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First Record of a Cavernous Land Leech *Sinospelaeobdella cavatuses* (Hirudinda: Haemadipsidae) from Thailand

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ABSTRACT.– Sixteen specimens of cavernous land leeches collected from a cave in Chiang Mai Province, northern Thailand, were examined by integrating morphological and molecular analyses. A phylogenetic tree based on *COI* sequences nested these specimens within the clade of the cavernous land leech *Sinospelaeobdella cavatuses* (Yang, Mo & Wang, 2009), bloodsucking leech that feeds exclusively on cave-dwelling bats, recently described from China and now representing the first record of a cavernous leech species in Thailand. Average genetic distance between Thai and Laotian specimens (4.02% P-distance) was higher than between Thailand and Chinese specimens (0.95% P-distance). Morphological description of Thai specimens is provided. Thai materials showed smaller body dimensions than the range reported in recent studies on this species.

KEYWORDS: cave-dwelling bat, DNA barcoding, land leeches, *Sinospelaeobdella*, Thailand

INTRODUCTION

Haemadipsidae is a family of terrestrial blood-feeding leeches. They have been reported throughout terrestrial habitats in tropical and subtropical Indo-Pacific continental regions, such as the damp jungles and humid forests of Southeast Asia, South Asia, and Australia (Borda and Siddall, 2010; Nakano et al., 2020). Although Thailand is regarded as a biodiversity hotspot (Singh et al., 2021), few studies have explored the biodiversity of land leeches in the country, and their ecology and biology are still poorly understood (Ngamprasertwong et al., 2005, 2007; Borda and Siddall 2010; Nakano et al., 2016, 2018). The *Sinospelaeobdella* Liu, Huang & Liu, 2019 is a genus of land leeches that primarily inhabit caves. This genus was described as lacking pigmentation, without spots or stripes, trignathous with tiny teeth, gonopores separated by two annuli, and ventral posterior sucker with about 78 indistinct friction rays (Liu et al., 2019). At present, only two species are known: *S. wulingensis* Liu, Huang & Liu, 2019, described from caves in the northern subtropical Wuling Mountains of Hunan, China; and *S. cavatuses* (Yang, Mo & Wang, 2009), described from a cave in Luxi City, Yunnan, China. The latter species has a wider distribution range. It has been reported from Hunan, China (Huang et al., 2019); Luang Namtha, Luang Prabang, and Bolikhamsai provinces, Laos (Borda and Siddall, 2010); and is probably distributed in Myanmar and Malaysia (Huang et al., 2019). These two species are distinguishable from each other by characteristics of their eyes: *S. cavatuses* has a larger first pair of eyes, while the fifth pair of eyes are usually absent (Huang et al., 2019).

Integrative approaches using morphological characteristics and molecular data were successfully used for species identification. DNA barcoding based on mitochondrial cytochrome c oxidase 1 (*COI*) gene sequencing has become highly popular for the molecular identification of species because it is a rapid, uncomplicated, and affordable method (Herbert et al., 2003; Smith et al., 2007; Frézal and Leblois, 2008). Mitochondrial DNA is popular as a target for species identification and the study of genetic diversity because it has high variation and lacks recombination. DNA barcoding has been used to differentiate between species based on genetic distance, and was reported to be useful for the identification of undescribed species of annelids, including leeches (Kutschera et al., 2007; Lai et al., 2011; Jeratthitikul et al., 2017, 2020; Chatterjee et al., 2018; Seesamut et al., 2018; Jiranuntskul et al., 2022).

TABLE 1. Specimens used in the molecular analysis with GenBank accession numbers, and collection countries.

