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SYSTEMATICS OF THE *CYPRINELLA LUTRENSIS* GROUP (CYPRINIDAE) FROM THE SOUTHWESTERN UNITED STATES AS INFERRED FROM VARIATION OF MITOCHONDRIAL DNA

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ABSTRACT—A total of 197 mitochondrial DNA (mtDNA) restriction sites was surveyed among samples representing the five species of the *Cyprinella lutrensis* group inhabiting the southwestern United States: *C. formosa*, *C. lepida*, *C.* cf. *lepida*, *C. lutrensis*, and *C. proserpina*. Average nucleotide sequence divergence between *C. proserpina* and the other four species was greater than that found between the other species and two species from the *Cyprinella whipplei* group (*C. galactura* and *C. venusta*) that were employed as outgroup taxa in phylogenetic analysis. These data coincide with other genetic data that suggest *C. proserpina* is not closely related to these species of the *C. lutrensis* group. Alternatively, *C. proserpina* may have experienced heterogeneous, perhaps rapid, genomic evolution. Maximum-parsimony analysis (employing unordered restriction-site characters) of the remaining four species (i.e., excluding *C. proserpina*) produced an unresolved tetrachotomy. Maximum-parsimony analysis that employed Dollo parsimony and neighbor-joining analysis of nucleotide sequence divergence estimates among the four species generated resolved but conflicting topologies. In the neighbor-joining analysis, branch lengths between species were short in comparison to branches to terminal taxa. Maximum-likelihood analysis generated a topology congruent with that generated by Dollo parsimony. Statistical comparisons (likelihood test of Kishino and Hasegawa), however, indicated that the conflicting topologies produced by Dollo parsimony (and maximum-likelihood) versus neighbor-joining were equally likely. The simplest interpretation of these data is that the four species evolved near-synchronously from a series of vicariant events that occurred in the western Gulf Coastal Plain. This interpretation is consistent with the hypothesis that ancestors to the *C. lutrensis* group entered the western Gulf Coastal Plain via connections with the western Great Plains before the onset of Pleistocene glaciation.

RESUMEN—Se muestrearon un total de 197 sitios de restricción ADN mitocondrial (mtDNA) entre muestras de cinco especies del grupo *Cyprinella lutrensis* habitantes del suroeste de los Estados Unidos: *C. formosa*, *C. lepida*, *C.* cf. *lepida*, *C. lutrensis*, y *C. proserpina*. La divergencia promedio de la secuencia nucleótida entre *C. proserpina* y las otras cuatro especies fue más grande que la encontrada entre las otras especies y dos especies del grupo *Cyprinella whipplei* (*C. galactura* y *C. venusta*) que fueron empleadas como los taxa del grupo-externo en un análisis filogenético. Estos datos coinciden con otros datos genéticos que sugieren que *C. proserpina* no está relacionada cercanamente a las especies del grupo *C. lutrensis*. Alternativamente, *C. proserpina* pudo haber experimentado una evolución genómica heterogénea, quizás rápida. El análisis "maximum-parsimony" (empleando carácteres no ordenados de sitios de restricción) de las cuatro especies restantes (i.e., excluyendo *C. proserpina*) produjo una tetracrotomía sin resolver. El análisis "maximum-parsimony" que emplea "Dollo-parsimony" y el de vecinos-ligados de la estimación de divergencia de secuencia nucleótida entre las cuatro especies generaron tipologías resueltas pero conflictivas. En el análisis de vecinos-ligados, las longitudes de las ramas entre especies fueron cortas en comparación a las ramas hacia los taxa terminales. El análisis "maximum-likelihood" generó una topología congruente con la generada por "Dollo-parsimony". Comparaciones estadísticas ("likelihood test" de Kishino y Hasegawa), sin embargo, indicaron que las topologías conflictivas producidas por "Dollo-parsimony" (y "maximum-likelihood") contra la de vecinos-ligados fueron igualmente probables. La interpretación más simple para estos datos es que las cuatro especies evolvieron casi sincrónicamente de una serie de eventos vicarios que ocurrieron en los planos costales ponientes del Golfo de México. Esta interpretación es consistente con la hipótesis de que los ancestros del grupo de *C. lutrensis* entraron a los planos costales ponientes del Golfo

de México vía conecciones con las Grandes Planicies ponientes antes del inicio de la glaciación del Pleistoceno.

