

## A NEW SITE RECORD OF STRIPED ASIAN TREEFROG (AMPHIBIA: RHACOPHORIDAE: *CHIRIXALUS TRILAKSONOI*) FROM SUMATRA, WITH REEVALUATION OF ITS CONSERVATION STATUS

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Submitted: November 8, 2023; Accepted: December 28, 2023; Published: December 31, 2023

### ABSTRACT

Two of the six *Chirixalus* species currently known are distributed in Indonesia. Both species are only known to be distributed in Java, and they are *Chirixalus trilaksonoi* and *Chirixalus pantaiselatan*. During two field expeditions conducted in 2022 in Palembang, South Sumatra, we encountered several individuals exhibiting morphological similarities to *C. trilaksonoi*. Subsequent analysis, encompassing molecular, morphological, and bioacoustic, was undertaken to ascertain the identity of these individuals. The analyses outcomes unequivocally confirm that the specimens from Palembang are indeed *C. trilaksonoi*. Nevertheless, slight variations were observed between the Javanese and Sumatran populations of *C. trilaksonoi*. These populations exhibited a low genetic distance of 0.6% in mitochondrial DNA and displayed three polymorphic sites. Furthermore, there are slight differences in various morphological characteristics, and in advertising calls. The IUCN red-list status of this species was also evaluated, and based on the available evidence, we propose *C. trilaksonoi* as Vulnerable according to criteria B1ab(iii). This study stresses the importance of comprehensive taxon studies, especially in neighboring island, and in one biogeographical region.

**Key words:** Bioacoustic, Conservation, Genetic diversity, Phylogenetic, Red-list status

### INTRODUCTION

Striped Asian Treefrog, genus *Chirixalus* Boulenger, 1893 currently comprises six nominal species distributed in northeastern India, Bhutan, Nepal, Bangladesh, Myanmar, Thailand, Vietnam, Cambodia, Laos, Indonesia, and China (Frost, 2023). In Indonesia, the genus is represented by two species: *Chirixalus pantaiselatan* Munir, Hamidy, Kusrini et al., 2021, and *Chirixalus trilaksonoi* (Riyanto & Kurniati, 2014). Both species are distributed in Java.

*Chirixalus trilaksonoi* is only found in two disjunct locations in the western part of Java (i) Bogor (based on five specimens), West Java Province, and (ii) Ujung Kulon National Park Banten Province, located in westernmost Java Island (Munir et al., 2021). There are no other records apart from those two places, hence it has been listed as an Endangered species (IUCN SSC Amphibian Specialist Group, 2018).

During two field trips to South Sumatra in March and June 2022, we encountered six individuals of *Chirixalus* frogs on the leaves of the palm tree (Arecaceae), in a suburb of Palembang City. From observations and examination of the live and subsequently preserved

specimen, the frog is indeed *C. trilaksonoi*. We use molecular analysis to analyze the phylogenetic, genetic divergence, and molecular variances of the Sumatran population (Palembang, South Sumatra) compared to the Javan population (Bogor, West Java). We also provide additional morphometric data and bioacoustic comparison based on the new population discovered from Sumatra, and re-evaluate the IUCN red list assessment of *C. trilaksonoi*.

## MATERIALS AND METHODS

### *Study sample*

We conducted fieldwork on 1<sup>st</sup> March, and 22<sup>nd</sup> June 2022 at Bukit Baru, Ilir Barat I District, Palembang, South Sumatra. A total of six individuals of *Chirixalus* frogs were collected during the fieldwork and a photo of a live male was taken (Fig. 1). The frogs were euthanized, fixed in 10% formalin, preserved in 70% ethanol, and stored in Museum Zoologicum Bogoriense (MZB), National Research and Innovation Agency. Liver tissues were sampled and preserved in 95% ethanol before fixation, and then tissue samples were stored in the freezer.



**Figure 1.** A live male of *C. trilaksonoi* (not collected) from Suburb of Palembang, South Sumatra. Photographed by Awal Riyanto.

### *Molecular sampling*

A total of 19 partial sequences of the 16S rRNA mitochondrial DNA gene were used for genetic analysis. We used four species of the genus *Chirixalus*, three species of the genus *Chiromantis* as ingroups, and one species of the genus *Polypedates* as an outgroup (Table 1).

