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Original Article

Species confirmation of freshwater prawns in Ternate Island, Indonesia, through DNA barcoding: Not *Macrobrachium rosenbergii*

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Abstract: Freshwater prawns of the genus *Macrobrachium* are widely distributed across tropical and subtropical regions. On Ternate Island, the local community refers to native freshwater prawns as “udang galah”, a name typically associated with *Macrobrachium rosenbergii*. This study aimed to accurately identify freshwater prawn species on Ternate Island using DNA barcoding. Prawn samples were collected from two different locations on Ternate Island: Togafo River, which has a steep gradient and flows directly into the sea without being affected by tidal salinity, and the river in Fitu Village, which consists of isolated pools with no connection to marine waters. Tissue samples were taken and preserved in 96% alcohol for molecular analysis. DNA barcoding results confirmed that the identified species is *Macrobrachium lar*, with genetic similarity ranging from 99.50% to 99.83% to reference data in GenBank. The presence of *M. lar* in isolated freshwater habitats raises questions about its dispersal patterns and life cycle, as this species is amphidromous and typically requires brackish water for larval development. These findings suggest the possibility of local adaptation or alternative dispersal mechanisms that warrant further investigation. Additionally, this study highlights the effectiveness of DNA barcoding for species identification, particularly for *Macrobrachium* species with high morphological variation or classified as cryptic.

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Introduction

Ternate Island, Indonesia, is part of an archipelago with high biodiversity, particularly in its freshwater ecosystems. Administratively, Ternate Island falls under the Fisheries Management Area of the Republic of Indonesia in Inland Waters (WPPNRI PD 413), which includes the waters surrounding Ternate Island and its vicinity. The freshwater habitats on this island support diverse aquatic species, including freshwater prawns, which have significant ecological and economic value. An exploration revealed the presence of freshwater prawns of the genus *Macrobrachium* Bate, 1868 (Crustacea: Palaemonidae) inhabiting small streams and isolated pools in the inland areas of Ternate Island. Some of these habitats are not directly connected to estuaries or lakes and are located in the foothills of Mount Gamalama, an active volcano. For years, the freshwater prawns found in Ternate Island have been identified as giant river prawns, referring to the species *Macrobrachium rosenbergii* (Abubakar et al., 2024; Muin et al., 2024; Samadan et al., 2022, 2023), which is widely recognized as a high-value fisheries commodity with a broad tropical distribution (Khair et al., 2000; Wowor and Ng, 2007; Wahidah et al., 2017). However, this claim is based solely on morphological identification, without genetic verification. This raises questions about the accuracy of such identification, considering the possibility of other species with similar morphological characteristics.

Morphological species identification often faces various challenges, particularly among organisms with high phenotypic variation or similar physical characteristics across species. In freshwater prawns, several species are cryptic (Cai et al., 2004; Jurniati et al., 2020), leading to frequent misidentifications. Therefore, a more precise and objective method is required to confirm the identity of freshwater prawn

