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The phylogenetic relationships of *Paramesotriton* (Caudata: Salamandridae) based on partial mitochondrial DNA gene sequences

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Abstract

We examined phylogenetic relationships among newst of the genus *Paramesotriton* using partial mitochondrial gene sequences, including the ND2-tRNA^{Tyr} region (1415 bp) and the 12S rDNA-tRNA^{Val}-16S rDNA region (1774 bp), from 42 individuals of 10 recognized *Paramesotriton* species and outgroups by Bayesian inference (BI), Maximum likelihood (ML) and Maximum Parsimony (MP) methods. We found that, (1) *Laotriton laoensis* is the sister group of *Paramesotriton*, (2) the genus *Paramesotriton* is monophyletic, composed of either the *P. caudopunctatus* species group and the *P. chinensis* species group, or the subgenera *Allomesotriton* and *Paramesotriton* (3) *P. longliensis* and *P. zhijinensis* should be placed in the *P. caudopunctatus* species group or subgenus *Allomesotriton*; (4) *P. fuzhongensis* is not a junior synonym of *P. chinensis*, and there is a close phylogenetic relationship between *P. fuzhongensis* and *P. guangxiensis*.

Key words: Mitochondrial DNA sequences, Phylogeny, *Paramesotriton*

Introduction

The genus *Paramesotriton* (Caudata: Salamandridae) contains 10 known species. Except *P. deloustali* is endemic to the Tam Dao region of Vietnam (Bourret) and *P. laoensis* is found in Laos (Stuart and Papenfuss), all other species occur in China, including *P. chinensis* (Gray), *P. fuzhongensis* (Wen), *P. guangxiensis* (Huang *et al.*), *P. hongkongensis* (Myers and Leviton), *P. caudopunctatus* (Hu *et al.*), *P. zhijinensis* (Li *et al.* 2008), *P. longliensis* (Li *et al.* 2008) and *P. ermizhaoi* (Wu *et al.* 2009).

However, researchers have disagreed on the validity of some species. For example, *P. fuzhongensis* was treated as a junior synonym of *P. chinensis* (Pang *et al.* 1992). Based on molecular data of six species of this genus, Lu *et al.* (2004) argued that *P. fuzhongensis* is indeed a legitimate species, distinguished from *P. chinensis,* but the species was still treated as a synonym of *P. chinensis* (Fei *et al.* 2006). *P. guanxiensis* was once treated as a junior synonym of *P. deloustali* (Pang *et al.* 1992), but it was treated as a legitimate species (Ye *et al.*, 1993; Fei *et al.* 2006). The phylogenetic relationship of [(*P. chinensis, P. hongkongensis*), (*P. fuzhongensis,* (*P. guangxiensis, P. deloustali*))] was well supported in the study of Lu *et al.* (2004). However, Weisrock *et al.* (2006) considered that the phylogenetic relationship of *P. fuzhongensis, P. guangxiensis* and *P. deloustali* is (*P. deloustali* (*P. fuzhongensis, P. guangxiensis*)). The recently described species, *P. ermizhaoi,* was considered as a sister species of the group of *P. chinensis* and *P. hongkongensis* (Wu *et al.* 2009).

P. zhijinensis (found in Zhijin, Guizhou, China) and *P. longliensis* (found in Longli, Guizhou, China), are recently described species. Zhao *et al.* (2008) considered that *P. zhijinensis* is a sister species of *P. caudopunctatus*, and *P. zhijinensis* represents the third evolutionary lineage of *Paramesotriton.* However, the phylogenetic placement of *P. longliensis* is unclear in the genus *Paramesotriton.*

Chan *et al*. (2001) reported that the genus *Paramesotriton* was monophyletic, with *P. caudopunctatus* as basal to *P. guangxiensis*, *P. deloustali* and *P. hongkongensis*. Based on mtDNA sequence data, Lu *et al.* (2004) considered that the six species of *Paramesotriton* (*P. caudopunctatus, P. chinensis, P. deloustal, P. fuzhongensis, P.*

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guangxiensis and *P. hongkongensis*) form a monophyletic group, with *P. caudopunctatus* as basal to the other five species. Weisrock *et al.* (2006) and Steinfartz *et al.* (2007) agreed with the results of Lu and colleagues. Sparreboom (1983) reported the morphological features, reproduction behavior and egg-laying of *P. caudopunctatus*. Freytag (1983) identified *P. caudopunctatus* as a species of the monotypic genus *Allomesotriton* based on its unique morphological characteristics and reproduction behavior. Pang *et al.* (1992) considered *Allomesotriton* to be a subgenus of *Paramesotriton* according to the morphological characteristics of the skull, skeleton, and hyoid apparatus of warty newts. Fei *et al.* (2006) divided the genus *Paramesotriton* into two species groups: the *P. caudopunctatu*s species group (only *P. caudopunctatu*s) and the *P. chinensis* species group (the remaining species). Dubois and Raffaelli (2009) placed *P. caudopunctatu*s into a distinct subgenus, *Allomesotriton*, which nomen is already available (Raffaeli 2007), and the remaining species of this genus into subgenus *Paramesotriton*.

