EVOLUTION, PHYLOGENY, BIOGEOGRAPHY, AND TAXONOMY OF CENTRAL TEXAS SPRING AND CAVE SALAMANDERS, *EURYCEA* AND *TYPHLOMOLGE* (PLETHODONTIDAE: HEMIDACTYLIINI)

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by

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DISSERTATION

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

THE UNIVERSITY OF TEXAS AT AUSTIN

December 1995

This dissertation is dedicated to my parents,

Sonia K. and Nigel K. Chippindale,

for their support, love and encouragement

throughout my life.

ACKNOWLEDGMENTS

Many people contributed to the success of this project. In addition to the valuable advice that he provided throughout, David Hillis often accompanied me with unbridled enthusiasm to even the slimiest of salamander caves. Andy Price was a great field companion and helped in more ways than I can count, including provision of much-needed moral support on many occasions. Jim Bull, my co-supervisor, was a wonderful source of ideas, and due to his unique thinking processes could be counted on for unconventional insights into many problems. David Cannatella provided key advice throughout this project, shared his taxonomic expertise, and allowed me access to the extensive herpetological collection of the Texas Memorial Museum. Mark Kirkpatrick was a great source of ideas and support and took a keen interest in many aspects of this study. Tim Rowe offered much-appreciated advice on project design, data analysis, and interpretation on many occasions. The caving expertise of Andy Grubbs made it possible for me to visit many subterranean salamander habitats that otherwise would have been inaccessible, and his knowledge of central Texas caves was invaluable. James Reddell was a fountain of knowledge regarding central Texas caves and cave and spring salamanders. George Veni provided many important insights into Edwards Plateau cave hydrogeology and arranged access to key caves. Bill Russell shared his experience and knowledge of Texas caves and also provided important specimens. Sam Sweet's detailed studies of the central Texas *Eurycea* and the information that he provided formed a large part

of the foundation of this study. David Wake was a great source of ideas, allowed me to examine crucial specimens from the herpetological collection of the Museum of Vertebrate Zoology at The University of California at Berkeley, and provided key tissue samples. Others who contributed to the project by providing specimens, access to important localities, field companionship, or valuable information include Bill Elliott, Basset Maguire, Lisa O'Donnell, Landon Lockett, Doyle Mosier, Robert Hansen, Glenn Longley, Joel Hunter, and Riley Nelson. I also thank the numerous central Texas landowners who cheerfully allowed me to search for salamanders on their properties; only once was I denied access to a salamander locality. Debbie Dorsey, Robert Sopryoni, and the Barton Springs Pool staff facilitated field studies of the Barton Springs salamander. Wayne Van Devender and Paul Moler kindly provided specimens of many key outgroup taxa. Jewel Husti was an invaluable assistant in the laboratory. I greatly appreciate Janet Young's help in map preparation. Marsha Berkman saw to it that I met the crucial deadlines and requirements that I so often forgot, and rescued me from the wrath of the Graduate School on more than one occasion. Jo Bunny enthusiastically organized scheduling of my defense and a myriad of associated details. Marty Badgett was a constant source of technical advice (and wisecracks) and facilitated many aspects of the lab work. John Wiens offered frequent advice on diverse topics ranging from phylogenetic analysis to osteology. In the course of this project I interacted with many other members of the Hillis-Bull laboratory and benefited greatly from their

ideas and support. These include (in no particular order) Barb Mable, Ken Halanych, Cliff Cunningham, Sharon Messenger, John Huelsenbeck, Jimmy McGuire, Rafael de Sa, Loren Ammerman, Mike Dixon, Tod Reeder, Wayne Crill, Keith Crandall, Brian Crother, Chris Austin, Mary White, Felix Breden and others too numerous to mention. Other members of the department whose insights and support were invaluable include Mike Singer, Mike Ryan, Gad Perry, and Marie Kerbeshian. My parents, Sonia and Nigel Chippindale, have been a tremendous source of support, and I can never thank them enough for all their help. I most especially thank Cynthia, my wife, for her tireless help in last-minute dissertation preparation and her loving devotion despite many trying times. This work was funded by a Cave Research Foundation Karst Fellowship, an NSF Dissertation Improvement Grant, and Section 6 cooperative funding from the US Fish and Wildlife Service and Texas Parks and Wildlife Department. I also received funding for personal support in the form of an NSERC postgraduate fellowship and several scholarships from the University of Texas at Austin Department of Zoology and Graduate School.

EVOLUTION, PHYLOGENY, BIOGEOGRAPHY, AND TAXONOMY OF CENTRAL TEXAS SPRING AND CAVE SALAMANDERS, *EURYCEA* AND *TYPHLOMOLGE* (PLETHODONTIDAE: HEMIDACTYLIINI)

Publication No. _____

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The University of Texas at Austin, 1995

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Numerous populations of aquatic salamanders of the genera *Eurycea* and *Typhlomolge* (Plethodontidae: Hemidactyliini) inhabit caves and springs in the Edwards Plateau region of central Texas. Species boundaries in the group are uncertain, in part because most members of the group are perennibranchiate (nontransforming) and most spring-dwellers from throughout the region are morphologically similar. Cave-dwellers exhibit a range of troglobitic morphologies

that may be due to parallelism or convergence. Relationships of the extreme troglobites of the genus *Typhlomolge* have been especially problematic. Data for 25 allozyme loci and up to 355 bp of the mitochondrial cytochrome b gene were used to investigate relationships and species boundaries in the group. High levels of molecular divergence are present, particularly among populations formerly assigned to the species *E. neotenes*, and numerous previously unrecognized species exist. Based on phenetic and phylogenetic analyses, the basal split is between populations north versus south of the Colorado River, and the southern group exclusive of *Typhlomolge* is further divided into southeastern and southwestern clades. *Typhlomolge* appears to be sister to other southern taxa; thus its members should be considered morphologically divergent *Eurycea*. Patterns of allozyme variation suggest localized bottlenecks, perhaps due to spring drying; many cave populations may have arisen from surface populations forced underground by such events. Low levels of sequence variation among southeastern populations may reflect a bottleneck for the ancestor of this group. Geological considerations suggest multiple origins of subterranean living throughout the region. However, distributions of cave- versus surface-dwellers on the preferred phylogenetic tree suggest the possibility of a subterranean ancestor. The evolutionary history of perennibranchiation in the group remains unclear due to the possibility of multiple genetic bases for this trait; from a strictly phylogenetic perspective, ancestral perennibranchiation is the most parsimonious hypothesis. Much more diversity likely remains to be discovered in the group, but loss of numerous species appears imminent.

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CHAPTER 1:

EVOLUTION, PHYLOGENY AND BIOGEOGRAPHY OF THE CENTRAL TEXAS HEMIDACTYLIINE PLETHODONTID SALAMANDERS

INTRODUCTION

The Edwards Plateau region of central Texas is characterized by Cretaceous limestones uplifted since at least mid-Tertiary times, dissected and eroded to form numerous springs and caves (see Sweet 1978a, Potter and Sweet 1981, Woodruff and Abbott 1986, and Veni 1994 for reviews of the geologic history of the area). These habitat islands are inhabited by a variety of endemic aquatic organisms, many with extremely restricted distributions. Predominant among the aquatic vertebrate fauna of the region are plethodontid salamanders of the genera *Eurycea* and *Typhlomolge* (tribe Hemidactyliini), almost all of which are perennibranchiate (i.e. retain gills and other larval morphological features throughout their lives; this condition is often termed paedomorphosis, but see Gould [1977] for a detailed discussion of heterochronic terminology). Members of this group exhibit a wide range of morphologies associated primarily with surface versus subterranean dwelling. *T. rathbuni*, the first member of the group to be described, was discovered after the drilling of the Artesian Well at San Marcos, Hays Co. in 1895 (Stejneger 1896). This large troglobitic salamander immediately captured the attention of the scientific community due to its seemingly bizarre morphology, including depigmentation, tiny nonfunctional vestiges of eyes, long slender legs, and a broad flattened head; this species and the presumed sister taxon *T. robusta*

continue to be regarded as possessing some of the most extreme cave-associated morphologies known among vertebrates (e.g. Potter and Sweet 1981, Sweet 1986) Recognized as a plethodontid by Emerson (1905) and later placed in the tribe Hemidactyliini by Wake (1966), the relationships of *Typhlomolge* to other hemidactyliines have remained controversial (e.g. Mitchell and Reddell 1965, Mitchell and Smith 1972, Potter and Sweet 1981, Lombard and Wake 1986, Chapter 2).

Additional central Texas hemidactyliines were not recognized until 1937, when Bishop and Wright described *E. neotenes* from a spring near Helotes in Bexar Co. In subsequent decades, several more spring and cave species were described: *E. nana* Bishop 1941 from San Marcos Springs in Hays Co., *E. latitans* Smith and Potter 1946 from Cascade Caverns in Kendall Co., *E. pterophila* Burger, Smith and Potter 1950 from Fern Bank Springs in Hays Co., *E. troglodytes* Baker 1957 from Valdina Farms Sinkhole in Medina Co, *E. tridentifera* Mitchell and Reddell 1965 from Honey Creek Cave in Comal Co., and *E. sosorum* Chippindale, Price and Hillis 1993 from Barton Springs in Travis Co. The status and relationships of these taxa have been problematic; most recently Sweet (1978a, b, 1984) regarded almost all spring and cave populations from throughout the region as *E. neotenes*, synonymizing under this name *E. pterophila* and relegating the morphologically variable cave-dwellers *E. latitans* and *E. troglodytes* to the status of a hybrid swarm derived from surface *E. neotenes* and subterranean *E. tridentifera* (*E. troglodytes*), and a population of *E. neotenes* that had experienced past introgression from *E. tridentifera* (*E. latitans*).

With the exception of Bogart's (1967) chromosomal studies, all inferences of relationships among the central Texas hemidactyliines have been based on morphology, and no truly phylogenetic analysis of the group has been attempted. Morphological variation in the group has proven confusing; most surface dwellers from throughout the region appear similar to one another based on external morphology (e.g. Mitchell and Smith 1972, Hamilton 1973, Sweet 1978a, 1982, Chippindale et al. 1993), while cave-dwellers display a spectrum of degrees of cave-associated morphologies and combinations of character states (Mitchell and Reddell 1965, Mitchell and Smith 1972, Sweet 1978a, 1984, Potter and Sweet 1981). Based on biogeographic and geologic considerations, multiple invasions of subterranean habitat are likely to have occurred (Mitchell and Smith 1972, Sweet 1978a, 1982, 1984, Potter and Sweet 1981), but in at least some cases (especially in the areas inhabited by *E. tridentifera*, *E. latitans*, and perhaps *E. troglodytes*) there is the potential for subterranean gene flow among cave populations (Sweet 1978a, 1984).

Given the nature of morphological variation in the group and the potential for parallel or convergent morphological evolution, I chose to use molecular markers (allozymes and mitochondrial DNA) to investigate the evolutionary history and relationships of the central Texas hemidactyliines. In doing so I assumed that these characters are less likely than the morphological features examined so far to be influenced in any particular direction by selection or drift (e.g. to fixation of loss of function mutations that yield similar phenotypes); see Moritz and Hillis

(1990) for further discussion of the neutrality of molecular variants. Work currently is underway by J.J. Wiens and me to investigate morphological variation in hemidactyliines in detail, and to determine the phylogenetic utility of this variation; here I will focus on the results of the molecular studies.

In this study, I sought to characterize genetic variation and diversity in the central Texas hemidactyliines, identify species boundaries, and determine the relationship between the phylogenetic history of the group and the biogeographic and geologic history of the Edwards Plateau region. A key issue is whether the group is monophyletic; Wake (1966) and Potter and Sweet (1981) suggested that *Typhlomolge* may be derived from an early Tertiary invasion of the area whereas *Eurycea* arrived later (perhaps in the late Miocene or Plio-Pleistocene). According to this scenario, the cave-associated morphology of the ancestor of *Typhlomolge* evolved independently in response to the subterranean environment, and perhaps the extreme nature of the troglobitic morphologies seen in this genus reflects the long period of time spent underground. The superficially similar but less extreme troglobitic morphologies seen in the central Texas *Eurycea* could then represent convergences with *Typhlomolge*, varying in degree according to the length of time spent underground and constrained by the common ancestry of members of the Edwards Plateau *Eurycea* group. For example, Potter and Sweet (1981) demonstrated that while the same general evolutionary trends in head morphology are apparent in *Typhlomolge* and some central Texas cave *Eurycea* (broadening and flattening of the skull), the osteological basis of these changes differs in the two genera. Perennibranchiation also could have evolved repeatedly in different central

Texas hemidactyliines, perhaps driven by late Tertiary and/or Pleistocene drying of the central Texas climate (Blair 1965, Wake 1966, Sweet 1977). A few naturally metamorphosing populations of *Eurycea* occur in the west-central Edwards Plateau region (Sweet 1977), suggesting either that in this area salamanders have simply retained the ability to transform (as suggested by Sweet 1977, 1978a, 1986), or that the metamorphosing condition is a reversal from an ancestral perennibranchiate state.

Extreme cave-associated morphologies are seen in some other non-Texan hemidactyliine plethodontids, including *Typhlotriton spelaeus* from the Ozark region and *Haideotriton wallacei* from northern Florida and Georgia. The latter is perennibranchiate, whereas the former transforms. I included these taxa in the present study; both are thought to have close affinities to *Eurycea* and/or *Typhlomolge* (Wake 1966, Lombard and Wake 1986). I also included representatives of all species groups currently recognized within *Eurycea*. Most described members of this eastern North American genus from outside of central Texas are transforming surface dwellers, but perennibranchiate forms are known in at least one of the loosely-defined species groups, the *E. multiplicata* group from the Ozark region. By including representatives of the full range of taxa thought or suspected to be closely related to the central Texas hemidactyliines, I was able to address monophyly of the central Texas group and at the same time investigate patterns of change in external morphology and life history (perennibranchiation versus transformation and surface- versus cave-dwelling) at a variety of phylogenetic levels, with the main emphasis on the central Texas group. This

system is ideal for such a study because it offers both a hierarchy of relationships (from higher level to interpopulation in central Texas), multiple replicates in the form of presumably repeated instances of cave colonization, and possible multiple origins of perennibranchiation. Recovery of a phylogeny for the group thus is fundamental to understanding the patterns of evolutionary change that have led to differentiation and life history mode shifts in the central Texas hemidactyliines.

MATERIALS AND METHODS

Salamanders were collected from springs and caves throughout the Edwards Plateau region and returned to the laboratory alive, where they were dissected for appropriate tissues (see below) after anaesthesia with MS-222 (Sigma). Specimens not destroyed in the course of sample preparation were, or will be, deposited into the collection of the Texas Memorial Museum (Austin, Texas). Precise localities for all populations sampled are given in Appendix 1, and localities are mapped in Fig. 1. Tissues were stored at - 80 C prior to preparation of samples for allozyme or DNA analysis.

Outgroup taxa

For all parsimony analyses (see below), eight outgroup taxa were used to root the trees. I chose taxa that span the range of morphological and genetic divergence in the genus *Eurycea*, plus other hemidactyliine genera that are suspected to be closely related to or nested within *Eurycea*. I based the choice of

outgroup members on published morphological work (Wake 1966, Sweet 1977, Lombard and Wake 1986) and molecular data from a study in progress of higher-level hemidactyliine relationships (Chippindale, unpublished). The outgroup consisted of *E. bislineata* (Renfrew Co. Ont.), *E. wilderae* (Watauga Co. NC), *E. quadridigitata* (yellow-bellied form, Tyler Co. TX), *E. quadridigitata* (silver-bellied form, Charleston Co. SC), *E. l. longicauda* (Baltimore Co. MD), *E. m. multiplicata* (Polk Co. AR), *Haideotriton wallacei* (Jackson Co. FL), and *Typhlotriton spelaeus* (Stone Co. MO).

Allozyme electrophoresis

I examined a total of 357 individual central Texas *Eurycea* for allozyme variation, representing 64 populations or taxa (see also *Population groups*, below). Early in the study, I homogenized many salamanders whole in a solution of 0.001 M EDTA and 0.010 M Tris (Sigma), pH 7.5, using approximately 1:1 w/v proportions of tissue to grinding solution. I subsequently found that destruction of entire specimens was unnecessary, and so for most later allozyme work I used a combination of skeletal muscle, heart, liver, and gut homogenized approximately 1:1 w/v in the above solution using an electric tissue grinder. Homogenates were spun for 3-5 minutes at 13,000 rpm, and 8-10 uL of the resulting supernatant was used to soak filter paper wicks for electrophoresis. Electrophoretic methods and staining procedures generally followed Murphy et al. (1990); electrophoretic conditions used for resolution of different enzyme-encoding loci are listed in Table 1. I screened 25 loci for which banding patterns were readily interpretable and activity was strong, and rejected numerous others for which activity levels were

highly variable among individuals, resolution was poor, or mobility patterns were not consistently reproducible. I made the following changes to the stain recipes described by Murphy et al. (1990):

1) Half volumes were used for all liquid stains except GR, G3PDH, IDH-1, IDH-2, and PGDH, and third or quarter volumes were used for agar overlays;
2) For the IDH stains, I substituted 0.08 g dry isocitric acid for the liquid form, and used pH 7.0 Tris/HCl buffer, because IDH-1 often showed little activity at pH 8.0;
3) In stains for AK, CK, and PK, 200 U of hexokinase were used (rather than the recommended 20 U);
4) Glucose-1-phosphate (Sigma G-1259) was used as the PGM substrate;
5) To stain for GR, I used 13 mL of Tris/HCl pH 8.0 , 0.5 mL 0.5 mg/mL DCIP, 0.002 g FAD, 0.01 g NADH, 0.02 g oxidized glutathione, and 0.5 mL 5 mg/mL MTT;
7) To stain for AAT, I used 12.5 mL of the following stock (to which we added 12.5 mL Tris/HCl pH 8.0 and 0.05 g Fast Blue BB): 500 mL water, 0.37 g alpha-ketoglutaric acid, 1.33 g L-aspartic acid, 2.50 g polyvinyl pyrrolidone, 0.5 g Na_2EDTA, and 14.20 g Na_2HPO_4.

DNA amplification and sequencing

Sequence data were gathered for 34 populations of central Texas *Eurycea*, including representatives from throughout the geographic range sampled for allozyme variation, populations that proved substantially divergent based on the

allozyme data, and all described species (whether currently recognized or not) except *T. robusta*, for which the presumed subterranean habitat is now inaccessible. Using PCR, I amplified a fragment of roughly 400 bp of the 5' end of the mitochondrial cytochrome b (cytb) gene , with primers described by Moritz et al. (1992) or slight modifications of these. The primers were:

light strand: primer MVZ 15: GAACTAATGGCCCACAC(AT)(AT)TACGNAA
heavy strand: primer CB2H: CCCCTCAGAATGATATTTGTCCTCA

A map of the cytb gene and the locations of these primers is provided by Moritz et al. (1992); CB2H is a truncated version of their cyt-b2 primer.

For most specimens, DNA was extracted from tail or liver tissue using the STE method described by Hillis et al. (1990); 1 -2 mL of the resulting solution was then diluted in 50 uL of 1X TE (e.g. Hillis et al. 1990) for PCR. For *E. troglodytes* (for which no allozyme data are available, and which may now be extinct) I used supernatant from an allozyme sample prepared in the mid-1970s by S. Sweet and provided by D. Wake, and applied a modification of the Chelex extraction method (Walsh et al. 1991). This method also was used for the Bat Well, Greenwood Springs, Cloud Hollow Spring, *E. tridentifera* (Ebert Cave) and *E. latitans* specimens. The method is as follows: A 5.0% solution of Chelex-100 (Biorad) was prepared using distilled water, autoclaved, and 500 uL was placed in each of several heat-resistant tubes. I then added 50 uL of allozyme supernatant (*E. troglodytes*) or a tiny fragment of liver or muscle (other specimens) to each tube

and placed the samples in a 55 C water bath for about 3 h, shaking occasionally. The samples were then vortexed briefly, heated to 95 C for 15 min, vortexed again, and centrifuged briefly to precipitate the Chelex.

PCR was performed using an Ericomp or MJ Research PTC-100 thermal cycler. PCR conditions that yielded the most consistent amplifications were as follows. Samples consisted of 3-6 uL dilute DNA (for Chelex extractions, 2 uL of DNA solution plus 2 uL of a 1 in 50 dilution of Chelex solution), 0.1 uM each primer, 40 uM dNTPs, standard Taq polymerase buffer (1.5 mM $MgCl_2$), and 1-2 U Taq polymerase in a total volume of 50 uL. Temperature cycling usually used was: Step 1: 94 C 1.5 min. (X 1)/ Step 2: 94 C 30 sec, 50 C 30 or 45 sec, 72 C 1 min. (X 34)/Step 3: 72 C 5 min. (X 1).

Greatest recovery of amplified DNA was accomplished using the method of Zhen and Swank (1994): 20-25 uL of the PCR sample was electrophoresed through a nonsubmerged gel of 1.5% agarose in TAE (Moritz et al. 1990) and the band of interest (stained with ethidium bromide) was allowed to run into a small well cut in the gel, which contained roughly 50 uL of a solution of 15% PEG 800 and 2X TAE. Initially, standard Sanger sequencing was performed using purified PCR product and a modification of the method described by Hillis et al. (1990). However, generation of single-stranded DNA required use of asymmetric PCR methods (e.g. Gyllensten and Ehrlich 1988), which did not work reliably. Therefore, most sequencing was done using cycle sequencing methods (e.g. Fulton and Wilson 1994), as follows. Six uL of gel-purified PCR product was added to a

tube containing 3 uL 0.4 uM sequencing primer [previously end-labelled with ¥-ATP (^{32}P) using polynucleotide kinase], 25 uL 1X cycle sequencing buffer (30 mM Tris/HCl pH 9.0, 50 mM $MgCl_2$, 300 mM KCl, 0.25% Tween 20, 0.25% NP40), and 1-2 U Taq polymerase, for a total volume of 35-36 uL. Eight uL of this mixture was then added to each of four tubes containing 2 uL A, G, C, or T termination solution for a total volume of 10 uL per tube. Termination solutions were 100 uM in dATP, dGTP, dTTP, and dCTP; the A solution was 2.0 mM in ddA, the G 0.2 mM in ddG, the T 2.0 M in ddT, and the C 1.0 M in ddC. A drop of sterile mineral oil was added as an overlay, and temperature cycling was performed in either of the thermal cyclers described above. Conditions that yielded the most consistent results were: Step 1: 94 C 1.5 min (X 1)/Step 2: 94 C 30 sec, 55 C 30 sec, 72 C 1 min (X 34)/Step 3: 94 C 30 sec, 72 C 1 min (X 5). However, successful results also were obtained with as few as 15 iterations of step 2 and as many as 15 iterations of step 3.

After adding 5 uL formamide/bromophenol blue/xylene cyanol stop dye to each reaction tube, 3-4 uL of each reaction mixture was used per well in a standard DNA sequencing gel (e.g. Hillis et al. 1990). Gels were dried into Whatman 3 filter paper without fixation and exposed to Kodak X-Omat AR or Biomax film for 1 - 4 days at room temperature without intensifying screens.

I sequenced each sample using both MVZ 15 and CB2H as sequencing primers, with substantial overlap in the middle region of the fragment for most samples. The Pedernales sample proved difficult to sequence using MVZ 15 (this

also was a problem for some more distantly related plethodontids not included in the present study). Therefore, I designed an internal primer which yielded more clearer sequence; its sequence is: 5'TC(ACT)TTTATTGA(CT)CTCCCAGC 3'. Sequences were aligned by eye, and initially were compared to published cytb sequence for plethodontid salamanders of the genus *Ensatina* (Moritz et al. 1992).

To confirm that I was working with the mitochondrial cytb gene, I purified mtDNA from a specimen from the Sutherland Hollow population using the modified alkaline lysis method of Timura and Aotsuka (1988) and compared its sequence to that of another individual from the same population. The sequences were identical for all readable base pairs.

Analysis of allozyme data

An IBM PC version of Swofford and Selander's (1981) Biosys-1 program was used to calculate measures of allozyme variation and genetic distances, and to perform phenetic clustering based on the allozyme data. To assess deviations from Hardy-Weinberg equilibrium, I used chi-square tests and applied Levene's (1949) correction for small sample size. I treated almost all localities as separate populations for phenetic analysis. However, due to the constraint of 60 as the maximum number of populations possible for analysis, I combined several populations that were geographically proximal and identical or near-identical in allelelic composition and frequency as single units to yield a total of 59 "populations". Populations combined were: Barrow Hollow + Stillhouse Hollow Springs; Knight + Cedar Breaks Springs; Pedernales Spring 1 + Spring 2; Murphy's

Spring + Sabinal Canyon Spring; Greenwood Springs 1 + 2 + 3; Cherry Creek Spring + Cloud Hollow Spring; and *E. rathbuni* from Ezell's Cave + Rattlesnake Cave + Diversion Spring.

No activity was observed for the Gr locus in any of the five *E. rathbuni* screened for allozyme variation, nor could I detect activity in any of the six Greenwood Springs individuals for Mdh-2. For the purposes of phenetic analyses and calculation of levels of variation, I treated these individuals as homozygous for unique alleles at these loci. My basis for doing so was the assumption that individuals in these populations possess a unique form of each enzyme, and that the differences have a genetic basis. The main effect of this approach will be a slight reduction in estimates of variability if these individuals were in fact heterozygous at these loci. For parsimony analyses (see below) these cases were simply treated as "missing data".

Phenetic clustering of allozyme data was performed using UPGMA (e.g. Sneath and Sokal 1973) with Manhattan (Prevosti) distances (e.g. Wright 1978); I also used Nei's (1978) unbiased distance and Rogers's (1972) distance for comparison. For parsimony analyses, I employed a method of frequency-based coding developed in conjunction with J. Wiens (personal communication and in press); a near-identical approach was developed independently and simultaneously by S. Berlocher and D. Swofford (D. Swofford, personal communication to PTC). Briefly, the method involves treatment of each different observed array of allele frequencies for a given locus (character) as a unique state. Manhattan distances

(Ds) among states are calculated, here using Biosys-1 (Swofford and Selander 1981). These Ds are then converted to whole numbers (we rounded to two digits) and used as the numbers of steps among states in a step matrix, implemented in Swofford's (1990) PAUP program (for these and all other parsimony analyses described here, I used PAUP version 3.1.2d5).

Analysis of sequence data

In order to assess the relative levels of sequence divergence among central Texas *Eurycea*, I calculated absolute distances among populations and taxa using PAUP and performed UPGMA clustering using Felsenstein's (1991) Phylip program. For presentation (Fig. 5), I converted these distances to approximate percent sequence divergence.

For phylogenetic analyses of the sequence data, I employed two approaches. The first was to simply weight all changes equally, without regard for transitions versus transversions, etc. The second approach involved partitioning of the sequence data into first, second, and third codon positions, followed by combinatorial weighting of changes among bases using Wheeler's (1990) method with Rodrigo's (1992) correction for invariant positions. Values in the resulting three transformation matrices were used as the numbers of steps among alternative bases in three PAUP step matrices (corresponding to first, second, and third codon positions). This approach allowed incorporation in the phylogenetic analysis of information on the frequency and direction of different kinds of changes, based on observed patterns in the data set. However, this method yields asymmetric step

matrices, which slows tree searching tremendously. Therefore, I averaged values across the diagonal of each matrix to produce symmetric step matrices (e.g. we used the mean of C to T and T to C changes in both the "C to T" and "T to C" cells of the step matrix). In most cases, the differential was relatively small (see Table 2), and thus this approach is unlikely to have had a strong effect on the outcome of the analyses.

Combined data analyses

I conducted two kinds of parsimony analyses in which I combined the allozyme and DNA data. First, I used the allozyme data (coded using loci as characters and Manhattan D step matrices, as described above) plus the DNA data with no combinatorial weighting. For these analyses, I weighted each DNA character (position) 100X the value of each allozyme character, because the treatment of the allozyme characters allowed up to 100 "steps" (Manhattan D units) among states (alternative frequency arrays at each locus). Thus, I allowed each sequence character to make a contribution to the analysis equal to that of each allozyme character. Second, I used the allozyme characters, coded as described above, plus the sequence data partitioned by codon position with the application of combinatorial weights. In these analyses, I scaled the DNA step matrix values to 100 for equivalence (with respect to relative weight in parsimony analyses) between changes at each allozyme locus and changes at each sequence position.

combinatorial weights or partitioning by codon position; 3) sequence data only with combinatorial weights, partitioned by codon position; 4) allozyme data with Manhattan D step matrices plus sequence data without combinatorial weights; and 5) allozyme data with Manhattan D step matrices plus sequence data partitioned by codon position with combinatorial weighting. I assessed confidence in selected nodes of the heuristic search trees using decay indices (Bremer 1988, Donoghue et al. 1992); i.e. the number of additional steps required (beyond the shortest tree or trees) before a given clade no longer appears. To determine decay indices, I successively added constraints which specified each node of interest to a given PAUP file. I then conducted a series of heuristic searches in which I saved only trees inconsistent with the chosen constraint and recorded the length of each. The difference between the length of the shortest tree(s) found without constraint and that of the shortest tree(s) found with the above "reverse" constraint represents the decay index. Note that many decay indices for all analyses (except DNA only, no combinatorial weights) are very high, due to the coding of the data and the step matrix approach, which also made the overall tree lengths very great. Therefore, for almost all trees shown, decay indices can be divided by 100 to yield values similar to those seen in more conventional analyses.

I also assessed phylogenetic confidence through use of nonparametric bootstrapping (Felsenstein 1985), implemented using PAUP. One hundred heuristic bootstrap pseudoreplicates were performed for all of the above-described data sets except DNA only, without combinatorial weights, and DNA only, with

combinatorial weights and codon position partitioning. For the latter two data sets, tree searching was so slow that bootstrapping proved impractical.

Population groups

The large number of populations included in the allozyme portion of this study made it impractical to include each as a separate unit in parsimony analyses, and sequencing of representatives from all populations was not possible. To reduce the number of working units, I constructed 24 population groups based on results of initial (phenetic) analyses plus consideration of geographic location and proximity. Membership of these groups is shown in Fig. 12, and locations are listed in Appendix 1. Whenever possible, I applied an existing name to members of the group (e.g. I resurrected the name *E. pterophila* for salamanders in the Blanco River drainage, a name previously synonymized with *E. neotenes* by Sweet [1978b]). For cases in which an appropriate name was not available, I used the name of the region or one of the localities sampled (e.g. Lake Georgetown group, Carson Cave group). While most of these groups likely represent real evolutionary units (species or monophyletic groups of species), several are somewhat arbitrary. Especially problematic assemblages of populations are the Carson Cave, *E. latitans*, and Buttercup Creek Caves groups. These issues, and the potential impact of the grouping approach on the analyses, will be addressed below (see *Discussion*).

RESULTS

Intrapopulation allozyme variation

Twenty-two of the 25 allozyme loci examined displayed polymorphism among and/or within populations of central Texas hemidactyliines (the exceptions were Cap, Ldh-B, and Sod; see Appendix 2). However, levels of intrapopulation genetic variation generally were low (Appendix 2). Direct-count heterozygosity (H) ranged from 0% in several populations to 12.0 % in the T.W.A.S.A. Cave population, represented by a single specimen; the next highest observed H was 10.8% in *E. tridentifera* from Badweather Pit (five specimens examined). Mean H across all populations was 2.92% (S.E. = 0.042). The percentage of polymorphic loci (P) ranged from 0 in several populations to 32 in *E. sosorum* with a mean of 9.69 (S.E. = 0.128), and the average number of alleles per locus (A) ranged from 1.0 to 1.2 with a mean of 1.09 (S.E. = 0.001). Eighteen significant deviations from Hardy-Weinberg equilibrium (HWE) were detected (chi-square test, $P < 0.05$), of a total of 1598 populations X loci examined. However, because many loci were invariant for any given population, this represents a total of only 153 chi-square tests, and thus (one could argue) a greater number of deviations than would be expected by chance. If I invoke the Bonferroni criterion (e.g. Sokal and Rohlf 1981) and divide the alpha level by the number of comparisons, only three deviations are significant, fewer than would be expected by chance.

Phenetic analysis of allozyme data

Here and in subsequent sections of the Results I will focus primarily on differentiation and relationships of the major groups of central Texas salamanders; systematics and taxonomy of the component members of these groups will be addressed elsewhere (Chapter 3 and in prep.). The UPGMA phenogram constructed from Manhattan Ds (Fig. 3) reveals a high degree of genetic differentiation among some populations and groups of populations. In particular, members of the "northern" group (populations from northeast of the Colorado River in Travis, Williamson, and Bell Counties) are extremely divergent from all other central Texas *Eurycea*. Average Manhattan Ds between the northern and other populations exceed 0.45, which correspond here to average Nei's (1978) Ds over 0.65 and Rogers's (1972) Ds over 0.45. These Ds reflect numerous differences in allelic composition (from all other central Texas *Eurycea* examined) that are fully or near fixed, or mutually exclusive. South of the Colorado River, *T. rathbuni* from the San Marcos region appears as the next most divergent member of the group with a Manhattan D exceeding 0.40, and *E. nana* (also from the San Marcos area) is next most divergent (Manhattan D over 0.30). Of the remaining populations, there is a division between a "southeastern group" (all populations east of extreme eastern Kerr Co., corresponding primarily to the southeastern drainages of the Edwards Plateau) and a "southwestern group" (corresponding primarily to southwestern drainages of the plateau). While *E. nana* and *T. rathbuni* each possess unique alleles at several loci (Appendix 2), differentiation among members of the southeastern and southwestern groups primarily is based upon allele frequency variation. UPGMA phenograms constructed using Nei's (1978) unbiased

D and Rogers's (1972) D had topologies nearly identical to that of the Manhattan D tree except at the very smallest Ds, and thus are not shown here. Manhattan Ds are plotted as "contour lines" on a map of central Texas in Fig. 4 for ease of interpretation; the choice of increments of 0.1 Manhattan D unit is only for convenience.

Levels of sequence variation and phenetic analyses of sequence data

Across the maximum 355 bp of cytb sequenced (see Appendix 3), 133 sites (37.5%) were variable including outgroup taxa; within the ingroup 101 sites (28.5%) varied. Of the 118 codons examined (Appendix 4), 15 (12.7%) exhibited amino acid variation, considering all taxa; excluding the outgroup, 13 (11.0%) were variable. The UPGMA phenogram constructed from absolute sequence distances (converted to percent sequence divergence) is shown in Fig. 5, and contour lines representing arbitrarily selected increments of 3% sequence divergence are plotted on the map in Fig. 6. In most respects, the major patterns of divergence seen in the allozyme-based phenogram also occur in the DNA-based phenogram. The northern populations are strongly differentiated from all others, exhibiting over 14% sequence divergence on average (Fig. 5 and Appendix 3). As in the allozyme-based phenogram, *T. rathbuni* appears as next most distinct, with an average sequence divergence of approximately 9% from other non-northern populations. The same division between southeastern and southwestern populations (exclusive of *T. rathbuni*) occurs as in the allozyme-based phenogram, with average sequence divergences over 7%. A key difference between the allozyme phenogram and that based on sequence data is that in the DNA-based tree, *E. nana* appears within the

southeastern group (the region in which it occurs geographically), whereas based on allozymes it is strongly differentiated and appears outside all other non-northern populations except *T. rathbuni*. The other major difference between the allozyme phenogram and that based on DNA is that the DNA phenogram reflects the near-total lack of sequence variation in the southeastern region, except with respect to *E. sosorum*, *E. nana*, and the Pedernales populations. In contrast, substantial allozyme variation is present in the southeastern group (Figs. 3, 4 and Appendix 2).

Biogeographic patterns of genetic differentiation and relationships of cave populations

As described above, phenetic analyses of both the allozyme and sequence data identify several major clusters of populations, most of which correspond to geographically circumscribed regions of the Edwards Plateau area: a northern group, a southeastern group, a southwestern group, *T. rathbuni* from San Marcos, and *E. nana* from San Marcos (*E. nana* is strongly differentiated based on allozymes only). The latter four, all from southwest of the Colorado River, will be referred to collectively here as the "southern group". The named taxa *E. neotenes*, *E. latitans*, *E. pterophila*, *E. sosorum*, and *E. tridentifera* all cluster phenetically within the southeastern region based on allozymes and DNA. The only remaining named taxon (exclusive of *T. rathbuni* and *E. nana*) is *E. troglodytes*, which clusters with members of the southwestern group based on sequence data. Cave populations from the northern, southeastern, and southwestern regions all cluster with spring populations in the same regions based on both allozymes and DNA, regardless of their degree of morphological divergence. The only exception to this

pattern is *T. rathbuni*, an extreme troglobite that is strongly differentiated both morphologically and based on allozymes and DNA from all other members of the southern assemblage.

I found no evidence that the cave-dwelling taxa *E. latitans* and *E. troglodytes* are of hybrid origin. None of the five *E. latitans* examined for allozyme variation displayed a unique MDHP allele that appears fixed or at very high frequency in the three populations of *E. tridentifera* that I examined (Appendix 2). Sequence data place *E. troglodytes* with populations in the southwestern region (see above) and do not support a close relationship between this population and the southeastern *E. tridentifera* (but see *Discussion* for caveats regarding use of mitochondrial sequence data).

Parsimony analyses and monophyly of the central Texas hemidactyliines

Results of all but one parsimony analysis indicate monophyly of the central Texas *Eurycea* with varying degrees of support (Figs. 7-11). The exception is the analysis of sequence data only without application of combinatorial weights (Fig. 10); in the resulting consensus tree, the central Texas group is rendered paraphyletic by the placement of *Typhlotriton* (from the Ozark region) as the sister taxon to the northern group. Based on the decay index, however, support for the node linking these taxa is weak, and trees in which the central Texas group appears as monophyletic are only one step longer. Application of codon position partitioning and combinatorial weighting to the DNA data alone resulted in a single tree in which the Texas group appears as monophyletic with a decay index of 156

"steps" (Fig. 9). Given the nature of the data coding, however, this decay index can be divided by 100 to yield a value more comparable to those seen in other studies; thus support for this node is not especially strong. In analyses in which only the allozyme data were used, the central Texas group appears monophyletic in the single tree resulting from a heuristic search (Fig. 11A), with a decay index of 152 (again, roughly equivalent to only 1.52). In the bootstrap analysis of the allozyme data alone (Fig.11B), the only node that is strongly supported (> 70%; see Hillis and Bull 1993) is that representing monophyly of the central Texas group, at 89%. Heuristic searches using the combined allozyme and DNA data (Figs. 7A and 8A) yielded decay indices of 488 "steps" (DNA without combinatorial weights) or 942 (with combinatorial weighting and partitioning by codon position), suggesting relatively strong support for monophyly of the central Texas group when all the relevant data are considered. Similarly, bootstrap values for this node in combined data analyses were 89% (allozymes plus DNA without combinatorial weighting or partitioning, Fig. 7B) and 98% (allozymes plus sequence data combinatorially weighted and partitioned by codon position, Fig. 8B).

Major groups based on parsimony analyses

Results of all parsimony analyses support monophyly of the northern group of populations. The weakest support occurs in the allozymes-only trees, with a decay index of 509 "steps" (roughly equivalent to 5) for this group based on the heuristic search (Fig. 11A) and a bootstrap value of 67% (Fig. 11B). However, note that support for all other nodes except ingroup monophyly is weak using the allozyme data alone. Decay indices for the node uniting members of the northern

group are high for heuristic searches involving all other subsets or combinations of the data (Figs. 7-10), and bootstrap values for this node are 100% in both combined data analyses (Figs. 7B and 8B). Members of the northern group not only are characterized by numerous diagnostic alleles at allozyme loci and unique sequence substitutions (Appendices 2, 3), but also by unique amino acid substitutions at codons 27, 44, 90, and 111 (Appendix 4). Unambiguous and potential synapomorphies for this and the other groups recognized here are listed in detail in Chapter 3.

