Tropical Natural History 20(1): 1–15, April 2020

Discovering a Population of *Tylototriton verrucosus* (Caudata: Salamandridae) from Thailand: Implications for Conservation

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Received: 20 August 2019; Accepted: 17 December 2019

ABSTRACT.— The Asian newt genus *Tylototriton*, is the only urodelan genus currently recorded in Thailand. For a long time in the past, *T. verrucosus* was the sole known urodelan species found in Thailand, but was recently shown to be comprised of three cryptic species, *T. uyenoi*, *T. anguliceps*, and *T. panhai*. However, in our molecular study, the newts from Doi Chang, Chiang Rai Province were different from these three recognized *Tylototriton* species, and nested within the clade of *T. verrucosus* from China. They also had morphological traits similar to *T. verrucosus*. These results lead to the confirmation of the existence of *T. verrucosus* in Thailand. The habitats of *T. verrucosus* need to be protected by future conservation plans.

KEY WORDS: conservation, cryptic species, newt, Thailand, *Tylototriton verrucosus*

INTRODUCTION

Thailand is a rather small country that has undergone extensive land development, but is still relatively rich in biodiversity (Baimai, 2010). Several new species of anurans have been discovered in unprotected areas (e.g. Suwannapoom et al., 2016, 2018). However, the majority of hotspot areas are inside government protected enclosures (Tantipisanuh and Gale, 2018), including national parks (NP) or wildlife sanctuaries (WS). Some of these protected areas house the newt genus *Tylototriton*, which have a small range distribution about which there is scarce information.

Tylototriton, is the only genus of salamandrid, Urodela, currently recorded in Thailand. In the past, *T. verrucosus* was the sole known representative species found in Thailand (Taylor, 1962; Wongratana, 1984; Nabhitabhata et al., 2000; Chan-ard, 2003). Their habitats are ponds that are covered with aquatic plants along with sand, stone debris, and organic materials scattered on the floor. They also reside terrestrially in moist areas, such as under rock piles and piles of dead leaves. They are distributed along high mountain ranges in the North and North-eastern regions of Thailand at an altitude of more than 1000 meters above medium sea level (m amsl) (Taylor, 1962; Wongratana, 1984; Chan-ard et al., 1999, Chan-ard, 2003; Pomchote et al., 2008; Hernandez et al., 2019). From 1962 to 2007, all *Tylototriton* found in Thailand were classified as *T. verrucosus*, although they were clearly distinct in terms of geographical separation and color patterns. Pomchote et al. (2008) reported two types of *T. verrucosus*: Type I had a yellow to orange body coloration and Type II had a dull body coloration. They were found along

mountain ranges in the Northern and North-eastern parts of Thailand, respectively.

The above research led to the subsequent studies that reported in fact *T. verrucosus* in Thailand was actually comprised of three species of newts: *T. uyenoi*, *T. anguliceps*, and *T. panhai*. These three species were distributed along the Ang Khang, Chang Kien, Inthanon, Pui, and Suthep mountains in Chiang Mai Province (*T. uyenoi*) (Nishikawa et al., 2013a), the Lahnga mountain in Chiang Rai Province (*T. anguliceps*) (Le et al., 2015), and the Phu Hin Rong Kla NP in Phitsanulok Province, and Phu Luang WS and Phu Suan Sai NP in Loei Province (*T. panhai*) (Nishikawa et al., 2013a). Despite confirmation of the existence of three newt species in Thailand, the question remained as to the existence of the true *T. verrucosus*. Should its existence be confirmed, where is it distributed? This question arose since, for example, a study conducted in 2013 assigned *T. uyenoi* to *T. verrucosus* (in Taylor, 1962) and *T. panhai* had been reported as *T. verrucosus* (in Wongratana, 1984; Chan-ard, 2003; Khonsue et al., 2010). This led to the emphasis of the question whether or not *T. verrucosus* does exist in Thailand, or simply represents misidentifications since 1962 of *T. uyenoi*, *T. panhai*, and *T. anguliceps* with no *T. verrucosus* in Thailand.

From our field survey in the Northern parts of Thailand, we found newts from Doi Chang, Chiang Rai Province, which is a new locality of *Tylototriton* in Thailand. However, this location is not in the protected area where the previous three *Tylototriton* species were observed. The newts found in Doi Chang had a rather similar color pattern to *T. uyenoi* and *T. anguliceps*, but were rather different in other external characters. Furthermore, Doi Chang (Chiang Rai Province) is separated from the locations where *T. uyenoi* and *T. anguliceps* were previously found. From the above lines of information, the possibility of a different *Tylototriton* species existing in Doi Chang, Chiang Rai Province, is suggested. Therefore, molecular and morphological analyses were performed to examine the taxonomic status of *Tylototriton* species from Doi Chang. The knowledge obtained in this study will be used in the implementation of future conservation plans of Thai *Tylototriton*.

MATERIALS AND METHODS

Sampling

Field surveys using the visual encounter survey method (Heyer et al., 1994) were conducted from the 23rd to the 24th and the 31st May 2018 in Namtok Mae Surin NP, Mae Hong Son Province and Khun Chae NP, Chiang Rai Province, respectively, and from the 2nd to the 3rd June 2018 in Doi Chang, Chiang Rai Province (Fig. 1). Ponds selected as survey sites were those that had bottoms covered with small pieces of debris on the mountains with an elevation of at least 1000 m amsl (Pomchote et al., 2008). Upon an encounter, adult newts were captured by either an aquatic dip net or by hand and kept in plastic boxes. The biological and physical data of their habitats were also recorded.

