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Genetic Relationship and the Putative Occurrence of A Species Complex Within the Indonesian *Calotes* (Daudin, 1802) (Squamata, Agamidae) Genus Based on COI Gene Sequences

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ABSTRACT

The *Calotes* genus presents a challenge due to the complexity of its species. However, research on the cryptic species complex within the Indonesian *Calotes* genus is still lacking. This study aims to determine the extent of genetic relationships and assess the potential existence of a species complex within the Indonesian genus *Calotes* (Daudin, 1802) (Squamata, Agamidae) using the partial Cytochrome c Oxidase Subunit 1 (COI) gene sequence as a molecular marker. Samples of the Indonesian *Calotes* genus in this study were collected from South Lampung (Lampung), Bogor (West Java), and Langkat (North Sumatra). By aligning 582 bp sequence similarities with reference sequences in GenBank, we confirmed that seven out of eight samples analyzed belonged to *Calotes vultuosus*, while one sample was identified as *Calotes versicolor*. The identity values ranged from 96 to 100%. The *C. vultuosus* samples in this study displayed lower genetic distances, ranging from 0 to 3%, with the reference *C. vultuosus* sequence from Indonesia compared to the reference sequence from India, which ranged from 6 to 9%. Phylogenetic tree reconstruction, utilizing both maximum likelihood with IQ-Tree and Bayesian Inference with BEAST methods, further supports these findings. It reveals distinct groupings between *C. vultuosus* samples from Indonesia and India. These results suggest the potential occurrence of a species complex within the Indonesian genus *Calotes*. Furthermore, the inclusion of eight COI gene sequences from two *Calotes* species in the GenBank database has the potential to confirm the existence of previously undocumented species in Indonesia.

1. Introduction

Calotes are cosmopolitan lizards that are easily found in plantation and garden ecosystems and often interact with humans. Most species within this genus have limited geographic ranges across the Oriental region (Hallerman 2000; Das 2010). Currently, the *Calotes* genus comprises 34 species (Midtgaard 2024). Of these 34 species, several *Calotes* species represent species complexes such as *C. jerdoni* Gunther 1870 (Wang *et al.* 2024), *C. mystaceus* Duméril & Bibron, 1837 (Hartmann *et al.* 2013; Wagner *et al.* 2021) and *C. versicolor* Daudin, 1802 (Zug *et al.* 2006; Gowande *et al.* 2021; Huang *et al.* 2023).

Zug *et al.* (2006) elevated two new species, *Calotes htunwini* Zug & Vindum 2006 (in Zug *et al.* 2006) and *Calotes irawadi* Zug *et al.* 2006, within the *C. versicolor* complex in Myanmar. *Calotes htunwini* differs from *C. versicolor* species group by the horizontal orientation of the scale rows on the side of the neck and adjacent supra-axillary area; scale-row orientation in the other *C. versicolor* members is obliquely posterior or vertical. *Calotes htunwini* 21.3% different from *C. versicolor* based on pairwise genetic distance. *Calotes irawadi* differs from *C. versicolor* (Pondicherry population) by a much smaller body size (female, male means 77.4, 82.4 mm SVL vs 93 mm, 119 mm, respectively), and more Dorsal (means

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48.9 vs 40.8) and Midbody (47.1 vs 42.8). Middorsal crest scales in *C. irawadi* are smaller (equal to tympanum diameter) and are straighter and more numerous than *C. versicolor* (Pondicherry) with crest scales of 2.5–3.0 × diameter of tympanum. Lengths of supratympanic spines in *C. irawadi* are half or less the diameter of the tympanum and 2 /3 or more tympanum diameter in *C. versicolor* (Pondicherry). Subsequently, *C. irawadi* is 9.5% different from *C. versicolor* based on pairwise genetic distance (Zug *et al.* 2006).

Gowande *et al.* (2021) elevated two new subspecies to species, *Calotes farooqi* Auffenberg & Rehman and 1995 *Calotes vultuosus* Harlan, 1825, within the same complex species in India. *Calotes farooqi* can be differentiated from *C. versicolor* by its smaller adult male body size (average male SVL 97 mm, vs 108 mm in *C. versicolor*, female SVL 82 vs 92 in *C. versicolor*), dorsal crest scales composed of comparatively shorter scales, which become shorter progressively to the base of the tail, lesser number of eyelid scales, Eyelid 9–11, the shape and the size of the scales between the nasal shield and the orbit, by the acuteness of the region between the nostril and the orbit. *Calotes farooqi* was at least 3.5% and 17.0% divergent from *C. versicolor* at 16S and COI, respectively.

Calotes vultuosus can be further differentiated by *C. versicolor* by its slightly smaller adult body size (average male SVL 106 mm vs 108 mm in *C. versicolor*, female SVL 77.5 vs 92.2 mm in *C. versicolor*), dorsal crest composed of comparatively smaller scales, which become progressively smaller to the base of the tail in both sexes (vs dorsal crest composed of large scales, which continues to the base of the tail in *C. versicolor*), supratympanic spines shorter in both sexes (vs longer in *C. versicolor*). The species has shorter crus than *C. versicolor* (average male CrusL 22.7 vs. 26.6 in *C. versicolor*). The species differ in the overall shape of the trunk, which tapers to a lesser extent in *C. vultuosus* (average PectW/PelvW 0.81) vs. trunk tapers to a greater extent in *C. versicolor* (average PectW/PelvW 0.70 in *C. versicolor*). *Calotes vultuosus* was at least 3.3% and 13.0% divergent from *C. versicolor* at 16S and COI, respectively (Gowande *et al.* 2021).