The southern group appears as monophyletic in all analyses except the heuristic search based on allozyme data alone (Fig. 11A), which places *T. rathbuni* as sister to the northern group and *E. nana* as sister to all central Texas *Eurycea*. The decay index for the node linking *T. rathbuni* and the northern group in this tree is 42 (roughly 0.4) and that for *E. nana* basal to other central Texas *Eurycea* is 152 (roughly 1.5), indicating little support for this arrangement. Bootstrapping of the allozyme data set (Fig. 11B) places *T. rathbuni* as sister to the remaining members of the southern group, but with a bootstrap value of only 42%. The other parsimony analyses also place *E. rathbuni* as sister to other members of the southern group; the strongest support for this relationship is seen in the combined data analyses, with bootstrap values of 98% (allozymes plus DNA, no combinatorial weighting or partitioning by codon position, Fig. 7B) and 87% (allozymes plus weighted and partitioned sequence data, Fig. 8B) for this node, respectively. In the bootstrap analysis of the allozyme data alone (Fig. 11B), *E. nana* appears as sister to the Camp Mystic population (from the southwestern

region), but with a bootstrap value of only 19%, which lends little credence to this hypothesized relationship. Support for *E. nana* as either embedded within or sister to the southeastern group is generally moderate to high in all analyses not based on allozymes alone (figs. 7-10).

Aside from *T. rathbuni* and *E. nana*, support for a group containing the remaining southern populations generally is moderate to high. The main exception is the heuristic search based on allozyme data alone; the resulting tree (Fig. 11A) shows most members of the southwestern group as sister to the northern group plus *T. rathbuni*, with the Carson Cave and Camp Mystic populations basal to the northern + *T. rathbuni*/southwest clade. Support for this arrangement is very low based on both decay indices and bootstrapping (Fig. 11B). In all analyses based on DNA alone or DNA plus allozymes, the southwestern populations form a monophyletic group; the strongest support for the monophyly of this group is provided by bootstrapping of the combination of allozymes plus weighted and partitioned DNA data (82%, Fig. 7B). The southeastern populations exclusive of *T. rathbuni* and (and *E. nana* in the allozymes-only analyses) always appear as a monophyletic group. The strongest support for the node linking these populations is provided by the combined data analyses, with bootstrap values of 97% in the allozymes plus combinatorially-weighted, partitioned sequence data analysis (Fig. 7B) and 99% in the allozymes plus unweighted, unpartitioned sequence data analysis (Fig. 8B).

Biogeographic and taxonomic patterns based on parsimony analyses

As detailed above, parsimony analyses generally support the division of the central Texas *Eurycea* into a monophyletic "northern group", or clade, from northeast of the Colorado River, and a less well supported "southern group", or clade, from southwest of the Colorado River. The southern clade is composed of *E. nana* and *T. rathbuni* from San Marcos, a "southeastern group" corresponding primarily to the eastern drainages on the southern portion of the Edwards Plateau exclusive of the San Marcos region (this group may include *E. nana* from San Marcos), and a "southwestern group" generally corresponding to the western drainages in the southern portion of the Edwards Plateau region. This major pattern of phylogenetic relationships is consistent with the pattern of similarities revealed by the phenetic analyses. As in the phenetic analyses, even the most morphologically divergent cave populations (with the exception of *T. rathbuni*) cluster with geographically proximal surface (spring) populations.

Relationships of outgroup taxa

I will devote little attention to relationships among other hemidactyliine plethodontids here; these issues will be addressed elsewhere. I note, however, that among the outgroup taxa used, there was relatively little consistency in inferred relationships among analyses. With the exception of the allozymes-only analyses, there generally was strong support for a sister relationship between *E. bislineata* and *E. wilderae*, consistent with previous inferences that these taxa are part of a distinct group within *Eurycea* (e.g. Jacobs 1987). *E. quadridigitata* from Texas and *E. quadridigitata* from South Carolina never clustered together, suggesting that this

taxon may not be monophyletic. Depending on the analysis, the monotypic cave-dwelling genera *Haideotriton* and *Typhlotriton* occur in various places in the tree, always imbedded within *Eurycea* based on the rootings implied by these analyses (however, use of further outgroups will be necessary to properly address the relationships of these taxa).

DISCUSSION

Intrapopulation allozyme variation

Shaffer and Breden (1989) found that nontransforming salamanders generally exhibit lower levels of genetic variation (as measured by allozyme markers) than do transforming species. My results for most populations of central Texas hemidactyliines are consistent with this observation, given the mean H across all populations of 2.9% (however, there are several notable exceptions, particularly among cave-dwellers ; see Appendix 2 and below). Shaffer and Breden (1989) suggested that this pattern may be due in part to the ephemeral nature of the habitats of many nontransforming species: perennibranchiate salamanders often inhabit bodies of water in areas that are relatively hot and arid, and periodic drying of these aquatic habitats may result in genetic bottlenecks. Anecdotal observations support this hypothesis for at least some populations of spring-dwelling central Texas hemidactyliines. For example, when D.M. Hillis, A.H. Price and I visited Helotes Creek Spring, the type locality of *E. neotenes,* in March 1990, we were told that the spring had been dry for approximately two years and had only started

to flow again the previous day. We found dozens of emaciated salamanders, some dead or dying, concentrated in the newly-formed spring pool; presumably these animals had retreated underground with the drying of the spring and had barely survived to reemerge with the rise in aquifer levels. Comal Springs, another habitat for members of the group, is known to have ceased flowing during a drought in the 1950s. While this caused local extirpation of the fountain darter *Etheostoma fonticola* (C. Hubbs pers. comm.), salamanders still exist there, and presumably retreated underground during the dry period, although effects of the event on population size are unknown. Sweet (1977) observed many instances of temporary drying in Edwards Plateau springs inhabited by *Eurycea*, during which the salamanders presumably were in subterranean waters.

These observations highlight the potential for periodic drastic reductions in population size, and lend support to Sweet's (1978a) hypothesis that many populations of cave hemidactyliines in central Texas may have originated from spring-dwellers that followed the water column underground when it dropped due to drought, or when erosion led to stream capture. It is interesting that some of the highest levels of heterozygosity observed were among cave dwellers from subterranean aquatic systems that likely are relatively extensive (especially *E. tridentifera* from caves of the Cibolo Sinkhole Plain in Comal, Kendall, and Bexar Co.'s); perhaps these systems support large numbers of individuals and/or are buffered against the drastic changes in water availability to which surface populations are subject. However, the relationship between heterozygosity and habitat type in general is far from clear-cut: a few spring populations exhibit

relatively high Hs (including the Helotes population described above!), and even within a given cave system, estimates of heterozyosity can vary widely from site to site (e.g. for *E. tridentifera* from Honey Creek Cave vs. Badweather Pit, and for the various populations of *E.* sp. from the Buttercup Creek Cave system in Williamson Co.). While some of this variability might be attributable to sampling error given the small sample sizes for many of the populations studied, a proper investigation of the relationship between levels of genetic variability and habitat type likely will depend on a much more detailed knowledge of factors such as spring reliability, the true extent of available habitat (subterranean versus surface), and the nature of interconnections among given springs and/or caves. Culver (1982) reviewed theoretical arguments that predict higher level of heterozygosity for cave versus surface-dwellers, and arguments for the reverse situation, but in his literature survey found no consistent pattern of differences. Kane et al. (1992) found no significant differences in heterozygosity between surface and cave-dwelling populations of the aquatic amphipod *Gammarus minus* (although surface populations did have significantly higher numbers of alleles per locus), and concluded that for this species recent bottlenecks or founder effects are unlikely to have played a major role in the evolution of cave populations. In a widely-cited study, Avise and Selander (1972) concluded that founder effect was responsible for the low levels of genetic variation seen in cave versus surface populations of Mexican tetras (*Astyanax*). For the Texas *Eurycea*, there are so many instances of cave-dwelling, and such variation in the nature and extent of spring and subterranean habitat, that one could invoke almost any explanation of levels of variation by citing selected examples.

Interpopulation sequence differentiation and variability of allozymes versus mitochondrial DNA

The levels of nucleotide variation seen for cytb here are very similar to those reported by Moritz et al. (1992) for plethodontine plethodontids of the genus *Ensatina* plus the outgroups *Aneides* and *Plethodon*. Including outgroups, I found 37.5% of the 355 positions surveyed positions variable (28.5% for ingroup members only), while Moritz et al. reported 37% of positions variable for the 681 bp they sequenced across all taxa. Possible implications of these results with respect to origins and divergence times of the central Texas hemidactyliines are discussed below. Moritz et al. (1992) found almost twice the amino acid sequence variation in their study as did I (22% of amino acids variable including ingroup and outgroups, compared to 12.7% here with outgroups and 11.0% without). The reason for the apparently greater level of conservation in hemidactyliine cytb amino acid sequences is unknown.

On a broad scale, the patterns of geographic variation exhibited by both allozymes and cytb sequences are similar (see discussion of phylogenetic and biogeographic implications below). However, considerably more variation was revealed by allozymes than cytb sequences in the southeastern region, where most populations exhibited identical or near-identical sequences. The very low levels of cytb nucleotide variation in most populations from the southeastern region are surprising given that the southwestern populations exhibit considerable variation for the same sequence, and salamanders from both regions exhibit similar levels of

interpopulation allozyme differentiation. Since the southeastern and southwestern clades appear to be sister groups, one might expect that both would show similar levels and patterns of sequence variation. This assumes that through stochastic processes and lineage sorting, polymorphisms present in the common ancestor of the two would be distributed among populations in both regions, plus whatever new mutations arose and persisted after separation of the southeastern and southwestern lineages. One might even predict higher levels of interpopulation variation in the southeast, because erosion on the surface of the Edwards Plateau is thought to have occurred from east to west (e.g. Sweet 1978a), presumably creating isolated islands of spring and cave habitat available for colonization earlier than most in the southwest. However, development of the fault zone aquifer likely proceeded from west to east (e.g. Woodruff and Abbott 1979), so if the ancestor of the southeastern and southwestern groups was associated with this feature one might hypothesize an early radiation into habitats in the southwest, followed by isolation and genetic divergence. This scenario is compromised by the relatively high levels of allozyme variation in both areas, plus the occurrence of the northern group adjacent to the current range of the southeastern one. The latter suggests that the ancestor of the entire central Texas *Eurycea* assemblage was distributed throughout the Edwards Plateau region, then split by vicariant events. The sequence homogeneity among most of the southeastern populations and taxa seems unlikely to be due to extensive gene flow, given that there is substantial nuclear (allozyme) evidence of substructuring or isolation for many southeastern populations and population groups.

This situation is the opposite of that seen in nontransforming North American cryptobranchid salamanders, in which there is a near-total lack of detectable allozyme variation (Merkle et al. 1977, Shaffer and Breden 1989), yet strong geographic substructuring based on mtDNA analysis (Routman 1993). Although the studies are not completely comparable (here I sequenced a portion of a single mt gene, whereas Routman used restriction sites from throughout the mt genome), this observation demonstrates that even a mt gene generally regarded as rapidly evolving (e.g. Graybeal 1993) may fail at some level to exhibit useful variation, while nuclear allozyme markers continue to be informative. Routman (1993) suggested that the reduced levels of allozyme variation in *Cryptobranchus* could be due to an ancestral bottleneck, after which the rapid rate of evolution of mt DNA allowed a more rapid accumulation of variants than in the nuclear genes encoding metabolic enzyme loci. In the present study, however, the low levels of mtDNA variation in the southeastern group suggest that if there was an ancestral bottleneck, the mt genome (or at least that portion examined) has not accumulated mutations as rapidly as have some of the genes encoding the loci used in the allozyme study. Routman (1993) found high mitochondrial restriction site variation relative to allozyme variation among populations of nontransforming tiger salamanders (*Ambystoma tigrinum mavortium*), and suggested that localized bottlenecks may have been extreme enough to reduce effective mt population sizes to the point where diversity was drastically diminished and particular haplotypes became fixed, while population sizes remained large enough to maintain allozyme diversity (since the effective population size of the mt genome is smaller than that of the nuclear genome; see Birky et al. 1983, 1989). This explanation is consistent

with the predictions of Nei et al. (1975) that the effect of a bottleneck in reducing levels of genetic variation is greatest at very small population sizes but rapidly diminishes as population sizes become even slightly larger. Thus perhaps the ancestor of most of the southeastern Edwards Plateau *Eurycea* underwent a bottleneck that reduced population size enough to drastically reduce mt variation, while substantial nuclear variation was retained.

Biogeographic history and patterns of genetic similarity

I present the results of phenetic (UPGMA) analyses of cytb sequences and allozyme frequency variation in order to illustrate patterns of differentiation among populations and groups of populations of central Texas hemidactyliines and to examine the relationship between genetic differentiation and geographic proximity. I draw no direct phylogenetic conclusions from these analyses (see *Phylogenetic Analyses*, below). I will make general inferences regarding the possibility or relative magnitude of gene flow among particular populations and groups of populations based primarily on occurrence of large genetic distances and especially fixed differences; more detailed, hierarchical analyses of gene flow are beyond the scope of this paper and will be presented elsewhere. UPGMA trees for both the allozyme and sequence data (Figs. 3 and 5) reflect the high degree of genetic subdivision present in the group, particularly with respect to the groups of populations and taxa from north versus south of the Colorado River. The large number of sequence substitutions between the northern and southern groups, coupled with numerous fixed or near-fixed allozyme differences, indicate that populations are isolated from each other and probably have been for a long period

of time. This conclusion is reinforced by the results of flow cytometric studies of nuclear genome size: C-values (nuclear DNA mass) are 12-13 % higher on average for members of the northern group than the southern group, and there is no overlap in C-value distributions for the two groups (Chippindale and Lowcock, unpublished, Licht and Lowcock, 1993 [note that Licht and Lowcock erroneously listed the mean C-value for members of the northern group as 25.8 rather than 28.5, and identified members of the northern group as native to "north Texas"]). While recognizing the potential of the Colorado River as a barrier to gene flow, Sweet (1978a, 1982) identified the few northern populations known then as *E. neotenes* (whose type locality is in Bexar Co., in the southern region) due to the high degree of morphological similarity of most surface-dwelling populations from the northern and southern areas. Based on the molecular data, members of the northern group represent strongly differentiated, long-isolated species (see *Taxonomic Implications*, below). The Colorado River is thought by many to be one of the oldest features of the Edwards Plateau (Abbott 1975, Sweet 1978a, Veni and Associates 1991), and has probably cut down through the elevated limestones of the plateau throughout its existence, dividing the Edwards (Balcones Fault Zone) Aquifer into two major sections with little or no hydrologic connection (Slade et al. 1986). Thus it is not surprising that salamanders from either side of the river are strongly differentiated; this pattern of vicariant isolation by waterway also has been observed in other groups of salamanders (e.g Good and Wake 1993). Veni and Associates (1992) examined distributions of troglobitic invertebrates (almost all terrestrial), and found that of 38 species found in the Jollyville Plateau region just north of the Colorado, only two occur south of the river as well. Many of the same

factors that limit the distributions of these organisms are likely to affect the central Texas salamanders, because the occurrence of salamanders is extremely closely tied to subterranean aquatic systems and the immediate areas of their spring outflows (e.g. Sweet 1982). Thus a major (deeply incised) surface waterway or other subterranean impermeable barrier likely will serve to prevent gene flow for aquatic troglobites, perhaps even more so than for terrestrial forms.

The above observations may explain the relatively high degree of divergence of salamanders from the Lake Georgetown spring cluster in the northern region; Veni and Associates (1991) identified Brushy Creek and the south fork of the San Gabriel River, both south of the Lake Georgetown area, as major barriers to gene flow in troglobitic invertebrates. However, this does not explain the relatively high degree of similarity of in allele frequencies and cytb sequences between populations from northeast of the Lake Georgetown area (Bat Well and Salado Springs) and populations south of the Lake Georgetown cluster (Jollyville Plateau, Round Rock, Cedar Park Caves). In this instance, similarity may not reflect close relationship, since phylogenetic analysis of combined allozyme and DNA data wiith differential weighting (Fig. 7) places the northeasternmost groups together, albeit with weak support.

The UPGMA trees also illustrate the relatively high levels of genetic divergence of the taxa from the San Marcos Pool of the Edwards Aquifer in Hays Co. (southern region). *T. rathbuni* and *E. nana* clearly are distinct species isolated from gene flow with other populations examined (for the latter species, this

conclusion is based primarily on allozymes). This result is consistent with the occurrence of numerous other endemic species of aquatic vertebrates, invertebrates, and plants at San Marcos (e.g. Holsinger and Longley 1980), and likely is related to the high degree of isolation of the San Marcos Pool of the Edwards Aquifer from the remaining southern portions of the aquifer (e.g. Potter and Sweet 1981).

The phenetic subdivision of members of the southern group exclusive of the San Marcos taxa (*T. rathbuni*, plus *E. nana* for allozymes) into southeastern and southwestern components corresponds roughly to the eastern versus western drainages of the southern plateau region, all of which are of more recent origin and less deeply incised than the Colorado River (e.g. see Sweet 1978a, 1982, and Veni 1994). The southeastern and southwestern groups are not as strongly differentiated from one another as are the northern and southern groups, or the San Marcos taxa compared all others: most of the allozyme-based differentiation constitutes allele frequency variation rather than fixed differences. However, lack of current gene flow between the regions is especially apparent based on the mitochondrial haplotype differences that separate all southeastern populations from all southwestern ones.

In nearly every case (excluding the San Marcos taxa) populations that display the highest degree of similarity in allozyme frequencies are those that are geographically close to one another, especially those that share a particular drainage system (e.g. the *E. pterophila* group of the Blanco river drainage and *E. tridentifera* of the subterranean Cibolo Creek drainage). This also is apparent for

many populations from the northern region, with the exception of Salado Springs and cave populations from this area. The latter appear based on phenetic analyses of allozyme data scattered throughout the northern portion of the tree (Fig. 3). The phenetic analysis of the sequence data yields more coarse-grained results, in part due to the more limited sampling and in part due to the lack of substantial variation in the southeastern region. Based on cytb sequences, the same major groups are present as in the allozyme-based analyses (except for *E. nana*, part of the southeastern group based on sequence data alone). In a few cases within the northern and southwestern groups sequence similarity does not correspond directly to geographic proximity. This occurs in the northern region for Salado Springs (as is the case in the allozyme-based phenogram) and Bat Well, and in the southwestern region for 176 Spring, Tucker Hollow Cave, and Greenwood Springs. However, in most cases the greatest sequence similarity is seen in geographically proximal populations regardless of whether they occur in springs or caves. Thus, if similarity reflects close relationship (and in most cases here it does appear to; see *Phylogenetic analyses*, below), both the allozyme and sequence data support multiple origins of subterranean dwelling throughout the Edwards Plateau region.

Of the non-San Marcos southeastern populations, only peripheral ones from the northeastern edge of the area display substantial sequence differentiation, *E. sosorum* from Barton Springs in Travis Co. and the recently-discovered populations from springs along the Pedernales River in Travis Co. *E. sosorum* likely is strongly isolated, due in part to the existence of a groundwater divide between the Barton Springs portion of the Edwards Aquifer and the remainder of the southern Edwards

Aquifer (Slade et al. 1986). The Pedernales populations are located in an isolated outcrop of Cow Creek limestone and there is little potential for direct connection of this aquatic system with other drainages known to be inhabited by *Eurycea.*

Time scale for origins and diversification of the central Texas hemidactyliines

Estimates for times of invasion of central Texas by plethodontids vary, but the earliest estimates for radiation of plethodontids from a presumed Appalachian center of origin are early Tertiary, with establishment of many North American genera by mid- to late-Tertiary (Wake 1966, Larson 1984). More recent fossil evidence supports the lower (oldest) end of this estimate for origins of genera in the tribe Plethodontini, indicating that *Plethodon* (presumably the western lineage) and *Aneides* had split from a common ancestor by the early Miocene (Tihen and Wake 1981). Sweet (1978a) and Potter and Sweet (1981) agreed with Wake (1966) that some Edwards Plateau cave habitats would have been available for invasion by salamanders in Miocene times, and summarized evidence that the Edwards Plateau was uplifted during the Eocene (and thus presumably available for colonization by hemidactyliines), much earlier than assumed by most previous workers. While I am skeptical of the existence of a "molecular clock" (or at least the ability to make meaningful calibrations given available data; see Moritz and Hillis 1990), I nonetheless compared the levels of allozyme and mtDNA divergence observed here with allozyme and albumin immunological distances (AID's), and estimated divergence times, calculated by Larson et al. (1981) for members of the tribe Plethodontini. I also considered cytb sequence divergences observed for plethodontines by Moritz et al. (1992). For allozymes, I compared the molecular

distance from this study corresponding to the inferred deepest split in the Texas hemidactyliines (northern versus southern groups; see also *Phylogenetic Analyses*, below) with mean Ds and the inferred time of splitting between western *Plethodon* and *Aneides*. The resulting estimate puts the north-south division at roughly 26 MYA. I also compared the maximum uncorrected sequence distance of Moritz et al. (corresponding to the division between *Ensatina* and *Aneides/Plethodon*) to the mean uncorrected sequence distance for northern to southern groups in the Texas hemidactyliines. I then calibrated this against the Larson et al. (1981) AID estimate of divergence time for the plethodontine genera; this places the north-south division at about 47 MYA. This (very large) range spans roughly the mid-Eocene through the mid-Oligocene, consistent with the biogeographic scenarios proposed by Wake (1966), Sweet (1978a), and Potter and Sweet (1981). However, the potential for compounding of errors is so great that this result should be viewed with great caution. Furthermore, an internal calibration using relatively robust estimates for the earliest development of subterranean habitat for *E. tridentifera* yields a very different result. To do this, I assumed that the earliest time of divergence of this taxon from other southeastern *Eurycea* was probably less than 1 MYA; I based this on times of cave development (see Veni 1994 and below) and made the assumption that *E. tridentifera* did not represent a distinct lineage before subterranean habitat became available. I then calibrated allozyme-based Manhattan Ds between the northern and southern groups against Ds between *E.tridentifera* and other southeastern populations. This places the north-south division at only about 4 MYA, assuming a linear relationship between genetic distance and time. Given the (more than tenfold) variation in estimates of divergence times described above, all

that I can suggest is that the deepest divergences in the Edwards Plateau hemidactyliines probably are old, certainly much older than the Plio-Pleistocene events suggested by Blair (1958, 1965) and Mitchell and Smith (1972) if the lower boundary of 4 MYA for the north-south split is correct.

Phylogenetic analyses , taxonomic implications, and hybridization

To assess the biogeographic scenarios proposed above, and to understand the evolution of life history strategies such as perennibranchiation and cave-dwelling, a phylogenetic framework is essential. I chose to use parsimony methods to reconstruct the evolutionary history of the central Texas hemidactyliines, because these methods are widely favored, easy to implement, and generally perform well under a wide range of conditions such as branch length inequalities (e.g. see Swofford and Olsen 1990, Huelsenbeck 1995). I believe that the best estimates of phylogeny are derived from treatments of the data that incorporate as much information as possible about both evolutionary processes and character state frequency variation. The former is borne out by simulation studies which suggest that, in general, the more realistic evolutionary parameters that can be incorporated into character coding and weighting, the more likely it is that the correct tree will be recovered (e.g. Hillis et al. 1994, Huelsenbeck 1995). While the application of frequency information in parsimony analysis has been controversial (e.g. see Buth 1984, Crother 1990), Swofford and Berlocher (1987) and Wiens (in press) make compelling arguments in favor of its use. The Manhattan distance/step matrix approach allows use of allele frequency information on a locus by locus (i.e. individual character) basis while avoiding the peculiar sampling properties of some

other commonly used genetic distances, such as Nei's (1972) D (Hillis 1984, Frost and Hillis 1990). D. Swofford (personal communication) has pointed out that this method (which he and S. Berlocher developed simultaneously and independently from J. J. Wiens, D.M. Hillis and me) is equivalent to use of Swofford and Berlocher's (1986) MANOB criterion, previously deemed computationally impractical. My choice of differential weighting schemes for the sequence data (Rodrigo's 1992 correction of Wheeler's 1990 combinatorial weighting method) allows incorporation of more detailed information on patterns of nucleotide change than would a simple transition versus transversion treatment. Examination of table 2 indicates that, while transitions apparently are more common than transversions (as is usually the case), within each class of changes not all substitutions occur with equal frequency. The fact that I had to render the step matrices symmetrical in order to attain sufficient speed of computation reduces the level of "fine-tuning" that is possible. However, Hillis et al. (1994) demonstrated in simulation studies that symmetric step matrices perform substantially better in recovering the true phylogeny than does no differential weighting of change probabilities (although not as well as asymmetric step matrices).

The issue of whether or not to combine data sets in phylogenetic analysis has been the subject of considerable recent controversy (reviewed by Miyamoto 1995 and Hillis 1995). I have argued strongly in favor of data combination, with application of appropriate differential weights (reflecting inferred probabilities of change among states) on an individual character basis (Chippindale and Wiens 1994, Wiens and Chippindale 1994). Most workers agree that it is desirable to

examine trees constructed based on both separate and combined analyses, and I consider the results of both kinds of analyses here. My "preferred" hypothesis of phylogeny based on these analyses is that derived from combination of the allozyme and DNA data, the former treated with frequency-based coding and the latter with combinatorial weighting and codon position partitioning.

In general, the major regional similarity groupings identified by UPGMA analyses of the allozyme and DNA data appear in the phylogenetic trees as monophyletic assemblages, and all analyses (except DNA only without differential weighting) provide moderate to very strong support for the monophyly of the central Texas group. There is very strong support based on nearly all treatments of the data for the monophyly of the northern group, and it is clear (as discussed above) that these populations do not represent the species *E. neotenes*. I believe (based on molecular, morphological, and biogeographic evidence) that there are at least three separate species in the northern group; more detailed information is presented in Chapter 3 and formal descriptions of these species will be presented by Chippindale et al.(in prep.). The basal split between populations of *Eurycea* from north versus south of the Colorado River in all analyses (except the allozymes-alone heuristic search) is consistent with the view of the Colorado as an ancient, strong barrier to gene flow that has divided the group into two major clades. *T. rathbuni* appears as embedded within the Texas *Eurycea* in all analyses, and thus I concur with Mitchell and Reddell (1965) and Mitchell and Smith (1972) that members of the genus *Typhlomolge* should be considered *Eurycea*, and will refer to them as such in the remainder of this chapter. (See Chapter 2 for a more detailed

treatment of the status of the genus *Typhlomolge*). All analyses based on either DNA alone or combined data indicate a sister taxon relationship between *E. rathbuni* (plus, presumably, *E. robusta*, which was unobtainable) and the remaining members of the southern group. This result supports an early divergence for the ancestor of *E. rathbuni* and *E. robusta*; implications of this with respect to morphology and life history will be discussed below. The other San Marcos taxon, *E. nana*, is more problematic (see Chapter 2 also), and its placement varies depending on which subset of the data is used. The sequence data place it within the southeastern group, not surprising given the minimal sequence divergence it displays, and all combined data treatments provide strong support for this species either within or sister to the southeastern group (San Marcos is located in the southeastern Edwards Plateau region but in many respects exhibits a unique aquatic fauna; e.g. see Holsinger and Longley 1980). However, its high level of divergence based on allozymes leads to an odd result in which it is placed outside all other central Texas hemidactyliines based on a heuristic search using the allozyme data alone. I place little faith in this result because the decay index for this node is low, and bootstrap analysis of this subset of the data yields a different placement. However, such a result suggests that the analysis I used for the allozyme data may be sensitive to long branch effects (a well-known problem in phylogenetic analysis; e.g. Felsenstein 1978), and invites further investigation of the properties of this method.

Nearly all phylogenetic analyses support the existence of monophyletic southeastern and southwestern groups (although actual character support for

monophyly of the southwestern group is weak; see Chapter 3 for details). Beyond this, there is little support based on the phylogenetic analyses for any particular pattern of relationships within the major groups that I have identified (for all treatments of the data, within-group bootstrap values are nearly all below 70%, the level identifed by Hillis and Bull [1993] as corresponding to a 95% probability that the clade is real). My intention here is not to address relationships or taxonomy at this level in great detail (see Chapter 3 for such a treatment). For this reason, I am not too concerned here about the above-described uncertainties, nor that a few of the population groupings that I used for phylogenetic analysis (listed in Appendix 1 and illustrated in Fig. 12) may not be monophyletic; the key point is that these groups cluster with others drawn from the same geographic region in nearly every analysis, and do so in every analysis for which there is strong support for the nodes uniting members of the major groups.

With respect to taxonomy, none of these analyses support the previous recognition (e.g. Baker 1961, Brown 1950, 1967, Sweet 1978a, 1982, 1984) of *E. neotenes* as widespread throughout the region, if the other currently accepted named taxa are also recognized. I restrict the *E. neotenes* group to springs in the area of the type locality at Helotes Creek, Bexar Co., resurrect the name *E. pterophila* Smith and Potter 1950 for Blanco River drainage populations, continue to recognize the species *E. tridentifera*, *E. sosorum*, and *E. nana*, and suggest that many more, as yet unnamed species exist. All named species in the group occur in the southeastern region except *E. troglodytes* Baker 1957, from Valdina Farms Sinkhole in Medina Co. The name of this taxon (which may now be extinct due to

habitat modification [Veni and Associates 1987, G. Veni pers. comm. to PTC]) was synonymized by Sweet (1978a, 1984) with both *E. neotenes* and *E. tridentifera* because he considered this population a hybrid swarm. Although allozyme data are not available, cytb sequences place this population within the southwestern region, where it occurs geographically. Given the maternal inheritance of the mt genome, there conceivably could be male-based flow of genes (in the form of *E. tridentifera* from the southeast, one of the putative parent species), and thus the population could still consist of hybrids. However, I doubt this, because the other putative hybrid taxon whose name was synonymized by Sweet, *E. latitans*, appears not to be a hybrid between *E. tridentifera* and surface *Eurycea* based on the allozyme data (although this is limited primarily to evidence from one locus). The geographic location of populations of *E. latitans* is much closer to the known range of *E. tridentifera* than is that of *E. troglodytes*, and gene flow in the hydrologic system of this area seems much more plausible than between this region and the portion of the southwestern region in which *E. troglodytes* occurs, 75 km distant from the known range of *E. tridentifera*. For these reasons, I resurrect the names *E. troglodytes* and *E. latitans*. At a minimum the name *E. troglodytes* should apply to the Valdina Farms locality, but could be extended to include all members of the southwestern group pending formal description of other species in the region. *E. latitans* applies at least to populations of the Cascade Caverns system of Kendall Co., and here I include several other populations in the area as members of an informal (and possibly non-monophyletic) "*E. latitans* group" (see also Chapter 3).

Patterns of morphological and molecular evolution and the origins of cave-dwelling and perennibranchiation

Wake et al. (1983), Larson (1984), Wake (1992), and Larson and Chippindale (1993) discussed the phenomenon of morphological evolutionary stasis (the situation in which there is lack of morphological differentiation despite substantial divergence as measured by molecular markers) in plethodontid salamanders. This apparent uncoupling of rates of evolution in genes that govern morphology versus others has been especially well documented in this group, and in almost every case in which a widespread "species" of plethodontid has been studied using molecular techniques, multiple species have been found to exist (for reviews see Larson 1984 and Larson and Chippindale 1993). Stasis appears to characterize many of the central Texas hemidactyliines, given the high degree of similarity in external morphology among many surface populations (Sweet 1978a, 1982, Chippindale et al. 1993, Chapter 3, Chippindale unpublished) despite molecular divergences that may be very large (especially those corresponding to the Colorado River division). From a parsimony perspective one can argue that the similarities in body form among many spring populations represent retention of ancestral features rather than parallelisms or convergences, consistent with the hypothesis of stasis. The conditions believed by Larson (1984) to promote stasis in plethodontids -- populations that are large and occupy similar habitat but are strongly isolated from one another -- typify the central Texas hemidactyliine assemblage. Because similar conditions appear to be present in most springs inhabitated by salamanders in the Edwards Plateau region (Sweet 1982), stabilizing selection may act to maintain a particular narrow range of morphologies.

However, the situation for the Texas hemidactyliines is particularly complicated, because in addition to the many instances of morphologically similar spring populations that exhibit varying degrees of molecular divergence, there also are a few spring populations that are substantially differentiated morphologically yet show relatively low levels of detectable molecular divergence; examples include *E. sosorum* from the southeastern region (Chippindale et al. 1993) and the Salado Springs population from the northern region (Chapter 3). There also is one spring-dwelling taxon (*E. nana*) that is highly divergent from all others at the molecular level based on allozymes (but not mtDNA) and substantially divergent in morphology (Chapter 2).

Mitchell and Smith (1972), Sweet (1978a, 1984) and Potter and Sweet (1981) provided detailed descriptions and morphological analyses of the cave populations, and here the situation becomes even more complex. While the taxa generally considered to exhibit the most extreme cave-associated morphologies (the two members of the former genus *Typhlomolge*, here considered *E. rathbuni* and *E. robusta*) appear based on the analyses presented here to have diverged from other southern Edwards Plateau *Eurycea* long ago, the taxon considered the next most extreme in troglobitic morphology, *E. tridentifera*, exhibits relatively little molecular divergence and phylogenetic analyses place it within or sister to the southeastern group. Morphological similarities between *E. rathbuni* and *E. tridentifera* are great enough that Wake (1966) initially considered *E. tridentifera* a member of the genus *Typhlomolge* (based on very limited numbers of specimens). As discussed above, Potter and Sweet (1981) believed there was ample geologic

evidence that the ancestor of *E. rathbuni and E. robusta* could have entered caves in Miocene times or earlier, as originally suggested by Wake (1966). Other workers (e.g. Mitchell and Smith 1972) have argued that caves could not have developed in the area until much later (Plio- or Pleistocene times), which would suggest a more recent origin for the morphological extremities exhibited by these taxa. However, the very diverse subterranean invertebrate fauna at San Marcos, which includes many taxa thought to be relicts of marine forms that occurred in the region prior to uplift of the Edwards Plateau (Holsinger and Longley 1980, Holsinger 1988), suggests that an underground aquatic system in the San Marcos region capable of supporting abundant life may indeed be very ancient.

Multiple methods for estimation of cave age place the lower (oldest) boundary for formation of caves inhabited by *E. tridentifera* in the Cibolo Creek/eastern Guadalupe River drainages at 1.2 MYA (Veni 1994), and development of a cave system suitable for invasion by aquatic organisms probably had not occurred until less than 1 MYA (Veni 1994 and pers. comm.). Thus, it appears that the complex of features associated with cave-dwelling (e.g. eye reduction, pigment loss, limb elongation, broadening and flattening of the skull, and shortening of the trunk) can evolve relatively rapidly, as suggested by Mitchell and Smith (1972). Estimates for ages of other salamander caves in the Edwards Plateau region vary, but most also appear to have been of Pleistocene or at most Pliocene origin (Veni 1994, Veni and Associates 1992, Mitchell and Smith 1972, Russell 1993), and Sweet (1978a, 1984) considered the caves inhabited by *E. tridentifera* the oldest in the southern region exclusive of those at San Marcos.

Nearly all northern cave salamander populations were discovered very recently, and thus their origins have not been addressed by previous authors. The most extreme troglobitic morphologies in the north are seen in members of the informal Buttercup Creek Cave group that I recognize here; estimates for the development of this cave system are rough, but openings to the surface may not have been present until 120 - 140 ka (Abbott 1984; see also Veni and Associates 1992). Another extreme troglobite existed in Salamander Cave in the Jollyville Plateau region of the north (Sweet 1978a, 1984; the cave is now built over and the specimens are lost); caves in this area may be older, some possibly even of Miocene origin (Veni and Associates 1992).

Previous workers (e.g. Mitchell and Smith 1972, Sweet 1978a, 1982, 1984) have argued that cave-dwelling central Texas hemidactyliines probably arose from surface-dwelling ancestral lineages that independently retreated underground as surface conditions became unsuitable. The relatively frequent occurrence of spring drying presumably leads to selection for the ability to survive extended periods in subterranean habitat (see below for details); thus arguments for multiple origins of subterranean living seem at least plausible. Geological considerations also suggest that numerous independent invasions of cave habitat have occurred. Cave systems in each of the major geographic regions inhabited by the central Texas hemidactyliines almost certainly developed independently, in many cases at different times (see above discussion of the geological history of the region for details). With the exception of some caves in the southeastern region, direct hydrological connections among cave systems are extremely unlikely ever to have

been present (see also Sweet 1978a). Thus, it seems likely that the ancestor of at least some of the Texas *Eurycea* was a relatively widespread surface-dweller.

Despite the apparent plausibility of the above scenario (repeated invasions of cave habitat by surface dwellers) given the geological history of the Edwards Plateau, it is difficult to support the argument that surface-dwelling is the primitive condition for the group based on phylogenetic considerations. Using the preferred phylogenetic hypothesis for the group, I plotted the minimum number of transitions to cave habitat assuming a surface-dwelling ancestor (Fig. 13). These suggest at least ten habitat shifts in total. I then plotted the minimum number of shifts to surface-dwelling, assuming a subterranean ancestor and allowing subsequent reversals to cave-dwelling (Fig. 14). Under this scenario, only nine shifts (steps) are required: two transitions from cave- to surface-dwelling and seven subsequent reversals to cave-dwelling. If reversals are not allowed, at least 14 steps are required to explain cave to surface transitions in the context of this phylogeny.

Thus, consideration of phylogeny alone fails to support the hypothesis that surface-dwelling is the primitive condition for the group; support for a subterranean ancestor is actually slightly better (i.e. requires one fewer step). Of course, in plotting hypothetical habitat shifts on the tree, I assumed that the probabilities of moving from surface to cave habitat, and from cave to surface, are equal, and for the reasons given above I doubt that the latter is as likely. In any case, the most parsimonious scenario still involves numerous reversals to cave-dwelling and thus

the potential for convergent or parallel evolution of morphologies after the hypothetical reentry into subterranean habitat.

The evidence for multiple origins of cave-dwelling (whether due to habitat shifts from an original surface-dwelling ancestor or multiple reversals to the subterranean condition) is consistent with Sweet's (1978a, 1984) hypothesis of widespread parallelism or convergence in morphology (including eye reduction, pigment loss, and in some cases broadening and flattening of the skull, lengthening of limbs, and shortening of the trunk) among cave-dwelling Edwards Plateau salamanders. (Although under the cave ancestor/later reversal hypothesis, this requires the assumption that cave-associated morphologies reevolved after the hypothetical second entries into subterranean habitat). On a broader phylogenetic scale in hemidactyliines, morphological parallelism or convergence also may have occurred: *E. rathbuni* bears a striking resemblance (externally) to *Haideotriton wallacei*, from caves in Florida and Georgia, yet probably is quite distantly related (and the likelihood of subterranean connections between the habitats of the two taxa is extremely low). Whether such convergences in morphology are the result of selective forces, relaxation of selection, or some combination of the two (all resulting in "regressive" morphological evolution; see Culver 1982, Holsinger 1988, and references therein) is unclear. There is evidence, however, of parallel selection for some morphological features associated with cave dwelling (especially eye reduction) in other troglobites (e.g. see Jones and Culver 1989, Jones et al. 1992, and review by Culver 1982).

