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Research Article

Genetic and Morphometric Variation of the Genus *Nyctixalus* in Indonesia Based on mtDNA 16S rRNA Gene

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ABSTRACT

The genus *Nyctixalus* was first described by Boulenger in 1882. Previous systematic studies of this group primarily focused on its relationships with other rhacophorid frogs and did not explore inter- and intraspecific variation in detail. This study aimed to determine the position of Indonesian species within their genus. We used DNA barcoding of the mitochondrial 16S rRNA gene and conducted a morphological analysis. Mitochondrial genealogies were constructed based on NJ, ML, and BI analyses, while uncorrected p-distance was also calculated. The Mann-Whitney U-test and PCA were used to analyse variations among species for each character component. Dissimilarity may contribute to measurable group structure. DNA barcoding revealed that *Nyctixalus* forms a monophyletic group. The PCA analysis revealed that *N. margaritifer* population from Java formed a group, while *N. pictus* could not be separated on the scatter plot. DNA barcoding revealed that Nyctixalus forms a monophyletic group. The genetic distance within the population of *N. margaritifer* (0–1.52 %) and *N. spinosus* (0–0.2 %) showed a variation of intraspecific genetic distance. However, in the two allopatric populations of *N. pictus* Kalimantan and Sumatra (p-distance = 4.3–4.6 %), there are high variations (6.9–9 %) between populations from Sumatra and Malaysia. These findings suggest that *N. anodon* van Kampen, 1907, from Kayu Tanam, Sumatra Barat, Indonesia, represents a distinct species from *N. pictus* Peters, 1871, originally described from Sarawak, Malaysia.

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INTRODUCTION

Genus *Nyctixalus* was first described based on adult morphological specimens *of Nyctixalus margaritifer* obtained from the *East Indies* (Boulenger 1882). However, the systematic status of the genus was not renewed and subsequent studies focused on the division of the family Ranidae (Boulenger 1888; Boulenger & Robinson 1912).There was also no discussion or improvement of the systematics of the genus, although some investigations used the genus as part of the identification keys of the family Ranidae (Cope 1889; Palacký 1898; Gadow 1901; Roux 1905).

Initially, genus *Nyctixalus* consisted of species *N. margaritifer* (Boulenger 1882) and *N. robinsoni* (Annandale 1917) (Annandale 1917; van Kampen 1923). *N. robinsoni* has a morphology of pupil shape with the same horizontal line axis as the specimen *Philautus aurifasciatus* (Schlegel), but it is not re-registered as part of genus *Nyctixalus* (Smith 1931). The discovery of *Ixalus pictus* (Peters 1871), *I. flavosignatus* (Boettger 1893), *Rhacophorus anodon* (van Kampen 1907), and *Hazelia spinosus* (Taylor 1920), also led to changes in both the nomenclature and the number of species within the genus

The *Nyctixalus*, which was initially transferred into the genus *Philautus*, also lacked vomer teeth (Smith 1931; Inger 1966). Similarities among *N. pictus*, *N. spinosus* and *N. margaritifer* include a warty dorsum, ossified scalp, and bony prominences along the frontoparietal region (Smith 1931; Inger 1966; Liem 1970). However, *N. margaritifer* can be easily distinguished from *N. pictus* and *N. spinosus* by toes half-webbed skin (Boettger 1893; Taylor 1920). In contrast, *N. margaritifer* has toes with a slight web at the base that extends as a narrow fringe along the sides (Boulenger 1882; van Kampen 1923) .

Their life cycles are similar, eggs hanging on a wooden wall filled with water (Taylor 1962). Subsequently, the genus was re-classified as *Hazelia* (Taylor 1962; Liem 1970) and *Edwardtayloria* (Marx 1975). The BMNH specimen 1885.12.31.35, a male adult obtained from Mount Willis (Java) by V. Huegel, was designated as a neotype of *N. margaritifer* (Boulenger 1882). These species were further re-classified as *Nyctixalus* (Dubois 1981; Segura-Delorme et al. 2005).

Recent molecular studies that utilized various genes, including 12S rRNA, tRNA val, and 16S rRNA mtDNA (Poyarkov et al. 2015; Sivongxay et al. 2016; Poyarkov et al. 2018; Chen et al. 2019), as well as cytochrome oxidase subunit I (COI), nuDNA brain-derived neurotrophic factor (BDNF), rhodopsin (RHO), seventh in absentia (SIA), and tyrosinase (TYR) (Chunskul et al. 2021) suggested that there may be significant genetic divergence within the *N. pictus* species.

The mitochondrial 16S rRNA gene has been widely utilized for DNA barcoding in amphibian research around the world. This approach is favoured due to the extensive reference database available for amphibians, which is significantly rich in taxa (Hebert et al. 2003; Vences et al. 2012) and has proven effective in identifying anuran species (Dubeux et al. 2022). The primary aim of this study was to conduct a comprehensive analysis of *Nyctixalus* samples collected from various locations throughout its distribution range. This involved employing DNA barcoding techniques using the 16S rRNA fragment, alongside morphometric analysis to better understand the systematic relationships of species within the genus, particularly in the context of Indonesia.

MATERIALS AND METHODS

Materials

This study examined 15 adult specimens of *N. margaritifer* (nine from West Java, six from Central Java) and 20 specimens *of N. pictus* (three from Aceh, four from North Sumatra, two from West Sumatra, one from Jambi, four from Bengkulu, one from West Kalimantan, three from East Kalimantan, and two

from North Kalimantan) housed in Museum Zoologicum Bogoriense (MZB), National Research and Innovation Agency (BRIN), and a specimen from the Mt. Wilis East Java stored in Universitas Negeri Malang (Priambodo et al. 2021). A total of 31 partial 16S mtDNA sequences were analysed, including sequences from *Nyctixalus*, *Rhacophorus reinwardtii*, *Chiromantis doriae*, *Theloderma licin*, *Theloderma horridum*, and *Theloderma pseudohorridum* (outgroup), as well as several populations of *N. margaritifer* (Table 1).

