

TITLE.- EYED CAVE FISH IN A KARST WINDOW

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ABSTRACT

Caballo Moro is a karst window cave in northeastern Mexico that supports a mixed population of cave *Astyanax mexicanus*: eyed and eyeless. The relationships of these sub-population to one another and to other populations of Mexican tetras were examined RAPD DNA fingerprint markers. The eyed tetras of Caballo Moro Cave are genetically closer to blind tetras from Caballo Moro and other caves in the region than they are to eyed tetras from the surface. The two forms are not genetically identical, however, and may represent distinct sub-populations.

Eyed and eyeless fish have a distributional bias in the cave, with eyed fish preferentially in the illuminated area and blind fish in the dark zone. Aggression of eyed towards blind fish in the illuminated area contributes to this bias and may serve to stabilize the eye-state polymorphism.

We considered four hypotheses for the origin of Caballo Moro eyed cave fish. The RAPD data rule out that the mixed population represents a transitional stage of evolution, or that the eyed fish are unmodified surface immigrants. We cannot rule out that the eyed fish are the direct descendants of surface fish that have acquired markers from blind fish by hybridization, although the apparent distinctness of the two sub-populations suggests otherwise. An alternative hypothesis, that the eyed fish of the cave are direct descendants of blind cave fish that re-acquired eyes with the opening of the karst window, is consistent with the data and tentatively accepted.

INTRODUCTION

The Mexican Tetra, *Astyanax mexicanus*, is a visually orienting, schooling fish widely distributed in surface streams of northern Mexico. In addition to the epigeal populations, numerous cave forms of this species occur in the Sierra de El Abra region of Northeast Mexico (Fig. 1; Mitchell *et al.* 1977). In contrast to the surface fish, these troglomorphic forms have rudimentary, non-functional eyes, and their melanin pigmentation is reduced or absent.

Generally, caves with troglomorphic Mexican tetras do not contain eyed tetras, except for the occasional doomed individual swept underground. One exception is El Sotano de El Caballo Moro Cave, which contains an apparently stable, mixed population of *A. mexicanus*, both eyed and eyeless.

The entrance of Caballo Moro Cave (CMC) is a karst window. Karst windows are habitats within cave systems that are exposed to light, and typically result from cave passage collapse. The 50-m deep entrance pit of CMC is found at the bottom of a 60-m doline, and leads directly to a large "lake" of approximately 18 X 90 m. (Cave "lakes" are wide stream pools.) Light reaches only the upstream half of the lake, while the downstream half remains in darkness. The lake contains both blind depigmented and eyed pigmented forms of *A. mexicanus*. The distribution of fish in the lake appears to be biased, with over-representations of blind fish in the dark area and eyed fish in the light area.

Mitchell *et al.* (1977) observed that the source of the eyed fish of Caballo Moro Cave was a mystery. The cave's entrance pit is 11 km away from the nearest potential resurgence and does not capture a surface stream. Furthermore, there is no permanent water nearby; the nearest recorded surface fish locality in the Rio Boquillas system is 4 km distant. They hypothesized that seasonal

flooding of Rio Boquillas tributaries affords occasional access to the cave through as yet undetected sinks.

As part of a larger study of the evolutionary history of the Mexican cave tetra, we investigated the relationships of the eyed fish of CMC. If they represent an unmodified surface population recently captured from a nearby sink, their presence in the karst window would be unremarkable. If, on the other hand, the population were of long standing, it would raise the question of the maintenance of its integrity in the face of potential hybridization with, and introgression of genes from, the troglobites. Alternatively, if the eyed fish of the cave originated from blind cave progenitors, they would make a good model for study of the effects of the reversal of selection pressures on populations.

MATERIAL and METHODS

The relationships among representative surface and cave populations of *Astyanax mexicanus* from the El Abra region were studied using RAPD data. RAPD (synonymous with AP-PCR) is a technique for generating a DNA fingerprint from genomic DNA using the polymerase chain reaction (Welsh and McClelland 1990; Williams *et al.*, 1990). RAPD fingerprints are species and population specific and carry significant amounts of taxonomic information (Borowsky *et al.* 1995).

The following populations were sampled (Fig. 1): caves: Molino, Vasquez, and Caballo Moro: surface: Rio Frio, Rio Boquillas and Rio Comandante. *Astyanax aeneus* from the Rio Granadas, a tributary to the Rio Amacuzac, northeast of Taxco, Guerrero, Mexico were used as the outgroup for phylogenetic analyses. Two individuals each were examined from Molino Cave, Vasquez Cave, all surface localities, and *A. aeneus*. Five blind individuals and six eyed

individuals were examined from CMC. RAPD amplification procedures followed Borowsky *et al.* (1995). Two primers were used: Mey7 (5'ggagtaggggatgatgatgga3') and Mey8 (5'cagcaaacagaaaccagtcag3'). Reactions were cycled five times in a Hybaid thermocycler: 94°C for 70 sec, 40C for 5 min, and 72C for 3 min, followed by 35 cycles at higher stringency: 94C for 70 sec, 50C for 1 min, and 72C for 90 sec. Reaction products were run on 6% polyacrylamide gels (29:1) and silver stained (after Gottlieb and Chavko, 1987). RAPD fragment distributions were compared among individuals using a size match criterion. Each uniquely-sized band was assumed to be a character, and character states were scored as "present" or "absent."

