# TITLE: Evolution in *Sinocyclocheilus* cavefish is marked by rate shifts, reversals and origin of novel traits

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## ABSTRACT

Epitomized by the well-studied *Astyanax mexicanus*, cavefishes provide important model organisms to understand adaptations in response to divergent natural selection. However, the

spectacular *Sinocyclocheilus* diversification of China, the most diverse cavefish clade in the world harboring nearly 75 species, demonstrate evolutionary convergence for many traits, yet remain poorly understood in terms of their morphological evolution. Here, using a broad sample of 49 species representative of this diversification, we analyze patterns of *Sinocylocheilus* evolution in a phylogenetic context. We categorized species into morphs based on eye-related condition: Blind, Micro-eyed (small-eyed), and Normal-eyed and we also considered three habitat types (Troglodytic – cave-restricted; Troglophilic – cave-associated; Surface – outside of caves). Geometric morphometric analyses show Normal-eyed morphs with fusiform shapes being segregated from Blind/Micro-eyed (Eye-regressed) morphs with deeper bodies along the first principal component ("PC") axis. The second PC axis accounts for shape complexity related to the presence of horns. Ancestral character reconstructions of morphs suggest at least three independent origins of Blind morphs, each with different levels of modification in relation to the typical morphology of ancestral Normal-eyed morphs. Interestingly, only some Blind or Micro-eyed morphs bear horns and they are restricted to a single clade (Clade B) and arising from a Troglodytic ancestral species. Our geophylogeny shows an east-to-west diversification spanning the Pliocene and the Pleistocene, with Troglodytic species dominating karstic subterranean habitats of the plains whereas predominantly Surface species inhabit streams and pools in hills to the west (perhaps due to the scarcity of caves). Integration of morphology, phylogeny and geography suggests *Sinocyclocheilus* are pre-adapted for cave dwelling. Analyses of evolutionary rates suggest that lineages leading to Blind morphs were characterized by significant rate shifts, such as a slowdown in body size evolution and a 3.3 to 12.5 fold increase in the evolutionary rate of eye regression. Furthermore, body size and eye size have undergone reversals, but horns have not, a trait that seem to require substantial evolutionary time to form. These results, compared to the *Astyanax* model

system, indicate *Sinocyclocheilus* fishes demonstrate extraordinary morphological diversity and variation, offering an invaluable model system to explore evolutionary novelty.

**KEYWORDS**: Phylomorphospace, Evolutionary Convergence, Blind fish, Troglodytism, Geophylogeny, Pre-adaptation

## INTRODUCTION

Due to the absence of light, stable mean temperatures, absence of primary productivity, and paucity of dissolved oxygen, subterranean habitats are among the most challenging environments for life on earth (Ginet and Decou, 1977, Camacho, 1992). From surface-dwelling ancestral species, cavefish have secondarily adapted to live in cave systems, often demonstrating a remarkable array of morphological and behavioral adaptations (Soares and Niemiller, 2013; Yoshizawa, 2015). These involve enhanced sensation, and also dispensing of traits that incur a developmental or energetic cost. Cavefish species can be divided into two forms: troglophiles are closely associated with caves, but do not entirely depend on them, and troglobites are obligate cave dwellers (Dowling et al., 2002; Jingcheng and Weicheng, 2015). Troglobite fish *may* harbor special adaptations, such as complete eye loss, loss of pigmentation, changes in cranial symmetry, proliferation of neuromast sensory organs, development of horns, and in some species flat, hollow heads (Strecker et al., 2004; Zhao, 2006; Gross et al., 2008). Despite the ~200 cavefish species from across the world, large diversification of cavefishes is rare. However, one extensive diversification occurs in *Sinocyclocheilus*, a monophyletic group of cyprinid fishes endemic to China, which allows a robust analysis of trait evolution relative to troglomorphism in a phylogenetic context.

70 The specialized traits cavefish bear have led them to be developed as models of evolution
71 especially with respect to adaptations to novel environments and evolutionary convergence
72 (Culver et al., 1995; Dowling et al., 2002; Jeffery, 2001; Li et al., 2008; Strecker et al., 2004;
73 Yang et al., 2016). A lion's share of knowledge on evolution and development in cavefishes has
74 come from *Astyanax mexicanus* (Mexican tetra), a species with both surface-dwelling (pigmented
75 and eyed) and cave-dwelling morphs (depigmented and blind), which can readily interbreed
76 (Borowsky, 2008). In contrast to this well-studied model system, *Sinocyclocheilus* species not only
77 include blind and normal-eyed morphs (Lan et al., 2013), but demonstrate a continuum from blind
78 to normal-eyed species. Indeed, members of the *Sinocyclocheilus* genus display remarkable
79 morphological evolution with divergent cave-dwelling, cave-associated, and surface-dwelling
80 species.

81 *Sinocyclocheilus* species are thought to have shared a common ancestor in the late Miocene,
82 undergoing a spectacular diversification spanning the Pliocene and Pleistocene across the
83 southwestern parts of China's 620,000 $km^2$ of karst habitats (Huang et al., 2008), with nearly 75
84 extant species (Jiang et al., 2019). This resulted in an adaptive diversification into subterranean
85 refugia traversing the intersection of the Guizhou, Guangxi and Yunnan provinces around the time
86 of the uplifting of Tibetan/Guizhou plateau (Li et al., 2008).

87 One of the most striking forms of cave adaptation in *Sinocyclocheilus* is variation in eye
88 morphology, categorized often into three morphs (Zhao and Zhang, 2009), ranging from Normal-
89 eyed to Micro-eyed (small-eyed) to Blind species. Of all Chinese hypogean fishes, 56 species show
90 troglomorphism such as reduction and/or loss of eyes, pigmentation, and the gas bladder. Presence
91 of a horn-like structure and hyper-development of the dorsal protuberance (similar to a humpback

92 whale) are two additional unique characters to certain Chinese hypogean species (Romero et al.,
93 2009). These dramatic adaptations to cave life are reflected in the unique morphology of these fish.
94 The morphology of *Sinocyclocheilus* is most likely attributed to their habitat and local
95 adaptations, however the precise function of certain morphologies (e.g., their horns and humps)
96 remains unknown (Ma and Zhao, 2012). For instance, many blind species are obligate cave
97 dwellers that have the ability to navigate along cave walls, cave-bottoms and within narrow
98 passages (Yoshizawa, 2015). Yet, others are open water species that navigate in the manner of
99 typical fish. There are also intermediate forms between these two principal morphs (Zhao and
100 Zhang, 2009). However, the morphology of these fishes is so extreme that substantial variation in
101 morphology is evident even within blind, intermediate and the open water species.
102 The pattern of body shape evolution in *Sinocyclocheilus* is a conundrum, which has not been
103 addressed in an evolutionary context. Here, we explore key patterns of morphological evolution in
104 these fishes, and demonstrate that the evolution in *Sinocyclocheilus* has been associated with
105 significant rate changes and trait reversals across their phylogenetic history.
106

107 **MATERIALS & METHODS**

108 *Phylogeny estimation*

109 We compiled sequence data from GenBank for two mtDNA fragments (*NADH4* and *cytb*) of
110 39 *Sinocyclocheilus* species, and the outgroup species *Linichthys laticeps* (Cyprinidae). In
111 addition, we generated sequence data for the *cytb* gene fragment of ten additional *Sinocyclocheilus*
112 species (Table S1). For these species, total genomic DNA was extracted using the DNeasy Blood
113 and Tissue Kit (Qiagen Inc., Valencia, CA) following the manufacturer's protocols. DNA was
114 amplified in 25-μL volume reactions: 3 mM $MgCl_2$, 0.4 mM of dNTP, 1X buffer, 0.06 U of Taq

115 DNA Polymerase, 2 mM of each primer. Thermocycling conditions included an initial step at 94
116 °C for 3 min, followed by 35 cycles at 45 s at 94 °C, 1 min at 46-50 °C and 45 s at 48-56 °C, and
117 a final step at 72 °C for 5 min. PCR products were electrophoresed in a 1.5% agarose gel, stained
118 with ethidium bromide and visualized under UV light. Successfully amplified products were
119 purified using MicroconTM Centrifugal Filter Units (Millipore, Billerica, MA, U.S.A.).
120 Sequencing reactions were carried out in 10 µl solutions including the following final
121 concentrations: 5 ng/µl of template DNA, 0.5 µl of Big DyeTM (Applied Biosystems Inc., Foster
122 City, CA, U.S.A.), 0.2 µM of each primer and 0.1X of reaction buffer. The final product was
123 purified using SephadexTM G-50 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) for
124 sequencing. Forward and reverse strands were reconciled using Staden v.1.6.0 (Staden, 1996).
125 Sequences from both genes were concatenated and aligned unambiguously using CLUSTALW
126 (Thompson et al., 2003), as implemented in MEGA v. 6.0 (Tamura et al., 2013), for a total
127 alignment length of 2155 bp. We used JMODELTEST v.2.1.10 (Santorum et al., 2014) to determine
128 the best models of evolution for each fragment, which were implemented in BEAST v.1.10.4
129 (Drummond and Rambaut, 2007) as a partitioned analysis to estimate the phylogenetic
130 relationships and relative divergence times within *Sinocyclocheilus*. We calibrated the tree using
131 the relative time period which diversification of *Sinocyclocheilus* initiated around 11.31 Mya, a
132 reference point obtained from (Chen et al., 2018). We used a strict molecular clock and a calibrated
133 Yule tree prior, as well as a GTR+I+G for each partition and ran the analysis for 20 million
134 generations using the Cipres Science Gateway Server (Miller et al., 2010). Convergence was
135 assessed by inspecting the log-output file in TRACER v.1.6 (Drummond et al., 2012), and by
136 ensuring ESS values were above 200. The first 10% of the trees were discarded, and the post burn-
137 in trees were used to infer the maximum clade credibility tree using TREEANNOTATOR v.1.4.4

138 (Rambaut and Drummond， 2019). The maximum clade credibility tree, as well as a set of 1000
139 post burn-in topologies, were retained for further analyses (see below).
140
141 *Morphometric data acquisition and analyses*
142 We assembled a database of images of referenced specimens of *Sinocyclocheilus* from scaled
143 photographs, which was complemented by species that we photographed (Table S2). The final
144 dataset included 90 images (54 from species descriptions and catalogues and 36 from photographs
145 by the authors) for 50 species, which included 70% of the total number of described species for
146 the genus. These images were used for geometric morphometrics analyses, which were based on
147 15 landmarks (See Fig. 1A) and 180 sliding semi-landmarks, obtained using tpsDig v. 2.16 (Rohlf,
148 2010). Semi-landmarks were collected as curves outlining the body. These data were subsequently
149 reduced to equidistant landmarks, and defined as semi-landmarks using tpsUtil v. 1.46 (Rohlf,
150 2010). We then slid the landmarks using the bending energy method (Gunz & Mitteroecker, 2013)
151 implemented in GEOMORPH v.3.2.0 (Adams & Otárola-Castillo, 2013). The landmark coordinates
152 were aligned using a generalized Procrustes superimposition analysis (Adams et al., 2013), and a
153 principal component analysis (PCA) was used to evaluate shape variation within the sample.
154 Multiple images for the same species were used to obtain the landmarks and the mean of their
155 Procrustes coordinates were calculated to be used in later analyses. We also obtained traditional
156 linear measurements, namely standard length (SL), eye diameter (ED) and standardized eye
157 diameter (sED, calculated as the ratio between ED and SL).
158
159 *Morphological and habitat evolution*

160 Since shape variation in *Sinocyclocheilus* cavefishes occurs mostly in the anterior end of the
161 fish and as one of the major features leading to this is eye-related, we considered the absence or
162 the size of the eye (when present) as a proxy for the categorization of morphs. Since the eye size
163 has an allometric association with body size, we used the standardized eye diameter (sED) in
164 placing them into three morphological categories: Blind (eye absent); Micro-eyed (0.0 – 3.0mm);
165 Normal-eyed (< 3.0mm) respectively. For ease of discussion, we considered Blind and Micro-eyed
166 together as Regressed-eyed; Micro-eyed and Normal-eyed together as Eyed species.

