-AAC-CAC- AAA-GAC- ATT GGC- AC	*Cytochrome Oxidase 1 (COI)*	680 bp	Ward *et al.,* 2005
Fish R1	TAG- ACT- TCT- GGG- TGG-CCA- AAG AAT- CA			
Fish F2	TCG-ACT-AAT-CAT-AAA-GAT-ATC-GGC-AC	*Cytochrome Oxidase 1 (COI)*	680 bp	Ward *et al.,* 2005
Fish R2	AC-TCA-GGG-TGA-CCG-AAG-AAT-CAG-AA			

The PCR applied in this study was pre-PCR (94ºC, 5 minutes), followed by denaturation (94ºC, 30 seconds), annealing (52ºC, 30 seconds), and extension (72ºC, 30 seconds) of 35 cycles, and post-PCR (72ºC, 5 minutes). Nucleotide sequences produced from PCR were further read using Applied Biosystems through Macrogen Korea.

Data Analyses

Phylogenetic Relationship and Genetic Diversity

According to Panijpan *et al.* (2014), *Trichopsis vittata* (KT250368.1) was used as an outgroup. A total of 92 *COI* sequences obtained from this study and 17 *COI* sequences from Genbank (there are ; KM485312.1, KM485402.1, KM485405.1, KM485407.1, KM485409.1, GQ911721.1; KM485461.1, KM485316.1, JN646094.1, KM485315.1, KM485320.1, GQ911722.1, KM485452.1, GQ911983.1, GQ911838.1, KM485443.1, KM485460.1) used as taxonomic references on phylogenetic trees construction. All of the DNA sequences were aligned using the Clustal W software and later checked by eye. Sequence regions in which the site homology was questionable in the alignment were omitted from the analysis. The molecular phylogenetic tree was constructed with the Molecular Evolution Genetic Analysis (MEGA ver. 5.1) software package (Tamura *et al.*, 2011). Neighbor-Joining (NJ) methods were chosen and the reliability of each branch was assessed by bootstraps with 1000 replications.

Nucleotide substitution was analyzed by the Hasegawa-Kishino-Yano (HKY) method using Maximum Likelihood by the following equation *HKY + G + I*; *G* is the rate of gamma evolution between sites, and *I* is the rate of evolution between sites that does not change. The ratio of transition and transversion substitution rates was calculated by the following equation $R = [A*G*k_1 + T*C*k_2]/[(A+G)*(T+C)]$, k_1 = substitution for purines and k_2 = substitution for pyrimidines (Tamura *et al.*, 2004). Genetic distance between species was measured using the model of Maximum Composite Likelihood according to Tamura & Nei (2004).

Species Status

Analysis of *COI* gene sequence was done for identification and barcoding specimens using BLAST method or sequence alignment according to the sequence in GenBank. The results of nucleotide sequences of *COI* gene were adjusted to the database of nucleotide sequences stored in GenBank, i.e. the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/blast) through BLAST program. Based on the percentage of homologous side of nucleotide sequences of mitochondrial-DNA *COI* gene of the species and the search result, percentage of similarity or identity similarity was obtained. An identity similarity value between 97-100% indicates the two sequences are from the same species. To confirm the species status of each specimen that has been determined through the BLAST programme, the results were traced through www.fishbase.org, www.ITIS.gov, and other scientific publications.

RESULTS AND DISCUSSION

Results

Analysis of Sequence

The amplification results of the *COI* gene in this study were shown in Figure 1. Based on the analyses of *COI* gene nucleotide sequences, the synthesis of phylogenetic relationships, betta fish species status, and intra-species diversity are presented and discussed below.

A total of 92 nucleotide sequences in this study were successfully read and aligned for further analyses. The analysis of 92 nucleotide sequences according to Tamura-Nei Model showed undistributed pattern of each nucleotide, namely Adenine (A) 25.08%, Thymine (T) 30.54%, Cytosine (C) 18.60%, and Guanine (G) 25.79% with pattern as follows: T > G > A > C. Several species had different pattern from the pattern obtained in this study, such as common

carp with pattern of T > C > A > G (Mohanty *et al*., 2013) and kalabau fish with pattern of: T > A > C > G (Asiah *et al.,* 2019). Estimation of substitution pattern between nucleotides was determined using the method of Maximum Composite Likelihood as presented in Table 2. The ratios of transition and transversion substitution were k_1 = 4.923 (for purin) and k_2 = 6.506 (for pyrimidine), with bias value of R = 3.016.

