Homoplastic evolution of external colouration in Asian stout newts (*Pachytriton*) inferred from molecular phylogeny

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The Asian stout newts of the genus *Pachytriton* (Salamandridae) inhabit montane streams in south-eastern China. Despite their abundance in the pet trade, the phylogeny and systematics of this genus are poorly understood. Colouration is often used to delimit species under the assumption that consistent chromatic differences characterize independent evolutionary lineages. We present the first phylogenetic study of *Pachytriton* that incorporates 2.35 kb of mitochondrial DNA (ND2, cyt*b*) and 1.2 kb of nuclear sequence data (RAG-1) along with morphometric characters to infer evolutionary relationships and patterns of colour evolution among the three described species: *Pachytriton brevipes*, *Pachytriton labiatus* and *Pachytriton archospotus*. Our results support the monophyly of *Pachytriton* and recover *P. archospotus* as the sister taxon to *P. brevipes*. Monophyly of *P. labiatus* is significantly rejected: south-western populations are sister to the group of *P. brevipes* plus *P. archospotus*, whereas north-eastern populations nest with *P. brevipes*. The two geographic units are further separated by multivariate morphological analyses. South-western *P. labiatus* is the type species; misidentification of north-eastern populations as *P. labiatus* results from their similar colouration. An unspotted, dark brown dorsum is the likely ancestral state for the genus, whereas black-spotted colouration characterized the common ancestor of *P. brevipes*, *P. archospotus*, and north-eastern *P. labiatus* and was secondarily lost in the latter group. Homoplastic evolution and intraspecific variation render colour pattern in *Pachytriton* an unreliable character for delimiting species boundaries. North-eastern populations of *P. labiatus* are declining as the result of human collection and habitat destruction and are in urgent need of conservation protection.

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Introduction

The booming utilization of molecular techniques and associated analytical tools has facilitated reassessment of evolutionary relationships that previously were inferred mainly by analysis of phenotypic variation. Phylogenies derived from molecular data may confirm, supplement and sometimes reject traditional taxonomic assessments. The latter instance often yields new evolutionary hypotheses to explain the incongruence between molecular data and other phenotypic characters (e.g. Schaefer *et al.* 2002; García-París *et al.* 2003; Babik *et al.* 2005). In vertebrate phylogenetics, mitochondrial DNA (mtDNA) is a frequently used marker for molecular analysis because it generally evolves faster than nuclear genes (Ballard & Whitlock 2004). The typically one-fourth coalescent time of mtDNA, in comparison with autosomal nuclear genes, offers an advantage for studies of closely related species in which lineage sorting of ancestral gene copies may

confound the gene tree with the true species tree (Moore 1995). However, in most cases, mitochondrial genes are all linked as a single molecule, which provides only one independent estimate of the phylogeny and such results may be misleading if there has been mitochondrial introgression. As advocated by integrative taxonomists, the combination of multiple lines of evidence, including independent molecular markers, morphology, behaviour, ecology, etc., yields the most informative and insightful evaluation of evolutionary relationships among species (Padial *et al.* 2009).

The Asian stout newts of the genus *Pachytriton* are popular in the global pet trade. In nature, they inhabit tiny shallow montane streams across south-eastern China, a large geographical area that is characterized by several mountain ranges that approach 2500 m in elevation. These mountains were uplifted by folding and deformation of the Earth's crust in polyphasic inherited orogenies from the Late Permian through the Cenozoic (Guo 1998). Between adjacent mountain ranges lie lower plains and large river systems, which constitute zoogeographical barriers to montane salamanders (Fu & Zeng 2008; Shepard & Burbrink 2008). Additionally, most areas of East Asia were not glaciated during the Pleistocene (Williams *et al.* 1998). In situations such as this, we can expect that the combination of complex topography and long-term persistence of populations on isolated mountain peaks may promote significant levels of interspecific and intraspecific genetic differentiation, as well as geographically based phenotypic divergence.

For a long time, *Pachytriton* was believed to comprise just two species: *Pachytriton brevipes* Sauvage, the black-spotted stout newt, and *Pachytriton labiatus* Unterstein, the unspotted stout newt. As their common names imply, these two species are distinguished mainly by external colour pattern. Recently, a third species, *Pachytriton archospotus* Shen *et al.*, was described from a population previously regarded as *P. brevipes*, which it resembles in colouration. And while phylogenetic relationships of *Pachytriton* with other Asian salamandrids are well resolved, the genus forms a well-supported, monophyletic group with *Paramesotriton* and *Cynops* (Titus & Larson 1995; Chan *et al.* 2001; Weisrock *et al.* 2006; Steinfartz *et al.* 2007); phylogenetic relationships within *Pachytriton* have not been assessed in detail, largely because of the difficulty of obtaining specimens with verified locality data. Interestingly, only two previous molecular studies analysed more than one specimen of *Pachytriton*, yet each study revealed substantial genetic divergence in the genus. In Chan *et al.* (2001), one commercially obtained *P. labiatus* was so different from two other conspecific specimens in cytochrome *b* (cyt*b*) sequences that Chan *et al.* concluded that *P. labiatus* could comprise more than one species. Another extensive work on Salamandridae by Weisrock *et al.* (2006) revealed the possible paraphyly of *P. brevipes*, with one specimen of this species grouping with *P. labiatus* rather than with other *P. brevipes*.

In this article, we present the first phylogenetic study of the genus *Pachytriton*, based on complete mitochondrial gene sequences that code for cty*b* and subunit two of NADH dehydrogenase (ND2), as well as partial nuclear-coding sequences from recombination activating gene 1 (RAG-1). We reconstruct a resolved phylogeny of samples from populations of *P. labiatus*, *P. brevipes* and *P. archospotus*. The current distribution of *P. labiatus* comprises two allopatric areas separated by a gap of at least 600 km (Fei *et al.* 2006). We assess the evolutionary relationship between populations of these two areas from molecular phylogenetic and additional morphometric data. On the basis of inferred phylogeny, we discuss how different colour patterns may have evolved among the three named *Pachytriton* species. Finally, we highlight the need for conservation efforts to end overexploitation and habitat destruction, which are the most serious factors leading to amphibian declines in east Asia (Collins & Storfer 2003; Stuart *et al.* 2004).

Materials and methods

Taxon sampling

We sampled three populations of *P. brevipes*, three *P. labiatus* populations from the north-eastern distribution (NE), three *P. labiatus* populations from the south-western distribution (SW) and one population of *P. archospotus* (Fig. 1; Table 1). Type localities of all three species were included to ensure correct phylogenetic inference. We also included five market-obtained *P. labiatus* to identify the source of commercial exploitation. Specimens were sampled from pet stores in Chengdu during May 2007 and in Hangzhou during August 2007. These five specimens are morphologically similar to *P. labiatus* from Mt. Tiantai in Zhejiang Province, which are characterized by red dorsolateral stripes (one on each side). Fresh liver or muscle was dissected from euthanized animals and preserved in 100% ethanol, then stored at 4 °C before final transfer to permanent storage at −80 °C. *Paramesotriton* was chosen as a phylogenetic outgroup based on its known sister relationship to *Pachytriton* (Chan *et al.* 2001; Weisrock *et al.* 2006; Steinfartz *et al.* 2007).