167 *Sinocyclocheilus* were also categorized based on their habitat as Troglodytic, Troglophilic,
168 and Surface species. Troglodytic species live in an obligatory association with caves, and are not
169 sampled outside of caves. Caves, as meant here represent roofed-caves, submerged caves, and
170 subterranean waterways that form windows intermittently with the surface. Troglophilic species
171 live in a close association with caves and are sampled both in the vicinity of cave entrances and
172 within caves. Finally, Surface species are found in habitats even when a cave is not found in close
173 proximity and live in normal streams ponds and lakes as typical fish do, but they could venture
174 into caves (underground water bodies) during unfavorable periods, when water is only available
175 in caves. It should be noted here that this categorization is strictly habitat based and not
176 morphology based (for instance, there are Normal-eyed species that are Troglodytic, Troglophilic
177 or Surface). These habitat associations are based on published literature (Zhao and Zhang, 2009;
178 Romero et al., 2009) and personal observations as outlined in Table S1.

179 To infer the number and timing of evolutionary shifts within eye-related morphs, horn
180 distribution and the habitat type, we used stochastic character mapping (Nielsen, 2002;
181 Huelsenbeck et al. 2003), as implemented in the make.simmap function in PHYTOOLS. On each of
182 the 1000 post burn-in trees obtained from BEAST, we used stochastic character mapping to

183 generate 100 potential histories. This approach therefore considers uncertainty both in the
184 evolutionary history of the traits as well as in the inferred topology of the phylogeny.

185 The landmark coordinates obtained were aligned using a generalized Procrustes
186 superimposition analysis (Adams et al., 2013), and a principal component analysis (PCA) was used
187 to explore shape variation within the sample. In addition, we described the eye-related
188 morphological variation in *Sinocyclocheilus* by estimating ancestral states of SL and ED and
189 visualizing them using traitgrams (Evans et al., 2009) as implemented in the phenogram function
190 in PHYTOOLS 0.7.20 (Revell, 2012) using the maximum clade credibility tree. We also visualized
191 the evolution of both traits simultaneously using a phylomorphospace (a projection of the tree into
192 morphospace, *sensu* Sidlauskas, 2008) using the phylomorphospace function in PHYTOOLS.

193

194 *Evolutionary rate variation in eye related morphs*

195 We tested whether the evolutionary rates of the studied continuous traits (SL, ED, sED) are
196 significantly different in different morphs. We used 100 potential trait histories from stochastic
197 character mapping and then fit two alternative models of evolution on each studied trait, one that
198 fixes the rate of evolution to be identical between morphs against an alternative model in which
199 the morphs have separate rates. We calculated the Akaike Information Criterion for small sample
200 size (AICc) from the maximum likelihood estimate on each tree using the brownie.lite function in
201 PHYTOOLS. Finally, if a multi-rate model provided a better fit to the data, we calculated model-
202 averaged estimates of evolutionary rates for each morph. Unless otherwise indicated, all analyses
203 were conducted using R v. 3.6.0 (R Core Team, 2019).

204

205 *Geophylogeny analyses*

206 We could not carry out a formal biogeographical analysis, given that their high endemism and
207 the complex pattern of underground connections between caves limits the establishment of
208 reasonable biogeographical areas. However, we assessed the geographical structuring
209 of *Sinocyclocheilus* diversification by building a geophylogeny on GenGIS v. 2.5.3 (Parks et al.,
210 2013) based on the maximum credibility tree.
211

212 **RESULTS**

213 The maximum credibility tree of *Sinocyclocheilus* is shown in Fig. 2 together with the
214 reconstruction of ancestral states for eye-related morphs; we consider four major clades (A,B,C,D),
215 as previously reported by other authors (Zhao and Zhang, 2009). Despite the inherent uncertainty
216 in ancestral state reconstructions, it is clear that Blind species evolved at least three times in
217 *Sinocyclocheilus*. Two of these events involved single species evolving from Normal-eyed
218 ancestors, namely *S. xunlensis* and *S. anophthalmus*. On the other hand, the third lineage of blind
219 *Sinocyclocheilus,* Clade B, includes several closely related species of Blind, Micro-eyed and a few
220 Normal-eyed species, with two cases of reversal from either Micro-eyed or Blind to Normal-eyed
221 morphs, namely *S. zhenfengensis* and *S. brevibarbatus* (Fig. 2). All four clades contain Regressed-
222 eyed species and comparatively, clade Blind contains the most cases. Interestingly, clade B
223 originated around the time of the beginning of the aridification process in China in the late Pliocene,
224 whereas the other two transitions to blind species were much more recent (Fig. 2).

225 Interestingly, the evolution of body size and eye diameter seem to have often involved
226 reversals, with little correspondence between body size (Fig. 3A) or eye diameter (Fig. 3B) and
227 their corresponding morphs, except for the case of blind species for which eye diameter is
228 necessarily zero. On the other hand, morphs are clearly distinguished when eye diameter and body

229 size are visualized simultaneously (Fig. 3C), which suggests that the evolution of different morphs
230 is achieved by altering the relationships between body size and eye diameter. Habitat associations
231 traced on the phylomorphospace (Fig. 3D) indicates that species with regressed eyes and small-to-
232 medium body sizes are obligate cave dwellers. However, normal eyed species can be Troglodytic,
233 Troglophilic or surface dwellers regardless of their body size. Interestingly, all horned species are
234 obligate cave dwellers while all cave species are not horned (Fig. 3E).

235 A more precise description of the overall changes associated with different morphs can be
236 visualized in the projections build from the geometric morphometrics analyses (Fig. 1B). The first
237 PC, which accounted for approximately 32% of the variance in the dataset (see Table S3), tended
238 to distinguish the slender Normal-eyed species on the left and Micro/Blind species on the right,
239 which were characterized by changes in shape and widening of the anterior dorsal area between
240 mouth and beginning of the dorsal fin of their body, resulting in a shift from the fusiform shape of
241 the Normal-eyed forms to a more "boxy" form of the Micro-eyed and Blind forms. The second
242 PC, which explained approximately 17% of the variance in the dataset, emphasized the differences
243 in the type of dorsoventral broadening of the mid-section between morphs, with a shortening of
244 the tail region (Fig. 1B). The variation in this axis is very high among the Micro-eyed and Blind
245 forms when compared to the Normal morphs.

246 The multiple-rate model of evolution provided the best fit to the data for all three quantitative
247 traits (ΔAIC=2.7, 10.7 and 7.9 respectively for ED, sED and SL; Table 1), indicating that the
248 evolution of different *Sinocyclocheilus* morphs was associated with significant changes in their
249 evolutionary rates. However, there were intriguing differences between traits in their rates (Table
250 2). The rates of evolution of eye diameter and standardized eye diameter were similar between

251 Normal-eyed and Micro-Eyed species, but increased between 3.3 to 12.56 times during shifts
252 towards Blind species.

253 Geophylogeny represents the phylogeny overlaid across the geographic location of each
254 species, where phylogenetic clustering is evident across the landscape. Considering the distribution
255 of *Sinocyclocheilus,* we mainly see a pattern where the basal, Normal-eyed morphs are placed in
256 the east, a substantial portion of Blind/Micro-eyed (Regressed-eyed) species are in the center, and
257 Normal-eyed morphs are predominant towards the western mountains (Fig. 4).

258

259 **DISCUSSION**

260 ***Habitat utilization in context of eye-morphology***

261 Integrating evolution of eye size and habitat manifests interesting and previously
262 unrecognized evolutionary patterns in the evolution of *Sinocyclocheilus*. The Eye-size based
263 ancestral reconstruction suggests the base of the phylogeny is most likely an Eyed species (i.e.
264 Normal- or Micro-), but habitat reconstructions places, with high probability, Troglodytic species
265 at the base (Fig. S1). This suggests an ancestral Eyed-species evolved a Troglodytic habit before
266 they became blind. This may be an example of preadaptation in *Sinocyclocheilus*, i.e., the
267 advancement of a functional change with little or no evolutionary modification (Ardila, 2016). In
268 *Astyanax* cavefish, surface-dwelling forms are scotophilic, they prefer to remain away from direct
269 light suggesting that scotophilia may be preadaptive for colonizing the dark, cave environment
270 (Espinasa et al., 2001). In *Sinocyclocheilus*, since a basal (eyed) species demonstrated preference
271 for the cave habitat, this preadaptation to darkness may hint towards why certain species tend to
272 become cave-dwellers while others do not. This pattern is supported by two principal lines of
273 evidence. First, most of the basal species are eyed, and Troglodytic (except for one species with

274 an unusual eye-related polymorphic condition that we discuss below). Second, the most westward
275 group (Clade D; Normal-eyed Surface fish), re-colonized caves whenever cave habitats were
276 available within that area, suggesting a strong predisposition for cave-dwelling across all
277 *Sinocyclocheilus*. In other words, when caves were present, members of *Sinocylocheilus,*
278 irrespective of eye-related condition, preferred the cave habitat.

279 The preference for caves may not be a preference for darkness, but in fact a preference for
280 depth, in search of water for survival. In a karstic environment where drying of surface running
281 water is common, a preference for such deeper habitats may have provided an evolutionary
282 advantage. In the presence of an array of subterranean waterways, such a predisposition would
283 have given rise to the eye-regressed forms living close to, or associated with, caves that are
284 characteristic of the genus.

285 Furthermore, apart from the Troglodytic and Troglophilic species of Clade D, some of the
286 putative Surface species of Clade D are often observed at the entrances of caves or at windows to
287 subterranean rivers (Zhao and Zhang, 2009). Hence, with more intensive ecological studies, some
288 species recognized as Surface species may indeed be Troglophilic species, bolstering the notion
289 that *Sinocyclocheilus* are predisposed to seek deeper waters of the karstic caves.

