JSE Journal of Systematics and Evolution

doi: 10.1111/jse.70003

Research Article

Evolutionary history of Middle American *Rhamdia* (Siluriformes: Heptapteridae) inferred from comparative mitogenomic data: Insights on historical biogeography and cave colonization in the group

Jairo Arroyave[1,2]*, Adán Fernando Mar-Silva[1], Bruno F. Melo[2], Sonia Gabriela Hernández-Ávila[1,3], Jesús M. López-Vila[4], Gabriel S. C. Silva[5], and Píndaro Díaz-Jáimes[6]

[1]Instituto de Biología, Universidad Nacional Autónoma de México, Cto. Zona Deportiva S/N, Ciudad Universitaria, Coyoacán, Ciudad de México 04510, Mexico

[2]Department of Ichthyology, American Museum of Natural History, New York, NY 10024, USA

[3]Instituto de Ciencias Biológicas, Universidad de Ciencias y Artes de Chiapas, Tuxtla Gutiérrez, Chiapas 29039, Mexico

[4]Colección de Peces, Departamento Conservación de la Biodiversidad, El Colegio de la Frontera Sur, Unidad San Cristóbal, Carretera Panamericana y Periférico Sur s/n, Barrio María Auxliadora, San Cristóbal de Las Casas, Chiapas 29290, Mexico

[5]Instituto de Biociências, Universidade Estadual Paulista Júlio de Mesquita Filho, Botucatu, São Paulo 18618-970, Brazil

[6]Instituto de Ciencias del Mar y Limnología, Universidad Nacional Autónoma de México, Circuito Exterior S/N, Ciudad Universitaria, Coyoacán, Ciudad de México 04510, Mexico

*Author for correspondence. E-mail: jarroyave@ib.unam.mx

Received 5 March 2025; Accepted 29 May 2025

Abstract Neotropical catfishes of the genus *Rhamdia* are divided into *cis*- and *trans*-Andean/Middle American reciprocally monophyletic components, the latter notable for its considerable cave-dwelling diversity. Despite previous research, uncertainties regarding the systematics and historical biogeography of the Middle American clade remain. To test previous phylogenetic hypotheses and improve our understanding of the evolutionary history of this group of Middle American freshwater fishes, we generated and analyzed comparative mitogenome-wide data from most valid species and known cave-dwelling forms. Our results corroborate this clade as divided into two reciprocally monophyletic groups (split dated at ~9 Ma): a clade representing the species *Rhamdia guatemalensis* (crown group dated at ~2.8 Ma) and a clade consisting of the remaining Middle American species (i.e., the *Rhamdia laticauda* species group; crown group dated at ~4 Ma). Our results also confirm the notion that *R. laticauda* is deeply paraphyletic and that phylogenetically scattered geographic lineages of this taxon could represent different species. Our divergence time estimates, coupled with present-day distribution patterns, support the biogeographic scenario in which northward dispersal and colonization of Central America and southern North America by *Rhamdia* was catalyzed by the emergence of the Panamanian Isthmus land bridge and stream captures across Lower Central America. Cave colonization in Middle American *Rhamdia* is widespread, convergent, relatively recent (dating from the Pleistocene), and most likely opportunistic, with established cave-dwelling populations possibly representing "evolutionary dead ends."

Key words: catfishes, heptapterids, hypogean, karst caves, mitochondrial genomes, troglomorphic.

1 Introduction

Catfishes (order Siluriformes) are a major component of the exceptionally diverse freshwater Neotropical ichthyofauna, and the three-barbeled catfishes (family Heptapteridae), with 240 valid species, rank as the third most species-rich family of Neotropical catfishes, only behind Loricariidae (1068 species) and Trichomycteridae (447 species) (Fricke et al., 2025). *Rhamdia* Bleeker 1858 is one of the 24 valid heptapterid genera (Silva et al., 2021) and arguably one of the most taxonomically complex within the family (Koerber & Reis, 2019). Distributed from southern Mexico to central Argentina, *Rhamdia* is effectively the most latitudinally widespread heptapterid genus. Despite its questionable monophyly (Bockmann, 1998; DoNascimiento et al., 2004; Garavello & Shibatta, 2016) and the considerable taxonomic upheaval that resulted from the only comprehensive

revisionary work (Silfvergrip, 1996) and subsequent studies that challenged several of the conclusions of this revision (Perdices et al., 2005; Hernández et al., 2015; Garavello & Shibatta, 2016), there is current consensus that *Rhamdia* consists of 27 valid species phylogenetically split into two reciprocally monophyletic groups: a *cis-* and a *trans*-Andean/Middle American clade (Perdices et al., 2002; Hernández et al., 2015; Arroyave & De La Cruz Fernández, 2021b; Buenavad-González et al., 2023; Fricke et al., 2024). The latter consists of 10 valid species and its distribution stretches from northwestern South America (NWSA) to southern Mexico. Although Middle America is traditionally defined as the whole of Central America and Mexico north of the Isthmus of Tehuantepec to the Rio Grande on the United States border (Chakrabarty & Albert, 2011; Winker, 2011), some biogeographic studies of Neotropical freshwater fishes consider NWSA as part of this region (Perdices et al., 2002; Elías et al., 2023). Therefore, the *trans*-Andean/Middle American clade of *Rhamdia*, which is the subject of this study, is hereafter simply referred to as the Middle American (MA) clade.

At the southern limit of its distribution, the MA clade includes species that inhabit *trans*-Andean basins on the Pacific versant of Colombia (e.g., Patía, San Juan) and Ecuador (e.g., Guayas) and on the Caribbean versant of Colombia (e.g., Magdalena, Atrato) and Venezuela (e.g., Catatumbo and Maracaibo). In Central America, the MA clade is distributed uninterruptedly from Panama to southern Mexico in basins of both Atlantic and Pacific slopes, with the Papaloapan and the Yucatán Peninsula aquifer basins in Mexico as the northern limits of its distribution (Silfvergrip, 1996; Perdices et al., 2002). Of the 10 species in the MA clade, two—*Rhamdia guatemalensis* and *R. laticauda*—are geographically widespread, with the former distributed virtually across the entire clade′s geographic range and the latter reported from southern Mexico to northern Panama (Silfvergrip, 1996; Arroyave, 2019). Conversely, four of the species in the MA clade—*R. reddelli*, *R. zongolicensis*, *R. macuspanensis*, and *R. laluchensis*—are hypogean (i.e., cave-dwelling) and microendemic of their respective caves in southern Mexico (Arroyave & De La Cruz Fernández, 2021b). Between these two extremes in extent of occurrence, there are four species with intermediate distribution ranges: *R. cinerascens* and *R. saijaensis* (Pacific versant of Ecuador and Colombia, respectively), *R. nicaraguensis* (Atlantic and Pacific versants of Costa Rica and Nicaragua), and *R. parryi* (Pacific slope of northern Guatemala and southern Mexico) (Bussing, 1985, 1998; Silfvergrip, 1996; Perdices et al., 2002; Miller, 2005).

The continental ichthyofauna of Middle America is interesting from a biogeographic perspective because it consists of groups of Nearctic and Neotropical origin that dispersed into and diversified in the region, eventually evolving into the assemblages seen today (Leroy et al., 2019; Rico et al., 2022). Early proposals of dispersal scenarios to explain current distribution patterns in Middle American primary freshwater fishes (i.e., physiologically intolerant to seawater) of Neotropical origin, such as *Rhamdia*, considered the Late Pliocene emergence of the Isthmus of Panama as the main catalyzer (Myers, 1966; Bussing, 1985). Although several empirical studies have tested this hypothesis in different groups of primary freshwater fishes including *Rhamdia* (Bermingham & Martin, 1998; Martin & Bermingham, 2000; Perdices et al., 2002; Reeves & Bermingham, 2006; Ornelas-García et al., 2008; Lovejoy et al., 2010; Picq et al., 2014; Melo et al., 2024), not all agree that the formation of the Panama land bridge is what ultimately allowed the dispersal of primary freshwater fishes from South America into Central and North America (i.e., some of the inferred colonization events are considerably older than the emergence of the isthmus). Further research is therefore needed to refine our understanding of the temporal context for the diversification of Middle American primary freshwater fishes, and the MA clade of *Rhamdia* offers an exceptional study system to address this topic.

Notably, almost half of the taxonomic diversity in this MA clade is represented by microendemic, cave-dwelling species that exhibit regressive troglomorphic traits such as integumentary depigmentation and eye reduction or loss. These troglobitic species were described in a span of ~20 years starting in the mid 1980s (Miller, 1984; Wilkens, 1993; Weber & Wilkens, 1998; Weber et al., 2003), although a recent phylogenetic study based on comparative DNA sequence data has cast doubt on their taxonomic validity, particularly on the grounds that their phylogenetic position renders the more widespread species *R. laticauda* deeply paraphyletic (Arroyave & De La Cruz Fernández, 2021b). Notwithstanding this taxonomic conundrum, it is undeniable that the genus *Rhamdia*, and particularly its Middle American component, is special among Neotropical freshwater fishes for its propensity to colonize caves in karstic regions. Besides the four Mexican microendemic cave-dwelling species, there are records of at least 10 populations of hypogean *Rhamdia* in Mexico (Mosier, 1984; Elliott, 2020; Arroyave & De La Cruz Fernández, 2021a; Buenavad-González et al., 2023) plus three more in Costa Rica (Arroyave et al., 2024). Most of these records correspond to subterranean populations of the epigean *R. laticauda,* but there are also a few from the epigean *R. guatemalensis* and *R. nicaraguensis*.

