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Undescribed Diversity in a Widespread, Common Group of Asian Mud Snakes (Serpentes: Homalopsidae: *Hypsiscopus*)

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Mud snakes (Serpentes: Homalopsidae) are a morphologically diverse family of aquatic snakes distributed from eastern Pakistan, eastward through South Asia, mainland and maritime Southeast Asia, and extending to New Guinea and northern Australia. Some species of homalopsids represent the most abundant tetrapods in aquatic systems in tropical Asia, but with few evolutionary studies investigating their diversity with dense geographic and taxonomic sampling. The genus *Hypsiscopus* includes two named species that inhabit freshwater systems throughout most of Southeast Asia: *H. matannensis* of Sulawesi, and the widespread *H. plumbea* found in rivers, lakes, and rice paddies in the remainder of Southeast Asia. We use a multilocus dataset of two mitochondrial and three nuclear genes with dense sampling of *H. plumbea* to elucidate the evolutionary history of this genus. We find that *H. plumbea* is paraphyletic with respect to *H. matannensis*, with populations around and north of Central Thailand's Khorat Plateau phylogenetically outside of a clade containing *H. matannensis* and *H. plumbea* south of the Khorat Plateau. This lineage differs morphologically and genetically from *H. plumbea sensu stricto* (south of the Khorat Plateau) and *H. matannensis*. We describe this lineage as a third species of *Hypsiscopus* based on its phylogenetic position and meristic and color pattern data. This study exemplifies the need to investigate widespread, abundant taxa to better understand the evolutionary histories of aquatic snakes in Southeast Asia.

SOUTHEAST Asia is home to a remarkable diversity of snakes in numerous families with vast varieties of morphologies, diets, and habitat preferences (Das, 2018). Over 400 species of snakes are found in mainland and maritime South and Southeast Asia, and new species are still being described at high rates, including from poorly known families (Miller et al., 2020; Weinell et al., 2020; Le et al., 2021; Lee, 2021). This regional diversity is owed to the complex geological histories that have formed heterogeneous landscapes and a multitude of different habitats. Tectonic uplift events have shifted the topology of the mainland (Hutchison, 1989; Rainboth, 1996), and river capture events have changed the courses of some of the largest rivers in Southeast Asia (Carbonnel, 1965; Workman, 1977). Additionally, one of the most notable geological processes, and most focused on with respect to biogeography, are the cyclic sea level fluctuations that have repeatedly connected and disconnected landscapes that are separated today (Hall, 2009). Sumatra, Borneo, Java, and other nearby smaller islands were connected to the Malay Peninsula and northern parts of mainland Southeast Asia (Singapore, Malaysia, Myanmar, Thailand, Cambodia, Laos, Vietnam, and China) in a region known as Sundaland (or the Sunda Shelf). As glacial cycles occurred in the Pleistocene, the last few hundred thousand years have seen concurrent cycles of land bridges that have allowed for the migration and homogenization of terrestrial populations of particular species, as well as the diversification of others (Voris, 2000; de Bruyn et al., 2014).

Homalopsid snakes (Asian Mud Snakes) are one of the many vertebrate groups whose diversity has been influenced by Southeast Asia's geological history (Alfaro et al., 2008; Bernstein et al., 2021). Taxonomic diversity of this family is exemplified by the recognition of 56 species amongst 29 genera, split into two major clades: the fangless and rear-fanged groups. Homalopsid snakes are found primarily in aquatic habitats, and they display a variety of morphologies and ecologies (Murphy, 2007; Brooks et al., 2009; Jayne et al., 2018). For example, some species groups prefer brackish waters and mudflats (e.g., *Cerberus*), while others have

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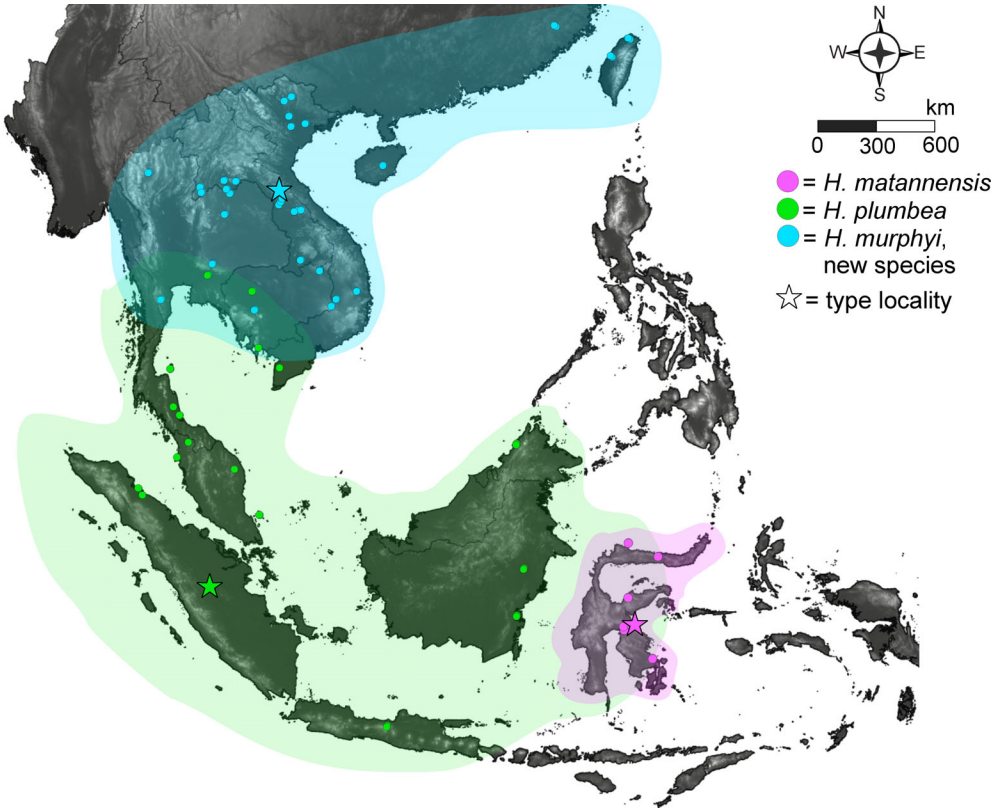

Fig. 1. Distribution map of *Hypsiscopus*. Shaded regions and points represent known ranges as of this study and sampling localities, respectively. Stars show type localities; note: type locality for *H. plumbea* is 'Java'; central coordinate given.

evolved to be freshwater specialists (e.g., *Enhydris*). These genera contribute to large proportions of the vertebrate biomass in some of the freshwater aquatic systems in Southeast Asia (Murphy, 2007). While new species of homalopsids have been recently described (Quah et al., 2017; Murphy and Voris, 2020; Köhler et al., 2021), the rates of species descriptions and evolutionary studies on this family of snakes pale in comparison to other caenophidians; this is due to a large number of homalopsid species living in hard-to-explore habitats (e.g., mangroves, tidal flats) with secretive, nocturnal habits. Furthermore, abundant, morphologically conserved, widespread species may contain undescribed diversity (Bickford et al., 2007).

Freshwater snakes of the genus *Hypsiscopus* are members of the rear-fanged homalopsid group that inhabit fresh waters of mainland and maritime Southeast Asia. They range from central Myanmar and coastal China (including Hainan and Taiwan) through the rest of mainland Southeast Asia, and throughout insular Southeast Asia, with Sulawesi as the eastern limit of their distribution (Murphy and Voris, 2014; Fig. 1). *Hypsiscopus* is currently composed of two described species: *H. matannensis*, endemic to Sulawesi, and *H. plumbea*, representing all other populations (including Sulawesi). These two species differ by few meristic characters, and only a few natural history or evolutionary studies have focused on the group. Bernstein et al. (2021) showed that *H. plumbea* is not monophyletic, but the lack of dense sampling, morphological data, and additional loci have precluded us from gaining a sufficient understanding of the species diversity within this group of freshwater specialists. In this study, we infer a phylogeny using a multilocus dataset of *Hypsiscopus* from throughout its range and recover patterns congruent with Bernstein et al. (2021). We find that *H. plumbea* is paraphyletic with respect to *H. matanennsis*, and specimens from central and northern mainland Southeast Asia represent a distinct lineage of *Hypsiscopus*. Combining these results with corroborating morphological data, we describe this species and discuss biogeographic hypotheses that may have led to this species diversity.

