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Evolution in the dark: Unexpected genetic diversity and morphological stasis in the blind, aquifer-dwelling catfish *Horaglanis*

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Abstract

The lateritic aquifers of the southern Indian state of Kerala harbour a unique assemblage of enigmatic stygobitic fishes which are encountered very rarely, only when they surface during the digging and cleaning of homestead wells. Here, we focus on one of the most unusual members of this group, the catfish *Horaglanis*, a genus of rarely-collected, tiny, blind, pigment less, and strictly aquifer-residing species. A six-year exploratory and citizen-science backed survey supported by molecular phylogenetic analysis reveals novel insights into the diversity, distribution and population structure of *Horaglanis*. The genus is characterized by high levels of intraspecific and interspecific genetic divergence, with phylogenetically distinct species recovered above a 7.0% genetic-distance threshold in the mitochondrial cytochrome oxidase subunit 1 gene. Contrasting with this deep genetic divergence, however, is a remarkable stasis in external morphology. We identify and describe a new cryptic species, *Horaglanis populi*, a lineage that is the sister group of all currently known species. All four species are represented by multiple haplotypes. Mismatch distribution reveals that populations have not experienced recent expansions.

Keywords

Cryptic species, groundwater, Kerala, molecular ecology, stygobitic, subterranean

Introduction

Data scarcity and knowledge shortfalls are two of the most important impediments to our ability to understand and conserve life on Earth (Hortal et al. 2015). Despite more than three centuries of natural history exploration and research, we continue to lack fundamental information on the diversity (the 'Linnean shortfall') and distribution (the 'Wallacean shortfall') of many plant and animal groups (Hortal et al. 2015). Such impediments to our knowledge of the living world are even more acute in the case of organisms inhabiting hidden or inaccessible environments, such as caves and subterranean waters (i.e., the Racovitzan shortfall) (Ficetola et al. 2019), which are

at the same time increasingly subjected to anthropogenic threats (Mammola et al. 2019).

Subterranean aquatic habitats often harbour unique assemblages of fishes with a high proportion of 'point endemics' or relic lineages, with no close relatives in surface waters (Galassi et al. 2014). This is especially true for bony fishes, with 289 valid species currently known from subterranean aquatic habitats on every continent except Antarctica (Proudlove 2022). These include some spectacular radiations of cave-adapted species (Mao et al. 2022), as well as lineages of 'living fossils' (Britz et al. 2020). These unusual fish species have been aptly called the 'wrecks of ancient life' (Darwin 1809-1822) and 'ghosts in the water' (Niemiller et al. 2019); many of them enjoy unusual scientific names (e.g., *Satan eurystomus* Hubbs & Bailey, *Aenigmachanna gollum* Britz, Anoop, Dahanukar & Raghavan), or have emerged as laboratory models (e.g., *Astyanax mexicanus* (de Filippi)) for understanding evolution, development, behaviour, and human health (Krishnan and Rohner 2017; McGaugh et al 2020).

Notwithstanding their interesting and often extraordinary fauna, subterranean aquatic habitats are good examples of biodiversity shortfalls (Ficetola et al. 2019), primarily because they are inaccessible and their inhabitants are rarely collected. Encounters with these subterranean animals are therefore often serendipitous, or happen when the gateways to the underground water-world are scrutinized – for example in the case of dug-out wells that are drained for maintenance (Ohara et al. 2016; Anoop et al. 2019). As a result of their unique habitat, information on diversity and distribution for most subterranean fish species is either highly incomplete or even absent, with most species known only from type material. Local communities interested in natural history, often the first or sometimes the only people to encounter these species (Ohara et al. 2016; Anoop et al. 2019), are thus potentially able to play a significant role in improving scientific knowledge of this unusual fauna.

A special area of subterranean fish diversity is the lateritic landscape in the southern Indian state of Kerala (Raghavan et al. 2021). It is recognized as a global hotspot for subterranean fishes, presently numbering 10 endemic species in five genera (*Aenigmachanna, Horaglanis, Kryptoglanis*, *Pangio* and *Rakthamichthys*) and two monotypic families (Aenigmachannidae and Kryptoglanidae) (Raghavan et al. 2021; Britz et al. 2022). Some of these fishes exhibit unusual morphological characters such as the absence of eyes and body pigments (*Horaglanis* spp., and *Rakthamichthys* spp.), as well as the absence of dorsal- (*Kryptoglanis shajii*) or pelvic-fins (*Aenigmachanna gollum*), or even both these fins (*Pangio bhujia* Anoop, Britz, Arjun, Dahanukar & Raghavan and *P. pathala* Sundar, Arjun, Sidharthan, Dahanukar & Raghavan).

Horaglanis (Fig. 1A) is a genus of catfishes, remarkable for their bizarre appearance (blind, pigmentless and of blood-red coloration), tiny size (< 35mm), occurrence in a unique habitat (lateritic aquifers) (Fig. 1B), rarity (appearing only occasionally in dug-out wells, Fig. 1C), paucity of museum specimens (known until recently just from a handful of examples), and unresolved phylogenetic and biogeographic affinities (Menon 1951; de Pinna 1993). Though three species are currently known (*Horaglanis krishnai* Menon, 1951, *H. alikunhii* Subhash Babu & Nair, 2004, *H. abdulkalami* Subhash Babu, 2012), the latter two were poorly described; the taxonomic and geographic boundaries between the three species have thus remained unclear. Fewer than ten published records of *Horaglanis* backed by voucher specimens are available (Fig. 1D), and to date no studies have attempted to understand the distribution and genetic diversity of the genus.

A six-year exploratory and citizen science-backed survey across the lateritic landscape of Kerala has resulted in an extensive biogeographic and molecular dataset – the largest ever assembled for *Horaglanis*. Utilizing new information derived from these samples, we significantly advance knowledge of, and reduce key biodiversity shortfalls for, these enigmatic catfishes. In particular, we unravel range sizes and boundaries, highlight their deep genetic divergence alongside remarkable morphological stasis, and describe a new cryptic species, recovered as the sister taxon of the three previously described members of the genus.

Materials and Methods

Surveys and sample/data collection

From May 2016 to March 2022, we toured the lateritic regions of the State of Kerala, India, from 12.7°N to 8.3°N, covering a north-south distance of approximately 600 km (Fig. 1E). Sampling sites included dug-out wells, bore wells, natural wetlands adjacent to lateritic zones, home-gardens and plantations, as well as lateritic caves. We conducted a series of workshops, focus-group discussions and informal interactions with communities at several localities, including the type locality of the three known species. Local villagers were informed of the importance of the species and their conservation needs, and they were asked to share information, photographs or videos if the species were encountered and/or collected. This citizen science approach was complemented by our own targeted collection efforts including the draining of wells and overhead storage tanks, the use of scoop nets in shallow wetlands and in water channels in home gardens and plantations, as well as the use of baited traps in dug-out wells in homesteads, ponds and caves.

Sample preservation and analysis of external morphology

All fishes collected for the study were photographed alive, euthanized with clove oil, fixed in 5% formalin, and preserved in 70% ethanol, or directly preserved in 100% ethanol. Specimens that were received dead were fixed in

