FINAL REPORT

As Required by

THE ENDANGERED SPECIES PROGRAM

TEXAS

Grant No. TX E-78-R

Endangered and Threatened Species Conservation

Population Genetic Analysis of the Texas Blind Salamander, *Eurycea rathbuni*

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FINAL REPORT

STATE: ____Texas_____ **GRANT NUMBER:** ___TX E – 78-R__

GRANT TITLE: Population Genetic Analysis of the Texas Blind Salamander, *Eurycea rathbuni*

REPORTING PERIOD: ____1 Aug 06 to 31 Aug 09_

OBJECTIVE(S):

To estimate migration rates, effective population size, cohesion, and genetic variation in *Eurycea rathbuni*, assess possible threats, and provide a genetic baseline for continued monitoring and management of this species.

Segment Objectives:

Task 1. Tissue samples will be obtained from live animals in the field as part of a mark-recapture study focusing on three putative populations (localities) of E. rathbuni (Project Statement: Attachment A, Appendix A). Year 1 and 2.

Task 2. Tissue samples from the other currently accessible localities (San Marcos Springs and possibly Fish Hatchery Well) will be obtained from salvaged specimens, captive populations, and museum collections (Texas State University, USFWS Fish Hatchery, and TNHC, respectively). Year 1 and 2.

Task 3. Genomic DNA will be extracted and numerous markers will be screened for appropriate levels of variation and utility in phyogenetic, phylogeographic, and population genetic analyses. Year 1 and 2.

Task 4. We will conduct phylogenetic and analytical analyses, and will explicitly assess metapopulation dynamics and test specific predictions regarding extinction, colonization, and migration using gene genealogies and the published models. Year 2.

Significant Deviation: None (Dr. Chippendale will update this report with further analyses as they become available).

Summary Of Progress: Please see Attachment A.

Location: Hays County, TX

Cost: ___Costs were not available at time of this report.__

Prepared by: _Craig Farquhar_____ **Date:** _23 October 2009

Approved by: _____ _____ **Date:**___23 October 2009___
C. Craig Farquhar

ATTACHMENT A

FINAL REPORT

Population genetic analysis of the Texas blind salamander, *Eurycea rathbuni*

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Introduction

The Texas blind salamander, *Eurycea* (formerly *Typhlomolge*) *rathbuni*, created tremendous scientific interest when it was discovered in 1895 in the outflow of the newly-drilled, 58 m deep Artesian Well on what is now the campus of Texas State University in Hays County, Texas. Its formal scientific description followed the next year (Stejneger 1896). This seemingly bizarre amphibian exhibits a combination of extreme features associated with cave dwelling, including highly reduced, nonfunctional eyes, lack of most dark pigmentation, long, thin legs, and skeletal modifications that include expansion of certain cranial elements to form an extremely broad, flattened head.

Although exclusively subterranean, *E. rathbuni* was the first of the central Texas *Eurycea* identified, despite the widespread occurrence of members of the group in springs and caves associated with the Edwards Aquifer. This recharging aquatic system results from erosion of the uplifted Cretaceous karst limestones of the Edwards Plateau, providing "islands" of aquatic habitat in a terrestrial environment generally inhospitable to these salamanders. Nearly all members of the group, including *E. rathbuni*, are paedomorphic, retaining juvenile features such as external gills and structure of the hyobranchial (feeding) apparatus and remaining aquatic throughout their lives. Thus, they are entirely dependent on quality and presumably quantity of water in the Aquifer and act as broader indicators of environmental health. *Eurycea rathbuni* was one of the first species to receive Federal protection under the Endangered Species Act in recognition of the potentially precarious nature of its existence. Until recently, *E. rathbuni* was thought to be limited to the San Marcos Pool of the Edwards Aquifer underlying the city of San Marcos, Hays County, but here we present evidence that its distribution may be more extensive than previously thought.

Status of the genus *Typhlomolge* long was controversial (e.g., Mitchell and Reddell, 1965; Mitchell and Smith, 1972; Potter and Sweet, 1981), but molecular studies by Chippindale et. al. (2000; further reviewed by Chippindale 2000) clearly established that these salamanders represent the sister group to Edwards Plateau *Eurycea* from south of the Colorado River, which bisects the Aquifer and forms an apparently impenetrable barrier to gene flow. *Eurycea* north of the Colorado River are highly divergent from and sister to the southern group; molecular-based dating indicates that the split between the southern and northern groups occurred approximately 15 MYA (Chippindale et al. 2004,

Wiens et al. 2006). By extension, divergence of "*Typhlomolge*" from other members of the southern group probably occurred roughly 8-9 MYA. Thus, retention of the genus *Typhlomolge* under the Linnaean system of classification would place a genus within a genus, and Chippindale et al. followed Mitchell and Smith (1972) in assigning *T. rathbuni* (and close relatives; see below) to *Eurycea*. However, Hillis et al. (2001) employed the Phylocode (an alternate classification system in which phylogenetic clades are recognized and named without hierarchical ranking) to retain the clade name *Typhlomolge*. Thus, depending on one's philosophy, it is not incorrect to use the name *T. rathbuni* for the Texas Blind Salamander, although use of the Linnaean-based *E. rathbuni* is much more prevalent.

Two other species currently are recognized within the subgroup that includes *E. rathbuni* (hereafter I will refer to this as the *Typhlomolge* clade for convenience, using Phylocode terminology, but will use the genus name *Eurycea*). *Eurycea robusta*, the Blanco Blind Salamander, is represented by the single remaining specimen of several collected in 1951, in the outflows of a hole drilled in the bed of the then-dry Blanco River adjacent to San Marcos, Hays County and very close to the known range of *E. rathbuni*. This is a very large, heavy-bodied salamander first described in a Master's thesis (Potter 1963; description invalid) and later redescribed by Potter and Sweet (1981). Morphologically it is very distinctive, but the range of variation is unknown and due to means of preservation of the specimen no molecular data are available. Especially given the information we provide in this report regarding the distribution of *T. rathbuni* and the possibility of additional species in the clade, obtaining additional specimens should be a high priority.

Hillis et al. (2001) described the Austin Blind Salamander (*Eurycea waterlooensis*) from the Barton Springs segment of the Edwards Aquifer underlying Austin, Travis County. Prior to this, only the Barton Springs Salamander (*Eurycea sosorum*) was known from this Aquifer subregion (Chippindale 1993, Chippindale 2000, Chippindale et al. 2000). *Eurycea waterlooensis* and *E. sosorum* appear to represent a subterranean/surface-dwelling species pair, as do *E. rathbuni* and *E. nana* at San Marcos Springs and potentially *E. robusta* and *E. pterophila* in the portion of the Aquifer associated with the Blanco River drainage. Hillis et al. noted that levels of mitochondrial sequence divergence among members of the surface-dwelling clade, and those between *E. rathbuni* and *E. waterlooensis* (data unavailable for *E. robusta*) are very similar, suggesting that the same geological phenomena led to speciation within both clades.

Further details of life history, distribution, historical background, and taxonomy of *E. rathbuni*, *E. robusta*, and *E. waterlooensis* are provided by Chippindale (2005) and are available online at http://amphibiaweb.org/search/index.html.

Barton Springs and San Marcos Springs lie on the southeastern edge of the Balcones Escarpment, the periphery of the Edwards Aquifer and the area in which water collects to form caverns and major spring outflows; the range of *E. robusta* is adjacent to that of *E. rathbuni*. Southwest of San Marcos, Comal Springs in the city of New Braunfels, Comal County and nearby Hueco Springs support large populations of predominantly surface-

dwelling members of the clade that also includes *E. nana*, *E. sosorum*, and *E. pterophila* plus additional species. The pattern of subterranean/surface species pairs was reinforced when in 2003, a juvenile specimen of a member of the *Typhlomolge* clade was found in the outflows of Comal Springs. Subsequently, several additional specimens (juvenile and adult) have been found in and near Comal Springs, in the outflows of Hueco Springs, and in a deep well that intersects this region of the Aquifer. As we show here, these may either represent an undescribed species or a major range extension for *E. rathbuni*. In either case, this has major implications for conservation efforts, watershed protection, and understanding of flow routes and gene dispersal patterns. In addition, we discuss implications of our and other recent data regarding the potential for *E. rathbuni* to range north from the San Marcos Pool of the Aquifer.

