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Incorporating New Datatypes to Enhance Species Delimitation: A Case Study in Rice Paddy Snakes (Homalopsidae: *Hypsiscopus*)

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Abstract

Homalopsids (Old World Mud Snakes) include 59 semiaquatic species in Asia and Australasia that display an array of morphological adaptations, behaviors, and microhabitat preferences. These attributes make homalopsids an ideal model system for broader questions in evolutionary biology, but the diversity of this understudied group of snakes is still being described. Recognized species diversity in rice paddy snakes (*Hypsiscopus*) has recently doubled after nearly 200 years of taxonomic stability. However, the evolutionary distinctiveness of some populations remains in question. In this study, we compare mainland Southeast Asian populations of *Hypsiscopus* east and west of the Red River Basin in Vietnam, a known biogeographic barrier in Asia, using an iterative approach with molecular phylogenetic reconstruction, machine-learning morphological quantitative statistics, and ecological niche modeling. Our analyses show that populations west of the Red River Basin represent an independent evolutionary lineage that is distinct in genetics, morphospace, and habitat suitability, and so warrants species recognition. The holotype of *H. wettsteini*, a species originally described in error from Costa Rica, grouped morphometrically with the population at the Red River Basin and eastward, and those west of the Red River Basin are referred to the recently described *H. murphyi*. The two species may have diversified due to a variety of geological and environmental factors, and their recognition exemplifies the importance of multifaceted approaches in taxonomy for downstream biogeographic studies on speciation scenarios.

Key words: China, East Asia, mud snakes, phylogenetics, systematics, Red River, Vietnam

Introduction

The heterogeneous topography of East and Southeast Asia has been formed by a mosaic of geological events over several millions of years (Hall 1996, 2009; Hutchison 1989; Mulch & Chamberlain 2006). The complex geological histories of these regions have repeatedly been shown as drivers of diversification in several organismal groups (Favre *et al.* 2015; Fu & Wen 2023; Qu *et al.* 2015). Aquatic species were likely influenced by the movement of water ways in Indochina (Breitfeld *et al.* 2020; Salles *et al.* 2021), and terrestrial taxa expanded their ranges via subaerial land bridges between now discontinuous landmasses (Voris 2000), leading to population separation in the Pleistocene. In addition to geological events like tectonic uplifts and changing paleo-river courses, environmental differences

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throughout the geographic ranges of widespread taxa also have the potential to further lineage diversification (Bernstein *et al.* 2024). Systematic studies routinely utilize molecular and morphological data to delimit species complexes (Ramos *et al.* 2019; Weijola *et al.* 2019), but in many cases these data types might not reveal clear species boundaries (Bernstein *et al.* 2022). Investigations into habitat suitability and niche differentiation can reveal further evidence of speciation scenarios along the speciation continuum (De Queiroz 2007) in otherwise difficult-to-delimit taxa (Chan *et al.* 2022; Raxworthy *et al.* 2007; Rissler & Apodaca 2007).

Old World Mud Snakes of the Family Homalopsidae are a group of 59 recognized species in 26 genera distributed throughout Asia, New Guinea, and Australia that show extensive variation in morphological (Catania *et al.* 2010; Murphy 2007) and behavioral (Fabre *et al.* 2016; Jayne *et al.* 2018) traits. Homalopsids have had a long history of taxonomic flux, with widespread species being found to be composed of distinct lineages at finer geographic scales (Karns *et al.* 2010; Murphy *et al.* 2012a,b). One of the most widespread groups in this family, rice paddy snakes of the genus *Hypsiscopus*, are found from Sulawesi through insular Southeast Asia and Indochina into East Asia. Recent studies have elevated the species richness in the genus from two to four, with *H. matannensis* (Boulenger, 1897) and *H. indonesiensis* Hamidy, Zakky, Fitriyana, & Endarwin 2023 endemic to Sulawesi, Indonesia, *H. plumbeus* (Boie 1827) found from Sulawesi to southern Indochina, and a fourth species, *H. murphyi* sensu Bernstein *et al.* (2022) distributed from central Indochina northward to Fujian, China (Bernstein *et al.* 2022; Hamidy *et al.* 2023). The latter taxon is the subject of this investigation.

Bernstein *et al.* (2022) demonstrated that the taxon distributed from central Indochina to eastern China was readily diagnosed with mitochondrial DNA, nuclear DNA, and morphology from *H. plumbeus*, and formally named this taxon *H. murphyi* Bernstein, Voris, Stuart, Phimmachak, Seateun, Sivongxay, Neang, Karns, Andrews, Osterhage, Phipps & Ruane, 2022. Bernstein *et al.* (2022) found a deep genetic break (~10% in cytochrome-b) within *H. murphyi* at the Red River Basin, a major biogeographic barrier in northern Vietnam and China, but the only morphological differences between populations east and west of the Red River Basin apparent to them was in coloration of lateral and ventral scales (dorsal-ventral color transition). As a result, Bernstein *et al.* (2022) noted this genetic divergence, but refrained from splitting *H. murphyi* into two taxa pending further study of morphology. Bernstein *et al.* (2022) overlooked the existence of *Helicops wettsteini* Amaral, 1929, a species described from "San Juan de Vinas (1000 ms. alt.), base of Volcano Turialba, central Costa Rica" (Amaral 1929) that was determined by Rossman and Scott (1968) to be a junior synonym of *H. plumbeus* (as *Enhydris plumbea*), with type specimens bearing egregiously erroneous locality data (Rossman and Scott 1968). David & Vogel (2024) expanded the descriptions of the types of *H. wettsteini*, demonstrated that their morphologies more closely resembled *H. murphyi* than *H. plumbeus*, and therefore transferred *H. murphyi* into the synonymy of *H. wettsteini*.

In this study, we compare molecular and morphological variation in populations of *H. wettsteini* (formerly *H. murphyi*) east and west of the Red River Basin using dense geographic sampling for a molecular dataset consisting of two mitochondrial and six nuclear genes. We use machine-learning quantitative statistics with morphological data to identify distinct groups in morphospace based on *a priori* groupings that reflect the study populations. While previous studies (Bernstein *et al.* 2022, 2024) have investigated the genetic and morphological diversity in this group, we also utilize environmental data to determine if there are differences in habitat suitability and niche space of the two populations, which can be critical for species delimitation (Raxworthy *et al.* 2007). Such use of environmental data, in conjunction with molecular and morphological data, might provide evidence for ecological divergence or differences in niche spaces for populations east and west of the Red River Basin (Richards *et al.* 2007).