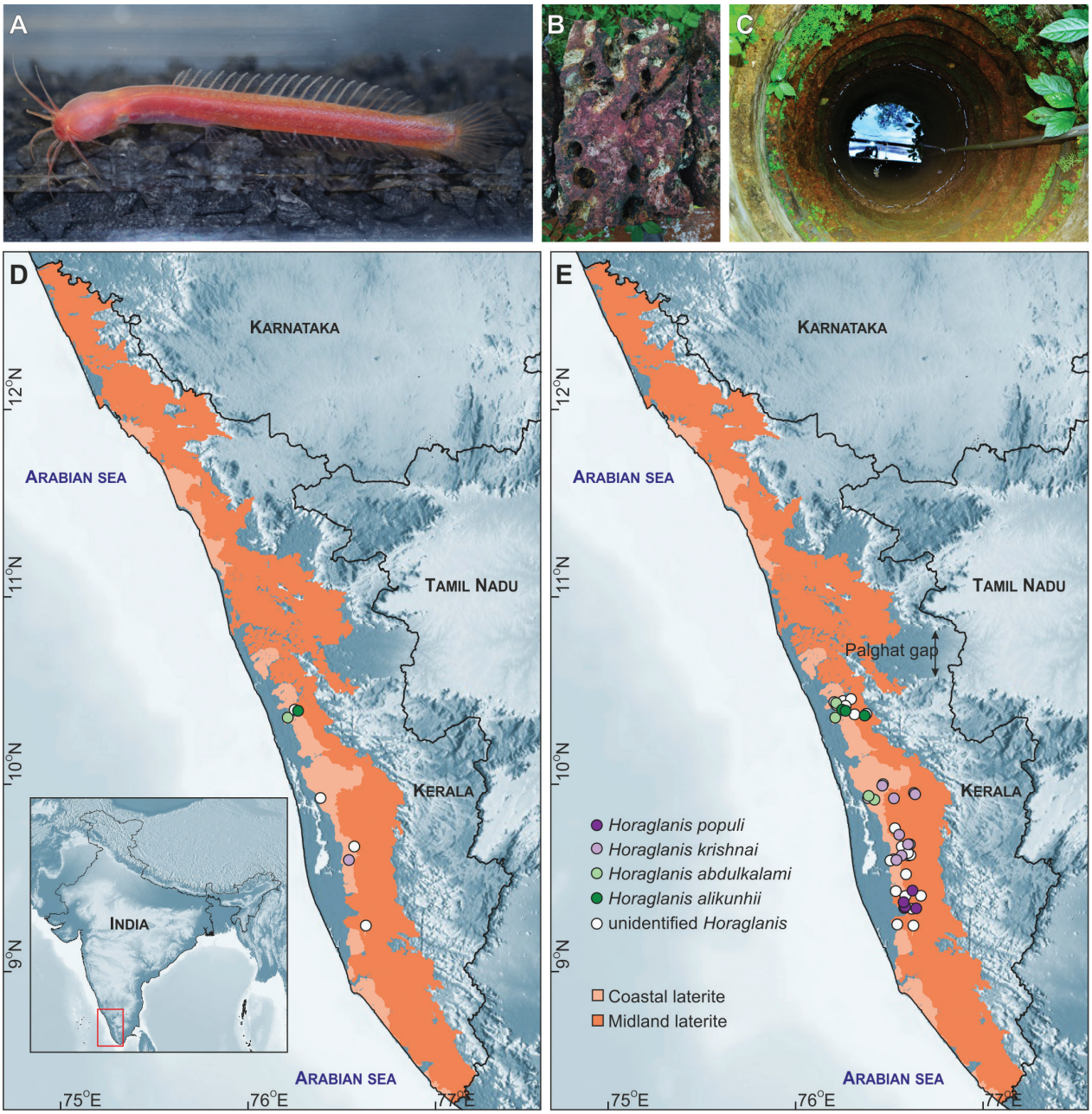

Figure 1. Habitus, habitat and distribution of *Horaglanis* in Kerala, southwestern India. **A** *Horaglanis* in life. **B** Typical laterite rock showing tiny pores. **C** Homestead lateritic dug-out well in Kerala – habitat of *Horaglanis*. **D** Range and species-specific localities within the lateritic soil zone of Kerala based on published distribution records prior to current study. **E** Current distribution records resulting from our citizen science campaign. Colored circles are genetically confirmed species, while unfilled/white circles indicate records available from social and print media that were not genetically analyzed.

formalin and subsequently transferred to ethanol. Specimens are deposited in the museum collection of Kerala University of Fisheries and Ocean Studies (KUFOS), Kochi, India. Characterization and analysis of morphometric and meristic information follow methods described in the original descriptions of the three species of *Horaglanis* (Menon 1951; Babu and Nayar 2004; Babu 2012), with the addition of several characters. Size adjusted multivariate morphometric data, expressed as percentage of standard length, were plotted using Principal Component Analysis (PCA) and the null hypothesis that there was no significant difference in the morphometric data among species was tested using PERMANOVA (Anderson 2001). PCA and PERMANOVA were performed in freeware PAST 4.12 (Hammer et al. 2001).

CT scanning

One paratype of *Horaglanis populi* (KUFOS.F.2022.106) was scanned with a Zeiss X-Radia Context CT-scanner in two segments (each 10:23 h), without filter at 50 kV and 4 W, with a voxel size of 2.05 micron, a cone angle of 7.08 degrees, using an exposure of 1.35 s and 8 frames, and 3201 projections. Volume was subsequently rendered in the software package Amira Pro.

Distribution range

We estimated Extent of Occurrence (EOO) and Area of Occupancy (AOO) as defined by the International Union for Conservation of Nature (IUCN Standards and Petitions Committee 2022) using the online tool GeoCat (Bachman et al. 2011) which considers a minimum spanning polygon and 2 km cell width respectively. In the case of *Horaglanis abdulkalami*, however, individuals were recorded from two wells, and therefore the distance between the wells was used as the EOO. The EOO and AOO calculations were done separately for the entire dataset, including all known locations, as well as for populations of species whose identity was confirmed by genetic analyses.

Genetic analysis

DNA was extracted from 20 freshly preserved specimens and or tissues using QIAamp® DNA Mini Kit – (QIAGEN, Germany) following the manufacturer's protocol. Four mitochondrial genes, (cytochrome oxidase subunit 1 [COI], cytochrome *b* [cyt *b*], the small [12S] and large [16S] subunit ribosomal ribonucleic acid) were amplified, purified and sequenced following published protocols (Rüber et al. 2006; Ali et al. 2013; Dahanukar et al. 2013; Verma et al. 2019). Chromatograms of DNA sequences were checked for the quality of base calls in FinchTV 1.4.0 (Geospiza, Inc.; Seattle, WA, USA; http://www.geospiza.com). A total of 65 new sequences of *Horaglanis* were generated (COI, 20; cyt *b*, 12; 12S, 17; and 16S, 16), and were combined with those already available on GenBank (Table 1). GenSeq nomenclature (Chakrabarty et al. 2013) for sequences generated in the current study is provided in Table 2. Sequences were aligned separately for each gene using MUSCLE 3.8.31 (Edgar 2004) implemented in MEGA 11 (Tamura et al. 2021) and then concatenated. These data were subsequently partitioned into four genes (COI, cyt *b*, 12S and 16S), as well as the respective three codon positions for COI and cyt *b*. Partition analysis (Chernomor et al. 2016) and ModelFinder (Kalyaanamoorthy et al. 2017) were used to identify the best partitioning scheme, and nucleotide substitution model based on the minimum Bayesian

Table 1. Locality, GenBank and haplotype details for cytochrome oxidase subunit 1 (COI), cytochrome *b* (cyt *b*), 12S rRNA and 16S rRNA gene sequences of *Horaglanis* species.

Species	Locality	COI	cyt *b*	12S	16S	COI haplotype
Horaglanis populi	Pathanamthitta, Edanad	OP825096**	OP832204**	OP824404**	OP824387**	Hp1
Horaglanis populi	Pathanamthitta, Mallappally	OP825097**	OP832205**	OP824405**	OP824388**	Hp2
Horaglanis populi	Pathanamthitta, Thiruvalla	OP825101**	OP832207**	OP824409**	OP824391**	Hp3
Horaglanis populi	Alappuzha, Chengannur	OP825098**	OP832206**	OP824406**	OP824389**	Hp3
Horaglanis populi	Alappuzha, Chengannur	OP825099**	–	OP824407**	–	Hp3
Horaglanis populi	Alappuzha, Chengannur	OP825100**	–	OP824408**	OP824390**	Hp4
Horaglanis populi	NA*	MZ820781	MZ802981	–	–	Hp5
Horaglanis populi	NA*	MZ820785	–	–	–	Hp6
Horaglanis populi	NA*	MZ820784	MZ802984	–	–	Hp7
Horaglanis abdulkalami	Thrissur, Cherpu	OP825092**	–	–	–	Hab1
Horaglanis abdulkalami	Ernakulam, Thuppampadi	OP825094**	OP832203**	OP824403**	OP824386**	Hab2
Horaglanis abdulkalami	Ernakulam, Chottanikara	OP825093**	–	OP824402**	OP824385**	Hab3
Horaglanis alikunhii	Thrissur, Parappukara	OP825095**	–	–	–	Hal1
Horaglanis alikunhii	Thrissur, Mankuttipadam	HE819391	HG937614	–	–	Hal2
Horaglanis alikunhii	Thrissur, Mankuttipadam	HE819392	–	–	–	Hal2
Horaglanis alikunhii	Thrissur, Mankuttipadam	HE819393	HG937613	–	–	Hal2
Horaglanis alikunhii	Thrissur, Mankuttipadam	HE819394	–	–	–	Hal2
Horaglanis alikunhii	NA*	MZ820782	MZ802982	–	–	Hal3
Horaglanis krishnai	Kottayam, Thiruvanchoor	OP825110**	OP832213**	OP824417**	OP824399**	Hk1
Horaglanis krishnai	Ernakulam, Pappukavala	OP825105**	OP832209**	OP824413**	OP824395**	Hk2
Horaglanis krishnai	Ernakulam, Avoly	OP825111**	OP832214**	OP824418**	OP824400**	Hk3
Horaglanis krishnai	Ernakulam, Kadayirippu	OP825102**	–	OP824410**	OP824392**	Hk4
Horaglanis krishnai	Ernakulam, Vazhakkulam	OP825104**	–	OP824412**	OP824394**	Hk5
Horaglanis krishnai	Ernakulam, Vazhakkulam	OP825108**	OP832212**	OP824416**	OP824398**	Hk5
Horaglanis krishnai	Ernakulam, Vazhakkulam	OP825109**	–	–	–	Hk5
Horaglanis krishnai	Kottayam, Kattachira	OP825103**	OP832208**	OP824411**	OP824393**	Hk6
Horaglanis krishnai	Kottayam, Kattachira	OP825106**	OP832210**	OP824414**	OP824396**	Hk7
Horaglanis krishnai	Kottayam, Kalathur	OP825107**	OP832211**	OP824415**	OP824397**	Hk8
Horaglanis krishnai	NA*	MZ820786	–	–	–	Hk9
Horaglanis krishnai	NA*	MZ820783	MZ802983	–	–	Hk10
Horaglanis krishnai	NA*	MZ820780	MZ802980	–	–	Hk11

Species	Locality	COI	cyt *b*	12S	16S	COI haplotype
Horaglanis krishnai	NA*	MZ820779	MZ802979	–	–	Hk11
Horaglanis krishnai	NA*	MZ820778	MZ802978	–	–	Hk11
Horaglanis krishnai	NA*	MZ820777	MZ802977	–	–	Hk11
Horaglanis krishnai	NA*	MZ820776	MZ802976	–	–	Hk11
Horaglanis krishnai	NA*	MZ820775	MZ802975	–	–	Hk11
Horaglanis krishnai	NA*	MZ820774	MZ802974	–	–	Hk12
* Location details not available; ** sequences generated in the current study						

Table 2. GenSeq nomenclature for sequences generated in the current study.