Extraction, amplification and sequencing

Genomic DNA was extracted from preserved liver or muscle tissue using a QIAGEN DNeasy blood and tissue kit (Valencia, CA, USA) following the manufacturer's protocol. Amplifications of mitochondrial fragments were

Fig. 1 Distribution of *Pachytriton brevipes* (lower right, solid line), *Pachytriton labiatus* (lower left and upper right, long-dashed line) and *Pachytriton archospotus* (upper left, short-dashed line) in southeastern China, with representative animals collected from the corresponding ranges (Zhao & Hu 1984; Fei *et al*. 2006; Shen *et al*. 2008). *Pachytriton labiatus* has a disjunct distribution. Solid circles denote sampled localities.

conducted under the condition of initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 45 s, extension at 72 °C for 90 s and a final extension at 72 °C for 5 min. Cycle annealing temperature was raised to 56 °C and final extension was increased to 7 min for the nuclear RAG-1 gene, with other conditions unchanged. Negative controls were added to detect contamination. Amplified products were examined on 1% agarose gels and successively purified with a QIAquick PCR purification kit (Valencia, CA, USA) and then sequenced on an ABI 3730 (DF/HCC DNA Resource Core, MA, USA). All regions were sequenced in both directions. PCR primers (Table S1) were designed specifically to *Pachytriton* and *Paramesotriton* for successful amplification and sequencing. Internal primers were applied to improve sequence quality. Sequences are deposited in GenBank (Table 1).

Phylogenetic analysis

Two mitochondrial sequences were amplified: 1181–1182 base pairs (bp) for the complete ND2 and flanking tRNAs (hereafter, 'ND2'); and 1172 bp for the complete cyt*b* and flanking tRNAs (cyt*b*). The nuclear fragment covered 1208 bp at the 3′ end of the RAG-1 gene. Sequences were imported into SE-AL 2.0 (Rambaut 1995) and aligned manually. Codon positions were determined according to reference sequences from the *Paramesotriton hongkongensis* mitochondrial genome (Zhang *et al*. 2005) and the complete RAG-1 coding sequence from *Pleurodeles waltl* (Fripiat *et al*. 2001). To assess the extent of substitution saturation, uncorrected pairwise distances (*p*-distance) were plotted against K2p-corrected distances (Kimura two-parameter model; Kimura 1980) for both transitions and transversions at each codon position in each gene. If a corrected distance is excessively larger than the corresponding uncorrected distance, then more than one substitution has occurred at some sites, thus increasing the chance of homoplasy. Saturation was further evaluated by Xia *et al*.'s (2003) saturation index in the program DAMBE (Xia & Xie 2001). Divergences among populations of three *Pachytriton* species are represented by net uncorrected pairwise distances in MEGA 4 (Tamura *et al*. 2007). To assess phylogenetic congruence between genes, Partition Homogeneity Tests (Farris *et al*. 1995) implemented in PAUP 4.0b10 (Swofford 2002) were conducted for 100 replicates.

Phylogenetic topologies were reconstructed under the maximum likelihood (ML) criterion. The best-fit evolutionary model was determined by Akaike Information Criterion (AIC) implemented in MODELTEST 3.7 (Posada & Crandall 1998). The resultant model parameters were input into GARLI v.0.951 (Zwickl 2006). The search for the ML tree was terminated when the likelihood score had not been improved for 500 000 generations. Bootstrap values were calculated for 100 replicates with the termination generation reducing to 100 000. For the maximum parsimony (MP) analysis, heuristic searches were performed for 1000 replicates of random sequence additions with tree bisection–reconnection (TBR) as the branch-swapping algorithm in PAUP 4.0b10. Gaps were treated as missing data and ambiguous nucleotides were interpreted as uncertainty. Nonparametric bootstrap values were calculated for 1000 replicates, each of which included 100 replicates of

Table 1 Taxon sampling and specimens used in this study.