Methods

Genetic Data and Taxon Sampling

Hypsiscopus tissues were obtained through field collection efforts and natural history collections for previous studies (Bernstein *et al.* 2022, 2024). Snakes collected in the field by the authors were 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

(NUOL), and Museum of Vertebrate Zoology, University of California, Berkeley (MVZ). Comparative material was examined in the holdings of these institutions 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 S1). Morphological measurements were taken from Bernstein *et al.* (2022). Institutional abbreviations follow Sabaj (2016). Experimental methods were approved by and in compliance with Institutional Animal Care and Use Committee (IACUC) protocols of the Field Museum of Natural History (FMNH) 06-4 to Harold K. Voris and North Carolina Museum of Natural Sciences 2011-01 to BLS. We included samples of *H. wettsteini* (formerly *H. murphyi*) from throughout its geographic range, both east and west of the Red River Basin (Fig. 1).

DNA extraction, polymerase chain reaction, and DNA sequencing protocols follow those of Bernstein *et al.* (2022, 2023). Briefly, we extracted whole genomic DNA from liver and muscle tissue using Qiagen DNeasy Blood & Tissue Kits, following the standard tissue protocol. We amplified two mitochondrial genes and six nuclear genes. For the mitochondrial genes, we sequenced ATPase (849–882 base pair [bp] fragment composed of the tRNA-Lys gene, the complete subunit 6 (*ATP6*) and subunit 8 (*ATP8*) genes, and part of the cytochrome oxidase c subunit III gene) and cytochrome-b (*cyt-b*; corresponding to the tRNA-Glu gene, the complete cytochrome *b* gene, and a portion of the tRNA-Thr gene). For the nuclear genes, we amplified the following genes: prolactin receptor (*PRLR*; 553 bp),

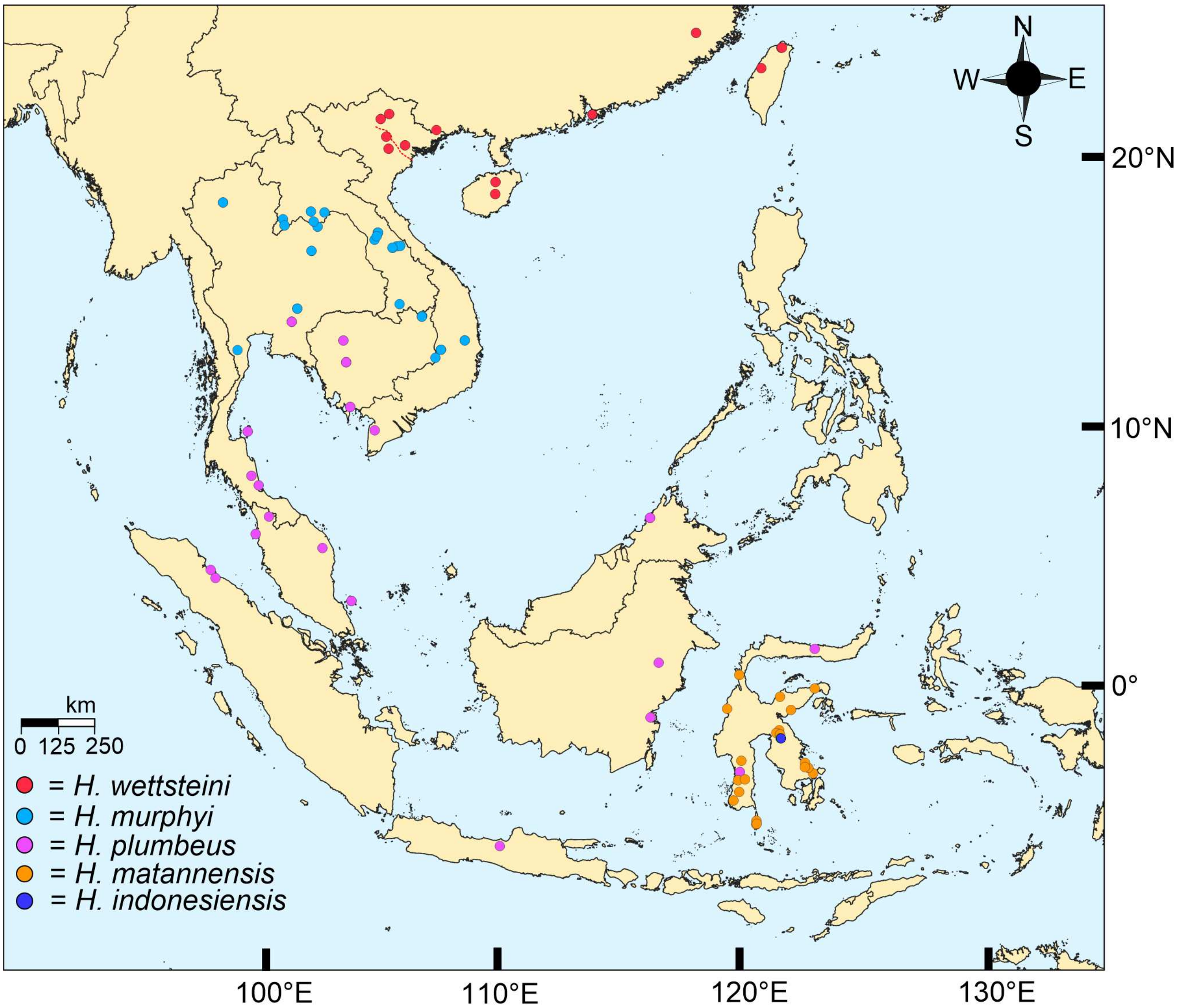

FIGURE 1. Distribution map of *Hypsicopus*. Points denote individual samples; records of *H. wettsteini* (= "eastern populations") and *H. murphyi* (= "western populations") represent the localities used for ecological niche models in this study. Coordinates for *H. plumbeus*, *H. matannensis*, and *H. indonesiensis* obtained from Bernstein *et al.* (2023) and Hamidy *et al.* (2023). Red dashed line in northern Vietnam represents the Red River.