The bright coloration of the dorsum in *P. laoensis* from Laos is remarkably different from the other representatives of the genus. Based on distinct morphological difference and well-supported molecular divergence, this species may be a sister-group to a clade containing the genera *Paramesotriton* and *Pachytriton* (Weisrock *et al.* 2006), or to the genus *Pachytriton* (Zhang *et al.* 2008). Weisrock *et al.* (2006) suggested that it ‘‘should not be placed in the genus *Paramesotriton*’’. Dubois & Raffaelli (2009) referred this species to its own genus, *Laotriton*, and this suggestion was followed by Frost (2011) in the Amphibian Species of the World database (ASW), and by Amphibiaweb (http://www.amphibiaweb.org, accessed November 2011).

Here, on the basis of newly collected *Paramesotriton* samples from different localities of Guizhou Province, Guangxi Province, Chongqing Municipality and Zhejiang Province, P. R. China, the phylogenetic relationships of all recognized *Paramesotriton* species and of a newly discovered population (*P.* sp) were reconstructed using newly determined and previously published mtDNA sequence data (3189 bp). In addition to conventional phylogenetic tree-building methods, we test the reliability of different phylogenetic hypotheses. Our goals are (i) to provide reliable evidence supporting or not whether all *Paramesotriton* species form a monophyletic group, (ii) to verify the phylogenetic position of the more recently described species, and (iii) to provide reliable evidence in support of the validity of some species.

Material and methods

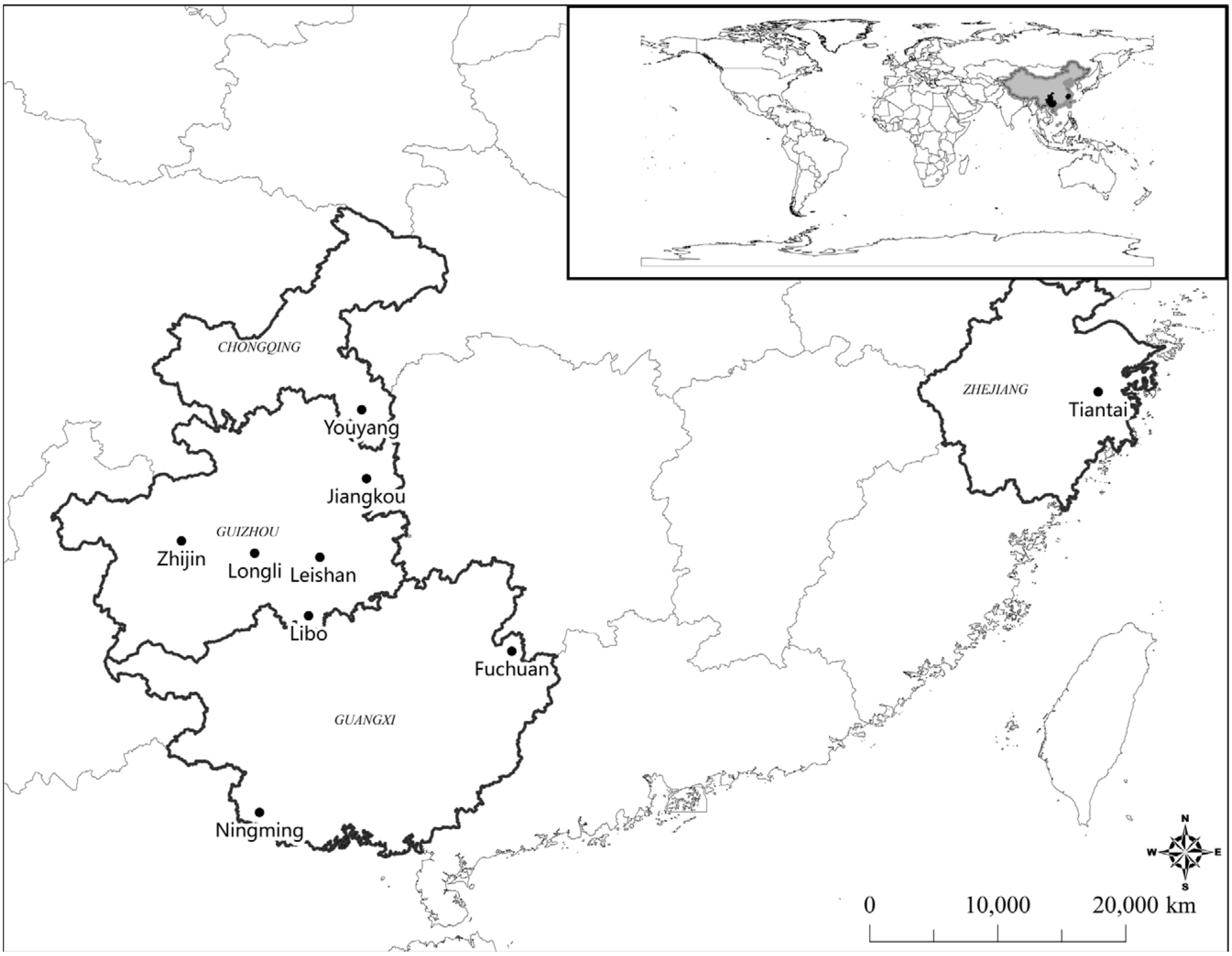
Taxon sampling and data collection. A total of 30 individuals of six species of *Paramesotriton*, including *P. guangxiensis*, *P. fuzhongensis*, *P. chineensis*, three populations of *P. caudopunctatus* (including Leishan, Jiangkou and Youyang populations), *P. zhijinensis*, *P. longliensis*, in addition to a newly discovered population (*P.* sp), were examined in this study (Table 1 and Figure 1).

