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Molecular Phylogeny of the Genus *Paramesotriton* (Caudata: Salamandridae)

Shunqing Lu,[1,2,3,4] **Zhi-Gang Yuan,**[1,4,5] **Junfeng Pang,**[1] **Datong Yang,**[1] **Fahong Yu,**[6] **Peter McGuire,**[6] **Feng Xie,**[2] **and Ya-Ping Zhang**[1,7,8]

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To elucidate the phylogeny of the genus Paramesotriton *(Caudata: Salamandridae), we investigated three mitochondrial DNA gene fragments (1207 bp in total) of cytochrome* b, *ND2, and ND4 for its six recognized species. The phylogenetic relationships within* Paramesotriton *were reconstructed by maximum parsimony (MP) and maximum likelihood (ML) methods. Phylogenetic trees (MP and ML trees) that were constructed from the combined data set of the three gene fragments indicated that all six species of* Paramesotriton *formed a monophyletic group, with* P. caudopunctatus *as basal to the other five species. This result suggests that* P. fuzhongensis *is a valid species in* Paramesotriton.

KEY WORDS: phylogeny; mitochondrial DNA; *Paramesotriton*.

[1] Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, Yunnan, People's Republic of China.
[2] Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu, Shichuan, People's Republic of China.
[3] Graduate School of the Chinese Academy of Sciences, Beijing, People's Republic of China.
[4] These authors contributed to this paper equally.
[5] Guangxi Medical University, Nanning, Guangxi, People's Republic of China.
[6] Department of Biochemistry and Molecular Biology, University of Florida, Gainesville, Florida.
[7] Laboratory for Conservation and Utilization of Bio-Resource, Yunnan University, Kunming, People's Republic of China.
[8] To whom correspondence should be addressed at Laboratory for Conservation and Utilization of Bio-Resource, Yunnan University, Kunming 650091, People's Republic of China; e-mail: zhangyp@public.km.yn.cn.

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INTRODUCTION

Paramesotriton (warty newt) was established as a valid genus of the family Salamandridae by Chang (1935) based on cranial morphology. Six species are currently widely accepted (Zhao and Adler, 1993), including *P. caudopunctatus* (Hu *et al.*, 1973), *P. chinensis* (Freytag, 1962; Freytag and Petzld, 1961), *P. deloustali*, *P. fuzhongensis* (Wen, 1989), *P. guangxiensis* (Huang *et al*., 1983), and *P. hongkongensis* (Myers and Leviton, 1962); however, some consider *P. hongkongensis* to be a subspecies of *P. chinensis* (Fei, 1999), *P. fuzhongensis* a synonymy of *P. chinensis*, and *P. guangxiensis* a synonymy of *P. deloustali* (Pang *et al.*, 1992). Except for *P. deloustali*, which is restricted to northern Vietnam, the five remaining species are distributed in eastern and southern China.

Warty newts are somewhat understudied, and there is much work to be done in terms of classification and phylogeny. Since the 1980s, various studies associated with the taxonomy and classification of *Paramesotriton* have been done based on morphology (Freytag, 1983; Pang *et al.*, 1992; Thorn and Raffaelli, 2001; Zhao and Hu, 1984), ecology (Sparreboom, 1981), and molecular data (Chan *et al.*, 2001; Titus and Larson, 1995). Sparreboom (1981) suggested that the systematic status of *P. caudopunctatus* should be revised because of its prominent differences in male caudal morphology, egg deposition behavior, and unusual behavior under captive breeding. Based on the combined analyses of morphological characters and molecular data (cytochrome *b* gene), Chan *et al.* (2001) suggested *Paramesotriton* as a monophyletic group with *P. caudopunctatus* as the basal branch to the other three species (*P. guangxiensis*, *P. deloustali*, *P. hongkongensis*). However, the monophyly of *Paramesotriton* received weak statistical support. To date, the phylogenetic relationships among *Paramesotriton* species are poorly understood and the taxonomic uncertainty is especially true for *P. caudopunctatus* and *P. fuzhongensis*.

In this study, *Paramesotriton* samples from different localities were compared by analyzing the sequence data from the mitochondrial cytochrome *b* gene, the ND2 gene, and the ND4 gene using maximum parsimony (MP) and maximum likelihood (ML) methods. Here we discuss the possibility of the monophyly of the genus *Paramesotriton* and reconstruct the phylogenetic relationships of species in *Paramesotriton* as well as the taxonomic status of *P. caudopunctatus* and *P. fuzhongensis*.

