COMPLEX DIVERSITY IN *STYGOBROMUS* AMPHIPODS OF THE TEXAS EDWARDS PLATEAU

THESIS

Presented to the Graduate Council of
Texas State University-San Marcos
in partial fulfillment
of the requirements

for the Degree

Master of SCIENCE

by

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San Marcos, Texas
December 2011

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ACKNOWLEDGEMENTS

I give thanks to my research advisor, Christopher C. Nice, without whom none of this would have been possible. Thank you very much Chris for your mentorship, expertise, and use of your laboratory. I am a more critical thinker and a better writer because of the knowledge and experience you have imparted.

I give thanks to my wife, Sonia R. Ethridge, who supported me in every way through this endeavor. Thank you very much Sonia for your love, support, and affection. May our fates be one forever.

I give thanks to my committee, Noland H. Martin and Benjamin F. Schwartz, who discussed ideas and reviewed manuscripts.

I give thanks to my USFWS collaborator, J. Randy Gibson, who provided a wealth of knowledge about *Stygobromus*, made all morphological identifications for this study, and reviewed manuscripts.

I give thanks to my lab partners, Laura Alberici de Barbiano and Michelle H. Downey, for sharing both their knowledge of molecular evolution and their experience working in the genetics lab.

From Texas through Arkansas to Tennessee, I give thanks to my family for the countless ways they have supported me over the years of my life.

This manuscript was submitted on October 18, 2011.

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ABSTRACT

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OF THE TEXAS EDWARDS PLATEAU

by

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Troglomorphic, spring-associated cave amphipods (genus *Stygobromus*) occupy discontinuous localities in the Edwards plateau region of the south-central United States. Given the prevalence of subterranean cryptic species diversity among widely disparate animal taxa and general patterns of subterranean dispersal and vicariance, *Stygobromus* may contain undetected biodiversity at the species and population levels, with conservation implications for *S. pecki*, a federal and state of Texas endangered short-range endemic. To explore *Stygobromus* evolutionary history, mitochondrial sequence (COI), nuclear sequence (ITS1), and AFLP data were collected for *S. pecki* and Edwards plateau congeners. The morphology-based taxonomy and proposed phylogeny of *Stygobromus* species and species groups were treated as hypotheses and tested with molecular data. *Stygobromus pecki*, which emerges from multiple spring flows at Landa Lake, was examined with population genetics tools to characterize population structure and diversity, with diversity measures compared to

congeners and previous findings for another spring endemic, the federally endangered Comal Springs riffle beetle, *Heterelmis comalensis*, which shares habitat with *S. pecki*. The taxonomy of *Stygobromus* species and species groups conflicted with molecular phylogenetic data and there is strong evidence of significant cryptic diversity. Within *S. pecki*, COI data contained two significantly diverged clades that may reflect a history of isolation succeeded by current sympatry and admixture. *S. pecki* genetic diversity was similar to that for congeners and significantly greater than for *H. comalensis*. This study demonstrates that Edwards plateau *Stygobromus* are a complex and genetically diverse group with substantially more diversity than currently recognized.

KEYWORDS: cryptic diversity, spring endemic, karst habitat, *Stygobromus*, *Stygobromus pecki*, conservation.

CHAPTER 1

EVOLUTION OF SUBTERRANEAN FAUNA

Introduction

Evolution is the universal mechanism of the proliferation of life. It is an iterative process that ultimately utilizes copy error of molecular information as the raw material of adaptation to a constantly changing environment. These basic conditions have created the multitudes of forms, functions, interdependencies, and cycles that comprise all of known biology, past and present. Our tree of life has been evolving for approximately 4.5 billion years and given the wide variety of environments it has penetrated (most dramatically, the habitats of the extremophiles), it comes as no surprise that global biodiversity lacks thorough characterization.

Characterization of hypogean (below-the-surface) evolution was historically more difficult because of lack of access to habitat and data, and as such were passed over for more accessible epigean (above ground) systems, or prematurely dismissed as uninteresting because uniformity among taxa suggested a relative absence of divergent processes. This historical limitation was overcome by the introduction of molecular biology tools which provided the data needed to better understand hypogean evolution. At present, a thorough examination of subterranean systems is underway in biology, and what has been learned so far suggests characteristic and sweeping differences from surface-dwellers. A more thorough

understanding of these differences is important for two reasons: (1) subterranean evolution may be contrasted with surface systems to elucidate more general statements on evolution. (2) The subterranean environment is habitat to numerous endangered and/or short-range endemic species, and information on how these groups evolve can inform conservation practices.

The Edwards plateau of the south-central United States is a unique karst environment that may be the most species-diverse limestone aquifer in the world (Longley 1981). There are numerous spring complexes throughout this region that contain unique biological systems and endangered species from widely disparate taxa. Molecular characterization of these systems is already underway (Gonzalez 2008, Lucas et al. 2009), but many taxa remain unstudied, particularly among the more obligate subterranean species. Evolutionary study of subterranean species in the Edwards plateau is important for three reasons: (1) General patterns of hypogean evolution differ from epigean patterns, a finding which is not extensively tested with molecular data from the Edwards plateau. (2) Karst environments are unique hypogean landscapes for the waters they contain which support numerous ecosystems below and above the surface (including human societies). (3) Cryptic species diversity and troglomorphic convergence are subterranean phenomena that can impact biodiversity estimates and phylogenetic inferences (see below and Chapter 2), but which have not been characterized for the Edwards plateau.

Hypogean evolution

Hypogean evolution proceeds in characteristic ways that distinguish it from epigean evolution. There are two primary factors at play: (1) limited capacity for dispersal, and (2)

vicariance, or limitation of gene flow by physical barriers. While these factors are observed in various epigean systems, they appear pervasive in hypogean systems, affecting widely disparate taxa from around the globe. Porter (2007) proposed a model of subterranean evolution in which dispersal from source populations occurs rarely and is punctuated by rapid vicariance. This is explained by the subterranean environment being a largely uninhabitable space presumably because of bedrock and other impenetrable features. Habitable zones occupy relatively small volumes of this space and are themselves heterogeneous.

Within subterranean habitable zones, the most salient environmental heterogeneity is that open spaces may be aquatic or terrestrial, with species obligated to each habitat. The border between aquatic and terrestrial ranges can change over time. Heterogeneities within aquatic and terrestrial regions, such as differences in solution chemistry and atmospheric composition over space and time, can affect local environments and potentially have fleeting, rhythmic, or permanent vicariant effects.

To illustrate with a hypothetical example, a chamber containing an aquatic population may have connectivity with another chamber that is also suitable habitat, but dispersal may be highly unlikely for a variety of reasons. One path may rise above the water table into terrestrial habitat. Another path may be aquatic, but contain an inhospitable extreme of pH, salinity, dissolved oxygen content, or other factor. A third path may have a flow gradient that opposes dispersal. Factors such as these may place limits on dispersal, but do not negate it altogether. Returning to the hypothetical aquatic population, flood conditions may raise the water table sufficiently for dispersal to the new range via the first path, dilute the offending chemistry for the second path, or reverse the direction of flow for

the third. An opportunistic founder group or gravid female may then disperse to the new chamber. In line with the model of Porter (2007), this may be followed by rapid vicariance when conditions return to normal, and dispersal back to the source population is no longer possible.

Karst habitat in the Edwards plateau

Located in the south-central United States, the Edwards plateau is an uplifted region bounded by the Balcones fault to the east and south, the Pecos River to the west, and the Llano Uplift & Llano Estacado to the north. Geologically, it consists of two karstic limestone aquifers dated to the late Cretaceous, the Edwards aquifer in the east and the Trinity aquifer in the west. These aquifers support numerous hypogean and epigean ecosystems including population-dense human societies at Austin and San Antonio, Texas.

In the Edwards plateau, karst landscapes form when mildly acidic groundwater reacts with limestone. With homogeneous limestone bedrock, karstification occurs only at very small scales in porous spaces. Over time, pore sizes increase and geologic activity creates cracks and fissures that can lead to the formation of directional flow paths. If hydrological gradients are strong (i.e. if water moves swiftly through a given space), the combined chemical and mechanical erosion of flowing groundwater leads to the formation of even larger channels. At the largest scale are underground rivers and sinkholes (or cenotes; the Devil's Sinkhole in the Edwards plateau region is an example). All of these karst features are potential habitat for a variety of hypogean animal taxa.