Species	Locality	Voucher	GenSeq
Horaglanis populi	Pathanamthitta, Mallappally	KUFOS.F.2022.101	genseq-1 COI, cyt *b*, 12S, 16S
Horaglanis populi	Pathanamthitta, Edanad	KUFOS.F.2022.103	genseq-2 COI, cyt *b*, 12S, 16S
Horaglanis populi	Pathanamthitta, Thiruvalla	KUFOS.F.2022.102	genseq-2 COI, cyt *b*, 12S, 16S
Horaglanis populi	Alappuzha, Chengannur	KUFOS.F.2022.106	genseq-2 COI, cyt *b*, 12S, 16S
Horaglanis populi	Alappuzha, Chengannur	KUFOS.F.2022.104	genseq-2 COI, 12S
Horaglanis populi	Alappuzha, Chengannur	KUFOS.F.2022.105	genseq-2 COI, 12S, 16S
Horaglanis abdulkalami	Thrissur, Cherpu	KUFOS.SFC.2022.01	genseq-3 COI
Horaglanis abdulkalami	Ernakulam, Thuppampadi	KUFOS.SFC.2022.02	genseq-4 COI, cyt *b*, 12S, 16S
Horaglanis abdulkalami	Ernakulam, Chottanikara	KUFOS.SFC.2022.03	genseq-4 COI, 12S, 16S
Horaglanis alikunhii	Thrissur, Parappukara		genseq-5 COI
Horaglanis krishnai	Kottayam, Thiruvanchoor	KUFOS.SFC.2022.09	genseq-4 COI, cyt *b*, 12S, 16S
Horaglanis krishnai	Ernakulam, Pappukavala	KUFOS.SFC.2022.10	genseq-4 COI, cyt *b*, 12S, 16S
Horaglanis krishnai	Ernakulam, Avoly	KUFOS.SFC.2022.13	genseq-4 COI, cyt *b*, 12S, 16S
Horaglanis krishnai	Ernakulam, Kadayirippu	KUFOS.SFC.2022.14	genseq-4 COI, 12S, 16S
Horaglanis krishnai	Ernakulam, Vazhakkulam		genseq-5 COI, 12S, 16S
Horaglanis krishnai	Ernakulam, Vazhakkulam		genseq-5 COI, cyt *b*, 12S, 16S
Horaglanis krishnai	Ernakulam, Vazhakkulam		genseq-5 COI
Horaglanis krishnai	Kottayam, Kattachira	KUFOS.SFC.2022.15	genseq-3 COI, cyt *b*, 12S, 16S
Horaglanis krishnai	Kottayam, Kattachira	KUFOS.SFC.2022.15	genseq-3 COI, cyt *b*, 12S, 16S
Horaglanis krishnai	Kottayam, Kalathur	KUFOS.SFC.2022.17	genseq-4 COI, cyt *b*, 12S, 16S

Information Criterion (BIC) (Schwarz 1978). Maximum likelihood (ML) analysis was performed in IQ-TREE 2.2.0 (Minh et al. 2020) with the best partition scheme and ultrafast bootstrap support for 1000 iterations (Hoang et al. 2018) (Supplementary Tables S1, S2). The phylogenetic tree was edited in FigTree v1.4.4 (Rambaut 2018). Genetic uncorrected p-distances for COI and cyt *b* were estimated in MEGA 11 (Tamura et al. 2021).

Because COI sequences were available for the largest number of samples, only this locus was used for population structure and species delimitation analyses (Barcode Gap Analysis and Poisson Tree Process). Assemble Species by Automatic Partitioning (ASAP) employing uncorrected genetic distances was used for barcode gap analysis (Puillandre et al. 2021), while the Poisson Tree Process (PTP) was performed using three different approaches, single-rate with maximum likelihood support (PTP), single-rate with Bayesian support (bPTP) and multi-rate (mPTP) (Zhang et al. 2013; Kapli et al. 2017). All PTP methods were performed using the maximum likelihood tree obtained from IQTREE 2.2.0 (Minh et al. 2020) using the best partition scheme and nucleotide substitution model.

Nucleotide diversity, number and diversity of haplotypes, Tajima's D, and demographic history (by constructing pairwise mismatch distributions) were estimated in DnaSP 6.12.03 (Rozas et al. 2017). The genetic network was constructed in the freeware POPART (Leigh and Bryant 2015) using the median joining method, with $\varepsilon = 0$ to derive the minimum spanning network (Bandelt et al. 2019).

Comparative material

Horaglanis krishnai (n = 10): KUFOS.SFC.2022.09, Thiruvanchoor, Kottayam, Kerala, India; KUFOS.SFC.2022.10–12, 3 ex., Pappukavala, Muvattupuzha, Ernakulam, Kerala, India; KUFOS.SFC.2022.13, Avoly, Muvattupuzha, Ernakulam, Kerala, India; KUFOS.SFC.2022.14, Kadayirippu, Ernakulam, Kerala, India; KUFOS.SFC.2022.15–16, 2 ex., Kattachira, Kottayam, Kerala, India; KUFOS.SFC.2022.17, Kalathur, Kottayam, Kerala, India; KUFOS.SFC.2022.18, Amayanoor, Kottayam, Kerala, India. *Horaglanis abdulkalami* (n = 3): KUFOS.SFC.2022.01, Cherpu, Thrissur, Kerala, In-

Table 3. Percentage genetic p-distances based on cytochrome oxidase subunit 1 (COI) gene. Values in bold are intraspecific distances.

Species	[1]	[2]	[3]	[4]
Horaglanis populi [1]	**0.0–4.1**			
Horaglanis abdulkalami [2]	15.6–17.4	**0.3–2.5**		
Horaglanis alikunhii [3]	15.3–16.5	7.0–8.3	**0.0–1.3**	
Horaglanis krishnai [4]	13.8–16.5	10.0–12.2	10.1–12.3	**0.0–5.3**

Table 4. Percentage genetic p-distances based on cytochrome *b* (cyt *b*) gene. Values in bold are intraspecific distances.

Species	[1]	[2]	[3]	[4]
Horaglanis populi [1]	**0.1–3.8**			
Horaglanis abdulkalami [2]	12.3–13.0	**0.0**		
Horaglanis alikunhii [3]	13.0–13.8	7.9	**0.0**	
Horaglanis krishnai [4]	13.0–14.0	11.2–12.1	12.8–13.6	**0.1–6.8**

dia; KUFOS.SFC.2022.02, Thuppampadi, Ernakulam, Kerala, India; KUFOS.SFC.2022.03, Chottanikara, Ernakulam, Kerala, India.

Results

Restricted range

Specimens of *Horaglanis* have hitherto been collected only from homestead dug-out wells (5-10 m deep) across the laterite soil formations in Kerala State, to which the genus is endemic. As a consequence of these unique sampling circumstances, *Horaglanis* was, until recently, known from only seven localities (Menon 1951; Mercy 1981; Babu and Nayar 2004; Babu 2012; Vincent 2012). Over the course of our study, we obtained 47 new vouchered location records for this genus (Fig. 1E), of which the vast majority were provided by interested members of the public as result of a citizen science campaign. Altitudinal distribution of *Horaglanis* ranged from wells located in villages close to mean sea level up to a maximum of 39 m above sea level (asl). The majority of records were confined to wells at 6 to 22 m asl with a median altitude of 12 m asl. Although the distribution range of *Horaglanis* has thus been expanded considerably, the genus still has a relatively small extent of occurrence of 3167 km^2 and an area of occupancy of 144 km^2 (between 9.3°N to 10.4°N). This range spreads across the lateritic zones of five districts in Kerala (Alappuzha, Pathanamthitta, Kottayam, Ernakulam and Thrissur) (Fig. 1E, Table 1), the northernmost and southernmost localities separated by a distance of ~150 km. Among the species, *H. krishnai* has the largest distribution, with an extent of occurrence of 429 km^2, with the southern and northern populations separated by an aerial distance of ~85 km. *Horaglanis abdulkalami* is the second-most widespread species, with an extent of occurrence of 73 km^2 and a distance of ~82 km separating the northern- and southernmost populations.

