What are the closest relatives of the hot-spring snakes (Colubridae, *Thermophis*), the relict species endemic to the Tibetan Plateau?

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ABSTRACT

We conducted phylogenetic analyses to identify the closest related living relatives of the Xizang and Sichuan hot-spring snakes (*T. baileyi* and *T. zhaoermii*) endemic to the Tibetan Plateau, using mitochondrial DNA sequences (cyt *b*, ND4) from eight specimens, together with sequences from 95 additional caenophidian and five henophidian genera that were downloaded from GenBank. Phylogenetic trees were obtained using Bayesian Inference and Maximum likelihood methods. Results suggest that hot-spring snakes, which are adapted to high and cold environments, were clustered in the monophyletic Xenodontinae. Xenodontinae is one of the largest subfamilies of colubrid snakes, with about 90 genera and more than 500 species known, and are primarily tropical snakes previously thought to be restricted to the New World. Our data failed to provide any evidence that the New World xenodontines diverged from *Thermophis* and dispersed into the New World, also failed to suggest a colonization of Asia by New World xenodontines by dispersal from the New World. An alternative plausible scenario may be that *Thermophis* and the New World xenodontines evolved independently in Asia and America, respectively, after the divergence of their common ancestor. The divergence of the two species in *Thermophis* was caused by the barrier of the Hengduan Mountains, and the speciation had almost occurred when Tibetan Plateau attained present elevation.

1. Introduction

The Tibetan Plateau, known as "the roof of the world", is the highest plateau on the Earth, and still in the process of uplift. Its geomorphology and climate show substantial interregional variation, leading to remarkable faunal and floral diversity and high levels of endemism (Peng et al., 2006). The Xizang and Sichuan hot-spring snakes, *T. baileyi* (Wall, 1907) and *T. zhaoermii* (Guo et al., 2008), are endemic to the Tibetan Plateau, and are two of only a few snakes found at such high altitudes (3700–4440 m).

Wall (1907) first described the Xizang hot-spring snake, a new snake from Xizang (Tibet) Autonomous Region (A.R.), as *Tropidonotus* (=*Natrix*) *baileyi*. Based on a study of the characters, Malnate (1953) removed this species from the genus *Natrix* and designated

it as the genotype of *Thermophis*, gen. nov. Most recently, Guo et al. (2008) conducted a detailed morphologically comparative study between *Thermophis* specimens from Litang County (Sichuan Province) and Xizang A.R. They concluded that Litang specimens should be a new species and named it *T. zhaoermii* (common name: Sichuan hot-spring snake). The formerly monotypic genus, *Thermophis*, now includes two species: *T. baileyi* and *T. zhaoermii*.

In recent decades, the exploitation of geothermal energy resources has resulted in environmental pollution and habitat destruction, and the wild populations of hot-spring snakes have been facing severe threats. In 2004, *T. baileyi* was listed on the China Species Red List as a Critically Endangered species (CR B1ab(iii,v) + D; http://www.chinabiodiversity.com/redlist). Up to the present, there are only eight sites at which the hot-spring snakes are known to occur (Fig. 1). Cogsum (CS), Litan County, Sichuan Province is the latest distribution site reported by Liu and Zhao (2004). This site is far away from the other seven sites in Xizang A.R. and separated from them by the Hengduan Mountains (Mts.) on the eastern margin of the Tibetan Plateau, a complex system of high parallel ridges and deep incised valleys. This
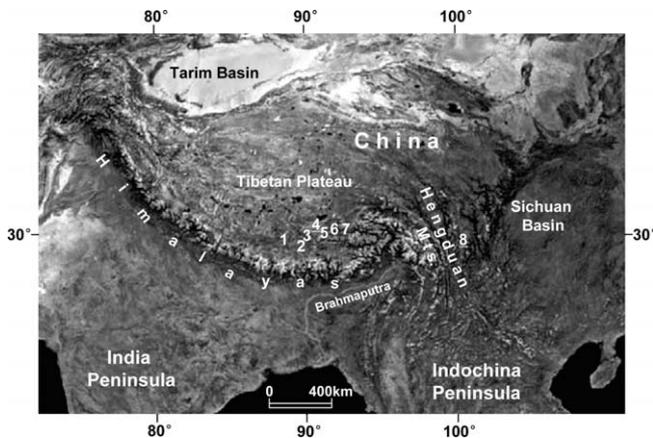
Fig. 1. Map of distribution (1–8) and sampling (4 and 8) sites of hot-spring snakes (*Thermophis*). 1, Shigatse; 2, Gyantse; 3, Nyemo; 4, Yangbajain (YBJ), Dangxung; 5, Lhasa; 6, Maizhokunggar; 7, Gongbogyamda; 8, Cogsum (CS), Litang.

population was recently confirmed as a distinct species by a morphologically comparative study (Guo et al., 2008).

We designate the hot-spring snakes as "relict" species, according to Pinou et al. (2004), due not only to their unique ecological adaptations to the environment and climate of the Tibetan Plateau, but also to the lack of their closely related living relatives, reflected in the assignation of this genus by Lawson et al. (2005) to Colubridae *insertae sedis*. Hot-spring snakes are often difficult to find in the field. Fortunately, we sampled eight individuals of *Thermophis* at two distribution sites: Yangbajain (YBJ), Dangxung, Xizang A.R. (being *T. baileyi*) and CS (being *T. zhaoermii*). In the present study we aim mainly to: (1) examine the systematic position of *Thermophis* within Caenophidia and (2) to evaluate the biogeographical significance of the Hengduan Mts. in separation of the two species of *Thermophis* .

