

Molecular Identification Based on COX1 Partial Gene of Red Shrimp from Anchialine Habitat at Buton Island, Indonesia

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Abstract: Anchialine ecosystems are unique coastal habitats that remain poorly explored in Indonesia and may support distinctive, endemic, and taxonomically unresolved fauna. In an anchialine pool in the Koguna Beach area on Buton Island, a conspicuous red shrimp has long been observed by local communities, yet its taxonomic identity has remained unresolved. This study aimed to provide the first molecular identification and genetic confirmation of this red shrimp using a partial mitochondrial COX1 gene sequence as a genetic marker. Shrimp tissue samples were collected from the study site and preserved in 96% ethanol. Total DNA was extracted using the DNeasy Blood & Tissue Kit, amplified by polymerase chain reaction (PCR), and sequenced using the Sanger method. The resulting COX1 sequences (713 bp) were trimmed and edited, yielding a final alignment of 570 bp for analysis. BLASTn results showed >99% sequence identity with *Parhippolyte uveae*. Intraspecific genetic distances were low (0.00173–0.00346), whereas interspecific distances between *P. uveae* and *P. misticia* (0.07313–0.07698), and between *P. uveae* and *Macrobrachium rosenbergii* (>0.25), supported species-level separation. Phylogenetic reconstruction further clustered the Buton Island samples within the *P. uveae* clade. In conclusion, molecular analysis supported the identification of the red shrimp from the study site as *P. uveae* and highlighted the importance of molecular approaches for biodiversity assessment and conservation planning in culturally protected and understudied anchialine habitats.

Keywords: Buton Island, mitochondrial partial gene, DNA barcoding, *Parhippolyte uveae*.

1. Introduction

Buton Island is one of the areas in Indonesia that supports endemic species. Klotz and von Rintelen (2013) reported the existence of a new endemic shrimp species on the island, named *Caridina butonensis*. It is similar to the previously described *C. boehmei*, found in Central Sulawesi, and *C. opaensis*, found in Southeast Sulawesi. Meanwhile, an unidentified shrimp species occurs in an anchialine pool in the Koguna Beach area, and its taxonomic identity has remained unresolved until the present study. Local communities refer to it as the red shrimp because of its bright red colouration. Anchialine habitats are unique, land-locked water bodies containing a mixture of freshwater and saltwater (mixohaline) and connected underground to both the ocean and groundwater. They typically form in karst, volcanic, or limestone settings and are characterised by strong stratification, most notably haloclines that separate water layers of differing salinity (Findra et al., 2024; Ghaly et al., 2023; Marrack et al., 2015; Shishido et al., 2022).

Species identification based on morphological characteristics, including morphometric and meristic traits, requires a large number of samples. This process may be subject to uncertainty because of cryptic and species-complex phenomena (Bickford et al., 2007). In contrast, molecular identification can provide evidence to support taxonomic status using only a small number of samples. This approach is particularly relevant because the red shrimp is considered sacred by the local community and should not be collected. The use of molecular markers can help resolve this taxonomic uncertainty (Arlyza et al., 2013; Findra et al., 2017, 2020; Su et al., 2014), thereby supporting appropriate resource management. This molecular approach is particularly crucial because the shrimp is considered sacred by the local community, limiting sample collection and making genetic identification the most suitable method.

A commonly used molecular marker for animal identification is the cytochrome c oxidase subunit I (COX1) gene (Findra et al., 2025; Hajibabaei et al., 2007; Hakim et al., 2022; Hebert et al., 2003). This mitochondrial gene fragment is commonly used to study species and animal populations (Findra et al., 2017; Samadan et al., 2024; Solihin, 1994). Furthermore, it is generally used as a DNA barcode because it evolves relatively slowly (Dhar et al., 2016; Hebert et al., 2003; Hernawati et al., 2020; Ratnasingham & Hebert, 2013; Sutrisno et al., 2013). Therefore, DNA barcoding is an efficient method for species-level identification and contributes to biodiversity taxonomy.

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Species identification in aquatic organisms often faces challenges arising from cryptic species. Cryptic species are two or more distinct species that are classified as a single species because of similar or ambiguous morphological characters (Bickford et al., 2007; Butet et al., 2019). Taxonomic certainty is essential for determining appropriate resource management, and molecular approaches offer one means of resolving this problem. Therefore, this study aimed to provide the first molecular identification and confirmation of the red shrimp species from Buton Island using a partial COX1 gene sequence.