290 Resource utilization plays a key survival role in harsh environments (Culver and Pipan, 2009).
291 Some of the Troglophilic, eyed-species are nocturnal, emerging from submerged caves,
292 presumably to feed at night to reduce competition from other non-cave inhabiting fish species
293 (personal observations). Some species like *S. altishoulderus, S. donglanensis* (Romero et al., 2009),
294 *S. bamaensis* (Su et al., 2003), *S. malacopterus* (Chen et al., 2017) and *S. longibarbatus* (personal
295 observation, video evidence as Supplementary information); are known to come out of caves
296 during the high water season, presumably to feed and breed. This explains dependence on the cave

297 as a diurnal refugium, from where these species can exploit the surface habitats at night. Strategies
298 such as this, where multiple resources are utilized simultaneously, points to the adaptability of
299 some *Sinocyclocheilus* species, resulting in their evolutionary success in a harsh and changing
300 environment. Hence, the cave entrances are possibly an important ecotone that is important in
301 *Sinocyclochelius* diversification and conservation.

302 Season and time of day seem to be important factors in determining habitat utilization patterns,
303 but this level of resolution in habitat data is not currently available for a majority of the species to
304 carry out a comprehensive habitat analysis across the diversification – indeed, many species are
305 known only from one or a few specimens (Zhao and Zhang, 2009).

306

307 ***Adaptations in the light of geophylogeny***

308 In combination with the data analyzed, basal *Sinocyclocheilus* (Clade A) are Normal-Eyed,
309 predominantly cave dwelling and non-horned species from the Eastern region of their distribution.
310 As pointed out, this suggests that the earliest ancestors of *Sinocyclocheilus* species where Normal-
311 eyed and but still lived in close association with caves. The ancestral reconstructions suggest that
312 the affinity to caves would have evolved early and is present in most *Sinocyclocheilus.* The clade
313 comprising basal species are from the east of the *Sinocyclocheilus* distribution, i.e. the He Jiang
314 and Gui Jiang river basin in northeastern Guangxi, and hence, it seems that the diversification of
315 these fish occurred from East to West (Fig. 4).

316 Within this predominantly Normal-eyed clade (Clade A), there is an exception,
317 *Sinocyclocheilus guanyangensis*, a species that we coded as Micro-eyed, has Normal-Eyed, Micro-
318 eyed and effectively Blind morphs within the same population – polymorphic for this trait. But
319 these blind morphs have their eyes completely covered by skin and the Micro-eye is not itself

320 degenerate. This kind of condition has been noted in several other taxa also (*S. xunlensis* and *S.*
321 *flexuodorsalis* – not available for our analysis), but is uncommon. This suggests a degree of
322 polymorphism for this trait, suggesting that the earliest ancestors of *Sinocyclocheilus* may have
323 been able to lose or gain eyes relatively easily as an adaptation to local conditions, this ability
324 appears several times within this cave-driven diversification.

325 The major adaptation for cave dwelling evolves predominantly in the expansive karstic area
326 in northwestern Guangxi (associated with the Liu Jiang basin and Hongshui river basin joining the
327 main Xijiang River system from the North), in Clade B, the southeastern corner of Guizhou
328 province (upper reaches of Hongshui River) and the northeastern plateau of Yunnan province. This
329 region can be considered the center for novel adaptations for *Sinocyclocheilus,* where these fishes
330 express their full morphological diversity, blindness, micro-eyedness, and their remarkable horns.
331 In the shape-related analyses, these species cluster on the right of morphospace (Fig. 1B). The
332 deeper caves and extensive subterranean river system associated with the Guangxi plains (Zhao
333 and Zhang, 2009) would have facilitated this extensive adaptive diversification (Fig. 4).

334 The karstic northwestern region that the Guangxi-dominated Clade (Clade B) experiences
335 drought conditions during much of the year, and one of the major sources of rain for the region is
336 through storms sweeping from the southeast that are strong enough to persist through the vast
337 plains of Guangxi, mainly from April to August (Zhao and Zhan, 2009). During unfavorable
338 periods, these fishes seem to have found refuge in the subterranean caves. The morphologically
339 most diverse clade being present in the region where the climatic conditions are most unfavorable
340 for surface fish, reinforces the notion that *Sinocyclocheilus* species adapted to life in caves as
341 climatic refuges (Zhao and Zhang, 2009).

342 The distribution of Clade C, characterized by mostly Normal-eyed but Troglodytic species
343 largely overlaps Clade B. In Clade C, the single Blind species (*S. xunlensis*) and the two Micro-
344 Eyed species (*S. cyphotergous* and *S. multipunctatus*) are shown within a narrow geographic area
345 on the Liu Jiang and Hongshui river system (Fig. 4).

346 Species in the clade that is found in the west (Clade D), predominantly on the hilly terrain of
347 Yunnan plateau, are predominantly Normal-eyed Surface species lacking horns (Fig. 1, Fig. 4).
348 However, wherever there are cave habitats and subterranean river systems, some of these putative
349 surface species have become facultative or obligate cave species. The obligate cave species found
350 within the region, *S. anophthalmus*, is blind. However, this Blind species stands clustered with the
351 Normal-eyed morphs in the morphospace, signifying that the shape of the species has not
352 extensively changed, possibly due to recent (Pleistocene) invasion of the cave habitat from a
353 Normal-eyed ancestor (Fig. 2) – time since becoming blind is not been long enough for change
354 into the box-like shape of the Blind species of Clade B.

355 Horn distributions show several peculiar trends. In most *Sinocyclocheilus* species, a prominent
356 hump is present (He et al., 2013). However, this hump is markedly low in the Normal-eyed surface
357 inhabiting species of the Yunnan clade (Fig. 4). For the species that bears a horn, the horn
358 represents the region in which the dorso-frontal hump is present, and always occurs before the
359 hump begins, at the boundary of the edge of the dorsal skull. The exception to this is *S.*
360 *cyphotergous* (Clade C), where a horn like structure is placed on top of the hump. *S. cyphotergous,*
361 a species found in clade C, is phylogenetically separate from other horned species, suggesting that
362 the origins of the "horn" for this species is evolutionarily different from the other horned species
363 (Fig. S2). Though the function of the horn remains unknown (protection of head, anchoring in
364 strong current and protection of head has been suggested; Zhao and Zhang, 2009), functionally

365 this structure may be similar to other species, if it is actually anchoring in strong current is the
366 main function.

367 The rates of evolution of various traits show some incongruent (non-allometric), but
368 interesting patterns that can be explained in the context to adaptations to a Troglodytic condition.
369 The rates of evolution in eye diameter are similar between Normal- and Micro-Eyed species but,
370 increases dramatically (3.3-12.56 times) with shifts towards blind forms. However, body size
371 evolution for these morpho groups shows a reversed pattern, with a 0.03 decrease in body size
372 evolution in the Blind morphs compared to the eyed-morphs. These patterns in rate variation
373 suggest that the evolution of Blind morphs to a Troglodytic habitat were simultaneously associated
374 with an increase in the rate of evolution of the eye-size degeneration and a decrease in the rate of
375 body size evolution. The smaller body size resulting from a sluggish rate of change will facilitate
376 both navigation within constricted spaces and sustenance on a limited supply of resources expected
377 to be experienced in subterranean habitats.

378 Much of our collective knowledge of the patterns and mechanisms of regressive evolution
379 come from studies of animals that have colonized the subterranean biome. Within this group, a
380 several studies have focused on the Mexican tetra, *Astyanax mexicanus* (Jeffery, 2009). This
381 natural animal model system comprises multiple cave-adapted morphs and a surface-dwelling
382 morph that resides in near the caves themselves (Gross, 2012). Since the discovery of *Astyanax*
383 cavefish in 1936, countless studies have provided insight to the developmental and genetic bases
384 for cave-associated traits (Hubbs and Innes, 1936). Indeed, much of this insight has emerged from
385 the interbreeding studies of conspecific cave and surface morphs (reviewed in Wilkens, 2016).
386 However, several aspects of regressive evolution and troglomorphic adaptation remain unresolved.
387 Owing to several of the differences with *Astyanax*, we argue that *Sinocyclocheilus* is well-

388 positioned to provide important new insights to broader patterns of diversification and adaptation
389 in cave-dwelling organisms.

390 For instance, *Astyanax* represents a single species with 30 different interfertile populations
391 from a relatively small geographic location (Wilkens and Strecker, 2017). In contrast,
392 *Sinocyclochelius* harbors about 75 species (49 in our study) inhabiting diverse geographic biomes
393 across a much larger geographic area. Moreover, while *Astyanax* cavefish converge on similar
394 phenotypes (regressed vision and pigmentation), they are not regarded as having numerous
395 morphological novelties. In contrast, *Sinocyclocheilus* species have recurrently evolved a unique
396 "horn" (Soares et al., 2019) from several eyeless species. Similarly, the larger number of
397 *Sinocyclocheilus* species allows keener resolution for understanding broad phylogenetic processes,
398 such as trait reversals and directions of diversification. Although a reversal from an eyeless to an
399 eyed form has been reported for one cave population in *Astyanax* (Caballo Moro; Krishnan and
400 Rohner, 2017), this phenomenon appears to be much less common than in *Sinocyclocheilus*.
401 Additionally, a clear polarity of diversification is lacking in *Astyanax* cavefish, rather ancient
402 stocks of surface-dwelling forms appear to have recurrently invaded caves to the east (i.e., Sierra
403 de El Abra caves), with more recent invasions having occurred in the northern (Sierra de Colmena)
404 and the western caves (Sierra de Guatemala; Bradic et al., 2012). However, the well-characterized
405 gene flow between the cave and surface waters obscures the ability to understand clear boundaries
406 between different cave groups. Further, most *Astyanax* cave populations are believed to have
407 diverged over the course of the last ~200 – 500 Ky (Herman et al., 2018). By contrast,
408 *Sinocyclocheilus* species are much older, and therefore one can determine how longer-term
409 processes unfold in these cave-dwelling animals. Thus, despite clear phylogenetic differences

410 between *Astyanax* and *Sinocyclocheilus*, both genera have the ability to provide complementary
411 and critical insights to the processes underlying cave evolution and diversification.
412 The integration of morphology, phylogeny, rate analyses, dating and distribution show not
413 only several remarkable patterns of evolution, but also interesting exceptions to these patterns that
414 signifies the diversification of *Sinocyclocheilus* as a unique model system to study evolutionary
415 novelty.
416

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423

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555

## Main Figures

**Fig. 1. (A) A specimen of *Sinocyclocheilus altishoulderus* indicating the position of 15 landmarks (red: larger points indicated by numbers 1-15) and 180 semi-landmarks (red: smaller point) used for the calculation of Procrustes coordinates and traditional linear measurements (SL: standard Length, ED: eye diameter and sED: standardized eye diameter) used in the geometric morphometric analyses.** (B) **PCA showing the variation in body shape of the genus *Sinocyclocheilus* traced with eye morphology and habitat occupation.** PC1 and PC2 accounts for 32% and 17% of the variance respectively. A shift from the fusiform shape of the Normal-eyed surface forms to a more "boxy" form of the Micro-eyed and Blind forms is evident.

