

The role of molecular taxonomy in uncovering local ornamental Palo Fish (*Betta* sp.: Osphronemidae) and other *Betta* based on Cytochrome b gene

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World Journal of Advanced Research and Reviews, 2021, 10(01), 030-040

Publication history: Received on 27 February 2021; revised on 28 March 2021; accepted on 31 March 2021

Article DOI: <https://doi.org/10.30574/wjarr.2021.10.1.0113>

Abstract

Cytochrome b gene mitochondrial DNA was used to study the Palo fish from Bukit Rangkak tributary, Harau Valley, West Sumatra. The study aimed to determine the taxonomy of Palo fish, which morphological suspected as the *Betta* group. Phylogenetic analysis was used to solve the relationship of Palo fish with other species of the *Betta*. The alignment of the DNA sequences was carried out with Clustal X version 2 and analysis of phylogenetic tree using MEGA 6 software program. Based on the analysis of the cytochrome b gene sequence (1047 bp), it is known that the genetic differences of Palo fish from two tributaries of Bukit Rangkak river is 0.0% and with other *Betta* fish ranges from 13.0-35.5%. The phylogenetic tree has shown that Palo fish has a close genetic relationship with the *Betta picta* (13.0%). The result showed that Palo fish is at the different species in the genus of *Betta* and proposed as a new species.

Keywords: *Betta* fish; Cytochrome b; Harau Valley; Palo fish; Phylogenetic

1. Introduction

In general, *Betta* is a tiny fish with a total length of 2.5-12.5 cm [1]. *Betta* fish has a finite habitat in lowland freshwater and some exist in Asia's highlands [2]. *Betta* has a unique color and the shape of the fins and as one of the export commodities as an ornamental fish. [3, 4, 5]. The ornamental fish in their original habitat, especially in the tropics area, has decreased in population because of over-exploitation, deforestation, forest conversion, and water pollution [6, 7, 8].

Some of the *Betta* species currently can be grouped into complex species and need studies for conservation purposes [9]. The IUCN Red List has classified some of the species in genus *Betta* as the endangered species; meanwhile, the group's information is limited [10]. *Betta* fish's grouping for a long time is based on the morphological characters [11, 12, 13, 14]. With the development of technology and the use of DNA, currently has developed a systematic study using molecular data, or a combination of both [15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25].

The taxonomic and systematic study of the endangered species is still limited and needs revision to get the information used as the base of the consideration of fish conservation [5]. Fish classifications are being transformed drastically with the development of molecular phylogenetic analysis. Several systematic studies that use molecular phylogenetic analysis have been carried out [26, 27, 28, 29, 30, 31, 32]. Several studies regarding phylogenetic analysis for the Sumatra fish have been reported [34, 35, 36, 37]. Based on the literature, it can be understood that the combination of morphological and molecular analysis provides more information in taxonomic and systematic studies.

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One of the genes that can be used in the phylogenetic analysis is cytochrome b. The cytochrome b gene has been widely used in systematic problems at the family to species level [15, 38, 39, 40, 41, 42, 43]. The cytochrome b gene is one of the mitochondrial DNA protein-coding genes [44]. Phylogenetic analysis is a grouping of taxa that illustrated the relationship among them. Brown [45] interpreted that phylogenetic is a organism classification method by showing its relationship between them. Weiss and Goker [46] state that the phylogenetic hypothesis can be described in a tree form, composed of branches that representing the relationship between them and the node as a species.

There are 79 known species in the genus of *Betta* [9]. One of them is found in the highlands of Sumatra island (Indonesia) in Harau Valley, which is part of the Bukit Barisan mountain range in West Sumatra. The local people named it Palo fish. Palo fish has an attractive color on the operculum, and it has small-sized with an average of 66.9 mm total length and 11.38 mm body width. The morphological appearance strengthens the suspicion that Palo was *Betta* fish

The limit of genetic information on Palo fish makes it difficult in the taxonomic grouping. The studies are needed regarding the genetic relationship between Palo fish and other *Betta* species using the cytochrome b gene in phylogenetic analysis. The phylogenetic information can later be used in determining the systematic and management of conservation of Palo fish in the Bukit Rangkak tributary.

