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

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Abstract

Cethosia myrina is a butterfly species belonging to the family Nymphalidae and is endemic to Sulawesi, Indonesia. The phylogenetic relationships of this species were investigated through molecular phylogenetic analysis. Phylogenetics is a method used to examine the evolutionary relationships among living organisms. This study aimed to describe the phylogenetic relationships of *C. myrina* based on the mitochondrial Cytochrome Oxidase Subunit I (COI) gene. Samples were collected using an exploratory survey method. Genomic DNA was isolated using the GS 100gSYNC™ DNA Extraction Kit. DNA amplification was performed using COI primers (LCO1490 forward and HCO2198 reverse) through the Polymerase Chain Reaction (PCR) technique. Amplified DNA fragments were visualized using 1% agarose gel electrophoresis, a UV transilluminator, and a gel documentation system. Subsequent bioinformatics analyses were conducted using Gene Studio, DnaSP, BLAST, DNASTAR, and MESQUITE software. Phylogenetic reconstruction was performed using the Neighbor-Joining (NJ) and Maximum Likelihood (ML) methods in MEGA version 11, applying the Kimura 2-parameter model with 10,000 bootstrap replicates. The results demonstrated that both the Neighbor-Joining and Maximum Likelihood methods produced phylogenetic trees that clearly illustrated the evolutionary relationships among the analyzed samples. Genetic variation analysis revealed the presence of two haplotypes, with a haplotype diversity (Hd) of 0.603 ± 0.165 and a nucleotide diversity (π) of 0.00053 ± 0.00013. Phylogenetic reconstruction formed a single monophyletic clade of *C. myrina* supported by a 99% bootstrap value. A genetic distance of 0.00% was observed among populations from Central Sulawesi (CMST1.1, CMST1.2, and CMST1.3), Central Sulawesi (EU275514.1), and South Sulawesi (HM998338.1), indicating an extremely close genetic relationship among these populations.

Keywords: *Cethosia myrina*; phylogenetic; COI (*Cytochrome oxidase* l), Central Sulawesi.

INTRODUCTION

The family Nymphalidae is one of the largest families within the order Lepidoptera, characterized by high species diversity and important ecological roles as pollinators and environmental bioindicators (Modeong et al., 2020). Members of Nymphalidae exhibit considerable variation in body size, ranging from small to large species (Ruslan & Andayaningsih, 2021). One species belonging to this family is *Cethosia myrina*, a butterfly endemic to Sulawesi (Vane-Wright, 2012). *C. myrina* can be readily distinguished by its serrated wing margins resembling lace, a characteristic feature of the genus *Cethosia*. The dorsal wing surface is predominantly bright orange with black markings, whereas the ventral surface displays intricate lace-like patterns in purple, white, and brown hues (Vane-Wright, 2012). Variations in wing patterns and morphological characteristics among butterfly populations on different islands suggest the influence of geographic isolation and long-term evolutionary processes. Previous studies on *C. myrina* have primarily focused on diversity, morphometric traits, and morphological variation. Consequently, molecular identification approaches are needed to overcome the limitations of morphology-based methods and to facilitate rapid and accurate taxonomic assessments (Muller & Beheregaray, 2010).

One of the most widely used approaches in molecular identification is phylogenetic analysis. Phylogenetics is a method used to investigate evolutionary relationships among organisms based on similarities in their characters. Organisms sharing similar characteristics are considered to have originated from a common ancestor and are therefore grouped into the same evolutionary lineage or monophyletic group (Astarini et al., 2021). Phylogenetic studies frequently utilize genetic markers to infer evolutionary relationships among species, one of the most common being the Cytochrome Oxidase Subunit I (COI) gene (Partiwi et al., 2023). The COI gene is a protein-coding mitochondrial DNA (mtDNA) gene involved in cellular respiration. A fragment of approximately 650 base pairs is widely recognized as the universal DNA barcode for animals because of its

relatively rapid mutation rate, high copy number within cells, and maternal inheritance pattern (Imtiaz et al., 2017). The COI gene contains both conserved and variable nucleotide regions, making it an effective genetic marker for a wide range of molecular analyses (Mamuaya et al., 2024). The use of the mitochondrial COI marker enables the examination of nucleotide variation among species, thereby facilitating the reconstruction of phylogenetic relationships and evolutionary histories (Kamal et al., 2019).

Mitochondrial DNA offers several advantages for molecular studies, including its high efficiency, with approximately 93% of its sequence representing coding regions (Chinnery & Hudson, 2013). In addition, mitochondrial DNA exhibits greater genetic variability than nuclear DNA because it is present in multiple copies per cell and generally accumulates mutations at a higher rate (Hendriari et al., 2020). Owing to these characteristics, mitochondrial DNA has been extensively applied in various fields, including population genetics, species identification, disease diagnosis, veterinary phylogenetics, and evolutionary biology (Wibowo et al., 2013). Despite the growing application of molecular techniques in butterfly research, information regarding the phylogenetic relationships of *C. myrina* based on the Cytochrome Oxidase Subunit I (COI) gene remains scarce. Therefore, investigating the phylogenetic relationships of *C. myrina* using the COI gene will contribute valuable genetic information and improve our understanding of the evolutionary history of this endemic Sulawesi butterfly. This study aimed to describe the phylogenetic relationships of *Cethosia myrina* based on sequences of the Cytochrome Oxidase Subunit I (COI) gene.

