Molecular Ecology (1995) 4, 163–171

Evolution of the *Cyprinella lutrensis* species group. III. Geographic variation in the mitochondrial DNA of *Cyprinella lutrensis* – the influence of Pleistocene glaciation on population dispersal and divergence

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Abstract

We employed restriction site variation in mitochondrial (mt)DNA to determine if significant phylogeographic structure occurs in the North American cyprinid fish *Cyprinella lutrensis*. Digestion patterns from 16 restriction endonucleases identified fifty mtDNA haplotypes among 127 individuals of *Cyprinella lutrensis* assayed from localities in the Gulf Coastal Plain, the Great Plains, and the Central Lowlands. Nucleotide sequence divergence among haplotypes was highly variable (mean ± SE: 2.87% ± 0.08; range: 0.14–9.24%). Maximum-parsimony analysis and the neighbour joining method of tree construction revealed three major groupings (clades) of haplotypes that differed in geographic distribution. Divergence estimates between the basal clade, comprised of haplotypes primarily from the Brazos River in east Texas, and the remaining two clades, place *C. lutrensis* in the western Gulf Coastal Plain prior to Pleistocene glaciation. Nucleotide sequence divergence between the second clade, comprised of haplotypes from the Trinity and Calcasieu rivers in east Texas and southwestern Louisiana, respectively, and the third clade (comprised primarily of haplotypes from localities north of Texas and affected directly by Pleistocene glaciation), suggest that *C. lutrensis* colonized glaciated regions to the north during the mid- to late Pleistocene. This hypothesis is supported by levels of intrapopulational nucleotide diversity in geographic localities outside of Texas and by geological evidence. Despite marked geographic variation in morphometrics, meristics, and nuptial coloration, mtDNA variation in glaciated regions was not geographically structured, and subspecies of *C. lutrensis* were not identifiable by phylogenetic analysis of mtDNA.

Keywords: colonization, *Cyprinella*, mitochondrial DNA, phylogeography, Pleistocene glaciation

Received 8 August 1994; revision received 26 September 1994; accepted 19 October 1994

Introduction

Genetic characters often have been used to determine whether populations within species or species within defined higher-level taxa are geographically structured (Avise *et al.* 1987; Dowling *et al.* 1990). In some instances, high levels of spatially partitioned genetic diversity within taxonomically or phylogenetically defined groups have been documented and interpreted to be the consequence of historical biogeographic or geological events (Neigel & Avise 1985; Avise *et al.* 1987). In other instances, the absence of significant geographic structure has been interpreted to be the result of recent population establishment following range expansion (Neigel & Avise 1985; Avise *et al.* 1987; Zink & Dittmann 1993). Complicating factors regarding the interpretation of these types of data are that population level phenomena (e.g. gene flow, deme size) may give rise to geographic structure in the

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absence of biogeographic or geological events (Avise *et al.* 1984). Nonetheless, documentation of geographic patterns of genetic diversification and variation forms the framework for inferring processes of geographic differentiation, and ultimately, speciation.

We examined phylogeographic structure in the red shiner, *Cyprinella lutrensis*, a cyprinid fish endemic to North America. The species is widely distributed throughout the central and south-central United States, and occurs extensively in areas outside of its native range, often flourishing where it has been introduced (McAda & Berry 1980; Lanigan & Berry 1981; Douglas *et al.* 1994). In this regard, *C. lutrensis* is a prime example of a pioneering or colonizing species. A major portion of the distribution of *C. lutrensis* is in areas that were directly or indirectly affected by Pleistocene glaciation. Pleistocene glaciation in North America profoundly affected the distribution of fishes and other aquatic organisms (Robison 1986), and has generated phylogeographic structure in several fish species and species-groups (Bermingham & Avise 1986; Bernatchez & Dodson 1990, 1991; Billington *et al.* 1990). Glaciation also has been shown to affect genetic diversity as a result of population fragmentation and the creation of new avenues for dispersal and gene flow. Because *C. lutrensis* is highly eurytypic and aggressive ecologically, a major question is whether *C. lutrensis* colonized glaciated regions after the glaciers receded, or whether it occurred in these regions prior to glaciation and persisted throughout the Pleistocene in glacial refugia. Gibbs (1957) and Metcalf (1966) hypothesized that *C. lutrensis* originated preglacially in the southwestern United States, and Metcalf (1966) was of the opinion that *C. lutrensis* occurred preglacially in his 'Ancient Plains Stream' centred in the Great Plains from central Nebraska south through southern Oklahoma.

