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CONSERVATION GENETICS OF CYPRINID FISHES IN THE UPPER NUECES RIVER BASIN IN CENTRAL TEXAS

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ABSTRACT—Sequences of the mitochondrial (mt) NADH dehydrogenase subunit 5 gene (ND5) were acquired to assess genetic diversity and female effective population size (N_{ef}) of two forms of *Cyprinella* (*C. lepida* and *C.* sp. cf *lepida*) and two species of *Dionda* (*D. serena* and *D. texensis*) in headwaters of three rivers in the upper Nueces River basin in central Texas. As documented in prior studies, two divergent clades of haplotypes of mtDNA were found in both genera: one in the Frio and Sabinal rivers, representing *C. lepida* and *D. serena*; one in the Nueces River, representing *C.* sp. cf *lepida* and *D. texensis*. Levels of variation in mtDNA from *C. lepida* in the Sabinal River and *D. serena* in the Frio and Sabinal rivers were comparable to or considerably lower than values documented for populations of several threatened or endangered cyprinids. Estimates of N_{ef} for *C. lepida* in the Frio River and *C.* sp. cf *lepida* in the Nueces River were low, suggesting that adaptive genetic variation through time may be compromised. Of all populations sampled, only *D. texensis* in the Nueces River appears at present to be genetically stable demographically. An unexpected finding was two individuals resembling *C. lepida* in the Frio River with a haplotype referable to *C.* sp. cf *lepida*; the origin of these individuals is unknown. Two other individuals resembling *C. lepida* but with haplotypes of mtDNA referable to *C. venusta* were found in the Frio River and presumably represent relatively recent hybrids. Results of our study indicate that *C. lepida*, C. sp. cf *lepida*, and *D. serena* in the upper Nueces River basin, especially in the Sabinal River drainage, are at appreciable genetic risk.

RESUMEN—Secuencias de la subunidad 5 del gen mitocondrial NADH deshidrogenasa (ND5) se tomaron para evaluar la diversidad genética y el tamaño efectivo de la población femenina de dos formas de *Cyprinella* (*C. lepida* y *C.* sp. cf *lepida*) y dos especies de *Dionda* (*D. serena* y *D. texensis*) en las cabeceras de tres ríos en la alta cuenta del río Nueces en el centro de Texas. Como en previos estudios, se observaron dos clades divergentes en los haplotypos mitocondriales en cada género: uno en los ríos Frio y Sabinal, representando *C. lepida* y *D. serena*, y otro en el río Nueces, representando *C.* sp. cf *lepida* y *D. texensis*. Niveles de variación en las secuencias mitocondriales de *C. lepida* en el río Sabinal y de *D. serena* en los ríos Frio y Sabinal fueron comparables o menores a los documentados de poblaciones de varias otras especies de cyprinidos en peligro de extinción. Estimaciones del tamaño efectivo de la población femenina (N_{ef}) para *C. lepida* en el río Frio y *C.* sp. cf. *lepida* en el río Nueces fueron pequeñas, lo cual sugiere que la variación genética adaptativa puede ser comprometida en el futuro. De todas las poblaciones muestreadas, sólo la población de *D. texensis* en el río Nueces parece demostrar una demografía actual estable a nivel genético. Un resultado inesperado fue encontrar dos individuos que se parecían a *C. lepida* en el río Frío con haplotypos referibles a *C.* sp. cf *lepida*; el origen de estos individuos es desconocido. Otros dos individuos que se parecían a *C. lepida* pero con haplotypos referibles a *C. venusta* también se encontraron en el río Frio y probablemente representan híbridos relativamente recientes. Los resultados de nuestro estudio indican que las poblaciones de *C. lepida*, *C.* sp cf *lepida*, y *D. serena* en la alta cuenca del río Nueces, especialmente en el desagüe del río Sabina, están comprometidas genéticamente.

The upper Nueces River basin in central Texas is an area of high priority for conservation because it hosts a high number of endemic plants and animals (The Nature Conservancy, http://www.nature.org/wherewework/northamerica/states/texas/files/edwardsplateauexecsum.pdf; Texas Wildlife Action Plan, http://www.tpwd.state.tx.us/publications/pwdpubs/pwd_pl_w7000_1187a/). The area is dominated by the Nueces, Frio, and Sabinal rivers, with the upper portions of the basin separated from middle and lower segments by the Balcones Escarpment, a geologic fault zone several kilometers wide that separates the Edwards Plateau from the Gulf Coastal Plain (P. L. Abbott and C. M. Woodruff, in litt., http://www.lib.utexas.edu/geo/balcomes_escarpment/balconesescarpment.html). These headwater systems are ecologically distinct from reaches below the escarpment, including the confluence of the three rivers. Endemic and apparently imperiled headwater species in the genera *Cyprinella* and *Dionda* are among the species limited by this ecological barrier.

