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Biogeography of “*Cyprinella lutrensis*”: intensive genetic sampling from the Pecos River ‘melting pot’ reveals a dynamic history and phylogenetic complexity

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Abstract

Thorough sampling is necessary to delineate lineage diversity for polytypic “species” such as *Cyprinella lutrensis*. We conducted extensive mtDNA sampling (cytochrome b and ND4) from the Pecos River, Rio Grande, and South Canadian River, New Mexico. Our study emphasized the Pecos River due to its complex geological history and potential to harbor multiple lineages. We used geometric-morphometric, morphometric, and meristic analyses to test for phenotypic divergence and combined nucDNA with mtDNA to test for cytonuclear disequilibrium and combined our sequences with published data to conduct a phylogenetic re-assessment of the entire *C. lutrensis* clade. We detected five co-occurring mtDNA lineages in the Pecos River, but no evidence for cytonuclear disequilibrium or phenotypic divergence. Recognized species were interspersed amongst divergent lineages of “*C. lutrensis*”. Allopatric divergence among drainages isolated in the Late Miocene and Pliocene apparently produced several recognized species and major divisions within “*C. lutrensis*”. Pleistocene re-expansion and subsequent re-fragmentation of a centralized lineage founded younger, divergent lineages throughout the Rio Grande basin and Edwards Plateau. There is also evidence of recent introductions to the Rio Grande, Pecos and South Canadian Rivers. Nonetheless, deeply divergent lineages have coexisted since the Pleistocene.

Keywords

allopatric speciation; cryptic diversity; lineage persistence; phylogenetic sampling; polytypic taxa; species introductions; widespread species

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Data Archiving: Genbank Accession numbers are provided for sequences obtained in this study. Geometric-morphometric and meristic data are available in Supplementary Table S2. Specimens used for geometric-morphometric are deposited at the Museum of Southwestern Biology (Catalog Numbers 95697-95705).

INTRODUCTION

Allopatric speciation depends on geographical separation of lineages, divergence in allopatry, and emergence of intrinsic barriers to gene flow allowing coexistence in secondary sympatry (Wiens, 2004). Divergence and reproductive incompatibility typically increase with time (Coyne & Orr, 1997; Bolnick & Near, 2005; Stelkens, Young & Seehausen, 2009), so probability of speciation should increase with duration of allopatry. However, lineage persistence and rate of divergence differ (Bolnick, Near & Wainwright, 2006; Dynesius & Jansson, 2014). Further, species of geographical regions that are geomorphically or climatically dynamic may diverge when gene flow is severed, but re-converge once gene flow resumes (Borden & Krebs, 2009; Bagley et al., 2011), creating potential for polyphyly and introgression among divergent lineages (Bossu & Near, 2009; Bagley et al., 2011). In these cases, comprehensive phylogeographic sampling is critical (Funk & Omland, 2003).

The river systems of North America have complex geomorphic histories that include periods of fragmentation, coalescence, and reorganization (e.g. Blum & Hattier-Womack, 2009; Galloway, Whiteaker & Ganey-Curry, 2011). Pre-historic fragmentation of river basins corresponds with patterns of fish endemism (Oberdorff, Lek & Guégan, 1999; Hoagstrom, Ung & Taylor, 2014). However, distributions of some North American fishes remain contiguous and transcend drainage divides. Some including *Ictalurus punctatus* (Hardman & Hardman 2008), *Percina caprodes* (Near & Benard, 2004), and *Micropterus dolomieu*, *M. punctulatus*, and *M. salmoides* (Near et al., 2003) are relatively young (i.e. Pleistocene), but others (e.g. *Pylodictis olivaris*) are ancient (i.e. Paleocene-Paleocene-Eocene, Hardman & Hardman, 2008). Widespread ancient species may harbor undetected cryptic diversity (e.g. Blanton, Page & Hilber, 2013) or if not, are interesting as exceptions to the general trend of allopatric speciation.

***Cyprinella lutrensis*, a widespread taxon of uncertain history**

Cyprinella lutrensis (Red Shiner) is a widespread generalist found across watersheds of south-central North America that exhibits broad ecological tolerances (Matthews, 1985) and exhibits molecular evidence of range expansion northward since the last glacial retreat (Richardson & Gold, 1995; Osborne et al., 2014). To the southwest, its distribution transcends geographic boundaries associated with endemism in other plains and prairie fishes (e.g. Hoagstrom, Brooks & Davenport, 2011), including members of the genus *Cyprinella* (Schönhuth & Mayden, 2010). It has also recently invaded northeastern areas modified for agriculture (Page & Smith, 1970) and has been widely translocated by humans within and outside of its native range (Husemann et al., 2012; Poulos et al., 2012). Although, its morphology is locally variable (Dugas & Franssen, 2011, 2012), there are no broad geographic breaks to indicate a history of allopatric divergence (Matthews, 1987). Further, *C. lutrensis* hybridizes with some congeners (e.g. Walters et al., 2008; Schönhuth & Mayden, 2010; Broughton et al., 2011) indicating gene flow can extend beyond taxonomic boundaries.

Richardson and Gold (1995) concluded the progenitor of *C. lutrensis* inhabited southern portions of the present range by the Late Miocene. There it was potentially exposed to

extended periods of population fragmentation within and among dynamic river systems (Hoagstrom et al., 2014). Matthews (1995) suggested such range fragmentation accounted for variation in nuptial coloration among southwestern populations. Further, southwestern taxa recognized as distinct species are nested within “*C. lutrensis*” (Schönhuth & Mayden, 2010), indicating either: (1) *C. lutrensis* is polytypic and includes peripheral “species”, (2) more cryptic “species” are present within “*C. lutrensis*”, or (3) *C. lutrensis* is a complex taxon that has both founded peripheral taxa appropriately recognized as species and maintained cryptic diversity within “*C. lutrensis*”.

To fully discover and define the taxonomic status of all populations within the *C. lutrensis* clade will require numerous detailed molecular and taxonomic studies. An important step is to better determine biogeographical patterns within and among southwestern populations. Schönhuth and Mayden (2010) tentatively elevated (or re-elevated) two putative southwestern species, that warranted further sampling. Broughton et al. (2011) found divergence of mtDNA sequences among *C. lutrensis* of adjacent, southwestern river basins, also needing further attention. Here we pair extensive new data from the Pecos River, Rio Grande, and South Canadian River, New Mexico with existing phylogenetic data (Schönhuth & Mayden, 2010; Broughton et al. 2011). The focal rivers are adjacent to those more intensively sampled by Schönhuth and Mayden (2010) and Broughton et al. (2011) and represent the western-most native populations (Fig. 1).

In this study, we combined data obtained herein with available Genbank sequences from Schönhuth and Mayden (2010) and those in Broughton et al. (2011) to help identify evolutionary origins of fishes in the Pecos River, and to place our results into deeper phylogenetic context. We adopted this strategy because preliminary sampling revealed evidence of deep phylogenetic divergence in this river system and hence was the focus of our study. We used mtDNA and nucDNA sequences to determine whether cytonuclear disequilibrium was evident and conducted morphometric, geometric-morphometric, and meristic comparisons to assess the presence of phenotypic divergence. We also estimated divergence times among lineages in our gene tree based on mtDNA. Our goals were to: (1) expand understanding of phylogenetic structure within the *C. lutrensis* clade, (2) use the expanded phylogeny to reconstruct the clades’ historical biogeography in light of geological evidence, and (3) use detailed data from Pecos River samples to study causes of lineage complexity, including the role of recent translocations, to gain a better understanding of the evolutionary processes that gave rise to this remarkably diverse and ecologically important species complex.

