

A New Species of the Genus *Tylototriton* (Urodela: Salamandridae) from Northeastern Hunan Province, China

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Abstract We describe a new species from the family Salamandridae from northeastern Hunan Province of China based on morphological and molecular genetic evidences. The new species, *Tylototriton liuyangensis*, is a member of the *T. asperrimus* group, and shares a number of similarities with *T. wenxianensis* and *T. broadoridgus*. The dorsal surface of the new *Tylototriton* species is completely black, with nodule-like warts distributed evenly along the lateral margin of dorsal body, and there is no transverse striae between the warts. There are several diagnostic characters of the new species that differ from those of *T. wenxianensis* and *T. broadoridgus* as follows: (1) the interorbital space (IOS) is comparatively wide; (2) there is a bony ridge present on the dorsal head surface, running along the upper eyelids; (3) the space between axilla and groin (AGS) is comparatively large, with the mean male AGS approximately equal to 50% of the snout-vent length (SVL) and the mean female AGS being 53% of SVL; and (4) there is a small genital armature on the anterior angle of labial folds present inside the anal fissure of adult males during the breeding season. The result from the molecular phylogenetic analysis of the genus *Tylototriton* (including the type specimen of the new species) based on the complete DNA sequence of the mitochondrial ND2 gene (1035 bp) indicated that the new species was similar to *T. wenxianensis*, *T. dabienicus*, and *T. broadoridgus*, but formed an independent clade. This result was consistent with the morphological analysis above, supporting that the Liuyang Population represented a distinct species.

Keywords new species, *Tylototriton liuyangensis*, Salamandridae, taxonomy, Liuyang Daweishan Provincial Nature Reserve of Hunan Province in China

1. Introduction

The genus *Tylototriton* (family Salamandridae) was created to accommodate a new species reported by Anderson in 1871. Between 1871 and 1983, four new species of the genus *Tylototriton* were described, and nine new species and two subspecies have been reported since 1984. Currently, the genus *Tylototriton* consists of fourteen species and one subspecies (*T. wenxianensis dabienicus*). This subspecies is likely to be reclassified as *T. dabienicus* based on molecular phylogenetic analysis

of the genus *Tylototriton* (Shen *et al.*, 2012). At present, the genus was divided into two clades: the *T. verrucosus* group and the *T. asperrimus* group (Fei *et al.*, 2005, 2006), corresponding to the two subgenera, *Tylototriton* and *Yaotriton*, named by Dubois and Raffaëlli (2009). Salamanders of the genus *Tylototriton* are distributed throughout southeastern Asia, mostly in China, and also in Vietnam, Laos, Nepal, Bhutan, Myanmar, Thailand, and India (Fei *et al.*, 2006, 2010; Böhme *et al.*, 2005; Stuart *et al.*, 2010; Chen *et al.*, 2010; Shen *et al.*, 2012; Hou *et al.*, 2012).

The *T. verrucosus* group or subgenus *Tylototriton*, is characterized by the presence of orange spots in the cranial region, on the body, tail, dorsal ridge, or lateral dorsum. It includes six species and one subspecies: *T.*

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verrucosus, *T. shanjing*, *T. kweichowensis*, *T. taliangensis*, *T. pseudoverrucosus*, *T. yangi*, and *T. verrucosus pulcherrima*. The *T. asperrimus* group, or *Yaotriton* subgenus, is characterized by lacking orange spots in the cranial region, body, tail, dorsal ridge, or lateral dorsum, and includes eight species: *T. asperrimus*, *T. hainanensis*, *T. lizhenchangi*, *T. wenxianensis*, *T. vietnamensis*, *T. notialis*, *T. dabienicus*, and *T. broadoridgus*.

Recent studies on the molecular phylogeny of *Tylosotriton* using partial DNA sequences from mitochondria have further strengthened identification of the species. Based on a phylogenetic study of known species from the *T. asperrimus* group, Yuan *et al.* (2011) concluded that this group consisted of four clades, of which *T. asperrimus*, *T. hainanensis*, *T. notialis*, *T. vietnamensis* and the two unnamed *Tylosotriton* populations from Vietnam were clustered together as one clade. Additionally, Yuan *et al.* (2011) suggested that the *T. asperrimus* group should be revised since the previous taxonomic assignment was questioned. By examining phylogenetic relationships among the subgenus *Yaotriton*, Gu *et al.* (2012) concluded that all species of the subgenus *Yaotriton* formed a monophyletic group, and both *T. hainanensis* and *T. wenxianensis* are valid species. Gu *et al.* (2012) also added that the Leishan Population should belong to *T. asperrimus* rather than *T. wenxianensis*, while the Suiyang Population should belong to *T. wenxianensis* rather than *T. asperrimus*. However, the studies mentioned above did not consider new morphological evidence in their classifications. A subsequent study by Hou *et al.* (2012) summarized the morphological differentiation and geographical variation of the genus *Tylosotriton*, and consequently reported three new species and one new subspecies.

Recently, we systematically studied the genus *Tylosotriton* and recovered that the specimens of *T. wenxianensis* from Liuyang Daweishan Provincial Nature Reserve in Hunan Province of China (Liuyang Population) were similar to its topotype and holotype, but distinct morphological and molecular genetic differences were found between Liuyang Population and others, as well as among other known species of the *T. asperrimus* group, supporting that the Liuyang Population represented a previously unrecognized species.