Studies of endemic aquatic vertebrates in the region primarily have involved species in the cyprinid genera *Cyprinella* and *Dionda*. Matthews (1987) described the plateau shiner, *Cyprinella lepida*, based primarily on specimens from the Nueces River. Subsequent studies (Richardson and Gold, 1995; Broughton and Gold, 2000) found that clades of mitochondrial (mt) DNA haplotypes of *C. lepida* in the upper basin were not monophyletic; one clade occurred in the Frio and Sabinal rivers, while a second, distantly related clade occurred in the Nueces River. Schönhuth and Mayden (2010) showed that the mtDNA clade in the Frio River was related to mtDNA of *Cyprinella formosa* and lineages of *Cyprinella lutrensis* from the Mississippi and upper Rio Grande river drainages, while the mtDNA clade in the Nueces River was related to mtDNA in lineages of *C. lutrensis* (now *Cyprinella suavis*) from the Gulf Slope. Phylogenetic analysis of sequences of the nuclear genes Rag1 (Schönhuth and Mayden, 2010) and Hoxc6a (Broughton et al., 2011) in a few individuals from the Nueces and Frio rivers, however, indicated monophyly of *C. lepida* from the two rivers, with that clade having affinities to *C. formosa* and lineages of *C. lutrensis*. In part because of nomenclatorial issues (Hubbs, 1954), *C. lepida* currently is used to refer to fish resembling *C. lepida* in the Frio and Sabinal rivers, whereas *C.* sp. cf *lepida* is used to refer to fish resembling *C. lepida* in the Nueces River (http://www.bio.txstate.edu/~tbonner/txfishes/cyprinella%20lepida.htm).

The systematics of *Dionda* in the upper Nueces River basin is less complex. Mayden (1992), based on allozymes, resurrected the name *Dionda serena* for specimens of *Dionda* from the Nueces and Frio rivers, and Schönhuth et al. (2012), based on mitochondrial and nuclear DNA sequences, resurrected the name *Dionda texensis* for *Dionda* in the Nueces River. Monophyly of *D. serena* and *D. texensis* is supported by sequences of mitochondrial and nuclear genes (Schönhuth et al., 2008; Schönhuth et al., 2012).

Threats to endemic fauna in the upper Nueces basin include many of the usual suspects: development; erosion; human disturbance; fragmentation (Texas Wildlife Action Plan). Many existing headwater and spring-associated communities in the region have been damaged by persistent drought and groundwater withdrawal (Garrett and Edwards, 2001), and the current, exceptional drought, which is the most severe drought in the recorded history of Texas (http://www.window.state.tx.us/specialrpt/drought/pdf/96-1704-Drought.pdf), has led to an even greater risk of deterioration of habitat and water-quality. One consequence of such impacts is the present decline in both *Cyprinella* and *Dionda* in the upper basin, especially in the Sabinal River (G. P. Garrett and R. J. Edwards, pers. observ.).

In our study, DNA sequences of the mitochondrial protein-coding NADH dehydrogenase subunit 5 gene (ND5) were acquired to assess the genetic diversity and female effective population size (N_{ef}) of populations of *C. lepida*, *C.* sp. cf *lepida*, and *Dionda* in headwaters of the three rivers in the upper Nueces basin. Effective population size (N_e) is the number of breeding individuals in an idealized population that experiences the same rate of genetic drift or inbreeding as the population under consideration (Wright, 1931); because mtDNA is maternally inherited, N_{ef} represents the female component of N_e. Consideration of effective size is of importance in conservation because low estimates of N_e can reflect fixation of deleterious alleles, loss of adaptive genetic variance, and the capacity to respond to natural selection or to environmental pressures such as degradation of habitat (Franklin, 1980; Frankham, 1995; Anderson, 2005). We chose to examine mtDNA, in part, because the genetic effective size of this locus in theory is four times less than that of nuclear DNA (Birky et al., 1989), meaning that population bottlenecks leading to reduced genetic variation and (female) effective size can be more easily detected than with nuclear-encoded DNA, in part because the mtDNA clades in the three rivers were thought to be fixed (Broughton et al., 2011) and, in part, because limited funding precluded more expensive microsatellite development and genotyping.

