# Interim reports: Genetic analyses of the Texas blind salamander (*Eurycea rathbuni*) and Jollyville Plateau salamander (*Eurycea tonkawae*)

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## Introduction

Here I present very brief interim summaries of two separately funded Section 6 projects, each addressing population structure and genetic variation in Texas cave and spring salamanders of the genus *Eurycea*. I combine the two because the methods used are similar, each study has been very successful to date, and each reveals a surprising result suggesting that earlier delineations of species boundaries in these taxa may need to be reassessed. In each case, conclusions are very preliminary; together with my colleague on these projects, Dr. Andrew Gluesenkamp, and my graduate student assistants, we are adding many more samples and data encompassing a wide range of mitochondrial and nuclear markers. Here we include only results based on data that are complete or near complete and have been thoroughly checked. These represent only a small fraction of the data that will be available at the conclusions of these projects.

*Eurycea rathbuni*: The goals of the study of *Eurycea* (formerly *Typhlomolge*) *rathbuni* are to determine levels of genetic variation, genetic cohesiveness, and population size of this enigmatic blind salamander. Since its discovery in 1896, *E. rathbuni* has been the subject of intensive interest, yet very little is known of population structure or life history despite its decades long listing as a Federally Endangered Species. Gluesenkamp is conducting a separate mark-recapture study of this species that will complement the work described here; results of that study will be described elsewhere.

Understanding of the distribution and status of *E. rathbuni* has been complicated by its subterranean occurrence in the San Marcos Pool of the Edwards Aquifer, the existence of a second recognized species in the group (*E. robusta*, represented by a single specimen collected in 1951 very close to the known distribution of *E. rathbuni*), the recent discovery of the closely related but clearly distinct Austin blind salamander (*E. waterlooensis*) in the Barton Springs segment of the Aquifer, and the even more recent discovery of a putatively new species of blind salamander associated with the subterranean system that feeds Comal Springs in New Braunfels, Comal County. No substantial molecular studies of *E. rathbuni* have been conducted, although I demonstrated in my dissertation work and subsequent publications (e.g., Chippindale, Price, Wiens, and Hillis 2000, Herpetological Monographs) that based on allozyme and limited mitochondrial data, it clearly is nested within the Texas *Eurycea* group as sister to all other species that occur south of the Colorado River. This was confirmed by further studies (Hillis, Chamberlain, Wilcox, and Chippindale 2001, Herpetologica) that showed

the newly described species *E. waterlooensis* to be sister to *E. rathbuni* (molecular data for *E. robusta* unobtainable), with the placement of these species among the Texas *Eurycea* the same as previously inferred. Results presented here and work in progress reveal high levels of genetic variation within *E. rathbuni* and the possibility that this species may exhibit substantial subdivision and possibly even a wider geographic range than previously suspected.

*Eurycea tonkawae*: The Jollyville Plateau salamander (*E. tonkawae*) was described by Chippindale, Price, Wiens and Hillis in 2000 (Herpetological Monographs) as one of three species from the aquifer regions north of the Colorado River. Although once regarded as members of the "southern" species *E. neotenes*, the "northern" group is extremely divergent based on multiple molecular data sets, sharing a most recent common ancestor with the southern species approximately 15 million years ago (Chippindale, Bonett, Baldwin, and Wiens 2004, Evolution; Wiens, Engstrom, and Chippindale 2006, Evolution). Chippindale et al. (2000) suggested that there might be additional species in the northern region, especially given the occurrence of morphologically divergent subterranean populations in the Buttercup Creek drainage of southern Williamson County. Currently, *E. tonkawae* is the focus of great interest given its occurrence in areas of extensive human impact in Travis and Williamson Counties and the near-complete lack of knowledge of its genetic structure. Here we present evidence of a major split within this species that, rather than reflecting cave vs. surface habitat, appears to correspond to major geologic and topographic features of the region.

