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## Genetic identification and reiterated captures suggests that the *Astyanax mexicanus* El Pachón cavefish population is closed and declining

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2

3 **Genetic identification and reiterated captures suggests that the *Astyanax***  
4 ***mexicanus* El Pachón cavefish population is closed and declining**

5

6 Running title: Demography of *Astyanax mexicanus* El Pachón cavefish population

7

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35

## 36 **Abstract**

37 The size of *Astyanax mexicanus* blind cavefish populations of North-East Mexico is a  
38 demographic parameter of great importance for investigating a variety of ecological,  
39 evolutionary and conservation issues. However, very few estimates have been obtained. For  
40 these mobile animals living in an environment difficult to explore as a whole, methods based  
41 on capture-mark-recapture are appropriate, but the feasibility of such approach and the  
42 interpretation of the results depend on several assumptions that must be carefully examined.  
43 Here, we provide evidence that minimally invasive genetic identification from captures at  
44 different time intervals can give insights on cavefish population size dynamics as well as  
45 other important demographic parameters of interest. We also provide tools to calibrate  
46 sampling and genotyping efforts necessary to reach a given level of precision. Our results  
47 suggest that the El Pachón cave population is currently very small, of an order of magnitude  
48 of a few hundreds of individuals, and is distributed in a relatively isolated area. The probable  
49 decline in population size in the El Pachón cave since the last census in 1971 raises serious  
50 conservation issues.

51

52 **Key words:** cavefish, population size, conservation, swabbing, genetic identification

53

## 54 **Introduction**

55 The Mexican tetra, or *Astyanax mexicanus*, is an outstanding freshwater fish model to study  
56 evolution. This species exists under two forms represented by cave-adapted and surface  
57 dwelling populations that can interbreed despite striking differences in morphology,  
58 physiology and behaviour (Keene et al., 2016). Today, there are 34 described cave locations  
59 which host *Astyanax* cavefish populations (Elliott, 2018; Espinasa et al., 2018; Espinasa et al.,  
60 2020; Mitchell et al., 1977). Genetic evidence suggests a recent origin of the cave populations  
61 of a few thousand years (< 20,000 years) according to some authors (Fumey et al., 2018;  
62 Policarpo et al., 2021) or up to about 150,000 years according to others (Herman et al., 2018).  
63 Whatever the date when extant troglomorphic populations first settled in caves and how many  
64 independent events were involved, genetic divergence between surface and cave fish is very  
65 low (Awise and Selander, 1972; Bradic et al., 2012; Fumey et al., 2018; Herman et al., 2018).  
66 The previous enlited characteristics make it possible to use genetic methods to search for  
67 loci, and eventually to identify mutations, involved in the evolution of cavefish phenotypes  
68 (Casane and Rétaux, 2016; Protas et al., 2006). However, the environmental and demographic  
69 context should also be considered to better understand the evolutionary mechanisms involved  
70 in the fixation of these mutations, that is, the relative roles of selection and genetic drift. For a  
71 given subterranean population, the main demographic parameters of interest are its size and  
72 the migration rates between this population and other subterranean and surface populations.  
73 These parameters can vary across the distribution area and can change through time.  
74 Estimates of population size, dispersal potential and level of hybridization with surface fish,  
75 and their variations, are also important for conservation purposes.  
76 For these small, mobile and relatively numerous animals that cannot be counted directly over  
77 their whole distribution area, population size can be estimated using capture-mark-recapture  
78 (CMR) methods (Bailey, 1951). The principle is as follows: we capture, mark and release *a*

79 animals out of a total population of size  $x$ . After the marked animals have freely mingled with  
80 the unmarked, we re-catch a random sample of size  $n$ , of which  $r$  are found to be marked.

81 Bailey obtained the maximum likelihood estimate of  $x$  and its variance:

$$82 \quad \hat{x} = an/r \quad (1)$$

$$83 \quad \text{var } \hat{x} = a^2n(n-r)/r^3 \quad (2)$$

84 If  $r = 0$  (no recapture of marked individuals), the population size cannot be estimated as  $\hat{x}$  has  
85 an infinite expectation. If  $r$  is very small (a few marked individuals are recaptured), the  
86 variance is very large and we can get the order of magnitude of the population size rather than  
87 a precise estimate.

88 Excluding the case  $r = 0$ , Bailey found that the expectation of  $\hat{x}$  is biased,  $E\hat{x} (r \neq 0) =$   
89  $x(1 + 1/m)$ , where  $m$  is the expectation of  $r$ . In order to take into account this bias when  $m$   
90 is small, he proposed an adjusted estimate and gave its variance:

$$91 \quad \check{x} = a(n+1)/(r+1) \quad (3)$$

$$92 \quad \text{var } \check{x} = a^2(n+1)(n-r)/(r+1)^2(r+2) \quad (4)$$

93 Equation (3) should not be used as a mathematical trick to solve the issue that a population  
94 size cannot be estimated if no marked animal is recaptured. Often, equation (3) and (4) are  
95 used instead of equations (1) and (2), not for obtaining precise and less biased estimates, but  
96 for getting an estimate with a smaller variance. However, the only valid solution to have a  
97 precise estimate, that is with a small variance, is to capture a large fraction of the total  
98 population in order to recapture many marked individuals. Moreover, the validity of the  
99 estimate depends on several assumptions that should be verified: 1) the population is closed,  
100 2) all animals are equally likely to be captured, 3) capture and marking do not affect  
101 catchability, 4) marks are not lost.

102 The last two assumptions are a matter of the methodology. Researchers should use permanent  
103 marks and marking should be as little invasive as possible to limit effect on catchability.

104 Whether the studied population is closed and restricted in a well-defined distribution area in  
105 which there is neither immigration nor emigration is often an unsettled issue. This is  
106 particularly true for cave animals for which we often can explore only a small part of their  
107 subterranean world. As marked and unmarked animals must have freely mingled before  
108 recapture, the animals must be mobile enough and the interval between sampling must be long  
109 enough to allow mixture. The validity of these assumptions depends on the model of the  
110 distribution of cavefish. We can imagine several possibilities, the two extremes being called  
111 here the “Oasis” and “Sea” model, and an intermediate “Lake” model (**Figure 1**). In the case  
112 of *Astyanax* cavefish populations, one model can be the most appropriate for a given region in  
113 the distribution area, but another model for another region. Moreover, in a given region, the  
114 best model can change through time. Even if models are oversimplifications of the reality,  
115 they help deciphering what is plausible from what is unlikely in a given case.

