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**Conservation Genetics of aN Undescribed Species of *Dionda* (Teleostei: Cyprinidae) in the Rio Grande Drainage in West Texas**

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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 in western Texas, was assessed using mtDNA sequences and nuclear-encoded microsatellites. Phylogenetic analyses of the mitochondrial cytochrome *b* (cyt*b*) gene revealed that the *Dionda* in Alamito Creek is likely conspecific with an undescribed species (*D*. sp. 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 in Alamito Creek *Dionda* is extremely low. All 18 fish assayed for 1,533 base pairs of mitochondrial mtDNA (cyt*b* and ND5 sequences) had the same haplotype, and 40 fish assayed for 34 nuclear-encoded microsatellites (25 monomorphic) averaged only 1.44 alleles per microsatellite (*HE* = 0.12). Estimates of current effective size (*Ne*) and effective number of breeders (*Nb*) were 22 and 28, respectively. A 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 *Nb* indicate that the population is genetically compromised and warrants monitoring and attention to its official conservation status. Expanded surveys of the Rio Grande drainage are needed to determine whether there are additional populations of *D*. sp. 1 in the basin.

Resumen— Evaluamos el estatus sistemático y la genética de conservación de una población del genero ciprínido *Dionda*, recolectado en el arroyo Alamito (un tributario del Río Grande) en el Big Bend Ranch State Park en el oeste de Texas usando secuencias de ADN mitocondrial y microsatélites nucleares. Análisis filogenéticos del gen mitocondrial citocromo *b* (cyt*b*) indican que los individuos de *Dionda* en el arroyo Alamito son probablemente conspecificos con una especie previamente no descrita (*D*. sp. 1), reportada solamente en las cuencas de los rios Conchos y Nazas en México y en los EEUU en la cuenca del riachuelo Cibolo, un tributario del Rio Grande. La variación genética en *Dionda* del arroyo Alamito es extremadamente baja. Los 18 individuos analizados exhiben el mismo haplotipo mitocondrial (1,533 bases secuenciadas); mientras los 40 individuos analizados usando 34 microsatélites nucleares (25 de los cuales fueron mono-morficos) promedian solo 1.44 alelos por microsatélite, con una diversidad genética de 0.12. Estimados del tamaño efectivo de la población (*Ne*) y del numero efectivo de individuos reproductivos (*Nb*) fueron, respectivamente, 22 y 28. Métodos coalescentes usando una inferencia Bayesiana indican que el tamaño efectivo de la población ha sido reducido por encima de tres ordenes de magnitud. El bajo nivel de variación genética y los bajos estimados de *Ne* y *Nb* indican que la población de *Dionda* del arroyo Alamito se enfrenta a posibles dificultades genéticas. Por ende, es necesario se establezca un monitoreo continuo y que se aclare su estado de conservación. Colectas mas expansivas en la cuenca del Río Grande son necesarias para determinar si existen mas poblaciones de *D*. sp. 1 en los Estados Unidos.

 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 are currently recognized (Schnöhuth et al., 2008, 2012): *D. argentosa* Girard, *D. diaboli* Hubbs and Brown, *D. episcopa* Girard, *D. flavipinnis* Cope, *D. melanops* Girard, *D. nigrotaeniata* Cope, *D. serena* Girard, and *D*. *texensis* Girard. Seven of these (all but *D. melanops*) inhabit central and western areas of 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* sp. 1 from the Conchos and Nazas river drainages, and *Dionda* sp. 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* sp. 3 from the San Saba and Concho rivers in the northern Colorado River drainage, and *Dionda* sp. 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 the undescribed species differ somewhat from those used in Schnöhuth et al. (2012).

