January 2010

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Revised version

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Introduction

The Jollyville Plateau salamander, *Eurycea tonkawae*, inhabits springs and caves in the complex aquifer system of the "northern" (north-northeast of the Colorado River) portion of the Edwards Aquifer in northern Travis and southern Williamson Counties. *Eurycea tonkawae*, *E. naufragia*, and *E. chisholmensis* (described simultaneously by Chippindale et al. 2000; see also Chippindale 2000 and 2005 for taxonomic summaries and additional information) comprise a monophyletic group once assigned to the species *E. neotenes*. *Eurycea neotenes* previously was thought to be widespread in the Edwards Plateau region of central Texas (e.g., Sweet 1982). However, based on Chippindale et al.'s (2000) molecular data and all subsequent studies, the "northern" group (clade *Septentriomolge*; Hillis et al. 2001) is extremely divergent from the "southern" group (clade *Notiomolge*). The latter includes *E. latitans*, *E. neotenes*, *E. nana*, *E. pterophila*, *E. rathbuni*, *E. robusta*, *E. sosorum*, *E.* sp. Pedernales, *E. tridentifera*, *E. troglodytes*, *E. waterlooensis*, and several other undescribed species. In addition, nuclear genome sizes of members of the *Septentriomolge* and *Notiomolge* clades differ by approximately 13% (Licht and Lowcock 1991; data in their publication are reported erroneously and were clarified by Chippindale et al. 2000). The most robust estimate of divergence time (Wiens et al. 2006) places the north-south split defined by the Colorado River at roughly 15 MYA, emphasizing the distinctiveness of the northern (*Septentriomolge*) clade. Multiple molecular data sets (Chippindale et al. 2000, Hillis et al. 2001, Chippindale et al. in prep [including limited data included herein]) support recognition of *E. tonkawae*, *E. naufragia*, and *E. chisholmensis* as distinct species, each with a very limited geographic range. Based on preliminary data from multiple nuclear and mitochondrial DNA markers that my colleagues and I have collected, additional species may be present in the northern region. However, all data indicate that *E. tonkawae* is a lineage or group of lineages restricted to the Jollyville Plateau region of Travis and Williamson Counties.

Reconstruction of phylogenetic relationships and identification of species boundaries in the central Texas *Eurycea* historically has been hampered by complex patterns of morphological variation. Almost all populations are paedomorphic (fail to undergo metamorphosis and reach reproductive maturity while retaining numerous juvenile characteristics). Many populations that are very distinct at the molecular level show little morphological differentiation. Additionally, cave dwelling has arisen multiple times and subterranean populations show varying degrees and kinds of cave-associated morphology such as eye and pigment reduction and skeletal modifications (e.g., Potter and Sweet 1984, Chippindale et al. 2000, Wiens et al. 2003). This combination of morphological conservatism and convergence created tremendous taxonomic

confusion, and although much of this was resolved using molecular data, a particular problem that Chippindale et al. (2000) encountered was that within *E. tonkawae*, some populations (primarily those in the Buttercup Creek Caves system of southern Williamson County; see map in Fig.1) exhibit both varying degrees of cave-associated morphological divergence and unusual patterns of allozyme (protein) and mitochondrial DNA (mtDNA) variation. Several of these populations provisionally were included within *E. tonkawae*, with the caveat that sampling was very limited and additional study was necessary. In addition, individuals from the population in Kretschmarr Salamander Cave (roughly 7 km southeast of the Buttercup Creek Caves complex) have unusual genetic composition and unique morphologies. These results, in part, led us (Chippindale et al. 2000, Chippindale 2000; Chippindale 2005; Chippindale, Nathan F. Bendik [City of Austin] and Andrew G. Gluesenkamp [TPWD], work in progress) to suspect that *E. tonkawae* might consist of genetically isolated units, but sampling was patchy, sample sizes (especially for the Buttercup Creek and Lake Travis drainages; Fig. 1) were small, and molecular techniques were much more limited in execution and application than at present.

Here I present results of analyses of mitochondrial and nuclear sequence data, together with those based on nuclear microsatellites, for representatives of *E. tonkawae* from localities throughout the known range. Gluesenkamp, Bendik and I will submit a more comprehensive manuscript to a peer-reviewed journal (likely Molecular Ecology). Some sampling gaps remain to be filled, but the results that I present here represent a very comprehensive survey of *Eurycea tonkawae*, and provide crucial insights into genetic structure based on a diverse range of molecular markers.

Key conclusions:

1) *Eurycea tonkawae* is distinct from *E. naufragia, E. chisholmensis*, and other "northern" populations that may represent additional species. *Eurycea tonkawae* is an isolated complex of evolutionary lineages that occupy a small geographic area.

2) *Eurycea tonkawae* consists of multiple fragmented genetic units, consistent with the complex nature of the northern Edwards Aquifer.

3) Based on analyses thus far, it seems likely that at least two ESUs (potentially different species) are present. One occurs in the Bull Creek, Walnut Creek, Shoal Creek, Brushy Creek, South Brushy Creek, and possibly southeastern Lake Travis drainages. A second lineage appears to occur in the Buttercup Creek and northern Lake Travis drainages and may also include salamanders from Kretschmarr Salamander Cave plus SAS Canyon Springs (essentially the same locality) in the southeastern Lake Travis drainage.

4) Although this study does not address anthropogenic effects, levels of urbanization in the region, combined with sensitivity of these amphibians to degradation of water quality (e.g., Bowles et al. 2006) present major threats to the survival of *E. tonkawae*, especially given the high degree of genetic structuring and extensive habitat fragmentation.

Methods

Sampling:

Salamanders, or tissue samples (tail tips) from animals that subsequently were released, were collected from throughout the known range of the species. Most samples were obtained in 2008-2010 (primarily by Gluesenkamp and Bendik), but some represent material that colleagues and I collected, mainly in the early to mid-1990s. All recently collected specimens were or will be deposited in the University of Texas at Arlington Amphibian and Reptile Diversity Research Center collection, and earlier specimens are held in the Texas Natural History Collection at the University of Texas at Austin. Georeferences for all localities are available from Bendik and will be added as an update to this report (currently he and I are clarifying several small points that have no impact on the results presented here), together with a list of "synonymies" for localities (many of the spring and cave sites inhabited by salamanders have been assigned multiple names and there has been confusion regarding several localities).

DNA sequence markers:

DNA from most specimens (roughly 250+) was extracted using Qiagen DNeasy kits; DNA samples from the early-mid 1990s were obtained using standard phenol-chloroform methods. Loci (here I use this term to mean any specific segment of DNA, mitochondrial [mt] or nuclear [nuc], coding or non-coding) were amplified via polymerase chain reaction (PCR) using a wide range of cycling conditions and in most cases, taxon-specific primers developed or modified by me and members of my laboratory group. I sequenced portions of a broad range of mt and nuc loci. Mt: cytochrome b (approximately 1.1 kilobase, kb), cytochrome oxidase I (approx. 700 base pairs, bp), and entire control region including a portion of the flanking 12S ribosomal RNA gene (approx. 2+ kb). Nuc: Portions of recombination-activating gene I (approx. 2 kb), melanocortin receptor gene (approx. 500 kb), pro-opiomelanocortin gene (approx. 450 bp), triosephosphate isomerase gene from end of exon 2 through exon 5 (including three introns; approx. 800 bp), and a portion of the ornithine decarboxylase (ODC) gene encompassing mid-exon 6 to mid-exon 8, thus including introns 6 and 7 (approx. 650 bp). Of the mt loci, cytochrome b (cyt b) was most variable overall (excluding short portions of the adjacent control region that could not reliably be aligned). I am also analyzing a smaller data set for the mt cytochrome oxidase I gene as a “check” on the inherently linked cyt b gene. These analyses are not yet complete, but thus far appear to support the results based on cyt b.

Of the nuc regions sequenced, nearly all proved invariable within *E. tonkawae* although most were variable and informative with respect to the northern central Texas *Eurycea* group as a whole, and also have shown informative variation for Texas *Eurycea* from south of the Colorado River. However, ODC exhibits a single two base-pair (bp) deletion mutation (indel) in intron 6 that appears to be unique to (although not fixed within) salamanders from a subset of drainages in the region (see below). This nuclear region is readily amplifiable and will be helpful for future assessments of species boundaries.

Microsatellites:

PCR primers for microsatellite loci were developed using modifications of the methods of Glenn and Schable (2006). PCR products were capillary-electrophoresed on an ABI 3130xl automated sequencer, generally multiplexed with combinations of HEX, FAM, and NED dyes and sized using ABI's 400 bp ROX-labeled size standard. Of roughly 60+ loci tested, I identified seven that are readily interpretable and show informative variation, and rejected numerous others for which amplification failed, was unreliable, or assessment of allelic variation was questionable. The loci used all vary primarily based on differences in numbers of tetranucleotide repeats. To ensure that I was examining genuine microsatellites (especially because some PCR primer pairs generate multiple peaks of widely varying sizes), I gel-extracted and sequenced bands from agarose gels to verify the identity of these markers. I obtained microsatellite data for a total of 225 individuals for the seven loci. Although some instances of missing data remain, the data set is extensive and comprehensive.