Data on geographic variation in *C. lutrensis* is primarily limited to morphological characters (Contreras-Balderas 1975; Matthews 1987; Mayden 1989). Four subspecies of *C. lutrensis* have been recognized (references in Matthews [1987]), and one (*C. l. blairi*) is presumed extinct (Miller *et al.* 1989). Matthews (1987), based on a study of nuptial coloration and meristic and morphometric character variation among ≥ 1000 *C. lutrensis*, identified geographic patterns of diversification in morphology, much of which was found in areas that were affected by glaciation. He found that levels of morphological diversity among *C. lutrensis* in the central and northern part of its range (i.e. in areas impacted by glaciation), were comparable to those in the southern portion of its range in the Gulf Coastal Plain. This pattern is consistent with the hypothesis that *C. lutrensis* persisted in glacial refugia. The isolation of populations in geographic space (e.g. in glacial refugia) often impacts a species' range of environmental tolerances, and can result in locally adapted diversification (Futuyama 1979). However, because the genetic basis of the morphological characters employed by Matthews (1987) is not known, the patterns observed may not necessarily reflect patterns of historical phylogeography.

We employed mitochondrial (mt)DNA analysis to determine if significant phylogeographic structure occurs in *C. lutrensis*. Several studies have used mtDNA to reveal strong patterns of phylogeographic structure attributable to historical biogeography (Bermingham & Avise 1986; Bernatchez & Dodson 1990, 1991; Riddle *et al.* 1993). In many of these studies, Pleistocene glaciation was inferred to be the underlying historical component responsible for the phylogeographic structure observed. Zink & Dittmann (1993), alternatively, employed mtDNA analysis in a study of geographic variation among song sparrows throughout glaciated areas in North America, but failed to identify phylogeographic structure. They interpreted the mtDNA homogeneity to be the result of recent episodes of colonization into previously unoccupied territory. The central questions of interest in our study were: (i) did *C. lutrensis* occur preglacially in the northern and central portions of its range (i.e. in areas directly or indirectly affected by Pleistocene glaciation), or did *C. lutrensis* colonize these areas after the glaciers receded? and (ii) do patterns of geographic diversity in mtDNA of *C. lutrensis* reflect observed patterns of morphological diversity?

Materials and methods

A total of 127 individuals of *C. lutrensis*, representing 11 sampling localities, were obtained from the three physiographic regions in which *C. lutrensis* occurs (Table 1, Fig. 1). Five of the geographic samples (three samples from Illinois, and one each from Nebraska and Kansas) are from areas that were under ice during one or more of the glacial episodes (Burr & Page 1986). Two of the geographic samples (both from Louisiana) are from the lower Mississippi Embayment and were affected indirectly during the Pleistocene by repeated incursions from the Gulf of Mexico (Robison 1986). The remaining four geographic samples (from Oklahoma and Texas) are from areas that presumably were not directly impacted by Pleistocene glaciation. *Cyprinella lepida* ($n = 22$), an undescribed species of *Cyprinella* (Richardson & Gold 1994) from the Nueces River ($n = 10$), and *Cyprinella venusta* ($n = 10$) were used as outgroups (Table 1, Fig. 1). The two *Cyprinella* from the Nueces River basin (*C. lepida* and the undescribed species) are members of the *C. lutrensis* species-group, a putatively monophyletic clade of ten or more species (Mayden 1989); *C. venusta* belongs to the *Cyprinella whipplei* species-group, the presumed sister clade to the *C. lutrensis* species-group (Mayden 1989).

Table 1 Geographic samples of *Cyprinella lutrensis* and other species examined in the study. Numbers refer to those shown in Fig. 1

Sample*		*N*	MtDNA haplotypes†
	Cyprinella lutrensis		
1	North Henderson Creek, Mercer Co., IL (Mississippi River)	12	14 (5),27 (2),28 (2),29 (2), 31 (1)
2	Mackinaw River, Tazewell Co., IL (Illinois River)	11	14 (6),32 (1),33 (2),34 (1), 35 (1)
3	Un-named tributary, Union Co., IL (Mississippi River)	11	14 (7),28 (2),30 (1),36 (1)
4	Bayou Macon, E/W Carrol Parish Line, LA (Mississippi River)	13	14 (5),37 (2),39 (2),41 (1), 42 (1),85 (1),86 (1)
5	Platte River, Buffalo Co., NB (Platte River)	12	45 (1),46 (8),47 (1),48 (1), 49 (1)
6	Smoky Hill River, Russel Co., KS (Kansas River)	12	14 (7),33 (1),50 (1),51 (1), 52 (1),53 (1)
7	Cimarron River, Kingfisher Co., OK (Arkansas River)	11	14 (7),54 (1),55 (1),56 (2)
8	Walnut Creek, McClain-Cleveland Co. Line, OK (Canadian River)	11	14 (2),31 (2),57 (2),58 (2), 59 (2),60 (1)
9	Bayou Serpent, Jefferson Davis Parish, LA (Calcasieu River)	10	14 (4),43 (5),44 (1)
10	Trinity River, Houston Co., TX (Trinity River)	12	35 (1),43 (3),64 (1),74 (2), 75 (1),76 (1),77 (1),78 (1), 79 (1)
11	Tehuacanna Creek, McLennan Co., TX (Brazos River)	12	62 (3),63 (1),64 (1),65 (1), 66 (2),67 (1),68 (1),69 (1), 70 (1)
	Cyprinella sp.		
12	Nueces River, Edwards Co., TX (Nueces River)	10	1 (4),2 (3),3 (2),4 (1)
	Cyprinella lepida		
13	Frio River, Bandera Co., TX (Frio River)	10	5 (2),6 (4),7 (1),8 (1), 9 (1),10 (1)
14	Sabinal River, Bandera Co., TX (Frio River)	12	9 (11),11 (1)
	Cyprinella venusta		
15	Little Brazos River, Burelson Co., TX (Brazos River)	10	18 (1),19 (1),20 (1),21 (1), 22 (1),23 (1),24 (1),25 (1), 26 (2)