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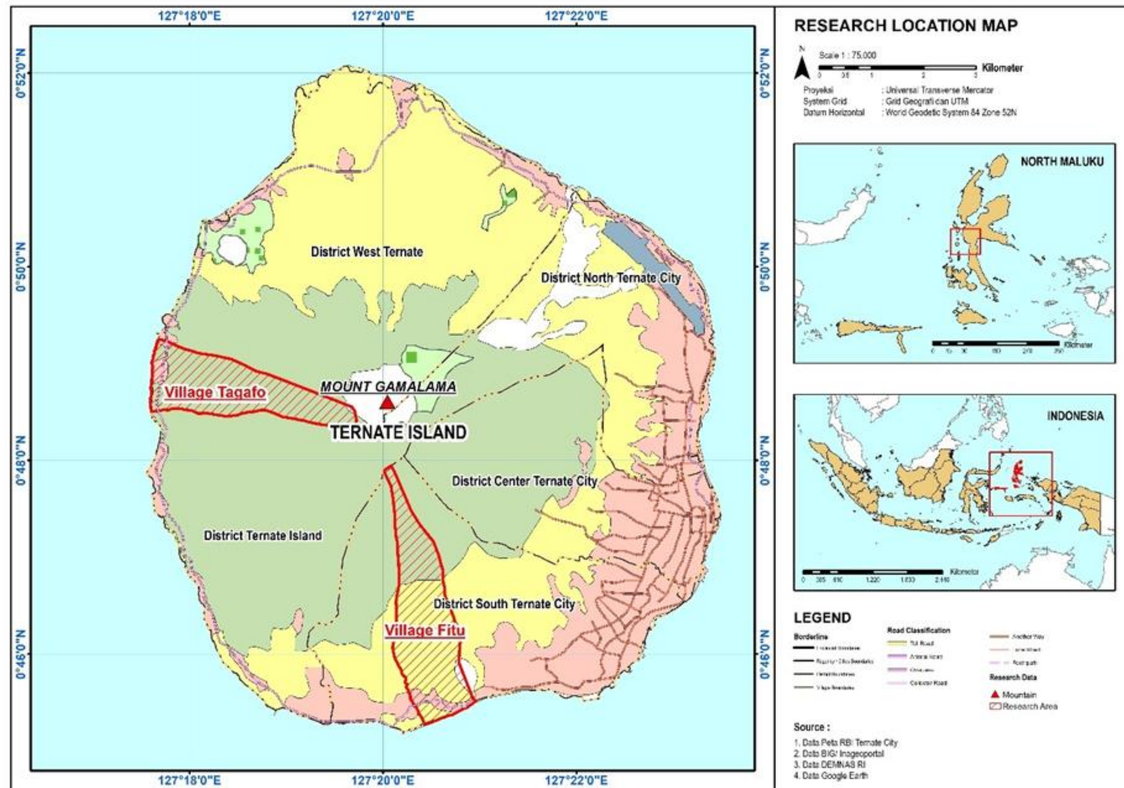

Figure 1. Sampling site of freshwater prawns in Ternate Island.

species in Ternate Island. One such method is DNA barcoding (Findra et al., 2020; Samadan et al., 2024; Syazili et al., 2024), a genetic sequence-based technique that is highly accurate for species identification.

DNA barcoding uses short sequences of target genes, such as the cytochrome c oxidase subunit I (COI) gene, to compare test specimens with reference databases (Hajibabaei et al., 2007; Ratnasingham and Hebert, 2013). This technique has been widely applied in various taxonomic and ecological studies to identify species, reveal phylogenetic relationships, and detect cryptic species that cannot be distinguished morphologically (Hebert et al., 2003; Siriwut et al., 2021). In the context of this study, DNA barcoding is highly relevant for testing the hypothesis of the presence of *M. rosenbergii* in freshwater habitats on Ternate Island.

Therefore, this study aims to confirm the identity of freshwater prawn species found on Ternate Island using DNA barcoding. Genetic analysis is expected to provide accurate information about the actual species inhabiting these waters. If the study reveals that the specimens found are not *M. rosenbergii*, this would have significant implications for taxonomy, conservation, and fisheries resource management in the region. According to Butet et al. (2019) and Findra et al. (2017), species misidentification can affect management and conservation strategies, underscoring the importance of DNA-based verification to ensure the accuracy of biodiversity data. Furthermore, the study's findings could offer new insights into the distribution and diversity of *Macrobrachium* species in Indonesia, as reported by Hernawati et al. (2020) and Siriwut et al. (2020). Thus, this research not only clarifies the taxonomic status of freshwater prawns on Ternate Island but also enhances understanding of freshwater biodiversity in Indonesia. The DNA barcoding approach employed in this study is expected to serve as a model for species identification in other studies, particularly for groups of organisms that pose challenges for morphological identification. Therefore, this study is a crucial step toward more accurate, effective, and sustainable

Figure 2. Natural habitat of freshwater prawns in Togafo (a) and Fitu (b) Villages.

management of freshwater fisheries resources.