Cave colonization in *Rhamdia*, like in most groups with hypogean diversity, has resulted in the evolution of troglomorphism, presumably as an adaptation to life in perpetual darkness (Wilkens, 2001). Troglomorphism in hypogean *Rhamdia*, however, is quite variable, ranging from minimal to complete (Arroyave & De La Cruz Fernández, 2021a, 2021b; Buenavad-González et al., 2023; Arroyave et al., 2024). This variability has been hypothesized to be correlated with the time since cave colonization and the degree of isolation from epigean populations, so that incomplete troglomorphism may potentially be explained by recency of colonization and/or ongoing gene flow with epigean forms (Arroyave & De La Cruz Fernández, 2021a, 2021b; Buenavad-González et al., 2023; Arroyave et al., 2024). Although the temporal context for the diversification of Middle American *Rhamdia* has been previously investigated in two studies (Perdices et al., 2002; Hernández et al., 2015), neither of them included hypogean populations/species, and thus, the timing of cave colonization in *Rhamdia* has yet to be investigated in a phylogenetic framework. Furthermore, recent phylogenetic hypotheses of Middle American *Rhamdia* including a comprehensive sampling of cave-dwelling diversity (Arroyave & De La Cruz Fernández, 2021b; Buenavad-González et al., 2023;

Arroyave et al., 2024) did not address the temporal context of diversification.

Besides not sampling hypogean populations/species or lacking information about the timing of diversification, these previous studies relied on comparative data from just a few mitochondrial (mtDNA) markers, mainly partial fragments of the *cytochrome oxidase c subunit I* (COI) and *cytochrome b* (CYTB) genes, totaling less than 2 kb in most cases (Perdices et al., 2002; Hernández et al., 2015; Arroyave & De La Cruz Fernández, 2021b; Buenavad-González et al., 2023; Arroyave et al., 2024). Moreover, those using molecular clocks to estimate divergence times relied on either a strict molecular clock approach that assumes a constant substitution rate—imported from a study conducted on a different group of fishes (Perdices et al., 2002)—or a more sophisticated approach such as Bayesian relaxed phylogenetics but with limitations in taxonomic and character sampling, as well as in the number of calibration nodes (Hernández et al., 2015). It is well established that an increase in the number of characters (e.g., nucleotides) generally leads to better resolution and support (Bremer et al., 1999). Therefore, an approach toward improving phylogenetic resolution while leveraging the power of mtDNA markers—by virtue of their lower coalescence times compared to slower-evolving nuclear genes—is to use complete mitochondrial genomes as comparative data. Thus, to overcome the limitations of previous investigations on the systematics and evolutionary history of Middle American *Rhamdia*, the objectives of this study are as follows: (i) to infer a time-scaled phylogeny for the MA clade based on comparative mitogenomic data from a broad taxon sampling encompassing most species in the clade and including samples from most cave-dwelling populations known to exist; and (ii) to establish the chronology of diversification using molecular dating so as to shed light on the tempo and mode of dispersal from South America and colonization of hypogean habitats in Central America and Mexico.

2 Material and Methods

2.1 Taxon sampling and specimen collection

Ingroup taxa consist of representatives of all species of *Rhamdia* from the MA clade, except the Colombian *Rhamdia saijaensis* and the Ecuadorian *R. cinerascens* (for which tissue vouchers were unavailable), totaling 26 terminals. Outgroup taxa consist of two *cis*-Andean species of *Rhamdia* plus representatives of five other heptapterid genera (*Brachyglanis*, *Cetopsorhamdia*, *Heptapterus*, *Imparfinis*, and *Pimelodella*), four pimelodid genera (*Phractocephalus*, *Pimelodus*, *Pseudoplatystoma*, and *Sorubim*), two pseudopimelodid genera (*Lophiosilurus* and *Pseudopimelodus*), and the ictalurid *Ictalurus punctatus* as root, totaling 18 terminals. In addition to the four Mexican troglobitic cave-dwelling species of *Rhamdia*, the ingroup includes individuals from most hypogean populations of epigean species documented to date (Elliott, 2020; Arroyave & De La Cruz Fernández, 2021a; Buenavad-González et al., 2023; Arroyave et al., 2024). Because of the costs associated with sequencing complete mitochondrial genomes (vs. partial gene fragments via Sanger sequencing), only one individual per species was sampled, except for the epigean species geographically widespread and with hypogean populations (i.e., *R. guatemalensis*, *R. laticauda*, and *R. nicaraguensis*), in which cases multiple individuals per species were sequenced (Table 1). The complete mitochondrial genomes from all sampled pimelodids (eight species), pseudopimelodids (two species), and *I. punctatus* (all part of the outgroup) were generated in previous studies (Nakatani et al., 2011; Rangel-Medrano et al., 2016; Restrepo-Escobar et al., 2016; Villela et al., 2017; Ren & Ma, 2019) and retrieved from GenBank. For the remaining 33 terminals (outgroup heptapterids and MA *Rhamdia*), complete mitochondrial genomes were newly generated (see next subsection below).

Voucher specimens and associated tissue samples from *Rhamdia* and other heptapterids used in this study were obtained through multiyear collecting efforts in Mexico and Costa Rica for ingroup terminals (Fig. 1) and in Brazil for outgroup taxa. Specimens were collected under collecting permits SGPA/DGVS/04259/17, SGPA/DGVS/05375/19, and SGPA/DGVS/08073/21 issued by the Secretaría de Medio Ambiente y Recursos Naturales (SEMARNAT), and R-SINAC-SE-DT-PI-029-2023 issued by the Sistema Nacional de Áreas de Conservación (SINAC). Access permit to genetic resources (CBio-54-2022-#359) was extended by the Comisión Institucional de Biodiversidad of the UCR. Sampling was conducted using a variety of fishing gear and techniques, including gill and cast nets, hooks and lines, baited minnow traps, and electrofishing. Fishes were handled in accordance with recommended guidelines for the use of fishes in research (Jenkins et al., 2014), euthanized using the anesthetic tricaine mesylate (MS-222), and tissued postmortem for genetic studies. Tissue samples were preserved in 96% ethanol and eventually frozen at −20°C. Voucher specimens were fixed using a 10% formalin solution for at least 1 week. Formalin-fixed specimens were eventually washed and gradually transferred to 70% ethanol for long-term storage. All vouchers used in this study for the generation of novel genetic data are cataloged and deposited in the following ichthyological collections: Colección Nacional de Peces (CNPE) of the Instituto de Biología, Universidad Nacional Autónoma de México (IBUNAM), Colección Biológica Regional de Peces de la Universidad de Ciencias y Artes de Chiapas (UNICACH), Colección Ictiológica del Museo de Zoología de la Universidad de Costa Rica (UCR), and Laboratório de Biologia e Genética de Peixes da Universidade Estadual Paulista, Botucatu, Brazil (LBP/UNESP) (Table 1).

2.2 Generation of novel complete mitochondrial genomes

2.2.1 DNA extraction and fragmentation

For the 26 samples corresponding to the ingroup (MA *Rhamdia*), high-molecular-weight genomic DNA was extracted using a standard phenol-chloroform DNA extraction protocol (Sambrook et al., 1989). Genomic DNA was fragmented via sonication using a Diagenode Bioruptor® Pico sonication device for 6–10 cycles (depending on the integrity of the DNA), where each cycle consisted of a 30 s phase of ultrasonic bursts followed by a 30 s pause in a 4°C water bath. A total of 200 ng of fragmented DNA per sample was quantified using a Qubit fluorometer (Invitrogen) and used for library preparation. For the seven heptapterid outgroup samples, DNA was extracted using the DNeasy

Table 1 Samples (including catalog, voucher, and locality/habitat type data) used to generate complete mitochondrial genomes

Species	Habitat	Catalog	Voucher	Locality	Latitude	Longitude	Basin	Country
				trans-Andean/Middle American *Rhamdia*				
Rhamdia guatemalensis	Epigean	CNPE-IBUNAM 23808	JA862	Río San Antonio	18.4605556	−96.63222222	Papaloapan	Mexico
Rhamdia guatemalensis	Epigean	CNPE-IBUNAM 24024	JA1303	Río La Venta	16.8323333	−93.53147194	Grijalva	Mexico
Rhamdia guatemalensis	Epigean	CNPE-IBUNAM 23287	JA182	Cenote San Miguel	20.6813889	−89.17833333	YP Aquifer	Mexico
Rhamdia guatemalensis	Epigean	CNPE-IBUNAM 23812	JA897	Río Agua Blanca	17.0666667	−93.87055556	Grijalva	Mexico
Rhamdia guatemalensis	Hypogean	UCR 3330-01	JA2068	Cueva Corredores	8.6564444	−82.91225	Río Coto	Costa Rica
Rhamdia guatemalensis	Hypogean	CNPE-IBUNAM 24021	JA1412	Cueva El Encanto	16.7574444	−93.52516667	Grijalva	Mexico
Rhamdia guatemalensis	Hypogean	CNPE-IBUNAM 24020	JA1294	Cueva Los Bordos	16.8301944	−93.52602778	Grijalva	Mexico
Rhamdia guatemalensis	Hypogean	CNPE-IBUNAM 23815	JA943	Grutas de Cocona	17.5636111	−92.92916667	Grijalva	Mexico
Rhamdia laluchensis	Hypogean	CNPE-IBUNAM uncat.	JA744	Sotano de La Lucha	17.0591667	−93.89555556	Grijalva	Mexico
Rhamdia laticauda	Epigean	UCR 3328-01	JA1804	Quebrada Altamira	10.7336111	−85.05944444	Río Zapote	Costa Rica
Rhamdia laticauda	Epigean	CNPE-IBUNAM 23809	JA864	Río San Antonio	18.4605556	−96.63222222	Papaloapan	Mexico
Rhamdia laticauda	Epigean	CNPE-IBUNAM 24026	JA1320	Río Sabinal	16.8259167	−93.27047222	Grijalva	Mexico
Rhamdia laticauda	Epigean	CNPE-IBUNAM 23810	JA886	Nacimiento Río Tonto (Huixtla)	18.5900583	−96.87728889	Papaloapan	Mexico
Rhamdia laticauda	Hypogean	CNPE-IBUNAM 24023	JA1280	Cueva Chorro Grande	16.5202778	−93.24416667	Grijalva	Mexico
Rhamdia laticauda	Hypogean	UCR 3323-01	JA1913	Cueva Gabinarraca	10.5547222	−84.76694444	Río Frío	Costa Rica
Rhamdia laticauda	Hypogean	CNPE-IBUNAM 23803	JA802	Cueva Naranjal	18.7763889	−96.95694444	Papaloapan	Mexico
Rhamdia laticauda	Hypogean	CNPE-IBUNAM 24022	JA1309	Cueva Paso Burro	16.8313889	−93.27472222	Grijalva	Mexico
Rhamdia laticauda	Hypogean	CNPE-IBUNAM 23801	JA792	Cueva Piedras Blancas	18.6661111	−96.90222222	Papaloapan	Mexico
Rhamdia laticauda	Hypogean	CNPE-IBUNAM 23799	JA763	Sumidero de Cotzalostoc	18.7047	−96.9186	Papaloapan	Mexico
Rhamdia laticauda	Hypogean	CNPE-IBUNAM 23817	JA748	Sotano de Popocatl	18.7575	−96.95444444	Papaloapan	Mexico
Rhamdia macuspanensis	Hypogean	CNPE-IBUNAM 23814	JA933	Grutas de Agua Blanca	17.6216667	−92.47388889	Usumascinta	Mexico
Rhamdia nicaraguensis	Epigean	UCR 3329-01	JA1734	Quebrada Palma	10.4983333	−84.68861111	San Carlos	Costa Rica
Rhamdia nicaraguensis	Hypogean	UCR 3323-02	JA1902	Cueva Gabinarraca	10.5547222	−84.76694444	Frío	Costa Rica
Rhamdia parryi	Epigean	CBR-P-UNICACH-0291	JA1634	Río Fortuna	15.8772222	−93.47222222	Chiapas Coast	Mexico
Rhamdia reddelli	Hypogean	CNPE-IBUNAM 23807	JA855	Cueva del Nacimiento del Río San Antonio	18.4755556	−96.63972222	Papaloapan	Mexico
Rhamdia zongolicensis	Hypogean	CNPE-IBUNAM 23800	JA785	Cueva Ostoc	18.6119444	−96.88722222	Papaloapan	Mexico
				cis-Andean *Rhamdia* + other heptapterids				

