Section 6 (Texas Traditional) Report Review

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Project Title: Genetic status of San Felipe Gambusia

Final or Interim Report? Final

Grant #: E-109-R

Reviewer Station: Austin ESFO

Lead station concurs with the following comments: NA (reviewer from lead station)

Interim Report (check one):

- ☐ Acceptable (no comments)
- ☐ Needs revision prior to final report (see comments below)
- ☐ Incomplete (see comments below)

Final Report (check one):

- ☒ Acceptable (no comments)
- ☐ Needs revision (see comments below)
- ☐ Incomplete (see comments below)

Comments:

FINAL REPORT

As Required by

THE ENDANGERED SPECIES PROGRAM

TEXAS

Grant No. TX E-109-R

Endangered and Threatened Species Conservation

Genetic status of San Felipe *Gambusia*

Prepared by:

Dr. Anthony Echelle

Carter Smith
Executive Director

Clayton Wolf
Director, Wildlife

22 November 2011

FINAL REPORT

STATE: Texas **GRANT NUMBER:** TX E-109-R

GRANT TITLE: Genetic status of San Felipe Gambusia

REPORTING PERIOD: 1 Oct 08 to 30 Sep 2011

OBJECTIVE(S):

To conduct a two-year study of the population dynamics and genetic status of the San Felipe Gambusia and Spotfin Gambusia.

Segment Objectives:

Task 1. Sept 2008-Dec 2008. Fish collections--

- For the analysis of population structure and hybridization in San Felipe Creek, we will make two large collections (n = 100) of *Gambusia* at locations where *G. clarkhubbsi* and *G. speciosa* occur together.
- We will make two reference collections (n = 30 each) of *G. speciosa*, one from Devils River and one from Sycamore Creek (Fig. 1).
- For genetic comparison with *G. clarkhubbsi*, we will make one collection of *G. krumholzi* (n = 40) from Rio de Nava, Coahuila.

Task 2. Sept 2008 and Sept 2009. Status of *G. krumholzi* in Rio de Nava--

- During two separate visits to Rio de Nava, we will document the extent of the distribution of *G. krumholzi* and we will obtain estimates of catch per unit effort. The distribution and abundance has never been examined for this species. Specimens will be deposited in the vertebrate collection at Universidad Autonoma de Nuevo Leon.
- We will evalulate the stability of flow in the springs feeding Rio de Nava. To our knowledge, there is no record of historical discharge, but we will search for such information in an attempt to document trends in discharge. At selected locations during both visits, we will compute stream discharge from measures of current speed and cross-sectional area of the watercourse.

Task 3. Dec. 2008-Mar. 2010. Genetic/morphological assays--

- The collections of co-occurring *G. clarkhubbsi* and *G. speciosa* will be scored for several morphological traits indicative of hybridization.
- All specimens of *G. clarkhubbsi*, *G. speciosa*, and *G. krumholzi* will be genotyped for nine microsatellite loci using primers developed for *Gambusia* by Zane et al. (1999) and Spencer et al. (1999). We successfully employed these primers in a recent study of hybridization in *Gambusia* (Davis et al., 2006).
- All specimens will be sequenced for the first 400 base pairs of the mitochondrial ND2 gene. Each variant detected will then be sequenced for the entire gene (1047 bp).

Task 4. Feb 2010-Aug 2010. Data analysis--

- To detect evidence of recent population expansion, we will use Beaumont's (1999) approach for microsatellites and Roger's (1995) approach for mtDNA.

- STRUCTURE software will be used to assign the multilocus microsatellite genotype of each individual to species and to identify first-generation hybrids and backcross progeny.
- Using PAUP and MRBAYES, we will obtain, respectively, maximum parsimony and Bayesian analyses of phylogenetic relationship based on ND2 sequence variation.
- Standard software will be used to obtain estimates of population genetic structure.

Significant Deviations:

None.

Summary Of Progress:

Please see Attachment A.

Location: Val Verde County, Texas; and, Nava, Coahuila, Mexico.

Cost: Costs were not available at time of this report. they will be available upon completion of the Final Report and conclusion of the project.