Fig. 2. Ancestral state reconstruction of morphology (Blind, Mico-eyed & Normal-eyed) on a time calibrated phylogeny. Posterior probabilities of node support also shown on the tree. **Maximum-likelihood reconstructions for the ancestral state of the eye-trait morphology (Blind, Micro and Normal-eyed morphs) on a time calibrated phylogeny.** A, B, C and D are four major clades. Posterior probabilities of node support values of 100% are indicated by *. Key events of *Sinocyclocheilus* evolution includes; at least three independent evolutionary events for Blind morphs; Blind, Micro-eyed and a few Normal-eyed species in clade B, with two cases of reversal from either Micro-eyed or Blind to Normal-eyed species.

Fig. 3. **Temporal patterns of body size and eye diameter in *Sinocyclocheilus* from the perspective of their eye trait related morphs.** The traitgrams suggests that the evolution of different morphs is attained by altering the allometric relationships between body size and eye

579 diameter. A. Traitgram of body size ; B. traitgram of eye size; C. Eye related morphs traced on
580 the phylomorphospace indicating clear separation of the three morphs in the morphospace.
581 (D)Habitat associations traced on the phylomorphospace showing species having eye diameter
582 <3mm and small to medium body sizes are obligate cave dwellers whereas species with eye
583 diameter >3mm can be Troglodytic, Troglophilic or Surface dwellers regardless of their body
584 size (E) Horn related morphs traced on the phylomorphospace indicating the presence of a horn
585 in smaller fish with reduced eye size. Horned species are Troglodytes.
586
587 **Fig. 4. Geophylogeny, the phylogeny laid across the geographic distribution of the species**
588 **considered in the analysis, with eye-related morphology, habitats, and horn-existence of the**
589 **species traced**. A pattern where basal, Normal-eyed, Troglodytic species are placed in the east,
590 predominantly Blind/Micro-eyed/Normal-Eyed, Troglodytic species in the center and Normal-
591 eyed, Surface dwelling species towards the western mountains is evident, indicating an East to
592 west dispersion of the genus *Sinocyclocheilus* across South and South Western China. Eye
593 specializations mostly occurred in Clade B, and horn evolution occurred exclusively in Clade B,
594 within the Central range of the *Sinocyclocheilus* distribution.
595
596

597 ## Main Tables:

598 **Table 1. Model fit and estimated Brownian rate parameters for three traits (ED, sED and**
599 **SL) in eye related morphs of *Sinocyclocheilus*.** Multiple-rate models of evolution providing the
600 best fit to the data for all three quantitative traits with ΔAIC >2.

| Trait | Single rate model | | Multiple rate model | | ΔAIC |
|---|---|---|---|---|---|
| | AICc | AIC weights | AICc | AIC weights | |
| ED | 253.9337 | 0.19991263 | 251.1600 | 0.800088 | 2.773681 |
| sED | 246.1558 | 0.004509138 | 235.3616 | 0.995491 | 10.794260 |
| SL | 538.3145 | 0.018806041 | 530.4053 | 0.981194 | 7.909184 |

601

602 **Table 2. Model averaged rate parameters for the measured traits in eye related morphs of**
603 ***Sinocyclocheilus.*** Normal-eyed and Micro-Eyed species indicate similar evolutionary rates with
604 marked shifts towards Blind species.

| Trait | Model Averaged Rate | | |
|---|---|---|---|
| | Blind | Micro-eyed | Normal-eyed |
| SL | 45.3506 | 1597.9230 | 1275.5080 |
| ED | 11.3935 | 2.9984 | 3.8872 |
| sED | 24.9441 | 1.6925 | 2.2773 |

605

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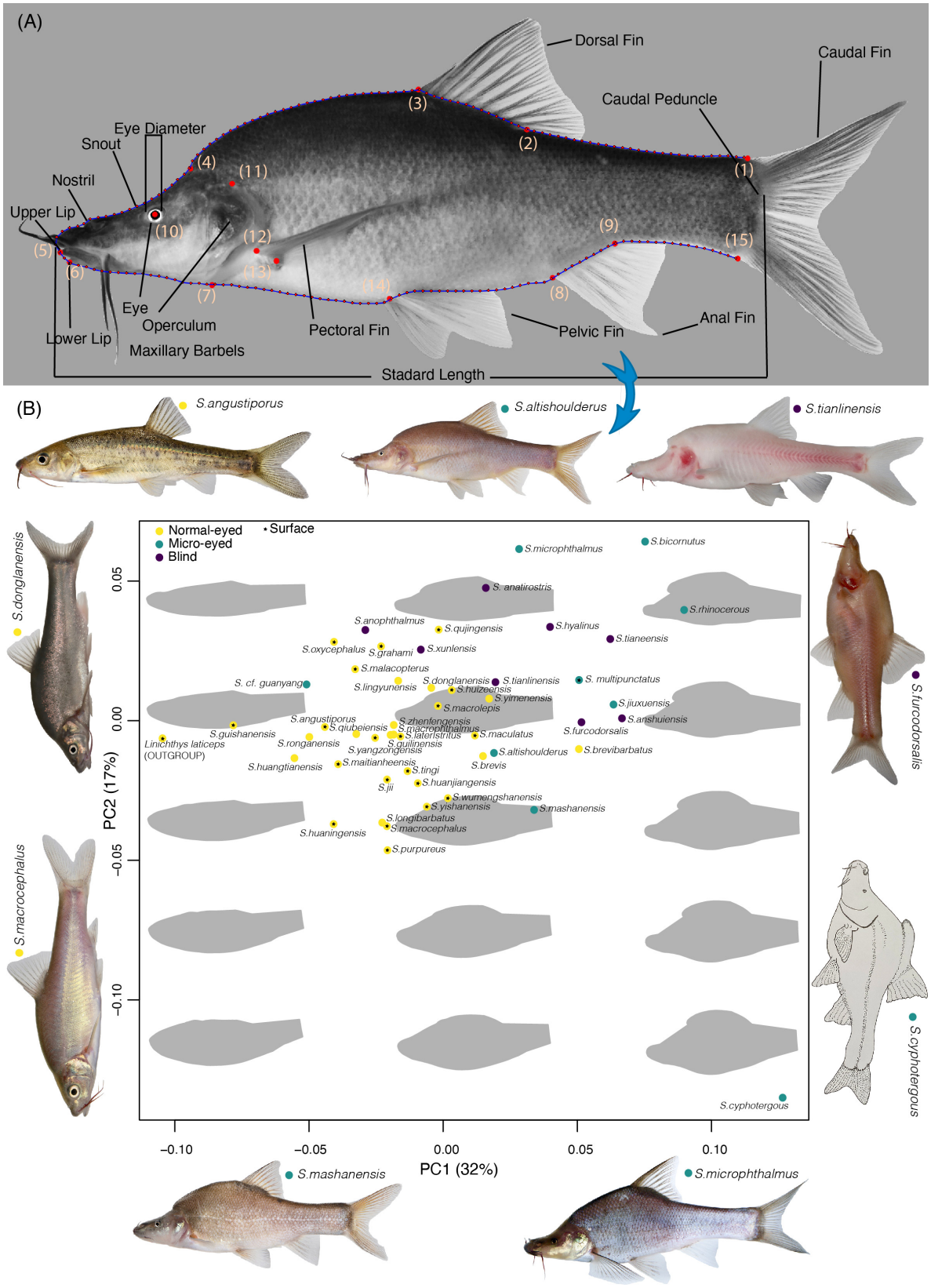
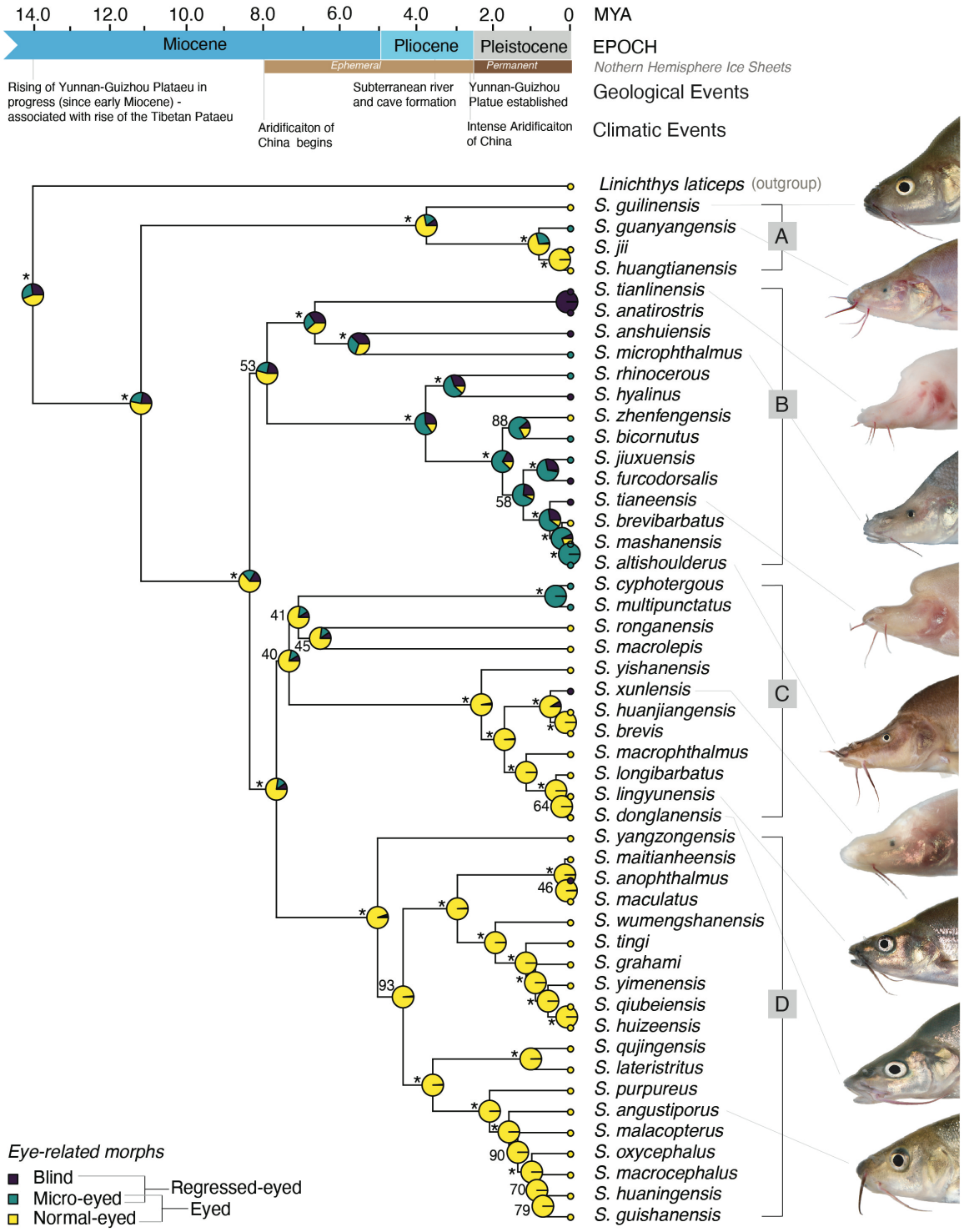
611 **Fig.1**

