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MOLECULAR ASSESSMENT OF PITVIPER POPULATIONS (GENUS *TRIMERESURUS*) IN LAOS AND VIETNAM REVEALS NEW COUNTRY RECORD AND OVERLOOKED DIVERSITY

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SUMMARY

Vietnam is home to at least ten species of the genus *Trimeresurus* (Asian green pitvipers) and at minimum five members are found in Laos. The number has been increasing in recent years because of new species descriptions, e.g., *Trimeresurus guoi*, and new records of recently discovered taxa, such as *T. cardamomensis*. However, the genus has still been poorly studied in Laos and Vietnam as many areas in the two countries have not been sufficiently surveyed. In addition, the taxonomic status of several populations in the two countries has not been thoroughly investigated. In this study, we sequenced 18 new samples of the Asian green pitvipers collected from various sites in Vietnam and from Khammouane province in Laos. Our analyses based on a short fragment of the mitochondrial *COI* gene confirm the occurrence of *T. stejnegeri* in Laos and thus increase the species richness in the country to at least six, but more study needs to be undertaken to better understand the diversity of the species group. Two other populations from Khammouane province potentially constitute cryptic species, although further investigation is warranted. In Vietnam, *T. albolabris* is broadly distributed and divided into two clades with unknown taxonomic status. Moreover, *T. gumprechti* is discovered

in two new localities from the Northeastern provinces of Bac Giang and Cao Bang. It is apparent that this species might represent a species complex, which requires more detailed taxonomic treatment.

Keywords: *COI* gene, taxonomic status, *Trimeresurus albolabris*, *T. gumprechti*, *T. stejnegeri*

INTRODUCTION

The Asian green pitvipers of *Trimeresurus* constitute one of the most diverse snake genera within the family Viperidae with 42 recognized species. This group is widely distributed from India to China and Southeast Asia (Uetz *et al*., 2021). Over the last ten years, 11 new species have been described from the region, consisting of *Trimeresurus arunachalensis*, *T. cardamomensis*, *T. caudornatus*, *T. davidi*, *T. gunaleni*, *T. guoi*, *T. kuiburi*, *T. phuketensis*, *T. salaza*, *T. sichuanesis*, *T. yingjiangensis* (Guo, Wang, 2011; Malhotra *et al*., 2011; Sumontha *et al*., 2011; Vogel *et al*., 2014; Captain *et al*., 2019; Chen *et al*., 2019; Chandramouli *et al*., 2020; Chen *et al*., 2020; Mirza *et al*., 2020; Chen *et al*., 2021; Sumontha *et al*., 2021). The type localities of the new species are mostly situated in China and India with others from Cambodia and Thailand.

Figure 1. *Trimeresurus albolabris* from Me Linh station, Vinh Phuc province.

In Laos, at minimum five species, i.e., *T. albolabris*, *T. gumprechti*, *T. macrops*, *T. popeiorum*, and *T. vogeli*, have been recorded (Nguyen *et al*., 2020; Uetz *et al*., 2021). Vietnam has at least ten members of the genus, namely *T. albolabris*, *T. cardamomensis*, *T. gumprechti*, *T. guoi*, *T. honsonensis*, *T. macrops*, *T. rubeus*, *T. stejnegeri*, *T. truongsonensis*, and *T. vogeli* (Malhotra, Thorpe, 2004; Orlov *et al*., 2004; Grismer *et al*., 2008; Malhotra *et al*., 2011; Ziegler *et al*., 2018; Chen *et al*., 2021; Uetz *et al*., 2021) (Fig. 1). The population from Phu Quoc

island in Kien Giang Province of Vietnam was formerly identified as either *T. albolabris, T. cardamomensis, T.* cf. *cardamomensis, T. macrops* or *T. rubeus* (Stuart *et al*., 2012b, Ziegler *et al.*, 2018). Using molecular comparison of two mitochondrial genes, 12S and ND4, between this population and others with data available from GenBank, Ziegler *et al.* (2018) concluded that the Phu Quoc population is definitely *T. cardamomensis*. Although molecular data from several populations of the genus have been incorporated in previous work, no molecular review of *Trimeresurus* in Vietnam has been undertaken. In this study, we compiled all available samples collected across Vietnam and from Khammouane province in Laos by our group and sequenced a fragment of the Cytochrome c oxidase subunit I (*COI*) gene. We analyzed the newly generated data with those available from GenBank. Based on the results, we provide hypotheses on the distribution and taxonomic status of the populations in Laos and Vietnam.

