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A phylogeny of the *Tylototriton asperrimus* group (Caudata: Salamandridae) based on a mitochondrial study: suggestions for a taxonomic revision

YUAN Zhi-Yong[1], JIANG Ke[1], LÜ Shun-Qing[2], YANG Jun-Xiao[1], NGUYEN Quang Truong[3], NGUYEN Thien Tao[4], JIN Jie-Qiong[1], CHE Jing[1, *]

(1. *State Key Laboratory of Genetic Resources and Evolution, Kunming Institute of Zoology, the Chinese Academy of Sciences, Kunming Yunnan* 650223, *China*; 2. *College of Life and Environment Sciences, Huangshan University, Huangshan* 245021, *China*; 3. *Institute of Ecology and Biological Resources,* 18 *Hoang Quoc Viet St., Hanoi, Vietnam*; 4. *Vietnam National Museum of Nature,* 18 *Hoang Quoc Viet St., Hanoi, Vietnam*)

Abstract: A phylogenetic hypothesis for the Asian newts of the *Tylototriton asperrimus* group was generated using data from two mitochondrial fragments including COI and the ND1-ND2 regions. Four distinct clades (A, B, C, D) were resolved with high nodal support within this monophyletic group. Clade A included *T. asperrimus*, *T. hainanensis*, *T. notialis*, "*T. vietnamensis*", and two unnamed salamander populations from Vietnam. Clade A, constituted the sister group of clades B + C. Newly identified clade C likely represents a new cryptic species. Clade C was the sister group of *T. wenxianensis*. The true *T. vietnamensis* exclusively constituted clade D. Our results bring into question some previous taxonomic decisions, and a revision is required. This study illustrates the necessity to include samples from type localities in taxonomic studies, and highlights the importance of fine-grained geographical sampling.

Key words: *Tylototriton notialis*; *Tylototriton hainanensis*; Salamander; Southeast Asia; Tonkin; Cryptic diversity

基于线粒体基因对细痣疣螈种组系统发育关系的探讨及物种修订建议

袁智勇[1], 蒋 柯[1], 吕顺清[2], 杨军校[1], NGUYEN Quang Truong[3], NGUYEN Thien Tao[4], 金洁琼[1], 车 静[1,*]

（1. 中国科学院昆明动物研究所 遗传资源与进化国家重点实验室, 云南 昆明 650223; 2. 黄山学院 生命与环境科学学院, 安徽 黄山 245021; 3. *Institute of Ecology and Biological Resources, 18 Hoang Quoc Viet St., Hanoi, Vietnam*; 4. *Vietnam National Museum of Nature, 18 Hoang Quoc Viet St., Hanoi, Vietnam*）

摘要：该文基于两段线粒体基因 COI 和 ND1-ND2 部分序列片段构建了细痣疣螈种组的系统发育关系, 结果显示该单系群内包含了四个明显的进化支系(A、B、C、D)。A 支系与 B、C 支系构成姐妹群关系; A 支系包含了细痣疣螈、海南疣螈、老挝疣螈和“越南疣螈”以及另外两个来自越南未被命名的种群; C 支系代表了一个新的独立进化支, 可能是一个隐存种; C 支系与 B 支系构成姐妹群关系。此外，来自模式产地的越南疣螈单独构成支系 D。该文结果不支持以往细痣疣螈种组部分物种的划分, 建议对该种组进行新的分类修订。该文强调了在分类研究中应该重视使用来自模式产地的样品并进行广泛的地理居群采样。

关键词：老挝疣螈; 海南疣螈; 蝾螈; 东南亚; 东京湾; 隐存多样性
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*通讯作者（Corresponding author），女，副研究员。 Tel: +86-871-5199030; Fax: +86-871-5133185; E-mail: chej@mail.kiz.ac.cn
第一作者简介：袁智勇，男，硕士研究生，主要从事东亚和东南亚蝾螈科物种遗传多样性及演化研究

The Asian newt genus *Tylototriton* Anderson, 1871 occurs from Nepal, through north-central India, Sikkim, Myanmar, northern Thailand, Laos, and Vietnam into southern China. Presently it contains nine species (AmphibiaWeb, 2011). The monophyly of the genus and two groups (or subgenera: Dubois & Raffaëlli, 2009), the *T. verrucosus* group and *T. asperrimus* group, have been consistently supported in recent studies (Stuart et al, 2010; Weisrock et al, 2006).

