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Influence of hydrogeographic history and hybridization on the distribution of genetic variation in the pupfishes *Cyprinodon atrorus* and *C. bifasciatus*

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Abstract

The evolutionary importance of hybridization in animals has been subject of much debate. In this study, we examined the influence of hydrogeographic history and hybridization on the present distribution of nuclear and mitochondrial DNA variation in two pupfish species, *Cyprinodon atrorus* and *Cyprinodon bifasciatus*. Results presented here indicate that there has been limited introgression of nuclear genes; however, mtDNA introgression has been substantial, with complete replacement of the *C. bifasciatus* mitochondrial genome by that of *C. atrorus*. Subsequent to this replacement, there has been diversification of mitochondrial haplotypes along major geographic regions in the basin. Evidence was also found that mitochondrial replacement follows a predictable, cyclical pattern in this system, with isolation and diversification followed by re-contact and replacement of *C. bifasciatus* mitochondrial haplotypes by those of *C. atrorus*. This pattern is best explained by a combination of a numeric bias towards *C. atrorus* and mating site selection rather than selection for *C. atrorus* mitochondrial genome. These results demonstrate the important role hybridization can play in evolution.

Keywords: *Cyprinodon*, hydrogeography, introgressive hybridization, mitochondrial DNA, nuclear DNA, phylogeography

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Introduction

A central challenge for evolutionary biologists is to synthesize observed evolutionary processes and patterns into general explanations of organismal diversity. While hybridization has generally been considered to be of limited relevance to animal evolution (Barton & Hewitt 1985; Arnold 1997), potential evolutionary consequences of hybridization are numerous, including transfer of adaptive variation between species, production of novel adaptations (Anderson 1949; Stebbins 1959; Lewontin & Birch 1966; Grant 1981), and creation of new species through recombination or reinforcement (Howard 1993; Arnold 1997; Dowling & Secor 1997). Introgressive hybridization can also be important to studies that aim to interpret broad-scale biogeographic and population genetic patterns within species as it can lead to confusion in interpreting population genetic structure by inflating genetic variance within species and reducing perceived divergence between species (Bernatchez *et al*. 1995; Glemet *et al*. 1998; Wilson & Bernatchez 1998; Redenbach & Taylor 2002). Quantification of the impact of introgressive hybridization is particularly critical in many cases involving endangered species because effective management may depend on accurate assessment of historical influences on present population genetic patterns.

In this study, we consider how present patterns of genetic variation in two distantly related pupfish species, *Cyprinodon atrorus* and *Cyprinodon bifasciatus*, endemic to Cuatro Cienegas, Coahuila, Mexico, has been shaped by hydrogeography and introgressive hybridization. Cuatro Cienegas is a small (~840 km^2) intermontane basin in the Chihuahuan desert that exhibits exceptional biodiversity and high levels of endemism. The basin is divided into eastern and western lobes that are partially separated by the Sierra de los Pinos (de San Marcos) (Fig. 1). Associated with the bajada (lower

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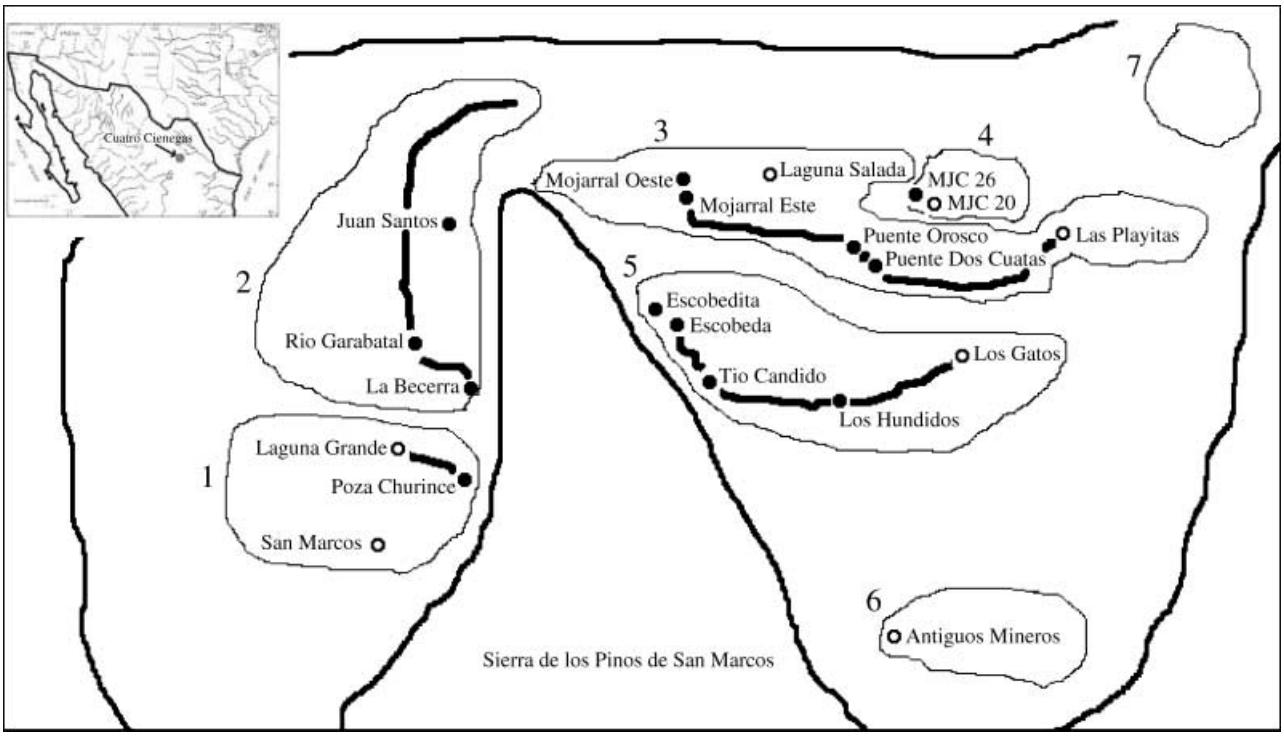

Fig. 1 Map of the Cuatro Cienegas basin, Coahuila, Mexico. Numbers indicate major drainages hypothesized by Minckley (1969): (1) Rio Churince; (2) La Becerra/Rio Garabatal/Juan Santos; (3) Rio Mesquites; (4) Rio Puente Chiquito; (5) Escobeda/Tio Candido/Los Hundidos; (6) Antiguos Mineros; and (7) Rio Salado de los Nadadores. Precise locality information upon request from the corresponding author. Names represent collection localities for *Cyprinodon atrorus* and *Cyprinodon bifasciatus*. Open circles represent *C. atrorus* populations, whereas closed circles represent *C. bifasciatus* populations.

mountain slope) of the northern tip of this mountain is a series of thermal springs that arise from fissures along an active fault (Minckley 1969). Springs are represented by either isolated pools or outflow into riverine systems that terminate in large evaporative lagunas (lakes) or cienegas (marshes) on the basin floor (barrial). Along the transition from spring to terminal habitats, there is typically a steep environmental gradient, ranging from near physicochemical constancy (pH, salinity, temperature) of thermal springs and upper, environmentally buffered riverine habitats to severe daily and/or seasonal physicochemical fluctuations within peripheral environments and terminal evaporative lagunas/marshes. Middle and lower riverine reaches, as well as interfaces with peripheral habitats, are environmentally intermediate.

While locally abundant, these pupfishes are considered endangered due to their limited geographic ranges. *Cyprinodon atrorus* and *C. bifasciatus* occupy opposite extremes along steep environmental gradients, with *Cyprinodon atrorus* typically found in the most severe and fluctuating environments associated with the basin floor and its distribution generally surrounds that of *C. bifasciatus*, which is limited to physicochemically benign thermal springs and environmentally buffered riverine habitats. These species are vastly different in their faunal associations (Miller 1968; Minckley 1969), environmental tolerances (Carson, unpublished), and behavioural ecology (Arnold 1972). Although *C. atrorus* and *C. bifasciatus* generally are found at opposite ends of this environmental continuum, their ranges overlap in environmentally intermediate habitats. In these regions, hybridization is extensive and is predominated by advanced backcrosses (Carson, unpublished).

