FINAL REPORT

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THE ENDANGERED SPECIES PROGRAM

TEXAS

Grant No. E - 67

Endangered and Threatened Species Conservation

Genetic Isolation of Comal Springs Riffle Beetle Populations

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19 May 2008

FINAL REPORT

STATE: Texas **GRANT NUMBER:** E - 67

GRANT TITLE: Endangered and Threatened Species Conservation

REPORTING PERIOD: 8/01/05 to 7/31/08

PROJECT TITLE: Genetic Isolation of Comal Springs Riffle Beetle Populations

OBJECTIVE(S):

To determine the degree of genetic isolation of *H. comalensis* at San Marcos Springs and Comal Springs.

Segment Objectives:

Task 1 – Utilize mitochondrial DNA analysis to determine species level and large-scale population differences.

Task 2 – Utilize the Inter-Transcribed Spacer of Ribosomal RNA Gene (ITS dcN gene) to assist in providing estimates of male-mediated versus female-mediated gene flow/ migration.

Task 3 – Utilize Amplified Fragment Length Polymorphism (AFLP) for a fine scale investigation of population genetic structure in the Landa Lake population, and to test hypotheses about population connectivity and cohesion, and provide data for estimating rates of gene flow and migration.

Significant Deviation:

None.

Summary Of Progress:

Please see Attachment A (pdf document)

Location: San Marcos Springs, Comal Springs, San Marcos River, Comal River, Guadalupe River, Big Bend National Park.

Cost: available upon completion of project

Prepared by: Craig Farquhar **Date:** 19 May 2008

Approved by: _____ **Date:** 19 May 08
C. Craig Farquhar

TITLE **GENETIC ISOLATION OF COMAL SPRINGS RIFFLE BEETLE POPULATIONS**

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Abstract.--We examined genetic variation within the endangered Comal Springs riffle beetle, *Heterelmis comalensis*, to assess population structure within the species. We estimated the degree to which the sampling localities of this endangered species were differentiated or isolated from each other. We also examined genetic variation and structure within three congeneric species: *H. glabra*, *H. obesa* and *H. vulnerata*. Data from these three species were used as a basis of comparison with *H. comalensis* and to examine the phylogenetic relationships among these species. Data from mitochondrial DNA (mtDNA) sequences indicated high levels of differentiation among most *H. comalensis* localities. *H. comalensis* populations generally contained similar or higher levels of genetic variation compared to populations of the other *Heterelmis* species. However, four sampling localities within Comal Springs were fixed for a single mitochondrial haplotype (Spring Runs 1-3 and Backwater Spring), while the three other sampling localities contained high levels of mtDNA variation that included private haplotypes (West Shore, Spring Island and San Marcos Springs). *H. comalensis* populations appear to be significantly isolated from each other despite relatively little geographic isolation and no obvious barriers to gene flow in most cases. There is no evidence of a pattern of Isolation-By-Distance in the mtDNA data. A survey of nuclear genomic variation using Amplified Fragment Length Polymorphism markers (AFLPs) revealed some structure, but the patterns of genomic divergence also showed no evidence of Isolation-By-Distance. Instead, Bayesian clustering analysis of the AFLP data grouped populations into two main groups: one group consisted of *H. comalensis* populations that contain high levels of mtDNA variation (West Shore, Spring Island and San Marcos Springs), the other group included populations that were relatively depauperate with respect to mtDNA variation (Spring Runs 1-3 and Backwater Spring). The three populations of *H. comalensis* with high mtDNA diversity do not appear to have suffered the loss of mtDNA genetic variation expected with small population sizes. Instead, these *H. comalensis* populations contain surprising amounts of genetic variation, including several private haplotypes, which indicates that either 1) these localities support unexpectedly large populations, perhaps existing within the Edwards aquifer, or 2) the signature of a significant population decline associated with a very recent bottleneck may not yet be detectable by surveys of variation at presumed neutral genetic markers. The other, genetically depauperate populations may have suffered a recent decline in effective population size, perhaps associated with recent droughts that reduced spring flows, especially at the Spring Run sites in Comal Springs. Phylogenetic analyses of mtDNA variation from the four *Heterelmis* species indicate that *H. comalensis* mitochondrial haplotypes are monophyletic with moderate to strong support. However, *H. glabra* haplotypes are paraphyletic with respect to *H. comalensis*. There is some evidence that the taxon *H. glabra* may contain cryptic taxonomic units. *H. vulnerata* and *H. obesa* are distantly related to the *H. comalensis* and *H. glabra* complex with average sequence divergences between species approaching 13%.

Introduction

Heterelmis comalensis, first described in 1988 (Bosse et al. 1988), and subsequently listed as a federally endangered species (USFWS, 1997), is endemic to central Texas. *H. comalensis* is known to occur in at least six different spring run locations in the Comal River and Landa Lake (Comal Springs), New Braunfels, Texas. These sites may contain distinct populations corresponding to different sources of flow from the Edwards Aquifer (Bosse, 1979). In 2004 *H. comalensis* was confirmed at a seventh locality in the San Marcos Springs complex of the San Marcos River, San Marcos, Texas, some 30 kilometers from the Comal Springs complex.

There is only one known population of *H. comalensis* in the San Marcos Springs (R. Gibson, USFWS, personal communication).