Approximately 3189 bp of mtDNA sequences (including the ND2 and the 12S-16S rDNA) of these species were amplified in this study. We acquired corresponding sequences of other three species of *Paramesotriton* (*P. deloustali*, *P. hongkongensis* and *P. ermizhaoi* (only the ND2)) and *Laotriton laoensis*, from GenBank for phylogenetic analysis. Because *Cynops* was reported to be the sister group of a clade containing both *Pachytriton* and *Paramesotriton* (Titus and Larson 1995; Chan *et al.* 2001; Weisrock *et al.* 2006; Steinfartz *et al.* 2007; Zhang *et al.* 2008), we included *C. pyrrhogaster*, *C. cyanurus* (*Hypselotriton cyanurus*, Dubois & Raffaelli 2009), *Pachytriton labiatus* and *P. brevipes* as outgroup taxa in this study (Table 1). The sequences of the outgroup taxa were acquired from GenBank. All samples in this study were deposited in the School of Life Sciences, Guizhou Normal University.

DNA extraction, amplification and sequencing. Genomic DNA was extracted from muscle tissues preserved in 90% alcohol followed the standard phenol-chloroform protocol. A 1415 bp segment including the genes encoded the ND2 (one subunit of NADH dehydrogenase), $tRNA^{Trp}$, $tRNA^{Ala}$, $tRNA^{Asn}$, O_L, $tRNA^{Cys}$ and part of $tRNA^{Tyr}$ (hereafter ND2) was amplified using the primer pair L4437-H5934 (Macey *et al.* 1997). A 1774 bp segment including the genes encoded 12SrRNA, $tRNA^{Val}$ and 16S rRNA (hereafter 12S-16S rDNA) was amplified by primers A and I (Titus and Larson 1995). The amplification was performed in a 50μl volume reaction with the following procedures: 5 min initial denaturation step at 95 ℃, 32 cycles of 30 seconds denaturation at 94 ℃, annealing for 30 seconds at 50 ℃ , extension for 1 min 50 seconds for the ND2 and a final extension at 72 ℃ for 10 min. Cycle annealing temperature was raised to 56 °C and extension was increased to 2 min10 seconds for the 12S-16S rDNA, with other conditions unchanged.

The PCR products were purified and sequenced in both directions using primer pairs L4437-H5934 (Macey *et al.* 1997) and the primers A, I and F (Titus and Larson 1995), respectively, at Shanghai DNA BioTechnologies Co, Ltd (Shanghai, China).

The fragments obtained were input into the NCBI database to search for homologous sequences. The sequences were aligned separately using the program Clustal X 1.81 (Thompson *et al.* 1997) with default parameters. Subsequently, the alignments were verified by eye and regions of ambiguous alignments were removed from the sequences. The accession numbers of all sequences we used were given in Table 1.



FIGUER 1. Map showing the distribution of the species of *Paramesotriton* used in this study. For information on species, see Table 1.

Phylogenetic reconstruction. To assess phylogenetic congruence between the ND2 and the 12S-16SrDNA, Partition Homogeneity Tests (Farris *et al*. 1995) implemented in PAUP4.0b10 (Swofford 2003) were conducted for 100 replicates. The concatenated datasets and the ND2 were analyzed using the Bayesian inference (BI), Maximum-likelihood (ML) and Maximum parsimony (MP) method, respectively. Bayesian analyses were carried out using MrBayes 3.0 (Ronquist & Huelsenbeck 2003) with the TVM+I+G model of DNA evolution, including 1×10^6 generations of Markov Chain Monte Carlo (Altekar *et al.* 2004). After omitting 25% of the generations (250000 generations) as burn in, a 50% majority rule consensus tree was calculated to generate a posterior probability for each node. Maximum likelihood analyses were carried out in PAUP*4.0b10 using the TVM+I+G model of DNA evolution. Heuristic search methods were used with TBR branch swapping. Analyses were repeated 200 times with a random order of sequence addition. Bootstrap tests were performed using 500 replicates with nearest neighbor interchange (NNI) branch swapping. We conducted MP analyses (using heuristic searches with 10 random input orders of taxa, TBR branch swapping, and equally weighted character) for all species with PAUP4.0b10 (Swofford

2003). Levels of repeatability of the branching patterns were assessed with 1000 bootstrap replicates. The strict consensus tree generated was with CI = 0.721, RI = 0.792, RC = 0.571 and L = 545.

Some alternative phylogenetic topologies were tested using the parsimony-based Templeton test (Templeton, 1983) and the Likelihood-based Approximately unbiased (AU) test (Shimodaira 2002). The Templeton test was done by using 1000 RELL bootstrap replicates, implemented in PAUP*4.0b10. To perform the AU test, we calculated site-wise log-likelihoods scores of trees constrained by topological conflicts using PAUP*4.0b10 and test P-values using the program CONSEL (Shimodaira & Hasegawa, 2001).