Present-day hydrogeographic relationships among major systems are poorly understood due to limited mapping of epigean connectivity and uncharted, but presumably extensive, subterranean networks are thought to characterize the karstic subsurface. A preliminary hypothesis of surficial drainage patterns (Minckley 1969) divides the basin into seven major drainage systems (Fig. 1): (i) Rio Churince, (ii) La Becerra/Rio Garabatal/Juan Santos, (iii) Rio Mesquites, (iv) Rio Puente Chiquito/Las Playitas, (v) Escobeda/Tio Candido/Los Hundidos/Los Gatos, (vi) Antiguos Mineros, and (vii) Rio Salado de los Nadadores (currently dry and therefore not relevant). Based on Minckley's hypothesized drainage relationships, we predict the following patterns of connectivity and relationships among locations (Fig. 2). For *C. atrorus*, we expect two major regions that should include populations from the western basin (Laguna Grande

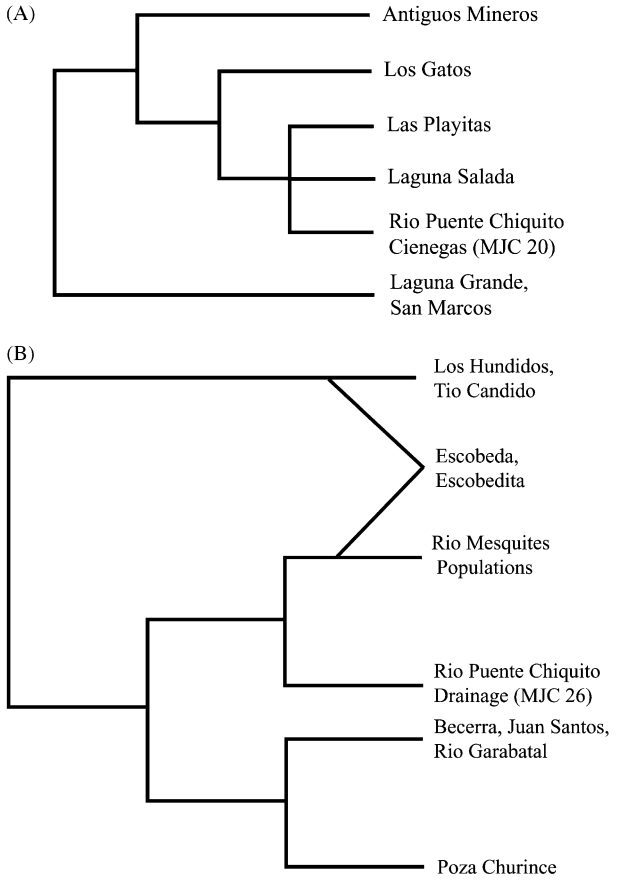

Fig. 2 Hypothesized drainage relationships for populations of *Cyprinodon atrorus* (A) and *Cyprinodon bifasciatus* (B).

and San Marcos) and the central-southeastern basin [Antiguos Mineros, Las Playitas, Los Gatos, Laguna Salada, and Rio Puente Chiquito marshes (MJC 20)]. For *C. bifasciatus*, populations should be largely divided into western-central basin [Rio Churince, La Becerra, Juan Santos, Rio Mesquites, and an unnamed river associated with the Rio Puente Chiquito drainage (MJC 26)] and southeastern basin (Los Hundidos and Tio Candido) components. Importantly, Escobeda and Escobedita should show evidence of connectivity to both the central basin and the southeastern basin, as these sites sit at a divide between these two major regions and exhibit evidence of past surface flow into both regions.

Materials and methods

Specimen collection

We collected 212 *Cyprinodon atrorus* and 461 *Cyprinodon bifasciatus* from 7 and 13 localities, respectively, representing the known distribution of both species (Fig. 1). Specimens were preserved in 95% ethanol and whole genomic DNA was extracted from muscle tissue using the proteinase K/phenol–chloroform as described in Tibbets & Dowling (1996).

Analysis of single-stranded conformational polymorphisms

Sequence variation for mitochondrial (cytochrome *b* — cyt *b*) and nuclear (creatine kinase, CK-A intron 7; recombination activation gene, RAG-1; and triosephosphate isomerase, TPI-B intron 4) genes within and among populations and species was characterized through analysis of single-stranded conformational polymorphisms (SSCPs — Glavac & Dean 1993; Takeda *et al*. 1995) using [α^{32}P]-dATP (Cetus–PerkinElmer Corp.) labelled DNA fragments as described in Dowling *et al*. (2005). For cyt *b*, the SSCP primers LC_{Gila} (5′-GCATCATTCTTCATCTGTAT-3′; Dowling, unpublished) and HD (Schmidt *et al*. 1998) were used to amplify a 312-bp segment of the gene under the following polymerase chain reaction (PCR) conditions: (94 °C, 1 min; 48 °C, 1 min; 72 °C, 2 min; 25 cycles). For CK-A7, the primers CK-F1 and CK-R1 (Quattro & Jones 1999) were used to amplify a 282-bp fragment using the following PCR conditions: (94 °C, 30 s; 60 °C, 30 s; 72 °C, 30 s; 30 cycles). For RAG-1, the primers Rag-B (5′-TTGGAAGTGTAGAGCC-AGTGGT-3′, Dowling, unpublished) and RAG-Cpup (5′-ATGGCGTGCTACCTGTCC-3′; Carson, unpublished) were used to amplify a 355-bp fragment using the following PCR conditions: (94 °C, 30 s; 55 °C, 30 s; 72 °C, 30 s; 30 cycles). For TPI-B4, the primers TPI-B4F2 and TPI-B4R1 (Merritt & Quattro 2001) were used to amplify a 213-bp fragment using the following PCR conditions: (94 °C, 30 s; 55 °C, 30 s; 72 °C, 30 s; 35 cycles). SSCP gels were run at room temperature for 18 h at 6 W on 6% (cyt *b* and RAG-1) or 8% (CK-A7 and TPI-B4) acrylamide gels. Ambiguous haplotypes or haplotypes that were difficult to score across the gel were re-run on additional gels (or sequenced) until all haplotypes were resolved.

Sequencing

At least one representative of each SSCP variant per gel was sequenced using an ABI 377 automated sequencer. Amplification parameters to generate PCR products for sequencing were the same as those described above except [α^{32}P]-dATP was not used and reaction volumes were larger (25 μL). The cyt *b* primers LA (Schmidt *et al*. 1998) and HD (Schmidt *et al*. 1998) were used to amplify the first half of the gene, including the SSCP fragment. The primers LD_{RBS} (5′-ACCCTAACACGATTCTTTGC-3′, Dowling, unpublished) and HA (Schmidt *et al*. 1998) were used to amplify the remainder of the gene for each confirmed haplotypic variant, and resulting products sequenced with the LD primer to characterize the remaining portion of the gene. For CK-A7, RAG-1, and TPI-B4, PCR protocol for sequencing was identical to that of SSCP and allelic variants were sequenced in both directions. All sequences were aligned by eye in MACDNASIS (Hitachi Corp.).

Population genetic analysis

To place mitochondrial and nuclear gene variation in a phylogenetic context, maximum parsimony (as implemented in PAUP, Swofford 1998) was used to determine relationships among cyt *b* SSCP haplotypes (based on sequences of the entire gene) and nuclear gene alleles. Population genetic parameters for SSCP haplotypes were examined using ARLEQUIN (Schneider *et al*. 2000). *P* values for analyses of Hardy–Weinberg and linkage disequilibrium were corrected for multiple comparisons using the Bonferroni correction (Sokal & Rohlf 1995). AMOVA (Excoffier *et al*. 1992) was used to quantify population genetic structure for all genes. Geographic structure of mitochondrial SSCP haplotypes was also examined using nested clade analysis (reviewed in Templeton 2001). TCS 1.18 (Clement *et al*. 2000) was used to determine clade structure and GEODIS 2.0 (Posada *et al*. 2000) was used to test for significant associations between clades and geography.