In addition, we discuss the results of our molecular analyses (involving a wide range of mitochondrial and nuclear markers) with respect to levels of genetic variation, genetic structure, potential for genetic "fingerprinting" (e.g., in captive breeding efforts or mark-recapture studies), species boundaries, and possible hybridization.

Materials and Methods

Samples for this study were obtained from various sources and encompass a broad range of quality with respect to both morphological condition and availability/quality of DNA. Figure 1 (map) illustrates sampling sites, which encompass the great majority of known localities for *E. rathbuni*, plus localities for the recently discovered members of the *Typhlomolge* clade in the New Braunfels region. For live animals, in most cases small tail clips were collected and the animals were released (this appears to cause no harm based on my experience and that of others who work with this and other salamander groups). In a few cases (live specimens to be preserved for museum deposition) liver samples were collected after euthanasia. Sampling of live animals (conducted primarily by A.G. Gluesenkamp) was severely limited by drought conditions, rendering collection at key localities (especially Rattlesnake Cave, a reliable locality at which *E. rathbuni* almost always are present) impossible or very dangerous. However, Gluesenkamp was able to obtain several samples from Rattlesnake Cave in 2006-2007, augmented by others collected by Chippindale, D.M. Hillis, and A.H. Price at this site in 1991. Additional specimens were obtained by Gluesenkamp and colleagues at Ezell's Cave, an historic locality for this species. Sessoms Creek Spring is an ephemeral spring that appeared along the banks of Sessoms Creek between Artesian Well and its confluence with the San Marcos River. A single specimen was collected by Victor Castillo in 2007 during a period of exceptionally high groundwater levels. No specimens have been observed at this locality since. There are historic accounts of this species occurring in the Wishing Well in Johnson's Well, San Marcos. The location of Johnson's Well was lost between 1917 and 2006. Johnson's Well has been re-discovered and it is less than 200 m from Primer's Well. Arrangements to sample it are currently underway. Primer's Well was inaccessible to researchers until very recently and a single specimen was collected from Primer's Well by Gluesenkamp in 2009 and included in our analyses.

The vast majority of specimens represent animals salvaged by nets placed at Diversion Spring over the course of approximately ten years by a team led by Dr. Glenn Longley at Texas State University, in conjunction with workers at the National Fish Hatchery and Technology Center in San Marcos. Diversion Spring is a pipe that releases aquifer water directly into Spring Lake, the body of water at the head of the San Marcos River fed by San Marcos Springs. A small group of animals was obtained by similar methods at the nearby Artesian Well, the original locality for the species. Many of these samples (generously provided by Longley and colleagues at Texas State University and obtained for this study by Gluesenkamp) represent salvages of dead or dying specimens, which yielded DNA ranging from good to very poor. Many additional samples were provided by workers at the Fish Hatchery, many representing animals that died in captivity. In addition to the range of conditions of the animals themselves, storage methods varied, with some animals preserved in formalin, which usually renders DNA unobtainable. Thus, often we were unable to use these samples or were limited to obtaining only a subset of molecular markers, and we used extreme caution to avoid possible cross-sample contamination. However, overall, we were successful in obtaining molecular data for large numbers of specimens and these data provide great insights into genetic diversity, gene flow, population structure, and species boundaries.

A single specimen of an unidentified *Typhlomolge* was collected from Comal Spring #3 in New Braunfels in the summer of 2003. During this same period of high spring flow, Randy Gibson (USFWS) collected juvenile specimens from driftnets placed over Comal Spring #3 and Hueco Spring, 4.5 km NW of Comal Springs. Edwards Aquifer Authority staff recorded video of two *Typhlomolge* in Mission Valley Bowling Well, am 86 m deep monitoring well 3 km W of Comal Springs. Two adult specimens were collected by AGG from a trap placed in the well in June, 2006. Randy Gibson collected a single adult from a trap placed in Panther Canyon Well (approx. 200 m w of Comal Spring #3) in August, 2009. Most of these were included in our analyses; samples from the New Braunfels region encompass a wide range of quality but we have been very successful in obtaining molecular data for several individuals. Samples of *E. waterlooensis* were provided by the City of Austin, and supplementary mitochondrial sequences were obtained from GenBank.

DNA was extracted using standard methods (Qiagen column purification). Polymerase chain reaction (PCR) amplification of DNA involved a wide range of cycling protocols and DNA polymerases, most often HotStart ExTaq (Takara-Mirus). Sequencing and microsatellite analyses were performed on an ABI 3130xL capillary sequencer following standard protocols. Details of all primer sequences and methods are available from Chippindale.

Data

We used three categories of molecular data: 1) mitochondrial DNA sequences; 2) nuclear DNA sequences; and 3) nuclear microsatellite markers.

Mitochondrial DNA:

The mitochondrial (mt) cytochrome *b* (cytb) gene is widely established as a marker in phylogenetic, phylogeographic, and population genetic studies of a huge range of eukaryotes, and has proven extremely useful in elucidating species boundaries, levels of genetic variation, and gene flow in the Texas *Eurycea* (e.g., Chippindale et al. 2000, Hillis et al. 2001, Wiens et al. 2003, Bendik and Chippindale, in prep.). Therefore, we have an excellent baseline for comparison and a wide variety of outgroup and other relevant sequences. We obtained cytb sequences for approximately 70 individuals (shown in Figs. 2 and 3, mt phylogenetic trees), typically 800+ - 1150 base-pairs (bp). In a few cases, we were only able to obtain approximately 450-600 bp of data, but this appears to have little effect on our analyses and conclusions as phylogenetic/phylogeographic signal appears to be consistent and roughly evenly distributed throughout the gene. Initially, we also examined roughly 1000+ bp of the mt cytochrome oxidase I gene (relatively distantly located on the mt chromosome from cyt b) for numerous individuals, but not surprisingly (as part of the same genetic linkage group), it revealed the same major patterns as cytb but with less variation (in contrast to its typical status as one of the fastest-evolving mt genes in amphibians; e.g., Vences et al. 2005). Therefore, we discontinued its use although it was very helpful in corroborating our cytb-based results. We also explored use of the mt control region, but this portion of the mt genome is very complex and difficult to interpret in the Texas *Eurycea* (Bendik and Chippindale, in prep) and proved unnecessary given the information available from cytb and cytochrome oxidase 1. Here we present mt results based on our extensive analyses of cytb. We will add considerably more sequences for publication (primarily those that require cleanup or independent confirmation), but there is no indication that this will have any substantial effects on our interpretations or conclusions.

Nuclear sequences:

We explored a wide array of nuclear genes using PCR primers from the literature and dozens developed by Chippindale and colleagues. Of these, the genes that yielded the most consistent results were those encoding portions of the proteins melanocortin receptor 1 (Mc1r; approximately 500 bp); ornithine decarboxylase (ODC; approximately 200-700 bp); pro-opiomelanocortin (POMC; approximately 450 bp); recombination-activating gene 1 (RAG-1, up to approximately 2000 bp); and triosephosphate isomerase (TPI; up to approximately 1000+ bp), plus introns if present. Our general approach was to sequence a representative set of individuals encompassing *E. rathbuni* (including members of the most mitochondrially divergent haplotype groups), other members of the *Typhlomolge* clade, and a representative range of species within the southern *Eurycea* group. If no or very little informative variation was found, we did not gather additional data. In some cases, amplification proved very difficult or confounding factors such as pseudogenes complicated reliability of the markers, and we put some aside as promising but not worthy of immediate follow-up. If potentially informative variation was identified (even a small number of informative sites) and amplification was straightforward, we sequenced additional individuals to characterize the extent and nature of variation for potential use in genetic fingerprinting and gene flow studies.