Materials and Methods—Specimens from the Frio, Sabinal, and Nueces rivers (Fig. 1) were collected by seine and preserved whole in 95% ethanol. Collections of *Cyprinella* were made at single localities in the Frio (26 specimens at 29°50′14.48″N, 99°46′40.66″W), Nueces, (23 specimens at 29°48′42.24″N, 100°0′56.45″W), and Sabinal (20 specimens at 29°31′0.59″N, 99°30′31.37″W) rivers. Collections of *D. serena* in the Frio River were made at two localities ca. 25 river-km apart (four specimens at 29°50′14.48″N, 99°46′40.66″W and 17 specimens at 29°37′49.08″N, 99°44′41.50″W); collections from the Nueces (24 specimens at 29°48′42.24″N, 100°0′56.45″W) and Sabinal (20 specimens at ca. 29°48′27.72″N, 99°34′14.26″W) rivers were

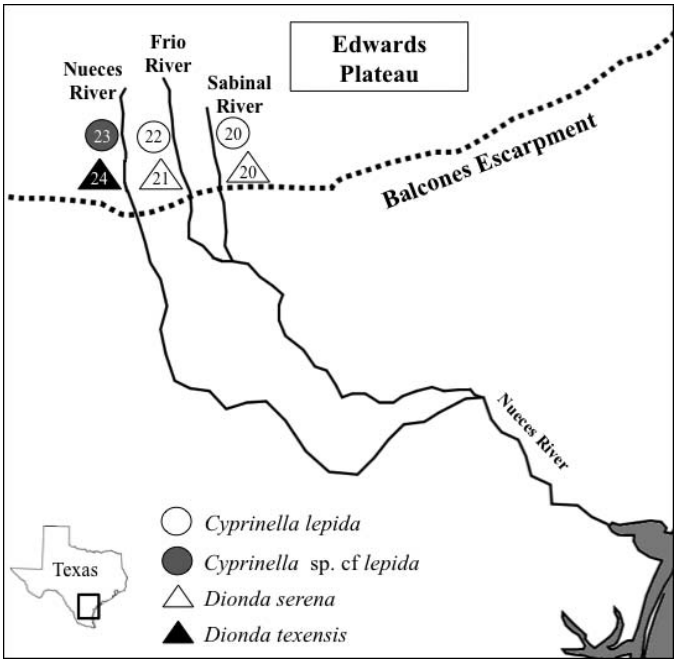

Fig. 1—Map of the upper Nueces River basin, including headwaters of the Frio, Nueces, and Sabinal rivers. Circles denote collections of *Cyprinella lepida* (open) and *C.* sp. cf *lepida* (solid); triangles identify collections of *Dionda serena* (open) and *D. texensis* (solid). Numbers within shapes represent sample sizes within rivers obtained for each species.

made at single locations. Substantial effort was made to collect fish at various locations in each headwater area, but low abundance of *Cyprinella* and *Dionda* restricted geographic coverage in each system. In fact, sampling at each locality required multiple hauls of seines at each primary site just to obtain at least 15–20 individuals at most sites. Representative specimens were deposited in the Biodiversity Research and Teaching Collections at Texas A&M University, College Station (voucher numbers given in Appendix). Samples of *D. serena* from the Sabinal River were procured nondestructively (finclips) due to concerns over the small census-size of this population.

Genomic DNA was extracted using the phenol-chloroform protocol of Sambrook et al. (1989). A 597 base-pair (bp) fragment of the mitochondrial protein-coding NADH dehydrogenase subunit-5 gene (ND-5) was amplified from each fish, using polymerase chain reaction (PCR) amplification. Primers L12328 (5′-AACTCTTGGTGCAAMTCCAAG-3′) and H13393 (5′-CCTATTTTKCGGATGTCTTGYTC-3′), developed by Miya et al. (2006), were used to amplify fragments of ND-5 from *Cyprinella*; primers L12328 (Miya et al., 2006) and DS-H (5′-AAAAATTTGTTGAATTTCTCAGGA-3′, developed in our laboratory) were used for *Dionda*. The terminal 12 bp at the 3′-end of the fragment were difficult to score consistently; therefore, sequences were trimmed to yield 585-bp fragments that could be scored reliably. Conditions of amplification were 95°C for 3 min, 35 cycles of 95°C for 45 s, 50°C for 30 s, 72°C for 1 min, with a final 10-min extension at 72°C. Products of amplification were cleaned with ExoSap-It (US Biological, Swampscott, Massachusetts) and electrophoresed on 2% agarose gels; target fragments were then obtained via band-cutting and cleaned using a QIAquick Gel Extraction kit (Qiagen, Valencia, California). Sequencing reactions were conducted with the L12328 (forward) primer and Big Dye terminators (Applied Biosystems, Foster City, California); an ABI 3100 (Applied Biosystems, Foster City, California) was used for DNA-sequencing. SEQUENCHER 4.1 (Gene Codes, Ann Arbor, Michigan) was used to align sequences; protein-coding was verified using MEGA4 (Tamura et al., 2007).