2. Materials and Methods

2.1 Specimens and data collection In 1989, 2010, and 2011, we collected adults, eggs, and juveniles of *Tylosotriton* from Liuyang Daweishan Provincial Nature

Reserve in Hunan Province of China and reared them in captivity for observation. Some specimens were processed using conventional methods. Two specimens were fixed in 95% ethanol and 16 specimens (9 ♂, 7 ♀) were fixed and preserved in 8% formalin. Morphological measurements were made using a slide caliper to the nearest 0.01 mm.

Following the methods of Fei *et al.* (2006), thirteen morphological measurements were recorded: total length (TOL), snout-vent length (SVL, from tip of snout to posterior edge of vent), head length (HL, from jugular fold to snout tip), head width (HW, maximum head width), snout length (SL, from tip of snout to the anterior corner of eye), interorbital space (IOS), diameter of eye (ED), space between axilla and groin (AGS), length of fore leg (FLL, from axilla to anterior limb tip of longest toe), length of hind leg (HLL, from groin to tip of longest toe), tail length (TL, from posterior edge of vent to tail tip), maximum tail height (TH), and width of tail-head (TW).

2.2 Examination of specimens of allied species We compared the morphology of these specimens of new form with the holotype and the plesiotype of *T. wenxianensis*, the type specimen series of *T. broadoridgus*, and the topotype of *T. asperrimus*. In addition, we reviewed the literature related to *Tylosotriton* and, particularly, compared the descriptions of the specimens with others of the genus.

Specific information on the specimens of the examined allied species is as follows:

***Tylosotriton wenxianensis*:** CIB 638164 (holotype, ♂, collected from Wenxian County, Gansu, China) deposited in the Chengdu Institute of Biology, Chinese Academy of Sciences in Sichuan, China; and NWS090500 a-b (plesiotypes, ♀, ♂, from Qingchuan County, Sichuan, China) deposited in Northwest Normal University in Gansu, China.

***Tylosotriton broadoridgus*:** HNUL 840513527 (holotype, ♂), HNUL 841000606 (allotype, ♀), HNUL 88050001, 840513506–840513508, 080721A6, 840513513–840513524, 840513528–840513533, 840600601–840600607, 820800806, 820800844, 820800859, 820800862 (paratype: 34 ♂), HNUL 88050002, 88050003, 84051352 (paratype: 3 ♀), and twenty juveniles (from Tianpingshan in Sangzhi County, northwestern Hunan, China) deposited in the College of Life Sciences, Hunan Normal University in Changsha, Hunan, China.

***Tylosotriton asperrimus*:** GXSD 82067001–82067007, GXSD 8206-7A–8206-7E (topotype, 12 ♂, from Dayaoshan in Jinxiu County, Guangxi, China) deposited

in the College of Life Sciences, Guangxi Normal University in Guilin, Guangxi, China.

2.3 Molecular systematics analyses

2.3.1 Sampling and gene sequencing A complete DNA sequence of the ND2 gene (1035 bp) from the mitochondria of one sample from the new species was newly sequenced (Table 1). The ND2 gene sequences of 10 congeneric species were downloaded from GenBank, of which *Tylotriton taliangensis*, *T. kweichowensis*, and *T. shanjing* were used as the outgroup based on the methodology of Shen *et al.* (2012). Total DNA extraction, PCR (Polymerase chain reaction), and ND2 gene sequencing were carried out as described by Wang *et al.* (2009).

2.3.2 Reconstruction of phylogenetic tree and calculation of genetic distance All the sequences were aligned using ClustalX 1.83 (Thompson *et al.*, 1997) with default settings. Maximum likelihood (ML) and Bayesian inference (BI) analyses were used to reconstruct the phylogenetic relationships (Figure 1). Under the Corrected Akaike Information Criterion (AICc; Hurvich and Tsai, 1989), the TrN + G model was selected as the best-fit model for the data set by likelihood ratio tests implemented in jModelTest 0.1.1 (Posada, 2008; Guindon and Gascuel, 2003). Under this model, ML analysis was performed using the program PhyML v 3.0 (Guindon and Gascuel, 2003). We set the proportion of invariant sites and the shape of the Gamma distribution in PHYML as 0.207 and 0 estimated by jModelTest, respectively. The base frequency and ratio of transitions/transversions were optimized by the ML criterion in PHYML. To assess the confidences of branches in ML trees, we used non-parametric bootstrapping with heuristic searches of 1000 replicates (Felsenstein, 1985; Felsenstein and Kishino, 1993; Hedges, 1992). Tree topologies with bootstrap values 70% or greater were regarded as sufficiently resolved (Huelsenbeck and Hillis, 1993).

Bayesian analyses using the Markov-chain Monte-Carlo technique (MCMC) were conducted for the combined data by using MrBayes 3.0b4 (Ronquist and Huelsenbeck, 2003). We used the TrN + G model under the Bayesian Information Criterion (BIC) estimated in jModelTest and initiated two dependent runs, each with four simultaneous MCMC chains, for 1×10^7 generations and sampled trees every 1000 generations. We determined the burn-in period graphically by using the parameter log-likelihood scores, which were found to be stabilized after 2 500 000 generations within and among the four independent analyses. The first 25 000 trees were discarded as burn-in, and from the remaining

trees we obtained a 50% Bayesian consensus tree and posterior probability values (BPP) for nodes. The BPP of 95% or greater were considered to be a significantly strong support (Leaché and Reeder, 2002; Parra-Olea *et al.*, 2004). Genetic distances among taxa were calculated using the Kimura 2-Parameter model in MEGA (Tamura *et al.*, 2007).

3. Results

3.1 Morphological comparison The Liuyang Population has a series of morphological characters matching those of the subgenus *Yaotriton* in the genus *Tylotriton*, including rows of “Λ” shaped vomerine teeth, a pair of prominent lateral bony ridges on the head, extremely rough dorsal and ventral skin covered with warts, a completely black body and tail, and lack of an orange circular spot on the dorsal surface of the body.

