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Author(s): A. H. Hanna , K. W. Conway , E. W. Carson , G. P. Garrett , and J. R. Gold

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CONSERVATION GENETICS OF AN UNDESCRIBED SPECIES OF *DIONDA* (TELEOSTEI: CYPRINIDAE) IN THE RIO GRANDE DRAINAGE IN WESTERN TEXAS

A. H. HANNA, K. W. CONWAY, E. W. CARSON, G. P. GARRETT, AND J. R. GOLD*

Center for Biosystematics and Biodiversity, Texas A&M University, College Station, TX 77843-2258 (AHH, KWC, EWC, JRG)
Inland Fisheries Division, Heart of the Hills Fisheries Science Center, Texas Parks and Wildlife Department, 5103 Junction Highway,
Mountain Home, TX 78058 (GPG)

*Correspondent: goldfish@tamu.edu

ABSTRACT—The systematic and conservation-genetic status of a population of the cyprinid genus *Dionda* in Alamito Creek, a tributary of the Rio Grande in Big Bend Ranch State Park, Presidio County, Texas, was assessed using mtDNA sequences and nuclear-encoded microsatellites. Phylogenetic analyses of the mitochondrial cytochrome *b* (*cytb*) gene revealed that the *Dionda* in Alamito Creek likely is conspecific with an undescribed species (*Dionda* species 1) known only from the Río Conchos and Río Nazas basins of Mexico and in the USA from Cibolo Creek, a small tributary of the Rio Grande drainage. Genetic variation of *Dionda* in Alamito Creek is extremely low. All 18 fish assayed for 1,533 base pairs of mitochondrial mtDNA (*cytb* and ND5 sequences) had the same haplotype, and 40 fish assayed for 34 nuclear-encoded microsatellites (25 monomorphic) averaged only 1.44 alleles/microsatellite ($H_E = 0.12$). Estimates of current effective size (N_e) and effective number of breeders (N_b) were 22 and 28, respectively. Bayesian coalescent analysis indicated that the population has undergone a greater than three-orders-of-magnitude decline in effective size. Low genetic diversity and low estimates of N_e and N_b indicate that the population is compromised genetically and warrants monitoring and attention to its official conservation status.

RESUMEN—Evaluamos el estatus sistemático y la genética de conservación de una población del género ciprínido (*Dionda*) colectada en el arroyo Alamito, un tributario del río Grande en el Big Bend Ranch State Park, condado de Presidio, Texas, usando secuencias de ADN mitocondrial y microsatélites nucleares. Análisis filogenéticos del gen mitocondrial citocromo *b* (*cytb*) indicaron que los individuos de *Dionda* en el arroyo Alamito son probablemente conspecíficos de una especie previamente no descrita (*Dionda* species 1), reportada solamente en las cuencas de los ríos Conchos y Nazas en México y en los USA en la cuenca del riachuelo Cibolo, un tributario pequeño del río Grande. Variación genética en *Dionda* del arroyo Alamito es extremadamente baja. Los 18 peces analizados exhibieron el mismo haplotipo mitocondrial para las 1,533 bases secuenciadas (*cytb* y ND5), y los 40 individuos analizados usando 34 microsatélites nucleares (25 de los cuales fueron mono-mórficos) promediaron sólo 1.44 alelos/microsatélite ($H_E = 0.12$). Estimaciones del tamaño efectivo actual de la población (N_e) y del número efectivo de individuos reproductivos (N_b) fueron 22 y 28, respectivamente. Métodos coalescentes Bayesianos indicaron que el tamaño efectivo de la población ha sido reducido por encima de tres órdenes de magnitud. El bajo nivel de variación genética y estimaciones bajas de N_e y N_b indican que la población está comprometida genéticamente y merece un monitoreo y una clarificación de su estado de conservación oficial.

