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Comparative morphometrics and ecology of a newly discovered population of *Tylototriton vietnamensis* from northeastern Vietnam including remarks on species conservation

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The genus *Tylototriton* has a restricted distribution in the eastern Himalayas region, central and southern China, and Indochina (Nishikawa et al. 2014, Yang et al. 2014, Le et al. 2015). The number of newly discovered *Tylototriton* species has been continuously increasing (Nishikawa et al. 2013a, b, Nishikawa et al. 2014, Yang et al. 2014, Le et al. 2015, Phimmachak et al. 2015) although the taxonomic status of some species is still under debate (e.g., Zhang et al. 2007, Nishikawa et al. 2013b, Nishikawa et al. 2015).

In Vietnam, five species of this genus are recognized and two of them, *T. vietnamensis* and *T. ziegleri*, are endemic to this country. The Vietnamese crocodile newt *T. vietnamensis* is currently known only from three localities in northern Vietnam, i.e., the Tay Yen Tu Nature Reserve located in Bac Giang Province (the type locality), the Mau Son Mountain in Lang Son Province, and the Xuan Son National Park in Phu Tho Province (Nguyen et al. 2009, Yuan et al. 2011). Bernardes et al. (2013) also reported this species from the eastern side of Yen Tu Mountain, within the Yen Tu Nature Reserve, Uong Bi District, Quang Ninh Province, located opposite of the type locality. These authors also used the Species Distribution Model (SDM) to predict the potential occurrence of further populations in northern Vietnam. Herein we report on a new population of *T. vietnamensis* from Dong Son – Ky Thuong Nature Reserve in Quang Ninh Province, whose existence was predicted by the SDM. In addition, we analysed the genetic distance between the newly discovered population and already known ones, and evaluated morphological characters and ecological adaptations at the new site in comparison with those from the type locality.

Field research was conducted by M. Bernardes, C. T. Pham and H. T. An in the Dong Son – Ky Thuong Nature Reserve (DSKT NR), Dong Son Commune, Hoanh Bo District, Quang Ninh Province, on 9 and 10 June 2012 and on 27 and 28 May 2014. Fieldwork was also conducted at the type locality in the Tay Yen Tu Nature Reserve, Son Dong and Luc Nam Districts, Bac Giang Province, between April and July of 2010 and 2012–2014 (Fig. 1). Seven forest ponds in the DSKT NR and another 22 in the Tay Yen Tu NR were visited for monitoring the presence of *T. vietnamensis*. Altitudes and coordinates were recorded with a Garmin GPS MAP62. The area of each pond was calculated by multiplying its maximum width with its maximum length using a rope of known length, which was also used for measuring the maximum pond depth. The canopy cover above the water was estimated visually as percentage of water covered. Environmental parameters (e.g., air temperature, rel-

ative humidity, and atmospheric pressure) were recorded with a mobile weather station (Krestel 3500), and pH and water temperature with a handheld pH-meter (Hanna HI 98129) calibrated to 25°C. Drop-by-drop colour tests from JBL (Testlab, Germany) were used to measure the chemical water parameters, including carbonate (KH), total hardness (gH), concentration of silicic acid [SiO_2^{2-}], concentration of ammonium [NH_4^+], concentration of nitrite [NO_2^-], concentration of nitrate [NO_3^-], and concentration of iron [Fe^-].

Adults and larvae were sampled by two persons using a 25-cm dip-net (JBL, Germany) in the water for a period of 20 minutes each. Clutches were searched for up to a maximum distance of 3 m around the banks of the ponds by carefully turning the vegetation. Egg sizes (including the jelly envelope) were measured in seven randomly selected eggs for each clutch. Morphological measurements were taken to the nearest 0.1 mm from live specimens using a digital calliper. Abbreviations are as follow: snout–vent length (SVL) from tip of snout to posterior margin of vent; head length (HL) from posterior end of parotoid to snout; maximum head width (HW); parotoid width (PW); maximum parotoid height (PH); eye length (EL); inter-eye distance (IE); inter-nostril distance (IN); eye–nostril distance (EN) from interior end of eye to nostril; humerus length (HUM) from axilla to exterior end of elbow joint; radius length (RAD) from exterior end of elbow joint to the tip of longest finger; femur length (FEM) from groin to exterior end of knee joint; tibia length (TIB) from exterior end of knee joint to the tip of longest finger; tail length (TL) from posterior end of vent to tail tip; and maximum tail height (TH). Besides the measured morphometric variables, additional ratios were calculated: the relative length of radius to humerus (RAD/HUM); tibia to femur (TIB/FEM); total forelimb length (FORE); total hindlimb length (HIND); hindlimb to forelimb lengths (HIND/FORE); and tail length to tail height (TL/TH). Weights were measured to the nearest 0.01 g with a digital scale. Since no females were