Microsatellites:

Microsatellites (msats) are noncoding regions of DNA that consist of strings of short repeats (e.g., ACACAC…; GTCAGTCAGTCA…). These also are referred to as STRs (short tandem repeats), especially in the human genetic literature. Such sequence regions are highly prone to mutation due to replication errors by DNA polymerase, in which repeats are gained or added, usually in multiple repeat sizes (e.g., a CTGCTGCTG… region can easily grow or shrink in increments of CTG when the polymerase "slips"). An allele of a microsatellite region represents a variant of a given length whose size is identified by gel or capillary electrophoresis. Given the hypermutability of these regions, their (usually) selectively neutral nature, and the ability to isolate loci from throughout the genome (thus allowing sampling of evolutionarily independent units) they have been widely used for very fine-scale phylogeographic and population studies. We used microsatellite-enriched genomic libraries developed for another member of the southern *Eurycea* group (*E. sosorum*) by N. Bendik (then a UTA graduate student) and colleagues at the Savannah River Ecology Lab using protocols refined by Travis Glenn and colleagues. Specifically, we focused on tri- and tetra-nucleotide repeats, which are much less prone to artifacts than mono- or dinucleotide repeats. We examined approximately 50 loci, many of which amplified via PCR using the range of primer pairs available. We were limited in part by complications in interpretation and especially inconsistency in amplification success (likely a consequence of the wide range of quality of our DNA samples). For the analyses presented here, we chose four loci of the many that we were able to amplify, representing the most reliable and readily interpretable of the array examined.

Results and Discussion

Mitochondrial differentiation and correspondence of nuclear markers:

Our survey of mitochondrial (mt) variation in *E. rathbuni* (summarized by the cytb trees, Figs. 2&3) reveals two main mt clades based on neighbor joining analysis (Fig. 2), excluding a problematic subset of the Artesian Well samples and one from Diversion Spring (see below). Only one of these clades (A) is well supported in the Bayesian analysis (Fig. 3), but clade B also forms a monophyletic group in this analysis. The level of divergence between members of these clades is substantial, typically about 2.5 - 3.0% (almost as high as the levels of cytb divergence seen among the commonest haplotypes in morphologically distinct, well-recognized species in the region such as *E. nana* and *E. sosorum* -- but see caveat below regarding these two species). With the exception of Artesian Well, Sessom Creek, and Primer's Well, the latter two of which only include one sample each, individuals from each haplotype group occur sympatrically at Rattlesnake Cave, Ezell's Cave, and presumably Diversion Spring (for Diversion Spring, "sympatry" is based upon salvage of members of both main haplotype groups from the outflow and we cannot determine with certainty their origins prior to ejection from the aquifer). This initially led us to question whether two reproductively isolated, cryptic species might be

present. However, retention of ancestral mt polymorphisms is not unusual (e.g., Weisrock et al. 2005) and this appears to be most likely based on our nuclear data, which do not indicate the presence of two distinct reproductively isolated units corresponding to the two major mt clades. Interestingly, Chippindale et al. (2000) found, based on nuclear allozyme markers, that *E. rathbuni* is one of the most genetically variable of the Texas *Eurycea*, so it would not be surprising if this extended to mt DNA and also is suggestive of large population size.

The nuclear gene sequences that we examined are relatively conserved evolutionarily. This often leads researchers to discard such markers as uninformative. In contrast, they can be highly informative: if changes are rare, then differences that are present likely represent single mutational events, as opposed to "multiple hits" (situations in which changes at a nucleotide site have occurred repeatedly, and the chances of distantly related lineages converging on a given change are high). Of the nuclear genes examined, POMC (approximately 24 individuals) possesses a "doublet" (two neighboring sites that covary) at which members of both clades possess heterozygotes and homozygotes at frequencies roughly concordant with Hardy-Weinberg equilibrium both within clades and with members of both clades pooled; RAG-1 (approximately 35 individuals) exhibits three sites within a region of approximately 2000 bp which show the same pattern, Mc1r (11 individuals) exhibits one site with similar variation in both mt clades; ODC is uninformative for the specimens of the *Typhlomolge* group examined (although informative for other subgroups of Texas *Eurycea*); and for TPI coverage is patchy, amplification and alignment are often difficult, and interpretation thus far is questionable, although this gene shows considerable promise for future studies, especially given Chippindale's previous experience with this gene in other salamanders.

The four msat loci on which we focused for the analyses presented here reveal a similar pattern with respect to the two major mt clades within *E. rathbuni* (Figures 4A-D). Sample sizes are relatively small and amplification is not completely consistent: we lack data for some individuals for all loci. However, simple frequency plots reveal no substantially different frequencies in alleles identified within either mt clade, and F_{st} values (calculated using Arlequin; Excoffier et al. 2005) are very low (maximum 0.039; locus u55; Table 1). F_{st} is a measure of structure among subpopulations within a "total" population and can range in value from 0-1, with 1 representing complete genetic isolation and 0 representing random interbreeding. The low Fsts and the consistency among presumably independent loci indicates a lack of genetic isolation between the mt clades identified and again suggests retention of ancestral mt polymorphisms. Currently we are expanding our sampling of these and other msat loci for publication (and checking via cloning the occurrence of primarily low-frequency alleles that do not conform to the general rule of differences in multiples of repeat type, perhaps due to presence of complex repeats and/or mutations in flanking sequences). However, at this stage we have no nuclear evidence of the presence of reproductively isolated entities in sympatry or close geographic proximity within *E. rathbuni*.

Status of the New Braunfels region cave dwellers:

The few samples of "new" members of the *Typhlomolge* clade from Comal Springs, Hueco Springs, Panther Canyon Well near Comal Springs, and "Bowling Alley Well" appear (with the exception of the "Hueco chunk", a salamander fragment of very poor quality and questionable identification obtained by netting spring outflows) as successively sister to one of the two mt clades (B) of *E. rathbuni* from the San Marcos region in the distance-based tree and minimally diverged (maximum uncorrected cytb divergence approximately 1.6% for the Bowling Alley Well specimens). They do not form a monophyletic group in the Bayesian tree, although their placements are not well supported in that analysis. Sampling is unavoidably minimal for these, and it is crucial that efforts to obtain additional material be intensified, both for molecular work and for morphological study. Based on the extremely limited nuclear data, these fall within the range of variation in *E. rathbuni* for the few variable sites in the conserved nuclear genes, yet exhibit msat alleles that are rare or absent in the samples of *E. rathbuni* examined. Assessment of species status would be preliminary at this stage, but whether these represent an undescribed species or a major range extension for *E. rathbuni*, either scenario is of major importance to conservation of members of the *Typhlomolge* clade in the region. Numerous additional wells in the region are accessible and comprehensive screening of the outflows of Comal and Hueco Springs appears promising.

Evidence for mt introgression:

A small subset of our samples (H1, H5, and H7 from the Artesian Well and H59A from Diversion Spring; "H" numbers represent samples from the Federal Fish Hatchery at San Marcos, for most of which collection and captive maintenance data are available) exhibit mt haplotypes that are extremely divergent from those seen in any other members of the *Typhlomolge* clade, and very closely related, but not identical to, those in members of the southern surface clade included in our analyses (Fig. 2). An additional sample from Artesian Well (H9) falls within one of the two major *E. rathbuni* mt clades. These are low-quality samples, but DNA has been extracted independently twice (and currently is being extracted for a third time). Although contamination is a possibility, it seems unlikely given repeated extractions and lack of contact of the DNA samples with members of the group that would represent the source of potential contamination. N. Bendik (City of Austin; former graduate student of Chippindale) and I (Chippindale) recently have found strong evidence of mt introgression from *E. waterlooensis* into *E. sosorum* at Barton Springs, and approximately 1/5 of *E. sosorum* possess a cytb haplotype very similar (but not identical) to the commonest cytb haplotype in "true" *E. nana* from San Marcos Springs. This raises the question, does hybridization occur among even distantly related members of the Texas *Eurycea* group? Introgression of mt DNA in salamanders (and many other eukaryotes) is not uncommon and can occur in the absence of "dilution" of the nuclear genome (e.g., Weisrock et al. 2005). Past or occasional introgression can lead to the presence of rare haplotypes representing mt variants of the introgressing species or, with substantial time elapsed since introgression, varying degrees of divergence of the introgressed haplotype. Further study involving

additional fast-evolving nuclear markers (which we are pursuing) should help to clarify whether there has been past hybridization between *E. nana* (or another species) and *E. rathbuni*, and to what extent this may have affected the evolutionary histories of these species.