With the addition of two newly described species, *T. vietnamensis* Böhme et al, 2005 and *T. notialis* Stuart et al, 2010, the *T. asperrimus* group includes five species in total, along with *T. asperrimus* Unterstein, 1930, *T. hainanensis* Fei et al, 1984, and *T. wenxianensis* Fei et al, 1984. However, despite recent progress, uncovering the dimensions of amphibian diversity and inferring evolutionary history remain difficult, in part because of extreme morphological conservation and associated homoplasy (Bossuyt & Milinkovitch, 2000; Wake, 1991). Identifying species is challenging for the *T. asperrimus* group, and determining the exact distributions of some species has been confusing (Fei et al, 2005, 2009; Nguyen et al, 2009), for example, *T. asperrimus*, *T. wenxianensis*, and *T. vietnamensis*. Moreover, previous molecular phylogenetic studies on this group are chiefly based on limited samples and at the same time, unfortunately, materials from the type localities are often absent (Stuart et al, 2010; Weisrock et al, 2006).

Here we report results of our use of mitochondrial data to investigate the phylogenetic relationships of *Tylototriton*, with an emphasis on understanding the evolutionary history of the *T. asperrimus* group.

1 Material and Methods

1.1 Sampling

Tissue samples from adults, including toe tips, muscle, and liver, and whole juvenile specimen were collected in the field following Animal Use Protocols approved by the Kunming Institute of Zoology Animal Care and Ethics Committee. The tissues were preserved in 95% ethanol. A total of 36 individuals of *Tylototriton* from 17 localities, representing all 9 currently recognized species in this genus, were examined in this study. Nineteen sequences were downloaded from GenBank for certain comparisons and validations. Outgroup taxa were *Echinotriton andersoni* and *E. chinhaiensis*, chosen mainly based on the study of Weisrock et al (2006). Fig. 1 provides an overview on the distribution of our samples. Details of taxonomic sampling and GenBank accession numbers are provided in Tab. 1. Species identity was based on morphological characters determined in the field. Some specimens could not be

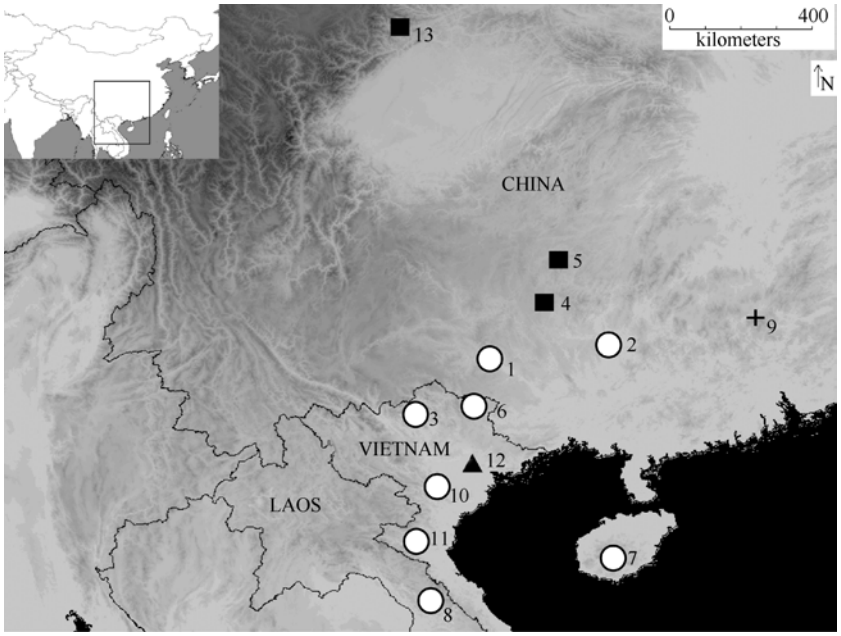

Fig. 1 Map showing the geographic locations of samples representing the *Tytolotriton asperrimus* group used in present analyses
The site numbers correspond to those in Tab. 1. Different species are represented by different symbols: empty circle for clade A, solid square for clade B, cross for clade C, and solid triangle for clade D.

