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EXAMINATION OF PHYLOGENETIC RELATIONSHIP AND SEXUAL DIMORPHISM IN THE VIETNAM WARTY NEWT (*Paramesotriton deloustali* (Bourret, 1934)) IN VIETNAM

Thinh Van Nguyen[1] Thien Tao Nguyen[2,✉], Hai Ngoc Ngo[2,✉], Hoa Thi Ninh[2], Huy Quoc Nguyen[2], Cuong The Pham[3], Truong Quang Nguyen[3], Marta Bernardes[4,5] and Thomas Ziegler[4,5]

[1]*Graduate University of Science and Technology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Nghia Do, Cau Giay, Hanoi 10000, Vietnam.*

[2]*Institute of Genome Research, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Nghia Do, Cau Giay, Hanoi 10000, Vietnam.*

[3]*Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Nghia Do, Cau Giay, Hanoi 10000, Vietnam.*

[4]*Institute of Zoology, University of Cologne, Zülpicher Strasse 47b, 50674 Cologne, Germany.*

[5]*Cologne Zoo, Riehler Straße 173, 50735, Cologne, Germany.*

✉To whom correspondence should be addressed. E-mail: nguyenthientao@gmail.com; ngohai2709@gmail.com

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ABSTRACT

Two warty newt species, *Paramesotriton deloustali* and *P. guangxiensis*, are known from Vietnam for a long time. However, the taxonomic status of some Vietnamese populations was recently clarified. In this study, we conducted surveys in northern Vietnam to collect samples for molecular and morphological analyses of *Paramesotriton* species. Our phylogenetic analyses based on two mitochondrial genes (16S and ND2) agreed well with previous findings that populations from Bac Kan, Ha Giang, Lao Cai, Son La and Vinh Phuc provinces were regarded as *P. deloustali*, and populations from Cao Bang and Quang Ninh provinces were identified as *P. guangxiensis*. Regarding sexual dimorphism, it is a prevalent phenomenon in many salamanders, including *P. deloustali*. There exists a female-biased pattern of size dimorphism with longer snout-vent length and trunk length in females than in males of *P. deloustali*. On the other hand, a male-biased pattern is documented for cloaca sizes. Furthermore, only males display a unique color of light green on the lateral sides of the tail.

Keywords: Color; morphology; natural selections; genetic divergences; populations.

INTRODUCTION

Asian warty newts of the genus *Paramesotriton* currently consist of 15 species inhabiting restricted distributions in southern China and northern Vietnam (Gu *et al.*, 2012; Wang, Tian & Gu, 2013; Sparreboom, 2014; Yuan *et al.*, 2014, 2016; Frost, 2022). Recently, taxonomic issues of *Paramesotriton* have been intensely investigated and new species have been discovered based on integrative approaches of morphological and molecular analyses. For example, five recently described species were previously hidden under the name *P. chinensis*, a species previously stated to be widely distributed in China, which now consists of *P. longliensis* from Chongqing

Province (Wu *et al.*, 2010), *P. yunwuensis* from Guangdong Province (Wu *et al.*, 2010), *P. fuzhongensis* and *P. labiatus* from Guangxi Province (Wu *et al.*, 2009; Wu *et al.*, 2010), and *P. qixilingensis* from Jiangxi Province. Luo *et al*. (2022) combined mitochondrial genomes and nuclear genes to reconstruct the molecular systematics among 14 warty newt species, but five potential cryptic taxa were noted, supporting the ongoing taxonomic complexity within the genus.

In Vietnam, only two *Paramesotriton* species have been found so far, namely the Vietnam Warty Newt – *P. deloustali* and the Guangxi Warty Newt – *P. guangxiensis* (Weisrock *et al.*, 2006; Nguyen *et al.*, 2009). *Paramesotriton deloustali* was originally described by Bourret (1934) from the Tam Dao Mountain, Vinh Phuc Province and subsequently recorded in several northern provinces of Vietnam, viz. in Bac Kan, Ha Giang, Lang Son, Quang Ninh, Son La, Thai Nguyen, Tuyen Quang and Yen Bai (Lu *et al.*, 2004; Weisrock *et al.*, 2006; Bour *et al.*, 2009; Nguyen *et al.*, 2009; Pham *et al.*, 2022). Zhang *et al*. (2018) first recorded *P. deloustali* from southern Yunnan, China, and sequenced the entire mitochondrial genome of the species. Using molecular data, Tran *et al*. (2023) evaluated phylogenetic relationships among *Paramesotriton* populations in Vietnam and determined their distribution. Accordingly, *P. deloustali* was confirmed to occur in Vinh Phuc, Thai Nguyen, Tuyen Quang, Yen Bai, and Lao Cai provinces, and another warty newt – *P. guangxiensis* was found in Cao Bang and Quang Ninh provinces.

Given the intraspecific difference between males and females in phenotypic characteristics, such as coloration, vocal sacs, skin patterns, body size and other morphological traits, sexual dimorphism has been widely documented in many terrestrial organisms, including salamanders (Darwin, 1871; Shine, 1979; Andersson, 1994; Fairbairn, Blackenhorn & Szekely, 2007; Kupfer, 2007). This phenomenon might be a result of evolutionary processes under various selections (e.g., natural, sexual or fecundity selection) that biasedly affect one sex. For example, the fecundity selection may particularly favor larger females to enhance the reproductive capacity, whereas male-male contests or mating choice driven by the sexual selection tend to prefer large males. Regarding the niche divergence, the sexual dimorphism evolves to reduce inter-sexual competition for food resources and micro-habitat preference. Studying the sexual dimorphism is ergo crucial for understanding the causes of morphological variations (Darwin, 1871; Shine, 1979; Andersson, 1994; Fairbairn *et al.*, 2007; Kupfer, 2007).

In this study, we collected molecular data of *Paramesotriton* to review phylogenetic relationships among surveyed populations in Vietnam, versus the analysis of Tran *et al*. (2023). We further used the extensive morphological data of *P. deloustali* to examine the variation among populations and the phenomenon of sexual dimorphism. Like many salamander species, we hypothesize that there is an inter-sexual variation in coloration and the female-biased morphology, referring to the sexual dimorphism of *P. deloustali* (Shine, 1979).