MATERIALS AND METHODS

Study Area

This study employed a descriptive-exploratory research design using both qualitative and quantitative approaches. Exploratory research aims to describe and investigate the characteristics of a particular phenomenon. Qualitative data consisted of descriptive information obtained from DNA isolation, amplification, sequencing, and bioinformatic analyses of Cethosia myrina using the Cytochrome Oxidase Subunit I (COI) gene as a molecular marker. Quantitative data consisted of numerical values derived from sequence analyses, including the percentage identity obtained from BLAST (Basic Local Alignment Search Tool) comparisons. The study was conducted in March 2026 in Bakubakulu Village, located in the Palolo District of Sigi Regency, Indonesia (Figure 1). The study area was selected because it represents a suitable habitat for *C. myrina* and provides access to natural butterfly populations for molecular and phylogenetic investigations.

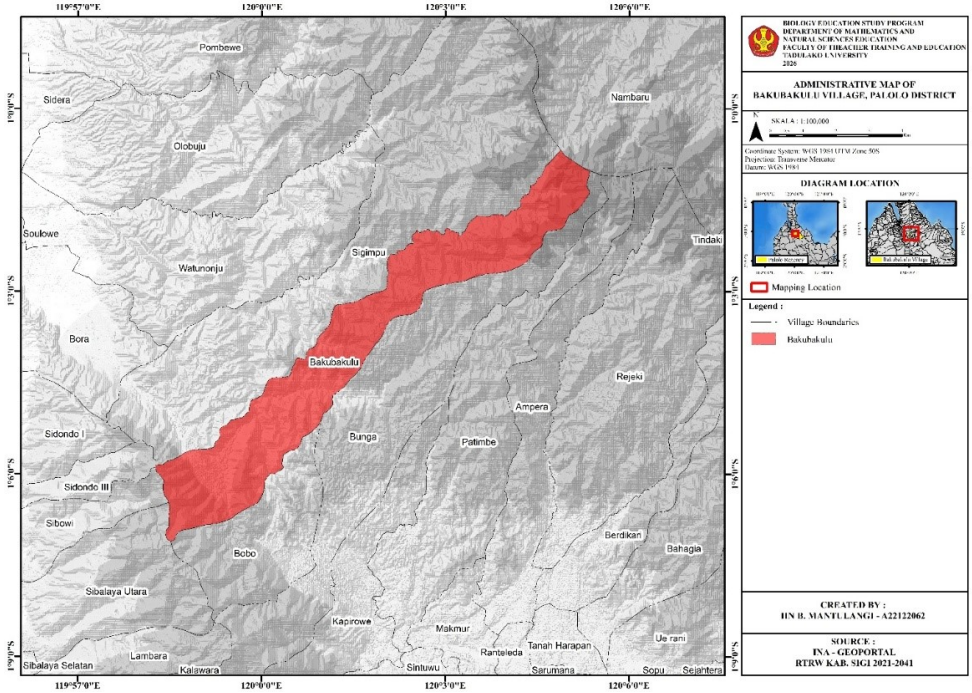

Figure 1. Map of the research location in Baku-bakulu Village, Palolo District, Sigi Regency, Central Sulawesi, Indonesia.

Procedures

Sample Collection

Specimens of *Cethosia myrina* were collected using an exploratory survey method. The geographic coordinates of each sampling location were recorded using a Global Positioning System (GPS) device. Butterflies were captured using an insect net to minimize morphological damage to the specimens. Each collected specimen was

subsequently labeled according to the sampling location and collection date and then preserved for subsequent molecular analyses.

DNA Isolation

Genomic DNA was extracted from *Cethosia myrina* specimens after removing the head and wings, using the GS 100gSYNC™ DNA Extraction Kit following the manufacturer's protocol. Samples were incubated at 60°C for 2.5 hours until complete lysis was achieved and the lysate became clear. The resulting supernatant was then processed with GSB buffer, absolute ethanol (E1QD), and a GS column to facilitate DNA binding. Washing and drying steps were performed to remove residual contaminants, followed by DNA elution at 60°C. The extracted DNA was stored at −20°C until further use in PCR amplification.

Amplification and Sequencing

Extracted DNA was amplified using the Polymerase Chain Reaction (PCR) technique with mitochondrial COI primers, namely LCO1490 (forward) and HCO2198 (reverse), in a total reaction volume of 25 µL. The PCR program consisted of an initial denaturation step at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 35 s, annealing at 50°C for 30 s, and extension at 72°C for 30 s. A final extension step was carried out at 72°C for 7 min. Successfully amplified PCR products were sent to the Integrated Research and Testing Laboratory (LPPT), Universitas Gadjah Mada, for DNA sequencing using an Applied Biosystems 3500 Genetic Analyzer.

Gel Electrophoresis

PCR products were visualized by electrophoresis on 1% agarose gel at 50 V for 17–20 min until distinct DNA bands were observed. The gel was examined using a UV transilluminator, and images were recorded using a gel documentation system to verify the presence and size of the amplified DNA fragments. Electrophoretic profiles were subsequently visualized and documented following the procedure described by Fitrian and Madduppa (2020).

Data Analysis

Forward and reverse sequencing chromatograms (*.ab1* files) were edited and assembled using GeneStudio and DNASTAR software to generate consensus sequences. The resulting sequences were compared against reference sequences using Nucleotide BLAST at the National Center for Biotechnology Information (NCBI) database to confirm species identity. Sequence alignment was performed using MESQUITE, and aligned sequences were converted into FASTA format for further analysis in MEGA version 11. Genetic distances were estimated using the Kimura 2-Parameter (K2P) model. Phylogenetic trees were reconstructed using both the Neighbor-Joining (NJ) and Maximum Likelihood (ML) methods with 10,000 bootstrap replicates to assess node support. Genetic variation analyses, including haplotype diversity (Hd) and nucleotide diversity (π), were conducted using DnaSP version 6. These analyses were used to evaluate the genetic diversity and phylogenetic relationships of *C. myrina* populations from different localities in Sulawesi.

