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PHYLOGEOGRAPHY OF THE PLAINS KILLIFISH, *FUNDULUS ZEBRINUS*

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Abstract.—Drainage systems of the Great Plains and western Gulf Slope underwent substantial changes through diversions and stream captures during the Pleistocene, either as the result of the glacial advances or through independent geologic processes. The distributions of a variety of fishes that range across west-central North America, such as the plains killifish (*Fundulus zebrinus*), are thought to be the product of this Pleistocene influence. We examined the geographic pattern of genetic variation in *F. zebrinus* using three allozyme loci ($n = 793$), mitochondrial DNA restriction fragment length polymorphisms (RFLPs, $n = 352$), and sequencing of the mitochondrial cytochrome oxidase I (COI, $n = 23$) in an attempt to understand the roles of dispersal and vicariance. The phylogeographic patterns were concordant between the allozyme and mitochondrial data with the exception of the population in the North Canadian River. The populations fell into three geographic assemblages, which we designated as northern, central, and southern. A large phylogenetic break (average Roger's $D = 0.702$; average sequence divergence in RFLPs = 4.6%; average sequence divergence in COI = 5.5%) separated the northern/central and southern assemblages. The northern region was likely colonized sometime during the mid-Pleistocene. Fish in the Brazos and Pecos Rivers probably reached these drainages through stream captures of the Red River. The large phylogenetic break between the northern/central and southern clades supports previous attempts to recognize two species of plains killifish: *F. zebrinus* and *F. kansae.*

Key words.—Allozymes, *Fundulus kansae, Fundulus zebrinus,* mitochondrial DNA, phylogeography, Pleistocene, systematics.

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The effect of the Pleistocene glaciations on the biogeography of organisms from northern latitudes is readily recognized and extensively documented (e.g., Pielou 1991; Hewitt 1996). However, the distribution of organisms in regions not covered by ice may have also been altered during the Pleistocene either through indirect effects of the glaciers or geologic processes. For example, the drainage systems of the Great Plains and western Gulf Slope, a region that we will refer to as west-central North America, experienced dramatic alterations during the Pleistocene. Drainage basins across the Great Plains were joined as northern rivers were diverted southward during the glacial advances (Metcalf 1966; Cross et al. 1986). Drainage systems of the western Gulf Slope, which includes the rivers entering the Gulf of Mexico between the Mississippi River and the Rio Grande, were shaped by a number of diversions and stream captures (Conner and Suttkus 1986).

Recognition that the Pleistocene drainage alterations shaped the biogeographic patterns of freshwater organisms in central North America can be found as far back as Williams's (1954) work on the crayfish *Orconectes neglectus.* Similar processes have influenced the distribution of the fish fauna, including *Fundulus zebrinus, Phenacobius mirabilis, Hybognathus placitus, Cyprinella lutrensis,* and *Etheostoma spectabile* (Metcalf 1966; Cross et al. 1986). The ranges of these fishes tend to be restricted to the western portion of Great Plains and western Gulf Slope rivers (see Fig. 1), although a few species are also widespread in the northern Mississippi Basin. The discontinuous distribution has been viewed as evidence for historical connections among drainage systems.

Considerable insight into the biogeography and systematics of freshwater fishes has resulted from studies of geographic patterns of genetic variation. For example, phylogeographic studies of the fishes of the southeastern United States (Bermingham and Avise 1986), Nearctic North America (Bernatchez and Wilson 1998), Central America (Bermingham and Martin 1998), and Australia (Hurwood and Hughes 1998) have all contributed to our understanding of regional ichthyofaunas. Within North America a great deal of research has focused on the taxonomically diverse aquatic fauna of the Central Highlands region, which includes the Eastern Highlands east of the Mississippi River and the Ozarks and Ouachitas west of the Mississippi (e.g., Mayden 1988; Turner and Trexler 1998; Crandall and Templeton 1999). However, little attention has been devoted to the fishes of west-central North America, with the exception of a series of studies of the *C. lutrensis* species group (Richardson and Gold 1995a, b, 1999; Broughton and Gold 2000).

The plains killifish, *F. zebrinus*, is a cyprinodontid that has a native range restricted entirely to the Great Plains and western Gulf Slope (Fig. 1). *Fundulus zebrinus* inhabits shallow, sandy-bottom streams or the backwaters and sloughs of larger rivers (Woodling 1985) and is tolerant of harsh environmental conditions including high temperature, high salinity, and low oxygen concentrations (Woodling 1985; Corah 1994; Cross and Collins 1995). The biogeography of *F. zebrinus* would have been strongly influenced by the vicariance events and dispersal opportunities produced by changes in rivers during the Pleistocene. Additionally, the taxonomy of the plains killifish suggests that these historical factors warrant closer examination. Various authorities recognize two species of plains killifish: *F. zebrinus* and *F. kansae* (reviewed in Poss

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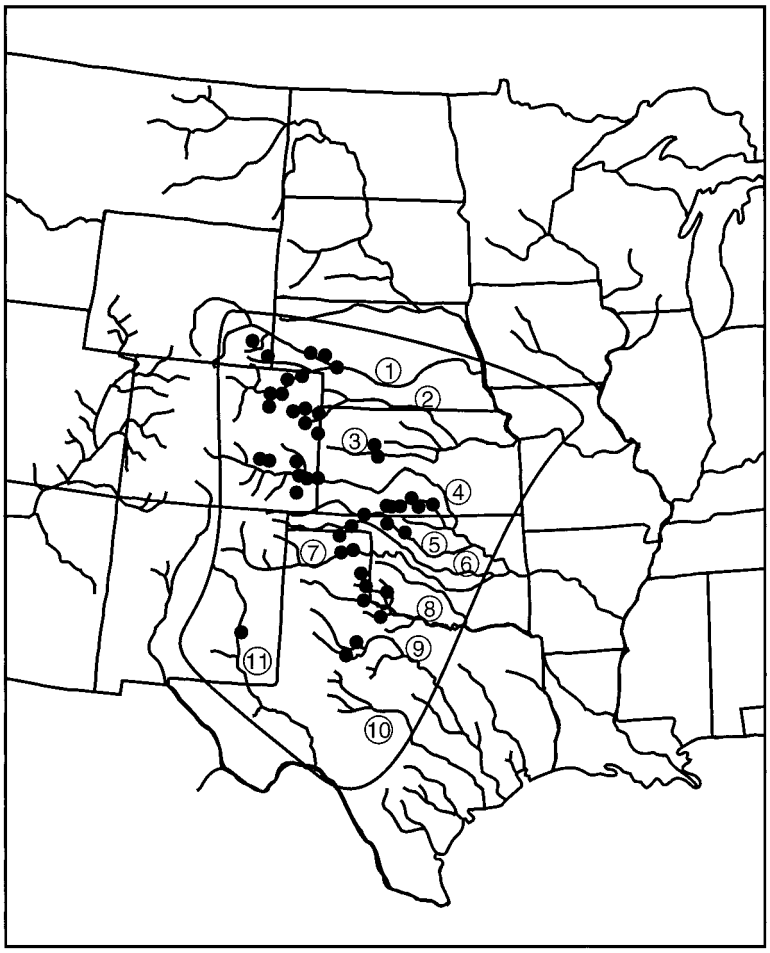