Continued

Table 1 Continued

Species	Habitat	Catalog	Voucher	Locality	Latitude	Longitude	Basin	Country
Rhamdia aff. *quelen*	Epigean	LBP 13273	69432	Lagoa Carraqueal, Rio Araguaia	−13.316667	−50.616667	Amazonas	Brazil
Rhamdia aff. *quelen*	Epigean	LBP 10264	47876	Rio São Francisco	−20.264361	−46.349306	São Francisco	Brazil
Brachyglanis microphthalmus	Epigean	LBP 7106	34644	Rio Negro	0.05635	−66.850117	Amazonas	Brazil
Cetopsorhamdia iheringi	Epigean	LBP 8053	37803	Rio Grande, Rio Paraná	−22.448944	−45.346444	La Plata	Brazil
Heptapterus mustelinus	Epigean	LBP 13129	55150	Rio Uruguai	−28.534222	−53.967750	La Plata	Brazil
Imparfinis minutus	Epigean	LBP 19494	76359	Rio São Francisco	−17.349056	−44.952417	São Francisco	Brazil
Pimelodella montana	Epigean	LBP 12595	54298	Rio Marañon	−4.254167	−73.500556	Amazonas	Peru

Tissue Kit (Qiagen) and quantified using the Qubit dsDNA Broad-Range Assay Kit (Invitrogen).

2.2.2 Library preparation and sequencing

Fragmented DNA from the ingroup samples was end-repaired and end-polished via A-tailing and subsequently ligated to custom TruSeq dual-indexed adapters (Glenn et al., 2016) via short PCR. Products were size-selected in a ~300–500 bp range using magnetic beads, enriched through PCR, purified, and normalized. Library preparation was carried out using a KAPA Biosystem Hyper Kit (Kapa, Biosystem Inc.). Mitogenome libraries were sequenced in an Illumina™ HiSeq X, resulting in paired-end sequences of 150 bp in length. Illumina sequencing was conducted at the Georgia Genomics and Bioinformatics Core of the University of Georgia (Athens, GA) and at Daicel Arbor Biosciences (Ann Arbor, MI).

2.2.3 Assembly and annotation

Raw data quality was first evaluated in FastQC (Andrews, 2010). Sequences with ambiguous nucleotides and low average quality were removed, and the remaining sequences trimmed and merged using Geneious Prime v2024.0.3 (Kearse et al., 2012a). Two strategies were employed to assemble the 26 ingroup novel mitogenomes. First, a *de novo* assembly strategy was implemented for sample JA886 (*Rhamdia laticauda*) using MITObim v1.9 (Hahn et al., 2013). A second strategy consisted of using two sequences deposited in GenBank as seeds (CYTB: PQ458964 and COX1: PQ451496). A consensus mitogenome was generated from both results, which was then used as a reference to assemble the other 25 novel complete mitochondrial genomes using a ref-map module in Geneious Prime under a medium sensitivity assembly. Identification and annotation of the 13 protein-coding genes (PCGs), the 22 transfer RNAs (tRNAs), the two ribosomal RNAs, and the noncoding Control Region of each mitochondrial genome were accomplished using the online MitoFish and MitoAnnotator pipeline (Iwasaki et al., 2013).

The mitogenomes of the seven outgroup heptapterids were obtained from raw reads of ultraconserved elements (UCEs) generated and published by two of the co-authors (BFM and GSCS) as part of a recent phylogenomic study of the family (Silva et al., 2021). Assembly of fastq reads into fasta contigs was obtained in Geneious v6.0.3 Read Mapper (Kearse et al., 2012b), where samples were mapped to the reference mitochondrial genome of *Pseudoplatystoma reticulatum* (GenBank NC_033859.1) (Villela et al., 2017) using the medium-sensitivity parameter with up to five iterations.

2.3 Assessment of mitogenome-level genetic divergences

Mitogenome-level genetic divergences among MA *Rhamdia* samples were estimated and visualized via *p*-distances using Mega X (Kumar et al., 2018) as well as mean Kimura-2-parameter (K2P) distances for 60 bp windows covering all 26 ingroup mitogenomes using the approach and customized R script of Santos et al. (2021). A multiple sequence alignment of ingroup mitogenomes was used as input data, and the species *Rhamdia zongolicensis* (GenBank accession PV662383) was used as the base for comparisons.

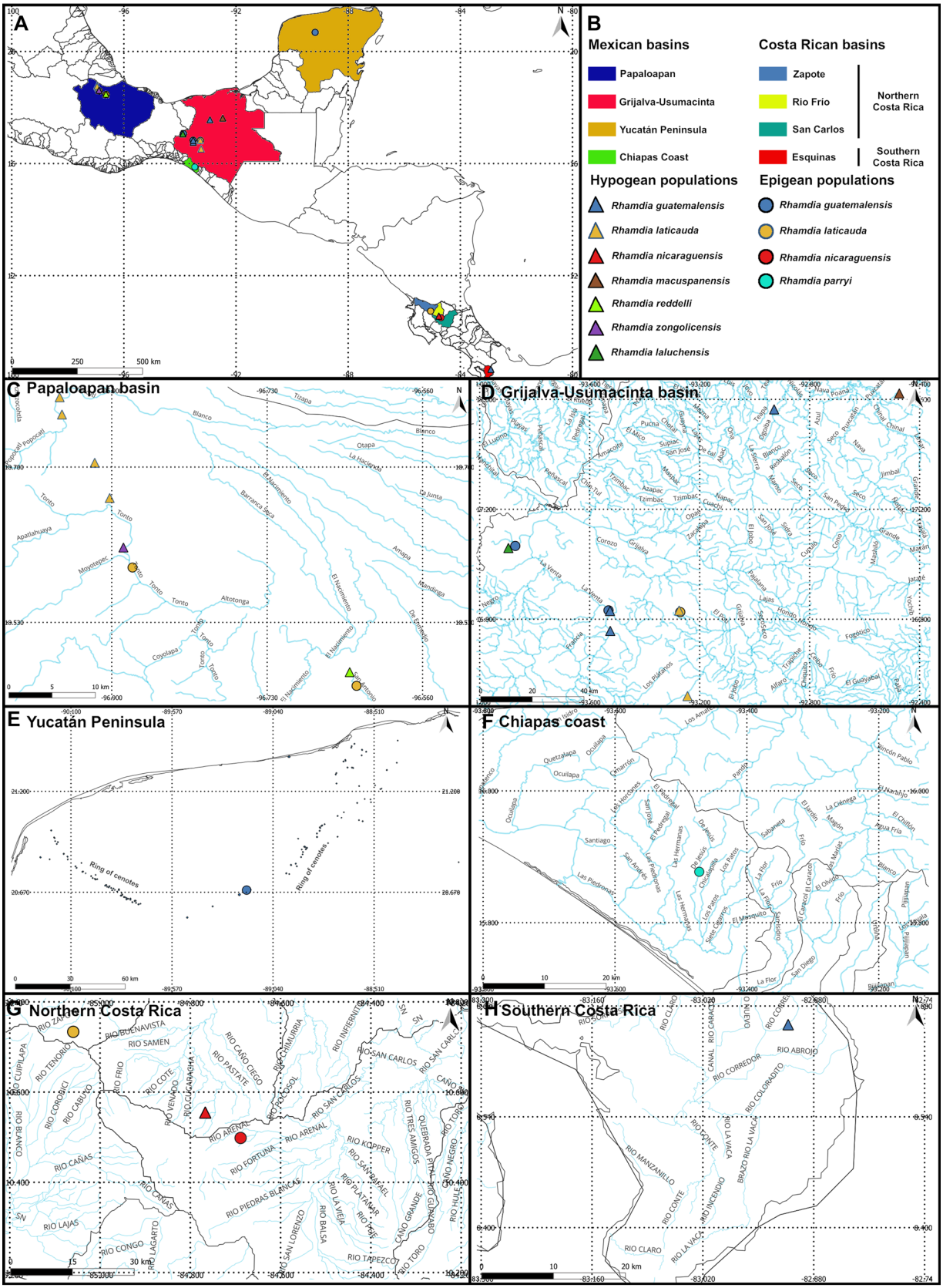

Fig. 1. Collecting localities of ingroup samples. **A,** Map of Middle America (partial) displaying sampling localities from Mexico and Costa Rica. **B,** Color- and shape-coding of samples by basin and habitat (epigean and hypogean). **C**–**H,** Close-up hydrological maps (by basin/region) displaying sampling localities at a finer scale.