TABLE 1. Taxon sampling for all ingroup and outgroup samples used in this study. GZNU = Guizhou Normal University, the Zoology Museum Collection (Guiyang, China)

Taxon	Voucher Specimen No.	GenBank Accession No.		Locality description
		12S-16SrDNA	ND2- tRNA^{Tyr}	
Paramesotriton				
P. chinensis 1	GZNU200806087	FJ938048	JF438979	Tiantai, Zhejiang, China
P. chinensis 2	MVZ 230360		DQ517800	Yongjia, Zhejiang, China
P. hongkongensis 1	Not mention	AY458597	AY458597	Shenzhen,Guangdong, China
P. hongkongensis 2	MVZ230367		DQ517805	Violet Hill, Hong Kong Island, Hong Kong, China
P. deloustali 1	MVZ223628	EU880327	EU880327	Tam Dao, Vinh Phu, Province, Vietnam
P. deloustali 2	MVZ223627		DQ517802	Tam Dao, Vinh Phu Province, Vietnam
P. guangxiensis 1	GZNU2006001	FJ938041	FJ169604	Ningming,Guangxi, China
P. guangxiensis 2	GZNU2006002	JF712657	JF438980	Ninming, Guangxi, China
P. fuzhongensis 1	GZNU2007052001	FJ938044	FJ169605	Fuchuan, Guangxi, China
P. fuzhongensis 2	GZNU2007052002	HQ711551	JF438981	Fuchuan, Guangxi, China
P. fuzhongensis 3	GZNU2006067003	HQ711552	JF438982	Fuchuan, Guangxi, China
P. ermizhaoi 1	CIB 88141		FJ744601	Jinxiu, Guangxi, China
P. ermizhaoi 2	CIB 88140		FJ744602	Jinxiu, Guangxi, China
P. caudopunctatus(LS) 1	GZNU2009042501	HQ702557	JF438983	Leishan, Guizhou, China
P. caudopunctatus(LS) 2	GZNU07072001	HQ702556	FJ938040	Leishan, Guizhou, China
P. caudopunctatus(LS) 3	GZNU20050727002	FJ938042	JF438984	Leishan, Guizhou, China
P. caudopunctatus(LS) 4	GZNU20050727001	HQ702555	JF438985	Leishan, Guizhou, China
P. caudopunctatus(LS) 5	GZNU07072005	HQ702558	JF438986	Leishan, Guizhou, China
P. caudopunctatus(JK) 1	GZNU2007071001	FJ938043	FJ169606	Jiangkou, Guizhou, China
P. caudopunctatus(JK) 2	GZNU2007071002	HQ702559	JF438987	Jiangkou, Guizhou, China
P. caudopunctatus(JK) 3	GZNU2007071003	HQ702560	JF438988	Jiangkou, Guizhou, China
P. caudopunctatus(JK) 4	GZNU2007071004	HQ702561	JF438989	Jiangkou, Guizhou, China
P.caudopunctatus(YY) 1	GZNU08072602	HQ702562	JF438990	Youyang,Chongqing, China
P. caudopunctatus(YY) 2	GZNU08072603	HQ702563	JF438991	Youyang,Chongqing, China
P. caudopunctatus(YY) 3	GZNU08072604	HQ702564	JF438992	Youyang,Chongqing, China
P. sp 1	GZNU2006030003	FJ938045	FJ169607	Libo, Guizhou, China
P. sp 2	GZNU2006030004	HQ711542	JF438993	Libo, Guizhou, China
P. sp 3	GZNU2006030005	HQ711543	JF438994	Libo, Guizhou, China
P. sp 4	GZNU2006030006	HQ711544	JF438972	Libo, Guizhou, China
P. longliensis 1	GZNU20070421001	FJ938046	FJ169608	Longli, Guizhou, China

continued next page

TABLE 1. (continued)

