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Rediscovery of Andrea's keelback, *Hebius andreae* (Ziegler & Le, 2006): First country record for Laos and phylogenetic placement

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Abstract: For more than a decade, the keelback snake *Hebius andreae* was only known from the holotype from the limestone forest in the central Truong Son (the Annamite Mountain Range) of Quang Binh Province in Vietnam. As the adult male was formaldehyde-fixed, the description was based on morphological characters only. During recent herpetological surveys in the karst forest of central Laos, opposite to the type locality of *H. andreae* on the other side of the Annamite Range, the Andrea's keelback is rediscovered. Based on a juvenile male from Bualapha District, Khammouane Province, within Hin Nam No National Protected Area, we herein report the first country record of this species from Laos, provide an expanded morphological definition, and for the first time recover the phylogenetic relationship of *H. andreae*, based on the sequences of four genes, including one mitochondrial, cytochrome b, and three nuclear markers, Cmos, NT3, and Rag1. The phylogenetic placement of *H. andreae* reveals it to be a member of *Hebius*, in fact the most basal representative of the genus. The rediscovery of the beautiful but still poorly known and obviously rare species underlines the conservation importance of the Annamite Mountain Range as habitat and refugium for the regional unique biodiversity, which only occurs in this karst massif.

Keywords: Khammouane Province - Hin Nam No National Protected Area - Natricidae - morphology - phylogeny.

INTRODUCTION

Amphiesma andreae was discovered in 2004 and officially described two years later by Ziegler & Le (2006). The original description was based on an adult male collected in the limestone forest in the central Truong Son (the Annamite Mountain Range) of Quang Binh Province in Vietnam. As the adult male individual was formaldehyde-fixed, the description was based on morphological characters only. *A. andreae* was diagnosed as a representative of the genus *Amphiesma* because of the head being distinct from the body, the large eyes with round pupils, the anteriorly broadly truncated internasals, the laterally positioned nostrils, the keeled dorsal scales in 19 rows, the divided precloacal scute, the paired subcaudals, less than 35 maxillary teeth arranged in a continuous series with the two posteriormost enlarged, and the undivided hemipenis and sperm groove (Ziegler & Le, 2006).

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A recent molecular study showed that the genus *Amphiesma* sensu lato is not monophyletic, and that there exist three distinct, monophyletic lineages, i.e., *Amphiesma* sensu stricto, *Hebius*, and *Herpetoreas* (Guo *et al.*, 2014). The genus *Amphiesma* is now monotypic (*A. stolatum*). *Herpetoreas* contains three species (*H. burbrinki*, *H. platyceps*, and *H. sieboldii*), and *Hebius* comprises the remaining species formerly listed under *Amphiesma* (*H. andreae*, *H. arquus*, *H. atemporalis*, *H. beddomei*, *H. bitaeniatus*, *H. boulengeri*, *H. celebicus*, *H. clerki*, *H. concelarus*, *H. craspedogaster*, *H. deschauenseei*, *H. flavifrons*, *H. frenatus*, *H. groundwateri*, *H. inas*, *H. ishigakiensis*, *H. johannis*, *H. kerinciensis*, *H. khasiensis*, *H. leucomystax*, *H. metusius*, *H. miyajimae*, *H. modestus*, *H. monticola*, *H. nicobariensis*, *H. octolineatus*, *H. optatus*, *H. parallelus*, *H. pealii*, *H. petersii*, *H. popei*, *H. pryeri*, *H. sanguineus*, *H. sarasinorus*, *H. sarawacensis*, *H. sauteri*, *H. taronensis*, *H. venningi*, *H. vibakari*, *H. viperinus*, and *H. xenura*), resulting in the new combination *H. andreae* (Guo *et al.*, 2014); for taxonomic authorities and the original publication of species names see Uetz *et al.* (2018) and for updated species names see Kizirian *et al*. (2018). For more than a decade, the Andrea's keelback was only known from the holotype. In the original description (Ziegler & Le, 2006) the type locality was given as adjacent to Phong Nha - Ke Bang National Park, Thuong Hoa Commune, Minh Hoa District. Since then, the area has been included in the extension of Phong Nha – Ke Bang National Park (e.g., Ziegler *et al.*, 2010; Luu *et al.*, 2013).