Additional markers:

I explored use of TE-AFLP (a method based on fragment size variation that potentially allows examination of numerous markers from throughout the genome; see van der Wurff et al. 2000). However, I abandoned this approach primarily because fragment profiles were dominated by a few very large peaks that obscured or reduced intensity of the others. I suspect that this is due to the very large genome sizes of plethodontid salamanders (e.g., Licht and Lowcock 1991) with lengthy blocks of repeat sequence that generate recurring restriction enzyme profiles. There also are issues of interpretation (AFLPs generally are considered dominant markers but may not be, and the loci are essentially anonymous). I chose to focus on microsatellites, which allow assessment of variation from throughout the genome and, as implemented here, were verifiable with respect to identity and allelic variation.

Population designations:

Given the complex structure of the northern Edwards Aquifer and very limiting understanding of its hydrogeology, it is often difficult to determine what constitutes a "population". Thus, I treated each collection locality as a population for purposes of DNA sequence analysis. For microsatellite analyses (which primarily are based on allele frequencies and can be sensitive to small sample sizes and missing data), I combined data for nearby localities in a few cases (these are shown in the microsatellite data tables). Choice of "regions" for analysis by AMOVA (see below) corresponds mainly to watersheds recognized by City of Austin (Fig. 1) but two additional regions were added to address possible structuring within the Lake Travis watershed. Regional designations were: 1) Walnut Creek watershed; 2) Bull Creek watershed; 3) Shoal Creek watershed; 4) Upper Brushy Creek watershed; 5) South Brushy Creek watershed; 6) southeastern Lake Travis watershed (Kretschmarr Salamander Cave and SAS Canyon Springs only; essentially the same locality); 7) northern Lake Travis watershed 1 (House, Kelly Hollow and MacDonald Well Springs); 8) northern Lake Travis watershed 2 (Wheless Springs only; I will add very recently obtained material from Blizzard Spring, for which I have mt data but microsatellite data are incomplete) and 9) Buttercup Creek watershed.

Analyses:

Sequences of mt genes were aligned using Sequencher (GeneCodes Corp). The cytochrome b-based phylogenetic/phylogeographic tree shown here (Fig. 2) is based on neighbor-joining clustering of HKY85- (Hasegawa et al. 1985) corrected genetic distances. Additional analyses (not shown) using MrBayes 3.1 (Ronquist et al. 2005) thus far show very similar patterns of relationship (I am redoing these computationally intensive analyses using additional data, and can provide this information as an update to this report).

Allelic designations for microsatellites, defined by relative fragment size, were determined using GeneMarker (Softgenetics), and population genetic analyses primarily were conducted using GenAlEx 6 (Peakall and Smouse 2006). Loci were treated as codominant markers, and results of analyses shown here (with respect to population differentiation and potential gene flow) are based largely on Wright's F statistics (Wright 1951, 1969). I also applied Slatkin's (1995) analogous R statistics, which take into account the stepwise nature of microsatellite evolution (i.e., loss or gain of discrete numbers of repeat units). I used AMOVA (Analysis of Molecular Variance; Excoffier et. al. 1992.) to determine the extent to which variation in microsatellites is partitioned among and within "populations" and "regions" relative to total variation. I am conducting additional analyses using the program Structure v. 6 (Pritchard et al. 2000, since then updated multiple times), which implements a Bayesian algorithm to identify the most probable number of genetically distinct units; this will be an extremely valuable adjunct to the results described here.

Results

MtDNA:

Analysis of mtDNA (cytochrome b [cyt b]) reveals two main clades that correspond to 1) the Bull Creek, Walnut Creek, Shoal Creek (but see below) and Upper and Southern Brushy Creek drainages, and 2) the Lake Travis and Buttercup Creek drainages (Fig. 2). Hereafter I will refer to the latter group as "peripheral"; i.e., peripheral to the Jollyville Plateau proper (note that this is not entirely consistent given that the Brushy Creek drainage is also "peripheral" to the Plateau; we need to develop better terms that informally describe these regional groupings). The only exception to this pattern is the placement of two individuals from Spicewood Springs (Shoal Creek drainage) as sister to (but very divergent from) the Lake Travis/Buttercup Creek group. A third individual from Spicewood Springs clusters within the Bull/Walnut/Brushy Creek group (Spicewood Springs is geographically adjacent to the Bull Creek drainage). The two apparently anomalous samples have proven difficult to amplify; I consider the data for these questionable and am clarifying this issue with additional samples and laboratory tests.

Mitochondrial DNA divergences within each clade generally are low, particularly within the Bull Creek watershed where individuals from many localities exhibit identical or near-identical

sequences (the maximum uncorrected sequence difference is approximately 1.0% between an individual from Lower Ribelin Springs and one from Barrow Hollow Springs; nearly all other divergences among individuals from the Bull Creek watershed are below 0.5%). Even the relatively distant (from Bull, Shoal, and Walnut Creek drainages) populations in the Brushy Creek and South Brushy Creek drainages exhibit relatively little mt differentiation from the Bull/Shoal/Walnut Creek group (maximum approximately 0.6%). Within the "peripheral" mt-based clade, nearly all divergences within and among populations are below 1.0%, with the exception of three individuals from House Spring that differ from others in the region by up to 2.2%. All three are difficult samples that lack a substantial amount of sequence data (but exhibit nucleotide substitutions characteristic of the peripheral group); two other individuals from this site fall within the normal range of variation seen in the peripheral group.

Uncorrected pairwise divergences between populations from each of the two major clades are roughly 2.5 - 3.0% (minimum 2.2%), very similar to levels of mtDNA divergence among some of the most closely related, recognized species of *Eurycea* from south of the Colorado River. Surprisingly, using the geographically and morphologically distinct outgroup taxa *E. naufragia* and *E. chisholmensis*, it is not possible to root the tree using the relatively simple clustering algorithm employed here such that *E. tonkawae* is monophyletic. This requires (and is undergoing) further testing. Mitochondrial divergences of *E. chisholmensis* and *E. naufragia* from members of the *E. tonkawae* group range from about 2.3% to about 4.3% and 3.9%, respectively.

ODC:

Sequences of the nuclear ornithine decarboxylase (ODC) gene for 99 individuals from throughout the range of *E. tonkawae* were identical among individuals and populations with the exception (as described above) of a two bp deletion (indel) in intron 6 that occurs at moderate to high frequency in the Bull Creek and Walnut Creek drainages (Table 1). Although not detected in the Upper Brushy Creek or South Brushy Creek drainages, and subject to further testing for the Shoal Creek drainage (Spicewood and Indian Springs) sampling is very limited for the latter three regions. This indel-based allele also was not seen in a very small sampling of *E. naufragia* and *E. chisholmensis*, the other two recognized members of the *Septentriomolge* clade, or in numerous other species of Texas *Eurycea* analyzed by me and colleagues in separate, ongoing studies. In contrast to the pattern of differentiation for mtDNA, this allele is at highest frequency in the southeasternmost portion of the Lake Travis drainage at the Kretschmarr Salamander Cave and SAS Canyon localities (essentially the same site; cave vs. springs), the sites geographically closest to those in the main Bull Creek drainage. Mitochondrially, Kretschmarr/SAS fall within the peripheral clade. This indel appears to be absent from populations in the remainder of the Lake Travis and Buttercup Creek drainages based on sampling from throughout the known range of this group and is a useful, readily interpretable marker that potentially serves as a lineage-specific "barcode".

Microsatellites:

As is typical for microsatellites, some loci exhibit large numbers of alleles, with a maximum of 19 for the u55 locus and minima of four alleles each for the u6 and u46 loci. Among populations,

the lowest average heterozygosity across loci (by far) was approximately 9.5% for the Testudo Tube Cave samples. The next lowest value, 25.0%, was for the combined samples from several of the Buttercup Creek caves (treated as a single population); these are extremely close geographically to Testudo Tube but the Testudo Tube animals are morphologically distinct (personal observation; Gluesenkamp pers. comm.). The highest value, 51.2%, occurred in the sample from Avery Deer Spring (Lower Brushy Creek drainage), possibly indicating large populations in this barely sampled region. Tests for deviation from Hardy-Weinberg equilibrium, uncorrected for multiple tests (e.g., Bonferroni criterion) revealed 16 significant deviations out of 161 (i.e., 23 "populations" multiplied by seven loci). This includes 35 comparisons in which a locus appeared monomorphic (=apparently fixed) for a single allele. The number of deviations exceeds the number expected by chance with an alpha level of 0.05 (although only four tests were significant at an alpha level of 0.01 or below). These deviations are not concentrated at any single locus; however, they are most frequent for u9 (five cases) and u55 (three cases).