### *Preparation of DNA, PCR and DNA sequencing*

We obtained tissues preserved in 95% ethanol. We used Qiagen extraction kit to extract the DNA from the small amount of tissue. The method used for extraction follows extraction kit procedures. Amplifications were done by Polymerase Chain Reaction (PCR). Amplifications of the 16S rRNA gene were performed using the forward primer 16 L2240 5'-AAAGTGGGCCTAAAAGCAGCCA-3' (Matsui et al., 2016) and the reverse primer 16H1

5'-CTCCGGTCTGAACTCAGATCACGTAGG-3' (Hedges, 1994). We followed Matsui et al. (2016) for PCR procedures. Successful PCR products were sent to 1<sup>st</sup> Base Asia (Singapore) for direct sequencing.

**Table 1.** Samples of four species of *Chirixalus*, including *C. trilaksonoi* from Sumatra and outgroups generated for 16S mtDNA analysis. Abbreviations for museum voucher specimens: FMNH = Field Museum of Natural History, KIZ = Kunming Institute of Zoology, KUHE = Kyoto University, Human and Environmental Studies, MVZ = Museum of Vertebrate Zoology, MZB = Museum Zoologicum Bogoriense, SN = Shunqing Lu Field Number Series, USNM = National Museum of Natural History, ZMKU = Kiev Zoological Museum

Species	Location	GenBank	Voucher	Reference
<i>Chirixalus doriae</i>	Thailand	KC357618	ZMKU Am 00604	Aowphol et al., 2013
<i>Chirixalus doriae</i>	China	GQ285682	KIZ 005 Rao	Li et al., 2009
<i>Chirixalus doriae</i>	China	EU215527	SN 030051	Li et al., 2008
<i>Chirixalus doriae</i>	Laos	DQ283135	FMNH 255213	Frost et al., 2006
<i>Chirixalus doriae</i>	Thailand	AB813159	KUHE:19301	Matsui et al., 2014
<i>Chirixalus nongkhorensis</i>	Viet Nam	KC465788	KIZ 1056	Li et al., 2013
<i>Chirixalus nongkhorensis</i>	Thailand	KC357610	ZMKU Am 00624	Aowphol et al., 2013
<i>Chirixalus nongkhorensis</i>	Thailand	KC357604	ZMKU Am 00620	Aowphol et al., 2013
<i>Chirixalus nongkhorensis</i>	Thailand	AB813158	KUHE:19498	Matsui et al., 2014
<i>Chirixalus pantaiselatan</i>	Java	MT155988	MZB Amph 30551	Munir et al., 2021
<i>Chirixalus pantaiselatan</i>	Java	MT155987	MZB Amph 30547	Munir et al., 2021
<i>Chirixalus pantaiselatan</i>	Java	MT155986	MZB Amph 30548	Munir et al., 2021
<i>Chirixalus trilaksonoi</i>	Java	MT155989	MZB Amph 27237	Munir et al., 2021
<i>Chirixalus trilaksonoi</i>	Sumatra	PP051251	MZBAmph32737	This study
<i>Chirixalus trilaksonoi</i>	Sumatra	PP051252	MZBAmph32738	This study
<i>Chiromantis petersii</i>	Africa	GQ204733	MVZ 234168	Meegaskumbura et al., 2010
<i>Chiromantis rufescens</i>	Africa	KY080107	USNM: Herp:578114	Deichmann et al., 2017
<i>Chiromantis xerampelina</i>	Africa	GQ204734	MVZ 234606	Meegaskumbura et al., 2010
<i>Polypedates leucomystax</i>	Java	AB728137	MZB: Amp12864	Kuraishi et al., 2013

### *Phylogenetics and genetic diversity analyses*

Fragments of the 16S rRNA genes (469 bp) were obtained for molecular analyses. DNA sequences obtained in this study were checked and edited using the ChromasPro software (Technelysium Pty Ltd., Tewntin, Australia) and aligned with published data from GenBank using Clustal W in MEGA XI (Tamura et al., 2021). The mitochondrial genealogies were reconstructed using Maximum Likelihood (ML), and Bayesian Inference (BI) analyses. A general time-reversible (GTR) with gamma shape parameter (G) was selected as the best-fitting model for both analyses using the Akaike criterion as implemented in the software Kakusan 4 (Tanabe, 2007).