MATERIALS AND METHODS

Field surveys

Field surveys were conducted in June 2012, July 2013, June 2014, April 2017, June 2018 and August 2023 in four provinces in northern Vietnam, including Ba Be National Park (NP) (N 22.412, E 105.616, Elv: 750 m), Bac Kan Province; Xin Man District, Ha Giang Province (N 20.5097, E 104.4808, Elv 700 m); Van Ban District, Lao Cai Province (N 22.144, E 104.1, Elv: 650 m); and Tam Dao NP, Vinh Phuc Province (N 21.4261, E 105.635, Elv: 1.200 m). Additional field surveys were conducted in Ba Che District, Quang Ninh Province in November 2014 and August – September 2023, Phia Oac – Phia Den NP, Cao Bang Province in April 2017 and Muong La District, Son La Province in October 2019 to obtain tissue samples/voucher specimens of warty newts (Fig. 1A, Table 1). The surveyed streams with low gradient and clear basins were located within evergreen forests and some sections were along rice fields. Stream bottoms contained leaves, dead wood, moss, sand, fine gravel, and scattered large rocks (Fig. 1B). The width of these streams was estimated from 1 to 5 m. In surveyed streams, individuals of *P. deloustali* were mainly observed in shallow and slow-moving sections (Ngo pers obs.).

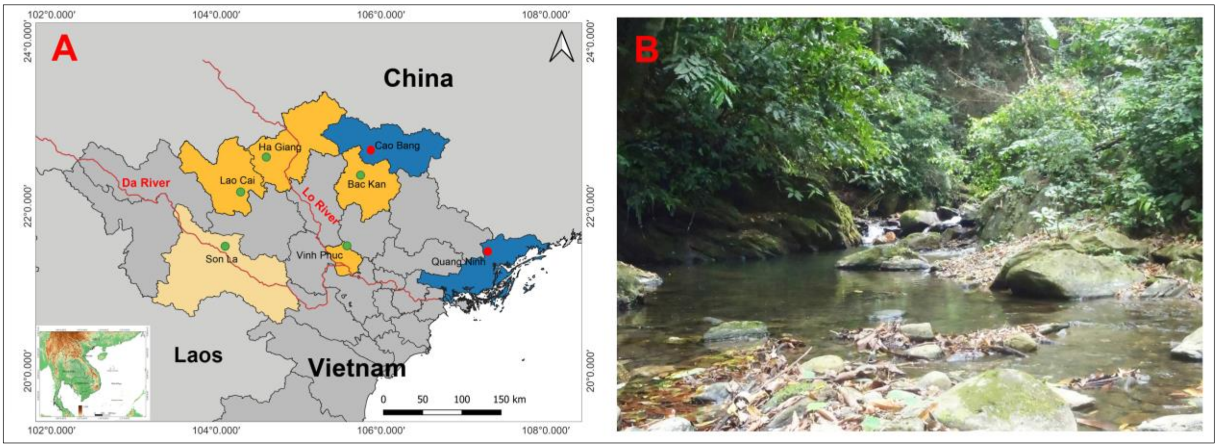

Figure 1. A. Map of northern Vietnam, including study sites (green circles for *Paramesotriton deloustali*, n = 5; red circles for *Paramesotriton guangxiensis*, n = 2); B. Warty newt microhabitat (*P. deloustali*) in Bac Kan Province (Photo by Hai Ngoc Ngo).

Additional tissue samples of *Paramesotriton* were collected from preserved specimens in the collections of the Institute of Ecology and Biological Resources (IEBR), Vietnam National Museum of Nature (VNMN), and Institute of Genome Research (IGR), Hanoi, Vietnam, for molecular analyses (Table 1).

Table 1. Samples of *Paramesotriton* species used in the molecular analyses of this study.

No.	Scientific name	Voucher number	Locality	Genbank no ND2	Genbank no 16S	Reference
1	*Paramesotriton deloustali*	MVZ223628	Tam Dao, Vinh Phuc, Vietnam		EU880327	Zhang *et al.*, 2008
2	*P. chinensis*	-	Guangxi, China	NC-35008	NC-35008	Yang *et al.*, 2017
3	*P. chinensis*	-	Guangxi, China	KY609177	KY609177	Yang *et al.*, 2017
4	*P. deloustali*		Yunnan, China		KY744236	Zhang *et al.*, 2017
5	*P. deloustali*	CAU1507046	Yunnan, China		NC 037713	Zhang *et al.*, 2017
6	*P. deloustali*	TD03	Tam Dao, Vinh Phuc, Vietnam		This study	
7	*P. deloustali*	VNMN 06615	Tam Dao, Vinh Phuc, Vietnam	This study		
8	*P. deloustali*	VNMN 06616	Tam Dao, Vinh Phuc, Vietnam	This study		
9	*P. deloustali*	VNMN 6617	Tam Dao, Vinh Phuc, Vietnam	This study		

10	*P. deloustali*	VNMN 6621	Tam Dao, Vinh Phuc, Vietnam	This study		
11	*P. deloustali*	VNMN 6622	Tam Dao, Vinh Phuc, Vietnam	This study		
12	*P. deloustali*	BK (2) BB	Ba Be, Bac Kan, Vienam	This study	This study	
13	*P. deloustali*	BK (1) BB	Ba Be, Bac Kan, Vienam		This study	
14	*P. deloustali*	BK (4) BB	Ba Be, Bac Kan, Vienam		This study	
15	*P. deloustali*	BK (3) BB	Ba Be, Bac Kan, Vienam	This study	This study	
16	*P. deloustali*	XM 10 HG	Xi Man, Ha Giang, Vietnam	This study		
17	*P. deloustali*	XM 12 HG	Xi Man, Ha Giang, Vietnam	This study		
18	*P. deloustali*	Laocai 02	Lao Cai, Vietnam	This study		
19	*P. deloustali*	Son La 03	Son La, Vietnam	This study		
20	*P. deloustali*	Son La 04	Son La, Vietnam	This study		
21	*P. guangxienesis*	CB (1)	Cao Bang, Vietnam	This study	This study	
22	*P. guangxienesis*	CB (2)	Cao Bang, Vietnam		This study	
23	*P. guangxienesis*	CB (3)	Cao Bang, Vietnam		This study	
24	*P. guangxienesis*	PR 10	Ba Che, Quang Ninh, Vietnam	This study	This study	
25	*P. guangxienesis*	PR 11	Ba Che, Quang Ninh, Vietnam	This study		
26	*P. guangxiensis*	MVZ220905	Linming County, Guangxi, China	DQ517804		David *et al.*, 2006
27	*P. guangxiensis*	KIZ09285	Ningming, Guangxi, China	KU375035		Yuan *et al.*, 2016