## Methods

For each species (and relevant outgroup members) DNA was obtained from dozens of samples using standard methods. In the case of *E. rathbuni*, most samples represent animals that died in captivity at the Federal fish hatchery in San Marcos and although localities of capture are known, dates of capture are uncertain in most cases. Additional samples were obtained by Gluesenkamp and colleagues from Rattlesnake and Ezell's Caves; in addition, several samples obtained from Rattlesnake Cave by Chippindale and colleagues in the early 1990s are being studied. The range of quality of DNA for *E. rathbuni* is tremendous, from excellent for several dozen specimens to unusable for some that were dead for extended periods prior to preservation or were preserved in formalin, which renders DNA extraction extremely difficult or impossible. Many samples have proven problematic for molecular studies and have required extensive precautions and repeated PCR amplification to guard against contamination. Hundreds of samples of *E. tonkawae* from over two dozen localities encompassing most of the known range of the species are available, primarily collected by Gluesenkamp and colleagues, Nathan Bendik (City of Austin), and Chippindale and colleagues over the course of almost 20 years. DNA quality for these generally is very high, and samples from many additional localities will be analyzed.

Briefly, numerous individuals of both species and close relatives have been examined for several mitochondrial DNA (mtDNA) regions. The mt genome represents a single

genetic linkage group, and as expected, conclusions based on different portions of the mt genome yield the same major results with respect to relationships and diversification. Thus, here we focus on the mt cytochrome b gene (1+ kilobases, kb), which has proven most tractable and informative at a variety of scales and is representative of the mt genome as a whole. However, for *E. tonkawae* in particular, the mt gene cytochrome oxidase 1 is sufficiently informative (and substantially augments phylogenetic/phylogeographic reconstruction based on cytochrome b) that we are continuing to obtain sequence data for nearly all samples. For *E. rathbuni*, there is such close agreement between cytochrome b and cytochrome oxidase 1 that our efforts are better directed toward exploring nuclear markers. In other words, there is no disagreement with respect to relationships among mt genes in either species; cytochrome b is sufficient to answer our questions (mitochondrially) in *E. rathbuni*, but addition of variable sequence sites in cytochrome oxidase 1 for the more recent radiation in what currently is considered *E. tonkawae* enhances our phylogenetic/phylogeographic reconstructions and understanding of gene flow.

In addition, currently we are screening individuals of both species for variation at several nuclear protein-coding loci and their introns (where applicable). These include portions of the recombination-activating gene (RAG-1), three exons and two introns of the ornithine decarboxylase gene (ODC), several exons and introns of the triosephosphate isomerase gene (TPI), an exon of the pro-opiomelanocortin gene (POMC), three exons and two introns of the rhodopsin gene, and the single-exon melanocortin receptor 1 gene. Results of these studies are very preliminary, but encouraging for some of these nuclear genomic regions. However, RAG-1 and POMC are very conserved evolutionarily and show almost no variation in *E. tonkawae*, although both genes have proven informative in other species and subgroups of Texas *Eurycea*; thus, we will not pursue these further for the studies of *E. tonkawae* described here, or POMC for *E. rathbuni*. Although extremely conserved, RAG-1 does provide information on differentiation in the taxon currently considered *E. rathbuni* (substantial variation occurs at third codon positions given enough sequence data), and we will obtain data for all possible individuals for this species. I will elaborate on the results of analyses of nuclear sequence data in subsequent reports.

The most informative results for very fine-scale study are being obtained from nuclear microsatellite (msat) loci, extremely fast-evolving regions consisting of varying numbers of adjacent tri- or tetranucleotide repeats (we have avoided the artifact-prone mono- and dinucleotide repeats although generally they are much more abundant in vertebrate genomes). Based on our screening so far, approximately 10-12 loci of about 60 examined are variable and interpretable for *E. rathbuni*, and we are in the midst of a comprehensive screening of this species. Several loci for which we have developed msat PCR primers for "southern" species amplify with varying degrees of success and interpretability in *E. tonkawae*, but given the level of phylogenetic divergence of this species, we are developing an msat "genomic library" specific to *E. tonkawae* to provide additional markers. This is a relatively straightforward though time-consuming process, and we expect that the results will be very informative.