It is often difficult to find unique characters (plesiomorphies and/or apomorphies) to characterize and group some snake taxa, as limited morphological information can be obtained from their specialized and therefore simple body structure. Thus, molecular data appear to be more useful in snake systematics to some extent. Over the last two decades, the analyses of fragment(s) of mtDNA have more and more frequently been used as a versatile and effective tool to investigate the phylogenetic relationships of snakes. The most frequently used fragments are mitochondrial cytochrome *b* (cyt *b*) and/or NADH dehydrogenase subunit 4 (ND4) genes (e.g. Kraus and Brown, 1998; Kelly et al., 2003; Malhotra and Thorpe, 2004; Lawson et al., 2005; Burbrink and Lawson, 2007). The abundance of both gene sequences of snakes in the GenBank database offers us large datasets for our comparative analyses to determine what are the closest related living relatives of the hot-spring snakes. In the present study, we tried to include most caenophidian cyt *b* and ND4 sequences available in the GenBank database, as well as five henophidian snakes as outgroups in the phylogenetic analysis.

The systematic nomenclature used in this paper is based on the updated classification recommended by Lawson et al. (2005), which derived from their analyses from mitochondrial and nuclear genes, and the evidence from other research using morphological, immunological, and DNA sequence data.

2. Materials and methods

2.1. Downloaded sequences and supraspecific terminals

Table 1 shows 156 species of Alethinophidia (primitive + advanced snakes) retrieved from GenBank for use in this study, with the accession numbers of cyt *b* and/or ND4 gene sequences, as well as their references. To summarize, 130 species have cyt *b* sequences, 112 species have ND4 sequences, and of these 85 species (from 85 genera) have both genes. In addition, 10 genera are represented by two different species with different gene sequences. In order to maximize our dataset of combined genes, we combined two different sequences from congeneric two species to form supraspecific terminals at the generic level (according to Bininda-Emonds et al., 1998; Kelly et al., 2003).

2.2. DNA extraction, amplification, and sequencing

Six individuals of *T. baileyi* were sampled from Yangbajain, Dangxung, Xizang A.R. (N: 30.0638; E: 90.4876; 4100 m.), two individuals of *T. zhaoermii* were sampled from Cogsum, Litang, Sichuan Provence (N: 29.6921, E: 100.3842; 3700 m). Snakes collected for this study were humanely euthanized using lethal injection. The fresh liver tissues or tail tip biopsies were removed and immediately preserved in 95% ethanol for sequencing their mitochondrial cyt *b* and ND4 genes. Specimens were deposited in the museums of Sichuan University and Sichuan Forest Academy. Total genomic DNA was extracted according to the phenol/chloroform extraction procedure (Sambrook et al., 1989). The entire cyt *b* and partial ND4 genes were amplified from the total DNA extracts using the polymerase chain reaction (PCR) with primer pairs L14910/H16064 (Burbrink et al., 2000) and ND4/Leu (Arévalo et al., 1994), respectively. The PCR contained approximately 100 ng of template DNA, 1 μL of each primer, 5 μL of 10× reaction buffer, 2 μL dNTPs (each 2.5 mM), and 2.0 U of *Taq* DNA polymerase in total 50 μL volume. The reactions were cycled at the following temperatures: an initial 94 °C denaturation for 3 min, 30 cycles of 94 °C denaturation for 1 min, 46–55 °C annealing for 1 min, 72 °C extension for 1 min, and a final 72 °C extension for 5 min. Amplified DNA was fractionated by electrophoresis through 0.8% low-melting agarose gels, recovered from the gels, and purified using BioStar Glassmilk DNA purification Kit according to manufacturer's instructions. Purified DNA was sequenced with the Perkin–Elmer BigDye DNA Sequencing Kit according to the manufacturer's protocol with the primers used in PCR.

2.3. Phylogenetic reconstruction

The newly determined cyt *b* and ND4 sequences of *Thermophis* (homologous protein-coding genes) were aligned with other retrieved sequences for the same gene regions, respectively, and to the combined gene sequences. These three datasets were aligned using Clustal X (Thompson et al., 1997), and translated into amino acid sequences to check for the presence of stop codons within the sequences which might indicate the amplification of pseudogene(s). The aligned sequences have been submitted to TreeBASE (www.treebase.org) under submission number SN4262. Gaps resulting from the alignment were treated as missing data.

Bayesian inference (BI) and Maximum likelihood (ML) approaches were used to reconstruct a phylogenetic bifurcating trees in the present study, using MrBayes v3.0 (Huelsenbeck and Ronquist, 2001) and PAUP∗ v4b10 (Swofford, 2002), respectively. Modeltest 3.7 (Posada and Crandall, 1998) was used to find the model of nucleotide evolution with Akaike Information Criterion (AIC) for ML and Bayesian Information Criterion (BIC) for BI. In the Bayesian analysis, a starting tree was obtained using the neighbor-joining algorithm. Posterior probabilities (PP) were obtained by Markov chain Monte Carlo (MCMC) analysis with one cold chain and three heated chains. Samples of

Table 1
The information of sequences retrieved from GenBank and sequenced in this study. The systematic nomenclature according to the updated classification recommended by Lawson et al. (2005).