2. Material and methods

2.1. Study area

Samples were collected from two Bukit Rangkak tributary locations, Harau Valley, West Sumatra (Figure. 1) using survey methods, direct collection [47]. The sample was fixed with 4% formalin for several days and transferred to 70% alcohol for long-term preservation. The tissue samples of Palo fish were stored in a microtube (1.5 ml) filled with 96% ethanol PA as a DNA resource.

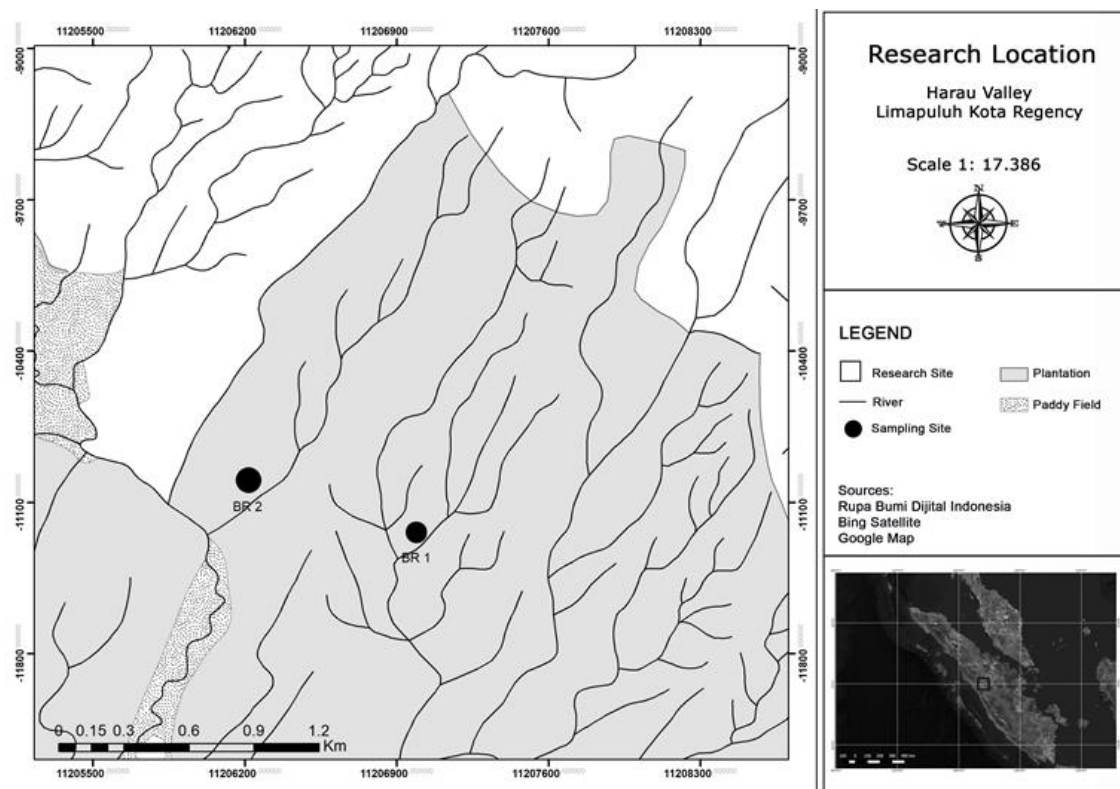


Figure 1 Research Location Map

2.2. Procedures

DNA isolation was done based on the PureLink™ Genomic DNA Kit protocol INVITROGEN Kit. PCR amplification was done using the reaction of a mixture with a total volume of 25 µl, consisting of 12.5 µl Gotaq green, 3.5 µl ddH₂O, two µl each primer and five µl DNA template. The primers for amplification referred to as DonGlu F and DonThr R [15].

PCR was carried out for 35 cycles. The PCR process starts initial denaturation at 94°C for 2 min, followed by denaturation at 94°C for 60 s, annealing at 51°C for 60 s, and 72°C for 90 s for elongation. The final extension phase at 72°C for 5 min. The quality of the PCR product examined the 2% agarose gel electrophoresis. The PCR product was sent for sequencing at the 1st Base Malaysia.

