BIOLOGY, MEDICINE, & NATURAL PRODUCT CHEMISTRY ISSN 2089-6514 (paper)
Volume 15, Number 1, April 2026 | Pages: 705-713 | DOI: 10.14421/biomedich.2026.151.705-713 ISSN 2540-9328 (online)

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

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Manuscript received: 06 January 2026. Revision accepted: 13 April 2026, Published: 26 May 2026.

Abstract

Papilio polytes is a butterfly belonging to the family Papilionidae and is endemic to Sulawesi. The evolutionary relationships of this species were investigated through phylogenetic analysis based on mitochondrial DNA, specifically the *COI* (Cytochrome Oxidase I) region, which was subsequently reconstructed into a phylogenetic tree. This study aimed to analyze the phylogenetic relationships of *P. polytes* collected from Bakubakulu Village, Palolo District, Sigi Regency, Central Sulawesi, in relation to other members of the genus *Papilio* from different regions. Specimens of *P. polytes* were collected using a roaming (exploratory) method, followed by DNA isolation, DNA amplification, sequencing, electrophoresis, and bioinformatics analyses using GeneStudio, DNASTAR, MESQUITE, and MEGA 11. The phylogenetic tree analyses using the Neighbor Joining (NJ) and Maximum Likelihood (ML) methods revealed clear evolutionary relationships. *Papilio polytes* from Central Sulawesi showed a closer genetic relationship with *P. polytes* from other regions and clustered within the same clade, whereas other species formed separate clades. Genetic distance analysis based on the *COI* gene indicated that *P. polytes* from Central Sulawesi exhibited a genetic distance of 0.00% among individuals, suggesting very high genetic similarity within the population. The genetic distance between *P. polytes* from Central Sulawesi and *P. polytes* populations from other regions was relatively low, ranging from 0.00% to 3.86%, while the distance to the outgroup species *Eurytides marcellus* and *Battus polydamas* was much higher, ranging from 13.15% to 16.25%. These findings indicate that the phylogenetic relationships among *P. polytes* populations are very close and consistent.

Keywords: *Papilio polytes*; Phylogenetics; COI (Cytochrome c oxidase subunit I); mtDNA (mitochondrial DNA); phylogenetic.

INTRODUCTION

Butterflies (Lepidoptera: Papilionoidea) are scale-winged insects that play an essential ecological role as pollinators (Handayani & Rahayuningsih, 2022). Through their pollination activities, butterflies indirectly contribute to plant reproductive processes and the maintenance of plant populations, thereby functioning as important ecosystem service providers that support the sustainability and conservation of various plant species. In addition to their role in pollination, butterflies are widely recognized as effective biological indicators of environmental change. Their life cycles, habitat specificity, and sensitivity to microclimatic conditions make them highly responsive to habitat alteration, pollution, and climate change, allowing their presence and diversity to reflect ecosystem health (Azahra, 2021).

Papilio polytes (Papilionidae) is a butterfly species well known for its distinct sexual dimorphism, particularly in females that exhibit remarkable mimicry of other unpalatable butterfly species. This mimicry is considered an adaptive strategy to reduce predation pressure. Morphologically, male *P. polytes* individuals are characterized by white streak patterns on the hindwings, whereas females possess elongated tails with conspicuous red spots on the hindwings (Aprilia et al., 2018). The wingspan of *P. polytes* reaches approximately five centimeters, placing it among medium-sized swallowtail butterflies. These morphological variations have attracted considerable attention in ecological and evolutionary studies, especially in relation to adaptation and survival strategies (Ilhamdi et al., 2019).

The geographical distribution of *P. polytes* is remarkably wide, encompassing Indo-China, India, Sri Lanka, the Malay Peninsula, Sumatra, Java, Bali, Nusa Tenggara, Kalimantan, Sulawesi, Maluku, and the Philippines (Mustari et al., 2016). Such a broad distribution suggests the existence of genetic variation among populations from different regions and highlights the importance of molecular approaches to understand population structure and evolutionary history. The availability of comprehensive mitochondrial DNA (mtDNA) data is therefore crucial for supporting studies

on genetic diversity, population differentiation, and phylogeography (Choiriyah, 2020).