2. Material and Methods

Sample Collection

Sample collection was conducted at an anchialine pool in the Koguna Beach area, Buton Island, Southeast Sulawesi, Indonesia, in 2021 (Figure 1). Shrimp tissue samples were collected from the study site. Subsequently, the tissue samples were placed in tubes and preserved in 96% ethanol.

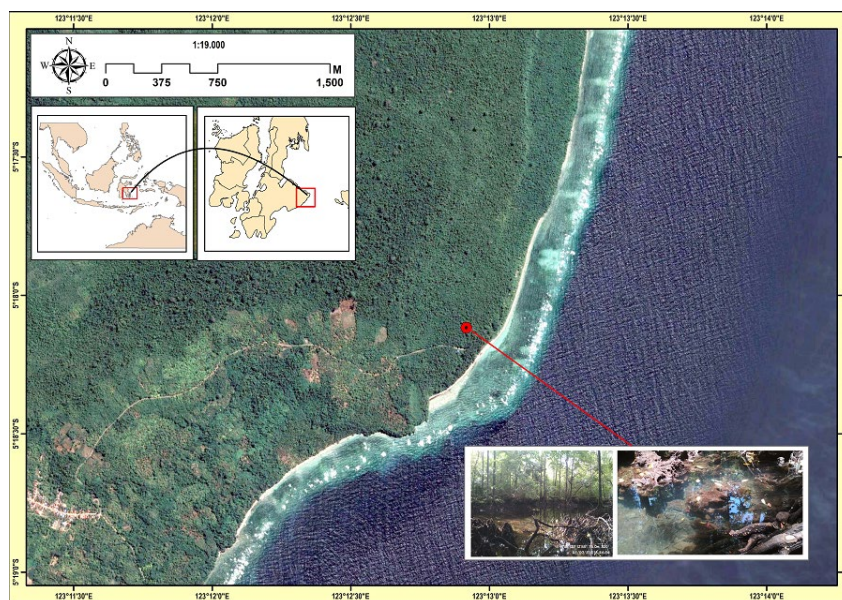


Figure 1. Sample collection site on Buton Island.

Isolation and Extraction of Total DNA

Prior to DNA extraction, the samples were washed with low-TE buffer to remove the ethanol solution. Subsequently, total DNA was isolated and extracted using the DNeasy Blood & Tissue Kit, following the manufacturer's protocol. The concentration and purity of the extracted DNA were measured using a spectrophotometer.

Visualisation, PCR Amplification, and Sequencing of Total DNA

The extracted and purified DNA products were electrophoresed on a 1.2% agarose gel in Tris-borate-EDTA (TBE) buffer. The gel was stained with 0.5 g/L ethidium bromide (EtBr) and visualised using a UV transilluminator ($\lambda = 300$ nm). This visualisation was performed to confirm the successful extraction of DNA.

The PCR reaction was performed in a total volume of 26 μ L, comprising 2 μ L of template DNA, 1.25 μ L of forward primer jgLCO1490, 1.25 μ L of reverse primer jgHCO2198, 9 μ L of nuclease-free water, and 12.5 μ L of Ready Mix. PCR amplification was performed using an Applied Biosystems 2720 Thermal Cycler. The PCR cycling conditions comprised denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 45 s for

38 cycles, followed by a final extension at 72 °C for 5 min. The forward primer jgLCO1490 and reverse primer jgHCO2198 were used, with nucleotide sequences of TITCIACIAAYCAYAARGAYATTGG and TAIACYTCIGGRTGICCRAARAAYCA, respectively (Geller et al., 2013). The amplified products were subsequently sequenced using the Sanger sequencing method at a sequencing service facility.

Sequence Alignment and Data Analysis

The sequence of each sample was analysed using BLASTn against the GenBank database to determine species identity and sequence similarity to other GenBank records. The sequencing results were then edited and aligned using MEGA 11 software (Tamura et al., 2021). Phylogenetic tree reconstruction was performed using the neighbour-joining (NJ) method with the Kimura two-parameter model and 1,000 bootstrap replicates in MEGA 11. Subsequently, genetic-distance analysis and phylogenetic-tree reconstruction were conducted using selected GenBank sequences as ingroup and outgroup taxa.

