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## *Parahelicops*, *Pararhabdophis*, Paraphyly: Phylogenetic Relationships among Certain Southeast Asian Natricine Snakes (*Hebius*)

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### ABSTRACT

We investigate the phylogenetic relationships of two poorly known Natricinae, *Parahelicops* and *Pararhabdophis*, for which we obtained nucleotide sequence data from one mitochondrial gene (cytochrome *b*) and three nuclear genes (CMOS, NT3, and RAG1). Maximum parsimony, maximum likelihood, and combined and partitioned Bayesian analyses suggest that both *Parahelicops* and *Pararhabdophis* are embedded within the genus *Hebius*. To align classification with phylogeny, we synonymize *Parahelicops* and *Pararhabdophis* with *Hebius*.

### INTRODUCTION

*Parahelicops annamensis* Bourret, 1934, has a history of entanglement with *Amphiesma* (e.g., Stuart, 2006; Teynie et al., 2013; David et al., 2015) and *Opisthotropis* (e.g., Bourret, 1934b; Smith, 1943; Stuart, 2006; Stuart and Chuaynkern, 2007; Murphy et al., 2008; Teynie et al., 2013; David et al., 2015). Citing unpublished data, Teynie et al. (2013) thought *Parahelicops annamensis*

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seemed “referable to the genus *Amphiesma*” but ultimately decided to retain the original name (despite using “*Amphiesma annamense*” in their key to species). David et al. (2015) cited unpublished molecular data that supported the monophyly of *Amphiesma* with respect to *Parahelicops* and, hence, retained the latter. At the same time, however, they listed specific morphological character states in *Parahelicops* also found in the *A. venningi* complex. Stuart (2006) recognized the validity of the genus *Parahelicops*, at least for *P. annamensis*, and suggested a close relationship of *Parahelicops* with *Opisthotropis*. The poorly known *Pararhabdophis chapaensis* Bourret (1934a) also exhibits morphological similarity to *Parahelicops annamensis* (David et al., 2015).

More inclusive phylogenetic studies have resulted in rearrangements of taxa relevant to the positions of *Parahelicops* and *Pararhabdophis*. After finding *Amphiesma* to be polyphyletic, Guo et al. (2014) resurrected the genus *Hebius* Thompson, 1913, for all species except *Amphiesma stolatum*. Guo et al. (2012) and Figueroa et al. (2016) found *Opisthotropis* to be outside the clade that includes *Hebius* and *Amphiesma* and closer to *Sinonatrix* and New World Natricinae. None of the previous molecular studies, however, addressed *Parahelicops* or *Pararhabdophis*. Herein, we reevaluate the phylogenetic relationships of *Parahelicops* and *Pararhabdophis* with respect to other natricines using nucleotide sequence data.

#### MATERIALS AND METHODS

**MOLECULAR DATA:** Tissue samples (appendix) were obtained from the American Museum of Natural History, New York (AMNH), the Field Museum, Chicago (FMNH), and the North Carolina Museum of Natural Sciences, Raleigh (NCSM). In total, we included six new samples (fig. 1), from two genera *Pararhabdophis* and *Parahelicops*, in the matrix published by Guo et al. (2014). In addition, we included *Opisthotropis cheni* and *O. lateralis* in phylogenetic analyses to root phylogenetic trees. Extracted DNA from the fresh tissue was amplified by PCR Master Mix (Fermentas, Burlington, ON, Canada) using the same primers and conditions employed by Guo et al. 2014. PCR products were subjected to electrophoresis through a 1% agarose gel (UltraPure™, Invitrogen, La Jolla, CA). Gels were stained for 10 min in 1 X TBE buffer with 2 µg/ml ethidium-bromide, and visualized under UV light. Successful amplifications were purified to eliminate PCR components using a GeneJET™ PCR Purification kit (Fermentas). Purified PCR products were sent to FirstBase Malaysia for sequencing.

**PHYLOGENETIC ANALYSES:** The sequences were aligned in Clustal X v2 (Thompson et al., 1997) with default settings. Data were analyzed using maximum parsimony (MP) and maximum likelihood (ML) as implemented in PAUP 4.0b10 (Swofford, 2001), and Bayesian analysis in MrBayes 3.2 (Ronquist et al., 2012). For MP analysis, heuristic analysis was conducted with 100 random taxon-addition replicates using tree-bisection and reconnection (TBR) branch-swapping algorithm, with no upper limit set for the maximum number of trees saved. Bootstrap support (BP) (Felsenstein, 1985) was calculated using 1,000 pseudoreplicates and 100 random taxon-addition replicates. All characters were equally weighted and unordered. For ML analysis, we used the optimal evolution model as selected by ModelTest v3.7 (Posada and Crandall, 1998). To estimate BP in the ML analysis, a simple taxon-addition option and 100

