

## Closure Report

**File Number :** CRG/2018/000790  
**Project Title :** Biogeography of Eastern Borderland in India: Evidence from Ectotherms.  
**Principal Investigator :** Dr. Abhijit Das  
Wildlife Institute of India (WII)  
Dehradun, UT,UTTARAKHAND-248007  
**Total Sanctioned Amount :** 42,46,499 (INR)  
**Total Released Amount :** 36,72,000 (INR)  
**Start Date of the Project:** 30 Mar, 2019  
**Date of completion:** 29 Mar, 2023 ( 48 months )  
**Approved Objectives :**

- To determine the biogeographic borderline separating the Indian and Indo-Chinese sub-regions in India
- To determine if this biogeographic borderline vary across taxonomic groups
- To gather evidence of hybridization between or within groups from India and Indo-Chinese subregions in the NEI (which is a transition zone between subregions)
- Research question: Whether it is Brahmaputra River or the Brahmaputra Valley, or any other river or mountain range that acting as biogeographic barriers for herpetofauna
- Research question: Which are the taxa that show dispersal or vicariant event in NEI? When and how Southeast Asian species dispersed across NEI?
- Research question: Where be the hybrid zones in NEI? Is there a relationship between hybridization and colonization in NEI?

### Deviation made from original objectives (If Any) :

For the third objective, we could only sample one taxonomic group of Rhacophorid frog of the genus *Raorchestes*. Due to fund constrain we could not carry our robust sampling needed for population genetic analysis. We will need atleast 20 samples from one locality and cover throughout the eastern borderland for hybridization study. However, we are trying to generate more resources for extensive sampling across the region to answer the question pertaining to Hybridization. We have added another aquatic frog taxa for the hybridization experiment.

**Ph.D. Produced/ Likely to be Produced** : 2

**Technical Personnel Trained** : 8

**Total Expenditure :** 37,46,710 (INR)

### Concise Research Accomplishment :

So far we have published three new species of reptiles and amphibian (*Rhabdophis bindi*, *Smithophis arunachalensis*, *Micryletta aishani*) in international peer reviewed journals. Few more species are under review process or accepted. Our study also reported rediscovery of lost species of snake such as Blue-bellied Kukri Snake (*Oligodon melaneus*) from Brahmaputra valley. This project also helped us conduct first ever systematic inventory of 20 protected areas in Northeast India. We generated a list of 234 species of herpetofauna which includes 136 amphibians and 98 species of reptiles. Our inventory also covered biogeographic units from north and south of Brahmaputra River including Dafla Hills, Adi Hills, Mishmi Hills, Patkai hills, Khasi-Garo hills, Lushai Hills, Barail Hills. We provided a strong taxonomic basis of our study by collecting at least 20 topotypic materials. As part of our integrative taxonomic approach we developed bioacoustics database from 90 individuals of Amphibian belonging to the genera *Raorchestes*, *Xenophrys*, *Hyla*, *Polypedates*, *Kurixalus*, *Rhacophorus*, *Minervarya*, and ranid frogs. This study is first of its kind that addressed the Darwinian shortfall in a most comprehensive way. We generated over 230 DNA sequences from tissue samples of amphibians (116 samples) and reptiles (38 samples) collected from Northeast India. This project enable us to enrich global database such as National Centre for Biotechnology Information (NCBI) by submitting ~30 newly generated DNA sequences already. Atleast 500 specimens collected during the project has been registered at the National Repository of Wildlife Institute of India, Dehradun Museum and is available for further research and comparative studies. We delineated geographical distribution range of morphologically similar species based on molecular study. As well as we reported new distributional record of agamid lizard (*Calotes medogensis*) from India based on morphological and molecular study. Our study likely to report more new distributional record of herpetofaunal species from the families Rhacophoridae, Viperidae and Colubridae. Laboratory work on this is undergoing. First ever genetic database has been generated for atleast 20 northeast endemic and poorly known species of herpetofauna. Updated data on morphology and phylogeny will help in redescription of these poorly known species. Putative new species of amphibians from the genera such as *Xenophrys*, *Amolops*, *Odorana*, *Raorchestes*, *Ingerana*, *Liurana*, *Nanorana*, *Minervarya*, and reptile species from the genera *Rhabdophis*, *Sphenomorphus*, *Ptyctolaemus* and *Cyrtodactylus*. Laboratory work (morphology and molecular) is underway for these species.



## Closure Details

### Experimental/ Theoretical Investigation carried out

During 2019 and 2021-2022, we selected 20 protected areas located north and south of river Brahmaputra as our sampling units. We have sampled at least 120 localities between an elevation of 50-3000 m in the region. We carried out nocturnal visual encounter survey (Crump and Scott, 1994) between 1800 – 2300 h aiding with torch light to record the herpetofaunal species. Surveys were carried out along possible habitats such as flowing streams, dry streambeds, forest trails, roadside vegetation, ponds and lakes, agriculture fields aided by flashlights. During day time searched for basking reptiles following forest trails, forest edge and river bank. Information on locality, date, habitat and microhabitat, natural history and behavioural observation were recorded. Geo-coordinates were taken using a Garmin etrex 20X GPS. Live specimens were photographed in nature for details of colouration and characters. Representative samples as per research permit were collected for morphological and molecular analysis. Metric and meristematic characters were studied using stereomicroscope. Morphometric measurements were taken using digital slide calliper (Mitutoyo™) to nearest 0.1 mm. Sex and maturity were determined by the presence of vocal sac and nuptial pad in males or by examining the gonads through a ventral incision. Measurements and associated terminologies follow Das et al. (2019) for amphibians and Das et al. (2021) and Boruah et al. (2022) for reptiles. For our taxonomic study we are studying historical museum specimens of amphibians and reptiles deposited in Natural History Museum, London, Bombay Natural History Society museum and Zoological Survey of India. For bioacoustics studies we recorded the advertisement calls of amphibian species using Sennheiser unidirectional handheld microphones and Marantz PMD 620 MK-II digital audio recorders between 1800 and 2300 hours. The calls were recorded at a distance approximately 0.1-0.3 m and ambient temperatures were recorded using a Kestrel weather meter. For the Molecular data and phylogenetic analysis, Genomic DNA was extracted from the collected muscle tissue samples using the DNeasy (Qiagen™) blood and tissue kit following the manufacturer's protocols. We amplified and sequenced six mitochondrial gene 12S, 16S, Cytochrome-b, Cytochrome c Oxidase subunit- I, NADH dehydrogenase- 4 and three nuclear genes such as Neurotrophin 3, Rhodopsin and Brain-derived neurotrophic factor (BDNF) gene using the previously published primers and protocols. Amplified PCR products were run on a 2% agarose gel and viewed under UV transilluminator. Purified PCR product was sequenced directly in an Applied Biosystems Genetic Analyzer 3500 XL in both directions using BigDye v3.1 kits. Sequences were assembled in MEGA v7.1 (Kumar et al. 2016) and aligned with the ClustalW algorithm (Higgins et al. 1994) with default settings. We performed a Maximum Likelihood (ML) analysis using IQ-TREE (Nguyen et al. 2015), implemented in the web server version (<http://iqtree.cibiv.univie.ac.at/>) (Trifinopoulos et al. 2016). We used Bayesian information criterion (BIC) as implemented in JMODELTEST v2.1.6 (Darriba et al. 2012) to select the best-fit model of nucleotide substitution, which was then used in model-based phylogenetic inference. Bayesian Inference (BI) was performed in MRBAYES v3.2 (Ronquist et al. 2012). The uncorrected pairwise distances (P-distance) were calculated in MEGA v7.1 with pairwise deletion of missing data and gaps. For molecular dating, we are aligning a concatenated dataset following our Natricine trait paper already published in Zoological Journal of the Linnean Society 2021. We selected fossil calibrations as recommended. We are using PartitionFinder for best-fitting partition scheme and model(s) of sequence evolution. We assigned a relaxed log-normal clock for each partition of the concatenated BEAST2 analysis. Markov chain Monte Carlo (MCMC) will be used for 200 000 000 generations. We are using sample size (ESS) values using Tracer v.1.7. We are estimating divergence dates using penalized likelihood as implemented in treePL applying fossil calibrations. Penalized likelihood uses a tree with branch lengths and calibrations without prior parametric distributions. We used the ML tree from RAxML-HPC2 v.8.2.12, built using the concatenated. We are using BAMM v.2.5.0 (Bayesian analysis of macroevolutionary mixtures) to investigate shifts in diversification rates across our phylogeny. Four parallel MCMC chains for 1 000 000 iterations will be applied, discarding the first 10% as burn-in and sampling every 10 000 iterations. The function set BAMMpriors from the BAMM tools package in R v.4.0 (reproducible R code) will be used for prior distribution parameters. Convergence checks will be performed by visual analysis of trace plots and by using the effective Size function in the coda package, with all parameters showing an ESS > 200. We will use BAMM tools to extract credible shift sets and best shift configuration and to create speciation-rate-through-time plots to visualize the variation in speciation rate. For biogeographic analysis we will maintain same genus level sampling. Historical biogeography will be investigated by estimating ancestral areas for the dated phylogeny using dispersal-extinction-cladogenesis and dispersal-vicariance analysis models implemented in BioGeoBEARS v.1.1 in R v.4.0 (reproducible R code is available). Best fit model will be decided using Akaike information criterion (AIC) scores and weights.

## Detailed Analysis of result

Role of Brahmaputra as Biogeographic Barrier: We are analyzing our dataset to understand barrier effect of the Brahmaputra river at generic and species level. We have sampled genera such as *Limnocytes*, *Micryletta*, *Nanorana*, *Smithophis* from the hill ranges north and south of the barrier. Our study (Boruah et al. 2022) shows that Brahmaputra valley is a potential barrier at species level such as Agamid lizard of the genus *Calotes*. Based on molecular and morphological tool we observed that two closely related species of green lizard (*Calotes medogensis* and *Calotes jerdoni*) are separated by the Brahmaputra valley. Our findings revealed that *Calotes medogensis* is distributed in north of Brahmaputra valley in Arunachal Pradesh in India and Medog County, China, which was earlier misidentified as *Calotes jerdoni*, which is now restricted to south of Brahmaputra valley in Meghalaya and Nagaland. In addition, we have also provided clear diagnostic characters for *C. jerdoni* and *C. medogensis* that were previously unknown. Similar results also shown in *Smithophis* and *Micryletta* which are already published. One of our recently published study (Köhler et al. 2023) provides evidence that the Brahmaputra might be acting as a barrier for arboreal snake of the genus *Boiga*. The study shows Brahmaputra as a barrier for typical Himalayan species such as *Boiga stoliczkae* and widespread Southeast Asian species such as *Boiga multomaculata* that meets its northwestern distributional limit in northeast India. The "filter" effect of the barrier is rather shown by our phylogenetic study involving Natricine snake of the genus *Rhabdophis* (Das et al. 2021). We used genetic sample of *Rhabdophis himalayanus* complex collected from north (Sikkim) and south (Meghalaya) of Brahmaputra river. These two samples had very low genetic differences (2% in *cytb* and 0.2% in *16s*) suggesting that the river may not be as strong a barrier for some species compared to others. While in the arboreal agamid lizard, this genetic difference among cryptic species is 13-14%. Our integrative taxonomic taxonomy described a new species of Natricine snake, *Rhabdophis bindi* from south of Brahmaputra valley and Khasi-Garo hill ranges. This species is so far restricted to lowland areas (below elevation 100 m asl) in Barak valley of Assam, Mizoram, Tripura and western and southern Bangladesh. This suggest elevation may be a potential barrier for dispersal of this lowland terrestrial snake.