Tab. 1 Voucher specimens, localities, and GenBank accession numbers for sample used for the *Tylototriton*

Species name	Name suggested	Voucher	Locality (No. in Fig. 1)	GenBank Accession No.		Source
				ND1-ND2	COI	
Ingroup						
***T. asperrimus* group**						
T. asperrimus	—	KIZ08080215	Baise, Guangxi, China (1)	JQ046340	JQ046316	This study
T. asperrimus	—	KIZ08080216	Baise, Guangxi, China (1)	JQ046341	JQ046317	This study
T. asperrimus	—	KIZ08080217	Baise, Guangxi, China (1)	JQ046338	JQ046314	This study
T. asperrimus	—	KIZ08080219	Baise, Guangxi, China (1)	JQ046339	JQ046315	This study
T. asperrimus	—	Tissue ID: YPX9918	Mt. Dayaoshan, Guangxi, China (2)	JQ046344	JQ046320	This study
T. asperrimus	—	Tissue ID: YPX9919	Mt. Dayaoshan, Guangxi, China (2)	JQ046345	JQ046321	This study
T. asperrimus	—	Tissue ID: YPX9920	Mt. Dayaoshan, Guangxi, China (2)	JQ046347	JQ046323	This study
T. asperrimus	—	Tissue ID: YPX9922	Mt. Dayaoshan, Guangxi, China (2)	JQ046346	JQ046322	This study
T. asperrimus	—	Tissue ID: YPX9923	Mt. Dayaoshan, Guangxi, China (2)	JQ046348	JQ046324	This study
T. asperrimus	—	VNMN1200 A	Bac Quang, Ha Giang, Vietnam (3)	JQ046350	JQ046326	This study
T. asperrimus	*T. wenxianensis*	MVZ237103	near Libo, Guizhou, China (4)	DQ517849	no	Weisrock et al (2006)
T. cf. asperrimus	*T. wenxianensis*	KIZ0805197	leishan, Guizhou, China (5)	JQ046354	JQ046330	This study
T. cf. vietnamensis	*T. asperrimus*	ROM35330	Quang Thanh, Cao Bang, Vietnam (6)	DQ517856	no	Weisrock et al (2006)
T. cf. vietnamensis	*T. asperrimus*	ROM35364	Quang Thanh, Cao Bang, Vietnam (6)	HM462056	no	Stuart et al (2010)
T. cf. vietnamensis	*T. asperrimus*	ROM26519	Quang Thanh, Cao Bang, Vietnam (6)	JQ046337	JQ046328	This study
T. cf. vietnamensis	*T. asperrimus*	ROM26520	Quang Thanh, Cao Bang, Vietnam (6)	JQ046351	JQ046327	This study
T. hainanensis	*T. asperrimus*	Tissue ID: YPX1304	Hainan, China (7)	JQ046336	JN700845	This study and Che et al (2011)
T. hainanensis	*T. asperrimus*	MVZ 230352	near Jianfengling, Hainan, China (7)	DQ517850	no	Weisrock et al (2006)
T. notialis	*T. asperrimus*	FMNH271120	Boualapha, Khammouan, Laos (8)	HM462061	no	Stuart et al (2010)
T. notialis	*T. asperrimus*	FMNH271121	Boualapha, Khammouan, Laos (8)	HM462062	no	Stuart et al (2010)
T. notialis	*T. asperrimus*	FMNH271122	Boualapha, Khammouan, Laos (8)	HM462063	no	Stuart et al (2010)
T. sp.	—	KIZ0808024	Mt. Mangshan, Hunan,China (9)	JQ046357	JQ046333	This study
T. sp.	—	KIZ0808027	Mt. Mangshan, Hunan,China (9)	JQ046355	JQ046331	This study
T. sp.	*T. asperrimus*	VNMN1214	Hoa Binh, Vietnam (10)	JQ046342	JQ046318	This study
T. sp.	*T. asperrimus*	VNMN3014	Hoa Binh, Vietnam (10)	JQ046343	JQ046319	This study
T. sp.	*T. asperrimus*	VNMN1230	Nghe An, Vietnam (11)	JQ046349	JQ046325	This study
T. vietnamensis	—	VNMN121F	Bac Giang, Vietnam (12)	JQ046352	JQ046329	This study
T. vietnamensis	—	NCSM77330	Son Dong, Bac Giang, Vietnam (12)	HM770088	no	Stuart et al (2010)
T. vietnamensis	—	NCSM77331	Son Dong, Bac Giang, Vietnam (12)	HM770089	no	Stuart et al (2010)
T. wenxianensis	—	MVZ236638	Longmenshan, Sichuan, China (13)	EU880341	EU880341	Zhang et al (2008)
T. wenxianensis	—	MVZ236632	Longmenshan, Sichuan, China (13)	DQ517855	no	Weisrock et al (2006)
T. wenxianensis	—	Tissue ID: YPX1339	unknown	JQ046353	JN700846	This study and Che et al (2011)
***T. verrucosus* group**						
T. taliangensis	—	KIZ05148	Liziping, Shimian, Sichuan	JQ046360	JN700850	This study and Che et al (2011)
T. kweichowensis	—	GZ070653	Longjiexiang, Weining, Guizhou	JQ046356	JQ046332	This study
T. shanjing	—	YN0705203	Huangcaoling, Jingdong, Yunnan	JQ046358	JQ046334	This study
T. verrucosus	—	Tissue ID: SH 2805	Nepal	JQ046359	JQ046335	This study
Outgroup						
Echinotriton andersoni	—	MVZ232187	Kagoshima Prefecture, Kyushu, Japan	EU880314	EU880314	Zhang et al (2008)
Echinotriton chinhaiensis	—	TP26195	Ningbo, Zhejiang, China	EU880315	EU880315	Zhang et al (2008)