Evolution of perennibranchiation in the central Texas *Eurycea* also remains problematic. Bruce (1976) suggested that for these salamanders there is selection for attainment of reproductive maturity at an early age, and thus mature animals are small and have a high surface:volume ratio. Given the arid conditions surrounding most Edwards Plateau region springs, this could in turn lead to selection for permanent aquatic living. Sweet (1977, 1978a) suggested that salamanders with larval morphologies are better equipped to retreat into underground waters when surface springs dry, especially with respect to mechanics of the feeding apparatus. Sweet (1977, 1978a, 1986) regarded the few naturally transforming populations of central Texas *Eurycea* (all from what we call here the southwestern region) as relicts that retained the ability to metamorphose due to their occurrence in an area of mesic canyons, where movement on land presumably is not severely limited by extremes of dryness as it is elsewhere in the Edwards Plateau region, and springs flow reliably throughout the year. This view clearly is not parsimonious (based on the phylogenetic reconstructions presented here) if transformation is viewed as a single character and loss of the ability to do so is considered a single state of this character. Under this scenario, and given our preferred phylogenetic hypothesis of ((north)(*E. rathbuni*(*nana*(southeast) (southwest)))))), retention of the ability to transform in some southwestern populations would require numerous independent origins of perennibranchiation in the other members of the group. In the context of the preferred phylogenetic hypothesis (Fig. 7A) at least nine independent origins of perennibranchiation would need to have occurred, as opposed to two reversals to transformation given a perennibranchiate ancestor for the group. However, the view of transformation as a single character probably is unrealistic. Duellman and

Trueb (1986) reviewed the phenomenon of perennibranchiation (which in salamanders they generally attributed to neoteny, i.e. retarded somatic maturity) and pointed out that transformation involves a pathway which can fail at various steps. Shaffer (1984) demonstrated that perennibranchiation in Mexican ambystomatids had arisen independently in multiple lineages (although the genetic basis was not known), while Harrison et al. (1990) showed that the genetic basis of perennibranchiation varies among populations of *Ambystoma talpoideum.* In the central Texas *Eurycea*, different populations and taxa exhibit a range of responses to thyroid hormones, the inducers of metamorphosis. Potter and Rabb (1960) induced metamorphosis in *E. nana*, and Kezer (1952) did so for what he considered *E. neotenes* (the status of the Bexar Co. populations that he used is uncertain, and could represent members of what we call the *E. latitans* group; based on their location these populations almost certainly belong to the southeastern clade). Other members of the group (*E. rathbuni, E. tridentifera*) undergo only partial metamorphosis when treated with thyroxin (Dundee 1957, Wake 1966, Bogart 1967). Among the many populations that Sweet (1978a, 1982, 1984) assigned to *E. neotenes*, there was considerable variation in response to thyroid hormones in preliminary experiments (Sweet, pers. comm.). All of this suggests that there may be multiple genetic bases for loss of transformation in the central Texas *Eurycea*, and precludes a simple interpretation of the evolution of perennibranchiation even in light of a relatively robust phylogeny for the group, although the simplest (most parsimonious) explanation given currently available information is that perennibranchiation is the primitive condition. It might be possible to identify the number of different mutations responsible for loss of

transformation by relatively simple genetic studies, but this will involve captive breeding of these salamanders. Various workers (e.g. Roberts et al. 1995) are developing methods for captive maintenance and breeding of members of the group, so prospects for future genetic and developmental studies are encouraging.

Species diversity and conservation issues

My investigations of the spring and cave-dwelling *Eurycea* of the Edwards Plateau region of central Texas have revealed considerable diversity in the group, and indicate that there are many distinct species inhabiting the numerous islands of cave and spring habitat that are scattered through the area. Many are characterized primarily by the molecular markers that I have identified, a few are distinguished largely based on morphology, some are differentiable based on both molecular and morphological criteria, and others likely are isolated from gene flow based primarily on geographic and hydrologic considerations. I consider any reasonable evidence that a population or interbreeding group of populations has embarked on a unique evolutionary trajectory (and is unlikely to substantially exchange genes with other such lineages in the foreseeable future) as potential evidence that it is a distinct species. In doing so, I follow the evolutionary species concept as advocated by Wiley (1978) and Frost and Hillis (1990); see Chapter 3 for further discussion of species concepts.

In the Texas *Eurycea*, geographic isolation in islands of suitable habitat has facilitated identification of species boundaries in some cases. Lineages that are restricted to one locality or a few nearby interconnected sites and exhibit diagnostic

markers or combinations of markers clearly represent distinct species; examples include *E. nana, E. sosorum*, and *E. rathbuni*, all of which satisfy nearly any criterion for recognition as separate species (except reproductive compatibility, which has not yet been tested). The highly differentiated northern populations definitely warrant taxonomic recognition as a distinct group that appears to consist of several species. I believe that there probably are many additional species within several of the groups that we informally recognize, particularly the relatively widespread "*E. latitans* group" in the southeast and the even more widespread "Carson Cave group" in the southwest. These are informal groupings based primarily on phenetic criteria, and additional sampling and study of molecular and morphological characters is needed to clarify species boundaries in these assemblages.

Unfortunately, even as we are discovering the rich diversity in the Edwards Plateau *Eurycea*, the existence of many members of the group is becoming increasingly threatened. Within the northern group, most populations of the Jollyville Plateau salamander occur in rapidly expanding northwest Austin, and most are likely to be destroyed by human activities in coming decades, while other newly discovered species in the north also face serious threats to their existence (Price et al. 1995). *Eurycea sosorum*, known only from a popular spring-fed swimming hole in downtown Austin, appears to have undergone a precipitous decline since the 1960's (Chippindale et al. 1993), and recent surveys (R. Hansen, pers. comm.) suggest a large reduction in numbers even since 1992; its proposed listing as a Federally Endangered Species still is pending after three years of

consideration. Increasing human demands on the waters of the southern Edwards Aquifer threaten to stop the flows of San Marcos and Comal Springs in the relatively near future, which likely would cause the extinction of the species of salamanders that inhabit these springs. Effects of a drop in aquifer levels on cave-dwellers, including the unique troglobite *E. rathbuni*, are uncertain but could be disastrous. The loss of salamander species diversity through habitat modification and destruction undoubtedly will be accompanied by loss of other organisms (many still undiscovered) that depend on the same aquatic habitats. Therefore, further study of species boundaries and relationships in the central Texas *Eurycea* is not only essential for an understanding of the evolutionary history of this group, but fundamental to identification of unique cave and spring ecosystems of the region that are most critically in need of protection.

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Table 1. Electrophoretic conditions used to resolve products of 25 enzyme-encoding loci in central Texas hemidactyliine plethodontid salamanders, plus outgroup taxa. Refer to Murphy et al. (1990) for composition of buffers 1, 3, 4, and 5, and Chippindale (1989) for composition of buffer 2. 1 = Tris-citrate II; 2 = "Poulik" pH 9.5; 3 = Tris-borate-EDTA pH 8.6; 4 = Tris-citrate-EDTA pH 7.0; and 5 = Tris-borate.

Locus	Abbreviation	E.C. number	Electrophoretic conditions
Aconitate hydratase 1	ACOH-1	4.2.1.3	1
Adenylate kinase	AK	2.7.4.3	1,4
Aspartate aminotransferase (cytosolic)	AAT-S	2.6.1.1	5
Creatine kinase 1	CK-1	2.7.3.2	1
Creatine kinase 2	CK-2	2.7.3.2	1
Cytosol aminopeptidase	CAP	3.4.11.1	1
Glucose-6-phosphate isomerase	GPI	5.3.1.9	2
Glutathione reductase	GR	1.6.4.2	1
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	1.2.1.12	3
Glycerol-3-phosphate dehydrogenase	G3PDH	1.1.1.8	3
Isocitrate dehydrogenase 1	IDH-1	1.1.1.42	1
Isocitrate dehydrogenase 2	IDH-2	1.1.1.42	1
Lactate dehydrogenase A	LDH-A	1.1.1.27	5
Lactate dehydrogenase B	LDH-B	1.1.1.27	5
Malate dehydrogenase 1	MDH-1	1.1.1.37	1
Malate dehydrogenase 2	MDH-2	1.1.1.37	1
NADP-dependent malate dehydrogenase 1	MDHP-1	1.1.1.40	1
Mannose-6-phosphate isomerase	MPI	5.3.1.8	2
Peptidase A (glycyl-L-leucine substrate)	PEP-A	3.4.?.?	3
Peptidase B (L-leucylglycylglycine sub.)	PEP-B	3.4.?.?	1
Peptidase D (L-phenylalanyl-L-proline sub.)	PEP-D	3.4.13.9	1
Phosphoglucomutase	PGM	5.4.2.2	3
6-Phosphogluconate dehydrogenase	PGDH	1.1.1.44	4
Pyruvate kinase	PK	2.7.1.40	4
Superoxide dismutase (cytosolic)	SOD-S	1.15.1.1	2

Table 2. A. Occurrence of observed alternative nucleotides in partial mitochondrial cytochrome b sequence, summarized by codon position. Values in cells represent total number of observations of combinations of alternative nucleotides (or invariant nucleotides) summed across the indicated positions. B. Transformation matrices calculated from the observed nucleotide changes, scaled to 100 for equivalence with allozyme frequency data; i = undefined value. To construct step matrices for parsimony analysis, values were averaged across the diagonal to yield symmetric step matrices (see text for details).

A.

Alternative nucleotide combination	Codon position 1	2	3
A	24	21	15
C	16	25	4
G	25	18	0
T	31	41	4
AC	3	0	0
AG	4	2	11
AT	1	1	1
CG	0	0	0
CT	8	6	48
GT	0	0	0
ACG	1	1	4
ACT	1	0	15
AGT	1	0	7
ACGT 0	0	7	

B.

Codon position 1:

	Nucleotide A	C	G	T
A	----	50	46	70
C	78	----	99	39
G	70	100	----	100
T	100	32	100	----

Codon position 2:

	Nucleotide A	C	G	T
A	----	100	58	100
C	100	----	100	54
G	62	100	----	i
T	89	44	i	----

Codon position 3:

	Nucleotide A	C	G	T
A	----	64	29	65
C	100	----	100	17
G	80	100	----	100
T	89	13	84	----

Figure 1.1. Localities sampled for molecular studies of central Texas *Eurycea*. Filled circles are springs and gray squares are caves. The hatched line along the margin of the area inhabited by salamanders represents the Balcones Fault Zone, the southern and eastern margin of the Edwards Plateau region.

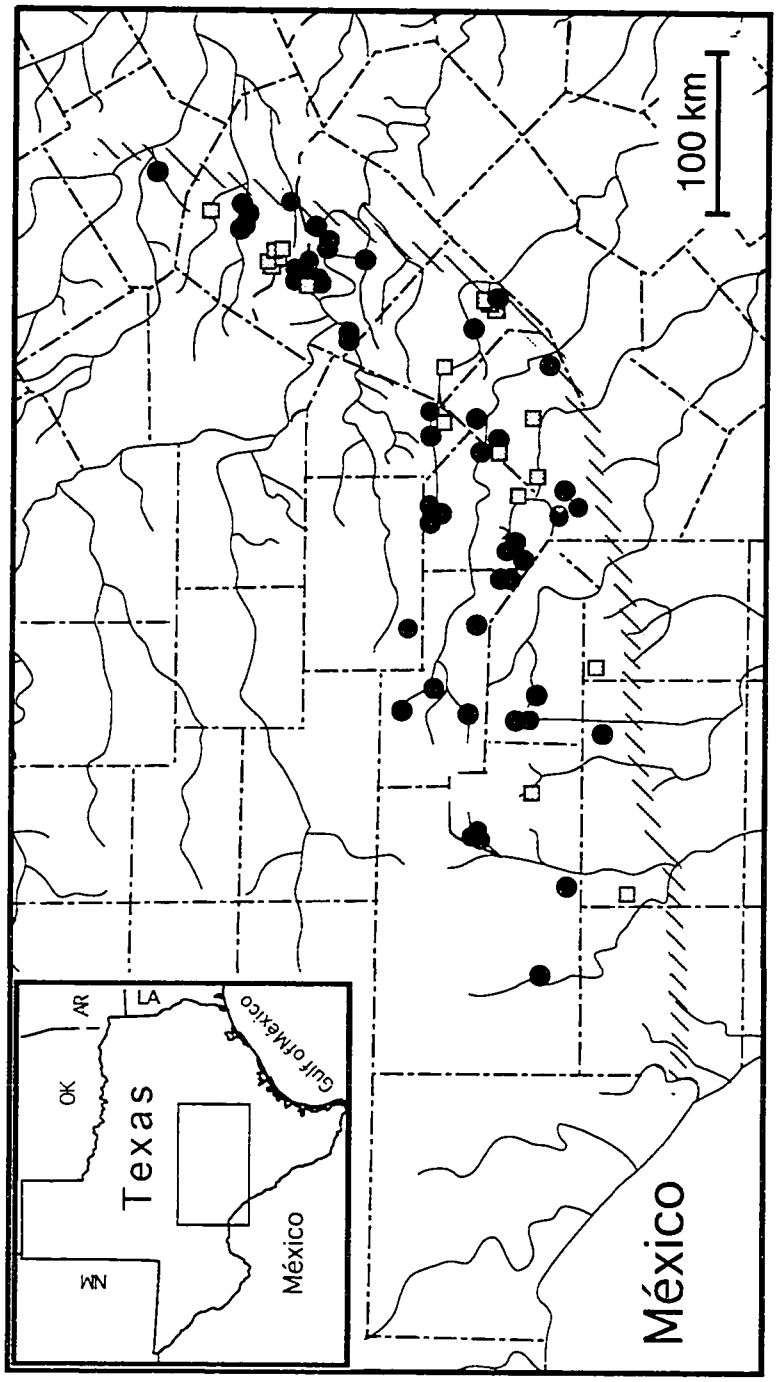

100 km
México
Texas
AR
LA
OK
NM
México
Gulf of Mexico

Figure 1.2. Central Texas counties inhabited by perennibranchiate *Eurycea*. Locations of major cities or salamander localities in the region are shown; city outlines are greatly simplified.

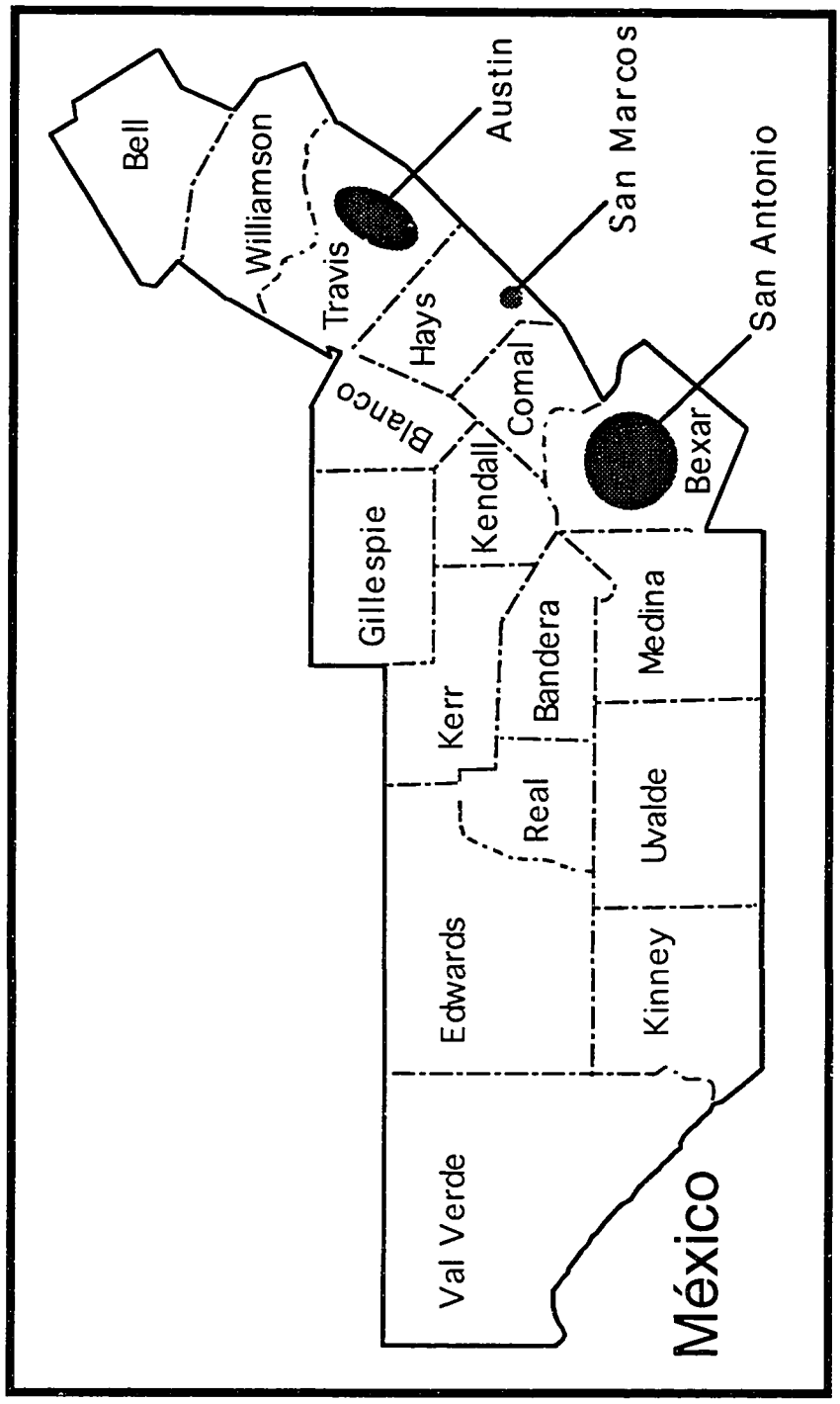

Bell
Williamson
Travis
Hays
Blanco
Gillespie
Kendall
Comal
Bexar
Austin
San Marcos
San Antonio
Kerr
Bandera
Medina
Real
Uvalde
Edwards
Kinney
Val Verde
México

Figure 1.3. UPGMA clustering of allozyme-based Manhattan distance for central Texas *Eurycea*. Major geographic regions are indicated. Numbers in parentheses correspond to the species and informal species groups that I recognize; these are shown in Figure 12 and listed in the legend to that figure.

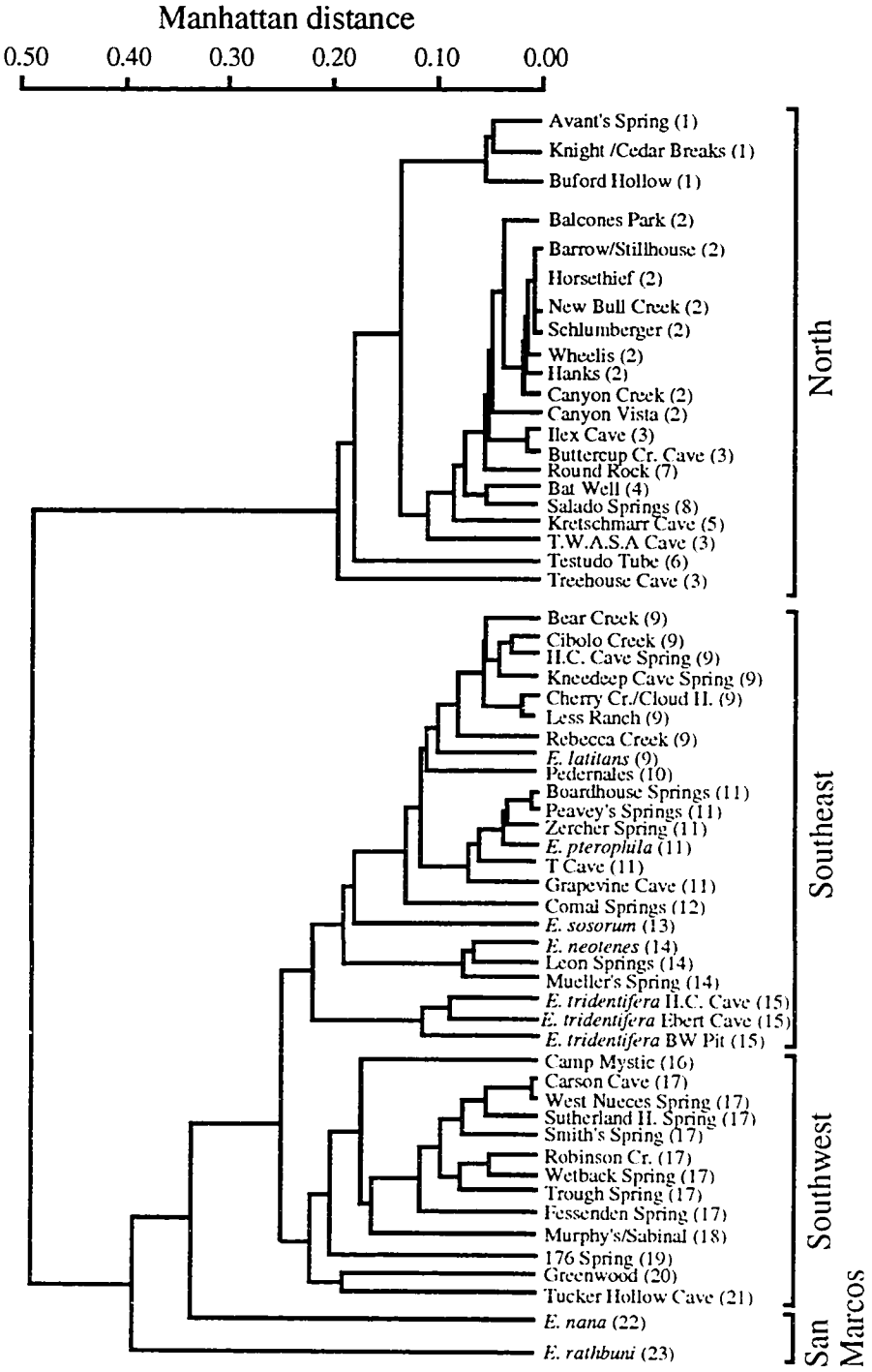

Manhattan distance
0.50
0.40
0.30
0.20
0.10
0.00
Avant's Spring (1)
Knight /Cedar Breaks (1)
Buford Hollow (1)
Balcones Park (2)
Barrow/Stillhouse (2)
Horsethief (2)
New Bull Creek (2)
Schlumberger (2)
Wheelis (2)
Hanks (2)
Canyon Creek (2)
Canyon Vista (2)
Ilex Cave (3)
Buttercup Cr. Cave (3)
Round Rock (7)
Bat Well (4)
Salado Springs (8)
Kretschmarr Cave (5)
T.W.A.S.A Cave (3)
Testudo Tube (6)
Treehouse Cave (3)
Bear Creek (9)
Cibolo Creek (9)
H.C. Cave Spring (9)
Kneedeep Cave Spring (9)
Cherry Cr./Cloud H. (9)
Less Ranch (9)
Rebecca Creek (9)
E. latitans (9)
Pedernales (10)
Boardhouse Springs (11)
Peavey's Springs (11)
Zercher Spring (11)
E. pterophila (11)
T Cave (11)
Grapevine Cave (11)
Comal Springs (12)
E. sosorum (13)
E. neotenes (14)
Leon Springs (14)
Mueller's Spring (14)
E. tridentifera H.C. Cave (15)
E. tridentifera Ebert Cave (15)
E. tridentifera BW Pit (15)
Camp Mystic (16)
Carson Cave (17)
West Nueces Spring (17)
Sutherland H. Spring (17)
Smith's Spring (17)
Robinson Cr. (17)
Wetback Spring (17)
Trough Spring (17)
Fessenden Spring (17)
Murphy's/Sabinal (18)
176 Spring (19)
Greenwood (20)
Tucker Hollow Cave (21)
E. nana (22)
E. rathbuni (23)
North
Southeast
Southwest
San Marcos

Figure 1.4. "Topographic" map of Manhattan distances among populations and taxa of central Texas *Eurycea*, based on the phenetic analysis shown in Fig. 3. The outline on which genetic distance contour lines are superimposed corresponds to counties inhabited by these salamanders, as shown in Fig. 2. Choice of distance increments is arbitrary.

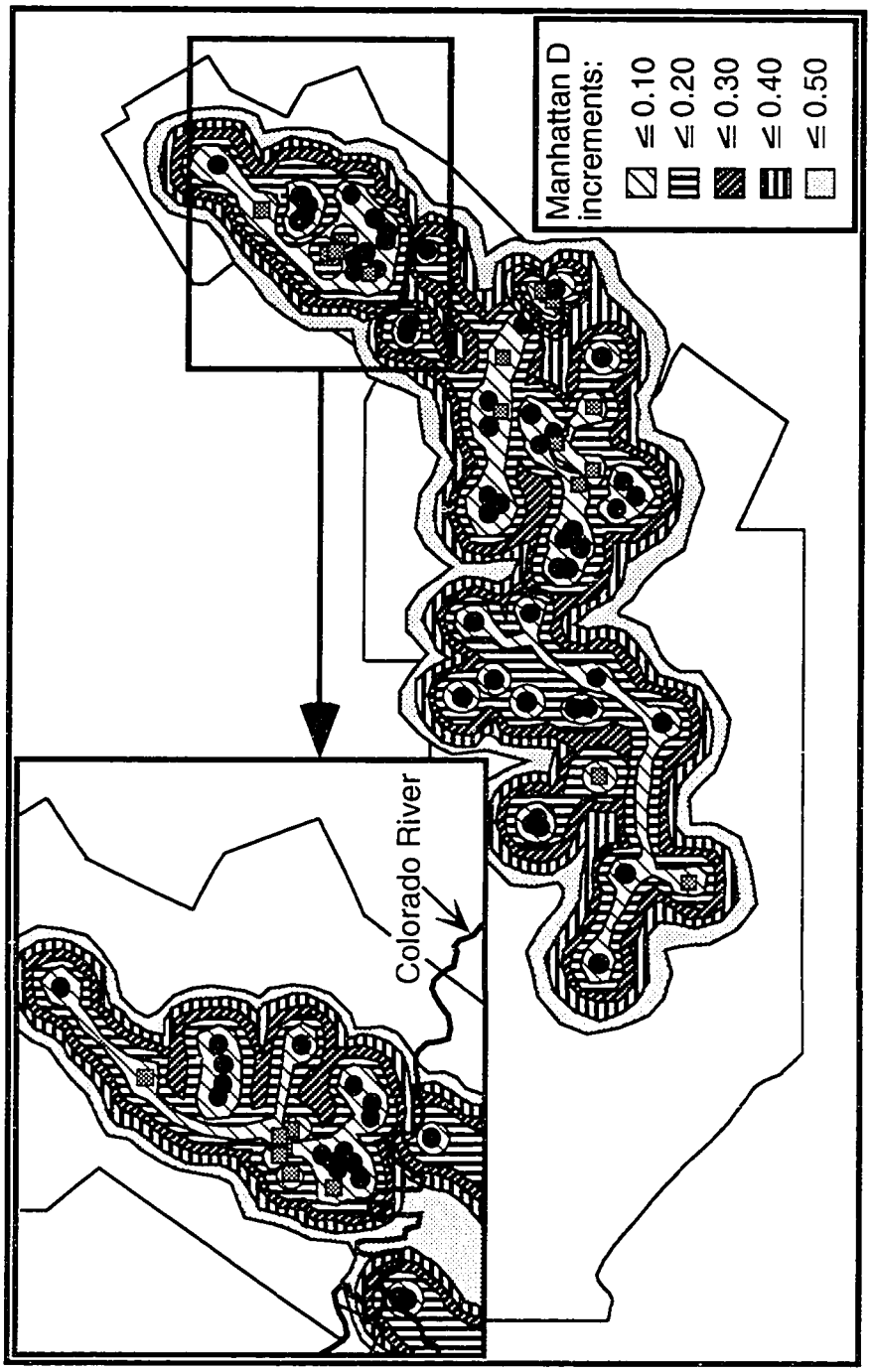

Manhattan D
increments:
≤ 0.10
≤ 0.20
≤ 0.30
≤ 0.40
≤ 0.50
Colorado River

Figure 1.5. UPGMA clustering of approximate sequence divergence for central Texas *Eurycea*, based on a maximum of 355 bp of cytochrome b sequence. Major geographic regions are indicated. Numbers in parentheses correspond to the species and informal species groups that I recognize; these are shown in Figure 12 and listed in the legend to that figure.

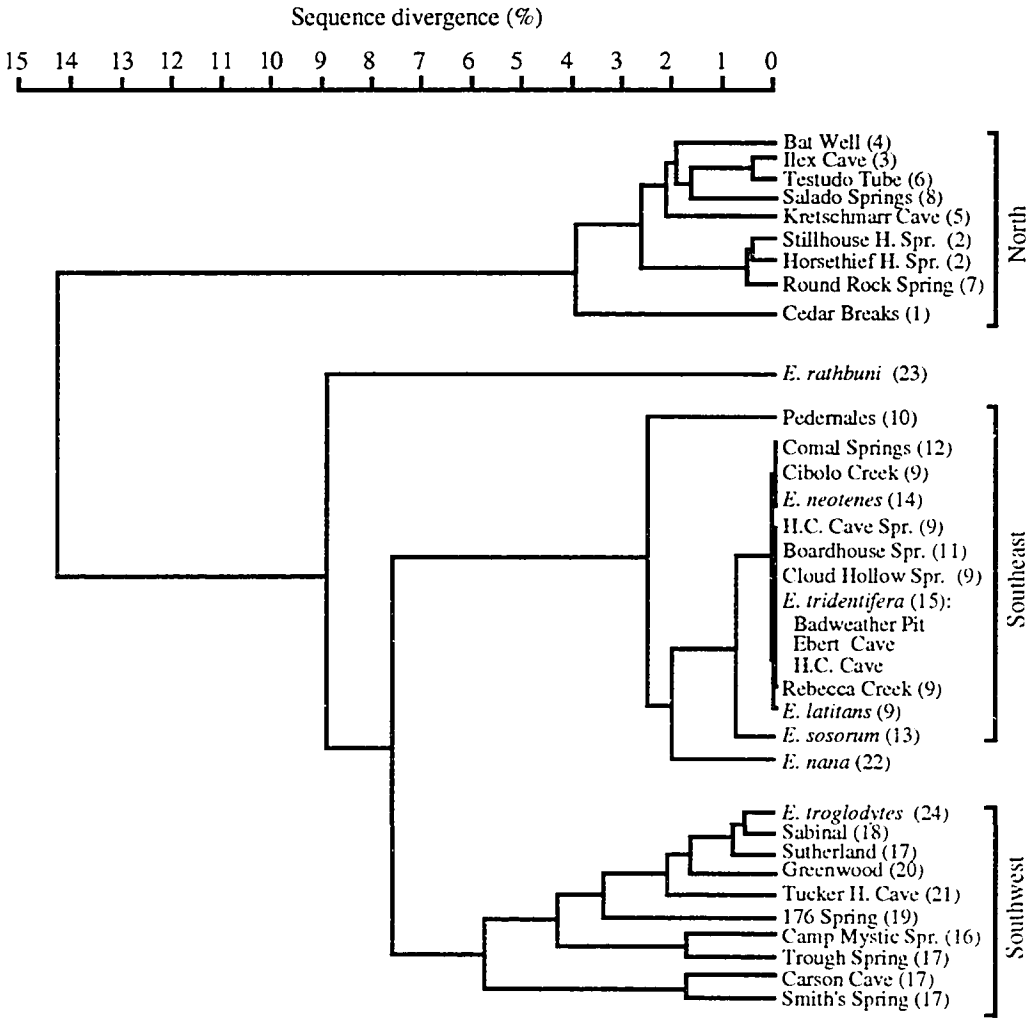

Sequence divergence (%)
15 14 13 12 11 10 9 8 7 6 5 4 3 2 1 0
Bat Well (4)
Ilex Cave (3)
Testudo Tube (6)
Salado Springs (8)
Kretschmarr Cave (5)
Stillhouse H. Spr. (2)
Horsethief H. Spr. (2)
Round Rock Spring (7)
Cedar Breaks (1)
North
E. rathbuni (23)
Pedernales (10)
Comal Springs (12)
Cibolo Creek (9)
E. neotenes (14)
H.C. Cave Spr. (9)
Boardhouse Spr. (11)
Cloud Hollow Spr. (9)
E. tridentifera (15):
Badweather Pit
Ebert Cave
H.C. Cave
Rebecca Creek (9)
E. latitans (9)
E. sosorum (13)
E. nana (22)
Southeast
E. troglodytes (24)
Sabinal (18)
Sutherland (17)
Greenwood (20)
Tucker H. Cave (21)
176 Spring (19)
Camp Mystic Spr. (16)
Trough Spring (17)
Carson Cave (17)
Smith's Spring (17)
Southwest

Figure 1.6. "Topographic" map of sequence divergences among populations and taxa of central Texas *Eurycea*, based on the phenetic analysis shown in Fig. 5. The outline on which sequence divergence contour lines are superimposed corresponds to counties inhabited by these salamanders, as shown in Fig. 2. Choice of sequence divergence increments is arbitrary.

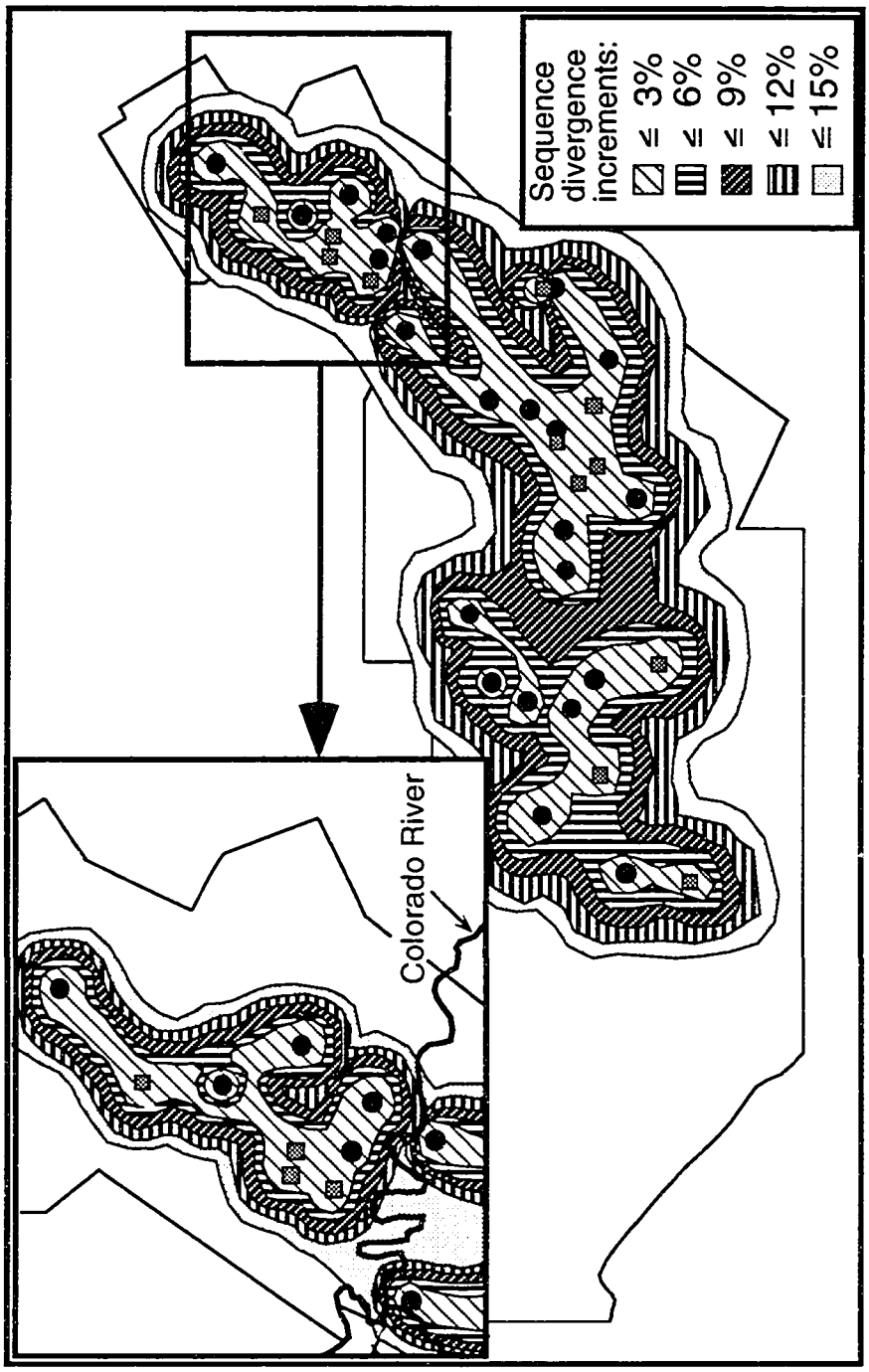

Sequence divergence increments:
≤ 3%
≤ 6%
≤ 9%
≤ 12%
≤ 15%
Colorado River

Figure 1.7A. Maximum parsimony tree based on a combination of allozyme data (coded using the Manhattan distance/step matrix approach described in the text) and sequence data (with application of combinatorial weights and codon position partitioning; see text for details). This is the single most parsimonious tree that resulted from a heuristic search using PAUP, and represents my "preferred" hypothesis of relationships in the group. Decay indices are shown for selected nodes.

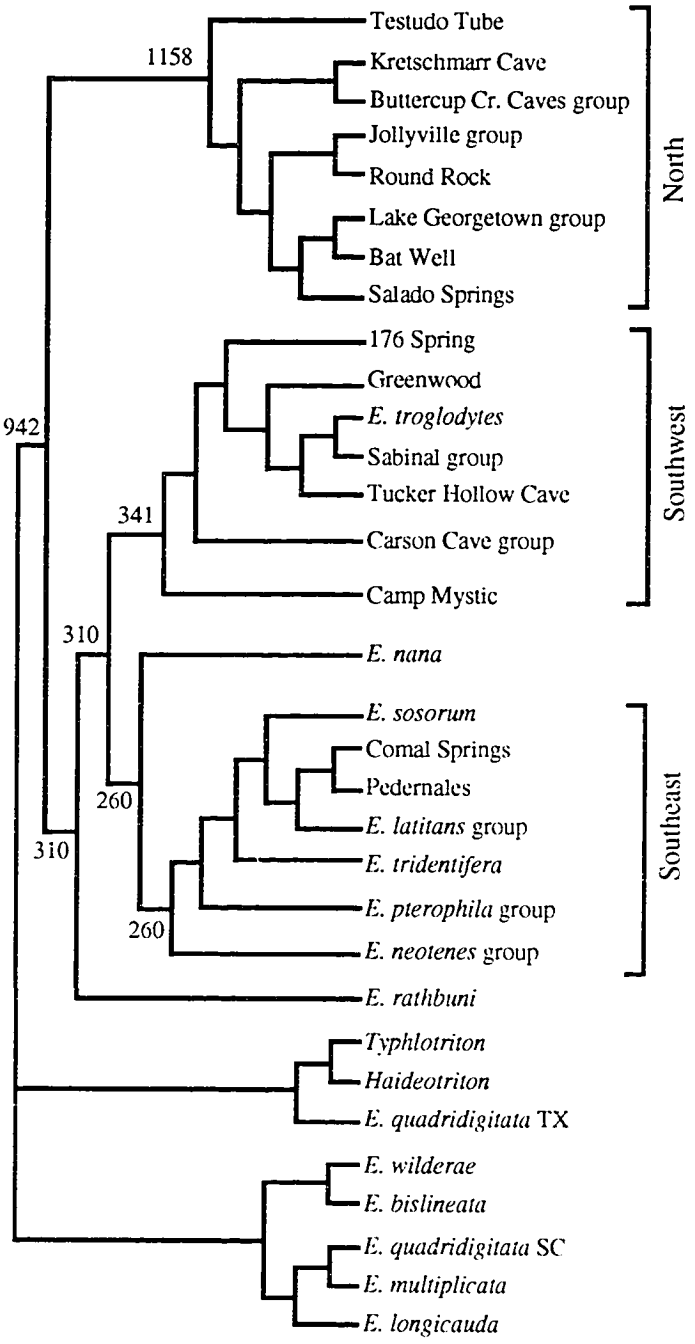

Testudo Tube
1158
Kretschmarr Cave
Buttercup Cr. Caves group
Jollyville group
Round Rock
Lake Georgetown group
Bat Well
Salado Springs
North
176 Spring
Greenwood
E. troglodytes
942
Sabinal group
Tucker Hollow Cave
341
Carson Cave group
Camp Mystic
Southwest
310
E. nana
E. sosorum
Comal Springs
Pedernales
260
E. latitans group
310
E. tridentifera
E. pterophila group
260
E. neotenes group
Southeast
E. rathbuni
Typhlotriton
Haideotriton
E. quadridigitata TX
E. wilderae
E. bislineata
E. quadridigitata SC
E. multiplicata
E. longicauda

Figure 1.7B. Bootstrap majority rule consensus tree based on a parsimony analysis of a combination of allozyme data (coded using the Manhattan distance/step matrix approach described in the text) and sequence data (with application of combinatorial weights and codon position partitioning; see text for details). Numbers at nodes represent the percentage of trees (of 100 bootstrap pseudoreplicates) in which a particular group appeared.

