Article

Troublesome Trimes: Potential cryptic speciation of the *Trimeresurus* (*Popeia*) *popeiorum* complex (Serpentes: Crotalidae) around the Isthmus of Kra (Myanmar and Thailand)

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Abstract

The taxonomic identity of the *Trimeresurus* (*Popeia*) *popeiorum* complex from the Isthmus of Kra and to the north was investigated. Several studies over the last decade have produced several specimens and associated mtDNA sequence data for a variety of individuals of the *T. popeiorum* and "*T. sabahi*" complexes. Here, we combine four mitochondrial genes (12S, 16S, ND4, and CytB) from all available specimens in GenBank with the addition of five new specimens collected from the mainland, Tanintharyi Region of Myanmar. Maximum Likelihood and Bayesian analyses identified that *T. popeiorum sensu lato* is paraphyletic with two geographically distinct clades: a northern clade representing populations from northern Myanmar, Laos and northern Thailand and a southern clade representing samples from the Tanintharyi Region and adjacent west Thailand. While the two clades have considerable genetic distance, they appear to be morphologically identical, leading to the hypothesis that the southern clade represents a cryptic, undescribed species. Because they appear to be cryptic species and the limitation of only five specimens from the southern lineage, this does not permit us to formally describe the new species. In accordance to past molecular studies, we uncovered paraphyly and lack of genetic support for the validity of taxa within the *T. sabahi* complex. However, we suggest recognizing these populations as subspecies within *T. sabahi*.

Key words: Cryptic speciation, Myanmar, Southeast Asia, Subspecies, Tanintharyi Region, Thailand, *Trimeresurus*

Introduction

Often times new species are described based on few individual specimens available, sometimes only from the type series, which can be problematic for interspecific comparisons. This can be particularly problematic in species with sexual dimorphism and/or from cryptic species complexes. Additionally, sequences in GenBank are often not represented by voucher specimens and sequences can be misidentified, which confounds resolution of relationships. Cryptic species, morphologically indistinguishable but genetically and/or reproductively isolated (Bickford *et al.* 2006; Jörger *et al.* 2013), can confound taxonomic matters even further (Funk *et al.* 2012). Molecular sequencing methods can be extremely useful for determining cryptic species (Hebert *et al.* 2004), particularly in groups with limited samples for morphological comparisons.

Southeast Asian Green Pitvipers (Genus: *Trimeresurus*) are notoriously difficult to classify. Despite the abundance of specimens in museum collections for some species, morphological conservatism in the genus makes taxonomic studies challenging and some species are only represented by few museum specimens. Such limited samples have resulted in misidentifications within the genus (e.g. Orlov & Helfenberger, 1997) that were subsequently corrected by others (Malhotra & Thorpe 2000; Giannasi *et al.* 2001; Tillack *et al.* 2003). Nevertheless, species diversity in *Trimeresurus* is likely underestimated, as detailed examination of several groups has revealed undescribed or revalidated species (Vogel *et al.* 2004; David *et al.* 2006; Grismer *et al.* 2006; David *et*

al. 2009; Guo & Wang, 2011; Malhotra *et al.* 2011; Sumontha *et al.* 2011; Vogel *et al.* 2014a; Vogel *et al.* 2014b). Molecular phylogenetic analyses also have identified cryptic diversity in some species (Guo *et al.* 2015; Thorpe *et al.* 2015).

Recent (2015–2016) rapid assessment surveys in southern Tanintharyi Region, Myanmar by one of us (DGM) yielded three specimens, which we assigned to the subgenus *Popeia* (Malhorta & Thorpe, 2004), specifically to *Trimeresurus popeiorum* Smith, 1937. Recently, several new species in this and the *T. sabahi* complexes have been described (e.g. Vogel *et al.* 2004; Grismer *et al.* 2006; David *et al.* 2009). Additional studies using mitochondrial DNA (mtDNA) sequence data have attempted to unravel these complexes (Malhorta and Thorpe 2004; Sanders *et al.* 2006). A recent study claims to resolve most taxonomic issues for this group (Wostl *et al.* 2016); however, a comprehensive analysis has yet to be conducted, and questions remain as to what species occur in the Tanintharyi.

Briefly, the *Popeia* subgeneric group occurs from northern India to southern China (Guo *et al.* 2015) southward through Myanmar, Thailand, Indochina, and Malay Peninsula into Borneo and Sumatra. The type species, *Trimeresurus popeiorum*, was described in 1937 by Malcolm A. Smith without any precise type locality or type specimen. This was subsequently corrected by Taylor & Elbel (1958). Later, Regenass & Kramer (1981) described two new subspecies (*T. p. barati* and *T. p. sabahi*) in the complex. In 2004, Malhotra & Thorpe performed a revision of Southeast Asian *Trimeresurus*. Their study included both morphological (hemipenes, scalation) and genetic (mtDNA) characters. They suggested the subdivision of the *Trimeresurus* species into several "new" genera. One of these genera was *Popeia* for *T. popeiorum*. In the same year, Vogel *et al.* (2004) revised *T. popeiorum* and, on the basis of morphological characters, recognized two new species in Peninsular Malaysia: *Trimeresurus fucatus* Vogel, David & Pauwels, 2004 in southern Thailand and Peninsular Malaysia and *Trimeresurus nebularis* Vogel, David & Pauwels, 2004 restricted to the Cameron Highlands of Peninsular Malaysia. They also elevated *Trimeresurus barati* (Regenass and Kramer, 1981) for the Sumatran populations and *T. sabahi* (Regenass and Kramer, 1981) for the "*popeiorum*" of Borneo. This new taxonomy resulted in the distribution *T. popeiorum* being the northern portion of the previous Southeast Asian-wide range but also identified the populations as far south as Myeik, (Tanintharyi, Myanmar), as *T. popeiorum*. Their distributional concept, however, disagreed with their identification of several specimens, i.e., they considered a female BMNH 1924.5.20.38 from "Taok Plateau, Tenasserim" (now Mt. Pya Taung, Tanintharyi, Myanmar) as *T. popeiorum*, and two males: BMNH 56.5.6.105 from Myeik, Tanintharyi, Myanmar and BMNH 1940.3.9.43 from Kanmaw Kyun Island (= Kisseraing Island), Tanintharyi, Myanmar as *T. fucatus*. Subsequently, Pauwels and Chan-ard (2006) identified *Trimeresurus* (*Popeia*) from Keang Krachan National Park, Thailand as *T. fucatus*.