Mitochondrial introgression, gene flow, and the geographic range of *Eurycea rathbuni*:

Above, we have presented tentative evidence (obviously in need of further testing) of mt introgression from a member of the southern (south of Colorado River) surface Texas *Eurycea* clade into *E. rathbuni*, reinforced the potential for this using well-supported evidence of mt introgression in the *E. waterlooensis*/*E. sosorum* subterranean/surface species pair at Barton Springs (time since common ancestor approximately 8-9 MYA), and introduced as-yet unpublished evidence gathered by Chippindale and N. Bendik (and supported independently by analyses by D. Hillis and colleagues at UT Austin; pers. comm.) that *E. nana* - like mt haplotypes are present at relatively high frequency in *E. sosorum* at Barton Springs. The latter suggests the possibility of some form of gene flow from salamanders in the San Marcos Pool of the Edwards Aquifer north to Barton Springs. This is consistent with the hypothesis of an intermittent hydrologic connection between the Barton Springs segment of the aquifer and the San Marcos pool, as suggested by Slade et al. (1986). A final, and potentially telling, piece of evidence from our study has implications for gene flow from *E. rathbuni* not only southward to the New Braunfels region but perhaps even northward to Barton Springs. One of the four samples of *E. waterlooensis* (Barton Springs *Typhlomolge* clade) included in this study exhibits a mt cytb haplotype nearly identical to those of one of the two major mt haplotype clades within *E. rathbuni* (based on multiple amplifications and sequencing of a very high-quality DNA sample). Given the substantial mt divergence between most (= 3 of 4) *E. waterlooensis* and the dozens of *E. rathbuni* examined here, and their morphological differentiation, it is suggestive that a haplotype barely diverged from that of *E. rathbuni* would be present in *E. waterlooensis*, barring strong selection on an ancestral haplotype. We expect to receive a larger sample of *E. waterlooensis* in the near future and will examine this issue further, independently of the current study. Existing evidence suggests that there may be more gene exchange among species of central Texas *Eurycea* than previously suspected. This does not mean that we are dealing with a single species, or a "continuum" -- the integrity of most recognized species appears to be well supported, and hybridization among species is common in nature. But the potential for identification of past and ongoing hybridization events provides the opportunity for numerous insights into biotic exchange and hydrogeology of the Edwards Aquifer, and conservation prioritization and management of this complex and critical region.

Direct applications of data obtained in this study to species management:

The array of mt and nuclear markers that we have developed provides a set of tools from multiple parts of the nuclear and organellar genomes to genetically fingerprint individuals, and potentially could be central to captive breeding, parentage, and mark-recapture studies. This has important implications for future management, especially in

the event of catastrophic changes in aquifer conditions. Based on the data now available, a readily applicable and comprehensive set of genetic tests could readily be designed to maximize monitoring, representation, preservation, and propagation of diversity.

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Figure 1. Sampled localities for members of the "*Typhlomolge*" blind salamander clade. 1 = *Eurycea waterlooensis* (Barton Springs, City of Austin, Travis County); 2 - 6 = *E. rathbuni* (City of San Marcos, Hays County; 2: Rattlesnake Cave, 3: Diversion Spring [Aquarena Springs], 4: Artesian Well and Sessom's Creek Spring, 5: Ezell's Cave, 6: Primer's Well); 7-10 = new localities for species of uncertain status (7: Hueco Springs, 8: Mission Valley Bowling Alley Well, 9: Comal Spring #3, 10: Panther Canyon Well).

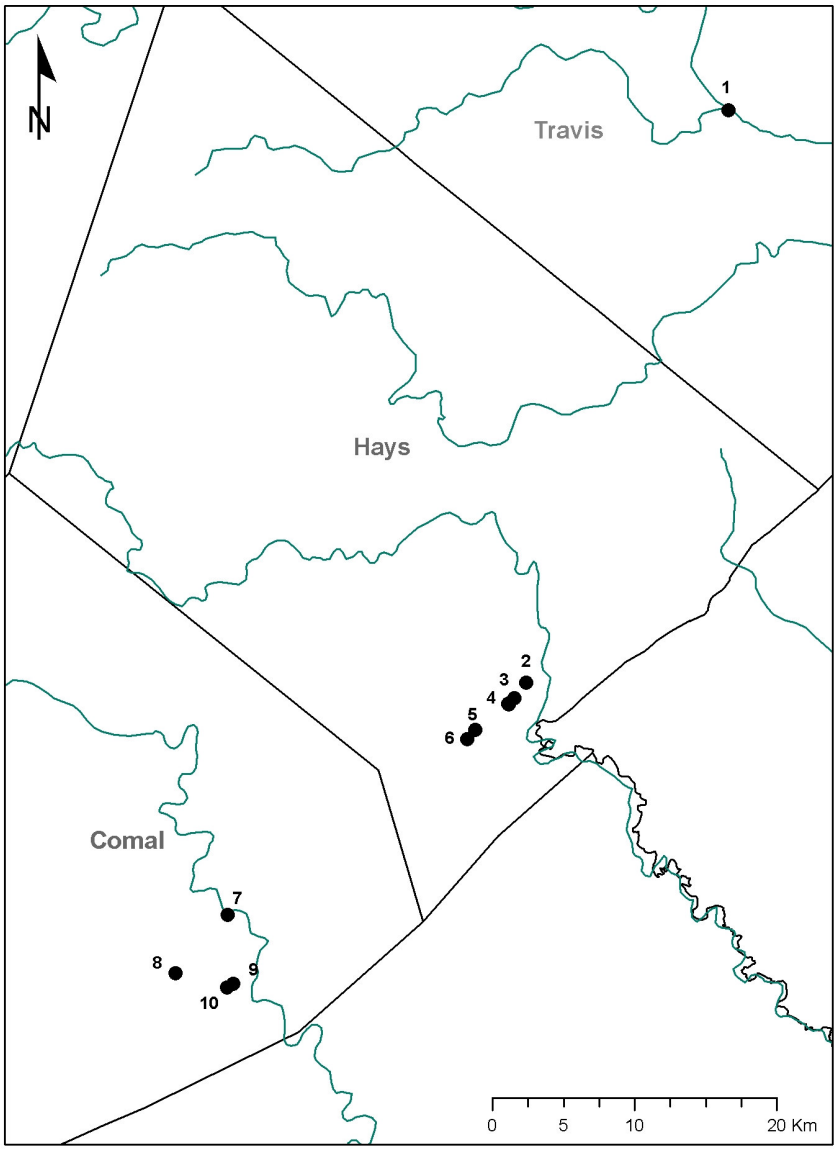


Figure 2. Neighbor-joining phylogram based on cytochrome b sequences and Kimura 3-parameter distances. All samples represent *Eurycea rathbuni* except where shown or noted otherwise. H = Fish Hatchery samples and TS = Texas State University samples. Samples indicated by asterisks represent subterranean specimens from the New Braunfels area, with "Hueco chunk" left as of questionable identification. Locality abbreviations: DS = Diversion Spring, EC = Ezell's Cave, and RC = Rattlesnake Cave.