FMNH: Field Museum of Natural History, Chicago, USA; KIZ: Kunming Institute of Zoology, the Chinese Academy of Sciences, China; MVZ: Museum of Vertebrate Zoology, University of California, Berkeley, USA; NCSM: North Carolina State Museum of Natural Sciences, Carolina, USA; ROM: Royal Ontario Museum, Toronto, Canada; VNMN: Vietnam National Museum of Nature, Hanoi, Vietnam. "—"means no taxonomic change.

reliably identified to species and we refer to them as "sp.".

1.2 Extraction, amplification and sequencing

Genomic DNA was extracted using the phenol-

chloroform method (Sambrook et al, 1989). Two mitochondrial sequences were amplified. One fragment covers a 1 014 bp region that encodes part of the NADH dehydrogenase subunit 1, tRNA Ile, tRNA Gln, tRNA Met, and part of the NADH dehydrogenase subunit 2 genes (hereafter, 'ND1 to ND2'). PCR primers KIZ1S (5'-*TGACCAATAGCARCAATA*-3') and KIZ1A (5'-*GTGGGCRATAGATGARTA*-3') were designed specifically for successful amplification. Internal primers KIZL4437 (5'-*AAGCTTTCGGGCCCATACC*-3') and KIZH4419 (5'-*AAGCTTTTGGGCCCATACC*-3') were applied to sequencing. Another gene is a 561 bp region from cytochrome oxidase subunit I (COI) which was amplified following the standard protocol (Che et al, in press). Amplification was performed in a 25 μL volume reaction with the following procedures: initial denaturation step for 5 min at 94 °C, 35 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 59 °C for ND1 to ND2 and 45 °C for COI, extension for 1 min at 72 °C. Final extension at 72 °C was conducted for 10 min.