Pairwise estimates of F_{ST} and R_{ST}, and corresponding values for Nm (number of migrants per generation) are given in Table 3. Values of F_{ST} and R_{ST} vary widely and in some cases are based on very small sample sizes (primarily because almost every locality was treated as a "population" in these analyses, potentially leading to high levels of sampling error with respect to estimation of allele frequencies). However, some generalizations are possible. First, most values of F_{ST} (and R_{ST}) are high to very high (see discussion below), indicating extensive genetic fragmentation throughout much of the range. The main region in which numerous values are low is the central and western portion of the Bull Creek watershed, with the lowest estimated value above zero for F_{ST}, 0.011, between the Lanier Spring/Lanier Riffle and Lower Ribelin + Horsethief Hollow sites. This corresponds to roughly 23 migrants per generation (Nm), assuming that gene flow is occurring (in the peripheral region, the lowest value is 0.008 between House and nearby MacDonald Well Springs, corresponding to Nm of about 31). Most of the highest values for F_{ST} and R_{ST} are between geographically distant sites, particularly those in different watersheds; for example, comparison of the Schlumberger Spring site (Bull Creek drainage) and Testudo Tube Cave (northern Lake Travis drainage) yields an F_{ST} of 0.857, indicating complete or near-complete genetic isolation (puzzling, however, is the high degree of apparent isolation between Testudo Tube and the other nearby Buttercup Creek caves). Similarly, both the Round Rock (Upper Brushy Creek) and Avery Deer Spring (South Brushy Creek) are strongly differentiated from most other populations and from one another based on microsatellites. Although individuals from the Kretschmarr/SAS Canyon site(s) possess mt haplotypes most similar to those from the Buttercup Creek and remaining Lake Travis drainages (peripheral group), they appear based on microsatellites to be substantially isolated from both while sharing the characteristic "Plateau" ODC indel. Individuals from Balcones Park Spring, the only known locality in the Walnut Creek drainage, exhibit surprisingly low levels of differentiation from other populations, especially given the relatively distant and potentially isolated nature of this site. However, sample sizes are very low, some missing data are present, and the samples (collected in the early 1990s) are of poor quality. Very recently, salamanders have reappeared at this site after not having been seen for years (Bendik, pers. comm.). We expect to obtain new material in the near future that should enable us to better understand the status of this population. It appears minimally diverged mitochondrially from those in the Bull Creek and Brushy Creek drainages, and one of three individuals from the Shoal Creek drainage.

Analysis of molecular variance (AMOVA; Table 2) using F-and R- statistics in which nearly all localities were treated as separate populations (23 in total) and subdivided into the nine regions described above (primarily watersheds/drainages) shows the following. 1) Based on F_{ST} approximately 28% of the total genetic variance is accounted for by differences among regions, 13% by differences among populations within regions, and 59% by variation within populations. 2) R-statistics (which implement a model specific to microsatellite evolution, as described above) suggest much greater isolation among regions: 73% of the variance is accounted for by inter-region differentiation, 6% among populations within regions, and 21% within populations. Thus, there appears to be major structuring (subdivision) at the regional geographic scale using either measure. The difference in estimates likely is due in part to the difference between a simple model of evolution of codominant allelic markers (F_{ST}) and one that differentially weights divergences among codominant alleles according to the magnitude of their difference in size (R_{ST}), i.e., under a stepwise model of microsatellite evolution, the greater the difference in number of repeat units, the longer the time since allelic divergence.

Currently I am conducting more extensive and comprehensive analyses that will shed further light on patterns and sources of geographic variation, as part of preparation of a manuscript for submission to a peer-reviewed journal. However, the results presented here leave little doubt that *E. tonkawae* represents a highly genetically fragmented group of evolutionary lineages that exhibits strong barriers to gene flow even on a very fine geographic scale.

Discussion

This study reveals moderate levels of regional mitochondrial differentiation within *E. tonkawae* (including levels of uncorrected sequence divergence similar to those associated with species-level status among other central Texas *Eurycea*), and strong geographic fragmentation based on nuclear markers. The mitochondrial data divide *E. tonkawae* into two primary clades, corresponding to the Bull, Shoal, Walnut, and Upper and South Brushy Creek drainages (with the exception of two individuals from the Shoal Creek drainage for which data are questionable); and the Buttercup Creek plus Lake Travis drainages, the latter pair informally referred to here as the "peripheral" clade. Although sample sizes for the nuclear ODC locus are low on a site-by-site basis, geographic coverage is very comprehensive. The presence of a very distinctive indel throughout the Bull Creek, Walnut Creek, and possibly Shoal Creek drainages (and in the Kretschmarr Salamander Cave and SAS Canyon localities of the southeastern Lake Travis drainage, geographically very close to populations in the main Bull Creek drainage) suggests ongoing or past gene flow. A possible interpretation is that Kretschmarr/SAS populations belong to the "main Plateau" group but have experienced mt introgression from the peripheral group (this is a common phenomenon in vertebrates that we now are detecting within the *Notiomolge* clade of the Texas *Eurycea*). Or, an ancestral polymorphism has been retained within the Kretschmarr/SAS and main Plateau populations but lost in all other Texas *Eurycea*. Regardless, variation at the ODC locus suggests strong separation between the main Plateau populations and the peripheral populations (northern Lake Travis and Buttercup Creek watersheds), with the exception of Kretschmarr/SAS. The status of populations in the Brushy Creek and South Brushy

Creek drainages with respect to this marker is uncertain, in that sample sizes are so small that detection of the indel would be unlikely unless it occurs at high frequency.

As described above, the occurrence of many high to very high F_{ST} and R_{ST} values based on microsatellites is indicative of strong geographic structuring. Essentially, these statistics measure the departure (or lack thereof) from Hardy-Weinberg equilibrium when data for subpopulations (equivalent here to "populations") are combined to form a hypothetical freely interbreeding unit (although interpretations vary; e.g., see Wright 1969, Slatkin 1985, and Neigel 2002). The greater the deficiency of heterozygotes, the less likely it is that the populations actually are interbreeding. F_{ST} and R_{ST} can vary between 0 (completely free interbreeding among individuals in a geographic context) and 1 (completely different allelic composition for the markers studied, reflecting near-unambiguous isolation from gene flow). Although such measures are not necessarily directly comparable across studies (or different kinds of data), as a very rough rule of thumb, values of F_{ST} from 0.0-0.05 indicate low levels of genetic fragmentation (and thus high levels of gene flow, expressed as Nm, the number of migrants per generation); from 0.05-0.15 moderate differentiation; from 0.15-0.25 high differentiation, and from 0.25 - 1.0 very strong differentiation (e.g., Slatkin 1985, Kindt et al. 2009). By these standards, most pairwise comparisons (Table 3) indicate high levels of fragmentation. This previously has been observed in a range of species of plethodontid salamanders, particularly terrestrial species whose vagility is likely to be low (Larson et al. 1984). For the semiaquatic plethodontid *Desmognathus quadramaculatus* in the southern Appalachians, Stiven and Bruce (1988) found (using allozyme markers) an average F_{ST} of 0.146 across samples from the the Coweeta and Great Smoky Mountains National Parks watersheds. They interpreted this as a moderate level of differentiation; here we see numerous instances of greater differentiation on a much smaller geographic scale. Across amphibians, the general pattern (based primarily on studies of frogs; see review by Newman and Squire 2001 and discussions by Hitchings and Beebee 1997 and Pröhl et. al. 2010) is one of relative genetic homogeneity on a local (< about a few km) scale, and more extensive fragmentation on larger scales. In a very recent example, Pröhl et al. (2010) found a microsatellite-based F_{ST} of approximately 0.22 (and corresponding R_{ST} of 0.72) in eleutherodactylid frogs separated by a gap of well over 100 km; here we see values of similar magnitude on a scale of as little as a kilometer or less. Obviously, levels of gene flow are determined by the vagility of a given species and the nature and distribution of barriers. The results presented here suggest that for *E. tonkawae*, barriers based on the complex structure of the aquifer system in the region likely are the major limiting factors for gene flow (ongoing studies of other members of the Texas *Eurycea* group by myself and colleagues are consistent with this hypothesis).

Although I provide Nm values (which can be calculated directly from F_{ST} or R_{ST}; e.g., Slatkin (1981, 1995), it is important to realize that these measures estimate the number of migrants per generation IF gene flow is occurring. In other words, historical events that may have completely separated populations can leave a "signature" that suggests some level of gene flow, even if similarities in allele frequencies are remnants of past connection. Larson et al. (1984) suggested that for plethodontid salamanders, F_{ST} (and therefore by extension Nm) may be more informative about population history than current genetic exchange.

With respect to *E. tonkawae*, it appears that even under the assumption that these measures predominantly reflect ongoing gene flow, this occurs primarily on a very localized scale. Although this is the case for many other amphibians, here restrictions to gene flow appear particularly strong, and levels and patterns of divergence indicate the occurrence of at least two evolutionary lineages that have at most extremely limited contact. However, patterns of nuclear variation suggest past or ongoing gene flow between the Kretschmarr Salamander Cave/SAS Canyon sites (southeastern Lake Travis drainage) and nearby populations in the Bull Creek watershed. This requires further investigation, especially in light of the apparently conflicting mitochondrial results. Additional sampling also is especially critical in the Brushy Creek drainages given the apparent geographic separation of these populations from other members of the group, substantial nuclear although not mitochondrial divergence, and extremely limited knowledge of their distribution in the rapidly urbanizing, northernmost portion of the range of *E. tonkawae*. In fact, their supposed geographic isolation could be a simple matter of inadequate sampling; in general, Gluesenkamp, Bendik and I are finding that many distributional gaps are the result of lack of survey effort, which emphasizes the need for intensive and immediate field study.