Cryptic diversity and troglomorphic convergence

Cryptic diversity is recognizable when a significant molecular divergence between samples (usually allopatric) has no correlate(s) in morphology, physiology, or behavior. The prevalence of cryptic diversity within subterranean fauna causes problems for phylogenetic reconstruction, which assumes homology of characters.

Cryptic diversity is related to troglomorphic convergence, a well-documented example of evolutionary convergence among subterranean animals from widely disparate taxa. Troglomorphic species exhibit convergence to a cave type that includes atrophy or loss of eyes, loss of pigmentation, hypertrophy of non-optic senses, elongation of appendages, increased lifespan and development times, and reduced metabolic rates (Porter 2007). The scale of troglomorphic convergence makes homoplasy a concern in any phylogenetic examination of subterranean fauna. This concern may be addressed by examination of molecular variation, because it presumably accumulates in living systems independent of troglomorphic convergence.

In molecular evolutionary studies, cryptic diversity is indicated by the simultaneous presence of molecular divergence and absence of morphological differences. In phylogenetic terms, two samples that identify to a single morphological species may have a polyphyletic molecular relationship. For the *Stygobromus* amphipods of the current study, this was found to be the case with substantially more diversity indicated by molecular data than has previously been suggested by morphological data (see Chapter 2).

CHAPTER 2

ORIGINAL RESEARCH

Introduction

The cave amphipods of genus *Stygobromus* (Amphipoda: Crangonyctidae) are distributed in subterranean aquatic ecosystems across North America and Eurasia (Wang and Holsinger 2001). Holsinger (1967, see also 1966, 1973, 1978), considered the authority on *Stygobromus*, used morphological data to formulate species groups for North America. Four species groups are found in the Edwards plateau of the south-central United States, two being endemic to that region. This study examined species in the widespread *tenuis* species group and the Edwards plateau endemic *flagellatus* and *hadenoecus* species groups (Table 1) from 19 localities in the Edwards plateau (Figure 1). Holsinger (1967) described the *tenuis* species group as less pronounced in troglomorphic features (most notably, shorter appendages) and associated with surface water bodies from Texas to the United States eastern seaboard. The *flagellatus* and *hadenoecus* species groups exhibit more pronounced troglomorphy, and have restricted species ranges typically associated with caves, phreatic zones, and groundwater spring flows within the Edwards plateau region. Holsinger (1967) used morphological data to construct a phylogeny for the *flagellatus* species group (Figure 2A).

Within the *flagellatus* group is the Peck's cave amphipod, *S. peckae*, a federal and state of Texas endangered, short-range spring endemic known from Landa Lake and Hueco Springs (Table 1). At Landa Lake, *S. peckae* emerges from multiple spring flows (1a-1 on Table 1, Figure 3). Molecular data can be used to assess *S. peckae* population structure and form the basis for the recognition of evolutionarily significant units (Crandall et al. 2000). *Stygobromus peckae* shares habitat at Landa Lake with the federally endangered spring-endemic Comal Springs riffle beetle, *Heterelmis comalensis* (Coleoptera: Elmidae), for which Gonzales (2008) characterized molecular structure and diversity. Gonzales (2008) detected greater *H. comalensis* genetic diversity from localities at the bottom of Landa Lake than from localities at slightly higher elevations, and hypothesized that the pattern reflects bottlenecks or extirpations resulting from the record drought of the 1950s, which caused baseflow to the higher-elevation localities to cease. Here, we compare measures of *S. peckae* genetic diversity with the findings of Gonzales (2008).

This study used mitochondrial sequence, nuclear sequence, and AFLP data to answer the following questions: (1) do nominal species and species groups comprise monophyletic groups using molecular data? (2) Are molecular data congruent with the *flagellatus* species group phylogeny proposed by Holsinger? (3) Is there evidence of barriers to gene flow between *S. peckae* populations? (4) Given conservation concerns for *S. peckae*, how do its levels of genetic variation compare with its regional, non-endangered congeners and the endangered *H. comalensis*?

Methods

Using drift nets placed over spring flows and the cloth-capture technique described by Gibson et al. (2008), *Stygobromus* individuals were sampled from 19 localities in the Edwards plateau region between 2004 and 2011 (*S. pecki* collected under permits USFWS #TE876811 and TPWD #SPR-0390-045). Specimens were typically stored in 95% ethanol immediately after collection. Localities were chosen to represent species groups. Localities are numbered 1-19 (Table 1, Figure 1) and reported as "(locality #) locality name" throughout this text.

Specimens were collected and species identifications were made following the keys and descriptions of Holsinger (1967). Identifications were made using combinations of morphological characters in adult specimens. The use of "unknown" to describe two groups (bottom of Table 1) occurred because those specimens had sufficient characters to identify the individual to a particular species group but not to a single species. Three groups were designated as "*near*" a given species because character combinations were sufficient to rule out other closely-related species, but insufficient for positive identification. For all cases, lack of definitive identifications occurred because specimens were not mature adults.

For specimens with a body length greater than 4mm, tissue samples were dissected from the middle of the organism to preserve the taxonomically-relevant head and tail regions. Smaller specimens were vouchered by photograph and digested whole during extraction to maximize DNA yield. DNA extractions used the Gentra Systems Purgene DNA Isolation Kit (Minneapolis, MN) followed by rehydration with 100 µl ddH_2O.

The mitochondrial gene cytochrome oxidase C subunit 1 (COI) was amplified because it has levels of variation appropriate to the analysis of evolutionary relationships

among species groups and species (Folmer et al. 1994). It is the most common locus previously used in studies of crangonyctid amphipods, allowing use of archived sequences for outgroups, and it is part of the mitochondrial region that Gonzales (2008) used to assess diversity in *H. comalensis*. Most mitochondrial sequence data were collected using primers designed by E. Sotka (personal communication, Table 2). Some individuals failed to amplify with these primers, so species group-specific internal primers were designed from sequence alignments and used to amplify COI from these individuals (Table 2). Initial PCR reactions used standard protocols with annealing temperatures of 50-58°C. A touchdown protocol was followed for reactions with internal primer pairs: the initial annealing temperature was 56-61°C and reduced by 1°C for each of the next 5 PCR cycles, after which the annealing temperature held at 51-56°C for 35 cycles.

To address concerns about the accuracy of evolutionary inferences based on a single locus (Forister et al. 2008, Gompert et al. 2006, Johnston et al. 2011), the internal transcribed spacer region 1 (ITS1) was amplified using primers developed for the amphipod *Gammarus minus* (Amphipoda:Gammarus, Carlini et al. 2009). ITS1 is a non-coding nuclear region flanked by highly conserved ribosomal DNA sequences, which served as priming sites. PCR reactions used standard protocols with an annealing temperature of 66.5°C. For most specimens, the desired PCR product was isolated from 2% agarose gels.

PCR clean-up used Promega Wizard SV Gel Kits (Madison, WI). Sequencing was performed in both directions for each individual and gene at the Nevada Genomics Center (Reno, NV) using Applied Biosystems Prism 3730 Analyzers (Carlsbad, CA). GENEIOUS v5.3 (Drummond et al. 2011) was used to edit and align sequences.

Using an alignment of all haplotypes for each gene, likelihood scores for 56 models of evolution were generated with MODELTEST v3.7 (Felsenstein 2005, Guindon and Gascuel 2003, Posada and Crandall 1998) within PAUP v4.0b (Swofford 2002). To select the best-fit model, Akaike Information Criterion (AIC) scores were assigned to likelihood values using MRMODELTEST v2.3 (Nylander 2004). Mitochondrial sequences were fully partitioned by codon position and a model of evolution was selected for each position.

Using parameters for the best-fit model of evolution, Bayesian phylogenies were generated for COI (Figure 4) and ITS1 (Figure 5) using MRBAYES v3.1.2 (Ronquist and Huelsenbeck 2003) with confidence assessed by posterior probabilities. Phylogenies were examined for monophyly of species groups and species, and topology compared with Holsinger's *flagellatus* phylogeny.