RESULTS AND DISCUSSION

Amplification and Sequence Similarity of *Cethosia myrina*

Amplification of the mitochondrial Cytochrome Oxidase Subunit I (COI) gene was successfully performed on three *Cethosia myrina* specimens collected from Bakubakulu Village, Palolo District, Sigi Regency, Central Sulawesi, using the primer pair LCO1490-F (forward) and HCO2198-R (reverse). Successful amplification was confirmed through agarose gel electrophoresis. DNA bands can be evaluated based on the intensity and clarity of the bands produced during electrophoresis (Tanzil & Fanata, 2024). High-quality DNA amplification is indicated by the presence of a single, distinct DNA band without smearing, reflecting the specificity of amplification and the integrity of the DNA template (Wasdili & Gartinah, 2018). The electrophoresis results of the mitochondrial COI gene from *C. myrina* are presented in Figure 2.

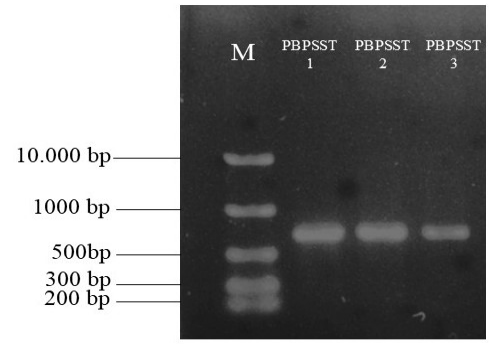

Figure 2. PCR amplification of the mitochondrial COI gene of *Cethosia myrina*. PBPSST represents *C. myrina* specimens collected from Bakubakulu Village, Palolo District, Sigi Regency, Central Sulawesi, Indonesia, while M denotes the DNA molecular weight marker (DNA ladder).

The DNA sequences obtained were analyzed using Nucleotide BLAST provided by the National Center for Biotechnology Information (NCBI) to determine the levels of similarity based on query coverage and percentage identity among the three DNA sequences obtained. This analysis was conducted to confirm species identity by comparing the generated sequences with reference sequences available in the GenBank database. The results of the BLAST analysis, including query coverage and sequence similarity values, are presented in Table 1.

Table 1. Identification results using BLATS (NCBI) mitochondrial gene sequence COI C. myrina Central Sulawesi with GenBank database.

Code	BLAST			Verification	Location
	% Identity	% *Query Cover*	Accession Number *GenBank*		
CMST1.1	100,00	100	EU275514.1	*Cethosia myrina*	Central Sulawesi
CMST1.2	100,00	100	EU275514.1	*Cethosia myrina*	Central Sulawesi
CMST1.3	100,00	100	EU275514.1	*Cethosia myrina*	Central Sulawesi

Genetic Variation of *Cethosia myrina*

Genetic variation analysis of *Cethosia myrina* was conducted using five COI gene sequences, each comprising 648 base pairs (bp). The analysis aimed to assess the level of genetic diversity among *C. myrina* populations from different regions of Sulawesi. Genetic variation parameters were estimated using DnaSP software, including 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 CMST1.1, CMST1.2, and CMST1.3, representing *C. myrina* from Central Sulawesi. Haplotype 2 included sequences EU275514.1 (*C. myrina* from Central Sulawesi) and HM998338.1 (*Cethosia myrina myrina* from South Sulawesi). A total of one variable site and one parsimony-informative site were identified across all analyzed sequences. The estimated haplotype diversity was Hd = 0.603 ± 0.165, while the nucleotide diversity was π = 0.00053 ± 0.00013 (Table 2). These values indicate a relatively low level of genetic variation among the analyzed *C. myrina* populations from Sulawesi.

Table 2. Genetic variation of *Cethosia myrina* based on the mitochondrial cytochrome oxidase subunit I (COI) gene.

Sample Code	bp	Number of Individuals	Number of Haplotypes	*Variabel Site*	*Parsimony Site*	*Haplotype Diversity* (Hd)	*Nucleotide Diversity* (π)
CMST1.01 CMST1.02 CMST1.03 EU275514.1 HM998338.1	648	5	3	1	1	0,603±0,165	0,00053±0,00013

Nucleotide Composition of *Cethosia myrina*

DNA consists of four nucleotide bases: adenine (A), thymine (T), guanine (G), and cytosine (C). Adenine and guanine belong to the purine group, whereas thymine and cytosine are classified as pyrimidines. In DNA base pairing, adenine pairs with thymine, while guanine pairs with cytosine (Anisa et al., 2016). Based on the analysis of five *Cethosia myrina* COI sequences using MEGA version 11, the average nucleotide composition was dominated by thymine (T) and adenine (A). The mean percentages of nucleotide bases were 41.11% T, 16.87% C, 28.72% A, and 13.28% G. The combined A+T content accounted for 69.83%, whereas the G+C content was 30.16%. This pattern indicates a marked A+T bias, with A+T content substantially exceeding G+C content. Such a nucleotide composition is a common characteristic of mitochondrial genes, particularly the Cytochrome Oxidase Subunit I (COI) gene (Table 3). The predominance of adenine and thymine bases is consistent with the typical nucleotide composition observed in insect mitochondrial genomes and reflects the evolutionary characteristics of mitochondrial DNA.

Table 3. Average nucleotide composition of *Cethosia myrina*.