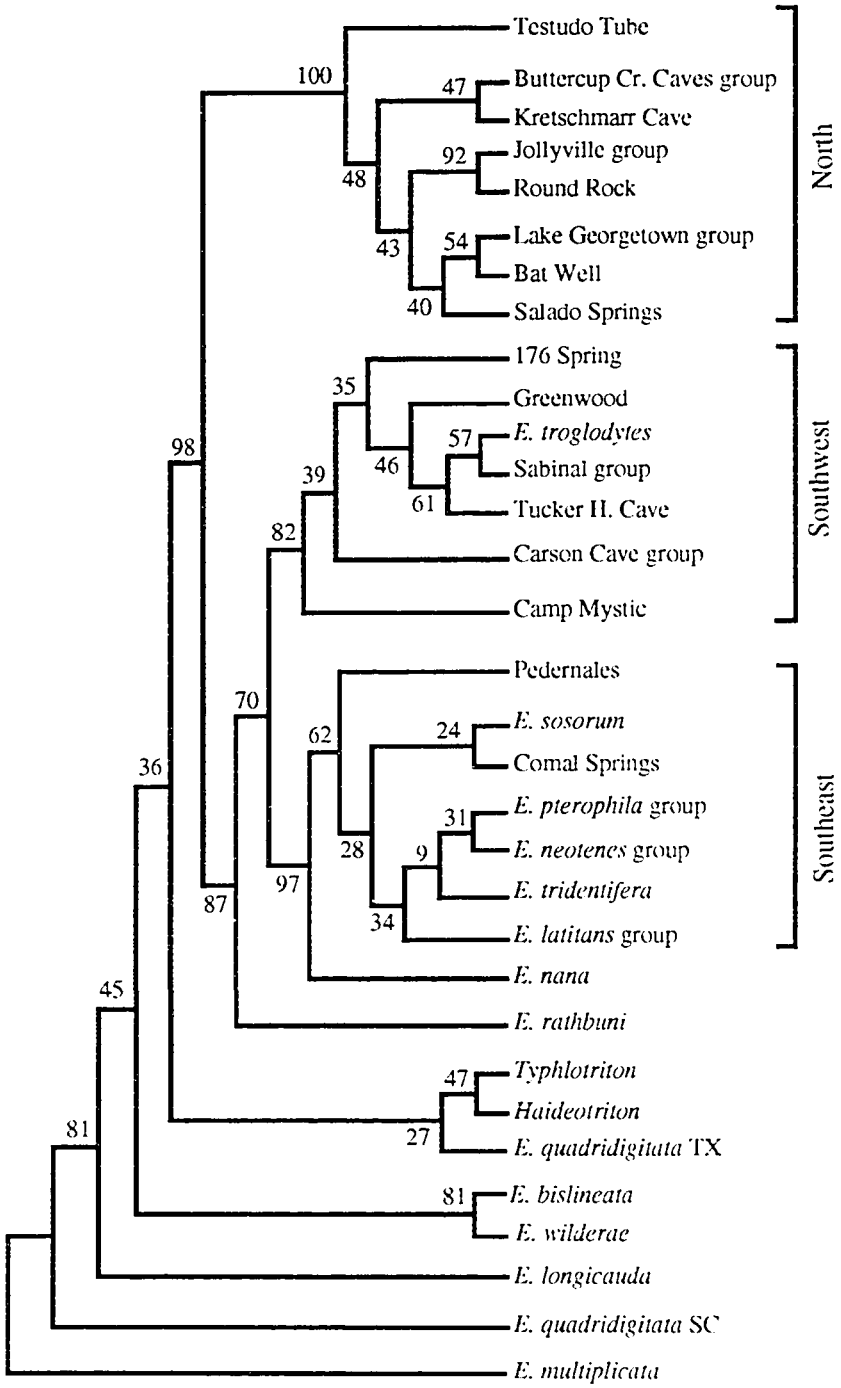

Testudo Tube
Buttercup Cr. Caves group
Kretschmarr Cave
Jollyville group
Round Rock
Lake Georgetown group
Bat Well
Salado Springs
North
176 Spring
Greenwood
E. troglodytes
Sabinal group
Tucker II. Cave
Carson Cave group
Camp Mystic
Southwest
Pedernales
E. sosorum
Comal Springs
E. pterophila group
E. neotenes group
E. tridentifera
E. latitans group
Southeast
E. nana
E. rathbuni
Typhlotriton
Haideotriton
E. quadridigitata TX
E. bislineata
E. wilderae
E. longicauda
E. quadridigitata SC
E. multiplicata
100
47
48
92
43
54
40
35
57
46
61
39
82
98
70
62
24
36
31
28
9
97
34
87
45
47
27
81
81

Figure 1.8A. Maximum parsimony tree based on a combination of allozyme data (coded using the Manhattan distance/step matrix approach described in the text) and sequence data (without application of differential character weighting). This is the single most parsimonious tree that resulted from a heuristic search using PAUP. Decay indices are shown for selected nodes.

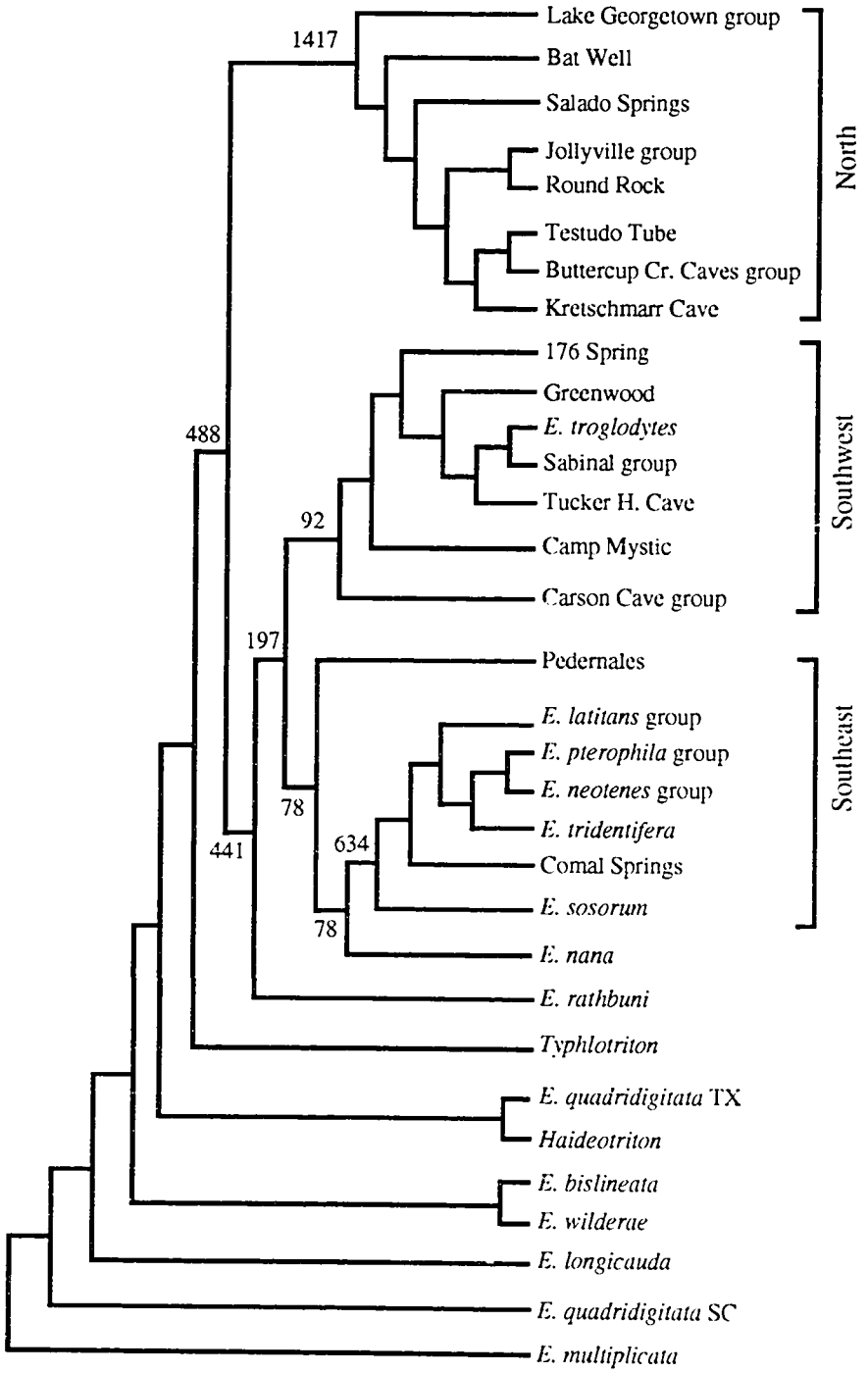

Lake Georgetown group
Bat Well
Salado Springs
Jollyville group
Round Rock
Testudo Tube
Buttercup Cr. Caves group
Kretschmarr Cave
176 Spring
Greenwood
E. troglodytes
Sabinal group
Tucker H. Cave
Camp Mystic
Carson Cave group
Pedernales
E. latitans group
E. pterophila group
E. neotenes group
E. tridentifera
Comal Springs
E. sosorum
E. nana
E. rathbuni
Typhlotriton
E. quadridigitata TX
Haideotriton
E. bislineata
E. wilderae
E. longicauda
E. quadridigitata SC
E. multiplicata
1417
488
92
197
78
441
634
78
North
Southwest
Southeast

Figure 1.8B. Bootstrap majority rule consensus tree based on a parsimony analysis of a combination of allozyme data (coded using the Manhattan distance/ step matrix approach described in the text) and sequence data (without application of differential character weighting). Numbers at nodes represent the percentage of trees (of 100 bootstrap pseudoreplicates) in which a particular group appeared.

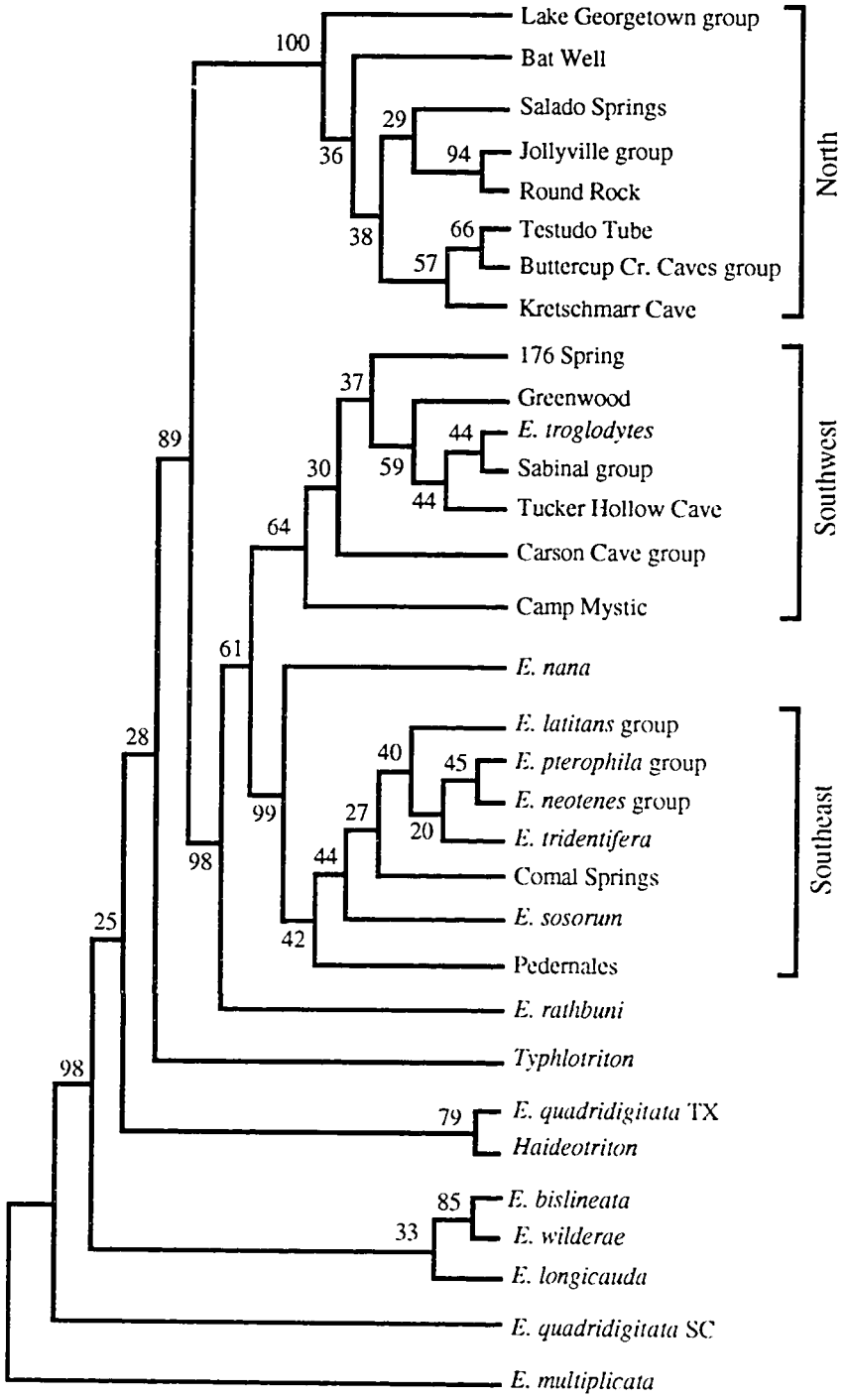

Lake Georgetown group
Bat Well
Salado Springs
Jollyville group
Round Rock
Testudo Tube
Buttercup Cr. Caves group
Kretschmarr Cave
North
176 Spring
Greenwood
E. troglodytes
Sabinal group
Tucker Hollow Cave
Carson Cave group
Camp Mystic
Southwest
E. nana
E. latitans group
E. pterophila group
E. neotenes group
E. tridentifera
Comal Springs
E. sosorum
Pedernales
Southeast
E. rathbuni
Typhlotriton
E. quadridigitata TX
Haideotriton
E. bislineata
E. wilderae
E. longicauda
E. quadridigitata SC
E. multiplicata
100
36
29
94
38
66
57
37
44
59
44
30
64
89
61
99
98
40
45
27
20
44
42
28
25
98
79
85
33

Figure 1.9. Maximum parsimony tree based on sequence data alone (with application of combinatorial weights and codon position partitioning; see text for details). This is a strict consensus of 90 equally parsimonious tree that resulted from a heuristic search using PAUP. Decay indices are shown for selected nodes.

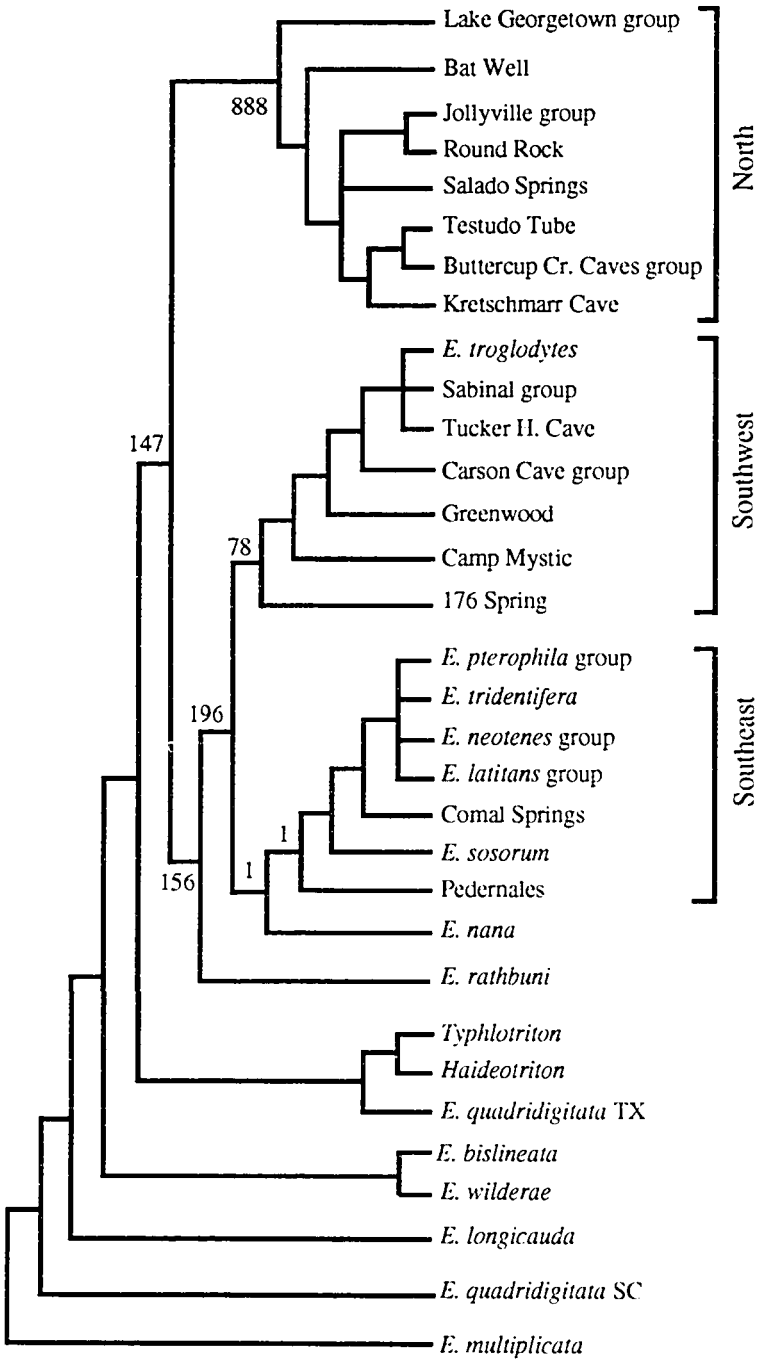

Lake Georgetown group
Bat Well
888
Jollyville group
Round Rock
Salado Springs
Testudo Tube
Buttercup Cr. Caves group
Kretschmarr Cave
North
E. troglodytes
Sabinal group
Tucker H. Cave
147
Carson Cave group
Greenwood
78
Camp Mystic
176 Spring
Southwest
E. pterophila group
E. tridentifera
196
E. neotenes group
E. latitans group
Comal Springs
1
E. sosorum
1
156
Pedernales
E. nana
Southeast
E. rathbuni
Typhlotriton
Haideotriton
E. quadridigitata TX
E. bislineata
E. wilderae
E. longicauda
E. quadridigitata SC
E. multiplicata

Figure 1.10. Maximum parsimony tree based on sequence data alone (without application of differential character weighting). This is a strict consensus of 88 equally parsimonious tree that resulted from a heuristic search using PAUP. Decay indices are shown for selected nodes.

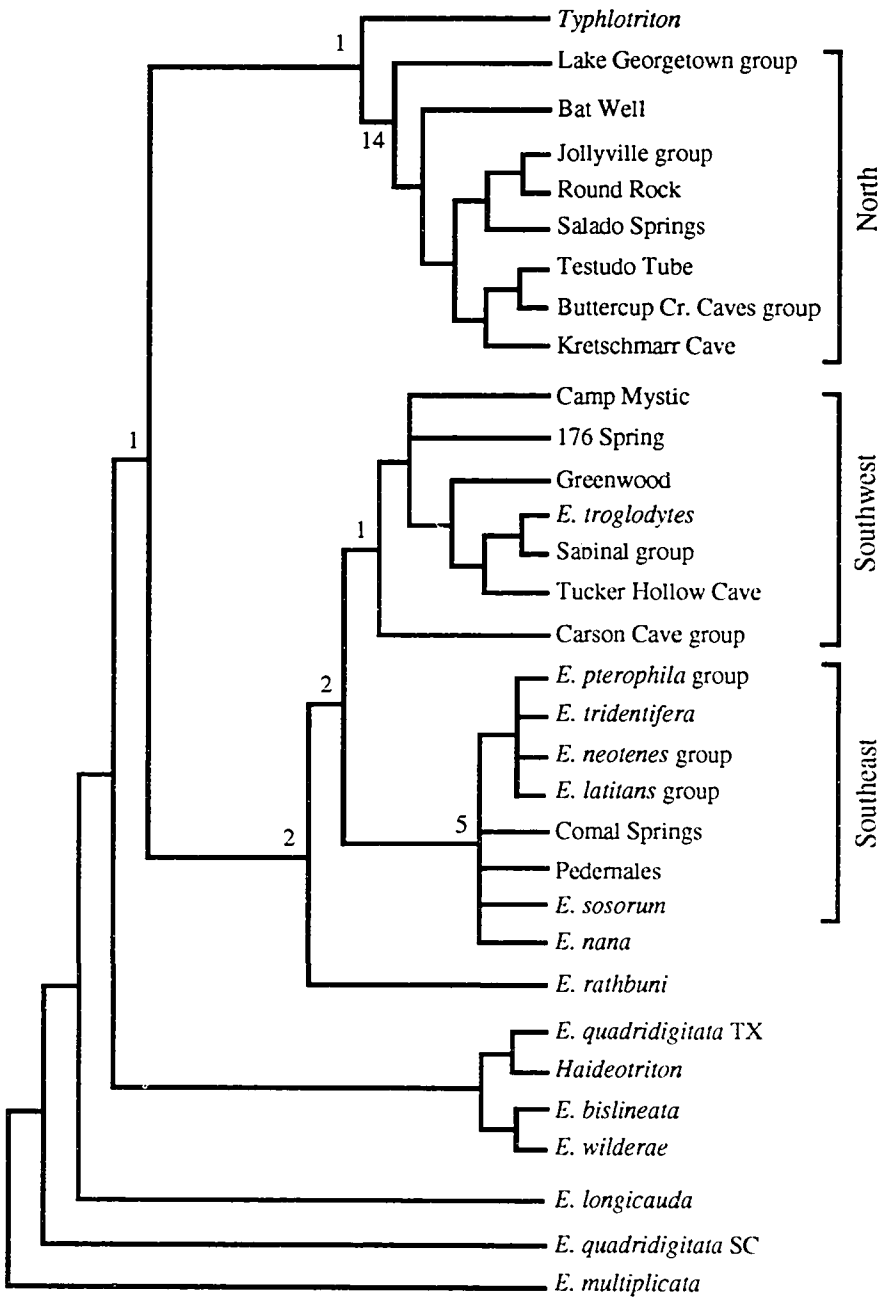

Typhlotriton
Lake Georgetown group
Bat Well
Jollyville group
Round Rock
Salado Springs
Testudo Tube
Buttercup Cr. Caves group
Kretschmarr Cave
North
Camp Mystic
176 Spring
Greenwood
E. troglodytes
Sabinal group
Tucker Hollow Cave
Carson Cave group
Southwest
E. pterophila group
E. tridentifera
E. neotenes group
E. latitans group
Comal Springs
Pedernales
E. sosorum
E. nana
Southeast
E. rathbuni
E. quadridigitata TX
Haideotriton
E. bislineata
E. wilderae
E. longicauda
E. quadridigitata SC
E. multiplicata
1
14
1
1
2
2
5

Figure 1.11A. Maximum parsimony tree based on allozyme data alone (coded using the Manhattan distance/step matrix approach described in the text). This is the single most parsimonious tree that resulted from a heuristic search using PAUP. Decay indices are shown for selected nodes.

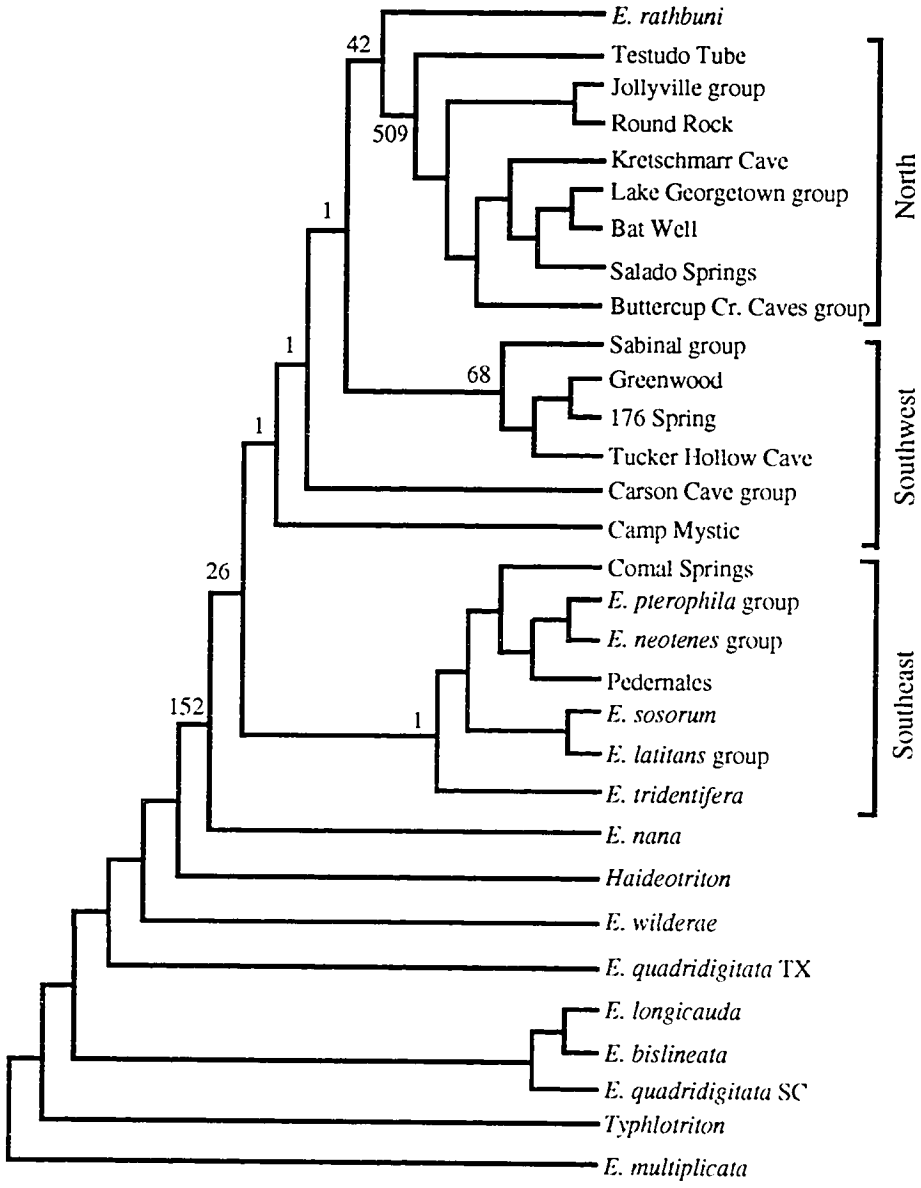

E. rathbuni
42
Testudo Tube
Jollyville group
509
Round Rock
Kretschmarr Cave
Lake Georgetown group
1
Bat Well
Salado Springs
Buttercup Cr. Caves group
North
1
Sabinal group
68
Greenwood
1
176 Spring
Tucker Hollow Cave
Carson Cave group
Camp Mystic
Southwest
26
Comal Springs
E. pterophila group
E. neotenes group
Pedernales
152
1
E. sosorum
E. latitans group
E. tridentifera
Southeast
E. nana
Haideotriton
E. wilderae
E. quadridigitata TX
E. longicauda
E. bislineata
E. quadridigitata SC
Typhlotriton
E. multiplicata

Figure 1.11B. Bootstrap majority rule consensus tree based on a parsimony analysis of allozyme data alone (coded using the Manhattan distance/step matrix approach described in the text). Numbers at nodes represent the percentage of trees (of 100 bootstrap pseudoreplicates) in which a particular group appeared.

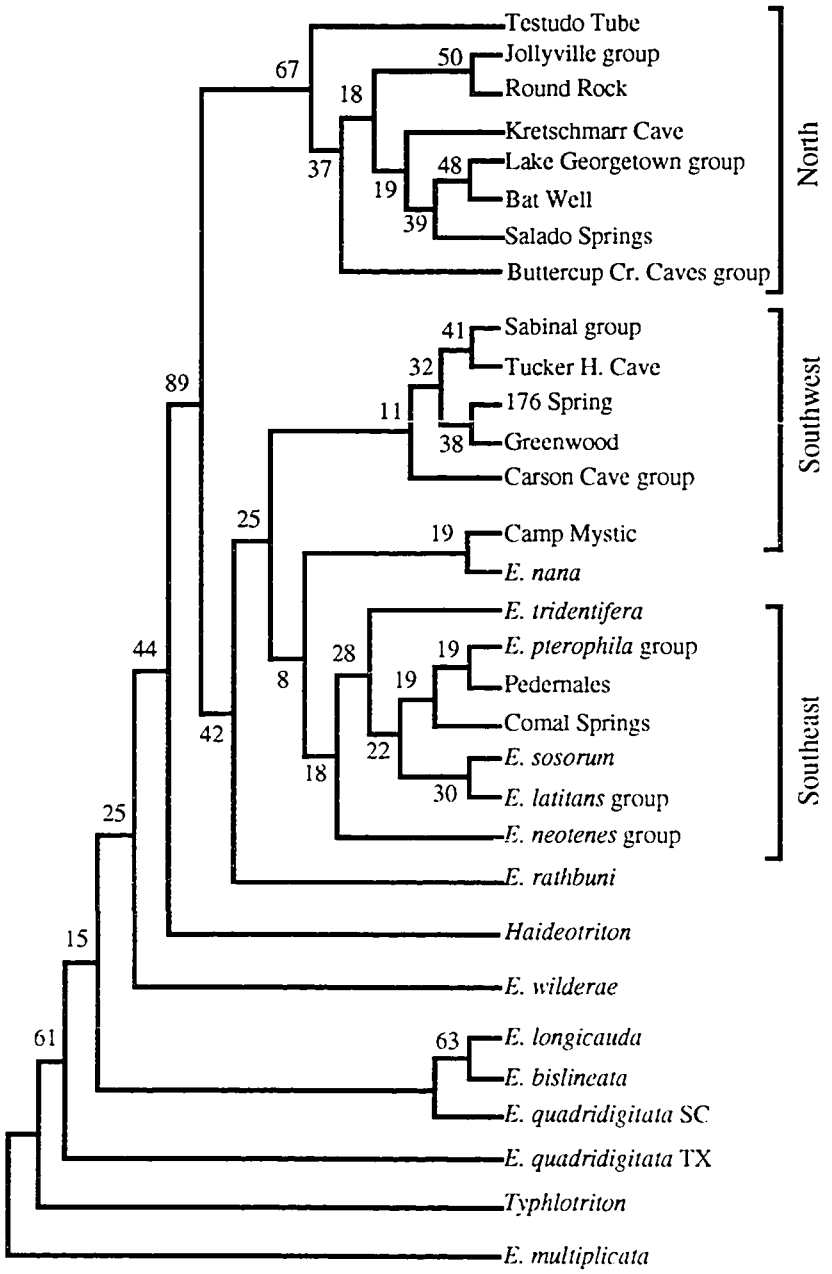

Testudo Tube
Jollyville group
Round Rock
Kretschmarr Cave
Lake Georgetown group
Bat Well
Salado Springs
Buttercup Cr. Caves group
North
Sabinal group
Tucker H. Cave
176 Spring
Greenwood
Carson Cave group
Southwest
Camp Mystic
E. nana
E. tridentifera
E. pterophila group
Pedernales
Comal Springs
E. sosorum
E. latitans group
E. neotenes group
Southeast
E. rathbuni
Haideotriton
E. wilderae
E. longicauda
E. bislineata
E. quadridigitata SC
E. quadridigitata TX
Typhlotriton
E. multiplicata
67
50
18
37
48
19
39
41
32
11
38
89
25
19
8
28
19
19
22
18
30
42
44
25
15
61
63

Figure 1.12. Species and informal groups to which I assign the central Texas *Eurycea* studied here. Filled circles represent springs and gray squares represent caves. The outline within which groups are shown represents the central Texas counties in which these salamanders are known to occur, as shown in Fig. 2. Group numbers correspond to those shown in the phenograms in Figures 3 and 5; specific details of these localities are given in Appendix 1. Groups are as follows: 1) Lake Georgetown; 2) Jollyville Plateau; 3) Buttercup Creek Caves; 4) Bat Well; 5) Kretschmarr Salamander Cave; 6) Testudo Tube Cave; 7) Round Rock; 8) Salado Springs; 9)*E. latitans* group; 10) Pedernales Springs; 11) *E. pterophila* group; 12) Comal Springs; 13) *E. sosorum*; 14) *E. neotenes* group; 15) *E. tridentifera*; 16) Camp Mystic Spring; 17) Carson Cave group; 18) Sabinal group; 19) 176 Spring; 20) Greenwood Ranch Springs; 21) Tucker Hollow Cave; 22) *E. nana*; 23) *E. rathbuni*; 24) *E. troglodytes*.

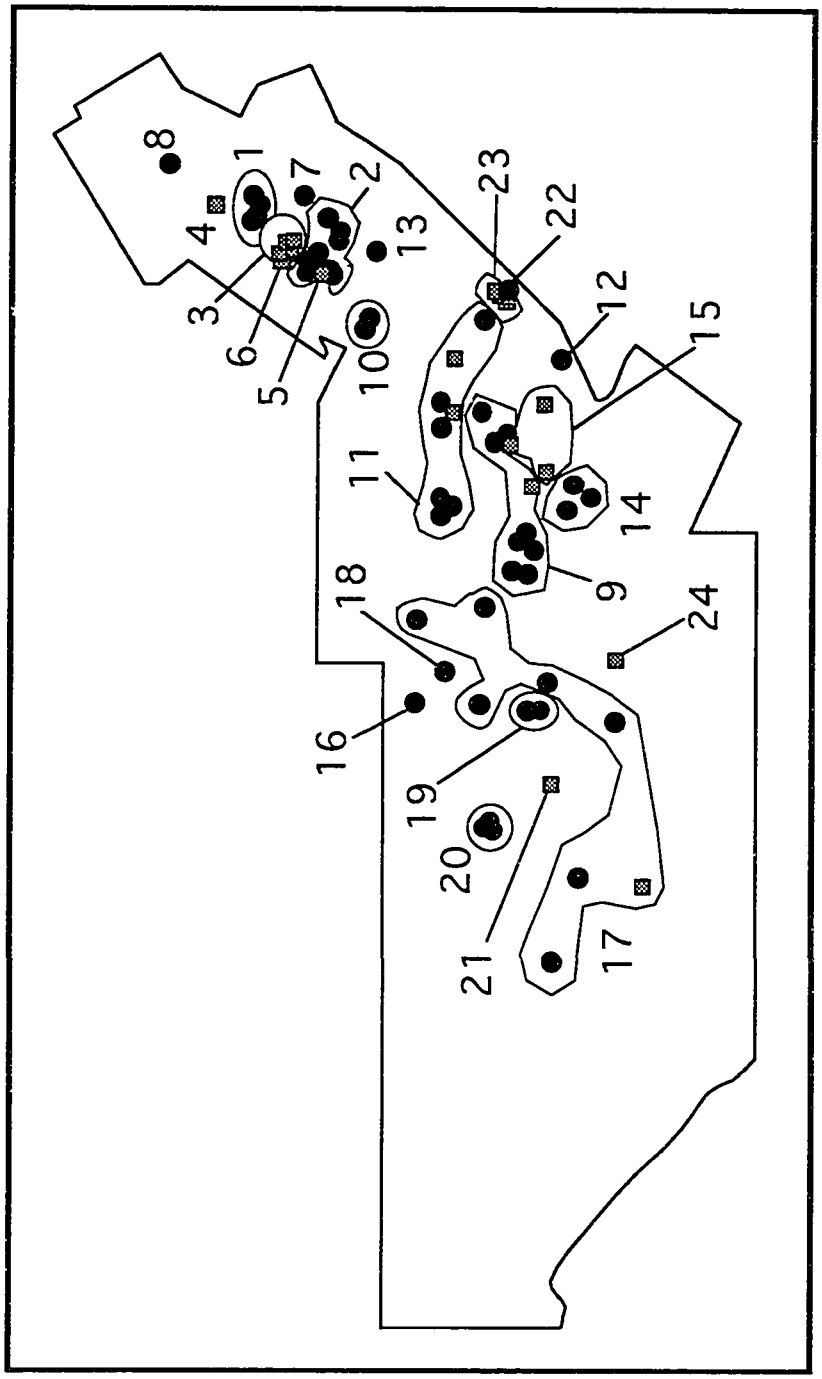

8
1
7
2
4
13
23
22
3
6
5
10
12
15
11
14
9
18
24
16
19
20
21
17

Figure 1.13. Number of habitat shifts required to explain the distribution of cave-dwelling in the central Texas *Eurycea*, given the preferred phylogenetic hypothesis shown in Fig. 1.7A. Black bars indicate minimum numbers of invasions of cave habitat assuming that surface-dwelling is primitive for the group and cave-dwelling arose independently in multiple lineages. Bars marked with an asterisk are groups for which only one member (of those examined) is a cave-dweller.