Species	GenBank ID (COI)	Collection country	Reference
Sinospelaeobdella cavatuses	OQ707890	Thailand	This study (CUMZ 3835, T1)
Sinospelaeobdella cavatuses	OQ707891	Thailand	This study (CUMZ 3836, T2)
Sinospelaeobdella cavatuses	MG195989	China	Huang et al., 2019
Sinospelaeobdella cavatuses	MG195988	China	Huang et al., 2019
Sinospelaeobdella cavatuses	MG195987	China	Huang et al., 2019
Sinospelaeobdella cavatuses	MG195993	China	Huang et al., 2019
Sinospelaeobdella cavatuses	HQ203168	Laos	Borda and Siddall, 2010
Sinospelaeobdella wulingensis	MG195994	China	Huang et al., 2019
Sinospelaeobdella wulingensis	MG195986	China	Huang et al., 2019
Sinospelaeobdella wulingensis	MG195990	China	Huang et al., 2019
Sinospelaeobdella wulingensis	MG195992	China	Huang et al., 2019
Sinospelaeobdella wulingensis	MG195991	China	Huang et al., 2019
Tritetrabdella kinabaluensis	KF839949	Malaysia	Merckx et al., 2015
Chtonobdella tanae	HQ203164	Australia	Borda and Siddall, 2010
Neoterrabdella australis	HQ203162	Australia	Borda and Siddall, 2010
Haemadipsa zeylanica	HQ203180	Nepal	Borda and Siddall, 2010
Haemadipsa zeylanica	HQ203173	Thailand	Borda and Siddall, 2010
Haemadipsa japonica	HQ203171	Japan	Borda and Siddall, 2010
Haemadipsa ornata	HQ203178	Indonesia	Borda and Siddall, 2010
Haemadipsa montana	HQ203182	Nepal	Borda and Siddall, 2010
Chtonobdella fallax	EU100096	Madagascar	Borda et al., 2008
Haemadipsa japonica	LC424190	Japan	Retrieved from GenBank
Xerobdella anulata	OP270248	Croatia	Brigić et al., 2022
Xerobdella lecomtei	EU100099	Slovenia	Borda et al., 2008

In this study, sixteen specimens of land leeches collected in 2018–2019 from a cave in Chiang Mai Province, northern Thailand, were examined by integrative taxonomy, combining the comparison of morphological characters and molecular-based analyses of *COI* sequences. This is the first study of cavernous land leeches in Thailand.

MATERIALS AND METHODS

Sample collection, preservation, and morphological examination

In December 2018 and July 2019, sixteen specimens of cavernous land leeches were collected from a limestone cave in northern Thailand. The GPS coordinates of the sampling locality were recorded, and the habitat was photographed. Specimens were relaxed by the gradual addition of absolute ethanol to freshwater. The fully relaxed leeches were photographed before being fixed in 95% (v/v) ethanol for morphological and molecular studies. Morphological observations were made under an OLYMPUS SZX16 stereo-microscope, and the identification was based on available taxonomic literature concerning land leeches from this region (Yang et al., 2009; Huang et al., 2019). Drawings were made of external characters and the reproductive system. The voucher specimens were deposited in Chulalongkorn University Zoological Museum (CUMZ) in Bangkok.

DNA extraction, PCR and sequencing

The genomic DNA was extracted from a piece of the caudal sucker using a Geneaid^{TM} DNA extraction kit. DNA was eluted in elution buffer and kept at -20 °C until use. A 658-bp region of the *COI* mito-chon-drial gene was amplified using PCR. Each PCR amplify-cation mixture consisted of 1 µl of DNA template, 2.5 µl (5 µM) each of LCO1490 (forward) and HCO2198 (reverse) universal primers (Folmer et al., 1994), 25 µl of Ultra-Pure Taq PCR Master Mix with emerald dye and 19–19.4 µl of double distilled H_2O. The PCR reaction mixture was heated to 94 °C for 2 min, followed by 35 cycles of 94 °C for 1 min, 48 °C for 1 min and 72 °C for 2 min, and then followed by a final extension at 72 °C for 5 min. The PCR products were separated with 1% (w/v) agarose gel electrophoresis in 0.5x TBE buffer and detected with SYBR Safe DNA gel staining under UV transillumination. Non-purified PCR products were sent for commercial purification and sequencing using an Applied Biosystems Automatic Sequencer (ABI 3730XL) at Macrogen, Inc. (Korea).