MATERIALS AND METHODS

Study area history

The Pecos River is a textbook example of a river basin formed by stream piracy (Hunt, 1974; DiPietro, 2013). Its formation began in the Late Miocene as streams flowing southeast across the Great Plains, from the easternmost mountains of the Basin and Range Province, were diverted south, into the subsiding Roswell and Delaware basins (Gustavson & Winkler, 1988; Hawley, 1993; Hill, 1996). The stream system of these basins was integrated, flowing from north to south (Hill, 1996), so the collective name “Capitan basins” is used to refer to

this Late Miocene-Pliocene drainage (Hoagstrom et al., 2014, Fig. 2A). The name comes from prominent geological features surrounding the Roswell and Delaware basins: El Capitan peak, Capitan Reef, Capitan Escarpment, Capitan Platform, and Capitan Mountains.

The early history of the Capitan basins was one of isolation (Fig. 2A). Streams diverted into these basins were previously part of the Middle Miocene Guadalupe River, which flowed across the Great Plains and Edwards Plateau toward the Gulf of Mexico (Galloway et al., 2011). Although diversion into the Capitan basins directed surface flow toward the lower Rio Grande, a lack of sediments from this region in deposits in the Gulf of Mexico suggests the developing river basin remained small and isolated through the Pliocene (Galloway et al., 2011). Severe aridity (Chapin, 2008) likely limited runoff and dissolution subsidence in the Delaware Basin may have outpaced surface flow and alluviation. Karstification in the eastern Delaware Basin diverted surface waters underground (Hill, 1996; Stafford et al., 2009).

Isolation ceased in the early Pleistocene (Fig. 2B). Accelerated incision in the lower Pecos River and adjacent Rio Grande canyons (Kastning, 1983; Veni, 1994) caused headward advance toward the Delaware Basin. Simultaneously, increased streamflow in the Capitan basins promoted basin filling and overflow, forming the Pecos River. By the late Pleistocene (Fig. 2C) the Pecos River extended from the Sangre de Cristo Mountains to the lower Rio Grande (Hill, 1996; Galloway et al., 2011).

Sample collection

Our study focused on *C. lutrensis* present in the Pecos River within the Roswell Basin portion of the Capitan basins (Fig. 2A) and upstream to the Fort Sumner Valley. Upstream sites are from reaches captured from the Colorado and Brazos rivers in the Pleistocene (Fig. 2B). *Cyprinella lutrensis* specimens ($n = 222$) were collected from eight sites, using a 1.2×1.8 m seine with 3mm mesh. Whole fish ≥ 30 mm standard length (SL) were retained and euthanized. Caudal-fin clips for DNA extraction were removed and preserved in 95% ethanol. Clipped specimens were fixed in 10% formalin for morphometric analysis.

Fin clips were also collected from: (i) the Middle Rio Grande between Albuquerque and Elephant Butte Reservoir, which is in the central portion of the Rio Grande Rift ($n = 36$), (ii) the South Canadian River between Ute Dam and the Texas border ($n = 19$), and (iii) the Delaware River near its confluence with the Pecos River ($n = 31$, this was a disjunct location within the Capitan basins). These specimens were intended only for the phylogenetic portion of the study. Samples from the South Canadian River included locations associated with recent (within the last 100 years) introductions of other fish species to the Pecos River (Moyer, Osborne & Turner 2005; Osborne, Diver & Turner, 2013).

Molecular methods

DNA was isolated and polymerase chain reactions (PCR) were performed for partial mitochondrial NADH dehydrogenase subunit 4 gene (ND4, 314 base pairs [bp]) for 308 specimens from the Pecos River and surrounding basins using primers and methods outlined in Osborne, Carson & Turner (2012). This locus has been used previously to identify shared

haplotypes among drainages in *Hybognathus placitus* and *Notropis girardi* (Moyer et al., 2005; Osborne et al., 2013). Individuals with unique ND4 haplotypes were sequenced for a 962 bp fragment of mitochondrial cytochrome b to allow phylogenetic analysis with other data available on Genbank (see above). Cytochrome b was amplified using the primers LA and HA (Schmidt, Bielawski & Gold, 1998) with an annealing temperature of 50°C. Following initial screening, and identification of two major lineages using mitochondrial data (called ‘Central’ and ‘Peripheral’) a subset of individuals (Table 1) from each lineage (n = 57 individuals total) were sequenced for partial nuclear recombination-activating 1 exon (RAG-1, 359 bp) to test for cytonuclear disequilibrium between nucDNA and mtDNA. We conducted this test because we hypothesized that divergent mtDNA lineages were reproductively isolated. RAG-1 was sequenced using PCR primers designed for *C. lutrensis* (RAG-1 F: 5’ TGGCAGCCGGCTTTAAAAACG and RAG-1 R: 5’ AAACATGAGGCACAAAGGTCT) with the following protocol: template DNA, 1x reaction buffer, 2 mM MgCl₂, 125 μM dNTPs, 0.5 μM of each primer, and 0.375 units Promega GoTaq® and the following PCR conditions: initial denaturation at 95°C for 3 minutes (min), 20 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 40 seconds (s), and extension at 72°C for 40 s, followed by 20 cycles of denaturation at 95°C for 1 min, annealing at 48°C for 40 s, extension at 72°C for 40 s, and final extension at 72°C for 10 min. PCR products were purified with Omega CyclePure kits, sequenced with the Big Dye sequencing kit (ver.1.1), and analyzed on an ABI3130 automated DNA sequencer (Applied Biosystems, Inc.). Genbank accession numbers for ND4 and cytochrome b are provided in Table 2 and 3, and for RAG-1 were KM389180-KM389197 (Table S1).

Molecular data analyses

Nucleotide sequences were aligned using Sequencher Version 4.9 (Genecodes). The most appropriate model of nucleotide substitution was selected using jModeltest2 (Darriba et al., 2012) for both cytochrome b and ND4. Median-joining networks were constructed for ND4 haplotypes with Network Ver. 4.5.1.6 (Bandelt, Forster & Röhl, 1999). Relationships among cytochrome b haplotypes are shown in the phylogenetic tree (see below).

Phylogenetic update for the “*Cyprinella lutrensis*” clade (cytochrome b)

To revise the gene tree of the *C. lutrensis* clade, our cytochrome b sequences and those reported in Broughton et al. (2011) (Broughton pers. comm.) were aligned with Genbank Popset (number: 283147685, Schönhuth & Mayden, 2010). Phylogenetic trees were estimated using MrBayes (Huelsenbeck & Ronquist, 2001) and the GTR+G model of nucleotide substitution (six general time-reversible substitution rates, assuming gamma rate heterogeneity). The analysis was run for 1×10^7 generations; sampling the Markov chain every 100 generations resulting in 100,000 trees of which 10,000 were discarded as burn-in. Support for nodes was determined by posterior probabilities obtained from the majority-rule consensus tree. Three runs were conducted with different starting trees to ensure consistency. We included representative *Cyprinella* species from the most closely related clades (I, II, III) identified by Schönhuth and Mayden (2010) and rooted with the *C. analostana*. We interpreted the evolutionary history of the *C. lutrensis* clade with the updated phylogeny and a review of relevant geological and biogeographical evidence.