Genetic diversification

Genetic analysis of 65 DNA sequences generated specifically for this study, in addition to 18 sequences already available in GenBank, revealed high intraspecific and interspecific genetic divergence between the different *Horaglanis* lineages in both the COI (Table 3) and the cyt *b* (Table 4) genes. Maximum likelihood analysis based on the COI gene (Fig. 2B) recovered a topology similar to that of the concatenated dataset (Fig. 2A). The barcode gap analysis using ASAP and three different approaches of PTP clearly delineate four species of *Horaglanis* (Fig. 2B). The best score for ASAP had four partitions, which separated the clades with a genetic uncorrected p distance of at least 7.0% (Supplementary Fig. S1). The greatest intraspecific genetic divergence in the COI barcoding region was observed in *H. krishnai* (5.3%), while the smallest intraspecific divergence was 7.0% (*H. abdulkalami* vs. *H. alikunhii*) (Table 3). As a result, a minimum genetic barcode gap of 5.3–7.0% separates the different species (Fig. 2C).

Three of the four lineages correspond to described species, *Horaglanis krishnai*, *H. alikunhii*, and *H. abdulkalami*. The unnamed southernmost lineage is described below as a new species, *Horaglanis populi*. All four species identified via genetic delimitation had multiple haplotypes (Fig. 3E–H), with *H. krishnai* possessing the largest number of unique haplotypes, followed by the new species *H. populi*. The greatest haplotype diversity was observed in *H. abdulkalami*, followed by *H. populi* and *H. krishnai*, while the greatest nucleotide diversity was observed in *H. krishnai*, followed by *H. populi* and *H. abdulkalami* (Supplementary Table S3). Mismatch distribution of all four species is multimodal (Fig. 3A–D), indicating a lack of evidence for recent population expansion.

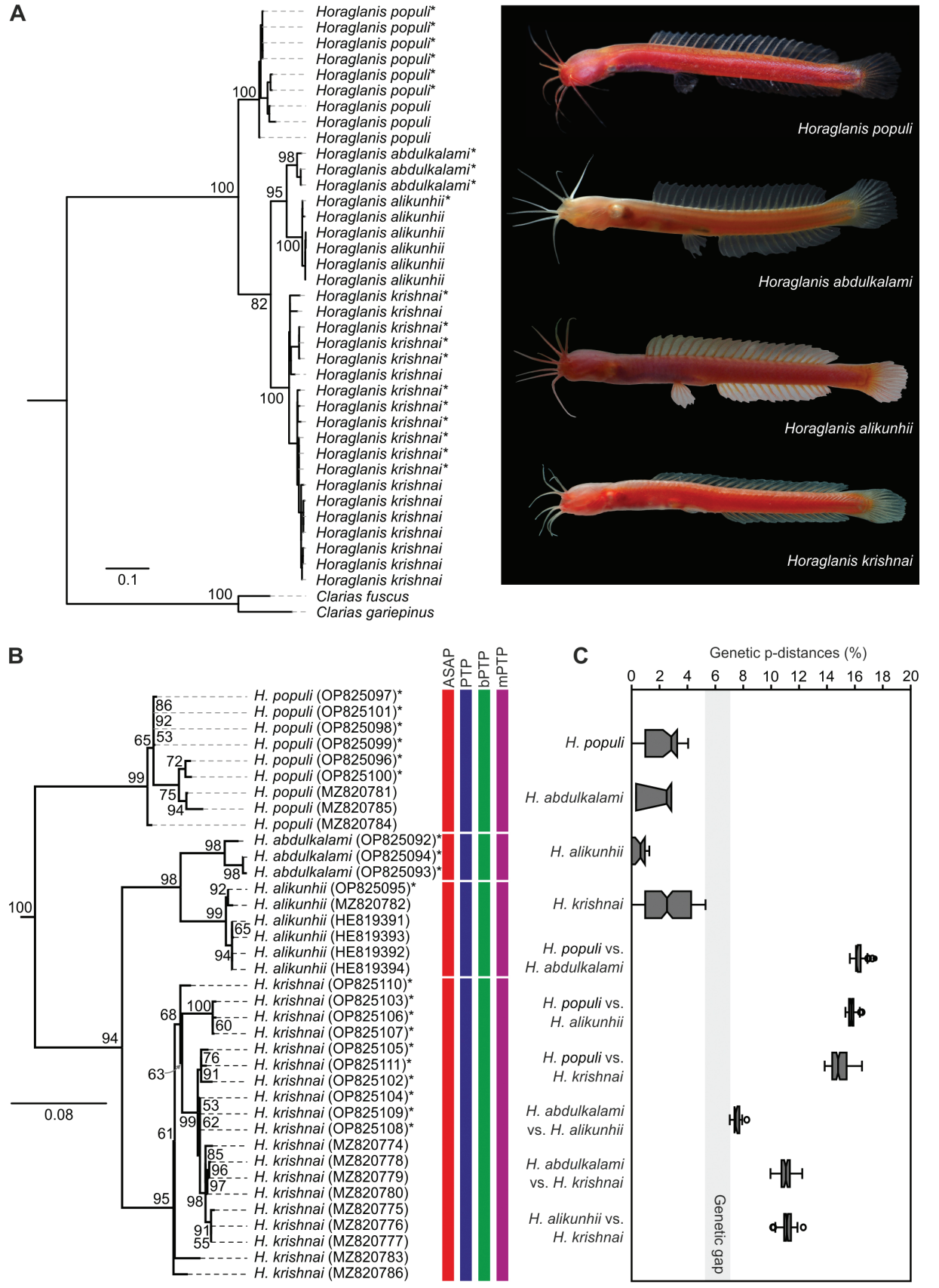

Figure 2. Phylogenetic tree of species of *Horaglanis* and their delimitation. **A** Maximum likelihood phylogenetic tree based on concatenated mitochondrial COI, cyt *b*, 12S rRNA and 16S rRNA gene sequences employing best partition scheme and nucleotide substitution models. **B** Maximum likelihood phylogenetic analysis based on COI gene employing best partition scheme and nucleotide substitution models. Species delimitation based on ASAP, PTP, bPTP and mPTP processes shown as bars adjacent to species names. **C** Box plots of intraspecific and interspecific genetic p-distances in COI gene. Genetic gap between greatest intraspecific (5.3%) and smallest interspecific (7.0%) genetic distance shown in light grey. **A**, **B** *Clarias* species used as outgroups. Values along nodes are bootstrap supports based on 1000 iterations. Asterisks indicate sequences generated in the current study.