116 The “Oasis” model states that the fish distribution is very patchy, with few small isolated  
117 populations that are not permanently connected. It fits well the definition of closed  
118 populations. Some populations are accessible, so that population size and its variation through  
119 time can be estimated. In addition, if these populations have a relatively high extinction rate  
120 and if some empty areas are sporadically invaded, this system has the dynamic of a  
121 metapopulation. At the other extreme stands the “Sea” model. There, we have access to a few  
122 subterranean pools that are well connected to a single network over a large distribution area.  
123 In this case, the population of cavefish is large and homogeneous, and it is almost impossible  
124 to study its size using a CMR approach. The intermediate “Lake” model proposes the  
125 existence of clusters of caves, with caves well connected within a cluster, but clusters poorly  
126 interconnected. A cluster of caves fits the definition of a closed population, but a cave only  
127 gives access to a fraction of the population. Yet not impossible, it is difficult to study the  
128 demography of such large population. This discouraging outlook may have limited the

129 research activity on cavefishes in this domain (Bichuette and Trajano, 2021; Elliott, 2018).  
130 Indeed, only two CMR studies have been performed on *Astyanax mexicanus* cavefish so far  
131 (Elliott, 2018). In March 1971, Elliot caught and marked 201 fish in Sótano de Yerbaniz and  
132 230 in Cueva de El Pachón. A caudal fin clip was used to mark captured fish. One day and  
133 three days later, respectively, 226 and 242 fish were caught, among which 4 and 3 were  
134 marked. In El Pachón cave, he could see about 3 to 5 cavefish per m<sup>2</sup>, but the water was  
135 murky deeper than 60 - 90 cm, and he assumed there were more fish deeper. The visual  
136 estimate alone would have provided a minimal estimate of 950-1600 cavefish, but we now  
137 know that the full extent of the El Pachón pool is not visible to humans because the so-called  
138 Maryland extension is not accessible when water level is high. He kept 20 marked fish in a 19  
139 L aquarium in the cave for the duration of the work to gauge the deleterious effects of the fin  
140 clip. In Yerbaniz, one control fish died and was eaten by the others after 48 hours. In El  
141 Pachón, five control fish died after 24 hours and one was nearly dead, but none showed  
142 obvious signs of attack by their fellows. Taking these death rates into account (1/20 and 6/20),  
143 the numbers of marked fish were corrected (191 and 161, respectively) and using equation (3)  
144 and (4), population size estimated to be 9,781 (95% confidence interval =  $\hat{x} \pm 1,96\sqrt{\text{var } \hat{x}} =$   
145 1,179 - 18,283) in El Pachón and 8,671 (1,810 - 15,534) in Yerbaniz. In 2009, Reynoso et al.  
146 captured and marked, by clipping the lower lobule of the caudal fin, 50 individuals in the El  
147 Pachón cave and 36 individuals in the Chica cave, but no marked fish were found among the  
148 54 and 14 captured two days later, respectively, making it impossible to estimate the  
149 population sizes (Elliott, 2018). The absence of marked fish in the second sample in El  
150 Pachón and Chica could be the result of too small samples compared to the population sizes,  
151 but also the consequence of a high mortality of marked fish.  
152 Undoubtedly, there is room to improve our knowledge of the demography of these  
153 subterranean fish as well as to develop novel non-invasive methods for CMR. Here, we

154 examined the demography of the population in the El Pachón cave using a modified CMR  
155 method to better fit some assumptions and identify the most appropriate population model.

156

## 157 **Materials and Methods**

### 158 **Fish sampling**

159 Since 2004, we have maintained a laboratory stock of *Astyanax mexicanus* cavefish  
160 originating from the El Pachón cave (Sierra de El Abra, Mexico), initially obtained from W.  
161 R. Jeffery (University of Maryland, USA). In 2018 and 2022, 16 fish were sampled at random  
162 in our breeding facility for genotyping.

163 During field expeditions in the El Pachón cave, we sampled 20 individuals in 2016 and 35  
164 individuals in 2019. In 2022, we sampled 30 and 29 individuals, respectively, at 3 days of  
165 interval (22nd and 25th February 2022), collected from the main pool.

166 In addition, in 2016, we sampled 38 surface fish individuals in the river Tampemole  
167 (thereafter called Arroyo Tampemole) and 8 surface fish individuals in a water extraction well  
168 close to the El Pachón cave (thereafter called Pozo Pachón Praxedis Guerrero) (**Figure 2**).

169

### 170 **Fish genotyping**

171 Up to 2022, all fish were fin-clipped and DNA extracted later from the fin fragments  
172 conserved in ethanol. Instead of this invasive fin clip procedure, which is the traditional way  
173 to get DNA, in 2022 we used a novel, non-invasive procedure. Gentle swabbings were  
174 performed on each flank of the fish in order to obtain two independent samples per individual.  
175 This procedure does not generate any physical damage to the fish and probably reduces stress  
176 significantly as compared to fin clipping. In all cases, fin-clipped or swabbed individuals were  
177 rapidly released in their natural pond after sampling.



178 The procedure of sample storage and DNA extraction was optimized to take into account the  
179 conditions of fieldwork, as follows.

180 DNA was extracted from swabs (FLOQSwabs<sup>R</sup>, COPAN Diagnostics Inc.) stored in tubes  
181 with silica gel beads at room temperature. Lysis with a mix of buffer (Tris HCl pH 8 100 mM,  
182 EDTA 2 mM, triton 0.2%) and proteinase K (250 µg/ml) overnight at 55°C was followed by  
183 inactivation of proteinase K for 10 min at 98°C. Aliquots of DNA were transferred in new  
184 tubes and stored at -20°C.

185 Among 26 microsatellite loci that proved to be highly polymorphic in this species (Bradic et  
186 al., 2012), we selected 18 loci on the basis that 1) they were polymorphic in El Pachón cave,  
187 2) they could be easily amplified by PCR, 3) the amplicon sizes allow the amplification of  
188 these 18 loci through only three multiplexed PCR (**Supplementary Table S1**). Before setting  
189 up the PCR reactions, we prepared 10X primer mix with 2 µM of each primer (Multiplex1,  
190 Multiplex2 and Multiplex3). The PCR reactions were carried out in 10 µl of final volume  
191 with: 1 µl of template DNA, 5 µl 2X Platinum<sup>TM</sup> Multiplex PCR Master Mix (ThermoFisher  
192 Scientific), 1 µl 10X primer mix and 3 µl H<sub>2</sub>O. The program used was: 2 min 95°C, followed  
193 by 30 cycles of 30 sec 95°C, 90 sec 60°C, 60 sec 72°C), and a final extension for 30 min at  
194 60°C.