Preliminary results and discussion

*Eurycea rathbuni*: The preliminary phylogenetic tree for *E. rathbuni* based on cytochrome b sequences reveals a high level of mt variation (interestingly, my early work with allozymes in this species revealed very high levels of protein-level variation relative to those in nearly all other species of Texas *Eurycea*, completely consistent with these results). One possible interpretation is that population size is very large, although this requires much more rigorous analysis. Furthermore, there are two very distinct and well-supported mt haplotype clades (Fig. 1). The uncorrected sequence divergences between haplotypes in the two clades range from roughly 2.8 to 3.1%, and intraclade divergences peak at about 0.6% within the more variable of the two clades and about 0.4% in the less variable clade (see discussion of sequence divergence and species boundaries below). *Eurycea waterlooensis* appears in the very preliminary tree as distinct from but sister to the less variable of the two haplotype clades (although there is no substantial bootstrap support for this placement), and the levels of divergence between members of the two clades within *E. rathbuni* are in the same range as the divergence of *E. waterlooensis* from the clade to which it appears sister. There is no apparent geographic pattern to the distribution of haplotypes in *E. rathbuni*: specimens from the three key sampling sites (Diversion Spring, Ezell's Cave, and Rattlesnake Cave) appear in both clades. Gluesenkamp has repeatedly attempted to gain access to two additional sites in San Marcos known to harbor *E. rathbuni*, Primer's Well and Johnson's Well, but still is awaiting landowner permission (we hope that this will be facilitated by USF&W personnel in the near future). We have very limited, degraded material from the Artesian Well at Texas State University for which we may be able to obtain data, and are adding a sample from the single known specimen from a recently discovered locality on Sessum Creek.

Although assessment of species boundaries based on mt data alone can be difficult, the mitochondrial pattern is striking and suggests the possibility that two distinct units occur within *E. rathbuni*. Alternatively, this may simply represent retention of ancestral mt polymorphisms, but even if this is the case, the haplotype diversity between and to a much lesser extent within clades is striking. It will be extremely important to compare the patterns of variation shown by nuclear data (especially msats) to those based on mtDNA. In addition (data not shown), the new blind salamander associated with the portion of the aquifer that feeds Comal Springs also appears (mitochondrially) to be sister to one of the haplotype clades within *E. rathbuni*, raising the intriguing possibility that *E. rathbuni* has a much broader geographic range than previously suspected. Like *E. waterlooensis*, it appears to be morphologically distinct from *E. rathbuni* and we anticipate that it will prove to be a distinct species. Obviously further study is necessary, and the data that we are collecting from fast-evolving nuclear loci will be critical to resolving these issues.

Although not a component of this project, sampling of the numerous accessible test wells in the region between and near San Marcos and New Braunfels is central to understanding relationships and diversity in the blind salamander group. Furthermore, the area of the type locality for the long-lost *E. robusta* is readily accessible, and drilling

of sampling wells is absolutely essential to unravel the complexity of genetic variation in the group. The technology is readily available and relatively inexpensive, and this recognized yet barely known species must be sampled in future work, especially given a previous (rejected due to insufficient information) attempt at Endangered Species listing. Increased knowledge of relationships in the blind salamander group is critical to understanding of the entire aquifer ecosystem in the region, and protection of water resources that are highly susceptible to degradation and eventual loss.

We have strong reason to question whether a species listed as Federally Endangered for 31 years is a single entity, and whether its range is as limited as previously thought. There is no question of the need for protection of the blind salamander group, unique to Texas. The key questions are: 1) How many species exist; and 2) How do we preserve the Texas blind salamanders and the water resources that support them and many other organisms, including humans?

*Eurycea tonkawae*: Analyses of cytochrome b (Fig. 2) reveal a surprising pattern, also supported by analysis of other mt sequence regions plus preliminary nuclear data. There are two major, moderately to very well supported clades: 1) Jollyville Plateau proper (primarily Bull Creek drainage) plus mid to eastern Brushy Creek drainage; and 2) A "peripheral Plateau" group associated with the northern and western edges of the Plateau (Buttercup Creek and Lake Travis drainages). The peripheral group includes all sampled populations from the Buttercup Creek Caves complex, many of which contain individuals that exhibit extreme cave-associated morphologies, plus those at Wheless, House, MacDonald Well, and Kelly Hollow Springs, and SAS Canyon Springs and Kretschmarr Cave (Kretschmarr not shown in tree). The phylogenetic placement of the latter two populations is especially striking, as their geographic location is very close to that of members of the Plateau group (e.g., WTP4 Spring). There, Highway 620 appears to mark the divide between the two groups, and the "edge" of the Plateau.