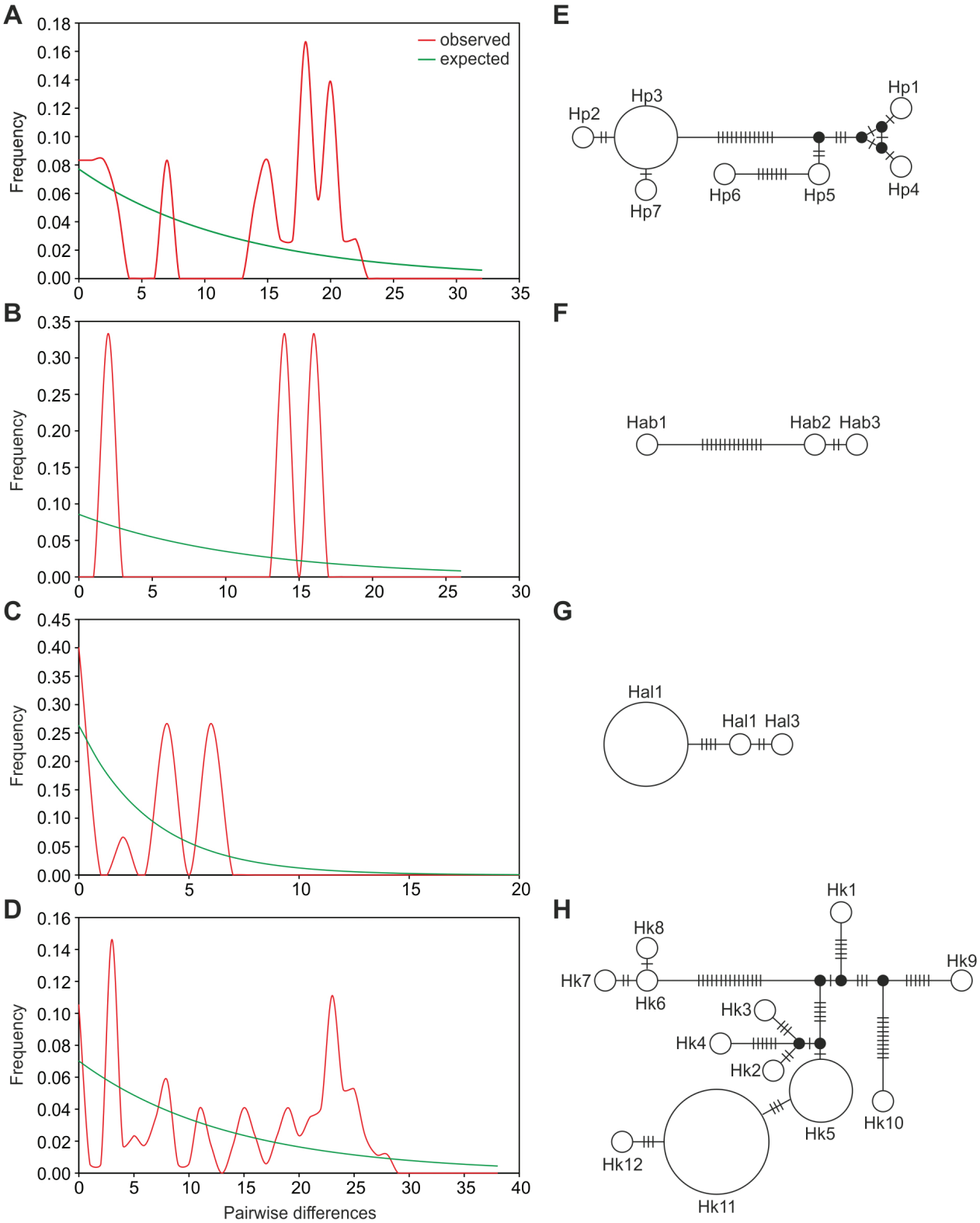

Figure 3. Mismatch distribution and median joining genetic network based on cytochrome oxidase subunit 1 for **A**, **E** *Horaglanis populi*; **B**, **F** *H. abdulkalami*; **C**, **G** *H. alikunhii*; **D**, **H** *H. krishnai*. Haplotype labels as in Table 1.

The intraspecific genetic distances in the COI gene for *Horaglanis krishnai, H. populi* and *H. alikunhii* were significantly correlated with the geographical distance separating the different localities/populations (Supplementary Fig. S2D). Whether the observed positive relationship between the genetic and geographical distance for *H. abdulkalami* (Supplementary Fig. S2C) was significant could not be determined as the number of occurrence points was fewer than four. The genetic network for all four species showed larger numbers of mutations separating the haplotypes (Fig. 3E–F), even from localities that were geographically adjacent. Despite this large genetic variation, Tajima's D was non-significant ($D = 0.7700$, $P > 0.10$), suggesting that the COI gene is under neutral evolution.

Table 5. Intra-specific variation in meristic counts across four different species of *Horaglanis*.

Species/Locations	Dorsal-fin rays	Pelvic-fin rays	Anal-fin rays	Caudal-fin rays
Horaglanis krishnai				
Kottayam[1]	23–24	6	16–17	22–24
Kottayam[2]	23–24	6	15–18	26
Ettumanoor[3]	23	-	17	24
Amayanoor	22	6	15	22
Kattachira (N = 2)	23–24	6	16–17	30–31
Kalathur	24	6	17	23
Thiruvanchoor	23	6	17	22
Pappukavala (N = 3)	23–24	6	16–17	23–27
Avoly	23	6	16	28–30
Kadayirippu	24	6	17	28
Horaglanis alikunhii				
Parappukara[3]	24	6	17	30
Pudukkad[4]	24	6	16	20
Kodakara[4]	23	6	16	20
Kodaaly[5]	23	-	16	-
Horaglanis abdulkalami				
Irinjalakuda[6]	21	6	15	28
Cherpu	23	6	16	22
Kodaaly[5]	20	-	15	-
Thuppampadi	22	6	16	28
Chottanikara	26	6	18	26
Horaglanis populi				
Edanadu	23	6	17	27
Malapally	26	6	17	29
Thiruvalla	24	6	16	25
Chengannur (N = 3)	21–24	6	14–17	23–28

[1] Menon (1951); [2] Mercy (1981); [3] Babu and Nayar (2004); [4] Based on photographs; [5] Vincent (2012); [6] Babu (2012)

Table 6. Intraspecific variation in morphometric characters across three different species of *Horaglanis* from our collection. Comparative material of *Horaglanis alikunhii* was not available for morphometric analysis.

Characters	*Horaglanis populi* (n = 6)			*Horaglanis krishnai* (n = 10)		*Horaglanis abdulkalami* (n = 3)	
	Holotype	Mean (sd)	Range	Mean (sd)	Range	Mean (sd)	Range
Total Length	37.0	31.9 (3.2)	27.1–37.0	36.2 (5.5)	28.7–43.4	32.4 (0.4)	32–32.8
Standard Length	32.5	28.2 (2.9)	23.9–32.5	32.5 (5.2)	25.0–39.9	28.7 (0.2)	28.5–28.9
% SL							
Head length	16.9	17.9 (1.9)	15.7–20.4	16.0 (0.7)	14.9–16.9	17.3 (1.9)	15.1–18.5
Pre-dorsal length	31.3	34.4 (2.3)	31.3–36.9	34.1 (6.1)	27.5–47.8	31.9 (1)	30.9–32.9
Dorsal-fin length	8.9	8.0 (0.8)	6.8–8.9	7.5 (0.7)	6.5–8.7	10 (0.2)	9.8–10.2
Dorsal-fin base length	58.5	61.4 (3.9)	57.4–68.2	58.7 (4.7)	52.1–64.3	59.7 (4.4)	56–64.5
Length from origin of dorsal fin to origin of anal fin	29.0	27.8 (0.9)	26.4–29.0	23.8 (3.3)	18.4–27.8	23.3 (0.3)	23.1–23.6
Length from origin of dorsal fin to origin of pelvic fin	9.0	11.9 (2.1)	9.0–14.1	10.1 (1.7)	7.3–12.6	9.8 (0.5)	9.3–10.2
Anal-fin length	8.8	8.2 (1.0)	7.2–9.9	6.9 (1.2)	5.2–9.0	8.9 (0.3)	8.6–9.1
Anal-fin base length	37.5	36.1 (3.1)	31.6–39.0	39.2 (2.5)	34.8–43.5	39.5 (3.2)	36.2–42.6
Pelvic-fin length	7.8	8.5 (1.4)	7.2–10.9	7.5 (2.8)	4.4–12.1	9.2 (1.2)	7.8–10
Caudal-fin length	15.7	14.7 (1.5)	13.2–16.7	12.5 (1.9)	8.7–15.6	14.2 (0.7)	13.5–14.9
Caudal-peduncle length	9.3	11.9 (2.9)	9.3–16.9	10.8 (2.1)	8.3–15.6	10.5 (0.8)	9.8–11.4

Morphological stasis

All four species of *Horaglanis* show a remarkable level of morphological reduction, with the pectoral fin reduced to a single fin spine and several bones missing in addition to the absence of eyes and the lateral-line canal system. Surprisingly, the external characters that can be observed in *Horaglanis*, and the meristic (Table 5) and morphometric (Table 6) data, show large intraspecific variation and no significant differences between the species, and

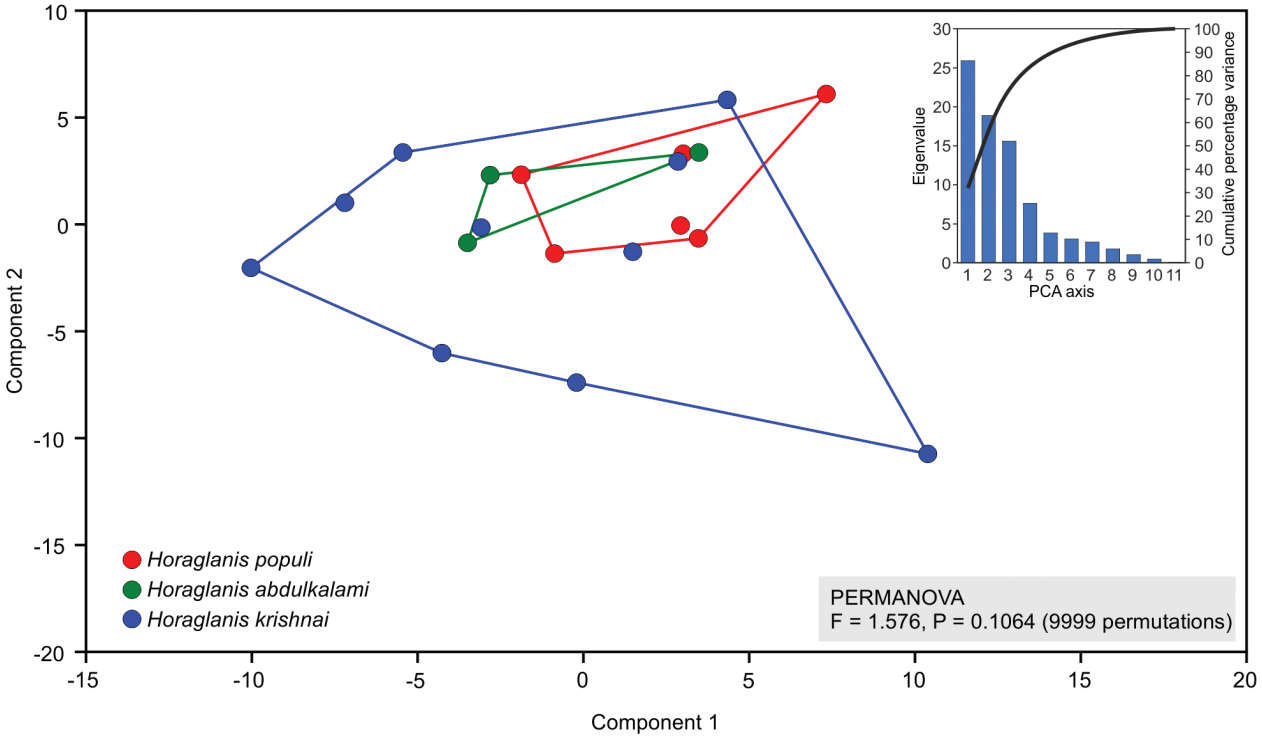

Figure 4. Principal Component Analysis of multivariate morphometric data presented in Table 4. Factor scores of observations are plotted on the first two components that together explained 55.28% of the total variation in the data. Scree plot for factor loadings is provided in the inset. There were no significant morphometric differences in the three species (PERMANOVA, 9999 permutations, F = 1.576, P = 0.1064).

