

# New records of cave-dwelling populations of *Rhamdia* catfishes (Siluriformes, Heptapteridae) from Chiapas, Mexico

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## Abstract

Dedicated ichthyological surveys in four active karstic caves in the Mexican state of Chiapas (Grijalva River drainage basin) resulted in the discovery of the same number of hypogean populations of *Rhamdia* catfishes assignable to two different species: *R. laticauda* and *R. guatemalensis*. The taxonomic identity of these populations was initially determined based on morphological traits and subsequently corroborated with molecular data in a phylogenetic framework. For the most part, these newly discovered populations exhibit partial and variable troglomorphism (vs. fixed), a pattern that has been observed in most other cave-dwelling species/populations of Mexican *Rhamdia*, and possibly caused by gene flow with and/or incipient speciation from epigeal lineages. Since most hypogean forms of Mexican *Rhamdia* derive from/are part of a larger *R. laticauda* clade, our discovery of cave-dwelling populations assignable to *R. guatemalensis* is noteworthy and includes the very first record of a *R. guatemalensis* population with pronounced and widespread troglomorphism. Our discovery of hitherto unrecorded populations of hypogean *Rhamdia* highlights the continued importance of exploration in the process of documenting subterranean biodiver-

sity, particularly in regions of the world rich with cave systems. Our findings corroborate the notion that, among Neotropical fishes, the catfish genus *Rhamdia* is one of the most prone and effective at colonizing subterranean habitats and establishing viable hypogean populations.

### Keywords

Cavefishes, hypogean populations, troglobitic fishes, troglomorphism

## Introduction

Catfishes of the genus *Rhamdia* Bleeker, 1858 are a clade of Neotropical freshwater fishes widely distributed throughout the continent—from Mexico to Argentina—and with considerable taxonomic diversity (27 currently valid species) (Silfvergrip 1996; Perdices et al. 2002; Hernández et al. 2015; Angrizani and Malabarba 2020; Fricke et al. 2020). Remarkably, *Rhamdia* catfishes have a propensity to colonize subterranean habitats, particularly in the form of active karst caves, as evidenced by the fact that about a quarter of their overall species diversity, plus several populations lacking definite species adscription, are stygobitic (living exclusively in groundwater) (Arroyave and De La Cruz Fernández 2021a, b).

The six valid species of hypogean (cave-dwelling) and troglobitic (displaying phenotypic adaptations to cave life) *Rhamdia* are: the Brazilian *Rhamdia enfiurnada* (Bichuette and Trajano 2005), the Venezuelan *Rhamdia guasarensis* (DoNascimento et al. 2004), and the Mexican *Rhamdia reddelli* (Miller 1984), *Rhamdia zongolicensis* (Wilkens 1993), *Rhamdia macuspanensis* (Weber and Wilkens 1998), and *Rhamdia laluchensis* (Weber et al. 2003). Notably, all of them are characterized by being fully troglomorphic and microendemic to their respective type-locality caves, at least based on their original descriptions. Meanwhile, cave-dwelling *Rhamdia* populations of dubious taxonomic designation and with varying degree of troglomorphism have so far only been reported for Mexico (Robertson 1983; Mosier 1984; Arroyave and De La Cruz Fernández 2021a, b).

As evidenced by the above, the bulk of the known diversity of hypogean *Rhamdia* resides in Mexico, which is rather unsurprising, given that roughly 20% of Mexican territory is karstic landscape (Bautista 2023). In Mexico, *Rhamdia* catfishes have colonized karst formations in the southeast, with most populations (at least five taxonomically unassigned, although probably *R. laticauda*) and species (*R. zongolicensis* and *R. reddelli*) reported from the mountainous karst region of the Sierra de Zongolica, state of Veracruz, bordering with the state of Oaxaca, Papaloapan River basin (Arroyave and De La Cruz Fernández 2021a, b). Only after ~350 km southeast of Zongolica do other cave *Rhamdia* species occur, in the state of Chiapas (*R. laluchensis*) and further east in Tabasco (*R. macuspanensis*), in active caves that are part of the Grijalva-Usumascinta River basin (Arroyave and De La Cruz Fernández 2021b).

Besides cave-dwelling forms, Mexican *Rhamdia* include the surface species *Rhamdia laticauda*, *Rhamdia guatemalensis*, and *Rhamdia parryi* (Miller 2005). Existing research on the evolutionary history of *Rhamdia* in Middle America suggests that the diversity

of cave-dwelling lineages in the region derives from the epigeal and more widespread species *R. laticauda*, with the possibility that all Mexican cave-dwelling forms (including all four valid species) are in fact cave-adapted populations of *R. laticauda* (Perdices et al. 2002; Arroyave and De La Cruz Fernández 2021a, b). To date, the only known exception to this pattern (*R. laticauda* being the “ancestor” of all cave *Rhamdia* in Mexico) would be a non-troglophic cave-dwelling population of *Rhamdia* from the Grutas de Coconá (Tabasco), assignable to the surface species *R. guatemalensis* (Arroyave and De La Cruz Fernández 2021b).