Materials and Methods

Sample collection: Prawn samples were collected from two locations on Ternate Island (Fig. 1): (1) Togafo Village, where a freshwater stream flows directly into the sea but remains unaffected by tidal salinity due to its steep gradient (Fig. 2a), and (2) Fitu Village, a small, isolated river system composed of disconnected pools with no direct connection to seawater (Fig. 2b). Both locations represent exclusively freshwater environments. Tissue samples were obtained by excising a small portion of the prawn's tissue using sterile scissors. The samples were then placed in tubes and preserved in 96% ethanol for further genetic analysis.

DNA analysis: DNA was extracted using the Qiagen kit following the manufacturer's protocol. The extracted DNA was then analyzed through amplification using the Polymerase Chain Reaction (PCR) method. The process followed the protocol of BIONESIA Laboratory and used a pair of primers recommended by Geller et al. (2013), namely jgLCO (5'-TIT CIA CIA AYC AYA ARG AYA TTG-3') and jgHCO (5'-TAI ACY TCI GGR TGI CCR AAR AA-3'). PCR reaction mixture consisted of 2 µL DNA

template, 1.25 µL primers (at a concentration of 10 mM), 9 µL of ddH_2O, and 12.5 µL of ready mix. The reaction was amplified using an Applied Biosystems™ 2720 Thermal Cycler. PCR conditions were as follows: initial denaturation at 94°C for three minutes, denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 60 seconds, repeated for 38 cycles. The final extension was carried out at 72°C for 2 minutes. PCR results were visualized on a 1% Agarose gel stained with Nucleic Acid Gel Stain (GelRed®). High-quality samples were then subjected to Sanger sequencing at PT. Genetika Science Indonesia.

Data analysis: The sequencing results were edited and aligned using ClustalW in MEGA 12 (Kumar et al., 2024). Each base arrangement was manually checked to ensure high data quality. The sequences were compared with the existing database in the National Center for Biotechnology Information (NCBI) GenBank using the Basic Local Alignment Search Tool (BLASTn) on the NCBI website. Furthermore, a phylogenetic tree was constructed to determine relationships among samples using the Neighbour-Joining (NJ) method with 1000 bootstrap replicates in MEGA 12. For genetic distance analysis, the p-distance method was used. Genetic distance analysis and phylogenetic tree reconstruction included several sequences from GenBank as both ingroup and outgroup. The sequences used were *M. rosenbergii* (MF563571.1, MF563572.1), *M. lar* (PP702365.1, PP702367), *M. spelaeus* (MW845631, MW845632), *M. sintangense* (MW845621, MW845622), *M. thai* (MW845633, MW845634), *M. sirindhorn* (MW845627.1, MW845628), and *Cherax cairnsensis* (EU921142) as the outgroup.

Results

PCR product and species validation: The isolation and extraction of prawn samples from Ternate Island yielded high-quality total DNA, which PCR successfully amplified. The six amplicons (T1, T2, T3, F1, F2, and F3) were of good quality, with sizes ranging from 678-686 bp. The nucleotide sequences of the COI gene samples uploaded to BLASTn on the NCBI website confirmed that the prawn samples were not *M. rosenbergii*. Instead, all samples exhibited a genetic similarity of 99.50-99.83% to *Macrobrachium lar* (Table 1). These results provide strong molecular evidence that the freshwater prawns in Ternate Island belong to *M. lar* rather than *M. rosenbergii*, contrary to previous morphological assumptions.

Genetic distance: The genetic distance of the COI gene fragment (571 bp) between samples of *M. lar* prawn originating from the study site averaged 0.0055. There was also a close relationship with several sequences from GenBank and the genus *Macrobrachium*, which were obtained as ingroup members with average genetic distances of 0.0070 and 0.1346, respectively. Meanwhile, the genetic distance to *Cherax cairnsensis* (EU921142.1) as an outgroup averaged 0.1346 (Table 2).