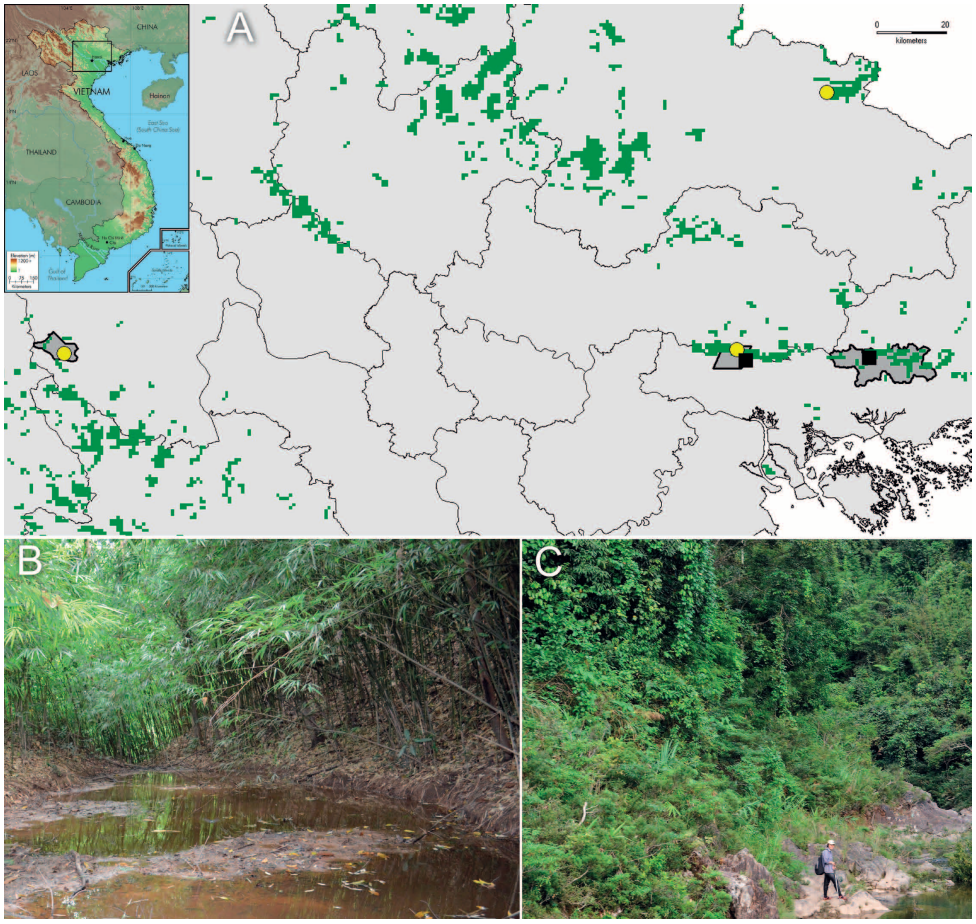

Figure 1. (A) Detailed map of records of *Tylototriton vietnamensis* in northern Vietnam represented by yellow circles in already-known localities (from left to right: Phu Tho, Bac Giang, and Lang Son provinces) and black squares within the province of Quang Ninh. The remaining forest cover in the region (from Global Land Cover 2000) is indicated by the scarce and fragmented green patches. The three areas delimited by a stronger line and darker grey indicate (from left to right) Xuan Son National Park, Tay Yen Tu and Yen Tu and Dong Son – Ky Thuong Nature Reserves; (B) Breeding site of *Tylototriton vietnamensis* in the Dong Son – Ky Thuong Nature Reserve in Quang Ninh Province; (C) Forest type in the province of Quang Ninh.

found in DSKT NR, we compared only the morphological characters of adult males from DSKT in Quang Ninh Province with those from the type locality in Bac Giang Province.

