Repeated colonization of caves leads to phenotypic convergence in catfishes (Siluriformes: *Trichomycterus*) at a small geographical scale

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Across various animal groups, adaptation to the challenging conditions of cave environments has resulted in convergent evolution. We document a Neotropical cavefish system with ample potential to study questions related to convergent adaptation to cave environments at the population level. In the karstic region of the Andes of Santander, Colombia, cave-dwelling catfish in the genus *Trichomycterus* exhibit variable levels of reduction of eyes and body pigmentation relative to surface congeners. We tested whether cave-dwelling, eye-reduced, depigmented *Trichomycterus* from separate caves in Santander were the result of a single event of cave colonization and subsequent dispersal, or of multiple colonizations to caves by surface ancestors followed by phenotypic convergence. Using mitochondrial DNA sequences to reconstruct phylogenetic relationships, we found that caves in this region have been colonized independently by two separate clades. Additional events of cave colonization – and possibly recolonization of surface streams – may have occurred in one of the clades, where surface and cave-dwelling populations exhibit shallow differentiation, suggesting recent divergence or divergence with gene flow. We also identify potentially undescribed species and likely problems with the circumscription of named taxa. The system appears promising for studies on a wide range of ecological and evolutionary questions.

ADDITIONAL KEYWORDS: adaptation – Andes – freshwater fishes – Neotropics – phylogeny – taxonomy.

INTRODUCTION

Animals living in caves have long been of interest in the study of adaptation by natural selection and evolutionary convergence (Juan *et al.*, 2010). Across various groups – including insects in different orders (Barr, 1962; Vidlicka *et al.*, 2003; Espinasa & Fisher, 2006), arachnids (Chamberlin & Malcolm, 1960; Lourenco & Pham, 2010; Arnedo *et al.*, 2008), crustaceans (Banta, 2001; Messana *et al.*, 2002; Campos, 2017) and several vertebrates (Wiens *et al.*, 2003) – adaptation to the particularly challenging conditions of cave environments has resulted in multiple instances of convergent evolution, involving a variety of morphological, physiological and behavioral traits (Jeffery, 2001; Protas *et al.*, 2011; Romero, 2011; Christiansen, 2012). Some of the most striking examples of convergent evolution are found in cave-dwelling fish of various distantly related lineages, which have consistently lost eyes and body pigmentation in comparison to their surface-living relatives (Proudlove, 2010; Borowsky, 2018; Niemiller *et al.* 2019).

Convergent evolution resulting from adaptation to cave environments has also been observed at the population level in fish, with a notable example being the Mexican tetra, *Astyanax mexicanus* (De Filippi, 1853), a well-studied taxon in which polyphyletic cave populations have reduced eyes and pigmentation, and unique aspects of metabolism and behaviour, compared to closely related surface populations

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of the same species (Espinasa & Borowsky, 2001; Yoshizawa *et al.*, 2010; Kowalko *et al.*, 2013; Moran *et al.*, 2015; Riddle *et al.*, 2018). Despite their striking phenotypic differences, surface and cave populations of *A. mexicanus* are connected by gene flow, suggesting that divergence in cave specialists has been driven and maintained by selection counteracting the homogenizing effects of migration (Bradic *et al.*, 2012; Herman *et al.*, 2018). The Mexican tetra has emerged as a model system to study the genetic and epigenetic basis of adaptation (Jeffery, 2001, 2009; Gross, 2012; Rohner *et al.*, 2013; Gross *et al.*, 2015; Gore *et al.*, 2018). Whether insights from the Mexican tetra system have broad applicability to other clades adapting to caves remains to be seen. We here document a Neotropical cavefish system with ample potential to study questions related to adaptation to cave environments at the population level and to more broadly test for evolutionary convergence (Blount *et al.*, 2018).

The eastern Cordillera of the Andes of Colombia harbours a vast karst limestone region with hundreds of caves, most of them in the Department of Santander (Muñoz-Saba *et al.*, 2013). Recent biological explorations of caves in this region have documented a wide diversity of animals restricted to cave environments, several of them endemic, including crabs, arachnids and fish (Villareal & García, 2016; Campos, 2017; Mesa S. *et al.*, 2018). Among the most peculiar organisms reported in these caves are seven species of catfish in the genus *Trichomycterus* Valenciennes, 1832, which coexist regionally with 12 surface species of the same genus (Ardila-Rodríguez, 2007, 2018; Castellanos-Morales *et al.*, 2011; Castellanos-Morales, 2018; Mesa S. *et al.,* 2018; Fig. 1; Table 1). Cave-dwelling species of *Trichomycterus* show a variable degree of reduction of eyes and body pigmentation relative to surface species (Fig. 2); some subterranean species are pigmented and have small eyes, whereas other species lack eyes entirely and are depigmented. Besides, the degree of eye atrophy may vary within species, among different caves (Ardila-Rodríguez, 2006; Castellanos-Morales, 2007, 2008, 2010, 2018).

Two alternative evolutionary scenarios may explain the existence of cave-dwelling *Trichomycterus* in separate caves in Santander and their phenotypic similarity involving eye atrophy and depigmentation. First, cave specialists may be the result of a single event of cave colonization, adaptation to cave environments and subsequent subterranean vicariance or dispersal leading to their currently disjunct distributions. Alternatively, multiple colonizations to caves by surface ancestors followed by evolutionary convergence towards cave-adapted phenotypes may have occurred. We here address these alternative hypotheses using a phylogenetic approach.

Previous phylogenetic studies of the family Trichomycteridae have considered only two cave species, both from Santander: *Trichomycterus sandovali* Ardila-Rodríguez, 2006 was sampled by Ochoa *et al.* (2017, 2020), whereas *T. rosablanca* Mesa S. *et al.*, 2018 was included in analyses by Mesa S. *et al.* (2018). Because in those studies each cave species was independently found to be sister to the surface species *Eremophilus mutisi* Humboldt, 1805, *T. sandovali* and *T. rosablanca* may be close relatives, which would imply a single cave colonization event by *Trichomycterus* in Santander. However, available information is insufficient to reach such a conclusion owing to limited geographic and taxonomic sampling. Here, new sequence data, generated from specimens collected in multiple localities of Colombia, allow for a first robust test of hypotheses posed to trace the history of colonization events in the group.