Sampling locality	Code in phylogenetic analysis	Musuem voucher	GeneBank Accession no.		
			ND2	cyt*b*	RAG-1
Pachytriton labiatus					
Mt. Dayao, Jin Xiu, Guangxi Province	Mt. Dayao Guangxi	CIB88165	GQ303602	GQ303639	GQ303680
Mt. Mao'er, Xing'an, Guangxi Province	Mt. Mao'er Guangxi1	CIB88182	GQ303594	GQ303631	GQ303672
Mt. Mao'er, Xing'an, Guangxi Province	Mt. Mao'er Guangxi2	CIB88173	GQ303595	GQ303632	GQ303673
Mt. Mao'er, Xing'an, Guangxi Province	Mt. Mao'er Guangxi3	CIB88158	GQ303596	GQ303633	GQ303674
Mt. Mao'er, Xing'an, Guangxi Province	Mt. Mao'er Guangxi4	CIB88156	GQ303597	GQ303634	GQ303675
Mt. Leigong, Leishan, Guizhou Province	Mt. Leigong Guizhou1	CIB88162	GQ303598	GQ303635	GQ303676
Mt. Leigong, Leishan, Guizhou Province	Mt. Leigong Guizhou2	CIB88148	GQ303600	GQ303637	GQ303678
Mt. Leigong, Leishan, Guizhou Province	Mt. Leigong Guizhou3	CIB88170	GQ303601	GQ303638	GQ303679
Mt. Leigong, Leishan, Guizhou Province	Mt. Leigong Guizhou4	CIB88147	GQ303599	GQ303636	GQ303677
Mt. Xi'tianmu, Lin'an, Zhejiang Province	Mt. Xi'tianmu Zhejiang1	CIB88145	GQ303606	GQ303643	GQ303684
Mt. Xi'tianmu, Lin'an, Zhejiang Province	Mt. Xi'tianmu Zhejiang2	CIB88137	GQ303607	GQ303644	GQ303685
Mt. Xi'tianmu, Lin'an, Zhejiang Province	Mt. Xi'tianmu Zhejiang3	CIB88152	GQ303608	GQ303645	GQ303686
Mt. Xi'tianmu, Lin'an, Zhejiang Province	Mt. Xi'tianmu Zhejiang4	CIB88139	GQ303609	GQ303646	GQ303687
Mt. Tiantai, Ninghai, Zhejiang Province	Mt. Tiantai Zhejiang1	CIB88143	GQ303610	GQ303647	GQ303688
Mt. Tiantai, Ninghai, Zhejiang Province	Mt. Tiantai Zhejiang2	CIB88161	GQ303611	GQ303648	GQ303689
Mt. Tiantai, Ninghai, Zhejiang Province	Mt. Tiantai Zhejiang3	CIB88169	GQ303612	GQ303649	GQ303690
Mt. Dapan, Jinhua, Zhejiang Province	Mt. Dapan Zhejiang1	CIB95997	GQ303624	GQ303661	GQ303702
Mt. Dapan, Jinhua, Zhejiang Province	Mt. Dapan Zhejiang2	CIB95996	GQ303625	GQ303662	GQ303703
Pet Market, Chengdu, Sichuan Province	Pet Trade Chengdu1	CIB88138	GQ303603	GQ303640	GQ303681
Pet Market, Chengdu, Sichuan Province	Pet Trade Chengdu2	CIB88181	GQ303604	GQ303641	GQ303682
Pet Market, Chengdu, Sichuan Province	Pet Trade Chengdu3	CIB88135	GQ303605	GQ303642	GQ303683
Pet Market, Hangzhou, Zhejiang Province	Pet Trade Hangzhou1	CIB88142	GQ303613	GQ303650	GQ303691
Pet Market, Hangzhou, Zhejiang Province	Pet Trade Hangzhou2	CIB88168	GQ303614	GQ303651	GQ303692
Pachytriton brevipes					
Mt. Junfeng, Nanfeng, Jiangxi Province	Mt. Junfeng Jiangxi1	CIB95926	GQ303626	GQ303663	GQ303704
Mt. Junfeng, Nanfeng, Jiangxi Province	Mt. Junfeng Jiangxi2	CIB95930	GQ303627	GQ303664	GQ303705
Mt. Wuyi, Wuyi Shan, Fujian Province	Mt. Wuyi Fujian1	CIB88221	GQ303615	GQ303652	GQ303693
Mt. Wuyi, Wuyi Shan, Fujian Province	Mt. Wuyi Fujian2	CIB88194	GQ303616	GQ303653	GQ303694
Mt. Wuyi, Wuyi Shan, Fujian Province	Mt. Wuyi Fujian3	CIB88188	GQ303617	GQ303654	GQ303695
Mt. Wuyi, Wuyi Shan, Fujian Province	Mt. Wuyi Fujian4	CIB88197	GQ303618	GQ303655	GQ303696
Mt. Wuyi, Wuyi Shan, Fujian Province	Mt. Wuyi Fujian5	CIB88192	GQ303619	GQ303656	GQ303697
Mt. Daiyun, Youxi, Fujian Province	Mt. Daiyun Fujiang1	CIB88207	GQ303620	GQ303657	GQ303698
Mt. Daiyun, Youxi, Fujian Province	Mt. Daiyun Fujiang2	CIB88189	GQ303621	GQ303658	GQ303699
Mt. Daiyun, Youxi, Fujian Province	Mt. Daiyun Fujiang3	CIB88190	GQ303623	GQ303660	GQ303701
Mt. Daiyun, Youxi, Fujian Province	Mt. Daiyun Fujiang4	CIB88185	GQ303622	GQ303659	GQ303700
Pachytriton archospotus					
Mt. Qiyun, Guidong, Hunan Province	Mt. Qiyun Hunan1	CIB95953	GQ303628	GQ303665	GQ303706
Mt. Qiyun, Guidong, Hunan Province	Mt. Qiyun Hunan2	CIB95950	GQ303629	GQ303666	GQ303707
Mt. Qiyun, Guidong, Hunan Province	Mt. Qiyun Hunan3	CIB95949	GQ303630	GQ303667	GQ303708
Paramesotriton ermizhaoi					
Mt. Dayao, Jin Xiu, Guangxi Province	*Paramesotriton ermizhaoi*1	CIB88141	FJ744601	GQ303670	GQ303711
Mt. Dayao, Jin Xiu, Guangxi Province	*Paramesotriton ermizhaoi*2	CIB88140	FJ744602	GQ303671	GQ303712
Paramesotriton deloustali					
Vinh Yen District, Tam Dao, Vinh Phu Province, Vietnam	*Paramesotriton deloustali*1	MVZ223628	FJ744599	GQ303668	GQ303709
Vinh Yen District, Tam Dao, Vinh Phu Province, Vietnam	*Paramesotriton deloustali*2	MVZ223629	FJ744600	GQ303669	GQ303710

Sequences will be deposited in GenBank upon acceptance.

random sequence additions. Bayesian inference (BI) was performed in MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001). We partitioned the mitochondrial data according to gene functions and codon positions, following the suggestion from Zhang *et al.* (2008) based on a mitogenomic study of Salamandridae. Nuclear RAG-1 data were not partitioned for its low level of variation. The best-fit evolutionary model was selected by AIC implemented in MrModeltest 2.2 (Nylander 2004). Metropolis-coupled Markov Chain Monte Carlo analyses were carried out simultaneously in two independent runs with default heating for two million generations. Convergence between runs is determined by the average standard deviation of split frequencies. The first one-fourth of the runs

was discarded as burn-in. A 50% majority-rule consensus tree was summarized over all postburn-in phylogenies.

To evaluate competing phylogenetic hypotheses, alternative phylogenies of highest likelihood were reconstructed under topological constraints in GARLI v.0.951 with the same evolutionary model used in the ML analysis. These topologies were then loaded into PAUP 4.0b10 and statistically compared with the ML tree by using a Templeton (1983) test, a one-tailed KH test (Kishino & Hasegawa 1989), and a one-tailed SH test (Shimodaira & Hasegawa 1999). Tests were performed for both mitochondrial and nuclear genes. The KH and SH tests were performed with 100 bootstrap replicates under full optimization. To trace the evolutionary history of colour pattern changes in *Pachytriton*, we coded the observed patterns of sampled populations into binary characters and reconstructed the putative ancestral character state for major nodes on the inferred phylogenetic tree using both parsimony and likelihood criteria in MESQUITE (Maddison & Maddison 2006). We further employed a statistical approach using Bayesian mutational mappings in the ancestral state reconstruction to accommodate uncertainty in the phylogeny by sampling Bayesian trees and associated parameters in proportion to their posterior probabilities (PP) (Bollback 2006). The posterior distributions of ancestral states are calculated from 20 sets of substitution rate priors drawn from the prior distribution for 20 000 postburn-in phylogenies from MRBAYES. By setting the bias prior on two-state character to the bell-shaped beta distribution ($\alpha = 5$), we assumed that the prior probability for each state is close to 0.5. This analysis was carried out using the program SIMMAP (Bollback 2006).

Morphometric analysis

Fifteen linear measurements were taken from specimens from the Chengdu Institute of Biology, Chinese Academy of Sciences: total length, snout-to-vent length, head length, head width, head depth, snout-to-axilla length, snout length, interocular distance, internostril distance, shoulder width, tail length, tail depth, tail width, forelimb length and hind limb length. Approximately 15 sexually mature specimens were measured per population. These populations correspond to those used in molecular analyses and are available from museum collections (Table S3). Animals obtained from the pet trade were also measured. Each linear measurement was taken multiple times using a digital caliper to minimize error. To reduce dominance of a few variables, such as total length (Manly 2004), measurements were normalized prior to principal-components (PC) analysis (SPSS ver. 13, Chicago, IL, USA). PC scores were plotted in orthogonal space. We conducted Levene's (1960) to assess the equality of variances of PC scores among different populations. One-factor analysis of variance (ANOVA) was performed to test if populations are significantly different in PC scores and individual measurements. When variances were heterogeneous among PC scores, Welch's (1938) test, which assumes unequal variance, was used instead. Additionally, we applied a multivariate analysis of variance (MANOVA) to determine if sexual dimorphism contributes to differences among populations.