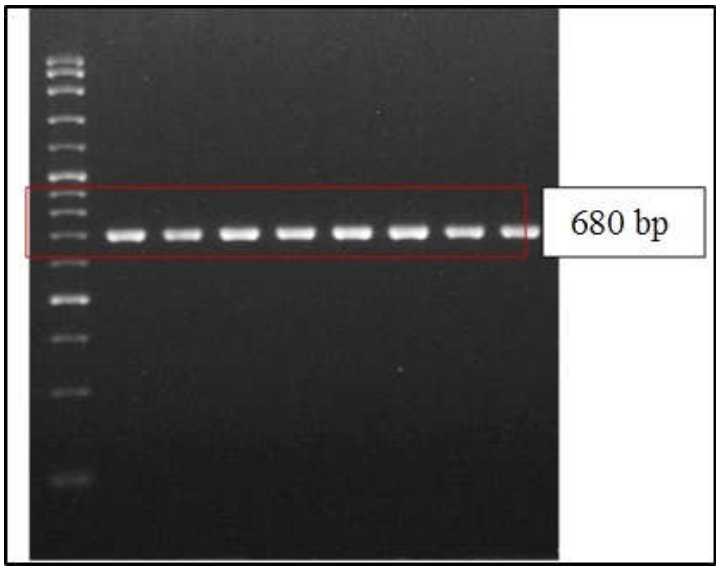

Figure 1. The bands of *COI* gene amplification.

Table 2. The nucleotide substitution Estimated*)

	A	T	C	G
A	-	*3.91*	*3.3*	**11.72**
T	*3.21*	-	**21.47**	*2.38*
C	*3.21*	**25.43**	-	*2.38*
G	**15.8**	*3.91*	*3.3*	-

*Note *) based on Maximum Composite Likelihood transition substitution: (**bold**),transversion substitution: (italic)*

Measurement result of genetic distance shows the genetic distance between species ranged from 0.02 to 0.30 (Table 3). The closest genetic distance was found between *B. rubra* and *B. dennisyongi.* Before 2013, both species were categorized as one species, i.e. *B. rubra*, yet following re-description conducted by Tan (2013), *B. rubra* was established to be different from *B. dennisyongi*. The basic differences between both species include the operculum pattern, head width, and bar pattern in fish chin. Furthermore, the populations of these two species was separately described since the northern part of West Sumatra was found to be the distribution area of *B. rubra*, whereas *B. dennisyongi* occupies the western North Sumatra. This finding also depicted that *B. rubra* was included in another group that was separated from the previous group, namely the group of *B. foerschi*.

Besides *B. rubra* and *B. dennisyongi*, the closest distance is also between *Betta sp.* and *B. pugnax that* is supported by a phylogenetic tree that shows both species in the same tree branch. While the farthest genetic distance is between *B. bellica* and *B. uberis*, the construction of phylogenetic trees between all of specimens indicated that *B. uberis* has the farthest distance or branch compared to all betta fish species.

The standard error estimates are shown above the diagonal in Table 3. Analyses were conducted using the Maximum Composite Likelihood model.

Table 3. The estimates of Genetic Distance between betta fish species that inhabit Indonesian waters.