Most specimens were released at their original sampling sites after taking morphological measurements and photographs. For genetic analysis, however, five adult males were collected as voucher specimens. These were first anaesthetized with ethyl acetate, fixed in 50% ethanol for a few hours, subsequently preserved in 70% ethanol, and deposited at the Institute of Ecology and Biological Resources (IEBR), Hanoi, Vietnam. Their catalogued numbers and date of collection are the following: IEBR A.2014.41, collected on 12 June 2012 in Tuan Mau Commune, Son Dong District, Bac Giang Province; IEBR A.2014.42, A.2014.43, collected on 9 June 2012 in Dong Son – Ky Thuong NR, Dong Son Commune, Hoanh Bo District, Quang Ninh Province; and IEBR A.2014.44, A.2014.45 collected on 24 July 2012 in Mau Son Commune, Loc Binh District, Lang Son Province.

We sequenced a partial mitochondrial gene, the NADH dehydrogenase subunit 2 (ND2), from the collected voucher specimens using the primer pair Sal_ND2_F1 and Sal_ND2_R2 (Nishikawa et al. 2013b). Sequences of *T. hainanensis*, *T. taliangensis*, *T. vietnamensis*, and *T. verrucosus* were obtained from GenBank. Data were analysed using maximum parsimony (MP) as incorporated in PAUP 4.0b10 (Swofford 2001). Uncorrected pairwise divergences were calculated with PAUP*4.0b10. Laboratory protocols and phylogenetic analyses followed Le et al. (2006).

Data for statistic analyses were organized in a bivariate scatter plot matrix and bivariate scatter plots and analysed for normal distribution and the presence of outliers, in which case the latter ones were excluded. Each measurement was calculated as a proportion of SVL (variable measurement/SVL) and log-transformed. The data were organized in three different sub-sets referring to head, limbs and tail morphology and analysed separately. For each sub-set of data, a Principal Component Analysis (PCA) was conducted to identify which variables were generally responsible for morphological variation, followed by a Redundancy Analysis (RDA) with 'Province' as the environmental variable, to specifically identify which character differed between the two provinces. A significance test for the RDA was performed with a One-way Analysis of Variance (ANOVA) for a significance level of 95%. Significance levels are presented in the text as following: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; ns = $p > 0.05$). The most important morphological characters resulting from a significant RDA were furthermore tested with ANOVAs and linear regressions. All statistic analyses were performed with RStudio v. 0.98.1102.

In June of 2012, specimens of *T. vietnamensis* were found in two of seven surveyed ponds within the DSKT NR, about 35 km air-line distance from the type locality of the species. These ponds were situated 2 km apart at altitudes of 419 and 456 m a.s.l., respectively. In one pond (approximately 400 m² surface of water and 30 cm maximum depth), a total of 20 adult males were collected from the water, along with two larvae at stage 44 (following Grosse 2013) and eight unattended clutches at the banks. Half of the clutches had empty eggs and were decomposing, and the other half consisted of 48–75 eggs (mean ± SD 53.25 ± 14.77). Egg sizes varied between 9.37 and 13.37 mm (11.34 ± 0.94) and distanced between 125 and 267 cm (206 ± 58.95) from the water. In the second pond (approximately 42 m² surface of water and 40 cm maximum depth), only two adult males were encountered. In May 2014, these ponds were revisited. The 'larger pond' had a remaining water surface of 15 m² and 20 cm depth and the 'smaller pond' 17 m² and a depth of 20 cm. A total of 14 and 10 adult males were found, respectively, in these ponds, but there was no evidence of reproductive activity.

These two ponds were characterized by pH values from 6.1 to 7.6, KH = 4°, gH = 1°, $[SiO_2^{2-}] > 6$ mg/l, $[NH_4^+] = 0$ mg/l, $[NO_2^-] < 0.05$ mg/l, $[NO_3^-] < 5$ mg/l, and $[Fe^-] > 1.5$ mg/l. The canopy cover above the water varied from 50 to 100%. The environmental parameters at the sites were: air temperature between 27.8 and 28.9°C, water temperature 26°C, humidity from 97 to 99% and atmospheric pressure from 28.08 to 28.25 in Hg.