Phylogenetic tree: The phylogenetic tree reconstructed in MEGA 12 was based on genetic distance, with 1000 bootstrap replicates. The results showed that prawns from Ternate Island and GenBank data clustered together, suggesting that the sample belonged to the *M. lar* species. Furthermore, *M. lar* was separated from other prawn species, including *M. rosenbergii, M. spelaeus*, *M. sintangense, M. thai,* and *M. sirindhorn*, as well as the outgroup *Cherax cairnsensis* (Fig. 3).

A phylogenetic tree was constructed using the Neighbor-Joining (NJ) method with 1,000 bootstrap replicates to infer relationships among the analyzed samples. The results showed that *M. lar* specimens from Ternate Island clustered together with *M. lar* reference sequences from GenBank, forming a monophyletic group with high bootstrap support (Fig. 3). The phylogenetic analysis further revealed two major clades among the *Macrobrachium* species examined. Clade 1 included *M. lar*, *M. rosenbergii*, *M. spelaeus*, *M. sintangense*, and *M. thai*, whereas Clade 2 contained only *M. sirindhorn*. The genetic distance between Clades 1 and 2 was 0.1662, indicating a substantial evolutionary divergence.

Discussions

Genetic studies serve as a rapid and reliable method

Table 1. Nucleotide-based BLASTn results at NCBI sites.

Code of sample	Query Cover (%)	Identity (%)	Species validation	Accession number
T1	87	99.67	*Macrobrachium lar*	PP702367
	97	85.07	*Macrobrachium hirsutimanus*	MW845478
	97	84.05	*Macrobrachium naiyanetri*	MW845513
	86	83.78	*Macrobrachium rosenbergii*	KX585725
	97	83.76	*Macrobrachium malayanum*	MT235964
T2	87	99.83	*Macrobrachium lar*	PP702367
	97	85.07	*Macrobrachium hirsutimanus*	MW845478
	97	84.05	*Macrobrachium naiyanetri*	MW845513
	97	83.90	*Macrobrachium malayanum*	MT235964
	86	83.78	*Macrobrachium rosenbergii*	KX585725
T3	89	99.83	*Macrobrachium lar*	PP702367
	98	85.07	*Macrobrachium hirsutimanus*	MW845478
	86	83.93	*Macrobrachium rosenbergii*	KX585725
	98	83.90	*Macrobrachium naiyanetri*	MW845513
	98	83.61	*Macrobrachium malayanum*	MT235964
F1	87	99.67	*Macrobrachium lar*	PP702367
	97	85.07	*Macrobrachium hirsutimanus*	MW845478
	97	84.05	*Macrobrachium naiyanetri*	MW845513
	86	83.78	*Macrobrachium rosenbergii*	KX585725
	97	83.61	*Macrobrachium malayanum*	MT235964
F2	87	99.50	*Macrobrachium lar*	PP702367
	98	85.67	*Macrobrachium hirsutimanus*	MW845478
	98	84.65	*Macrobrachium naiyanetri*	MW845513
	86	83.78	*Macrobrachium rosenbergii*	KX585725
	97	83.76	*Macrobrachium malayanum*	MT235964
F3	87	99.66	*Macrobrachium lar*	PP702367
	98	85.52	*Macrobrachium hirsutimanus*	MW845478
	98	84.50	*Macrobrachium naiyanetri*	MW845513
	86	83.93	*Macrobrachium rosenbergii*	KX585725
	98	83.61	*Macrobrachium malayanum*	MT235964

for species identification (Butet et al., 2019; Hakim et al., 2022). In this study, DNA sequence data were validated through the NCBI database to determine their similarity to reference sequences in GenBank. The BLASTn results confirmed that the prawns collected from the study sites belong to *Macrobrachium lar*, with a genetic similarity of 99.50-99.83% to sequences stored in GenBank (Accession: PP702365.1). These findings clarify previous misidentifications of the species as *M. rosenbergii* based solely on morphological characteristics.