Unlike other aquatic beetles, riffle beetles (Family Elmidae) do not swim and do not breathe at the surface. *Heterelmis* breathe through an "underwater respiratory device" called a plastron (Brown, 1987). A plastron is a thin sheet of air held by small water-repellent hairs beneath the elytra and serves as a gill. As oxygen is used for respiration, it is replaced through diffusion from the surrounding water. A consequence of plastron respiration is that *Heterelmis* requires the near-saturated oxygen levels associated with cool, fast-flowing shallow streams (Brown, 1987). Therefore, the greatest threat to this species' habitat is a loss or reduction of either adequate water quantity or quality, "due primarily to human withdrawal of water from the San Antonio segment of the Edwards (Balcones Fault Zone) Aquifer" (USFWS, 1997), in combination with natural droughts in central Texas. In addition, because the areas around Comal Springs and San Marcos Springs are highly urbanized, there may an additional threat from human recreational activities.

Riffle beetle larvae live for many months and the larvae and adults are found in the same environment (Bosse et al., 1988). In this study, *Heterelmis* adults have been found between April and November, which may be a sign of seasonality. Elmids crawl about slowly on surfaces where algae grow, specifically on surfaces "rough enough to be grasped by their claws," such as submerged rocks, logs, or roots. Elmid larvae and adults feed upon "minute algae encrusted on submerged rocks or wood" (Bosse, 1979) or "upon algae and detritus scraped from the substrate" (Brown, 1987). According to Brown (1987), although little is known about Elmid courtship behavior, "it seems minimal." Few observations have been reported about Elmid oviposition, but it is assumed that elmid females attach eggs to rocks, plants or woody debris below the surface of the water (Brown, 1987).

Heterelmis comalensis is morphologically different from the other species of *Heterelmis* in that it is the smallest of the species in the United States (about 2mm) (Bosse et al., 1988) and its hind wings are vestigial; therefore it cannot fly. By moving down into the available subsurface water, *H. comalensis* apparently survived the drought of the middle 1950s when Comal Springs ceased flowing from June 13 to November 3, 1956; however, considering that this is a fully aquatic species, the populations were likely to have been negatively impacted.

Given the potential threats to this endangered and restricted endemic species, information about the population structure of *H. comalensis* and identification of units for conservation will be necessary for conservation management. Here we determine if population genetic structure is present in the restricted range of the *H. comalensis* and ask whether the genetic variation present in *H. comalensis* is depauperate compared to other *Heterelmis* species. We surveyed molecular genetic variation in two mitochondrial DNA (mtDNA) genes (cytochrome oxidase subunit I (COI) and cytochrome oxidase subunit II (COII), one single copy nuclear gene (the Inter-Transcribed Spacer of Ribosomal RNA Gene (ITS)), and at Amplified Fragment Length Polymorphism (AFLP) markers.

Objective: To determine the degree of genetic isolation of *H. comalensis* at San Marcos Springs and Comal Springs.

Locations

Heterelmis were collected directly by searching on woody debris and the undersides or rocks, or by placing rags near spring openings for up to 3 weeks and collecting beetles directly from retrieved rags. This latter technique was developed by Randy Gibson (USFWS, San Marcos

National Fish Hatchery and Technology Center) and proved to be especially effective for *H. comalensis*.

For *H. comalensis*, beetles were collected at all seven known localities (Table 1, Fig. 1). Surveys at other possible sites in the Comal Springs complex and within the San Marcos springs complex did not yield beetles.

Collections of *H. vulnerata* were made at seven localities (Table 1). Collections of *H. glabra* were made at five localities (Table 1). Collections of a fourth species, *H. obesa*, were also made under permit from west Texas in Guadalupe National Park. These specimens of *H. obesa* (which are outside of the scope of the original project objectives) were used as an outgroup for phylogenetic analyses and also to provide an additional comparison to patterns in *H. comalensis*.

Upon capture, *Heterelmis* were placed in 95% ethanol for storage until DNA isolation. Photographs of each specimen were taken using a dissecting microscope equipped with a digital camera. Genitalia were dissected from males and preserved on microscope slides using standard methods. DNA was then extracted using the Purgene DNA Isolation Kit (Gentra Systems, Minneapolis, MN, USA).

Methods

Dr. Nice and a graduate student will conduct genetic analyses. Preparation of specimens, isolation of total DNA, Polymerase Chain Reaction amplifications of all genetic markers and genotyping using an Applied Biosystems 377 Automated Sequencer will be performed in the laboratory of Dr. Nice, Department of Biology, Texas State University. Dr. Nice will assist and oversee the project and train the graduate student in all procedures. Dr. Nice will also oversee and guide all statistical analyses and interpretation of data as well as the preparation of publications.

Procedure I - Specimen Collection (See Figures 1-3) Collection will be conducted by Randy Gibson of the San Marcos National Fish Hatchery in cooperation with this project (512-353-0011, randy_gibson@fws.gov). The following will be collected in support of this project:

- *H. comalensis* from Comal and San Marcos Springs (30 specimens from San Marcos Springs (Spring Lake); 60 specimens from Comal Springs (30 from Landa Lake/30 from springruns).
- *H. glabra* (the nearest relative to *H. comalensis*) from Big Bend National Park (30 specimens). This species is found in west Texas, Arizona, Mexico, and Central America (Brown and Barr, 1988).
- *H. vulnerata* from Comal, San Marcos, Guadalupe Rivers 180 specimens (60 each site). This species is widely distributed in Texas and Oklahoma (Brown, 1972).

