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Research Article

Genetic Variation of Baram River Frog, *Pulchrana baramica* (Boettger, 1900), In Java, Sumatra, and Kalimantan based on *16S* Mitochondrial Gene

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ABSTRACT

Baram River Frog (*Pulchrana baramica*) is a ranid species distributed in the Malay Peninsula, Borneo, Sumatra and represents the sole species from the genus *Pulchrana* on Java Island. Cryptic species are commonly encountered within the amphibian group which can cause confusion in the identification process. Due to the broad distribution range of *P. baramica* and the frequent occurrence of cryptic species within the amphibian group, it is important to evaluate the taxonomic status of *P. baramica*. Therefore, we investigated the taxonomic position of *P. baramica* from three populations (Kalimantan, Sumatra, and Java) and identified the interpopulation genetic variation based on molecular data of the *16S* mitochondrial gene. We reconstructed phylogenetic relationships using Neighbour Joining, Maximum Likelihood, and Bayesian Inference. The research results revealed that *Pulchrana baramica* is a monophyletic group and nested within a group together with *P. glandulosa* and *P. laterimaculata*. The monophyletic group of *P. baramica* consisted of four distinct lineages that molecularly showed interspecific genetic variation. Clade 1 represents the population of Sumatra and Borneo (Kalimantan), clade 2 comprises the population from Borneo (Sarawak), clade 3 consists of population from Java, and clade 4 represents the population from Sumatra. Further research is required with the addition of morphological and acoustic data as supportive evidence to obtain more extensive comprehension of species identification.

INTRODUCTION

Baram River Frog or *Pulchrana baramica* is a ranid species and was first described from the Baram River in Sarawak, Malaysia, and described in 1900 by Boettger. Boettger identified the diagnostic characteristics of the Baram River Frog as having reduced webbing on the hind limbs, a granular dorsum, lacking folds on the glands, and expanded toe tips, which are comparable to *Rana signata* Gunther, 1872. This species can be found in areas with elevations up to 600 m asl (Iskandar 1998; Leong & Lim

2003; Das et al. 2007; Chan et al. 2014).

The distribution of the *Pulchrana baramica* species includes Johor, Selangor, Peninsular Malaysia, Singapore, Kalimantan, Sumatera (Riau and Bangka Island), and based on Iskandar's records in 1998, the species was found in Cilebut, Bogor Regency, and 2018 in Haurbentes Village, Bogor Regency on Java Island. Additionally, recent records by Herlambang et al. (2022) reported the presence of *P. baramica* in Bunguran and Tiga Island of the Kepulauan Natuna, Indonesia. To date, the Genus *Pulchrana* comprises 18 species and *Pulchrana baramica* (Boettger, 1900) is known as the only species of the *Pulchrana* genus found on Java Island (Frost 2021).

The determination of taxonomic status for amphibian species generally employs morphometric, molecular, or vocal characteristics. However, taxonomic determination using morphometric approaches can encounter obstacles when it is known that the species in question is a cryptic species that is morphologically similar. The molecular data was proven to be an effective tool for solving taxonomic problems within some cryptic species of amphibians in Sunda land regions (Hamidy et al. 2011; Hamidy et al. 2012; Munir et al. 2018; Munir et al. 2021). In amphibians, the species delimitation process frequently used is the non-tree-based method: genetic distance. In this method, species separation is determined from the value of genetic distance. For amphibian groups, Fouquet et al. (2007) proposed an uncorrected p-distance value of >3% as a species delimitation threshold for the mitochondrial genes *12S* and *16S* rRNA.

According to Inger (1966), frogs in the *Pulchrana* genus can be identified by looking at the webbing on their hind legs. The frog species *P. baramica* is closely related to *P. glandulosa*. Those two species can be distinguished by the webbing on their hind legs, with *P. baramica* having lack of developed webbing than *P. glandulosa* (Van Kampen 1923). However, identifying amphibians based on morphological characteristics has limitations because the morphological characteristics used to differentiate between species are very limited, and require detailed observations and special skills. Therefore, molecular data is needed to strengthen previous identifications for inter and intraspecific populations.