MATERIALS AND METHODS

Data collection

Specimen collection and sampling.—Specimens of *Hypsiscopus* were collected in the field by the authors and humanely euthanized by cardiac injection of aqueous sodium pentobarbital or tricaine methanesulfonate (MS-222; Simmons, 2015). Specimens were fixed in 10% buffered formalin after preserving liver or muscle in DMSO/EDTA salt-saturated tissue buffer, RNAlater (Invitrogen), or 95–100% ethanol for molecular analyses. Specimens were later transferred to 70% ethanol for permanent storage and deposited at the Field Museum of Natural History (FMNH), North Carolina Museum of Natural Sciences (NCSM), Department of Biology, Faculty of Natural Sciences, National University of Laos (Lao National History Museum; NUOL), and Museum of Vertebrate Zoology, University of California, Berkeley (MVZ). Comparative material was examined from the holdings of these and the American Museum of Natural History (AMNH), Florida Museum of Natural History (UF), La Sierra University Herpetology Collection (LSUHC), Museum of Comparative Zoology (MCZ), Royal Ontario Museum (ROM), Sabah State Museum (SSM), and University of Kansas Biodiversity Institute & Natural History Museum (KU; Supplemental Table 1; see Data Accessibility). Institutional abbreviations follow Sabaj (2020). The distribution map (Fig. 1) was made

using QGIS v3.4.3 Madeira and layers downloaded from DIVA-GIS (https://www.diva-gis.org/).

DNA extraction, amplification, and sequencing.—Whole genomic DNA was extracted from liver or muscle using the PureGene Animal Tissue DNA Isolation Protocol (Gentra Systems, Inc.) or the DNeasy Blood & Tissue Kit (Qiagen). Two mitochondrial genes and three nuclear genes were amplified by polymerase chain reaction (PCR). An 849–882 bp fragment of mitochondrial DNA that encodes part of the tRNA-Lys gene, the complete ATPase subunit 6 gene, the complete ATPase subunit 8 gene, and part of the part of the cytochrome oxidase *c* subunit III gene (hereafter as "ATPase") was amplified using the forward primer LYS2F and the reverse primers H-COXIIIb (most samples) or CO31R. A 1,136–1,145 bp fragment of mitochondrial DNA that encodes part of the tRNA-Glu gene, the complete cytochrome *b* gene, and part of the tRNA-Thr gene (hereafter as "cyt-b") was amplified using the forward primers L14910 (most samples) or L14919 and the reverse primer H16064. A 553 bp fragment of nuclear DNA that encodes part of the prolactin receptor (PRLR) nuclear gene was amplified using the primers PRLR_f1 and PRLR_r3. These three genes were amplified using an initial denaturation of 94°C for 3 min, followed by 35 cycles of 94°C for 45 s, 50–60°C for 30 s, and 72°C for 1 min, with a final extension of 72°C for 7 min. A ~1,300 bp fragment of nuclear DNA that encodes part of the WAP, Follistatin/Kazal, Immunoglobulin, Kunitz and Netrin Domain Containing 2 (WFIKKN2) gene was amplified using a nested PCR reaction following Shen et al. (2013). An 885 bp fragment of nuclear DNA that encodes part of the intron of the vacuolar protein sorting-associated protein 13B (VPS13B) gene was amplified using a nested PCR reaction following Li et al. (2017).

PCR products were cleaned using GELase (Epicentre Technologies) or ExoSAP-IT (Applied Biosystems). Cycle sequencing products were sequenced in both directions on a Prism 3100 Genetic Analyzer (Applied Biosystems) or a 3730 DNA Analyzer (Applied Biosystems) using Big Dye Terminator version 3 chemistry (Applied Biosystems) and the amplifying primers. The internal primers L-ATPint and H-ATPint were also used in the ATPase sequencing reactions, and L15584, H-cytbEpint, H15149, and H15716 were also used in the cyt-b sequencing reactions. Primers, corresponding sequences, and their source publications are given in Supplemental Table 2 (see Data Accessibility). Sequences were edited using Sequencher version 4.1 (Gene Codes Corporation) or Geneious Prime 2020.2.4 (Biomatters Ltd.) and deposited in GenBank under the following accession numbers: OM479860, OM479866, OM479876, OM479878, OM489881, OM479882, OM479884–479927, OM479930–OM479932, OM479934, OM479935, OM479937, OM479939, OM479947, OM479948, OM479950–OM479994, OM479997, OM480002, OM480007, OM480011, OM480022, OM480023–OM480055, OM480057, OM480060–OM480067, OM480069, OM480071, OM480074–OM480099, OM480101, OM480106, OM480111, OM480114, OM480118, OM480125–OM480132, OM480134–OM480160, OM480166, OM480167, OM480171, OM480176, OM480177, OM480179, OM480180–OM480182, OM480184–OM480199, OM480202, OM480203, OM480205, OM480206, OM480209–OM480252, OM480255–OM480263, OM480265, OM480267, OM480276–OM480298, OM480300, OM480303, OM480304, OM480306–OM480315, OM480317–OM480327.

Phylogenetic analyses.—Raw sequence reads were assembled in Geneious v7.1.9 (https://www.geneious.com) using the MUSCLE function with default settings, and then checked by eye. We used sequences of *Enhydris chanardi* and *Homalopsis buccata* as outgroups based on the homalopsid phylogeny of Bernstein et al. (2021); for ATPase, due to sequence availability, we used *Myrrophis chinensis*. Outgroup sequences were obtained from NCBI's GenBank (Karns et al., 2010; Wiens et al., 2012; Bernstein et al., 2021; Supplemental Table 1; see Data Accessibility). We calculated uncorrected pairwise distances for each gene using the 'distance' calculate function under default parameters in Geneious. To estimate gene trees, we used IQ-TREE v1.6.1 (Nguyen et al., 2015), using the best model implemented by ModelFinder (Kalyaanamoorthy et al., 2017). For nuclear gene trees, we partitioned analyses by gene and codon position; mitochondrial genes were left unpartitioned. We also generated cyt-b+ATPase and mitochondrial+nuclear (all genes) concatenated trees (hereafter referred to as 'mitochondrial' and 'concatenated,' respectively), the latter using the same nuclear partitioning scheme for the individual nuclear gene trees. We implemented 1000 ultrafast (UF) bootstraps (Minh et al., 2013; Hoang et al., 2018) to assess branch support. As recommended by Guindon et al. (2010), we also calculated Shimodaira-Hasegawa-approximate likelihood ratio tests (SH-alrt); relationships were considered strongly supported if SH-alrt and UF bootstraps were $\geq$ 80% and 95%, respectively. We ran additional analyses on our nuclear loci using phased datasets to better understand discordance amongst gene trees and alleles. Heterozygotes were called using the 'Find Heterozygotes' plugin in Geneious, followed by manual identification of heterozygotes in sequence chromatograms. We then phased heterozygotes using the PHASE function under default parameters in DnaSP6 (Rozas et al., 2017). Nuclear gene trees of both alleles, and a concatenated tree consisting of phased nuclear loci (one random allele chosen) and mitochondrial genes were reconstructed in IQ-TREE using the same parameters for unphased alignments. Parsimony-informative sites were calculated using the *ips* (Heibl, 2008) package in R.