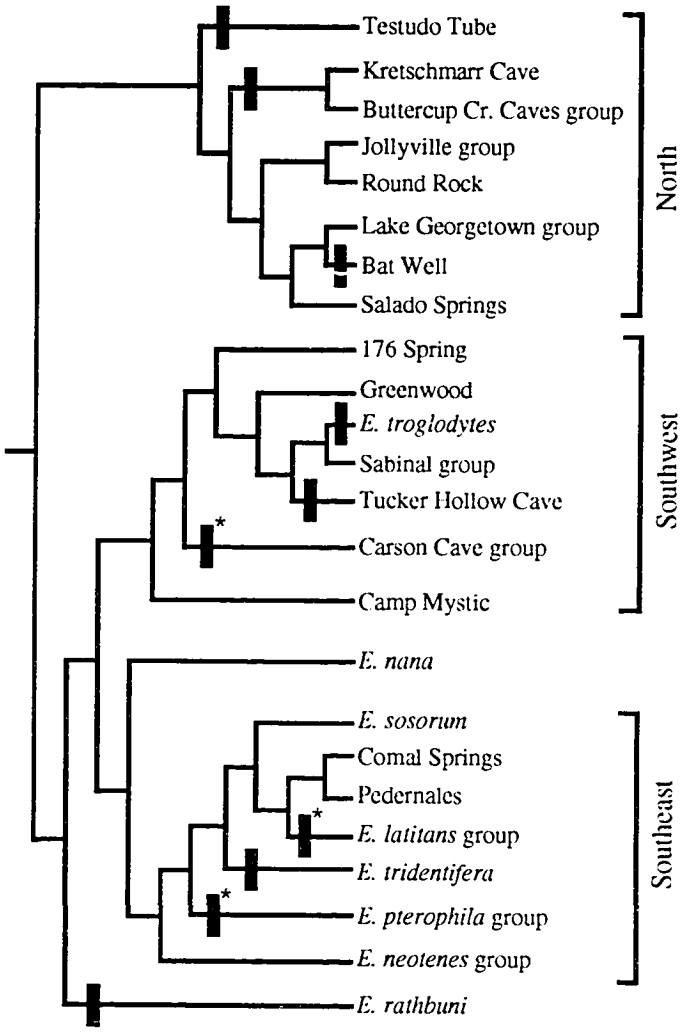
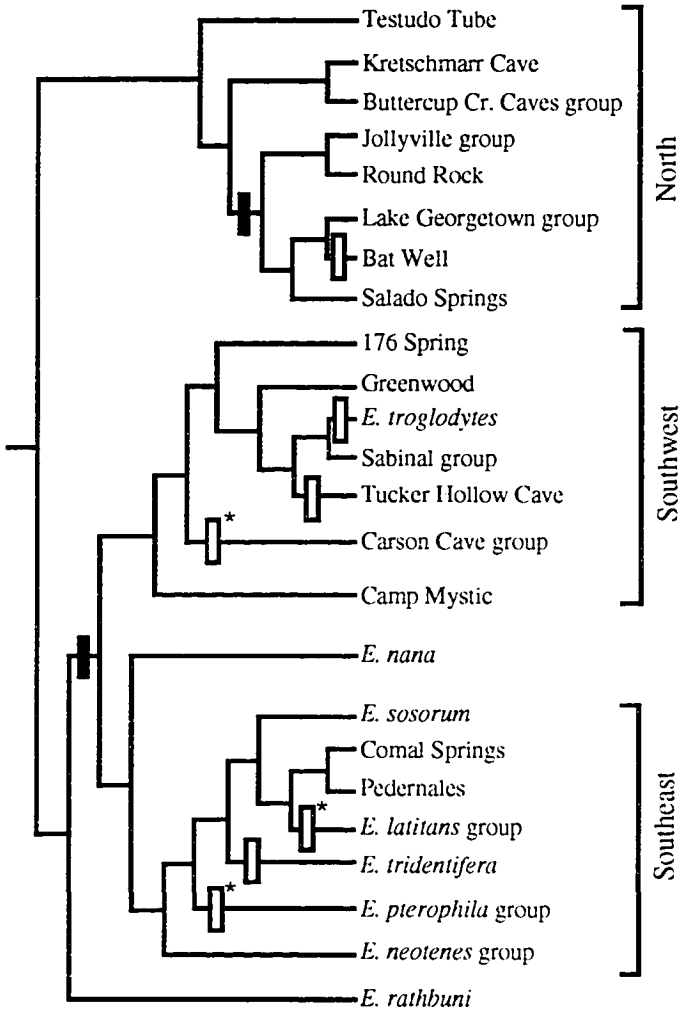

Testudo Tube
Kretschmarr Cave
Buttercup Cr. Caves group
Jollyville group
Round Rock
Lake Georgetown group
Bat Well
Salado Springs
North
176 Spring
Greenwood
E. troglodytes
Sabinal group
Tucker Hollow Cave
Carson Cave group
Camp Mystic
Southwest
E. nana
E. sosorum
Comal Springs
Pedernales
E. latitans group
E. tridentifera
E. pterophila group
E. neotenes group
Southeast
E. rathbuni

Figure 1.14. Number of habitat shifts required to explain the distribution of cave-dwelling in the central Texas *Eurycea*, given the preferred phylogenetic hypothesis shown in Fig. 1.7A. Black bars indicate minimum numbers of invasions of surface habitat assuming that cave-dwelling is primitive for the group, surface-dwelling arose subsequently, and reversals to cave-dwelling have occurred. Open bars indicate hypothesized reversals to cave-dwelling. Bars marked with an asterisk are groups for which only one member (of those examined) is a cave-dweller.


Testudo Tube
Kretschmarr Cave
Buttercup Cr. Caves group
Jollyville group
Round Rock
Lake Georgetown group
Bat Well
Salado Springs
North
176 Spring
Greenwood
E. troglodytes
Sabinal group
Tucker Hollow Cave
Carson Cave group
Camp Mystic
Southwest
E. nana
E. sosorum
Comal Springs
Pedernales
E. latitans group
E. tridentifera
E. pterophila group
E. neotenes group
Southeast
E. rathbuni

CHAPTER 2:

TAXONOMIC STATUS AND DISTRIBUTION OF THE SAN MARCOS SALAMANDER, *EURYCEA NANA*, WITH COMMENTS ON THE TAXONOMIC STATUS OF THE GENUS *TYPHLOMOLGE* (PLETHODONTIDAE: HEMIDACTYLIINI)

INTRODUCTION

The San Marcos Pool of the Edwards (Balcones Fault Zone) Aquifer in Hays Co., central Texas is a well delineated hydrologic system characterized by a large number of endemic species of vertebrates, invertebrates, and plants (e.g. Holsinger and Longley 1980, Longley 1986). These include perennibranchiate hemidactyliine plethodontid salamanders of the genera *Eurycea* and *Typhlomolge*. *E. nana* Bishop 1941 was described from specimens collected from the outlets of San Marcos Springs at the headwaters of the San Marcos River, while the extreme troglobite *T. rathbuni* Stejneger 1896 was described from specimens collected from the outflow of the nearby, 188 foot deep Artesian Well. Later, a second species of *Typhlomolge*, *T. robusta*, was recognized from a subterranean water system northeast of San Marcos (Potter 1963, Potter and Sweet 1981). The status and distribution of both *E. nana* and the two recognized species of *Typhlomolge* continue to be problematic; here I review the confusion and controversy in the

literature and attempt to resolve these issues based on molecular and morphological work that I have conducted with these taxa and other central Texas hemidactyliines.

MATERIALS AND METHODS

Allozyme and DNA sequence data presented here are drawn from a broad-scale study of relationships of central Texas hemidactyliines; for data and details of the methodology used for molecular analyses, refer to Chapter 1. Descriptions of measurements and morphometric analyses used here for investigation of the status of *E. nana* are given in Chippindale et al. (1993) along with summary statistics for the populations used and precise localities. Note that of the 11 measures used, HLB refers to the distance from the tip of the snout to the anterior margin of the eye and HLC refers to the distance from the posterior margin of the eye to the first gill insertion, incorrectly reported by Chippindale et al. (1993). In the morphometric analyses, I included a total of 127 specimens of *Eurycea*, of which 21 were from San Marcos Springs and 19 were from Comal Springs. The remainder, consisting of five groups (each from a separate locality) represent what I consider to be several distinct species from the southeastern Edwards Plateau region, the geographic area in which both San Marcos Springs and Comal Springs are located. These include the currently recognized named taxa *E. neotenes* and *E. sosorum*, plus what I consider *E. pterophila*, and a member of what I informally call the *E. latitans* species group (Chapter 3). Molecular analyses (Chapter 1, summarized in Fig. 1 of this chapter) indicate that the Comal Springs population, and probably the San Marcos population named as *E. nana*, belong to a clade of

hemidactyliines that consists of the populations from the southeastern region (exclusive of *T. rathbuni*), and thus *Eurycea* from this area are the most appropriate taxa for comparison.

RESULTS AND DISCUSSION

Eurycea nana

Since its description by Bishop (1941), *E. nana* has continued to be regarded by most authors as a distinct species, although Schmidt (1953) relegated this salamander to the status of a subspecies of the supposedly widespread species *E. neotenes*, a taxonomic change that has not been followed by most subsequent authors and was strongly protested by Brown (1967a). Bishop (1941) limited the known distribution of this salamander to the lake at the head of the San Marcos River in Hays Co. (fed by the outflows of San Marcos Springs); however, Baker (1961) mis-cited Bishop's description of the type locality as "the Comal Springs at San Marcos, Hays County." Baker clearly intended to refer to San Marcos Springs, as Comal Springs are located approximately 50 km southwest of San Marcos Springs at the head of the Comal River in New Braunfels, Comal Co., and later in the same paper, he referred to the population at Comal Springs in New Braunfels as *E. neotenes*. Sweet (1978) suggested informally that *E. nana* might occur at both San Marcos and Comal Springs, but did not elaborate except to say that animals from the two localities are "very similar" (and the Comal Springs population was listed as *E. neotenes* in Appendix 1 of Sweet 1978). Citing Sweet (personal communication), Dixon (1987) also extended the distribution of this species to

include Comal Co. Edwards et al. (1984) cited Bogart (1967) as believing that *E. nana* occurred at other sites in central Texas, but a rereading of Bogart (1967) shows that this was a misinterpretation (verified by personal communications with J.P. Bogart and D. Tupa), and Bogart actually regarded only the San Marcos Springs population as *E. nana*.

I found the San Marcos Springs *Eurycea* to be extremely distinct based on allozymes. Of the 25 enzyme-encoding loci examined for 357 specimens of central Texas hemidactyliines (plus outgroups representing three genera and eight species of hemidactyliines) the Comal Springs (12 specimens examined) and San Marcos Springs (13 specimens examined) populations were differentiated from each other by apparently fixed or mutually exclusive differences at eight loci. Of these, the San Marcos population exhibited fixed unique alleles (not seen in other central Texas hemidactyliines or outgroups) at five loci, clear evidence of the high level of divergence of this taxon and the lack of gene flow between it and other central Texas *Eurycea*. The Comal Springs population exhibited no unique alleles, but did possess an aconitate hydratase 1 allele at medium (45.5%) frequency otherwise seen only in the otherwise very distinct taxon *Typhlomolge rathbuni*. Sequence data for an approximately 355 bp portion of the mitochondrial cytochrome b gene (Chapter 1) also distinguish the San Marcos Springs population from other central Texas hemidactyliines (including the Comal Springs population). Although the level of differentiation based on these data was relatively low (sequence divergence of roughly 2% from other southeastern Edwards Plateau *Eurycea*; Chapter 1), the San Marcos population was distinguished from the other 33 populations of central

Texas *Eurycea* examined, plus outgroups, by unique substitutions at sequence positions 66 and 259. Phylogenetic analysis of the molecular data (Fig. 1 and Chapter 1) generally places *E. nana* from San Marcos as sister to other southeastern Edwards Plateau *Eurycea* exclusive of *T. rathbuni*, further illustrating the distinctiveness of this taxon.

Most San Marcos *E. nana* also are distinguishable from other central Texas hemidactyliines, including the Comal Springs population, based on external morphology. Body proportions and coloration have allowed me and others (D. Roberts, Dallas Zoo Aquarium, personal communication) to differentiate individuals from the San Marcos Springs and Comal Springs populations on sight. Discriminant analysis of 11 external morphological measures (Fig. 2) illustrates some differences in body form between the San Marcos Springs population (21 specimens examined) and the Comal Springs population (19 specimens examined). While both populations overlap substantially with each other (and some or all of the remaining populations) on canonical variate axes one and two (see also Chippindale et al. 1993), along canonical variate axes three and four there is very little overlap between the San Marcos Springs and Comal Springs specimens (Fig. 2; note that both populations do overlap on these axes with other southeastern Edwards Plateau *Eurycea*). Axis three accounts for about 8% of the total dicrimination among groups (on all axes), and axis four accounts for about 5%. Discrimination among the San Marcos, Comal Springs, and other southeastern Edwards Plateau *Eurycea* along each canonical variate axis is described by the following equations:

Canonical variate three = -52.838(SL) + 29.498(AG) + 7.419(TL) - 0.254(HLA) - 15.796(HLB) + 1.651(HLC) + 0.762(HW) - 8.856(IOD) + 12.335(HLL) + 13.234(ALL) + 2.115(ED)

Canonical variate four = -7.646(SL) - 17.721(AG) - 2.271(TL) - 7.511(HLA) - 0.936(HLB) + 14.112(HLC) + 28.194(HW) - 20.226 (IOD) + 20.279(HLL) - 17.012(ALL) + 7.272(ED)

Along axis three, most discrimination appears related to body size, with standard length (SL) accounting for 37.7% of the total discrimination among groups, AG (axilla-groin length) 34.4%, and TL (tail length) 20.6%. This likely reflects the relatively small adult size of mature San Marcos animals. Axis four appears to reflect the relatively narrow head of the San Marcos animals, with the highest loadings (35.4% and 35.1%) on HW (head width), and IOD (interocular distance), respectively; HLC (head length C; see Materials and Methods) accounts for 22.3%, and another 22.3% is attributable to HLL (hind limb length).

In addition to body proportions, Brown (1967a) listed several features of coloration and dentition that distinguish the San Marcos population from other Edwards Plateau *Eurycea*. This included the Comal Springs population, which he regarded as *E. n. neotenes* based on the distribution map that he provided. While I have not examined dentition in these salamanders in detail, I agree with Brown (based on casual observations) that the combination of coloration characters seen in

the San Marcos population (in particular the light brown color, which often appears to me as reddish, coupled with a dark eye ring) is unique among central Texas *Eurycea*.

Given that *E. nana* from San Marcos Springs readily can be differentiated from all other central Texas *Eurycea* examined based on allozymes, mitochondrial DNA, and at least some morphological features, and appears to be sister to a clade that consists of all other *Eurycea* species in the southeastern Edwards Plateau region (except "*T.*" *rathbuni*; see below), it seems reasonable to restrict the name of this taxon to the population at San Marcos Springs. The status of the Comal Springs population remains problematic, although it is clear that it does not represent *E. nana*. I regard this population as a distinct, undescribed species (following the evolutionary species concept sensu Wiley 1978 and Frost and Hillis 1990), but it exhibits considerable overlap with other southeastern Edwards Plateau *Eurycea* based both on molecular markers and morphology and thus many will not accept this interpretation. Additional study of this putative taxon is necessary to determine its relationship to other central Texas *Eurycea* and further clarify species boundaries in the group.

The genus *Typhlomolge*

Three species have at various times been regarded as members of the genus *Typhlomolge*. *T. rathbuni* Stejneger 1896 was the first to be described, and is known from several caves and wells that intersect the San Marcos Pool of the Edwards Fault Zone Aquifer (Russell 1976, Potter and Sweet 1981, Longley 1986).

In 1981, Potter and Sweet described another species of *Typhlomolge*, *T. robusta*, based on a single specimen collected in 1951 from a hole drilled in the bed of the Blanco River northeast of San Marcos. Longley (1978) sometimes is credited with an unintentional original description of this taxon (e.g. see Potter and Sweet 1981) because in a government report he used the name *T. robusta*, originally applied in a master's thesis by Potter (1963), and offered an extremely brief description of the type specimen (while acknowledging Potter as the author of the name). Dixon (1987) rejected Longley's description and cited several ways in which it deviated from taxonomic practices specified by the 1984 revised Code of Zoological Nomenclature. I concur with Dixon, and regard Potter and Sweet (1981) as the authors of the name. Wake (1966) initially recognized a third species in the genus, *T. tridentifera*, originally described by Mitchell and Reddell (1965) as *Eurycea tridentifera*. Sweet (1977) listed additional applications of this combination. However, Wake's assessment was based on a very limited number of specimens, and subsequent morphological work using additional material led Mitchell and Smith 1972) to place members of the genus *Typhlomolge* in the synonymy of the genus *Eurycea*. This conclusion also is strongly supported by the molecular work (Chapter 1, Fig. 1 of this chapter, and below).

Since the discovery of *Typhlomolge rathbuni*, relationships of the genus have remained controversial. Emerson (1905) first recognized that *T. rathbuni* was a plethodontid, and Wake (1966) assigned the genus to the tribe Hemidactyliini. Based on pre-phylogenetic analyses, Wake (1966) regarded *Typhlomolge* as part of a "pre-*Eurycea*" Miocene radiation of hemidactyliine plethodontids in southeastern

and south-central North America. Mitchell and Reddell (1965) and Mitchell and Smith (1972) viewed members of *Typhlomolge* simply as extremes in a continuum of cave-associated morphological changes in the Texas *Eurycea*. Potter and Sweet (1981) concurred with Wake's (1966) interpretation of the time scale for invasion of the Edwards Plateau region by ancestors of modern *Typhlomolge*. They demonstrated that while superficially, evolution of head morphology in *Typhlomolge* and central Texas cave *Eurycea* appeared to have followed the same trends, the osteological bases for broadening and flattening of the anterior portion of the skull differ in the two putative genera. Therefore, they made the suggestion (reasonable in light of their analyses) that the genus *Typhlomolge* should continue to be recognized. Using parsimony methods, Lombard and Wake (1986 and D.B. Wake, pers. comm.) investigated relationships among genera of plethodontids based primarily on characters of the feeding apparatus (especially the tongue), but were unable to reliably reconstruct the position of *Typhlomolge* except to verify that it belonged within the Hemidactyliini.

Allozyme and mitochondrial sequence data (Chapter 1, summarized in Fig. 1 of this chapter) strongly support the monophyly of the central Texas hemidactyliines inclusive of *T. rathbuni* (*T. robusta* was unobtainable for molecular studies, but I assume based on morphology that it is the sister species to *T. rathbuni* [see Potter and Sweet 1981 and Russell 1976 for further details]). However, the most basal split in the central Texas group clearly is between the groups of populations northeast versus southwest of the Colorado River, to which I refer here and elsewhere (Chapters 1 and 3) as the "northern" and "southern"

groups. Some members of both clades have until now been regarded as *E. neotenes* (e.g. Baker 1961, Brown 1967b, Sweet 1978, 1982), whose type locality is in Bexar Co. in the southern region. In nearly all analyses (summarized in Fig. 1; see Chapter 1) *T. rathbuni* is strongly supported as sister to all other southern populations and taxa; thus according to the current taxonomy, the "species" *E. neotenes* is rendered paraphyletic by the placement of the genus *Typhlomolge*. I am in the process of revising the taxonomy of central Texas hemidactyliines, recognizing additional species, and limiting use of the name *E. neotenes* (Chapters 1 and 3 and in prep.), but under any taxonomic arrangement the genus *Typhlomolge* cannot be retained if it is phylogenetically nested within *Eurycea*. In order to minimize the number of taxonomic changes, I therefore synonymize the genus *Typhlomolge* under *Eurycea*, and henceforth will refer to the members of this former genus as *Eurycea rathbuni* and *E. robusta*. Such a move is consistent with the recommendations of Mitchell and Reddell (1965) and Mitchell and Smith (1972), and thus simply represents a reapplication of this combination for *E. rathbuni*. However, as far as I am aware this is the first use of this combination for *E. robusta* since its formal description.

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Figure 2.1. Individual scores on canonical axes three and four based on discriminant analysis for *Eurycea nana* from San Marcos (open circles) and *E.* sp. from Comal Springs (filled circles). The filled background cluster labelled "other" illustrates scores for other southeastern Edwards Plateau *Eurycea*, including topotypical *E. neotenes*, topotypical *E. sosorum*, and members of what I consider the *E. pterophila* and *E. latitans* species groups.

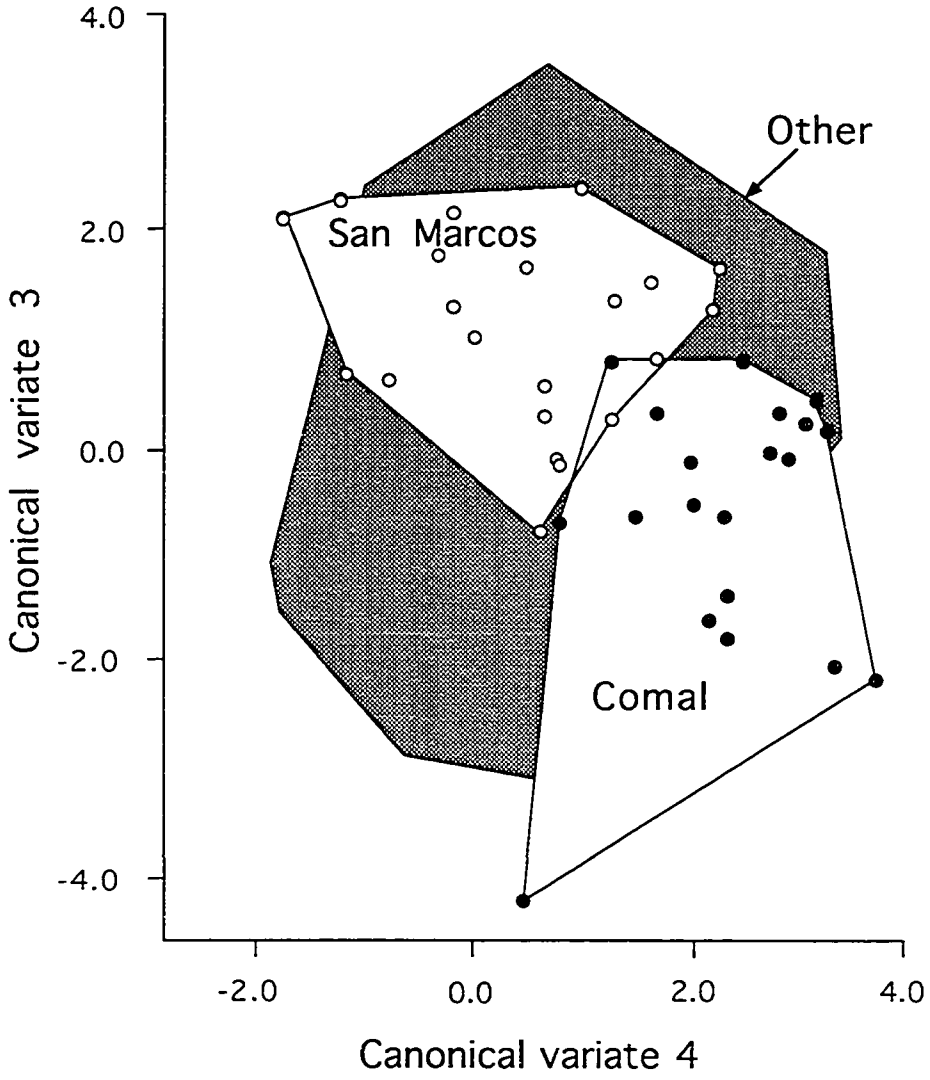
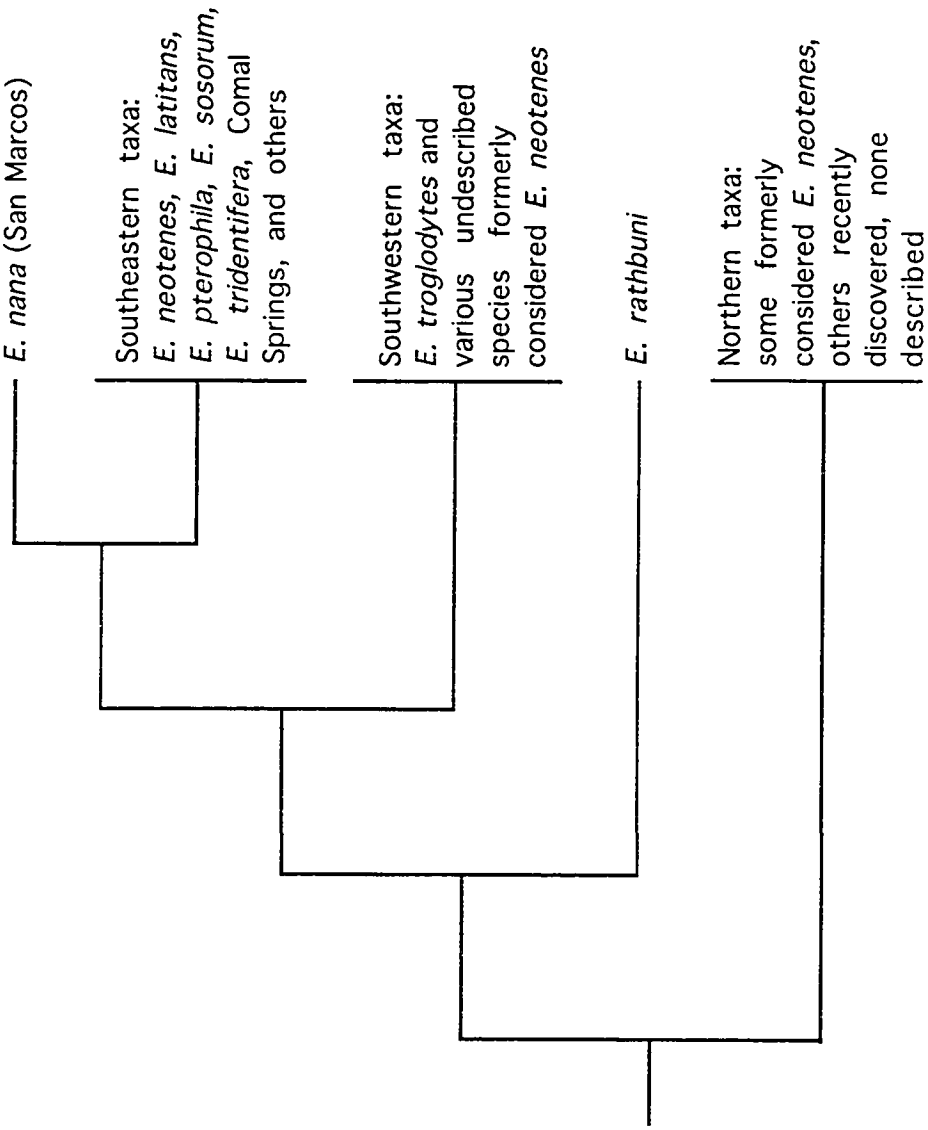

4.0
2.0
0.0
-2.0
-4.0
Canonical variate 3
-2.0
0.0
2.0
4.0
Canonical variate 4
Other
San Marcos
Comal

Figure 2.2. Simplified summary of phylogenetic hypotheses for the Texas *Eurycea* based on allozymes and mitochondrial cytochrome b sequences. See Chapter 1 for details of these analyses.


E. nana (San Marcos)
Southeastern taxa:
E. neotenes, E. latitans,
E. pterophila, E. sosorum,
E. tridentifera, Comal
Springs, and others
Southwestern taxa:
E. troglodytes and
various undescribed
species formerly
considered E. neotenes
E. rathbuni
Northern taxa:
some formerly
considered E. neotenes,
others recently
discovered, none
described

CHAPTER 3:

TAXONOMIC ASSESSMENT OF THE CENTRAL TEXAS *EURYCEA*

INTRODUCTION

The central Texas hemidactyliine plethodontid salamanders constitute a monophyletic assemblage (Chapter 1), highly fragmented geographically by geologic and climatic events. Isolation in springs and cave waters has led to considerable differentiation among many populations and groups of populations. In some cases, the primary evidence of divergence is morphological, and prior to my molecular work (detailed in Chapter 1) nearly all assessments of species diversity and boundaries were based on morphological criteria. This approach resulted in the recognition of a relatively small number of taxa in the group, at most six species or subspecies of *Eurycea* (e.g. see Baker 1961, Brown 1967a,b) plus two species of *Typhlomolge*. Several authors suggested, however, that additional species likely remained to be discovered in the group (e.g. Brown 1950, 1967b, Baker 1961, Bogart 1967, Mitchell and Smith 1972). In contrast, Sweet (1978a,b, 1982, 1984) took a "lumping" view of morphological diversity in the group, assigning nearly all spring and cave populations from throughout the Edwards Plateau region to the species *E. neotenes* and synonymizing the names *E. pterophila*, *E. latitans*, and *E. troglodytes* (the first he considered *E. neotenes* and the latter two he considered hybrids between *E. neotenes* and the cave-dweller *E. tridentifera*). This approach left

three recognized species of *Eurycea* in the group, *E. neotenes*, *E. tridentifera*, and *E. nana*, plus a fourth taxon from Barton Springs in Travis Co. that Sweet (1978a, 1984) considered a distinct species but did not formally describe (it later was described by Chippindale et al. 1993 as *E. sosorum*). Potter and Sweet (1981) also recognized two species in the genus *Typhlomolge*, *T. rathbuni* and *T. robusta* (I and some others consider these *Eurycea*; see Chapter 2).

Identification of species boundaries in the group is complicated (as is the case for all taxonomic groups) by different taxonomists' ideas of what constitutes a species. I follow the evolutionary species concept sensu Wiley (1978) and Frost and Hillis (1990). According to this view of species, one seeks evidence that a population or interbreeding group of populations constitutes a distinct evolutionary lineage that will continue to maintain a separate identity from other such lineages until it disappears or splits into two new species. This evidence can be based on any kinds of characters (e.g. morphological, molecular, behavioral etc.) and geographic considerations (i.e. the potential for isolation from gene flow) also are relevant. I reject species concepts that attempt to define species based on their reproductive compatibility with others (e.g the biological species concept of Mayr 1982 and the recognition species concept of Paterson 1985), their autapomorphies (e.g. phylogenetic species concepts such as that of Cracraft 1989), or their average level of differentiation from others (e.g. Highton et al. 1989, Highton 1990). All of these criteria can be helpful in identifying species boundaries, but none are sufficient. Rather than defining species, I believe that they are real entities which must be discovered. Sometimes the tools that we use are adequate to do so, but overall we

probably miss many, especially those that have diverged recently and thus have not accumulated many (or any) diagnostic markers.

The molecular evidence that I have gathered alone argues for the recognition of numerous species of Edwards Plateau *Eurycea*; here I use this information, together with morphological and geographic information, to perform an initial revision of the taxonomy of the group. In many cases, considerable ambiguity remains, and this is reflected by my tentative recognition of such heterogeneous (and possibly non-monophyletic) assemblages as the "Carson Cave group" in the southwestern Edwards Plateau region and the "*E. latitans* group" in the southeastern plateau region. My goal is not to apply a formal name to every single population, because for most, much more work is necessary before I could do so reliably. Instead, I will provide skeleton synonymies for each of the species and informal groups that I recognize, detail the evidence that led me to separate them, recommend a formal name for members of the group if one currently is in use or is available for resurrection, indicate what taxonomic problems remain, and suggest ways to solve these problems. My primary aims are to highlight the remarkable diversity in the group, identify populations or groups of populations that are particularly likely to represent distinct species, and provide a framework within which future studies of this diversity can proceed.

In the following accounts, I have organized the species and informal groups that I recognize by geographic region. These regions, and the distributions of salamanders within them, are detailed in Chapter 1; Appendix 1 provides precise

locality information for all populations examined. The northern and southern groups (populations northeast versus southwest of the Colorado River) each appear to be monophyletic clusters of species, and within the southern group are the apparently monophyletic southeastern and southwestern groups (see Chapter 1 and lists of synapomorphies below). The designation of a San Marcos region (inhabited by *E. nana*, *E. rathbuni*, and *E. robusta*) is simply one of convenience, because the taxa from this area are highly divergent and the placement of *E. nana* is uncertain (Chapters 1 and 2); no close relationship between *E. nana* and the other two species at San Marcos is implied. I also list character evidence for the monophyly of the central Texas *Eurycea* below. Sequence data refer to the maximum 355 bp of mitochondrial cytochrome b described in Chapter 1, and allozyme data are for the 25 enzyme-encoding loci listed in Chapter 1. For each account, I give the name that I recommend, followed by a skeleton synonymy (i.e. a list of each name or combination that has been applied to that taxon).

MONOPHYLY OF THE CENTRAL TEXAS *EURYCEA*, MAJOR GROUPS, AND TAXONOMIC ACCOUNTS

In Chapter 1, I show the results of phenetic and phylogenetic analyses of the molecular data and discuss the strong support for monophyly of the central Texas *Eurycea*. Here I will list unambiguous and potential synapomorphies for the group. These are:

Allozymes: All members of the group examined were fixed for the unique SOD a allele not seen in any other species of hemidactyliines examined; all share the unique

PK a allele except *E. rathbuni* and Camp Mystic Springs (*E. rathbuni* has a unique allele at this locus and Camp Mystic has an allele with the same mobility as that seen in *Haideotriton wallacei*); and all share the unique MDH-2 a allele except *E. rathbuni* (which has a unique allele) and Greenwood Valley Ranch Springs (for which no activity was detected at this locus in any of the six specimens examined).

Sequence: All populations share a T at position 216 except Greenwood and *E. rathbuni*, which both have C (all outgroup members have an A at this position); and all share an A at position 264 except the Carson Cave and Smith's Spring populations from the southwest, which have a G (outgroup members all have T or C).

Southern Region

This assemblage includes all populations of *Eurycea* from southwest of the Colorado River, ranging from western Travis County to eastern Val Verde County. I have examined specimens from 12 of the 14 counties in the southern region in which salamanders are known or thought to occur. The exceptions are Kinney Co., in which salamanders are likely to occur but no populations are known, and Val Verde Co. For the latter, repeated visits to the two known salamander localities at Del Rio (Fourmile Cave and San Felipe Springs; see Sweet 1978a) failed to yield specimens. I expect that these salamanders would fall within the southwestern group that I recognize (see below) based on their location. Very late in the study, *Eurycea* were discovered by Riley Nelson and co-workers north of Del Rio in springs in the Devil's River drainage. Superficially, these appear similar to other surface

populations from the southwest, but I have not yet examined them for molecular markers.

Only two unambiguous molecular synapomorphies (see *Allozymes* and *Sequence* below) unite members of the southern group, but this group is nearly always strongly supported as monophyletic in phylogenetic analyses of the molecular data (Chapter 1). Unambiguous or potential synapomorphies include the following:

Allozymes: For IDH-2 in all populations except *E. rathbuni* and Smith's Spring the b allele is fixed (an allele with the same mobility is seen in the outgroup taxon *Haideotriton*, however); at LDH-A the b allele is fixed or predominates in all populations (but an allele with this mobility is seen in the outgroup taxon *E. multiplicata*); and at MDHP the unique c allele is fixed or predominates in all populations except those from the Sabinal River drainage and *E. tridentifera* (each of these also have high frequency unique alleles).

Sequence: All populations except *E. rathbuni*, Carson Cave and Smith's Spring share a unique G at position 143; all except *E. rathbuni* share a unique C at 267; and all are united by a unique C at 270.

Amino acid sequence: N at codon 27 (also seen in one outgroup member, *E. bislineata*).

San Marcos Region

This area is located within Hays Co. in the southeastern region, but displays a unique aquatic fauna in many respects, and the salamanders found here (*E. rathbuni* and *E. robusta* underground and *E. nana* on the surface) are highly divergent from others in the Edwards Plateau region based both on morphology and molecular markers. I will not provide detailed accounts of these species here because I already have done so in Chapter 2. Synonymies and basic information are as follows:

Eurycea nana

Eurycea nana Bishop, 1941, Occas. Papers Mus. Zool. Univ. Michigan 451:6.

Eurycea neotenes nana Schmidt, 1953:55.

HOLOTYPE: UMMZ 89759.

TYPE LOCALITY: "Lake at the head of the San Marcos River, Hays County, Texas".

COMMENTS: As discussed in Chapter 2, there has been a great deal of confusion in the literature regarding the status and distribution of this taxon. All recent authors have used the name *E. nana*, which I support.

Eurycea rathbuni

Typhlomolge rathbuni Stejneger, 1896 Proc. U.S. Natl. Museum 18:620.

Eurycea rathbuni (Mitchell and Reddell, 1965:23).

HOLOTYPE: USNM 22686.

TYPE LOCALITY: "subterranean waters near San Marcos, Texas" (the specimens examined by Stejneger were from the 188 foot deep Artesian Well at what is now Southwest Texas State University in San Marcos).

COMMENTS: In Chapter 2, I discuss recognition of the genus *Typhlomolge* and recommend use of the name *Eurycea rathbuni* for this taxon. Frost (1985) gave the range of this species as "Underground waters in Hays, Kendall, and Comal counties, central Texas, USA", but there is no evidence that this species is known from anywhere but the San Marcos Pool of the Edwards Aquifer (see Potter and Sweet 1981 and Chapter 2 for further discussion).

Eurycea robusta

Typhlomolge robusta Potter and Sweet, 1981, Copeia 1981:70.

HOLOTYPE: TNHC 20255.

TYPE LOCALITY: "Beneath the Blanco River, 178 m elevation, 5 airline km NE of the Hays County Courthouse, San Marcos, Hays County, Texas".

COMMENTS: In Chapter 2 I recommend synonymization of the genus *Typhlomolge* under *Eurycea*, which renders this taxon *Eurycea robusta*. I recognize Potter and Sweet (1981) as authors of the original name for reasons detailed in Chapter 2.

Southeastern Region

This region encompasses western Travis, Hays (except San Marcos, which I treat separately; see above and Chapter 2), Blanco, Comal, Kendall, Bexar, and extreme eastern Kerr Counties. It contains the type localities for most of the central Texas *Eurycea* that have been recognized: *E. neotenes*, *E. latitans*, *E. pterophila*, *E. tridentifera*, and *E. sosorum*. It is not clear whether the problematic taxon *E. nana* (from San Marcos Springs) should be considered part of a monophyletic

southeastern clade: as discussed in Chapters 1 and 2, this species is highly divergent based on allozymes but falls within or sister to the southeastern populations based on cytochrome b sequence. My preferred hypotheses of relationships (based on combined allozyme and sequence data) suggest that *E. nana* is the sister species of all southeastern taxa. In listing potential synapomorphies and distinguishing molecular character states for the southeastern group I will specify which include this species.

Allozymes: For AAT-S, the a allele predominates in most southeastern populations except *E. neotenes*, *E. tridentifera*, Comal Springs, and Pedernales (and *E. nana*); however, this allele also is found in some other central Texas *Eurycea*.

Sequence: Unique C at position 117 (includes *E. nana*); unique C at 261 (includes *E. nana*); T at 303 (includes *E. nana*, but also shared with *E. rathbuni*); unique G at 328 (includes *E. nana*); unique G at 360.

Southeastern taxa and informal groups

For the purposes of analysis (see Chapter 1) I divide the southeastern clade into seven groups: *E. neotenes* group, *E. pterophila* group, *E. sosorum*, *E. tridentifera*, Comal Springs, Pedernales Springs, and *E. latitans* group. It is likely that all except the last (which is a heterogeneous and wide-ranging assemblage; see below) represent distinct species. The evidence of species status is particulary strong for *E. tridentifera*, *E. sosorum*, and the Pedernales populations, each of which exhibit diagnostic molecular markers and (for the former two) unique morphological features (the Pedernales populations have not yet been examined in detail morphologically). Members of the *E. neotenes*, *E. latitans*, and *E. pterophila* groups

plus the Comal Springs population are distinguished primarily based on substantial allele frequency differences at allozyme loci.

Southeastern group members

Eurycea neotenes

Eurycea neotenes Bishop and Wright 1937, Proc. Biol. Soc. Wash. 50:142.

Eurycea neotenes neotenes, Schmidt, 1953:55.

HOLOTYPE: USNM 10161.

TYPE LOCALITY: "Culebra Creek, 5 miles north of Helotes, Bexar County, Texas"; corrected by Brown (1942) to the headspring of Helotes Creek in Bexar Co.

COMMENTS: Schmidt (1953) first suggested that *E. neotenes* from the type locality, plus most other populations of *Eurycea* in the Edwards Plateau region, be considered the subspecies *E. n. neotenes*, and he also recognized the subspecies *E. n. nana* and *E. n. pterophila* (see Chapter 2 and below for detailed accounts of these taxa). The subspecific designation was ignored by some authors (e.g. Baker 1961); Brown (1967a) argued for recognition of *E. nana* as a full species but continued to recognize the subspecies *E. n. neotenes and E. n. pterophila*. Sweet (1978b) formally synonymized the names *E. pterophila* or *E. n. pterophila* under *E. neotenes*, and subspecies within *E. neotenes* have not generally been recognized since. Many authors (e.g. Brown 1950, Schmidt 1953, Conant 1958, Baker 1961, Mitchell and Smith 1972, Sweet 1977b, 1978a,b, 1982, 1984, Conant and Collins 1991) have regarded *E. neotenes* (or *"E. n. neotenes"*) as widespread in springs and caves of the Edwards Plateau region. Based on the molecular evidence presented in Chapter 1, I

disagree, and recommend restriction of the name *E. neotenes* to spring populations from the vicinity of the type locality. The three populations that I examined which I place in the "*E. neotenes*" group are those from the type locality at Helotes Creek Spring, Bexar Co., plus that at Leon Springs, Bexar Co., and Mueller's Spring, Kendall Co. (here and throughout, precise localities are given in Appendix 1 of Chapter 1). My decision to do so is based primarily on phenetic criteria and geographic considerations, as I found no unambiguous autapomorphies (among-population synapomorphies) for this taxon. Evidence for the distinctiveness of *E. neotenes* is based on allozymes, as follows:

Allozymes: These populations exhibit the b allele at high frequency at AAT-S, otherwise absent or at low frequency in other southeastern populations and taxa (except *E. tridentifera*, *E. sosorum*, and the Comal Springs and Pedernales populations); possess the b allele at PGM at high frequency, otherwise seen only in the Comal Springs and Camp Mystic (southwestern group) populations and the outgroup member *E. quadridigitata* from Texas; and exhibit the MPI b allele at high frequency, otherwise seen only in the Pedernales populations, some members of the "*E. pterophila* group", and the Fessenden Springs population from the southwest. Additional study of members of the southeastern clade will be necessary to more clearly delineate the boundaries of this and other species in the group.

