

**A contemporary analysis of a loss-of-function *oculocutaneous albinism type II (Oca2)* allele within the Río Subterráneo *Astyanax* cavefish population**

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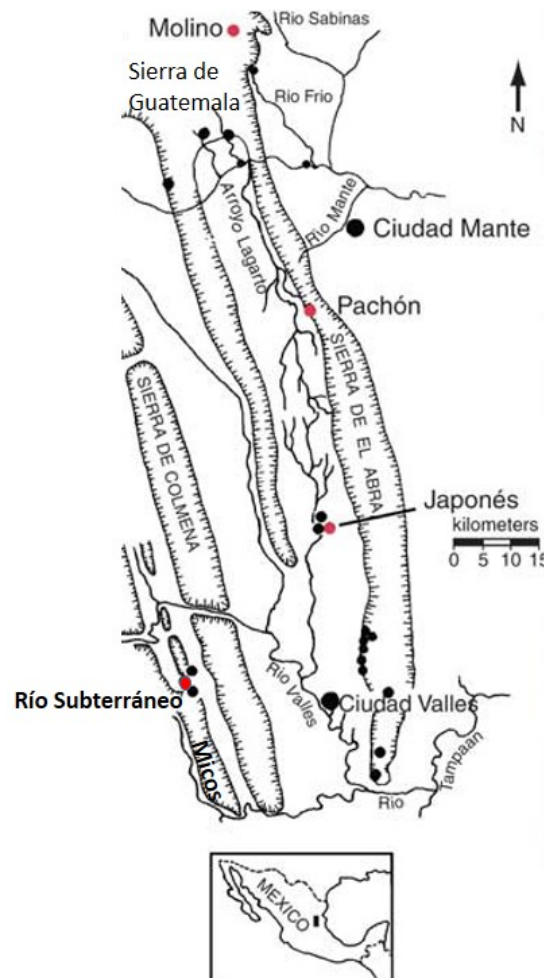
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The Mexican tetra fish, *Astyanax mexicanus* (= *A. fasciatus mexicanus* De Filippi, 1853) has an eyed, pigmented surface morph and a blind, depigmented cave morph. Populations of the cave morph are found in 29 caves in three geographically distinct mountain ranges of northern Mexico (**Figure 1**): the Sierra de El Abra, the Sierra de Guatemala, and the Micos area (Mitchell et al. 1977). While all cavefish populations have depigmented individuals, not all populations are albino. True albinism is defined by the inability to synthesize melanin. In multiple populations, the skin may be completely pale, but the pigmented epithelium in their retina is black (Espinasa and Jeffery 2006) and produces melanin, thus indicating that the white skin coloration is not due to true albinism, and depigmentation is caused by other genetic or developmental processes. Most localities do not harbor albinism (e.g., Chica, Curva, Piedras and Sabinos cave populations). Localities where albinism has been reported include Molino, Pachón, Yerbaniz and Japonés caves (Gross et al. 2009). Albinism has evolved independently at least three different times due to a mutation in the Ocular and Cutaneous Albinism Type II (*Oca2*) gene (Protas et al. 2006). Within the Sierra de Guatemala, the Molino population harbors a coding sequence deletion (in exon 21) of the *Oca2* allele, which results in a non-functional protein. In the northern Sierra de El Abra, the Pachón population harbors a destructive deletion in a portion of exon 24, as well as a base change in exon 13 at position 1252 where adenine is substituted by a guanine residue. In southern Sierra de El Abra, the Japonés population has alterations that suggest a third, independent mutation in the *Oca2* gene, perhaps in a regulatory sequence (Protas et al. 2006). When homozygous, all of these mutations result in albinism, but when heterozygous, pigmentation occurs.