Molecular analysis

For the molecular analyses, two fragments of mitochondrial genes, the NADH dehydrogenase subunit 2 (ND2) and 16S rRNA, were selected for DNA sequencing. The primer pair Sal_ND2_F1 (5’-AAGCTTTTGGGCCCATACC-3’) and Sal_ND2_R2 (5’-GGTTGCATTCAGAAGATGTG-3’) (Nishikawa, Matsui & Nguyen, 2013) were used to amplify a fragment of approximately 545 bps of the ND2 gene for 15 voucher specimens, and the primer pair L2204 (AAAGTGGGCCTAAAAGCAGCCA) (Matsui *et al.*, 2006) and H3056 (CTCCGGTCTGAACTCAGATCACGTAGG) (Hedges 1994) were used for the fragment of approximately 871 bps of 16S rRNA for nine voucher specimens (table 1). For mtDNA, the PCR conditions included an initial denaturation at 94°C for 5 min and 35 cycles of the 30s at 94 °C, 15 s at 53°C and 2 min 40 s at 72°C. PCR products were purified using the Genomic DNA Purification Kit (Germany) and then sent to 1stBase company (Malaysia) for sequencing.

Sequences of *Paramesotriton chinensis* were used as an outgroup in the phylogenetic analyses (Yang *et al.*, 2017; Table 1). Chromas Pro Software (Technelysium Pty Ltd., Tewantin, Australia) was used to edit the sequences, which were aligned using MEGA version 7 (Kumar, Stecher & Tamura, 2016) with default settings. We then checked the initial alignments by eye and adjusted them slightly. Phylogenetic trees were constructed using IQ-Tree (Nguyen *et al.*, 2015) with maximum likelihood bootstrap support (MLBS) evaluated by ultrafast bootstrap approximation with 1000 replicates (Hoang *et al.*, 2018) (ML) and Bayesian inference (BI). Prior to running ML and Bayesian analyses, we chose the optimum substitution models using Kakusan 4 (Tanabe, 2011) based on the Akaike information criterion (AIC). The best model selected for ML was the general time reversible model (GTR: Tavaré, 1986) with a gamma shape parameter (G: 0.100 in ML and 0.483 in BI) in the 16S rRNA fragment analysis and (G: 0.9339 in ML and 0.985 in BI) in the ND2 fragment analysis. The BI phylogenetic construction was done in MrBayes version 3.2.7a (Ronquist *et al.*, 2012) in two independent runs with four Markov Chains for 10,000,000 generations. A tree was sampled every 100 generations and a consensus topology was calculated for 70,000 trees after discarding the first 30,001 trees (burn-in = 3,000,000). We checked parameter estimates and convergence using Tracer version 1.6 (Rambaut & Drummond, 2013). The strength of nodal support in the ML tree was analyzed by using non-parametric bootstrapping (MLBS) with 1,000 replicates. We regarded tree nodes in the ML tree with bootstrap values of 70% or greater as sufficiently resolved (Hillis & Bull, 1993), and nodes with a BPP of 95% or greater as significant in the BI analysis (Leaché & Reeder, 2002). Pairwise comparisons of uncorrected sequence divergences (p-distance) were calculated for 16S rRNA and ND2 fragments only between species of the genus *Paramesotriton* using Mega version 7.

Morphological data

Animals were captured by hand and subsequently released at the same sites after measured and photographed. A total of 19 morphometric variables of four *Paramesotriton* populations from Bac Kan, Ha Giang, Lao Cai and Vinh Phuc provinces were measured with dial calipers to the nearest 0.1 mm at the right side of each individual (Table 2). Values were presented as mean ± standard deviation and ranged from minimum to maximum. The coloration variation was assessed between males and females, by taking photos from four directions (e. g., left, right, ventral, and dorsal sides). Sex of each individual was determined by the presence of the large swollen cloaca in male, while the non-swollen cloaca was absent in female (Fig. 3).

Table 2. Definitions of the morphological character sets and abbreviations

Characters	Definition
SVL	Snout-vent length: from the tip of snout to the posterior margin of the cloaca
HL	Head length: from the tip of the snout to the gular fold

HH	Head height: height of the head at its highest point
HW	Head width: width of the head at its widest point
MW	Mouth width: the distance between mouth angles
JL	Upper jaw length
END	Eye–naris distance
ESD	Eye–snout distance
OD	Distance from posterior dorsal cranial edge to the posterior corner of the eye.
ED	Diameter of the eye
IN	Internarial distance from nostril to nostril
IC	Intercanthal distance: the minimum distance between anterior corners of the eyes
FLL	Forelimb length: from the base of the forelimb to the tip of the longest finger
HLL	Hindlimb length: from the base of the hindlimb to the tip of the longest toe
AGS	Space between axilla and groin: the space between the posterior base of the forelimb and the anterior base of the hindlimb on the same side
CL	Cloacal length
CW	Cloacal width
TL	Tail length: from the posterior margin of the cloaca to the tip of the tail
TW	Tail width

Statistical analysis

The sexual dimorphism index (SDI) was calculated to assess sexual size dimorphism (SSD) by the formula suggested by Lovich & Gibbons (1992), in which SDI = (size of larger sex / size of smaller sex) ± 1; +1 if males are larger or -1 if females are larger, and the results are arbitrarily defined as positive when females are larger than males and negative on the contrary. A Student t-test was performed to determine the difference in the Snout-Vent length (SVL) between males and females. All statistical analyses were performed by using the software environment R.3.1.2 (RStudio Team, 2018).