Sanders *et al.* (2006) using an expanded molecular and morphological data from Malhotra and Thorpe (2004), defined two clades within the *Popeia* complex. The Northern Clade contained all specimens north of the Isthmus of Kra and one specimen (B467) near Phang-nga (south Thailand). The Southern Clade contained all specimens further south in the Malay Peninsula and the islands of Indonesia. Sanders *et al.* (2006) recommended a more conservative taxonomy, placing all *Popeia* species from the Sundaland region into *T. sabahi,* except for *T. nebularis* and retaining *T. popeiorum* for specimens north of the Malay Peninsula. Sanders *et al.* (2006) also examined the morphology of a specimen from Tanintharyi, identified by Vogel *et al.* (2004) as *T. fucatus* (BMNH 1940.3.9.43), and it was identified as part of the "Northern Clade". However, they did not examine any other individuals from Tanintharyi and provided no meristic data from the BMNH specimen. They included uncatalogued specimens from Phetcheburi Province (AMB52; B34) in their statistical sample and identified them as *T. popeiorum*; these specimens were paraphyletic with respect to other *T. popeiorum* specimens in their molecular phylogenetic analysis. Another uncatalogued specimen from Phang-nga Province, Thailand (B467) was also included in the northern clade, and this inclusion also rendered *T. popeiorum* paraphyletic.

The taxonomy suggested by Sanders *et al.* (2006) remains controversial. Subsequent studies focusing on the *Popeia* subgenus continue to follow the taxonomy suggested by Vogel *et al.* (2004). Grismer *et al.* (2006) described *T. buniana* from Pulau Tioman Island off the coast of Peninsular Malaysia. David *et al.* (2009) described northern Sumatran specimens as *T. toba* and recommended that the genus *Popeia* (Malhotra & Thorpe, 2004) be used as a subgenus in order to preserve the definitive nature of the genus *Trimeresursu*s. David *et al.* (2011) expanded on this recommendation and identified the nucleospecies (= type species) of the genus *Trimeresurus* as *T. viridis* Lacépède, 1804 (= *T. insularis* Kramer, 1977) and officially recognized all genera proposed by Malhotra & Thorpe (2004) as subgenera with the exception of *Ovophis* and *Protobothrops*. Based on morphometrics, Sumontha *et al.* (2011) described *T. phuketensis*, a species endemic to Phuket Island, Thailand. It is unique among *Popeia* in that both

males and females contain a bicolored postocular and ventrolateral stripe. Most recently, Wostl *et al.* (2016) added molecular data for two previously un-sampled taxa (*T. barati* and *T. toba*), but used only two (of four) mtDNA genes used by Sanders *et al.* (2006). Wostl *et al.* (2016) also identified all *Popeia* from the Sundaland biogeographic region as *T. sabahi,* including *T. buniana* and *T. toba.* However, they did not evaluate the taxonomic status of *T. phuketensis* as there were no genetic sequences available; they also did not include the south (B467) and west (AMB52; B34) Thai samples of *T. popeiorum*—which rendered the species paraphyletic in Sanders *et al.* (2006).

In spite of the large number of taxonomic studies focused on the *Popeia* subgenus, the identity of populations in the Tanintharyi region and the Isthmus of Kra remains uncertain. No comprehensive molecular dataset has been used to examine the affinities of the green *Trimeresurus* from this area. Here, we use recently collected specimens, augmented with additional California Academy of Sciences (CAS) specimens from the Tanintharyi Region, to determine which species occur in this region. We investigate the identity and relationships of these specimens with those in adjacent areas using molecular and morphological data by including all available specimens available in GenBank, all four mtDNA loci, and morphological data provided in previous studies.

Material and methods

All three specimens from the 2015–2016 survey were deposited in the National Museum of Natural History, Smithsonian Institution (USNM). The first specimen (USNM 587588) was collected in Lenya in May 2015. Two specimens, both adult females, (USNM 587918 and USNM 587919) were collected in Ywahilu in May 2016. One of these (USNM 587918) was found dead on the road and partially skeletonized, hence unavailable for morphological study. However, tissue samples were taken and the specimen is vouchered as a skeleton. Two specimens (a juvenile female USNM 587920, adult male USNM 587921) collected from Kawthaung, Tanintharyi, Myanmar, were included in the morphological analysis. Our molecular analyses also included specimens from the California Academy of Sciences: Dawei Township (a male CAS 245932) and Kawthaung (a female CAS 247754).

