CONSERVATION GENETICS OF FIVE SPECIES OF *DIONDA* IN WEST TEXAS

A Thesis

by

ASHLEY HELEN HANNA

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2011

Major Subject: Wildlife and Fisheries Sciences

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Approved by:

Chair of Committee,	John R. Gold
Committee Members,	J. Spencer Johnston
	R. Neal Wilkins
Head of Department,	John B. Carey

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ABSTRACT

Conservation Genetics of Five Species of *Dionda* in West Texas. (December 2011)

Ashley Helen Hanna

Chair of Advisory Committee: Dr. John R. Gold

Minnows of the genus *Dionda* (Cyprinidae, Teleostei) inhabit spring-fed streams in the southwestern United States and Mexico. Five nominal species of *Dionda* (*D. argentosa*, *D. diaboli*, *D. episcopa*, *D. nigrotaeniata* and *D. serena*) are found in streams and rivers in central and west Texas. Because *Dionda* require clean, flowing water, they serve as aquatic indicator species of biological impacts of drought and human water use. Consequently, the ecological and conservation status of species of *Dionda* are important relative to monitoring habitat deterioration. This study used genetic data from geographic samples of the five nominal species of *Dionda* in Texas waters to document the conservation-genetics status of populations in each species. Fish were collected in cooperation with the Texas Parks and Wildlife Department and the U.S. Fish and Wildlife Service. Data from 585 base pairs of the mitochondrially encoded, protein coding ND-5 gene and from 21 to 33 nuclear-encoded microsatellites were used to assess genetic variation, population structure, historical demography, and genetic effective size of samples of each of the five species. The sample from Independence Creek, initially assumed to be *D. episcopa* because of its location, was found to be *D. argentosa*. Results of genetic assays indicate that each geographic sample in each

species should be treated as a separate population and managed in a way that preserves the natural diversity found within each species. Genetic data revealed that all of the populations evaluated may be compromised genetically and should be monitored further.

DEDICATION

To my fiancé, Brett, for all his patience, support, and love. He is always there to remind me that fretting yesterday won't improve tomorrow, focusing on today will.

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INTRODUCTION

Minnows of the genus *Dionda* (Cyprinidae; Teleostei) are found in the southwestern United States and Mexico. Five nominal species are found in central and west Texas: *D. argentosa*, *D. diaboli*, *D. episcopa*, *D. nigrotaeniata*, and *D. serena* (Scharpf 2005). Species of *Dionda* typically inhabit springs and spring-fed streams (Edwards et al. 2004; Hubbs and Brown 1956; Hubbs et al. 1991) and thus rely on limited underground water sources. As a consequence, species of *Dionda* are of particular interest to conservation and management as indicator species of water quality (Harvey 2005; Edwards et al. 2004). Unfortunately, overexploitation of water resources and drought in west Texas, especially in recent decades, has depleted natural aquifers, causing the springs and spring-fed streams in which *Dionda* reside to desiccate, thus threatening native *Dionda* populations (Brune 2002). Populations of *Dionda* are also threatened by pollution and invasive species (TWAP 2005; López-Fernández and Winemiller 2005). In order to preserve and manage the biodiversity represented by the species of *Dionda*, state and federal management plans require information on the genetic status of each species, including genetic diversity, genetic differences between localities, effective population size, and population growth or decline.

Conservation genetics and genetic markers

Genetic analysis is an important tool that aids in establishing the conservation status of populations and guiding the steps taken to handle imperiled populations. A

This thesis follows the style of *Conservation Genetics.*

basic understanding of both the quantity of genetic variation and its spatial distribution is important for management decisions, which ultimately preserve unique genetic resources and the adaptive ability of populations (Meffe 1990). Geographically defined populations, however, do not always correlate with genetically distinct populations. Waples (1991) defined an evolutionarily significant unit (ESU) in order to interpret the Endangered Species Act (ESA) term 'distinct population segment'. According to Waples (1991), an ESU is reproductively isolated and evolutionarily important to the species. Moritz (1994) refined the definition of an ESU to include only samples that are reciprocally monophyletic for mitochondrial haplotypes, but added that genetic distance and diversity could lead to identification of different management units (MU). Palsbøll et al. (2007) argued that although current criteria for defining MUs focus on rejecting panmixia, demographic independence, defined through interpretation of genetic divergence, better defines MUs for conservation. A point to note, however, is that even when populations experience sufficient gene flow to homogenize allele frequencies, geographical differences can cause demographic changes that may distinguish populations for conservation and management (Emerson et al. 2001). With the units of conservation defined by factors such as genetic diversity, effective population size, and historical demography, management actions can be taken. A genetic study of *Dionda* may thus determine if the different geographic localities of different species represent genetically defined populations and assess how those populations differ from one another.

Measurement of genetic variation and diversity in conservation genetics typically utilizes both mitochondrial (mt)DNA and nuclear-encoded DNA sequences (Avise 1994; Avise et al. 1995; Haig 1998; Sunnucks 2000). Assessment of mtDNA focuses on individual mtDNA haplotypes, where each haplotype represents a unique mtDNA sequence. Genetic variation of mtDNA is assessed by number of haplotypes, haplotype richness, haplotype diversity, and nucleotide diversity (Hedrick 2005; Nei and Li 1979; Nei and Tajima 1981). Assessment of nuclear-encoded DNA sequences in diploid species focuses on allelic and genotypic variation and is assessed by allelic richness and gene diversity, where the latter is the expected number of heterozygous genotypes under Hardy-Weinberg expectations (Nei 1987; Nei and Tajima 1981; Petit et al. 1998).

Another important measure of the conservation-genetics status of a population is effective population size (N_e). By definition (Wright 1931; Lande and Barrowclough 1987), effective population size represents the size of an ideal population which matches the level of genetic drift found in an actual population. Effective population size can be estimated using both maternally inherited sequences such as mtDNA and nuclear-encoded sequences; estimates based on mtDNA, however, generate female effective size (N_{ef}). As a contributing factor to the genetic viability of a population, effective population size is a central aspect to consider when assessing the conservation status of a population (Lande and Barrowclough 1987).

Status, range, and ecology of Dionda *in Texas*

The five species of *Dionda* in Texas are currently placed into different conservation categories. *Dionda diaboli* is considered threatened by both the United States and the

State of Texas (USFWS 1999). The federal Recovery Action Plan for *D. diaboli* includes evaluation of geographic variation of genetic structure and the development of a genetics management plan (USFWS 2005). Scharpf (2005) listed both *D. argentosa* and *D. serena* as imperiled, whereas *D. nigrotaeniata* and *D. episcopa* were considered secure (Scharpf 2005). The present-day range of *Dionda* in Texas is shown in Figure 1. *Dionda argentosa* is found in tributaries in the Rio Grande drainage, namely the Devils River, San Felipe Creek, and Sycamore Creek (Hubbs et al. 1991). However, Carson et al. (2010) extended the range of *D. argentosa* to include at least part of the lower Pecos river basin. Extant populations of *D. diaboli* are found in the Rio Grande basin, in the Devils River, San Felipe Creek, and Pinto Creek (Scharpf 2005). The population of *D. diaboli* in Las Moras Creek appears to have been extirpated (Garrett et al., 1992) and the status of *D. diaboli* in Sycamore Creek is uncertain, as Garrett et al. (1992) only collected *D. diaboli* from a single, stagnant pool. *Dionda episcopa* inhabits the Pecos River in Texas and New Mexico, the Rio Grande near Big Bend, and Rio Grande tributaries in Mexico (Scharpf 2005). This range has been reduced by the report of Carson et al. (2010), as populations in Independence Creek, previously assumed to be *D. episcopa* (Scharpf 2005; Sublette et al. 1990), were found to be *D. argentosa*. *Dionda nigrotaeniata* occupies the Colorado and Guadalupe river drainages (Edwards et al. 2004), as well as the San Antonio River Basin (Scharpf 2005). *Dionda serena* inhabits the Frio, Nueces, and Sabinal rivers in the Nueces River basin (Scharpf 2005).

The spring-dwelling nature of *Dionda* contributes to the relative isolation of current populations. The samples of *Dionda* used in this study are included in drainages of the

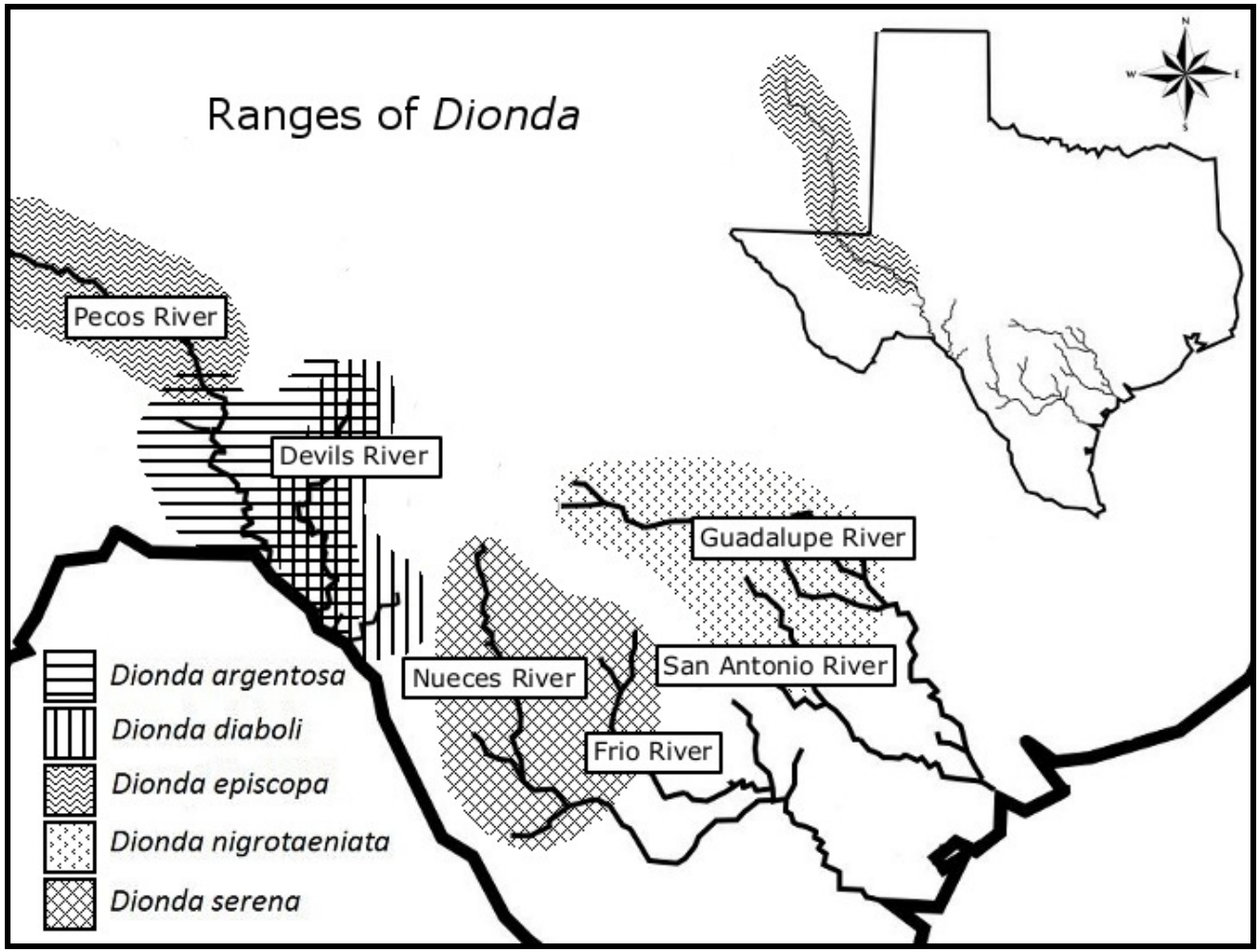

Figure 1. Approximate ranges of species of *Dionda* in Texas. Each species is sparsely distributed within the range area, often confined to springs or small streams. The boundary between the ranges of *D. argentosa* and *D. episcopa* in the Pecos River is unknown.

lower Rio Grande River and the western Gulf Coast. While the lower Rio Grande has followed much the same course since the Tertiary, the Pecos River changed its course and increased in size due to stream capture (Mayden 1992). The histories of smaller tributaries, such as Pinto Creek, are not well known, but flooding and stream capture may have occurred (Hubbs 1957). The Colorado, Guadalupe, Nueces and Frio rivers may once have been connected during times of lower sea levels, allowing for fish dispersal between rivers (Mayden 1992).

The five species of *Dionda* in Texas are similar in ecological preferences and life history. All five require clear, spring-fed headwaters and spring runs with little temperature variation (Hubbs and Brown 1956; Hubbs et al. 1991), and all five are algivores with a long, coiled intestinal tract and a subterminal mouth useful for substrate grazing (Hubbs et al. 1991). Gibson et al. (2004) found that captive *D. diaboli* spawned over gravel substrate in pool and riffle areas and did not construct nests or egg clusters. Similarly, Hubbs (1951) reported that spawning females of *D. serena* in the wild released heavy, non-adhesive eggs which became lodged in the gravel. Wayne (1979) found that the number of separated mature ova among 76 female *Dionda* sampled from Fessenden Spring, Comal Spring, and the San Marcos River ranged from 165 to 350.

Prior genetic studies of Dionda *and other imperiled freshwater fishes*

Prior genetics-based studies of *Dionda* have focused mainly on systematics. Mayden et al. (1992) used allozyme products from 32 protein-coding genes to infer a phylogeny of the genus. Samples from the Guadalupe and Colorado drainages, previously identified as *D. episcopa*, were hypothesized by Mayden et al. (1992) to be a

diagnosable species. Specimens from these drainages are currently considered to be *Dionda nigrotaeniata* (Gilbert 1998). Gold et al. (1992) documented chromosome numbers, chromosomal nucleolar organizer regions (NORs), and genome sizes for the five species of *Dionda* in Texas waters and found that these species differed less from one another than is common among groups of other cyprinid species. A recent study by Schönhuth et al. (2008) compared sequences from the mitochondrial cytochrome *b* gene and three nuclear genes (S7, Rhodopsin, *Rag*1) across 15 species of *Dionda* and 32 related cyprinid species. Morphological, osteological, and allozyme studies (Mayden et al. 1992) supported the hypothesis that *Dionda* is a monophyletic assemblage; results of the study by Schönhuth et al. (2008), however, indicated that *Dionda* was polyphyletic and therefore an artificial classification. They argued that the southern species of *Dionda* belonged in their own genus, *Tampichthys*. The species of *Dionda* that are the focus of this study belong to the monophyletic northern clade of *Dionda* (Schönhuth et al. 2008).