Male newts had obvious sexual cloacal characters. During the breeding season, the male cloaca is swollen and the cloacal opening is oval shaped (Pomchote et al., 2008; Phimmachak et al., 2015b). From these criteria, all the newts found in this study were sexed as breeding males. Twenty newts (three from Khun Chae NP, seven from Doi Chang, and 10 from Namtok Mae

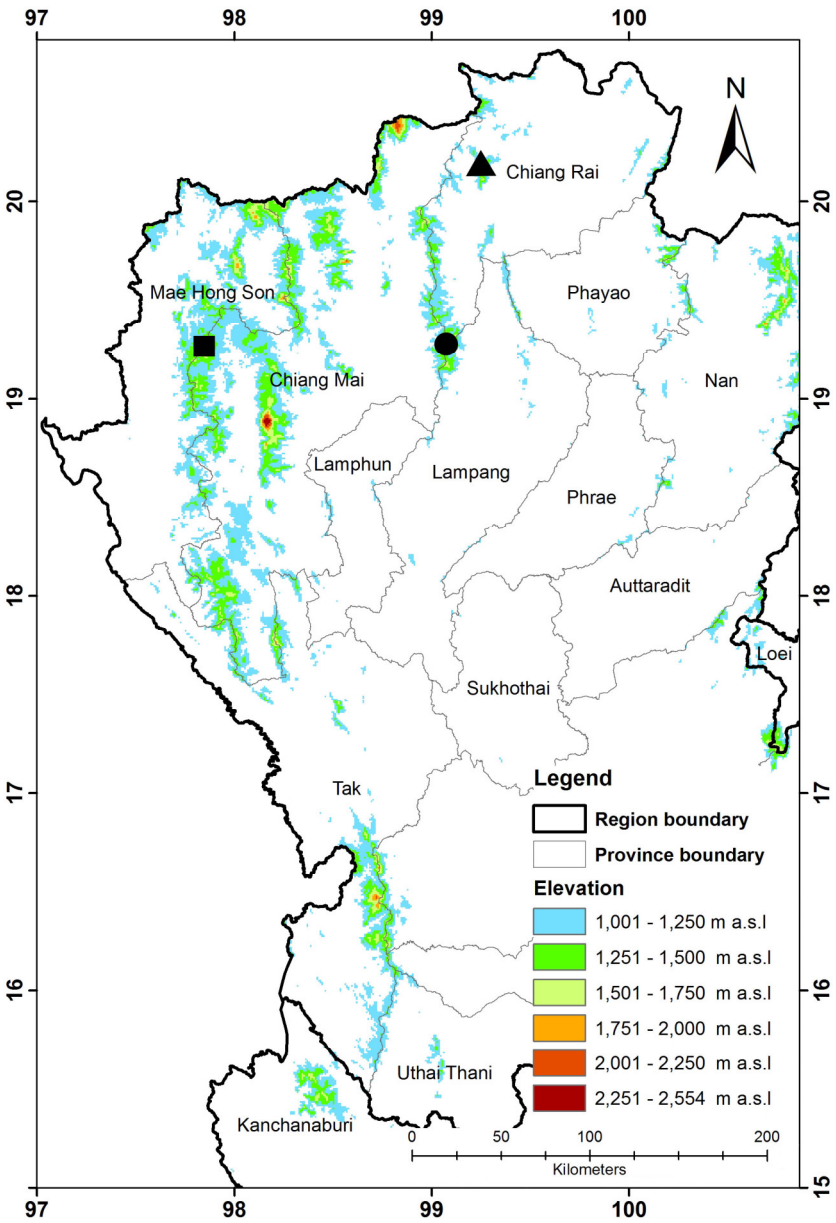

FIGURE 1. Distribution map of the three *Tylototriton* species in this study: *Tylototriton anguliceps* (circle), *T. uyenoi* (square), and *T. verrucosus* (triangle). The map is modified by Praphatsorn Punsompong.

Surin NP) were used for the morphological study, while 15 newts were used for the molecular analyses (Table 1).

The samples were anesthetized by immersion in a solution of tricaine methane sulfonate (MS-222; 5 g/L) for about five minutes (modified from Mitchell, 2009). All

TABLE 1. Specimens for molecular analyses. CAS = California Academy of Sciences; CIB = Chengdu Institute of Biology; HNUE = Hanoi National University of Education; IEBR = Institute of Ecology and Biological Resources, Hanoi; KIZ = Kunming Institute of Zoology; KUHE = Graduate School of Human and Environmental Studies, Kyoto University; LSUHC = La Sierra University Herpetological Collection; MVZ = Museum of Vertebrate Zoology, University of California, Berkeley; NMNS = National Museum of Natural Science, Taiwan; TBU = Tay Bac University, Son La; VNMN = Vietnam National Museum of Nature; ZDUM = Zoology Department of University of Mandalay; ZMMU = Zoological Museum of Moscow State University. *topotype