Morphological examination.—We collected morphological data from specimens using the following meristic characters: dorsum color; venter color; ventral color pattern (pattern/spotted vs. immaculate); ventral tail pattern, the pattern of pigmentation on the ventral side of the tail; dorsal–ventral color change, the color transition of the dark dorsum to the light venter (sharp vs. gradual); chin pigmentation, measured as the amount of melanistic pigmentation on the mental, infralabial, and anterior chin shield scales (light vs. heavy); number of dorsal scale rows 10 ventral scales posterior to the head, at midbody, and five ventral scales anterior to the cloaca (DSR #-#-#); number of ventral scales, following Dowling (1951; V); cloacal plate (complete vs. divided); subcaudal type (complete vs. divided); number of subcaudal scales on left and right sides (Sc_left, Sc_right); temporal formula; number of supraocular scales; number of preocular scales; number of subocular scales; number of postocular

Table 1. Uncorrected (*p*) pairwise intra- and interspecific distance ranges within *Hypsiscopus*. All values are given as a percent (%); single numbers represent data for which only two specimens were available.

	ATPase	cyt-b	PRLR	VPS13B	WFIKKN
H. matannensis	3.85	0.28–7.87	0.26	0–1.27	0.49
H. plumbea	0–2.78	0–3.43	0–0.72	0–0.32	0–0.79
H. murphyi	0–6.36	0–7.2	0–2.17	0–1.97	0–1.01
H. matannensis vs. *H. plumbea*	5.67–6.96	4.75–8.11	0.09–0.9	0.06–1.42	0.39–1
H. matannensis vs. *H. murphyi*	10.54–13.06	7.12–13.06	0.09–2	0–1.98	0.43–1.27
H. plumbea vs. *H. murphyi*	10.43–13.53	10.08–11.71	0–2.48	0.06–2.31	0–1.25

scales; supralabials contacting the loreal (starting with supralabial I as the scale adjacent to the rostral scale); number of supralabials contacting the eye; number of infralabials; infralabials contacting the anterior chin shields (starting with infralabial I as the scale adjacent to the mental scale); infralabials contacting the posterior chin shields. We also recorded the following continuous measurements using a Mitutoyo digital caliper or soft measuring tape: snout–vent length (SVL); tail length (TL); total length (TtL); TL:TtL ratio; head length (HL), measured from the tip of the snout straight back to behind the jaw; head width (HW), measured as the widest part of the head; head length–width ratio (HL:HW); body width (BW), taken as the average of three measurements at midbody; body circumference (BC), measured using the formula $2\pi r$, with r (radius) measured as half the body width; anterior chin shield length (AchL); anterior chin shield width (AchW); posterior chin shield length (PchL); posterior chin shield width (PchW); intergenial length (length of scales between the posterior chin shields; IL); intergenial width (IW). Characters occurring bilaterally were measured on both side of the specimen, and numbers represent equal values for both sides unless indicated otherwise. All measurements are given in millimeters (mm).

Isolation by distance (IBD).—Geographically widespread species may exhibit population structure due to nonadjacent populations being unable to breed with each other, leading to a higher correlation of genetic distances with geographic distances (Wright, 1943) and leading to false conclusions of true diversity reflective of speciation. Thus, to test for IBD, we conducted a Mantel test in R on the cyt-b dataset, the gene that shows the most structure of the five used in this study, for *H. plumbea* and the new species. Geographic and genetic pairwise distances for the IBD analyses were calculated using the *geodist* (Padgham et al., 2021) and *ape* v5.5 (Paradis and Schliep, 2019) packages, respectively. We then ran the Mantel test using the 'mantel.randtest' function with 999 permutations using the *ade4* package (Thioulouse et al., 2018). Indeed, Mantel tests may not always detect IBD (Teske et al., 2018), but we include them here for hypothesis testing; we also plot our geographic and genetic distances for a more transparent view of our data.

Morphological quantitative statistics.—To identify the distribution of our morphological data in morphospace, we used multidimensional scaling (principal coordinates analysis [PCoA]) using the *vegan* (Oksanen et al., 2020) and *labdsv* (Roberts, 2019) packages in R v4.1.2 (R Core Team, 2020). We included all scalation and continuous data measurements in our PCoA (DSR, V, Sc_left, Sc_right, SVL, TL, TtL, TL:TtL, HL, HW, HL:HW, BW, BC, AchL, AchW, PchL, PchW, IL, IW). Because little is known on the size ranges of neonates and juveniles vs. adults (neonates 122–160 mm TtL; Pope, 1935), we only included specimens that were ≥300 mm TtL. We also excluded any damaged specimens from our analysis, or those with missing data for any characters. Although methods exist for allometric adjustment in morphological data (e.g., Chan and Grismer, 2022), we instead ran our analysis using size-standardized and non-standardized datasets. Dividing by SVL is a common practice to standardize continuous values and take allometry into account during quantitative statistical analysis. However, SVL includes head length, which may vary between species and populations as well. We used a Fisher's *F*-test to determine equal or unequal variance amongst head shapes between specimens of *H. plumbea sensu stricto* and those of the new species (described below), and subsequently determined if head lengths were significantly different between the two populations with a student's t-test ($\alpha = 0.05$). Because no significant differences in head length were found (see Results: Morphological quantitative statistics), we standardized all continuous measurements in the 'standardized dataset' by dividing measurements by SVL, and then excluding SVL from the subsequent analysis.

RESULTS

Phylogenetic analyses.—The datasets for cyt-b+ATPase, PRLR, VPS13B, and WFIKKN contained 2,030, 610, 940, and 1,041 aligned characters, with 105, 88, 71, and 62 taxa, respectively. The concatenated alignment contained 4,621 characters and 112 taxa. Mitochondrial genes had uncorrected pairwise distances within species (*H. matannensis, H. plumbea*, and the new species described below) ranging from 0–7.87%, but among species from 4.75–13.53% (Table 1). Nuclear genes had uncorrected pairwise distances within species ranging from 0–2.17%, but among species from 0–2.48% (Table 1).

All phylogenetic analyses recovered a monophyletic *Hypsiscopus* (Supplemental Figs. 1–5; see Data Accessibility). The mitochondrial dataset recovered *H. matannensis* as sister to *H. plumbea sensu stricto*, and this clade in turn was sister to the new species (below), all with strong support using both support metrics; nuclear trees showed species-level groupings but had lower support. The VPS13B tree had the same topology as the mitochondrial tree, but poor support for the *H. matannensis* clade and high support for both *H. plumbea* and a clade we describe as a new species below. The PRLR and WFIKKN trees had low topological resolution and support compared to the other genes. The unphased concatenated (mitochondrial+nuclear) tree was broadly congruent with the mitochondrial tree, but species-level clades had high SH-alrt support values and low UF bootstraps, with the exception of *H. matannensis* as strongly supported. Two specimens of *H. plumbea* (KU 328517 and LSUHC 5581) usually grouped with

other specimens of *H. plumbea*, but they were placed in the new species clade in the VPS13B and concatenated trees, respectively (Supplemental Figs. 1–5; see Data Accessibility).

The phased analyses showed similar topological structure to unphased analyses, and primarily supported three groups consisting of *H. matannensis*, *H. plumbea*, and the new species (Supplemental Figs. 6–9; see Data Accessibility). The phased VPS13B tree strongly supported *H. matannensis* as sister to *H. plumbea*, except one specimen of *H. plumbea* from southern Thailand (KU 328517) was recovered in a clade with the new species. The phased WFIKKN tree showed three clades corresponding to each species (unlike the unphased WFIKKN analysis, which showed little genus-wide structure), but one specimen of *H. plumbea* (FMNH 259223) was recovered sister to *H. matannensis* (outside of the *H. plumbea* clade), two specimens of *H. plumbea* (FMNH 328517–8) were recovered within the new species, and one specimen (FMNH 98995) had one allele recovered in the new species and the other with *H. plumbea sensu stricto*. The PRLR unphased and phased analyses were identical and unresolved. The concatenated analysis with phased data strongly supported all species-level and interspecific nodes (Fig. 2C); similar to the unphased concatenated analysis, one specimen of *H. plumbea* (LSUHC 5581) was recovered within the new species clade.