Genetic analysis, using mtDNA sequences and microsatellites, has been applied to management efforts for other small headwater and spring fish. Burridge and Gold (2003) used ten microsatellites and one anonymous nuclear locus to assess genetic diversity in the critically endangered Cape Fear shiner, *Notropis mekistocholas*. Their goals were to measure genetic diversity, evaluate if that diversity was affected by small population sizes, ascertain if different geographical groups exhibited genetic differences, and assess changes in the effective population size of each sample. Saillant et al. (2004) extended the work on Cape Fear shiners by using 22 microsatellites, one anonymous

(nuclear) locus, and sequences from the mitochondrial ND-5 (258bp) and ND-6 (367bp) genes. They found significant genetic heterogeneity between Cape Fear shiners at two localities; Bayesian coalescent analysis of the microsatellite data indicated a recent decline in effective population size. Parker et al. (1999) compared genetic variation among samples of the endangered Gila topminnow (*Poeciliopsis o. occidentalis*) in the four watersheds where it still occurs. They concluded that populations in all four watersheds should be considered distinct ESUs based on genotypes at five polymorphic microsatellites and results from previous studies of allozymes, a major histocompatability-complex locus, ecology and biology (Parker et al. 1999 and references therein). Parker et al. (1999) asserted that while microsatellites alone should not determine whether populations are ESUs, microsatellites are a valuable tool in the decision making process. Stockwell et al. (1998) recommended two ESUs for the White Sands pupfish (*Cyprinodon tularosa*), using a 482 base-pair (bp) segment of the mtDNA control region. Alves et al. (2001) assessed variation of the mtDNA cytochrome *b* gene in the endangered Iberian cyprinid *Anaecypris hispanica*, defining three ESUs and finding evidence of additional conservation units. Salgueiro et al. (2003) assessed microsatellite diversity in *Anaecypris hispanica*, finding the overall genetic diversity of the species was distributed across several populations. Osborne and Turner (2006) used microsatellites and sequences from the mitochondrial ND-4 gene (322bp) to evaluate the diversity, population structure, and historical demography of the threatened bluntnose shiner (*Notropis simus pecosensis*). They found reasonably high levels of genetic diversity and a high number of rare haplotypes. They also concluded that the population

of bluntnose shiner in the Pecos River was panmictic and had experienced demographic decline. Alò and Turner (2005) evaluated the effects of river fragmentation on the endangered Rio Grande silvery minnow (*Hybognathus amarus*) through assessment of patterns of genetic diversity. They concluded that, as a result of interactions between life history and river fragmentation, the genetic effective size of the Rio Grande silvery minnow was too small to preserve long term genetic viability.

Goal of this study

The goal of this study was to assess the population-genetic status of populations of the five species of *Dionda* that inhabit Texas waters. This study employed statistical analysis of genetic data derived from nuclear-encoded microsatellites and a fragment of a protein-coding mitochondrial gene. Genetic variation and diversity, effective size, and growth or decline of each population was assessed.

MATERIALS AND METHODS

Sampling, ND-5 sequencing, and microsatellite genotyping

Samples of *Dionda argentosa*, *D. diaboli*, *D. nigrotaeniata* and *D. serena* were obtained by seine, with the assistance of personnel from the Texas Parks and Wildlife Department (TPWD) and the United States Fish and Wildlife Service (USFWS); samples were obtained between March and August of 2008 under permits SPR-0390-045 (TPWD) and TE676811 (USFWS). Samples of *D. episcopa* from a March 2007 collection were provided by personnel of the Museum of Southwestern Biology at the University of New Mexico. A listing of the samples, by collection locality and species, and sample sizes may be found in Table 1. A map of the collection localities is given in Figure 2. Individual specimens were stored in 95% ethanol, with the exception of the samples of *D. episcopa*, which were provided as fin clips in 70% ethanol. Tissues (muscle and/or fin) were removed from each fish and DNA was extracted using either the standard phenol-chloroform protocol of Sambrook et al. (1989) or the DNEASY Blood and Tissue Kit (QIAGEN, www.qiagen.com).

A portion of the mitochondrial ND-5 gene was sequenced for a subset of individuals from each of the 10 sample localities. Polymerase chain reaction (PCR) primers L12328 (5'-AACTCTTGGTGCAAMTCCAAG-3') and H13393 (5'- CCTATTTTKCGGATGTCTTGYTC-3'), designed from ND-5 sequences of the cyprinid *Cyprinus carpio* by Miya et al. (2006), were used to obtain preliminary sequences from three individuals of each species of *Dionda*. PCR primers DS-H (5'- AAAAATTTGTTGAATTTCTCAGGA-3') and

Table 1. Species, sample localities, and sample sizes of *Dionda* examined in the study. Based on drainage, the sample of *D. argentosa* from Independence Creek (Pecos River drainage) initially was thought to be *D. episcopa*. Genetic data (Carson et al. 2010; this study) revealed this population to be *D. argentosa*, not *D. episcopa*. Voucher specimens are stored in the Texas Cooperative Wildlife Collection (TCWC). Voucher numbers of specimens, from each sample, used for mtDNA data, microsatellite data, or both 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. nigrotaeniata* Fessenden Spring (14786.01-14846.01), *D. nigrotaeniata* Comal Springs (15064.01-15123.01), *D. serena* Nueces River (14273.01-14286.01, 14475.01-14485.01, 14489.01-14515.01, 14517.01), *D. serena* Frio River (14268.01-14272.01, 14461.01-14474.01, 14974.01-14978.01). As specimens of *D. episcopa* from El Rito Creek were provided by the Museum of Southwestern Biology, voucher samples (MSB054.21-61) remain there.

Species	Sample location	Drainage	# Individuals	Date sampled	Coordinates
Dionda argentosa	Devils River (TX)	Rio Grande	71	3/13/2008	29°53′N 100°59′W
	San Felipe Creek (TX)	Rio Grande	33	4/25/2008	29°21′N 100°53′W
	Independence Creek (TX)	Pecos	34	8/31/2008	30°28′N 101°48′W
Dionda diaboli	Devils River (TX)	Rio Grande	56	3/13/2008	29°53′N 100°59′W
	Pinto Creek (TX)	Rio Grande	50	7/1/2008	29°24′N 100°27′W
Dionda episcopa	El Rito Creek (NM)	Pecos	41	3/23/2007	33°18′N 104°41′W
Dionda nigrotaeniata	Fessenden Spring (TX)	Guadalupe	61	3/12/2008	30°10′N 99°20′W
	Comal Springs (TX)	Guadalupe	60	8/6/2008	29°43′N 98°7′W
Dionda serena	Nueces River (TX)	Nueces	56	7/3/2008	29°48′N 100°0′W
	Frio River (TX)	Nueces	24	7/3/2008	29°50′N 99°46′W

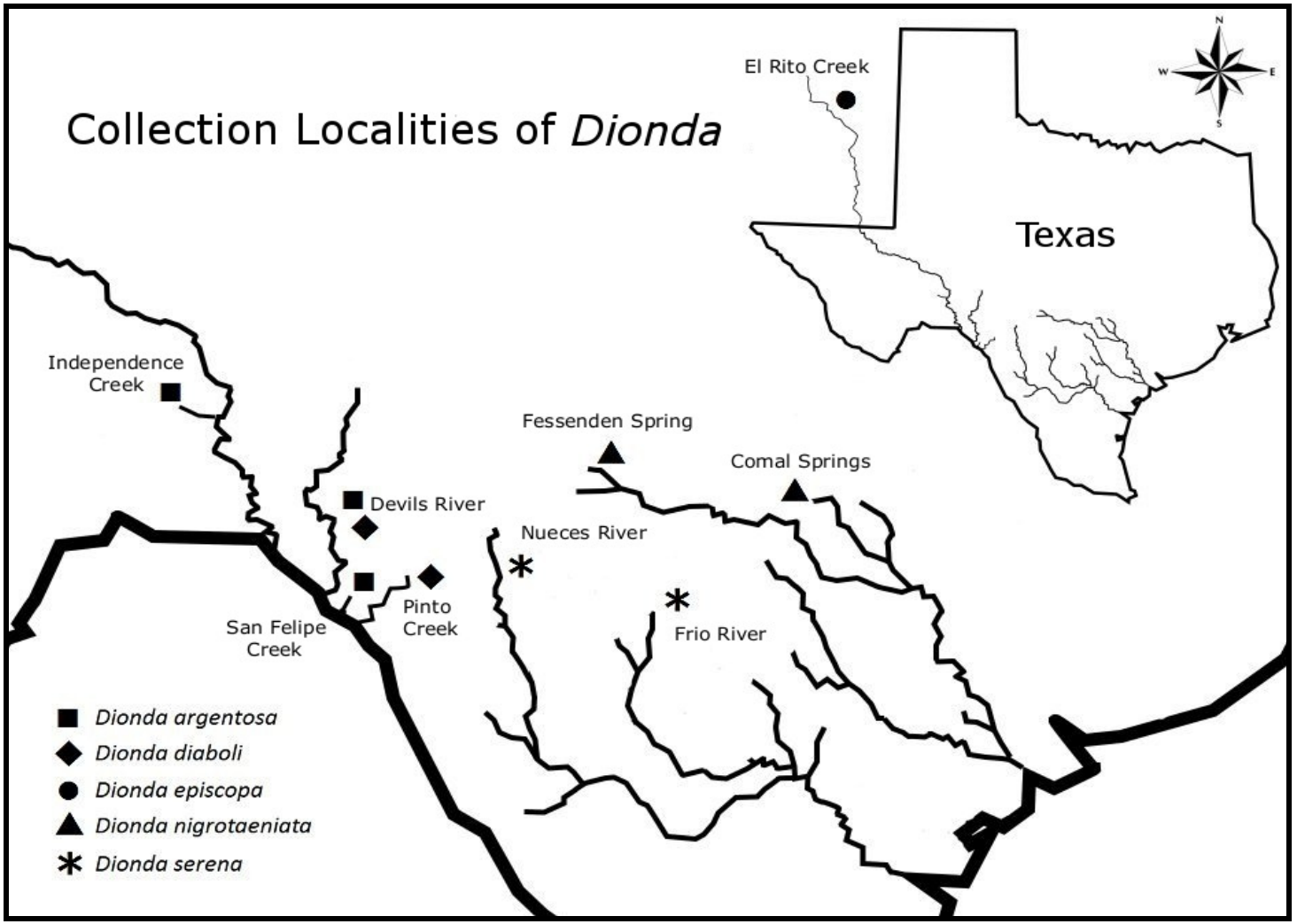

Figure 2. Collection localities of *Dionda* examined in this study.

AEN-H (5'- CAGGAGTTTATATTTATTGCAAAT-3') were then developed from ND-5 sequences of all five species. The L12328 (Miya et al. 2006) and DS-H primers were ultimately used to amplify a 597 bp segment of ND-5 from all five species, with L12328 being the forward primer. PCR amplifications were conducted using a PTC-200 thermal cycler (MJ Research) and 50 µL reactions containing 100 ng DNA, 1x PCR buffer, 0.5 U *Taq* DNA polymerase (GoTaq Flexi DNA Polymerase, Promega), 0.5 µM of each primer, 0.8 mM dNTPs, and 1.5 mM $MgCl_2$. Amplifications used a profile of initial denaturation at 95°C for 3 min, 35 cycles of 95°C for 30 sec, 48°C for 1 min and 72°C for 1 min, and final extension of 72°C for 10 min. Double-stranded PCR amplification products from single individuals were band-cut from 2% agarose gels and purified with QIAquick Gel Extraction Kit (QIAGEN, www.qiagen.com). Sequencing was carried out with the BigDye Terminator Kit (Applied Biosystems), on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Sequences were analyzed in SEQUENCHER v. 3.0 (Gene Codes, http://www.genecodes.com/). Computer-generated sequences were checked by eye to ensure accurate base calling. 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 ND-5 fragment. Unique haplotypes were identified using MEGA v. 4.0.2 (Kumar et al. 1994), http://www.megasoftware.net/, and assigned a haplotype number.

Variation at 28-34 nuclear-encoded microsatellites was assessed for individuals at each sample locality. PCR reaction conditions and primers for each microsatellite are given in Renshaw et al. (2009); microsatellites assessed for each sample can be found in Table 2. 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, using an ABI PRISM 377 DNA Sequencer (Applied Biosystems). Sizes of microsatellite fragments were assessed 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.

Table 2. A list of microsatellites used to genotype samples of five species of *Dionda*. Primers were developed from clones of genomic DNA of *Dionda episcopa*. Primer sequences and other information regarding each microsatellite may be found in Renshaw et al. (2009).

Samples	Microsatellites
All *Dionda*	*Dep* 1, 10, 21, 30, 32, 33, 38, 44, 51, 53, 61, 67, 73, 74, 85, 90, 91, 100, 103, 105, 106
D. argentosa	*Dep* 2, 7, 9, 13, 28, 65, 101, 108
D. diaboli	*Dep* 2, 3, 7, 8, 13, 18, 101
D. episcopa	*Dep* 2, 3, 7, 8, 9, 18, 20, 28, 40, 57, 65, 93, 108
D. nigrotaeniata	*Dep* 2, 3, 13, 18, 20, 28, 40, 57, 65, 93, 101, 102
D. serena	*Dep* 3, 7, 8, 9, 12, 18, 40, 101, 108

MtDNA and microsatellite data analysis

For mtDNA, number of haplotypes, haplotype richness, and haplotype diversity were generated for each sample locality, using FSTAT v. 2.9.3.2 (Goudet 1995), http://www2.unil.ch/popgen/softwares/fstat.htm. Nucleotide diversity was measured using DNASP v. 5.10.00 (Rozas et al. 2003), http://www.ub.edu/dnasp/. Haplotype richness, haplotype diversity, and nucleotide diversity were compared across samples by generating 95% confidence intervals, using coalescent modeling in DNASP (Rozas et al.

2003). Homogeneity of haplotype distributions between or among samples within each species was tested via global exact tests and analysis of molecular variance (AMOVA), using GENEPOP v. 4.0.10 (Raymond and Rousset 1995; http://kimura.univ-montp2.fr/~rousset/Genepop.htm) and ARLEQUIN v. 3.11 (Excoffier et al. 1992; Schneider et al. 2000; http://cmpg.unibe.ch/software/arlequin3/), respectively. Pair-wise exact tests and pair-wise Φ_{ST} values also were used to test homogeneity of haplotype distributions between or among localities within each species and to assess the magnitude of genetic difference. Pair-wise exact tests were carried out using GENEPOP (Raymond and Rousset 1995), while Φ_{ST} values were determined using ARLEQUIN (Excoffier et al. 1992; Schneider et al. 2000). Genetic distances between samples within species were calculated as pair-wise Φ_{ST} values, using FSTAT.

Tests of selective neutrality, measured as Fu and Li's D^* and F^* (1993) and Fu's F_S (1997) metrics, were performed for each sample, using DNASP. 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).