Methods

Morphological Analysis

Morphometric analysis included precise measurement of 24 characters using a dial calliper, calibrated to an accuracy of 0.01 mm (Matsui 1984; Matsui et al. 2013). The specific morphometric characters measured include: Snout-vent length (SVL): the distance from the tip of the snout to the vent. Tibia length (TL): the distance from the external margin of the knee joint to the external margin of the heel articulation. Tarsal length (TR): the distance from the external margin of the heel articulation to the proximal edge of the inner metatarsal tubercle. Femur foot length (F): The distance from the midpoint of the cloacal gap to the external margin of the knee joint, measured with the thighs and shins oriented perpendicular to the body axis First toe length (T1): the distance from the distal edge of the inner metatarsal tubercle to the tip of the first toe. Fourth toe length (T4): measured from the distal edge of the inner metatarsal tubercle to the tip of the fourth toe. Third toe length (T3): determined from the distal edge of the inner metatarsal tubercle to the tip of the third toe. Fourth toe disc diameter (TD4): the horizontal width of the fourth toe disk. Upper arm length (H) and forearm length (A): these measurements contribute to an understanding of limb proportions. Wrist width (Lt. m): this metric assesses the structural integrity of the limbs. Third finger length (F3): measured from the distal edge of the palmar tubercle to the tip of the third finger. Third finger disc diameter (F3D): the diameter representing the horizontal width of the third finger disk. Second finger length (F2): measured from the distal edge of the palmar tubercle to the tip of the second finger. Head length (HL): the distance from the tip of the head to the posterior margin of the tympanum. Head width (HW): the widest distance across the head, measured at the commissure of the jaws. Additionally, distances were measured for posterior eyes distance (EPD), inter-orbital distance (IOD), anterior eyes distance (EAD), inter-nostril distance (IND), eye-nostril distance (EN), eye diameter (EY), and horizontal tympanum diameter (TD) (Figure 1).

To compare the variations among the 24 characters, ratio values of morphometric datasets were calculated by dividing each measurement by the standard length (SVL) to minimize the body size influence (Munir et al. 2018). The Mann-Whitney U test assessed variance between species for each character, while principal components analysis (PCA) examined potential group structures. Characters were analysed using the Bartlett test for homogeneity of variance, with significance set at $p < 0.05$. Predictor variables from PCA were retained based on the Kaiser-Meyer-Olkin measure (>0.5) and eigenvalues (>1) (Kaiser 1960; Gabriel 1971). Mean ± standard deviation (SD) was calculated for 24 characters of each species, with statistical significance set at $P < 0.05$. All analyses were conducted using R software, ensuring precision and reliability in our results (R Core Team 2019).

Molecular work

Mitochondrial 16S rRNA gene sequences were obtained from five adult *N. margaritifer* specimens collected from Mt. Salak (West Java), Mt. Slamet (Central Java), and Mt. Wilis (East Java), as detailed in Table 1. DNA extraction, amplification, and sequencing were conducted following the procedures

Table 1. Samples mitochondrial 16S rRNA gene from *Nyctixalus* and outgroup species analysed along with voucher information, collection location, and GenBank accession number.

No.	Species	Specimen vouchers	Country	Accession No.	Source
In group					
1.	*N. pictus*	FMNH231094	Malaysia: Borneo, Sabah (Lahad Datu)	GQ204726	Meegaskumbura et al. 2011
2.	*N. pictus*			AF458135	Wilkinson et al. 2002
3.	*N. pictus*	FMNH 231095	Malaysia: Borneo, Sabah (Lahad Datu)	DQ283133	Frost et al. 2006
4.	*N. pictus*			AF268255	Wilkinson et al. 2002
5.	*N. pictus*	KUHE53517	Malaysia: Borneo, Sarawak, Bario	LC012863	Nguyen et al. 2015
6.	*N. pictus*	NMBE1056413	Malaysia: Borneo, Sarawak, Batang Ai_National Park	JN377342	Das & Haas 2010
7.	*N. pictus*	MVZ239460	Indonesia: Sumatra, Bengkulu	GQ204732	Meegaskumbura et al. 2011
8.	*N. pictus*			KU561880	Dever 2017
9.	*N. pictus*	AH07001	Malaysia: Borneo, Sarawak, Mt. Mulu National Park	GU154888	Das & Haas 2010
10.	*N. pictus*	ZFMK 61922	Indonesia: Borneo, West Kalimantan, Kapuas Hulu, Rantau Prapat	AF215349	Vences 1999
11.	*N. pictus*	ZCAK SEA0005	Indonesia	LC640580	Kambayashi et al. 2022
12.	*N. pictus*	MNHN 1999.7718	Thaïland: Phang-Nga Province, Takva Thung District Raman Forest Park	AY880502	Segura-Delorme & Dubois 2004
13.	*N. pictus*	CAS247868	Myanmar: Tanintharyi	KU244380	Dever 2017
14.	*N. spinosus*	ACD 1043	Philippines: Mindanao	DQ283114	Frost et al. 2006
15.	*N. spinosus*	Nsp1	Philippines: Mindanao	KT461916	Poyarkov et al. 2015
16.	*N. spinosus*	ACA 940	Philippines: Mindanao	AF458136	Wilkinson et al. 2002
17.	*N.margaritier*	MZB.Amph.33355	Indonesia: West Java, Mt. Salak, Sukamantri Village, Taman Sari, Kabupaten Bogor	PP987095	This study
18.	*N. margaritier*	MZB.Amph.33356	Indonesia: West Java, Mt. Salak, Sukamantri, Taman Sari, Kabupaten Bogor	PP987094	This study
19.	*N. margaritier*	MZB.Amph. 33353	Indonesia: Central Java, Mt. Slamet, Kabupaten Banjarnegara District	PP986996	This study
20.	*N. margaritier*	MZB.Amph. 33354	Indonesia: Central Java, Mt. Slamet, Kabupaten Banjarnegara	PP987054	This study
21.	*N. margaritier*	KUHE:26135	Indonesia: Central, Java, Mt. Ungaran	LC012864	Nguyen et al. 2015
22.	*N. margaritier*	BP UM11	Indonesia: East Java, Mt. Wilis, Sigogor Pupus Village, Ngebel Subdistrict, Ponorogo District	PP987108	Priambodo et al. 2021
23	*N. margaritier*	NK2114	Indonesia: East Java, BTSNP, Senduro, Mt. Semeru	OQ555086.1	Hanifa et al. 2023
24	*N. margaritier*	NK2122	Indonesia: East Java, BTSNP, Senduro, Mt. Semeru	OQ555087.1	Hanifa et al. 2023
25.	*Nyctixalus sp.*	CAS247492	Myanmar	KU561882	Dever 2017

Table 1. Contd.

