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Molecular Phylogenetic Assessment of *Trimeresurus gunaleni* Vogel, David & Sidik, 2014 (Serpentes: Viperidae: Crotalinae)

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The Asian pit vipers of the genus *Trimeresurus* Lacepède, 1804 represent one of the most taxonomically challenging snake groups in Southeast Asia, with over 56 recognized species—not including species now referred to the genus *Craspedocephalus*—and numerous unresolved complexes (David *et al*. 2011; Vogel *et al*. 2014; Mirza *et al*. 2020, 2023; Idiiatullina *et al*. 2024a, b). A major source of historical confusion stems from the morphological conservatism and intraspecific variation across the members of the group (Malhotra & Thorpe 2004). To mitigate taxonomic instability, David *et al*. (2011) proposed the use of subgenera—*Craspedocephalus, Himalayophis, Parias*, *Popeia*, *Trimeresurus, Viridovipera*—which retained phylogenetic structure while avoiding nomenclatural disruption. Among these, *Parias* Gray, 1849 encompasses several montane and insular taxa from the Sunda Shelf and the Philippines.

Trimeresurus gunaleni was described by Vogel, David & Sidik (2014) from montane habitats of Mt. Sibayak, northern Sumatra (1,500–2,200 m a.s.l.). It was separated and differentiated from the lowland species *T. sumatranus* based on lower ventral scale counts, a longer relative tail length, green iris coloration, and distinct elevational and ecological niches. Subsequent morphological studies (e.g., Vogel *et al*. 2022) have acknowledged *T. gunaleni* as a distinct species, but robust molecular support has remained lacking.

A female specimen identified as *Trimeresurus gunaleni* stored at The University of Texas at Arlington, USA (specimen UTA R-66247; Fig. 1), was collected along the trail to Mount Leuser (3.7256° N, 97.1558° E; 2,314 m a.s.l.) in Gayo Lues Regency, Aceh Province, northern Sumatra, Indonesia. The specimen was photographed in life before preservation, and liver tissue was sampled and stored in 95% ethanol for molecular analysis. We compared the external morphology of this specimen with the holotype of *T. gunaleni* (Museum Zoologicum Bogoriense, Cibinong; MZB.Ophi.5452) to confirm its identification based on diagnostic scalation, coloration, and pattern characters.

Here, we expand the molecular dataset for *Trimeresurus gunaleni* by incorporating mitochondrial 16S rRNA and cytochrome *b* gene sequences and infer its phylogenetic position relative to congeners within the subgenus *Parias*. The analyses aim to reassess the distinctiveness of *T. gunaleni* through a combined-gene approach, quantify its genetic divergence from closely related taxa, and clarify its phylogenetic placement within *Parias* based on maximum-likelihood phylogenetic inference.