Microsatellite data were organized by sample locality and formatted into appropriate input files. Each microsatellite in each sample was tested for conformance to Hardy-Weinberg equilibrium; significance testing of departure from Hardy-Weinberg equilibrium was carried out in GENEPOP, using a Markov chain method of 500 batches of 5000 iterations (Guo and Thompson 1992). Sequential Bonferroni correction (Rice

1989) was applied for all multiple tests performed simultaneously. Occurrence of allelic dropout, large-allele dropout, short-allele dominance, stuttering, and null alleles was assessed via analysis with MICROCHECKER (van Oosterhout et al. 2004), http://www.microchecker.hull.ac.uk/. Microsatellites were considered problematic based on two criteria: significant deviation from Hardy-Weinberg equilibrium (after Bonferroni correction), and possible amplification errors and/or null alleles as indicated by MICROCHECKER. Based on those criteria, a set of experimentally tractable microsatellites was determined for each species, and subsequent analyses were carried out using those experimentally tractable microsatellites.

Each sample locality in each species was assessed for number and frequency of alleles, allelic richness, gene diversity (expected heterozygosity), and F_{IS} (inbreeding coefficient), measured as *f* of Weir and Cockerham (1984) and using FSTAT. Confidence intervals (95%) around mean observed values for allelic richness and gene diversity in each sample were generated in SPSS v. 16 (SPSS Inc.), compared across samples, and compared to measures of mtDNA diversity. Homogeneity of allelic richness and gene diversity between or among samples of the same species was tested using Wilcoxon signed-rank tests as implemented with SPSS (SPSS Inc.). Exact tests of homogeneity in microsatellite allele and genotype distributions between or among samples of each species were carried out using GENEPOP (Raymond and Rousset 1995). Significance testing used a Markov chain method of 500 batches of 5,000 iterations; probability values were adjusted with a sequential Bonferroni approach. The three samples of *D. argentosa* were assessed further for homogeneity of allelic and genotypic distributions,

using pair-wise exact tests. Tests of homogeneity employing AMOVA also were carried out within each species, using ARLEQUIN; 10,000 permutations were used to test significance. Genetic distances between samples within species were calculated as pair-wise F_{ST} values, using FSTAT. Threshold F_{ST} values for further assessment of demographic independence (Palsbøll et al. 2007) were defined based on minimum estimates of N_e (see below) and a 10% dispersal rate between populations (Hastings 1993).

Tests of genotypic independence between pairs of microsatellites was carried out using the linkage disequilibrium test in GENEPOP (Raymond and Rousset 1995). Monomorphic loci were excluded. Significance was assessed using a Markov chain method of 500 batches of 5,000 iterations per batch. Results of significance testing were then evaluated using sequential Bonferroni correction.

Estimation of effective size

The linkage disequilibrium method (LDNE) of Waples and Do (2008) was used to generate an estimate of contemporaneous number of breeders (N_b). The 2% threshold for exclusion of rare alleles was used, as recommended by Waples and Do (2010), and the jackknife method was used to calculate 95% confidence intervals. Confidence intervals using the 1% and 5% thresholds for exclusion of rare alleles, and parametric 95% confidence intervals, are available from the author. Maximum-likelihood estimation of average, long-term effective population size (N_{eLT}) was carried out in MIGRATE v.3.0.3 (Beerli and Felsenstein 1999; 2001), http://popgen.sc.fsu.edu/Migrate/Migrate-n.html. Initial runs were performed to

determine an initial estimate of theta (Θ), which then served as starting parameters for longer runs. Long runs used ten short chains with 10,000 sampled gene trees, four long chains with 5,000,000 sampled gene trees, and a burn-in of 50,000. Estimation of the average mutation rate (μ) across microsatellites, generated by MSVAR (see below), were used to calculate effective population size, using the equation $\Theta = 4N_e\mu$.

Average, long-term effective population size and time since divergence were estimated using the Bayesian coalescent approach in MSVAR v.4.1b (Beaumont 1999; Storz and Beaumont 2002), available at http://www.rubic.rdg.ac.uk/~mab/software.html. Parameters of current effective size (N_1), ancestral effective size (N_0), average mutation rate per generation (μ), and generations since the population size change began (t_a) were estimated. Initial parameters were set to a generation time of two 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 5,000 years. Runs used 20,000 data points and a burn in of 2,000. Output from MSVAR was assessed, using SAS v.9.2 (SAS Institute), for density estimated mode, 2.5 percentile, and 97.5 percentile values.

Possible 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 (http://swfsc.noaa.gov/textblock.aspx?Division=FED&id=3298); critical values of *M*, referred to as M_C, were estimated using Critical_M (Garza and Williamson 2001). 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 two and theta values generated using MIGRATE (Beerli and Felsenstein 1999; 2001) were tested.

RESULTS

Analysis of mtDNA variation

Sequence alignment of ND-5 fragments was straightforward as conserved regions facilitated alignment across species; no insertions or deletions (indels) were detected in any sequence. Based on locality, the sample from Independence Creek initially had been assumed to be *D. episcopa*. When sequences were aligned, all individuals from Independence Creek matched closely to *D. argentosa*, not *D. episcopa*. Microsatellite genotypes also indicated that the sample from Independence Creek was *D. argentosa*. Carson et al. (2010) further explored this finding.

A total of 41 haplotypes were found across the five species; none of the haplotypes were shared among any of the species. The spatial distribution of haplotypes among samples and GenBank accession number for each haplotype are given in Table A1. Summary statistics for mitochondrial sequences acquired from each sample are shown in Table 3. These statistics consist of sample size, number of haplotypes, haplotype richness, haplotype diversity, and nucleotide diversity. No mtDNA variation was found for the El Rito Creek sample of *D. episcopa* or the Comal Springs sample of *D. nigrotaeniata*. Median-joining, haplotype networks for the remaining four species are presented in Figure 3. Two of the samples of *D. argentosa* (Devils River and San Felipe Creek) and both samples of *D. diaboli* and *D. nigrotaeniata* (Figures 3a-c) shared mtDNA haplotypes; the third sample of *D. argentosa* (Independence Creek) possessed unique haplotypes (Figure 3a) as did both samples of *D. serena* (Figure 3d). In the

Table 3. Summary statistics for mtDNA data. Abbreviations: n = sample size, H = number of haplotypes, H_R = haplotype richness, H_E = haplotype diversity, π = nucleotide diversity.

Sample	n	H	H_R	H_E	π
D. argentosa					
Devils River	26	7	6.89	0.692	0.0015
San Felipe Creek	20	3	3.00	0.626	0.0015
Independence Creek	26	3	2.95	0.280	0.0005
D. diaboli					
Devils River	23	7	6.97	0.700	0.0020
Pinto Creek	21	3	3.00	0.567	0.0010
D. episcopa					
El Rito Creek	22	1	1.00	0	0
D. nigrotaeniata					
Fessenden Spring	20	2	2.00	0.337	0.0006
Comal Springs	20	1	1.00	0	0
D. serena					
Nueces River	24	14	13.87	0.906	0.0044
Frio River	21	5	5.00	0.352	0.0008

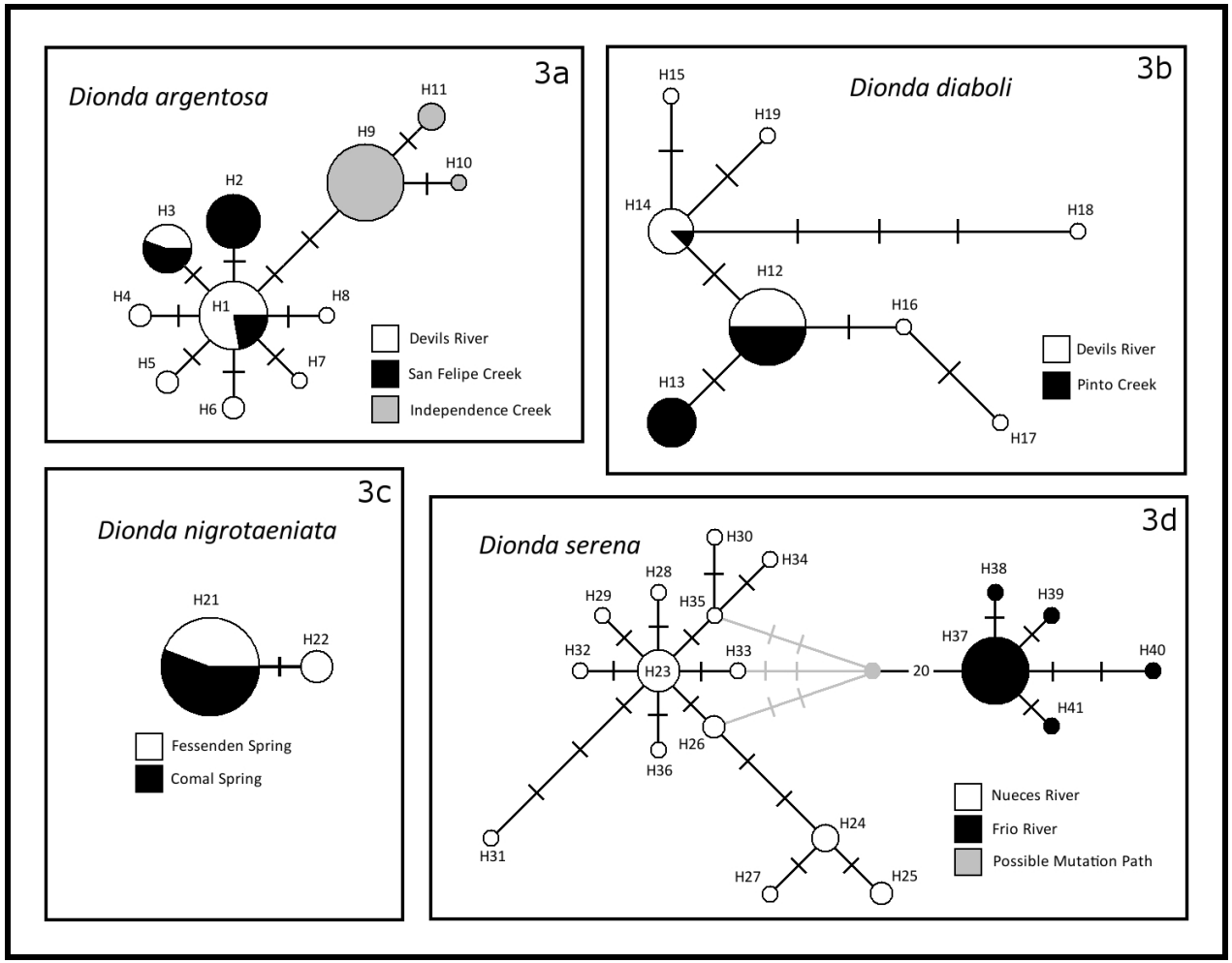

Figure 3. Median-joining networks of mtDNA haplotypes in each of four species of *Dionda*. A network is not shown for *D. episcopa* as only a single haplotype was found in the sample from El Rito Creek. Each hash mark indicates a single base-pair substitution between adjacent haplotypes.

latter, the clade of haplotypes found in *D. serena* from the Nueces River were separated from the clade of haplotypes found in *D. serena* from the Frio River by at least 22 single nucleotide substitutions.

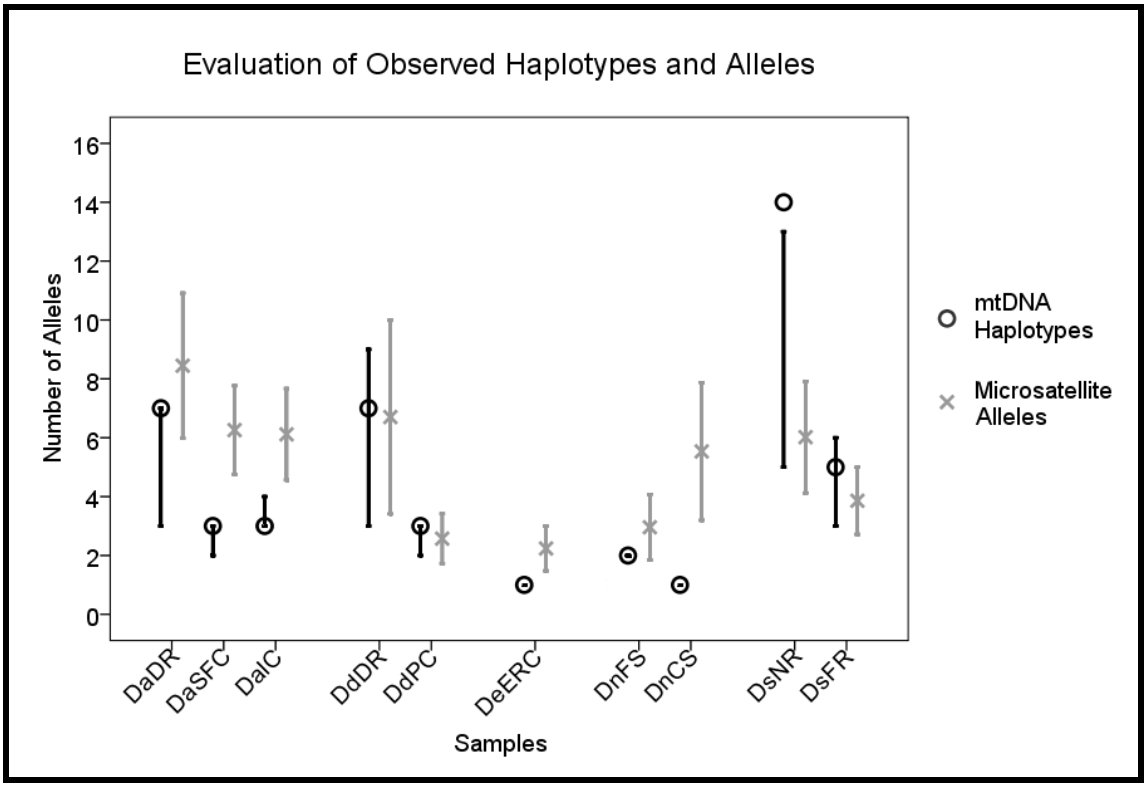

Figure 4. Comparison among samples within each species of number of mtDNA haplotypes (alleles) and average number of alleles per microsatellite. Confidence intervals for number of haplotypes were based on coalescent evaluation with DNASP (Rozas et al., 2003). Average number of alleles was evaluated using 95% confidence intervals calculated in SPSS. 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, DeERC = *D. episcopa* from El Rito Creek, DnFS = *D. nigrotaeniata* from Fessenden Spring, DnCS = *D. nigrotaeniata* from Comal Springs, DsNR = *D. serena* from Nueces River, and DsFR = *D. serena* from Frio River.