Code	T (U)	C	A	G	A+T	G+C	Location	References
CMST1.1	41,11	16,87	28,72	13,28	69,83	30,16	Central Sulawesi	Research Data
CMST1.2	41,11	16,87	28,72	13,28	69,83	30,16	Central Sulawesi	Research Data
CMST1.3	41,11	16,87	28,72	13,28	69,83	30,16	Central Sulawesi	Research Data
EU275514.1	41,11	16,87	28,72	13,28	69,83	30,16	Central Sulawesi	Silva *et al.* (2008)
HM998338.1	41,11	16,87	28,72	13,28	69,83	30,16	South Sulawesi	Muller *et al.* (2010)
Average	41,11	16,87	28,72	13,28	69,83	30,16		

Phylogenetic Tree Reconstruction and Genetic Distance Analysis

Phylogenetic analysis was conducted to determine the genetic relationships among the *Cethosia myrina* sequences examined in this study. Phylogenetic trees were reconstructed using MEGA version 11 (Kumar et al., 2018) employing two approaches: the Neighbor-Joining (NJ) method (Figure 3) and the Maximum Likelihood (ML) method (Figure 4). Both analyses were performed using the Kimura 2-Parameter (K2P) model with 10,000 bootstrap replicates. The K2P model estimates transition and transversion substitution rates within DNA sequences while assuming equal frequencies of the four nucleotide bases (Daniyati, 2021). The analysis included 31 COI sequences, comprising three *C. myrina* sequences from Central Sulawesi, additional reference sequences retrieved from GenBank, and the outgroup species *Papilio demoleus* and *Papilio fuscus*. The Neighbor-Joining method reconstructs phylogenetic relationships based on genetic distance differences among sequences. Furthermore, pairwise genetic distance analysis was performed to support the interpretation of the inferred phylogenetic relationships (Table 4).

The genetic distance analysis revealed that *Cethosia myrina* individuals from Central Sulawesi exhibited genetic distances ranging from 0.00% to 0.00%, indicating the absence of detectable genetic variation among these samples. Similarly, the genetic distances between *C. myrina* from Central Sulawesi and populations from other regions of Sulawesi also ranged from 0.00% to 0.00%, confirming that all analyzed specimens belong to the same species. In contrast, genetic distances between *C. myrina* and the outgroup species, *Papilio demoleus* and *Papilio fuscus*, were substantially higher, ranging from 13.56% to 14.22%, indicating clear species-level differentiation and evolutionary divergence. A genetic divergence threshold of approximately 3% is commonly used for species delimitation; therefore, genetic distances exceeding 3% generally indicate distinct species, whereas values below this threshold suggest conspecific populations (Zhang & Bu, 2022).

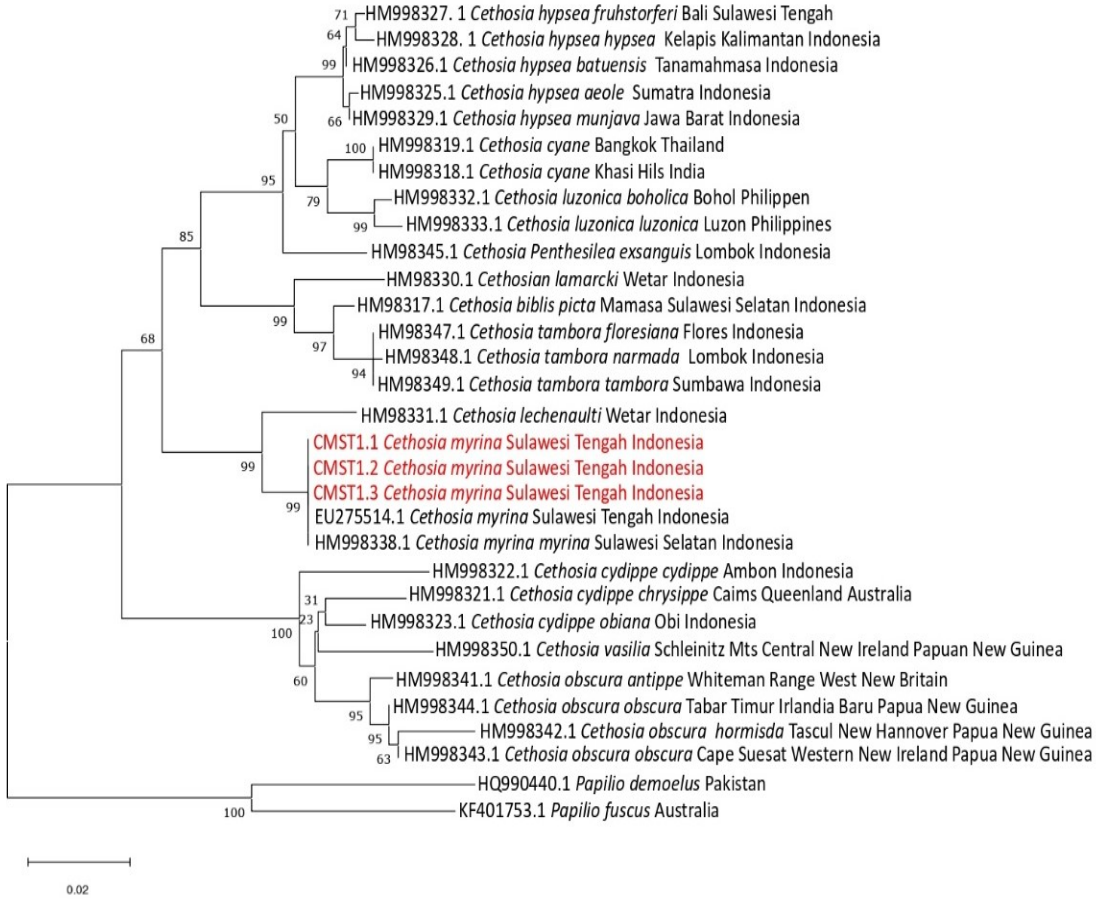

Figure 3. Phylogenetic tree of *Cethosia myrina* reconstructed using the Neighbor-Joining (NJ) method based on the Kimura Two-Parameter (K2P) Model with 10,000 bootstrap replicates.