According to Glässer-Trobisch & Trobisch (2014), *C. versicolor* can be found very easily in Java, especially in the western and central parts of the island. In that observation, there was a greenish-coloured *C. versicolor* population group in Karangpucung, Central Java (Indonesia). Greenish-coloured *C. versicolor* was never found in any other *C. versicolor* population group in Indonesia. Glässer-Trobisch & Trobisch (2014) added that *C. versicolor*, with a green colour, has been found in southern Myanmar, and the unique colour only appears during the rainy season in Myanmar. The appearance of different morphological characters in different populations further indicates the potential for species-complex phenomena in *C. versicolor* populations in Indonesia (Glässer-Trobisch & Trobisch 2014). Until the end of 2022, Indonesia has been known to harbour two species of *Calotes*, namely *C. versicolor* and *Calotes nigriplicatus* Hallerman 2000. However, in December 2022, a sequence of *C. vultuosus* from East Java (Indonesia) was added to the GenBank, making it the first discovery of *C. vultuosus* in Indonesia.

The studies by Zug *et al.* (2006) and Gowande *et al.* (2021) shed light on the *Calotes versicolor* complex species, primarily in India and Myanmar, attributing their differentiation to vicariance factors like geographical isolation and environmental variations. While these studies utilized different genetic markers such as part of ND1, ND2, 16S, and COI genes, they provide crucial insights into the evolutionary dynamics of *Calotes* species in specific geographic regions. However, in Indonesia, there exists a research gap concerning the genetic characterization of *Calotes* species. Despite the prevalence of these species in the region, there is a lack of standardized molecular identification methods. The existing techniques primarily rely on mitochondrial DNA (mtDNA) COI gene analysis, employing PCR amplification with universal primers followed by sequence alignment. The COI gene, known for its utility as a phylogenetic marker (Ariyanti *et al.* 2015), has been widely used across various taxa for species identification, genetic differentiation assessment, and the discovery of cryptic species across various taxa, including Crustacea (Belyaeva & Taylor 2009), Hemiptera (Wang *et al.* 2011; Hu *et al.* 2017; Song *et al.* 2021), as well as Amphibians and Reptiles (Zangl *et al.* 2020).

Therefore, this study aims to investigate the genetic relationships of *Calotes* species in Indonesia. Focusing on the partial COI gene, this study seeks to analyze the genetic relationship within the Indonesian *Calotes* genus, contributing to a better understanding of its evolutionary history and potentially uncovering any cryptic species within the region.

2. Materials and Methods

2.1. Sampling

The samples used in this study were acquired through both self-collection and from wet collection specimens stored at the Zoologicum Bogoriense Museum. These collection samples originated from four main locations, as depicted in Figure 1: South Lampung (Sumatra Island), Bogor 1 (West Java), Langkat (North Sumatra), and Bogor 2 (West Java).

Samples were captured during the daytime using a net in garden and park areas. The captured samples were brought to the laboratory alive and then morphologically identified based on Gowande *et al.* (2021). The samples were then euthanized using chloroform crystals. The deceased samples were then dissected using professional surgical equipment to isolate liver tissue samples. The isolated liver tissue was then macerated using ethanol in sample tubes.

The collection specimens and reference sequences used in this study are described in Table 1 as follows: two samples (SL_1 and SL_2) from the Jati Agung District (South Lampung), four samples (BGR1_1, BGR1_2, BGR1_3, and BGR1_4) from Jonggol (Bogor 1), one specimen (LKT) from the Museum's collection originating from Stabat (Langkat) and one sample (BGR2) from Cibinong (Bogor 2).

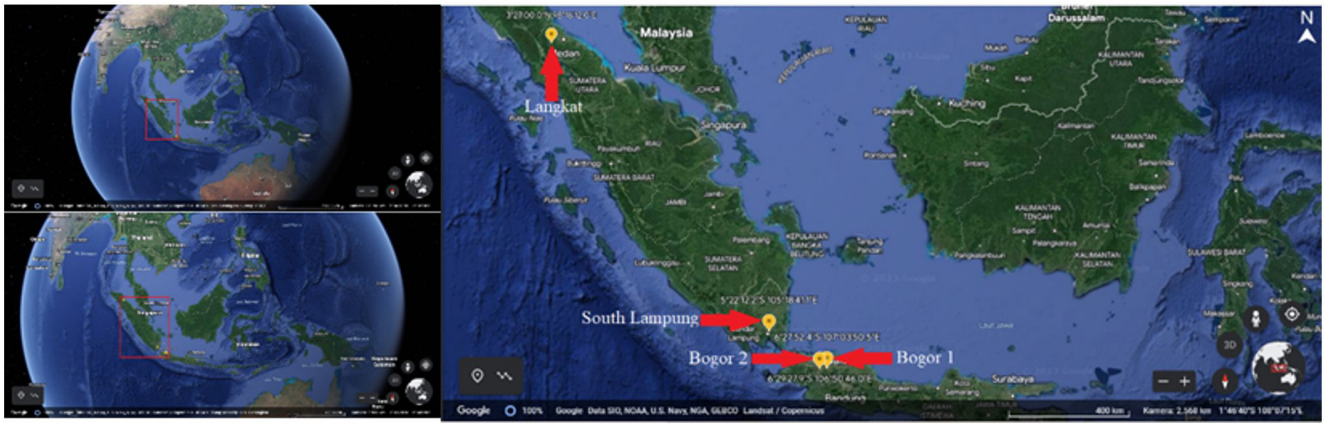


Figure 1. Sampling map (yellow circle indication sampling site location)

Table 1. Collection specimen and mt DNA COI sequences, along with their accession numbers

