

# Molecular phylogeny reveals cryptic diversity in *Sibynophis* from China (Serpentes: Sibynophiidae)

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## Abstract

Illumination of species diversity and their distribution is key to evolution, genetics and conservation. The genus of *Sibynophis* is a group of rare snakes with less attentions. Based on more extensive sampling, we use both mitochondrial fragments and nuclear gene to explore the species diversity of the species of *Sibynophis* occurring in China. The results showed that *S. c. miyiensis* is a synonym of *S. c. grahami*, and *S. c. grahami* should be gave a specific rank as *S. graham*. *Sibynophis triangularis* was uncovered to be new to China and Myanmar. On basis of our specimens and molecular phylogeny results, the species distribution boundaries of each Chinese species were redefined.

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Running title: Phylogeny of *Sibynophis* from China

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## KEY WORDS

taxonomy, distribution, snakes, *Sibynophis grahami* , Asia

## 1 INTRODUCTION

Species diversity is central to biodiversity. Exploration of species diversity and identification of species distribution boundaries are pivotal to the preservation and management of biodiversity. The accumulation of multi-locus data and development of related analytical technologies have greatly advanced our understanding of cryptic species diversity. In recent decades, considerable progress has been made in the phylogeny of Asian snakes. Notably, various systematic issues have been addressed (Guo, Zhu, Liu, Wang, & Nguyen, 2020; Hou et al., 2021; Liu et al., 2021; Poyarkov et al., 2022) and an increasing number of new taxa have been described (Hou et al., 2021; Liu, Zhong, Wang, Liu, & Guo, 2018; Ren, Jiang, Huang, David, & Li, 2022). However, in comparison to well-studied common snake groups, such as *Hebius* and *Lycodon* (Guo et al., 2013; Guo et al., 2014; Hou et al., 2021; Ren et al., 2022), little attention has been paid to rare snake species, such as those in the genus *Sibynophis* .

The family Sibynophiidae is comprised of only two genera, including the non-venomous genus *Sibynophis* . These snakes are characterized by their small body size, loose attachment of their dentary to the articular bone, and the presence of numerous small maxillary teeth of uniform size (Pope, 1935). Currently, nine species are recognized within *Sibynophis* , with primary distribution in South and Southeast Asia (Uetz, Freed, Aguilar, & Hošek, 2022; Wallach, Williams, & Boundy, 2014). Two species, *S. collaris* and *S. chinensis* , are known to occur in China (Wang et al., 2020; Zhao, 2006). *Sibynophis chinensis* was initially described based on a sole specimen from Yichang, Hubei, China (Günther, 1889), but is now known to occur in China, Korea, and Vietnam (Wallach et al., 2014). In China, the species exhibits broad distribution, ranging from southern Xizang in the west to Jiangsu in the east (Zhao, 2006). Three subspecies are currently recognized (Zhao, 2006; Zhao, Huang, & Zong, 1998), including *S. c. chinensis* , *S. c. grahami* , and *S. c. miyiensis* . In addition to the nominal species, which broadly occurs in Southwest, Central, and East China, *S. c. miyiensis* is endemic to Southwest Sichuan and Northwest Yunnan, while *S. c. grahami* is endemic to northeastern Yunnan and Guizhou (Zhao et al., 1998; Zhao & Yang, 1997). This taxonomical arrangement is followed by most authors (Uetz et al., 2022; Wallach et al., 2014; Wang et al., 2020). The type locality of *S. collaris* is in Assam, India (Gray, 1853), but the species is restricted to Xizang and Yunnan in China (Zhao, 2006; Zhao et al., 1998). Morphologically, the two species differ from one another by number of supralabials and anterior temporal scales only (Zhao, 2006; Zhao et al., 1998).

Due to their rare occurrence and elusive nature, few *Sibynophis* specimens have been collected since their initial description and few studies have been conducted on their biology, particularly their systematics (Li et al., 2020; Pyron, Burbrink, & Wiens, 2013; Zaher et al., 2019). Although *Sibynophis* species have been included in certain phylogenetic studies (Chen et al., 2013; Li et al., 2020; Pyron et al., 2013; Zaher et al., 2019), only a limited number of samples have been examined. For example, Zaher et al. (2019) conducted a large-scale molecular phylogenetic study of advanced caenophidian snakes, which included five *Sibynophis* species, each with only one individual. Thus, given the small number of species and specimens studied, the diversity and evolution of the genus remain poorly understood.

