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CONSERVATION GENETICS OF SIX SPECIES OF GENUS *DIONDA* (CYPRINIDAE) IN THE SOUTHWESTERN UNITED STATES

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ABSTRACT.—We examined allelic variation at nuclear-encoded microsatellites and sequences of mitochondrial (mt)DNA in 10 geographic samples representing 6 nominal species of the cyprinid genus *Dionda.* Species of *Dionda* are found in springs and spring-fed headwaters in the southwestern United States and Mexico and are of particular interest to conservation and management, in part because of their limited distribution and habitat specificity, and in part as indicator species of habitat quality. All 10 samples examined appear to be discrete, demographically independent populations, with greater observed F_{ST} values between or among samples within species (0.123–0.280) than threshold values above which demographic independence is indicated. All 10 exhibited microsatellite and mtDNA variation comparable to or lower than that found in other cyprinids considered to be threatened or endangered; across microsatellites, average number of alleles across populations ranged from of 2.09 to 9.76, allelic richness from 2.24 to 8.45, and gene diversity from 0.0211 to 0.606; for mtDNA, the number of haplotypes across populations ranged from 1 to 14. Estimates of historical and present-day genetic demography indicated that all 10 populations have experienced order-of-magnitude declines in effective population size, with lower bounds of time intervals for the declines in 9 of the populations ranging from 6 to 65 years. Estimates of average long-term effective population size (536 in *Dionda argentosa* from San Felipe Creek to 2335 in *D. texensis*) and effective number of breeders (22 in *D. flavipinnis* from Fessenden Spring to 555 in *D. diaboli* from Devils River) also indicated recent declines in effective size, and at least 5 of the populations appear to have undergone recent, severe bottlenecks (mean M_c range 0.806–0.848, *P* value range 0.000–0.0350). The observation that all 10 populations are demographically independent indicates that local extirpations likely would not be replaced by new migrants and that loss of any of the populations would represent loss of a unique genetic entity. Conservation recommendations for each of the populations are briefly discussed.

RESUMEN.—Examinamos la variación alélica para microsatélites nucleares y secuencias mitocondriales en diez muestras geográficas representantes de seis especies nominales del género ciprínido *Dionda*. La distribución de este género incluye manantiales y sus cabeceras en México y el suroeste de los Estados Unidos, y dada su distribución limitada y preferencia de hábitat, las especies de este género son de particular interés para la conservación ya que pueden ser usadas como indicadores de calidad de hábitat. Las diez muestras estudiadas parecen corresponder a poblaciones discretas y demográficamente independientes; observamos niveles de F_{ST} entre las muestras de las especies (0.123–0.280) superiores a los valores del umbral indicado por la independencia demográfica. Las 10 muestras indicaron variación microsatelital y de ADNmt comparable o inferior a las previamente reportadas para otras especies de ciprínidos amenazados o en peligro; a través de los microsatélites, el número promedio de alelos entre poblaciones osciló entre 2.09–9.76, 2.24–8.45 en riqueza alélica, yo 0.0211–0.606 en diversidad genética; para el ADNmt el número de haplotipos entre las poblaciones variaron entre 1–14. Estimaciones en la demografía genética histórica y contemporánea indican que todas las poblaciones han sufrido declives en orden de magnitud en los tamaños efectivos de población, con límites de intervalo de tiempo más bajos para las pérdidas de nueve de las poblaciones, variando entre seis y 65 años. Estimaciones del tamaño efectivo promedio a largo plazo (536 en *Dionda argentosa* del San Felipe Creek a 2335 en *D. texensis*) y el número efectivo de individuos reproductivos (22 en *D. flavipinnis* de Fessenden Spring a 555 en *D. diaboli* de Devils River) también indican recientes declives en los tamaños efectivos de las poblaciones, y en al menos cinco de las poblaciones parecen haber sufrido severos cuellos de botella recientemente (rango medio M_c de 0.806–0.848, valores *P* de 0.000–0.0350). La observación de que las diez poblaciones son independientes demográficamente sugiere que extirpaciones locales probablemente no serían reemplazadas por nuevos migrantes, por lo cual representaría la pérdida de una entidad genética única. Discutimos recomendaciones para la conservación de cada población brevemente.

Many species of freshwater fish, especially those restricted to spring-dependent waterways in arid or semiarid regions, face continuing challenges of habitat loss and degradation (Garrett and Edwards 2001, Edwards et al. 2004, Jelks et al. 2008). Studies of habitat preference, for example, indicate that at least 70% of spring-dwelling fishes may be seriously jeopardized, with contributing factors including non-point-source pollution (e.g.,

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siltation), alteration of water flow by impoundments, development, invasive species, and the small native range or high endemism of the species themselves (Etnier 1997, Rahel 2002, López-Fernández and Winemiller 2005). Several fish species in these habitats in the southwestern United States are now either endangered or threatened (USFWS 2012), including, in Texas, 2 that are threatened (*Dionda diaboli* and *Notropis girardi*) and 8 that are endangered (*Cyprinodon bovinus, Cyprinodon elegans, Etheostoma fonticola, Gambusia gaigei, Gambusia georgei, Gambusia heterochir, Gambusia nobilis,* and *Hybognathus amarus*). Compounded upon anthropogenic threats are effects of drought, which reduce or eliminate spring flows and consequently degrade associated downstream tributary and river habitats. While the aquifer-fed nature of many springs often allows for continued flow at times of reduced rainfall, even large springs are at risk, especially when drought is combined with human water use (Brune 2002, Cook et al. 2004). Of particular concern is the effect of recent, severe drought (Neilson-Gammon 2011, Combs 2012) on fish and wildlife in Texas and other parts of the southwestern United States.

In order to preserve and manage biodiversity and genetic resources represented by aquatic species living in these habitats, conservation planning requires information on the genetic status of individual species (Meffe 1990, Frankham 1995, Vrijenhoek 1998); parameters of interest include genetic variation, effective population size, population growth or decline, and genetic divergence between or among populations. Reduced genetic variation stemming from reduced numbers of individuals poses a significant threat, especially in small, isolated populations (Soulé 1980, Lynch et al. 1995, Frankham 1996), and often is a consequence of environmental deterioration (Caro and Laurenson 1994).

At the request of the Texas Parks and Wildlife Department (TPWD) and the U.S. Fish and Wildlife Service (USFWS), we evaluated the conservation-genetic status of 10 populations representing 6 species of roundnose minnows (Cyprinidae: genus *Dionda*) from spring-fed headwaters in Texas and New Mexico. Species of *Dionda* are found in the southwestern United States and Mexico; 7 nominal and 2 undescribed species occur in central and west Texas; 1 nominal and 1 undescribed species occur in New Mexico; and 3 nominal and 2 undescribed species occur in Mexico (Schönhuth et al. 2012, Hanna et al. 2013). Roundnose minnows typically inhabit springs and spring-fed streams (Hubbs and Brown 1956, Hubbs et al. 1991, Edwards et al. 2004) and are of particular interest to conservation and management by TPWD and USFWS, in part because of their limited distribution in spring-fed headwaters, and in part as indicator species of habitat quality (Harvey 2005, Edwards et al. 2004). The species examined in this study were *Dionda argentosa, D. diaboli, D.* sp. 4 (until recently, *D. episcopa*), *D. flavipinnis* (until recently, *D. nigrotaeniata*), *D. serena,* and *D. texensis* (until recently, *D. serena* from the Nueces River). The recent taxonomic revisions may be found in Schönhuth et al. (2012). *Dionda diaboli* is considered threatened by both the United States and the state of Texas (USFWS 1999) and endangered by the Endangered Species Committee of the American Fisheries Society (Jelks et al. 2008). The federal recovery action plan (USFWS 2005) for *D. diaboli* includes evaluation of geographic variation and population genetic structure. Scharpf (2005) listed both *D. argentosa* and *D. serena* (the latter was split into *D. serena* and *D. texensis* by Schönhuth et al. 2012) as imperiled, whereas *D. episcopa* and *D. flavipinnis* (listed then as *D. nigrotaeniata*) were listed as secure. With support from TPWD and USFWS, we acquired sequences of mitochondrial (mt)DNA and genotypes at nuclear-encoded microsatellites to evaluate the conservation-genetic status of these species.

METHODS

Samples of adult *Dionda* were obtained by seine (4 × 6 ft., 0.25-in^2 mesh) in 2008 from 10 localities (Fig. 1, Table 1). Collections of all species were made, when possible, at multiple sites at each locality. Whole specimens or caudal fin clips were preserved in 95% ethanol. Voucher specimens were donated to the Biodiversity Research and Teaching Collections (BRTC) at Texas A&M University. Sampling in Texas was conducted according to collection protocols of the Texas Parks and Wildlife Department (details available from GPG). Tissue samples (muscle) of *Dionda* sp. 4 (*D.* sp. 4) from the upper Pecos River in New Mexico were provided by the Museum of Southwestern

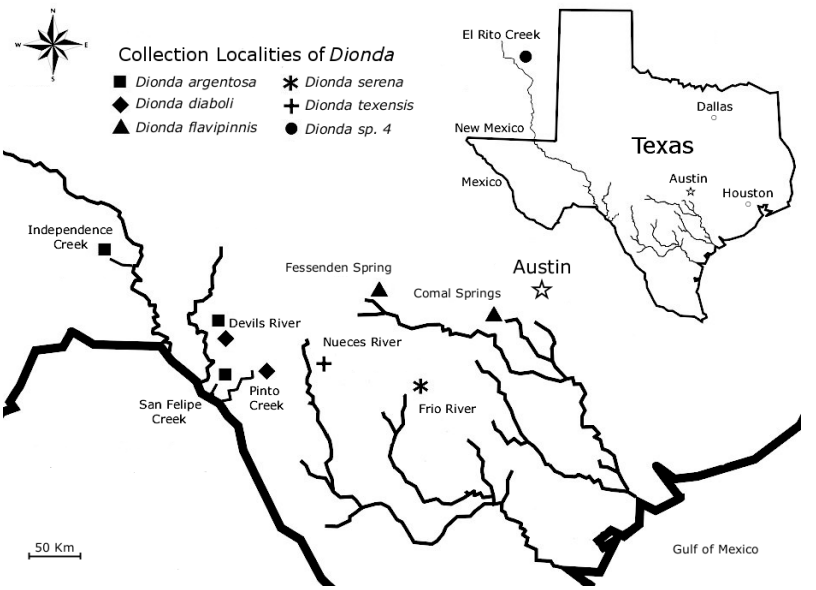

Fig. 1. Collection localities of *Dionda* examined in this study.