Phylogenetic analysis

Two individuals of the newly obtained specimens were sequenced. The *COI* sequences were edited with MEGA X (Kumar et al., 2018) and adjusted manually. The sequences were checked and compared with other sequences available in the GenBank database at the

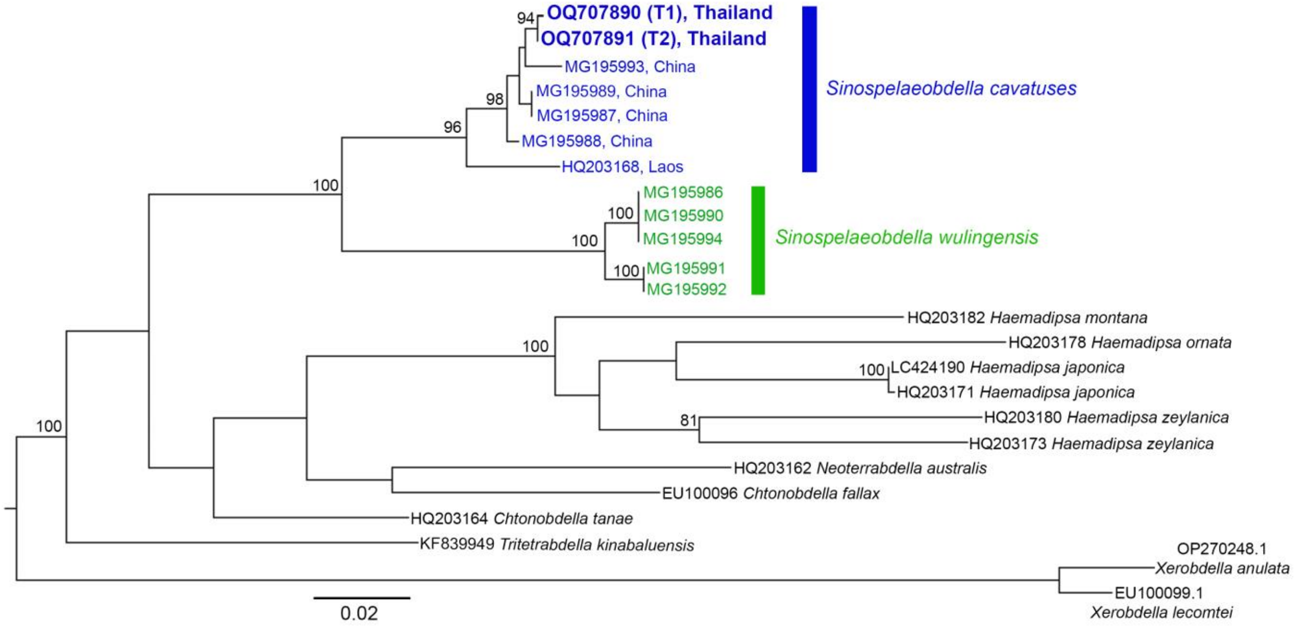

FIGURE 1. Maximum likelihood tree based on *COI* gene sequences (658 bp). Bootstrap values of >70% are considered as sufficiently supported and are shown on the tree. T1 (OQ707890) and T2 (OQ707890) are the samples of *S. cavatuses* newly collected from Thailand in this study.

TABLE 2. Pairwise corrected genetic distances among *Sinospelaeobdella* specimens based on the p-distance method.

