PERFORMANCE REPORT

State: New Mexico **Project Number:** E-54-4

Project Title: Comparative morphological assessment of the *Gammarus pecos* complex (Crustacea: Amphipoda) of New Mexico and Texas

Study Title: Endangered Species

Contract Period: 7 November 2007 **To:** 31 March 2008

I. Program Narrative Objectives

1. Characterize morphological attributes of all known populations of the *Gammarus pecos* complex Cole, 1985 from New Mexico and Texas using quantitative morphometrics.

2. Use molecular genetic techniques to determine the taxonomic relationships among closely related gammarid populations in the Pecos River Valley of New Mexico.

3. Record ecological data (habitat characteristics, physicochemical conditions) and behavioral observations of gammarid amphipods from spring systems in the Pecos River Valley of New Mexico.

4. Document population status (distribution, abundance, threat assessments) of gammarid amphipods in the Pecos River Valley of New Mexico.

5. Prescribe management actions applicable to taxonomically discrete populations of the *Gammarus pecos* complex.

II. Procedures

A. Obtain voucher material for morphologic and genetic studies of gammarid amphipods in the Pecos River Valley of New Mexico.

1. Estimate amphipod population densities from benthic samples.

2. Measure water depth and velocity, substrate type, and physicochemical parameters (water temperature, salinity, specific conductance, total dissolved solids, dissolved oxygen, and pH) at sample sites.

3. Record field observations of habitat use and behavior of gammarid amphipods.

During this segment, voucher material for genetic studies was collected from the gammarid amphipod population (n = 23 specimens) located in the Rio Hondo, South Tract, Bitter Lake National Wildlife Refuge. In March 2008, the project biologist also conducted presence/absence surveys for amphipods at two sites of the Bitter Creek system: Dragonfly Spring run and Lost River pool. No *Gammarus desperatus* were found at either site, but quantitative benthic sampling in June, September, and December of 2007 did reveal the presence of this species at relatively low densities at the Lost River site.

B. Morphologic analysis will be based on field collections from extant populations (BLNWR and Sitting Bull Spring), and museum collections for taxa determined during field surveys as extirpated.

1. Temporary slide mounts of dissected body parts will allow for accurate morphometrics (± 0.01 mm), meristics of appendage and antennal segmentation, and setae characterization (type, counts) on mouth parts, gnathopods, pereopods, uropods, and brood plates. Permanent mounts will facilitate comparison of interspecific morphological characters and will serve as future reference.

2. Species-specific characters will be photographed and/or illustrated. If possible employ scanning electronic microscopy of diagnostic structures*.

3. Analysis of morphologic data will follow Cole (1985) with additional tests where appropriate.

4. Deposit voucher material in a nationally recognized invertebrate collection.

(* Pending permission for use from the Department of Biology, University of New Mexico.)

Progress on morphometric study has been hampered by inability of the project biologist to discern diagnostic character traits for members of the *Gammarus pecos* species complex that Cole and Bousfield (1970) and Cole (1976, 1981, 1985) considered to be population specific, namely: setation type and patterns on the mandibular palps and morphology of the epimera of the first three abdominal side plates (Cole 1970). After considerable effort, the project biologist consulted in-person with Dr. Jullian Lewis, Borden, IN, one of North America's leading amphipod taxonomists. Dr. Lewis confirmed that such difficulty is normal and that morphologic study of cryptic amphipod species is hampered by several sources of variation (e.g., ecophenotypic, ontogenetic, sexual dimorphism): "setation is like human hair…not often consistent within or among individuals at any life stage." Further progress on morphologic study will be detailed in the Final Report for E-54.

C. Molecular genetic study will entail sequencing the 16s rRNA and the cytochrome oxidase I (COI) mitochondrial genes. The choice of these two genes will provide the best opportunity to ascertain degrees of relationship among the five populations

within the complex in New Mexico. Forty individuals will be sampled from each population, including the two populations suspected of containing cryptic species (BLNWR, Sitting Bull Spring). The criteria advocated by Moritz (1994) will be used to identify significant genetic differences among populations.

The NMDGF issued a 3-year professional services contract to Miami University (MU) to conduct a genetic study using two regions of the amphipod mitochondrial genome: cytochrome oxidase 1 (COI) and 16S rRNA. During this segment, the project biologist collaborated with MU on a draft manuscript for publication in the peer-reviewed literature (Molecular Ecology). The manuscript compares within- and among-population genetic affinities of gammarid populations of New Mexico and Texas (see Appendix A). MU is currently running genetic assays for the Rio Hondo *Gammarus* population.

D. Submit annual reports summarizing activities during the reporting period. These activities will include preliminary analysis of results, identification of threats, and management recommendations.

Segment 4 activities are reported here for the period 7 November 2007 to 31 March 2008. During Segment 3, the Department provided the U. S. Fish and Wildlife Service, Ecological Service Office (Albuquerque, New Mexico) the "*Recovery and Conservation Plan for Four Invertebrate Species of Chaves County.*" This plan includes population status, threats, and management recommendations for Noel's amphipod and three prosobranch snails (*Assiminea pecos, Juturnia kosteri, Pyrgulopsis roswellensis*).

III. Geographic Location

Field surveys will occur at known site occurrences of gammarid amphipods in the Pecos River Valley of New Mexico (see Table 1). Laboratory studies and report preparation will be conducted at the NMDGF headquarters, Santa Fe, NM. Genetic studies will be conducted by Dr. David Berg, Miami University (MU), Ohio. Lab facilities at the University of New Mexico will be required for scanning electron microscopy pending permission for use of this equipment.

All field work was conducted in New Mexico. Lab work occurred at the NMDGF headquarters in Santa Fe, New Mexico, and at the Department of Zoology, Miami University, Oxford, Ohio.

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Table 1. Members of the *Gammarus pecos* complex Cole, 1985 in New Mexico and Texas.