Phylogenetic hypotheses from sequences of ND-5 were generated using neighbor-joining and maximum-parsimony methods, as implemented in Mega4. The Jukes-Cantor model of nucleotide substitution was used for neighbor-joining; the heuristic search option, with 10 random-addition replicates, was used for maximum parsimony. Robustness of inferred relationships was assessed via 1,000 bootstrap pseudo-replicates. Out-

Table 1—Spatial distribution of mtDNA haplotypes among *Cyprinella* and *Dionda* sampled from the Sabinal, Frio, and Nueces rivers in central Texas. Haplotype 5 recovered in *Cyprinella* from the Frio River has phylogenetic affinity to the mtDNA clade of *C.* sp. cf *lepida* from the Nueces River; haplotypes 11 and 12 are of *C. venusta* origin.

	River			
Haplotype	Sabinal	Frio	Nueces	GenBank
Cyprinella				
1	20	9	-	HQ338512
2	-	7	-	HQ338513
3	-	5	-	HQ338514
4	-	1	-	HQ338515
5	-	2	8	HQ338516
6	-	-	10	HQ338517
7	-	-	1	HQ338518
8	-	-	2	HQ338519
9	-	-	1	HQ338520
10	-	-	1	HQ338521
11	-	1	-	HQ338522
12	-	1	-	HQ338523
Dionda				
1	-	-	7	GU252323
2	-	-	3	GU252324
3	-	-	2	GU252325
4	-	-	2	GU252326
5	-	-	1	GU252327
6	-	-	1	GU252328
7	-	-	1	GU252329
8	-	-	1	GU252330
9	-	-	1	GU252331
10	-	-	1	GU252332
11	-	-	1	GU252333
12	-	-	1	GU252334
13	-	-	1	GU252335
14	-	-	1	GU252336
15	20	17	-	GU252337
16	-	1	-	GU252338
17	-	1	-	GU252339
18	-	1	-	GU252340
19	-	1	-	GU252341

group taxa (and their GenBank Accession Numbers) are given in Appendix.

Number and diversity of haplotypes and diversity of nucleotides at each sampled locality were generated using DNASP version 5.10.01 (Rozas et al., 2003); haplotype richness was estimated using FSTAT version 2.9.3.2 (Goudet, 1995). Pairwise genetic distances between haplotypes were calculated in MEGA4, using the Jukes-Cantor model of nucleotide substitution. Homogeneity of number and diversity of haplotypes was tested through the bootstrap method of Dowling et al. (1996), with resampling conducted in PopTools (http://www.poptools.org/). Homogeneity in distribution of mtDNA haplotypes was tested using exact tests and analysis of molecular variance (AMOVA), as implemented in ARLEQUIN version 3.5 (Excoffier and Lischer, 2010). Pairwise estimates of Φ_{ST}, an analogue of F_{ST}, were generated using ARLEQUIN, with significance determined by exact tests (Raymond and Rousset, 1995; Goudet et al., 1996). Sequential Bonferroni correction (Rice, 1989) was used to adjust for multiple tests executed simultaneously.

Maximum-likelihood estimates of average, long-term N_{ef} were generated using the coalescent-based Markov chain, Monte Carlo approach in LAMARC version 2.1.5 (Kuhner, 2006; Kuhner and Smith, 2007), under the assumption of a mutation rate of 1% per million years and using the formula $N_{ef} = \theta/2\mu$ as appropriate for a haploid, maternally-inherited locus. Coalescent-based estimates of N_e are fairly insensitive to small sample sizes comparable to those obtained for this study (https://biotech.inbre.alaska.edu/fungal_portal/program_docs/lamarc/index.html) but are nonetheless subject to relatively large variances when derived from single loci such as mtDNA. Initial analyses implemented default settings to explore parameters suitable for final sampling strategies of the coalescent-based Markov chain, Monte Carlo approach. Final analyses included three replicates, using the following Markov-chain parameters: an initial analysis of 10 short chains, with 20,000 genealogies sampled, the first 2,000 of which were discarded to ensure parameter stability; a final analysis of three long chains, with 2.5×10^6 genealogies sampled and the first 25,000 trees discarded. Although generation times for *Cyprinella* and *Dionda* are not well established, life-history data on other North American cyprinids (Harrell and Cloutman, 1978; Cloutman and Harrell, 1987) indicate that a generation time between 2 and 3 years is reasonable; therefore, estimates of N_{ef} were based on 2-year and 3-year generation times.