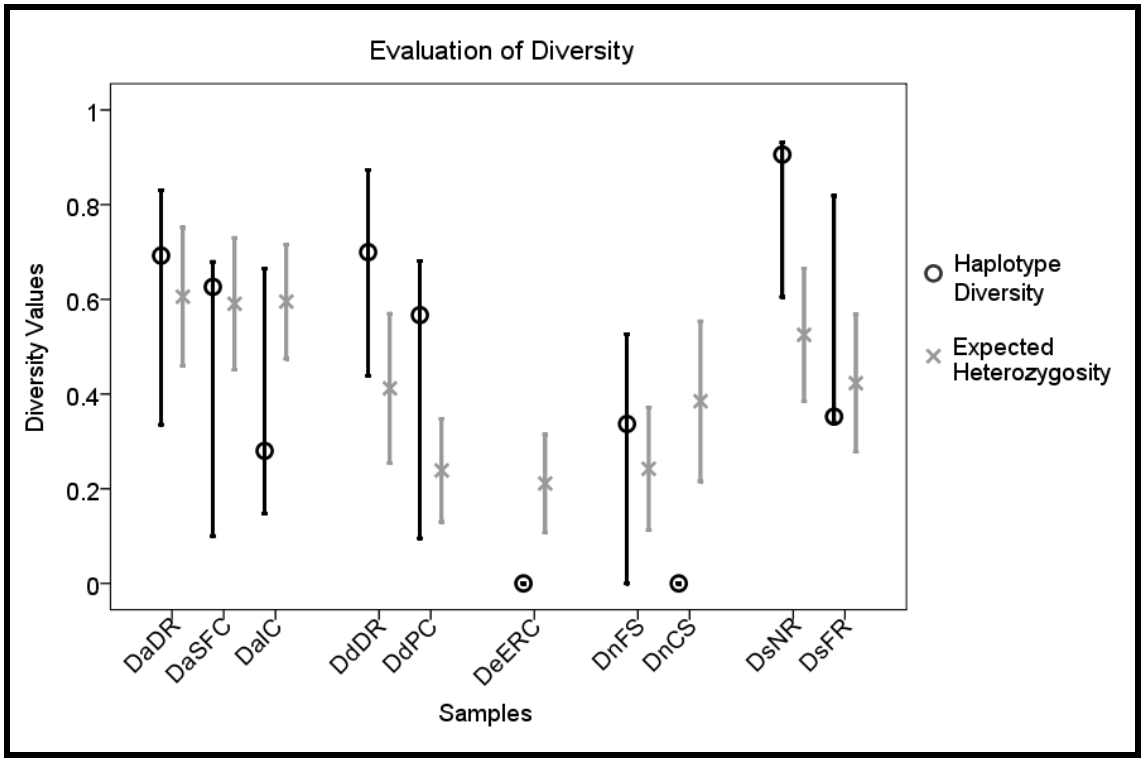

Figure 5. Comparison among samples within each species of haplotype diversity of mtDNA sequences and expected heterozygosity of microsatellites. Confidence intervals for measures of haplotype diversity were based on coalescent evaluation with DNASP (Rozas et al., 2003). Expected heterozygosity was evaluated using 95% confidence intervals (calculated using SPSS). 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, DeERC = *D. episcopa* from El Rito Creek, DnFS = *D. nigrotaeniata* from Fessenden Spring, DnCS = *D. nigrotaeniata* from Comal Springs, DsNR = *D. serena* from Nueces River, and DsFR = *D. serena* from Frio River.

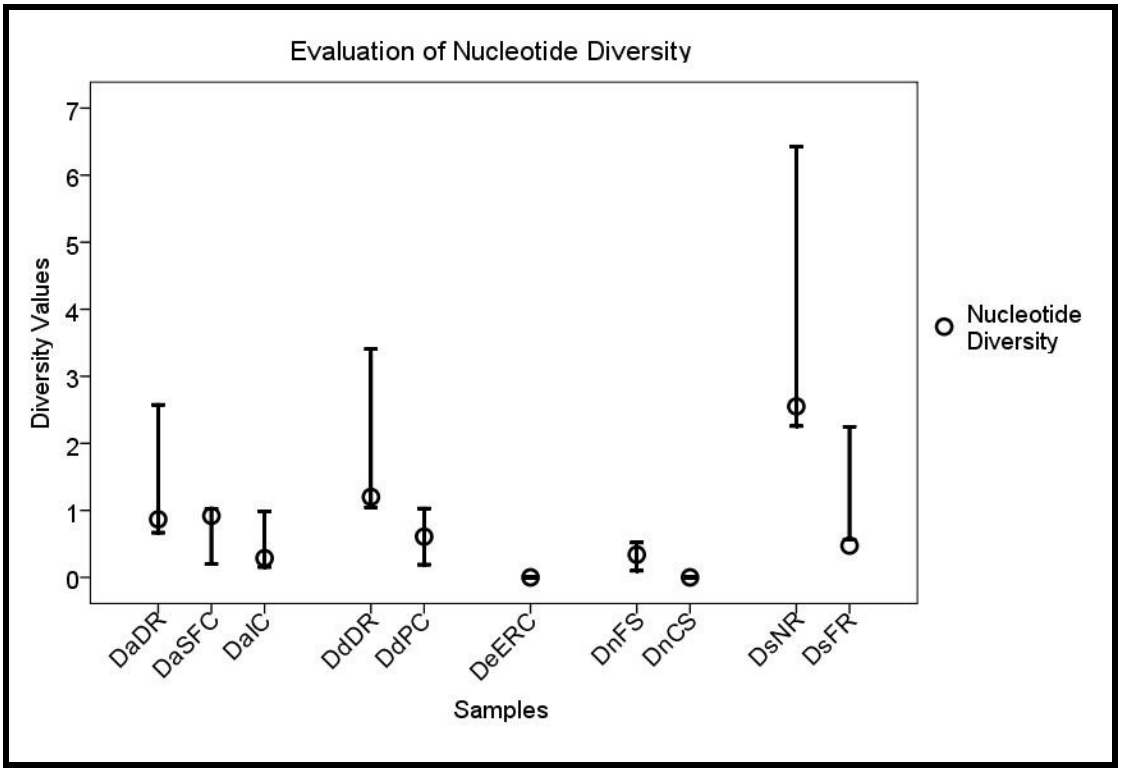

Figure 6. Comparison of nucleotide diversity of mtDNA sequences among samples. Confidence intervals were based on coalescent evaluation with DNASP (Rozas et al., 2003). Acronyms for samples are as follows: DaDR = *D. argentosa* from Devils River, DaSFC = *D. argentosa* from San Felipe Creek, DaIC = *D. argentosa* from Independence Creek, DdDR = *D. diaboli* from Devils River, DdPC = *D. diaboli* from Pinto Creek, DeERC = *D. episcopa* from El Rito Creek, DnFS = *D. nigrotaeniata* from Fessenden Spring, DnCS = *D. nigrotaeniata* from Comal Springs, DsNR = *D. serena* from Nueces River, and DsFR = *D. serena* from Frio River.

Confidence intervals for measures of homogeneity in number of haplotypes (Figure 4), haplotype diversity (Figure 5), and nucleotide diversity (Figure 6) were estimated using coalescent simulations (Rozas et al. 2003). Significant differences in the number of mtDNA haplotypes (Figure 4) between or among samples within species were found in *D. argentosa* (fewer haplotypes in San Felipe Creek and Independence Creek), *D. diaboli* (fewer haplotypes in Pinto Creek), and *D. serena* (fewer haplotypes in the Frio River). No haplotype variation was found in the sample of *D. episcopa* from El Rito Creek or in the sample of *D. nigrotaeniata* from Comal Spring; only two haplotypes were found in the sample of *D. nigrotaeniata* from Fessenden Springs. Significant differences in mtDNA haplotype diversity (Figure 5) were found in *D. argentosa* (lower diversity in Independence Creek) and *D. serena* (lower diversity in the Frio River). Average haplotype diversity in *D. diaboli* from Pinto Creek was less than the lower 95% interval of haplotype diversity in *D. diaboli* from the Devils River, and there was no haplotype diversity in the lone sample of *D. episcopa* or in the sample of *D. nigrotaeniata* from Comal Springs. Significant differences in nucleotide diversity (Figure 6) were found in *D. argentosa* (lower diversity in Independence Creek), *D. diaboli* (lower diversity in Pinto Creek), *D. nigrotaeniata* (lower diversity in Comal Springs), and *D. serena* (lower diversity in the Frio River). There was no nucleotide diversity found in the sample of *D. episcopa* or in the sample of *D. nigrotaeniata* from Comal Springs.

Estimates of Fu and Li's (1993) F^* and D^* (Table 4) metrics were negative and differed significantly from zero prior to Bonferroni correction for the sample of

Table 4. Selective neutrality of *Dionda* samples. Selective neutrality was measured by Fu and Li's (1993) *F** and *D** and Fu's (1997) F_S metrics. Significance probabilities (*P*) were estimated using coalescent simulations in DNASP (Rozas et al., 2003). † and ‡ indicate significance before and after Bonferroni correction, respectively.

Sample	*D**	*P*	*F**	*P*	F_S	*P*
D. argentosa						
Devils River	-0.273	0.510	-0.676	0.263	-3.535	0.021†
San Felipe Creek	0.866	0.514	1.179	0.910	1.020	0.846
Independence Creek	-0.689	0.467	-0.881	0.232	-1.046	0.213
D. diaboli						
Devils River	-2.201	0.035†	-2.300	0.044†	-2.579	0.103
Pinto Creek	-0.612	0.500	-0.443	0.484	0.204	0.606
D. episcopa						
El Rito Creek	-	-	-	-	-	-
D. nigrotaeniata						
Fessenden Spring	0.650	0.309	0.653	0.582	0.721	0.583
Comal Springs	-	-	-	-	-	-
D. serena						
Nueces River	-2.258	0.037†	-2.348	0.028†	-8.090	0.002‡
Frio River	-2.865	0.016†	-3.022	0.015†	-3.068	0.022†

D. diaboli from Devils River and for both samples of *D. serena*; neither metric in these samples differed significantly from zero following correction. All other *F** and *D** values did not differ significantly from zero prior to Bonferroni correction. Fu's (1997) F_S (Table 4) metric was negative and differed significantly from zero both before and after Bonferroni correction for the sample of *D. serena* from the Nueces River. This metric was negative and differed significantly from zero before, but not after, Bonferroni correction for the sample of *D. argentosa* from the Devils River and for the sample of *D.*

serena from the Frio River. All other F_S values did not differ significantly from zero prior to Bonferroni correction.

Analysis of microsatellite variation

Summary statistics, including the number of individuals scored (*n*), number of alleles (#A), allelic richness (A_R), expected heterozygosity or gene diversity (H_E), probability of conformance to Hardy-Weinberg equilibrium expectations (P_{HW}), and the inbreeding coefficient (F_{IS}), by microsatellite for each sample are presented in Table A2. The average number of alleles per microsatellite ranged from 2.09 ± 2.15 in the El Rito Creek sample of *D. episcopa* to 9.76 ± 6.52 in the Devils River sample of *D. argentosa*; average allelic richness per microsatellite ranged from 2.24 ± 0.76 in the El Rito Creek sample of *D. episcopa* to 8.45 ± 2.47 in the Devils River sample of *D. argentosa*; and average expected heterozygosity per microsatellite ranged from 0.211 ± 0.104 in the El Rito Creek sample of *D. episcopa* to 0.606 ± 0.146 in the Devils River sample of *D. argentosa*.

Results of tests for conformance to Hardy-Weinberg equilibrium are shown for each microsatellite in each sample in Table A2. Significant deviations from Hardy-Weinberg were found both before and after Bonferroni correction in several samples (Table A3). Genotypic disequilibrium between pairs of microsatellites (3,899 pair-wise comparisons) also was assessed within each sample. A total of 145 (3.72%) pair-wise comparisons were significant before Bonferroni correction; only the comparison of *Dep*20 and *Dep*32 in the sample of *D. nigrotaeniata* from Fessenden Springs remained significant after Bonferroni correction. Potential amplification errors identified, in several samples, by

MICROCHECKER also are given in Table A3. A set of microsatellites (Table 5) considered experimentally tractable in each species were then chosen for subsequent analyses. Experimentally tractable microsatellites were those that did not deviate significantly from Hardy-Weinberg equilibrium expectations following Bonferroni correction and which showed no evidence of amplification errors (null alleles and/or stuttering) in one or more samples within each species. Results of subsequent analyses of microsatellite data are reported only for the experimentally tractable microsatellites unless otherwise noted.

Table 5. Experimentally tractable microsatellites for each species. Only microsatellites that were in Hardy-Weinberg equilibrium and which showed no evidence of amplification error were considered usable in subsequent analysis.

Samples	Number	Microsatellites
D. argentosa	21	*Dep* 1, 2, 7, 9, 10, 13, 21, 28, 30, 32, 33, 38, 44, 51, 53, 65, 73, 74, 100, 105, 106
D. diaboli	23	*Dep* 1, 2, 3, 7, 8, 10, 13, 18, 21, 30, 33, 38, 44, 51, 53, 67, 73, 74, 85, 100, 101, 103, 105
D. episcopa	33	*Dep* 1, 2, 3, 7, 8, 9, 10, 18, 20, 21, 28, 30, 32, 33, 38, 40, 44, 51, 53, 57, 61, 65, 67, 73, 74, 85, 90, 91, 100, 103, 105, 106, 108
D. nigrotaeniata	33	*Dep* 1, 2, 3, 10, 13, 18, 20, 21, 28, 30, 32, 33, 38, 40, 44, 51, 53, 57, 61, 65, 67, 73, 74, 85, 90, 91, 93, 100, 101, 102, 103, 105, 106
D. serena	21	*Dep* 1, 3, 7, 8, 9, 12, 30, 38, 44, 51, 53, 61, 73, 85, 90, 100, 101, 103, 105, 106, 108

Homogeneity of microsatellite variation was assessed graphically and using non-parametric statistical tests. Confidence intervals around the average allelic richness and average expected heterozygosity (over all microsatellites) are shown in Figures 4 and 5, respectively. Results of homogeneity testing are presented in Table 6. Significant differences in allelic richness were found between or among samples in all four species, as follows: *D. argentosa* (Devils River > San Felipe Creek, Independence Creek); *D. diaboli* (Devils River > Pinto Creek); *D. nigrotaeniata* (Comal Springs > Fessenden Springs); and *D. serena* (Nueces River > Frio River). Tests of gene diversity revealed significant differences in *D. diaboli* (Devils River > Pinto Creek) and *D. nigrotaeniata* (Comal Springs > Fessenden Springs).

Table 6. Results (probability [*P*] values) of spatial homogeneity in microsatellite variation between/among samples of each species of *Dionda*. Tests include pair-wise Wilcoxon signed-rank tests of allelic richness and gene diversity.

Sample	*Allelic richness*	*Gene diversity*
D. 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
D. diaboli	0.001	0.004
D. nigrotaeniata	0.000	0.002
D. serena	0.006	0.089

Comparison of patterns of variation between mtDNA and microsatellites

Differences between mtDNA (female) and microsatellite variation were observed within a few samples. In *D. argentosa* from Independence Creek, *D. episcopa*, and *D. nigrotaeniata* from Comal Springs, mtDNA haplotype diversity was considerably less than microsatellite gene diversity (Figure 5), whereas in both samples of *D. diaboli* and in *D. serena* from the Nueces River, mtDNA haplotype diversity was considerably greater than gene diversity (Figure 5). In all other samples, mtDNA haplotype diversity and microsatellite gene diversity fell within the same range (Figure 5).