FIG. 1. The native range of *Fundulus zebrinus* (enclosed within the line) and collections sites (indicated by dots). Major drainages are labeled by numbers as follows: 1, Platte; 2, Republican; 3, Smoky Hill; 4, Arkansas; 5, Cimarron; 6, North Canadian; 7, Canadian; 8, Red; 9, Brazos; 10, Colorado; 11, Pecos.

and Miller 1983). The proposed morphological differentiation, albeit questioned by some, indicates that an ancient vicariance event may characterize the evolution of this species group. Our goal was to examine the geographic patterns of genetic variation in *F. zebrinus* to understand the historic events that have shaped the evolution of this species. To do this we conducted a rangewide survey of genetic variation in *F. zebrinus* using both nuclear (allozymes) and mitochondrial loci. Multiple markers were used to obtain independent measures of the historical signal that might be present in the genome of *F. zebrinus.*

MATERIALS AND METHODS

Fish were collected either by seining or electroshocking from 51 sites from most of the major drainage systems within the native range of *F. zebrinus* (Fig. 1; for a complete list of site localities, see Kreiser 1999). Specimens were placed on dry ice, returned to the laboratory, and stored at −70°C. Preserved collections are housed at the Colorado Division of Wildlife (Denver). We collected the northern studfish, *F. catenatus,* from Brushy Fork, Miller County, Missouri to serve as an outgroup in some of the analyses. Unfortunately, no clear choice of outgroup existed because the basal relationships within the genus *Fundulus* are not well resolved (Parenti 1981; Wiley 1986; Bernardi 1997). *Fundulus zebrinus* has been regarded as a basal lineage of the Fundulidae and sometimes placed into its own genus *Plancterus* (Parenti 1981).

Molecular Techniques

Allozymes

Muscle tissue from the caudal peduncle was processed for starch gel electrophoresis using standard methods (see Kreiser 1999) and stored at −70°C. Twenty-one putative loci were initially screened using horizontal starch gel electrophoresis on one or more of seven buffer systems. Of these, three loci were well resolved and highly polymorphic. Poulik's (1957) discontinuous buffer system was used to resolve glucose-6-phosphate isomerase (*Gpi,* EC 5.3.1.9), phosphoglucomutase (*Pgm,* EC 5.4.2.2), and mannose-6-phosphate isomerase (*Mpi,* EC 5.3.1.8). Gels were made at a concentration of 12% (starch from Sigma, St. Louis, MO) and run for 3.5–4.0 h at 50 mA, 150 V. Enzymes were stained using standard procedures (Selander et al. 1971; Harris and Hopkinson 1976).

Genotypic data for each population were analyzed with BIOSYS-1 (Swofford and Selander 1981) to generate variability measures and to test for departures from Hardy-Weinberg equilibrium expectations. In addition, populations were pooled by drainage system and the same analyses were repeated. For the pooled populations, modified Rogers distances (Wright 1978) were calculated and the relationships among the drainages were represented with a UPGMA phenogram (Sneath and Sokal 1973).

Mitochondrial DNA

Total genomic DNA was extracted from muscle tissue using standard methods (Kreiser 1999). The polymerase chain reaction (PCR) was used to amplify regions of the mitochondrial (mtDNA) genome. Genetic variation was assayed through restriction fragment length polymorphisms (RFLPs) and sequences.

Restriction site variation was examined in a region of the mtDNA genome that included a portion of the 3′ end of the 12S rRNA, the tRNA-Val gene, and a portion of the 5′ end of the 16S rRNA. The 12S-16S region was amplified using the 12S light (L1091) primer of Kocher et al. (1989) and the 16sar-H-myt of Lydeard et al. (1996), which produced a fragment that was approximately 2.1-kb long, hereafter referred to as the 2.1-kb fragment. Amplifications were conducted in a total volume of 50 μl using: 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.01% gelatin, 2 mM $MgCl_2$, 200 μM dNTPs, 1.5 units *Taq* polymerase, 0.3 μM of each primer, 100–300 ng template DNA, and water to the final volume. PCR cycling conditions consisted of an initial denaturing step of 95°C for 1 min followed by 30 cycles of 1 min at 95°C, 1 min at 52°C, and 3 min at 72°C. A final elongation step of 7 min at 72°C ended the cycle. Variation in the 2.1-kb fragment was assayed with restriction digests using four restriction enzymes (four-base cutters: *Dpn*II, *Nla*III, and *Rsa*I; six-base cutter: *Eco*RI) following the manufacturer's recommendations (New England Biolabs, Beverly, MA). Each digestion was allowed to proceed for 4–6 h at 37°C. The fragments produced by these digests were separated on 2% agarose gels stained with ethidium bromide (0.5 μg/ml) and visualized under ultraviolet light. Each band produced by a restriction digest was scored for size by comparison to known size standards (Gibco BRL, Grand Island, NY, 1-kb DNA ladder and ØX174 RF DNA/*Hae*III fragments).

Restriction sites were inferred from the digest patterns as well as the complete sequence of the 2.1-kb fragment. Initially, one individual was chosen for sequencing and primer design. The PCR product was cleaned using the QIAquick PCR purification kit (Qiagen, Inc., Santa Clarita, CA). Both strands of the 2.1-kb fragment were then cycle sequenced with the original primers using ABI Big Dye (Perkin Elmer Applied Biosystematics, Foster City, CA) terminator chemistry and visualized on an ABI Prism model 377 automated DNA sequencer (MCDB sequencing facility, Univ. of Colorado). Selected homologous sequences from other fish species in GenBank (*Cyprinus carpio*, X61010; *Oncorhynchus mykiss*, L29771; *Gadus morhua*, X99772) were aligned with this sequence using the program Sequencher 3.0 (Gene Codes Corp., Ann Arbor, MI). Primers were designed with the aid of Primer version 1.0 (Resnick 1996) to amplify the 2.1-kb fragment in three separate fragments. Fragment 1 corresponded to base pairs 1404–2168 in the complete mtDNA genome of carp (*C. carpio*) and was amplified using 12S light (L1091) of Kocher et al. (1989) and the new primer 12S 3′R (5′-ATTTCCCTTGCGGTACTTTTTCT-3′). Fragment 2 corresponded to base pairs 2142–2944 in carp and was amplified using the new primers 12S 3′ F (5′-CAATAGAAATAG-TACCGCAAGGG-3′) and 16S 5′R (5′-GAGGCGA-TGTTTTTGGTAAACA-3′). Fragment 3 corresponded to base pairs 2919–3508 in carp and was amplified with 16sar-L-myt of Lydeard et al. (1996) and the new primer 16S 3′R (5′-CGTTGAACAAACGAACCCTTAA-3′). PCR conditions were the same as those used for the 2.1-kb fragment.