Mitochondrial DNA is extensively used in population genetics and phylogenetic research because it is maternally inherited, has a relatively rapid evolutionary rate, and lacks recombination, allowing clear resolution of evolutionary lineages (Tavakoli et al., 2017). Among the mitochondrial genes, the cytochrome c oxidase subunit I (*COI*) gene is one of the most widely employed molecular markers. This gene plays a key role in cellular energy production and possesses a relatively conserved structure, while still containing sufficient nucleotide variation for distinguishing closely related species (Hermawan et al., 2022). Moreover, the *COI* gene exhibits low levels of insertions and deletions, making it particularly suitable for species identification, DNA barcoding, and phylogenetic reconstruction (Tindi et al., 2017; Mamuaya et al., 2024).

Phylogenetic analysis is a powerful approach for investigating evolutionary relationships among organisms based on shared morphological or genetic characteristics. Species that share similar traits are considered to have closer evolutionary relationships and are often grouped into monophyletic clades derived from a common ancestor (Astarini et al., 2021). This analytical framework also provides insights into evolutionary processes by revealing patterns of divergence and character change among species over time. Despite extensive studies on the diversity, morphometrics, and morphological variation of *P. polytes*, phylogenetic analyses based on the mitochondrial *COI* gene remain limited (Shakya et al., 2020). This study aims to analyze the phylogenetic relationships of *P. polytes* using the mitochondrial *COI* gene and to reconstruct its evolutionary history through phylogenetic tree analysis, providing a scientific basis for taxonomic clarification and molecular-based conservation strategies.

MATERIAL AND METHODS

Study area

This study was conducted from November 2025 in Bakubakulu Village, Palolo District, Sigi Regency, Central Sulawesi, Indonesia (Figure 1). The study area is characterized by a mosaic of agricultural land, secondary forest, and residential areas, which provide suitable habitats for various butterfly species, including *Papilio polytes*. The selection of this location was based on the frequent occurrence of *P. polytes* populations and its ecological suitability for sampling activities. Environmental conditions such as temperature, vegetation composition, and availability of host plants support the life cycle and distribution of swallowtail butterflies in this region.

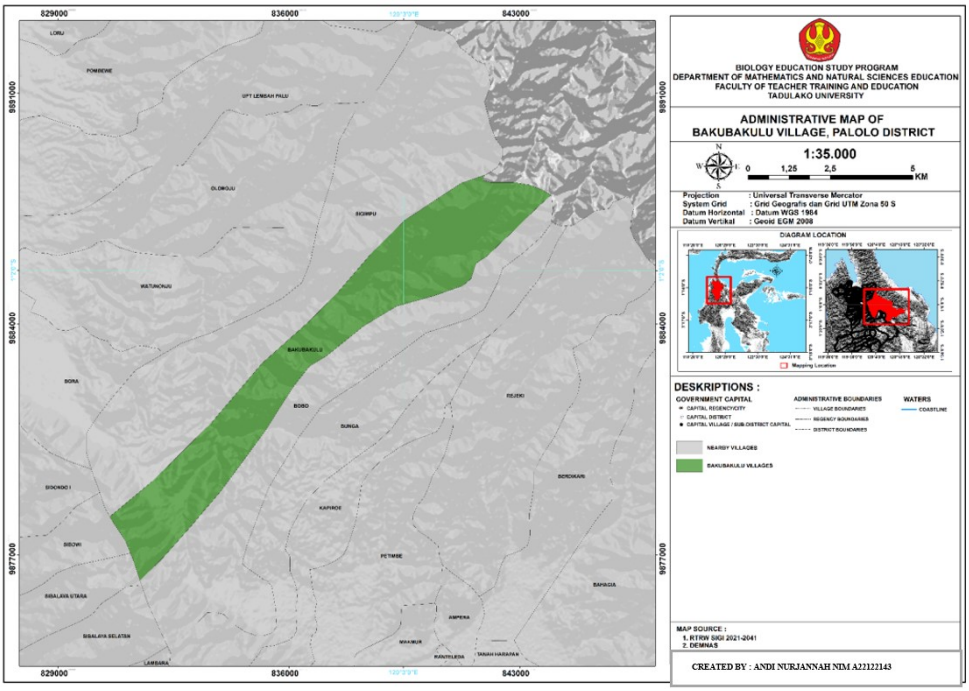

Figure 1. Map of the research location in Bakubakulu Village, Palolo District, Sigi Regency, Central Sulawesi, Indonesia.