During a recent herpetological survey by our team in the karst forests of the Hin Nam No National Protected Area in Khammouane Province in central Laos, just opposite to Phong Nha – Ke Bang on the other side of the Annamite Range, an individual of a keelback snake was found, showing the unique and characteristic colour pattern of *H. andreae*. Based on this juvenile individual we herein are able to report the first country record of this poorly known species for Laos, provide an expanded morphological definition, and recover the phylogenetic relationship of *H. andreae*.

MATERIAL AND METHODS

Sampling: This study is based on a newly collected *Hebius andreae*, a juvenile male, VNUF R.2017.25 (field number NM 17.25), from Nong Ma Village, Bualapha District, Khammouane Province, within Hin Nam No National Protected Area, collected by Vinh Quang Luu and Ngoan Van Ha, at an elevation of 537 m a.s.l., on 9 April 2017. The specimen was anaesthetized with ethyl acetate, fixed in approximately 85% ethanol, and then transferred to 70% ethanol for permanent storage. A tissue sample was preserved separately in 95% ethanol. The *H. andreae* from Laos was subsequently deposited in the collection of the Vietnam National University of Forestry (VNUF), Hanoi, Vietnam. The holotype of *H. andreae* (ZFMK 83747) is deposited in the Zoologisches Forschungsmuseum Alexander Koenig (ZFMK), Bonn, Germany (see Ziegler & Le, 2006).

Morphological examination: Identification of sex was performed by inspection of presence of hemipenes (which have been tried to be everted before fixation and thus are partially protruding from the cloaca). Measurements were taken after preservation with a measuring tape. The number of ventral scales was counted according to Dowling (1951). The numbers of dorsal scale rows are given at one head length behind head, at midbody, and at one head length before vent, respectively. Due to the uniqueness and fragility of the new record from Laos and due to its small size we refrained from invertedly dissecting hemipenes as well as dissect the upper jaw for maxillary teeth count. Scalation was studied by using a binocular. We herein use the term precloacal instead of anal. Bilateral values were given as left / right.

Abbreviations of morphological characters used in the text / Table 1 are as follows: – *Measures and ratios*: SVL: snout-vent length. – TaL: tail length. – TL: total length (SVL + TaL). – TaL/TL: ratio tail length/total length. – *Meristic characters*: ATem: anterior temporal scales (in contact with postocular scale / scales). – BodySc: body scales. – InN/Lor: Internasal and loreal in contact. – DSR:

Table 1. Sex, morphometry and scalation of the holotype of *Hebius andreae* from Vietnam (after Ziegler & Le, 2006) and the newly collected specimen from Laos.

	ZFMK 83747 holotype	VNUF R.2017.25
Sex	male	male
TL	608	290
SVL	420	209
TaL	188	81
TaL/TL	0.31	0.28
SL	9/9	9/9
SL/orbit	4-6	4-6
IL	9/9	9/9
PreOc	1/1	1/1
PostOc	3/3	3/3
Lor	1/1	1/1
Atem	1/1	1/1
PTem	1/1	2/2
DSR	19-19-17	19-19-17
PreVen	2	2
Ven	179	180
Prec	divided	divided
Subc	99	103

dorsal scale rows. – IL: infralabial scales. – Lor: loreal scales. – PreOc: preocular scales. – PreVen: preventral scales. – PostOc: postocular scales. – PTem: posterior temporal scales (in contact with anterior temporal scale / scales). – SL: supralabial scales. – SL/orbit: supralabial scale / scales touching the orbit. – Subc: subcaudal scales (without terminal scute). – SubOc: subocular scales. – TailSc: tail scales. – Ven: ventral scales.

Molecular analyses: Extracted DNA from the fresh tissue was amplified by PCR mastermix (Fermentas, Burlington, ON, Canada) using the same primers and conditions employed by Guo *et al.* (2014). In total, we sequenced four genes, including one mitochondrial, the cytochrome b, and three nuclear, Cmos, NT3, and Rag1, markers. PCR products were separated by electrophoresis through a 1% agarose gel (UltraPure™, Invitrogen, La Jolla, CA). Gels were stained for 10 min in 1 X TBE buffer with 2 pg/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 (see Table in Appendix).