WAP, follistatin/kazal, immunoglobulin, kunitz and netrin domain containing 2 (*WFIKKN2*; ~1,300 bp), vacuolar protein sorting-associated protein 13B (*VPS13B*; 855 bp, ATP/GTP binding protein-like 5 (*AGBL5*; ~627 bp), zinc finger protein basonuclin-1 (*BCN1*; ~1,949 bp), and a fragment of recombination activating gene 2 (*RAG2*; ~1,100 bp). We followed the standard PCR and primer protocols of Bernstein *et al.* (2021) for cyt-b and PRLR, and the nested PCR protocols of Shen *et al.* (2013) and Li *et al.* (2017) to amplify *WFIKKN*, *VPS13B*, *AGBL5*, *BNC1*, and *RAG2*. PCR products were visualized on a 1.5% agarose gel, and amplicons were cleaned with ExoSAP-IT (Applied BioSystems). Purified PCR products were sequenced on a 3730 DNA Analyzer (Applied Biosystems) using Big-Dye Terminator version 3 chemistry and amplifying and sequencing primers. All amplification and sequencing primers were developed from previous studies (Karin *et al.* 2020; Li *et al.* 2017, 2020; Shen *et al.* 2013) and can be found in Supplementary Table S1. We supplemented our sequencing efforts with additional sequences of cyt-b, *ATP6*, *ATP8*, *PRLR*, *WFIKKN2*, and *VPS13B* from previous studies on *Hypsiscopus* (Bernstein *et al.* 2021, 2022) through NCBI's GenBank. GenBank Accession IDs for all samples used in this study can be found in Supplementary Table S1. Supplementary information, sampling locations, morphological data, DNA alignment files, and code for all subsequent analyses can be found at https://github.com/jbernst.

Phylogenetic Analyses

Raw sequence data were aligned, concatenated, and edited by eye in Geneious R11.1.5 using default parameters of the Geneious alignment algorithm. We manually edited sequencing to check for ambiguous base calls or sequencing errors. We phased nuclear data by using the PHASE function in DnaSP6 (Rozas *et al.* 2017) under default parameters to obtain better resolution of allele placement amongst our populations. We used PartitionFinder2 (Lanfear *et al.* 2017) to identify the best model of evolution for our partitions, which were separated by gene and codon position (mitochondrial genes were considered one locus). Following previous phylogenetic studies on homalopsids, sequences of *Homalopsis buccata* and *E. chanardi* (Bernstein *et al.* 2021, 2022) were used as outgroups. For complete sampling of the genus *Hypsiscopus*, we included sequences of *H. plumbeus*, *H. matannensis*, and *H. indonesiensis,* the latter of which was recently described based only on morphological data (Hamidy *et al.* 2023) but was placed in a molecular phylogeny prior to many recent molecular works (Alfaro *et al*. 2008). Phylogenetic reconstruction was performed using IQ-TREE v1.6.1 (Nguyen *et al.* 2015). Maximum likelihood trees were run with 1,000 ultrafast bootstrap (UFB) iterations (Minh *et al.* 2013). Additionally, we computed 1,000 bootstrap replicates of the SH-like approximate likelihood-ratio test (SH-aLRT; Guindon *et al.* 2010) in our IQ-TREE analysis. We considered tree nodes as strongly supported if UFB ≥ 95 and SH-aLRT values ≥ 80. In addition to the maximum likelihood tree, we also ran Bayesian Inference (BI) on the same dataset using MrBayes v3.2.7a (Ronquist *et al.* 2012), partitioning the data by gene and codon position (mitochondrial genes as one locus), and model selection using PartitionFinder2. We ran the analysis for 20 million generations (25% burn-in) and considered the runs converged as the average standard deviation in split frequencies had dropped below 0.01. All effective sample sizes (ESS) were greater than 400, suggesting a sufficient sampling of tree space (ESS values > 200; Ronquist *et al.* 2012). We considered Bayesian Posterior Probabilities > 0.95 to be strong relationship support in the tree. Phylogenies were visualized using FigTree v1.3.1 (Rambaut 2014). To compute genetic (uncorrected pairwise) distances, we used the *genetic distance* function in Geneious for *cyt-b* because it was the gene with the most complete taxonomic sampling in our dataset.

Quantitative Statistics of Morphology

Bernstein *et al.* (2022) failed to identify morphological characters that diagnosed eastern and western populations of *H. wettsteini* (as *H. murphyi*) other than color pattern (Bernstein *et al.* 2022). We used a morphological dataset of 86 individuals to run a linear discriminant analysis (LDA). The LDA uses a machine-learning algorithm on *a priori* groups to create a confusion matrix, determining if the morphological data training set was able to accurately identify the input groups. We used three *a priori* groupings: *H. wettsteini* east of the Red River Basin ("eastern population"), *H. wettsteini* at and west of the Red River Basin ("western population"), and *H. plumbeus*. Our morphological data were based on meristic and mensural characters from Bernstein *et al.* (2022), totaling 31 continuous or discrete variables in nature. Discrete characters were converted to numerical values representing the discrete states, and the entire dataset was log-transformed and non-normal variables were removed. Only adult individuals were used for the LDA; snakes with snout-vent-lengths < 250 mm were considered juveniles (Murphy *et al.* 1999) and excluded from the analysis. Our final morphological dataset consisted of 21 individuals of the eastern population, 44 individuals of the western population, and 21 individuals of *H. plumbeus*. To incorporate data for the adult holotype of *H. wettsteini* from the expanded description by David & Vogel (2024; the paratype was deemed a juvenile), we also reran this analysis with a reduced dataset of 13 characters that overlapped between our dataset and that of David & Vogel (2024): number of ventrals, color transition from dorsum to venter, ventral color pattern, dorsal scale row count at midbody (SVL / 2), dorsal scale row count posteriorly, number of left subcaudals, number of right subcaudals, head length, head width, total length, snout-vent-length, tail length, and total length/tail length ratio. In total, two analyses were performed: an analysis with 31 characters (full dataset, without the *H. wettsteini* holotype), and an analysis with 13 characters (containing the *H. wettsteini* holotype). Morphological data were placed in Supplementary Table S1 and all code and input files at https://github.com/jbernst.

