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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 NADH dehydrogenase subunit 5 gene (ND5) were acquired to assess genetic diversity and female effective population size (*Nef*) 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. The region is of high ecological significance and of increasing conservation concern. As documented in prior studies, two divergent clades of mtDNA haplotypes were found in both genera: one in the Frio and Sabinal rivers, representing *C. lepida* and *D. serena*, and one in the Nueces River, representing *C*. sp. cf *lepida* and *D*. *texensis*. Levels of mtDNA variation in *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 *Nef* 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 *C. lepida*-like individuals in the Frio River with a haplotype referable to *C*. sp. cf *lepida*; the origin of these individuals is unknown. Two other *C. lepida*-like individuals but with mtDNA haplotypes referable to *Cyprinella 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, accentuating the growing concern for biota living in headwater streams and the observations that headwater fish species are particularly vulnerable to extirpation.

Resumen—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*) fueron asesados usando secuencias de la subunidad 5 del gen mitocondrial NADH deshidrogenasa (ND5) en las cabeceras de tres rios en la alta cuenta del Rio Nueces en Texas. Esta región es de alta importancia ecológica y de valor conservativo. Como en previos estudios, se observaron dos clades divergentes en los haplotypos mitocondriales en cada genero: 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 son comparables o menores a los documentados para poblaciones de varias otras especies de cyprinidos en peligro de extinción. Estimados del tamaño efectivo de la población femenina (*Nef*) para *C. lepida* en el Río Frio y *C.* sp. cf. *lepida* en el Río Nueces fueron pequeños, lo cual sugiere que la variación genética adaptativa de estas poblaciones esta posiblemente comprometida. De todas las poblaciones muestreadas, solo la población de *D. texensis* en el Río Nueces parece demostrar una demografía estable al nivel genético.  Un resultado inesperado fue encontrar dos individuos similares a *C. lepida* con haplotypos referibles a *C.* sp. cf *lepida* en el Río Frio; el origen de estos individuos es desconocido. Otros dos individuos similares a *C. lepida* pero con haplotypos referibles a *Cyprinella venusta* también fueron detectados 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 Río Sabina, están comprometida genéticamente, lo cual acentúa el creciente interés por la biota de los riachuelos en las cabeceras de ríos y las observaciones que peces en estos hábitats son particularmente vulnerables a la extirpación.

The upper Nueces River basin in Central Texas is an area of high priority for conservation, as it hosts a high number of endemic plants and animals (TNC, 2004; TWAP, 2005). 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 miles wide that separates the Edwards Plateau from the Gulf Coastal Plain (Abbott and Woodruff, 1986). 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 *Rag*1 (Schönhuth and Mayden, 2010) and *Hox*c6a (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 *Cyprinella formosa* and lineages of *C. lutrensis*. In part because of nomenclatorial issues (Hubbs, 1954), *C.* *lepida* currently is used to refer to *C. lepida*-like fish in the Frio and Sabinal rivers, whereas *C*. sp. cf *lepida* is used to refer to *C. lepida*-like fish in the Nueces River ([http://www.bio.txstate.edu/~tbonner/txfishes/cyprinella%20lepida.htm)](http://www.bio.txstate.edu/~tbonner/txfishes/cyprinella%20lepida.htm%29).

The systematics of *Dionda* in the upper Nueces River basin is less complex. Mayden (1992), based on allozyme data, 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 sequence data, resurrected the name *Dionda texensis* for *Dionda* in the Nueces River. Monophyly of *D. serena* and *D. texensis* is supported by sequences of both mitochondrial and nuclear-encoded genes (Schönhuth et al., 2008, 2012).

Threats to endemic fauna in the upper Nueces basin include many of the usual suspects: development, erosion, human disturbance, and fragmentation (TWAP, 2005). Many existing headwater and/or 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 recorded Texas history (<http://www.window.state.tx.us/>[specialrpt/drought/pdf/96-1704-Drought.pdf](http://www.window.state.tx.us/specialrpt/drought/pdf/96-1704-Drought.pdf)), has led to an even greater risk of habitat and water-quality deterioration. One consequence of such impacts is the present decline in both *Cyprinella* and *Dionda* in the upper basin, especially in the Sabinal River (G. Garrett and R. Edwards, unpublished).