Tissue samples were taken from the liver and heart and preserved in salt-saturated DMSO/EDTA buffer for genetic analyses. Extractions of genomic DNA were conducted on small pieces of liver or muscle tissue and run on an Auto-Genprep 965 (2011 AutoGen, Inc.), using standard phenol manufacturer protocols. Genomic DNA was eluted in 100 µl of AutoGen R9 re-suspension buffer. We sequenced four mitochondrial genes CytB, ND4, 16S and 12S. Primers used for each gene are identified in Table 1. Cycle-sequence reactions were performed in both directions, using the PCR primers using BigDye Terminator v3.1 Cycle Sequencing Kit's in 0.25 × 10 µl reactions run on and ABI3730 Sequencer (2011 Life Technologies) using the 950 chemistry. Raw trace files were edited in Geneious 9.1.5 (Biomatters Ltd 2005–2016), complementary strands were aligned, edited, and inspected for translation. All sequences were deposited in GenBank under accession numbers MF476856–MF476874. Outgroups were chosen based on close phylogenetic relationship between taxa (Alencar *et al.* 2016). Additional genetic material along with our outgroups came from published records in GenBank (see Table 2). We performed maximum-likelihood (ML) analyses on the concatenated mtDNA using RAxML (v8.2.9, Stamatakis, 2014) with the rapid bootstrap inferences (1000 replicates) and subsequent GTRCAT thorough ML search, with each gene as a separate partition. We also conducted Bayesian analyses using MrBayes (v3.2.6; Ronquist *et al.* 2012). We partitioned our dataset by locus, applied the GTR+I+G model, and unlinked all partitions. We ran our analyses for 10×10^6 generations with four chains, sampling every 1000 generations. Stationarity was assessed by the average standard deviation of split frequencies (ASDSF < 0.01) and visual plots of log-likelihood by generation in Tracer v1.2 (Rambaut and Drummond, 2004); the first 1,000 trees (of 10,000) were discarded as the burn-in. A 50% majority-rule with compatible groups consensus was taken from the remaining trees and posterior probabilities (pp) of 0.95 or above were considered significant.

We examined morphological characters considered diagnostic to the *Popeia* subgenus based on previous studies (Pope & Pope, 1933; Regenass & Kramer, 1981; Vogel *et al.* 2004). Although the most recent taxonomic treatment of the subgenus *Popeia* (Wostl *et al.* 2016) indicated that all Sundaic populations should be recognized as *T. sabahi*, we only compared the morphology of our specimens to the Thai-Malaysian populations of *T. sabahi* recognized as *T. fucatus* by Vogel *et al.* (2004) (see Table 2). This decision makes it easier for us to compare our specimens on a local basis, as the allopatric populations of *T. sabahi* defined by Wostl *et al.* (2016) as well as

Sanders *et al.* (2006) each contain relatively stable morphologies, sexual dimorphism and ecology. Ventral scale count methodology follows Dowling (1951). Color pattern vocabulary follows Vogel *et al.* (2004).

TABLE 1. List of primers used to amplify each mitochondrial gene in our study.

Locus	Primer	Direction	Temp.	Sequence 5' to 3'	Reference
12S	12SI	Forward	48	TGCCAGCAGYCGCGGTTA	Puillandre *et al.* 2009
12S	12SIII	Reverse	48	AGAGYGRCGGGCGATGTGT	Puillandre *et al.* 2009
16S	16Sar–L	Forward	54	CGCCTGTTTATCAAAAACAT	Palumbi *et al.* 1991
16S	16Sbr–H	Reverse	54	CCGGTCTGAACTCAGATCACGT	Palumbi *et al.* 1991
CytB	Gludge	Forward	48	TGACTTGAARAACCAYCGTTG	Parkinson *et al.* 2002;
CytB	ATRCB3	Reverse	48	TGAGAAGTTTTCYGGGTCRTT	Parkinson *et al.* 2002;
ND4	HypLeu2r.1	Forward	48	TACCACTTGGATTTGCACCA	Mulcahy 2008 MPE
ND4	HypNad4f.1	Reverse	48	TGCCTAGCAGCCTTYATAGCTA	Mulcahy 2008 MPE

Results

We obtained alignments of the mitochondrial genes CytB (826 bp), ND4 (846 bp), 16S (539 bp) and 12S (410 bp) for a total of 2621 bp of aligned sequence data. Our ML analyses placed specimens from northern Myanmar sister to *T. nebularis*, with poor bootstrap value support (<50%, Fig. 1). An outlying specimen (B467), from Phang-nga Province, south Thailand, initially identified as *T. popeiorum* was placed at the base of a clade containing the northern Myanmar *T. popeiorum* + *T. nebularis* specimens with strong support (91%). The latter two were sister to one another, but by a very short branch length with poor support (<50%). The Tanintharyi Region and western Thailand specimens (AMB52 and B34 of Sanders *et al.* 2006) were placed sister to this clade (Fig. 1). However, similar to the taxonomy of Wostl *et al.* (2016) and Sanders *et al.* (2006), we recovered a single, well-supported clade containing all Sundaic populations of *Popeia* with strong support (100%). Our Bayesian results were very similar, with the main difference being the south Thai sample (B467) was placed sister to *T. nebularis* clade (albeit with poor posterior probability support and short branch; <.50 pp), and they were placed sister to the northern *T. popeiorum* samples, with strong support (pp = 0.99). The *T. sabahi* clade was resolved with strong support (pp = 0.99), relationships among the lineages in this clade were slightly different from the ML topology, but were also poorly supported (values shown in Fig. 1).

The morphology is summarized in Table 3. All specimens are described as followed: TailL/TotalL ratio 20.9% in the male, 15.0–17.0% in females. The dorsal pattern in all specimens was solid green, except for the juvenile specimen (USNM 587920), which had faint irregular vertebral crossbars. It is unclear what color they were in life, but they are dark green in preservative. Postocular striping in females is faint but present in all specimens as a thin white line; in the male (USNM 587921), the postocular streak is bicolored with the thin section (bottom) plain white and the wide section (top) red. Ventrolateral striping in females is extremely faint, less than half a dorsal scale wide, visible as margins on the dorsal scales and is plain white. In the male (USNM 587921), the ventrolateral stripe is bicolored with the bottom being deep red and the top plain white, extending to the tail where it becomes sporadic. The eye color in life (available from photographs of female specimens USNM 587588 and USNM 587919) is red. The tail is mottled in rusty-red in all specimens with no clear distinction between the two colors, but females appear to have a green border laterally. Snout truncated; distinct but no sharp canthus rostralis; rostral visible from above; occipital scales distinctively keeled in the male (USNM 587921), slightly keeled in females; temporals only slightly keeled in all specimens. Loreal pit in contact with second labial; nostril always distinct from first labial; two preoculars in contact with loreal pit; single subocular always long and crescent shaped; one or two rows of scales between subocular and supralabials; first infralabial largest; two chin shields; mental never in contact with chin shields. Dorsal scales keeled and in 21 rows at midbody; Ventral scales in females 165–171, 169 in male specimen; subcaudals 57–65 in females, 72 in male specimen, and all have a single anal plate.