Ecological Niche Modeling

To determine and compare the habitat suitability and niche space of eastern and western populations, we constructed ecological niche models using Maxent v. 3.4.3 (Phillips *et al.* 2004, 2006). We used custom R code (Soto-Centeno 2022) that uses the R packages *rJava* (Urbanek 2021), *dismo* (Hijmans *et al.* 2021), *dplyr* (Wickham *et al.* 2020), *ecospat* (Di Cola *et al.* 2017), *ENMeval* (Kass *et al.* 2021), ggplot2 (Wickham 2011), *maptools* (Bivand & Lewin-Koh 2021), *maps* (Becker *et al.* 2018), *raster* (Hijmans *et al.* 2022), *rasterVis* (Lamigueiro & Hijmans 2022), *RColorBrewer* (Neuwirth 2022), *rgdal* (Bivand *et al.* 2021), *sf* (Pebesma 2018), *spThin* (Aiello-Lammens *et al.* 2015), *tidyverse* (Wickham *et al.* 2019), and *viridis* (Garnier *et al.* 2021). We obtained the geographic coordinates of specimens of 70 western and 47 eastern *H. wettsteini* and rarefied these sets to remove duplicate values; our final rarefied datasets for niche modeling consisted of unique coordinates for 23 western and 12 eastern *H. wettsteini*. We used the 19 bioclimatic variables from WorldClim2 (Fick & Hijmans 2017) at a 2.5 minute resolution as predictor variables; we choose a 2.5 minute resolution to maximize model fit for our data and to make our study's results comparable to those of Bernstein *et al.* (2024). We explored species-specific parameter tuning in ENMeval v2.0 (Kass *et al.* 2021) using five different feature class combinations: L, LQ, H, LQH, and LQHP (L = linear, Q = quadratic, H = hinge, P = product), and used regularization multiplier values of 1 through 5. The best combination of feature classes was chosen based on the model with the lowest ΔAICc value (*H. wettsteini* western: LQH, rm = 2; *H. wettsteini* eastern: LQ, rm = 5). Models of climate and habitat suitability were projected using the present uncorrelated bioclimatic variables. Layers were tested for correlation using a Pearson correlation with a threshold value of 0.80. In addition to our niche models, we plotted the range of climatic conditions where eastern and western populations are geographically distributed ("climate envelopes;" Hijmans and Graham 2006). We constructed climate envelopes using all 19 bioclimatic variables to understand differences in environmental niche space between the eastern and western populations.

Results

Phylogenetic Reconstruction and Genetic Distances

Our maximum likelihood and Bayesian phylogenies of *Hypsiscopus* included 86 *Hypsiscopus* from a concatenated dataset of 7,152 bp. Our BI tree was congruent in topology and support with the ML tree (Supplementary Fig. 1), and thus we focus the rest of our results and discussion on the ML tree. All specimens assigned to *Hypsiscopus* formed a monophyletic group with strong support. All species-level and interspecific nodes were strongly supported, with *H. plumbeus* recovered as sister to *H. indonesiensis* and *H. matannensis*, and this group recovered as sister to *H. wettsteini* (Fig. 2A). *Hypsiscopus wettsteini* was strongly recovered as two clades representing the eastern and western populations. Uncorrected pairwise distances between the eastern and western populations were greater (5.6–7.1%) than interspecific distances of sister taxa in *Hypsiscopus* (4.5–5.2% for *H. matannensis* and *H. indonesiensis*; Table 1). No geographic substructure of eastern *H. wettsteini* was observed, as individuals from Hainan Island were found in the same clades as individuals from the mainland.

TABLE 1. Uncorrected pairwise distances of *cytochrome-b* for species of *Hypsiscopus* and populations in this study. A "-" represents no value for intraspecific distances due to only one specimen. All values represented in percent difference (%).

	H. plumbeus	*H. matannensis*	*H. indonesiensis*	*H. wettsteini* (='eastern')	*H. murphyi* (= 'western')
H. plumbeus	0–3.2				
H. matannensis	5–7.4	0–2			
H. indonesiensis	6–7.3	4.5–5.2	-		
H. wettsteini (='eastern')	9.8–10.9	9.9–11.1	10.2–10.8	0–1.8	
H. wettsteini (='western')	9.3–11.6	8.3–10.9	9.5–10.4	5.6–7.1	0–3