Morphologically, the specimens of *T. vietnamensis* from the DSKT NR agreed well with the diagnostic characters provided by Böhme et al. (2005).

The final data matrix contained 12 terminals and 1035 aligned characters. Our phylogenetic analysis produced three most parsimonious trees (tree length = 571, consistency index = 0.64, retention index = 0.88). All samples of *T. vietnamensis* were strongly supported as monophyletic (bootstrap value = 100, Fig. 2). Genetic divergence between the samples was small, lower than 1.5%, while the genetic distance between *T. vietnamensis* and the closely related *T. hainanensis* was greater than 10%. These data show that the population of *Tylototriton* from DSKT in Quang Ninh Province is conspecific with *T. vietnamensis* from Bac Giang and Lang Son provinces.

A simple t-test of SVL showed no significant differences between Bac Giang and Quang Ninh populations ($F_{1, 314} = 1.775$, ns [Table 1]; BG: 67.464 ± 4.214, N = 282; QN: 68.445 ± 3.096, N = 35 [Table 2]). The SVL-corrected body mass on the other hand was by 6.78% higher in the Quang Ninh population ($F_{1, 288} = 37.69$, ***; BG: 8.5 ± 1.21, N = 258; QN: 8.966 ± 1.02, N = 32). The mean body condition index (100 X (log weight/log(SVL+TL)) was also higher in the Quang Ninh population (47) compared to the Bac Giang population (44).

Our PCAs analysis indicated that the most important characters to explain morphological variation were HL, PW, PH, HUM, TIB, HIND/FORE, RAD/HUM, TL and TH. The RDA dealing with the head morphology was not significantly different between the two populations from Bac Giang and Quang Ninh provinces ($F_{1, 56} = 0.929$, ns). The RDA for the limbs on the other hand identified FEM, RAD and TIB/FEM as the most important characters to distinguish limb morphology between sites ($F_{1, 114} = 7.497$, **). The SVL-corrected and log-transformed ratio of TIB/

FEM was by 23% lower in the Quang Ninh population than that in the Bac Giang population ($F_{1, 114}$ = 7.497, **). The lengths of tibia and femur were linearly related (y = 0.324 x - 0.603; $F_{1, 114}$ = 19.63, ***), but explain only 14% of the variation. This might be due to a higher variation in the Bac Giang population (-1.143 < TIB < -0.177; -0.695 < FEM < 0.148) than that in Quang Ninh (-0.966 < TIB < -0.374; -0.459 < FEM < 0.135) population. Femur length was by 29.8% longer in the Bac Giang population ($F_{1, 114}$ = 7.646, **) and tibia length by 7.4% longer in the Quang Ninh population ($F_{1, 114}$ = 7.581, **) (Fig. 3A).

The ratio of RAD/HUM, however, showed no significant inter-population difference ($F_{1, 114}$ = 0.294, ns). There was a linear relationship between the two morphological characters in Bac Giang specimens (y = 0.460 x + 0.292; $F_{1, 80}$ = 31.43, ***) but not in Quang Ninh specimens ($F_{1, 32}$ = 0.087, ns) (Fig. 3B). However, this proportionality only explained 27.3% of the variation. The relation between the length of the radius and the SVL was significant in both the Bac Giang (y = 0.05 x + 11.346; $F_{1, 80}$ = 7.797, **) and Quang Ninh populations (y = 0.135 x + 6.727; $F_{1, 32}$ = 10.58, **), while the humerus length, although related to SVL, in the Quang Ninh population (y = 0.155 x - 0.91; $F_{1, 32}$ = 5.696, *) was not statistically significant in the Bac Giang population ($F_{1, 80}$ = 0.381, ns). Humerus length was by 28.2% longer in the Bac Giang population ($F_{1, 114}$ = 13.04, ***), while the radius length was by 8.9% longer in the Quang Ninh population ($F_{1, 114}$ = 30.44, ***) (Fig. 3C).

The proportion between hind and fore limbs did not differ between the two populations ($F_{1, 114}$ = 1.056, ns). Nevertheless, both hind ($F_{1, 114}$ = 11.92, ***) and fore limbs ($F_{1, 114}$ = 29.99, ***) were longer in the Quang Ninh population than those in the Bac Giang population by 7.23 and 9.13%, respectively (HIND: BG = 23.31 ± 1.82, N = 83; QN = 24.88 ± 2.3, N = 34; FORE: BG = 23.65 ± 1.61, N = 83; QN = 25.72 ± 1.66, N = 34).