195 Genotypes were scored using an ABI 3130 XL Genetic Analyzer, with GeneScan<sup>TM</sup> 500  
196 LIZ<sup>TM</sup> size standard (ThermoFisher Scientific) and GeneMapper<sup>TM</sup> software v4.1 (Applied  
197 Biosystems<sup>TM</sup>). For each locus, several alleles with different sizes were sequenced using  
198 homozygous specimens. These sequences allowed us to translate allele relative sizes obtained  
199 with GeneMapper into real allele sizes.

200 For each specimen captured in 2022, two independent samples (swabs) were genotyped.

201 Globally, with most DNA samples (obtained by fin clip or swabbing, and from cave, river,  
202 well or lab individuals) we could obtain the genotype at each of the 18 loci, but for 8 fish the

203 genotype at one or several loci was missing. These incomplete genotypes were not used in  
204 statistical analyses (**Supplementary Table S2**).

205 For statistical analyses, only complete genotypes were used. This included 16 samples from  
206 our breeding facility (2022); 19, 35, 29 and 29 samples from El Pachón cave collected in the  
207 field (2016, 2019, 22nd and 25th February 2022, respectively); 34 samples from Arroyo  
208 Tampemole, (2022), and 6 samples from Pozo Pachón Praxedis Guerrero (2022).

209

## 210 **Statistical analyses**

### 211 **Polymorphism variation through time**

212 Changes in allele frequencies at each locus between each pair of sampling dates were tested  
213 using the Fisher's exact test, using the `fisher.test` function from the stats package in R 4.1.0 (R  
214 Core Team, 2021). As 180 tests were performed, we used the Bonferroni correction (threshold  
215  $\alpha = 0.05/180$ ) and Benjamini-Hochberg procedure in order to decrease the false discovery rate  
216 using the `p.adjust` function from the stats package in R (R Core Team, 2021).

217

### 218 **Multidimensional scaling**

219 To represent the matrix of genetic distances, the genetic distance between two individuals  
220 being the number of allelic differences, as a two-dimensional scatter plot, we performed a  
221 metric multidimensional scaling (MDS) using the `cmdscale` function from the stats package in  
222 R (R Core Team, 2021). We used only individuals for which a complete genotype was  
223 available.

224

### 225 **Distribution of pairwise genetic distances according to kinship**

226 First, we estimated the probability that two unrelated individuals would have the same  
227 genotype ( $P_{uni}$ ) using the following formula:

$$P_{id} = \sum_{i=1}^n (f_i^2)^2 + \sum_{i=1}^{n-1} \sum_{j>i}^n (2f_i f_j)^2$$

$$P_{uni} = \prod_{k=1}^L P_{id_k} \quad (5)$$

where  $P_{id}$  is the probability that two unrelated individuals have the same genotype at a given locus,  $n$  is the number of alleles at a given locus,  $f_i$  is the frequency of allele  $i$ , and  $L$  is the number of loci analyzed.

Although it is simple to compute the probability that two unrelated individuals have the same genotype, it is more difficult to compute the expected distribution of the genetic distance, that is the number of different alleles between two individuals, according to their kinship (*i.e.* unrelated, parent-descendant, full siblings, half siblings). These distributions were obtained using simulations. Pairs of genotypes were generated for different kinships. For two unrelated individuals, at each locus, the genotype of each individual was generated by randomly sampling two alleles, considering current allele frequencies in the population. For a parent and its descendant, the genotype of the parent was generated as described above, and the genotype of the descendant was generated by sampling at random one allele from this parent and the other allele from an unknown parent, that is from the population, considering allele frequencies. For full siblings, two unrelated parents were first generated; then, each descendant was generated by randomly sampling one allele from each parent. For half siblings, one mother and two unrelated fathers were first generated; then, one descendant was generated by randomly sampling one allele from the mother and one allele from one father. The other descendant was generated by randomly sampling one allele from the same mother and one allele from the other father. For each kind of relationship, one million simulations were performed to estimate the empirical distribution of pairwise distances according to a given kinship. Simulations and data analyses were carried out in the R statistical environment

251 (R Core Team, 2021). The R script “GenerateIndividual\_astyanax.r” can be found in GitHub  
252 “jmorode/Genetics\_Astyanax”.

253

## 254 **Estimation of genealogical relationships**

255 The software ML Relate (Kalinowski et al., 2006) was used to find evidence of relatedness  
256 between cavefish. The accuracy of inferred genealogical relationships by the software was  
257 evaluated using simulated families of individuals of known genotypes. One thousand families  
258 were generated using the R script described above. Each family was composed by a mother,  
259 two unrelated fathers, two full siblings and two pairs of half siblings. This family composition  
260 allowed us to test the four relationships assessed by ML Relate: parent-offspring, full siblings,  
261 half siblings and unrelated. The genotypes of the members of these 1,000 families were  
262 written in an input file for ML Relate. For each pair of individuals in each family, the known  
263 relationship was compared with the one inferred by ML Relate. In each family, 6 unrelated  
264 individuals, 1 pair of full siblings, 2 pairs of half siblings and 6 pairs of parent-offspring were  
265 expected. The percentage of known relationships found by ML Relate over the 1,000 families  
266 was interpreted as an estimation of the accuracy of this software when identifying  
267 genealogical relationships in our observed population of cavefish.

268

## 269 **Results**

### 270 **Genetic polymorphism in the El Pachón cave and nearby surface locations**

271 Individuals collected in the El Pachón cave from 2016 to 2022 were genotyped at 18  
272 microsatellite loci. In addition, El Pachón individuals from our lab stock, derived from El  
273 Pachón cavefish collected before 2004 (probably in the 1990’s), and surface fish from two  
274 locations, a well and a river close to the El Pachón cave, were also genotyped (**Figure 2**). All  
275 genotypes are reported in **Supplementary Table S2**.

276 The number of alleles at each locus was smaller in the El Pachón cave samples than in the two  
277 surface samples (**Table 1**). In total, among the 18 microsatellite loci analysed, only 54 alleles  
278 were recovered from 113 El Pachón cavefish whereas 203 alleles were recovered from 43  
279 surface fish. The mean heterozygosity in El Pachón cave (0.2) is about 4 times lower than in  
280 river-dwelling fish (0.66 and 0.80 in Pozo Pachón Praxedis Guerrero and Arroyo Tampemole  
281 respectively).

282 Allele frequencies are reported in **Table 2** for El Pachón cave samples and **Supplementary**  
283 **Table S3** for surface populations.