Once sequence data were obtained for selected individuals, the fragments were aligned and edited with Sequencher 3.0 to produce a composite sequence of the 2.1-kb fragment. Sequencher 3.0 also was used to determine the exact location of restriction sites for the enzymes of interest. The location of additional restriction sites were inferred from the fragment data from the population survey. Within a population, DNA polymorphism was measured by haplotype diversity (h) and the nucleotide diversity (π) as estimated by equations (8.5) and (10.5) respectively of Nei (1987, pp. 179 and 256). RESTSITE version 1.2 (Miller 1991) was used to estimate the amount of nucleotide divergence among populations corrected for intrapopulation variation (number of nucleotide substitutions per site = d; Nei and Miller 1990). The resulting distance matrix was used in a UPGMA clustering algorithm (Sneath and Sokal 1973).

Twenty-three individuals were sequenced for a portion of 3′ end of the cytochrome oxidase I gene (COI). Individuals were selected to represent the major drainage systems as well as the haplotypes identified by the RFLP analysis of the 2.1-kb fragment. One individual of *F. catenatus* was sequenced to serve as an outgroup. The LCO1490 and HCO2198 primers of Folmer et al. (1994) were used to amplify a 718-bp fragment using the same reaction conditions as the 2.1-kb fragment with the exception that the concentration of $MgCl_2$ was 4 mM. PCR products were sequenced with the same procedures as described above. Sequences were edited and aligned using the program Sequencher 3.0. Phylogenetic relationships were inferred with unweighted maximum parsimony (MP) and neighbor joining (NJ, Saitou and Nei 1987) using PAUP* version 4.01b (Swofford 1998). NJ was performed on both uncorrected distance (Nei 1987) and Kimura two-parameter (Kimura 1980) distances. A branch-and-bound search was run on the COI sequences rooting the tree with sequence from *F. catenatus.* Additionally, a bootstrap analysis with 1000 rounds of resampling was performed for each phylogenetic method (Felsenstein 1985). NJ bootstrapping used uncorrected distance as the metric. MP bootstrapping was performed using a full heuristic search with random addition using 20 replicates per round.

Analysis of Molecular Variance

We used Arlequin version 2.000 (Schneider et al. 2000) to perform an analysis of molecular variance (AMOVA; Excoffier et al. 1992) on both the RFLP and allozyme data to

examine the geographic partitioning of genetic variation. Three geographic regions were designated as defined by the differentiation revealed in the mtDNA data: northern (Platte, Republican, and Smoky Hill drainages); central (Arkansas, Cimarron, North Canadian, and Canadian drainages); and southern (Red, Brazos, and Pecos drainages). We tested the significance of partitioning among the three geographic regions using nonparametric permutation tests (Excoffier et al. 1992; 1000 permutations).

Results

Allozymes

Levels of genetic variation

A total of 793 fish were scored for *Gpi, Pgm,* and *Mpi.* Five alleles were found to be segregating at *Gpi,* seven alleles at *Pgm,* and four alleles at *Mpi* (Table 1). Some regions exhibited little genetic variability within populations. Populations in the northern portion of the range were monomorphic for all loci, with the exception of six individuals heterozygous for *Gpi* (Table 1). Similarly, populations from the western Arkansas drainage also tended to be monomorphic, with only six individuals heterozygous for *Gpi,* and only one site was polymorphic for *Pgm.* The other central region populations were typically polymorphic at two or three loci and possessed direct-count heterozygosity levels ranging from 0.153 to 0.270 (Kreiser 1999). Populations in the southern portion of the range also tended to be polymorphic. The Red River population was polymorphic at all three loci, whereas the Brazos and Pecos River populations were both polymorphic at two loci. Direct-count heterozygosity at sites in the southern region was relatively high, with values ranging from 0.261 to 0.312 (Kreiser 1999).

Geographic distribution of alleles

Gpi.—The A allele was found at a high frequency in the northern portion of the range (Table 1). In contrast, the central region had high frequencies of the B allele and low frequencies of the A allele, with the C allele present at mid to low frequencies. The D allele was found in the North Canadian, but at low frequencies. In the southern portion of the range, the B allele was still found at high frequencies, but the A allele was only found in the Red River drainage. A new allele (E) was present in both the Red and Brazos River drainages at low frequencies.

Pgm.—Sites in the northern region were monomorphic for the D allele, which was also at a high frequency in the central region (Table 1). The B and C alleles were found at low frequencies in the central region, except for the North Canadian drainage, which had a high frequency of the C allele. In the southern region, the C allele was found at high frequencies. Several low-frequency but unique alleles were also present. The E allele was unique to the Pecos, and the G allele was unique to the Red River. The F allele was nearly restricted to the Red with the exception of a single heterozygous individual found in the Cimarron.

Mpi.—The B allele was the only allele detected in the northern populations and it had high frequencies in the central region (Table 1). The A and C alleles also occurred in the central region but at very low frequencies. The exception was the North Canadian drainage, where the C allele was the most common allele. In the southern region, the B allele was also present at high frequencies, with the A allele also being common in the Brazos and Pecos. The only drainage-specific allele for *Mpi* was the D allele found in the Brazos.

Geographic differentiation

Three regional assemblages of populations were evident in the phenogram (Fig. 2). The northern drainages clustered together with very little differentiation among populations. The central drainages formed a distinct group with the exception of the North Canadian, which clustered with the southern drainages. The northern and central regions were more similar to one another than either was to the southern region. Although the absolute values of the branch lengths are of marginal utility given the small number of loci sampled, the values provide an indication of the differentiation between the northern and central regions versus the southern region. The average value of genetic distance between the northern and central regions was $D = 0.522$, whereas the average distances between the southern/northern and southern/central were larger with values of $D = 0.821$ and 0.617, respectively.