TABLE 2. Voucher information for specimens sequenced for this study, along with their GenBank accession numbers for each gene. See holding institution for additional locality information. (Abbreviations: CAS: California Academy of Sciences, San Francisco, USA; FMNH: Field Museum of Natural History, Chicago, USA; MZB-OPHI: Museum Zoologicum Bogoriense, Bogor à Cibinong, Java, Indonesia; USNM: United States National Museum, Washington DC, USA; all other abbreviations represent unvouchered wild-caught specimens sampled from Malhotra & Thorpe [2004] and Sanders et al. [2006]).

Taxon	Voucher Number	CytB	ND4	16S	12S	Country	Locality	Source
Trimeresurus albolabris	AMA 157	AF171884	AY352839	AY352744	AY352805	Hong Kong	n/a	Malhotra & Thorpe (2004)
Trimeresurus insularis	AMA 109	AY352767	AY352833	AY352738	AY352799	Indonesia	East Java	Malhotra & Thorpe (2004)
Trimeresurus tibetanus	ZMB 65641	AY352749	AY352810	AY352715	AY352776	Nepal	Helambu Prov.	Malhotra & Thorpe (2004)
Trimeresurus stejnegeri	AMA 160	AF171896	AY059593	AY059555	AY059539	Taiwan	Taipei County	Malhotra & Thorpe (2004)
Trimeresurus barati	MZB-OPHI 5197	KP899264	KP939322	–	–	Indonesia	Sumatra, Jambi Prov.	Wostl *et al.* (2016)
Trimeresurus barati	UTA-R 61640	KP899263	KP939321	–	–	Indonesia	Sumatra, Jambi Prov.	Wostl *et al.* (2016)
Trimeresurus barati	MZB-OPHI 5199	KP899262	KP939320	–	–	Indonesia	Sumatra, Lampung Prov.	Wostl *et al.* (2016)
Trimeresurus barati	UTA-R 61639	KP899261	KP939319	–	–	Indonesia	Sumatra, Lampung Prov.	Wostl *et al.* (2016)
Trimeresurus barati	AMB 361	AY371801	AY371837	AY371769	AY371753	Indonesia	Sumatra, Bengkulu Prov.	Malhotra & Thorpe (2004)
Trimeresurus buniana	AMB 519	AY371818	AY371853	AY371778	AY371752	Malaysia	Pulau Tioman	Sanders *et al.* (2006)
Trimeresurus fucatus	B246	AY059570	AY059589	AY059556	AY059540	Malaysia	Fraser's Hill	Malhotra & Thorpe (2004)
Trimeresurus fucatus	B278	AY371821	AY371857	AY371780	AY371750	Malaysia	Fraser's Hill	Malhotra & Thorpe (2004)
Trimeresurus fucatus	AMA 203	AY371796	AY059588	AY059553	AY059537	Thailand	Nakhon si Thammarat Prov.	Malhotra & Thorpe (2004)
Trimeresurus fucatus	AMA 202	AF171904	AY371840	AY371770	AY371739	Thailand	Thung Song Prov.	Sanders *et al.* (2006)
Trimeresurus fucatus	A246	AY371820	AY371856	–	AY371749	Thailand	Thung Song Prov.	Sanders *et al.* (2006)
Trimeresurus fucatus	B19	AY371804	AY371844	AY371779	–	Thailand	Thung Song Prov.	Malhotra & Thorpe (2004)
Trimeresurus nebularis	A197	AY371808	AY371846	AY371773	AY371746	Malaysia	Cameron Highlands	Sanders *et al.* (2006)
Trimeresurus nebularis	B235	AY371812	AY371838	–	AY371740	Malaysia	Cameron Highlands	Sanders *et al.* (2006)
Trimeresurus nebularis	B236	AY371819	AY371847	–	AY371747	Malaysia	Cameron Highlands	Sanders *et al.* (2006)
Trimeresurus nebularis	B237	AY371813	AY371848	–	AY371748	Malaysia	Cameron Highlands	Sanders *et al.* (2006)
Trimeresurus nebularis	B238	AY371814	AY371839	AY371774	AY371737	Malaysia	Cameron Highlands	Sanders *et al.* (2006)
Trimeresurus nebularis	B345	AY371811	AY371849	AY371775	–	Malaysia	Cameron Highlands	Malhotra & Thorpe (2004)

TABLE 2. (Continued)