MATERIAL AND METHODS

Sample Collection

The samples used in this study comprised tissue samples from voucher specimens of *Pulchrana baramica* that were deposited in *Museum Zoologicum Bogoriense* (MZB), the National Research and Innovation Agency (BRIN). All ingroup samples represent the population of *P. baramica* from Sumatra, Kalimantan, and Java in Indonesia. The sample collection locations can be seen in Figure 1, and the detailed location and voucher specimen number are presented in Table 1.

DNA Extraction

The complete DNA genomic samples were isolated from muscle using the kit (dneasy Blood and tissue kit from QIAGEN, Valencia, CA, USA) following the manufacturer's protocols.

DNA Amplification, Electrophoresis, and Sequencing

The DNA isolation product was subjected to amplification using *16S* rRNA primers consisting of forward primer L2606 5' CTG ACC GTG CAA AGG TAG CGT AAT CACT-3' and reverse primer H3056 5' CTC CGG TCT GAA CTC AGA TCA CGT-3' (Hedges et al. 1993). The PCR amplification utilized 25 µl reaction consisting of 12.5 µl Redmix PCR

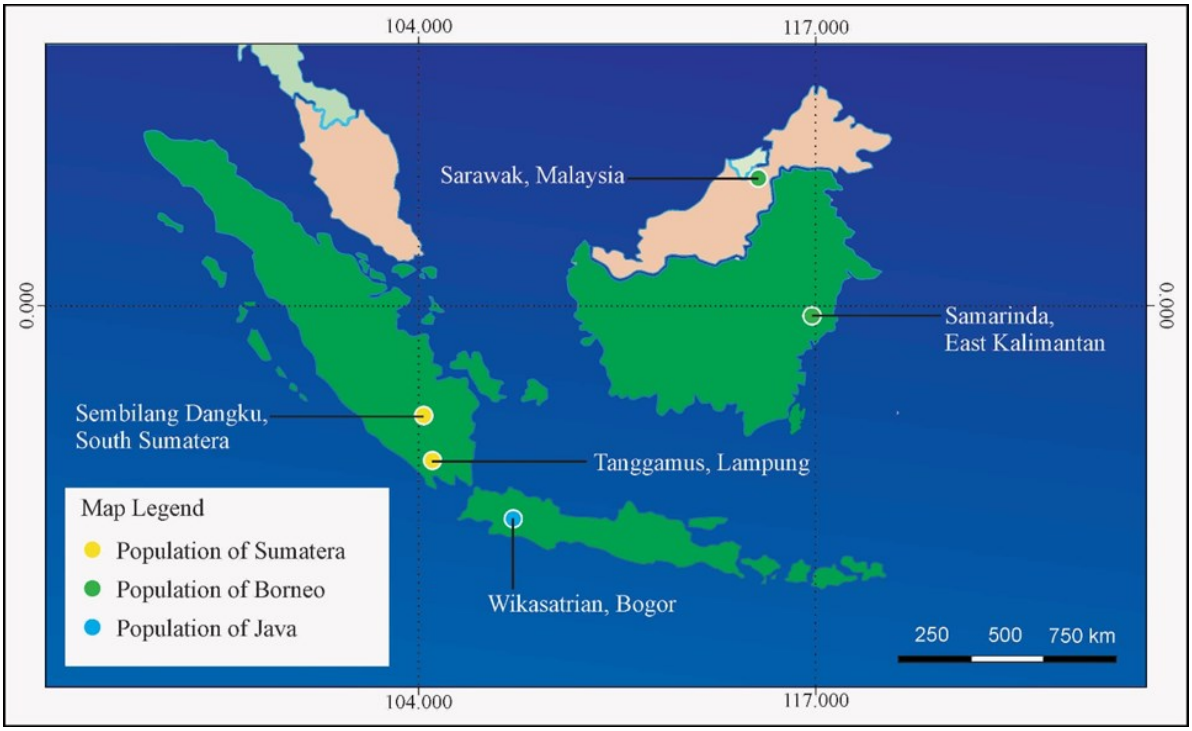

Figure 1. Map of frog sampling locations in this study.

