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Systematics molecular investigation of Palo fish (*Betta* sp.) in the Harau Valley, West Sumatra using the COI gene

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Abstract. *Putri UK, Roesma DI, Tjong DH. 2025. Systematics molecular investigation of Palo fish (*Betta *sp.) in the Harau Valley, West Sumatra using the COI gene. Biodiversitas 26: 698-705.* Palo fish (*Betta* sp.) is a local ornamental fish in the Harau Valley, Lima Puluh Kota, West Sumatra, Indonesia, which was suspected to be a new species from the *Betta* of the *Pugnax* group based on the Cytochrome b gene. Molecular investigations using more reliable genes were needed to validate the taxonomic status of Palo fish and determine the phylogenetic relationship with other *Betta* species. The cytochrome oxidase I (COI) gene has been recognized for identification at the species level. Liver tissue samples were taken from eight Palo fish individuals from four Rangkak Hill tributaries in Harau Valley. Eight Palo fish and 44 comparison sequences were analyzed using the Aliview, IQ Tree, and MEGA VII programs. The 641 bp COI gene sequences analysis showed that Palo fish from four tributaries in Harau Valley had 100% nucleotide base similarity (identical), which shared the same haplotype. Palo fish have a close relationship with *Betta stigmosa* and have the least genetic distance to *Betta* cf. *apollon* (2.6%), followed by *Betta ferox* (3.1%) and *Betta apollon* (3.7%). The genetic distance values show the differences at the subspecies level within the same species. Palo fish from Harau Valley confirmed as *Betta* cf. *stigmosa*. Comprehensive studies on *Betta* species need to be carried out to complete the systematics of the *Betta* group.

Keywords: *Betta* cf. *stigmosa*, barrier, COI gene, phylogenetic, *Pugnax*

INTRODUCTION

Betta is a fish genus with the most significant species in the Osphronemidae family (Kottelat et al. 1993; Nelson 2016; Van der Lann 2018). *Betta* generally lives in lowland areas, and some are also found in the highlands of Asia (Kottelat et al. 1993; Tan and Ng 2005; Kowasupat et al. 2014; Goldstein 2015). The high level of *Betta* fishing in the wild and land clearing for settlements, agriculture, industry, and tourism has resulted in a decline in the population of this fish in its natural habitat (Chan 2015; Rahman and Matthew 2021; Nur et al. 2022a, b). This is evidenced by the high percentage of *Betta* fish conservation status, which is critically endangered based on IUCN (IUCN 2024). The decline in *Betta* fish populations in their natural habitat has encouraged cultivation efforts and artificial selection activities that sometimes do not pay attention to the type of broodstock used because of morphological similarities. Thus, interspecies mating can result in the genetic mixing of different species and produce hybrid species (Saint-Pe et al. 2018; Beer et al. 2019).

Currently, 74 *Betta* species have been described morphologically, of which 51 are found in Indonesian waters (Nelson 2016; Van der Lann 2018 and Fishbase 2024). New species are discovered on average every 5-10 years, and most show morphological similarities (Kowasupat et al. 2014; Pammanasut et al. 2018; Nur et al. 2022a, b). This indicates the existence of cryptic diversity that occurs in the *Betta* lineage (Srikulnath et al. 2021; Zhang et al. 2022; Panthum et al. 2023). One species morphologically included in the *Betta* group is known locally as the Palo fish (*Betta* sp.), found in the tributary of Rangkak Hill, Harau Valley, West Sumatra (Putri et al. 2021). Excessive fishing activities of Palo fish to be used as souvenirs have caused a decline in the population, which is thought to have an impact on the extinction of the fish. Therefore, the initial steps in conservation efforts must be taken by providing taxonomic and genetic information on Palo fish. Molecular studies using the cytochrome b gene have been carried out by Putri et al. (2021), which show that the Palo fish has the closest relationship to *Betta picta* Valenciennes 1846 with a genetic distance of 13%. This value indicates that the Palo fish is a different species from *B. picta*. The limited Cyt b gene data of *Betta* species available in GenBank, NCBI, results in a lack of comparative data for identifying Palo fish to the species level.

More reliable molecular markers are needed to validate the taxonomic status and provide genetic information on Palo fish (*Betta* sp.) and its relationship to other *Betta* species. The cytochrome oxidase I (COI) gene is an internationally agreed gene used for species identification known as DNA barcoding (Hubert et al. 2015; Kartavtsev 2021; Naz et al. 2023). Several studies using molecular markers of the COI gene for species identification and analysis of kinship relationships have been conducted on various groups of fish (Roesma et al. 2018; Roesma et al. 2019; Roesma et al. 2020; Roesma et al. 2022; Tsoupas et al. 2022; Roesma et al. 2024) including the *Betta* group (Kowasupat et al. 2014; Panijpan et al. 2014; Fahmi et al.