Tests of Population Expansion- (ND4 and cytochrome b)

To examine mitochondrial sequences for signals of recent demographic expansion or bottlenecks, we used DnaSP (Librado & Rozas, 2009) to calculate Tajima's D (Tajima, 1989), Fu's F_s (Fu, 1997), and R_2 (Ramos-Onsins & Rozas, 2002). These statistics employ different aspects (including mutation frequency and haplotype distribution) of the data and vary in their power to detect departures from a constant population size. Significantly negative Tajima's D and Fu's F_s are indicative of recent population expansion (or purifying selection) as are small values of R_2 . Positive values of Tajima's D and Fu's F_s suggest population bottlenecks (or a selective sweep). For small sample size and low number of segregating site the R_2 statistic is the most powerful for detecting population growth (Ramos-Onsins & Rozas 2002). Statistical significance was assessed by creating a test distribution with 10,000 coalescent simulations. We conducted these analyses using cytochrome b and ND4 haplotypes for all clade and subclades with at least four haplotypes, including the Central and Peripheral groups and the Capitan basins, Mississippi, and Red River sub-clades.

Divergence time estimates (cytochrome b)

Divergence-time estimates among lineages identified herein were estimated from cytochrome b sequences using the program BEAST 2.1.2 (Bouckaert et al., 2014). Dates derived from fossil data are best used to calibrate an external node to estimate time of divergence for a split of interest, but *Cyprinella* has no known sister taxa within the fossil record. Spencer, Smith & Dowling (2008) estimated ages of drainage isolation events based on fish cytochrome b distances between allopatric sister lineages, calibrated with Miocene and Pliocene fossil ages (Smith et al., 2002). Using this methodology an average substitution rate for western minnows of 1.1% per million years (myr) (equivalent to 0.55 per my single lineage clock rate) was estimated by Spencer et al. (2008). However, dates of divergence among the major clades identified here (Central and Peripheral) would predate the origin of genus *Cyprinella* (estimated at ~24 mya by Hollingsworth et al., 2013) if this clock rate is used. Moreover, Estabrook, Smith & Dowling (2007) suggested that this rate based on fossils of large, northern cyprinid fish underestimated evolutionary rates in small-bodied and southern fishes. A higher level of divergence in small-bodied fishes (located in warmer climates) is supported by the observation that variability in rates of sequence change occurs as a function of metabolic rate, which is affected by temperature and body size (Martin & Palumbi, 1993; Gillooly et al., 2005). Recently, Hollingsworth et al. (2013) used cytochrome b sequences and fossil data to calibrate the molecular clock and obtain divergence dates for members of the open posterior myodome cyprinids, including *Cyprinella*. Using dates of Hollingsworth et al. (2013, available in TreeBase) and percent sequence divergence among species, a per lineage clock rate of 1% per myr was obtained for members of *Cyprinella*. We employed this rate using an uncorrelated lognormal relaxed clock to account for rate variation across nodes (Ho & Phillips 2009) and the Yule tree prior. We used the RBS (reversible base-jump model) Beast add-on; which automatically adjusts the analysis to select the best substitution model for a number of specified partitions (Bouckaert, Alvarado-Mora & Pinho, 2013). We conducted three independent runs using 50 million generations for each, sampling every 5000 generations. Tracer v.1.6 (<http://>

Beast.bio.ed.ac.uk/Tracer) was used to check for convergence of model parameter estimates. Log and tree files from multiple runs were combined using LogCombiner v.2.1.2 (<http://Beast.bio.ed.ac.uk/LogCombiner>) with the first 10% of trees discarded as burnin. Trees were annotated using TreeAnnotator v. 2.1.2 and mean heights and 95% highest posterior density of divergence times were visualized on the chronogram with Figtree v.1.4 (<http://Beast.bio.ed.ac.uk/FigTree>). Divergence-time estimates were presented as a framework for understanding patterns divergence among lineages but should be viewed with the appropriate level of caution as they are derived from a single gene tree which may overestimate the age of lineage divergence.

NucDNA diversity and tests of cytonuclear disequilibrium

We used PHASE (as implemented in DnaSP v5, Librado & Rozas, 2009) to determine RAG-1 alleles from heterozygous individuals. A median-joining network was constructed for RAG-1 alleles with Network Ver. 4.5.1.6 (Bandelt et al., 1999) to assess whether there was concordance with the mtDNA network. Nonrandom association of alleles at nuclear loci with mitochondrial haplotypes is referred to as cytonuclear disequilibrium (Asmussen, Arnold & Avise, 1987; Schnabel & Asmussen 1989) and is equivalent to linkage disequilibrium between nuclear loci. Cytonuclear disequilibrium can be influenced by a variety of factors including migration between genetically distinct sources, hybridization (e.g. Asmussen, Arnold & Avise, 1989), selection (e.g. Cruzan & Arnold, 1993), and drift (Fu & Arnold, 1992). To test for cytonuclear disequilibrium, we examined 41 individuals sampled from the Pecos River. Individuals were selected to represent the two most divergent mtDNA lineages ($n = 21$ Central lineage, $n = 20$ Peripheral lineage) and these were cross-tabulated with genotype frequencies at RAG-1 after binning alleles into groups to improve statistical robustness (i.e., to reduce the number of cells with zero values in the contingency table). The binning procedure grouped alleles that differed by no more than two mutational steps. Thus, from a total of twelve RAG-1 alleles observed in the Pecos River (A through L, Fig S1), we obtained five allelic groups: A = 1; B, C, D, E = 2; F = 3; G, H, I, J, K = 4; and L = 5. This resulted in a two row (mtDNA lineage) by 15 column (RAG-1 genotype) contingency table. We then followed the approach of Basten and Asmussen (1997), using the program CNDm (<http://statgen.ncsu.edu/cnd/CNDm.php>) to estimate a standardized estimate an allelic D' between RAG-1 allelic groups and mtDNA lineages. In CNDm we employed Fisher's exact test for significant deviations from the null hypothesis of no allelic association (Basten & Asmussen, 1997).

Morphological diversity in the Pecos River

We used geometric-morphometric analysis, meristic characters, and morphometric measurements on samples from the Pecos River to test whether morphological variation was concordant with molecular results. Two-dimensional images of each fish from each major group (i.e. Central and Peripheral, $n = 19$ each) were taken for geometric-morphometric analysis using tps software (life.bio.sunysb.edu/morph). Photos were randomized prior to digitization to reduce bias associated with digitization order. Sixteen homologous landmarks were set based on easily-identified features (i.e. fin origin, snout tip, center of the eye, etc.). Generalized Procrustes Analysis (GPA) was performed to remove variation in landmark configurations due to differences in size, position, and orientation in tpsRelw (Rohlf & Slice

1990). Shape variables consisting of relative warps and uniform components (i.e. weight matrix) and centroid size (i.e. fish size; the square root of the summed squared distances of each landmark from the centroid) were calculated and retained for statistical analyses.

We tested for shape differences between the Central and Peripheral groups using multivariate analysis of covariance (MANCOVA) implemented in R (R Development Core Team, 2008). Because we had more shape variables ($n = 28$) than individuals per group, we first reduced dimensionality of the shape data using the covariance matrix from a principal components analysis (PCA). Only axes with eigenvalues >1.0 were retained for interpretation. Nine PCA axes (explaining 91% of the variance) were entered as dependent variables in a MANCOVA model with centroid size as a covariate to control for effects of allometry and lineage set as a fixed factor to test for morphological differences between mitochondrial lineages. We tested for heterogeneity of slopes with the group $\times$ centroid interaction, but it was not significant and was thus removed from the final model. To visualize shape variation between groups, we conducted another PCA on size-corrected shape variables (i.e. residuals of a preparatory MANCOVA with the nine PC axes as dependent variables and centroid size as a covariate) and visualized variation between the groups on the first two PC axes.