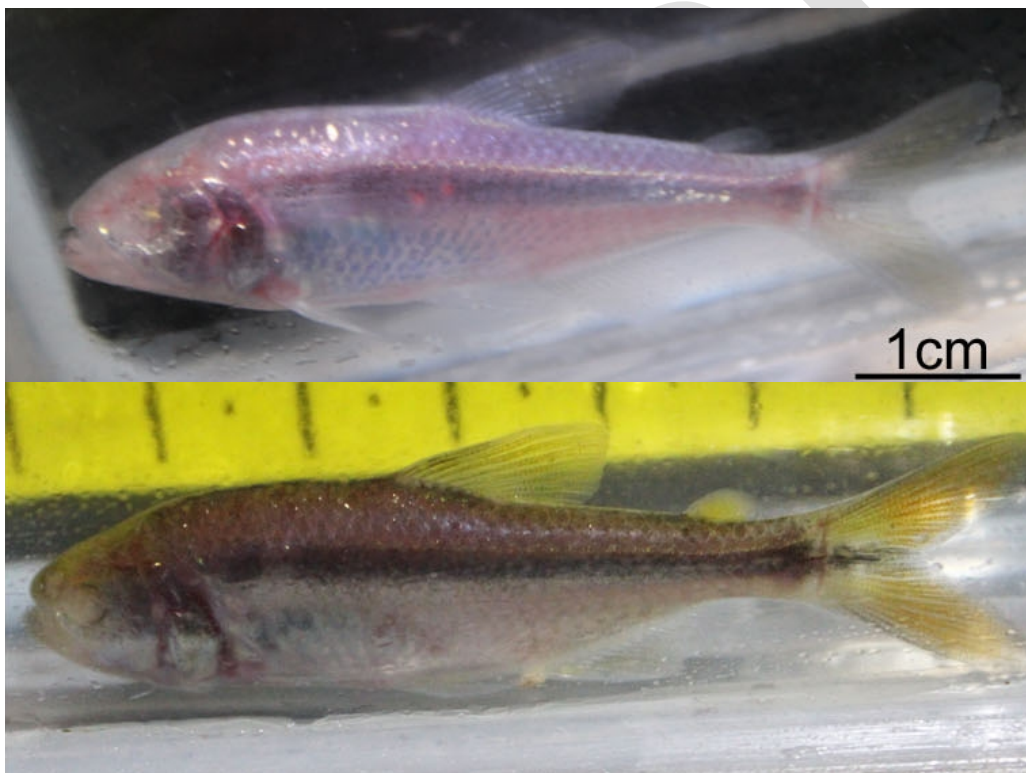


**Figure 1.** Map showing cave localities inhabited by *Astyanax mexicanus* populations. Mountain ranges include Sierra de Guatemala, Sierra de El Abra, and Micos region. The small dots represent the reported caves inhabited by troglomorphic fish and the large dots represent nearby cities. Red dots highlight Río Subterráneo, Molino, Pachón, and Japonés caves. The Pachón and Río Subterráneo caves are over 60 km apart, separated by mountain ranges, and in different river drainages (adapted from Protas et al. 2006).

A particularly interesting population occurs in Río Subterráneo Cave, in the Micos area. This cave contains a wide array of intermediate, hybrid phenotypes that are living alongside more troglomorphic fish. This is probably due to seasonal migration of surface fish into the cave after flooding and subsequent introgression in the existing cave population. Near the entrance of the cave but already in the dark zone, there is a small pool with tens of fish at most. In multiple trips to this cave, the authors of this paper have observed both surface and troglomorphic fish here, but the surface fish are very thin, show signs of starvation, and it is not uncommon to find dead surface specimens. Troglomorphic fish on the contrary appear healthy. Deeper into the cave, three more

pools are found that are inhabited by hundreds of fish each. The pools harbor a higher ratio of troglomorphic fish the deeper they are located within the cave. The population in Río Subterráneo Cave graphically illustrates the selective advantage of troglomorphic fish over surface fish within the cave environment. A detailed description of the cave and its fish population can be found in Wilkens and Burns (1972) and Mitchell et al. (1977).

When originally studying the *Astyanax* cavefish, Horst Wilkens collected 15 fish from Río Subterráneo Cave in 1969, presumably of the most troglomorphic type and from the deepest pools, and bred the captured fish for tens of generations over a span of about 45 years (Gross and Wilkens 2013). He also captured individuals from Pachón Cave in Sierra de El Abra and stored them in the same laboratory, but kept the fish from both populations isolated in separate tanks to avoid contamination and accidental crossbreeding. While the Pachón cavefish exhibit true albinism, Río Subterráneo Cavefish are highly variable in coloration, but even the palest fish always have at least some traces of melanin pigment in their body in the wild (**Figure 2**) and thus do not express an albino phenotype in the wild (Gross and Wilkens 2013).



**Figure 2.** Two wild Río Subterráneo cavefish demonstrate phenotypic variation in pigmentation. Photos were taken in the field. Extreme depigmentation can be caused by reasons other than albinism. Note that although one individual is highly reduced in body pigmentation, neither is a true albino and traces of melanin pigment in the caudal spot can be seen in both specimens.