Isolation by distance (IBD).—Mantel tests indicated evidence of IBD ($P = 0.001$, $P = 0.001$, and $P = 0.012$) within the new species (below), within *H. plumbea*, and between *H. plumbea* and the new species, respectively. However, plotting genetic and geographic distances revealed poor evidence for an IBD scenario between the new species and *H. plumbea*, as well as within *H. plumbea* (see Discussion; Fig. 2B), but still shows support for IBD when considering disparate sampling sites across the wide geographic range of the new species.

Morphological quantitative statistics.—After removing juveniles/neonates and damaged specimens, our dataset for morphological quantitative statistics included 81 specimens. Fisher's *F*-test showed equal variance between the head lengths of *H. plumbea* and the new species ($F_{60,19} = 0.82$, $P = 0.55$), thus we used a t-test and found no significant difference in head lengths between the two species ($P = 0.25$) and standardized our continuous measurements by SVL. The final datasets used in PCoA included 81 and 83 specimens for the standardized and non-standardized analyses, respectively. Both *H. plumbea* and the new species occupied similar regions of morphospace along PC1 and PC2 when using the standardized dataset, with some separation into two groups. The non-standardized PCoA run showed both species occupying a similar morphospace along PC1, but primarily distinct regions along PC2 (Fig. 2A).

Taxonomy.—Based on corroborating lines of evidence from the multilocus phylogenetic analyses, meristic morphological differences, and qualitative color pattern differences, we describe the northern populations of *H. plumbea sensu lato* as a distinct species, as follows.

***Hypsiscopus murphyi*, new species**
urn:lsid:zoobank.org:act:4CD2E1F2-E747-45AA-9F56-01F089033F09
Murphy's Mud Snake
Figures 1–4, Supplemental Figures 1–9

Holotype.—NCSM 85490 (field number BLS 16458; Fig. 3), adult male, Laos, Khammouan Province, Gnommalath District, Ban Phak Phoung, 17.58191°N, 105.21997°E, 167 m elev., collected in water in a rice paddy near karst forest at 2110 h , Bryan L. Stuart, Niane Sivongxay, Sengvilay Seateun, and Monekham Davanhkham, 11 July 2014.

Paratypes.—NCSM 85491, 1 female, same data as holotype; NCSM 85493, 1 female, same data as holotype except 17.58174°N, 105.23250°E, 160 m elev., 15 July 2014; NUOL 00537, 1 female, same data as holotype except 17.58212°N, 105.22979°E, 166 m elev., 12 July 2014; FMNH 255229, 1 female, Laos, Champasak Province, Mounlapamok District, Dong Khanthung National Protected Area, near Xe Lepou River at Cambodian border, 14.12°N, 105.48°E, 60 m elev., Bryan L. Stuart, 14 July 1998; FMNH 259222, 1 male, Cambodia, Mondolkiri Province, Pichrada District, Phnom Nam Lyr Wildlife Sanctuary, 12.49694°N, 107.49250°E, 700 m elev., Bryan L. Stuart, Dara An, and Phalla Suon, 22 June 2000; FMNH 259225, 1 female, FMNH 259226, 1 male, Laos, Vientiane Province, Phou Phanang, 300 m elev., 18.07194°N, 102.44611°E, Bryan L. Stuart and Troy E. Hansel, 12 September 2000; FMNH 262422–24, 3 females, Thailand, Nong Bua Lamphu Province, Ban Wang Muan, 16.841519°N, 102.546383°E, Daryl R. Karns, John C. Murphy, and Harold K. Voris, 8 July 2003; FMNH 265800, 1 female, Thailand, Nong Khai Province, Se Ga District, Phu Wua Wildlife Sanctuary, road to Chet Si Waterfall, 18.15208°N, 103.95086°E, 220 m elev., Bryan L. Stuart and Yodchaiy Chuaynkern, 10 September 2004; MVZ 258174, 1 female, Cambodia, Ratanakiri Province, Veunsai District, Virachey National Park, Veal Thom, 14.2203°N, 107.01023°E, 601 m elev., Bryan L. Stuart, Jodi J. L. Rowley, and Thy Neang, 8 October 2007; MVZ 258175–77, 3 females, Cambodia, Ratanakiri Province, Veunsai District, Virachey National Park, Veal Thom, 14.20422°N, 107.01332°E, 661 m elev., Bryan L. Stuart, Jodi J. L. Rowley, and Thy Neang, 2 October 2007; NCSM 76557–58, 2 females, Laos, Savannakhet Province, Vilabouli District, Sepon Mining Tenement, Ban Nonsomphou, 16.96706°N, 105.81369°E, 200 m elev., Ken Aplin, 25 November 2008; NCSM 78627, 1 female, Laos, Bolikhamxay Province, Thaphabhat District, Phou Khao Khouay National Protected Area, Tad Leuk, 18.39501°N, 103.07153°E, 205 m elev., Bryan L. Stuart, Somphouthone Phimmachak, Sengvilay Seateun, and Niane Sivongxay, 27 May 2011; NCSM 85517, 1 female, Laos, Vientiane Province, Xaythany District, Ban Sivilay, 18.01037°N, 102.63216°E, 185 m elev., Somphouthone Phimmachak, 20 June 2013; NUOL 00534, 1 male, Savannakhet Province, Vilabouli District, Sepon Mining Tenement, Ban Nonsomphou, 16.97431°N, 105.81017°E, 218 m elev., Bryan L. Stuart, Sengvilay Seateun, Niane Sivongxay, Derin Henderson, Singthong Sanvixay, and Soupha Khamlounvilayvong, 29 September 2014; ROM 25391, 1 male, Vietnam, Gia Lai Province, Krong Pa District, 13.23368°N, 108.72690°E, Ilya S. Darevsky and Nikolai L. Orlov, 6 November 1993; ROM 32373–32374, ROM 32376, 3 unsexed specimens, ROM 32378, 1 male, Vietnam, Gia Lai Province, Krong Pa District, 13.23368°N, 108.72692°E, Robert W. Murphy, Nikolai L. Orlov, Amy Lathrop, and Leslie A. Lowcock, 26 September 1997; ROM 32386, ROM 32391, 2 unsexed specimens, Vietnam, Dac Lac Province, Buon Don District, Yok Don National Park, 12.86051°N, 107.76637°E,

