

ORIGINAL ARTICLE

Albinism in phylogenetically and geographically distinct populations of *Astyanax* cavefish arises through the same loss-of-function *Oca2* allele

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The Mexican tetra, *Astyanax mexicanus*, comprises 29 populations of cave-adapted fish distributed across a vast karst region in northeastern Mexico. These populations have a complex evolutionary history, having descended from ‘old’ and ‘young’ ancestral surface-dwelling stocks that invaded the region ~6.7 and ~2.8 MYa, respectively. This study investigates a set of captive, pigmented *Astyanax* cavefish collected from the Micos cave locality in 1970, in which albinism appeared over the past two decades. We combined novel coloration analyses, coding sequence comparisons and mRNA expression level studies to investigate the origin of albinism in captive-bred Micos cavefish. We discovered that albino Micos cavefish harbor two copies of a loss-of-function *ocular and cutaneous albinism type II (Oca2)* allele previously identified in the geographically distant Pachón cave population. This result suggests that phylogenetically young Micos cavefish and phylogenetically old Pachón cave fish inherited this *Oca2* allele from the ancestral surface-dwelling taxon. This likely resulted from the presence of the loss-of-function *Oca2* haplotype in the ‘young’ ancestral surface-dwelling stock that colonized the Micos cave and also introgressed into the ancient Pachón cave population. The appearance of albinism in captive Micos cavefish, caused by the same loss-of-function allele present in Pachón cavefish, implies that geographically and phylogenetically distinct cave populations can evolve the same troglomorphic phenotype from standing genetic variation present in the ancestral taxon.

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INTRODUCTION

Regressive phenotypes represent those traits that are reduced or lost in a derived lineage. Several hypotheses seek to explain the evolution of regressive traits through various mechanisms such as direct selection (Fong *et al.*, 1995), neutral forces (Wilkens, 1988) or indirectly through pleiotropy (Jeffery, 2005). Past studies have offered mixed support for each of these hypotheses. However, the mechanism that explains the evolution of these traits, and whether one mechanism governs all regressive phenotypes, remains unclear (Culver and Wilkens, 2000; Borowsky and Wilkens, 2002; Jeffery, 2009).

The freshwater fish, *Astyanax mexicanus*, is a powerful model system for understanding regressive phenotypic evolution. This species includes both surface- and cave-dwelling fish (Alvarez, 1946; Schlagel and Breder, 1947; Wilkens, 1988, 2010; Jeffery, 2001). These fish can be bred to produce viable offspring, enabling classical genetic and quantitative trait loci (QTL) studies (Şadoğlu, 1957; Wilkens, 1971; Protas *et al.*, 2006). La Cueva Chica, one of the southern El Abra caves, was the first cave locality in which *Astyanax* cavefish were discovered (Hubbs and Innes, 1936); however, over the past several decades many additional cave-dwelling populations have been discovered (Mitchell *et al.*, 1977). All of the known cave populations are located in northeastern Mexico and are distributed across three principal geographical regions: the eastern Sierra de El Abra populations, the northern Sierra de Guatemala populations and the western

Sierra de Colmena populations (Bradic *et al.*, 2012). Many of these populations have converged on the same troglomorphic phenotypes, such as eye loss and pigmentation regression (Wilkens and Strecker, 2003).

The consensus view is that two ancestral ‘stocks’ of epigeal fish invaded from the south and colonized these limestone caves (reviewed in Gross, 2012). The older stock seeded the caves of the El Abra region, including the Pachón cave, ~6.7 MYa, and the younger stock (which is related to the local, extant epigeal population) seeded the northern Guatemala caves and the western Colmena caves ~2.8 MYa (Dowling *et al.*, 2002; Strecker *et al.*, 2004; Ornelas-García *et al.*, 2008; Hausdorf *et al.*, 2011; Bradic *et al.*, 2012; Strecker *et al.*, 2012).

In this study, we sought to determine the genetic origin for albinism that appeared spontaneously in a captive stock of Micos cavefish from the Colmena region. Micos is the most geographically isolated population of *Astyanax* cavefish (Wilkens and Burns, 1972; Peters *et al.*, 1975). This locality includes three cave entrances to an underground river (Río Subterráneo) that are ~35 km west of the southern El Abra cave network, separated by the Sierra de Nicolás Perez and Sierra de Colmena mountain ranges (Mitchell *et al.*, 1977). This cave population was discovered in the late 1960s. Shortly thereafter, a set of pigmented Micos cavefish were collected and bred for several generations over the past 40+ years. The initial stock of individuals was uniformly pigmented, developing the same number of

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melanophores under daylight as surface-dwelling fish (Wilkins, 1988). However, unpigmented (albino) individuals spontaneously arose within this laboratory stock after approximately six or seven generations.

Albinism in *Astyanax* is a recessive trait (Şadoğlu, 1955, 1957; Şadoğlu and McKee, 1969) that was mapped to the *ocular and cutaneous albinism type II (Oca2)* locus (Protas *et al.*, 2006). This gene encodes a 12-transmembrane domain protein channel that is believed to mediate small-molecule transport within pigment-producing cells (Lee *et al.*, 1995). Pachón cavefish represent one of four *Astyanax* cave populations, along with Molino, Yerbániz and Japonés cavefish populations, which express albinism in the wild (Protas *et al.*, 2006; Wilkins, 1988; Wilkins and Strecker, 2003). Interestingly, different coding sequence alterations affecting *Oca2* are present between the Pachón and Molino populations. Both coding sequence mutations, however, completely eliminate *Oca2* protein function based on functional expression assays using a melan-*P* cell line assay (Protas *et al.*, 2006). The other two cave localities, Yerbániz and Japonés, harbor presumed *cis*-regulatory alterations affecting transcriptional abundance of *Oca2*. This is based on complementation analyses between albino Pachón cavefish and Japonés cavefish in which all offspring displayed the albino phenotype (Protas *et al.*, 2006).

In this report, we analyzed albino Micos individuals and determined that they express no melanin and did not differ in this regard from individuals derived from the Pachón cave. We found that albinism in these fish is not caused by *cis*-regulatory mutations affecting expression levels of the albinism gene, *Oca2*. Rather, the same coding sequence alterations first characterized in Pachón cavefish were also present in albino Micos cavefish. This implies that the *Oca2* haplotype discovered in Pachón cavefish was part of the standing genetic variation in the epigeic stock that seeded the phylogenetically young Micos cave population and introgressed into the phylogenetically old Pachón cave population.