PCR products were purified with a Gel Extraction Mini Kit (Watson BioTechnologies, Shanghai), and sequenced in both directions using a BigDye Terminator Cycle Sequencing Kit (version 2.0, Applied Biosystems) and a ABI PRISM 3730 automated DNA sequencer (Applied Biosystems). The sequence data were translated into amino acids to confirm the absence of premature stop codons, i.e. to preclude the sequenced of nuclear DNA pseudogenes, and also submitted to a BLAST search in GenBank to confirm that the required sequences had been amplified. Nucleotide sequences were aligned using Clustal X 1.81 (Thompson et al, 1997) with default parameters, and then optimized by eye in MEGA 4.0. (Tamura et al, 2007）

1.3 Molecular analyses

Considering that all mtDNA gene sequences are virtually inherited as one linkage group, the two mtDNA gene segments were concatenated into a single partition. The ten samples for which COI sequences are lacking were treated as missing data when combined. Mixed-model Bayesian phylogenetic inference was performed using Bayesian inference (BI) with MrBayes version 3.1.2 (Ronquist & Huelsenbeck, 2003). Data were partitioned by ND1 codon position, tRNA, ND2 codon position, and COI codon position. The model of sequence evolution that best described each of the data partitions was inferred using the Akaike Information Criterion as implemented in MrModeltest 2.3 (Posada & Crandall, 1998). These were TIM+G for ND1 first codon position; HKY+I for ND1 second codon position; GTR+I for ND1 third codon position and tRNA; TRN+G for ND2 first codon position; HKY+G for ND2 second codon position; GTR+G for ND2 third codon position and COI third codon position; and F81 for COI first and second codon positions. Four independent Bayesian analyses were performed on each dataset. In each analysis, four chains were run for 5 000 000 generations using the default priors, trees were sampled every 100 generations, log-likelihood scores were tracked for stabilization and the first 25% of the trees were discarded as burn-in.

2 Results and Discussion

2.1 Molecular analyses

The aligned sequences yielded 1 014 base pairs of ND1 to ND2 (305 variable, 233 parsimony-informative sites) and 561 bp base pairs of COI (169 variable, 133 parsimony-informative sites). The expected bias against guanine nucleotides was observed (G=12.0%, A= 35.1%, T=25.6 %, and C=27.3 % for ND1 to ND2; and A=25.9%, T=27.4%, C=29.4%, G=17.3% for COI). These data indicated the mitochondrial gene was sequenced, and not a nuclear copy. Forty-seven new sequences were determined and deposited in GenBank (Tab. 1).

The BI tree is shown in Fig. 2. Consistent with Weisrock et al (2006) and Stuart et al (2010), the monophly of the genus of *Tylototriton* is strongly supported. A major difference is that our data did not recover the two major clades within *Tylototriton* reported in previous studies. This is not surprising given our sequencing of a shorter fragment and our far denser sampling for *T. asperrimus* group. The monophyly of the *T. asperimmus* group as shown by Stuart et al (2010) collapsed into two subclades (A+B+C and D) in the present analyses. Clade A including seven strongly supported subclades (A1−A7) was recovered as the sister group of B+C. The position of clade D was not resolved in the present study.

Our increased sampling reveals previously unrecorded phylogenetic structure. On the one hand, *T. asperrimus* does not contain a single maternal lineage (Fig. 2). The nominal form of *T. asperrimus* (type locality: Dayao shan, Guangxi; locality 2) is in lineage A6, which is the sister taxon to the two apparently