Specimens code	Coordinates point	Origin	Genetic references	GenBank accession
SL_1	-5.37 LU; 105.31 BT		-	OR229794.1
SL_2		South Lampung	-	OR229795.1
BGR1_1			-	OR229796.1
BGR1_2	-6.46 LS; 107.06 BT	Bogor 1	-	OR229797.1
BGR1_3			-	OR229798.1
BGR1_4			-	OR229799.1
LKT	-3.45 LU; 98.27 BT	Langkat	-	OR229800.1
BGR2	-6.49 LS; 106.84 BT	Bogor 2	-	OR229801.1
-	-	Vietnam	*C. bachae* (Hartmann *et al.* 2013)	MW817625.1
-	-	Cambodia	*C. bachae*	MW817626.1
-	-	Myanmar	*C. emma* (Gray, 1845)	MG935451.1
-	-	Myanmar	*C. emma*	MT609352.1
		Pakistan	*C. farooqi*	MZ489213.1
-	-	Myanmar	*C. goetzi* (Wagner *et al.* 2021)	MW817592.1
-	-	Myanmar	*C. goetzi*	MW817594.1
-	-	Myanmar	*C. irawadi*	MZ489212.1
-	-	Cambodia	*C. mystaceus* (Duméril & Bibron, 1837)	KC016060.1

Table 1. Continued

Specimens code	Coordinates point	Origin	Genetic references	GenBank accession
-	-	Thailand	*C. versicolor*	MT438521.1
-	-	Thailand	*C. versicolor*	MT438522.1
-	-	Thailand	*C. versicolor*	MT438523.1
-	-	Laos	*C. versicolor*	MT438524.1
-	-	Myanmar	*C. vindumbarbatus* (Wagner *et al.* 2021)	MW817602.1
-	-	Myanmar	*C. vindumbarbatus*	MW817603.1
-	-	Indonesia	*C. vultuosus*	OQ118405.1
-	-	Indonesia	*C. vultuosus*	OQ118406.1
-	-	Indonesia	*C. vultuosus*	OQ118407.1
-	-	India	*C. vultuosus*	MZ489201.1
-	-	India	*C. vultuosus*	MZ489202.1
-	-	India	*C. vultuosus*	MZ489203.1
-	-	Myanmar	*Bronchocela burmana* (Blanford, 1878)	MT607997.1

2.2. DNA Extraction and Amplification

DNA extraction and isolation were performed using the Thermo Fisher Scientific™ Genomic DNA Purification extraction kit. The DNA from the samples collected in South Lampung, Bogor 1 and Bogor 2, originated from liver tissue, while the DNA from the Langkat samples was extracted from muscle tissue at the base of the tail. This method was done because the Langkat sample was a wet collection in the form of the base of the tail at the Museum. To ensure consistency and accuracy, DNA extraction and isolation procedures were conducted following the Tissue and Tail Genomic DNA Purification Protocol.

DNA amplification of the COI gene segment of mitochondrial DNA was performed using COI-ReptBC primers (Castañeda & de Queiroz 2011). The PCR reaction was prepared with a total volume of 50 µL, comprising of the following components: template DNA (1 µL), forward primer (1 µL) and reverse primer (1 µL) at a 10 µM concentration, PCR mix (25 µL) and distilled water (22 µL). The mixing process of template DNA and PCR cocktail was carried out in a laminar air flow hood.

PCR was performed using a Fisher Scientific™ Thermal Cycler (ABI 2720 model) with the following temperatures and times: Pre-denaturation (94°C, for 4 minutes), Denaturation (94°C, for 45 seconds), Annealing (49°C, for 30 seconds), Elongation (72°C, for 45 seconds), Finish (72°C, for 5 minutes), Hold (4°C) and with 30 cycles. Target genes were amplified using primer pairs of 23 and 26 bp (Table 2). Visualization of DNA bands was done by electrophoresis using agarose gel with 2% agarose concentration. The DNA migration process was carried out for 25 minutes and used a voltage of 80 volts.

Table 2. Nitrogen base sequence and attachment temperature of the two primer pairs

Primer	Sequence 5'→3'	Attachment temperature
COI-ReptBCF	TCAACAAACCAYAAAGAYATY GG	49°C
COI-ReptBCR	TAAACTTCAGGGTGGCCRAAR AATCA	49°C

2.3. DNA Purification

DNA purification was conducted using the alcohol precipitation method (Green & Sambrook 2012). Initially, 20 µL of amplicons were combined with 20 µL of distilled water, 40 µL of isopropanol, and 3 µL of 5M NaCl in a microtube. This mixture was then left to incubate at room temperature for 10 minutes. Following incubation, it was vigorously vortexed for 5 minutes at 14,000 g. Subsequently, the supernatant was carefully discarded. Next, 100 ml of 70% ethanol was introduced, and the solution was vortexed again for 3 minutes at 14,000 g. Once more, the supernatant was removed. The microtube was then allowed to air dry for 30 minutes. After the drying period, 80 µL of distilled water was added as the final solvent.

2.4. Nucleotide Sequencing

Target DNA samples displaying a single band during visualization were selected as templates for

PCR amplification prior to sequencing. The nucleotide sequencing process took place at the Sequencing Laboratory located in the Genomics Building of The National Research and Innovation Agency (BRIN). The sequencing procedure itself was executed utilizing the Sanger sequencing method.