## Conclusions

This project helped us determining the previously underexplored cryptic diversity and delineating distribution range based on phylogenetic approach. After further analysis of result we expect that we will be able to describe atleast ten new species of reptiles and amphibians collected during this study. Our preliminary study shows that Brahmaputra is acting as a strong barrier for arboreal taxa and not for semiaquatic taxa such as natricine snakes. However, in next two months, we will be able to complete our "Divergence dating analysis" to explicitly determine the historical biogeography of the herpetofauna of eastern borderland. We expect to publish most of our results within 2023 with due acknowledgement to SERB for financial support. We will be happy to communicate all the publications.

## Scope of future work

Addressing the Wallacean shortfall for cryptic herpetofaunal species based on our genetic sample collected from across Northeast India. The IUCN status of most of the herpetofaunal species from Northeast India is either not evaluated (NE) or data deficient (DD). One of the reasons behind this is lack of information on their geographical distribution. This will help in accessing the IUCN status of herpetofaunal species of the region. The study will provide a strong basis for future taxonomic research as most of the cryptic group will have their phylogenetic data published. The specimen and tissue repository at the museum of WII will help researchers to access the database and initiating further research initiative. Further sampling across multiple elevational gradients available in the region will help in determine the gene flow across the eastern Himalayan mountain chain and northeast hills. Sampling across widespread shallow divergence group will help generate information for reticulate evolution.

**List of Publications (only from SCI indexed journals) :**

Title of the Paper	List of Authors	Journal Details	Month & Year	Volume	Status	DOI No	Imp Fact
A new species of <i>Micryletta</i> frog ( <i>Microhylidae</i> ) from Northeast India	Abhijit Das, Sonali Garg, Amir Hamidy, Eric N. Smith and S. D. Biju	PeerJ (International)	Jun-2019	(32)	Published	10.7717/peerj.7012	2.35
A taxonomic revision of <i>Boiga multomaculata</i> (Boie, 1827) and <i>B. ochracea</i> (Theobald, 1868), with the description of a new subspecies ( <i>Squamata, Serpentes, Colubridae</i> )	GUNTHER KÖHLER, PANUPONG THAMMACHOTI CHARUNROCHANA, LINDA MOGK, NI LARTHAN3, NIA KURNIAWAN, AHMAD MUAMMAR KADAFI, ABHIJIT DAS, FRANK TILLACK and MARK O' SHEA	ZOOTAXA (International)	Apr-2023	5270 (151–193)	Published	<a href="https://doi.org/10.11646/zootaxa.5270.2.1">https://doi.org/10.11646/zootaxa.5270.2.1</a>	1.09
A multilocus molecular perspective on the systematics of the poorly known Northeast Indian colubrid snakes <i>Blythia reticulata</i> (Blyth, 1854), <i>B. hmuifang</i> Vogel, Lalremsanga & Vanlalhrima, 2017, and <i>Hebius xenura</i> (Wall, 1907). <i>Zootaxa</i> 4768(2):193-200	Lalronunga S, Lalrinchhana C, Vanramliana, Das, A., Gower D.J, Deepak V.	ZOOTAXA (International)	May-2020	2 (193-200)	Published	<a href="https://doi.org/10.11646/zootaxa.4768.2.2">https://doi.org/10.11646/zootaxa.4768.2.2</a>	0.93
Description of a new species of <i>Smithophis</i> Giri et al. 2019 ( <i>Serpentes: Colubridae: Natricinae</i> ) from Arunachal Pradesh, India	ABHIJIT DAS, V. DEEPAK, ASHOK CAPTAIN, EDWARD O. Z. WADE & DAVID J. GOWER	ZOOTAXA (International)	Oct-2020	4860 (2) (267–283)	Published	<a href="https://doi.org/10.11646/zootaxa.4860.2.8">https://doi.org/10.11646/zootaxa.4860.2.8</a>	0.94

**List of Papers Published in Conference Proceedings, Popular Journals :**

Title of the Paper	List of Authors	Journal Details	Month & Year	Volume	Status	DOI No	Imp Fact
An updated checklist of reptiles from Dampa Tiger Reserve, Mizoram, India, with sixteen new distribution records.	Malsawmdawngliana, Boruah, B., Patel, N.G., Lalronunga, S., Zosangliana, I., Lalmangaiha, K. & Das, A.	Journal of Threatened Taxa (National)	Oct-2022	14 (21946)	Published	<a href="https://doi.org/10.11609/jott.8004.14.10.21946">https://doi.org/10.11609/jott.8004.14.10.21946</a>	
Multilocus phylogeny, natural history traits and classification of natricine snakes ( <i>Serpentes: Natricinae</i> ).	Deepak, V., Cooper, N., Poyarkov, N.A., Kraus, F., Burin. G., Das, A., Narayanan, S., Streicher, J.W., Smith, S.J., and Gower, D.J. (2022).	Zoological Journal of the Linnean Society (International)	Oct-2021	195 (279-298)	Published	<a href="https://doi.org/10.1093/zoolinnean/zlab099">https://doi.org/10.1093/zoolinnean/zlab099</a>	3.28
Hidden in the plain sight: a new species of <i>Rhabdophis</i> ( <i>Serpentes: Natricinae</i> ) from the <i>Rhabdophis himalayanus</i> complex.	Das, A., Smith, E. N., Sidik, I., Sarker, G. C., Boruah, B., Patel, N. G., Murthy, B. & Deepak, V.	Zootaxa (International)	Aug-2021	5020 (401-433)	Published	10.11646/zootaxa.5020.3.1	1.09

Title of the Paper	List of Authors	Journal Details	Month & Year	Volume	Status	DOI No	Imp Fact
Rediscovery and systematics of the rarely encountered Blue-bellied kukri snake ( <i>Oligodon melaneus</i> Wall, 1909) from Assam, India.	Das, A., Gower, D. J., Narayanan, S., Pal, S., Boruah, B., Magar, S., Das, S., Moulick, S. & Deepak, V.	Zootaxa (International)	May-2022	5138 (417-430)	Published	<a href="https://doi.org/10.11646/zootaxa.5138.4.4">https://doi.org/10.11646/zootaxa.5138.4.4</a>	1.09

**List of Patents filed/ to be filed :**

Patent Title	Authors	Patent Type	Country/Agency Name	Patent Status	Application No.
Not Available					

**Equipment Details :**

Equipment Name	Cost (INR)	Procured	Make & Model	Utilization %	Amount Spent (INR)	Date of Procurement
stereo microscope	5,35,130	Yes	Olympus SZX10	80	6,76,000	12 Dec, 201
Headlamps	22,875	No		0	0	
Call Recorder	54,870	No		0	0	
External Hard Disc	15,104	No		0	0	
Refrigerator	48,280	No		10	0	

**Plans for utilizing the equipment facilities in future:**

Stereo Microscope is crucial for taxonomic studies. thus this equipment is available for full utilization by the researcher and scientists of the Institute. Currently three PhD students and four interns are utilizing this equipment for specimen study and photography.

# Multilocus phylogeny, natural history traits and classification of natricine snakes (Serpentes: Natricinae)

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Natricine snakes are geographically widespread, species rich (with ~250 extant species) and both morphologically and ecologically diverse. We present a multilocus DNA sequence phylogeny for 249 natricine specimens representing 189 named species, including 69 specimens and 21 species not previously sampled. Our inferred Bayesian and maximum likelihood trees form the basis for evaluations of genus-level classification, historical biogeography, lineage diversification, and dietary, habit and reproductive-mode diversity and evolution, although several, mostly deeper, relationships remain poorly resolved. The optimal trees support natricine origins in Asia, with dispersals to Australo-Melanesia, sub-Saharan Africa (including Seychelles Archipelago, excluding Aldabra), Europe and North Africa and into North and Central America. Viviparity appears to have evolved independently three times in Natricinae but was not significantly associated with an aquatic habit. We found limited associations between habit and diet categories. We propose generic reallocations for four natricine species and highlight other points of uncertainty in natricine classification.

**ADDITIONAL KEYWORDS:** biogeography – colubrid snakes – diet – diversification – evolution – habit – Natricidae – systematics.

## INTRODUCTION

Natricine colubrid snakes (Natricinae, in the sense used by, e.g. [Pyron \*et al.\*, 2013](#); [Figueroa \*et al.\*, 2016](#); [Uetz \*et al.\*, 2020](#); equivalent to Natricidae of [Zaher \*et al.\*, 2009](#); [Burbrink \*et al.\*, 2020](#)) originated in Asia ~35.0–47.1 Mya ([Guo \*et al.\*, 2012](#); [Zheng & Wiens, 2016](#); [Zaher \*et al.\*, 2019](#); [Burbrink \*et al.\*, 2020](#)), and many more extant species occur in Asia than in all other major regions (~185 vs. 65 species; [Uetz \*et al.\*, 2020](#)).

Most extant natricine diversity lies north of the equator, with no evidence that natricines in the Americas ever dispersed into South America. Extant American natricines are monophyletic, and it is estimated that this lineage dispersed to North America via Beringia during the Miocene (~23 Mya; [Guo \*et al.\*, 2012](#); [McVay \*et al.\*, 2015](#)). Sub-Saharan African (including Seychelles Archipelago) natricines are monophyletic and are estimated to have dispersed there during the Miocene from Asia (~43–25 Mya; [Deepak \*et al.\*, 2021b](#)). The timing and number of dispersals of natricines to the east of Wallace's Line is unknown.

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Since their origin during the Miocene, natricines have adapted to a wide diversity of niches and lifestyles. Most extant natricines are terrestrial or semi-aquatic in habit, often having a generalist diet, although some species are specialists, feeding on anurans [e.g. *Hebius beddomei* (Günther, 1864)], crayfish [e.g. *Regina septemvittata* (Say, 1825)], molluscs (e.g. *Storeria* spp.) or earthworms [e.g. *Thamnophis brachystoma* (Cope, 1892)]. Fully aquatic natricines are typically generalists, but some feed only on crayfish [e.g. some species of *Liodytes* (Cope, 1885) and *Regina* Baird & Girard, 1853], worms and small crustaceans (e.g. species of *Opisthotropis* Günther, 1872, *Rhabdops* Boulenger, 1893 and, possibly, *Hydrablades* Boulenger, 1891) or fish (e.g. *Trimerodytes balteatus* Cope, 1895). All known American natricines are viviparous (Tinkle & Gibbons, 1977), and, as far as is known, all other natricines except the Asian *Trimerodytes annularis* (Hallowell, 1856) and *Pseudagkistrodon rudis* (Boulenger, 1906) are oviparous. Natricines range in maximum size from ~11.5 to 119.5 cm snout–vent length, with many of the smallest species burrowing in soil.