Procedures

Sample Collection

Samples of P. polytes were collected using an exploratory (roaming) method to maximize encounters with individuals in their natural habitat. Butterflies were actively captured using insect nets during daylight hours when butterflies are most active. Each captured specimen was carefully handled to avoid physical damage and

subsequently preserved for molecular analysis. This method allowed for representative sampling of P. polytes individuals from the study area.

DNA Isolation

DNA isolation was performed on *P. polytes* specimens using the GS 100gSYNC™ DNA Extraction Kit, following the manufacturer's protocol with minor modifications. One individual specimen, excluding the head and wings, was placed into a 1.5 mL microcentrifuge tube. A total of 200 µL of GST buffer and 200 µL of Proteinase K were added, followed by vortexing and spin-down to obtain a homogeneous solution. The mixture was incubated at 60°C for 2.5 hours until complete lysis occurred and the solution became clear. The resulting supernatant was then processed using GSB buffer, absolute ethanol (EtOH), and a GS column. The DNA was subsequently washed, dried, eluted at 60°C, and stored at −20°C until further analysis.

DNA Amplification and Sequencing

The extracted DNA was amplified using mitochondrial COI gene primers, namely LCO1490 (forward) and HCO2198 (reverse), in a total PCR reaction volume of 25 µL. The PCR cycling conditions consisted of pre-denaturation at 95°C for 5 minutes, followed by denaturation at 94°C, annealing at 50°C for 30 seconds, extension at 72°C for 30 seconds, post-extension at 72°C for 7 minutes, and a final hold at 4°C. The amplified products were then sequenced using a Genetic Analyzer 3500 to obtain nucleotide sequences of the *COI* gene.

Electrophoresis

Electrophoresis was carried out using 1% agarose gel with a DNA ladder as a molecular size marker. The process was conducted at 50 volts for 17–20 minutes to separate DNA fragments based on their size. After electrophoresis, the gel was visualized under a UV transilluminator using a gel documentation system to observe and document the presence of DNA bands, confirming successful amplification.

Data Analysis

COI gene sequencing data (forward and reverse *.ab1* files) were edited and assembled using GeneStudio and DNASTAR software to obtain consensus sequences. Species identification was performed by comparing the consensus sequences with reference data using Nucleotide BLAST (NCBI). Sequence alignment was conducted using MESQUITE version 3.51 (Aji & Arisuryanti, 2021), and the aligned sequences were converted into FASTA format for further analysis in MEGA 11. Genetic distance estimation was calculated using the Kimura 2-Parameter model, while phylogenetic trees were reconstructed using the Neighbor Joining (NJ) and Maximum Likelihood (ML) methods implemented in MEGA 11 (Tamura et al., 2021). These analyses were used to infer the evolutionary relationships and genetic affinities among *P. polytes* populations.

RESULTS AND DISCUSSION

Amplification and Sequence Similarity of *Papilio polytes*

Three mitochondrial *COI* gene samples of *P. polytes* collected from Bakubakulu Village, Palolo District, Sigi Regency, Central Sulawesi, were successfully amplified using the COI gene with the forward primer LCO1490-F and the reverse primer HCO2198-R. Agarose gel electrophoresis revealed a DNA fragment of approximately 609 bp, corresponding to the expected size of the *COI* gene region (Figure 2). PCR conditions were considered optimal when a single, thick, and clear DNA band of the target size was observed on the agarose gel without smearing or non-specific amplification products, indicating that the samples were suitable for subsequent sequencing analysis (Setyawati & Zubaidah, 2021).