284 The polymorphism in El Pachón cave appears stable since 2004, as all alleles recovered from  
285 lab individuals (i.e., derived from fish taken from the cave before 2004) are still present in the  
286 cave in 2022. In the lab stock, genetic drift may have led to the loss of some rare alleles  
287 (**Table 2, Figure 3**). Indeed, Fisher's exact tests, with no correction for multiple tests  
288 (**Supplementary Figure S1A**), with a Bonferroni correction (**Supplementary Figure S1B**)  
289 and applying the Benjamini-Hochberg procedure (**Supplementary Figure S1C**) indicate that  
290 significant allele frequencies differences observed between pairs of El Pachón samples  
291 involve most often the lab sample. However, these differences are minimal as compared to  
292 those observed between El Pachón samples and surface fish samples, or even between the two  
293 different surface fish locations (**Figure 3**, orange and green dots).

294 Of note, the polymorphism observed in our El Pachón cavefish samples is different from the  
295 one estimated in individuals collected in 2008 at the same place (Bradic et al., 2012)  
296 (**Supplementary Table S4**). The lengths of the alleles are different, and even doing a  
297 translation, we could not align the data. Moreover, higher polymorphism was found in Bradic  
298 et al. (2008). They often found two alleles differing by one repeat and with similar  
299 frequencies, while we find that for most loci one allele had a high frequency (> 80%). It is

300 currently difficult to understand this discrepancy and these data were not further considered in  
301 our analyses.

302

### 303 Genetic evidence of recaptures and population size estimation in El Pachón cave

304 The genetic capture-recapture method is based on the assumption that two fish have a very  
305 low probability of having the same genotype. The probability that, in our samples, two  
306 unrelated individuals have the same genotype was estimated using equation (5). For surface  
307 populations which display high allelic diversity, the probability is extremely low,  $2.1 \times 10^{-15}$   
308 and  $1.5 \times 10^{-23}$  in Pozo Pachón Praxedis Guerrero and Arroyo Tampemole, respectively. For  
309 El Pachón cave, the probability is  $2.1 \times 10^{-4}$ ,  $2.8 \times 10^{-4}$ ,  $2.2 \times 10^{-4}$  and  $2.2 \times 10^{-4}$  for samples  
310 obtained in 2016, 2019 and 22 Feb 2022 and 25 Feb 2022, respectively. Thus, it is virtually  
311 impossible to catch unrelated surface fish with the same genotype, and it is unlikely for two  
312 unrelated cavefish to have the same genotype - despite the lower genetic polymorphism in the  
313 El Pachón cave population. One must also take into account that closely related fish, like full  
314 sibs, have more similar genotypes than unrelated individuals. Simulations of the genotypes of  
315 pairs of individuals with various relatedness were realized in order to estimate the probability  
316 of finding a given genetic distance, in the range of 0 to 36, according to kinship  
317 (**Supplementary Figure S2**). For full sibs (the most closely related individuals), in our  
318 simulations, we did not observe identical genotypes in surface populations, but about 2% in  
319 the El Pachón population. These results indicate that even if there were some closely related  
320 individuals in our samples of cavefish, the probability is low that they share the same  
321 genotype. In accordance with this assumption, we found four pairs of identical genotypes  
322 from different El Pachón samples, but none within a given sample. We therefore considered  
323 that identical genotypes in different samples corresponded to the same individual that had  
324 been recaptured.

325 On the 22nd February 2022, 29 fish were sampled by swabbing in El Pachón main pool and  
326 genotyped, and 29 fish were sampled 3 days later in the same place and genotyped. We  
327 identified 3 pairs of identical genotypes that we assumed to be recaptured individuals  
328 (**Supplementary Table S5**). Using equation (3) and (4), the estimated population size in El  
329 Pachón cave is therefore of 218 cavefish (95% CI = 40 - 395).  
330 Interestingly, among 55 genotypes observed in 2022, we found three genotypes that were  
331 identical to genotypes observed in 2019, suggesting that three individuals have been  
332 recaptured three years after their first capture (**Supplementary Table S5**). Taking into  
333 account that 35 were captured in 2019 and assuming a low mortality between 2019 and 2022  
334 of the fishes captured in 2019, using equation (3) and (4), we obtained another estimate of the  
335 population size in El Pachón cave, 490 (95% CI = 76 – 904). Both estimates point to a small  
336 population consisting of a few hundreds of individuals.

337

### 338 **Genealogical Relatedness**

339 Individual genotypes from a small population give an opportunity to examine kinship.  
340 However, the reliability of kinship inferences depends on the level of polymorphism. We  
341 assessed the possibility to infer kinships among surface fish on the one hand and El Pachón  
342 cavefish on the other hand using the software ML Relate. First, pairs of individuals with  
343 different relatedness (unrelated individuals, offspring-parent, full sibs and half sibs) were  
344 simulated taking into account allele frequencies. Then the true kinship was compared to the  
345 one inferred by ML Relate. For surface populations, the accuracy was 81% and 92% for Pozo  
346 Pachon Praxedis Guerrero and Arroyo Tampemole respectively, suggesting that the  
347 polymorphism is sufficient to infer relatedness with a good confidence (**Supplementary**  
348 **Table S6**). However, for cavefish the accuracy was only about 50% (**Supplementary Table**

349 **S6**). The polymorphism at the 18 microsatellite loci in El Pachón cave is thus not sufficient to  
350 identify related individuals.

351

## 352 **Discussion**

### 353 **Genetic tags for long-term population surveys**

354 Two assumptions about the experimental conditions, which should be verified for CMR  
355 analyses, are that capturing and marking have a minimal effect on the probability of  
356 recapturing. Several reports indicate that fin clip can affect their behaviour and be lethal for a  
357 substantial percentage of fish (Elliott, 2018). To circumvent this problem that is both technical  
358 and ethical, in 2022 we developed and used a method with swabbing instead of fin-clipping to  
359 collect DNA samples, which has been successfully applied for population estimates in  
360 amphibians including the flagship cave species, *Proteus anguinus* (Prunier et al., 2012;  
361 Trontelj and Zaksek, 2016). This approach is likely much less invasive if not completely  
362 neutral, if we do not take into account the stress induced by net capture and human handling  
363 during the swabbing procedure – which was performed as gently as possible. Yet it does  
364 “mark” captured fish genetically. Genotyping is then necessary to identify captured  
365 individuals. Although more time-consuming and costly, the great advantage of marking by  
366 genotyping is that we have access to a unique and stable tag that allows the identification of  
367 each animal over its entire life. However, the uniqueness of a genotype depends on the genetic  
368 diversity in the population. Microsatellite loci are particularly useful because their high  
369 mutation rate leads to several alleles at a given locus when the population size is relatively  
370 large, and individuals can be identified based on the combination of a small number of loci.  
371 Conversely, in small populations, the polymorphism can be low and genotyping more loci  
372 might be necessary to associate a genotype with a unique individual. Here, we found that a  
373 combination of 18 microsatellite loci is sufficient to identify each El Pachón cavefish and



374 compute population size, but the population is not polymorphic enough to infer kinship. The  
375 more reliable kinships inferred with surface fish polymorphism, which is about four times  
376 larger than in El Pachón cave, suggest that the use of four times more microsatellite markers  
377 would be necessary to get reliable inferences of kinship between cavefish.