Outgroup sequences for COI were obtained from GenBank for the following taxa: *Stygobromus emarginatus* (northern West Virginia and western Maryland), *Crangonyx floridanus* (Florida), *Crangonyx pseudogracilis* (Ontario, Canada), *Crangonyx islandicus* (Iceland), and *Gammarus minus* (Iceland). Outgroups were not used for ITS1 because the only candidate outgroup that would align, *Gammarus minus* (Carlini et al. 2009), caused loss of informative ingroup variation after removal of indel variable sites.

To more finely examine evolutionary relationships, *S. pecki* COI data was analyzed in a population genetics context. To test for barriers to gene flow between *S. pecki* localities, ARLEQUIN v3.5 (Excoffier et al. 2005) was used to conduct an analysis of molecular variance (AMOVA, Excoffier et al. 1992, Table 3A), and to estimate pairwise Φ statistics (F_{ST}-based genetic distances) and pairwise exact test probabilities (Raymond and Rousset 1995). To assess levels of genetic diversity, ARLEQUIN v3.5 was used to estimate unbiased

haplotype diversity (h, Nei 1987) and within-group nucleotide diversity (π, Tajima 1983, 1993). DNASP v5.10 (Librado and Rozas 2009) was used to estimate among-group nucleotide diversity (π, percent sequence divergence). Because Gonzales (2008) did not report all information relevant to the current comparison with *S. pecki*, parameters and standard errors for *H. comalensis* were estimated using the alignment of *H. comalensis* haplotypes and frequencies reported by Gonzales.

Unexpected monophyletic COI diversity within *S. pecki* in the form of two divergent clades (see Results) led to additional analysis of *S. pecki* data: (1) to test geographic partitioning of diversity, all AMOVA and pairwise tests were conducted again with haplogroups within locality considered separately. (2) to describe the depth of the COI haplotype divergence, ARLEQUIN v3.5 was used to conduct an AMOVA with COI haplogroup as a factor (Table 3B), which partitioned molecular variance into a nested hierarchy among haplogroups, among localities within haplogroups, and within localities. (3) TCS v1.21 (Clement et al. 2000, Templeton et al. 1992) was used to produce a parsimony network of haplotypes to illustrate COI diversity as a gene genealogy (Figure 6). (4) To rule out a *Wolbachia* endosymbiont as cause of the *S. pecki* COI divergence, the presence of *Wolbachia* was tested using the methods of Nice et al. (2009) on 3 individuals from each COI haplogroup.

Unexpected polyphyletic COI diversity was detected in *S. dejectus*, *S. longipes*, *S. flagellatus*, and *near S. russelli* (see Results). To test if cryptic species explained this polyphyletic diversity better than nominal species alone, a null AMOVA grouped individuals by nominal species (Table 3C), and an alternative AMOVA grouped individuals by nominal species and

where present, polyphyletic COI haplogroups within nominal species (Table 3D). Results were examined for how well they explained molecular variation.

To estimate genome-wide population structure, AFLP data (Meudt and Clarke 2006, Vos et al. 1995) was collected for *S. pecki*, *S. longipes*, and *S. dejectus* following the methods of Gompert et al. (2006, 2008). *Stygobromus longipes* and *S. dejectus* were included to estimate the amount of differentiation among these closely-related nominal species. Two selective primer pairs, mCAGCA (5' GAT GAG TCC TGA GTA ACA GCA 3') and mCAGAT (5' GAT GAG TCC TGA GTA ACA GAT 3'), were each paired with EcoR1. Size fragment analysis of selective PCR products was conducted at the Nevada Genomics Center (Reno, NV) using the Applied Biosystems Prism 3730 Analyzer (Carlsbad, CA).

PeakScanner v1.0 (Applied Biosystems, Carlsbad, CA) was used to format raw AFLP data for automated scoring by RawGeno v2.0 (Arrigo et al. 2009), a CRAN package for R Statistical Software v2.12.0 (R Development Core Team 2011). Scored AFLP data was analyzed with Structure v2.3 (Falush et al. 2003, Falush et al. 2007, Pritchard et al. 2000), which assigns individuals probabilistically to populations using a Bayesian clustering algorithm. An admixture model was used, allowing for gene flow between populations. Runs used a Markov Chain Monte Carlo (MCMC) of 250,000 generations/25,000 initial burn-in, and the number of clusters (K) was evaluated from 1 to 15 (number of sampling localities plus one) for 10 iterations each. Two approaches were used to select K: (1) K over mean log likelihood of K was plotted (Figure 7A), with the asymptote of the plot corresponding to the K value that best explains the data (Pritchard et al. 2000). (2) The ad hoc statistic ΔK, based on the rate of change in the log probability of data between successive K values, was

calculated for each K and plotted (Figure 7B), with the best value of K corresponding to the highest value in the plot (Evanno et al. 2005).

Results

COI sequencing reactions yielded a 501 base pair (bp) product with 76 haplotypes obtained from 128 individuals. Of 501 bases in the alignment, 247 were variable (254 invariable). The protein translation was 166 amino acids with 37 variable amino acid sites (22%). ITS1 sequencing reactions yielded a 212-bp product obtained from 32 individuals producing 11 haplotypes with sequence length variation due to indel history. Of 212 bases, 34 or 16% were variable (178 invariable). AFLP data for the two selective primer pairs were combined to yield a dataset of 428 loci from 92 individuals.

A common pattern in molecular data was the detection of two distinct clades within five nominal taxa: *S. pecki*, *S. dejectus*, *S. longipes*, *S. flagellatus*, and *near S. russelli*. These clades are referred to as haplogroups A and B and their species name (i.e. *S. pecki* haplogroup A).

Excluding outgroups and two unexpectedly disparate *S. flagellatus* individuals (see below), there were two major clades that arose from COI data (Figure 4). The first consisted entirely of *flagellatus* species group specimens (*S. pecki*, *S. dejectus* haplogroup A, *S. longipes*, and *near S. pecki*), while the second contained *flagellatus, tenuis*, and *hadenoecus* species group specimens.

The *flagellatus* and *tenuis* species groups were not monophyletic for COI or ITS1. Though strictly monophyletic, sequence results for the *hadenoecus* species group were obtained from one locality only, and were nested within the *tenuis* portion of the phylogeny (Figure 4). The proposed *flagellatus* species group phylogeny (Figure 2A) conflicted with

molecular data, which suggested different relationships between *S. pecki*, *S. dejectus* haplogroup A, and *S. longipes*, and cast doubt on the inclusion of *S. dejectus* haplogroup B and *S. flagellatus* (Figure 2B).

Species monophyletic for COI were *S. pecki*, *S. bifurcatus*, and *near S. hadenoecus* (100%, 99%, and 100% bootstrap support, respectively), and all were either monophyletic or monomorphic for ITS1 (*S. pecki* shared its ITS1 haplotype with *S. longipes* and *S. dejectus* haplogroup A). *Stygobromus dejectus* and *near S. russelli* were polyphyletic for both genes. *Stygobromus longipes, S. russelli*, and *S. flagellatus* were polyphyletic for COI only.

Stygobromus dejectus was polyphyletic for both genes with weak bootstrap support for the monophyly of haplogroup A (82%) and strong support for haplogroup B (100%). Without bootstrap support, *S. dejectus* haplogroup A collapses into a polytomy with two individuals identified to other species (*S. longipes* and *near S. pecki*). The closest relative of *S. dejectus* haplogroup B are the unknown specimens from (18) Artesian Well for both COI and ITS1, with 96% and 100% bootstrap support, respectively. AFLP results also supported the presence of *S. dejectus* cryptic diversity (see below).

Stygobromus longipes was polyphyletic for COI, with *S. longipes* haplogroup A being monophyletic with 100% bootstrap support, while *S. longipes* haplogroup B (a single, disparate sequence) grouped with *S. dejectus* haplogroup A (Figure 4). Among-group sequence divergence (π) values for *S. longipes* and *S. dejectus* haplogroup B contradicted the nominal taxonomy: 0.10037 between *S. longipes* haplogroups, but only 0.01065 between *S. longipes* haplogroup B and *S. dejectus* haplogroup A (Table 4).