The subgenus *Yaotriton* can be morphologically classified into two distinctly different groups. One group consists of *T. wenxianensis*, *T. dabienicus*, *T. broadoridgus*, and the Liuyang Population. The salamanders in this group are characterized by the dense tubercles on the lateral trunk that form continuous nodule-like warts, with thin transverse striae present between the warts. The second *Yaotriton* group consists of *T. asperrimus*, *T. hainanensis*, *T. vietnamensis*, *T. notialis*, and *T. lizhenchangi*. This group is characterized by the presence of relatively large tubercles among the nodule-like warts on the lateral dorsum, and a relatively wide or relatively narrow gap, but obvious, between large warts.

The morphological differences between the Liuyang Population and the other three species, *T. wenxianensis*, *T. dabienicus*, and *T. broadoridgus*, are as follows:

- (1) The IOS of the Liuyang Population is wider than that of *T. wenxianensis* and *T. broadoridgus* (Figure 2). The IOS of their holotypes are 11.7%, 9.9%, and 10.3% of their SVL, respectively. The IOS of the paratypes of the new one and *T. broadoridgus* are 12.4% ($n = 6 \text{ ♂}$) and 10.5% ($n = 29 \text{ ♂}$) of their SVL, respectively.
- (2) Bony ridges on the lateral head surface of the Liuyang Population extend through the upper eyelids (Figure 2), whereas those of *T. wenxianensis* and *T. broadoridgus* extend across the inner side of the eyelids.
- (3) The AGS of the Liuyang Population is relatively long, accounting for more than 50% of the SVL (the average AGS of 8 males and 3 females was 51% and 53% of the SVL, respectively, and the AGS of the holotype and paratypes are 47.6% and 55% of the SVL, respectively). The AGS of *T. broadoridgus* is almost equal to the SVL

(the average AGS of males and females is 43.4% and 47.6% of the SVL, respectively, and the AGS of the holotype (♂) and the allotype (♀) is 41.8% and 49.3% of the SVL, respectively). The AGS of the holotype (♂) of *T. wenxianensis* is 41.2% of the SVL.

(4) The cloacal region of the Liuyang Population male is slightly bulbous during the breeding season, but there is no villous genital papilla present inside the anal fissure. The cloacal region of male *T. wenxianensis* is clearly bulbous during the breeding season, and villous genital papillae are present inside the anal fissure. The cloacal region of male *T. broadoridgus* is slightly bulbous, and villous genital papillae are not present inside the anal fissure. Small genital armatures are present inside the anal fissure of male in the Liuyang Population on the anterior angle of two labial folds, whereas both *T. wenxianensis* and *T. broadoridgus* lack this structure.

Based on these analyses mentioned above, we conclude that the specimens collected from Liuyang belong to the *T. asperrimus* group (*Yaotriton* subgenus) of *Tylosotriton*, and that they are distinctly different from any of the known species in the genus *Tylosotriton*. Thus, we suggest that this specimen should be recognized as a new species.

3.2 Molecular systematics comparison All phylogenetic trees derived from ML and BI using the ND2 sequence data (aligned ND2 sequences of all 11 samples referenced in this article yielded a total 1035 bps) essentially showed the same topologies (Figure 1). The *T. asperrimus* group is monophyletic, corresponding to the subgenus *Yaotriton* (Dubois and Raffaëlli, 2009), and consists of two lineages: *T. vietnamensis*, *T. asperrimus*, *T. hainanensis*, and *T. notialis* cluster together as one lineage (Lineage I, ML/BI: 100/1.00), while *T. broadoridgus*, *T. dabienicus*, *T. wenxianensis*, and the Liuyang Population of *Tylosotriton* form another lineage (Lineage II, ML/BI: 96/0.98). The Liuyang Population is the sister group to that consisting of the other three species within Lineage II (Figure 1).

The genetic distances among the 11 samples of *Tylosotriton* (Table 2) show that interspecies genetic distances in the outgroup (*T. shanjing*, *T. kweichowensis*, *T. taliangensis*) are 0.060–0.079, and those in Lineage I (*T. asperrimus*, *T. hainanensis*, *T. notialis*, and *T. vietnamensis*) within the ingroup are 0.043–0.051. In Lineage II they are 0.035–0.080, while the genetic distance between the Liuyang Population and the others studied in this work are 0.073–0.109 (Table 2). These genetic distances together with their phylogenetic relationships described above indicate that the genetic differentiation of the Liuyang Population may have reached species level differentiation (Stuart *et al.*, 2010).

The result of molecular phylogenetic analysis of the genus *Tylosotriton* was consistent with that of the morphological analysis, and suggested that the Liuyang Population was similar to *T. wenxianensis*, *T. dabienicus*, and *T. broadoridgus*, but be a distinct species.

3.3 New species description

Tylosotriton liuyangensis sp. nov. (Figures 2–6).

Holotype: HNUL 11053108, adult male (Figure 3 A), SVL 74.3 mm, was collected by Dongbo FEI and Daode YANG from Chuandiwo (28°25' N, 114°06' E, at an elevation of 1386 m) in Hunan Liuyang Daweishan Provincial Nature Reserve on 31 May, 2011.

Allotype: HNUL 11053110, adult female (Figure 3 B), SVL 88 mm, was collected by the same people from the same place at the same time as the holotype.