2.5. Sequence Analysis

The sequencing results provided readable nucleotide sequences for all eight samples. The readable nucleotide sequences were then edited with Bioedit version 7.2.5 to remove empty nucleotide bases or revise nucleotide columns with determinations of more than one nucleotide. Subsequently, the edited DNA sequences were used as input for a nucleotide sequence similarity search on the BLAST website (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

This search aimed to determine the similarity and alignment of the obtained sequences with those available in the GenBank database. The nucleotide sequences of eight samples, Then as many as 21 *Calotes* COI gene sequences from other species, and a COI gene sequence of *Bronchocela burmana* from Myanmar MT607997.1 were added to maximize comparison results through phylogenetic trees and genetic distance. A total of 30 sequences, with 582 bp each, were aligned using the MUSCLE (Edgar 2004) method in the MEGA 11 program (Tamura *et al.* 2021).

Phylogenetic tree reconstruction was performed using the IQ-TREE v. 2 (Minh *et al.* 2020) and BEAST v.1 (Suchard *et al.* 2018) with the Yule process speciation tree model (Udny 1925; Gernhard 2008). The p-distance model (Sneath & Sokal 1973) was used because p-distance generally yields better results in comparing the proportions of differences in nucleotide sequences compared to other more complicated models (Filipski *et al.* 2015).

Additionally, the reconstruction of the phylogenetic tree involved the inclusion of sequences from an outgroup. The chosen outgroup sequence was sourced from *Bronchocela burmana* (MT607997), a lizard belonging to the same subfamily as *Calotes*, namely draconinae (Uetz *et al.* 2017). This selection of an outgroup helps to provide a broader perspective for the phylogenetic analysis. For the genetic distance analysis, the pairwise distance method was employed, using the p-distance model (Sneath & Sokal 1973). This analysis allowed for the comparison of genetic distances between the sequences further to understand the evolutionary relationships among the species under study.

3. Results

A total of eight samples were successfully amplified and produced a single band when on a 2% agarose gel. All eight samples have a DNA band size according to the partial COI gene sequence target, which is around (600-700 bp) (Figure 2). Based on the results of alignment between samples, 582 nucleotides (nt) DNA segments were obtained that were aligned with each other, with 293 nt that were the same (conserve), 289 nt that were different (variable), and 198 nt optimal criteria (parsimony informative sites) between samples.

3.1. Genetic Distance Analysis

Genetic distances were analyzed using the pairwise distance method with the p-distance model on MEGA 11. The result of the genetic distance between collection samples (lower diagonal) and their associated standard errors (upper diagonal) compared with samples from GenBank can be seen in Table 3.

The genetic distance in samples SL_1, SL_2, BGR1_1, BGR1_2, BGR1_3, BGR1_4, and BGR2 is in the range of 0%-3% with other *C. vultuosus* from Indonesia (Table 3). This distance score indicates that all these individuals are closely related and conspecific. Nonetheless, the available and the newly generated sequences from Indonesia had higher genetic distances in the range of 6-9% with *C. vultuosus* from India. The greatest intraspecific genetic distance was at 13% between the sample SL_1 from Indonesia and MZ48920 from India1 (Table 3). These results indicate that *C. vultuosus* is a species complex with Indonesian and Indian populations representing two distinct lineages.

The genetic distance between the LKT sample sequences was compared with the other seven sample sequences in this study, revealing a difference of 15%. However, when compared with *C. versicolor* (MT438521, MT438522, MT438523, and MT438524), the genetic distance was approximately 2%. This strong evidence indicates that the LKT samples most likely belong to the species *C. versicolor*.

All eight sequences from this study were subsequently deposited into GenBank. The accession

Figure 2. Visualization of sample PCR results on 2% agarose gel

Table 3. Genetic distance between samples (lower diagonal) with standard error (upper diagonal) using the p-distance model