Results

Sequence composition

No length variation was observed in either mitochondrial or nuclear protein-coding sequences. Only three single-base indels were detected in the mitochondrial *tRNA-Trp* gene. When K2p-corrected distances were plotted against uncorrected *p*-distances for the mtDNA and RAG-1 fragments, neither transitions nor transversions showed evidence of excessive multiple substitutions, although the third mitochondrial codon position suggested some overprinted substitutions (results not shown). Saturation at the third codon position was significantly rejected by Xia *et al.*'s. (2003) saturation index (ND2: $P_{\text{symmetrical}} = 0.000$ and $P_{\text{asymmetrical}} = 0.000$; cyt*b*: $P_{\text{symmetrical}} = 0.000$ and $P_{\text{asymmetrical}} = 0.000$). Furthermore, downweighting the third codon position did not alter the topology of phylogenetic reconstructions. Both ND2 and cyt*b* exhibited a compositional bias against guanines (12.5% and 14.8% respectively), while RAG-1 had a relatively even base composition.

Mitochondrial genes presented high levels of interspecific divergence among the three *Pachytriton* species (Table 2). South-western *P. labiatus*, which included a population sample from its type locality, diverged from *P. brevipes* and *P. archospotus* by more than 12% and 10.8% in ND2 and 7.4% and 8% in cyt*b* respectively. The latter two species were separated by moderate genetic distances (~7%). Surprisingly, *P. labiatus* from the two allopatric ranges exhibited remarkably large genetic differences (12–13.4% in ND2 and 7.4–8% in cyt*b*), similar to the observed levels of interspecific divergence. Among-population variation was also high within south-western *P. labiatus* (2.1–4.7%), within north-eastern *P. labiatus* (0.4–4.5%), and within *P. brevipes* (0.9–7%). In contrast, little within-population variation was observed. For RAG-1 sequences, the average net uncorrected sequence divergence was very limited both among and within *Pachytriton* populations (<0.9%).

Phylogenetic inference

For ingroup taxa, there were 266 (22.5%) and 206 (17.6%) parsimony-informative sites in the ND2 (1183 bp) and cyt*b* (1172 bp) alignments respectively. Of

Table 2 Net average uncorrected *p*-distance (in percentage) between and within samples. Lower diagonal: between-group *p*-distance of ND2; upper diagonal: between-group *p*-distance of cyt*b*. On diagonal before slash: within-group *p*-distance of ND2; after slash: within-group *p*-distance of cyt*b*.

		Mt. Mao'er	Mt. Leigong	Mt. Dayao (T)	Mt. Xi'tianmu	Mt. Tiantai	Mt. Dapan	Mt. Wuyi (T)	Mt. Daiyun	Mt. Junfeng	Mt. Qiyun (T)
Pachytriton labiatus (SW)	Mt. Maoer	0.1/0.1	2.1	2.8	7.6	7.9	7.8	8.7	8.1	8.3	8.5
	Mt. Leigong	2.8	0.3/0.5	2.7	7.4	7.6	7.5	8.5	7.9	8.1	8.1
	Mt. Dayao (T)	4.7	4.3	N/A*	7.5	8.0	8.0	8.3	8.1	8.4	8.0
Pachytriton labiatus (NE)	Mt. Xitianmu	12.5	12.1	12.0	0.1/0.1	2.5	2.4	6.4	2.2	5.9	6.4
	Mt. Tiantai	12.8	12.4	12.1	3.8	1/0.7	0.4	6.2	2.8	5.7	7.3
	Mt. Dapan	13.3	13.2	13.0	4.5	0.8	0.2/0.3	6.1	2.8	5.6	7.2
Pachytriton brevipes	Mt. Wuyi	12.1	12.2	12.5	7.3	7.9	8.3	0.2/0.2	6.6	0.9	6.8
	Mt. Daiyun	12.7	12.0	12.0	3.1	3.7	4.5	7.0	0.0/0.0	6.2	6.7
	Mt. Junfeng (T)	12.0	12.3	12.8	7.2	7.9	8.0	1.1	6.9	0.3/0.6	7.0
Pachytriton archospotus	Mt. Qiyun (T)	11.1	10.8	11.0	7.4	8.3	8.5	7.4	7.5	7.3	1.5/1.2

T, type locality.

*Only one specimen is available from Mt. Dayao.

1208 characters in the nuclear RAG-1 sequences, only 37 were variable and 21 (1.7%) were parsimony-informative. Results from the Partition Homogeneity Test suggested that mitochondrial ND2 and cyt*b* genes were congruent with each other ($P = 0.36$). However, significant incongruence was revealed among ND2, cyt*b* and RAG-1 ($P = 0.01$). Removing the third codon position in mitochondrial genes did not result in a homogeneous data set ($P = 0.01$). Therefore, we concatenated the two mitochondrial genes (total 2355 bp) in the phylogenetic inference but analysed RAG-1 separately.

The best-fit substitution model was selected as TIM with a proportion of 0.6338 invariable sites for the mitochondrial data in the ML analysis. Nucleotide frequencies were estimated as follows: $A = 0.3344$, $C = 0.2601$, $G = 0.1225$ and $T = 0.2829$. The ML tree resolved three major clades in *Pachytriton* with moderate-to-strong support (Fig. 2). Clade A corresponded to south-western populations of *P. labiatus*, which diverged early from other species. Clade B represented the newly described *P. archospotus*. The third lineage (Clade C), which was sister to Clade B, presented an unexpected grouping of north-eastern *P. labiatus* with *P. brevipes*. Monophyly of north-eastern *P. labiatus* was not supported statistically. Specimens from the pet trade formed a less-resolved group with north-eastern *P. labiatus* from Mt. Tiantai and Mt. Dapan. Animals collected at the same locality were either monophyletic or nested with nearby populations. BI recovers an identical topology under the GTR+I model for each data partition. Posterior probabilities were high for most major groups, but low again regarding the monophyly of north-eastern *P. labiatus*. The MP heuristic search against mtDNA found 40 equally parsimonious trees with 1113 steps (CI = 0.6739, RI = 0.9202). The topology of the strict consensus tree resembled that of the ML tree, and relationships between north-eastern *P. labiatus* and *P. brevipes* from Mt. Daiyun were unresolved.

The substitution model of TVM (transversional model) with a proportion of 0.9042 invariable sites was selected for RAG-1 sequences. The ML analysis produced a less-resolved phylogeny than the mtDNA-derived tree. Of the outgroups, *Paramesotriton ermizhaoi* (Wu *et al*. 2009) had a long-branch connection to the ingroup taxa (result not shown); the same pattern occurred in the mtDNA ML tree (Fig. 2). Long branches reduce the accuracy of outgroup rooting (Graham *et al*. 2002), especially when sequence variation is low, because substitutions on long outgroup branches may converge with those on ingroup branches, resulting in spurious rooting. Therefore, we re-ran the analysis without *Paramesotriton ermizhaoi* and rooted the RAG-1 phylogenies using only two *Paramesotriton deloustali* (Fig. 3). The RAG-1 ML tree recognized two main clades: one included south-western *P. labiatus* and *P. archospotus*; the other grouped north-eastern *P. labiatus* with *P. brevipes*. MP and Bayesian analyses produced similar topologies, although most nodes had low statistical support.