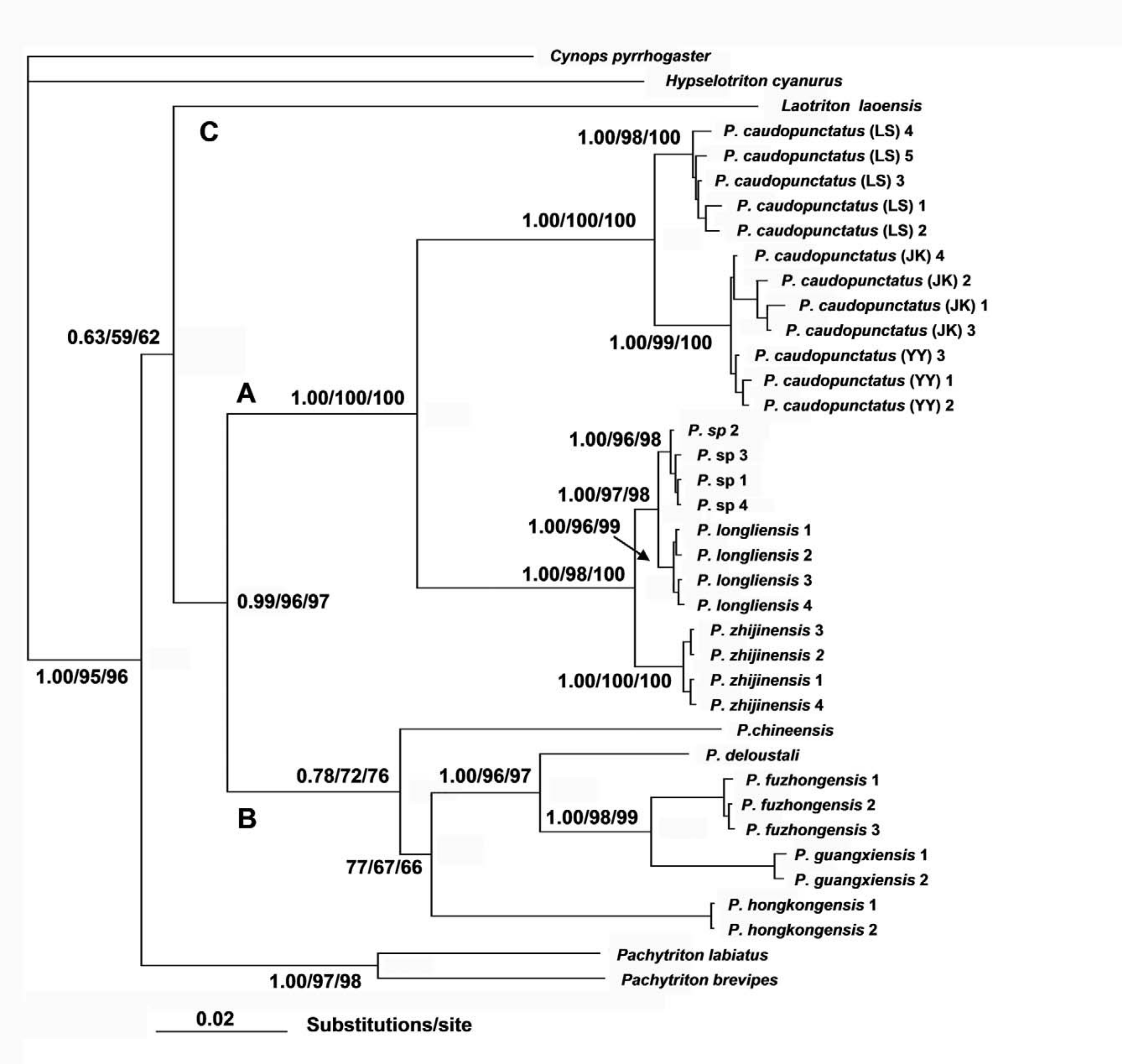
Taxon	Voucher Specimen No.	GenBank Accession No.		Locality description
		12S-16SrDNA	ND2- $tRNA^{Tyr}$	
P. longliensis 2	GZNU20070421002	HQ711548	JF438973	Longli, Guizhou, China
P. longliensis 3	GZNU20070421003	HQ711549	JF438974	Longli, Guizhou, China
P. longliensis 4	GZNU20070421004	HQ711550	JF438975	Longli, Guizhou, China
P. zhijinensis 1	GZNU20070415001	FJ938047	FJ169609	Zhijin, Guihzou, China
P. zhijinensis 2	GZNU20070415002	HQ711545	JF438976	Zhijin, Guihzou, China
P. zhijinensis 3	GZNU20070415003	HQ711546	JF438977	Zhijin, Guihzou, China
P. zhijinensis 4	GZNU20070415004	HQ711547	JF438978	Zhijin, Guihzou, China
Laotriton laoensis	FMNH255452	EU880328	EU880328	Phou Sang Kat mountain, Saysamboun Special Zone, Laos
Outgroup				
Pachytriton labiatus	MVZ230355	EU880325	EU880325	Not mention
Pachytriton brevipes	MVZ231167	EU880324	EU880324	Not mention
*Cynops pyrrhogaste*r	TP-MVZ03	EU880313	EU880313	Not mention
Hypselotriton cyanurus (*Cynops.cyanurus*)	TP-MVZ02	EU880309	EU880309	Not mention

Results

No length variation was observed in the ND2 gene region of 30 individuals of the six species of *Paramesotriton*. The sequence length of the ND2 fragment is 1415 bp after alignment. Among them, 438 are variable and 274 are potentially phylogenetically informative. Most of the variable and the phylogenetically informative sites are from protein-coding region, only 63 variable and 35 phylogenetically informative sites are from tRNA genes and non-coding regions. Of the 239 phylogenetically informative characters from protein-coding regions, 115 are from third position of codons. The third-position sites amount to near half of the phylogenetically informative sites in the genetic region. Average level of base are T = 25.80%, C = 25.90%, A = 35.40%, G = 12.80%, with a G + C content of 38.70%. The sequence length of the 12S-16SrDNA of 30 individuals of the six *Paramesotriton* species used for alignment is 1774 bp. Among them, there are 36 insertion or deletion sites, and the variable sites and parsimony informative sites are 347 and 193 bp, respectively. Average proportions of bases are T = 25.97%, C = 25.97%, A = 36.40%, G = 11.66%, with a G + C content of 37.63%.

The ND2 fragment presents higher levels of interspecific divergence than the 12S-16S rDNA fragment among ingroup and outgroup. Surprisingly, remarkably small genetic differences, 1.5% in the ND2 and 0.7% in the 12S-16SrDNA, were exhibited between *P. zhijinensis* and *P. longliensis*, less than those of *P. caudopunctatus*' inter-population distance (1.7% ~2.3% in the ND2 and 1.2~2.6% in the 12S-16S rDNA).