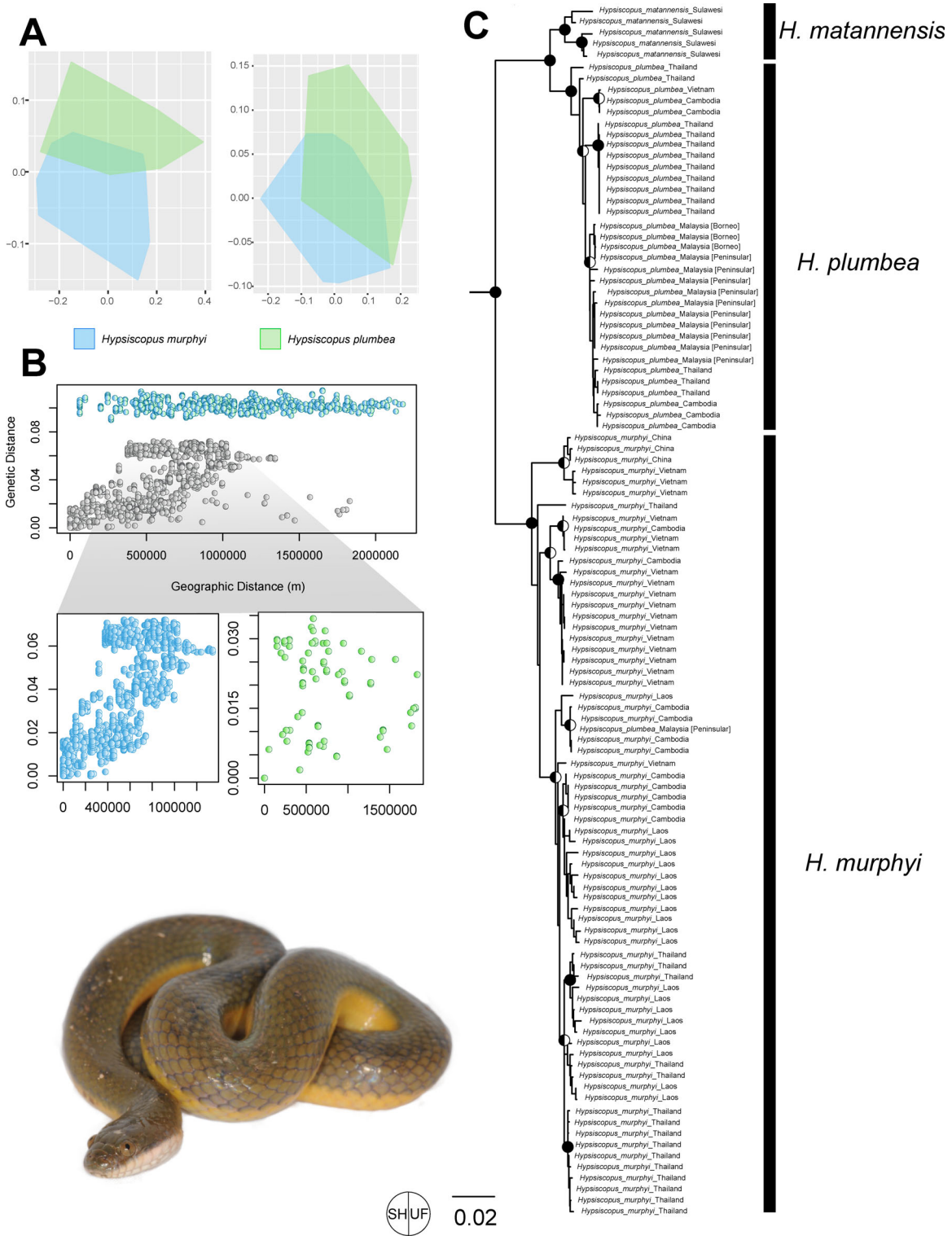

Fig. 2. Phylogeny and quantitative statistics of *Hypsiscopus*. (A) Principal coordinates analysis of *H. murphyi* (blue) and *H. plumbea* (green); non-standardized (left) and standardized (right) runs are shown. Both X- and Y-axes represent PC1 and PC2, respectively. (B) Isolation by distance (IBD) plot showing specimen points for *H. murphyi* and *H. plumbea* in separate plots, which comprise the gray points in the larger chart. The blue-green points at the top of the larger chart represent interspecific comparisons of *H. murphyi* and *H. plumbea*. (C) Concatenated (phased nuclear+mitochondrial) phylogeny of all specimens of *Hypsiscopus* used in this study. Circles at nodes represent support values: left side = SH-alrt, right side = UF bootstraps. Black halves of a circle indicate high support for the respective support value at that node. See Data Accessibility for tree file. Photo credit of live *H. murphyi* holotype (NCSM 85490): BLS.

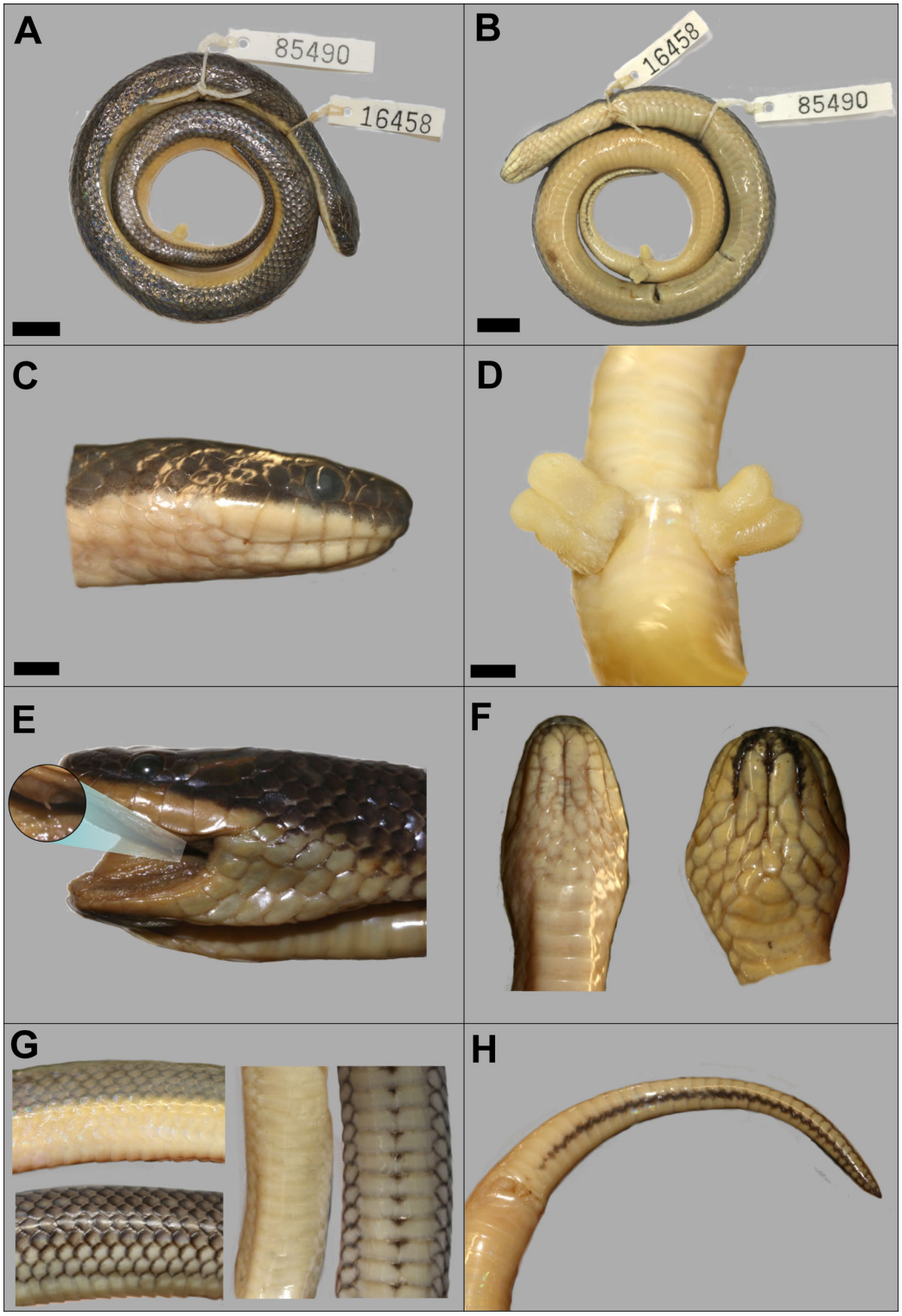

Fig. 3. Morphological characters and variation of *Hypsiscopus murphyi*. (A–D) Holotype of *H. murphyi* (NCSM 85490) in preservative showing dorsal (A), ventral (B), and right lateral head (C) views, and hemipenes (D). Black scale bars represent 1 cm in panels A and B, and 2 mm in panels C and D. (E) Close-up of rear-fang of *H. murphyi* (AMNH R-33908). (F) Variation in melanin pigmentation of the infralabials and anterior chin shields: slightly pigmented (left; NCSM 85490) and heavily pigmented (right; ROM 32374). (G) Left two images, variation in dorsal-to-ventral color patten change: sharp (NCSM 85490) and gradual (ROM 30818). Right two images, variation in ventral color pattern: immaculate (NCSM 85490) and mid-ventral halfmoons (ROM 30818). (H) Variation in extent of mid-ventral caudal stripe, with stripe beginning on second subcaudal pair (AMNH R-33908) rather than the 13th subcaudal pair in the holotype (NCSM 85490; panel B).