378

### 379 **Size and isolation of the cavefish population in the El Pachón cave**

380 There are four pools in El Pachón cave with sizable numbers of fish, but only two are usually  
381 accessible to humans: the main pool, which measures about 25 x 5 m and in which the study  
382 was conducted, and a small pool on a side lateral gallery about 3 x 1.5 m. These two pools  
383 connect during the rainy season. Fish are concentrated in the small pool at higher densities.  
384 We have visited the El Pachón cave multiple times in the past 14 years and with photos and *in*  
385 *situ* observations, we estimate that in the small pool there are usually about 50-150  
386 individuals. Often, a large part of the “main pool” is hidden to humans as water continues into  
387 the “Maryland extension” under a sump (Elliott, 2018). Only in 2003, 2009 and in February  
388 2022 when we performed the genetic mark and recapture experiment have we seen the water  
389 level so extremely low as to give access to the Maryland extension galleries. In 2022, we  
390 visited the Maryland extension galleries and in particular the perched pool named UAEM  
391 pool (Elliott, 2018). This pool is higher and does not connect with the main pool. We did not  
392 do genetic mark and recapture experiments at the UAEM pool, but observations *in situ*  
393 suggest that the density of fish is similar to the main pool. The fourth pool was not visited in  
394 2022, but in 2003 there may have been an estimate of about 50 individuals based on *in situ*  
395 observations. Given that during extreme rainy seasons all four pools may exchange  
396 individuals, the population size obtained for the main pool (218 fish) should be multiplied by  
397 ~3 for El Pachón cave as a whole.

398 Our analysis therefore suggests that the El Pachón cavefish population size in 2022 was in the  
399 order of magnitude of a few hundreds. The capture in 2022 of three fish that were first  
400 captured in 2019 (with samples of 35 and 55 fishes, respectively) gives a population size of  
401 490 fishes, which is in line with the estimate 654 (3 x 218) obtained with the CMR in 2022.  
402 Together, these results suggest that the El Pachón population corresponds to the Oasis model,  
403 i.e., a small population poorly connected with other subterranean populations. Indeed,  
404 movements of fish between subterranean populations should prevent the recapture of fish over  
405 several years. Moreover, and importantly, the low genetic diversity and the stability of allele  
406 frequencies over time support the absence of a gene flow from surface or other genetically  
407 differentiated cave populations between ~1990/2000 and 2022. In 1986 and 1988, some  
408 individuals with variable eye sizes and melanin pigmentation were observed in the albino and  
409 eye-reduced El Pachón population (Langecker et al., 1991). Thus, it is possible that sporadic  
410 migrations of surface fish occurred but the low and stable genetic polymorphism in the cave  
411 during the last 20 years when compared to the high polymorphism in close surface  
412 populations shows that surface fish migrations had an undetectable impact on the genetic  
413 diversity in the cave during the last few decades.

414

### 415 Population genetic issues

416 Several researchers used genetic polymorphism to estimate the effective population size ( $N_e$ )  
417 and the migration rate in El Pachón cave. The first one, examining the allozyme variations at  
418 17 loci, found no polymorphism in this population, suggesting a small  $N_e$  and isolation from  
419 surface populations (Avisé and Selander, 1972). Later on, the analysis of 26 microsatellite  
420 loci also pointed out a small  $N_e$  (< 1000) and limited gene flow (Bradic et al., 2012). A re-  
421 analysis of this data set, combined with transcriptomic data and using other statistical  
422 approaches led to the same conclusion (Fumey et al., 2018). Finally, a study using genomic

423 data estimated that  $N_e$  could be higher (median = 32,000, min = 3,000 and max = 46,000)  
424 (Herman et al., 2018).  $N_e$  is a population genetic parameter that depends on the long term  
425 census population size ( $N_c$ ) but also on a series of other biological and demographic  
426 parameters (Charlesworth, 2009). In the case of *Astyanax* cavefish, two parameters could lead  
427 to a  $N_e$  much lower than  $N_c$ , potentially several order of magnitude lower. First, episodes of  
428 low population size are known to have a disproportionate effect on the overall value of  $N_e$   
429 (Charlesworth, 2009). Moreover, when females have the potential to lay thousands of eggs  
430 during their reproductive lifespan, like in this species, this can lead to a much larger variance  
431 in offspring number than expected with purely random variation, reducing  $N_e$  much below  $N_c$ .  
432 Indeed, if many individuals from only a few egg laying events survive during exceptional and  
433 favorable environmental conditions whereas most individuals from most egg laying die, then  
434 the variance of reproductive success can be very large, a process known as sweepstakes  
435 reproductive success (Hedgewood, 1994). In a small population, this could result in many full-  
436 or half-siblings in a cohort sample. The hypothesis might be tested using genetic  
437 polymorphism. Even if the variance of reproductive success is not large, we can assume that  
438  $N_e$  is at best in the order of magnitude of the smallest  $N_c$ , that is a few hundreds.

439

#### 440 Conservation issue

441 In 1971, Elliott estimated the El Pachón cavefish population to ~10,000 individuals (Elliott,  
442 2018). Our 2022 estimate of the number of cavefishes in El Pachón cave to a few hundreds of  
443 fish, i.e., an order of magnitude lower, is very worrying, and questions the long-term  
444 maintenance of this population. The causes of the apparent decline in population size in this  
445 cave may be manifold. They might include variations in water quality parameters or water  
446 levels, human impacts like phreatic contamination, habitat disturbing like paintings of the  
447 cave walls above the main pool and plastic waste inside the caves, water pumping out of the

448 cave for human consumption, and too frequent and too important samplings involving lethal  
449 experiments or removal of specimens from the cave by researchers, which are all facilitated  
450 by the ease of access to this cave. The effect by researchers should be easily and collectively  
451 handled and corrected by the *Astyanax* cavefish research community. Given all the intellectual  
452 benefit it has received from this single population, scientists should be actively and  
453 collectively engaged in its conservation. A longitudinal monitoring of the El Pachón cave  
454 population and the ecological parameters in the cave will also be paramount to counteract  
455 population decline and avoid extinction. Unfortunately and sadly, the El Pachón cavefish  
456 population, which has been the most studied and emblematic since its first scientific  
457 description in 1946 as “*Astyanax antrobius*” (Elliott, 2018) could well be the victim of its own  
458 success.