Meristic characters and morphological measures were collected from the same individuals used for geometric-morphometric analysis. Counts and measurements were made on formalin-fixed specimens (Museum of Southwestern Biology Catalog Numbers 95697-95705) following Hubbs and Lagler (1964). Eleven morphometric characters were used: SL, body depth, caudal peduncle depth, caudal peduncle length, pre-dorsal length, head length, eye width, snout length, and pelvic and pectoral fin length. Morphometric characters were measured to the nearest 0.01 mm using digital-slide calipers. Meristic measures included counts of dorsal-fin rays, anal-fin rays, pelvic-fin rays, pectoral-fin rays, lateral-line scales, scales above lateral line, and scales below lateral line.

We used MANCOVA of $\log_{10}$ -transformed morphometric and standard-length variables to test for differences in morphometric and meristic variation between lineages. After transformation, we reduced the dimensionality of the data by PCA (using correlation a matrix) and retained four axes explaining 100% of the variance. The MANCOVA model included the four PCA axes as dependent variables, SL as a covariate, and lineage as a fixed factor. The lineage $\times$ SL term was not significant and removed from the final model. We then visualized morphometric and meristic variation between lineages by assessing variation along the first two PCA axes from a PCA on the size-corrected morphometric and meristic variables (as described above).

RESULTS

MtDNA (ND4) haplotype diversity

Twenty-eight haplotypes were identified from 308 ND4 sequences obtained. A median-joining haplotype network indicated two major groups with mean sequence divergence of 20% (GTR+G) (Fig. 3A). We refer to these groups as “Central” and “Peripheral” based on comparative geographical distributions determined by cytochrome b relationships (Fig. 1).

The Peripheral group is ancient (late Tertiary, Fig. 3B) and its distribution (as presently known) includes disjunct representatives north, south, east, and west of areas inhabited by the Central group. Peripheral group haplotypes mainly persist in drainages that were isolated from, or peripheral to, streams of the Rio Grande basin and Edwards Plateau in the Pleistocene including Brazos, Red and Mississippi Rivers.

ND4 haplotypes of the Peripheral lineage occurred at low frequencies in the Pecos River, being more prevalent in the South Canadian River (Table 2). Three Peripheral sub-clades were designated in our samples: (i) Red River (haplotypes U-CC) (cytochrome b haplotypes E,G-K, T) (Table 3), found in the Pecos and South Canadian rivers, (ii) Brazos River (haplotypes P1-P3, cytochrome b haplotypes D, DD, DDD) found in Pecos River, and (iii) Mississippi River (haplotypes P-S, cytochrome b haplotypes A-C, CB2), found in the Middle Rio Grande and South Canadian Rivers. Sub-clade names are explained below.

Divergence within the Central group is more recent (Pleistocene, Fig. 3B, see below) and representatives only occur in basins accessible from the Rio Grande or Edwards Plateau in the Pleistocene. Three sub-clades were identified within the Central group based on ND4 sequences and are consistent with cytochrome b data. Listed in order of haplotype number (Fig. 3A) and frequency (Table 2) within the Pecos River, we designated them: (i) Capitan basins sub-clade (haplotypes A-I) found in samples from the Pecos River basin, including the Delaware River (cytochrome b haplotypes CB1, CB3-CB5, Q), (ii) Colorado River sub-clade (haplotypes L-N) found in the Pecos River (cytochrome b haplotypes CBL, CBM, CBN) and (iii) Chihuahuan Desert sub-clade (haplotypes J and K) found in the Rio Grande (cytochrome b haplotypes M and BB).

Phylogenetic update for the “*Cyprinella lutrensis*” clade (cytochrome b)

We identified 23 haplotypes from 962 base pairs of cytochrome b obtained from the same 28 individuals used in the analysis of ND4. These differed by 15.5% (GTR+G). All haplotypes grouped within the *C. lutrensis* clade of Schönhuth and Mayden (2010), but were distributed broadly therein and expanded the clade (Fig. 4).

Cytochrome b sequences revealed that branches of the Peripheral group represented divergent basal clades. Within the Peripheral group, a Red River sub-clade was well supported (posterior probability = 1.0) (Fig. 4). Here, representatives of the Red River sub-clade were present in the Pecos and South Canadian rivers and in the Wichita River, a Red River tributary. Broughton et al. (2011) found these haplotypes widespread in the Red River basin. Members of the Red River sub-clade have not been previously included in a whole-clade phylogeny, so these results also expand the *C. lutrensis* clade. A Brazos River sub-clade of the Peripheral group was also well supported (posterior probability = 1.0) in the branch immediately above those of Frio River *C. lepida*, *C. forlonensis*, and the *C. bocagrande*-*C. formosa* clade. The Brazos sub-clade haplotypes occurred in the Brazos and Pecos Rivers, and Middle Rio Grande (Fig. 4).

The next higher branch on the cytochrome b tree was a Mississippi River sub-clade (Fig. 4). Being most inclusive, it included samples of Broughton et al. (2011) from the North Canadian River, with relatively low branch support (62% posterior probability). With these

excluded, samples from the Middle Rio Grande, Pecos River, and throughout the Mississippi River basin formed a well-supported branch (posterior probability = 98%); concordant with the phylogenies of Schönhuth and Mayden (2010) and Broughton et al. (2011).

The Central group (Fig. 3) corresponded to the crown of the cytochrome b tree (Fig. 4), as in Broughton et al. (2011) and Schönhuth and Mayden (2010). The haplotypes identified here included three well-supported branches that formed an unresolved polytomy (Fig. 4). First, the Chihuahuan Desert sub-clade with haplotypes from the Middle Rio Grande, Río Conchos and adjacent Rio Grande, and from tributaries to the lower Rio Grande. Second, the Capitan basins sub-clade is an expansion of a singleton branch presented in Schönhuth and Mayden (2010). So far, it is only known from the Pecos River (Figs. 3 and 4). Third, the Colorado River sub-clade comprised haplotypes from the Pecos and Colorado rivers (Fig. 4). Haplotypes on this branch were found in both locations sampled in the upper Colorado River basin by Broughton et al. (2011).

Tests of Constant Population Size

Fu's F_S and R_2 statistics were significantly negative for cytochrome b and ND4, suggesting recent population expansion for the Capitan basin subclade (Table 4). In contrast, there was no evidence of departures from constant population sizes in the Peripheral subclades.

Divergence time estimates (cytochrome b)

Central and Peripheral groups were estimated to have diverged in the Miocene (15.99 to 7.63 mya) (Fig. 3B). Within the Peripheral group, the Red River sub-clade diverged from the Brazos and Mississippi river sub-clades between the Middle Miocene and Pliocene (14.15 to 6.69 mya), whilst the Brazos and Mississippi river sub-clades diverged in the Late Miocene to Pliocene (9.74 to 4.34 mya) (Fig. 3B). Central-group sub-clades diverged in the Pleistocene: the Colorado sub-clade diverged from the Chihuahuan and Capitan basins sub-clades 2.22 to 1.32 mya (Fig. 3B).