 In this 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 (*Nef*, and hereafter effective size) of populations of *C. lepida*, *C*. sp. cf *lepida*, and *Dionda* in headwaters of all three rivers in the upper Nueces basin. Effective population size (*Ne*) 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, *Nef* represents the female component of *Ne*. Consideration of effective size is of importance in conservation because low estimates of *Ne* 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 habitat degradation (Frankham, 1995; Franklin, 1980; Anderson, 2005). We chose to examine mtDNA, in part because the genetic effective size of this locus in theory is four times less than 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 of limited funds precluding more expensive microsatellite development and genotyping. Conservation implications of our findings are discussed.

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 approximately 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 ~ 29°48'27.72" N, 99°34'14.26" W) rivers were made at single locations. Substantial effort was made to collect fish at various locations in each headwater area, but low abundance of both *Cyprinella* and *Dionda* restricted geographic coverage in each system. In fact, sampling at each locality required multiple seine hauls 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 (BRTC) at Texas A&M University. BRTC voucher numbers are given in Material Examined. Samples of *D. serena* from the Sabinal River were procured non-destructively (fin-clips) 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 ND-5 fragments of *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. Amplification conditions were 95°C for 3 min, 35 cycles of 95°C for 45 sec, 50°C for 30 sec, 72°C for 1 min, with a final 10 min extension at 72°C. Amplification products were cleaned with ExoSap-It (US Biological, Swampscott, MA) and electrophoresed on 2% agarose gels; target fragments were then obtained via band cutting and cleaned using a QIAquick Gel Extraction kit (Qiagen, Valencia, CA). Sequencing reactions were conducted with the L12328 (forward) primer and Big Dye terminators (Applied Biosystems, Foster City, CA); an ABI 3100 (Applied Biosystems, Foster City, CA) was used for DNA sequencing. Sequencher 4.1 (Gene Codes, Ann Arbor, MI) was used to align sequences; protein coding was verified using Mega4 (Tamura et al., 2007).

 Phylogenetic hypotheses of ND-5 sequences were generated using neighbor joining (NJ) and maximum parsimony (MP) methods, as implemented in Mega4. The Jukes-Cantor model of nucleotide substitution was used for NJ; the heuristic search option, with 10 random-addition replicates, was used for MP. Robustness of inferred relationships was assessed via 1,000 bootstrap pseudo-replicates. Outgroup taxa (and GenBank Accession Numbers) are given in Material Examined.

 Number of haplotypes, haplotype diversity, and nucleotide diversity at each sample locality were generated using DnaSP v 5.10.01 (Rozas et al., 2003); haplotype richness was estimated using Fstat v 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 haplotype number and haplotype diversity was tested through the bootstrap method of Dowling et al. (1996), with resampling conducted in PopTools ([http://www.poptools.org/](http://www.cse.csiro.au/poptool/index.html)). Homogeneity in mtDNA haplotype distribution was tested using exact tests and analysis of molecular variance (Amova), as implemented in Arlequin v 3.5 (Excoffier and Lischer, 2010). Pairwise estimates of ФST, an analogue of FST, were generated using Arlequin, with significance determined by exact tests (Raymond and Rousset, 1995; Goudet et al., 1996).

 Maximum-likelihood estimates of average, long-term female effective size (*Nef*) were generated using the coalescent-based Markov chain, Monte Carlo (MCMC) approach in Lamarc v 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 *Nef* = θ/2μ as appropriate for a haploid, maternally-inherited locus. Coalescent-based estimates of *Ne* are fairly insensitive to small sample sizes comparable to those obtained for this study (

[https://biotech.inbre.alaska.edu/](https://biotech.inbre.alaska.edu/fungal_portal/program_docs/lamarc/index.html)