Species[†]	**Location**
Gammarus desperatus, Cole, 1981	Bitter Lake National Wildlife Refuge, Chaves Co., NM
Gammarus hyalelloides, Cole, 1976	Phantom Lake Spring, Jeff Davis Co., TX
Gammarus pecos, Cole and Bousfield, 1970	Diamond Y Spring, Pecos Co., TX
Gammarus sp. form C	Jeff Davis Co., TX
Gammarus sp. form E	Eddy Co., NM
Gammarus sp. form M	Reeves Co., TX
Gammarus sp. form S	San Solomon Spring, Reeves Co., TX
Gammarus sp.[1]	Giffin Spring, Reeves Co., TX
Gammarus sp.[2]	East Sandia Spring, Reeves Co., TX
Gammarus sp.[3]	Caroline Spring, Terrell Co., TX
Gammarus sp.[4]	Bitter Lake National Wildlife Refuge, Hunter Marsh, Chaves Co., NM
Gammarus sp.[5]	Malpais Spring, White Sands Missile Range, Sierra Co., NM

[†] All Texas species and morphotypes (forms) designated by Cole (1985) are Federal Species of Concern. *Gammarus desperatus* is state-listed as Endangered in New Mexico; the species is under a federal proposed rule to list as endangered with critical habitat.

[1, 2, 3, 4, 5] Gammarid populations discovered in June 2000, May 2001, June 2003, April 2004, and April 2005, respectively.

Appendix A. Phylogeographic analysis reveals multiple cryptic species of amphipods (Crustacea: Amphipoda) in Chihuahuan Desert springs.

Phylogeographic analysis reveals multiple cryptic species of amphipods (Crustacea: Amphipoda) in Chihuahuan Desert springs

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Key words: conservation genetics, endemic species, aquatic invertebrates, biogeography

Running title: Phylogeography reveals cryptic diversity in amphipods

ABSTRACT

Biodiversity conservation and the identification of conservation units among invertebrates are complicated by low levels of morphological difference, particularly among aquatic taxa. Accordingly, biodiversity is often underestimated in communities of aquatic invertebrates, as revealed by high genetic divergence between cryptic species. We analyzed PCR-amplified portions of the mitochondrial cytochrome *c* oxidase I (COI) gene and 16S rRNA gene for amphipods in the *Gammarus pecos* species complex, endemic to springs in the Chihuahuan Desert of southeast New Mexico and west Texas. Our analyses uncover the presence of eight separate species in this complex, in which only three nominal taxa are described. The distribution of these species in highly correlated with geography, with many present only in one spring or one spatially-restricted cluster of springs, indicating that each species likely merits protection under the U.S. Endangered Species Act. We show that patterns detected in the *G. pecos* species complex also correlate with endemic fish (*Gambusia* spp., pupfish) and hydrobiid snails. Our results provide clues important for future biodiversity investigations in geographyically isolated aquatic habitats, and shed light on the understudied and underestimated levels of biodiversity present in desert spring systems.

INTRODUCTION

Biodiversity conservation relies heavily on the identification and description of units of conservation. Conceptual development and research aimed at defining appropriate and widely applicable conservation units have produced a body of work centered around "evolutionarily significant units" (ESUs). An ESU is described as a group of organisms that has been isolated from conspecific groups for sufficiently long time periods such that meaningful genetic divergence has occurred separating the focal population from the other groups (Ryder 1986; Waples 1991). From an operational standpoint, the ESU should be a group of organisms representing the minimal unit targeted for conservation management (Vogler & DeSalle 1994). The determination of these minimal units brings the field of conservation biology into close association with systematic biology, which involves the discovery of monophyletic groups at higher levels, and the delineation of distinct lineages at lower levels (Dimmick *et al.* 1999; Wheeler & Meier 2000).

In recent decades, the use of genetic data has greatly increased the speed and reliability at which biodiversity can be assessed (Avise 2004). Quantification and analysis of molecular data can help uncover important patterns in genetic variability which underpin the long term viability of populations and entire species. Genetic investigations have helped identify appropriate conservation units and also the geographical locations where appropriate management actions would be most effectively implemented in organisms as varied as Komodo dragons (Ciofi, *et al.* 1999) and southwest Australian plants (Coates 2000). Moritz (1994) proposed that ESUs could be

identified via genetic markers in situations where mitochondrial DNA lineages were reciprocally monophyletic: i.e., all lineages within a particular group share a more recent common ancestor than any lineage in one group shares with lineages in other groups (Paetkau 1999).

One practical challenge in assessing biodiversity involves the difficulty of identifying the units of diversity in the field, which undermines the reliability of estimates of distribution (McNeely *et al.* 1990). The challenge becomes even more acute when the task focuses on aquatic invertebrates, which often display low levels of morphological distinctiveness (Müller 2000; Pfenninger *et al.* 2003; Witt *et al.* 2003). This might be because the actual cues used by aquatic taxa for conspecific recognition may not involve the same morphological characters used by taxonomists for species determination (Knowlton 1993). Given these relatively low levels of morphological difference, we would expect traditional taxonomy to underestimate marine and freshwater biodiversity (Thorpe & Solé-Cava 1994; Gómez *et al.* 2002). With the advent of molecular techniques, conservation biologists fortunately have additional means for discovering diagnostic characters in organisms that are indistinguishable based on morphology alone. Molecular genetic techniques now reveal substantial hidden diversity within morphologically delimited species (Remerie *et al.* 2006), and unusually high levels of genetic divergence between cryptic species (Bucklin *et al.* 1995; Knowlton & Weigt 1998; Lee 2000). Identification of species boundaries is particularly crucial in situations involving endangered species assessments. For 38 recent endangered species petitions, 81% of those showing genetic distinction were granted protection status (Fallon 2007),

which underscores the importance of using genetic markers to reveal important differences among morphologically similar taxa, particularly among aquatic invertebrates.