Since the sampling always took place during the breeding season (during May–June in the DSKT NR and during April–July in Bac Giang Province), we considered that the tail morphology (length and height) would not change significantly. The RDA for tail morphology indicated that tail height and TL/TH were the most important variables to explain morphological differences between the two populations ($F_{1, 304}$ = 11.232, ***). The SVL-corrected and log-transformed ratio TL/TH was by 10.96% greater in the DSKT NR population than that in the population from Bac Giang ($F_{1, 304}$ = 9.793, **). Tail height was linearly related to tail length (y = 0.223 x - 0.166; $F_{1, 304}$ = 18, 51, ***), although only 5.5% of the variation could be explained. While the relation between tail length and SVL was significant in the Bac Giang (y = 0.403 x + 31.406; $F_{1, 272}$ = 27.33, ***) and Quang Ninh populations (y = 0.515 x + 25.561; $F_{1, 32}$ = 8.205, **), tail height was only linearly related to SVL in the Bac Giang population (y = 0.083 x + 3.967; $F_{1, 272}$ = 26.29, ***) and not in the one in Quang Ninh ($F_{1, 32}$ = 0.058, ns). The tail length was by 18.86% longer in the Quang Ninh population (BG = 58.59 ± 5.29, N = 280; QN = 60.78 ± 3.54, N = 34; $F_{1, 306}$ = 4.453, *), while tail height was 70% higher than in the Bac Giang population (BG = 9.61 ± 1.12, N = 277; QN = 10.68 ± 0.95, N = 35; $F_{1, 306}$ = 6.086, **) (Fig. 3D).

Although our molecular analysis showed no significant genetic differences in the mitochondrial DNA-sequenced data between the newly discovered population of *T. vietnamensis* in the DSKT NR and other populations, morphological comparisons indicated some intraspecific variation. Males of the new population have slightly longer tails and longer fore and hind limbs. Although the ratio between hind and fore limbs was the same for the two populations,

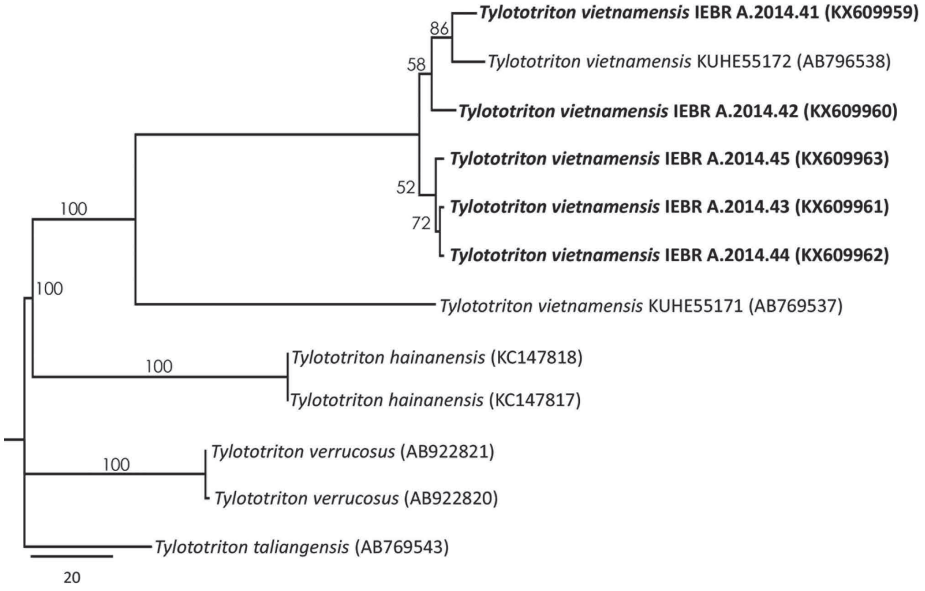

Figure 2. One of three most parsimonious trees based on the partial ND2 gene. Numbers above branches are bootstrap values. Newly collected samples are shown in bold.