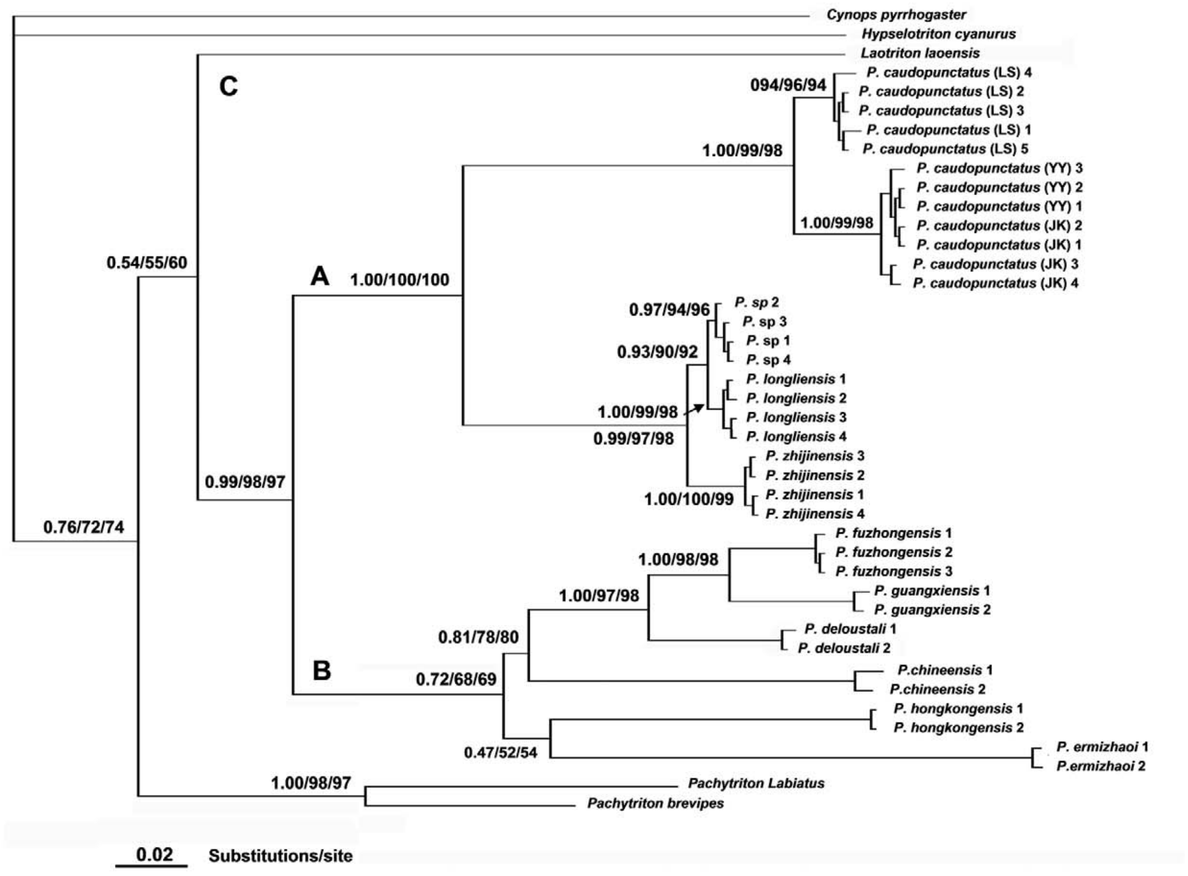
Results from the Partition Homogeneity Test suggested that the ND2 and 12S-16S rDNA are congruent with each other ($P = 0.35$). Therefore, we concatenated the two fragments (total 3144bp) in the phylogenetic inference and analyzed the phylogenetic relationships mainly based on BI, ML and MP trees obtained from the concatenated dataset. The BI, ML and MP analyses from concatenated datasets produced identical topologies. The Bayesian posterior probabilities (PP) of all ingroup clades of the BI tree are greater than 0.8 except for three clades (*L. laoensis* clade (clade C) and the first, the second sub-clade in clade B), and the parsimony boot strap values (MPBS) and likelihood boot strap values (MLBS) of all ingroup clades of the MP tree are over 70% except for two clades (*L. laoensis* clade and the second sub-clade in clade B). The results of the three analyses suggest that all eight species of *Paramesotriton* and *L. laoensis* form a monophyletic group, in which *L. laoensis* is sister to these eight *Paramesotriton* species, but the clade is only weakly supported (PP/MLBS/MPBS = 0.63/59/62). These eight *Paramesotriton* species are recovered as a monophyletic group strongly supported (PP/ MLBS/MPBS = 0.99/96/97), and could be divided into clades A and B. Clade A includes three populations of *P. caudopunctatus* (Leishan, Jiangkou and Youyang populations), *P. zhijinensis*, *P. longliensis* and the newly discovered population (*P.* sp), and can be further divided into two sub-clades: [*P. zhijinensis* (*P. longliensis*, *P.* sp)] sub-clade and *P. caudopunctatus* sub-clade. Clade B consists of *P. hongkongensis, P. chinensis, P. deloustali* , *P. fuzhongensis* and *P. guangxiensis*. Among these species, *P. chinensis*, *P. hongkongensis* and *P. deloustali* are positioned as the first, the second and the third sub-clade, respectively. And *P. deloustali* is a sister species to the fourth sub-clade consisting of *P. guangxiensis* and *P. fuzhongensis* (Fig 2).



