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Current and historical hybridization with differential introgression among three species of cyprinid fishes (genus *Cyprinella*)

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Abstract Hybridization is common among freshwater fishes, particular among the Cyprinidae. We used two mitochondrial genes and one nuclear gene to characterize hybridization among two species pairs of *Cyprinella* in southwestern North America. Genalogical patterns revealed that *C. lutrensis* and *C. venusta* are currently hybridizing in several localities producing apparent F_1, F_2 and backcross generations, yet there was no evidence for introgression outside of local hybrid zones. Alternatively, mitochondrial haplotypes from *C. lutrensis* appear to have introgressed into a *C. lepida* population in the Nueces River completely replacing the native *C. lepida* haplotype. There was no evidence of introgression of nuclear DNA and there does not appear to be ongoing hybridization. The population of *C. lepida* from the nearby Frio River exhibits no evidence of hybridization with *C. lutrensis*. Thus, contact between *C. lutrensis* and *C. venusta* results in the formation of localized hybrid swarms, while contact between *C. lutrensis* and *C. lepida* has resulted in complete mitochondrial introgression in the Nueces River or no apparent hybridization in the Frio River. The three different outcomes of contact between these species illustrate the variable nature of interspecific reproductive interactions and provide an excellent system in which to better understand the factors influencing hybridization among freshwater fishes.

Keywords Cyprinidae · Hybridization · Introgression

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Introduction

Natural zones of hybridization have been called windows on the evolutionary process (Harrison 1990) because they represent cases where speciation is incomplete and thus may reveal the genetic and ecological factors that lead to reproductive isolation while they are still in progress. Hybridization appears to be more common among freshwater fishes than in other vertebrate groups and hybrids are relatively common in nature (Hubbs 1955; Campton 1987; Scribner et al. 2001). Hybridization may increase diversity at the population level, at least temporarily, through development of hybrid swarms and via reciprocal introgression of alleles into the genetic background of parental populations. Alternatively, strongly asymmetric introgression may reduce diversity by overwhelming the genomes of recipient species (potentially leading to extinction), particularly when there is a corresponding asymmetry in population size (e.g., Echelle and Connor 1989; Dowling and Childs 1992). If hybridizing populations merge into a single interbreeding unit, taxonomic diversity is reduced (generating one species from two). Another possible outcome is isolation of a hybrid population from its parent species yielding an entirely new taxon, increasing taxonomic diversity (three species from two) (e.g., DeMarais et al. 1992). The nature of reproductive barriers appears to be dynamic, as reflected by spatial and temporal variation in the degree of reproductive (e.g., Dowling et al. 1997; Aboim et al. 2010). While hybridization appears to be an important component of freshwater fish evolution (Smith 1992; Dowling and Secor 1997; Gerber et al. 2001), our understanding of the factors that promote or allow frequent interspecific mating remains limited.

Hybridization has been documented in a variety of freshwater fish taxa, including black basses and sunfishes

(Centrarchidae; Bolnick and Near 2005), darters (Percidae; Keck and Near 2010), mollies and guppies (Poeciliidae; Turner et al. 1980, Scribner et al. 2001), and minnows and chubs (Cyprinidae; Dowling et al. 1989, Alves et al. 2001; Aboim et al. 2010). Cyprinidae is the worlds largest clade of freshwater fishes and is also the largest family in North America with 275–280 species (Mayden et al. 1992). Interspecific hybridization is common in the diverse North American cyprinid fauna and is not restricted to matings among closely related species. For example, Hubbs (1955) reported extensive hybridization between members of different cypinrid genera and subgenera. Sixty-eight species pairs found east of the continental divide produce intergeneric or inter-subgeneric hybrid offspring (Hubbs 1955). More recently, Smith (1992) observed that morphological divergence provides little constraint on the ability of North American cyprinids to interbreed. The extent of hybridization among more closely related cyprinid congeners has not been completely documented but anecdotal observations suggest it is likely to number in the hundreds of species pairs. This suggests that much of the divergence that leads to "species-level" differences occurs in the absence of reproductive isolation and that intrinsic barriers to gene exchange may often arise independently of morphological differences.