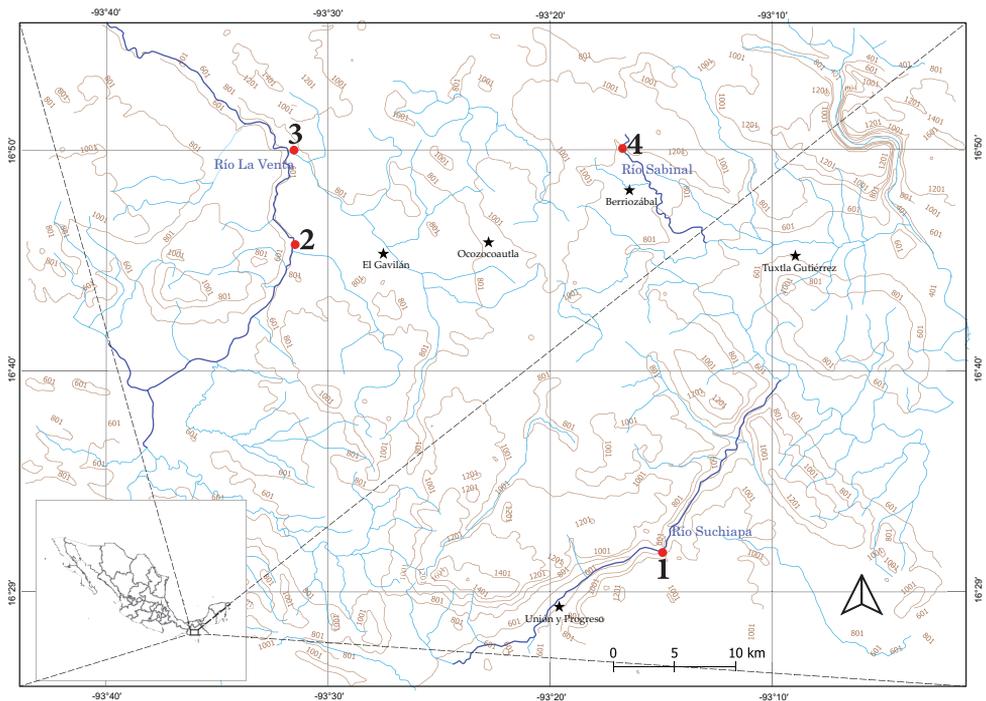
While a renewed interest and recent research including field surveys and exploration has resulted in improved understanding of the taxonomic diversity and evolutionary history of Mexican cave *Rhamdia* (Arroyave and De La Cruz Fernández 2021a, b), vast extensions of karstic landscape remain to be surveyed for the presence of hypogean populations. The state of Chiapas, with its rich karstic topography that includes the Sierra Madre de Chiapas, the Chiapas Highlands, and the Chiapas Depression, offers a promising target for the discovery and documentation of previously unknown and/or understudied hypogean populations of *Rhamdia*. Besides the formally described troglobitic species *Rhamdia laluchensis* (Weber et al. 2003), existing records of hypogean catfishes in caves of Chiapas are limited to a few reports from historical international speleological expeditions in the region and from anecdotal accounts from local spelunkers, namely: Sistema Pecho Blanco (Cintalapa de Figueroa) (Sbordoni et al. 1986; Sbordoni and Lucarelli 1989), Cueva de los dos Hermanos (Berriozabal) (Sbordoni et al. 2004), an unnamed cave in the Selva del Mercadito (Sbordoni and Lucarelli 1989), El Chorro Grande cave (Río Suchiapa) (<http://www.oztotl.com/ps/reports/El%20Chorro%20Grande.pdf>, <http://eksa.free.fr/chiapas2008/chiapas2008.pdf>), Paso Burro cave (Berriozabal), and Los Bordos and El Encanto caves (Río La Venta). None of these gray-literature and anecdotal reports, however, has been previously investigated and documented from a taxonomic standpoint. Consequently, we embarked on an ichthyological expedition devoted to survey and sample some of these caves. This study presents our findings, documenting hitherto unknown cave-dwelling populations of *Rhamdia* catfishes from the state of Chiapas, and shedding light on their taxonomic nature and evolutionary history based on analyses of morphological and genetic comparative data.

## Methods

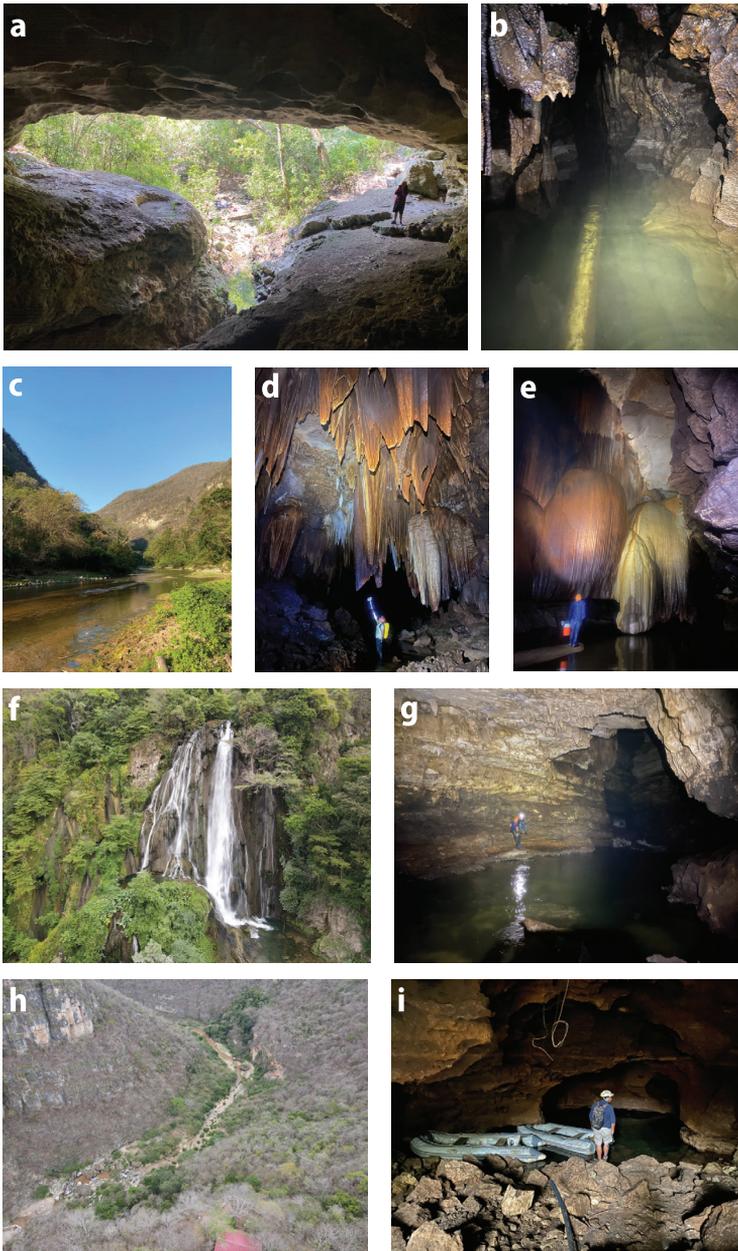
### Area of study

From March 17<sup>th</sup> to 25<sup>th</sup>, 2022, we conducted ichthyological surveys in four active, resurgence, semidry (vs. submerged), and relatively horizontal karstic caves located in the Mexican state of Chiapas, within the Grijalva River drainage basin, including three of its tributaries. Two of the surveyed caves, Los Bordos (16°49'48.70"N, 93°31'33.70"W) and El Encanto (16°45'26.8"N, 93°31'30.6"W), are part of the Río La Venta basin,