No.	Species	Specimen vouchers	Country	Accession No.	Source
26.	*Nyctixalus sp.*	CAS247476	Myanmar	KU561881	Dever 2017
Out group					
27.	*C. doriae*	FMNH 255213	Laos: Huaphahn Province, Vieng Tong District, Phou Louey National Biodiversity Conservation Area, nr. Nam Puong River	DQ28315	Frost et al. 2006
28.	*R. reinwardtii*	US 16179	Indonesia: Patuha, Bandung, West Java	KX398930.1	O'Connell et al. 2016
29.	*T. licin*	KUHE 19426	Thailand: Nakon Sri Tamarat	LC012859	Nguyen et al. 2015
30.	*T. horridum*	KUHE 52582	Malaysia: Negeri Sembilan, Kenaboi	LC012861	Nguyen et al. 2015
31	*T. pseudohorridum*	MZB:Amph 23883	Indonesia: Central Java, Nusa Kambangan Island	ON571716.1	Kurniawan et al. 2023

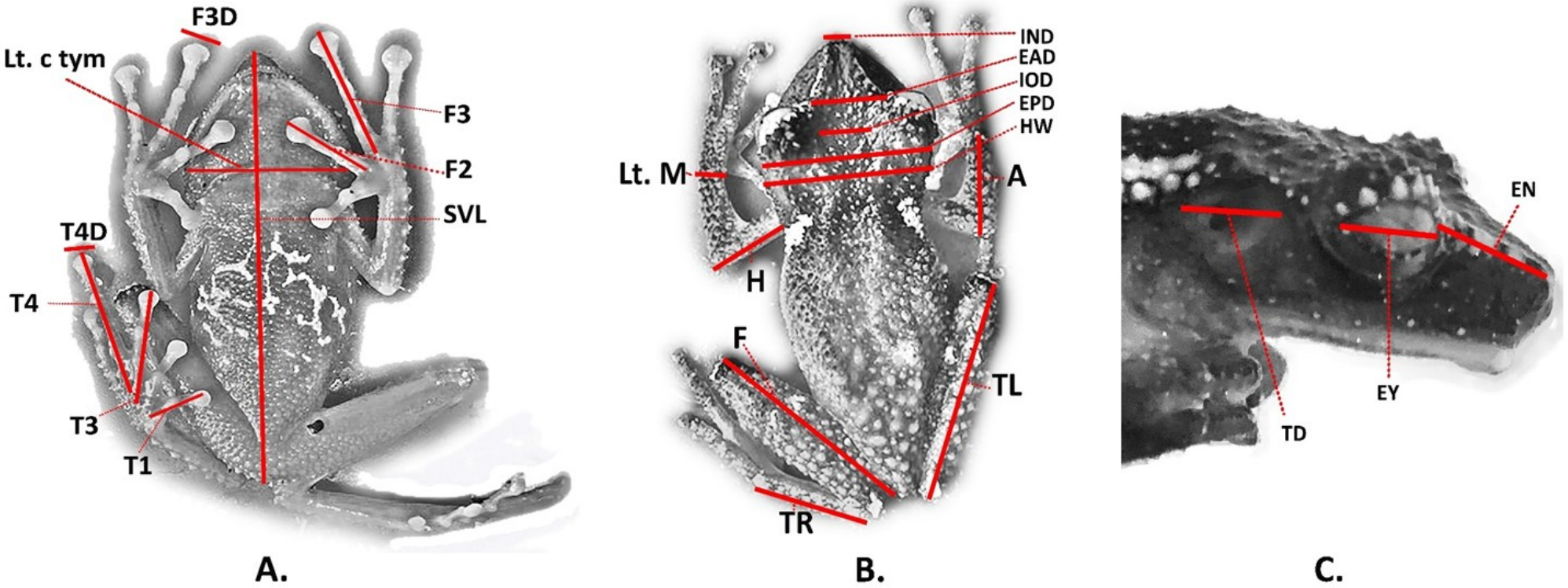

Figure 1. An illustration showing physical location for the 24 morphometric measurements (A) ventral, (B) dorsal, (C) lateral view. See the main text for the definitions of these characters.

outlined by Sambrook et al. (1989), Matsui et al. (2010), Hamidy et al. (2012), and Munir et al. (2018).

Total DNA was extracted from small amounts of tissue diluted in a buffer solution using the standard phenol-chloroform method. DNA amplifications of the 16S rRNA gene were carried out in polymerase chain reaction (PCR) using the primer set 16L-1 (CTGACCGTGCAAAGGTAGCGTAATCACT) and primer 16H-1 (CTCCGGTCTGAACTCAGATCACGTAGG) (Hedges 1994) to obtain a round 540 bp-length fragment of the gene. The PCR reactions were performed in 30 µL total volumes comprised of 1 µL DNA template, 3 µL PCR buffer (NH_2SO_4), 0.6 µL dNTP, 4 µL $MgCL_2$, 1 µL of each primer, 0.5 µL Bovine serum albumin (BSA), 0.2 µL *Taq* DNA polymerase with appropriate buffer and ddH_2O to volume.

The PCR process began with an initial denaturation phase at 94 °C for 5 minutes. This was followed by 33 amplification cycles, each consisting of three key phases: denaturation at 94 °C for 30 seconds, annealing the primer for 30 seconds at temperatures between 48 °C and 50 °C, and extension at 72 °C for 1 minute and 30 seconds. For DNA sequencing, BigDye® Terminator reagents from 1st Base Asia were used to sequence the PCR products in both directions with the same primers to ensure the accuracy. Sequence chromatograms were checked, edited, and assembled using ChromasPro software (Technelysium Pty Ltd., Tewantin, Queensland, Australia).

