Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 29(5): 963 – 977 (2025)
www.ejabf.journals.ekb.eg

Molecular Evidence and Phylogenetic Analysis of the Mitochondrial COI Gene for Species Confirmation of Mitre Squid *Uroteuthis chinensis* (Gray, 1849) from Bangka Island

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ARTICLE INFO

Article History:
Received: June 5, 2025
Accepted: Aug. 28, 2025
Online: Sep. 22, 2025

Keywords:
DNA barcoding,
COI gene,
U. chinensis,
Species identification,
Bangka Island,
Phylogenetic analysis

ABSTRACT

Bangka Island is recognized as one of the key production areas for premium-quality squid, renowned for its superior flavor and higher market value in both domestic and international markets. Accurate species identification is essential to ensure the commercial value of this resource while supporting sustainable fisheries management. This study aimed to analyze the mitochondrial cytochrome c oxidase subunit I (COI) gene sequences of *U. chinensis* collected from Bangka Island, Indonesia, using DNA barcoding for species confirmation. Fresh mantle tissue samples were extracted using the Geneaid Genomic DNA Extraction Kit, followed by PCR amplification with universal primers LCO1490 and HCO2198, and bidirectional sequencing. The resulting 658 bp COI fragment exhibited 99–100% sequence similarity with reference *U. chinensis* sequences available in GenBank. Phylogenetic analysis using the neighbor-joining method (Kimura 2-parameter model) clustered the Bangka specimens within the *U. chinensis* clade with high bootstrap support (99%). These findings confirm the species identity of *U. chinensis* from Bangka Island and provide essential molecular data for broodstock assessment, particularly to support breeding programs and the sustainable management of squid resources.

INTRODUCTION

Mitre squid *Uroteuthis chinensis*, a member of the family Loliginidae **(Pratasik *et al.*, 2022)**, is a commercially important species with a broad distribution across the Indo-Pacific region **(Ervinia *et al.*, 2024; Liu *et al.*, 2024)**. In Indonesia, Bangka Island is recognized as one of the primary production centers for premium-quality squid, nationally renowned as "Bangka squid" **(Oktariza *et al.*, 2015; Robin *et al.*, 2025)**. This reputation is supported by its distinctive flavor profile, firm and elastic flesh texture, pronounced fresh ocean aroma, and the ability to retain taste quality after cooking **(Robin**

***et al.*, 2025)**. These attributes contribute to the higher market value of *U. chinensis* from Bangka compared to similar products from other regions.

The superior quality of Bangka squid is closely linked to the island's geographical setting, being surrounded by the Natuna Sea, the South China Sea, and the Bangka-Belitung Sea **(Puspasari *et al.*, 2025)**. These waters exhibit oceanographic characteristics favorable for squid populations, including relatively shallow depths, sandy–muddy substrates, stable currents, and an abundant supply of natural prey **(Puspasari *et al.*, 2025; Robin *et al.*, 2025)**. Such conditions provide an optimal habitat for the growth and reproduction of *U. chinensis*, reinforcing Bangka's role as one of Indonesia's leading sources of high-quality squid.

However, species identification of Uroteuthis based on morphological characters is often challenging due to the high degree of similarity among congeners, such as *U. duvaucelii*, *U. sibogae* and *U. edulis* **(Jin *et al.*, 2022; Zamroni *et al.*, 2024)**. Misidentification not only compromises the accuracy of biodiversity data but also poses risks to stock management, potentially leading to misdirected breeding programs and affecting product authenticity in the market **(Stephenson, 1999; Tillett *et al.*, 2012; Helgoe *et al.*, 2020; Carreiro, *et al.*, 2023)**. Therefore, accurate scientific identification is essential to ensure product authenticity, maintain trade quality, and support the sustainable utilization of resources **(Syarif *et al.*, 2023; Nazran *et al.*, 2025; Valen *et al.*, 2025)**.

Furthermore, molecular identification in this study aims to provide precise verification of the species identity of "Bangka squid" in the marketplace, thereby preventing mislabeling, safeguarding product reputation, and ensuring quality assurance for consumers **(Robin *et al.*, 2025)**. Molecular approaches, particularly DNA barcoding based on the mitochondrial cytochrome c oxidase subunit I **(**COI**)** gene, have proven effective in overcoming the limitations of morphological identification **(Robin *et al.*, 2023; Valen *et al.*, 2023; Syarif *et al.*, 2025)**.

