Molecular Ecology (2003) **12**, 699–710

Genetic divergence between cave and surface populations of *Astyanax* in Mexico (Characidae, Teleostei)

U. STRECKER,* L. BERNATCHEZ† and H. WILKENS*
Zoological Institute and Zoological Museum, University of Hamburg, Martin-Luther-King-Platz 3, 20146 Hamburg, Germany, †Département de Biologie, Pavillon Vachon, Université du Laval, Sainte-Foy, QC, Canada G1K 7P4

Abstract

A study of genetic diversity at microsatellite loci and the mitochondrial DNA (mtDNA) cytochrome *b* gene was carried out to assess genetic relationships among four Mexican cave (Pachon, Sabinos, Tinaja, Chica) and four surface populations of *Astyanax fasciatus* (Characidae) from northeast Mexico and the Yucatan. With the exception of Chica, the cave populations were all characterized by extremely low microsatellite variability, which most likely resulted from bottleneck events. Population analyses of the microsatellite data indicated no measurable levels of gene flow between all cave and surface populations (F_{ST} > 0.0707). Phylogenetic analyses of mtDNA data showed that only two cave populations — Sabinos and Tinaja — group together to the exclusion of surface populations. From the microsatellite data these cave populations cluster with the Pachon cave fish population. The mtDNA thus appears to have been replaced in Pachon because of introgressive hybridization. It is likely that these three cave populations have descended from a surface ancestor in common with current surface populations, rather than evolving recently from one of the extant surface populations. Like Pachon, the Chica population clustered with the surface populations according to mtDNA data, but was not clearly associated with either the surface or the other cave populations according to the microsatellite data. Our data indicate that the Chica population evolved recently from a surface population, and subsequently hybridized with a phylogenetically older cave population. In conclusion, both the microsatellite and mtDNA data suggest multiple origins of cave populations and the Chica and Sabinos/Tinaja/Pachon were founded after at least two independent invasions from surface populations.

Keywords: cytochrome *b*, microsatellites, multiple origin, population genetics, population structure, troglobites

Received 21 June 2002; revision received 25 November 2002; accepted 25 November 2002

Introduction

Cave animals represent unique models for studying the evolutionary loss of eyes and pigmentation and gain of specialized traits (e.g. taste and olfaction) (Culver 1982; Wilkens 1988; Culver *et al*. 1995). When closely related surface sister forms coexist, the direction of evolutionary processes can be inferred and comparative morphological, developmental and genetic studies of the ontogeny and phylogeny are possible. Interfertility between cave and surface forms also enables analysis of the genetic basis of complex traits [see Wilkens (1988) and Culver & Wilkens (2000) for detailed reviews]. Furthermore, cave animals often provide good model systems for studying the genetics of speciation processes (Allegrucci *et al*. 1987; Sbordoni *et al*. 2000).

The neotropical fish *Astyanax fasciatus* (syn. *A. mexicanus*) is widely distributed in Mexican surface waters (Miller 1986). Within a comparatively restricted karst area in northeastern Mexico, more than 20 blind and pigmentless populations are known (Mitchell *et al*. 1977). In the laboratory all of them are interfertile, both between each other and with the surface form.

It is currently unknown whether different cave populations of *Astyanax* originated by single or multiple founder

Correspondence: U. Strecker. Fax: 49 40 42838 3937; E-mail: Strecker@uni-hamburg.de

© 2003 Blackwell Publishing Ltd

events and whether there is gene flow among populations (Avise & Selander 1972; Mitchell *et al*. 1977; Wilkens 1988; Espinasa & Borowsky 2001; Dowling *et al*. 2002). This information is necessary to determine if troglobitic features, such as eyelessness and pigment reduction, have evolved only once or are convergent.

Allozyme analyses by Avise & Selander (1972) favoured a single founder event for the Pachon, Sabinos and Chica populations, and suggested that eyes and pigment were lost, totally or partly, by cave *Astyanax* populations before they became entirely isolated from each other. In contrast, Dowling *et al*. (2002) proposed, based on sequence data from the mitochondrial DNA (mtDNA) *ND2* gene, that at least two separate founder events occurred, with independent evolution of troglobitic characters in each lineage.

Little is known of the occurrence or the extent of introgression of surface fish genes into the cave fish gene pool, although in several caves they occur syntopically (Wilkens & Burns 1972; Mitchell *et al*. 1977). Knowledge of population genetic differentiation in cave-living species and how they compare to their surface sister forms is limited (Avise & Selander 1972; Caccone & Sbordoni 1987; Culver *et al*. 1995). The size of the founding populations as well as the occurrence and frequency of bottleneck events are also poorly documented. In this study, we performed a gene diversity analysis based on six microsatellite loci among cave and surface populations of *Astyanax* to address these issues. Furthermore, a mtDNA cytochrome *b* analysis was used to establish a phylogenetic framework.

Materials and methods

Samples

A total of 260 *Astyanax* specimens were sampled in March 1996 and fin clips were immediately preserved in 95% ethanol. Abbreviations of *Astyanax* populations and number of individuals sampled are given in parenthesis. The cave fish studied in this analysis were collected using handnets in the Cueva de El Pachón (Pachon, 40), Cueva de los Sabinos (Sabinos, 25), Sótano de la Tinaja (Tinaja, 30) and Cueva Chica (Pool II, Breder 1942) (Chica, 21), all located in the Sierra de El Abra (San Luis Potosi) (Fig. 1). The surface fish from the cave region were sampled in a tributary of the Arroyo Lagarto, which is situated near the Cueva de El Pachón (Pachon-S, 30), and a creek draining into the Micos cave (Cueva del Rio Subterraneo; Mitchell *et al*. 1977) (Micos-S, 32). Sampling in the Micos cave was not possible because it had been blocked by falling rocks. An overview of the hydrology of cave and surface locations is presented by Mitchell *et al*. (1977). Surface fish from Yucatan were caught using baited hooks in the lagoon alongside the road leading from Cafetal to Majahual north of the Chetumal Bay (Majahual, 51) and in the Laguna Chichancanab (Chichancanab, 31), where they were observed for the first time in 1996 (U.S. and H.W., unpublished data).

Microsatellite analysis

Fish samples were screened for genetic variation at six microsatellite loci. DNA extraction, polymerase chain reaction (PCR) amplification and scoring procedures were performed as described in Strecker (2003). The mean number of alleles per locus (A), observed heterozygosity (H_O) and gene diversity (H_E), were calculated using GENEPOP software 3.0 (Raymond & Rousset 1995a). GENEPOP was also used to assess heterogeneity in genotype frequencies among pairwise population comparisons and to test for deviations from Hardy–Weinberg equilibrium within populations for each locus and over all loci (Raymond & Rousset 1995b). Values of significance were estimated by the Markov chain method with 1000 iterations (Guo & Thompson 1992). Heterozygote deficiencies in microsatellite loci may result from the occurrence of null alleles. We estimated the expected frequency of a presumed null allele based on the formulae presented by Chakraborty *et al*. (1992) and Brookfield (1996) for each population at each locus (r_c and r_b, respectively).