MATERIALS AND METHODS

Fish collection and husbandry

A sample of 15 pigmented fish was originally collected in the Micos cave (named 'La Cueva del Río Subterráneo' in Mitchell *et al.*, 1977) in 1970. Live fish were transported and maintained at the Zoological Museum fish facility at the University of Hamburg in Germany. To avoid contamination with other populations reared in the same laboratory, all fish were maintained in separate rearing tanks, unconnected from one another, and observed daily throughout all periods of husbandry and breeding. Fish were maintained under a Middle European light:dark photic schedule at 24 °C and fed once daily. Adults were fed tetra flake food, supplemented periodically with live brine shrimp (*Artemia*) and water fleas (*Daphnia*). Larvae were fed brine shrimp hatchlings. All Micos fish analyzed in this study were descended from the original wild-caught stock that has been maintained at the Wilkins laboratory since the early 1970s.

Fish breeding

Since the original collection, the stock of Micos fish was bred roughly once every 4 years. Although *Astyanax* cavefish are sexually mature by ~1 year, these captive Micos cavefish were bred less frequently only in order to maintain the stocks. Fish were maintained as a group and bred together within a single tank since they were brought into the lab. All fish were bred naturally through group matings.

Albino Micos individuals first appeared within a single generation in the 1990s. As these fish were maintained in captivity since the early 1970s, breeding at a rate of roughly once every 4 years, the albino offspring first appeared after approximately six or seven generations in the lab. The albino fish used in this study were derived from a group breeding of four female and five male specimens. Two separate breedings were carried out using these nine adult fish.

The first breeding resulted in ~1000 offspring, of which 8 were albino. The second breeding resulted in roughly the same number of offspring, of which three or four were albino. All fish used in this study have been reared and maintained under identical lighting conditions and the same 100-liter glass tanks.

Coloration analysis

We developed a novel technique to provide quantitative measures of coloration differences between specimens. This technique involved isolation of protein extract from fin clips on to a filter, high-resolution microscopic analysis of the filters and digital assessment of filter color using the Adobe Photoshop software (San Jose, CA, USA; Supplementary Figure S1). We also utilized a parallel measure of melanic darkness on each filter using ImageJ software (NIH, Bethesda, MD, USA), similar to that used in Gross *et al.* (2009).

The dorsal portion of the caudal fin was excised from live, anesthetized fish. Each fin clip was digested and processed from each specimen in 180 µl Buffer ATL (each supplemented with 20 µl proteinase K solution) using the DNeasy genomic DNA extraction kit (Qiagen, Valencia, CA, USA). Tail fin clips were collected from each of seven different individuals from the following five groups: surface fish, Pachón cavefish, surface × Pachón cavefish F₁ hybrids, pigmented Micos cavefish, and albino Micos cavefish. All procedures involving live animals were approved by the University of Cincinnati Institutional Animal Care and Use Committee.

Following the final spin filtration step and drying, the central filter membrane was carefully isolated from each QiaSpin column following extraction and imaged at ×13.7 magnification (Supplementary Figure S1A). This kit can yield variable levels of genomic DNA from the same starting material, therefore we standardized several parameters before microscopy and imaging (see Supplementary Figure S1).

The membrane-bound filtrate was comprised of protein extract, including melanin, providing a metric for analyzing coloration in tail fin clips (Supplementary Figure S1). Photomicrographs (Supplementary Figure S1B) were digitally overlaid with a circular grid (Supplementary Figure S1C) of 13 concentric 'dots' distributed evenly around the 360° area of each filter (Supplementary Figure S1D). Each dot was assessed for coloration in each of the four channels of the CMYK mode using the Adobe Photoshop software program (Supplementary Figure S1E). This was accomplished by placing the 'eye dropper' tool over each portion of the circular grid and recording the values (ranging from 0% to 100%) for the pixel set within each dot (Supplementary Figure S1E). Values for each channel were averaged (Supplementary Figure S1F) and compared across all the seven specimens within each group (Supplementary Figures S1G and H).

The amount of melanin pigmentation present on each filter membrane was also quantified using ImageJ analysis (as described in Gross *et al.*, 2009). Accordingly, each image was rendered in grayscale to discard color information and inverted before processing. This provided a quantifiable measure of darkness ranging in value from 0 to 255 (0 = white; 255 = black). Each image was loaded into ImageJ, the filter image was encircled and the mean darkness value was recorded in triplicate and averaged. As above, all mean values were compared across each of the five groups. All statistical analyses were carried out using SPSS v. 18 (Armonk, NY, USA; Supplementary Table S1).

Quantitative PCR analysis

Quantitative PCR analyses were carried out using a Bio-Rad MiniOpticon light cycler (Bio-Rad, Hercules, CA, USA). cDNA template was generated using the Transcriptor RT kit (Roche, Indianapolis, IN, USA). Briefly, total RNA was extracted from the tail fin clips of albino Micos cavefish (*n* = 5) and representative (non-albino) surface fish (*n* = 5) using the RNeasy RNA extraction kit (Qiagen). Isolated RNA (1 µg) was hybridized to Oligo dT primers (Invitrogen; Grand Island, NY, USA) at 65 °C, cooled on ice for 5 min, to which was added 4 µl 5 × RT buffer, 0.5 µl Protector RNase Inhibitor, 2 µl dNTP mixture and 0.5 µl Transcriptor RT for each reaction. The reaction mixture was incubated at 50 °C for 1 h and then inactivated at 85 °C for 5 min. For control experiments, all primers were tested using genomic DNA pools from both albino Micos cavefish and surface fish as templates for positive controls of amplification. Each template was tested in triplicate across two

separate experiments. The cycling threshold ($C(t)$) values were collected using cDNA pools as templates and normalized to the expression of the house-keeping gene *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase; primers: forward = 5'-TGTGTCCGTGGTGGATCTTA-3', reverse = 5'-TGTCGCCAATGAAGTCAGAG-3'). The primers used to assess *Oca2* expression were the following: *Oca2* forward primer: 5'-GGTAATGGGACACTGATTGG-3'; *Oca2* reverse primer: 5'-CCTGAAGAATTCCATGAAAGAGA-3'. These primers amplified a 96-bp fragment in surface and Pachón cavefish cDNA. Cycling parameters were as follows: step 1—95 °C for 30 min, step 2—95 °C for 5 min, step 3—55.1 °C for 10 min, plate read, go to step 2 for 39 additional cycles. Experiments were carried out using the EvaGreen supermix dye (Bio-Rad). All data were collected and analyzed using the Bio-Rad CFX Manager v.1.6 software program (Bio-Rad).