Cyprinella is a monophyletic group of 30 cyprinid species that are among the most locally abundant fishes in streams and rivers throughout eastern North America (Mayden 1989). *Cyprinella lutrensis* is the most widespread species, occurring throughout the Great Plains and arid southwest. *Cyprinella venusta* is also broadly distributed, occurring in most Gulf-coastal drainages from Florida to northern Mexico. The ranges of *C. lutrensis* and *C. venusta* overlap in Texas, southern Oklahoma, and Louisiana (Mayden 1989). Although not frequently encountered at the same locality, individuals of mixed ancestry have been observed at several localities in Texas where they co-occur (Hubbs and Strawn 1956). Experimental crosses between *C. lutrensis* and *C. venusta* indicated that hybrid individuals are fertile, capable of backcrossing with both parental species and producing F_2 hybrids (Hubbs and Strawn 1956). Hybridization between these species has been observed more recently where *C. lutrensis* has been introduced outside its native range in the Coosa River system of Alabama (Walters et al. 2008; Blum et al. 2010). Figure 1 illustrates phenotypes of each species.

Cyprinella lepida has a very restricted range, occurring only in the Nueces, Frio, and Sabinal Rivers on the Edwards Plateau of southwest Texas (Matthews 1987). This distribution is contained entirely within the ranges of *C. lutrensis* and *C. venusta*. *Cyprinella lepida* has been variously described as a subspecies of *C. lutrensis* (Jordan and Evermann 1896; Hubbs 1972) and as a distinct species (e.g., Lytle 1972; Matthews 1987; Mayden 1989). Phylogenetic studies revealed two distinctive mtDNA haplotypes from *C. lepida*, one found in the upper Nueces River and the other from the Frio and Sabinal Rivers which form a tributary to the Nueces (Richardson and Gold 1995; Broughton and Gold 2000). Despite occurring as little as 50 km apart, individuals from these two areas were so different from each other that Richardson and Gold (1995) suggested that the Nueces population was an undescribed species. Haplotypes from *C. lepida* were phylogenetically similar to those from different populations of *C. lutrensis*, rendering the latter paraphyletic (Broughton and Gold 2000). Although not initially apparent, this pattern is consistent with genetic exchange between *C. lepida* and *C. lutrensis* (Schonhuth and Mayden 2010; this paper). Photographs of C. lepida from the Frio and Nueces Rivers appear in Fig. 1.

Here, we describe the genealogical history of mitochondrial and nuclear genes from several southwestern populations of *C. lutrensis, C. venusta* and *C. lepida*. Population samples were drawn mainly from three drainages in close proximity in south Texas where the three species occur. The history of hybridization may be revealed by the extent of introgression among recent hybrid individuals and the depth at which haplotype or allelic lineages are resolved on the genealogy. If hybridization is recent and/or ongoing individuals of mixed ancestry should possess cross-specific gene variants reflecting local variation in parental populations. Alternatively, historical hybridization events may be marked by discordance among loci reflecting differential introgression and sorting among lineages that arose deeper in genealogical history. Our results clarify the evolutionary history of *C. lepida* and characterize genetic aspects of hybridization between *C. lutrensis* and *C. venusta*, provide new insights in the dynamic nature and variable results of hybridization in North American cyprinid fishes.

Materials and methods

Individual fishes were collected by seine and preserved in 95% ethanol in the field. Table 1 lists sample localities and species collected as well as the number of individuals sequenced for nuclear and mitochondrial genes. Rivers and sampling localities are illustrated in Fig. 2. Sampling focused on *C. lepida* from the Nueces River and Frio/Sabinal Rivers as well as *C. venusta* and *C. lutrensis* (and their hybrids) from the Guadalupe River and tributary creeks of the Rio Grande. In order to assess which haplotypes and alleles were unambiguously characteristic of particular species, additional *C. lutrensis* from outside the range of *C. venusta* in Oklahoma and *C. venusta* from the

Fig. 1 Photographs showing phenotypic characteristics of some *Cyprinella*. **a** *C. lutrensis*; **b** *C. venusta*; **b** phenotypic intermediates of *C. lutrensis* and *C. venusta*; **d** *C. lepida* from the Frio River; **e** *C. lepida* from the Nueces River. Photos: A, N. Franssen; B, W. Matthews; C-E, REB

Suwanee River of Georgia were included. *Cyprinella formosa* and *C. bocagrande* appear to be closely related to *C. lutrensis* and *C. lepida*, so several of these individuals were also included for phylogenetic perspective. *Cyprinella spiloptera* from the Mississippi River basin was used as the outgroup.