MATERIAL AND METHODS

STUDY SYSTEM

Trichomycterus is one of the most taxonomically challenging groups of Neotropical fish. The genus comprises *c*. 170 species (half the richness of its family Trichomycteridae; Fricke *et al.*, 2020), and it ranges from Costa Rica to northern Patagonia. Species of *Trichomycterus* inhabit lowland to mountain freshwater streams, with an important fraction of its taxonomic diversity concentrated in the Colombian Andes (DoNascimiento & Prada Pedreros, 2020). To date, 12 species living in cave environments have been described from disjunct regions, including Bolivia (one species), Brazil (three), Venezuela (one) and the eastern Cordillera of Colombia (seven; Fig. 1; Bichuette & Rizzato, 2012; Ardila-Rodríguez, 2018; Castellanos-Morales, 2018; Mesa S. *et al.,* 2018). *Trichomycterus* is considered a non-diagnosable, non-monophyletic genus, where species failing to meet diagnoses for other genera are often placed (Ochoa *et al.*, 2017; Katz *et al.*, 2018; Ochoa *et al.*, 2020). One of the main issues complicating the taxonomy of the species of *Trichomycterus* is their simplified morphology, which features relatively few diagnostic characters. However, a recent analysis using data from multiple genes partly clarified phylogenetic relationships in the genus, sorted species into clades largely matching large-scale geographical distributions and made hundreds of sequences available to be employed in additional analyses (Ochoa *et al.*, 2017). We here capitalize on these existing data, complemented with newly generated sequences from specimens we collected, to test hypotheses about evolution in cave environments in Colombia. As a first approximation to
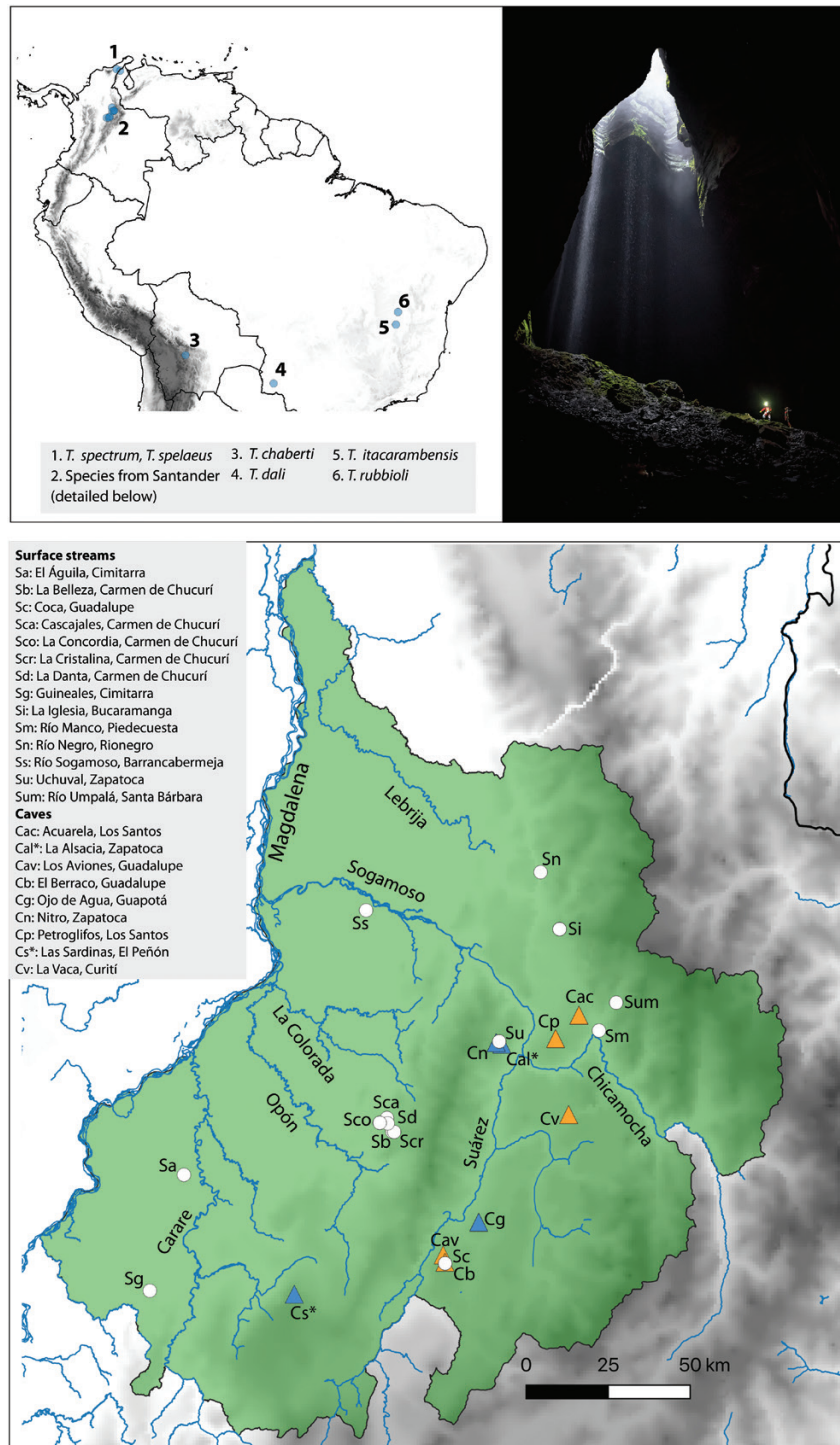
Figure 1. Type localities of cave species of *Trichomycterus* in the Neotropical region (top left), a photograph showing an example of habitats occupied by fishes in this system (Las Sardinas Cave, the type locality of *T. rosablanca*; photo by Felipe Villegas) and map of the Department of Santander showing the collection sites of specimens of *Trichomycterus* analysd in this study (bottom). Triangles correspond to caves, circles to surface streams. Orange triangles correspond to cave localities where fishes show reduced eyes and coloration, and blue triangles to caves where fishes have regular eyes and coloration. Cave localities are designated with letter C and surface streams with the letter S. The type localities of cave species *T. rosablanca* (Cs) and *T. sandovali* (Cal) are indicated with asterisks. Photograph (El Peñón, Santander) by Felipe Villegas-Instituto Humboldt.