while the other two caves, Paso Burro ( $16^{\circ}49'53.3''\text{N}$ ,  $93^{\circ}16'29.2''\text{W}$ ) and El Chorro Grande ( $16^{\circ}31'13.0''\text{N}$ ,  $93^{\circ}14'39.0''\text{W}$ ), are part of the Río Sabinal and Río Suchiapa basins, respectively (Figs 1, 2). Los Bordos, a relatively high discharge cave containing rather deep ponds ( $-2$  m), develops a total of 5211 m in length and has a vertical gradient of  $+58$  m from the entrance (Barbe and Morenas 1989). El Encanto is a much shorter cave (364 m) and with a much smaller vertical gradient ( $-4$ ,  $+9$  m) (Whitaker 1988; Barbe and Morenas 1989). The resurgence of El Chorro Grande opens at the bottom of the Río Suchiapa canyon, and, in the rainy season, the water gushes violently from the porch, hence its name (The Great Jet). El Chorro Grande cave develops 9650 m and has a vertical gradient of  $+175$  m throughout its entire length (<http://eksa.free.fr/chiapas2002/chiapas2002.pdf>). The resurgence of Paso Burro feeds the Sabinal River and provides water for the municipality of Berriozabal (Whitaker 1988). Los Bordos and El Chorro Grande are relatively remote, hard-to-reach, non-touristy caves that require considerable hiking time and effort (mostly through the Río La Venta riverbed) followed by a rather steep ascent through the canyon wall up to the cave entrance. Conversely, El Encanto (also known as Cueva del Aguacero) is a touristy cave easily accessible by motor vehicle and therefore barely requiring any hiking. In between these extremes is Paso Burro cave, which only requires a relatively short and effortless hike from the point of closest car access.



**Figure 1.** Map of the area of study showing the location of the surveyed caves with hitherto undocumented populations of hypogean *Rhambdia*. Cave localities are indicated by red dots and correspond to: **1** El Chorro Grande cave (Río Suchiapa basin) **2** El Encanto cave (Río La Venta basin) **3** Los Bordos cave (Río La Venta basin), and **4** Paso Burro cave (Río Sabinal basin).



**Figure 2.** Images of hypogean and epigean localities surveyed in this study **a** main entrance of Paso Burro cave **b** interior of Paso Burro cave where it can be seen an underwater aqueduct pipe traversing the main channel **c** Río Suchiapa riverbed near the access point to El Chorro Grande cave **d, e** Inside of El Chorro Grande cave, showing its massive cross-section size and different types of correspondingly enormous speleothems **f** aerial view of La Conchuda waterfall, which flows into the Río La Venta from the Los Bordos cave **g** interior of the Los Bordos cave showing a pool where fishes were collected **h** aerial view of the Río La Venta canyon at El Aguacero waterfall, near El Encanto cave (located in the same premises as the red roof house seen at the bottom of the photograph **i** inside El Encanto cave at entrance point, showing boats normally used by tourists who want to navigate the interior of the cave.

## Specimen sampling and preservation

We collected cave-dwelling *Rhamdia* specimens using baited minnow traps deployed along the main longitudinal axis of the cave, up until our point of maximum penetration (approximately a few hundred meters in all four caves). For comparative purposes, in addition to hypogean populations, we sampled epigeal *Rhamdia* by means of electrofishing at river locations in the vicinity of the sampled caves. We were able to sample epigeal populations nearby all surveyed caves except El Chorro Grande (Río Suchiapa basin). After capture, we euthanized the fishes using the anesthetic tricaine mesylate (MS-222) and then took tissue samples (fin clips) for genetic studies. Tissues were preserved in 96% ethanol and eventually cryopreserved at  $-80\text{ }^{\circ}\text{C}$ . After tissueing, we fixed voucher specimens using a 10% formalin solution. Back in the lab, we washed formalin-fixed specimens and then transferred them to 70% ethanol for long-term storage in the Colección Nacional de Peces (CNPE) of the Instituto de Biología, Universidad Nacional Autónoma de México (IBUNAM), where they have been catalogued and deposited (Table 1). Fishes were handled in accordance with recommended guidelines for the use of fishes in research (Jenkins et al. 2014). Specimens were collected under permit SGPA/DGVS/08073/21 issued by the Mexican Ministry of Environment and Natural Resources (Secretaría de Medio Ambiente y Recursos Naturales; SEMARNAT).

## Comparative data generation and analysis

To document patterns of phenotypic and genetic variation, and to shed light on the taxonomic nature of these newly discovered cave-dwelling *Rhamdia* populations, we collected morphometric and meristic data from all hypogean specimens sampled, as well as DNA sequence data from a reduced subset. Traditional morphometric measurements and meristic counts follow previous taxonomic studies of *Rhamdia* (Silfvergrip 1996; Hernández et al. 2015). All measurements were taken on the left side of the specimen using a Mitutoyo digital caliper (precision = 0.1 mm; accuracy =  $\pm 0.02$  mm). To generate comparative genetic data, we first extracted total genomic DNA from fresh tissue samples using the Qiagen DNeasy Tissue Extraction Kit, following the manufacturer's protocol. Subsequently, we amplified and sequenced a partial fragment ( $\sim 650$  bp) of the mitochondrial marker cytochrome c oxidase subunit I (COI) using the primer pairs LCO1490/HCO2198 (Folmer et al. 1994). DNA extraction, amplification, and sequencing were carried out at Laboratorio de Secuenciación Genómica de la Biodiversidad y de la Salud (Instituto de Biología, UNAM), in-house Sanger sequencing facilities. Contig assembly, sequence editing, and multiple sequence alignment were accomplished using Geneious Prime 2023.0.2 (<https://www.geneious.com>). We sequenced a total of 18 individuals: eight representing all newfound hypogean populations and 10 from epigeal populations immediately adjacent to three of the four caves surveyed (Los Bordos, El Encanto, and Paso Burro) (Table 1). To broaden the taxonomic and geographic coverage of samples used for phylogenetic analysis, we also mined additional *Rhamdia* COI sequences from GenBank ([www.ncbi.nlm.nih.gov/Genbank](http://www.ncbi.nlm.nih.gov/Genbank)) and BOLD ([www.barcodelifelife.org](http://www.barcodelifelife.org)) (37) and from unpublished data (26) previously generated by the senior au-