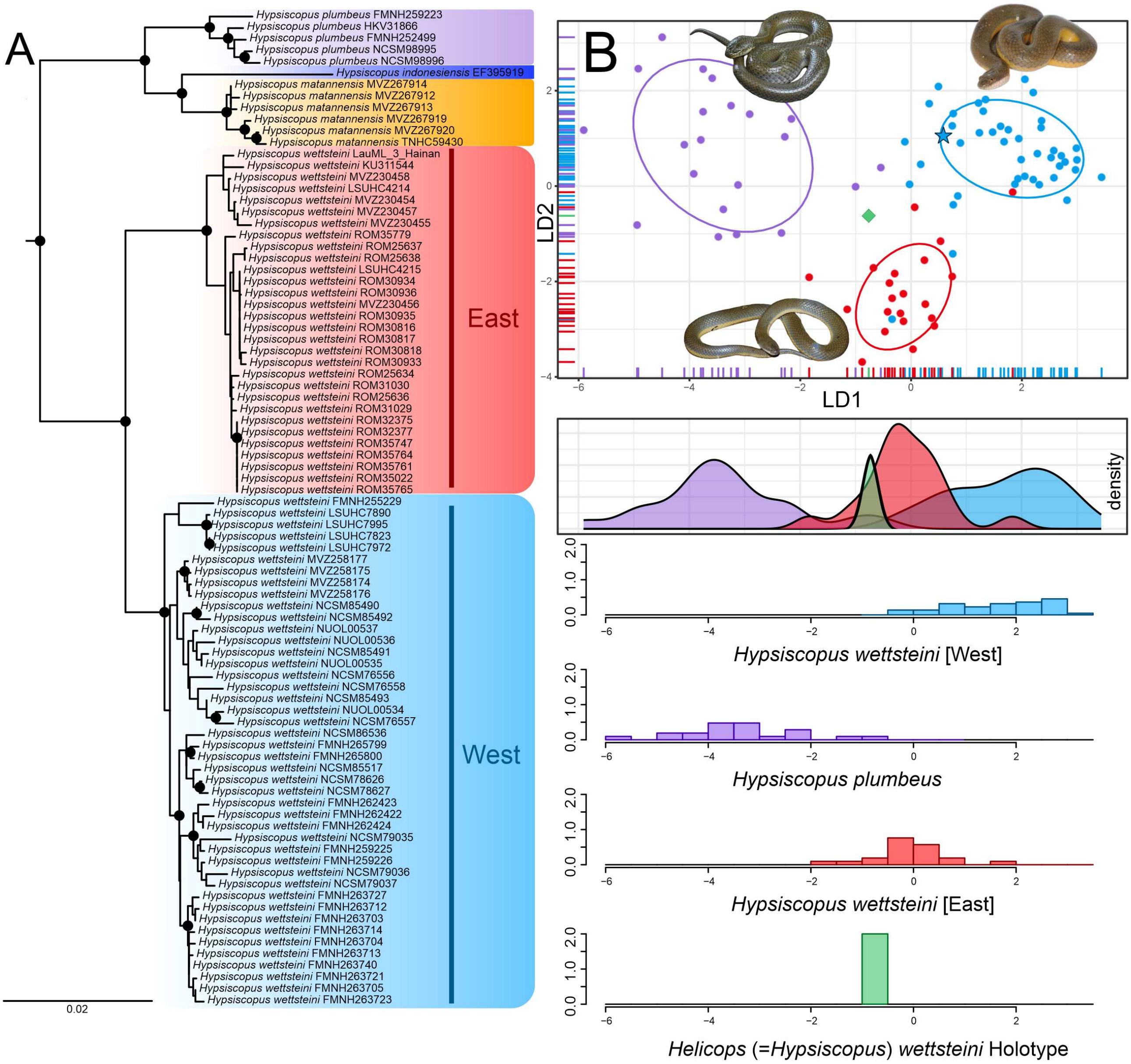

FIGURE 2. A) Maximum likelihood concatenated phylogeny of *Hypsiscopus* (A). Black dots represent strongly supported nodes; scale bar in substitutions per site. B) Linear discriminant analysis of *H. wettsteini* (western population; blue), *H. wettsteini* (eastern population; red), and *H. plumbeus* (purple). Axes represent linear discriminants 1 and 2 (LD1 and LD2), with respective density rugs and density plots on the margins of the plot corresponding to the points in within morphospace. Green diamond and blue star in the LDA represents the holotype of *Helicops* (=*Hypsiscopus*) *wettsteini* (NMW 18726-1) and *H. murphyi* sensu Bernstein *et al.* 2022 (NCSM 85490), respectively.

Linear Discriminant Analysis

Our LDA was performed on a dataset of 27 variables after removing non-normal and invariable characters. These 27 characters were ventral scale count, color pattern transition, ventral color pattern, ventral tail color, number of left subcaudals, number of right subcaudals, head length, head width, left anterior chin shield length, right anterior chin shield length, left anterior chin shield width, right anterior chin shield width, left posterior chin shield length, right posterior chin shield length, left posterior chin shield width, right posterior chin shield width, left intergenial scale length, left intergenial scale width, right intergenial scale length, right intergenial scale width, infralabials contacting the posterior chin shields, total length, snout-vent-length, tail length, body width average at mid-body, and circumference at mid-body.

Our analysis had a classification accuracy of 97.7% (95% CI 91.85–99.72%; $p = <2.2x10^{-16}$). Only two individuals were misclassified, with one specimen of *H. plumbeus* being identified as western *H. wettsteini*, and one specimen of western *H. wettsteini* being classified as *H. plumbeus*. The first linear discriminant axis (LD1) showed 58.6% group separation, with specimens of eastern *H. wettsteini* sharing a similar region of morphospace with *H. plumbeus*, and western *H. wettsteini* occupying their own region in morphospace (Fig. 2B). The second discriminant axis (LD2) showed 41.4% group separation, where *H. plumbeus*, eastern *H. wettsteini*, and western *H. wettsteini* occupied their own regions in morphospace (Fig. 2B). Running our analysis to include the holotype of *H. wettsteini* showed similar results to when we did not include the specimen. The holotype of *H. wettsteini* was recovered within the morphospace of the eastern population of *H. wettsteini* along LD1 (69.59% group separate) and with some overlap in morphospace with eastern *H. wettsteini* and *H. plumbeus* along LD2 (26.13% group separation). The confusion matrix, which included the three previously defined groups, plus the holotype of *H. wettsteini* as a fourth group, only misidentified one *H. plumbeus* as a western *H. wettsteini* and two eastern *H. wettsteini* as a western *H. wettsteini*. The classification accuracy of this second analysis was 93.1% (95% CI 85.59–97.43%; $p = <2.2x10^{-16}$).

Ecological Niche Models

Our ecological niche models revealed differing habitat suitability between eastern and western populations of *H. wettsteini* (Fig. 3A). Western *H. wettsteini* had suitable habitat restricted to Indochina and the southern edge of East Asia (and the Philippines, Lesser Sunda Islands, and northern Australia, all of which they have never been recorded from), which reflected its known present-day distribution (average area under the curve [AUC] = 0.897; Boyce index [BI] = 0.839). Within Indochina, western *H. wettsteini* had its highest suitability in moderate to high elevations near mountains, with lower suitability at coastlines. Bioclimatic variables 18 (~10%; Precipitation of Warmest Quarter), 4 (~14%; Temperature Seasonality [standard deviation ×100]), 8 (~16%; Mean Temperature of Wettest Quarter), 6 (~20%; Minimum Temperature of Coldest Month), and 9 (~27%; Mean Temperature of Driest Quarter) had the highest contributions (with all other variables ≤ ~5%).

In contrast to western *H. wettsteini*, the eastern population had primarily moderate to high suitability throughout all East and Southeast Asia (and the Philippines, New Guinea, and northern Australia), with highest suitability found at the lower elevation coastlines on mainland and insular Southeast Asia (AUC = 0.825; BI = 0.716). The only bioclimatic variables that contributed to the niche model were 7 (~24%; Temperature Annual Range [Maximum Temperature of Warmest Month—Min Temperature of Coldest Month]) and 2 (~75%; Mean Diurnal Range (Mean of monthly [maximum temperature—minimum temperature]); all other variables had no contribution.

Climate envelopes that had no overlap in values or error were considered significantly different. The climate envelopes between eastern and western populations showed significant differences for bioclimatic variables 3 (Isothermality [Mean Diurnal Range/Temperature Annual Range]), 4 (Temperature Seasonality), and 11 (Mean Temperature of Coldest Quarter) (Fig. 3B).