Table 7. Results of spatial homogeneity testing of mtDNA haplotypes between/among samples of each species of *Dionda*. Exact tests were based on mtDNA haplotype distributions; Φ_{ST} values are from analysis of molecular variance (AMOVA).

Sample comparisons	*Exact tests (P)*	*AMOVA - Φ_{ST} (P)*
D. argentosa	-	0.705 (0.000)
Devils River – San Felipe Creek	$P < 0.001$	-
Devils River – Independence Creek	$P < 0.001$	-
San Felipe Creek – Independence Creek	$P < 0.001$	-
D. diaboli	$P < 0.001$	0.252 (< 0.001)
D. nigrotaeniata	$P = 0.106$	0.158 (0.108)
D. serena	$P < 0.001$	0.933 (0.000)

Genetic divergence between/among samples of each species

Spatial genetic homogeneity between or among samples in each species was assessed for mtDNA sequences and microsatellites. Results of exact tests and analysis of molecular variance (AMOVA) of mtDNA sequences are shown in Table 7. Significant heterogeneity was detected in all comparisons between or among samples in each species except for the samples of *D. nigrotaeniata* from Comal Springs and Fessenden Spring. Results of exact tests of microsatellite allele and genotype distributions, and of AMOVA, are shown in Table 8. Significant heterogeneity was detected in all comparisons, including the two samples of *D. nigrotaeniata*. Genetic distances between

Table 8. Results of spatial homogeneity testing of microsatellites between/among samples of each species of *Dionda*. Tests include exact tests of microsatellite allele and genotype distributions and analysis of molecular variance (AMOVA).

Sample	*Alleles*	*Genotypes*	*AMOVA - F_{ST} (P)*
D. argentosa	$P < 0.001$	$P < 0.001$	0.123 (0.000)
Devils River – San Felipe Creek	$P < 0.001$	$P < 0.001$	-
Devils River – Independence Creek	$P < 0.001$	$P < 0.001$	-
San Felipe Creek – Independence Creek	$P < 0.001$	$P < 0.001$	-
D. diaboli	$P < 0.001$	$P < 0.001$	0.230 (0.000)
D. nigrotaeniata	$P < 0.001$	$P < 0.001$	0.280 (0.000)
D. serena	$P < 0.001$	$P < 0.001$	0.376 (0.000)

samples in each species, based on pair-wise Φ_{ST} values of mtDNA sequences and pair-wise F_{ST} values of microsatellites, are shown in Table A4. Probability values for all tests of $\Phi_{ST} = 0$ were significant ($P < 0.05$) except for the estimated Φ_{ST} value between the two samples of *D. nigrotaeniata* ($P = 0.104$). Estimates of Φ_{ST} between samples of each species ranged from 0.248 between *D. argentosa* from the Devils River and San Felipe Creek to 0.933 between *D. serena* from the Nueces and Frio rivers. Probability values for all tests of $F_{ST} = 0$ were significant ($P < 0.05$). Estimates of F_{ST} between samples of the same species ranged from 0.045 between *D. argentosa* from the Devils River and San Felipe Creek to 0.457 between *D. serena* from the Nueces and Frio rivers. Threshold F_{ST} values, based on minimum estimates of N_e (see below) and a 10% dispersal rate between populations (Hastings, 1993), were calculated for *D. argentosa* (F_{ST} threshold = 0.015), *D. diaboli* (F_{ST} threshold = 0.026), *D. nigrotaeniata* (F_{ST} threshold = 0.105), and *D. serena* (F_{ST} threshold = 0.007). All observed F_{ST} values (Table A4) were higher than threshold values.

Genetic demography

Minimum and maximum estimates (based on 95% confidence intervals from jackknifing across microsatellites) of the effective number of breeders (N_b) are presented in Table 9 and were generated using the linkage disequilibrium approach in LDNE (Waples and Do, 2010). Several point estimates and upper limits to all but one of the confidence intervals were returned either as errors (negative numbers) or as infinity (∞); minimum confidence intervals, however, are still considered informative (Waples and

Table 9. Estimates of 95% confidence intervals of the effective number of breeders (N_b) in each sample. Estimates were generated using LDNE and with alleles at a frequency less than 0.02 excluded.

Sample	*Estimated N_b*
D. argentosa	
Devils River	442.4 – ∞
San Felipe Creek	319.9 – ∞
Independence Creek	169.8 – ∞
D. diaboli	
Devils River	547.2 – ∞
Pinto Creek	94.6 – ∞
D. episcopa	
El Rito Creek	50.7 – 1552.6
D. nigrotaeniata	
Fessenden Spring	21.3 – ∞
Comal Springs	169.0 – ∞
D. serena	
Nueces River	340.0 – ∞
Frio River	357.0 – ∞

Do, 2010), particularly for populations or species of conservation concern. Minimum estimates of N_b ranged from 21.3 in the sample of *D. nigrotaeniata* from Fessenden Spring to 547.2 in the sample of *D. diaboli* from the Devils River. Marked variation in minimum estimates of N_b 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. nigrotaeniata* (Comal Springs > Fessenden Spring); minimum estimates of *D. serena* were similar between samples. Only the minimum estimate of N_b for *D. diaboli* from the Devils River was greater than 500 and estimates for *D. diaboli* from Pinto Creek, *D. episcopa* from El Rito Creek, and *D. nigrotaeniata* from Fessenden Spring were less than 100.

Assessment with MSVAR provided estimates of average mutation rate (over all microsatellites) per generation, an estimate (r) of long-term population growth or decline, and the time period, t_a, since the growth or decline occurred (Table 10). Estimates of average mutation rate 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* from the Frio river to -3.21 in the sample of *D. nigrotaeniata* from Fessenden Spring. Of the ten samples, six (60%) experienced a decline of more than two orders of magnitude. Assuming a generation time of 1-3 years, modal estimates of t_a ranged from 508-1524 years in *D. argentosa* from the Devils River to 3211-9632 years in *D. diaboli* from Pinto Creek. Minimum estimates, however, were less than 100 years for nine of the ten samples (90%).

Table 10. Results of analysis with MSVAR. Modal values and their 95% quartiles are given for mutation rate (μ) and $\log_{10} r$. Time since expansion/decline began, t_a, is given for a range of generation times from one to three years (Harrell and Cloutman, 1978; Cloutman and Harrell, 1987).

	Mode	0.025 quartile	0.975 quartile
D. argentosa			
Devils River			
μ	2.4×10^{-4}	2.8×10^{-5}	2.1×10^{-3}
$Log_{10}(r)$	-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}
$Log_{10}(r)$	-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}
$Log_{10}(r)$	-2.31	-2.44	-2.22
t_a *(years)*	514 - 1542	37 - 111	7147 - 21440
D. diaboli			
Devils River			
μ	2.5×10^{-4}	2.7×10^{-5}	2.2×10^{-3}
$Log_{10}(r)$	-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}
$Log_{10}(r)$	-2.98	-3.05	-2.85
t_a *(years)*	3211 - 9632	206 - 618	40651 - 121954
D. episcopa			
El Rito Creek			
μ	2.3×10^{-4}	2.6×10^{-5}	2.1×10^{-3}
$Log_{10}(r)$	-2.32	-2.36	-2.35
t_a *(years)*	1163 - 3488	60 - 181	15686 - 47057
D. nigrotaeniata			
Fessenden Spring			
μ	2.3×10^{-4}	2.6×10^{-5}	2.0×10^{-3}
$Log_{10}(r)$	-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}
Log10(r)	-2.31	-2.40	-2.17
t_a *(years)*	569 - 1706	13 - 40	21747 - 65241

Table 10 continued.

	Mode	0.025 quartile	0.975 quartile
D. serena			
Nueces River			
μ	2.5×10^{-4}	2.8×10^{-5}	2.3×10^{-3}
$Log_{10}(r)$	-1.56	-1.65	-1.51
t_a *(years)*	1507 - 4522	45 - 136	30860 - 92581
Frio River			
μ	2.3×10^{-4}	2.7×10^{-5}	2.1×10^{-3}
$Log_{10}(r)$	-1.35	-1.69	-1.34
t_a *(years)*	927 - 2781	6 - 17	86497 - 259490

Table 11. 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. An estimate of N_{eLT} for *D. episcopa* could not be generated as Θ failed to converge.

Sample	*Theta*(Θ)	N_{eLT}
D. 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)
D. diaboli		
Devils River	1.364	1371.0 (1282.0 – 1452.8)
Pinto Creek	0.501	503.5 (475.5 – 534.0)
D. episcopa		
El Rito Creek	--	--
D. nigrotaeniata		
Fessenden Spring	0.624	685.6 (657.3 – 716.1)
Comal Springs	1.285	1434.6 (1372.6 – 1498.1)
D. serena		
Nueces River	2.351	2335.2 (2209.1 – 2489.5)
Frio River	1.372	1485.1 (1375.0 – 1641.5)

Analysis of microsatellite data with MIGRATE provided estimates of theta (Θ), which then were used to derive estimates of N_{eLT}, the average, long-term genetic-effective population size for each sample (Table 11). Estimates of theta for the El Rito Creek sample of *D. episcopa* failed to converge. Estimates of average, long-term effective population size ranged from 503 in *D. diaboli* from Pinto Creek to 1485 individuals in *D. serena* from the Nueces River. The sample of *D. argentosa* from San Felipe Creek, *D. diaboli* from Pinto Creek, and *D. nigrotaeniata* from Fessenden Spring had N_{eLT} estimates of less than 1000. Estimates of N_{eLT} for all samples were higher than minimum estimates of N_b.

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 12. Estimation of *M* included a probability value (*P*) that represents the percentage of the time a lower value of *M*-ratio would be found under equilibrium conditions. 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. episcopa* from El Rito Creek, and *D. nigrotaeniata* from Fessenden Spring were all significant, indicating that recent bottlenecks had occurred in those samples. When theta values based on analysis with MIGRATE were used, *M*-ratios for these same samples as well as for *D. nigrotaeniata* from Comal Springs were significant.

Table 12. 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.

		Theta Value of 2		*Theta Value Based on MIGRATE*		
Sample	*Mean M*	M_c	*P*	*Theta*	M_c	*P*
D. 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
D. 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
D. episcopa						
El Rito Creek	0.783	0.795	0.024			
D. nigrotaeniata						
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
D. serena						
Nueces River	0.802	0.784	0.122	2.351	0.776	0.148
Frio River	0.912	0.773	0.947	1.372	0.794	0.861

DISCUSSION

At the core of conservation genetics is the evaluation of genetic diversity within and among populations in order 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 conservation risks, while evaluation of genetic diversity (divergence) between or among geographic populations can identify populations that may be considered as distinct evolutionarily significant units or management units (Waples 1991; Moritz 1994). Sufficient levels of genetic diversity within a population ensure a good suite of different alleles to deal with different environmental situations (Frankham 1995; Lynch et al. 1995). Finally, most studies of genetic diversity utilize genetic markers that are considered to be selectively neutral (Avise 1994; McKay and Latta 2002; Reed and Frankham 2003); while such markers do not necessarily correlate to levels of diversity found in genes that would impact fitness of individuals (McKay and Latta 2002), surrogates of fitness, such as heterozygosity in selectively neutral markers, are important tools for evaluating the conservation status of populations (Reed and Frankham 2003).

The historical demography of a population is another factor to consider in conservation biology as effective population size plays a critical role in the continued survival of populations (Lande and Borrowclough 1987). Patterns of long-term population growth or decline can be determined through estimation of ancestral and

contemporary effective size (Storz and Beaumont 2002). Decline in a population may produce a small effective population size, which increases the probability of inbreeding and exposes the population to genetic drift (Wright 1931); genetic drift can in turn lead to increased levels of deleterious recessive alleles and reduced genetic diversity (Wright 1931). The time frame of decline in population size may also provide information to conservation efforts. Severe droughts within the last 2000 years (Cook et al. 2004; Fye et al. 2003) and more recent anthropogenic factors such as water use and pollution (TWAP 2005; Vrijenhoek 1998) are factors that could be considered as potentially affecting populations of *Dionda* in Texas waters.

In this study, differing levels of mtDNA and nuclear-encoded DNA diversity were observed among geographic samples of four of the five species of *Dionda* examined; in the fifth species, *D. episcopa,* only a single geographic sample was evaluated. Conversely, in the endangered cyprinids *Notropis mekistocholas* (Burridge and Gold 2003), *Hybognathus amarus* (Alò and Turner 2005), and *Anaecypris hispanica* (Salguerio et al. 2003), levels of diversity did not differ between or among geographic samples. In addition, each geographic sample in the four species of *Dionda* where different samples were studied differed genetically from one another, indicating that each sample is a genetically defined population. Similarly, other samples of the endangered cyprinid *Anaecypris hispanica* (Alves et al. 2001) and geographic samples of the endangered, spring-dwelling cyprinodontid, *Cyprinodon tularosa* (Stockwell et al. 1998) differed genetically and were considered separate populations. Finally, all of the populations of *Dionda* assayed demonstrated historical declines in effective population

size. Several populations of *Dionda* assessed also experienced recent bottlenecks, which can greatly reduce effective size and contribute to a loss of genetic variation (Avise 1994). Further discussion of genetic variation, genetic diversity, and demography in each of the five species of *Dionda* studied is presented below.

Evaluation of Dionda argentosa

The three samples of *D. argentosa* (Devils River, San Felipe Creek, and Independence Creek) represent three genetically distinct populations. Significant heterogeneity was detected, both by exact tests and AMOVA, in both mtDNA haplotype distribution and microsatellite allele and genotype distributions. All three geographic samples of *D. argentosa* thus are genetically unique populations that could be considered as distinct management units (*sensu* Moritz 1994). In addition, pair-wise F_{ST} values were greater than a threshold of ten percent dispersal rate between localities, providing genetic evidence of demographic independence**.** Finally, populations of *D. argentosa* from the Devils River and San Felipe Creek shared three of eight mtDNA haplotypes; whereas none of the mtDNA haplotypes found in the population from Independence Creek were shared with the other two populations. Because the population in Independence Creek appears to be reciprocally monophyletic for a clade of mtDNA haplotypes, it could be considered as an evolutionarily significant unit, especially because it occurs in the Pecos River drainage, whereas the other two populations are in the Devils River drainage.

Heterogeneity in allelic richness (microsatellites) was detected between the population from the Devils River and populations from both San Felipe Creek and

Independence Creek (Devils River > San Felipe Creek/Independence Creek). The number of mtDNA haplotypes and microsatellite alleles, along with haplotype diversity and nucleotide diversity (mtDNA) and gene diversity (microsatellites), were average to high in the population of *D. argentosa* from the Devils River relative to all other samples of *Dionda*. Comparatively, the number of mtDNA haplotypes was low in *D. argentosa* from both San Felipe Creek and Independence Creek; mtDNA haplotype diversity also was comparatively low in *Dionda* from Independence Creek. Populations in both San Felipe Creek and Independence Creek had average levels of gene diversity (microsatellites) compared to all other samples of *Dionda* studied. The lower estimates of mtDNA haplotype diversity in Independence Creek relative to average levels of gene diversity (microsatellites) may suggest a disproportionate reduction in the effective number of females.