MATERIALS AND METHODS

Samples

A total of 19 individuals of six recognized species of *Paramesotriton* was examined in this study. The number of samples and sampling localities are presented in Table I and Fig. 1. The tissues used in this study are deposited in the Kunming Institute

Table I. Samples of *Paramesotriton* and Outgroup Species Used in This Study

Species	Locality	No.	Voucher number
Cynops cyanurus	Kunming, Yunnan, China	2	SN02041, SN02042
Pachytriton labiatus	Ziyuan, Guangxi, China	2	SN02027, SN02028
Paramesotriton caudopunctatus	Fuchuan, Guangxi, China	4	SN02019, SN02020 SN02021, SN02022
Paramesotriton fuzhongensis	Fuchuan, Guangxi, China	4	SN02008, SN02009 SN02010, SN02011
Paramesotriton guangxiensis	Ningming, Guangxi, China	4	SN02050, SN02051 SN02052, SN02053
Paramesotriton deloustali	Lao Cai, Vietnam	1	ROM35433
Paramesotriton chinensis	Jinxiu, Guangxi, China	4	SN02060, SN02061 SN02062, SN02063
Paramesotriton hongkongensis	Hong Kong, China	2	20020481, 20020482

of Zoology and the Chengdu Institute of Biology at the Chinese Academy of Sciences.

DNA Extraction, Amplification, and Sequencing

Genomic DNA was extracted from muscle tissues that were preserved in 70% alcohol using the standard phenol–chloroform method. The partial sequences of

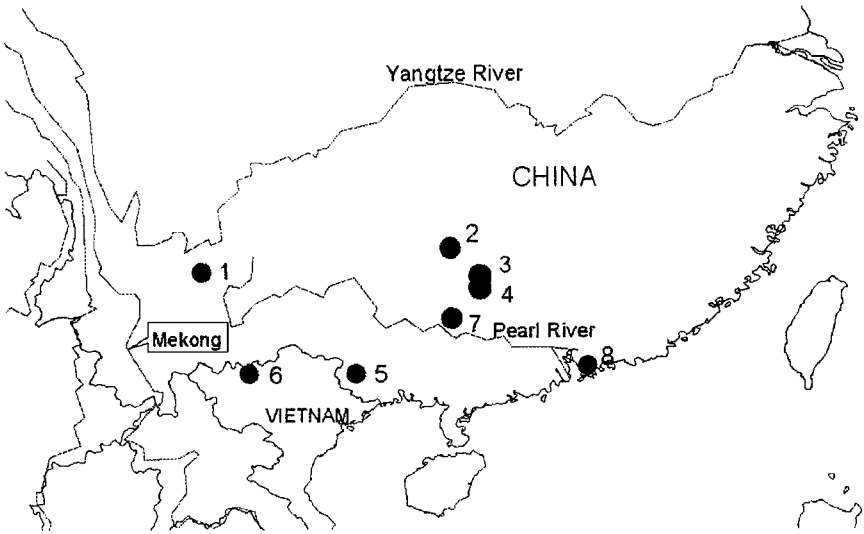

Fig. 1. The sampling sites of this study: 1. Kunming, Yunnan (*Cynops cyanurus*); 2. Ziyuan, Guangxi (*Pachytriton labiatus*); 3. Fuchuan, Guangxi (*Paramesotriton caudopunctatus*); 4. Fuchuan, Guangxi (*Paramesotriton fuzhongensis*); 5. Ningming, Guangxi (*Paramesotriton guangxiensis*); 6. Lao Cai, Vietnam (*Paramesotriton deloustali*); 7. Jinxiu, Guangxi (*Paramesotriton chinensis*); and 8. Hong Kong (*Paramesotriton hongkongensis*).

Table II. The Primer Pairs Used for Amplification and Sequencing

Region	Primer[a]	Primer sequence (5′-3′)
Cytochrome *b*	L14841	5′AAAAAGCTTCCATCCAACATCTCAGCATGAAA3′
	H15149	5′AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA3′
ND2	L4437	5′AAGCAGTTGGGCCCATRCC3′
	H4980	5′ATTTTTCGTAGTTGGGTTTGRTT3′
ND4	ND4F	AAAAACCTAAACCTACTACAATG
	ND4R	5′TGT TCC TGC GGT TAG TCG TT3′

[a] L and H refer to the light and heavy strands, respectively. Positions with mixed bases are labeled with the standard one-letter code: R = G or A.