Results—Twelve unique mtDNA haplotypes were found among 69 specimens of *Cyprinella*, whereas 19 haplotypes were found among 65 specimens of *Dionda* (Table 1). Most haplotypes of *Cyprinella* were recovered in two strongly supported clades (Fig. 2a); one clade contained four haplotypes found in the Sabinal and Frio rivers (*C. lepida*), while the other was composed of six haplotypes found in the Nueces River (*C.* sp. cf *lepida*), one of which (haplotype 5) also was found in two individuals from the Frio River. In addition, two haplotypes recovered from the Frio River (haplotypes 11 and 12, Table 1) aligned (100% bootstrap support) with a haplotype of *Cyprinella venusta*. The three distinct haplotypes recovered from the Frio River (haplotype 5,

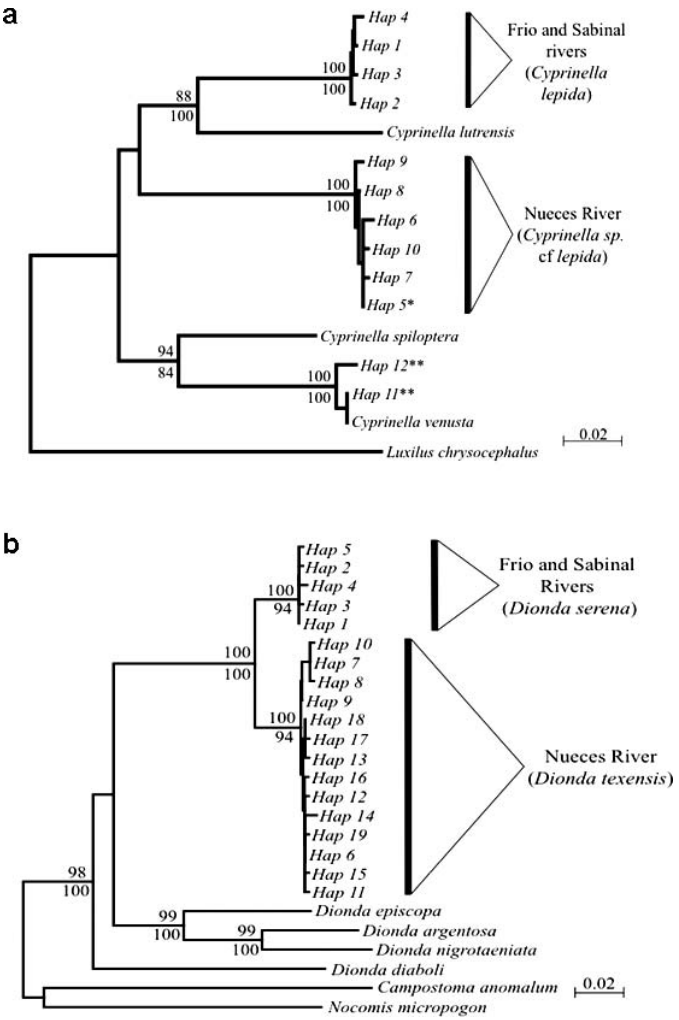

Fig. 2—Neighbor-joining topologies for a) *Cyprinella* and b) *Dionda*. Numbers above and below branches indicate bootstrap support for neighbor-joining and maximum-parsimony trees, respectively. Genetic distance is indicated by the scale in the lower right corner of each tree.

related to haplotypes in the Nueces River, and haplotypes 11 and 12, related to *C. venusta*) were omitted from subsequent analyses.

Sequence-divergence among haplotypes within clades ranged from 0.2–0.5% (*C. lepida*) and 0.2–0.9% (*C.* sp. cf *lepida*); sequence-divergence between haplotypes in the two clades ranged from 14.5–15.3%. The two clades were not sister to one another, because a haplotype of *C. lutrensis* from Kansas, which belongs to a clade of *C. lutrensis* from the Mississippi and upper Rio Grande drainages (Schönhuth and Mayden, 2010), was sister to the clade containing haplotypes from the Frio and Sabinal rivers (*C. lepida*). Haplotypes of *Dionda* also separated into two distinct, strongly supported clades (Fig. 2b); one contained five haplotypes recovered from the Frio and Sabinal rivers (*D. serena*), whereas the other contained 14 haplotypes from the Nueces River (*D. texensis*). Sequence-divergence among haplotypes within

each clade ranged from 0.2–0.5% (*D. serena*) and 0.2–1.2% (*D. texensis*); sequence-divergence between haplotypes in the two clades ranged from 3.9–4.9%. A sister-group relationship between two clades was strongly supported (100% bootstrap).