Table 1. List of ingroup samples used in this study.

No	Voucher Number	Genbank Number	Species name	Location	Sources
1	MZB Amph 32583		*Pulchrana baramica*	Java, West Java, Bogor, Pasir Angin, Wikansatrian	This study
2	MZB Amph 32586		*Pulchrana baramica*	Java, West Java, Bogor, Pasir Angin, Wikansatrian	This study
3	MZB Amph 31578		*Pulchrana baramica*	Borneo, East Kalimantan, Samarinda	This study
4	MZB Amph 31579		*Pulchrana baramica*	Borneo, East Kalimantan, Samarinda	This study
5	MZB Amph 32662		*Pulchrana baramica*	Sumatra, South Sumatra, Sembilang Dangku	This study
6	MZB Amph 32665		*Pulchrana baramica*	Sumatra, South Sumatra, Sembilang Dangku	This study
7	MZB Amph 32666		*Pulchrana baramica*	Sumatra, South Sumatra, Sembilang Dangku	This study
8	MZB Amph 32673		*Pulchrana baramica*	Sumatra, South Sumatra, Sembilang Dangku	This study
9	MZB Amph 22327		*Pulchrana baramica*	Sumatra, Lampung, Tanggamus	This study
10	MZB Amph 22329		*Pulchrana baramica*	Sumatra, Lampung, Tanggamus	This study
11	KUHE 53640	AB719234	*Pulchrana baramica*	Borneo, Sarawak, Mulu	Matsui et al. 2012
12	KUHE 53617	AB719232	*Pulchrana baramica*	Borneo, Sarawak, Mulu	Matsui et al. 2012
13	KUHE 53623	AB719233	*Pulchrana baramica*	Borneo, Sarawak, Mulu	Matsui et al. 2012
14	KUHE 53678	AB719231	*Pulchrana baramica*	Borneo, Sarawak, Mulu	Matsui et al. 2012

kit, 1 mm $MgCl_2$, 0.6 mm of forward primer L2606 and reverse primer H3056, 5.5 µl ddH_2O, and 3 µl (10-100 ng) of DNA template. All solutions were mixed in a PCR tube for each sample. The solution mixture was vortexed and centrifuged using the spin-down mode before being placed in a Thermocycler with 1 cycle of reaction for 5 minutes of predenaturation at 95°C, 35 cycles of reaction for 35 seconds of denaturation at 95°C, 30 seconds of annealing at 50°C, 30 seconds of extension at 72°C and 7 minutes of final extension at 72°C.

The PCR products were subjected to agarose gel electrophoresis on a 1% gel at 100 volts for a duration of 15 minutes. Florosafe (Bioline) was used for staining, while Tris-acetate EDTA (TAE) 1X served as the buffer. The amplification products were visualised under UV light. Purification and sequencing of each amplification sample in the forward and reverse directions were carried out using the Big Dye Terminator (Applied Biosystems) and the Genetic Analyser ABI 3730xl, respectively. These procedures were conducted by P.T. Genetika Science in Jakarta and delivered to First Base (Malaysia).

Sequence Editing

Genestudio software was used to create and to modify the consensus sequence, which was then verified for accuracy through the use of DNASTAR software's seqman and editseq menus (DNASTAR Inc., Madison, USA). While conducting this procedure, careful inspection of the chromatograms was carried out to ensure the absence of any uncertain bases.