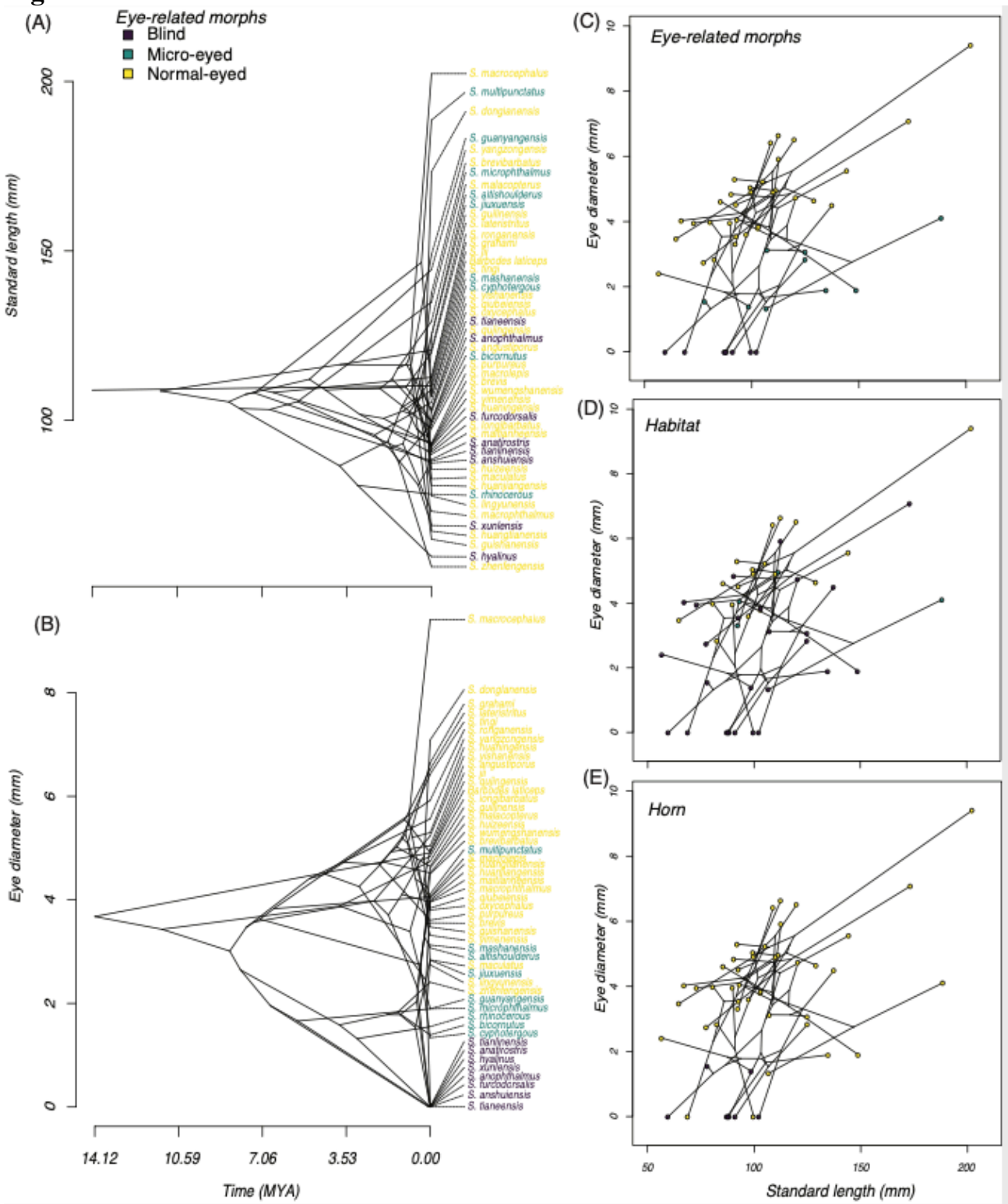

(A)
Dorsal Fin
Caudal Fin
Caudal Peduncle
Eye Diameter
Snout
Nostril
Upper Lip
Lower Lip
Eye
Operculum
Maxillary Barbels
Pectoral Fin
Pelvic Fin
Anal Fin
Stadard Length
(1)
(2)
(3)
(4)
(5)
(6)
(7)
(8)
(9)
(10)
(11)
(12)
(13)
(14)
(15)
(B)
S.angustiporus
S.altishoulderus
S.tianlinensis
S.donglanensis
S.macrocephalus
S.furcodorsalis
S.cyphotergous
S.mashanensis
S.microphthalmus
Normal-eyed
Micro-eyed
Blind
Surface
PC2 (17%)
PC1 (32%)
0.05
0.00
−0.05
−0.10
−0.10
−0.05
0.00
0.05
0.10
S.microphthalmus
S.bicornutus
S. anatirostris
S.rhinocerous
S.anophthalmus
S.qujingensis
S.hyalinus
S.tianeensis
S.oxycephalus
S.grahami
S.xunlensis
S.malacopterus
S. cf. guanyang
S.lingyunensis
S.donglanensis
S.tianlinensis
S. multipunctatus
S.huizeensis
S.yimenensis
S.jiuxuensis
S.macrolepis
S.angustiporus
S.zhenfengensis
S.anshuiensis
S.guishanensis
S.qiubeiensis
S.macrophthalmus
S.furcodorsalis
S.lateristritus
S.maculatus
Linichthys laticeps
(OUTGROUP)
S.ronganensis
S.guilinensis
S.yangzongensis
S.altishoulderus
S.brevibarbatus
S.huangtianensis
S.maitianheensis
S.brevis
S.tingi
S.ji
S.huanjiangensis
S.wumengshanensis
S.yishanensis
S.mashanensis
S.longibarbatus
S.macrocephalus
S.huaningensis
S.purpureus
S.cyphotergous

612

**Fig.2.**


14.0
12.0
10.0
8.0
6.0
4.0
2.0
0
MYA
Miocene
Pliocene
Pleistocene
EPOCH
Ephemeral
Permanent
Nothern Hemisphere Ice Sheets
Rising of Yunnan-Guizhou Plataeu in progress (since early Miocene) - associated with rise of the Tibetan Pataeu
Subterranean river and cave formation
Yunnan-Guizhou Platue established
Geological Events
Aridificaiton of China begins
Intense Aridificaiton of China
Climatic Events
Linichthys laticeps (outgroup)
S. guilinensis
S. guanyangensis
S. jii
S. huangtianensis
S. tianlinensis
S. anatirostris
S. anshuiensis
S. microphthalmus
S. rhinocerous
S. hyalinus
S. zhenfengensis
S. bicornutus
S. jiuxuensis
S. furcodorsalis
S. tianeensis
S. brevibarbatus
S. mashanensis
S. altishoulderus
S. cyphotergous
S. multipunctatus
S. ronganensis
S. macrolepis
S. yishanensis
S. xunlensis
S. huanjiangensis
S. brevis
S. macrophthalmus
S. longibarbatus
S. lingyunensis
S. donglanensis
S. yangzongensis
S. maitianheensis
S. anophthalmus
S. maculatus
S. wumengshanensis
S. tingi
S. grahami
S. yimenensis
S. qiubeiensis
S. huizeensis
S. qujingensis
S. lateristritus
S. purpureus
S. angustiporus
S. malacopterus
S. oxycephalus
S. macrocephalus
S. huaningensis
S. guishanensis
A
B
C
D
53
88
58
41
45
40
64
46
93
90
70
79
Eye-related morphs
Blind
Micro-eyed
Normal-eyed
Regressed-eyed
Eyed

615 **Fig. 3.**




616
617
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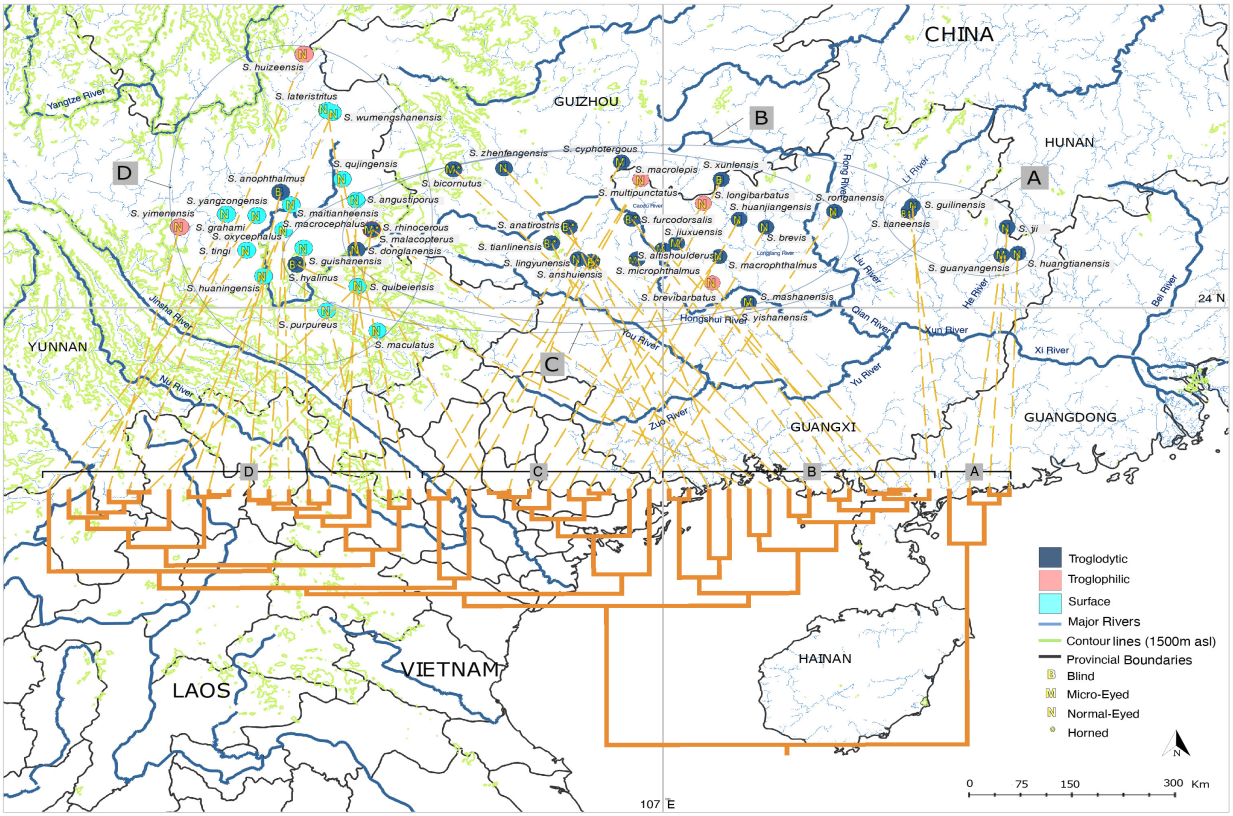
619