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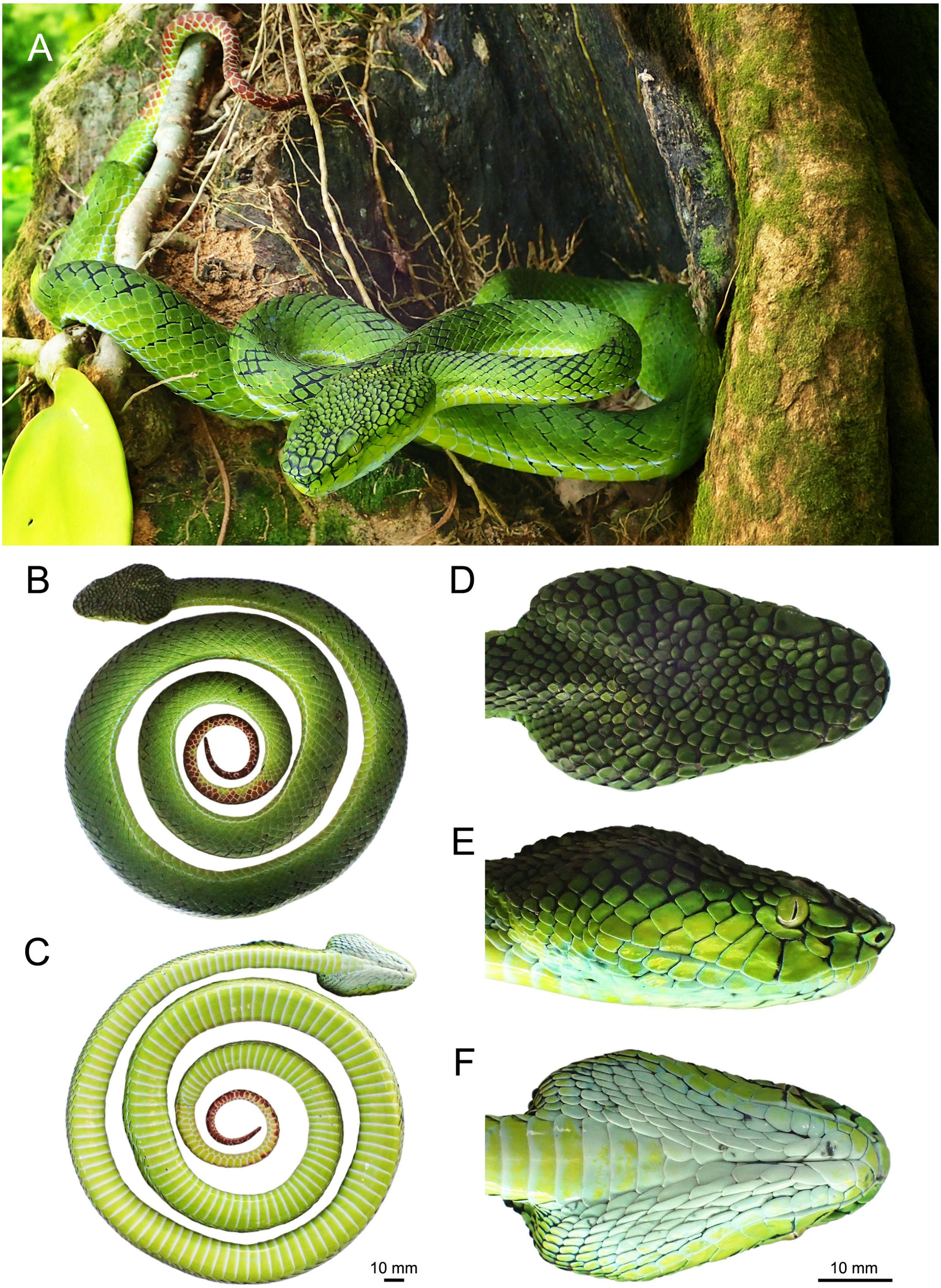

FIGURE 1. Life and preserved specimen of *Trimeresurus gunaleni* (UTA R-66247) from the trail to Mt. Leuser at an elevation of 2,314 m a.s.l. in Gayo Lues, Aceh, Sumatra, Indonesia, that was used for tissue sampling: **(A)** live individual in natural resting position; preserved individual's **(B)** dorsal and **(C)** ventral views of the full body; and **(D)** dorsal, **(E)** lateral, and **(F)** ventral views of the head.

Total genomic DNA was extracted from ethanol-preserved liver tissue using the Qiagen DNeasy™ Blood & Tissue Kit (Qiagen, Hilden, Germany). Partial fragments of two mitochondrial genes,16S rRNA (16S) and cytochrome *b* (Cyt *b*), were amplified using gene-specific primers. The 16S fragment was amplified with primers L2606 (5′-CTGACCGTGCAAAGGTAGCGTAATCACT-3′) and H3056 (5′-CTCCGGTCTGAACTCAGATCACGTAGG-3′) following Hedges *et al*. (1993). The Cyt *b* fragment was amplified using primers L14910 (5′-GACCTGTGATMTGAAAACCAYCGTTGT-3′) and H16064 (5′-CTTTGGTTTACAAGAACAATGCTTTA-3′) following Malhotra & Thorpe (2004), which have been widely used for *Trimeresurus* and related pitvipers. PCR amplifications were carried out in 25 µL reactions containing approximately 25 ng genomic DNA, 0.5 µM of each primer, 12.5 µL of 2× DreamTaq™ PCR Master Mix (Thermo Scientific), and nuclease-free water to volume. Thermal cycling conditions consisted of an initial denaturation at 94 °C for 5 min; 35 cycles of 94 °C for 30 s, annealing at 52–55 °C for 30 s, and extension at 72 °C for 90 s; and a final extension at 72 °C for 10 min. PCR products were visualized on 1% agarose gels, purified, and bidirectionally sequenced by Macrogen (Singapore) using the same primers. Sequence chromatograms were inspected, edited, and assembled in ChromasPro (Technelysium Pty Ltd.).