The most species-rich extant natricine genera are *Hebius* Thompson, 1913 (43 species) and *Thamnophis* Fitzinger, 1843 (35 species) from Asia and North America, respectively. The lowest extant continental diversity of natricines is in Australia with a single species in northern Australia and in mainland sub-Saharan Africa (16 species; Uetz *et al.*, 2020). There are multiple monotypic genera among Asian and African natricines (*Amphiesmoides* Malnate, 1961, *Isanophis* David *et al.*, 2015, *Helophis* Witte & Laurent, 1942, *Lycognathophis* Boulenger, 1893 and *Afronatrix* Rossman & Eberle, 1977) and among North and Central American natricines (*Clonophis* Cope, 1889, *Tropidoclonion* Cope, 1860, *Haldea* Baird & Girard, 1853 and *Virginia* Baird & Girard, 1853). Several Asian natricine genera have recently been relegated as junior synonyms (e.g. *Balanophis*, *Pararhabdophis*, *Parahelicops* and *Macropisthodon*), transferred to other (sub)families [e.g. *Hologerrhum* Günther, 1858 (Lamprophiidae)] or removed from Natricinae entirely (e.g. *Iguanognathus* Boulenger, 1898). Thirty-nine new species of natricine snakes have been described since 2000, of which only four are from the Americas (Conant, 2000; Rossman & Burbrink, 2005), one from Australo-Melanesia (Kraus & Allison, 2004), one from Africa (Conradie *et al.*, 2020) and the rest from Asia (David & Das, 2003; Ziegler & Le Khac, 2006; David *et al.*, 2007, 2011, 2021; Stuart & Chuaynkern, 2007; Ziegler *et al.*, 2008, 2018, 2019; David & Vogel, 2010; Doria *et al.*, 2013; Yang *et al.*, 2013; Guo *et al.*, 2014, 2019; Teynie *et al.*, 2014; Zhu *et al.*, 2014; Giri *et al.*, 2017, 2019; Ren *et al.*, 2017; Vogel *et al.*, 2017, 2020; Wang *et al.*, 2017;

Wickramasinghe *et al.*, 2017, 2019; Qinliu *et al.*, 2018; Raha *et al.*, 2018; Bhosale *et al.*, 2019; Purkayastha & David, 2019; Zhou *et al.*, 2019; Conradie *et al.*, 2020; Das *et al.*, 2020a, 2021; Piao *et al.*, 2020). This highlights that the diversity of Asian natricines has probably been underestimated and there is a need for ongoing systematic revision. A substantial component of recent systematic revisions has been underpinned by molecular phylogenetic studies (Guo *et al.*, 2014; Giri *et al.*, 2017, 2019; Kizirian *et al.*, 2018; Ren *et al.*, 2018, 2019; Takeuchi *et al.*, 2018; Lalronunga *et al.*, 2020; Das *et al.*, 2020b; Conradie *et al.*, 2020), although many species have yet to be sampled for DNA sequence data.

Given their broad geographical range, high taxonomic and ecomorphological diversity and ongoing taxonomic flux, a more densely sampled molecular phylogeny of natricines will substantially aid our understanding of the diversity and evolution of this group. Here, we present the results of a new molecular phylogenetic analysis for 249 natricine specimens representing 202 species (named and unnamed). We map key natural history traits onto the resulting trees, investigate historical biogeography and test hypotheses associated with diversification rates and reproductive evolution. We also address implications for the generic-level classification of natricines.

## MATERIAL AND METHODS

### SPECIMEN PREPARATION AND DNA SEQUENCE GENERATION

Tissue samples (muscle, liver or tail tips) came from 69 individuals of natricine snakes (Supporting Information, Table S1). We extracted genomic DNA from tissues using DNeasy (Qiagen) blood and tissue kits and amplified partial sequences of three mitochondrial (mt) and four nuclear (nu) genes. The mt genes were 16S ribosomal RNA, cytochrome *b* (*cytb*) and NADH dehydrogenase subunit 4 (*nd4*); and the nu markers were oocyte maturation factor (*cmos*), neurotrophin-3 (*nt3*), brain-derived neurotrophic factor (*bdnf*) and recombination activating gene 1 (*rag1*). Polymerase chain reaction (PCR) conditions followed previously reported protocols [16S, primers 16Sar-L and 16Sbr-H (Palumbi *et al.*, 1991); *cytb*, primers GluDG L (Palumbi, 1996) and H16064 (Burbrink *et al.*, 2000); *nd4*, primers ND4 and Leu (Arévalo *et al.*, 1994); *cmos*, primers S77 and S78 (Lawson *et al.*, 2005); *nt3*, primers nt3f and nt3r (Townsend *et al.*, 2008); *bdnf*, primers BDNF-f and BDNF-r (Noonan & Chippindale, 2006); *rag1*, primers R13 and R18 (Groth & Barrowclough, 1999)]. Sanger sequencing was carried out using the same primers that were used for PCR amplification. In total, we generated 432 new sequences in this



study and deposited them in GenBank ([Supporting Information, Table S1](#)). We assembled contigs from bidirectional sequence chromatograms and edited in SNAPGENE VIEWER ([http://www.snapgene.com/products/snapgene\\_viewer](http://www.snapgene.com/products/snapgene_viewer)).

#### PHYLOGENETIC INFERENCE

We generated sequences for 69 samples of natricines from South Asia, Southeast Asia, New Guinea and Central and North America, then assembled a concatenated sequence dataset (5040 bp in length) with 251 tips, which included 249 natricines and two outgroups: *Sibynophis subpunctatus* Duméril, Bibron & Duméril, 1854 and *Grayia ornata* (Bocage, 1866) ([Supporting Information, Table S1](#)). We checked for stop codons in unexpected regions by translating nucleotide alignments to amino acids for protein-coding genes (*cytb*, *nd4*, *cmos*, *nt3*, *bdnf* and *rag1*) using MEGA7 ([Kumar et al., 2016](#)). We aligned sequences using CLUSTALW ([Thompson et al., 1997](#)) in MEGA7 ([Kumar et al., 2016](#)) with default settings (alignments available online from the Natural History Museum London, data portal). Most of the sequences (95.4%) for each species in the concatenated dataset came from the same tissue sample; 18 samples were chimeric, as indicated in the [Supporting Information \(Table S1\)](#). Data coverage for each of the genes in this dataset, excluding the outgroups, is as follows: *cytb* 88%, 16S 43%, *nd4* 59%, *cmos* 64%, *nt3* 56%, *bdnf* 47% and *rag1* 47%.

We used the CIPRES Science Gateway v.3.3 ([Miller et al., 2010](#)) for all phylogenetic analyses. We used PARTITIONFINDER2 ([Lanfear et al., 2017](#)) in XSEDE (xsede.org) to identify the best-fitting partition scheme for the concatenated dataset and the best-fitting model of sequence evolution for each partition as determined by the Bayesian information criterion (BIC) using the default greedy algorithm linked to

branch lengths ([Lanfear et al., 2012](#)). The best-fitting scheme comprises nine partitions by gene and by codon position ([Table 1](#)).

We then partitioned the data by gene and codon position and performed maximum likelihood (ML) analyses ([Felsenstein, 1981](#)) in RAXML-HPC2 v.8.2.12 ([Stamatakis, 2014](#)) on XSEDE, using the GTRGAMMA model of sequence evolution, which is recommended over GTR+G+I, because the 25 rate categories account for potentially invariant sites ([Stamatakis, 2006](#)). For Bayesian inference (BI) analyses, we used MRBAYES v.3.2.6 ([Ronquist et al., 2012](#)) in XSEDE, with default prior settings and with all five partitions assigned their best-fitting model as determined by PARTITIONFINDER ([Table 1](#)). We set up two separate runs with four Markov chains each, initiated from random trees and allowed to run for one million generations, sampling every 100 generations and discarding the first 25% of trees as burn-in. We terminated the analyses when the standard deviation of split frequencies was < 0.005, then constructed 50% majority rule consensus trees. We quantified support for internal branches in ML and BI trees using bootstrap (BS; 1000 replicates) and posterior probability, respectively. We rooted the trees with the two outgroups, *Sibynophis subpunctatus* and *Grayia ornata*, based on evidence that these are close relatives of a monophyletic Natricinae ([Figueroa et al., 2016](#); [Zaher et al., 2019](#); [Lalronunga et al., 2020](#)).

#### MOLECULAR DATING

We assembled a second dataset of 310 tips, comprising 249 natricines and 59 other snakes including representatives from all subfamilies of Alethinophidia, using two scolecophidians [*Gerrhopilus mirus* (Jan, 1860) and *Liotyphlops albirostris* (Peters, 1857)] as outgroups. This concatenated dataset comprised 5047 bp, including the same seven genes described in the phylogenetics section above

**Table 1.** Partitions and models of sequence evolution used in the maximum likelihood and Bayesian inference analyses for the 251-tip dataset

Partition	Sites	Bayesian inference	Maximum likelihood
1	<i>nd4</i> <sup>1st</sup> , <i>cytb</i> <sup>1st</sup>	GTR+I+G	GTR+G
2	<i>cytb</i> <sup>2nd</sup> , <i>nd4</i> <sup>2nd</sup>	GTR+I+G	GTR+G
3	<i>nd4</i> <sup>3rd</sup> , <i>cytb</i> <sup>3rd</sup>	GTR+G	GTR+G
4	16S	GTR+I+G	GTR+G
5	<i>cmos</i> <sup>2nd</sup> , <i>cmos</i> <sup>1st</sup> , <i>nt3</i> <sup>2nd</sup>	K80+I+G	GTR+G
6	<i>cmos</i> <sup>3rd</sup> , <i>rag1</i> <sup>3rd</sup>	HKY+G	GTR+G
7	<i>nt3</i> <sup>1st</sup> , <i>rag1</i> <sup>1st</sup> , <i>bdnf</i> <sup>1st</sup> , <i>bdnf</i> <sup>2nd</sup> , <i>rag1</i> <sup>2nd</sup>	HKY+I+G	GTR+G
8	<i>nt3</i> <sup>3rd</sup>	K80+I+G	GTR+G
9	<i>bdnf</i> <sup>3rd</sup>	K80+G	GTR+G

1st, 2nd and 3rd refer to codon positions

(Supporting Information, Table S2). We aligned these data again using the same methods outlined above (alignments available from Deepak *et al.*, 2021a).