Specimens code/accession number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
SL_1		0.00	0.01	0.01	0.01	0.01	0.02	0.01	0.02	0.02	0.02	0.02	0.02	0.02	0.02
SL_2	0.00		0.01	0.01	0.01	0.01	0.02	0.01	0.02	0.02	0.02	0.02	0.02	0.02	0.02
BGR1_1	0.03	0.02		0.00	0.00	0.00	0.02	0.00	0.02	0.02	0.02	0.02	0.02	0.02	0.02
BGR1_2	0.03	0.03	0.01		0.00	0.00	0.01	0.00	0.02	0.02	0.02	0.02	0.02	0.02	0.02
BGR1_3	0.03	0.02	0.00	0.01		0.00	0.02	0.00	0.02	0.02	0.02	0.02	0.02	0.02	0.02
BGR1_4	0.03	0.03	0.01	0.01	0.00		0.02	0.00	0.02	0.02	0.02	0.02	0.02	0.02	0.02
BGR2	0.03	0.03	0.01	0.01	0.01	0.01		0.01	0.02	0.02	0.02	0.02	0.02	0.02	0.02
LKT	0.15	0.15	0.15	0.15	0.15	0.15	0.15		0.02	0.02	0.02	0.02	0.02	0.02	0.02
*Calotes_bachae*_Vietnam_MW817625.1	0.22	0.22	0.21	0.21	0.21	0.21	0.21	0.21		0.01	0.02	0.02	0.02	0.01	0.01
*Calotes_bachae*_Vietnam_MW817626.1	0.23	0.22	0.21	0.21	0.21	0.21	0.21	0.22	0.04		0.02	0.02	0.02	0.01	0.01
*Calotes_emma*_Myanmar_MG935451.1	0.23	0.23	0.22	0.23	0.22	0.22	0.20	0.22	0.18	0.19		0.00	0.02	0.02	0.02
*Calotes_emma*_Myanmar_MT609352.1	0.23	0.23	0.22	0.23	0.22	0.22	0.20	0.21	0.18	0.19	0.01		0.02	0.02	0.02
*Calotes_farooqi*_India_MZ489213.1	0.19	0.19	0.19	0.19	0.18	0.18	0.16	0.18	0.19	0.20	0.18	0.18		0.02	0.02
*Calotes_goetzi*_Myanmar_MW817592.1	0.20	0.20	0.18	0.18	0.18	0.19	0.19	0.18	0.15	0.14	0.17	0.17	0.19		0.01
*Calotes_goetzi*_Myanmar_MW817594.1	0.19	0.19	0.18	0.18	0.18	0.19	0.19	0.18	0.14	0.14	0.16	0.16	0.19	0.02	
*Calotes_irawadi*_Myanmar_MZ489212.1	0.16	0.16	0.16	0.15	0.16	0.16	0.05	0.15	0.21	0.21	0.21	0.20	0.16	0.19	0.18
*Calotes_mystaceus*_Vietnam_KC016060.1	0.20	0.20	0.19	0.19	0.19	0.20	0.19	0.19	0.15	0.14	0.16	0.17	0.20	0.05	0.04
*Calotes_versicolor*_Thailand_MT438521.1	0.16	0.16	0.16	0.15	0.16	0.15	0.02	0.15	0.21	0.20	0.20	0.20	0.16	0.19	0.18
*Calotes_versicolor*_Thailand_MT438522.1	0.16	0.16	0.16	0.15	0.16	0.15	0.02	0.15	0.21	0.20	0.20	0.20	0.16	0.19	0.18
*Calotes_versicolor*_Thailand_MT438523.1	0.16	0.16	0.16	0.15	0.16	0.15	0.02	0.15	0.21	0.20	0.20	0.20	0.16	0.19	0.18
*Calotes_versicolor*_Laos_MT438524.1	0.16	0.16	0.16	0.15	0.16	0.15	0.02	0.15	0.21	0.20	0.20	0.20	0.16	0.19	0.18
*Calotes_vindumbarbatus*_Myanmar_MW817602.1	0.21	0.21	0.19	0.19	0.19	0.19	0.20	0.19	0.14	0.14	0.18	0.18	0.19	0.07	0.06
Calotes_vindumbarbatus _Myanmar_MW817603.1	0.20	0.20	0.19	0.18	0.19	0.19	0.19	0.18	0.14	0.14	0.18	0.18	0.19	0.07	0.05
*Calotes_vultuosus*_Indonesia_OQ118405.1	0.03	0.02	0.00	0.01	0.00	0.00	0.15	0.01	0.21	0.21	0.22	0.22	0.18	0.18	0.18
Calotes_vultuosus _Indonesia_OQ118406.1	0.03	0.02	0.00	0.01	0.00	0.00	0.15	0.01	0.21	0.21	0.22	0.22	0.18	0.18	0.18
*Calotes_vultuosus*_Indonesia_OQ118407.1	0.03	0.02	0.00	0.01	0.00	0.00	0.15	0.01	0.21	0.21	0.22	0.22	0.18	0.18	0.18
*Calotes_vultuosus*_India_MZ489201.1	0.09	0.08	0.07	0.06	0.06	0.06	0.14	0.07	0.20	0.20	0.22	0.22	0.17	0.18	0.17
*Calotes_vultuosus*_India_MZ489202.1	0.08	0.08	0.07	0.07	0.06	0.06	0.13	0.07	0.19	0.19	0.21	0.21	0.17	0.17	0.17
*Calotes_vultuosus*_India_MZ489203.1	0.08	0.08	0.07	0.07	0.06	0.06	0.13	0.07	0.19	0.19	0.21	0.21	0.17	0.17	0.17
*Bronchocela_burmana*_Myanmar_MT607997.1	0.26	0.26	0.25	0.25	0.25	0.25	0.26	0.25	0.25	0.24	0.23	0.23	0.24	0.23	0.23