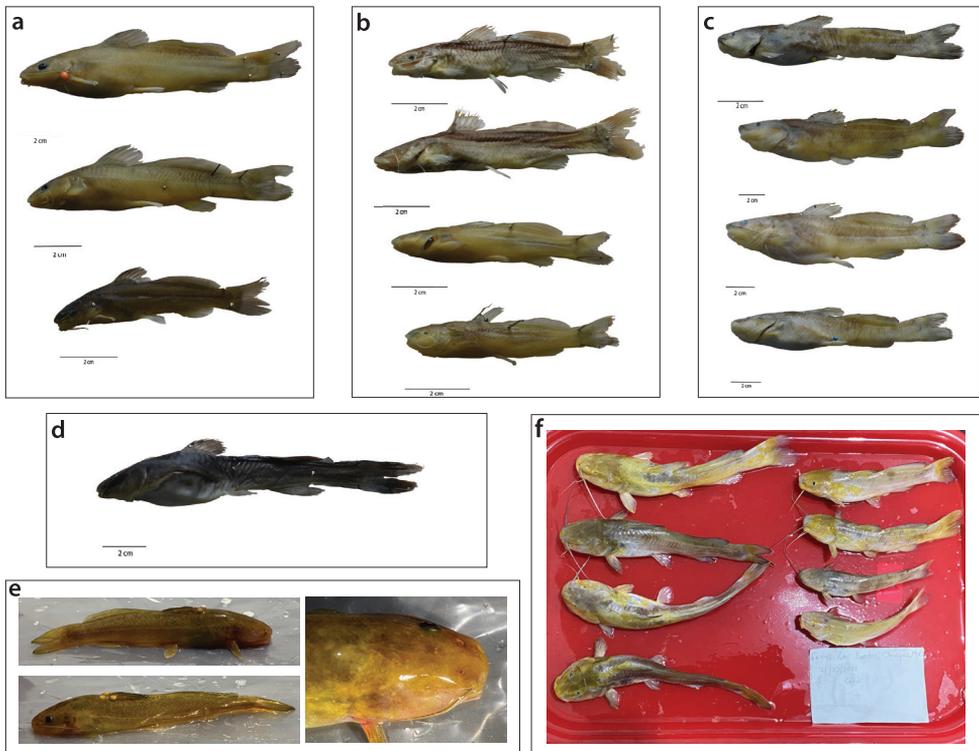
**Table 1.** *Rhamdia* specimens collected during the fieldwork component of this study, including samples from four newly discovered hypogean populations and from four epigeal populations in the vicinity of three of the surveyed caves, with their respective catalog and voucher numbers. GenBank accession numbers correspond to COI sequenced data generated herein and used for phylogenetic analysis.

Habitat	Locality	Coordinates	Basin	Species	Catalog	Voucher	COI GenBank accession
Hypogean	Los Bordos cave	16°49'48.70"N, 93°31'33.70"W	La Venta	<i>Rhamdia guatemalensis</i>	CNPE-IBUNAM 24020	JA1294	OR512373
					CNPE-IBUNAM 24020	JA1295	OR512374
					CNPE-IBUNAM 24020	JA1296	OR512375
					CNPE-IBUNAM 24020	JA1297	n/a
					CNPE-IBUNAM 24020	JA1298	n/a
					CNPE-IBUNAM 24020	JA1299	n/a
					CNPE-IBUNAM 24020	JA1300	n/a
		CNPE-IBUNAM 24020	JA1301	n/a			
	El Encanto cave	16°45'26.8"N, 93°31'30.6"W	La Venta	<i>Rhamdia guatemalensis</i>	CNPE-IBUNAM 24021	JA1412	OR512387
	Paso Burro cave	16°49'53.3"N, 93°16'29.2"W	Sabinal	<i>Rhamdia laticauda</i>	CNPE-IBUNAM 24022	JA1272	OR512370
					CNPE-IBUNAM 24022	JA1273	OR512371
					CNPE-IBUNAM 24022	JA1309	OR512379
					CNPE-IBUNAM 24022	JA1310	n/a
					CNPE-IBUNAM 24022	JA1311	n/a
El Chorro Grande cave	16°31'13.0"N, 93°14'39.0"W	Suchiapa	<i>Rhamdia laticauda</i>	CNPE-IBUNAM 24023	JA1275	n/a	
				CNPE-IBUNAM 24023	JA1276	OR512372	
				CNPE-IBUNAM 24023	JA1277	n/a	
				CNPE-IBUNAM 24023	JA1278	n/a	
				CNPE-IBUNAM 24023	JA1279	n/a	
				CNPE-IBUNAM 24023	JA1280	n/a	
				CNPE-IBUNAM 24023	JLP3R3	n/a	
	CNPE-IBUNAM 24023	JLP1R1	n/a				
Epigeal	Río La Venta at La Conchuda waterfall	16°49'57.42"N, 93°31'52.3"W	La Venta	<i>Rhamdia guatemalensis</i>	CNPE-IBUNAM 24024	JA1302	OR512376
					CNPE-IBUNAM 24024	JA1303	OR512377
					CNPE-IBUNAM 24024	JA1304	OR512378
	Río La Venta at El Aguacero waterfall	16°45'40.16"N, 93°31'33.1"W	La Venta	<i>Rhamdia guatemalensis</i>	CNPE-IBUNAM 24027	JA1359	OR512384
					CNPE-IBUNAM 24027	JA1360	OR512385
					CNPE-IBUNAM 24027	JA1361	OR512386
	Río Sabinal at Paso Burro cave outflow	16°49'33.3"N, 93°16'13.7"W	Sabinal	<i>Rhamdia guatemalensis</i>	CNPE-IBUNAM 24025	JA1314	OR512380
					CNPE-IBUNAM 24025	JA1315	OR512381
					CNPE-IBUNAM 24025	JA1316	OR512382
			<i>Rhamdia laticauda</i>	CNPE-IBUNAM 24026	JA1320	OR512383	