Genotypic and molecular analysis of *Oca2*

We utilized oligo dT-primed cDNA libraries (as above) generated from total RNA pools extracted from tail fin clips. The *Oca2* coding sequence was amplified from albino Micos cavefish using the full-length primers (described in Protas *et al.*, 2006) as well as the overlapping primer sets (*Oca2* tiling primers: set 1 forward 5'-ATGTATTGGAGAACAAGAACAC-3', set 1 reverse 5'-GAAGCTTCAATAAGGCATTATCAC-3', set 2 forward 5'-GCCCTTCATGACCGGAAT-3', set 2 reverse 5'-CACACCTGATGGTAACAGGA-3', set 3 forward 5'-CCACCATGATGCTTCTACT-3', set 3 reverse 5'-TCAGCCAAAA TAAGCAACCA-3', set 4 forward 5'-TGGTTGCTTATTTGGCTGA-3', set 4 reverse 5'-TCGGGCTGGGCTGGAACACCTAA-3'). Overlapping primer sets allowed sequenced tiles to be assembled from multiple PCR runs. PCR

fragments were subcloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) using standard protocols, as described in Gross *et al.* (2009).

DNA preparations were sequenced via the Sanger method, utilizing the universal M13F (forward) and M13R (reverse) sequencing primers (Operon, Huntsville, AL, USA). Raw sequences were tiled together and analyzed as consensus sequences using the LaserGene software suite (DNASTar, Madison, WI, USA; see below). In total, $n=6$ individuals from both pigmented and albino Micos cavefish were assessed for sequencing analyses. For the genomic screen (Figure 4), specific regions of the *Oca2* gene were amplified and sequenced from multiple representative individuals from each group (Figure 4) using the diagnostic primers as described in Protas *et al.* (2006). These included the following: exon 14 (control): forward primer 5'-GCGAGGTGCTGAATCTGG-3', reverse primer 5'-CGCAGGTTTTGGTTGGAC-3'; exon 21: forward primer 5'-TGTGCCAGAGCGGAGCGG-3', reverse primer 5'-ATGGTGGCGGTGAAGGG-3'; exon 23 (control): forward primer 5'-ACGCTTGGAACGAGCTTCAT-3', reverse primer 5'-AAGCCAGTCGGGTGTGTTTC-3'; and exon 24–3'UTR (3' untranslated region): forward primer 5'-GGCTCCCATGATGCTGAT-3', reverse primer 5'-GCGTTCCTTGTTTGAATGT-3'. Control primers were predicted to amplify in every specimen as these regions did not demonstrate alterations in previous analyses (Protas *et al.*, 2006). The A1252G single nucleotide polymorphism (SNP) was amplified from Micos cavefish using the following: forward primer 5'-GCGTATCAGGTGTCCAGAGG-3', reverse primer 5'-AGAGCATCATGGTGGTCACA-3'. PCR fragments were directly sequenced, using the Sanger method, utilizing the reverse primer as the sequencing primer (Operon) in $n=6$ individuals from each group (that is, surface fish, Pachón cavefish, albino Micos cavefish and pigmented Micos cavefish). The LaserGene v.9.1.1 software suite (EditSeq,

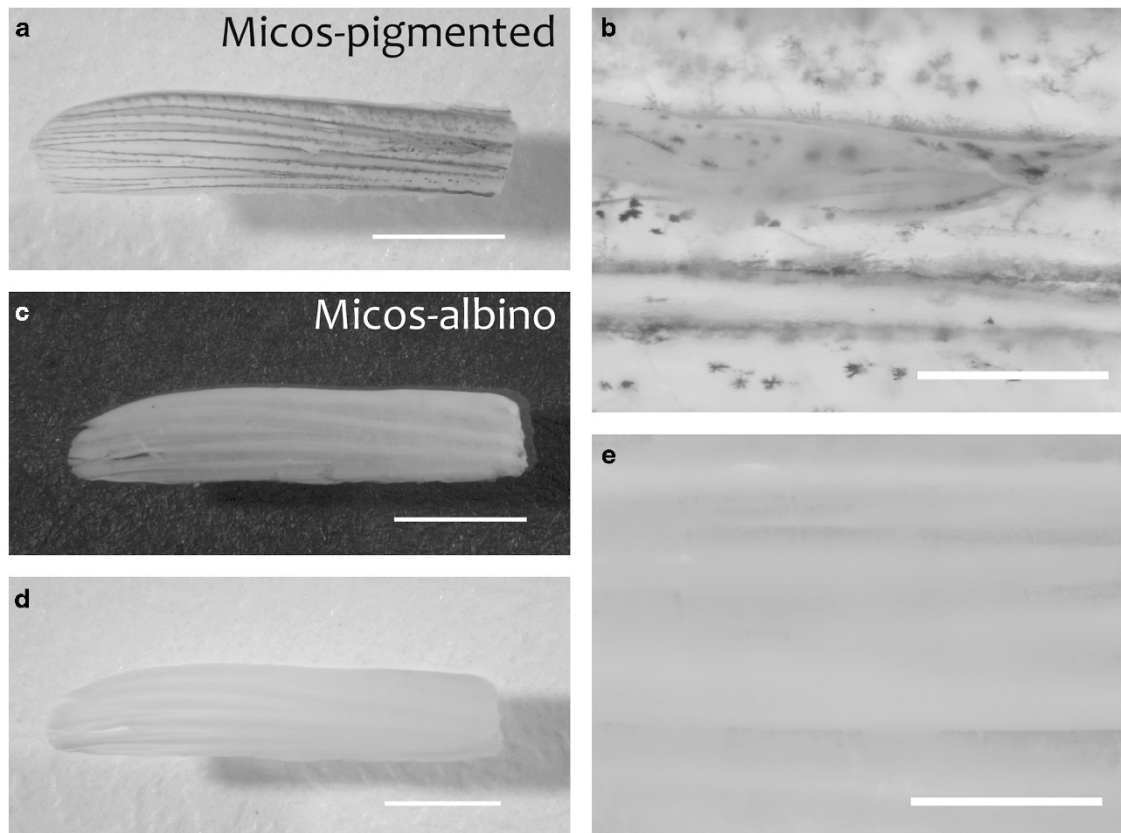


Figure 1 Albino individuals appeared spontaneously within captive stocks of pigmented Micos cavefish. A caudal fin clip derived from a representative pigmented individual demonstrated melanic pigmentation under low (**a**; $\times 16$) and high (**b**; $\times 100$) magnification. Pigment-producing melanophores with a characteristic 'spindly' appearance are present in the pigmented form (**b**). A caudal fin clip derived from a representative unpigmented (albino) individual harbored no eumelanin (**c–e**), as observed against a dark (**c**; $\times 14$) or light (**d**; $\times 14$) background. Higher magnification images demonstrated total absence of pigmented melanophores from any part of the tissue (**e**; $\times 100$). Bars = 2 mm in **a**, **c** and **d**; 50 μ m in **b** and **e**.