2020 Valen et al. 2023; Syarif et al. 2023). Therefore, it is necessary to conduct molecular studies to determine the taxonomic status and systematics of Palo fish (*Betta* sp.) in other *Betta* groups. This information is primary data in determining the right strategy for conservation and cultivation efforts of Palo fish (*Betta* sp.) as a local fish with the potential as a source of germplasm resources.

MATERIALS AND METHODS

Study area

Palo fish samples were collected in four tributaries of Rangkak Hill, located in the primary forest, Harau Valley, Lima Puluh Kota, West Sumatra, Indonesia (Figure 1). The four tributaries include Rangkak Hill 1 (BR 1, 0°06'24"S 100°40'31"E), Rangkak Hill 2 (BR 2, 0°06'02"S 100°40'04"E), which is a tributary above Rangkak Hill and a tributary under Rangkak Hill including Sarasah Bunta (SB, 0°06'36"S 100°40'43"E), and Air Putih (AP, 0°05'19"S 100°39'43"E). Between the tributaries of Rangkak Hill, there is a barrier in the form of a waterfall. The tributary of Rangkak Hill has characteristics in the form of a depression/pool of water with a width of around 1.5-3 meters, with a water base consisting of sand, rocks, litter, and tree roots. The tributary current's depth and speed depend on the rainfall's intensity.

Procedures

Samples collection

Individual samples were collected using survey methods and direct individual collection using fishing gear. Tissue samples of several individuals were collected and placed in microtubes containing absolute ethanol. The total number of tissue samples collected was eight, consisting of two BR1, two BR2, two SB and two AP. Before the samples were preserved and photographed in the aquarium, morphological characteristics of the fish, such as body color, fin color, and other characteristics that would be lost or altered, were noted. All individual samples were labeled and preserved in 10% formalin. After being preserved for several weeks, the samples were washed with running water until the smell of formalin disappeared. All samples were stored in a sample box containing 70% alcohol solution for long-term storage at the Genetics and Biomolecular Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Andalas, Padang, West Sumatra, Indonesia.

DNA isolation, DNA amplification, and DNA sequencing

DNA isolation was carried out on eight samples of Palo fish tissue in the Harau Valley. Samples were isolated using the Extragene GeneAll genomic DNA Kit protocol. The DNA isolation steps consist of lysis, binding, washing, and elution processes. The isolated DNA results were amplified using universal primers COI Fish FI and Fish RI (Ward et al. 2005). The composition of the DNA amplification solution consisted of Supermix Bioline 11 µL; ddH_2O 9 µL; forward primer 1 µL; reverse primer 1 µL; and isolate DNA 3 µL, with a total volume of 25 µL. PCR cycle with the predenaturation process at 95°C for 2 minutes. Next, the denaturation stage at 94°C for 30 seconds, annealing at 52°C for 30 seconds, extension at 72°C for 1 minute for 35 cycles, and final extension at 72°C for 10 minutes. DNA sequencing was performed after the DNA amplification sample was purified. Good-quality DNA samples were sent to First Base Malaysia for sequencing.