Sample no.	Species	Voucher no.	Locality	GenBank accession no.	Source
1	*T. verrucosus**	KIZ 201306055	Husa, Yunnan, China	AB922818	Nishikawa et al. (2014)
2	*T. verrucosus*	No voucher	Doi Chang, Chiang Rai, Thailand	LC505605	This study
3	*T. verrucosus*	No voucher	Doi Chang, Chiang Rai, Thailand	LC505606	This study
4	*T. verrucosus*	No voucher	Doi Chang, Chiang Rai, Thailand	LC505607	This study
5	*T. verrucosus*	No voucher	Doi Chang, Chiang Rai, Thailand	LC505608	This study
6	*T. verrucosus*	No voucher	Doi Chang, Chiang Rai, Thailand	LC505609	This study
7	*T. verrucosus*	No voucher	Doi Chang, Chiang Rai, Thailand	LC505610	This study
8	*T. verrucosus*	No voucher	Doi Chang, Chiang Rai, Thailand	LC505615	This study
9	*T. verrucosus*	No voucher	Doi Chang, Chiang Rai, Thailand	LC505616	This study
10	*T. verrucosus*	No voucher	Doi Chang, Chiang Rai, Thailand	LC505614	This study
11	*T. verrucosus*	No voucher	Doi Chang, Chiang Rai, Thailand	LC505617	This study
12	*T. anguliceps**	VNMN A.2014.3	Muong Nhe, Dien Bien, Vietnam	LC017832	Le et al. (2015)
13	*T. nguliceps**	HNUE A.I.1.111	Muong Nhe, Dien Bien, Vietnam	LC017836	Le et al. (2015)
14	*T. anguliceps*	TBU PAE.671	Thuan Chau, Son La, Vietnam	LC017833	Le et al. (2015)
15	*T. anguliceps*	IEBR A.2014.10	Thuan Chau, Son La, Vietnam	LC017834	Le et al. (2015)
16	*T. anguliceps*	No voucher	Doi Lahnga, Chiang Rai, Thailand	AB830728	Nishikawa et al. (2013a)
17	*T. anguliceps*	No voucher	Khun Chae NP, Chiang Rai, Thailand	LC505611	This study
18	*T. anguliceps*	No voucher	Khun Chae NP, Chiang Rai, Thailand	LC505613	This study
19	*T. uyenoi**	KUHE 19146	Doi Suthep, Chiang Mai, Thailand	AB830732	Nishikawa et al. (2013a)
20	*T. uyenoi**	KUHE 19147	Doi Suthep, Chiang Mai, Thailand	AB830733	Nishikawa et al. (2013a)
21	*T. uyenoi**	No voucher	Doi Suthep, Chiang Mai, Thailand	AB830734	Nishikawa et al. (2013a)
22	*T. uyenoi*	KUHE 19037	Doi Inthanon, Chiang Mai, Thailand	AB830730	Nishikawa et al. (2013a)

TABLE 1. continue

23	*T. uyenoi*	KUHE 19038	Doi Inthanon, Chiang Mai, Thailand	AB830731	Nishikawa et al. (2013a)
24	*T. uyenoi*	No voucher	Namtok Mae Surin NP, Mae Hong Son, Thailand	LC505612	This study
25	*T. uyenoi*	No voucher	Namtok Mae Surin NP, Mae Hong Son, Thailand	LC505618	This study
26	*T. uyenoi*	No voucher	Namtok Mae Surin NP, Mae Hong Son, Thailand	LC505619	This study
27	*T. uyenoi*	No voucher	Doi Ang Khang, Chiang Mai, Thailand	AB830729	Nishikawa et al. (2013a)
28	*T. panhai**	No voucher	Phu Luang WS, Loei, Thailand	AB830736	Nishikawa et al. (2013a)
29	*T. panhai*	No voucher	Phu Suan Sai NP, Loei, Thailand	AB830737	Nishikawa et al. (2013a)
30	*T. panhai*	No voucher	Phu Hin Rong Kla NP, Phitsanulok, Thailand	AB830735	Nishikawa et al. (2013a)
31	*T. shanjing**	NMNS 3682	Jingdong, Yunnan, China	AB830721	Nishikawa et al. (2013a)
32	*T. pulcherrima*	KUHE 46406	Yunnan, China (pet trade)	AB830738	Nishikawa et al. (2013a)
33	*T. podichthys*	KUHE 34399	Xam Neua, Houa Phan, Laos	AB830727	Nishikawa et al. (2013a)
34	*T. podichthys*	IEBR A.2014.11	Xam Neua, Houa Phan, Laos	LC017835	Le et al. (2015)
35	*T. panwaensis**	CAS 245418	Panwa, Myitkyina, Myanmar	KT304279	Grismer et al. (2018)
36	*T. yangi*	KUHE 42282	China (pet trade)	AB769546	Nishikawa et al. (2013b)
37	*T. yangi*	NMNS 3114	Mt. Dawei, Pingbian, Yunnan, China	LC017831	Nishikawa et al. (2015)
38	*T. shanorum**	CAS 230940	Taunggyi, Shan, Myanmar	AB922823	Nishikawa et al. (2014)
39	*T. ngarsuensis**	LSUHC 13762	Ywangan, Shan, Myanmar	MH836585	Grismer et al. (2018)
40	*T. ngarsuensis**	LSUHC 13763	Ywangan, Shan, Myanmar	MH836584	Grismer et al. (2018)
41	*T. himalayanus*	MVZ no number	Nepal	DQ517854	Weisrock et al. (2006)
42	*T. kachinorum**	ZMMU A5953	Indawgyi, Kachin, Myanmar	MK097273	Zaw et al. (2019)
43	*T. kachinorum**	ZDUM-0103	Indawgyi, Kachin, Myanmar	MK097274	Zaw et al. (2019)
44	*T. kweichowensis*	MVZ 230371	Daguan, Yunnan, China	DQ517851	Weisrock et al. (2006)
45	*T. taliangensis*	KUHE 43361	Unknown (pet trade)	AB769543	Nishikawa et al. (2013b)
46	*T. asperrimus**	CIB 200807055	Jinxiu, Guangxi, China	KC147815	Shen et al. (2012)
47	*Echinotriton andersoni**	KUHE no number	Nago, Okinawa, Japan	AB769545	Nishikawa et al. (2013b)
48	*Pleurodeles waltl*	MVZ 162384	Rabat, Morocco	DQ517813	Weisrock et al. (2006)
49	*Notophthalmus viridescens*	MVZ 230959	St. Charles, Missouri, USA	DQ517795	Weisrock et al. (2006)