SeqMan and MegAlign modules) was used for all sequencing assembly and analysis (DNASTar).

Microscopy and imaging analysis

High-resolution images were collected using a Leica M205 FA stereomicroscope equipped with a DFC310FX camera (Leica, Wetzlar, Germany). Photomicrographs were collected utilizing the MultiFocus module within the Leica Application Suite (LAS) v3.8 software package. Accordingly, multiple focal steps were photographed, aligned and assembled into a single MultiFocus file. All photomicrographs were saved as TIFF files and compiled into composite figures using Adobe Photoshop CS3.

RESULTS

Albino individuals appeared spontaneously within captive stocks of pigmented *Micos cavefish*

Cavefish specimens collected from the Micos cave locality in 1970 demonstrated melanic pigmentation (Figures 1a and b). None of the specimens from the initial collection demonstrated the albino phenotype. Over the course of 40+ years, following numerous matings between groups of individuals derived from the wild stocks, albinism appeared in certain individuals (Figures 1c–e). We

determined that these specimens, rather than demonstrating faint pigmentation, were albino (that is, completely absent melanin). Light microscopic analysis of tail fin tissues failed to reveal any pigmented melanophores (Figures 1c and d). In comparison to pigmented individuals derived from the same stock, no pigment-producing cells were observed in albino *Micos cavefish* (Figure 1e).

A digital analysis of coloration revealed a shared absence of melanic pigmentation in albino *Micos* and *Pachón cavefish*

To further investigate the melanic pigmentation in *Micos cavefish* forms, we performed a digital coloration analysis (Supplementary Figure S1). This analysis was carried out on protein extracts derived from tissues extracted from multiple *Astyanax* specimens. We utilized Qiagen DNeasy spin columns to isolate tissue extracts from multiple groups of fish (for example, surface, *Pachón cavefish*, *Micos cavefish*; Figures 2a–e). Using this technique, we measured the average level of channel representation (C = cyan; M = magenta; Y = yellow; K = key) at 13 discrete points around the filter membrane (see Materials and methods). We discovered that the average coloration profiles for filter membranes produced from surface fish and surface × *Pachón* F₁ fish