The population genetic structure was quantified using an analysis of variance framework, which involves computing correlations of a pair of alleles drawn from the same subpopulation relative to that randomly drawn from a group of subpopulations (Weir & Cockerham 1984). Fixation indices were also computed in an analysis of molecular variance framework (AMOVA), using information on the mutational differences among alleles, which was entered as a matrix of Euclidean square distances (Michalakis & Excoffier 1996). Comparing the extent of population structure indicated by allele frequency and mutational information allows an assessment of the relative role of long-term separation and contemporary genetic drift in populations (Slatkin 1995; Goodman 1998). By this procedure, genetic structure indices were computed at three hierarchical levels: within individuals, among individuals within populations (F_{IS}), and among populations (F_{ST}) using the program ARLEQUIN (Schneider *et al*. 1997). The significance of variance components associated with the different levels of genetic structure were tested using nonparametric permutation procedures (1000 permutations). Pairwise estimates among all population combinations were computed in the same manner. Whenever pertinent, the significance of P-values was adjusted following Bonferroni sequential corrections for multiple simultaneous statistical tests (Rice 1989), with an initial alpha value of $0.05/k$, k being the number of tests.

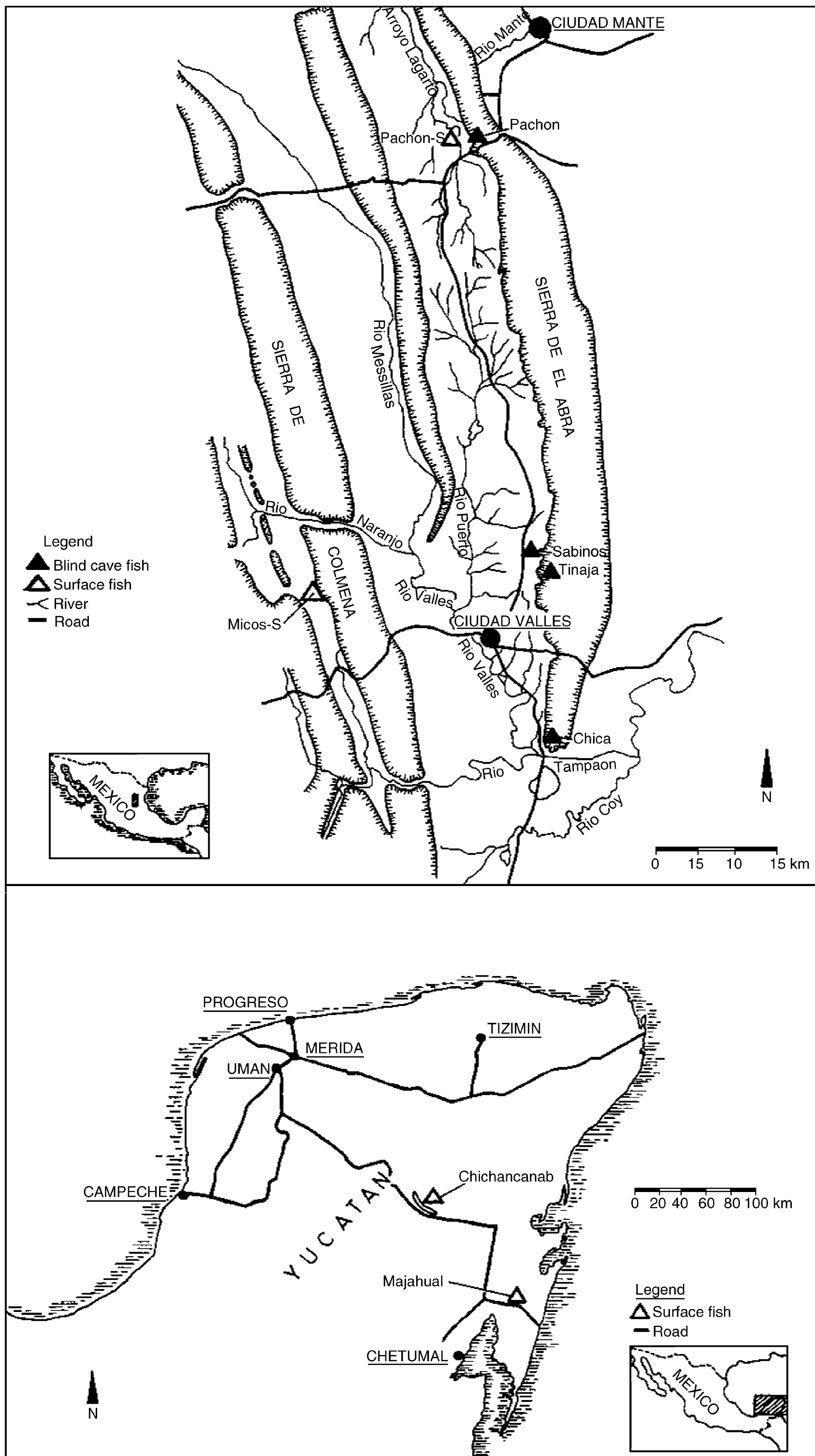

Fig. 1 Sampling localities of *Astyanax* populations in Mexico.

Population relationships

Because of uncertainty regarding what constitutes the most appropriate method for quantifying population differences based on microsatellite polymorphism, three measures were used to quantify pairwise genetic distances among populations: Chakraborty & Jin's distance (D_{AS}), which is the simplest estimation based on the proportion of shared alleles (Chakraborty & Jin 1993); Nei's DA distance (Nei *et al.* 1983) which assumes pure genetic drift; $(\delta\mu)^2$,

which takes into account size differences between alleles and theoretically fits linearity with time better than nonstepwise-mutation model-based distance measures when microsatellites follow a strict stepwise mutation model (SMM; Goldstein *et al*. 1995). The strength of support for each node in the tree was tested by 2000 bootstrap replications each over loci and individuals using the program NJBPOP developed by J. M. Cornuet (Laboratoire de Modélization et de Biologie Evolutive, INRA-URLB, Montpellier, France).

We also estimated relationships among single individuals by calculating the pairwise shared allele distance among individual multilocus genotypes as described by Chakraborty & Jin (1993). The resulting matrix was used to reconstruct an individual tree using the neighbour-joining algorithm. These procedures were applied using a program provided by J. M. Cornuet.

Mitochondrial DNA sequencing and analysis

We analysed a partial sequence of the cytochrome *b* mitochondrial gene using three individuals per population. An *Astyanax* sp. from Brazil was used as an outgroup. PCR were carried out in a 25-μL reaction volume containing 50–100 ng of template DNA, 0.5 units Pharmacia *Taq* polymerase, 1× *Taq* polymerase buffer (50 mM KCL, 1.5 mM $MgCl_2$, 10 mM Tris–HCL, pH 9.0), 0.2 mM of each dNTP, 1.25 μg bovine serum albumin and 0.4 μM of each primer. Specific primers, AstCytB-L 5′-CTGCCCCCTCAAACATTTCA-3′ and AstCytB-H 5′-GGTTGGGGGAGAATAAGGCTAA-3′ were designed from the whole cytochrome *b* sequence of *Astyanax fasciatus* (EMBL/GenBank accession number AF045997; Zardoya & Doadrio 1998). PCR profiles started with 1 min at 95 °C, followed by 35 cycles of 95 °C denaturation for 15 s, 53 °C annealing for 30 s and 72 °C extension for 30 s. PCR products were cleaned using a High Pure PCR Product Purification Kit (Roche). Double-stranded PCR products were sequenced directly using the BigDye Terminator kit (Applied Biosystems Inc.) on an automated DNA sequencer (Applied Biosystems 377) following the manufacturer's instructions using the PCR primers. Sequences determined here have been deposited in GenBank under the accession numbers AY177205–AY177221. They were aligned using SEQUENCHER 3.1.1 (Gene Codes Corporation) and gaps were not necessary to align the haplotypes. Pairwise distances between populations were estimated following the Kimura two-parameter model (Kimura 1980) with the MEGA program version 1.02 (Kumar *et al*. 2001). Phylogenetic analyses were carried out using the maximum-parsimony (MP) and neighbour-joining (NJ) approaches in PAUP 4.0 (Swofford 2002). MP analysis was performed using a heuristic search with 50 random stepwise additions of taxa. The NJ tree was calculated based on the Kimura two-parameter model (Kimura 1980) and robustness of tree topology was assessed using 1000 bootstrap pseudoreplicates.