 Given their specific habitat requirements, species of *Dionda* are vulnerable to habitat alteration (Garrett et al., 1992; Edwards et al. 2004). Of the seven species in Texas, only one (*D. diaboli*) is considered threatened (USFWS, 1999); the remaining six species, however, likely face challenges (Brune 2002; López-Fernández and Winemiller, 2005; Texas Wildlife Action Plan, <http://www.tpwd.state.tx.us/publications/>), particularly during periods of extended drought. An understanding of the geographic distribution of species 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 the 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 survey work in portions of Alamito Creek, a tributary of the Rio Grande located in Big Bend Ranch State Park in western 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 Depart (TPWD) for several years, but no biological assessment has been carried out (G. P. Garrett, unpubl.). An analysis of sequences of the mitochondrial ND5 gene from a few individuals indicated that the Alamito Creek fish were distinct from all other nominal *Dionda* occurring in Texas. We then acquired sequences of the mitochondrial cytochrome *b* (cyt*b*) gene to investigate the species identity of the Alamito Creek population and genotypes at 34 nuclear-encoded microsatellites to evaluate the conservation-genetic status of the population. Morphometric and meristic character states were documented for future comparisons with other species of *Dionda*.

Materials and Methods—A total of 94 specimens were collected from a stretch of Alamito Creek in Big Bend Ranch State Park. Sample localities occurred between 29°42ʹ36ʺN, 104°7ʹ42ʺW and 29°42ʹ23ʺN, 104°7ʹ55ʺW. Specimens were preserved in 95% ethanol (for DNA analyses) or fixed in 10% formalin, with subsequent transfer to 70% ETOH, 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 (5ʹ-AACTCTTGGTGCAAMTCCAAG-3ʹ; Miya et al., 2006) and DS-H (5ʹ-AAAAATTTGTTGATTTCTCGGA-3ʹ; 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 *D*. sp. 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*. sp. 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 Schönhuth et al. (2012). Laboratory methods were as in Carson et al. (2010). Phylogenetic analyses (not shown) revealed that the Alamito Creek fish 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 cyt*b* gene (1,141 bp). PCR primers Glu-F and Thr-R (5ʹ-GAAGAACCACCGTTGTTATTCAA-3ʹ and 5ʹ-ACCTCCRATCTYCGGATTACA-3ʹ, respectively; Zardoya and Doadrio, 1998) were used to amplify cyt*b* 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). 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 v. 3.0 (Gene Codes, http://www.genecodes.com/) and truncated to 969 orthologous bp for comparison across species. Haplotypes were identified using Mega v. 4.0.2 (Kumar et al., 1994).

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

 Maximum-parsimony (MP) analysis of the cyt*b* data set employed heuristic searches in PAUP\* v. 4.0b10 (Swofford, 2002), utilizing TBR branch swapping with the MULTREES option and 1000 random-addition sequence replicates. Bootstrapping with 1000 pseudoreplicates (random addition sequence and TBR branch swapping) was used to evaluate nodal support. Maximum-likelihood (ML) analysis and non-parametric bootstrapping (1000 pseudoreplicates) were conducted using Garli v.0.951 (Zwickl, 2006), utilizing 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 (*Dep*2, *Dep*44, *Dep*57, and *Dep*102) were discarded due to scoring inconsistency. The remaining 34 microsatellites were genotyped using fluorescently labeled DNA (following Renshaw et al., 2009) and an ABI PRISM 377 DNA Sequencer (Applied Biosystems). Alleles were sized using the 400 HD Rox size-standard (Applied Biosystems). Chromatograms were analyzed in Genescan (v. 3.1.2, Applied Biosystems); alleles were scored using Genotyper (v 2.5, Applied Biosystems).

 Exact probability tests as implemented in Genepop v. 4.0.10 (Raymond and Rousset, 1995) were used to test genotypes for conformance to Hardy-Weinberg expectations (HWE) 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 and/or null alleles, using Microchecker (van Oosterhout et al., 2004). F-stat v. 2.9.3.2 (Goudet, 1995) was used to obtain number of alleles, gene diversity (expected heterozygosity), and the inbreeding coefficient *FIS* (Weir and Cockerham’s (1984) *f*); SPSSTM was used to compute confidence intervals (95%) around means for number of alleles and gene diversity.