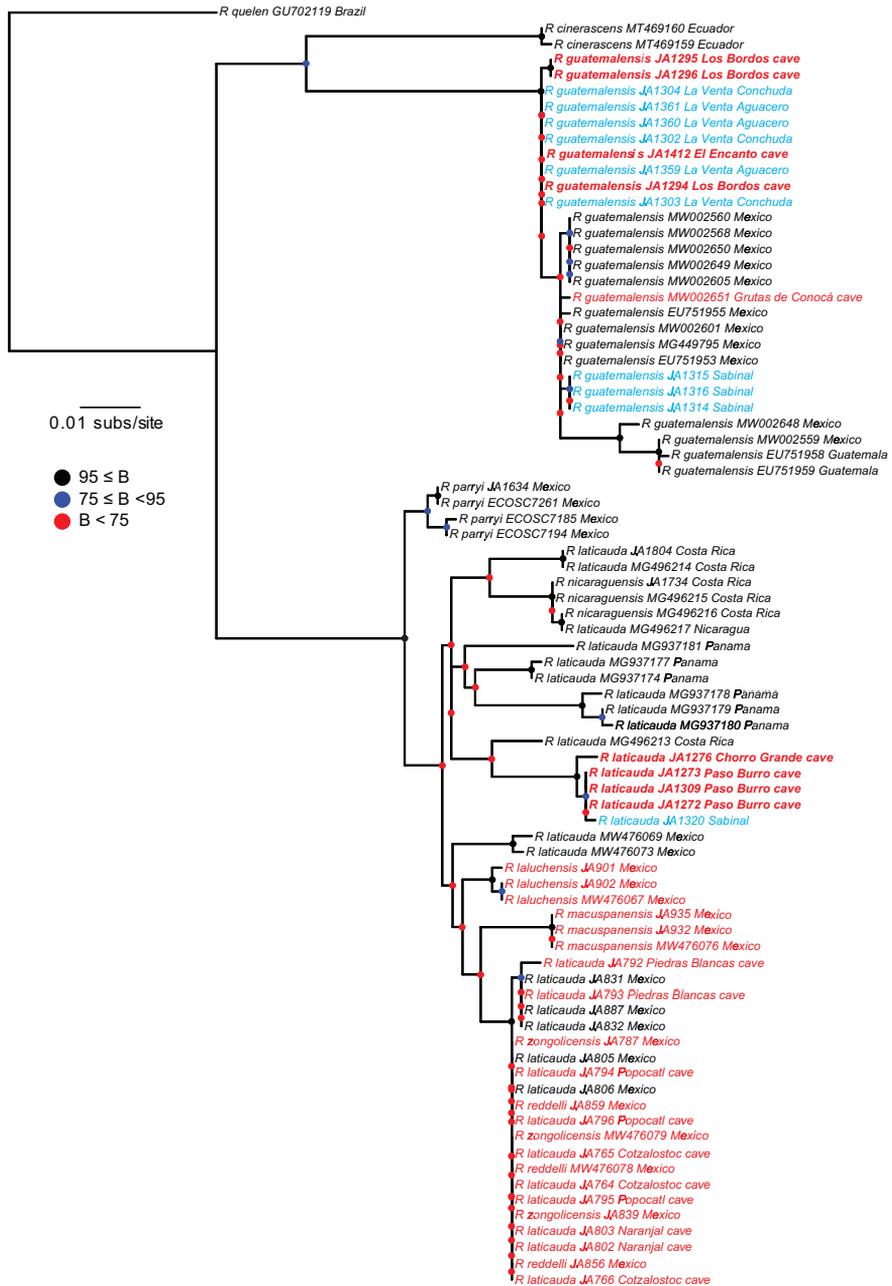
thor (JA). Ultimately, we assembled a molecular data matrix that included most species (90%) of the trans-Andean/Middle American *Rhamdia* clade, totaling 81 terminals and 640 aligned positions. We inferred a phylogenetic tree based on the resulting COI matrix (with *Rhamdia quelen* as outgroup) using the software RAXML-NG (v. 1.0.1) (Kozlov et al. 2019) under the HKY+I+G model of molecular evolution. Statistical selection of the best-fit model of nucleotide substitution was implemented with the software jModelTest2 (v. 2.1.10) (Darriba et al. 2012) under the following likelihood settings: number of substitution schemes = 3; base frequencies = +F; rate variation = +I and +G with nCat = 4; base tree for likelihood calculations = ML optimized; and base tree search = NNI, effectively evaluating 24 models. Clade support was estimated using the bootstrap character resampling method (Felsenstein 1985) based on 1000 pseudoreplicates.

## Results

Our ichthyological surveys in four cave systems in the state of Chiapas, Mexico, resulted in the discovery of four hitherto undocumented hypogean populations of catfishes of the genus *Rhamdia*, three of which with individuals only partially (vs. completely) troglomorphic, and to a varying degree (Fig. 3). Despite its cave-dwelling nature, the only specimen collected at El Encanto cave displayed normal (non-troglomorphic) phenotype (Fig. 3). Specimens from these four newly discovered populations were initially ascribed, based on external morphology, to the epigeic species *R. guatemalensis* (Los Bordos and El Encanto caves) and *R. laticauda* (Paso Burro and El Chorro Grande caves), a result that was further corroborated by comparative molecular data in a phylogenetic context (Fig. 4). Morphological and meristic data from the specimens collected at the surveyed caves are presented in Tables 2, 3. The phylogeny presented in Fig. 4 clearly shows that



**Figure 3.** Specimens of newly discovered populations of hypogean *Rhamdia* from Chiapas, showing observable external morphological features, including variation in the degree of troglomorphy (eye reduction and depigmentation) **a** preserved specimens of *R. laticauda* from Paso Burro cave **b** preserved specimens of *R. laticauda* from El Chorro Grande cave **c** preserved specimens of *R. guatemalensis* from Los Bordos cave **d** preserved specimen of *R. guatemalensis* from El Encanto cave **e** fresh specimen (prior to euthanasia and formalin fixation) of *R. laticauda* from Paso Burro cave in lateral (body, both sides) and dorsal (head) views, displaying partial troglomorphy characterized by depigmentation and complete absence of the right eye **f** fresh specimens of *R. guatemalensis* from Los Bordos cave, displaying a wide spectrum of variation in the degree of troglomorphy, including complete loss of eyes and pigmentation.