Table 1. Cave and surface populations of *Trichomycterus* sampled for this study

Terminal taxon	Habitat	Cave phenotype	Catalog number	Locality	Geographic coordinates	GenBank/BOLD accession codes
Bullockia maldonadoi	Surface	no	LBP3112	La Laja, Biobio, Chile	−37.2152, −72.4469	KY857926.1
Cambeva davisi	Surface	no	LBP7130	Paranapanema, Brazil		KY857988.1
Cambeva diatropoporos	Surface	no	LBP14694	Jacuí, Laguna dos Patos, Brazil		KY857996.1
Cambeva zonata	Surface	no	LBP2653	Ribeira de Iguape, Atlantic, Brazil	−24.6866, −48.9945	KY857986.1
Copionodon pecten	Surface	no	LBP2653	Paraguaçu, Atlantic, Brazil		KY857986.1
Eremophilus mutissii	Surface	no	ANSP11306	Magdalena drainage, Colombia		KY857931.1
Ituglanis goya	Surface	no	LBP17131	das Almas, Tocantins, Brazil	-13.7551, −47.4555	KY857945.1
Ituglanis ramiroi	Surface	no	LBPV-63262	Paraná, Tocantins, Brazil		KY857998.1
Ituglanis eichorniarum	Surface	no	LBP4686	Paraguay, Paraná, Brazil	−14.6955, −57.2597	KY857939.1
Nematogenys inermis	Surface	no	LBP3105	Concepción, Chile		KY857952.1
Scleronema minutum	Surface	no	LBP13185	Laguna dos Patos, Atlantic, Brazil		KY857962.1
Scleronema minutum	Surface	no	LBP3310	Laguna dos Patos, Atlantic, Brazil		KY857957.1
Trichomycterus banneaui	Surface	no	LBP19537	Magdalena drainage, Colombia		KY858001.1
Trichomycterus cachiraensis	Surface	no	LBP19832	Galvanes, Magdalena drainage, Colombia	7.70626, −73.0359	KY857971.1
Trichomycterus guianensis	Surface	no	LBP17444	Potaro, Essequibo, Guyana	5.33777, −59.5658	KY857974.1
Trichomycterus itatiayae	Surface	no	LBP16356	Paraiba do Sul, Atlantic, Brazil		KY857977.1
Trichomycterus knerii	Surface	no	LBP18717	Meta, Orinoco drainage, Colombia	3.88101, −73.7822	KY857987.1
Trichomycterus punctulatus	Surface	no	ANSP180733	Pisco, Pacific, Perú	−13.6666, −75.7716	KY857983.1
Trichomycterus piratymbara	Surface	no	LBP9004	Grande, Paraná, Brazil	−20.6237, −46.2304	KY857970.1
Trichomycterus reinhardti	Surface	no	LBP16302	Paraopeba, São Francisco, Brazil	−20.1945, −44.1014	KY857997.1
Trichomycterus rosablanca	Cave	yes	0502-I	Sardinas cave, Carare River drainage, El Peñón	6.09333, −73.8285	MH407228.1
Trichomycterus ruitoquensis	Surface	no	LBP19838	Rio Frio, Magdalena drainage, Colombia	7.06902, −73.0704	KY857984.1
Trichomycterus sandovali	Cave	yes	LBP19833	Magdalena drainage, Colombia	6.80375, −73.2730	KY857985.1
Trichomycterus spilosoma	Surface	no	LBP19339	Pacific, Ecuador		KY857942.1
Trichomycterus straminius	Surface	no	LBP19834	Manco River, Sogamoso River drainage, Piedecuesta	6.84947, −72.9886	KY858006.1
Trichomycterus striatus	Surface	no	LBP19846	Magdalena, drainage, Colombia		KY858003.1
Trichomycterus transandianus	Surface	no	LBP19844	Magdalena, drainage, Colombia		KY858004.1
Trichogenes longipinnis	Surface	no	LBP3862	Camburi, Atlantic, Brazil	−23.3518, −44.7635	KY857961.1

Table 1. Continued

Terminal taxon	Habitat	Cave phenotype	Catalog number	Locality	Geographic coordinates	GenBank/BOLD accession codes
Trichomycterus aff. transandianus IAvH-P 17683	Surface	no	IAvH-P 17683	Colorada River drainage, vereda La Belleza, Carmen de Chucurí	6.55800, −73.55900	SBIOF015-19
Trichomycterus areolatus	Surface	no	LBP3118	Tolten, Pacific, Chile		KY857964.1
Trichomycterus calai IAvH-P 17803	Surface	no	IAvH-P 17803	Carare River drainage, Cimitarra	6.58200, −73.59330	SBIOF014-19
Trichomycterus cf. mogotensis IAvH-P 21069	Surface	no	IAvH-P 21069	Umpalá River, Chicamocha River drainage, Santa Bárbara	6.91303, −72.94260	SBIOF071-19
Trichomycterus sp. Curití ID 422	Cave	no	IAvH-P-23284	Vaca cave, Fonce River drainage, Curití	6.60458, −73.07350	MT682739
Trichomycterus sp. Curití ID 425	Cave	no	IAvH-P-23285	Vaca cave, Fonce River drainage, Curití	6.60458, −73.07350	MT682738
Trichomycterus sp. Curití ID 426	Cave	no	IAvH-P-23286	Vaca cave, Fonce River drainage, Curití	6.60458, −73.07350	MT682740
Trichomycterus latistriatus ("guacamayoensis")	Cave	no	CBIHS010-17	Los Aviones cave, Suárez River drainage, Guadalupe	6.21789, −73.41890	ADI1985
Trichomycterus ruitoquensis IAvH-P 12397	Surface	no	IAvH-P 12397	Río Negro, Rionegro	6.11954, −74.22620	SBIOF102-19
Trichomycterus ruitoquensis ID 02 La Iglesia	Surface	no	IAvH-P-23251	Iglesia stream, Lebrija River drainage, Pan de Azúcar, Bucaramanga	7.11537, −73.09845	MT682734
Trichomycterus ruitoquensis ID 03 La Iglesia	Surface	no	IAvH-P-23252	Iglesia stream, Lebrija River drainage, Pan de Azúcar, Bucaramanga	7.11537, −73.09845	MT682735
Trichomycterus ruitoquensis ID 04 La Iglesia	Surface	no	IAvH-P-23253	Iglesia stream, Lebrija River drainage, Pan de Azúcar, Bucaramanga	7.11537, −73.09845	MT682736
Trichomycterus ruitoquensis ID 05 La Iglesia	Surface	no	IAvH-P-23254	Iglesia stream, Lebrija River drainage, Pan de Azúcar, Bucaramanga	7.11537, −73.09845	MT682737
Trichomycterus sandovali ID 402	Cave	yes	IAvH-P-23261	Alsacia cave, Suárez River drainage, Zapatoca	6.79839, −73.25728	MT682749
Trichomycterus sandovali ID 403	Cave	yes	IAvH-P-23262	Alsacia cave, Suárez River drainage, Zapatoca	6.79839, −73.25728	MT682757
Trichomycterus sandovali ID 404	Cave	yes	IAvH-P-23263	Alsacia cave, Suárez River drainage, Zapatoca	6.79839, −73.25728	MT682758
Trichomycterus sp. Carare IAvH-P 20905	Surface	no	IAvH-P 20905	Carare River drainage, Cimitarra	6.43868, −74.13230	SBIOF097-19

