Zootaxa 3873 (4): 425–440
www.mapress.com/zootaxa/

Article

ISSN 1175-5326 (print edition)
ZOOTAXA
ISSN 1175-5334 (online edition)

http://dx.doi.org/10.11646/zootaxa.3873.4.5
http://zoobank.org/urn:lsid:zoobank.org:pub:3B3F6C1E-BBD9-4ED3-91CC-4B89FBC9E348

A taxonomic revision of the Asian keelback snakes, genus *Amphiesma* (Serpentes: Colubridae: Natricinae), with description of a new species

PENG GUO[1,2,6], FEI ZHU[1,3], QIN LIU[1,2], LIANG ZHANG[4], JIAN X. LI[1], YU Y. HUANG[1] & R. ALEXANDER PYRON[5]

[1]*College of Life Sciences and Food Engineering, Yibin University, Yibin 644007, China*
[2]*Biology Institute, Yibin University, Yibin 644007, China*
[3]*College of Life Sciences, Sichuan University, Chengdu 610064, China*
[4]*South China Institute of Endangered Animals, Guangzhou 510260, China*
[5]*Department of Biological Sciences, 2023 G St. NW, The George Washington University, Washington, D.C. 20052 USA*
[6]*Corresponding author. E-mail: ybguop@163.com*

Abstract

The Asian keelback snakes (genus *Amphiesma*) are a widely distributed group of Old World natricines, inhabiting a variety of niches and exhibiting significant morphological variation. Recent molecular phylogenies suggest that this genus is not monophyletic, and that additional cryptic diversity is also likely present. We conducted a phylogenetic analysis of the group based on 3162 bp of one mitochondrial gene (Cyt. *b*) and three nuclear genes (C-mos, Rag1, NT3), sampling 18 species in addition to those sequenced in previous works. All analyses consistently show that *Amphiesma* consists of three distinct, monophyletic lineages with strong support. We divide *Amphiesma* into three genera, *Amphiesma, Hebius,* and *Herpetoreas*. The genus *Amphiesma* is monotypic, *Herpetoreas* contains three species, and *Hebius* comprises the remaining 39 species. On the basis of a combination of molecular analyses and external morphological comparisons, we describe a new species in the *Herpetoreas* group from China as *H. burbrinki* sp. nov. Several other species are shown to be non-monophyletic or contain significant levels of intraspecific genetic diversity. Another Old World natricine genera, *Xenochrophis* is also found to be non-monophyletic. Our results indicate that further taxonomic revisions are needed in Natricinae, at multiple levels.

Key words: Natricinae, *Amphiesma*, *Hebius, Herpetoreas*, Snakes, new species, Southeastern Asia, systematics

Introduction

The genus *Amphiesma* sensu lato is one of the largest and most diverse groups in Natricine, with at least 42 species (Pyron *et al*. 2011; Guo *et al*. 2012; Uetz 2013). The members of this group are generally small- or medium-sized, with total lengths not exceeding one meter (Zhao 2006). They are terrestrial to semiaquatic, oviparous, and generally considered harmless (non-venomous). Species in this group also have a wide distribution throughout southern, eastern, and southeastern Asia, ranging from Pakistan and India to eastern China, north into southernmost Russia and Japan, and southwards to Sumatra and Sulawesi (Fig. 1; see Uetz 2013).

Based on morphological characters including hemipenial morphology, dentition, and external scalation, Malnate (1960) divided the genus *Natrix* sensu lato into several genera, revalidating the genus *Amphiesma,* which had been erected by Duméril, Bibron, and Duméril (1854) with the type species *A. stolatum*. The diagnostic characters of *Amphiesma* are defined as: hemipenes and sulci spermaticus simple; maxillary teeth in continuous series, gradually becoming larger posteriorly in the series or the last two teeth abruptly enlarged; terrestrial; internasals broad anteriorly, nostrils lateral; apical pits present or absent (Malnate 1960).

Due to their wide distribution, secretive habits, rarity of many species, and cryptic diversity, it is very difficult to collect the samples needed for a comprehensive systematic study on this group. A recent molecular study of Natricinae found that this genus is paraphyletic (Guo *et al*. 2012). A similar result was also found in subsequent works (Pyron *et al*. 2013a, b). However, incomplete sampling in these works precluded a taxonomic

Accepted by Z. Nagy: 17 Sept. 2014; published: 17 Oct. 2014

recommendation. Sampling of additional species and characters are thus needed to resolve taxonomic issues in the group.

The development of DNA sequencing technology and application of robust analytical methods have provided new evidence for examining the relationships between organisms, in particular for those whose morphological characteristics are highly convergent. Increasingly, molecular phylogenies indicate that taxonomies and evolutionary relationships based solely on morphological traits have possibly been mislead by morphological convergence (Herrmann *et al.* 2004; Guo *et al*. 2007, 2013; Huang *et al*. 2007). Natricine snakes, and *Amphiesma* in particular, seem to be a prime example of this phenomenon.

In this work, we sample 18 species and multiple individuals per species (about half of the 42 species known in the genus) to resolve relationships and taxonomy in *Amphiesma* sensu lato. We used one mitochondrial gene and three nuclear genes to evaluate the relationship of the taxa in the genus *Amphiesma*. We use this phylogeny to revise taxonomy within *Amphiesma,* describe a new species, and suggest avenues for future investigation.

Material and methods

Sampling. All samples sequenced were obtained through fieldwork or tissue loans from colleagues or Museums. Fifty-four individuals representing 18 known species and several unidentified species of the *Amphiesma* complex were sequenced and analyzed in this work (Table 1). To investigate monophyly of *Amphiesma* sensu lato, some representatives of its relatives were also included from previous studies (Pyron *et al*. 2011; Guo *et al*. 2012). In total, an additional 10 genera and 16 species were included, with most sequences newly generated in this work and some retrieved from Genbank (Table 1). *Natrix natrix* was chosen as outgroup in all phylogenetic analyses (Pyron *et al*. 2011; Guo *et al*. 2012).

Sequencing. Total DNA was extracted from liver or muscle tissues preserved in 85% alcohol, using standard methods (Sambrook and Russell, 2002). The entire gene sequences for the mitochondrial cytochrome *b* (Cyt. *b*) and partial sequences of three nuclear genes (oocyte maturation factor mos [C-mos], recombination-activating gene 1 [Rag1], and neurotrophin 3 [NT3]) were amplified by polymerase chain reaction (PCR). Primer sequences for these loci are listed in Table 2. The cycling parameters were identical to previous studies (Groth & Barrowclough 1999; Burbrink *et al*. 2000; Lawson *et al*. 2005; Noonan & Chippindale 2006). Prior to sequencing, PCR products were purified using various commercial kits. The double-stranded product was sequenced in a commercial company (BGI, Beijing, China).