We performed ML analysis using raxmlGUI 2.0 (Edler et al. 2021) with 1,000 bootstrap replicates. BI analysis was estimated using MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) for 10 million generations with parameters and topology sampling every 1,000 generations, Markov chain Monte Carlo (MCMC) diagnosis frequency of 100,000. We discarded 25% of the first analysis as burn-in. Nodal support characterized by Bootstrap values (BS)  $\geq 70$  in Maximum Likelihood (ML) tree analysis, as established by Hillis & Bull (1993), along with Posterior

Probability values (PP)  $\geq 0.95$  in Bayesian analyses following the criteria set by Huelsenbeck & Ronquist (2001), are considered as strong support (Huelsenbeck & Ranala, 2004; Mulcahy et al., 2011).

The constructed tree was then exported into FigTree™ version 1.4.1 (Rambaut, 2010) and visualized. Genetic distance (uncorrected p-distance) was estimated using MEGA XI (Tamura et al., 2021). Genetic diversity parameters were computed using the DnaSp v6.12.03 (Rozas et al., 2017) and Arlequin v.3.5.2.2 software (Excoffier & Lischer, 2010).

### ***Morphological data***

Body measurements were taken after fixation under a stereo microscope to the nearest 0.1 mm using a dial caliper following Grismer et al. (2007), Riyanto & Kurniati (2014), and Matsui et al. (2014). We follow Watters et al. (2016) for abbreviations used for body measurements: (1) snout-vent length (SVL), distance between tip of snout to posterior margin of vent; (2) head length (HL), distance between tip of snout to posterior of the jaw; (3) head width (HW), distance between posterior corners of the eyes; (4) snout length (SL), distance from the tip of the snout to the anterior corner of the eye; (5) eye to nostril distance (EN), distance from anterior corner of the eye to the posterior margin of the nostril; (6) eye diameter (ED), distance from the anterior to posterior corner of the eye; (7) eye to tympanum distance (ETD), distance from the anterior margin of the tympanum to the posterior corner of the eye; (8) tympanum diameter (TD), greatest horizontal width of the tympanum; (9) internarial distance (IND), distance between the inner margins of the nostrils; (10) interorbital distance (IOD), distance between the anterior corners of the orbits; (11) lower arm length (LAL), distance from the elbow to the tip of third finger; (12) third finger length (Fin3L), distance from the base of the first sub articular tubercle to the tip of the third finger; (13) third finger disk width (Fin3DW), measured across the widest points on the third finger disk; (14) thigh length (THL), distance from the vent to the flexed knee; (15) tibia length (TL), distance from the knee to heel on flexed leg; (16) foot length (FL), distance from the proximal edge of the inner metatarsal tubercle to the tip of the fourth toe; (17) fourth toe length (Toe4L), distance from the base of the first sub articular tubercle to the tip of the toe; (18) disk width of the fourth toe (Toe4DW), measured across the widest points on the fourth toe disk. The males were identified by having vocal slits near the commissure of jaws.

We used eight *Chirixalus trilaksonoi* samples deposited at MZB for the morphometric analysis, and followed Leonart et al. (2000) to reduce allometric bias due to ontogeny using the *GrupStruck* package (Chan & Grismer, 2022). The allometric formula defined by Thorpe (1975) is as follows:  $X_{adj} = \log(X) - b[\log(BL) - \log(BL_{mean})]$ . Where  $X_{adj}$  = size corrected variable;  $X$  = unadjusted trait variable;  $b$  = regression coefficient or slope of the relationship between  $\log(X)$  and  $\log(BL)$ ;  $BL$  = standard measurement of body length/size (we use snout-vent-length);  $BL_{mean}$  = the grand mean of all BLs. We perform a student t-test utilizing data that adheres to the assumptions of parametric tests (normality and homogeneity) using only male specimens to compare the means of variables between the two populations of *C. trilaksonoi* from Java (n=4) and Sumatra (n=4). Only male was used for analysis due to the lower number of female samples for statistical analyses. All statistical analyses were analyzed using R (R Core Team, 2023). Specimens used for morphological comparison and analyses: Male specimens of *C. trilaksonoi*:

MZB Amph 32737, 32739, 32740, 32741 (Palembang, South Sumatra), MZB Amph 17932, 17933, 17934, 28441 (Bogor, West Java), and female specimens of *C. trilaksonoi*: MZB Amph 32696, 32738 (Palembang, South Sumatra), MZB Amph 27237 (Bogor, West Java).