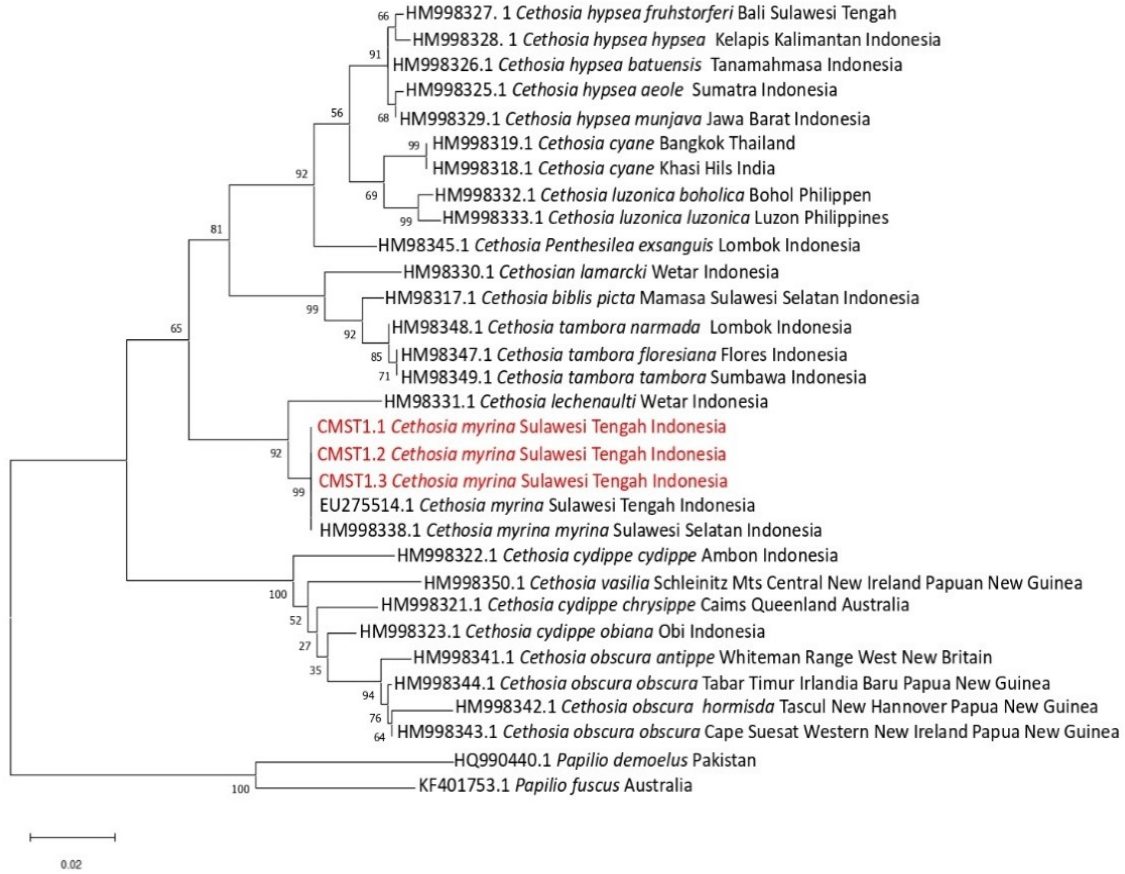

Figure 4. Phylogenetic tree of *Cethosia myrina* reconstructed using the Maximum Likelihood (ML) method based on the Kimura Two-Parameter (K2P) Model with 10,000 bootstrap replicates