FIGUER 2. Phylogenetic relationships of extant *Paramesotriton* species (except *P. ermizhaoi*) inferred from concatenated datasets. The DNA dataset (3189 sites) was analyzed with Maximum parsimony, partitioned maximum likelihood, and Bayesian inference. All approaches produce identical topology, and their branch-support measures are mapped to the phylogram. Left numbers along branches represent weighted maximum parsimony bootstrap values, middle numbers represent partitioned maximum likelihood bootstrap values and right numbers represent Bayesian posterior probabilities. Branch lengths were estimated by Bayesian inference analysis.

The results of the BI, ML and MP analyses from the ND2 are basically consistent. All nine species of *Paramesotriton* and *L. laoensis* form a monophyletic group, in which *L. laoensis* is sister to the nine species of *Paramesotriton*, but the clade (clade C) is only weakly supported, (PP/MLBS/MPBS = 0.54/55/60). These nine *Paramesotriton* species are also recovered as a monophyletic group strongly supported (PP/MLBS/MPBS = 0.99/ 98/97), and could be divided into clades A and B. Species *P. ermizhaoi* is a sister species of *P. hongkongensis*, but the clade is weakly supported ,too (PP/MLBS/MPBS = 0.47/52/54), and (*P. hongkongensis*, *P. ermizhaoi*), *P.*

chineensis are the first and the second sub-clades, respectively, in clade B in th phylogenetic tree from the ND2, the positions of other species are identical to that from concatenated datasets (Fig 3).



FIGUER 3. Phylogenetic relationships of extant *Paramesotriton* species inferred from the ND2. The DNA dataset (1415 sites) was analyzed with Maximum parsimony, partitioned maximum likelihood, and Bayesian inference. All approaches produce identical topology, and their branch-support measures are mapped to the phylogram. Left numbers along branches represent weighted maximum parsimony bootstrap values, middle numbers represent partitioned maximum likelihood bootstrap values and right numbers represent Bayesian posterior probabilities. Branch lengths were estimated by Bayesian inference analysis.

The ambiguous parts of the two phylogenetic trees are the placement of *L. laoensis, P. hongkongensis, P. chinensis* and *P. ermizhaoi* because the clades are only weakly or moderately supported. Topological tests based on both likelihood and parsimony were unable to reject the alternative hypotheses on the phylogenetic positions of these species ($P > 0.05$; Table 3).

TABLE 2. Statistical comparisons among alternative hypotheses of salamandrid relationships using the AU test and Templeton test.

Alternative topology tested	Likelihood-based		Parsimony-based
	ΔlnL[a]	AU test	Templeton test Δsteps (*P* value)
Best tree	—	—	—
L.laoensis sister to *Pachytriton*	1.29834	$P = 0.237$	N = 17 $P = 0.4669$
L.laoensis sister to *Pachytriton*+ *Paramesotriton*	0.73161	$P = 0.379$	N = 15 $P = 0.7963$
P. hongkongensis sister to all remaining Newts in *P. chinensis* species group	2.88313	$P = 0.125$	N = 20 $P = 0.3711$
P. hongkongensis sister to *P. chinensis*	1.68247	$P = 0.310$	N = 24 $P = 1$
P. ermizhaoi sister to (*P. chinensis* + *P. hongkongensis*)	1.28161	$P = 0.219$	N = 27 $P = 0.5637$
P. ermizhaoi sister to *P. chinensis*	1.10305	$P = 0.265$	N = 23 $P = 0.1444$

Discussion

***L. laoensis* and all species of *Paramesotriton* form a monophyletic group.** Based on the combined result of molecular and morphological data, Chan *et al.* (2001) proposed the monophyly of *Paramesotriton*. But their tree contained only four *Paramesotriton* species. With partial sequences data of mitochondrial genes, Lu *et al.* (2004) argued that all six species of *Paramesotriton* (*P. deloustali*, *P. caudopunctatus, P. chinensis*, *P. fuzhongensis*, *P. guangxiensis* and *P. hongkongensis*) included in their study formed a monophyletic group. Weisrock *et al.* (2006) argued that all six species of *Paramesotriton* except *P. laoensis* (*L. laoensis*) formed a monophyletic group. Our results suggest that *L. laoensis* and eight species (in phylogenetic trees from the concatenated datasets) or nine species (in phylogenetic trees from the ND2) of *Paramesotriton* form a monophyletic group, and *L. laoensis* is the sister group of *Paramesotriton,* albeit *L. laoensis* clade (clade C) is only weakly supported (PP/MLBS/MPBS = 0.63/ 59/62, in the concatenated datasets, PP/MLBS/MPBS = 0.47/52/54. in the ND2) in the monophyletic group. Owing to the low PP/MLBS/MPBS, the phylogenetic status of *L. laoensis* is still not fully resolved.

The topological tests of our study could not refuse *L. laoensis* sister to (*Pachytriton*, *Paramesotriton*) and *L. laoensis* sister to *Pachytriton* ($p > 0.05$, Table 3). *L. laoensis,* in fact, may be a sister-group to the cluster of genera *Paramesotriton* and *Pachytriton* (Weisrock *et al.* 2006), or to the genus *Pachytriton* (MPBS = 97; alternative hypotheses $P < 0.05$; the ML-corrected sequence divergence matrix also indicated that *L. laoensis* is closer to *Pachytriton* than to *Paramesotriton.* Zhang *et al.* 2008). Weisrock *et al.* (2006) suggested that it ‘‘should not be placed in the genus *Paramesotriton*’’, Zhang *et al.* (2008) thought this species likely represents a transitional form when the increasingly specialized *Pachytriton* stock evolved from *Paramesotriton* to become aquatic, Dubois & Raffaelli (2009) referred this species to its own genus, *Laotriton*. All standard online references (ASW and Amphibiaweb) have accepted *Laotriton* by now. Our data do not contradict the conclusion of these authors of placing *P. laoensis* (*L. laoensis*) in an own genus, because the placement of *L .laoensis* sister to *Paramesotriton* in our analysis was not strongly supported. However, we cannot provide conclusive new evidence for *L. laoensis* to be a separate placement.