NucDNA diversity and tests of cytonuclear disequilibrium

Eighteen RAG-1 alleles were identified for individuals used for detailed morphometric and meristic comparisons between the Central and Peripheral groups (Table 1). Seven of these were shared between mtDNA groups (Figure S1) and were randomly distributed in the RAG-1 network with respect to mtDNA lineage (Figure S1). In contrast, four were restricted to the Central group and seven to the Peripheral group. Fisher's exact tests as employed in CNDm revealed scant evidence of cytonuclear disequilibrium. Only group 4 genotypes (RAG-1 allele K is the most frequent) showed marginal association with mtDNA lineage (nominal $P = 0.048$, Bonferroni-adjusted $\alpha = 0.003$). Otherwise, P -values were greater than 0.18. This supports the hypothesis of random mating amongst divergent mtDNA lineages in the Pecos River.

Morphological diversity in the Pecos River

Multivariate Analysis of Covariance (MANCOVA) of geometric-morphometric data revealed substantial overlap of body-shape dimensions with no significant difference between Central and Peripheral groups ($F_{9,27}=1.06$, $P = 0.42$). Visual inspection of the first

two PCA axis scores of the shape variables demonstrated broad overlap (Fig. 5A). Likewise, MANCOVA results of morphometric measures and meristic characters indicated no difference between groups as ($F_{4,32}$ 0.44, $P=0.78$) with the first two PCA scores explaining 67% of total variation (Fig. 5B).

Discussion

Phylogenetic expansion of the “*C. lutrensis*” clade

This study adds to abundant evidence that “*C. lutrensis*” is a complex taxon with a long evolutionary history throughout a broad, geologically dynamic region (Matthews, 1995; Richardson & Gold, 1995). As concluded by Schönhuth and Mayden (2010), precise delimitation of clades within *C. lutrensis* requires comprehensive study. Inclusion of our samples and those of Broughton et al. (2011) in a cytochrome b phylogeny expanded the *C. lutrensis* clade of Schönhuth and Mayden (2010), adding the well-supported basal Red River sub-clade to their phylogeny along with another well-supported crown lineage (Colorado River sub-clade). In addition, two “singleton” branches reported by Schönhuth and Mayden (2010) were supported as distinct lineages and the known geographical distribution of each was expanded. Further, the geographic distribution of the *C. suavis* and *C. sp.1* cf. *lutrensis* branches is greater than reported by Schönhuth and Mayden (2010), with geographical overlap between them. Importantly, our most thoroughly sampled area, the Capitan basins, produced the most haplotypes. Hence, more sampling elsewhere (including all portions of the Rio Grande, Brazos and Red River basins, and drainages of the Edwards Plateau) is required to accurately delimit lineage distributions and phylogenetic relationships and to identify cryptic diversity. Thus, the hypotheses presented below are provisional, for the purposes of directing future research. Putative geographic lineages identified thus far may eventually be found to be more broadly distributed and additional lineages likely remain undetected.

Pre-historic biogeography (river-basin evolution)

Deep divergence between the Central and Peripheral groups and among sub-clades of the Peripheral group indicates range fragmentation in the Late Miocene and Pliocene. Divergence-time estimates produced by Hollingsworth et al. (2013, available in TreeBase) indicate the *C. lutrensis* clade diverged from other *Cyprinella* roughly 16 mya (Early-Middle Miocene). Divergence from the *C. spiloptera*-*C. venusta* clade at this time may reflect an east-west split caused by separation of the ancestral Mississippi River basin from the ancestral Tennessee River basin (sensu Galloway et al., 2011; Hoagstrom et al., 2014).

The progenitor of the *C. lutrensis* clade must have been widespread in the western Gulf of Mexico basin during the Middle-Late Miocene in order to found populations that became *C. forlonensis* (Río Pánuco, Mexico), the *C. bocagrande*-*C. formosa* clade (Cabeza de Vaca Basin, Mexico), and *C. lepida* (Frio River, Texas) along with the Central and Peripheral groups of *C. lutrensis* (Fig. 1). The basal position of the Red River sub-clade indicates it is the oldest known extant lineage of the clade (i.e. older than recognized species). Presumably, this lineage diverged while isolated in the ancient Red River basin, which arose in the Early Miocene (Galloway et al., 2011). The fact that these lineages presently

demonstrate random mating where sympatric suggests divergence must have occurred in allopatry.

The lineage that gave rise to all other (i.e. non-Red River) sub-clades likely occupied the Miocene Guadalupe River, Rio Bravo, and Rio Grande basins of Galloway et al. (2011). These basins were dynamic and interconnected. Tectonic activity and increasing aridity eventually isolated peripheral populations that gave rise to *C. forlonensis*, *C. bocagrande*-*C. formosa*, and *C. lepida* (sensu Hoagstrom et al., 2014). Isolation of the Brazos River sub-clade apparently occurred during the Mio-Pliocene transition as the Brazos River formed on the Edwards Plateau's northern flank. This pattern of basal lineages persisting in peripheral drainages was originally noted by Contreras-Balderas (1977). In this case, the late Miocene to early Pliocene divergence estimate between the Mississippi and Brazos river lineages generally agrees with estimated Late Miocene divergence between *C. lepida* and *C. bocagrande*-*C. formosa* (Hollingsworth et al., 2013) and estimated Pliocene divergence between Brazos and Trinity river *C. lutrensis* (Richardson & Gold, 1995).

Increasing aridity through the Late Miocene (Chapin, 2008) and eastward expansion of Great Plains Ogallala apron (Seni, 1980; Galloway et al., 2011) filled river valleys and buried the eroded landscape (Harrell 1993; Cather, Chapin & Kelley, 2012). Rivers traversing the Great Plains were sediment laden and perched upon porous alluvium (Menzer & Slaughter, 1971; Seni, 1980; Gustavson & Finley, 1985). High rates of infiltration presumably combined with aridity to reduce or eliminate freshwater habitats. If so, then the marginalization of river valleys around the periphery of the Great Plains surface (Galloway et al., 2011) could have corresponded with depopulation of ancestral *C. lutrensis* from the High Plains, explaining the lack of evidence for pre-Pleistocene lineages (Fig. 2A). Late Tertiary and early Pleistocene volcanism in the region (Galloway et al., 2011; Nereson et al., 2013) may have reduced habitat suitability if (for instance) it produced frequent ash deposition.

The sister relation of the Mississippi River sub-clade and Central group suggests the common ancestor was subdivided within the Gulf Coastal Plain. The Mississippi River sub-clade persisted to the east and eventually invaded the Mississippi River basin. Accordingly, Richardson and Gold (1995) found that some *C. lutrensis* in the Trinity and Calcasieu rivers were close relatives of northern individuals. The Mississippi Embayment and adjacent Gulf Coast was likely a climatic refugium (Richardson & Gold, 1995). Similarly, the ancestor of the Central group apparently survived the Late Miocene and Pliocene in the Rio Grande Embayment and Burgos Basin region (Fig. 2A), which was a climatic refugium for vascular plants (Sorrie & Weakley, 2001) and Neotropical hymenopterans (Porter, 1977, 1981).

Short branch lengths within the Central group are consistent with inferences of recent population (and hence possible population/range) expansion of the Central-group ancestor in the Pleistocene. The basal sub-clade of the Central group has been called *C. suavis* (Schönhuth & Mayden, 2010), but we refer to it only as the Rio Grande-Burgos Basin (RGB) sub-clade because this branch had poorer support and a broader distribution compared to previous studies. More sampling is needed to determine the distribution and phylogeny of these haplotypes.