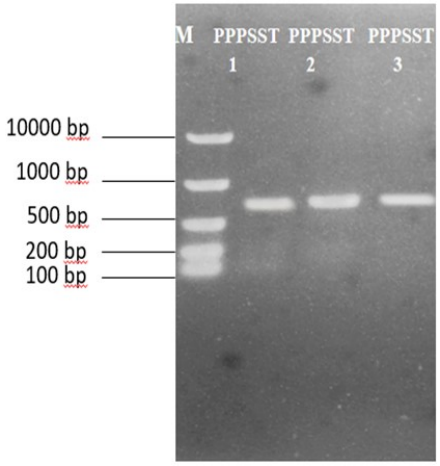

Figure 2. Results of mitochondrial *COI* gene amplification. Sample PPPSST represents *P. polytes* collected from Bakubakulu Village, Palolo District, Sigi Regency, Central Sulawesi, while M denotes the DNA marker (ladder)

The obtained sequences were analyzed using Nucleotide BLAST (NCBI) to determine sequence similarity and query coverage against reference data in GenBank. BLAST analysis of the mitochondrial *COI* gene of *Papilio polytes* from Central Sulawesi showed a similarity level of 99–100% with sequences deposited in GenBank (Table 1). A higher percentage of identity indicates a greater similarity between the query sequence and the reference sequence (Nuraini & Purwanto, 2021).

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

Code	BLAST			Species Verification	Location
	% Identity	% Query Cover	Accession Numbern *GenBank*		
PPPSST. 01	99,68%	100%	MW168164.1	*Papilio polytes*	Central Sulawesi
PPPSST. 02	99,68%	100%	MW168164.1	*Papilio polytes*	Central Sulawesi
PPPSST. 03	99,68%	100%	MW168164.1	*Papilio polytes*	Central Sulawesi

Genetic Variation of *Papilio polytes*

The genetic variation of *Papilio polytes* showed a haplotype diversity (Hd) of 0.786 ± 0.113, with four haplotypes, and a nucleotide diversity (π) of 0.0207 ± 0.00447, indicating a high level of nucleotide diversity. A total of 24 variable sites and 23 parsimony-informative sites were identified (Table 2). Genetic variation reflects the level of diversity within a species. A high similarity index indicates low genetic variation or a close genetic distance among individuals, whereas a low similarity index suggests high genetic diversity (Hanum et al., 2025). Haplotype diversity values of Hd < 0.5 indicate low genetic diversity, while values of Hd > 0.5 to ≤ 1.0 indicate high genetic diversity. The relatively high haplotype diversity observed in *P. polytes* therefore reflects substantial genetic diversity within the studied population.

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

Sample Code	bp	Number of Individual	Number of Haplotypes	Variable Site	Parsimony Site	Haplotype Diversity (Hd)	Nucleotide Diversity (π)
PPPSST.01 PPPSST.02 PPPSST.03 MG892099.1 KC158441.1 KR006994.1 AB969795.1 KR006999.1	609	8	4	24	23	0,786± 0,113	0,02070± 0,00447

Nucleotide Composition

Each nucleotide consists of a deoxyribose sugar, a phosphate group, and a nitrogenous base, namely adenine (A), thymine (T), guanine (G), and cytosine (C). Purine bases (A and G) always pair with pyrimidine bases (T and C), following the base-pairing rules A = T and G = C (Alberts et al., 2017). Based on the analysis of eight *Papilio polytes* sequences using MEGA 11, the average nucleotide composition was 39.2% thymine (T), 16.4% cytosine (C), 30.2% adenine (A), and 14.3% guanine (G). The A+T base pair composition was 8.66%, whereas the G+C composition was 3.83% (Table 3).