459

## 460 **Data Availability**

461 The R script “GenerateIndividual\_astyanax.r” can be found in GitHub “jmorode/Genetics\_Astyanax”.

462

## 463 **Supplementary Material**

464 Supplementary data are available at Zoological Research

465

## 466 **Acknowledgments**

467 This work was supported by a CNRS MITI (Mission pour les Initiatives Transverses et  
468 Interdisciplinaires) grant “Expérimentation en Milieux Extrêmes » to SR and collaborative  
469 grants from Agence Nationale de la Recherche (BLINDTEST) and Institut Diversité Ecologie  
470 et Evolution du Vivant to S.R. and D.C.

471

## 472 **Ethics approval**

473 Animals were treated according to the French and European regulations for handling of  
474 animals in research. SR's authorization for use of animals in research including *Astyanax*  
475 *mexicanus* is 91-116 and the Paris Centre-Sud Ethic Committee protocol authorization  
476 number related to this work is 2012-0052.

477

### 478 **Sampling authorization**

479 *Astyanax mexicanus*: fin clips from surface and cave morphs of *A. mexicanus* were sampled  
480 during field expeditions, under the auspices of the field permits SGPA/DGVS/02438/16,  
481 SGPA/DGVS/1893/19 and SGPA/DGVS/03334/22 delivered to P Ornelas-García and S  
482 Rétaux by the Secretaría de Medio Ambiente y Recursos Naturales of Mexico  
483 (SEMARNAT).

484

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540

541

542

**Table 1. Allelic diversity in surface locations and El Pachón cave.**

H-e5	L-b1	V-f3	A8-g5	X-a3	W-f6	A13-e5	L-b9	A2-a7	V-c10	A13-f8	A6-f1	A6-h6	A14-d8	A4-g11	W-d11	W-c12	A5-f9
154	303	201	150	198	273	242	213	104	228	107	257	103	115	161	154	169	194
156	305	205	160	200	277	244	215	108	232	109	265	105	121	153	162	171	196
158	307	207	162	202	281	246	223	110	234	113	267	107	123	167	166	173	198
160	309	209	164	204	282	248	223	112	236	115	269	111	125	169	170	177	200
162	311	211	166	206	283	250	227	114	238	117	271	113	127	171	172	179	204
164	313	213	168	208	285	252	229	116	240	119	273	115	129	173	174	185	206
166	315	215	170	210	287	254	233	118	242	121		121	131	177	176	193	207
168	317		172	212	289	256	259	120	244	123		123	133	179	178		
170	319		174		291	258	271	122	246	125		125	135	183	180		
172	329		176		293	262		124	248	127		127	137		182		
174			178		295	264		126		129		129	143		184		
178					297	272		128		131		131	145		186		
								130		133		135	147		188		
								134		135		139			190		
								138		137					192		
								140		139					194		
								142		143					196		
								144		147					200		
								148		149					202		
								150		161					204		
								156									
								164									
								172									

The names of the 18 microsatellite loci analyzed are indicated in the first line. For each locus, allele sizes are indicated in columns. Alleles in red are found in El Pachón, those highlighted in green are unique to El Pachón.



**Table 2. Allele frequencies in El Pachón cave.**

2022 (2)	2022 (1)	2019	2016	lab	V-43	2022 (2)	2022 (1)	2019	2016	lab	I-b1	2022 (2)	2022 (1)	2019	2016	lab	H-c5
0.02	0.02	0.01	1.00	1.00	213	0.10	0.08	0.06	0.10	0.09	305	0.02	0.02	0.04	0.03	0.03	154
0.98	0.98	0.99	1.00	1.00	215	0.03	0.02	0.03	0.02	0.03	311	0.02	0.02	0.04	0.03	0.03	172
0.03	0.03	0.03	0.03	0.00	He	0.17	0.19	0.18	0.22	0.23	He	0.17	0.19	0.18	0.23	0.22	He
0.02	0.02	0.03	1.00	1.00	289	0.03	0.02	0.03	0.02	0.03	202	0.29	0.29	0.03	0.03	0.03	160
1.00	0.98	0.97	1.00	1.00	295	0.03	0.02	0.03	0.02	0.03	206	0.04	0.02	0.03	0.03	0.03	168
0.00	0.03	0.06	0.00	0.00	He	0.30	0.45	0.31	0.41	0.40	He	0.30	0.45	0.31	0.40	0.41	He
0.02	0.12	0.13	0.24	0.56	106	0.05	0.05	0.09	0.05	0.05	213	0.03	0.03	0.16	0.10	0.10	242
0.19	0.33	0.32	0.37	0.28	108	0.05	0.95	0.91	0.95	0.95	225	0.03	0.91	0.06	0.13	0.13	246
0.40	0.45	0.44	0.34	0.16	112	0.03	0.03	0.06	0.03	0.03	He	0.45	0.10	0.06	0.10	0.10	252
0.36	0.10	0.11	0.44	0.58	114	0.03	0.03	0.06	0.03	0.03	He	0.45	0.10	0.06	0.10	0.10	254
0.03	0.67	0.68	0.05	0.69	124	0.03	0.03	0.06	0.03	0.03	He	0.45	0.10	0.06	0.10	0.10	256
0.67	0.67	0.67	0.69	0.68	He	0.45	0.10	0.06	0.03	0.03	He	0.45	0.10	0.06	0.10	0.10	He
0.31	0.20	0.14	0.41	0.16	265	0.02	0.02	0.05	0.02	0.05	121	0.03	0.02	0.06	0.03	0.03	228
0.69	0.02	0.05	0.81	0.84	267	0.02	0.98	0.88	0.93	0.93	125	0.03	0.97	0.06	0.13	0.07	246
0.43	0.35	0.31	0.49	0.26	271	0.03	0.03	0.06	0.03	0.03	127	0.07	0.97	0.06	0.13	0.07	248
0.43	0.35	0.31	0.49	0.26	He	0.13	0.03	0.21	0.13	0.13	He	0.07	0.03	0.21	0.13	0.13	He
0.98	0.98	0.94	1.00	1.00	165	0.00	1.00	1.00	1.00	1.00	129	0.07	0.97	0.03	0.07	0.07	103
0.02	0.02	0.06	0.11	0.00	171	0.00	0.00	0.00	0.00	0.00	He	0.07	0.97	0.03	0.07	0.07	115
0.03	0.03	0.03	0.03	0.00	He	0.00	0.00	0.00	0.00	0.00	He	0.07	0.97	0.03	0.07	0.07	He
0.97	1.00	0.96	0.97	1.00	198	0.07	0.07	0.01	0.07	0.03	173	0.22	0.22	0.01	0.07	0.07	162
0.03	0.03	0.04	0.03	0.03	200	0.03	0.02	0.01	0.02	0.02	177	0.21	0.09	0.01	0.16	0.16	166
0.03	0.03	0.04	0.03	0.00	He	0.03	0.02	0.01	0.02	0.02	185	0.21	0.09	0.01	0.16	0.16	172
0.03	0.03	0.04	0.03	0.00	He	0.03	0.02	0.01	0.02	0.02	185	0.21	0.09	0.01	0.16	0.16	176
0.03	0.03	0.04	0.03	0.00	He	0.03	0.02	0.01	0.02	0.02	185	0.21	0.09	0.01	0.16	0.16	186
0.03	0.03	0.04	0.03	0.00	He	0.03	0.02	0.01	0.02	0.02	185	0.21	0.09	0.01	0.16	0.16	188
0.03	0.03	0.04	0.03	0.00	He	0.03	0.02	0.01	0.02	0.02	185	0.21	0.09	0.01	0.16	0.16	200
0.07	0.07	0.08	0.05	0.00	He	0.07	0.16	0.06	0.07	0.07	He	0.07	0.16	0.06	0.07	0.07	He