Most specimens were readily identifiable to species in the field based on diagnostic morphological and coloration characters. However, in the Guadalupe River and Sycamore Creek individuals of mixed ancestry between *C. lutrensis* and *C. venusta* were observed. In comparison, *C. venusta* exhibits a long shallow body (typically 9–10 cm standard length) that is mostly silver in coloration with a large black spot at the base of the caudal fin in both sexes. *Cyprinella lutrensis* has a shorter deeper body (typically 6–7 cm standard length), red colored fins (except the dorsal fin), and distinctive blue and red colored post-opercular bars in breeding males. The black caudal spot is absent in *C. lutrensis*. A range of individuals with different combinations of these characters were collected. Some appeared to be pure of one species or the other, while many intergrades appeared to have more or less characteristics of a particular species. Some individuals exhibited what appeared to be equal parts of each species as might be expected of F1 hybrids. We designated such individuals of mixed ancestry as more *C. lutrensis*-like (lut/ven), more *C. venusta*-like (ven/lut), or F1-like (lut=ven).

DNAs were isolated with the DNeasy Kit (Qiagen, Inc.). We sequenced mitochondrial NADH dehydrogenase subunit 2 (ND2) and Cytochrome b (Cyt b) genes and the intron 1 of the nuclear Hox c6a gene. PCR primers for ND2 were from Broughton and Gold (2000) and for Cyt b were from Schmidt et al. (1998). We designed species-specific primers in several cases where apparent nucleotide substitutions in the priming site inhibited primer binding (available from R.E.B. on request). Primers for the Hoxc6a gene were designed for this study using the annotated genome of *Danio rerio* on the Ensemble Database. These were located in exons flanking the single 610 bp intron, yielding an amplicon of approximately 800 bp (Hoxc6a-int-F 5′: gaaccaggtgaagacatttgc 3′, Hoxc6a-int-R: 5′ cattggcgatytcgatgcgtc 3′). Templates for sequencing were amplified in 50 µl reactions containing: 1× reaction buffer (10 mM Tris–HCl (pH 8.5), 50 mM KCl, 1.5 mM $MgCl_2$), 200 µM each dNTP, 0.5 µM each primer, 2.5 U Taq, approx. 100 ng template DNA, and ultrapure water. Thermal cycling conditions were as follows: initial denaturation at 94° for 1 min., followed by 35 cycles of denaturation at 94° for 10 s., annealing at 48–55° for 15 s., and polymerization at 72° for 90 s. Amplification products were purified for sequencing by filtration with Multiscreen PCR96 filter plates (Millipore Inc.).

DNA sequencing reactions contained the following in a total volume of 10 µl: 1 µl BigDye version 3.1 reaction

Table 1 Locality information for specimens used in this study

Sample site	Species collected	Nuclear	Mito
Terlingua Creek, Brewster Co., TX	*C. lutrensis*	0	5
Tornillo Creek, Brewster Co., TX	*C. lutrensis*	0	3
Elm Creek, Maverick Co., TX	*C. lutrensis, C. venusta*	1	4
Sycamore Creek, Cooke Co., TX	*C. lutrensis, C. venusta,* hybrids	5	4
Pinto Creek, Kinney Co., TX	*C. lutrensis*	0	4
Nueces River, Real/Edwards Co., TX	*C. lepida*	3	6
Frio River, Real Co., TX	*C. lepida*	4	3
Sabinal River, Bandera Co., TX	*C. lepida*	2	2
N Fork Guadalupe River, Kerr Co., TX	*C. lutrensis, C. venusta,* hybrids	4	6
Concho River, Tom Green Co., TX	*C. lutrensis*	2	5
Colorado River, Concho Co., TX	*C. lutrensis*	0	1
Croton Creek, Stonewall Co., TX	*C. lutrensis*	1	1
S Fork Wichita River, Knox Co., TX	*C. lutrensis*	1	2
N Fork Red River, Greer/Kiowa Co., OK	*C. lutrensis*	1	0
Stinking Creek, Jackson Co., OK	*C. lutrensis*	0	2
Washita River, Johnston Co., OK	*C. lutrensis*	0	3
Little River, Cleveland Co., OK	*C. lutrensis*	1	0
N Canadian River, Woodward Co., OK	*C. lutrensis*	1	2
Morris Creek, Le Flore Co., OK	*C. lutrensis*	0	3
Arkansas River, Muskogee Co., OK	*C. lutrensis*	1	1