B. belica		0,03	0,04	0,03	0,04	0,03	0,04	0,03	0,04	0,03	0,04	0,04	0,03	0,04	0,04	0,03	0,04	0,03	0,04	0,04	0,04	0,03	0,04	0,03	0,03	0,04	0,04
B. albimarginata	0,23		0,03	0,01	0,03	0,03	0,03	0,02	0,02	0,02	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,02	0,02	0,03	0,03	0,02	0,03	0,02
B. burdigala	0,25	0,19		0,03	0,02	0,04	0,03	0,03	0,03	0,03	0,04	0,02	0,03	0,03	0,03	0,03	0,04	0,04	0,03	0,03	0,03	0,03	0,01	0,04	0,03	0,02	0,03
B. channoides	0,24	0,07	0,20		0,03	0,03	0,03	0,02	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03
B. coccina	0,25	0,20	0,14	0,21		0,04	0,03	0,03	0,03	0,03	0,03	0,02	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,02	0,04	0,03	0,00	0,03
B. ideii	0,24	0,22	0,24	0,21	0,25		0,03	0,03	0,03	0,03	0,02	0,03	0,02	0,03	0,03	0,02	0,02	0,02	0,02	0,03	0,03	0,03	0,03	0,03	0,03	0,04	0,03
B. dennisyongi	0,26	0,20	0,19	0,19	0,19	0,23		0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,01	0,03	0,03	0,03	0,03	0,03	0,03	0,03
B.dimidiata	0,24	0,15	0,21	0,15	0,23	0,24	0,22		0,02	0,02	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,02	0,02	0,03	0,03	0,02	0,03	0,02
B. enisae	0,29	0,16	0,19	0,18	0,21	0,24	0,22	0,13		0,02	0,03	0,03	0,03	0,03	0,02	0,03	0,03	0,03	0,03	0,03	0,02	0,02	0,03	0,03	0,02	0,03	0,02
B. fusca	0,24	0,17	0,21	0,18	0,22	0,23	0,22	0,12	0,13		0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,02	0,01	0,03	0,03	0,02	0,03	0,02
B. gladiator	0,30	0,20	0,25	0,20	0,25	0,14	0,24	0,22	0,18	0,22		0,03	0,02	0,03	0,03	0,01	0,00	0,01	0,01	0,03	0,03	0,03	0,04	0,03	0,03	0,03	0,03
B. hendra	0,26	0,19	0,13	0,19	0,13	0,23	0,20	0,20	0,19	0,21	0,25		0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,02	0,04	0,03	0,02	0,03
Betta sp	0,25	0,21	0,24	0,21	0,22	0,15	0,22	0,20	0,20	0,19	0,09	0,24		0,03	0,03	0,01	0,01	0,01	0,01	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03
B.miniopinna	0,27	0,18	0,19	0,19	0,18	0,20	0,21	0,21	0,18	0,20	0,22	0,17	0,23		0,01	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03
Betta persephone	0,26	0,18	0,18	0,19	0,18	0,20	0,21	0,22	0,17	0,21	0,22	0,17	0,23	0,02		0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03
Betta unimaculata	0,25	0,21	0,24	0,21	0,24	0,13	0,23	0,21	0,20	0,22	0,09	0,24	0,06	0,22	0,22		0,01	0,01	0,01	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03
B. ocellata	0,30	0,20	0,25	0,20	0,25	0,14	0,24	0,22	0,18	0,22	0,00	0,24	0,09	0,22	0,22	0,09		0,01	0,01	0,03	0,03	0,03	0,04	0,03	0,03	0,03	0,03
B. pollifina	0,25	0,20	0,24	0,20	0,23	0,14	0,23	0,20	0,21	0,20	0,09	0,24	0,06	0,21	0,20	0,05	0,09		0,02	0,03	0,03	0,03	0,03	0,03	0,03	0,03	0,03
B. patoti	0,26	0,22	0,24	0,21	0,24	0,12	0,24	0,21	0,20	0,22	0,08	0,24	0,09	0,20	0,21	0,09	0,08	0,09		0,03	0,03	0,03	0,04	0,03	0,03	0,03	0,03
B. rubra	0,25	0,20	0,20	0,19	0,19	0,22	0,02	0,22	0,22	0,22	0,24	0,20	0,22	0,21	0,21	0,22	0,24	0,22	0,24		0,03	0,03	0,03	0,03	0,03	0,03	0,03
B. raja	0,25	0,16	0,19	0,18	0,21	0,22	0,21	0,12	0,13	0,12	0,19	0,20	0,18	0,18	0,19	0,20	0,18	0,19	0,19	0,21		0,02	0,03	0,03	0,00	0,03	0,00
B. stigmosa	0,25	0,16	0,21	0,17	0,22	0,24	0,22	0,12	0,13	0,02	0,21	0,21	0,19	0,21	0,21	0,22	0,21	0,20	0,22	0,21	0,12		0,03	0,03	0,02	0,03	0,02
B. uberis	0,26	0,19	0,04	0,20	0,13	0,24	0,19	0,21	0,20	0,22	0,26	0,13	0,24	0,18	0,17	0,25	0,26	0,25	0,26	0,19	0,20	0,22		0,04	0,03	0,02	0,03
B. smaragdina	0,24	0,23	0,24	0,21	0,26	0,24	0,23	0,23	0,22	0,25	0,24	0,26	0,22	0,24	0,23	0,23	0,24	0,22	0,22	0,23	0,22	0,25	0,25		0,03	0,04	0,03
B. pugnax	0,25	0,16	0,20	0,18	0,21	0,21	0,21	0,12	0,13	0,12	0,19	0,20	0,18	0,18	0,19	0,20	0,18	0,18	0,19	0,20	0,02	0,12	0,21	0,22		0,03	0,00
B. livida	0,25	0,20	0,14	0,21	0,02	0,25	0,19	0,22	0,21	0,22	0,24	0,13	0,22	0,18	0,18	0,23	0,24	0,23	0,24	0,19	0,20	0,22	0,13	0,25	0,20		0,03
Betta spp	0,25	0,16	0,20	0,18	0,21	0,22	0,21	0,12	0,13	0,12	0,18	0,20	0,18	0,18	0,19	0,20	0,18	0,19	0,19	0,20	0,01	0,12	0,21	0,22	0,02	0,20	

Phylogenetic Relationship of Betta Fish Species

This is the first study that constructed a phylogenetic relationship among wild betta fish that inhabit Indonesian waters based on genetic data. The phylogenetic tree among betta fish species is presented in Figure 2. The values of branches in the determination of clade phylogenetic tree depend on permutation nonparametric Maximum Likelihood bootstrapping. Bootstrap value higher than 90% was able to group species into one clade with one ancestral species. Species of *Betta sp, B. pugnax, B. raja, B. dimidiata, B. fusca, B. stigmosa*, and *B. enisae* were classified as the members of Clade 1. Moreover, the members of Clade 2 consisted of *B. albimarginata* and *B. channoides;* Clade 3 consisted of *B. rubra* and *B. dennisyongi*; Clade 4 consisted of *B. miniopina, B. burdigala, B. uberis, B. livida,* and *B. coccina;* Clade 5 consisted of *B. ideii, B. unimaculata, Betta sp-1, B.pollifina, B. patoti, B. gladiator,* and *B. ocellata.* Furthermore, other species formed their own clade that consisted of one species, namely clade of *B. bellica,* and *B. smaragdina.* The phylogenetic showed that phylogenetic genetic relationships among species was able to form one clade (Figure 2). Species with different phenotypes in terms of morphological characteristics will be grouped under the same clade if they have similar genetic information (Panijpan *et al.,* 2014).