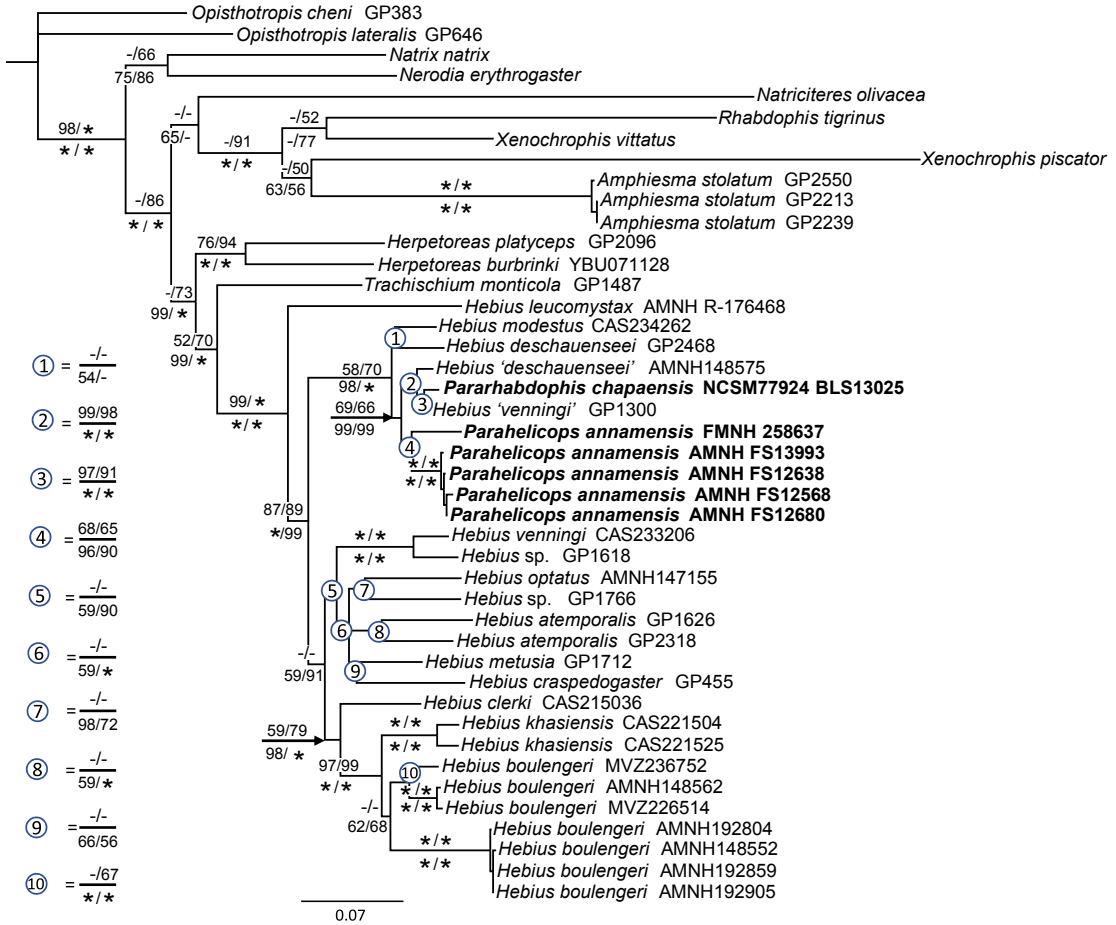


FIGURE 1. Combined Bayesian phylogram based on all concatenated data. Numbers above and below branches are MP/ML bootstrap values and combined/partitioned Bayesian posterior probabilities (>50%), respectively. Hyphen and asterisk denote <50% and 100% values, respectively. Bold text indicates samples sequenced for this study.

pseudoreplicates were employed. We arbitrarily assumed bootstrap values of  $\geq 70$  % to represent strong support and values of <70 % as weak support (Hillis and Bull, 1993).

For Bayesian analyses, we used the optimal model determined by Modeltest with parameters estimated by MrBayes 3.2.1. Two simultaneous analyses with four Markov chains (one cold and three heated) were run for 10 million generations with a random starting tree and sampled every 1000 generations. Log-likelihood scores of sample points were plotted against generation time to determine stationarity of Markov chains. Trees generated before log-likelihood scores reached stationarity were discarded from the final analyses using the burn-in function. The posterior probability (PP) values for all clades in the final majority-rule consensus tree are provided. We ran analyses using both combined and partitioned datasets to examine the robustness of the tree topology (Nylander et al., 2004; Brandley et al., 2005). In the mixed-model analysis, we partitioned the data into 12 sets based on gene codon positions (first,

Table 1. Models used in Bayesian analyses

Data analysis	Model determined by Modeltest
<b>Combined Bayesian analysis</b>	
Concatenated matrix	TIM2+I+R
<b>Partitioned Bayesian analysis</b>	
Cytochrome <i>b</i> 1st position	TrN+I+G
Cytochrome <i>b</i> 2nd position	TIM+I+G
Cytochrome <i>b</i> 3rd position	TVM+I+G
Cmos 1st position	K80
Cmos 2nd position	JC
Cmos 3rd position	HKY
NT3 1st position	JC
NT3 2nd position	K80+G
NT3 3rd position	K80+I
Rag1 1st position	HKY
Rag1 2nd position	TrNef+I
Rag1 3rd position	HKY

second, and third) of cytochrome *b*, CMOS, NT3, and RAG1. Optimal models of molecular evolution for the partitions were calculated using Modeltest, and then assigned to these partitions in MrBayes 3.2 using the command APPLYTO. Model parameters were inferred independently for each data partition using the UNLINK command. All models employed in Bayesian analyses are shown in table 1.