Two right-hand columns indicate the number of specimens sequenced for nuclear and mitochondrial genes, respectively

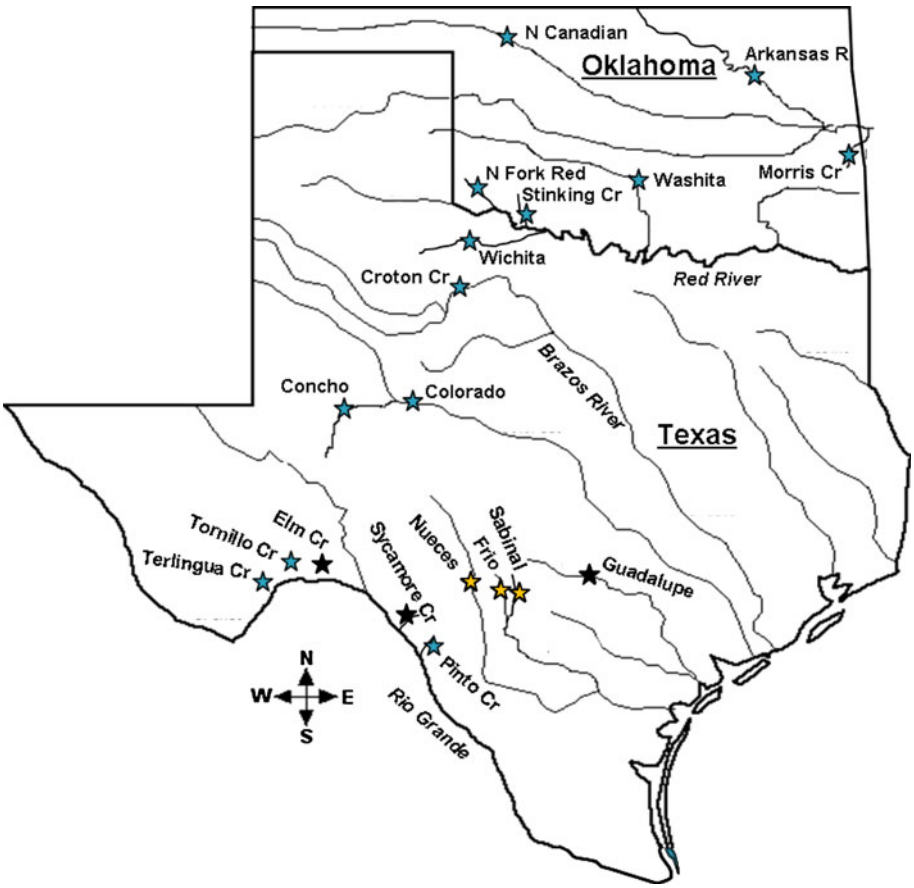

Fig. 2 Map of major river drainages of Oklahoma and Texas, USA, showing collection localities (with star symbol) for this study. *Star fill* color indicates species collected: blue = *C. lutrensis*, black = *C. venusta* and C. lutrensis, and yellow = *C. lepida*. State names are underlined; italicized river names are for reference