For each of the 18 loci analyzed, allele frequencies are shown for each session of sampling in the lab (2018 + 2021) and in the cave (2016, 2019 and 2022). Yellow highlighting indicates the frequency of dominant alleles over the years. Lab stock was obtained with fish sampled before 2004. He: expected heterozygosity.

543 **Figure legends**

544

545 **Figure 1. Hypothetical models for the distribution of cavefish populations**

546 Dark blue represents water bodies that are accessible to humans and where fishes can be  
547 observed and sampled (arrows). Light blue represents large or restricted, permanent or  
548 temporary water conducts and bodies that connect accessible pieces of water. The “Oasis”  
549 model corresponds to a patchy distribution of small populations with little connections  
550 (dashed lines). At the other end, the “Sea” model corresponds to a large population of  
551 cavefishes distributed over a large area in fully connected aquifers. The “Lake” model is an  
552 intermediate. See text for details.

553

554 **Figure 2. Map of the Sierra de El Ebra region in North East Mexico and sampling**

555 **locations**

556 Red dots indicate cave locations where cavefish populations have been described. P: El  
557 Pachón cave. Blue dots indicate sampling stations for surface fish, A: the well Pozo Pachón  
558 Praxedis Guerrero, B: the river Arroyo Tampemole.

559

560 **Figure 3. MultiDimensional Scaling (MDS) showing genetic distances between cave and**

561 **surface fish.**

562 Each dot represents an individual. The color code for each sampling location is given in the  
563 inset. PPP = Pozo Pachón Praxedis Guerrero; AT = Arroyo Tampemole.

564

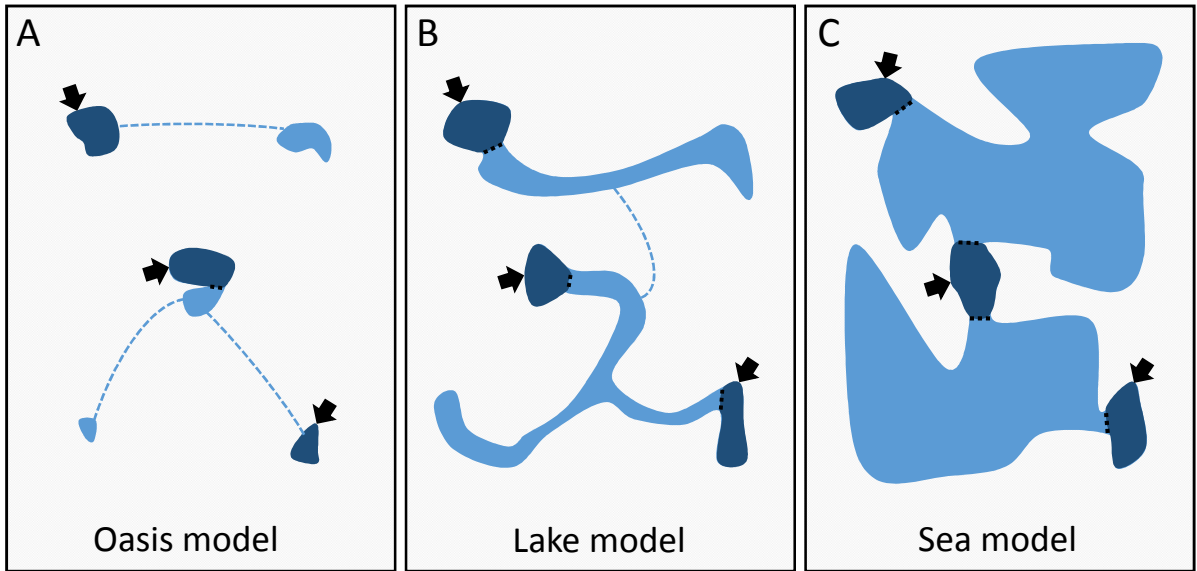
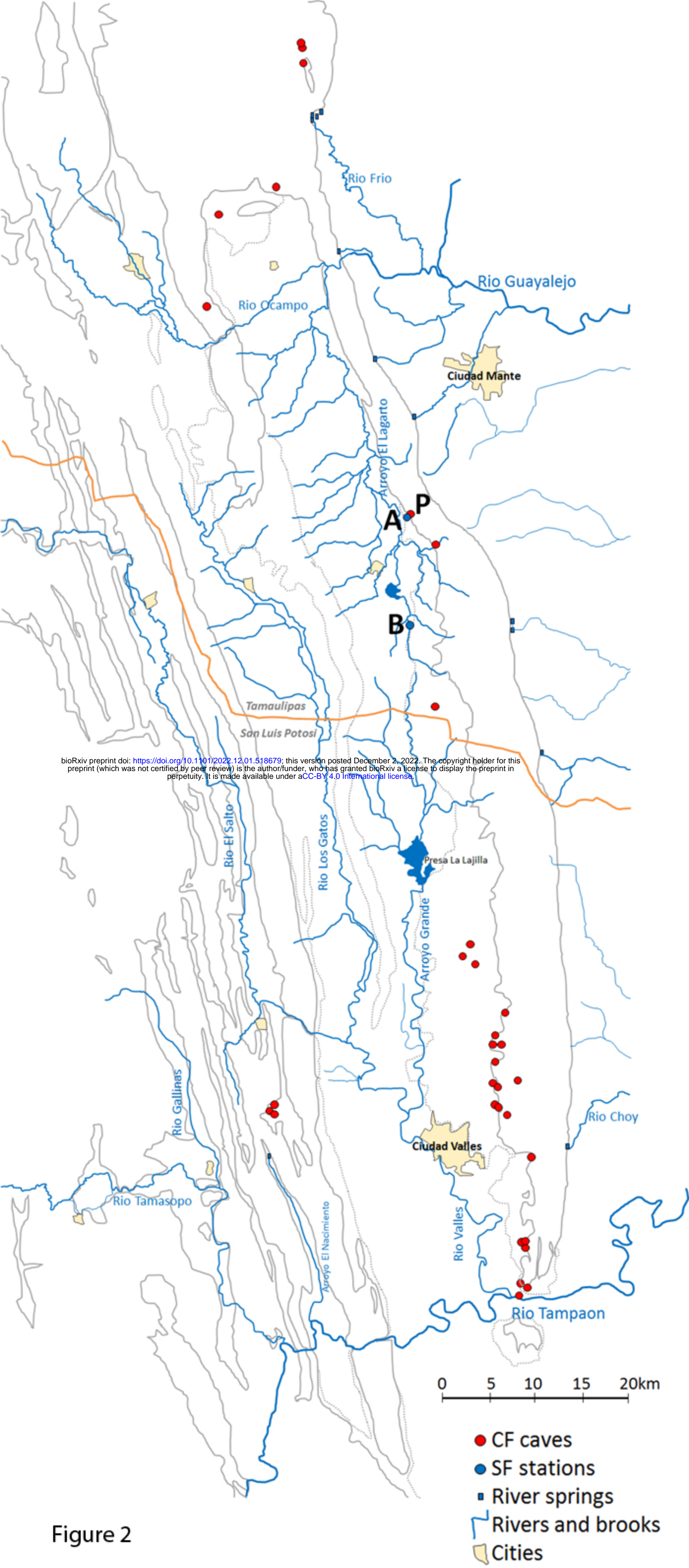