Alternative phylogenetic topologies were compared statistically with the ML trees under both parsimony and likelihood criteria. A monophyletic *P. labiatus* as currently recognized was significantly rejected by the Templeton test, the one-tailed KH test and the one-tailed SH test for both mtDNA and RAG-1 data (each $P < 0.005$). As a monophyletic north-eastern *P. labiatus* was not supported in any of the phylogenetic analyses (Figs 2 and 3), we also tested the non-monophyly of north-eastern *P. labiatus*. Under this topological constraint, the highest-likelihood phylogeny placed the Mt. Xi'tianmu population basal to *P. brevipes* from Mt. Daiyun plus all other north-eastern *P. labiatus*. Topological tests were unable to reject this

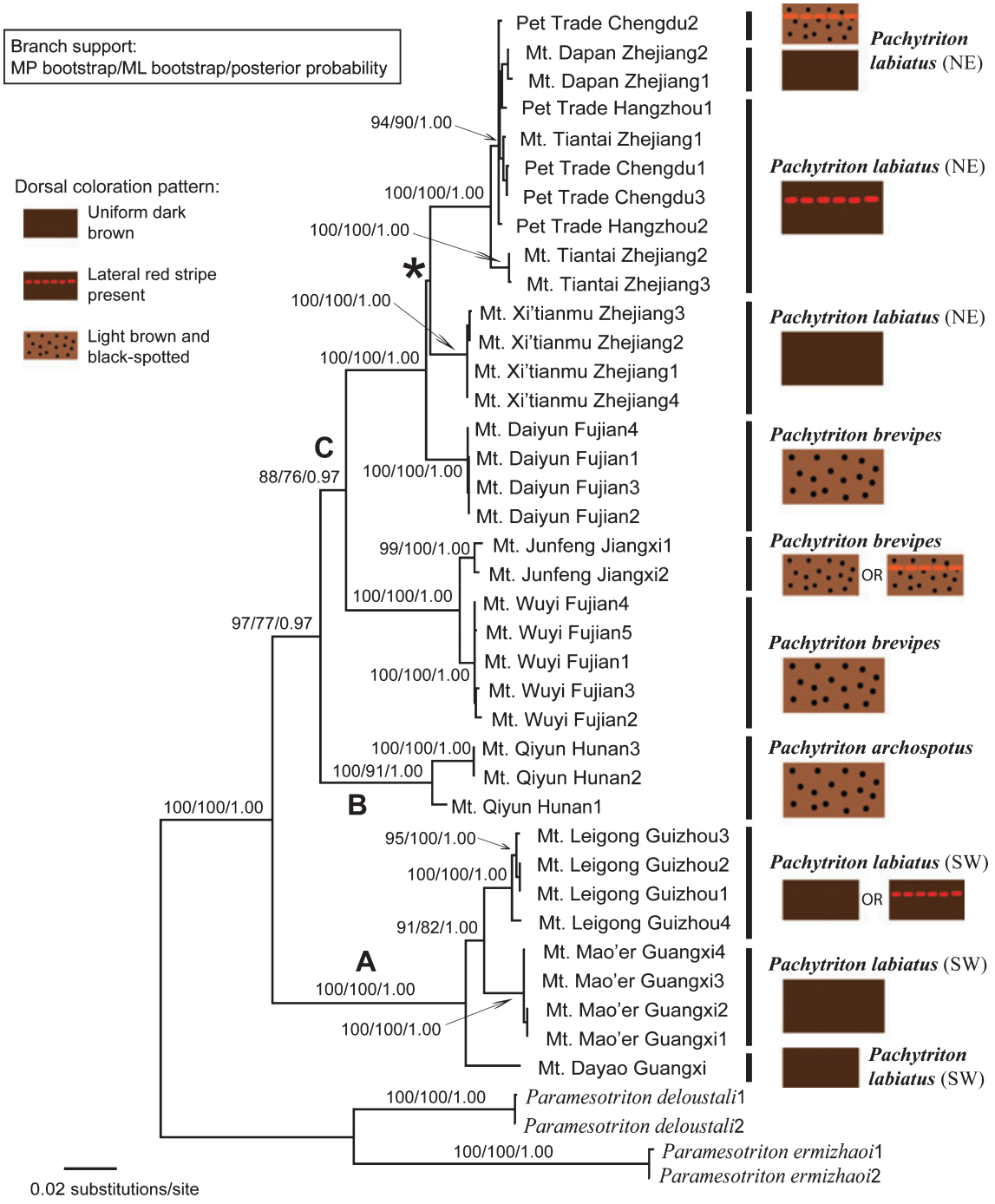

Fig. 2 Maximum-likelihood tree of the genus *Pachytriton* based on combined mitochondrial ND2 and cyt*b* sequence data with bootstrap values and posterior probabilities. Maximum parsimony and Bayesian inference produced very similar topologies. Three principal clades are evident (A, B, C). Dorsal color patterns are mapped to each locality; two localities have two patterns each. The asterisk denotes low statistical support for that node. SW, south-western *Pachytriton labiatus*; NE, north-eastern *P. labiatus*.

phylogeny (each $P > 0.05$). Reconstruction of the ancestral colour pattern within *Pachytriton* suggested that an unspotted, dark-brown dorsum was the likely ancestral state for the genus ($PP_{unspotted} = 0.75$, $PP_{black\text{-}spotted} = 0.25$), which was retained in south-western *P. labiatus* ($PP_{unspotted} = 1.00$, $PP_{black\text{-}spotted} = 0.00$). Black spots possi-