Stygobromus russelli, the unknown *tenuis* specimens from (12) Cold Spring, and *near S. russelli* haplogroup A formed a single clade in the COI phylogeny (Figure 4). The unknown

tenuis specimens produced 3 haplotypes, 2 of which were shared with positively-identified *S. russelli* specimens, suggesting that these unknowns are *S. russelli*. *Near S. russelli* haplogroup A did not share any haplotypes with positively identified *S. russelli*, but did exhibit close molecular relationships with *S. russelli* for COI and ITS1, suggesting they are also *S. russelli*. If both are accepted as *S. russelli*, the group exhibits monophyly with 98% bootstrap support.

Stygobromus flagellatus produced three polyphyletic COI haplogroups. *Stygobromus flagellatus* haplogroup A paired with *S. dejectus* haplogroup B for both COI and ITS1. Four haplotypes from the unknown *flagellatus* individuals had a close molecular relationship with *S. flagellatus* haplogroup A, and both groups were collected from (18) Artesian Well at the same time, raising the possibility that the unknown *flagellatus* specimens are actually *S. flagellatus*. *Stygobromus flagellatus* haplogroup B grouped with the predominantly *tenuis* portion of the phylogeny. *Stygobromus flagellatus* haplogroup C grouped with the conspecific outgroup *S. emarginatus* (northern West Virginia and western Maryland, species group *emarginatus*) in the COI phylogeny and were responsible for 21 of the 37 variable amino acid sites in the COI translation. ITS1 results contradicted COI: the two specimens sequenced (one from haplogroup B and one from haplogroup C) shared a single ITS1 haplotype that was closely related to the haplotypes for *S. pecki* and *near S. russelli* haplogroup B.

AMOVA analysis of *S. pecki* molecular data revealed no significant structure among localities (Table 3A, Φ_{ST}=0.01496, p=0.32942). All pairwise Φ statistics and exact tests were non-significant except for the Φ statistic between (1d) Spring run 3 and (1e) Upwelling (Φ_{ST}=0.19951, p=0.04505). Relative to each other, (1d) Spring run 3 had a disproportionate number of haplogroup A (13 of 15) and (1e) Upwelling had a disproportionate number of haplogroup B (5 of 7). When the analyses were run again but with COI haplogroups

considered separately at each locality, all Φ_{ST} values and within-haplogroup pairwise comparisons were non-significant. Despite the presence of two distinct mitochondrial clades, there is no apparent geographic population structure for *S. pecki* at (1) Landa Lake.

Haplotype diversity (h) measures for *S. pecki* were not significantly different from estimates for its regional congeners (Table 1). Within-group nucleotide diversity (π - Table 1) for *S. pecki* was not significantly different from *S. dejectus* haplogroup A, and was significantly less than for *S. dejectus* haplogroup B, *S. longipes* haplogroup A, and *S. russelli*. Compared to *H. comalensis*, *S. pecki* haplotype and nucleotide diversity measures were significantly greater except haplotype diversity within the (1f) West Shore locality (Table 5), which was not significantly different. Generally, *S. pecki* did not follow the *H. comalensis* pattern of reduced genetic diversity at the higher-elevation localities (spring runs 1 and 3). It is however noted that the sole significant pairwise Φ statistic reported in the previous paragraph was between (1d) Spring run 3 and (1e) Upwelling.

The *S. pecki* COI haplogroups did not sort geographically (see above). Sequence divergence between the *S. pecki* haplogroups is 2.772% (Table 4) with eight nucleotide differences between them as visualized in a parsimony network of haplotypes (Figure 6). An AMOVA using haplogroup as a factor (Table 3B) yielded a Φ_{ST}=0.8257 ($p<0.00001$), meaning 82.57% of COI genetic variation is attributed to differences between haplogroups. A *Wolbachia* endosymbiont was ruled out as cause after no *S. pecki* from either haplogroup tested positive for infection.

Regarding the AMOVA hypothesis test, it was found that treating polyphyletic COI haplogroups as cryptic species diversity (Table 3D, Φ_{ST}=0.8068, $p<0.00001$) explained COI variation better than nominal species alone (Table 3C, Φ_{ST}=0.6872, $p<0.00001$).

Both approaches to select K indicated that four clusters best explain the AFLP data (Figure 7). The majority of *S. pecki* COI haplogroup B individuals assigned to a single cluster (1 – Figure 8), while *S. pecki* haplogroup A individuals had mixed probabilistic assignments to two different clusters (1 and 2 – Figure 8). Under the admixture model, an individual's cluster assignment probability can be interpreted as the proportion of that individual's genome originating in that cluster (Pritchard et al. 2000). Using this interpretation, the distribution of clusters 1 and 2 between COI haplogroups A and B suggests that *S. pecki* consists of two previously isolated groups that have reestablished asymmetric gene flow, as indicated by the admixture observed for COI haplogroup A that is relatively absent in haplogroup B (Figure 8).

Stygobromus dejectus haplogroup A and *S. longipes* mostly assigned to cluster 3. Cluster 3 also included smaller proportions of some *S. pecki* genomes, a finding which is not surprising given the close phylogenetic relationship between these three species indicated by COI and ITS1. Six *S. dejectus* individuals assigned to cluster 4, three of which were *S. dejectus* COI haplogroup B, further supporting cryptic diversity within *S. dejectus*. The remaining three from cluster 4 failed to produce COI or ITS1 sequence.

Discussion

Phylogenetic relationships and patterns of geographic variation observed in Edwards plateau *Stygobromus* are complex. The species group taxonomic framework and *flagellatus* species group phylogeny (Holsinger 1967, Figure 2A) were largely unsupported by molecular data generated by the current study. Nominal species had varying levels of support, and there were strong indications that more species diversity may be present in Edwards plateau

Stygobromus than is currently recognized. *Stygobromus pecki* from Landa Lake contained unexpected mitochondrial diversity in the form of two divergent clades separated by 2.3% sequence divergence (Table 4, Figures 4 and 6). This partitioning of *S. pecki* COI diversity was paralleled to some extent in the AFLP data though less distinctly (Figure 8). The explanation offered here is that *S. pecki* was historically split into two substantially isolated populations followed by restoration of gene flow and asymmetric admixture. The picture presented by current data is complex, and expanded geographical and taxonomic sampling will be required to create a complete picture of *Stygobromus* biodiversity and evolutionary history.

Given the confounding effects of troglomorphic convergence on phylogenetic assumptions, it is not surprising that the morphology-based species group taxonomy and *flagellatus* species group phylogeny proposed by Holsinger (Figure 2A) were contradicted by molecular findings. The *flagellatus* and *tenuis* species groups were polyphyletic for both genes, and the *hadenoecus* species group was nested within the *tenuis* portion of the COI phylogeny (Figure 4). *Stygobromus dejectus* haplogroup B and the unknown species from (18) Artesian Well were distantly related to other *flagellatus* species group members at both genes. Surprisingly, two *S. flagellatus* COI sequences grouped close to archived *S. emarginatus* sequences (species group *emarginatus*) from the middle Atlantic region of the United States, but the signal from ITS1 contradicted this. An alternative to nominal species groups is suggested by the current study. The two major COI clades (Figure 4) suggest a widespread group that includes representatives of all nominal species groups, and a much shorter-range and possibly endemic species group that consists of *S. pecki*, *S. longipes*, *S. dejectus* haplogroup A, and *near S. pecki*.

Regarding proposed phylogenetic relationships (Figure 2A), the hypothesis of sister species relationship between *S. pecki* and *S. dejectus* was contradicted by two molecular findings: (1) *S. dejectus* is a polyphyletic group with strong evidence of cryptic species diversity (Table 4, Figures 4, 5, and 8), and (2) The COI phylogeny suggests that *S. longipes* haplogroup A is the sister taxa to *S. pecki*. The placement of *S. flagellatus* on a revised phylogeny is uncertain (Figure 2B) because of polyphyletic diversity, small sample sizes, and contradicting signals from COI and ITS1.