Sequences were edited manually using Seqman in DNAstar (DNASTAR, Inc.), aligned in Mega 6 using the ClustalW algorithm with default parameters (Tamura *et al.* 2013), and checked by eye for ambiguous alignments. We translated protein-coding fragments into amino acid sequences using Mega 6 (Tamura *et al*. 2013), and aligned them with the published sequences to confirm an open reading frame was maintained and that we had not amplified likely pseudogenes (Zhang & Hewitt 1996). Average divergence estimates between species of interest were calculated from mitochondrial sequences.

Phylogenetic inference. Phylogenetic analyses were performed using Bayesian Inference (BI) and Maximum Likelihood (ML). For the three nuclear genes, heterozygous sequences were phased using Seqphase (Flot 2010) and Phase (Stephens *et al*. 2001), and we selected at random one of the phased nuclear gene copies to represent each individual on this and subsequent analyses to avoid extremely time-consuming computations.

Phylogenetic trees were estimated separately for the mitochondrial locus, nuclear-gene data set (C-mos, NT3 and Rag 1), and the concatenated data set of four loci (mtDNA and nDNA). The optimal partitioning scheme for each analysis was chosen by PartitionFinder under BIC (Lanfear *et al.* 2012).

We used MrBayes 3.2.2 (Ronquist & Huelsenbeck 2003; Ronquist *et al*. 2012) for the BI analyses, and all searches were run with three independent runs and each initiated with a random tree. Each run consisted of four Markov chains (three heated chains and a single cold chain) estimated for 2 billion generations and sampled every 2000 generations with 25% initial samples discarded as burn-in.

Substitution parameters were unlinked and rates were allowed to vary across partitions. Convergence was assessed by examining effective sample sizes (ESS values >200 are recommended) and likelihood plots through time in TRACER v1.4 (Rambaut & Drummond 2007). After confirming that three analyses reached stationarity at a similar likelihood score and the topologies were similar, the resultant trees were combined to calculate posterior probabilities (PP) for each node in a 50% majority-rule consensus tree.

TABLE 1. Samples and sequence sources for the analyses used in this study (Abbreviations: AMNH: American Museum of Natural History, New York; CAS: California Academy of Science, San Francisco, USA; MVZ: Museum of Vertebrate Zoology, Berkeley; USNM: United States National Museum, Washington; YBU: Yibin University; GP: Guo Peng, own catalogue number).

Taxon	Voucher number	Locality	GenBank accession number			
			Cyt.*b*	C-mos	NT3	Rag1
Amphiesma stolatum	GP 2239	Guangdong, China	KJ685694	KJ685644	KJ685746	KJ685586
Amphiesma stolatum	GP 2550	Taiwan, China	KJ685702	KJ685652	KJ685755	KJ685593
Amphiesma stolatum	GP 901	Guizhou, China	JQ687450	JQ687432	KJ685768	KJ685606
Amphiesma stolatum	USNM 524073	Myanmar	KJ685711	KJ685661	KJ685771	KJ685610
Amphiesma stolatum	-	-	AF471030	AF471097	-	-
Amphiesma stolatum	GP 2213	Guangdong, China	KJ685693	KJ685643	KJ685745	KJ685585
Atretium yunnanensis	GP 842	Yunnan, China	JQ687448	GQ281787	KJ685764	KJ685602
Hebius atemporale	GP 1626	Guangdong, China	KJ685680	KJ685630	KJ685732	KJ685572
Hebius atemporale	GP 2318	Yunnan, China	KJ685695	KJ685645	KJ685747	KJ685587
Hebius bitaeniatum	CAS 215037	Yunnan, China	KJ685667	KJ685616	KJ685717	KJ685560
Hebius bitaeniatum	GP 1863	Guizhou, China	KJ685686	KJ685636	KJ685738	KJ685578
Hebius bitaeniatum	GP 1940	Guangxi, China	KJ685688	KJ685638	KJ685740	KJ685580
Hebius bitaeniatum	GP 2402	Guizhou, China	KJ685698	KJ685648	KJ685750	-
Hebius boulengeri	GP 1789	Guangdong, China	KJ685684	KJ685634	KJ685736	KJ685576
Hebius boulengeri	GP 2134	Hainan, China	KJ685691	KJ685641	KJ685743	KJ685583
Hebius boulengeri	GP 2433	Fujian, China	KJ685699	KJ685649	KJ685751	KJ685589
Hebius cf. boulengeri	AMNH 148562	Ha Giang, Vietnam	KJ685664	KJ685613	KJ685714	KJ685557
Hebius cf.boulengeri	MVZ 236752	Hainan, China	KJ685710	KJ685660	KJ685770	KJ685609
Hebius craspedogaster	GP 1240	Guizhou, China	KJ685672	KJ685622	KJ685723	KJ685565
Hebius craspedogaster	GP 139	Sichuan, China	JQ687437	JQ687429	KJ685730	KJ685569
Hebius craspedogaster	GP 1636	No detail	KJ685681	KJ685631	KJ685733	KJ685573
Hebius craspedogaster	GP 1963	Sichuan, China	KJ685689	KJ685639	KJ685741	KJ685581
Hebius craspedogaster	GP 336	Sichuan, China	KJ685703	KJ685653	KJ685756	KJ685594
Hebius craspedogaster	GP 455	Sichuan, China	KJ685704	KJ685654	KJ685759	KJ685597

……*continued on the next page*

TABLE 1. (Continued)