Clade 1 and Clade 5 obtained from the phylogenetic construction of *COI* gene in this study have more members compared to the other two clades. Phylogenetic relationship based on the *COI* gene was similar to a previous study that was conducted by Panijpan *et al.* (2014). Out of 26 species examined by Panijpan *et al.* (2014), two main clades consisting of 5-6 species were obtained, while other clades consisted of 1-2 species. Generally, seven phylogenetic clades that formed in this study indicated the beta group representative, clade 1 represented the *Betta pugnax* Group, clade 2 represented the *Betta albimarginata* Group, clade 3 represented the *Betta foerschi* Group, clade 4 represented the *Betta coccina* Group, clade 5 represented the *Betta unimaculata* Group and two last group are *Betta bellica* Group and *Betta splenden* Group.

Phylogenetic construction in this study placed four species that were not successfully identified by using morphological characters, they were classified as the members of clades 1 and 5. *Betta sp-1, Betta sp-2, Betta sp-3* are members of clade 1 (*Betta pugnax* Group), while *Betta sp-4* are members of clade 5 (*Betta unimaculata* Group). A certainty of species names, given after being aligned with the nucleotide sequence that deposit in GenBank by using BLAST program, with the index of similarity between 98-100% have the same name as species in GenBank but using "confer (*cf*)" due to genetically the same only .

The construction of phylogenetic trees using *Trichopsis vittata* as an outgroup showed that *B. smaragdina* was the ancestral species of genus *Betta* in Indonesian waters. Similar result was also found by Kowasupat *et al.* (2014) that *B. smaragdina* was the ancestral species of bubblenested group and *B. macrostoma* was the ancestral species of mouthbrooder group, where the phylogenetic relationship was constructed based on *COI* and *ITS1*. Panijpan *et al.* (2014) showed that the ancestral species of genus *Betta* was *B. macrostoma* and *B. bellica* was the generation after *B. macrostoma*. This study did not include *B. macrostoma* sequence in the analysis process since this species does not live in Indonesian waters, but the same results obtained

in this study were the species after the basal species is *B. bellica*. A study related to the evolution process of mouthbrooding betta fish was conducted by Rüber *et al*. (2004) indicated that *B. macrostoma* as the ancestral of mouthbrooding betta fish, followed by *B. unimaculata* and *B. ocellata*. In detail explanation, the evolution processes of bubblenested and mouthbrooder betta fish were not significantly correlated. The form of parental care in *Betta* is correlated to offspring size only, with mouthbrooders having significantly bigger offspring than bubblenesters, but is not correlated to egg volume, clutch size, and brood-care duration, nor with any of the three habitat variables tested and their evolution processes (Rüber *et al.,* 2004).

In general, the construction of phylogenetic trees in this study was in line with phylogenetic trees constructed in the previous studies conducted by Rüber *et al. (*2004) and Panijpan *et al.* (2014) with the addition of several species endemic to Indonesian waters. The similarity between both study results included the grouping of several species in one cluster, such as a clade consisted of *B. pugnax, B. anabatoides, B. fusca,* and *B. dimidiata* and a clade consisted of *B. unimaculata, B. patoti,* and *B. ocellata*. Phylogenetic relationship among species in one clade is supported by data and extremely small genetic distance of 0.02 as well as the same ancestral species in one cluster of phylogenetic trees.

Mueller *et al.* (2006) mentioned that a clade with ratio of inter and intra-clade higher than 10% might be categorized as a new clade, while intra-species differences of higher than 3% could show speciation or the formation of new species. In this study, there were five polyphyletic clades and three monophyletic clades, namely *B. miniopina, B. bellica,* and *B. smaragdina*. The fish in a monophyletic group require great attention considering the conservation since these species are vulnerable to extinction. Moreover, the fish included into a polyphyletic group will easily obtain gene flow from their closest relative, hence this condition will maintain the sustainability (fitness) of betta fish in nature (Frankham, 2003).

Status of Betta Fish Species

Re-identifying wild betta fish that inhabit Indonesian waters by using a molecular approach became the main reason for this study. The specimen names in this study were according to the common names by ornamental fish traders and hobbies. Alignment by BLAST program allows to detect the identical or similarity of a sequence that was found in this study. Alignment results between nucleotide sequences in present study and nucleotide sequences existing in the GenBank library were presented in Appendix 2. Out of 680 base pairs obtained, only 648 bp were analyzed further to reduce doubt on the result of sequence analysis. The list in Appendix 2 shows the number of nucleotides matched between the nucleotide sequence in present study and existing in GenBank library. Percentage identity obtained was in a range of 89-100 percent.

Out of 62 sequences matched to the data in GenBank through the BLAST program, there were 34 sequences includes 14 species high similarity to the species recorded in GenBank, among others: *B. albimarginata* (2 sequences), *B. coccina* (2 sequences), *B. enisae* (1 sequence), *B. gladiator* (4 sequences), *B. ideii* (3 sequences), *B. ocellata* (2 sequences), *B. patoti* (3 sequences), *B. rubra* (3 sekuen), *B. stigmosa* (2 sequences), *B. uberis* (2 sequences), *B. unimaculata* (2 sequences), *B. smaragdina* (2 sequences), *B. pugnax* (2 sequences), and *B. bellica* (3 sequences) with percentage identity similarity of sequence in a range of 97-100 percent. This finding was supported by the phylogenetic trees in Figure 2. While the other of 24 sequences includes 9 species were found to have weak similarity to species in the GenBank, there were *B. burdigala, B. channoides, B. dennisyongi, B. fusca, B. hendra, B. miniopina, B. polifina, B. raja,* and *B. livida* with percentage identity similarity of sequence between 89-96 percent. Hereafter four sequences included two morphologically unidentified species have similarity index with *B. anabatoides* and *B. unimaculata*, there are 99% and 94% respectively.