Complete mitogenomes were aligned using MUSCLE (Edgar, 2004) as implemented in Mega X (Kumar et al., 2018).

2.4 Co-estimation of phylogeny and divergence times and reconstruction of ancestral mode of life (epigean/hypogean)

Divergence time estimation (DTE) analysis was conducted in BEAST v2.4.8 (Bouckaert et al., 2019) based on 13 protein-coding genes, 11 508 bp, 44 terminals, a relaxed lognormal clock, and GTR + I + G site model (gamma category = 4; invariant sites = 0.5). We used a birth–death tree model and four calibration nodes (three primary (i.e., fossil-based) and one secondary (i.e., based on a divergence time estimate from a previous study)). The first calibration fossil was cf. *Phractocephalus* sp. from the Late Oligocene (28.4–23.0 Ma) Contamana Formation of Loreto in Peru (Antoine et al., 2016). The genus *Phractocephalus* is hypothesized to be sister to *Leiarius* in the *Leiarius-Phractocephalus* clade, which is sister to all remaining pimelodids except *Steindachneridion* (Silva et al., 2024). Thus, this fossil was used to constrain the most recent common ancestor (MRCA) crown node of Pimelodidae during the Chattian of Late Oligocene (mean = 25.5; sigma = 1.5; 95%–5%; quantiles: 28.0–23.0). The second calibration fossil was cf. *Pseudopimelodus* or *Cephalosilurus* (LACM 128374) of Middle Miocene (15.9–11.5 Ma) Río Acre fauna (Acre VI locality) of Solimões/Pebas formation of Peru (Lundberg et al., 2009). Due to generic uncertainty, this fossil was used to constrain the node representing the MRCA of Pseudopimelodidae, including *Lophiosilurus* and *Pseudopimelodus* (mean = 13.0; sigma = 1.0; 95%–5%; quantiles: 14.6–11.4). The third calibration fossil was *Sorubim* sp. (MACN Pv-14224) from Late Miocene (9–6 Ma) of the Ituzaingó Formation of Entre Ríos in Argentina (Bogan & Agnolin, 2021). This fossil was used to constrain the age of the MRCA of *Sorubim* that includes *Sorubim cuspicaudus* and *Sorubim lima* (mean = 7.5; sigma = 1.0; 95%–5%; quantiles: 9.14–5.86). Finally, we used the divergence time between Ictaluridae and Pimelodoidea estimated by Betancur et al. (2017) as a secondary calibration to constrain the age of the root (mean = 80.0; sigma = 5.0; 95%–5%; quantiles: 88.2–71.8). Three independent BEAST runs of 100 million MCMC generations were conducted, sampling at every 10 000 generations until convergence of all parameters (effective sample size > 200) as observed in Tracer v1.7 (Rambaut et al., 2018). We used the last 27 001 trees after a 10% burn-in procedure in TreeAnnotator and visualized the maximum clade credibility (MCC) in FigTree v1.4.

Ancestral state reconstruction (ASR) of mode of life (epigean vs. hypogean) was performed in BEAST v2.6 (Bouckaert et al., 2019) based on a coalescent constant population tree model and a relaxed clock lognormal model, using the same mitochondrial matrix (28 taxa; 11 508 bp) and a habitat matrix classifying terminals (species and populations) into two categories: epigean (inhabiting surface waters such as rivers, streams, lagoons, and cenotes) and hypogean (inhabiting subterranean aquatic habitats such as active caves) (Table 1). A pruned version of the tree resulting from the DTE analysis, to include only species/populations of *Rhamdia* as terminals, was used as the starting topology. BEAST ran for 100 M generations, saving trees every 10 000 generations. Tracer v1.7 (Rambaut et al., 2018) was used to check for convergence of all parameters, and TreeAnnotator filtered the last 9001 trees (10% burn-in) and saved the MCC tree.

3 Results

3.1 Novel complete mitochondrial genomes

Genomic libraries from ingroup samples (MA *Rhamdia*) resulted in 268 444 778 paired reads, with individual sample reads ranging between 1 508 868 and 20 249 978 (Table 2). The complete mitochondrial genomes from the 26 samples of MA *Rhamdia* are very similar in size, averaging ~16 480 bp in total length and ranging between 16 461 bp in *Rhamdia laluchensis* (GenBank accession PV662366) and 16 491 bp in a sample of *R. laticauda* from Costa Rica (GenBank accession PV662367) (Table 2). Conversely, the mitogenomes from the two samples of *trans*-Andean *Rhamdia,* as well as most from the other heptapterid genera, could only be partially recovered and assembled, resulting in incomplete mitogenomes ~500–1000 shorter than the expected total size. GenBank accession numbers for all novel mitogenomes generated in this study are included in Table 2.

Barring the few instances of incomplete sequencing/assembly, all newly generated heptapterid mitogenomes follow the typical arrangement reported for vertebrate mitochondrial genomes, which consists of 37 genes, including 13 protein-coding genes (PCGs), two ribosomal RNA genes (rRNAs), 22 transfer RNAs genes (tRNAs), and one noncoding Control Region, with 28 genes encoded in the H-strand (12 PCGs, 2 rRNAs, 14 tRNAs) and nine in the L-strand (NAD6 and eight tRNAs). Likewise, all novel mitochondrial genomes have a similar overall base composition (within the following percentage ranges: T = 25.4%–25.7%, C = 28%–28.2%, A = 30.8%–31.4%, and G = 15%–15.5%) and, like most teleost mitogenomes, exhibit bias toward A + T (56.3%–56.9%), positive AT skewness (0.0934, 0.1026) and negative GC skewness (−0.3010, −0.2903) (Table 2).

3.2 Mitogenome-wide genetic divergences

Genetic distances (both uncorrected and corrected) among all 26 MA *Rhamdia* complete mitochondrial genomes ranged from 0% to ~6.5%. In the species for which multiple individuals were sampled and sequenced (*R. guatemalensis*, *R. laticauda*, and *R. nicaraguensis*), intraspecific divergences ranged from 0% to ~3%, with the largest intraspecific variation observed in *R. laticauda*. Interspecific genetic divergences varied from 0.3% to ~6.5%, with the cave-dwelling pair *R. zongolicensis* and *R. reddelli* displaying the smallest genetic differentiation (Table S1). As expected, pairwise distances for each region compared to the base mitogenome revealed that the Control Region/D-loop exhibits the highest number of polymorphisms, followed by ATP6, ND2, ND5, CYTB, and COX2. Except for ATP8, most of the remaining PCGs exhibit intermediate levels of variation. Conversely, variation is lowest in the rRNA ribosomal genes (Fig. 2).

3.3 Phylogenetic relationships of Middle American *Rhamdia*

Our phylogeny derived from mitogenomic comparative data (Fig. 3; File S1) resolves the MA clade into two well-supported and reciprocally monophyletic groups: a clade consisting of the epigean species *R. guatemalensis* and a clade consisting

Table 2 Size and nucleotide composition of the complete mitochondrial genomes analyzed in this study and their corresponding GenBank accession numbers. Included is the number of paired reads (Illumina sequencing output) used for mitogenome assembly