Paratype: two adult males (HNUL 89070505 and 89070501) and four adult females (HNUL 89070502–504 and 89062900) were collected by Youhui SHEN from a place near Chuandiwo (28°25' N, 114°06' E, at an elevation of 1386 m) in the same nature reserve on 29 June and 5 July, 1989. Seven adult males (HNUL 110601F02–F04, 110601F06, 11053108–109 and 11053116) and two adult females (HNUL 11053110 and 110601F01) were collected by Daode YANG and Dongbo FEI from the same place as the holotype on 31 May and 1 June, 2011.

The holotype and some paratypes were deposited in the Animal Specimens Museum of Hunan Normal University, Changsha, Hunan, China. The allotype and some of the paratypes were deposited in the Animal Specimens Museum of Central South University of Forestry and Technology, Changsha. Two paratypes were deposited in the Herpetological Museum of Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu, Sichuan, China.

Diagnosis: This new species has a series of morphological characters different from those of other species of the genus *Tylosotriton*: 1) comparatively wide interorbital space (IOS) (Figure 2); 2) bony ridges presented on dorsal head, going through the upper eyelids; 3) comparatively large space between axilla and groin (AGS), with mean male AGS being approximately equal to 50% of SVL and mean female AGS to 53% of SVL; and 4) a small genital armature on the anterior angle of labial folds found inside the anal fissure in male adults during the breeding season.

Description of the holotype (HNUL 11053108): adult male, TOL 130.7 mm, SVL 74.3 mm (other measurements are given in Table 3). Head compressed slightly and HL is subequal to HW. Snout is relatively narrow in front of eyes while the head is the widest at the eye postcornu.

Table 1 Specimens for molecular systematics analyses on the 11 species of the genus *Tylototriton*.

No.	Species or subspecies	Voucher	Locality	GenBank No.**
1	<i>Tylototriton taliangensis</i>	CIB-GG 200110183	Liziping, Shimian County, Sichuan Province, China	KC147819
2	<i>T. kweichowensis</i>	MVZ 230371	Daquan County, Yunnan Province, China	DQ517851
3	<i>T. shanjing</i>	MVZ 219763	Jingdong, Yunnan Province, China	DQ517852
4	<i>T. hainanensis</i>	MVZ 230352	12 km NE Jianfengling, Hainan Province, China	DQ517850
5	<i>T. vietnamensis</i>	ROM 35364	Quang Thanh, Cao Bang Province, Vietnam (22°37'43" N, 105°54'46" E)	HM462056
6	<i>T. notialis</i>	FMNH 271122	Boualapha District, Khammouan Province, Laos (17°38'39.6" N, 105°44'12.3" E)	HM462063
7	<i>T. asperrimus</i>	CIB-200807055	Xianglu Shan, Jinxiu County, Guangxi Province, China	KC147815
8	<i>T. broadoridgus</i>	CIB-200085	Tianping Shan, Sangzhi County, Hunan Province, China	KC147814
9	<i>T. dabienicus</i>	HNNU 1004II024	Dabie Mountains, Anhui Province, China	KC147812
10	<i>T. wenxianensis</i>	CIB 20090527	Wenxian County, Gansu Province, China	KC147813
11	<i>T. liuyangensis</i> sp. nov.*	CSUFT 20100108	Liuyang County, Hunan Province, China	KJ205598 #

CIB: Chengdu Institute of Biology, Chinese Academy of Sciences; HNNU: Henan Normal University; ROM: Royal Ontario Museum; FMNH: Field Museum of Nature and History; MVZ: the Museum of Vertebrate Zoology; CSUFT: Central South University of Forestry and Technology.

*: The new species *T. liuyangensis* sp. nov., was previously identified as Liuyang population of *T. wenxianensis* or *T. asperrimus*.

** : The DNA sequence with # was newly sequenced for this work, and the others were downloaded from GenBank.

Table 2 Genetic distances among the 11 species of the genus *Tylototriton*.

No.	Taxon	1	2	3	4	5	6	7	8	9	10
1	<i>Tylototriton taliangensis</i>										
2	<i>T. kweichowensis</i>	0.066									
3	<i>T. shanjing</i>	0.079	0.06								
4	<i>T. hainanensis</i>	0.093	0.091	0.108							
5	<i>T. vietnamensis</i>	0.108	0.102	0.12	0.043						
6	<i>T. notialis</i>	0.101	0.106	0.119	0.048	0.05					
7	<i>T. asperrimus</i>	0.099	0.107	0.121	0.051	0.046	0.049				
8	<i>T. broadoridgus</i>	0.095	0.097	0.106	0.082	0.086	0.086	0.083			
9	<i>T. dabienicus</i>	0.1	0.109	0.12	0.097	0.103	0.1	0.1	0.035		
10	<i>T. wenxianensis</i>	0.094	0.105	0.106	0.096	0.103	0.096	0.094	0.038	0.052	
11	<i>T. liuyangensis</i> sp. nov.*	0.098	0.101	0.109	0.087	0.088	0.094	0.096	0.073	0.08	0.076

*: The new species *T. liuyangensis* sp. nov. was identified as *T. wenxianensis* or *T. asperrimus* in the past.