MAFFT was used for sequence alignment with E-INS-I parameters (Katoh et al. 2002). MEGA X performed the Neighbor Joining (NJ) analysis and calculated the uncorrected p-distance (Kumar et al. 2018). The NJ tree was generated with 1,000 replications using the two-parameter Kimura model, applying pairwise deletion for gaps (Kimura 1980). A genetic distance of ≥ 3 % was set for species differentiation (Fouquet et al. 2007). The Maximum Likelihood (ML) analysis was performed using the IQ-Tree Web Server, incorporating ultrafast bootstrap replicates (Trifinopoulos et al. 2016). The general time-reversible (GTR) model, featuring empirical base frequencies (+F) and four discrete gamma shape rate categories (+G4), was selected as the model of evolution that is based on the Bayesian Information Criterion (BIC).

A Bayesian Inference (BI) analysis was conducted using MrBayes version 3.1.2 (Huelsenbeck & Ronquist 2001; Ronquist et al. 2012). This analysis employed a Monte Carlo Markov Chain (MCMC) approach over 20 million generations, with sampling occurring every 1,000 generations. The GTR+G model was chosen based on the Akaike Information Criterion (AIC). Bootstrap values of 70 % or higher were considered significant for confirming the nodes (Huelsenbeck & Hillis 1993; Leaché & Reeder 2002; Minh et al. 2013). The optimal models for both maximum likelihood (ML) and Bayesian inference (BI) analyses were determined using jModelTest, ensuring reliable and accurate results (Posada 2008; Darriba et al. 2014). The resulting trees were visualized using FigTree. v1.4.3 (Rambaut 2016).

RESULTS AND DISCUSSION

The morphometric analysis results revealed no significant difference in morphological character between *N. pictus* specimens from Sumatra and Kalimantan ($P > 0.05$). A comparison of morphology character ratios between *N. margaritifer* and *N. pictus* from Sumatra, Indonesia, showed no significant differences ($P > 0.05$) in the characters T1, T4, T3, H, A, Lt. c. tym, EN, EY, TD, and HW. Similarly, the comparison morphology character ratios with N. pictus from Kalimantan presented no significant variances among characters T4, T3, H, F3D, A, EPD, IOD, IND, EN, EY, TD, and HW (Table 2).

Morphometric analysis indicated no significant differences in morphological characters between *N. pictus* specimens from Sumatra and Kalimantan ($P > 0.05$). A comparative analysis of morphological ratios between *N. margaritifer* and *N. pictus* from Sumatra revealed no significant differences ($P > 0.05$) in the characters T1, T4, T3, H, A, Lt. c. tym, EN, EY, TD, and HW. Furthermore, no significant differences were observed in the following morphological measurements among N. pictus specimens from Kalimantan: T4, T3, H, F3D, A, EPD, IOD, IND, EN, EY, TD, and HW as seen in Table 2.

The PCA analysis used characters based on the Kaiser-Meyer-Olkin (KMO) test results. The overall KMO value was 0.73, derived from the morphological characters SVL, TL, T1, TD4, left wrist (lt. M-wrist), F3, and F2. The Bartlett test results of the ratio of morphological characters showed that variances were equal across groups (homogeneous) as indicated by $P = 0.000$ ($P < 0.05$). PCA analysis used PCI and PC2 components with eigenvalues > 1, as shown in Table 3. The results showed a PC I of 53.41 %, which had positive contribution values for SVL, F3, and F2. Meanwhile, PC II of 15.32 % had positive contribution values for TL, T1, TD4, and Lt. M. These two principal components in Table 3 explained 68.73 % of the morphometric variation.

Furthermore, the PCA analysis showed that the *N. margaritifer* populations were separated from *N. pictus* from Kalimantan, while there was a slight overlap between the populations of both species from Sumatra. However, the *N. pictus* population from the two regions did not show a clear separation (Figure 2).

The alignment in this study consisted of 31 sequences (Table 1) with targeted gene fragment of ~542 bp. The phylogeny tree reconstruction using NJ, ML, and BI methods revealed no significant differences in tree topology (Figure 3, S1, S2), with consistent genetic distances and bootstrap values observed among the tree branches between populations.