procedures were then conducted once the sample was fully anesthetized. After completing the morphological measurements and tissue sampling for molecular analysis (see below), all anesthetized newts were rinsed in water to help them regain consciousness. They were assessed by observation of their movements, kept and monitored in a captive box for 24 h to allow full recovery before being released on land near their respective capture site.

Molecular Study

Tissue samples were obtained by removing the tail tips, approximately 8% of the full tail length (Arntzen et al., 1999; Polich et al., 2013), using surgical scissors sterilized with 70% (v/v) ethanol (Fellers and Freel, 1995). Then an antiseptic liquid was used to clean the wound. The tissue samples were preserved in absolute ethanol for DNA extraction and further analysis.

Total DNA was extracted using a DNeasy Blood & Tissue kit (QIAGEN, Hilden, Germany) and used to PCR amplify and sequence the mitochondrial NADH dehydrogenase subunit 2 region (ND2) for molecular analyses as reported previously (Nishikawa et al., 2013a). Sequence data obtained were deposited in GenBank (accession Nos. LC505605–619). Combining them with sequences of the other *Tylototriton* species (Table 1), molecular phylogenetic trees were reconstructed by using the maximum likelihood (ML) and Bayesian inference (BI) methods. We used Kakusan 4 (Tanabe, 2011), RAxML v8.2.8 (Stamatakis, 2014), and MrBayes v3.2.6 (Ronquist et al., 2012) for model selection, ML, and BI, respectively.

The robustness of the ML tree was tested using bootstrap analysis (Felsenstein, 1985) with 1000 replicates, and we regarded tree topologies with bootstrap values (BS) of 70% or greater as well supported. For BI, two independent runs of four Markov chains were conducted for 10 million generations. The first three million generations were discarded as burn-in, and we considered topologies with posterior probabilities (bpp) of 0.95 or greater as significant support. Pairwise comparisons of uncorrected sequence divergences (p-distance by 400 bps) were calculated using MEGA X (Kumar et al., 2018).

Morphological Study

The morphology of the newts was compared among the three locations (total N = 20). The following 27 measurements, with character definitions are described in Nishikawa et al. (2011), were taken for comparisons: SVL (snout-vent length); HL (head length); HW (head width); MXHW (maximum head width); SL (snout length); LJL (lower jaw length); ENL (eyelid-nostril length); IND (internarial distance); IOD (interorbital distance); UEW (upper eyelid width); UEL (upper eyelid length); OL (orbit length); AGD (axilla-groin distance); TRL (trunk length); TAL (tail length); VL (vent length); BTAW (basal tail width); MTAW (medial tail width); BTAH (basal tail height); MXTAH (maximum tail height); MTAH (medial tail height); FLL (forelimb length); HLL (hindlimb length); 2FL (second finger length); 3FL (third finger length); 3TL (third toe length); and 5TL (fifth toe length). All measurements were taken using a digital sliding caliper to the nearest 0.01 mm. Each measurement was conducted three times and the average was used for further analyses. While the newts were anesthetized, their body weights (BW) were recorded using a digital weighing scale. The sex was checked from observing the cloacal characters (noted in Results).

The SVL, BW, and 26 ratio values to SVL (R, %) were compared among the

newts from the three locations. Differences in morphological characters between *T. anguliceps* and *T. verrucosus*, and between *T. uyenoi* and *T. verrucosus* were analyzed by the Mann-Whitney U test. The relationships of all characters were examined using principal component analysis (PCA). All statistical analyses were performed using SPSS version 22 for Windows. Statistical significance was accepted at the $P < 0.05$ level.