Snout point is blunt, clearly extending beyond the lower lip. The bony ridges on the head are notable, extending from the dorsal region of the rostral side, and through the dorsal region of the eyeballs (but not the interior side of the eyeball) to the occiput, and the top of the head is slightly concave. The two bony ridges on the dorsal head surface form a “V” shape, which is low and flat. Although often termed the parotid gland by some scholars, the posterior horn (postcornu) on the lateral head surface is actually a tergoleural triangular protuberance consisting of skin and muscle and is supported by the epibranchial bone of the hyoid apparatus. The nostrils on the side of the snout form a relatively small hiatus. The IOS is very wide (11.7% of SVL) and is wider than the inter-nasal space. Eyes are rounded at the top on the lateral side of the head, and the eyelid is circular in shape around the eye socket. The oral fissure is flat and straight, and

extends a distance greater than half of the HL. The eyes are in the middle of the upward side of the oral fissure. The labial margin is smooth. The posterior of the upper jaw is slightly dilated, while the posterior of the lower jaw is slightly contracted. Tiny teeth are present on the edge of the jaw. The ventral surface of the throat is covered with tubercles. Rows of vomerine teeth are arranged in “Λ” shape that converges at the anterior. The tongue lies anterior to the oropharyngeal cavity (cavum oropharyngeum), which is oval, and nearly entirely fixed at the base, but free at both lateral edges.

The body is fully covered with tubercles. The neck is obvious. The bony ridge running along the middle of the dorsum is relatively narrow, smaller than the diameter of the eye, and its anterior width is subequal to the posterior width.

Running laterally along the dorsum of the body are

Table 3 Measurements of the adult specimens of *T. liuyangensis* sp. nov.

Item	Holotype ♂ HNUL 11053108		Allotype ♀ HNUL 11053110		Paratype 8♂		Paratype 6♀	
	Mean	%	Mean	%	Mean ± SD (Range)	%	Mean ± SD (Range)	%
TOL	130.7	175.9	154.2	175.2	127.6 ± 10.5 (110.1–146.5)	183.8	145.2 ± 6.9 (138.6–154.2)	172.9
SVL	74.3		88		69.4 ± 5.5 (64.2–82.0)		84.0 ± 2.5 (80.8–88.0)	
HL	17.3	23.3	19.1	21.7	16.5 ± 1.4 (15.2–19.8)	23.8	19.5 ± 0.3 (18.5–19.1)	22
HW	16.6	22.3	19	21.6	16.1 ± 1.2 (14.8–18.4)	18.5	19.2 ± 1.2 (17.4–21.0)	22.9
HH	7	9.4	8.7	9.9	7.0 ± 0.6 (6.0–7.7)	13.3	8.7 ± 0.5 (8.4–9.4)	10.3
SL	7.3	9.8	6.4	7.3	5.9 ± 0.5 (5.4–6.6)	8.6	6.6 ± 0.5 (5.8–7.3)	7.8
ED	4.5	6.1	4.7	5.3	4.0 ± 0.6 (3.1–4.8)	5.8	4.7 ± 0.3 (4.4–5.7)	5.6
IOS	8.7	11.7	10.5	11.9	8.6 ± 0.8 (7.2–9.6)	12.4	9.6 ± 0.5 (8.7–10.1)	11.4
DRW	3	4	3.6	4.1	2.9 ± 0.6 (2.0–3.8)	4.1	3.6 ± 0.4 (3.1–4.4)	4.3
AGS	35.4	47.6	48.5	55.1	35.4 ± 1.9 (31.4–38.6)	51	44.5 ± 2.6 (42.2–48.5)	53
FLL	20.3	27.3	23.5	26.7	19.9 ± 1.7 (18.0–23.5)	28.7	22.8 ± 1.5 (20.5–23.5)	27
HLL	19.3	26	23.9	27.2	19.8 ± 1.0 (18.3–21.2)	28.4	23.3 ± 2.0 (20.8–26.2)	27.7
TL	56.4	75.9	78.7	66.7	58.2 ± 6.6 (43.6–64.5)	83.8	61.9 ± 4.2 (56–66.5)	73.7
TH	7.5	10.1	9.9	9.7	7.5 ± 0.4 (7.0–7.8)	10.8	8.6 ± 1.0 (7.6–9.6)	10.2
TW	9.5	12.8	11.5	9.3	7.8 ± 0.7 (7.0–8.6)	11.3	9.6 ± 0.7 (8.5–10.6)	11.1

% is the ratio of each measurement to the SVL.

