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Phylogenetic Analysis of Sulawesi Endemic Butterfly *Papilio blumei* Using the COI (Cytochrome Oxidase I) Gene

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Abstract

Phylogenetics is a method used to study and analyze evolutionary relationships among living organisms. In phylogenetic studies, organisms that share similar traits or characteristics are considered to have close evolutionary relationships, as they are assumed to have originated from a common ancestor. *Papilio blumei* is characterized by wings with a bright, iridescent green coloration. This study aimed to describe the phylogenetic relationship of *P. blumei* based on the cytochrome oxidase subunit I (*COI*) gene. Sampling was conducted using a roaming (exploratory) method. DNA was isolated using the GS 100gSYNCTM DNA Extraction Kit. DNA amplification was performed using *COI* primers (LCO1490 forward and HCO2198 reverse) through polymerase chain reaction (PCR). DNA electrophoresis was carried out using 1% agarose gel, a UV transilluminator, and a gel documentation system. Data were analyzed using GeneStudio, DnaSP, BLAST, DNASTAR, and MESQUITE software, and phylogenetic reconstruction was performed using the Neighbor-Joining and Maximum Likelihood methods in MEGA 11 with the Kimura 2-parameter model and 10,000 bootstrap replications. The results showed that the DNA samples had a 99.50% identity with the reference sequences in GenBank. Genetic variation analysis revealed two haplotypes with haplotype diversity (hd = 0.600 ± 0.175) and nucleotide diversity (π = 0.00051 ± 0.00015). Phylogenetic tree reconstruction formed a single monophyletic cluster of *P. blumei* with bootstrap values ranging from 99% to 100%. A genetic distance of 0.00% among populations from Central Sulawesi (PBPSST.1, PBPSST.2, PBPSST.3), North Sulawesi (JQ982056.1), and South Sulawesi (JQ982058.1) confirms that all *P. blumei* samples have very close genetic relationships.

Keywords: Phylogenetic analysis; Biodeversity; *Chytochrome Oxidase subunit* I (COI); *Papilio blumei*; Sulawesi.

INTRODUCTION

The family Papilionidae comprises large-sized butterflies with striking and attractive coloration (Makhzuni & Dahelmi, 2013). One species within this family is *Papilio blumei*, an endemic butterfly of Sulawesi (Alias & Soesilohadi, 2015). *P. blumei* wings has a bright iridescent green coloration (Kolle et al., 2010). This species exhibits adaptive survival behaviors, including countershading, Batesian mimicry, and the use of an osmeterium as a defensive mechanism against predators. *P. blumei* employs a reproductive strategy known as patrolling, in which males actively fly to search for females in open areas (Alias & Soesilohadi, 2015). The unique morphology of *P. blumei* makes it a highly interesting species for scientific investigation (Condamine et al., 2013). However, existing studies have been largely limited to morphological and morphometric analyses, while molecular analyses of the Sulawesi-endemic butterfly *P. blumei* using the COI gene remain scarce.

One molecular identification method that can be applied is phylogenetic analysis. Phylogenetics is a method used to study and analyze evolutionary relationships among living organisms. In phylogenetic studies, organisms that share similar traits or characteristics are considered to have close evolutionary relationships, as they are assumed to originate from a common ancestor. Such similarities form monophyletic groups, which consist of a single ancestor and all of its descendants (Fietri et al., 2020). Phylogenetic analyses often involve the use of genes to determine evolutionary relationships among species, one of which is the COI gene marker (Partiwi et al., 2023). The COI gene is part of mitochondrial DNA (mtDNA) and plays an important role in energy production processes, making it relatively conserved (Hermawan et al., 2022). The COI gene, located within the mitochondrial genome and known to be an effective genetic marker, has advantageous characteristics such as a low rate of deletions and insertions in its sequence (Tindi et al., 2017). The use of the COI gene marker located in the mitochondrial segment allows for the analysis of nucleotide base

variation within species, thereby enabling the determination of phylogenetic relationships among species (Kamal et al., 2019).

Mitochondrial DNA has advantages in genetic diversity studies due to its much higher copy number in cells compared to nuclear DNA and its higher mutation rate (Hendiari et al., 2020). Currently, mitochondrial DNA has been widely utilized in various fields, including disease detection, population genetic analysis, species identification, and phylogenetic studies. This is supported by its maternal inheritance pattern, relatively rapid mutation rate, and continuous replication ability (Mohapatra et al., 2019). However, no phylogenetic analysis of *P. blumei* butterflies from Bakubakulu Village, Palolo District, Sigi Regency, Central Sulawesi, using the COI (Cytochrome Oxidase subunit I) gene has been reported to date. This study aimed to describe the phylogenetic relationship of *P. blumei* based on the cytochrome oxidase subunit I (COI) gene.

MATERIALS AND METHODS

Study area

This study was conducted in November 2025 in Bakubakulu Village, Palolo District, Sigi Regency, Central Sulawesi, Indonesia (Figure 1). The study area is characterized by heterogeneous environmental conditions, dominated by hilly landscapes and agricultural land adjacent to forested areas. The region has relatively diverse vegetation cover, sufficient food resources, and a tropical climate that supports the presence and activity of butterflies, particularly endemic species. The samples used in this study consisted of three individuals of *P. blumei*.