*Rivers in brackets refer to major river drainages for each locality.
†Numbers in parentheses correspond to the number of individuals of a corresponding haplotype.

Details of genomic DNA extraction, precipitation, and storage may be found in Gold & Richardson (1991). Sixteen restriction endonucleases were used to digest mtDNA molecules according to manufacturer's specifications: *Apa*I, *Bcl*I, *Bgl*II, *Bst*EII, *Eco*RI, *Eco*RV, *Hpa*I, *Kpn*I, *Nco*I, *Nsi*I, *Pst*I, *Pvu*II, *Sac*I, *Sac*II, *Sca*I, and *Xho*I. Methods of agarose electrophoresis, Southern blots, hybridization, and autoradiography may be found in Gold & Richardson (1991). Hybridization employed an intact mtDNA molecule from *C. lutrensis* labelled with [α^{32}P]-dCTP (New England Nuclear, sp. act. = 3000 Ci/mM) by random priming (Feinberg & Volgelstein 1984). A description of the mtDNA probe used may be found in Richardson & Gold (1991). MtDNA fragments were sized by fitting migration distances to a least-squares regression line of lambda DNA *Hin*dIII fragment migration distances. When necessary, homology of fragments was verified with side-by-side comparisons. Relative map positions of all restriction sites were determined by double-digestion (Sambrook *et al.* 1989), and by side-by-side restriction digests of polymerase-chain-reaction (PCR) amplified mtDNA fragments. Procedures used for PCR were similar to those outlined in Schmidt & Gold (1992). A mtDNA gene map depicting the relative map position of all restriction sites and the size and orientation of the PCR fragments amplified is shown in Fig. 2.

Fig. 1 Map depicting area (shaded) and major physiographic provinces in which *C. lutrensis* occurs. Numbers enclosed in circles refer to sample localities of *Cyprinella* examined in the study (Table 1). Sample localities 1–8 refer to drainages of the Mississippi River basin, while localities 9–15 are Gulf Coast drainages.

A restriction-site presence/absence matrix for individual mtDNA composite genotypes (haplotypes) was constructed using the GENERATE program in the REAP v4.0 package (McElroy *et al.* 1992). Maximum-parsimony analysis of mtDNA haplotypes was carried out using PAUP v3.0 (Swofford 1991). All autapomorphic and sym-