Amphipods comprising the *Gammarus pecos* species complex (Cole 1985) are endemic to spring systems associated with the Pecos River of New Mexico and Texas (Figure 1). It has been hypothesized that these freshwater amphipods are derived from a broadly distributed marine progenitor that became isolated inland upon the recession of the Western Interior Seaway from the North American continent during the Late Cretaceous (Bousfield 1958; Holsinger 1976; Baldridge 2004). Members of this complex likely speciated in response to diverse ecological conditions that developed in the various aquatic environments differing in elevation, substrate mineral composition, drainage patterns, and local hydrochemical conditions. This complex consists of the three nominal species *Gammarus pecos* Cole and Bousfield, 1970, *Gammarus desperatus* Cole, 1981, and *Gammarus hyalelloides* Cole, 1976, differentiated by morphology, at least 6 populations of undetermined taxonomic affinity that may represent several undescribed species, and at least two other populations presumed to be extirpated (Cole & Bousfield 1970; Cole 1976, 1981, 1985). Presently, this group of endemic amphipods is confronted with a high rate of imperilment related to habitat modification and groundwater withdrawal (Lang *et al.* 2003). Loss of spring habitat by groundwater mining and habitat alterations (e.g. diversions, damming, dewatering, channelization) is a major threat to aquatic biodiversity in arid regions of the western United States (Glennon 2002), where isolated spring systems often harbor unique assemblages of narrowly

endemic biota (Minckley & Unmack 2000; Hershler & Sada 2002; Sada & Vinyard 2002; Sada *et al.* 2005).

We conducted phylogeographic analysis of all 12 extant populations of this species complex, using mitochondrial DNA sequences for the cytochrome *c* oxidase subunit I (COI) gene and the 16S rRNA (16S) gene. Our objective in this study was to obtain and evaluate these high resolution genetic data to clarify the number of species present in a faunal group of conservation concern. While a previous investigation of this species complex using allozymes allowed some degree of resolution in detecting differences among amphipod populations (Gervasio *et al.* 2004), our sequencing of the two mitochondrial genes provides greatly increased resolution, revealing the presence of previously undetected species based on discrete clustering of mitochondrial haplotypes by spring habitat location. We show clear separations between haplotype clusters, corresponding to the populations occurring at each spring in this system. Because each cluster is restricted to a single spring system, they likely represent individual ESUs, each of which will merit protection. Patterns uncovered here provide valuable clues about the biogeographic patterns that can be expected among other desert spring fauna, based on similar geographic isolation followed by adaptive radiation.

METHODS

Amphipods from extant populations in the *G. pecos* species complex were obtained from 12 spring sites (Figure 1; Table 1) associated with the Pecos River basin in

southeastern New Mexico and western Texas. Eleven of these spring sites are located in the Pecos River watershed of the Permian Basin (Cartwright 1930), while Malpais Spring is located within the endorheic Tularosa Basin, west of the Permian Basin. Two populations sampled during previous investigations are presumed extirpated (Cole 1981, 1985), while we were unable to reconcile the locality of Cole's population "M." Hand nets were used to collect amphipods (30-200 animals per site) from the water column, macrophytes, or substrata. All samples were preserved in 95% ethanol.

Complete genomic DNA was extracted from 10 - 40 individuals per spring site. DNA extraction involved dissecting either intact pleon or pereopods from each individual and followed a standard extraction protocol involving proteinase K, ribonuclease, and several Promega® reagents (Nuclei Lysis Solution, cat.# A7943; Protein Precipitation Solution, cat.# A7953). A 680-bp region of the COI gene was amplified using the primers LCO1490: GGTCAACAAATCATAAAGATATTGG and a shortened version of HCO2198: TCAGGGTGACCAAAAAATCA (Folmer *et al.* 1994). Also for COI, the custom internal primers CBL4f: GTGAAGAGAGAAAATAGCTA and CBL4r: ATYATAATTGGGGGGTTC were developed for use in amplifying shorter COI gene fragments when the Folmer *et al*. (1994) "universal" primers failed to produce amplification of the full 680-bp fragment. Each 50-µL polymerase chain reaction (PCR) contained 25 µL Taq PCR Master Mix (3.7 U Taq DNA polymerase, 1.5 mM $MgCl_2$ and 200 µM each dNTP; Qiagen® cat.# 201443), 0.05 nmol each primer, ca. 2 mM additional $MgCl_2$, 5 ng DNA template and 6.5 µL molecular water. PCR conditions consisted of 3 min at 94 ^{o}C followed by 5 cycles of 1 min at 94 ^{o}C, 1 min at 45 ^{o}C, 1 min at 72 ^{o}C;

followed by 35 cycles of 1 min at 94 ^{o}C, 1 min at 50 ^{o}C, 1 min at 72 ^{o}C; followed by 5 min at 72 ^{o}C. COI gene products were isolated using electrophoresis in a 2% agarose gel and extracted using Qiagen® QIAquick Spin Columns and buffers (cat.# 28106). Cycle sequencing reactions were performed using ABI BigDye terminator v3.1 sequencing kits (25 cycles, annealing temperature: 50 ^{o}C). PCR products were sequenced in both directions using the PCR primers mentioned above and an ABI 3130 automated sequencer (Applied Biosystems). Forward and reverse sequences were aligned using BioEdit (Hall 1999) to verify basecall accuracy. While the final COI alignment for nine of the focal populations contained sequences 620-bp in length, the populations BLBC, BLSS, and BLU6 (those requiring the custom internal primers) produced COI sequences 140-bp in length.

For the 16S rRNA gene, a 480-bp region was amplified using the primers 16STf: GGTAWHYTRACYGTGCTAAG (Macdonald *et al.* 2005) and 16Sbr: CCGGTTTGAACTCAGATCATGT (Palumbi *et al.* 1991). The 50-µL PCR reactions contained the same reagent quantities as listed above for the COI gene. PCR conditions for 16S consisted of 4 min at 95 ^{o}C followed by 40 cycles of 1 min at 95 ^{o}C, 1 min at 42 ^{o}C, 2.5 min at 72 ^{o}C; followed by 7 min at 72 ^{o}C. PCR products for 16S were sequenced in both directions and aligned as for COI. The final 16S alignment for all individuals contained sequences 476-bp in length. Combination mitochondrial sequences were assembled by joining the COI and 16S gene sequences end-to-end for all animals that sequenced successfully for both genes.