Prepared by: Craig Farquhar **Date:** 22 November 2011

Approved by: _____ **Date:** 22 November 2011
C. Craig Farquhar

Genetic Status of San Felipe *Gambusia*

Section 6 Grant TX E-109-R

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Date

17 November 2011

1. SUMMARY

Variation in mtDNA (cyt*b*) and six microsatelllite loci were used to address the conservation genetics of *Gambusia krumholzi*, a species previously considered endemic to the Río de Nava of the Río La Compuerta, a tributary of the Rio Grande in Coahuila, Mexico. The results and a reevaluation of morphology suggest that *G. clarkhubbsi*, recently described from San Felipe Creek, Texas, should be treated as a junior synonym of *G. krumholzi*. The San Felipe Creek population had only the common cyt*b* haplotype of *G. krumholzi*, and its microsatellite alleles largely composed a subset of those in Mexican populations of the species. A survey of 22 springfed situations in Coahuila found *G. krumholzi* in two localities well outside the previously understood range of the species, one near the tailwaters of the Río La Compuerta and one in the Río San Diego, a drainage connected to the Río Grande about 80 km upstream of the Río La Compuerta. Diversity attributable to among-population differences was six-fold greater when the San Felipe Creek population was included in the AMOVA than when it was excluded (38.5% versus 6.5%). The level of divergence (F_{ST} = 0.38-0.41) between the San Felipe Creek and Mexican populations suggests a long history of isolation. A search for evidence of genetic introgression by *G. speciosa* in the San Felipe Creek population of *G. krumholzi* found only three hybrids in 198 fish; these included an F_1 hybrid and two progeny of backcrossing, one to each of the parental species. There was no evidence of introgression, indicating that hybridization has played no role in the divergence of the San Felipe Creek population from the Mexican populations of *G. krumholzi*. Based on the genetic structure and restricted distribution of *G. krumholzi*, the San Felipe Creek population and four populations in Mexico are recommended as management units for future monitoring.

2. INTRODUCTION

In this report we use variation in mitochondrial DNA (mtDNA) sequences and microsatellite DNA loci to assess the genetic structure and species-level taxonomy of a complex of closely related populations, one of which is treated as a separate species. The latter is the San Felipe Gambusia (*Gambusia clarkhubbsi*) in San Felipe Creek, a springfed tributary of the Rio Grande in Val Verde County, Texas (Garrett and Edwards 2003). The population was recognized as a species distinct from the Spotfin Gambusia (*G. krumholzi*) in several qualitative characters, including pigmentation and aspects of the male gonopodium. Prior to this study, *G. krumholzi* was considered endemic to the Río de Nava, Coahuila, Mexico (Minckley, 1963; Miller et al., 2005), an upstream, springfed section of the Río La Compuerta drainage, which connects with the Rio Grande about 150 km downstream of the mouth of San Felipe Creek. *Gambusia clarkhubbsi* is listed as threatened by the American Fisheries Society (Jelks et al., 2008) and the State of Texas; *G. krumholzi* is considered vulnerable to extinction (Jelks et al., 2008; http://www.iucnredlist.org). The genetic results presented here, together with a re-evaluation of morphological distinctiveness, indicate synonymy for *G. clarkhubbsi* and *G. krumholzi*.

Therefore, we use *G.* cf. *clarkhubbsi* to distinguish the San Felipe Creek population from Mexican populations of *G. krumholzi*.

One objective of this report is to address the enigmatic historical record for *G.* cf. *clarkhubbsi*. The population went undetected until 1997 despite a long history of collecting in San Felipe Creek (Garrett and Edwards, 2003). Since 1997, it has occurred in large numbers alongside the historically abundant Tex-Mex Gambusia (*G. speciosa*). The change in abundance was associated with a dramatic increase in another spring-associated species, the federally threatened Devils River Minnow (*Dionda diaboli*). These changes might have been caused by a 500-yr flood that scoured the streambed in 1998 and implementation, in 1997, of a multifaceted management plan to improve the headsprings area for *D. diaboli* (Garrett and Edwards, 2003). It is possible that the San Felipe Creek population "has long been present . . . but in low numbers and perhaps associated with an as yet unidentified . . . rare habitat" (Garrett and Edwards, 2003:787). An alternative is recent colonization of San Felipe Creek by *G. krumholzi*. Recent colonization predicts that genetic markers in *G.* cf. *clarkhubbsi* will be a reduced subset of those present in *G. krumholzi*, whereas a long history in San Felipe Creek predicts novel markers.

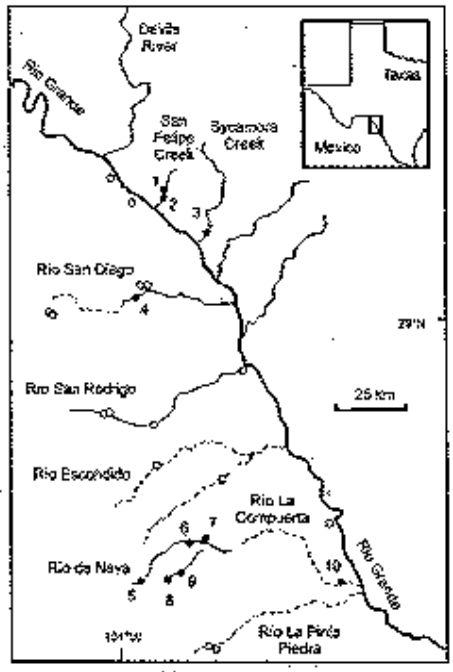

Fig. 1. Collection localities. *Gambusia* cf. *clarkhubbsi* was taken from sites 1-3; *G. krumholzi* from localities 4-10. Open circles = localities in Mexico that did not yield *G. krumholzi*. *G. speciosa*, which is relatively widespread on both sides of the Rio Grande in this region (Miller et al., 2005), was sampled from localities 1 and 2. In the text, Mexican collections are named for the municipality of collection; locality 4 = Los Cristales, 5 = Los Alamos, 6 and 7 = Nava, 8 and 9 = Allende, and 10 = Guerrero.