Procedure II - Mitochondrial DNA (MtDNA) Analysis

Dr. Chris Nice (512-245-2178, ccnice@txsate.edu) and Research Assistant will utilize MtDNA Analysis to determine species level and large-scale population differences. Specifically, this approach will be used to answer the following questions and make the following comparisons: 1) Is there detectable evolutionary divergence between

populations within the Comal Springs area and between Spring Lake and the populations at Comal Springs? 2) Is *H. glabra* the nearest relative of *H. comalensis*? 3) What is the timing of divergence between *H. glabra* and *H. comalensis* and does this timing correspond to hypothesized historical biogeographical/ hydrological events? (This involves calculation of molecular clock estimates of time since divergence.) 4) Does the timing of divergence between Comal Springs and Spring Lake populations correspond to hypothesized historical biogeographical/ hydrological events?

Procedure IIII – ITS scNgene

Dr. Chris Nice (512-245-2178, ccnice@txsate.edu) and Research Assistant will utilize the Inter-Transcribed Spacer of Ribosomal RNA Gene (a single copy nuclear gene commonly employed in insect systematics and population genetics studies) to discriminate between sex biased dispersal when examined in combination with mtDNA and to corroborate results of AFLP analyses. The comparison of the geographical distribution of genetic variation in mtDNA (which is maternally inherited and non-recombining) and a nuclear gene (which is biparentally inherited) will provide estimates of male-mediated versus female-mediated gene flow/ migration.

Procedure IV – Amplified Fragment Length Polymorphism (AFLPs)

Dr. Chris Nice (512-245-2178, ccnice@txsate.edu) and Research Assistant will utilize AFLPs to generate multi-locus genotypes for a fine scale investigation of population genetic structure in the Landa Lake population. This will quantify the fine-scale genetic structure within and among populations, provide multi-locus genotype data for assignment tests to test hypotheses about population connectivity and cohesion, and provide data for estimating rates of gene flow and migration.

Results

Mitochondrial DNA sequence variation – Phylogenetic Analyses

A total of 1014 nucleotide base pairs (bp) were sequenced from mtDNA (457bp from COI and 557bp from COII). A total of 8 unique mtDNA haplotypes for the combined mitochondrial genes were observed in *H. comalensis* (Table 1). 20 unique haplotypes were observed in *H. glabra*. *H. vulnerata* contained only four unique sequences and *H. obesa* contained five unique haplotypes (Table 1). Thus, mitochondrial diversity, in terms of the number of haplotypes observed, is as high or higher in *H. comalensis* compared to the other three species. Phylogenetic reconstructions of the mitochondrial gene tree using Neighbor-Joining, Maximum Likelihood and partitioned Bayesian Maximum Likelihood methods produced trees with congruent topologies (Fig. 2). *H. comalensis* is monophyletic with moderate bootstrap and high Bayesian posterior probability support. *H. glabra* is clearly the closest relative to *H. comalensis*, however *H. glabra* is paraphyletic with respect to *H. comalensis* in this data set. *H. glabra* contains significant sequence divergence that appears to be organized geographically (Table 1, Fig. 2). MtDNA haplotypes from *H. glabra* specimens from Fern Bank (Hays County) are significantly divergent from haplotypes from *H. glabra* collected at Dolan Springs (Val Verde County) and nearby sites. Relatively low mtDNA diversity was observed in *H. vulnerata* and *H. obesa*, and it is clear that these species are very distantly related to *H. comalensis* and *H. glabra*, with up to 13% sequence divergence between *H. vulnerata*, *H. obesa* and the other two species (Fig. 2, Table 2).

Mitochondrial DNA sequence variation – Population Genetic Analyses
H. comalensis populations contain a surprising amount of genetic diversity. Haplotype diversity, h (Nei 1987) in *H. comalensis*, ranged from 0.00 at Spring Runs 1-3 and Backwater Spring, to 0.746 at West Shoreline (mean h = 0.254) (Table 1). Furthermore, this variation within *H. comalensis* was highly structured. The exceptions were Spring Runs 1-3 and Backwater Spring, which contained little variation, but the other three *H. comalensis* localities (West Shore Line, Spring Island and San Marcos Springs) contained high amounts of variation, including several private haplotypes observed in each of these three sites. For example, while the San Marcos Springs population shares two haplotypes with populations at Comal Springs (haplotypes 1 and 3), this site also contains a private allele (haplotype 8) (Table 1, Fig. 3). West Shore Line contains one private allele and Spring Island has three private alleles.

Analysis of Molecular Variance (AMOVA) (Excoffier et al. 1992) indicated that most of the genetic variation within *H. comalensis* was distributed within populations, but there was also significant among-population variation (Table 3A). There was little differentiation between spring complexes when populations were grouped into Comal Springs (6 populations) and San Marcos Springs (1 population) (Table 3B), however, the populations with high haplotypic variation (West Shoreline, Spring Island and San Marcos) were strongly and significantly differentiated from each other (Table 3C). This reflects the high diversity and the presence of private alleles in each.

A Mantel test was used to test for a pattern of Isolation-By-Distance among *H. comalensis* populations using the R-PACKAGE software (Legendre and Vaudor, 1991). The Mantel test was used to determine the correlation between pairwise Phi-statistics (F-statistic analogues) based on mtDNA variation (Table 3C) and pairwise geographic distance between populations. No significant correlation was detected (Mantel's r = 0.237, 999 replications, p = 0.185).

Significant diversity and structure among populations was also observed in *H. glabra*. *H. vulnerata* and *H. obesa* contained fewer unique haplotypes, but haplotype diversity within populations was comparable or slightly lower compared to *H. comalensis* and *H. glabra* (Table 1). Overall, the comparisons of mtDNA variation across species give no indication that *H. comalensis* is depauperate despite its smaller range.