cytochrome *b*, ND2, and ND4 were determined by three primer pairs: L14841-H15915 (Kocher *et al.*, 1989) for cytochrome *b*; L4437-H4980 (Macey *et al.*, 1997) for ND2; and ND4F-ND4R (designed for this study) for ND4 (Table II). PCR amplifications were performed in 25 μL volumes containing 60 ng genomic DNA, 2.5 μL 10$\times$ reaction buffer (Sino-American), 1.5 mM $MgCl_2$, 0.2 mM dNTPs (Amresco), 0.2 μM each primer, and 0.8 unit Taq polymerase. Amplification consisted of predenaturation at 95°C for 4 min followed by 35 cycles of denaturation for 50 s at 94°C, annealing for 1 min at 50°C, and extension for 40 s at 72°C. Amplification was terminated with a postextension of 10 min at 72°C. The profiles of PCR amplification for the three target genes were identical. PCR products were sequenced using a BigDye Terminator Kit (Perkin-Elmer) in both directions. Sequencing products were analyzed on an ABI 377 automated sequencer (Applied Biosystems, Costa Mesa, CA).

All sequence data were edited and aligned using the program DNASTAR (DNASTAR Inc.). The variant sites were rechecked by comparing the four-color electromorph of sequencing data against the computer results. Amino acid translations of the sequences were compared with those of *Xenopus laevis* (Roe *et al.*, 1985) to ensure that there were no frame shifts or premature stop codons. All sequences were deposited in Genbank and the accession numbers are AY327524, AY327525, and AY233134–AY233200.

Phylogenetic Analysis

Phylogenetic analyses were based on the partial sequences of the cytochrome *b*, ND2, and ND4 genes and the combined data set of these three gene fragments. The compatibility of the three gene fragments included in this study was examined using the partition homogeneity test (Farris *et al.*, 1995). Maximum parsimony (MP) and maximum likelihood (ML) methods were applied for inferring the phylogenetic relationships of *Paramesotriton*, which were reconstructed by using PAUP

4.0b8a (Swofford, 2001) with *Cynops cyanurus* and *Pachytriton labiatus* as the outgroup taxa.

Paramesotriton is closely related to the genera *Cynops* and *Pachytriton*, with which many similar characteristics are shared (Zhao and Hu, 1984), including breeding behavior, secondary sexual characteristics (during the breeding season), and habitat. The shared morphological and molecular characters constructed a well-supported polytomy of *Cynops*, *Paramesotriton*, and *Pachytriton*, forming a *Cynops–Pachytriton–Paramesotriton* monophyletic clade (Chan *et al.*, 2001; Titus and Larson, 1995). We selected *Cynops cyanurus* and *Pachytriton labiatus* as the outgroup taxa to increase the reliability of the phylogenetic reconstruction.

In the phylogenetic analysis, each nucleotide was treated as an unordered character with four alternative states, gaps were considered as missing data in both methods, and data were treated with equal weight (Allard and Carpenter, 1996). In the MP analysis, the sequence data from three genes were separately analyzed. Then a phylogenetic tree was generated based on the combined data set of three gene fragments with equally weighted parsimony. A heuristic search algorithm with TBR branch swapping, 100 random sequence addition replicates, and "Multrees" was performed. The bootstrap values were derived from 1000 replicates of the analysis (Farris *et al*., 1995; Felsenstein, 1985; Swofford, personal communication).

In the ML analysis, the combined data set of three gene fragments was used to reconstruct the phylogenetic relationship. Modeltest3.06 (Posada and Crandall, 1998) was used to select the substitution model. Likelihood ratio tests indicated that TrN+I was the most appropriate model for subsequent analyses. Settings for the TrN+I model were as follows: R-matix = (1.0000, 33.0145, 1.0000, 1.0000, 12.2453, 1.0000); base frequencies = (A = 0.3407, C = 0.2670, G = 0.1108, T = 0.2815); proportion of invariant sites = 0.6005. A heuristic ML search algorithm with 100 random additional sequences and TBR branch swapping was performed with the TrN+I model. The bootstrap values in the ML tree were derived from 500 replicates.