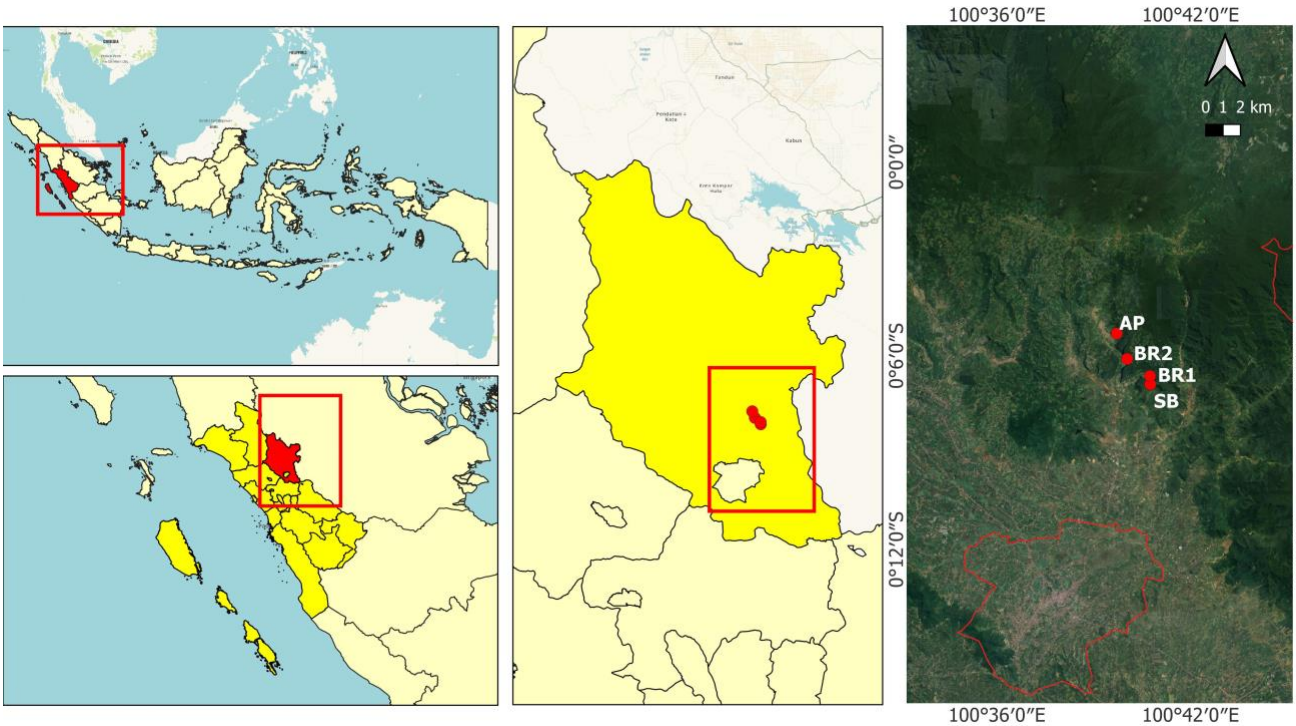

Figure 1. Map of the sampling location of Palo fish (*Betta* sp.) in Harau Valley, Lima Puluh Kota, West Sumatra, Indonesia

Data analysis

The DNA sequencing results were analyzed using several programs, including DNA Star, BLAST, Aliview, DNA SP, MEGA 11, and IQ Tree. The total DNA sequences of palo fish analyzed were seven sequences because one sequence failed. The forward and reverse DNA sequences of Palo fish were contig using the DNA Star program. All Palo fish sequences contig were BLAST to compare with sequences in the GenBank, NCBI, and BOLD System databases. Several sequences that had the closest similarity to Palo fish were used as comparison sequences (44 sequences) for identification. Aliview was used to combine and align all sequences according to the similarity of nucleotide base sequences. After aligning all sequences, the DNA sequences were translated into amino acid sequences using the online DNA to Protein Translation website. DNA SP and MEGA 11 were used to check the polymorphism of the entire DNA sequence (haplotype type, haplotype diversity, and nucleotide diversity). The DNA sequence of the COI gene of Palo fish was registered with the Barcode of Life Data System (BOLD System) as the DNA Barcode of Palo fish to obtain the Barcode Index Number (BIN) according to its match with the DNA sequence in the BOLD System (Kartavtsev 2018). The phylogenetic tree was reconstructed using the IQ Tree with the Maximum Likelihood method with 5000 bootstraps.

RESULTS AND DISCUSSION

Analysis of nucleotide base variation

The BLAST analysis results indicate that the Palo fish (*Betta* sp.) from Harau Valley shares a DNA sequence similarity with other *Betta* genera ranging from 99% to 88%. Based on the BLAST results, 44 reference sequences were used for analysis, including 42 sequences from species within the Osphronemidae family and two species as outgroups from the Cyprinidae, and Pomacentridae families. The total of all sequences analyzed included seven sequences of Palo fish (*Betta* sp.) Harau Valley is 51 sequences. The obtained alignment of all sequences resulted in a total of 641 analyzed base pairs. A total of 641 base pairs of the COI gene were located at nucleotide positions 5524-6165 bp of the complete mitochondrial genome of one of the *Betta* species, *B. pi*. The COI gene position in the Palo fish (*Betta* sp.) corresponded to the COI gene positions in other *Betta* species, including *B. pi* at positions 5485-7044 bp (Prakhongcheep et al. 2018), COI gene in *Betta simplex* Kottelat 1994 at positions 5521-7080 bp, and *Betta apollon* Schindler and Schmidt 2006 at positions 5498-7057 bp from the complete mitochondrial genome (Ponjarat et al. 2019). Among the 641 bp analyzed, 362 bp (56.47%) were conserved sites, and 279 bp (43.52%) were variable sites. The number of nucleotide bases in the COI gene analyzed in this study was almost the same as that analyzed by Panijpan et al. (2014), which was 652 base pairs of the COI gene.