The North American cyprinid genus *Cyprinella* consists of 27 or so species of minnows that inhabit rivers and streams of eastern North America from the Atlantic slope west to central Mexico. Monophyly of *Cyprinella* is supported by numerous morphological and osteological characters (Mayden, 1989). Hypotheses of relationships based on morphological similarities among various species of *Cyprinella* were proposed over thirty years ago by Gibbs (1957). Mayden (1989) was the first to generate a phylogenetic hypothesis of all described species of *Cyprinella* by using a cladistic analysis of morphological characters. He divided *Cyprinella* into two major assemblages: the eastern (*whipplei*) clade comprised of at least 17 species, and the western (*lutrensis*) clade comprised of at least 11 species, six of which are restricted to northern and/or central Mexico. The five species of the *lutrensis* clade (hereafter, *C. lutrensis* group) found in the southwestern United States are: *C. formosa* (restricted to the endorheic Guzmán basin and a few drainages in northwestern Mexico and southern Arizona); *C. lepida* (restricted to the Nueces River basin in west-central Texas); *C. lutrensis* (widespread, occurring in the Great Plains, Central Lowlands, and western Gulf Coastal Plain); *C. proserpina* (restricted to tributaries of the lower Rio Grande River, including the Pecos and Devil's rivers); and *C.* cf. *lepida,* an undescribed species also restricted to the Nueces River basin (Matthews, 1987; Mayden, 1989; Richardson and Gold, 1995*a*).

Mayden's (1989) hypothesis of relationships among the four nominal species of the *C. lutrensis* group found in the southwestern United States is as follows: (*C. lutrensis* (*C. formosa* (*C. lepida, C. proserpina*))). None of these four species formed "actual" sister groups in Mayden's hypothesis when species of *Cyprinella* restricted to Mexico were included. As examples, Mayden (1989) hypothesized that *C. panarcys* was sister to *C. proserpina, C. bocagrande* was sister to *C. formosa,* and *C. garmani* was sister to *C. lutrensis.* Other data containing information on relationships within *Cyprinella* include those on nucleolar organizer region (NOR) chromosomes and genomic DNA melting-rate-profiles. Briefly, *C. formosa, C. lepida,* and *C. lutrensis* possess a *C′* NOR phenotype, as do *C. spiloptera* and *C. venusta,* species placed by Mayden (1989) in the eastern or *whipplei* clade (hereafter, *C. whipplei* group). Alternatively, *C. proserpina* possesses an *H* NOR phenotype (Amemiya and Gold, 1990). Genomic DNA melting-rate profiles revealed that *C. lepida, C. lutrensis, C. spiloptera,* and *C. venusta* possess a distinct, heavy DNA component (identified in *C. lutrensis* as a family of highly-repeated, satellite DNAs) not found in *C. proserpina* and other shiners (Karel and Gold, 1987, in litt.; Moyer et al., 1988). These data suggest that *C. proserpina* may not belong to an assemblage that includes *C. formosa, C. lepida, C. lutrensis, C. spiloptera,* and *C. venusta.*

In this study, we surveyed variation in restriction enzyme sites of mitochondrial (mt)DNA in the five species of the *C. lutrensis* group inhabiting the southwestern United States and in two members (*C. galactura* and *C. venusta*) in Mayden's (1989) *C. whipplei* group. Objectives of the study were to examine Mayden's (1989) hypothesis of relationships among the southwestern United States representatives of the *C. lutrensis* group, and explore further genetic similarity/dissimilarity between *C. proserpina* and other members of the *C. lutrensis* group. We also were interested in examining further the hypothesis of Richardson and Gold (1995*a*) that ancestors to the *C. lutrensis* group entered the western Gulf Coastal Plain before the onset of Pleistocene glaciation and diverged as a consequence of near-synchronous vicariant events.