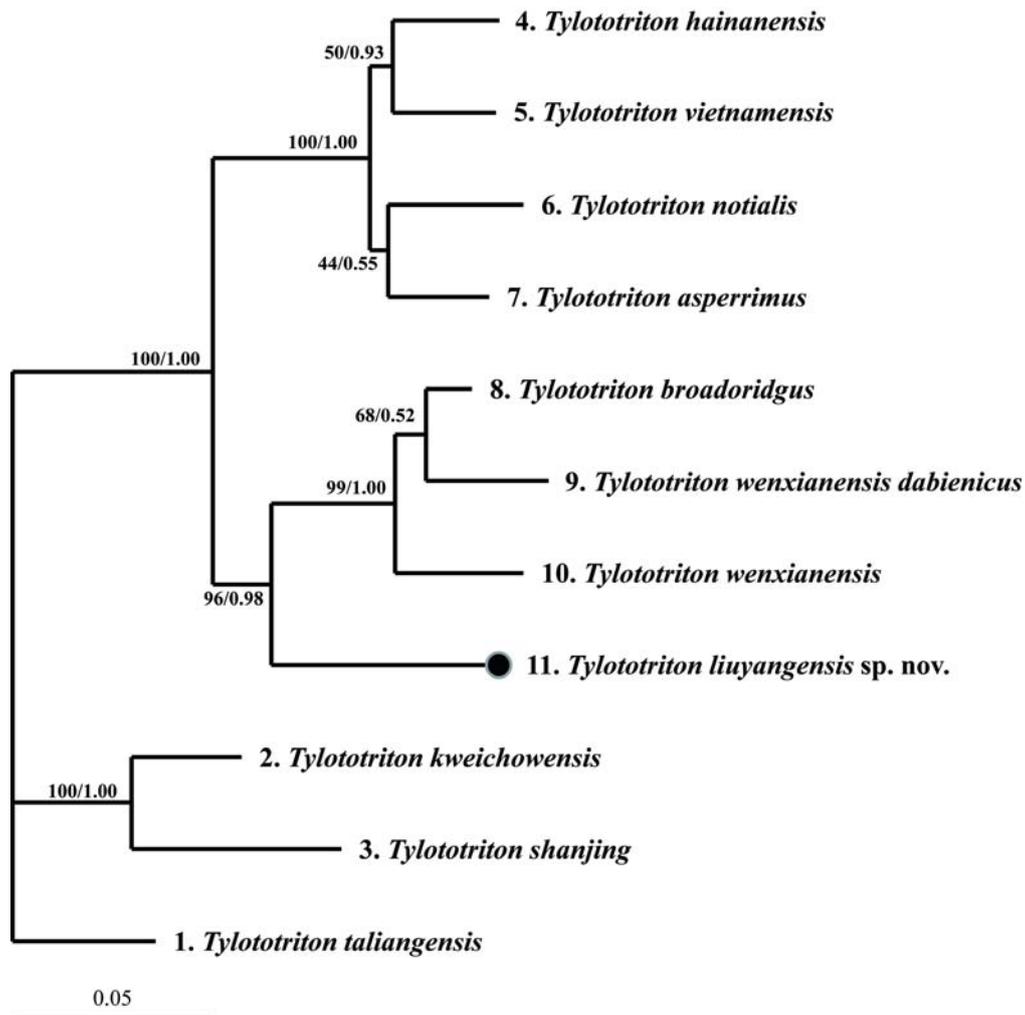


Figure 1 The phylogenetic relationships among the *T. asperrimus* group inferred from ND2 of mtDNA. The numbers above branches represent bootstrap supports for maximum likelihood (ML) and Bayesian posterior probabilities.

tubercles that form a line of small warts. The nodule-like tubercles are continuously distributed with no clear separation between neighboring tubercles. Unlike the dorsal tubercles, the ventral tubercles are relatively flat and have many transverse striae. The AGS is 38.6 mm, which is 47.1% of the SVL.

The four limbs are well-proportioned. The length of the hind limbs is almost equal to that of forelimbs, but the hind limbs are slightly stouter. The fingertips are flush to the eye while the forelimbs are stretched forward. The tips of fingers and toes cannot touch each other when forelimbs and hind limbs are adpressed along the trunk. All digits are flat with obtuse ends, without subarticular tubercles.

There are four fingers on the forelimbs, with their relative lengths being $II > III > I > IV$ (the length of finger II is nearly equal to that of finger III). There are 5 toes on the hind limbs, and the relative length of each toe is $III > IV > II > V > I$ (the length of toe III is nearly equal to that of toe IV). The palm and metatarsus are relatively wide and thick, but the tubercles of the metacarpal and metatarsal are indistinct. The tail is compressed laterally. The base of the tail is relatively wide, and the dorsal fin fold of tail is comparatively high, while the ventral fin fold of the tail is low, short, and smooth. In general, the appearance of the tail is relatively low and flat. Tail height is less than the width at the tail base, and the tail tip is rounded.

The body of live individuals is completely black, and the ventral surface is slightly flat and somewhat grey black. The iris is black brown. The interior and margin of the anal fissure are orange. The ventral sides of both fingers and toes, and the ventral tail fin are all orange. Following liquid immersion of specimens the orange coloration fades to a milky white.

The cloacal region is slightly bulbous (Figure 4). Smooth labial folds are present on the inner wall at the anterior of the anal fissure. The left and right labial folds converge on the anterior angle, on which a small genital armature is present (Figure 4). The posterior inner wall is covered with tubercles, and lacks villous genital papillae.

Description of the allotype: The cloacal region of females is slightly bulbous and thickened at the periphery. The inner cloacal wall has protuberances, which are similar to the tubercles around the peripheral cloacal region and protrude radially from the cloaca to the periphery. The apertura cloacalis expands, and becomes circular during the breeding season. The female has no labial fold on the inner cloacal wall and no villous genital papillae.

Secondary sexual characteristics: The female body is relatively large and robust, and becomes more prominent in the abdomen during the breeding season. The AGS of the female is relatively large, and that of the male is relatively small. The size of the AGS is positively correlated with the size of the abdominal cavity. A larger abdominal cavity in females benefits ovarian development, and allows for a larger brood and abundant vitellus, increasing the fitness of the offspring. Though many females belonging to *Urodela* have this feature, the ratio of AGS to SVL is relatively large in *T. liuyangensis*.

Variables: All type specimens were collected from the type locality during the breeding season, and their morphological characters are basically the same and have not obvious mutation. The individual differences because of the sexuality, size, and growth factors, have been detailedly recorded in Table 3.

Color: The body dorsum is almost black except that three specimens (HNUL89070501, 89070504, and 89070505) are black brown. The ventral surface is a bit lighter except the ventral tail fin folds, peripheral area of cloaca, the fingertip and toe-end are orange. Among them, the metacarpal protrusion of HNUL 110601F01 and 11053108 are orange-red while others are not. The skin color around their anal fissure can be divided into three types: one with narrow orange edge around anal pore (HNUL 89070501, 89070505, 110601F04, and 11053109); the second with wider orange edge around anal pore (HNUL 11053110, 11053108, 110601F01, and the four female specimens collected in 1989); and the third with pure black edge around anal pore (including the remaining seven specimens). The orange color of the specimens would become milky white after being stored in formalin, even become brown or brownish black such as the four specimens collected in 1989 and having been stored in formalin since then.