Results

Nuclear gene variation

Standard measures of genetic variation in *Cyprinodon atrorus* and *Cyprinodon bifasciatus* indicated that allelic variation at the three nuclear loci is generally low in both species (Table 1) in comparison to mitochondrial variation (see below). Populations were generally in Hardy–Weinberg equilibrium at each locus and there was no evidence of linkage disequilibrium among loci (data not shown). In *C. atrorus*, CK-A7 was most variable, segregating for three alleles typical of the species (cA1-cA3; Table 2), with a single individual from Laguna Salada carrying an allele typical of *C. bifasciatus* (cB1; Table 2). At this locus, cienega and playa lake systems associated with the central basin [Laguna Salada, Las Playitas, Los Gatos, and cienegas associated with the origin of Canal Tio Julio (MJC 20)] contained more variation than small or geographically restricted populations (Antiguos Mineros, Laguna Grande, and San Marcos). At RAG-1, most populations of *C. atrorus* were fixed for a single allele (rA1; Table 2) characteristic of the species, although three populations also carried a low frequency of an allele typical of *C. bifasciatus* (rB1; Table 2). Similarly, at TPI-B4 *C. atrorus* populations were generally fixed for a typical *C. atrorus* allele (tA1; Table 2), although three populations carried the typical *C. bifasciatus* allele (tB1; Table 2) at low frequency.

In *C. bifasciatus*, CK-A7 showed the greatest variation (12 alleles), followed by RAG-1 (four alleles) then TPI-B4 (three alleles) (Table 2). At CK-A7, most populations were fixed or nearly fixed for the common *C. bifasciatus* allele (cB1; Table 2), although the common *C. atrorus* allele (cA1) was found in four populations. Four populations also segregated alleles unique to *C. bifasciatus* at low frequency (Table 2). At CK-A7, variation in *C. bifasciatus* was highest in Juan Santos and La Becerra/Rio Garabatal. Minimal variation was found in the Rio Mesquites system. The remaining smaller, isolated populations of *C. bifasciatus*, such as Escobeda, Los Hundidos, Poza Churince, and Tio Candido, contained no variation at CK-A7. At the remaining loci, RAG-1 and TPI-B4, variation in *C. bifasciatus* was more limited. At RAG-1 (Table 2), most populations were fixed for the common *C. bifasciatus* allele (rB1), with minor variation contributed by rare alleles unique to *C. bifasciatus* (rB2 and rB3), and a low frequency of an allele typical of *C. atrorus* (rA1). Variation at RAG-1 was highest in Juan Santos, segregating for a common allele as well as an allele unique to this system. However, interpretation of variation at RAG-1 was confounded by one *C. bifasciatus* allele (rB2; Table 2) that could not be reliably differentiated from the common *C. bifasciatus* allele (rB1) on SSCP gels. These two common alleles were easily distinguishable from *C. atrorus*-specific alleles and therefore counted as a single allele. At TPI-B4 there were only three alleles in *C. bifasciatus* (Table 2). One of these alleles (tB1; Table 2) predominated, one was rare (tB2), and one (tA1) likely represented an introgressed allele from nearby *C. atrorus* populations.

There was clear differentiation between *C. atrorus* and *C. bifasciatus* (Table 2) at the nuclear gene level. Minimum nucleotide differences between alleles typical of *C. atrorus* and *C. bifasciatus* varied from one in CK-A7, three in RAG-1, and two in TPI-B4, with all changes represented by nucleotide substitutions. Three separate analyses (one for each gene) were run, with geographic samples nested within species. Most variation in CK-A7 ($F_{CT} = 0.928$; $P < 0.001$), RAG-1 ($F_{CT} = 0.960$; $P < 0.001$), and TPI-B4 ($F_{CT} = 0.987$; $P < 0.001$) was partitioned among species rather than by geography. Variation among populations within species was low in TPI-B4 ($F_{SC} = 0.0002$; $P < 0.001$), moderate in CK-A7 ($F_{SC} = 0.101$; $P < 0.001$), and high in RAG-1 ($F_{SC} = 0.490$; $P < 0.001$), with the elevated value at RAG-1 generated by a high frequency (69%) of a unique allele (rB3) at Juan Santos. In six separate analyses (one analysis for each gene by species combination), variation was not distributed among hydrogeographic regions as defined by Minckley (1969) for either *C. atrorus* ($F_{CT} = -0.0714$, $P = 0.696$; $F_{CT} = -0.033$, $P = 0.848$; $F_{CT} = 0.003$, $P = 0.704$ for CK-A7, RAG-1, and TPI-B4, respectively) or *C. bifasciatus* ($F_{CT} = 0.007$, $P = 0.223$; $F_{CT} = -0.026$, $P = 0.200$; $F_{CT} = 0.002$, $P = 0.532$ for CK-A7, RAG-1, and TPI-B4, respectively).

Mitochondrial variation

Surveys of variation in a mitochondrial gene (cyt *b*) identified 10 and 15 haplotypes in *C. atrorus* and *C. bifasciatus*, respectively. Sequence divergence was low between any pair of haplotypes (< 1%); however, only four were shared

Table 1 Standard measures of genetic variation in CK-A7, RAG-1, and TPI-B4 for each *Cyprinodon atrorus* and *Cyprinodon bifasciatus* population (as labelled in Fig. 1). Standard errors are in parentheses