Here, based on more extensive sampling, we explored species diversity of the genus *Sibynophis* and revised the taxonomy and distribution boundaries of each taxon within China.

## 2 MATERIALS AND METHODS

In total, 24 specimens previously identified as *S. collaris* and *S. chinensis* were collected from China (Fig. 1), then sequenced and analyzed. Additional samples from the two species and their congeners, were included and their sequences were retrieved from GenBank (Table 1). *Scaphiodontophis annulatus* was chosen as the outgroup based on previous work (Pyron et al., 2013).

Total DNA was extracted from 85% alcohol-preserved liver or muscle tissues using M5 HiPer Universal DNA Mini Kit (Mei5 Biotechnology Co., Ltd., Beijing, China) following the manufacturer's protocols. Two

mitochondrial gene fragments, cytochrome *b* (*cyt.b*) and NADH subunit 2 (ND2), and nuclear gene oocyte maturation factor *mos* (*c-mos*), were amplified by polymerase chain reaction (PCR) using primers L14910/H16064 (Burbrink, Lawson, & Slowinski, 2000), ND2L49/ND2H50 (Alfaro & Arnold, 2001), and S77/S78 (Lawson, Slowinski, Crother, & Burbrink, 2005). Cycling parameters were identical to those described in the above studies. Prior to sequencing, PCR products were purified using various commercial kits. The double-stranded product was sequenced by a commercial company (GENEWIZ Company, Suzhou, China).

The sequences were manually edited using SeqMan in Lasergene v15.1 (DNASTAR Inc., USA), aligned using Muscle with default settings, and quality-checked using MEGA v7.0 (Kumar, Nei, Dudley, & Tamura, 2008; Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). Phylogenetic analyses were performed based on the two mitochondrial DNA (mtDNA) fragments using Bayesian inference (BI) and maximum-likelihood (ML). BI was executed in MrBayes v3.2.2 (Ronquist et al., 2012) using evolutionary models selected in PartitionFinder v2.1.1 under Bayesian information criterion (BIC) (Lanfear, Calcott, Ho, & Guindon, 2012). All searches were performed with three independent runs, each initiating a random tree. Each run consisted of four Markov chains (three heated and one cold chain), with  $2 \times 10^6$  generations, sampling every 1 000 generations, and 25% of initial samples discarded as burn-in. Convergence was assessed by examining effective sample size (ESS) ( $> 200$ ) and likelihood plots through time in Tracer v1.7 (Rambaut, Drummond, Xie, Baele, & Suchard, 2018). The resulting trees were combined to calculate posterior probabilities (PP) for each node in a 50% majority-rule consensus tree. The ML trees were constructed using RAxML v7.2.6 (Stamatakis, 2006) with the GTMMAGTRCAT model under the same partitioning scheme as BI analysis. Branch support was assessed using 1 000 non-parametric bootstrap (BS) topological replicates.

We constructed a haplotype network to depict interspecific/clade relationships based on *c-mos* sequences. Analyses were executed using PopART v1.7 (Leigh & Bryant, 2015) with the parameter epsilon set to 0.

Pairwise genetic distances (*p*-distances) between species or mtDNA clades were also calculated using Mega v7.0 (Kumar et al., 2008; Tamura et al., 2013).

### 3 RESULTS

The BI and ML analyses depicted relatively consistent topologies (Fig. 2). All species of *Sibynophis* formed a highly supported lineage. Sister species *S. bistrigatus* and *S. subpunctatus* were basal to the lineage, while all other specimens formed another monophyly with strong support. The Chinese specimens were positioned in four distinct but highly supported clades (A-D). Clade A was composed of specimens from eastern Yunnan and Guizhou, China; clade B consisted of samples from China (Xizang) and Myanmar; clade C contained specimens from China (Yunnan), Vietnam, Myanmar, and Cambodia; and clade D contained specimens from Vietnam and China (Sichuan, Shaanxi, and southern China). The four clades formed a highly supported monophyly with well-resolved interclade relationships (D, (A, (B, C))). A sample previously identified as *S. collaris* (CAS 240150) is much distinct from the other *S. collaris* identified samples (B clade).