Family	Genus and species	Accession No.		Source
Subfamily		Cyt *b*	ND4	
Acrochordidae	*Acrochordus granulatus*	AB177879	AB177879	Dong and Kumazawa (2005)
Colubridae				
Calamariinae	*Calamaria pavimentata*	AF471081		Lawson et al. (2005)
	Pseudorabdion oxycephalum	AF471073		Lawson et al. (2005)
Colubrinae	*Arizona elegans*	DQ902101	AF138750	Burbrink and Lawson (2007); Rodriguez-Robles and De Jesus-Escobar (1999)
	Bogertophis subocularis	DQ902103	DQ902281	Burbrink and Lawson (2007)
	Boiga dendrophila	AF471089	U49303	Lawson et al. (2005), Kraus and Brown (1998)
	Cemophora coccinea	AF471091	DQ902282	Lawson et al. (2005), Burbrink and Lawson (2007)
	Coelognathus erythrurus	DQ902108	DQ902288	Burbrink and Lawson (2007)
	Coluber constrictor priapus	AY486913	AY487040	Nagy et al. (2004)
	Coronella girondica	AF471088	AY487066	Lawson et al. (2005), Nagy et al. (2004)
	Crotaphopeltis tornieri	AF471093		Lawson et al. (2005)
	Dasypeltis atra	AF471065		Lawson et al. (2005)
	Dinodon semicarinatus	AB008539	AB008539	Kumazawa et al. (1996)
	Dipsadoboa unicolor	AF471062		Lawson et al. (2005)
	Drymarchon corais	AF471064	DQ902314	Lawson et al. (2005), Burbrink and Lawson (2007)
	Eirenis modestus	AY486933	AY487072	Nagy et al. (2004)
	Elaphe carinata	DQ902133	DQ902284	Burbrink and Lawson (2007)
	Euprepiophis mandarinus	DQ902115	DQ902294	Burbrink and Lawson (2007)
	Gastropyxis smaragdina	DQ112075		Lawson et al. (2005)
	Gonyosoma oxycephalum	AF471084	DQ902309	Lawson et al. (2005), Burbrink and Lawson (2007)
	Hemorrhois hippocrepis	AY486916	AY487045	Nagy et al. (2004)
	Hierophis viridiflavus	AY486925	AY487057	Nagy et al. (2004)
	Lampropeltis caligaster	DQ902129	DQ902311	Burbrink and Lawson (2007)
	Lycodon zawi	AF471040		Lawson et al. (2005)
	Lycodon capucinus		U49317	Kraus and Brown (1998)
	Lytorhynchus diadema	DQ112076		Lawson et al. (2005)
	Macroprotodon cucullatus	AF471087	AY487064	Lawson et al. (2005), Nagy et al. (2004)
	Masticophis flagellum	AY486927	AY487060	Nagy et al. (2004)
	Oligodon cinereus	AF471033	U49316	Lawson et al. (2005), Kraus and Brown (1998)
	Opheodrys aestivus	AF471057		Lawson et al. (2005)
	Oocatochus rufodorsatus	DQ902123	DQ902301	Burbrink and Lawson (2007)
	Oreophis porphyraceus	DQ902118	DQ902298	Burbrink and Lawson (2007)
	Orthriophis taeniurus	EF076709	EF076708	Burbrink and Lawson (2007)
	Oxybelis aeneus	AF471056		Lawson et al. (2005)
	Pantherophis slowinskii	DQ523162	DQ523162	Jiang et al. (2007)
	Philothamnus heterodermus	AF471055		Lawson et al. (2005)
	Phyllorhynchus decurtatus	AF471083		Lawson et al. (2005)
	Pituophis melanoleucus	DQ902130	DQ902312	Burbrink and Lawson (2007)
	Platyceps karelini	AY486918	AY487047	Nagy et al. (2004)
	Ptyas mucosus	AF471054	AY487063	Lawson et al. (2005), Nagy et al. (2004)
	Rhinechis scalaris	AY486932	AY487068	Nagy et al. (2004)
	Rhinocheilus lecontei	AF337109	AF138774	Fetzner and Miller (Unpubl.), Rodriguez-Robles and De Jesus-Escobar (1999)
	Rhynchophis boulengeri	AF471053		Lawson et al. (2005)
	Salvadora mexicana	AY486934	AY487075	Nagy et al. (2004)
	Senticolis triaspis	DQ902127	AF138775	Burbrink and Lawson (2007); Rodriguez-Robles and De Jesus-Escobar (1999)
	Sonora semiannulata	AF471048		Lawson et al. (2005)
	Spalerosophis diadema	AF471049	AY487059	Lawson et al. (2005), Nagy et al. (2004)
	Spilotes pullatus	AF471041		Lawson et al. (2005)
	Stilosoma extenuatum	DQ902131	AF138776	Burbrink and Lawson (2007); Rodriguez-Robles and De Jesus-Escobar (1999)
	Tantilla relicta	AF471045		Lawson et al. (2005)
	Telescopus fallax	AF471043		Lawson et al. (2005)
	Thelotornis capensis	AF471042		Lawson et al. (2005)
	Thrasops jacksoni	AF471044		Lawson et al. (2005)
	Trimorphodon biscutatus		DQ497542	Devitt (2006)
	Zamenis longissimus	DQ902138		Burbrink and Lawson (2007)
	Zamenis lineatus		DQ902319	Burbrink and Lawson (2007)
Natricinae	*Afronatrix anoscopus*	AF420073	AF420076	de Queiroz et al. (2002)
	Amphiesma stolata	AF471030		Lawson et al. (2005)
	Clonophis kirtlandii	AF402908		Alfaro and Arnold (2001)
	Natriciteres olivacea	AF471058		Lawson et al. (2005)
	Natrix natrix	AF471059	AY487800	Lawson et al. (2005), Guicking et al. (2006)
	Nerodia fasciata	AY866529	AY873705	Guicking et al. (2006)
	Regina rigida	AF471052		Lawson et al. (2005)
	Rhabdophis subminiatus		U49325	Kraus and Brown (1998)
	Rhabdophis tigrinus	AF471051		Lawson et al. (2005)
	Storeria dekayi	AF471050	EF417365	Lawson et al. (2005); de Queiroz and Lawson (Unpubl.)

Table 1 (continued)