Assimilation of isolated Pliocene river basins to form the modern Rio Grande (Galloway et al., 2011; Hoagstrom et al., 2014) opened new dispersal corridors to Central-group *C. lutrensis* (Fig. 2B). The Middle Rio Grande, within the Rio Grande Rift, overflowed the Hueco Bolson approximately 2.25 mya (Gustavson, 1991). Overflow of the Río Conchos into the Rio Grande likely occurred in this period (King & Adkins, 1946; Hawley, 1969) and river valleys were filled with alluvium and canyons were shallow (Hawley, 1969; Veni, 1994; Mack et al., 2006). These conditions may have favored range expansion of Central-group *C. lutrensis*, given that all *C. lutrensis* favor aggraded, plains-like environments with turbid streams (Matthews, 1995). Inclusion of Nueces River *C. lepida* in the Central group indicates that ancestral Central-group *C. lutrensis* were the ones that interbred with resident *C. lepida*, causing mitochondrial introgression (Broughton et al., 2011; Carson et al., 2014). To the east, the Mississippi River sub-clade also expanded its distribution (Richardson & Gold, 1995; Osborne et al., 2014).

Central-group *C. lutrensis* apparently invaded the Parras Basin and founded a disjunct population giving rise to *C. garmani*. Both nucDNA (Schönhuth & Mayden, 2010) and mtDNA (Schönhuth & Mayden, 2010; this study) nested *C. garmani* within the Central Group of *C. lutrensis*. Arellano (1951) provided evidence that pluvial lakes of the Parras Basin flowed toward the Rio Grande via the Río Salado (a Rio Grande tributary) in the Pleistocene (see also Contreras-Balderas, 1977). This connection was likely severed due to tectonic activity and increasing aridity. Distributions of several aquatic reptiles (Conant 1963), a mesic-adapted lizard (Montanucci, 1974), and pupfishes (Echelle et al., 2005) support pre-historic existence of this fluvial corridor. Alternatively, it is possible ancestral *C. garmani* reached the Parras Basin via exchanges with the Río Conchos and Río Mezquital (Minckley et al., 2005), but in this scenario *C. garmani* would be expected to be sister with the Chihuahuan Desert sub-clade, which it is not. Hence, we favor the former hypothesis.

Canyon formation could account for divergence of Chihuahuan Desert *C. lutrensis* from Capitan basins-Colorado River *C. lutrensis* ~1.98 mya. Pluvial climates and glacial runoff accelerated valley incision throughout the basin and narrowed the Rio Grande corridor (Hawley, 1969; Kastning, 1983; Veni, 1994; Mack et al., 2006). As streamflow increased and canyon incision progressed, canyon reaches likely became less suitable for *C. lutrensis* perhaps restricting gene flow (Fig. 2C). Although characteristic of low-gradient streams, *C. lutrensis* is relatively rare in larger rivers (Quist, Hubert & Rahel, 2004; Hoagstrom et al., 2006a, 2006b). Sustained high flows suppress *C. lutrensis* populations (Gido & Propst, 2012) and floods in steep-walled canyons incised into bedrock displace them (Minckley & Mefee, 1987). Populations become fragmented where unsuitable habitats are interspersed amidst suitable ones (e.g. Lynch & Roh, 1996). The Capitan basins sub-clade was possibly isolated upstream of the lower Pecos River canyon in a similar fashion.

The unresolved relation between the Capitan basins and Colorado River (Texas) sub-clades presents two possible scenarios for their divergence. In one scenario, Central group *C. lutrensis* may have colonized the ancestral Colorado River from the Capitan basins via the Simanola Valley, during the period when the Pecos River captured Arroyo del Macho from the Colorado River basin (sensu Gustavson & Finley, 1985; Fig. 2B). In this case, formation of the Caprock Escarpment and Llano Estacado (Figs. 2B and C) created separation. In the

alternative scenario, Central group *C. lutrensis* may have independently colonized the proto Colorado River through east-flowing rivers of the Edwards Plateau that paralleled the Balcones Escarpment (sensu Woodruff & Abbott, 1986). These rivers were later fragmented by stream captures across the escarpment (Woodruff & Abbott, 1986), isolating the Colorado River population. These alternatives need not be mutually exclusive and it is possible progenitors of the Capitan basins sub-clade reached the Pecos River via the Simanola Valley (Fig. 2B). Regardless, both sub-clades were isolated in the Pleistocene (Fig. 2C).

Fish faunas of the Capitan basins, Edwards Plateau, Rio Grande, and Río Conchos have clear affinities that demonstrate close biogeographical relations consistent with their inclusion within the Central group. The genus *Dionda* (Schönhuth et al., 2012), *Gambusia nobilis* species group (Rauchenberger, 1989), and *Etheostoma* subgenus *Austroperca* (Near et al., 2011), exhibit largely overlapping distributions with the Central group of *C. lutrensis* and each is represented by geographically divergent taxa. This suggests similar patterns of range expansion and fragmentation have affected each clade. The distribution of Clade III of *Cyprinella* (a lineage basal to the *C. lutrensis* clade) is also largely nested within the range of Central group *C. lutrensis* (Schönhuth & Mayden, 2010), suggesting the process of river drainage expansion and fragmentation, which has occurred repeatedly over time (Galloway et al., 2011), has facilitated sequential bouts of allopatric speciation.

Recent biogeography (human-mediated translocation)

It is well established that humans have broadly distributed *C. lutrensis* outside its native range (e.g. Hubbs, 1954; Poulos, et al., 2012). Until recently, there has been limited interest in translocations within the native range, but Husemann et al. (2012) found multiple highly divergent haplotypes coexisting in the Trinity and Brazos River basins and attributed this pattern to anthropogenic translocations. Our study documents similar conditions in the Pecos, Rio Grande, and South Canadian basins and provides insight into the sources of translocated haplotypes.

Red River (ND4 and cytochrome b) haplotypes shared between the South Canadian and Pecos rivers suggest representatives of these sub-clades were recently transferred to the Pecos River. We infer transfer direction because it mirrors introductions of other fishes (Moyer et al., 2005; Osborne et al., 2013). Further, if the Pecos River was the source of haplotypes shared with the South Canadian and Red rivers, then the more common Capitan basins haplotypes would be expected in samples from those rivers (i.e. 73% of Pecos River individuals had Capitan basins haplotypes). But, Red river haplotypes were prevalent in samples from the South Canadian River (65% and 35% respectively) while Capitan basins haplotypes were absent. Red River sub-clade haplotypes in the South Canadian River could also reflect recent translocations as *Notropis bairdi* (Red River shiner) and *Cyprinodon rubrufluvialis* (Red River Pupfish) have both been introduced there (Ashbaugh, Echelle & Echelle, 1994; Luttrell et al., 1995; Pigg, Gibbs & Luttrell, 1995). In contrast, non-native invaders are relatively rare in the Red River basin (Gido, Schaefer & Pigg, 2004) and Capitan basins haplotypes of *C. lutrensis* have yet to be found there (Broughton et al., 2011).

The presence of Brazos and Colorado River (ND4 and cytochrome b) sub-clade haplotypes in the Pecos River is also likely the results of human mediated translocations, as biogeographic evidence does not support a link between them (e.g. distinct ichthyofaunas found in the Brazos and Pecos Rivers). Sequence divergence (cytochrome b) between the Brazos clade haplotypes found in the Pecos and the natal Brazos haplotype is 0.3%–1.1% (p-distance) however only a single Brazos individual was included. One haplotype was identical between the Colorado and the Pecos (CBL, Delaware River) consistent with recent translocation and two others were slightly divergent. We cannot therefore exclude the possibility that further sampling in the Brazos and Colorado will reveal other shared haplotypes between these systems and the Pecos, supporting recent human-aided translocation. It is worth noting that other fishes from the Colorado River basin (Texas) have been inadvertently distributed throughout New Mexico (Mueller & Brooks, 2004; Davenport & Remshardt, 2008).