Results

Microsatellite overall diversity

The microsatellite primers successfully amplified all *Astyanax* populations and their overall level of polymorphism was generally high. The observed number of alleles per locus was 11 in Ast9, 13 in Ast10, 20 in Ast1, 25 in Ast2, 27 in Ast13 and 29 in Ast4, with an overall gene diversity (H_E) between 0.7895 in Ast1 and 0.8876 in Ast13, with that for Ast9 (0.7308) being lower (mean 0.8187). Mean intrapopulation diversity was similar among loci, both in terms of mean number of alleles per locus (A_m) and mean heterozygosity values (Table 1).

Most loci showed nearly continuous allelic size distributions (Appendix). In locus Ast4, however, a major gap of approximately 100 base pairs (bp) separated the prevalent allele size (range = 169–243 bp) from a larger allele group (341, 343 and 345 bp). The latter was exclusively found in the Chichancanab population, which lacked the shorter alleles. A major gap was also observed at Ast2, for which 268 bases separated the 477 allele from the next smallest one (209), with the large allele found only in a single individual of the Majahual population. Two other large alleles differing by 34 bp and 36 bp from Ast2–477 (511 and 513 bp) were found in two individuals of the surface-dwelling Micos population. The large alleles of loci Ast2 and Ast4 were not included in the analyses based on SMM assumptions.

Interpopulation diversity varied considerably (Table 1), with both gene diversity estimates and number of alleles per locus generally lower in the three cave populations Tinaja, Sabinos and Pachon (means 0.2604, respectively, 3.0) compared to surface fish from the same area Micos-S and Pachon-S (means 0.6936, respectively, 8.6). The Chica cave population had intermediate values at most loci compared to cave and surface populations. Populations from the Yucatan had higher (Majahual) as well as lower (Chichancanab) genetic diversity compared to the northern populations.

Hardy–Weinberg disequilibria

Significant departure from Hardy–Weinberg equilibrium was detected in 19 out of 48 tests for loci within populations (39.6%, Table 1), which is more than expected by chance alone (two or three deviations predicted from type I error at alpha = 0.05). Ten of those represented statistically significant heterozygote deficiencies after Bonferroni adjustments. These involved locus Ast2

Table 1 Variation of six microsatellite loci in eight *Astyanax* cave and surface populations. Number of different alleles (A), total number of specimens scored for each sample and locus (N), expected (H_E) and observed (H_O) heterozygosity by locus and population and mean number of alleles per population (A_m) are listed

	Surface populations				Cave populations			
	Chichancanab	Majahual	Micos-S	Pachon-S	Chica	Pachon	Sabinos	Tinaja
Ast1								
N	31	51	32	30	21	40	25	30
A	5	19	4	9	8	3	2	2
H_E	0.3184	0.9142	0.3953	0.6785	0.7213	0.1845	0.0784	0.0655
H_O	0.2581	0.8824	0.4375	0.7333	0.7619	0.2000	0.0000	0.0667
P	*	NS	NS	NS	NS	NS	*	NS
Ast2								
N	31	50	30	30	21	40	25	30
A	4	12	11	10	5	2	2	2
H_E	0.3411	0.9000	0.8046	0.8819	0.6585	0.0962	0.2457	0.4520
H_O	0.3871	0.6471	0.4375	0.6000	0.6190	0.1000	0.2800	0.4667
P	NS	**	**	**	*	NS	NS	NS
Ast4								
N	31	50	32	29	21	40	25	30
A	3	20	11	13	6	4	4	1
H_E	0.3342	0.9184	0.7892	0.9008	0.6585	0.4630	0.4318	—
H_O	0.3226	0.6600	0.5000	0.5862	0.6667	0.2250	0.0800	—
P	NS	**	**	**	NS	**	**	—
Ast9								
N	31	51	32	30	21	40	25	30
A	3	9	4	2	1	3	1	1
H_E	0.3559	0.8214	0.5551	0.3045	—	0.0737	—	—
H_O	0.3226	0.6471	0.4688	0.3667	—	0.0250	—	—
P	NS	*	NS	NS	—	*	—	—
Ast10								
N	31	51	32	30	21	40	25	30
A	4	10	8	9	5	5	3	6
H_E	0.4209	0.7012	0.7108	0.6819	0.5714	0.3066	0.3984	0.4520
H_O	0.1613	0.5686	0.6250	0.7667	0.6190	0.3500	0.1600	0.4333
P	**	**	**	NS	NS	NS	**	NS
Ast13								
N	31	51	31	30	21	40	25	30
A	2	20	8	14	6	3	6	4
H_E	0.0635	0.9334	0.7308	0.8893	0.7549	0.2434	0.6629	0.5328
H_O	0.0645	0.8627	0.4516	0.8667	0.9048	0.2750	0.4800	0.5333
P	NS	NS	**	NS	NS	NS	**	NS
A_m	3.5	15	7.67	9.5	5.17	3.33	3	2.67
Mean H_E	0.3057	0.8648	0.6643	0.7228	0.5608	0.2279	0.3029	0.2504

NS = not significant; *$P < 0.05$; **$P < 0.01$.

(Majahual, Micos-S, Pachon-S), Ast4 (Majahual, Micos-S, Pachon-S, Sabinos), Ast10 (Chichancanab, Sabinos) and Ast13 (Micos-S).

It is possible that the presence of null alleles and/or the mis-scoring of heterozygotes as homozygotes were responsible for these deviations from Hardy–Weinberg equilibrium. The estimate of the expected frequency of null alleles based on the method of Chakraborty *et al*. (1992) gave values from negative or zero to a high of 1.00 for r_c for Ast1 in the Sabinos cave population and r_b (Brookfield 1996) values from negative or zero to a high of 0.2034 for Ast2 in the Micos surface population. Averaged values across loci ranged from 0.036 to 0.206 for r_c and from 0.004 to 0.109 for r_b. The highest values for both r_c and r_b were

found for locus Ast4. We estimated that null alleles would have an expected frequency of either 0.05 or 0.11, based on the methods of Brookfield (1996) and Chakraborty *et al.* (1992), respectively. These values would predict expected frequencies of null homozygotes varying between 0.0025 and 0.0121. However, we did not observe any nonamplifying (putative null homozygous) individuals. Therefore the null alleles frequencies should be estimated from the apparent heterozygote deficiency after Brookfield (1996) which resulted in lower estimates.

These estimates suggest that if heterozygote deficiencies are entirely a result of nonamplifying alleles, then such alleles are present at relatively moderate frequencies averaging approximately 5%. However, this method assumes that all of the observed heterozygote deficiencies are a result of null alleles (Brookfield 1996), which is unlikely for the *Astyanax* populations. The observed deviations from Hardy–Weinberg equilibrium were spread among the six loci and were not concentrated at any particular locus, as would be expected if they resulted from null alleles alone. Other explanations for Hardy–Weinberg deficiencies include a Wahlund effect and/or nonrandom mating or selection against heterozygotes. It is not possible to clarify which combination of these potential causes is responsible for the heterozygote deficiencies observed.