Taxon	Voucher number	Locality	GenBank accession number			
			Cyt.*b*	C-mos	NT3	Rag1
Hebius deschauenseei	AMNH 148575	Ha Giang, Vietnam	KJ685665	KJ685614	KJ685715	KJ685558
Hebius johannis	GP 1569	Yunnan, China	KJ685678	KJ685628	KJ685731	KJ685571
Hebius johannis	GP 897	Yunnan, China	KJ685708	KJ685658	KJ685767	KJ685605
Hebius khasiensis	AMNH 148552	Quang Nam, Vietnam:	KJ685663	KJ685612	KJ685713	KJ685556
Hebius khasiensis	CAS 221504	KaChin state, Myanmar	KJ685668	KJ685617	KJ685718	KJ685561
Hebius khasiensis	CAS 221525	KaChin state, Myanmar	KJ685669	KJ685618	KJ685719	KJ685562
Hebius metusium	GP 1712	Sichuan, China	KJ685682	KJ685632	KJ685734	KJ685574
Hebius metusium	GP 871	Sichuan, China	KJ685707	KJ685657	KJ685766	-
Hebius modesta	CAS 234262	Yunnan, China	KJ685671	KJ685620	KJ685721	KJ685564
Hebius modesta	MVZ 226514	Vinh Phu, Vietnam	KJ685709	KJ685659	KJ685769	KJ685608
Hebius octolineatum	GP 1242	Guizhou, CHina	KJ685673	KJ685623	KJ685724	-
Hebius octolineatum	GP 1244	Guizhou, China	KJ685674	KJ685624	KJ685725	-
Hebius optatum	AMNH 147155	Vinh Phu, Vietnam	KJ685662	KJ685611	KJ685712	KJ685555
Hebius optatum	GP 1048	-	-	KJ685621	KJ685722	-
Hebius optatum	GP 1885	Guizhou, China	KJ685687	KJ685637	KJ685739	KJ685579
Hebius parallela	CAS 215036	Yunnan, China	KJ685666	KJ685615	KJ685716	KJ685559
Hebius popei	GP 2169	Hainan, China	KJ685692	KJ685642	KJ685744	KJ685584
Hebius popei	GP 2386	Guizhou, China	KJ685697	KJ685647	KJ685749	KJ685588
Hebius popei	GP 63	Hainan, China	KJ685705	KJ685655	KJ685763	KJ685601
Hebius sauteri	GP 2382	Sichuan, China	KJ685696	KJ685646	KJ685748	-
Hebius sauteri	GP 2549	Taiwan, China	KJ685701	KJ685651	KJ685754	KJ685592
Hebius sauteri	GP 864	Sichuan, China	KJ685706	KJ685656	KJ685765	KJ685603
Hebius sp.	GP 1618	Myanmar	KJ685679	KJ685629	-	-
Hebius sp.	GP 1766	Jiangxi, China	KJ685683	KJ685633	KJ685735	KJ685575
Hebius sp.	GP 1790	Guangdong, China	KJ685685	KJ685635	KJ685737	KJ685577
Hebius venningi	CAS 233206	KaChin state, Myanmar	KJ685670	KJ685619	KJ685720	KJ685563

……*continued on the next page*

TABLE 1. (Continued)

Taxon	Voucher number	Locality	GenBank accession number			
			Cyt.*b*	C-mos	NT3	Rag1
Hebius venningi	GP 1300	Yunnan, China	KJ685675	KJ685625	KJ685727	-
Hebius venningi	GP 2468	Thailand	KJ685700	KJ685650	KJ685752	KJ685590
Hebius vibakari	GP 1345	Liaoning, China	KJ685676	KJ685626	KJ685728	KJ685567
Hebius vibakari	GP 1352	Heilongjiang, China	KJ685677	KJ685627	KJ685729	KJ685568
Herpetoreas burbrinkii	GP 600/YBU 071128	Xizang, China	JQ687443	GQ281781	KJ685761	KJ685599
Herpetoreas platyceps	GP 2096	Xizang, China	KJ685690	KJ685640	KJ685742	KJ685582
Afronatrix anoscopus	-	-	AF420073	AF471123	EU390906	EU402832
Atretium schistosum	-	-	KC347487	KC347383	-	KC347421
Lycognathophis seychellensis	-	-	-	FJ387220	-	-
Macropisthodon rudis	GP 1266	Guizhou, China	JQ687452	JQ687434	KJ685726	KJ685566
Macropisthodon rudis	GP 384	Guangdong, China	JQ687442	GQ281780	KJ685758	KJ685596
Natriciteres olivacea	-	-	AF471058	AF471146	-	-
Natrix natrix	-	-	AY487756	AF471121	EU390931	EU402858
Opisthotropis cheni	GP 383	Guangdong, China	JQ687441	GQ281779	KJ685757	KJ685595
Rhabdophis nuchalis	GP 251	Sichuan, China	JQ687438	GQ281786	KJ685753	KJ685591
Rhabdophis subminiata	GP 57	Hainan, China	JQ687436	GQ281777	KJ685760	KJ685598
Rhabdophis tigrinus	GP 613	Liaoning, China	JQ687444	GQ281785	KJ685762	KJ685600
Sinonatrix annularis	GP889	Jiangxi, China	JQ687431	JQ687449	-	KJ685604
Sinonatrix percarinata	GP956	Chengdu, China	JQ687433	JQ687451	-	KJ685607
Trachischium monticola	GP1487	Xizang,China	JQ687428	JQ687435	-	KJ685570
Xenochrophis asperrimus	-	-	KC347480	KC347414	-	KC347451
Xenochrophis flavipunctatus	-	-	-	AF544714	FJ434102	-
Xenochrophis piscator	-	-	GQ225659	GQ225669	EU390941	EU402868
Xenochrophis punctulatus	-	-	AF471079	AF471106	-	-
Xenochrophis schnurrenbergeri	-	-	GQ225660	GQ225668	-	-
Xenochrophis vittatus	-	-	EF395895	EF395930	-	-

ML trees were constructed in the program RaxMLv7.2.6 (Stamatakis 2006) with GTRGAMMA model under the same partitioning scheme as the BI analysis. Branch support was assessed by performing 1000 non-parametric bootstrap (BS) replicates of the topology. For the sake of discussion, nodes with 95% Bayesian posterior probabilities were considered to be strongly supported (Felsenstein 2004); in the ML analysis, nodes with 70% bootstrap support were considered strongly supported (Hillis & Bull 1993).

TABLE 2. Primers Used for DNA Amplification and Sequencing.

Primers	Primer sequences	Use	Reference
Cyt. *b*			
L14919	5'-AACCACCGTTGTTATTCA ACT-3'	Amp./Seq.	Burbrink *et al*. 2000
H16064	5'-CTTTGGTTTACAAGA ACAATGCTTTA-3'		
C-mos			
S77	5-CATGGACTGGGATCAGTTATG-3'	Amp./Seq.	Lawson *et al*. 2005
S78	5-CCTTGGGTGTGATTTTCTCACCT-3		
Rag1			
R13	5'-TCTGAATGGAAATTCAAGCTGTT-3'	Amp./Seq.	Groth & Barrowclough 1999
R18	5'-GATGCTGCCTCGGTCGGCCACCT TT-3'		
NT3			
NT3F	5'-ATATTTCTGGCTTTTCTCTGTGGC-3'	Amp./Seq.	Noonan & Chippindale 2006
NT3R	5'-GCGTTTCATAAAAATATTGTTTGACCGG-3'		
NTF3_F2	5'- TTCTCTGTGGCATCCAATCRACCA -3'	Amp./Seq.	This study
NTF3_R2	5'- TATTGTTTGACCGGAGAYGGGCCCA -3'		

Monophyly. Our phylogenetic analyses failed to recover all putative species of *Amphiesma* sensu lato as monophyletic (three different clades; see below). We therefore evaluated support for the alternate hypothesis that all *Amphiesma* sensu lato species sampled (clades A, B, and C) form a group, and that the species in clades A and B form a group. We tested this for mtDNA, nDNA and concatenated muti-locus (mtDNA and nDNA) trees by enforcing two monophyletic constraints, (A+B+C) and (A+B), for these lineages. The best scoring ML trees from the constrained and unconstrained analyses were compared using S-H tests (Shimodaira & Hasegawa 1999) in RAxML v7.2.6. In BI analysis, Bayes factors (BF) calculated from the log files of the unconstrained and constrained analyses were used to test alternative hypotheses. A log difference in the range of 3-5 is typically considered minimum evidence in favor of the unconstraint tree based on the guidelines of Kass and Raftery (1995).