MATERIALS AND METHODS—Specimens were obtained by seine from the following localities (number of specimens examined): *C. formosa,* Dexter National Fish Hatchery, Dexter, New Mexico (10); *C. galactura,* North Fork Holston River, Scott Co., Tennessee (3); *C. lepida,* Frio River, Bandera Co., Texas (10); *C. proserpina,* Devil's River, Val Verde Co., Texas (10); *C. venusta,* Little Brazos River, Burleson Co., Texas (10); and *C.* cf. *lepida,* Nueces River, Edwards Co., Texas (10). MtDNA haplotypes of *C. lutrensis* were selected from those reported in previous studies (Richardson and Gold, 1995*a*, 1995*b*) in order to reflect accurately major mtDNA phylogenetic assemblages thus far identified in *C. lutrensis.* Included

were haplotypes 14, 46, and 56 (*C. lutrensis* clade A), haplotype 41 (an independent lineage), haplotype 43 (*C. lutrensis* clade B), and haplotype 62 (*C. lutrensis* clade C). Relationships among these clades inferred from maximum-parsimony analysis of variable restriction sites (Richardson and Gold, 1995*a*, 1995*b*) are: (clade C (clade B (haplotype 41, clade A))). A listing of these haplotypes and collection localities of individuals of *C. lutrensis* that possessed these haplotypes may be found in Richardson and Gold (1995*a*, 1995*b*).

Genomic DNA isolation, digestion with restriction endonucleases, Southern transfer to nylon membranes, hybridization with ^{32}P-labelled mtDNA of *C. lutrensis*, and autoradiography followed methods in Richardson and Gold (1991) and Gold and Richardson (1991). Sixteen restriction endonucleases (*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) were used to digest mtDNA according to manufacturer's recommendations. Restriction sites were mapped by using single- and double-digestions (Sambrook et al., 1989) or by digesting polymerase chain reaction (PCR-) generated mtDNA fragments from known regions of mtDNA (Kristmundsdóttir and Gold, 1996). Side-by-side comparisons of mtDNA fragments and digested, PCR-generated mtDNA fragments were made to confirm homology of individual restriction sites.

Relationships among taxa were inferred using maximum-parsimony analysis and maximum-likelihood analysis of presence/absence restriction-site matrices of composite mtDNA genotypes (haplotypes) and by neighbor-joining analysis of pairwise nucleotide sequence divergence estimates between individual haplotypes. Maximum-parsimony analysis employed Phylogenetic Analysis Using Parsimony (PAUP), version 3.1 (Swofford, 1991). Maximum-parsimony analysis was carried out treating characters as unordered or ordered under Dollo parsimony. The latter is consistent with requirement that each character be derived uniquely, and is appropriate for restriction-site data in that convergent, restriction-site losses are more likely than convergent, restriction-site gains. Bootstrapping (Felsenstein, 1985) was used to assess stability of branches inferred from maximum-parsimony analysis. Maximum-likelihood analysis employed the RESTML option in Phylogenetic Inference Package (PHYLIP), version 3.4 of Felsenstein (1992). Distances for neighbor-joining analysis were estimated as described in Nei and Tajima (1981) by using the DSE program in the Restriction Enzyme Analysis Package (REAP) of McElroy et al. (1992). Neighbor-joining trees were reconstructed by using the NEIGHBOR program in PHYLIP (version 3.4). Statistical tests of alternate topologies were conducted using the test of Kishino and Hasegawa (1989), as implemented in the RESTML option of PHYLIP (version 3.4). The presence/absence restriction-site matrix for individual haplotypes and the matrix of nucleotide sequence divergences are available upon request from the first author. The two species (*C. galactura*, *C. venusta*) placed by Mayden (1989) in the *C. whipplei* group were used as outgroups in both maximum-parsimony and neighbor-joining analysis.

RESULTS—A total of 197 unique restriction sites representing 34 mtDNA haplotypes was employed in the analysis. The number of haplotypes (in parentheses) for each taxon was: *C. formosa* (2), *C. lepida* (6), *C.* cf. *lepida* (4), *C. lutrensis* (6), *C. proserpina* (5), *C. galactura* (2), and *C. venusta* (9). Restriction sites for *Apa*I in *C. galactura* (18 total) and for *Nsi*I and *Sca*I in *C. proserpina* (27 total) could not be mapped accurately, even after repeated efforts. Restriction sites for these enzymes in the two taxa were coded as "missing" in maximum-parsimony analysis. Estimates of nucleotide sequence divergence (for neighbor-joining analysis) were based on a reduced data set that did not include *Apa*I, *Nsi*I, and *Sca*I restriction sites.