Analysis of historical demography revealed that all three populations of *D. argentosa* have experienced historical, order-of-magnitude declines in effective population size, with the declines being steeper in San Felipe Creek and Independence Creek. Comparison of estimates of long-term effective population size (N_{eLT}) and minimum estimates of the effective number of breeders (N_b) also indicated relatively steep declines in the populations in the Devils River and Independence Creek (~1,500 vs ~440 and ~1,230 vs ~170, respectively) and a moderate decline in San Felipe Creek (~540 vs ~320). The lower estimate of N_{eLT} in the population from San Felipe Creek suggests that historically the effective size of this population has not been as large as the

other two. Finally, results of the *M*-ratio test indicated significant, recent bottlenecks in both San Felipe Creek and Independence Creek.

Minimum estimates of N_b in all three populations of *D. argentosa* were less than 500, suggesting possibly that the equilibrium between the loss of adaptive genetic variance from genetic drift and its replacement by mutation might be compromised. This inference is based on the '50/500' rule (Rieman and Allendorf 2001) for genetic effective size (N_e), where an N_e of less than 50 indicates a population is highly vulnerable to inbreeding depression, while an N_e average of 500 or more allows a population to maintain adaptive genetic variation through time. The relationship between N_e and N_b, however, is complex, particularly in iteroparous species (Waples 2011). Alternatively, Waples and Do (2010) suggested that an estimate of N_b from a random sample of mixed-age individuals might approximate N_e per generation if the sample contained age classes equal to a generation length. Whether this is the case in the samples of *Dionda* examined in this study is not known. All indications, however, are that the populations of *D. argentosa* (and most of the other populations of the other *Dionda* examined in this study – see below) may well be genetically compromised.

Of the three populations of *D. argentosa* examined, the one in the Devils River appears the least compromised genetically; the populations in San Felipe Creek and Independence Creek have lower genetic variation, reduced minimum N_b, and appear to have experienced recent bottlenecks. The population in Independence Creek is of particular concern as it has the lowest minimum N_b (170) and to date is the only fully substantiated population of *D. argentosa* in the Pecos river drainage (Carson et al. 2010).

It is recommended that all three populations of *D. argentosa* be monitored, but close attention be paid to the populations in San Felipe Creek and, especially, Independence Creek.

Evaluation of Dionda diaboli

The two samples of *D. diaboli* (Devils River and Pinto Creek) are genetically distinct in both mtDNA haplotype and microsatellite allele and genotype distributions, meaning that both are distinct genetic populations that could be considered as separate management units (*sensu* Moritz 1994). Pair-wise F_{ST} values were greater than a threshold of ten percent dispersal rate between localities, providing genetic evidence of demographic independence. Of the eight mtDNA haplotypes found in the Devils River and Pinto Creek populations of *D. diaboli*, two were shared. This suggests that there has been insufficient time for lineage sorting (Avise et al. 1984) to occur between these two populations of *D. diaboli*.

Significant heterogeneity of allelic richness and gene diversity was detected between the two populations. The number of mtDNA haplotypes and microsatellite alleles, mtDNA haplotype diversity and nucleotide diversity, and (microsatellite) gene diversity in *D. diaboli* from the Devils River were average to high as compared to the other samples of *Dionda*. Gene diversity and nucleotide diversity in the population in Pinto Creek were average (compared to other samples of *Dionda*), but all other measures of genetic variation were comparatively low. Interestingly, mtDNA haplotype diversity in both populations was considerably higher than microsatellite gene diversity,

suggesting that a disproportionate reduction in the effective number of males may have occurred recently in both populations.

Analysis of historical demography revealed that both populations of *D. diaboli* have experienced a two order-of-magnitude decline in effective population size, with the decline in the population in Pinto Creek reaching nearly three orders of magnitude. Comparison of estimates of long-term effective population size (N_{eLT}) and minimum estimates of the effective number of breeders (N_b) also indicated relatively steep declines in both populations (~1,370 vs ~550 in the Devils River and ~500 vs ~95 in Pinto Creek). The lower estimate of N_{eLT} in the population of *D. diaboli* in Pinto Creek suggests that historically the effective size of this population has been smaller than the effective size of the population in the Devils River. Results of the *M*-ratio test indicated a significant, recent bottleneck in the population in Pinto Creek. Finally, the minimum estimate of N_b of the population in the Devils River was ~550, near the upper bound of the '50/500' rule, whereas the minimum estimate of N_b for the population in Pinto Creek was ~95.

As *D. diaboli* is considered threatened at the federal and state level (USFWS 1999), its genetic status is of particular interest. Between the two populations of *D. diaboli* evaluated, the Devils River population appears to be less impaired genetically than the Pinto Creek population. The Pinto Creek population has lower genetic variation, reduced minimum N_b, and has experienced a recent bottleneck. It is recommended that both populations of *D. diaboli* be monitored, but the Pinto Creek population should be observed especially closely.

Evaluation of Dionda episcopa

After the sample of *Dionda* from Independence Creek sample was reassigned to *D. argentosa*, only one sample of *D. episcopa* (from El Rito Creek in New Mexico) remained in the study. All measures of genetic variation, including number of mtDNA haplotypes, average number of microsatellite alleles, mtDNA haplotype diversity and nucleotide diversity, and average gene diversity, were lower in this sample of *D. episcopa* than in any other sample of *Dionda* in the study. The finding of only a single mtDNA haplotype in this sample, and much lower mtDNA haplotype diversity as compared to microsatellite gene diversity, suggests a severe, recent reduction (bottleneck) in the effective number of females. Analysis of historical demography of this population revealed a more than two-order-of magnitude decline in effective size, while the minimum estimate of the effective number of breeders (N_b) in the population was ~50. Results of the *M*-ratio test were significant, indicating a recent bottleneck in both males and females.

The population of *D. episcopa* in El Rito Creek appears severely compromised genetically and evaluation of *D. episcopa* at other localities is clearly warranted. As other samples of *Dionda* from the lower Pecos River have been shown to be *D. argentosa* (Schönhuth et al. 2008; Carson et al. 2010), the range of *D. episcopa* may be constrained to the northern Pecos River drainage. If levels of variation and genetic demography in other populations of *D. episcopa* are comparable to those of the population in El Rito Creek, it is possible that *D. episcopa* could be threatened or endangered.

Evaluation of Dionda nigrotaeniata

The two samples of *D. nigrotaeniata* (Fessenden Spring and Comal Springs) are genetically distinct in microsatellite allele and genotype distributions; divergence was not detected in mtDNA haplotype distributions. The latter was undoubtedly due to the extremely reduced mtDNA diversity in both samples. Both samples shared a common haplotype, and a second, low-frequency haplotype was found in *Dionda* from Fessenden Spring. Based on the heterogeneity in microsatellites allele and genotype distributions, the two samples should be considered as genetically distinct populations that could be separate management units (*sensu* Moritz 1994). Pair-wise F_{ST} values were greater than a threshold of ten percent dispersal rate between localities, providing genetic evidence of demographic independence.

Significant heterogeneity of allelic richness and gene diversity was detected between the two populations. The number of mtDNA haplotypes and nucleotide diversity, along with the number of alleles and (microsatellite) gene diversity, were low for the Fessenden Spring population of *D. nigrotaeniata* relative to all other samples of *Dionda*, while haplotype diversity was comparatively average. The population in Comal Springs was monomorphic for the mtDNA sequence, while the number of microsatellite alleles and gene diversity were average relative to other samples of *Dionda*. The lower estimates of mtDNA haplotype diversity in the population in Comal Springs relative to average levels of gene diversity (microsatellites) may suggest a disproportionate reduction in the effective number of females.

Analysis of historical demography of *D. nigrotaeniata* revealed that both populations have experienced historical declines of three (Fessenden Spring) and two (Comal Springs) orders of magnitude. Comparison of estimates of long-term effective population size (N_{eLT}) and minimum estimates of the effective number of breeders (N_b) also indicated relatively steep declines in the populations in Fessenden Spring (~690 vs ~20) and Comal Springs (~1400 vs ~170). The lower estimate of N_{eLT} in the population of *D. nigrotaeniata* in Fessenden Spring suggests that historically the effective size of this population has been smaller than the effective size of the population in Comal Springs. Results of the *M*-ratio test indicated significant, recent bottlenecks in both populations. Finally, the minimum estimate of N_b for the population in Fessenden Spring was ~20, well below the lower bound of the '50/500' rule, whereas the minimum estimate of N_b of the population in the Comal Springs was ~170, below the upper bound.

While the Comal Springs population of *D. nigrotaeniata* appears less compromised genetically than the Fessenden Spring population, both populations have low genetic variation, small minimum N_b, and appear to have experienced recent bottlenecks. Of particular concern is the low mtDNA (female) diversity of the population in Comal Springs and the small N_b (~20) of the population in Fessenden Springs. It is recommended that both populations of *D. nigrotaeniata* be monitored closely.

Evaluation of Dionda serena

The two samples of *D. serena* (Nueces River and Frio River) represent genetically distinct populations. Significant heterogeneity was detected in mtDNA haplotype distribution and microsatellite allele and genotype distributions, meaning that both could

be considered as distinct management units (*sensu* Moritz 1994). Pair-wise F_{ST} values were greater than a threshold of ten percent dispersal rate between localities, providing genetic evidence of demographic independence. The Nueces River and Frio River populations were reciprocally monophyletic for clades of mtDNA haplotypes, meaning minimally the two populations could be considered as evolutionarily significant units (*sensu* Waples 1991). Additionally, mtDNA ND-5 sequences between the two populations differed by a minimum of 22 single nucleotide polymorphisms, representing a difference in sequence of between 3.8% and 4.6%. Studies of other cyprinids (Broughton et al. 2000; Schönhuth and Mayden 2010; Richardson and Gold 1995) indicate that minimum pair-wise species divergence for mtDNA sequences ranged between 0.7% in 297bp of ND-4L in *Cyprinella* (Broughton et al. 2000) and 12.9% in 1140bp of cytochrome *b* in *Pimphales* (Schönhuth and Mayden 2010). Therefore, the Nueces River and Frio River populations of *D. serena* should be investigated to explore the possibility that the populations represent two different species.

The two populations of *D. serena* differed significantly in number of haplotypes and haplotype diversity (mtDNA) and in number of alleles and allelic richness (microsatellites). The number of mtDNA haplotypes and microsatellite alleles, mtDNA haplotype diversity and nucleotide diversity, and microsatellite gene diversity in *D. serena* from the Nueces River were high as compared to all other populations of *Dionda* sampled. The number of haplotypes, haplotype diversity and gene diversity in the Frio River were average (compared to other samples of *Dionda*), but all other measures were comparatively low. In the Nueces River, mtDNA haplotype diversity was considerably

higher than microsatellite gene diversity, suggesting that a disproportionate reduction in the effective number of males may have occurred recently in that population. Also, high nucleotide diversity in the Nueces River population indicates historical stability of the female population, and a significant, negative value for Fu's *Fs* (1997) indicates an excess of alleles caused by population expansion or genetic hitchhiking.

Analysis of historical demography revealed that both populations of *D. serena* have experienced historical declines in effective size, with a two order-of-magnitude decline in the Nueces River and a one order-of-magnitude decline in the Frio River. Comparison of estimates of long-term effective population size (N_{eLT}) and minimum estimates of the effective number of breeders (N_b) also indicated relatively steep declines in the Nueces River (~2,300 vs ~340) and Frio River (~2,800 vs ~360). Estimates of N_{eLT} suggest that historically the effective sizes of both populations have been comparable. Results of the *M*-ratio test indicated that neither population has experienced a recent bottleneck. Finally, the minimum estimates of N_b of both populations were ~350, below the upper bound of the '50/500' rule.

Of the two populations of *D. serena*, the Nueces River population appears less compromised genetically. While both populations exhibit low minimum N_b, the population in the Frio River appears to have reduced genetic variation. It is recommended that both populations of *D. serena* be monitored, but close attention should be paid to the population in the Frio River.

SUMMARY

Each sample of *Dionda* examined in this study was genetically distinct, meaning that all ten samples represent genetically distinct populations that could be considered as separate management units. In addition, the clade of mtDNA haplotypes in the population of *D. argentosa* in 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 each clade could represent an evolutionarily significant unit. MtDNA haplotype clades in the two populations of *D. serena* were reciprocally monophyletic and differed from one another by 22 base-pair substitutions. The two populations of *D. serena* clearly are different evolutionary significant units and could be specifically distinct.

Several of the populations of *Dionda* evaluated appear to be compromised genetically because of low genetic variation, small (minimum) contemporaneous effective size, and evidence of recent bottlenecks; these include *D. argentosa* in San Felipe Creek and Independence Creek, *D. diaboli* in Pinto Creek, *D. episcopa* in El Rito Creek, *D. nigrotaeniata* in Fessenden Spring and Comal Springs, and *D. serena* in the Frio River. These populations should be monitored closely and further study should evaluate the conservation needs of each population.

Scharpf (2005) reported conservation statuses based on analysis by NatureServe (http://www.natureserve.org/), which accounts for demography, range and habitat of a species, but does not account for genetic factors (NatureServe 2011). Also, with the

exception of *D. diaboli*, the status of these species of *Dionda* was last examined in 1996. Given this lapse of time and the genetic analysis provided by this study, the conservation status of *D. argentosa*, *D. episcopa*, and *D. nigrotaeniata* should be re-evaluated. The species *D. argentosa* was considered imperiled based on its small range in Texas (NatureServe 2011), but of the three populations evaluated in this study, two (San Felipe Creek and Independence Creek) have experienced recent bottlenecks and exhibit low (minimum) contemporaneous effective population size. Future evaluation should also take into account the low diversity in maternally inherited mtDNA found in the Independence Creek population. The species *D. episcopa* was considered secure (NatureServe 2011), but the El Rito Creek population appears to be severely compromised genetically with low mtDNA and nuclear variation, recent bottlenecks, a decline in effective size, and a low (minimum) contemporaneous effective size. If other populations of *D. episcopa* exhibit similar characteristics, the species could be severely compromised. The species *D. nigrotaeniata* was considered to be apparently secure (NatureServe 2011), but both populations of *D. nigrotaeniata* examined in this study appear compromised genetically, with low mtDNA diversity, recent bottlenecks, decline in effective size, and low (minimum) contemporaneous effective size. Future evaluation should take the genetic status of these populations into consideration and seek to evaluate other populations of the species. Finally, the species *D. serena* was considered imperiled based on its limited range in Texas river systems. The population of *D. serena* in the Nueces river appears genetically stable, whereas the population in the Frio River had fewer mtDNA haplotypes and microsatellite alleles and lower haplotype diversity.