thus cannot be used to distinguish them. The unexpectedly large genetic divergence between the species is thus not mirrored by any significant morphological diversification (Fig. 4); we therefore had to rely on molecular characters to diagnose the new species, *H. populi.*

Horaglanis populi, sp. nov.

https://zoobank.org/64F96C39-BC11-44B6-807B-A0DF-8D76A97C

Fig. 5

Holotype. KUFOS.F.2022.101, 32.5mm SL. from a dug-out well at Malapally, Kerala, India (21 m asl), collected by Remya L. Sundar, Arya Sidharthan and C.P. Arjun on 6 Dec 2020.

Paratypes (n = 5). KUFOS.F.2022.102, 23.9mm SL, from a dug-out well at Thiruvalla, Kerala, India (7 m asl), collected by V.K. Anoop on 11 Dec 2019; KUFOS-.F.2022.103, 26.8mm SL, from a dug-out well at Edanadu, Kerala, India (18 m asl), collected by Remya L. Sundar and Arya Sidharthan on 03 Dec 2020; KUFOS.F.2022.104, 27.4mm SL, from a dug-out well at Thiruvanvandoor, Chengannur, Kerala, India (5 m asl), collected by Remya L. Sundar on 10 Mar 2022; KUFOS.F.2022.105, 29.0mm SL, from a dug-out well at Thiruvanvandoor, Chengannur, Kerala, India (5 m asl), collected by Arya Sidharthan on 14 Dec 2020; KUFOS.F.2022.106, 29.4mm SL, from a dug-out well in Chengannur, Kerala, India (5 m asl), collected by Remya L. Sundar and Arya Sidharthan on 01 Dec 2021.

Etymology. The species name *populi*, genitive of the Latin noun *populus* = people, honours the invaluable contributions made by interested members of the public in the southern Indian state of Kerala, helping to document the biodiversity of subterranean and groundwater systems, including the discovery of this new species.

Diagnosis. A species of *Horaglanis* as evidenced by the absence of eyes and pigment, a blood-red body in life, a highly reduced pectoral fin in which only a shortened spine is present, an elongate body with long dorsal and anal fins extending to the base of the caudal peduncle, and four pairs of well-developed barbels. Genetically, *Horaglanis populi* forms a distinct clade, the sister group to the other three congeners (Fig. 2), from which it differs by a genetic uncorrected p distance of 13.8–17.4% in the COI gene, and between 12.3–14.0% in the cyt *b* gene. Specifically, *H. populi* differs from all three known species in the barcoding gene (Supplementary Table S4) in positions 106 (C vs. T), 115 (T vs. C), 142 (T vs. C), 171 (G vs. A), 183 (T vs. C), 216 (A vs. C or T), 234 (C vs. T), 237 (G vs. A), 265 (T vs. G), 270 (C vs. A), 312 (A vs. C or T), 324 (A vs. C), 325 (T vs. C) 330 (G. vs. A or T), 350 (G vs. T), 363 (T vs. G), 421 (C vs. G), 448 (C vs. T), 481 (G vs. T), 489 (C vs. T), 496 (A vs. G), 517 (c vs. T), 528 (G vs. T), 533 (G vs. A), 538 (A vs. C), 539 (A vs. G), 542 (T vs. C), 565 (T vs. A), 576 (G

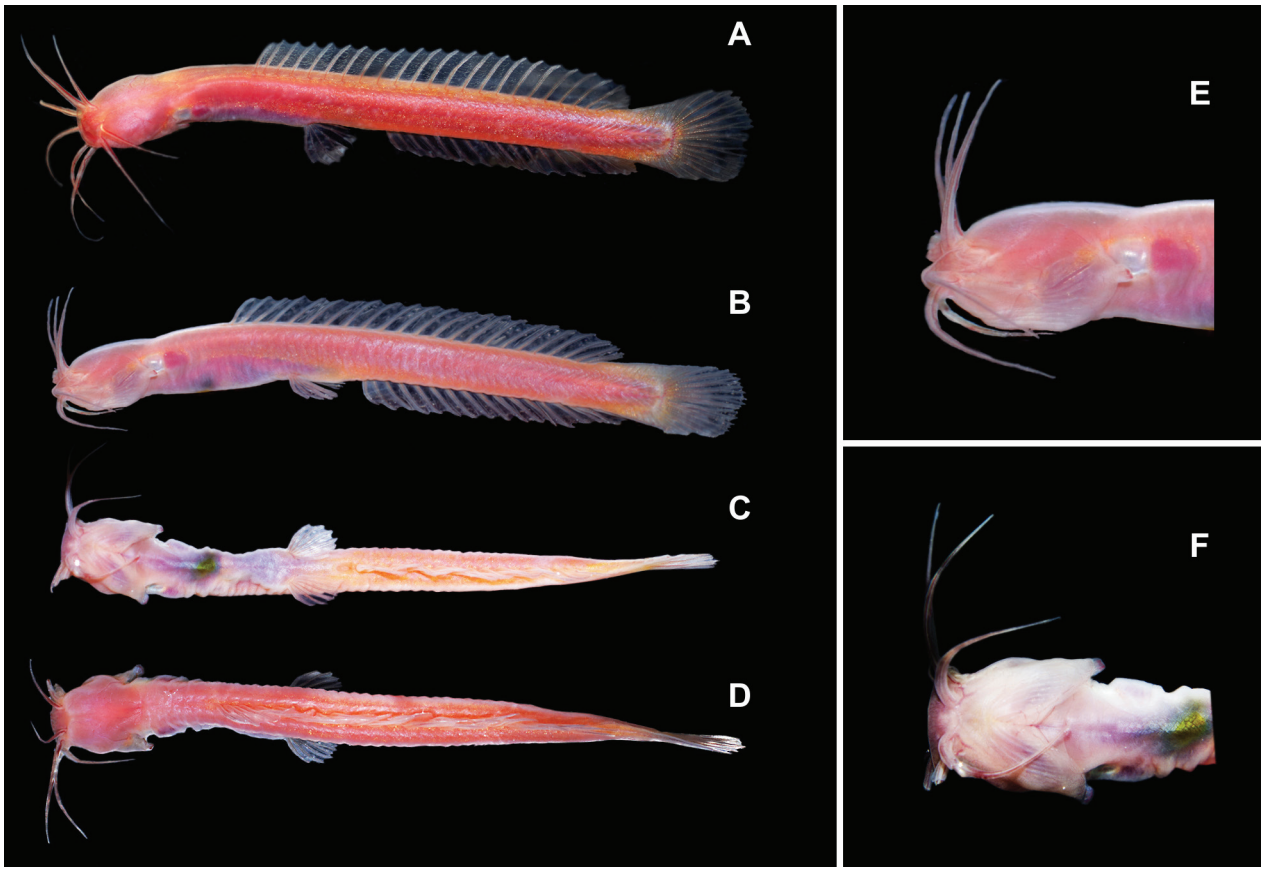

Figure 5. *Horaglanis populi* holotype (KUFOS.F.2022.101, 32.5 mm standard length) in **A** life and **B**–**F** immediately after preservation. **A**, **B** Lateral view; **C** ventral view; **D** dorsal view; **E** lateral view of head; **F** ventral view of head.

vs. T or C), 597 (A vs. C), 618 (C vs. T), 633 (G vs. A) and 636 (C vs. T).

Description. Body elongated (Fig. 5), round in cross section anteriorly, laterally compressed posteriorly, dorsal profile slightly convex to start of dorsal fin, straight more posteriorly. Ventral profile convex in head region, then straight posteriorly. Head large, 15.7–20.4% standard length (Table 6), with dorsally and laterally bulging adductor muscles. Snout truncated. Mouth wide, terminal. Eye absent. Four pairs of barbels: two mandibular, one maxillary and one nasal barbel pair; nasal and inner mandibular barbels shorter than maxillary and outer mandibular barbels. Maxillary and outer mandibular barbels reaching posterior border of pectoral fins when folded back. Gill opening large, extending to slightly above pectoral-fin base; gill membranes united with isthmus. Scales absent. Caudal peduncle laterally compressed, 9.3–16.9% of standard length. Dorsal fin long, with 22–23 soft rays (xiii–xiv unbranched/8–9 branched), originating in advance of pelvic fin origin. Anal fin long, with xiii–xvii unbranched rays, starting opposite dorsal fin ray number 9, ending opposite base of last dorsal fin ray. Pectoral fin vestigial, consisting only of modified pectoral fin spine covered by thickened skin. Pelvic fin short, wide, with rounded margin, with ii–iv unbranched and 2–4 branched rays. Caudal fin with rounded posterior margin, with 8–9 branched and 2–4 dorsal unbranched and 2–4 ventral unbranched rays.