Locality	Locus	*N*	Number of alleles	Theta	Gene diversity	Mean number of pairwise differences	Nucleotide diversity
C. atrorus populations							
Antiguos Mineros	CK-A7	28	1	0 (0)	0 (0)	0 (0)	0 (0)
	RAG-1	29	1	0 (0)	0 (0)	0 (0)	0 (0)
	TPI-B4	29	1	0 (0)	0 (0)	0 (0)	0 (0)
Laguna Grande	CK-A7	40	1	0 (0)	0 (0)	0 (0)	0 (0)
	RAG-1	40	2	0.6057 (0.3709)	0.0250 (0.0242)	0.0750 (0.1481)	0.0003 (0.0006)
	TPI-B4	40	2	0.4038 (0.2943)	0.0494 (0.0332)	0.0987 (0.1711)	0.0006 (0.0011)
Laguna Salada	CK-A7	30	4	0.8578 (0.4716)	0.4847 (0.0477)	0.5540 (0.4611)	0.0024 (0.0022)
	RAG-1	30	2	0.6433 (0.3965)	0.0655 (0.0433)	0.1966 (0.2496)	0.0007 (0.0010)
	TPI-B4	30	2	0.4289 (0.3137)	0.0333 (0.0320)	0.0667 (0.1397)	0.0004 (0.0009)
Las Playitas	CK-A7	30	3	0.4289 (0.3137)	0.0977 (0.0519)	0.0992 (0.1721)	0.0004 (0.0008)
	RAG-1	30	1	0 (0)	0 (0)	0 (0)	0 (0)
	TPI-B4	30	1	0 (0)	0 (0)	0 (0)	0 (0)
Los Gatos	CK-A7	32	3	0.4230 (0.3091)	0.2009 (0.0632)	0.2045 (0.2550)	0.0009 (0.0012)
	RAG-1	32	1	0 (0)	0 (0)	0 (0)	0 (0)
	TPI-B4	32	1	0 (0)	0 (0)	0 (0)	0 (0)
MJC 20	CK-A7	29	2	0.2160 (0.2160)	0.4217 (0.0505)	0.4229 (0.3899)	0.0018 (0.0018)
	RAG-1	30	1	0 (0)	0 (0)	0 (0)	0 (0)
	TPI-B4	30	2	0.4289 (0.3137)	0.0333 (0.0320)	0.0667 (0.1397)	0.0004 (0.0009)
San Marcos	CK-A7	10	1	0 (0)	0 (0)	0 (0)	0 (0)
	RAG-1	10	2	0.8456 (0.5403)	0.1000 (0.0880)	0.3000 (0.3263)	0.0011 (0.0013)
	TPI-B4	10	1	0 (0)	0 (0)	0 (0)	0 (0)
C. bifasciatus populations							
Becerra	CK-A7	37	7	1.0257 (0.5143)	0.2966 (0.0690)	0.3938 (0.3723)	0.0017 (0.0018)
	RAG-1	31	2	0.2129 (0.2129)	0.0635 (0.0420)	0.0635 (0.1361)	0.0002 (0.0006)
	TPI-B4	36	2	0.2063 (0.2063)	0.0548 (0.0366)	0.0548 (0.1259)	0.0003 (0.0008)
Escobeda	CK-A7	37	1	0 (0)	0 (0)	0 (0)	0 (0)
	RAG-1	39	1	0 (0)	0 (0)	0 (0)	0 (0)
	TPI-B4	38	1	0 (0)	0 (0)	0 (0)	0 (0)
Juan Santos	CK-A7	40	3	0.4038 (0.2943)	0.1421 (0.0513)	0.1441 (0.2097)	0.0006 (0.0010)
	RAG-1	40	2	0.2019 (0.2019)	0.4351 (0.0396)	0.4351 (0.3953)	0.0016 (0.0016)
	TPI-B4	38	1	0 (0)	0 (0)	0 (0)	0 (0)
MJC 26	CK-A7	30	1	0.2145 (0.2145)	0.0333 (0.0320)	0.0334 (0.0979)	0.0001 (0.0005)
	RAG-1	30	1	0 (0)	0 (0)	0 (0)	0 (0)
	TPI-B4	30	2	0.4354 (0.3187)	0.0357 (0.0341)	0.0714 (0.1449)	0.0004 (0.0009)
Mojarral Este	CK-A7	30	2	0 (0)	0 (0)	0 (0)	0 (0)
	RAG-1	30	1	0 (0)	0 (0)	0 (0)	0 (0)
	TPI-B4	28	1	0 (0)	0 (0)	0 (0)	0 (0)
Mojarral Oeste	CK-A7	35	1	0.6226 (0.3823)	0.0567 (0.0381)	0.1423 (0.2085)	0.0006 (0.0010)
	RAG-1	35	1	0 (0)	0 (0)	0 (0)	0 (0)
	TPI-B4	30	1	0 (0)	0 (0)	0 (0)	0 (0)
Los Hundidos	CK-A7	35	3	0 (0)	0 (0)	0 (0)	0 (0)
	RAG-1	36	1	0 (0)	0 (0)	0 (0)	0 (0)
	TPI-B4	36	1	0 (0)	0 (0)	0 (0)	0 (0)
Poza Churince	CK-A7	39	1	0 (0)	0 (0)	0 (0)	0 (0)
	RAG-1	39	1	0 (0)	0 (0)	0 (0)	0 (0)
	TPI-B4	40	1	0 (0)	0 (0)	0 (0)	0 (0)
Puente Dos Cuatas	CK-A7	59	2	0.3743 (0.2717)	0.0336 (0.0230)	0.0676 (0.1398)	0.0003 (0.0007)
	RAG-1	59	2	0.5614 (0.3413)	0.0336 (0.0230)	0.1008 (0.1725)	0.0004 (0.0007)
	TPI-B4	60	2	0.3731 (0.2708)	0.0331 (0.0226)	0.0661 (0.1381)	0.0004 (0.0009)
Puente Orosco	CK-A7	39	2	0.6088 (0.3730)	0.0506 (0.0340)	0.1532 (0.2168)	0.0007 (0.0010)
	RAG-1	37	1	0 (0)	0 (0)	0 (0)	0 (0)
	TPI-B4	39	1	0 (0)	0 (0)	0 (0)	0 (0)
Rio Garabatal	CK-A7	30	4	0.4289 (0.3137)	0.3701 (0.0727)	0.4161 (0.3860)	0.0018 (0.0018)
	RAG-1	30	2	0.2145 (0.2145)	0.0966 (0.0507)	0.0966 (0.1698)	0.0004 (0.0007)
	TPI-B4	29	1	0 (0)	0 (0)	0 (0)	0 (0)
Tio Candido	CK-A7	30	1	0 (0)	0 (0)	0 (0)	0 (0)
	RAG-1	30	1	0 (0)	0 (0)	0 (0)	0 (0)
	TPI-B4	22	1	0 (0)	0 (0)	0 (0)	0 (0)

Table 2 Numbers and distribution of CK-A7 (c), RAG-1 (r), and TPI-B4 (t) alleles in samples of *Cyprinodon atrorus* (A) and *Cyprinodon bifasciatus* (B). Alleles are designated numerically by locus and species in which they are found (as indicated above)

	CK-A7 Alleles															RAG-1 Alleles					TPI-B4 Alleles			
Locality	cA1	cA2	cA3	cA4	cB1	cB2	cB3	cB4	cB5	cB6	cB7	cB8	cB9	cB10	Total	rA1	rB1	rB2	rB3	Total	tA1	tB1	tB2	Total
C. atrorus populations																								
Antiguos Mineros	56														56	58				58	58			58
Laguna Grande	80														80	79	1			80	78	2		80
Laguna Salada	39	19	1		1										60	58	2			60	59	1		60
Las Playitas	57	2		1											60	60				60	60			60
Los Gatos	57	6		1											64	64				64	64			64
MJC 20	41	17													58	60				60	59	1		60
San Marcos	20														20	19	1			20	20			20
															0					0				0
C. bifasciatus populations															0					0				0
Becerra	2				62	1		4	1	2	2				74		60	2		62		70	2	72
Escobeda					74										74		78			78		76		76
Juan Santos					74							1	5		80		25		55	80		76		76
Los Hundidos					60										60		60			60		60		60
MJC 26					59									1	60		60			60	1	55		56
Mojarral Este					70										70		70			70		60		60
Mojarral Oeste	1	1			68										70		72			72		72		72
Poza Churince					78										78		78			78		80		80
Puente Dos Cuatas	2				116										118	2	116			118	2	118		120
Puente Orosco		2			76										78		74			74		78		78
Rio Garabatal					47	4	1	8							60		57	3		60		58		58
Tio Candido					60										60		60			60		44		44
Total	355	47	1	2	845	5	1	12	1	2	2	1	5	1	1280	400	814	5	55	1274	401	851	2	1254

Table 3 Numbers and distribution of cyt *b* SSCP haplotypes (identified by letters) in populations of *Cyprinodon atrorus* and *Cyprinodon bifasciatus*

	Mitochondrial haplotype																					
Locality	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	Total
C. atrorus populations																						
Antiguos Mineros		9															21					30
Laguna Grande	40																					40
Laguna Salada		30																				30
Las Playitas		29	1																			30
Los Gatos		29		1	5	1						1	3				1			1		42
San Marcos	10																					10
Poza Churince	40																					40
C. bifasciatus populations																						
Becerra	40																					40
Escobedita												30										30
Escobeda							29				2	7							1			39
Juan Santos									40													40
Los Hundidos							30															30
MJC 26	1	15					14															30
MJC 20		30																				30
Mojarral Oeste	1						4			8				15	5			3				36
Mojarral Este	8	1					7			5				13	2							36
Puente Orosco	6	14			1		4	3				10				2						40
Puente Dos Cuatas	8	7			6							7				2						30
Rio Garabatal	29																				1	30
Tio Candido							30															30
Total	183	164	1	1	12	1	118	3	40	13	2	55	3	28	7	4	22	3	1	1	1	663

between species (Table 3; Fig. 3). In *C. atrorus* most variation (Table 3) was concentrated in the Los Gatos population, which resides in a large, stable marsh and laguna complex, whereas other *C. atrorus* populations were fixed or nearly fixed for single haplotypes (Table 3). Standard measures of population genetic variation (Table 4) showed that *C. atrorus* populations had an average of 2.3 haplotypes per population (range 1–8) and gene diversity of 0.15 (range 0.00–0.51). Estimated nucleotide diversity in *C. atrorus* was 0.001 (range 0.000–0.004) and the average pairwise comparison revealed 0.32 differences (range 0–10). Theta ranged from 0.00 to 2.09 (average 0.44). In *C. bifasciatus*, standard population genetic measurements (Table 4) showed that this species had an average of three haplotypes per population (range 1–7). Most of this variation was harboured in the Rio Mesquites populations (Mojarral Este and Oeste, Puente Dos Cuatas, and Puente Orosco; Table 3), with three other populations (Escobeda, MJC 26, and Rio Garabatal) exhibiting low to moderate variation (2–4 haplotypes). The remaining six populations of *C. bifasciatus* were fixed for a single haplotype. Gene diversity in *C. bifasciatus* ranged from 0.00 to 0.80 (average 0.32), whereas nucleotide diversity ranged from 0.000 to 0.006 (average 0.002) and the average pairwise comparison yielded 0.67 differences (range 0–12). Theta ranged from 0.00 to 1.69 (average 0.65).