Due to high collinearity, the raw differences in other morphological characteristics may be intrinsically affected by the differences in overall body size. To independently assess the differences and limit the correlation effects, a normalization equation following an allometric growth model first suggested by Thorpe (1975) was applied to adjust raw morphological data in the R package GroupStruct through the allom () function, handling multipopulation datasets (Chan & Grismer, 2021). Accordingly, the allometric formula calculates the value: $X_{adj} = \log_{10}(X) - b[\log_{10}(SVL) - \log_{10}(SVL_{mean})]$, where X_{adj} = size corrected variable; X = unadjusted dependent variable; b = regression coefficient or slope of the relationship between $\log_{10}(X)$ and $\log_{10}(SVL)$. The slope is calculated for each population and SVL_{mean} is the grand mean averaged across all populations. Subsequently, we used the Student t-test approach to examine the inter-sexual differences in these adjusted variables. For all of these tests, we applied a significance level of P = 0.05.

We further performed a Multiple Factor Analysis (MFA) for the morphological dataset, comprising five groups – “Head” (i.e., head length, head width, head height, mouth width, and jaw length), “Trunk Length” (i.e., axilla-groin length), “Limbs” (i.e., forelimb and hindlimb), “Cloaca” (i.e., cloacal length) and “Tail” (i.e., tail length). The MFA test was applied to identify active groups and paramount variables accounting for the variation in the morphology dataset and measure their contribution. In addition, we used a Principal Component Analysis (PCA) of these ten selected variables between the combined eastern population (i.e., from Bac Kan and Vinh Phuc provinces) and the western population (i.e., from Ha Giang, Lao Cai provinces) to assess their variation in morphology (Tran *et al.* 2023). Morphological spaces between sexes, and East – West populations were visually illustrated by clustering all individual points of similarly coded colors in the ordination of the Dim1 and Dim2 axes in the MFA and PCA analyses, respectively, to evaluate the possibility of overlap. Their "Dims" were individually extracted to examine the inter-sexual difference and the variation between "East – West" populations by using the student t-test. Tests of MFA and PCA were performed using the packages "factoextra" (Kassambara & Mundt, 2020) and "FactoMinerR" (Le *et al.*, 2008).

RESULTS

Molecular phylogeny

Using two mitochondrial genes (16S and ND2), both ML and BI analyses presented similar topologies for the phylogenetic relationships of Vietnamese *Paramesotriton*, and showed a strong nodal support of two clades with low intraspecific genetic divergences representing *P. deloustali* (16S: 0 – 0.48 %; ND2: 0 – 1.12 %) and *P. guangxiensis* (16S: 0 – 0.75 %; ND2: 0 – 0.76 %) (fig. 2, Supplementary tables S1, S2). Their interspecific genetic divergence was significantly different in both genes (16S: 2.66 – 3.41 %; ND2: 3.18 – 5.42 %; Supplementary tables S1, S2). Like the result of Tran *et al.* (2023), populations of *Paramesotriton* from Bac Kan, Ha Giang, Lao Cai and Son La provinces were conspecific to the topotypic population of *P. deloustali* (Vinh Phuc Province), whereas populations from Cao Bang and Quang Ninh provinces clustered together with *P. guangxiensis* from China.

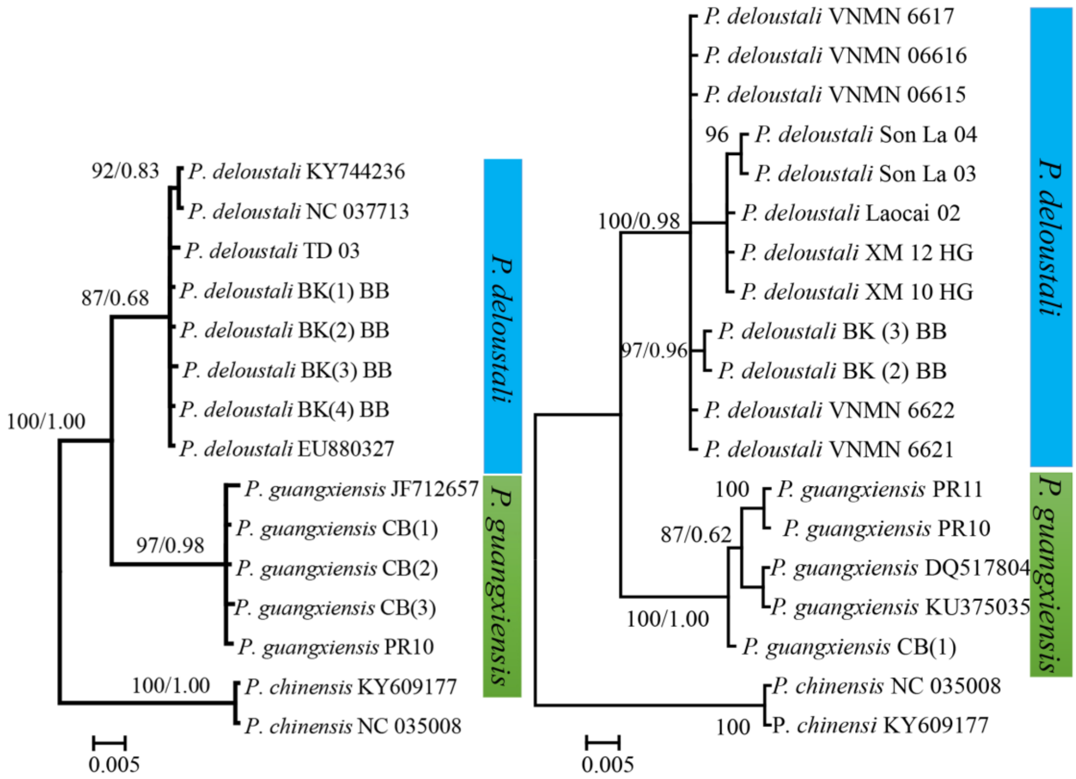

Figure 2. BI tree based on a 871 bp fragment of the mitochondrial 16S rRNA gene (left) and BI tree based on a 545 bp fragment of the mitochondrial ND2 gene (right) among populations of *Paramesotriton*. ML bootstrap values (ML-BS) and Bayesian posterior probabilities (BPP) are shown near the node, respectively.