GenBank no./Species	1	2	3	4	5	6	7	8	9	10	11	12
1. OQ707890 *S. cavatuses*												
2. OQ707891 *S. cavatuses*	0.2											
3. MG195989 *S. cavatuses*	0.8	0.9										
4. MG195988 *S. cavatuses*	0.8	0.9	0.9									
5. MG195987 *S. cavatuses*	0.8	0.9	0.0	0.9								
6. MG195993 *S. cavatuses*	1.2	1.4	1.4	1.7	1.4							
7. HQ203168 *S. cavatuses*	4.0	4.1	3.8	3.3	3.8	4.0						
8. MG195994 *S. wulingensis*	9.7	9.9	10.0	9.7	10.0	10.2	10.5					
9. MG195986 *S. wulingensis*	9.7	9.9	10.0	9.7	10.0	10.2	10.5	0.0				
10. MG195990 *S. wulingensis*	9.7	9.9	10.0	9.7	10.0	10.2	10.5	0.0	0.0			
11. MG195992 *S. wulingensis*	9.8	10.0	10.2	9.9	10.2	10.4	10.6	1.7	1.7	1.7		
12. MG195991 *S. wulingensis*	9.8	10.0	10.2	9.9	10.2	10.4	10.6	1.7	1.7	1.7	0.0	

National Center for Biotechnology Information (NCBI) using BLAST similarity search tool (http://www.ncbi.nlm.nih.gov). Other sequences of related species were retrieved from GenBank database and included in phylogenetic analysis (Table 1). These sequences included *Sinospelaeobdella* and other land leech species as outgroups. A phylogenetic tree was constructed using maximum likelihood analysis (ML) as implemented in RAxML-HPC2 on XSEDE (Stamatakis, 2014), through the CIPRES Science Gateway portal (Miller et al., 2012). One thousand bootstrap replicates were used to assess branch support and the default parameters were used. Bootstrap values lower than 75% for each node were considered as insufficiently supported (Okanishi et al., 2018). The resulting tree was plotted using FigTree v.1.4.4 (http://tree.bio.ed.ac.uk/software/figtree) and the tree diagram was created in Adobe Photoshop 2020. Moreover, pairwise genetic divergences were examined using a distance-based analysis (uncorrelated p-distance) as implemented in MEGA X with a bootstrap re-analysis of 1,000 pseudoreplicates.

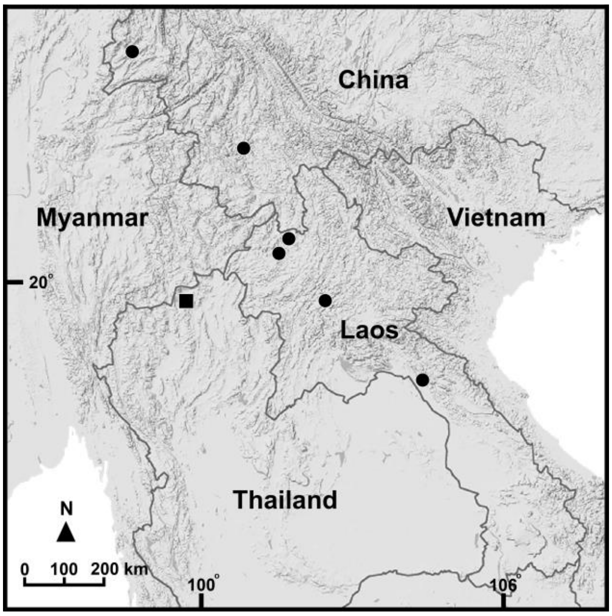

FIGURE 2. Distribution map of *Sinospelaeobdella cavatuses*. The square symbol represents the locality of *S. cavatuses* collected in this study.

RESULTS

Molecular analysis

The 658-base pair sequences of mitochondrial *COI* from the two specimens collected for this study had 99.24% nucleotide identity with *S. cavatuses* (MG 195989) according to the BLAST analysis. The nucleotide sequences were deposited in the GenBank database under accession numbers OQ707890 (sample ID, T1) and OQ707891 (sample ID, T2). The phylogenetic tree reveals *Sinospelaeobdella* as a genus was 100% supported, *S. wulingensis* as a species was 100% supported and the sequences of *S. cavatuses* had 96% supported (Fig. 1). The genetic distance revealed that specimen T1 differed by 1 substitution (0.20%) from T2. Both specimens differed by 5 substitutions (0.95%) from a *S. cavatuses* specimen from Yunnan, China (MG195989); by 26 substitutions (4.02%) from a *S. cavatuses* specimen from Luang Namtha, Laos (HQ 203168); and by 65 substitutions (9.9%) from *S. wulingensis* (MG195991) (Table 2). Overall, the mean intraspecific distance for *COI* in *S. cavatuses* was 1.76%, whereas the mean interspecific distance between *S. cavatuses* and *S. wulingensis* was 10.07%.