MATERIAL AND METHODS

Samples of *Trimeresurus* sp.1 and *Trimeresurus* sp.2, *T. albolabris, T. gumprechti, T. macrops, T. medoensis, T. popeiorum, T. purpureomaculatus, T. stejnegeri, T. sichuanensis, T. tibetanus,* and *T. yunnanensis* from GenBank along with 18 newly collected samples of *T. albolabris, T. gumprechti, Trimeresurus* sp., *T. stejnegeri*, and *T. vogeli* from Laos and from Vietnam were included in the analyses. *Protobothrops xiangchengensis* and *P. trungkhanhensis* were used to provide outgroup polarity (Table 1). Samples were extracted using the DNeasy blood and tissue kit (Qiagen, USA). A fragment *COI* in the mitochondrial genome, was amplified using the primer pair VF1-d and VR1-d (Ivanova *et al*., 2006).

Table 1. Samples used in the analyses of this study. New sequences are highlighted in bold.

Voucher/Code/Field Number	Species	Locality	GenBank accession No.
GP27	*Protobothrops xiangchengensis*	China: Jiulong, Sichuan	KP403676
CB2012.74	*Protobothrops trungkhanhensis*	Vietnam: Cao Bang prov., Trung Khanh	KP403674
GV102	*Trimeresurus albolabris*	Vietnam: Vinh Phuc prov., Tam Dao NP	KC291123
Rom Herps 35299	*Trimeresurus albolabris*	Vietnam: Hai Duong prov., Chi Linh vicinity, Hoang Hoa Tham village	KU986327
ZFMK 97350	*Trimeresurus albolabris*	Vietnam: Vinh Phuc prov., Me Linh	MG978117
IEBR 4335	*Trimeresurus albolabris*	Vietnam: Hai Phong City, Bach Long Vy island	MG978113
IEBR 4336	*Trimeresurus albolabris*	Vietnam: Hai Phong City, Bach Long Vy island	MG978114
ZFMK 101038	*Trimeresurus albolabris*	Vietnam: Hai Phong City, Bach Long Vy Island	MG978118
IEBR 4338	*Trimeresurus albolabris*	Vietnam: Kien Giang prov., Hon Me	MG978115
IEBR 4339	*Trimeresurus albolabris*	Vietnam: Kien Giang prov., Hon Me	MG978116
ZFMK 101039	*Trimeresurus albolabris*	Vietnam: Kien Giang prov., Hon Me	MG978119
GP3286	*Trimeresurus* cf. *gumprechti*	China: Daweishan, Yunnan	KP403704
KIZ05089	*Trimeresurus "gumprechti"*	China: Baoshan, Yunnan	KP403706
CHS644	*Trimeresurus "gumprechti"*	-	MK064795