The COI gene exhibits high sequence conservation within species but sufficient variability between species, providing strong resolution for taxonomic confirmation **(Liu *et al.*, 2020)**. This study aimed to analyze COI gene sequences of *U. chinensis* from Bangka Island, scientifically verify its species identity, and generate essential molecular data for broodstock assessment to support breeding programs and the sustainable management of squid fisheries.

MATERIALS AND METHODS

1. Sample collection

This study was conducted in the coastal waters surrounding the Bangka Belitung Islands, Indonesia, a region recognized as a potential habitat for various cephalopod species. Field sampling was carried out from July to August 2025, coinciding with the peak fishing season for *U. chinensis*. Specimens were collected from five major fishing

grounds: Tuing and Deniang Villages (Bangka Regency), Sadai Village (South Bangka Regency), and Kampak and Air Nyatoh Villages (West Bangka Regency) (Fig. 1).

Geographically, these sites are situated along the transitional waters of the Natuna Sea, the South China Sea, and the Bangka Strait. The fishing grounds are characterized by relatively shallow depths (15– 40m), sandy–muddy substrates, and moderate to stable currents, with sea surface temperatures ranging between 27– 30°C and salinity levels of 30– 33ppt during the sampling period. These oceanographic features, combined with the high abundance of natural prey, create favorable conditions for the occurrence, growth, and reproduction of *U. chinensis*, making the region one of Indonesia's most productive squid fishing areas.

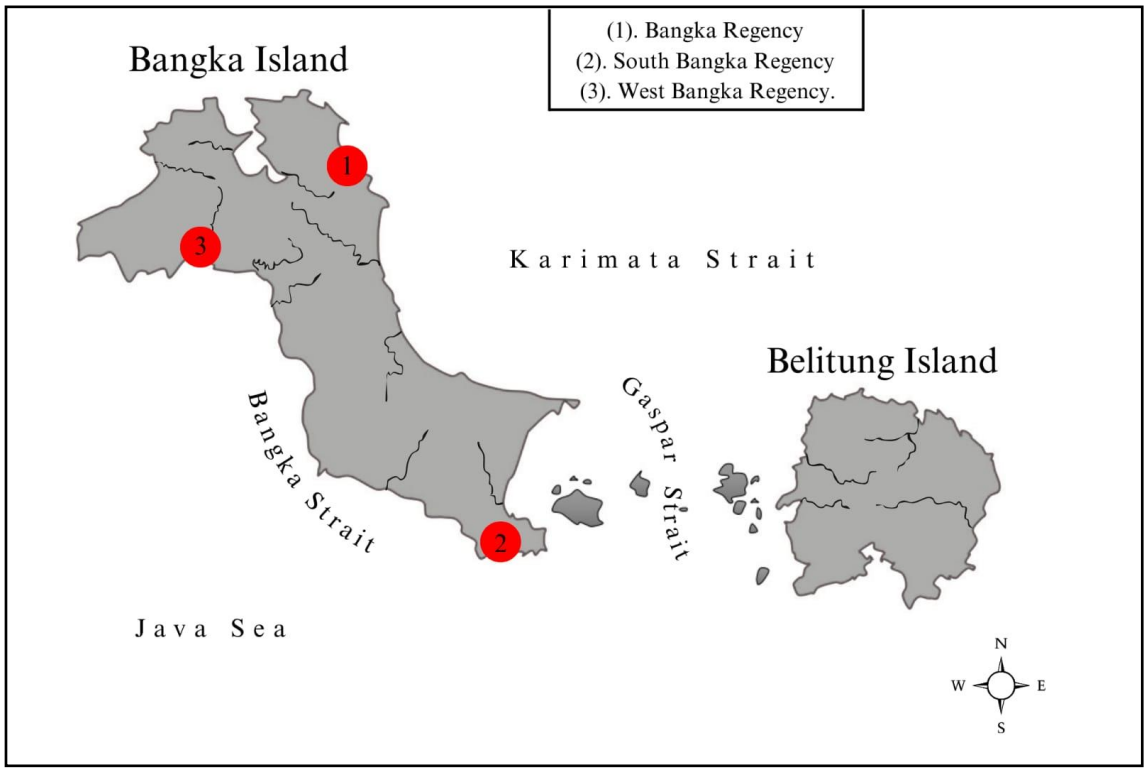

Fig. 1. Map showing the sampling locations for *U. chinensis* on the Bangka Belitung Islands, (1). Bangka Regency, (2). South Bangka Regency, and (3). West Bangka Regency