2.3. Data Analysis

The DNA sequence of Palo fish arranged by forward and reverse contig with DNA STAR program [48] to get the DNA sequence. Compared with the 33 accessions of cytochrome b gene sequences from Osphronemidae, Cyprinidae, and Pomacentridae family, the DNA sequence of Palo fish, was download from GenBank NCBI (National Center of Biotechnology Information). The alignment of the DNA sequence used Clustal X 1.8 version and edited by Bioedit. The polymorphism sequence analyses used DNA sequence polymorphism 5.10. [49]. The phylogenetic tree was constructed and analyzed by MEGA (Molecular Evolutionary Genetics Analysis) 6 [50].

3. Results and discussion

3.1. Result

3.1.1. Blast Analysis

The BLAST analysis results were used to determine the similarity value of the Palo fish sequences with their close relatives. In the present study, eight cytochrome b mitochondrial DNA sequences of Palo fish were used. The BLAST results showed that the similarity value of Palo fish with the *Betta* genus was 88-99% (Table 1). Palo fish sequences have the highest similarity value to *Betta picta* species (99%).

Based on BLAST results, 33 cytochrome b gene sequences were downloaded from NCBI GenBank as a comparison. The downloaded sequences consist of species from the family of Osphronemidae and two species from the family Cyprinidae and Pomacentridae. The total of all sequences that were analyzed included sequences Palo fish are 41 sequences.

The number of nucleotide bases resulting from the alignment of all sequences analyzed was 1047 bp, which in the position of 14468–15500 bp of *Betta pi* complete genome. Prakhongcheep et al. [24] reported that the *Betta pi* cytochrome b gene sequence at the full genome site at the range from 14388–15543 bp. From 1047 bp sequences, 470 bp (44.89%) as conserved site, 577 bp (55.10%) as variable site, 498 bp (47.56%) as parsimony site and 79 bp (7.54%) as a singleton site.

The nucleotide base compositions of 1047 bp cytochrome b gene are A (Adenine) 25.9%, T (Thymine) 30.6%, C (Cytosine) 28.8%, and G (Guanin) 14.7%. The average percentage of Adenine+Thymine (A+T) nucleotide base was 56.5%, and Guanin+Cytosine (G+C) nucleotide bases were 43.5%. The number of bases in Palo fish (A+T) is higher than (G+C). According to the previous researchers [51, 52, 53, 54, 55] the number of bases (A+T) in vertebrates is higher than (G+C).

Based on the polymorphism sequence analysis using the DNA Sequence Polymorphism program [51] 34 haplotypes were obtained from the 41 sequences analyzed (Table 2). Eight individuals Palo fish from two tributary locations, belonging to the same haplotype, namely Haplotype 1. Based on the haplotype analysis results, it can be stated that there is no genetic variation in the two Palo fish populations. Geographically, two tributaries of sampling sites are from the same upstream.

Table 1 Species List taxonomy, Location and Genbank Accession Number of samples.