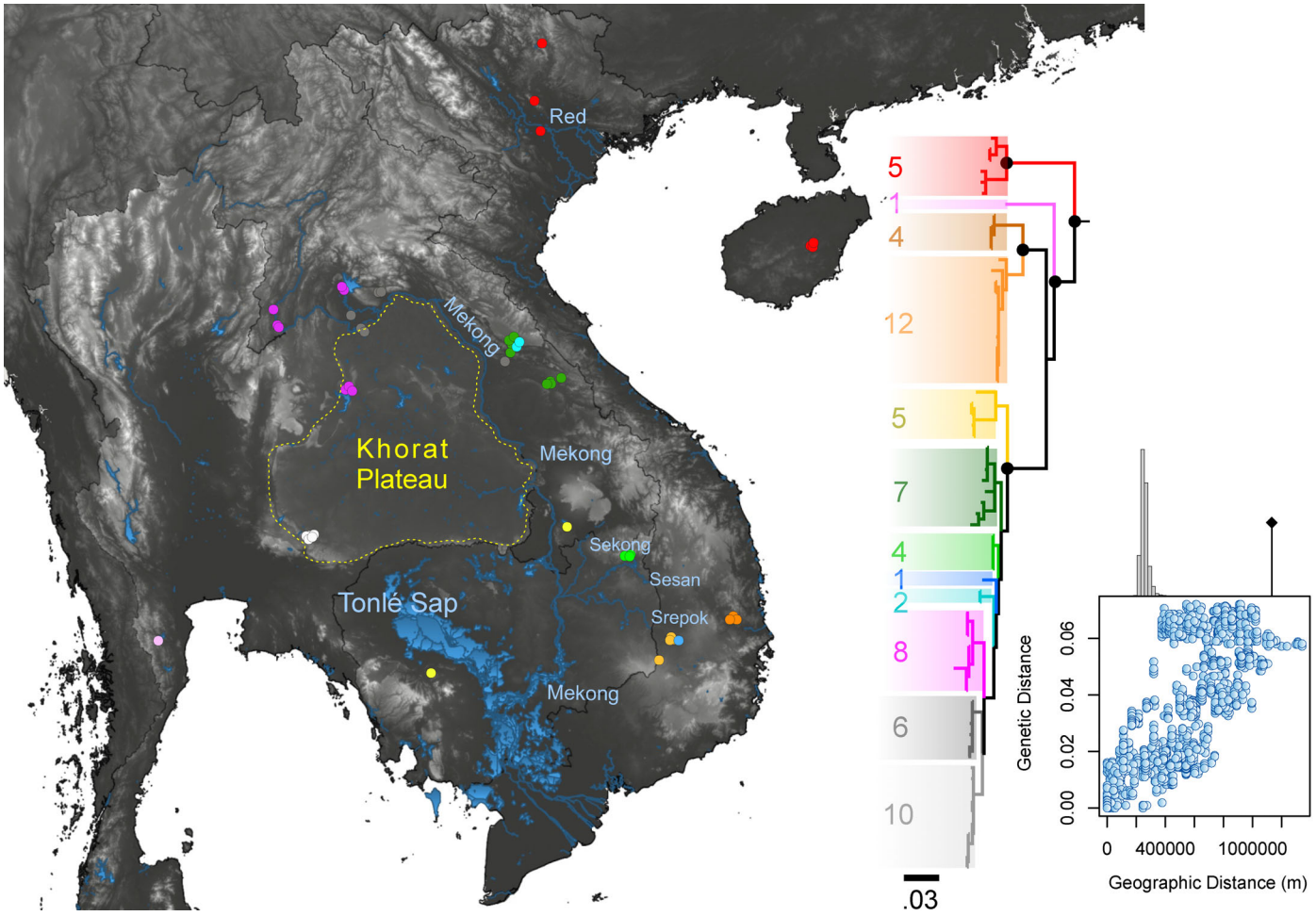

Fig. 4. Populations of *Hypsiscopus murphyi* in Indochina with sampling localities (points). Mitochondrial phylogenetic tree shown on right; filled node circles represent strong support; number of individuals for the respective clade are given to the left of the tree. Inset plot shows results of the Mantel test (IBD scenario); histogram on top shows the original values of the observed data (diamond) outside of the gray bars that represent the absence of spatial structure. Major lakes and rivers/tributaries (blue) are labeled on map. Light and dark gray areas on map depict higher and lower elevation, respectively. See Data Accessibility for tree file.

Robert W. Murphy, Amy Lathrop, Raoul H. Bain, and L. Tuey, 31 May 1997.

Diagnosis.—A small to moderately sized (holotype 355.9 mm total length), rear-fanged (opisthoglyphous) homalopsid with 19–19–(15–17) dorsal scale rows, 22–42 subcaudal scales, and 122–136 ventral scales; nasals with crescent-shaped nares; a singular internasal scale; a pair of anterior and posterior chin shields, with a small pair of intergenial scales separating the posterior chin shields; prefrontals unfused; one supraocular scale, one (rarely two) preocular scales, subocular scales absent, two (rarely one) postocular scales; eight (rarely nine) supralabials, supralabials II–III (rarely I–III) contacting loreal, supralabials IV–V (rarely III–V) contacting the eye; ten (rarely eight, nine, or eleven) infralabials, infralabials I–V (rarely I–IV or I–VI) contacting the anterior chin shields, infralabials V–VI (sometimes IV–V, V, or VI–VII) contacting the posterior chin shields (Fig. 3A–E). All morphological data and measurements are found in Supplemental Table 1 (see Data Accessibility).

Hypsiscopus murphyi can be distinguished from its congeners by having a higher range of ventral scales which is nearly non-overlapping with that of *H. plumbea* (113–123), a lower minimum number of subcaudal scales than *H. plumbea* (30–44), a lower range of subcaudals than *H. matannensis* (43–48), and a lower number of scales at mid-body than *H. matannensis* (21 DSR at midbody).

Description of the holotype.—Adult male, based on size data (Pope, 1935; Murphy, 2007; Fig. 2A–D, F–G). Rostral broader (3.1) than tall (1.3); nasals in contact with each other, and right wider (2.4) than long (1.5), left wider (2.1) than long (1.8), surrounding the entirety of the nares, with fold in scale from lateral edge of nare to dorsoposterior corner of supralabial I; nares crescent-shaped; internasal triangular, not divided, wider (2.6) than long (1.3), not in contact with loreals; prefrontals unfused, right wider (1.8) than long (1.4), left wider (1.9) than long (1.5), bordered anteriorly by internasal, laterally by loreals, posterolaterally by supraoculars, and posteriorly by frontal; frontal whole, longer (4.1) than wide (2.5); parietals undivided, right longer (4.7) than wide (2.6), left longer (5.2) than wide (3.0); eyes cloudy, with elliptical pupils, right eye as wide (2.1) as tall (2.1), left eye as wide (2.0) as tall (2.0), both bordered by one supraocular, one preocular, no suboculars, two postoculars; right (1.5) and left (1.6) preoculars about as tall as right (1.5)

and left (1.7) postoculars combined, respectively; single anterior temporal, margin contacting postoculars slightly shorter than height of both postoculars combined, right (1.1), left (1.1); two posterior temporals, right (1.3), left (1.6), dorsal-most posterior temporal shorter than right (2.4) and left (2.3) ventral-most posterior temporal, respectively; eight supralabials, rectangular, gradually increasing in length and height anterior to posterior, II–III contacting the loreal, IV–V contacting the eye; rostral as wide (1.3) as long (1.3); ten infralabials, rectangular, gradually increasing in length and height from I to VI and then decreasing in length and height from VII to X, I–V contacting anterior chin shields, IV–V contacting posterior chin shields; posterior chin shields separated by pair of intergenials, both longer (1.2) than wide (0.4); posterior-most maxillary tooth enlarged ("rear-fanged"; Fig. 3E); dorsal scale rows 19–19– 15; subcaudals 41 (right and left), divided; cloacal plate divided. Hemipenes bilobed, with small spines (Fig. 3D).