 The Bayesian coalescent approach in Msvar v.4.1b (Beaumont, 1999; Storz and Beaumont, 2002) was used to estimate parameters *N0* and *N1* (effective number of chromosomes at sampling and at the beginning of an expansion/decline phase, respectively), *ta* (number of generations since effective size change began), and *μ* (average mutation rate across all microsatellites). Run parameters are available from the first author. Effective number of breeders (*Nb*) 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% confidence intervals were estimated using the jackknife method. Finally, average, long-term effective population size (*NeLT*) was estimated using the maximum-likelihood approach in Migrate v.3.0.3 (Beerli and Felsenstein, 1999, 2001). A short run was used to provide an initial estimate of theta (*Θ*) for the 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 *Θ* = 4*Neμ*, where *μ* was generated using Msvar (see above).

 A total of 15 morphometric characters were taken from 12 specimens; six of these were cleared and double-stained (after Taylor and van Dyke, 1985) for fin-ray and vertebrae counts. Measurements and counts followed Hubbs and Lagler (1958). Males and females were identified by the 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 both male and female specimens may be found at <http://agrilife.org/wfsc/doc/> under the file names ‘Morphological data’ and ‘*Dionda* from Alamito Creek.’

Results—No variation in cyt*b* (969 bp) or ND5 (564 bp) sequences was observed among the 18 individuals sequenced. GenBank Accession Numbers are JQ412818 (cyt*b*) and JQ412817 (ND5). Topologies resulting from the MP and ML analyses of cyt*b* sequences recovered a monophyletic group comprising *Dionda* from Alamito Creek and *Dionda* sp. 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 *D.* sp. 1 by two or seven substitutions, and from all other nominal species of *Dionda* by 64 to 143 substitutions. Both topologies may be found <http://agrilife.org/wfsc/doc/> under the file name ‘Phylogenetic topologies’. The topologies may be of interest as the GenBank Accession Numbers for all species (described and undescribed) of *Dionda* suggested by Schönhuth et al. (2012) are provided.

 Twenty-five of 34 microsatellites assayed were monomorphic. One microsatellite, *Dep*3, deviated significantly from HWE 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 were 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, the averages were 1.4 and 0.116, respectively.

 Bayesian coalescent analysis (Table 2) revealed a negative, posterior distribution of log10 (*r*) value of −3.295, consistent with a three orders-of-magnitude decline in effective size in the Alamito Creek population; the modal estimate of current effective size (*N0*) in the population was 21.8. Given possible generation times of one and three years, the estimated time since decline ranged from 13 to 38,550 years ago (mode between 538 and 1,614 years ago). The estimated effective number of breeders in the Alamito Creek population was 28 (CI = 7.9 – infinity); the estimate of average, long-term effective size was 660 (CI = 585 – 755).

Discussion—Cytochrome *b* sequences of *Dionda* in Alamito Creek are essentially the same as those from *D.* sp. 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 *D.* sp. 1, but the cyt*b* data support the hypothesis that Alamito Creek *Dionda* are conspecific with *D*. sp. 1. Additional survey of spring habitats in the vicinity of Big Bend Ranch State Park and Big Bend National Park may lead to the discovery of additional populations of *D.* sp. 1 in Texas or even undiscovered populations of other described and/or undescribed species of *Dionda*.

 Genetic diversity in Alamito Creek *Dionda* is extremely low. Only a single mtDNA haplotype (1,533 bp) was recovered among 18 individuals assayed. In contrast, the average (± SE) number of ND5 haplotypes (585 bp) and haplotype diversity 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 Alamito Creek population. Including the monomorphic microsatellites, the average number of alleles and gene diversity were 1.4 and 0.116, respectively. In comparison, the average number of alleles and gene diversity, based on 28-34 microsatellites, in 10 populations representing five of the described species of *Dionda* in waters in the USA were 6.2 ± 1.0 and 0.463 ± 0.159, respectively (Hanna et al. unpubl.).