Taxonomy

Owing to corroborated lines of evidence in genetics (including *cyt-b* divergences similar to those of interspecific values in *Hypsiscopus*), distinctiveness in qualitative morphological characters and especially morphospace, and differences in environmental suitability, we hypothesize that the eastern and western populations of *H. wettsteini* represent separate species. The holotype of *H. wettsteini* lacks provenance but in morphospace it clusters with the eastern population (Fig. 2B). While our analysis shows a point in morphospace that only slightly overlaps along LD2, this point has complete overlap along LD1 which explains a majority of the variation in the dataset. The holotype of *H. murphyi* from Khammouan Province, Laos, clusters morphologically with the eastern populations (Fig. 2). *Hypsiscopus wettsteini* can be distinguished from its congeners by having a higher range (123–129) of ventral scales than *H. plumbeus* (113–123); a lower range (123–129) of ventrals than *H. indonesiensis* (152–159) and *H. murphyi* (122–136); a lower range (28–42) of subcaudal scales than *H. plumbeus* (30–44), *H. indonesiensis* (36–41), and *H. matannensis* (43–48); a lower number of scales at mid-body (19) than *H. matannensis* (21) and *H. indonesiensis* (25–27); a gradual color change from dark dorsal scales to light lateral/ventral scales (vs. distinct, sharp change in color in *H. murphyi*); and a halfmoon-shaped marking on anterior edge of some or most ventral scales (ventrals immaculate in *H. murphyi*) (Table 2).

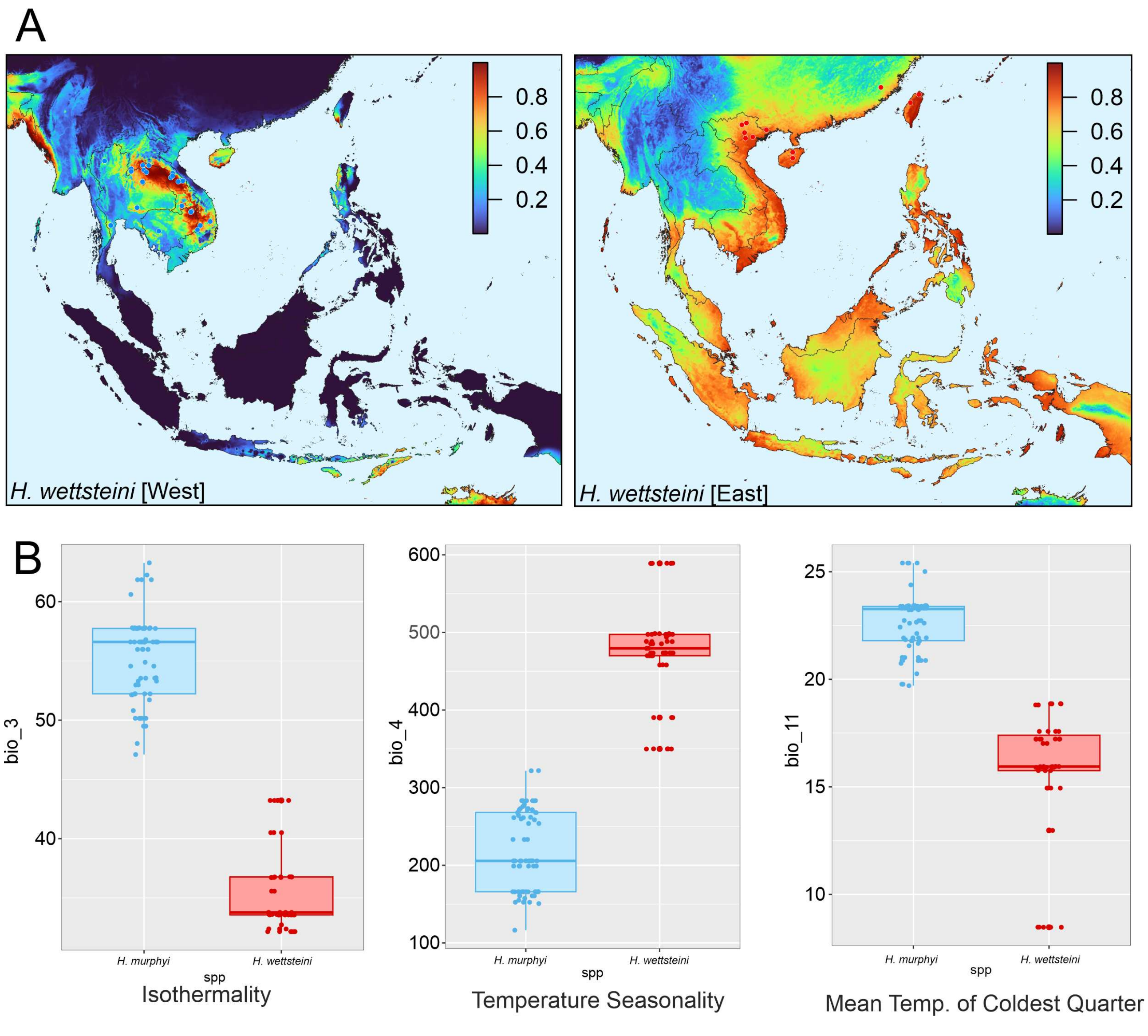

FIGURE 3. A) Ecological niche models using the 19 bioclimatic variables from WorldClim2. Warmer and colder colors represent higher and lower habitat suitability, respectively. Points on map are identical to those in Figure 1. B) Climatic envelopes of the environmental variables that had no overlap between *H. wettsteini* (= "western populations") and *H. wettsteini* (= "eastern populations").

TABLE 2. Morphological diagnostic characters for species of *Hypsicopus*: Dorsal scale rows (DSR) at midbody, number of ventrals and subcaudals, presence or absence of halfmoon-shaped pigmentation at anterior edge of ventral scales, and color change from dorsum to venter.