Table 4. Genetic distance of the COI gene in *Cethosia myrina.*

	CMST1.1_Cethosia_myrina_Sulawesi_Tengah_Indonesia	CMST1.2_Cethosia_myrina_Sulawesi_Tengah_Indonesia	CMST1.3_Cethosia_myrina_Sulawesi_Tengah_Indonesia	EU275514.1_Cethosia_myrina_Sulawesi_Tengah_Indonesia	HM998338.1_Cethosia_myrina_myrina_Sulawesi_Selatan_Indonesia	HM998317.1_Cethosia_biblis_picta_Mamasa_Sulawesi_Selatan_Indonesia	HM998319.1_Cethosia_cyane_Bangkok_Thailand	HM998318.1_Cethosia_cyane_Khasi_Hils_India	HM998321.1_Cethosia_cydippe_chrysippe_Cairns_Queenland_Australia	HM998322.1_Cethosia_cydippe_cydippe_Ambon_Indonesia	HM998323.1_Cethosia_cydippe_obiana_Obi_Indonesia	HM998325.1_Cethosia_hypsea_aeole_Sumatra_Indonesia	HM998326.1_Cethosia_hypsea_batuensis_Tanahmasa_Indonesia	HM998327.1_Cethosia_hypsea_fruhstorferi_Bali_Indonesia	HM998328.1_Cethosia_hypsea_hypsea_Kelapis_Kalimantan_Indonesia	HM998329.1_Cethosia_hypsea_munjava_Jawa_Barat_Indonesia	HM998330.1_Cethosia_lamarcki_Wetar_Indonesia	HM998331.1_Cethosia_lechenaulti_Wetar_Indonesia	HM998332.1_Cethosia_luzonica_boholica_Bohol_Philippines	HM998333.1_Cethosia_luzonica_luzonica_Luzon_Philippines	HM998350.1_Cethosia_vasilia_Schleinitz_Mts_Central_New_Ireland_Papua_New_Guinea	HM998345.1_Cethosia_penthesilea_exsanguis_Lombok_Indonesia	HM998347.1_Cethosia_tambora_floresiana_Flores_Indonesia	HM998348.1_Cethosia_tambora_narmada_Lombok_Indonesia	HM998349.1_Cethosia_tambora_tambora_Sumbawa_Indonesia	HM998341.1_Cethosia_obscura_obscura_Cape_Suesat_western_New_Ireland_Papua_New_Guinea	HM998344.1_Cethosia_obscura_obscura_Tabar_Timur_Irlandia_Baru_Papua_New_Guinea	HQ990440.1_Papilio_demoleus_Pakistan	KF401753.1_Papilio_fuscus_Australia
CMST1.1_Cethosia_myrina_Sulawesi_Tengah_Indonesia																													
CMST1.2_Cethosia_myrina_Sulawesi_Tengah_Indonesia	0.00%																												
CMST1.3_Cethosia_myrina_Sulawesi_Tengah_Indonesia	0.00%	0.00%																											
EU275514.1_Cethosia_myrina_Sulawesi_Tengah_Indonesia	0.00%	0.00%	0.00%																										
HM998338.1_Cethosia_myrina_myrina_Sulawesi_Selatan_Indonesia	0.00%	0.00%	0.00%	0.00%																									
HM998317.1_Cethosia_biblis_picta_Mamasa_Sulawesi_Selatan_Indonesia	6.60%	6.60%	6.60%	6.60%	6.60%																								
HM998319.1_Cethosia_cyane_Bangkok_Thailand	6.43%	6.43%	6.43%	6.43%	6.43%	5.83%																							
HM998318.1_Cethosia_cyane_Khasi_Hils_India	6.43%	6.43%	6.43%	6.43%	6.43%	5.83%	0.00%																						
HM998321.1_Cethosia_cydippe_chrysippe_Cairns_Queenland_Australia	9.89%	9.89%	9.89%	9.89%	9.89%	9.84%	10.70%	10.70%																					
HM998322.1_Cethosia_cydippe_cydippe_Ambon_Indonesia	10.51%	10.51%	10.51%	10.51%	10.51%	10.87%	10.67%	10.67%	4.46%																				
HM998323.1_Cethosia_cydippe_obiana_Obi_Indonesia	8.84%	8.84%	8.84%	8.84%	8.84%	9.22%	9.64%	9.64%	2.39%	3.89%																			
HM998325.1_Cethosia_hypsea_aeole_Sumatra_Indonesia	7.23%	7.23%	7.23%	7.23%	7.23%	6.03%	2.76%	2.76%	10.92%	11.12%	10.51%																		
HM998326.1_Cethosia_hypsea_batuensis_Tanahmasa_Indonesia	7.23%	7.23%	7.23%	7.23%	7.23%	5.64%	2.38%	2.38%	10.49%	10.69%	10.08%	0.36%																	
HM998327.1_Cethosia_hypsea_fruhstorferi_Bali_Indonesia	7.64%	7.64%	7.64%	7.64%	7.64%	5.83%	2.76%	2.76%	10.92%	11.12%	10.51%	0.72%	0.36%																
HM998328.1_Cethosia_hypsea_hypsea_Kelapis_Kalimantan_Indonesia	7.85%	7.85%	7.85%	7.85%	7.85%	6.23%	2.94%	2.94%	10.92%	11.12%	10.51%	0.91%	0.54%	0.54%															
HM998329.1_Cethosia_hypsea_munjava_Jawa_Barat_Indonesia	7.03%	7.03%	7.03%	7.03%	7.03%	5.83%	2.57%	2.57%	10.71%	10.90%	10.29%	0.18%	0.18%	0.54%	0.72%														
HM998330.1_Cethosia_lamarcki_Wetar_Indonesia	7.20%	7.20%	7.20%	7.20%	7.20%	3.14%	7.03%	7.03%	10.48%	11.52%	10.28%	6.44%	6.03%	6.44%	6.64%	6.23%													
HM998331.1_Cethosia_lechenaulti_Wetar_Indonesia	2.76%	2.76%	2.76%	2.76%	2.76%	7.81%	7.23%	7.23%	10.92%	12.21%	10.08%	8.25%	8.25%	8.67%	8.88%	8.04%	8.43%												
HM998332.1_Cethosia_luzonica_boholica_Bohol_Philippines	6.45%	6.45%	6.45%	6.45%	6.45%	7.03%	2.19%	2.19%	10.30%	9.85%	8.42%	3.32%	2.94%	3.32%	3.51%	3.13%	7.64%	7.05%											
HM998333.1_Cethosia_luzonica_luzonica_Luzon_Philippines	6.66%	6.66%	6.66%	6.66%	6.66%	7.23%	2.38%	2.38%	10.30%	9.43%	8.42%	3.51%	3.13%	3.51%	3.70%	3.32%	8.26%	7.67%	0.91%										
HM998350.1_Cethosia_vasilia_Schleinitz_Mts_Central_New_Ireland_Papua_New_Guinea	9.45%	9.45%	9.45%	9.45%	9.45%	11.55%	11.55%	11.55%	3.89%	5.23%	3.32%	11.76%	11.32%	11.76%	11.76%	11.54%	11.77%	11.13%	9.86%	9.44%									
HM998345.1_Cethosia_penthesilea_exsanguis_Lombok_Indonesia	6.43%	6.43%	6.43%	6.43%	6.43%	7.03%	4.09%	4.09%	9.86%	9.84%	8.82%	2.94%	2.94%	3.32%	3.52%	2.76%	7.65%	6.42%	3.71%	3.90%	10.90%								
HM998347.1_Cethosia_tambora_floresiana_Flores_Indonesia	7.20%	7.20%	7.20%	7.20%	7.20%	1.27%	6.63%	6.63%	9.84%	10.87%	9.64%	6.03%	5.64%	5.64%	6.23%	5.83%	3.14%	8.43%	7.64%	7.85%	11.55%	7.24%							
HM998348.1_Cethosia_tambora_narmada_Lombok_Indonesia	7.41%	7.41%	7.41%	7.41%	7.41%	1.09%	6.83%	6.83%	10.06%	11.08%	9.85%	6.23%	5.83%	5.83%	6.44%	6.03%	3.33%	8.63%	7.85%	8.05%	11.77%	7.44%	0.18%						
HM998349.1_Cethosia_tambora_tambora_Sumbawa_Indonesia	7.20%	7.20%	7.20%	7.20%	7.20%	1.27%	6.63%	6.63%	9.84%	10.87%	9.64%	6.03%	5.64%	5.64%	6.23%	5.83%	3.14%	8.43%	7.64%	7.85%	11.55%	7.24%	0.00%	0.18%					
HM998341.1_Cethosia_obscura_antippe_Whiteman_Range_West_New_Britain	9.26%	9.26%	9.26%	9.26%	9.26%	9.64%	10.28%	10.28%	3.70%	4.45%	2.56%	10.72%	10.29%	10.72%	10.72%	10.51%	10.28%	10.94%	10.10%	9.46%	4.26%	9.88%	9.64%	9.85%	9.64%				
HM998342.1_Cethosia_obscura_hormisda_Tascul_New_Hannover_Papua_New_Guinea	11.13%	11.13%	11.13%	11.13%	11.13%	11.71%	11.95%	11.95%	4.64%	6.00%	3.49%	12.21%	11.77%	12.21%	12.21%	11.99%	12.37%	12.85%	11.33%	11.12%	5.02%	11.54%	11.71%	11.93%	11.71%	2.37%			
HM998343.1_Cethosia_obscura_obscura_Cape_Suesat_western_New_Ireland_Papua_New_Guinea	9.49%	9.49%	9.49%	9.49%	9.49%	10.06%	10.29%	10.29%	3.13%	4.46%	2.01%	10.73%	10.30%	10.73%	10.73%	10.52%	10.70%	11.17%	9.90%	9.69%	3.69%	9.89%	10.06%	10.27%	10.06%	0.90%	1.45%		
HM998344.1_Cethosia_obscura_obscura_Tabar_Timur_Irlandia_Baru_Papua_New_Guinea	9.27%	9.27%	9.27%	9.27%	9.27%	10.06%	10.08%	10.08%	3.13%	4.27%	2.01%	10.52%	10.09%	10.52%	10.52%	10.30%	10.70%	10.95%	9.69%	9.47%	3.69%	9.67%	10.06%	10.27%	10.06%	0.90%	1.63%	0.18%	
HQ990440.1_Papilio_demoleus_Pakistan	13.56%	13.56%	13.56%	13.56%	13.56%	15.52%	17.12%	17.12%	17.38%	18.77%	16.69%	16.69%	16.23%	16.69%	16.69%	16.46%	16.20%	14.87%	16.21%	16.44%	17.13%	15.99%	15.98%	16.20%	15.98%	17.15%	18.32%	16.93%	16.70%
KF401753.1_Papilio_fuscus_Australia	14.22%	14.22%	14.22%	14.22%	14.22%	15.73%	16.41%	16.41%	16.25%	16.00%	15.12%	15.52%	15.30%	15.75%	15.75%	15.52%	15.29%	15.54%	15.75%	15.75%	16.47%	15.76%	16.41%	16.64%	16.41%	16.70%	17.89%	16.50%	16.27%	8.39%