During initial stages of restriction-site mapping, it became apparent that mtDNA from *C. proserpina* differed markedly from mtDNA of other members of the *C. lutrensis* group. Single digestions with most of the enzymes yielded similar fragment patterns among *C. formosa*, *C. lepida*, *C.* cf. *lepida*, and *C. lutrensis*, whereas fragment patterns produced upon digestion of mtDNA from *C. proserpina* bore little to no resemblance to those in the other species. Comparison of fragment lengths indicated that differences between *C. proserpina* and the other species did not involve mtDNA length variation. Comparison of nucleotide sequence divergence among mtDNA haplotypes of each taxon revealed the same pattern: haplotypes of *C. proserpina* differed from haplotypes of other members of the *C. lutrensis* group by 17.2 ± 0.1% (mean ± *SE*), whereas haplotypes among *C. formosa*, *C. lepida*, *C.* cf. *lepida*, and *C. lutrensis* differed by 10.5 ± 0.1%. The difference between *C. proserpina* and other members of the *C. lutrensis* group, in fact, was greater than nucleotide-sequence divergence (13.3 ± 0.1%) between other members of the *C. lutrensis* group and the two outgroup species, *C. galactura* and *C. venusta*. Based on data from pri-

mates (Brown et al., 1979) and cyprinid fishes (Dowling et al., 1992), the probability of convergent mutation in mtDNA restriction sites increases rapidly at sequence-divergence values greater than 10–15%, resulting in a corresponding reduction in resolution in phylogenetic analysis. The comparatively large mtDNA sequence divergence between *C. proserpina* and other members of the *C. lutrensis* group strongly suggests that restriction sites in *C. proserpina* have become saturated, and that phylogenetic analysis that included data from *C. proserpina* would be seriously compromised. In addition, a neighbor-joining tree generated from estimates of nucleotide-sequence divergence among haplotypes revealed that the branch leading to the cluster of haplotypes of *C. proserpina* was, on average, nearly three times the length of branches leading to the cluster of haplotypes representing other members of the *C. lutrensis* group. This further indicated that phylogenetic analysis including haplotypes of *C. proserpina* would be compromised, in this case because of "long-branch attraction" (Felsenstein, 1978; Hendy and Penny, 1989). Therefore, we removed haplotypes of *C. proserpina* from subsequent phylogenetic analysis.

Maximum-parsimony analysis (heuristic search) of unordered characters (exclusive of *C. proserpina* and using *C. galactura* and *C. venusta* as outgroups) resulted in three, equally parsimonious trees of 214 steps ($CI = 0.673$; $RI = 0.910$). The bootstrapped (500 replicates) strict-consensus tree (Fig. 1a) revealed strong support for clades of haplotypes representing *C. formosa*, *C. lepida*, and *C.* cf. *lepida*. The clades of haplotypes representing *C. lutrensis* were supported in 69% of boostrapped replicates when haplotype 62 was included. The clade of all other haplotypes representing *C. lutrensis*, i.e., haplotype 62 excluded, was supported in 100% of bootstrapped replicates. Haplotype 62 represents a basal and strongly differentiated clade of haplotypes within *C. lutrensis* that is hypothesized to have diverged from other haplotype lineages of *C. lutrensis* well before the Pleistocene (Richardson and Gold, 1995*b*). Relationships among the four taxa, i.e., *C. formosa*, *C. lepida*, and *C.* cf. *lepida*, and *C. lutrensis*, were unresolved. In a 50% majority-rule, maximum-parsimony tree, a sister-group relationship between haplotypes of *C. lepida* and haplotypes of *C. formosa* was supported in 56% of bootstrapped replicates.