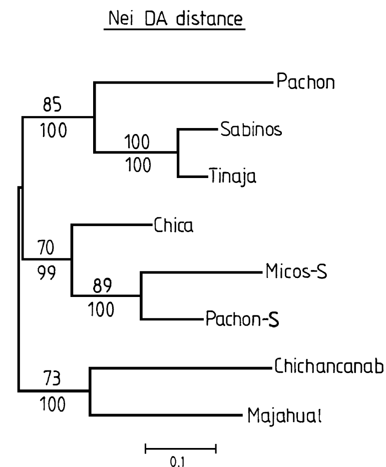

Fig. 2 Neighbour-joining tree illustrating the relationships among eight cave and surface *Astyanax* populations based on Nei's DA genetic distance measure based on six microsatellite loci. Values along branches indicate bootstrap values (as a percent of bootstrap pseudoreplicates) either based on locus (above line) or individual (below line).

Genetic differentiation among populations

Significant heterogeneity in genotypic frequency distributions was found in all pairwise comparisons among populations, confirming their genetic divergence. Three of the comparisons were not informative because of the presence of a monomorphic locus Ast9 in Chica, Sabinos and Tinaja. Altogether, 146 out of 165 pairwise comparisons (88.5%) showed significant heterogeneity in genotype frequencies among populations ($P < 0.000001$). Nine comparisons had P-values < 0.001, three had $P < 0.01$, and two had $P < 0.05$. Only five comparisons were not significant (3%), for the locus Ast1 Sabinos vs. Tinaja, for Ast9 Chica vs. Pachon, Pachon vs. Sabinos and Pachon vs. Tinaja, as well as for Ast10 Chica vs. Micos-S.

Accordingly, gene diversity analysis revealed large genetic variance among populations, based on both allelic (mean $F_{ST} = 0.3914$, range = 0.0707–0.7242) and molecular variance (mean $R_{ST} = 0.3522$, range = −0.0092–0.8055). F_{ST} and R_{ST} estimated values were not always congruent.

Based on F_{ST} values, the lowest level of divergence was observed between the Sabinos and Tinaja cave populations, and the Micos and Pachon surface populations were also only moderately differentiated. Divergence between the Micos and Pachon surface populations and the geographically remote surface population Majahual from the Yucatan were lower than those from the geographically much closer cave populations Pachon, Sabinos and Tinaja (Fig. 2). Divergence between the cave populations, e.g. Pachon vs. Sabinos/Tinaja, was as high as that between the cave populations and the Micos and Pachon surface samples. In contrast, divergence between cave and surface populations from the same locality (Pachon) was greater than that between the Micos and Pachon surface populations vs. Majahual from Yucatan, suggesting more recent genetic exchange between remote geographical localities (about 1000 km distance) than between cave and surface forms from the same geographical area. In contrast to the other cave populations, the Chica cave population was less divergent from the surface populations (Micos and Pachon). This suggests recent genetic exchange between Chica and surface populations.

R_{ST} values had an incongruent pattern of population structuring relative to that using F_{ST}. It seems very unlikely, for example, that the Chica cave population groups with Majahual (0.0829) and that divergence values between Majahual and Micos and Pachon surface populations were dissimilar (0.3500 and 0.1596, respectively). Such inconsistencies suggest that the F_{ST}-estimates of population divergence, based on the microsatellite loci used here, are more accurate than the SMM-based R_{ST} values.

A hierarchical analysis of gene diversity based on variance of allele frequency resulted in significant levels of genetic variance when comparing the surface with the cave populations, excluding the Chica population (Table 2a). The AMOVA revealed that nearly 11% of the total microsatellite DNA diversity was explained by variance among the surface and cave population groups. A higher proportion of the variance (16.49%) was attributable to interpopulation

Table 2 AMOVA analyses of genetic variation between cave and surface *Astyanax* populations (Chichancanab excluded) with significance levels

Source of variation	d.f.	Variance components	Percentage variation	*P* value
(a) cave fish without Chica population vs. epigean forms				
Among groups	1	9.83945	11.22	< 0.000001
Among populations within groups	4	14.45562	16.49	< 0.000001
Within populations	410	63.36895	72.29	< 0.000001
(b) cave fish including Chica population vs. epigean forms				
Among groups	1	6.05765	6.94	0.09971
Among populations within groups	5	17.48796	20.03	< 0.000001
Within populations	451	63.75992	73.03	< 0.000001
(c) cave fish vs. epigean forms and Chica population				
Among groups	1	9.36102	10.54	< 0.000001
Among populations within groups	5	15.69485	17.67	< 0.000001
Within populations	451	63.75992	71.79	< 0.000001

differences (within groups) whereas the largest proportion of the variance (72.29%) was found within populations. When adding Chica to the cave group, the genetic variance between the cave and surface groups was not significant ($P = 0.0997$) (Table 2b), whereas this component of genetic variance remained significant when including Chica with the surface group ($P = 0.000001$) (Table 2c). The Chica population was subjected to this procedure because of its ambiguous grouping with cave or surface populations.

Population relationships

The topology of the $(\delta\mu)^2$ population tree (not shown) was poorly supported by bootstrap replications and showed little correspondence with geographical locations. For instance, the Pachon cave population clustered with the surface population, and the Chica cave population clustered close to Yucatan populations. In contrast, the topology of the population tree reconstructed from the DA distance matrix revealed population assemblages that were supported by considerably higher bootstrap values (Fig. 2). One cluster was composed of the two Yucatan populations Majahual and Chichancanab. Populations from the cave area clustered in two groups corresponding to cave and surface populations, with the exception that the Chica cave population grouped closer to the two surface populations. The tree topology obtained from the net shared allele distance matrix was largely congruent with the DA tree, except that the Chica population grouped with the other cave populations. It appears from the DA and the D_{AS} trees that the Chica population cannot clearly be associated with either surface populations or other cave populations.

This unclear gouping of the Chica population was also reflected in the analysis of relationships based on individual D_{AS} among all fish from the cave area. Fish from the surface populations (Micos and Pachon) all clustered distinctively from those in cave populations, with the exception of Chica fish. Within the cave and surface groups, fish generally tended to group by population with little mixing except between Sabinos and Tinaja. Fish from Chica generally clustered together. However, five individuals from the Pachon surface population clustered with those from Chica and three Chica individuals clustered closer to the cave group, supporting the intermediate nature of this population.

The intermediate position of Chica was also supported by its allele composition. Surface and cave populations from the cave area were generally characterized by strong differences in allele frequencies and sometimes the presence of diagnostic alleles between both groups (Appendix). For instance, allele Ast1–140 was found at a relatively high frequency in the Micos and Pachon (surface) populations but was completely absent from the Pachon, Sabinos and Tinaja cave populations. Similarly, the three cave populations were characterized by a high frequency of the Ast1–150 allele which was absent from surface populations from the cave area. In contrast, the Chica population possessed both alleles at intermediate frequencies. Allele Ast2–163 was found at a greater frequency in the Chica population than in the surface populations whereas –177 was found at high frequencies in all cave populations and was at a low

frequency in the Chica population. For the locus Ast13, the allele –137 was found at high frequencies in the Sabinos and Tinaja cave populations and occurred at a low frequency only in Chica. The Chica population also shared several other low-frequency alleles with either the cave or surface groups, with three shared exclusively with the other cave populations and 10 with the surface populations. The Pachon cave population shared these most frequent alleles at the Ast1, Ast2 and Ast9 loci with Sabinos and Tinaja, and differed at three loci. In comparison to Sabinos and Tinaja, the Pachon population was slightly more variable and shared five alleles exclusively with the Micos and Pachon surface populations.