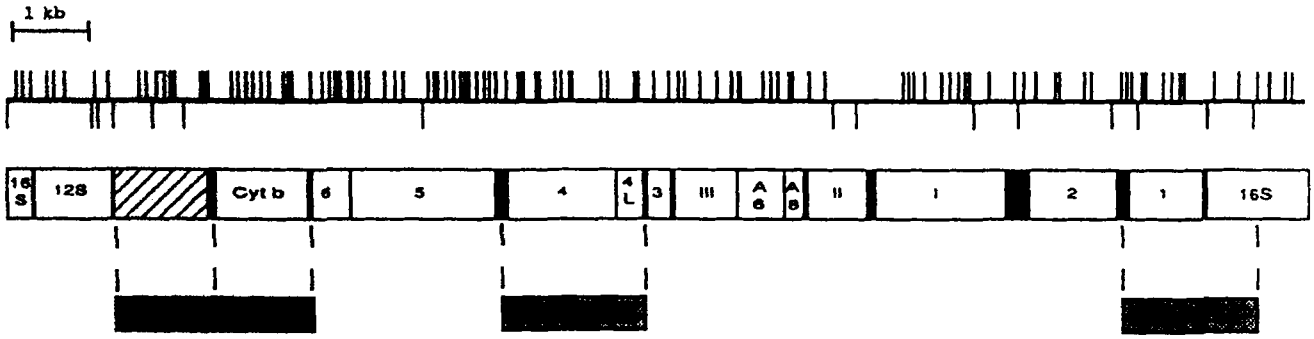

Fig. 2 Mitochondrial (mt)DNA gene map showing relative placement of 164 restriction sites surveyed in the study. Polymorphic sites are shown above the line; conserved sites are shown below the line. Size and orientation of fragments (shaded regions) amplified using the PCR reaction are shown below the map. Mitochondrial gene map is oriented to the human mtDNA gene map (Anderson *et al.* 1981). Abbreviations of mtDNA genes are as follows: small and large rRNA genes (12S and 16S), NADH dehydrogenase subunits (1–6,4L), cytochrome oxidase subunits (I-III), ATPase subunits (A6, A8), and cytochrome *b* (cyt b); transfer RNA genes appear as black areas, and the D-loop or control region is shaded.

plesiomorphic characters were removed prior to parsimony analysis using the REDUCE program in REAP. Phylogenetic relationships among geographic samples of *C. lutrensis* also were assessed using estimates of mtDNA nucleotide sequence divergence. Sequence divergence (based on haplotype frequencies) among pairs of sample localities was calculated using equation 10.20 of Nei (1987). Based on maximum-parsimony analysis of individual haplotypes, if haplotypes contained within individual sampling localities grouped into different phylogenetic assemblages, sample localities were partitioned into separate operational units. This was done in order to remove confounding effects caused by phylogenetically divergent haplotypes occurring at the same geographic locality. The underlying rationale of the latter is that co-occurrence of phylogenetically distinct mtDNA lineages at the same sample locality is likely the result of secondary dispersal or admixture (Avise *et al.* 1987). Trees were generated from distance matrices using the neighbour-joining algorithm (Saitou & Nei 1987) and the NEIGHBOUR program in PHYLIP v3.4 (Felsenstein 1992). Finally, in order to quantify mtDNA diversity within and among sampling localities, we computed both nucleon diversity (the probability that any two individuals drawn at random will differ in mtDNA haplotype) and intrapopulational nucleotide sequence diversity (the average nucleotide difference between any two individuals drawn at random) using equations in Nei & Tajima (1981).

Results

The mean genome size of all complete digestions was 16.7 ± 0.2 kilobases (kb); however, one individual from the Cimarron River was found to possess a tandem duplication (Richardson & Gold 1991). The 16 restriction enzymes used detected an average of 55.8 sites per mtDNA genome of *C. lutrensis*. Because the restriction enzymes utilized in this study cleave six-base recognition sites, an average of 332 nucleotides or approximately 2.0% of the mtDNA genome was surveyed. Digestion patterns from the 16 restriction enzymes revealed 70 composite mtDNA genotypes (haplotypes): 50 were found among the 127 individuals of *C. lutrensis* assayed, 11 were found among the 32 individuals representing the two species of *Cyprinella* from the Nueces River basin, and nine were found among the 10 individuals of *C. venusta*. Estimated nucleotide sequence divergence (p) among the fifty haplotypes from *C. lutrensis* was 2.87% ± 0.08 (mean ± SE) and ranged from 0.14% to 9.24%.

Maximum-parsimony analysis of mtDNA haplotypes generated over 4500 equally parsimonious trees (length = 228 steps, CI = 0.553, RI = 0.913). The majority rule consensus tree generated from these trees (Fig. 3) revealed at least three ingroup (*C. lutrensis*) clades that were phylogeographically informative. The first (clade A, Fig. 3) comprised 34 haplotypes that were widely distributed throughout the central and northern part of the species range. With one exception, all of the haplotypes in clade A were found in localities outside of Texas. The exception was haplotype 35 which was found in one individual from the Mackinaw River in south-central Illinois, and in one individual from the Trinity River in east Texas. Within clade A, haplotypes from the Platte River (haplotypes 45 through 49, Fig. 3) formed a well-supported, monophyletic lineage. The second clade (clade B, Fig. 3) comprised five haplotypes that were restricted to two river drainages in the Gulf Coastal Plain, i.e. the Trinity River in east Texas and Bayou Serpent (Calcasieu River drainage) in south-western Louisiana. The last clade (clade C, Fig. 3) comprised 10 haplotypes found in the Brazos and Trinity river drainages in east Texas. Two of the sample localities from the Gulf Coastal Plain (viz.