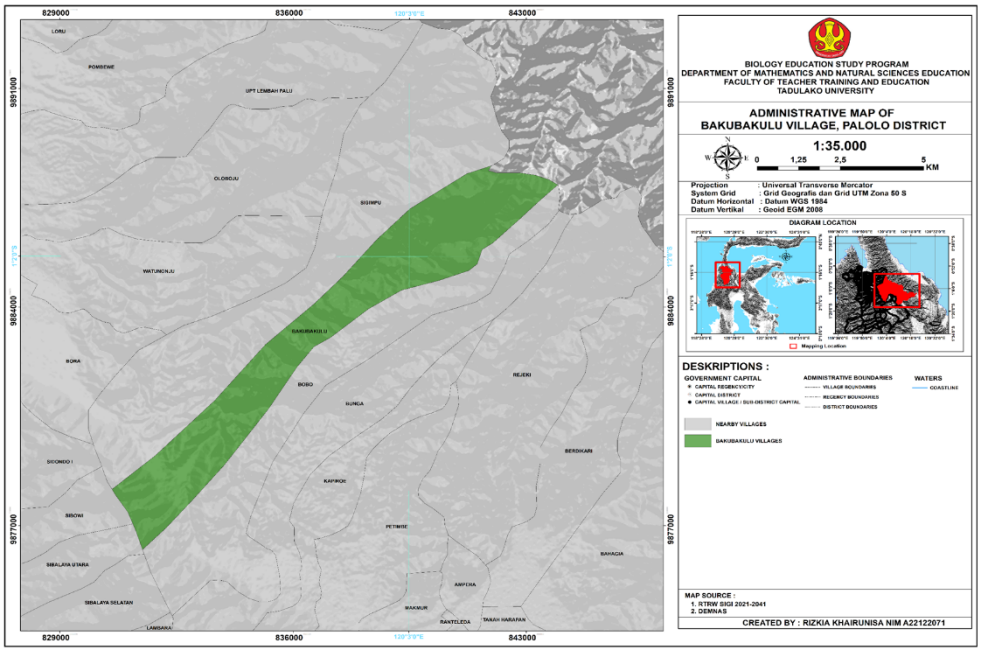

Figure 1. Map of the research location in Bakubakulu Village, Palolo District, Sigi Regency, Central Sulawesi, Indonesia.

Procedures

Sample Collection

Samples of *Papilio blumei* were collected using an exploratory (roaming) method by surveying areas considered potential butterfly habitats. Each *P. blumei* individual encountered was captured using an insect net to minimize morphological damage. The geographic coordinates of each sampling location were recorded using a Global Positioning System (GPS) to ensure accurate spatial data and facilitate species distribution mapping. Collected specimens were properly labeled according to location and sampling time and subsequently prepared for further molecular analysis.

DNA Isolation

DNA isolation was performed on *Papilio blumei* specimens after removing the head and wings to reduce contamination and obtain optimal DNA quality. The isolation process was carried out using the GS 100gSYNCTM DNA Extraction Kit following the manufacturer's protocol. Tissue samples were incubated at 60°C for 2.5 hours to enhance cell lysis. The resulting supernatant was then processed using GSB solution, absolute ethanol (EtOH), and a GS column to bind the DNA. Washing and drying steps were conducted to remove residual contaminants, followed by DNA elution at 60°C. The extracted DNA was stored at −20°C until further use in the amplification process.

Amplification and Sequencing

DNA amplification was conducted using the Polymerase Chain Reaction (PCR) technique with COI primers, namely LCO1490 (forward) and HCO2198 (reverse), in a total reaction volume of 25 µL. The PCR program consisted of a pre-denaturation step at 95°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 35 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 30 seconds. A final post-extension step was performed at 72°C for 7 minutes. Successful PCR products were subsequently sent to the Integrated Research and Testing Laboratory (LPPT), Universitas Gadjah Mada, for sequencing using a Genetic Analyzer 3500 (Applied Biosystem).

Electrophoresis

DNA electrophoresis was performed to verify the success of PCR amplification using a 1% agarose gel prepared from 0.2 g agarose dissolved in 20 mL of 1× TAE buffer and stained with FloroSafe. The gel was cast in an electrophoresis tray equipped with wells. Each well was loaded with a DNA ladder as a molecular size marker and 2 µL of PCR product from each sample. Electrophoresis was conducted at 50 volts for 17–20 minutes until distinct DNA bands were observed. The results were visualized using a UV transilluminator and documented with a gel documentation system to confirm the presence of amplified DNA fragments.

Data analysis

Sequencing data in the form of forward and reverse ab1 files were edited and assembled using GeneStudio and DNASTAR software to generate consensus sequences. The obtained sequences were then analyzed using Nucleotide BLAST (NCBI) to confirm species identity based on similarity to reference sequences. Sequence alignment was performed using MESQUITE and converted into FASTA format prior to further analysis in MEGA 11. Genetic distances were estimated using the Kimura 2-parameter model, while phylogenetic trees were reconstructed using the Neighbor-Joining and Maximum Likelihood methods with 10,000 bootstrap replications. Genetic variation analyses, including haplotype and nucleotide diversity, were conducted using DnaSP version 6.

RESULTS AND DISCUSSION

Amplification and Sequence Similarity of *Papilio blumei*

Amplification of the mitochondrial *Cytochrome Oxidase subunit I* (COI) gene of *P. blumei* collected from Bakubakulu Village, Palolo District, Sigi Regency, Central Sulawesi, was successfully achieved using PCR with the forward primer LCO1490-F and the reverse primer HCO2198-R (Figure 2). The DNA bands produced during electrophoresis showed good quality with clear and well-defined patterns. The quality of the DNA bands reflects the quality of the PCR products obtained (Dailami et al., 2022).

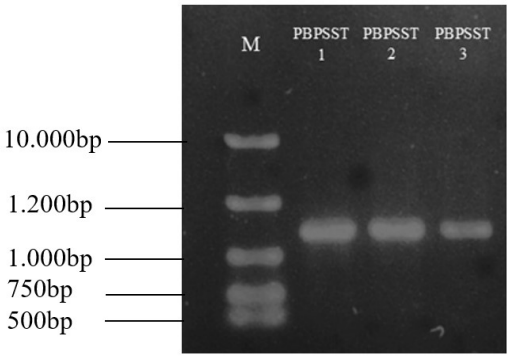

Figure 2. The results of mitochondrial COI gene amplification show that samples labeled PBPSST represent *P. blumei* collected from Bakubakulu Village, Palolo District, Sigi Regency, Central Sulawesi, while M denotes the molecular size marker.

The obtained DNA sequences were analyzed using the Nucleotide BLAST tool provided by the National Center for Biotechnology Information (NCBI) to evaluate query coverage and sequence similarity for the three DNA sequences generated in this study. This analysis was conducted to confirm species identity by comparing the obtained sequences with reference sequences available in the GenBank database (Table 1).