Nuclear DNA sequence variation – ITS region
Sequencing of the single-copy nuclear gene ITS revealed no variation within *H. comalensis*. All individuals sequenced contained identical 502 base pair sequences (haplotye A). Furthermore, these sequences were also observed in *H. glabra* (Table 1). Thus, the ITS gene is invariant across both *H. comalensis* and *H. glabra*. A substantially divergent sequence (haplotype B) was obtained from *H. vulnerata* that contained significant insertion/deletion variation compared to *H. comalensis* and *H. glabra* sequences. However, *H. vulnerata* was fixed for this one sequence (i.e. there was also no variation within *H. vulnerata* (Table 1)). This was unexpected given the apparent utility of this gene in other Coleopterans (e.g Vogler and DeSalle, 1994, Szalanski et al. 2000) and invertebrates generally (e.g. Jie et al. 2003).

The completely unexpected lack of diversity at the ITS locus prompted a search for other single-copy nuclear genes with informative variation. The nuclear gene *wingless* also proved to be invariant within *H. comalensis* and *H. glabra*, though a BLAST search of similar sequences

deposited on GenBank revealed sequence similarity to *wingless* sequences from other beetles in the family Carabidae. Unfortunately, universal insect primers for the genes *elongation factor 1-alpha*, *tektin*, *tubulin*, and *white* (all genes that have demonstrated utility for population genetic analyses in other insects) failed to produce PCR products. Future work with single-copy nuclear gene sequences will require the discovery of unique and variable nuclear genes, specific to *Heterelmis*.

Multi-locus genomic markers – AFLP analyses
AFLP marker profiles were produced for 5-14 individuals from each of the 7 populations of *H. comalensis* sampled. AFLP profiles were generated using the selective primer pair *EcoRI*-ACA and *MseI*-CTTG. Amplicons were separated and visualized on Beckman Coulter CEQ 8800 automated DNA sequencer using 6% denaturing polyacrylamide. Beckman Coulter fragment analysis software was used to visualize AFLP bands, which were sized by comparison to a standard ladder (600bp, Beckman Coulter) added to each lane. Band selection and quality control were performed following previously described methods (Gompert *et al.* 2006), which were shown to yield highly reproducible results. This procedure generated 159 polymorphic AFLP markers.

The program STRUCTURE v2.2 (Pritchard *et al*. 2000; Falush *et al*. 2007) was used to cluster individuals based on their AFLP banding profiles, which facilitated identification of groups of populations of genomically similar individuals. STRUCTURE v2.2 employs a model-based Bayesian clustering algorithm to assign individuals probabilistically to clusters to minimize deviations from linkage equilibrium. Analyses were conducted using a model allowing for recessive alleles, which is appropriate for dominant molecular markers such as AFLPs (Falush *et al*. 2007). The admixture model was used, which allows for gene exchange among populations, and runs were conducted using a Markov chain Monte Carlo (MCMC) with 500,000 generations with an initial burnin of 50,000 generations. Prior information regarding the population or species from which an individual was sampled was ignored. The number of clusters (k) was evaluated from 1 to 7 and 10 independent MCMC runs were conducted for each k to provide an assessment of the variance in likelihood estimates for each value of k. We then plotted k versus the mean log likelihood for each k to aid in selecting the number(s) of clusters that best explained the AFLP data, which is assumed to be the value of k at which the log likelihood reaches an asymptote (Pritchard *et al*. 2000).

The number of clusters (k) that best explained the data for *H. comalensis* was three clusters (Figure 4). However, this Bayesian clustering analysis at k=3 revealed two main groups with individuals scattered across several populations being assigned to a third group with low probability. The first major group corresponded to the four populations with low mtDNA variation (Spring Runs 1-3 and Backwater Spring). The other group consisted of the three high diversity populations (West Shore Line, Spring Island and San Marcos Springs) (Figure 4). These AFLP results partially corroborate the analysis of mtDNA variation. Both the mtDNA and AFLP data sets show no indication of isolation by distance and both identify 2 groups of populations (i.e. high vs low diversity). However, the AFLP data did not recover substantial genomic divergences among the West Shore Line, Spring Island and San Marcos Springs populations. It is likely that more AFLP markers generated from additional primer pairs is required for finer discrimination of these populations.

Discussion

Phylogenetic analysis of mtDNA sequence variation indicates that *H. comalensis* and *H. glabra* are closely related and probably sister taxa. In the current analysis, *H. glabra* is paraphyletic with respect to *H. comalensis*. Indeed, *H. glabra* appears to contain significant mitochondrial variation, which has some significant geographical structure. It is possible that *H. glabra* may contain cryptic species diversity. For example, the Fern Bank population of *H. glabra* contains mitochondrial haplotypes (haplotyes 9, 10, 11 and 12) that are greater than 1% divergent from other *H. glabra* sequences, more divergent than the other *H. glabra* haplotypes are from *H. comalensis* haplotypes, which average about 0.8% sequence divergence. Further sampling within *H. glabra* and data from nuclear markers will be required to test the hypothesis of cryptic species diversity within *H. glabra*.

H. comalensis contains a surprising amount of genetic variation as measured by mtDNA sequence variation. The haplotypic diversity observed in population samples from West Shoreline, Spring Island and San Marcos vastly exceeds *a priori* expectations of low diversity associated with small effective population sizes for *H. comalensis*. In fact, the haplotypic diversity in these populations is greater than that observed for many other, non-endangered invertebrates (e. g. Vogler & DeSalle, 1993; Dobler & Farrell, 1999; Nice et al. 2005). This high level of within-population mitochondrial diversity is especially surprising upon comparison with diversity observed in *H. vulnerata*. Because of this species capacity for flight, widespread distribution (relative to *H. comalensis*) and apparent larger population sizes, we expected to see more diversity maintained in *H. vulnerata* populations. However, *H. vulnerata* populations contain slightly lower amounts of mtDNA diversity (Table 1).