Taxon	Voucher Number	CytB	ND4	16S	12S	Country	Locality	Source
Trimeresurus nebularis	B346	AY371810	AY371850	–	–	Malaysia	Cameron Highlands	Malhotra & Thorpe (2004)
Trimeresurus popeiorum	AMB 196	AY059571	AY059590	AY059554	AY059538	Laos	Phongsali Prov.	Sanders *et al.* (2006)
Trimeresurus popeiorum	FMNH 258950	AY059571	AY059590	AY059554	AY059538	Laos	Phongsali Prov.; Phongsali Dist.	Malhotra & Thorpe (2004)
Trimeresurus popeiorum	CAS 205847	AY371816	AY371855	AY371783	AY371751	Myanmar	Bago Div.: Bago Yoma	Sanders *et al.* (2006)
Trimeresurus popeiorum	CAS 222195	AY371806	AY371841	AY371777	AY371738	Myanmar	Mon State: Kyaihto Twp.	Sanders *et al.* (2006)
Trimeresurus popeiorum	CAS 216609	AY371805	AY371845	AY371776	AY371738	Myanmar	Mon State: Kyaihto Township	Sanders *et al.* (2006)
Trimeresurus popeiorum	CAS 245932	MF476865	MF476870	MF476860	MF476856	Myanmar	Tanintharyi Div.; Dewei Dist.	This study
Trimeresurus popeiorum	CAS 247754	MF476866	MF476871	MF476861	MF476857	Myanmar	Tanintharyi Div.; Kawthaung Dist.	This study
Trimeresurus popeiorum	USNM 587988	MF476867	MF476872	MF476862	MF476858	Myanmar	Tanintharyi Div.; Lenya	This study
Trimeresurus popeiorum	USNM 587918	MF476868	MF476873	MF476863	–	Myanmar	Tanintharyi Div.; Ywahilu	This study
Trimeresurus popeiorum	USNM 587919	MF476869	MF476874	MF476864	MF476859	Myanmar	Tanintharyi Div.; Ywahilu	This study
Trimeresurus popeiorum	A204	AF171902	AY371843	AY371784	AY371742	Thailand	Chiang Rai Prov.	Malhotra & Thorpe (2004)
Trimeresurus popeiorum	A205	AF171906	AY371854	AY371767	AY371741	Thailand	"North"	Sanders *et al.* (2006)
*Trimeresurus popeiorum**	B467	AY371807	AY371851	AY371781	AY371744	Thailand	Phang-nga Prov.	Sanders *et al.* (2006)
Trimeresurus popeiorum	AMB 52	AY371800	AY371836	AY371768	AY371754	Thailand	Phetcheburi Prov.	Sanders *et al.* (2006)
Trimeresurus popeiorum	B34	AY059572	AY059591	AY059558	AY059542	Thailand	Phetcheburi Prov.	Malhotra & Thorpe (2004)
Trimeresurus sabahi	AMB 344	AY371815	AY371842	AY371771	AY371736	Malaysia	Borneo, Sabah	Malhotra & Thorpe (2004)
Trimeresurus sabahi	AMB 341	AY371803	AY371834	AY371772	AY371734	Malaysia	Borneo (presumably Sabah)	Sanders *et al.* (2006)
Trimeresurus toba	MZB-OPHI 5342	KP899266	KP939324	–	–	Indonesia	Sumatra, Sumatera Utara Prov.	Wostl *et al.* (2016)
Trimeresurus toba	UTA-R 61641	KP899265	KP939323	–	–	Indonesia	Sumatra, Sumatera Utara Prov.	Wostl *et al.* (2016)

*We consider these sequences to represent *T. phuketensis* (see Discussion).

TABLE 3. Variation of morphological characters between *Trimeresurus (Popeia) popeiorum* and *Trimeresurus (Popeia) fucatus* (data based from Vogel et al. 2004) in comparison to four specimens collected from the Tanintharyi Division, Myanmar.