TABLE 1. Species, sample localities, and sample sizes of *Dionda* examined in the study. For number of individuals, the number before the slash is the total number collected and analyzed for microsatellite variation in microsatellite genotypes; whereas the number after the slash is the subsample of individuals sequenced for variation in mtDNA sequence.

Species	Sample location	Drainage	Number of individuals	Date sampled	Coordinates
Dionda argentosa	Devils River (TX)	Rio Grande	63/26	13 Mar 2008	29°53′ N, 100°59′ W
	San Felipe Creek (TX)	Rio Grande	33/20	25 Apr 2008	29°21′ N, 100°53′ W
	Independence Creek (TX)	Pecos	34/26	31 Aug 2008	30°28′ N, 101°48′ W
Dionda diaboli	Devils River (TX)	Rio Grande	56/23	13 Mar 2008	29°53′ N, 100°59′ W
	Pinto Creek (TX)	Rio Grande	50/21	1 Jul 2008	29°24′ N, 100°27′ W
Dionda flavipinnis	Fessenden Spring (TX)	Guadalupe	61/20	12 Mar 2008	30°10′ N, 99°20′ W
	Comal Springs (TX)	Guadalupe	60/20	6 Aug 2008	29°43′ N, 98°7′ W
Dionda serena	Frio River (TX)	Nueces	24/21	3 Jul 2008	29°50′ N, 99°46′ W
Dionda texensis	Nueces River (TX)	Nueces	53/24	3 Jul 2008	29°48′ N, 100°0′ W
Dionda sp. 4	El Rito Creek (NM)	Pecos	41/22	23 Mar 2007	33°18′ N, 104°41′ W

Biology (MSB) at the University of New Mexico. Voucher numbers of specimens used to acquire mtDNA sequences, microsatellite genotypes, or both are listed in Appendix 1.

DNA was isolated using the phenol-chloroform protocol of Sambrook et al. (1989) or the DNeasy Blood and Tissue Kit (QIAGEN, www.qiagen.com). A 597–base pair (bp) fragment of the mitochondrial ND-5 gene was sequenced for a subset (n = 20–26) of individuals from each of the 10 sample localities. Polymerase chain reaction (PCR) primers, amplification conditions, and sequencing were the same as those outlined in Carson et al. (2010). Sequences were aligned and protein coding verified in SEQUENCHER 4.1 (Gene Codes, www.genecodes.com). The 597-bp fragment obtained was trimmed to a homologous set of 585 bp due to consistently poor sequence readability at the 3′ end of the fragment. Unique haplotypes were identified using MEGA v. 4.0.2 (Kumar et al. 1994) and assigned a haplotype number. Variation at 28–34 nuclear-encoded microsatellites, depending on species, was assessed from 24 to 63 individuals across all sample localities (Table 1). PCR primers and amplification conditions for each microsatellite and species are given in Renshaw et al. (2009). Amplified DNA from each PCR reaction was combined with a fluorescent dye and

a 400 HD ROX size-standard (Applied Biosystems) DNA ladder and electrophoresed on a 5% acrylamide gel by using an ABI PRISM 377 DNA Sequencer (Applied Biosystems). Sizes of microsatellite fragments were assessed by using GENOTYPER v. 2.5 (Applied Biosystems) and visually confirmed by viewing the gel image in GENESCAN v. 3.1.2 (Applied Biosystems). Alleles at each microsatellite were documented for each individual.

Number of mtDNA haplotypes and haplotype diversity were generated for each sample locality, using FSTAT v. 2.9.3.2 (Goudet 1995). Nucleotide diversity was measured using DNASP v. 5.10.00 (Rozas et al. 2003). For comparisons among populations within species (i.e., for *D. argentosa, D. diaboli,* and *D. flavipinnis*), the number of haplotypes of each sample was corrected for sample size (H_R) by using ANALYTIC RAREFACTION 1.3 (Holland 2003) and after pooling samples of each species, as conducted by Carson et al. (2011). Homogeneity of haplotype distributions among samples within each species was tested via global exact tests in GENEPOP v. 4.1 (Raymond and Rousset 1995, Rousset 2008) and analysis of molecular variance (AMOVA) in ARLEQUIN v. 3.5.1.3 (Excoffier and Lischer 2010). Pairwise exact tests (using GENEPOP) were used to test homogeneity of haplotype distributions between or among localities within species, and pairwise Φ_{ST} values (generated with ARLEQUIN) were used to assess the magnitude of genetic difference. In order to test for changes in historical demography (e.g., population expansion or decline), tests of selective neutrality, measured as Fu and Li's (1993) D^* and F^* metrics and Fu's (1997) F_S statistic, were performed for each sample, using DNASP. In cases where deviations from neutral expectation are detected, comparison of results (among tests) can be used to distinguish between results consistent with historical demographic change and those consistent with selection (Fu and Li 1993). Significance of each metric was assessed using coalescent simulation, with 10,000 iterations, as implemented in DNASP and assuming the segregating-sites model. Haplotype networks were constructed for each species, using the median-joining algorithm in NETWORK 4.5.1.6 (Bandelt et al. 1999).

Departure of genotypic proportions from Hardy–Weinberg (HW) expectations for each microsatellite within each sample was measured as Weir and Cockerham's (1984) f as implemented in F-STAT. Significance of f was evaluated using an exact probability test as implemented in GENEPOP. The exact probability was estimated using a Markov Chain approach (Guo and Thompson 1992) that employed 5000 dememorizations, 500 batches, and 5000 iterations per batch. Genotypic disequilibrium between pairs of microsatellites also was evaluated using exact tests in GENEPOP; the exact probability was estimated via a Markov Chain method using the same parameters as above. Sequential Bonferroni correction (Rice 1989) was applied for all multiple tests performed simultaneously. Occurrence of large-allele dropout, short-allele dominance, stuttering, and null alleles was assessed via analysis with MICROCHECKER (van Oosterhout et al. 2004).

Number and frequency of alleles, allelic richness, gene diversity (expected heterozygosity), and F_{IS} (inbreeding coefficient) were obtained using FSTAT. Exact tests (global or pairwise) of homogeneity in microsatellite allele and genotype distributions between or among samples of each species were carried out using GENEPOP; exact probabilities were estimated via the Markov Chain method (using the same parameters as above) and corrected using the sequential Bonferroni approach. Homogeneity of allelic richness and gene diversity between or among samples of the same species also were tested using Wilcoxon's signed-rank tests and AMOVA, as implemented in SPSS v. 16 (SPSS Inc.) and ARLEQUIN, respectively; for AMOVA, 10,000 permutations were used to test significance. Genetic distances between pairs of samples within species were calculated as pairwise F_{ST} values by using FSTAT. Because demographic independence of populations depends, in part, on contemporaneous dispersal rates (rather than historical averages of gene flow), threshold F_{ST} values were used to further assess distinction among populations (Palsbøll et al. 2007). Threshold F_{ST} values were defined based on estimates of contemporaneous N_e (see LDNE below) and the dispersal rate (10%) above which populations become correlated demographically (Hastings 1993).

The demographic history of each of the 10 samples was investigated using the microsatellite data and the Bayesian coalescent approach

in MSVAR v.4.1b (Beaumont 1999, Storz and Beaumont 2002). This method is useful in conservation because genetic evidence of population decline and its timing may be evaluated with respect to a potential correlation with recent habitat degradation. Demographic parameters, inferred assuming a stepwise mutation model, were N_0, N_1, μ, and t_a. N_0 and N_1 are the effective number of chromosomes at sampling and at the beginning of an expansion/decline phase, respectively; μ is the average mutation rate over all microsatellites per generation; and t_a is the time since the beginning of an expansion/decline phase. Initial parameters were set to a generation time of 2 years (Harrell and Cloutman 1978, Cloutman and Harrell 1987), current and ancestral effective sizes of 10,000, a mutation rate of 0.0005, and a time since decline or expansion of 5000 years. Runs used 20,000 data points and a burn-in of 2000. Output from MSVAR was assessed for density-estimated mode, 2.5 percentile, and 97.5 percentile values, using SAS v.9.2 (SAS Institute). As a complement to the MSVAR analyses, maximum-likelihood estimates of theta (Θ) in each sample were generated using MIGRATE v.3.0.3 (Beerli and Felsenstein 1999, 2001). Initial runs were performed to generate estimates of Θ, which then served as starting parameters for longer runs. Long runs employed 10 short chains with 10,000 sampled gene trees, 4 long chains with 5,000,000 sampled gene trees, and a burn-in of 50,000. Estimates of the average mutation rate (μ) across microsatellites were obtained by using MSVAR and then used to estimate average long-term effective population size (N_eLT) by the following equation: $\Theta = 4N_e\mu$. Estimates of N_eLT provide information about the harmonic mean of the effective size of a population over approximately the past $4N_e$ generations and is, therefore, disproportionately influenced by small effective population size, including genetic bottlenecks, of past generations. Finally, the linkage disequilibrium method (LDNE) of Waples and Do (2008) was used to generate raw estimates of the contemporaneous number of breeders ($\widehat{N}_b$) in each sample. The 2% threshold for exclusion of rare alleles, as recommended by Waples and Do (2010), was used in all samples except for *D. serena*, where use of the 3% threshold was required because the small sample size ($n = 24$) limited the observed frequency of sampled alleles to 2.1% and above (i.e., $1/2n = 0.021$); see Waples and Do (2010) for a thorough explanation and general recommendations for cases where $n < 25$. For all estimates, the jackknife method was used to calculate 95% confidence intervals of $\widehat{N}_b$. To correct for bias of overlapping generations (Waples et al. 2013, 2014), estimates of raw $\widehat{N}_b$ were adjusted using the equation

$$N_{b(\mathrm{Adj})} = \frac{N_b}{1.26 - 0.323\,(N_b/N_e)}.$$

The ratio N_b/N_e was determined by using the equation

$$N_b/N_e = 0.485 + 0.758\log(AL/\alpha),$$

where AL is adult life span and α is age at maturity (Waples et al. 2014). Based on studies of other small cyprinids (Harrell and Cloutman 1978, Cloutman and Harrell 1987), we used 3 years for AL and 1 year for α. Because $\widehat{N}_{b(\mathrm{Adj})}$ shows a close relationship to true N_e in species where mixed cohorts approximate a generation (Waples et al. 2014), estimates of $\widehat{N}_{b(\mathrm{Adj})}$ for populations of *Dionda* should closely approximate N_e. Estimates of $\widehat{N}_{b(\mathrm{Adj})}$ were used as contemporaneous values of N_e in estimation of threshold F_{ST} values (above).