The mtDNA variation within *H. comalensis* is also highly structured. Differentiation among populations within the Comal Springs complex (Table 3C) is quite high and greater than pairwise measures from other invertebrates separated by much greater distances (e.g. Nice et al. 2005). This is reflective of high diversity and private alleles contained within each of three variable populations (West Shore Line, Spring Island and San Marcos Springs). The presence of these private alleles and high Φ_{ST} values is indicative of very low levels of gene flow among populations, or none at all. The San Marcos population also contains high haplotypic diversity and private alleles, indicating that, despite its recent discovery, this population has probably existed as a relatively large population for a long time. Clustering of AFLP marker data indicates that the San Marcos Springs population is similar to, and clusters with, West Shore Line and Spring Island.

No evidence of a pattern of Isolation-By-Distance was observed in either the mtDNA or AFLP data sets. This suggests that *H. comalensis* populations are likely not exchanging migrant individuals, which supports the interpretation of little or no gene flow based on pairwise Phi-statistics among these populations (Table 3). This also suggests that *H. comalensis* populations have been isolated for a substantial period of time, especially given the significantly large values of pairwise Phi-statistics.

The three Spring Run populations are an interesting contrast to the populations discussed above, These populations contain no haplotypic diversity and no private alleles. These three are not differentiated from each other (Table 3C) and contain haplotye 1 (the most common haplotype) exclusively. The Spring Run populations appear to have much smaller effective population sizes

than the other *H. comalensis* populations and/or have experienced a recent and severe bottleneck event. Perhaps the drought of the 1950's had a more devastating impact on the Spring Run and Backwater Spring populations, while West Shoreline and Spring Island populations some how escaped this event.

These preliminary findings from our survey of mtDNA sequence and AFLP variation must be considered cautiously until corroborated by data from nuclear sequence markers. It is possible that a recent and drastic decline in effective population size may not yet be detectable using mitochondrial markers. Data from nuclear markers may be especially relevant given recent concerns about the neutrality of mtDNA variation (Bazin et al. 2006a,b). However, adaptive evolution of mitochondrial genes appears to be an unlikely explanation for the high mitochondrial diversity observed in *H. comalensis*.

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Table 1: Sample sizes and genetic data summary for *Heterelmis* collection localities. Mitochondrial DNA (mtDNA) haplotypes are designated by letter (i.e. hA) with the number of individuals possessing each haplotype in parentheses. Haplotype diversity, h (Nei 1987), is provided for mitochondrial data for populations with six or more individuals sequenced. Genotypes for the single copy nuclear gene ITS are followed by the number of individuals possessing each genotype in parentheses. Gene diversity is not reported for ITS because there was no variation detected within populations.

Nominal taxonomic designation	**Population/locality**	**Number collected**	**Number sequenced**	**mtDNA haplotypes (number of individuals)**	**h**	**nuclear ITS haplotypes (number of individuals)**
H. comalensis	Run 1, Comal Springs, Comal Co.	24	20	1(20)	0.000	A(2)
	Run 2, Comal Springs, Comal Co.	25	21	1(21)	0.000	A(2)
	Run 3, Comal Springs, Comal Co.	22	21	1(21)	0.000	A(5)
	Backwater Spring, Comal Springs, Comal Co.	12	12	1(12)	0.000	
	Spring Island, Comal Springs, Comal Co.	26	21	1(13), 2(7), 3(1)	0.529	A(2)
	West Shoreline, Landa Lake, Comal Co.	30	29	1(9), 4(7), 5(10), 6(2), 7(1)	0.746	A(2)
	San Marcos Springs, Hays Co.	32	28	1(18), 3(1), 8(9)	0.500	A(2)
H. glabra	Fern Bank, Little Arkansas Spring, Hays Co.	74	25	9(9), 10(1), 11(14), 12(1)	0.577	A(2)
	Fessenden Spring, Kerr Co.	50	11	13(5), 14(3), 15(2), 16(1)	0.746	A(2)
	Caroline Spring, Independence Creek, Terrell Co.	60	10	17(1), 18(3), 19(1), 20(2), 21(1), 22(1), 23(1)	0.911	A(2)
	Finegan Spring, Devils R., Val Verde Co.	60+	10	24(9), 25(1)	0.200	A(1)
	Dolan Springs, Val Verde Co.	40+	21	26(7), 27(13), 28(1)	0.527	A(2)

Table 1 (continued)

Nominal taxonomic designation	Population/locality	Number collected	Number sequenced	mtDNA haplotypes (number of individuals)	h	nuclear ITS haplotypes (number of individuals)
H. vulnerata	Plum Creek at Hwy 183, Caldwell Co.	60+	19	29(4), 30(14), 32(1)	0.433	B(2)
	Guadalupe R. off Hwy 183, Gonzales Co.	11	10	29(3), 30(7)	0.467	B(2)
	Guadalupe R. on Hwy 474, Kendall Co.	24	10	29(10)	0.000	
	Guadalupe R. - EAST, off Hwy 474, Kendall Co.	37	10	29(8), 30(2)	0.356	
	Old Channel, Comal Springs, Comal Co.	14	10	29(6), 30(4)	0.533	B(1)
	San Marcos R., WWTP, Hays Co.	52	10	29(5), 30(4), 31(1)	0.644	B(1)
	San Marcos R., East Dam of Spring Lake, Hays Co.	1	1	29(1)	0.000	
H. obesa	McKittrick Creek, GMNP, Culberson Co.	12	10	33(5), 34(1), 35(3), 36(1)	0.711	
	Smith Spring, GMNP, Culberson Co.	33	11	33(4), 37(7)	0.509	

Table 2: Mitochondrial sequence divergence (uncorrected p-distances) within- and among-species of *Heterelmis*. Values on diagonal are within-species sequence divergence.