Polymorphism sequence analysis obtained 43 haplotypes from 51 COI gene sequences analyzed. Overall, the Haplotype and nucleotide diversity for the 51 analyzed sequences are Hd = 0.983 ± 0.012 and Pi = 0.154 ± 0.008. The total number of base mutations was 279, with a transition/transversion bias value of R = 2.9. Among the mutations occurring in all sequences, 199 amino acid changes were observed. One haplotype was shared by eight sequences of Palo fish (*Betta* sp.), and the other 42 haplotypes belong to the reference sequences. Thirty-eight haplotypes were found in the other species of the *Betta* genus, and four haplotypes were found in the outgroup species. Eight individual Palo fish (*Betta* sp.) from four tributaries of the Harau Valley have the same haplotype (H1). The haplotypes possessed by eight individuals of Palo fish (*Betta* sp.) from four tributaries in the Harau Valley showed no differences in their nucleotide bases. Waterfalls are a barrier that separates the four tributaries of the Harau Valley. The tributary of Rangkak Hill I (BR I) is separated from the tributary of Sarasah Bunta (SB) by the Sarasah Bunta waterfall, and the tributary of Rangkak Hill 2 (BR 2) is separated from the tributary of the Air Putih (AP) by the waterfall of Air Putih. The four tributaries in the Harau Valley are suspected to have come from the same river. Based on this, it can be assumed that the Palo fish (*Betta* sp.) in the four tributaries of the Harau Valley come from the same river population. This result was in line with previous research by Putri et al. (2021), who reported that Palo fish (*Betta* sp.) from the upper tributaries of the Harau Valley hills have 100% genetic similarity in the nucleotide base sequence of the CYTB gene. Roesma et al. (2020) explained that low variation between populations in a species can occur because they come from the same lineage. The presence of the waterfall as a barrier that separated the tributaries in the Harau Valley does not affect the nucleotide base differences of Palo fish (*Betta* sp.) Harau Valley.

Palo fish (*Betta* sp.) has the lowest nucleotide base difference with *Betta* cf. *apollon* (16 bases), followed by *B. ferox* (19 bases), *B. stigmosa* (22 bases), and *B. apollon* (22 bases). Palo fish (*Betta* sp.), *B.* cf. *apollon*, *Betta ferox* Schindler and Schmidt 2006, *B. stigmosa*, and *B. apollon* are grouped in the *Pugnax* group. Among the nucleotide base variations in the Palo fish (*Betta* sp.), and three other species in the *Pugnax* group, 31 are transition mutations, and five are transversion mutations. The mutations have caused the changes of 26 amino acids. Meanwhile, for all members of the *Betta* species, there were 266 nucleotide bases variations that has resulted in changes in 189 amino acids.

Phylogenetic tree analysis

The relationship between Palo fish (*Betta* sp.) and other *Betta* species based on the COI gene was demonstrated by the phylogenetic tree reconstruction in IQ Tree with Maximum Likelihood method as well as 5000 bootstrap (Figure 2). The most suitable analysis model used was TIM2+F+I+G4, according to BIC. The phylogenetic tree shows that the members of the *Betta* group have a monophyletic relationship (from the same ancestor). In the phylogenetic tree, members of the *Betta* species were grouped based on similarities in egg care behavior and morphological characteristics. Based on egg care behavior,

Betta species were divided into two groups (Mouth brooders and Bubble nest builders). The Mouth brooders group consists of the *Pugnax* complex and the *Unimaculata* complex. Furthermore, the classification of the Bubble nest builders group, which includes the *Coccina* and the *Splendens* complexes, aligns with the previous report by Ruber et al. (2004). The phylogenetic tree reconstruction shows that the Palo fish (*Betta* sp.) clusters together with *B. stigmosa*, *B. ferox*, *B. apollon*, and *B.* cf. *apollon* in the Mouth Brooders group.

Based on the morphological characteristics, the *Betta* group analyzed was classified into five complex groups (i) *Pugnax*; (ii) *Coccina*; (iii) *Unimaculata*; (iv) *Splendens*; and (v) *Albimarginata*. In the phylogenetic tree, the comparative COI sequence data used originated from several previous studies that have been combined. Various authors have separated the species within the *Pugnax* complex group into different groups. Therefore, after being merged in this study's phylogenetic analysis, these species are divided into four groups (i) *Pugnax*; (ii) *Picta*; (iii) *Waseri*; and (iv) *Anabatoides*. The classification of *Betta* species as members of each group aligns with previous studies that were reported by Ruber et al. (2004), Tan and Ng (2005), Schindler and Schmidt (2006), and Panijpan et al. (2014).