Reduction(s) in effective population size or bottlenecks at each sample locality were assessed using the M test (Garza and Williamson 2001), where M is equal to the mean ratio of the number of alleles to the range in allele size across microsatellites. Values of M were estimated using M_P_VAL; critical values of M (designated as M_c), were estimated using Critical_M. The observed value of M was assessed using a 10,000-replicate Monte Carlo analysis to determine the probability of an M value smaller than the M_c value. Calculations of M and M_c and assessment of probability used the recommended assumption (Garza and Williamson 2001) of 10% non-single steps, with the average non-single step being 3.5 steps. Both an assumed theta value of 2 and theta values generated using MIGRATE were tested.

RESULTS

A total of 41 mtDNA haplotypes were found across the 6 species; none of the haplotypes

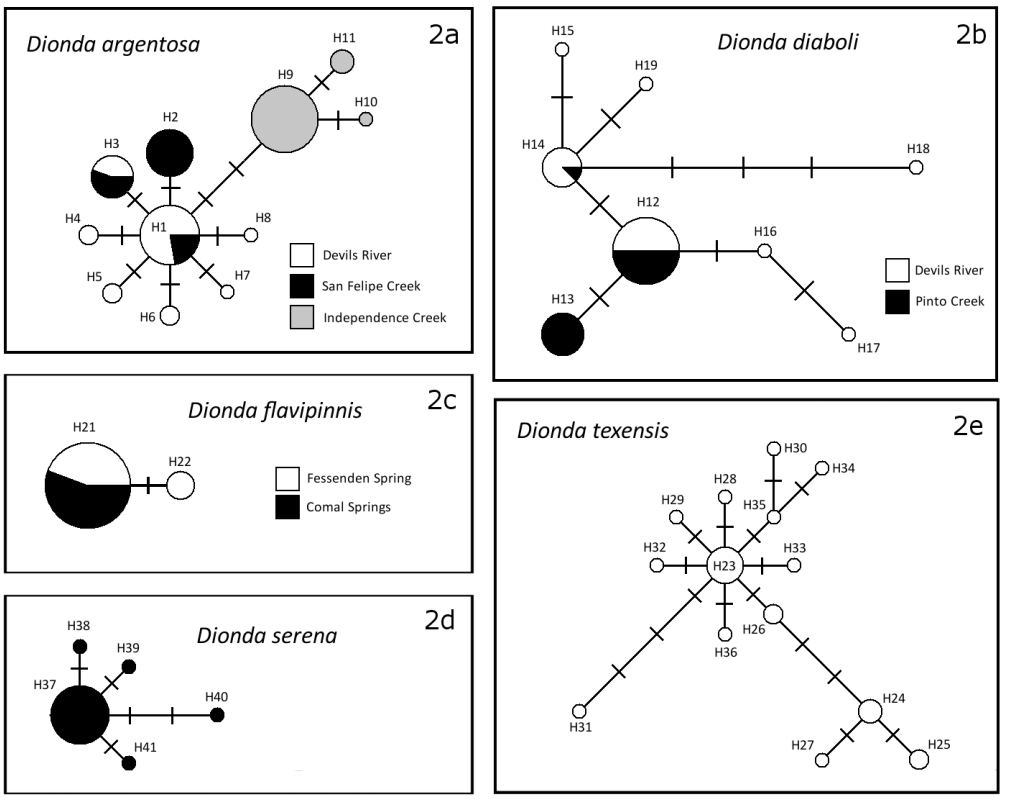

Fig. 2. Median-joining networks of mtDNA haplotypes in each of 5 species of *Dionda*. A network is not shown for *Dionda* sp. 4, as only a single haplotype was found in the sample from El Rito Creek. Each hash mark indicates a single base pair substitution between adjacent haplotypes.

were shared among any of the species. The spatial distribution of haplotypes among samples and the GenBank accession number for each haplotype are given in Appendix 2. No mtDNA variation was found in either *D.* sp. 4 from El Rito Creek or *D. flavipinnis* from Comal Springs, and only 2 haplotypes were found in *D. flavipinnis* from Fessenden Spring. Median-joining haplotype networks are presented in Fig. 2. The number and diversity of haplotypes was greatest in the sample of *D. texensis* from the Nueces River, while haplotypes in the sample of *D. argentosa* from Independence Creek were reciprocally monophyletic relative to the other 2 samples, one from the Devils River and one from San Felipe Creek (Appendix 3).

Based on rarefaction of total haplotype diversity within species, haplotype number was lower than expected for *D. argentosa* from San Felipe Creek (3 observed, 6.8 ± 2.4 expected) and from Independence Creek (3 observed, 7.6 ± 2.4 expected); for *D. diaboli* from Pinto Creek (3 observed, 5.4 ± 2.1 expected); and for *D. flavipinnis* from Comal Springs (1 observed, 1.9 ± 0.4 expected). Significant differences in nucleotide diversity (data not shown) were found in *D. argentosa* (lower in Independence Creek), *D. diaboli* (lower in Pinto Creek), and *D. flavipinnis* (lower in Comal Springs); nucleotide diversity in *D. texensis* from the Nueces River was over 2 times greater than that in any other sample. Estimates of Fu and Li's *F** and *D** metrics were negative but did not differ significantly from zero following Bonferroni correction in any of the 10 samples. Fu's F_S metric was negative and differed significantly from zero after Bonferroni correction in the sample of *D. texensis;* F_S metrics in the remaining samples were negative but did not differ significantly from zero following Bonferroni correction.

TABLE 2. Results (probability [*P*] values) of spatial homogeneity in microsatellite variation between/among samples of each species of *Dionda.* Tests include pairwise Wilcoxon's signed-rank tests of allelic richness and gene diversity.

Sample	Allelic richness	Gene diversity
Dionda argentosa		
Devils River–San Felipe Creek	0.002	0.433
Devils River–Independence Creek	0.008	0.191
San Felipe Creek–Independence Creek	0.554	0.879
Dionda diaboli	0.001	0.004
Dionda flavipinnis	0.000	0.002

Significant deviations from HW expectations before and after Bonferroni correction and potential amplification errors and/or possible null alleles identified by MICROCHECKER were found at various microsatellites in several samples; no deviations from genotypic disequilibrium were found after correction. The microsatellite data set was then reduced to 21–33 experimentally tractable microsatellites that did not deviate significantly from HW equilibrium expectations, following Bonferroni correction, and that showed no evidence of amplification errors or null alleles in any sample. A list of all microsatellites omitted from subsequent analyses may be found in Appendix Table 3 in Hanna (2011). Summary statistics for each experimentally tractable microsatellite in each sample are presented in Appendix 4. Mean number of alleles, allelic richness, and gene diversity (expected heterozygosity) were lowest in *D*. sp. 4 (2.09, SE 0.37; 2.24, SE 0.13; and 0.211, SE 0.018, respectively) and highest in *D. argentosa* from the Devils River (9.76, SE 1.23; 8.45, SE 0.47; and 0.606, SE 0.028, respectively). Pairwise Wilcoxon's signed-rank tests of average number of alleles and average gene diversity (expected heterozygosity) over all microsatellites (Table 2) indicated significant differences in number of alleles among samples of *D. argentosa* (Devils River > San Felipe Creek, Independence Creek) and between samples of *D. diaboli* (Devils River > San Felipe Creek) and *D. flavipinnis* (Comal Springs > Fessenden Spring); corresponding differences in average gene diversity were indicated between samples of *D. diaboli* and samples of *D. flavipinnis.*

Significant heterogeneity in mtDNA haplotype distributions was detected in all comparisons between or among samples in each species ($P < 0.001$), except for the comparison of *D. flavipinnis* ($P = 0.106$) from Comal Springs and Fessenden Spring. Significant heterogeneity in microsatellite allele ($P < 0.001$) and genotype ($P < 0.001$) distribution was detected in all comparisons between samples within each species, including the 2 samples of *D. flavipinnis*. Genetic distances between samples in each species, based on pairwise Φ_{ST} values of mtDNA sequences and pairwise F_{ST} values of microsatellites, may be found in Appendix 5. Probability values for tests of $\Phi_{ST} = 0$ were significant among samples of *D. argentosa* ($\Phi_{ST} = 0.705$, $P = 0.000$) and among samples of *D. diaboli* ($\Phi_{ST} = 0.252$, $P < 0.001$), but were nonsignificant between the 2 samples of *D. flavipinnis* ($\Phi_{ST} = 0.158$, $P = 0.108$). Probability values for all tests of $F_{ST} = 0$ were significant ($P < 0.001$). Threshold F_{ST} values, based on minimum estimates of $N_{b(\mathrm{Adj})}$[$= N_e$; see below] and a 10% dispersal rate between populations (Hastings, 1993), were estimated for *D. argentosa* (F_{ST} threshold = 0.014), *D. diaboli* (F_{ST} threshold = 0.025), and *D. flavipinnis* (F_{ST} threshold = 0.104). All observed F_{ST} values (0.123 for *D. argentosa*, 0.230 for *D. diaboli*, and 0.280 for *D. flavipinnis*) were higher than threshold values, indicating demographic independence of each population relative to others evaluated.