Family	Genus and species	Accession No.		Source
Subfamily		Cyt *b*	ND4	
	Thamnophis sirtalis infernalis	AF420193	AF420196	de Queiroz et al. (2002)
	Tropidoclonion lineatum	AF402931	AF420208	Alfaro and Arnold (2001), de Queiroz et al. (2002)
	Xenochrophis punctulatus	AF471079	AY487074	Lawson et al. (2005)
Natricinae *insertae sedis*	*Amplorhinus multimaculatus*	DQ486352	DQ486327	Kelly (Unpubl.)
Pseudoxenodontinae				
	Pseudoxenodon karlschmidti	AF471080		Lawson et al. (2005)
Xenodontinae (Dipsadinae)				
	Atractus elaps		EF078584	Mulcahy (2007)
	Carphophis amoenus	AF471067		Lawson et al. (2005)
	Coniophanes fissidens		EF078586	Mulcahy (2007)
	Cryophis hallbergi		EF078544	Mulcahy (2007)
	Dipsas catesbyi		EF078585	Mulcahy (2007)
	Diadophis punctatus	AF471094	AF258899	Lawson et al. (2005), Feldman and Spicer (2006)
	Eridiphas slevini		EF078547	Mulcahy (2007)
	Hypsiglena torquata	AF471038	EF078548	Lawson et al. (2005), Mulcahy (2007)
	Imantodes cenchoa		EF078556	Mulcahy (2007)
	Rhadinaea flavilata	AF471078		Lawson et al. (2005)
	Rhadinaea fulvivittis		EF078587	Mulcahy (2007)
	Sibon sartorrii		EF078588	Mulcahy (2007)
	Leptodeira septentrionalis		EF078573	Mulcahy (2007)
	Pseudoleptodeira latifasciata		EF078583	Mulcahy (2007)
Dipsadinae *insertae sedis*	*Tantalophis discolor*		EF078589	Mulcahy (2007)
Xenodontinae (Xenodontinae)				
	Alsophis portoricensis	AF471085	U49308	Lawson et al. (2005)
	Arrhyton exiguum	AF471071		Lawson et al. (2005)
	Contia tenuis	AF471095	AF258879	Lawson et al. (2005)
	Farancia abacura	U69832	DQ902307	Campbell (1997); Burbrink and Lawson (2007)
	Helicops angulatus	AF471037		Lawson et al. (2005)
	Helicops pictiventris		U49310	Kraus and Brown (1998)
	Heterodon simus	AF217840	DQ902310	Slowinski and Keogh (2000), Burbrink and Lawson (2007)
	Hydrops triangularis	AF471039		Lawson et al. (2005)
Colubridae *insertae sedis*				
	Thermophis zhaoermii (CS1)	EU864145	EU864150	This study
	Thermophis zhaoermii (CS2)	EU864146	EU864150	This study
	Thermophis baileyi (YBJ1)	EU864147	EU864149	This study
	Thermophis baileyi (YBJ2)	EU864148	EU864149	This study
Elapidae				
Atractaspidinae	*Amblyodipsas dimidiata*	DQ486346	DQ486322	Kelly (Unpubl.)
	Aparallactus werneri	AF471035	U49315	Lawson et al. (2005), Kraus and Brown (1998)
Boodontinae				
	Bothrophthalmus lineatus	AF471090		Lawson et al. (2005)
	Dipsina multimaculata	DQ486357	DQ486332	Kelly (Unpubl.)
	Dromophis lineatus	DQ486428	DQ486265	Kelly (Unpubl.)
	Grayia tholloni	DQ486351	DQ486326	Kelly (Unpubl.)
	Lamprophis fuliginosus	AF471060	DQ486315	Lawson et al. (2005); Kelly (Unpubl.)
	Lycodonomorphus laevessimus	DQ486338	DQ486314	Kelly (Unpubl.)
	Lycophidion ornatum	AF471086		Lawson et al. (2005)
	Lycophidion capense	DQ486344	DQ486320	Kelly (Unpubl.)
	Pythonodipsas carinata	AY188036		Nagy et al. (2003)
	Pseudaspis cana	DQ486343	DQ486319	Kelly (Unpubl.)
	Pseudoboodon lemniscatus	DQ486350	DQ486325	Kelly (Unpubl.)
	Scaphiophis albopunctatus	DQ486345	DQ486321	Kelly (Unpubl.)
Elapinae				
	Bungarus fasciatus	AJ749350	AJ830224	Kuch (Unpubl.)
	Dendroaspis polylepis	AF217832	AY058974	Slowinski and Keogh (2000); Slowinski and Lawson (Unpubl.)
	Homoroselaps lacteus	AF217833	AY058976	Slowinski and Keogh (2000); Slowinski and Lawson (Unpubl.)
	Mehelya unicolor	AF471077	DQ486324	Lawson et al. (2005); Kelly (Unpubl.)
	Micrurus fulvius		AY058980	Slowinski and Lawson (Unpubl.)
	Micruroides euryxanthus	AF217823	EF137408	Slowinski and Keogh (2000), Castoe et al. (2007b)
	Naja kaouthia	AF217835	AY058982	Slowinski and Keogh (2000); Slowinski and Lawson (Unpubl.)
	Notechis ater	AF217836	AY058981	Slowinski and Keogh (2000); Slowinski and Lawson (Unpubl.)
	Ophiophagus hannah	AF217842	AY058984	Slowinski and Keogh (2000); Slowinski and Lawson (Unpubl.)
Hydrophiinae				
	Pseudonaja guttata		DQ098471	Skinner et al. (2005)
	Laticauda colubrina	AF217834	AY058977	Slowinski and Keogh (2000); Slowinski and Lawson (Unpubl.)
Xenodermatinae				
	Oxyrhabdium leporinum	AF471029		Lawson et al. (2005);

Table 1 (continued)