Mitochondrial DNA Restriction Fragment Length Polymorphisms

Four individuals, representing four haplotypes, were sequenced for the entire 2.1-kb fragment (Table 2; GenBank accessions AF221747–AF221758). The 5′ end of the sequence begins within the 12S rRNA and corresponds to site 1418 in carp (*C. carpio*; Chang et al. 1994). The four individuals sequenced were 2091 or 2092 bp long. The individuals of haplotypes 2 and 3 each possessed a single nucleotide deletion at position 1145, the representative for haplotype 1 had a deletion at 1046, and two deletions were present (638 and 1147) in the individual of haplotype 7.

Levels of genetic variation

Nineteen restriction sites existed for the four restriction enzymes, and of these restriction sites nine were polymorphic. A total of 352 individuals were surveyed (Table 2). All the drainages in the northern region were monomorphic for haplotype 1. Intrapopulation variation was low within the central region, with haplotype 2 possessing the highest frequency. Only two central region drainages demonstrated moderate haplotype diversity, the West Arkansas ($h = 0.506$) and the Canadian ($h = 0.459$). Additionally, only two individuals from East Arkansas possessed haplotype 4. Nucleotide diversity within the central region ranged from 0.0000 to 0.0048. Haplotype 5 was widespread in the southern region and it was the only haplotype found in the Red River drainage. Haplotype 8 was restricted to the Brazos, and haplotype 7 was restricted to the Pecos. Nucleotide diversity for the Brazos and Pecos were similar with values of 0.0020 and 0.0017, respectively.

TABLE 1. Allelic frequencies for pooled populations by drainage. The West Ark. (Arkansas) includes populations within Colorado, and the East Ark includes populations in Kansas; *n* indicates sample size.

Locus	North Platte	South Platte	Republican	Smoky Hill	West Ark.	East Ark.	Cimarron	North Canadian	Canadian	Red	Brazos	Pecos
Gpi												
(*n*)	52	80	34	24	173	143	50	42	30	92	62	11
A	0.981	1.000	0.971	0.958	0.017	0.042	0.030	0.000	0.000	0.120	0.000	0.000
B	0.010	0.000	0.029	0.042	0.983	0.678	0.850	0.798	0.850	0.788	0.798	1.000
C	0.010	0.000	0.000	0.000	0.000	0.276	0.120	0.024	0.117	0.000	0.000	0.000
D	0.000	0.000	0.000	0.000	0.000	0.003	0.000	0.179	0.033	0.060	0.008	0.000
E	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.033	0.194	0.000
Pgm												
(*n*)	52	80	34	24	173	143	50	42	30	92	62	11
A	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.048	0.000	0.022	0.000	0.000
B	0.000	0.000	0.000	0.000	0.029	0.000	0.060	0.012	0.000	0.000	0.000	0.000
C	0.000	0.000	0.000	0.000	0.014	0.000	0.040	0.774	0.067	0.918	1.000	0.909
D	1.000	1.000	1.000	1.000	0.957	1.000	0.890	0.167	0.933	0.005	0.000	0.000
E	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.091
F	0.000	0.000	0.000	0.000	0.000	0.000	0.010	0.000	0.000	0.043	0.000	0.000
G	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.011	0.000	0.000
Mpi												
(*n*)	52	80	34	24	173	143	50	42	30	92	62	11
A	0.000	0.000	0.000	0.000	0.000	0.003	0.010	0.048	0.017	0.005	0.589	0.591
B	1.000	1.000	1.000	1.000	1.000	0.972	0.990	0.071	0.850	0.821	0.258	0.409
C	0.000	0.000	0.000	0.000	0.000	0.024	0.000	0.881	0.133	0.174	0.008	0.000
D	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.145	0.000

Geographic differentiation

A UPGMA phenogram based on the average number of nucleotide substitutions per site (*d*) revealed three geographically distinct groups (Fig. 3). As with the allozyme data, the northern and central populations grouped together at the exclusion of the southern populations. However, unlike the allozyme data, the North Canadian population clustered with the other central region drainages. The average amount of nucleotide divergence between the northern/central and southern groups was 4.6% (SE = 0.11), whereas the average nucleotide divergence between the northern and central populations was 1.0% (SE = 0.14).

Mitochondrial DNA Cytochrome Oxidase I Sequence

From the 718-bp COI fragment, we obtained 618 bp of sequence for each of 23 *F. zebrinus* and the one outgroup, *F. catenatus* (GenBank accessions AF208264–AF208287). In the 618 bp of sequence from *F. zebrinus,* 51 sites were variable. Forty-seven transitions and five transversions were observed, and all but three changes occurred in the third codon position.

Levels of genetic variation

Although the lack of extensive population sampling with the sequence data precluded the estimation of any formal measures of genetic variation, the data did allow a qualitative assessment of regional levels of genetic variation. Of the five individuals from the northern region sequenced for COI, only one possessed a distinct haplotype (Smoky Hill-1), which differed by a single transition (0.16% sequence divergence). Eight unique haplotypes were found among the nine individuals sampled from the central region. The average (uncorrected) sequence divergence among these samples was p = 0.9% (SE = 0.05). However, as discussed below (see Geographic differentiation), haplotypes within the central region formed two distinct clades (clades C1 and C2; see Fig. 4), with an average sequence divergence of p = 1.3% (SE = 0.2). The average sequence divergence within the two clades was 0.5% (SE = 0.004) and 0.4% (SE = 0.07) for clades C1 and C2, respectively. Within the southern region, six distinct haplotypes were found among the nine samples sequenced, and the average sequence divergence was 0.6% (SE = 0.004).

Geographic differentiation

NJ analyses of uncorrected distance and Kimura two-parameter distance produced trees with only slight differences in topology, so we only discuss the tree derived from uncorrected distances (Fig. 4). The MP analysis recovered two most parsimonious trees (length = 134). We are only going to be referring to the strict consensus of these two trees (Fig. 4). The overall topologies of the NJ and MP trees were concordant, and were similar to the phenograms produced from the allozyme (Fig. 1) and mtDNA RFLP data (Fig. 2).

