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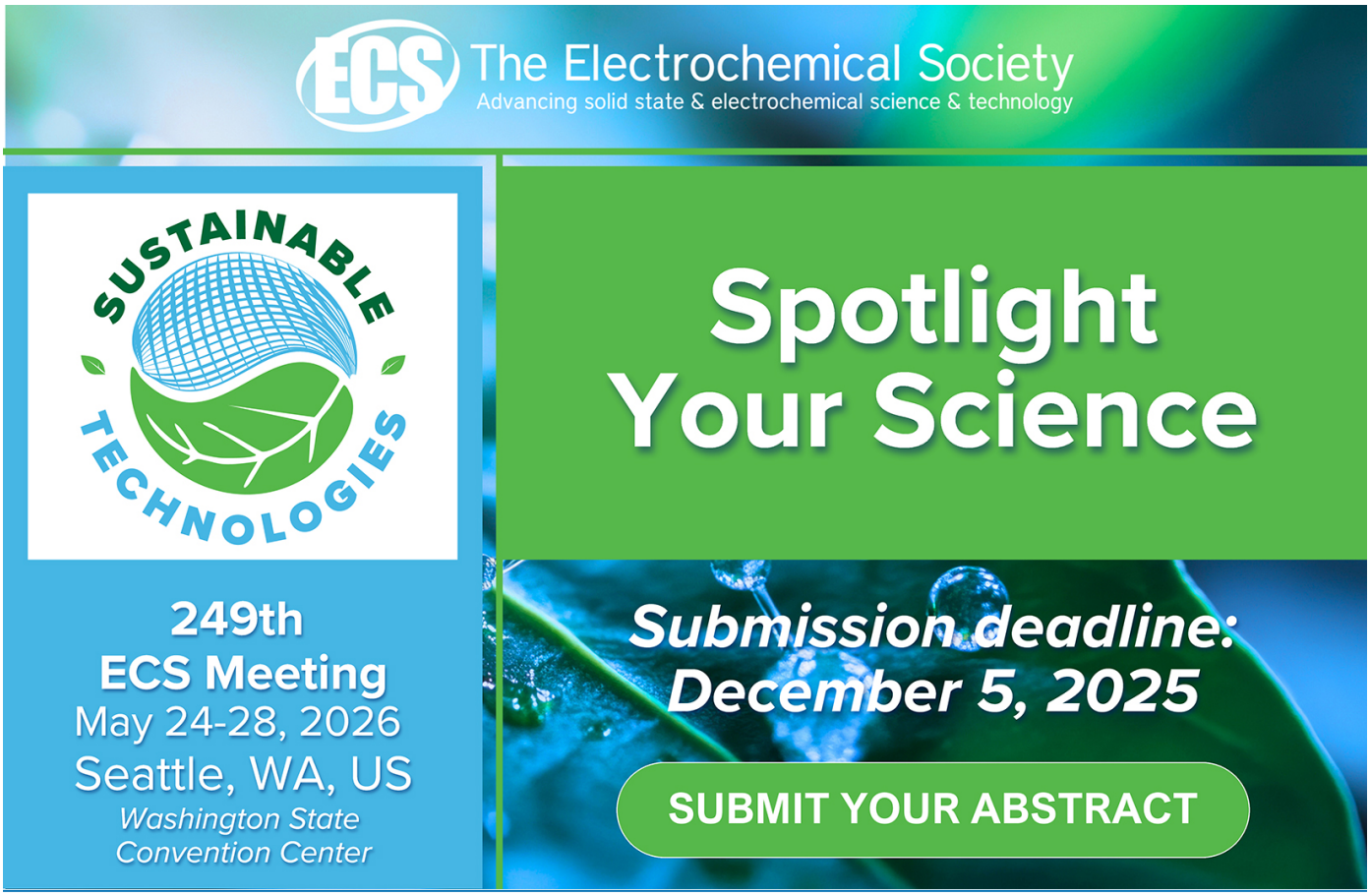

Unveiling the close relationship between *Betta burdigala* and *Betta uberis* through DNA barcoding based on COI Gene

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Abstract. A Wild *Betta burdigala* is only known from Bangka Island, while a Wild *Betta uberis* resides in Kalimantan Tengah and Kalimantan Barat in Indonesia. Both species share numerous physical characteristics, particularly in the meristic count, body color, and specific habitat. In this study, we estimate the degree of similarity between *Betta burdigala* and *Betta uberis* using DNA barcoding based on the Cytochrome Oxidase Sub Unit I (COI) gene. The COI gene is a unique and small gene which found in mitochondrial DNA and is used in the DNA barcoding approach to identify species. This approach allows for the analysis of species similarities and the learning of the evolutionary histories of those species. Based on the COI gene, *Betta burdigala* and *Betta uberis* have a close genetic distance and a DNA similarity of 96.66%, much higher than other bettas. They differ by eight nucleotide bases, and their genetic distance is 0.04, while a genetic distance between 0.010 and 0.099 is considered to be low and indicative of high similarity. According to the phylogenetic tree, these species are descended from a single, closely related ancestor on the same branch. Based on the COI gene, we assume that they are identical. Additionally, we advise conducting additional research using the mitochondrial DNA complex and in-depth morphological examination to confirm the accuracy of the study's findings.