An especially important follow-up to this work will be detailed morphological examination of salamanders from throughout the Jollyville Plateau and adjacent areas. This is in part a practical consideration for field identification of new populations or cases in which material for molecular analysis is not available, and heritable morphological traits obviously have the potential to serve as key markers for studies of gene flow and population structure. There is a strong possibility that *E. tonkawae* represents more than one species, and morphological diagnosis is a fundamental part of species description. Very recently, funding has become available that will allow morphological analysis of members of this group (by C. Roelke, a former graduate student in my lab).

The results of the molecular-based analyses presented here show that *Eurycea tonkawae* is a genetically diverse, geographically and genetically fragmented complex of evolutionary lineages restricted to a very small area. Whether one, two, or multiple species, there is an urgent need for protection given the inherent vulnerability of these salamanders and the intensive, rapidly growing threats to habitat and water resources in the Jollyville Plateau region.

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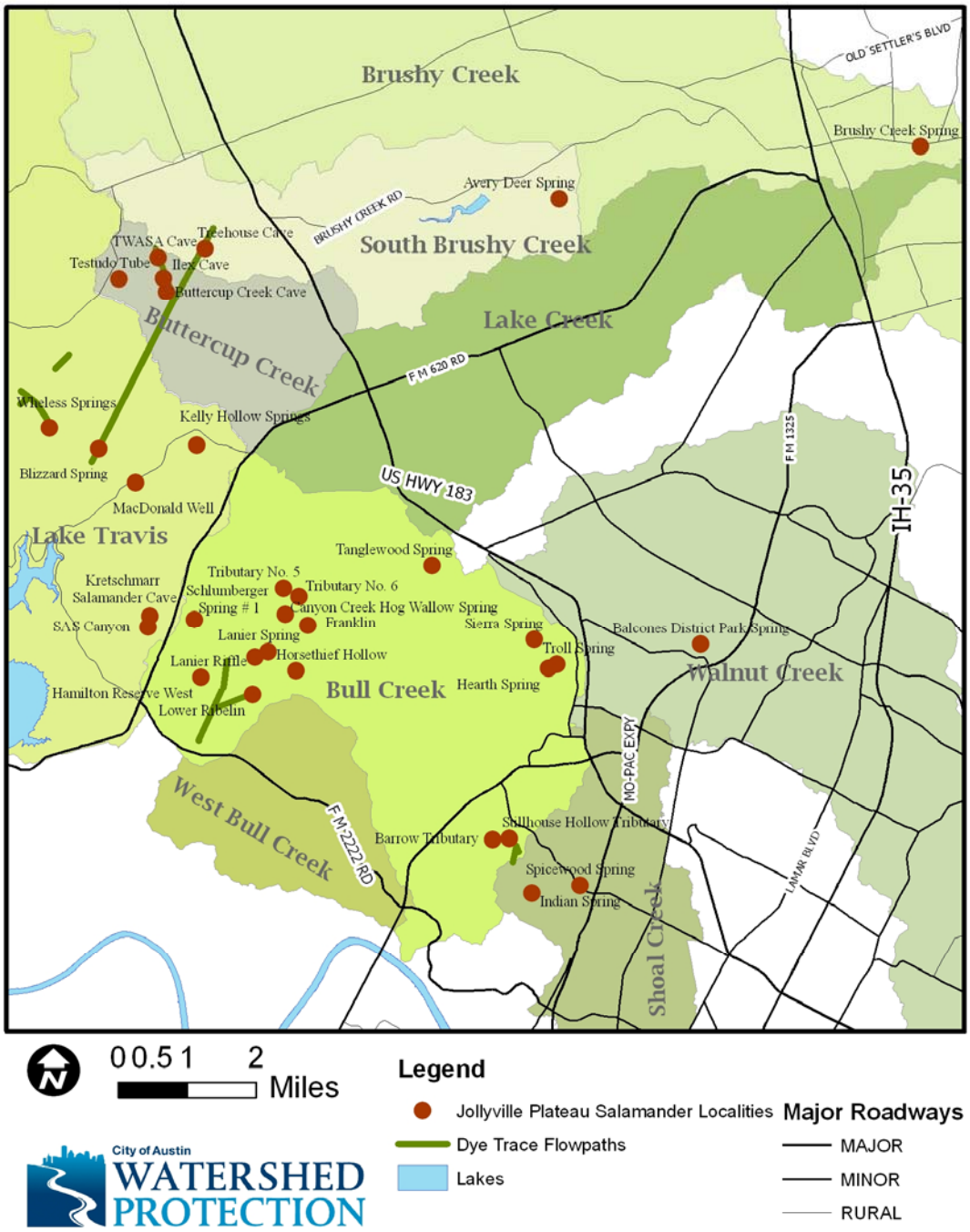

Figure 1. Sampling localities for *E. tonkawae* used in this study. Major drainage systems (as recognized by City of Austin) are shown. The break between the two putative ESUs or species primarily is defined by FM 620 ("Plateau" group east/southeast of 620, "peripheral" group west/northwest); the Kretschmarr and SAS Canyon populations are of uncertain status. The Avery Deer and Brushy Creek Springs populations (South and Upper Brushy Creek drainages, respectively) appear most closely related to the "Plateau" group based on mtDNA; their status is uncertain based on nucDNA. This map was prepared by Nathan F. Bendik, City of Austin.

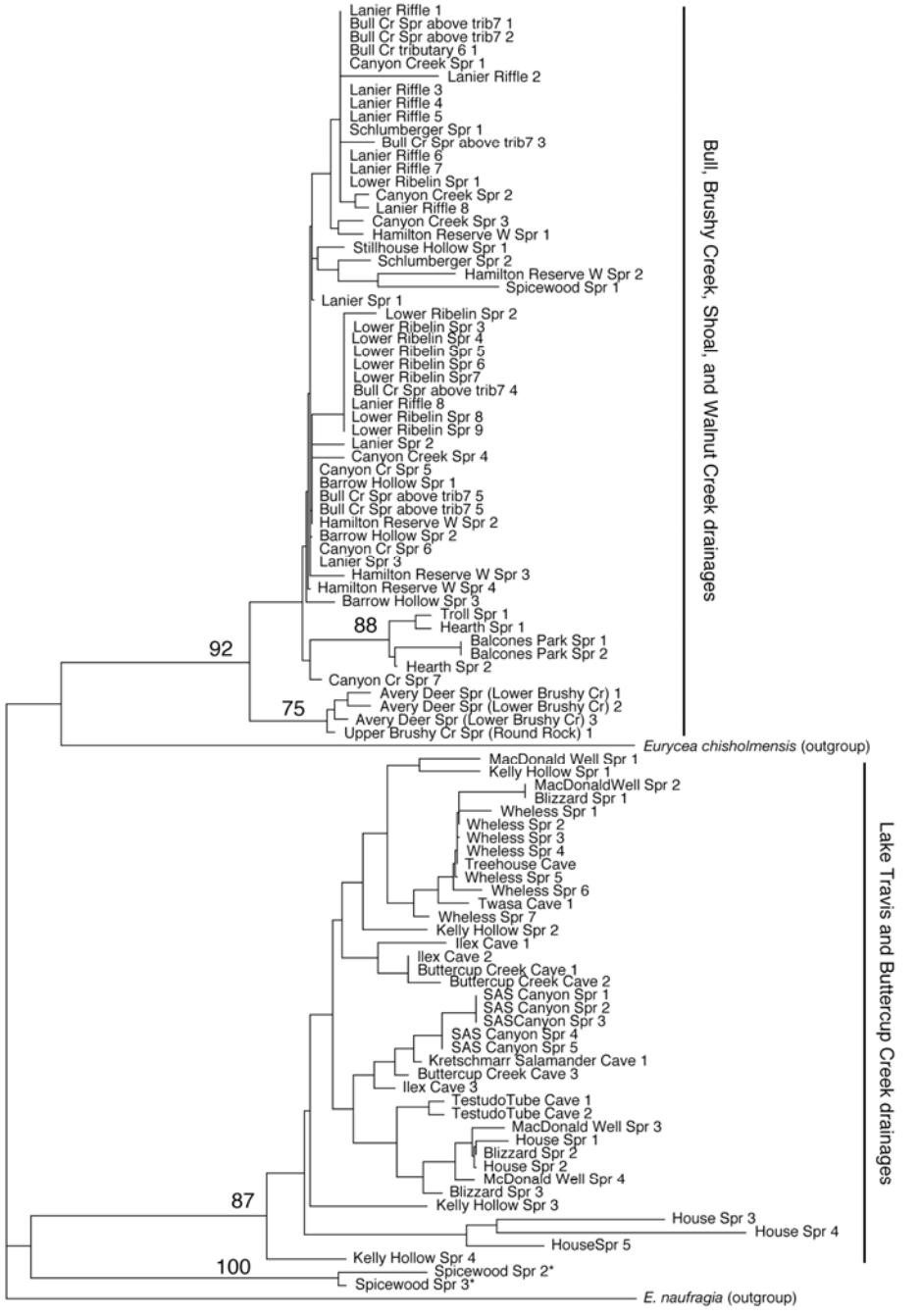

Figure 2. Neighbor joining tree for populations of *Eurycea tonkawae* based on up to 1066 basepairs of the mitochondrial cytochrome b gene, using HKY-corrected genetic distances. Numbers following locality names represent individuals from the site. Numbers at key nodes represent nonparametric bootstrap values (1000 pseudoreplicates) greater than 70%. Note that the tree could not be rooted such that the ingroup is monophyletic with respect to *E. chisholmensis* and *E. naufragia*. Sequences for the two highly divergent individuals from Spicewood Springs (marked with *) are considered questionable (see text and note placement of a third individual from this population, which is in the Shoal Creek drainage).