Phylogenetic placement of *P. zhijinensis*, *P. longliensis* and *P.* sp. Because of the unique morphological features, *P. caudopunctatus* was once considered to be the only a species of the new genus, *Allomesotriton* (Freytag 1983). However, based on the study of morphological structure, Pang *et al.* (1992) suggested that *Allomesotriton* is a subgenus of the genus *Paramesotriton* and *P. caudopunctatus* is the most basal species of the genus *Paramesotriton*. This proposal was supported by phylogenetic studies (Chan *et al.* 2001; Lu *et al.* 2004; Weisrock *et al.* 2006) and *P. caudopunctatus* was considered as the sister lineage to all remaining species of *Paramesotriton* in these studies. In the study, the most basal species of the genus include *P. caudopunctatus, P. longliensis, P. zhijinensis* and *P.* sp. The genus *Paramesotriton* is divided into two species groups by Fei *et al.* (2006), the *P. caudopunctatus* species group(only *P. caudopunctatus*) and the *P. chinensis* species group (the remaining species). Dubois and Raffaelli (2009) promoted the *P. caudopunctatus* species group and the *P. chinensis* species group to subgenera, as *Allomesotriton* and *Paramesotriton*, respectively. The former corresponds to clade A, the latter corresponds to the clade B of our results. The species *P. zhijinensis*, *P. longliensis* and *P.* sp could be assigned to *P. caudopunctatus* group or the subgenus *Allomesotriton*.

P. sp, native to Libo county of Guizhou Province, China, is different from other species of the genus morphologically, such as enormous body size (TOL: ♀ ,approximately 200 cm; ♂ ,approximately 184 cm), a pair of horn protuberance on the upper laterals post head (Fig.4), but *P.* sp is recovered as sister to *P. longliensis* in our phylogenetic trees. The pairwise sequence divergence of *P.* sp to *P. longliensis* and *P. zhijinensis* are 0.7% and 1.6% in the ND2, 0.6% and 1.1% in the 12S-16S rDNA, respectively. This suggests that *P.* sp may be a new population of the rare newt *P. longliensis.*

The phylogenetic relationships of the *P. chinensis* species group. The relationships of the five species in the clade B in the phylogenetic tree from concatenated datasets are not identical to those reconstructed by Lu *et al.* (2004), Weisrock *et al.* (2006) and Zhao *et al.* (2008), in which *P. hongkongensis* is a sister taxon of *P. chinensis.* The phylogenetic relationship among *P. fuzhongensis, P. guangxiensis* and *P. deloustali* is (*P. fuzhongensis* (*P. guangxiensis, P. deloustali*)) in studies of Lu *et al.* (2004) and Zhao *et al.* (2008), but the phylogenetic relationship of this three species is (*P. deloustali* (*P. fuzhongensis, P. guangxiensis*)) in study of Weisrock *et a*l. (2006). Our result suggests that *P. chinensis* is a sister taxon of [(*P. hongkongensis*), (*P. deloustali,* (*P. guangxiensis, P. fuzhongensis*)] (only moderately supported, PP/MLBS/MPBSs are 0.78/72/76 in the concatenated datasets), *P. fuzhongen-*

sis is not a junior synonym of *P. chinensis*, and there is a close phylogenetic relationship between *P. fuzhongensis* and *P. guangxiensis* instead of *P. deloustal,* which is identical to the result of Weisrock *et al*. (2006).

FIGUER 4. Dorsal, lateral view of *P.* sp (GZNU2008070001, ♀), a newly discovered population from Libo county of Guizhou Province, China.

P. ermizhaoi is recovered as a sister species of the group of *P. chinensis* and *P. hongkongensis* with a low PP and MPBS (0.54/53) (Wu *et al.* 2009), but *P. ermizhaoi* is recovered as sister to *P. hongkongensis*, also having a low PP, MLBS and MPBS (0.47/52/54), and (*P. hongkongensis*, *P. ermizhaoi*), *P. chineensis* are the first and the second sub-clades, respectively, in our study. Because of the low PP, MLBS and MPBS, the phylogenetic placement of *P. ermizhaoi* is unclarified.

Templeton tests and AU tests could not reject that *P. chinensis* is sister species to all remaining newts in this species group and *P. hongkongensis* is a sister specie to *P. chinensis* (*p* > 0.05, Table 3).The two tests could also not reject that *P. ermizhaoi* is a sister specie to *P. chinensis* and *P. ermizhaoi* is a sister species of the group of *P. chinensis* and *P. hongkongensis* (*p* > 0.05, Table 3) . Therefore, also the precise phylogenetic position of *P. hongkongensis*, *P. chinensis* and *P. ermizhaoi* is left with uncertainty. To clarify the phylogenetic positions of these three species, a more comprehensive study based on morphological and molecular analysis is needed.

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