Phylogenetic analysis of the data was done using Paup 4.0b2 software (Swofford, 1999). Maximum parsimony analysis (character states unordered) was done by bootstrapping the data (1000 replicates) using full heuristic search to produce a 50% majority-rule consensus tree. For analysis of distance ("mean character difference"), neighbor-joining trees were generated from bootstrapped data (1000 replicates) and used to obtain a 50% majority-rule consensus tree.

A supplementary analysis was done using a Monte Carlo procedure to estimate the variance of distances among individuals within and between the sets of eyed and eyeless fish from CMC. Individual phenotypes for distance comparisons were created by sampling, based on the frequencies of bands in each set. Twenty such pairs of phenotypes were generated for each simulation and the calculated distances were used to estimate means and their standard deviations. For this analysis, distances were calculated as the sum of the absolute differences in band frequencies among taxa or individuals divided by the number of bands.

RESULTS

One hundred and fifty-eight bands were scored, of which 127 were variable and of value

in distance analysis, and 58 were parsimony informative. The number of bands observed in any individual ranged from 55 to 69. The raw data matrix is presented as Table 1, which is organized in the style of a “sequence alignment.” As such, it arrays the character states of the outgroup species along the top row (+, -, and “P” for polymorphic). The character states for the other taxa are arrayed below, using “.” to denote state identity with the outgroup, and the other symbols, where different from the outgroup. The data were sorted by character states in the cave fish, putting “-“ towards the left and “+” towards the right. This arrangement makes apparent a series of derived bands shared among all cave fishes or among all individuals of Caballo Moro cave. These synapomorphies imply a closer relationship of the eyed fish of Caballo Moro cave to other cave fish than to epigeal fish.

This implication is supported by both parsimony and distance analyses, which gave essentially the same result: consensus trees with two clusters, one consisting of the epigeal populations and the other of the cave populations. The tree produced by distance analysis (Fig. 2) had a little more structure than the one based on parsimony and may be more appropriate for analysis of populations which can hybridize. The relationship of the eyed and blind fish of Caballo Moro cave is strongly supported by a bootstrap value of 0.83 as is the clustering of all fish of Caballo Moro cave with the other cave fish (bootstrap value of 0.82). The tree also shows a clustering of four of the five blind fish within Caballo Moro, which suggests that the eyed and blind fish of the cave may comprise two distinct sub-populations, in spite of their closeness.

The supplemental distance analysis lends some support to these hypotheses. Distances calculated among populations showed the eyed and eyeless fish of CMC to be closer to each

other (0.101) than either was to surface fish (0.337 and 0.359, respectively) or to the other blind cave fish (0.253 and 0.240, respectively). The distance between the eyed and eyeless fish of CMC was investigated in more detail by Monte Carlo simulation.. The average distance between simulated eyed and eyeless individuals (0.095 ± 0.015) was significantly greater than the average distance between simulated eyed individuals (0.067 ± 0.016 , $t_{38} = 3.83$, $P < 0.05$) or simulated eyeless individuals (0.031 ± 0.012 , $t_{38} = 9.60$, $P < 0.05$). The means and standard deviations of all the distances measured among the real individuals in the two groups are very similar to those in the simulation (between sets: 0.1226 ± 0.0264 ; eyed: 0.0965 ± 0.0215 ; eyeless: 0.0513 ± 0.168) and the t values are high ($t_{38} = 9.60$ and $t_{43} = 3.24$), but only the t tests in the simulation are valid.

Of 41 fish collected from Caballo Moro Cave, 21 were eyed and pigmented, and eighteen had eye rudiments completely covered by muscle and scales and were depigmented. Two were intermediate in phenotype. The collection made from the dark side of the lake had eight fish, one with eyes. The collection made from the illuminated side of the lake had seventeen fish, ten with eyes (locations of other specimens had not been recorded). The biased distribution is statistically significant (Fisher's exact test, $P < 0.05$). We observed eyed fish nipping and chasing blind fish on the illuminated side, and this behavior may contribute to the distributional bias within the lake.