Sequence Identification

The sequence of samples was identified using the Nucleotide BLAST analysis available on the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The similarity and the query cover of the sample were used to estimate the species of *P. baramica* compared to the available data in genbank.

Sequence Alignment

The sample sequences were aligned using the Mesquite v.3.70 software on the Opal menu (Maddison & Maddison 2021) and the MEGAX software on the clustalw menu (Kumar et al. 2018).

Intrapopulation and Intraspecies Analysis

The primary objective of the intrapopulation analysis was to determine the nucleotide composition of each sample belonging to the same population. Additionally, the intraspecies analysis aimed to extend the inquiry by examining the nucleotide composition, genetic distance, and phylogenetic relationship across diverse populations.

Nucleotide Composition & Genetic Distance

The nucleotide composition was computed using the MEGAX program. The genetic distance was analysed with the *p*-distance model and visualized in a Neighbour-Joining (NJ) tree, which is a widely employed approach in DNA barcoding research, as described by Hebert et al. (2003).

Phylogenetic Relationship

In this analysis, we used 14 sequences of *P. baramica* as ingroup members, ten of them are from this study and four from the genbank (Table 1). For outgroups, we involved *P. glandulosa* and *P. laterimaculata* from genbank data. The MEGAX software (Kumar et al. 2018) was used to

evaluate the reconstruction of the phylogenetic tree using three methods, Neighbour-Joining, and Maximum Likelihood, both with 1,000 bootstraps, and Bayesian Inference method through the BEAST program (Suchard et al. 2018). The optimal evolutionary model was identified using the Bayesian Information Criterion (BIC) implemented in jmodeltest 2.1.10 (Darriba et al. 2012). The HKY with a gamma value of 0.170 (HKY + G) was determined to be the best sequence substitution model based on the Bayesian Inference Criterion. The method of Markov Chain Monte Carlo (MCMC) was employed to estimate the posterior probabilities distribution over 10^7 generations with a sampling frequency of 1,000 generations.

RESULTS AND DISCUSSION

Result

Species verification

We successfully amplified the *16S* mitochondrial gene from 10 samples of Baram River Frogs, yielding fragments of 500 bp (Figure 2). Sequence editing was performed using genestudio software, resulting in consensus sequences ranging from 508 bp to 547 bp in length. Genetic similarity analysis of the samples with reference species in genbank revealed that out of the 10 samples studied, 8 samples exhibited a similarity range of 93.93 to 96.68% with species *Pulchrana baramica*, while 2 samples showed a similarity range from 96.09 to 96.27% with species *Pulchrana laterimaculata* (Table 2).