Head skeleton well ossified (Fig. 6); neurocranium with a single large cranial fontanelle, no epiphyseal bridge connecting frontals in dorsal midline; lateral neurocranium wall with large trigeminofacial foramen; supraoccipital with long, narrow and pointed crest; opercle small and subtriangular. Jaws massive, dentary and premaxilla studded with numerous rows of closely set, recurved villiform teeth.

Distribution. *Horaglanis populi* is restricted to the lateritic aquifer systems in the Alappuzha and Pathanamthitta Districts of Kerala, southern India, where it has been collected from dug-out wells in the towns of Malapally, Edanadu, and Chengannur, and the nearby village of Thiruvanvandoor (Fig. 1E).

Discussion

Aquifers are unique subterranean microhabitats owing to their strong hydrographical isolation, limited connectivity with surface waters (mostly through springs, small pools and dug-out wells), and reduced possibilities for long-range dispersal (Trontelj et al. 2009; Juan and Emerson 2010; Galassi et al. 2014; Segherloo et al. 2018). Life in these microhabitats is constrained by ecological conditions including darkness, reduced concentration of nutri-

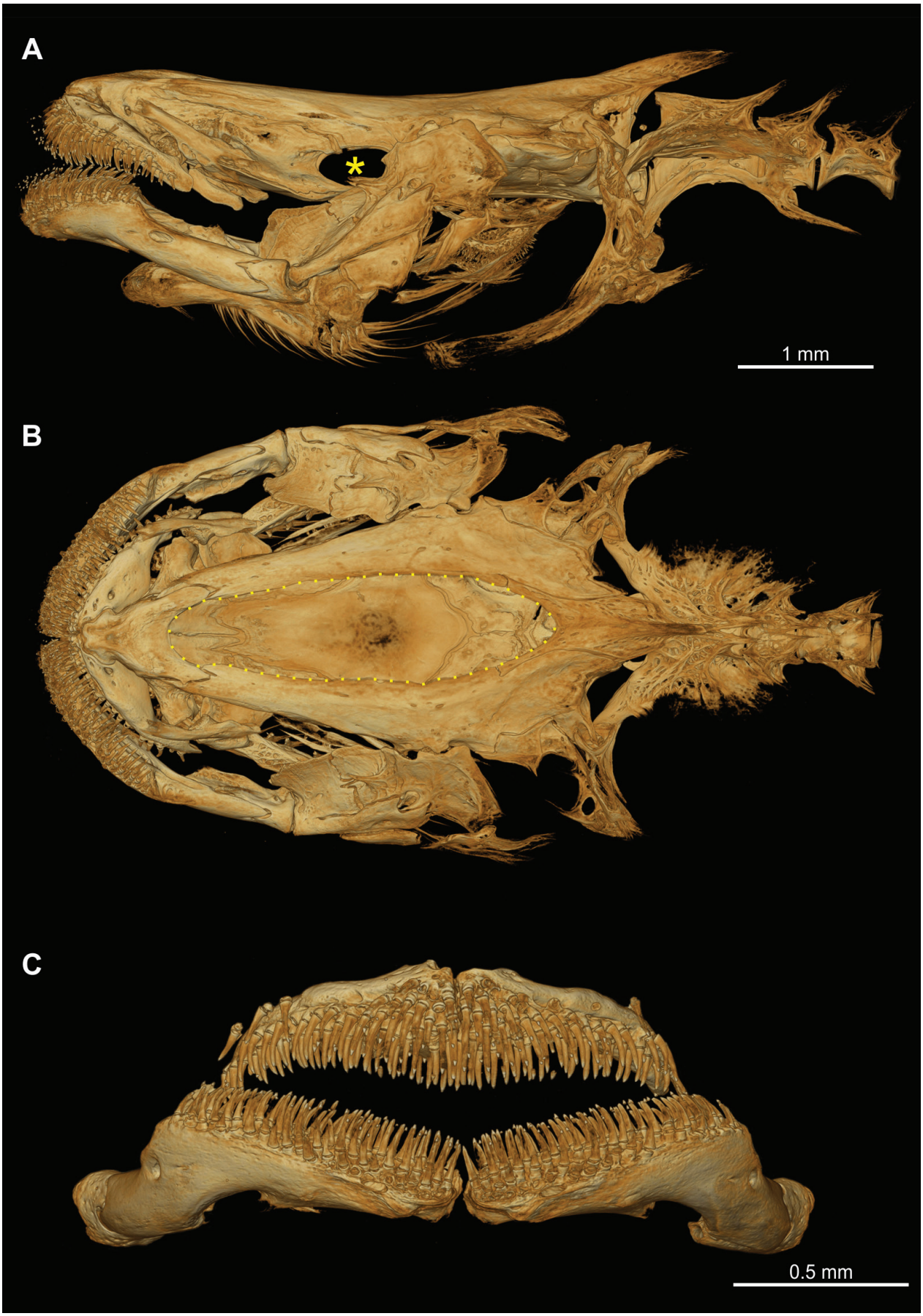

Figure 6. 3-D reconstructed CT-images of head and anterior vertebrae of *Horaglanis populi*, KUFOS.F.2022.106, 29.3 mm. **A** Lateral view, note large trigeminofacial foramen (marked by asterisk) in lateral wall of neurocranium; **B** dorsal view illustrating lack of epiphyseal bridge and large cranial fontanelle (margin marked by line of dots); **C** anterior view of upper and lower jaws, showing rows of sharply pointed recurved, villiform teeth.

ents, carbon and dissolved oxygen, and highly restricted free space (Hancock et al. 2005). Of the 289 known subterranean fish species, which include 53 catfishes (Proudlove 2022), fewer than 10% reside in aquifers. Examples include the enigmatic blind catfishes *Trogloglanis pattersoni* Eigenmann and *Satan eurystomus* of the Edwards Aquifer in Texas (Langecker and Longley 1983), the *Phreatobius* catfishes of South America (Muriel-Cunha and de Pinna 2005), the blind gobies of the genus *Typhleotris* from southwestern Madagascar (Vences et al. 2018), and the blind species of *Garra* from the Zagros mountains of Iran (Vatandoust et al. 2019). *Horaglanis* represents the only example of a genus of stygobitic fishes associated exclusively with lateritic aquifers – all other aquifer-dwelling fishes inhabit limestone formations.

Interestingly, no aquifers elsewhere in the world appear to have evolved as diverse a fish fauna as that associated with the laterite soil formations in the coastal area of southwest peninsular India. With the discovery and description of *Horaglanis populi*, four species of this catfish genus, as well as three species of the swamp-eel genus *Rakthamichthys* and two species of the eel loach genus *Pangio* have been discovered from aquifer-fed wells. All these show the typical troglomorphies associated with life underground (Raghavan et al. 2021). Two additional subterranean species, *Aenigmachanna gollum* and *Kryptoglanis shajii* Vincent & Thomas, collected also from rice paddies and adjoining wetlands, show no obvious troglomorphies: they may not be strictly aquifer-dwelling species (Raghavan et al. 2021). Overall, the diversity of subterranean fishes in southern peninsular India is rivalled only by the radiation of cave fishes of the genus *Sinocyclocheilus* in the karstic regions of southwest China (Zhou et al. 2022).

Much like the subterranean habitat in which the genus is found, *Horaglanis* has received very little scientific attention, despite being arguably one of the most unusual genera of catfishes known. Of the three nominal species previously known, only *Horaglanis krishnai* has been studied in any detail (Menon 1952; Mercy et al. 1982; Mercy and Pillai 1985; Mercy et al. 2001; Mercy and Pillai 2001). The remaining two are known only from their original descriptions (Babu and Nayar 2004; Babu 2012). Our extensive dataset of 47 new location records and 65 new genetic sequences shows that *Horaglanis* is endemic to the part of Kerala State south of the Palghat Gap (Fig. 1E), a well-known biogeographic barrier. With the exception of the species pair *H. abdulkalami* and *H. alikunhii,* the species of *Horaglanis* occur in allopatry. The genus is restricted to lateritic aquifers and has been encountered solely in groundwater-fed wells. While we have no information on the number, size and extent of the aquifers populated by *Horaglanis*, we found that the northern extent of its range is limited by the Bharathapuzha (the second largest river basin in the region), as well as a wide zone of rock formations in which laterite rock is absent. This zone (i.e., the northern extremity of the distribution of the genus) coincides with the Palghat Gap. Similarly, the major barrier separating the southern *H. krishnai* and *H. populi* from the other two species is likely the Periyar-Chalakudy River basin (the largest river basin in the region). However, some populations of *H. abdulkalami*, though substantially divergent genetically from those close to the type locality of this species, occur south of the Periyar.