### **Bioacoustics**

We recorded advertisement calls of *C. trilaksonoi* from Bukit Baru, Ilir Barat I District, Palembang, South Sumatra on 22<sup>nd</sup> June 2022. The calls were recorded shortly after a rain at an air temperature of 25.0°C, in a temporary small pond. The species were harmoniously vocalizing alongside *Polypedates leucomystax*, *Microhyla sriwijaya*, *Duttaphrynus melanostictus*, and *Fejervarya limnocharis*. We analyzed one consecutive call from a single individual (uncollected) using Raven Pro 1.6 for Windows software (<http://www.birds.cornell.edu/raven>). We followed Pettitt et al. (2012) for the definition of the calls, the parameters that we examined and measured included the following: Consecutive call duration (s; the duration of consecutive calls), Call duration (s; the duration of single call), Pulse duration (ms; a duration of a single pulse), Pulse rate (pulse/s), and Calling rate (calls/s).

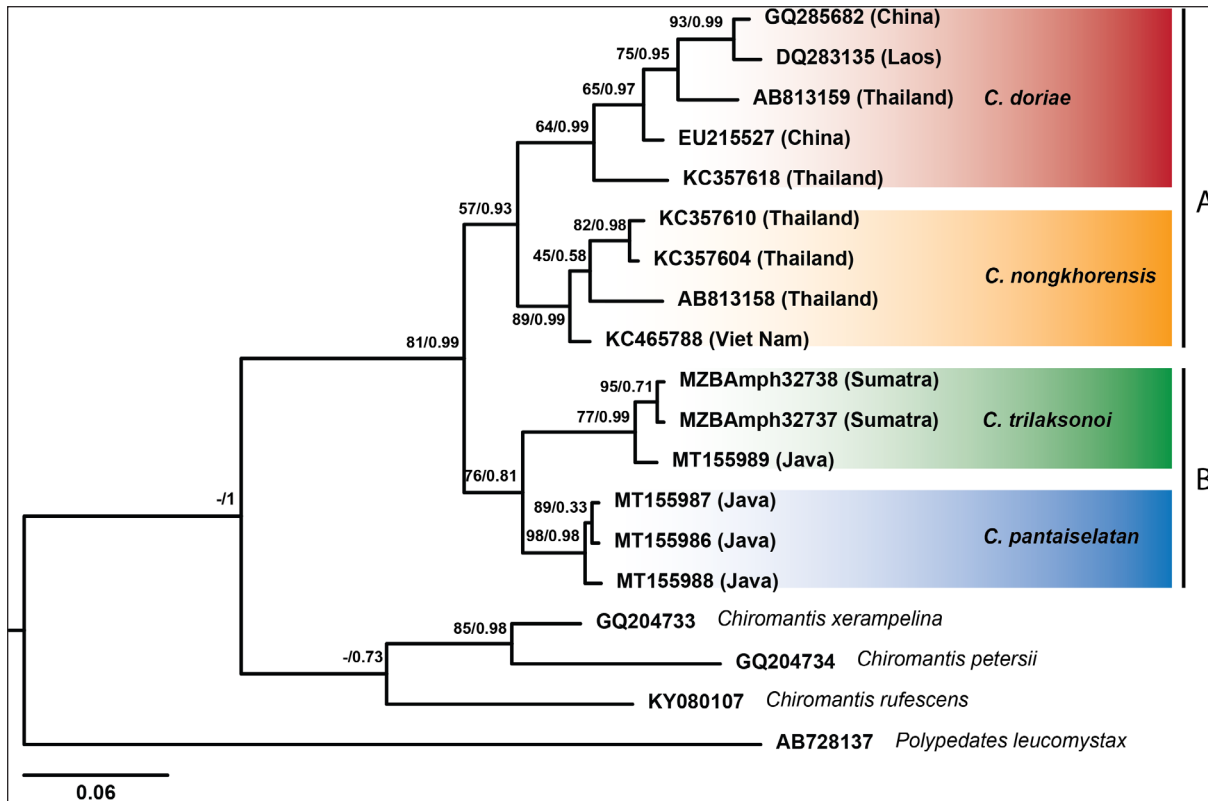
## **RESULTS**

### **Phylogenetic analysis**

Both ML and BI phylogenetic tree analyses resulted in nearly the same topologies. The Bayesian tree (Fig. 2) infers the following sets of relationships: Monophyly of *Chirixalus* concerning *Chiromantis* was supported in the Bayesian tree (PP = 1). *Chirixalus* clade was mainly divided into two clades. Clade A contained two species from China and Indochina populations, and Clade B contained Javan and Sumatran populations. The two allopatric populations of *C. trilaksonoi* exhibit a low genetic distance of 0.6% (Table 2) confirming both populations belong to the same lineage.