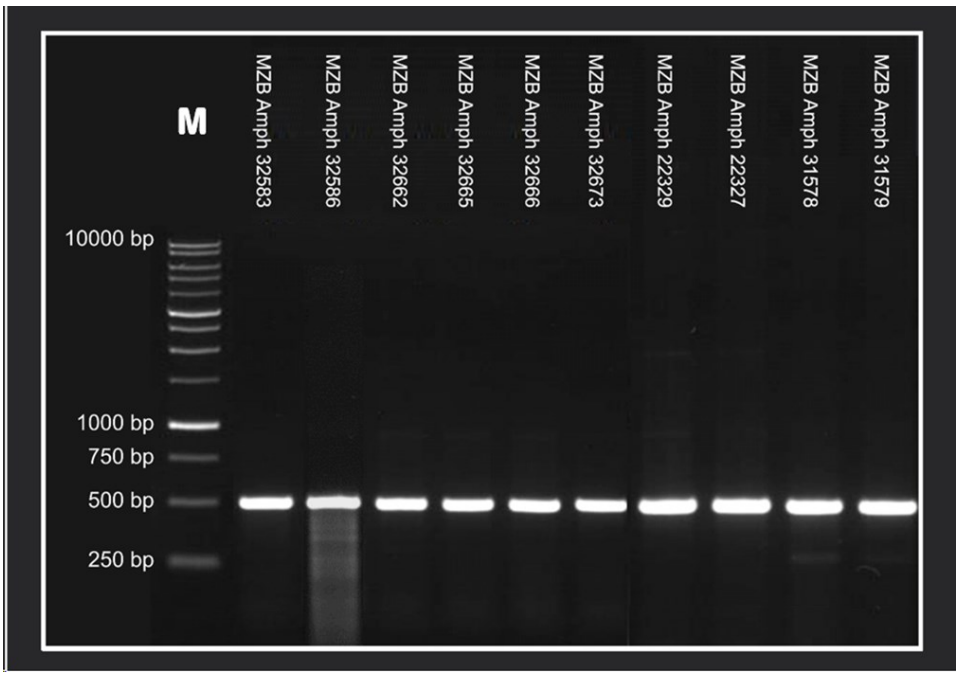

Figure 2. The PCR amplification results of the *16S* mitochondrial gene from frog samples collected from Java, Sumatra, and Kalimantan. The samples were run on 1% agarose gel electrophoresis and identified by the samples *P. baramica* from Indonesia. A 1kb DNA ladder (GENEAID) was included as a reference marker, labelled as "M" in the figure.

We conducted a *16S* mitochondrial gene alignment of in group samples collected from Java, Sumatra, and Kalimantan. The alignment yielded fragment length of 456 base pairs for each species. These sequences were subsequently utilised to conduct intrapopulation analysis for each species. Additionally, we performed intraspecies analysis by comparing the *16S* sequences of Sarawak samples from Mulu, which located in one river system with the Baram River (type locality) as record-

Table 2. Identification of species based on the database of genbank using BLAST.

Specimen number (MZB)	Sample Code	Fragment Length (bp)	BLAST			Genbank Species Identified
			% *Identity*	% *Query Cover*	*Accession Number Genbank*	
MZB Amph 32583	L.J.1	545	95.50	93	DQ835351.1	*Pulchrana baramica*
MZB Amph 32586	L.J.2	508	93.93	99	DQ835351.1	*Pulchrana baramica*
MZB Amph 32662	L.S.1	547	96.29	93	DQ835352.1	*Pulchrana baramica*
MZB Amph 32665	L.S.2	543	96.09	94	EU604193.1	*Pulchrana laterimaculata*
MZB Amph 32666	L.S.3	543	96.27	93	EU604193.1	*Pulchrana laterimaculata*
MZB Amph 32673	L.S.4	546	96.68	93	DQ835352.1	*Pulchrana baramica*
MZB Amph 22329	L.S.5	539	96.48	94	DQ835352.1	*Pulchrana baramica*
MZB Amph 22327	L.S.6	536	96.68	95	DQ835352.1	*Pulchrana baramica*
MZB Amph 31578	L.K.1	538	96.48	94	DQ835352.1	*Pulchrana baramica*
MZB Amph 31579	L.K.2	546	96.48	93	DQ835352.1	*Pulchrana baramica*

ed in genbank and species from Genus *Pulchrana* in Indonesia. To comprehend the evolutionary relationships of the samples, we aligned the sequences from Java (West Java), Sumatera (South Sumatra and Lampung), and Kalimantan (Samarinda) with outgroups member (*P. glandulosa* and *P. laterimaculata*) sequences from genbank. The resulting aligned sequences were then employed to construct a phylogenetic tree.

For intrapopulation analysis, we analysed a total of 10 frog samples from Wikasatrian (West Java), Sembilang Dangku (South Sumatra), Lampung (Sumatra), and Samarinda (East Kalimantan). Our results showed that the average nucleotide composition of samples consisted of 21.96% thymine (T), 27.17% cytosine (C), 30.34% adenine (A), and 20.53% guanine (G), with variations ranging from 0 to 0.9%, 0 to 1.05%, 0 to 0.30%, and 0 to 0.45%. The average composition of nucleotides A+T was found to be 52.30%, while G+C accounted for 47.70%.