Nominal species had varying levels of support. *Stygobromus pecki* and *S. bifurcatus* were monophyletic for both genes and with strong bootstrap support. If five individuals lacking positive identification to *S. russelli* are accepted as such given molecular evidence (see Results), *S. russelli* was monophyletic as well. *Stygobromus dejectus* and *near S. russelli* exhibited the strongest evidence of cryptic species diversity from COI and ITS1. *Stygobromus longipes* and *S. flagellatus* were polyphyletic, and while they lacked sufficient sample sizes for in-depth explorations, each produced a curious finding: (1) *S. longipes* haplogroup B and *S. dejectus* haplogroup A had a smaller sequence divergence (π=0.01065) than the sequence divergence between *S. pecki* haplogroups (π=0.02272). (2) *Stygobromus flagellatus* haplogroup B showed a distant relationship to all other Edwards plateau *Stygobromus* sampled.

The *S. pecki* COI haplogroups prompted further inquiry about causation. They could reflect an ongoing process, such as a scenario in which two groups of closely related amphipods experienced a substantial period of allopatry followed by secondary contact and admixture. The two COI clades remain distinct due to non-recombination in mitochondria, but there is clear evidence of some admixture in the nuclear AFLP data. This scenario seems tenable given two findings: (1) the complete sympatry of the two haplogroups (i.e. the two

haplogroups were detected in every *S. pecki* sampling locality and at nearly equal frequencies at each locality), and (2) the presence of two less distinct but nevertheless discernible clusters in the AFLP data (Figure 8), which suggests that admixture is not complete or that some partial or asymmetric barrier to gene flow between haplogroups is maintaining differentiation as observed in the barplots. These patterns are highly unusual. Niemiller et al. (in press) have previously discovered cryptic variation within an endangered species, but the sympatric, cryptic variation observed in the endangered *S. pecki* may be unprecedented. Alternatively, endosymbionts could produce the discordance observed for COI and ITS1. We found no evidence of *Wolbachia*, but other endosymbionts, such as *Rickettsia*, *Cardinium*, and *Spiroplasma*, could be important agents (Moran et al. 2008).

COI genetic diversity estimates for the endangered *S. pecki* are similar to estimates for its regional congeners and significantly higher than estimates for the endangered Comal Springs riffle beetle, *H. comalensis*. The *H. comalensis* pattern of differential genetic diversity based on elevation was not observed for *S. pecki*, suggesting that the cause of reduced diversity in *H. comalensis* did not similarly affect *S. pecki*. Assuming the reduced diversity was caused by the record drought of the 1950's as Gonzales (2008) suspected, we may infer that *S. pecki* is capable of surviving in deeper habitats than *H. comalensis*.

The molecular data collected for Edwards plateau *Stygobromus* can serve as a baseline for future studies of these spring-endemic species. This study raises as many questions as it answers and there is much work to be done to delimit species boundaries and to provide an accurate accounting of the taxonomic diversity in this group of amphipods. The current study strongly supports the presence of more species diversity than is currently recognized. To gain a more accurate assessment of *Stygobromus* biodiversity and evolutionary history,

increased sampling in the Edwards plateau is needed. This includes sampling of the endangered *S. pecki* from (2) Hueco Springs, the only place other than (1) Landa Lake from which *S. pecki* has been described, and from which the current study provided data from only one individual.

Table 1: Sample information and within-group COI diversity. Species group and species determinations made following the keys of Holsinger (1967), considered the authority on Edwards plateau *Stygobromus* taxonomy. Species samples that were phylogenetically disparate for COI are designated "haplogroups" with measures reported for each. Within each species, sample sizes are reported for each of the 3 datasets (COI, ITS1, AFLP) with haplogroup and locality sample sizes reported in brackets and parentheses, respectively. Within-group haplotype diversity (h) and nucleotide diversity (π) with their standard errors (SE) are reported.

Species (species group)	Sampling Location	N_{COI}	N_{ITS}	N_{AFLP}	COI h±SE	COI π±SE
S. pecki (flagellatus)	Pooled	71	8	66	0.8632±0.0367	0.010764±0.005819
	S. pecki COI haplogroup A	[49]	[4]	[46]	0.7355±0.0680	0.002896±0.001987
	S. pecki COI haplogroup B	[22]	[4]	[20]	0.8745±0.0672	0.004830±0.003038
	(1a) Canyon Well	(5)	(1)	(5)	0.7000±0.2184	0.011178±0.007548
	(1b) Spring Run 1	(11)	(2)	(11)	0.8000±0.1138	0.010016±0.005946
	(1c) Kiddy Pool	(3)		(2)	1.0000±0.2722	0.013307±0.010774
	(1d) Spring Run 3	(15)	(1)	(15)	0.7810±0.1016	0.006767±0.004114
	(1e) Upwelling	(9)		(8)	0.9722±0.0640	0.014774±0.008728
	(1f) West shore	(3)		(2)	1.0000±0.2722	0.013307±0.010774
	(1g) Spring Island	(15)	(3)	(14)	0.9238±0.0530	0.010341±0.005947
	(1h) Spring Run 5	(6)		(6)	1.0000±0.0962	0.013706±0.008682
	(1i) Spring Run 4	(3)		(3)	1.0000±0.2722	0.017299±0.013757
	(2) Hueco Springs	(1)			n/a	n/a
near S. pecki (flagellatus)	(3) Bowling Well	1			n/a	n/a
S. dejectus (flagellatus)	Pooled	12	6	18	0.8636±0.0716	0.074427±0.039259
	S. dejectus COI haplogroup A	[9]	[4]	[9]	0.7778±0.1100	0.003881±0.002748
	S. dejectus COI haplogroup B	[3]	[2]	[3]	0.6667±0.3143	0.027944±0.021703
	(4) Cascade Caverns	(10)	(6)	(13)	0.8889±0.0754	0.085163±0.045748
	(5) Stealth Cave	(2)		(5)	1.0000±0.5000	0.001996±0.002823
S. longipes (flagellatus)	Pooled	8	4	8	0.9643±0.0772	0.046051±0.025895
	S. longipes COI haplogroup A	[7]	[4]	[7]	0.9524±0.0955	0.027944±0.016388
	S. longipes COI haplogroup B	[1]		[1]	n/a	n/a
	(6) Cave without a name	(4)	(2)	(4)	1.0000±0.1768	0.011643±0.008406
	(7) CM Cave	(2)	(2)	(2)	1.0000±0.5000	0.007984±0.008926
	(8) Honey Creek Cave	(1)		(1)	n/a	n/a
	(9) Magic Springs	(1)		(1)	n/a	n/a
S. flagellatus (flagellatus)	Pooled	4	2			
	S. flagellatus COI haplogroup A	[1]			n/a	n/a
	S. flagellatus COI haplogroup B	[1]	[1]		n/a	n/a
	S. flagellatus COI haplogroup C	[2]	[1]		1.0000±0.5000	0.003992±0.004889
	(10) Diversion Spring	(3)	(2)		1.0000±0.2722	0.181637±0.136274
	(18) Artesian Well	(1)			n/a	n/a
S. russelli (tenuis)	Pooled	14	4		0.8791±0.0788	0.049133±0.025798
	(11) Blowing Sink Cave	(7)	(1)		0.5238±0.2086	0.035168±0.020427
	(12) Cold Spring	(4)	(2)		0.8095±0.1298	0.032697±0.019046
	(13) Barton Creek Well	(1)			n/a	n/a
	(14) Salamander Cave	(1)	(1)		n/a	n/a
	(15) Onion Creek Well	(1)			n/a	n/a
near S. russelli (tenuis)	(16) San Gabriel Springs	5	2		1.0000±0.1265	0.095409±0.058591
	near S. russelli COI haplogroup A	[3]	[1]		1.0000±0.2722	0.017299±0.013757
	near S. russelli COI haplogroup B	[2]	[1]		1.0000±0.5000	0.009980±0.010933
S. bifurcatus (tenuis)	Pooled	2	2		1.0000±0.5000	0.115768±0.116762
	(15) Onion Creek Well	(1)	(1)		n/a	n/a
	(17) Adobe Springs	(1)	(1)		n/a	n/a

Table 1 continued

near S. hadenoecus (hadenoecus)	(19) Devil's River	4	4		0.5000±0.2652	0.007984±0.006000
Unknown species *(tenuis)*	(12) Cold Spring	3			1.0000±0.2722	0.052562±0.040060
Unknown species *(flagellatus)*	(18) TSU Artesian Well	4	2		1.0000±0.1265	0.003194±0.002632
	TOTAL	128	32	92	0.9548±0.0129	0.116907±0.056254

TABLE 2: Primers. Reported 5' to 3'.