In contrast to nuclear genes, mitochondrial variation was not structured according to species ($F_{CT} = 0.090$; $P = 0.078$), but was significantly partitioned among populations within species ($F_{SC} = 0.727$; $P < 0.001$). Two follow-up AMOVA analyses were used to determine if genetic variation within each species was distributed according to geography, specifically Minckley's (1969) hypothesis of surficial drainage relationships in the basin. Analysis of *C. atrorus* indicated that genetic variation was associated with hydrogeography ($F_{ST} = 0.813$; $P < 0.001$), with most of this explained by drainage regions ($F_{CT} = 0.821$; $P < 0.001$). Hydrogeography was also important for *C. bifasciatus* ($F_{ST} = 0.722$; $P < 0.001$); however, variation was partitioned among drainage regions ($F_{CT} = 0.403$; $P = 0.006$) and among populations within regions ($F_{SC} = 0.534$; $P < 0.001$).

Nested clade analysis indicated that the two primary lineages, 'A' and 'B' (Fig. 3), were subdivided into three major nested clades (2-1, 2-2, and 2-3; Fig. 4). Clade 2-1 showed evidence of restricted gene flow with limited dispersal, whereas clade 2-2 was subject to past fragmentation. Clade 2-3 showed evidence of range expansion with long-distance colonization. Clade 2-1 primarily comprised populations of *C. atrorus* from Antiguos Mineros, Laguna Salada, Las Playitas, Los Gatos, and MJC 20, as well as *C. bifasciatus* populations from MJC 26, Mojarral Este, Puente

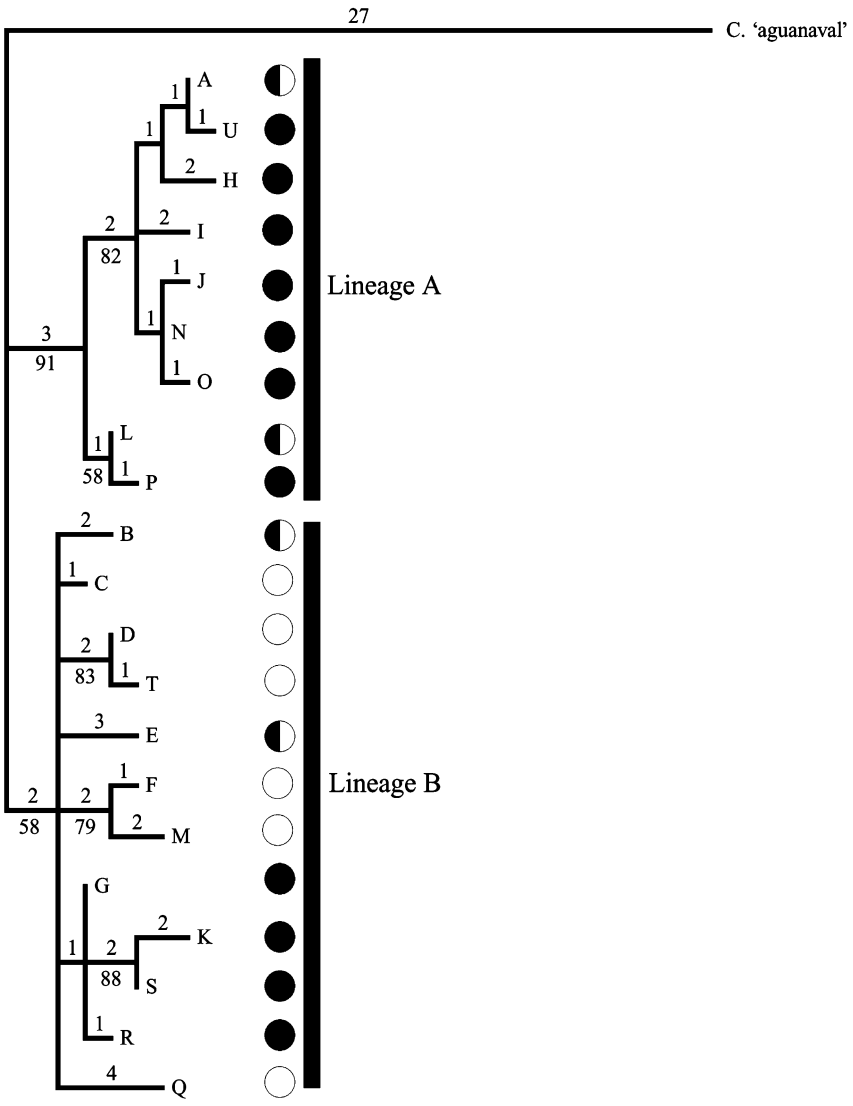

Fig. 3 One of three maximum-parsimony trees obtained from analysis of sequence from the SSCP fragment of the mitochondrial cyt *b* gene (length = 70 steps, CI = 0.80, RI = 0.932, uninformative characters excluded from the analysis but included in branch lengths of the figure). The outgroup is represented by *Cyprinodon* sp. from the Rio Aguanaval, Mexico. Upper numbers represent number of steps, whereas lower numbers represent level of bootstrap support. Circles beside lineages 'A' and 'B' represent presence or absence of each haplotype in *Cyprinodon atrorus* (white) and *Cyprinodon bifasciatus* (black).

Orosco, and Puente Dos Cuatas. Clade 2-2 contained *C. bifasciatus* populations from Escobeda, Los Hundidos, MJC 26, Mojarral Este, Mojarral Oeste, Puente Orosco, and Tio Candido. Finally, Clade 2-3 included *C. atrorus* populations from Laguna Grande and San Marcos, and *C. bifasciatus* populations from Becerra, Escobedita, Escobeda, Juan Santos, MJC 26, Mojarral Este, Mojarral Oeste, Poza Churince, Puente Orosco, Puente Dos Cuatas, and Rio Garabatal.

Discussion

Genetic variation

The distribution of genetic variation in Cuatro Cienegas *Cyprinodon* shows a complex interplay between hydrogeography, species, and introgressive hybridization. At the nuclear gene level, *C. atrorus* and *C. bifasciatus* were clearly distinctive, with only minor influence of introgression between species. There were insufficient levels of variation at nuclear genes to examine hydrogeographic relationships hypothesized by Minckley (1969); however, these markers were useful for discrimination between *C. atrorus* and *C. bifasciatus*.

Introgressive hybridization has had a significant impact on patterns of mtDNA variation. Mitochondrial DNA indicates a close phylogenetic relationship between the two Cuatro Cienagas taxa (Echelle *et al*. 2005); however, analyses of allozymes and morphology indicate that *C. atrorus* is member of a clade including other species from the old Nazas drainage while *C. bifasciatus* is a divergent basal lineage (Miller 1968; Lozano *et al*. 1993; Echelle & Echelle 1998). Given the placement of *C. bifasciatus* as a member of the old Nazas lineage based on mtDNA, the *C. bifasciatus* mitochondrial genome has been replaced by that of *C. atrorus*. This replacement has impacted all populations of *C. bifasciatus* as all haplotypes were very similar, forming

Table 4 Standard measures of genetic variation in the SSCP fragment from cyt *b* for each population (as labelled in Fig. 1). Standard errors are in parentheses