We applied seven fossil calibrations (Table 2), largely those recommended by Head (2015) and Head *et al.* (2016) as recently used by Deepak *et al.* (2021b). The best-fitting partition scheme and model(s) of sequence evolution identified by PARTITIONFINDER had ten partitions (Table 3). Initially, we carried out divergence dating, analysing this dataset and partition scheme with BEAST v.2.5 (Bouckaert *et al.*, 2019) using XSEDE in CIPRES Science Gateway v3.3 (Miller *et al.*, 2010) under a Yule tree process. We assigned a relaxed log-normal clock for each partition of the concatenated BEAST2 analysis. We set up two independent runs, each using the Markov chain Monte Carlo (MCMC) for 200 000 000 generations, sampling every 5000 trees. We obtained effective sample size (ESS) values using TRACER v.1.7 (Rambaut *et al.*, 2014). We also repeated the analysis implementing the less complex HKY model for the partitions. Neither of these analyses attained ESS values > 100 for the priors and posteriors (for

BEAUTI settings, see Supporting Information, Table S3). Therefore, we estimated divergence dates using penalized likelihood (Sanderson, 2002) as implemented in TREEPL (Smith & O'Meara, 2012), applying the same seven fossil calibrations, but changing any soft minimum and maximum ages to hard values (Table 2) and setting the root of the tree at a maximum age of 128 Mya and minimum age of 123 Mya (i.e. Early Cretaceous, to correspond to the approximate age of the Serpentes root, based on point or mean values from Zheng & Wiens, 2016; Miralles *et al.*, 2018; Burbrink *et al.*, 2020). Penalized likelihood uses a tree with branch lengths and calibrations without prior parametric distributions. We executed TREEPL with a smoothing value of 10 000. We used the ML tree from RAXML-HPC2 v.8.2.12, built using the 310-tip dataset as the input tree for TREEPL analysis (Supporting Information, Fig. S1).

#### DIVERSIFICATION

We used BAMM v.2.5.0 (Bayesian analysis of macroevolutionary mixtures; Rabosky, 2014) to

**Table 2.** Fossil calibrations and root calibration used in the TREEPL divergence dating analysis

Calibration	Node calibrations	Minimum age (Mya)	Maximum age (Mya)
1	Oldest divergence within crown Alethinophidia	93.9	95.0
2	Oldest divergence between non-xenodermid colubroids and their closest living relative (Xenodermidae)	50.5	72.0
3	Divergence between Boinae and the sister taxon (Erycinae + Candoiinae)	58.0	64.5
4	Divergence between <i>Corallus</i> and ( <i>Chilabothrus</i> + ( <i>Epicrates</i> + <i>Eunectes</i> ))	50.0	64.0
5	Divergence between Viperinae and Crotalinae	20.0	23.5
6	Divergence between <i>Acrochordus javanicus</i> and ( <i>A. ararfurae</i> + <i>A. granulatus</i> )	18.1	23.5
7	Oldest divergence between <i>Naja (Afronaja)</i> and <i>Naja (Boulengerina)</i>	17.0	20.5
8	Root age of Serpentes	123	128

**Table 3.** Partitions and models of sequence evolution used in the maximum likelihood and Bayesian inference analyses for the 310-tip dataset

Partition	Sites	Bayesian inference	Maximum likelihood
1	<i>nd4</i> <sup>1st</sup> , <i>cytb</i> <sup>1st</sup>	GTR+I+G	GTR+G
2	<i>cytb</i> <sup>2nd</sup> , <i>nd4</i> <sup>2nd</sup>	GTR+I+G	GTR+G
3	<i>nd4</i> <sup>3rd</sup> , <i>cytb</i> <sup>3rd</sup>	GTR+G	GTR+G
4	16S	GTR+I+G	GTR+G
5	<i>cmos</i> <sup>2nd</sup> , <i>cmos</i> <sup>1st</sup> , <i>nt3</i> <sup>2nd</sup> , <i>rag1</i> <sup>1st</sup> , <i>rag1</i> <sup>2nd</sup>	HKY+I+G	GTR+G
6	<i>cmos</i> <sup>3rd</sup> , <i>rag1</i> <sup>3rd</sup>	GTR+G	GTR+G
7	<i>nt3</i> <sup>1st</sup>	SYM+G	GTR+G
8	<i>nt3</i> <sup>3rd</sup>	SYM+G	GTR+G
9	<i>bdnf</i> <sup>1st</sup> , <i>bdnf</i> <sup>2nd</sup>	HKY+I+G	GTR+G
10	<i>bdnf</i> <sup>3rd</sup>	K80+G	GTR+G

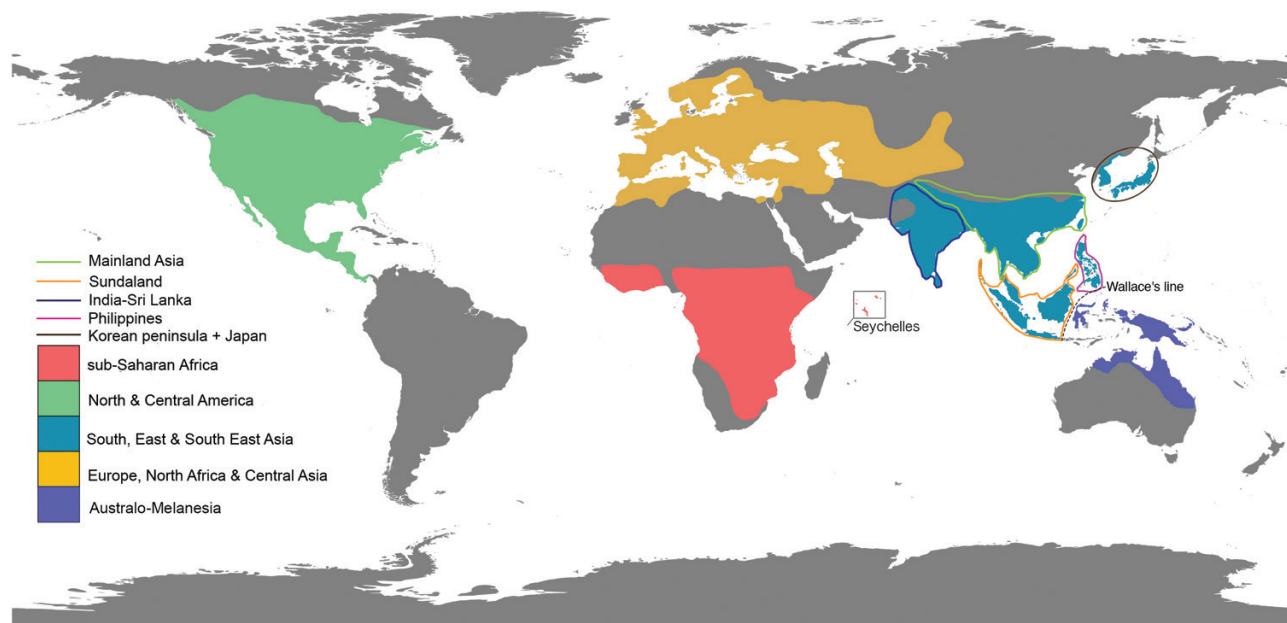
1st, 2nd and 3rd refer to codon positions

investigate shifts in diversification rates across our phylogeny. We ran four parallel MCMC chains for 1 000 000 iterations, discarding the first 10% as burn-in and sampling every 10 000 iterations, resulting in a posterior distribution of 900 values. We set the prior distribution parameters using the function *setBAMMpriors* from the BAMMTOOLS package (Rabosky *et al.*, 2014) in R v.4.0 (R Core Development Team, 2020; reproducible R code is available from Deepak *et al.*, 2021a). Convergence checks were performed by visual analysis of trace plots and by using the *effectiveSize* function in the *coda* package (Plummer *et al.*, 2006), with all parameters showing an ESS > 200. We also used BAMMTOOLS to extract credible shift sets and best shift configuration and to create speciation-rate-through-time plots to visualize the variation in speciation rate for lineages from four continental-scale regions: (1) Americas; (2) sub-Saharan Africa (including Seychelles); (3) Europe, North Africa and Central Asia; and (4) South, East and Southeast Asia and Australo-Melanesia.

#### BIOGEOGRAPHY

The historical biogeography of natricines has been assessed previously based on less-densely sampled phylogenies (Guo *et al.*, 2012; Deepak *et al.*, 2021b). We retained representative taxa from each biogeographical region and removed closely related taxa in particular biogeographical regions, maintaining the same genus-level sampling. We investigated historical biogeography

by estimating ancestral areas for the dated phylogeny using dispersal–extinction–cladogenesis (DEC; Ree *et al.*, 2005, 2008) and dispersal–vicariance analysis (DIVALIKE; Ronquist, 1997; Ronquist & Sanmartín, 2011) models implemented in BIOGEOBEARS v.1.1 (Matzke, 2013) in R v.4.0 (R Core Team, 2020; reproducible R code is available from Deepak *et al.*, 2021a). A subset of the phylogeny with 120 tips was selected for this analysis to reduce computational time (Supporting Information, Table S1). We also implemented these models using the ‘jump dispersal’ parameter, which, although criticized by Ree & Sanmartín (2018), is often considered the most biologically realistic scenario for colonization and subsequent divergence on remote islands (Tsang *et al.*, 2020). We compared the fit of these four models (DEC, DEC+*j*, DIVALIKE and DIVALIKE+*j*) using Akaike information criterion (AIC) scores and weights. We defined eight broad areas based on geological history, size and, to some extent, numbers of extant natricine species: (1) mainland Asia and Japan; (2) Sri Lanka and India excluding north-east India and the Himalayas; (3) Sundaland and Wallacea; (4) Australo-Melanesia; (5) the Philippines; (6) sub-Saharan Africa including Seychelles; (7) North and Central America; and (8) Europe, Central Asia and North Africa (Fig. 1). We set the maximum number of ancestral areas to three, because this was the maximum number of areas occupied by any single species [*Xenochrophis piscator* (Schneider, 1799), in our analysis]. A second analysis was carried out using ten biogeographical regions by



**Figure 1.** Global distribution of natricine snakes. Broad areas of distribution in different regions of the world are coloured and labelled. Important biogeographical regions used in the analysis are highlighted with borders of different colours.

disaggregating India plus Sri Lanka and mainland Asia plus Japan into four separate entities.

#### NATURAL HISTORY TRAITS

We extracted information on diet, reproductive mode and habits of natricine snakes from the published literature, including articles in journals, magazines and books (particularly field guides) and International Union for Conservation of Nature Red List species accounts. We also compiled natural history information from personal observations. In total, we mined 197 literature and personal data sources for information (Deepak *et al.*, 2020; Supporting Information, Table S4). We used these data to formulate and score three characters: (1) reproductive mode, scored as either viviparous or oviparous; (2) habit, scored as terrestrial burrower (species known to burrow and/or often found under leaf litter or fallen logs or rocks), aquatic burrowers (species known to burrow in stream beds or under rocks in water bodies), aquatic (species that are predominantly aquatic and rarely seen outside water; note that some of these species might also be aquatic burrowers, but no evidence for that was found in the sources examined), semi-aquatic (species typically found in both aquatic and terrestrial habitats), terrestrial non-burrower (species never or rarely entering water bodies); and (3) diet, divided into generalist (species that prey on both aquatic and terrestrial vertebrate and invertebrate prey), aquatic generalist (species whose diet includes a broad range of aquatic vertebrates or invertebrates), molluscivore (species specializing in feeding on gastropods), vermivore (species reported to eat only 'worms', generally assumed to be oligochaetes), crayfish specialist (species reported to feed predominantly on crayfish), lizard specialist (species reported to feed predominantly on lizards), anuran specialist (species reported to feed only on frogs and toads) and piscivore (species reported to feed only or predominantly on fish).

We saved the resulting tree files from ML, BI and TREEPL analyses and imported them into R (R Core Development Team, 2020) to visualize natural history traits and distribution, as defined in the biogeography section above, on the phylogeny using the packages APE (Paradis & Schliep, 2018), treeio (Yu, 2019a), GGTREE (Yu *et al.*, 2017) and tidytree (Yu, 2019b). We also used the ace function of APE (Paradis & Schliep, 2018) to estimate ancestral states for each natural history trait using maximum likelihood, and we visualized these on the phylogeny.