No	Family	Genus	Species	Accession Numbers	Location
1	Osphronemidae	<i>Betta</i>	<i>Betta breviobesus</i>	AF519668.1	Indonesia
2			<i>Betta pi</i>	AF519672.1	Thailand
3			<i>Betta waseri</i>	AF519671.1	Sumatra
4			<i>Betta hipposideros</i>	AF519673.1	Selangor
5			<i>Betta chloropharynx</i>	AF519675.1	Banka
6			<i>Betta anabatooides</i>	AF519674.1	Borneo
7			<i>Betta edithae</i>	AF519663.1	Borneo
8			<i>Betta prima</i>	AF519664.1	Thailand
9			<i>Betta imbellis</i>	AF519690.1	Sumatra
10			<i>Betta splendens</i>	AF519689.1	Mekong
11			<i>Betta smaragdina</i>	AF519688.1	Mekong
12			<i>Betta rutilans</i>	AF519682.1	Borneo
13			<i>Betta brownorum</i>	AF519681.1	Borneo
14			<i>Betta cf. burdigala</i>	AF519678.1	Banka
15			<i>Betta tussyaie</i>	AF519679.1	Malaysia
16			<i>Betta coccina</i>	AF519684.1	Borneo
17			<i>Betta pugnax</i>	AF519665.1	Singapore
18			<i>Betta fusca</i>	AF519666.1	Sumatra
19			<i>Betta dimidiata</i>	AF519667.1	Borneo
20			<i>Betta picta</i>	AF519670.1	Malaya
21			<i>Betta simplex</i>	AF519669.1	Thailand
22			<i>Betta cf. albimarginata</i>	AF519676.1	Borneo
23			<i>Betta ocellata</i>	AF519693.1	Borneo
24			<i>Betta patoti</i>	AF519691.1	Borneo
25			<i>Betta unimaculata</i>	AF519692.1	Borneo
26		<i>Trichogaster</i>	<i>Trichogaster pectoralis</i>	AY763758.1	Borneo
27			<i>Trichogaster trichopterus</i>	AY763759.1	Thailand
28		<i>Pseudosphromenus</i>	<i>Pseudosphromenus dayi</i>	AY763764.1	India
29			<i>Pseudosphromenus cupanus</i>	AF519699.1	Srilanka
30		<i>Parosphromenus</i>	<i>Parosphromenus paludicola</i>	AY763763.1	Malaysia
31			<i>Parosphromenus anjunganensis</i>	AY763761.1	Borneo
32	Cyprinidae	<i>Cyprinus</i>	<i>Cyprinus carpio</i>	AY347294.1	India
33	Pomacentridae	<i>Chrysiptera</i>	<i>Chrysiptera traceyi</i>	JQ707176.1	West Pacific

Table 2 Haplotypes of Palo fish and other species

No	Haplotype	Species
1	Haplotype 1	Palo
2	Haplotype 2	<i>Betta breviobesus</i>
3	Haplotype 3	<i>Betta pi</i>
4	Haplotype 4	<i>Betta waseri</i>
5	Haplotype 5	<i>Betta hipposideros</i>
6	Haplotype 6	<i>Betta chloropharynx</i>
7	Haplotype 7	<i>Betta anabatoides</i>
8	Haplotype 8	<i>Betta edithae</i>
9	Haplotype 9	<i>Betta prima</i>
10	Haplotype 10	<i>Betta imbellis</i>
11	Haplotype 11	<i>Betta splendens</i>
12	Haplotype 12	<i>Betta smaragdina</i>
13	Haplotype 13	<i>Betta rutilans</i>
14	Haplotype 14	<i>Betta brownorum</i>
15	Haplotype 15	<i>Betta cf. Burdigala</i>
16	Haplotype 16	<i>Betta tussyae</i>
17	Haplotype 17	<i>Betta coccina</i>
18	Haplotype 18	<i>Betta pugnax</i>
19	Haplotype 19	<i>Betta fusca</i>
20	Haplotype 20	<i>Betta dimidiata</i>
21	Haplotype 21	<i>Betta picta</i>
22	Haplotype 22	<i>Betta simplex</i>
23	Haplotype 23	<i>Betta cf. Albimarginata</i>
24	Haplotype 24	<i>Betta ocellata</i>
25	Haplotype 25	<i>Betta patoti</i>
26	Haplotype 26	<i>Betta unimaculata</i>
27	Haplotype 27	<i>Cyprinus carpio</i>
28	Haplotype 28	<i>Chrysiptera traceyi</i>
29	Haplotype 29	<i>Trichogaster pectoralis</i>
30	Haplotype 30	<i>Trichogaster trichopterus</i>
31	Haplotype 31	<i>Pseudosphromenus dayi</i>
32	Haplotype 32	<i>Pseudosphromenus cupanus</i>
33	Haplotype 33	<i>Parosphromenus paludicola</i>
34	Haplotype 34	<i>Parosphromenus anjunganensis</i>

3.2. Phylogenetic Analysis

The phylogenetic tree constructed by cytochrome b gene (Figure 2) separated into four main clusters based on genus with sequence divergences 13.0–50.0%. The first cluster was placed by species from the *Betta* genus with sequence divergences 13.0–35.5%. The second cluster placed by two species from the *Pseudosphromenus* genus with sequence divergences 32.7–42.9% to the first cluster. The third cluster was placed by two species from the *Parosphromenus* genus with sequence divergences 35.1–50.0% to the first and second clusters. The fourth cluster was placed by two species from the *Trichogaster* genus with sequence divergences 31.1–40.9% to the other three clusters.