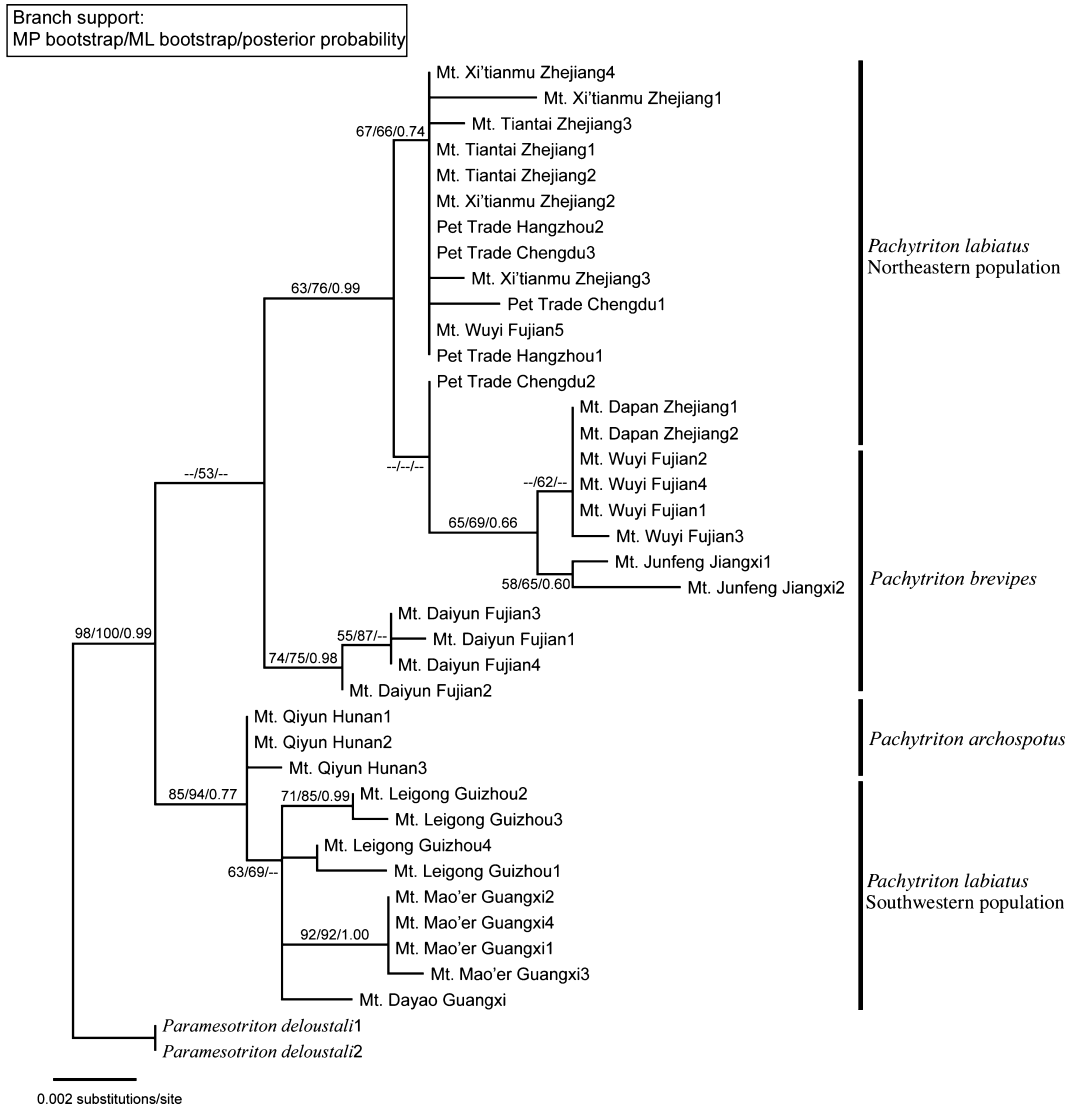

Fig. 3 Maximum-likelihood tree of the genus *Pachytriton* based on RAG-1 sequences. Levels of statistical support are indicated above or below branches. Double hyphens denote nodes with bootstrap values or posterior probabilities lower than 50 or 0.5. The inferred phylogeny fails to identify the three named *Pachytriton* species as monophyletic clades. The tree is rooted by *Paramesotriton deloustali*.

bly evolved in the most recent common ancestor of *P. archospotus*, *P. brevipes* and north-eastern *P. labiatus* ($PP_{unspotted}$ = 0.21, $PP_{black\text{-}spotted}$ = 0.79), and was retained in the common ancestor of *P. brevipes* and north-eastern *P. labiatus* ($PP_{unspotted}$ = 0.19, $PP_{black\text{-}spotted}$ = 0.81). Bright dorsolateral flecks, which could be found in various groups (Fig. 2), were reconstructed as independent acquisitions under parsimony, likelihood and Bayesian criteria.

Morphometric variation

The first three PC explained 90% of the total variance among the fifteen variables. These components represented overall body size, body shape (i.e. length vs. girth) and relative head size respectively (Fig. 4). South-western and north-eastern *P. labiatus* were significantly different in PC1 and PC3 scores in one-factor ANOVA and Welch's tests (each $P < 0.005$), which indicated that south-western *P. labiatus* have a significantly larger body size and relatively larger head than north-eastern *P. labiatus*. *Pachytriton brevipes* were intermediate, but still significantly different from the two groups of *P. labiatus*. North-eastern *P. labiatus* was the most slender *Pachytriton* of all. It had the largest ratio of forelimb length to total length ($P = 0.001$) and the smallest ratio of head width to total length ($P < 0.001$). MANOVA suggested no detectable sexual dimorphism among the pooled specimens (Wilks' Lambda $P = 0.306$), and measurement differ-

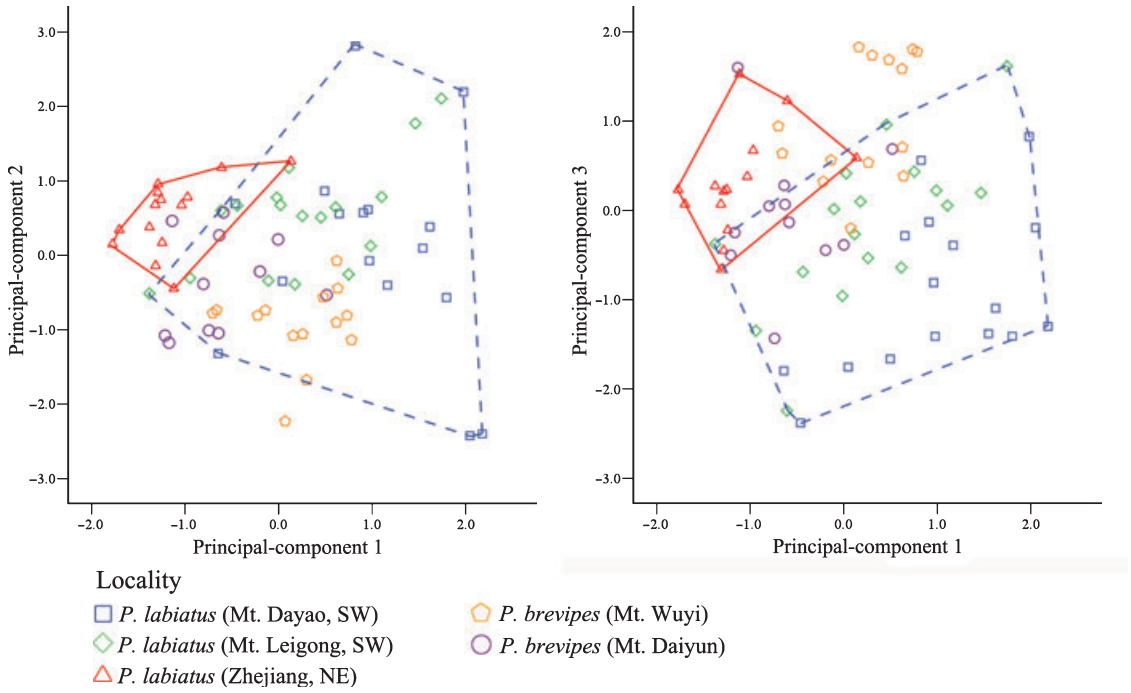

Fig. 4 Principal-components analysis of morphometric measurements of five populations of *Pachytriton*. South-western *Pachytriton labiatus* (bounded by dashed line) are separated from north-eastern *P. labiatus* (solid line) in the morphospace. Eigenvectors are listed in Table S2. *Pachytriton archospotus* is not available from museum collections.

ences between sexes did not contribute significantly to the separation of populations in the PC morphospace. Box's (1949) *M*-test rejected the equality of covariance matrices, however, so this result should be interpreted with caution.