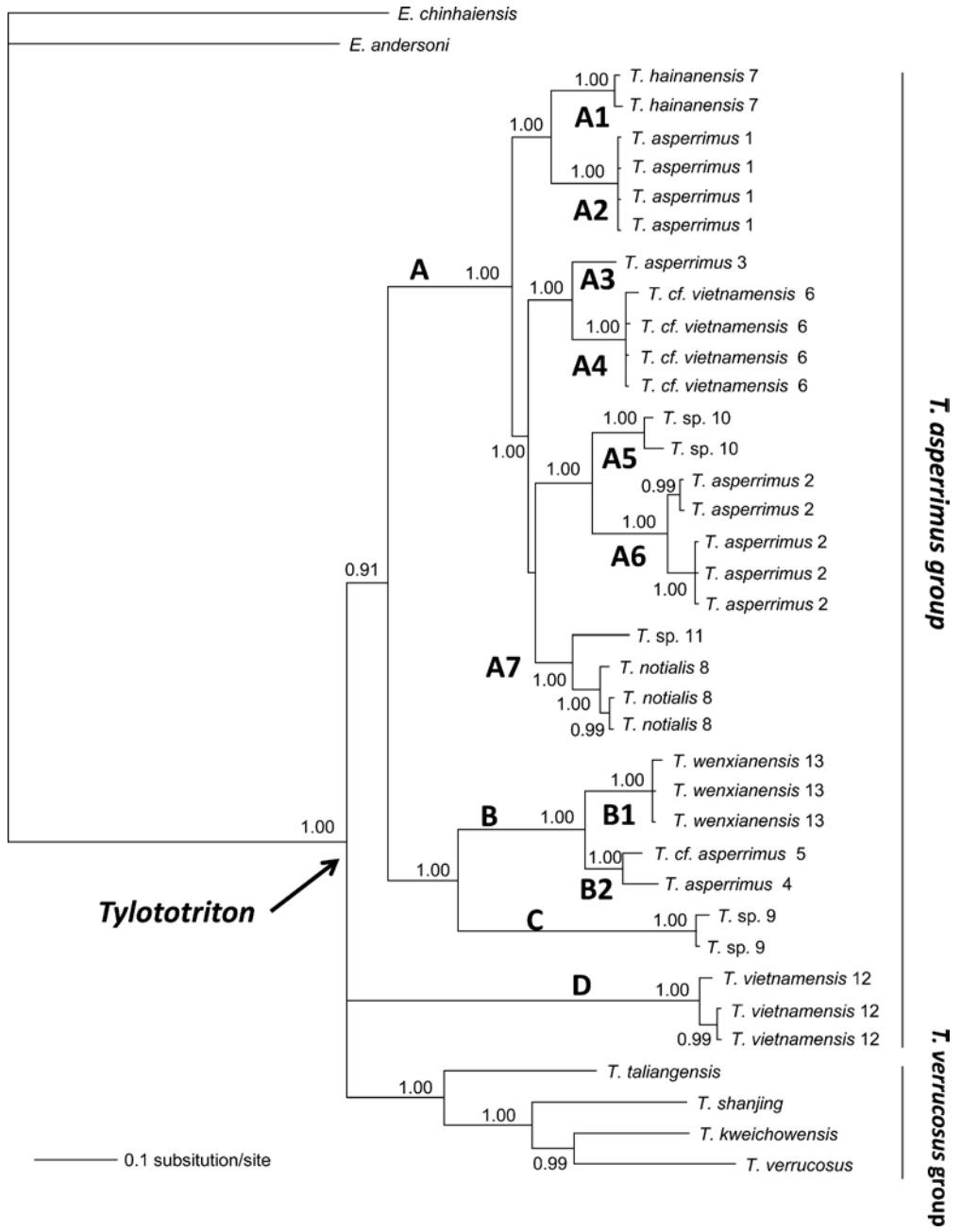

Fig. 2 A Bayesian tree resulting from mixed-model analysis of mitochondrial DNA

Numbers near nodes are Bayesian posterior probabilities. Vertical bars show the clade assignment. Numbers following the species name refer the locality number as shown in Tab. 1.

unnamed samples *T.* sp. from Hoa Binh, Vietnam (A5). Lineages A2, A3, and B2 have also been assigned to *T. asperrimus*. Salamanders from Baise, Guangxi (locality 1) form lineage A2, which is closely related to *T. hainanensis* (lineage A1). Lineage A3 is from Ha Giang, Vietnam (locality 3), and it is the sister group of "*T. vietnamensis*" from Cao Bang, Vietnam (locality 6). Lineage B2 is from Libo and Leigongshan, Guizhou, China (localities 4 and 5), is the sister group of *T. wenxianensis* (lineage B1). Additionally, the true *T. vietnamensis* from Bac Giang, Vietnam, exclusively constitutes clade D, which is distantly related to the population from Cao Bang (locality 6).