Locality	*N*	Number of haplotypes	Theta	Gene diversity	Mean number of pairwise differences	Nucleotide diversity
C. atrorus						
Antiguos Mineros	30	2	0.7573 (0.4762)	0.4345 (0.0699)	1.3035 (0.8379)	0.0041 (0.0029)
Laguna Grande	30	1	0 (0)	0 (0)	0 (0)	0 (0)
Laguna Salada	30	1	0 (0)	0 (0)	0 (0)	0 (0)
Las Playitas	30	2	0.2524 (0.2524)	0.0667 (0.0613)	0.0667 (0.1416)	0.0002 (0.0005)
Los Gatos	42	8	2.0916 (0.8939)	0.5134 (0.0892)	0.8583 (0.6186)	0.0027 (0.0022)
MJC 20	30	1	0 (0)	0 (0)	0 (0)	0 (0)
San Marcos	10	1	0 (0)	0 (0)	0 (0)	0 (0)
C. bifasciatus						
Becerra	40	1	0 (0)	0 (0)	0 (0)	0 (0)
Escobeda	39	4	1.1826 (0.6104)	0.4224 (0.0860)	0.9501 (0.6644)	0.0030 (0.0023)
Escobedita	30	1	0 (0)	0 (0)	0 (0)	0 (0)
Juan Santos	40	1	0 (0)	0 (0)	0 (0)	0 (0)
MJC 26	30	3	1.0097 (0.5710)	0.5494 (0.0381)	0.7149 (0.5515)	0.0022 (0.0019)
Mojarral Este	36	6	1.4469 (0.7063)	0.7810 (0.0375)	1.7429 (1.0373)	0.0055 (0.0036)
Mojarral Oeste	36	6	1.6881 (0.7855)	0.7587 (0.0487)	1.7810 (1.0547)	0.0056 (0.0037)
Los Hundidos	30	1	0 (0)	0 (0)	0 (0)	0 (0)
Poza Churince	40	1	0 (0)	0 (0)	0 (0)	0 (0)
Puente Dos Cuatas	30	5	1.2621 (0.6612)	0.8023 (0.0264)	1.7770 (1.0579)	0.0056 (0.0037)
Puente Orosco	40	7	1.6457 (0.7600)	0.7936 (0.0377)	1.7013 (1.0159)	0.0053 (0.0035)
Rio Garabatal	30	2	0.2524 (0.2524)	0.0667 (0.0613)	0.0667 (0.1416)	0.0002 (0.0005)
Tio Candido	30	1	0 (0)	0 (0)	0 (0)	0 (0)

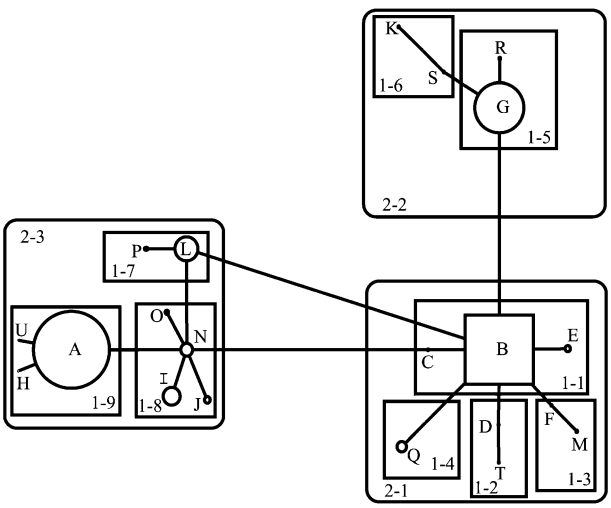

Fig. 4 Cyt *b* tree produced by TCS 1.18 for nested clade analysis of mitochondrial variation (SSCP fragment). Boxes represent nesting levels and letters denote mitochondrial haplotype as in Fig. 3.

a monophyletic group. Likewise, AMOVA indicated that variation was partitioned not by species, but by hydrogeography. Although there have been demonstrations of mitochondrial replacement in parts of species' ranges (Ferris *et al*. 1983; Tegelstrom 1987; Dowling *et al*. 1989; Dowling & Hoeh 1991; Bernatchez *et al*. 1995; Wilson & Bernatchez 1998), to our knowledge this is the first demonstration of complete replacement of an entire species' mitochondrial genome. Although there are other possible cases in pupfishes, as indicated by conflicting phylogenetic hypotheses from allozyme and mtDNA (Echelle *et al*. 2005), these require further corroboration.

Local mitochondrial replacement, however, is common and thus broad-scale population genetic analyses are appropriate for detecting such replacements and their population genetic effects (Taylor 2004). For example, in numerous salmonid species there has been local replacement of the mitochondrial genome of one species by that of another. Often, these replacements are ancient and the donating species no longer occurs in the region where replacement has taken place. For example, Wilson & Bernatchez (1998) found an allopatric population of lake trout with Arctic char mtDNA. Similarly, Redenbach & Taylor (2002) observed several populations of Dolly Varden with bull trout mtDNA in regions where bull trout no longer occur. Based on such findings, these researchers concluded that ancient hybridization events were responsible for the observed population genetic patterns.

Despite replacement of *C. bifasciatus* mitochondrial genome, there was substantial genetic variation and structure at cyt *b* in both species, indicating that mitochondrial replacement was sufficiently ancient to allow for divergence among populations. As predicted, there were two main regions in

which mitochondrial variation was partitioned. First, for *C. atrorus* there was a clear distinction between western and central-southeastern populations. Similarly, for *C. bifasciatus*, populations in the southeastern basin were distinct from populations in the western-central basin, with the exception of MJC 26, a population from an unnamed river in the Rio Puente Chiquito drainage. Here, however, it is likely that this departure from expectation resulted in part from recent introgressive hybridization with *C. atrorus* (MJC 20), which predominates in marshes that surround MJC 26 and is known to hybridize with *C. bifasciatus* in this region (see below).

Population subdivision was also evident within at least one of the two major regions noted for each species, although we found only partial correspondence with Minckley's hypothesis of drainage relationships. As predicted, the southeastern population of *C. atrorus* at Los Gatos was closely related to populations from the central basin, although Los Gatos harboured more variation than the other populations. Although these populations are not connected today, it is likely that they would have been in contact during pluvial periods pre-dating the Holocene. In addition, human activity (canal construction) may have recently diminished connectivity between these populations through a general lowering of the water table in the basin (Minckley 1969). Thus, there is strong evidence that *C. atrorus* from the central and southeastern basin inhabits a historically broadly interconnected drainage region. The population of *C. atrorus* from Antiguos Mineros, however, was distinct, as predicted by Minckley. In fact, nested clade analysis suggests that the geographic distribution of haplotypes in clade 2-1 (Fig. 4) is best explained by restricted gene flow with some long distance dispersal. This result is plausible, as during times of higher water level in the basin *C. atrorus* from Los Gatos could easily have expanded its range across the barrial via large lagunas and cienegas that would have likely existed between Los Gatos and Antiguos Mineros. Also consistent with our predictions, *C. atrorus* populations from the western basin contained cyt *b* haplotypes that were identical to each other, but divergent from those in the central and southeastern basin.

A more complex picture emerged from analysis of mtDNA variation in *C. bifasciatus*. In this species, populations in the western basin did show local divergence. One population, Juan Santos, was distinct, whereas the Rio Churince and La Becerra systems were identical. While there is no present surface connection between the La Becerra and the Rio Churince systems, our results suggest that there has probably been relatively recent contact between these two major systems. This connection could have occurred through *C. atrorus*, which inhabits marshes and lagunas on the barrial and would have easily communicated between these two systems during periods of higher water, perhaps during the period that pre-dated the Holocene. Juan Santos in contrast is isolated high on the bajada of the Sierra de los Pinos y San Marcos and may not have had a connection with the latter two systems during periods of higher water levels. Thus, patterns in the western basin were not in strict concordance with Minckley's (1969) hypothesis, but still point to local geographic variation in the western region.

We also predicted that *C. bifasciatus* populations in the western basin should show a close relationship with populations in the central basin, with southeastern populations more divergent. This prediction was largely met. For example, samples from the Rio Mesquites were most closely related to populations from the western basin. While Minckley (1969) considered the Rio Mesquites as a single unit, our results suggest that the upper and lower reaches of this system are divergent, with the upper system (Mojarral East and Mojarral West) mainly composed of haplotypes from lineage 'A' and clade 2-3, and the lower segment (Puente Orosco and Puente Dos Cuatas) harbouring haplotypes from lineages 'A' and 'B' and clades 2-1, 2-2, and 2-3. Two explanations can account for this pattern of differentiation between the upper and lower reaches of the Rio Mesquites in *C. bifasciatus*. First, lower reaches of the Rio Mesquites are thought to be separated from the upper Rio Mesquites by a large intervening marsh system that may serve as a partial barrier to gene flow between these river segments. Second, the upper Rio Mesquites populations of *C. bifasciatus* are not generally surrounded by *C. atrorus*, and thus hybridization and introgression of typical *C. atrorus* haplotypes into *C. bifasciatus* from the upper Rio Mesquites is very limited. This contrasts with the lower Rio Mesquites where *C. atrorus* is abundant in the surrounding marshland and it hybridizes extensively with *C. bifasciatus* (Carson, personal observation). Thus, hybridization with *C. atrorus* likely explains the presence of common central and southeastern *C. atrorus* haplotypes (B and E) in *C. bifasciatus* from the lower Rio Mesquites.