**Table 2.** Uncorrected p-distances (in percent) in the genus *Chirixalus* based on the ~469 bp mitochondrial 16S rRNA gene sequences

No	Species	1	2	3	4	5
1	<i>C. trilaksonoi</i> Sumatra	0				
2	<i>C. trilaksonoi</i> Java	0.65	–			
3	<i>C. pantaiselatan</i>	3.68–3.90	3.03–3.25	0.0–0.21		
4	<i>C. doriae</i>	4.55–6.07	5.20–6.50	5.20–6.29	0.65–3.68	
5	<i>C. nongkhorensis</i>	4.83–5.26	5.49–5.92	3.94–5.04	3.50–5.93	1.31–2.19



**Figure 2.** Bayesian tree of ~469 bp fragment of 16S rRNA gene for Sumatran population of *C. trilaksonoi*, with representatives of four *Chirixalus* species. Values above the branch indicate Bootstrap Value (BS) and Posterior Probability values (PP).

### Genetic diversity

Sequences of *C. trilaksonoi* (n=3) from Java and Sumatra resulted in 16S r-RNA mitochondrial DNA gene (469 bp) being added to genetic analysis. The frequencies of the nucleotides are: A= 0.3254, T= 0.2285, G= 0.2061, C= 0.2401. The sequences resulted in two haplotype variations with three polymorphic sites. The genetic diversity of two populations of *C. trilaksonoi* uncovers the nearness of variety. The haplotype diversity (Hd) of the sample was 0.6667, and the nucleotide diversity ( $\pi$ ) of the sample was 0.0044 (Table 3). The distance between Javan and Sumatran populations is formed by three mutation steps.

**Table 3.** Genetic diversity of *C. trilaksonoi*

Species	bp	Number of Samples (n)	Haplotype number (Hn)	Haplotype diversity (Hd)	Nucleotide Diversity ( $\pi$ )	Number of polymorphic sites
<i>C. trilaksonoi</i>	469	3	2	0.67 ± 0.31	0.0044 ± 0.0041	3

**Table 4.** Polymorphic sites of two haplotype of *C. trilaksonoi* from two localities

Nucleotide position	n	8	11	20
Haplotype 1–Java	1	C	G	A
Haplotype 2–Sumatra	2	T	A	G

### Morphological information

We identified *Chirixalus* specimens from Palembang as *Chirixalus trilaksonoi* by showing smooth skin; head skin not co-ossified to the skull; digital disks with a circummarginal and transverse ventral groove; white yellowish patch on the upper jaw beginning bellows middle portion of the eye and extending posteriorly to the shoulder region. Besides the higher morphological similarity between the *C. trilaksonoi* population from Java and Sumatra, they also have slight infraspecific variations in body coloration. Some individuals have light green bodies lacking dorsal longitudinal stripes, while others have stripes on yellowish bodies. However, both populations have a significant difference in body sizes. This difference is shown in the results of the student t-test ( $\alpha = 0.05$ ) in the following characters: HL, HW, SNL, TD, IND, IOD, and 4Toel (Table 5).

**Table 5.** Measurement of adult *C. trilaksonoi* from Java and Sumatra (in mm) with P-values of the student t-test. Asterisks (\*) denote significant result based on  $\alpha=0.05$