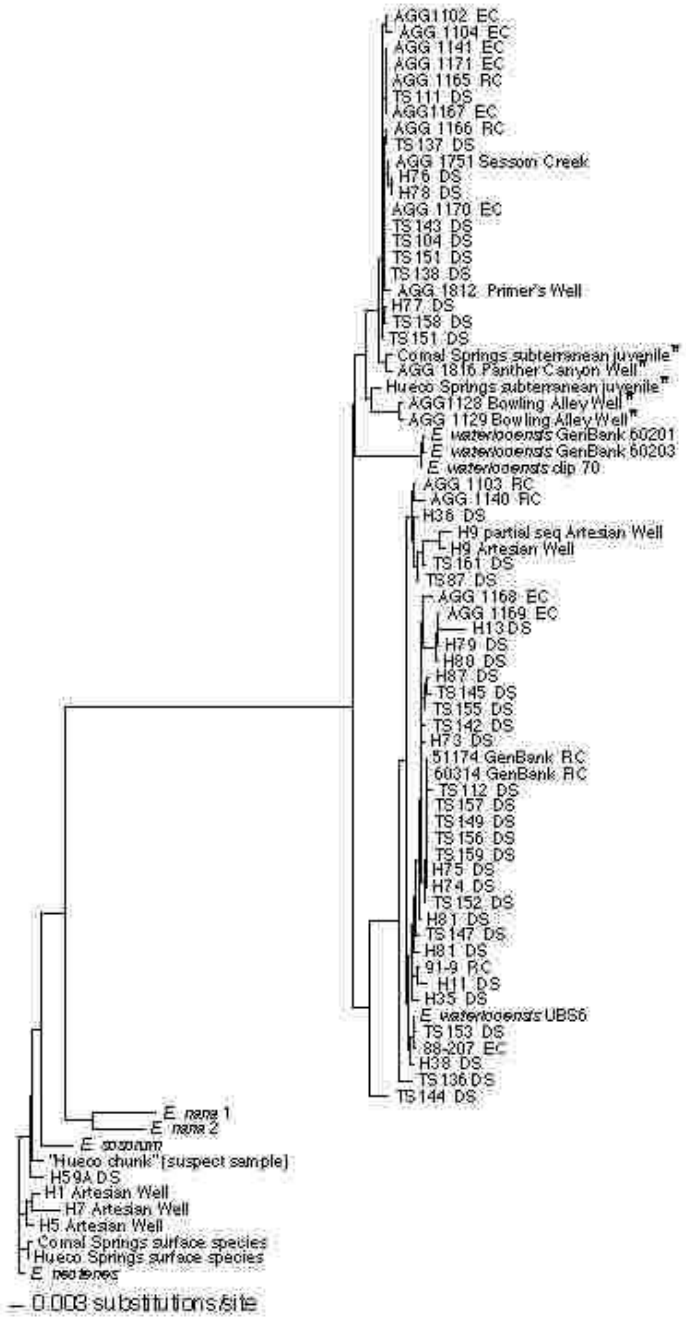
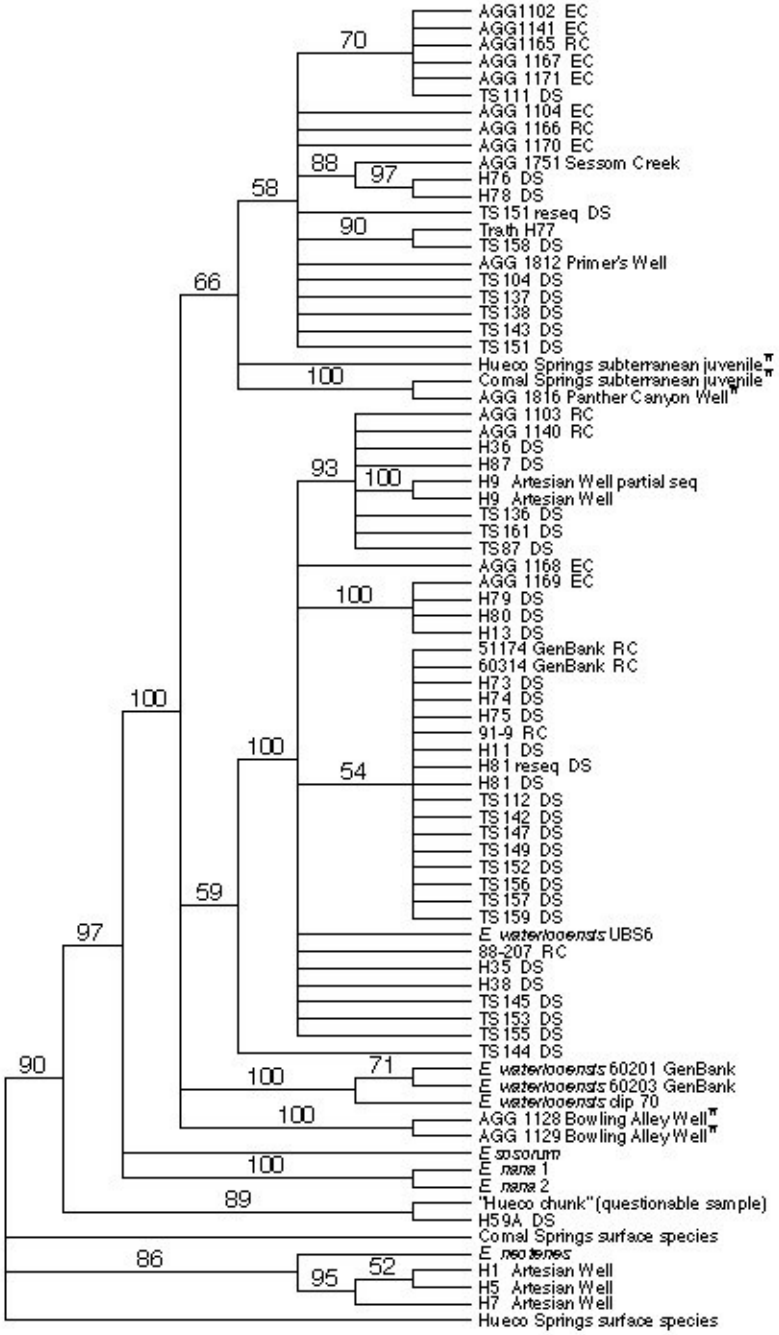
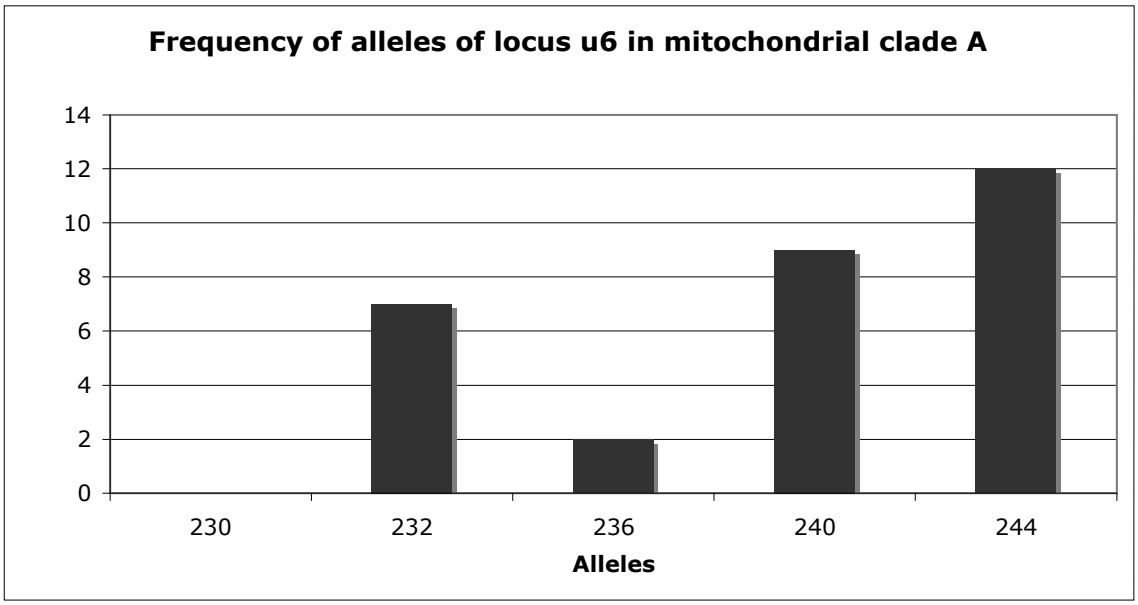