Species	Voucher	Total paired reads	GenBank #	Length (bp)	T (%)	C (%)	A (%)	G (%)	AT (%)	GC (%)	AT-skew	GC-skew
Rhamdia guatemalensis	JA862	13 363 486	PV662358	16483	25.6	28.1	30.9	15.4	56.54310502	43.45689498	0.0942	−0.2933
Rhamdia guatemalensis	JA1303	14 438 758	PV662359	16481	25.5	28.2	30.8	15.5	56.29512772	43.70487228	0.0938	−0.2897
Rhamdia guatemalensis	JA182	5 822 430	PV662360	16482	25.5	28.2	30.8	15.5	56.2735105	43.7264895	0.0946	−0.2910
Rhamdia guatemalensis	JA897	17 933 028	PV662361	16481	25.5	28.2	30.8	15.5	56.25265457	43.74734543	0.0948	−0.2913
Rhamdia guatemalensis	JA2068	14 814 676	PV662362	16479	25.5	28.2	30.8	15.5	56.32623339	43.67376661	0.0942	−0.2916
Rhamdia guatemalensis	JA1412	12 398 516	PV662363	16481	25.5	28.2	30.8	15.5	56.28906013	43.71093987	0.0941	−0.2901
Rhamdia guatemalensis	JA1294	20 249 978	PV662364	16480	25.5	28.2	30.8	15.5	56.30461165	43.69538835	0.0934	−0.2895
Rhamdia guatemalensis	JA943	16 733 204	PV662365	16479	25.5	28.2	30.8	15.5	56.24127678	43.75872322	0.0941	−0.2903
Rhamdia laluchensis	JA744	5 246 130	PV662366	16461	25.4	28.2	31.0	15.3	56.41509434	43.58490566	0.1007	−0.2959
Rhamdia laticauda	JA1804	13 047 808	PV662367	16491	25.6	28.0	31.3	15.1	56.9098296	43.0901704	0.1003	−0.2981
Rhamdia laticauda	JA864	15 787 942	PV662368	16483	25.4	28.2	31.1	15.3	56.51277073	43.48722927	0.1006	−0.2955
Rhamdia laticauda	JA1320	13 685 068	PV662369	16487	25.5	28.0	31.4	15.1	56.89330988	43.10669012	0.1026	−0.3010
Rhamdia laticauda	JA886	4 311 768	PV662370	16481	25.4	28.2	31.1	15.3	56.50142588	43.49857412	0.1001	−0.2950
Rhamdia laticauda	JA1280	14 103 800	PV662371	16486	25.5	28.1	31.4	15.0	56.89069514	43.10930486	0.1044	−0.3029
Rhamdia laticauda	JA1913	5 904 694	PV662372	16489	25.7	27.9	31.3	15.2	56.95312026	43.04687974	0.0983	−0.2959
Rhamdia laticauda	JA802	17 585 124	PV662373	16483	25.4	28.2	31.1	15.3	56.50063702	43.49936298	0.1013	−0.2965
Rhamdia laticauda	JA1309	7 959 636	PV662374	16487	25.5	28.0	31.4	15.1	56.89330988	43.10669012	0.1026	−0.3010
Rhamdia laticauda	JA792	10 870 978	PV662375	16482	25.4	28.1	31.1	15.3	56.52833394	43.47166606	0.0999	−0.2949
Rhamdia laticauda	JA763	4 195 864	PV662376	16483	25.4	28.2	31.1	15.3	56.50063702	43.49936298	0.1013	−0.2965
Rhamdia laticauda	JA748	1 508 868	PV662377	16483	25.4	28.2	31.1	15.3	56.50670388	43.49329612	0.1014	−0.2967
Rhamdia macuspanensis	JA933	3 733 730	PV662378	16486	25.3	28.3	31.0	15.4	56.33871163	43.66128837	0.1008	−0.2942
Rhamdia nicaraguensis	JA1734	13 139.51	PV662379	16489	25.4	28.1	31.1	15.3	56.59530596	43.40469404	0.1007	−0.2955
Rhamdia nicaraguensis	JA1902	13 092 492	PV662380	16488	25.5	28.1	31.1	15.3	56.59267346	43.40732654	0.1004	−0.2952
Rhamdia parryi	JA1634	12 797 720	PV662381	16488	25.6	27.9	31.3	15.2	56.88985929	43.11014071	0.0987	−0.2957
Rhamdia reddelli	JA855	4 182 472	PV662382	16484	25.4	28.2	31.1	15.3	56.50934239	43.49065761	0.1014	−0.2961
Rhamdia zongolicensis	JA785	4 663 468	PV662383	16483	25.4	28.2	31.1	15.3	56.51883759	43.48116241	0.1015	−0.2959
Rhamdia aff. *quelen*	69432	1 117 469	PV662384	15750	25.7	27.8	31.0	15.5	56.71111111	43.28888889	0.0949	−0.2837
Rhamdia aff. *quelen*	47876	617 547	PV662385	15048	25.2	28.3	30.7	15.8	55.93671054	44.06328946	0.0989	−0.2842
Brachyglanis microphthalmus	34644	1 019 946	PV662386	16121	24.8	28.7	30.5	16.1	55.27352298	44.72647702	0.1019	−0.2812
Cetopsorhamdia iheringi	37803	2 067 287	PV662387	15858	26.0	27.8	29.8	16.4	55.80630887	44.19369113	0.0696	−0.2599
Heptapterus mustelinus	55150	477 250	PV662388	15979	27.7	26.1	31.4	14.8	59.04225352	40.95774648	0.0628	−0.2765
Imparfinis minutus	76359	1 040 416	PV662389	16142	27.4	26.6	30.9	15.1	58.25279277	41.74720723	0.0605	−0.2754
Pimelodella montana	54298	1 463 220	PV662390	16575	26.3	27.3	31.4	15.0	57.65075149	42.34924851	0.0878	−0.2893

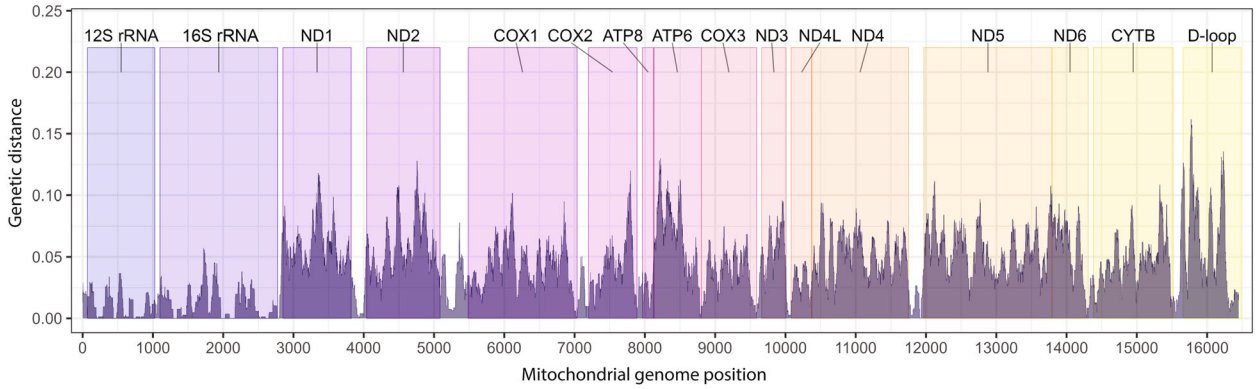

Fig. 2. Kimura-2-parameter (K2P) distances for 60 bp windows covering all 26 ingroup mitogenomes. Colored boxes highlight rRNAs, coding genes, and the control region. Variations of peak heights indicate the distance related to the base mitogenome of *R. zongolicensis*.

of the remaining MA species (i.e., the *R. laticauda* species-group (spp.-grp.)), including a deeply paraphyletic *R. laticauda*. The monophyly of *R. guatemalensis* is strongly supported by our results, with some hypogean populations from this species almost genetically indistinguishable from epigean ones, particularly those in sympatry. Conversely, samples morphologically identified as *R. laticauda* are phylogenetically spread over the *R. laticauda* spp.-grp. clade, associated with three distinct geographic lineages/clades: one consisting of Costa Rican samples (both epigean and hypogean) that is sister to a well-supported *R. nicaraguensis* clade, another consisting of Mexican samples from the Grijalva basin (both epigean and hypogean), and the last one consisting of Mexican samples from the Papaloapan basin (both epigean and hypogean) that also includes the troglobitic and cave-dwelling *R. reddelli* and *R. zongolicensis* (Fig. 3).

3.4 Time-scaled phylogeny of Middle American *Rhamdia* and the evolution of cave colonization

The results of the DTE analysis (Fig. 3) indicate that the genus *Rhamdia* dates from the Middle Miocene, with the node representing the crown group of the genus, and the split between *cis-* and *trans*-Andean lineages, aged at around 12.42 Ma (16.6–8.7 Ma 95% highest posterior density [HPD] interval). The age of the node representing the crown group of the MA clade, and the split between *R. guatemalensis* and the *R. laticauda* spp.-grp. clade, is estimated to have occurred during the Late Miocene (8.9 Ma; 12.3–6.2 Ma 95% HPD). According to our results, the crown group of *R. guatemalensis* most likely originated in the Late Pliocene (2.77 Ma; 4.1–1.7 Ma 95% HPD), whereas the clade containing all the remaining sampled MA species (i.e., the *R. laticauda* spp.-grp. clade) dates back to the Early Pliocene (4.0 Ma; 5.3–2.9 Ma 95% HPD). The four currently recognized hypogean and troglobitic species of MA *Rhamdia* (all endemic of Mexico) are part of a very recent lineage within the *R. laticauda* spp.-grp. clade that dates from the Pleistocene (2 Ma), with *R. laluchensis* and *R. macuspanensis* as the earliest diverging ones (originating in the Gelasian and Calabrian, respectively), and with *R. reddelli* and *R. zongolicensis* as the most recent ones, having evolved during the Chibanian (~200 ka). Additionally, when considering all cave-dwelling populations (i.e., not only exclusively hypogean species but also subterranean populations of more widespread primarily epigean species), our results imply that cave colonization in MA *Rhamdia* essentially dates from the Pleistocene, with most colonization events occurring during the last 500 ka (Fig. 3).

The results of the ASR analysis (Fig. 4; File S2) imply a total of 13 independent instances of cave colonization in MA *Rhamdia*, almost as numerous as the total number of cave-dwelling populations included in the analyses (16). Results indicate that cave colonization in *R. guatemalensis* (with four hypogean populations) evolved independently at least three times, including a relatively ancient one (~2.7 Ma) that coincides with the MRCA of the crown group. Such an optimization (a hypogean ancestor for *R. guatemalensis*), however, may represent an artifact of limited phylogeographic sampling (see Discussion). The other two instances of cave colonization in *R. guatemalensis* are estimated to have occurred during the Late Pleistocene (<400 ka) and restricted to a few cave systems of the Grijalva River drainage in southern Mexico: Grutas de Coconá in the state of Tabasco and two caves in the state of Chiapas (El Encanto and Los Bordos caves). In contrast to our findings for *R. guatemalensis*, colonization of hypogean habitats in the *R. laticauda* spp.-grp. clade appears more widespread and common, with the 12 cave-dwelling populations included in the analyses being the result of at least six lineages independently colonizing hypogean habitats and several reversals to the epigean condition during the Quaternary (Figs. 3, 4). For most nodes, including those corresponding to the MRCA of MA *Rhamdia* and of each major subclade (*R. guatemalensis* and the *R. laticauda* spp.-grp. clade), the posterior probabilities of the inferred ancestral states (epigean vs. hypogean) were only moderate, which implies almost equal probabilities for the ancestral condition being either epigean or hypogean and therefore considerable