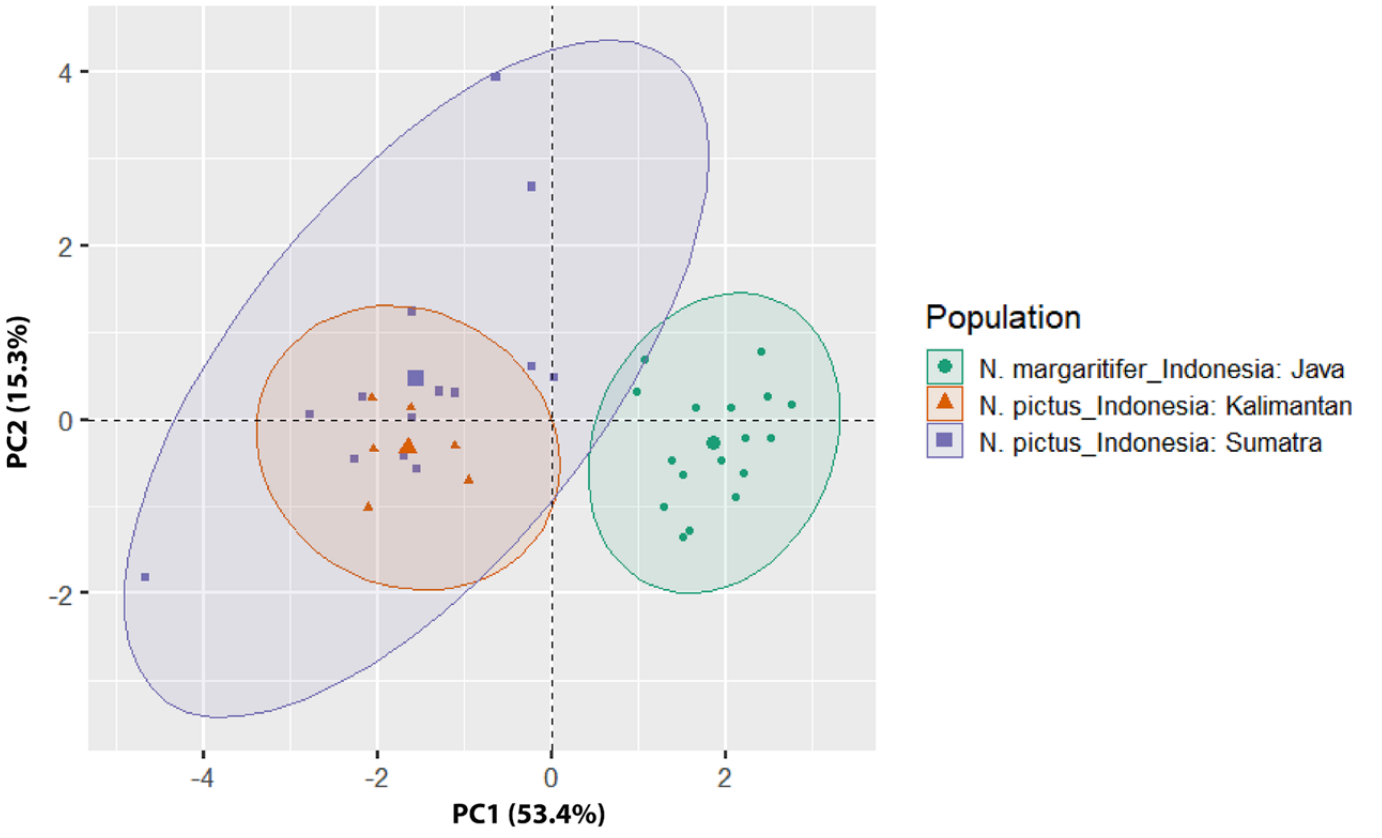

Figure 2. Principal Component Analysis plot on seven size-corrected morphological characters *Nyctixalus* from Indonesia.

Table 2. Measurements morphological character male *N. margaritifer* and *N. pictus*. SVL (Mean ± SD, in mm). *$P < 0.05$ See text for character abbreviations.

Body part	Measurement			U test		
	*N. margaritifer*_Indonesia: Java (Nm_J) n = 17	*N. pictus*_Indonesia: Sumatra (Np_S) n = 14	*N. pictus*_Indonesia: Kalimantan (Np_K) n = 6	Nm_J vs Np_S	Nm_J vs Np_K	Np_S vs Np_K
SVL	**42.03 ± 2.03**	**31 ± 2.02**	**31.87 ± 2.09**	**0.000***	**0.000***	**0.621**
TL	22.02 ± 0.92	17.82 ± 1.01	18.53 ± 1.52	0.000*	0.001*	0.409
TR	12.54 ± 1.16	9.92 ± 0.84	10.22 ± 0.56	0.010*	0.021*	0.680
F	19.05 ± 1.51	15.55 ± 1.25	16.33 ± 1.3	0.000*	0.002*	0.284
T1	4.08 ± 0.26	2.9 ± 0.67	2.79 ± 0.14	0.234	0.006*	0.409
T4	12.66 ± 1.01	9.1 ± 0.94	9.59 ± 0.59	0.405	0.944	0.621
T3	9.3 ± 1.02	6.81 ± 0.57	7.05 ± 0.52	0.937	0.889	0.564
TD4	1.56 ± 0.19	0.96 ± 0.16	0.97 ± 0.17	0.004*	0.006*	0.741
H	8.24 ± 0.51	5.64 ± 0.82	5.96 ± 1.04	0.062	0.107	0.741
A	10.28 ± 0.89	7.42 ± 0.73	7.78 ± 0.99	0.190	0.674	0.621
lt. M	2.19 ± 0.27	1.32 ± 0.29	1.17 ± 0.17	0.002*	0.001*	0.099
F3	11.15 ± 0.73	6.89 ± 0.78	7.39 ± 0.78	0.000*	0.002*	0.284
F3D	2.2 ± 0.44	1.31 ± 0.25	1.7 ± 1.15	0.004*	0.123	0.934
F2	6.88 ± 0.64	4.37 ± 0.69	4.66 ± 0.81	0.000*	0.010*	0.869
HL	14.17 ± 1.43	8.1 ± 0.52	8.52 ± 0.92	0.000*	0.002*	0.680
Lt.c. tym	12.3 ± 0.89	9.41 ± 0.91	10.12 ± 0.66	0.062	0.003*	0.117
EPD	13.04 ± 0.89	9.21 ± 0.53	9.67 ± 0.8	0.015*	0.363	0.138
IOD	6.26 ± 0.49	4.4 ± 0.43	4.4 ± 0.26	0.017*	0.093	0.458
EAD	7.21 ± 0.47	5.62 ± 0.51	6.02 ± 0.5	0.012*	0.008*	0.409
IND	2.34 ± 0.39	1.52 ± 0.23	1.58 ± 0.22	0.017*	0.161	0.805
EN	5.06 ± 0.55	3.82 ± 0.4	4.14 ± 0.35	0.302	0.107	0.187
EY	4.81 ± 0.41	3.5 ± 0.34	3.32 ± 0.37	0.874	0.141	0.138
TD	3.04 ± 0.68	2.51 ± 0.27	2.48 ± 0.24	0.112	0.441	0.409
HW	14.74 ± 0.6	11.38 ± 1.25	11.64 ± 1.1	0.074	0.123	0.458

Table 3. Variabel PC1 and PC2 morphological character male *N. margaritifer* and *N. pictus*. Bold figures indicate the highest loadings.

Variable	PC1	PC2
Snout-vent length (SVL)	**0.4647223**	-0.32533093
Tibia length (TL)	-0.4100977	**0.30999856**
First toe length (T1)	0.2565834	**0.69352190**
Fourth toe disc diameter (TD4)	0.3160115	**0.32218328**
Wrist width (Lt. M)	0.3574805	**0.36893181**
Third finger length (F3)	**0.4258582**	-0.27430144
Second finger length (F2)	**0.3755093**	-0.04397765
Eingen value	1.9335	1.0357
% of varians	53.41	15.32
Cumulative varians (%)	53.41	68.73

The phylogeny tree revealed a significant discovery *Nyctixalus* (clade *Nyctixalus* genus) is in a monophyletic position against the outgroup. This finding, a cornerstone of our research, underscores the unique evolutionary path of the *Nyctixalus* genus. In the clade, genus *Nyctixalus* formed a monophyletic group divided into two subclades (NJBS = 88 %, UFB = 94 %, and BPP = 1.00). The subclade I consisted of *N. margaritifer* and *N. pictus* groups (NJBP = 90 %, UFB = 83 %, BPP = 0.96), while subclade II consisted of *N. spinosus from the* Philippines (NJBP = 100 %, UFB = 98 %, BPP = 1.00).