Table 1. Results of analysis of variance of morphological characters of *Tylototriton vietnamensis* from two different populations, Bac Giang (BG) and Quang Ninh (QN). F – F-statistics; DF – degree of freedom; P – p-value; N – number of specimens analysed. Significance levels are presented as * = p < 0.05; ** = p < 0.01; *** = p < 0.001.

Variation between populations	F	DF	P	N
SVL	1.78	314	0.184	317
Body mass	37.69	288	<0.001***	290
RDA head	0.93	56	0.401	58
RDA limbs	9.47	114	0.001***	116
TIB/FEM	7.50	114	0.007**	116
FEM	7.65	114	0.006**	116
TIB	7.58	114	0.007**	116
RAD/HUM	0.29	114	0.58	116
HUM	13.04	114	<0.001***	116
RAD	30.44	114	<0.001***	116
HIND/FORE	1.06	114	0.306	116
HIND	11.92	114	<0.001***	116
FORE	29.99	114	0.001***	116
RDA tail	11.23	304	0.001***	306
TL/TH	9.79	304	0.002**	306
TL	4.45	306	0.036*	308
TH	6.09	306	0.014**	308
Variation between combinations				
TIB on FEM	19.63	114	<0.001***	116
RAD on HUM BG	31.43	80	<0.001***	82
RAD on HUM QN	0.09	32	0.769	34
TH on TL	18.51	304	<0.001***	306
Variation relative to SVL within population from ...				
... Bac Giang: RAD	7.80	80	0.007**	82
HUM	0.38	80	0.539	82
TL	27.33	272	<0.001***	274
TH	26.29	272	<0.001***	274
... Quang Ninh: RAD	10.58	32	0.003**	34
HUM	5.70	32	0.023*	34
TL	8.21	32	0.007**	34
TH	0.06	32	0.8	34

the longer limbs in the population from Quang Ninh Province might be related to an increase in weight-bearing related to the longer tail.

Homologous structures present in limbs (e.g., radius–tibia and humerus–femur) showed a stronger relationship between them than between the skeletal elements of each limb: radius and tibia lengths were by ca 30% longer in the Bac Giang population, while humerus and femur lengths were by ca 10% longer than those in the Quang Ninh population. Morphological differences observed in geographically distinct populations might reflect different environmental conditions (Zhang et al. 2014), i.e., biotic factors such as food availability (Maerz et al. 2006), or abiotic factors such as climate and altitude (Tilley 1980; Miaud

Table 2. Morphological measurements of live males of *Tylototriton vietnamensis* from Bac Giang Province (type locality) and Quang Ninh Province (new population). Mean ± standard deviation (SD) above range in parentheses and number of individuals (N).

Character	Bac Giang	Quang Ninh
SVL (mm)	67.46±4.21 (49.22–86.53) N=281	68.44±3.1 (62.66–73.63) N=35
Body mass (g)	8.51±1.22 (5.9–13.25) N=259	9.97±1.02 (8.1–12.42) N=32
HIND (mm)	23.31±1.82 (19.11–27.29) N=83	24.88±2.3 (18.15–28.16) N=34
FORE (mm)	23.65±1.61 (20.89–28.08) N=83	25.72±1.66 (21.46–28.67) N=34
TL (mm)	58.59±5.29 (33.70–71.34) N=280	60.78±3.54 (53.54–70.19) N=34
TH (mm)	9.61±1.12 (6.58–13.13) N=277	10.68±0.95 (8.50–12.84) N=35