 Bayesian estimates of *Ne* (current effective population size) ranged from 0.4 to 525.8, with highest probability of modal *Ne* of 21.8. This modal value was similar to the effective number of breeders (*Nb* = 28) obtained from the linkage disequilibrium approach in LdNe. The latter (*Nb*) provides information about the effective number of breeding adults that produced the sampled cohort(s) (Waples and Do, 2010); relating *Nb* to *Ne*, however, is problematic for iteroparous species because of the potential for overlap between sets of parents producing offspring in successive years (Waples, 2010). Nonetheless, both *Ne* and *Nb* are estimates on a recent time scale (Beaumont, 2003) and their near identity for Alamito Creek *Dionda* is striking. Finally, the Msvar-derived N0/*N*1 ratio indicated a three orders-of-magnitude decline in effective size of the Alamito Creek population, occurring between 13 and 38,550 years ago, with modal estimates ranging from 538-1,614 years ago. A decline in effective size also was indicated by the estimate of average, long-term effective size (*NeLT* = 660), where *NeLT* represents a harmonic mean of *N*e over approximately 4*N*e generations (Hare et al., 2011).

 The minimum effective size 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 less than 50, as in Alamito Creek *Dionda*, indicates high vulnerability to inbreeding depression (Rieman and Allendorf, 2001) and extinction risk due to fixation of deleterious alleles and loss of adaptive genetic variance (Franklin 1980; Anderson 2005). The low estimates of *Ne* (22) and *Nb* (28) for Alamito Creek *Dionda*, together with low levels of genetic diversity, clearly indicate the population is compromised genetically.

 Factors affecting the decline in effective size of Alamito Creek *Dionda* undoubtedly include habitat and water quality deterioration. 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 vertebrate species of conservation concern, and Alamito Creek alone contains three other fish species (*Campostoma ornatum*, Mexican stoneroller; *Notropis* *chihuahua*, Chihuahua shiner; and *Cyprinodon eximus*, Conchos pupfish) that are listed as threatened by the State of Texas. Persistent drought and groundwater withdrawal 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 habitat and water quality deterioration. In addition to the more than 10 km of Alamito Creek that occur on Big Bend Ranch State Park, an additional 5.5 km segment upstream of the park is protected by the Trans Pecos Water & Land Trust. This upstream segment has been recognized by the State of Texas as meeting the 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 the low estimates of *Ne*and *Nb* 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 *D*. sp. 1 in the basin. It also would be important for *D*. sp. 1 to be described as a means to assist in its conservation and protection.

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Table 1—Summary statistics for nine polymorphic microsatellites in 40 individuals of *Dionda* sampled from Alamito Creek. *FIS* is an inbreeding coefficient; *P* is the probability that *FIS*= 0.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Microsatellite | # Alleles | Gene Diversity | *FIS* | *P* |
| *Dep*3 | 5 | 0.592 | -0.141 | 0.007† |
| *Dep*7 | 2 | 0.444 | -0.127 | 0.489 |
| *Dep*9 | 2 | 0.491 | 0.136 | 0.513 |
| *Dep*20 | 3 | 0.535 | 0.114 | 0.764 |
| *Dep*38 | 2 | 0.096 | -0.040 | 1.000 |
| *Dep*40 | 2 | 0.468 | -0.232 | 0.181 |
| *Dep*91 | 3 | 0.344 | -0.165 | 0.088 |
| *Dep*93 | 3 | 0.488 | 0.026 | 0.487 |
| *Dep*100 | 2 | 0.475 | -0.161 | 0.495 |

 † Non-significant following Bonferroni correction

Table 2—Summary statistics for posterior distributions of the parameters *µ*, *N0*, *N1*, and *ta*. The parameter *r* is the ratio *N0*/*N1*. Estimates of *ta* are given for generation times of one and three years.

|  |  |  |  |
| --- | --- | --- | --- |
| Parameter | Mode | 0.025 quartile | 0.975 quartile |
| *µ* | 2.2 × 104 | 2.6 × 105 | 1.9 × 103 |
| *N0* | 21.8 | 0.4 | 525.8 |
| *N1* | 43,052.7 | 3,270.4 | 617,731.9 |
| log10(*r*) | -3.295 | -3.916 | -3.062 |
| *ta* (years)† | 538-1,614 | 13-39 | 12,850-38,550 |