Finally, we include an analysis of hybridization between *G.* cf. *clarkhubbsi* and the co-occurring Tex-Mex Gambusia (*G. speciosa*). The purpose was to assess the possibility that hybridization might explain genetic differences between *G.* cf. *clarkhubbsi* and *G. krumholzi*. Despite representing different species groups within the genus (Rauchenberger, 1989), the *G. nobilis* group (*G. krumholzi*) and the *G. affinis* group (*G. speciosa*), representatives of the two groups are known to hybridize (Hubbs, 1971; Davis et al., 2006), and there is morphological evidence of genetic introgression (Hubbs, 1971). Finally, we include the results of a survey for *G. krumholzi* in springfed waters of Coahuila outside of the Río de Nava.

3. MATERIALS AND METHODS

Samples.—We collected *G. krumholzi-G.* cf. *clarkhubbsi* from a total of 10 localities (Fig. 1; Table 1) in Texas, U.S.A. (sites 1-3), and Coahuila, Mexico (sites 4-10). Collections of *G.* cf. *clarkhubbsi* were from two localities (sites 1 and 2) separated by 1.5 stream-km. For the analysis of hybridization, we also collected *G. speciosa* from sites 1 and 2. The Mexican localities are designated by the municipality of collection (see legend, Fig. 1). Fin clips from the Mexican collections and whole fish from the Texas collections were placed in 95% ethanol and shipped to Dexter National Fish Hatchery and Technology Center for analysis. In addition, one of us (MLLV) attempted, without success, to collect *G. krumholzi* from 15 additional springfed sites in Coahuila (Fig. 1). For reasons explained in Results, we attempted, in July 2011, to collect *Gambusia* from five localities in Sycamore Creek and five in its largest tributary (Mud Creek), including Mud Springs, the only spring mentioned from Sycamore Creek in Brune's (2002) review of Texas springs. We also searched the Fishes of Texas database (http://www.fishesoftexas.org) for records of *Gambusia* from the Sycamore Creek drainage, and the museum lots for such records were examined by one of us for the possible presence of mis-identified *G.* cf. *clarkhubbsi*.

Molecular methods.—Genomic DNA was extracted using Qiagen DNeasy® Blood and Tissue Kits and stored at -80ºC. We assayed most specimens for 10 microsatellite loci (all with dinucleotide repeat motifs); three developed by Zane et al. (1999) for *G. holbrooki* (*Mf*-1, *Mf*-6, and *Mf*-13) and seven developed by Spencer et al. (1999) for *G. affinis* (*Gafu*1, *Gafu*2, *Gafu*3, *Gafu*4, *Gafu*5, *Gafu*6, and *Gafu*7). Three of these (*Mf*1, *Mf*6, and *Gafu*1) were monomorphic across all species and one (*Gafu*6) was not reliably scoreable. All specimens were genotyped for the remaining six loci.

Microsatellite amplification was done in 10-µl PCR reactions: 0.175 µl AmpliTaq Gold® DNA polymerase; 1X GeneAmp® 10X PCR buffer; 2.5 mM $MgCl_2$; 1.5 mM dNTPs; 0.5 µl each, forward and reverse primers; 3.5 µl ddH_2O; 2 µl DNA. Forward primers were labeled with one of four fluorescent dyes: *Mf*-13 (VIC); *Gafu*-2 (6-FAM); *Gafu*-3 (PET); *Gafu*-4 (NED); *Gafu*-5 (PET); and *Gafu*-7 (VIC). All PCR reagents and primers were purchased from Applied Biosystems. The PCR conditions involved a touchdown protocol that began with a denaturing step of 95ºC for 9 min to activate the Amplitaq Gold®; this was followed by 33 cycles of 94ºC for 45 s, an initial annealing temperature of 56ºC for 45 s, and an extension interval of 72ºC for 60 s. The annealing temperature decreased by 0.2ºC for every cycle. The final extension interval was 15 min at 70ºC. The products were processed on an ABI 3130xl genetic analyzer using GeneScan™ 500 LIZ® size standard. Multilocus genotypes were compiled with GeneMapper™ 4.0 software (Applied Biosystems). About 90% of the genotypes were scored by two separate investigators.

Amplification (10-µl reactions) of mtDNA cytochrome b (*cytb*) followed the PCR conditions outlined above except the extension step was increased to 120 s. We used primers LA15058 and H15149 (Schmidt et al., 1998) to amplify a 402-bp fragment of *cytb*. For selected specimens, the

entire gene (1,149 bp) was amplified using primers LA15058 and HA16249 (Schmidt et al., 1998); the specimens so treated were *G. cf. clarkhubbsi* (n = 5) and *G. krumholzi* from Guerrero (n = 12) and Río San Diego (n = 13). PCR products were purified using the Exo-SAP (Fermentas) procedure using 1/4 reactions following manufacturer instructions; sequencing reactions (both strands) used the Big Dye® v3.1 cycle sequencing kit (ABI) with 1/8 reactions and were run on an ABI 3130xl Genetic Analyzer. Sequences were edited using Sequencher v4.9 (Gene Codes) and aligned by hand in Se-Al v2.0a11 (http://tree.bio.ed.ac.uk/software/seal).