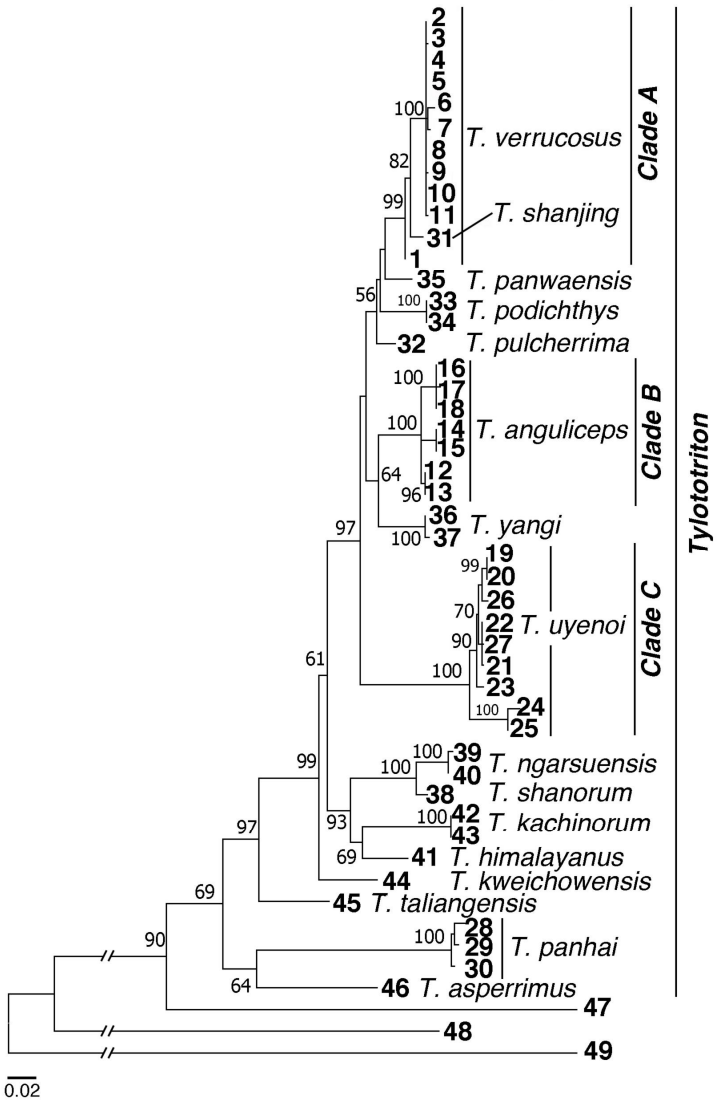

FIGURE 2. Maximum likelihood phylogenetic tree based on the partial ND2 mtDNA gene for *Tylototriton* and related species (see Table 1). Numbers at nodes show bootstrap supports for ML inference (bs). Numbers at branches tips are sample numbers shown in Table 1. Scale bar = 0.02 substitutions/site.

RESULTS

Molecular Study

We analyzed ~1035 bp sequences of the partial ND2 region for 49 specimens, including an outgroup from GenBank data (Table 1). Of the 1035 nucleotide sites sequenced in this study, 369 were variable and 262 were parsimony informative. The mean likelihood score of the BI analyses for all trees sampled at stationarity was -6078.038. The likelihood value of the ML tree was -6074.

Phylogenetic analyses employing two different optimality criteria (BI and ML) yielded nearly identical topologies. We, therefore, present only the ML tree in Figure 2. However, whilst monophyly of *Tylototriton* was supported in the BI tree (bpp = 0.96), it was not supported in the ML tree (BS = 69%). Within *Tylototriton*, we recognized many clades but their relationships did not tend to be well supported (genetic distances available upon request from the senior author). Thai samples from Doi Chang (Samples 2–11) were nested within Clade A (see Fig. 2) of *T. verrucosus* and *T. shanjing* (Samples 1 and 31) with an average genetic p-distance of 1% (range: 0–2%), those from Khun Chae NP (Samples 17 and 18) were nested within Clade B (see Fig. 2) of *T. anguliceps* (Samples 12–16) with an average p-distance of 1% (range: 0–2%), and those from Namtok Mae Surin NP (Samples 24–26) were nested within Clade C (see Fig. 2) of *T. uyenoi* (Samples 19–23) with an

TABLE 2. Morphological comparison of adult males of the three *Tylototriton* species from this study (mean ± SD of SVL (in mm), mean ± SD of BW (g), and median of ratios of characters (R: %SVL), with range in parentheses). Character abbreviations refer to text.