Family	Genus and species	Accession No.		Source
Subfamily		Cyt *b*	ND4	
Psammophiinae				
	Hemirhagerrhis kelleri	DQ486335	DQ486311	Kelly (Unpubl.)
	Malpolon monspessulanus	AY058965	AY058989	Slowinski and Lawson (Unpubl.)
	Mimophis mahfalensis	AY188032	DQ486202	Nagy et al. (2003); Kelly et al. (Unpubl.)
	Rhamphiophis acutusacutus	DQ486425	DQ486262	Kelly et al. (Unpubl.)
	Psammophis condanarus	AF471075	AY058987	Lawson et al. (2005); Slowinski and Lawson (Unpubl.)
	Psammophylax rhombeatus	DQ486361	DQ486200	Kelly et al. (Unpubl.)
Pseudoxyrhophiinae				
	Alluaudina bellyi	AY188005		Nagy et al. (2003)
	Ditypophis vivax	AY188013		Nagy et al. (2003)
	Duberria lutrix	AF471061	DQ486240	Lawson et al. (2005); Kelly et al. (Unpubl.)
	Leioheterodon modestus	AY058967	AY058978	Slowinski and Lawson (Unpubl.)
	Ithycyphus miniatus	AY188019		Nagy et al. (2003)
Elapidae *insertae sedis*	*Prosymna visseri*	AY188033		Nagy et al. (2003)
	Prosymna ruspolii		DQ486323	Kelly (Unpubl.)
	Psammodynastes pulverulentus	AF471031		Lawson et al. (2005)
Pareatidae				
	Pareas macularius	AF471082		Lawson et al. (2005)
	Pareas nuchalis		U49311	Kraus and Brown (1998)
Viperidae				
Azemiopinae	*Azemiops feae*		U41865	Kraus et al. (1996)
Crotalinae	*Agkistrodon piscivorus*	DQ523161	DQ523161	Jiang et al. (2007)
	Crotalus viridis	AF471066		Lawson et al. (2005)
	Crotalus atrox		DQ679870	Castoe et al. (2007a)
	Gloydius brevicaudus		AY352815	Malhotra and Thorpe (2004)
	Ovophis okinavensis	AB175670	AB175670	Dong and Kumazawa (2005)
	Protobothrops mucrosquamatus		AY223629	Parkinson et al. (2002)
	Trimeresurus flavoviridis		U41894	Parkinson et al. (2002)
	Deinagkistrodon acutus	DQ343647	DQ343647	Yan et al. (Unpubl.)
Viperinae	*Atheris nitschei*	AF471070	AY223618	Lawson et al. (2005), Parkinson et al. (2002)
	Cerastes cerastes	AF471028		Lawson et al. (2005)
	Daboia russellii	AF471076	DQ305477	Lawson et al. (2005), Castoe and Parkinson (2006)
Homalopsidae				
	Cerberus rynchops	AF471092	U49327	Lawson et al. (2005), Kraus and Brown (1998)
	Enhydris plumbea	DQ343650	DQ343650	Yan et al. (Unpubl.)
Outgroups:				
Boidae	*Boa constrictor*	AB177354	AB177354	Dong and Kumazawa (2005)
Cylindrophiidae	*Cylindrophis ruffus*	AB179619	AB179619	Dong and Kumazawa (2005)
Pythonidae	*Python regius*	AB177878	AB177878	Dong and Kumazawa (2005)
Uropeltidae	*Uropeltis phillipsi*	AF471034		Lawson et al. (2005)
	Uropeltis sp.		DQ904396	Shouche (Unpubl.)
Xenopeltidae	*Xenopeltis unicolor*	AB179620	AB179620	Dong and Kumazawa (2005)

trees and parameters were drawn every 100 steps from a total of 1,000,000 MCMC steps. The first 100,000 generations (1000 trees) were discarded as burn in (representing the generations in which the chain had not yet reached stationarity) and only the results from the last 900,000 generations (9000 trees) were used to compute a consensus tree. We ran three additional analyses starting with random trees. Consensus of all of the post-burn in generations (3,600,000 generations resulting in 36,000 trees) was computed from all four runs. We did not conduct a bootstrap analysis for ML because of the calculation time constraints.

In phylogenetic analyses, a low character to terminal taxa ratio (i.e., short sequences and many taxa) tends to result in low node supports, especially for internal nodes (stated by Kelly et al., 2003). In the present study, we firstly reconstructed three "big" phylogenetic trees to find the closest related living relatives of *T. baileyi* within Caenophidia using the three datasets described above. Then, we conducted further analyses on a subset of this data which contained only the sequences of *Thermophis* and the subfamily identified as its closest relative as ingroups, and selected outgroup(s) according to the results from the first analaysis. These "smaller" datasets further increase the character: terminal taxon

ratio, and allows a clearer understanding of the relationships among *Thermophis* and its relatives.

3. Results

3.1. Sequence characteristics

Alignment by eye of the cyt *b* and ND4 sequences of hot-spring snakes generated by this study resulted in 1100 and 688 sites, respectively. The cyt *b* sequence of all snakes used in this study commenced with the ATG methionine codon, which is apparently universal in squamates (Lawson et al., 2005). Because of length variation in codon numbers at the 3' terminus of cyt *b* gene of snakes, the alignment at the end of the cyt *b* sequences was ambiguous. Following Lawson et al. (2005), only the first 1100 bp of all cyt *b* sequences were used in the present study for the phylogenetic analyses.

Six cyt *b* sequences of *T. baileyi* generated in this study share two haplotypes, two cyt *b* sequences of *T. zhaoermii* share two haplotypes. Six ND4 sequences of *T. abileyi* and two of *T. zhaoermii*, respectively, share one haplotype. These sequences of haplotypes were deposited in GenBank (their accession numbers is listed in

Table 2
Average nucleotide composition (%), numbers of variable (V) and parsimony informative (PI) sites for cyt *b* and ND4 gene fragment, based on the data used in the present study.

Taxa	Cyt *b* (1 100 aligned sites)							ND4 (696 aligned sites)						
		T	C	A	G	V	PI		T	C	A	G	V	PI
Caenophidia	n = 128	27.6	29.8	31.6	11.0	718	645	n = 109	25.5	29.6	33.4	11.5	471	414
Xenodontinae	n = 11	27.5	31.3	30.1	11.1	493	344	n = 18	25.1	30.2	33.7	11.1	345	236
Thermophis	n = 4	26.2	32.6	29.8	11.4	115	113	n = 2	24.2	30.5	33.0	12.3	63	

n, numbers of sequences of each group.

Table 3
Sequence divergences between species of Caenophidia, Xenodontinae, and individuals from site 4 (*T. baileyi*) vs. site 8 (*T. zhaoermii*) for cyt *b* and ND4 sequences.

Taxa	Uncorrected *p*-distance					
	Cyt *b*			ND4		
	Maximum	Minimum	Mean	Maximum	Minimum	Mean
Overall ingroup taxa	0.288	0.057	0.213 (*n* = 128)	0.307	0.059	0.200 (*n* = 109)
Xenodontinae	0.232	0.132	0.188 (*n* = 11)	0.240	0.096	0.170 (*n* = 18)
Thermophis (site 4 vs. site 8)			0.104 (*n* = 4)			0.092 (*n* = 2)

n, numbers of sequences of each group.