To test for associations between habit and reproductive mode and between habit and dietary specialism, we used models of discrete character evolution in the R package APE (Paradis & Schliep, 2018). We were primarily interested in whether

reproductive mode was associated with an aquatic habit, given that the most aquatic extant snakes, sea snakes and Acrochordidae, are viviparous (Tinkle & Gibbons, 1977; Sanders *et al.*, 2013); therefore, we collapsed our habit categories down to aquatic (aquatic plus aquatic burrower plus semi-aquatic) and non-aquatic (terrestrial burrower plus terrestrial non-burrower). We repeated the analysis including semi-aquatic species in the non-aquatic group.

We also wanted to determine whether habit and habitat determine the extent to which natricines are dietary specialists, given that most burrowing natricines are vermivores and aquatic natricines are piscivores (Deepak *et al.*, 2020). For the test between habit and dietary specialism, we collapsed our diet categories into generalists (generalists plus aquatic generalists) and specialists (the six other diet categories). Next, we combined the traits of interest [habit plus reproductive mode, habit plus diet, habit(alternative) plus diet] to generate a new four-state trait for each test, in which each state represented one of the possible pairwise combinations between the states of the original traits. For example, for habit plus reproductive mode, the trait state was aquatic–oviparous, aquatic–viviparous, non-aquatic–oviparous or non-aquatic–viviparous. We then used these new traits to estimate the transition rates between states by fitting a *Mk* model (where the 'M' stands for 'Markov' and 'k' refers to the number of states observed), with which we tested three different scenarios: (1) equal rates of transition between all states; (2) symmetrical transition rates between pairs of states; and (3) different transition rates for each pair of states. We then compared the different models using corrected Akaike information criterion (AICc) scores, with a  $\Delta$ AICc difference of two units as the threshold for selecting the best model. If the best-fitting model is one where transition rates among all states are equal, or one with symmetrical rates between pairs of states, this suggests that there is no association between the two traits. If the best model is one where transition rates among states differ, this might suggest an association between the traits, although the interpretation of the results will depend on which transition rates are highest. For example, we expect an association between aquatic habits and viviparity; hence, transition rates towards this state should be higher than those away from this state. Reproducible R code for these analyses is available from Deepak *et al.* (2021a).

## RESULTS

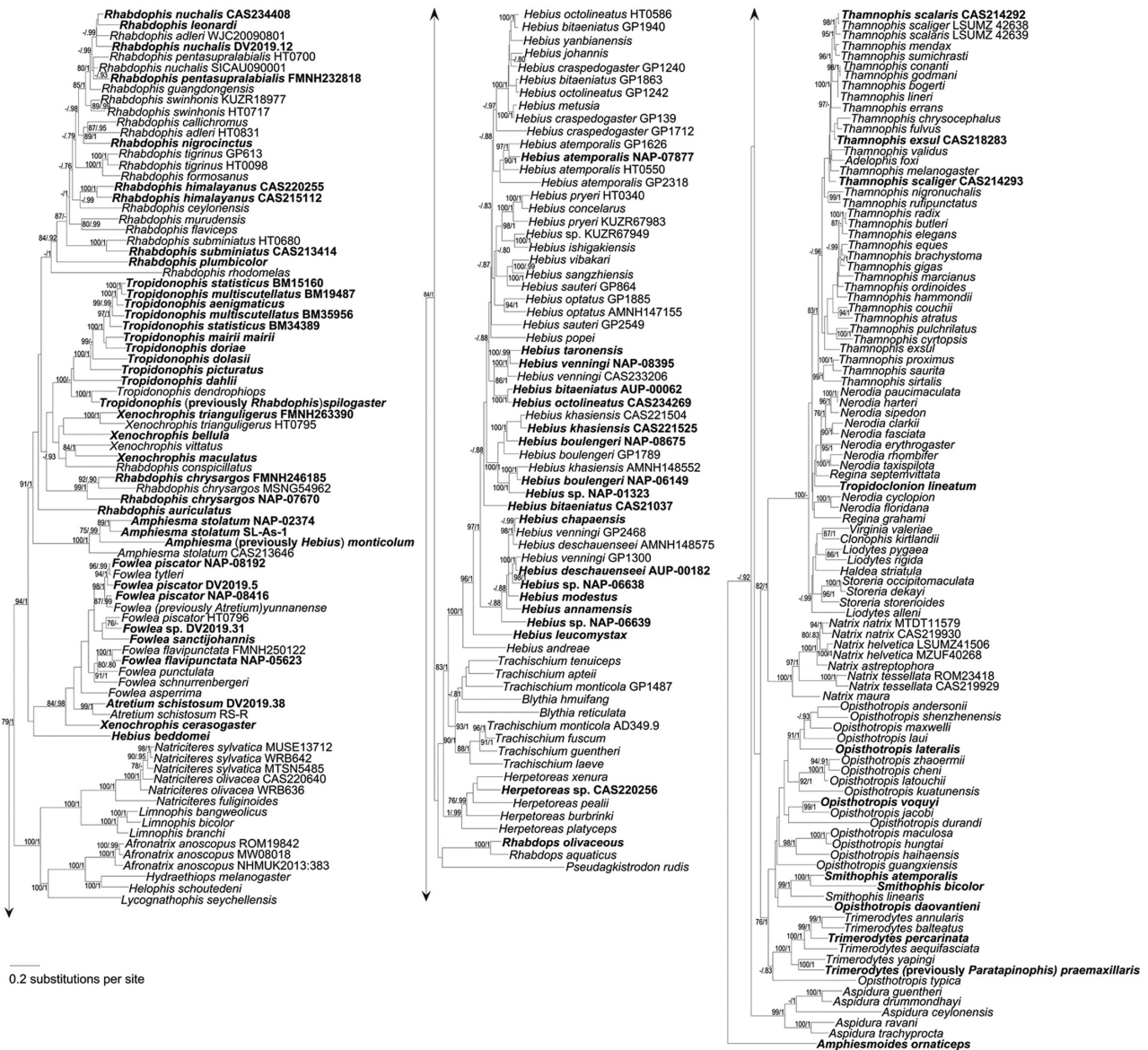
### PHYLOGENY

The ML analysis recovered a moderately well-supported phylogeny, although many of the internal



branches in the deepest parts of the tree were poorly supported (Fig. 2). Of the 248 internal branches, 103 had BS support < 75, and 135 had BS support between 76 and 90. Bayesian analysis recovered a similar tree topology except for relationships that were less well supported in the ML tree (Fig. 2), with 169 of the 248 internal branches having posterior probability values > 0.75. *Amphiesmoides ornaticeps* (Werner, 1924), which we sequenced here for the first time, was recovered as the sister to all other extant natricines (less well supported by ML than by BI).

*Tropidonophis* Jan, 1863 + *Rhabdophis spilogaster* (Boie, 1827) formed a well-supported clade in the BI tree (Fig. 2). The recently resurrected genus *Fowlea* Theobald, 1868 (Purkayastha *et al.*, 2018) was non-monophyletic, with *Atretium yunnanensis* Anderson, 1879 nested inside it (for nomenclatural changes, see the Discussion subsection, *Nomenclatural changes*), with *Atretium schistosum* (Daudin, 1803) and *Xenochrophis cerasogaster* (Cantor, 1839) forming a clade that was the sister group of *Fowlea* + *Atretium yunnanensis*. The Asian genus



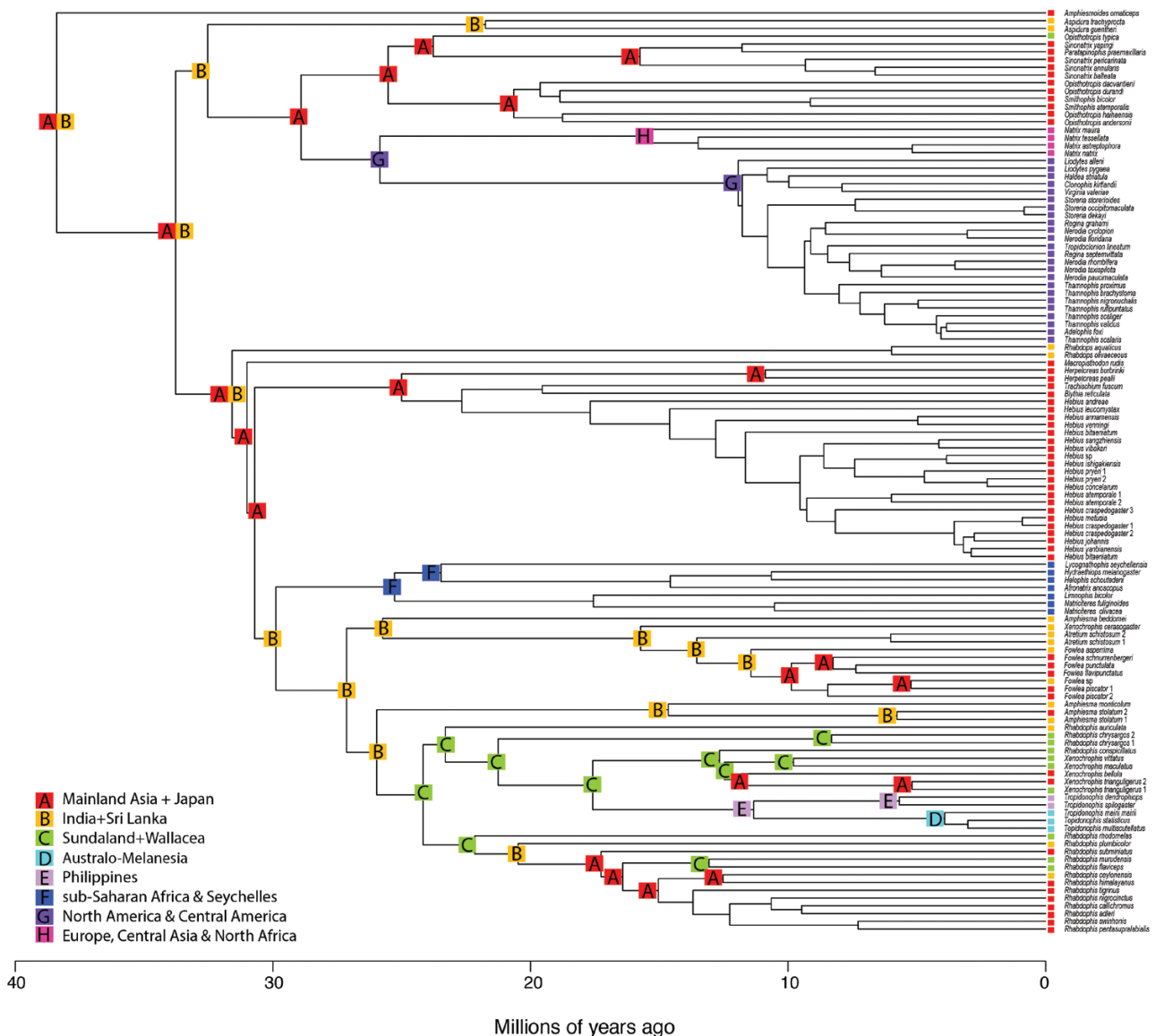
**Figure 2.** Maximum likelihood (ML) tree showing inferred relationships among natricine snakes. Maximum likelihood bootstrap support (BS) and Bayesian posterior probability (PP) values are shown at each internal branch. Posterior probability values < 0.75 and BS values < 75 are not shown. Tips for which sequence data were newly generated in the present study are shown in bold.