Uncorrected *p*-distances between species and clades are listed in Table 2. The interspecific/clade genetic distances ranged from 8.3% (clades B and C) to 21.2% (clade B and *S. bistrigatus*) based on *cyt.b* and from 7.9% (clades B and C) to 18.3% (clade D and sample CAS 240150) based on ND2 (Table 2).

The network inferred from the *c-mos* gene showed each species/clade exhibited unique haplotype, except clade B (*S. collaris*), which shared a haplotype with clade C (*S. triangularis*). *Sibynophis c. grahami*, *S. c. chinensis*, and *S. triangularis* contained three, two, and two unique haplotypes, respectively (Fig. 3).

### 4 DISCUSSION

This study represents the first comprehensive investigation of *Sibynophis* systematics and diversity in China. The phylogenetic relationships reconstructed using the two methods generated mostly identical topologies and highly similar support indices for major clades, with slight differences in support for several shallow nodes. Phylogenetic analyses further revealed that Chinese species of *Sibynophis* consisted of four clades (A-D), with well-supported interclade relationships. Network analyses showed similar results.

#### 4.1 Systematics of *S. chinensis*

*Sibynophis chinensis* is widely distributed in China. Here, the mtDNA-based phylogeny indicated that *S. chinensis* was composed of clades A and D. Clade D consisted of specimens from Vietnam and Southwest, Central, and Southeast China, corresponding to the *S. c. chinensis* subspecies. Clade A consisted of specimens from Yunnan, Guizhou, and Sichuan, which are the distribution regions of the *S. c. miyiensis* and *S. c. grahami* subspecies; three specimens (YBU 15152, GP 10544, and KIZ 028322) attributed to *S. c. miyiensis* were nested within the other specimens, thereby rendering *S. c. grahami* non-monophyletic. Therefore, based on priority rule of nomenclature we concluded that clade A should be represented by *S. c. grahami*, and *S. c. miyiensis* should be considered a synonym of *S. c. grahami*.

Unexpectedly, although clades A and D were highly supported, neither formed a monophyletic lineage (Fig. 2). The genetic distance between the two clades was 12.6% (cyt. *b* -based), which exceeds that between certain species (e.g., 9.4% between *S. subpunctatus* and *S. bistrigatus*) (Table 2). In addition, network analyses revealed that the two clades did not share any nDNA haplotype. These findings suggest that both clades represent two distinct species, i.e., clade A represents *S. grahami* and clade D represents *S. chinensis*.

Given the taxonomic revision of *S. chinensis* (*sensu lato*), the distribution of *S. grahami* should be redefined accordingly. Thus, based on previous records (Zhao, 2006; Zhao et al., 1998) and our data, we conclude that *S. grahami* is present in southwestern Sichuan, Yunnan, and Guizhou in China.

#### 4.2. Distribution of *S. triangularis* and *S. collaris* in China

*Sibynophis triangularis* was originally described based on a single specimen from Thailand (Taylor & Elbel, 1958). Although initially considered a subspecies of *S. collaris*, it was subsequently elevated to a separate species, i.e., *S. triangularis* (Taylor, 1965). At present, this species is known to occur in Thailand and Cambodia (Stuart, Sok, & Neang, 2006; Uetz et al., 2022; Wallach et al., 2014). However, our molecular phylogenetic reconstruction revealed that a particular sample (FMNH 263023, Cambodia), previously identified as *S. triangularis* (Stuart et al., 2006), formed a well-supported clade (C) with specimens from Vietnam, Myanmar, and China (southern and northwestern Yunnan). Hence, we confirm that clade C represents the species *S. triangularis*, which is newly reported in China and Myanmar. Currently, *S. triangularis* is only known to occur in Fugong and Cangyuan in Yunnan of China.