Summary statistics of mtDNA variation for the two genera in each river system are given in Table 2. *Cyprinella lepida* from the Sabinal River had significantly lower number (1) and diversity (0.0) of haplotypes than expected in comparable, random samples of *C. lepida* from the Frio River (expected $H_N = 3.6$, 95% confidence interval, CI, = 3–4; expected $H_D = 0.86$, 95% CI = 0.69–0.96) and *C.* sp. cf *lepida* from the Nueces River (expected $H_N = 4.6$, 95% CI = 3–6; expected $H_D = 0.77$, 95% CI = 0.61–0.89). Number (4) and diversity (0.71) of haplotypes from *C. lepida* in the Frio River did not differ significantly from that in a comparable, random sample of *C.* sp. cf *lepida* from the Nueces River (expected $H_N = 4.7$, 95% CI = 3–6; expected $H_D = 0.78$, 95% CI = 0.62–0.91). The same approach revealed that *D. serena* from the Sabinal River had a significantly lower number (1) and diversity (0.0) of haplotypes than expected in comparable, random samples of *D. serena* from the Frio River (expected $H_N = 3.5$, 95% CI = 2–5; expected $H_D = 0.39$, 95% CI = 0.12–0.72) and *D. texensis* from the Nueces River (expected $H_N = 9.3$, 95% CI = 7–12; expected $H_D = 0.94$, 95% CI = 0.85–0.99) and that *D. serena* from the Frio River had a significantly lower number (5) and diversity (0.35) of haplotypes than expected in a comparable, random sample of *D. texensis* from the Nueces River (expected $H_N = 9.5$, 95% CI = 7–12; expected $H_D = 0.94$, 95% CI = 0.84–0.99). Although difficult to test statistically, nucleotide diversity (π_D) in *D. serena* from the Frio River ($\pi_D = 0.0008$) was slightly less than one-fifth of that observed for *D. texensis* ($\pi_D = 0.0044$).

Significant heterogeneity in distributions of haplotypes among the three rivers (both lineages) was detected by exact tests ($P < 0.001$, *Cyprinella*; $P = 0.001$, *Dionda*) and by AMOVA ($\Phi_{ST} = 0.983$, $P < 0.001$, *Cyprinella*; $\Phi_{ST} = 0.934$, $P < 0.001$, *Dionda*). Pairwise estimates of Φ_{ST} and probabilities of Φ_{ST} (<0.001) indicated highly divergent distributions of haplotypes (both genera) between fish from either the Frio or Sabinal rivers compared to fish in the Nueces River (Φ_{ST} values of 0.984 and 0.989 in *Cyprinella* and 0.933 and 0.941 in *Dionda*). Values of Φ_{ST} in comparisons between the Frio and Sabinal rivers were significant for *Cyprinella* ($\Phi_{ST} = 0.200$, $P < 0.001$) but nonsignificant for *Dionda* ($\Phi_{ST} = -0.002$, $P > 0.05$).

Estimates of average, long-term N_{ef} for *C. lepida* (Frio River) and *C.* sp. cf *lepida* (Nueces River) ranged from 112.5–75.0 and 298.9–199.3, respectively; whereas estimates for *D. texensis* ranged from 5,725.1–3,816.7. The absence of variation in mtDNA haplotypes of *C. lepida* and *D. serena* from the Sabinal River and the skewed distribution and low diversity of haplotypes of *D. serena* in the Frio River (Table 2) precluded reliable estimation of N_{ef} for these three samples. The 95% confidence intervals for each estimate of N_{ef} were fairly broad, such that the estimates for *C. lepida* (Frio River; 33.6–722.9 and 22.4–481.9) and *C.* sp. cf *lepida* (81.7–1,758.3 and 54.5–1,172.2) did not differ significantly from each other; the estimates of N_{ef} for *C. lepida* (Frio River) and *C.* sp. cf *lepida*, however, were well outside the lower 95% confidence limit of the estimate of N_{ef} for *D. texensis* ($905.8-\infty$ and $603.8-\infty$). The lower 95% confidence limits for *Cyprinella* from the Frio and Nueces rivers were <100 for both estimates of generation time.

Table 2—Summary statistics of variation in mtDNA among samples of *Cyprinella* and *Dionda* from the Sabinal, Frio, and Nueces rivers in central Texas. Abbreviations denote sample size (n), number of haplotypes (H_N), haplotype richness (H_R), haplotype (nucleon) diversity (H_D), and nucleotide diversity (π_D).