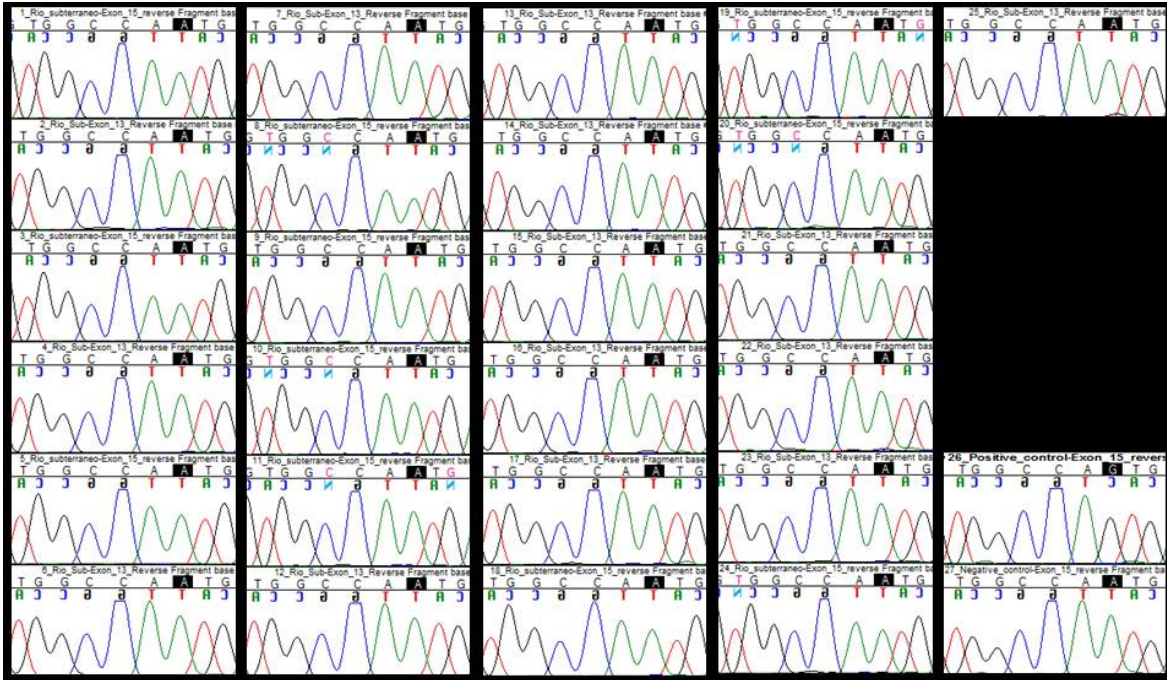
The initial offspring's phenotypes of the Río Subterráneo fish kept in Wilkens' laboratory were consistently non-albino. Yet, spontaneously, a few albino fish appeared within the laboratory stock after about seven generations. Using DNA analysis, coloration studies and real-time PCR analyses, Gross and Wilkens (2013) surprisingly found the same genetic lesion in these captive Río Subterráneo albino fish as the one first identified in wild caught contemporary Pachón cavefish. They suggested that some of the original 15 individuals collected in the field must have been heterozygous carriers of the albino allele and that the bottleneck effect of captive breeding allowed the phenotype to become evident. They also proposed gene flow between the Pachón and Río Subterráneo cavefish populations via surface dwelling *Astyanax mexicanus* to explain the presence of the exact same allele, despite considerable geographic barriers and a distance of about 64 km between the populations. Gross and Wilkens (2013) did not survey extant individuals collected directly in the wild from Río Subterráneo Cave.

To test whether the *Oca2* allele, first identified in Pachón cavefish and then in captive-bred Río Subterráneo cavefish, is found in the contemporary wild Río Subterráneo fish population, DNA from fish directly caught from the wild during March 2013 were obtained from the Río Subterráneo cave population. We specifically tested whether any specimen field collected from the contemporary population at Río Subterráneo had the same single nucleotide polymorphism (guanine instead of adenine) within exon 13 at position 1252 of the *Oca2* gene as the laboratory stock albino Río Subterráneo cavefish and the wild Pachón cavefish.

DNA from the fin clippings of 25 wild caught Río Subterráneo cavefish, one Pachón cavefish and one surface *Astyanax* fish was extracted using the DNeasy Kit by QIAGEN®. The Río Subterráneo specimens came from the deepest pool, where the most troglomorphic fish can be found in the wild, and were released alive after the fin clipping. Pachón and surface specimens came from Dr. William Jeffery's captive breed stock at the University of Maryland, which have the same allele as contemporary wild caught individuals from those localities. A polymerase chain reaction (PCR) amplification of a 66 bp *Oca2* gene fragment was performed using the primers 5'-GCGTATCAGGTGTCCAGAGG-3' and 5'-AGAGCATCATGGTGGTCACA-3' with an annealing temperature of 55 °C. PCR amplicons were purified using the QIAquick PCR Purification Kit by QIAGEN® and sent to SeqWright DNA Technology Services® for sequencing. Using the computer program *Sequencher 5.1*, chromatograms were visualized for analysis.

All 25 wild fish collected from the Río Subterráneo Cave were homozygous for adenine (A) at base position 1252 (**Figure 3**). No specimen had a double peak, characteristic of heterozygous genotypes. Chromatograms also showed that the Pachón cavefish was homozygous for guanine (G) at base position 1252 and that the *Astyanax* surface fish was homozygous for adenine (**Figure 3**).