Table 1. Continued

Terminal taxon	Habitat	Cave phenotype	Catalog number	Locality	Geographic coordinates	GenBank/ BOLD accession codes
Trichomycterus sp. Carare IAvH-P 20978	Surface	no	IAvH-P 20978	Carare River drainage, Cimitarra	6.11954, −74.22620	SBIOF099-19
Trichomycterus sp. Guadalupe ID 410	Surface	no	IAvH-P-23269	Coca stream, Suárez River drainage, Guadalupe	6.19471, −73.41343	MT682761
Trichomycterus sp. Guadalupe ID 411	Surface	no	IAvH-P-23270	Coca stream, Suárez River drainage, Guadalupe	6.19471, −73.41343	MT682762
Trichomycterus sp. Guadalupe ID 413	Surface	no	IAvH-P-23273	Coca stream, Suárez River drainage, Guadalupe	6.19471, −73.41343	MT682765
Trichomycterus sp. Guadalupe ID 414	Cave	no	IAvH-P-23274	Berraco cave, Suárez River drainage, Guadalupe	6.19802, −73.41380	MT682764
Trichomycterus sp. Guadalupe ID 416	cave	no	IAvH-P-23276	Berraco cave, Suárez River drainage, Guadalupe	6.19802, −73.41380	MT682763
Trichomycterus sp. Guapotá ID 417	Cave	yes	IAvH-P-23278	Ojo de Agua cave, Suárez River drainage, Guapotá	6.30815, −73.32090	MT682741
Trichomycterus sp. Guapotá ID 420	Cave	yes	IAvH-P-23281	Ojo de Agua cave, Suárez River drainage, Guapotá	6.30815, −73.32090	MT682743
Trichomycterus sp. Guapotá ID 421	Cave	yes	IAvH-P-23282	Ojo de Agua cave, Suárez River drainage, Guapotá	6.30815, −73.32090	MT682742
Trichomycterus sp. La Colorada IAvH-P 17686	Surface	no	IAvH-P 17686	La Cristalina stream, Colorada River drainage, Carmen de Chucurí	6.55697, −73.55370	SBIOF020-19
Trichomycterus sp. La Colorada IAvH-P 17716	Surface	no	IAvH-P 17716	Cascajales river, Colorada River drainage, Carmen de Chucurí	6.59519, −73.57340	SBIOF024-19
Trichomycterus sp. La Colorada IAvH-P 17723	Surface	no	IAvH-P 17723	La Danta stream, Colorada River drainage, Carmen de Chucurí	6.58036, −73.57130	SBIOF027-19
Trichomycterus sp. La Colorada IAvH-P 17734	Surface	no	IAvH-P 17734	La Concordia stream, Colorada River drainage, Carmen de Chucurí	6.58200, −73.59330	SBIOF030-19
Trichomycterus sp. Sogamoso IAvH-P20797	Surface	no	IAvH-P20797	Magdalena River basin, Barrancabermeja	7.16718, −73.63080	SBIOF081-19
Trichomycterus sp. Zapatoca ID 06	Cave	yes	IAvH-P-23255	Nitro cave, Suárez River drainage, Zapatoca	6.80313, −73.27282	MT682755
Trichomycterus sp. Zapatoca ID 07	Cave	yes	IAvH-P-23256	Nitro cave, Suárez River drainage, Zapatoca	6.80313, −73.27282	MT682750
Trichomycterus sp. Zapatoca ID 08	Cave	yes	IAvH-P-23257	Nitro cave, Suárez River drainage, Zapatoca	6.80313, −73.27282	MT682746

Table 1. Continued

| Terminal taxon	Habitat	Cave phenotype	Catalog number	Locality	Geographic coordinates	GenBank/BOLD accession codes
Trichomycterus sp. Zapatoca ID 09	Cave	yes	IAvH-P-23258	Nitro cave, Suárez River drainage, Zapatoca	6.80313, −73.27282	MT682756
Trichomycterus sp. Zapatoca ID 10	Cave	yes	IAvH-P-23259	Nitro cave, Suárez River drainage, Zapatoca	6.80313, −73.27282	MT682744
Trichomycterus sp. Zapatoca ID 408	Surface	no	IAvH-P-23267	Uchuval stream, Sogamoso River drainage, Zapatoca	6.80594, −73.26463	MT682759
Trichomycterus sp. Zapatoca ID 409	Surface	no	IAvH-P-23268	Uchuval stream, Sogamoso River drainage, Zapatoca	6.80594, −73.26463	MT682745
Trichomycterus sp. ID 01 La iglesia	Surface	no	IAvH-P-23250	Iglesia stream, Lebrija River drainage, Pan de Azúcar, Bucaramanga	7.11537, −73.09845	MT682733
Trichomycterus uisae ID 427	Cave	no	IAvH-P-23288	Petroglifos cave, Sogamoso River drainage, Los Santos	6.81385, −73.10935	MT682754
Trichomycterus uisae ID 428	Cave	no	IAvH-P-23289	Petroglifos cave, Sogamoso River drainage, Los Santos	6.81385, −73.10935	MT682753
Trichomycterus uisae ID 429	Cave	no	IAvH-P-23290	Petroglifos cave, Sogamoso River drainage, Los Santos	6.81385, −73.10935	MT682760
Trichomycterus uisae ID 430	Cave	no	IAvH-P-23291	Petroglifos cave, Sogamoso River drainage, Los Santos	6.81385, −73.10935	MT682752
Trichomycterus uisae ID 431	Cave	no	IAvH-P-23293	Acuarela cave, Sogamoso River drainage, Piedecuesta	6.87845, −73.04520	MT682748
Trichomycterus uisae ID 433	Cave	no	IAvH-P-23294	Acuarela cave, Sogamoso River drainage, Piedecuesta	6.87845, −73.04520	MT682747
Trichomycterus uisae ID 435	Cave	no	IAvH-P-23295	Acuarela cave, Sogamoso River drainage, Piedecuesta	6.87845, −73.04520	MT682751
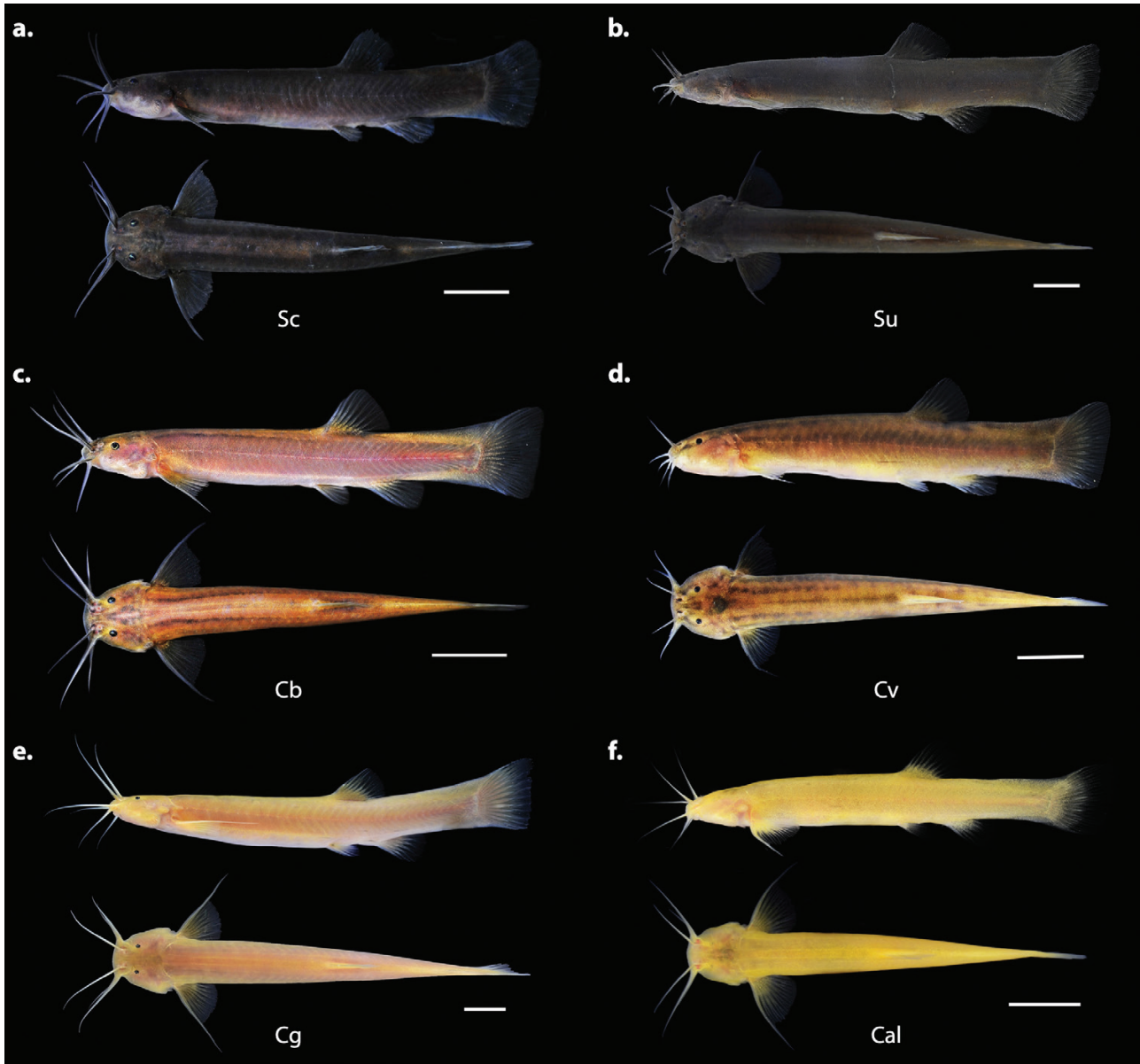
Figure 2. Morphological variability of some species of *Trichomycterus* included in this study. Lateral and dorsal view of the same individual from cave localities (designated with letter C) and surface streams (designated with letter S). Sc = Coca, Guadalupe; Su = Uchuval, Zapatoca; Cb = El Berraco, Guadalupe; Cv = La Vaca, Curití; Cg = Ojo de Agua, Guapotá; Cal = La Alsacia, Zapatoca. Bar indicates 1 cm.