Morphological examination. One of the specimens (YBU 071128) analyzed in molecular phylogeny showed strong genetic divergence from the other species, and is found in a remote location that does not harbor any other known *Amphiesma* species. In order to explore whether it was an undescribed species, we examined and recorded some external morphological data and compared to its relatives.

We followed the methods used in Guo *et al*. (2009) to record a number of characters relating to scalation, color pattern, and body proportions from the specimen. Ventral scale count was done according to Dowling (1951). The other characters recorded are described as below. SVL: distance between the tip of the snout and the cloaca (snout-vent length); TL: distance between the cloaca and the tip of the tail (tail length); SC8TO6: subcaudal scale position of the reduction of the tail dorsal scales from 8 to 6 scale rows; DV8TO6: dorsoventral scale position of reduction of the tail dorsal scales from 8 to 6 scale rows; SC6TO4: subcaudal scale (SC) position of the reduction of the tail dorsal scales from 6 to 4 scale rows; DV6TO4: dorsoventral scale position of reduction of the tail dorsal scales from 8 to 6 scale rows; VS19TO17: ventral scale position of the reduction of dorsal scales from 19 to 17 scale rows; DV19TO17: the dorsoventral scale position of the reduction (the lowest of the two merging scale rows) of dorsal scales from 19 to 17 scale rows.

Results

Sequence data and alignment. The final alignment of four gene fragments consisted of 3162 base pairs: 1074 bps

from Cyt. *b*, 584 bps from c-mos, 499 bps from NT3, and 1005 bps from Rag1. The sequence completeness for ingroup taxa was relatively high: Cyt. *b* (54 taxa), cmos (55 taxa), NT3 (54 taxa) and Rag1 (47 taxa) representing 98%, 100%, 98%, and 85% respectively. We translated the protein-coding sequences into amino acid, and no stop codons were detected, indicating that unintentional amplification of pseudogenes was unlikely. Base frequencies were estimated as A = 0.3168, C = 0.2733, G = 0.1654, T=0.2445. New sequences generated here were deposited in GenBank (Table 1, Accession Nos. KJ685555-KJ685771).

Phylogenetic relationships. The optimal models of sequence evolution of each partition are identified by Partitionfinder and listed in Table 3. Bayesian analysis based on the mitochondrial data, nuclear genes, and the concatenated data set recovered effective samples sizes above 200 for all parameters indicating adequate mixing. All three BI analyses had similar tree topologies with previous studies to some extend (Guo *et al*. 2012; Pyron *et al*. 2013 a, b). In BI trees, the putative species of *Amphiesma* sensu lato did not form a monophyletic group, but instead consist of three distinct clades with very high support values (Fig. 2). The first clade (clade C) is composed of six individuals of *Amphiesma stolatum* with strong support (1.00 PP in three trees). This lineage is part of an unresolved clade containing the genera *Xenochrophis* (which is also paraphyletic), *Atretium*, and *Rhabdophis*.

TABLE 3. The best Partitioning scheme for the entire data set found in Partitionfinder under BIC.

Partition	Best model
Cyt. *b*-pos. 1	GTR+I+G
Cyt. *b*-pos. 2	HKY+I+G
Cyt. *b*-pos. 3	TIM+G
C-mos-pos 1 & pos. 3, NT3-pos.1 & pos.2, Rag1-pos.1 & pos.3	HKY+I+G
C-mos-pos.2 & Rag1-pos.3	HKY+G
NT3-pos.3	HKY+I+G

The second clade includes only two individuals: one is *A. platyceps*, and the other is a sample from Xizang, China (YBU 071128). This is unexpected, as this specimen was previously identified as *A. craspedogaster* (Guo *et al*. 2008). This clade was strongly supported with more than 0.97 PP in all BI trees. This group is separated from the remaining species of *Amphiesma* sensu lato by *Trachischium* in the nuclear and combined analyses.

The last clade (clade A) comprises the remaining *Amphiesma* sensu lato species. This clade also received support values of ~1.0 PP for all analysis. Of these trees, three distinct and highly supported subclades A1, A2, and A3 were detected for nDNA data. However, these subclades have not been recovered in the other two trees. In addition, several species in clade A are not monophyletic; these species are: *A. bitaeniatum*, *A. craspedogaster*, *A. sauteri*, *A. modesta*, *A. venningi*, *A. khasiensis*.

ML trees showed similar topologies with corresponding BI trees. Three same clades were consistently uncovered with high support values in mtDNA and the concatenated data trees, but low in clade B in nDNA ML tree (53% BS). However, most shallow nodes showed very week support (Fig. 2).

TABLE 4. Hypothesis testing. DLH = difference of the likelihood scores of best unconstrained and constrained ML trees. ML = maximum likelihood, BI = Bayesian Inference. "+" indicates strong rejection of monophyly, "-" indicates that monophyly is neither strongly supported or rejected.

Analysis	S–H test (ML)		Bayes factors (BF)	Results	
	DLH	P value	Cyt. *b*	ML	BI
Multilocus (A, B, C)	1379	P<0.01	696	+	+
Multilocus (A, B)	27	P>0.05	35.5	-	+
nDNA (A, B, C)	544	P<0.01	62	+	+
nDNA (A, B)	449	P<0.01	37	+	+
Cytb (A, B, C)	35	P>0.05	18	-	+
Cytb (A, B)	4.4	P>0.05	3.5	-	+

Monophyly test. In the ML analyses, S-H tests rejected ($P < 0.01$) the monophyly of all putative species of *Amphiesma* sensu lato for mtDNA, nDNA, and concatenated data set (mtDNA and nDNA), but not against ($P>0.05$) the hypothesis that the species in clades A and B are monophyletic for mtDNA and concatenated data set (Table 4). In the Bayesian tests, monophyly of all putative species of *Amphiesma* sensu lato and those in clades A and B was rejected (log10 Bayes factor >3) for all BI trees (Table 4).