Table 1. Occurrence of a two base-pair deletion in intron 6 of the nuclear ornithine decarboxylase (ODC) gene in *Eurycea tonkawae* from throughout the Jollyville Plateau region. Numbers in parentheses represent totals that include individuals for which genotype requires verification. "Present" means that the indel (deletion allele) was detected. "Homoz" = homozygous and "heteroz" = heterozygous.

Locality	**Watershed/drainage**	**Indel present?**	**N homoz. present**	**N homoz. absent**	**N heteroz.**
Balcones Park Spring	Walnut Creek	Y	0	2	1
Bull Cr. trib. 5 Spring	Bull Creek (western main watershed)	Y	1	0	0
Franklin Springs	Bull Creek (western main watershed)	Y	8	0	1
Lanier Spring	Bull Creek (western main watershed)	Y	1	0	3
Lower Ribelin Springs	Bull Creek (western main watershed)	Y	2	3	1
Hamilton Reserve W Spring	Bull Creek (western main watershed)	Y	0	0	1
Schlumberger Spring	Bull Creek (western main watershed)	Y	0	0	1
Hearth Spring	Bull Creek (eastern main watershed)	N	0	1	0
Troll Spring	Bull Creek (eastern main watershed)	Y	0	1	1
Tanglewood Spring	Bull Creek (N-central main watershed)	Y	3	0	2
Barrow Hollow Spr	Bull Creek (southeastern main watershed)	N	0	1	0
Stillhouse Hollow Spr	Bull Creek (southeastern main watershed)	Y	0	1 (2)	1 (2)
Spicewood Springs	Shoal Creek	(N)	0	2 (3)	0 (1)
Indian Spring	Shoal Creek	N	0	1	0
Round Rock Spring	Upper Brushy Creek	N	0	2	0
Avery Deer Spring	South Brushy Creek	N	0	2	0
SAS Canyon Springs	Southeastern Lake Travis	Y	5	0	0
Kretschmarr Salamander Cave	Southeastern Lake Travis	Y	3 (4)	0	0 (1)
Blizzard Spring	Northern Lake Travis	N	0	3	0
House Spring	Northern Lake Travis	N	0	4	0
Kelly Hollow Spring	Northern Lake Travis	N	0	4	0
MacDonald Well Spr	Northern Lake Travis	N	0	5	0

Wheless Springs	Northern Lake Travis	N	0	15	0
Buttercup Creek Cave	Buttercup Creek	N	0	4	0
Ilex Cave	Buttercup Creek	N	0	2	0
Treehouse Cave	Buttercup Creek	N	0	1	0
Testudo Tube Cave	Buttercup Creek	N	0	4	0
E. naufragia – Cedar Breaks Spring, Williamson County	Non-Jollyville	N	0	2	0
E. naufragia – Booty Spring, Williamson County	Non-Jollyville	N	0	2	0
E. chisholmensis – Salado Springs, Bell County	Non-Jollyville	N	0	2	0
E. cf. *chisholmensis* – Cobb Springs, Bell County	Non-Jollyville	N	0	1	0

Table 2. A. Partitioned molecular variance (based on microsatellite data) among regions, among populations within regions, and within populations, determined via AMOVA using F-statistics (significance determined by 999 random data permutations). F_{RT} represents variance of regions relative to total, F_{SR} represents subpopulations (here "populations") relative to regions, and F_{ST} represents subpopulations (populations) relative to total. B. Partitioned molecular variance as described above, based on R-statistics.

A.
Summary AMOVA Table

Source	df	SS	MS	Estimated variance	Percentage of total	Statistic
AmongRegions	8	244.705	30.588	0.674	28%	$\mathbf{F_{RT}}$ = 0.278 (P<0.001)
Among Pops/Regions	14	100.450	7.175	0.306	13%	$\mathbf{F_{SR}}$ = 0.175 (P<0.001)
Within Pops	427	616.207	1.443	1.443	59%	$\mathbf{F_{ST}}$ = 0.404 (P<0.001)
Total	449	931.302	39.206	2.423		

B.
Summary AMOVA Table

Source	df	SS	MS	Estimated variance	Percentage of total	Statistic
AmongRegions	8	170114.149	21264.269	577.335	73%	$\mathbf{R_{RT}}$ = 0.725 (P<0.001)
Among Pops/Regions	14	15771.177	1126.513	51.163	6%	$\mathbf{R_{SR}}$ = 0.234 (P<0.001)
Within Pops	427	71553.877	167.573	167.573	21%	$\mathbf{R_{ST}}$ = 0.789 (P<0.001)
Total	449	257439.203	22558.355	796.071		

Table 3. Pairwise divergences and estimates of gene flow among populations of *E. tonkawae* based on F_{ST} and R_{ST}. Probabilities are derived from 999 permutations of the microsatellite allele frequency data. Cases in which estimates of gene flow (Nm, number of migrants per generation) are undefined (based on division by zero) are designated by "undf". "Buttercup Cr. Caves" comprises a subterranean karst complex thought to be part of the same interconnected system. Testudo Tube Cave is treated separately; although geographically adjacent to this system, salamanders from this site exhibit distinct morphologies and allelic composition.

Population 1	Population 2	F_{ST}	Nm	Prob	R_{ST}	Nm	Prob	Pop 1	Pop 2
								N	N
Balcones Park Spr	Bull Creek trib. 5 Spr	0.250	0.749	0.006	0.536	0.216	0.001	4	2
Balcones Park Spr	Bull Creek trib. 6 Spr	0.325	0.520	0.003	0.645	0.137	0.002	4	2
Bull Creek trib. 5 Spr	Bull Creek trib. 6 Spr	0.083	2.750	0.313	0.217	0.902	0.211	2	2
Balcones Park Spr	Franklin Springs	0.201	0.991	0.001	0.524	0.227	0.001	4	43
Bull Creek trib. 5 Spr	Franklin Springs	0.000	undf	0.420	0.000	undf	0.376	2	43
Bull Creek trib. 6 Spr	Franklin Springs	0.108	2.075	0.022	0.360	0.444	0.002	2	43
Balcones Park Spr	Canyon_Creek Spr	0.239	0.797	0.001	0.537	0.215	0.001	4	4
Bull Creek trib. 5 Spr	Canyon_Creek Spr	0.000	undf	0.383	0.000	undf	0.366	2	4
Bull Creek trib. 6 Spr	Canyon_Creek Spr	0.113	1.960	0.135	0.316	0.540	0.037	2	4
Franklin Springs	Canyon_Creek Spr	0.000	undf	0.399	0.022	11.355	0.249	43	4
Balcones Park Spr	Lanier Spr & Riffle	0.340	0.484	0.001	0.613	0.158	0.001	4	24
Bull Creek trib. 5 Spr	Lanier Spr & Riffle	0.000	undf	0.422	0.000	undf	0.394	2	24
Bull Creek trib. 6 Spr	Lanier Spr & Riffle	0.105	2.138	0.050	0.259	0.717	0.016	2	24
Franklin Springs	Lanier Spr & Riffle	0.053	4.471	0.001	0.042	5.740	0.005	43	24
Canyon Creek Spr	Lanier Spr & Riffle	0.030	8.119	0.181	0.000	undf	0.396	4	24
Balcones Park Spr	L. Ribelin&Horsethief	0.359	0.447	0.001	0.601	0.166	0.001	4	27
Bull Creek trib. 5 Spr	L. Ribelin&Horsethief	0.000	undf	0.409	0.000	undf	0.377	2	27
Bull Creek trib. 6 Spr	L. Ribelin&Horsethief	0.127	1.724	0.036	0.267	0.686	0.022	2	27
Franklin Springs	L. Ribelin&Horsethief	0.088	2.580	0.001	0.031	7.770	0.019	43	27
Canyon Creek Spr	L. Ribelin&Horsethief	0.090	2.539	0.018	0.000	undf	0.404	4	27
Lanier Spr & Riffle	L. Ribelin&Horsethief	0.011	22.567	0.128	0.000	undf	0.444	24	27
Balcones Park Spr	Schlumberger Spr	0.306	0.566	0.006	0.531	0.221	0.002	4	2
Bull Creek trib. 5 Spr	Schlumberger Spr	0.100	2.250	0.278	0.000	undf	0.357	2	2
Bull Creek trib. 6 Spr	Schlumberger Spr	0.278	0.650	0.054	0.495	0.255	0.039	2	2
Franklin Springs	Schlumberger Spr	0.056	4.241	0.128	0.050	4.748	0.204	43	2
Canyon Creek Spr	Schlumberger Spr	0.076	3.049	0.208	0.000	undf	0.403	4	2
Lanier Spr & Riffle	Schlumberger Spr	0.063	3.719	0.130	0.000	undf	0.393	24	2
L. Ribelin&Horsethief	Schlumberger Spr	0.092	2.482	0.085	0.009	28.174	0.357	27	2
Balcones Park Spr	Hamilton Reserve W	0.189	1.076	0.002	0.436	0.324	0.001	4	5
Bull Creek trib. 5 Spr	Hamilton Reserve W	0.025	9.685	0.331	0.103	2.173	0.201	2	5
Bull Creek trib. 6 Spr	Hamilton Reserve W	0.187	1.085	0.013	0.404	0.368	0.010	2	5
Franklin Springs	Hamilton Reserve W	0.061	3.835	0.019	0.097	2.335	0.032	43	5
Canyon Creek Spr	Hamilton Reserve W	0.000	undf	0.431	0.058	4.063	0.206	4	5
Lanier Spr & Riffle	Hamilton Reserve W	0.126	1.741	0.001	0.092	2.482	0.042	24	5
L. Ribelin&Horsethief	Hamilton Reserve W	0.154	1.369	0.001	0.067	3.461	0.078	27	5
Schlumberger Spr	Hamilton Reserve W	0.232	0.826	0.002	0.207	0.957	0.057	2	5
Balcones Park Spr	Sierra&Troll&Hearth	0.107	2.078	0.011	0.109	2.036	0.078	4	7
Bull Creek trib. 5 Spr	Sierra&Troll&Hearth	0.459	0.294	0.001	0.437	0.323	0.005	2	7
Bull Creek trib. 6 Spr	Sierra&Troll&Hearth	0.486	0.264	0.001	0.471	0.281	0.005	2	7
Franklin Springs	Sierra&Troll&Hearth	0.367	0.431	0.001	0.650	0.135	0.001	43	7
Canyon Creek Spr	Sierra&Troll&Hearth	0.443	0.314	0.001	0.457	0.298	0.001	4	7
Lanier Spr & Riffle	Sierra&Troll&Hearth	0.488	0.262	0.001	0.641	0.140	0.001	24	7
L. Ribelin&Horsethief	Sierra&Troll&Hearth	0.513	0.237	0.001	0.643	0.139	0.001	27	7
Schlumberger Spr	Sierra&Troll&Hearth	0.508	0.242	0.002	0.428	0.335	0.003	2	7
Hamilton Reserve W	Sierra&Troll&Hearth	0.409	0.361	0.001	0.381	0.405	0.001	5	7
Balcones Park Spr	Stillhouse&Barrow	0.159	1.320	0.001	0.149	1.428	0.048	4	13
Bull Creek trib. 5 Spr	Stillhouse&Barrow	0.336	0.493	0.001	0.395	0.384	0.008	2	13
Bull Creek trib. 6 Spr	Stillhouse&Barrow	0.425	0.339	0.001	0.462	0.291	0.009	2	13