Table 1). The average nucleotide composition (%), numbers of variable (V) and parsimony informative (PI) sites for cyt *b* and ND4 gene fragment of Caenophidia, Xenodontinae (*Thermophis* clusters in this subfamily, see below), and *Thermophis* used in the present study are listed in Table 2. The percentages of the four nucleotides were not significantly different across taxa groups, and showed strong bias against guanidine, typical for mitochondrial DNA (e.g. de Queiroz et al., 2002; Malhotra and Thorpe, 2004).

Sequence divergences of uncorrected *p*-distance between ingroup taxa (excluding *Thermophis*), and individuals from site 4 (*T. baileyi*) vs. site 8 (*T. zhaoermii*) for both genes are listed in Table 3. For cyt *b* sequences, the divergence within all ingroup taxa (Caenophidia) ranged from 0.057 (*Coronella girondica* vs. *Gastropyxis smaragdina*) to 0.288 (*Pareas macularius* vs. *Acrochordus granulatus*); the divergence within Xenodontinae (*Thermophis* clusters within this subfamily, see below) ranged from 0.132 (*Alsophis portoricensis* vs. *Arrhyton exiguum*) to 0.232 (*Heterodon simus* vs. *Rhadinaea flavilata*). For ND4 sequences, the divergence within all ingroup taxa ranged from 0.059 (*Nerodia fasciata* vs. *Tropidoclonion lineatum*) to 0.307 (*Prosymna ruspolii* vs. *Pareas nuchalis*); the divergence within Xenodontinae ranged from 0.096 (*Eridiphas slevini* vs. *Hypsiglena torquata*) to 0.240 (*Pseudoleptodeira latifasciata* vs. *Alsophis portoricensis*). For both genes, the divergence between the two species of *Thermophis* was more than the minimum divergences within all ingroup taxa, and less than (but very similar to) the minimum of divergences within Xenodontinae (Table 3). Based on this fact, the validity of *T. zhaoermii* is further affirmed. For each group in Table 3, the maximum, minimum, and mean values of cyt *b* were very close to, (very slight more) than those of ND4. These suggest that cyt *b* and ND4 genes evolve at comparable rates (agreeing with the finding of Kelly et al., 2003), and possibly indicating that the evolutionary rate of cyt *b* is slightly faster than ND4 in caenophidian snakes.

No stop codons were found in any sequence used in the present study.

3.2. Topological results

The topologies of BI and ML bifurcating trees derived from each "big" dataset were nearly identical. The general grouping pattern of terminal taxa between each "big" datasets were identical. Fig. 2 showed the BI tree with Posterior probabilities (PP) of each clade derived from the combined dataset. The clades recovered and their PP

values of BI trees of the trees based on individual cyt *b* and ND4 sequences are listed in Table 4 and 5, respectively (trees not shown).

Based on each "big" datasets, and using both BI and ML approaches, *Acrochordus* always forms the root to the monophyletic Colubroidea, with very high supporting PP values (see Fig. 2, Tables 4 and 5). This result is in agreement with many earlier studies of morphological analyses and DNA sequence data (e.g., Lawson et al., 2005). All trees in this study reveal that the Viperidae form a monophyletic group, with 100% PP in BI trees. Based on cyt *b* sequences, Table 4 shows that *Pareas* is the sister genus to Viperidae with a 99% PP. Based on ND4 sequences, supported by a 99% PP in BI tree, *Pareas* forms the sister genus to all ingroup terminal taxa excluding *Acrochordus* (Table 5) which is the root to Colubroidea. In the combined dataset, the relationship of *Pareas* and Viperidae is not resolved. Regardless of the exact relationship of *Pareas* and Viperidae, they always form the outgroup to Clade A + Clade B (i.e., all remaining caenophidian taxa excluding *Acrochordus*, Viperidae, and *Pareas* in this study).

In Fig. 2, Tables 4 and 5, the components of Clade A and Clade B each nearly identical. Clade A include most snakes of Boodontinae, Psammophiinae, Pseudoxyrhophiinae, Atractaspidinae, Elapinae, and Hydrophiinae. Clade B include Colubrinae, Natricinae, and Xenodontinae. The position of Homalopsidae (*Cerberus rynchops* and *Enhydris plumbea* used in our datasets) is not certain with very low PP values. Based on cyt *b* and combined gene datasets, Homalopsidae cluster in Clade A, but based on the ND4 dataset it clusters in Clade B. The phylogenetic results based on the three "big" datasets all show that the focal taxon, *Thermophis*, clusters in the Xenodontinae clade with very high supporting PP values. A southern Chinese species, *Pseudoxenodon karlschmidti*, previously assigned to Pseudoxenodontinae also clusters in this clade based on the cyt *b* dataset (only cyt *b* sequence of this snake is available), similar to the results of Lawson et al. (2005).