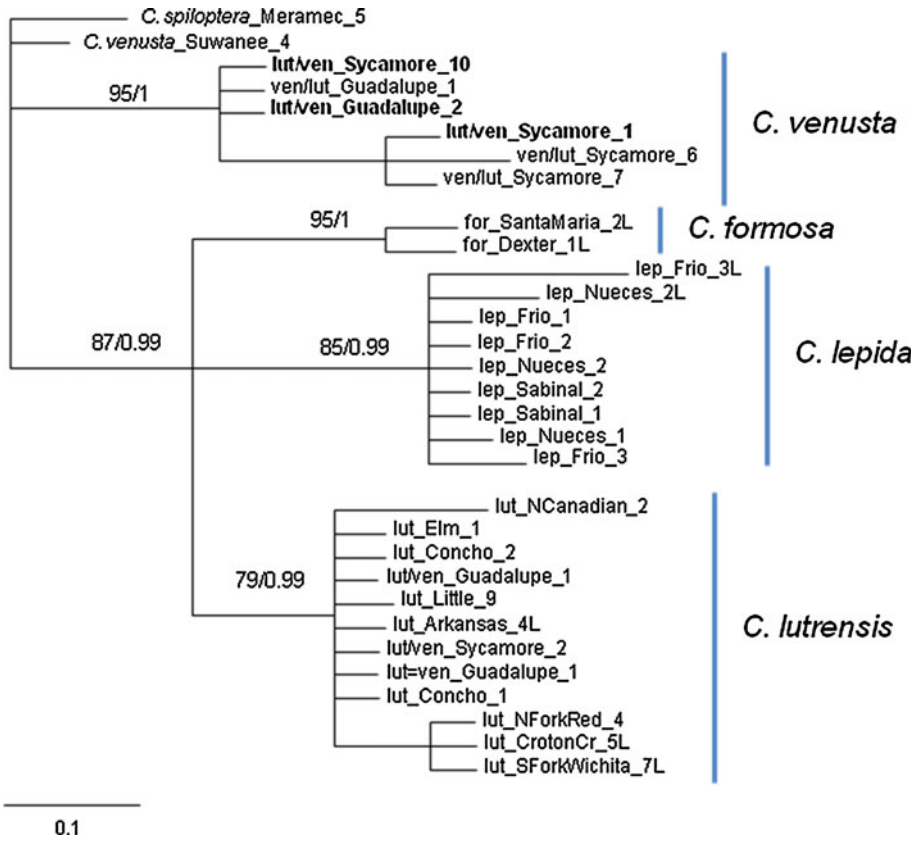

Fig. 3 Maximum likelihood tree for the nuclear Hoxc6a intron. Numbers on nodes indicate maximum likelihood bootstrap and Bayesian posterior probabilities, respectively. Individuals are labeled with a species designation based on phenotype followed by the collection locality (from Table 1) and a specimen number. Fishes of mixed phenotype from the Guadalupe River and Sycamore Creek are given the designation of ven/lut if they had more phenotypic characteristics typical of *C. venusta*, lut/ven if they were phenotypically more like *C. lutrensis*, or lut=ven if they exhibited characteristics of both species in approximately equal proportions. *Bold face* indicates individuals with a nuclear genotype different from the species they most resembled phenotypically

mix (Applied Biosystems Inc.), 1.5 µl 5× sequencing dilution buffer (400 mM Tris–HCl pH 9.0, 10 mM $MgCl_2$), 0.32 µl of 10 µM primer stock, 3 µl (approx. 50 ng) template DNA, and ultrapure water. Cycling conditions were as follows: initial denaturation at 96° for 30 s., followed by 40 cycles of 96° for 10 s., 55° for 15 s., and 60° for 4 min. Sequence reaction products were purified via precipitation with 95% ethanol and 3 M sodium acetate, washed with 70% ethanol, and dried. Electrophoresis and base-calling were performed with an Applied Biosystems 3130xl Genetic Analyzer. Sequences were assembled and aligned with Geneious Pro 5.0 (Biomatters Ltd.).

Phylogenetic analysis of DNA sequences utilized maximum likelihood and Bayesian methods. Models of DNA sequence evolution were selected with Modeltest 3.06 (Posada and Crandall 1998). Maximum likelihood was conducted with the sequential version of RAxML-VI-HPC ver. 7.0 (Stamatakis 2006). We employed the GTRMIX model wherein sites are partitioned into 25 discrete rate categories for tree searching and topological comparisons, and then a discrete gamma distribution is utilized to estimate final tree likelihoods. The "-a" option was used in RAxML which performs non-parametric bootstrapping (1,000 pseudoreplicates), followed by a search for the maximum likelihood tree. Bayesian analyses were performed with MrBayes 3.1 (Ronquist and Huelsenbeck 2003) using default settings for 2 million generations. For the mitochondrial genes, three data partitions corresponding to the three codon positions were used for both maximum likelihood and Bayesian analyses; the nuclear intron was not partitioned. Separate and combined analyses of Cytb and ND2 were performed to assess congruence between mitochondrial genes.

Results

Aligned data matrices contained 1,047 nucleotides (420 phylogenetically informative) for ND2, 1137 (322 phylogenetically informative) for Cyt b, and 800 (53 phylogenetically informative) for Hoxc6a intron 1. No heterozygous sites were observed in the intron sequences. The best models indicated by ModelTest using both likelihood ratio tests and AIC were GTR + G + I for each mitochondrial gene and HKY + G for the nuclear intron. Results of separate phylogenetic analyses of ND2 and Cyt b were identical except for a few differences among individuals within the major groups (not shown) so we combined these genes for all subsequent analyses.