Estimates of average microsatellite mutation rate (μ) per generation, long-term population growth of decline (r, where r is the ratio N_0/N_1 and is expected to be <1 in a declining population, equal to 1 in a stable population, and >1 in an expanding population), and the period (t_a) since growth or decline occurred are given in Table 3. Estimates of μ ranged from 2.2×10^{-4} to 2.5×10^{-4} and were consistent across samples. Modal estimates of $\log_{10}(r)$ were negative for all samples, indicating declines in effective size, and ranged from –1.35 in the sample of *D. serena* (Frio River) to –3.21 in the sample of *D. flavipinnis* from Fessenden Spring. Of the 10 samples, 6 appear to have experienced a decline of more than 2 orders of magnitude. Assuming a generation time of 1–3 years, modal estimates of t_a ranged from 508 to 1524 years in *D. argentosa* from the Devils River and from 3211 to 9632 years in *D. diaboli*

TABLE 3. Modal values and their 95% quantiles for mutation rate (μ) and $\log_{10} r$ for 10 samples of *Dionda;* time since expansion/decline began (t_a) is given for a range of generation times from 1 to 3 years.

Sample	Mode	0.025 quantile	0.975 quantile
Dionda argentosa			
Devils River			
μ	2.4×10^{-4}	2.8×10^{-5}	2.1×10^{-3}
$\text{Log}_{10}(r)$	–1.54	–2.13	–1.36
t_a (years)	508–1524	6–19	13344–40033
San Felipe Creek			
μ	2.4×10^{-4}	2.8×10^{-5}	2.0×10^{-3}
$\text{Log}_{10}(r)$	–2.13	–2.28	–2.02
t_a (years)	961–2882	65–196	12882–21440
Independence Creek			
μ	2.4×10^{-4}	2.7×10^{-5}	2.0×10^{-3}
$\text{Log}_{10}(r)$	–2.31	–2.44	–2.22
t_a (years)	514–1542	37–111	7147–21440
Dionda diaboli			
Devils River			
μ	2.5×10^{-4}	2.7×10^{-5}	2.2×10^{-3}
$\text{Log}_{10}(r)$	–2.11	–2.08	–1.75
t_a (years)	1482–4446	39–116	10325–120976
Pinto Creek			
μ	2.5×10^{-4}	2.7×10^{-5}	2.2×10^{-3}
$\text{Log}_{10}(r)$	–2.98	–3.05	–2.85
t_a (years)	3211–9632	206–618	40651–121954
Dionda sp. 4			
El Rito Creek			
μ	2.3×10^{-4}	2.6×10^{-5}	2.1×10^{-3}
$\text{Log}_{10}(r)$	–2.32	–2.36	–2.35
t_a (years)	1163–3488	60–181	15686–47057
Dionda flavipinnis			
Fessenden Spring			
μ	2.3×10^{-4}	2.6×10^{-5}	2.0×10^{-3}
$\text{Log}_{10}(r)$	–3.21	–3.64	–3.03
t_a (years)	749–2247	22–67	14251–43563
Comal Springs			
μ	2.2×10^{-4}	2.5×10^{-5}	2.0×10^{-3}
$\text{Log}_{10}(r)$	–2.31	–2.40	–2.17
t_a (years)	569–1706	13–40	21747–65241
Dionda serena			
Frio River			
μ	2.3×10^{-4}	2.7×10^{-5}	2.1×10^{-3}
$\text{Log}_{10}(r)$	–1.35	–1.69	–1.34
t_a (years)	927–2781	6–17	86497–259409
Dionda texensis			
Nueces River			
μ	2.5×10^{-4}	2.8×10^{-5}	2.3×10^{-3}
$\text{Log}_{10}(r)$	–1.56	–1.65	–1.51
t_a (years)	1507–4522	45–136	30860–92581

from Pinto Creek. Minimum estimates of t_a were less than 100 years for 9 of the 10 samples.

Estimates of theta (Θ), generated using MIGRATE, and average long-term effective size (N_{eLT}) for each sample are presented in Table 4. Estimates of N_{eLT} were based on the relationship $\Theta = 4N_e\mu$; average values of μ were from MSVAR. Estimates of theta for the sample of *D.* sp. 4 failed to converge. Estimates of N_{eLT} ranged from 503 in *D. diaboli* from Pinto Creek to 2335 in *D. texensis.* Minimum and maximum estimates (based on 95% confidence intervals from jackknifing across microsatellites) of the effective number of breeders (N_b) and of $N_{b(\text{Adj})}$ are given in Table 5. Several point estimates were returned as errors (negative numbers) and upper limits to all but one of the confidence intervals were returned as infinity (∞); minimum confidence intervals, however, are considered informative (Waples

TABLE 4. Estimates of average long-term genetic effective size (N_{eLT}) and 95% confidence intervals; estimates of N_{eLT} were based on estimates of theta (Θ), obtained from MIGRATE, and mutation rate (μ), obtained from MSVAR. Estimates of μ are not shown but may be obtained from AHH. An estimate of N_{eLT} for *Dionda* sp. 4 could not be generated as Θ failed to converge.

Sample	Theta (Θ)	N_{eLT}
Dionda argentosa		
Devils River	1.396	1449.9 (1384.3–1517.5)
San Felipe Creek	0.523	536.0 (499.5–606.6)
Independence Creek	1.156	1227.8 (1161.1–1302.0)
Dionda diaboli		
Devils River	1.364	1371.0 (1282.0–1452.8)
Pinto Creek	0.501	503.5 (475.5–534.0)
Dionda flavipinnis		
Fessenden Spring	0.624	685.6 (657.3–716.1)
Comal Springs	1.285	1434.6 (1372.6–1498.1)
Dionda serena		
Frio River	1.372	1485.1 (1375.0–1641.5)
Dionda texensis		
Nueces River	2.351	2335.2 (2209.1–2489.5)
Dionda sp. 4		
El Rito Creek	—	—

TABLE 5. Estimates (and 95% confidence intervals) of the effective number of breeders before (N_b) and after ($N_{b(\mathrm{Adj})}$) correction for bias introduced by overlapping generations. Estimates from LDNE are based on the 2% threshold for removal of rare alleles, except for *D. serena* (see Methods for further details).

Sample	Estimated N_b	$N_{b(\mathrm{Adj})}$
Dionda argentosa		
Devils River	442–∞	448–∞
San Felipe Creek	320–∞	324–∞
Independence Creek	170–∞	172–∞
Dionda diaboli		
Devils River	547–∞	555–∞
Pinto Creek	96	96–∞
Dionda flavipinnis		
Fessenden Spring	21–∞	22–∞
Comal Springs	169–∞	171–∞
Dionda serena		
Frio River	101–∞	103–∞
Dionda texensis		
Nueces River	340–∞	345–∞
Dionda sp. 4		
El Rito Creek	51–1553	51–∞

and Do 2010) for populations or species of conservation concern. Minimum estimates of N_b (after correction for overlapping generations, i.e., $N_{b(\mathrm{Adj})}$) ranged from 22 in the sample of *D. flavipinnis* from Fessenden Spring to 555 in the sample of *D. diaboli* from the Devils River. Marked variation in minimum estimates of $N_{b(\mathrm{Adj})}$ was observed among samples of *D. argentosa* (Devils River > San Felipe Creek > Independence Creek), between samples of *D. diaboli* (Devils River > Pinto Creek), and between samples of *D. flavipinnis* (Comal Springs > Fessenden Spring). Only the minimum estimate of $N_{b(\mathrm{Adj})}$ for *D. diaboli* from the Devils River was greater than 500, and estimates of $N_{b(\mathrm{Adj})}$ for *D. diaboli* from Pinto Creek, *D.* sp. 4, and *D. flavipinnis* from Fessenden Spring were <100, with the estimate for *D.* sp. 4 near the effective size of 50 at which there may be immediate concern over loss of fitness as a result of inbreeding depression (Rieman and Allendorf 2001). Estimates of $N_{b(\mathrm{Adj})}$ for all samples were less than minimum estimates of N_{eLT}.

Estimates of *M*, the mean ratio of the number of alleles to the range in allele size, and M_c, the critical (95%) value for *M*, are presented in Table 6. With an assumed theta value of 2, *M* values for *D. argentosa* from San Felipe Creek and Independence Creek, *D. diaboli* from Pinto Creek, *D.* sp. 4, and *D. flavipinnis* from Fessenden Spring were significant, indicating occurrence of recent bottlenecks in those samples. When theta values based on analysis with MIGRATE were used, *M*-ratios for these same samples, as well as for *D. flavipinnis* from Comal Springs, were significant.

DISCUSSION

At the core of conservation genetics is the evaluation of genetic diversity within and among populations to provide information for maintenance of natural levels and patterns of genetic diversity and to mitigate anthropogenic effects on that diversity (Meffe 1990, Vrijenhoek 1998). Evaluation of genetic diversity (variation) present within populations can highlight potential conservation risks, while evaluation of genetic diversity (divergence) between or among geographic populations can identify populations that may be considered distinct evolutionarily significant units or management units (Waples 1991, Moritz 1994). Sufficient levels of genetic diversity within a

TABLE 6. Results of the *M* test. The *M* test was performed using a theta value of 2 and theta values based on results from MIGRATE. Critical values (M_c) and the probability (*P*) of a smaller *M* are also shown.

Sample	Mean *M*	Theta value of 2		Theta value based on MIGRATE		
		M_c	*P*	Θ	M_c	*P*
Dionda argentosa						
Devils River	0.837	0.783	0.331	1.396	0.800	0.204
San Felipe Creek	0.740	0.772	0.007	0.523	0.839	0.000
Independence Creek	0.677	0.777	0.000	1.156	0.806	0.000
Dionda diaboli						
Devils River	0.843	0.785	0.383	1.253	0.807	0.206
Pinto Creek	0.748	0.784	0.006	0.538	0.844	0.000
Dionda flavipinnis						
Fessenden Spring	0.716	0.798	0.000	0.654	0.848	0.000
Comal Springs	0.811	0.797	0.098	1.330	0.817	0.035
Dionda serena						
Frio River	0.912	0.773	0.947	1.372	0.794	0.861
Dionda texensis						
Nueces River	0.802	0.784	0.122	2.351	0.776	0.148
Dionda sp. 4						
El Rito Creek	0.783	0.795	0.024			

population ensure a suite of different alleles that potentially can respond to different environmental situations (Frankham 1995, Lynch et al. 1995). Finally, most studies of genetic diversity have utilized genetic markers that are considered selectively neutral (Avise 1994, McKay and Latta 2002, Reed and Frankham 2003); although such markers do not necessarily correlate to levels of diversity found in genes that would impact fitness of individuals (McKay and Latta 2002), estimates of variability (e.g., heterozygosity) in selectively neutral markers are, at present, extensively used to evaluate the conservation status of populations (Reed and Frankham 2003).