The sequences were aligned in ClustalX 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 1000 pseudo-replicates 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 & Crandall, 1998). To estimate BP in the ML analysis, a simple taxon addition option and 100 pseudo-replicates were employed. We assumed bootstrap values of $\geq$ 70 % to represent strong support and values of < 70% as weak support (Hillis & Bull, 1993). To verify the ML results, we also performed an analysis in IQ-TREE v.1.6.7.1 (Nguyen *et al.*, 2015) using a single model, GTR+I+G, as selected by ModelTest with 10,000 ultrafast bootstrap replications.

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. Two independent analyses were run simultaneously. 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, 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 2.

RESULTS

The juvenile male (Figs 1-4) was found during daytime actively crawling on a limestone forest path under dry leaves. The surrounding habitat was karst forest, dominated by species of Ebenaceae, Dracaenaceae, Arecaeae, Poaceae, Meliaceae, and Moraceae.

Morphological assessment

Measurements, ratios and scalation data of the new record of *Hebius andreae* from Laos compared to the holotype from Vietnam are presented in Table 1.

The newly collected specimen from Hin Nam No National Protected Area largely agreed with the description of the holotype of *H. andreae* from Phong Nha – Ke Bang National Park (see Fig. 5), except for the following deviations:

Supranasals slightly wider than long (*vs.* slightly longer than wide in the holotype). Suture between the supranasals slightly shorter than the suture between the prefrontals (*vs.* suture being slightly longer in the holotype). Two posterior temporals (*vs.* one posterior temporal in the holotype). Supralabials 8 and 9 being the largest (*vs.* the eighth being the largest in the holotype). The first 5/6 infralabials border the anterior chin-shields (*vs.* the first five infralabials in the holotype). 180 ventrals (*vs.* 179 in the holotype); 103 subcaudals (*vs.* 99 in the holotype). Dorsal scale rows keeled, with a narrow, sharp keel, except for outermost 1-2 rows; keels in part become indistinct towards the second body half; somewhat beyond midbody also outermost dorsal scale rows in part with keel (*vs.* dorsals distinctly keeled; somewhat beyond midbody also outermost dorsal scale row with distinct keel in the holotype).

From pictures of the underside of the juvenile male from Laos taken alive, it becomes obvious that the venter in the juvenile male was of cream colour in the head and neck region in life, but later turning into pinkish orange including the lower tail surface.

Table 2. Models used in Bayesian analyses.

Data set	Model determined by ModelTest
Combined Bayesian analysis	
Concatenated matrix	GTR+I+G
Partitioned Bayesian analysis	
Cytochrome b 1st position	TVM+I+G
Cytochrome b 2nd position	HKY+I+G
Cytochrome b 3rd position	TrN+I+G
Cmos 1st position	K80+I
Cmos 2nd position	HKY
Cmos 3rd position	K80
NT3 1st position	JC
NT3 2nd position	HKY+80
NT3 3rd position	TrNef+I
Rag1 1st position	HKY
Rag1 2nd position	HKY
Rag1 3rd position	HKY

Fig. 1. Portrait of the first record of *Hebius andreae* from Laos in life. Photo V. Q. Luu.

Fig. 2. General views of the juvenile male of *Hebius andreae* from Laos in life. Photos V. Q. Luu.

Fig. 3. Head views of the juvenile male of *Hebius andreae* (VNUF R.2017.25) from Laos in preservative. Photos T. Ziegler.

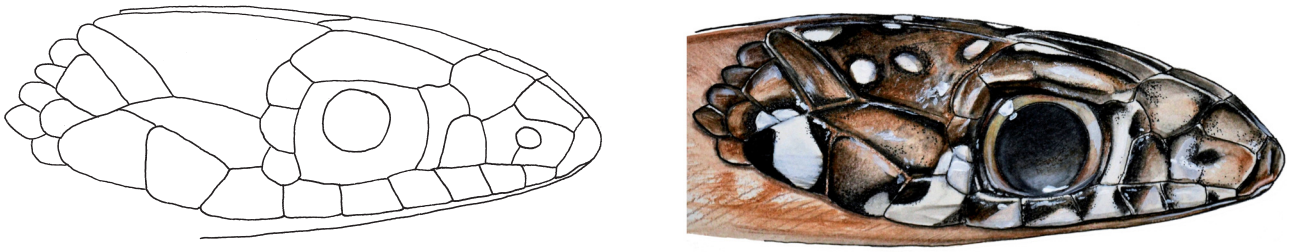

Fig. 4. Line drawing and colour painting of the right head side of *Hebius andreae* (VNUF R.2017.25) from Laos. Drawing T. Ziegler, painting C. Niggemann.