Discussion

Phylogenetic relationships within Pachytriton

Our analysis yields a well-resolved phylogeny for species and populations of *Pachytriton*. The combined mitochondrial data are congruent with Chan *et al*. (2001) and Weisrock *et al*. (2006) in supporting the monophyly of the genus. Three distinct lineages are identified. The first clade, A, comprises three populations of *P. labiatus* from its south-western distribution, which includes Mt. Dayao, the species' type locality. This clade, which should retain the name *P. labiatus*, was the first branching clade of *Pachytriton*. Monophyly of this clade is well supported by mitochondrial data. When *P. labiatus* was described (Unterstein 1930), many authors regarded it as a synonym of *P. brevipes*, which is similar morphologically except for colouration (Pope 1931; Chang 1936). Later, Hu *et al*. (1973) confirmed the colour difference between the two forms, but they chose instead to classify *P. labiatus* as a subspecies of *P. brevipes*. Then Zhao & Hu (1984) once again elevated *P. labiatus* to species level. In our study, south-western *P. labiatus* exhibited considerable mitochondrial sequence divergence from other populations of *Pachytriton*. Uncorrected pairwise distances to Clade B (*P. archospotus*) are 10.8–11.1% for ND2 and 8.0–8.5% in cyt*b*. It differs from clade C by more than 12% and 7.4% in ND2 and cyt*b* respectively. These levels of mitochondrial divergences are comparable to or greater than interspecific differences within other salamander genera, such as species of *Lyciasalamandra* (7.6–10.1%; Weisrock *et al*. 2001), *Triturus* (5–6.8%; Steinfartz *et al*. 2007), *Lissotriton* (~5%; Babik *et al*. 2005) and another Asian stream salamander *Batrachuperus* (6.05–10.89%, Fu & Zeng 2008). The geographical distribution of south-western *P. labiatus* generally abuts other *Pachytriton* species. In a few localities where ranges of two species overlap, co-occurrence of both species in a given part of stream has not been observed. There is no direct evidence of gene flow between *P. labiatus* and congeners. According to the general metapopulation lineage species concept, species are regarded as separately evolving metapopulations (de Queiroz 2005, 2007). Therefore, we consider *P. labiatus*, which comprises only its south-western populations, a valid species based on available evidence.

Clade B, the second lineage in the mtDNA phylogeny, corresponds to *P. archospotus*, a recently described species

that was long misidentified as *P. brevipes* because of similar external morphology and colouration (Shen *et al*. 2008). The new species exhibits a unique osteological feature, straight epibranchial bones in the hyobranchial skeleton, which resembles the ancestral state observed in *Pachytriton*'s sister genera, *Paramesotriton* and *Cynops*. Epibranchial bones are highly curved and flared in both *P. labiatus* and *P. brevipes* (Shen *et al*. 2008). Our mitochondrial data indicate that *P. archospotus* forms the sister taxon to the group of north-eastern *P. labiatus* and *P. brevipes*. On the basis of the above osteological feature and its large genetic divergence from congeners (Table 2), we accept the validity of *P. archospotus*. Interestingly, our phylogenetic hypotheses, which place *P. archospotus* in a non-basal position within *Pachytriton*, suggest the independent evolution of straight epibranchial bones in this species. A detailed comparative osteological study shows that this feature in *P. archospotus* is different from its counterparts in *Paramesotriton* and *Cynops*, and most likely represents an autapomorphy (Y. Wu *et al*., unpublished data).

Phylogenetic relationships of clade C are more complicated. *Pachytriton brevipes* from Mt. Junfeng, the type locality of *P. brevipes*, and Mt. Wuyi, are split from a lineage comprising *P. brevipes* from Mt. Daiyun and north-eastern *P. labiatus*. The result indicates that *P. labiatus*, as current taxonomy defined, is paraphyletic. Monophyly of this species is significantly rejected in parsimony-based and likelihood-based topological tests. North-eastern *P. labiatus* diverges from south-western *P. labiatus*, which is the name-bearing species, by more than 12% in ND2 and 7.4% in cyt*b* sequences. Morphometric analysis further supports the separation of the two groups and their recognition as distinct lineages. South-western *P. labiatus* are significantly larger than north-eastern *P. labiatus* and have a much wider head. North-eastern *P. labiatus*, in turn, have a slender body shape. These results agree with the overall impression that north-eastern *P. labiatus* is a small and gracile newt even as adults, whereas adults of congeners are larger and more bulky. However, north-eastern *P. labiatus* may in fact consist of several lineages that are similar phenotypically, because monophyly of this clade is not supported by either molecular data set.

The phylogeny based on RAG-1 sequence data failed to identify the same three clades revealed by analysis of mtDNA. Instead, this nuclear gene groups clades A and B and yields low support for clade C. Incongruence between RAG-1 and mitochondrial data is not rare in amphibian phylogenetics, where low levels of genetic variation in RAG-1 sequences among closely related taxa often defy attempts to obtain a robust phylogeny (e.g. San Mauro *et al*. 2004; Martinez-Solano *et al*. 2007; Kotaki *et al*. 2008). Furthermore, because nuclear autosomal genes typically have an effective population size that is four times larger than maternally inherited mtDNA, lineage sorting of ancestral polymorphisms may be incomplete in recently diverged species (Moore 1995). Consequently, groupings of nuclear alleles do not necessarily correspond to species boundaries. Nevertheless, our RAG-1 data provide an independent test of the possibility of interspecific gene flows. The affinity between north-eastern *P. labiatus* and *P. brevipes* in the mtDNA-derived phylogeny could be a result of mitochondrial introgression from the latter species followed by fixation. But an introgressed nuclear allele is less likely to reach fixation if assumed neutral (Funk & Omland 2003) and would reveal the true relationship between source and sink species. Despite low level of support at major nodes, south-western *P. labiatus* and north-eastern *P. labiatus* are consistently placed in separate parts of the nuclear-gene trees in the MP, ML and BI analyses. In contrast, moderate bootstrap values and posterior probabilities support the grouping of north-eastern *P. labiatus* with *P. brevipes*. Topological tests significantly reject the monophyly of the two *P. labiatus* groups. Several unambiguous substitutions are shared between *P. brevipes* and north-eastern *P. labiatus*, to the exclusion of south-western *P. labiatus*. A second, independent nuclear POMC gene also unequivocally supports the close relationship between *P. brevipes* and north-eastern *P. labiatus* (Y. Wu, unpublished data). Hence, our analyses are unlikely to reflect past hybridization events and the inferred relationship among south-western *P. labiatus*, north-eastern *P. labiatus* and *P. brevipes* should be robust.

Colour pattern evolution

Until the discovery of *P. archospotus*, the other two species of *Pachytriton*, are identified and differentiated mainly by their external colourations (Fei *et al*. 2006). In general, *P. brevipes* exhibits numerous black, rounded spots on a yellow to light brown dorsum, flanks and tail (Sauvage 1876; Fei *et al*. 2006). On the other hand, *P. labiatus* has a chocolate-to-dark-brown dorsum that lacks any black spots (Unterstein 1930; Fei *et al*. 2006), but may have bright orange to red flecks laterally. South-western and north-eastern *P. labiatus* have nearly identical colouration , which includes bright dorsolateral flecks that are visible on museum specimens of both groups. This shared colouration is perhaps the most compelling reason for recognizing the two allopatric *P. labiatus* as a single species. *Pachytriton archospotus* is chromatically similar to *P. brevipes*. A systematic key based on colour pattern was provided by Fei *et al*. (2006).