Macrobrachium lar, commonly known as the "monkey river prawn" or "Tahitian prawn", is an amphidromous freshwater species widely distributed across tropical and subtropical regions (Lal et al., 2012, 2014; Sethi et al., 2012; Fadli et al., 2018). Due to high morphological variation within the *Macrobrachium* genus, taxonomic identification based solely on morphology can be challenging (Ghazi and Hassan, 2021). However, Fadli et al. (2018) found that 8 out of 11 morphometric characteristics of *M. lar* effectively distinguished populations, with body weight, total length, and rostrum length being the most reliable traits. Like other *Macrobrachium* species, *M. lar* possesses a well-developed second pair of pereiopods, featuring distinctive chelae with two sharp-tipped fingers

Table 2. Genetic distance of COI genes of *Macrobrachium lar*, *M. rosenbergii*, *M. spelaeus*, *M. sirindhorn*, *M. sintangense*, *M. thai* and *Cherax cairnsensis* using the pairwise distance method.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1																		
2	0.0035																	
3	0.0105	0.0105																
4	0.0035	0.0035	0.0105															
5	0.0053	0.0053	0.0088	0.0018														
6	0.0035	0.0035	0.0105	0.0000	0.0018													
7	0.0123	0.0123	0.0018	0.0123	0.0105	0.0123												
8	0.0018	0.0018	0.0088	0.0018	0.0035	0.0018	0.0105											
9	0.1646	0.1646	0.1646	0.1646	0.1646	0.1646	0.1664	0.1629										
10	0.1646	0.1646	0.1646	0.1646	0.1646	0.1646	0.1664	0.1629	0.0000									
11	0.1594	0.1594	0.1594	0.1576	0.1559	0.1576	0.1611	0.1576	0.1786	0.1786								
12	0.1594	0.1594	0.1594	0.1576	0.1559	0.1576	0.1611	0.1576	0.1786	0.1786	0.0000							
13	0.1646	0.1646	0.1646	0.1646	0.1629	0.1646	0.1629	0.1629	0.1769	0.1769	0.1454	0.1454						
14	0.1629	0.1629	0.1594	0.1629	0.1611	0.1629	0.1576	0.1611	0.1751	0.1751	0.1436	0.1436	0.0053					
15	0.1716	0.1716	0.1734	0.1681	0.1664	0.1681	0.1734	0.1699	0.1839	0.1839	0.1664	0.1664	0.1734	0.1734				
16	0.1594	0.1629	0.1611	0.1629	0.1611	0.1629	0.1611	0.1611	0.1769	0.1769	0.1646	0.1646	0.1751	0.1716	0.0525			
17	0.1926	0.1926	0.1891	0.1891	0.1874	0.1891	0.1909	0.1909	0.1804	0.1804	0.1699	0.1699	0.1821	0.1839	0.1489	0.1699		
18	0.1909	0.1909	0.1874	0.1874	0.1856	0.1874	0.1891	0.1891	0.1839	0.1839	0.1716	0.1716	0.1856	0.1874	0.1506	0.1716	0.0070	
19	0.2504	0.2487	0.2487	0.2487	0.2487	0.2487	0.2469	0.2487	0.2329	0.2329	0.2417	0.2417	0.2172	0.2137	0.2242	0.2294	0.2347	0.2382

1 = *Macrobrachium lar* T1; 2 = *M. lar* T2; 3 = *M. lar* T3; 4 = *M. lar* F1; 5 = *M. lar* F2; 6 = *M. lar* F3; 7 = *M. lar* PP702365; 8 = *M. lar* PP702367; 9 = *M. rosenbergii* MF563571; 9 = *M. rosenbergii* MF563572; 11 = *M. spelaeus* MW845630; 12 = *M. spelaeus* MW845631; 13 = *M. sirindhorn* MW845627; 14 = *M. sirindhorn* MW845628; 15 = *M. sintangense* MW845621; 16 = *M. sintangense* MW845622; 17 = *M. thai* MW845633; 18 = MW845634; 19 = *Cherax cairnsensis* EU921142.