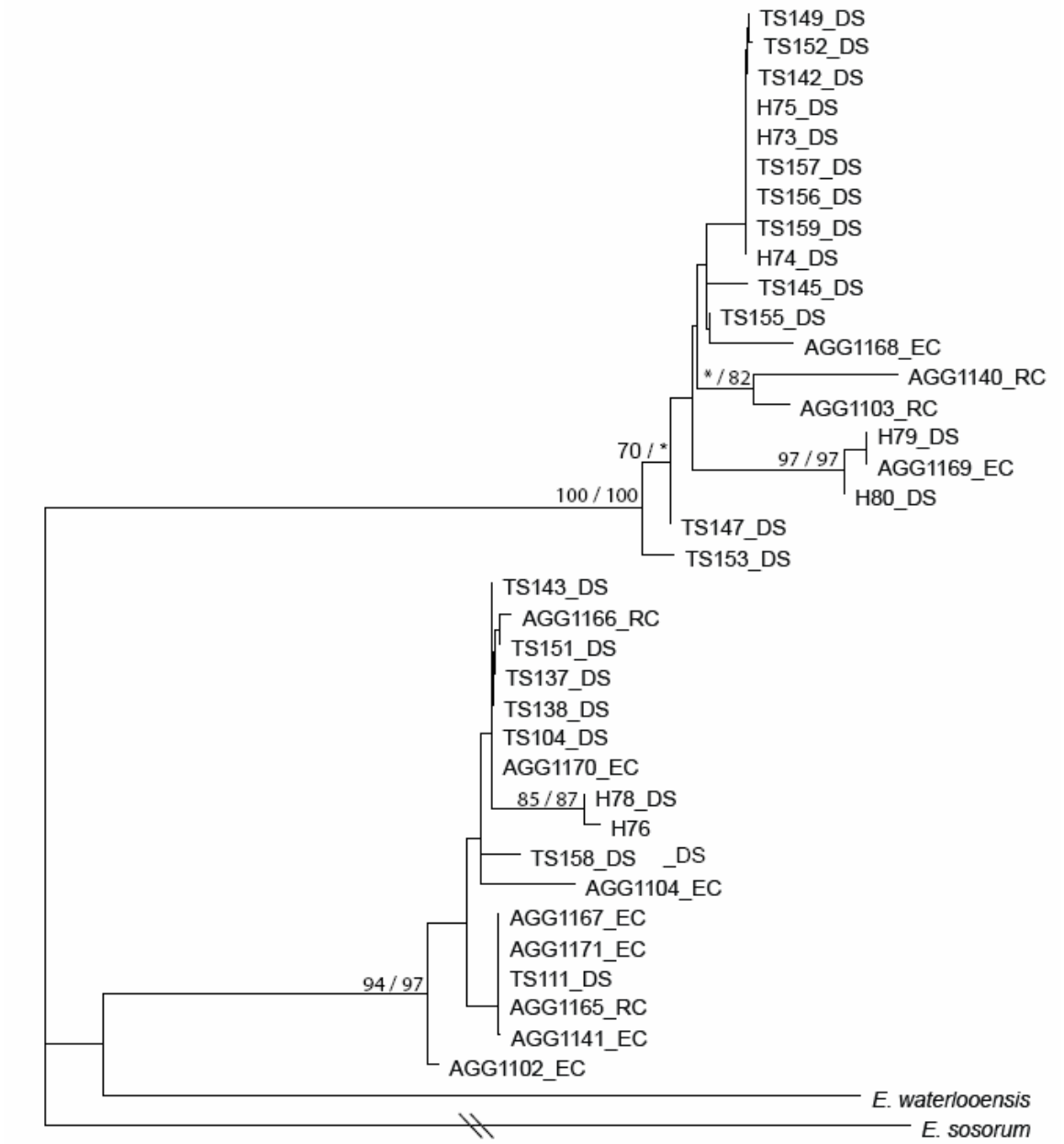
Our sampling is not yet complete with respect to all of the material that we already have or anticipate receiving, but the pattern appears clear based on a fairly comprehensive sampling and we are in the process of conducting a much more thorough analysis using a wide range of molecular markers. For example, the easternmost population in Round Rock is not shown in our tree due to the need for minor data checking, although it clearly belongs in the "Plateau" group. One key locality is Balcones Park Spring, in the Walnut Creek drainage; we expect to obtain data for specimens from that population very soon (I have several samples from this locality although they no longer appear to be accessible or perhaps even extant in the wild), and also will obtain material from Spicewood Springs in the Shoal Creek drainage collected by Andy Price (TPWD) several years ago. Some populations of these salamanders appear to be declining or disappearing quickly, even before species boundaries can be established.