GENE	PRIMER NAME	SEQUENCE	CITATION
ITS1	CarliniF	TCC GTA GGT GAA CCT GCG G	Carlini et al. (2009)
	CarliniR	AGT GAT CCA CCG CTC AGA G	Carlini et al. (2009)
COI	SotkaF	GGT CWA CAA AYC ATA AGA YAT TGG	Sotka (unpublished)
	SotkaR	TAA ACY TCA GGR TGA CCR AAR AAY CA	Sotka (unpublished)
	flagellatusF	TCA TCC GAT CCG AAC TAT CCT G	Current study
	flagellatusR	TCG GTA AGT AAT ATA GTA ATA GCA CC	Current study
	tenuisF	TTA TCC GCT CTG AGT TAT CTT G	Current study
	tenuisR	TCA GAA CGT AGT ATT GTA ATA GCT CC	Current study

TABLE 3: COI AMOVA. (A-B) consider *S. pecki* haplotypes only, (C-D) consider all *Stygobromus* haplotypes. Within *S. pecki*, (A) considers locality only and shows that *S. pecki* genetic variation is not geographically partitioned. Illustrating the striking COI divergence within *S. pecki*, (B) found that 82.57% of *S. pecki* COI variation is explained by differences between haplogroups. Supporting cryptic diversity in *Stygobromus*, the alternative condition (D) explained a larger proportion of the variance than the null (C) by considering polyphyletic COI haplogroups in addition to nominal species.

(A) *S. pecki* grouped by locality: Φ_{ST}=0.01496 (p=0.32942)

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	P value
Among localities	9	26.400	0.04041	1.50	0.32942
Within localities	61	162.346	2.66142	98.5	

(B) *S. pecki* grouped by COI haplogroup: Φ_{ST}=0.8257 (p<0.00001)

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	P value
Among groups	1	128.727	4.20546	82.57	<0.00001
Among localities within groups	17	16.654	0.03675	0.72	0.53861
Within localities	52	44.253	0.85102	16.71	<0.00001

(C) All *Stygobromus* grouped by nominal species: Φ_{ST}=0.6872 (p<0.00001)

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	P value
Among groups	8	2772.257	28.27780	68.72	<0.00001
Among localities within groups	12	264.271	6.49085	15.77	0.03519
Within localities	107	682.690	6.38028	15.51	<0.00001

(D) All *Stygobromus* grouped by species + polyphyletic haplogroups: Φ_{ST}=0.8068 (p<0.00001)

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	P value
Among groups	13	3191.244	33.31205	80.68	<0.00001
Among localities within groups	11	314.910	5.90671	14.31	<0.00001
Within localities	103	213.064	2.06858	5.01	<0.00001

TABLE 4: COI sequence divergences (π) among species. Measures are the same above and below the diagonal.

	S. pecki	*S. pecki A*	*S. pecki B*	*S. longipes*	*S. longipes A*	*S. longipes B*	*S. dejectus A*	*S. dejectus B*	*S. russelli*
S. pecki	-	n/a	n/a	0.11725	0.11376	0.14174	0.14101	0.18395	0.18770
S. pecki A	n/a	-	0.02272	0.11163	0.10794	0.13744	0.13589	0.18648	0.19001
S. pecki B	n/a	0.02272	-	0.11941	0.11699	0.13636	0.13571	0.18603	0.18316
S. longipes	0.11725	0.11163	0.11941	-	n/a	n/a	0.08746	0.17033	0.17799
S. longipes A	0.11376	0.10794	0.11699	n/a	-	0.10037	0.09844	0.16976	0.17942
S. longipes B	0.14174	0.13744	0.13636	n/a	0.10037	-	0.01065	0.17432	0.16795
S. dejectus A	0.14101	0.13589	0.13571	0.08746	0.09844	0.01065	-	0.17365	0.16819
S. dejectus B	0.18395	0.18648	0.18603	0.17033	0.16976	0.17432	0.17365	-	0.17979
S. russelli	0.18770	0.19001	0.18316	0.17799	0.17942	0.16795	0.16819	0.17979	-

TABLE 5: Comparison of genetic diversity for *S. pecki* and *H. comalensis*. Within-group haplotype diversity (h) and nucleotide diversity (π) with standard error (SE) reported. In all interspecies comparisons except h for West shore, *S. pecki* exhibited significantly greater molecular diversity that *H. comalensis*. Within species, *S. pecki* did not show the pattern of relatively impoverished diversity for spring runs 1 and 3 that *H. comalensis* did, suggesting that whatever caused the impoverished diversity for *H. comalensis* did not similarly affect *S. pecki*.

	Haplotype diversity (h±SE)		Nucleotide diversity (π±SE)	
	S. pecki	***H. comalensis***	***S. pecki***	***H. comalensis***
(1b) Spring Run 1	0.8000±0.1138	0.0000	0.010016±0.005946	0.0000
(1d) Spring Run 3	0.7810±0.1016	0.0000	0.006767±0.004114	0.0000
(1f) West shore	(1.0000±0.2722)	(0.7463±0.0382)	0.013307±0.010774	0.001700±0.001136
(1g) Spring Island	0.9238±0.0530	0.5286±0.0788	0.010854±0.006209	0.001475±0.001034
All specimens	0.8632±0.0367	0.4278±0.0493	0.010764±0.005819	0.000904±0.000696

Figure 1: Sampling localities. (1) Landa Lake, (2) Hueco Springs, (3) Bowling Well, (4) Cascade Cavern, (5) Stealth Cave, (6) Cave without a name, (7) CM Cave, (8) Honey Creek Cave, (9) Magic Springs, (10) Diversion Spring, (11) Blowing Sink Cave, (12) Cold Spring, (13) Barton Creek Well, (14) Salamander Cave, (15) Onion Creek Well, (16) San Gabriel Springs, (17) Adobe Springs, (18) Texas State Artesian Well, (19) Devil's River. Straight-line distance between Devil's River and central Texas is approximately 275 km.