Color of the holotype in life.—Dorsum uniform olive green, with darker, gray scale margins, venter mustard yellow, immaculate, with dark gray mid-ventral stripe below tail that traces the medial division of the subcaudals, starting at subcaudal pair 13. Dorsal and ventral colorations sharply divided laterally at dorsal scale row III. Iris olive green, slightly lighter in color than dorsum, with hues of orange surrounding a black, elliptical pupil (Fig. 3).

Color of the holotype in preservative.—Dorsum faded to uniform gray, with some areas of darker coloration, venter faded to cream/light yellow. Rust-coloration on parietals and left ventral-most postocular (Fig. 3A–D).

Variation.—In preservative, variation from the holotype of *H. murphyi* was observed in the following coloration: pigmentation on mental scale, infralabials, and anterior chin shields (no melanistic pigment or lightly pigmented with melanin; Fig. 3F); dorsal-ventral coloration (gradual change of dark pigmentation on the dorsal and lateral scales to lighter pigmentation on the ventral scales; Fig. 2G); ventral pattern (melanistic pigmentation from lateral scales, sometimes with halfmoon pattern at anterior or posterior edge of middle of the ventrals; Fig. 3G); caudal, melanistic mid-ventral stripe (broken up or not continuous; Fig. 3H), extends entire length of tail or starts anywhere from subcaudal pair 2–14 and to the tip of the tail; and caudal mid-ventral stripe width/shape (the melanistic stripe color 'bleeds' into the subcaudal scales).

Etymology.—The specific epithet is a patronym for John C. Murphy, who has dedicated decades of his research career to investigating and describing homalopsid snakes.

Distribution.—*Hypsiscopus murphyi* is distributed from Taiwan and southern China (Zhejiang Province) at its northernmost limit (but see Discussion), extending southward into China (including Hainan Island), into Vietnam, Laos, Cambodia, and Thailand. The southern limit of this species based on our sampling occurs in Phetchaburi, Thailand. Specimens from adjacent and nearby islands will need to be identified pending future molecular and morphological comparisons (e.g., Andaman Islands and other parts of India and Myanmar [Murphy, 2007; Murphy and Voris, 2014]).

Natural history notes.—This species, similar to *H. plumbea*, is primarily a freshwater homalopsid and typically found in sluggish streams, ponds, rice fields, and marshlands at elevations from sea level up to at least 1200 m (Mell, 1922; Deuve, 1970; Murphy, 2007). However, the species can be found some distance from the water, such as underneath logs, especially during the dry season (Saint Girons, 1972). Paratype FMNH 259222 was found during the day (1420 h) 2 m above the ground inside a rotted, hollow, termite-infested vertical tree in gallery evergreen forest near a stream. The species is nocturnal or cathemeral (Murphy, 2007). *Hypsiscopus murphyi* is likely syntopic with its congener, *H. plumbea*, in regions south of the Khorat Plateau in central and eastern Thailand and western Cambodia (Fig. 1), up to latitudes of approximately 12.4°N. Like other members of the genus, this species feeds on fishes, frogs, and sometimes crustaceans (Schmidt, 1927; Gressitt, 1941). *Hypsiscopus murphyi* is ovoviviparous, containing 2–18 young (Murphy, 2007); averages of nine young per litter have been reported (Cox, 1991). Two examined specimens of *H. murphyi* (ROM 30818 and ROM 30933) in this study contained seven and nine ova, respectively. The venom toxicity of this species is not considered dangerous to humans, usually only causing localized effects (if any) such as burning sensations and local swelling (Karsen, 1986).

DISCUSSION

Hypsiscopus murphyi is the third species of *Hypsiscopus* and the 57th species of Homalopsidae to be described. *Hypsiscopus plumbea sensu lato* has, until now, been considered to inhabit nearly all of Southeast Asia, including Sulawesi. Previous research on homalopsid snakes has recovered *H. plumbea* as paraphyletic with respect to the Sulawesi-endemic *H. matannensis* (Bernstein et al., 2021), a finding confirmed here with additional sampling. Our molecular and morphological analyses show evidence that the populations around and north of central Thailand's Khorat Plateau represent a distinct lineage that warrants species-level recognition. Additionally, although we do not use genetic distances to diagnose this species, our genetic divergences (mitochondrial genes: 4.75–13.53%; nuclear genes: 0–2.48%; Table 1) exceed that of species-level distances seen in studies on homalopsids (Köhler et al., 2021) and other snakes (Ruane et al., 2018). These genetic distances, as well as the biogeography of the group, corroborate our recognition of the species described here.

Our phylogenies consistently recovered clades that represent the currently described species of *Hypsiscopus*: *H. plumbea*, *H. matannensis*, and *H. murphyi*. Strongly supported clades were observed in the mitochondrial tree, but the nuclear data and concatenated analyses had lower support, likely due to a lower number of parsimony-informative sites in the nuclear dataset (28, 31, and 55 for WFIKKN, PRLR, and VPS13B, respectively) compared to the mitochondrial dataset (253 and 312 for ATPase and cyt-b, respectively). However, we observed both quantitative and qualitative morphological distinctiveness of specimens that corresponded to clades that were recovered in the mitochondrial, VPS13B, and concatenated trees, notably the number of ventrals and subcaudals in *H. murphyi* and *H. plumbea* and number of mid-body DSR in *H. matannensis* (Figs. 2–3). Genomic data consisting of ultraconserved elements, anchored hybrid enrichment loci,

and additional nuclear genes (Bernstein et al., unpubl. data) also strongly support the topology observed in this study: (*H. murphyi*, (*H. plumbea*, *H. matannensis*)).