All of the geographic samples of *Dionda* examined in this study appear to be discrete, demographically independent populations. Conspecific samples of *D. argentosa*, *D. diaboli*, and *D. flavipinnis* differed significantly from one another in microsatellite allele and genotype distributions, and except for the 2 samples of *D. flavipinnis*, where the only haplotype found in 20 individuals from Comal Springs occurred in 16 of 20 individuals in Fessenden Spring, all differed significantly in mtDNA haplotype frequencies. Based on the approach and suggested criteria outlined in Palsbøll et al. (2007), all of the samples of *Dionda* should be considered discrete genetic populations and separate management units (MUs). In addition, the clade of mtDNA haplotypes in the population of *D. argentosa* from Independence Creek was reciprocally monophyletic relative to the clade of mtDNA haplotypes in the populations of *D. argentosa* in the Devils River and San Felipe Creek, suggesting that the population of *D. argentosa* in Independence Creek could represent an evolutionarily significant unit (ESU). Based on the work of Schönhuth et al. (2012), this form of *D. argentosa* also occurs farther south in the Pecos River and is related to samples of *D. argentosa* found in several localities in Mexico. Finally, the observation that all 10 populations are demographically independent indicates that local extirpations likely would not be replaced by new migrants and that loss of any of the populations would represent loss of a unique genetic entity.

All 10 populations of *Dionda* examined in this study exhibited mtDNA and microsatellite variation comparable to or lower than that found in other threatened or endangered cyprinids (Tables 7, 8). A particularly relevant comparison is with the Cape Fear shiner, *Notropis mekistocholas*, a species listed as endangered (Jelks et al. 2008) or critically endangered (Hilton-Taylor 2000). Except for the population of *D. texensis*, the populations of *Dionda* examined in this study had fewer mtDNA haplotypes and lower haplotype diversity (Table 7) and generally fewer microsatellite alleles and lower gene diversity (Table 8) than reported for *N. mekistocholas*. The low level of genetic variation observed in the populations of *Dionda* is of concern given that reduced genetic diversity may negatively impact the capability

TABLE 7. Summary of mtDNA variation in *Dionda* (this study) and in other imperiled cyprinids. Values are within-population averages, ranging across populations.

Species	Source	Conservation status	mtDNA	Base pairs	Samples	Individuals per sample	Haplotypes	Haplotype diversity
Dionda								
Dionda argentosa	This study	Imperiled	ND-5	585	3	24	3–7	0.280–0.692
Dionda diaboli	This study	Threatened	ND-5	585	2	22	3–7	0.567–0.700
Dionda flavipinnis	This study	Secure	ND-5	585	2	20	1–2	0.000–0.337
Dionda serena	This study	Imperiled	ND-5	585	1	21	5	0.352
Dionda texensis	This study	Imperiled	ND-5	585	1	24	14	0.906
Dionda sp. 4	This study	Secure	ND-5	585	1	22	1	0.000
Other cyprinids								
Anaecypris hispanicia	Alves et al. (2001)	Endangered	Cyt *b*, Control	1818	9	15.4	2–5	0.600–1.00
Hybognathus amarus	Alò and Turner (2005)	Endangered	ND-4	295	8	49.6	2–9	0.119–0.667
Gila cypha	Garrigan et al. (2002)	Endangered	ND-2	790	1	18	5	—
Gila elegans	Garrigan et al. (2002)	Endangered	ND-2	763	1	16	3	—
Notropis mekistocholas	Gold et al. (2004)	Critically endangered	ND-5, ND-6	625	3	13.3	5–9	—
Notropis mekistocholas	Saillant et al. (2004)	Critically endangered	ND-5, ND-6	625	2	27.5	11–14	0.80–0.85
Notropis simus pecosensis	Osborne and Turner (2009)	Threatened	ND-4	322	3	108.3	20	0.603–0.650

TABLE 8. Summary of microsatellite variation in *Dionda* (this study) and in other imperiled cyprinids. Values are within-population averages, ranging across populations.

Species	Source	Conservation status	Micro-satellites	Samples	Individuals per sample	Alleles	Gene diversity
Dionda							
Dionda argentosa	This study	Imperiled	21	3	43.3	6.10–9.76	0.591–0.606
Dionda diaboli	This study	Threatened	23	2	53	2.17–6.17	0.240–0.392
Dionda flavipinnis	This study	Secure	33	2	60.5	2.52–4.94	0.255–0.378
Dionda serena	This study	Imperiled	21	1	24	3.71	0.423
Dionda texensis	This study	Imperiled	21	1	53	7.67	0.525
Dionda sp.4	This study	Secure	33	1	41	2.09	0.257
Other cyprinids							
Anaecypris hispanicia	Salgueiro et al. (2003)	Endangered	5	8	39.4	7.4–13.4	0.59–0.78
Hybognathus amarus	Alò and Turner (2005)	Endangered	7	8	49.6	9.3–13.0	0.684–0.752
Notropis mekistocholas	Burridge and Gold (2003)	Critically endangered	11	3	13.3	5.1–5.3	—
Notropis mekistocholas	Gold et al. (2004)	Critically endangered	11	3	13.3	6.2–7.9	0.77–0.79
Notropis mekistocholas	Saillant et al. (2004)	Critically endangered	22	2	27.5	8.18	0.701
Notropis simus pecosensis	Osborne and Turner (2009)	Threatened	7	3	108.3	13.3–23.7	0.816–0.846

of a population to respond to environmental perturbations (Frankham et al. 2002). In addition, the finding that genetic diversity in these *Dionda* is less than that in other threatened or endangered cyprinids suggests that their conservation status may need to be reevaluated.

Estimates of historical and present-day genetic demography indicated that all 10 populations of *Dionda* examined have experienced relatively large declines in effective population size, with 6 having experienced declines of over 2 orders of magnitude and one (*D. flavipinnis* from Fessenden Spring) having experienced a decline of over 3 orders of magnitude. Modal estimates of the time (in years) of the declines were >500 years; however, the lower bounds of the time intervals for 9 of the populations ranged from 6 to 65 years (average of 32.5 years), compatible with a number of recent, anthropogenic changes to typical *Dionda* habitat (Garrett and Edwards 2001). Comparison of the estimates of both average long-term effective size (N_{eLT}) and the effective number of breeders ($N_{b(\mathrm{Adj})}$) in the present-day populations also are consistent with relatively recent declines in effective size. Estimates of N_{eLT} ranged from 503 (*D. diaboli* in Pinto Creek) to >2000 (*D. texensis* in the Nueces River) and averaged 1225.4 (SE 183.8). Lower 95% confidence intervals for estimates of $N_{b(\mathrm{Adj})}$ (effective number of breeders) ranged from 22 (*D. flavipinnis* from Fessenden Spring) to 555 (*D. diaboli* from the Devils River), and averaged 228.7 (SE 55.8). Estimates of $N_{b(\mathrm{Adj})}$ are based on the principle that genetic drift increases the incidence of nonrandom associations among alleles at different loci in the parents of the sampled cohort (Luikart et al. 2010), and as such represent an estimate of inbreeding effective size (N_{eI}) on a recent timescale (Beaumont 2003, Waples and Do 2010). The differences between the estimates of N_{eLT} and $N_{b(\mathrm{Adj})}$ are consistent with the inference that most of these *Dionda* populations have experienced large declines in the relatively recent past. This inference also is supported by results of the *M* test in that significant recent bottlenecks appear to have occurred in *D. argentosa* from San Felipe Creek and Independence Creek, *D. diaboli* from Pinto Creek, *D.* sp. 4, *D. flavipinnis* from Fessenden Spring, and possibly *D. flavipinnis* from Comal Springs. One final point is that minimum estimates of $N_{b(\mathrm{Adj})}$ in all of the populations except for *D. diaboli* from the Devils River were <500, suggesting that the equilibrium between the loss of adaptive genetic variance from genetic drift and its replacement by mutation might be compromised. This suggestion is based on the "50/500" benchmark (Rieman and Allendorf 2001) for genetic effective size (N_e), where an N_e of <50 indicates a population is highly vulnerable to inbreeding depression, while an N_e average of ≥500 allows a population to maintain adaptive genetic variation through time. Thus, most of the populations of *Dionda* appear to be compromised genetically.

Conservation Recommendations

Of the 3 populations of *D. argentosa* examined, the one in the Devils River appears the least compromised genetically, whereas the populations in San Felipe Creek and Independence Creek have lower genetic variation and reduced minimum $N_{b(\mathrm{Adj})}$, and also appear to have experienced recent bottlenecks. All 3 populations should be monitored, but close attention should be paid to the populations in San Felipe Creek and Independence Creek, especially as the latter can be categorized as an ESU. Because *D. diaboli* is listed as either threatened or endangered (USFWS 1999, Jelks et al. 2008), its genetic status was of particular interest. The population in Pinto Creek has low genetic variation and a very low minimum $N_{b(\mathrm{Adj})}$, and has experienced a significant recent bottleneck. The population in the Devils River appears among the least compromised genetically of the 10 populations examined and was the only population where the minimum estimate of $N_{b(\mathrm{Adj})}$ was >500. Both populations likely will be monitored given the official conservation status of the species. We recommend that specimens from other known localities of *D. diaboli* be examined genetically; these include San Felipe Creek (Scharpf 2005) and both Las Moras and Sycamore creeks, although the latter two may be extirpated (Garrett et al. 1992). Both populations of *D. flavipinnis* examined have very little genetic variation and a small minimum $N_{b(\mathrm{Adj})}$, and have experienced significant recent bottlenecks. Of particular concern is the low mtDNA diversity of both populations and the small $N_{b(\mathrm{Adj})}$ (22) of the population in Fessenden Spring. Clearly, the conservation status of this species is no longer "secure" as

listed in Scharpf (2005), and both populations should be closely monitored. The remaining 3 populations examined represent 3 different species: *D. serena* from the Sabinal and Frio rivers, *D. texensis* from the Nueces River, and *D*. sp. 4 from El Rito Creek. The number of haplotypes, haplotype diversity, and gene diversity in the population of *D. serena* were average (compared to the other populations examined), and all measures of genetic diversity were comparatively high in the population of *D. texensis.* Both have experienced historical declines in effective size, and the minimum estimates of $N_{b(\mathrm{Adj})}$ in both were <500. Both probably warrant continued monitoring. The population of *D.* sp. 4 in El Rito Creek appears severely compromised genetically, and evaluation of *D.* sp. 4 at other localities is clearly warranted. If levels of variation and genetic demography in other populations of *D.* sp. 4 are comparable to those of the population in El Rito Creek, it is probable that *D.* sp. 4 is threatened or endangered. Additionally, proper definition of *D.* sp. 4 as a nominal species will be imperative in moving forward with further study and management of this species.