A similar pattern was observed in *C. bifasciatus* from an unnamed river in the Rio Puente Chiquito drainage (MJC26). Here *C. bifasciatus* was expected to be distinct as it inhabits an isolated, unnamed river that is surrounded by marshes with large *C. atrorus* populations. This riverine system is also in close proximity to lower reaches of the Rio Mesquites. In this unnamed river system, *C. bifasciatus* exhibited a high frequency (50%) of the 'B' haplotype (lineage 'B'). This site also had a high frequency of the 'G' haplotype (47%; lineage 'B') that is common in nearby Puente Orosco (35%) and Puente Dos Cuatas (23%) and fixed in the remaining southeastern *C. bifasciatus* populations (Los Hundidos and Tio Candido). Thus, cyt *b* haplotypes in this unnamed river system appear to share a close affinity with those from central basin *C. atrorus* and southeastern *C. bifasciatus*.

In support of Minckley's hypothesis, *C. bifasciatus* from the southeastern basin (Escobeda, Los Hundidos, and Tio Candido) were more closely related to each other than to populations from other regions. This is supported by fixation

of the 'G' haplotype in Los Hundidos and Tio Candido, and a high frequency of this haplotype in Escobeda (~74%). Nested clade analysis revealed that the geographic distribution of haplotypes from clade 2-2 (Fig. 4) is best explained by past fragmentation. This is also supported by apparent past surface connection between Escobeda, Escobedita, Tio Candido, and Los Hundidos, which is visible on satellite images as a remnant riverbed connecting these areas (Minckley, personal communication).

Presence of the 'L' haplotype at Escobeda and Escobedita suggests that these populations may also have had a former connection to the Rio Mesquites, as this haplotype is more similar to haplotypes from the central and western basin ('A' lineage) than those from the southeastern portion of the basin ('B' lineage). The hypothesized connection between Escobeda/Escobedita and the lower Rio Mesquites is also supported by presence of a spring-deposited travertine field originating at Escobeda and running toward the lower Rio Mesquites. This suggests that these sites occupy a central, connecting position between the southeastern portion of the basin and the lower Rio Mesquites.

Similarity to other systems

These complex patterns of population subdivision and introgressive hybridization in Cuatro Cienegas *Cyprinodon* have much in common with other systems. For example, broad-scale population subdivision is found in a variety of other organisms, including European fire-bellied toads (Szymura *et al*. 2000), in which introgressive hybridization also occurs, and brown trout (Machordom *et al*. 2000) and western pond turtles (Spinks & Shaffer 2005). In each of these cases population subdivision occurred across major hydrogeographic regions, with generally weak subdivision within regions. Similarly, introgressive hybridization has played a major role in structuring of genetic variation in numerous other taxa, including pocket gophers (Ruedi *et al*. 1997), chipmunks (Good *et al*. 2003), and European newts (Babik *et al*. 2005). In many systems where introgressive hybridization is common, it is often asymmetric (reviewed in Wirtz 1999), as occurs in Cuatro Cienegas *Cyprinodon*. Such introgression has often led to replacement of the mitochondrial genomes of local populations (e.g. Dowling *et al*. 1989; Bernatchez *et al*. 1995; Wilson & Bernatchez 1998). While complete replacement in these pupfishes provides an extreme example of the impacts of introgressive hybridization, it likely reflects the limited range of these species.

Dynamics of mitochondrial replacement

Several hypotheses have been constructed to explain asymmetrical mitochondrial gene flow (reviewed in Wirtz 1999) and could explain the complete mitochondrial replacement observed in this system. Wirtz (1999) outlined the sexual selection hypothesis for unidirectional hybridization, which predicts that when there is female choice females of the rarer species preferentially hybridize with males of the more common species, thus leading to unidirectional hybridization. Hybridization between *C. atrorus* and *C. bifasciatus* does not appear to conform to the sexual selection model proposed by Wirtz, but relates instead to mating site preferences of males and females of these species in regions of sympatry (Ludlow 2000). In regions of contact, male *C. atrorus* and *C. bifasciatus* generally mate in shallow pools. *C. atrorus* females are common and congregate in these pools whereas female *C. bifasciatus* usually inhabit laguna or riverine habitats. Thus, the hybrid offspring are generally produced by mating between *C. bifasciatus* males and *C. atrorus* females, and this pattern could have resulted in the observed pattern of mitochondrial replacement.

Despite the expectation derived from knowledge of mating behaviour, it is surprising that *C. atrorus* from the central cienegas showed no evidence of mitochondrial introgression from surrounding *C. bifasciatus* populations given hybridization still occurs. In regions of the basin where the two taxa co-occur, such as La Becerra and Poza Churince, *C. bifasciatus* populations are fixed for cyt *b* haplotypes that are also fixed in a connected or nearby *C. atrorus* population. In contrast, isolated populations of *C. bifasciatus* are typically fixed for or segregate for alleles unique to *C. bifasciatus*. These patterns suggest that when *C. bifasciatus* and *C. atrorus* come into contact, there is a predictable replacement of *C. bifasciatus* mitochondrial haplotypes by those of *C. atrorus*, with a cyclical pattern of isolation and divergence followed by re-contact and mtDNA replacement.

While the dynamics of such replacement remain unknown, the general pattern suggests a scenario in which *C. bifasciatus* mitochondrial genome was outnumbered and consequently replaced by that of *C. atrorus*. Because *C. atrorus* is more abundant than *C. bifasciatus* where they are sympatric, mitochondrial replacement has potentially occurred due to the overwhelming number of *C. atrorus* in the system and the direction of mating preferences within the hybrid zone as is typical for fishes (Hubbs 1955). If such patterns have been a general trend throughout the history of contact between these species, then the original replacement of *C. bifasciatus* mitochondrial genome may also have been effected by mating site selection and a numeric bias towards *C. atrorus* mitochondria, rather than selection for the *C. atrorus* mitochondrial genome.

Conclusions

Results from this study suggest that both hydrogeography and hybridization have played critical roles in the evolution of pupfish in Cuatro Cienegas. At the nuclear gene level, these species are easily distinguishable, with allelic variation

reflecting morphological species differences, although there was minor introgression into some populations of each species. Patterns at the mitochondrial level provide a stark contrast to those observed at nuclear loci. At cyt *b*, ancient hybridization has led to the complete replacement of *Cyprinodon bifasciatus* mitochondrial genome by that of *Cyprinodon atrorus*. Subsequent diversification at the mitochondrial level has occurred with respect to hydrogeography that roughly corresponds to Minckley's (1969) seven drainage hypothesis. There is also evidence that mitochondrial replacement acts in a predictable manner, with isolation and divergence followed by re-contact between *C. atrorus* and *C. bifasciatus* that leads to mitochondrial replacement of *C. bifasciatus* haplotypes. These findings compliment a growing body of evidence that demonstrates introgressive hybridization has had an important and common evolutionary influence in animal systems. Results from this study in particular demonstrate the significant role introgressive hybridization may have in both animal evolution and in the interpretation of phylogeographic history among closely related, hybridizing organisms.

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References

Anderson E (1949) *Introgressive hybridization*. John Wiley and Sons, New York, New York, USA.

Arnold ET (1972) *Behavioral ecology of two pupfishes (Cyprinodontidae, genus* Cyprinodon*) from northern Mexico*. Unpublished PhD Dissertation. Arizona State University, Tempe, Arizona.

Arnold ML (1997) *Natural Hybridization and Evolution*. Oxford University Press, Oxford, UK.

Babik W, Branicki W, Crnobrnja-Isailovic J *et al.* (2005) Phylogeography of two European newt species — discordance between mtDNA and morphology. *Molecular Ecology*, **14**, 2475–2491.

Barton NH, Hewitt GM (1985) Analysis of hybrid zones. *Annual Review of Ecology and Systematics*, **16**, 113–148.

Bernatchez L, Glemet H, Wilson CC, Danzmann RG (1995) Introgression and fixation of arctic char (*Salvelinus alpinus*) mitochondrial genome in an allopatric population of brook trout (*Salvelinus fontinalis*). *Canadian Journal of Fisheries and Aquatic Sciences*, **52**, 179–185.

Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealities. *Molecular Ecology*, **9**, 1657–1659.

Dowling TE, Hoeh WR (1991) The extent of introgression outside the contact zone between *Notropis cornutus* and *Notropis chrysocephalus* (Teleostei: Cyprinidae). *Evolution*, **45**, 944–956.

Dowling TE, Secor CL (1997) The role of hybridization and introgression in the diversification of animals. *Annual Review of Ecology and Systematics*, **28**, 593–619.

Dowling TE, Smith GR, Brown WM (1989) Reproductive isolation and introgression between *Notropis cornutus* and *Notropis chrysocephalus* (family Cyprinidae): comparison of morphology, allozymes, and mitochondrial DNA. *Evolution*, **43**, 620–634.

Dowling TE, Marsh PC, Kelsen AT, Tibbets CA (2005) Genetic monitoring of wild and repatriated populations of endangered razorback sucker (*Xyrauchen texanus*, Catostomidae, Teleostei) in Lake Mohave, Arizona-Nevada. *Molecular Ecology*, **14**, 123–135.

Echelle AA, Echelle AF (1998) Evolutionary relationships of pupfishes in the *Cyprinodon eximius* complex (Atherinomorpha: Cyprinodontiformes). *Copeia*, **1998**, 852–865.

Echelle AA, Carson EW, Echelle AF, Van Den Bussche RA, Dowling TE, Meyer A (2005) Historical biogeography and the role of genetic introgression in the evolution of the New World pupfish genus *Cyprinodon* (Teleostei: Cyprinodontidae). *Copeia*, **2005** (2), 320–339.

Excoffier L, Smouse P, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics*, **131**, 470–491.

Ferris SD, Sage RD, Huang C, Nielsen JT, Ritte U, Wilson AC (1983) Flow of mitochondria across a species boundary. *Proceedings of the National Academy of Sciences, USA*, **80**, 2290–2294.

Glavac D, Dean M (1993) Optimization of the single-strand conformation polymorphism (SSCP) technique for detection of point mutations. *Human Mutation*, **2**, 404–414.

Glemet H, Blier P, Bernatchez L (1998) Geographical extent of Arctic char (*Salvelinus alpinus*) mtDNA introgression in brook char populations (*S. fontinalis*) from eastern Quebec, Canada. *Molecular Ecology*, **7**, 1655–1662.

Good JM, Demboski JR, Nagorsen DW, Sullivan J (2003) Phylogeography and introgressive hybridization: chipmunks (genus *Tamias*) in the northern Rocky Mountains. *Evolution*, **57** (8), 1900–1916.

Grant V (1981) *Plant Speciation*. Columbia University Press, New York.

Howard DJ (1993) Reinforcement: origin, dynamics, and fate of an evolutionary hypothesis. In: *Hybrid Zones and the Evolutionary Process* (ed. Harrison RG), pp. 46–69. Oxford University Press, Oxford.

Hubbs CL (1955) Hybridization between fish species in nature. *Systematic Zoology*, **4** (1), 1–20.

Lewontin RC, Birch LC (1966) Hybridization as a source of variation for adaptation to new environments. *Evolution*, **20**, 315–336.

Lozano-V MDL, Contreras-B S (1993) Four new species of *Cyprinodon* from southern Nuevo Leon, Mexico, with a key to the *C. eximius* complex (Teleostei: Cyprinodontidae). *Ichthyological Exploration of Freshwaters*, **4**, 295–308.

Ludlow AM (2000) *Sexual selection in the Cuatro Cienegas pupfish: mate choice and hybridization between* Cyprinodon atrorus *and* Cyprinodon bifasciatus. Unpublished PhD Dissertation. Lehigh University, *Bethlehem, Pennsylvania*.

Machordom A, Suarez J, Almodovar A, Bautista JM (2000) Mitochondrial haplotype variation and phylogeography of Iberian brown trout populations. *Molecular Ecology*, **9**, 1325–1338.

Merritt TJS, Quattro JM (2001) Evidence for a period of directional selection following gene duplication in a neurally expressed locus of triosephosphate isomerase. *Genetics*, **159**, 689–697.

Miller RR (1968) Two new species of the genus *Cyprinodon* from the Cuatro Cienegas basin, Coahuila, Mexico. *Occasional Papers of the Museum of Zoology Michigan*, **659**, 1–15.

Minckley WL (1969) Environments of the bolson of Cuatro Cienegas, Coahuila, Mexico, with special reference to the aquatic biota. *University of Texas, El Paso Sci. Ser.*, **2**, 1–65.

Posada D, Crandall KA, Templeton AR (2000) GEODIS: a program for the cladistic nested analysis of the geographical distribution of genetic haplotypes. *Molecular Ecology*, **9**, 487–488.

Quattro JM, Jones WJ (1999) Amplification primers that target locus-specific introns in actinopterygian fishes. *Copeia*, **1999**, 191–196.

Redenbach Z, Taylor EB (2002) Evidence for historical introgression along a contact zone between two species of char (Pisces: Salmonidae) in northwestern North America. *Evolution*, **56**, 1021–1035.

Ruedi M, Smith MF, Patton JL (1997) Phylogenetic evidence of mitochondrial DNA introgression among pocket gophers in New Mexico (family Geomyidae). *Molecular Ecology*, **6**, 453–462.

Schmidt TR, Bielawski JP, Gold JR (1998) Molecular phylogenetics and evolution of the cytochrome *b* gene in the cyprinid genus *Lythrurus* (Actinopterygii: 43 Cypriniformes). *Copeia*, **1998**, 14–22.

Schneider SD, Roessli D, Excoffier L (2000) *ARLEQUIN: A software for population genetics data analysis. Version 2.000*. Genetics and Biometry Laboratory, Department of Anthropology, University of Geneva, Switzerland. http://anthropologie.unige.ch/arlequin/.

Sokal RR, Rohlf FJ (1995) *Biometry*. WH Freeman, New York.

Spinks PQ, Shaffer HB (2005) Range-wide molecular analysis of the western pond turtle (*Emys marmorata*): cryptic variation, isolation by distance, and their conservation implications. *Molecular Ecology*, **14**, 2047–2064.

Stebbins GL (1959) The role of hybridization in evolution. *Proceedings of the American Philosophical Society*, **103**, 231–251.

Swofford DL (1998) *PAUP* 4.0. Phylogenetic Analysis Using Parsimony (*and Other Methods)*. Sinauer Associates, Sunderland, Massachusetts.

Szymura JM, Uzzell T, Spolsky C (2000) Mitochondrial DNA variation in the hybridizing fire-bellied toads *Bombina bombina* and *B. variegate*. *Molecular Ecology*, **9**, 891–899.

Takeda KA, Onishi N, Ishida K, Kawakami M, Komatsu M, Inumaru S (1995) SSCP analysis of pig mitochondrial DNA D-loop region polymorphism. *Animal Genetics*, **26**, 321–326.

Taylor EB (2004) Evolution in mixed company: evolutionary inferences from studies of natural hybridization in Salmonidae. In: *Evolution Illuminated: Salmon and Their Relatives* (eds Hendry AP, Stearns SC), pp. 232–263. Oxford University Press, Oxford.

Tegelstrom H (1987) Transfer of mitochondrial DNA from the northern red-backed vole (*Clethrionomys rutilus*) to the black vole (*C. grareolus*). *Journal of Molecular Evolution*, **24**, 218–227.

Templeton AR (2001) Using phylogeographic analyses of gene trees to test species status and processes. *Molecular Ecology*, **10**, 779–791.

Tibbets CA, Dowling TE (1996) Effects of intrinsic and extrinsic factors on population fragmentation in three species of North American minnows (Teleostei: Cyprinidae). *Evolution*, **50**, 1280–1292.

Wilson CC, Bernatchez L (1998) The ghosts of hybridization past: fixation of arctic char (*Salvelinus alpinus*) mitochondrial DNA in an introgressed population of lake trout (*S. namaycush*). *Molecular Ecology*, **7**, 127–132.

Wirtz P (1999) Mother species–father species: unidirectional hybridization in animals with female choice. *Animal Behaviour*, **58**, 1–12.