Results of phylogenetic analyses of the Hoxc6a intron are shown in Fig. 3. Maximum likelihood and Bayesian

analyses recovered identical topologies. Several well-supported groups corresponding to recognized species were recovered although the limited variation in these sequences did not resolve relationships of individuals within species groups. Notably, all individuals of putative *C. lepida* from the Nueces and Frio drainages formed a monophyletic group which is consistent with the traditional taxonomy recognizing *C. lepida* as a single taxon. Monophyletic groups of *C. formosa*, *C. lepida*, and *C. lutrensis* were well defined but relationships among these three species were not resolved. While not strictly monophyletic, the *C. venusta*-like alleles appear to be closely related to the allopatric specimen from the Suwanee River in Georgia. Individuals recovered in the *C. lutrensis* clade were initially identified as pure *C. lutrensis* or as having more *C. lutrensis*-like characters, in addition to one individual of intermediate phenotype. However, three individuals (one from the Guadalupe R. and two from Sycamore Cr.) exhibiting *C. lutrensis*-like morphology possessed *C. venusta* Hox alleles. Note that while not all the individuals are the same as for analysis of the nuclear versus mitochondrial genes, the same set of major drainages are represented and several of the same specimens were used for populations suspected of hybridation.

Phylogenetic analysis of mitochondrial genes also yielded several well-supported groups, however these tended to correspond to major river drainages rather than taxonomic designations (Fig. 4). Major clades included *C. venusta*, *C. formosa*, *C. lepida* from the Frio and Sabinal Rivers, *C. lutrensis* from the Red River, Arkansas River, and south Texas region, and *C. lepida* from the Nueces River. There is a large clade that is essentially a four-way polytomy including a *C. formosa*-*C. lepida* Frio/Sabinal clade, two populations of *C. lutrensis* from the Arkansas River and Red River drainages and a single individual from the Brazos River of Texas. The *C. formosa* clade is monophyletic and includes an individual from the closely related species *C. bocagrande*. The relationship between at least some *C. lepida* and the formosa group is not unexpected based on previous studies (Broughton and Gold 2000; Schonhuth and Mayden 2010). The northern populations of *C. lutrensis* are distinct from those from more southern drainages in Texas which flow into the Gulf of Mexico. The south Texas group forms a clade with the monophyletic *C. lepida* Nueces population embedded within it.

A monophyletic *C. venusta* clade contains one individual with a *C. lutrensis* phenotype and one with an intermediate phenotype, whereas the C. lutrensis South Texas group contains two individuals that were phenotypically *C. venusta*-like. Taken with the nuclear result, this suggests that both the Guadalupe River and Sycamore Creek harbor individuals of mixed ancestry between *C. lutrensis* and *C. venusta*.

Discussion

We examined genealogical patterns focusing on three species of *Cyprinella* with ranges that overlap in the American Southwest. Two of these species, *C. lutrensis* and *C. venusta*, have been known to hybridize in some areas of contact and another pair, *C. lutrensis* and *C. lepida*, has been suspected of possible hybridization but previously available data have been inconclusive. Our approach was to use a phylogenetic framework to compare patterns of possible interbreeding between different species pairs that are within close geographic proximity.

Interbreeding between *C. lutrensis* and *C. venusta* is characterized by the occurrence of individuals of mixed ancestry in several river drainages, indicating multiple areas of contact and hybridization. In these cases individuals of both parental species are present along with individuals with intermediate phenotypes (apparent F1 s or recent backcrosses). The presence of individuals with *C. lutrensis* nuclear alleles and *C. venusta* mtDNA haplotypes as well as individuals with *C. venusta* nuclear alleles and *C. lutrensis* mtDNA haplotypes confirms the existence of hybrid individuals and that hybridization is reciprocal between these species. Bidirectional interbreeding appears to produce fertile offspring of both sexes consistent with the experimental results of Hubbs and Strawn (1956). Heterospecific mtDNA haplotypes found in both of these species are diverse and broadly representative of the pool of parental haplotypes found in the same geographic region suggesting that hybridization is not limited to a few rare occurrences. Interspecific genotypic combinations are found within a full range of phenotypic intermediates (from very lutrensis-like to very venusta-like) suggesting that hybridization is ongoing and that backcrossing may be extensive. Analyses with additional nuclear loci may further characterize the filial identities of individuals in these hybrid zones but there would appear to be separate hybrid swarms where the species come into contact in these two south Texas rivers. Nonetheless, there appears to be little introgression outside of the immediate areas of contact. This situation appears to be somewhat typical of hybrid zones in other taxa.