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

Code	T (U)	C	A	G	A+T	G+C	Location	References
PPPSST.01	39,6	15,8	30,4	14,3	8,75	3,76	Central Sulawesi	Research Data
PPPSST.01	39,6	15,8	30,4	14,3	8,75	3,76	Central Sulawesi	Research Data
PPPSST.01	39,6	15,8	30,4	14,3	8,75	3,76	Central Sulawesi	Research Data
MG892099.1	38,9	16,7	30,0	14,3	8,61	3,87	Bangladesh	Hossain *et al*. (2018)
KC158441.1	38,9	16,7	30,0	14,3	8,61	3,87	Pakistan	Ashfaq *et al*. (2013)
KR006994.1	38,9	16,7	30,0	14,3	8,61	3,87	India	Rekha *et al*. (2015)
AB969795.1	38,8	16,9	30,0	14,3	8,60	3,90	Japan	Yamda *et al*. (2014)
KR006999.1	39,1	16,6	30,0	14,3	8,63	3,86	India	Rekha *et at*. (2015)
Average	39,2	16,4	30,2	14,3	8,66	3,83		

Phylogenetic Tree and Genetic Distance

The phylogenetic tree of *P. polytes* was constructed using MEGA 11 software with two analytical methods, namely Neighbor-Joining (NJ) (Figure 3) and Maximum Likelihood (ML) (Figure 4), employing the Kimura 2-parameter model with 10,000 bootstrap replicates. The NJ method was selected because it provides representative estimates of branch lengths, while the ML method infers phylogenetic relationships based on variations in evolutionary branch lengths (Oktafia & Badruzsaufari, 2021). The Kimura 2-parameter model is widely applied due to its computational efficiency and accuracy for DNA sequence analysis (Anafarida & Badruzsaufari, 2020).

The analysis included 39 *COI* sequences, comprising three *P. polytes* sequences from Central Sulawesi, additional *Papilio* sequences retrieved from GenBank, and two outgroup species, *Eurytides marcellus* and *Battus polydamas*. The outgroup species were used as references to determine evolutionary relationships within the ingroup, serving as a basis for phylogenetic tree construction and aiding in the identification of shared ancestral characteristics (Sahadeva & Pertiwi, 2023).