**Fig.4.**




**Supplementary Information:**

641 Fig. S1. Maximum-likelihood reconstructions for the ancestral states of the habitat occupation
642 (Troglodytic, Troglophilic and Surface) on the phylogeny of the genus *Sinocyclocheilus*.
643
644 Fig. S2. Maximum-likelihood reconstructions for the ancestral state of the horn related trait
645 (presence/absence of horn) on the phylogeny of the genus *Sinocyclocheilus*.
646
647 Table S1. Species information and GenBank accession numbers of two mtDNA fragments
648 (*NADH4* and *cytb*) of 39 *Sinocyclocheilus* species. The information of the outgroup species
649 *Linichthys laticeps* (Cyprinidae) is also indicated along with calculated standard length (SL), eye
650 diameter (ED), standard eye diameter (sED), discrete trait categories related to eye morphology
651 (Blind, Micro-eyed, Normal eyed), presence or absence of a horn and habitat occupation
652 (Troglodytic, Troglophilic and surface) for all species used in the analysis. Accession numbers
653 indicated as XXXX will be accessible upon the acceptance of the manuscript.

| **Species name** | **SL** | **ED** | **SED** | **CYTB** | **NADH4** | **Morph** | **Habitat** | **Horn** |
|---|---|---|---|---|---|---|---|---|
| *Linichthys laticeps* | 110.20 | 4.91 | 4.45 | AY854739 | AY854796 | Normal-eyed | Surface | Absent |
| *S. altishoulderus* | 125.10± 16.74 | 3.07±0.35 | 2.46 | AY854724 | AY854781 | Micro-eyed | Troglodytic | Absent |
| *S. anatirostris* | 88.40 | 0.00 | 0 | AY854708 | AY854765 | Blind | Troglodytic | Present |
| *S. angustiporus* | 99.57±19.79 | 5.05±0.77 | 5.07 | AY854702 | AY854759 | Normal-eyed | Surface | Absent |
| *S. anophthalmus* | 99.80 | 0.00 | 0 | AY854715 | AY854772 | Blind | Troglodytic | Absent |
| *S. anshuiensis* | 87.40 | 0.00 | 0 | NC_027169 | NC_039769 | Blind | Troglodytic | Present |
| *S. bicornutus* | 98.80 | 1.39 | 1.41 | AY854730 | AY854787 | Micro-eyed | Troglodytic | Present |
| *S. brevibarbatus* | 137.55±18.17 | 4.50±0.84 | 3.27 | XXXX | - | Normal-eyed | Troglophilic | Absent |
| *S. brevis* | 92.91±6.39 | 3.55±0.14 | 3.83 | XXXX | - | Normal-eyed | Troglodytic | Absent |
| *S. guanyangensis* | 148.92±7.39 | 1.90±1.14 | 1.27 | AY854711 | AY854768 | Micro-eyed | Troglodytic | Absent |
| *S. cyphotergous* | 106.95±13.08 | 1.34±0.61 | 1.25 | AB196440 | - | Micro-eyed | Troglodytic | Absent |
| *S. donglanensis* | 173.37±15.94 | 7.09±0.69 | 4.09 | AY854709 | AY854766 | Normal-eyed | Troglodytic | Absent |
| *S. furcodorsalis* | 91.29±16.36 | 0.00 | 0 | AY854694 | AY854751 | Blind | Troglodytic | Present |
| *S. grahami* | 112.60 | 6.64 | 5.9 | XXXX | - | Normal-eyed | Surface | Absent |

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|---|---|---|---|---|---|---|---|---|
| *S. guilinensis* | 120.68±15.04 | 4.74±0.33 | 3.93 | XXXX | - | Normal-eyed | Troglodytic | Absent |
| *S. guishanensis* | 64.90 | 3.48 | 5.35 | AY854722 | AY854779 | Normal-eyed | Surface | Absent |
| *S. huangtianensis* | 67.32±20.48 | 4.03±0.72 | 5.99 | XXXX | - | Normal-eyed | Troglodytic | Absent |
| *S. huaningensis* | 92.20 | 5.30 | 5.75 | AY854718 | AY854775 | Normal-eyed | Surface | Absent |
| *S. huanjiangensis* | 80.79±17.68 | 3.99±0.45 | 4.94 | XXXX | - | Normal-eyed | Troglodytic | Absent |
| *S. huizeensis* | 85.60 | 4.61 | 5.39 | NC_044072 | NC_039769 | Normal-eyed | Troglophilic | Absent |
| *S. hyalinus* | 59.80±28.43 | 0.00 | 0 | AY854721 | AY854778 | Blind | Troglodytic | Present |
| *S. jii* | 111.43±17.21 | 4.97±0.47 | 4.46 | AY854727 | AY854784 | Normal-eyed | Troglodytic | Absent |
| *S. jiuxuensis* | 125.10 | 2.84 | 2.27 | AY854736 | AY854793 | Micro-eyed | Troglodytic | Absent |
| *S. lateristritus* | 120.00 | 6.52 | 5.43 | AY854703 | AY854760 | Normal-eyed | Surface | Absent |
| *S. lingyunensis* | 77.69±9.35 | 2.75±0.60 | 3.53 | AY854691 | AY854748 | Normal-eyed | Troglodytic | Absent |
| *S. longibarbatus* | 90.63±1.82 | 4.84±0.24 | 5.34 | AY854714 | AY854771 | Normal-eyed | Troglophilic | Absent |
| *S. macrocephalus* | 202.30 | 9.42 | 4.65 | AY854683 | AY854740 | Normal-eyed | Surface | Absent |
| *S. macrolepis* | 93.30 | 4.06 | 4.35 | AY854729 | AY854786 | Normal-eyed | Troglophilic | Absent |
| *S. macrophthalmus* | 73.16±11.50 | 3.96±0.71 | 5.41 | AY854735 | AY854792 | Normal-eyed | Troglodytic | Absent |
| *S. maculatus* | 82.70 | 2.84 | 3.43 | EU366193 | EU366183 | Normal-eyed | Surface | Absent |
| *S. maitianheensis* | 90.00 | 3.96 | 4.4 | AY854710 | AY854767 | Normal-eyed | Surface | Absent |
| *S. malacopterus* | 129.20 | 4.65 | 3.6 | AY854697 | AY854754 | Normal-eyed | Surface | Absent |
| *S. mashanensis* | 107.36±5.72 | 3.13±0.34 | 2.91 | XXXX | - | Micro-eyed | Troglodytic | Absent |
| *S. microphthalmus* | 134.89±10.51 | 1.89±0.38 | 1.4 | AY854687 | AY854744 | Micro-eyed | Troglodytic | Absent |
| *S. multipunctatus* | 188.60 | 4.11 | 2.18 | AY854712 | AY854769 | Micro-eyed | Troglophilic | Absent |
| *S. oxycephalus* | 103.10 | 3.80 | 3.69 | AY854685 | AY854742 | Normal-eyed | Surface | Absent |
| *S. purpureus* | 97.60 | 3.61 | 3.7 | EU366194 | EU366177 | Normal-eyed | Surface | Absent |
| *S. qiubeiensis* | 103.30 | 3.84 | 3.72 | EU366195 | EU366181 | Normal-eyed | Surface | Absent |
| *S. qujingensis* | 99.90 | 4.91 | 4.92 | AY854719 | AY854776 | Normal-eyed | Surface | Absent |
| *S. rhinocerous* | 78.20 | 1.55 | 1.98 | AY854720 | AY854777 | Micro-eyed | Troglodytic | Present |
| *S. ronganensis* | 112.72 | 5.92 | 5.25 | NC_032385 | NC_039769 | Normal-eyed | Troglodytic | Absent |
| *S. tianeensis* | 102.34±12.43 | 0.00 | 0 | AY854717 | AY854774 | Blind | Troglodytic | Present |
| *S. tianlinensis* | 87.96±27.97 | 0.00 | 0 | XXXX | - | Blind | Troglodytic | Present |
| *S. tingi* | 109.00 | 6.42 | 5.89 | AY854701 | AY854758 | Normal-eyed | Surface | Absent |
| *S. wumengshanensis* | 92.80 | 4.52 | 4.87 | NC_039769 | NC_039769 | Normal-eyed | Surface | Absent |
| *S. xunlensis* | 69.01±21.44 | 0.00 | 0 | EU366187 | EU366184 | Blind | Troglodytic | Absent |
| *S. yangzongensis* | 144.50 | 5.56 | 3.85 | AY854725 | AY854782 | Normal-eyed | Surface | Absent |
| *S. yimenensis* | 92.50 | 3.31 | 3.58 | EU366192 | EU366179 | Normal-eyed | Troglophilic | Absent |
| *S. yishanensis* | 105.34±8.15 | 5.23±0.23 | 4.97 | XXXX | - | Normal-eyed | Troglodytic | Absent |

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|---|---|---|---|---|---|---|---|---|
| *S. zhenfengensis* | 56.78 | 2.41 | 4.24 | MK610342 | MK610347 | Normal-eyed | Troglodytic | Absent |

Table S2. Information of digitized images used in the morphometric geometric analysis. Table indicates the voucher number of the specimen used for the analysis and the reference from which the image was obtained. Images photographed during the current study are also indicated with voucher numbers stated as GXUXXX (GXU: Guangxi University, China).