[fungal\_portal/program\_docs/lamarc/index.html](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 MCMC sampling strategies. Final runs included three replicates, using the following Markov chain parameters: (i) an initial run of 10 short chains, with 20,000 genealogies sampled, the first 2000 of which were discarded as burn-in to ensure parameter stability; and (ii) a final run of three long chains, with 2.5 x 106 genealogies sampled and the first trees 25,000 trees discarded as burn-in. 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 two and three years is reasonable; therefore, *Nef* estimates were based on two-year and three-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* (Appendix Table 1). Most haplotypes of *Cyprinella* were recovered in two strongly supported clades (Figure 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, Appendix Table 1) aligned (100% bootstrap support) with a haplotype of *Cyprinella venusta*. The three distinct haplotypes recovered from the Frio River (Haplotype 5, 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 to 0.5% (*C*. *lepida*) and 0.2 to 0.9% (*C*. sp. cf *lepida*); sequence divergence between haplotypes in the two clades ranged from 14.5 to 15.3%. The two clades were not sister to one another, as 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 fell into two distinct, strongly supported clades (Figure 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 to 0.5% (*D. serena*) and 0.2 to 1.2% (*D. texensis*); sequence divergence between haplotypes in the two clades ranged from 3.9 to 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 1. *Cyprinella* *lepida* from the Sabinal River had significantly lower haplotype number (1) and diversity (0.0) than expected in comparable, random samples of *C. lepida* from the Frio River (expected HN = 3.6, 95% CI=3-4; expected HD=0.86, 95% CI=0.69-96) and *C*. sp. cf *lepida* from the Nueces River (expected HN = 4.6, 95% CI=3-6; expected HD=0.77, 95% CI=0.61-0.89). Haplotype number (4) and diversity (0.71) of *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 HN = 4.7, 95% CI=3-6; expected HD=0.78, 95% CI=0.62-0.91). The same approach revealed that *D. serena* from the Sabinal River had a significantly lower haplotype number (1) and diversity (0.0) than expected in comparable, random samples of both *D. serena* from the Frio River (expected HN =3.5, 95% CI=2-5; expected HD=0.39, 95% CI=0.12-0.72) and *D*. *texensis* from the Nueces River (expected HN =9.3, 95% CI=7-12; expected HD=0.94, 95% CI=0.85-0.99), and that *D. serena* from the Frio River had significantly lower haplotype number (5) and diversity (0.35) than expected in a comparable, random sample of *D. texensis* from the Nueces River (expected HN =9.5, 95% CI=7-12; expected HD=0.94, 95% CI=0.84-0.99). Although difficult to test statistically, nucleotide diversity (πD) in *D. serena* from the Frio River (πD = 0.0008) was slightly less than one-fifth of that observed for *D. texensis* (πD = 0.00436).

 Significant heterogeneity in haplotype distributions among the three rivers (both lineages) was detected by exact tests (*P* = <0.001, *Cyprinella*; *P* = 0.001, *Dionda*) and by Amova (*ФST*= 0.983**,** *P*<0.001, *Cyprinella*; *ФST*= 0.934**,** *P*<0.001, *Dionda*). Pairwise estimates of *ФST* and probabilities of *ФST* = 0 (Table 2) indicated highly divergent haplotype distributions (both genera) between fish from either the Frio or Sabinal rivers compared to fish in the Nueces River (*ФST* values of 0.984 and 0.989 in *Cyprinella* and 0.933 and 0.941 in *Dionda*). *ФST* values in comparisons between the Frio and Sabinal rivers were significant for *Cyprinella* (*ФST* =0.200, P<0.001) but non-significant for *Dionda* (*ФST* =0.999, P > 0.05).

 Estimates of average, long-term female effective size (*Nef*) for *C. lepida* (Frio River) and *C.* sp. cf *lepida* (Nueces River) ranged from 112.5 to 75.0 and 298.9 to 199.3, respectively; whereas estimates for *D. texensis* ranged from 5,725.1 to 3,816.7 (Table 3). The absence of mtDNA haplotype variation in both *C. lepida* and *D. serena* from the Sabinal River and the skewed distribution and low haplotype diversity of *D. serena* in the Frio River (Table 1) precluded reliable estimation of *Nef* for these three samples. The 95% confidence intervals for each *Nef* estimate were fairly broad (Table 3), such that the estimates for *C. lepida* (Frio River) and *C*. sp. cf *lepida* did not differ significantly from each other; the estimates of *Nef*for *C. lepida* (Frio River) and *C*. sp. cf *lepida*, however, fell well outside the lower 95% confidence limit of the *Nef* estimate for *D. texensis*. The lower 95% confidence limits for *Cyprinella* from the Frio and Nueces rivers were less than 100 for both estimates of generation time.