Mitochondrial data

We found 13 mtDNA haplotypes in 24 individuals and 42 polymorphic sites. Only four of them were in the first codon position resulting in two amino acid changes, whereas all others were found at the third position. Five transversions were detected and the ratio of transitions to transversion was 7.4. Three of the eight populations (Chichancanab, Mahajual and Pachon) contained a single haplotype. Two of the haplotypes were found in more than one population, in Sabinos and Tinaja as well as in Chica and Pachon-S. The lowest haplotype pairwise distances (0.11%) occurred between the Sabinos and Tinaja populations, and the greatest were between the two cave populations Sabinos/Tinaja vs. Pachon, Chica, Pachon-S and Micos-S (4.38–4.49%).

The MP analysis was performed with equally weighted characters as well as with weighting of transversions and first codon positions by a factor of two and three, respectively. These trees had identical topologies with 124 steps (CI 0.863) and 176 steps (CI 0.886) for the unweighted and weighted analyses, respectively (Fig. 3). Both the NJ and MP trees were largely congruent with three clusters formed by the Yucatan populations, the Tinaja and Sabinos cave populations, and the Micos and Pachon surface fish together with the Chica and Pachon cave populations.

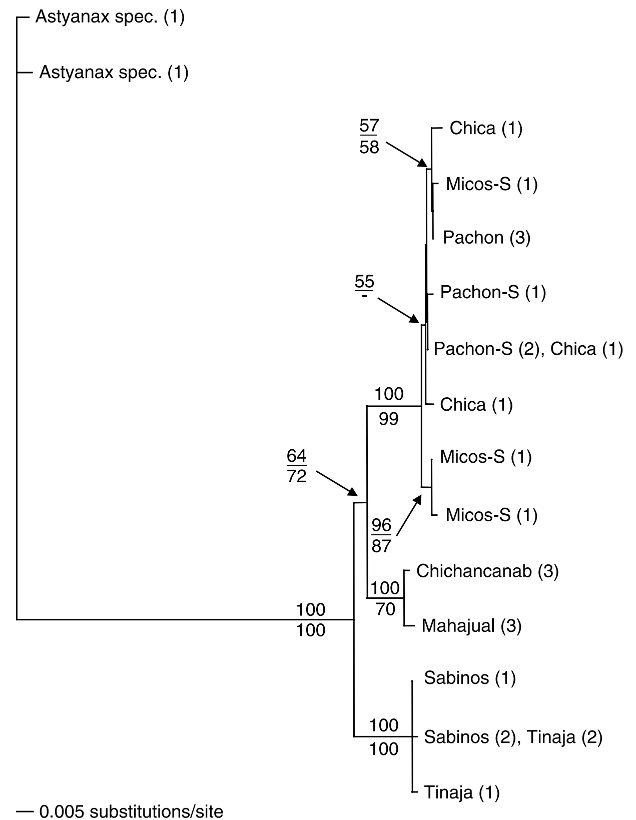

Fig. 3 Neighbour-joining tree based on mtDNA sequence data. The numbers above branches represent the bootstrap values obtained for 1000 replicates for the neighbour-joining and below for the maximum parsimony analysis. The dash indicates a branch not recovered by one of the methods. Numbers in brackets after the population name refer to the number of individuals with that particular sequence.

Discussion

Microsatellite variability

The overall microsatellite diversity of the populations studied was comparable to that found in other freshwater fish (Tessier *et al*. 1997; Angers & Bernatchez 1998; Brunner *et al*. 1998; Largiadèr *et al*. 1999). However, the diversity was highly variable among populations, and was significantly lower in all cave populations except Chica compared to surface populations from the cave area. The low variability found in the Tinaja, Sabinos and Pachon cave populations is congruent with results obtained using allozymes (Avise & Selander 1972) as well as mtDNA sequences (Dowling *et al*. 2002). Low genetic variability is also typical of other aquatic cave animals, including the amphipod *Gammarus minus* (Culver 1982), the isopod *Typhlocirolana* species (Caccone *et al*. 1986) and in amblyopsid cave fish (Swofford *et al*. 1980). However, relatively high genetic diversity was found for the cave bivalve *Congeria kusceri* (Stepien *et al*. 2001).

Lower diversity in *Astyanax* cave populations may be the result of founder effects. However, given the rapid mutation rate of microsatellites, one might expect higher diversity. The current cave population sizes are estimated to be in the order of several to many thousands of individuals (Mitchell *et al*. 1977). Small contemporary population size is therefore an unlikely explanation for the lower genetic diversity observed in cave populations (except Chica). The most likely explanation for reduced diversity in *Astyanax* cave populations is the occurrence of periodic population bottlenecks, possibly related to changes in environmental conditions such as food supply and/or

water level fluctuations. Bottlenecking is also assumed to be the reason for the low genetic diversity found in some other cave animals (Laing *et al*. 1976; Kane *et al*. 1992; Culver *et al*. 1995).

The intermediate variability (including three mtDNA haplotypes) found in the Chica population is comparable with that based on allozymes (Avise & Selander 1972). In contrast, Dowling *et al*. (2002) found no variability in the mtDNA ND2 gene which is more highly conserved than cytochrome *b*. The greater amount of genetic variability in the Chica population than other cave populations indicates that the Chica population has remained more constant in size and may not have experienced appreciable bottlenecking. Because of a large bat roost, the Chica cave has the most abundant food recources known in the area, which may have circumvented bottlenecks.

Epigean ancestry

In accordance with the assumption that cave populations originated from the local surface form, one should expect populations to cluster by localities rather than by form (cave vs. surface populations). This hypothesis was not supported by our microsatellite data because the Pachon cave fish grouped with the Sabinos and Tinaja cave populations rather than with the surface fish from the same area (Fig. 2). This grouping is not, however, because of current gene flow among these cave populations as shown by the high F_{ST} estimates.

Exept for the Pachon cave fish the genetic differentiation based on microsatellites is generally concordant with the genetic distances provided by mtDNA data despite the expected level of homoplasy of microsatellite alleles, especially in longer separated populations. For example, the level of genetic differentiation between cave and surface populations from the cave area is higher than that between the surface populations (cave area and Yucatan) for both microsatellite and mtDNA data (Figs 2, 3). This is suggestive of a relatively long period of separation between cave and surface populations which is also supported by mtDNA *ND2* data (Dowling *et al*. 2002). Therefore, we propose that cave populations originated from an ancestral group different from the contemporary surface populations.