this complex system, we focused our sampling efforts in the Department of Santander, the area with the most extensive karstic landscape of the country (Mendoza, Murillo, & Orjuela, 2009; Muñoz-Saba *et al.*, 2013).

The eastern Cordillera of Colombia is a mountain chain *c*. 500 km long, 150–200 km wide and reaching elevations of *c*. 1.3–5.0 km on average (Horton, Parra & Mora, 2020). Its exposed area of 100 000 km² is composed mostly of sedimentary rocks from the Upper and Lower Cretaceous (50% and 30%, respectively;

Horton *et al.*, 2020). During the Cretaceous, the area was a shallow ocean where sediments formed sandstones, mudstones and carbonates (Hoorn *et al.*, 2010; Blandin & Purser, 2013; Horton *et al.*, 2020). These carbonate-limestone formations resulted in a karstic landscape due to meteoric and erosional processes caused by rock fracturing during the uplift of the eastern Cordillera since the Miocene (~13.5–11.8 Mya; Albert & Reis, 2011) and by the high precipitation in this tropical region (Kay & Madden, 1997).

Cave and surface trichomycterids have been found across karstic landscapes of the eastern Cordillera of Colombia in several departments, especially Santander (Castellanos-Morales & Galvis, 2012; DoNascimiento *et al.*, 2017). The limestone formations in Santander include the Cumbre, Rosablanca, Paja and Tablazo geological formations (Gaona-Narváez, 2015; Gómez-Tapia *et al.*, 2015), which have hundreds of caves spread in a relatively small area (~170 km) that remains largely scientifically unexplored (Mendoza *et al.*, 2009; Muñoz-Saba *et al.*, 2013). Geological and ecological information is only available for the El Peñón cave system (the type locality of *Trichomycterus rosablanca*; Lasso *et al.*, 2019), where caves form a complex with more than 100 km of passages. Caves in this system comprise a wide array of aquatic microhabitats, from small occasional pools to streams, tall waterfalls (some > 155 m) and large pools reaching > 145 m in diameter (Lasso *et al.*, 2019). The El Peñón system is perhaps the largest in Santander, but other caves have hundreds of meters of passages with water bodies composed of stream-pool systems (e.g. Cueva Ojo de Agua in Guapotá; pers. obs.).

SAMPLING

We sampled populations of *Trichomycterus* catfish in caves and surface streams, seeking to collect specimens from caves and adjacent streams whenever possible (Table 1). We collected 49 specimens from several populations in 2017, using hand nets or electrofishing (Samus-725 M), following methods for cavefish sampling proposed by Muñoz-Saba *et al.* (2013). Fish were photographed, euthanized by immersion in roxicaine and preserved in 96% ethanol. Taxonomic identification of specimens was based on comparison with original descriptions and geographic correspondence with the type locality of nominal species, followed by comparative morphological examination of specimens available at the Freshwater Fish Collection of the Instituto Alexander von Humboldt (IAvH-P). We followed the nomenclature of DoNascimiento & Prada-Pedreros (2020) regarding synonymization of some recently described taxa from Santander. All samples were deposited at IAvH-P. We also analysed specimens collected in different drainages in Santander in 2018 during the 'Santander Bio' expeditionary project (Torres & Quiñones, 2019), several of which likely correspond to undescribed species of *Trichomycterus* (Table 1). Our sampling also included *Copionodon pecten* de Pinna, 1992 (Copionodontinae), *Nematogenys inermis* Guichenot, 1848 (Nematogenyidae) and *Trichogenes longipinnis* Britski & Ortega, 1983 (Trichogeninae), which we included as outgroups for phylogenetic analyses based on previous studies (Ochoa *et al.*, 2017, 2020).

DNA EXTRACTION AND SEQUENCING

We used sequences of the mitochondrial *COI* gene to reconstruct the phylogenetic relationships of *Trichomycterus* from Santander. This rapidly evolving marker was one of the genes used by Ochoa *et al.* (2017) to reconstruct the phylogeny of Trichomycteridae, and genealogies constructed using *COI* data are consistent with multilocus phylogenies of the group (Ochoa *et al.*, 2017; Ochoa *et al.*, 2020). We extracted total genomic DNA from the right pelvic fin using a phenol-chloroform-isoamyl alcohol protocol (Wasko *et al.*, 2003). We amplified a 652-bp segment of the *COI* mitochondrial gene, using primers FishF1 and FishR1 (Ward *et al.*, 2005). We conducted PCRs in a volume of 25 µL, containing 2.5 µL of Buffer 10X, 1.5 µL of $MgCl_2$ (50 mmol/L), 0.5 µL of dNTPs (10 mmol/L), 0.2 µL of Taq Polymerase (5U/µL), 1.25 µL of each primer, 1 µL of BSA (0.066 mmol/L), 4 µL of template DNA and 12.8 µL of ddH_2O. The cycling included an initial denaturation at 94 °C for 120 s; 34 cycles of denaturation at 94 °C for 240 s, annealing at 54 °C for 30 s, hybridization at 72 °C for 45 s and extension at 72 °C for 600 s. Polymerase chain reaction (PCR) products were sequenced at Macrogen Inc. and the sequencing facilities of the Universidad de Los Andes (Bogotá, Colombia), resulting in 41 *COI* gene sequences, which we visualized, edited and aligned using the MUSCLE algorithm (Edgar, 2004) in GENEIOUS v.10.1.3 (Kearse *et al.*, 2012). Combining our new data with existing sequences resulted in an alignment comprising a total of 74 sequences (Table 1).