	River		
Statistic	Sabinal	Frio	Nueces
Cyprinella			
n	20	22	23
H_N	1	4	6
H_R	1	3.99	5.96
H_D	0	0.710	0.708
π_D	0	0.0017	0.0028
Dionda			
n	20	21	24
H_N	1	5	14
H_R	1	4.99	13.75
H_D	0	0.352	0.906
π_D	0	0.0008	0.0044

Discussion—All studies to date of mtDNA of fishes resembling *C. lepida* in the upper Nueces basin have found two nonmonophyletic mtDNA clades: one in the Frio and Sabinal rivers (*C. lepida*); one in the Nueces River (*C.* sp. cf *lepida*). Haplotypes in the two clades differ in ND5 sequences by 14.5–15.3%. Assuming, for heuristic purposes, an evolutionary rate of ND5 between 0.75 and 1.00% per lineage per million years based on estimates for cytochrome b in cyprinids (Dowling et al., 2002) and the observation (Meyer, 1994) that NADH-dehydrogenase subunit genes evolve faster than other mitochondrial protein-coding genes, the two mtDNA clades likely have been evolving independently for over 7 million years. Phylogenetic analyses of mtDNA and the nuclear genes Rag1 (Schönhuth and Mayden, 2010) and Hoxc6a (Broughton et al., 2011) from several lineages of *Cyprinella* are consistent with the hypothesis that the mtDNA clade in the Frio and Sabinal rivers represents the ancestral mtDNA of *C. lepida*, while that in the Nueces River represents introgression of mtDNA from a lineage resembling *C. lutrensis* inhabiting Gulf Slope drainages,

most likely *Cyprinella suavis* (Schönhuth and Mayden, 2010).

By our count from all published papers, the 42 fish resembling *C. lepida* thus far examined from the Nueces River belong to one mtDNA clade, whereas 72 of 76 fish resembling *C. lepida* examined from the Frio and Sabinal rivers belong to the second mtDNA clade. The four exceptions, found in samples from the Frio River in this study, are two individuals that possessed a haplotype of *C.* sp. cf *lepida* and two individuals with haplotypes referable to *C. venusta*. The latter is not surprising because *C. venusta* occurs in the Frio River and hybridization between *C. venusta* and fish resembling *C. lutrensis* is common and well documented (Hubbs and Strawn, 1956; Broughton et al., 2011). Occurrence of a haplotype of *C.* sp. cf *lepida* in the Frio River is another matter as prior studies (e.g., Broughton et al., 2011) generally have assumed that the two mtDNA clades are fixed in their respective localities and that the putative hybridization between *C. lepida* (or its direct progenitor) and a fish resembling *C. suavis* occurred only in the Nueces River. Alternatively, the haplotype of *C.* sp. cf *lepida* could represent a remnant in the Nueces River from transplants by anglers using cyprinids as bait for sunfish (*Lepomis*), largemouth bass (*Micropterus*), and channel catfish (*Ictalurus punctatus*). Anecdotally, such transplants in the Nueces basin were not uncommon in the past (GPG, pers. observ.), and the two rivers in the upper basin are generally <25 km apart by road. One other possibility is historical capture of headwaters between the two rivers (Hill, 1898). Testing any of these possibilities will be problematic.

Mitochondrial DNA haplotypes in *Dionda* formed two distinct, strongly supported mtDNA clades, one in the Frio and Sabinal rivers (*D. serena*) and one in the Nueces River (*D. texensis*). These results are fully consistent with separation into two species as proposed by Schönhuth et al. (2012).

Number (normalized in relation to sample size) and diversity of haplotypes were significantly reduced in *C. lepida* and *D. serena* from the Sabinal River where only a single haplotype was found in each species. Estimates of both parameters did not differ significantly between *C. lepida* (Frio River) and *C.* sp. cf *lepida*, whereas estimates of both parameters did differ significantly in pairwise comparisons among *Dionda* from all three rivers and following the pattern Nueces > Frio > Sabinal. Number (14 versus 5) and diversity (0.906 versus 0.352) of haplotypes were nearly three times greater for *D. texensis* than for *D. serena* (Frio River). In addition, diversity of nucleotides (the average number of nucleotide differences per site between any two DNA sequences chosen randomly) was more than five times greater in *D. texensis*, indicating that *Dionda* in the Nueces River has been more stable demographically in recent times, is undergoing expansion relative to *Dionda* in the other two rivers, or both. Also, the estimates of diversity of mtDNA haplotypes in *C. lepida* and *D. serena* from the Sabinal River and *D. serena* from the Frio River are comparable to or considerably lower than values documented for populations of several threatened or endangered cyprinids, including *Anaecypris hispanica* (Alves et al., 2001), *Notropis mekistocholas* (Saillant et al., 2004), *Hybognathus amarus* (Alò and Turner, 2005), *Notropis simus pecosensis* (M. Osborne and T. F. Turner, in litt., http://www.wildlife.state.nm.us/conservation/share_with_wildlife/documents/06Osborne.pdf), and several species in the genus *Gila* (T. Dowling, pers. comm.).