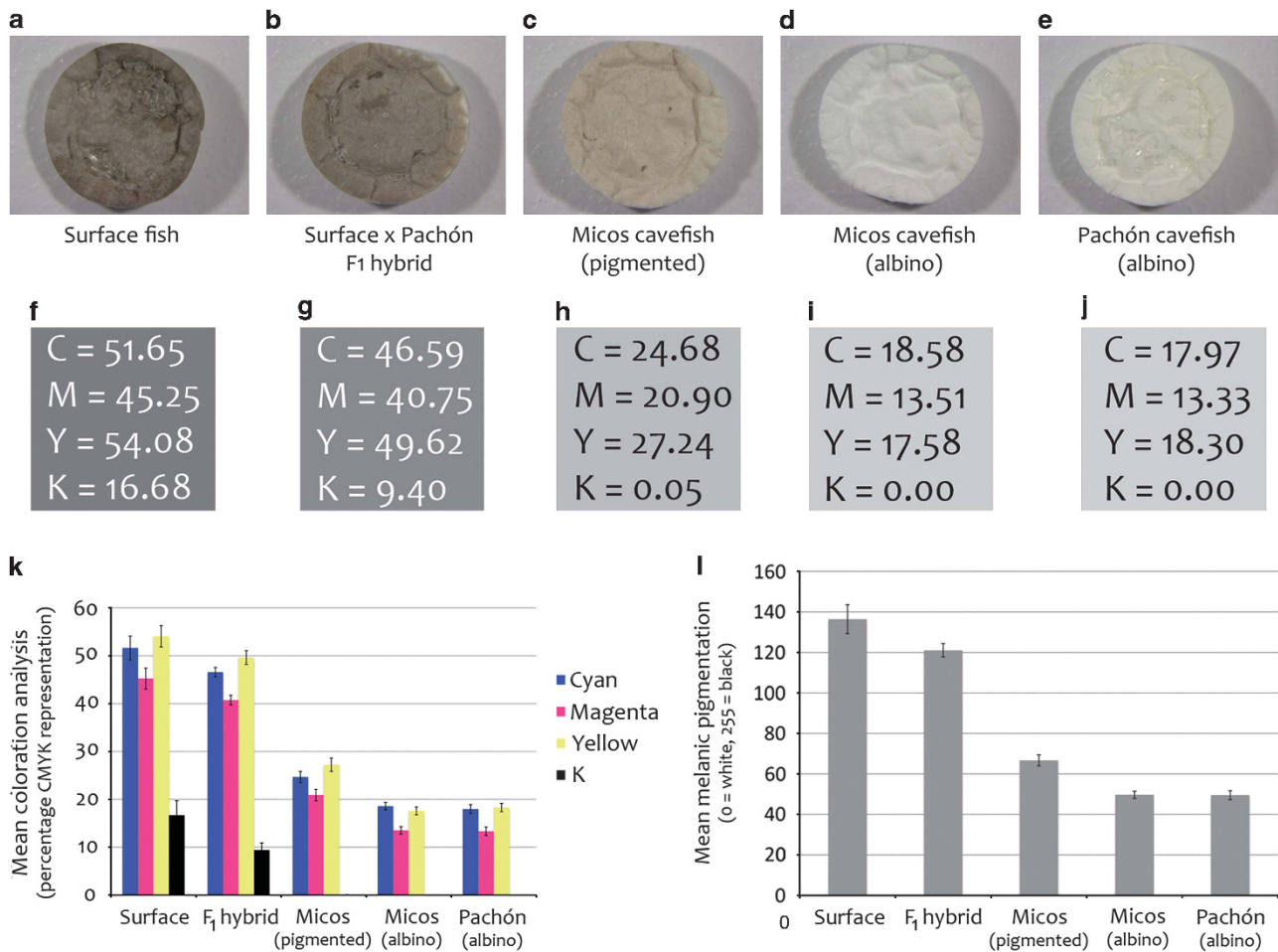


Figure 2 A digital analysis of coloration reveals a shared absence of melanic pigmentation in albino *Micos* and *Pachón cavefish*. A representative photograph of each filter membrane is presented for surface (a), surface × *Pachón* F₁ (b), pigmented *Micos* (c), albino *Micos* (d) and albino *Pachón* (e) morphs. Thirteen separate regions of each filter image were averaged for the four coloration channels in the CMYK mode (see Materials and methods). The average values for each are presented within a palette (f–j) depicting the average coloration for each category. Both albino *Micos* and albino *Pachón cavefish* had a value of 0 for the K (key) channel (i–k). We assessed the darkness of each filter membrane in grayscale (l; see Materials and methods). Albino *Micos* and *Pachón cavefish* specimens demonstrated an absence of melanic pigmentation (l). CMYK values range from 0 to 100 and are expressed as a percentage in panel (k). Grayscale values range from 0 to 255 in panel (l).

were highly similar (Figures 2f, g and k). The profile from pigmented Micos individuals was slightly lower than surface fish (Figures 2h and k). The profiles derived from albino Micos (Figures 2i and k) and albino Pachón cavefish (Figures 2j and k) were nearly identical in the all channels.

To assess for group coloration differences in each channel, we conducted a one-way analysis of variance using group identity (for example, Pachón cavefish) as the grouping variable and the mean score for each color channel as the dependent variable. For each of the cyan, magenta and yellow channels, the omnibus F-test was significant, indicating significant coloration differences between groups (cyan: $F(4,30) = 130.53$, $P < 0.0001$; magenta: $F(4,30) = 133.63$, $P < 0.0001$; yellow: $F(4,30) = 144.88$, $P < 0.0001$; Supplementary Table S1). *Post hoc* pair-wise comparisons using Tukey's Honestly Significant Difference test yielded a pattern of results that was identical across all the three channels, in which albino Micos cavefish and albino Pachón cavefish did not differ from each other (Supplementary Table S1). We were unable to perform an analysis of variance for the keyed (K) channel in our CMYK analysis because two of the groups harbored no variation for this measure. Specifically, all albino Micos cavefish and all albino Pachón cavefish had a value of 0 for this channel, as a consequence of having no melanic pigmentation. As the K channel measures the contribution of white and black to a colored pixel, we measured overall darkness, conferred by melanic pigmentation, of each filter using a grayscale analysis (Figure 2l).

A significant level of melanic pigmentation was observed in surface fish, with a slight decrease in surface \times Pachón F₁ hybrids (Figure 2l). Somewhat lower levels of melanic pigmentation were present in pigmented Micos cavefish derived from the wild-caught stocks (Figure 2l) compared with surface fish. The measure of melanic pigmentation in albino Micos fish, however, was highly similar to albino cavefish derived from the Pachón cave (Figure 2l). The results of a one-way analysis of variance using the same parameters described above indicated significant group-wide differences ($F(4,30) = 109.48$, $P < 0.0001$; Supplementary Table S1). A *post hoc* pair-wise comparison using Tukey's Honestly Significant Difference test yielded the same results as our coloration analysis, indicating that albino Micos cavefish and albino Pachón cavefish did not differ from each other (Supplementary Table S1). Taken together, these results supported the qualitative observation (Figure 1) that there is no difference between albino Pachón cavefish and albino Micos cavefish with respect to coloration and darkness measurements. In summary, gross phenotypic assessment, high-resolution microscopy and comparative digital analyses of darkness/coloration indicated that depigmented Micos cavefish were formally albino.

Quantitative PCR analysis demonstrated significant levels of *Oca2* expression in albino Micos cavefish

Previous genomic analyses revealed that the gene leading to albinism in *Astyanax cavefish* is *Oca2* (Protas *et al.*, 2006). These studies investigated the genetic basis of albinism in three caves: the Pachón cave, the Molino cave and the Japonés cave. Although coding sequence mutations were present in the Pachón and Molino caves, presumed *cis*-regulatory alterations affecting *Oca2* explained the albino phenotype in Japonés fish (Protas *et al.*, 2006). Therefore, we assessed the expression level of this gene in albino Micos cavefish compared with surface form fish, which did not express the albino phenotype (Figure 3). We reasoned that a lower level of *Oca2* expression in albino Micos cave forms might explain the inability to produce eumelanin in these fish. Interestingly, rather than expressing a reduced level of transcripts compared with surface fish,

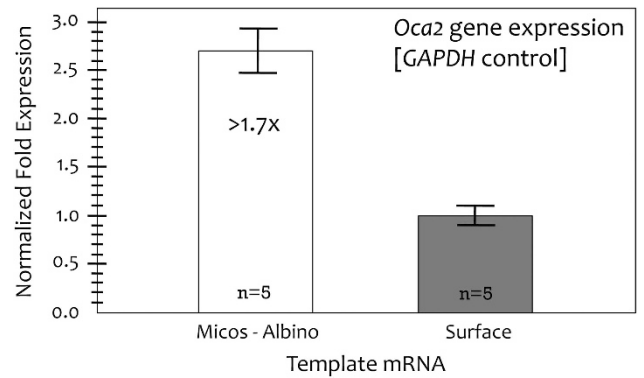


Figure 3 Quantitative PCR analysis demonstrates normal levels of *Oca2* expression in albino Micos cavefish. We compared expression levels of *Oca2* in cDNA pools derived from albino Micos cavefish ($n = 5$) and surface fish ($n = 5$) tail fin clips. Expression levels were assessed using real-time PCR analysis (see Materials and methods) and normalized to the housekeeping gene *GAPDH*. We observed moderately higher levels of expression ($> 1.7 \times$ fold difference) of this gene in albino Micos cavefish compared with surface fish.

the Micos cave forms expressed a slightly higher level of *Oca2* (Figure 3). Given this increased expression level, we next investigated the coding sequence of *Oca2* to determine whether coding sequence alterations may be present within the open reading frame of the transcript.