Figure 1



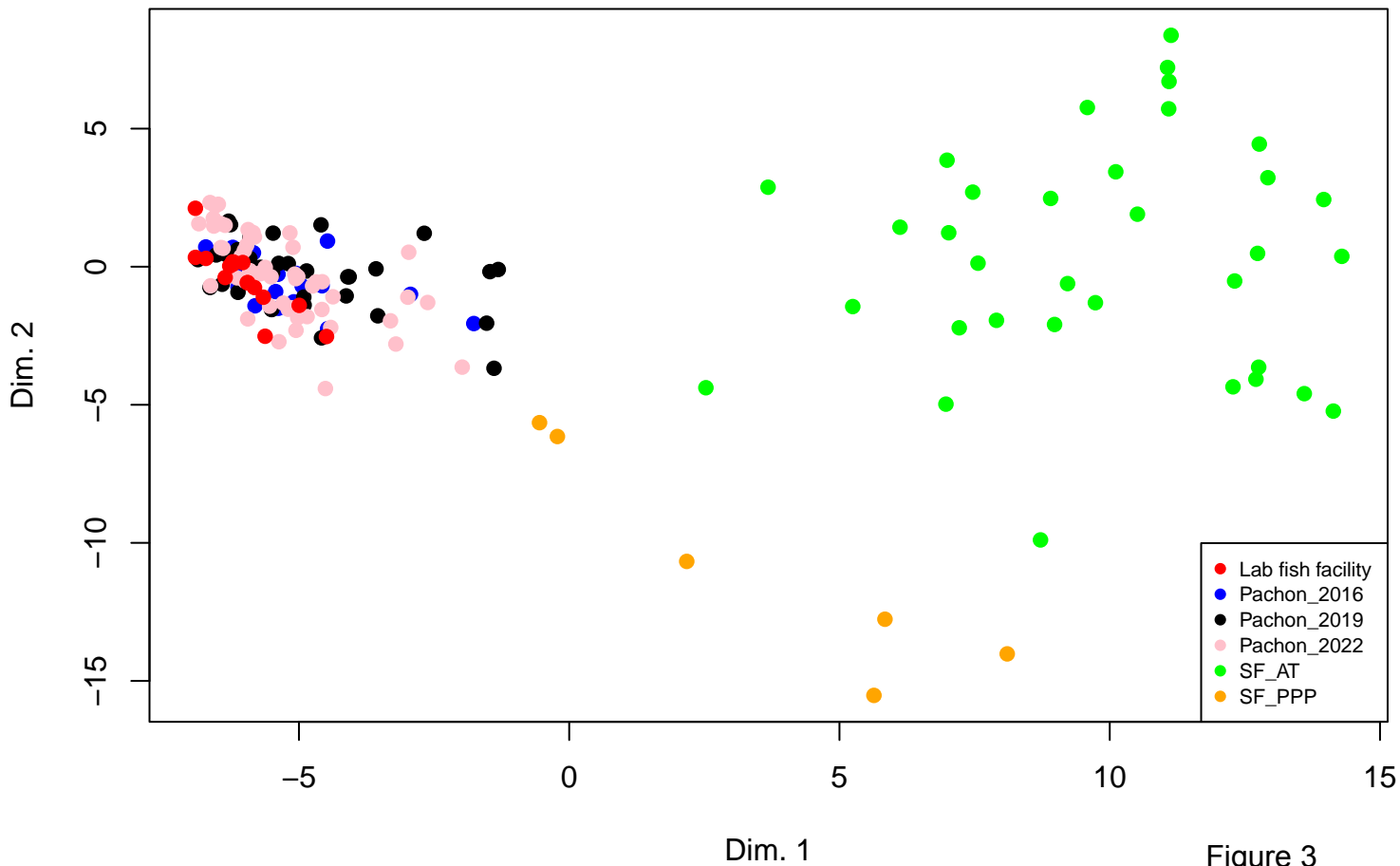


Figure 3