**Figure 4.** Phylogenetic tree of Middle American *Rhambdia* based on comparative COI sequence data, highlighting the phylogenetic position of individuals from the newly discovered hypogean populations as well as from epigeal populations near the surveyed caves. Cave-dwelling species/populations in red (newfound in bold). Epigeal species/populations in black, except for localities surveyed herein, in blue. Terminals corresponding to COI sequences obtained from GenBank ([www.ncbi.nlm.nih.gov/Genbank](http://www.ncbi.nlm.nih.gov/Genbank)), BOLD ([www.barcodinglife.org](http://www.barcodinglife.org)), or from unpublished data previously generated by the senior author (JA), include the corresponding accession or voucher/catalog number in the label (between the species name and the country of origin). Colored circles on nodes indicate degree of clade support as determined by bootstrap values (B).

the newly discovered cave-dwelling populations of *Rhamdia* documented herein (colored in bold red) are well nested within two major species-level clades that correspond to the epigean *R. guatemalensis* (Los Bordos and El Encanto caves) and *R. laticauda* (Paso Burro and El Chorro Grande caves), respectively. The phylogeny also shows that samples from Los Bordos and El Encanto are closely associated with and almost genetically indistinguishable from epigean samples from the Río La Venta (colored in blue) collected at El Aguacero and La Conchuda waterfalls. Within the *R. guatemalensis* clade, the only other known hypogean population is found in the Grutas de Coconá cave system, in the state of Tabasco. As shown in the phylogeny, the bulk of the diversity of hypogean Middle American *Rhamdia* is however represented by populations of *R. laticauda*, including two documented for the first time in this study: El Chorro Grande and Paso Burro caves. Unsurprisingly, the most closely related sample to those from Paso Burro cave is from an epigean population found in the river flowing out of the cave (Río Sabinal). While there is strong nodal support for the clade consisting of El Chorro Grande, Paso Burro, and Sabinal samples, its relationship to other components of the *R. laticauda* radiation is rather uncertain (due to the low support of the nodes involved). Despite this fact, it appears that this clade is more closely related to *R. laticauda* Central American lineages (including *R. nicaraguensis*) than to those occurring further north in Mexico, including cave-dwelling species and populations found in Veracruz (*R. zongolicensis* and hypogean *R. laticauda*), Oaxaca (*R. reddelli*), Chiapas (*R. laluchensis*), and Tabasco (*R. macuspanensis*).

**Table 2.** Meristic comparative data from specimens from the newly discovered hypogean populations. Meristic traits abbreviations as follows: PFR = pectoral-fin rays, PvFR = pelvic-fin rays, DFR = dorsal-fin rays, ARF = anal-fin rays, uCFR = upper caudal-fin rays, and lCFR = lower caudal-fin rays. Caudal-fin rays numbers (x,y,z) correspond to unsegmented (x), unbranched segmented (y), and branched segmented (z) rays.

Cave	Species	Voucher	PFR	PvFR	DFR	ARF	uCFR	lCFR
Paso Burro	<i>Rhamdia laticauda</i>	JA1272	1-7	6	1-7	8	4,2,6	4,2,5
		JA1273	1-8	6	1-6	7	4,2,6	4,2,5
		JA1309	1-9	6	1-7	9	5,2,6	4,2,5
		JA1310	1-10	6	1-7	10	5,2,6	4,2,5
		JA1311	1-10	6	1-7	10	4,2,6	4,2,5
		JA1312	1-9	6	1-7	10	4,2,6	4,2,5
		JA1313	1-9	5	1-7	10	4,2,6	4,2,5
El Chorro Grande	<i>Rhamdia laticauda</i>	JA1275	1-8	5	1-5	8	4,2,6	4,2,4
		JA1276	1-8	6	1-7	9	4,2,6	4,2,5
		JA1277	1-7	5	1-6	8	4,2,6	4,2,5
		JA1278	1-7	6	1-6	9	4,2,6	4,2,4
		JA1279	1-8	6	1-6	9	4,2,6	4,2,5
		JA1280	1-8	5	1-6	7	4,2,6	4,2,5
		JLP1R1	1-8	7	1-6	10	4,2,6	4,2,5
Los Bordos	<i>Rhamdia guatemalensis</i>	JLP3L3	1-6	6	1-6	10	4,2,6	4,2,5
		JA1294	1-9	6	1-6	10	4,2,6	4,2,6
		JA1295	1-7	6	1-6	10	4,2,5	3,2,5
		JA1296	1-7	6	1-6	11	4,2,6	4,2,5
		JA1297	1-7	6	1-6	10	4,2,6	4,2,6
		JA1298	1-7	6	1-6	10	4,2,6	4,2,6
		JA1299	1-7	6	1-6	10	4,2,6	4,2,6
El Encanto	<i>Rhamdia guatemalensis</i>	JA1300	1-7	6	1-6	11	5,2,6	4,2,5
		JA1301	1-7	6	1-6	10	4,2,6	4,2,6
		JA1412	1-9	6	1-7	6	7,2,5	6,2,4

**Table 3.** Morphometric comparative data from specimens from the newly discovered hypogean populations. Measurements abbreviations as follows: SL = Standard Length, HL = Head Length, HL = Head Length, BW = Body Width, DFH = Dorsal Fin Height, DSH = Dorsal-fin Spine Height, AFL = Anal Fin Length, AdFL = Adipose Fin Length, PFL = Pectoral Fin Length, PSL = Pectoral-fin Spine Length, PvFL = Pelvic Fin Length, ISL = Interdorsal Space Length, CPL = Caudal Peduncle Length, CPD = Caudal Peduncle Depth, IOW = Interorbital Width, ORB = Orbital Diameter, SNT = Snout Length, MBL = Maxillary Barbel Length, MdBL = Mandibular Barbel Length, MeBL = Mental Barbel Length.