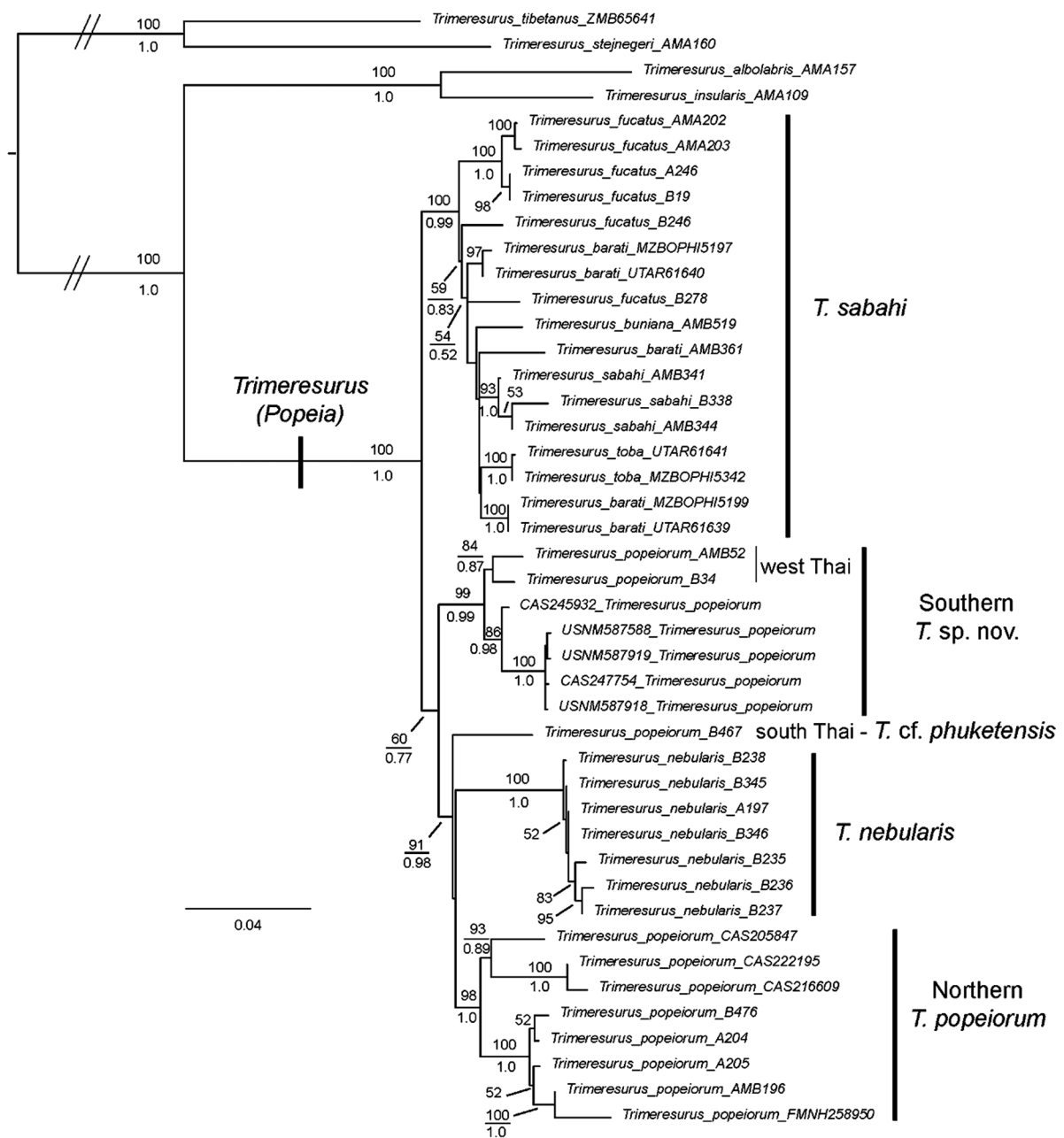| Catalog	USNM 587588	USNM 587919	USNM 587920	USNM 587921	T. (P.) popeiorum	T. (P.) popeiorum	T. (P.) fucatus	T. (P.) fucatus
Sex	♀	♀	♀	♀	♀	♀	♀	♀
SVL	406	452	246	495	–	–	–	–
TailL	78	80	47	131	–	–	–	–
TotalL	484	532	293	626	–	–	–	–
HeadL	22.8	22.9	15.4	26.5	–	–	–	–
HeadW	16.6	14.9	10.5	17.6	–	–	–	–
TailL/TotalL ratio	0.170	0.150	0.160	0.209	0.181–0.211	.149–.173	0.201–0.241	.159–.189
Ventrals	169	165	171	169	151–166	154–168	156–171	157–170
Subcaudals	60	57	65	72	59–75	56–64	69–84	59–73
Supralabials	10/11	10/11	11/10	11/11	9–11	9–11	9–12	9–12
Infralabials	13/14	13/13	13/12	13/13	10–15	10–15	10–15	10–15
Ventrolateral stripe	Present	Present	Present	Present	Present	Present	Present	Present
Color of ventrolateral stripe	White	White	White	Bicolored red/white	Bicolored deep red/White	White	Bicolored or white	White
Postocular streak	Present	Absent	Present	Present	Present/rarely absent	Present/rarely absent	Present/absent	Absent
Color of postocualr streak	White	Absent	White	Bicolored red/white	Bicolored deep red/White	White	Bicolored or white or absent	Absent
Blotches	Absent	Absent	Present	Absent	Absent	Absent	Present	Absent
Eye Color	Red	Red	Unknown	Unknown	Red	Red	Yellow/Copper/Green	Yellow/Copper/Green
Tail color	Rusty red	Rusty red	Rusty red	Rusty red	Rusty red	Rusty brown	Rusty brown	Rusty brown
Tail pattern	Mottled, green laterally	Mottled, green laterally	Mottled, green laterally	Mottled, no bordering	Mottled, no bordering	Mottled, no bordering	Mottled, no bordering	Mottled, no bordering

FIGURE 1. Maximum-Likelihood phylogeny of the *Trimeresurus* (*Popeia*) subgenus based on 2621 base–pairs of mtDNA from four loci (ND4, CytB, 12S, and 16S). Major clades found are labeled using vertical lines with their designated taxonomy. Maximum-Likelihood bootstrap values are shown above and Bayesian posterior-probabilities are shown below, for relevant nodes.

Discussion

Species assignment in the subgenus *Popeia* is challenging because of previous name assignments to specimens do not match their "identity" in molecular phylogenies. For example, the specimen (B467) from Phang-nga Province, Thailand, identified as "*Popeia popeiorum*" (sensu Sanders *et al.* 2006), is placed sister to the *T. nebularis* + northern *T. popeiorum* populations, which is enigmatic. The sequence data for B467 have no further locality information, nor a voucher specimen. Possibly, it was examined in the field for morphological data and blood

samples, and was not collected as a voucher. Sumontha *et al.* (2011) described *T. phuketensis* from nearby Phuket Province, Thailand, but they did not provide any tissue for a molecular analysis. It is possible that the south Thai sample (B467) represents *T. phuketensis*, which was not described at the time it was sampled. Presently, *T. phuketensis* is only known from Phuket Island. Recent studies of the agamid lizard *Bronchocela rayanesis* showed that species, initially described from Langkawi Island, Malaysia (Grismer *et al.* 2015), also occurs on Phuket Island and mainland Phang-nga Province, Thailand (Grismer *et al.* 2016; Zug *et al.* 2017). The tree agamid lizard *Acanthosaura phuketensis* (Pauwels *et al.* 2015), also seems to occur on both Phuket Island and nearby adjacent Phang-nga, Province. Until sequences from the type locality of *T. phuketensis* are analyzed, the status of B467 remains problematic. Our data supports *T. phuketensis* as valid; and we tentatively identify the sequences of B467 to represent this species.