Over the past several decades, many researchers have studied higher-level snake phylogeny using osteological, soft anatomical and ecological characters, and more recently using molecular data, e.g. the studies of Kelly et al. (2003) and Lawson et al. (2005) which are based on larger datasets than the others. Although taxon sampling varied between the above-mentioned two studies and this study, and details concerning the positions of several individual snake species vary, the basic relationships of the commonly recognized families and/or subfamilies are similar: i.e., *Acrochordus* (Viperidae ((Atractaspidinae, Boodontinae, Elapinae, Hydrophiinae,
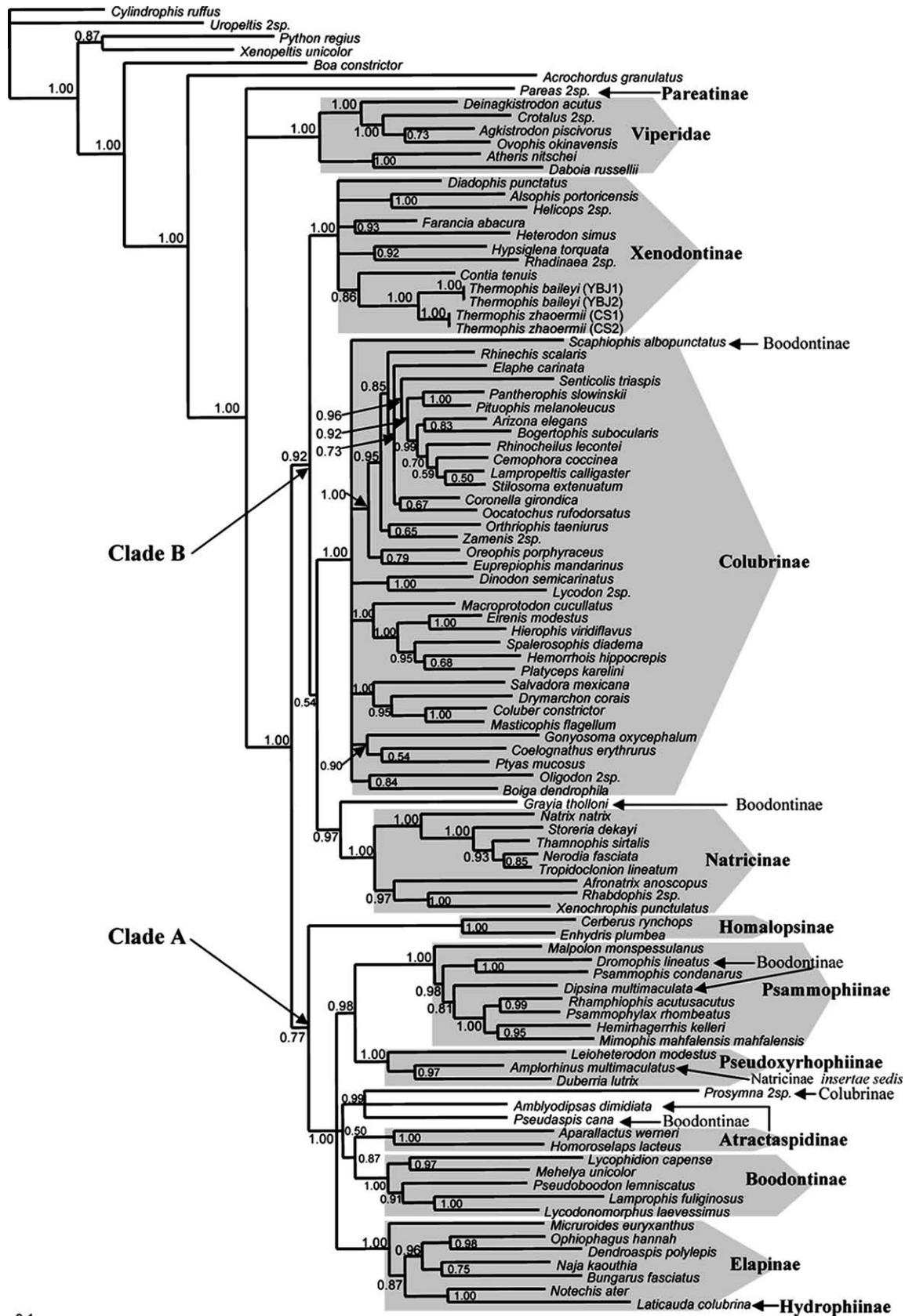
Fig. 2. The 50% majority-rule consensus tree from Bayesian analysis based on cyt *b* and ND4 combined sequences. Posterior probability values are shown at the corresponding nodes. Likelihood settings from best-fit model (TVM + I + Γ) selected by BIC in Modeltest 3.7 (−lnL = 69016.1). Base = (0.4039 0.3650 0.0437), Nst = 6, Rmat = (0.1140 4.0316 0.3851 0.2554 4.0316), Rates = gamma, Shape = 0.4572, Pinvar = 0.3075. Supraspecific terminals are labeled with generic names + 2sp.

Table 4
The clades and their posterior probabilities in the 50% majority-rule BI consensus tree based on cyt *b* sequences (total: 134 sequences) used in this study.

Clade	Posterior probability
Acrochordus + Colubroidea (in group)	1.00
Viperidae	1.00
Viperidae + *Pareas*	0.99
(Viperidae + *Pareas*) + (Clade A + Clade B)	0.95
Clade A + Clade B	0.95
Clade A	0.76
Elapinae (with *Laticauda*)	0.64
Boodontinae	1.00
Psammophiinae (with *Dipsina*, *Dromophis*)	1.00
Pseudoxyrhophiinae (with *Amplorhinus*)	0.75
Xenodermatinae	–
Homalopsidae	–
Psammodynastes	–
Atractaspidinae	–
Clade B	0.85
Xenodontinae (with *Thermophis*, *Pseudoxenodon*)	0.96
Colubrinae (with *Scaphiophis*)	0.97
Natricinae	1.00
Colubrinae + Natricinae	0.57
Calamariinae + *Grayia*	0.97
(Colubrinae + Natricinae) + (Calamariinae + *Grayia*)	0.52

Likelihood settings from best-fit model (GTR + I + G) selected by BIC in Modeltest 3.7 (−ln L = 56618.0195). Base = (0.3921 0.3718 0.0400), Nst = 6, Rmat = (0.1122 4.0439 0.2953 0.2704 3.4561), Rates = gamma, Shape = 0.4300, Pinvar = 0.3099.

Table 5
The clades and their posterior probabilities in the 50% majority-rule BI consensus tree based on ND4 sequences (total: 114 sequences) used in this study.