Discussion—All studies to date of mtDNA of *C. lepida*-like fishes in the upper Nueces basin have found two non-monophyletic mtDNA clades: one in the Frio and Sabinal rivers (*C. lepida*) and one in the Nueces River (*C*. sp. cf *lepida*). Haplotypes in the two clades differ in ND5 sequences by 14.5 to 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 seven million years. Phylogenetic analyses of mtDNA and the nuclear genes *Rag*1 (Schönhuth and Mayden, 2010) and *Hox*c6a (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 *C. lutrensis*-like lineage inhabiting Gulf Slope drainages, most likely *C.* *suavis* (Schönhuth and Mayden, 2010).

 By our count from all published papers, the 42 *C. lepida*-like fish thus far examined from the Nueces River belong to one mtDNA clade, whereas 72 of 76 *C. lepida*-like fish 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 *C.* sp. cf *lepida* haplotype and two individuals with haplotypes referable to *C. venusta*. The latter is not surprising as *C. venusta* occurs in the Frio River and hybridization between *C. lutrensis*-like fish and *C. venusta* 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 *C*. *suavis*-like fish occurred only in the Nueces River. Alternatively, the *C*. sp. cf *lepida* haplotype could represent a remnant from 'bait-bucket’ transplants from the Nueces River by anglers using cyprinids as bait for sunfish, largemouth bass, and channel catfish. Anecdotally, such ‘bait-bucket’ transplants in the Nueces basin were not uncommon in the past (G. Garrett, personal observation) and the two rivers in the upper basin are generally less than 25 km apart by road. One other possibility is historical headwater stream capture between the two rivers as the region has been a ‘…scene of a continuous warfare between the courses of the minor head-stream water drainage, whereby…streams have been deflected from one course into another’ (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 and et al. (2012).

*Mitochondrial Variation and Female Effective Size*—Number of haplotypes (normalized in relation to sample size) and haplotype diversity were significantly reduced in both *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. Both number of haplotypes (14 vs. 5) and haplotype diversity (0.906 vs. 0.352) were nearly three times greater in *D. texensis* as compared to *D. serena* (Frio River). In addition, nucleotide diversity (the average number of [nucleotide](http://en.wikipedia.org/wiki/Nucleotide) differences per site between any two [DNA](http://en.wikipedia.org/wiki/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 and/or is undergoing expansion relative to *Dionda* in the other two rivers. Finally, the estimates of mtDNA haplotype diversity 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* (Osborne and Turner, 2006), and several species in the genus *Gila* (T. Dowling, personal communication).

 The absence of mtDNA haplotype variation in *C. lepida* and *D. serena* in the Sabinal River and the low and asymmetric haplotype variation in *D. serena* from the Frio River precluded estimation of average, long-term *Nef* for these populations. Estimates of *Nef* 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 less than 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 *Nef* for *D. texensis* exceeded 3,000, consistent with a presently stable population demography, given that long-term estimates of *Ne* represent an average of *Ne* over approximately 2*Ne* generations (Hare et al., 2011).

*Mitochondrial Diversity*— MtDNA haplotype distributions differed significantly among *Cyprinella* sampled from each of the three rivers, with the degree of difference (FST ≥ 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 FST 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 *C. lepida*-like fish 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 management units (*sensu* Moritz, 1994). Homogeneity tests of mtDNA haplotype distributions among the three rivers yielded essentially the same results for *Dionda*. FST estimates between *D. serena* (Frio and Sabinal rivers) versus *D. texensis* were ≥ 0.933, whereas the FST estimate 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*), *C. lepida*-like fish and *D. serena* in the upper Nueces river basin, especially in the Sabinal River, are at considerable genetic risk, as indicated by significantly reduced mtDNA diversity and low, long-term female effective size. 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 (TWAP, 2005) 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 fish species are particularly vulnerable to extirpation (Warren et al., 2000).