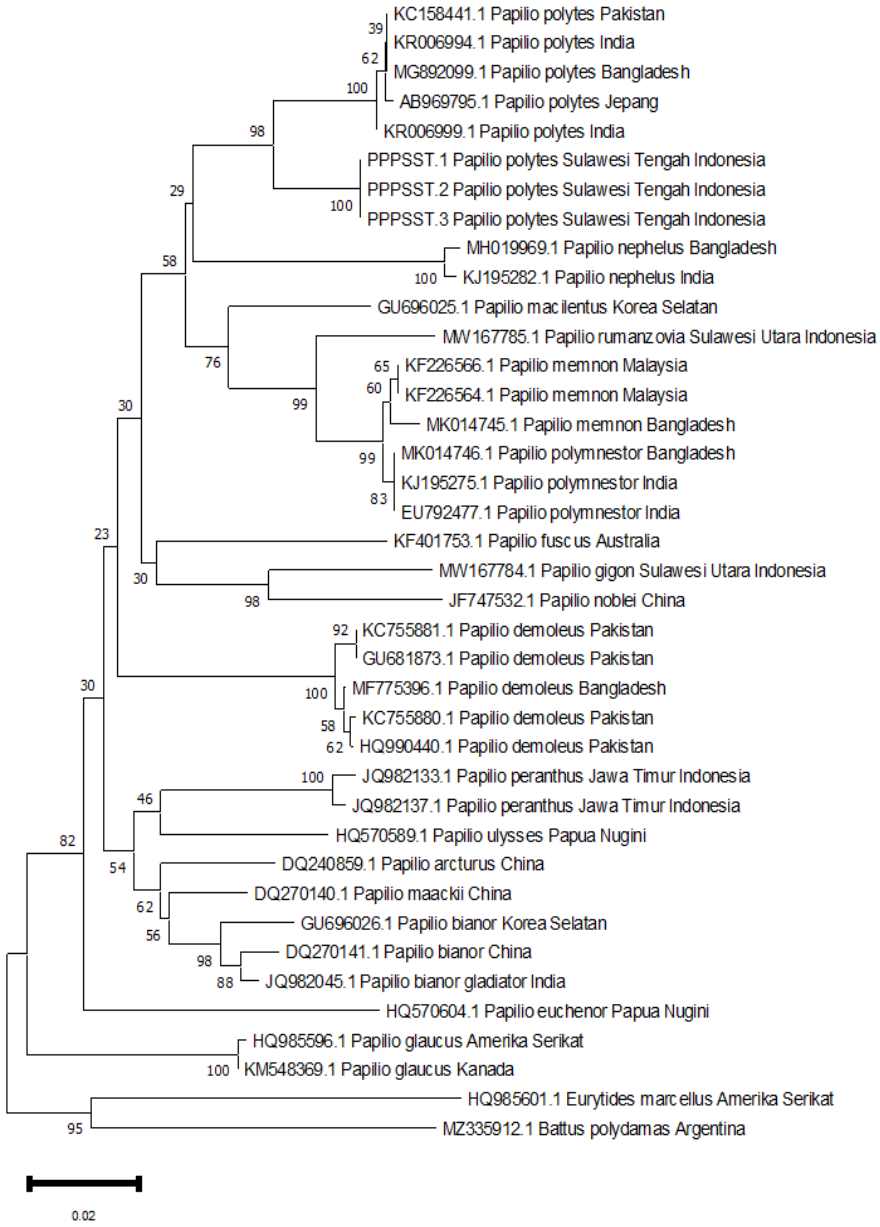

Figure 3. Phylogenetic tree constructed using the Neighbor-Joining (NJ) method with the Kimura 2-Parameter model and 10,000 bootstrap replicates.