Franklin Springs	Stillhouse&Barrow	0.271	0.671	0.001	0.516	0.234	0.001	43	13
Canyon Creek Spr	Stillhouse&Barrow	0.332	0.502	0.001	0.384	0.401	0.004	4	13
Lanier Spr & Riffle	Stillhouse&Barrow	0.336	0.494	0.001	0.515	0.235	0.001	24	13
L. Ribelin&Horsethief	Stillhouse&Barrow	0.343	0.479	0.001	0.509	0.241	0.001	27	13
Schlumberger Spr	Stillhouse&Barrow	0.352	0.460	0.001	0.398	0.378	0.005	2	13
Hamilton Reserve W	Stillhouse&Barrow	0.278	0.650	0.001	0.245	0.770	0.008	5	13
Sierra&Troll&Hearth	Stillhouse&Barrow	0.271	0.673	0.001	0.063	3.701	0.081	7	13
Balcones Park Spr	Tanglewood Spring	0.320	0.530	0.001	0.395	0.382	0.001	4	4
Bull Creek trib. 5 Spr	Tanglewood Spring	0.000	undf	0.411	0.000	undf	0.447	2	4
Bull Creek trib. 6 Spr	Tanglewood Spring	0.191	1.057	0.012	0.098	2.290	0.145	2	4
Franklin Springs	Tanglewood Spring	0.056	4.245	0.039	0.073	3.188	0.088	43	4
Canyon Creek Spr	Tanglewood Spring	0.000	undf	0.412	0.000	undf	0.371	4	4
Lanier Spr & Riffle	Tanglewood Spring	0.090	2.536	0.013	0.032	7.505	0.228	24	4
L. Ribelin&Horsethief	Tanglewood Spring	0.160	1.314	0.002	0.043	5.537	0.153	27	4
Schlumberger Spr	Tanglewood Spring	0.279	0.645	0.014	0.000	undf	0.417	2	4
Hamilton Reserve W	Tanglewood Spring	0.040	5.946	0.165	0.048	4.933	0.141	5	4
Sierra&Troll&Hearth	Tanglewood Spring	0.489	0.261	0.001	0.424	0.339	0.001	7	4
Stillhouse&Barrow	Tanglewood Spring	0.391	0.389	0.001	0.372	0.421	0.001	13	4
Balcones Park Spr	Spicewood&Indian	0.140	1.536	0.003	0.118	1.866	0.048	4	9
Bull Creek trib. 5 Spr	Spicewood&Indian	0.253	0.740	0.003	0.024	10.004	0.289	2	9
Bull Creek trib. 6 Spr	Spicewood&Indian	0.303	0.576	0.001	0.173	1.196	0.100	2	9
Franklin Springs	Spicewood&Indian	0.247	0.764	0.001	0.156	1.350	0.001	43	9
Canyon Creek Spr	Spicewood&Indian	0.231	0.830	0.001	0.071	3.280	0.151	4	9
Lanier Spr & Riffle	Spicewood&Indian	0.342	0.481	0.001	0.201	0.993	0.001	24	9
L. Ribelin&Horsethief	Spicewood&Indian	0.369	0.427	0.001	0.204	0.978	0.001	27	9
Schlumberger Spr	Spicewood&Indian	0.321	0.530	0.001	0.000	undf	0.383	2	9
Hamilton Reserve W	Spicewood&Indian	0.179	1.148	0.001	0.093	2.447	0.091	5	9
Sierra&Troll&Hearth	Spicewood&Indian	0.289	0.614	0.001	0.305	0.569	0.001	7	9
Stillhouse&Barrow	Spicewood&Indian	0.249	0.756	0.001	0.283	0.634	0.001	13	9
Tanglewood Spring	Spicewood&Indian	0.307	0.564	0.001	0.062	3.798	0.156	4	9
Balcones Park Spr	Round Rock Spring	0.138	1.563	0.009	0.575	0.185	0.003	4	3
Bull Creek trib. 5 Spr	Round Rock Spring	0.481	0.269	0.004	0.762	0.078	0.006	2	3
Bull Creek trib. 6 Spr	Round Rock Spring	0.507	0.243	0.004	0.766	0.077	0.006	2	3
Franklin Springs	Round Rock Spring	0.378	0.412	0.001	0.853	0.043	0.001	43	3
Canyon Creek Spr	Round Rock Spring	0.461	0.293	0.001	0.793	0.065	0.001	4	3
Lanier Spr & Riffle	Round Rock Spring	0.511	0.239	0.001	0.860	0.041	0.001	24	3
L. Ribelin&Horsethief	Round Rock Spring	0.537	0.216	0.001	0.857	0.042	0.001	27	3
Schlumberger Spr	Round Rock Spring	0.564	0.193	0.007	0.771	0.074	0.003	2	3
Hamilton Reserve W	Round Rock Spring	0.425	0.338	0.001	0.749	0.084	0.001	5	3
Sierra&Troll&Hearth	Round Rock Spring	0.108	2.059	0.013	0.136	1.587	0.095	7	3
Stillhouse&Barrow	Round Rock Spring	0.285	0.626	0.001	0.371	0.425	0.005	13	3
Tanglewood Spring	Round Rock Spring	0.518	0.233	0.002	0.705	0.104	0.001	4	3
Spicewood&Indian	Round Rock Spring	0.294	0.599	0.001	0.549	0.205	0.001	9	3
Balcones Park Spr	Avery Deer Spring	0.395	0.383	0.001	0.688	0.114	0.002	4	4
Bull Creek trib. 5 Spr	Avery Deer Spring	0.507	0.243	0.003	0.619	0.154	0.011	2	4
Bull Creek trib. 6 Spr	Avery Deer Spring	0.507	0.243	0.003	0.580	0.181	0.014	2	4
Franklin Springs	Avery Deer Spring	0.427	0.335	0.001	0.833	0.050	0.001	43	4
Canyon Creek Spr	Avery Deer Spring	0.467	0.286	0.001	0.672	0.122	0.002	4	4
Lanier Spr & Riffle	Avery Deer Spring	0.538	0.215	0.001	0.807	0.060	0.001	24	4
L. Ribelin&Horsethief	Avery Deer Spring	0.588	0.175	0.001	0.811	0.058	0.001	27	4
Schlumberger Spr	Avery Deer Spring	0.561	0.196	0.002	0.620	0.153	0.010	2	4
Hamilton Reserve W	Avery Deer Spring	0.453	0.301	0.001	0.664	0.127	0.001	5	4
Sierra&Troll&Hearth	Avery Deer Spring	0.410	0.359	0.001	0.495	0.255	0.001	7	4
Stillhouse&Barrow	Avery Deer Spring	0.526	0.225	0.001	0.559	0.197	0.001	13	4
Tanglewood Spring	Avery Deer Spring	0.494	0.256	0.001	0.608	0.161	0.003	4	4
Spicewood&Indian	Avery Deer Spring	0.394	0.384	0.001	0.584	0.178	0.001	9	4
Round Rock Spring	Avery Deer Spring	0.473	0.278	0.001	0.533	0.219	0.005	3	4
Balcones Park Spr	Kretschmarr Sal Cv	0.237	0.804	0.001	0.758	0.080	0.001	4	7
Bull Creek trib. 5 Spr	Kretschmarr Sal Cv	0.395	0.383	0.001	0.812	0.058	0.002	2	7
Bull Creek trib. 6 Spr	Kretschmarr Sal Cv	0.401	0.373	0.001	0.810	0.059	0.001	2	7
Franklin Springs	Kretschmarr Sal Cv	0.262	0.705	0.001	0.913	0.024	0.001	43	7
Canyon Creek Spr	Kretschmarr Sal Cv	0.342	0.482	0.001	0.831	0.051	0.001	4	7