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

Material Examined—Specimens vouchered at the Biodiversity Research and Teaching Collections (BRTC): *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 14273.01-14286.01, 14475.01-14482.01, and 14484.01-14485.01 (Nueces River); because samples were taken non-destructively (fin-clips) from the Sabinal River drainage (Lost Maples State Park) this collection was not vouchered. Sequences obtained from GenBank: Outgroups to *Cyprinella* – *Cyprinella lutrensis* (NC008643.1), *Cyprinella spiloptera* (NC008103.1), *Cyprinella venusta* (HQ338524), and *Luxilus chrysocephalus* (EF452753.1). Outgroups to *Dionda* – *Dionda* *argentosa* (GU252302), *Dionda* *diaboli* (GU252318), *Dionda* sp.4 (GU252320), *Dionda* *flavipinnis* (GU252321), *Campostoma anomalum* (GU252342), and *Nocomis micropogon* (GU252343).

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Table 1—Summary statistics of mtDNA variation among samples of *Cyprinella* and *Dionda* from the Sabinal, Frio, and Nueces rivers. Abbreviations denote sample size (*n*), number of haplotypes (HN), haplotype richness (HR), haplotype (nucleon) diversity (HD), and nucleotide diversity (πD).

|  |  |  |  |
| --- | --- | --- | --- |
| *Cyprinella* | Sabinal | Frio | Nueces |
| *n* | 20 | 22 | 23 |
| HN | 1 | 4 | 6 |
| HR | 1 | 3.99 | 5.96 |
| HD | 0 | 0.710 | 0.708 |
| πD | 0 | 0.0017 | 0.0028 |
|   |  |  |  |
| *Dionda* | Sabinal | Frio | Nueces |
| *n* | 20 | 21 | 24 |
| HN | 1 | 5 | 14 |
| HR | 1 | 4.99 | 13.75 |
| HD | 0 | 0.352 | 0.906 |
| πD | 0 | 0.0008 | 0.0044 |

Table 2—Pairwise fixation indices (*ФST*, above diagonal) for *Cyprinella* and *Dionda* sampled from the Sabinal, Frio, and Nueces rivers. Probability (*P*) values (below diagonal) based on pairwise exact tests of homogeneity in mtDNA haplotype distribution.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| *Cyprinella* |  | River | Sabinal | Frio | Nueces |
|  |  | Sabinal | -- | 0.200 | 0.989 |
|  |  | Frio | <0.001\* | -- | 0.984 |
|  |  | Nueces | <0.001\* | <0.001\* | -- |
|  |  |  |  |  |  |
|  |  |  |  |  |  |
| *Dionda* |  | River | Sabinal | Frio | Nueces |
|  |  | Sabinal | -- | -0.002 | 0.941 |
|  |  | Frio | 0.999 | -- | 0.933 |
|  |  | Nueces | <0.001\* | <0.001\* | -- |

\* Significant following Bonferroni correction (Rice 1989)

Table 3—Estimates of average, long-term female effective population size (*Nef*) and their 95% confidence intervals (in parentheses) for *Cyprinella* in the Nueces and Frio rivers and *Dionda* in the Nueces River, based on two- and three-year generation times.

|  |  |
| --- | --- |
|  | Generation time |
| Taxon/Locality | Two years | Three years |
| *Cyprinella*/Frio River | 112.5 (33.6-722.9) | 75.0 (22.4-481.9) |
| *Cyprinella*/Nueces River | 298.9 (81.7-1758.3) | 199.3 (54.5-1172.2) |
| *Dionda*/Nueces River | 5,725.1 (905.8-∞) | 3,816.7 (603.8-∞) |

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



Figure 2. Neighbor-joining topologies for *Cyprinella* (a) and *Dionda* (b). 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.

a)

a)

b)



Appendix Table 1—Spatial distribution of mtDNA haplotypes among *Cyprinella* and *Dionda* sampled from the Sabinal, Frio, and Nueces rivers. †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.

|  |  |  |
| --- | --- | --- |
|  | *Cyprinella* |  |
| Haplotype | Sabinal | Frio | Nueces | GenBank |
| 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* |  |
| Haplotype | Sabinal | Frio | Nueces |  |
| 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 |