Table 1. BLAST analysis of mitochondrial *COI* gene sequences of *P. blumei* from Central Sulawesi.

Code	BLAST			Species Verification	Location
	% Identity	% Query Cover	Accession Numbern *GenBank*		
PBPSST.1	99,50	100	JQ982058.1	*Papilio blumei*	Central Sulawesi
PBPSST.2	99,50	100	JQ982058.1	*Papilio blumei*	Central Sulawesi
PBPSST.3	99,50	100	JQ982058.1	*Papilio blumei*	Central Sulawesi

Genetic Variation of *Papilio blumei*

Genetic variation analysis of *P. blumei* sequences was conducted on five sequences with a nucleotide length of 1188 bp. This analysis aimed to assess the level of genetic diversity among *P. blumei* populations originating from different regions of Sulawesi. Genetic variation was analyzed using DnaSP software to determine the number of haplotypes (h), variable sites,

parsimony-informative sites, haplotype diversity (hd), and nucleotide diversity (π). The results revealed the presence of two haplotypes. Haplotype 1 consisted of samples PBPSST 1, PBPSST 2, and PBPSST 3, representing *P. blumei* from Central Sulawesi, whereas haplotype 2 included sequences JQ982058 (*P. blumei* from South Sulawesi) and JQ982056 (*P. blumei* from North Sulawesi). In addition, one variable site and one parsimony-informative site were identified across all analyzed sequences. The estimated haplotype diversity was hd = 0.600 ± 0.175, while the nucleotide diversity was π = 0.00051 ± 0.00015 (Table 2).

Table 2. Intraspecific genetic variation of *P. blumei* based on mitochondrial COI gene sequences compared with *P. blumei* from GenBank.

Sample Code	bp	Number of Individual	Number of Haplotypes	Variable Site	Parsimony Site	Haplotype Diversity (Hd)	Nucleotide Diversity (π)
PBPSST.1 PBPSST.2 PBPSST.3 JQ982058 JQ982056	1188	5	2	1	1	0,600±0,175	0,00051±0,00015

Nucleotide Composition of *Papilio blumei*

DNA is composed of four nitrogenous bases, namely thymine (T), cytosine (C), adenine (A), and guanine (G). The base pair adenine (A) and thymine (T) is connected by two hydrogen bonds, whereas cytosine (C) and guanine (G) are linked by three hydrogen bonds, making the C–G pair more stable and stronger than the A–T pair (Saleky & Dailami, 2021). These differences in bonding strength play an important role in maintaining DNA structural stability and contribute to genetic variation within organisms. Based on the analysis of five *P. blumei* sequences, the average nucleotide composition indicated a dominance of thymine (T) and adenine (A). The mean percentages of nitrogenous bases were 40.70% T, 14.00% C, 31.20% A, and 14.10% G. This pattern reflects a higher A–T content compared to C–G, which is a common characteristic of mitochondrial genes, particularly the COI gene (Table 3).

Table 3. Average nucleotide composition of *P. blumei.*

Code	T (U)	C	A	G	A+T	G+C	Location	References
PBPSST.1	40,60	14,00	31,20	14,10	71,80	28,10	Central Sulawesi	Research Data
PBPSST.2	40,60	14,00	31,20	14,10	71,80	28,10	Central Sulawesi	Research Data
PBPSST.3	40,60	14,00	31,20	14,10	71,80	28,10	Central Sulawesi	Research Data
JQ982058	40,80	14,00	31,20	14,10	72,00	28,10	South Sulawesi	Condamine *et al.* (2013)
JQ982056	40,80	14,00	31,20	14,10	72,00	28,10	North Sulawesi	Condamine *et al.* (2013)
Rata-rata	40,70	14,00	31,20	14,10	71,88	28,10		

Phylogenetic Tree and Genetic Distance

Phylogenetic tree analysis was conducted to determine the evolutionary relationships and genetic affinities among the analyzed *P. blumei* sequences. Phylogenetic reconstruction was performed using MEGA 11 software by applying two analytical methods, namely Neighbor-Joining (NJ) (Figure 3) and Maximum Likelihood (ML) (Figure 4). The Kimura 2-parameter (K2P) nucleotide substitution model was employed, as it is commonly used in mitochondrial gene–based phylogenetic studies, particularly those involving the COI gene. The reliability of the phylogenetic tree topology was assessed through bootstrap analysis with 10,000 replications. Higher bootstrap values indicate greater confidence in the branching patterns and taxon groupings within the phylogenetic tree; thus, increasing bootstrap values reflect improved robustness of the inferred clades and evolutionary relationships (Sianturi et al., 2021). In addition, genetic distance analysis among sequences was conducted to support the interpretation of the phylogenetic relationships obtained (Table 4).

Genetic distance analysis using the Kimura 2-Parameter (K2P) model revealed that the genetic distances among *Papilio blumei* individuals from Central Sulawesi ranged from 0.00% to 0.00%, indicating no detectable genetic divergence among these samples. Similarly, the genetic distances between *P. blumei* from Central Sulawesi and *P. blumei* populations from other regions of Sulawesi also ranged from 0.00% to 0.00%. These results indicate a very high level of genetic homogeneity among *P. blumei* populations across Sulawesi. In contrast, the genetic distances between *P. blumei* and the comparative species *Troides helena* and *Tirumala hamata* were considerably higher, ranging from 14.79% to 15.37%. Such substantial genetic divergence reflects clear evolutionary separation between *P. blumei*

and these species (Table 4). A commonly accepted threshold for species delimitation based on genetic divergence is 3%. When the genetic distance between two individuals or groups exceeds 3%, they are considered to belong to different species. Conversely, if the genetic distance is equal to or less than 3%, the individuals or groups are regarded as belonging to the same species (Zhang & Bu, 2022).