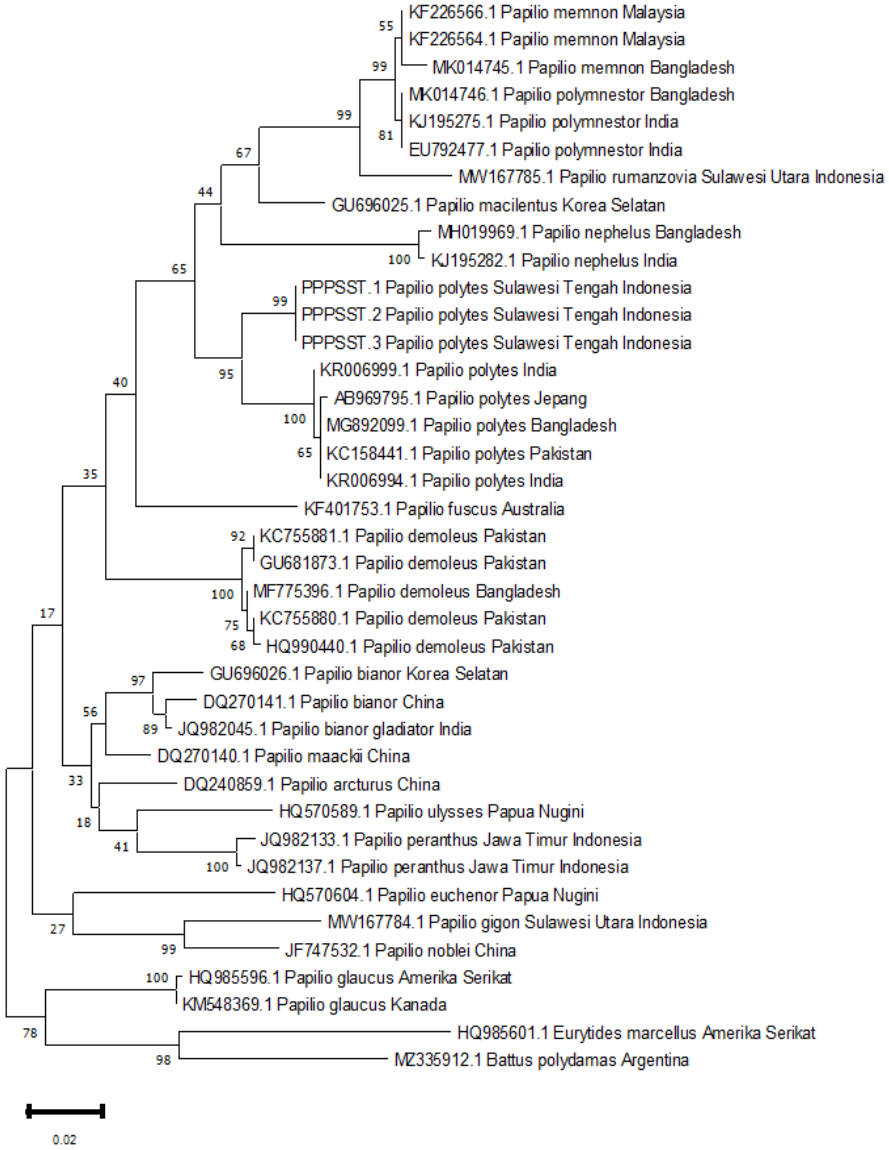

Figure 4. Phylogenetic tree constructed using the Maximum Likelihood (ML) method with the Kimura 2-Parameter model and 10,000 bootstrap replicates.

Based on Figures 3 and 4, the phylogenetic tree analyses of the *COI* gene within the genus *Papilio* were reconstructed using the Neighbor-Joining (NJ) and Maximum Likelihood (ML) methods. The phylogenetic trees show that all *P. polytes* sequences from Central Sulawesi (PPPSST.1, PPPSST.2, and PPPSST.3) clustered within the same clade as *P. polytes* from India, Japan, Bangladesh, Pakistan, and other regions, with high bootstrap values indicating strong statistical support. Other *Papilio* species formed separate clades, while *Eurytides marcellus* and *Battus polydamas* were positioned outside the ingroup as outgroup taxa. A phylogenetic tree is considered to have high reliability when bootstrap values exceed 70% (Subari et al., 2021). Furthermore, genetic distance analysis revealed that *P. polytes* from Central Sulawesi exhibited a genetic distance of 0.00% among individuals, indicating very high genetic similarity within the population. The genetic distance between *P. polytes* from Central Sulawesi and *P. polytes* populations from other regions was relatively low, ranging from approximately 0.00% to 3.86%, confirming that all samples belong to the same species. In contrast, the genetic distance between *P. polytes* and other *Papilio* species was considerably higher, ranging from 7.50% to 11.80%, while the distance to the outgroup species *Eurytides marcellus* and *Battus polydamas* ranged from 13.15% to 16.25%, indicating clear species differentiation and evolutionary divergence. Based on a species delimitation threshold of 3% (Zhang &Bu, 2022), the *P. polytes* population from Central Sulawesi is therefore confirmed to belong to the species *Papilio polytes*.

Discussion

Papilio polytes is a butterfly species belonging to the family Papilionidae that is widely distributed across

tropical Asia, including South and Southeast Asia. This species is recognized as a habitat generalist, as it can persist under a wide range of environmental conditions, from natural forests to areas affected by anthropogenic disturbance (Lim et al., 2022). One of the most distinctive characteristics of *P. polytes* is the presence of Batesian mimicry expressed exclusively in females, while males retain a non-mimetic wing pattern. Female *P. polytes* are able to mimic the coloration and patterns of toxic butterfly species from other genera, thereby reducing predation risk. Recent genetic studies have demonstrated that this polymorphic mimicry is controlled by complex genetic regulation, particularly involving developmental regulatory genes such as *doublesex* (Palmer & Kronforst, 2020).

Amplification of the mitochondrial *COI* gene from *P. polytes* specimens collected in Bakubakulu Village, Central Sulawesi, successfully produced DNA fragments of approximately 609 bp. The presence of a single, distinct DNA band without smearing on agarose gel electrophoresis indicates that the PCR conditions were optimal. This successful amplification confirms that the primers LCO1490 and HCO2198 specifically targeted the *COI* gene, rendering the PCR products suitable for subsequent sequencing. These results also indicate that the isolated DNA was of good quality and free from PCR inhibitors.

The high sequence similarity between the *COI* sequences of *P. polytes* from Central Sulawesi and reference sequences in GenBank confirms the accuracy of molecular species identification. An identity percentage of 99.68% suggests that the observed nucleotide variation remains within the range of intraspecific variation, as commonly reported in global DNA barcoding studies of Lepidoptera (Hebert et al., 2016). The consistency of BLAST results further supports the reliability of the *COI* gene as an effective molecular marker for taxonomic verification of butterflies, particularly in groups with high morphological similarity.