Systematics

Family Haemadipsidae Blanchard, 1893

Genus *Sinospelaeobdella* Liu, Huang & Liu, 2019

Sinospelaeobdella cavatuses (Yang, Mo & Wang, 2009)

(Figes 2–4)

Haemadipsa cavatuses Yang, Mo & Wang, 2009: 125–129, figs. 1–6.
Sinospelaeobdella cavatuses—Huang et al., 2019: 259, fig. 3b.

Material examined.– CUMZ 3833 (6 specimens in ethanol) from Wat Tham Huay Bon forest monastery, Fang District, Chiang Mai Province, Thailand (19°59' 00.7"N 99°11'05.4"E), 8th December 2018. CUMZ 3834 (10 specimens in ethanol) with the same locality data, 16th July 2019.

Description of new material.– Body medium in size, slenderly cylindrical, convex dorsally and flat ventrally. Body length 23.28 to 32.00 mm (average 26.16±3.64 mm; n = 16), caudal diameter 3.18 to 5.39 mm (average 3.92±0.57 mm), maximum body breadth 2.21 to 5.64 mm (average 3.39±0.86 mm), oral breadth 1.88 to 3.29 mm (average 2.25±0.34 mm). Body surface without pigment or dorsal stripes, colorless and transparent (milky white in ethanol). Head region slightly expanded, sub-triangular. Anterior sucker deep, wide and triangular. Eyespots 5 pairs, arranged on the back of the head, the first pair largest, the second to the fourth pair gradually reduced in size to small dots, the fifth pair absent or very small. Three jaws present with tiny teeth. Caudal sucker nearly circular, slightly longer than wide, diameter little larger than maximum body width, dorsally with one pair of auricles located. Anus located at the middle of transverse depression on top of the projection.

Male gonopore in somite XII b2/a2, female gonopore in XII b5/b6. Gonopores separated by two annuli. Male reproductive system with 10 pairs of testisacs, the first pair of testes located on both sides of ganglion XIV. Vas deferens connected to epididymis. Epididymis consisting of multiple convoluted tubes, located on side of ganglia XII and XIII, anteriorly connected to large ejaculatory bulb. Penis sac located on the left side of ganglion XI. Female reproductive system with a pair of ovisacs located at ganglion XII. Ovaries connected to large vagina sac via long, sigmoid, and slender chief common oviduct. Vaginal sac circular in shape, 3–4 times larger than penis sac, connected to female gonopore by a thick and bent vaginal duct.

Distribution.– This species is found exclusively in caves in East and Southeast Asia (Fig. 2): Yunnan and Hunan, China; Luang Namtha, Luang Prabang, and