3. Results And Discussion

The resulting sequences (713 bp) were trimmed and edited to remove low-quality bases, resulting in a final alignment of 570 bp for downstream analyses. Furthermore, the quality of the PCR products was assessed by 1.2% agarose gel electrophoresis and visualisation under a UV transilluminator, confirming successful amplification (Figure 2).

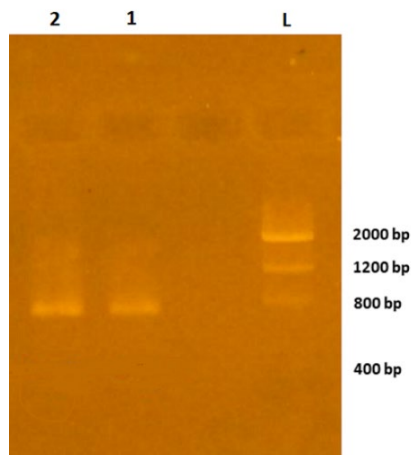


Figure 2. Successfully amplified PCR products (L = DNA ladder; 1 = red shrimp sample 1; 2 = red shrimp sample 2).

The sequences were analysed using BLASTn against the National Center for Biotechnology Information (NCBI) GenBank database to verify their molecular identities (Table 1). BLASTn results showed that the red shrimp samples from the study site matched *Parhippolyte uveae*, with percentage identities of 99.65% for sample 1 and 99.83% for sample 2. These results supported the identification of both samples as *P. uveae*. The BLASTn results for both red shrimp sequences also showed lower similarity to *P. misticia* (approximately 92% identity) and *Macrobrachium* sp. (approximately 80% identity). *Parhippolyte uveae* is a member of the family Barbouriidae within the order Decapoda (De Grave & Fransen, 2011) and is commonly referred to as the sugarcane shrimp or caridean shrimp. It is a bright-red species with a wide geographic distribution, including Aldabra in the western Indian Ocean; Tiniguiban Island, Philippines; Halmahera, Northern Maluku, Indonesia; Uvea and Sayawa Islet in the Loyalty Islands; Eniwetok Atoll, Vatulale Island, Fiji Archipelago; Vanua Vatu and Vanua Levu Islands in the Ellice Islands; Oahu, Hawaii, and Maui in the Hawaiian Islands; and Kakaban Island, East Kalimantan, Indonesia (reviewed by Fransen & Tomascik, 1996), as well as Tanjung Sanjangan, Central Sulawesi, Indonesia (Arisuryanti et al., 2020).

Table 1. BLASTn results for nucleotide sequences searched against the NCBI GenBank database.

Sample	Query cover (%)	Identity (%)	Species identification	Accession number
Red shrimp 1	88	99.65	<i>P. uveae</i>	MT524351.1 (NCBI, 2020a)
	88	92.75	<i>P. misticia</i>	MT524350.1 (NCBI, 2020b)
	88	92.40	<i>P. misticia</i>	MT524349.1 (NCBI, 2020c)
	88	92.23	<i>P. misticia</i>	MT524348.1 (NCBI, 2020d)
	99	80.09	<i>Macrobrachium</i> sp.	MW845576.1 (NCBI, 2021)
Red shrimp 2	97	99.83	<i>P. uveae</i>	MT524351.1 (NCBI, 2020a)
	88	92.92	<i>P. misticia</i>	MT524350.1 (NCBI, 2020b)
	88	92.57	<i>P. misticia</i>	MT524349.1 (NCBI, 2020c)
	88	92.40	<i>P. misticia</i>	MT524348.1 (NCBI, 2020d)
	99	80.25	<i>Macrobrachium</i> sp.	MW845576.1 (NCBI, 2021)

Genetic distances among *P. uveae* sequences from the 570-bp COX1 gene alignment and reference sequences retrieved from GenBank were analysed using the Kimura two-parameter model (Table 2).

Table 2. Pairwise genetic distances among *Parhippolyte uveae* from Buton Island and reference sequences, estimated using the Kimura two-parameter model.