Sequence of *B. hendra* and *B. burdigala* have similarity identity between 90-93% with the sequence of *B. uberis* (access code GQ911983.1), referred to the phylogenetic tree of the three species within the same clade, namely clade 4 (*Betta coccina* Group). According to Schindler & Linke (2013), *B. hendra* is a new species in *Betta coccina* group, geographically living in a close area to *B. uberis* and both species were found to have a similar phenotype. After the revision of taxonomy performed by Schindler & Linke (2013), *B. hendra* was observed to be quite different from *B. uberis* in terms of less number of dorsal fins and brighter green operculum. The results of this study assumed that the data of *COI* gene sequence of *B. hendra* are not yet recorded in GenBank.

Moreover, *B. dennisyongi* was found to have 99% similarity to *B. rubra.* Tan (2013) mentioned that *B. rubra* has similarity both in terms of genetics and morphology to *B. dennisyongi*. Before 2013, both species were categorized as one species of *B. rubra*.

Several specimens were also found to have a weak similarity index with species registered in GenBank included *B. channoides, B. fusca, B. raja*, and *B. pugnax*, with similarity value of DNA sequence of 93-99 %. Generally, naming of specimens is confirmed with species that has the closest phylogenetic relationship or high similarity index sequence. Krauthammer *et al.* (2000) describe BLAST is a system or tool for DNA sequence comparison which automatically identifies gene names, by using database of DNA sequence that deposited in GenBank library. Moreover, Krauthammer *et al*. (2000) show this approach is feasible, the system matches sequence names with a recall of 78.8% and a precision of 71.7%, which includes names that are not part of the system database. BLAST results illustrated that the similarity index of *B. channoides* sequence in this study is closer to the sequence of *B. albimarginata* in GenBank. The international organization of betta fish hobbies (International Betta Congress) have mentions *B. channoides* and *B. albimarginata* are similar species, likewise *B. raja* and *B. pugnax* are similar species (Thorup, 2017).