| Species name | Reference | Voucher # |
|---|---|---|
| *Linichthys laticeps* | Zhang, E., and F. Fang. 2005. Linichthys: A New Genus of Chinese Cyprinid Fishes (Teleostei: Cypriniformes). Copeia 2005:61–67. | *IHB 78X6242* |
| *S. altishoulderus* | Guangxi University (this study). | *GXU001, GXU002, GXU003* |
| *S. anatirostris* | Romero, A., Y. Zhao, and X. Chen. 2009. The Hypogean fishes of China. Environ Biol Fish 86:211–278. | *IHB 84VII255,* |
| *S. angustiporus* | Guangxi University (this study). | *GXU007, GXU008 , GXU009* |
| *S. anophthalmus* | Romero, A., Y. Zhao, and X. Chen. 2009. The Hypogean fishes of China. Environ Biol Fish 86:211–278. | *KIZ 865949* |
| *S. anshuiensis* | Xi, G., W. Tie-Jun, W. Mu-Lan, and Y. Jian. 2013. A new blind barbine species, *Sinocyclocheilus anshuiensis* sp. nov.(Cypriniformes: Cyprinidae) from Guangxi, China. Kunming Institute of Zoology, Chinese Academy of Sciences. | *12070276* |
| *S. bicornutus* | Romero, A., Y. Zhao, and X. Chen. 2009. The Hypogean fishes of China. Environ Biol Fish 86:211–278. | *IHB 12209043-9o5o241* |
| *S. brevibarbatus* | Guangxi University (this study). | *GXU010, GXU011, GXU012* |
| *S. brevis* | Guangxi University (this study).<br>Romero, A., Y. Zhao, and X. Chen. 2009. The Hypogean fishes of China. Environ Biol Fish 86:211–278. | *GXU013, GXU014*<br>*IHB12209033-* |

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| | | *87087496* |
| *S. cyphotergous* | Romero, A., Y. Zhao, and X. Chen. 2009. The Hypogean fishes of China. Environ Biol Fish 86:211–278.<br>Huang, J., A. Gluesenkamp, D. Fenolio, Z. Wu, and Y. Zhao. 2017. Neotype designation and redescription of *Sinocyclocheilus cyphotergous* (Dai) 1988, a rare and bizarre cavefish species distributed in China (Cypriniformes: Cyprinidae). Environ Biol Fish 100:1483–1488. | *IHB 12209040*<br>*ASIZB 204678* |
| *S. donglanensis* | Guangxi University (this study). | *GXU015, GXU016, GXU017* |
| *S. furcodorsalis* | Guangxi University (this study). | *GXU018, GXU019, GXU020* |
| *S. grahami* | Romero, A., Y. Zhao, and X. Chen. 2009. The Hypogean fishes of China. Environ Biol Fish 86:211–278. | *ASIZB 03496* |
| *S. guanyangensis* | Guangxi University (this study). | *GXU004,GXU005, GXU006* |
| *S. guilinensis* | Guangxi University (this study). | *GXU021, GXU022, GXU023* |
| *S. guishanensis* | Romero, A., Y. Zhao, and X. Chen. 2009. The Hypogean fishes of China. Environ Biol Fish 86:211–278. | *Li980514005* |
| *S. huangtianensis* | Guangxi University (this study). | *GXU024, GXU025, GXU026* |
| *S. huaningensis* | Romero, A., Y. Zhao, and X. Chen. 2009. The Hypogean fishes of China. Environ Biol Fish 86:211–278. | *ASIZB79228* |
| *S. huanjiangensis* | Guangxi University (this study). | *GXU027, GXU028, GXU029* |
| *S. huizeensis* | Cheng, C., P. Xiao-Fu, C. Xiaoyong, J. Li, L. Ma, and J. Yang. 2015. A new species of the genus *Sinocyclocheilus* (Teleostei:Cypriniformes), from Jinshajiang Drainage, Yunnan, China. Cave Research 1:1–4. | *KIZ2013001246* |
| *S. hyalinus* | Romero, A., Y. Zhao, and X. Chen. 2009. The Hypogean fishes of China. Environ Biol Fish 86:211–278. | *KIZ916001*<br>*Photograph in life* |

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|---|---|---|
| | You, H., C. Xiaoyong, X. Ti-Qao, and Y. Jun-Xing. 2013. Three-dimensional morphology of the *Sinocyclocheilus hyalinus* (Cypriniformes : Cyprinidae) horn based on synchrotron X-ray microtomography. Zoological research 34:128–134. | |
| *S. jii* | Romero, A., Y. Zhao, and X. Chen. 2009. The Hypogean fishes of China. Environ Biol Fish 86:211–278.<br>Baradi, W., Z. Yahui, Z. Chunguang, D. Rongji, and A. Abdul. 2013. Anatomical Studies of the Olfactory Epithelium of Two Cave Fishes *Sinocyclocheilus jii* and *S. furcodorsalis* (Cypriniformes: Cyprinidae) from China. Pakistan Journal of Zoology 45:1091 – 1101. | *ASIZB62726*<br>*Photograph in life* |
| *S. jiuxuensis* | Romero, A., Y. Zhao, and X. Chen. 2009. The Hypogean fishes of China. Environ Biol Fish 86:211–278. | *ASIZB102260* |
| *S. lateristritus* | Romero, A., Y. Zhao, and X. Chen. 2009. The Hypogean fishes of China. Environ Biol Fish 86:211–278. | *IHB12209036-865027* |
| *S. lingyunensis* | Guangxi University (this study).<br>Romero, A., Y. Zhao, and X. Chen. 2009. The Hypogean fishes of China. Environ Biol Fish 86:211–278. | *GXU030*<br>*ASIZB 73038* |
| *S. longibarbatus* | Guangxi University (this study). | *GXU031, GXU032, GXU033* |
| *S. macrocephalus* | Romero, A., Y. Zhao, and X. Chen. 2009. The Hypogean fishes of China. Environ Biol Fish 86:211–278. | *IHB12209012-662001* |
| *S. macrolepis* | Romero, A., Y. Zhao, and X. Chen. 2009. The Hypogean fishes of China. Environ Biol Fish 86:211–278. | *IHB12209035-87IV457* |
| *S. macrophthalmus* | Guangxi University (this study). | *GXU034,GXU035,GXU036* |
| *S. maculatus* | Romero, A., Y. Zhao, and X. Chen. 2009. The Hypogean fishes of China. Environ Biol Fish 86:211–278. | *Li870808001* |

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| *S. maitianheensis* | Romero, A., Y. Zhao, and X. Chen. 2009. The Hypogean fishes of China. Environ Biol Fish 86:211–278. | *IHB12209039-874001* |
| *S. malacopterus* | Romero, A., Y. Zhao, and X. Chen. 2009. The Hypogean fishes of China. Environ Biol Fish 86:211–278. | *KIZ775831* |
| *S. mashanensis* | Guangxi University (this study). | *GXU037, GXU038, GXU039* |
| *S. microphthalmus* | Guangxi University (this study). | *GXU040, GXU041, GXU042* |
| *S. multipunctatus* | Romero, A., Y. Zhao, and X. Chen. 2009. The Hypogean fishes of China. Environ Biol Fish 86:211–278. | *ASIZB73000* |
| *S. oxycephalus* | Romero, A., Y. Zhao, and X. Chen. 2009. The Hypogean fishes of China. Environ Biol Fish 86:211–278. | *IHB12209013-652047* |
| *S. purpureus* | Romero, A., Y. Zhao, and X. Chen. 2009. The Hypogean fishes of China. Environ Biol Fish 86:211–278. | *IHB12209015-731004* |
| *S. qiubeiensis* | Romero, A., Y. Zhao, and X. Chen. 2009. The Hypogean fishes of China. Environ Biol Fish 86:211–278. | *Li990527002* |
| *S. qujingensis* | Romero, A., Y. Zhao, and X. Chen. 2009. The Hypogean fishes of China. Environ Biol Fish 86:211–278. | *ASIZB78790* |
| *S. rhinocerous* | Romero, A., Y. Zhao, and X. Chen. 2009. The Hypogean fishes of China. Environ Biol Fish 86:211–278. | *ASIZB93907* |
| *S. ronganensis* | FuGuang, L., H. Jie, L. Xia, L. Tong, and W. YanHong. 2016. *Sinocyclocheilus ronganensis* Luo, Huang et Wen sp. nov., a new species belonging to *Sinocyclocheilus* Fang from Guangxi (Cypriniformes: Cyprinidae). Guangxi Academy of Agricultural Sciences 47:650–655. | *20151114001* |
| *S. tianeensis* | Guangxi University (this study). | *GXU043, GXU044, GXU045* |
| *S. tianlinensis* | Guangxi University (this study). | *GXU052, GXU053, GXU054* |
| *S. tingi* | Romero, A., Y. Zhao, and X. Chen. 2009. The Hypogean fishes of China. Environ Biol Fish 86:211–278. | *ASIZB60227* |

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| *S. wumengshanensis* | Romero, A., Y. Zhao, and X. Chen. 2009. The Hypogean fishes of China. Environ Biol Fish 86:211–278. | *KIZ82100006* |
| *S. xunlensis* | Guangxi University (this study). | *GXU046, GXU047, GXU048* |
| *S. yangzongensis* | Romero, A., Y. Zhao, and X. Chen. 2009. The Hypogean fishes of China. Environ Biol Fish 86:211–278. | *KIZ6351069* |
| *S. yimenensis* | Romero, A., Y. Zhao, and X. Chen. 2009. The Hypogean fishes of China. Environ Biol Fish 86:211–278. | *Li030509009* |
| *S. yishanensis* | Romero, A., Y. Zhao, and X. Chen. 2009. The Hypogean fishes of China. Environ Biol Fish 86:211–278. | *GXU049, GXU050, GXU051* |
| *S. zhenfengensis* | Liu, T., H. Q. Deng, L. Ma, N. Xiao, and J. Zhou. 2018. *Sinocyclocheilus zhenfengensis* , a new cyprinid species (Pisces: Teleostei) from Guizhou Province, Southwest China. J Appl Ichthyol 34:945–953. | *GZNU20120701001* |

Table S3. Calculated Principal Component values (PC1, PC2 and PC3) of all the specimens used in the current analysis

| Spname | PC1val | PC2val | PC3cal |
|---|---|---|---|
| *Linichthys laticeps* | 0.1069618 | -0.006044939 | -0.032010383 |
| *S. altishoulderus* | -0.01804998 | -0.010081536 | -0.046423134 |
| *S. anatirostris* | -0.01766386 | 0.046175863 | 0.051169448 |
| *S. angustiporus* | 0.04334953 | -0.002675304 | 0.034186136 |
| *S. anophthalmus* | 0.02908059 | 0.031606384 | 0.00917709 |
| *S. anshuiensis* | -0.06639039 | -0.000250595 | -0.004873197 |
| *S. bicornutus* | -0.07667729 | 0.060886565 | 0.012973894 |
| *S. brevibarbatus* | -0.05048176 | -0.011045867 | 0.006805293 |
| *S. brevis* | -0.0142754 | -0.011768784 | -0.001763996 |