A total of 25 *U. chinensis* specimens were collected, of which three were selected for molecular analysis of their DNA characteristics. Prior to preservation, each specimen was documented photographically (Fig. 2). The three specimens for genetic analysis were preserved in 96% ethanol **(Valen *et al.*, 2022)** and subsequently processed at the Genetics Science Laboratory, Jakarta. The remaining 20 squid were transported alive to the Fisheries Hatchery of Bangka Belitung University for further studies on breeding, reproduction, and domestication. Additionally, two specimens were preserved as voucher

specimens in 10% formalin solution **(Jatayu *et al.*, 2023; Pramono *et al.*, 2025)** and deposited in the Aquaculture Laboratory **(**Ichthyological Collection**)**, University of Bangka Belitung.

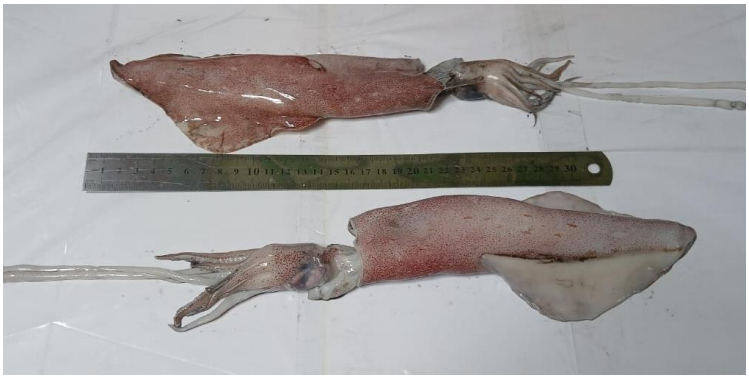

Fig. 2. Freshly caught specimens of *U. chinensis* from Bangka Island photographed prior to preservation for genetic and voucher specimen preparation

2. DNA extraction, PCR amplification and sequencing

DNA extraction and PCR amplification were carried out between 5 and 10 August 2025. Genomic DNA was isolated using the gSYNCTM DNA Extraction Kit (Geneaid, GS300) following the manufacturer's protocol, which includes four main steps: cell lysis, DNA binding, washing, and final elution. PCR amplification was performed with MyTaq HS Red Mix (Bioline, BIO-25048) in a 25µL reaction mixture containing 9.5µL nuclease-free water, 12.5µL MyTaq HS Red Mix, 10µM of the forward primer LCO1490 (5′-GGTCAACAAATCATAAAGATAATTGG-3′) and reverse primer HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) **(Folmer *et al.*, 1994)**, along with the DNA template. Amplification was conducted in a BioRad T100TM thermal cycler under the following conditions: initial denaturation at 95°C for 3min**;** 35 cycles of denaturation at 96°C for 10s, annealing at 53°C for 30s, and extension at 72°C for 45s, followed by a final hold at 4°C **(Valen *et al.*, 2024a)**. The amplified products were visualized on 1% agarose gels stained with GelRed® Nucleic Acid Gel Dye. The gel was prepared with 5mL TAE buffer, 45mL distilled water, and 8µL GelRed®. Each well was loaded with 5µL PCR product mixed with 1µL loading dye. Samples producing clear, luminous DNA bands were subsequently sent to PT Genetics Science, Jakarta, for sequencing using the Sanger dideoxy method.

3. Sequence analysis

Chromatogram files (Table 1) from the sequencing results were visually inspected using BioEdit v7.2.6. The initial and terminal regions of the sequences exhibiting weak signals or high background noise were trimmed to ensure optimal data quality. Forward and reverse sequences were aligned using MEGA X **(Kumar *et al.*, 2018)** to generate a single consensus sequence for each sample. The consensus sequences were then

compared with reference sequences downloaded from GenBank. Sequence alignment was performed using ClustalW to maintain positional homology of nucleotides.