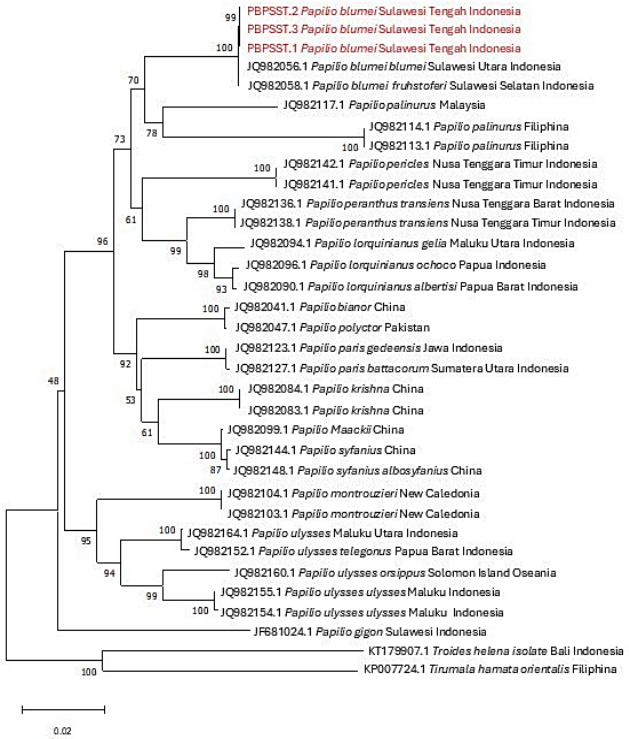

Figure 3. Phylogenetic tree constructed using the Neighbor-Joining (NJ) method with the Kimura 2-Parameter model and 10,000 bootstrap replicates.

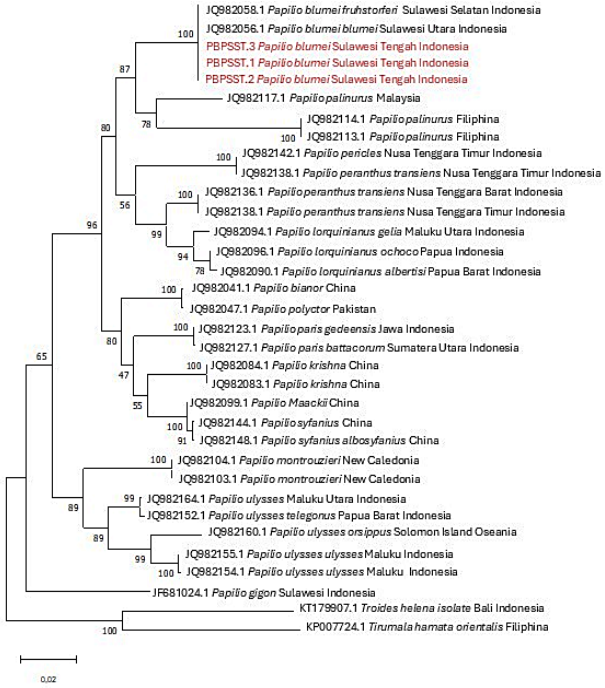

Figure 4. Phylogenetic tree constructed using the Maximum Likelihood (ML) method with the Kimura 2-Parameter model and 10,000 bootstrap replicates.