The nucleotide composition of A+T is more dominant compared to the composition of nucleotides C+G. In the population of Java Island, the composition of nucleotide T was 22.42%, C ranged from 26.59 to 26.81%, A ranged from 30.33 to 30.55%, and the composition of nucleotide G was 20.44%. In the population of Sumatra Island (clade 1), the composition of nucleotide T ranged from 21.54 to 22.20%, the composition of nucleotide C ranged from 26.75 to 27.91%, A ranged from 30.11 to 30.55%, and G ranged from 20.44 to 20.88%. In the population of Kalimantan Island (clade 1), the composition of nucleotide T ranged from 21.76 to 21.98%, C was 27.25%, A ranged from 30.11 to 30.55%, and G ranged from 20.44 to 20.66%.

The resulting phylogenetic tree revealed the division of the 10 samples into four separate clades, supported by high bootstrap values of 95-100% (NJ), 92-98% (ML), and a posterior probability of 1 (BI). The phylogenetic tree reconstruction is presented in Figure 3, highlighting the distinct separation of clades 1, 2, 3, and 4. Next, our genetic distance analysis results in the estimation of genetic distances between sequences. The sequence fragments used for analysis had a length of 456 bp. The lowest genetic distance among all clades was observed between clade 1 and clade 2, measuring 0.22%. Conversely, the highest genetic distance among all clades was observed between clade 3 and clade 4, measuring 5.45% (Table 3).

Discussion

Genetic identification methods can be employed to rapidly and accurately determine individuals based on their unique genetic information. We ob-