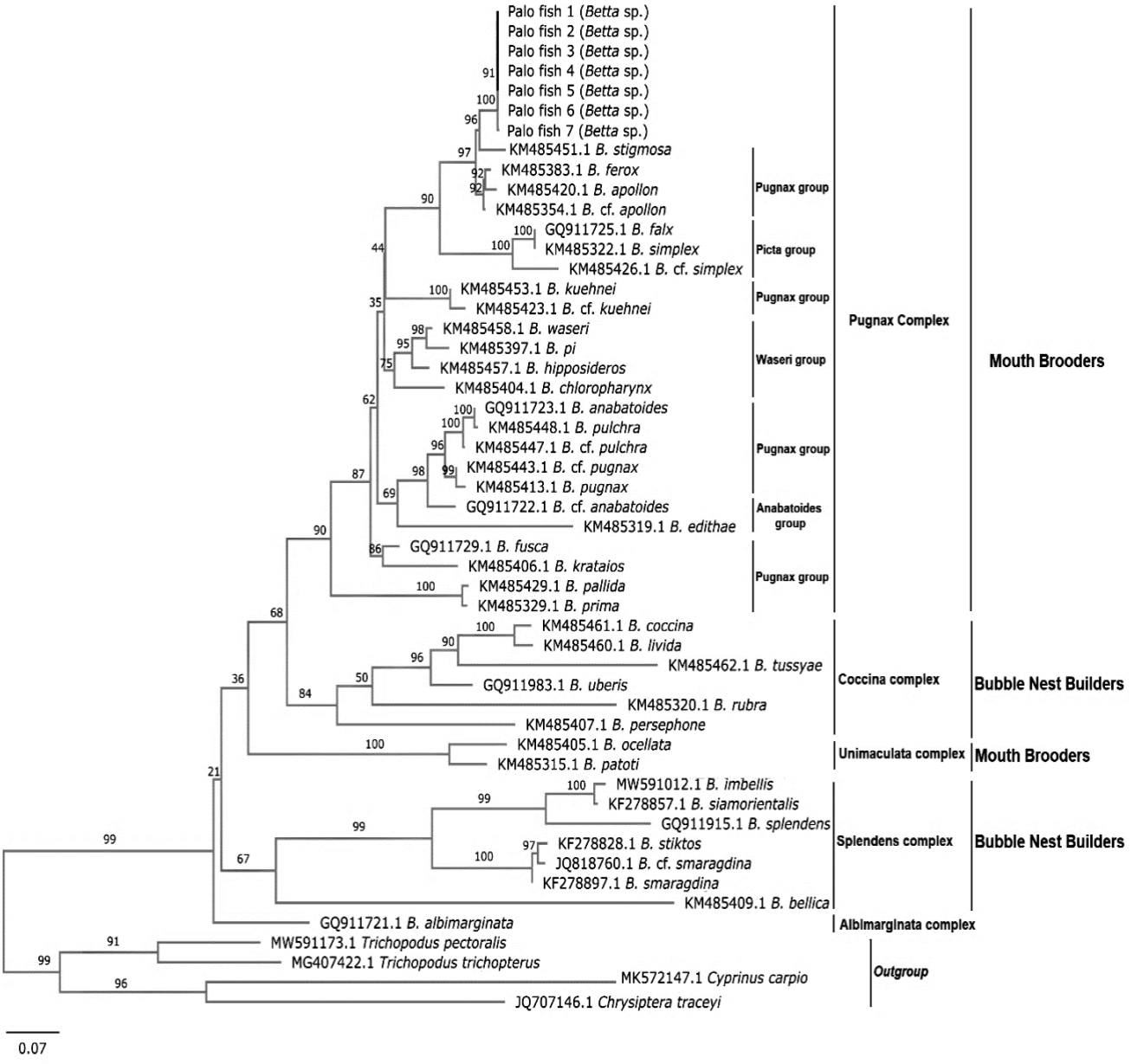

Figure 2. The phylogenetic tree of the Palo fish (*Betta* sp.) and its close relatives based on the COI gene, the numbers present on the branching of the tree shows a bootstrap value of 5000 times (ML)

Phylogenetic tree reconstruction shows the Palo fish (*Betta* sp.) grouped with members of the *Pugnax* group *B. stigmosa*, *B. ferox*, *B. apollon* and *B.* cf. *apollon*. This data indicates that the Palo fish (*Betta* sp.) is a *Betta* species in the *Pugnax* group. Meanwhile, *B. simplex* grouped with *Betta falx* Tan and Kottelat 1998 in the *Picta* group to become sister taxa of Palo fish (*Betta* sp.), *B. stigmosa*, *B. ferox*, and *B. apollon* (*Pugnax* group). The results showed that species classification in phylogenetic trees was the same as the grouping based on egg care behavior and morphology.