Mitochondrial and nuclear sequence data reveal unexpected evolutionary relationships by grouping north-eastern *P. labiatus* with *P. brevipes* and separating

south-western *P. labiatus* as a different species. According to this scheme, the colour pattern shared by the two groups of *P. labiatus* does not constitute a synapomorphy by which this species can be delimited. Colour pattern variation has been studied in other salamander species, mostly as intraspecific polymorphisms (e.g. Baird *et al*. 2006; Wake 2006; McKnight & Nelson 2007). There are fewer documented instances of interspecific homoplasy of external colouration. One nicely illustrated example involves European *Salamandra lanzai* and *Salamandra atra*, which achieve melanistic colour by convergent evolution (Veith 1996; Bonato & Steinfartz 2005). We provide the first example of interspecific colour homoplasy in Asian newts. As discussed earlier, chromatic similarity between south-western and north-eastern *P. labiatus* is unlikely to be the result of hybridization. Natural selection, on the other hand, may favour certain types of colouration due to environmental and biotic interactions (Schaefer *et al*. 2002; Chiari *et al*. 2004). Little is known regarding the life history of *Pachytriton* in its native habitat; hence, it is difficult to evaluate the selective force that may be driving colour pattern evolution. Both crypsis and aposematism are possible explanations of the shared colour pattern, which involves a dark-toned dorsum with bright dorsolateral flecks. Phylogenetic reconstruction of the ancestral colour pattern suggests that the common ancestor of *Pachytriton* had an unspotted dark dorsum and that the black-spotted pattern possibly was acquired later in the common ancestor of *P. brevipes*, *P. archospotus* and north-eastern *P. labiatus*. Given that most species in the sister genera *Paramesotriton* and *Cynops* have an unspotted dark dorsum and may develop various spots during breeding season, the implied ancestral states for *Pachytriton* are plausible. The results further suggest that black spots were lost secondarily in north-eastern *P. labiatus*, a reversal to the ancestral state that is retained in south-western *P. labiatus*. We found one north-eastern *P. labiatus* (Pet Trade Chengdu 2; Fig. 2) with apparent black spots that resembles *P. brevipes*. As the monophyly of north-eastern *P. labiatus* is not supported statistically, multiple reversals to the unspotted colouration may have occurred.

Alternatively, black spots may have evolved independently in different populations of *P. brevipes* and *P. archospotus* from the unspotted ancestral colouration, which is retained in both groups of *P. labiatus*. However, this hypothesis is challenged by the ecological characteristics of this genus. *Pachytriton brevipes* and *P. archospotus* (so do south-western *P. labiatus*) are restricted to montane streams at high elevations and populations are isolated by intervening lowlands. Under such a sky island distribution, several independent acquisitions of the same chromatic trait are unlikely. In comparison, north-eastern *P. labiatus* can be found at lower foothills, which implies potential gene flow between mountain ranges. This is supported by the mixture of mitochondrial haplotypes from different localities (Fig. 2), although a demographic recovery from a population bottleneck can produce a similar phylogenetic pattern. But in either case, colour change may easily sweep across the entire population. Therefore, in conjunction with the results from posterior ancestral state reconstruction, we favour the first hypothesis that north-eastern *P. labiatus* acquired the unspotted dorsal colouration through evolutionary reversals.

Evolution of bright dorsolateral flecks presents a similar situation. Even though ancestral state reconstruction suggested independent acquisition of this trait in different lineages, we favour, for the same reason concerning ecological traits, the alternative scenario, which involves independent loss of bright dorsolateral flecks. We propose that the common ancestor of the genus *Pachytriton* had an unspotted dark brown dorsum with bright dorsolateral flecks.

Before recognizing the likely homoplastic evolution of dorsal colouration in *Pachytriton*, some authors have proposed using both dorsal and ventral colour pattern to diagnose species (Fei *et al*. 2006). We regard this strategy as problematic, however, because ventral colour pattern is subject to large intraspecific variation. In both groups of *P. labiatus*, it varies with age of an animal. Juveniles have clearly defined red ventral blotches, which become connected and then blurred in subadults and gradually fade away in older animals. On the other hand, whereas *P. brevipes* generally possesses black ventral spots, they vary greatly in density. Many specimens are spotless, which renders them less distinct from older *P. labiatus*. In addition, subadults of *P. brevipes* from Mt. Qiyun have a very similar ventral colour pattern to subadults of *P. labiatus*. Overall, external colouration is not a reliable character to delimit species boundaries in *Pachytriton*.

Conservation implications

Pachytriton is a widely available newt in the commercial pet trade. Large numbers of animals are collected in the wild each year and exported from China to Europe, North America and other continents. It also is one of the most popular amphibian pets in China (Fei *et al*. 2006). In addition, many animals are collected from certain localities and deposited in the nation's museums. Overexploitation has been the most serious crisis faced by East Asian amphibians (Stuart *et al*. 2004) and it is worth considering if *Pachytriton* is susceptible to a similar fate.

In our study, *Pachytriton* purchased in several Chinese cities on different dates are genetically similar to north-eastern *P. labiatus* from Zhejiang Province. Analysis of other published DNA sequences in GenBank further

implicates mountains in eastern Zhejiang as the major source of traded north-eastern *P. labiatus* (data not shown). Tourism and tunnel construction have damaged the natural environment in this region. Montane streams, the vital habitats for *Pachytriton* and other aquatic organisms, have been polluted or dried out. Yet, at the same time, as *Pachytriton* populations are declining and disappearing, we are just beginning to resolve phylogenetic relationships and evolutionary histories in this genus, which are much more complex than previously thought. Adult size decrease in north-eastern *P. labiatus*, for example, which typically occur at much lower elevation than other *Pachytriton*, provides excellent opportunities to study the interactions among amphibian ontogeny, morphology and environmental factors. Sustaining, and in some instances restoring north-eastern *P. labiatus* populations are crucial for continuing research. We, therefore, urge the imposition of legal restrictions that would limit human exploitation and habitat destruction.

Conclusions

Phylogenetic relationships within *Pachytriton* are inferred for the first time with molecular and morphometric data. Mitochondrial genes reveal considerable interspecific divergence and identified three major clades that correspond to the three known species in *Pachytriton*. Nuclear RAG-1 data yielded a less resolved phylogeny due to limited genetic variation. The monophyly of *Pachytriton* is supported. *Pachytriton labiatus* and *P. archospotus* are recognized as valid species. However, the two allopatric *P. labiatus* groups do not form a monophyletic clade. North-eastern *P. labiatus* is nested within *P. brevipes* and south-western *P. labiatus* represents the authentic nominal species. The shared colour pattern between the two *P. labiatus* groups is homoplastic, probably through reversed evolution in north-eastern *P. labiatus*. The black-spotted colour pattern evolved in the common ancestor of *P. brevipes*, *P. archospotus* and north-eastern *P. labiatus*. Incongruence of molecular phylogeny and colouration, as well as large intraspecific variation, suggests that chromatic traits alone are not sufficient to diagnose species in this genus.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Primers designed in this study. mtDNA primers are named by their 5′ position in the mitochondrial genome of *Paramesotriton hongkongensis* (AY458597; Zhang *et al*. 2005); nuclear primers are named by their 5′ position in the RAG-1 coding sequence of *Pleurodeles waltl* (AJ010258; Frippiat *et al*. 2001)

Table S2 Principal components matrix consisting of component eigenvectors. The first PC represents overall body size, the second PC measures overall body shape (i.e. length versus girth), and the third PC evaluates head size relative to postcranial body size

Table S3 Measurements from museum specimens

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