The genealogical patterns observed for *C. lutrensis* and *C. lepida* suggest a very different scenario. The nuclear data suggest that *C. lepida* represents a single monophyletic species that has diverged to an extent similar to other recognized species. It is distinct from but related to *C. lutrensis* and *C. formosa*, as expected based on previous phylogenetic studies (Mayden 1989; Broughton and Gold 2000; Schonhuth and Mayden 2010). The contrasting mitochondrial data suggest that the Frio/Sabinal River populations may represent original mtDNA lineages of *C. lepida*, because their relationship with *C. formosa* and

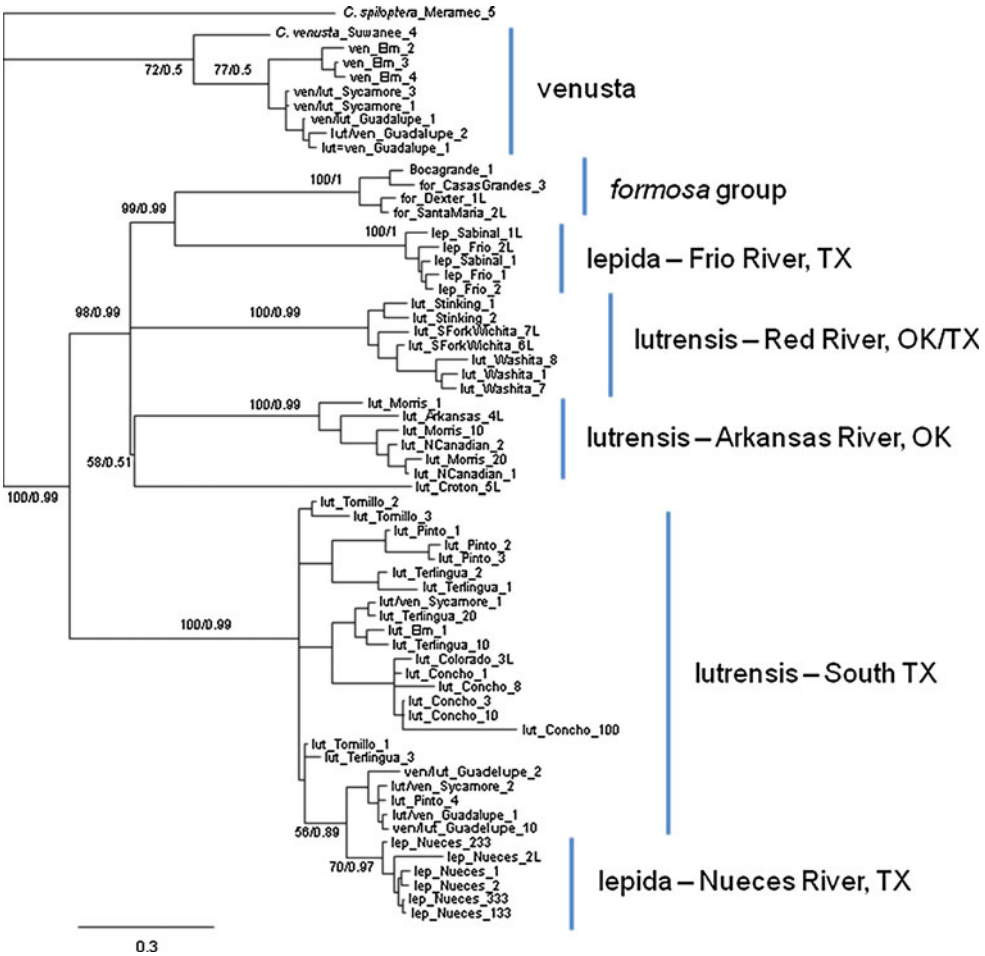

Fig. 4 Maximum likelihood tree for mitochondrial ND2 and Cyt b genes. Numbers on nodes indicate maximum likelihood bootstrap and Bayesian posterior probabilities, respectively. Individuals are labeled with a species designation based on phenotype followed by the collection locality (from Table 1) and a specimen number. Fishes of mixed phenotype from the Guadalupe River and Sycamore Creek are given the designation of ven/lut if they had more phenotypic characteristics typical of *C. venusta*, lut/ven if they were phenotypically more like *C. lutrensis*, or lut=ven if they exhibited characteristics of both species in approximately equal proportions. *Bold face* indicates individuals with a mitochondrial haplotype different from their species phenotypic designation