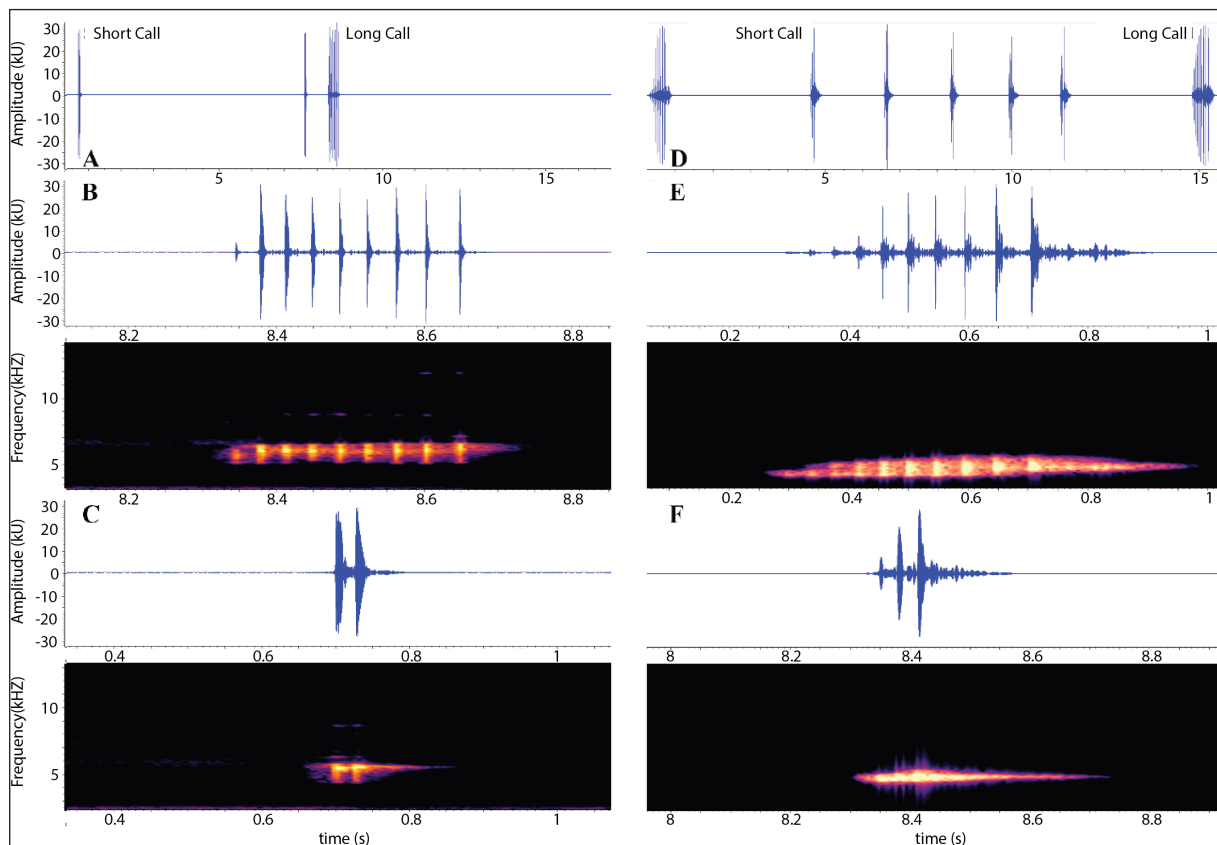
Characters	<i>C. trilaksonoi</i> (Java)		<i>C. trilaksonoi</i> (Sumatra)		Student t-test
	Male (n=4)	Female (n=1)	Male (n=4)	Female (n=2)	
SVL	25.75 ± 0.72	30.26	25.07 ± 0.48	25.67 ± 3.29	0.12
HL	7.92 ± 0.48	8.75	9.24 ± 0.15	9.11 ± 0.64	0.00*
HW	7.44 ± 0.34	9.38	7.69 ± 0.05	8.02 ± 1.48	0.02*
SNL	3.31 ± 0.13	3.62	3.62 ± 0.24	3.70 ± 0.47	0.04*
NEL	2.11 ± 0.14	2.62	2.06 ± 0.10	2.36 ± 0.38	0.48
ED	3.46 ± 0.26	4.30	3.31 ± 0.15	3.38 ± 0.02	0.29
TEL	0.57 ± 0.11	0.74	0.70 ± 0.10	0.69 ± 0.16	0.06
TD	1.51 ± 0.12	1.88	1.68 ± 0.16	1.60 ± 0.50	0.04*
IND	1.99 ± 0.16	2.02	2.52 ± 0.15	2.25 ± 0.14	0.00*
IOD	2.95 ± 0.18	3.55	3.56 ± 0.13	3.45 ± 0.15	0.00*
LAL	10.03 ± 0.41	12.80	10.53 ± 0.44	11.19 ± 1.46	0.10
3FL	3.50 ± 0.35	4.08	3.66 ± 0.38	4.48 ± 0.69	0.51
3FDW	0.97 ± 0.35	1.02	1.10 ± 0.22	1.10 ± 0.04	0.19
FML	11.68 ± 0.65	14.42	11.71 ± 0.48	13.14 ± 2.12	0.90
TBL	12.87 ± 0.23	15.74	12.68 ± 0.45	13.59 ± 2.04	0.28
FL	9.15 ± 0.36	11.17	9.03 ± 0.27	10.09 ± 2.00	0.43
4Toel	4.86 ± 0.34	6.32	5.46 ± 0.02	5.81 ± 0.76	0.01*
4TDW	0.87 ± 0.05	0.90	0.855 ± 0.14	0.92 ± 0.05	0.76

### Bioacoustic

Advertisement calls of *C. trilaksonoi* are identified to have a similar structure and tempo between populations of Java and Sumatra. In the following section, we provide a comparison of advertisement call of *C. trilaksonoi* from Java and Sumatra. Our description of the call of *C. trilaksonoi* is based on a single consecutive call from a male holotype specimen (MZB Amph 17932; Bogor, West Java), and a male specimen (uncollected, Palembang, South Sumatra). The calls are recorded in its natural habitat.