TMA31	*Trimeresurus macrops*	Thailand: Nakhon Ratchasima	AB920206
CHS824	*Trimeresurus medoensis*	-	MK064903
GV37	*Trimeresurus popeiorum*	Vietnam: Dong Nai prov., Cat Tien NP	KC291070
CHS527	*Trimeresurus popeiorum*	-	MK064748
CHS893	*Trimeresurus purpureomaculatus*	-	MK064941
GP2952	*Trimeresurus sichuanensis*	China: Jiangkou, Guizhou	KP403679
CHS750	*Trimeresurus sichuanensis*	-	MK064852
GV59	*Trimeresurus stejnegeri*	Vietnam: Kon Tum prov. Ngoc Linh	KC291086
GP230	*Trimeresurus stejnegeri*	China: Hejiang, Sichuan	KP403710
CHS715	*Trimeresurus stejnegeri*	-	MK064832
GP149	*Trimeresurus tibetanus*	China: Zhangmu, Xizang	KP403631
GP150	*Trimeresurus tibetanus*	China: Zhangmu, Xizang	KP403632
GP38	*Trimeresurus yunnanensis*	China: Huili, Sihcuan	KP403720
Tr6	***Trimeresurus albolabris***	**Vietnam: Vinh Phuc prov., Me Linh**	**OQ525951**
NA2014.1	***Trimeresurus albolabris***	**Vietnam: Nghe An prov.**	**OQ525949**
QNG2014.1	***Trimeresurus albolabris***	**Vietnam: Quang Ngai prov.**	**OQ525952**
PY2014.1	***Trimeresurus albolabris***	**Vietnam: Phu Yen prov., Tay Hoa District**	**OQ525950**
HB2014.31	***Trimeresurus "gumprechti"***	**Vietnam: Hoa Binh prov.**	**OQ525936**
HB2014.31 (2)	***Trimeresurus "gumprechti"***	**Vietnam: Hoa Binh prov.**	**OQ525937**
YT2012.29	***Trimeresurus "gumprechti"***	**Vietnam: Bac Giang prov., Yen Tu NR**	**OQ525938**
CB2015.27	***Trimeresurus "gumprechti"***	**Vietnam: Cao Bang prov.**	**OQ525939**
TK16.04	***Trimeresurus* sp.1**	**Laos: Khammouane prov.**	**OQ525947**
HNN155	***Trimeresurus* sp.1**	**Laos: Khammouane prov.**	**OQ525948**
AT2011	***Trimeresurus* sp.1**	**Laos: Khammouane prov.**	**OQ525946**
HNN121	***Trimeresurus* sp.2**	**Laos: Khammouane prov.**	**OQ525935**
HNN3.14	***Trimeresurus stejnegeri***	**Laos: Khammouane prov.**	**OQ525940**
HNN44.14	***Trimeresurus stejnegeri***	**Laos: Khammouane prov.**	**OQ525941**
HNN72	***Trimeresurus stejnegeri***	**Laos: Khammouane prov.**	**OQ525942**
HNN73	***Trimeresurus stejnegeri***	**Laos: Khammouane prov.**	**OQ525944**
HNN118	***Trimeresurus stejnegeri***	**Laos: Khammouane prov.**	**OQ525943**
MNHN199.9036	***Trimeresurus vogeli***	**Vietnam (no specific location)**	**OQ525945**

The PCR volume consisted of 10 μL of Dream Taq PCR mastermix (ThermoFischer Scientific, Lithuania), 5 μL of water, 2 μL of each primer at 10 pmol/μL and 2 μL of DNA or higher depending on the quantity of DNA in the final extraction solution). The following temperature profile for PCR was used: 95°C for 5 min; 40 cycles at 95°C for 30 s, 58°C for 45 s, 72°C for 60 s; and the final extension at 72 °C for 6 min. PCR products were subjected to electrophoresis through a 1% agarose gel (Invitrogen, USA). Gels were stained for 10 min in 1 X TBE buffer with 2 pg/ml ethidium-bromide and visualized under UV light (Fig.

2A, 2B). Successful amplifications were purified to eliminate PCR components using a GeneJET PCR Purification kit (ThermoFischer Scientific, Lithuania). Purified PCR products were sent to FirstBase (Malaysia) for sequencing.

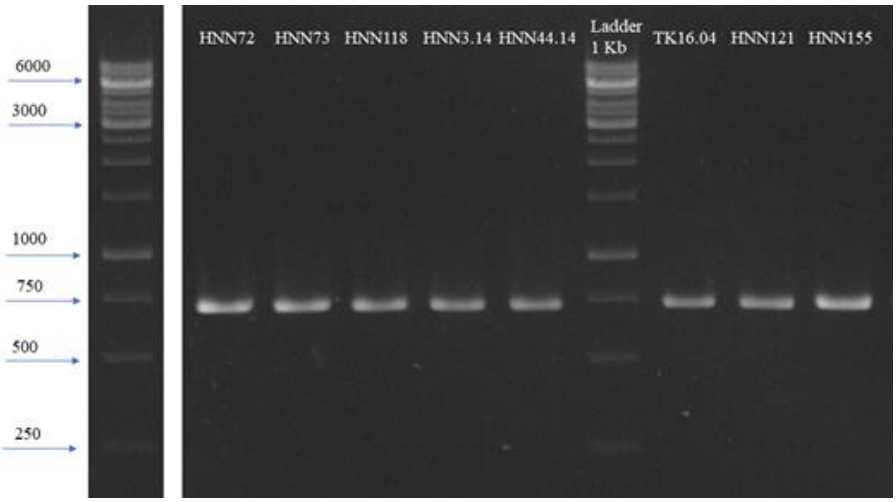

Figure 2A. Agarose gel electrophoresis of PCR products of samples collected in Laos. Annotated ladder size is shown in bp.