While additional, fine-scale sampling is needed to determine the environmental and genomic factors that contribute to the diversity within these snakes, population-level structure is recovered that provides insights into how Southeast Asia's heterogeneous landscape may have resulted in the isolation and subsequent speciation of *H. murphyi* (Fig. 4). Indochina consists of hundreds of river systems and a variety of mountain ranges that have been found to drive herpetofaunal diversity (Bain and Hurley, 2011), as well as Pleistocene sea level fluctuations that have connected and disconnected now-discontinuous landscapes (i.e., Hainan Island and China; Sundaland) over the last 400,000 years (Voris, 2000; Hall, 2009; Husson et al., 2020). Hypotheses of riverine vicariance (Wallace, 1854) are commonly suggested when breaks in population structure of terrestrial organisms are observed at or near major rivers. For example, the enormous river systems of Indochina, such as the Mekong and Ayeyarwady (Irrawaddy) and their tributaries, have been proposed as barriers to gene flow in *Draco* lizards (Klabacka et al., 2020), agamids (Hartmann et al., 2013), amphibians (Geissler et al., 2015), and mammals (Meijaard and Groves, 2006). However, it is less clear how large or small rivers could prove to be an effective barrier to semi-aquatic snakes that may disperse more like fishes than terrestrial species. This seems particularly applicable in the flood-prone monsoonal areas of Southeast Asia where *Hypsiscopus* is widespread. While region-wide studies suggest the Mekong River is not a biogeographic barrier for all herpetofauna (Bain and Hurley, 2011), the change in course of this river during the Quaternary (Carbonnel, 1965; Workman, 1977) has changed patterns of gene flow and subsequent population structure in the homalopsid snake *Enhydris subtaeniata* (Lukoschek et al., 2011). Additionally, morphological data of *E. subtaeniata* has been found to reflect the genetic diversity observed between drainage basins (Voris et al., 2012). In our trees, we see structure that may be reflective of the Mekong River facilitating isolation (Savannakhet and Khammouan Provinces, Laos; NCSM 76556–8, 85491, NUOL 00534–7). Despite this structure, some of our samples in northern Thailand and northern Laos were found on both sides of the Mekong River (or even on small land platforms in the middle of the river); dispersal across rivers is a likely scenario given the aquatic nature of these snakes. In southern parts of our sampling of *H. murphyi*, we see geographic-based structure that is hypothesized to be from other river systems like the Srepok, Sesan, and Sekong major tributaries of the Mekong River, as well as Indochina's largest freshwater lake, Tonlé Sap (Fig. 4). The river catchment event that changed the East–West trajectory of the Mekong occurred ~2.5 million years ago due to the tectonic uplift of the 180,000 km^2 Khorat Plateau in central Thailand (Hutchison, 1989; Rainboth, 1996). The Khorat Plateau, bordered on two sides by the Mekong River, has been suspected to act as a filter or partial barrier for *Hypsiscopus* (Bernstein et al., 2021) and in other taxonomic groups (e.g., birds; Pereira and Wake, 2015), and the plateau may also inhibit gene flow in these snakes. This is evidenced by our data in which *H. plumbea* does not extend past the southern margin of the plateau, and we also see substructure between northern and southern Khorat Plateau specimens of *H. murphyi* (Fig. 4). The Khorat Plateau rim sites, while not particularly high, may have a slope sufficient enough to isolate homalopsid populations on and off the plateau (Karns et al., 2005). Additionally, in our dataset, the northernmost populations of *H. murphyi* east of the Red River (Vietnam and China), which is considered a major biogeographic barrier (Bain and Hurley, 2011; Yuan et al., 2016), are genetically distinct from other populations of *H. murphyi* (Fig. 2C). The cause of this disjunction is unclear, as no diagnostic characters were apparent to us in these specimens, and thus they are tentatively assigned here to *H. murphyi* pending further sampling. We also note that the Hainan and mainland China populations of *H. murphyi* are closely related, likely a result of sea level fluctuations connecting these areas during the Pleistocene, followed by subsequent dispersal. It is likely that the genetic structure seen within populations of *H. murphyi* is the result of IBD (Fig. 4), as the range of this taxon is continuous. This contrasts with what is seen in *H. plumbea* (based on geographic-genetic distance plots; Fig. 2), in which the pairwise genetic distances are poorly correlated with geographic distance due to the disjunct distribution of *H. plumbea* (Fig. 2B). The discontinuous distribution through mainland Southeast Asia and the Greater and Lesser Sunda Islands may have led to higher levels of genetic differentiation in *H. plumbea* due to periods of isolation during high sea levels.

We also find evidence for incomplete lineage sorting (ILS) and/or introgression. Our VPS13B phased gene tree groups a specimen of *H. plumbea* from southern Thailand closely with *H. murphyi* from Hainan, China (~1600 km away; Supplemental Fig. 7; see Data Accessibility); because of the large distance between these localities, this likely indicates a scenario of ILS. This may also be evidenced by our observation on phenotype; color patterns of distantly related lineages in this group are more similar to each other. For example, the Chinese populations of *H. murphyi* are more phenotypically similar to *H. plumbea* rather than other populations of *H. murphyi* (ventral scale pattern present; gradual color change from dorsum to venter). Additionally, our phased WFIKKN tree recovers scenarios in which both or only one allele from three specimens of *H. plumbea* from southern Thailand and Cambodia group with specimens from *H. murphyi* (Supplemental Fig. 8; see Data Accessibility). Cambodia is part of the range where these two species overlap. This region may be a zone of hybridization, which would be supported by the Cambodian specimen (FMNH 98995) having one allele from *H. plumbea* and another from *H. murphyi*. Our concatenated analyses show strong support for *H. murphyi* being a distinct lineage of *Hypsiscopus*, and it is not surprising to see a small number of individuals group within a clade of a closely related, parapatric species. Such scenarios can reflect true biological phenomena that occur between distinct species (e.g., migration, ILS, introgression), and it has been strongly supported that many parts of the tree of life are better represented as reticulating, rather than a bifurcating, tree (Edwards et al., 2016; Burbrink and Gehara, 2018). More sampling will provide accurate testing of these hypotheses in future studies (in progress) and determine i) if rivers, drainage systems, the Khorat Plateau, and other

geographic features such as the Annamite Mountain Range of eastern Indochina, are the primary factors shaping the structure of these and other homalopsid snakes (or alternatively, environmental factors), and ii) if ILS, gene flow/hybridization, or migration are responsible for the patterns seen in the phased data.

More work remains to be done on *Hypsiscopus*. The treatment of *H. plumbea sensu lato* as a single widespread species muddies taxonomic and natural history accounts in the literature. Populations of *H. plumbea sensu stricto* are reported from the Malay Peninsula, Greater Sundas, and Wallacea, including Sulawesi, the latter of which is the island that *H. matannensis* is endemic to (de Lang and Vogel, 2005; Murphy, 2007; Murphy and Voris, 2014; de Lang, 2017). Morphological data from region or locality-specific field guides report some of the low numbers of ventrals we observed in *H. plumbea sensu stricto* (e.g., de Lang and Vogel, 2005; Stuebing et al., 2014; Lang, 2017), or, conversely higher numbers of ventrals in Vietnam (142 *sensu* Murphy [2007], likely corresponding to a specimen of *H. murphyi*). An in-depth morphological investigation of all islands and regions in Southeast Asia, with dense sampling, is needed to accurately determine i) the true variation in scale counts between populations, and ii) if *H. murphyi* is present on Borneo or other islands of the Greater Sundas. This biogeographic scenario would indeed be possible due to land bridges having connected Indochina to Sumatra, Java, and Borneo as recently as the Pleistocene (Voris, 2000; de Bruyn et al., 2014). Additionally, secondary investigation of specimens from previous studies can provide insight into the latitudinal range of *Hypsiscopus*. One specimen examined by Ko Ko Gyi (1970) from the Stanford University Natural History Collection (*Enhydris* [= *Hypsiscopus*] *plumbea* SU 71955 [now CAS 71955]) is reported with a locality in China of 'Nanking, Kiangsu Province, China,' (= Nanjing, Jiangju Province), which is ~700 km north from some of the northernmost specimens of *H. plumbea sensu lato*. Due to our inability to examine this specimen, we consider the northernmost extent of the geographic range of *H. murphyi* to be near Zhejiang Province, China, pending morphological data from CAS 71955 and future sampling efforts. However, it is possible that *Hypsiscopus* ranges much more northward than previously thought. Increased numbers of samples and loci in our future analyses, as well as integration of other data types such as geospatial and ecological data, will likely broaden our understanding of the biogeography and diversity within *Hypsiscopus*.

DATA ACCESSIBILITY

Supplemental material is available at https://www.ichthyologyandherpetology.org/h2022015. Unless an alternative copyright or statement noting that a figure is reprinted from a previous source is noted in a figure caption, the published images and illustrations in this article are licensed by the American Society of Ichthyologists and Herpetologists for use if the use includes a citation to the original source (American Society of Ichthyologists and Herpetologists, the DOI of the *Ichthyology & Herpetology* article, and any individual image credits listed in the figure caption) in accordance with the Creative Commons Attribution CC BY License. ZooBank publication urn:lsid:zoobank.org:pub:9D09C686-02CE-46AA-8BB0-77E746239DE8.

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