Minimum estimates of N_b of both populations, however, were below the upper bound of the '50/500' rule. It is possible that all five species of *Dionda* in Texas are imperiled.

Several populations of *Dionda* examined in this study exhibited mtDNA and microsatellite variation comparable to or lower than that found in other, 'officially' threatened or endangered cyprinids. A summary of mtDNA variation in the species of *Dionda* studied here and in five endangered cyprinids, one threatened cyprinid, and two other endangered, North American freshwater fish is presented in Table 13. MtDNA variation in several of the species of *Dionda* was approximately the same or less than observed in the other threatened or endangered species. A particularly relevant comparison is with the Cape Fear shiner, *Notropis mekistocholas*, a species officially listed (Hilton-Taylor 2000) as critically endangered. Except for *D. serena*, the species of *Dionda* examined in this study had fewer haplotypes and lower haplotype diversity than reported by Saillant et al. (2004) and Gold et al. (2004) for *N. mekistocholas*. However, the seemingly higher number of haplotypes and haplotype diversity in *D. serena* was due to the population in the Nueces River; values for *D. serena* in the Frio River (five haplotypes, haplotype diversity of 0.352, Table 3) are comparable to or lower than values for the other threatened or endangered cyprinids (Table 13). Observed number of haplotypes and haplotype diversities for *D. episcopa* and *D. nigrotaeniata* (Table 13) certainly indicate that the conservation status of these two species is no longer 'secure.'

Microsatellite variation in the populations of *Dionda* studied here and in populations of three endangered cyprinids, one threatened cyprinid, and three other endangered North American freshwater fish are summarized in Table 14. As with mtDNA variation,

Table 13. Summary of mtDNA variation in *Dionda* (this study) and in imperiled cyprinids and non-cyprinids. Values are within-population averages, ranging across populations.

Species	Source	Conservation status	mtDNA	#Base pairs	#Popu-lations	#Individuals/ population	#Haplo-types	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 episcopa	This study	Secure	ND-5	585	1	22	1	0.000
Dionda nigrotaeniata	This study	Secure	ND-5	585	2	20	1 – 2	0.000 – 0.337
Dionda serena	This study	Imperiled	ND-5	585	2	22.5	5 – 14	0.352 – 0.906
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	Saillant et al. (2004)	Endangered	ND-5, ND-6	625	2	27.5	11 - 14	0.80 - 0.85

Table 13 continued.

Species	Source	Conservation status	mtDNA	#Base pairs	#Popu-lations	#Individuals/ population	#Haplo-types	Haplotype diversity
	Gold et al. (2004)		ND-5, ND-6	625	3	13.3	5 - 9	
Notropis simus pecosensis	Osborne and Turner (2006)	Threatened	ND-4	322	3	108.3	20	0.603 - 0.650
Non-cyprinids								
Cyprinodon tularosa	Stockwell et al. (1998)	Endangered	Control	482	3	40	3	
Xyrauchen texanus	Garrigan et al. (2002)	Endangered	Cyt *b*	311	1	49	10	

Table 14. Summary of microsatellite variation in *Dionda* (this study) and in imperiled cyprinids and non-cyprinids. Values are within-population averages, ranging across populations.

Species	Source	Conservation status	#Micro-satellites	#Popu-lations	#Individuals/ population	#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 episcopa	This study	Secure	33	1	41	2.09	0.257
Dionda nigrotaeniata	This study	Secure	33	2	60.5	2.52 – 4.94	0.255 – 0.378
Dionda serena	This study	Imperiled	21	2	38.5	3.71 – 7.67	0.423 – 0.525
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)	Endangered	11	3	13.3	5.1 - 5.3	
	Saillant et al. (2004)		22	2	27.5	8.18	0.701
	Gold et al. (2004)		11	3	13.3	6.2 - 7.9	0.77 - 0.79

Table 14 continued.

Species	Source	Conservation status	#Micro-satellites	#Popu-lations	#Individuals/ population	#Alleles	Gene diversity
Notropis simus pecosensis	Osborne and Turner (2006)	Threatened	7	3	108.3	13.3 - 23.7	0.816 - 0.846
Non-cyprinids							
Cyprinodon tularosa	Stockwell et al.(1998)	Endangered	4	3	40	2.0 – 4.5	0.251 - 0.534
Poeciliopsis o. occidentalis	Hedrick et al. (2001)	Endangered	5	13	27.5	1.2 - 4.4	0.042 - 0.437
Poeciliopsis. o. sonorinensis	Hedrick et al. (2001)	Endangered	7	2	19.5	2.4 - 2.9	0.393 - 0.425

microsatellite variation, measured as the range in the number of alleles and gene diversity, in the species of *Dionda* was comparable to or less than values in the threatened or endangered cyprinids. The observed range in number of alleles and gene diversity in *D. episcopa*, *D. nigrotaeniata*, and *D. diaboli* were lower than in the other cyprinids and on a par with values reported for the endangered non-cyprinids *Cyprinodon tularosa* and the two subspecies of *Poeciliopsis occidentalis* (Table 14). Also, as with mtDNA variation, the range in number of alleles (3.71) and gene diversity (0.423) for *D. serena* in the Frio River was comparable to or less than values in the other cyprinids (Table 14). That several of the species (and populations) of *Dionda* examined in this study exhibit comparable or lower variation in mtDNA and microsatellites than other threatened or endangered cyprinids is cause for concern and an indication that further study, monitoring, and consideration of a change in conservation status are clearly warranted.

Implications

Results of this study have larger implications for *Dionda* in Texas, including the need for further investigation and the need for conservation action. While genetic status comprises only part of the challenge that each species or population faces, it is a significant component of viability and survival. Genetic data also can direct further taxonomic, demographic, or ecological study. This study represents the first genetic evaluation of *Dionda* and provides a conservation genetic perspective that should serve as a baseline against which to compare future data. Each species of *Dionda* in Texas needs both further study and conservation management.

Genetic findings for *D. argentosa*, based on three populations, indicate the species is imperiled and needs further evaluation to determine if it is critically imperiled (threatened). The combination of several factors, including low mtDNA diversity, decline in effective population size, and recent bottlenecks, illustrate the compromised genetic status of these populations. Additionally, the population in Independence Creek exhibited a unique genetic situation (monophyly of mitochondrial haplotypes) compared to the two populations in the Devils river drainage. There are several steps which could be taken to manage *D. argentosa*: (i) the Pecos river drainage should be surveyed for other populations of *D. argentosa*, especially downstream from the confluence of Independence Creek with the Pecos River; (ii) any additional populations should be evaluated from a genetic perspective; and (iii) the loss of further genetic diversity in the three populations examined in this study should be mitigated by protecting the spring and steam ecosystems they inhabit from anthropogenic desiccation, pollution, and invasive species.

Genetic findings for *D. diaboli*, based on two populations, are consistent with its threatened status. Populations of *D. diaboli* face a combination of factors, including low diversity, decline in effective population size, and a recent bottleneck in Pinto Creek. Understanding the full range of *D. diaboli* is important, and surveying Sycamore Creek and other habitats in the Devils River drainage to ascertain the presence of *D. diaboli* will contribute to that understanding. If additional populations of *D. diaboli* exist, evaluation of their genetic status should follow. Ideally, the status of *D. diaboli* in Mexico also should be thoroughly evaluated. Finally, the loss of further genetic

diversity should be mitigated by protecting the spring and steam ecosystems inhabited by *D. diaboli* from anthropogenic desiccation, pollution, and invasive species.

Of all the species evaluated, *D. episcopa* deviated most from expectations, as it was thought to be a wide-spread, secure species. The possible range reduction of *D. episcopa* (Carson et al. 2010) and a history of re-defining nominal species of *Dionda* from populations once considered *D. episcopa* (Gilbert, 1998) weaken the assumption that the species is wide-spread. Results of genetic assessment of the population in El Rito Creek, including low variation, decline in effective population size, low effective number of breeders, and a recent bottleneck, weaken the assumption that *D. episcopa* is secure. While it is unwise to base species management on the status of one population, it would be equally unwise to ignore the genetic status of the population in El Rito and not gather more data on the species as a whole. It is imperative that all populations thought to be *D. episcopa* be evaluated genetically, both to ascertain that they are, in fact, *D. episcopa*, and to assess the variation and demography of those populations. If the population in El Rito Creek represents a typical population of *D. episcopa*, the species is most certainly critically imperiled (threatened), if not endangered.

Genetic findings for *D. nigrotaeniata*, based on two populations, indicate that *D. nigrotaeniata* is imperiled, perhaps critically imperiled, rather than secure. The combination of several factors, including low mtDNA variation, declines in effective size, low effective number of breeders, and recent bottlenecks highlight the threat to viability that *D. nigrotaeniata* faces. Assessment of other populations of *D. nigrotaeniata* will help management efforts gain more perspective. If the genetic status

of the two populations evaluated in this study is typical of *D. nigrotaeniata*, management actions should prevent the loss of further genetic diversity by protecting the spring and steam ecosystems inhabited by *D. nigrotaeniata* from anthropogenic desiccation, pollution, and invasive species.

Given the degree of divergence found between populations of *D. serena* in the Nueces and Frio rivers, further genetic and morphological study is needed. Further genetic study should evaluate the distance between these two populations of *Dionda* using more DNA sequences, both mtDNA and nuclear DNA. Morphological differences between the populations also should be assessed, as genetic data do not present the whole picture. If the two populations are found to represent different species, other populations in the drainage need to be assessed to determine where they fall within any new taxonomic classification. Once the taxonomic status of *Dionda* in the Nueces river drainage is better assessed, the genetic status of additional populations should be evaluated. Both populations evaluated in this study had declined in effective size and had a low effective number of breeders; the population in the Frio River also exhibited low genetic variation. The combination of these factors indicates that the habitat of *Dionda* in the Nueces River drainage should be protected from threats such as anthropogenic desiccation, pollution, and invasive species.

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APPENDIX

Table A1. Observed mtDNA haplotypes, arranged by species and sample locality, and GenBank accession numbers.

MtDNA Haplotype	*D. argentosa*			*D. diaboli*		*D. episcopa*	*D. nigrotaeniata*		*D. serena*		GenBank Accession #
	Devil's River	San Felipe Creek	Independence Creek	Devil's River	Pinto Creek	El Rito Creek	Fessenden Springs	Comal Springs	Nueces River	Frio River	
1	14	4									GU252301.1
2		11									GU252302.1
3	2	5									GU252303.1
4	2										GU252304.1
5	2										GU252305.1
6	1										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

Table A2. Summary statistics for 38 nuclear-encoded microsatellites applied to five species of *Dionda* (28-34 per species). Values for sample size (*n*), number of alleles (#A), allelic richness (A_R), gene diversity (expected heterozygosity [H_E]), probability the locus conforms to Hardy-Weinberg equilibrium (P_{HW}), and the inbreeding coefficient (F_{IS}) measured as Weir and Cockerham's (1984) *f*.

Locus and Statistic	*D. argentosa*			*D. diaboli*		*D. episcopa*	*D. nigrotaeniata*		*D. serena*	
	Devil's River	San Felipe Creek	Independence Creek	Devil's River	Pinto Creek	El Rito Creek	Fessenden Springs	Comal Springs	Nueces River	Frio River
Dep1										
n	63	33	34	56	50	41	61	60	53	24
#A	3	2	2	1	1	1	2	2	1	3
A_R	2.94	2.00	2.00	1.00	1.00	1.00	2.00	2.00	1.00	3.00
H_E	0.204	0.088	0.276	0.000	0.000	0.000	0.138	0.081	0.000	0.657
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	41	61	60		
#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	41	61	60	53	24
#A				20	3	2	10	10	27	7
A_R				19.81	3.00	2.00	9.82	9.86	16.88	6.63
H_E				0.92	0.402	0.137	0.712	0.552	0.917	0.738
P_{HW}				0.571	0.026	1.000	0.022	0.631	0.803	0.716

FIS				-0.028	-0.094	-0.067	0.126	-0.057	0.033	0.040
Dep 7										
n	63	33	34	56	50	41			53	24
#A	13	8	8	8	4	1			12	8
AR	11.20	7.93	7.90	7.99	3.98	1.00			10.28	7.83
HE	0.795	0.810	0.807	0.834	0.511	0.000			0.864	0.684
PHW	0.552	0.729	0.121	0.382	0.799	-			0.067	0.946
FIS	-0.038	-0.047	0.089	-0.006	0.099	-			0.040	-0.036
Dep 8										
n				56	50	41			53	23
#A				1	2	3			2	2
AR				1.00	2.00	3.00			1.40	1.91
HE				0.000	0.059	0.357			0.019	0.043
PHW				-	0.031	0.774			-	-
FIS				-	0.662	0.043			0.000	0.000
Dep 9										
n	63	33	34			41			53	24
#A	9	5	5			1			8	5
AR	7.21	5.00	4.91			1.00			6.40	5.00
HE	0.570	0.706	0.567			0.000			0.761	0.784
PHW	0.754	0.532	0.295			-			0.482	0.575
FIS	-0.114	0.013	0.170			-			0.058	-0.062
Dep 10										
n	63	33	34	56	50	41	61	60	53	24
#A	9	6	7	1	1	2	1	2	7	2
AR	8.16	5.94	6.91	1.00	1.00	2.00	1.00	1.93	6.37	2.00
HE	0.826	0.713	0.803	0.000	0.000	0.198	0.000	0.017	0.674	0.156

P_{HW}	0.141	0.465	0.695	-	-	0.388	-	-	0.003	1.000
F_{IS}	-0.058	-0.147	-0.063	-	-	0.138	-	0.000	0.300	-0.070
Dep 12										
n									53	24
#A									3	1
A_R									1.79	1.00
H_E									0.038	0.000
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	41	61	60		
#A				26	8	2	3	6		
A_R				24.84	7.92	2.00	2.99	5.93		
H_E				0.944	0.551	0.252	0.281	0.573		
P_{HW}				0.730	0.146	0.570	0.034	0.400		
F_{IS}				-0.021	0.092	-0.159	0.068	0.098		
Dep 20										
n						41	61	60		
#A						3	8	15		
A_R						3.00	7.99	15.00		