The grouping of *Betta* species on the phylogenetic tree using the COI gene was generally similar to the findings of Ruber et al. (2004) using nuclear DNA and mitochondrial DNA and Panijpan et al. (2014) using mitochondrial DNA (COI and ITS), with some differences in the placement of *Betta* species on the phylogenetic tree. These differences were due to variations in the genes used and the availability of sequences in GenBank, NCBI. In the study by Ruber et al. (2004), there was a species *B. picta* that was not present in the work of Panijpan et al. (2014) and in this study. This absence was due to the lack of COI gene data for the *B. picta* species in GenBank, NCBI. In the phylogenetic tree of Ruber et al. (2004), *B. picta* is seen within the *Pugnax* group and is the sister taxon to *B. simplex*. Meanwhile, in Panijpan et al. (2014) and this study, *B. picta* was absent, but species *B. ferox* and *B. apollon* cluster together within the *Pugnax* group and were the sister taxa to *B. simplex*. This data indicated the differences in comparative gene sequences, which can affect the positions and relationships among species in the phylogenetic tree.

The Palo fish (*Betta* sp.) from Harau Valley have a 100% (identical) COI gene sequence similarity, resulting in the same haplotype with a genetic distance of 0.0%. This data was evident in the phylogenetic tree, where all individual Palo fish (*Betta* sp.) specimens from the four tributaries of Rangkak Hill were grouped. The genetic similarity in all Palo fish individuals suggested that all four tributaries might come from the same river population, a finding that sets a new standard in genetic research. This finding was supported by the topography of the Harau Valley region, which was believed to have been a single landmass with a shared river system in the past. However, geological processes led to the separation of land through horst and graben mechanisms, forming valleys and hills. This event caused the formation of a waterfall so that the river flow was cut off, which separated it into a tributary above and a tributary under Rangkak Hill, Harau Valley.

The assumption of the formation of the Harau Valley was based on the statements of Ngadenin (2013) that geologically, the Harau Valley was located on the edge of the Ombilin basin, precisely in the Payakumbuh sub-basin, which was formed from the complete graben process. Although there was a geographical barrier in the form of waterfalls separating the tributaries, it did not have an impact on the emergence of genetic variations in the COI gene sequence of Palo fish (*Betta* sp.). This statement was suspected because the current formation time of Harau Valley was insufficient for speciation or the emergence of genetic variations within a species. This statement was supported by the report of Allio et al. (2017), which stated that mitochondrial DNA in vertebrates requires 10^6 years to undergo a 0.0199 per site change. In addition, the Palo fish was identical to the COI gene due to the similarity of habitat and environmental factors such as substrate type, water currents, water temperature, and vegetation around the tributary.

The phylogenetic tree (Figure 2) shows that Palo fish (*Betta* sp.) have the closest relationship with *B. stigmosa*, where the branching line of the Palo fish group is directly related to *B. stigmosa*, followed by *B. ferox* and *B. apollon* in the *Pugnax* group. Based on genetic distance values (Table 1) Palo fish (*Betta* sp.) are related as sister taxa to *B.* cf. *apollon*, *B. ferox*, *B. stigmosa*, and *B. apollon*. According to Kartavtsev et al. (2016), the differences in genetic distance values between Palo fish (*Betta* sp.) and *B.* cf. *apollon*, *B. ferox*, *B. stigmosa*, and *B. apollon* indicate differences at the same species level. In the previous study, Putri et al. (2021) used the Cyt b gene and reported that the Palo fish has the closest relationship to *B. picta*, with a genetic distance of 13.00%.

This value led Putri et al. (2021) to assume that the Palo fish (*Betta* sp.) from Harau Valley is a potential new species within the genus *Betta*. The difference in results between the analyses using the COI and the Cyt b genes occurs due to using different reference sequences. In the previous study, the Cyt b gene sequences were unavailable for *B. stigmosa*, *B. ferox*, and *B. apollon*, closely related to the Palo fish (*Betta* sp.) in the COI gene analysis. Additionally, in the COI gene analysis, reference sequence data for *B. picta* was unavailable in GeneBank, NCBI, making it impossible to determine the relationship between the Palo fish (*Betta* sp.) and *B. picta*.