We calculated descriptive statistics of genetic diversity (number of unique and shared haplotypes, mean number of pairwise differences) within each population using Arlequin v2.000 (Schneider *et al.* 2000). Divergence of populations was measured by calculating F_{ST} for pairwise combinations of populations. We measured geographic distances, distances along permanent and ephemeral streams connecting springs sites, for all pairs of populations and tested for isolation-by-distance by examining the correlation of geographic distance and genetic distance by population using a Mantel test.

We used the program TCS (Clement *et al*. 2000) to generate networks for clusters of haplotypes separated by 19 steps or fewer. Given the high sequence divergence between some of the populations, we were unable to produce a single unified haplotype network using only TCS v1.18 (Clement *et al*. 2000). To determine the fewest number of hypothesized intermediates necessary for connecting the various subnetworks into a single grand network, the most common haplotypes from each subnetwork were identified and analyzed separately using Arlequin v2.000 (Schneider *et al.* 2000). The shortest calculated paths of hypothesized intermediates were then added manually to the grand network.

RESULTS

Our investigation recovered combination sequences for 134 individuals from 12 populations of *Gammarus*. A total of 91 haplotypes were identified; the number of haplotypes per site ranged from 3-34, with all but two of these being confined to a single

site (Table 2). Where haplotypes were shared between spring sites (BLBC and BLSS; ESS and GS), the shared haplotype was also the most common one in both populations. Haplotype richness was correlated with sample size ($r = 0.964$, $n = 12$, $p < 0.001$). The Mantel test between geographic distance along river (km) and pairwise % nucleotide mismatch between haplotypes showed a strong positive correlation between the two matrices ($r = 0.8552$, $P = 0.001$, for log-transformed data; Figure 2). The calculated F_{ST} values (most significantly > 0) for all possible pairs of populations ranged from 0.00148 – 0.98944 (arithmetic mean = 0.88242; Table 3).

Using the method of Templeton *et al.* (1992), we estimated the genealogical relationships among combined sequences and displayed the structure of those relationships using a haplotype network (Figure 3). This network visually depicts the relationships among the same 91 combined sequence haplotypes described on a spring-by-spring basis and shows a strong association of diversity and divergence based on spring location (Table 2). The distinctive nature of the haplotype clusters found in each spring is visually apparent in the haplotype network (Figure 3). Of the 12 springs, eight contain genetic groups that share no haplotypes with other springs in the study area. The pairwise F_{ST} estimates among these eight springs are all well above $F_{ST} = 0.2$ (Table 3). The two remaining pairs of springs (BLBC and BLSS; ESS and GS) display much lower F_{ST} values.

The genetic data for the second pair of springs (ESS and GS) indicate these amphipods are considerably more similar to SSS, even though SSS and DY are referable

to *Gammarus pecos* (Cole 1985). The F_{ST} for SSS vs. ESS and SSS vs. GS are much lower than DY vs. ESS and DY vs. GS (Table 3). Based on these data and evidence from the haplotype network, SSS is far more genetically similar to ESS and GS, than to "conspecific" DY. These data are consistent with the geographic clustering of ESS, GS and SSS from the Toyah Basin, all of which are considerably closer to one another than to DY, located approximately 86 km east of these Toyah Basin springs. Accordingly, our genetic data suggests that *Gammarus pecos* is restricted to the Diamond Y Spring system whereas the Toyah Basin harbors an undescribed gammarid (ESS, SSS, GS) and *Gammarus hyalleloides* from Phantom Lake Spring. The undescribed gammarid at CS also shows distinctiveness from all other populations in the study area.

In summary of results for all populations, our genetic study has resulted in the detection of at least eight separate species in this complex: 1) BLBC, BLSS, and BLU6; 2) BLHM; 3) CS; 4) DY; 5) ESS, GS, and SSS; 6) MS; 7) PL; 8) SB.

DISCUSSION

The strong positive correlation between haplotype difference and geographic distance (Figure 2) indicates allopatric speciation of the *G. pecos* complex via isolation-by-distance (Vrijenhoek 1998). The majority of F_{ST} values in Table 3 are greater than 0.9, indicating a high degree of differentiation (Hartl & Clark 2005) and the strong likelihood of continued divergence over time (Lowe *et al.* 2004). Furthermore, high F_{ST} values reveal the presence of distinct species or subspecies (Hogg *et al.* 2000). Only in

situations where $F_{ST} < 0.2$ are populations expected to maintain genetic connectivity (Lowe *et al.* 2004). Thus, it is likely that most of these populations have been isolated for substantial periods of time.

Gammarus desperatus, a state and federally endangered species, is present on Bitter Lake National Wildlife Refuge at BLBC and BLSS. The BLU6 population is likely considered conspecific with these, due to relatively small separation (3 hypothesized intermediates) between BLU6 and the pair BLBC & BLSS. Pairwise FST values for BLU6 vs. BLBC and BLU6 vs. BLSS are 0.41194 and 0.43564, respectively, indicating far less differentiation and more recent gene flow relative to all pairs among the remaining populations. Ironically, the amphipod present at BLHM is nominally considered *G. desperatus* (Federal Register 2005), but given the F_{ST} values of BLHM vs. BLBC, BLHM vs. BLSS, and BLHM vs. BLU6, we interpret these data to indicate that the BLHM population is a different species than those present at the three other Bitter Lake sites. This interpretation is supported by the haplotype network, which shows ≥ 48 hypothesized intermediates between BLHM and other populations (Figure 3). While current management of Bitter Lake National Wildlife Refuge amphipods assumes only the presence of *G. desperatus*, our results indicate the presence of at least two allopatric species within the relatively close confines of the refuge.