Table 3. Continued

Specimens code/accession number	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
SL_1	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.02
SL_2	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.02
BGR1_1	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.00	0.00	0.00	0.01	0.01	0.01	0.02
BGR1_2	0.01	0.02	0.01	0.01	0.01	0.01	0.02	0.02	0.00	0.00	0.00	0.01	0.01	0.01	0.02
BGR1_3	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.00	0.00	0.00	0.01	0.01	0.01	0.02
BGR1_4	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.00	0.00	0.00	0.01	0.01	0.01	0.02
BGR2	0.01	0.02	0.01	0.01	0.01	0.01	0.02	0.02	0.00	0.00	0.00	0.01	0.01	0.01	0.02
LKT	0.01	0.02	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.02
*Calotes_bachae*_Vietnam_MW817625.1	0.02	0.02	0.02	0.02	0.02	0.02	0.01	0.01	0.02	0.02	0.02	0.02	0.02	0.02	0.02
*Calotes_bachae*_Vietnam_MW817626.1	0.02	0.01	0.02	0.02	0.02	0.02	0.01	0.01	0.02	0.02	0.02	0.02	0.02	0.02	0.02
*Calotes_emma*_Myanmar_MG935451.1	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
*Calotes_emma*_Myanmar_MT609352.1	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
*Calotes_farooqi*_India_MZ489213.1	0.02	0.02	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
*Calotes_goetzi*_Myanmar_MW817592.1	0.02	0.01	0.02	0.02	0.02	0.02	0.01	0.01	0.02	0.02	0.02	0.02	0.02	0.02	0.02
*Calotes_goetzi*_Myanmar_MW817594.1	0.02	0.01	0.02	0.02	0.02	0.02	0.01	0.01	0.02	0.02	0.02	0.02	0.02	0.02	0.02
*Calotes_irawadi*_Myanmar_MZ489212.1		0.02	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.02
*Calotes_mystaceus*_Vietnam_KC016060.1	0.18		0.02	0.02	0.02	0.02	0.01	0.01	0.02	0.02	0.02	0.02	0.02	0.02	0.02
*Calotes_versicolor*_Thailand_MT438521.1	0.05	0.18		0.00	0.00	0.00	0.02	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.02
*Calotes_versicolor*_Thailand_MT438522.1	0.05	0.18	0.00		0.00	0.00	0.02	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.02
*Calotes_versicolor*_Thailand_MT438523.1	0.05	0.18	0.00	0.00		0.00	0.02	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.02
*Calotes_versicolor*_Laos_MT438524.1	0.05	0.18	0.00	0.00	0.00		0.02	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.02
*Calotes_vindumbarbatus*_Myanmar_MW817602.1	0.19	0.07	0.19	0.19	0.19	0.19		0.00	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Calotes_vindumbarbatus _Myanmar_MW817603.1	0.19	0.07	0.19	0.19	0.19	0.19	0.01		0.02	0.02	0.02	0.02	0.01	0.01	0.02
*Calotes_vultuosus*_Indonesia_OQ118405.1	0.16	0.19	0.16	0.16	0.16	0.16	0.19	0.19		0.00	0.00	0.01	0.01	0.01	0.02
Calotes_vultuosus _Indonesia_OQ118406.1	0.16	0.19	0.16	0.16	0.16	0.16	0.19	0.19	0.00		0.00	0.01	0.01	0.01	0.02
*Calotes_vultuosus*_Indonesia_OQ118407.1	0.16	0.19	0.16	0.16	0.16	0.16	0.19	0.19	0.00	0.00		0.01	0.01	0.01	0.02
*Calotes_vultuosus*_India_MZ489201.1	0.15	0.18	0.14	0.14	0.14	0.14	0.18	0.18	0.06	0.06	0.06		0.01	0.01	0.02
*Calotes_vultuosus*_India_MZ489202.1	0.14	0.18	0.13	0.13	0.13	0.13	0.17	0.16	0.06	0.06	0.06	0.05		0.00	0.02
*Calotes_vultuosus*_India_MZ489203.1	0.14	0.18	0.13	0.13	0.13	0.13	0.17	0.16	0.06	0.06	0.06	0.05	0.00		0.02
*Bronchocela_burmana*_Myanmar_MT607997.1	0.25	0.24	0.25	0.25	0.25	0.25	0.23	0.23	0.25	0.25	0.25	0.25	0.25	0.25	

*The red line indicates the genetic distance value with the smallest number, which is found between the collection sample and the sample from GenBank
*The blue line indicates intraspecific genetic distance between samples
*The green line indicates interspecific genetic distance between samples

numbers and corresponding codes for each sequence are as follows: SL_1 (OR229794), SL_2 (OR229795), BGR1_1 (OR229796), BGR1_2 (OR229797), BGR1_3 (OR229798), BGR1_4 (OR229799), LKT (OR229800), BGR2 (OR229801).