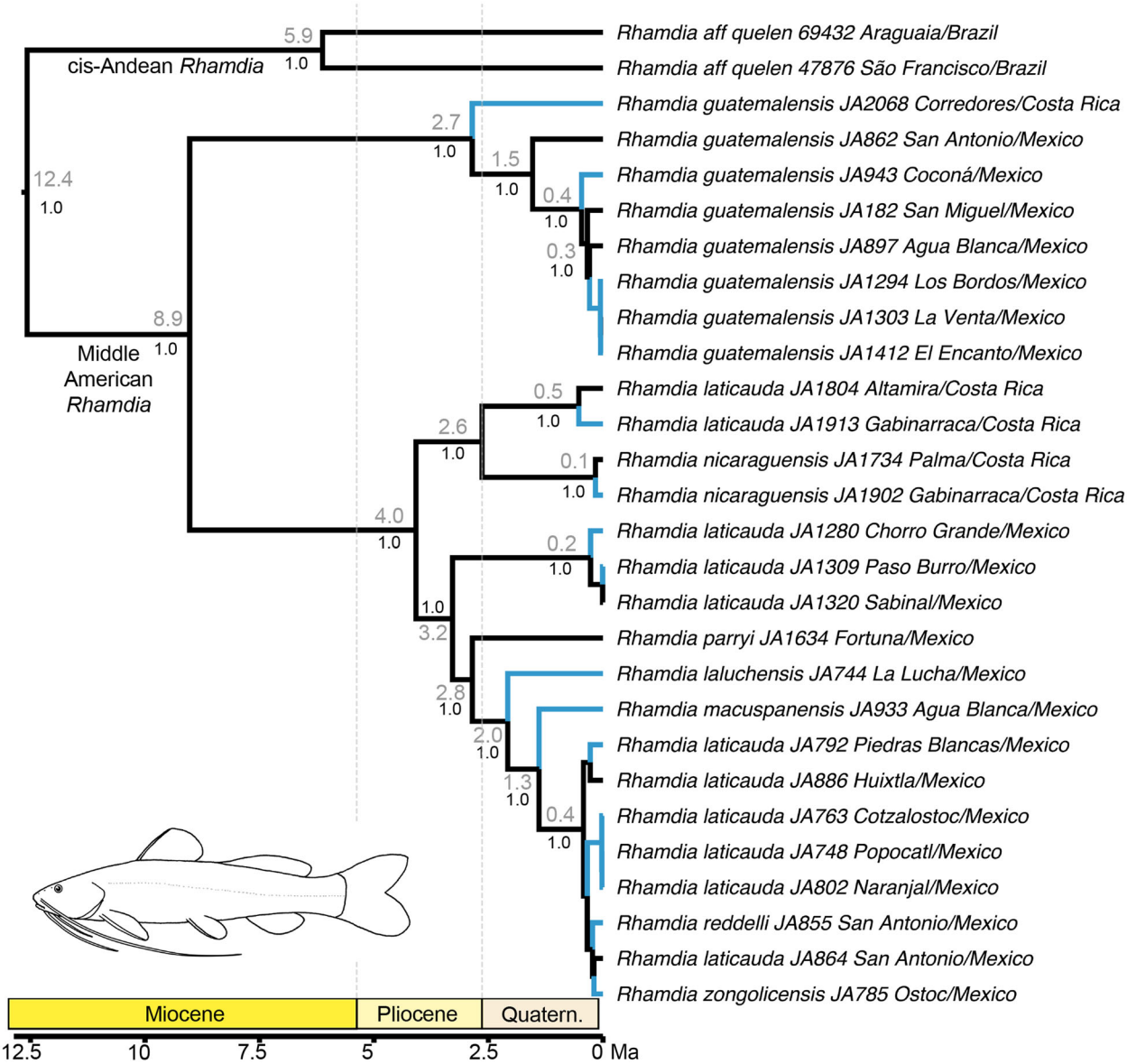

Fig. 3. Time-calibrated phylogeny of Middle American *Rhamdia* based on the BEAST analysis of mitochondrial genomes (44 terminals, 11 508 bp). Outgroup taxa not shown. Numbers in gray and black represent estimated mean ages and posterior probabilities, respectively, of each relevant node discussed in the text. Color-coded terminal branches indicate epigean (black) and hypogean (blue) populations/species.

uncertainty in the inferred evolutionary history of cave colonization (Fig. 4).

4 Discussion

4.1 Systematics of Middle American *Rhamdia*

Since the recognition that *Rhamdia* consists of two reciprocally monophyletic groups corresponding to the *cis*- and *trans*-Andean clades (Perdices et al., 2002; Hernández et al., 2015), only a few studies, using partial fragments of mitochondrial markers as comparative data (totaling less than 2 kbp in all cases), have contributed to improving our understanding of the phylogenetic relationships in the MA clade (inclusive of *trans*-Andean species) (Arroyave & De La Cruz Fernández, 2021b; Buenavad-González et al., 2023; Arroyave et al., 2024). While these recent works have offered support to some previous ideas about species monophyly and interrelationships in the clade, such as the sister-group relationship between a monophyletic *Rhamdia guatemalensis* and a clade consisting of the remaining MA species, they have also uncovered some phylogenetic patterns that are in conflict with the current taxonomy, particularly with the validity of *R. laticauda* and of some of the Mexican troglobitic species.

Our phylogenetic results, based on a much larger character sampling than any previous work (~11.5 kbp), corroborate, for the most part, the findings of these previous studies, while

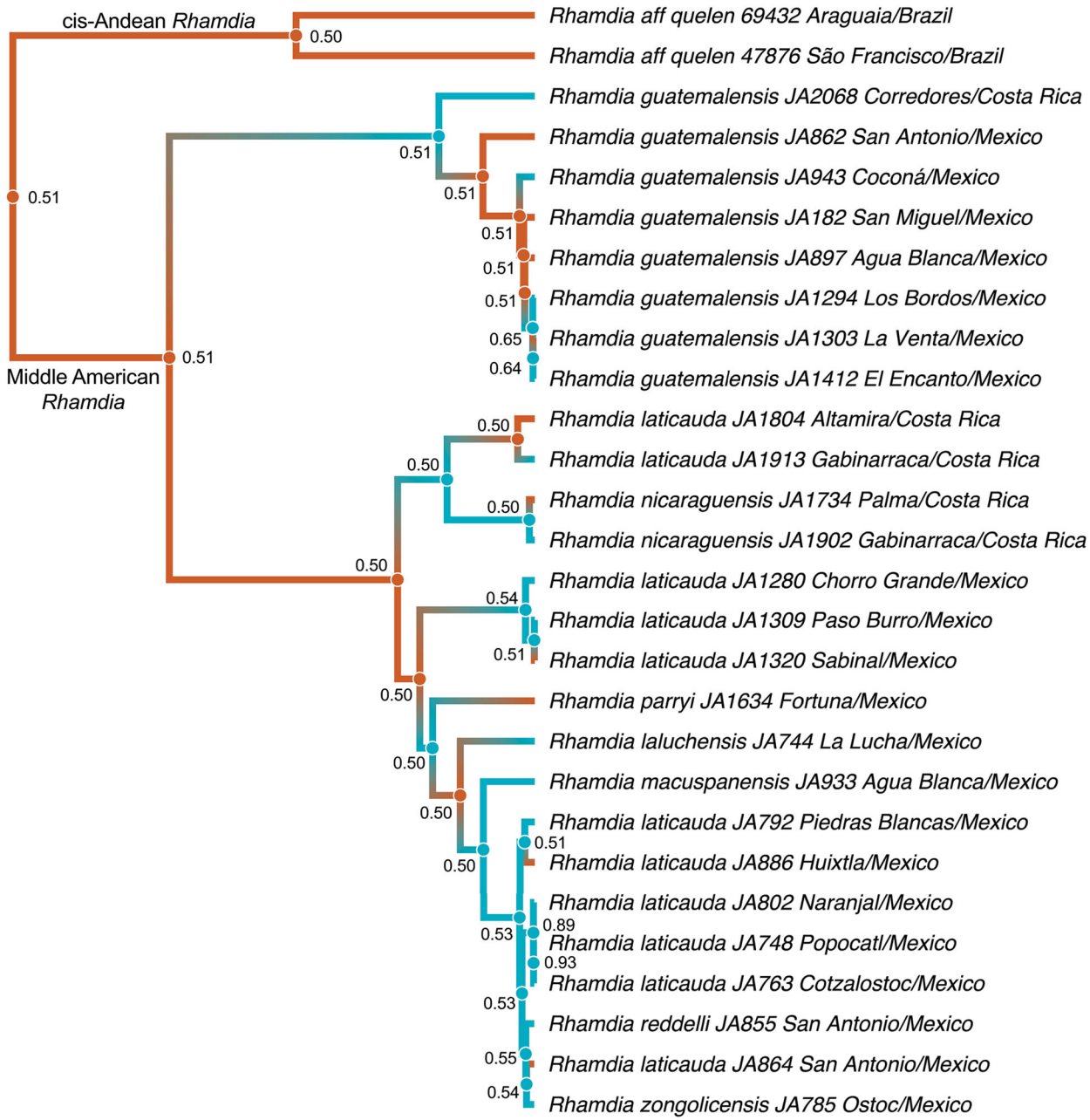

Fig. 4. Ancestral state reconstruction (ASR) of the mode of life (epigean vs. hypogean) in Middle American *Rhamdia*. The most likely ancestral condition at each node and along branches is based on the resulting posterior probabilities (numbers in black) and indicated using differential color-coding, with brown for epigean and blue for hypogean.

offering a comparatively exceptionally high nodal support to the inferred topology. Perhaps the main issue that plagued previous hypotheses of relationships in the MA clade was the ubiquity of poorly supported nodes inside the *R. laticauda* spp.-grp. clade, likely a result of attempting to resolve rapid and relatively recent divergences with limited—and, therefore, not variable enough—comparative DNA sequence data. We believe that, despite being effectively a single locus (the circular mitochondrial DNA), the almost tenfold increase in the amount of comparative data entailed by our mitogenomic dataset with respect to most previous studies explains the overall improved level of resolution and clade support throughout the phylogeny. Notwithstanding the above-mentioned improvement in resolution and support offered by our mtDNA dataset, reliance on a single locus limits our ability to accommodate the confounding effects of gene tree discordance, horizontal gene transfer, and locus-specific biases in phylogenetic inference (Degnan & Rosenberg, 2006; Fournier et al., 2009). Therefore, while informative and a considerable improvement upon previous research, our phylogenetic results should be interpreted with consideration of these potential limitations.