Genetic Analysis.—We used GenAlEx (v6.3; Peakall and Smouse, 2006) to compute expected and observed heterozygosity (H_E and H_O), number of alleles per locus (A_N), and number of private alleles (A_P); FSTAT (v2.9.3.2; Goudet, 1995) for allele richness (A_R) corrected for a sample size of 12; GENEPOP on the web (Raymond and Rousett, 1995) for exact tests of Hardy-Weinberg equilibrium (HWE), pairwise linkage disequilibrium (10,000 iterations), and an analysis of isolation by distance based on microsatellite F_{ST}; and Arlequin (v3.5; Excoffier and Lischer, 2010) to compute analyses of molecular variance (AMOVAs), pairwise F_{ST} values and, for mtDNA, haplotype (h) and nucleotide (π) diversity. To visualize microsatellite variation, we used GenAlEx for a principal coordinates analysis (PCA) of genetic distances among individual multilocus genotypes (Smouse and Peakall, 1999). The sequential Bonferroni correction (α = 0.05) was used for significance in multiple tests of the same hypothesis.

We used GeneClass2 (Piry et al., 2004) with the leave-one-out option and the Bayesian MCMC re-sampling method for allele frequencies to assess the most likely species assignment for individual multilocus genotypes. To search for hybrids between *G. cf. clarkhubbsi* and *G. speciosa* in San Felipe Creek, we used STRUCTURE (v2.2.3; Pritchard et al., 2000) with K = 2, generations back = 2, POPINFO with admixture, and 1.1 million iterations (burnin = first million). The initial species designation for each fish was based on its GeneClass2 assignment. Two separate runs gave essentially identical results.

Morphology.—We examined three and five museum collections of, respectively, *G. cf. clarkhubbsi* (60 males, 60 females) and *G. krumholzi* (56 males, 96 females) for potentially distinguishing characters. Collections chosen were based on availability and the need to encompass variation by including populations from more than one season or locality (see Material Examined).

Qualitative codes were assigned to the following pigmentation characters (range of code values in parentheses) that Garrett and Edwards (2003) used to distinguish *G. cf. clarkhubbsi* from *G. krumholzi*: width of predorsal streak (1-2), development of lateral band on the body (0-3), dark bar on dorsal fin margin (0-2), rows of spots on dorsal fin (0-2), body spotting (= degree of cross-

hatching; 0-4), presence-absence of postanal streak (0,1), darkness of caudal fin margin (0-2) and darkness of anal fin (0-2). Male gonopodial characters included number of fused elbow elements on ray 4a, location of elbow tip (opposite first spinous segment on ray 3 versus opposite segments proximal to the spinous segment), and length of ray-4p hook stepped into its basal length (the length of the ray segment).

For each character and, keeping the sexes separate, we used nested ANOVAs provided by J. McDonald (http://udel.edu/~mcdonald/statnested.html) to test for differences between groups (*G.* cf. *clarkhubbsi* and *G. krumholzi*) and to partition total variance into three components: between groups, among collections within groups, and within collections. OpenStat™ (v. 30.06.10; http://www.Statprograms4U.com) was used to test for normality and equality of variances. No characters showed inequality of variances among collections (Levene tests, $P > 0.05$), but all within-collection tests for normality showed significant deviations (Wilks-Shapiro tests, $P < 0.05$). The AMOVA tests of significance are subject to error because of non-normality and large sample-size differences, but variance-partitioning gives perspective into the strength of between-group differences (McDonald, 2009), the primary concern for morphology in this paper.

4. RESULTS

Distributions.—Both *G.* cf. *clakhubbsi* and *G. speciosa* were abundant in San Felipe Creek. Unexpectedly, a single individual (male, 31 mm SL) with the morphology of *G. krumholzi*-*G.* cf. *clarkhubbsi* was found in a collection of 30 *G. speciosa* preserved in ethanol by one of us (RJE) on 10 September 2009 at the U.S. Highway 277 crossing of Sycamore Creek, Kinney-Val Verde county line, Texas (site 3; Fig. 1). Visits to that site and elsewhere in the Sycamore Creek drainage in July 2011 encountered only dry streambed except for Mud Springs near the headwaters of Mud Creek, where we sampled three springfed sites over a reach of about 1 km; *G. speciosa* was the only poeciliid found. Collections of *Gambusia* from the Highway 277 locality in 1990 (TNHC 22218; $n = 9$) and 1999 (TNHC 27411; $n = 117$) contained only *G. speciosa* as did a combined collection ($n = 508$) from the Sycamore Creek crossings of Highways 277 and 90 in 2002 (TNHC 29476) and a collection from Mud Creek about 10 km downstream from Mud Springs (TNHC 30527; $n = 15$).