The presence of Mississippi River (ND4 and cytochrome b) and Brazos (cytochrome b) sub-clade haplotypes in the Middle Rio Grande is highly likely a result of recent translocation as there is no known “natural” connection between these basins and the Rio Grande Rift since the Early Miocene (Galloway et al., 2011), so shared and similar haplotypes with individuals from the South Canadian River suggests recent translocation. Consistent with these conclusions, molecular study of non-native White Sucker (*Catostomus commersonii*) also indicated multiple introductions to the Middle Rio Grande from multiple sources including the Pecos River (McPhee & Turner, 2009).

Evolutionary and biogeographic implications

“*Cyprinella lutrensis*” is a complex lineage owing to: (i) a late Tertiary expansion-fragmentation sequence that founded peripheral southwestern populations, giving rise to divergent species and cryptic lineages (Hoagstrom et al., 2014; this study), (ii) later Pleistocene expansions that led to independent colonization of areas integrated into the Rio Grande basin (Central group, this study), (iii) secondary contact between some late Tertiary isolates and Pleistocene invaders (Broughton et al., 2011), (iv) fragmentation of Central group populations in the Pleistocene (this study), and (v) most recently human mediated translocations. Persistence of deeply divergent but morphologically similar “*C. lutrensis*” lineages contrasts with isolated clades that are phenotypically distinct (e.g. *C. bocagrande*; *C. forlonensis*; *C. formosa*; *C. garmani*; *C. lepida*), especially given that the *C. lutrensis*-like Red River sub-clade is basal to all of these recognized species. While molecular and morphological distinctiveness apparently justify recognition of divergent peripheral species (Schönhuth, Doadrio & Mayden, 2006), this perspective confounds the taxonomic status of undifferentiated Peripheral lineages with similar antiquity. Thus, if recognized species of the *C. lutrensis* clade are valid, then “*C. lutrensis*” is polyphyletic without recognizable phenotypic differentiation or apparent reproductive barriers. In other words, allopatric speciation has not occurred in these lineages despite ample opportunity.

To determine the taxonomic status of undifferentiated Peripheral lineages, it will be critical to understand how they differ. It may be worthwhile to conduct more extensive comparisons of morphology (Berendzen, Olson & Barron, 2009) and nuptial coloration (Matthews,

1995). Chihuahuan Desert and Capitan basins sub-clades remain numerically dominant in their putative native ranges, which is somewhat surprising given that, in other cases, introduced congeners have rapidly displaced some sympatric native fishes (Childs, Echelle & Dowling, 1996; Moyer et al., 2005; Hoagstrom et al., 2010). Hence, it is critical to understand the nature of interbreeding amongst “*C. lutrensis*” lineages. Persistence of introduced mtDNA lineages suggests maternal contribution is conserved, but contrary to other studies (e.g. Garrett, 1988; Holtmeier, 2001; Ward et al., 2012) this did not increase morphological diversity within the Pecos River population.

In this context, the basal Red River sub-clade is of particular interest. Detailed comparative study of its distribution, physiology, and ecology relative to other members of the *C. lutrensis* clade is warranted. Declines of native populations have primarily been detected within the Red River basin (Matthews & Marsh-Matthews, 2007). Although the haplotypes of declining populations are unknown, if they were representatives of the basal Red River sub-clade, it is possible that this lineage may be more vulnerable to anthropogenic disturbance than for *C. lutrensis* as a whole (Poulos et al., 2012). However, it is possible declines occurring elsewhere may have gone unnoticed.

Given that *C. lutrensis* demonstrates potential for rapid adaptation to changing environments (King, Zimmerman, & Beiting, 1985), the *C. lutrensis* clade exhibits some characteristics of a phylogenetic raceme (Williams, 1992) (phylogeny with many transient branches that die out quite quickly, sensu Bell & Foster, 1994) where centralized lineages are persistent and evolutionarily conservative, whereas remote, peripheral isolates diverge. This creates instances in which speciation does not alter the stem (i.e. parental) species (Bell, 1979). However, the longevity of isolates in the *C. lutrensis* clade greatly exceeds the expectations of this paradigm. Clades with prolonged histories of peripheral isolation from a stable stem are said to speciate via peripheral budding or centrifugal speciation (Hodge et al., 2012; Puckridge, Last & Andreakis, 2015). However, the *C. lutrensis* clade does not fit this paradigm either because multiple conservative lineages are interspersed phylogenetically with divergent peripheral isolates.

Broughton et al. (2011) suggested that potential for outbreeding may be conserved in freshwater fishes because extrinsic barriers to movement among river drainages preclude a need for intrinsic reproductive barriers during allopatric speciation. The conservative lineages of *C. lutrensis* have broad distributions likely to preclude genetic drift and all still inhabit ecologically similar, turbid streams of grasslands and deserts (Matthews, 1987, 1995). In contrast, divergent lineages have narrow distributions in isolated habitats with much higher potential for genetic drift (especially where climatic conditions are highly variable) and many are confined to spring-fed habitats with different ecological conditions than streams typically occupied by *C. lutrensis* (Miller, 2005; Carson et al., 2014). These distinct environments are associated with peripheral isolates in other genera (e.g. *Gambusia*, Hubbs, 2001). Thus, for *C. lutrensis*, geographical isolation over an extended time appears inadequate to ensure allopatric speciation. Thus, other ecological divergence (Funk, Nosil & Etges, 2006) or genetic drift may be required to initiate speciation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Map of the southwestern United States and northern Mexico indicating tentative boundaries of the lineages identified in this study. Open polygons enclosed with solid lines indicate Peripheral sub-clades of estimated Miocene to Pliocene origin. Shaded polygons enclosed with dashed lines indicate Central group sub-clades of estimated Pleistocene origin.

Figure 2.

Putative scenario of expansion and fragmentation of southwestern *Cyprinella lutrensis*. (A) Beginning Pliocene: mountain ranges (gray polygons) border High Plains (i.e. Ogallala surface); mountain-fed rivers cross High Plains to Edwards Plateau and Coastal Plain (east and south of mapped area), except for endorheic drainage into Delaware (DB) and Roswell (RB) subsidence basins (collectively Capitan basins); sediments fill deep dissolution basins (e.g. Toyah Basin, TB); Central group *C. lutrensis* (CG) isolated in lower Rio Grande (shiner icon). (B) Beginning Pleistocene: dissolution subsidence forms Caprock Escarpment and High Plains is desiccated; Rio Grande Rift drainage (northwest of mapped area) and Rio Conchos overflow basins and connect; Capitan basins overflow Delaware Basin to join Rio Grande; incipient Pecos River expands headward, capturing Arroyo del Macho (Macho) of Colorado River; Central Group *C. lutrensis* (CG) expand north and west into interconnected rivers, colonizing Colorado River via Pecos River or Coastal Plain; incipient South Canadian River expands headward, capturing headwaters of east-flowing rivers; Mississippi River sub-clade (checkerboard shiner icon) invades South Canadian River. (C) Late Pleistocene: Caprock Escarpment prominent around desiccated Llano Estacado (e.g. Portales and Simanola valleys abandoned); swollen Conchos, Pecos, and Rio Grande form canyons