H_E						0.379	0.815	0.906		
P_{HW}						1.000	0.511	0.044		
F_{IS}						-0.030	-0.086	0.073		
Dep 21										
n	63	33	34	56	50	41	61	60	53	24
#A	2	1	1	7	2	1	2	1	25	8
A_R	1.87	1.00	1.00	6.63	2.00	1.00	1.92	1.00	18.81	7.72
H_E	0.047	0.000	0.000	0.718	0.229	0.000	0.016	0.000	0.940	0.799
P_{HW}	1.000	-	-	0.336	1.000	-	-	-	0.000	0.488
F_{IS}	-0.016	-	-	0.005	0.038	-	0.000	-	0.257	0.009
Dep 28										
n	63	33	34			41	61	60		
#A	6	7	5			3	2	3		
A_R	5.43	6.94	4.91			3.00	2.00	3.00		
H_E	0.741	0.760	0.681			0.453	0.357	0.417		
P_{HW}	0.570	0.036	0.571			1.000	0.493	0.041		
F_{IS}	-0.071	-0.275	0.007			0.031	0.081	0.241		
Dep 30										
n	63	33	34	56	50	41	61	60	53	24
#A	1	1	1	2	2	1	6	17	18	2
A_R	1.00	1.00	1.00	2.00	2.00	1.00	5.75	16.91	11.89	2.00
H_E	0.000	0.000	0.000	0.388	0.059	0.000	0.543	0.906	0.780	0.120
P_{HW}	-	-	-	0.483	1.000	-	0.760	0.181	0.620	1.000
F_{IS}	-	-	-	0.125	-0.021	-	-0.116	-0.085	-0.016	-0.045
Dep 32										
n	63	33	34	56	50	41	61	60	53	24
#A	28	11	12	19	6	4	5	13	20	14

AR	23.56	10.76	11.56	18.17	5.98	4.00	4.99	12.86	14.84	13.08
HE	0.951	0.846	0.844	0.803	0.597	0.184	0.715	0.864	0.920	0.847
PHW	0.365	0.792	0.019	0.000	0.280	1.000	0.720	0.051	0.005	0.375
FIS	0.032	-0.039	0.059	0.400	0.129	-0.061	-0.170	0.074	0.221	0.016
Dep 33										
n	63	33	34	56	50	41	61	60	53	24
#A	8	6	6	1	1	1	1	1	21	8
AR	6.92	5.99	6.00	1.00	1.00	1.00	1.00	1.00	13.52	7.83
HE	0.759	0.754	0.754	0.000	0.000	0.000	0.000	0.000	0.835	0.68
PHW	0.962	0.705	0.259	-	-	-	-	-	0.004	0.582
FIS	-0.046	-0.045	-0.053	-	-	-	-	-	0.118	0.081
Dep 38										
n	63	33	34	56	50	41	61	60	52	24
#A	15	9	4	2	2	2	1	1	1	1
AR	12.77	8.99	4.00	2.00	2.00	2.00	1.00	1.00	1.00	1.00
HE	0.875	0.857	0.635	0.387	0.298	0.252	0.000	0.000	0.000	0.000
PHW	0.178	0.872	0.239	1.000	0.327	0.570	-	-	-	-
FIS	0.057	-0.096	0.074	0.032	-0.210	-0.159	-	-	-	-
Dep 40										
n						41	61	60	53	21
#A						8	3	10	40	22
AR						8.00	2.92	9.99	23.52	22.00
HE						0.743	0.232	0.788	0.958	0.965
PHW						0.026	0.308	0.688	0.000	0.002
FIS						0.146	0.152	-0.036	0.153	0.260
Dep 44										
n	63	33	34	56	50	41	61	60	53	24

#A	12	6	4	14	5	3	3	10	12	9
AR	10.53	5.94	3.99	13.73	5.00	3.00	3.00	9.93	9.54	8.87
HE	0.850	0.783	0.668	0.731	0.651	0.529	0.648	0.694	0.832	0.880
PHW	0.198	0.102	1.000	0.485	0.176	0.645	0.004	0.461	0.840	0.630
FIS	-0.120	0.033	-0.012	-0.051	0.078	0.078	0.038	0.063	-0.020	0.005
Dep 51										
n	63	33	34	56	50	41	61	60	53	24
#A	15	11	6	5	2	1	4	8	8	2
AR	12.55	10.87	5.82	4.86	2.00	1.00	4.00	8.00	5.97	2.00
HE	0.845	0.867	0.462	0.231	0.416	0.000	0.266	0.781	0.495	0.424
PHW	0.774	0.822	0.137	0.608	1.000	-	0.012	0.009	0.858	0.346
FIS	0.005	-0.049	0.045	-0.006	-0.010	-	0.199	0.039	-0.030	0.214
Dep 53										
n	63	33	34	56	50	41	61	60	53	24
#A	7	5	5	3	1	1	4	7	10	8
AR	6.49	4.99	5.00	3.00	1.00	1.00	3.91	6.87	7.51	7.86
HE	0.804	0.704	0.672	0.136	0.000	0.000	0.328	0.798	0.823	0.861
PHW	0.179	0.508	0.981	1.000	-	-	0.660	0.293	0.496	0.275
FIS	-0.026	0.139	-0.006	-0.048	-	-	0.100	-0.086	0.014	0.177
Dep 57										
n						41	61	60		
#A						4	1	1		
AR						4.00	1.00	1.00		
HE						0.452	0.000	0.000		
PHW						0.580	-	-		
FIS						-0.187	-	-		
Dep 61										

n	63	32	34	56	50	41	61	60	53	24
#A	4	3	5	10	3	2	1	1	11	5
Ar	3.99	3.00	5.00	9.84	3.00	2.00	1.00	1.00	8.21	5.00
He	0.502	0.254	0.646	0.760	0.542	0.494	0.000	0.000	0.583	0.600
Phw	0.010	0.097	0.003	0.000	0.563	0.200	-	-	0.422	0.209
Fis	0.178	0.260	0.362	0.366	-0.144	-0.235	-	-	-0.101	0.166
Dep 65										
n	63	33	34			41	61	60		
#A	3	2	4			1	1	1		
Ar	2.94	1.94	3.91			1.00	1.00	1.00		
He	0.242	0.030	0.633			0.000	0.000	0.000		
Phw	0.712	-	0.917			-	-	-		
Fis	0.016	0.000	-0.069			-	-	-		
Dep 67										
n	63	33	34	56	50	41	61	60	53	24
#A	14	6	11	3	1	5	1	2	14	7
Ar	10.37	5.94	10.65	2.86	1.00	5	1.00	2.00	9.44	6.625
He	0.743	0.672	0.829	0.135	0.000	0.563	0.000	0.168	0.796	0.739
Phw	0.517	0.077	0.054	0.234	-	0.425	-	0.396	0.000	0.284
Fis	0.060	0.189	0.220	0.208	-	0.047	-	0.108	0.431	0.098
Dep 73										
n	63	33	34	56	50	41	61	60	53	24
#A	15	8	12	22	5	2	1	1	4	1
Ar	13.08	7.94	11.90	21.08	4.96	2.00	1.00	1.00	3.17	1.00
He	0.898	0.810	0.864	0.911	0.365	0.158	0.000	0.000	0.224	0.000
Phw	0.251	0.647	0.173	0.218	0.027	1.000	-	-	0.567	-
Fis	0.010	-0.122	-0.021	0.039	-0.096	-0.081	-	-	0.075	-

Dep 74										
n	63	33	34	56	50	41	61	60	53	24
#A	15	8	9	9	5	2	3	10	26	12
AR	13.16	7.87	8.82	8.86	4.98	2.00	3.00	10.00	18.84	11.46
HE	0.860	0.677	0.807	0.794	0.623	0.302	0.532	0.727	0.944	0.861
PHW	0.920	0.189	0.074	0.535	0.951	0.312	0.232	0.399	0.000	0.225
FIS	0.021	-0.119	-0.020	-0.011	0.006	-0.212	-0.140	0.083	0.580	0.032
Dep 85										
n	63	33	34	56	50	41	61	60	53	24
#A	6	6	6	5	2	3	2	2	11	5
AR	5.74	6.00	5.99	4.86	2.00	3.00	2.00	2.00	8.51	4.86
HE	0.704	0.649	0.621	0.417	0.078	0.597	0.374	0.417	0.826	0.591
PHW	0.966	0.017	0.670	0.002	1.000	0.122	0.486	0.544	0.890	0.905
FIS	-0.060	0.299	0.006	0.058	-0.032	0.182	0.124	0.081	-0.074	-0.129
Dep 90										
n	63	33	34	56	50	41	61	60	53	23
#A	8	9	6	22	7	3	6	16	8	3
AR	6.98	8.82	5.99	21.12	6.96	3.00	5.91	15.79	7.04	2.91
HE	0.795	0.781	0.752	0.940	0.780	0.499	0.654	0.896	0.641	0.126
PHW	0.825	0.771	0.002	0.000	0.396	0.234	0.212	0.925	0.051	1.000
FIS	-0.018	-0.048	0.374	0.202	-0.052	-0.173	0.047	-0.005	0.000	-0.031
Dep 91										
n	63	33	34	56	50	41	61	60	53	24
#A	20	14	15	16	8	3	1	2	23	10
AR	18.22	13.76	14.72	15.73	8.00	3.00	1.00	2.00	17.99	9.72
HE	0.941	0.895	0.910	0.910	0.848	0.523	0.000	0.049	0.935	0.871
PHW	0.018	0.854	0.290	0.002	0.003	0.003	-	1.000	0.107	0.512

FIS	0.123	0.052	-0.002	0.039	0.151	-0.260	-	-0.017	0.092	-0.004
Dep 93										
n						41	61	60		
#A						3	4	7		
AR						3.00	3.91	6.93		
HE						0.14	0.385	0.649		
PHW						0.003	0.124	0.568		
FIS						0.651	-0.022	-0.052		
Dep 100										
n	63	33	34	56	50	41	61	60	53	24
#A	4	4	6	2	1	2	1	2	3	1
AR	3.36	4.00	5.90	2.00	1.00	2.00	1.00	2.00	2.93	1.00
HE	0.23	0.409	0.552	0.193	0.000	0.071	0.000	0.168	0.545	0.000
PHW	0.662	0.105	1.000	1.000	-	1.000	-	0.397	0.081	-
FIS	0.034	0.110	-0.065	-0.111	-	-0.026	-	0.108	-0.073	-
Dep 101										
n				56	50		61	60	53	24
#A				6	3		3	5	9	3
AR				5.73	3.00		3.00	4.93	6.98	2.88
HE				0.475	0.524		0.140	0.506	0.609	0.512
PHW				0.835	0.013		1.000	0.455	0.720	0.804
FIS				-0.128	-0.030		-0.053	0.045	0.009	-0.140
Dep 102										
n							61	60		
#A							2	3		
AR							2.00	3.00		
HE							0.288	0.512		

P_{HW}							0.358	0.028		
F_{IS}							0.145	-0.301		
Dep 103										
n	63	33	34	56	50	41	61	60	53	24
#A	8	6	9	3	3	4	2	3	4	5
A_R	7.69	5.93	8.74	3.00	2.98	4.00	2.00	3.00	4.00	4.75
H_E	0.739	0.582	0.823	0.374	0.512	0.510	0.485	0.386	0.732	0.582
P_{HW}	0.554	0.370	0.288	0.867	0.479	0.046	0.287	0.839	0.750	0.139
F_{IS}	-0.095	0.063	0.178	-0.051	-0.094	-0.099	0.155	0.050	0.072	0.285
Dep 105										
n	63	33	34	56	50	41	61	60	53	24
#A	6	4	3	1	1	1	3	1	4	6
A_R	4.87	3.94	2.91	1.00	1.00	1.00	2.84	1.00	3.42	5.95
H_E	0.248	0.530	0.190	0.000	0.000	0.000	0.033	0.000	0.383	0.659
P_{HW}	1.000	0.278	1.000	-	-	-	1.000	-	0.148	0.721
F_{IS}	-0.086	0.199	-0.085	-	-	-	-0.004	-	0.114	-0.012
Dep 106										
n	63	33	34	56	49	41	61	60	53	23
#A	8	9	7	4	1	4	3	3	4	3
A_R	7.23	8.94	6.82	3.88	1.00	4.00	3.00	3.00	3.78	2.91
H_E	0.806	0.745	0.721	0.185	0.000	0.532	0.430	0.081	0.559	0.126
P_{HW}	0.457	0.142	0.624	0.002	-	0.270	0.001	1.000	0.819	1.000
F_{IS}	0.055	0.064	0.061	0.421	-	-0.100	0.123	-0.024	0.020	-0.031
Dep 108										
n	63	33	31			41			53	22
#A	6	4	5			3			3	2
A_R	5.45	3.94	5.00			3.00			2.88	2.00

H_E	0.653	0.616	0.493		0.433		0.396	0.496
P_{HW}	0.947	0.641	0.000		0.638		0.075	1.000
F_{IS}	0.028	0.163	0.607		0.042		0.125	0.083

Table A3. Microsatellites in each sample found either to deviate significantly from Hardy-Weinberg equilibrium (before and after Bonferroni correction) or to show evidence of null alleles and/or amplification errors based on analysis with MICROCHECKER.

Sample	*Significant Deviations From Hardy-Wienberg Equilibrium*		*Possible Null alleles or Amplification Errors*
	Before Bonferroni Correction	*Before and After Bonferroni Correction*	
D. argentosa			
Devils River	*Dep*61, 91, 101	*Dep*101	*Dep*91, 101
San Felipe Creek	*Dep*28, 85, 101	*Dep*101	*Dep*85, 101
Independence Creek	*Dep*32, 61, 67, 90, 101, 108	*Dep*101, 108	*Dep*61, 67, 90, 101, 103, 108
D. diaboli			
Devils River	*Dep*32, 61, 85, 90, 91, 106	*Dep*32, 61, 90, 91, 106	*Dep*32, 61, 90, 106
Pinto Creek	*Dep*8, 73, 91, 101	None	*Dep*91
D. episcopa			
El Rito Creek	*Dep*40, 91, 93, 103	None	*Dep*93
D. nigrotaeniata			
Fessenden Spring	*Dep*3, 18, 44, 51, 106	*Dep*106	None
Comal Springs	*Dep*51, 102	None	None
D. serena			
Nueces River	*Dep*10, 18, 21, 32, 33, 40, 67, 74	*Dep*18, 21, 33, 40, 67, 74	*Dep*10, 18, 21, 32, 33, 40, 67, 74, 91
Frio River	*Dep*8, 18, 40	None	*Dep*18, 40

Table A4. Above the diagonal: Distance between samples as measured by pair-wise Φ_{ST} values between homologous mtDNA sequences. Probability (P) values for all tests of $\Phi_{ST} = 0$ were significant ($P < 0.05$) except for the pair-wise distance between the samples of *D. nigrotaeniata* from Fessenden Springs and Comal Springs ($P = 0.104$). Below the diagonal: Distance between samples as measured by pair-wise F_{ST} values. All 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, DnFS = *D. nigrotaeniata* from Fessenden Spring, DnCS = *D. nigrotaeniata* from Comal Springs, DsNR = *D. serena* from Nueces River, and DsFR = *D. serena* from Frio River.

	DaDR	DaSFC	DaIC	DdDR	DdPC	DnFS	DnCS	DsNR	DsFR
DaDR	-	0.248	0.779						
DaSFC	0.052	-	0.808						
DaIC	0.160	0.172	-						
DdDR				-	0.252				
DdPC				0.230	-				
DnFS						-	0.158*		
DnCS						0.280	-		
DsNR								-	0.933
DsFR								0.376	-

VITA

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