Species	*T. anguliceps*	*T. verrucosus*	*T. uyenoi*	Species	*T. anguliceps*	*T. verrucosus*	*T. uyenoi*
No. and character	**3**	**7**	**10**	**No. and character**	**3**	**7**	**10**
SVL	64.8 ± 3.5 (60.9-67.4)	66.6 ± 2.7^{+} (62.6-70.3)	71.8 ± 2.4 (68.9-75.8)	RTRL	71.2 (70.8-71.5)	73.6^{*+} (72.1-74.5)	75.0 (71.8-98.0)
BW	14.0 ± 2.3 (12.2-16.6)	10.7 ± 0.6^{*+} (10.2-12.0)	14.8 ± 1.8 (11.2-17.0)	RTAL	87.3 (87.2-87.8)	94.3* (93.5-110.4)	98.0 (88.8-110.4)
RHL	26.1 (25.7-26.4)	26.4^{+} (24.9-27.5)	25.1 (24.2-26.3)	RVL	11.4 (9.6-11.4)	10.1 (9.7-11.9)	12.4 (7.4-15.3)
RHW	19.6 (19.5-20.3)	17.6^{*+} (17.5-18.4)	18.8 (17.5-19.3)	RBTAW	7.5 (7.3-7.7)	5.9* (5.1-7.0)	5.8 (4.4-6.2)
RMXHW	24.4 (24.4)	24.1^{+} (23.2-25.1)	25.8 (24.5-26.4)	RMTAW	4.6 (3.8-4.7)	3.7* (3.0-4.0)	3.7 (2.9-4.3)
RSL	7.4 (7.1-8.2)	8.1^{+} (7.6-9.0)	8.8 (8.1-9.4)	RBTAH	13.1 (12.7-14.1)	9.8^{*+} (8.6-11.0)	12.1 (11.5-12.9)
RLJL	20.5 (20.1-21.4)	20.7^{+} (19.7-21.5)	22.0 (20.7-22.5)	RMXTAH	14.4 (13.5-14.6)	10.0^{*+} (9.8-11.2)	12.7 (11.0-14.2)
RENL	6.2 (6.1-6.3)	6.2 (5.4-7.9)	6.8 (6.0-7.5)	RMTAH	14.2 (13.5-14.3)	8.6^{*+} (8.0-10.3)	11.8 (11.0-13.3)
RIND	7.8 (6.8-7.9)	6.2* (5.3-6.7)	6.8 (5.6-7.5)	RFLL	40.3 (39.8-41.2)	38.6^{*+} (37.6-39.1)	43.7 (42.6-44.6)
RIOD	12.3 (12.1-12.4)	13.3 (12.1-13.5)	13.0 (12.6-14.4)	RHLL	44.2 (43.9-45.4)	41.4^{*+} (40.2-42.5)	44.8 (42.3-48.1)
RUEW	3.2 (2.7-3.4)	2.6^{+} (2.2-3.1)	3.1 (2.2-3.8)	R2FL	6.1 (5.6-6.8)	6.0 (5.6-6.8)	5.5 (4.5-6.8)
RUEL	7.3 (6.8-7.3)	6.2 (5.6-7.4)	6.4 (5.8-7.1)	R3FL	7.6 (7.1-7.9)	7.3 (5.9-8.4)	6.8 (5.5-7.4)
ROL	4.2 (4.2-4.4)	4.4 (3.6-5.7)	4.2 (3.5-4.8)	R3TL	9.6 (9.6-11.1)	8.6* (7.7-9.5)	8.3 (7.0-9.2)
RAGD	52.3 (51.1-52.4)	55.1^{*+} (53.9-56.4)	49.9 (45.7-52.3)	R5TL	4.8 (4.3-4.9)	4.2 (3.7-4.7)	4.1 (2.8-6.1)

$^{*}P < 0.05$ compared to *T. anguliceps* (Mann-Whitney U test)
$^{+}P < 0.05$ compared to *T. uyenoi* (Mann-Whitney U test)

average p-distance of 2% (range: 0–3%). From these genetic results, the Doi Chang samples are regarded as conspecific with *T. verrucosus* or *T. shanjing* and heterospecific to the other Thai species.

Morphological Study

A total of 20 adult males were used for the morphological comparisons (Table 2). For the SVL, the Doi Chang samples (*T. verrucosus*) were smaller than *T. uyenoi*, but nearly similar to *T. anguliceps*. For the BW, the Doi Chang samples were the lightest. In character ratios, the Doi Chang samples had the smallest values for RHW, RBTAH, MXTAH, RMTAH, RFLL, and RHLL, but, in contrast, the RAGD of the Doi Chang samples was the largest. For the RTRL, the Doi Chang samples were smaller than *T. uyenoi* but larger than *T. anguliceps*.

The overall morphological differences in males were examined using PCA for three *T. anguliceps*, seven Doi Chang samples and 10 *T. uyenoi*. The first two principal components accounted for 52.59% of the total variation. The two-dimensional plot of PC2 versus PC1 showed that all three species were completely separated from one another (Fig. 3).