RESULTS

Partial mitochondrial DNA gene sequences (1207 bp) were successfully determined for the cytochrome *b* gene (307 bp), ND4 gene (401 bp), and ND2 gene (499 bp). No intraspecific sequence variation was found within the samples that were collected from the same locality. The partition-homogeneity test revealed that there was no significant incongruence among these three genes ($P = 0.189$). The MP analyses based on the cytochrome *b*, ND2, ND4, and the combined data sets showed that the ND2 gene had the most parsimony-informative sites, and the cytochrome *b* gene had the least (Table III). Both ND4 and cytochrome *b* gene

Table III. Comparison of Indices for the Phylogenetic Trees of the Genus *Paramesotriton* Based on ND2, ND4, Cytochrome *b*, and the Combined Data Sets

Comparison	ND2	ND4	cytochrome *b*	Combined data
Number of characters	499	401	307	1207
Number of variable characters	153	141	84	378
Number of parsimony-informative characters	74	72	45	191
Number of most parsimonious trees	1	2	1	1
Tree length	222	213	116	557
Consistency Index (CI)	0.757	0.761	0.759	0.750
Retention Index (RI)	0.550	0.553	0.659	0.560
Rescaled Consistency Index (RC)	0.416	0.420	0.500	0.420

Note. Tree length, CI, RI, and RC include uninformative characters.

data sets showed six *Paramesotriton* species as monophyletic, while the phylogenetic tree reconstructed using the ND2 gene fragment indicated that *Pachytriton labiatus* was nested within *Paramesotriton*, with *P. caudopunctatus* diverged from the other five *Paramesotriton* species.

When the fragments of these three genes were combined as a single data set, there were 378 variable sites and 191 parsimony-informative sites over 1207 nucleotides. The MP analysis generated a single most parsimonious tree with CI = 0.750, RI = 0.560, RC = 0.420, $L = 557$ (Fig. 2(a)). The ML analysis yielded one most likely tree with a score of ln = 3987.77442 (Fig. 2(b)). In both trees, all six species of *Paramesotriton* constructed a moderately supported monophyletic clade (MP tree, BP = 72; ML tree, BP = 84) with *P. caudopunctatus* as the basal branch. *P. fuzhongensis*, *P. deloustali*, and *P. guangxiensis* formed a well-supported monophyletic group with a very high bootstrap value (MP BP = 100, ML BP = 100). The slight difference was that in the MP tree, *P. chinensis* and *P. hongkongensis* are in successive basal branches, while in the ML tree, *P. chinensis* and *P. hongkongensis* were clustered together and then formed a monophyletic clade with the remaining species.

DISCUSSION

The Possibility of Monophyly of *Paramesotriton*

Paramesotriton species have rough skin, granular warts, prominent vertebral ridges, V-shape ridges on the back of the skull, and a lateral ridge along each side of the back (e.g., Chan *et al.*, 2001; Zhao and Adler, 1993; Zhao and Hu, 1984). Based on the combined study of molecular and morphological data, Chan *et al.* (2001) suggested the monophyly of *Paramesotriton*. But their monophyletic tree consisted

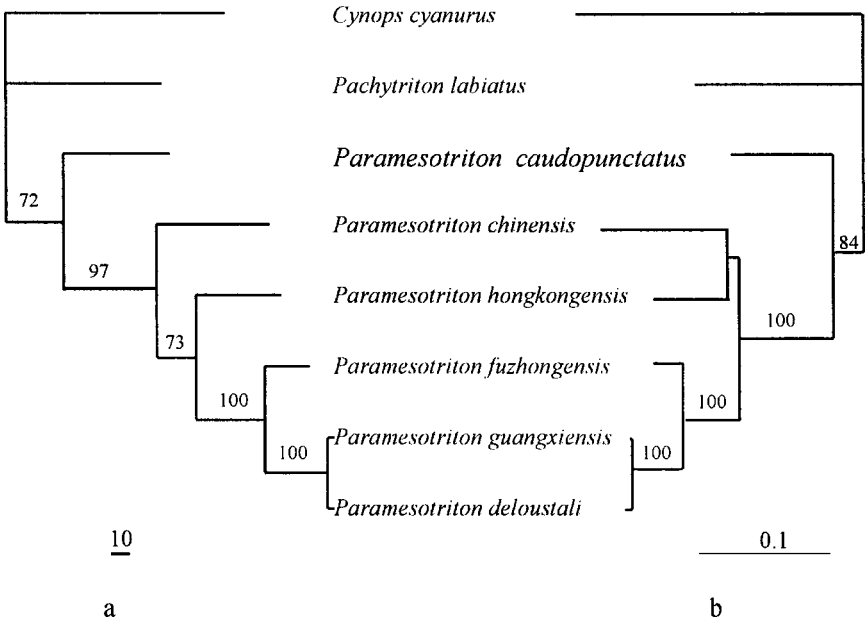

Fig. 2. (a) A single most parsimonious tree derived from the MP analysis based on the combined mitochondrial DNA sequence data set. The number above each branch is the bootstrap value (1000 replicates). (b) Maximum likelihood phylogram derived from the ML analysis. Numbers above branches are ML bootstraps (500 replicates).