Samples from the northern region formed a monophyletic clade with moderate to strong bootstrap support (MP = 69; NJ = 92). The central region was a paraphyletic assemblage. The majority of samples from the central region formed a weakly supported clade (bootstrap MP = 55; NJ = 55) that was sister to the northern clade. However, the sister group status of this central clade (clade C1) and the northern clade possessed stronger support (bootstrap MP = 69; NJ = 85). The remaining three samples from the central region formed a clade (clade C2, bootstrap MP = 76; NJ = 92) basal to the northern and C1 clades. Clade C1 was found in the populations of the Arkansas drainage, whereas clade C2 was

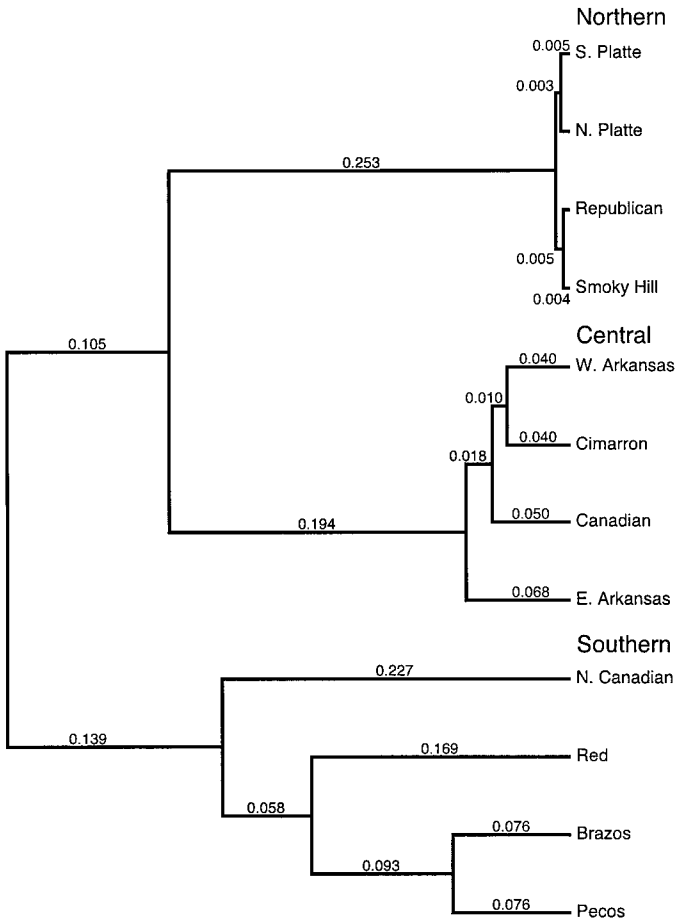

FIG. 2. UPGMA phenogram of populations of *Fundulus zebrinus* produced from the modified Rogers distances (Wright 1978) calculated from the allozyme data. Distances are indicated along the branches, and the populations are labeled according to geographic locale (northern, central, and southern).

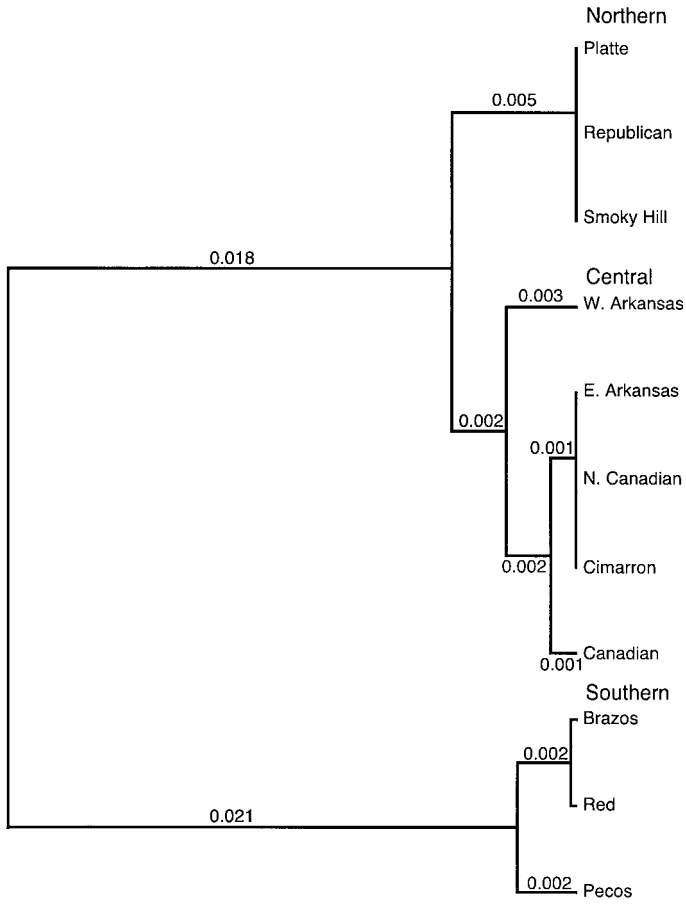

FIG. 3. UPGMA phenogram of populations of *Fundulus zebrinus* produced from the average number of nucleotide substitutions per site (*d*; Nei and Miller 1990) calculated from the 2.1-kb fragment restriction-fragment-length-polymorphism data. Branch lengths are indicated, and the populations are labeled according to geographic locale (northern, central, and southern).

found in populations of the Cimarron, Beaver, and Canadian. However, the geographic partitioning of the two clades was not complete. Of the two samples that were sequenced from the Canadian drainage, one was a C1 haplotype and the other was a C2 haplotype. The southern region formed a monophyletic clade of six closely related haplotypes. The North Fork of the Red (NF Red) sample was basal to the remaining haplotypes, which formed a weakly supported clade (bootstrap MP = 64; NJ = 85) with poorly resolved relationships. Each drainage appeared to harbor unique haplotypes, but one haplotype was observed in both the Brazos and the Pecos drainages.

TABLE 2. Frequency of 2.1-kb fragment restriction-fragment-length-polymorphism haplotypes by drainage. Haplotypes labeled with an asterisk have been sequenced for a representative individual. *h* is the haplotype diversity (Nei 1987, p. 179; eq. 8.5) and π is the nucleotide diversity (Nei 1987, p. 256; eq. 10.5).

Drainage	*n*	1*	2*	3*	4	5	6	7*	8	*h*	π
N. Platte	28	1.0	—	—	—	—	—	—	—	0.000	0.0000
S. Platte	29	1.0	—	—	—	—	—	—	—	0.000	0.0000
Republican	15	1.0	—	—	—	—	—	—	—	0.000	0.0000
Smoky Hill	11	1.0	—	—	—	—	—	—	—	0.000	0.0000
W. Arkansas	82	—	0.5	0.5	—	—	—	—	—	0.506	0.0048
E. Arkansas	36	—	0.94	—	0.06	—	—	—	—	0.116	0.0006
Cimarron	35	—	1.0	—	—	—	—	—	—	0.000	0.0000
N. Canadian	20	—	1.0	—	—	—	—	—	—	0.000	0.0000
Canadian	19	0.32	0.68	—	—	—	—	—	—	0.459	0.0024
Red	45	—	—	—	—	1.0	—	—	—	0.000	0.0000
Brazos	20	—	—	—	—	0.75	0.2	—	0.05	0.416	0.0020
Pecos	12	—	—	—	—	0.25	—	0.75	—	0.409	0.0017