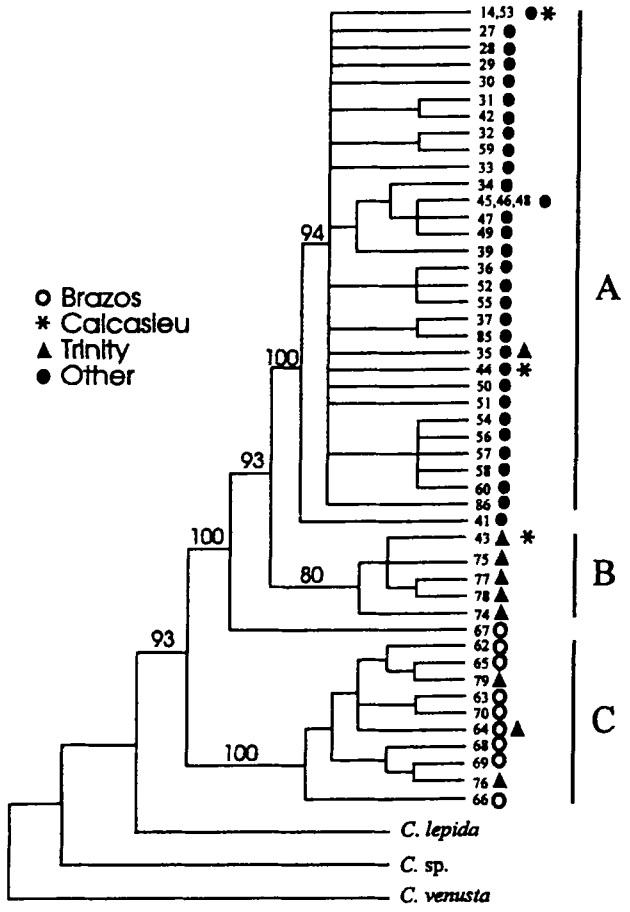

Fig. 3 Majority rule (50%) consensus tree of mtDNA haplotypes produced by maximum-parsimony analysis. Numbers above nodes represent the proportion (in percent) of trees from over 4500 trees that identified a given clade. Haplotypes refer to those listed in Table 1. Symbols represent geographic regions where mtDNA haplotypes were found. Major phylogenetic clades within *C. lutrensis* are denoted by the letters A, B, and C. Relationships among haplotypes found within outgroup taxa are not shown. Branch lengths are not accurate representations of the number of restriction site changes between operational units.

Bayou Serpent in southwestern Louisiana and the Trinity River in east Texas) contained haplotypes found in more than one of the three clades. Haplotypes 14, 44 (clade A), and 43 (clade B) were found in the sample from Bayou Serpent; whereas haplotypes 35 (clade A), 43, 74, 75, 77, 78 (clade B), and 64, 76, 79 (clade C) were found in the sample from the Trinity River. Two haplotypes (41 and 67, found in Bayou Macon in northeastern Louisiana and in the Brazos River drainage, respectively) were not included in clades A–C (Fig. 3). Haplotype 41 was placed as a sister to clade A, and haplotype 67 was placed as a sister to a clade that included clades A and B. Finally, haplotype 14 (clade A) was the most common haplotype encountered in the survey, occurring in 43 of 127 individuals (34%) and in all sample localities except for the Platte River in Nebraska, and the Trinity and Brazos river drainages in east Texas.

Relationships among the major clades of *C. lutrensis* haplotypes were as follows: clade A (plus haplotype 41) was sister to clade B, and a lineage of clade A, haplotype 41, clade B, and haplotype 67 was sister to clade C (Fig. 3). The number of restriction site changes between clades differed dramatically. Haplotypes within clades A and B, and haplotypes 41 and 67 differed from one another by an average of 6.1 restriction sites (range 1–17); whereas haplotypes in clade C differed from haplotypes in clades A and B, and haplotypes 41 and 67, by an average of 35.8 restriction sites (range 30–46).

Because maximum-parsimony analysis of mtDNA haplotypes revealed that haplotypes occurring in Bayou Serpent (Calcasieu River drainage), the Trinity River, and the Brazos River drainage belonged to different clades or lineages, these three sample localities were subdivided into separate operational units as follows: Bayou Serpent (Calcasieu1: haplotypes 14 and 44; Calcasieu2: haplotype 43); Trinity River (Trinity1: haplotypes 43, 74, 75, 77, 78; Trinity2: haplotypes 64, 76, 79; and Trinity3: haplotype 35); and Brazos River drainage (Brazos1: haplotypes 62–66, 68–70; Brazos2: haplotype 67). This resulted in a total of 15 operational units for which pairwise estimates of (interpopulational) mtDNA nucleotide sequence divergence were calculated. The neighbour joining tree (Fig. 4), generated from the interpopulational distance matrix, was virtually identical in all major features to the