4. BLAST analysis

Species identification was performed using BLASTn (Basic Local Alignment Search Tool) searches against the NCBI GenBank database. The resulting percent identity and E-value were recorded, with emphasis placed on reference sequences exhibiting the highest similarity. A percent identity of ≥98% was interpreted as strong evidence that the sample and the reference belong to the same species **(Hebert *et al.*, 2003; Ward *et al.*, 2005)**.

5. Nucleotide composition and sequence characteristics analysis

The nucleotide composition and sequence characteristics were analyzed to understand the basic structure of the COI gene and to ensure data quality prior to its use in taxonomic and phylogenetic analyses. The proportions of nucleotides (A, T, G, C) and GC content were calculated using MEGA X **(Kumar *et al.*, 2018)**. Sequences were also examined to confirm the absence of insertions, deletions, or stop codons that could indicate the presence of nuclear mitochondrial DNA segments, which may interfere with subsequent analyses.

6. Genetic distance calculation

Genetic distances were calculated using the Kimura 2-Parameter **(**K2P**)** model implemented in MEGA X **(Kumar *et al.*, 2018)**. The K2P substitution model was selected as it accounts for differences in transition rates (purine ↔ purine or pyrimidine ↔ pyrimidine) and transversion rates (purine ↔ pyrimidine), making it widely applicable in DNA barcoding studies **(Hebert *et al.*, 2003)**. Genetic distances were expressed as substitutions per site. Intraspecific and interspecific distances were compared to determine species boundaries. A genetic distance of < 2% (0.020) was generally interpreted as indicative of intraspecific variation in marine invertebrates, including cephalopods, whereas values ≥ 2% were considered to reflect interspecific divergence or the potential presence of cryptic species **(Hebert *et al.*, 2003; Ward *et al.*, 2005)**.

7. Phylogenetic analysis

Phylogenetic analysis was conducted to visualize the evolutionary relationships between *U. chinensis* specimens from Bangka Island and reference sequences from GenBank, as well as other species within the family Loliginidae. A phylogenetic tree was constructed using the Neighbor-Joining (NJ) method with the Kimura 2-Parameter (K2P) substitution model, which is commonly applied in DNA barcoding studies **(Hebert *et al.*, 2003)**. Bootstrap values were calculated with 1000 replicates to assess the reliability of the branching nodes **(Felsenstein, 1985)**. For comparative purposes, COI sequences from other species in the genus *Uroteuthis*, such as *U. duvaucelii* and *U. edulis*, along with additional members of the Loliginidae, were included to clarify interspecific boundaries. *Sepia officinalis* was used as an outgroup for tree rooting.

RESULTS

1. COI sequence characteristics

An analysis of the Cytochrome C Oxidase Subunit I (COI) gene sequence characteristics was conducted to describe the molecular profile of *U. chinensis* from Bangka Island prior to genetic distance and phylogenetic analyses. Three haplotypes were identified among the analyzed samples, each with a fragment length of 446 base pairs (bp), corresponding to a portion of the standard COI barcode region (Table 1).

Table 1. COI sequence characteristics of *U. chinensis* from Bangka Island

Sample	DNA Length **(**bp**)**	GenBank Accession Code
U. chinensis Bangka 1	446	EU349429.1
U. chinensis Bangka 2	446	EU349430.1
U. chinensis Bangka 3	446	EU349431.1

Nucleotide composition for each haplotype is presented in Table (2). Thymine (T) content was the highest among all nucleotides, followed by adenine (A), cytosine (C), and guanine (G), indicating an AT-rich composition, a common feature of mitochondrial genes in marine animals, including cephalopods.

Table 2. Nucleotide composition of *U. chinensis* from Bangka Island

Sample Code	**T/U (%)**	**C (%)**	**A (%)**	**G (%)**	**Length (bp)**
1	33,4	22,6	28,0	15,9	446
2	33,2	22,9	28,3	15,7	446
3	33,2	22,9	28,3	15,7	446

2. Sequence similarity analysis

BLASTn searches of the COI sequences against the NCBI GenBank database revealed that *U. chinensis* specimens from Bangka Island exhibited very high similarity (99–100%) to reference *U. chinensis* sequences deposited in GenBank (Table 3). Query coverage was 100% in all comparisons, indicating complete overlap of the compared fragment lengths. A 100% identity was recorded with accession number EU349429.1, while three other references (LC552692.1, MG192387.1, LC552693.1) showed 99% identity, consistent with intraspecific variation in cephalopods **(Yang *et al.*, 2025)**. These results confirm that all analyzed specimens belong to *U. chinensis*