Species	***H. comalensis***	***H. glabra***	***H. vulnerata***	***H. obesa***
H. comalensis	0.00303			
H. glabra	0.01092	0.00928		
H. vulnerata	0.12019	0.12194	0.00263	
H. obesa	0.13417	0.13609	0.12998	0.00217

Table 3: Analysis of Molecular Variance (AMOVA). A) AMOVA of *H. comalensis* populations. B) AMOVA of *H. comalensis* populations, examining differentiation between San Marcos Springs (1 population) and the Comal Springs populations (6 populations). C) Pairwise Φ_{ST} values (F_{ST} analogs) among all *H. comalensis* populations.

A.

Source of Variation	df	Sum of Squares	Variance Components	Percentage of Variation
Among Populations	6	23.009	0.16353	33.93%
Within Populations	145	46.162	0.31836	66.07%
Total	151	63.171	0.48188	

B. Comal Springs vs San Marcos Springs

Source of Variation	df	Sum of Squares	Variance Components	Percentage of Variation
Between Spring Complexes	1	4.245	-0.01290	-2.72% (n.s.)
Among Populations within Spring Complexes	5	18.675	0.16809	35.50%
Within Populations	145	46.162	0.31836	67.23%
Total	151	63.171	0.47355	

C. Pairwise Φ_{ST} – statistics

	Spring Run 1	Spring Run 2	Spring Run 3	Backwater Spring	Spring Island	West Shoreline	San Marcos
Spring Run 1	0.000						
Spring Run 2	0.000	0.000					
Spring Run 3	0.000	0.000	0.000				
Backwater Spr.	0.000	0.000	0.000	0.000			
Spring Island	**0.208**	**0.286**	**0.286**	**0.220**	0.000		
West Shoreline	**0.409**	**0.415**	**0.415**	**0.355**	**0.129**	0.000	
San Marcos	**0.229**	**0.234**	**0.234**	**0.181**	**0.298**	**0.419**	0.000