Table 4. Genetic distance of *Papilio blumei.*

	PBPSST.1_Papilio_blumei_Sulawesi_Tengah_Indonesia	PBPSST.2_Papilio_blumei_Sulawesi_Tengah_Indonesia	PBPSST.3_Papilio_blumei_Sulawesi_Tengah_Indonesia	JQ982058.1_Papilio_blumei_fruhstorferi_Sulawesi_Selatan	JQ982056.1_Papilio_blumei_blumei_Sulawesi_Utara_Indonesia	JQ982164.1_Papilio_ulysses_Maluku_Utara_Indonesia	JQ982160.1_Papilio_ulysses_crassus_Solomon_Island_Oseania	JQ982155.1_Papilio_ulysses_ulysses_Maluku_Indonesia	JQ982154.1_Papilio_ulysses_ulysses_Maluku_Indonesia	JQ982152.1_Papilio_ulysses_telegonus_Papua_Barat_Indonesia	JF681024.1_Papilio_gigon_Sulawesi_Indonesia	JQ982117.1_Papilio_palinurus_Malaysia	JQ982114.1_Papilio_palinurus_Filiphina	JQ982113.1_Papilio_palinurus_Filiphina	JQ982041.1_Papilio_bianor_China	JQ982047.1_Papilio_polyctor_Pakistan	JQ982144.1_Papilio_syfanius_China	JQ982148.1_Papilio_syfanius_albosyfanius_China	JQ982123.1_Papilio_paris_gedeensis_Jawa_Indonesia	JQ982127.1_Papilio_paris_battacorum_Sumatera_Utara_Indonesia	JQ982136.1_Papilio_peranthus_transiens_Nusa_Tenggara_Barat	JQ982138.1_Papilio_peranthus_transiens_Nusa_Tenggara_Timur	JQ982096.1_Papilio_lorquinianus_ochoco_Papua_Indonesia	JQ982094.1_Papilio_lorquinianus_gelia_Maluku_Utara_Indonesia	JQ982090.1_Papilio_lorquinianus_albertisi_Papua_Barat_Indonesia	JQ982084.1_Papilio_krishna_China	JQ982083.1_Papilio_krishna_China	JQ982104.1_Papilio_montrouzieri_New_Caledonia	JQ982103.1_Papilio_montrouzieri_New_Caledonia	JQ982142.1_Papilio_pericles_Nusa_Tenggara_Timur_Indonesia	JQ982141.1_Papilio_pericles_Nusa_Tenggara_Timur_Indonesia	JQ982099.1_Papilio_maackii_China	KT179907.1_Troides_helena_isolate_Bali_Indonesia	KP007724.1_Tirumala_hamata_orientalis_Filiphina
PBPSST.1_Papilio_blumei_Sulawesi_Tengah_Indonesia																																		
PBPSST.2_Papilio_blumei_Sulawesi_Tengah_Indonesia	0,00%																																	
PBPSST.3_Papilio_blumei_Sulawesi_Tengah_Indonesia	0,00%	0,00%																																
JQ982058.1_Papilio_blumei_fruhstorferi_Sulawesi_Selatan_Indonesia	0,00%	0,00%	0,00%																															
JQ982056.1_Papilio_blumei_blumei_Sulawesi_Utara_Indonesia	0,00%	0,00%	0,00%	0,00%																														
JQ982164.1_Papilio_ulysses_Maluku_Utara_Indonesia	7,23%	7,23%	7,23%	7,23%	7,23%																													
JQ982160.1_Papilio_ulysses_crassus_Solomon_Island_Oseania	8,31%	8,31%	8,31%	8,26%	8,26%	4,37%																												
JQ982155.1_Papilio_ulysses_ulysses_Maluku_Indonesia	8,09%	8,09%	8,09%	8,06%	8,06%	3,69%	2,92%																											
JQ982154.1_Papilio_ulysses_ulysses_Maluku_Indonesia	8,20%	8,20%	8,20%	8,16%	8,16%	3,78%	3,02%	0,09%																										
JQ982152.1_Papilio_ulysses_telegonus_Papua_Barat_Indonesia	7,37%	7,37%	7,37%	7,34%	7,34%	0,28%	4,47%	3,69%	3,78%																									
JF681024.1_Papilio_gigon_Sulawesi_Indonesia	9,44%	9,44%	9,44%	9,40%	9,40%	8,15%	9,50%	8,56%	8,67%	8,15%																								
JQ982117.1_Papilio_palinurus_Malaysia	5,41%	5,41%	5,41%	5,38%	5,38%	8,06%	8,90%	9,53%	9,63%	8,27%	10,35%																							
JQ982114.1_Papilio_palinurus_Filiphina	7,86%	7,86%	7,86%	7,82%	7,82%	10,46%	11,01%	11,22%	11,33%	10,68%	12,21%	7,72%																						
JQ982113.1_Papilio_palinurus_Filiphina	7,86%	7,86%	7,86%	7,82%	7,82%	10,46%	11,01%	11,22%	11,33%	10,68%	12,21%	7,72%	0,00%																					
JQ982041.1_Papilio_bianor_China	5,47%	5,47%	5,47%	5,44%	5,44%	6,63%	8,06%	7,55%	7,65%	6,94%	8,89%	6,67%	8,41%	8,41%																				
JQ982047.1_Papilio_polyctor_Pakistan	5,47%	5,47%	5,47%	5,44%	5,44%	6,63%	7,85%	7,34%	7,45%	6,94%	8,68%	6,67%	8,41%	8,41%	0,18%																			
JQ982144.1_Papilio_syfanius_China	5,97%	5,97%	5,97%	5,95%	5,95%	7,15%	8,59%	7,76%	7,87%	7,36%	8,89%	7,18%	8,72%	8,72%	4,68%	4,48%																		
JQ982148.1_Papilio_syfanius_albosyfanius_China	5,87%	5,87%	5,87%	5,94%	5,94%	7,15%	8,58%	7,76%	7,86%	7,35%	8,89%	7,17%	8,72%	8,72%	4,67%	4,48%	0,18%																	
JQ982123.1_Papilio_paris_gedeensis_Jawa_Indonesia	5,97%	5,97%	5,97%	5,95%	5,95%	7,14%	7,96%	7,86%	7,96%	7,35%	8,78%	6,56%	9,03%	9,03%	4,57%	4,57%	3,79%	3,79%																
JQ982127.1_Papilio_paris_battacorum_Sumatera_Utara_Indonesia	6,08%	6,08%	6,08%	6,05%	6,05%	7,25%	8,07%	7,97%	8,07%	7,45%	8,89%	6,66%	9,14%	9,14%	4,67%	4,67%	3,89%	3,88%	0,09%															
JQ982136.1_Papilio_peranthus_transiens_Nusa_Tenggara_Barat_Indonesia	5,17%	5,17%	5,17%	5,15%	5,15%	6,63%	7,65%	6,84%	6,94%	6,84%	8,89%	6,57%	8,30%	8,30%	5,75%	5,75%	6,26%	6,26%	5,55%	5,65%														
JQ982138.1_Papilio_peranthus_transiens_Nusa_Tenggara_Timur_Indonesia	5,17%	5,17%	5,17%	5,15%	5,15%	6,63%	7,65%	6,84%	6,94%	6,84%	8,89%	6,57%	8,30%	8,30%	5,75%	5,75%	6,26%	6,26%	5,55%	5,65%	0,00%													
JQ982096.1_Papilio_lorquinianus_ochoco_Papua_Indonesia	5,47%	5,47%	5,47%	5,44%	5,44%	7,14%	8,38%	8,18%	8,28%	7,35%	9,10%	6,36%	8,40%	8,40%	5,85%	5,85%	6,06%	6,05%	5,15%	5,25%	2,63%	2,63%												
JQ982094.1_Papilio_lorquinianus_gelia_Maluku_Utara_Indonesia	5,57%	5,57%	5,57%	5,54%	5,54%	7,45%	8,37%	8,17%	8,27%	7,55%	9,62%	6,67%	8,50%	8,50%	5,85%	5,85%	5,95%	5,95%	5,25%	5,35%	2,54%	2,54%	1,21%											
JQ982090.1_Papilio_lorquinianus_albertisi_Papua_Barat_Indonesia	5,47%	5,47%	5,47%	5,44%	5,44%	6,94%	8,17%	7,97%	8,07%	7,15%	8,89%	6,16%	8,40%	8,40%	5,65%	5,65%	5,85%	5,85%	5,15%	5,25%	2,44%	2,44%	0,28%	1,49%										
JQ982084.1_Papilio_krishna_China	5,58%	5,58%	5,58%	5,55%	5,55%	7,04%	8,59%	8,07%	8,18%	7,25%	8,79%	6,36%	9,15%	9,15%	4,58%	4,58%	3,80%	3,80%	4,88%	4,99%	6,27%	6,27%	6,36%	6,67%	6,16%									
JQ982083.1_Papilio_krishna_China	5,58%	5,58%	5,58%	5,55%	5,55%	7,04%	8,59%	8,07%	8,18%	7,25%	8,79%	6,36%	9,15%	9,15%	4,58%	4,58%	3,80%	3,80%	4,88%	4,99%	6,27%	6,27%	6,36%	6,67%	6,16%	0,00%								
JQ982104.1_Papilio_montrouzieri_New_Caledonia	8,52%	8,52%	8,52%	8,48%	8,48%	5,06%	6,66%	5,75%	5,85%	5,07%	8,68%	9,44%	11,01%	11,01%	8,38%	8,17%	7,05%	7,05%	7,56%	7,66%	7,56%	7,56%	7,68%	8,09%	7,47%	8,19%	8,19%							
JQ982103.1_Papilio_montrouzieri_New_Caledonia	8,52%	8,52%	8,52%	8,48%	8,48%	5,06%	6,66%	5,75%	5,85%	5,07%	8,68%	9,44%	11,01%	11,01%	8,38%	8,17%	7,05%	7,05%	7,56%	7,66%	7,56%	7,56%	7,68%	8,09%	7,47%	8,19%	8,19%	0,00%						
JQ982142.1_Papilio_pericles_Nusa_Tenggara_Timur_Indonesia	5,98%	5,98%	5,98%	5,95%	5,95%	8,37%	9,41%	9,21%	9,21%	8,38%	10,36%	7,09%	9,15%	9,15%	7,38%	7,38%	6,87%	6,87%	6,76%	6,86%	5,36%	5,36%	6,06%	5,66%	5,86%	7,59%	7,59%	9,12%	9,12%					
JQ982141.1_Papilio_pericles_Nusa_Tenggara_Timur_Indonesia	5,98%	5,98%	5,98%	5,95%	5,95%	8,37%	9,41%	9,21%	9,21%	8,38%	10,36%	7,09%	9,15%	9,15%	7,38%	7,38%	6,87%	6,87%	6,76%	6,86%	5,36%	5,36%	6,06%	5,66%	5,86%	7,59%	7,59%	9,12%	9,12%	0,00%				
JQ982099.1_Papilio_maackii_China	5,77%	5,77%	5,77%	5,74%	5,74%	6,94%	8,38%	7,56%	7,66%	7,15%	8,68%	6,97%	8,51%	8,51%	4,38%	4,18%	0,28%	0,28%	3,59%	3,69%	6,06%	6,06%	5,85%	5,75%	5,63%	3,51%	3,51%	6,95%	6,95%	6,87%	6,87%			
KT179907.1_Troides_helena_isolate_Bali_Indonesia	15,45%	15,45%	15,45%	15,37%	15,37%	13,03%	14,47%	13,69%	13,80%	13,13%	14,82%	15,04%	17,13%	17,13%	14,25%	14,25%	14,14%	14,14%	13,69%	13,81%	14,25%	14,25%	14,26%	14,37%	14,14%	14,47%	14,47%	13,58%	13,58%	13,58%	13,58%	13,80%		
KP007724.1_Tirumala_hamata_orientalis_Filiphina	14,87%	14,87%	14,87%	14,79%	14,79%	12,69%	14,02%	13,80%	13,91%	12,80%	14,46%	15,13%	16,52%	16,52%	14,13%	14,13%	14,01%	14,01%	14,24%	14,35%	14,23%	14,23%	13,79%	14,35%	13,68%	14,12%	14,12%	13,90%	13,90%	13,79%	13,79%	13,68%	12,60%	