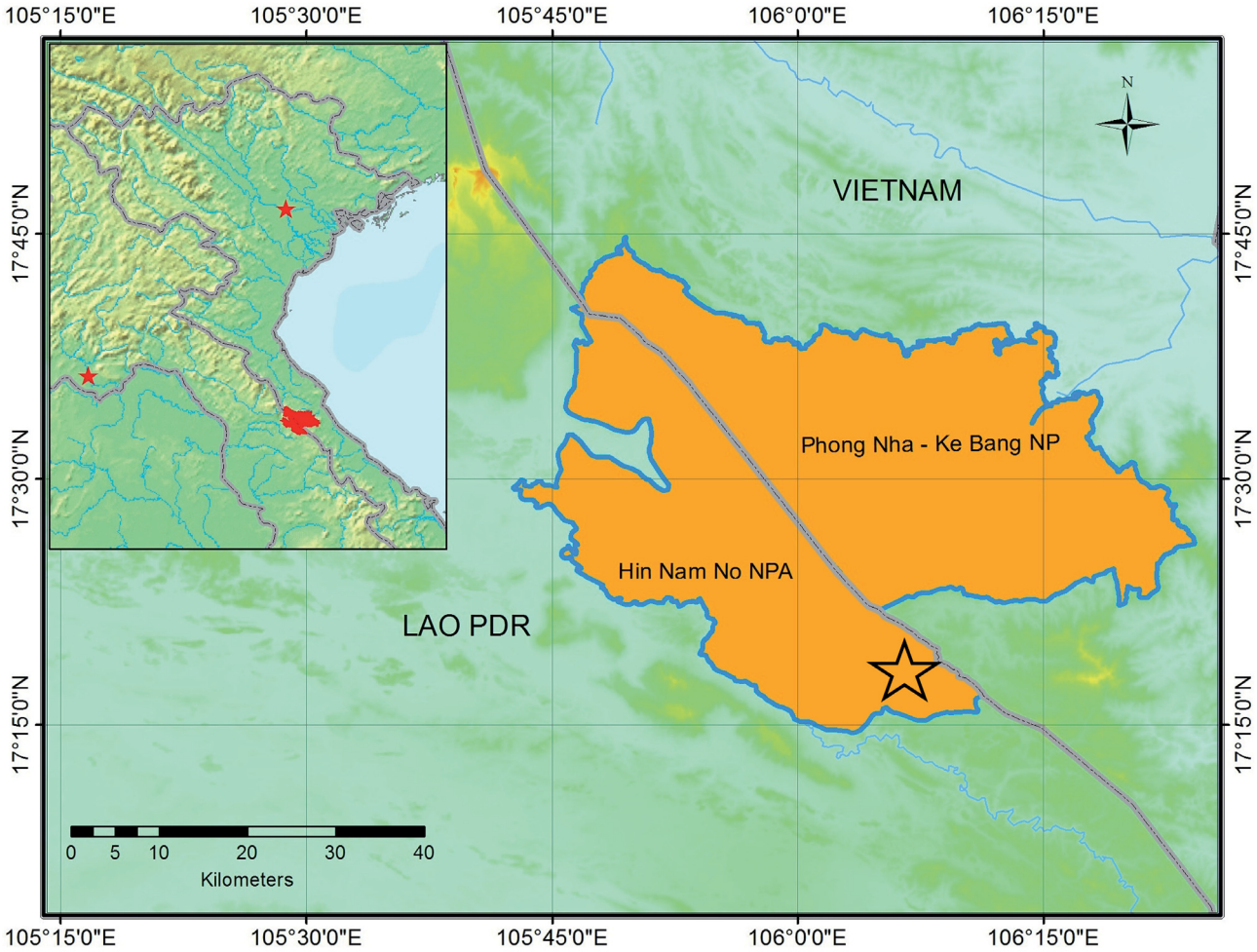

Fig. 5. Location of the Hin Nam No National Protected Area in central Laos, where the new record (star) of *Hebius andreae* took place, which is located opposite to the type locality in Vietnam, viz. Phong Nha – Ke Bang National Park in Vietnam.