The differences in position and relationships of the Palo fish (*Betta* sp.) with other *Betta* species due to the incomplete availability of comparative sequence data for different genes (COI and Cyt b) indicate the need for further study to provide the sequence data for other *Betta* species. Hence, the systematics within the *Betta* genus can be resolved. Study on the other *Betta* species also shows that the relationship between *Betta enisae* Kottelat 1995 and *Betta edithae* Vierke 1984 is still being determined due to GenBank's lack of *Betta* species sequence data (Panijpan et al. 2014). So, more than the sequence data is needed to determine the relationship between closely related species. The close relationship between Palo fish (*Betta* sp.) and *B. stigmosa*, *B. ferox*, *B. apollon*, and *B.* cf. *apollon* has also been suspected to be related to the proximity of their distribution areas. *B. stigmosa* was reportedly distributed in Peninsular Malaysia (Tan and Ng 2005), while *B. ferox*, *B. apollon*, and *B.* cf. *apollon* have been distributed in Thailand (Schindler and Schmidt 2006; Panijpan et al. 2014).

Table 1. Comparison of the genetic distance of Palo fish (*Betta* sp.) with its sister taxa based on the COI gene

Genetic distance (%)			
B.* cf. *apollon	***B. ferox***	***B. stigmosa***	***B. apollon***
2.6	3.1	3.6	3.7

Meanwhile, Palo fish (*Betta* sp.) has been reported by Putri et al. (2021) to be found in the Harau Valley, West Sumatra, Indonesia. This data is the first distribution information reported for Palo fish (*Betta* sp.). However, geographical isolation separates the distribution areas of Palo fish (*Betta* sp.), *B.*cf. *apollon*, *B. ferox*, *B. stigmosa*, and *B. apollon*, this does not significantly change the nucleotide base sequence of their COI gene. Thus, there are still differences in genetic distance at the same species level. Previous research by Roesma et al. (2018) has reported that *Puntius* cf. *binotatus* Valenciennes 1842 from Lake Diatas Solok, West Sumatra, Indonesia has a close genetic distance to *Puntius* cf. *binotatus* from the Batang Lembang River, Solok, West Sumatra, Indonesia while there is no connecting access between the lake and the river. These two areas are considered to be connected in the past, allowing genetic mixing between lake and river populations.

Panijpan et al. (2014) have reported that *Betta prima* Kottelat 1994 and *Betta pallida* Schindler and Schmidt 2004 have separate distribution areas between southern and eastern Thailand but have identical COI gene sequences. The separation of Thailand's southern and eastern regions has provided little time for changes in the nucleotide bases of the COI gene. Based on the case of the *Betta* group, it is estimated that the tributaries in the Harau Valley were once connected to rivers in Malaysia and Thailand. This statement is supported by geological records by Voris (2000), Hall and Morley (2004), and Sathiamurthy and Voris (2006) who reported that Sumatra Island was once connected to the mainland with Malaysia and Thailand known as Sundaland. These geological records play a crucial role in supporting the genetic relationships among Palo fish (*Betta* sp.) with *B.* cf. *apollon*, *B. ferox*, *B. stigmosa*, and *B. apollon*, suggesting they may have originated from the same ancestor.

Palo fish (*Betta* sp.) with *B.* cf. *apollon*, *B. ferox*, *B. stigmosa*, and *B. apollon*, which have close genetic distances for different species levels, are estimated to be due to living in similar habitat conditions. This research reports that Palo fish (*Betta* sp.) were collected in depressions formed from tree roots or large rocks in Rangkak Hills tributaries with a substrate of a mixture of sand and gravel in water conditions with a pH of 6.2-6.5 and temperature of 22-28°C. This report is in accordance with previous reports by Putri et al. (2021). The same thing was found in *B. stigmosa* which lives in hilly swamp streams that lead to waterfalls (Tan and Ng 2005). *B. apollon* is found among plant roots, leaf litter, and sometimes under river rocks in shaded hilly forest areas, with a pH of 6.2 and water temperature of 24-26°C (Schindler and Schmidt 2006). *B. ferox* is found among tree roots in small rivers near waterfalls, with a substrate of mixed gravel and sand, according to Schindler and Schmidt (2006).