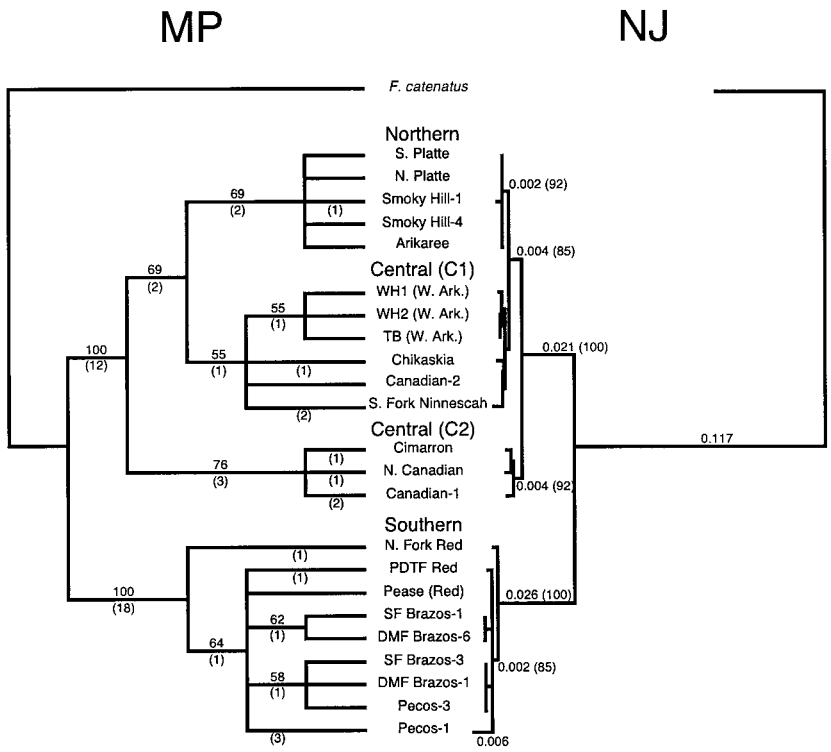

FIG. 4. Consensus tree from the maximum parsimony (MP) bootstrap analysis of the cytochrome oxidase I sequence data on the left. Bootstrap values are indicated above the branches, and the number of nucleotide substitutions are indicated in parentheses below the branch. The neighbor-joining (NJ) phylogram is on the right. Branch lengths and bootstrap values (in parentheses) are indicated for selected branches. Site abbreviations are as follows: WH, Wild Horse Creek in the West Arkansas drainage; TB, Two Butte Creek in the West Arkansas drainage; PDTF Red, Prairie Dog Town Fork of the Red; SF Brazos, Salt Fork of the Brazos; DMF Brazos, Double Mountain Fork of the Brazos. Haplotypes are labeled according to geographic locale (northern, central, and southern), and the two clades (C1 and C2) within the central region are identified.

As with the allozyme and mtDNA RFLP data, the COI sequence data revealed two distinct geographic assemblages, with the northern and central samples forming one highly supported clade (bootstrap MP = 100; NJ = 100) and the southern samples forming a second highly supported clade (bootstrap MP = 100; NJ = 100). The phylogenetic break corresponded to an average of 5.5% (SE = 0.02) sequence divergence. Additional sequence data from the mitochondrial cytochrome *b* gene (Kreiser 1999) revealed identical geographic patterns and also supported the major phylogenetic break between the northern/central and southern fish.

Analysis of Molecular Variance

The analysis of molecular variance (Table 3) indicated that most of the genetic variation was found between regions for both the mtDNA RFLP data (83.19%) and allozyme data (56.73%). Additionally, regional differentiation (Φ_{CT}) was highly significant for both datasets ($P < 0.001$; Table 3). Relatively little variation was distributed within populations or among populations within regions, a result that is also reflected in the other measures of genetic diversity. The geographic structure that we tested for both the mtDNA and allozyme data was based on the pattern seen in the mtDNA data. Although the structure revealed by the allozyme data was not entirely concordant (Fig. 2), the removal of the North Canadian population or placement within the southern region had little effect on the results of the AMOVA (results not shown).

DISCUSSION

Our survey of genetic variation in the plains killifish revealed three regional groups, which we designated as northern, central, and southern. The mtDNA data revealed shallow phylogeographic structuring within regions and a low level of differentiation between the northern and central regions. However, a large phylogenetic break distinguished populations of the southern region. Sequence divergence between the two clades was high and bootstrap support for each clade was robust. The allozyme data also supported the recognition

TABLE 3. Results of analysis of molecular variance (AMOVA). The variance components are as follows: AR, among regions; AP/WR, among populations within regions; WP, within populations. An asterisk indicates a significance level of <0.001.

Variance component	MtDNA RFLP		Allozyme	
	% Variation	Φ Statistic	% Variation	Φ Statistic
AR	83.19	$\Phi_{CT} = 0.8319$*	56.73	$\Phi_{CT} = 0.5673$*
AP/WR	7.43	$\Phi_{SC} = 0.4420$*	17.72	$\Phi_{SC} = 0.4097$*
WP	9.38	$\Phi_{ST} = 0.9062$*	25.54	$\Phi_{ST} = 0.7446$*

of three regional groups, with the exception of the placement of the North Canadian population. The generally concordant patterns seen in the mtDNA and allozyme data strengthen hypotheses based on these patterns. Concordance across unlinked loci suggests that these patterns reflect the biogeographic forces that shaped the distribution of genetic diversity in this species (Avise and Ball 1990; Avise 1998). In the discussion that follows, we will interpret the phylogeographic patterns in light of the geologic history of the Great Plains and western Gulf Slope drainages, as well as compare our results with previous studies of the ichthyofauna of these regions.

Biogeography of the Great Plains

The northern/central clade was characterized by limited differentiation, as evidenced by the short branch lengths on the NJ tree (Fig. 4). This pattern suggests that contemporary gene flow has been restricted, resulting in divergence between the northern and central regions. Furthermore, the northern populations were characterized by a near lack of variation in both mtDNA and allozymes. Hewitt (1996) and Bernatchez and Wilson (1998) described a general pattern of lower genetic diversity in terrestrial animals and fish from higher latitudes compared with more southern populations. These authors attributed the decline in genetic diversity with latitude to the effects of drift and population bottlenecks occurring during the colonization of the northern areas subsequent to Pleistocene glaciations. Based on this characterization, the northern populations of *F. zebrinus* possess a population genetic structure that is indicative of recent colonization, potentially by a small number of founders.