Molecular results

The final matrix consisted of 3162 aligned characters, of which 503 were parsimony informative. The alignment contained no gap. MP analysis of the dataset recovered a single most parsimonious tree with 2568 steps (CI = 0.42; RI = 0.43). In the ML analysis, the -Ln likelihood score of the single best tree found was 15,553.39. The cutoff point for the burn-in function was set to 20 in combined and 21 in 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). Our phylogenetic results revealed that *H. andreae* is strongly corroborated as a member of the genus *Hebius* in all analyses (BP_{MP} = 96, all other values = 100, including the BP value derived from the ML analysis using IQ-TREE). In addition, it represents the most basal taxon of the genus (Fig. 6).

DISCUSSION

The first phylogenetic evaluation of *Hebius andreae* based on the new finding from Laos revealed it to be a member of *Hebius*. Ziegler & Le (2006) already expected future records of *H. andreae* from Laos due to the close proximity of the type locality to the border with Laos. Unless molecular comparison with topotypic *Hebius andreae* from Phong Nha – Ke Bang shows otherwise, we assess the new record of this species from Laos for the time being as conspecific. At this stage of knowledge, the minor morphological deviations can be explained by 1) individual variation, in concert with 2) juvenile age of the individual from Laos and 3) suboptimal preservation condition. The new record from Laos also was found in the same kind of biotope, on the ground of limestone forest at a similar elevation compared with the holotype (537 m vs. 450 m).

The original diagnosis of *H. andreae* thus can be expanded as follows:

1) Body and tail slender, tail cylindrical and tapering; tail/total length ratio in males 0.28-0.31;
2) the eye diameter, if projected forward, reaches beyond the suture of first and second supralabial;
3) a single loreal;
4) a single preocular;
5) three postoculars;
6) a single anterior temporal followed by 1 or 2 posterior temporals;