Discussion

DNA amplification is a method used to increase the quantity of DNA strands obtained after the extraction stage, thereby enabling clearer and more accurate molecular analysis (Rahmadhan et al., 2019). The objective of amplification using the polymerase chain reaction (PCR) technique is to synthesize DNA at specific temperatures through repeated cycles. Short DNA primers bind to single-stranded DNA templates to initiate replication (Adhiyanto et al., 2020). Primers used in amplification must be specific to the target DNA to enhance amplification success. Each primer has its own optimal annealing temperature; therefore, optimization through PCR temperature cycling is required to determine the most suitable conditions. The amplified DNA products are subsequently subjected to electrophoresis to evaluate amplification quality (Aulia et al., 2021).

The amplification results of *Papilio blumei* specimens from Sulawesi produced clear and uniform DNA bands corresponding to the target fragment size. This indicates that the PCR process was optimal, with no evidence of DNA degradation or contamination. Additionally, no extra bands or smearing were observed, demonstrating the high specificity of the primers for the COI gene. Similarity analysis using Nucleotide BLAST (NCBI) revealed that the three *P. blumei* sequences from Central Sulawesi exhibited 100% query coverage and sequence identity values exceeding 98%, indicating a high level of accuracy in identifying *P. blumei* from Central Sulawesi. Higher query coverage and similarity values reflect greater homology between the sample sequences and reference sequences in the database (Aprilianto & Sembiring, 2016).

Genetic variation refers to changes at the nucleotide to genomic level that influence an organism's ability to adapt to environmental conditions; higher genetic variation generally increases the survival potential of a population (Rani et al., 2024). In this study, genetic variation parameters were analyzed using DnaSP software, including the number of haplotypes, variable sites, parsimony-informative sites, haplotype diversity (Hd), and nucleotide diversity (π). Analysis of the COI gene sequences (1,188 bp in length) revealed the presence of two haplotypes. Haplotype 1 consisted of samples PBPSST.1, PBPSST.2, and PBPSST.3 from Central Sulawesi, while Haplotype 2 included samples JQ982058.1 from South Sulawesi and JQ982056.1 from North Sulawesi. Furthermore, one variable site and one parsimony-informative site were identified.

The genetic variation analysis of *P. blumei* from Central Sulawesi yielded a haplotype diversity value of $Hd = 0.600 \pm 0.175$, indicating high haplotype diversity, as the value falls within the range of $0.5 < Hd \leq 1$. In contrast, the nucleotide diversity value was $\pi = 0.00051 \pm 0.00015$, indicating low nucleotide diversity, as the value is less than 0.10. A high Hd value suggests substantial variation among individuals within the *P.*

blumei population, while low nucleotide diversity may result from population decline due to natural events followed by rapid population expansion (Dewana et al., 2025).