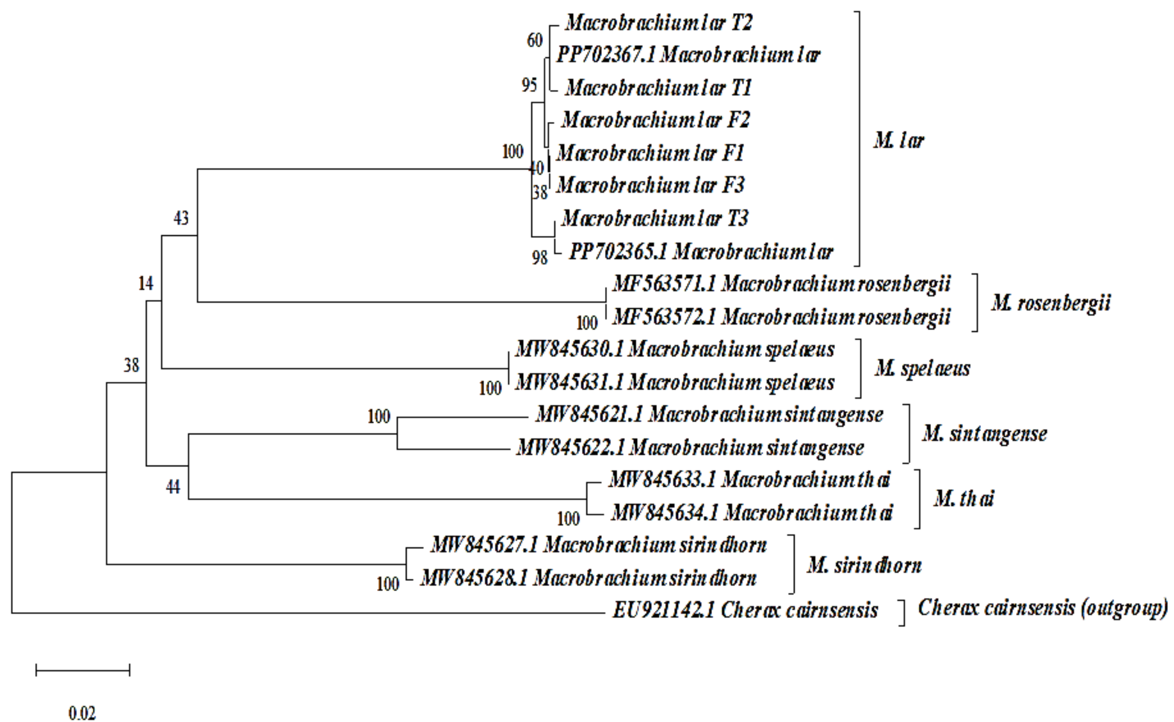

Figure 3. Reconstruction of phylogenetic trees based on COI genes in *Macrobrachium lar*, *M. rosenbergii*, *M. spelaeus*, *M. sintangense*, *M. thai*, *M. sirindhorn*, and *Cherax cairnsensis* using the Neighbour-Joining method with the p-distance model, with a bootstrap value of 1000 replications.

(Wowor et al., 2004).

Ecological adaptations and life cycle: Freshwater prawns can be categorized into two ecological groups: those that complete their entire life cycle in freshwater and those that require brackish water for larval development before migrating upstream as juveniles (Wowor et al., 2004). *Macrobrachium lar* belongs to the latter group, requiring relatively high salinity for larval development. Lal et al. (2012) reported that newly hatched *M. lar* larvae can tolerate freshwater or low-salinity brackish water (~10‰) but require a gradual increase in salinity to 30-35‰ for successful development. The optimal salinity ranges for larval stages are: stages I–II (10-15‰), III–IV (15-25‰), V–

VI (25-30‰), and VII–VIII (30-35‰). Once they reach the post-larval (PL) stage, salinity can be gradually reduced. Larvae reared outside these optimal salinity conditions exhibit significantly lower survival and growth rates, with those kept in freshwater (0‰) typically not surviving beyond four days unless transferred to brackish water.