Table 3. Sequence similarity of *U. chinensis* from Bangka Island

Specimen	**GenBank Similarity (Species outcome)**	**Gene**	**Accession Number**	**Query Cover**	**Identity (%)**

				(%)	
	U. chinensis	CO1	EU349429.1	100	100
U. chinensis	*U. chinensis*	CO1	LC552692.1	100	99
Bangka Island	*U. chinensis*	CO1	MG192387.1	100	99
	U. chinensis	CO1	LC552693.1	100	99

3. Genetic distance

Genetic distance analysis using the Kimura 2-Parameter (K2P) model for the three haplotypes of *U. chinensis* from Bangka Island revealed extremely low values, ranging from 0.000 to 0.005, equivalent to a 0.50% nucleotide difference (Table 4).

Tabel 4. Genetic distance of *U. chinensis* from Bangka Island

		1	2	3	4	5	6	7	8
1	*U. chinensis* Bangka 1								
2	*U. chinensis* Bangka 2	0,005							
3	*U. chinensis* Bangka 3	0,005	0,005						
4	*U. chinensis* China	0,000	0,005	0,005					
5	*U. duvauceli*	0,236	0,236	0,232	0,236				
6	*U. edulis*	0,179	0,172	0,179	0,179	0,208			
7	*U. singhalensis*	0,240	0,241	0,241	0,240	0,143	0,231		
8	*U. etheridgei*	0,137	0,137	0,137	0,137	0,231	0,190	0,214	

These values are well below the commonly accepted interspecific divergence threshold for marine invertebrates (≥ 2%) **(Hebert *et al.*, 2003; Ward *et al.*, 2005)**, while distances to other *Uroteuthis* species exceeded 13%, demonstrating a pronounced "barcode gap." Specifically, genetic distances between *U. chinensis* and *U. duvaucelii* ranged from 0.232–0.236 (23.2–23.6%), whereas between *U. chinensis* and *U. edulis* ranges were from 0.172–0.179 (17.2–17.9%), and between *U. chinensis* & *U. singhalensis* they fluctuated from 0.240–0.241 (24.0–24.1%), and between *U. chinensis* and *U. etheridgei* genetic distances were recorded at 0.137 (13.7%).

4. Phylogenetic analysis

The neighbor-joining (NJ) phylogenetic tree constructed using the Kimura 2-Parameter (K2P) model with 1000 bootstrap replicates showed that the three *U. chinensis* samples from Bangka Island clustered in a single monophyletic clade together with reference *U. chinensis* sequences from various locations, including Karimunjawa, Muara Angke, China, and Hong Kong (Fig. 3).

This clade displayed relatively high bootstrap support for several branching points (63–90), indicating strong grouping stability. No clustering with other species in the genus *Uroteuthis*, such as *U. duvaucelii* or *U. edulis*, was observed. The outgroup species

Sepioteuthis australis was clearly separated from all *Uroteuthis* clades, consistent with previously reported taxonomic relationships.