The first cluster consists of 26 species of the *Betta* genus, divided into two sub-clusters. The first sub-cluster consists of 23 species with sequence divergences 13.0-20.4%, and the second sub-cluster consists of 3 species with a sequence of 29.4-46.5% to the first sub-cluster. Based on the sequence divergences, the first cluster is divided into four groups, namely the *pugnax*, *albimarginata*, *coccina*, and *unimaculata*. The lower the sequence divergences value, the closer the position in the phylogenetic tree and vice versa. According to Kartavtsev et al. [43] and Kartavtsev et al. [56] the sequence divergences value of a species will increase with the taxon's increasing level. This separating group is in line with another result [15] who divided the *Betta* genus into seven groups, *pugnax*, *albimarginata*, *coccina*, *foerschi*, *splendens*, *unimaculata*, and *macrostoma*. Morphological differences were also detected in these four groups by looking at a sketch from Ruber et al. [15] used in phylogenetic trees (Figure 2). The illustration shows the different characteristics of the caudal fin shape of each group. The *pugnax* group has lancet-shaped caudal fins; the *coccina* group is perfectly rounded while the *albimarginata* and *unimaculata* caudal fins are rounded modified with a flat caudal tip. Differences in morphological characters in the four groups are also explained by Tan and Ng [11]. The *pugnax* group has characteristics of lancet-shaped on caudal fins. The *albimarginata* group has wide and falcate pelvic fins, rounded caudal fins. The *coccina* groups are identical with small body sizes, and the *unimaculata* group has pelvic, dorsal, tapered fins and lancet-shaped caudal fins.

4. Discussion

Palo fish are in the first group (*pugnax* group) and *B. picta*, *B. simplex*, *B. breviobesus*, *B. anabatoides*, *B. chloropharynx*, *B. hipposideros*, *B. pi*, *B. waseri*, *B. dimidiata*, *B. pugnax*, *B. fusca*, *B. edithae*, *B. prima*. The value of the sequence divergences in this sub-cluster is 13.0%-20.8%. Based on the Authors [43, 56] that value, has grouped the species in this group into the same genus. Therefore, Palo fish is one of the species of the genus *Betta*. Ruber et al. [15] supported the grouped of *B. picta*, *B. simplex*, *B. breviobesus*, *B. anabatoides*, *B. chloropharynx*, *B. hipposideros*, *B. pi*, *B. waseri*, *B. dimidiata*, *B. pugnax*, *B. fusca*, *B. edithae*, *B. prima* in the same cluster, the *pugnax* group. This grouping is also supported by morphological forms that are similar to each other. Tan and Ng [11] describe that the *Betta pugnax* group has the characteristics. They have a black line chin bar from under the eye to the lower jaw, there are transverse bars in the form of black transverse stripes on the dorsal, anal and caudal fins in adult male individuals and have caudal fins lancet-shaped. These characteristics are also found in Palo fish (Figure 3). This explanation confirms that the Palo fish is a species of the *Betta* genus which belongs to the *pugnax* group.

In the second group, the *albimarginata* group is consists of *Betta* cf. *albimarginata*, with 28.0%. Sequence divergences to Palo fish. Referring to Kartavtsev et al. [43] and Kartavtsev et al. [56] Palo fish is a different species in the same genus as *Betta* cf. *albimarginata*. Ruber et al. [15] also show that *Betta* cf. *Albimarginata* forms a separate group, the *albimarginata* group.

The *coccina* group is in the third group consist of *B. coccina*, *B. tussyae*, *B. cf. burdigala*, *B. rutilanss*, and *B. brownorum*. These species are found in Indonesia, Thailand, and Malaysia. The sequence divergences between Palo fish and these species ranges from 28.7-33.1%. Following Kartavtsev et al. [43] and Kartavtsev et al. [56] it was concluded that Palo fish is a species of a different family from other species in the *coccina* group.