Sequence divergence between the two clades (Plateau and peripheral) is substantial, ranging from approximately 1.9 to 2.8%, even for those in very close proximity to one another (as little as about <2 km). Although it is preliminary to recognize two different

species in the *tonkawae* group, our results indicate that this may be warranted. The levels of differentiation are within the range of mt divergences consistent with species status in many other Texas *Eurycea* recognized as separate lineages based on multiple criteria. Although sequence divergence alone is insufficient for species recognition, it is indicative of degree of current or recent gene flow; this appears low or nonexistent among these units. Many would argue that degree of divergence reflects taxonomic group-specific evidence of speciation. Within major groups identified here, divergences range from 0.0 to 0.5% and 0.0 to 0.6% (within the "Bull Creek clade" and the "peripheral clade", respectively), versus roughly 2-3% between clades. The hierarchical levels of genetic variation and divergence warrant careful consideration of conservation strategy, especially given the precarious status of all populations in the group and the scattered and as yet poorly understood distribution and sampling of peripheral populations in particular. I recommend extensive sampling of additional likely sites of occurrence in the peripheral region to avoid the loss of at least one potentially new species, endemic to an area that is subject to major anthropogenic threats.

Likely there are numerous other populations within the peripheral and Plateau areas that remain to be discovered, and could easily disappear in the near future in the absence of further study and action. We are successfully establishing molecular criteria based on multiple genomic segments for identification of species and evolutionarily significant units in the region. These markers will become available to all researchers for diagnostic work and future study.

The results presented here represent a small fraction of the data that we have collected for both *E. rathbuni* and *E. tonkawae*. The preliminary results strongly indicate the need for both revised conservation policies and additional studies. We will provide and publish more conclusive results once we finish our current studies, but our findings to date and the remarkably patchy sampling of each species or species complex highlight the need for extensive future work that encompasses a wide range of approaches. Salamanders of the genus *Eurycea* are indicators of the quality of aquatic ecosystems and water resources in central Texas, providing the basis for protection of other organisms that inhabit these fragile aquatic habitats, and preservation of water resources upon which humans also depend.




0.01 substitutions/site

Figure 1. Relationships of individuals of *Eurycea rathbuni* and outgroups based on 1096 bp of the mt cytochrome b gene. Tree topology and branch lengths are based on neighbor joining using the Kimura 3-parameter distance correction; confidence values at major nodes are bootstrap support numbers based on 1000 pseudoreplicates from NJ analysis and maximum parsimony analysis, respectively. Asterisks indicate values below 50% for nodes supported by only one type of analysis. Branch length for *E. sosorum* (outgroup) is truncated to roughly 1/3 actual length. Topological results are nearly identical to those of a separate Bayesian analysis, except that support values here are more conservative. DS = Diversion Spring, EC = Ezell's Cave, and RC = Rattlesnake Cave.