Cyprinids of the genus *Dionda* inhabit springs and spring-fed streams in New Mexico and Texas in the United States and in Mexico (Mayden et al., 1992; Schnöhuth et al., 2012). Eight described species currently are recognized (Schnöhuth et al., 2008, 2012): *D. argentosa*, *D. diaboli*, *D. episcopa*, *D. flavipinnis*, *D. melanops*, *D. nigrotaeniata*, *D. serena*, and *D. texensis*. Seven of these (all but *D. melanops*) inhabit central and western Texas, including spring-fed headwaters of the San Antonio, Colorado, Guadalupe, and Nueces river drainages, and

portions of the lower Rio Grande drainage and its tributaries, including the Pecos and Devils rivers (Hubbs et al., 1991; Edwards et al., 2004; Schnöhuth et al., 2008, 2012; Carson et al. 2010); *D. melanops* occurs in Mexico in various parts of the lower Rio Grande drainage (Schnöhuth et al., 2008, 2012). Four additional, undescribed species of *Dionda* have been reported (Schnöhuth et al., 2012): two from Mexico (*Dionda* species 1 from the Conchos and Nazas river drainages, and *Dionda* species 2 from Ojo de Agua de San Juan in the Mezquital River

drainage and from El Vergel Spring, also in the Mezquital drainage), and two from the USA (*Dionda* species 3 from the San Saba and Concho rivers in the northern Colorado River drainage, and *Dionda* species 4 from the upper Pecos River drainage in New Mexico). These undescribed species apparently are being described by R. L. Mayden and colleagues (Schnöhuth et al., 2008, 2012). Interested readers should be aware that the numbers used in Schnöhuth et al. (2008) to annotate undescribed species differ somewhat from those used in Schnöhuth et al. (2012).

Given their specific habitat requirements, species of *Dionda* are vulnerable to alteration of habitat (Garrett et al., 1992; Edwards et al. 2004). Of the seven species in Texas, only one (*D. diaboli*) is considered threatened (United States Fish and Wildlife Service, 1999); however, the remaining six species likely face challenges (Brune, 2002; López-Fernández and Winemiller, 2005; Texas Wildlife Action Plan, <http://www.tpwd.state.tx.us/publications/>), particularly during extended drought. Understanding the geographic distribution of *Dionda* in the United States and Mexico has been a central effort in the laboratory of R. L. Mayden (Schnöhuth et al., 2008, 2012) and is critical to managing and conserving the biodiversity they represent. Recent fieldwork has led to discovery of new populations of *Dionda* in Texas, extending the known range of *D. diaboli* (Garrett et al., 2004) and *D. argentosa* (Carson et al., 2010).

Recent surveys in portions of Alamito Creek, a tributary of the Rio Grande in Big Bend Ranch State Park, Presidio County, Texas, uncovered a population of *Dionda* inhabiting small permanent pools. *Dionda* referred to as *D. episcopa* has been recorded in other parts of Alamito Creek (<http://www.fishesoftexas.org>). Populations of *Dionda* in Big Bend State Park have been monitored sporadically by the Texas Parks and Wildlife Department for several years, but no biological assessment has been conducted. An analysis of sequences of the mitochondrial ND5 gene from a few individuals indicated that the fish in Alamito Creek were distinct from all other nominal *Dionda* in Texas. To investigate identity of the population in Alamito Creek, we acquired sequences of the mitochondrial cytochrome *b* (*cytb*) gene; genotypes at 34 nuclear-encoded microsatellites were used to evaluate the conservation-genetic status of the population. Morphometric and meristic characters were documented for future comparisons with other species of *Dionda*.

MATERIALS AND METHODS—A total of 94 specimens was collected from Alamito Creek in Big Bend Ranch State Park, Presidio County, Texas. Sampling localities were between 29°42'36"N, 104°7'42"W and 29°42'23"N, 104°7'55"W. Specimens were preserved in 95% ethanol (for analyses of DNA) or fixed in 10% formalin, with subsequent transfer to 70% ethanol, for morphometric and meristic evaluation.