Sexual dimorphism

In terms of coloration in life, the side of the tail of *P. deloustali* males bears a slight green middle stripe on the brownish skin during the breeding season, whereas the tail sides in females remain reddish brown only. The ventral side of the Vietnam Warty Newts displays various colors with orange, yellow or red, divided with black or grey lines and scattered black dots (Fig. 3). These colors on the belly were noted in both sexes.

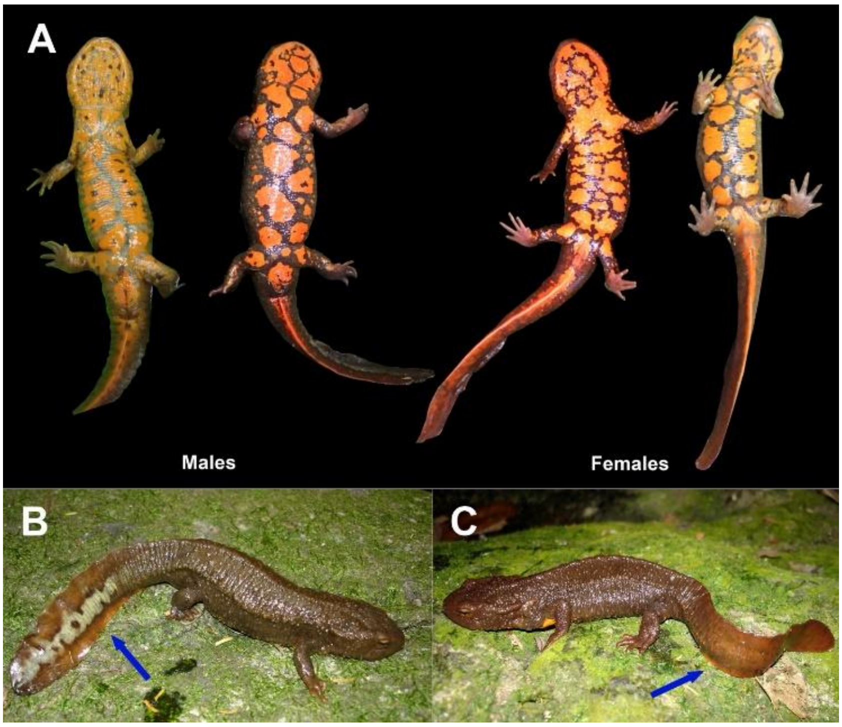

Figure 3. A. Ventral sides of *Paramesotriton deloustali* individuals showing the different cloacal characteristics between sexes; B. Adult male, and; C. Adult female of *P. deloustali* from Tam Dao National Park, Vinh Phuc Province with different tail side color patterns between sexes (blue arrows) (Photos by Huy Quoc Nguyen and Truong Quang Nguyen).

A total of 240 individuals (127 males and 113 females) from four populations were captured to obtain morphological data before releasing, whereof 23 males and 29 females from Ha Giang Province, 22 males and 26 females from Lao Cai Province, 30 males and 35 females from Bac Kan Province, and 52 males and 23 females from Vinh Phuc Province. The mean values and ranges of the 19 phenotypic characteristics of *P. deloustali* are fully presented in Table 3. The mean SVL of females (88.4 ± 7.4 mm, n = 113) was significantly greater than that of males (85.5 ± 7.2 mm, n = 127) (t-test, t = 3.1, df = 233, P value < 0.01; Table 3). This intersexual difference was noted in four surveyed provincial populations (P-values < 0.01). Furthermore, the value of SDI was estimated to be 0.034 > 0, following the female-biased pattern in *P. deloustali*. Using adjusted data for the remaining morphological characteristics, our inter-sexual comparisons in *P. deloustali* showed that females have significantly longer axilla-groin length (AGS), longer tail (TL), wider head width (HW), mouth width (MW), internarial distance (IN), forelimb (FLL) and hindlimb (HLL) than males, indicating the female-biased pattern for these characteristics. On the other hand, males have a significantly longer and wider cloaca (CL and CW) than females, suggesting the male-biased pattern for these characteristics (Fig. 4, Table 3).