Discussion

DNA amplification is the process of generating multiple copies of a DNA fragment *in vitro* using the Polymerase Chain Reaction (PCR) technique, which employs repeated temperature cycles to produce millions of copies of a target DNA sequence. PCR is a fundamental method in molecular biology because it enables rapid and specific amplification of DNA within a relatively short period. The technique consists of three main stages: denaturation, annealing, and extension (Karic, 2023). PCR amplification aims to replicate target DNA under controlled thermal conditions through repeated cycles. DNA primers, which are short nucleotide sequences, function by binding to complementary regions of single-stranded DNA and initiating DNA synthesis (Adhiyanto et al., 2020). To ensure successful amplification, primers must exhibit high specificity toward the target DNA sequence. Because each primer pair has a unique optimal annealing temperature, PCR conditions often require optimization through testing a range of temperatures to determine the most effective amplification conditions. The resulting PCR products are subsequently evaluated using electrophoresis to assess amplification quality and specificity (Aulia et al., 2021).

Amplification of the mitochondrial Cytochrome Oxidase Subunit I (COI) gene from *Cethosia myrina* specimens collected in Bakubakulu Village, Central Sulawesi, produced a DNA fragment of approximately 648 bp. The presence of a single, distinct DNA band without smearing on agarose gel electrophoresis indicated that the PCR reaction proceeded successfully and specifically, with no evidence of DNA degradation or contamination. This result demonstrates that the primer pair LCO1490 and HCO2198 effectively amplified the target COI region, producing PCR products suitable for subsequent sequencing analysis. Furthermore, the successful amplification suggests that the extracted DNA was of high quality and free from substances that could inhibit PCR reactions. Sequence similarity analysis using Nucleotide BLAST (NCBI) revealed that the three *C. myrina* sequences from Central Sulawesi showed 100% query coverage and 100.00% sequence identity with reference sequences available in GenBank, confirming the accurate identification of *C. myrina* specimens from the study area. Higher query coverage and percentage identity values indicate a greater degree of homology between sample sequences and reference sequences deposited in public databases (Aprilianto & Sembiring, 2016). Similarity values are calculated based on the degree of nucleotide sequence correspondence between the analyzed samples and sequences available in GenBank (Pangsuma & Hidayat, 2023).