Bold indicates $p < 0.05$

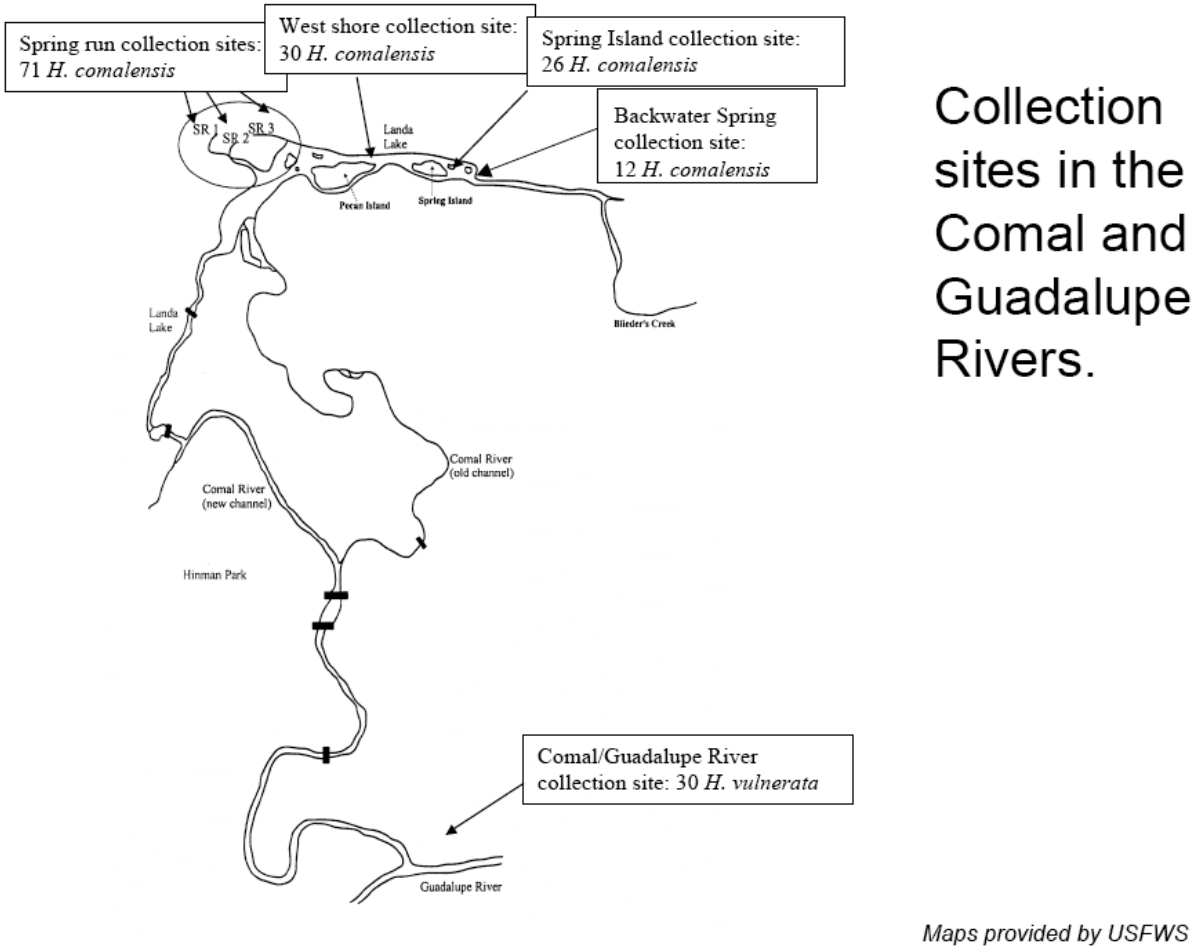

Figure 1: Sampling locations for *H. comalensis* within the Comal Springs complex (New Braunfels, TX).

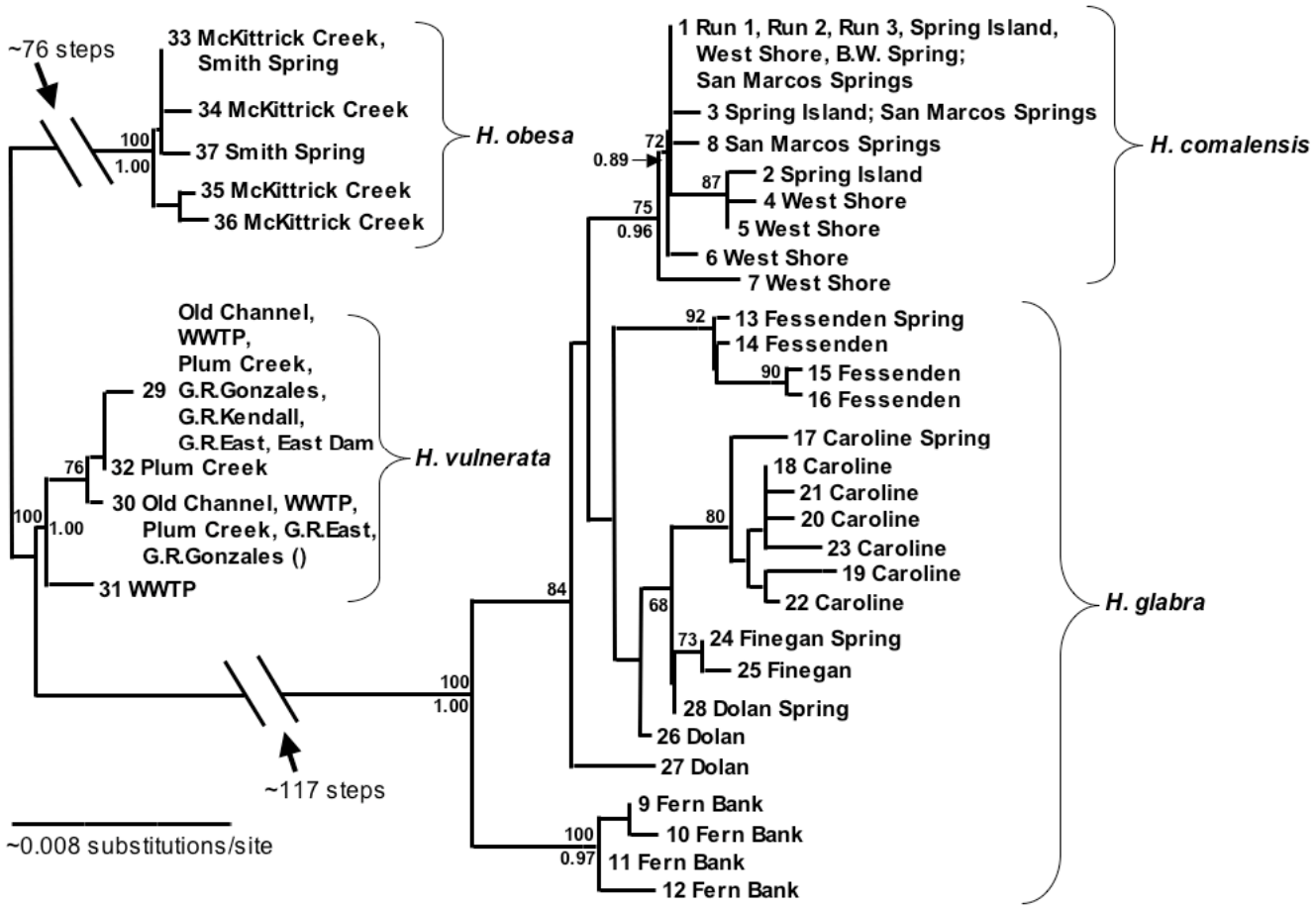

Figure 2: Phylogenetic reconstruction of mtDNA variation (1014bp) within *Heterelmis comalensis*, *H. glabra*, *H. obesa* and *H. vulnerata*. This phylogram is the consensus of the Neighbor-Joining tree, the Maximum Likelihood tree and partitioned Bayesian Maximum Likelihood tree. Confidence at major nodes is indicated as Maximum Likelihood bootstrap value above the node, and Bayesian posterior probability below the node.