Analysis of nucleotide composition showed proportions of T = 40.70%, A = 31.20%, C = 14.00%, and G = 14.10%, resulting in an A+T content of 71.88% and a C+G content of 28.10%. This indicates that the GC content is lower than the AT content. Adenine (A) always pairs with thymine (T) through two hydrogen bonds, which are weaker than the three hydrogen bonds connecting guanine (G) and cytosine (C). DNA sequences with lower GC content generally have lower denaturation temperatures compared to sequences with higher GC content (Dailami et al., 2022). A low GC percentage may reduce primer-binding efficiency to target DNA, as GC base pairs provide greater stability due to stronger hydrogen bonding (Nova et al., 2023). Variations in nucleotide composition are useful for detecting genetic similarities and differences among individuals for species identification purposes (Triandiza et al., 2021).

Phylogenetic analysis based on the mitochondrial COI gene was conducted using MEGA software with the Neighbor-Joining and Maximum Likelihood methods under the Kimura 2-parameter model with 10,000 bootstrap replications. Both methods produced similar tree topologies, with no significant differences in bootstrap values, and formed distinct clades in which each species clustered within the same clade. Phylogenetic reconstruction revealed that *Papilio blumei* from Central Sulawesi, *Papilio blumei fruhstorferi* from South Sulawesi, and *Papilio blumei blumei* from North Sulawesi exhibited very close evolutionary relationships, supported by bootstrap values ranging from 99% to 100%. Higher bootstrap values indicate greater confidence in the accuracy of the reconstructed phylogenetic topology (Oktafia & Badruzsaufari, 2021).

Genetic distance analysis showed that the highest genetic distances occurred between *P. blumei* and the outgroup species *Troides helena* and *Tirumala hamata*, ranging from 14.79% to 15.37%. These results are supported by the phylogenetic tree topology, which places *P. blumei* and the outgroup species far apart. Greater genetic distance values indicate more distant evolutionary relationships, which are reflected by increased separation in the phylogenetic tree (Bramasta et al., 2021). The lowest genetic distance value (0.00%) was observed among *Papilio blumei* from Central Sulawesi, *Papilio blumei fruhstorferi* from South Sulawesi, and *Papilio blumei blumei* from North Sulawesi. A genetic distance of 0.00% indicates that these taxa belong to the same genus and share very close genetic relationships. In general, lower genetic distance values correspond to closer evolutionary relationships. A commonly used threshold for species delimitation based on genetic divergence is 3% (Sahadeva & Pertiwi, 2023).

CONCLUSIONS

The phylogenetic analysis of *P. blumei* using the Neighbor-Joining (NJ) and Maximum Likelihood (ML) methods revealed that endemic butterfly specimens from Central Sulawesi are very closely related to *P. blumei* populations from South and North Sulawesi. This close relationship is supported by high bootstrap values (99–100) in the phylogenetic tree reconstruction, a query cover of 100%, and a sequence identity of 99.50%, indicating a clear and consistent phylogenetic relationship among *P. blumei* populations.

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REFERENCES

Adhiyanto, C., Hendarmin, L., dan Puspitaningrum, R. (2020). Pengenalan Dasar Teknik Bio-Molekuler. Penerbit Deepublish. Sleman.

Alias, S., & Soesilohadi, R.H. (2015). Perilaku dan Musuh Alami Kupu Endemik Sulawesi *Papilio blumei*: Acuan dalam Konservasi. *Jurnal Bioedukasi: Jurnal Pendidikan Biologi*, 8(1), 52-56. https://doi.org/10.20961/bioedukasi-uns.v8i1.3488.

Aprilianto, V., & Sembiring, L. (2016). Filogenetik Molekuler: Teori dan Aplikasi. *Innosain*, Yogyakarta.

Aulia, S. L., Suwignyo, R. A., dan Hasmeda, M. (2021). Optimasi Suhu Annealing untuk Amplifikasi DNA Padi Hasil Persilangan Varietas Tahan Terendam dengan Metode Polymerase Chain Reaction. Jurnal Ilmiah Matematika dan Ilmu Pengetahuan Alam, 18(1): 44-54. https://doi.org/10.31851/sainmatika.v18i1.5805

Bramasta, R. C., Faiqoh, E., Hendrawan, I. G., Sembiring, A., & Yusmalinda, N. L. A. (2021). Identifikasi Hiu yang Diperdagangkan di Bali Menggunakan Metode DNA Barcoding dan Analisis Filogenetik. Journal of Marine and Aquatic Sciences, 7(1), 84. https://doi.org/10.24843/jmas.2021.v07.i01.p12

Condamine, F. L., Toussaint, E. F. A., Cotton, A. M., Genson, G. S., Sperling, F. A. H., & Kergoat, G. J. (2013). Fine-scale biogeographical and temporal diversification processes of peacock swallowtails (Papilio subgenus Achillides) in the Indo-Australian Archipelago. *Cladistics*, 29(1), 88–111. https://doi.org/10.1111/j.1096-0031.2012.00412.x.

Dailami, M., Saleky, D., Toha, A. H. A., & Agamawan, L. P. I. (2022). Identifikasi Genetik Udang Mantis Dengan Pendekatan DNA Barcoding Gen *Sitokrom Oksidase 1* (*CO1*). *Acropora : Jurnal Ilmu Kelautan dan Perikanan Papua*, 5(1), 37-43.

Dewana, I. G. J., Putu, N., Pertiwi, D., Luh, N., & Yusmalinda, A. (2025). Genetic Diversity and Species Identification of Unhatched Sea Turtle Eggs from Southern Bali Hatcheries. *Jurnal Biologi Tropis*.

Fietri, W. A., Razak, A., & Ahda, Y. (2020). Analisis Filogenetik Ikan Tuna (*Thunnus* spp) di Perairan Maluku Utara Menggunakan *COI* (*Cytocrome Oxydase I*). *Jurnal Biologi Makasar*, 5(1), 69–78.