Lanier Spr & Riffle	Kretschmarr Sal Cv	0.407	0.364	0.001	0.904	0.027	0.001	24	7
L. Ribelin&Horsethief	Kretschmarr Sal Cv	0.455	0.299	0.001	0.905	0.026	0.001	27	7
Schlumberger Spr	Kretschmarr Sal Cv	0.354	0.456	0.001	0.814	0.057	0.001	2	7
Hamilton Reserve W	Kretschmarr Sal Cv	0.360	0.445	0.001	0.814	0.057	0.001	5	7
Sierra&Troll&Hearth	Kretschmarr Sal Cv	0.343	0.478	0.001	0.585	0.177	0.001	7	7
Stillhouse&Barrow	Kretschmarr Sal Cv	0.357	0.450	0.001	0.698	0.108	0.001	13	7
Tanglewood Spring	Kretschmarr Sal Cv	0.421	0.343	0.001	0.808	0.060	0.001	4	7
Spicewood&Indian	Kretschmarr Sal Cv	0.321	0.530	0.001	0.745	0.086	0.001	9	7
Round Rock Spring	Kretschmarr Sal Cv	0.352	0.460	0.001	0.322	0.527	0.004	3	7
Avery Deer Spring	Kretschmarr Sal Cv	0.350	0.465	0.001	0.661	0.128	0.001	4	7
Balcones Park Spr	SAS Canyon Springs	0.228	0.846	0.001	0.683	0.116	0.001	4	14
Bull Creek trib. 5 Spr	SAS Canyon Springs	0.350	0.465	0.001	0.739	0.088	0.001	2	14
Bull Creek trib. 6 Spr	SAS Canyon Springs	0.360	0.445	0.001	0.734	0.090	0.001	2	14
Franklin Springs	SAS Canyon Springs	0.254	0.733	0.001	0.875	0.036	0.001	43	14
Canyon Creek Spr	SAS Canyon Springs	0.302	0.578	0.001	0.752	0.082	0.001	4	14
Lanier Spr & Riffle	SAS Canyon Springs	0.374	0.419	0.001	0.848	0.045	0.001	24	14
L. Ribelin&Horsethief	SAS Canyon Springs	0.418	0.348	0.001	0.852	0.043	0.001	27	14
Schlumberger Spr	SAS Canyon Springs	0.330	0.508	0.001	0.739	0.088	0.001	2	14
Hamilton Reserve W	SAS Canyon Springs	0.303	0.575	0.001	0.729	0.093	0.001	5	14
Sierra&Troll&Hearth	SAS Canyon Springs	0.322	0.527	0.001	0.537	0.216	0.001	7	14
Stillhouse&Barrow	SAS Canyon Springs	0.318	0.535	0.001	0.635	0.144	0.001	13	14
Tanglewood Spring	SAS Canyon Springs	0.365	0.435	0.001	0.745	0.085	0.001	4	14
Spicewood&Indian	SAS Canyon Springs	0.287	0.622	0.001	0.719	0.098	0.001	9	14
Round Rock Spring	SAS Canyon Springs	0.327	0.514	0.001	0.252	0.740	0.013	3	14
Avery Deer Spring	SAS Canyon Springs	0.295	0.597	0.001	0.574	0.185	0.001	4	14
Kretschmarr Sal Cv	SAS Canyon Springs	0.000	undf	0.400	0.000	undf	0.360	7	14
Balcones Park Spr	Buttercup Cr. caves	0.380	0.408	0.001	0.763	0.078	0.001	4	8
Bull Creek trib. 5 Spr	Buttercup Cr. caves	0.485	0.265	0.001	0.846	0.045	0.001	2	8
Bull Creek trib. 6 Spr	Buttercup Cr. caves	0.531	0.221	0.001	0.854	0.043	0.001	2	8
Franklin Springs	Buttercup Cr. caves	0.393	0.386	0.001	0.902	0.027	0.001	43	8
Canyon Creek Spr	Buttercup Cr. caves	0.457	0.297	0.001	0.857	0.042	0.001	4	8
Lanier Spr & Riffle	Buttercup Cr. caves	0.487	0.264	0.001	0.902	0.027	0.001	24	8
L. Ribelin&Horsethief	Buttercup Cr. caves	0.522	0.229	0.001	0.900	0.028	0.001	27	8
Schlumberger Spr	Buttercup Cr. caves	0.483	0.268	0.001	0.853	0.043	0.001	2	8
Hamilton Reserve W	Buttercup Cr. caves	0.454	0.301	0.001	0.830	0.051	0.001	5	8
Sierra&Troll&Hearth	Buttercup Cr. caves	0.459	0.295	0.001	0.592	0.172	0.001	7	8
Stillhouse&Barrow	Buttercup Cr. caves	0.437	0.322	0.001	0.661	0.128	0.001	13	8
Tanglewood Spring	Buttercup Cr. caves	0.496	0.254	0.001	0.822	0.054	0.001	4	8
Spicewood&Indian	Buttercup Cr. caves	0.427	0.336	0.001	0.741	0.087	0.001	9	8
Round Rock Spring	Buttercup Cr. caves	0.486	0.265	0.001	0.463	0.290	0.001	3	8
Avery Deer Spring	Buttercup Cr. caves	0.540	0.213	0.001	0.765	0.077	0.001	4	8
Kretschmarr Sal Cv	Buttercup Cr. caves	0.434	0.326	0.001	0.487	0.263	0.001	7	8
SAS Canyon Springs	Buttercup Cr. caves	0.408	0.363	0.001	0.415	0.352	0.001	14	8
Balcones Park Spr	Testudo Tube	0.509	0.241	0.002	0.916	0.023	0.002	4	3
Bull Creek trib. 5 Spr	Testudo Tube	0.738	0.089	0.008	0.972	0.007	0.003	2	3
Bull Creek trib. 6 Spr	Testudo Tube	0.771	0.074	0.006	0.970	0.008	0.002	2	3
Franklin Springs	Testudo Tube	0.497	0.253	0.001	0.935	0.017	0.001	43	3
Canyon Creek Spr	Testudo Tube	0.641	0.140	0.002	0.964	0.009	0.001	4	3
Lanier Spr & Riffle	Testudo Tube	0.612	0.158	0.001	0.942	0.015	0.001	24	3
L. Ribelin&Horsethief	Testudo Tube	0.643	0.139	0.001	0.939	0.016	0.001	27	3
Schlumberger Spr	Testudo Tube	0.805	0.061	0.005	0.981	0.005	0.011	2	3
Hamilton Reserve W	Testudo Tube	0.616	0.156	0.001	0.946	0.014	0.001	5	3
Sierra&Troll&Hearth	Testudo Tube	0.539	0.214	0.001	0.720	0.097	0.001	7	3
Stillhouse&Barrow	Testudo Tube	0.558	0.198	0.001	0.776	0.072	0.001	13	3
Tanglewood Spring	Testudo Tube	0.686	0.114	0.001	0.895	0.029	0.001	4	3
Spicewood&Indian	Testudo Tube	0.519	0.232	0.001	0.787	0.068	0.001	9	3
Round Rock Spring	Testudo Tube	0.600	0.167	0.003	0.748	0.084	0.005	3	3
Avery Deer Spring	Testudo Tube	0.670	0.123	0.003	0.604	0.164	0.001	4	3
Kretschmarr Sal Cv	Testudo Tube	0.597	0.169	0.001	0.569	0.190	0.001	7	3
SAS Canyon Springs	Testudo Tube	0.542	0.211	0.001	0.396	0.381	0.001	14	3
Buttercup Cr. caves	Testudo Tube	0.427	0.336	0.001	0.721	0.097	0.001	8	3
Balcones Park Spr	Kelly Hollow Spring	0.154	1.371	0.003	0.613	0.158	0.001	4	4