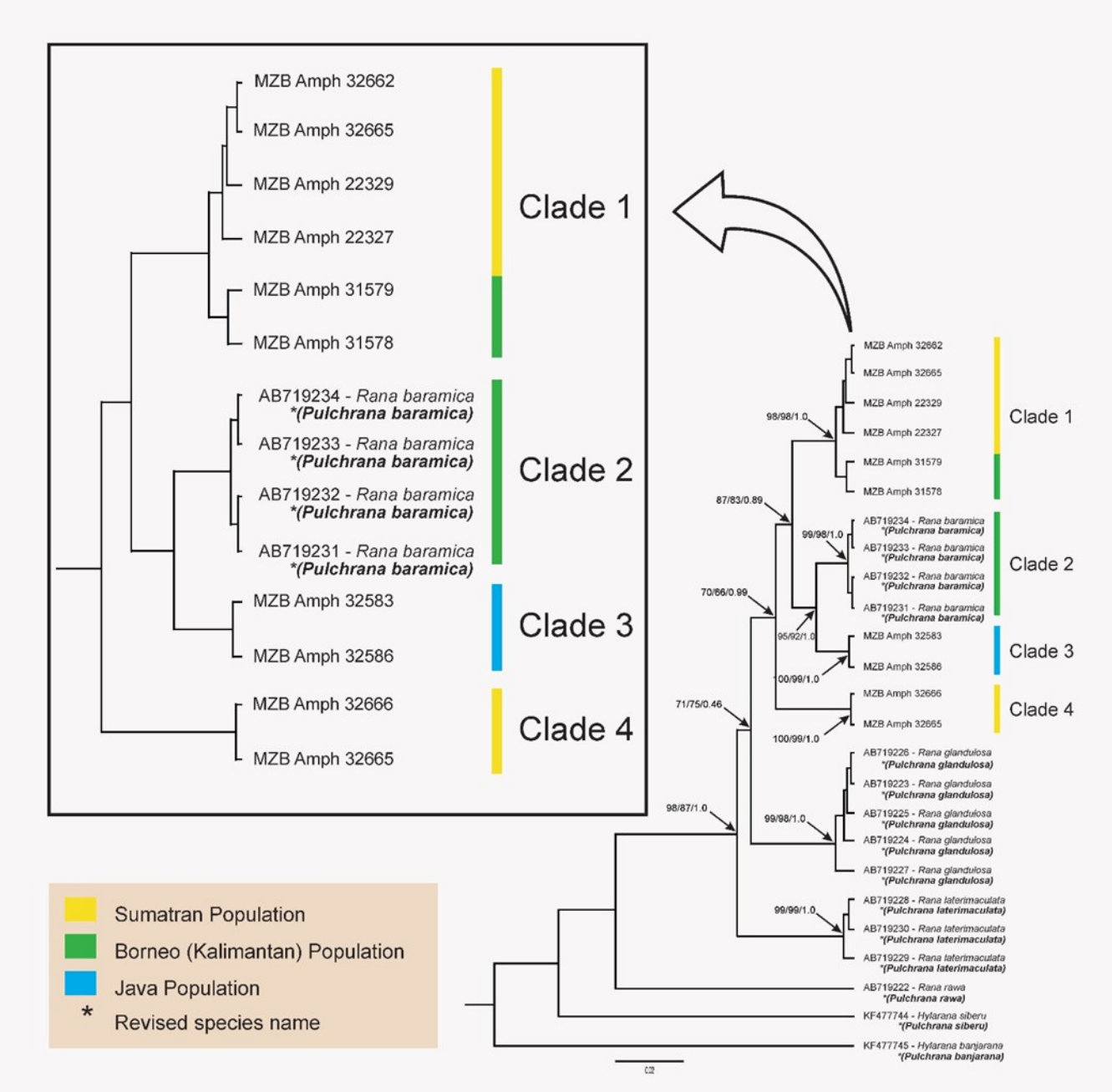

Figure 3. Phylogenetic tree reconstruction based on Neighbour-Joining (NJ), Maximum-Likelihood (ML), Bayesian Inference (BI) topology species sample, species of *Pulchrana baramica* and its outgroup members based on *16S* gene sequences (456 bp). The node represented the number bootstrap (NJ and ML) and Bayesian Posterior Probability (Bayesian Inference).

served that the analysis of nucleotide composition in the *16S* gene of the ten studied frog samples exhibited varying compositions of nucleotides T, C, A, and G. The respective mean nucleotide compositions of T, C, A, and G in the ten studied frog samples were 21.96, 27.17, 30.34, and 20.53%. The average composition of nucleotides A+T was 52.30%, while G+C was 47.70%. In this study, it is evident that the nucleotide base composition follows the order A>C>T>G in descending order. When compared to the research conducted by Chowdhury et al. (2021) on the frog species *Hydrophylax leptoglossa*, which exhibited a nucleotide base composition pattern of A>C>G>T in descending order, it indicates that frogs within the same family may exhibit different patterns of nucleotide base composition.

Our phylogenetic tree revealed the presence of four distinct clades. Clade 1 represents the population from Sumatra and Borneo

Table 3. The percentage (%) of interpopulation genetic distances observed in this research and sample *Pulcrana baramica from* genbank data based on the *16S* rRNA gene.

	Clade 1	Clade 2	Clade 3	Clade 4
Clade 1	0.59% (0.22%-1.10%)			
Clade 2	3.41% (2.08%-3.74%)	0.00% 0.00%		
Clade 3	4.40% (3.96%-4.84%)	2.75% (2.64%-2.86%)	0.22% (0.22%)	
Clade 4	4.07% (3.74%-4.40%)	4.62% (4.62%)	5.16% (5.05%-5.27%)	0.00% 0.00%

(Kalimantan), clade 2 represents a population from Mulu, clade 3 represents a population from Java, and clade 4 comprises a population from Sumatra (South Sumatra). Clade 2 (Borneo, Sarawak, Mulu population) is located in one river system with Baram River (type locality of *P. baramica*), so this clade likely represents "true" *P. baramica*. Therefore, other lineages (clade 1, 3, and 4) are remaining unnamed species. We also found the existence of two distinct lineages from Sumatra Island (clade 1 and clade 4).