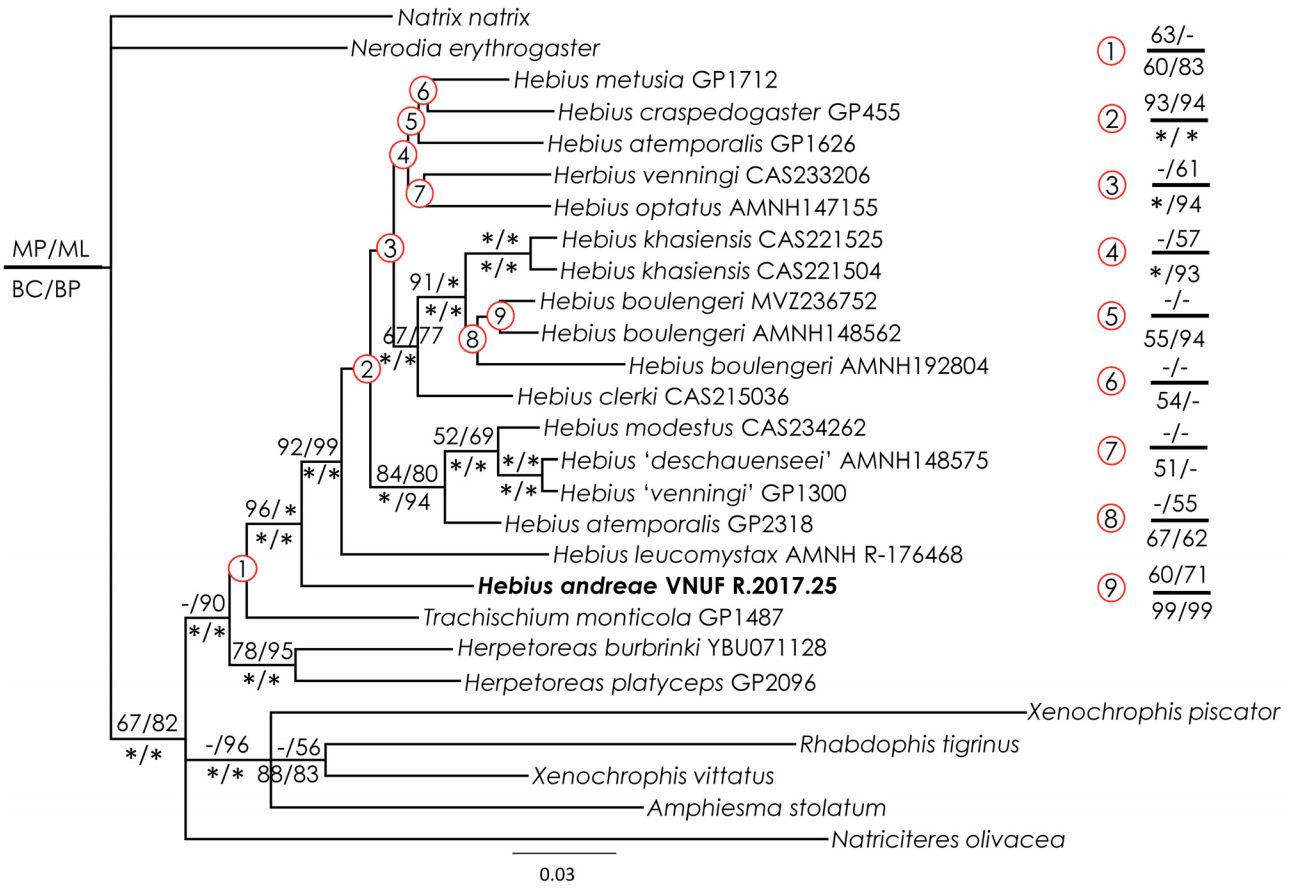

Fig. 6. Bayesian phylogram based on all data combined and a single model. Numbers above and under branches are MP/ML bootstrap values and combined/partitioned Bayesian posterior probabilities (>50%), respectively. Hyphen and asterisk denote <50% and 100% values, respectively.

7) nine supralabials, fourth to sixth in contact with the eye, 8th or 8th and 9th supralabials largest;
8) nine infralabials, first pair in contact with each other behind the mental, the first five or six bordered by the anterior chin-shields;
9) posterior chin-shields longer than anterior ones, separated from each other on their entire length by gular scales;
10) 179-180 ventrals (plus two preventrals);
11) precloacal plate divided;
12) 99-103 divided subcaudals;
13) dorsal scales in 19-19-17 rows, keeled, except outermost row(s) in the anterior body, with a narrow, sharp keel;
14) dorsal ground coloration brownish-olive, with a pale, black-edged bar before and behind the eye; head and neck with several pale, dark-edged blotches that turn into pale and black-edged transversal bars on the anterior body; such a transversal bar pattern dissolves anterior to the midbody region and then turns into a series of small pale blotches that build each a dorso-lateral stripe that ends at the dorsal tail base;
15) venter light, laterally with dark spots in the forebody region; cream in the anterior body, then turning into pinkish orange at least in juvenile males;
16) 34 maxillary teeth, arranged in a continuous series, the two posteriormost distinctly enlarged, without diastema;
17) hemipenis simple, with undivided sperm groove; the outer genital organ is covered with small spines except for a single, strongly enlarged spine next to the sperm groove at the hemipenis base and except for irregularly arranged medium-sized spines that encircle the hemipenis horizontally at the trunco-pedicel area.