Morphology of YBU071128. External morphological examination indicated that the specimen YBU071128 was very different from those species in clades A, C and its close relative *A. platyceps*. Combining the molecular data, we concluded that this specimen was not a representive of any other described species of *Amphiesma* sensu lato, and represented an undescribed species (see below).

Discussion

Implications for the taxonomy of *Amphiesma* sensu lato. Phylogenetic analyses based on four independent molecular loci consistently indicate that Asian keelback snakes *Amphiesma* sensu lato consist of three distinct clades (A, B, and C) that do not form a monophyletic group, suggesting that the genus *Amphiesma* sensu lato as defined presently represents an extreme case of convergent phenotypic evolution, and requires substantial taxonomic revision. This result is congruent with previous results (Guo *et al*. 2012; Pyron *et al*. 2013a, b).

Several generic names have historically been applied to species in this group, presenting a bit of confusion. The genus *Amphiesma* (Dumeril *et al*. 1854) is represented by the type species *A. stolatum*. Günther (1860) erected the genus *Herpetoreas* for *A. sieboldii,* a close relative of *A. platyceps* (Malnate 1966). Thompson (1913) erected the genus *Hebius* for *A. vibakari*, on the basis of unique hemipenial characters. These three names correspond to the three clades recovered here.

As the three clades of *Amphiesma* sensu lato do not form a monophyletic group, we propose to rectify this by recognizing these three genera. As noted above, *A. stolatum* is the type species of *Amphiesma* Duméril, Bibron and Duméril, 1854. Thus *Amphiesma* sensu stricto will be restricted to member of the clade C, including only *A. stolatum* presently.

For clade B, the type species *A. sieboldii* was not included in this work, but we sampled *A. platyceps*, with which *A. sieboldii* was synonymized previously, and which is considered a close relative (see Smith 1943; Malnate 1966). Thus we took a conservative and tentative strategy to designate the generic name *Herpetoreas* for the species in clade B, which now contains *H. platyceps*, *H. sieboldii,* and the new species (see below). While non-monophyly of B+C is not strongly supported in all analyses, their genetic distinctiveness is on par with other genera in Natricinae, and thus recognition of *Herpetoreas* would not be unwarranted even if it were found to represent the sister-group of Clade C in future analyses.

Clade C contains species with two available generic synonyms. The species *Amphiesma modestus* is the type species of *Paranatrix* Mahendra, 1984. However, the older name *Hebius* Thompson, 1913 is available for *Amphiesma vibakari*, and thus has priority for this clade (Ride 1999). We resurrect *Hebius* for this clade, which now contains the remaining 39 species.

Note that many of these species are not monophyletic, and diversity in this genus is likely much higher. It is also possible that some of the 24 species not sampled in this study are not part of *Hebius*, and may be found to represent additional distinct lineages in the future. We group them here conservatively, as *Hebius* contains the most species and widest distribution of taxa sampled in our analyses. Previously used morphological characters are clearly inadequate to resolve genus-level taxonomy in this group, so we cannot place un-sampled species based on morphology. It is also possible that some species will be found to group with *Herpetoreas* or *Amphiesma* in the future. However, we believe that placing them in the largest group, *Hebius*, is the most conservative and stable solution for the time being.

As a final note, *Amphiesma* sensu stricto is part of an unresolved clade that contains *Xenochrophis*, *Atretium*, and *Rhabdophis*, and the latter two render *Xenochrophis* non-monophyletic in most analyses. Thus, further taxonomic revision is likely needed to resolve the genus-level nomenclature of this group. In any event, *Amphiesma* is valid as a monotypic genus in all results thus far, as the species *A. stolatum* is monophyletic. We await further sampling (e.g., the type species *X. craspedogaster*) before making additional taxonomic changes, noting that any potential resolutions would preserve *Amphiesma*, as it is the oldest name. There is apparently deep intraspecific genetic diversity in *A. stolatum* based on our results, and further broadly sampling may find additional species in this lineage.

Description of a new species. Both morphological comparison and molecular phylogenetic revealed that the specimen YBU071128 is much different from the other species of *Amphiesma* sensu lato. Thus we describe it here as a new species:

***Herpetoreas burbrinki* sp. nov.**
(Figs. 1 & 3)

Holotype. YBU 071128, an adult male, collected in Sep. 2007 in Zayu County, Xizang A. R., China, at an elevation of 1889 m above sea level (Fig.1).

Diagnosis. *Herpetoreas burbrinki* can be distinguished from its relatives on the basis of the following combination of characters: 1) TL/SVL ratio in the single male 0.26; 2) one or two preoculars; 3) three postoculars; 4) three anterior temporals followed by two posterior temporals; 5) eight supralabials, 3rd to 5th in contact with the eye, 7th supralabial largest; 6) ten infralabials, the first five bordering the anterior chinshields; 7) 172 ventrals (plus two preventrals) in the one single male; 8) anal plate divided; 9) 96 divided subcaudals in the one single male; 10) reduction of dorsal scales rows from 19 to 17 scale (VS19TO17) occurring above ventral scale position 108; 11) reduction of the tail dorsal scales rows from 8 to 6 (SC6TO4) occurring above subcaudal 63; 12) dorsal scales in 19-19-17 rows, all distinctly keeled.