PHYLOGENETIC ANALYSES

We used Bayesian inference as implemented in MrBayes v.3.2.6 (Huelsenbeck & Ronquist, 2001) and maximum likelihood in RAxML v.8.2.10 (Stamatakis, 2014) to infer gene trees based on *COI* sequences. Bayesian analyses were conducted under the GTR+I+G model of nucleotide substitution, identified as the best-fit to the *COI* data as per the Akaike information criterion in PartitionFinder v.2.1.1 (Lanfear *et al.*, 2012). We ran 30 million generations, with two runs of four independent Markov chain Monte Carlo (MCMC) chains (three heated, one cold), sampling trees every 1000 generations. We evaluated convergence of MCMC analysis by checking values of the standard deviation of split frequencies, potential scale reduction factor and effective sample size using software TRACER 1.7 (Rambaut *et al.*, 2018). We discarded 25% of generations as burn-in and the remaining parameters in BI analysis were set by default. The maximum-likelihood analysis was performed under the GTR+GAMMA model and bootstrap resampling was applied to assess nodal support using the autoMRE criterion. To

further examine relationships among *COI* haplotypes, we constructed a median-joining haplotype network using PopArt v.1.7 (Leigh & Bryant, 2015) and redrew it manually. Finally, we asked whether a phylogeny enforcing the monophyly of cave species was less likely than unconstrained topologies using a Shimodaira–Hasegawa test (Shimodaira, 2002), implemented in the *phangorn* package (Schliep, 2011) for R (R Development Core Team, 2018).

RESULTS

We analysed 522 base pairs of the *COI* gene, of which 162 were variable (22 singletons, 140 parsimony-informative sites). Bayesian and maximum-likelihood analyses resulted in nearly identical topologies (see Supporting Information, Fig. S1-S2), showing that cave forms of *Trichomycterus* in Santander have evolved independently at least twice. One evolutionary event corresponds to *Trichomycterus rosablanca*, which was recovered with strong support (0.96 posterior probability, 72% maximum likelihood bootstrap) as sister to *Eremophilus mutisii* Humboldt, 1805, a species that lives in surface streams in the Bogotá River basin, has conspicuous eyes and shows profusely vermiculated dark coloration (Fig. 3, cade 1). The second evolutionary event corresponded to a clade including the species *T. sandovali* and specimens from the Sogamoso River drainage, including surface populations and all the remaining cave species and populations analysed (Fig. 3, clade 2). Relationships among the clades including *T. rosablanca* and *E. mutisii*, *T. sandovali* and allies, and other species are not strongly supported, but the Shimodaira–Hasegawa test shows that unconstrained and constrained topologies differ significantly. The topology in which cave populations are monophyletic was less fit to the data than the unconstrained topology in which troglomorphic traits are homoplasious (*P* = 0.002). We thus reject the hypothesis of a single origin for the evolution of cave-living and associated phenotypes (i.e. loss of eyes and pigmentation).

We found that cave populations referred to *Trichomycterus sandovali* were more closely allied to surface taxa than to the cave species *T. rosablanca*. Moreover, the genetic divergence between cave-dwelling *T. sandovali* and other taxa from both surface streams and caves was shallow to non-existent. For example, we observed no genetic divergence between cave and surface populations from Zapatoca: the surface population showed eyes and a spotted to homogeneously dark coloration, whereas cave populations (one of them collected at the type locality of *T. sandovali*) had reduced eyes or lacked them and their coloration is faint. There was no genetic

divergence between populations from Zapatoca and Los Santos (the latter collected close to the type locality of *T. uisae* Castellanos-Morales, 2008), which are separated by the deep Sogamoso River basin (Fig. 3, clade 2). Likewise, cave and surface populations collected in Guadalupe and Guapotá (Suárez River drainage) and Curití (Fonce River drainage) differed only by two substitutions from those collected at the type locality of *T. sandovali*. The haplotype network for clade 2 showed that cave and surface populations share haplotypes, suggesting recent divergence or gene flow (Fig. 3, median-joining network). Furthermore, we found no genetic differences in *COI* sequences between *T. sandovali* and two other cave species, namely '*T. guacamayoensis*' Ardila Rodríguez, 2018 [recently shown to be a junior synonym of *T. latistriatus* (Eigenmann, 1917); see DoNascimiento & Prada-Pedreros, 2020] and *T. uisae* (Fig. 3).

Despite their geographic proximity to surface and cave populations from Santander, several surface taxa from our study area were more closely related to species from other regions (Fig. 3). The species *Trichomycterus ruitoquensis* Ardila Rodríguez, 2007, *T. straminius* (Eigenmann, 1917) and undescribed taxa recently collected in the Carare, Colorada and Sogamoso rivers, belonged to clades with species not occurring in Santander, like *T. striatus* (Meek & Hildebrand, 1913) (restricted to Costa Rica and Panamá) and *T. banneaui* (Eigenmann, 1912) (from tributaries in the upper Magdalena basin, Department of Tolima, Colombia).

We find two additional unexpected results. First, our data revealed a potentially undescribed species in the La Iglesia surface stream in the city of Bucaramanga, Santander. Most of the specimens we collected at this locality corresponded genetically and morphologically to *Trichomycterus ruitoquensis* (Fig. 3, clade 1). However, one of the individuals collected in the same stream differed genetically from *T. ruitoquensis* by five substitutions in *COI* and morphologically by an irregular spotted pattern (vs. regular in *T. ruitoquensis*). Second, we observed atypical coloration among some surface individuals collected in the Uchuval stream, near the Nitro and Alsacia caves in Zapatoca. Some individuals showed a homogeneously dark pigmentation, which differed from other individuals from the same stream exhibiting a spotted pigmentation pattern on the body (Fig. 2).