Coding sequence analyses demonstrated that the same exon deletion present in albino Pachón cavefish was also present in albino Micos cavefish

To determine whether the *Oca2* allele present in albino Micos cavefish represented a previously discovered coding deletion, we performed a PCR-based analysis of the two coding mutations identified from the Molino and Pachón cave populations. Protas *et al.* (2006) identified two different non-functional allelic variants of *Oca2* in the Pachón and Molino cavefish populations and an intact, functional allele in surface-dwelling *Astyanax* populations. The Molino population harbored a deletion of exon 21. The presence of exon 21 was determined by the amplification of a 102-bp fragment (Protas *et al.*, 2006). We confirmed that this exon was absent from genomic DNA derived from representative Molino cave individuals based on the absence of this 102-bp amplicon (amplicon 'B', Figure 4). This exon, however, was present in both the albino and pigmented Micos cavefish (Figure 4). The second *Oca2* lesion evaluated was the Pachón allele that represents a deletion in a portion of exon 24 through the 3' UTR. Presence of exon 24–3'UTR was determined by the successful amplification of a 235-bp fragment (Protas *et al.*, 2006). We discovered that this fragment did not amplify in albino Micos cavefish (amplicon 'D', Figure 4), suggesting that the Pachón deletion was also present in the albino Micos cavefish. This coding sequence alteration, when translated, leads to an altered *Oca2* protein that is truncated at the C-terminus, missing 19 amino-acid residues compared with the functional protein expressed by surface-dwelling fish (Protas *et al.*, 2006). In a cell-based assay, this allelic variant caused the inability to produce melanin when transfected into a melan-*P* cell line (Protas *et al.*, 2006). In contrast to albino Micos cavefish, the exon 24–3'UTR deletion was not observed in pigmented Micos forms (Figure 4). As expected, the intact form of the *Oca2* allele—present in the surface-dwelling fish—harbored expected amplicons for both the exons (Figure 4).

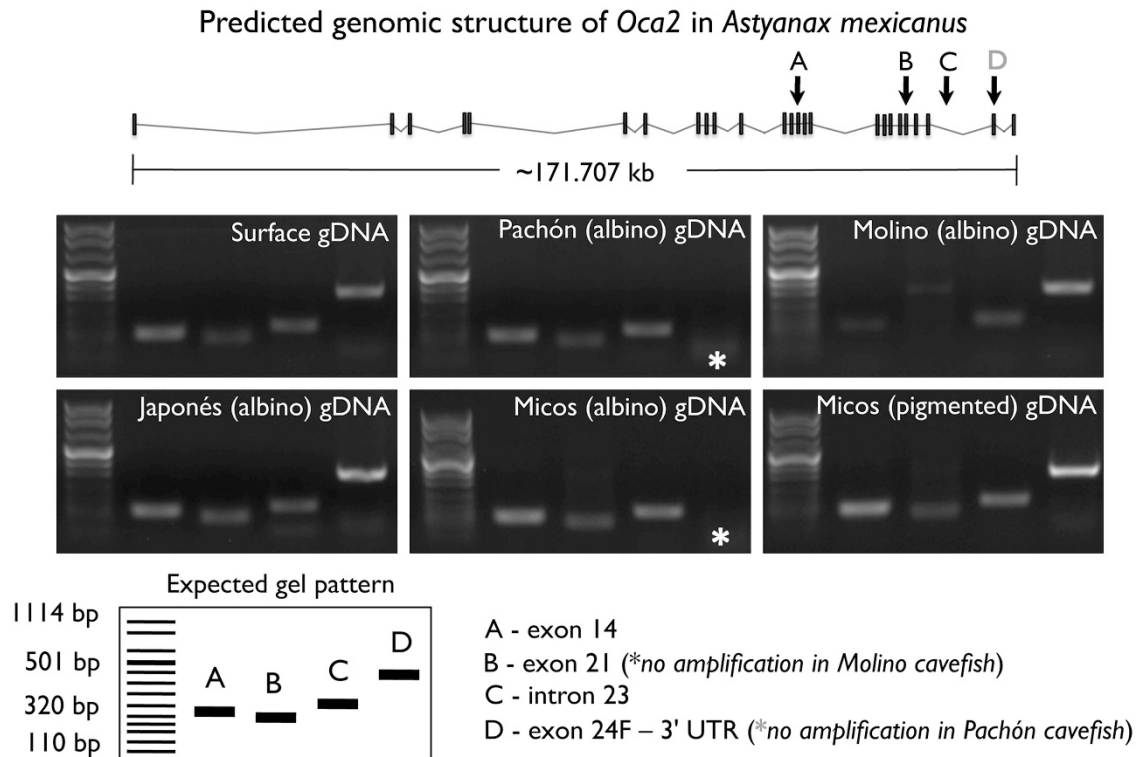


Figure 4 Coding sequence analyses demonstrate that the same exon deletion present in albino Pachón cavefish is found in albino Micos cavefish. The genomic structure of *Oca2*, based on the physical genome of *Danio rerio*, predicts a multi-exon gene >170 kb in length. The Pachón cave population harbors a deletion of exon 24 through a portion of the 3'UTR (gray 'D'). The Molino cave population harbors a deletion of exon 21 ('B'). We utilized additional primer sets (primer set A—exon 14; set B—exon 21; set C—intron 23; set D—exon 24–3'UTR) to control for amplification artifacts. Primer set D, which should yield a ~300-bp fragment, failed to amplify in both Pachón and albino Micos individuals (white asterisks). Each primer set amplified a robust band in representative pigmented Micos cavefish, surface fish and Japonés cavefish. Fragment size was scored using the Roche VIII ladder (110–1114 bp). A schematic representation of the expected gel pattern is depicted in the lower left.

Genotypic analyses suggested that pigmented Micos cavefish harbor at least one intact, functional copy of *Oca2*

Among the nonsynonymous coding sequence changes discovered in the Pachón *Oca2* allele was a SNP at nucleotide position 1252 within exon 13 (Supplementary Figure S2A; Protas *et al.*, 2006). This point mutation leads to the substitution of a valine residue for a methionine residue at amino-acid position number 418. This position is highly conserved across vertebrates. However, unlike the exon deletion in Pachón cavefish, previous studies indicated that the A1252G SNP does not impair *Oca2* protein function, based on functional analysis of this allele in a melan-*P* cell culture assay (Protas *et al.*, 2006).