1. Introduction

The variety of freshwater fish in Indonesia is quite high, currently, there are 1,266 species of freshwater fish in Indonesian inland waters in 2022 [1], and more than 8.500 fish species categorized based on habitat features (e.g., salty, brackish, and freshwater) [2,3]. The diversity of Indonesian freshwater fish consists of endemic, native, introduced, and reintroduced [4,5]. However, the diversity of freshwater species will be greatly influenced by human activities around the waters, directly and indirectly. Human

activities like habitat modification, overfishing, and the introduction of alien species, can all pose threats to an area's ichthyofauna diversity [6-8].

Bangka Belitung province is a western part of Indonesia known for its high biodiversity [9,10]. However, The existence of freshwater fish in Bangka Belitung Island has recently moderately decreased because of the negative effects of tin mining which caused a decline in environmental quality due to chemical contamination and physical alteration of the river ecosystems, making the rivers susceptible to degradation and biodiversity loss [11,12]. One of the freshwater species which endemic to the Bangka Islands is a wild *Betta burdigala*. This species was listed as Critically Endangered (CR) on the IUCN Red List of Threatened Species [13]. Furthermore, in the other western region of Indonesia, called Borneo Island, there is also a *Betta* species, namely *Betta uberis* with a conservation status was Vulnerable (VU) according to the IUCN Red List of Threatened Species [14]. The population of those species has significantly declined based on their conservation category.

Betta budigala and *Betta uberis* are the wild betta from the Osphronemidae's Family and Betta's genus. The two species have a strong morphological resemblance, at first glance they are the same species; in fact, they are different species [15]. However, the life history of those species can be traced by molecular analysis using DNA barcoding [16]. In addition, DNA barcoding can also be used as a tool for rapid species identification by using standardized genetic regions, and one or more genes in mitochondrial DNA [17]. One approved gene used to identify species is the cytochrome c oxidase subunit I (COI) gene found in mitochondrial DNA [18]. The use of the COI gene as a species identification tool has successfully identified freshwater fish in Indonesia [19,20]. This study aims to identified the close relationship between *Betta burdigala* and *Betta uberis* Through DNA barcoding based on COI Gene. We will discuss the percentage of similarity between the two species, then analyze the polymorphic sites between species, then the genetic distance between the two species and finally we will present the evolution of the two species using a phylogenetic tree.

2. Material and Method

Specimens of Wild *Betta burdigala* were in Bikang Stream, Toboali Area, Kabupaten Bangka Selatan, Indonesia amid the fieldwork from 7 to 13 April 2023, and the sequence of the COI gene of *Betta uberis* was obtained from the NCBI Genbank with the Accession number GQ911984.1 and GQ911983.1. DNA of the specimen was extracted using the 10% Chelex protocol following the BIONESIA method with FISH-F1 and FISH-R1 primers of COI gene [21]. The reaction mixture was then amplified using an Applied BiosystemsTM 2720 Thermal Cycler machine. PCR cycling parameters included an initial denaturing phase of 3 minutes, denaturing at 94^{o}C for 30 seconds, annealing at 48^{o}C for 30 seconds, and extension at 72^{o}C for 45 seconds for 38 cycles. The PCR results were then visualized in 1% agarose gel via electrophoresis by staining Nucleic Acid Gel Stain (GelRed®) [18]. A positive sample (sparkling DNA bands) was then processed for DNA reading (sequencing) using the Sanger dideoxy method [37].

Species Identification was calculated using the BLASTn (Basic Local Alignment Search Tool-nucleotide) in NCBI GenBank (https://blast.ncbi.nlm.nih.gov). The sequences were aligned using the Muscle algorithm [23]. The evolutionary history was inferred using the Neighbor-Joining method [24] with the bootstrap test about 1000 replicates [25]. The evolutionary distances were computed using the Maximum Composite Likelihood method [26] and are in the units of the number of base substitutions per site. Evolutionary analyses, Nucleotide composition and polymorphic sites were conducted in MEGA X [27].