Because of the moderate level of differentiation uncovered between at least two pairs within both species #1 and species #5 above, our estimate of the number of provisional species present in this system is probably conservative. Further evidence of

the conservative nature of our hypothesis comes from the analysis of a larger 16S dataset for the BLHM population. Even though combined sequences were not possible for the additional samples, a total of 12 16S haplotypes showed a strong bimodal distribution, suggesting the presence of two distinct lineages at BLHM (RAS, BKL, & DJB, unpublished data). However, bimodality was not observed among 12 COI haplotypes, although there were only three individuals from BLHM which could be successfully sequenced for both COI and 16S genes. Thus, only three combined haplotypes for BLHM were available for our analysis presented here, meaning that the bimodal mismatch distribution revealed in the complete 16S data was not shown in this analysis.

Moritz (1994) proposed the combination of sampling nuclear genes and mitochondrial genes to identify significant genetic differences among populations, an approach particularly useful in clarifying relationships that lie close to the boundary between conspecific populations and distinct species. All of our provisional species are reciprocally monophyletic (*sensu* Moritz 1994), and most were shown to exhibit significant divergence in nuclear allele frequencies at allozyme loci (Gervasio *et al.* 2004), which was the second criterion suggested for genetic determination of ESU (Moritz 1994). We recommend that the species present at these eight locations (or location sets, in the cases of species #1 and species #5) be managed as separate units of conservation, due to their discreteness and distinctness, both genetically and geographically. While each of these species is locally abundant, each is also endemic to a single spring system.

The act of delimiting species among groups with few differentiating morphological characters can be daunting. While several species within this complex were originally described morphologically (Cole & Bousfield, Cole 1976, Cole 1981), a variety of crustacean studies have used molecular evidence as verification or refutation of the earlier morphology-based conclusions about species identity (Sotela *et al*. 2008). Recent molecular studies have both verified morphological taxonomies in crustaceans (Geller *et al.* 1997, Mathews *et al.* 2002, Sotela *et al.* 2008), and refuted them (Tsoi *et al.* 2005; Reuschel & Schubart 2006; Cook *et al.* 2006). Indeed, degree of genetic divergence has been diagnostic in detecting distinct species (Sotela *et al.* 2008), with interspecific divergences for crustaceans ranging from 1-5% for 16S, and 2-11% for COI (Schubart *et al.* 2001; Mathews *et al.* 2002; Schubart & Koller 2005). Even though we assembled combined sequences by linking fragments for the generally more conservative 16S gene with COI fragments, the provisional species we propose show divergences in the ranges of those found in these other studies.

Our detection of relatively high levels of cryptic diversity informs the larger discussion among conservation biologists and aquatic ecologists about the proportion of biodiversity harbored in freshwater systems. It has been suggested that freshwater biodiversity has been underestimated due to several factors, including relatively less research effort directed towards aquatic invertebrates (Strayer 2006), unexplored and poorly known groundwater habitats (Strayer 2006), and the common presence of morphologically cryptic species in aquatic ecosystems (Lee & Frost 2002; Witt *et al.* 2003; Lefébure *et al.* 2006). Furthermore, freshwater spring habitats occurring in hot

deserts support a disproportionate level of species diversity (Minckley & Unmack 2000) and high genetic diversity (Thomas *et al.* 1997, 1998; Witt *et al.* 2003, 2006) due to extreme habitat patchiness, stable environmental conditions, and long-term isolation (Thomas *et al.* 1998; Minckley & Unmack 2000). Even with reports pointing towards high diversity in desert springs, most of the diversity found in these geographically isolated habitats is not well documented. In the case of native fauna in the western United States, species dwelling in spring and spring-brook habitats are also in greater danger of extinction than organisms associated with more hydrologically integrated habitat types (Sada & Vinyard 2002). Despite insufficient research effort directed at the arid landscapes typical of hot deserts, we know that spring systems within these regions are geographically isolated and that a single system can often be the only remaining habitat for endemics like hydrobiid snails (Hershler *et al.* 1999) and pupfishes (Echelle *et al.* 2005).

For members of the *G. pecos* complex, we report very distinct genetic patterns which are likely to be shared among diverse aquatic taxa distributed across desert landscapes. These results further underscore the increasing role of phylogeographic analysis in studies of regional biogeography (Crews & Hedin 2006). Considerable diversity has been reported for odonates (K. Gaines, pers. comm.), ostracodes (A. Smith, pers. comm.), and hydrobiid snails (Hershler *et al.* 2002) from the northern Chihuahuan Desert. Farther south in the Chihuahuan Desert, at Cuatro Ciénegas in Coahuila, México, a spring complex supports at least 70 endemic aquatic vertebrates, diverse microbes (Souza *et al.* 2006), endemic snails (Moline *et al.* 2004), and rare living stromatolites

(Dinger *et al.* 2006). Given the cryptic diversity reported among aquatic invertebrates (Müller 2000; Pfenninger *et al.* 2003; Witt *et al.* 2003), coupled with the high endemism encountered at desert springs (Hershler *et al.* 1999; Echelle *et al.* 2005), we would expect the widespread occurrence of undescribed, endemic taxa of conservation concern in desert springs. The patterns we present can guide future biodiversity assessment efforts, based on the expectation of similar trends among diverse taxa. One example of this expected trend is the correlation of our mtDNA results and the allozyme data (Gervasio *et al.* 2004) for the *G. pecos* complex, with genetic data for the endemic fish *Gambusia pecosensis* (Echelle *et al.* 1989). We also note similar patterns comparing our genetic data to those for cyprinid pupfishes (Echelle *et al.* 1987) and hydrobiid snails (Hershler *et al.* 1999). Similar biogeographic trends are recognized among terrestrial invertebrate taxa of this geographic region (i.e., land snails, see Bequaert & Miller 1973; Metcalf 1997; Metcalf & Smartt 1997). Even terrestrial vertebrates like the ridge-nose rattlesnake mirror the biogeographic pattern found among the amphipods we investigated; i.e. distinct geographic areas harboring distinct genetic entities (Holycross & Douglas 2007).