The population of *N. pictus group exhibited six distinct lineages* (NJBS = 90 %, UFB = 83 %, BPP = 0.96). The six lineages in *N. pictus* included *N. pictus* from Malaysia, specifically Sabah, Lahad Datu, Sarawak, Bario (Lineage 1, NJBS = 100 %, UFB = 97 %, BPP = 1.00), Mt. Mulu (Lineage 2, NJBS = 84 %, UFB = 87 %, BPP = 0.99), and Sarawak, Batang Ai, also recognized as true *pictus* (Lineage 6), Indonesia from Kapuas Hulu, West Kalimantan (Lineage 3, NJBS = 99 %, UFB = 94 %, BPP = 1.00) and Bengkulu, Sumatra (Lineage 4, NJBS = 100 %, UFB = 99 %, BPP = 1.00), Myanmar and Thailand (Lineage 5, NJBS = 99 %, UFB = 99 %, BPP = 1.00), and lineage 6 from Sarawak, Batang Ai, also known as true pictus.

In our results, *N. margaritifer* from Java demonstrated low genetic distances of only 0–1.5 % (Table 4) consistent with morphology characteristics. Our study results confirmed the aforementioned by Priambodo et al. (2021) and Hanifa et al. (2023). Furthermore, the rediscovery of *N. margaritifer* in Mt. Willis, East Java (Priambodo et al. 2021) also supported the identification of a topotype of *N. margaritifer* collected in Mount Willis (Java) by V. Huegel (Dubois 1981).

The genetic distances among *N. pictus* populations were substantial (*N. pictus* lineage 1–6, p-distance = 4.3–9.9 %; Table 4), strongly supporting their distinct species status. This finding is consistent with the established understanding that uncorrected p-distances exceeding 3 % between taxa often indicate distinct species (Fouquet et al. 2007). The diverse genetic distances observed among various lineages in the analyses can help clarify their distinct species relationships (Hamidy et al. 2011).

However, in the two allopatric populations *N. pictus* lineage 3 (Kalimantan) and 4 (Sumatra) (p-distance = 4.3–4.6 %), there are high variations (8.26–9.01 %) between population *N. pictus* from Sumatra and Malaysia (Table 4). It can be assumed that *N. anodon* van Kampen, 1907 from Kayu Tanam, Sumatra Barat, Indonesia, is a different species to *N. pictus* Peters, 1871 (type locality) from Sarawak, Malaysia, which has been recognized as a synonym of *N. pictus* (Smith 1931; Inger 1966).

The ancestral genus *Nyctixalus* is believed to have a significant connec-

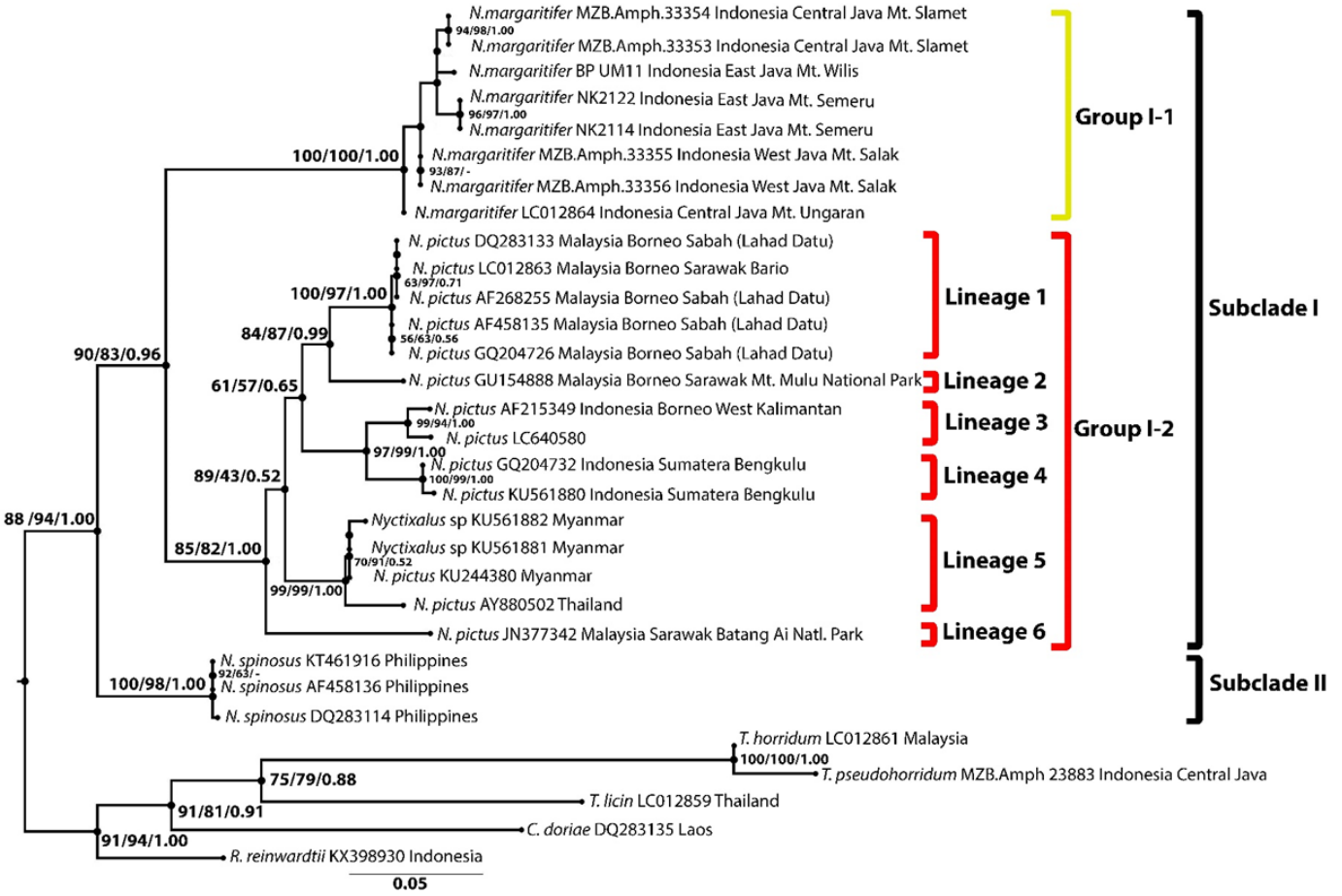

Figure 3. Maximum Likelihood phylogram showing the relationship within genus *Nyctixalus*. Values at the branches point to Neighbour Joining probability (NJBP), Ultrafast Bootstrap Support (UFB), and Bayesian Posterior Probabilities (BPP).