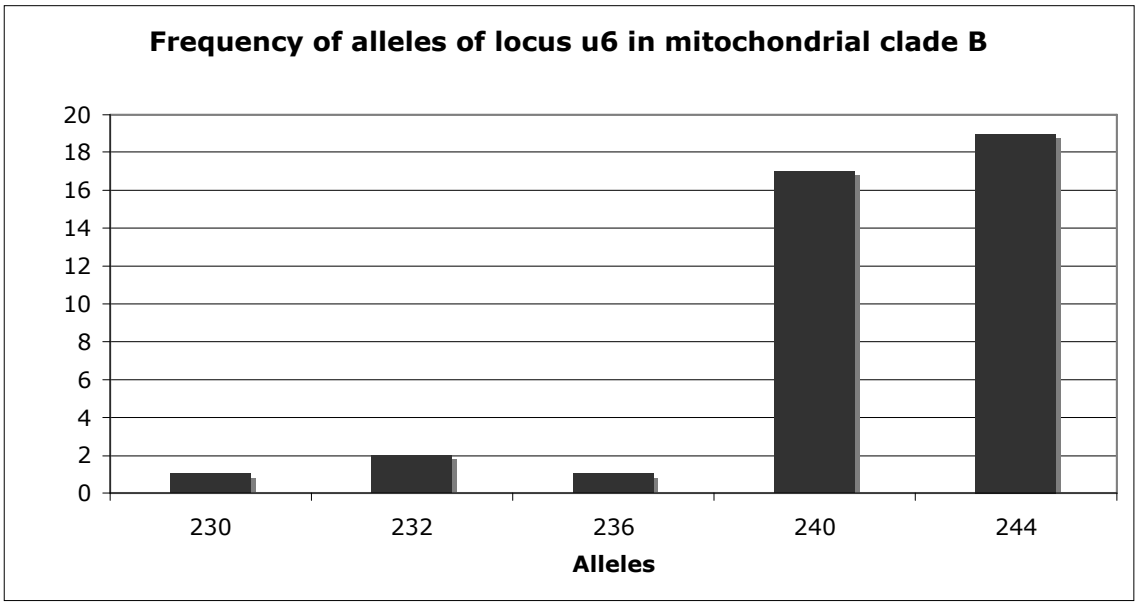
Figure 3. Cladogram based on cytochrome b sequences and Bayesian analysis (two million generations; summary of two independent analyses). Numbers at nodes are posterior probabilities (probability of correctness of relationship). All samples represent *Eurycea rathbuni* except where shown or noted otherwise. H = Fish Hatchery samples and TS = Texas State University samples. Samples indicated by asterisks represent subterranean specimens from the New Braunfels area, with "Hueco chunk" left as of questionable identification. Locality abbreviations: DS = Diversion Spring, EC = Ezell's Cave, and RC = Rattlesnake Cave.



Figures 4A-D. Frequencies of alleles at the four focal microsatellite loci in *E. rathbuni*. Each subfigure illustrates allele frequencies for members of the two mitochondrial clades identified by neighbor-joining analysis, followed by a table of allelic composition by individual. Individuals indicated by * represent samples from new localities in the New Braunfels region. All other individuals are considered *E. rathbuni*, where TS indicates Texas State University samples, H indicates Fish Hatchery samples, RC represents Rattlesnake Cave, EC represents Ezell's Cave, and DS represents Diversion Spring.

Fig. 4A. Locus u6:

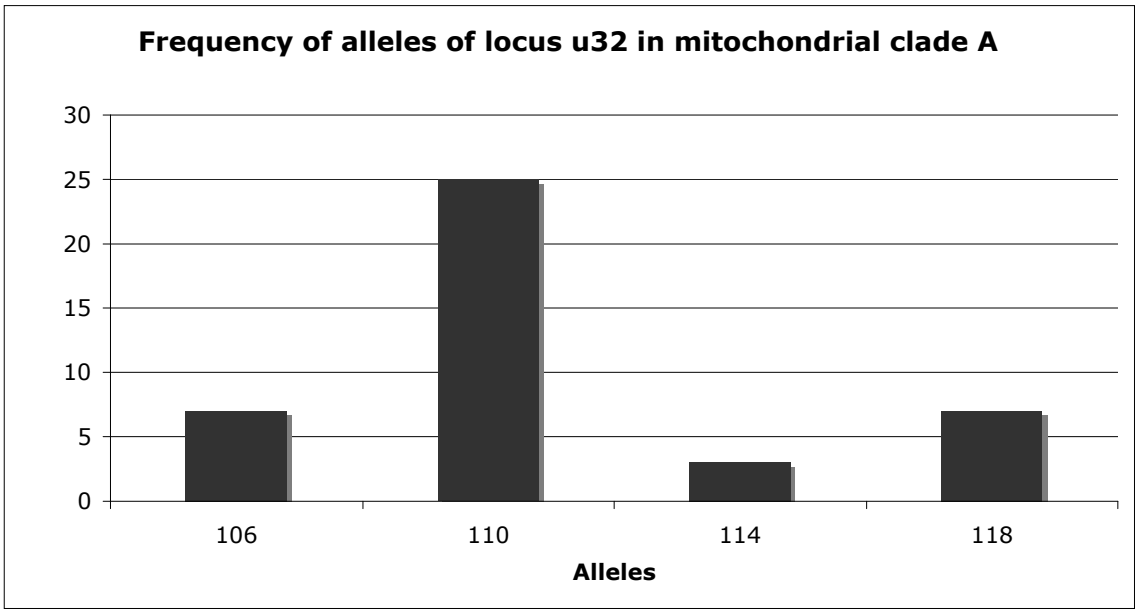


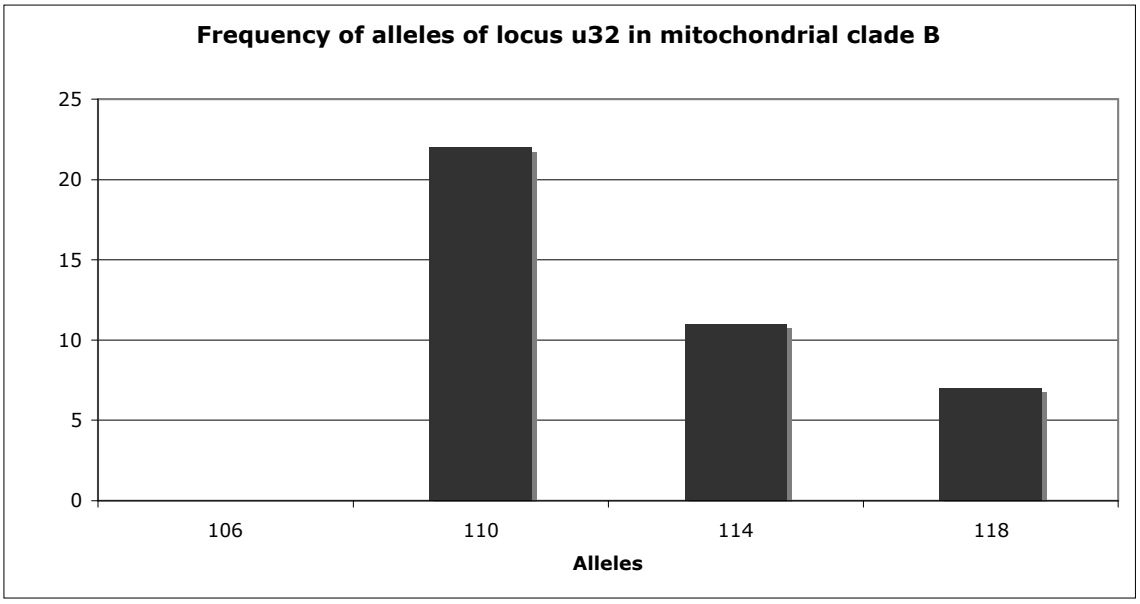


Locus u6

Sample	Genotype	Locality	Group
H79	244	DS	A
H75	232, 236	DS	A
H80	232	DS	A
TS145	232, 244	DS	A
TS147	236, 244	DS	A
AGG 1103	240, 244	RC	A
AGG 1140	240, 244	RC	A
H81	232, 240	DS	A
Artesian Well H9	240	AW	A
TS152	244	DS	A
TS156	240	DS	A
TS136	244	DS	A
TS142	232, 240	DS	A
TS145	232, 244	DS	A
TS152	244	DS	A
TS157	232, 244	DS	A
TS159	240, 244	DS	A
AGG 1166	240	RC	B
Sessom Creek AGG1751	240	SC	B
TS151	244	DS	B
H76	230, 244	DS	B
TS143	240, 244	DS	B
TS158	240, 244	DS	B
AGG 1102	244	EC	B
AGG 1104	240	EC	B
AGG 1141	240, 244	EC	B
AGG 1165	240	RC	B
AGG 1166	240	RC	B
AGG 1167	244	EC	B
AGG 1171	244	EC	B
H77	236, 240	DS	B
H87	240, 244	DS	B
TS104	232, 244	DS	B
TS111	232, 240	DS	B
UTA58512 (H78)	244	DS	B
TS137	240	DS	B
TS138	240, 244	DS	B
Primer's Well AGG 1812	240, 244	PW	B
Bowling Alley Well AGG 1128*	240, 248	BAW	C
Bowling Alley Well AGG 1129*	240, 248	BAW	C
Panther Canyon Well AGG 1816*	236	PC	C
TS146	232	DS	?
H72	244	DS	?
TS160	256	DS	?
E. waterlooensis UBS6	240, 244		W
E. waterlooensis clip70	240		W