DISCUSSION

At least four hypotheses could account for the presence of eyed fish in Caballo Moro Cave. The first is that the eyed individuals are surface fish recently swept underground. As such, their residency might be short-lived and they would not necessarily be part of the

troglobitic population. A second hypothesis is that the eyed fish represent one phenotypic extreme of a variable cave fish population in evolutionary transition towards eyelessness. A third is that they are the descendants of surface fish swept underground that had interbred with the blind fish and acquired their RAPD marker set by hybridization. A fourth is that the eyed fish are descendants of blind, depigmented cave fish that reacquired eyes and pigmentation through an evolutionary process. The reacquisition of eyes and pigment in troglobites reintroduced to light has been suggested before, for karst window populations of the amphipod *Gammarus minus* (Culver *et al.*, 1995).

We reject the first hypothesis because it predicts that the eyed fish of CMC should be genetically closer to surface fish than to the blind cave fish. Our results showed the opposite to be true; both distance and parsimony analyses clustered the eyed fish of the cave with blind cave fish rather than surface fish. This clustering was well supported by bootstrap analysis (Fig. 2).

What of the second hypothesis? Is the CMC population in transition from an eyed to a blind condition? Wilkens (1988) hypothesized such a situation in the isolated cave populations of the Micos area, to the west of the El Abra. Micos fish have reduced eyes, but the rudiments are better developed than in the cave tetras of the Sierra de El Abra region, and Micos fish are not fully depigmented. Wilkens suggested that the Micos cave tetras are in transition because they are "phylogenetically younger" than other populations of troglobitic Mexican Tetras, and our (unpublished) RAPD data support this.

Nevertheless, we think it unlikely that the CMC population is in transition between the eyed and blind conditions, as in the Micos fish. First, Caballo Moro Cave is centrally located within the range of other populations of cave tetras, none of which appear to be in a transitional

state. Second, the fish of the Micos caves are fairly uniformly intermediate in eye size and pigmentation phenotype (Wilkens, 1988; our unpublished observations), while most (95%) of the Caballo Moro Cave fish fall into two distinct morphological groups -- eyes functional *versus* blind. Thus, any intermediate "transitional" quality of the CMC population exists primarily as a statistical average of two phenotypic extremes.

We cannot yet distinguish between the third and fourth hypotheses: the eyed fish of the cave may have descended from a captured surface population having interbred extensively with the blind fish or it may have descended from blind cave ancestors by reacquisition of eyes and pigment. Both hypotheses predict extensive sharing of character states among eyed and eyeless fish from CMC and might prove difficult to distinguish in practice.

A test based on distance data may be possible. Our results show that the average distance between eyed and eyeless individuals of CMC is significantly greater than the average distances within these sets. A biologically significant genetic distance between the two groups of fish would arise in different ways according to the two hypotheses. Hypothesis three is one of introgressive hybridization, and would view distance as evidence of a mixing process not yet complete. Hypothesis four is one of centripetal evolution and would view distance as a derived state, as one subset splits from the other. Thus, hypothesis three predicts the eyed fish of CMC to be closer than their eyeless companions to the fish of the surface and more distant from the fish of the other caves. Instead, our data show both groups in CMC to be equally far from surface fish and equally far from the other cave fish. Thus, the current data support hypothesis four, but more will be necessary for a definitive test.

The data presented here confirm the status of the CMC population as one worth further

study for the light it can shed upon evolutionary processes. Karst windows, in general, should provide unique opportunities to study the effects of the alteration of selective pressures on troglobites and the ecological and evolutionary interactions between troglobitic and surface species.

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Figure Captions

Figure 1, Map of the Sierra de El Abra region showing the collection sites. 1) Caballo Moro cave, 2) Molino cave, 3) Vasquez cave, 4) Rio Subterraneo cave (a "Micos" cave), 5) the Rio Frio surface locality at the Nacimiento de la Florida, 6) a surface locality in the Rio Boquillas system, 1 km upstream of La Servilleta Canyon, and 7) the Rio Comandante surface locality, just upstream of its confluence with the Rio Frio. Other caves in the area are shown as unlabeled solid circles. (Redrawn from Wilkens, 1988.)

Figure 2. Bootstrapped Neighbor-Joining tree showing the relationships among cave and surface populations of *Astyanax mexicanus*. Figures on branches are the percentage of bootstrapped trees that had identical clusters and measure the reliability of the associations. The Guerrero population of *Astyanax anaeus* served as outgroup in the parsimony analyses.

Table Caption

Table 1. Character states for the 127 variable RAPD bands. The top line gives the states in the *Astyanax aeneus* outgroup: “+” = band present, “-“ = band absent, “P” = population polymorphic. Character states for the other groups are aligned below those of the outgroup. The symbol “.” denotes a state identical to that in the outgroup. Characters were sorted from left to right, putting characters with “-“ states in cave fish first.