One final comment is that while there may be other populations of these species in Texas and New Mexico, finding and sampling them is problematic. More than 94% of Texas is privately owned or operated (http://www.tpwd.state.tx.us/landwater/land/private/lone_star_land_steward/), and large portions of the rivers in the western part of the state run through private land. Obtaining permission from landowners to sample what might be imperiled or threatened species is difficult, and even representatives of the state management agency are generally unable obtain permission to sample. It is possible that our samples are among the few that can be legally obtained in headwater areas of the rivers and creeks sampled.

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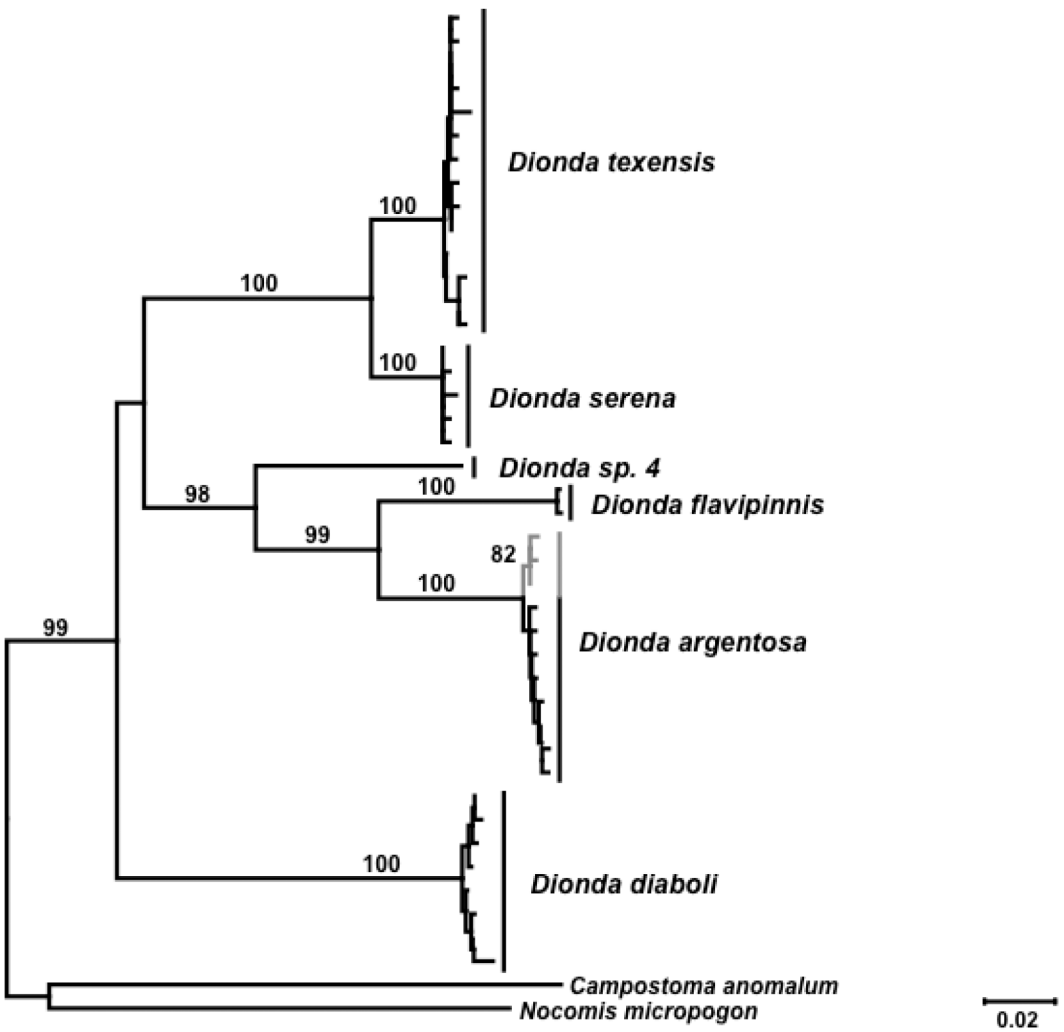
APPENDIX 1. Voucher specimens for 6 species of *Dionda*.

Voucher specimens for all samples except *Dionda* sp. 4 from El Rito Creek are stored in the Biodiversity Research and Teaching Collections (BRTC) at Texas A&M University. Voucher numbers for specimens from BRTC specimens are as follows: *D. argentosa* Devils River (14847.01–14904.01, 14908.01–14912.01), *D. argentosa* San Felipe Creek (14981.01–15013.01), *D. argentosa* Independence Creek (15124.01–15157.01), *D. diaboli* Devils River (14905.01–14907.01, 14921.01–14973.01), *D. diaboli* Pinto Creek (15014.01–15050.01, 15051.01–15063.01), *D. flavipinnis* Fessenden Spring (14786.01–14846.01), *D. flavipinnis* Comal Springs (15064.01–15123.01), *D. serena* Frio River (14268.01–14272.01, 14461.01–14474.01, 14974.01–14978.01), *D. texensis* Nueces River (14273.01–14286.01, 14475.01–14485.01, 14489.01–14515.01, 14517.01). Specimens of *D.* sp. 4 are stored in the Museum of Southwestern Biology under voucher number MSB054.21-61.

APPENDIX 2. Observed *Dionda* mtDNA haplotypes and GenBank accession numbers, arranged by species and sample locality.

	D. argentosa			*D. diaboli*		*D.* sp. 4	*D. flavipinnis*		*D. texensis*	*D. serena*	
MtDNA Haplotype	Devils River	San Felipe Creek	Independence Creek	Devils River	Pinto Creek	El Rito Creek	Fessenden Spring	Comal Springs	Nueces River	Frio River	GenBank Accession #
1	14	4									GU252301.1
2		11									GU252302.1
3	4	5									GU252303.1
4	2										GU252304.1
5	2										GU252305.1
6	2										GU252306.1
7	1										GU252307.1
8	1										GU252308.1
9			22								GU252309.1
10			1								GU252310.1
11			3								GU252311.1
12				11	11						GU252312.1
13					9						GU252313.1
14				7	1						GU252314.1
15				1							GU252315.1
16				1							GU252316.1
17				1							GU252317.1
18				1							GU252318.1
19				1							GU252319.1
20						22					GU252320.1
21							16	20			GU252321.1
22							4				GU252322.1
23									7		GU252323.1
24									3		GU252324.1
25									2		GU252325.1
26									2		GU252326.1
27									1		GU252327.1
28									1		GU252328.1
29									1		GU252329.1
30									1		GU252330.1
31									1		GU252331.1
32									1		GU252332.1
33									1		GU252333.1
34									1		GU252334.1
35									1		GU252335.1
36									1		GU252336.1
37										17	GU252337.1
38										1	GU252338.1
39										1	GU252339.1
40										1	GU252340.1
41										1	GU252341.1



APPENDIX 3. Neighbor-joining tree based on ND-5 haplotypes of 10 samples of *Dionda* species. Numbers above branches indicate levels of bootstrap support, and the corresponding scale of genetic distance is shown at the bottom of the figure. Branch lengths in gray designate samples of *D. argentosa* from Independence Creek. Modified from Fig. 1 of Carson et al. (2010). Used with permission from the *Southwestern Naturalist*.

APPENDIX 4. Summary statistics for nuclear-encoded microsatellites in 10 samples (from 6 species) of *Dionda*. Parameters are sample size (n), number of alleles (#A), allelic richness (A_R), gene diversity (expected heterozygosity, H_E), probability that the locus conforms to Hardy–Weinberg equilibrium (P_{HW}), and inbreeding coefficient (F_{IS}) measured as Weir and Cockerham's (1984) *f*.

	D. argentosa			*D. diaboli*		*D. flavipinnis*		*D. serena*	*D. texensis*	*D.* sp. 4
Locus and statistic	Devils River	San Felipe Creek	Independence Creek	Devils River	Pinto Creek	Fessenden Spring	Comal Springs	Frio River	Nueces River	El Rito Creek
Dep 1										
n	63	33	34	56	50	61	60	24	53	41
#A	3	2	2	1	1	2	2	3	1	1
A_R	2.94	2.00	2.00	1.00	1.00	2.00	2.00	3.00	1.00	1.00
H_E	0.204	0.088	0.276	0.000	0.000	0.138	0.081	0.657	0.000	0.000
P_{HW}	0.557	1.000	0.180	—	—	1.000	1.000	0.822	—	—
F_{IS}	0.068	–0.032	0.255	—	—	–0.071	–0.035	0.048	—	—
Dep 2										
n	63	33	34	56	50	61	60			41
#A	9	6	13	1	1	1	1			1
A_R	6.39	5.94	12.63	1.00	1.00	1.00	1.00			1.00
H_E	0.301	0.439	0.718	0.000	0.000	0.000	0.000			0.000
P_{HW}	0.763	0.111	0.354	—	—	—	—			—
F_{IS}	–0.055	0.172	0.017	—	—	—	—			—
Dep 3										
n				56	50	61	60	24	53	41
#A				20	3	10	10	7	27	2
A_R				19.81	3.00	9.82	9.86	6.63	16.88	2.00
H_E				0.92	0.402	0.712	0.552	0.738	0.917	0.137
P_{HW}				0.571	0.026	0.022	0.631	0.716	0.803	1.000
F_{IS}				–0.028	–0.094	0.126	–0.057	0.040	0.033	–0.067
Dep 7										
n	63	33	34	56	50			24	53	41
#A	13	8	8	8	4			8	12	1
A_R	11.20	7.93	7.90	7.99	3.98			7.83	10.28	1.00
H_E	0.795	0.810	0.807	0.834	0.511			0.684	0.864	0.000
P_{HW}	0.552	0.729	0.121	0.382	0.799			0.946	0.067	—
F_{IS}	–0.038	–0.047	0.089	–0.006	0.099			–0.036	0.040	—
Dep 8										
n				56	50			23	53	41
#A				1	2			2	2	3
A_R				1.00	2.00			1.91	1.40	3.00
H_E				0.000	0.059			0.043	0.019	0.357
P_{HW}				—	0.031			—	—	0.774
F_{IS}				—	0.662			0.000	0.000	0.043

APPENDIX 4. Continued.