DNA was extracted from muscle tissue, using a Chelex-resin-extraction protocol (Estoup et al., 1996). Initially, polymerase-

chain-reaction (PCR) primers L12328 (5'-AACTCTTGGTG-CAAMTCCAAG-3'; Miya et al., 2006) and DS-H (5'-AAAAATTTGTTGATTCTCGGA-3'; Carson et al. 2010) were used to sequence a 585 base-pair (bp) fragment of the ND5 gene from three individuals; these were compared to ND5 sequences in Carson et al. (2010) of six species of *Dionda* (*D. argentosa*, *D. diaboli*, *D. flavipinnis*, *D. serena*, *D. texensis*, and *Dionda* species 4) known from Texas and New Mexico. The change, relative to nomenclature used in Carson et al. (2010), from *D. episcopa* sampled in the upper Pecos River to *D. species 4*, *D. nigrotaeniata* to *D. flavipinnis* (*Dionda* in the Guadalupe River drainage), and *D. serena* (*Dionda* in the upper Nueces River) to *D. texensis* reflects nomenclatorial changes suggested by Schönuth et al. (2012). Laboratory methods were as in Carson et al. (2010). Phylogenetic analyses (not shown) revealed that fish from Alamito Creek possibly represented a new species of *Dionda*. Fifteen additional specimens were then sequenced for the 585 bp ND5 fragment and all 18 specimens were sequenced for the complete mitochondrial *cytb* gene (1,141 bp). PCR primers Glu-F and Thr-R (5'-GAAGAACCACCGTTGTTATTCAA-3' and 5'-ACCTCCRATCTYCGGATTACA-3', respectively; Zardoya and Doadrio, 1998) were used to amplify *cytb* sequences, using amplification protocols described in Carson et al. (2010). PCR products from each individual were band-cut from 2% agarose gels and purified using the QIAquick Gel Extraction Kit (Qiagen, Inc., Valencia, California). Sequencing was carried out by the Interdisciplinary Center for Biotechnology Research at the University of Florida (<http://www.biotech.ufl.edu/>), using the forward primer, Glu-F. Sequences were analyzed using Sequencher version 3.0 (Gene Codes, <http://www.genecodes.com/>) and truncated to 969 orthologous bp for comparison across species. Haplotypes were identified using Mega version 4.0.2 (Kumar et al., 1994).

Cytochrome *b* sequences of *Dionda* from Alamito Creek were compared to available sequences of described and undescribed species of *Dionda* from Texas, New Mexico, and Mexico (Mayden et al., 2007; Schönuth et al., 2008, 2012). Sequences (GenBank accession numbers) examined were: *D. argentosa* (EU082498.1, EU082499.1), *D. diaboli* (DQ324085.1, DQ324086.1, EU082493.1, EU082494.1), *D. episcopa* (DQ324077.1, EU082490.1), *D. flavipinnis* (EU082501.1, EU082502.1), *D. melanops* (EU082495.1, EU082496.1, EU082497.1), *D. serena* (DQ324080.1), *D. texensis* (EU082504.1, EU082505.1), *Dionda* species 1 (DQ324084.1, EU082492.1), *Dionda* species 2 (DQ324081.1, DQ324082.1, DQ324083.1, EU082491.1), *Dionda* species 3 (EU082503.1), and *Dionda* species 4 (DQ324078.1). Sequences for *Dionda* species 4, *D. flavipinnis*, and *D. texensis* are in GenBank under the names *D. episcopa*, *D. nigrotaeniata*, and *D. serena*, respectively; the change to *Dionda* species 4 (*Dionda* in the upper Pecos River), *D. flavipinnis* (*Dionda* in the Guadalupe River drainage), and *D. texensis* (*Dionda* in the upper Nueces River) reflects nomenclatorial changes suggested by Schönuth et al. (2012). Sequences (GenBank accession numbers) from five species of *Camptostoma* (DQ324062.1, DQ324063.1, DQ324064.1, DQ324065.1, EU082476.1, EU082477.1) and *Nocomis leptcephalus* (EU082468.1) were included as outgroup taxa. Sequences were aligned by eye, using TextWrangler version 2.3 (<http://www.barebones.com/products/textwrangler/>) and McClade version 4.05 (Maddison and Maddison, 1997).

Maximum-parsimony analysis of the *cytb* dataset employed heuristic searches in PAUP* version 4.0b10 (Swofford, 2002),

using TBR branch swapping with the MULTREES option and 1,000 random-addition sequence replicates. Bootstrapping with 1,000 pseudoreplicates (random-addition sequence and TBR branch swapping) was used to evaluate nodal support. Maximum-likelihood analysis and non-parametric bootstrapping (1,000 pseudoreplicates) were conducted using Garli version 0.951 (Zwickl, 2006), using a GTR model of nucleotide substitution with all parameters set to default.