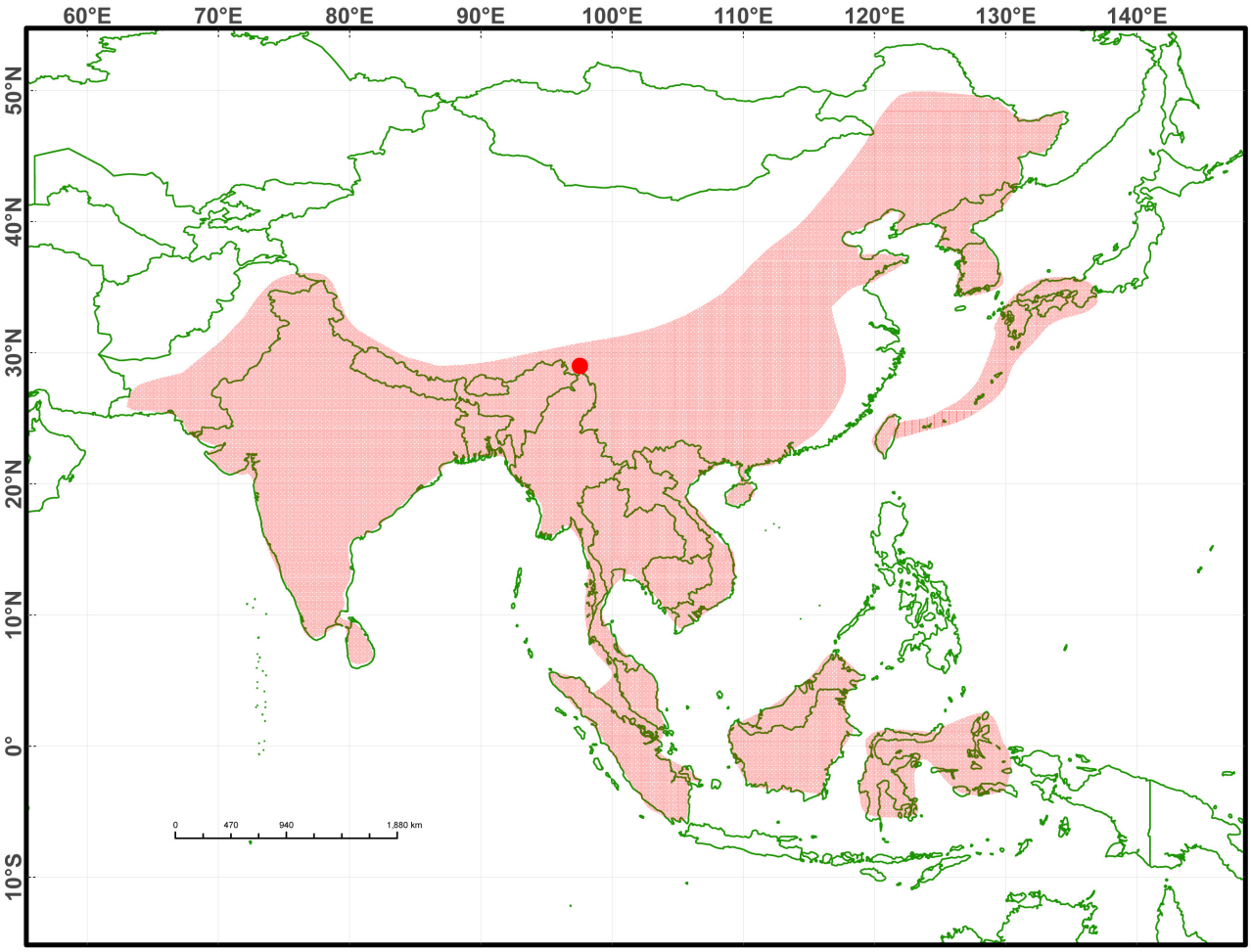

FIGURE 1. Approximately distribution of *Amphiesma* (*sensu lato*). Red dot indicates the type locality of the new species *Herpetoreas burbrinki* **sp. nov.**

Description of the holotype. Dorsal scale rows 19-19-17, all distinctly keeled. Scales on posterior part of head and temporal region keeled. Reduction from 19 to 17 dorsal scale rows occurs at the level of the 3rd and 4th dorsal

scale rows and between the 107th and 109th ventrals. There are 172 ventrals and 2 preventrals with 96 pairs of subcaudals. Anal divided. The scale rows reduction formula on the tail is:

$$10\frac{9(4+5)}{9(4+5)}8\frac{23(3+4)}{23(3+4)}6\frac{63(2+3)}{63(2+3)}4$$

Rostral visible dorsally. Internasals subtriangular, wider than long. Prefrontals large, extending laterally onto side of head. Frontal longer than wide. Supraoculars broadly in contact with prefrontal, with supraocular two-thirds as broad as frontal. Single loreal. Preoculars 2 (left) or 1 (right); postoculars 3/3. Temporals 3+2 on both sides. Supralabials 8/8, the 3rd to 5th bordering orbit. Infralabials 10/10, first 5 contacting anterior chinshields. Nostrils lateral, nasals are undivided. In preservative, the dorsal body and upper tail surfaces are dark gray. Labials and ventral surface of head faint white. A faint white stripe is present on each side of the body. SVL 495 mm, TL130 mm, ratio TL / SVL 0.26.

Etymology. This species is named after the herpetologist Frank T. Burbrink of Peoria, Illinois, in recognition of his contributions to snake systematics and evolution. We suggest the common name of this new species be Burbrink's Keelback in English and Chayu Fulianshe (Chinese).

Distribution. This species is currently known only from the type locality (Fig.1). The specimen was collected on a road located in an evergreen forest close to a town (Fig. 3). It is likely that the new species also occurs in northern Burma and/or extreme eastern India given the proximity of the type locality to their borders. No data on its feeding and reproduction are available.