Most of the Tanintharyi specimens in our dataset are morphologically identical to *T. popeiorum.* In all females, a faint plain white postocular stripe is always present; adults lack vertebral spots; eye color of specimens photographed in life is deep red. These characters match the descriptions of Vogel *et al.* (2004). There is some overlap with features of *T. fucatus*. Most ventral scale counts of the specimens examined are at the high end of *T. popeiorum.* In the juvenile female specimen USNM 587920 for example, the ventral scale count is 171 (which is higher than in both species), the number of subcaudals is higher than *T. popeiorum* and within the range of *T. fucatus*, and vertebral spots are usually absent in *T. popeiorum.* In the male (USNM 587921), the number of ventral scales is 169, higher than the known ventral scale counts of male *T. popeiorum* (151–166; Vogel *et al.* 2004). However, the ventral and caudal scale counts in these specimens are not significantly different from *T. popeiorum* and may represent clinal variation. Similar trends have been reported in other snakes (Mulcahy & Archibald, 2003; Lee *et al.* 2016), including pit vipers (Ashton, 2001). We have not located in-life photographs of these specimens or any other specimens from Kawthaung, or field notes documenting coloration. Therefore, characteristics such as eye color, presence of white vertebral spots, and other important characters that diagnose species of the *Popeia* subgenus, cannot be determined and affect the accuracy of our identification. Nevertheless, all Tanintharyi and the west Thai specimens (AMB52, B34) of Sanders *et al.* (2006) do not form a clade with any other *Popeia* in our molecular dataset from the Thai–Malay Peninsula and Indonesia. Instead, they form a well-supported lineage of their own from the Tanintharyi Region and neighboring Thailand that is tentatively (60% ML and pp = 0.77) placed sister to *T. nebularis* + northern *T. popeiorum*, + south Thai specimen (B467 of Sanders *et al.* 2006), the latter sister to *T. nebularis* + northern *T. popeiorum* in our ML phylogeny (nested among them in our Bayesian analysis). Our topology is similar to the results of Sanders *et al.* (2006) and Wostl *et al.* (2016), both of which also recovered a paraphyletic *T. popeiorum* with respect to the samples from west Thailand. However, the species *T. fucatus* in our tree is paraphyletic. This may be because Wostl *et al.* (2016) only sampled two closely-related *T. fucatus* (A202–3), and used only two mitochondrial genes, while our phylogenetic analysis used four genes and all available genetic samples.

Herein, our molecular results lead us to consider three possible taxonomic solutions: (A) recognize all clades as a single species—*T. popeiorum*; (B) consider the two most diverged clades as species—*T. popeiorum* (including *T. nebularis*) and *T. sabahi*; or (C) consider all clades as distinct species. Indeed, solutions A and B are conservative approaches that stabilize the taxonomy of the *Popeia* subgenus significantly. However, both approaches ignore genetic diversity and distinctiveness of *T. nebularis*, which, under multiple sources, is morphologically and ecologically distinct (Vogel *et al.* 2004; Sanders *et al.* 2006). Because of this, we believe that solution C—recognizing both northern and southern clades of *T. popeiorum*, as well as *T. nebularis*, *T. phuketensis,* and *T. sabahi* (sensu Sanders *et al.* 2006 and Wostl *et al.* 2016) is the most suitable decision. In this case, it may be applicable to recognize the allopatric, yet closely related populations of *T. sabahi* as subspecies.

Interest in the application of the subspecies designation has been recently discussed within snakes and other reptiles (Mulcahy, 2008; Hawlitschek *et al.* 2012; Tolstrom *et al.* 2014; Kaito *et al.* 2017). Nevertheless, unanimous criteria for the recognition and designation of subspecies in reptiles and amphibians are lacking. While we acknowledge that the assignment of subspecies will likely subside on a case-by-case basis, we offer the following justifications for why we believe designating taxa in the *T. sabahi* clade as subspecies is the appropriate taxonomic decision following similar arguments for the generalized lineage concept and species criteria discussed in Mulcahy (2008). First, all of the previously recognized taxa (*T. sabahi*, *T. fucatus*, *T. barati*, *T. buniana* and *T. toba*) are all geographically-cohesive (allopatric) with respect to one another (therefore, likely no current gene-flow). The populations do not exhibit intergradation, they are morphologically distinct, yet they are not genetically

FIGURE 2. Live specimens of *Trimeresurus* (*Popeia*) collected from the Tanintharyi Division, Myanmar examined in our study. (A) Adult female specimen of *Trimeresurus* (*Popeia*) **sp. nov** from Lenya, Tanintharyi Division, Myanmar (USNM 587588). (B-C) Adult female specimen of *Trimeresurus* (*Popeia*) **sp. nov** from Ywahilu, Tanintharyi Division, Myanmar (USNM 587919). Photographs by Daniel G. Mulcahy.
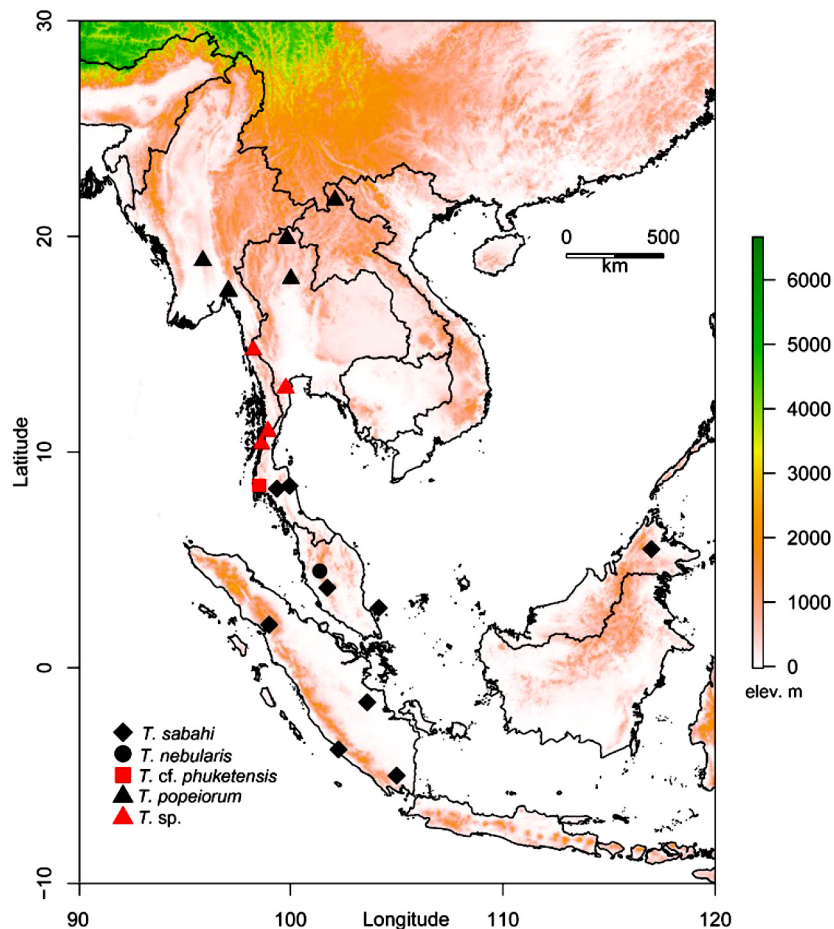
FIGURE 3. Distribution map showing the molecular sampling of *Trimeresurus* (*Popeia*) in Southeast Asia. See symbols for species identification.