Hendiari, I. G. A. D., Sartimbul, A., Arthana, I. W., & Kartika, G. R. A. (2020). Keragaman genetik ikan lemuru (*Sardinella lemuru*) di wilayah perairan Indonesia. *Acta Aquatica: Aquatic Sciences Journal*, 7(1), 28. https://doi.org/10.29103/aa.v7i1.2405.

Hermawan, I., Amin, M., & Suhadi, S. (2022). Genetic diversity of Springtails (*Collembola Subclass*) Based on *Cytochrome oxidase Subunit I* (*COI*) Genes in Malang. *Biotropika: Journal of Tropical Biology*, 10(1), 67–77. https://doi.org/10.21776/ub.biotropika.2022.010.01.09.

Kamal, M. M., Hakim, A. A., Butet, N. A., Fitrianingsih, Y., & Astuti, R. (2019). Autentikasi Spesies Ikan Kerapu Berdasarkan Marka Gen Mt-*Coi* Dari Perairan Peukan Bada, Aceh. *Jurnal Biologi Tropis*, 19(2), 116–123. https://doi.org/10.29303/jbt.v19i2.1245.

Kolle M, Salgard-Cunha PM, Scherer MR, Huang F, Vukusic P, Mahajan S, Baumberg JJ, Steiner U (2010). Mimicking the colourful wing scale structure of the *Papilio blumei* butterfly. Nat Nanotechnol, 5(7), 511-5.

Makhzuni, R., & Dahelmi, S. (2013). Variasi Morfometri *Papilio polytes* L . (Lepidoptera : Papilionidae) di Beberapa Lokasi di Sumatera Barat Morphometry variation of *Papilio polytes* L . (Lepidoptera : Papilionidae) in several places in West Sumatra. *Jurnal Biologi Univwrsitas Ansalas,* 2(1), 50–56.

Mohapatra, S., Nayak, V., Paul, A., & Adhikary, S. (2019). Mitochondrial DNA: A Molecular Tool for Assessment of Genetic Diversity. *International Journal of Livestock Research*, 9(8), 49-58. https://doi.org/10.5455/ijlr.20190404113609.

Oktatia, R. E., & Badruzsaufari. (2021). Analisis Filogenetik *Garcinia* Spp. Berdasarkan Sekuens Gen rRNA *Ziraa'ah: Majatah Umiah Pertanian*. 46(2), 259-264. https://doi.org/10.31602/ZMIP.V4612.4526

Nova, B., Wardi, E. S., Rahmi, M., & Zikri, F. (2024). Desain Primer dan Deteksi Gen CHS (*Chalcone Synthase*) pada Tanaman Gambir (*Uncaria gambir* (Hunter) Roxb.) Tipe Riau Mancik. *Baselang*, 4(1), 1–12. https://doi.org/10.36355/bsl.v4i1.124.

Partiwi, S., Al Idrus, A., Zulkifli, L., Mahrus, & Sedijani, P. (2023). Isolation and Molecular Characterization of Brotowali (*Tinospora crispa*) Rhizosphere Bacteria Producing Siderophore from Dry Lands of Lombok Island. *Jurnal Biologi Tropis*, 23(2), 275–284. https://doi.org/10.29303/jbt.v23i2.6138.

Rahmadhan, D., Sari, R., & Apridamayanti, P. (2019). Pengaruh suhu *annealing* terhadap amplifikasi gen tem menggunakan primer dengan %GC rendah. *Jurnal Mahasiswa Farmasi Fakultas Kedokteran UNTAN*, 4(1):1-7.

Rani, W. M., Dea Puspita, R., Sefina, N., Sa'adah, N., & Achyar, A. (2024). Analisis Variasi Genetik Gen L1 HPV-52 Menggunakan RFLP secara in Silico. *Indonesian Journal of Pharmaceutical Education*, 4(1), 82–96. https://doi.org/10.37311/ijpe.v4i1.24496.

Sahadeva, M. L., & Pertiwi, N. P. D. (2024). Konstruksi Pohon Filogenetik Spesies dalam Famili Orchidaceae Berdasarkan Marka Gen matK Kloroplas: Studi in Silico. *Wahana Matematika dan Sains: Jurnal Matematika, Sains, Dan Pembelajarannya*, 17(3), 12–27. https://doi.org/10.23887/wms.v17i3.87986.

Saleky, D., & Dailami, M. (2021). Konservasi Genetik Ikan Kakap Putih *(Lates calcarifer*, Bloch, 1790) Melalui Pendekatan DNA Barcoding dan Analisis Filogenetik di Sungai Kumbe Merauke Papua. *Jurnal Kelautan Tropis*, 24(2), 141–150. https://doi.org/10.14710/jkt.v24i2.10760.

Sianturi, R., Dailami, M., & Saleky, D. (2021). Identifikasi dan Analisis Filogenetik Ikan Ekonomis Penting *Oreochromis* sp. dengan Pendekatan DNA Barcoding. *Bioscientist : Jurnal Ilmiah Biologi*, 9(2):465-476.

Tindi, M., Mamangkey, N. G. F., & Wullur, S. (2017). The DNA Barcode and molecular phylogenetic analysis several Bivalve species from North Sulawesi Waters based on *COI* gene. *Jurnal Pesisir dan Laut Tropis*, 1(2), 32–38.

Triandiza, T., Kusnadi, A., Sari, N., Persilette, R.N., Ainarwoman, A., Suparmo., Sapulate, S. (2021) Keragaman Genetik Kima Kecil (*Tridacna maxima*) Di Pulau Kur, Pulau Biak , Dan Manado Serta Implikasinya Untuk Konservasi. *Jurnal Penelitian Perikanan Indonesia*, 167–179.

Zhang, H., & Bu, W. (2022). Exploring Large-Scale Patterns of Genetic Variation in the *COI* Gene among Insecta: Implications for DNA Barcoding and Threshold-Based Species Delimitation Studies. *Insects*, 13(5). https://doi.org/10.3390/insects13050425.