DISCUSSION

Our study provides evidence for the repeated evolution of cave-living forms from surface ancestors in trichomycterid catfish in a restricted geographic region in Colombia. At least two lineages (*Trichomycterus*
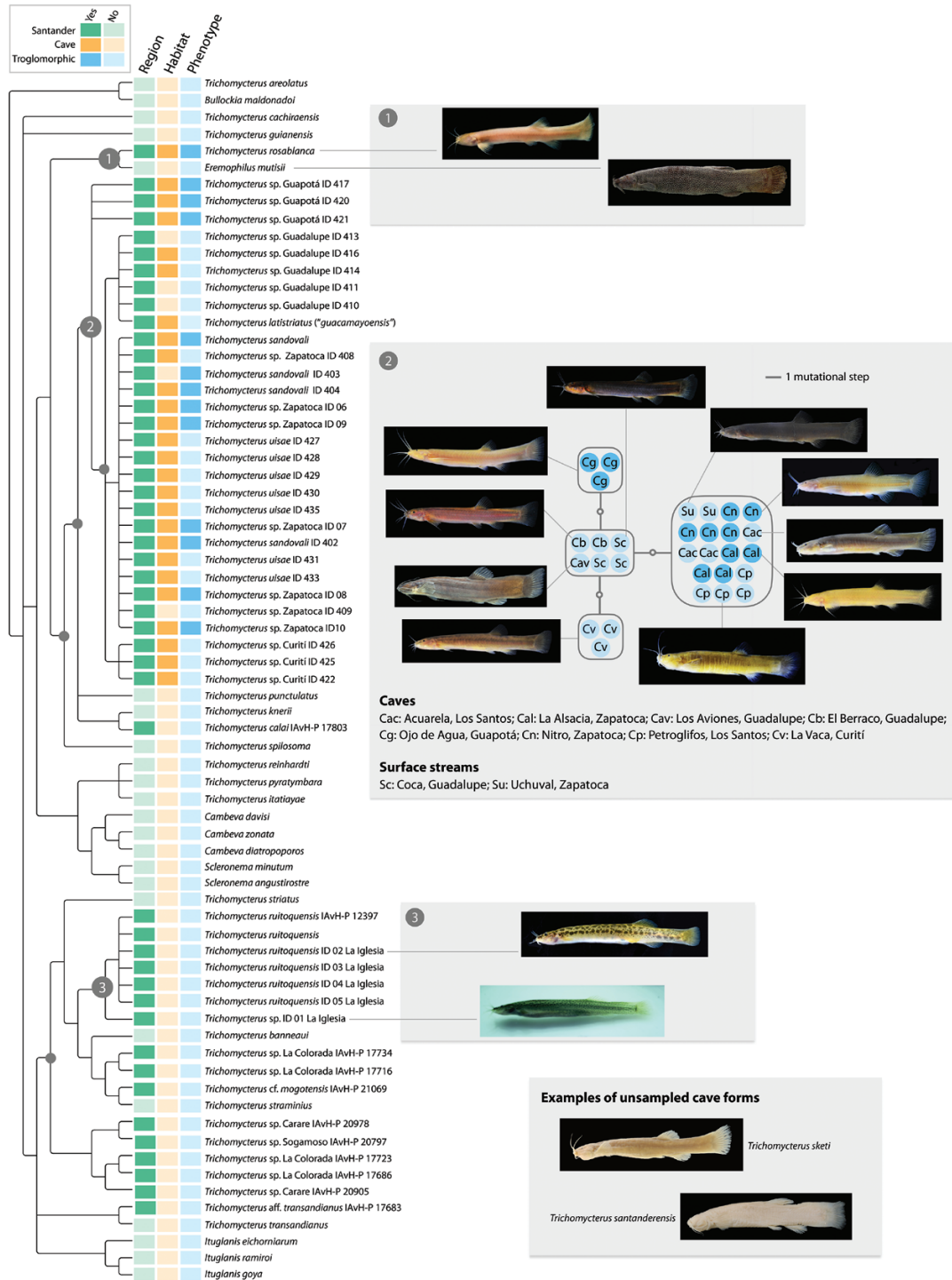
Figure 3. Relationships among catfish in *Trichomycterus* and other genera reveal repeated colonization of caves and phenotypic convergence in the karst region of Santander, Colombia. The Bayesian gene tree on the left is an overview of phylogeny based on sequences of the *COI* gene (outgroups not shown). Colours at the tips indicate whether specimens were collected in the western slope of the Eastern Andes of Santander, whether they were found in cave habitats, and whether they lacked eyes or body pigmentation (i.e. troglomorphism). Strongly supported nodes (posterior probability ≥ 0.95, maximum likelihood bootstrap ≥ 70%) relevant to the phylogenetic position of specimens from Santander are indicated with circles; numbered nodes are mentioned in the text and panels on the right. Node 1 defines a clade formed

rosablanca and *T. sandovali* and allies) colonized caves independently, and convergently evolved loss of eyes and body pigmentation in the karst region of Santander. These two lineages occur in separate drainages (Suárez and Carare rivers, respectively) that are not connected by river courses, further supporting the hypothesis that cave-living forms are derived from independent colonization events of subterranean environments in different areas. We lack DNA sequences from cave species of *Trichomycterus* from other countries in South America (Fig. 1A), but the vast geographical distances separating them, and overall congruence between geography and phylogeny in the group (Ochoa *et al.*, 2017; Katz *et al.*, 2018; Ochoa *et al.*, 2020), suggest that species of *Trichomycterus* have independently colonized caves multiple times. Also, it remains possible that cave species from Santander, which we did not sample, may have independently colonized these environments (Fig. 3). Examples include *T. santanderensis* Castellanos-Morales, 2007 from the Lebrija River drainage, which we hypothesize belongs to the clade formed by *T. sandovali* and allies, and *T. sketi* Castellanos-Morales, 2011, which occurs in a separate drainage (Opón River) and may represent a distinct lineage.

Convergent evolution associated with cave living has been documented in other fish systems, including *Astyanax mexicanus* (Dowling *et al.*, 2002; Bradic *et al.*, 2012), *Garra barreimiae* Fowler & Steinitz 1956 (Kirchner *et al.*, 2017) and amblyopsids (Hart *et al.*, 2020). In all cases, cave-dwelling fish exhibit similar phenotypic traits, including absence or reduction of eyes and body pigmentation, suggesting adaptation to cave environments along similar trajectories. More broadly, such a pattern speaks to the repeatability of evolution by natural selection owing to common selective pressures and potentially to shared genetic and developmental constraints (Losos, 2017).

In addition to our finding of independent colonization of caves by *Trichomycterus rosablanca* and *T. sandovali*, our work raises the intriguing possibility that different populations closely allied to the latter (i.e. members of clade 2) may have independently colonized and adapted to caves, in drainages along the Sogamoso drainage. Genetic similarity and haplotype sharing between surface and cave populations in this clade suggest that these populations diverged recently, that they still experience gene flow or both. Gene flow in these karstic systems is likely because the water flows in and, in some cases, out of caves, potentially allowing dispersal of adult fish, larvae or eggs. This result is consistent with studies in other cave organisms (*Astyanax mexicanus*, *Garra barreimiae* and cave salamanders) in which cave and surface populations are also connected by gene flow (Niemiller *et al.*, 2008; Bradic *et al.*, 2012; Kirchner *et al.*, 2017), possibly due to sporadic flooding. Given genetic exchange between surface and cave environments, selective pressures strong enough to counteract the effects of migration are needed to account for phenotypic evolution in cavefish (Cartwright *et al.*, 2017). Accordingly, some studies suggest that divergence in the face of gene flow is a major driver of the evolution of cave-dwellers (Juan *et al.*, 2010; Bradic *et al.*, 2012). Limited genetic divergence between surface and cave populations is also consistent with scenarios in which the origin of cave phenotypes is initially promoted by phenotypic plasticity (Rohner *et al.*, 2013; Bilandžija *et al.*, 2019) or via epigenetic mechanisms (Gore *et al.*, 2018).