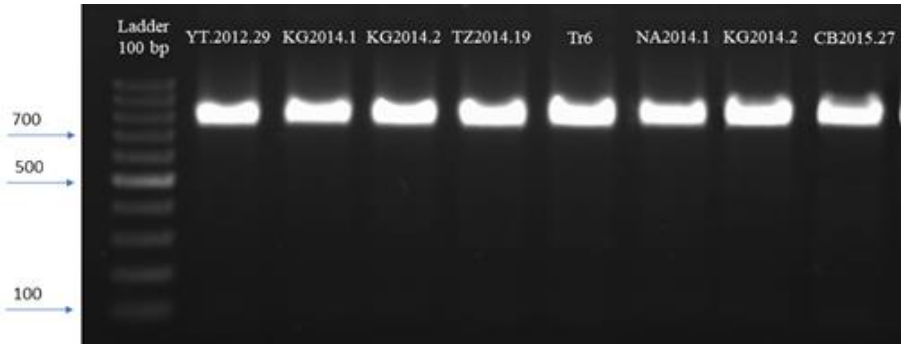

Figure 2B. Agarose gel electrophoresis of PCR products of samples collected in Vietnam.

After sequences were aligned using Clustal X v2 (Thompson *et al*., 1997), data were analyzed using Maximum Likelihood (ML) as implemented in IQ-TREE v1.6.7.1 (Nguyen *et al*., 2015), and Bayesian Inference (BI) as implemented in MrBayes v3.2.7 (Ronquist *et al*., 2012) because they are the most commonly used methods in phylogenetic reconstruction. Both BI and ML were run using a single best model, TIM3+I+G, as selected by jModelTest v2.1.4 (Posada, 2008). For BI, two simultaneous analyses with four Markov chains (one cold and three heated) were run for 10 million generations with a random starting tree and sampled every 1,000 generations. Log-likelihood scores of sample points were plotted against generation time to determine stationarity of Markov chains. Trees generated before log-likelihood scores reached stationarity were discarded from the final analyses using the burn-in function. Two independent analyses were run simultaneously. The cutoff point for the burn-in function was set to 43 in the Bayesian analysis, as -lnL scores reached stationarity after 43,000 generations in both runs. Nodal support was evaluated using 10,000 ultrafast bootstrap replications (UFB) (Minh *et al*., 2013) in IQ-TREE v1.6.7.1, and posterior probabilities (PP) in MrBayes v3.2. and

PP, UFB ≥ 95% are regarded as strong support for a clade (Ronquist *et al.*, 2012; Nguyen *et al.*, 2015). Uncorrected pairwise divergences were calculated in PAUP*4.0b10 (Swofford, 2001).

RESULTS AND DISCUSSION

The combined matrix contained 668 aligned characters with no gap. The phylogenetic results recovered by our analyses are similar to those reported by Malhotra and Thorpe (2004) and Cheng *et al.* (2020) in that *Trimeresurus albolabris* was placed in the same clade as *T. purpureomaculatus* with strong statistical support and *T. tibetanus* with *T. popeiorum* despite with insignificant support value. In addition, *T. macrops* was also grouped in the clade with high statistical support from the BI analysis only. However, positions of several species were unresolved, including *T. gumprechti*, *T. stejnegeri*, and *T. medoensis* probably because only limited data are available from a short fragment of the mitochondrial *COI* gene. *T. popeiorum* was paraphyletic and it is likely that the sequence with GenBank accession number KC291070 was mislabeled (Fig. 3A, 3B). The sample was collected in Cat Tien National Park, Dong Nai province, Southern Vietnam (Table 1). However, *T. popeiorum* has never been reported from Vietnam. Instead, it has been recorded in Northeastern India, Nepal, Myanmar, northern Thailand, northern Laos, China, and Malaysia (Uetz *et al.*, 2021). The sample from Vietnam is closely related to *T. macrops*, which occurs in Cambodia, Laos, Thailand, and Vietnam (Uetz *et al.*, 2021). However, it is 8.2% divergent from the sample of *T. macrops* from Thailand.