Fig. 4B. Locus u32:

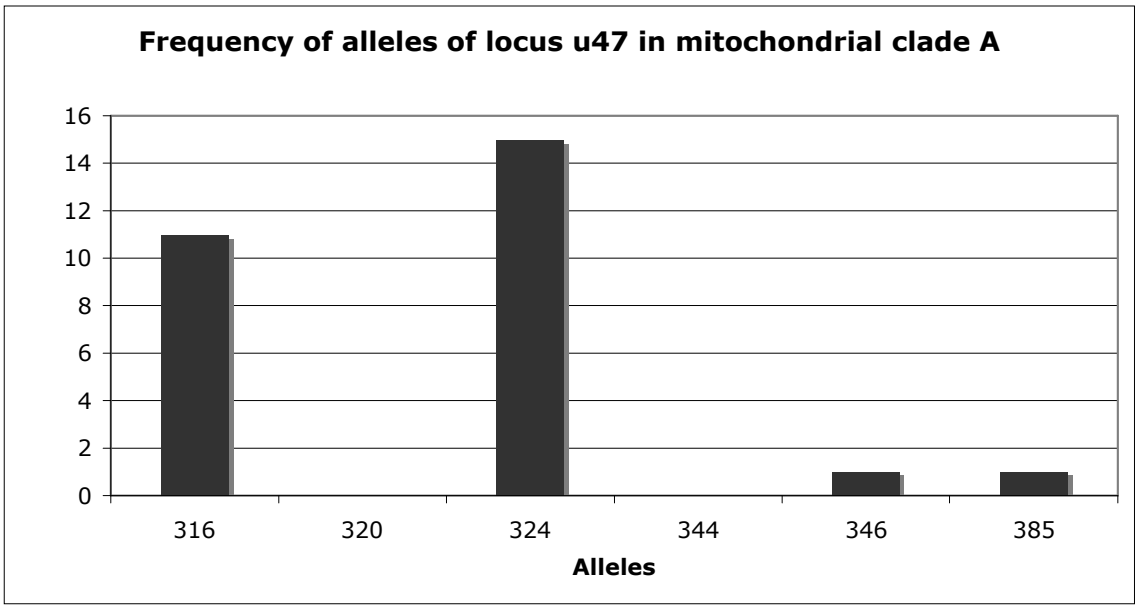


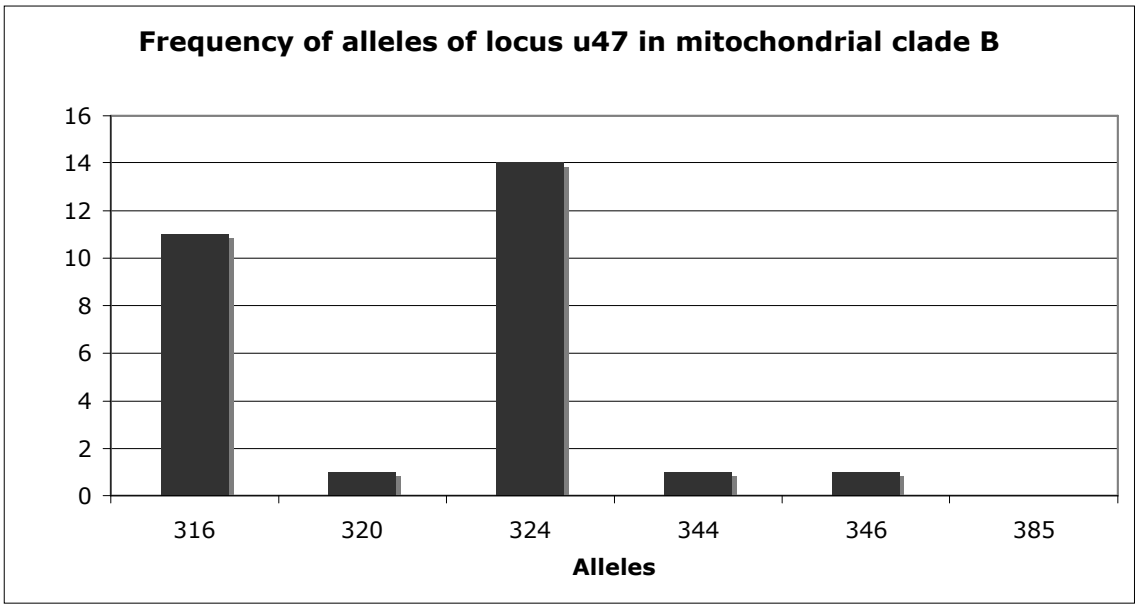


Locus u32

Sample	Genotype	Locality	Group
AGG 1103	110	RC	A
AGG 1140	118	RC	A
AGG 1169	106, 114	EC	A
H73	110, 118	DS	A
H74	110, 114	DS	A
H75	110	DS	A
H79	110	DS	A
H80	110	DS	A
H81	118	DS	A
H87	106	DS	A
H9	106, 114	AW	A
TS112	106, 110	DS	A
TS136	106, 110	DS	A
TS142	110	DS	A
TS145	110	DS	A
TS147	118	DS	A
TS149	110	DS	A
TS152	110	DS	A
TS156	110	DS	A
TS157	110	DS	A
TS159	106, 110	DS	A
AGG 1102	114	EC	B
AGG 1104	114	EC	B
AGG 1141	110, 114	EC	B
AGG 1165	114	RC	B
AGG 1166	118	RC	B
AGG 1167	114, 118	EC	B
AGG 1170	110	EC	B
AGG 1171	110, 114	EC	B
Sessom Creek AGG 1751	110	SC	B
H76	110	DS	B
TS104	110, 118	DS	B
TS111	110	DS	B
TS137	110	DS	B
TS138	110	DS	B
TS143	110, 118	DS	B
TS151	110	DS	B
TS158	110	DS	B
UTA58512 (H78)	110	DS	B
H77	118	DS	B
Primer's Well AGG 1812	114	PW	B
Bowling Alley Well AGG 1128*	114, 139	BAW	C
Bowling Alley Well AGG 1129*	139	BAW	C
Hueco Chunk (* questionable)	139	HC	C
Hueco subterranean juvenile*	110, 114	HT	C
Panther Canyon Well AGG 1816*	110, 114	PC	C
H2	110	AW	?
H67	135	DS	?
H72	118	DS	?
TS100	110	DS	?
TS141	106, 110	DS	?
TS160	110	DS	?
TS84	110	DS	?
TS85	110, 123	DS	?
TS86	110	DS	?
TS90	106	DS	?
TS92	110	DS	?
TS97	110	DS	?
TS98	106	DS	?
TS99	120	DS	?
E. waterlooensis UBS6	110, 123		W
E. waterlooensis clip70	123, 127		W

Fig. 4C. Locus u47:

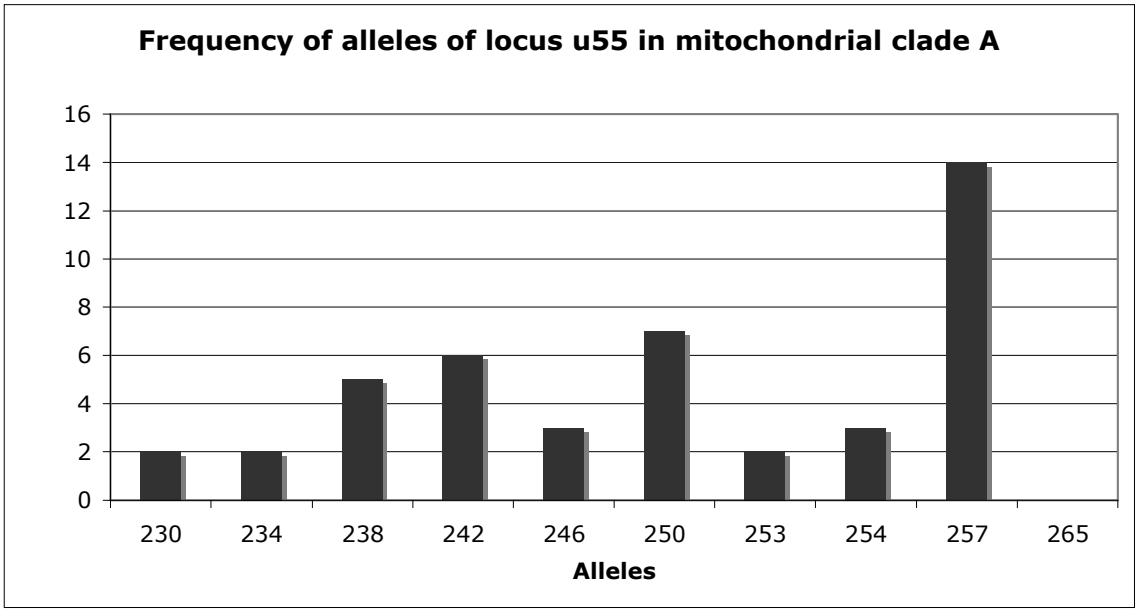


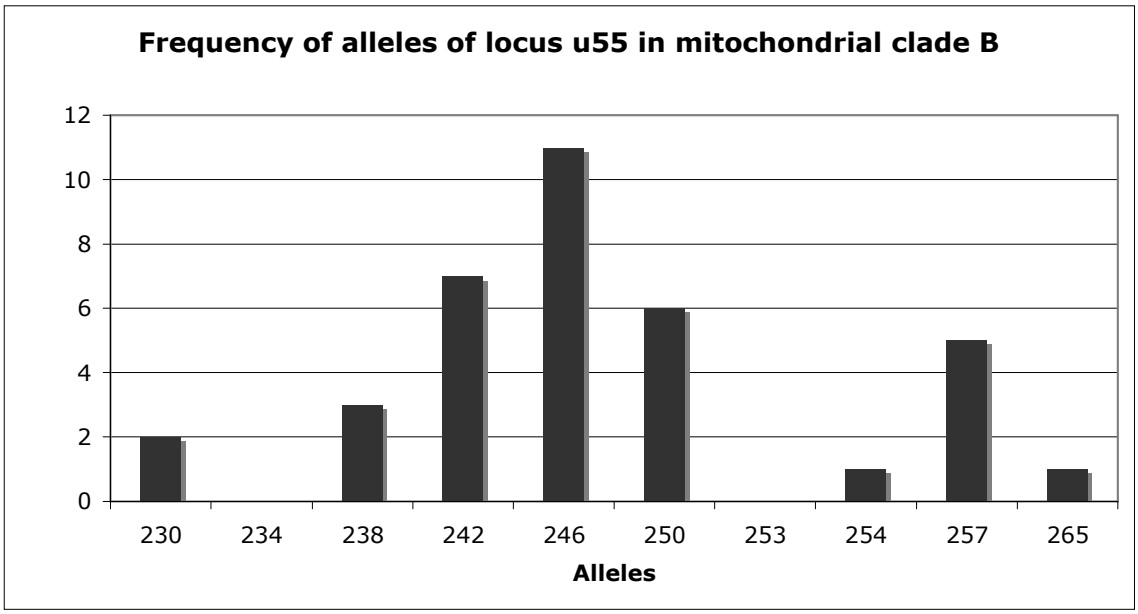


Locus u47

Sample	Genotype	Locality	Group
AGG 1169	316, 324	EC	A
AGG 1103	324	RC	A
AGG 1140	324	RC	A
H74	324	DS	A
H75	324	DS	A
H76	316, 324	DS	A
H77	316, 324	DS	A
H80	324	DS	A
H81	324, 385	DS	A
H87	316, 324	DS	A
Artesian Well H9	316	AW	A
TS112	316, 346	DS	A
TS145	316	DS	A
TS87	316	DS	A
AGG 1102	324, 344	EC	B
AGG 1104	324	RC	B
AGG 1141	324, 346	EC	B
AGG 1165	316, 324	RC	B
AGG 1166	324, 316	RC	B
AGG 1170	316, 324	EC	B
AGG 1171	316	EC	B
TS104	316, 324	DS	B
TS111	316, 324	DS	B
TS137	316, 324	DS	B
TS143	324	DS	B
TS147	324	DS	B
TS151	316, 320	DS	B
TS158	316	DS	B
E. waterlooensis UBS6	337		W
E. waterlooensis clip70	337, 360		W
TS100	324	DS	C
TS146	316, 324	DS	C
TS92	316	DS	C

Fig. 4D. Locus u55:





Locus u55

Sample	Genotype	Locality	Group
AGG1103	238, 253	RC	A
AGG1140	238, 242	RC	A
AGG1169	254	EC	A
H73	250	DS	A
H74	234, 254	DS	A
H75	257	DS	A
H79	230, 242	DS	A
H80	250, 257	DS	A
H81	242, 257	DS	A
Artesian Well H9	250, 257	AW	A
TS112	238, 257	DS	A
TS136	246, 250	DS	A
TS142	246, 250	DS	A
TS145	230, 257	DS	A
TS147	257	DS	A
TS149	238, 242	DS	A
TS152	234, 257	DS	A
TS155	257	DS	A
TS157	246, 257	DS	A
TS159	253, 257	DS	A
TS87	238, 242	DS	A
H87	242, 250	DS	A
AGG1102	242	EC	B
AGG1104	246, 250	EC	B
AGG1141	242, 246	EC	B
AGG1165	238, 242	RC	B
AGG1166	230, 250	RC	B
AGG1167	250, 257	EC	B
AGG1170	246	EC	B
AGG1171	242, 265	EC	B
AGG1751	257	SC	B
H76	230, 246	DS	B
H77	242, 246	DS	B
TS104	246, 257	DS	B
TS111	242, 246	DS	B
TS137	246, 250	DS	B
TS138	246, 250	DS	B
TS151	238, 254	DS	B
TS158	238, 250	DS	B
Primer's Well AGG 1812	246, 257	PW	B
BowlingAlley Well AGG 1128*	246, 250	BAW	C
Hueco Chunk (*questionable)	250	HS	C
Hueco subterranean juvenile*	250, 260	HS	C
PantherCanyonWellAGG1816*	246	PC	C
Artesian Well H1	238	AW	?
Artesian Well H2	246, 250	AW	?
H57	238	DS	?
H59	246, 250	DS	?
H64	234, 242	DS	?
H72	238, 246	DS	?
H8	238	AW	?
TS100	238, 242	DS	?
TS141	246, 250	DS	?
TS146	246, 250	DS	?
TS84	238, 242	DS	?
TS85	238, 242	DS	?
TS89	238, 242	DS	?
TS90	238, 242	DS	?
TS92	246, 250	DS	?
TS97	246, 250	DS	?
TS98	246, 250	DS	?
TS99	246, 250	DS	?
E. waterlooensis UBS6	230, 238		W
E. waterlooensis clip70	257, 264		W

Table 1. Summary F statistics for loci u6, u32, u47, and u55 for mitochondrial clades A and B.

Fst:

Locus	**Fst**
u6	0.012
u32	0.026
u47	0.017
u55	0.039