The mechanisms that drove the drastic morphological changes borne by *Trichomycterus* that colonized caves are unknown. Several studies on cave fish support the hypothesis of directed selection against eyes or indirect selection due to pleiotropy (Krishnan & Rohner, 2017). However, these phenotypic changes can also be explained by neutral evolution, with regressive mutations arising and accumulating without the 'razor of selection' (Wilkens, 2020). Future work extending our analyses to genome-wide assays of genetic variation will allow us to better characterize

by phenotypically contrasting species from caves in Santander (*T. rosablanca*) and surface streams in the Bogotá River basin (*Eremophilus mutisii*). Node 2 defines a large clade for which genealogical relationships are shown on the right with a median-joining network. Rectangles correspond to haplotypes and circles to individuals, with colours indicating whether or not specimens lacked eyes or pigmentation following the colour scheme in the phylogeny; upper-case letters indicate habitat (C = cave, S = surface) and lower-case letters define localities. Note that all specimens differ at most by two substitutions in *COI*, that individuals sharing two of the haplotypes occur in multiple localities including both caves and surface streams and that haplotypes are not consistently associated with phenotypes. The most common haplotype occurred both in caves and surface streams, and was shared by individuals with various phenotypes and by populations assigned to at least three different species: *T. sandovali*, *T. uisae* and '*T. guacamayoensis*' (i.e. *T. latistriatus*). Finally, node 3 defines a clade from Santander more closely allied to species from other regions in the Neotropics than to taxa from the karst region; photographs of live fishes illustrate differences in phenotype between specimens from the same surface stream, which also differed genetically, highlighting a potentially undescribed species. The panel on the bottom illustrates two species from cave habitats in Santander lacking eyes and pigmentation, which we were unable to sample and may represent other instances of convergence. Photographs by the authors and by Felipe Villegas (*T. rosablanca*), Cesar Castellanos (*T. santanderensis* and *T. sketi*) and from DoNascimiento *et al.* (2014; *E. mutisii*).

gene flow, elucidate relationships and estimate the timing of divergence among populations, assess the genetic basis of phenotypes and the importance of the action of selection in this system.

In addition to suggesting that caves were likely colonized repeatedly and that phenotypic evolution in *Trichomycterus* may occur rapidly and with gene flow, our finding of no genetic differentiation in the *COI* gene between cave-dweller and surface populations in the *T. sandovali* group (Fig. 3, clade 2) may have taxonomic implications. In particular, we found no genetic divergence among populations currently assigned to *T. sandovali*, '*T. guacamayoensis*' (i.e. *T. latistriatus*) and *T. uisae*, as well as specimens from the municipalities of Guadalupe, Guapotá and Zapatoca. Although the populations we sampled are separated by up to nearly a couple hundred kilometers and by deep canyons, and although some of them lack eyes and pigments while others have these traits, they all are genetically too similar to distinguish them using the mitochondrial marker we employed, which is often used as a barcode for species identification (Hebert *et al.*, 2003; Ward *et al.*, 225, 2009). While lack of *COI* divergence may simply reflect recent diversification or gene flow, an alternative interpretation is that *T. sandovali* might not correspond to a cavefish species restricted to the Alsacia cave in Zapatoca as traditionally believed, but rather to a taxon with a much broader distribution consisting of hypogean and epigean populations throughout the Sogamoso River drainage, much like the case of the Mexican tetra (Bradic *et al.*, 2012). A taxonomic review is needed to assess whether some lineages included in our analysis correspond to undescribed species and others – currently considered valid species – should be synonymized.

The importance of more extensive sampling to better understand patterns of cave colonization and evolution in our study system is illustrated by comparing our results to those implied by previous phylogenetic studies of trichomycterids. It appeared likely that cave species from Santander had a single origin, based on work showing that two cave species lacking eyes and pigmentation (*T. sandovali* and *T. rosablanca*) occupied similar phylogenetic positions in analyses with partly overlapping and incomplete sampling (Mesa S. *et al.*, 2018; Ochoa *et al.*, 2017, 2020). By sampling multiple additional populations from the Santander karst region, we were able to determine (1) that *T. sandovali* and *T. rosablanca* belong to different clades, implying separate colonization of caves; and (2) that more than one event of cave colonization may have occurred among *T. sandovali* and its close relatives. Because multiple cave populations exist in the Santander karst region that remain unstudied, additional work has the potential to reveal other examples of convergence associated with cave living. Our phylogeny is generally congruent with trees based on thousands of loci and a

broader taxonomic sampling (Ochoa *et al.* 2017, 2020). Nevertheless, greater taxonomic and geographic sampling in future analyses should be coupled with sequencing genome-wide markers to obtain more robust inferences of phylogeny and test alternative demographic scenarios to account for the origin and phenotypic diversification of cave-living populations in the group.

We are intrigued by our finding of individuals with homogeneously dark pigmentation differing from other individuals in the same surface stream exhibiting a spotted pigmentation pattern. A potential explanation is that homogeneously pigmented individuals might be the result of re-colonizations of the surface environment from caves. Because cave-dweller populations are uniformly depigmented, individuals recolonizing the surface may have recovered dark pigmentation yet not in a spotted pattern likely lost following cave invasion. Although this scenario is speculative and would need to be tested, we note that cave fish may be common exceptions to Dollo's law (Collin & Miglietta, 2008), which argues that once a complex trait is lost it is unlikely to be re-acquired. Although cave-dwellers have been considered evolutionary 'dead-ends' (Stern *et al.*, 2017) owing to their specialization to underground life, involving the loss of eyes and pigmentation, evidence from several lineages of organisms adapted to caves, including fish (Dillman *et al.*, 2011), scorpions (Prendini *et al.*, 2010) and amphipods (Copilaş-Ciocianu *et al.*, 2018), indicates that reacquisition of surface phenotypes upon recolonization from caves is indeed possible.

To conclude, we have shown that trichomycterids from Santander have experienced convergent evolution as a likely consequence of repeated colonization of cave environments and that such convergence may have occurred in the face of gene flow and within a relatively restricted geographic area. More broadly, our study has barely scratched the surface of what we anticipate to be a promising system to address many questions about the evolutionary, ecological and developmental processes leading to the divergent phenotypes that characterize cave-living.

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SUPPORTING INFORMATION

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

Figure S1. Bayesian gene tree of *Trichomycterus* and other genera based on sequences of the *COI* gene. Branch length proportional to genetic divergence and node support indicated.
Figure S2. ML gene tree of *Trichomycterus* and other genera based on sequences of the *COI* gene. Branch length proportional to genetic divergence and node support indicated.