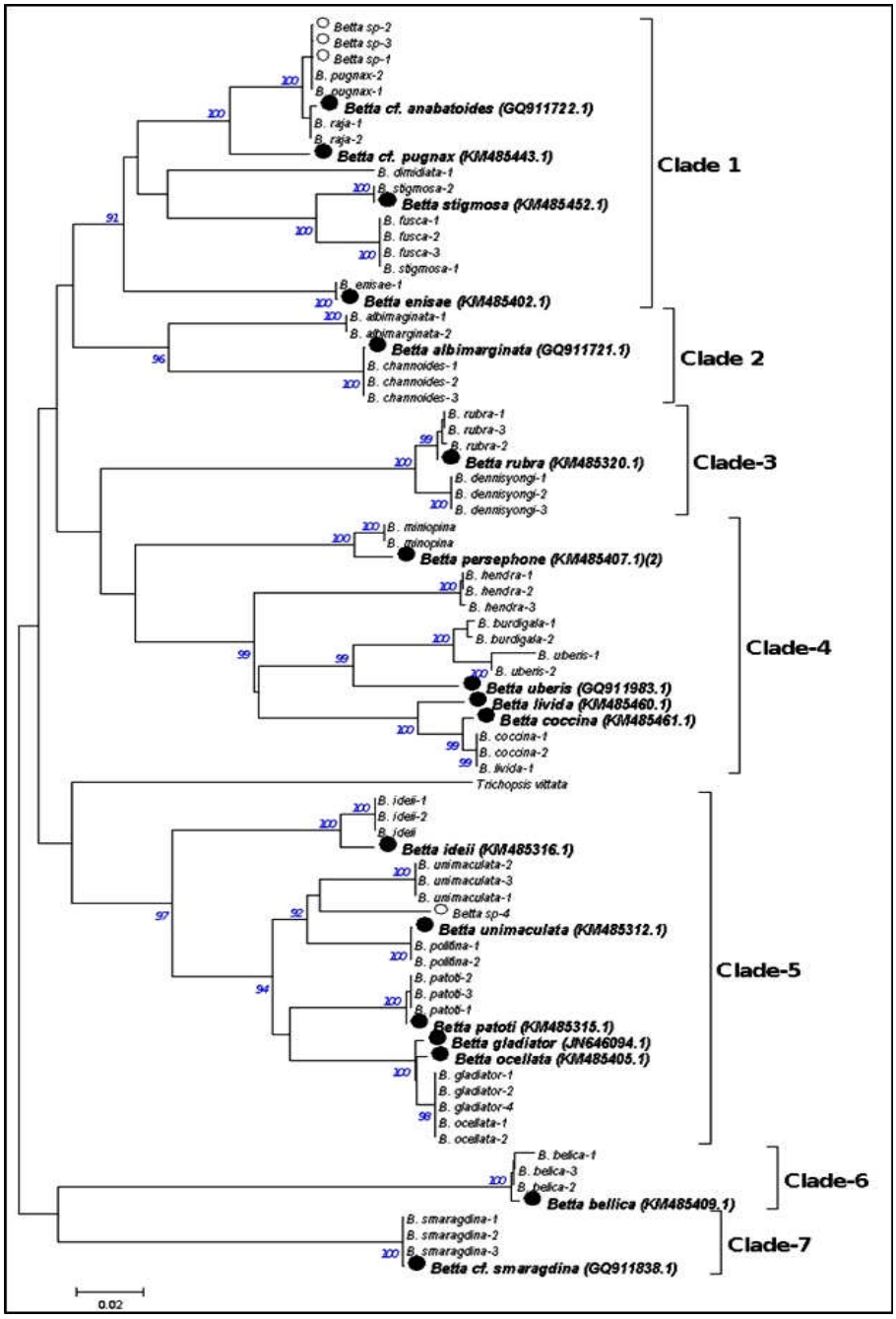

Figure 2. The phylogenetic relationship among betta fish species in Indonesian waters. The tree was constructed based on Neighbor-Joining (NJ) *methods.*

Species *B. miniopinna* was observed to have similarity value of DNA sequence of 98% to species *B. persephone.* Tan & Ng (2005a) reported that *B. persephone* is betta fish endemic to the waters of Johor Malaysia, and its existence in nature is threatened to extinction (Chan, 2015). For the last decade, this species has been included in the Red List of Threatened Species by IUCN (Low, 2019). Similarity of nucleotide sequence between *B. persephone* and *B. miniopinna* inhabit Sumatera waters is expected to be such attractive information considering conservation efforts of this species.

This study presents the DNA sequence *COI* gene as an identification tool. A molecular approach to identify species becomes popular nowadays since the identification based on a morphological approach has a limitation due to convergent and divergent adaptations that lead to changes in the morphological characteristics of fish species (Bingpeng *et al.,* 2018). The different specimen naming on *B. channoides, B. burdigala, B. pallifina, B. fusca, B. raja,* and *B. pugnax* cannot be decided at this time cause need more supports of data to decide, by using other primers, morphological and osteological approaches.ne of characters of the wil bBetta fish commonly inhabit peat waters is a high level of endemicity, so this situation will be a rising level of divergence and convergence of genes for fish adaptation process. The adaptation on gene level will eventually trigger changes at the morphological level, while the morphological changes over a long period of time will create new species or sub-species (Frankham, 2003). Betta fish have a sedentary or non-migratory character, which is the hypothesis of high speciation or species divergence on genus betta fish. The high number of new species found by hobbies or collectors is not followed by the taxonomic side. The high speciation level and limited process of identification or taxonomy in betta fish are the main obstacles in naming these fighting fish.

The challenge of DNA barcoding technique is not only trying to correctly identify the species but also to make and build a standardized global reference library based on the identification of target specimens such as sequences deposited in GenBank (Imtiaz *et al.,* 2017). Nowadays, several researchers recommended utilizing DNA barcoding techniques because it is cost effective, fast, and authentic for species conservation. So, it is most necessary to improve the genetic data of Indonesian betta fish on GenBank to establish the standard global betta fish reference. Besides that, the identification using morphological characters also needs to be updated and improved, especially betta fish which are of economic value and widely traded by collectors and hobbies, but their taxonomic and biological information is very limited.

This study found 5 specimens were included into two species (*Betta sp 1-3* and *Betta sp 4-5*) that were unidentified by morphological approach. The results of phylogenetic relationship analysis and alignment by BLAST program showed *Betta sp1-3* had a highly relationship and similarity to the *B. pugnax* sequence, whereas *Betta sp* 4-5 do not have a highly relationship and similarity to the sequences in GenBank. Based on this study and interviews with the collectors, *Betta sp 4-5* were recommended as new species but need furthure analysis.

Approximately 75 species of betta fish have been registered in Fishbase.org and 27 species of them were only identified after 2000. The revolution in the molecular biology field is expected to identify many new species. Around two-thirds of 75 species members of *Betta* genus inhabit Indonesian waters, and some of them are endemic species. Indonesian waters is considered as the center of origin of betta fish as mentioned by Frankham (2003) with one characteristic of high diversity in the region as in the case of eel fish (Fahmi, 2013).

Intra-species Diversity

The intraspecies diversity was calculated only on species that have more than 3 specimens and measured using Maximum Composite Likelihood model based on the substitution of nucleotides between sequences analyzed (Table 4). According to the result of analysis, several species obtained zero intra-species diversity. It was expected due to the low number of sequences analyzed thus it was difficult to obtain different sites as found in species *B. pollifina*. Moreover, zero value of intra-species diversity between *B. channoides* and *B. coccina* was possibly caused by the situation that fish were produced from the same offspring or spawning in the collecting site. Both species have been successfully cultured, yet their population in nature is extremely low. The highest intra-species diversity was obtained in species *Betta sp 1-3* originated from two different locations, namely Kalimantan and Jambi.