Origins of cave populations and introgression

Since the beginning of research on *Astyanax* cave fish the occurrence of morphologically variable populations has led to speculation on the extent of introgression of surface genes into the cave gene pool (Breder 1942). Our cytochrome *b* mtDNA data showed that the Chica cave fish group with the surface populations from the cave area to the exclusion of the other cave populations (Fig. 3). However, according to the microsatellite data introgression into the Chica population cannot clearly be attributed to either the cave or surface populations from the cave area (Fig. 2). The Chica population shares alleles which are unique to either cave or surface populations from the cave area (Appendix). Our findings may be explained by two hypotheses. First, Chica is a phylogenetically old cave fish population, similar to Sabinos, Tinaja, or Pachon, that may have hybridized with a recent surface population. This hypothesis was proposed by Avise & Selander (1972) from allozyme studies, who also inferred that surface gene flow into the Chica cave population continues today. However, our microsatellite data show that although Chica has diverged from the surface populations, it contains surface alleles at a high frequency and is lacking the high-frequency cave alleles (Appendix). In addition, Chica has an F_{ST} of only about 0.17 with surface populations but 0.40 with cave populations. In the tree of individual fish only three of the 21 Chica fish clustered with the cave fish. The mtDNA sequences and the hierarchical analysis of gene diversity indicate a closer relationship of Chica to the surface fish (Fig. 3, Table 2a–c). On the basis of our data we favour a second hypothesis that the Chica population has, in contrast to Sabinos, Tinaja and Pachon, diverged from the recent surface population and is a phylogenetically young cave population which has subsequently been partly introgressed with microsatellite alleles from a phylogenetically old cave population.

For the Pachon population the microsatellite and mtDNA data are in contradistinction. Based on the mtDNA analysis, the Pachon cave population clusters with the surface fish from the cave area (Fig. 3). This grouping was also found by Dowling *et al*. (2002). In contrast, our microsatellite data revealed that the Pachon population groups with the phylogenetically older cave populations Sabinos and Tinaja (Fig. 2). This latter finding is concordant with the high degree of morphological and behavioural adaptation of Pachon fish to the cave environment, in eye and pigment reduction as well as in feeding behaviour (Wilkens 1988). This contradistinction could be explained by an introgressive hybridization event, as hypothesized by Dowling *et al*. (2002), whereby the original mtDNA haplotype was partially or totally replaced. Such an event has been proposed for other fish species (Gerber *et al*. 2001; Lu *et al*. 2001).

In 1986, individuals with variable eye sizes and melanin pigmentation were observed in the former albinotic and eye-reduced Pachon population (Langecker *et al*. 1991). In 1996, only traces of this introgression were found (U.S. and H.W., unpublished data). The assumption presented by Dowling *et al*. (2002) that selective collecting of hybrids could explain the alleged absence of hybrid phenotypes seems very unlikely in a population that, according to Mitchell *et al*. (1977), consists of 5000–12 000 individuals. It

is much more probable that the introgressed genes were distributed through and diluted within the larger Pachon gene pool leading to a rapid decrease in the number of hybrid phenotypes. However, our microsatellite data suggest that gene flow between surface and cave Pachon populations is unlikely. Laboratory crosses between Pachon and other cave populations revealed hybrid specimens with externally visible eyes, which surpass the size of parental eyes (Wilkens & Strecker, accepted). Therefore, we hypothesize that the Pachon population may not have been introgressed from the surface population, but by another yet undiscovered phylogenetically young cave population already better adapted to the troglobitic environment and favoured by a low level of selection. This would better explain the replacement of the mtDNA haplotypes. However, we cannot yet determine whether this replacement was because of an earlier hybridization event.

It has long been disputed whether the phenotypically alike cave populations of *Astyanax*, showing extreme reduction of eyes and pigmentation, have evolved independently after multiple colonization events or if they resulted from a single invasion with subsequent dispersal in the underground water system secondarily (Avise & Selander 1972; Mitchell *et al*. 1977). Our data indicate at least two independent invasions into the cave environment, namely Chica and the phylogenetically old cave populations Sabinos, Tinaja and Pachon. Also, Dowling *et al*. (2002) found '… two genetically distinctive cave fish lineages with similar eyeless phenotypes'. The high F_{ST} values between Pachon and the other cave populations suggest an extended period in which gene flow has been absent. The Pachon cave is situated about 60 km away from the Sabinos and Tinaja caves and is isolated above groundwater level (Mitchell *et al*. 1977). This may indicate its longer existence perhaps in separate troglobitic evolution.

The *Astyanax* system offers an extraordinary tool for the study of genetic and developmental mechanisms underlying the evolution of surface fish into cave fish. Here we present data on its population genetic and phylogenetic background. Furthermore it is shown that microsatellite data not only provide insight into relationships between closely related populations but may be more reliable for interpreting patterns of gene flow (e.g. introgression) between populations than mtDNA data.

Acknowledgements

We are grateful to W. F. Eanes (State University of New York) for providing laboratory facilities to perform a large part of the practical work. We also thank A. Meyer. We acknowledge V. Faúndez for performing the mtDNA sequencing. The constructive comments of S. Cooper (South Australian Museum, Adelaide) and two anonymous reviewers on earlier versions of this manuscript are much appreciated. This project was financially supported by Deutsche Forschungsgemeinschaft (Wi 302/16–1).

References

Allegrucci G, Cesaroni D, Sbordoni V (1987) Adaptation and speciation of Dolichopoda cave crickets (Orthoptera, Rhaphidophoridae): geographic variation of morphometric indices and allozyme frequencies. *Biological Journal of the Linnean Society*, **31**, 151–160.

Angers B, Bernatchez L (1998) Combined use of SMM and non-SMM methods to infer fine structure and evolutionary history of closely related brook charr (*Salvelinus fontinalis*, Salmonidae) populations from microsatellites. *Molecular Biology and Evolution*, **15**, 143–159.

Avise JC, Selander RK (1972) Genetics of cave-dwelling fishes of the genus *Astyanax*. *Evolution*, **26**, 1–19.

Breder CM (1942) Descriptive ecology of La Cueva Chica, with special reference to the blind fish *Anoptichthys*. *Zoologica*, **27**, 7–15.

Brookfield JFY (1996) A simple new method for estimating null allele frequency from heterozygote deficiency. *Molecular Ecology*, **5**, 453–455.

Brunner PC, Douglas MR, Bernatchez L (1998) Microsatellite and mitochondrial DNA assessment of population structure and stocking effects in Arctic charr *Salvelinus alpinus* (Teleostei: Salmonidae) from central Alpine lakes. *Molecular Ecology*, **7**, 209–223.

Caccone A, Allegrucci G, Cesaroni D, Sbordoni MC, Matthaeis ED, Rosa GL (1986) Genetic variability and divergence between cave dwelling populations of *Typhlocirolana* from Majorca and Sicily. *Biochemical Systematics and Ecology*, **14**, 215–221.

Caccone A, Sbordoni V (1987) Molecular evolutionary divergence among North American cave crickets. I. Allozyme variation. *Evolution*, **41**, 1198–1214.

Chakraborty R, Andrade MD, Daiger SP, Budowle B (1992) Apparent heterozygote deficiencies observed in DNA typing data and their implications in forensic applications. *Annals of Human Genetics*, **52**, 922–927.

Chakraborty R, Jin L (1993) A unified approach to study hypervariable polymorphism: statistical considerations of determining relatedness and population distances. In: *DNA Fingerprinting: State of the Science, Volume* (eds Pena SDJ, Chakraborty R, Epplen JT, Jeffreys AJ), pp. 153–175. Birkhauser Verlag, Basel.

Culver DC (1982) *Cave Life Evolution and Ecology*. Harvard University Press, Cambridge and London.

Culver DC, Kane TC, Fong DW (1995). *Adaptation and Natural Selection in Caves*. Harvard University Press, Cambridge and London.

Culver DC, Wilkens H (2000) Critical review of the relevant theories of the evolution of subterranean animals. In: *Ecosystems of the World*, Vol. 30 (eds Wilkens H, Culver DC, Humphreys WF), pp. 381–398. Elsevier Science Ltd, Oxford.