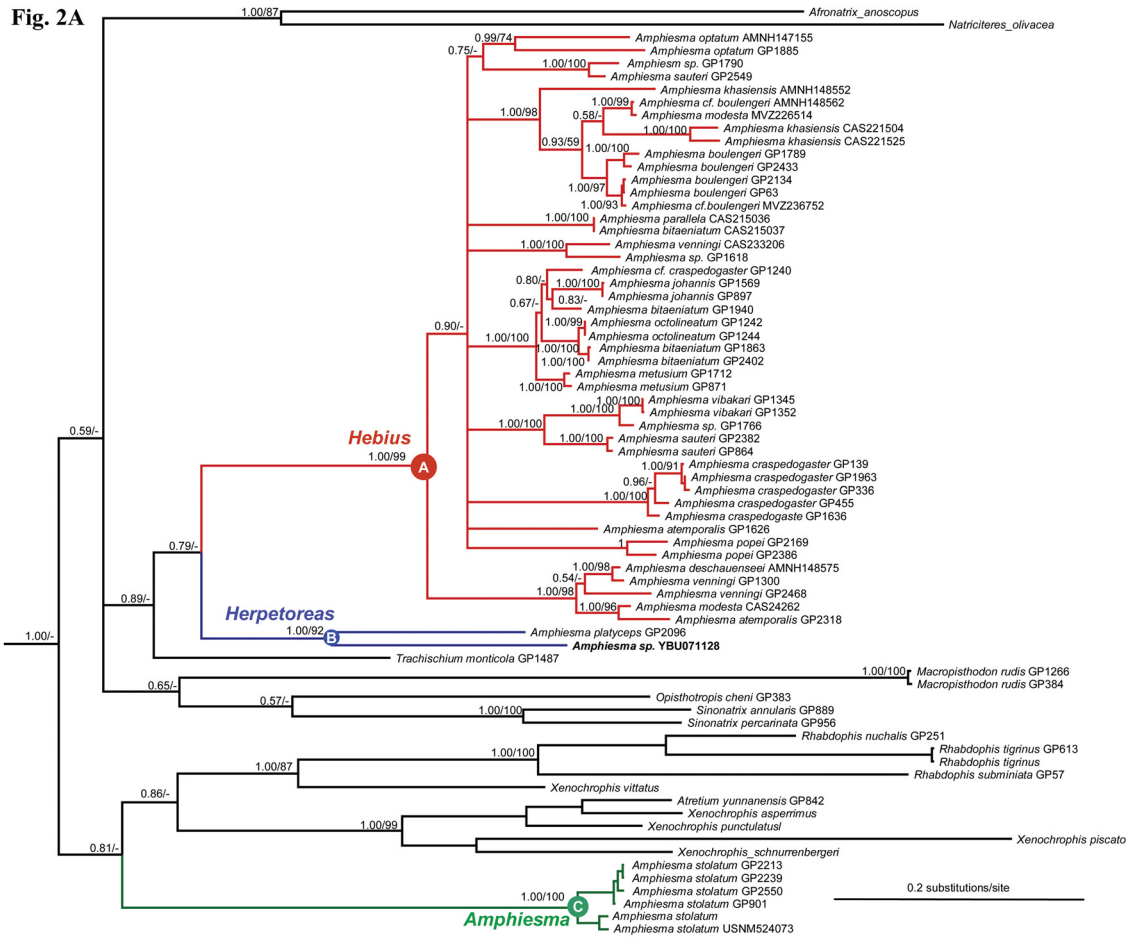

FIGURE 2A. Bayesian 50% majority-rule consensus tree inferred from mtDNA data (A)in MrBayes 3.2. The values assigned to the internodes indicate posterior probability support (before the slash) and ML bootstrap (after the slash). A node with support value <50% was indicated by "-". Branch-support indices are not given for some internodes to preserve clarity. The partition strategy is to see the main text.

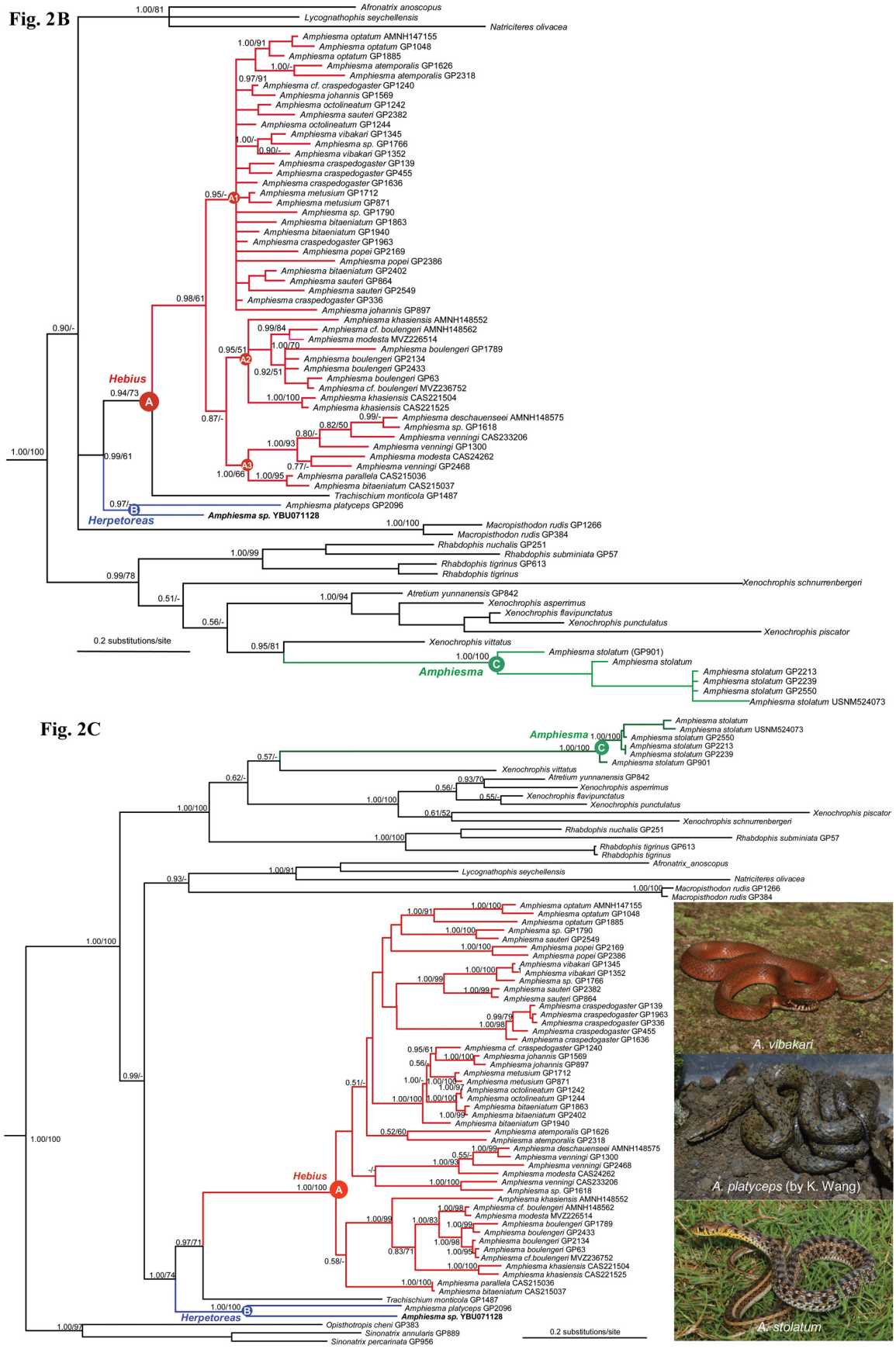

FIGURE 2B, 2C. Bayesian 50% majority-rule consensus tree inferred from nDNA data (B) and concatenated data set (C) in MrBayes 3.2. The values assigned to the internodes indicate posterior probability support (before the slash) and ML bootstrap (after the slash). A node with support value <50% was indicated by "-". Branch-support indices are not given for some internodes to preserve clarity. The partition strategy is to see the main text.

FIGURE 3. Dorsal view (A) and ventral view (B) of the holotype of *Herpetoreas burbrinki* **sp. nov.**, and its habitat (C).

Remarks. In molecular phylogenies, *H. burbrinki* is closely related to *H. platyceps* (Fig. 2). Besides their differences in external morphology, these species were significantly different in genetic distance. Based on cyt *b* sequences, the average divergence (P-distance) between them (13.6%) is much higher than those between some other species in clade A (e.g. 13.1% between *H. optatum* and *H. parallelum*, 4.6% between *H. octolineatum* and *H. metusium*).