3. Result and Discussion

3.1 DNA Barcoding and Species Identification

Cytochrome C Oxidase Subunit 1 (COI) gene sequences were used for species identification. Utilization of the COI gene has been proven informative for species identification on several freshwater species [28]. COI gene sequence of *B. burdigala* and *B. uberis* were 670 and 665 base pairs. The minimum of 658 bp long fragment using the COI gene can be used as a basis for differentiation between animals and species identification [29], and comparing the genetic distance between species. This information is very important to enrich science, especially to understand the taxonomy and improve knowledge in

biotechnology. This sequence also aims to register eukaryotic biodiversity in support of the Barcode of Life (iBOL) project [30]. Alignments of *Betta burdigala* and *Betta uberis* were obtained using the BLASTn method (Basic Local Alignment Search Tool-Nucleotides) (https://blast.ncbi.nlm.nih.gov) to analyze sequence homology, we also ensure species validity through the BOLD SYSTEM (https://www.boldsystems.org) by Specimen Identification tools to check the species level of similarity (Tabel 1).

Table 1. Species Identification and Similarity

Specimen	Simlarity GenBank	Species Outcome	Accession Number (GenBank)	Distribution
Betta burdigala (Bangka Island)	96.66	*Betta uberis*	GQ911983.1	Borneo
	88.30	*Betta Coccina*	KM485461.1	Malaysia & Indonesia
	81.96	*Betta anabatoides*	GQ911723.1	Borneo

The sequence of *Betta burdigala* from Bangka has around 96.66% similarity to *Betta uberis* from Borneo, which is almost 97%. According to Hebert et al., (2003) [29], sequences with 97-100% similarity are spoken to be identical, and species with 3% or more differences are different species. Based on DNA BLAST results from the COI gene, between *Betta burdigala* and *Betta uberis*. Based on geographical distribution (Fig 1), *Betta burdigala* was found on Bangka Island, Indonesia, and *Betta uberis* were found on Borneo Island Indonesia. Based on history, Borneo and the Bangka Islands were connected to the ancient river which existed thousands of years ago called Sundaland. Belitung Island is located in the Greater Sunda Islands region of Indonesia [31]. In addition, Bangka Island is up to 500 km southeast of the nearest locality in Peninsular Malaysia, about 450 km southeast of the nearest locality in sumatra, and about 750 km southwest from the nearest locality in Borneo. The extent of the Sundaland is approximately 1,800,000 km 2 including the Malay Peninsula, Sumatra, Java, and the islands of Borneo [32-35].

3.2 Polymorphic sites

Differences in the nucleotide bases of a species can be identified by conducting polymorphic site analysis. This analysis aims to determine the location of sites that experience variations (changes) within the same species. There are 18 nucleotide bases out of 687 nucleotide bases between *Betta burdigala* and *Betta uberis* (Table 2).

Table 2. Polymorphic Sites of *Betta burdigala* and *Betta uberis*

Sequences	**Nucleotide site**								
	25	**43**	**82**	**85**	**86**	**94**	**230**	**231**	**256**
Betta burdigala	C	A	A	A	T	G	C	C	G
Betta ubesis	T	G	G	G	C	C	T	T	A
Sequences	**262**	**271**	**297**	**411**	**417**	**483**	**528**	**639**	**655**
Betta burdigala	T	A	G	G	T	T	A	T	C
Betta ubesis	C	G	A	A	C	C	G	C	T

There are 18 nucleotide bases that differ between *B. burdigala* and *B. uberis* out of a total of 687 base pairs. The mutations were dominated by transition mutations (A↔G and C↔T), 17 mutations and 1 transversion mutation (G↔C). Transition mutations are the replacement of purines (A, G) with fellow purines or the replacement of pyrimidines (C and T) with fellow pyrimidines. A transversion replaces of a purine into a pyrimidine or vice versa. Transversion is the replacement of a purine to a pyrimidine or a pyrimidine to a purine. Transversions usually result in a greater probability of protein change than transitions because there is a more drastic change in the process of forming amino acids [30]. There are

four possible mutations in transition (A↔G, C↔T) and eight possible mutations in transversion (A↔C, A↔T, G↔C, G↔T).

3.3 Genetic Distance and phylogenetic tree

Genetic distance is the degree of difference in a gene which is calculated based on differences between species or populations. The closest distance between species occurs in *Betta burdigala* and *Betta uberis* about 0.04 (Table 3), which means that out of 100 base pairs, there are 4 different base pairs. According to Nei (1972) [36], a genetic distance of 0.010-0.099 is included in the low category, 0.1-0.99 is included in the medium category, and a genetic distance of 1.00-2.00 is included in the high category.