Dowling TE, Martasian DP, Jeffery WR (2002) Evidence for multiple genetic forms with similar eyeless phenotypes in the blind cevefish, *Astyanax mexicanus*. *Molecular Biology and Evolution*, **19**, 446–455.

Espinasa L, Borowsky R (2001) Origins and relationship of cave populations of the blind Mexican tetra, *Astyanax fasciatus*, in the Sierra de El Abra. *Environmental Biology of Fishes*, **62**, 233–237.

Gerber AS, Tibbets CA, Dowling TE (2001) The role of introgressive hybridization in the evolution of the *Gila robusta* complex (Teleostei: Cyprinidae). *Evolution*, **55**, 2028–2039.

Goldstein DB, Linares AR, Cavalli-Sforza LL, Feldman MW (1995) An evaluation of genetic distances for use with microsatellite loci. *Genetics*, **139**, 463–471.

Goodman S (1998) Patterns of extensive genetic differentiation and variation among European harbor seals (*Phoca vitulina vitulina*) revealed using microsatellite DNA polymorphisms. *Molecular Biology and Evolution*, **15**, 104–118.

Guo SW, Thompson WA (1992) Performing the exact test of Hardy–Weinberg proportions for multiple alleles. *Biometrics*, **48**, 361–372.

Kane TC, Barr TC, Badaracca WJ (1992) Cave beetle genetics: geology and gene flow. *Heredity*, **68**, 277–286.

Kimura M (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, **16**, 111–120.

Kumar S, Tamura K, Jakobsen IB, Nei M (2001) *MEGA2: Molecular Evolutionary Genetics Analysis Software*. Arizona State University, Tempe, AZ.

Laing CD, Carmodi GR, Peck SB (1976) Population genetics and evolutionary biology of the cave beetle *Ptomaphagus hirtus*. *Evolution*, **30**, 484–494.

Langecker TG, Wilkens H, Junge P (1991) Introgressive hybridization in the Pachon cave population of *Astyanax fasciatus* (Teleostei: Characidae). *Ichthyological Exploration of Freshwaters*, **2**, 209–212.

Largiadèr CR, Fries V, Kobler B, Bakker CM (1999) Isolation and characterization of microsatellite loci from the three-spined stickleback (*Gasterosteus aculeatus* L.). *Molecular Ecology*, **8**, 342–344.

Lu G, Basley DJ, Bernatchez L (2001) Contrasting patterns of mitochondrial DNA and microsatellite introgressive hybridization between lineages of lake whitefish (*Coregonus clupeaformis*); relevance for speciation. *Molecular Ecology*, **10**, 965–985.

Michalakis Y, Excoffier L (1996) A generic estimation of population subdivision using distances between alleles with special reference to microsatellite loci. *Genetics*, **142**, 1061–1064.

Miller RR (1986) Composition and derivation of the fresh water fish fauna of Mexico *Annales Escuela*. *Nacional Ciencia Biologica*, **30**, 121–153.

Mitchell RW, Russell WH, Elliott WR (1977) Mexican eyeless characin fishes, genus *Astyanax*: environment, distribution, and evolution. *Special Publications of the Museum of Texas Technical University*, **12**, 1–89.

Nei M, Tajima F, Tateno Y (1983) Accuracy of estimated phylogenetic trees from molecular data. *Journal of Molecular Evolution*, **19**, 153–170.

Raymond M, Rousset F (1995a) GENEPOP (Version 1.2): population genetics software for exact test and ecumenism. *Journal of Heredity*, **86**, 248–249.

Raymond M, Rousset F (1995b) Testing heterozygote excess and deficiency. *Genetics*, **140**, 1413–1419.

Rice WR (1989) Analyzing tables of statistical tests. *Evolution*, **43**, 223–225.

Sbordoni V, Allegrucci G, Cesaroni D (2000) Population genetic structure, speciation and evolutionary rates in cave dwelling organisms. In: *Ecosystems of the World*, Vol. 30 (eds Wilkens H, Culver DC, Humphreys WF), pp. 453–477. Elsevier Science Ltd, Oxford.

Schneider S, Jueffer J-M, Roessli D, Excoffier L (1997) *ARLEQUIN, Version 1.1: an exploratory population genetics software environment*. Genetics and Biometry Laboratory, Department of Anthropology, University of Geneva, Geneva.

Slatkin M (1995) A measure of population subdivision based on microsatellite allele frequencies. *Genetics*, **139**, 457–462.

Stepien CA, Morton B, Dabrowska KA, Guarnera RA, Radja T, Radja B (2001) Genetic diversity and evolutionary relationships of the troglodytic 'living fossil' *Congeria kusceri* (Bivalvia: Dreissenidae). *Molecular Ecology*, **10**, 1873–1879.

Strecker U (2003) Polymorphic microsatellites isolated from the cave fish *Astyanax fasciatus*. *Molecular Ecology Notes*, in press.

Swofford DL (2002) *PAUP* Phylogenetic Analysis Using Parsimony (*and Other Methods)*. Sinauer Associates, Sunderland, MA.

Swofford DL, Branson BA, Sievert GA (1980) Genetic differentiation of cavefish populations. *Isozyme Bulletin*, **13**, 109–110.

Tessier N, Bernatchez L, Wright JM (1997) Population structure and the impact of supportive breeding inferred from mitochondrial and microsatellite DNA analyses in landlocked Atlantic salmon, *Salmo salar*. *Molecular Ecology*, **6**, 735–750.

Weir BS, Cockerham CC (1984) Estimating F-statistics for the analysis of population structure. *Evolution*, **38**, 1358–1370.

Wilkens H (1988) Evolution and genetics of epigean and cave *Astyanax fasciatus* (Characidae, Pisces). Support for the neutral mutation theory. In: *Evolutionary Biology*, Vol. 23 (eds Hecht MK, Wallace B), pp. 271–367. Plenum Publishing Corporation, New York and London.

Wilkens H, Burns RJ (1972) A new *Anoptichthys* cave population (Characidae, Pisces). *Annales de Spéléolgie*, **27**, 263–270.

Wilkens H, Strecker U (accepted) Convergent evolution of the cavefish *Astyanax* (Characidae, Teleostei): Genetic evidence from eye and pigment reduction. *Biological Journal of the Linnean Society*.

Zardoya R, Doadrio I (1998) Phylogenetic relationships of Iberian cyprinids: systematic and biogeographical implications. *Proceedings of the Royal Society of London B*, **265**, 1365–1372.

The work was carried out in a research group dealing with the evolution of cave animals, focusing on the genetics and rates of evolution of regressive traits and examining the cave and surface populations of Mexican *Astyanax fasciatus*. U. Strecker is a postdoctoral fellow with interests in the analysis of population structure and speciation processes in fish. H. Wilkens is a curator of Ichthyology and Herpetology at the Zoological Museum in Hamburg. L. Bernatchez's research program focuses on the understanding of pattern and processes of molecular and organismal evolution, as well as their significance to conservation.

Appendix

Frequencies of the six microsatellite loci in the *Astyanax* populations studied. *N* refers to the number of specimens scored for each population and locus.

Locus Ast1

Chichancanab, *N* = 31: 146: 0.081, 150: 0.032, 158: 0.823, 160: 0.016, 162: 0.048.