We sequenced exon 13 from surface (Supplementary Figure S2B), Pachón (Supplementary Figure S2C), albino Micos (Supplementary Figure S2D) and pigmented Micos (Supplementary Figures S2E and F) cavefish. We discovered that the same mutation (1252G) was present in albino Micos cavefish as well as in albino Pachón cavefish, strictly in the homozygous condition (Supplementary Figures S2C and D). Micos cavefish that harbor pigmentation (Supplementary Figures S2E and F) were either homozygous (1252A) or heterozygous (1252A/G) at this locus. Assuming that the exon 24 deletion and the A1252G SNP are present on the same allele (as reported by Protas *et al.*, 2006), this suggests that the 1252G variant is associated with albinism. This association, consistent with the findings of Protas *et al.* (2006), implies that one copy of an intact *Oca2* allele is critical for the expression of melanic pigmentation, explaining the presence of pigmented Micos cavefish that are heterozygous at this locus (1252A/G).

Based on the genomic structure of *Oca2* in *Danio rerio* (Figure 3), we estimate the physical distance between the A1252 locus (within exon 13) and exon 24 is ~44.1 kb. The gametic phase of these two loci in the Micos parental fish that gave rise to albino offspring is unknown, because these studies were carried out through a series of group breedings involving a small number of individuals (see Materials and methods). However, we presume 1252G and the exon 24–3'UTR deletion are in the *cis* configuration and on the same chromosome, as was originally described for the *Oca2* allele identified in Pachón cavefish (Protas *et al.*, 2006). As albinism in *Astyanax* is a recessive Mendelian trait (Şadoğlu, 1955), albino Micos cavefish likely appeared in our captive stocks through random matings between (heterozygous) pigmented Micos cavefish that harbored one intact, functional allele and one loss-of-function *Oca2* allele.

DISCUSSION

Astyanax cave populations were colonized by two waves of ancestral surface-dwelling fish (Bradic *et al.*, 2012). Caves of the El Abra region, including Pachón, were colonized by the first (older) wave of ancestral surface fish (Strecker *et al.*, 2004). Caves of the western Micos (that is, Colmena) region and northern Guatemala region were colonized by a more recent wave of surface-dwelling fish (Hausdorf *et al.*, 2011; Bradic *et al.*, 2012). Our discovery of the same *Oca2* allele in Micos and Pachón cavefish implies that this allele was inherited from standing genetic variation present in the epigeal fish populations.

This finding is supported, in part, by early population genetic studies that clustered Pachón cavefish with the geographically distant

Micos populations, based on mtDNA evidence (Dowling *et al.*, 2002). This result was at odds with geographical evidence (Mitchell *et al.*, 1977) and subsequent microsatellite analyses (Strecker *et al.*, 2003; Hausdorf *et al.*, 2011) that grouped Pachón with the older El Abra populations. This discordance has been explained as the consequence of introgressive hybridization between Pachón cavefish and surrounding epigeal populations, resulting in 'mitochondrial capture' of the epigeal mtDNA by this cave population (Hausdorf *et al.*, 2011; Strecker *et al.*, 2012). These data, along with the findings we present here, imply historical and/or recent gene flow between these geographically distinct populations (Bradic *et al.*, 2012) that has resulted in the presence of the same loss-of-function *Oca2* allele in two geographically distant populations.

This is surprising, in part, because until now genetic analyses have suggested that different lesions in the same gene govern the convergent evolution of pigmentation phenotypes in *Astyanax*. For instance, two separate pigmentation QTL studies identified alterations to *Oca2* leading to albinism (Protas *et al.*, 2006) and to *Mc1r* leading to the brown phenotype (Gross *et al.*, 2009). Neither of these studies, however, found cave alleles associated with reduced pigmentation present in the epigeal populations.

Further, past complementary studies demonstrated that different genes account for eye loss in different cave populations (Wilkens and Strecker, 2003). For instance, Wilkens (1971) reported that crosses between the eyeless Pachón and Sabinos cave populations produced offspring with larger and more differentiated eyes than the parents. More recently, Borowsky (2008) demonstrated that hybrid offspring bred from Pachón and Tinaja cavefish harbored a functional visual system that was not present in either parental stock. In both studies, however, the populations being investigated were drawn from the same geographical region (Sierra de El Abra) that was colonized by the same (older) ancestral stock (Bradic *et al.*, 2012). By contrast, we report here that the same phenotype in two phylogenetically and geographically distinct cave populations is mediated by the same loss-of-function allele.

The appearance of albinism in Micos cavefish

Different cave-associated phenotypes may evolve at different rates in nature. For instance, Culver *et al.* (1995) argued that eye size reduction evolved more quickly than antennae size in the cave-adapted crustacean, *Gammarus minus*. This may be a consequence of antennae size evolving under selective forces, whereas eye size reduction evolved through the additive effects of both selective and neutral forces (Culver *et al.*, 1995).

In *Astyanax*, eye size reduction may have evolved as an indirect (pleiotropic) consequence of natural selection for constructive traits, such as increased lateral line sensitivity (Jeffery, 2005; Porter *et al.*, 2007). However, some evidence suggests eyes and pigmentation regression operate through different evolutionary mechanisms. A polarity analysis revealed that cave alleles at every lens and eye QTL studied within the context of an F₂ pedigree were associated with smaller sizes (Protas *et al.*, 2007). By contrast, QTL polarities for pigmentation (melanophore numbers) demonstrated mixed results. The authors concluded that while eye regression evolved under negative selection in *Astyanax*, melanophore number evolved through neutral forces (genetic drift) or pleiotropy (Protas *et al.*, 2007). Future studies will clarify if younger *Astyanax* populations evolved all regressive phenotypes (for example, eye loss) through standing genetic variation inherited from the ancestral surface taxon or whether this only applies to albinism.

An interesting finding from our study is that while the Micos cave population harbors a loss-of-function *Oca2* allele, this population of

cavefish does not express albinism in the wild. This may imply that pigmentation is not under selection in Micos. Rather, albinism may not confer a selective advantage, and therefore be evolving neutrally, consistent with the interpretation that reduced melanophore numbers may evolve through neutral forces (Protas *et al.*, 2007).