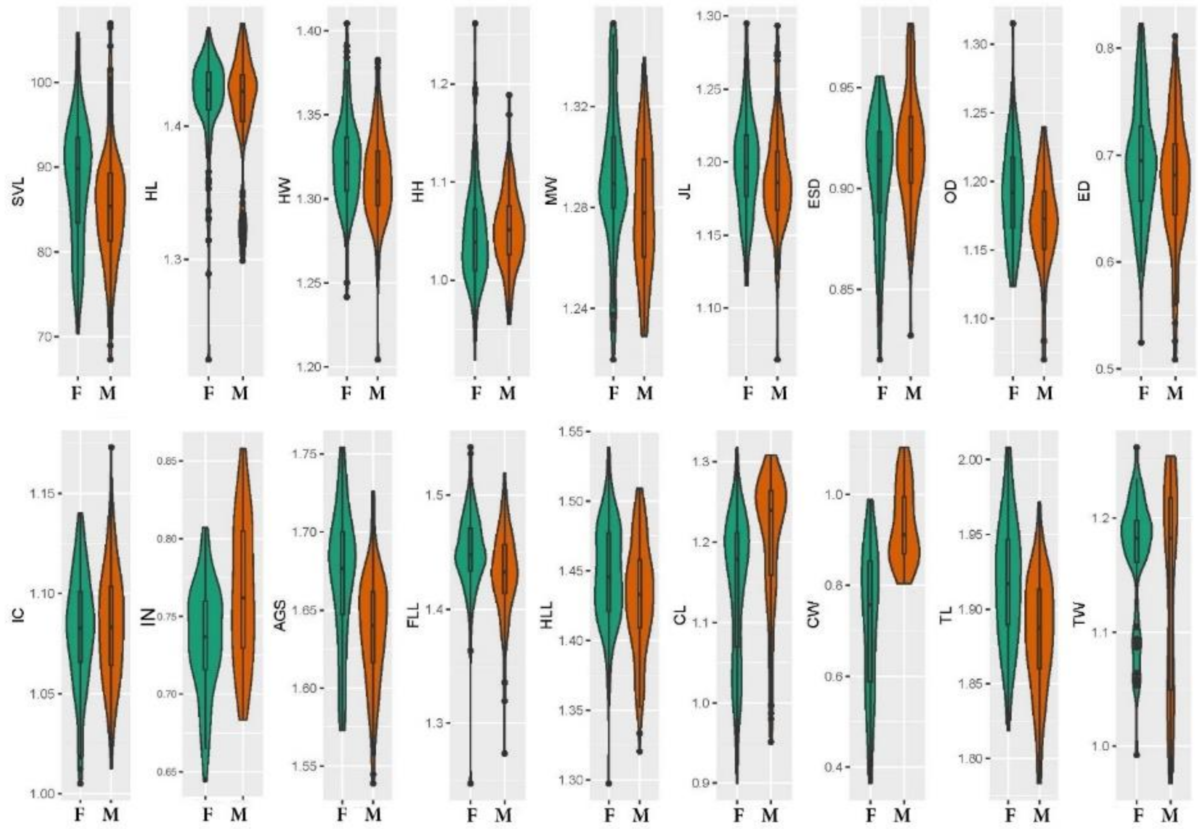

Figure 4. Violin plots overlaid with box plots showing the raw size of snout-vent length (SVL) and remaining size-adjusted morphological characteristics between males and females of *Paramesotriton deloustali* (Abbreviations of characters in Table 2).

The MFA analysis identified morphological data sets of Cloaca, Trunk length and Limbs as active groups (Fig. 5A). We found that axilla-groin length (AGS) and cloacal length (CL) are the most important variables accounting for Dim1 (47.4%) and Dim2 (26%) of the overall variation in morphology of *P. deloustali*, respectively (Figs. 5B, C, D). This test further showed that the morphological space of females relatively overlaps with that of males (Fig. 5E). However, the averages of Dim1 ($t = 9.715$; df = 133; P value < 0.001) and Dim2 ($t = -2.1$; df = 127; P value < 0.05) between males and females are both significantly different.

Given the morphological variation between populations following the genetic separation reported by Tran *et al*. (2023) and confirmed by our phylogenetic analysis, characteristics of SVL, HW, ESD, ED, IC, HLL, TW are significantly different between the East and West groups. In the MFA analysis, the phenotypic space of the East population mostly overlapped with that of the West population (Dim 1: $t = 0.47$, df = 116, P-value = 0.6 > 0.05; Dim 2: $t = 2.38$, df = 126, P-value = 0.02 < 0.05) (Fig. 5). Two PCA analyses between males and females of the two populations showed that their phenotypic spaces are relatively separated from each other (Fig. 5). In statistical comparisons, Dim1-PCA values between populations are significantly different (Males: $t = 3.54$, df = 72, P-value < 0.001; Females: $t = 2.79$, df = 60.5, P-value < 0.01), whereas Dim2-PCA values are slightly consistent (Males: $t = 1.91$, df = 69.3, P-value = 0.06 > 0.05; Females: $t = -0.23$, df = 55.5, P-value = 0.82 > 0.05).