Locus and statistic	*D. argentosa*			*D. diaboli*		*D. flavipinnis*		*D. serena*	*D. texensis*	*D.* sp. 4
	Devils River	San Felipe Creek	Independence Creek	Devils River	Pinto Creek	Fessenden Spring	Comal Springs	Frio River	Nueces River	El Rito Creek
Dep 9										
n	63	33	34					24	53	41
#A	9	5	5					5	8	1
A_R	7.21	5.00	4.91					5.00	6.40	1.00
H_E	0.570	0.706	0.567					0.784	0.761	0.000
P_{HW}	0.754	0.532	0.295					0.575	0.482	—
F_{IS}	–0.114	0.013	0.170					–0.062	0.058	—
Dep 10										
n	63	33	34	56	50	61	60	24	53	41
#A	9	6	7	1	1	1	2	2	7	2
A_R	8.16	5.94	6.91	1.00	1.00	1.00	1.93	2.00	6.37	2.00
H_E	0.826	0.713	0.803	0.000	0.000	0.000	0.017	0.156	0.674	0.198
P_{HW}	0.141	0.465	0.695	—	—	—	—	1.000	0.003	0.388
F_{IS}	–0.058	–0.147	–0.063	—	—	—	0.000	–0.070	0.300	0.138
Dep 12										
n								24	53	
#A								1	3	
A_R								1.00	1.79	
H_E								0.000	0.038	
P_{HW}								—	1.000	
F_{IS}								—	–0.005	
Dep 13										
n	63	32	34	56	50	61	60			
#A	18	13	10	5	2	3	5			
A_R	14.33	12.90	9.74	4.87	2.00	2.84	4.93			
H_E	0.865	0.872	0.846	0.437	0.243	0.033	0.535			
P_{HW}	0.678	0.702	0.600	0.502	1.000	1.000	0.118			
F_{IS}	–0.009	0.032	–0.148	–0.185	0.013	–0.004	0.003			
Dep 18										
n				56	50	61	60			41
#A				26	8	3	6			2
A_R				24.84	7.92	2.99	5.93			2.00
H_E				0.944	0.551	0.281	0.573			0.252
P_{HW}				0.730	0.146	0.034	0.400			0.570
F_{IS}				–0.021	0.092	0.068	0.098			–0.159

APPENDIX 4. Continued.

Locus and statistic	*D. argentosa*			*D. diaboli*		*D. flavipinnis*		*D. serena*	*D. texensis*	*D.* sp. 4
	Devils River	San Felipe Creek	Independence Creek	Devils River	Pinto Creek	Fessenden Spring	Comal Springs	Frio River	Nueces River	El Rito Creek
Dep 20										
n						61	60			41
#A						8	15			3
A_R						7.99	15.00			3.00
H_E						0.815	0.906			0.379
P_{HW}						0.511	0.044			1.000
F_{IS}						–0.086	0.073			–0.030
Dep 21										
n	63	33	34	56	50	61	60	24	53	41
#A	2	1	1	7	2	2	1	8	25	1
A_R	1.87	1.00	1.00	6.63	2.00	1.92	1.00	7.72	18.81	1.00
H_E	0.047	0.000	0.000	0.718	0.229	0.016	0.000	0.799	0.940	0.000
P_{HW}	1.000			0.336	1.000	—	—	0.488	0.000	—
F_{IS}	–0.016			0.005	0.038	0.000	—	0.009	0.257	—
Dep 28										
n	63	33	34			61	60			41
#A	6	7	5			2	3			3
A_R	5.43	6.94	4.91			2.00	3.00			3.00
H_E	0.741	0.760	0.681			0.357	0.417			0.453
P_{HW}	0.570	0.036	0.571			0.493	0.041			1.000
F_{IS}	–0.071	–0.275	0.007			0.081	0.241			0.031
Dep 30										
n	63	33	34	56	50	61	60	24	53	41
#A	1	1	1	2	2	6	17	2	18	1
A_R	1.00	1.00	1.00	2.00	2.00	5.75	16.91	2.00	11.89	1.00
H_E	0.000	0.000	0.000	0.388	0.059	0.543	0.906	0.120	0.780	0.000
P_{HW}	—	—	—	0.483	1.000	0.760	0.181	1.000	0.620	—
F_{IS}	—	—	—	0.125	–0.021	–0.116	–0.085	–0.045	–0.016	—
Dep 32										
n	63	33	34	56	50	61	60	24	53	41
#A	28	11	12	19	6	5	13	14	20	4
A_R	23.56	10.76	11.56	18.17	5.98	4.99	12.86	13.08	14.84	4.00
H_E	0.951	0.846	0.844	0.803	0.597	0.715	0.864	0.847	0.920	0.184
P_{HW}	0.365	0.792	0.019	0.000	0.280	0.720	0.051	0.375	0.005	1.000
F_{IS}	0.032	–0.039	0.059	0.400	0.129	–0.170	0.074	0.016	0.221	–0.061

APPENDIX 4. Continued.

Locus and statistic	*D. argentosa*			*D. diaboli*		*D. flavipinnis*		*D. serena*	*D. texensis*	*D.* sp. 4
	Devils River	San Felipe Creek	Independence Creek	Devils River	Pinto Creek	Fessenden Spring	Comal Springs	Frio River	Nueces River	El Rito Creek
Dep 33										
n	63	33	34	56	50	61	60	24	53	41
#A	8	6	6	1	1	1	1	8	21	1
A_R	6.92	5.99	6.00	1.00	1.00	1.00	1.00	7.83	13.52	1.00
H_E	0.759	0.754	0.754	0.000	0.000	0.000	0.000	0.68	0.835	0.000
P_{HW}	0.962	0.705	0.259	—	—	—	—	0.582	0.004	—
F_{IS}	–0.046	–0.045	–0.053	—	—	—	—	0.081	0.118	—
Dep 38										
n	63	33	34	56	50	61	60	24	52	41
#A	15	9	4	2	2	1	1	1	1	2
A_R	12.77	8.99	4.00	2.00	2.00	1.00	1.00	1.00	1.00	2.00
H_E	0.875	0.857	0.635	0.387	0.298	0.000	0.000	0.000	0.000	0.252
P_{HW}	0.178	0.872	0.239	1.000	0.327	—	—	—	—	0.570
F_{IS}	0.057	–0.096	0.074	0.032	–0.210	—	—	—	—	–0.159
Dep 40										
n						61	60	21	53	41
#A						3	10	22	40	8
A_R						2.92	9.99	22.00	23.52	8.00
H_E						0.232	0.788	0.965	0.958	0.743
P_{HW}						0.308	0.688	0.002	0.000	0.026
F_{IS}						0.152	–0.036	0.260	0.153	0.146
Dep 44										
n	63	33	34	56	50	61	60	24	53	41
#A	12	6	4	14	5	3	10	9	12	3
A_R	10.53	5.94	3.99	13.73	5.00	3.00	9.93	8.87	9.54	3.00
H_E	0.850	0.783	0.668	0.731	0.651	0.648	0.694	0.880	0.832	0.529
P_{HW}	0.198	0.102	1.000	0.485	0.176	0.004	0.461	0.630	0.840	0.645
F_{IS}	–0.120	0.033	–0.012	–0.051	0.078	0.038	0.063	0.005	–0.020	0.078
Dep 51										
n	63	33	34	56	50	61	60	24	53	41
#A	15	11	6	5	2	4	8	2	8	1
A_R	12.55	10.87	5.82	4.86	2.00	4.00	8.00	2.00	5.97	1.00
H_E	0.845	0.867	0.462	0.231	0.416	0.266	0.781	0.424	0.495	0.000
P_{HW}	0.774	0.822	0.137	0.608	1.000	0.012	0.009	0.346	0.858	—
F_{IS}	0.005	–0.049	0.045	–0.006	–0.010	0.199	0.039	0.214	–0.030	—

APPENDIX 4. Continued.