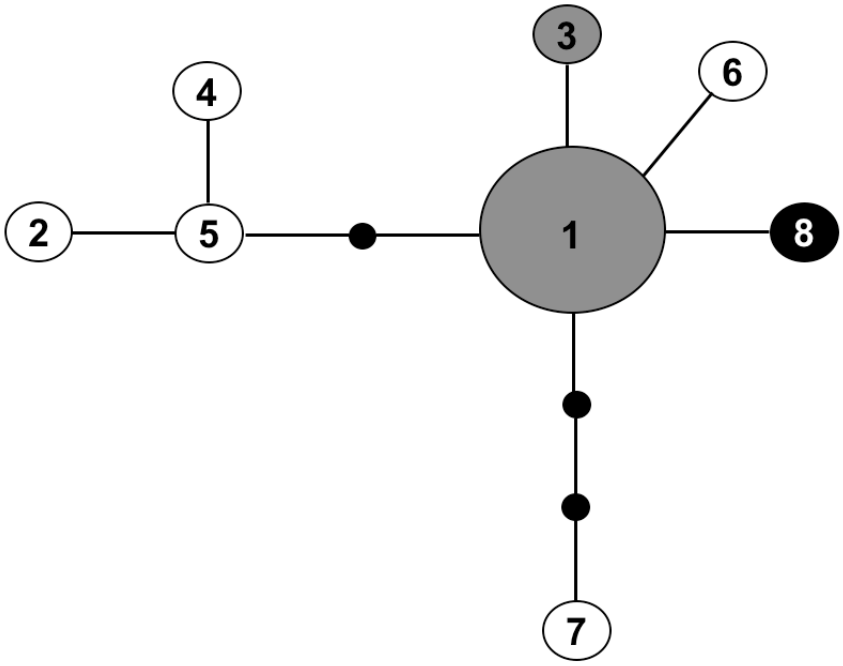

Figure 3: Mitochondrial DNA haplotype network for *Heterelmis comalensis*. Circles represent unique haplotypes; their size is approximately proportional to their frequency in the total data set. Small black circles represent missing haplotypes. Lines indicate one mutational step. Haplotypes are colored by location: white = haplotypes in Comal Springs, black = haplotype in San Marcos Springs, grey = haplotypes found in both springs complexes.

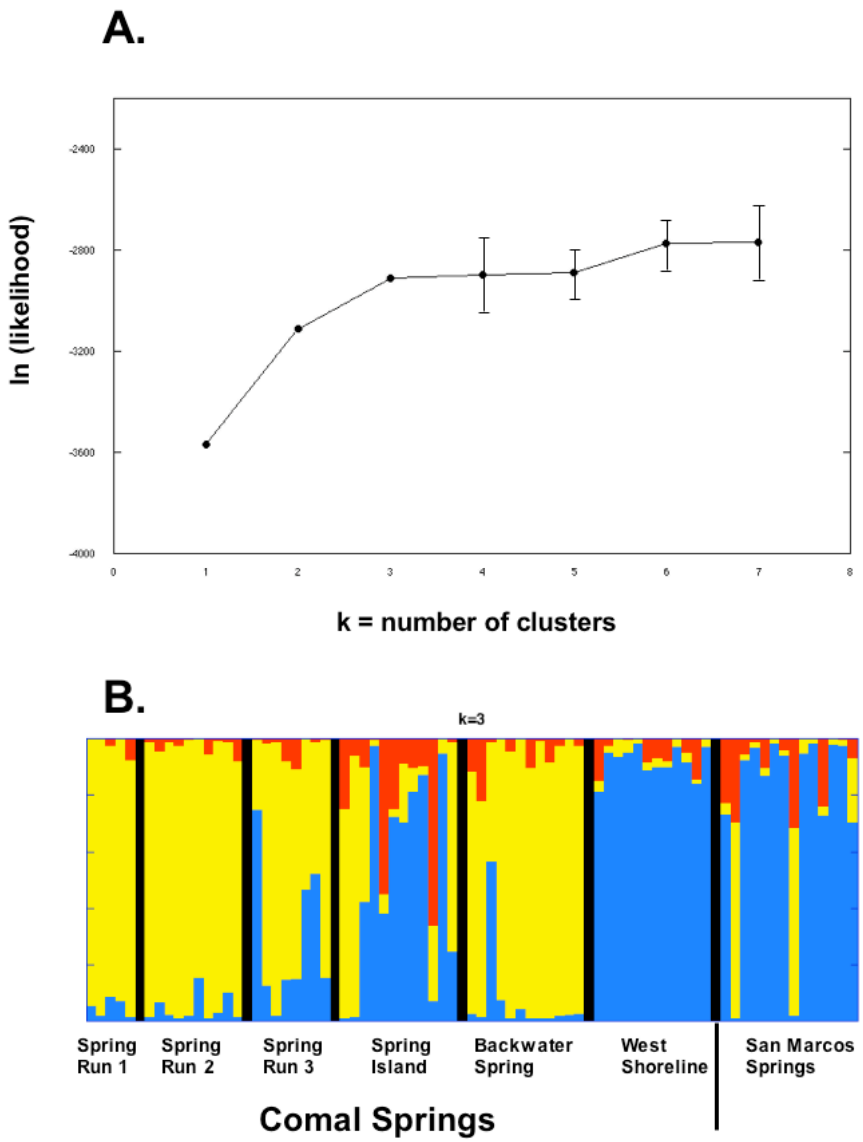

Figure 4: AFLP data. A) Plot of mean log likelihood from 10 MCMC runs vs k = the number of inferred clusters. Lines represent standard deviations. B) Barplot for three clusters. Each vertical bar represents a single individual and is colored in accordance with the Bayesian estimate of the proportion of that individuals genome that originated in a given cluster or population based on STRUCTURE v2.2 under the admixture model.