Future biodiversity investigations in the Chihuahuan Desert will almost certainly uncover species meriting protection among the region's highly endemic fauna, given previously undetected variation and high degree of crypsis among invertebrates (Remerie *et al.* 2006), particularly among aquatic habitats (Müller 2000; Pfenninger *et al.* 2003; Witt *et al.* 2003), coupled with the distinct genetic structure uncovered here. Other

deserts deserve biodiversity reevaluations also, based on the patterns revealed in the northern Chihuahuan Desert.

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Figure Legends:

Figure 1. Collection sites for *G. pecos* complex populations from the Chihuahuan Desert. See Table 1 for population codes.

Figure 2. Geographic distance along river vs. pairwise % nucleotide mismatch. Values are positively correlated (Mantel test, ln (x+1)-transformed data, $r = 0.8552$, $P = 0.001$).

Figure 3. A haplotype network constructed from combination sequences for COI and 16S. Each unique haplotype is displayed as an oval, and the size of each oval corresponds to the haplotype frequency, with frequencies higher than 1 denoted by number (e.g. n = 3). Small yellow circles correspond to hypothesized intermediate haplotypes not detected in the sample. Relatively large gaps have number of intermediates indicated inside lightning bolt symbol.

Table 1. Sample sites and population codes for the *Gammarus pecos* species complex. All individuals analyzed during this study were collected within 300 m of the coordinates shown. The last three sites either, 1) harbor no amphipods presently, or 2) cannot be located based upon earlier descriptions given. Extant populations which were not considered by Cole (1985) are indicated in the second column by the character "-".

Population Code	Cole (1985) Designation	Sample Site Location	Latitude / Longitude	Nominal Species
BLBC	"D"	Bitter Creek, Bitter Lake National Wildlife Refuge, Chaves County, NM	33° 28′ 46″ N / 104° 25′ 39″ W	*Gammarus desperatus*
BLHM	"D"	Hunter Marsh, Bitter Lake National Wildlife Refuge, Chaves County, NM	33° 24′ 52″ N / 104° 25′ 16″ W	*Gammarus desperatus*
BLSS	"D"	Sago Spring, Bitter Lake National Wildlife Refuge, Chaves County, NM	33° 28′ 41″ N / 104° 25′ 11″ W	*Gammarus desperatus*
BLU6	"D"	Unit 6, Bitter Lake National Wildlife Refuge, Chaves County, NM	33° 26′ 46″ N / 104° 24′ 16″ W	*Gammarus desperatus*
CS	-	Caroline Spring, Independence Creek, Terrell County, TX	30° 26′ 40″ N / 101° 43′ 13″ W	*Gammarus* sp.
DY	"P"	Diamond Y Spring, Diamond Y Draw, Pecos County, TX	31° 02′ 12″ N / 102° 53′ 27″ W	*Gammarus pecos*
ESS	-	East Sandia Spring, Toyah Creek, Jeff Davis County, TX	30° 59′ 28″ N / 103° 43′ 44″ W	*Gammarus* sp.
GS	-	Giffin Spring, Toyah Creek, Jeff Davis County, TX	30° 56′ 45″ N / 103° 47′ 23″ W	*Gammarus* sp.
MS	-	Malpais Spring, White Sands Missile Range, Cibola County, NM	33° 17′ 18″ N / 106° 18′ 33″ W	*Gammarus* sp.
PL	"H"	Phantom Lake Spring, Toyah Creek, Jeff Davis County, TX	30° 56′ 05″ N / 103° 50′ 58″ W	*Gammarus hyalelloides*
SB	"E"	Sitting Bull Falls, Lincoln National Forest, Eddy	32° 14′ 12″ N / 104° 42′	*Gammarus* sp.