Bull Creek trib. 5 Spr	Kelly Hollow Spring	0.365	0.435	0.002	0.796	0.064	0.004	2	4
Bull Creek trib. 6 Spr	Kelly Hollow Spring	0.380	0.408	0.002	0.816	0.056	0.003	2	4
Franklin Springs	Kelly Hollow Spring	0.311	0.553	0.001	0.882	0.033	0.001	43	4
Canyon Creek Spr	Kelly Hollow Spring	0.358	0.448	0.001	0.822	0.054	0.001	4	4
Lanier Spr & Riffle	Kelly Hollow Spring	0.444	0.312	0.001	0.888	0.031	0.001	24	4
L. Ribelin&Horsethief	Kelly Hollow Spring	0.490	0.261	0.001	0.886	0.032	0.001	27	4
Schlumberger Spr	Kelly Hollow Spring	0.442	0.315	0.002	0.799	0.063	0.002	2	4
Hamilton Reserve W	Kelly Hollow Spring	0.355	0.454	0.001	0.791	0.066	0.001	5	4
Sierra&Troll&Hearth	Kelly Hollow Spring	0.237	0.807	0.001	0.387	0.395	0.003	7	4
Stillhouse&Barrow	Kelly Hollow Spring	0.355	0.455	0.001	0.553	0.202	0.001	13	4
Tanglewood Spring	Kelly Hollow Spring	0.417	0.349	0.001	0.762	0.078	0.001	4	4
Spicewood&Indian	Kelly Hollow Spring	0.296	0.595	0.001	0.616	0.156	0.001	9	4
Round Rock Spring	Kelly Hollow Spring	0.233	0.823	0.004	0.285	0.627	0.021	3	4
Avery Deer Spring	Kelly Hollow Spring	0.359	0.446	0.002	0.735	0.090	0.001	4	4
Kretschmarr Sal Cv	Kelly Hollow Spring	0.273	0.667	0.001	0.467	0.285	0.003	7	4
SAS Canyon Springs	Kelly Hollow Spring	0.273	0.664	0.001	0.422	0.342	0.001	14	4
Buttercup Cr. caves	Kelly Hollow Spring	0.417	0.349	0.001	0.334	0.499	0.001	8	4
Testudo Tube	Kelly Hollow Spring	0.521	0.230	0.002	0.816	0.056	0.002	3	4
Balcones Park Spr	House Spring	0.159	1.324	0.001	0.667	0.125	0.001	4	7
Bull Creek trib. 5 Spr	House Spring	0.391	0.389	0.001	0.772	0.074	0.001	2	7
Bull Creek trib. 6 Spr	House Spring	0.435	0.324	0.002	0.777	0.072	0.001	2	7
Franklin Springs	House Spring	0.318	0.535	0.001	0.895	0.029	0.001	43	7
Canyon Creek Spr	House Spring	0.393	0.387	0.001	0.794	0.065	0.001	4	7
Lanier Spr & Riffle	House Spring	0.455	0.300	0.001	0.885	0.032	0.001	24	7
L. Ribelin&Horsethief	House Spring	0.482	0.268	0.001	0.886	0.032	0.001	27	7
Schlumberger Spr	House Spring	0.422	0.343	0.002	0.775	0.073	0.001	2	7
Hamilton Reserve W	House Spring	0.365	0.435	0.001	0.770	0.075	0.001	5	7
Sierra&Troll&Hearth	House Spring	0.257	0.722	0.001	0.471	0.281	0.001	7	7
Stillhouse&Barrow	House Spring	0.317	0.538	0.001	0.623	0.151	0.001	13	7
Tanglewood Spring	House Spring	0.446	0.310	0.001	0.768	0.076	0.001	4	7
Spicewood&Indian	House Spring	0.301	0.581	0.001	0.702	0.106	0.001	9	7
Round Rock Spring	House Spring	0.233	0.824	0.001	0.168	1.239	0.052	3	7
Avery Deer Spring	House Spring	0.411	0.358	0.001	0.678	0.119	0.001	4	7
Kretschmarr Sal Cv	House Spring	0.245	0.772	0.001	0.165	1.263	0.031	7	7
SAS Canyon Springs	House Spring	0.245	0.772	0.001	0.173	1.196	0.011	14	7
Buttercup Cr. caves	House Spring	0.309	0.559	0.001	0.251	0.745	0.001	8	7
Testudo Tube	House Spring	0.470	0.282	0.001	0.629	0.147	0.001	3	7
Kelly Hollow Spring	House Spring	0.121	1.809	0.004	0.168	1.234	0.035	4	7
Balcones Park Spr	MacDonald Well Spr	0.142	1.515	0.006	0.629	0.148	0.001	4	5
Bull Creek trib. 5 Spr	MacDonald Well Spr	0.412	0.357	0.003	0.767	0.076	0.001	2	5
Bull Creek trib. 6 Spr	MacDonald Well Spr	0.432	0.329	0.003	0.777	0.072	0.002	2	5
Franklin Springs	MacDonald Well Spr	0.325	0.518	0.001	0.891	0.030	0.001	43	5
Canyon Creek Spr	MacDonald Well Spr	0.394	0.384	0.001	0.796	0.064	0.001	4	5
Lanier Spr & Riffle	MacDonald Well Spr	0.462	0.291	0.001	0.887	0.032	0.001	24	5
L. Ribelin&Horsethief	MacDonald Well Spr	0.504	0.246	0.001	0.887	0.032	0.001	27	5
Schlumberger Spr	MacDonald Well Spr	0.435	0.325	0.002	0.770	0.075	0.002	2	5
Hamilton Reserve W	MacDonald Well Spr	0.386	0.397	0.001	0.770	0.075	0.001	5	5
Sierra&Troll&Hearth	MacDonald Well Spr	0.227	0.851	0.001	0.404	0.369	0.001	7	5
Stillhouse&Barrow	MacDonald Well Spr	0.347	0.469	0.001	0.584	0.178	0.001	13	5
Tanglewood Spring	MacDonald Well Spr	0.452	0.303	0.001	0.756	0.081	0.001	4	5
Spicewood&Indian	MacDonald Well Spr	0.301	0.580	0.001	0.666	0.125	0.001	9	5
Round Rock Spring	MacDonald Well Spr	0.264	0.696	0.002	0.131	1.659	0.100	3	5
Avery Deer Spring	MacDonald Well Spr	0.396	0.382	0.001	0.687	0.114	0.001	4	5
Kretschmarr Sal Cv	MacDonald Well Spr	0.213	0.923	0.001	0.284	0.629	0.005	7	5
SAS Canyon Springs	MacDonald Well Spr	0.223	0.873	0.001	0.272	0.670	0.004	14	5
Buttercup Cr. caves	MacDonald Well Spr	0.327	0.514	0.001	0.244	0.774	0.003	8	5
Testudo Tube	MacDonald Well Spr	0.537	0.215	0.001	0.699	0.108	0.002	3	5
Kelly Hollow Spring	MacDonald Well Spr	0.128	1.703	0.010	0.031	7.845	0.289	4	5
House Spring	MacDonald Well Spr	0.008	30.509	0.329	0.000	undf	0.400	7	5
Balcones Park Spr	Wheless Spring	0.359	0.447	0.001	0.733	0.091	0.001	4	24
Bull Creek trib. 5 Spr	Wheless Spring	0.506	0.244	0.001	0.850	0.044	0.001	2	24
Bull Creek trib. 6 Spr	Wheless Spring	0.546	0.208	0.001	0.863	0.040	0.001	2	24

Franklin Springs	Wheless Spring	0.415	0.352	0.001	0.881	0.034	0.001	43	24
Canyon Creek Spr	Wheless Spring	0.491	0.259	0.001	0.848	0.045	0.001	4	24
Lanier Spr & Riffle	Wheless Spring	0.504	0.246	0.001	0.880	0.034	0.001	24	24
L. Ribelin&Horsethief	Wheless Spring	0.531	0.221	0.001	0.879	0.035	0.001	27	24
Schlumberger Spr	Wheless Spring	0.548	0.206	0.001	0.851	0.044	0.001	2	24
Hamilton Reserve W	Wheless Spring	0.480	0.271	0.001	0.819	0.055	0.001	5	24
Sierra&Troll&Hearth	Wheless Spring	0.369	0.427	0.001	0.605	0.164	0.001	7	24
Stillhouse&Barrow	Wheless Spring	0.440	0.318	0.001	0.669	0.124	0.001	13	24
Tanglewood Spring	Wheless Spring	0.529	0.222	0.001	0.835	0.049	0.001	4	24
Spicewood&Indian	Wheless Spring	0.460	0.293	0.001	0.763	0.078	0.001	9	24
Round Rock Spring	Wheless Spring	0.445	0.312	0.001	0.494	0.256	0.003	3	24
Avery Deer Spring	Wheless Spring	0.558	0.198	0.001	0.829	0.052	0.001	4	24
Kretschmarr Sal Cv	Wheless Spring	0.483	0.267	0.001	0.586	0.177	0.001	7	24
SAS Canyon Springs	Wheless Spring	0.456	0.298	0.001	0.544	0.209	0.001	14	24
Buttercup Cr. caves	Wheless Spring	0.372	0.422	0.001	0.258	0.718	0.001	8	24
Testudo Tube	Wheless Spring	0.485	0.266	0.001	0.813	0.057	0.001	3	24
Kelly Hollow Spring	Wheless Spring	0.283	0.635	0.001	0.042	5.664	0.158	4	24
House Spring	Wheless Spring	0.382	0.405	0.001	0.283	0.634	0.001	7	24
MacDonald Well Spr	Wheless Spring	0.336	0.494	0.001	0.180	1.139	0.013	5	24