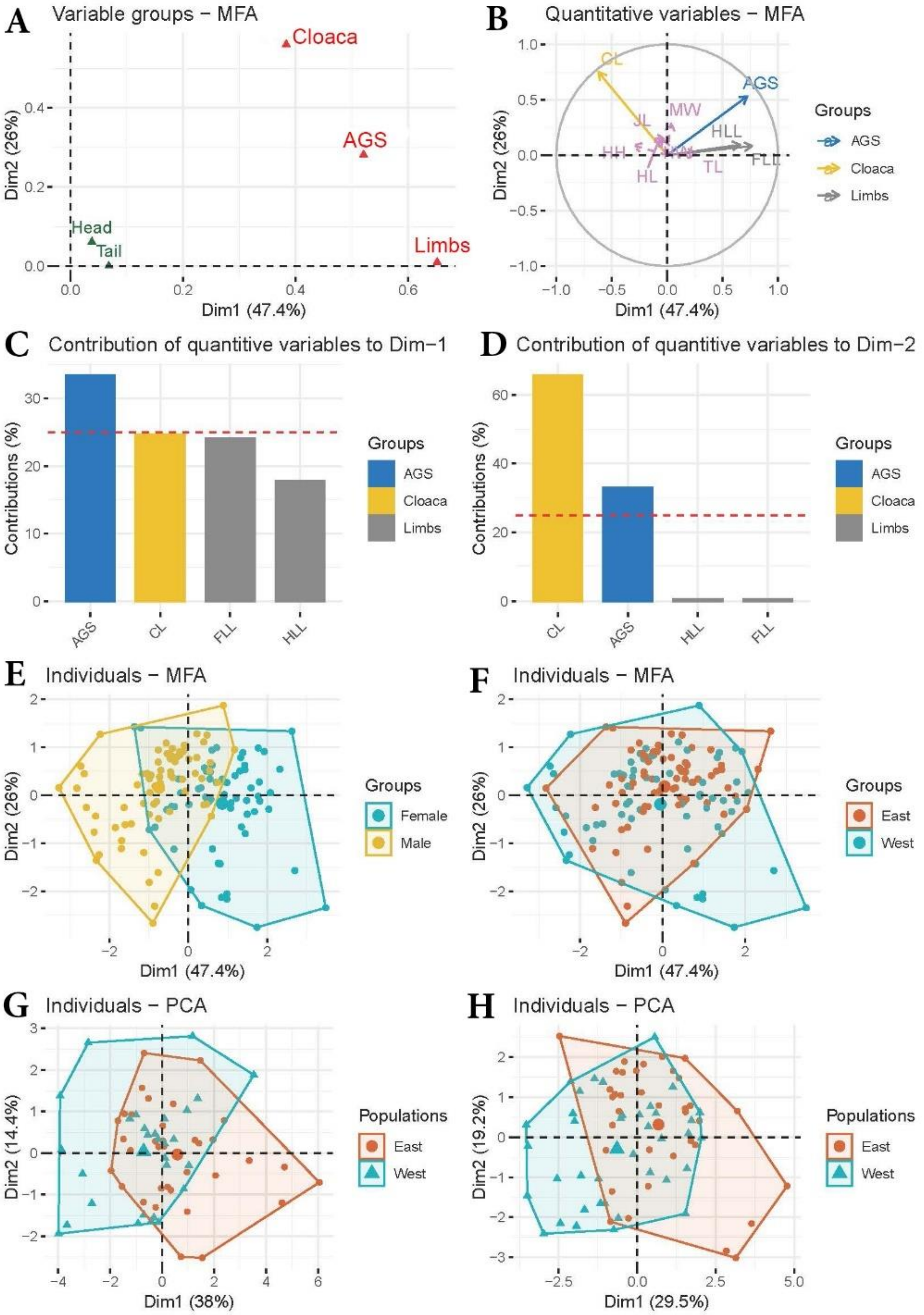

Figure 5. A. Scatterplot of all variable groups for the first (Dim1) and second (Dim2) axes in the Multiple Factor Analysis (MFA) (green triangles as inactive groups, red triangles as active groups or variables); B. Contribution of variable groups along the first two axes (Dim1 and Dim2); C. The first four important variables of the Dim1; and D. of the Dim2; E. Scatter diagram illustrating the morphological spaces between males and females; F. between East and West populations in the MFA analysis; G. between females of East and West populations H between males of East and West populations of *Paramesotriton deloustali* in the PCA analysis.

Table 3. Measurements of morphological characteristics (mm) in males and females of *Paramesotriton deloustali* presented as mean ± standard deviation and range (minimum-maximum) and results from inter-sexual variation based on Student t-tests (t). df: degrees of freedom; P: p–value; *: significant values. For abbreviations see Table 2.

	Male		**Female**		**Tests – adjusted data**	
	Mean ± SD	Range	Mean ± SD	Range	Total	East-West
SVL	85.5±7.2 (127)	67.3-106.9	88.4±7.4 (113)	70.4-105.9	t = 3.1; df = 233; p < 0.01	*
HL	25.9±2.8 (127)	20.2-32.1	26.4±2.8 (113)	15.2-33.0	t = 1.2; df = 238; P > 0.05	NS
HW	20.6±2.03 (127)	15.6-25.2	20.8±2.1 (113)	14.1-24.7	t = 2.4; df = 232; P < 0.05	***
HH	11.3±1.2 (94)	8.8-15.6	11.3±1.6 (73)	7.9-14.8	t = -0.6; df = 130; P > 0.05	NS
MW	18.9±1.5 (94)	15.6-22.3	19.6±1.8 (73)	14.4-24.8	t = 2.9; df = 147; P < 0.05	NS
JL	15.4±1.4 (94)	11.7-19.7	15.8±1.5 (73)	11.4-19.99	t = 1.6; df = 156; P > 0.05	NS
ESD	7.7±1.2 (53)	5.9-9.9	7.2±1.2 (49)	5.5-10.1	t = -2.4; df = 129; P < 0.05	***
OD	14.8±1.2 (74)	11.8-17.6	15.6±1.6 (64)	12.3-20.7	t = 3.4; df = 129; P < 0.001	NS
ED	4.8±0.7 (107)	3.3-6.7	4.8±0.6 (104)	3.3-6.5	t = 2.1; df = 208; P < 0.05	***
IN	5.9±0.8 (33)	4.8-7.6	5.5±0.6 (40)	4.8-7.3	t = -3.1; df = 60; P < 0.05	NS
IC	12.2±1.1 (107)	10.2-14.7	12.0±1.0 (102)	9.4-14.3	t = -0.8; df = 206; P > 0.05	**
FLL	27.3±2.8 (127)	18.7-35.3	28.4±2.9 (113)	17.1-35.8	t = 4.0; df = 237; P < 0.001	NS
HLL	27.4±3.3 (127)	20.9-35.4	28.3±3.2 (113)	19.1-35.6	t = 3.4; df = 237; P < 0.001	***
AGS	43.2±4.6 (74)	30.3-52.9	46.9±6.6 (73)	33.2-65.5	t = 5.2; df = 139; P < 0.001	NS
CL	16.0±2.9 (126)	8.9-19.9	13.9±2.7 (113)	7.99-18.5	t = -4.9; df = 207; P < 0.001	NS
CW	8.8±2.0 (33)	6.5-13.2	5.6±1.9 (40)	2.3-9.9	t = -7.2; df = 62; P < 0.001	NS
TL	76.3±8.6 (126)	56.9-96.1	82.0±10.8 (113)	57.0-108.2	t = 6.6; df = 230; P < 0.001	NS
TW	14.4±3.4 (53)	9.6-21.4	14.9±2.6 (46)	9.9-19.7	t = 1.4; df = 90; P > 0.05	***

DISCUSSION

In this study, the genetic difference between the western group of Ha Giang and Lao Cai provinces, and the eastern group of Bac Kan and Vinh Phuc provinces (Vietnam) resembled the phylogenetic analysis of Tran *et al*. (2023), including populations from Son La Province (Vietnam) and Yunnan Province (China) for the eastern group. Multiple tests also recorded a slight difference in terms of morphology between these geographical units of *P. deloustali* (Figs 5F, G, H, Table 4). Tran *et al*. (2023) noted that adapting to specific niches separated by riverine barriers (i.e., Da River and Lo River; Fig. 1A) might limit genetic flow between western and eastern populations of *P. deloustali*. However, we herein consider individuals from the western population are conspecific to the type population of *P. deloustali* in Vinh Phuc Province from the eastern site.

The female-biased pattern of SSD in *P. deloustali* was first noted by Nguyen *et al*. (2019) in a population from Vinh Phuc Province. However, there were some limitations in that inter-sexual comparison, such as all morphological dimensions were not adjusted to reduce correlative influences of the body size and the number of males and females from only one population was not statistically significant (Nguyen *et al*., 2019). These matters were addressed in this study by using standardized data of four populations of *P. deloustali*. When comparing the inter-sexual morphology of *P. deloustali*, consequently, the pattern of female-biased SSD, as well as longer trunk length (AGS) in females, were recognized in the Vietnam Warty Newt. Shine (1979) documented 61% of 79 newt species showing the female-biased pattern of SSD and many studies also recorded the bias in other salamanders (Joly & Giacoma, 1992; Castanet *et al*., 2000; Seglie *et al.*, 2010; Liao & Chen, 2012; Cadeddu *et al.*, 2012; Liao, 2013; Liao *et al.*, 2015). The fecundity selection might particularly favor the female-biased pattern in the SSD and trunk length to enhance the success of reproductive capacity by increasing the abdominal volume of females for clutches/eggs (Shine, 1979; Hedrick & Temeles, 1989; Griffith, 1990; Jockusch, 1997; Fairbairn *et al.*, 2007; Marvin, 2009).