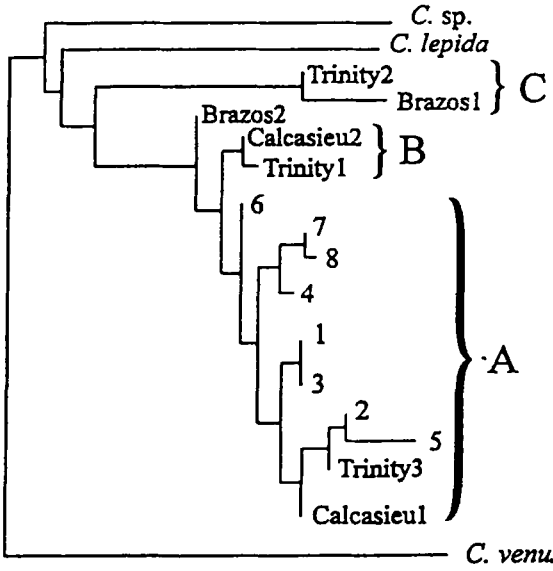

Fig. 4 Neighbour-joining tree generated from interpopulational nucleotide sequence divergence estimates. Numbers (1–8) refer to *C. lutrensis* samples listed in Table 1. Additional samples refer to groupings of haplotypes identified from maximum-parsimony analysis of mtDNA haplotypes (see text).

Table 2 Nucleon and mean (± SE) intrapopulational nucleotide sequence diversities (as a percentage) within individual sample localites of *C. lutrensis*

Sample	Nucleon Diversity	Nucleotide sequence diversity
1 North Henderson Creek	0.803	0.338 ± 0.029
2 Mackinaw River	0.709	0.258 ± 0.035
3 Tributary; Upper-Mississippi	0.545	0.281 ± 0.038
4 Bayou Macon	0.846	0.417 ± 0.029
5 Platte River	0.575	0.114 ± 0.015
6 Smoky Hill River	0.682	0.247 ± 0.031
7 Cimarron River	0.600	0.227 ± 0.029
8 Walnut Creek	0.909	0.428 ± 0.035
9 Bayou Serpent	0.644	0.361 ± 0.046
10 Trinity River	0.939	3.331 ± 0.407
11 Tehuacanna Creek	0.939	1.644 ± 0.267

(maximum- parsimony) consensus tree generated from mtDNA haplotypes: clade A included all samples (and haplotypes) found outside of Texas (except for haplotype 43 from Bayou Serpent), plus haplotype 35 from the Trinity River; clade B included haplotypes from Bayou Serpent (Calcasieu1) and the Trinity River (Trinity1); and clade C included haplotypes from the Trinity River (Trinity2) and the Brazos River drainage (Brazos1). Inferred relationships among clades A–C in the neighbour-joining tree also were identical to those in the (maximum-parsimony) consensus tree.

Nucleon and intrapopulational nucleotide sequence diversities within individual sample localities of *C. lutrensis* ranged from 0.55–0.94 and from 0.11–3.3%, respectively (Table 2). Both estimates of mtDNA variation were considerably higher in samples from east Texas (i.e. the Trinity River and the Brazos River drainage). The distribution of nucleotide sequence divergence estimates among all pairwise comparisons of *C. lutrensis* mtDNA haplotypes (Fig. 5) is clearly bimodal with a sharp discontinuity between 3 and 5%. This type of pattern is consistent with significant phylogeographic structuring (Avise & Ball 1991).

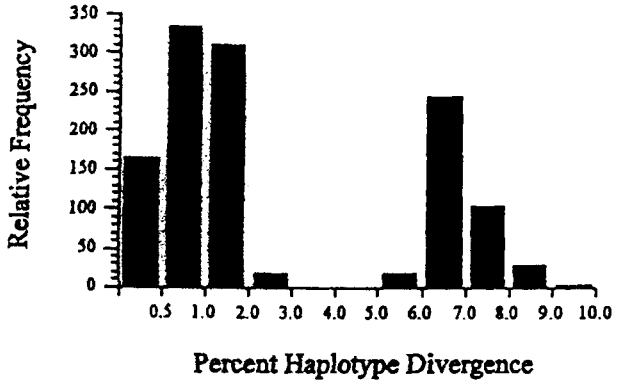

Fig. 5 Distribution of nucleotide sequence divergence estimates among mtDNA haplotypes of *C. lutrensis*.

Discussion

Phylogenetic analysis of mtDNA haplotypes of *C. lutrensis* revealed three major clades (A–C). Clade A contained haplotypes from sample localities throughout the study area except for the Brazos River. Clade B contained haplotypes from the Trinity and Calcasieu rivers (two Gulf Coastal Plain drainages), and clade C comprised haplotypes found exclusively in the Brazos and Trinity rivers in east Texas. Clade C was inferred to be sister to a clade comprising clades A and B. MtDNA sequence difference between clade C vs. (A–B), and clade B vs. A, was 6.80% (30–46 restriction site differences) and 0.97% (1–17 restriction site differences), respectively.