Our results identified clade B as sister to clade C, representing *S. collaris* based on geographical origin. Unexpectedly, sample CAS 240150 collected from Myanmar and originally identified as *S. collaris* in Chen et al. (2013) formed a distinct mtDNA lineage from clade B, with genetic distances of 8.3% (cyt. *b* -based) and 7.9% (ND2-based), respectively (Table 2; Fig. 2). These findings suggest that this specimen was misidentified and may represent an undescribed taxon. Thus, further examination of this specimen is required. In addition, in a previous large-scale systematic study of Chinese snakes, Li et al. (2020) identified one specimen (CHS 244) from Honghe, southern Yunnan, China, as *S. collaris*, and two specimens (CHS 879 and CHS 880) from Motuo, Xizang, China, as *S. chinensis*. Our results showed the first specimen was nested within the clade A and the latter two positioned in the clade B. Thus we concluded that these samples were misidentified, with the former being *S. grahami* and the latter two being *S. collaris*.

Zhao and Yang (1997), Zhao et al. (1998), Zhao (2006), and Yang and Rao (2008) proposed that *S. collaris* is distributed in southern Xizang and northwestern Yunnan in China. Based on our molecular phylogenetic analyses, however, several specimens collected from northwestern Yunnan, which is geographically close to Motuo, Xizang, were identified as *S. grahami* and *S. triangularis* (Fig. 2). Thus, it is most like that *S. collaris* is endemic to Motuo in Xizang, with no occurrence in Yunnan.

### CONCLUSIONS

Based on more extensive sampling, we conducted a comprehensive study of Chinese species of *Sibynophis*, providing new insights into their systematics and distribution. Our results revealed that *S. c. miyiensis* is synonymous with *S. c. grahami*, which should be elevated to a species level. Furthermore, *S. triangularis* is

reported as new to China and Myanmar. In total, four species, *S. chinensis*, *S. collaris*, *S. grahami*, and *S. triangularis*, are distributed in China. Consequently, we revised their distribution boundaries in China and re-examined previously misidentified specimens.

## AUTHOR CONTRIBUTIONS

**Peng Guo** : Conceptualization (Lead); Data curation (Lead); Formal analysis (Lead); Funding acquisition (Lead); Investigation (Lead); Methodology (Lead); Project administration (Lead); Writing – original draft (Lead); Writing – review & editing (Lead). **Ping Wang** : Data curation (Supporting); Methodology (Supporting); Resources (Supporting). **Bing Lyu**: Data curation (Supporting); Resources (Supporting); Software (Supporting); Visualization (Supporting). **Qin Liu**: Data curation (Supporting); Formal analysis (Supporting); Resources (Equal). **Jieyu Zheng**: Data curation (Supporting); Formal analysis (Supporting); Resources (Supporting). **Chunmei Fu**: Data curation (Supporting); Formal analysis (Supporting); Resources (Supporting). **Yayong Wu**: Data curation (Supporting); Resources (Supporting); **Guocheng Shu**: Data curation (Supporting); Resources (Supporting). **Shaobing Hou**: Data curation (Supporting); Resources (Supporting).

## CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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None.

## DATA AVAILABILITY STATEMENT

All DNA sequences newly generated are deposited in Genbank (Accessions No: OQ981548-OQ981602)