The call of *C. trilaksonoi* from Java was described by Riyanto & Kurniati (2014). It comprises a short and a long call type, and we observed similar calls from Sumatra with only a few structure and tempo variations (Fig. 3, Table 6). Generally, the short calls of *C. trilaksonoi* consist of 2–3 pulses per call. However, the pulse rate of the Javan population remains constant across the calls, while the Sumatran population exhibits a gradient from a lower to a higher rate (Figs. 3C & 3F) The duration of short calls in the Javan population is slightly shorter ( $0.16 \pm 0.02$  ( $0.14–0.18$ ) s;  $n=3$ ) than in the Sumatran population ( $0.40 \pm 0.02$  ( $0.37–0.42$ ) s;  $n=16$ ).

Additionally, the pulse duration of short calls in the Javan population is slightly longer ( $70 \pm 20$  ( $20–120$ ) ms;  $n=7$ ) than in the Sumatran population ( $60 \pm 50$  ( $20–230$ ) ms;  $n=34$ ). The long calls of *C. trilaksonoi* in Javan and Sumatran population have similar characteristics to the short calls, consisting of 9–12 pulses per call. The Javan population has a constant pulse rate across the calls, while the Sumatran population exhibits a gradient from a lower to a higher pulse rate (Figs. 3B & 3E). Javan population also has a shorter long call duration ( $0.44 \pm 0.03$  ( $0.42–0.46$ ) s;  $n=2$ ) compared to the Sumatran population ( $0.71 \pm 0.02$  ( $0.70–0.73$ ) s;  $n=2$ ). Additionally, the pulse duration of long calls of the Javan population is slightly shorter ( $40 \pm 20$  ( $20–110$ ) ms;  $n=19$ ) than that of the Sumatran population ( $60 \pm 40$  ( $30–200$ ) ms;  $n=22$ ). The calling rate of the Javan population is slightly lower (0.21 calls/s) than that of the Sumatran population (0.39 calls/s).



**Figure 3.** Comparison of advertisement calls. (A) Oscillograms of long and short call type, Oscillograms and spectrograms of (B) long call, (C) short call of *C. trilaksonoi* from Java, and (D) Oscillograms of long and short call type, Oscillograms and spectrograms of (E) long call, (F) short call of *C. trilaksonoi* from Sumatra.



**Table 6.** Advertisement call characteristics of *C. trilaksonoi*

Sound parameters	<i>C. trilaksonoi</i> (Java)			<i>C. trilaksonoi</i> (Sumatra)		
	Mean	SD	(min-max)	Mean	SD	(min-max)
Consecutive calls duration (s)	23.3	–	–	15.2	–	–
Calling rate (calls/s)	0.21	–	–	0.39	–	–
Call duration of short calls (s)	0.16	0.02	(0.14–0.18)	0.40	0.02	(0.37–0.42)
Call duration of long calls (s)	0.44	0.03	(0.42–0.46)	0.71	0.02	(0.70–0.73)
Pulse duration of short calls (ms)	70	20	(20–120)	60	50	(20–230)
Pulse duration of long calls (ms)	40	20	(20–110)	60	40	(30–200)
Pulse rate of short calls (pulse/s)	14.9	5.12	(11.1–20.7)	16.6	2.13	(14.2–19.4)
Pulse rate of long calls (pulse/s)	21.5	0.09	(21.4–21.6)	15.4	1.62	(14.2–16.5)

## DISCUSSION

*Chirixalus trilaksonoi* population rediscovered from Palembang inhabits in a suburban area, and the individuals we collected were perched on a palm leaf near an artificial pond. This population is sympatric with other amphibians such as *D. melanostictus*, *F. limnocharis*, *M. sriwijaya*, and *P. leucomystax*. Riyanto & Kurniati (2014) also reported *C. trilaksonoi* from Bogor residing in a paddy field near residential areas. With these records of this species in suburban and residential habitats, and wide distribution in two major islands, it is indicating their ability of potential resistance to habitat changes. Although, the natural history of *C. trilaksonoi* remains largely unknown, it is supposed that, similar to other *Chirixalus* congeners such as *Chirixalus simus* (Deuti, 2001; Banerjee, 2014; Mandal et al., 2020), *Chirixalus trilaksonoi* also construct nests under leaves near bodies of water during the rainy season (Alhadi et al., 2021).