The new species is very similar to *H. sieboldii* in external morphology. But the former can be distinct from the latter by having: fewer ventrals (172 vs. 216), more subcaudals (96 vs. 90), more postoculars (3 vs. 2), and a lower TL / SVL ratio (0.26 vs 0.32). In addition, a faint white stripe is present on each side of the body in *H. burbrinki*, but is absent in *H. sieboldii*.

Notes on *Hebius*. In this work, it is not our main aim to explore and address species level taxonomy, but several species are apparently non-monophyletic, and should be examined in future studies. Previously, morphological work indicated that some species of *Amphiesma* sensu lato were poorly diagnosed and quite variable, contributing to frequent misidentification and confusion between these species (David *et al*. 2007, 2013).

This misidentification problem was also apparent in this work based on multilocus molecular sampling. It is particularly true for the species within the genus *Hebius*. These species include *H. bitaeniatum*, *H. sauteri*, *H. craspedogaster*, *H. venningi*, and *H. modestum* (Fig. 2C).

The species *H. sauteri* is widely distributed in China (including Taiwan) and Vietnam (Zhao *et al*. 1998). Based on the counts of ventals and subcaudals, Malnate (1962) divided it into three subspecies (see Zhao *et al*. 1998). The populations from Taiwan and southeastern China are assigned to *H. s. sauteri*, and those from Sichuan belong to *H. s. maxima*. However, molecular results presented here suggest they are not close relatives. So, it is likely that these populations represent two different species, and that *H. s. maxima* should be given a full species rank as *H. maxima*. Another example is *H. modestum*. A sample of *H. modestum* from Yunnan, China (CAS 234262) is much distinct from another one from Vietnam (MVZ 226514). Because most samples analyzed here were loaned from museums and colleagues, we are not sure whether these questions have arisen from misidenfification or they represent undescribed new taxa. A comprehensive study with more complete samples as well as various methods is highly desirable in resolving the taxonomies at species level.

Finally, taxonomic information on the revised genera *Amphiesma, Hebius,* and *Herpetoreas* are provided below. However, we note that the generic diagnoses of these genera have been modified from published sources cited herein, and thus may not be precise. More complete diagnoses will depend on comprehensive morphological studies, but even then, the evident degree of convergence may complicate this substantially (Pyron & Burbrink, 2009).

Amphiesma Duméril, Bibron, and Duméril, 1854

Type species: *Coluber stolatus* Linnaeus, 1758

Content. *A. stolatus*

Diagnosis. Solid-toothed non-venomous snakes with keeled scales on the body in 19 dorsal rows mid-body, anal usually divided, and all subcaudals divided. Everted hemipenis is Y shape with simple sulcus.

Notes. This is a restricted definition of this genus. Gender is neuter. This group may contain additional species here referred to *Hebius*, and likely presents a continuing problem with respect to *Xenochrophis*. Further taxonomic revision will likely be necessary in the future.

Herpetoreas Günther, 1860

Type species: *Herpetoreas sieboldii* Günther, 1860

Diagnosis. Semi-aquatic snakes with keeled dorsal scales. The posterior maxillary tooth longest, in a continuous series with the anterior ones. Body and tail slender, compressed. Two nasals, one loreal, one anterior, two posterior oculars. Scales moderately elongate, keeled, in nineteen rows. Eye is moderate size (Günther, 1860).

Contents. *H. sieboldii*, *H. platyceps*, *H. burbrinki*.

Notes. This group may contain additional species here referred to *Hebius*. Further taxonomic revision may be necessary in the future. Gender of name is unclear; likely feminine.

Hebius Thompson, 1913

Type species: *Tropidonotus vibakari* Boie, 1826

Diagnosis. Semi-aquatic snakes with keeled dorsal scales, hemipenes and sulci spermaticus simple; maxillary teeth in continuous series, gradually becoming larger posteriorly in the series or the last two teeth abruptly enlarged; internasals broad anteriorly, nostrils lateral; apical pits present or absent. Color pattern commonly consists of a series dark dots dorsolaterally, forming two longitudinal stripes.

Contents. *H. andreae, H. arquus,* ***H. atemporale****, H. beddomei,* ***H. bitaeniatum****,* ***H. boulengeri****, H. celebicum,*

H. concelarum, ***H. craspedogaster****,* ***H. deschauenseei****, H. flavifrons, H. frenatum, H. groundwateri, H. inas, H. ishigakiense,* ***H. johannis****, H. kerinciense,* ***H. khasiense****, H. leucomystax,* ***H. metusium****, H. miyajimae,* ***H. modestum****, H. monticola, H. nicobariense,* ***H. octolineatum****,* ***H. optatum****,* ***H. parallelum****, H. pealii, H. petersii,* ***H. popei****, H. pryeri, H. sanguineum, H. sarasinorum, H. sarawacense,* ***H. sauteri****,* ***H. venningi****,* ***H. vibakari****, H. viperinum,* and *H. xenura.*

Notes. Species in bold are sampled in the molecular phylogeny, and confidently placed in *Hebius*. The remaining 23 species are placed tentatively, as this group contains the majority of morphological variation and geographic coverage of the former *Amphiesma*, and thus it seems likely that most of these species are allied with *Hebius*. However, it is possible that future studies will show that some are actually placed in *Amphiesma* sensu stricto or *Herpetoreas*. Gender of name is masculine. Several of these taxa are not monophyletic, and thus likely contain multiple cryptic species.

Conclusion

This study is the first presenting an overview of the relationships of Asian keelback snakes of *Amphiesma* sensu lato in the framework of a multi-locus molecular phylogeny. Although not all species were included and analyzed, the data generated and results presented here will benefit subsequent work on the systematics and taxonomy of this group. Future studies should focus on addressing the specific boundaries and distribution, and resolving the genus-level taxonomy of *Amphiesma* sensu stricto, *Atretium, Rhabdophis*, and *Xenochrophis*.

Acknowledgments

This project was funded in part by the National Natural Science Foundation of China (NSFC31372152) and the Scientific Research Fund of Sichuan Provincial Education Department (13TD0027) to PG, and by U.S. NSF grant DBI-0905765 to RAP. H. Chen, L. Ding, G. L. Wang, K. Jiang, H. Zhao, G. H. Zhong, J. Hu, Y. M. Yang, G. S. Xiang, R. Wilson, K. de Queiroz, J. Vindum, D. Kizirian, C. L. Spencer, and C. J. Raxworthy are gratefully acknowledged for their assistance with obtaining samples. Tissues were provided by the American Museum of Natural History (New York), the California Academy of Science (San Francisco), the Museum of Vertebrate Zoology (Berkeley), and the United States National Museum (Washington). We think K. Wang for providing a photo. G. Vogel and K. Jiang are acknowledged for providing documents. We thank Z.T. Nagy and two anonymous reviewers for comments.

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