	DSR Midbody	Ventrals	Subcaudals	Mid-Ventral Halfmoon	Color Pattern
H. wettsteini	19	123–129	28–42	Present	Gradual
H. murphyi	19	122–136	22–41	Absent	Sharp
H. plumbeus	19	113–123	30–44	Present	Gradual
H. matannensis	21	137	43–48	Present	Gradual
H. indonesiensis	25–27	152–159	36–41	Present	Gradual

Natural History

Like *H. murphyi*, *H. wettsteini* is usually found in sluggish streams, ponds, marshlands, and rice and other cultivated fields (Mell 1922, Pope 1929, Kuntz 1963, Karsen *et al.* 1986, Murphy 2007), but may wander overland on rainy nights in search of food (Karsen *et al.* 1986). Our elevational records spanned from sea level to 813 m elevation. Field data from some specimens used in this study shed additional light on natural history. The specimen KU 311544 was found at night (21:00–24:00) in a recently ploughed, wet field. The species is nocturnal or cathemeral (Karsen *et al.* 1986, Murphy 2007) and feeds on fish, frogs, and sometimes crustaceans (Schmidt 1927, Pope 1929, Gressitt 1941, Kuntz 1963, Karsen *et al.* 1986). Specimen KU 222704 contained a barely digested frog (Fig. 4). The venom toxicity of this species is likely not dangerous to humans given the lack of concern from bites of other *Hypsiscopus* species, which usually only causes localized effects (if any) such as local swelling and burning sensations (Karsen *et al.* 1986). Like *H. murphyi*, *H. wettsteini* is ovoviviparous and gives birth to live young (Schmidt 1927, Pope 1929, Karsen *et al.* 1986). Specimen KU 222704 contained nine developing embryos (Fig. 4), which is the average litter size reported by Cox (1991).

Discussion

We recognize five species of *Hypsiscopus*, with *H. wettsteini* and *H. murphyi* recognized as two distinct species; this marks the fourth named species of rice paddy snake in less than a year. This demonstrates that the diversity of this group is still in need of investigating with various datasets and datatypes, and dense sampling schemes. Our knowledge of the evolutionary histories of homalopsids snakes has increased drastically in recent years (Bernstein *et al.* 2021, 2022, 2023, 2024; Hamidy *et al.* 2023; Murphy & Voris 2021; Quah *et al.* 2017, 2018), and this study marks the 59th species of the family Homalopsidae. Our integrative data approach has shown that while traditional morphological examinations may not always lead to the delimitation of closely related species (Bernstein *et al.* 2022), more rigorous analyses like LDA and analyses of environmental data can reveal additional lines of evidence for delimiting species.

Our phylogeny (Fig. 2A) represents the most comprehensive evolutionary history of *Hypsiscopus* to date. We note that the phylogenetic structure of *H. wettsteini* reflects Pleistocene land bridges between the mainland and Hainan Island, in which individuals from Hainan are in the same clades as those from mainland China and Vietnam. Future studies that include genetic data from Taiwan specimens will likely reveal similar structure due to land bridges that also connected the mainland to Taiwan during the Pleistocene (Voris 2000).

The most notable biogeographic pattern in our data is the split between *H. murphyi* and *H. wettsteini* at the Red River Basin in northern Vietnam. The region where this river exists represents a major strike-slip fault zone between the South China and Indochina tectonic plates (Hall 1998; Leloup *et al.* 1995). The Red River has been found to be a major biogeographic barrier for several organismal groups, especially in reptiles and amphibians, some of which are restricted to aquatic habitats (Zhang *et al.* 2010a,b; Bain & Hurley 2011; Yuan *et al.* 2016). However, while the Red River Basin seems to be a geological feature that delineates the distribution of these two species, our study lacks sampling west of the Red River Basin and near other significant geological features (e.g., the Black River, the Hoang Lien Son Mountains) to determine what drove the speciation of these lineages. Our records show occurrences on both sides of the river (maximum distance west of the Red River = ~3.5 km), and while this geological feature may