To infer the phylogenetic position of *Trimeresurus gunaleni*, we combined the new mitochondrial 16S and Cyt b sequences from the recently collected specimen with homologous 16S and Cyt b sequences of congeners available in GenBank (Supplementary Table 1). We also retrieved ND4 sequences from GenBank to maximize character and taxon overlap across the *Parias* clade, where some taxa lack Cyt b but are represented by ND4. To avoid conflating signal across loci with different evolutionary dynamics, all concatenated analyses were conducted under a partitioned scheme and we additionally report interspecific distances and single-gene trees for 16S and Cyt b. These steps allow us to leverage available ND4 information while keeping gene-specific inferences transparent. The final dataset comprised a total of 122 sequences (16S, 70 taxa; Cyt b, 35 taxa; ND4,17 taxa) representing 65 individuals from all major *Trimeresurus* lineages, including new 16S and Cyt b sequences for *T. gunaleni*. Sequences were aligned using MAFFT v7.480 (Katoh & Standley 2013) under default parameters. The concatenated alignment of 1788 bp included 603 parsimony-informative sites.

TABLE 1. Comparative summary of key morphological characters between the holotype of *Trimeresurus gunaleni* (MZB. Ophi.5452), additional specimens reported by Vogel *et al*. (2014), and the newly collected specimen (UTA R-66247) from Gayo Lues, Aceh Province, Sumatra, Indonesia. The data serve to confirm the identification of UTA R-66247 as *T. gunaleni* based on consistency with the holotype and published variation.

Characters	Holotype (MZB. Ophi.5452)	Other specimens (fide Vogel *et al*., 2014)		Our specimen (UTA R-66247)
Sex	Female	Males (*n*=3)	Females (*n*=3)	Female
Ventrals	171	162–179	164–174	168
Subcaudals	60	71–72	58–66	63
Mid DSR	21	21	21	21
Supralabials (SUP)	9/9	9/9	9/9	9/9
Infralabials	11/11	11/10-11	11/11	11/11
Suboculars (touch SUP)	2 (6)	2 (6)	2 (6/7)	2 (6)
loreals	1	1	1	1
preoculars	2	2	2	2
postoculars	2	2	2	2
Tail Ratio (TaL/TL)	16.7%	20.1–21.0%	14.4–18.0%	16.2%
Reddish tail	No	Yes/No		Yes
Ventrals with black margins		No		No
Subcaudal with black margins		No		No
Eye colour in life		Green		Green