	1	2	3	4	5	6	7
1							
2	0.00173						
3	0.00346	0.00173					
4	0.07698	0.07501	0.07313				
5	0.08085	0.07887	0.07698	0.00695			
6	0.08275	0.08076	0.07887	0.00869	0.00173		
7	0.25749	0.26015	0.25985	0.25956	0.25720	0.25956	

Notes:

1. *P. uveae* Buton Island 1
2. *P. uveae* Buton Island 2
3. *P. uveae* (MT524351.1)
4. *P. misticia* (MT524350.1)
5. *P. misticia* (MT524349.1)
6. *P. misticia* (MT524348.1)
7. *Macrobrachium rosenbergii* (MW845595.1)

For the 570-bp COX1 gene fragment, the intraspecific genetic distance among *P. uveae* sequences ranged from 0.00173 to 0.00346, whereas the interspecific distance between *P. uveae* and *P. misticia*, which belong to the same family, ranged from 0.07313 to 0.07698. In contrast, the genetic distance between *P. uveae* and *M. rosenbergii*, which belong to different families, ranged

from 0.25720 to 0.26015 (Table 2). Genetic distance provides an indication of evolutionary divergence among sequences and may assist in distinguishing taxa. According to Hebert et al. (2003), genetic divergence exceeding 3% may indicate distinct species in DNA-barcoding analyses. The phylogenetic reconstruction is presented in Figure 3.

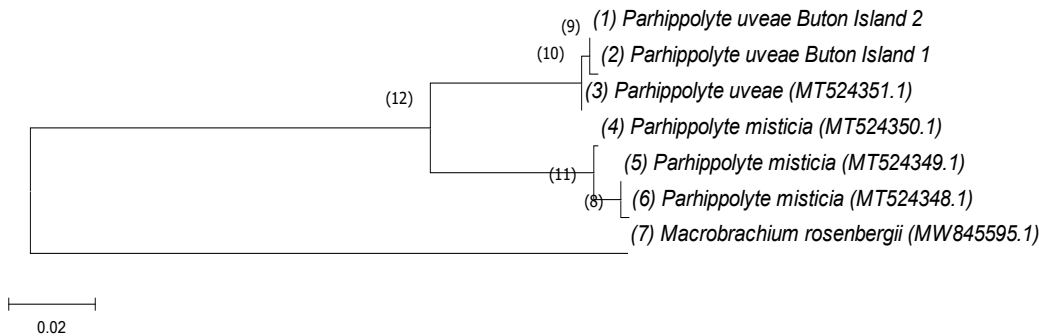


Figure 3. Neighbour-joining phylogenetic tree based on the Kimura two-parameter model, with 1,000 bootstrap replicates.

The phylogenetic analysis showed that each species formed a separate group. The *P. uveae* sequences obtained in this study clustered with the *P. uveae* reference sequence from Aldabra (Ditter et al., 2020) and were separated from *P. misticia* sequences. This result supports the identification of the red shrimp inhabiting the anchialine pool on Buton Island as *P. uveae*. The phylogenetic tree also showed that *P. uveae* is closely related to *P. misticia* (accession numbers MT524350.1, MT524349.1, and MT524348.1). This relationship is consistent with the classification of both species in the family Barbouriidae (De Grave & Fransen, 2011). Meanwhile, *M. rosenbergii*, used as the outgroup, formed a separate clade from *P. uveae* and *P. misticia* and belongs to the family Palaemonidae (De Grave & Fransen, 2011; Wowor et al., 2009; Wowor & Ng, 2007).

Molecular identification plays a critical role in understanding anchialine biodiversity, particularly in ecosystems characterised by high levels of endemism and cryptic diversity. Anchialine habitats are coastal, land-locked brackish-water systems with subterranean connections to the ocean that host unique and often endemic species. Molecular tools, such as DNA barcoding and next-generation sequencing (NGS), enable the identification of morphologically indistinguishable or poorly studied species and can reveal cryptic diversity and genetic differentiation among populations (Pérez-Moreno et al., 2016; Santos, 2006; Santos et al., 2023). For example, molecular analyses of the Hawaiian anchialine shrimp *Halocaridina rubra* revealed distinct genetic lineages, highlighting regional endemism and restricted gene flow that are important for understanding population structure and biogeographic patterns (Santos, 2006; Santos et al., 2023).

4. Conclusion

Molecular analysis supported the identification of the red shrimp collected from an anchialine habitat on Buton Island. Based on analysis of the COX1 gene fragment, the shrimp was identified as *Parhippolyte uveae*.

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