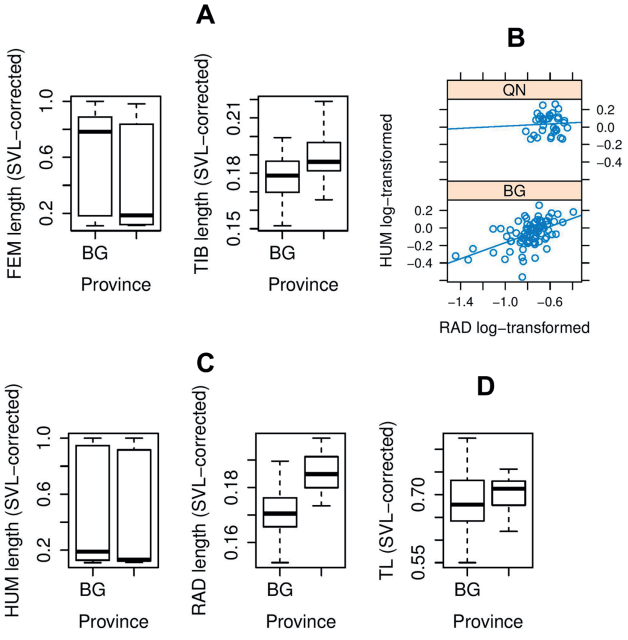

Figure 3. Variation of the following morphological characters of *Tylototriton vietnamensis* between Bac Giang (BG) and Quang Ninh (QN) provinces: (A) SVL-corrected femur (FEM) and tibia (TIB) lengths; (B) XY-plot of the log-transformed relation between humerus (HUM) and radius (RAD) and variation of the SVL-corrected humerus and radius lengths; (C) SVL-corrected tail length (TL).

et al. 2000). Since the population from the DSKT NR is distributed at similar altitudes and not far apart from the population at the type locality (see Bernardes et al. 2013), we can exclude potential effects from climate and altitude. However, our results revealed a higher pH and carbonate hardness in ponds in the DSKT NR, while in the province of Bac Giang, ponds are characterized by more acidic water and lower carbonate hardness. In fact, the parent rock material in Bac Giang Province is igneous (granite), whereas the coastal area in Quang Ninh is characterized by the presence of sedimentary rock, such as limestone (Sterling et al. 2006). We expect that these differences in the type of bedrock influence both microhabitat characteristics and associated prey communities, although dietary studies are still necessary to evaluate the existence of a true difference between populations. Our data was not conclusive in differentiating the two populations in terms of other ecological factors or explaining the absence of *T. vietnamensis* from five ponds in the DSKT NR. As Bernardes et al (2013) noted, the ponds' hydroperiods during the breeding season seem to be a reasonable variable that limits the distribution of *T. vietnamensis* throughout its range. Concentrations of ammonium and nitrites were low, but the higher concentration of nitrates might be an indicator of eutrophication and the presence of organic pollutants. However, these values, and the high concentration of silicic acid and iron in the DSKT NR, are still compatible with amphibian life and not toxic for amphibians (Marco et al. 1999).

Coal mining is a particular threat to forest integrity and biodiversity in the region, and with 98% of the coal deposits in Vietnam being located in Quang Ninh, this province is the main supplier for the country (Sterling et al. 2006). In Bac Giang Province, coal mining also extends to the buffer zone of the Tay Yen Tu Nature Reserve and constitutes a major threat to *T. vietnamensis* and the associated fauna and flora due to direct forest destruction, fragmentation and water pollution (van Schingen et al. 2015). Despite of the newly discovered occurrences of *T. vietnamensis* being situated inside a nature reserve, we noticed an increase in human disturbance during the research period. In 2012, there was no evident disturbance in the vicinity, but two years later, we found trails in the surrounding forests and evidence that the ponds were used by cattle. Furthermore, we have witnessed that local people collect newts from the wild, either to use them privately as traditional medicine, or for selling them at Chinese markets, to the international pet trade, or to local tourists at the pagoda of Mount Yen Tu. Rowley et al. (2016) reported that pet shops in Hanoi claimed to sell wild-caught newts from Bac Giang Province.

Tylototriton vietnamensis currently is evaluated as Endangered in the IUCN Red List (2016) and in the Red Book of Vietnam (Tran et al. 2007). Despite the proximity between this newly discovered population and the type locality, suitable habitat between them is so fragmented that it is virtually nonexistent, as is reflected by the scarcity of forested areas (Fig. 1). Efforts should be increased to reduce habitat fragmentation and avoid the isolation of *T. vietnamensis* populations in particular and the entire associated fauna in general. It could be devastating for the continued existence of *T. vietnamensis* if its genetic diversity was diminished due to high levels of isolation between populations. We therefore recommend that the already existing green-corridor network between the individual protected areas be strengthened to facilitate exchange between populations.

A population monitoring programme is required for the area to clarify the health and viability of the resident populations. As this site harbours the largest known population of *T. vietnamensis* in the wild, the survival of the entire species might rely on present conservation efforts and good management plans.

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