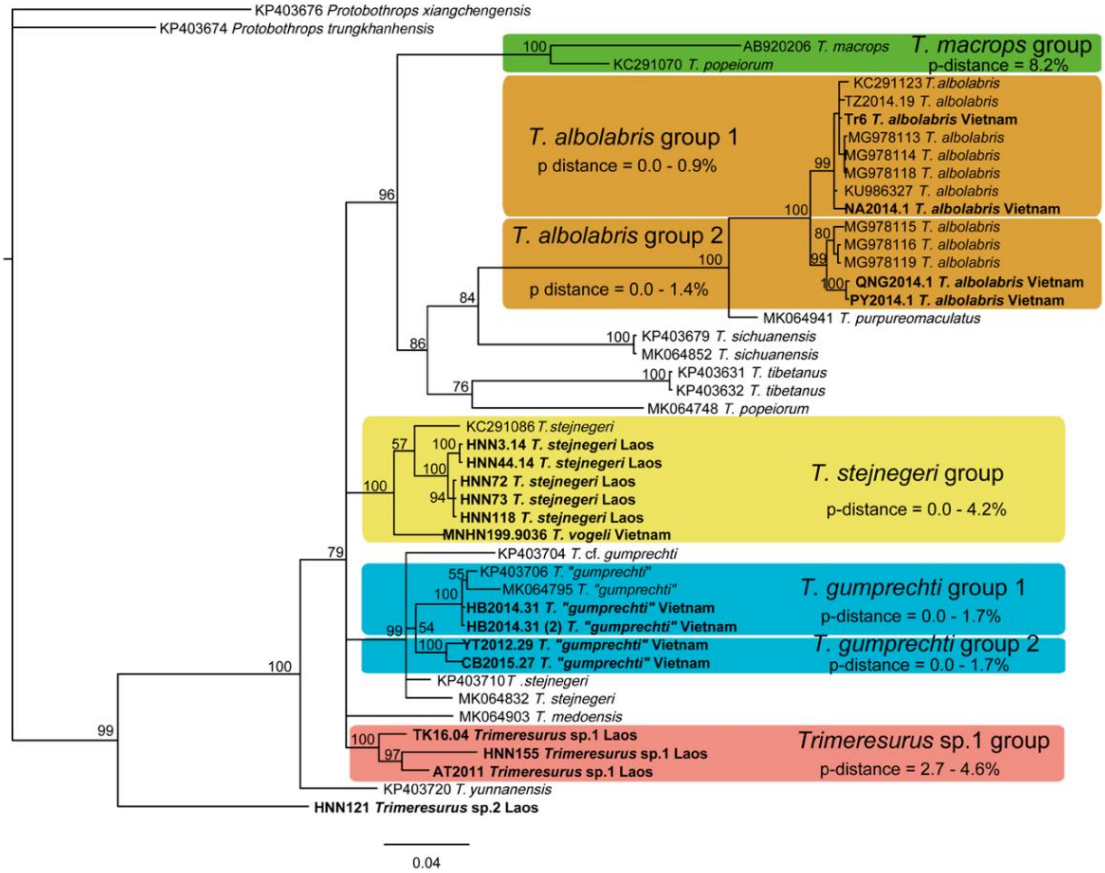

Figure 3A. Cladogram estimated by the Bayesian analysis. Numbers above branches are Bayesian posterior probabilities (>50%). Highlighted in bold are the sequences generated by this study.