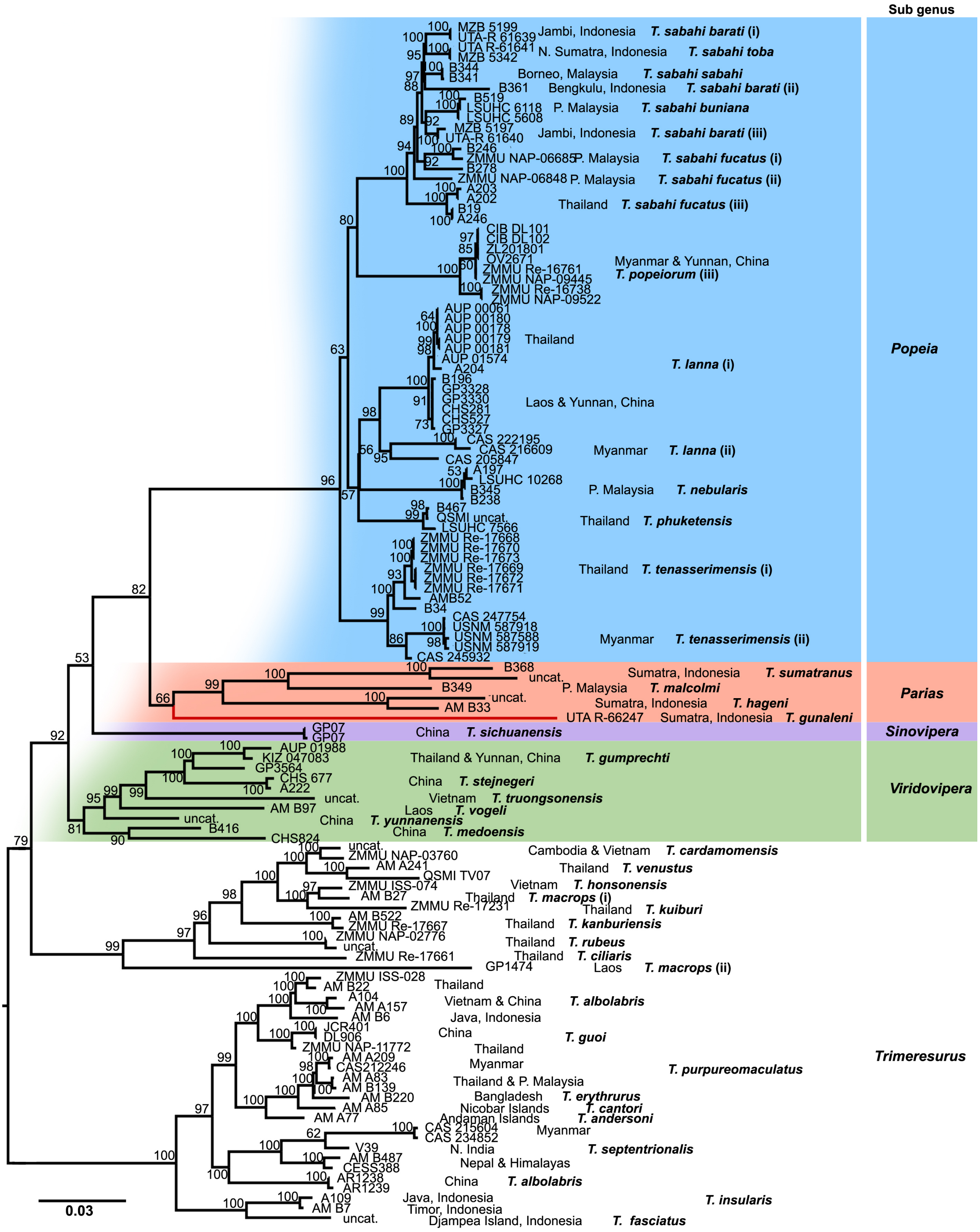

FIGURE 2. Maximum-likelihood (ML) phylogeny of *Trimeresurus* inferred from the concatenated mitochondrial dataset (16S, Cyt b, and ND4; total length 1,788 bp). Bootstrap (UFboot) support values are shown at nodes. The outgroups, *Craspedocephalus borneensis* and *C. puniceus*, are not shown.

Maximum-likelihood (ML) phylogenetic inference was conducted in IQ-TREE v2.4.0 (Nguyen *et al*. 2015; Minh *et al*. 2020) under a partitioned scheme: 16S as a single partition and Cyt b and ND4 were each partitioned by codon position. ModelFinder (Kalyaanamoorthy *et al*. 2017) selected the best-fitting substitution model under BIC, and partitions with indistinguishable fit were merged (IQ-TREE v2, -m MFP+MERGE). The final models were: 16S = GTR+I+G; Cyt b 1st = GTR+G; Cyt b 2nd = HKY+I+G; Cyt b 3rd = HKY+I; ND4 1st = HKY+I+G; ND4 2nd = GTR+I+G; ND4 3rd = HKY+I+G. Node support was assessed with 1,000 ultrafast bootstrap replicates (Minh *et al*. 2013). We considered nodes with UFboot ≥ 90% as strongly supported. In addition to the concatenated analysis, single-gene ML trees for 16S and Cyt b are provided in the Supplementary Material. Genetic distances (uncorrected *p*-distances) were computed separately for 16S and for Cyt b in MEGA v11 (Tamura *et al*. 2021).