Because of our sampling strategy, we were only able to test the monophyletic status of *R. laticauda*, *R. guatemalensis*, and *R. nicaraguensis*, corroborating previous findings in this regard for these species. Our results strongly support the monophyly of *R. guatemalensis* and *R. nicaraguensis* and confirm that hypogean populations from these species belong to their respective species-level clades. The hypothesis of a paraphyletic *R. laticauda*, initially suggested over 20 years ago due to the phylogenetic placement of *R. nicaraguensis* and *R. reddelli* (Perdices et al., 2002), and further advanced by more recent studies considering also the position of the remaining troglobitic species as well as of the epigean *R. parryi* (Arroyave & De La Cruz Fernández, 2021b; Arroyave et al., 2024), is also strongly supported by our results. An emerging pattern, likely discernible due to the improved levels of nodal support and resolution offered by our tree, is the distribution of samples morphologically identified as *R. laticauda* into several genetically distinct geographic lineages/clades, including a Costa Rican lineage (sister to *R. nicaraguensis*) and two Mexican lineages from the Grijalva and Papaloapan basins, respectively. Besides epigean populations, the Papaloapan lineage comprises many hypogean forms, including the troglobitic species *R. reddelli* and *R. zongolicensis*. A possible solution to the recalcitrant paraphyly of *R. laticauda* would, therefore, be to elevate each of these geographic lineages to a species-level clade, which, in the case of the Papaloapan lineage, would also imply the synonymy of *R. reddelli* and *R. zongolicensis* with the species name that applies for this clade, considering all relevant available names and the principle of priority. Such a nomenclatural action, however, would first require refining our understanding of *R. laticauda*'s type locality, which is currently simply referred to as "Mexico" (Fricke et al., 2025). Similarly, elevating the Costa Rican lineage of *R. laticauda* to species level would first require assessing if it corresponds to any of the Costa Rican species "long-lost synonymized" (*sensu* Gippoliti et al. (2024)) by Silfvergrip (1996) (i.e., *R. regani* Meek 1907, *R. rogersi* Regan 1907, and *R. underwoodi* Regan 1907). It should be noted that the number of geographic lineages recognized within *R. laticauda* would likely be influenced by geographic sampling, and therefore, it is probable that a more extensive coverage of populations across its range would result in the discovery of additional geographic lineages potentially representing different species. Regardless of the degree of geographic sampling, implementing such a taxonomic and nomenclatural rearrangement is certainly beyond the scope of this study and would require a proper systematic revision. Our results, however, could serve as a guideline for future phylogenetic and revisionary work by targeting genetic lineages unique to geographic areas with the potential to be recognized, delimited, and diagnosed as different species.

Given our topology and branch lengths, coupled with the results from previous phylogenetic studies sampling multiple individuals per species (Arroyave & De La Cruz Fernández, 2021b; Buenavad-González et al., 2023; Arroyave et al., 2024), we suggest that the epigean *R. parryi* and the hypogean *R. laluchensis* and *R. macuspanensis* are maintained as valid despite the paraphyly of *R. laticauda*. These species certainly represent distinct geographic and ecological lineages, and therefore, most likely separately evolving metapopulations. The phylogenetic position of *R. parryi* inside the *R. laticauda* spp.-grp., however, remained unsettled, with conflicting results likely due to poorly supported nodes (Perdices et al., 2002; Arroyave & De La Cruz Fernández, 2021b; Buenavad-González et al., 2023; Arroyave et al., 2024). Our inference of a robust sister-group relationship between *R. parryi* and the clade consisting of all Mexican troglobitic species and the Papaloapan lineage of *R. laticauda* thus offers resolution to this phylogenetic puzzle. Similarly, whereas not as problematic as that of *R. parryi*, the phylogenetic positions of *R. laluchensis* and *R. macuspanensis* had yet to be established with better support and more certainty. Our resultant phylogeny achieves this, resolving *R. laluchensis* as the earliest diverging cave-dwelling species, followed by *R. macuspanensis* as sister to the Papaloapan clade of *R. laticauda,* inclusive of *R. reddelli* and *R. zongolicensis*. The phylogenetic positions of *R. cinerascens* and *R. saijaensis*, South American species not included in this study, remain to be reliably established. A recent molecular phylogeny based on CYTB sequence data (Arroyave et al., 2024), nonetheless, suggests that these species are more closely related to the *R. laticauda* spp.-grp. clade than to *R. guatemalensis* (albeit with moderate support), with *R. cinerascens* as the earliest diverging lineage, followed by *R. saijaensis* as sister to the *R. laticauda* spp.-grp. clade.

Our results demonstrate that a considerable increase in the amount of comparative sequence data, even when coming from the same locus, can lead to a substantial improvement in the degree of resolution and nodal support in the phylogeny of the MA clade of *Rhamdia*. Notwithstanding this progress, future research on the systematics of this group of fishes can move towards the use of genome-scale comparative data (e.g., genome-wide single nucleotide polymorphisms generated via RADSeq) coupled with state-of-the-art analytical approaches to species delimitation (e.g., Bayesian coalescent-based methods) and ensuring an even more comprehensive sampling of the species in the clade and of populations of *R. laticauda* from its entire geographic range.

4.2 Temporal and biogeographic context for the diversification of *Rhamdia* in Middle America

The current diversity of Middle American freshwater fishes (primary and secondary) is considered to be the result of multiple asynchronous waves of South American invasions occurring both before and after the Pliocene formation of the Panamanian Isthmus (Bussing, 1985; Bermingham & Martin, 1998; Chakrabarty & Albert, 2011), for which there is broad consensus that it dates back to ~3.2–2.8 Ma (O'Dea et al., 2016) despite geological (Montes et al., 2012) and biological (Bacon et al., 2015) studies suggesting an otherwise much earlier complete emergence. Bussing (1985) tentatively considered *Rhamdia* as a member of the "Paleoichthyofauna" or "Old Southern Element," which allegedly first dispersed and became established in Central America from South America by means of ancient (Cretaceous or Paleogene) Earth history events (e.g., a proto-Antillean land bridge) or marine dispersal. Such an ancient origin and historical biogeographic scenario for *Rhamdia*, however, has been refuted by the two studies that have previously used molecular dating analysis to

investigate the systematics and biogeography of Middle American *Rhamdia* (Perdices et al., 2002; Hernández et al., 2015). Both studies inferred a Late Miocene origin for *Rhamdia*, although with slightly different node ages (~10.5 vs. ~8 Ma). Our inferred Middle Miocene (~12.5 Ma) origin of the genus (Fig. 3) offers further support to the notion that *Rhamdia* is not a very ancient lineage and that the split between *cis*- and *trans*-Andean clades was likely promoted by Miocene tectonism and the ensuing Andean uplift in NWSA, specifically by the rise of the Eastern Cordillera (~12 Ma), which isolated the Pacific Slope and Magdalena basins from its *cis*-Andean counterparts (Albert et al., 2006; Bedoya et al., 2021).

Based on our divergence time estimates coupled with present-day distribution patterns of the species in the MA clade, we hypothesize a biogeographic scenario in which northward dispersal and colonization of Central America and southern North America by *Rhamdia* was catalyzed by the emergence of the Panamanian Isthmus land bridge and stream captures across Lower Central America (LCA). Under this scenario, an ancient lineage of *Rhamdia* leading to the MA crown clade most likely evolved in South America during the Middle and Late Miocene, at which time it split into the lineages that eventually led to the contemporary species *R. guatemalensis* and those in the *R. laticauda* spp.-grp. These ancient lineages therefore evolved in South America, as probably did *R. guatemalensis*. Although our results indicate a Pliocene (~3 Ma) age for the *R. guatemalensis* crown group (Fig. 3), this result might be an artifactual underestimate caused by our incomplete intraspecific geographic sampling, limited to populations from Mexico and Costa Rica and thus lacking samples from the southern limit of its distribution. Several studies, including the present one, have shown that *R. guatemalensis* displays a phylogeographic structure largely consistent with a latitudinal (south-to-north), isolation-by-distance gradient (Arroyave et al., 2021, 2024; Arroyave & De La Cruz Fernández, 2021b). It is, therefore, likely that, had we sampled populations from NWSA, we would have inferred an older crown group age for *R. guatemalensis*, like that inferred for the *R. laticauda* spp.-grp. clade. Considering that *R. guatemalensis* is the most geographically widespread of all species in the MA clade, it is not surprising that it is also the oldest, originating in South America and dispersing into Central America once the Panamanian Isthmus was fully formed. Contrary to Chakrabarty & Albert (2011), who based on the findings of Perdices et al. (2002) proposed a potential Isthmian biogeographic reversal (north-to-south dispersal) for *R. guatemalensis*, and thus a new take on the Great American Biotic Interchange, we posit that the South American lineages are indeed the oldest and therefore the distribution of this species can be simply explained as the result of a one-way northward dispersal and colonization (without speciation) from NWSA.

While the lineage leading to *R. guatemalensis* persisted in NWSA, the lineage leading to the *R. laticauda* spp.-grp. clade did not remain in South America, but diversified in Central America and Mexico after its dispersal through the Panamanian Isthmus. This scenario is supported by our time-scaled phylogeny (Fig. 3), which indicates that the species in the *R. laticauda* spp.-grp. clade diversified only until the Pleistocene. Like the phylogeographic pattern seen in *R. guatemalensis*, diversification in the *R. laticauda* spp.-grp. clade seems to follow a latitudinal, south-to-north trajectory, with most of the species-level diversity being of very recent origins (<500 ka) and concentrated in Mexican basins and hypogean habitats at the northern limit of the distribution of the genus.

Despite our estimated node ages being older than those from previous studies, and contrary to Perdices et al. (2002), we consider that the diversification of MA *Rhamdia* better conforms to the second—not the first—wave of invasion into LCA that took place during the Middle Pliocene *sensu* Bermingham & Martin (1998). Of the few recent studies investigating the historical biogeography of other *trans*-Andean/Middle American clades of primary freshwater fishes, Melo et al. (2024) arrived at a similar conclusion to explain the expansion into LCA by the curimatid genus *Cyphocharax*. Conversely, while resorting to the Panamanian land bridge to explain the dispersal of *Hyphessobrycon*—a *trans*-Andean acestrorhamphid genus—Elías et al. (2023) did so under the assumption of a much earlier (Early Miocene) emergence of the Isthmus (Montes et al., 2012; Bacon et al., 2015) and in that way reconciling much older lineage origins and divergences with dispersal via freshwater corridors on exposed land. Discrepancies between the tempo and mode of evolution of co-occurring *trans*-Andean fishes are not necessarily unexpected, yet patterns of co-diversification are not rare either and usually interpreted as a shared response to the same historical processes (Rincon-Sandoval et al., 2019). It appears, therefore, that dispersal into LCA by South American primary freshwater fish lineages was indeed promoted by the emergence of the Isthmus, although contingent on the taxon age as well as on both stochastic and deterministic processes in the eco-evolutionary dynamics of range expansion (Miller et al., 2020). Thus, it could be stated that the Isthmus literally paved the way, and it was up to the species to jump at the opportunity for northward dispersal.