Table 4. Genetic diversity intra-species of betta fish that inhabit Indonesian waters

No	Specimens	Genetic *diversity* intraspecies	standard deviation
1	*Betta sp*	7,288	1,67
2	*B. albimarginata*	0,125	0,01
3	*B. bellica*	0,003	0,00
4	*B. burdigala*	0,165	0,02
5	*B. channoides*	0,000	0,00
6	*B. coccina*	0,004	0,00
7	*B. dennisyongi*	0,000	0,00
8	*B. enisae*	0,003	0,00
9	*B. fusca*	0,051	0,01
10	*B. gladiator*	0,102	0,01
11	*B. hendra*	0,001	0,00
12	*B. livida*	0,032	0,01
13	*B. minopina*	0,000	0,00
14	*B. ocellata*	0,056	0,01
15	*B. patoti*	0,001	0,00
16	*B. pallifina*	0,000	0,00
17	*B. pugnax*	3,018	0,91
18	*B. raja*	0,003	0,00
19	*B. rubra-*	0,002	0,00
20	*B. smaragdina*	0,000	0,00
21	*B. stigmosa*	0,025	0,01
22	*B. uberis*	0,052	0,01

CONCLUSIONS

The analyses of *COI* gene sequence in this study concluded that (a) The ancestral of Indonesian wild betta fish is *B. smaragdina*, (b) 14 species have a high similarity index with sequences deposited in GenBank, 10 species have a weak similarity index, and one species is recommended as a new species, in this study initialed as *Betta sp*. The issues arising from this study should be settled by experts' collaboration (on morphology, molecular genetics, bioinformatics, and taxonomy) in renaming all species on genus *Betta*. The collaboration should make and build a standardized global reference library for identification of wild betta sequence and morphology.

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Appendix 1. List of specimens used in this study

No	Species Group	Species	Classification	Location of sample collection
1	*Betta bellica* group	*B. bellica* Sauvage, 1884	*bubblenester*	Sumatra (Jambi)
2	*Betta pugnax* group	*B. pugnax* Cantor, 1850	*mouthbrooder*	Sumatera (Anambas)
		B. fusca Regan, 1910	*mouthbrooder*	Sumatera (Sumatera Utara)
		B. enisae Kottelat, 1995	*mouthbrooder*	Kalimantan Barat
		B. stigmosa Tan & Ng, 2005a	*mouthbrooder*	Sumatera (Bangka)
		B. raja Tan & Ng, 2005a	*mouthbrooder*	Sumatera (Jambi)
3	*Betta akarensis* group	*B. akarensis* Regan, 1910	*mouthbrooder*	Sumatera (Riau), Kalimantan
4	*Betta unimaculata* group	*B. unimaculata* Popta, 1905	*mouthbrooder*	Kalimantan Timur (Kutai)
		B. patoti Weber de & Beaufort 1992	*mouthbrooder*	Kalimantan Timur
		B. ocellata de Beaufort, 1933	*mouthbrooder*	Kalimantan Timur
		B. pallifina Tan & Ng, 2005a	*mouthbrooder*	Kalimantan Tengah (Barito atas)
		B. ideii Tan & Ng, 2005a	*mouthbrooder*	Kalimantan Selatan
		B. gladiator Tan & Ng, 2005a	*mouthbrooder*	Kalimantan
6	*Betta splendens* group	*B. smaragdina* Ladiges, 1972	*mouthbrooder*	Kalimantan
7	*Betta coccina* group	*B. coccina* Vierke, 1979	*bubblenester*	Sumatra (Riau);
		B. miniopinna Tan & Tan, 1994	*bubblenester*	Sumatera (Riau-Bintan)
		B. livida Ng & Kottelat, 1992	*bubblenester*	Kalimantan Tengah
		B. hendra Schindler & Linke, 2013	*bubblenester*	Palangkaraya, sungai Sebagau
		B. burdigala Kottelat & Ng, 1994	*bubblenester*	Sumatera (Bangka)
8	*Betta foerschi* group	*B. rubra* Perugia, 1893	*mouthbrooder*	Aceh
		B. dennisyongi	*mouthbrooder*	Aceh
9	*Betta albimarginata* group	*B. albimarginata* Kottelat & Ng, 1994	*mouthbrooder*	Kalimantan Tengah
		B. channoides Kottelat & Ng, 1994	*mouthbrooder*	Kalimantan Barat
10	*Betta dimidiata* group	*B. dimidiata* Roberts, 1989	*mouthbrooder*	Kalimantan Barat (Kapuas)

Appendix 2. Results of BLAST gene sequences of *COI* betta fish from Indonesian waters