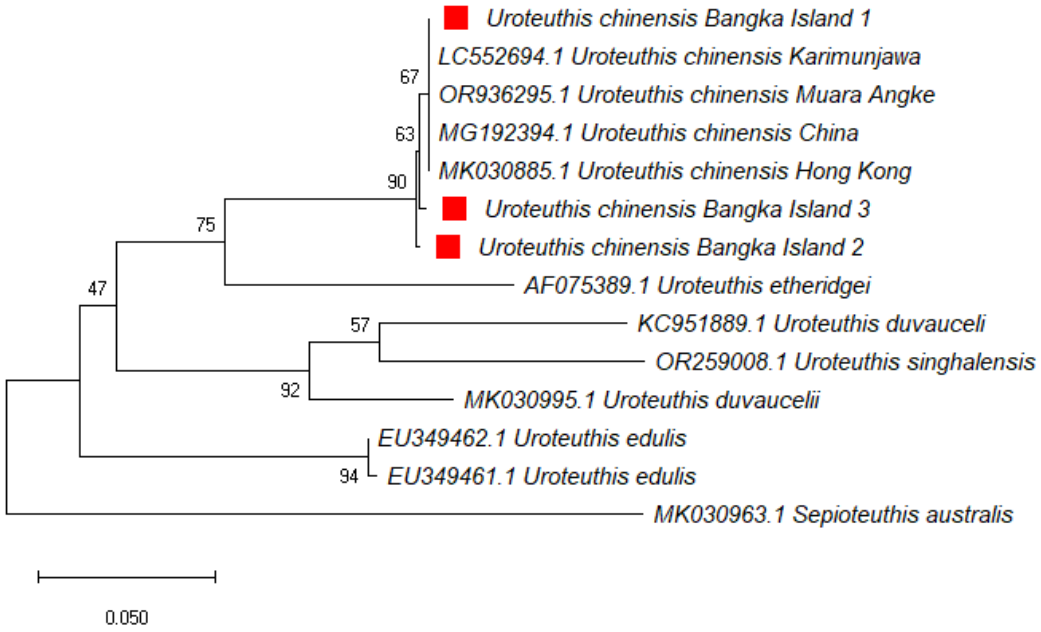

Fig. 3. Phylogenetic analysis of *U. chinensis* : Insights into evolutionary relationships within the loliginidae family

DISCUSSION

The consistent COI fragment length (446 bp) indicates that all samples were successfully amplified from the same gene region without insertions, deletions, or fragment degradation. This length is appropriate for molecular analysis and species delimitation using DNA barcoding **(Hasan *et al.*, 2021; Robin *et al.*, 2022; Valen *et al.*, 2024b; Pertesi *et al.*, 2025)**. The uniformity of nucleotide composition among haplotypes suggests a relatively homogeneous population, although further analysis using additional genetic markers is required to verify the level of population diversity **(Afiati *et al.*, 2022; La Torre *et al.*, 2024)**. The results revealed a high AT content, ranging from 61.2 to 61.4%. Such elevated AT content is a typical feature that can serve as a molecular signature for cephalopod identification **(Cheng *et al.*, 2013; Li *et al.*, 2023)**. High AT content is thought to contribute to the stability of the mitochondrial genome in marine environments with fluctuating temperatures **(Cheng *et al.*, 2013)**. GC content ranged from 38.6 to 38.8%, which is relatively low and influences melting temperature and PCR primer design **(Insani *et al.*, 2022)**. The difference in nucleotide composition among haplotypes was minimal (< 0.3%), indicating a high degree of genetic homogeneity among *U. chinensis* samples from Bangka Island **(Jin *et al.*, 2022; Robin *et al.*, 2025)**.

Sequence similarity analysis was performed using the BLASTn algorithm against the NCBI GenBank database to compare COI sequences of *U. chinensis* specimens from Bangka Island with valid reference sequences. All Bangka Island specimens exhibited

100% query coverage with reference sequences, indicating complete alignment of the analyzed fragment without unaligned regions. Percent identity ranged from 99 to 100, with the highest match (100%) to reference accession EU349429.1. The complete identity with EU349429.1 confirms that Bangka specimens are genetically identical to the reference *U. chinensis* in GenBank, providing strong molecular evidence for species confirmation **(Robin *et al.*, 2025)**. The 99% identity observed with three other reference sequences (LC552692.1, MG192387.1, and LC552693.1) falls within the expected intraspecific variation range for COI in cephalopods **(Jin *et al.*, 2022; Yang *et al.*, 2025)**. This 1% difference likely reflects natural haplotype variation among populations or geographic differences in reference specimen origin **(Li *et al.*, 2023)**. The uniform 100% query coverage across all matches demonstrates the high quality of the COI fragments used in this study, without missing data, thereby increasing the reliability of the analysis. These findings show strong molecular consistency between Bangka specimens and *U. chinensis* references from various Indo-Pacific locations **(Zamroni *et al.*, 2024; Robin *et al.*, 2025)**. The 99–100% similarity supports the interpretation that genetic variation among samples represents intraspecific diversity, with no indication of distinct species **(Valen *et al.*, 2023b; Yang *et al.*, 2025)**. This confirms that the tested specimens are indeed *U. chinensis*, thereby supporting the authenticity of the "Bangka squid" label in the market with scientific evidence.