In terms of cloacal dimensions, conspicuous differences were observed in the Vietnam Warty Newt as being longer and wider cloaca in males. In urodeles, the well-developed swollen cloaca in males likely improves the reproductive advantage. In particular, the larger cloaca is favored in males to produce spermatophores and pheromones during courtship performance (Verrell, 1989; Sever & Trauth, 1990; Sever, 2003; Kupfer, 2007). Given the color variation of *P. deloustali*, we only recorded the inter-sexual difference on the lateral tail in males during the breeding season. This exceptional color may be used to attract the attention of females in during the breeding season.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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SUPPLEMENTARY MATERIALS

Table S1. Mean uncorrected (p) distance (%) based on 549 bp fragments of 16S rRNA of *Paramesotriton deloustali*.

		1.	***2.***	***3.***	***4.***	***5.***	***6.***	***7.***	***8.***	***9.***	***10.***	***11.***	***12.***	***13.***	***14.***
1.	*P. deloustali* TD 03	0.00													
2.	*P. deloustali* EU880327	0.24													
3.	*P. deloustali* KY744236	0.48	0.23												
4.	*P. deloustali* NC 037713	0.48	0.23	0.00											
5.	*P. deloustali* BK (1) BB	0.00	0.00	0.23	0.23										
6.	*P. deloustali* BK (2) BB	0.00	0.00	0.25	0.25	0.00									
7.	*P. deloustali* BK (3) BB	0.24	0.12	0.35	0.35	0.12	0.12								
8.	*P. deloustali* BK (4) BB	0.24	0.00	0.23	0.23	0.00	0.00	0.12							
9.	*P. guangxiensis* CB (1)	3.25	3.14	3.14	3.14	3.14	3.21	3.02	3.15						
10.	*P. guangxiensis* CB (1)	2.78	2.66	2.78	2.78	2.66	2.74	2.54	2.69	0.12					
11.	*P. guangxiensis* CB (1)	2.93	2.93	2.93	2.93	2.93	2.93	2.79	2.93	0.27	0.13				
12.	*P. guangxiensis* PR10	3.37	3.16	3.16	3.16	3.16	3.29	2.95	3.18	0.00	0.00	0.00			
13.	*P. guangxiensis* JF712657	3.41	3.18	3.19	3.19	3.04	3.13	2.99	3.19	0.23	0.46	0.75	0.00		
14.	*P. chinensis* KY609177	3.88	3.46	3.46	3.46	3.50	3.69	3.36	3.53	3.63	3.51	4.00	3.80	3.19	
15.	*P. chinensis* NC035008	3.88	3.46	3.46	3.46	3.50	3.69	3.36	3.53	3.63	3.51	4.00	3.80	3.19	0.00

Table S2. Mean uncorrected (p) distance (%) based on 545 bp fragments of ND2 of *Paramesotriton deloustali*.

	1.	***2.***	***3.***	***4.***	***5.***	***6.***	***7.***	***8.***	***9.***	***10.***	***11.***	***12.***	***13.***	***14.***	***15.***	***16.***	***17.***	***18.***
1. *P. deloustali* VNMN 6617																		
2. *P. deloustali* VNMN 06616	0.00																	
3. *P. deloustali* VNMN 06615	0.00	0.00																
4. *P. deloustali* VNMN 6621	0.00	0.00	0.00															
5. *P. deloustali* VNMN 6622	0.00	0.00	0.00	0.00														
6. *P. deloustali* Son La 03	0.78	0.80	0.78	0.81	0.80													
7. *P. deloustali* Son La 04	0.66	0.68	0.66	0.68	0.65	0.00												
8. *P. deloustali* Laocai 02	0.48	0.49	0.48	0.49	0.47	0.23	0.23											
9. *P. deloustali* XM 12 HG	0.58	0.60	0.58	0.60	0.60	0.19	0.21	0.00										
10. *P. deloustali* XM 10 HG	0.58	0.60	0.58	0.60	0.60	0.19	0.21	0.00	0.00									
11. *P. deloustali* BK (3) BB	0.19	0.20	0.19	0.20	0.20	1.15	0.87	0.71	0.95	0.95								
12. *P. deloustali* BK (2) BB	0.19	0.20	0.19	0.20	0.20	1.15	0.87	0.71	0.95	0.95	0.00							
13. *P. guangxiensis* DQ517804	4.35	4.02	4.36	3.84	4.23	5.37	5.15	4.82	5.32	5.12	4.65	4.61						
14. *P. guangxiensis* KU375035	4.35	4.02	4.36	3.84	4.26	5.38	5.23	4.84	5.36	5.16	4.69	4.64	0.00					
15. *P. guangxiensis* PR10	4.39	4.06	4.40	3.88	4.27	5.42	4.95	4.57	5.36	5.17	4.69	4.68	0.75	0.76				
16. *P. guangxiensis* PR11	4.39	4.06	4.40	3.88	4.28	5.42	4.96	4.57	5.38	5.18	4.70	4.67	0.75	0.76	0.00			
17. *P. guangxiensis* CB (1)	3.70	3.36	3.71	3.18	3.60	4.73	4.48	4.04	4.71	4.51	4.05	4.01	0.56	0.56	0.57	0.57		
18. *P. chinensis* KY609177	9.15	9.17	9.18	8.68	7.95	10.03	7.97	8.50	10.42	10.22	9.62	9.53	10.82	10.97	11.05	11.03	10.20	
19. *P. chinensis* NC035008	9.15	9.17	9.18	8.68	7.95	10.03	7.97	8.50	10.42	10.22	9.62	9.53	10.82	10.97	11.05	11.03	10.20	0.00