On the other hand, compared with previous studies, our data unexpectedly identify a distinct evolutionary clade C from Mangshan, Hunan (locality 9), which is the sister group of clade B.

2.2 Taxonomic implications

Species identification within the *T. asperrimus* group is challenging because all members are superficially

very similar and conservative in morphology (Stuart et al, 2010). Except for *T. hainanensis* and *T. notialis*, which occur in limited geographic regions, Hainan Island and Laos, respectively, *T. wenxianensis*, *T. asperrimus*, and *T. vietnamensis* have more dispersed distributions (Chen, 1991; Dai et al, 2011; Fei et al, 2005, 2009; Nguyen et al, 2009) and species borders are not clear.

Tylototriton wenxianensis was reported to occur in Gansu, Sichuan, Chongqing, southeastern Guizhou, Anhui, and nothern and central Hunan provinces in China (Fei et al, 2005, 2009). Our limited samples supported the monophyly of *T. wenxianensis* as a separate species, which can be interpreted as support for the taxonomic revision by Fei et al (1984). Moreover, we consider two samples from southeastern and extremely southern Guizhou, originally identified as "*T. asperimmus*" by Weisrock et al (2006) and our team, to be conspecific with *T. wenxianensis*, based on the phylogeny and genetic distance estimation. Among these, one sample from Guizhou (locality 4) was unfortunately used to represent the nominal *T. asperimmus* in comparisons of genetic distances with other species, for example, *T. notialis* (Stuart et al, 2010). Based on our data, we refer clade B to be *T. wenxianensis* (Tab. 1, Fig. 3). The level of genetic divergence between *T. wenxianensis* (B) and other clades is relatively higher (0.073−0.103) than other comparisons within *Tylototriton* (Tab. 2). Denser sampling within this clade is required, including investigation of the recent report of a population of *T. wenxianensis* relatively far to the north in Henan province, China (Chen et al, 2010).

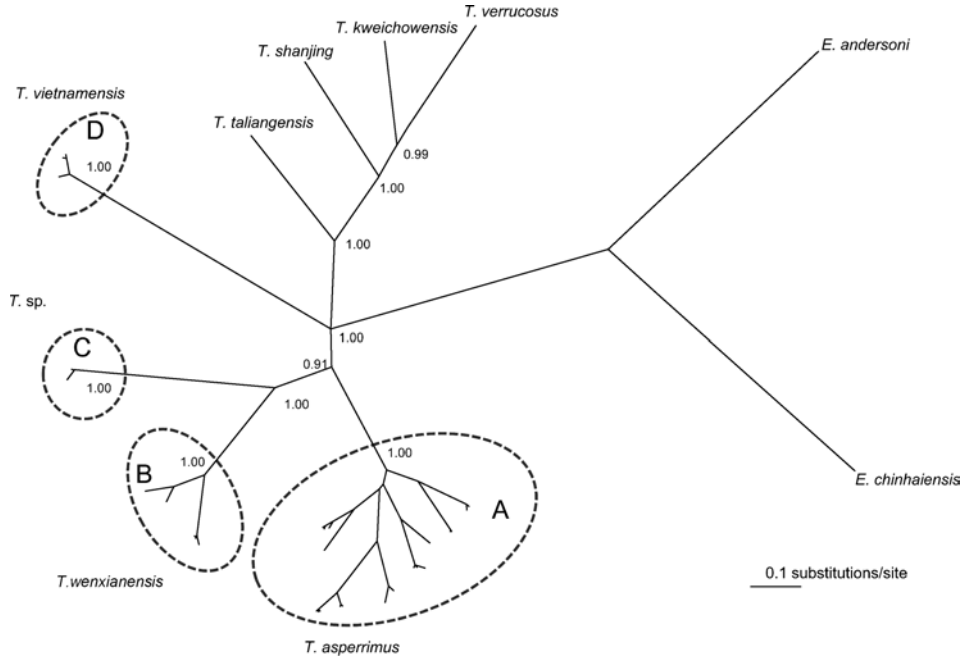

Fig. 3 Unrooted phylogenetic tree of mitochondrial gene sequences reconstructed using Bayesian methods for the *Tylototriton*
Posterior probabilities are displayed only at the major nodes. Taxonomic change suggestions were indicated on this tree.