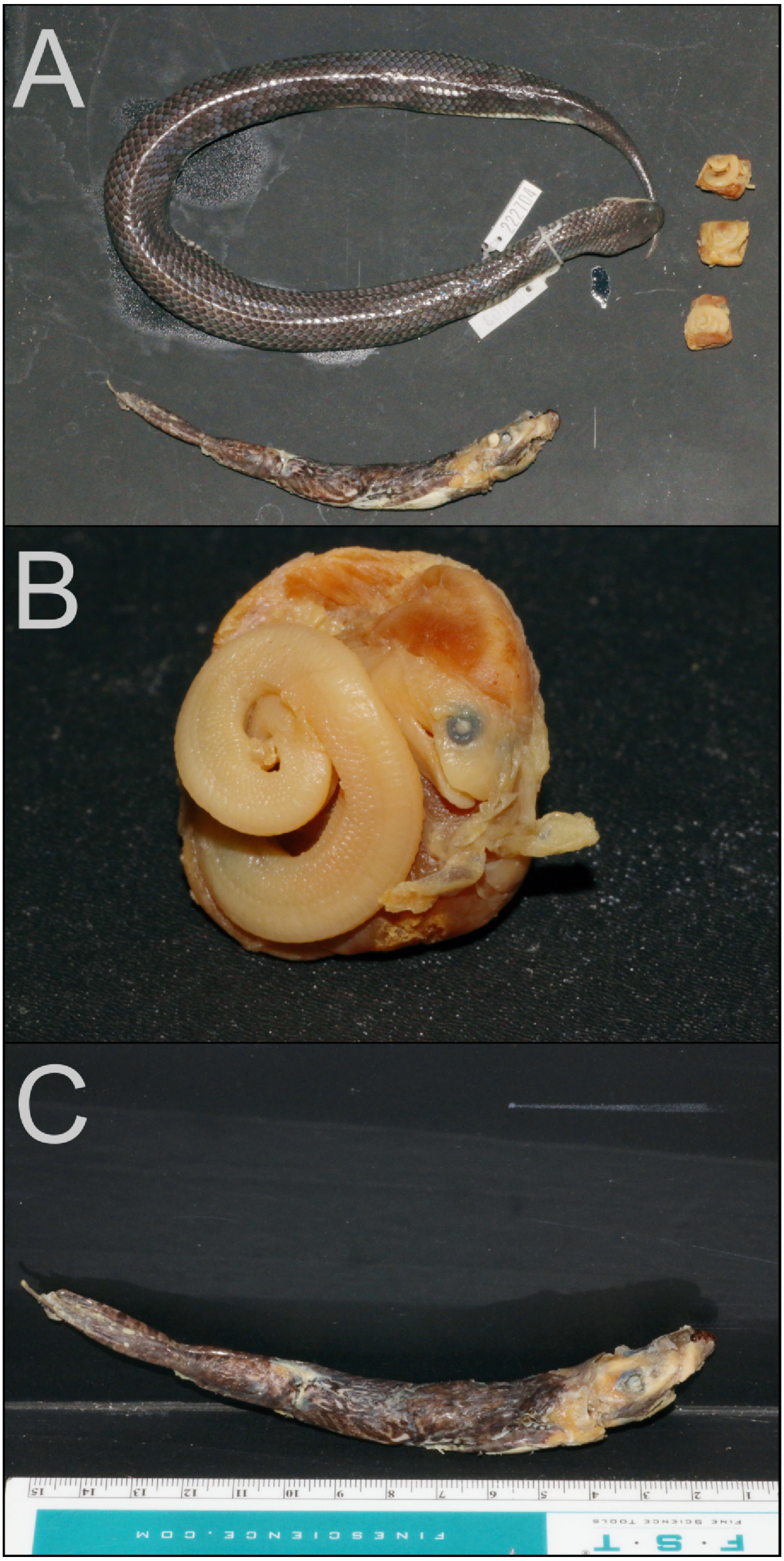

FIGURE 4. Specimen of *H. wettsteini* (KU 222704) from Lantau Island, Hong Kong, China. A) Dorsal view, with embryos and stomach contents shown. B) Close up dorso-lateral view of embryo. C) Partially digested frog from stomach contents. Photo credits: JMB.

limit gene flow between *H. murphyi* and *H. wettsteini*, we confine the conclusions of this study to the recognition of two distinct lineages that were, prior to this study, considered *H. wettsteini*.

Interestingly, the color pattern of *H. wettsteini* is essentially identical to that of *H. plumbeus* despite *H. wettsteini* being more closely related to *H. murphyi*. Our LDA sufficiently separates *H. murphyi*, *H. wettsteini*, and *H. plumbeus* and accurately identifies them (Fig. 2B), clearly demonstrating the morphological distinctiveness of these taxa. Indeed, we can only use a limited number of characters and compare our specimens to a single *H. wettsteini*, the name-bearing type (NMW 18726), in our LDA. However, our results support that there is a greater morphological similarity of the *H. wettsteini* holotype to the populations of *H. wettsteini* sensu David & Vogel (2024) at or east of the Red River Basin, ultimately making the western *H. wettsteini* populations distinct (here resurrected as *H. murphyi*). *Hypsiscopus murphyi* is the only species of rice paddy snake to have a sharp transition of dark to light coloration from dorsum to venter, and it is possible that this is a novel mutation in gene(s) coding to color pattern for this taxon. However, there is a possibility that similar environmental conditions between *H. wettsteini* and *H. plumbeus* have led to similar coloration (Wiens 2004; Wiens & Graham 2005). Our niche models reveal that *H. wettsteini* has a similar habitat suitability (broadly throughout Southeast Asia, highest in low elevation coastal areas) to *H. plumbeus* (not shown; Bernstein *et al.* 2024) and it is possible that similar niche space has led to a convergence in color pattern in these snakes. Contrarily, a lack of niche conservatism has the potential to lead to niche divergence (Ahmadzadeh *et al.* 2013; Enriquez-Urzelai *et al.* 2022). *Hypsiscopus murphyi* shows a more limited distribution than *H. wettsteini* and suitability towards higher elevations. Divergent climatic niches have previously been found in closely related species of reptiles and amphibians (Burbrink *et al.* 2021; Hua & Wiens 2010; Knouft *et al.* 2006; Pyron & Burbrink 2009), and even populations within the same species (Muñoz *et al.* 2013; Ogden & Thorpe 2002; Schneider *et al.* 1999). Homalopsids are primarily considered low-elevation species, but *H. murphyi* has adapted to higher elevations compared to its congeners. We find it likely that while the Red River may have been a significant factor in the diversification of the lineage ancestral to *H. murphyi* and *H. wettsteini,* environmental factors likely contributed to differentiation between these two species, especially temperature variables as seen in our climate envelopes (see *Results*: *Ecological Niche Models*). This is a likely scenario given that the Red River also demarcates a transition zone between subtropical and tropical climates (Chen & Chen 2013; Peel *et al.* 2007), and temperature and precipitation vary between these regions.

It remains uncertain how the type specimens of *H. wettsteini* became erroneously associated with a collection of specimens from Costa Rica housed in the Museum of Natural History in Vienna, Austria (David & Vogel 2024), and their actual provenance remains unknown. However, our data strongly posit that they originated from somewhere in northeastern Vietnam, southeastern China, or Taiwan. With greater success rates in obtaining molecular data from voucher and type specimens (Bernstein *et al.* 2023; Bernstein & Ruane 2022; O'Connell *et al.* 2021; Ruane & Austin 2017), future studies might be able to obtain a finer-scale geographic resolution of the type specimens. In addition to determining more accurate locality information for type specimens, filling in geographic sampling gaps, such as those between the ranges of *H. murphyi* and *H. wettsteini*, will likely provide more information on how these lineages differ from each other and what factors have led to their diversification.

Widespread species such as *H. plumbeus* sensu Murphy and Voris (2014) and *H. murphyi* sensu Bernstein *et al.* (2022) represent opportunistic systems to identify hidden diversity and investigate the drivers of diversification amongst lineages. While future studies should focus on obtaining samples from understudied regions, such as Taiwan or other regions of mainland East Asia, we find evidence that vicariant and environmental influences led to the extant diversity of *Hypsiscopus*. Our study shows that integrative datasets are necessary for systematics and taxonomy, and the incorporation of multiple data types may be crucial for identifying additional lines of evidence for taxonomy-based decisions.

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Supplementary Materials. The following supporting information can be downloaded at the DOI landing page of this paper:

Supplementary Fig. 1: Concatenated Bayesian Inference tree of *Hypsiscopus*. Numbers at nodes represent Bayesian Posterior Probabilities (black circles at nodes indicate strongly supported relationships, ≥ 0.95). Scale bar in substitutions per site.

Supplementary Table S1: Metadata for all samples used in this study. GenBank accession numbers, locality data, morphological data for linear discriminant analysis, and primer information for DNA amplification are provided.