The fourth group ist the *unimaculata* group consisting of *B. unimaculata*, *B. ocellata*, and *B. patoti*. These species are *Betta* fish found in East Kalimantan. The sequence divergences between Palo fish and these species ranges from 27.3-28.3%. Referring to Kartavtsev et al. [43] and Kartavtsev et al. [56] means that Palo fish are a different species in other families of the *unimaculata* group.

The phylogenetic tree also shows that *B. picta* is the closest branch of the Palo fish, with a 13.0% sequence divergences. Based on Kartavtsev et al. [43] and Kartavtsev et al. [56] Palo fish are a different species of *B. picta*. The difference in the distribution of these two species supports the high level of genetic variation. *B. picta* has a distribution area on the island of Java [11]. Meanwhile, Palo fish was found in the tributaries of Bukit Rangkak, West Sumatra. Ruber et al. [15] grouping the *B. picta* into the *pugnax* group based on nuclear and mitochondrial DNA analysis and the evolutionary history of parenting mouthboarding in the *Betta* genus.

On the other hand, Tan and Ng [11] did not include the *B. picta* into the *pugnax* group; rather, it has its group, the *picta* group (based on morphometric and meristic characters, with striking differences in the anal and caudal fin patterns). Witte and Schmit [57] have described *B. picta* as having a rounded caudal fin pattern and the anal fin sometimes tapered. This character difference is also seen in Palo fish, which have lancet caudal fin patterns and tapered anal fins, which are the *pugnax* group's characters. However, based on the analysis of the cytochrome b gene, it can be stated that the *B. picta* is a sister taxon of the Palo fish because it has the closest genetic distance compared to other *Betta* species used in

this analysis. In line with the opinion of Gregory's opinion [55] which states that sister taxa are a close relative of a species that comes from the same branch in a phylogenetic tree.

Based on cytochrome b gene analysis, it is also known that there is no genetic variation between Palo fish populations from two locations of the Bukit Rangkak tributaries with a sequence divergences of 0.0%. That value is presumably due to the two sites of tributaries, which are branches of the Bukit Rangkak tributary (Figure 1). The possibility of inter-population breeding still occurs. This possibility is supported by Roesma et al. [35] which states that if genetic mixing between species with separate locations can still occur, then genetic similarities between species are always maintained. In line with the statements of Garg and Mishra [54] which states that gene flow has the potential to reduce genetic differentiation. Zero genetic variation between Palo fish populations based on the cytochrome b gene is also predicted because these two populations come from the same ancestor. The fragmentation between tributaries does not affect reproductive isolation because two populations come from the same ancestor. Song et al. [59] and Song et al. [60] states that mtDNA's genetic component is inherited maternally and there is no recombination, the genetic variation will arise if there is a separation of common ancestor components due to genetic drift or bottleneck effect.