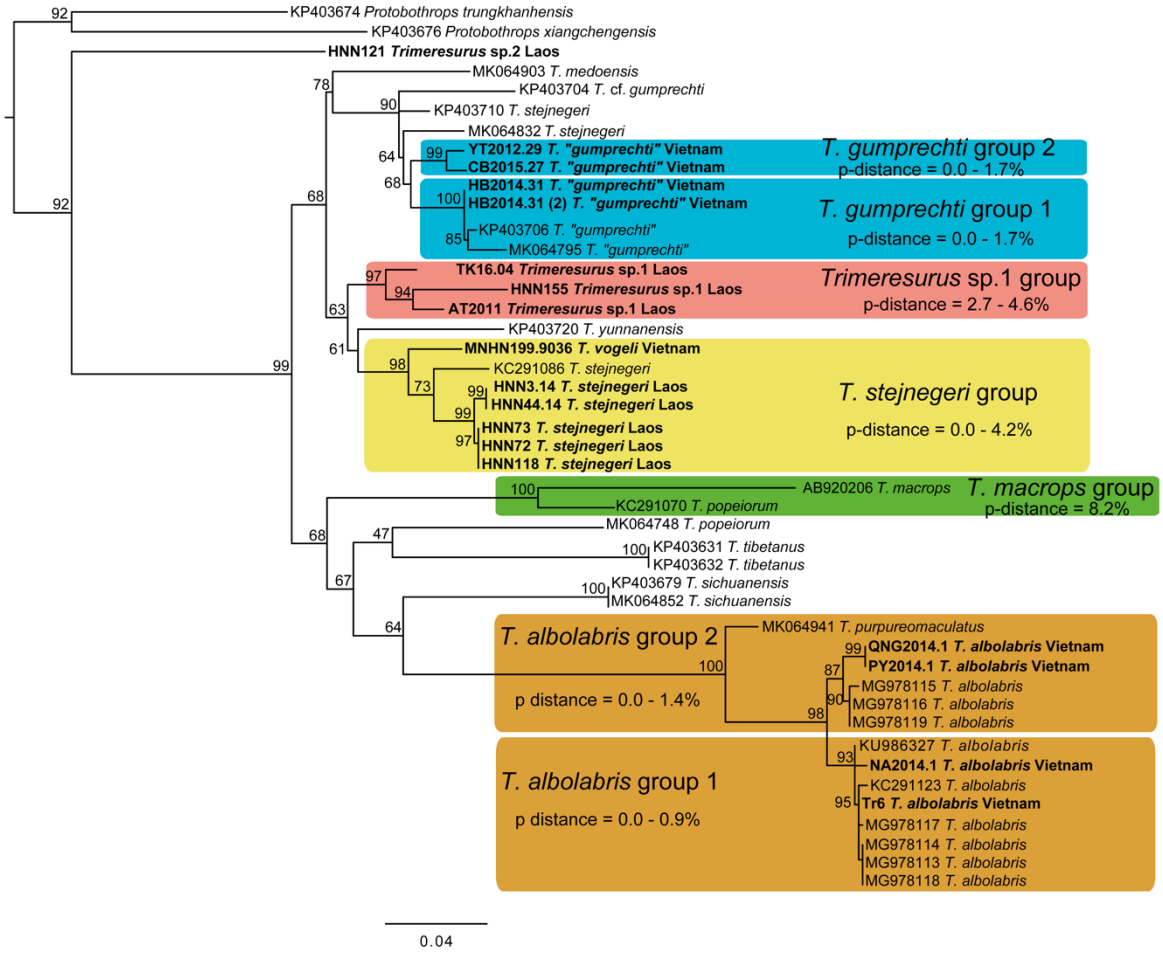

Figure 3B. Cladogram estimated by the Maximum Likelihood analysis. Numbers above branches are ML ultrafast bootstrap values. Highlighted in bold are the sequences generated by this study.

Samples of *T. albolabris* clustered into two main clades with significant support values from the Bayesian analysis only. Genetic distance between samples of *T. albolabris* group 1 ranges from 0.0 to 0.9% and group 2 from 0.0 to 1.4%. The genetic divergence between the two lineages is a little more than 2% (Table 2, see also Ziegler *et al.*, 2018). The two lineages show distinctly different distribution ranges. The first one is distributed from Nghe An Province to the North and the second one is from Quang Ngai Province to the South (Fig. 3A, 3B, Table 1). It is apparent that *T. albolabris* is distributed broadly across Vietnam. Taxonomic status of the two lineages in Vietnam should be further examined with a focus on morphological variations.

T. gumprechti was recovered as paraphyletic. Samples collected from Hoa Binh, Bac Giang, and Cao Bang provinces were placed in this clade although they belong to two different lineages with high statistical values from both BI and ML. The highest pairwise genetic divergence between samples within *T. gumprechti* group 1 and group 2 is approximately 1.7%. Genetic distance between the two clades ranges from 3.4 to 3.7% (Table 2). Previously, this species was known from Hoa Binh (Uetz *et al.*, 2021) and from Lai Chau, Lao Cai, and Nghe An provinces (Stuart *et al.*, 2012a). The occurrences of the species in Bac Giang and Cao Bang have never been reported. This clade also includes two sequences identified

as *T. stejnegeri* with GenBank accession numbers KP403710 and MK064832. The genetic divergence between two sequences and other members of *T. gumprechti* group from 2.7 to 5.5%. Therefore, the identities of the sequences need to be checked for correctness. Their positions in the tree can be a result of misidentified sequences from GenBank or the potential existence of an unknown cryptic species. Our results show that *T. gumprechti* may represent a species group with the highest genetic divergence of 6.2 % (Table 2), which warrants further taxonomic investigation.