	<i>Rhamdia laticauda</i>						<i>Rhamdia guatemalensis</i>			
	Paso Burro (N = 7)			El Chorro Grande (N = 8)			Los Bordos (N = 8)			El Encanto (N = 1)
	Range	Mean	SD	Range	Mean	SD	Range	Mean	SD	Value
<b>mm</b>										
SL	57.3–122.2	88.07	23.15	48.8–89.9	67.41	14.18	116.6–183.2	150.26	25.45	125.70
HL	13.2–22.2	17.56	3.48	11.1–17.7	13.80	2.38	25.6–42.7	33.93	6.89	23.70
<b>% SL</b>										
HL	17.87–23.73	20.30	2.12	18.41–21.17	20.16	1.04	20.76–24.52	22.47	1.36	18.85
BW	17.04–18.32	17.72	0.40	17.38–18.80	18.16	0.45	17.84–19.94	18.79	0.75	17.42
DFH	9.72–15.36	12.10	1.82	7.30–13.25	10.49	2.29	9.32–11.90	10.47	0.99	12.89
DSH	2.79–6.34	4.84	1.26	4.04–7.34	6.02	0.99	6.25–8.83	7.79	0.84	9.31
AFL	4.03–6.26	5.22	0.86	3.51–7.22	4.86	1.38	4.29–6.90	5.83	0.94	3.26
AdFL	31.73–36.36	34.39	1.59	22.02–32.22	26.51	3.35	27.77–34.29	31.36	2.19	43.91
PFL	10.61–15.01	12.23	1.36	12.53–14.76	13.38	0.85	11.88–13.88	12.82	0.66	13.92
PSL	3.26–5.76	4.49	0.79	5.62–9.45	6.84	1.30	6.65–10.26	9.09	1.26	8.99
PvFL	11.64–13.78	12.47	0.89	10.43–12.39	11.76	0.57	10.15–13.29	11.30	1.03	12.81
ISL	8.59–9.95	9.47	0.48	9.30–17.79	14.49	2.68	5.95–9.69	7.12	1.32	1.75
CPL	20.19–23.21	21.75	1.20	17.40–23.41	20.89	2.07	23.04–24.64	23.88	0.51	24.26
CPD	9.78–10.41	10.07	0.23	7.73–10.90	9.62	1.07	9.52–11.89	10.60	0.91	7.16
<b>% HL</b>										
IOW	31.61–38.86	36.45	2.80	36.44–47.75	41.95	3.82	33.59–44.00	37.84	3.54	51.90
ORB	14.07–20.00	17.67	2.24	2.50–11.41	7.68	3.49	5.86–13.96	9.34	2.66	18.14
SNT	34.37–43.43	39.45	3.31	39.84–47.75	42.54	2.81	38.53–48.80	42.72	3.64	38.40
MBL	100–172	129.11	25.14	114.69–177.48	153.99	23.31	151.54–201.04	178.54	19.27	228.69
MdBL	60.57–81.82	69.54	6.93	38.46–86.18	68.41	15.75	65.48–98.62	78.88	11.68	91.56
MeBL	32.16–47.75	39.01	6.73	34.23–59.89	43.42	8.55	36.72–49.48	43.18	5.19	55.70

### Discussion

The catfish genus *Rhamdia* is unarguably one of the most successful groups of freshwater fishes at colonizing hypogean habitats, particularly in Mexico, where most cave-dwelling species/populations are found, mainly in the Sierra de Zongolica, a karstic mountainous region that drains tributaries of the Papaloapan River basin in the state of Veracruz (Arroyave and De La Cruz Fernández 2021a). Prior to this study, the only taxonomically authoritative record of a cave-dwelling form of *Rhamdia* from the state of Chiapas corresponded to the species *R. laluchensis*, microendemic from the Sótano de La Lucha, a pit cave on the Grijalva River basin (Weber et al. 2003). The few grey-literature and anecdotal records of hypogean *Rhamdia* from Chiapas had neither been corroborated nor further investigated to establish the taxonomic nature of these populations. Our ichthyological surveys of four of these purportedly cavefish-harboring caves resulted in the rather unsurprising discovery of what appear to be well-established populations of hypogean *Rhamdia*, thus bringing the number of taxonomi-

cally verified (including catalogued vouchers) cave-dwelling populations of *Rhamdia* for the state of Chiapas up to five.

A first notable discovery from this study is that the *Rhamdia* specimens collected inside the surveyed caves do not represent undescribed species but populations of more widespread epigeal species, specifically of *R. laticauda* and *R. guatemalensis*, and that each cave only harbors a single catfish species. Both morphological and molecular evidence strongly support this conclusion (Figs 3, 4). Whereas morphological identification of these populations was relatively straightforward (primarily based on pectoral spine serration pattern), characters traditionally used to distinguish between *R. laticauda* and *R. guatemalensis*, such as interdorsal space and adipose fin length, were not as clear-cut as expected (*R. laticauda* normally having a shorter adipose fin and correspondingly longer interdorsal space) (Table 3). Regardless of this potentially confounding morphological observation, phylogenetic analysis of comparative COI sequence data unequivocally resolved the populations from Paso Burro and El Chorro Grande as *R. laticauda* and those from Los Bordos and El Encanto as *R. guatemalensis* (Fig. 4). Furthermore, COI pairwise genetic distances between the newly discovered hypogean populations and their respective epigeal species never exceeded the traditionally employed ~3% sequence divergence heuristic threshold for conspecifics (Hebert et al. 2003), providing additional support for the abovementioned species-level designation. Although our phylogenetic results also expose the non-monophyly of *R. laticauda* samples with respect to *R. nicaraguensis*, this pattern has been previously reported and discussed (Perdices et al. 2002; Arroyave and De La Cruz Fernández 2021b), and while deserving of further scrutiny, it is not the topic of our present study. This is, however, the subject of ongoing research by the senior author (JA) and collaborators, who are using genome-wide molecular markers and denser taxonomic and geographic sampling to address the systematics of the genus in Middle America.