The characteristics of the *Pugnax* group that inhabit non-migratory nests (Ayyubi et al. 2020) and aggressively oppose individuals from different populations (Panijpan et al. 2020) also contribute to the persistence of COI gene sequences in the Palo fish (*Betta* sp.), *B.* cf. *apollon*, *B. ferox*, *B. stigmosa*, and *B. apollon*. This characteristic can also be seen in *B.* cf. *apollon*, *B. ferox*, *B. stigmosa*, and *B. apollon*, which are grouped in the *Pugnax* group (Panijpan et al. 2014). Therefore, based on molecular systematic studies using the COI gene, it can be stated that the Palo fish (*Betta* sp.) from Harau Valley, West Sumatra, is the same species as *B.* cf. *apollon*, *B. ferox*, *B. stigmosa*, and *B. apollon*. Panijpan et al. (2014), and Panijpan et al. (2020) stated that *B. ferox* and *B. apollon* are suspected to be the same species based on the low differences in the genetic distance values of the COI gene DNA sequence.

Based on morphological characters there are several differences in morphological characters between Palo fish (*Betta* sp.) and *B. stigmosa*, *B. apollon*, and *B. ferox* (Figure 3). These differences include body characteristics, according to Schindler and Schmidt (2006) *B. apollon* has the characteristic elongation of the branchiostegal to the posterior operculum, which the Palo fish (*Betta* sp.) does not have. Schindler and Schmidt (2006) and Tan and Ng (2005) reported that *B. stigmosa* is a new species within the *Pugnax* group due to its dark-edged posterior fin.

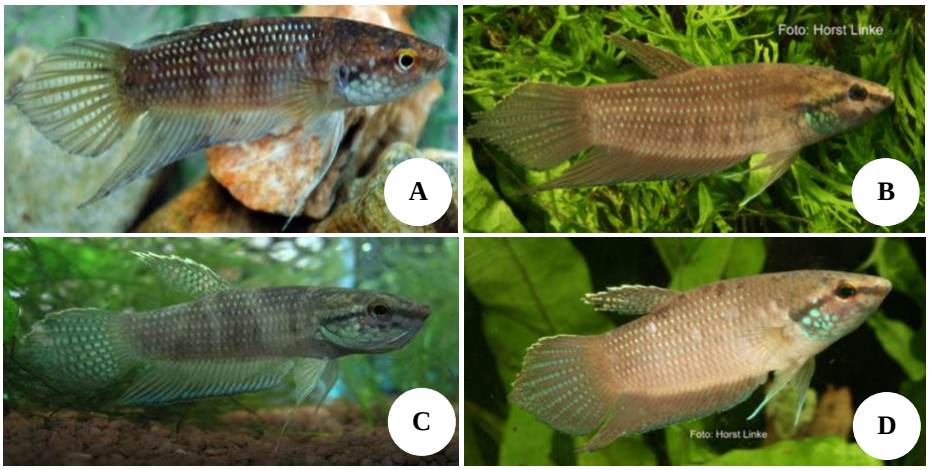

Figure 3. A. The figure of Palo fish (*Betta* sp.) (personal documentation); B. *B. apollon* (source: Fishbase); C. *B. stigmosa* (source: Fishbase); D. *B. ferox* (source: Fishbase)

Betta ferox was reported by Schindler and Schmidt (2006) to have a more intense dark pattern than *B. apollon* and *B. stigmosa*. However, the differences in morphological characteristics that make it a distinct species are inconsistent with the results of molecular analysis of the COI gene. This shows that morphological characteristics cannot be used as a reference in distinguishing between Palo fish (*Betta* sp.) with *B. stigmosa*, *B. apollon*, and *B. ferox*. Figure 3 also shows that the differences in morphological characters between them are not very clear. Panijpan et al. (2020) and Fahmi et al. (2020) also state that the taxonomic study for the genus *Betta* is still limited, and further study on the phylogenetic relationship of *Betta* species is needed. Therefore, advanced comparative studies were still essential to explain *Betta*'s inter-species relationships

Based on the results of molecular analysis (nucleotide base variations and phylogenetic trees) supported by morphological data, it can be stated that the Harau Valley Palo fish has the scientific name *Betta* cf. *stigmosa*. This study confirms that Palo fish (*Betta* sp.) is a sub-species of *B. stigmosa*, *B. apollon*, or *B. ferox*, which belongs to the *Pugnax* group. However, our understanding of the *Betta*'s group is not yet complete. It's urgent that further molecular systematic studies of other *Betta* species are conducted to fully explain the relationships within the group, underlining the importance and urgency of this research.

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