Gambusia krumholzi was found at seven of the 21 springfed sites sampled in Coahuila (Fig. 1). They include five sites in irrigation canals of the upper Río La Compuerta, three from Río de Nava (site 5, "Los Alamos", and sites 6 and 7, "Nava") and two (sites 8 and 9, "Allende") from a separate system of irrigation canals. The species also was found in the lower Río La Compuerta

in a spring (site 10, "Guerrero") separated from the Nava-Allende area by about 60 km of normally dry arroyos. Outside of the Río La Compuerta drainage, *G. krumholzi* was found only at a locality (site 4, "Los Cristales") in the Río San Diego drainage. Efforts at four other springfed sites in the drainage yielded no *G. krumholzi*.

Genetic markers.—We combined the two collections of *G.* cf. *clarkhubbsi* for all genetic analyses except the AMOVAs. For all analyses, two other pairs of collections were combined, the two from Nava and the two from Allende. The members of the three lumped pairs were geographically proximal and lumping caused no microsatellite deviation from HWE.

Numbers of alleles for the six microsatellite loci were (total number/number for *G.* cf. *clarkhubbsi-G. krumholzi*) *Mf*13 (5/3); *Gafu*2 (9/7); *Gafu*3 (16/4); *Gafu*4 (28/26); *Gafu*5 (9/4), and *Gafu*7 (14/5). There was no evidence of deviation from HWE or pairwise linkage disequilibrium among loci. Six mtDNA haplotypes representing six substitutions (transitions; none shared by haplotypes) were detected in the 402-bp *cyt*b fragment from *G.* cf. *clarkhubbsi* and *G. krumholzi* (Table 1); the predominant haplotype (A) differed from the others at one (haplotypes B-E) or two (F) positions (uncorrected divergence = 0.2-0.5%). Haplotypes B-E differed from F at three positions (0.7% divergence). In contrast, the three haplotypes detected in *G. speciosa* (Table 1) included two highly divergent (7.0-7.2%) groups (G and J versus I) with 28-29 substitution differences (23-24 transitions, 5 transversions). Haplotypes I and J were rare (frequency = 1-2%). BLAST searches of GenBank showed that Haplotype G was identical to a haplotype (GenBank JF437631) reported from *G. speciosa* (Langerhans et al., in review) and haplotype J differed by only two substitutions; both of these haplotypes were 2.2% divergent from a haplotype (U18207) reported for *G. geiseri* (Lydeard et al., 1995). Haplotype I was identical to a haplotype (DQ075681) reported from *G. affinis* in central Texas (Davis et al., 2006).

Table 1. Distribution of mtDNA haplotypes in *G.* cf. *clarkhubbsi*, *G. krumholzi*, and *G. speciosa*. Asterisk for haplotype I signifies identity with a haplotype from *G. affinis* (see text).

	Haplotype								
Population	A	B	C	D	E	F	G	I	J

PCA analysis and G. *cf.* clarkhubbsi *x* G. speciosa *hybridization.*—The GeneClass2 assignments based on microsatellites showed high probabilities ($q > 0.94$) for placement of all specimens in one or the other of the three groups (*G.* cf. *clarkhubbsi*, *G. speciosa*, and *G. krumholzi*). The one Sycamore Creek specimen with the morphology of *G. krumholzi*-*G.* cf. *clarkhubbsi* classified as *G.* cf. *clarkhubbsi*. One San Felipe Creek specimen (SFG003) classified as *G. speciosa* although it had the common haplotype (A) of *G. krumholzi* and *G.* cf. *clarkhubbsi*. The two putative *G. speciosa* carrying the rare mtDNA haplotype (I) from *G. affinis* classfied as *G. speciosa*; all of their alleles were present in other *G. speciosa*.

With four exceptions, the clusters in the PCA biplot of axes 1 and 2 closely agreed with mtDNA haplotype (Fig. 2). The four exceptions had the common mtDNA haplotype (A) of *G.* cf. *clarkhubbsi* and *G. krumholzi*, but fell well outside the microsatellite clusters for those groups. One of these (SFG001) was the one *G.* cf. *clarkhubbsi* from Sycamore Creek. The remaining three were classified as hybrids in the STRUCTURE analysis of ancestry: an F_1 hybrid (SFG062; q = 0.91), one product of hybrid backcrossing to *G.* cf. *clarkhubbsi* (SFG074; q = 1.00), and one product of backcrossing to *G. speciosa* (SFG003; q = 0.92). The three hybrids and the one Sycamore Creek *G.* cf. *clarkhubbsi* were eliminated from subsequent analyses.