The resulting phylogeny (Fig. 4) also makes absolute sense biogeographically, with samples from Paso Burro closely related—and almost genetically identical—to the epigeal sample from Río Sabinal (river flowing out of the cave), and these samples altogether forming a clade sister to the sample from El Chorro Grande cave. Similarly, samples from Los Bordos and El Encanto caves were mainly resolved well nested within epigeal samples from El Aguacero and La Conchuda waterfalls, an unsurprising result given that all four localities are part of the Río La Venta basin and in close proximity (no more than 10 km apart from each other). Furthermore, El Aguacero and La Conchuda waterfalls are located almost immediately outside of El Encanto and Los Bordos caves, respectively. Notably, the branch subtending the clade consisting of Paso Burro and El Chorro Grande samples (inclusive of the Río Sabinal sample) is particularly long, even longer than that of any of the troglotic species currently recognized as distinct and valid (although nested within the *R. laticauda* radiation). While the causes behind such large genetic divergence are unclear at this point, this pattern certainly deserves attention and future scrutiny.

Another noteworthy finding of our study has to do with patterns of troglomorphy in cave *Rhamdia*. Except for El Encanto, for which no troglomorphic individuals were sampled (likely because of the small sample size,  $N = 1$ ), all surveyed caves contained

fishes with varying degree of troglomorphism (Fig. 3). This finding offers further support to the notion that hypogean fishes will eventually evolve a troglobitic phenotype characterized by eye reduction/loss and depigmentation, likely as adaptation to life in aphotic subterranean environments (Wilkins and Strecker 2017). Notably, complete troglomorphism (complete eye loss and depigmentation) is not fixed in any of the newly discovered hypogean populations. Instead, this condition is variable and only partial in most individuals sampled (Fig. 3). Partial troglomorphism in these populations is characterized by a varying degree of depigmentation and eye reduction (from normal to completely absent eyes). Remarkably, a specimen of hypogean *R. laticauda* from Paso Burro cave was found to display asymmetrical troglomorphism, lacking the right eye while still having a seemingly normal left one (Fig. 3e). The herein observed pattern of partial and variable troglomorphism conforms with recent research on Mexican cave-dwelling *Rhamdia* (Arroyave and De La Cruz Fernández 2021b), which uncovered that species believed to be completely troglomorphic, as per their original descriptions (Miller 1984; Wilkins 1993; Weber and Wilkins 1998; Weber et al. 2003), in reality display a spectrum of variation in the degree of troglomorphism. Such variable troglomorphism was also explicitly reported in the original description of the Brazilian *R. enfernada* (Bichuette and Trajano 2005). Similarly, hypogean populations of *R. laticauda* from the Sierra de Zongolica have also been shown to exhibit clinical morphological variation in regressive troglomorphic traits (Arroyave and De La Cruz Fernández 2021a). Altogether, these findings suggest that varying (vs. fixed) troglomorphism might be the norm rather than the exception in hypogean *Rhamdia*. Although the exact causes behind this pattern have yet to be uncovered, time since cave colonization (incipient speciation) and crossings between hypogean and epigeal individuals (gene flow) might be the main drivers constraining fixation of complete troglomorphism in these populations, a hypothesis recently advanced by previous authors (Arroyave and De La Cruz Fernández 2021b).

Admittedly, the discovery of hypogean populations of *R. guatemalensis* was contrary to our expectations, given that almost all known lineages of Mexican cave-dwelling *Rhamdia* derive from/are part of the *R. laticauda* radiation (Arroyave and De La Cruz Fernández 2021a, b). To our knowledge, the only prior record of a fully subterranean population of *R. guatemalensis* corresponds to the Grutas de Coconá cave system in the state of Tabasco (CNPE-IBUNAM 23815), with all three sampled specimens displaying a normal, non-troglomorphic phenotype (Arroyave and De La Cruz Fernández 2021b). As in the case of El Encanto cave, given this small sample size ( $N = 3$ ), the existence of troglomorphic individuals in the population from Grutas de Coconá cannot be completely discounted. In any case, our discovery of two novel cave-dwelling populations of *R. guatemalensis* is noteworthy, particularly the one from Los Bordos cave, which effectively constitutes the very first record of a hypogean population of *R. guatemalensis* with pronounced and widespread troglomorphism (Fig. 3c, f). While some populations of *R. guatemalensis* from cenotes (karstic sinkholes) in the Yucatán Peninsula aquifer are considered somewhat hypogean, to the extent that they have been assigned subspecies status due to their incipient troglomorphism (i.e., *R. g. decolor* and *R. g. stygaea*) (Hubbs 1936), these populations are, strictly speaking, not troglobitic but troloxenes, for the cenote is

in most cases not a subterranean but an epigeal habitat, and these fishes appear to only rarely venture into the cavern and cave zones of the aquifer (Arroyave et al. 2021).

We believe that the existence of hypogean fish populations, regardless of their taxonomic/phylogenetic distinctiveness, should eventually result in conservation efforts aimed at protecting them and their habitats. While the taxonomic rank of subspecies has been proposed to be of conservation utility (Phillimore and Owens 2006), it is our view that subspecies should be recognized on the basis of evidence beyond ecological/environmental singularity (e.g., cave-dwelling nature), such as discontinuities in the geographical distribution of phenotypic traits and some degree of phylogenetic distinctiveness and phylogeographic substructure. For this reason, as tempting as it is to propose subspecies names for each of the hypogean populations documented herein (with the ensuing conservation implications), we refrain from this course of action, at least until future research results in conclusive evidence in support of not only ecological but also evolutionary uniqueness. Notwithstanding this taxonomic resolve, we urge environmental policy-makers to acknowledge the ecological uniqueness of subterranean populations as diversity worth preserving.

Our discovery and documentation of hitherto taxonomically non-verified populations of hypogean *Rhamdia* highlights the continued importance of exploration in the process of documenting subterranean biodiversity, particularly in regions of the world rich with cave systems. Our findings corroborate the notion that, among Neotropical fishes, *Rhamdia* catfishes are one of the most prone and effective genera at colonizing subterranean habitats and establishing viable hypogean populations. One implication of this being that the diversity of cave-dwelling *Rhamdia* is most likely grossly underestimated, not only in Mexico but also throughout the distribution range of the genus. Consequently, much more exploration and taxonomically sound documentation work are needed to reach a more accurate picture of its hypogean diversity and ultimately about its evolutionary history.

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