distinct, which could be caused by incomplete lineage sorting or recent introgression. Regarding the morphological distinctiveness of the Sundaland taxa, Wostl *et al.* (2016) argued that these populations exhibited minimal morphological differences; however, they only examined a few specimens from Sumatra (five *T. barati* and three *T. toba*), and did not examine comparative material from museum collections. Instead, Wostl *et al.* (2016) recorded morphological characters from photographs for *T. fucatus* and *T. nebularis* and did not account for the sexual dimorphism present in populations referable to *T. buniana* and *T. fucatus*. By not recognizing these taxa, one undermines the potential diversity present in this group. The subspecies designation may be of particular importance in *T. buniana*, endemic to Pulau Tioman, Malaysia, because it is listed as "Endangered" under the IUCN Red List. A flagship species, *T. buniana* and the island itself are under threat by habitat loss and purchase of the island by a private company. Treating this taxon as a synonym would undermine its conservation status and potentially result in its extinction. Designating this population as a subspecies, will enable it to maintain protection better than conservation management definitions, such as, an evolutionary significant unit (ESU) or management unit (MU), particularly in this case where only one genetic sample is available where monophyly cannot be assessed; see Mulcahy *et al.* (2006 and references therein) for discussion of terms.

T. popeiorum was described by Smith (1937) without any precise type locality or type specimen. Taylor & Elbel (1958) corrected this, by designating a lectotype (BMNH 72.4.17.137) with the type locality "Khasi Hills, Assam, [State of Meghalaya], India". Since the specimens from the northern clade (from northern Myanmar, Thailand and Laos) are closest to the type locality, we assign *T. popeiorum sensu stricto* to the northern clade. The southern clade has significant genetic distance from the northern clade and is restricted to a distinct geographic area (Tanintharyi, Myanmar and western Thailand). Therefore, it is likely that these populations represent a cryptic, yet

undescribed species. Recent studies focusing on the Isthmus of Kra region of Myanmar and Thailand have discovered several endemic reptile species (Vogel *et al.* 2012; Pauwels *et al.* 2016; Zug *et al.* 2017; Connette *et al.* 2017). As yet, we have discovered no diagnostic character that separates the northern clade of *T. popeiorum* from the southern one. Cryptic species that are molecularly distinct yet morphologically similar may sometimes be the result of a poor morphological effort (Grismer *et al.* 2014). Furthermore, datasets for pit vipers may suffer from issues such as introgression and incomplete lineage sorting (Guo *et al.* 2015). Although we contend that the populations of the southern clade are cryptic, we refrain from formally describing it as our sample size is limited and prevents us from preparing a formal taxonomic description. Instead, we prefer to wait for a study that addresses these issues by incorporating nuclear DNA (preferably RadSeq or UCE; e.g. Leaché *et al.* 2015), additional morphological characters, and new material from potential sampling gaps. Until then, we refer to the populations representing the southern clade as *Trimeresurus* (*Popeia*) **sp. nov.**

In conclusion, we suggest researchers studying the herpetofauna of Southeast Asia continue to investigate the subgenus *Popeia* and to obtain more specimens from this region to allow for morphometric analyses. The possibility of an undescribed species in Malaysia (Sumarli *et al.* 2015) also suggests that the systematics of this group are still unresolved. A study that incorporates our samples with other newly collected material, along with additional morphological characters and nuclear DNA will hopefully provide a more insightful look at the phylogenetic relationships of the *Popeia* subgenus and resolve debates surrounding recent systematic interpretations within this group. We recommend the use of subspecies in the *T. sabahi* complex, recognizing the following taxa within the *Popeia* subgenus: *Trimeresurus* **sp. nov**, (Mulcahy *et al.* 2017), *Trimeresurus popeiorum* (Smith, 1937)*, Trimeresurus nebularis* (Vogel, David and Pauwels, 2004)*, Trimeresurus phuketensis* (Sumontha, Kunya, Pauwels, Nitikul, Punnadee, 2011)*, Trimeresurus sabahi sabahi* (Regenass & Kramer, 1981)*, Trimeresurus sabahi barati* (Regenass & Kramer, 1981)*, Trimeresurus sabahi buniana* (Grismer, Grismer & McGuire, 2006)*, Trimeresurus sabahi fucatus* (Vogel, David & Pauwels, 2004)*,* and *Trimeresurus sabahi toba* (David, Petri, Vogel & Doria, 2009).

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