Lao samples from Khammuane Province, including HNN3.14, HNN44.14, HNN72, HNN73 and HNN118, clustered in a single clade with strong support from both analyses (Fig. 3). These samples are likely the true *T. stejnegeri*. As a result, our study confirms the presence of *T. stejnegeri* in Laos. In terms of genetic distance, these samples differed from the one with GenBank accession number KC291086 about 3%. Genetic distance between samples of Laos ranges from 0.4 to 0.6%. *T. vogeli* is the most divergent from the rest in the clade with more than 4% of genetic distance (Table 2).

Four remaining samples from Laos, HNN121, HNN155, AT-2011, and TK16.04, are all labeled as *Trimeresurus* sp. because they were not paired with any existing species in the tree (Fig. 3A, 3B). Three samples, HNN155, AT-2011, and TK16.04, clustered in a single clade with strong support from both analyses, while HNN155 and AT-2011 formed a lineage with significant support only from BI. HNN155 and AT-2011 are around 4% divergent, HNN155 and TK16.04 around 4.6 %, and AT-2011 and TK16.04 approximately 2.7%. HNN121 is the most distinct one with at least 11% genetic divergence from other species of *Trimeresurus* included in this study (Table 2). The populations from Laos are interesting taxonomically and should be further investigated, especially with a focus on morphological distinction from other species within the genus. It is also important that more specimens be collected for comparative study.

Table 2. Uncorrected ("p") distance matrix showing percentage pairwise genetic divergence (COI) between different *Trimeresurus* clades

Species name	1	2	3	4	5	6	7	8	9	10	11	12
1. *T. albolabris*	*(0.0-2.85)*											
2. *T. gumprechti* group	10.2-13.7	*(0.0-6.2)*										
3. *T. macrops* group	12.3-15.3	9.5-14.6	*(8.2)*									
4. *T. medoensis*	10.5-12.2	5.8-8.1	9.9-12.8	-								
5. *T. popeiorum*	12.5-13.6	9.9-11.9	11.2-13.7	11.4	-							
6. *T. purpureomaculatus*	4.5-5.6	10.9-12.5	13.1-15	11.1	12.9	-						
7. *T. sichuanensis*	11.7-13.4	9.5-12.0	11.3-13.4	10.5	10.5	12.0	*(0.0)*					
8. *T.* sp.1	11.6-13.9	5.4-8.4	10.4-14.2	7.2-8.2	10.7-11.6	11.1-12.9	9.4-10.2	*(2.7-4.6)*				
9. *T.* sp.2	12.6-14.7	11.2-13.1	12.3-14.5	13.2	13.7	14.0	12.0	11.4-12.7	-			
10. *T. stejnegeri* group	10.7-13.3	5.9-8.5	9.3-13.3	6.8-7.8	9.8-11.1	9.9-11.7	9.0-10.1	5.1-7.3	11.1-12.1	*(0.0-4.2)*		
11. *T. tibetanus*	12.5-13.8	10.5-11.7	13.0-15.3	11.4	10.7-10.8	12.5	11.4-11.6	9.8-12.4	12.4	10.4-11.4	*(0.0)*	
12. *T. yunnanensis*	13.2-14.5	7.5-8.9	10.0-12.8	7.7	12.3	12.8	10.5	6.6-7.7	12.0	6.7-7.7	11.4	-

Note: *T. gumprechti* group including *T. "gumprechti"* and *T. stejnegeri* (Genbank accession number: KP403710 and MK064832); *T. macrops* group including *T. macrops* and *T. popeiorum; T. stejnegeri* group including *T. stejnegeri* and *T. vogeli;* Pairwise distance within clades is italicized and shown in parenthesis.

CONCLUSION

Our phylogenetic analyses of pitviper samples collected from Vietnam and Laos show that the diversity of the genus *Trimeresurus* in the region has not been well understood. Taxonomic status of several cryptic populations discovered in this study should be investigated in future work. In particular, morphological analyses need to be conducted to compare collected specimens from genetically divergent populations with those from existing congeners. As our taxonomic sampling only represents a small subset of areas where the widely distributed group occurs in both countries, further studies into poorly studied regions might reveal additional distinct populations, and potentially new taxa.

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