Locus and statistic	*D. argentosa*			*D. diaboli*		*D. flavipinnis*		*D. serena*	*D. texensis*	*D.* sp. 4
	Devils River	San Felipe Creek	Independence Creek	Devils River	Pinto Creek	Fessenden Spring	Comal Springs	Frio River	Nueces River	El Rito Creek
Dep 53										
n	63	33	34	56	50	61	60	24	53	41
#A	7	5	5	3	1	4	7	8	10	1
A_R	6.49	4.99	5.00	3.00	1.00	3.91	6.87	7.86	7.51	1.00
H_E	0.804	0.704	0.672	0.136	0.000	0.328	0.798	0.861	0.823	0.000
P_{HW}	0.179	0.508	0.981	1.000	—	0.660	0.293	0.275	0.496	—
F_{IS}	–0.026	0.139	–0.006	–0.048	—	0.100	–0.086	0.177	0.014	—
Dep 57										
n						61	60			41
#A						1	1			4
A_R						1.00	1.00			4.00
H_E						0.000	0.000			0.452
P_{HW}						—	—			0.580
F_{IS}						—	—			–0.187
Dep 61										
n	63	32	34	56	50	61	60	24	53	41
#A	4	3	5	10	3	1	1	5	11	2
A_R	3.99	3.00	5.00	9.84	3.00	1.00	1.00	5.00	8.21	2.00
H_E	0.502	0.254	0.646	0.760	0.542	0.000	0.000	0.600	0.583	0.494
P_{HW}	0.010	0.097	0.003	0.000	0.563	—	—	0.209	0.422	0.200
F_{IS}	0.178	0.260	0.362	0.366	–0.144	—	—	0.166	–0.101	–0.235
Dep 65										
n	63	33	34			61	60			41
#A	3	2	4			1	1			1
A_R	2.94	1.94	3.91			1.00	1.00			1.00
H_E	0.242	0.030	0.633			0.000	0.000			0.000
P_{HW}	0.712	—	0.917			—	—			—
F_{IS}	0.016	0.000	–0.069			—	—			—
Dep 67										
n	63	33	34	56	50	61	60	24	53	41
#A	14	6	11	3	1	1	2	7	14	5
A_R	10.37	5.94	10.65	2.86	1.00	1.00	2.00	6.625	9.44	5
H_E	0.743	0.672	0.829	0.135	0.000	0.000	0.168	0.739	0.796	0.563
P_{HW}	0.517	0.077	0.054	0.234	—	—	0.396	0.284	0.000	0.425
F_{IS}	0.060	0.189	0.220	0.208	—	—	0.108	0.098	0.431	0.047

APPENDIX 4. Continued.

Locus and statistic	*D. argentosa*			*D. diaboli*		*D. flavipinnis*		*D. serena*	*D. texensis*	*D.* sp. 4
	Devils River	San Felipe Creek	Independence Creek	Devils River	Pinto Creek	Fessenden Spring	Comal Springs	Frio River	Nueces River	El Rito Creek
Dep 73										
n	63	33	34	56	50	61	60	24	53	41
#A	15	8	12	22	5	1	1	1	4	2
A_R	13.08	7.94	11.90	21.08	4.96	1.00	1.00	1.00	3.17	2.00
H_E	0.898	0.810	0.864	0.911	0.365	0.000	0.000	0.000	0.224	0.158
P_{HW}	0.251	0.647	0.173	0.218	0.027	—	—	—	0.567	1.000
F_{IS}	0.010	–0.122	–0.021	0.039	–0.096	—	—	—	0.075	–0.081
Dep 74										
n	63	33	34	56	50	61	60	24	53	41
#A	15	8	9	9	5	3	10	12	26	2
A_R	13.16	7.87	8.82	8.86	4.98	3.00	10.00	11.46	18.84	2.00
H_E	0.860	0.677	0.807	0.794	0.623	0.532	0.727	0.861	0.944	0.302
P_{HW}	0.920	0.189	0.074	0.535	0.951	0.232	0.399	0.225	0.000	0.312
F_{IS}	0.021	–0.119	–0.020	–0.011	0.006	–0.140	0.083	0.032	0.580	–0.212
Dep 85										
n	63	33	34	56	50	61	60	24	53	41
#A	6	6	6	5	2	2	2	5	11	3
A_R	5.74	6.00	5.99	4.86	2.00	2.00	2.00	4.86	8.51	3.00
H_E	0.704	0.649	0.621	0.417	0.078	0.374	0.417	0.591	0.826	0.597
P_{HW}	0.966	0.017	0.670	0.002	1.000	0.486	0.544	0.905	0.890	0.122
F_{IS}	–0.060	0.299	0.006	0.058	–0.032	0.124	0.081	–0.129	–0.074	0.182
Dep 90										
n	63	33	34	56	50	61	60	23	53	41
#A	8	9	6	22	7	6	16	3	8	3
A_R	6.98	8.82	5.99	21.12	6.96	5.91	15.79	2.91	7.04	3.00
H_E	0.795	0.781	0.752	0.940	0.780	0.654	0.896	0.126	0.641	0.499
P_{HW}	0.825	0.771	0.002	0.000	0.396	0.212	0.925	1.000	0.051	0.234
F_{IS}	–0.018	–0.048	0.374	0.202	–0.052	0.047	–0.005	–0.031	0.000	–0.173
Dep 91										
n	63	33	34	56	50	61	60	24	53	41
#A	20	14	15	16	8	1	2	10	23	3
A_R	18.22	13.76	14.72	15.73	8.00	1.00	2.00	9.72	17.99	3.00
H_E	0.941	0.895	0.910	0.910	0.848	0.000	0.049	0.871	0.935	0.523
P_{HW}	0.018	0.854	0.290	0.002	0.003	—	1.000	0.512	0.107	0.003
F_{IS}	0.123	0.052	–0.002	0.039	0.151	—	–0.017	–0.004	0.092	–0.260

APPENDIX 4. Continued.

Locus and statistic	*D. argentosa*			*D. diaboli*		*D. flavipinnis*		*D. serena*	*D. texensis*	*D.* sp. 4
	Devils River	San Felipe Creek	Independence Creek	Devils River	Pinto Creek	Fessenden Spring	Comal Springs	Frio River	Nueces River	El Rito Creek
Dep 93										
n						61	60			41
#A						4	7			3
A_R						3.91	6.93			3.00
H_E						0.385	0.649			0.14
P_{HW}						0.124	0.568			0.003
F_{IS}						–0.022	–0.052			0.651
Dep 100										
n	63	33	34	56	50	61	60	24	53	41
#A	4	4	6	2	1	1	2	1	3	2
A_R	3.36	4.00	5.90	2.00	1.00	1.00	2.00	1.00	2.93	2.00
H_E	0.23	0.409	0.552	0.193	0.000	0.000	0.168	0.000	0.545	0.071
P_{HW}	0.662	0.105	1.000	1.000	—	—	0.397	—	0.081	1.000
F_{IS}	0.034	0.110	–0.065	–0.111	—	—	0.108	—	–0.073	–0.026
Dep 101										
n				56	50	61	60	24	53	
#A				6	3	3	5	3	9	
A_R				5.73	3.00	3.00	4.93	2.88	6.98	
H_E				0.475	0.524	0.140	0.506	0.512	0.609	
P_{HW}				0.835	0.013	1.000	0.455	0.804	0.720	
F_{IS}				–0.128	–0.030	–0.053	0.045	–0.140	0.009	
Dep 102										
n						61	60			
#A						2	3			
A_R						2.00	3.00			
H_E						0.288	0.512			
P_{HW}						0.358	0.028			
F_{IS}						0.145	–0.301			
Dep 103										
n	63	33	34	56	50	61	60	24	53	41
#A	8	6	9	3	3	2	3	5	4	4
A_R	7.69	5.93	8.74	3.00	2.98	2.00	3.00	4.75	4.00	4.00
H_E	0.739	0.582	0.823	0.374	0.512	0.485	0.386	0.582	0.732	0.510
P_{HW}	0.554	0.370	0.288	0.867	0.479	0.287	0.839	0.139	0.750	0.046
F_{IS}	–0.095	0.063	0.178	–0.051	–0.094	0.155	0.050	0.285	0.072	–0.099

APPENDIX 4. Continued.

Locus and statistic	*D. argentosa*			*D. diaboli*		*D. flavipinnis*		*D. serena*	*D. texensis*	*D.* sp. 4
	Devils River	San Felipe Creek	Independence Creek	Devils River	Pinto Creek	Fessenden Spring	Comal Springs	Frio River	Nueces River	El Rito Creek
Dep 105										
n	63	33	34	56	50	61	60	24	53	41
#A	6	4	3	1	1	3	1	6	4	1
A_R	4.87	3.94	2.91	1.00	1.00	2.84	1.00	5.95	3.42	1.00
H_E	0.248	0.530	0.190	0.000	0.000	0.033	0.000	0.659	0.383	0.000
P_{HW}	1.000	0.278	1.000	—	—	1.000	—	0.721	0.148	—
F_{IS}	–0.086	0.199	–0.085	—	—	–0.004	—	–0.012	0.114	—
Dep 106										
n	63	33	34	56	49	61	60	23	53	41
#A	8	9	7	4	1	3	3	3	4	4
A_R	7.23	8.94	6.82	3.88	1.00	3.00	3.00	2.91	3.78	4.00
H_E	0.806	0.745	0.721	0.185	0.000	0.430	0.081	0.126	0.559	0.532
P_{HW}	0.457	0.142	0.624	0.002	—	0.001	1.000	1.000	0.819	0.270
F_{IS}	0.055	0.064	0.061	0.421	—	0.123	–0.024	–0.031	0.020	–0.100
Dep 108										
n	63	33	31					22	53	41
#A	6	4	5					2	3	3
A_R	5.45	3.94	5.00					2.00	2.88	3.00
H_E	0.653	0.616	0.493					0.496	0.396	0.433
P_{HW}	0.947	0.641	0.000					1.000	0.075	0.638
F_{IS}	0.028	0.163	0.607					0.083	0.125	0.042

APPENDIX 5. Pairwise Φ_{ST} values between samples within species as measured between homologous mtDNA sequences (above diagonal). Probability (*P*) values for all tests of $\Phi_{ST} = 0$ were significant ($P < 0.05$) except for the pairwise distance between the samples of *Dionda flavipinnis* from Fessenden Springs and Comal Springs ($P = 0.104$). Distance between samples within species as measured by pairwise F_{ST} values for microsatellites (below diagonal). Probability (*P*) values for all tests of $F_{ST} = 0$ were significant ($P < 0.05$). Acronyms for samples are as follows: DaDR = *D. argentosa* from Devils River, DaSFC = *D. argentosa* from San Felipe Creek, DaIC = *D. argentosa* from Independence Creek, DdDR = *D. diaboli* from Devils River, DdPC = *D. diaboli* from Pinto Creek, DfFS = *D. flavipinnis* from Fessenden Spring, DfCS = *D. flavipinnis* from Comal Springs.

	DaDR	DaSFC	DaIC	DdDR	DdPC	DnFS	DnCS
DaDR	—	0.248	0.779				
DaSFC	0.052	—	0.808				
DaIC	0.160	0.172	—				
DdDR				—	0.252		
DdPC				0.230	—		
DfFS						—	0.158
DfCS						0.280	—