Eurycea pterophila

Eurycea pterophila Burger, Smith, and Potter, 1950, Proc. Biol. Soc. Washington 63:51.

Eurycea neotenes pterophila, Schmidt, 1953:56.

Eurycea neotenes, Sweet, 1978b: 106 (in part).

HOLOTYPE: Floyd Potter Coll. No. A993 (private collection, now presumed lost).

TYPE LOCALITY: "shallow stream flowing from Fern Bank Spring, 6.3 miles northeast of Wimberley on the Blanco River Road, Hays County, Texas".

COMMENTS: Sweet (1978b) demonstrated that the morphological character states used by Burger et al. (1950) to distinguish *E. pterophila* from other Edwards Plateau *Eurycea* were either more widespread than previously thought or were erroneous (e.g. supposedly short digits were actually due to loss of tips through bacterial infection). Sweet concluded that there was no reason to recognize the Fern Bank Spring population as a separate taxon, and relegated this taxon to synonymy with *E. neotenes*. Based on the information then available this was a reasonable assessment. Hamilton (1973) also was unable distinguish Fern Bank Spring *Eurycea* from other populations in the Blanco River drainage and elsewhere in the southeastern Edwards Plateau region based on morphometric analyses. However, the allozyme evidence presented in Chapter 1 shows that the Fern Bank population plus all others in the Blanco River drainage share a high degree of similarity in allele frequencies (although they are characterized by no autapomorphies shared by all populations), suggesting the possibility of recent or ongoing gene flow. This, coupled with the restriction of this phenetic grouping to a single drainage, leads me to resurrect the name *E. pterophila* , especially since the allozyme and geographic evidence indicates that these populations almost certainly are isolated from gene flow with true (topotypical) *E. neotenes*. Populations that I have examined and assign to this taxon are: Fern Bank Springs, Zercher Spring, Boardhouse Springs, Peavey's Springs, Grapevine Cave, and T Cave.

Eurycea sosorum

Eurycea neotenes, Brown, 1950 (in part).

Eurycea neotenes neotenes, Brown, 1967b (in part; mapped locality only).

Eurycea sp., Sweet, 1978a, 1984.

Eurycea sosorum Chippindale, Price, and Hillis, 1993, Herpetologica 49:249.

HOLOTYPE: TNHC 51184.

TYPE LOCALITY: "outflow of Parthenia (Main) Springs in Barton Springs Pool, Zilker Park, Travis Co., Texas ($30^{O}15'49"$ N, $97^{O}46'14"$ W)".

COMMENTS: A detailed account of the morphological and molecular features that distinguish this species from other central Texas *Eurycea* was provided in the original description (Chippindale et al. 1993). Subsequent molecular work has reinforced the conclusion that this population represents a distinct species isolated from gene flow from all others, and the taxon continues to be known only from the immediate vicinity of Barton Springs, Travis Co. My recognition of this taxon as a distinct species is consistent with Sweet's (1978a, 1984) conclusions based solely on morphology. Molecular autapomorphies and characters that reflect the isolation of this species are:

Allozymes: At AAT-S, the b allele predominates, otherwise rare in the southeastern group except for *E. tridentifera*, the Pedernales populations, Comal Springs, and *E. neotenes*; for PEP-A the unique e allele is present at 83% frequency, and the other (d) allele present is otherwise seen only in members of the northern group, *E. nana*, and the Pedernales populations; and at PEP-D the a allele is fixed, otherwise seen only in

geographically distant and otherwise very divergent members of the northern and southwestern groups.

Sequence: C at position 237 is shared only with the otherwise distant Pedernales, Smith's Spring and Carson Cave populations and *E. nana*.

Eurycea tridentifera

Eurycea tridentifera Mitchell and Reddell, 1965, Texas J. Sci. 17:14.

Typhlomolge tridentifera, Wake, 1966:64.

HOLOTYPE: USNM 153780.

TYPE LOCALITY: "Honey Creek Cave, Comal Co., Texas".

COMMENTS: Mitchell and Reddell (1965), Wake (1966), and Sweet (1977a, 1978a, 1984) regarded *E. tridentifera* as exhibiting a cave-associated morphology second only to that of *E. rathbuni* (and *E. robusta*) in extremity among the Edwards Plateau hemidactyliines. The morphological features of this taxon led Wake (1966) to transfer it to the genus *Typhlomolge*, although later work by Mitchell and Smith (1972) suggested that the members of the genus *Typhlomolge* actually belong within *Eurycea*, a move that I also support based on the molecular evidence (Chapters 1, 2, and above). The molecular evidence also indicates that *E. tridentifera* is not especially closely related to *E. rathbuni*, or (presumably) *E. robusta* (for which no molecular data are available). Sweet (1977a, 1978a, 1984) suggested that the caves of northern Bexar Co. and the Cibolo Sinkhole Plain in which most known populations of this species occur are among the oldest in the plateau region, which would have allowed a long time for the evolution of cave-associated features. In Chapter 1 I discuss hypotheses of cave origins with respect to this species. Sweet

(1978a, 1984) demonstrated using morphometric analyses that populations from throughout the known range of the species cluster together, and recommended recognition of this taxon as a single species with a relatively wide subterranean range. The molecular evidence supports this view for the populations that I was able to sample (see Chapter 1, but note the exception for the G3PDH locus, below); in addition, Bogart (1967) identified a chromosomal nondisjunction that appeared to be unique to the two populations of *E. tridentifera* that he examined, Badweather Pit and Honey Creek Cave. Sweet (1977a) listed six known localities, and suggested that more likely would be found in the Cibolo Sinkhole Plain and northern Bexar Co. As he predicted, salamanders that appear to be this species have been seen at Genesis Cave in northern Bexar Co. (D. Pearson, pers. comm.; see Veni 1988 for details of the cave), and A.G. Grubbs and I have collected this species at Ebert Cave in Comal Co. (near Sweet's [1977a, 1978a, 1984] Kappelman Salamander Cave locality); *E. tridentifera* was first collected there by J. Reddell and M. Reyes (pers. comm.; see Appendix 1 of Chapter 1 for details of location). There also are rumors of blind salamanders in a cave on the Fair Oaks subdivision adjacent to the Grosser's Sinkhole locality (A.G. Grubbs, pers. comm.). Conant and Collins (1991) restricted the distribution of this species to the type locality, Honey Creek Cave, Comal Co., but this clearly was in error.

In addition to the distinctive morphological features exhibited by this taxon (see Mitchell and Reddell 1965, Wake 1966, Mitchell and Smith 1972, Sweet 1977a, 1978a, 1984) and the potential chromosomal autapomorphy described above,

potential molecular autapomorphies (here, synapomorphies that unite populations) and distinguishing features are as follows:

Allozymes: At AAT-S, the b allele is at medium to high frequency (this allele otherwise is rare in the southeastern group, except in *E. sosorum*, Comal Springs, Pedernales, and *E. neotenes*); at G3PDH the b allele appears fixed in the Honey Creek Cave and Ebert Cave populations (otherwise this allele is seen at low frequency in *E. sosorum*, *E. rathbuni*, and members of the southwestern group; however, it does not appear to be present in Badweather Pit *E. tridentifera*, suggesting that this population may in fact be isolated); and all three populations examined possess the unique MDHP d allele at high or 100% frequency.

Sequence: No sequence autapomorphies were found.

Eurycea latitans group

Synonymies are given below for *E. latitans* from the type locality only. I include numerous populations in an informal *E. latitans* group; all (except the previously unknown Less Ranch Spring, Cherry Creek Spring, and Cloud Hollow Spring populations) were assigned by Sweet (1978a, 1982) to *E. neotenes*. Some of these may have been assigned to species earlier by Baker (1961) or Brown (1967b), but for the populations in question these authors provided only maps, so the precise localities to which they referred are uncertain.

Synonymy for *E. latitans* from the subterranean Cascade Caverns system:

Eurycea neotenes, Wright and Wright, 1938 (assumed by Wright and Wright to be *E. neotenes* based on a second-hand report; location given only as "a cave near Boerne", assumed by Smith and Potter 1946 to be Cascade Caverns; Bishop, 1943 reported the presence of *E. neotenes* in Cascade Caverns).

Eurycea latitans Smith and Potter, 1946, Herpetologica 3:105.

Eurycea neotenes latitans, Schmidt, 1953:55.

Eurycea neotenes (in part)/*Eurycea tridentifera* (in part), Sweet, 1978a, 1984 (Sweet 1984 actually relegated *E. latitans* to the synonymy of *E. neotenes*, but believed that the population contained introgressed *E. tridentifera* genes).

HOLOTYPE: USNM 123594.

TYPE LOCALITY: "the first large pool deep within the recesses of Cascade Cavern, 4.6 miles by road (3 1/2 miles by airline) southeast of Boerne, Kendall County, Texas."

The status of this taxon is especially problematic. Sweet (1978a, 1984) demonstrated substantial morphological variation in this population and showed that specimens from Cascade Caverns presented a morphological spectrum from surface-like morphologies to extreme troglobitic morphologies similar to those of *E. tridentifera*. He hypothesized that this was the result of past introgression of the advanced troglobite *E. tridentifera* into a cave population of *E. neotenes*. Our molecular data provide no evidence of a hybrid origin for this population, or the other putative hybrid, *E. troglodytes* from the southwestern region (see Chapter 1 and below). Since the population that lives in the underground system associated with

Cascade Caverns also does not appear to represent *E. neotenes* based on the molecular evidence, it seems reasonable to resurrect the name *E. latitans* for salamanders in this cave system. Here I assign several other populations (Rebecca Creek Spring, Hays Co., Bear Creek Spring, Cibolo Creek Spring, Less Ranch Spring, and Kneedeep Cave Spring, all in Kendall Co., Cherry Creek and Cloud Hollow Springs, Kerr Co., and Honey Creek Cave Spring, Comal Co.) to an informal, heterogeneous and widespread "*E. latitans* group" based on similarity in allele frequencies and cytochrome b sequences. This group likely is a catch-all for members of the southeastern group whose affinities are uncertain, and definitely needs further investigation. No autapomorphies characterize all members of the group, although the Rebecca Creek population and topotypical *E. latitans* each exhibit potential sequence autapomorphies. Specifically, Rebecca Creek exhibits a T at position 41 otherwise seen only in *Haideotriton*, and has a unique F at codon 47. Topotypical *E. latitans* have a T at position 123 (also seen in the southwestern Carson Cave population and outgroup members *E. bislineata* and *E. wilderae*).

Eurycea sp. -- Comal Springs

Eurycea neotenes, Baker, 1961 (in part).

Eurycea neotenes neotenes, Brown, 1967b (in part; mapped locality only).

Eurycea nana, Sweet, 1978a (in part; tentative designation).

COMMENTS: The many characters that distinguish this population from true *E. nana* (from San Marcos Springs, Hays Co.) are discussed in Chapter 1, and this population clearly does not represent *E. nana*. I believe that it is a separate species, but it overlaps substantially based on morphometric analyses and molecular data with

other southeastern populations (Chapter 2); thus my view of the Comal Springs population as a distinct lineage is based largely on its apparent geographic isolation. Further study of this putative taxon is critical, because its spring habitat is threatened by human demands on the waters of the southern Edwards Aquifer. Potential molecular autapomorphies and distinguishing features are as follows:

Allozymes: At ACOH-1, the c allele (otherwise seen only in *E. rathbuni*) is at medium frequency; at AAT-S, the b allele (rare in the southeast except in *E. tridentifera*, *E. sosorum*, *E. neotenes*, and Pedernales) is at medium frequency; and at PGM, the b allele is at medium frequency (otherwise seen only in *E. neotenes*, Camp Mystic from the southwest, and the outgroup member *E. quadridigitata* from Texas).

Sequence: No diagnostic sequence characters were found.

Eurycea sp. -- Pedernales populations

No previous taxonomic history (discovered in the course of this study).

COMMENTS: D.M. Hillis and I first found the two known populations of this salamander in 1990, in two small springs on the northeast side of the Pedernales River in extreme western Travis Co. These springs are located in an isolated band of Cow Creek limestone and are well separated from all other known populations of central Texas *Eurycea*. Although no detailed morphological studies have yet been conducted, these salamanders appear to mature at a very small size, and exhibit a relatively high frequency of "gold" morphs (individuals in which the melanophores are widely separated, yielding a light yellowish color). These salamanders also possess unique combinations of allozyme and sequence character states (see below)

and almost certainly represent a distinct species; we intend to formally name them as such. Distinguishing molecular character states are as follows:

Allozymes: At ACOH-1 the c allele (otherwise characteristic of the northern group) occurs at low frequency; at AAT-S, the b allele is at medium frequency (otherwise rare in the southeast except in *E. tridentifera*, *E. sosorum*, *E. neotenes*, and Comal Springs); at LDH-A the a allele is at medium frequency (otherwise seen only in the T Cave population, a member of the *E. pterophila* group); and at MDHP the unique f allele occurs at low frequency.

Sequence: T at position 117 (shared only with Smith's Spring in the southwestern group); C at 237 (shared only with the southwestern Smith's Spring and Carson cave populations); unique G at 287; T at 288 (shared with some northern and southwestern group members as well as members of the outgroup); C at 324 (shared with *E. rathbuni* and members of the northern and southwestern groups and outgroup); and C at 353 (shared with members of the southwestern and northern groups, *E. rathbuni*, and outgroup members).

Southwestern Region

This region encompasses southern Gillespie Co., most of Kerr Co. (except the easternmost extreme), Bandera, Real, and Edwards Counties, northern Medina, Uvalde, and probably Kinney Co., and likely the populations in Val Verde Co. (not yet examined) belong in the southwestern clade as well. My molecular studies are only the beginning for discovery of diversity in this putative clade, and much more intensive sampling will be necessary to reliably identify species boundaries in this group. Only a single species has been recognized in the southwestern region, *E.*

troglodytes Baker, 1957 (see below), and most other known populations have been considered *E. neotenes* (e.g. Sweet 1978a, 1982). Both allozyme and sequence data (Chapter 1 and below) suggest that many members of this group are isolated from gene flow and probably represent distinct species. My recognition of a "Carson Cave group" (see below) is based on phenetic criteria and it may not represent a monophyletic assemblage of populations or species. The southwestern group usually is quite strongly supported as monophyletic in phylogenetic analyses of the molecular data (Chapter 1) based on bootstrapping and decay indices. However, "real" character evidence for the monophyly of this group is lacking: character states that separate members of the southwestern clade from those of the southeastern clade are likely to be primitive, since they are shared with the northern group and/or *E. rathbuni* and/or members of the outgroup. I have identified no unambiguous synapomorphies for the group. Character states that distinguish this group to varying extents are:

Allozymes: At AAT-S the b allele, absent or at low frequency in most southeastern populations, appears fixed in all southwestern populations.

Sequence: A at position 117 in all except Smith's Spring,which has a T (A shared with northern group and outgroup members); G at 261 (shared with *E. rathbuni*, some northern populations, and members of the outgroup); A at 328 (shared with *E. rathbuni*, members of the northern group, and members of the outgroup), and A at 360 (shared with *E. nana*, *E. rathbuni*, and some outgroup members).

Southwestern group members

E. troglodytes

E. troglodytes Baker, 1957, Texas J. Sci. 9:328.

E. neotenes (in part)/*E. tridentifera* (in part), Sweet, 1978a, 1984.

HOLOTYPE: TNHC 21791.

TYPE LOCALITY: "a pool approximately 600 feet from the entrance of the Valdina Farms Sinkhole, Valdina Farms, Medina County, Texas."

COMMENTS: Although Sweet (1978a, 1984) considered this taxon a hybrid swarm derived from *E. tridentifera* and what he considered *E. neotenes* based on the morphological variability that it displays, this seems very unlikely based on molecular evidence and geographic and hydrologic considerations (see Chapter 1). Therefore, I recommend continued recognition of this species, especially since it is the only named member of the southwestern group. Unfortunately, this species may be extinct due to human modification of its only known habitat (Veni and Associates 1987, G. Veni pers. comm.). I was unable to examine this species for allozyme variation; no distinguishing sequence characters were detected.

Eurycea sp. -- Tucker Hollow Cave

Eurycea neotenes, Sweet 1978a, 1984 (in part)

COMMENTS: This is one of the few cave populations of *Eurycea* distant from the Balcones Fault Zone, and almost certainly is isolated from all or most other populations. These salamanders, known only from two shallow pools in a hillside cave, exhibit strong troglobitic morphologies, including reduced eyes and pigmentation and broadened heads. Sweet (1978a) provided a detailed description of

the animals, and Sweet (1984) included this population in a morphometric analysis of central Texas cave *Eurycea*. J.R. Reddell (pers. comm.) considered this population a distinct species, although a formal description was never published. I concur with Reddell, given the distinctive morphologies and apparent geographic isolation of animals from this population; the following molecular markers also distinguish this population from others to varying extents:

Allozymes: The unique c allele appears fixed at IDH-1; the a allele appears fixed at GAPDH (also seen at medium frequency in populations of the Sabinal group, below); the MPI c allele appears fixed (otherwise seen only in the Sabinal group and some outgroup members).

Sequence: C at position 91 (shared with the southwestern Smith's Spring and Carson Cave populations, southeastern populations, and some outgroup members); C at 228 (shared with *E. rathbuni*, Smith's Spring, Carson Cave, and some outgroup members); unique G at 268.

Eurycea sp. -- Greenwood Ranch Springs

No previous taxonomic history, although some of the nearby populations assigned by Baker (1961) and Sweet (1978a, 1982) to *E. neotenes* , and by Brown (1967b) to *E. n. neotenes*, could represent the same species.

COMMENTS: Superficially, these salamanders appear similar to other southwestern spring populations. Additional sampling in this area of the range is very desirable, as the status of these populations is uncertain; they easily could represent an undescribed species. Distinguishing molecular character states are:

Allozymes: Lack of detectable activity at MDH-2.

Sequence: C at position 216 (shared with *E. rathbuni*); C at 264 (shared with Carson Cave).

Eurycea sp. -- 176 Spring

Eurycea neotenes, Sweet, 1982 (in part).

COMMENTS: Like many southwestern populations, this population appears to be distinct based on molecular evidence, but sampling in the region is very limited and thus the status of this population is uncertain. Distinguishing molecular character states are:

Allozymes: Unique LDH-A e allele at high (88%) frequency; a allele at PEP-A (also seen in nearby Fessenden Springs).

Sequence: Unique T at position 213; unique T at 238; C at 345 (shared with northern populations, *E. rathbuni*, and some outgroup members).

Eurycea sp. -- Camp Mystic Springs

Eurycea neotenes, Sweet, 1978a, 1982 (in part; Sweet called this locality Edmunson Creek Spring).

COMMENTS: This is another southwestern spring population that does not appear morphologically distinct, but which has many molecular character states that indicate isolation from gene flow, at least from the other populations examined. Thus it may represent another distinct species. Several sequence characters suggest the possibility of close relationship with the Trough Spring population, here placed in the informal (and problematic) Carson Cave group (below). Distinguishing molecular character states are:

Allozymes: Unique c allele at 90% frequency at MDH-1; a allele at PEP-D (shared with some northern populations, *E. sosorum*, and nearby Fessenden Springs); b allele fixed at PK (an allele with the same mobility is seen in the outgroup member *Haideotriton*).

Sequence: C at position 129 (shared with some outgroup members); T at 180 (shared with members of the northern group and the outgroup member *E. longicauda*); T at 192 (shared with members of the northern group, some outgroup members, and nearby Trough Springs); A at 202 (shared with Trough Springs); T at 203 (shared with Trough Springs); G at 245 (shared with Cedar Breaks Spring from the north and the outgroup member *E. wilderae*); A at 245 (shared with Cedar Breaks Spring, Trough Spring, and some outgroup members; and T at 252 (shared with some outgroup members).

***Eurycea* sp. -- Sabinal group**

Eurycea neotenes, Sweet,1978a, 1982 (Baker 1961 and Brown 1967b listed localities for *E. neotenes* that may include this species).

COMMENTS: These populations include some of the few known naturally metamorphosing *Eurycea* in the Edwards Plateau region (discussed by Bruce 1976 and Sweet 1977b). They may represent a distinct species. For the two localities that I examined (Murphy's Spring and Sabinal Canyon Spring), distinguishing molecular character states are:

Allozymes: At GAPDH the a allele (otherwise seen only in Tucker Hollow Cave) is at medium frequency; at MDHP the unique c allele is at high frequency; and at MPI

the c allele (otherwise seen in Tucker Hollow Cave and some outgroup members) is at high frequency.

Sequence: No diagnostic markers found.

***Eurycea* sp. -- Carson Cave group**

Eurycea neotenes, Sweet 1978a, 1982, 1984 (in part).

COMMENTS: Here I omit earlier authors' taxonomic assignments of populations which may be closely related to members of this group, because the group's composition is highly problematic. Thus I cite only Sweet because he actually assigned most of the same populations that I examined to *E. neotenes*. This phenetic assemblage (based on allozyme allele frequencies) definitely needs further study. Even the patterns of relationship suggested by the sequence data are at odds with this grouping in some respects (e.g. for Trough Spring and Camp Mystic Spring), and there probably are several species involved. In future studies, the best strategy probably will be to address relationships within the southwestern group alone, treating as many populations as possible as separate units of analysis.

The population for which this group is named, Carson Cave, consists of morphologically distinctive troglobites similar in some respects to those from Tucker Hollow Cave (see Sweet 1978a, 1984 for more detailed morphological information). It may represent a distinct species (as believed by J.R. Reddell, pers. comm.), although sequence data suggest a close relationship between this population and the nearby but morphologically dissimilar Smith's Spring population (see Chapter 1 and below). The populations included in this informal group range widely in the

southwestern region and do not correspond to any single drainage. In this group I have included the populations from West Nueces Spring and Smith's Spring (Edwards Co.), Carson Cave and Wetback Spring (Uvalde Co.), Sutherland Hollow Spring (Bandera Co.), Robinson Creek Spring and Fessenden Spring (Kerr Co.), and Trough Spring (Gillespie Co.). Below I will list characters that exhibit autapomorphies for particular members of the group, or suggest possible relationships among its components; no unambiguous synapomorphies unite all members of this assemblage.

Allozymes: Unique MDH-1 b allele in Smith's Spring at 61% frequency; Fessenden Spring has PEP-A a allele (shared with 176 Spring).

Sequence: C at position 91 in Carson Cave, Smith's Spring, and Tucker Hollow Cave (shared with southeastern populations and some outgroup members); T at 124 in Carson Cave (shared with topotypical *E. latitans* and the outgroup members *E. bislineata* and *E. wilderae*); unique C at 125 in Carson Cave; A at 144 in Carson Cave and Smith's Spring (shared with northern populations and outgroup taxa); C at 228 in Carson Cave, Smith's Spring, and Tucker Hollow Cave (shared with *E. rathbuni* and some outgroup members); G at 264 in Carson Cave and Smith's Spring (shared with *E. rathbuni*); C at 269 in Carson Cave and Smith's Spring only; C at 272 in Sutherland Hollow Spring, Trough Spring, and some outgroup members; C at 279 in Carson Cave and Smith's Spring (shared with southeastern, San Marcos, and some northern populations); A at 309 in Carson Cave and Smith's Spring (shared with southeastern and northern populations, *E. nana*, and some outgroup members); C at 364 in Carson Cave and Greenwood Ranch Springs.

Northern Region

As detailed in Chapter 1, populations of *Eurycea* from northeast of the Colorado River in Travis, Williamson, and Bell Counties are extremely divergent from all others in central Texas (and elsewhere) based on a host of molecular markers. The Colorado River appears to represent a strong barrier that has isolated these salamanders for a long period of time, and evidence of the monophyly of the northern group is overwhelming. Despite their high level of divergence, no members of the group have been formally described as distinct species, mainly because animals from the northern surface populations known to previous workers appear very similar morphologically to those from southern spring populations. Also, the majority of northern populations were discovered during the course of this study, and thus their diversity and the extent of their range previously was very poorly known.

In addition to the characters listed below, members of the northern group are characterized (among the central Texas *Eurycea*) by substantially larger genome sizes than all others (see Chapter 1 for details), and all members examined so far exhibit a diagnostic ApaLI restriction site near the 5' end of the 28S nuclear ribosomal DNA repeat unit (Chippindale, unpublished). Identification of species boundaries in this group has proven difficult in some cases, especially with respect to the cave populations. I have treated most subterranean populations in the region as separate units for analysis because of the uncertainty of their placement; clearly additional sampling, and the use of new molecular markers, is desirable to further elucidate relationships and species boundaries in the group. Evidence for the monophyly

and/or distinctiveness of the northern group is provided by the following allozyme and sequence characters:

Allozymes: All populations examined appear fixed for the unique LDH-A c allele; all populations appear fixed for the unique GAPDH b allele except Salado Springs (which also has the c allele seen in many southern populations); all populations appear fixed for the unique IDH-1 a allele; all populations appeared fixed for the IDH-2 b allele (also seen in *E. rathbuni* and *Haideotriton*); all populations appear fixed for the unique a allele at CK-1 except the Lake Georgetown populations, which also possess the unique c allele at medium frequency; all appear fixed for the unique MDHP b allele except Round Rock and some Jollyville Plateau populations, which also have the unique a allele at varying frequencies; all are characterized by a fixed ACOH-1 c allele (also seen at low frequency in *E. sosorum* and in the outgroup member *E. quadridigitata* from Texas) except Testudo Tube and members of the Buttercup Creek Cave group (which also have the southern b allele) and the Lake Georgetown populations (which also have the unique f allele);); and all appear fixed for the PEP-A d allele (also seen at medium frequency in *E. nana* and at low frequency in *E. sosorum* and Pedernales) except T.W.A.S.A Cave (which has the southern b allele).

Sequence: Unique T at position 75, unique C at 80, unique C at 105 (except in Cedar Breaks Spring, in the Lake Georgetown group); unique T at 130; unique T at 156; unique A at 268; unique A at 285 (except in Testudo Tube and Ilex Cave, which have a unique G); unique A at 291; unique G at 378.

Amino acid sequence: Unique T at codon 27; unique S at 44; unique L at 90; unique F at 109.

Northern group members

Eurycea sp. -- Jollyville Plateau

Eurycea neotenes, Baker, 1961 (in part).

Eurycea neotenes neotenes, Brown, 1967b (in part).

COMMENTS: Animals from springs of the Jollyville Plateau in Travis Co. all appear similar morphologically and are almost identical to one another based on molecular evidence. Thus I believe that these populations (plus, probably, that from Round Rock, below) constitute a single species, especially since they are readily distinguishable based on the molecular evidence from the closest populations, in the vicinity of Lake Georgetown (see below). Sweet (1978a, 1982) considered these peripheral populations *E. neotenes*, and commented that densities of salamanders in springs of the Jollyville Plateau region generally are very low. However, I have found these salamanders to be abundant at some localities, especially in the headwaters of the Bull Creek drainage and at the Stillhouse and Barrow Hollow Spring locations (unfortunately, most of these springs are likely to be altered or obliterated by development in the near future; see Price et al. 1995). I suspect that the discrepancies in my versus Sweet's observations may be due at least in part to the fact that D.M. Hillis, A.H. Price and I visited the localities year-round, whereas Sweet did most field work in the summer. My qualitative observations are that members of the northern group generally are most common on the surface during winter months, whereas the southern taxa seem to exhibit less seasonality in activity patterns. This suggests that there may be major behavioral differences between the northern and southern groups, and invites further study.

D.M. Hillis, A.H. Price and I are in the process of formally describing the Jollyville Plateau spring populations, plus that at Round Rock, as a distinct species. As indicated above, such recognition is timely, as many populations of this species likely will be extirpated in the near future and thus formal protection as a distinct taxon is warranted. Distinguishing molecular character states are:

Allozymes: MDHP a allele (shared with Round Rock) present at varying frequencies.

Sequence: Unique G at position 69 in Stillhouse Hollow; C at 99 (shared with Round Rock and members of the southern group and outgroup); G at 240 (shared with Round Rock and members of the southeastern group, *E. nana*, *E. rathbuni*, and some outgroup members); unique G at 261; A at 289 (shared with Round Rock); T at 324 (shared with Round Rock and members of the southern group and outgroups).

***Eurycea* sp. -- Round Rock**

Eurycea neotenes, Baker, 1961 (in part).

Eurycea neotenes neotenes, Brown, 1967b (in part).

COMMENTS: I assume here that the Round Rock population (Krienke Spring on Brushy Creek) to which the above authors referred was conspecific with the nearby Brushy Creek spring population discovered in the course of this study; the Krienke Spring population was destroyed by quarrying operations (Sweet 1978a). These salamanders appear very similar based on morphology and molecular markers to the Jollyville Plateau populations and probably are conspecific, although I treated them as a separate unit for the purposes of the phylogenetic analyses presented in Chapter

1. This conclusion is reinforced by the faunal similarities between the Jollyville Plateau region and the Brushy Creek drainage (which probably originates from the plateau; see Veni and Associates 1992). Distinguishing molecular character states are:

Allozymes: Low frequency MDHP a allele (also seen in Jollyville Plateau populations); and high frequency PEP-D c allele (also seen in many members of the southern group).

Sequence: C at 99 (shared with Jollyville and members of the southern group and outgroup);); G at 240 (shared with Jollyville and members of the southeastern group, *E. nana*, *E. rathbuni*, and some outgroup members); A at 289 (shared with Jollyville); T at 324 (shared with Jollyville and members of the southern group and outgroups).

***Eurycea* sp. -- Kretschmarr Salamander Cave**

No previous taxonomic history.

COMMENTS: Although this population occurs in a tiny stream cave on the Jollyville Plateau in the vicinity of known Jollyville Plateau salamander spring localities, salamanders at this site are somewhat distinct at the molecular level and could represent an undescribed species (although they exhibit no diagnostic autapomorphies). Morphologically, these salamanders appear similar to those from nearby surface populations. At the molecular level, they are distinguished from nearby populations by the following:

Allozymes: At GPI, the b allele appears fixed (otherwise seen in Lake Georgetown and southern populations).

Sequence: C at position 177 (shared with Cedar Breaks Spring in the Lake Georgetown group, members of the southern group, and the outgroup); C at 180 (shared with members of the southern group and outgroup); C at 183 (shared with members of the southern group and outgroup).

***Eurycea* sp. -- Testudo Tube Cave**

No previous taxonomic history.

COMMENTS: The status of this recently-discovered cave population fom Williamson Co. is problematic. The few known specimens from this cave appear similar to surface animals, and salamanders which could represent the Jollyville Plateau salamander are known from springs on the nearby Audubon Preserve (personal observations; I was unable to collect at the Audubon locality). Testudo Tube may be separated hydrologically to some extent from caves of the nearby Buttercup Creek Cave system (Russell 1993) and thus salamanders from Testudo Tube may not be conspecific with those of the Buttercup Creek Caves. Testudo Tube salamanders are distinguished primarily by the following:

Allozymes: High frequency AAT-S c allele (otherwise seen in some members of the nearby (and possibly conspecific) Buttercup Creek Caves group, one member of the Jollyville Plateau group, *E. rathbuni* , and outgroup member *E. quadridigitata* from Texas).

Sequence: G at position 285 (shared with the Ilex Cave population in the Buttercup Creek Caves group).

***Eurycea* sp. -- Buttercup Creek Caves group**

No previous taxonomic history (populations were discovered by W.H. Russell, J.R. Reddell, and others prior to or during the course of this study).

COMMENTS: The recently discovered Buttercup Creek Cave system, in the Cedar Park area of Williamson Co., is a relatively extensive and probably hydrologically interconnected subterranean system (e.g. see Russell 1993). However, the *Eurycea* that inhabit these caves display a great deal of molecular variability and do not always appear as most similar to one another in phenetic analyses based on allozyme allele frequencies (Chapter 1); this could simply be an artifact of sampling, however. Most salamanders that have been observed in the Buttercup Creek caves exhibit strong troglobitic morphologies including depigmentation, eye reduction, and broadening and flattening of the head, but so few specimens are available that generalizations about the morphologies of these salamanders are difficult. I treat the nearby Testudo Tube cave population separately (see above) because salamanders from that locality appear quite "surface-like", and hydrologic connection between this cave and the others may be limited (Russell 1993 and pers. comm.). However, one potential sequence synapomorphy (see below) does unite the Testudo Tube population with that from Ilex Cave, the single member of the Buttercup Creek caves group for which sequence data are available. At present, I am reluctant to formally describe these or other northern cave populations as new species because I believe that further study is necessary; however, I strongly suspect that salamanders of the Buttercup Creek Caves system represent a distinct evolutionary lineage.

Allozymes: AAT-S c allele in some component populations (otherwise seen in Testudo Tube, one member of the Jollyville Plateau group, *E. rathbuni* , and outgroup member *E. quadridigitata* from Texas).

Sequence: G at position 285 (shared with Testudo Tube).

***Eurycea* sp. -- Lake Georgetown group**

Eurycea neotenes, Sweet, 1978a, 1982 (in part).

COMMENTS: I assume here that the salamanders reported by Sweet (1978a, 1984) from springs in the city park at Georgetown are conspecific with those that we examined from nearby springs in the vicinity of Lake Georgetown. The single individual that I found at the Georgetown park site was a larva that failed to yield successful results for key molecular markers. Salamanders of the Lake Georgetown group (represented here by Avant's Spring, Knight Spring, Buford Hollow Spring, and Cedar Breaks Hiking Trail Spring, all discovered in the course of this study) are quite divergent from all other central Texas *Eurycea* based on molecular markers, and may also exhibit characteristic iridophore patterns (Chippindale et al., in prep.). The distinctiveness of members of this group from populations at Round Rock and the Jollyville Plateau region to the south is consistent with the observation by Veni and Associates (1992) that the south fork of the San Gabriel River is a strong barrier to migration, at least by terrestrial troglobitic invertebrates. D.M. Hillis, A.H. Price and I intend to describe the Lake Georgetown populations as a distinct species (Chippindale et al. in prep.). Recently discovered populations in the Cowan Creek drainage, slightly north of the populations in the Lake Georgetown vicinity (Price et al. 1995) probably represent this species, although they have not yet been examined

for molecular or morphological characters. Distinguishing molecular character states are:

Allozymes: Unique ACOH-1 f allele at medium frequency in all populations except Avant's Spring; CK-1 c allele at medium frequency (also seen in *E. quadridigitata* from Texas); PEP-D a allele common (also seen in Bat Well, Salado, *E. sosorum*, and the southwestern Fessenden Springs and Camp Mystic Springs populations); fixed d allele at G3PDH (otherwise seen only in T.W.A.S.A. Cave, in the Buttercup Creek Caves group).

Sequence: C at position 63 (shared with Bat Well and members of the southern group and outgroup); unique T at 105; G at 222 (shared with Camp Mystic in the southwestern group, and outgroup member *E. wilderae*); C at 255 (shared with outgroup members); C at 288 (shared with members of the southern group); unique G at 342.

Eurycea sp. -- Bat Well

No previous taxonomic history.

COMMENTS: A single specimen is available from this cave. It appears morphologically similar to northern surface populations, and spring populations of *Eurycea* are likely to occur in the area. I treated this population separately for purposes of analysis, because its affinities are uncertain. Some molecular characters suggest a close relationship with members of the geographically close Lake Georgetown group, and perhaps also Salado Springs (see Chapter 1), but additional sampling in the area is highly desirable. Molecular characters that distinguish this population from all or most others in the northern region are:

Allozymes: PEP-D a allele (shared with Salado, Lake Georgetown group, *E. sosorum*, and Fessenden Springs in the southwest).

Sequence: C at position 63 (shared with Cedar Breaks Spring in the Lake Georgetown group and members of the southern group); unique G at 188; unique T at 289.

Eurycea sp. -- Salado Springs

Eurycea neotenes, Sweet 1978a, 1982 (in part; the single previously known, larval specimen was not examined by Sweet but was assumed to represent *E. neotenes*).

COMMENTS: This is the northeasternmost known population of *Eurycea* in the Edwards Plateau region, and salamanders from these springs are very elusive. Prior to this study, only a single specimen (unavailable for examination) was known; D.M. Hillis, A.H. Price and I found several others in 1989-1991 but have been unable to find any others since, despite numerous attempts. These salamanders are immediately distinguishable based on external morphology: they have long, broad, almost rectangular heads, very reduced eyes, long slender limbs, elongate bodies, and a dark gray-brown coloration that under magnification appears as a series of tiny reticulations. In many respects the morphologies of these salamanders are unlike those of any others in the Edwards Plateau region. The morphological distinctiveness of the Salado population also was demonstrated using discriminant function morphometric analyses by Chippindale et al. (1990). This was a preliminary analysis and results are not presented here, because currently I am modifying and expanding the morphometric work on this and other northern taxa in the course of formally describing them (including this population) as distinct species.

Very recently, salamanders have been found in springs of nearby Buttermilk Creek (G. Longley, pers. comm.); these could represent this species, although specimens are not yet available for examination. Distinguishing molecular character states are:

Allozymes: PEP-D a allele fixed (shared with Bat Well, most Lake Georgetown populations, *E. sosorum*, and Fessenden and Camp Mystic Springs in the southwest).

Sequence: T at position 294 (shared with members of the southern group and some outgroup members).

CONCLUSIONS

As should be apparent from the above, the taxonomy of the central Texas *Eurycea* remains largely unresolved. This is not surprising, given the large number of populations, extreme geographic fragmentation, and high potential for morphological parallelism or convergence. Some may find my treatment of diversity in the group unsatisfying, as I have resisted the temptation to assign every population to a particular species. I believe that it is better at this stage to recognize the diversity in the group than try to force each of its members into a categorization which may prove artificial. I see the work described here as the basis for more detailed studies of relationships and species boundaries within and among the species groups that I have recognized. The identification of the major monophyletic groups in this assemblage will facilitate future systematic and taxonomic work, as it will be possible to focus on particular subsets of the Texas *Eurycea* (e.g. the northern populations)with confidence that one is dealing with evolutionarily real

(monophyletic) units. Previously, for example, a study of "*E. neotenes*" would necessarily have had to encompass the whole region and what have proven to be numerous distinct species. Future work on the group should include both morphological study and use of rapidly-evolving molecular markers (e.g. mitochondrial D-loop sequences, microsatellites, and perhaps intron sequences); some of this research is planned or in progress by me and J. Wiens. I am optimistic that further study will clarify many of the systematic and taxonomic problems that remain in the Texas *Eurycea*, and hope that it will be possible to characterize and preserve the diversity in the group before much of it is lost due to human activities.

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APPENDIX 1

Localities for populations and taxa of central Texas hemidactyliine salamanders examined in this study. Watersheds in which each locality is located are given where not obvious based on the locality name. In cases in which formal taxon names have been assigned to populations from particular localities, those names are used. Informal groups to which populations have been assigned based on this study are listed where appropriate.

BANDERA COUNTY

Murphy's Spring (Wedgeworth Creek South Spring), Sabinal River watershed, 29°48'00" N, 98°33'31" W. (Sabinal group).

Sabinal Canyon Spring, Sabinal River watershed, 29°49'26" N, 99°34'01" W. (Sabinal group).

Sutherland Hollow Spring, west prong Medina River, 29°44'58" N, 99°25'36" W. (Carson Cave group).

BELL COUNTY

Salado (Big Boiling, Main, or Siren) Springs, Salado Creek, 30°56'37" N, 97°32'31" W. (Salado group).

Salado (Robertson) Springs, Salado Creek, 30°56'37" N, 97°32'39" W. (Salado group).

BEXAR COUNTY

Helotes Creek Spring, Medina River watershed, 29°38'15" N, 98°41'40" W. (*E. neotenes* -- type locality).