A more detailed understanding of the biogeographic history of MA *Rhamdia,* considering vicariance, range expansion, and stream capture events, has yet to be offered and was beyond the scope of this study. We, therefore, recommend that future research implementing phylogenetic and probabilistic model-based biogeographic analyses (e.g., BioGeoBEARS) based on a broader geographic sampling of widespread species and comparative genomic data (such as ultraconserved elements or genome-wide single nucleotide polymorphisms) be conducted to arrive at such levels of understanding and to test the biogeographic scenario proposed here.

4.3 Evolutionary history of cave colonization

The time since cave colonization in cavefishes varies greatly among species and lineages, with estimated ages ranging between tens and hundreds of thousands of years (e.g., *Astyanax mexicanus* (Herman et al., 2018), *Barbatula barbatula* (Behrmann-Godel et al., 2017)) to millions of years (e.g., *Rhamdiopsis krugi* (Bichuette et al., 2015), *Sinocyclocheilus* (Mao et al., 2021)). Invasion of hypogean habitats by members of the MA clade of *Rhamdia* appears to be a rather relatively recent evolutionary trend considering that, while this group of fishes has been around since the Middle

Miocene, most instances of cave colonization date from the Pleistocene, particularly from the last 500 ka (Fig. 3). Although we could not find any information in the geologic literature about the speleogenesis and hydrogeology of the cave systems with documented presence of *Rhamdia*, we hypothesize that the relative recency of cave colonization in the group is correlated with the age of the caves that harbor these hypogean populations and species. In other words, *Rhamdia* has opportunistically dispersed into the hypogean realm soon after caves were formed and became available for colonization by fishes inhabiting adjacent epigean waters. Notably, most records of cave-dwelling MA *Rhamdia* are from southern Mexico, specifically from karstic caves in the Sierra Madre del Sur (SMS) and the Sierra Madre de Chiapas (SMC) mountain ranges (Arroyave & De La Cruz Fernández, 2021a, 2021b; Buenavad-González et al., 2023), an unsurprising fact given that Mexico contains >90% of Middle American karst, particularly in the southern and eastern states (Day & Kueny, 2004). While bedrocks throughout the SMS and SMC are rather old (Mesozoic and Early Cenozoic) (Morán-Zenteno et al., 1999; González-Torres et al., 2013; Eguiluz y de Antuñano et al., 2023), the overlaying karst caves must be considerably younger, given that the processes of karst cave formation, enlargement, and modification are dynamic and ongoing; even if a cave system has ancient origins, the features and passages observed today may be the result of more recent activity (Bosák, 2002; Smith, 2002). Indeed, the Pleistocene appears to be a major period for cave formation and karstification in Mexico, with several documented karst caves dating back to this epoch (Weidie, 1985; Arroyo-Cabrales et al., 1995; Smith, 2002; Méjean et al., 2015; Ardelean et al., 2023). Furthermore, cave colonization in other notable cavefish of the region, the blind Mexican tetra (genus *Astyanax*), is hypothesized to date from the Pleistocene through entrapment by stream capture (Jeffery, 2001), with evidence suggesting even more recent (<25 ka) colonization events for a number of hypogean populations, indicating rapid adaptation to the cave environment (Simon et al., 2019). It is, therefore, reasonable to presume that the Mexican karst caves colonized by *Rhamdia* are also Quaternary in origin and that their formation allowed the opportunistic invasion of these newly developed hypogean habitats by epigean fishes that were already preadapted to cave life (i.e., nocturnal, elongated barbels for enhanced chemo- and mechanoreception, increased fat storage capacity) (Wilkens, 2001). Whether cave colonization in MA *Rhamdia* was promoted by paleoclimatic pressures (with caves acting as refugia from episodic climatic changes) or simply by the possibility to move into newly developed hypogean habitats to exploit resources otherwise unavailable on the surface (Howarth's "adaptive shift" model (Howarth, 1973)) remains to be empirically tested. Although Wilkens (2001) briefly speculated on the subject, he did so based on the wrong timeframe, for his proposed ages—not empirically estimated—were vastly younger than our inferred divergence times between *R. reddelli* and *R. zongolicensis* (~8–10 ka vs. ~230 ka).

Besides being geologically recent, cave colonization in MA *Rhamdia* is phylogenetically widespread and convergent (i.e., evolved independently multiple times) (Fig. 4). Our ASR of mode of life (epigean vs. hypogean), however, resulted in mostly poorly supported ancestral reconstructions (PP = ~0.5), including a few rather counterintuitive ones, such as hypogean ancestors for the MRCA of *R. guatemalensis* and for the MRCA of *R. parryi* and *R. zongolicensis* (Fig. 4; File S2), quite geographically widespread clades. The inference—albeit poorly supported—of hypogean ancestors for rather large clades that include epigean descendants is, in our opinion, likely an artifact of insufficient geographic sampling across the range of species and lineages. The idea that incomplete and/or biased sampling (taxonomic and/or geographic) can significantly influence the inference of ancestral states, and that increased sampling density generally improves the accuracy of ASR, is both intuitive and well documented in the literature (Salisbury & Kim, 2001; Hardy, 2006; Sanderson et al., 2010; Bahl et al., 2016; Liu et al., 2022). As a case in point, a hypogean ancestor for *R. guatemalensis* (a species widely distributed from Mexico to Colombia and Venezuela) was probably inferred as a result of having sampled only one population south of Mexico, which happened to be hypogean (Corredores Cave, Costa Rica); had we sampled additional epigean populations south of Costa Rica, the inferred ancestral state would have most likely been epigean. Despite our efforts to investigate for the first time the evolution of cave colonization in MA *Rhamdia* in a phylogenetic framework, we realize that our ancestral reconstructions of mode of life are likely compromised by limited intraspecific geographic sampling. Therefore, caution must be taken when interpreting these results.

Notwithstanding our arguably problematic ancestral reconstructions, the inferred recent, convergent, and phylogenetically widespread nature of cave colonization in MA *Rhamdia* (Figs. 3, 4), coupled with our knowledge of the group from years of research (Arroyave & De La Cruz Fernández, 2021a, 2021b; Arroyave et al., 2021, 2024; Buenavad-González et al., 2023), lead us to hypothesize that, for the most part, cave-dwelling populations of MA *Rhamdia* may represent "blind alleys" or "evolutionary dead ends" (*sensu* Luo (2007); i.e., terminal lineages with no direct ancestor–descendant relationship to the emergent clades in the succeeding episode of diversification). In most caves with resident populations of *Rhamdia*, entry of fishes (from the surface) appears much more probable than their exit, decreasing the likelihood of dispersal and allopatric speciation from cave-dwelling ancestors. Moreover, many of these caves are rather vulnerable ecosystems, making resident fauna unable to disperse and quite susceptible to extinction (Elliott et al., 2023). As such, cave-dwelling populations of MA *Rhamdia* could represent lineages ecologically constrained to reduced diversification and even extinction. We hope this evolutionary hypothesis can be tested in future research based on denser and more comprehensive sampling of epigean populations across the geographic range of species, as well as on genome-wide comparative data to include representation of the nuclear genome and minimize potential issues of individual locus bias.

Acknowledgements

This research was financially supported by the Dirección General de Asuntos del Personal Académico (DGAPA) de la

Universidad Nacional Autónoma de México (UNAM) through the Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica (PAPIIT) grants IA202119 and IN214922 to JA and through a postdoctoral grant (Programa de Becas Elisa Acuña) to AFMS. Additional funding was provided by the Consejo Nacional de Ciencia y Tecnología (CONACyT) through a "Ciencia Básica" grant (A1-S-28293) to JA, the AMNH Axelrod Research Curatorship, and FAPESP #20/13433-6 grant to BFM, and FAPESP proc. 2021/12979-8 and 2022/13025-0 grants to GSCS. The authors would like to thank the Laboratorio Nacional de Cómputo de Alto Desempeño (LANCAD), the Secretaría de Ciencia, Humanidades, Tecnología e Innovación, México (SECIHTI), and the Universidad Nacional Autónoma de México (UNAM) for granting computing time on the computer cluster Miztli. Additional thanks to Heron O. Hilário (PUC-MG) for help with the customized R script for genetic divergences. Special thanks to all the people who have supported JA throughout the years during fieldwork in Mexico and Central America devoted to collecting specimens of *Rhamdia*. The fish specimens used in this research were collected under permits SGPA/DGVS/04259/17, SGPA/DGVS/05375/19, and SGPA/DGVS/08073/21 issued by the Secretaría de Medio Ambiente y Recursos Naturales (SEMARNAT, México) and R-SINAC-SE-DT-PI-029-2023 issued by the Sistema Nacional de Áreas de Conservación (SINAC, Costa Rica). Access permit to genetic resources (CBio-54-2022-#359) was extended by the Comisión Institucional de Biodiversidad of the UCR, Costa Rica. Open access funding provided by UNAM.

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Supplementary Material

The following supplementary material is available online for this article at http://onlinelibrary.wiley.com/doi/10.1111/jse.70003/suppinfo:

File S1. Tree file (in Newick format) corresponding to the time-calibrated phylogeny presented in Fig. 3.

File S2. Tree file (in Newick format) corresponding to the ancestral state reconstruction presented in Fig. 4.

Table S1. Pairwise genetic distances among the 26 complete mitochondrial genomes of MA *Rhamdia* species and populations generated in this study. Uncorrected (*p*-distances) and corrected (K2P) distances below and above the diagonal, respectively.