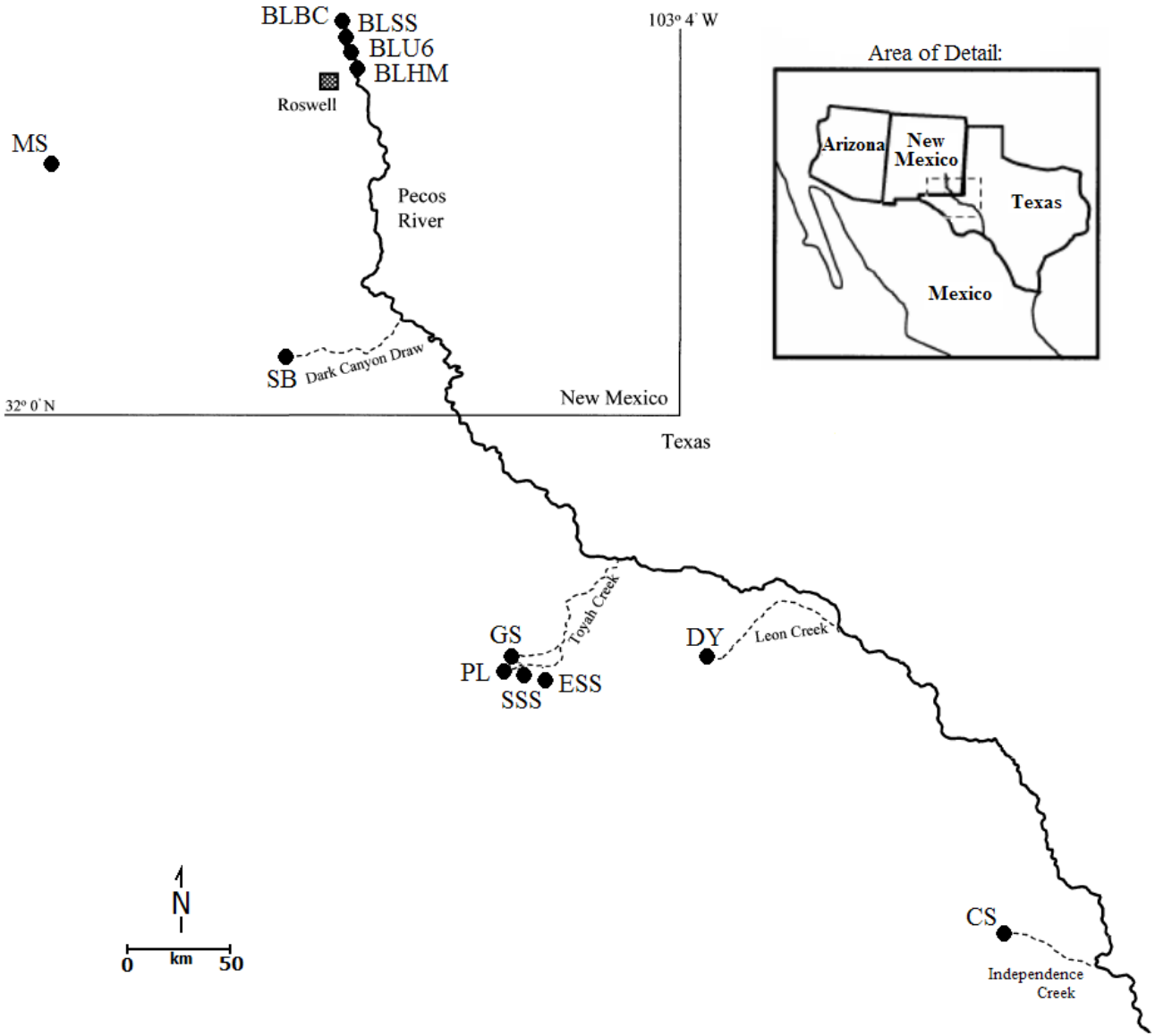
		County, NM	08″ W	
SSS	“S”	San Solomon Spring, Toyah Creek, Jeff Davis County, TX	30° 56′ 41″ N / 103° 47′ 11″ W	*Gammarus pecos*
(extirpated)	“D”	North Spring, Roswell, Chaves County, NM	33° 25′ 30″ N / 104° 29′ 20″ W	*Gammarus desperatus*
(extirpated)	“C”	Irrigation Canal, Jeff Davis County, TX	30° 56′ 00″ N / 103° 50′ 40″ W	*Gammarus* sp.
?	“M”	Reeves County, TX	unknown	*Gammarus hyalelloides*

Table 2. Summary of descriptive genetic data for combined sequences.

	No. of combined sequences per population	No. of unique haplotypes per spring	No. of haplotypes shared with other spring(s)	Mean population diversity (0 no. of pairwise differences)
BLBC	6	3	1	1.00000
BLHM	3	3	0	8.00000
BLSS	9	7	1	1.94444
BLU6	13	12	0	5.38462
CS	10	8	0	3.53333
DY	11	3	0	1.09091
ESS	9	3	1	0.44444
GS	8	4	1	1.42857
MS	8	5	0	2.50000
PL	12	7	0	1.95455
SB	36	34	0	8.88413
SSS	9	4	0	2.44444

Table 3. Pairwise F_{ST} for all populations (* = not significantly different from 0, at $\alpha = 0.05$).

	BLBC	**BLHM**	**BLSS**	**BLU6**	**CS**	**DY**	**ESS**	**GS**	**MS**	**PL**	**SB**	**SSS**
BLBC	-											
BLHM	0.94381	-										
BLSS	0.04448*	0.94083	-									
BLU6	0.41194	0.88869	0.43564	-								
CS	0.94836	0.93849	0.94503	0.91477	-							
DY	0.98258	0.97366	0.97601	0.94631	0.96055	-						
ESS	0.98944	0.97678	0.98097	0.94735	0.95723	0.98797	-					
GS	0.98009	0.96595	0.97302	0.93971	0.94685	0.98194	0.00148*	-				
MS	0.98536	0.95607	0.98284	0.96655	0.97192	0.98625	0.98791	0.98337	-			
PL	0.97363	0.96492	0.96874	0.94164	0.94388	0.97789	0.92934	0.90912	0.98177	-		
SB	0.94148	0.90117	0.94379	0.94049	0.93285	0.94422	0.94082	0.93911	0.33157	0.94254	-	
SSS	0.97030	0.95856	0.96565	0.93600	0.93948	0.97512	0.34637	0.28673	0.97910	0.89486	0.93861	-