Leon Springs, Leon Creek, Medina River watershed, 29°39'46" N, 98°38'14" W. (*E. neotenes* group).

BLANCO COUNTY

Boardhouse Springs, Blanco River watershed, 30°06'40" N, 98°18'07" W. (*E. pterophila* group).

T-Cave, Blanco River watershed, 30°04'36" N, 98°19'46" W. (*E. pterophila* group).

Zercher Spring, Blanco River watershed, 30°06'10" N, 98°27'25" W. (*E. pterophila* group).

COMAL COUNTY

Badweather Pit, Cibolo Creek watershed, 29°45'21" N, 98°37'13" W. (*E. tridentifera*).

Comal Springs, headwaters of the Comal River, 29°42'49" N, 98°08'13" W.

Ebert Cave, Cibolo Creek watershed, 29°45'06" N, 98°23'28" W. (*E. tridentifera*).

Honey Creek Cave, Guadalupe River watershed, 29°50'50" N, 98°29'30" W. (*E. tridentifera* -- type locality).

Rebecca Creek Spring, Guadalupe River watershed, 29°55'28" N, 98°22'22" W. (*E. latitans* group).

EDWARDS COUNTY

Smith's (= Dutch Creek) Spring, Nueces River watershed, 29°39'09" N, 100°06'12" W. (Carson Cave group).

West Nueces River Spring, 29°43'20" N, 100°24'51" W. (Carson Cave group).

GILLESPIE COUNTY

Trough Spring, Pedernales River watershed, 30°08'36" N, 99°04'40" W. (Carson Cave group).

HAYS COUNTY

Ezell's Cave, San Marcos River watershed, 29°52'27" N, 97°57'34" W. (*E. rathbuni*).

Fern Bank (Little Arkansas) Springs, Blanco River watershed, 29°59'00" N, 98°00'49" W. (*E. pterophila* -- type locality).

Grapevine Cave, Blanco River watershed, approximately 30°02'30" N, 98°12'45" W. (*E. pterophila* group).

Rattlesnake Cave, San Marcos River watershed, 29°54'07" N, 97°55'17" W. (*E. rathbuni*).

San Marcos (Aquarena) Springs, pipe outflow at submarine theater, headwaters of the San Marcos River, 29°53'35" N, 97°55'50" W. (*E. rathbuni*).

San Marcos (Aquarena) Springs, headwaters of the San Marcos River, 29°53'35" N, 97°55'50" W. (*E. nana* -- type locality).

KENDALL COUNTY

Bear Creek Spring, Medina River watershed, 29°48'15" N, 98°52'10" W. (*E. latitans* group).

Cibolo Creek Tributary Spring, Cibolo Creek watershed, 29°49'03" N, 98°51'43" W. (*E. latitans* group).

Kneedeep Cave Spring, Guadalupe River State Park, 29°52'31" N, 98°29'05" W. (*E. latitans* group).

Less Ranch Spring, Guadalupe River watershed,29°46'40" N, 98°50'52" W. (*E. latitans* group).

Mueller's Spring, Medina River watershed, approximately 29°44' N, 98°47'30" W. (*E. neotenes* group).

Peavey's Springs, headwaters of the Blanco River, approximately 30°05'30" N, 98°39'30" W. (*E. pterophila* group).

Pfeiffer's Water Cave, Guadalupe River watershed, 29°45'44" N, 98°39'59" W. (*E. latitans* -- subterranean extension of type locality).

KERR COUNTY

176 Spring, Guadalupe River watershed, 30°05'18" N, 99°19'14" W.

Cherry Creek Spring, Guadalupe River watershed, 29°50'41" N, 98°56'52" W. (*E. latitans* group).

Cloud Hollow Spring, Medina River watershed, 29°50'36" N, 98°57'14" W (*E. latitans* group).

Edmunson Creek (Camp Mystic) Springs, Guadalupe River watershed, 30°00'21-3" N, 99°21'43-54" W.

Fessenden Springs, Guadalupe River watershed, 30°10'00" N, 99°20'32" W. (Carson Cave group).

Robinson Creek (Highway 16 South) Spring, north prong Medina River watershed, 29°54'55" N, 99°15'08" W.

REAL COUNTY

Greenwood Valley Ranch Spring #1, east prong Nueces River, 29°57'20" N, 99°58'17" W.

Greenwood Valley Ranch Spring #2, east prong Nueces River, 29°59'11" N, 99°57'51" W.

Greenwood Valley Ranch Spring #3, east prong Nueces River, 29°59'22" N, 99°57'13" W.

Tucker Hollow Cave, Frio River watershed, 29°44'33" N, 99°46'42" W.

TRAVIS COUNTY

Balcones Community Park Spring, Walnut Creek watershed, 30°24'45" N, 97°43'02" W. (Jollyville Plateau group).

Barrow Hollow Spring, Bull Creek watershed, 30°22'33" N, 97°46'02" W. (Jollyville Plateau group).

Barton Springs, Barton Creek, 30°15'49" N, 97°46'14" W. (*E. sosorum* -- type locality).

Bull Creek (Hanks Tract) Spring, north fork Bull Creek, 30°25'38" N, 97°49'08" W. (Jollyville Plateau group).

Bull Creek Spring Pool (New Bull Creek Spring), west fork Bull Creek, 30°24'59" N, 97°49'00" W. (Jollyville Plateau group).

Canyon Creek Spring, north fork Bull Creek, 30°25'33" N, 97°48'51" W. (Jollyville Plateau group).

Canyon Vista Spring, Bull Creek watershed, 30°25'51" N, 97°46'55" W. (Jollyville Plateau group).

Hammett's Crossing Spring #1 (Pedernales Spring #1), Pedernales River, 30°20'28" N, 98°08'14" W. (Pedernales group)

Hammett's Crossing Spring #2 (Pedernales Spring #2), Pedernales River, 30°20'23" N, 98°08'15" W. (Pedernales group).

Horsethief Hollow Spring, Bull Creek watershed, 30°24'31" N, 97°49'00" W. (Jollyville Plateau group).

Kretschmarr Cave, Colorado River watershed, 30°24'47" N, 97°51'10" W.

Schlumberger Spring, headwaters west fork Bull Creek, 30°25'15" N, 97°50'24" W. (Jollyville Plateau group).

Stillhouse Hollow Springs, Bull Creek watershed, 30°22'28" N, 97°45'55" W. (Jollyville Plateau group).

Wheelis Springs, Long Hollow Creek, Colorado River watershed, 30°27'42" N, 97°52'28" W. (Jollyville Plateau group).

UVALDE COUNTY

Carson Cave, West Nueces River watershed, 29°28'50" N, 100°04'44" W. (Carson Cave group).

Wetback Spring, Sabinal River watershed, 29°35'12" N, 99°36'14" W. (Carson Cave group).

WILLIAMSON COUNTY

Avant's (Capitol Aggregates) Spring, middle fork San Gabriel River, 30°38'44" N, 97°44'11" W. (Lake Georgetown group).

Bat Well, Cowan Creek watershed, San Gabriel River drainage, 30°42'10" N, 97°42'59" W.

Brushy Creek (Round Rock) Spring, 30°31'00" N, 97°39'38" W.

Buford Hollow Springs, just below Lake Georgetown Dam, north fork San Gabriel River, 30°39'39" N, 97°43'36" W. (Lake Georgetown group).

Buttercup Creek Cave (Buttercup River Cave), Buttercup Creek Karst, Brushy Creek watershed, approximately 30°29'33" N, 97°50'44" W. (Buttercup Creek Cave group).

Cedar Breaks Hiking Trail Spring, south shore of Lake Georgetown, north fork San Gabriel River, 30°39'36" N, 97°45'02" W. (Lake Georgetown group).

Ilex Cave, Buttercup Creek Karst, Brushy Creek watershed, approximately 30°29'28" N, 97°50'50" N. (Buttercup Creek Cave group).

Knight (Crockett Garden) Spring, south shore of Lake Georgetown, north fork San Gabriel River, 30°39'50" N, 97°45'04" W. (Lake Georgetown group).

T.W.A.S.A. Cave, Buttercup Creek Karst, Brushy Creek watershed, approximately 30°29'49" N, 97°50'48" W. (Buttercup Creek Cave group).

Testudo Tube, Buttercup Creek Karst, Brushy Creek watershed, approximately 30°29'35" N, 97°51'23" W.

Treehouse Cave, Buttercup Creek Karst, Brushy Creek watershed, approximately 30°29'55" N, 97°50'07" W. (Buttercup Creek Cave group).

APPENDIX 2

Allele frequencies in populations of central Texas *Eurycea* and outgroup members. N is the number of individuals from a given population in which a particular locus was resolved. Loci are numbered from least to most anodally migrating, and alleles are designated by letters that in most cases increase with increasing anodal mobility. H = direct-count heterozygosity per locus per individual, P = percentage polymorphic loci, and A = number of alleles locus. Standard errors are in brackets. NC = not calculated.

		Locus									
		ACO-1	AK	AAT-S	CK-1	CK-2	CAP	GPI	GR	GAPDH	G3PDH
NORTH											
Jollyville Plateau											
Balcones Park Spring	N=	5	5	5	5	5	5	4	4	4	5
		c: 1.000	b: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	a: 1.000	a: 0.625 b: 0.375	b: 1.000	a: 1.000
Barrow Hollow Spring	N=	5	5	5	4	4	4	5	5	4	5
		c: 1.000	b: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	b: 1.000	a: 1.000
Bull Creek Spring	N=	5	5	5	4	5	5	5	5	5	5
		c: 1.000	b: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	b: 1.000	a: 1.000
Canyon Creek Spring	N=	3	3	3	3	3	3	3	3	3	3
		c: 1.000	b: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	b: 1.000	a: 1.000
Canyon Vista Spring	N=	4	4	4	3	3	3	4	4	2	4
		c: 1.000	b: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	b: 1.000	a: 1.000
Horsethief Hollow Spring	N=	1	1	1	1	1	1	1	1	1	1
		c: 1.000	b: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	b: 1.000	a: 1.000

		IDH-1	IDH-2	LDH-A	LDH-B	MDH-1	MDH-2	MDHP	MPI	PEP-A	PEP-B
NORTH											
Jollyville Plateau											
Balcones Park Spring	N=	5	5	5	5	5	5	5	5	5	5
		a: 1.000	b: 1.000	c: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	c: 1.000	d: 1.000	b: 1.000
Barrow Hollow Spring	N=	5	5	5	5	4	4	5	4	5	5
		a: 1.000	b: 1.000	c: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	c: 1.000	d: 1.000	b: 1.000
Bull Creek Spring	N=	5	5	5	5	5	5	5	5	5	5
		a: 1.000	b: 1.000	c: 1.000	a: 1.000	a: 1.000	a: 1.000	a: 0.100 b: 0.900	c: 1.000	d: 1.000	b: 1.000
Canyon Creek Spring	N=	3	3	3	3	3	3	3	3	2	3
		a: 1.000	b: 1.000	c: 1.000	a: 1.000	a: 1.000	a: 1.000	a: 0.333 b: 0.667	c: 1.000	d: 1.000	b: 1.000
Canyon Vista Spring	N=	4	4	4	4	4	4	4	4	4	4
		a: 1.000	b: 1.000	c: 1.000	a: 1.000	a: 1.000	a: 1.000	a: 0.875 b: 0.125	c: 1.000	d: 1.000	b: 1.000
Horsethief Hollow	N=	1	1	1	1	1	1	1	1	1	1
		a: 1.000	b: 1.000	c: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	c: 1.000	d: 1.000	b: 1.000

		PEP-D	PGM	PGDH	PK	SOD-S	H	P	A
NORTH									
Jollyville Plateau									
Balcones Park Spring	N=	5	5	5	5	5			
		e: 0.900	a: 1.000	e: 1.000	a: 1.000	a: 1.000	0.018	8.0	1.1
		f: 0.100					[0.013]		[0.1]
Barrow Hollow Spring	N=	4	4	5	4	5			
		e: 1.000	a: 1.000	e: 1.000	a: 1.000	a: 1.000	0.000	0.0	1.0
							[0.000]		[0.0]
Bull Creek Spring	N=	5	5	5	5	5			
		e: 0.900	a: 0.900	e: 1.000	a: 1.000	a: 1.000	0.024	12.0	1.1
		f: 0.100	e: 0.100				[0.013]		[0.1]
Canyon Creek Spring	N=	2	3	3	3	3			
		e: 1.000	a: 1.000	e: 1.000	a: 1.000	a: 1.000	0.027	4.0	1.0
							[0.027]		[0.0]
Canyon Vista Spring	N=	4	4	4	4	4		4.0	1.0
		e: 1.000	a: 1.000	e: 1.000	a: 1.000	a: 1.000	0.010		[0.0]
							[0.01]		
Horsethief Hollow Spring	N=	1	1	1	1	1		0.0	1.0
		e: 1.000	a: 1.000	e: 1.000	a: 1.000	a: 1.000	0.000		[0.0]
							[0.000]		

		ACO-1	AK	AAT-S	CK-1	CK-2	CAP	GPI	GR	GAPDH	G3PDH
New Bull Creek Spring	N=	2	2	2	2	2	2	2	2	1	1
		c: 1.000	b: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	b: 1.000	a: 1.000
Schlumberger Spring	N=	6	6	6	6	6	6	6	6	6	6
		c: 1.000	b: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	b: 1.000	a: 1.000
Stillhouse Hollow Springs	N=	14	14	14	13	14	14	14	14	14	14
		c: 1.000	b: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	b: 1.000	a: 1.000
Wheelis Springs	N=	5	5	5	5	5	5	5	5	5	5
		c: 1.000	b: 1.000	b: 0.900	a: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	b: 1.000	a: 1.000
				c: 0.100							
Round Rock Spring	N=	9	9	9	9	9	9	9	9	9	9
		c: 1.000	b: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	b: 1.000	a: 1.000
Lake Georgetown Area	N=	4	4	5	5	4	5	3	4	3	4
Avant's Springs		c: 1.000	b: 1.000	b: 1.000	a: 0.700	a: 1.000	a: 1.000	a: 0.667	a: 0.375	b: 1.000	d: 1.000
					c: 0.300			b: 0.333	b: 0.625		
Buford Hollow Springs	N=	8	8	9	8	9	9	9	7	7	9
		c: 0.688	b: 1.000	b: 1.000	a: 0.688	a: 1.000	a: 1.000	a: 0.944	a: 0.929	b: 1.000	d: 1.000
		f: 0.313			c: 0.313			b: 0.056	b: 0.071		

		IDH-1	IDH-2	LDH-A	LDH-B	MDH-1	MDH-2	MDHP	MPI	PEP-A	PEP-B
New Bull Creek Spring	N=	2	2	2	2	2	2	2	2	2	2
		a: 1.000	b: 1.000	c: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	c: 1.000	d: 1.000	b: 1.000
Schlumberger Spring	N=	6	6	6	6	6	6	6	6	6	6
		a: 1.000	b: 1.000	c: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	c: 1.000	d: 1.000	b: 1.000
Stillhouse Hollow	N=	14	14	14	14	14	14	14	14	14	14
		a: 1.000	b: 1.000	c: 1.000	a: 1.000	a: 1.000	a: 1.000	a: 0.214 b: 0.786	c: 1.000	d: 1.000	b: 1.000
Wheelis Springs	N=	5	5	5	5	4	4	5	5	2	5
		a: 1.000	b: 1.000	c: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	c: 1.000	d: 1.000	b: 1.000
Round Rock Spring	N=	9	9	9	9	9	9	9	9	9	9
		a: 1.000	b: 1.000	c: 1.000	a: 1.000	a: 1.000	a: 1.000	a: 0.063 b: 0.938	c: 1.000	d: 1.000	b: 1.000
Lake Georgetown Area	N=	5	5	5	5	5	5	4	5	4	5
Avant's Spring		a: 1.000	b: 1.000	c: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	c: 1.000	d: 1.000	b: 1.000
Buford Hollow Springs	N=	9	9	9	9	9	9	9	9	9	9
		a: 1.000	b: 1.000	c: 1.000	a: 1.000	a: 1.000	a: 0.944 c: 0.056	b: 1.000	c: 1.000	d: 1.000	b: 1.000

		PEP-D	PGM	PGDH	PK	SOD-S	H	P	A
New Bull Creek Spring	N=	1	2	2	2	2			
		e: 1.000	a: 1.000	e: 1.000	a: 1.000	a: 1.000	0.000	0.0	1.0
							[0.000]		[0.0]
Schlumberger Spring	N=	6	6	6	6	6			
		e: 1.000	a: 1.000	e: 1.000	a: 1.000	a: 1.000	0.000	0.0	1.0
							[0.000]		[0.0]
Stillhouse Hollow Springs	N=	14	14	14	14	14			
		e: 1.000	a: 1.000	e: 1.000	a: 1.000	a: 1.000	0.006	4.0	1.0
							[0.006]		[0.0]
Wheelis Springs	N=	4	4	5	5	5		8.0	1.1
		e: 0.875	a: 1.000	e: 1.000	a: 1.000	a: 1.000	0.018		[0.1]
		f: 0.125					[0.013]		
Round Rock Spring	N=	9	9	9	9	9			
		e: 0.944	a: 1.000	b: 0.056	a: 1.000	a: 1.000	0.014	12.0	1.1
		e: 0.056		e: 0.944			[0.008]		[0.1]
Lake Georgetown Area	N=	2	5	5	3	5			
Avant's Springs		a: 1.000	a: 1.000	e: 1.000	a: 1.000	a: 1.000	0.038	12.0	1.1
							[0.031]		[0.1]
Buford Hollow Springs	N=	9	9	9	9	9		24.0	1.2
		a: 0.944	a: 1.000	e: 1.000	a: 1.000	a: 1.000	0.049		[0.1]
		e: 0.056					[0.022]		

		ACO-1	AK	AAT-S	CK-1	CK-2	CAP	GPI	GR	GAPDH	G3PDH
Cedar Breaks Spring	N=	5	5	5	4	5	5	5	5	4	5
		c: 0.900	b: 1.000	b: 1.000	a: 0.625	a: 1.000	a: 1.000	a: 1.000	a: 0.100	b: 1.000	d: 1.000
		f: 0.100			c: 0.375				b: 0.900		
Knight Spring	N=	5	5	5	5	5	5	5	4	4	5
		c: 0.200	b: 1.000	b: 1.000	a: 0.500	a: 1.000	a: 1.000	a: 1.000	a: 0.375	b: 1.000	d: 1.000
		f: 0.800			c: 0.500				b: 0.625		
Salado Springs	N=	8	8	8	8	8	8	8	8	8	8
		c: 1.000	b: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	b: 0.875	a: 1.000
										c: 0.125	
Bat Well	N=	1	1	1	1	1	1	1	1	1	1
		c: 1.000	b: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	a: 1.000
Testudo Tube	N=	2	2	2	2	2	2	2	2	2	2
		b: 1.000	b: 1.000	b: 0.250	a: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	b: 1.000	a: 1.000
				c: 0.750							
Kretschmarr Cave	N=	4	4	4	4	4	4	3	4	4	4
		c: 1.000	b: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	b: 1.000	b: 1.000	a: 1.000
Buttercup Creek Caves											
Buttercup Creek Cave	N=	3	3	3	3	3	3	3	3	2	3
		b: 0.167	b: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	b: 1.000	a: 1.000
		c: 0.833									

		IDH-1	IDH-2	LDH-A	LDH-B	MDH-1	MDH-2	MDHP	MPI	PEP-A	PEP-B
Cedar Breaks Spring	N=	5	5	5	5	5	5	5	5	5	5
		a: 1.000	b: 1.000	c: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	c: 1.000	d: 1.000	b: 1.000
Knight Spring	N=	5	5	5	5	5	5	5	5	4	5
		a: 1.000	b: 1.000	c: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	c: 1.000	d: 1.000	b: 1.000
Salado Springs	N=	8	8	8	8	8	8	8	8	8	8
		a: 1.000	b: 1.000	c: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	c: 1.000	d: 1.000	b: 1.000
Bat Well	N=	1	1	1	1	1	1	1	1	1	1
		a: 1.000	b: 1.000	c: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	c: 1.000	d: 1.000	b: 1.000
Testudo Tube	N=	2	2	2	2	2	2	2	2	2	2
		a: 1.000	b: 1.000	c: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	c: 1.000	b: 1.000	b: 1.000
Kretschmarr Cave	N=	4	4	4	4	4	4	3	4	4	4
		a: 1.000	b: 1.000	c: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	c: 1.000	d: 1.000	b: 1.000
Buttercup Creek Caves											
Buttercup Creek Cave	N=	1	3	3	3	3	3	3	3	3	3
		a: 1.000	b: 1.000	c: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	c: 1.000	d: 1.000	b: 1.000

		PEP-D	PGM	PGDH	PK	SOD-S	H	P	A
Cedar Breaks Spring	N=	4	5	5	5	5			
		a: 1.000	a: 1.000	e: 1.000	a: 1.000	a: 1.000	0.026	12.0	1.1
							[0.014]		[0.1]
Knight Spring	N=	5	5	5	5	5			
		a: 1.000	a: 1.000	e: 1.000	a: 1.000	a: 1.000	0.034	12.0	1.1
							[0.020]		[0.1]
Salado Springs	N=	8	8	8	7	8			
		a: 1.000	a: 1.000	e: 1.000	a: 1.000	a: 1.000	0.010	4.0	1.0
							[0.010]		[0.0]
Bat Well	N=	1	1	1	1	1			
		a: 1.000	a: 1.000	e: 1.000	a: 1.000	a: 1.000	0.000	0.0	1.0
							[0.000]		[0.0]
Testudo Tube	N=	2	2	2	2	2			
		e: 1.000	a: 1.000	e: 1.000	a: 1.000	a: 1.000	0.020	4.0	1.0
							[0.020]		[0.0]
Kretschmarr Cave	N=	3	4	4	4	3			
		f: 1.000	a: 1.000	e: 1.000	a: 1.000	a: 1.000	0.000	0.0	1.0
							[0.000]		[0.0]
Buttercup Creek Caves									
Buttercup Creek Cave	N=	3	3	3	3	3			
		e: 0.167	a: 1.000	e: 1.000	a: 1.000	a: 1.000	0.027	8.0	1.1
		f: 0.833					[0.018]		[0.1]

		ACO-1	AK	AAT-S	CK-1	CK-2	CAP	GPI	GR	GAPDH	G3PDH
Ilex Cave	N=	2	2	2	2	2	2	2	2	2	2
		c: 1.000	b: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	b: 1.000	a: 1.000
Treehouse Cave	N=	1	1	1	1	1	1	1	1	1	1
		b: 1.000	b: 1.000	b: 0.500	a: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	b: 1.000	a: 1.000
				c: 0.500							
T.W.A.S.A. Cave	N=	1	1	1	1	1	1	1	1	1	1
		c: 1.000	b: 1.000	b: 0.500	a: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	b: 1.000	a: 0.500
				c: 0.500							d: 0.500
SAN MARCOS											
E. nana	N=	12	13	13	13	13	13	13	13	11	13
		d: 1.000	a: 1.000	c: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	c: 1.000	c: 1.000	c: 1.000
E. rathbuni	N=	5	5	5	4	5	5	5	0	5	5
		c: 1.000	b: 1.000	b: 0.600	b: 1.000	b: 1.000	a: 1.000	a: 1.000		c: 1.000	b: 0.700
				c: 0.400							c: 0.300
SOUTHEAST											
***E. latitans* group**											
E. latitans	N=	3	4	5	4	4	3	5	5	2	2
		b: 1.000	b: 1.000	a: 0.900	b: 1.000	a: 1.000	a: 1.000	a: 1.000	a: 1.000	c: 1.000	c: 1.000
				b: 0.100							

		IDH-1	IDH-2	LDH-A	LDH-B	MDH-1	MDH-2	MDHP	MPI	PEP-A	PEP-B
Ilex Cave	N=	2	2	2	2	2	2	2	2	2	2
		a: 1.000	b: 1.000	c: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	c: 1.000	d: 1.000	b: 1.000
Treehouse Cave	N=	1	1	1	1	1	1	1	1	1	1
		b: 1.000	a: 1.000	c: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	c: 1.000	d: 1.000	b: 1.000
T.W.A.S.A. Cave	N=	1	1	1	1	1	1	1	1	1	1
		a: 1.000	b: 1.000	c: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	c: 1.000	b: 0.500	b: 1.00
										d: 0.500	
SAN MARCOS											
E. nana	N=	13	13	13	13	13	13	13	12	12	13
		b: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	c: 1.000	c: 1.000	b: 0.375	d: 1.000
										d: 0.625	
E. rathbuni	N=	5	5	5	5	5	5	5	5	5	5
		b: 1.000	b: 1.000	b: 1.000	a: 1.000	a: 1.000	b: 1.000	c: 1.000	c: 1.000	b: 1.000	b: 0.900
											d: 0.100
SOUTHEAST											
***E. latitans* group**											
E. latitans	N=	3	5	5	5	5	5	5	4	2	3
		b: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	c: 1.000	c: 1.000	b: 1.000	a: 1.000

		PEP-D	PGM	PGDH	PK	SOD-S	H	P	A
Ilex Cave	N=	2	2	2	2	2			
		e: 0.250	a: 1.000	e: 1.000	a: 1.000	a: 1.000	0.020	4.0	1.0
		f: 0.750					[0.020]		[0.0]
Treehouse Cave	N=	1	1	1	1	1		4.0	1.0
		f: 1.000	a: 1.000	e: 1.000	a: 1.000	a: 1.000	0.040		[0.0]
							[0.040]		
T.W.A.S.A. Cave	N=	1	1	1	1	1		12.0	1.1
		f: 1.000	a: 1.000	e: 1.000	a: 1.000	a: 1.000	0.120		[0.1]
							[0.066]		
SAN MARCOS									
E. nana	N=	12	11	13	8	12			
		h: 1.000	a: 1.000	e: 1.000	a: 1.000	a: 1.000	0.017	4.0	1.0
							[0.017]		[0.0]
E. rathbuni	N=	4	5	5	4	4			
		d: 0.250	a: 0.800	a: 0.200	a: 0.750	a: 1.000	0.052	28.0	1.3
		g: 0.750	g: 0.200	e: 0.800	e: 0.250		[0.027]		[0.1]
SOUTHEAST									
***E. latitans* group**									
E. latitans	N=	4	5	5	5	4		8.0	1.1
		e: 0.625	a: 1.000	b: 1.000	a: 1.000	a: 1.000	0.018		[0.1]
		d: 0.375					[0.013]		

		ACO-1	AK	AAT-S	CK-1	CK-2	CAP	GPI	GR	GAPDH	G3PDH
Bear Creek Spring	N=	6	6	6	6	6	6	6	6	6	6
		b: 1.000	b: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	a: 1.000	c: 1.000	c: 1.000
Cherry Creek Spring	N=	2	2	2	2	2	1	2	2	2	2
		b: 1.000	b: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	a: 1.000	c: 1.000	c: 1.000
Cloud Hollow Spring	N=	5	5	5	5	5	5	4	5	4	5
		b: 1.000	b: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	a: 1.000	c: 1.000	c: 1.000
Cibolo Creek Spring	N=	8	8	8	8	8	8	7	8	8	8
		b: 1.000	b: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	a: 1.000	c: 1.000	c: 1.000
Kneedeep Cave Spring	N=	6	5	6	6	6	5	5	6	6	6
		a: 0.083	b: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 0.900	a: 1.000	c: 1.000	c: 1.000
		b: 0.917						b: 0.100			
Honey Creek Cave Spring	N=	1	1	1	1	1	1	1	1	1	1
		b: 1.000	b: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	a: 1.000	c: 1.000	c: 1.000
Less Ranch Spring	N=	2	2	2	2	2	2	2	2	2	2
		b: 1.000	b: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	a: 1.000	c: 1.000	c: 1.000

		IDH-1	IDH-2	LDH-A	LDH-B	MDH-1	MDH-2	MDHP	MPI	PEP-A	PEP-B
Bear Creek Spring	N=	5	6	6	6	6	6	6	6	6	6
		b: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	c: 1.000	c: 1.000	b: 1.000	b: 1.000
Cherry Creek Spring	N=	2	2	2	2	2	1	2	1	2	2
		b: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	c: 1.000	c: 1.000	b: 1.000	b: 1.000
Cloud Hollow Spring	N=	5	5	5	5	5	5	5	5	5	5
		b: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	c: 1.000	c: 1.000	b: 1.000	b: 1.000
Cibolo Creek Spring	N=	8	8	8	8	8	8	8	8	8	8
		b: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	c: 1.000	c: 1.000	b: 1.000	b: 1.000
Kneedeep Cave Spring	N=	6	6	6	6	6	6	6	6	6	6
		b: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 0.667 d: 0.333	a: 1.000	c: 1.000	c: 1.000	b: 1.000	b: 1.000
Honey Creek Cave Spring	N=	1	1	1	1	1	1	1	1	1	1
		b: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	c: 1.000	c: 1.000	b: 1.000	b: 1.000
Less Ranch Spring	N=	2	2	2	2	2	2	2	2	2	2
		b: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	c: 1.000	c: 1.000	b: 1.000	b: 1.000

		PEP-D	PGM	PGDH	PK	SOD-S	H	P	A
Bear Creek Spring	N=	6	6	6	6	6			
		c: 1.000	a: 0.917	d: 1.000	a: 1.000	a: 1.000	0.007	4.0	1.0
			c: 0.083				[0.007]		[0.0]
Cherry Creek Spring	N=	2	2	2	2	1			
		c: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	0.000	0.0	1.0
							[0.000]		[0.0]
Cloud Hollow Spring	N=	4	5	5	4	5			
		c: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	0.000	0.0	1.0
							[0.000]		[0.0]
Cibolo Creek Spring	N=	8	8	8	8	7			
		c: 1.000	a: 1.000	a: 0.063	a: 1.000	a: 1.000	0.015	4.0	1.2
				b: 0.063			[0.015]		[0.2]
				c:0.063					
				d: 0.250					
				e: 0.563					
Kneedeep Cave Spring	N=	6	6	6	5	5			
		c: 1.000	a: 1.000	b: 0.250	a: 1.000	a: 1.000	0.048	16.0	1.2
				e: 0.750			[0.028]		[0.1]
Honey Creek Cave Spring	N=	1	1	1	1	1			
		c: 1.000	a: 1.000	e: 1.000	a: 1.000	a: 1.000	0.000	0.0	1.0
							[0.000]		[0.0]
Less Ranch Spring	N=	2	2	2	2	2			
		c: 0.750	a: 1.000	b: 1.000	a: 1.000	a: 1.000	0.020	4.0	1.0
		d: 0.250					[0.020]		[0.0]

		ACO-1	AK	AAT-S	CK-1	CK-2	CAP	GPI	GR	GAPDH	G3PDH
Rebecca Creek Spring	N=	6	6	3	5	5	6	5	6	5	6
		b: 1.000	b: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	b: 1.000	a: 1.000	c: 1.000	c: 1.000
***E. neotenes* group**											
E. neotenes (Helotes Spring)	N=	11	9	11	11	10	9	10	11	6	9
		b: 1.000	b: 1.000	a: 0.136	b: 1.000	a: 1.000	a: 1.000	a: 0.700	b: 1.000	c: 1.000	c: 1.000
				b: 0.864				c: 0.100			
								d: 0.200			
Leon Springs	N=	2	2	2	2	2	2	2	2	2	2
		b: 1.000	b: 1.000	b: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 0.500	b: 1.000	c: 1.000	c: 1.000
								b: 0.500			
Mueller's Spring	N=	4	4	4	4	4	4	4	4	4	3
		b: 1.000	b: 1.000	b: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 0.875	b: 1.000	c: 1.000	c: 1.000
								d: 0.125			
***E. pterophila* group**											
E. pterophila (Fern Bank)	N=	10	10	10	10	10	10	10	10	10	10
		b: 1.000	b: 1.000	a: 0.950	b: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	c: 1.000	c: 1.000
				b: 0.050							
Boardhouse Springs	N=	11	11	11	11	11	11	11	11	11	11
		b: 1.000	b: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 0.955	b: 1.000	c: 1.000	c: 1.000
								b: 0.045			

		IDH-1	IDH-2	LDH-A	LDH-B	MDH-1	MDH-2	MDHP	MPI	PEP-A	PEP-B
Rebecca Creek Spring	N=	6	6	6	6	6	6	6	6	6	6
		b: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	c: 1.000	c: 1.000	b: 1.000	b: 1.000
***E. neotenes* group**											
E. neotenes (Helotes Spring)	N=	9	11	11	11	9	11	10	11	11	11
		b: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	c: 1.000	b: 1.000	b: 1.000	b: 1.000
Leon Springs	N=	2	2	2	2	2	2	2	2	2	2
		b: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	c: 1.000	b: 0.570 c: 0.250	b: 1.000	b: 1.000
Mueller's Spring	N=	4	4	4	4	4	4	4	4	4	4
		b: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	c: 1.000	b: 1.000	b: 1.000	b: 1.000
***E. pterophila* group**											
E. pterophila (Fern Bank)	N=	10	10	10	10	10	10	10	10	10	10
		b: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	c: 1.000	b: 0.600 c: 0.400	b: 1.000	b: 1.000
Boardhouse Springs	N=	11	11	11	11	11	11	11	11	11	11
		b: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	a. 1.000	c: 1.000	c: 1.000	b: 1.000	b: 1.000

		PEP-D	PGM	PGDH	PK	SOD-S	H	P	A
Rebecca Creek Spring	N=	5	6	6	6	6			
		c: 0.900	a: 1.000	c: 1.000	a: 1.000	a: 1.000	0.008	4.0	1.0
		d: 0.100					[0.008]		[0.000]
***E. neotenes* group**									
E. neotenes (Helotes Spring)	N=	9	10	10	10	11			
		c: 0.667	a: 0.400	c: 0.650	a: 1.000	a: 1.000	0.081	20.0	1.2
		d: 0.333	b: 0.600	c: 0.350			[0.036]		[0.1]
Leon Springs	N=	2	2	2	2	2			
		c: 1.000	a: 0.500	c: 0.750	a: 1.000	a: 1.000	0.040	16.0	1.2
			b: 0.500	c: 0.250			[0.028]		[0.1]
Mueller's Spring	N=	3	4	4	4	4			
		c: 1.000	a: 0.125	b: 0.500	a: 1.000	a: 1.000	0.020	12.0	1.1
			b: 0.875	c: 0.500			[0.014]		[0.1]
***E. pterophila* group**									
E. pterophila (Fern Bank)	N=	10	10	10	10	7			
		c: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	0.028	8.0	1.1
							[0.024]		[0.1]
Boardhouse Springs	N=	11	11	11	11	11			
		c: 0.955	a: 1.000	b: 1.000	a: 1.000	a: 1.000	0.007	8.0	1.1
		d: 0.045					[0.005]		[0.1]

		ACO-1	AK	AAT-S	CK-1	CK-2	CAP	GPI	GR	GAPDH	G3PDH
Grapevine Cave	N=	2	2	2	2	2	2	2	2	2	2
		b: 1.000	b: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	c: 1.000	c: 1.000
Peavey's Springs	N=	5	5	5	5	5	5	5	5	5	5
		b: 1.000	b: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	c: 1.000	c: 1.000
T Cave	N=	5	5	5	5	5	4	5	5	5	5
		b: 1.000	b: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	c: 1.000	c: 1.000
E. sosorum	N=	13	14	14	14	14	14	14	13	12	14
		b: 0.923	b: 1.000	a: 0.964	b: 1.000	a: 1.000	a: 1.000	a: 0.464	a: 1.000	c: 1.000	b: 0.107
		c: 0.077		b: 0.036				b: 0.536			c: 0.893
E. tridentifera											
Honey Creek Cave	N=	5	5	5	5	5	4	5	5	5	5
		b: 1.000	b: 1.000	a: 0.300	b: 1.000	a: 1.000	a: 1.000	a: 1.000	a: 1.000	c: 1.000	b: 1.000
				b: 0.700							
Badweather Pit	N=	5	5	5	5	5	5	5	5	5	5
		b: 1.000	b: 1.000	a: 0.400	b: 1.000	a: 1.000	a: 1.000	a: 1.000	a: 0.900	c: 1.000	c: 1.000
				b: 0.600					b: 0.100		
Ebert Cave	N=	6	6	6	6	6	6	6	6	6	6
		b: 1.000	b: 1.000	a: 0.500	b: 1.000	a: 1.000	a: 1.000	a: 0.583	a: 1.000	c: 1.000	b: 1.000
				b: 0.500				b: 0.417			

		IDH-1	IDH-2	LDH-A	LDH-B	MDH-1	MDH-2	MDHP	MPI	PEP-A	PEP-B
Grapevine Cave	N=	2	2	2	2	2	2	2	2	2	2
		b: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	c: 1.000	b: 1.000	b: 1.000	b: 1.000
Peavey's Springs	N=	5	5	5	5	5	5	5	5	5	5
		b: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	c: 1.000	c: 1.000	b: 1.000	b: 1.000
T Cave	N=	5	5	5	5	5	5	5	5	5	5
		b:1.000	a: 1.000	a: 0.500	a: 1.000	a: 1.000	a: 1.000	c: 1.000	c: 1.000	b: 1.000	b: 1.000
				d: 0.500							
E. sosorum	N=	12	14	14	14	14	14	14	14	12	14
		b: 1.000	a: 1.000	b: 0.964	a: 1.000	a: 1.000	a: 1.000	c: 1.000	c: 1.000	d: 0.167	a: 0.071
				d: 0.036						c: 0.833	b: 0929
E. tridentifera											
Honey Creek Cave	N=	4	4	5	5	5	5	5	5	3	4
		b: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	d: 1.000	c: 1.000	b: 1.000	a: 0.750
											b: 0.250
Badweather Pit	N=	5	5	5	5	5	5	5	5	5	5
		b: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 0.900	d: 1.000	c: 1.000	b: 1.000	a: 0.400
							c: 0.100				b: 0.600
Ebert Cave	N=	6	6	6	6	6	6	6	6	6	6
		b: 1.000	a: 1.000	b: 0.500	a: 1.000	a: 1.000	a: 1.000	c: 0.083	c: 1.000	b: 1.000	a: 1.000
								d: 0.917			

		PEP-D	PGM	PGDH	PK	SOD-S	H	P	A
Grapevine Cave	N=	2	2	2	2	2			
		c: 0.250	a: 1.000	b: 1.000	a: 1.000	a: 1.000	0.020	4.0	1.0
		d: 0.750					[0.02]		[0.0]
Peavey's Springs	N=	5	5	5	4	5			
		c: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	0.000	0.0	1.0
							[0.000]		[0.0]
T Cave	N=	5	5	5	5	5			
		c: 0.500	a: 1.000	b: 1.000	a: 1.000	a: 1.000	0.032	8.0	1.1
		d: 0.500					[0.025]		[0.1]
E. sosorum	N=	11	14	14	12	11			
		a: 1.000	a: 1.000	b: 0.214	a: 1.000	a: 1.000	0.053	32.0	1.3
				e: 0.786			[0.023]		[0.1]
E. tridentifera									
Honey Creek Cave	N=	5	5	5	5	4			
		a: 0.100	a: 1.000	b: 0.200	a: 1.000	a: 1.000	0.032	16.0	1.2
		d: 0.900		e: 0.800			[0.019]		[0.1]
Badweather Pit	N=	4	5	5	5	5			
		c: 0.250	a: 1.000	b: 0.700	a: 1.000	a: 1.000	0.108	24.0	1.2
		d: 0.750		e: 0.300			[0.040]		[0.1]
Ebert Cave	N=	4	6	6	6	6			
		d: 0.625	a: 1.000	b: 0.833	a: 1.000	a: 1.000	0.097	20.0	1.2
		e: 0.375		e: 0.167			[0.046]		[0.1]