Clade	Posterior probability
Acrochordus + Colubroidea (in group)	0.99
Pareas + [Viperidae + (Clade A + Clade B)]	0.99
Crotalinae	0.99
Crotalinae + Azemiopinae	1.00
(Crotalinae + Azemiopinae) + Viperinae	1.00
Viperidae + (Clade A + Clade B)	0.98
Clade A + Clade B	0.99
Clade A (with *Amplorhinus*)	1.00
Psammophiinae (with *Dipsina*, *Dromophis*)	1.00
Psammophiinae + *Leioheterodon*	0.95
Elapinae (with *Laticauda*, *Pseudonaja*)	1.00
Boodontinae	–
Atractaspidinae	–
Pseudoxyrhophiinae	–
Clade B	0.97
Natricinae	0.84
Natricinae + *Grayia*	0.75
(Natricinae + *Grayia*) + Homalopsidae	0.87
Xenodontinae (with *Thermophis*)	0.99
Colubrinae	0.94

Likelihood settings from best-fit model (TVM + I + G) selected by BIC in Modeltest 3.7 (−ln L = 30365.9648). Base = (0.4378 0.3355 0.0455), Nst = 6, Rmat = (0.1006 3.3675 0.3233 0.3477 3.3675), Rates = gamma, Shape = 0.5249, Pinvar = 0.2927.

Psammophiinae, Pseudoxyrhophiinae) + (Colubrinae, Natricinae, Xenodontinae))).

Even though the support for the monophyly of Xenodontinae (including *Thermophis*) is high in the three "big" trees, the internal nodes within Xenodontinae are generally poorly supported, and its internal structure is unclear. In order to further understand the relationships between *Thermophis* and other xenodontines, we grouped them in a smaller dataset combined with 1–3 outgroup(s) (*Ophiophagus hannah*, and/or *Deinagkistrodon acutus*, and/or *Acrochordus granulatus*) to conduct further phylogenetic analyses. We also use the sequences of *Thermophis* and other xenodontines to construct unrooted phylogenetic trees. Using cyt *b*, ND4 sequences, and combined genes, and using BI, ML tree reconstruction approaches, respectively, we found that the topology of all resulting

trees varied, and node support are still generally low (Modeltest results and the trees not shown).

4. Discussion

4.1. Position of Thermophis

Previously, *T. baileyi* was deemed to belong to the Colubrinae. However, some researchers considered that it did not fit into any well known snake clade; e.g., Lawson et al. (2005) placed it as *insertae sedis* within the Colubridae. Fortunately, the ability to re-trieve sequence data of all snake clades (families and subfamilies) of Caenophidia from previous studies has now made it possible to understand the phylogenetic position of this taxon, as successfully demonstrated by the study of relict North American snakes (Pinou et al., 2004).

Our phylogenetic analyses have clearly indicated that *Thermophis* clusters in the subfamily Xenodontinae. Xenodontinae is one of the largest subfamilies of snakes with about 90 genera and more than 500 species; all current members are restricted to the New World (Cadle and Greene, 1993). The habitat of *Thermophis* is dra-matically different to its closest relatives, being endemic to the high and cold Tibetan Plateau, whereas xenodontines are primarily tropical species, with most occurring in Central America, South America, and the West Indies (Vidal et al., 2000). The relationship between Xenodontinae and *Thermophis* is also supported by a for-merly morphological studies: Zhang et al. (1984) found that *T. bai-leyi* was very similar to xenodontines in hemipenial morphology.

4.2. Biogeography

For some groups of snakes distributed in the Holarctic Region, research concerning their origin, dispersal route, and divergence time have been conducted. These biogeographic studies have gen-erally accepted that some clades of snakes originated in the Palae-arctic and/or Oriental Realm, have subsequently dispersed into the Nearctic Realm. For example, the results of Burbrink and Lawson (2007) suggested that "the ratsnakes originated in tropical Asia in the late Eocene and subsequently dispersed to the Western and Eastern Palearctic by the early Oligocene", and "the monophy-letic New World ratsnakes (the Lampropeltini) diverged from Old World ratsnakes and dispersed through Beringia in the late Oligo-cene/early Miocene". As stated by Pinou et al. (2004), most of the Nearctic snake fauna can be placed in snake clades with Palearctic and Oriental snake species, and thus can explain the origin of North American crotalids, natricines, and colubrids.

All phylogenetic analyses in the present study failed to resolve the relationship between *Thermophis* and other xenodontines, even after increasing the ratio of character vs. terminal taxon. It is may necessary to attempt to use slow evolving gene(s) to resolve their relationship. Our data are not able to provide any evidence that the New World xenodontines diverged from *Thermophis* and dispersed into the New World, also failed to suggest a colonization of Asia by New World xenodontines by dispersal from the New World. An alternative plausible scenario may be that *Thermophis* and New World xenodontines evolved independently in Asia and New World, respectively, at a later time after the divergence of their common ancestor, a member of either an Asian-North American early Tertiary fauna or a Gondwanan-derived fauna (Cadle, 1985). Nevertheless, more work is needed to put forward robust hypotheses.

Most of the numerous mountain ranges in China run west to east, but the Hengduan Mts. stretch north to south. The name "Hengduan" means "to transect" and "cut downward" in Chinese. Topographically, in this area, parallel mountain ranges are sepa-

rated by deep, narrowly incised river valleys. Vertical patterns of species richness and distribution patterns of flora and fauna are very marked. The famous Three Parallel Rivers – Nujiang (Salween), Lantsang (Mekong), and Jinshajiang (upriver of Changjiang) – traverse this area from north to south, and partition the two sampling sites of this study. With such strong geographic heterogeneity, Hengduan Mts. have therefore became a refugia, and also naturally resulted in vicariance restricting gene flow.

Harrison et al. (1992) stated that "thermochronologic, sedimentologic, oceanographic, and paleoclimatic studies suggest that rapid uplift and unroofing of southern Tibet began about 20 million years ago and that the present elevation of much of the Tibetan plateau was attained by about 8 million years ago". Estimates of rates of mtDNA sequence divergence for reptile species range from 0.47 to 1.32% per million years (Zamudio and Greene, 1997). Using this wider range, the time of sequence divergence between the two species of *Thermophis* in this study is likely to fall within the range 7.0–22.1 million years ago. This time estimated here basically tallies with the rapid uplift of the Tibetan Plateau suggested by Harrison et al. (1992). The divergence of the two species in *Thermophis* was caused by the barrier of the Hengduan Mts., and the speciation had almost occurred when the Tibetan Plateau attained present elevation.

Acknowledgments

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