Increased resolution among the four taxa was obtained in maximum-parsimony analysis (heuristic search) when characters were ordered under Dollo parsimony. Eight, equally parsimonious trees of 258 steps ($CI = 0.558$; $RI = 0.960$) were produced, and the bootstrapped (500 replicates) strict-consensus tree (Fig. 1b) revealed moderate to strong support for a topology where *C. formosa* was sister to *C. lepida*, *C.* cf. *lepida* was sister to the *C. formosa–C. lepida* clade, and *C. lutrensis* was sister (and basal) to the clade of the other three taxa. These relationships were recovered in all eight, equally parsimonious topologies, and were supported by the same non-homoplasious and homoplasious character-state changes (data not shown). Maximum-parsimony analysis under Dollo parsimony also provided strong support (100% of bootstrap replicates) for clades of haplotypes representing *C. formosa*, *C. lepida*, and *C.* cf. *lepida*. The clade of haplotypes representing *C. lutrensis* was supported in 75% of bootstrapped replicates when haplotype 62 was included, and 100% of bootstrapped replicates when haplotype 62 was excluded. Maximum-likelihood analysis produced the same topology as that generated by Dollo parsimony. Branch lengths supporting a sister relationship between *C. formosa* and *C. lepida*, and between *C.* cf. *lepida* and the *C. formosa–C. lepida* clade differed significantly ($P < 0.05$) from zero.

The topology of the distance tree produced by neighbor-joining (Fig. 2) differed from the maximum-parsimony (Dollo parsimony) and maximum-likelihood topologies in that *C. lepida* formed an initial cluster with *C.* cf. *lepida*, followed successively by *C. formosa*, then *C. lutrensis*. Lengths of branches defining *C. formosa*, *C. lepida*, and *C.* cf. *lepida*, however, were far longer than branches supporting relationships among the three taxa. Branch lengths (in % nucleotide sequence difference) connecting *C. lepida* to *C.* cf. *lepida*, *C. formosa* to the *C. lepida–C.* cf. *lepida* cluster, and *C. lutrensis* to the *C. formosa*, *C. lepida*, and *C.* cf. *lepida* cluster, were 0.94%, 0.79%, and 1.47%, respectively. Branch lengths to terminal taxa were, on average, 4–5 times as long. The branch defining haplotype 62 of *C. lutrensis* was far longer than the branch uniting the remaining haplotypes of *C. lutrensis* (Fig. 2).

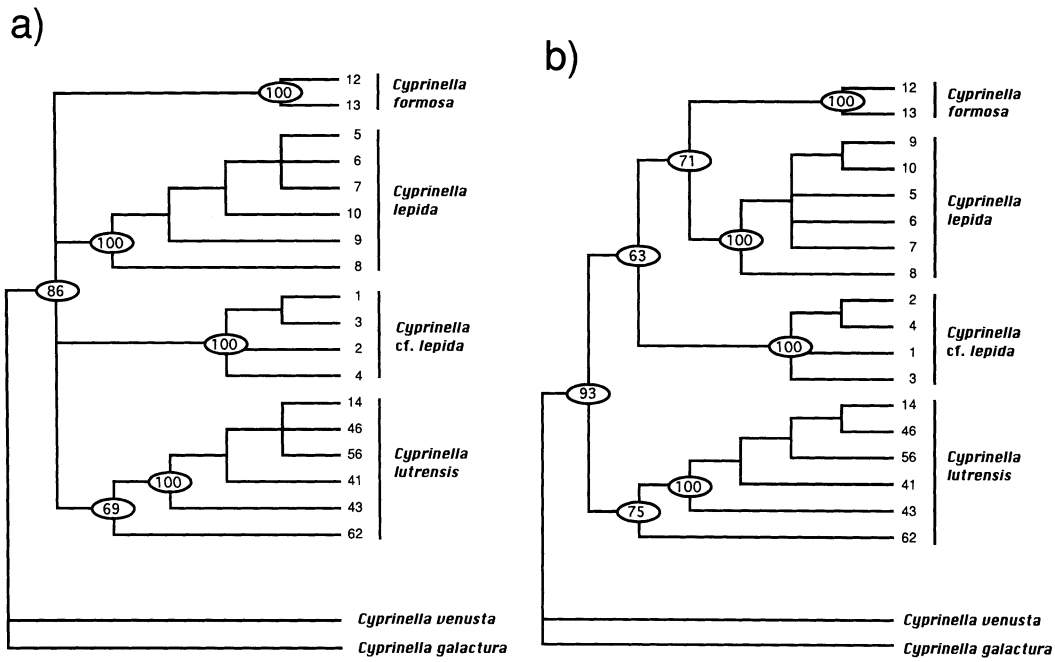

FIG. 1—a) Strict-consensus tree of mtDNA haplotypes produced by maximum-parsimony, and b) by maximum-parsimony with Dollo criteria. Numbers at nodes are bootstrap values. Relationships of haplotypes in outgroups are not shown. Branch lengths are not accurate representations of the number of restriction-site changes between operational units.