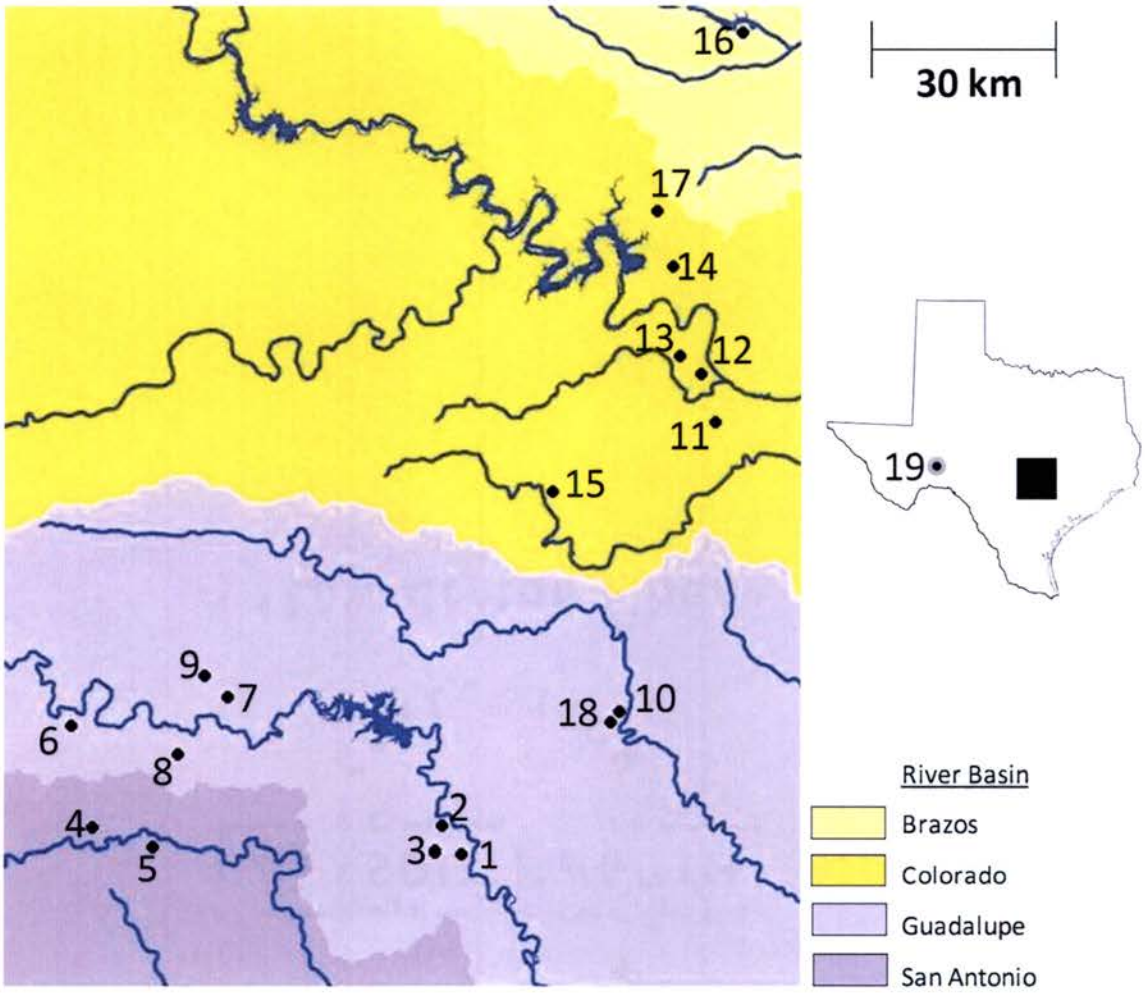


Figure 2: Phylogenetics of the *flagellatus* species group. (A) Proposed phylogeny for the *flagellatus* species group (Holsinger 1967). (B) Relationships between these taxa suggested by molecular data. The placement of *S. flagellatus* is uncertain due to small sample size and conflicting signals between COI and ITS1 sequence data.

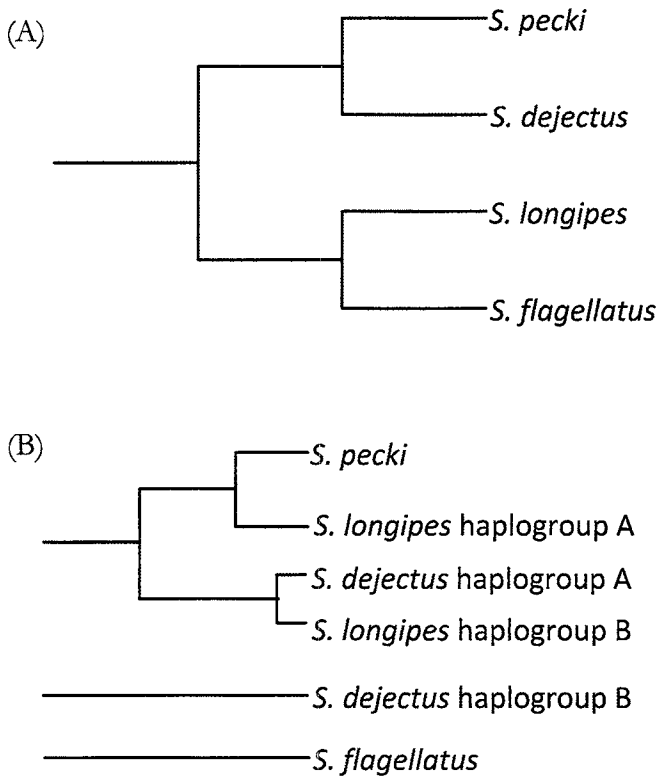


Figure 3: *S. pecki* sampling localities at (1) Landa Lake. (a) Panther Canyon Well, (b) Spring Run 1, (c) Kiddy pool, (d) Spring Run 3, (e) Upwelling, (f) West Shore, (g) Spring Island, (h) Spring Run 5, (i) Spring Run 4. Data for *H. comalensis* was available for localities b, d, e, and f.

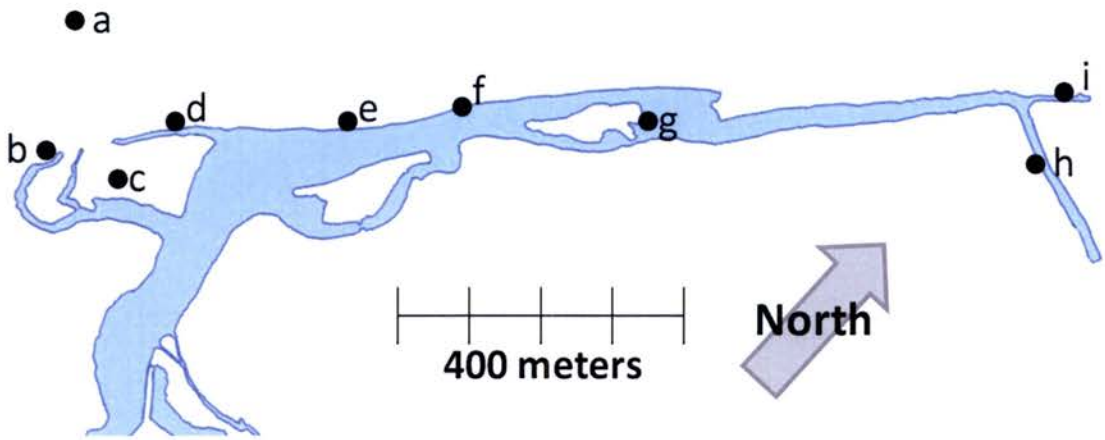


Figure 4: COI haplotype phylogeny. Each point represents a unique haplotype with its collection site(s) in parentheses. Haplogroups within *S. pecki, S. longipes, S. dejectus, S flagellatus,* and *near S. russelli* are labeled. Specimens for which the species is unknown report the species group. Outgroup is *Gammarus minus* from Iceland Confamilial outgroups are *Crangonyx islandicus* (Iceland), *C. floridanus* (Florida), and *C. pseudogracilis* (Ontario). Congeneric outgroup is *S emarginatus* (Virginia).

Figure 5: ITS1 haplotype phylogeny. ITS1 data for *S. pecki*, *S. dejectus*, and *near S. russelli* haplogroups supported COI findings. *S. flagellatus* signal conflicted with that found for COI. No outgroups were used in this phylogeny.

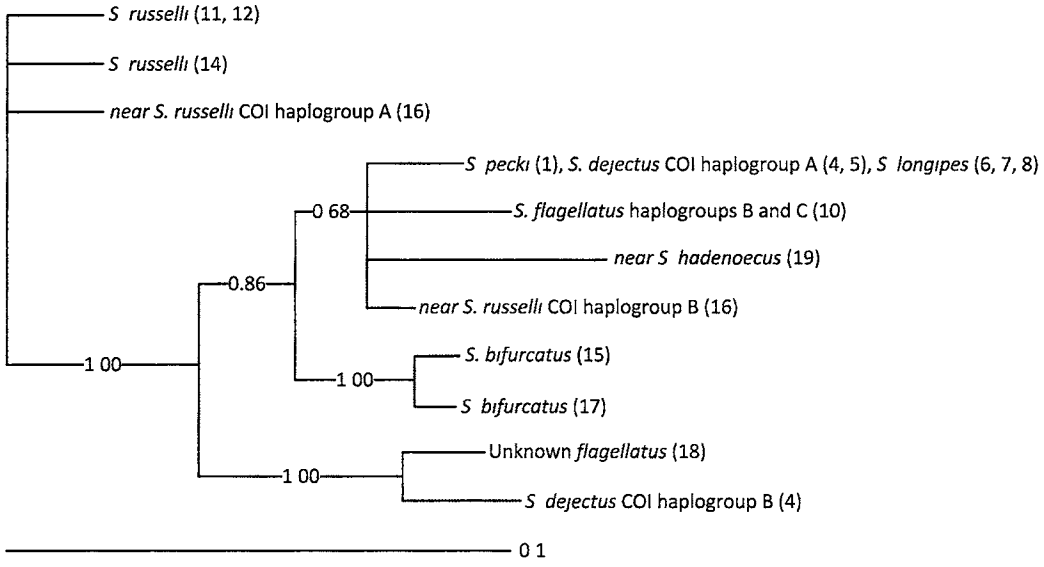


Figure 6: *S. pecki* COI haplotype network. Each circle reports haplotype number (sample size), and circle size approximately corresponds with sample size. Blank circles represent one nucleotide substitution. Three paths between haplogroups are depicted because they are equally parsimonious.