There exist other local endemic herpetofauna representatives in Hin Nam No and Phong Nha – Ke Bang, such as the tree frog *Gracixalus quyeti* (Nguyen, Hendrix, Böhme, Vu & Ziegler, 2008), which is known only from central Vietnam's Quang Binh Province and from Khammouane Province in central Laos (Nguyen *et al.*, 2008; Egert *et al.*, 2017), or the bent-toed gecko species *Cyrtodactylus cryptus* Heidrich, Rösler, Vu, Böhme & Ziegler, 2007, which also can be found only along the Annamite Mountain Range of central Vietnam and Laos (Heidrich *et al.*, 2007; Luu *et al.*, 2016). However, Luu *et al.* (2016) also could uncover cryptic *Cyrtodactylus* speciation along both sides of the Annamites, with five endemic karst-dwelling *Cyrtodactylus* occurring in a restricted area on opposite sides of the Range. In Vietnam, there are two endemic karst-adapted species, *C. phongnhakebangensis* Ziegler, Rösler, Herrmann & Vu, 2002 and *C. roesleri* Ziegler, Nazarov, Orlov, Nguyen, Vu, Dang, Dinh & Schmitz, 2010 as opposed to three endemic karst-adapted species in Laos, *C. calamei* Luu, Bonkowski, Nguyen, Le, Ngo, Schneider & Ziegler, 2016, *C. hinnamnoensis* Luu, Bonkowski, Nguyen, Le, Ngo, Schneider & Ziegler, 2016, and *C. sommerladi* Luu, Bonkowski, Nguyen, Le, Ngo, Schneider & Ziegler, 2016 (Ziegler *et al.*, 2002, 2010; Luu *et al.*, 2016). According to Luu *et al.* (2016), the rapid adaptation to isolated local karst conditions compared with generalist ground or tree-associated taxa might offer an explanation for such cryptic speciation processes on karst, as the environmental conditions in karst are known to accelerate evolutionary processes (Nicolas *et al.*, 2012; Le *et al.*, 2015).

The new record of the beautiful but still poorly known and rare species *H. andreae* in Laos underlines the importance of the Hin Nam No National Protected Area as habitat and refugium for the regional unique biodiversity, which only occurs in this karst massif. Phong Nha – Ke Bang already is UNESCO World Heritage Site whereas Hin Nam No currently is on the tentative list (UNESCO WHC 2018).

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Appendix Table

GenBank accession numbers of the four marker genes and associated voucher specimens/tissues that were used in this study. For more details see Guo *et al.* (2014).
Sequences of species in bold are unpublished and were provided by P. Guo as personal communication.

Species names	GenBank (cytb)	GenBank (Cmos)	GenBank (NT3)	GenBank (Rag1)	Voucher
Amphiesma stolatum	AF471030	AF471097	-	-	CAS:HERP:206560
Hebius andreae	MK253674	MK253675	MK253676	MK253677	VNUF R.2017.25
Hebius atemporalis	KJ685680	KJ685630	KJ685732	KJ685572	GP1626
Hebius atemporalis	KJ685695	KJ685645	KJ685747	KJ685587	GP2318
Hebius boulengeri	KJ685710	KJ685660	KJ685770	KJ685609	MVZ236752
Hebius boulengeri	KJ685664	KJ685613	KJ685714	KJ685557	AMNH148562
Hebius boulengeri	-	-	-	-	AMNH192804
Hebius clerki	KJ685666	KJ685615	KJ685716	KJ685559	CAS215036
Hebius craspedogaster	KJ685704	KJ685654	KJ685759	KJ685597	GP455
Hebius 'deschauenseei'	KJ685665	KJ685614	KJ685715	KJ685558	AMNH148575
Hebius khasiensis	KJ685669	KJ685618	KJ685719	KJ685562	CAS221525
Hebius khasiensis	KJ685668	KJ685617	KJ685718	KJ685561	CAS221504
Hebius leucomystax	-	-	-	-	AMNH R-176468
Hebius metusia	KJ685682	KJ685632	KJ685734	KJ685574	GP1712
Hebius modestus	KJ685671	KJ685620	KJ685721	KJ685564	CAS234262
Hebius optatus	KJ685662	KJ685611	KJ685712	KJ685555	AMNH147155
Hebius venningi	KJ685670	KJ685619	KJ685720	KJ685563	CAS233206
Hebius 'venningi'	KJ685675	KJ685625	KJ685727	-	GP1300
Herpetoreas burbrinki	GQ281781	JQ687443	KJ685761	KJ685599	YBU071128
Herpetoreas platyceps	KJ685690	KJ685640	KJ685742	KJ685582	GP2096
Natrix natrix	AF471059	AF471121	EU390931	-	-
Natriciteres olivacea	AF471058	AF471146	-	-	CAS:HERP:220640
Nerodia erythrogaster	GQ285504	JN090137	-	-	-
Rhabdophis tigrinus	AF471051	AF471119	-	-	LSUMZ:37418
Trachischium monticola	JQ687435	JQ687453	-	KJ685570	GP1487
Xenochrophis piscator	GQ225659	GQ225669	EU390941	-	-
Xenochrophis vittatus	EF395895	EF395920	-	-	FMNH257460