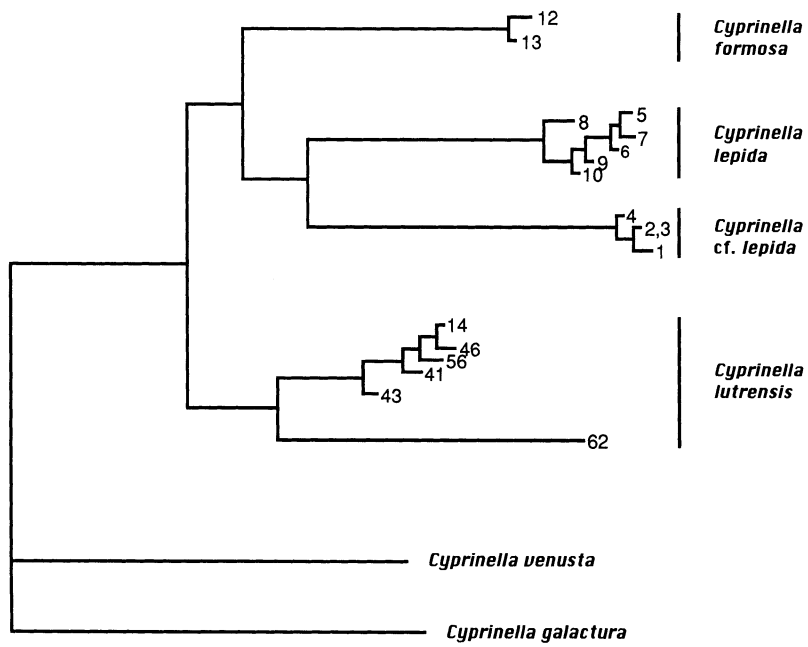

FIG. 2—Neighbor-joining tree generated from nucleotide-sequence-divergence estimates between mtDNA haplotypes. Relationships of haplotypes in outgroups are not shown. Branch lengths are proportional to estimates of nucleotide sequence divergence.

The resolved but conflicting topologies generated by maximum-parsimony-Dollo criteria (and maximum-likelihood) analysis and neighbor-joining analysis were compared statistically by using the likelihood test of Kishino and Hasegawa (1989). More than 95% of log-likelihood differences taken across sites between the two topologies overlapped zero, indicating that the difference in likelihood between the two trees did not differ significantly from zero, i.e., the null hypothesis that the two topologies were equally alike could not be rejected.

DISCUSSION—The distribution of restriction sites in mtDNA revealed that *C. proserpina* differs trenchantly from other southwestern United States members of the *C. lutrensis* group. This brings to three (i.e., mtDNA restriction sites, chromosomal NORs, and DNA melting-rate profiles) the data sets that document significant "genomic" divergence in *C. proserpina* relative to other members of the *C. lutrensis* group that have been examined for these characters. Unfortunately, the hypothesized closest relatives of *C. proserpina*, i.e., *C. panarcys*, *C. rutila*, and *C. xanthicara* (Mayden, 1989), are found only in Mexican waters, and thus far we have been unable to obtain suitable tissues from these species for mtDNA or other genetic analysis. Mayden (1989) identified 23 morphological and osteological synapomorphies that placed these latter four species in a terminal assemblage in the *C. lutrensis* group. Included among these synapomorphies was a long gular bar that represented a unique condition within *Cyprinella*. Examination of the remaining three species in this clade will be necessary to determine whether genomic divergence in *C. proserpina* is unique to that taxon. However, present data clearly suggest that *C. proserpina* is not closely related to *C. lutrensis* and other southwestern United States members of the *C. lutrensis* group. Alternatively, if *C. proserpina* is closely related to other southwestern United States members of the *C. lutrensis* group, it would appear that the species has experienced heterogeneous, perhaps rapid, genomic evolution.