The northern portion of the range of *F. zebrinus* was thought to have been colonized via the Ancestral Plains Stream (Metcalf 1966; Cross et al. 1986). The Ancestral Plains Stream (APS) was a north-south river that extended from the northern Great Plains southward across central Kansas and Oklahoma to the ancestral Red River. The APS served as the major drainage system of the Great Plains until after the Kansan glacial stage (~500,000 years ago). Subsequent to the Kansan, streams of the Mississippi River basin cut westward and captured portions of the APS producing the west-to-east flowing drainage systems that exist today.

The lack of phylogeographic structure in the northern portion of *F. zebrinus* and limited divergence of these populations from their closet neighbors in the central portion of the range supports this dispersal scenario. For the RFLP data, the average divergence between the northern and central populations was 1.0% (SE = 0.14%), and for the COI sequence data the divergence between the northern and C1 clades was 0.57% (SE = 0.02%). Using a calibration for the rate of evolution for COI in marine teleost fishes (1.2% per million years; Bermingham et al. 1997), the COI sequence data places the divergence between the northern and C1 clades as having occurred around 475,000 years ago. This estimated divergence time roughly corresponds to the Kansan glacial stage. A similar pattern of differentiation was revealed by the work of Richardson and Gold (1995b) on the red shiner, *Cyprinella lutrensis.* The red shiner has a range similar to *F. zebrinus* and would have been influenced by the same geologic history. In the populations of *C. lutrensis* from Nebraska, Kansas, and Illinois, Richardson and Gold reported a lack of phylogeographic structure and low levels of intrapopulation variation, leading them to suggest that colonization of these northern areas took place during the mid to late Pleistocene.

Biogeography of the Western Gulf Slope

No clear phylogeographic pattern was evident in the southern clade (Fig. 4). The North Fork Red haplotype was basal to a polytomy of haplotypes from the Red, Brazos, and Pecos Rivers. The haplotypes, many restricted to single drainages, were only slightly differentiated. Just one haplotype was shared between the Brazos and the Pecos drainages.

In general, the western Gulf Slope drainages harbor low levels of endemism and many fish species have extensive ranges outside of this area, leading Conner and Suttkus (1986) to conclude that much of this region's ichthyofauna evolved outside of the western Gulf Slope. For example, *F. zebrinus, Hybognathus placitus, C. lutrensis,* and *Phenacobius mirabilis* are found across the western Gulf Slope as well as across the Great Plains. Great Plains fishes could have reached the western Gulf Slope drainages either via stream captures or by dispersal via the Gulf of Mexico.

The drainage basins of the upper reaches of both the Colorado and Brazos Rivers seem to have been derived from stream captures of some unknown southern Great Plains river systems. Evidence supporting this includes the presence of diversion elbows or regions where the river abruptly changes direction (in this case from east to southeast). Both rivers probably obtained their current forms sometime before the Kansan glacial stage. Additional changes are thought to have occurred in the Brazos when part of this drainage was captured by the Red River. The Pecos River probably acquired its current drainage basin by capturing the headwaters of other rivers, including the Red and Canadian Rivers (Conner and Suttkus 1986). Alternately, Thomas (1972) describes evidence for the capture of the upper Canadian and Brazos by the Pecos during the late Pleistocene, followed by the loss of portions of the upper headwaters of the Pecos back to the Canadian River.

The mtDNA data (Figs. 3, 4) clearly indicated a close relationship of the Red River populations with those in the Brazos and the Pecos. This pattern suggests that minimally the dispersal of *F. zebrinus* into the Brazos, and Pecos was the result of stream captures from the Red River. Neither the mtDNA nor the allozyme data supports the postulated stream capture of the Canadian by a western Gulf Slope drainage (Conner and Suttkus 1986). However, further interpretations of the data are limited by the lack of resolution within the southern clade (Fig. 4). Several hypotheses of stream captures could be postulated to account for the distribution of haplotypes in the Red, Brazos, and Pecos Rivers. The main conclusion that we draw from the data is that *F. zebrinus* obtained access to the western Gulf drainages from the Great Plains via stream captures of the Red River and not some of the other southern Great Plains drainages.

The North Canadian

The grouping of the North Canadian population with the southern lineage is the only discordance between the allo-

zyme and mtDNA data. Table 1 illustrates the reason why the UPGMA clustering algorithm recovered this relationship. For *Pgm,* the C allele was found at a high frequency in the North Canadian as well as populations in the southern drainages. Furthermore, the North Canadian shares a rare allele (A) with the Red River population. This pattern could be the product of shared history, natural selection, genetic drift, convergence in mobility of electromorphs, or a combination of the these. If the pattern is indicative of biogeographic history, then the genetic contact was the result of the North Canadian capturing a portion of a southern drainage. The fish transferred between drainages during this capture would have possessed high frequencies of the C allele at *Pgm.* Additionally, the establishment of the C allele may have been enhanced if the migrants arrived at a time when the existing population was small (Childs et al. 1996), which is a possibility given the intermittent nature of many western streams. The lack of a southern mtDNA haplotype in the North Canadian might be a consequence of the smaller effective population size for mtDNA compared to nuclear loci leading to the loss of the southern haplotype through lineage sorting. However, none of the alternative explanations mentioned above can be supported or refuted with the current data.

Evolutionary History of Fundulus zebrinus

In each of the datasets (allozymes, mtDNA RFLP, and mtDNA COI sequences), *F. zebrinus* exhibits regional genetic differentiation. A substantial phylogenetic break splits the range of *F. zebrinus* into two clades composed of populations from the northern/central drainages and the southern drainages. Typically, a strong phylogenetic break, with the corresponding spatial separation of the clades, is attributed to the existence of a long-term barrier to gene flow. Certainly this is a plausible explanation, but in the case of *F. zebrinus* what barrier to gene flow led to the isolation and divergence of the two groups?

Undoubtedly the APS was a major factor shaping the distribution of fish across the northern Great Plains. However, drainage patterns of the APS south of southern Oklahoma are not well understood (Cross et al. 1986). The APS either joined the ancestral Mississippi or had an independent entry to the Gulf, perhaps via the current Trinity River valley in Texas. Regardless of the path of the APS, at some point there had to have been a confluence of the APS with the ancestral Red River, serving to connect the northern and southern portions of the range of *F. zebrinus.* One group was perhaps restricted to the upper portion of the ancestral Red River, whereas the other group was restricted to the drainages associated with the APS. A way to test this hypothesis would be to acquire collections along the lower portion of the Red River and within the Arkansas drainage in southern Oklahoma. If the phylogenetic break is truly indicative of a long-term barrier to gene flow, then the haplotypes observed would continue to fall into the two distinct clades. Alternatively, if the separation of the two groups was not complete, an isolation by distance pattern of differentiation would be expected. In this case, further collections would reveal more closely related haplotypes in the geographic regions closer to the ancestral confluence of the Red and Arkansas Rivers.