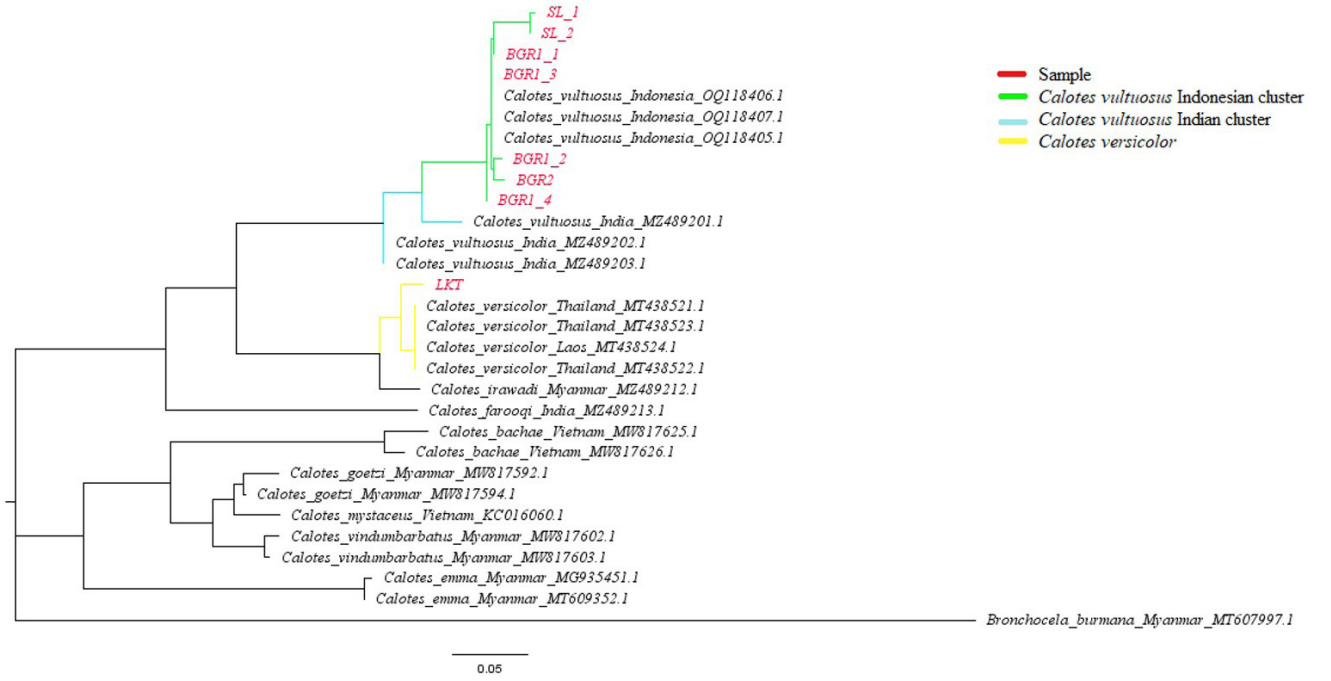
3.2. Phylogenetic Reconstruction

The phylogenetic relationship was analyzed through phylogenetic tree topology using both (a) maximum likelihood with IQ-Tree and (b) Bayesian Inference with BEAST methods (Figure 3). The phylogenetic tree grouped the samples into two clades, represented by black lines, and identified a total of 10 species. Two different models were used to create phylogenetic trees. However, both the Maximum-likelihood model and the Bayesian Inference model yielded similar results, suggesting that all eight samples belonged to the same clade and species group.

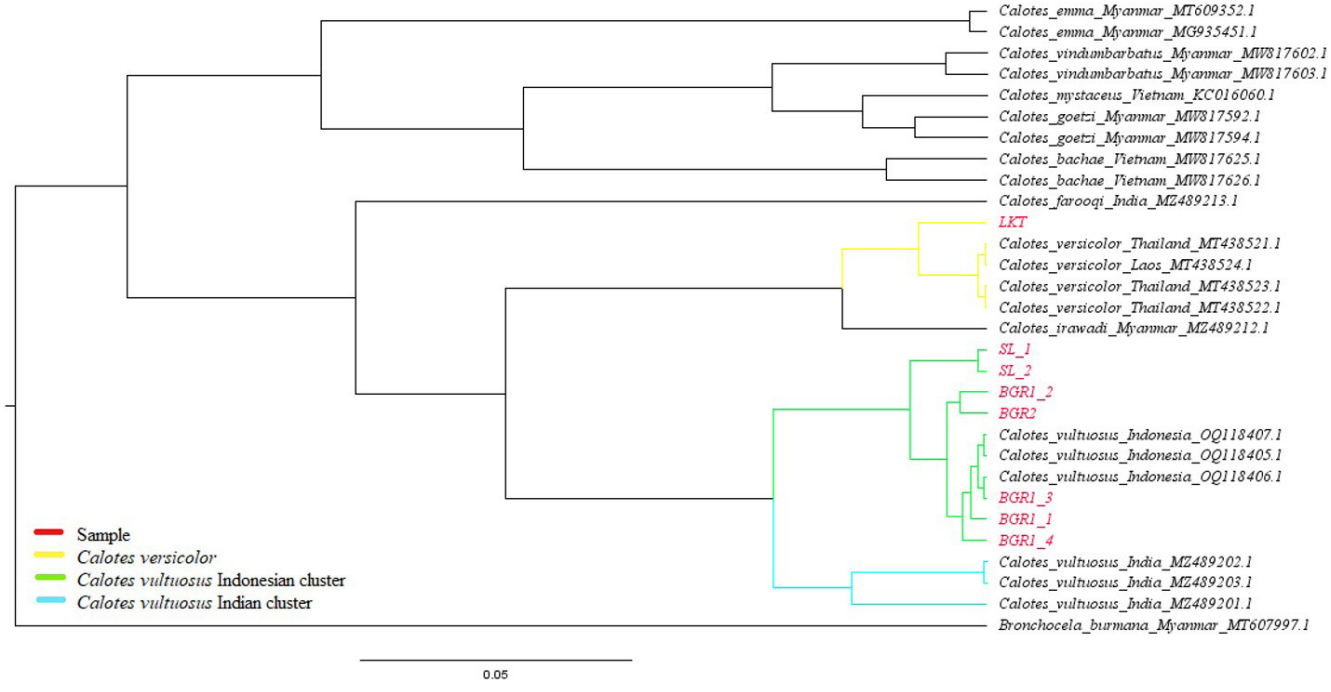
The results of the homologous sequence search for the eight nucleotide sequences confirmed that they indeed belonged to the COI gene sequences from the *Calotes* genus. This finding provides crucial evidence for the accuracy of the samples and their suitability for further analysis in studying the genetic relationships and diversity within the *Calotes* genus.

4. Discussion

The genetic distance analysis conducted in this study provides intriguing insights into the genetic relationships within the *Calotes* genus in Indonesia. Of particular interest is the discovery that samples SL_1 and SL_2 represent two distinct individuals of *C. vultuosus* collected from different locations within a relative proximity of 300 meters in the Sumatra Institut of Technology (ITERA) Botanical Garden. Similarly, samples BGR1_1, BGR1_2, BGR1_3, and BGR1_4 originate from *C. vultuosus* specimens collected in a plantation ecosystem within a 50-meter radius in Bogor point 1. In contrast, sample BGR2 is a *C. vultuosus* specimen collected from Bogor



(A)



(B)

Figure 3. Phylogeny tree reconstruction results based on mtDNA COI gene segments using the Maximum Likelihood with IQ-Tree (A) and Bayesian Inference with BEAST (B)

Point 2, situated 24 kilometres away from Bogor Point 1. The distance between these points includes various barriers such as highways, rivers, and dense residential areas.

Given their genetic distance and proximity, these six individuals are expected to have DNA sequences with very high similarity, possibly belonging to the same lineage (Pearson 2013). Overall, the genetic similarities between the samples and the *C. vultuosus* reference sequence indicate that the samples can be identified as *C. vultuosus*. The variations in similarity percentages among the samples could be attributed to differences in geographic locations and potential barriers affecting gene flow between populations. Low genetic distance indicates gene flow between populations (Trisyani & Rahayu 2020).