Genotypes at 38 nuclear-encoded microsatellites were acquired from 40 individuals. PCR primers and reaction conditions for each microsatellite are given in Renshaw et al. (2009). Four microsatellites (*Dep2*, *Dep44*, *Dep57*, and *Dep102*) were discarded due to inconsistency of scoring. The remaining 34 microsatellites were genotyped using fluorescently labeled DNA following Renshaw et al. (2009) and an ABI PRISM 377 DNA Sequencer (Applied Biosystems, Carlsbad, California). Alleles were sized using the 400 HD Rox size-standard (Applied Biosystems, Carlsbad, California). Chromatograms were analyzed in Genescan (version 3.1.2, Applied Biosystems, Carlsbad, California); alleles were scored using Genotyper (version 2.5, Applied Biosystems, Carlsbad, California).

Exact probability tests as implemented in Genepop version 4.0.10 (Raymond and Rousset, 1995) were used to test genotypes for conformance to Hardy-Weinberg expectations and for genotypic disequilibrium. Sequential Bonferroni correction (Rice, 1989) was applied for multiple tests of the same hypothesis. Each microsatellite was evaluated for amplification errors, null alleles, or both, using Microchecker (van Oosterhout et al., 2004). F-stat version 2.9.3.2 (Goudet, 1995) was used to obtain number of alleles, gene diversity (expected heterozygosity), and the inbreeding coefficient F_{IS} (f of Weir and Cockerham, 1984); SPSS software (International Business Machines, Armonk, New York) was used to compute 95% confidence intervals (CI) around means for number of alleles and gene diversity.

The Bayesian coalescent approach in Msvr version 4.1b (Beaumont, 1999; Storz and Beaumont, 2002) was used to estimate parameters N_0 and N_1 (effective number of chromosomes at sampling and at the beginning of an expansion-decline phase, respectively), t_a (number of generations since effective change in size began), and μ (average rate of mutation across all microsatellites). Run parameters are available from AHH. Effective number of breeders (N_b) was estimated using the linkage-disequilibrium method in LdNe (Waples and Do, 2008). Alleles were excluded using the 2% threshold recommended by Waples and Do (2010); 95% CIs were estimated using the jackknife method. Finally, average, long-term effective size of population (N_{eLT}) was estimated using the maximum-likelihood approach in Migrate version 3.0.3 (Beerli and Felsenstein, 1999, 2001). A short run was used to provide an initial estimate of theta (Θ) for longer runs, which used 10 short chains (10,000 sampled gene trees) and four long chains (5,000,000 sampled gene trees). Average, long-term effective size was then estimated as $\Theta = 4N_e\mu$, where μ was generated using Msvr version 4.1b (Beaumont, 1999; Storz and Beaumont, 2002).

A total of 15 morphometric characters was taken from 12 specimens; six of these were cleared and double-stained after Taylor and van Dyke (1985) for counts of fin-rays and vertebrae. Measurements and counts followed Hubbs and Lagler (1958). Males and females were identified by presence or absence, respectively, of snout tubercles and rows of tubercles along the

anterior-most pectoral fin-rays. Voucher specimens were deposited at the Texas Cooperative Wildlife Collection (TCWC 14782.01-17). Compiled morphological data and a photograph of male and female specimens are at <<http://agrillife.org/gold/doc/>> under morphological data and *Dionda* from Alamito Creek.

RESULTS—No variation in *cytb* (969 bp) or ND5 (564 bp) sequences was observed among the 18 individuals sequenced. GenBank accession numbers are JQ412818 (*cytb*) and JQ412817 (ND5). Topologies resulting from maximum-parsimony and maximum-likelihood analyses of *cytb* sequences recovered a monophyletic group comprising *Dionda* from Alamito Creek and *Dionda* species 1 (*sensu* Schönhuth et al., 2008) from the Río Conchos and Río Nazas basins of Mexico, with 100 and 98% bootstrap support, respectively. The mtDNA haplotype from Alamito Creek differed from those of *Dionda* species 1 by 2–7 substitutions, and from all other nominal species of *Dionda* by 64–143 substitutions. Both topologies are at <<http://agrillife.org/gold/doc/>> under phylogenetic topologies. Topologies may be of interest as GenBank accession numbers for all described and undescribed species of *Dionda* suggested by Schönhuth et al. (2012) are provided.