tion to the formation of the original Borneo landmass, which was separated from the larger Sundaland area. This geological history is mirrored in another Bornean frog genus, Leptobrachium. In this case, the divergence between the Bornean and Philippine Clades and the other related clades from Sundaland and Asia is likely occurred during a critical period spanning the late Eocene, approximately 42 to 35 million years ago (MYBP), extending into the early Oligocene, roughly 35 to 29 million years ago (MYBP). Subsequently, the pattern is similar when the ancestor of *Leptobrachium lumadorum* from Mindanao split from entire Bornean species in early Miocene, 23– 14 MYBP (Matsui et al. 2010), and this event is likely responsible for splitting event of *N. spinosus* from the entire ancestor of *N. pictus* and *N. margaritifer*. The evolutionary history of *Nyctixalus* species provides crucial insights into the region's biodiversity and biogeography.

The ancestor *N. margaritifer* split from the common ancestor of *N. pictus* is earlier than diversification within lineages of *N. pictus*. The ancestor of *N. pictus* lineage 4 (Sumatra) split from *N. pictus* lineage 3 (Borneo) probably occurred during the Pleistocene glacial periods when all Sunda land islands were connected as one landmass (Inger & Voris 2001; Hall 2013). The high genetic variation in the *N. pictus* in Southeast Asia, especially Borneo Island, involves multiple events. The long-term continental connection between Borneo and the Malay Peninsula occurred episodically twice: once in the mid-Miocene and again in the early Pliocene (Voris 2000; Inger & Voris 2001; Inger 2005; Hall 2013). The event might have isolated the ancestor of *N. pictus* in Borneo from a common ancestor of *N. pictus* in Sumatra and Peninsular (Myanmar and Thailand).

The regions of Sumatra (Riyanto & Kurniati 2014; Hamidy & Kurniati

Table 4. Average uncorrected p–distances (%) based on 542 bp fragment of the mitochondrial 16S rRNA gene within genus *Nyctixalus*.

No	Species	1	2	3	4	5	6	7	8	9	10	11	12
1.	*N. pictus* lineage 1 (Malaysia: Borneo, Sabah (Lahad Datu) and Sarawak, Bario)	0 -0.19											
2.	*N. pictus* lineage 2 (Malaysia: Borneo, Sarawak, Mt. Mulu)	4.55-4.88											
3.	*N. pictus* lineage 3 (Indonesia: Borneo, West Kalimantan)	6.46 -7.33	6.25-7.13	1.55									
4.	*N. pictus* lineage 4 (Indonesia: Sumatra, Bengkulu)	6.94-7.64	7.56-7.84	4.27-4.63	0.41								
5.	*N. pictus* lineage 5 (Myanmar and Thailand)	4.83-7.64	5.93-7.65	7.21-10.59	6.59-9.41	0.57-2.95							
6.	*N. pictus* lineage 6 (Malaysia: Borneo, Sarawak, Batang Ai)	8.83-9.29	9.48	9.42-9.62	8.26-9.01	7.4-9.88							
7.	*N. margaritifer* (Indonesia: Java)	11.29-12.99	13.8-14.91	13.24-14.72	12.94-14.32	10.62-14.24	11.65-12.45	0 – 1.52					
8.	*N. spinosus* (Philippines)	11.3 – 12.13	13.91-14.17	12.09-12.97	12.03-13.22	10.77-13.53	10.78-11.08	11.39-13.83	0-0.2				
9.	*R. reindwardtii*	13.95-14.56	15.7	16.62-16.69	15.19-16.28	12.84-16.01	14.55	15.58-17.9	11.8-12.41				
10.	*C. doriae*	20.05-21.7	19.99	20.13-20.29	19.39-20.4	17.4-20.72	18.03	19.02-20.52	15.91-16.22	14.02			
11.	*T. licin*	15.57-16.58	17.85	17.5-17.45	18.09-18.5	15.39-17.56	20.12	18.61-20.37	17.11-17.69	15.48	19.61		
12.	*T. horridum*	20.43-21.68	24.34	24.05-24.77	23.56-24.46	19.03-22.86	23.56	20.5-22.2	18.37-19.16	18.77	20.5	17.06	
13	*T. pseudohorridum*	21.79-22.57	25.17	25.72-25.75	23.51-24.41	20.57-23.13	23.26	21.07-23.08	18.36-23.26	19.05	21.31	18.56	2.84

2015; Munir et al. 2018) and Borneo (Matsui 2015) remained unexplored and faced threats from anthropogenic activities (Eprilurahman et al. 2021). Therefore, further investigations must be conducted to evaluate the taxonomic status of selected *N. pictus* populations. These investigations should include studying the call and morphological characteristics to identify any new lineage *of the N. pictus* group. Conservation efforts are also recommended to preserve the species from extinction.

CONCLUSIONS

This study showed that *N. margaritifer* population in Java, Indonesia, had no morphological differences and genetic distance. The morphometric analysis showed minimal differences between *N. margaritifer* and *N. pictus* from Sumatra and Kalimantan in body ratios and measurements, including toe lengths (T3 and T4), upper arm length (H), forearm length (A), eye-nostril distance (EN), eye diameter (EY), tympanum diameter (TD), and head width (HW). Given the high uncorrected p-distances above 3 % between the *N. pictus* population in Sumatra and those in Malaysia, it can be assumed that *N. anodon* from Kayu Tanam, Sumatra Barat, Indonesia is a different species to *N. pictus* (type locality) from Sarawak, Malaysia. Therefore, additional research is necessary to determine the taxonomic status *N. anodon* from Kayu Tanam Sumatra and new lineages of *N. pictus* in Indonesia.

AUTHOR CONTRIBUTION

A.H., and R.I., designed the research. R.I. collected, analysed data, and wrote the manuscript. Speciment Mt. Wilis, East Java, Indonesia collected by A.M.K., B.P. Manuscript supervised by A.H., A.F., D.P-F.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest in the preparation of this research article.