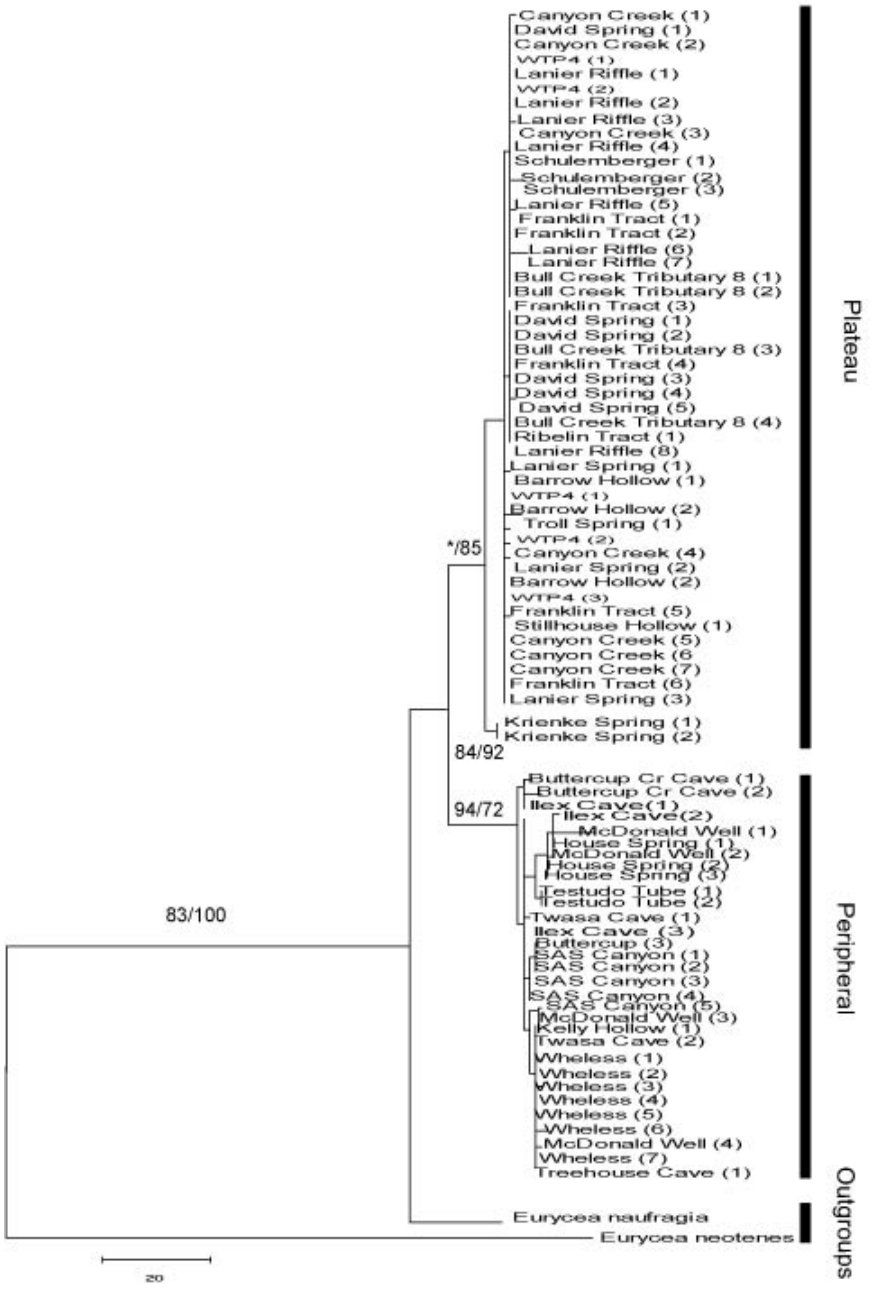



Figure 2. Relationships of populations of the *Eurycea tonkawae* group, based on maximum parsimony analysis of mt cytochrome b data. Support for major nodes above 50% is shown by maximum parsimony bootstrap values, 1000 pseudoreplicates, followed by neighbor joining bootstrap values for coinciding nodes using Kimura 3-parameter distance. Asterisks indicate values below 50% when . Numbers in parentheses indicate individuals sampled. Scale bar shows number of inferred substitutions under the parsimony criterion.