The presence of the substantial phylogenetic break reopens a long-standing taxonomic dispute as to whether there are actually two species of plains killifish: *F. zebrinus* and *F. kansae.* A confused history surrounds the taxonomy of this fish (see Poss and Miller 1983). Various authors have synomynized *F. zebrinus* and *F. kansae* (e.g., Jordan and Gilbert 1883; Everett 1972; Poss and Miller 1983), recognized them as distinct species (e.g., Hubbs 1926; Gosline 1949; Parenti 1981), or considered them to represent subspecies (e.g., Sigler and Miller 1963; Echelle et al. 1971; Duke 1981). Although morphological studies have been unable to conclusively resolve the issue, the extent of divergence between the northern/central and southern clades, the strong bootstrap support for these clades (Fig. 4), and additional data (mitochondrial cytochrome *b*; Kreiser 1999) all support the recognition of two species.

A discrepancy does exist between the ranges described for *F. zebrinus* and *F. kansae* in the literature and the geographic distribution of the clades (Fig. 4) putatively designated as *F. zebrinus* and *F. kansae.* Miller (1955) described the range of *F. zebrinus* as being restricted to the Brazos, Colorado, and Pecos Rivers, whereas *F. kansae* is found in the Red River and further north. However, the *F. kansae* and *F. zebrinus* groups recognized by this study do not follow the range descriptions proposed by Miller (1955). The molecular data places the clade putatively representing *F. zebrinus* in the Pecos, Brazos, and Red (the Colorado was not sampled) and the clade putatively representing *F. kansae* in drainages north of, but not including, the Red River. Although the existing taxonomic framework seems sufficient for including the molecular evidence, the ranges of *F. kansae* and *F. zebrinus* should be reexamined in light of the newly resolved phylogeographic relationships.

Comparisons with Other Species

Great Plains

Two classes of fish distributions seem to be present in the Great Plains based on geographic distribution and habitat preferences: species that are widespread and seemingly endemic to the Great Plains and others that are found in restricted areas with their main distributions in the Central Highlands (Metcalf 1966; Pflieger 1971; Cross and Moss 1987). The fish with affinities to the Central Highlands are likely relictual populations from a widespread distribution across the Great Plains during more favorable climatic conditions of the Pleistocene (Cross 1970). Insight into the paleohydrology of the Great Plains might be obtained through studies of these relictual populations. For example, the disjunct populations of the southern redbelly dace (*Phoxinus erythrogaster*) in Colorado, Kansas, and Oklahoma may be the result of dispersal through the APS. However, a better understanding of the Great Plains paleohydrology would also be obtained by examining fish thought to have evolved in the Great Plains.

One example of a fish with a very similar distribution to *F. zebrinus* is the plains minnow (*H. placitus*), although it is more widely distributed in the northern Great Plains. Al-Rawi and Cross (1964) examined meristic and morphological variation in *H. placitus* across its range and they found that scale

counts above and below the lateral line demonstrated clinal variation. A break in the cline was between populations from the Red and Arkansas River drainages. Al-Rawi and Cross chose not to recognize subspecies, but the fact that the geographic location of the break in the cline is identical to the phylogenetic break seen in the plains killifish suggests that a molecular study might reveal a similar pattern in *H. placitus.*

Western Gulf Slope

We examined studies of western Gulf Slope species, searching for concordant patterns that would support our geologic interpretations. The *C. lutrensis* species complex has a wide distribution across the Great Plains and into the western Gulf Slope. Like *F. zebrinus,* the ancestor to this group is thought to have originated in the Great Plains (Gibbs 1957) and have been present within the APS (Metcalf 1966). However, these fishes do not seem to have been influenced by the same events that shaped the biogeography of *F. zebrinus.*

Within the *C. lutrensis* species group, Richardson and Gold (1995a) determined that *Cyprinella* within the Nueces and Frio/Sabinal River systems of the western Gulf Slope were actually comprised of two distinct species that differed from *C. lutrensis* by 8.36% and 9.04% sequence divergence as measured by mtDNA RFLPs. They proposed that the ancestor to this group dispersed to the western Gulf Slope via a connection between the western Great Plains and ancestral Rio Grande/upper Pecos Rivers during the late Miocene–early Pliocene. This scenario is in contrast to a mid Pleistocene dispersal of fish across the western Gulf Slope via stream captures as seems to be the case for *F. zebrinus.* In their survey of variation across the range of *C. lutrensis,* Richardson and Gold (1995b) found 6.8% sequence divergence in mtDNA RFLPs between fish from the Brazos River (their clade C) and the clade containing fish from the Trinity River (clade B) and populations to the north (clade A). They suggested that this split was the result of a major vicariant event that separated the Brazos and Trinity Rivers during the mid Pliocene. The phylogenetic break between the two main clades of *C. lutrensis* does not correspond geographically to the one in *F. zebrinus,* but the timing of the split (pre-Pleistocene) is concordant between the two species. In addition, the vicariance pattern seen in *C. lutrensis* may provide some insight into the geologic history of the APS. The Trinity River clade was sister to the clade containing the haplotypes located in the northern portion of the range, which may be an indication that the APS did empty into the Trinity River valley.

Although both *C. lutrensis* and *F. zebrinus* are thought to have originated within the APS, their dispersal and divergence within the western Gulf Slope appears to be the product of different geologic events. The lack of concordance between these two species indicates that care should be used when choosing among competing geologic hypotheses to explain the phylogeographic patterns seen in a single species.

Conclusions

The Pleistocene glaciations were a major force in shaping the drainage systems and thus the ichthyofauna of west-central North America. We used the patterns and amount of genetic variation to make specific inferences regarding the paleohydrology of rivers in the range of *F. zebrinus.* Populations of *F. zebrinus* in the northern portion of its range are likely the result of dispersal via the APS. We were also able to make inferences about the history of stream captures among the western Gulf Slope drainages. The molecular data suggest that *F. zebrinus* gained access to the western Gulf slope via stream captures of the Red River and not the Canadian River. Additionally, the distribution of the C allele at the *Pgm* locus suggests the possibility of a recent historical connection (stream capture) between the central and southern drainages via the North Canadian River. Finally, the major phylogenetic break observed between the northern/central and southern populations suggests not only that two regions (the APS and ancestral Red River) likely served as the evolutionary centers for the plains killifish, but also that these two regions gave rise to distinct species of plains killifish. Attempts to support our interpretations with the phylogeographic patterns of other fishes are limited by the few available studies of fishes endemic to Great Plains. Our understanding of the evolution of west-central North American fishes would be strengthened by employing a comparative approach to test the generality of the biogeographic patterns detected in *F. zebrinus.*

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