## RESULTS

The final matrix consisted of 3162 aligned characters, of which 614 were parsimony informative. The alignment contained no gap. MP analysis of the dataset recovered nine most parsimonious trees with 3259 steps (CI = 0.38; RI = 0.59). In the ML analysis, the  $-\ln L$  likelihood score of the single best tree found was 18,729.82. The cutoff point for the burn-in function was set to 20 and 21 in combined and partitioned Bayesian analyses as  $-\ln L$  scores reached stationarity after 20,000 and 21,000 generations, respectively. The topologies derived from our study are similar to those in Guo et al. (2014). Most relevant here, we found *Parahelicops* and *Pararhabdophis* nested within *Hebius* with strong support in all analyses, and within the smallest clade including *H. deschauenseei*, *H. modestus*, and some *H. venningi* with high statistical values from all, but the MP analysis (fig. 1).

## DISCUSSION

Our analysis provides a phylogenetic explanation for the reported similarity between *Parahelicops*, *Hebius venningi*, and *H. deschauenseei* noted by David et al. (2015: 216). Specifically, we find *Parahelicops* and *Pararhabdophis* to be imbedded within *Hebius*, in a clade including *H. deschauenseei*, *H. modestus*, and some of the specimens identified as *H. venningi*. To align taxonomy with the recovered phylogeny of this group, we synonymize *Parahelicops* Bourret, 1934, and *Pararhabdophis* Bourret, 1934, with *Hebius* Thompson (1913), yielding the new combinations, *Hebius chapaensis* (Bourret, 1934) and *Hebius annamensis* (Bourret, 1934). We corroborated (not presented) Guo et al. (2012) and Figueroa et al. (2016) who found *Opisthotropis* to be outside *Hebius* and, therefore, not closely related to *Parahelicops* (contra Stuart, 2006).

Sequence divergence (approximately 6%) between *Hebius annamensis* from the Ca (Vietnam samples) and Mekong (Lao samples) drainages suggests that multiple species might exist under that binomial. Divergence between northern and southern *H. annamensis* might reflect isolation by low-elevation habitats characterized by a mixture of evergreen, semievergreen, and dry forest types between northern and central Annamite ranges discussed by Bain and Hurley (2011).

We also corroborate the findings of David et al. (2013) and Guo et al. (2014), who reported variation suggestive of additional unrecognized species diversity under the names *H. boulengeri* and *H. venningi*, the latter of which is polyphyletic in Guo et al. (2014) and herein. Stuart et al. (2006) suggested that there are no “geographically widespread, forest-dwelling frog species in Southeast Asia.” Such a pattern may also exist in snakes such as *Hebius*, some of which are known to be connected to anurans through trophic relations (e.g., Moriguchi and Naito, 1982; David et al., 2007).

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## APPENDIX

## MATERIAL EXAMINED

*Pararhabdophis chapaensis*: LAO PDR: Houaphan Province: Phou Louey National Protected Area, Viengthong District, near Tad Loi Waterfall (20.23253°N, 103.2108°E), 1186 m (NCSM 77924).

*Parahelicops anammensis*: VIETNAM: Ha Tinh: Huong Son District, Huong Son Reserve, Rao An region, near top of Po-mu Mountain (18° 20' 26" N, 105° 14' 13" E), 870 m (AMNH-R 147129 [corpus], AMNH-FS 13993 [field series], AMCC 106598 [tissue]). Nghe An Province: Pu Mat National Park: Anh Son District (near N 18.8177, E 104.9609), 170 m (AMNH-R 176469 [corpus], AMNH-FS 12568 [field series]), AMCC 192504–06 [tissue]). Nghe An Province: Pu Mat National Park: Anh Son District (N 18.8092, E 104.9499), 357 m (AMNH-R 176470 [corpus], AMNH-FS 12638 [field series], AMCC 192596–97 [tissue]). Nghe An Province: Pu Mat National Park: Anh Son District, Khe Suc River (N 18.8171, E 104.9484), 237 m (AMNH-R 176471 [corpus], AMNH-FS 12680 [field series], AMCC 192626–27 [tissue]). LAO PDR: Xekong Province: Kaleum District: Xe Sap National Biodiversity Conservation Area (16° 04' 10" N, 106° 58' 45" E), 1280–1500 m (FMNH 258637).



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