The low genetic distances (0.000–0.005) indicate a very high degree of genetic similarity among *U. chinensis* haplotypes from Bangka Island, with one haplotype (Bangka 1) being identical to the Chinese *U. chinensis* sample. In contrast, the 13.7–24.1% distances to other *Uroteuthis* species clearly demonstrate strong interspecific separation. These values are far above the interspecific threshold in marine invertebrate DNA barcoding (generally ≥ 2–3%) **(Xiao *et al.*, 2022; Zhang & Bu, 2022; Muhala *et al.*, 2024)**, providing robust support for the designation of the samples as *U. chinensis* . According to the literature, intraspecific genetic differences in cephalopods are typically below 1%, while interspecific differences can reach 3–10% or more **(Anderson *et al.*, 2011)**. The zero distance (0.000) between Bangka 1 and the Chinese *U. chinensis* sample indicates a shared haplotype for the analyzed COI fragment (446 bp), consistent with the possibility of extensive gene flow or the presence of a common haplotype across the western Indo-Pacific range **(Horne *et al.*, 2008; Zhang *et al.*, 2020)**. The 0.005 distance among the remaining Bangka haplotypes reflects low intraspecific diversity, in agreement with BLAST and phylogenetic results. The low intraspecific divergence reinforces the species-level identification of "Bangka squid" as *U. chinensis*. For broodstock and breeding stock assessments, this genetic consistency facilitates the selection of suitable brood individuals and serves as a baseline for resource sustainability monitoring **(Taniguchi, 2003; Horn *et al.*, 2022; Wu *et al.*, 2025)**. However, to maintain population resilience and prevent inbreeding, periodic genetic monitoring and the inclusion of

additional markers (e.g., nuclear loci or complete mitogenome) are recommended to assess fine-scale population structure.

The phylogenetic tree revealed that the three *U. chinensis* samples from Bangka Island clustered in a single monophyletic clade together with *U. chinensis* reference sequences from various locations, including Karimunjawa, Muara Angke, China, and Hong Kong. No admixture with clades of other species was observed. These phylogenetic results are consistent with the BLAST and genetic distance analyses, confirming that all "Bangka squid" samples are *U. chinensis*. The clustering of Bangka specimens with references from diverse locations reflects high genetic similarity, suggesting potential gene flow among *U. chinensis* populations in the western Indo-Pacific **(Jin *et al.*, 2022; Zamroni *et al.*, 2023; Robin *et al.*, 2025; Yang *et al.*, 2025)**.

The *U. chinensis* clade was clearly separated from *U. duvaucelii* and *U. edulis*, indicating substantial genetic distances and distinct taxonomic boundaries. This is consistent with DNA barcoding thresholds for cephalopods, in which interspecific divergence typically exceeds 3% **(Zamroni *et al.*, 2023; Robin *et al.*, 2025)**. This phylogenetic pattern reinforces the genetic consistency of "Bangka squid" across individuals, which is essential for ensuring product quality and authenticity in the market. Furthermore, these data are valuable for broodstock selection in breeding programs and for designing conservation strategies informed by genetic data **(Kobayashi *et al.*, 2025)**.

CONCLUSION

Mitochondrial COI gene–based molecular analysis confirmed the species identity of the "Bangka squid" as *U. chinensis* . BLAST comparisons showed 99–100% sequence similarity with *U. chinensis* references in GenBank, while intraspecific genetic distances among Bangka haplotypes were extremely low **(**≤ 0.005**)**, indicating high genetic homogeneity. Phylogenetic reconstruction placed all Bangka specimens in a single monophyletic clade with *U. chinensis* from multiple Indo-Pacific locations, distinctly separated from other *Uroteuthis* species. These findings provide robust molecular evidence supporting the authenticity of "Bangka squid" in the marketplace and establish a scientific basis for accurate product labeling, sustainable resource stock management, and broodstock selection in breeding programs. Furthermore, the generated molecular data constitute a valuable reference for conservation planning and the long-term sustainable management of this economically important squid resource from Bangka Island.

ACKNOWLEDGMENT

The authors express their sincere gratitude to Universitas Bangka Belitung for providing research facilities and technical support. This study was funded through the 2025 fiscal year Internal University-Level Research Grant (PDTU) of Universitas Bangka Belitung under Contract No. 2164F/UN50/M/PP/2025, which played a crucial role in facilitating and completing this research. The authors also extend their appreciation to the local fishers of Bangka Island for their assistance in sample collection, as well as to the

reviewers for their valuable feedback, which significantly improved the quality of this manuscript.

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