We confirmed the topotypical population (Java) and the newly discovered Sumatra population from Palembang represent a single species, *C. trilaksonoi*. Nevertheless, slight infraspecific variations in morphological characters and advertising calls were observed in two different populations isolated to two different islands, and the two populations of *C. trilaksonoi* exhibit low genetic distance and slight genetic diversity. Such low genetic distance was also shown by the other amphibians distributed between Java and Sumatra, e.g., *Rhacophorus reinwardtii* (Fajri et al., 2023). It hinted how ancestral populations of some of the widespread species dispersed recently between these two major islands. Nonetheless, the limited genetic diversity is likely influenced by ecological factors within their habitats (Hu et al., 2019). Environment is also a key element in phenotypic development, resulting in variation in morphology and behavior across species (Bertossa, 2011). On the other hand, significant differences may be present due to the limited number of specimens available for morphometric analysis (see Grabowski & Porto, 2016).

It is possible that the advertising calls variations in two populations of *C. trilaksonoi* is forced by evolutionary processes due to typical philopatric behavior and limited dispersal capacity observed in amphibians as shown by *Hyla japonica* (Jang et al., 2011). The predominant influence shaping population genetic structure and geographic variation in call characters may be restricted gene flow (Stebbins & Cohen, 1993). Furthermore, to accurately understand variation

in frog advertising calls, the number of individuals in the sample should be at least 20 (see Weaver et al., 2020).

### *Evaluation of IUCN red list status*

*Chirixalus trilaksonoi* was so far listed in IUCN red list as an Endangered species based on the category and criteria B1ab(iii) ver 3.1 (IUCN SSC Amphibian Specialist Group, 2018). This was mostly due to severely fragmented habitats and the least number of locations as *C. trilaksonoi* had fewer than five threat-defined locations, and continuing decline observed, estimated, inferred, or projected in any area, extent, and/or quality of habitat.

In this study, we re-evaluated the red list status of *C. trilaksonoi* and propose to be Vulnerable, according to the B1ab(iii) criteria, supported by the following evidence: *C. trilaksonoi* has been identified in eight distinct threat-defined locations. Six of these locations are in West Java and Banten: Bogor (Riyanto & Kurniati, 2014; INaturalist, 2023), Purwakarta, Bekasi, Serang, Lebak (INaturalist 2023), and Ujung Kulon National Park (Munir et al. 2021; INaturalist, 2023). Additionally, there are two locations in Sumatra: Bukit Tigapuluh National Park, Jambi (F. Kaprawi pers. comm. September 2023), and Palembang (this study). In addition, there is an unpublished record of this species presence in Lampung (F. Kaprawi pers. comm. September 2023), and its occurrence in Lampung is highly possible. However, as we lack specimen samples from Lampung, it has not been included in our distribution map (Fig. 4). With the current distribution evidence of the species, the Extent of Occurrence (EOO) exceeds 5000 km<sup>2</sup>, positioning *C. trilaksonoi* within the “Vulnerable” criteria under category B1, subpoint (a). Regarding subpoint (b)(iii), despite the species ability to adapt to environmental changes, the quality of its habitat may deteriorate, leading to potential habitat shifts or population declines. Land cover changes over time in Java and Sumatra could have adverse effects on the population. Furthermore, the overall population size remains unknown, and there are no documented instances of this species being exploited.



**Figure 4.** Currently known distribution of *C. trilaksonoi*. Created and edited from <http://www.simplemapp.net> (Shorthouse, 2010). The type locality is represented by a black square, and the distribution outside the type locality is represented by a red circle.

## ACKNOWLEDGMENTS

We thank the late Misbahul Munir for his valuable advice in constructing the manuscript at the beginning of the writing process; Fajar Kaprawi (Amfibi Reptil Sumatra) for providing insight information regarding the distribution of amphibians in Sumatra; We thank the reviewers: A.A. Thasun Amarasinghe (BRIN), Koshiro Eto (Kitakyushu Museum of Natural History and Human History, Japan) Ibuki Fukuyama (Kyoto University, Japan), and Vesti Atmaja (Bengkulu University) for their constructive and helpful comments, which have significantly improved the quality and clarity of our manuscript.

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