Genetic variation refers to differences occurring at the levels of nucleotides, genes, chromosomes, and genomes. Such variation arises through mutations, genetic recombination during meiosis, and gene flow among populations (Khairani et al., 2024). Genetic diversity plays a crucial role in enabling populations to adapt to environmental changes and contributes to their long-term evolutionary potential. In this study, genetic variation was analyzed using DnaSP software (Baksir et al., 2022), which was employed to estimate the number of haplotypes (h), variable sites, parsimony-informative sites, haplotype diversity (Hd), and nucleotide diversity (π). The analysis was performed on COI gene sequences with a length of 648 bp. The results revealed the presence of two haplotypes. Haplotype 1 consisted of samples CMST1.1, CMST1.2, and CMST1.3, representing *C. myrina* from Central Sulawesi, whereas Haplotype 2 included sequences EU275514.1 (*C. myrina* from Central Sulawesi) and HM998338.1 (*Cethosia myrina myrina* from South Sulawesi). In addition, one variable site and one parsimony-informative site were identified among the analyzed sequences.

The genetic variation analysis of *C. myrina* revealed a haplotype diversity (Hd) value of 0.603 ± 0.165, indicating a relatively high level of haplotype diversity, as the value falls within the range of $0.5 < Hd \leq 1.0$. In contrast, the nucleotide diversity (π) value was 0.00053 ± 0.00013, reflecting very low nucleotide variation among the analyzed sequences. The combination of relatively high haplotype diversity and low nucleotide diversity suggests the presence of several closely related haplotypes that differ by only a few nucleotide substitutions. Such a pattern is commonly associated with populations that have undergone recent expansion following a period of reduced population size or genetic bottleneck. The high Hd value indicates the existence of genetic variation among individuals within the *C. myrina* population, whereas the low nucleotide diversity suggests limited sequence divergence among haplotypes. This pattern may also be influenced by evolutionary and ecological factors, including adaptation processes and the potential occurrence of cryptic lineages that can complicate the interpretation of genetic diversity within populations (Kurniawan et al., 2023).

Analysis of the mitochondrial COI gene sequences of *C. myrina* revealed nucleotide proportions of 41.11% thymine (T), 28.72% adenine (A), 16.87% cytosine (C), and 13.28% guanine (G). The combined A+T content accounted for 69.83%, whereas the G+C content represented only 30.16%. These results indicate a pronounced A+T bias, a characteristic commonly observed in insect mitochondrial genomes. A lower GC content generally reduces DNA stability because GC base pairs contain fewer hydrogen bonds than AT base pairs, making the DNA molecule more susceptible to denaturation (Nova et al., 2023). Conversely, a high A+T content may reflect patterns of genetic variation and evolutionary processes within a species. Differences in nucleotide composition, particularly the proportions of A+T and G+C, are frequently used in phylogenetic studies to infer evolutionary relationships and to identify lineage-specific adaptations that distinguish one species from another (Anisa et al., 2016).

Phylogenetic analyses were performed using MEGA version 11. Phylogenetic trees were reconstructed using both the Neighbor-Joining (NJ) and Maximum Likelihood (ML) methods under the Kimura 2-Parameter (K2P) model with 10,000 bootstrap replicates. The resulting trees from both methods displayed highly similar topologies, although minor differences were observed in bootstrap support values. In both reconstructions, species were consistently grouped within the same clades, indicating stable phylogenetic relationships regardless of the analytical approach employed. The phylogenetic reconstruction demonstrated that *C. myrina* specimens from Central Sulawesi clustered closely with other *C. myrina* sequences from Central Sulawesi and *C. myrina myrina* from South Sulawesi, forming a well-supported monophyletic clade with bootstrap values of approximately 99%. Such high bootstrap support indicates strong confidence in the inferred evolutionary relationships and confirms the close genetic affinity among these populations. Generally, higher bootstrap values correspond to greater reliability and robustness of the reconstructed phylogenetic topology (Oktafia & Badruzsaufari, 2021).

Genetic distance is a measure of genetic divergence between species or populations within a species, expressed as the proportion of nucleotide sites that differ between two compared DNA sequences (Fassler & Cooper, 2011). The genetic distance analysis revealed that the highest genetic distances occurred between *Cethosia myrina* and the outgroup species *Papilio demoleus* and *Papilio fuscus*, ranging from 13.56% to 14.22%. These findings are consistent with the phylogenetic tree topology, which clearly placed *C. myrina* and the outgroup taxa in distantly separated branches. In general, greater genetic distance indicates a higher degree of genetic differentiation and a more distant evolutionary relationship. Genetic divergence accumulates over time through nucleotide substitutions driven by evolutionary processes and environmental influences that alter DNA sequences (Fitriani et al., 2022). In contrast, the lowest genetic distance (0.00%) was observed among *C. myrina* specimens from Central Sulawesi and *C. myrina myrina* from South Sulawesi. A genetic distance of 0.00% indicates the absence of detectable nucleotide differences within the analyzed COI fragment, suggesting an extremely close genetic relationship among these taxa. This result confirms that the analyzed populations belong to the same species and share a highly similar genetic background. The absence of genetic divergence also supports the phylogenetic reconstruction, which grouped all *C. myrina* sequences into a single well-supported monophyletic clade, reflecting a common evolutionary origin and minimal genetic differentiation among populations across Sulawesi.

CONCLUSIONS

Based on the results of this study, it can be concluded that phylogenetic analyses using both the Neighbor-Joining (NJ) and Maximum Likelihood (ML) methods successfully generated phylogenetic trees that clearly and systematically illustrated the evolutionary relationships among the analyzed taxa. Both methods produced consistent clustering patterns, demonstrating the reliability of the inferred phylogenetic relationships. *Cethosia myrina* specimens from Central Sulawesi exhibited a very close genetic relationship with *Cethosia myrina myrina* from South Sulawesi, as evidenced by the 99% bootstrap support obtained in the phylogenetic reconstruction. Furthermore, the 100% query coverage and 100.00% sequence identity obtained from BLAST analysis confirmed the high degree of genetic similarity among the analyzed populations. These findings provide strong molecular evidence of a close phylogenetic relationship and minimal genetic divergence among *C. myrina* populations across Sulawesi.

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