Of 34 microsatellites assayed, 25 were monomorphic. One microsatellite, *Dep3*, deviated significantly from Hardy-Weinberg expectations before, but not after, sequential Bonferroni correction. There was no evidence of amplification errors or null alleles at any microsatellite. Five of 45 tests of genotypic equilibrium were significant before Bonferroni correction; none was significant after correction. Average number of alleles and average gene diversity among the nine polymorphic microsatellites were 2.7 and 0.437, respectively (Table 1); considering all 34 microsatellites, averages were 1.4 and 0.116, respectively.

Bayesian coalescent analysis (Table 2) revealed a negative, posterior distribution of $\log_{10}(\tau)$ value of -3.295 , consistent with a three-orders-of-magnitude decline in effective size of the population in Alamito Creek; the modal estimate of current effective size (N_0) of the population was 21.8. Given possible generation times of 1 and 3 years, estimated time since decline was 13–38,550 years ago (mode, 538–1,614 years ago). Estimated effective number of breeders in the population in Alamito Creek was 28 (CI = 7.9–infinity); estimate of average, long-term effective size of population was 660 (CI = 585–755).

DISCUSSION—Cytochrome *b* sequences of *Dionda* in Alamito Creek are essentially the same as those from *Dionda* species 1 (*sensu* Schönhuth et al., 2008), a species known to date only from the Río Conchos and Río Nazas basins of Mexico and in the USA from Cibolo Creek, a small tributary of the Rio Grande. We have not had the opportunity to examine other specimens of *Dionda*

TABLE 1—Summary statistics for nine polymorphic microsatellites in 40 individuals of *Dionda* sampled from Alamito Creek, Presidio County, Texas: F_{IS} is an inbreeding coefficient; P is the probability that $F_{IS} = 0$.

Microsatellite	Number of alleles	Gene diversity	F_{IS}	P
<i>Dep3</i>	5	0.592	-0.141	0.007 ^a
<i>Dep7</i>	2	0.444	-0.127	0.489
<i>Dep9</i>	2	0.491	0.136	0.513
<i>Dep20</i>	3	0.535	0.114	0.764
<i>Dep38</i>	2	0.096	-0.040	1.000
<i>Dep40</i>	2	0.468	-0.232	0.181
<i>Dep91</i>	3	0.344	-0.165	0.088
<i>Dep93</i>	3	0.488	0.026	0.487
<i>Dep100</i>	2	0.475	-0.161	0.495

^a Non-significant following Bonferroni correction.

species 1, but the *cytb* data support the hypothesis that the *Dionda* in Alamito Creek is conspecific with *Dionda* species 1. Additional surveys of spring habitats in the vicinity of Big Bend Ranch State Park and Big Bend National Park may lead to discovery of additional populations of *Dionda* species 1 in Texas or even undiscovered populations of other described, undescribed, or both, species of *Dionda*.

Genetic diversity in *Dionda* from Alamito Creek is extremely low. Only one mtDNA haplotype (1,533 bp) was recovered among 18 individuals assayed. In contrast, average ($\pm SE$) number of ND5 haplotypes (585 bp) and diversity of haplotypes across 10 populations representing five of the described species in waters of the USA was 4.6 ± 1.3 and 0.446 ± 0.096 , respectively (Carson et al., 2010). In addition, 25 of 34 microsatellites assayed were monomorphic in the population in Alamito Creek. Including the monomorphic microsatellites, average number of alleles and gene diversity were 1.4 and 0.116, respectively. In comparison, average number of alleles and gene diversity, based on 28–34 microsatellites, in 10 populations representing five described species of *Dionda* in waters in the USA were 6.2 ± 1.0 and 0.463 ± 0.159 , respectively (A. H. Hanna et al., in litt.).