Some stygobitic fishes are known to have large distribution ranges, such as the catfish *Prietella phreatophila* Carranza, whose northern and southernmost populations in Mexico are separated by a span of 750 km (Hendrickson et al. 2001), or the blind, subterranean cave eel, *Ophisternon candidum* (Mees)*,* a north-western Australian endemic (>400 km) (Moore et al. 2018). This vast distribution range could be attributed to the 'interstitial highway' hypothesis (Ward and Palmer 1994), i.e., the presence of an extensive, continuous hypogean habitat. Compared to *P. phreatophila* and *O. candidum,* the distribution ranges of all four species of *Horaglanis* lie within a north-south span of only 150 km. The current distribution of the various species of *Horaglanis* is likely the result of vicariance events, or the traditional low-dispersal (movement within the aquifers) hypotheses (Trontelj et al. 2009) associated with most subterranean taxa – for example, the blind cave fishes of Iran (Segherloo et al. 2022). Though *Horaglanis* populations are able to move through the narrow pores of aquifers, they are likely confined by barriers such as the ones mentioned above, which limit longer distance movements. Another potential vicariance barrier may have resulted from historical changes of eustatic sea level. These have occurred frequently since the late Miocene and continued into the Pleistocene (Miller et al. 2005, 2020); they would have led to prolonged marine transgression of the coastal areas of Kerala, to which *Horaglanis* is endemic. These marine transgressions may also have played a role in forming distribution barriers leading to vicariant speciation (discussed for the case of Sri Lanka in Pethiyagoda & Sudasinghe 2021). The interesting and complex distribution pattern of *Horaglanis* is thus likely linked to successive isolation and reconnection events (Devitt et al. 2019), that can be further unraveled through integrative phylogenomic and hydrological studies.

By generating the first multi-gene phylogeny of *Horaglanis,* we discovered that *H. populi* comprises the sister group of the clade containing its congeners, from which it is separated by a genetic distance (in the barcoding region of COI) of 13.8–17.4%. With interspecific divergences of 7.0-17.4%, the four species of *Horaglanis* were unambiguously delimited into distinct species based on both the barcode gap analysis and the Poisson tree process. This large interspecific genetic distance in *Horaglanis* is in sharp contrast to the lower genetic divergence in the COI gene (3.8%) between the morphologically distinct *Garra typhlops* Bruun & Kaiser and *G. lorestanensis* Mousavi-Sabet & Eagderi, two sympatric, blind Iranian cave barbs (Segherloo et al. 2012), but comparable to the three obligate cave-dwelling gobies of the Malagasy genus *Typhleotris* (Vences et al. 2018), in which it is 6.3-9.8%. Although the data on genetic divergence in subterranean catfishes are limited, both the maximum intraspecific and minimum interspecific genetic divergence in *Horaglanis* is higher than those for most surface-dwelling catfishes

(see for example, Anjos et al. 2020; Bhattacharjee et al. 2012; Hashimoto et al. 2020; Zou et al. 2020).

While the descriptions of *H. abdulkalami* and *H. alikunhii* (Babu and Nayar 2004; Babu 2012), which were based on four specimens and a single specimen, respectively, provided several characters to distinguish them from *H. krishnai*, our study, based on larger series of specimens, indicates that all four species of *Horaglanis* are indistinguishable in external morphology. The meristic data showed strong overlap in the character states among the four species. Though the morphometric data were available only for three of these (Table 6, Fig. 4), the limited data available on *H. alikunhii* from its original description (Subhash Babu and Nair 2004) suggests that the species is not morphometrically different from its three congeners. *Horaglanis* thus provides a case of extreme morphological stasis, similar to that seen in the African freshwater butterfly fish *Pantodon* (Lavoué et al. 2010), the Lake Tanganyikan cichlid *Tropheus* (Sturmbauer and Meyer 1992), and the subterranean catfishes of the genera *Rhamdiopsis* and *Trichomycterus* (Trajano 2021). Morphological stasis is often attributed to stabilizing selection (Sturmbauer and Meyer 1992; Parson 1994) influenced by factors such as lack of interspecific competition (Sturmbauer and Meyer 1992; Trajano 2021), the high energetic costs of life in harsh environments that preclude major evolutionary change (Turner 1986), low metabolic rates leading to low fecundity as an adaptation to survive in stressful and low-energy environments that restrict further morphological changes (Howarth 1993), and to life in stable phreatic environments over a long period (Trajano 2021). We suspect that morphological stasis in *Horaglanis* may be the result of a combination of several of these factors. The small pore size of the lateritic rocks in the aquifers restricts access to this habitat for other subterranean predators such as *Aenigmachanna* (Britz et al. 2020), resulting in a predator-free environment for *Horaglanis,* thus severely limiting interspecific competition. The low number of just 25 to 30 comparatively large eggs in *Horaglanis* (Mercy 1981) may be a response to living in a nutrient poor habitat. Compared to other aquatic systems, which are influenced by numerous external factors, lateritic aquifers would have provided a stable and ecologically homogenous environment for *Horaglanis* over a long period of time, likely the most important factor in stabilizing its external morphology despite speciation and significant diversification at the genetic level.

Our decision to describe a new species of *Horaglanis* based exclusively, at this point, on differences in the COI barcoding gene has not been taken lightly. We were faced with the decision to either (1) synonymize *H. alikunhii* and *H. abdulkalami* with *H. krishnai*, given the lack of external diagnostic characters, and also include the southernmost *Horaglanis*, we here refer to *H. populi*, in this taxon, or (2) to make a name available for the latter. In view of the substantial genetic divergence of this southern *Horaglanis* from its already named congeners, a divergence otherwise not even encountered between genera of catfishes (see for example, Bhattacharjee et al. 2012; Zou et al. 2020), we decided to describe a new species based solely on molecular characters. Lending further support to our decision is the fact that the four species form reciprocally monophyletic clades in the multigene phylogeny. This is confirmed by genetic species delimitation based on two independent methods: genetic barcode gap analysis (using ASAP) and Poisson tree process (using PTP, bPTP and mPTP). It remains to be investigated whether the morphological stasis among species of *Horaglanis* that we encountered in relation to external characters applies also to internal anatomical characters.

Part of the reason why *Horaglanis* has received only cursory scientific attention in the past is the rarity of the occasions on which these fishes have been collected. The dearth of specimens has rendered detailed studies on their anatomy, ecology and life history impossible. Our ongoing 'citizen science' campaign has helped raise awareness of the subterranean fauna of southern India, and this has in turn dramatically increased the number of occurrence reports of these interesting fishes. It has also led to more specimens becoming available for research. Interested citizen naturalists have, no doubt, been at the forefront of aiding improvement of our knowledge of *Horaglanis*, especially through making available rare observations, photographs, videos and specimens. Our *Horaglanis* project is an excellent example of how the involvement of the general public can substantially increase our knowledge of rarely collected organisms that live in relatively inaccessible habitats, through multiplying eyes and ears of researchers by several orders of magnitude (Tricario 2022).

Cryptic species and evolutionarily distinct lineages with small distribution ranges are highly vulnerable to extinction, particularly if residing in groundwater and subterranean habitats (Niemiller et al. 2013). The species of *Horaglanis* have received little or no protection through local or regional legislation, and their habitats are embedded within densely populated human landscapes. The entrances (often as dug-out wells) to the lateritic aquifers inhabited by *Horaglanis* have hitherto been reported entirely from privately owned lands, where groundwater is extracted at high levels for both household and agricultural purposes, and laterite soil is extensively mined for developmental activities (Raghavan et al. 2021). Given that many localities in which *Horaglanis* occurs are within 30 km of the coast, an additional threat is the intrusion of seawater into these aquifer systems, from which water extraction is both substantial and unregulated (see Prusty and Farooq 2020). Ensuring the security of these enigmatic stygobitic catfishes in the lateritic aquifers of Kerala will therefore require a landscape-level planning and implementation approach involving a variety of stakeholders. These will have to include local communities that have played the most important role in helping bridge biodiversity knowledge shortfalls.

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Supplementary material 1

Supplementary informations

Authors: Raghavan R, Sundar RL, Arjun CP, Britz R, Dahanukar N (2023)
Data type: .docx
Explanation notes: Tables S1. Statistics for partition scheme and substitutional model analysis for maximum likelihood analysis provided in Figure 2a. — **Table S2.** Statistics for partition scheme and substitutional model analysis for maximum likelihood analysis provided in Figure 2b. — **Table S3.** Number of haplotypes (H), haplotype diversity (Hd), nucleotide diversity (π) and standard deviations of estimates (sd) for four *Horaglanis* species. — **Table S4.** Nucleotide character states in barcoding region of cytochrome oxidase 1 (COI) for molecular diagnosis of species of *Horaglanis* with respect to nucleotide positions in complete COI gene of *Clarias fuscus* (KM029965). — **Figure S1.** Barcode gap analysis using ASAP. — **Figure S2.** Genetic distance versus geographical distance.

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