Distribution of genetic diversity in G. *cf.* clarkhubbsi *and* G. krumholzi.—For mtDNA, *G. cf. clarkhubbsi* was fixed for the predominant haplotype (A) in *G. krumholzi* (Table 1). All fish sequenced for the entire *cytb* gene had the haplotype-A sequence for the first 402 bp, but there were two complete-gene haplotypes differing by two substitutions (transitions). One of the haplotype-A variants was in the five *G. cf. clarkhubbsi* assayed for the complete gene and in 16 of the 26 *G. krumholzi.*

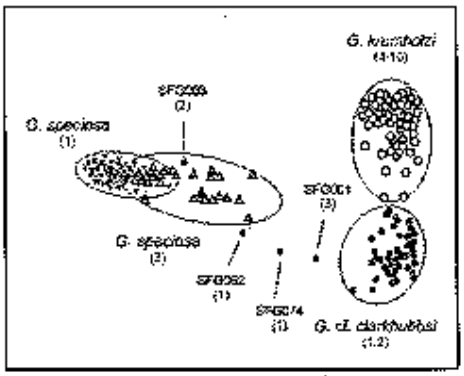

Fig 2. Scores of individual fish on principal coordinate axes 1 and 2. Numbers in parentheses are locality numbers in Figure 1. Solid black circles = haplotype A in fish from San Felipe Creek (locality 1, 2) and Sycamore Creek (locality 3); haplotype A also occurred in most *G. krumholzi.* SFG001 is the single individual of *G. clarkhubbsi* from Sycamore Creek. SFG003, SFG062, and SFG074 are hybrids (see text). All hybrids had the haplotype of *G. cf. clarkhubbsi.*

The lack of mtDNA diversity in *G. cf. clarkhubbsi* was associated with the lowest microsatellite diversity detected (A_R = 2.0, H_E = 0.27; Table 2). Two populations of *G. krumholzi* (Los Cristales and Guerrero, Fig. 1) had zero mtDNA diversity and the lowest microsatellite allele richness detected in the Mexican populations (2.9-3.7 versus 4.2-4.4), but heterozygosity (H_E = 0.33-0.36) was within the range for other Mexican populations (H_E = 0.32-0.37). The other Mexican populations of *G. krumholzi* had 2-3 mtDNA haplotypes.

For mtDNA, only one pairwise F_{ST} comparison, *G. cf. clarkhubbsi* versus the relatively small (n = 13) Los Alamos collection, was significant with the Bonferroni correction (F_{ST} = 0.27; P < 0.0001; Table 3). Without correction, only tests involving the Los Alamos collection were significant or approached significance (F_{ST} = 0.06-0.11; P = 0.03-0.05). Average F_{ST} was 0.06 (range = 0.00-0.16) across the five comparisons of *G. cf. clarkhubbsi* and *G. krumholzi.* AMOVA indicated that zero diversity was due to differences between the two species. With *G. cf. clarkhubbsi* excluded, 4.8% was attributable to differences among populations (P < 0.0001); 95.2% was within-population diversity.

For microsatellites, all except three alleles in *G. cf. clarkhubbsi* were present in *G. krumholzi.* The exceptions were (frequencies in parentheses) *Gafu3*^{243} (0.06), *Gafu4*^{187} (0.29), and *Gafu5*^{264} (0.10). With one exception (Guerrero; *Gafu2*^{127}, frequency = 0.16), and excluding *G. speciosa*, all other private alleles were rare (0.02-0.06; Table 2). All pairwise F_{ST} comparisons among populations were significant (P < 0.0001) except Allende versus Nava (F_{ST} = 0.01; Table 3); there was no microsatellite evidence of isolation by distance (P = 0.09). AMOVA indicated that 35.0% of the genetic diversity was attributable to differences between the two groups, 3.5% to differences among populations within groups, and 61.5% to within-population diversity. With *G. cf. clarkhubbsi* excluded, 6.5% was attributable to differences among populations (P < 0.0001); 93.5% was within-population diversity.

Table 2. Diversity indices for *G.* cf. *clarkhubbsi*, *G. krumholzi*, and *G. speciosa*. Numbers in parentheses = locality numbers in Figure 1. n = number of specimens, n_2 = average number of genotypes assayed per microsatellite locus, P = number of polymorphic loci, A_N = average number of alleles, A_R = allele richness corrected for a sample size of 12, A_P = number of private alleles (all species included/*G. speciosa* excluded), H_O = observed heterozyosity, H_E = expected heterozygosity, h = haplotype diversity, π = nucleotide diversity.

	Microsatellites								mtDNA	
Population	n	n_2	P	A_N	A_R	A_P	H_O	H_E	h	π
G. cf. *clarkhubbsi*										

Table 3. Genetic divergence (F_{ST}) among populations of *G. krumholzi*, including *G.* cf. *clarkhubbsi* from San Felipe Creek. Above diagonal = mtDNA; below = microsatellite loci. Asterisks signify significance with the Bonferroni correction.

Population	Locality number					
(locality number)	1,2	10	8,9	6,7	5	4

Morphology.—With one exception (body crosshatching in males), the nested ANOVAs indicated no significant morphological difference between *G.* cf. *clarkhubbsi* and *G. krumholzi*. Body crosshatching was more strongly developed in male *G.* cf. *clarkhubbsi* than in male *G. krumholzi* ($F_{1,111}$ = 17.4; P = 0.009). The codes for this character ranged from 1 (faintly crosshatched) to 4 (cross hatching strong above and below midlateral line) for *G.* cf. *clarkhubbsi* and 1 to 3 (cross hatching strong above and faint below midline) for *G. krumholzi*. Differences between the two groups accounted for 28% of the variation; 2% was explained by variation among collections within groups. For females, 0% of the variation ($F_{1,154}$ = 0.13; P = 0.73) in cross-hatching was attributable to differences between the two groups, whereas differences among collections within groups accounted for 29% ($F_{1,154}$ = 8.5; P < 0.0001).