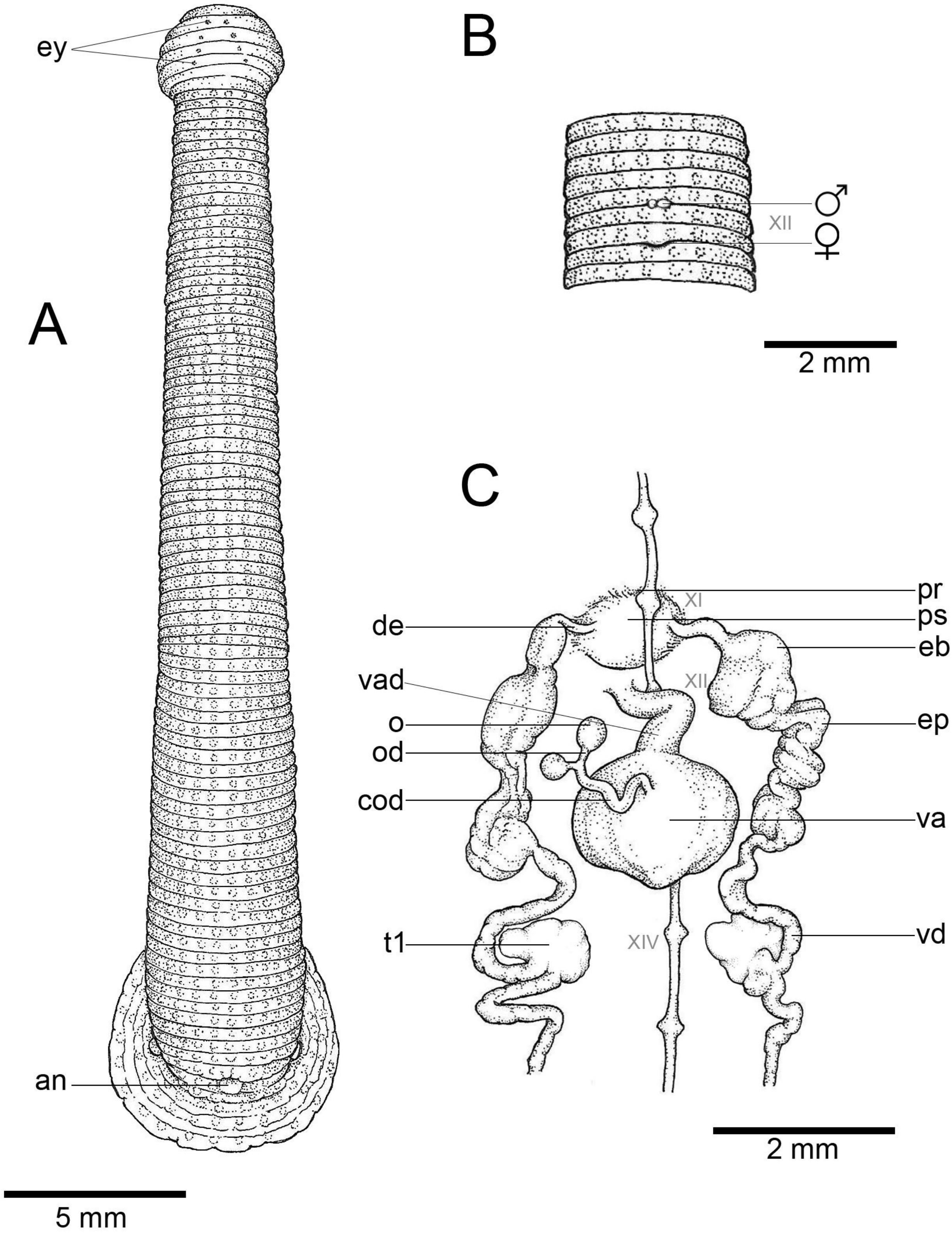

FIGURE 3. *Sinospelaeobdella cavatuses*, specimen CUMZ 3834. **A.** Dorsal view. **B.** Ventral view. **C.** Reproductive system. Abbreviations: an, anus; cod, chief oviduct; de, ejaculatory duct; eb, ejaculatory bulb; ep, epididymis; ey, eyespots; o, ovisac; od, oviduct; pr, prostate gland; ps, penis sac; t1, first pair of testes; va, vagina sac; vad, vaginal duct; vd, vas deferens; ♂, male gonopore; ♀, female gonopore.