C. lutrensis haplotypes is comparable to that of the nuclear gene. Alternatively, haplotypes of *C. lepida* from the Nueces River are highly similar to those of the South Texas *C. lutrensis* population, suggesting that *C. lutrensis* haplotypes have completely replaced the original *C. lepida* haplotypes in this population. Interactions between *C. lutrensis* and *C. lepida* would only seem to have occurred at a single locality, the Nueces River, and there is no evidence for hybridization ever occurring in the nearby Frio River. *Cyprinella lutrensis* in not known from the upper Nueces and individuals with intermediate phenotypes have not been observed. In fact, no hybrid zone between these two species is known from any locality. The genotypic combination of *C. lepida* nuclear alleles with *C. lutrensis* mtDNA haplotypes appears to be fixed in the Nueces River. Larger sample sizes will add greater confidence to this conclusion, but independent samples from the Nueces River by Richardson and Gold (1995) and Schonhuth and Mayden (2010) have in no case recovered individuals that are not consistent with a population fixed for heterospecific mtDNA.

Heterospecific mtDNAs that are fixed, monophyletic, and exclusive to *C. lepida* of the Nueces suggests the absence of ongoing hybridization where we would expect to observe haplotypes that are not each other's closest relatives. The exclusive group of haplotypes combined with a nucleotide diversity comparable to that of the Frio River population, suggests that introgression from *C. lutrensis* was a historic event and that a few introgressed haplotypes have been diverging in the absence of gene flow from *C. lutrensis* since that event. It is possible that other unsampled genes have introgressed from *C. lutrensis* into the Nueces population and/or that the Nueces and Frio populations have diverged from one another. The two populations do appear to exhibit differences in coloration with individuals from the Nueces exhibiting a more gold body coloration (Fig. 1d, e). The present data are not sufficient to address whether there some incompatibility between the Frio population and *C. lutrensis* or whether the lack of historical hybridization (or introgression) in the Frio is simply due to stochastic factors. It may be that the two populations of C. lepida have diverged and represent different species but the relatively conserved Hox intron does not reflect this divergence.

Ultimately, the taxonomic status of *C. lepida* populations remain controversial. Matthews (1987) noted

coloration differences between the Nueces and Frio populations and extensive divergence of mtDNA led Richardson and Gold (1995) to suggest that the Nueces population was a new species. In an extensive phylogenetic study of *Cyprinella*, Schonhuth and Mayden (2010) recovered two distinctive populations based on Cyt b sequences that they referred to as *C. lepida* (from the Frio) and *C. sp.* '*cf. lepida*' (from the Nueces). However, analysis of the nuclear Rag1 gene for two individuals from the Frio and one from the Nueces recovered all three as monophyletic with strong support. Schonhuth and Mayden (2010) suggested introgression of mtDNA in the Nueces population but its taxonomic status remains undefined. Resolution of this question will require characterization of additional nuclear genes in the context of whether this population represents a new species, possibly of hybrid origin. The central issue might be whether introgression of mtDNA alone is sufficient to describe a new species.

The frequency of hybridization among freshwater fishes may be due in part to functional divergence of populations in relative isolation in different streams or rivers. The ability to interbreed appears to be relatively conserved among freshwater taxa suggesting that traits that may affect post-zygotic reproductive isolation evolve more slowly and are more or less uncoupled from functionally adaptive traits. The habit of many cyprinid species to spawn in groups over crevices or the nests of other species reflects weak prezygotic barriers in many groups. It may be that physical separation is sufficient to keep most mid-reach and headwater species isolated and there has been little adaptive value to reproductive characters that evolve rapidly. This may explain the prevalence of hybridization where species come into contact. Human activities and habitat alterations may in fact be increasing contact between species that might otherwise remain separated and may contribute to the frequency of hybridization. We have shown that hybridization between different pairs of *Cyprinella* species may have different outcomes even in a narrow geographic area. Evidence for historical hybridization suggests that it must have occurred at some frequency prior to human influence. Further genetic characterization of individuals within current and historical hybrid zones as well as the ecological factors that characterize these contact zones will provide new insights on speciation and perhaps further illuminate the factors that make hybridization so common in freshwater fishes.

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