of only four *Paramesotriton* species with relatively low statistical support. In the present study, the partial sequences determined from three mitochondrial genes (cytochrome *b*, ND2, and ND4) were used to reconstruct the phylogenetic relationships of six species of *Paramesotriton*. Phylogenetic analyses indicated that all six species formed a monophyletic group with high statistical support (ML BP = 84). However, the bootstrap value to support the branching order was only 72 in the MP tree. Owing to this moderate bootstrap value and the branching order differences between MP and ML trees, the monophyletic relationship of *Paramesotriton* was not resolved in this study, suggesting that much remains to be done.

Phylogeny of *Paramesotriton*

In early studies, differences in external characteristics and morphological structures in the species of *Paramesotriton* were used to study phylogenetic relationships (e.g., Huang *et al.*, 1983; Pang *et al.*, 1992; Wen, 1989). Based on morphological comparisons, Pang *et al.* (1992) suggested that *P. fuzhongensis* is a synonymy of *P. chinensis*, and *P. guangxiensis* is a synonymy of *P. deloustali*. The morphological

relationship of ((*P. guangxiensis, P. hongkongensis*), ((*P. fuzhongensis,P. chinensis*), *P.caudopunctatus*))) indicated that *P. caudopunctatus* diverged earlier than *P. fuzhongensis* and *P. chinensis*. However, considering its specialized external characteristics, Pang *et al*. (1992) inferred that *P. caudopunctatus* is the most basal species of *Paramesotriton.* Our results are consistent with their hypothesis. Except for *P. caudopunctatus*, the monolyphyletic relationship of the other five *Paramesotriton* species was well supported in our study, although it differed from the earlier studies (Pang *et al.*, 1992; Wen, 1989).

In *Paramesotriton*, *P. caudopunctatus* is a rather large species and has highly specialized external characters, such as a long and narrow skull, flared epibranchials of the hyobranchial apparatus, and unique ecological behaviors (Sparreboom, 1981). During the breeding season, adult males develop distinct spots along the lateral side of the tail. This characteristic is unique to *P. caudopunctatus* and positively distinguishes this species from the others. Because of its unique morphological characteristics, *P. caudopunctatus* was once identified as a species of the monotypic genus *Allomesotriton* (Freytag, 1983). By comparing the morphological characteristics of the skull, skeleton, and hyoid apparatus of warty newts, Pang *et al.* (1992) considered *Allomesotriton* to be a subgenus of *Paramesotriton*. Our results also indicate *P. cuadopunctatus* as the most basal species of this monophyletic group.

P. guangxiensis is another controversial species whose taxonomic status remains uncertain in *Paramesotriton*. Huang *et al.* (1983) elevated *P. guangxiensis* as a valid species based on the morphological comparison with *P. chinensis*. Others regarded it as a conspecific form of *P. deloustali* because of similar morphological characteristics (Pang *et al.*, 1992). Our molecular data also reveal a close phylogenetic relationship between *P. guangxiensis* and *P. deloustali*, which are clustered together with very high bootstrap values in MP and ML trees (BP = 100). To clarify their taxonomic positions, a comprehensive study based on morphological and molecular analyses is needed.

Taxonomic Status of *P. fuzhongensis*

P. fuzhongensis is robust, often with varying dorsal patterns and markedly warty skin. It can be distinguished from most other *Paramesotriton* species by its extraordinarily warty skin and the presence of a larger fleshy fold of skin near the base of the mouth (Wen, 1989). Since *P. fuzhongensis* and *P. chinensis* had no obvious differences in external characters and skeletal structures, Pang *et al.* (1992) considered *P. fuzhongensis* to be a synonymy of *P. chinensis*. These revisions were based mainly on shared primitive morphological features and resulted in greater confusion regarding the phylogeny of *Paramesotriton*. The phylogenetic relationship of ((*P. fuzhongensis*, (*P.guangxiensis, P. deloustali*)) is well supported in our study (Fig. 2). In the MP analysis, *P. chinensis* was the second basal species; while

in the ML analysis it is a sister group of *P. hongkongensis*. This study finds that *P. fuzhongensis* is a valid species of *Paramesotriton*.

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