The classification of samples SL_1, SL_2, BGR1_1, BGR1_2, BGR1_3, BGR1_4, and BGR2 as *C. vultuosus* is further supported by the topology of the two phylogenetic trees. The two phylogenetic tree results support the grouping of these seven samples together with *C. vultuosus* from Indonesia (accession numbers OQ118405, OQ118406, and OQ118407) and *C. vultuosus* from India (MZ489201, MZ489202, and MZ489203). This clustering in the phylogenetic tree suggests a close genetic relationship between the collection samples and the reference *C. vultuosus* sequences. Moreover, the phylogenetic tree shows that the *C. vultuosus* samples from the collection form a distinct subgroup with *C. vultuosus* from Indonesia (OQ118405, OQ118406, and OQ118407).

The observed differences in genetic clusters between *C. vultuosus* populations in Indonesia and India can be largely attributed to geographical factors. The vast oceanic separation between these regions acts as a significant barrier to gene flow, making it difficult for *C. vultuosus* to move between them. Additionally, the imposing mountainous terrain surrounding India further restricts their movement, effectively isolating Indian populations. These geographical features create conditions conducive to allopatric speciation, contributing to the genetic distinctions seen in these two regions (Grismer *et al.* 2016).

Recent studies conducted by Gowande *et al.* (2021), Wagner *et al.* (2021), and Tantrawatpan *et al.* (2021) align with this hypothesis, reinforcing the idea that geographic distance and ecological barriers play pivotal roles in generating genetic differences among *Calotes* populations. This growing body of evidence emphasizes the significance of geographical and ecological factors in shaping the genetic diversity and evolutionary patterns of *Calotes* species, including *C. vultuosus.*

In contrast, during the initial stages of the study, all samples were initially considered as *C. versicolor*. However, based on the BLAST results of the sample sequences, it was found that only the LKT sample was classified as *C. versicolor*. The LKT sample exhibited a high similarity percentage with the sequence of *C. versicolor* from Laos (MT438524) found in GenBank. The phylogenetic tree topology also supported this classification, with the LKT sample forming a separate group along with *C. versicolor* from Thailand (MT438521, MT438522 & MT438523) and Laos (MT438524). These findings highlight the importance of using multiple lines of evidence, such as BLAST analysis and phylogenetic tree reconstruction, to identify and classify species based on their genetic sequences accurately.

On both topologies of the phylogenetic tree, all sequences were grouped into two distinct clades. The first clade includes species such as *C. calotes, C. farooqi, C. Irawadi, C. versicolor* and *C. vultuosus*. On the other hand, the second clade comprises species such as *C. bachae, C. emma, C. goetzi, C. mystaceus* and *C. vindumbarbatus*. The arrangement of individuals within each clade is consistent with the phylogenetic tree topology found in the studies by Gowande *et al.* (2021) and Wagner *et al.* (2021). This clustering of species into two distinct clades suggests a clear pattern of genetic relatedness and evolutionary relationships among these *Calotes* species.

Furthermore, in the phylogenetic tree topology, samples SL_1, SL_2, BGR1_1, BGR1_2, BGR1_3, BGR1_4, BGR2, and *C. vultuosus* from Indonesia form a distinct clade that is separate from *C. vultuosus* from India. This clear separation in the phylogenetic tree, coupled with the relatively high genetic distances observed between *C. vultuosus* populations in Indonesia and India, strongly suggests the presence of two distinct genetic clusters within this species. This cluster variation underscores the significant genetic diversity within *C. vultuosus*, which is a crucial factor for its survival and adaptation.

Based on the research findings, it is apparent that samples SL_1, SL_2, BGR1_1, BGR1_2, BGR1_3, and BGR1_4 share a very close genetic relationship with *Calotes vultuosus* from Indonesia. However, these

same samples exhibit higher genetic distance values when compared to *Calotes vultuosus* from India. This difference in genetic distances raises suspicions of the existence of two distinct clusters within *Calotes vultuosus* populations in Indonesia and India. The interspecific genetic distances between samples SL_1, SL_2, BGR1_1, BGR1_2, BGR1_3, and BGR1_4, when compared with the LKT samples, fall within the medium category. The LKT samples, in turn, display a very close genetic relationship with *Calotes versicolor* from Thailand and Laos. These observations indicate the likelihood of genetic differentiation between the *C. vultuosus* populations in Indonesia and the *C. versicolor* populations in Thailand and Laos.

The collected samples of *C. versicolor* sensu stricto and *C. vultuosus* are thought to live in the same habitat and overlap. Both are lizard species that are not native to Indonesia. The discovery of the first specimen of *C. versicolor* was made by de Rooij in 1915 in Banda Aceh, while the first record of the discovery of *C. vultuosus* was based on COI gene nucleotide data submitted in 2022 at NCBI (Hilhanif 2018). There is a possibility that *C. vultuosus* has been introduced to Indonesia for a longer period. However, since *C. vultuosus* is part of the *C. versicolor* species complex, *C. vultuosus* in Indonesia will be considered as *C. versicolor* since they are morphologically similar. In addition, research on the *C. versicolor* species complex molecularly in Indonesia is still very minimal, so all members of the genus *Calotes* found in Indonesia will be considered as *C. versicolor*.

Future research endeavours could be enriched by integrating additional data derived from morphometric and meristic measurements. The combination of genetic data alongside morphological measurements holds the potential to furnish a more comprehensive understanding of the evolutionary relationships, speciation processes, and adaptation patterns within the *Calotes* genus. Adopting such a multidisciplinary approach can facilitate a deeper exploration of the intricate complexities surrounding species diversity and distribution within these lizard populations.

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