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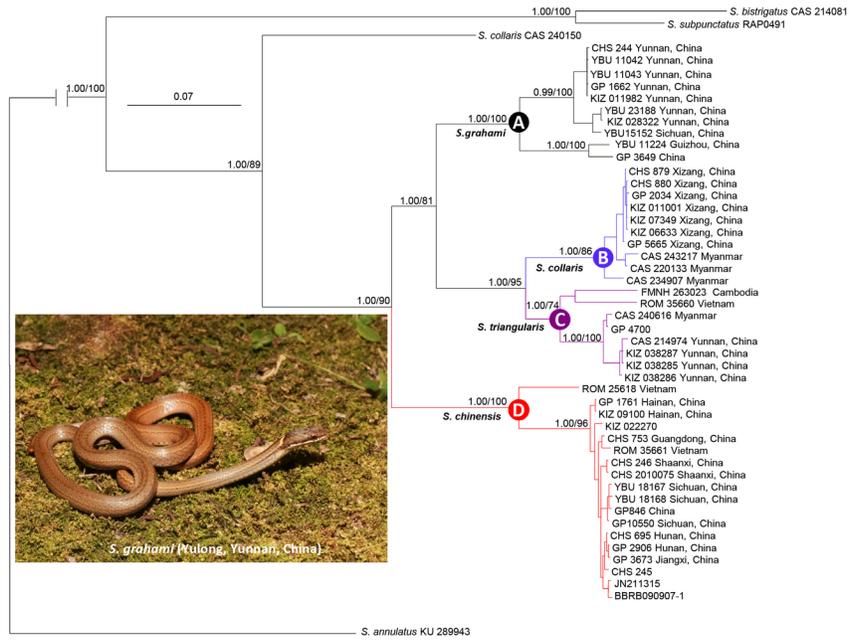
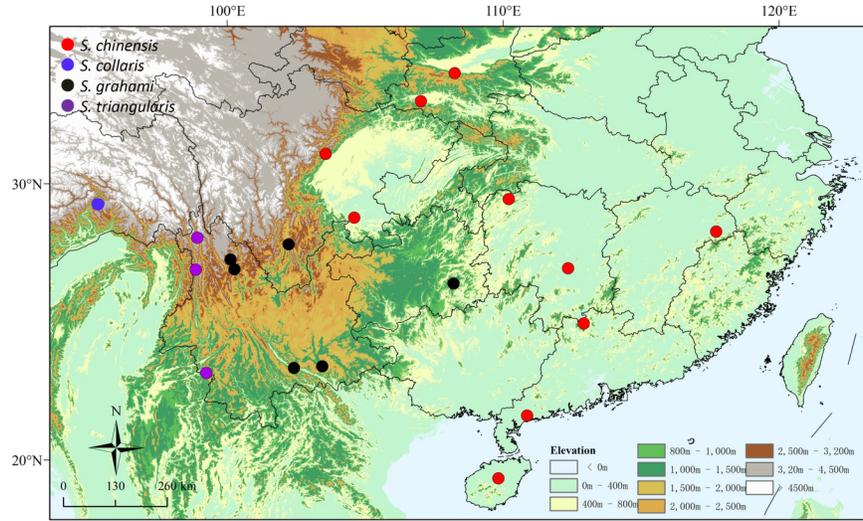
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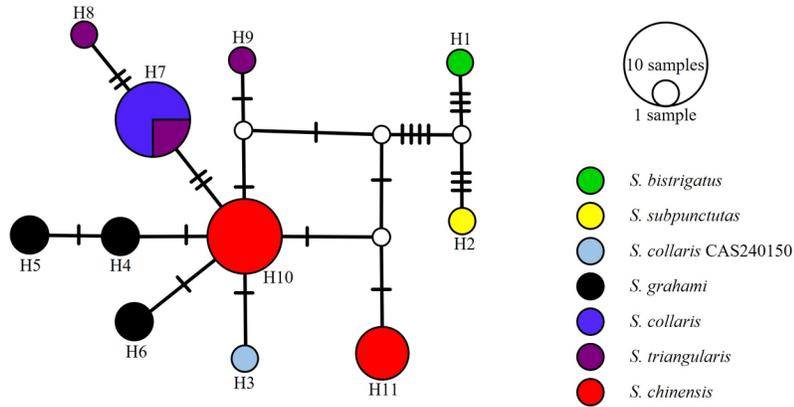
## FIGURE LEGENDS

Figure 1 Map showing localities of Chinese samples of *Sibynophis* used in this study.

Figure 2 Bayesian consensus tree of *Sibynophis* inferred from mitochondrial DNA using models detailed in text. Posterior probabilities (>0.50) and bootstrap support values (>50%) are given adjacent to respective nodes for major nodes. Branch support indices are not given for shallow nodes to preserve clarity.

Figure 3 The network based on c-mos gene (lines between the haplotypes represent one mutational step).





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Table 1\_Information for samples-new.docx available at <https://authorea.com/users/574992/articles/643917-molecular-phylogeny-reveals-cryptic-diversity-in-sibynophis-from-china-serpentes-sibynophiidae>

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