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APPENDICES

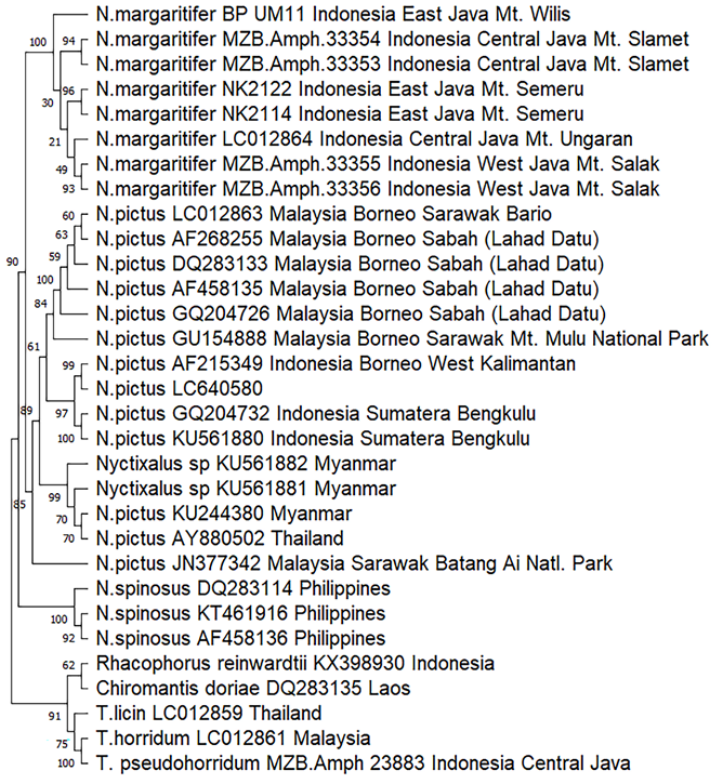

Figure S1. The neighbour-*joining* phylogram based on the studied 542 bp of the mitochondrial 16S rRNA gene. The value at the branch represents bootstrap support.

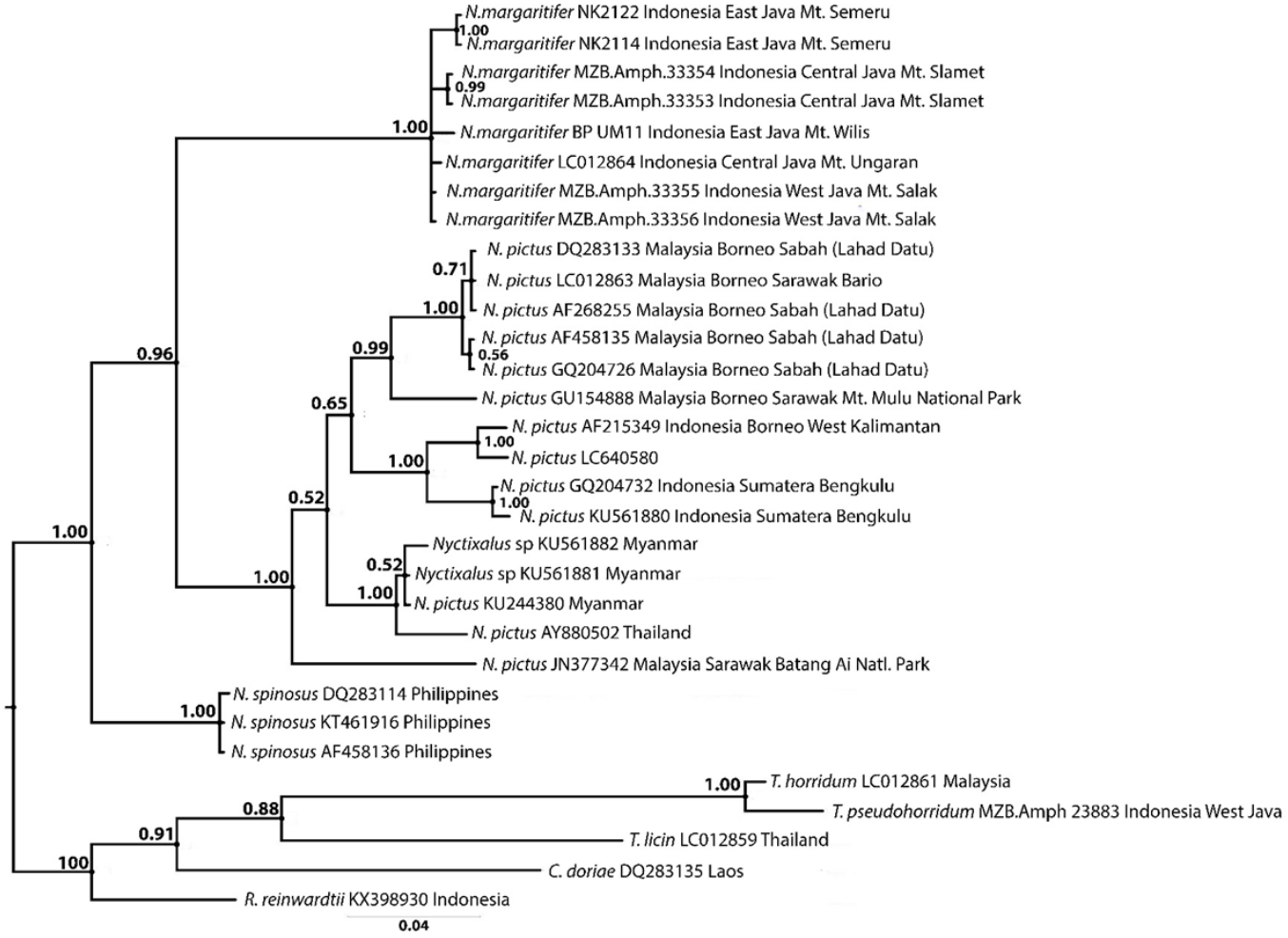

Figure S2. Bayesian consensus phylogram based on the studied 542 bp fragment of the mitochondrial 16S rRNA gene. Values at the branches indicate Bayesian Posterior Probabilities (BPP).