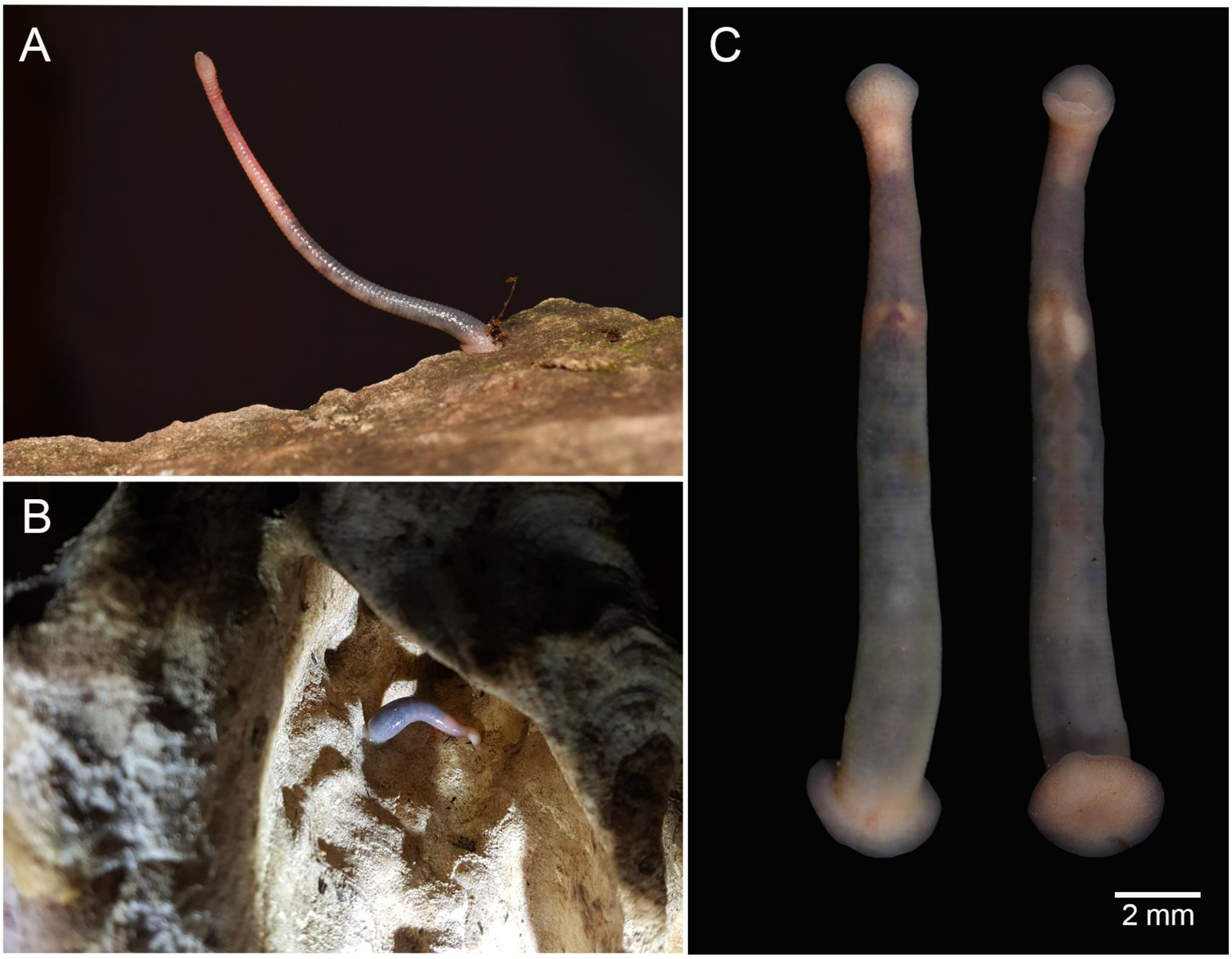

FIGURE 4. Living specimens and live coloration of *Sinospelaeobdella cavatuses* from Wat Tham Huay Bon forest monastery, Fang District, Chiang Mai Province, Thailand. **A.** Leech with full extension when attracted by a human collector. **B.** Leech in resting site in the hollow of a stalactite. **C.** Dorsal (left) and ventral (right) views of specimen after being relaxed with series of ethanol. **A** and **B** not to scale.

Bolikhamsai, Laos; Chiang Mai, Thailand (this study). The records from Myanmar and Malaysia are based solely on photographs with uncertain location data and without information of voucher specimens (De Vivo et al., 2005; Huang et al., 2019).