The first issue of interest in our study was whether *C. lutrensis* occurred preglacially in the northern and central portions of its range, or colonized previously inaccessible habitat after the glaciers receded. Perhaps the first question to ask is: did *C. lutrensis* occur preglacially? MtDNA nucleotide sequence difference between *C. lepida*, an outgroup used in this analysis, and *C. lutrensis*, was 8.49%. The nucleotide sequence difference of 8.49% is consistant with the hypothesis that these two lineages diverged preglacially. Divergence during the Pleistocene (beginning 1.8 Myr ago) would yield a minimal rate of mtDNA evolution of nearly 5% per million years; whereas divergence during the late Miocene – early Pliocene (6.0–4.5 Myr ago) would yield an mtDNA evolutionary rate of 1.4–1.9% per million years. The latter is far more consistant with rates of mtDNA evolution estimated in other poikilothermic vertebrates (Martin & Palumbi 1993), where average rates appear to be in the range of 0.20–0.75%, and in bony fishes, where estimated rates range between 0.22 and 1.91% (Bentzen *et al.* 1993; Brown & Chapman 1991; Martin & Palumbi 1993). In addition, the mtDNA nucleotide sequence difference of approximately 6.8% between clade C and clade [A+B] (both *C. lutrensis*) would indicate that *C. lutrensis* arose well before the presumed onset of Pleistocene glaciation.

Our data support the hypothesis that *C. lutrensis* entered the region directly affected by glaciation during the mid- to late-Pleistocene. First, there is almost no phylogeographic structure among haplotypes of *C. lutrensis* in the region directly affected by Pleistocene glaciation. This includes sample localities north of Texas and the sample from northeastern Louisiana (Bayou Macon). One exception to the above are the haplotypes from the Platte River that formed a well-supported monophyletic lineage within clade A. These haplotypes from the Platte River, however, only share a single, synapomorphic restriction

site change, indicating that divergence of this subclade within clade A is relatively recent. Secondly, levels of mtDNA variation, as measured by intrapopulational nucleotide sequence diversity, were uniformly low in sample localities that were directly affected by glaciation. Low levels of genetic variability indicate the effective absence of divergent haplotype lineages within individual sample localities, and further suggest that haplotypes within each locality have a relatively recent origin. Finally, the common occurrence of a single haplotype (haplotype 14) throughout most of the area directly affected by glaciation, indicates either that there is extensive gene flow among geographic samples, or that colonization was relatively recent.

We have interpreted patterns of mtDNA phylogeographic structure in *C. lutrensis* as evidence of colonization of glaciated regions during or after the Pleistocene. It also is possible that *C. lutrensis* occurred in these regions prior to the Pleistocene, but that all populations of the species were driven south by advancing ice fronts and fluctuating climatic conditions. Biogeographic patterns in other organisms impacted by Pleistocene glaciation have been interpreted in a similar way. Zink & Dittmann (1993), in a survey of mtDNA restriction site variation in song sparrows, interpreted the absence of geographic structure of mtDNA haplotypes to be the result of relatively recent (post-glacial) colonization and dispersal across the continent. Similarly, Bernatchez & Dodson (1991) found that a single mtDNA haplotype occurred in over 90% of all lake whitefish surveyed from glaciated regions, and that a single assemblage of mtDNA haplotypes occurred over most of the species range. They also found evidence of mtDNA phylogeographic structure at the periphery of the species range, and suggested that the regions from which these haplotypes (corresponding to separate lineages) originated, represented glacial refugia.

Phylogeographic analysis of mtDNA haplotypes of *C. lutrensis* also provides information relative to possible zones of vicariance and paths of dispersal. Based on mtDNA nucleotide sequence difference, divergence of clade C from the lineage leading to clades B and A was estimated to have occurred preglacially. Clade C contained haplotypes primarily from the Brazos River, whereas clade B contained haplotypes primarily from the Trinity River. This suggests that a major vicariant event separated *C. lutrensis* in these two rivers, perhaps during the mid-Pliocene. Knapp (1953), and others (Conner 1977), have noted that several endemic species occur in the Brazos River drainage, and that the Brazos River system represents the range termination of several species and is a poor 'fit' with adjacent drainages in rank correlation analysis of drainage faunal relations (Conner 1977). This pattern of endemism and poor 'fit' with adjacent drainages is consistent with the hypothesis that the Brazos River drainage has been (or was) isolated for a considerable period of time, and that the rise in sea level that presumably occurred during the mid-Pliocene (Riggs 1984) may have isolated *C. lutrensis* in the Brazos River. The few haplotypes from clade C sampled from the Trinity River probably represent secondary dispersal or admixture. Sea levels fell at the end of the Pliocene and at least three times during the Pleistocene (Vail & Mitchum 1979), and could have facilitated movement of *C. lutrensis* between the Brazos and Trinity rivers on a broadened Gulf Coastal Plain. It is widely believed that many, presently independent drainages of the Gulf slope were connected farther downstream on the exposed continental shelf during lower sea-level stands (Conner & Sutkus 1986).