Morphological examination confirmed conformity with the original description, particularly in the number of ventral scales and the tail ratio (Table 1). The absence of black margins on the ventral scales and tail (Fig. 1), along with green eye coloration in the live specimen (Fig. 1), further supports its identification as *T. gunaleni*. Thus, our newly collected specimen represents a new locality record for *Trimeresurus gunaleni* in Sumatra, extending its known distribution beyond the mountainous regions of Sumatra Utara and Sumatra Barat. The elevational range of the species is thereby revised to 1,500–2,400 m a.s.l. In addition to ventral and subcaudal counts and tail ratio, we recorded standard cephalic scalation and body measurements used for *Trimeresurus* taxonomy, including dorsal scale rows at one head length behind the head, at midbody, and one head length before the vent (DSR), supralabials and infralabials, internasals and prefrontals, loreals, preoculars, postoculars, and subocular contact with supralabials, as well as SVL, tail length, and total length (Table 1). These characters of UTA R-66247 fall within the ranges reported for *T. gunaleni* and differ from sympatric lowland congeners in combination.

The concatenated maximum-likelihood tree (Fig. 2) places our *T. gunaleni* individual within the subgenus *Parias*, but deep relationships within *Parias* are weakly resolved with mitochondrial data alone (UFboot values < 70 at relevant internal nodes). Within *Parias*, *T. gunaleni* forms a weakly supported grouping with *T. hageni*, *T. sumatranus*, and *T. malcolmi*, and we therefore refrain from assigning a definitive sister relationship. Accordingly, our conclusions are restricted to clade membership and interspecific divergence rather than to a specific placement within *Parias*. Despite limited nodal support at deeper levels, uncorrected *p*-distances show that *T. gunaleni* is genetically distinct from closely related *Parias* taxa (Table 2). For 16S and Cyt b, average divergence from *T. sumatranus* and other examined *Parias* species exceeds commonly observed intraspecific values for Asian pit vipers (3–4%; Malhotra & Thorpe 2004; Sanders *et al*. 2006; Idiiatullina *et al*. 2024a, b). We therefore maintain recognition of *T. gunaleni* as a distinct species, consistent with its diagnostic morphology.

TABLE 2. Uncorrected *p*-distance values (%) among selected members of the subgenus *Parias* based on concatenated mitochondrial 16S (lower triangle) and Cyt *b* (upper triangle) sequences. Only pairwise distances within the *Parias* clade are shown, including *T. gunaleni*, *T. sumatranus*, *T. hageni*, *T. malcolmi*, and *T. schultzei*.

Species	*T. gunaleni*	*T. sumatranus*	*T. hageni*	*T. malcolmi*	*T. schultzei*
T. gunaleni	-	4.78	4.53	4.91	5.29
T. sumatranus	0.42	-	3.62	3.94	4.23
T. hageni	0.37	0.33	-	3.48	3.71
T. malcolmi	0.39	0.36	0.31	-	3.85
T. schultzei	0.45	0.4	0.37	0.39	-

Our results provide preliminary molecular support for the species-level distinctiveness of *Trimeresurus gunaleni*, corroborating its original diagnosis based on morphology of scalation and coloration (Vogel *et al*. 2014). The observed genetic divergence from *T. sumatranus*, together with its ecological isolation in montane forest habitats, reinforces the validity of *T. gunaleni* under both the Phylogenetic Species Concept (Cracraft 1983; Mishler & Theriot 2000) and the Evolutionary Species Concept (Simpson 1951; Wiley 1978; Wiley & Mayden 2000).

Given its restricted montane range, *T. gunaleni* may be vulnerable to climate change, habitat loss, and trade (Marshall *et al*. 2020). We recommend its formal conservation assessment under IUCN Red List criteria. Additionally, the venom composition of *T. gunaleni* has not been studied and may differ significantly from lowland congeners.

This study highlights the value of molecular data in confirming taxonomic boundaries within *Trimeresurus*, as similarly demonstrated in recent work on Southeast Asian pitvipers (Pawangkhanant *et al*. 2025), and underscores the need for broader phylogeographic sampling across Sumatra and nearby islands.

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SUPPLEMENTARY MATERIALS. The following supporting information can be downloaded at the DOI landing page of this paper.

SUPPLEMENTARY TABLE 1. GenBank accession numbers, gene fragments, and localities for specimens of *Trimeresurus* and related taxa included in the molecular analyses. Newly generated sequences for *T. gunaleni* (UTA R-66247; 16S and Cyt b) are shown in bold. Other sequences (16S, Cyt b, and ND4) were obtained from GenBank.