Remarks.– These specimens are placed in *Sinospelaeobdella* on a basis of morphological characteristics by having three jaws with tiny teeth, two annuli separating gonopores, and no spot nor strip on body surface. The absence of a fifth pair of eyes in specimens from Thailand is the morphological character used to confirm its taxonomic status as *S. cavatuses.* This character also distinguishes this species from *S. wulingensis*. *Sinospelaeobdella cavatuses* has been reported to feed on bats in a deep cave in Yunnan, China (Yang et al., 2009). In addition, De Vivo et al. (2005) reported unidentified leeches hanging close to the roosting site of a bat from Shan State, Myanmar; the leeches were later identified as *S. cavatuses*.

Unfortunately, we could not observe blood-sucking behavior during this survey. However, bats are known to inhabit this cave based on field observation. Therefore, these bats could possibly be the host for *S. cavatuses*. One empty cocoon was collected in this study, suggesting that reproduction of *S. cavatuses* was successfully completed inside of this cave. We also include drawings (Fig. 3) and color plates (Fig. 4) of selected specimens.

DISCUSSION

This paper is the first study of cave-dwelling land leeches collected in Thailand. Based on morphological characteristics, the specimens collected from Fang, Chiang Mai, Thailand, were identified as *S. cavatuses*. The current geographical distribution of this species includes locations in China, Laos, Myanmar, Malaysia, and Thailand (this study). The *S. cavatuses* specimens from Thailand show variation in body dimensions. They are smaller than the range of measurements of specimens from Yunnan previously reported by Yang (2009). Measurements of the body length of Thai specimens were 23.28 to 32.00 mm (vs 31.0 to 44.0 mm in specimens from Yunnan), caudal sucker diameter 3.18 to 5.39 mm (vs 6.0 to 7.5 mm), the maximum body breadth 2.21 to 5.64 mm (vs 6.0 to 8.0 mm), and oral sucker breadth 1.88 to 3.29 mm (vs 2.6 to 3.0 mm). Although the *S. cavatuses* specimens from Thailand are generally smaller than the specimens from Yunnan, they still have greater average body length (26.2±3.64 mm) than *S. wulingensis* from Hunan, China (22.7±5.0 mm). Therefore, the body size is possibly useful for identifying *Sinospelaeobdella* species, as mentioned in Huang (2019).

In this study, the *COI* interspecific distance between cavernous land leeches *S. cavatuses* and *S. wulingensis* was 10.07%, lower than in other land leech genera, such as between *Haemadipsa rjukjuana* and *H. limuma* (14.6%; Won et al., 2014), or among *Tritetrabdella* species (13.34% average; Nakano et al., 2016); however, it is still higher than the genetic distance between the freshwater leeches (buffalo leeches) *Hirudinaria thailandica* and its congeners (9.21% average; Jeratthitikul et al., 2020), or among four cryptic species in the *Hirudinaria manillensis* species complex (7.37% average; Jiranuntskul et al., 2022). We suggest that land leeches, including cavernous land leeches, show a higher genetic distance between species than fresh-water leeches due to geographic isolation. The average *COI* genetic distance of *S. cavatuses* between Chiang Mai, Thailand and Luang Namtha, Laos samples was higher than between Thailand and Yunnan, China samples, even though Thailand and Laos are geographically closer than Thailand and China. Therefore, our results support the assumption of Huang et al. (2019), that the sample from Laos is possibly an undescribed distinct species or subspecies.

In conclusion, this is the first exploration of blood-sucking and cave-dwelling land leeches in Thailand, and reports the occurrence of *S. cavatuses* from Chiang Mai Province. More than 5,000 caves have been documented in the country (Bolger and Ellis, 2015); however, the knowledge of the cavernous land leech fauna of Thailand is still insufficient. More intensive surveys thus will increase the known taxonomic diversity, and future studies should include larger sample sizes to better represent the range of morphological variation.

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