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|---|---|---|---|
| *S. guanyangensis* | -4.38E-05 | 0.015156895 | -0.025200906 |
| *S. cyphotergous* | -0.1220022 | -0.139796596 | 0.008525168 |
| *S. donglanensis* | 0.004198408 | 0.013428914 | -0.021746042 |
| *S. furcodorsalis* | -0.050741 | -0.000898602 | -0.044412032 |
| *S. grahami* | 0.02231859 | 0.026236004 | 0.022098623 |
| *S. guilinensis* | 0.01963493 | -0.0033727 | -0.023009295 |
| *S. guishanensis* | 0.07886827 | 0.00257981 | -0.059167855 |
| *S. huangtianensis* | 0.05562456 | -0.011114954 | 0.021111628 |
| *S. huaningensis* | 0.04117411 | -0.037450418 | 0.031061221 |
| *S. huanjiangensis* | 0.009958768 | -0.021868616 | 0.007063138 |
| *S. huizeensis* | -0.003779219 | 0.010286192 | 0.031012201 |
| *S. hyalinus* | -0.03974731 | 0.030183657 | 0.039671171 |
| *S. jii* | 0.02155773 | -0.019701057 | -0.005284772 |
| *S. jiuxuensis* | -0.06337799 | 0.004932247 | -0.008080491 |
| *S. lateristritus* | 0.01609448 | -0.006156199 | 0.021918263 |
| *S. lingyunensis* | 0.01666848 | 0.015544267 | 0.002003128 |
| *S. longibarbatus* | 0.0238826 | -0.037134912 | 0.007645544 |
| *S. macrocephalus* | 0.0223646 | -0.03930768 | 0.034945546 |
| *S. macrolepis* | 0.002586613 | 0.0056233 | -0.005281302 |
| *S. macrophthalmus* | 0.01921085 | -0.004423179 | 0.011957836 |
| *S. maculatus* | -0.01164108 | -0.003338214 | -0.043022304 |
| *S. maitianheensis* | 0.04058493 | -0.015399253 | -0.004022456 |
| *S. malacopterus* | 0.03369873 | 0.016341474 | 0.002649988 |
| *S. mashanensis* | -0.03299028 | -0.033529198 | -0.005547116 |
| *S. microphthalmus* | -0.02936266 | 0.061223556 | -0.040563771 |
| *S. multipunctatus* | -0.05126049 | 0.014657848 | -0.010821531 |

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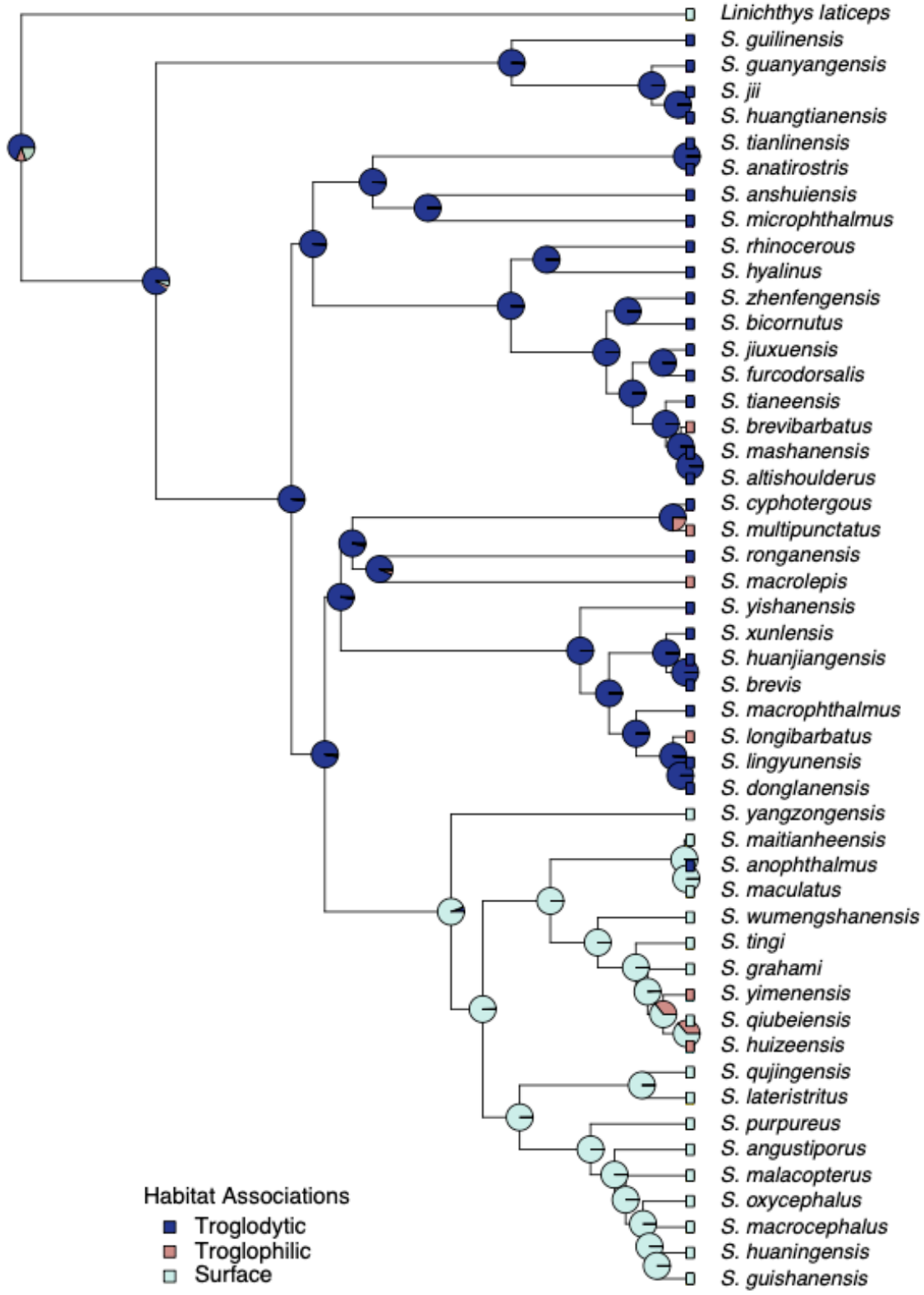
|---|---|---|---|
| *S. oxycephalus* | 0.04140438 | 0.030989746 | -0.036348165 |
| *S. purpureus* | 0.02288238 | -0.045035385 | -0.018448807 |
| *S. qiubeiensis* | 0.03248934 | -0.003880679 | 0.012191068 |
| *S. qujingensis* | 0.000644406 | 0.033555511 | 0.046291334 |
| *S. rhinocerous* | -0.08985807 | 0.03649387 | -0.020510521 |
| *S. ronganensis* | 0.05067357 | -0.004282673 | -0.02013066 |
| *S. tianeensis* | -0.06234116 | 0.02827463 | -0.030564848 |
| *S. tianlinensis* | -0.01905521 | 0.012948686 | -0.00406653 |
| *S. tingi* | 0.01508119 | -0.019031787 | -0.002178747 |
| *S. wumengshanensis* | -0.000171791 | -0.027155405 | 0.003238896 |
| *S. xunlensis* | 0.008420042 | 0.022462118 | 0.047598226 |
| *S. yangzongensis* | 0.02622031 | -0.003887167 | -0.006000758 |
| *S. yimenensis* | -0.01762049 | 0.008265106 | 0.019258774 |
| *S. yishanensis* | 0.007505367 | -0.029610044 | -0.01952727 |
| *S. zhenfengensis* | 0.01938822 | -0.002301361 | 0.003044235 |

679 **Fig.S1.**

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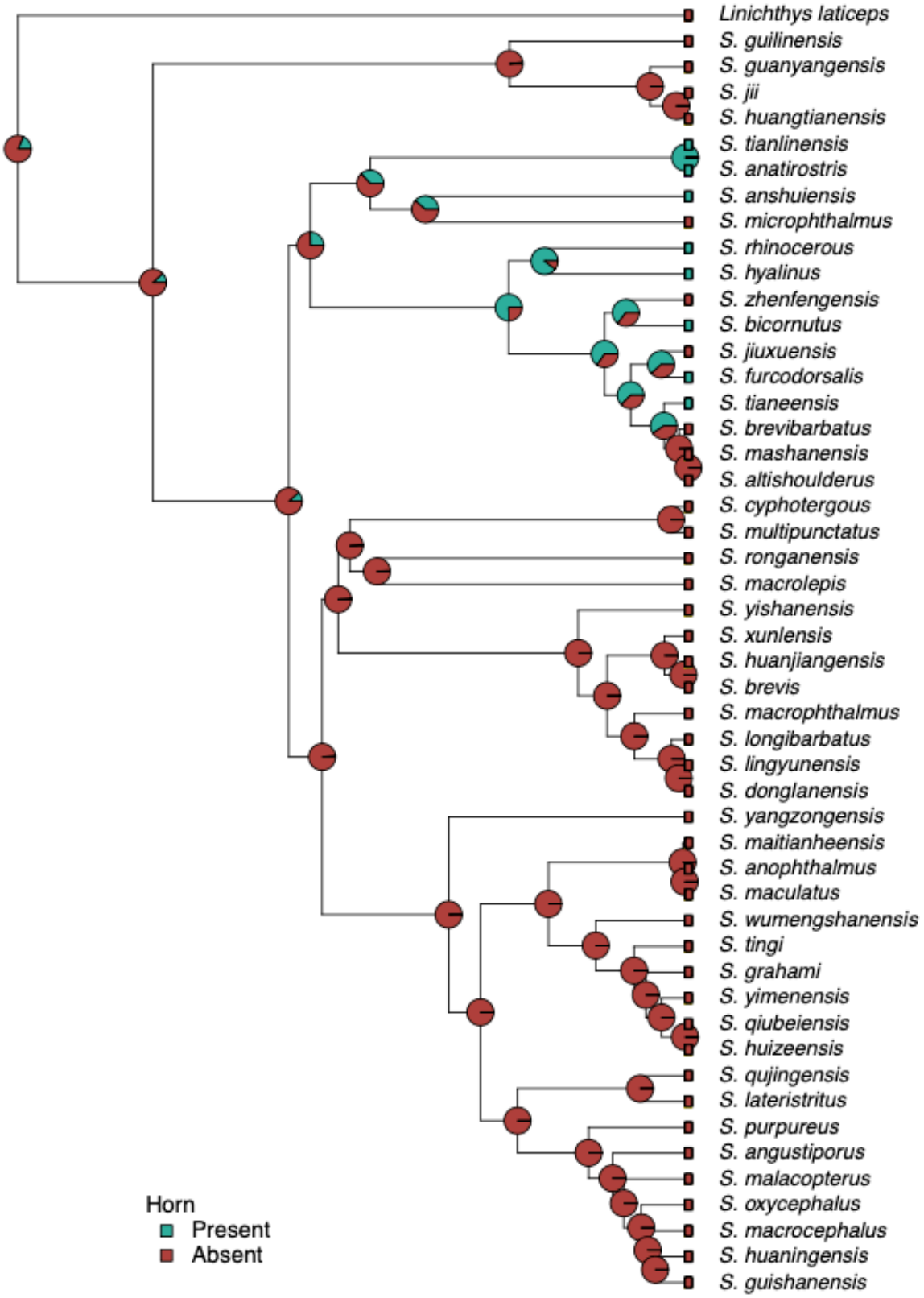
682 **Fig.S2.**

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