For the remaining characters, the ranges for *G.* cf. *clarkhubbsi* and *G. krumholzi* were identical, and the portion of the variance attributable to differences between the two groups was small: 10% and 15% for dorsal-fin spotting in, respectively, males and females, 12% for lateral stripe development in females, 0% to 7% for gonopodial characters, and 0% for the rest of the characters. Two pigmentation characters showed significant (P < 0.0001) variation attributable to differences among collections within groups: lateral stripe and dorsal-fin bar, both of which ranged from absent to distinct in both sexes of both groups. The three gonopodial characters also showed significant variation associated with differences among collections within groups: hook length (P = 0.003), position of elbow tip over spinous or non-spinous segment (0.003), and number of fused elbow segments (P = 0.004).

5. DISCUSSION

Morphological reassessment and the genetic results indicate that *G. clarkhubbsi* is a junior synonym of *G. krumholzi*. None of the genetic markers (mitochondrial cyt*b* and six microsatellite loci) was diagnostic of the two forms. For cyt*b*, the San Felipe Creek population (*G.* cf. *clarkhubbsi*) was fixed for the common haplotype in *G. krumholzi*, and, with three exceptions, its microsatellite alleles were a subset of those detected in *G. krumholzi*. The exceptions occurred at low (0.06-0.10) to moderate frequencies (0.29) alongside alleles shared with *G. krumholzi*. The previously perceived morphological differences were subtle and, in this study, generally showed no added variance associated with the species-level taxonomy. The one exception, degree of body crosshatching in males, differed in the direction (greater in *G. clarkhubbsi*) noted by Garrett and Edwards (2003). However, the males of the two groups overlapped broadly and the difference was not detected in females.

The observed genetic divergence of the San Felipe Creek population of *G. krumholzi* from Mexican populations is consistent with the hypothesis (Garrett and Edwards, 2003) that it has a long history in San Felipe Creek, despite its absence in collections prior to 1997. The presence of novel alleles not attributable to hybridization with *G. speciosa* shows that the population is not a result of recent colonization or introduction from the Mexican portion of the range. Diversity attributable to among-population differences was six-fold greater when the San Felipe Creek population was included in the AMOVA than when it was excluded (38.5% versus 6.5%). The level of divergence (F_{ST} = 0.38-0.41) between it and the five Mexican populations suggests that the population is functionally independent of other populations of *G. krumholzi*. Under selective neutrality and migration-drift equilibrium, F_{ST} values <0.20 reflect long-term migration rates of less than one individual per generation and such rates have negligible effect on allele frequencies (Mills and Allendorf, 1996).

Except for the San Felipe Creek population, there is no genetic evidence of strong barriers to gene flow among populations of *G. krumholzi*. The species is common in springfed waters of the upper Rio La Compuerta system, including the Río de Nava and a separate, springfed system of irrigation canals in the Allende area. The lack of divergence in microsatellite allele frequencies between Allende and Nava (F_{ST} = 0.01) suggests frequent genetic exchange. The Allende and Nava waterflows normally terminate in agricultural fields but they probably are occasionally confluent via downstream arroyos in both systems. The somewhat larger and statistically significant F_{ST} values (≈ 0.05) between the Allende-Nava pair and the Los Alamos collection, all from the upper Río La Compuerta, might reflect sampling error associated with the small (n = 13) size of the Los Alamos collection.

The collections of *G. krumholzi* from Guerrero and especially Los Cristales were well outside the previously understood range of the species in Mexico. The Guerrero site extends the distribution downstream in the Río La Compuerta to a springfed situation separated from the Río de Nava by about 60 km of usually dry arroyo. The Los Cristales population is separated from the Río La Compuerta by about 120 km of presumably inhospitable riverine habitat, including about 80 km of the Rio Grande. The low level of microsatellite divergence between Los Cristales and the Río La Compuerta populations (mean F_{ST} = 0.08) suggests that suitable habitats might have existed in the Rio Grande prior to anthropogenic changes in the river.

We tentatively consider the Los Cristales population to be native, although three observations are consistent with non-native status: (1) it carried no novel alleles and was no more divergent from the Río La Compuerta populations than some of the latter were from each other; (2) collecting efforts in other springfed waters in the Río San Diego drainage failed to yield the species; and (3) there is no extant record of occurrence in springs between the Rio San Diego and the Río La Compuerta. Peden (1970) mentioned, without giving locality details, an uncataloged collection from the Río Escondido, a Rio Grande tributary between Río La

Compuerta and Río San Diego. This collection appears to have been lost and attempts by one of us (MLLV) failed to find the species in the Río Escondido and other Mexican drainages except at Los Cristales and localities in the Río La Compuerta system. Native status for the Los Cristales population is not negated by its disjunct distribution. For example, the species is unknown from a number of well-sampled, Texas tributaries of the Rio Grande between the Río La Compuerta and San Felipe Creek, except for our collection of a single specimen from Sycamore Creek.