Tab. 2 K2P pairwise distances of the ND1-ND2 sequences used in this study for the *Tylototriton*

	T. asperrimus	*T. wenxianensis*	*T. vietnamensis*	*T.* sp	*T. kweichowensis*	*T. shanjing*	*T. verrucosus*	*T. taliangensis*
T. asperrimus	—							
T. wenxianensis	0.079							
T. vietnamensis	0.099	0.093						
T. sp.	0.087	0.073	0.109					
T. kweichowensis	0.088	0.097	0.118	0.097				
T. shanjing	0.086	0.092	0.117	0.102	0.057			
T. verrucosus	0.093	0.103	0.120	0.107	0.052	0.064		
T. taliangensis	0.076	0.076	0.100	0.083	0.064	0.071	0.077	—

Currently, *T. asperrimus* is reported to be distributed in Guangxi, Guangdong, northern, western, and southern Guizhou, China (Fei et al, 2005, 2009) and northern Vietnam, for example, Lao Cai and Ha Giang (Nguyen et al, 2009). The distributions of *T. asperrimus* and *T. vietnamensis* in Vietnam require further assessments. Presently, *T. vietnamensis* is only reported to occur in Cao Bang, Lang Song, and Bac Giang (Nguyen et al, 2009). Based on our new molecular analyses and calculated genetic distances (Tab. 2), *T. vietnamensis* from the type locality of Bac Giang was distinctly separated as our clade D, as hypothesized by Stuart et al (2010). However, the assumed "*T. vietnamensis*" (or *T. cf. vietnamensis*: Stuart et al, 2010; Weisrock et al, 2006) from Cao Bang (A4) was distantly separated from the true *T. vietnamensis*. Together, *T. hainanensis*, *T. notialis*, "*T. vietnamensis*", and three unnamed salamanders from Vietnam clustered with the true *T. asperrimus* from Daoyaoshan, China, which constituted clade A. Based on the evolutionary relationships and genetic distances, we assign all members of our clade A to one species, *T. asperrimus* (Tab. 1, Fig. 3). Accordingly, we assign *T. notialis* Stuart et al, 2010 and *T. hainanensis* Fei et al, 1984 as junior objective synonyms of *T. asperrimus* Unterstein 1930.

The level of genetic divergence among A1-A7 ranged from 0.021 to 0.047 (not shown here), which is much lower than the hypothesized inter-specific divergence (0.052−0.120, Tab. 2). Samples from clade A are distributed around the Gulf of Tonkin (Fig. 1). The closure of the Tonkin Strait in the past probably allowed gene flow exchanges between populations of clade A.

Samples from clade C could be treated as a cryptic species, and the level of genetic divergence between lineages C and other clades within *Tylototriton* ranged from 0.073−0.109; however, it also is similar to *T. asperrimus* in morphology and we could not easily identify it when we are in the field. Further detailed studies including a combination of various datasets should be done to ascertain whether it represents a distinct species (Hou et al, unpublished data), or, like clade B2, it should be assigned to *T. wenxianensis*. A denser sampling covering the entire distribution ranges regarding clade B and C is desirable.

Altogether, the description of amphibian diversity and evolutionary history remains a difficult task in some cases because of extremely conservative morphology and in other cases homoplasy (Bossuyt & Milinkovitch, 2000; Wake, 1991). In this case, molecular tools do help, however samples from type localities should be fully considered when formal taxonomic revisions and descriptions of new species are undertaken. Inclusion of additional genes, especially nuclear genes, may prove to be essential. MtDNA data alone cannot assess introgression and gene flow.

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