MtDNA sequence difference of clade B from clade A was estimated to be 0.97%. Both clades include haplotypes from the Trinity and Calcasieu river drainages, but clade A primarily contains haplotypes from areas to the north that were directly affected by Pleistocene glaciation. The divergence of these two clades is hypothesized to have occurred during the Pleistocene, and it is interesting to note that a connection between the Trinity River and the Red River to the north is thought to have existed prior to Kansan time (Slaughter *et al.* 1962; Cross *et al.* 1986). A physical connection between the Brazos and Red rivers has been dated to 600 000 years ago (Swineford *et al.* 1955), but no haplotypes related to clade C (primarily in the Brazos River) have yet been found outside of Texas. This suggests the possibility that dispersal of *C. lutrensis* into glaciated regions was through a Trinity-Red river connection.

The second issue of interest in our study was whether patterns of geographic diversity in mtDNA of *C. lutrensis* reflect observed patterns of morphological diversity. Taxonomically, three extant subspecies of *C. lutrensis* are recognized: *C. l. lutrensis*, widely distributed in the central United States; *C. l. forbesi*, in Illinois and Missouri; and *C. l. suavis*, a Gulf Coastal Plain form. These designations are based primarily on differences in a few morphological and meristic characters (Baird & Girard 1853; Girard 1857; Jordan 1878). Matthews (1987), in a thorough study of *C. lutrensis* from localities throughout its native range, combined meristic and morphometric character variation and suggested the existence of a Great Plains group, a Coastal Plain group, and a morphologically intermediate group that included drainages from Texas, the lower Red and Mississippi rivers, and the upper Mississippi, Missouri, and Illinois river drainages. Within the last group, Matthews (1987) suggested that samples from the upper Mississippi, Missouri, and Illinois river drainages could comprise a distinct subset. Morphologically, *C. lutrensis* from glaciated regions appear as diverse, if not more so, than *C. lutrensis* from the

Gulf Coastal Plain.

In our study, seven sample localities were from the range corresponding to *C. l. lutrensis* (samples 4–8, 10, and 11; Fig. 1), a single locality was from the range of *C. l. suavis* (sample 9; Fig. 1), and three sample localities were from the range corresponding to *C. l. forbesi* (samples 1–3; Fig. 1). Most of the mtDNA diversity within *C. lutrensis* was found among samples from the Gulf Coastal Plain. Areas north of Texas (regions presumably affected by Pleistocene glaciation), contained relatively little mtDNA diversity. In addition, neither the described subspecies nor the regional groups described by Matthews (1987) could be identified by phylogenetic analysis of mtDNA haplotypes. This suggests minimally that morphological diversification and mtDNA evolution are decoupled in *C. lutrensis*. Similar findings, i.e. morphological diversity in the absence of geographically structured mtDNA variation in glaciated areas, were reported by Zink & Dittmann (1993), and have been documented in a variety of organismal groups (Avise *et al.* 1975; Gold 1980; Novacek 1993).

Increases in morphological diversity subsequent to colonization of glaciated areas have been documented in a variety of organisms, including both animal and plant species (Stuart 1982; Huntley & Webb 1989). The general hypothesis is that environmental changes (climatic fluctuations) can often produce strong selective pressures that can result in rapid morphological diversification (Huntley & Webb 1989). Based on our work, *C. lutrensis* appears to be another example where morphological diversification appears to have occurred rapidly following colonization of newly opened habitat.

Acknowledgements

We thank J. Camper, C. Garrett, G. Garrett, L. Garrett, Jer. Gold, Jes. Gold, P. Powers, and C. Ragland for assistance in the field, and C. Aldridge, M. Birkner, A. Kristmundsdottir, and F. Sun for assistance in the laboratory. We also thank T. Schmidt for providing the mitochondrial DNA primers used in PCR amplification, and R. Honeycutt and T. Schmidt for critical comments on a draft of the manuscript. Work was supported by the National Science Foundation (Award BSR-90-20217) and the Texas Agricultural Experiment Station (Project H-6703). This paper represents Contribution number 39 of the Centre for Biosystematics and Biodiversity at Texas A&M University.

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This paper is taken from the MSc thesis of L. R. Richardson, and is part of a continuing series of studies on the molecular systematics and biogeography of the North American cyprinid genus *Cyprinella*. The work was carried out in the laboratory of J. R. Gold whose research interests include genetics and the phylogeny of fishes.