Majahual, *N* = 51: 130: 0.029, 132: 0.029, 134: 0.049, 136: 0.069, 138: 0.108, 140: 0.049, 142: 0.059, 144: 0.098, 146: 0.196, 148: 0.02, 150: 0.059, 152: 0.118, 156: 0.01, 158: 0.01, 160: 0.039, 162: 0.02, 164: 0.02, 170: 0.01, 172: 0.01.

Micos-S, *N* = 32: 134: 0.016, 136: 0.016, 138: 0.219, 140: 0.75.

Pachon-S, *N* = 30: 134: 0.183, 136: 0.033, 138: 0.083, 140: 0.533, 142: 0.017, 144: 0.05, 146: 0.033, 148: 0.05, 154: 0.017.

Chica, *N* = 21: 134: 0.119, 140: 0.214, 142: 0.024, 146: 0.048, 148: 0.024, 150: 0.476, 152: 0.071, 154: 0.024.

Pachon, *N* = 40: 134: 0.087, 142: 0.013, 150: 0.9.

Sabinos, *N* = 25: 150: 0.96, 154: 0.04.

Tinaja, *N* = 30: 150: 0.967, 152: 0.033.

Locus Ast2

Chichancanab, *N* = 31: 169: 0.806, 171: 0.032, 177: 0.081, 179: 0.081.

Majahual, *N* = 50: 161: 0.01, 165: 0.069, 167: 0.108, 169: 0.137, 171: 0.176, 173: 0.059, 175: 0.108, 177: 0.069, 179: 0.098, 181: 0.108, 183: 0.029, 189: 0.01, 477: 0.02.

Micos-S, *N* = 30: 163: 0.406, 165: 0.031, 167: 0.141, 169: 0.078, 171: 0.016, 181: 0.063, 183: 0.016, 187: 0.047, 203: 0.047, 207: 0.063, 209: 0.031, 511: 0.047, 513: 0.016.

Pachon-S, *N* = 30: 163: 0.15, 165: 0.033, 169: 0.183, 171: 0.117, 173: 0.183, 181: 0.033, 185: 0.05, 195: 0.133, 197: 0.067, 199: 0.05.

Chica, *N* = 21: 163: 0.548, 169: 0.119, 173: 0.143, 177: 0.143, 191: 0.048.

Pachon, *N* = 40: 171: 0.05, 177: 0.95.

Sabinos, *N* = 25: 171: 0.05, 177: 0.95.

Tinaja, *N* = 30: 177: 0.667, 179: 0.333.

Locus Ast4

Chichancanab, *N* = 31: 341: 0.129, 343: 0.806, 345: 0.065.

Majahual, *N* = 50: 169: 0.02, 171: 0.01, 181: 0.01, 183: 0.07, 185: 0.2, 187: 0.05, 189: 0.07, 191: 0.09, 193: 0.02, 195: 0.05, 199: 0.01, 201: 0.03, 203: 0.11, 205: 0.05, 207: 0.07, 209: 0.07, 211: 0.03, 213: 0.01, 227: 0.02, 243: 0.01.

Micos-S, *N* = 32: 169: 0.047, 181: 0.078, 183: 0.031, 185: 0.047, 189: 0.047, 191: 0.031, 195: 0.297, 197: 0.344, 199: 0.031, 205: 0.031, 223: 0.016.

Pachon-S, *N* = 29: 177: 0.069, 181: 0.086, 183: 0.052, 189: 0.017, 191: 0.121, 193: 0.034, 195: 0.172, 197: 0.121, 205: 0.155, 209: 0.017, 219: 0.017, 221: 0.017, 223: 0.121.

Chica, *N* = 21: 177: 0.31, 179: 0.071, 183: 0.024, 185: 0.071, 195: 0.5, 197: 0.024.

Pachon, *N* = 40: 191: 0.125, 193: 0.712, 195: 0.138, 197: 0.025.

Sabinos, *N* = 25: 183: 0.02, 195: 0.72, 197: 0.24, 209: 0.02.

Tinaja, *N* = 30: 195: 1.

Locus Ast9

Chichancanab, *N* = 31: 175: 0.065, 181: 0.79, 183: 0.145.

Majahual, *N* = 51: 165: 0.029, 171: 0.02, 173: 0.029, 175: 0.029, 177: 0.176, 179: 0.275, 181: 0.147, 183: 0.225, 185: 0.069.

Micos-S, *N* = 32: 167: 0.547, 175: 0.391, 177: 0.031, 189: 0.031.

Pachon-S, *N* = 30: 167: 0.183, 175: 0.817.

Chica, *N* = 21: 175: 1.

Pachon, *N* = 40: 167: 0.013, 173: 0.025, 175: 0.962.

Sabinos, *N* = 25: 175: 1.

Tinaja, *N* = 30: 175: 1.

Locus Ast10

Chichancanab, *N* = 31: 238: 0.726, 240: 0.242, 242: 0.016, 244: 0.016.

Majahual, *N* = 51: 234: 0.01, 236: 0.088, 238: 0.412, 240: 0.353, 242: 0.029, 244: 0.029, 248: 0.01, 252: 0.029, 254: 0.029, 256: 0.01.

Micos-S, *N* = 32: 222: 0.031, 238: 0.281, 242: 0.016, 244: 0.453, 248: 0.078, 250: 0.078, 252: 0.047, 254: 0.016.

Pachon-S, *N* = 30: 222: 0.017, 238: 0.533, 244: 0.167, 246: 0.017, 248: 0.05, 250: 0.017, 252: 0.083, 254: 0.05, 256: 0.067.

Chica, *N* = 21: 238: 0.286, 242: 0.071, 244: 0.595, 248: 0.024, 254: 0.024.

Pachon, *N* = 40: 222: 0.025, 238: 0.125, 240: 0.013, 252: 0.013, 254: 0.825.

Sabinos, *N* = 250: 236: 0.16, 248: 0.76, 254: 0.08.

Tinaja, *N* = 30: 238: 0.083, 240: 0.05, 244: 0.017, 248: 0.733, 250: 0.033, 252: 0.083.

Locus Ast13

Chichancanab, *N* = 31: 133: 0.032, 137: 0.968.

Majahual, *N* = 51: 119: 0.059, 127: 0.01, 129: 0.01, 131: 0.049, 133: 0.127, 135: 0.069, 137: 0.088, 139: 0.098, 141: 0.108, 143: 0.088, 145: 0.01, 147: 0.01, 149: 0.039, 151: 0.069, 153: 0.02, 155: 0.049, 157: 0.029, 159: 0.029, 161: 0.029, 163: 0.01.

Micos-S, *N* = 31: 101: 0.145, 103: 0.032, 119: 0.016, 125: 0.452, 127: 0.129, 143: 0.194, 145: 0.016, 149: 0.016.

Pachon-S, *N* = 30: 113: 0.033, 115: 0.2, 125: 0.2, 127: 0.117, 141: 0.067, 143: 0.067, 145: 0.117, 147: 0.017, 149: 0.017, 153: 0.067, 155: 0.017, 159: 0.017, 161: 0.05, 169: 0.017.

Chica, *N* = 21: 121: 0.095, 127: 0.167, 137: 0.024, 141: 0.119, 143: 0.167, 149: 0.429.

Pachon, *N* = 40: 129: 0.863, 141: 0.013, 153: 0.125.

Sabinos, *N* = 25: 137: 0.54, 139: 0.12, 141: 0.18, 143: 0.1, 145: 0.04, 149: 0.02.

Tinaja, *N* = 30: 137: 0.583, 141: 0.367, 145: 0.033, 149: 0.017.