(gray river outlines) that fragment Central group *C. lutrensis*: (i) Chihuahuan Desert sub-clade (Chi), (ii) Capitan basins sub-clade (Cap), (iii) Rio Grande - Burgos basins sub-clade (RGB), and (iv) Colorado River sub-clade (Col); Peripheral-group lineages expand westward: (i) Red River (polka dot shiner icon) and (ii) Brazos River (striped shiner icon). (D) Today: post-glacial aridity and human water uses diminish rivers; widespread translocations (pickup icons) include *C. lutrensis*: (i) Red River sub-clade introduced to South Canadian and Pecos, (ii) Brazos and Mississippi sub-clades introduced to Pecos (pickups) and Middle Rio Grande (arrows), and (iii) Colorado River sub-clade introduced to Pecos. State lines provided for reference. Other abbreviations mentioned in text: CM - Capitan Mountains; DR - Delaware River; EC - El Capitan Peak; GM-CP - Guadalupe Mountains- Capitan Platform; TCC = Terlingua Creek confluence.

Figure 3.

(A) Median-joining haplotype network based on ND4 sequence data. Accession numbers for ND4 haplotypes obtained here are given in Table 2. Circle size is proportional to haplotype frequencies. Distinct ND4 haplotypes are indicated by letters. Shading distinguishes distinct drainages: Pecos River in black, Rio Grande in gray and Canadian in white. Numeric values adjacent to branches indicate the number of base changes between haplotypes (single base changes are not accompanied by a number). (B) Chronogram from Beast analysis based on cytochrome b shows estimated time (mya) to most recent common ancestor. 95% HPD (highest posterior density) intervals are below these values, letters within triangles refer to cytochrome b haplotypes (MISSISSI—Mississippi subclade).

Figure 4. Bayesian tree from mtDNA cytochrome b sequences from *C. lutrensis* from the Rio Grande, Pecos River, and Canadian River and from the previously published sequences from Schönhuth and Mayden (2010) and those reported in Broughton (2011) (Broughton pers. comm.). Posterior probabilities are reported by nodes when greater than 0.70. Asterisks indicate sequences obtained in this study. Genbank accession numbers are also provided.

Fig. 5A.

Fig. 5B.

Figure 5. Principal components analysis of (A) geometric weight-matrix and (B) combined morphometric and meristic data. The Peripheral group is represented in black squares and the Central group is represented by gray triangles. Percent of variation explained is given within each axis title.

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Table 1

Number of individuals from each river sequenced for genetic and morphological analyses.

River	ND4	Cytb	RAG-1	Meristics	Geomorph
^ Pecos	255	18	41	38	38
Rio Grande	36	5	10	0	0
South Canadian	19	5	6	0	0
Total	308	28	57	38	38

^ Also includes samples from the Delaware River that is a tributary of the Pecos River.

Table 2

ND4 haplotype frequencies and Genbank accession numbers. Haplotype sub-clades as identified in Fig. 3 and Fig. 4 are also given. Asterisks indicate contemporary transfer of haplotypes between drainages.

	Haplotype	Genbank Accession	[^] Pecos	South Canadian	Rio Grande
Peripheral	U	KM199673	--	0.05	--
	V	KM199674	0.019	0.150*	--
	W	KM199675	--	0.05	--
	X	KM199676	0.004	--	--
	Y	KM199677	0.016	--	--
	Z	KM199678	0.035	0.250*	--
	BB	KM199680	0.019	0.100*	--
	CC	KM199681	--	0.05	--
	P1**	KM504967	0.004	--	--
	P2**	KM504968	0.004	--	--
Brazos	P3	KP987457	0.004	--	--
	P	KM199668	--	0.250*	0.087
	R	KM199670	--	0.1	-
	Q	KM199669	--	--	0.087
	S	KM199671	--	--	0.087
Mississippi	A	KM199653	0.728	--	--
	B	KM199654	0.031	--	--
	C	KM199655	0.004	--	--
	D	KM199656	0.004	--	--
	E	KM199657	0.008	--	--
	F	KM199658	0.031	--	--
	G	KM199659	0.004	--	--
	I	KM199661	0.004	--	--
	J	KM199662	--	--	0.043
	K	KM199663	--	--	0.696
Capitan					
Central					

	Haplotype	Genbank Accession	[^] Pecos	South Canadian	Rio Grande
Colorado	L	KM199664	0.008	--	--
	M	KM199665	0.062	--	--
	N	KM199666	0.012	--	--

Sequences obtained in an earlier study of Pecos River cyprinids are indicated by **, these were not detected in the present study but reflect a divergent lineage.

[^] Includes Pecos and Delaware River samples.

Table 3

Cytochrome-b Genbank accession numbers. Phylogenetic sub-clades corresponding to Fig. 3 and Fig. 4- are also given.

	Cytochrome b Haplotype	Genbank Accession	Pecos	Canadian	Rio Grande
PERIPHERAL	E	KM082996	x	--	--
	K	KM083001	x	--	--
	I	KM083000	x*	x	--
	G	KM082998	--	x	--
	H	KM082999	--	x	--
	T	KM083008	x	--	--
Brazos River	D	KM082994	x	--	--
	DD	KM082995	x	--	--
	DDD	KP769968	x	--	--
Mississippi River	A	KM082991	--	--	x
	B	KM082992	--	--	x
	C	KM082993	--	x	--
	CB2	KP769970	--	x	--
Colorado River	CBL	KP769965	x	--	--
	CBM	KP769966	x	--	--
	CBN	KP769967	x	--	--
Chihuahuan Desert	BB	KP769974	--	--	x
	M	KM083002	--	--	x
Capitan basins	CBQ	KM083006	x	--	--
	CB1	KP769969	x	--	--
	CB3	KP769971	x	--	--
	CB4	KP769972	x	--	--
	CB5	KP769973	x	--	--

Asterisks indicate probable contemporary transfer of haplotypes. x-- indicates presence of haplotype in the Pecos River, Canadian River or Rio Grande.

Table 4

Test statistics (Tajima's D, Fu's Fs and R2) and associated p-values for all cytochrome b (Cyt-b) and ND4 haplotypes, for Central and Peripheral haplotypes and for the Capitan, Mississippi and Red sub-clades. Grey shading indicates significant departures from a constant population size; that is consistent with recent population expansion/growth.

	Gene	All	Central	Peripheral	Capitan	Mississippi	Red
Tajima's	D	1.354	0.462	1.483	-1.295	1.254	-0.794
	P	0.95	0.72	0.959	0.061	0.91	0.269
Tajima's	D	1.746	-0.546	1.335	-1.284	0.18	-0.414
	P	0.976	0.322	0.943	0.126	0.71	0.371
Fu's Fs	Cyt-b	1.189	-0.512	1.684	-3.047	2.747	-1.157
	P	0.71	0.32	0.753	0.003	0.897	0.144
Fu's Fs	ND4	-4.867	-6.422	-0.817	-5.694	-1.082	-1.754
	P	0.049	0.002	0.289	0.0003	0.097	0.088
R2	Cyt-b	0.201	0.177	0.213	0.11	0.256	0.188
	P	0.996	0.759	0.989	0.003	0.677	0.379
R2	ND4	0.198	0.112	0.206	0.097	0.232	0.144
	P	0.993	0.147	0.972	0.001	0.258	0.084