BLBC
BLSS
BLU6
BLHM
Roswell
MS
Pecos
River
SB
Dark Canyon Draw
103° 4' W
32° 0' N
New Mexico
Texas
Area of Detail:
Arizona
New
Mexico
Texas
Mexico
GS
Toyah Creek
PL
SSS
ESS
DY
Leon Creek
CS
Independence
Creek
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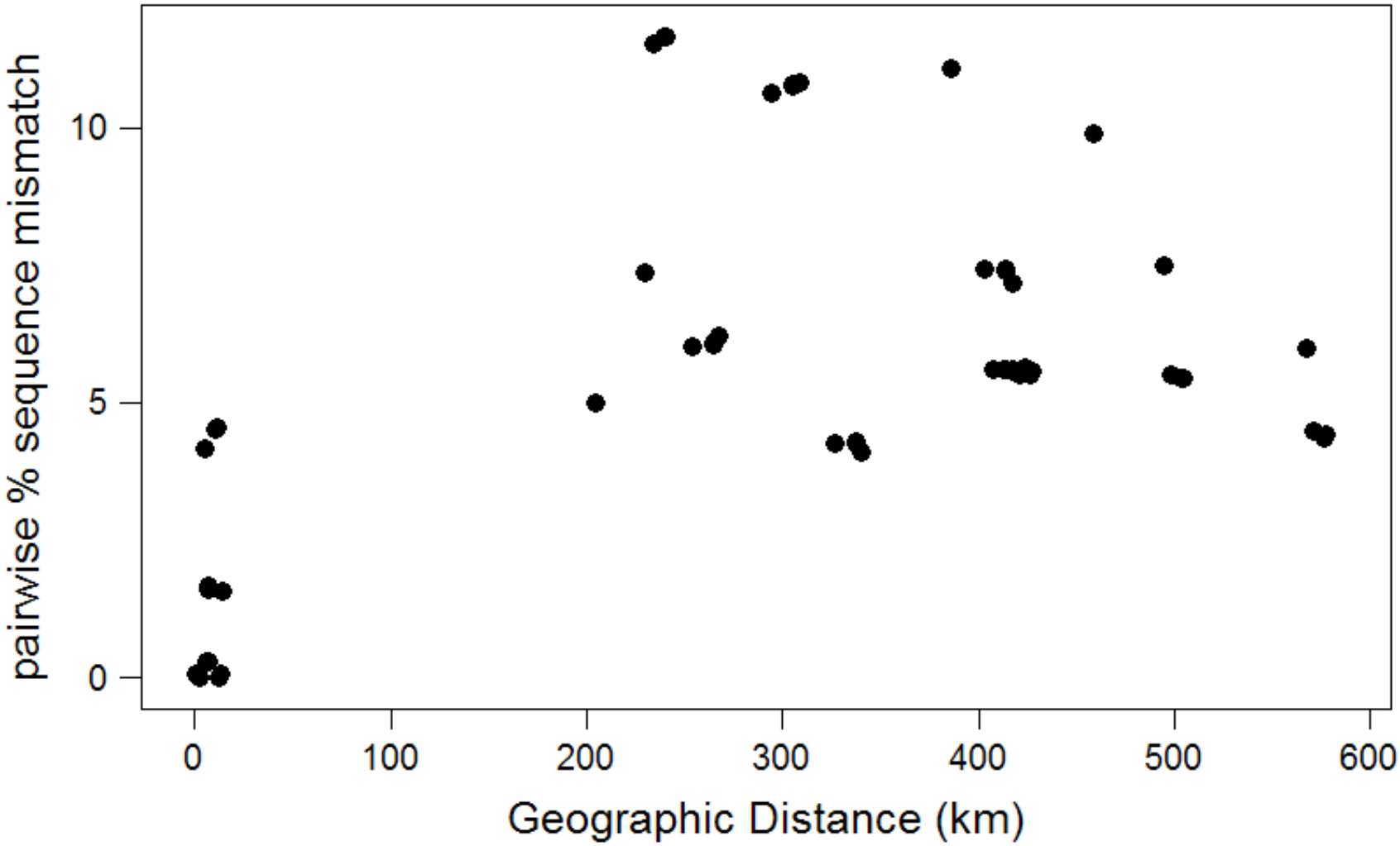
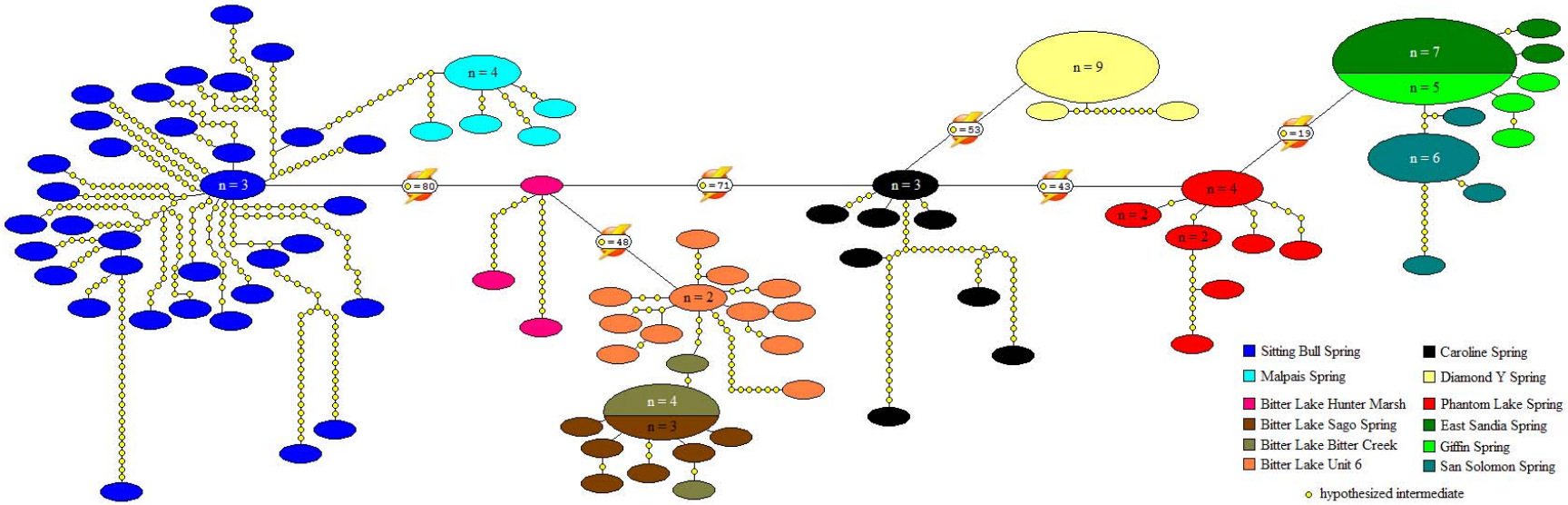
50


pairwise % sequence mismatch
0
5
10
0
100
200
300
400
500
600
Geographic Distance (km)


n = 3
n = 4
= 80
= 48
n = 2
n = 4
n = 3
= 71
n = 3
= 53
n = 9
= 43
n = 4
n = 2
n = 2
= 19
n = 7
n = 5
n = 6
Sitting Bull Spring
Malpais Spring
Bitter Lake Hunter Marsh
Bitter Lake Sago Spring
Bitter Lake Bitter Creek
Bitter Lake Unit 6
Caroline Spring
Diamond Y Spring
Phantom Lake Spring
East Sandia Spring
Giffin Spring
San Solomon Spring
hypothesized intermediate