Table 3. Estimation of Evolutionary Divergence between *Betta burdigala* and *Betta uberis*

		1	2	3	4
1	*Betta burdigala*				
2	*Betta anabatoides*	0,22			
3	*Betta uberis*	0,04	0,21		
4	*Betta coccina*	0,14	0,21	0,11	

Following the genetic distance, we reconstructed phylogenetic relationship of *Betta burdigala* and *Betta uberis* were based on the mitochondrial COI gene to analyze the evolutionary history of *Betta burdigala* and *Betta uberis* (Figure 1).

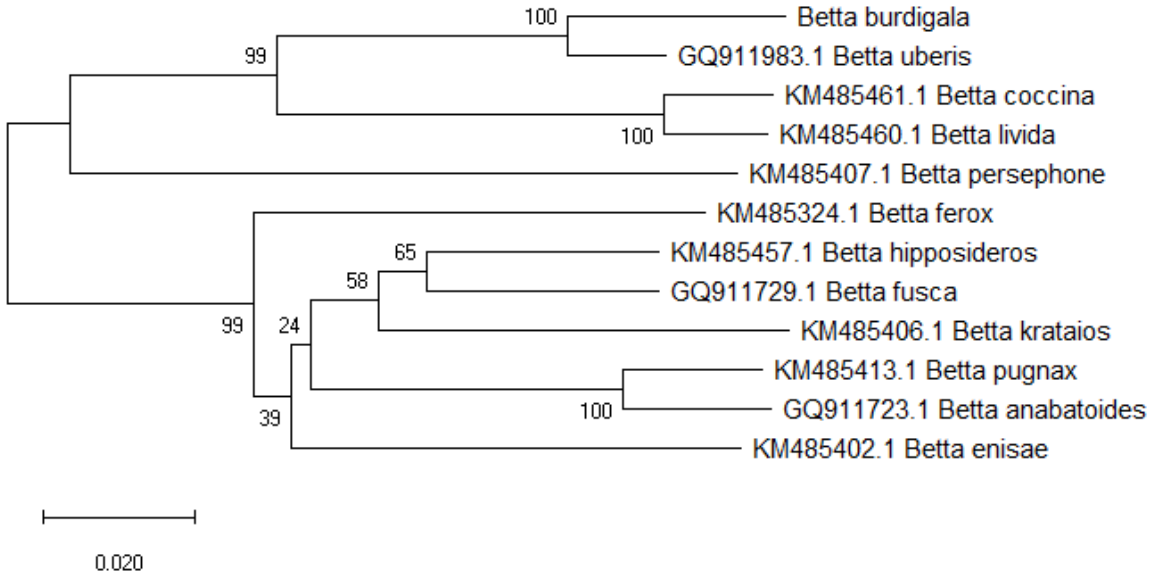

Figure 1. Evolutionary relationships of *Betta burdigala* and *Betta uberis* based on COI Gene

The bootstrap value on the evolutionary tree of *Betta burdigala* and *Betta uberis* is more than 100, this shows that the branching is very accurate, and consistent. A branch of a phylogenetic tree that is more than 70% is a branch that has truth with a 95% confidence interval [37]. The phylogenetic tree forms 2 branches and 2 clades. *Betta burdigala* and *Betta uberis* are in the same clade. This shows that they are very close in family tree and evolutionary history. This supports our speculation that these species are morphologically similar, and Tan & Ng [15] actually also grouped these species into one group, namely the coccina group. However, further evidence regarding the evolutionary history and genetics of *Betta uberis* and *Betta burdigala* is still required. To gain a comprehensive understanding,

we suggest analyzing the entire genome to determine the extent of similarity between these two species. Additionally, it is advisable to conduct morphological comparisons to support these hypotheses.

4. Conclusions

This study identified the close relationship between *Betta burdigala* and *Betta uberis* Through DNA barcoding based on COI Gene. Sequence *B. burdigala* was obtained from Bangka Island, Indonesia with the accession number of GenBank OQ281707 and *Betta uberis* was acquired from Borneo with the Accession number of GenBank GQ911983. The analysis of the evolutionary history of *B. burdigala* revealed its close relationship with *Betta uberis*, The genetic distance between the two species was found to be around 0.04. Those species had 18 nucleotide bases differences out of a total of 687 base pairs. The mutations were dominated by transition mutations (A↔G and C↔T) baout 17 mutations and one transversion mutation (G↔C). Moreover, the study highlighted the significant association between Borneo and Bangka Island, established through the ancient river, Sundaland, which explains the shared biodiversity between these two regions. It is noteworthy that Bangka Island, situated approximately 750 km southwest of the nearest Borneo locality, was previously part of this ancient river system. However, more research is still needed to understand the genetics and evolutionary history of *Betta uberis* and *Betta burdigala.* We recommend examining the full genome to assess the degree of similarity between these two species in order to acquire a thorough knowledge. To further support these hypotheses, morphological comparisons should be made.

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