Maximum-parsimony analysis (Dollo criteria) and maximum-likelihood analysis of mtDNA restriction sites among the remaining taxa indicated that *C. lutrensis* was sister to a clade comprised of *C. formosa*, *C. lepida*, and *C.* cf. *lepida*. Within the latter, *C.* cf. *lepida* was inferred to be sister to a clade composed of *C. formosa* and *C. lepida*. This hypothesis is compatible with that of Mayden (1989) who placed *C. lutrensis* as sister to a clade that included *C. formosa* and *C. lepida*. [Note: Mayden's (1989) sample of *C. lepida* was from the Nueces River and represents *C.* cf. *lepida* as used here. We employ *C. lepida* to refer to the species of *Cyprinella* in the Frio River where the type specimens of *C. lepida* (Girard, 1856) were obtained.] Neighbor-joining analysis, however, indicated a closer similarity between *C. lepida* and *C.* cf. *lepida*, with *C. formosa* joining them at the next level. These similarities are more concordant with current distributions, as *C. lepida* and *C.* cf. *lepida* are restricted to the upper reaches of the Nueces River basin, whereas *C. formosa* is restricted to northwestern Mexico and southern Arizona. However, branch lengths in the neighbor-joining tree that lead to clusters of haplotypes of each species were considerably longer than those connecting species. Differences between the relatively low estimates of nucleotide sequence divergence that supported relationships between species may not be significant. Alternatively, the Kishino-Hasegawa test indicated that the likelihood difference between conflicting topologies did not differ significantly from zero, i.e., both are equally likely.

Richardson and Gold (1995*a*) hypothesized that ancestors to the *C. lutrensis* group entered the western Gulf Coastal Plain via connections in the western Great Plains well before Pleistocene glaciation, perhaps as early as late Miocene-early Pliocene (6.0 to 4.5 million years ago). During this time, renewed uplift of the Rocky Mountains is thought to have created an ancestral Rio Grande–upper Pecos river system that could have drained into northwestern Mexico, the present-day lower Pecos River, and other systems emptying to the southeast into the Gulf of Mexico (Thomas, 1972; Belcher, 1975; Echelle and Echelle, 1978). This period also was a time of active uplift in both northern New Mexico and western Texas (Thomas, 1972; Belcher, 1975), and as argued by Richardson and Gold (1995*a*), could have generated several vicariant events where drainage connections across the Llano Estacado and what is now the southwestern United States were severed. If connections among various

drainages were disrupted at roughly the same geological time, and assuming a nearly equivalent rate of nucleotide sequence divergence among close relatives, mtDNA-based branch lengths among the species would essentially be identical and shorter than branch lengths to terminal taxa.

Based on the above, we are hesitant to promote a sister relationship between *C. lepida* and *C. formosa* (indicated by maximum-parsimony analysis under Dollo parsimony) or between *C. lepida* and *C.* cf. *lepida* (indicated by neighbor-joining analysis and consistent with current distributions). Additional molecular data (e.g., DNA sequences) may increase resolution, although preliminary data employing sequences of the mitochondrially-encoded ND2 gene (R. E. Broughton, in litt.) suggest that the number of base pairs needed may be relatively large. Additional morphological characters may prove useful in this case, although it is worth noting that in *C. lutrensis*, morphological diversification and mtDNA variation appear to be decoupled (Richardson and Gold, 1995*b*). We are thus content, at present, to leave relationships among the four species as unresolved.

Our study of mtDNA lineages in *C. lutrensis* and related species may only touch the tip of a "phylogenetic" iceberg. The hypothesis that preglacial geologic events severed drainage connections in the southwestern Great Plains and western Gulf Coastal Plain predicts that additional, divergent assemblages of *C. lutrensis* or *C. lutrensis*-like forms should be found in other drainages of the southwestern United States and in northwestern Mexico. The existence of several nominal species in northern Mexico that are related to *C. lutrensis* (Mayden, 1989) supports this prediction. Presently, we are assaying mtDNA variation in populations of *C. lutrensis* from throughout the western Great Plains and western Gulf Coastal Plain to examine the prediction further.

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