Reproduction: Based on examination of anatomic specimens and our field observations, we estimate that the breeding season of *T. liuyangensis* in the Dawei Mountains, Liuyang County, Hunan Province lasts from May to June. Egg masses were discovered and photographed on May 9, 2010, juveniles were observed and photographed on May 24, 2010, males and pregnant females were collected on May 31 and June 1, 2011, and developing fertilized eggs were photographed on June 8, 2011. The spawning site was surrounded by dense grasses (Figure 5) and was located in the puddle of a swale near the Chuandiwo of the Dawei Mountains. The eggs are solitary, and arranged in a sheet (Figure 6 A). The ova are yellow, and 3.2–3.8 mm in diameter. The egg diameter,

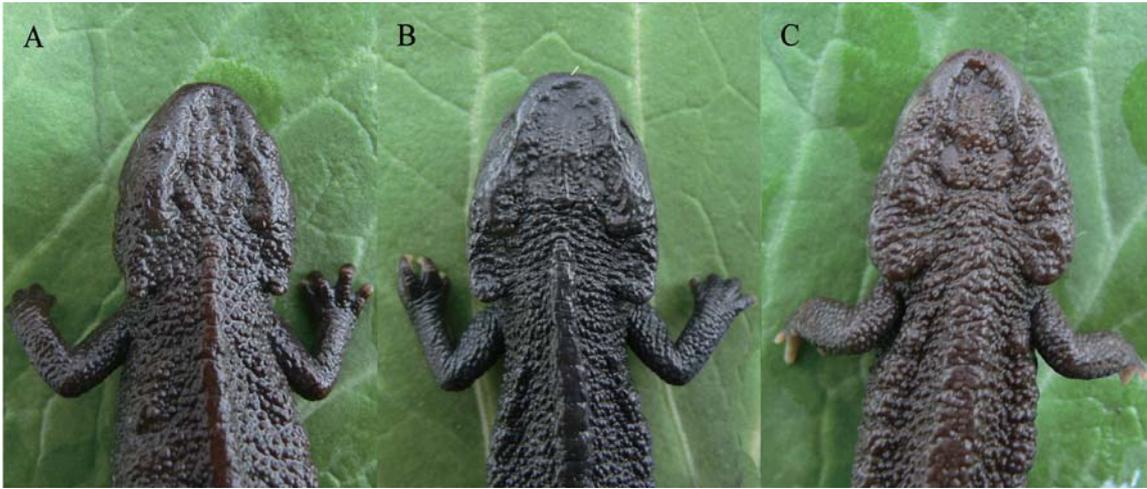


Figure 2 Interorbital space (IOS) differences among *T. wenxianensis*, *T. liuyangensis* sp. nov. and *T. broadoridgus*. A: *T. wenxianensis* CIB638164; B: *T. liuyangensis* sp. nov. HNUL11053108; C: *T. broadoridgus* HNUL 840513527.



Figure 3 Type specimens of *Tylotriton liuyangensis* sp. nov. A: Holotype HNUL 11053108 ♂; B: Allotype HNUL11053110 ♀.

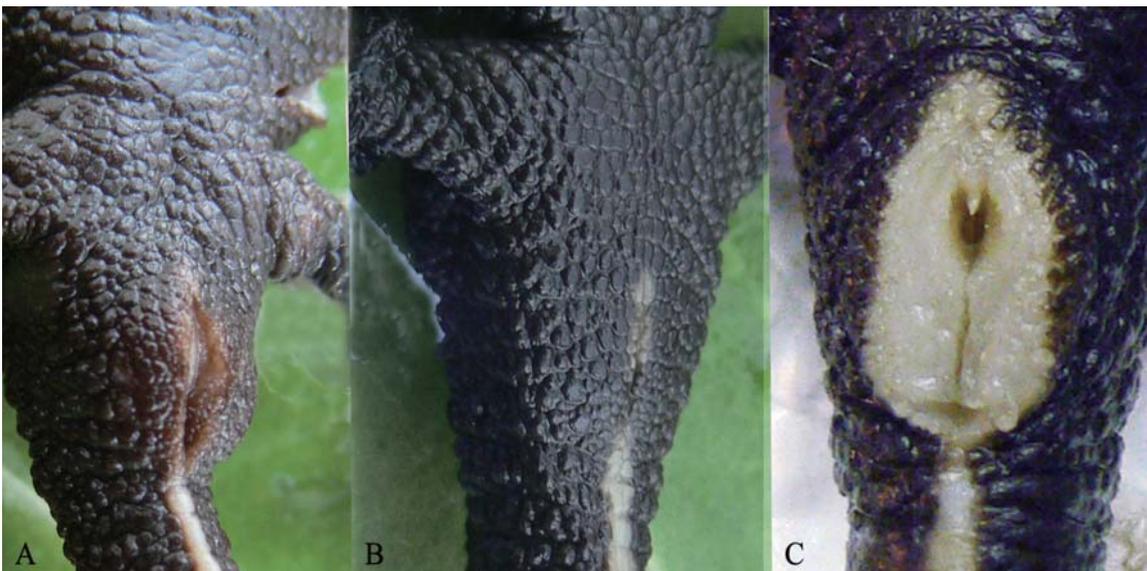


Figure 4 Male anal fissure of *T. wenxianensis* and *T. liuyangensis* sp. nov. A: Anal fissure of male *T. wenxianensis* CIB638164; B: Anal fissure (closed state) of male *T. liuyangensis* sp. nov. HNUL110601 F02; C: Internal structure at anal fissure (open state) of male *T. liuyangensis* sp. nov. HNUL11053108).