Bayesian estimates of N_e (current effective size of population) were 0.4–525.8, with highest probability of modal N_e of 21.8. This modal value was similar to the effective number of breeders ($N_b = 28$) obtained from the linkage-disequilibrium approach in LdNe. The latter (N_b) provides information about the effective number of breeding adults that produced the sampled cohort(s) (Waples and Do, 2010); however, relating N_b to N_e is problematic for iteroparous species because of the potential for overlap between sets of parents producing offspring in successive years (Waples, 2010). Nonetheless, both N_e and N_b are estimates on a recent time scale (Beaumont, 2003) and their near identity for *Dionda* in Alamito Creek is striking. Finally, the Msvr-derived $N_0:N_1$ ratio indicated a three-orders-of-magnitude decline in effective size of the population in Alamito Creek,

TABLE 2—Summary statistics for posterior distributions of parameters μ , N_0 , N_1 , and t_a . The parameter r is the ratio N_0/N_1 . Estimates of t_a are given for generation times of 1 and 3 years.

Parameter	Mode	0.025 quartile	0.975 quartile
μ	2.2×10^4	2.6×10^5	1.9×10^3
N_0	21.8	0.4	525.8
N_1	43,052.7	3,270.4	617,731.9
$\log_{10}(r)$	-3.295	-3.916	-3.062
t_a (years)	538–1,614	13–39	12,850–38,550

occurring 13–38,550 years ago, with modal estimates of 538–1,614 years ago. A decline in effective size of population also was indicated by the estimate of average, long-term effective size of population ($N_{eLT} = 660$), where N_{eLT} represents a harmonic mean of N_e over ca. $4N_e$ generations (Hare et al., 2011).

The minimum effective size of population needed to ensure long-term genetic integrity remains a matter of debate. In theory, the equilibrium between loss of adaptive genetic variance, stemming from genetic drift, and its replacement, by mutation, necessitates an effective size of a few hundred to a few thousand individuals (Schultz and Lynch, 1997; Lynch and Lande, 1998). An effective size of population < 50 , as in *Dionda* in Alamito Creek, indicates high vulnerability to inbreeding depression (Rieman and Allendorf, 2001) and risk of extinction due to fixation of deleterious alleles and loss of adaptive genetic variance (Franklin, 1980; Anderson, 2005). The low estimates of N_e (22) and N_b (28) for *Dionda* in Alamito Creek, together with low levels of genetic diversity, clearly indicate the population is compromised genetically.

Factors affecting decline in effective size of the population of *Dionda* in Alamito Creek undoubtedly include deterioration of habitat and water quality. Alamito Creek is an intermittent stream in the Chihuahuan Desert that contains segments of healthy riparian habitat and perennial pools that historically have supported populations of endemic fishes, amphibians, and aquatic invertebrates. This region of Texas has the highest percentage of species of vertebrates of conservation concern, and Alamito Creek alone contains three other species of fish (*Camptostoma ornatum* Mexican stoneroller, *Notropis chihuahua* Chihuahua shiner; *Cyprinodon eximus* Conchos pupfish) that are listed as threatened by the state of Texas. Persistent drought and withdrawal of groundwater have damaged many existing spring-associated communities in this region (Garrett and Edwards, 2001), and the current, exceptional drought in much of Texas raises an even greater risk of deterioration of habitat and water quality. In addition to > 10 km of Alamito Creek that occurs on Big Bend Ranch State Park, an additional 5.5-km segment upstream of the park is protected by the Trans Pecos Water and Land Trust. This upstream segment has been recognized by the state of Texas as meeting criteria of an ecologically unique river

and stream segment, and a coordinated plan for a holistic approach to watershed conservation is underway.

The paucity of genetic variation and low estimates of N_e and N_b for the population of *Dionda* in Alamito Creek clearly suggest a need for continued monitoring and perhaps assignment as an officially recognized population of conservation concern. Further, there is a need for additional surveys of tributaries and streams in the Rio Grande drainage to assess whether there are additional populations of *Dionda* species 1 in the basin. It also would be important for *Dionda* species 1 to be described as a means to assist in its conservation and protection.

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