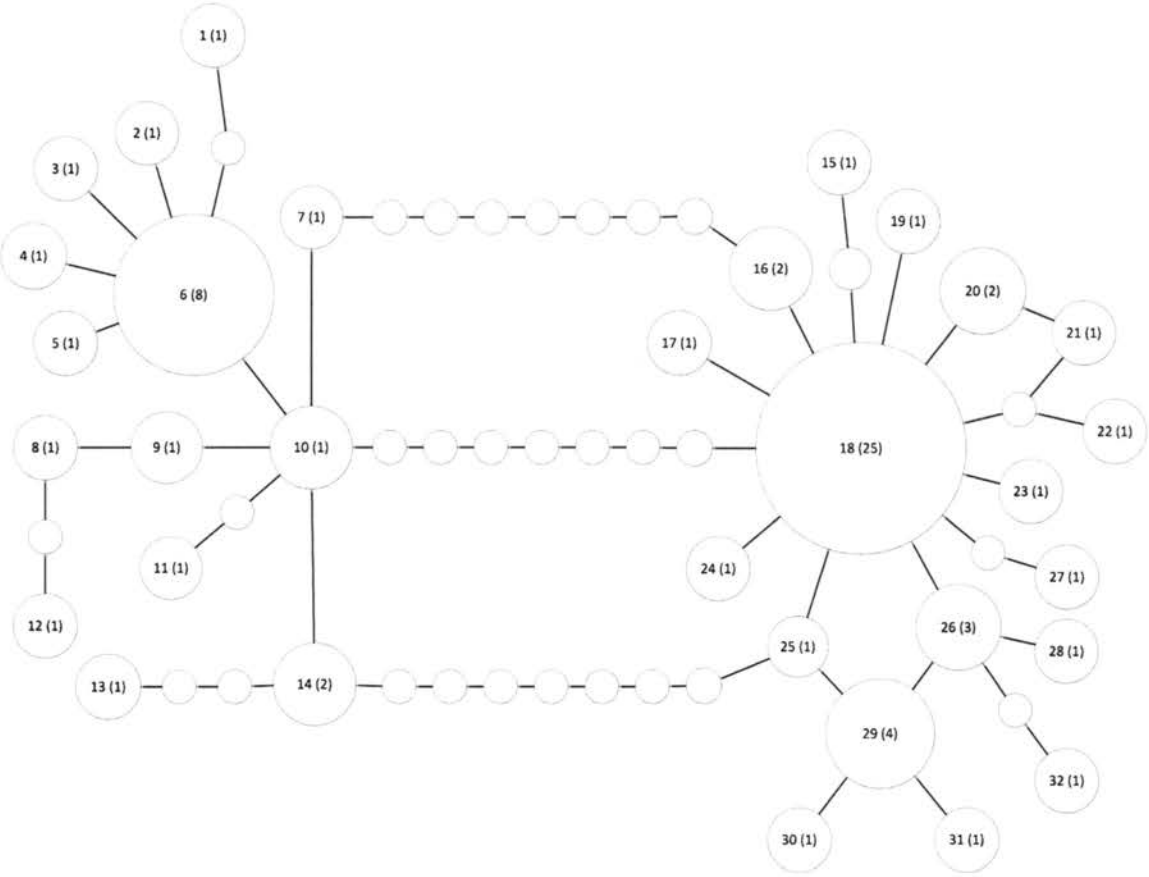
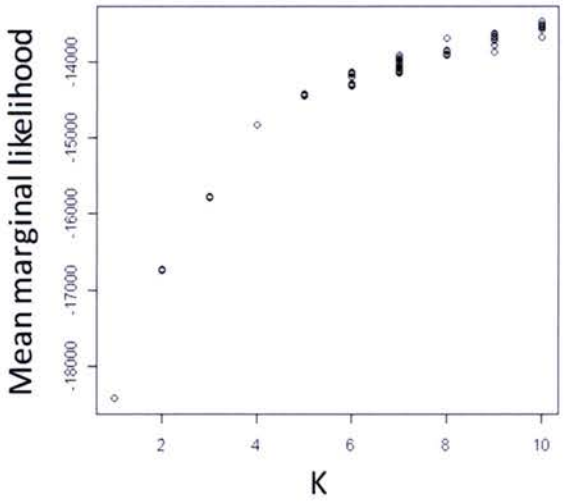


Figure 7: K estimation. Two methods were used to estimate the "true" K, (A) the mean marginal likelihood approach (Pritchard et al. 2000), and (B) the K method (Evanno et al. 2005). The mean marginal likelihood approach (A) estimates the mean marginal likelihood for each value of K. The best value of k corresponds to the point when the slope breaks toward (but does not necessarily achieve) a horizontal asymptote. The K approach (B) estimates the ad hoc K for each value of K, with the best value of K corresponds to peak K values on the plot.

(A)



(B)

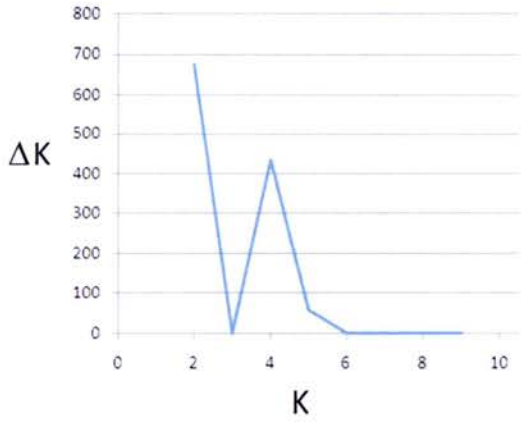
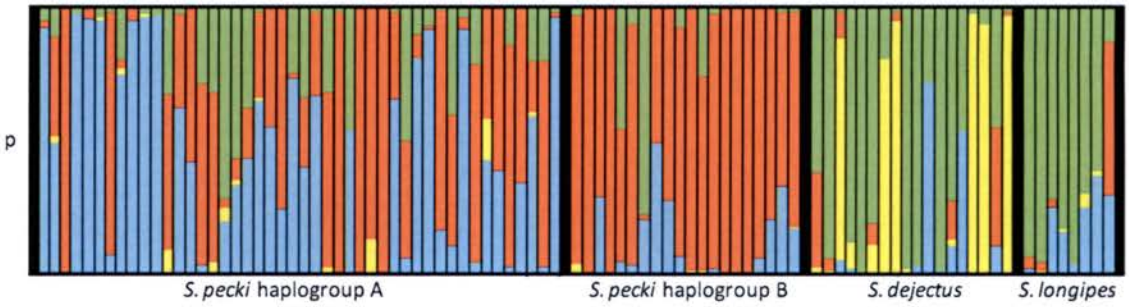


Figure 8: AFLP barplot (K=4). Clusters are (1) Red, (2) Blue, (3) Green, (4) Yellow. Each column represents an individual in the analysis, and the area of each color equals that individuals probabilistic assignment to that cluster. Under the admixture model, an individual's cluster assignment probability can be interpreted as the proportion of that individual's genome originating in that cluster (Gompert et al. 2006). Cluster 1 is most associated with *S. pecki* haplogroup B and cluster 2 with *S. pecki* haplogroup A. The distribution of genomes between the two suggests asymmetric gene flow. Cluster 3 appears in both *S. pecki* haplogroups because *S. dejectus* and *S. longipes* are its closest relations. Within *S. dejectus*, the genomic distance of cluster 4 individuals (3 of which are *S. dejectus* COI haplogroup B, the other 3 are unknown) from the others is indicated by their very high, and all others' very low, assignment probabilities to cluster 4.



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