*Hebius* formed a well-supported clade in both the ML and BI phylogenies (Fig. 2). *Trimerodytes* Cope, 1895, *Opisthotropis*, *Smithophis* Giri et al., 2019 and *Paratapinophis* Angel, 1929 formed a well-supported clade that was sister to the *Natrix* Laurenti, 1768 clade plus the North American natricine clade (Fig. 2). The sub-Saharan natricines were maximally supported as monophyletic (Fig. 2), and their sister group was a large clade comprising South, East and Southeast Asian and Australo-Melanesia taxa, which included the following monophyletic genera: *Rhabdophis* Fitzinger, 1843, *Tropidonophis*, *Xenochrophis* Günther, 1864, *Amphiesma* A.M.C.Duméril, Bibron & A.H.A.Duméril, 1854, *Fowlea* and *Atretium* Cope,

1861. This sister-group relationship was recovered in both ML and BI analyses with moderate to high support (Fig. 2).

#### DIVERSIFICATION

The estimated age of the crown node of Natricinae was ~39 Myr, which is similar to the estimated range in recently published studies (Zaher et al., 2019; Burbrink et al., 2020; Deepak et al., 2021b). All the oldest natricine lineages were found in Asia (Fig. 3). Extant sub-Saharan African natricines were estimated to have diverged from their closest (Asian) extant relatives ~30 Mya, and the Seychellean *Lycognathophis* was estimated to



**Figure 3.** Ancestral area estimations (DEC+j model) of natricines. Coloured boxes at tips indicate the current distribution of extant species. For the complete estimated dates at each node, see the Supporting Information (Fig. S1).

have diverged from its closest extant (African) relatives ~24 Mya, both slightly more recently than the lower end of the corresponding estimates (56.5–34.1 and 43.2–24.6 Mya, respectively) made by Deepak *et al.* (2021b). Extant European and North African natricines were estimated to have diverged ~26 Mya, with diversification among extant American lineages inferred to have occurred only from ~12 Mya onward (Fig. 3). The genus *Tropidonophis*, found in western Melanesia, the Philippines and northern Australia, was nested in one of the Asian lineages, and its split from Asian relatives occurred ~17 Mya. Within *Tropidonophis*, the split between the species in the Philippines and those east of Wallace's Line was inferred to have occurred ~11.5 Mya.

Results from BAMM indicated two major shifts in diversification rates, one within the North and Central American group and the other within one of the major Asian clades (Fig. 4). It is worth noting that, although Figure 4 shows only the best-shift configuration (with a posterior probability of 0.108), all nine shift configurations with highest posterior probabilities (which sum to a cumulative posterior probability of 0.553) represent qualitatively similar scenarios (Supporting Information, Fig. S2). Rate-through-time plots for the four major regions show clear shifts in speciation rate in the Americas and in the South, East and Southeast Asia and Australo-Melanesia clades (Fig. 5A, D) that correspond to the rate shifts identified by BAMM. The rate-through-time plots also show a general trend of decline of speciation rates toward the tips for the sub-Saharan Africa (including Seychelles) and the European, North African and Central Asian regions (Fig. 5B, C).

#### BIOGEOGRAPHY

Among the four models implemented in BIOGEOBEARS, DEC+*j* was the best fitting, closely followed by DIVALIKE+*j* (Table 4). These results were recovered for both the eight- and ten-area analyses, and with broadly similar geographical scenarios; more detailed results and discussion are provided only for the best-fitting model for the latter. The oldest extant lineages of natricines are found today in mainland Asia and India plus Sri Lanka (*Amphiesmoides* and *Aspidura* Wagler, 1830), and the ancestral-area estimation suggested that ancestral natricines were likely to be from one of these two areas. A single dispersal event was inferred from India plus Sri Lanka to sub-Saharan Africa (and from mainland sub-Saharan Africa to the Seychelles). Extant natricines from the Philippines were inferred to have originated from a dispersal from Sundaland plus Wallacea, and extant natricines from Australo-Melanesia were inferred to have originated from a primarily Philippine clade. The Sundaland plus Wallacean, Philippine and Australo-Melanesian natricines were all inferred as originating

from an initial dispersal from India plus Sri Lanka. Extant natricines from North and Central America, and from Europe, Central Asia and North Africa, were reciprocally monophyletic, and the best-fitting DEC+*j* model suggested that the dispersal route was from mainland Asia plus Japan to North and Central America to Europe, Central Asia and North Africa.

#### NATURAL HISTORY TRAITS

Natricine snakes are predominantly semi-aquatic (31%) and terrestrial (28%) in habit; fewer species are aquatic (22%), aquatic burrowers (10%) or terrestrial burrowers (8%) (Fig. 6). We did not find information on habit for the remaining 1% of natricine species. The ancestral habitat of all natricines was estimated to be predominantly terrestrial (~50%), followed by semi-aquatic/aquatic (Fig. 6; Supporting Information, Fig. S3). Many natricines are dietary generalists (37%) or aquatic generalists (25%). Some are reported to feed only on anurans (18%) and others only on worms (12%). Most vermivores are also burrowers or aquatic burrowers (Fig. 6). There are a few species (8%) that are dietary specialists feeding on fish, molluscs or crayfish (Fig. 6; Supporting Information, Table S4). The ancestral state estimation for diet of all natricines shows a higher proportion of anuran or aquatic generalists in comparison to the other six diet categories (Supporting Information, Fig. S3). All North American natricines are viviparous. The only non-American viviparous natricines (the Asian *Pseudagkistrodon rudis* and *Trimerodytes annularis*) represent independent origins of viviparity from oviparous ancestors (Fig. 5; Supporting Information, Fig. S3).

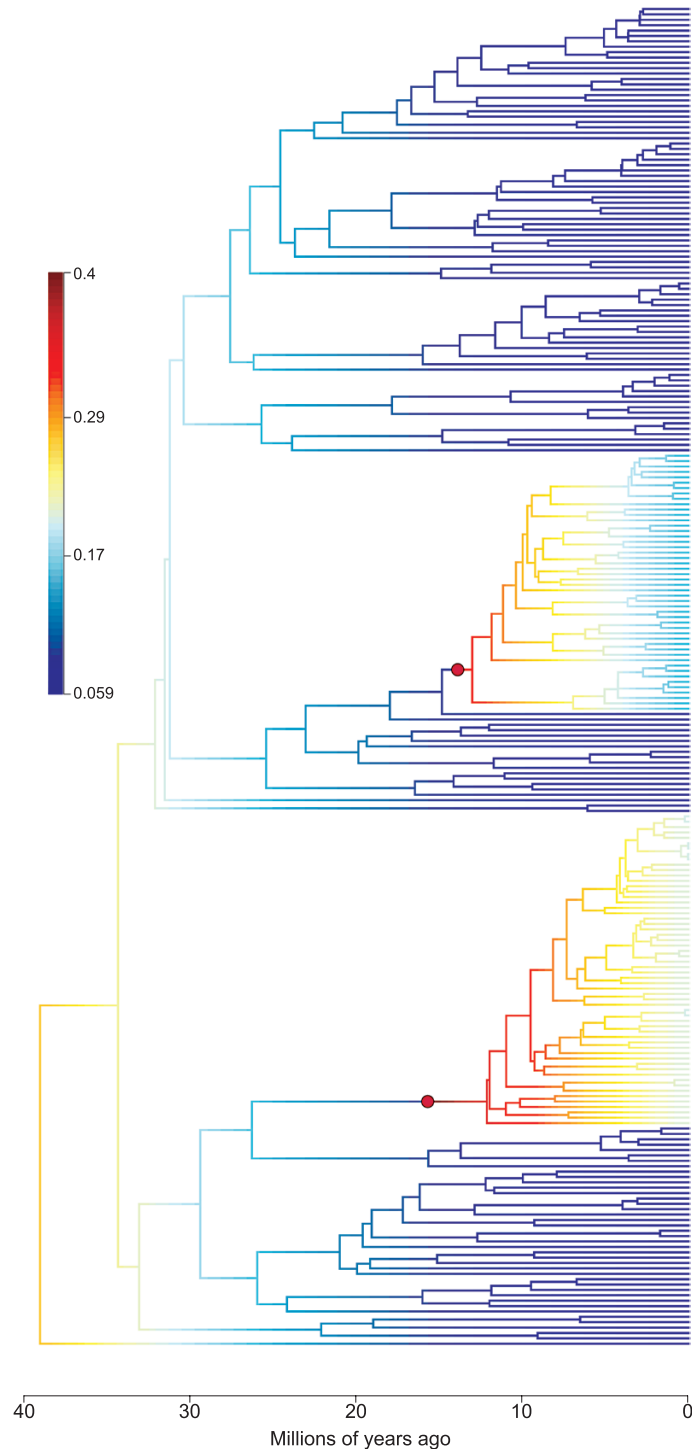
When testing for associations between habit and reproductive mode, the best-fitting model was one where transition rates among states were symmetric (Table 5). This suggests that there was no association between habit and reproductive mode, because the rates towards aquatic viviparity and terrestrial oviparity were equal to those away from these states (Supporting Information, Table S5). For habit and dietary specialism, our results showed weak support for both the equal-rates and the all-rates-different models (Table 5). However, when using the alternative classification of habits, we found that the symmetrical model had stronger support than the other two (Table 5). These results combined suggest there is limited association between habit and dietary specialization (Supporting Information, Table S6).