We assessed the robustness of our phylogenetic tree by examining the bootstrap values and posterior probabilities, and we found that the formation of clades in the phylogenetic tree had high bootstrap values and posterior probabilities, indicating the robustness of these clades. According to Apriliyanto and Sembiring (2016), if the bootstrap value approaches 100% in the Neighbour-Joining and Maximum Likelihood methods, and the posterior probability value is close to 1 in the Bayesian Inference method, the formation of clades can be deemed robust. In our study, we found that the result aligns with this criterion, providing additional evidence for the dependability and precision of our phylogenetic tree reconstruction.

Our genetic distance analysis revealed significant average genetic divergence ranging from 2.75 to 5.16% among clades. These values closely approach the proposed threshold of 3% for intraspecies genetic divergence, as suggested by Fouquet et al. (2007). Our investigation involved a comprehensive examination of ten carefully selected samples collected from three major island populations in Indonesia. Comparative analysis with the control dataset from the sequence data of *Pulchrana baramica* species obtained from genbank revealed noteworthy outcomes. Specifically, two allopatric populations of Java (clade 3) and Borneo, Mulu (clade 2) exhibited an average genetic divergence of 2.75%, thus supporting interspecific genetic divergence. Conversely, the two clades representing the populations from Sumatra Island and Kalimantan Island (identified as clades 1 and 4, respectively) exhibited genetic divergences of 3.50 and 4.83% respectively, suggesting that they do not belong to the same species as *Pulchrana baramica*. These findings demonstrated that the population from Kalimantan (Samarinda) within clade 1 represents a distinct species, separated from clade 2 Sarawak (Mulu) despite both being on one Island of Borneo.

Our study also revealed that genetic differentiation within Sumatra (clade 1 and clade 4) and Borneo (clade 1 and clade 2) are greater than two allopatric populations of Java and Borneo Mulu (clade 2 and 3). We hypothesised that this result can be attributed to the large population sizes and similar habitat and climate conditions at the sampling locations of clades 2 and 3, which may have limited natural selection. Moreover, larger populations have a higher likelihood of gene flow between individ-

uals within the population and a greater chance of gene exchange with other populations. In small populations, gene flow between individuals or with other populations may be more restricted, leading to genetic isolation and the accumulation of genetic differences between populations. The higher genetic within Borneo (clade 1 and clade 2) can be attributed to factors such as differences in population size and habitat, which could induce adaptation and natural selection, resulting in a higher genetic distance despite being on the same island. Additionally, significant genetic distance is observed for clade 4, whereas it has an average genetic distance ranging from 4.07 to 5.15% compared to other clades (clade 1, 2, 3), this is possibly due to various factors such as population size, habitat variations, and stochastic effects. It is important to note that the lack of supporting data regarding morphological data, habitat types, climate conditions, and population sizes, may have influenced the results of the phylogenetic tree analysis in this study. Future studies with larger sample sizes and comprehensive supporting data including morphological data may be necessary to gain a better understanding of the patterns of genetic diversity within each studied population location.

CONCLUSIONS

Our study provides compelling evidence for the existence of cryptic species within the *Pulchrana baramica* species through analysis of the *16S* mtDNA gene, while also exploring their genetic diversity and relationships. Our phylogenetic tree analysis revealed the existence of four distinct clades within the population of *P. baramica*. Furthermore, additional information on morphological and bioacoustic data is needed to determine the taxonomic status of several unnamed species within the population of the *P. baramica* group.

AUTHOR CONTRIBUTION

T.A. and A.H. were in charge of planning, designing, and supervising the entire research process. L.F. and K.W.A. worked in a laboratory (DNA extraction, DNA amplification, agarose gel electrophoresis, data analysis, and manuscript writing). A.R. and E.N.S. provided support for research materials.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest. The authors are responsible for the article's content and writing.

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