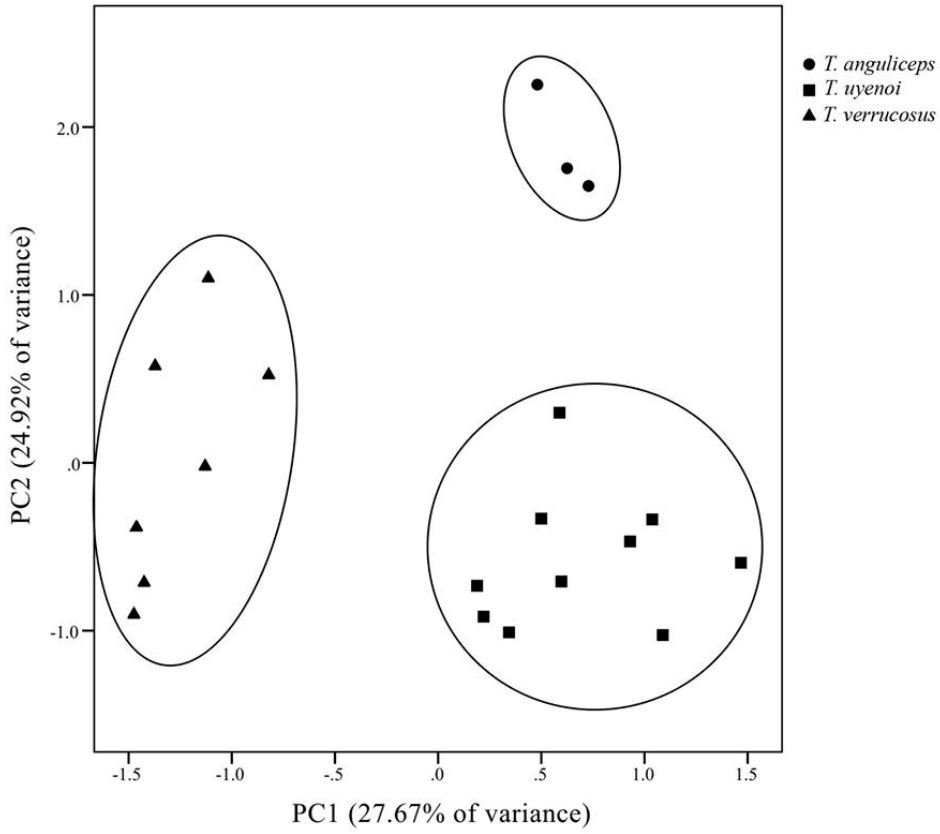

FIGURE 3. Principal component analysis of *Tylototriton* showing the two dimensional plots of the first principal component (PC1) versus the second component (PC2).

TABLE 3. Morphological differences in adult males of the three *Tylototriton* species from this study.

Character	*T. verrucosus*	*T. uyenoi*	*T. anguliceps*
Snout	Truncate	Rounded to blunt	Truncate
Cranial crest	Narrow, weakly curved medially at posterior end	Wide, weakly or rather curved medially at posterior end	Narrow, distinctly curved medially at posterior end
Surface of cranial crest	Rather smooth	Rough	Rough
Sagittal ridge on head	Rather distinct	Rather distinct	Prominent
Vertebral ridge	Narrow, segmented	Wide, segmented	Narrow, weakly segmented
Rib nodules	Isolated but few individuals connected, rounded but few individuals oval, 12–16	Isolated, rounded, 12–16	Isolated, rounded, 14–15
Glandular skin	Small granules, sparse	Large granules, dense	Large granules, dense
Adpressed limbs	Overlapping	Overlapping	Overlapping
Color of ventral trunk	Brown to dark brown	Brown to dark brown	Partly dull orange-brown to yellowish-brown
Color of digit tips	Dull orange-brown to yellowish-brown	Dark brown to black	Dull orange-brown to yellowish-brown

The three species had a rather similar coloration, with a brown to dark-brown background, although the Doi Chang samples tended to be the most blackish. The dorsal and ventral head, parotoids, vertebral ridge, rib nodules, limbs, vent, and tail were dull orange-brown to yellowish-brown, although *T. uyenoi* had darker orange markings than the other species. The ventral tail ridge was brighter in coloration among these three species. However, some parts of the bodies had different colorations (see Table 3). The morphological differences between the three species are summarized in Table 3.

When the Doi Chang samples (Fig. 4) were compared with *T. verrucosus*, using data taken from the neotype from Longchuan County, Yunnan Province, China, as described by Nussbaum et al. (1995), they both showed similar color patterns of a mostly blackish background but orange to reddish-brown markings on the head, trunk, limbs, vent, and ventral ridge of the tail. They had a distinctly segmented vertebral ridge. The rib nodules were round and distinct. However, the number of rib nodules in the Doi Chang samples ranged from 12–16, whereas in *T. verrucosus* (Nussbaum et al., 1995) the nodules amounted to 13. Both taxa had similar small and sparse granules that were denser on the dorsal side than the ventral side.

These morphological results conformed to the genetic ones mentioned above, where the Doi Chang samples are regarded as conspecific with *T. verrucosus* or *T. shanjing* and heterospecific to the other Thai species. Therefore, we provisionally identified the Doi Chang samples as *Tylototriton* cf. *verrucosus* at the moment.

Habitat Use

To date, in Thailand, *Tylototriton* cf. *verrucosus* is known only from a single locality at Doi Chang, Chiang Rai Province, which is located on the Khun Tan mountain range (Fig. 1). All adult males of *Tylototriton* cf. *verrucosus* were found at night between 21.00–22.00 h in the pond during the breeding season (Fig. 5). The bottom of the pond was covered with small pieces of debris; such as leaf litter. Surrounding areas were hill evergreen forest. The pond size was approximately 400 cm long, 290 cm wide, and 33 cm in maximum depth. At 21.34 h on the 2nd June 2019, the water and air temperatures were 20.4 °C and 22.4 °C, respectively, with a relative air humidity of 88%. The water quality parameters were: pH 6.75; dissolved oxygen 1.48 mg/L; conductivity 375 µS/cm; and total dissolved solid 164 mg/L. The altitude was 1500 m amsl. No fish were observed.

FIGURE 4. Male *Tylototriton verrucosus* from Doi Chang, Chiang Rai Province, Thailand.

FIGURE 5. Pond habitat of *Tylototriton verrucosus* at Doi Chang, Chiang Rai Province, Thailand.