Standing genetic variation versus *de novo* mutations

Albinism in a number of natural and captive animal systems arises through *de novo* genetic mutations (Brown and Norris, 1956; Frankham *et al.*, 1986; Laikre, 1999; Clark, 2002; McPhee, 2004; Pelletier *et al.*, 2009). Indeed *de novo* mutations could, in principle, explain the presence of albinism in Micos cavefish; however, we believe this is unlikely. A *de novo* origin of albinism through *Oca2* would require the identical exon 24 deletion and A1252G SNP to evolve independently in each *Astyanax* cavefish population. This is assuming that both the exon deletion and the A1252G SNP are present on the same chromosome in all individuals in each of the cave (Micos and Pachón) and surface populations.

It is also possible that the loss-of-function *Oca2* allele discovered in Pachón cavefish evolved from the older ancestral invasion and was subsequently passed to the Micos cave through direct contact between populations. We regard this scenario as less likely due to significant geographical barriers between the Pachón and Micos cave localities. For example, the shortest linear (map) distance between the Micos cave entrance (at Río Subterráneo) and the Pachón cave entrance is ~64 km (Mitchell *et al.*, 1977); however, the actual distance is much longer as fish would be limited by available surface streams.

Further, the Pachón cavefish reside in 'perched' pools that are isolated from surrounding rivers and streams (Mitchell *et al.*, 1977). The Pachón cave entrance (~210 m above sea level) is located at the western edge of the Sierra de El Abra region, while the Micos entrance (230 m above sea level) is located three valleys and two mountain ranges to the west (Mitchell *et al.*, 1977). It is unknown if an underground passage unites the two cave populations; however, the subterranean Pachón cave system is believed to be highly complex and maze-like (Bradic *et al.*, 2012). Additionally, hydrogeological evidence demonstrates that water in the Pachón cave collects to a subsurface drainage and discharges into the Río Choy (Mitchell *et al.*, 1977), on the eastern side of the southern El Abra range—over 40 km from the Micos cave entrance.

In light of these geographical limitations, it is more likely that the loss-of-function *Oca2* allele discovered in Pachón was part of the standing genetic variation in ancestral epigeal fish that subsequently became present in both the cave populations. Investigations in stickleback fish have demonstrated that recurrent evolution of lateral plate armor loss in freshwater populations is mediated by selection for ancient *Eda* alleles present at low frequency in the marine population (Colosimo *et al.*, 2005). Interestingly, Bradic *et al.* (2012) recently argued that genetic variation in cavefish is most likely the result of standing genetic variation from ancestral surface-dwelling stock and possible gene flow between populations, rather than the consequence of *de novo* mutations. Our study supports this interpretation and provides the first evidence, to our knowledge, that the causative regressive allele leading to a cave-associated phenotype (albinism) in different cave populations was inherited from surrounding epigeal populations.

Alternative explanations for shared *Oca2* genotypes in albino Micos and Pachón cavefish

We assume that the A1252G point mutation is a marker that is linked to the exon 24 deletion (Supplementary Figure S2). However, unlike

the exon 24–3'UTR deletion in Pachón cavefish that was demonstrated to be a loss-of-function mutation, the A1252G SNP did not impair *Oca2* function (Protas *et al.*, 2006). Therefore, the association we demonstrated here (Supplementary Figure S2) may be misleading if these two mutations are not completely linked to one another.

If these loci are unlinked, then other possibilities may explain our findings. For instance, one of the mutations may have been inherited through standing genetic variation, while the other evolved independently in both the lineages. Alternatively, Pachón cavefish and Micos cavefish may have each acquired one of the mutations independently and then exchanged them mutually through contact via gene flow between cave populations. We estimated that these two loci are ~44.1-kb apart, based on extrapolation of the *Oca2* genomic structure in *Danio rerio* (Figure 4); however, the precise distance between these loci is unknown. Further, given our small sample size, we cannot estimate linkage disequilibrium within our Micos cavefish. Nonetheless, given this estimated distance, the two loci may recombine enough to break linkage, rendering these alternatives plausible.

Additionally, the scenario that we present here suggests that the loss-of-function *Oca2* allele (originally identified in Pachón cavefish) persisted in the ancestral epigeal population at a high enough frequency to be detected in each cave colonization (or hybridization) event. These founder events would be predicted to reduce the probability of our sampling the presumably rare *Oca2* allele during the original collection at the Micos cave locality. 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, Pachón cavefish and surrounding surface populations would address this point by providing a clearer picture of the frequency of loss-of-function *Oca2* alleles in the natural *Astyanax* populations. This would, in turn, provide a better understanding of the origin and movement of loss-of-function alleles among geographically distant *Astyanax* cavefish.

CONCLUSIONS

The *Oca2* allele we identified in Micos cavefish is the same causative allele governing albinism in Pachón cavefish. The presence of this allele in two phylogenetically and geographically distinct cave populations suggests that cave associated traits in 'young' cave populations can arise from standing variation inherited from ancestral surface-dwelling fish. Thus, the appearance of existing, and rare, ancestral polymorphisms provide an additional mechanism for rapid evolution in cave populations. It is unclear whether this finding reflects a general feature of regressive trait evolution in 'young' *Astyanax* cave populations or whether it only applies to albinism. Until now, it has remained a mystery why the gene *Oca2* is repeatedly implicated in albinism in geographically distant populations. One explanation may be that natural selection favors the evolution of albinism. However, our findings here indicate the presence of a loss-of-function *Oca2* allele in a population that does not express albinism in the wild. This may indicate that albinism is not under selection but rather evolved through neutral forces in the Micos cave population. Our study also indicates that extreme cave-associated phenotypes such as albinism can arise remarkably quickly in captive-bred fish drawn from cave populations that do not express albinism in nature. It remains unclear, however, if other complex cave-associated phenotypes (such as eye loss) are similarly susceptible to rapid appearance

following a bottleneck event. This study provides the first demonstration of regressive phenotypic change in *Astyanax* occurring through standing genetic variation in the ancestral population and further underscores the complex origin of *Astyanax* cavefish.

DATA ARCHIVING

The following raw data has been deposited in the Dryad Digital Repository (doi:10.5061/dryad.jh8j7): (1) raw measurements from digital coloration analyses; (2) descriptive statistical analyses of coloration analyses; (3) raw filter images from coloration analyses; (4) filter images overlain with a 'digital' grid; (5) raw quantitative PCR measures for expression of *Oca2* and *GAPDH*; and (6) raw fin clip images of representative pigmented and albino Micos cavefish.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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