#### DISCUSSION

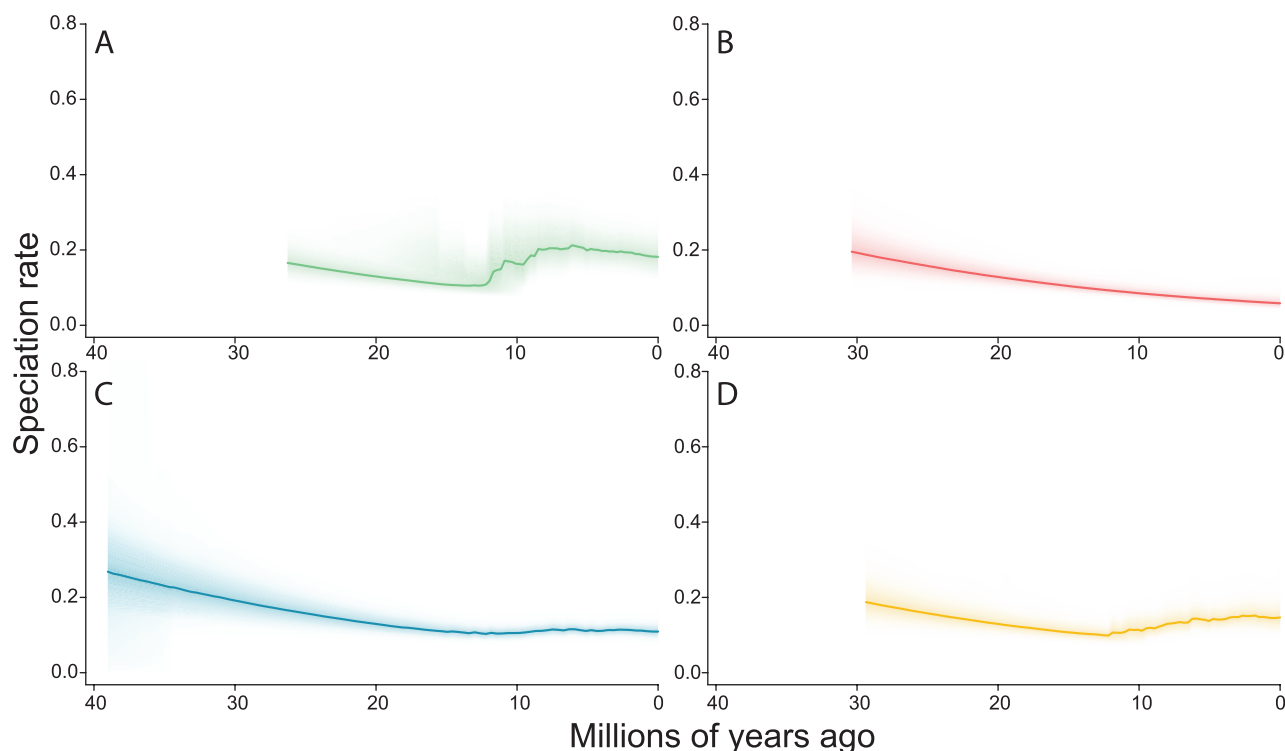
##### PHYLOGENY, DIVERSIFICATION AND BIOGEOGRAPHY

We have inferred the most taxonomically complete phylogeny for natricines to date, having sampled 75%





**Figure 4.** Best shift configuration sampled by Bayesian analysis of macroevolutionary mixtures (BAMM) for the naticrine phylogeny. Colours along the branches represent instantaneous rates of speciation, and the red circles represent shifts in the diversification regimes. These shifts are on branches leading to the North and Central American group (upper red circle) and one of the major Asian clades (lower red circle). For complete phylogeny, see [Figure 2](#). For complete geographical distribution information, see [Figure 6](#).



**Figure 5.** Mean speciation rates through time from Bayesian analysis of macroevolutionary mixtures (BAMM). A, Americas. B, sub-Saharan Africa (including Seychelles). C, South, East and Southeast Asia and Australo-Melanesia. D, Europe, North Africa and Central Asia.

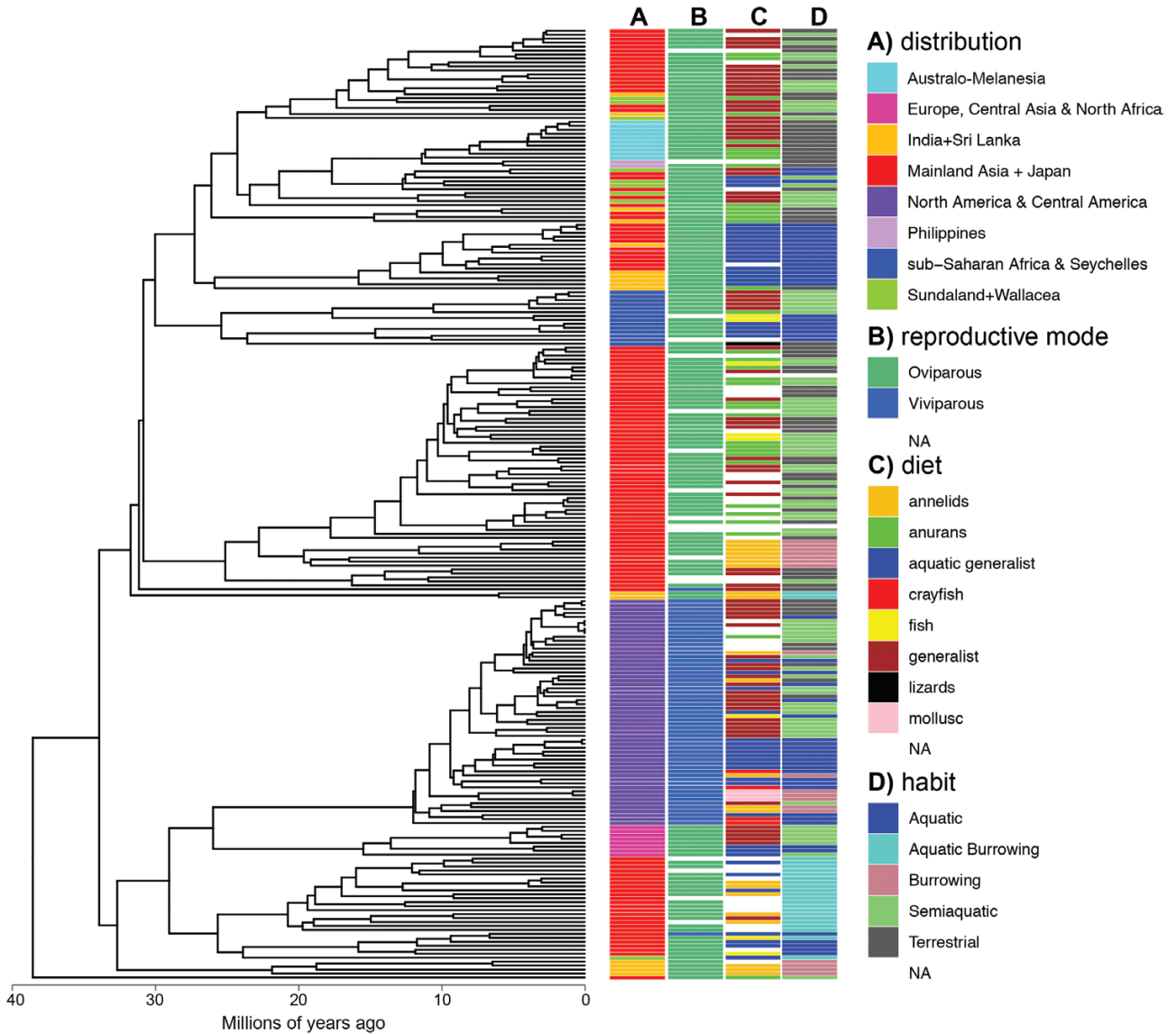
**Table 4.** Comparison of four models of ancestral area estimations implemented using BIOGEOBEARS. Models A and B were run defining eight and ten areas, respectively. Akaike information criterion scores and weights are reported. Abbreviations: AIC, Akaike information criterion;  $d$ , dispersal;  $e$ , extinction;  $j$ , jump parameter;  $K$ , number of parameters; LnL, log-likelihood

Model	LnL	$K$	$d$	$e$	$j$	AIC score	AIC weight
Model A							
DEC	-131.8	2	0.001	0.000	0.000	267.8	0.000
DEC+ $j$	-107.4	3	0.000	0.000	0.016	220.9	0.735
DIVALIKE	-148.2	2	0.002	0.000	0.000	300.4	0.000
DIVALIKE+ $j$	-108.7	3	0.000	0.000	0.017	223.6	0.194
Model B							
DEC	-259.4	2	0.010	0.010	0.000	523.0	0.000
DEC+ $j$	-144.2	3	0.000	0.000	0.017	294.6	0.691
DIVALIKE	-189.0	2	0.002	0.003	0.000	382.1	0.000
DIVALIKE+ $j$	-145.0	3	0.000	0.000	0.018	296.3	0.309

of the named species in the clade. The new phylogeny broadly agrees with previously published phylogenies in that: (1) Asian natricines are paraphyletic and include some of the oldest lineages; (2) all American natricines are monophyletic and sister to the European plus Central Asian plus North African lineage, the genus *Natrix* (McVay *et al.*, 2015); and (3) the sub-Saharan species are monophyletic (Guo *et al.*, 2012;

Pyron *et al.*, 2013; Figueroa *et al.*, 2016; Zaher *et al.*, 2019; Deepak *et al.*, 2021b).

The evolutionary lag that we detected in initial cladogenesis of extant American natricine lineages was also seen in previous natricine phylogenies (Guo *et al.*, 2012; McVay *et al.*, 2015). The speciation-rates-through-time plot for the American natricine lineages (Fig. 4) indicates relatively rapid lineage diversification



**Figure 6.** Geographical distribution and the three natural history traits plotted onto the dated natricine phylogeny (see Fig. 2). Abbreviation: NA, no information available (open bars).

after a prolonged initial lag in cladogenesis or from extinctions of most of the oldest American lineages (Fig. 4). The dated phylogeny from this study and others (e.g. Guo *et al.*, 2012) indicates that although the American natricines dispersed there ~26 Mya, increased cladogenesis among the extant lineages began only after 10 Mya in this new region.

The shifts in speciation rates found in both the North American and Asian clades are possibly the result of ecological opportunity. In the case of the North American clade, this might also be related causally to the transition to viviparity, which has been suggested to spur diversification in fish (Mank & Avise, 2006; Helmstetter *et al.*, 2016) and invertebrate

(platyhelminths; Boeger *et al.*, 2003) groups. However, this association is difficult to test for natricines, because it represents a single evolutionary event (Maddison & Fitzjohn, 2015; Uyeda *et al.*, 2018), and all North American natricines are viviparous. In contrast, there is no clear pattern in any of the compiled trait information that might explain the increased speciation rate within the Asian clade. For example, the *Hebius* clade comprises small-bodied semi-aquatic or terrestrial natricines, but the same is true of closely related Asian *Herpetoreas* Günther, 1860 and distantly related Asian *Amphiesma*, both of which are not characterized by exceptional speciation rates. This lack of clear potential explanation for shifts

**Table 5.** Model comparisons for the association between habit and reproductive mode and between habit and dietary specialism using the standard and alternative classifications for habit. The best-fitting model is shown in bold. Abbreviations: AICc, corrected Akaike information criterion; ARD, all-rates-different model; ER, equal rates model; SYM, symmetric rates model

Traits	Model	AICc	ΔAICc
Habit + reproductive mode	ER	371.9	79.62
	SYM	<b>292.3</b>	<b>0</b>
	ARD	298.2	5.893
Habit <sub>alternative</sub> + reproductive mode	ER	211.4	39.7
	SYM	171.7	0
	ARD	175.0	3.3
Habit + dietary specialism	ER	402.2	0
	SYM	407.8	407.8
	ARD	403.1	0.988
Habit <sub>alternative</sub> + dietary specialism	ER	388.4	25.32
	SYM	<b>363.1</b>	<b>0</b>
	ARD	0	2.379

in speciation rates based only on the natural history traits and distribution of extant taxa highlights the need for further studies using other information, such as (palaeo)environmental or other phenotypic variables, to help us gain a better understanding of the factors that promoted these radiations.