The absence of variation in haplotypes of mtDNA in *C. lepida* and *D. serena* in the Sabinal River and the low and asymmetric variation in haplotypes in *D. serena* from the Frio River precluded estimation of average, long-term N_{ef} for these populations. Estimates of N_{ef} for *C. lepida* (Frio River) and *C.* sp. cf *lepida* did not differ from one another based on 95% confidence limits. However, the lower 95% confidence intervals for both populations of *Cyprinella* and at both generation times considered were <100, and, to the extent that lower confidence intervals for estimates of effective size should be considered informative (Waples and Do, 2010), both populations may have suffered high rates of genetic drift in their past and may now lack the genetic diversity to successfully adapt to environmental change. Average, long-term estimates of N_{ef} for *D. texensis* exceeded 3,000, consistent with the demography of a presently stable population, given that long-term estimates of N_e represent an average of N_e over approximately $2N_e$ generations (Hare et al., 2011).

Distributions of haplotypes of mtDNA differed significantly among *Cyprinella* sampled from each of the three rivers, with the degree of difference ($F_{ST} \geq 0.980$) greatest between *Cyprinella* from the Nueces River (*C.* sp. cf *lepida*) versus those from the Sabinal and Frio rivers (*C. lepida*). The estimated F_{ST} for the comparison of *C. lepida* from the Sabinal River versus those from the Frio River was 0.200 and differed significantly from zero; the difference, however, was largely a reflection of haplotype 1 occurring in all 20 fish from the Sabinal River but in only nine of 22 (41%) fish from the Frio River. Minimally, there are two genetically distinct populations of fish resembling *C. lepida* in the upper Nueces River basin, one in the Nueces River (*C.* sp. cf *lepida*) and one in the Sabinal and Frio rivers (*C. lepida*); both should be considered as separate units of management (sensu Moritz, 1994). Homogeneity tests of distributions of haplotypes of mtDNA among the three rivers yielded essentially the same results for *Dionda*. Estimates of F_{ST} between *D. serena* (Frio and Sabinal rivers) and *D. texensis* were ≥0.933, whereas the estimate of F_{ST} for the comparison between *D. serena* in the Sabinal and Frio rivers did not differ from zero.

The foregoing indicates that, except for *Dionda* in the Nueces River (*D. texensis*), *D. serena* and fish resembling *C. lepida* in the upper Nueces River basin, especially in the

Sabinal River, are at considerable genetic risk, as indicated by significantly reduced diversity in mtDNA and low, long-term N_{ef}. Thus, present concern should place priority on continuation of surveys within the basin to identify and evaluate additional populations, should they exist. Also, given the challenges that human-induced perturbations typically present to imperiled species (Caro and Laurenson, 1994), it will be critical to assess how a predicted doubling of the human population in the basin over the next several decades (Texas Wildlife Action Plan) is likely to impact these unique natural resources. Finally, our study contributes to the growing concern for biota living in headwater streams (Meyer et al., 2007) and to the observations that headwater species of fish are particularly vulnerable to extirpation (Warren et al., 2000).

One final point regards whether *C. lepida* and *C.* sp. cf *lepida* warrant designation as distinct species. As noted by Broughton et al. (2011), the central issue is whether introgression (and replacement) of mtDNA is sufficient to merit designation as a species. In our view, regardless of their evolutionary history, *C. lepida* and *C.* sp. cf *lepida* warrant some level of protection because both appear genetically compromised and genetically unique.

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Appendix 1—Material examined.

Specimens vouchered at the Biodiversity Research and Teaching Collections (BRTC), Texas A&M University, College Station. *Cyprinella lepida*, 14249.01–14253.01, 14405.01, 14406.01, 14408.01, and 14412.01–14424.01 (Sabinal River) and 14254.01–14261.01 and 14424.01–14441.01 (Frio River); *Cyprinella* sp. cf. *lepida*, 14262.01–14267.01 and 14442.01–144457.01 (Nueces River); *Dionda serena*, 14268.01–14272.01 and 14461.01–14474.01 (Frio River), and *Dionda texensis* 14273.01–14286.01, 14475.01–14482.01, and 14484.01–14485.01 (Nueces River). Because samples were taken nondestructively (fin-clips) from the Sabinal River drainage (Lost Maples State Park), this collection was not vouchered.

Sequences obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank/). Outgroups to *Cyprinella*: *Cyprinella lutrensis* (NC008643.1); *Cyprinella spiloptera* (NC008103.1); *Cyprinella venusta* (HQ338524); *Luxilus chrysocephalus* (EF452753.1). Outgroups to *Dionda*: *Dionda argentosa* (GU252302); *Dionda diaboli* (GU252318); *Dionda* sp. 4 (GU252320); *Dionda flavipinnis* (GU252321); *Campostoma anomalum* (GU252342); *Nocomis micropogon* (GU252343).