Genetic variation analysis revealed that *Papilio polytes* exhibited a haplotype diversity (Hd) value of 0.786 ± 0.113 and a nucleotide diversity (π) of 0.02070 ± 0.00447. The relatively high Hd value indicates the presence of multiple haplotypes within the population, suggesting that although individuals are genetically closely related, intraspecific genetic variation is still maintained. The identification of 24 variable sites and 23 parsimony-informative sites demonstrates that the *COI* gene retains sufficient evolutionary information to resolve phylogenetic relationships at the population level.

The nucleotide composition of the mitochondrial *COI* gene in *P. polytes* shows a dominance of adenine (A) and thymine (T) over guanine (G) and cytosine (C), resulting in a higher A+T content than G+C. This pattern is typical of insect mitochondrial DNA and reflects the molecular stability of the COI gene. The predominance of A+T bases also supports the suitability of the *COI* gene as a phylogenetic marker, as it does not exhibit extreme compositional bias that could distort evolutionary reconstructions (Zhang & Bu, 2022).

The clustering of *P. polytes* from Central Sulawesi within a single clade together with populations from other regions indicates a very close phylogenetic relationship and a shared evolutionary origin. The Neighbor-Joining method effectively illustrates genetic relationships based on evolutionary distance, particularly at the species and population levels (Kumar et al., 2018). High bootstrap values reinforce the reliability of the resulting clusters and indicate that the phylogenetic signal of the *COI* gene is sufficiently strong to reconstruct the evolutionary relationships of *P. polytes*. The phylogenetic topology generated using the Maximum Likelihood method shows a pattern consistent with that obtained using Neighbor-Joining. The Maximum Likelihood approach is considered more robust because it statistically incorporates nucleotide substitution models, resulting in more accurate phylogenetic inference (Tamura et al., 2021). The congruence between the two methods strengthens the conclusion that *P. polytes* populations from different regions belong to a single evolutionary lineage.

The low genetic distances observed among *P. polytes* individuals indicate high genetic similarity and support its status as a single species. The relatively narrow range of intraspecific genetic distances is consistent with commonly accepted thresholds in Lepidoptera DNA barcoding studies, typically below 3–4% (Hebert et al., 2016). The close genetic relationship between *P. polytes* from Central Sulawesi and populations from other regions may be attributed to the relatively low mutation rate of the *COI* gene, which results in genetic stability across geographically separated populations. This is reflected in the consistent clustering of *P. polytes* within the same clade using both Neighbor-Joining and Maximum Likelihood methods with high bootstrap support, indicating evolutionary homogeneity across regions despite geographic separation. These findings are consistent with those of Hossain et al. (2022), who reported that the *COI* gene consistently groups *P. polytes* across different locations. Furthermore, the markedly higher genetic distances between *P. polytes* and outgroup species such as *Eurytides marcellus* and *Battus polydamas* clearly indicate species-level divergence. Overall, this study confirms that *Papilio polytes* populations exhibit very close phylogenetic relationships based on *COI* gene analysis and reinforces the effectiveness of the *COI* gene as a molecular marker for phylogenetic studies.

CONCLUSIONS

Mitochondrial *COI* gene based phylogenetic analysis revealed that *Papilio polytes* from Central Sulawesi is closely related to *P. polytes* populations from various

other regions, clustering within the same clade with high bootstrap support. Extremely low genetic distances among individuals (0.00%) and relatively small genetic distances to populations from other regions (0.00–3.86%) confirm that all samples belong to the same species.

Acknowledgements: The authors would like to express their sincere gratitude to Tadulako University for the institutional support and facilities provided during the conduct of this research.

Authors' Contributions: Conceptualization, Andi Nurjannah, Lestari M.P Alibasyah, and Manap Trianto; methodology, Manap Trianto and Yulia Windarsih; analysis, Masrianih and Amalia Buntu; writing original draft preparation, Andi Nurjannah, Lestari M.P Alibasyah, and Manap Trianto; writing review and editing, All authors.

Competing Interests: The authors declare that there are no competing interests.

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