Similar to previous molecular studies, we found the American genera *Regina* and *Thamnophis* to be non-monophyletic (Alfaro & Arnold, 2001; de Queiroz *et al.*, 2002; Pyron *et al.*, 2013; Figueroa *et al.*, 2016; Zheng & Wiens, 2016). Additionally, our analyses support non-monophyly of the genus *Liodytes* (Fig. 1). The genus *Opisthotropis* is non-monophyletic; in particular, *Opisthotropis typica* (Mocquard, 1890) from Sundaland is more closely related to *Trimerodytes* from mainland Asia. This highlights further that the addition of missing lineages from Sundaland might reveal new phylogenetic relationships, which, in turn, could influence the inferred biogeographical scenarios for Asian natricines.

The addition of previously unsampled lineages of natricines from the Philippines and New Guinea in the present study allowed us to include additional areas in the biogeographical analysis. The Philippine and New Guinean taxa are inferred to have arisen from within a primarily Sundaland lineage that dispersed initially into the Philippines and then to Melanesia. However, the phylogeny is still incomplete, and parts of it are not compellingly resolved (especially the deeper parts of the tree); hence, inferences might change with addition of other, as yet unsampled taxa.

There is clear evidence for exchange of natricines between India + Sri Lanka and mainland Asia during the early-mid Eocene (Fig. 3) as also inferred for other vertebrate and invertebrate taxa (Klaus *et al.*, 2010, 2016; Grismer *et al.*, 2016). The biogeographic inferences for the main lineages within the clades from sub-Saharan Africa + Seychelles, Europe, North Africa, Central Asia and the Americas are expected to remain unchanged given that each of them is a well-supported clade.

The East and Southeast Asian *Trimerodytes* is monophyletic, as inferred also by Guo *et al.* (2020), but we find strong support for *Trimerodytes yapingi* Guo, Zhu & Liu, 2019 being sister to *Trimerodytes praemaxillaris* Angel, 1929 (not sampled by Guo *et al.*, 2020) instead of being a lone sister to all other *Trimerodytes*. Given that *Trimerodytes praemaxillaris* occurs south-east of the Hengduan Mountains, our phylogeny erodes support for Guo *et al.*'s (2020) hypothesis that *Trimerodytes* originated in the Hengduan Mountains.

Deepak *et al.* (2021b) were unable to resolve relationships among the Seychellean endemic *Lycognathophis seychellensis* (Schlegel, 1837) and the mainland sub-Saharan natricines. In the present study, much stronger (maximal) support is found for the sister relationship between *Lycognathophis seychellensis* and the clade comprising *Afronatrix*, *Helophis* and *Hydraethiops* Günther, 1872, which was also the best-supported resolution found by Deepak *et al.* (2021b). The estimated age of the split between *Lycognathophis seychellensis* and its African sister (24 Mya; Fig. 1) is consistent with *Lycognathophis seychellensis* reaching the Seychelles via overseas dispersal, inasmuch as those islands last had a connection with Africa > 100 Mya and with other landmasses > 60 Mya (for further discussion, see Deepak *et al.*, 2021b).

Many Sri Lankan taxa have their closest extant relatives in peninsular India (e.g. Bossuyt *et al.*, 2004). However, we found the Sri Lankan endemic *Rhabdophis ceylonensis* (Günther, 1858) to be sister to the Indo-Burmese *Rhabdophis himalayanus* (Günther, 1864), rather than to the peninsular Indian *Rhabdophis plumbicolor* (Cantor, 1839) (see also Das *et al.*, 2021). Such a pattern of relationships is rare, although it has been reported for the gecko *Cnemaspis* Strauch, 1887 (Agarwal *et al.*, 2021).

Despite the inclusion of many new lineages and loci, several internal branches remain poorly supported under ML and/or BI analysis. *Pseudagkistrodon rudis* has long been difficult to classify (e.g. Maki, 1931; Bourret, 1936; Kelly *et al.*, 2003) and was identified previously as an unstable or rogue taxon in molecular phylogenies (Giri *et al.*, 2017), and we are not able to identify its closest relative(s) here with confidence. The closest relative of the Sri Lankan burrowing *Aspidura*

is still unclear, as in previously published phylogenies (Pyron *et al.*, 2013; Figueroa *et al.*, 2016; Zaher *et al.*, 2019). *Rhabdophis rhodomelas* (Boie, 1827), which was earlier identified as a rogue taxon (Figueroa *et al.*, 2016), is recovered here with moderate to good support as sister to the lineage of *Rhabdophis* species that have nuchal glands (Takeuchi *et al.*, 2018). The addition of more lineages and/or markers might provide better resolution to some of these relationships in future. Many taxa included in our analyses lacked data for some of the markers we used, and improving data completeness for taxa might also help. *Anoplohydrus* Werner, 1909, *Isanophis* and *Hydrablades* are the only three currently recognized natricine genera not sampled in molecular phylogenetic studies, and these should be a priority for additional studies.

#### NATURAL HISTORY TRAITS

With respect to diet and major habit, the limited amount and typically opportunistic nature of ecological data generally available for natricines limited us to formulating qualitative, somewhat subjective categories. Additionally, some species were scored for these categories based on small or very small sample sizes [e.g.  $N = 1$  for *Tropidonophis dolasii* Kraus & Allison, 2004 and *Hebius johannis* (Boulenger, 1908)], such that some records of dietary specialism require verification. In these aspects, our study is clearly preliminary. Dietary information on natricine snakes is patchy, generally with much better documentation for the American species, particularly for dietary specialists (Fig. 4; Supporting Information, Table S4). However, we found no clear evolutionary association between diet and habit, despite more specialized diets being observed more frequently in species that are aquatic and/or burrowing. Future research should test hypotheses that might explain this association, such as that it reflects varying dietary choices in water, soils and on land, or that specialized diets evolve in concert with shifts to derived habits in Colubridae (in the sense used by, e.g. Pyron *et al.*, 2013; Figueroa *et al.*, 2016; = Colubrinae of e.g. Zaher *et al.*, 2009; Burbrink *et al.*, 2020).

We were able to score almost all the sampled natricines for our habit categories. Although most natricine species are semi-aquatic or terrestrial, some are aquatic and/or burrowing. There are multiple field observations of aquatic burrowing habits for some Asian natricines [e.g. *Opisthotropis andersonii* (Boulenger, 1888), *Trimerodytes balteatus* and *Rhabdops aquaticus* Giri, Deepak, Captain & Gower, 2017; Deepak *et al.*, 2020]. Our new phylogeny indicates that this habit has evolved at least three times among Asian natricines. Burrowing and aquatic ecomorphs have evolved more recently in the North and Central American lineage

than among counterparts in Asia, Europe and Africa. Africa lacks extant burrowing natricines, having only (semi)aquatic species. Most terrestrial burrowing natricines are reported to consume earthworms, except *Storeria* spp., which are molluscivores.

The transition from oviparity to viviparity (or ovoviviparity) has occurred many times in reptiles, with these transitions occurring more times in squamates than in all other vertebrate lineages combined (Blackburn, 1982, 1985; Shine, 1985). Within the ancestrally oviparous Natricinae, viviparity has originated independently at least three times, in two monotypic Asian lineages (*Trimerodytes annularis* and *Pseudagkistrodon rudis*) and in the ancestor of the entire American radiation. Although previous studies differ slightly in their inferred relationships compared with our phylogeny, these two Asian lineages have never been recovered as sister species or closely related to the American radiation (Guo *et al.*, 2012; Figueroa *et al.*, 2016; Zaher *et al.*, 2019; Das *et al.*, 2020b; Lalronunga *et al.*, 2020). Viviparity is argued to be advantageous in cold climates (e.g. Blackburn, 1982, 1985; Shine, 1985; Qualla *et al.*, 1995; Pyron & Burbrink, 2014). Although all American natricines are viviparous, all other natricine species that live in colder climates in northern latitudes in Europe and Asia are oviparous. Generating and analysing precise, quantitative data on thermal regime and parity mode for all natricines was beyond the scope of our study. Many fully aquatic snakes (e.g. sea snakes, Acrochordidae) are viviparous, but we found no association between parity and habit for natricines, none of which is as obligately aquatic as any hydrophiine or acrochordid.

#### NOMENCLATURE CHANGES

The improved sampling in our phylogeny allows us to resolve some taxonomic uncertainties in Natricinae. We propose four nomenclatural changes based on well-supported relationships retrieved here, transferring: (1) *Rhabdophis spilogaster* to *Tropidonophis* as ***Tropidonophis spilogaster*** (Boie, 1827) **comb. nov.**; (2) *Hebius monticola* (Jerdon, 1854) to *Amphiesma* as ***Amphiesma monticola*** (Jerdon, 1854) **comb. nov.**; (3) *Paratapisophis praemaxillaris* to *Trimerodytes* as ***Trimerodytes praemaxillaris*** (Angel, 1929) **comb. nov.**; and (4) *Atrietium yunnanensis* to *Fowlea* as ***Fowlea yunnanensis*** (Anderson, 1879) **comb. nov.** We have also identified other taxa whose monophyly or generic classification is unclear and needs further study. Despite these changes, several non-monophyletic genera persist in Natricinae, notably *Regina* and *Thamnophis* (and, possibly, *Liodytes*). See Supporting Information, Appendix S1 for a more detailed



discussion of natricine taxonomy and justification for our proposed nomenclatural changes.

#### CONCLUSION

Natricine snakes are species-rich, geographically widespread and both ecologically and morphologically diverse, making them an interesting subject for evolutionary biology. We generated molecular phylogenetic data for 21 previously unsampled natricine species and inferred an updated phylogeny for this clade. This provides a framework for assessing natricine historical biogeography, evolutionary diversification and systematics, although support is unconvincing for several (mostly deeper) internal branches, and ecological data are generally coarse and patchy. Priority resources for future research are a more complete and better-resolved phylogeny and more refined ecological trait data.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Figure S1.** Dated phylogeny (time scale on x-axis in millions of years ago) of extant natricines using a maximum likelihood (ML) phylogeny time calibrated in TREEPL. Numbers at internal branches indicate node ages.

**Figure S2.** The nine shift configurations with the highest associated posterior probability for the natricine tree obtained from the Bayesian analysis of macroevolutionary mixtures (BAMM) analysis. The values at the top of each panel represent the posterior probability associated with each shift configuration. Colours along the branches represent instantaneous rates of speciation, whereas the grey circles represent shifts in the diversification regimes.

**Figure S3.** Ancestral-state estimations for the three natural history traits mapped onto the dated natricine phylogeny.

**Appendix S1.** Natricine systematics and proposed nomenclatural changes.

**Table S1.** GenBank accession numbers and voucher numbers for the 251-tip dataset. \*Chimeric sequences. ‘-’ = no data available.

**Table S2.** GenBank numbers for the additional 60 tips used in the 310-tip dataset that were used for estimating divergence dates. ‘-’ = no data available.

**Table S3.** Parameter values for fossil calibrations used in the BEAST divergence dating analysis. Ages are in millions of years ago. All maximum ages are soft, except for a hard maximum for calibration 6.

**Table S4.** Natural history information on 246 species (255 tips) of natricine snakes. For data sources, see [Deepak \*et al.\* \(2020\)](#).

**Table S5.** Model comparisons for association between habit and other variables.

**Table S6.** Transition rates between all pairwise combinations of habit and other variables.