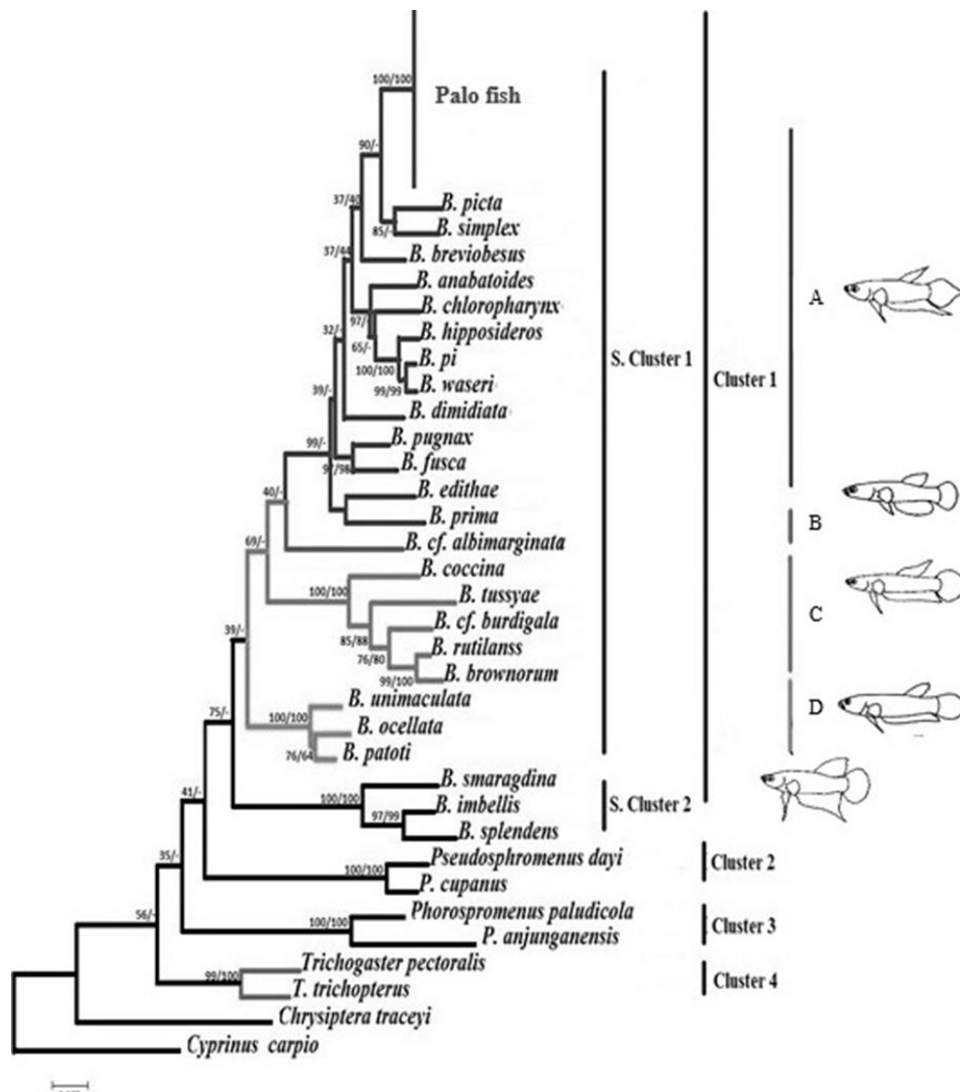


Figure 2 The ML phylogenetic tree of the cytochrome b gene with the bootstrap value of 1000 replicates (ML/NJ). Note: A. *pugnax* group; B. *albimarginata* group; C. *coccina* group; D. *unimaculata* group. Sketch of the picture based on Ruber et al. (2004)

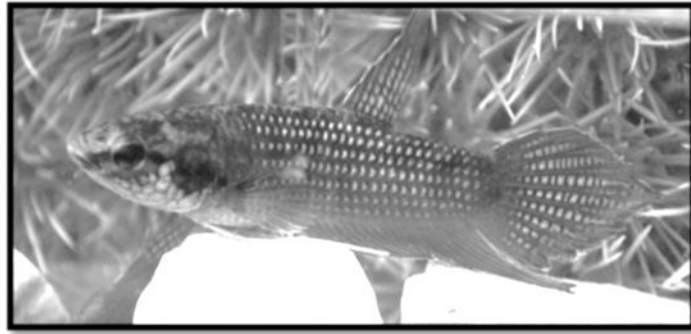


Figure 3 Adult male Palo fish

5. Conclusion

The present study reported that Palo fish from two populations of Bukit Rangkak tributaries are genetically identical. Palo fish belong to the genus *Betta* in the *pugnax* group. The Palo fish sequence's value is 13.0% with other *Betta* genus species; therefore, the present study proposed that the Palo fish is a new species in the *Betta* genus. Further research is being carried out.

Compliance with ethical standards

Acknowledgments

We want to thank the Ministry of Research, Technology, and Higher Education Management to assist PKM-P research grant assistance in 2018. Our thanks also expressed to the Biology Department of Andalas University for the field and Laboratory work permit. We also would like to thank our friends, who helped us in samples collection and laboratory works in Genetic and Biomolecular Laboratory, Faculty of Mathematics and Sciences, Andalas University, Padang, Indonesia.

Disclosure of conflict of interest

There is no interest in the conflict between the authors of this piece of research work. The authors agreed and assigned in hand to all matter arise to this piece of research work.

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