No	Specimen	Nucleotide analyzed	Species in Genbank	Access Code	Identities Match	Percentage
1	*B.albimarginata-1*	611	*Betta albimarginata*	GQ911721.1	607	99
2	*B.albimarginata-2*	616	*Betta albimarginata*	GQ911721.1	614	99
3	*B. burdigala-1*	615	*Betta uberis*	GQ911983.1	552	93
4	*B. burdigala-2*	615	*Betta uberis*	GQ911983.1	552	93
5	*B. channoides-1*	604	*Betta albimarginata*	GQ911721.1	562	93
6	*B. channoides-2*	615	*Betta albimarginata*	GQ911721.1	571	93
7	*B. channoides-3*	616	*Betta albimarginata*	GQ911721.1	604	98
8	*B. coccina-1*	615	*Betta coccina*	KM485461.1	615	100
9	*B. coccina-2*	619	*Betta coccina*	KM485461.1	616	99
10	*B. dennisyongi-1*	613	*Betta rubra*	KM485320.1	609	99
11	*B. dennisyongi-2*	613	*Betta rubra*	KM485320.1	609	99
12	*B. dennisyongi-3*	615	*Betta rubra*	KM485320.1	603	98
13	*B. dimidiata-1*	612	*Betta krataios*	KM485406.1	602	98
14	*B. dimidiata-2*	617	*Betta krataios*	KM485406.1	605	98
15	*B. enisae*	615	*Betta enisae*	KM485402.1	605	98
16	*B. fusca-1*	458	*B. stigmosa*	KM485451.1	411	97
17	*B. fusca-2*	392	*B. stigmosa*	KM485451.1	350	97
18	*B. fusca-3*	602	*B. stigmosa*	KM485451.1	597	97
19	*B. gladiator-1*	616	*Betta gladiator*	JN646094.1	600	97
20	*B. gladiator-2*	611	*Betta gladiator*	JN646094.1	595	97
21	*B. gladiator-3*	613	*Betta gladiator*	JN646094.1	597	97
22	*B. gladiator 4*	573	*Betta gladiator*	JN646094.1	541	94
23	*B. hendra-1*	617	*Betta uberis*	GQ911983.1	612	90
24	*B. hendra-2*	617	*Betta uberis*	GQ911983.1	612	90
25	*B. hendra-3*	617	*Betta uberis*	GQ911983.1	612	90
26	*B. ideii-1*	610	*Betta ideii*	KM485316.1	545	89
27	*B. ideii-2*	610	*Betta ideii*	KM485316.1	545	89
28	*B. ideii*	613	*Betta ideii*	KM485316.1	548	89
29	*Betta sp-5*	591	*Betta unimaculata*	KM485312.1	577	92
30	*Betta sp-4*	614	*Betta unimaculata*	KM485314.1	602	94
31	*B. miniopinna-1*	586	*Betta persephone*	KM485407.1	549	98
32	*B. miniopinna-2*	330	*Betta persephone*	KM485407.1	330	98
33	*B. ocellata-1*	629	*Betta ocellata*	KM485405.1	607	99
34	*B. ocellata-2*	620	*Betta ocellata*	KM485405.1	609	98
35	*B. palifina-1*	615	*Betta unimaculata*	KM485312.1	603	98
36	*B. palifina-2*	622	*Betta unimaculata*	KM485312.1	614	99
37	*B. palifina-3*	617	*Betta unimaculata*	KM485312.1	612	99
38	*B. patoti-1*	611	*Betta patoti*	KM485315.1	611	100
39	*B. patoti-2*	615	*Betta patoti*	KM485315.1	615	100
40	*B. patoti-3*	618	*Betta patoti*	KM485315.1	618	100
41	*B. rubra-1*	613	*Betta rubra*	KM485320.1	612	100
42	*B. rubra-2*	615	*Betta rubra*	KM485320.1	614	100
43	*B. rubra-3*	257	*Betta rubra*	KM485320.1	222	100
44	*B. raja-1*	613	*Betta pugnax*	KM485443.1	612	95
45	*B. rutilans*	549	*Betta coccina*	KM485461.1	548	99
46	*B. stigmosa-1*	615	*Betta stigmosa*	KM485451.1	614	99
47	*B. stigmosa-2*	616	*Betta stigmosa*	KM485452.1	615	100
48	*B. uberis-1*	610	*Betta uberis*	GQ911983.1	594	97
49	*B. uberis-2*	614	*Betta uberis*	GQ911983.1	614	100
50	*B. unimaculata-1*	471	*Betta unimaculata*	KM485314.1	445	94
51	*B. unimaculata-2*	585	*Betta unimaculata*	KM485314.1	549	94
52	*B. smaragdina-1*	613	*Betta smaragdina*	GQ911838.1	572	100
53	*B. smaragdina-2*	612	*Betta smaragdina*	GQ911838.1	573	100
54	*B. pugnax-1*	610	*Betta pugnax*	KM485443.1	573	94
55	*B. pugnax-2*	607	*Betta pugnax*	KM485424.1	585	94
56	*B. livida 1*	616	*Betta coccina*	KM485461.1	616	100

57	*Betta bellica-1*	615	*Betta bellica*	KM485409.1	611	99
58	*Betta bellica-2*	616	*Betta bellica*	KM485311.1	612	99
59	*Betta bellica-3*	615	*Betta bellica*	KM485311.1	611	99
60	*Betta sp-1*	612	*Betta cf. anabatoides*	GQ911722.1	598	99
61	*Betta sp-2*	605	*Betta cf. anabatoides*	GQ911722.1	578	99
62	*Betta sp-3*	598	*Betta cf. anabatoides*	GQ911722.1	580	99