The presence of *M. lar* in isolated freshwater habitats on Ternate Island raises important questions regarding its ability to complete its life cycle without direct access to brackish water. The Togafo River, although it flows into the sea, has a steep gradient that limits tidal influence, and the Fitu site consists of small, disconnected freshwater pools with no direct connection to the sea. These conditions suggest that *M. lar* populations in these areas may have adapted to complete their life cycle entirely in freshwater or may rely on alternative dispersal mechanisms. Further research on the reproductive biology, larval salinity tolerance, and population dynamics of *M. lar* in Ternate is needed to clarify these adaptations.

Phylogenetic analysis and genetic relationships: The genus *Macrobrachium* is highly diverse, with 105 species documented by Wowor et al. (2009) and 243 species listed by De Grave and Sakihara (2011). Phylogenetic analysis in this study revealed that *M. lar* is closely related to several other *Macrobrachium* species. The constructed phylogenetic tree indicates that *Macrobrachium* species form a monophyletic group distinct from the outgroup.

Two main clades were identified. Clade 1 consisted of *M. lar*, *M. rosenbergii*, *M. spelaeus*, *M. sintangense*, and *M. thai*, while Clade 2 comprised only *M. sirindhorn*. The genetic distance analysis revealed that members of Clade 1 had a mean genetic distance of 0.1272, whereas the genetic distance between Clades 1 and 2 was 0.1662. This suggests that the species within Clade 1 are more closely related to each other than to *M. sirindhorn*.

Locally, *M. lar* is referred to as “udang galah”, the same name used for *M. rosenbergii*. However, genetic distance indicates that *M. lar* and *M. rosenbergii* differ by 16.29-16.64%, confirming that they are distinct species. Hebert et al. (2003) suggested that species can be considered separate if their genetic distance exceeds 3%, while Ratnasingham and Hebert (2013) Stated that COI gene variations above 4% indicate reproductive isolation. These results strongly support that *M. lar* and *M. rosenbergii* are distinct species despite their similar local nomenclature.

Implications for DNA barcoding and cryptic species identification: This study highlights the effectiveness of DNA barcoding in confirming species identity, particularly in crustacean taxa with high morphological variation or cryptic species. Cryptic species are morphologically similar and thus often misidentified (Bickford et al., 2007). Such phenomena have been reported in various aquatic organisms, including crustaceans (Butet et al., 2019; Hakim et al., 2022), mollusks (Findra et al., 2017, 2020), and fish (Wang et al., 2020). Morphology-based identification alone is often insufficient for distinguishing closely related species, underscoring the need for molecular tools for accurate species differentiation.

The confirmation of *M. lar* as the freshwater prawn species found in Ternate contradicts previous assumptions that it was *M. rosenbergii*. This misidentification highlights the limitations of relying solely on morphological characteristics and underscores the importance of integrating genetic approaches for precise taxonomic classification. Additionally, the presence of *M. lar* in isolated freshwater habitats warrants further ecological and genetic investigations to elucidate its dispersal patterns, potential local adaptations, and evolutionary history.

Conclusion

This study confirms that the freshwater prawn found on Ternate Island is *M. lar*. Although the local community refers to it as “udang galah”, genetic analysis reveals that *M. lar* and *M. rosenbergii* differ by more than 16%, indicating that local nomenclature does not always reflect true taxonomic relationships. DNA barcoding results indicate high genetic similarity (99.50-99.83%) of *M. lar* to reference sequences in GenBank, thereby confirming its presence in the inland freshwater habitats of Ternate

Island.

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