DISCUSSION

The p-distance between species of *Tylototriton* was previously estimated at > 3.3% (Nishikawa et al., 2013a), which was confirmed in the present study except for *T. shanorum* and *T. ngarsuensis* with a smaller distance of 2%, the same as the intraspecific variation in *T. uyenoi*. Thus, the variation within the three clades (Clades A, B, and C) in this study could be judged as being at the intraspecific level and the taxonomic relationship between *T. shanorum* and *T. ngarsuensis* should be reexamined in a future study.

Clade A included topotypic samples of *T. verrucosus* and *T. shanjing*, and we tentatively treat this clade as *T. verrucosus*. Anderson (1871) did not specify the precise type locality, but only mentioned some areas located at Nantin, Momien, and Hotha valleys, in western Yunnan Province (China). Moreover, Anderson (1871) neither classified the type series nor mentioned where the described specimens were deposited. Nussbaum et al. (1995) designated a neotype of the species from Gongwa Village, Longchuan County, Yunnan Province, China, because the presumed Anderson's syntype was considered as *T. shanjing* and did not correspond to Anderson's original description of the dark brown-patterned specimen. *Tylototriton verrucosus* and *T. shanjing* occur in parapatry in Yunnan, but they share mitochondrial genomes (Zhang et al., 2007; Phimmachak et al., 2015a). Thus, further intensive study is needed to clarify their actual species identities. However, from the morphological and molecular data, we tentatively treat *T. verrucosus* and *T. shanjing* as the same species and use the former species name here, and so identify the *Tylototriton* species from Doi Chang, Chiang Rai Province, Thailand as *T. verrucosus*.

Clade B could be treated as *T. anguliceps* and Clade C as *T. uyenoi* without any doubt from the present results. Accordingly, we now confirm that four species of *Tylototriton* exist in Thailand. *Tylototriton anguliceps*, *T. uyenoi* and *T. verrucosus* are found in the Northern part, while *T. panhai* is only found in the North-eastern part, and they are all geographically separated. Although *T. verrucosus* is distributed on the Khun Tan mountain range, Chiang Rai Province, close to the range of *T. anguliceps*, until now no sympatry between the two species has been observed. Since the *Tylototriton* species use shallow and isolated ponds on mountain ranges at elevations ≥ 1000 m amsl for breeding, each population may be isolated owing to the presence of lowlands between the mountain ranges where they occur.

During the breeding season, adult males of *T. verrucosus* from Doi Chang use similar microhabitats in ponds at more than 1000 m amsl, as per previous reports in *T. anguliceps* (Le et al., 2015), *T. uyenoi* (Pomchote et al., 2008: as the Type I form of *T. verrucosus*; Dowwiangkan et al., 2018), and *T. panhai* (Pomchote et al., 2008; Khonsue et al., 2010: as the Type II form of *T. verrucosus*).

We did not find any female newts in the three sampled locations. Female newts may enter breeding ponds later or they may stay in the water for only a short period and then return to land (Grismer et al., 2018). Males were found in a greater number than females in *T. uyenoi* (Dowwiangkan et al., 2018) and in some populations of *T. himalayanus* (Wangyal and Gurung, 2012: as *T. verrucosus*). However, an equal sex ratio was observed in *T. podichthys* (Phimmachak et al., 2015b). Thus, a longitudinal study is needed for a population study in the Thai *Tylototriton* species.

In the amphibian wildlife protection list of Thailand, the only protected newt species is *T. verrucosus* (Department of National Parks, Wildlife and Plant Conservation). The other three candidates (*T. uyenoi*, *T. anguliceps*, and *T. panhai*) are not protected. Moreover, the *T. verrucosus* population, which is only found at Doi Chang, Chiang Rai Province, may be in risk of decline because the Doi Chang area is not protected by law. Thus, we suggest that this species is threatened because of habitat alteration and human activities, such as modifying the area into tourist attractions, tourist accommodation, and commercial crop cultivation plots, like Doi Chaang Coffee.

Our study confirms that *T. verrucosus sensu stricto* is still in existence, even after recent and drastic taxonomic changes, in Thailand and is expected to be found in other areas of Thailand. However, their habitats need to be protected by future conservation plans.

The experimental protocol was approved by the Animal Care and Use Committee of Faculty of Science, Chulalongkorn University (Protocol Review No. 1723008 Renew). This research was permitted by the National Research Council of Thailand (NRCT) and the Department of National Parks, Wildlife and Plant Conservation (DNP).

ACKNOWLEDGEMENTS

We express our thanks to two anonymous reviewers for their valuable suggestions on the manuscript, Robert Butcher for English language editing, and Songyot Kullasoot, Boonpiyathida Klongklaew, Parada Peerachi-dacho, Kotchanun Bunjerdluk, Nuttakorn Taewcharoen, Praphatsorn Punsompong, and Chitchol Phalaraksh for kind support. We

also thank the Animal Care and Use Committee of the Faculty of Science, Chulalongkorn University, NRCT and DNP. This research was supported by the Ratchadaphiseksomphot Endowment Fund Part of the "Research Grant for New Scholar CU Researcher's Project" (RGN_2559_061_19_23 to Porrawee Pomchote), and JSPS Core-to-Core Program B (to Kanto Nishikawa [coordinator: Masaharu Motokawa]).

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