The single Sycamore Creek specimen of *G. krumholzi* detected in this study probably represents anthropogenic introduction from San Felipe Creek. Genetically it was most similar to the San Felipe Creek population, but its multilocus microsatellite genotype fell well outside the PCA cluster for that population, suggesting that it originated from a genetically divergent parental genome. Our survey of the drainage and the historical absence of *G. krumholzi* from Sycamore Creek collections suggest a transient population that was extirpated during the severe drought of 2010-2011, when the majority of the Sycamore Creek system went dry. Founder effect associated with anthropogenic introduction would explain divergence from the San Felipe Creek population.

Conservation implications.—The San Felipe Creek population of *G. krumholzi* is not recognizable as an ESU based on Moritz's (1994) criterion of reciprocal monophyly for mtDNA. Also, it likely does not meet the criteria of genetic and (or) ecological non-exchangeability required for ESU recognition by Crandall et al. (2000). All populations of *G. krumholzi* are associated with springflows in a small geographic region and the virtual absence of mtDNA divergence indicates a short time for evolutionary divergence. Therefore, the San Felipe Creek population probably is not ecologically divergent from the remainder of the populations of *G. krumholzi*. Nonetheless, the population warrants special consideration from conservation agencies because it represents a major source of genetic diversity in the species.

To help guide conservation management for *G. krumholzi*, we recommend five management units (MUs) distributed as follows: (1) San Felipe Creek, (2) Los Cristales, (3) Guerrero, (4) Río de Nava (Los Alamos to Nava) and (5) the Allende system. Management units are portions of the species (ESU) warranting some degree of attention in terms of monitoring and management for their continued existence. Recognition of MUs can be based on significant divergence in gene frequencies (Moritz, 1994), but the required level of divergence is subjective (Taylor and Dizon, 1999). The San Felipe Creek population clearly warrants MU recognition, but the Mexican populations are more problematic.

Taylor and Dizon (1999) argue against MU recognition based solely on genetics and suggest that policy goals, such as maintenance at some percentage of historic abundance or maintenance of

the full historic range of the species, should play a role. Considering the restricted geographic range of *G. krumholzi*, we believe that the loss of any of the five MUs recommended here would be a significant step toward extinction. Ideally, and beyond maintenance of MUs, managers should aim to protect or restore the natural pattern of dispersal among populations, thereby preserving the processes that maintain diversity and evolutionary potential (Crandall et al. 2000).

6. MATERIAL EXAMINED

Institutional abbreviations are as listed at http://www.asih.org/codons.pdf.

Genetics.—Parentheses give locality number (Fig. 1), museum number for voucher specimens, and latitude-longitude. *Gambusia krumholzi* (*G. cf. clarkhubbsi*), Texas, Val Verde County: San Felipe Creek at highway 277 bridge in Del Rio (1; OSUS 27800; 29°22'11"N, 100°53'6.73"W); San Felipe Creek at Lions Park in Del Rio (2; OSUS 27799; 29°21'35"N, 100°53'30"W); Sycamore Creek at Highway 277 bridge (3; OSUS 27797; 29°15'15"N, 100°45'02"W). *Gambusia krumholzi*, Coahuila: Spring in Los Cristales, Río San Diego drainage (4; UANL 19496; 29°04'55"N, 101°00'07"W); Río de Nava SE Los Alamos (5; UANL 19549; 28°17'18"N, 101°00'11"W); irrigation canal 8.2 km W Nava (6; UANL 19544; 28°24'50"N, 100°51'55"W); irrigation canal in Nava (7; UANL 19540; 28°25'06"N, 100°46'30"W); water works canal in Allende (8; UANL 19490, 28°20'05"N, 100°52'09W); irrigation canal near Allende (9; UANL 19484; 28°19'39"N, 100°53'09"W); spring in Guerrero city park (10; UANL 19530; 28°18'40"N, 100°22'25"W). *Gambusia speciosa*, Texas, Val Verde County, San Felipe Creek: at highway 90 bridge in Del Rio (1; OSUS 27796; 29°22'11"N, 100°53'6.73"W); at Lions Park in Del Rio (2; OSUS 27798, 29°21'35"N, 100°53'30"W).

Morphology.—Collections are listed with locality number in parentheses followed by museum number, date, and sample size (male/female). *Gambusia cf. clarkhubbsi*: (2) OSUS 27801, 8 March 2008, 20/20; (2) OSUS 27802, 16 March 2009, 9/15; (1) OSUS 27803, 10 July 2011, 31/25. *Gambusia krumholzi*: (10) UANL 18545, 29 August 2007, 6/29; UANL 19530, 6 February 2010, 12/15; (4) UANL 18658, 28 September 2007, 7/17; UANL 19496, 6 February 2010, 4/5; (7) UANL19540, 7 February 2010, 26/30.

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