Figure 5 Habitat of *T. liuyangensis* sp. nov.

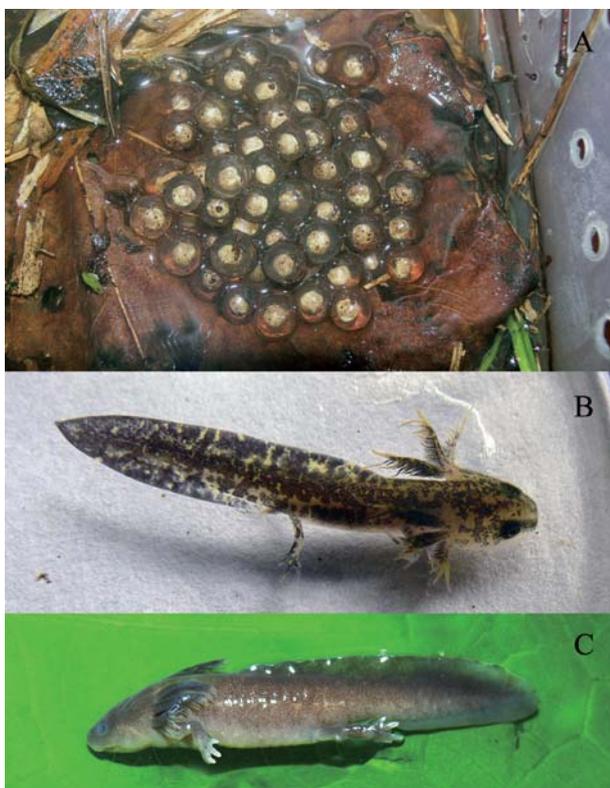


Figure 6 Eggs and juveniles of *T. liuyangensis* sp. nov. A: Egg mass; B: Juvenile 1; C: Juvenile 2

including egg film, is 7.8–8.1 mm.

Juvenile 1: A young larva was observed on 24 May, 2010 (Figure 6 B). Skin is smooth. The body is covered with dense black variegation, whereas the abdomen has less variegation and is lighter in color. Lacking a balancer and lateral line, the limbs appear to be quite weak, but clearly have four fingers and five toes. Three pairs of external gills are present with well-developed gill filaments. Tail is well-developed and tail end is blunt. The heights of the dorsal and ventral fin folds of tail are

approximately equal.

Juvenile 2: A mature larva (that hatched from fertilized eggs collected in early June, 2010) was observed on 5 September, 2010 (Figure 6 C). Skin is smooth without any tubercles, the body dorsum is somewhat dark but not black, and the abdomen is light in color. The four limbs and external gills are all well-developed. Thirteen pairs of costal grooves are present between the lateral axilla and groin. There are two pairs of lateral lines present. The upper pair is comparatively long, and extends laterally along the body from the posterior eye to the tail. The lower pair of lateral lines is divided into two sections. The anterior section extends from behind the gills to the third costal groove anterior to the crotch of the hind limbs, and the posterior section extends from the lower edge of the spine behind the hind limbs to the tail. The dorsal fin fold extends from the posterior body to the end of the tail, while the ventral fin fold of the tail extends from the cloaca to the end of the tail. The heights of the dorsal fin fold and the ventral fin fold of the tail are almost equal. The tail is very high, and its end is rounded. The cloacal region of the larva is obviously bulbous.

Etymology: The Latin name of the new species is derived from the locality where the new species was first found.

4. Discussion

The evidence from our molecular and morphological comparisons indicates that the Liuyang Population, which was previously known as a population of *T. wenxianensis*, is discernibly different from the other forms of the *T. wenxianensis* clade. This supports the hypothesis that the Liuyang Population represents a distinct species, and is described herein as a new species:

This new species, *T. liuyangensis*, is placed in the *T. asperrimus* group or the subgenus *Yaotriton*, which consists of nine species at present. Based on our observations and related reports that have evaluated the relationships within the genus *Tylototriton* using mitochondrial DNA sequences, the nine species are classified into two clades: Clade One includes *T. wenxianensis*, *T. dabienicus*, *T. broadoridgus*, and *T. liuyangensis* sp. nov., and Clade Two includes *T. asperrimus*, *T. hainanensis*, *T. vietnamensis*, *T. notialis*, and *T. lizhenchangi*. These two clades have clearly distinguishable morphological characteristics. For example, Clade One is characterized by the dorsum of live individuals being completely black, and having band-like warts on the lateral trunk which are arranged continuously and lack an obvious interval. Clade Two

does not exhibit any of these traits.

The two clades also differ in their known geographic distribution. The species included in Clade One are distributed throughout the Yangtze River Basin. *T. wenxianensis* is distributed in the Qinling Mountains in the upstream reaches of the Jialing River, *T. broadoridgus* in the Wuling Mountains in the upstream reaches of the Lishui River, and *T. liuyangensis* in the upstream reaches of the Liuyang River. All these rivers are within the Yangtze River Basin. *T. dabienicus* is distributed in the Dabie Mountains, a source of the Huai River that is adjacent to the Yangtze River Basin. These areas are all located within Central China, a region that has significant geographical barriers, such as the Yangtze River and Dongting Lake (Ancient Yun-meng Lakes). The species in Clade Two (including the recently reported *T. lizhenchangi*) are distributed in the southern China and adjacent areas. The two clades appear to be geographically isolated with no overlap.

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