**Figure 3.** *Oca2* chromatograms of field collected Río Subterráneo cavefish (samples 1 to 25). Pachón cavefish (sample 26 “positive control”) and surface *Astyanax* (sample 27 “negative control”) are shown at the bottom left corner. Exon 13 at position 1252 is highlighted in black. Note that none of the Río Subterráneo cavefish has either a single black peak (G) or a double green/black peak (A/G) indicating the presence of the guanine found in the Pachón allele. In sum, none of the contemporary wild Río Subterráneo specimens had the allele found in the Pachón cave population or in the captive breed Río Subterráneo fish.

Gross and Wilkens’ (2013) study suggested that the Pachón albino *Oca2* allele was present within the gene pool of the Río Subterráneo cavefish population. They remarked, “this raises the intriguing possibility that the *Oca2* allele is not rare in the darkness of the cave environment. Perhaps a loss-of-function *Oca2* allele harbors a ‘cryptic’ selective value for cave-dwelling fish. If this is the case, then the assumption that this allele is rare and deleterious may need to be revised. Future studies that include additional sampling from natural Micos cavefish would address this point by providing a clearer picture of the frequency of loss-of-function *Oca2* alleles in the natural *Astyanax* populations.”

Our results, based on 25 wild caught specimens, show that if the allele is present in the contemporary population, it must be rare. All 25 DNA sequences from wild Río Subterráneo cavefish that we collected were homozygous for adenine at base position 1252. Therefore, these Río Subterráneo cavefish all appear to have the same *Oca2* allele as the surface pigmented *Astyanax mexicanus* fish. Albino Pachón cavefish have guanine at base position 1252. This single nucleotide polymorphism was not found in any of the 25 wild Río Subterráneo cavefish *Oca2* gene copies. Since each individual

carries two *Oca2* alleles, 50 alleles were sequenced in total. If the Pachón albinism allele is present within the contemporary Río Subterráneo cave fish population, it likely occurs at a frequency of less than one out of fifty, i.e. less than 2%.

Based on the results of this study, several possibilities could explain the absence of the Pachón albino *Oca2* allele in contemporary Río Subterráneo cavefish. First, the allele may be present, but with a frequency below the level of detection in our study. A second alternative is that both phenotypic and genotypic frequencies are known to fluctuate over time. Indeed, classical observations by Breder (1943) noted that the ratio of pigmented and eyed fish in Chica Cave (formerly named *Anoptichthys jordani*) increased dramatically in just a six-year period. The presence of the loss-of-function *Oca2* allele in the natural cave population roughly 45 years ago, and its absence from the individuals sampled in this study, may indicate the allele has vanished from the Río Subterráneo population. This cave population is believed to hybridize with surrounding surface fish populations (Mitchell et al. 1977) due to seasonal flooding that washes surface-dwelling fish into the cave. This frequent introgression of surface *Oca2* alleles into the cave population, over the past 45 years, may explain (at least in part) these observations.

When discussing the reason for a shared albino allele in Pachón and Río Subterráneo cave populations, Gross and Wilkens (2013) mentioned that in light of the geographical limitations, it is likely that the shared loss-of-function *Oca2* allele was part of the standing genetic variation in the ancestral epigean fish that subsequently became present in both cave populations when the independent colonizations occurred. Perhaps albinism does not confer a selective advantage, and therefore is evolving neutrally, consistent with the interpretation that reduced melanophore numbers may evolve through neutral forces (Protas et al. 2007). If the founding surface population that colonized Río Subterráneo had a low loss-of-function *Oca2* allele frequency and if there is no selective advantage for albinism in Río Subterráneo, then we might expect a low frequency (or total absence) of the allele in this cave population, consistent with our findings. If this is true, DNA from a larger collection of Río Subterráneo cavefish may be necessary to detect the loss-of-function Pachón *Oca2* allele within the population.

A final explanation is that some error in the husbandry of the fish occurred in the laboratory. If a clerical mistake was made, a Pachón cavefish could have ended up in a Río Subterráneo tank and hybridized, resulting in the introduction of the Pachón *Oca2* allele into the captive-bred Río Subterráneo fish population. Another possibility is that eggs or sperm got accidentally transferred via a shared water system or in wet equipment such as nets. We consider these explanations less likely. The reason being that Horst Wilkens is recognized as a very thorough and cautious scientist whose laboratory is among the best managed in the field of *Astyanax*. As mentioned before, fish were kept isolated in separate tanks to avoid contamination and accidental crossbreeding. Furthermore, Pachón and Río Subterráneo fish are clearly morphologically different and would be easily detected if in the wrong tank. Finally,

regarding gamete transfer between fish tanks with *Astyanax*, it has never been reported despite many laboratories across the world having similar installations and continuously depending on genetic isolation for their studies.

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