		ACO-1	AK	AAT-S	CK-1	CK-2	CAP	GPI	GR	GAPDH	G3PDH
Comal Springs	N=	11	12	11	11	11	12	11	11	9	9
		b: 0.545	b: 1.000	a: 0.591	b: 1.000	a: 1.000	a: 1.000	a: 0.545	a: 1.000	c: 1.000	c: 1.000
		c: 0.455		b: 0.409				b: 0.455			
Pedernales											
Spring 1	N=	7	7	6	7	7	6	5	7	7	7
		b: 0.857	b: 1.000	a: 0.333	b: 1.000	a: 1.000	a: 1.000	a: 1.000	a: 1.000	c: 1.000	c: 1.000
		c: 0.143		b: 0.667							
Spring 2											
	N=	7	7	7	7	7	7	7	7	7	7
		b: 0.857	b: 1.000	a: 0.714	b: 1.000	a: 1.000	a: 1.000	a: 1.000	a: 1.000	c: 1.000	c: 1.000
		c: 0.143		b: 0.286							
SOUTHWEST											
Carson Cave Group											
Carson Cave	N=	5	5	5	5	5	5	5	5	4	5
		b: 1.000	b: 1.000	b: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	c: 1.000	b: 1.000
Fessenden Spring	N=	8	8	8	8	8	8	8	8	8	8
		a: 0.063	b: 1.000	b: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	c: 1.000	c: 1.000
		b: 0.938									
Robinson Creek Spring	N=	4	4	4	4	4	4	4	4	4	4
		b: 1.000	b: 1.000	b: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	c: 1.000	c: 1.000

		IDH-1	IDH-2	LDH-A	LDH-B	MDH-1	MDH-2	MDHP	MPI	PEP-A	PEP-B
Comal Springs	N=	11	12	12	11	11	12	12	11	12	11
		b: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	c: 1.000	c: 1.000	b: 1.000	b: 1.000
Pedernales											
Spring 1		7	7	7	7	7	7	7	7	6	7
		b: 1.000	a: 1.000	a: 0.429	a: 1.000	a: 1.000	a: 1.000	c: 0.929	b: 0.500	b: 0.917	b: 1.000
				b: 0.571				f: 0.071	c: 0.500	d: 0.083	
Spring 2	N=	7	7	7	7	7	7	7	7	7	6
		b: 1.000	a: 1.000	a: 0.429	a: 1.000	a: 1.000	a: 1.000	c: 0.929	b: 0.429	b: 1.000	b: 1.000
				b: 0.571				f: 0.071	c: 0.571		
SOUTHWEST											
Carson Cave Group											
Carson Cave	N=	5	5	5	5	5	5	5	5	5	5
		b: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	c: 1.000	c: 1.000	b: 0.900	a: 1.000
										c: 0.100	
Fessenden Spring	N=	8	8	8	8	8	8	7	8	7	8
		b: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	c: 1.000	b: 0.063	a: 0.929	a: 1.000
									c: 0.938	b: 0.071	
Robinson Creek Spring	N=	4	4	4	4	4	4	4	4	4	4
		b: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	c: 1.000	c: 1.000	b: 1.000	a: 1.000

		PEP-D	PGM	PGDH	PK	SOD-S	H	P	A
Comal Springs		12	12	11	11	12			
		c: 1.000	a: 0.375	b: 1.000	a: 1.000	a: 1.000	0.057	16.0	1.2
			b: 0.625				[0.030]		[0.1]
Pedernales									
Spring 1		7	7	7	5	6			
		c: 0.857	a: 1.000	b: 1.000	a: 1.000	a: 1.000	0.090	28.0	1.3
		d: 0.143					[0.038]		[0.1]
Spring 2		7	7	7	6	7	0.057	24.0	1.2
		c: 1.000	a: 1.000	b: 0.929	a: 1.000	a: 1.000	[0.027]		[0.1]
				c: 0.071					
SOUTHWEST									
Carson Cave Group									
Carson Cave	N=	5	5	5	5	5			
		c: 1.000	a: 1.000	c: 1.000	a: 1.000	a: 1.000	0.008	4.0	1.0
							[0.008]		[0.0]
Fessenden Spring	N=	8	8	8	8	8			
		a: 0.063	a: 1.000	c: 1.000	a: 1.000	a: 1.000	0.026	16.0	1.2
		d: 0.875					[0.013]		[0.1]
		e: 0.063							
Robinson Creek Spring	N=	3	4	4	4	4	0.027	4.0	1.0
		d: 0.333	a: 1.000	c: 1.000	a: 1.000	a: 1.000	[0.027]		[0.0]
		e: 0.667							

		ACO-1	AK	AAT-S	CK-1	CK-2	CAP	GPI	GR	GAPDH	G3PDH
Smith's Spring	N=	14	14	14	14	14	14	14	14	14	14
		b: 1.000	b: 1.000	b: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	c: 1.000	b: 0.536
											c: 0.464
Sutherland Hollow Spring	N=	11	11	11	11	11	11	11	11	11	11
		b: 1.000	b: 1.000	b: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 0.955	b: 1.000	c: 1.000	b: 0.455
								b: 0.045			c: 0.545
Trough Spring	N=	10	10	10	10	10	10	10	10	10	10
		b: 1.000	b: 1.000	b: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	c: 1.000	c: 1.000
West Nueces Spring	N=	1	1	1	1	1	1	1	1	1	1
		b: 1.000	b: 1.000	b: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	c: 1.000	b: 1.000
Wetback Spring	N=	4	4	4	4	4	4	4	4	4	4
		b: 1.000	b: 1.000	b: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 0.750	b: 1.000	c: 1.000	c: 1.000
								b: 0.250			
Camp Mystic Spring	N=	10	10	10	10	10	10	10	10	10	10
		b: 1.000	b: 1.000	b: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	c: 1.000	c: 1.000
Greenwood Ranch Springs	N=	6	6	6	6	6	6	6	6	6	6
		b: 1.000	b: 1.000	b: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	c: 1.000	b: 1.000

		IDH-1	IDH-2	LDH-A	LDH-B	MDH-1	MDH-2	MDHP	MPI	PEP-A	PEP-B
Smith's Spring	N=	12	14	14	14	14	14	14	14	13	14
		b: 1.000	a: 0.857 b: 0.143	b: 1.000	a: 1.000	a: 0.393 b: 0.607	a: 1.000	c: 1.000	c: 1.000	b: 0.654 c: 0.346	a: 1.000
Sutherland Hollow Spring	N=	11	11	10	11	11	11	11	11	11	11
		b: 1.000	a: 1.000	b: 0.650 d: 0.350	a: 1.000	a: 1.000	a: 1.000	c: 0.909 e: 0.091	c: 1.000	b: 1.000	a: 1.000
Trough Spring	N=	10	10	10	10	10	10	10	10	9	9
		b: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	c: 1.000	d: 1.000	b: 1.000	a: 1.000
West Nueces Spring	N=	1	1	1	1	1	1	1	1	1	1
		b: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	c: 1.000	c: 1.000	b: 1.000	a: 1.000
Wetback Spring	N=	4	4	4	4	4	4	4	4	4	4
		b: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	c: 1.000	c: 1.000	b: 1.000	a: 0.875 b: 0.125
Camp Mystic Spring	N=	10	10	10	10	10	10	10	10	10	10
		b: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 0.100 c: 0.900	a: 1.000	c: 1.000	c: 1.000	b: 1.000	a: 1.000
Greenwood Ranch Springs	N=	6	6	6	6	6	0	6	6	6	6
		d: 1.000	a: 1.000	b: 1.000	a: 1.000	e: 1.000		c: 1.000	c: 1.000	c: 1.000	a: 1.000

		PEP-D	PGM	PGDH	PK	SOD-S	H	P	A
Smith's Spring	N=	14	14	14	14	14			
		c: 0.929	a: 1.000	c: 1.000	a: 1.000	a: 1.000	0.044	20.0	1.2
		d: 0.071					[0.018]		[0.1]
Sutherland Hollow Spring	N=	10	11	11	11	11			
		c: 1.000	a: 1.000	c: 1.000	a: 1.000	a: 1.000	0.046	16.0	1.2
							[0.029]		[0.1]
Trough Spring	N=	9	10	10	10	10	0.013	8.0	1.1
		c: 0.778	a: 1.000	d: 0.050	a: 1.000	a: 1.000	[0.010]		[0.1]
		d: 0.222		e: 0.950					
West Nueces Spring	N=	1	1	1	1	1			
		c: 1.000	a: 1.000	c: 1.000	a: 1.000	a: 1.000	0.000	0.0	1.0
							[0.000]		[0.0]
Wetback Spring	N=	4	4	4	4	4			
		c: 0.625	a: 1.000	c: 1.000	a: 1.000	a: 1.000	0.060	12.0	1.1
		d: 0.375					[0.036]		[0.1]
Camp Mystic Spring	N=	4	10	10	10	10			
		a: 1.000	a: 0.900	c: 1.000	b: 1.000	a: 1.000	0.008	8.0	1.1
			b: 0.100				[0.008]		[0.1]
Greenwood Ranch Springs	N=	4	6	6	6	6			
		c: 0.250	a: 1.000	c: 1.000	a: 1.000	a: 1.000	0.020	4.0	1.0
		d: 0.750					[0.020]		[0.0]

		ACO-1	AK	AAT-S	CK-1	CK-2	CAP	GPI	GR	GAPDH	G3PDH
176 Spring	N=	4	3	4	4	4	4	4	4	4	4
		a: 0.125 b: 0.875	b: 1.000	b: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	b: 1.000	c: 1.000	b: 1.000
Sabinal Group											
Murphy's Spring	N=	4	4	4	4	4	2	4	4	4	4
		b: 1.000	b: 1.000	b: 1.000	b: 1.000	a: 1.000	a: 1.000	b: 1.000	b: 1.000	a: 0.500 c: 0.500	b: 1.000
Sabinal Canyon Spring	N=	11	11	11	11	11	11	11	11	10	11
		b: 1.000	b: 1.000	b: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 0.091 b: 0.909	b: 1.000	a: 0.950 c: 0.050	b: 0.955 c: 0.045
Tucker Hollow Cave	N=	6	5	6	6	6	6	6	6	6	6
		b: 1.000	b: 1.000	b: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 0.667 b: 0.333	b: 1.000	a: 1.000	b: 1.000
OUTGROUP											
Eurycea m. multiplicata	N=	2	2	2	2	2	2	2	2	2	2
		c: 1.000	b: 1.000	l: 1.000	d: 1.000	c: 1.000	c: 1.000	b: 1.000	e: 1.000	x: 1.000	a: 1.000
E. l. longicauda	N=	2	2	2	2	2	2	2	2	2	2
		x: 0.500 y: 0.500	b: 1.000	y: 1.000	d: 1.000	c: 1.000	a: 1.000	b: 1.000	a: 1.000	y: 1.000	a: 1.000

		IDH-1	IDH-2	LDH-A	LDH-B	MDH-1	MDH-2	MDHP	MPI	PEP-A	PEP-B
176 Spring	N=	4	4	4	3	4	4	4	4	3	4
		b: 1.000	a: 1.000	b: 0.125 e: 0.875	a: 1.000	a: 1.000	a: 1.000	c: 1.000	b: 1.000	a: 1.000	a: 1.000
Sabinal Group											
Murphy's Spring	N=	4	4	4	4	4	4	4	4	4	4
		b: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	c: 0.125 e: 0.875	c: 1.000	b: 1.000	a: 1.000
Sabinal Canyon Spring	N=	11	11	11	11	11	11	11	11	11	11
		b: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	c: 0.409 e: 0.591	a: 0.045 c: 0.955	b: 1.000	a: 1.000
Tucker Hollow Cave	N=	5	6	6	6	6	6	6	6	6	4
		c: 1.000	a: 1.000	b: 1.000	a: 1.000	a: 1.000	a: 1.000	c: 1.000	c: 1.000	c: 1.000	a: 1.000
OUTGROUP											
Eurycea m. multiplicata	N=	2	2	2	2	2	2	2	2	0	2
		b: 1.000	e: 1.000	b: 1.000	b: 1.000	a: 1.000	e: 1.000	x: 1.000	d: 0.750 i: 0.250		p: 1.000
E. l. longicauda	N=	2	2	2	2	2	2	2	2	0	2
		b: 1.000	e: 1.000	x: 1.000	a: 0.500 e: 0.500	a: 1.000	g: 1.000	k: 0.750 m: 0.250	e: 1.000		l: 1.000

		PEP-D	PGM	PGDH	PK	SOD-S	H	P	A
176 Spring	N=	2	4	4	4	4			
		d: 0.750	a: 1.000	e: 1.000	a: 1.000	a: 1.000	0.040	12.0	1.1
		e: 0.250					[0.024]		[0.1]
Sabinal Group									
Murphy's Spring	N=	4	4	4	4	4			
		e: 0.875	a: 1.000	e: 1.000	a: 1.000	a: 1.000	0.040	12.0	1.1
		d: 0.125					[0.024]		[0.1]
Sabinal Canyon Spring	N=	11	10	11	11	11			
		e: 0.909	a: 1.000	e: 1.000	a: 1.000	a: 1.000	0.037	24.0	1.2
		d: 0.091					[0.020]		[0.1]
Tucker Hollow Cave	N=	6	4	6	6	6			
		e: 0.500	a: 1.000	e: 1.000	a: 1.000	a: 1.000	0.040	8.0	1.1
		d: 0.500					[0.029]		[0.1]
OUTGROUP									
Eurycea m. multiplicata	N=	0	2	2	2	2			
			a: 0.750	e: 1.000	x: 0.75	f: 1.000	NC	NC	NC
			x: 0.250		d: 0.25				
E. l. longicauda	N=	0	2	2	2	2			
			a: 1.000	o: 1.000	d: 1.000	f: 1.000	NC	NC	NC

OUTGROUP (continued)		ACO-1	AK	AAT-S	CK-1	CK-2	CAP	GPI	GR	GAPDH	G3PDH
E. wilderae	N=	3 c: 1.000	3 b: 1.000	0	0	2 f: 1.000	3 a: 1.000	3 b: 1.000	3 a: 1.000	3 y: 1.000	3 a: 1.000
E. bislineata	N=	1 i: 0.500 k: 0.500	1 b: 1.000	1 y: 1.000	0	1 c: 1.000	1 a: 1.000	1 b: 1.000	1 a: 1.000	1 y: 1.000	1 a: 1.000
E. quadridigitata TX	N=	2 c: 1.000	2 b: 1.000	2 f: 0.500 g: 0.500	2 c: 1.000	2 a: 1.000	2 a: 1.000	2 b: 1.000	2 e: 1.000	2 y: 1.000	2 a: 1.000
E. quadridigitata SC	N=	2 k: 1.000	2 b: 1.000	2 b: 1.000	2 y: 1.000	2 a: 1.000	2 a: 1.000	2 b: 1.000	2 e: 1.000	2 y: 1.000	2 b: 1.000
Haideotriton wallacei	N=	1 d: 1.000	1 b: 1.000	1 x: 1.000	1 d: 1.000	1 c: 1.000	0	0	1 a: 1.000	1 j: 1.000	0
Typhlotriton spelaeus	N=	2 d: 0.500 e: 0.500	2 b: 1.000	2 b: 1.000	0	2 c: 1.000	2 c: 1.000	2 b: 1.000	2 b: 0.750 e: 0.250	2 d: 1.000	2 x: 1.000

OUTGROUP (continued)		IDH-1	IDH-2	LDH-A	LDH-B	MDH-1	MDH-2	MDHP	MPI	PEP-A	PEP-B
E. wilderae	N=	3	3	3	3	3	3	3	3	0	3
		b: 1.000	e: 1.000	f: 1.000	a: 1.000	a: 1.000	g: 1.000	m: 0.670	c: 0.250		b: 0.250
								n:0.330	y: 0.750		d: 0.750
E. bislineata	N=	1	1	1	0	1	1	1	1	0	1
		b: 1.000	e: 1.000	f: 1.000		a: 1.000	g: 1.000	g: 1.000	e: 1.000		b: 1.000
E. quadridigitata TX	N=	2	2	2	2	2	2	2	2	0	2
		e: 1.000	e: 1.000	f: 1.000	a: 1.000	a: 1.000	g: 1.000	g: 1.000	c: 0.500		j: 0.500
									e: 0.500		l: 0.500
E. quadridigitata SC	N=	2	2	2	2	2	2	2	2	0	2
		b: 1.000	x: 1.000	f: 1.000	c: 1.000	x: 1.000	d: 1.000	g: 1.000	d: 1.000		a: 1.000
Haideotriton wallacei	N=	1	1	1	0	1	1	1	1	0	1
		x: 1.000	b: 1.000	f: 1.000		a: 1.000	k: 1.000	m: 1.000	c: 1.000		i: 0.500
											k: 0.500
Typhlotriton spelaeus	N=	2	2	2	2	2	2	2	2	0	2
		b: 0.750	e: 1.000	f: 1.000	x: 1.000	a: 1.000	i: 1.000	x: 0.750	z: 1.000		l: 1.000
		g: 0.250						g: 0.250			

		PEP-D	PGM	PGDH	PK	SOD-S	H	P	A
OUTGROUP (continued)									
E. wilderae	N=	0	3	3	3	3			
			a: 1.000	e: 0.500	d: 1.000	f: 1.000	NC	NC	NC
				x: 0.333					
				y: 0.167					
E. bislineata	N=	0	1	1	1	1			
			a: 1.000	b: 1.000	d: 1.000	f: 1.000	NC	NC	NC
E. quadridigitata TX	N=	0	2	2	2	2			
			b: 1.000	e: 1.000	e: 1.000	f: 1.000	NC	NC	NC
E. quadridigitata SC	N=	0	2	2	2	2			
			a: 1.000	b: 1.000	x: 1.000	f: 1.000	NC	NC	NC
Haideotriton wallacei	N=	0	1	1	1	1			
			a: 1.000	e: 1.000	b: 1.000	f: 1.000	NC	NC	NC
Typhlotriton spelaeus	N=	0	2	2	2	2			
			a: 1.000	d: 0.250	d: 1.000	f: 1.000	NC	NC	NC
				e: 0.750					

APPENDIX 3. Cytochrome b sequence, from positions 44 to 398 within the gene, for central Texas *Eurycea* and outgroup members. Texas group members are listed by general area of occurrence. "*E. trid.*" = *E. tridentifera* and "*E. quad.*" = *E. quadridigitata*

Northern	
Testudo Tube	TTAACAACTCATTTATTGATCTCCCAGCCCCTTCTACCTTATCCTACT
Ilex Cave	???.....
Kretschmarr Cave	??????.....?.....
Stillhouse Hollow	??????.....G.....?.G.....
Horsethief Hollow	??????.....??G.....
Round Rock	????.....??.G.....
Cedar Breaks	????.....C.....G.....
Bat Well	????.....C.... .....???G.....
Salado	???.....?.G.....
San Marcos	
E. rathbuni	????...T..C.....C..A...A....C..C.A...?..T..T.
E. nana	????.....C.....C..T...?....G..A.A.C.?..T....
Southeastern	
E. sosorum	??.....C.....C.....G..A.A?.....T...C
E. latitans	?????.....C.....C.....C..A.AT.....T...C
E. trid. BW Pit	????.....C.....C.....?.....C.????T.....T...C
E. trid. H.C.Cave	??????....C.....C.....C..A.???....T...C
E. trid. Eb. Cave	?????.....C.....C.....C..A.A??....T...C
Boardhouse	????.....C.....C.....?.....C..A.AT?....T...C
Cibolo Creek	??????....C.....C.....G..A.??.....T...C
Cloud Hollow	????????..??.....C..?.....C..A.AT.....T...C
Comal Springs	????.....C.....C.....G..A.AT?....T...C
H.C.Cave Spring	????..?...C.....C.....C..A.AT.....T...C
E. neotenes	???????...C.....C.....G..A???.....T...C
Pedernales	?????????.C.....C....??.?...G.?A.A????..T...C
Rebecca Creek	??????....C.....C.....C.?A???.....T...C
Southwestern	
E. troglodytes	???????...C.....C.....C.....A..A.A??....T....
176 Spring	??????....T.....C.....?.....G..A.A?C....T....
Camp Mystic	????.....C.....C.....G..A.A.C....T....
Carson Cave	???????...C.....C.....C.....G..A.ATC.?.....C
Greenwood Valley	???????...C.....C.....G..A.A?C....T....
Sabinal Canyon	?????.....C.....C.....C.....A..A.A.C....T....
Smith's Spring	????????..C.....C.....?.?..?G.?A.???.?.....C
Sutherland Hollow	??????....C.....C.....?....?A..A.A??....T....
Trough Spring	??????....C.....C.....G..A.A?C....T....
Tucker H. Cave	????????..C.....C.....C.....A..A.??C....T...C
Outgroup	
E. bislineata	????.....T.....C.....C.....A....A.C.?..T...C
E. longicauda	????T.....C.....C.....A..A.G?....C....C
E. multiplicata	????.....C.....C.....C.....A..A.G.C.G.C....C
E. quad SC	????.....C.....C.....C.....A..A.G.C.G.C....C
E. quad TX	????T.....T.....CA....A....GTC.G.C.....
E. wilderae	??????....T.....C.....C.....A...?G??.?..T....
Haideotriton	.C..T.....T.....CA....A....GTC.G.C.....
Typhlotriton	?????..T.....C.....CA....G..A.G.C.G.CT..T.

Appendix 3 continued

```
Northern
Testudo Tube        TATGAAATTTTGGCTCTCTCTTAGGAGTCTGCCTAATTTCACAAATTCT
Ilex Cave           ...........................................
Kretschmarr Cave    ...........................................
Stillhouse Hollow   .......C...................................
Horsethief Hollow   .......C...................................
Round Rock          .......C...................................
Cedar Breaks        ............T.........................T.......
Bat Well            ...........................................
Salado              ...........................................
San Marcos
E. rathbuni         .......C.....A..C..A......C.T.........A........T.
E. nana             .......C.....A.....T.....CA...........A........T.
Southeastern
E. sosorum          .......C.....A.....T.....CA...........A........T.
E. latitans         .......C.....A.....T.....CA.....T.....A........T.
E. trid. BW Pit     .......C.....A.....T.....CA...........A........T.
E. trid. H.C.Cave   .......C.....A.....T.....CA...........A........T.
E. trid. Eb. Cave   .......C.....A.....T.....CA...........A........T.
Boardhouse          .......C.....A.....T.....CA...........A........T.
Cibolo Creek        .......C.....A.....T.....CA...........A........T.
Cloud Hollow        .......C.....A.....T.....CA...........A........T.
Comal Springs       .......C.....A.....T.....CA...........A........T.
H.C.Cave Spring     .......C.....A.....T.....CA...........A........T.
E. neotenes         .......C.....A.....T.....CA...........A........T.
Pedernales          .?..?..C.....A.....T.....TA...........A........T.
Rebecca Creek       .......C.....A.....T.....CA...........A........T.
Southwestern
E. troglodytes      .......C.....A..C..T..................A........T.
176 Spring          .......C.....A..C..T..................A........T.
Camp Mystic         .......C.....A..C..T..................CA.......T.
Carson Cave         .......C.....A..C..T.............TC....A........T.
Greenwood Valley    .......C.....A..C..T..................A........T.
Sabinal Canyon      .......C.....A..C..T..................A........T.
Smith's Spring      .......C.....A..C..T.....T.????.......A........T.
Sutherland Hollow   .......C.....A..C..T..................A........T.
Trough Spring       .......C.....A..C..T..................A........T.
Tucker H. Cave      .......C.....A..C..T..................A........T.
Outgroup
E. bislineata       .......C.....G..CC.T.............T.....A.......CT.
E. longicauda       .......C.....A..CC.........A.T.........CA.......CT.
E. multiplicata     ............A..CC.T..................CA....G..CT.
E. quad SC          ............A..CC.T..................CA....G..CT.
E. quad TX          .......C.....A..CC.T.......A.T.........A........T.
E. wilderae         .......C.....G..CA.T........A...T.....A...?...CT.
Haideotriton        .......C.....A..CC.T......A.T.........A.......CT.
Typhlotriton        .......C..C..A...C.A......A...........A.......C..
```

Appendix 3 continued

Northern	
Testudo Tube	AACAGGCCTATTTCTCGCAATACACTATACTGCAGATACTACTTCCGCA
Ilex Cave	.....
Kretschmarr Cave	.....C..C..C.....
Stillhouse Hollow	.....
Horsethief Hollow	.....
Round Rock	.....
Cedar Breaks	.....C.....
Bat Well	.....G.
Salado	.....
San Marcos	
E. rathbuni	.....T.....T.....C..C..C..A...
E. nana	...G...T.....T.....T..C.....C..C..C..A...
Southeastern	
E. sosorum	...G...T.....T.....T..C.....C..C..C..A...
E. latitans	...G...T.....T.....T..C.....C..C..C..A...
E. trid. BW Pit	...G...T.....T.....T..C.....C..C..C..A...
E. trid. H.C.Cave	...G...T.....T.....T..C.....C..C..C..A...
E. trid. Eb. Cave	...G...T.....T.....T..C.....C..C..C..A...
Boardhouse	...G...T.....T.....T..C.....C..C..C..A...
Cibolo Creek	...G...T.....T.....T..C.....C..C..C..A...
Cloud Hollow	...G...T.....T.....T..C.....C..C..C..A...
Comal Springs	...G...T.....T.....T..C.....C..C..C..A...
H.C.Cave Spring	...G...T.....T.....T..C.....C..C..C..A...
E. neotenes	...G...T.....T.....T..C.....C..C..C..A...
Pedernales	...G...T.....T.....T..C.....C..C..C..A...
Rebecca Creek	T..G...T.....T.....T..C.....C..C..C..A...
Southwestern	
E. troglodytes	...G...T.....T.....T.....C..C..C..A...
176 Spring	...G...T.....T.....T.....C..C..C..A...
Camp Mystic	...G...T.....T.....T.....C.....C..A...
Carson Cave	.....T.....T.....T.....C..C..C..A...
Greenwood Valley	...G...T.....T.....T.....C..C..C..A...
Sabinal Canyon	...G...T.....T.....T.....C..C..C..A...
Smith's Spring	.....T.....T.....T.....C..C..C..A...
Sutherland Hollow	...G...T.....T.....T.....C..C..C..A...
Trough Spring	...G...T.....T.....T.....C..C..C..A...
Tucker H. Cave	...G...T.....T.....T.....C..C..C..A...
Outgroup	
E. bislineata	.....A.....C..T.....T.....G.....C.....T...
E. longicauda	.....AT.....T.....T..C.....C.....C.....
E. multiplicata	.....GT....C..T.....C..C.....C..CGTC..A...
E. quad SC	.....GT....C..T.....C..C.....C..CT.C..A...
E. quad TX	.....T.....T.....C..C.....C..C..C.....
E. wilderae	.....A.....C..T.....G..C..C..C.....
Haideotriton	T.....AT.....T..T.....C..C..G..C..CT.C..T...
Typhlotriton	.....C..T.....C..C.....C..CTC.....

Appendix 3 continued

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Northern
Testudo Tube        TTTTCCTCCGTGGCCCACATTTGCCGTGATGTAAATTATGGCTGACTTG
Ilex Cave           ...........................................
Kretschmarr Cave    ...........................................
Stillhouse Hollow   ...........................................
Horsethief Hollow   ...........................................
Round Rock          ...........................................
Cedar Breaks        ..............................G............
Bat Well            ..........?....................................
Salado              ...........................................
San Marcos
E. rathbuni         ..C.....T..A.............C...........C.........A
E. nana             ..C.....T..A.....................................C.
Southeastern
E. sosorum          ..C.....T..A.....................................C.
E. latitans         ..C.....T..A.....................................
E. trid. BW Pit     ..C.....T..A.....................................
E. trid. H.C.Cave   ..C.....T..A.....................................
E. trid. Eb. Cave   ..C.....T..A.....................................
Boardhouse          ..C.....T..A.....................................
Cibolo Creek        ..C.....T..A.....................................
Cloud Hollow        ..C.....T..A.....................................
Comal Springs       ..C.....T..A.....................................
H.C.Cave Spring     ..C.....T..A.....................................
E. neotenes         ..C.....T..A.....................................?.
Pedernales          ..C.....T..A.....................................C.
Rebecca Creek       ..C.....T..A.....................................
Southwestern
E. troglodytes      ..C.....T.............................C............
176 Spring          ..C.....T..A...........T.........................T
Camp Mystic         ........T..AAT..................G.................
Carson Cave         ..C.....T..A.............................C........C.
Greenwood Valley    ..C.....T.....T...........C........C.............
Sabinal Canyon      ..C.....T.....T.....................C.............
Smith's Spring      ..C.....T..A.............................C..?.....C.
Sutherland Hollow   ..C.....T.....T.....................C.............
Trough Spring       ........T..AAT..................................
Tucker H. Cave      ..C.....T.....T.......................C..?.......
Outgroup
E. bislineata       ..C.....T..A..T..T........A..C.....C.....A.....G.
E. longicauda       ..C.....T..A.....T..C.....A..C..T........T.....A.
E. multiplicata     ..C..T..T..A.....T..C.....A..C..................A.
E. quad SC          ..C.....T..A.....T..C.....A..C..................A.
E. quad TX          ...........A..T.....C.....A...........C..T.......
E. wilderae         ..C........A.....T........A.....GG?......T.......
Haideotriton        ........T..T..T...........A..............T.....A.
Typhlotriton        .....T..A..A..A..T..C.....A...........C..A.....G.
```

Appendix 3 continued

Northern	
Testudo Tube	TACGCAGCATTCACACTAATGGAGCATCACTATTCTTTATTTGTATGTA
Ilex Cave	.....
Kretschmarr Cave	.....A..
Stillhouse Hollow	.G.....T.....G.....?..C.....A..
Horsethief Hollow	.G.....T.....G.....?..C.....A..
Round Rock	.G.....T.....C.....A..
Cedar Breaks	......AT.....C.....C.....A..
Bat Well	.....T.....A..
Salado	.....T.....C.....A..
San Marcos	
E. rathbuni	.G..T.AT.....C.....G...A.C.....C.....T..
E. nana	.G.....T.....CA.C.....CA.C.....C..C..T..
Southeastern	
E. sosorum	.G.....T.....C..C.....CA.C.....C..C..T..
E. latitans	.G.....T.....C..C.....CA.C.....C..C..T..
E. trid. BW Pit	.G.....T.....C..C.....CA.C.....C..C..T..
E. trid. H.C.Cave	.G.....T.....C..C.....CA.C.....C..C..T..
E. trid. Eb. Cave	.G.....T.....C..C.....CA.C.....C..C..T..
Boardhouse	.G.....T.....C..C.....CA.C.....C..C..T..
Cibolo Creek	.G.....T.....C..C.....CA.C.....C..C..T.?
Cloud Hollow	.G.....T.....C..C.....CA.C.....C..C..T..
Comal Springs	.G.....T.....C..C.....CA.C.....C..C..T..
H.C.Cave Spring	.G.....T.....C..C.....CA.C.....C..C..T..
E. neotenes	.G.....T.....C..C.....CA.C.....C..C..T..
Pedernales	.G.....T.....C..C.....CA.C.....C..C..T.G
Rebecca Creek	.G.....?.....C..C.....CA.C.....C..C..T..
Southwestern	
E. troglodytes	.....T.....CA.C.....T..
176 Spring	.....T.....CA.C.....T..
Camp Mystic	......A.....T.....CA.C.....T..
Carson Cave	.....T.....C.....G..CACC.....C.....T..
Greenwood Valley	.....T.....CA.C.....T..
Sabinal Canyon	.....T.....CA.C.....T..
Smith's Spring	.....T.....C.....G..CACC.....C.....T..
Sutherland Hollow	.....T.....CA.C.CT.....T..
Trough Spring	......A.....T.....CA.C.CT.....T..
Tucker H. Cave	.....T.....CG.C.....T..
Outgroup	
E. bislineata	.G..T.AT.....T.....C..TA.T..T.....T..
E. longicauda	.G.....T.....T..TA...CT..?.....C..T..
E. multiplicata	.....C..C.....C...A.....T..
E. quad SC	.....C..C.....C...A.....T..
E. quad TX	......AT.....C.....C..TA.T..T..C.....C..T..
E. wilderae	......AT.....T.....C.....C..TA.T..T.....T..
Haideotriton	.G....AT.....C..TA.T..T..C.....C..T..
Typhlotriton	......ATG.....C.....C...A.T.....T..

Appendix 3 continued

```
Northern
Testudo Tube        TCTACACATTGGACGGGGCCTATATTATGGCTCTTACATATTTAAAGAA
Ilex Cave           ........................................................
Kretschmarr Cave    ........................................................
Stillhouse Hollow   .A.....................................T.....................
Horsethief Hollow   .A.....................................T.....................
Round Rock          .A.....................................T.....................
Cedar Breaks        C.......................................................
Bat Well            .T......................................................
Salado              ......T.................................................
San Marcos
E. rathbuni         C..T..T........T.....G...........A.......A.......
E. nana             C..T..T........T.................A..T..G.A...G...
Southeastern
E. sosorum          C..T..T........T.................A..T..G.A...G...
E. latitans         C..T..T........T.................A..T..G.A...G...
E. trid. BW Pit     C..T..T........T.................A..T..G.A...G...
E. trid. H.C.Cave   C..T..T........T.................A..T..G.A...G...
E. trid. Eb. Cave   C..T..T........T.................A..T..G.A...G...
Boardhouse          C..T..T........T.................A..T..G.A...G...
Cibolo Creek        C..T..T........T.................A..T..G.A...G...
Cloud Hollow        C..T..T........T.................A..T..G.A...G...
Comal Springs       C..T..T........T.................A..T..G.A...G...
H.C.Cave Spring     C..T..T........T.................A..T..G.A...G...
E. neotenes         C..T..T........T.................A..T..G.A...G...
Pedernales          ...T..T........T.................A.....G.A...G...
Rebecca Creek       C..T..T........T.................A..T..G.A...G...
Southwestern
E. troglodytes      ...T..T.....G........G...........A..T....A.......
176 Spring          C..T..T.............G...........A.......A.......
Camp Mystic         C..T..T.....G........G...........A.......A.......
Carson Cave         C..T..T.............................A..T....A.......
Greenwood Valley    C..T..T.....G........G...........A..T....A.......
Sabinal Canyon      ...T..T.....G........G...........A.......A.......
Smith's Spring      C..T..T.............................A.......A.......
Sutherland Hollow   ...T..T.....G........G...........A..T....A.......
Trough Spring       C..T..T.....G........G...........A.......A.......
Tucker H. Cave      C..T..T.....G........G...........A.......A.......
Outgroup
E. bislineata       ...T..T.....C..A..............T..G..T.............
E. longicauda       ...T..T........A...T....C........A.....G..C......
E. multiplicata     ...T..................T...........A..T....A.......
E. quad SC          ...T...........A..............?..A..T.............
E. quad TX          ...G.....C.....A..................A..T.....?......
E. wilderae         ...C..T.....C..A....?...C.....T..A........?.....G
Haideotriton        ...T..T....CG..A.....G..............T...........G
Typhlotriton        ...T...........A..................A..T....A......G
```

Appendix 3 continued

```
Northern
Testudo Tube        ACCTGAAACATCGGAGTTATTCTTTTGTTTTTAGTAATAGCGACAGCAT
Ilex Cave           .............G.........?.......................
Kretschmarr Cave    .............G.......................?........?
Stillhouse Hollow   .............G.......................?.........
Horsethief Hollow   .............G.......................?.........
Round Rock          .............G................................
Cedar Breaks        .....G..................................?...?...
Bat Well            ..........................................????
Salado              .............G.........?...................?...
San Marcos
E. rathbuni         ..T........T.....C.....A..A........T.....A.......
E. nana             ..T.....T..T...........A..A........T.....A.......
Southeastern
E. sosorum          ..T.....T..T...........G..A........T.....A.......
E. latitans         ..T.....T..T...........G..A........T...?.A...?..?
E. trid. BW Pit     ..T.....T..T...........G..A........T...?.A...?..?
E. trid. H.C.Cave   ..T.....T..T...........G..A........T.....A...?.??
E. trid. Eb. Cave   ..T.....T..T...........G..A........T...?.A......C
Boardhouse          ..T.....T..T...........G..A........T.....A...????
Cibolo Creek        ..T.....T..T...........G..A........T.....?.......
Cloud Hollow        ..T.....T..T...........G..A........T...?.A......C
Comal Springs       ..T.....T..T...........G..A........T.....A.......
H.C.Cave Spring     ..T.....T..T.......  ...G..A........T.....A.......
E. neotenes         ..T.....T..T...........G..A........T.....A..??.??
Pedernales          ..T.....T..T.....C.....G..A........T.....A.......
Rebecca Creek       ..T.....T..T...........G..A........T.....A.....??
Southwestern
E. troglodytes      ..T.....T..T.....C...T.A...........C...???.......
176 Spring          ..T........T.....C...T.A.........A.C.....A.......
Camp Mystic         ..T.....T..T.....C...T.A...........T.....A...??.?
Carson Cave         ..T.....T..T.....C...T.A?..C.......T.....A.......
Greenwood Valley    ..T.....T..T.....C...T.A...C.......C...?.A.......
Sabinal Canyon      ..T.....T..T.....C...T.A...........C.....A.......
Smith's Spring      ..T.....T..T.....C...T.A...........T....??.......
Sutherland Hollow   ..T.....T..T.....C...T.A...........C.....A.......
Trough Spring       ..T.....T..T.....C...T.A...........T.....A...?..?
Tucker H. Cave      ..T.....T..T.....C...T.A...........C.....A.......
Outgroup
E. bislineata       ...........T.....A......C.A..............A.......
E. longicauda       ...........T........C?.AC.A........G.....A....???
E. multiplicata     ........T..T.....C..C..CC.A........C..G..T.......
E. quad SC          ........T..T.....C..C...C.A..............A.......
E. quad TX          ........T..T.....C..C..?C.A..............A.......
E. wilderae         ...........T..........?.C.A..............A.......
Haideotriton        ........T..T.....C.....CC................A....?..
Typhlotriton        ..T.....................A.....CC....C.....A.......
```

Appendix 3 continued

Northern	
Testudo Tube	TTGTTGGGTA???
Ilex Cave	.....?????
Kretschmarr Cave	????????????
Stillhouse Hollow	.....TGT
Horsethief Hollow	.....TGT
Round Rock	.....?????
Cedar Breaks	.????????????
Bat Well	????????????
Salado	????????????
San Marcos	
E. rathbuni	.C....???????
E. nana	.?...????????
Southeastern	
E. sosorum	....?????????
E. latitans	?C???????????
E. trid. BW Pit	?C???????????
E. trid. H.C.Cave	????????????
E. trid. Eb. Cave	????????????
Boardhouse	????????????
Cibolo Creek	.C???????????
Cloud Hollow	????????????
Comal Springs	.C....???????
H.C.Cave Spring	.C..C.???????
E. neotenes	?C..?????????
Pedernales	.C..C..A..TG?
Rebecca Creek	????????????
Southwestern	
E. troglodytes	????????????
176 Spring	.C.??????????
Camp Mystic	?C.??????????
Carson Cave	.C..C..A?????
Greenwood Valley	????????????
Sabinal Canyon	.C..C.???????
Smith's Spring	.C..C..??????
Sutherland Hollow	.C???????????
Trough Spring	????????????
Tucker H. Cave	????????????
Outgroup	
E. bislineata	......???????
E. longicauda	????????????
E. multiplicata	....C????????
E. quad SC	?.......?????
E. quad TX	.C.....??????
E. wilderae	????????????
Haideotriton	.C..A.....???
Typhlotriton	.C..A.....TG?

APPENDIX 4. Amino acid composition, from codons 16 to 134, of mitochondrial cytochrome b in central Texas *Eurycea* and outgroup members. "*E. quad.*" = *E. quadridigitata* and "*E. trid.*" = *E. tridentifera*.

Northern	
Testudo Tube	NNSFIDLPTPSTLSYLWNFGSLLGVCLISQIMTGLFLAMHYTADTTSAF
Ilex Cave	?.....
Kretschmarr Cave	??.....
Stillhouse Hollow	??.....?.....
Horsethief Hollow	.....?.....
Round Rock	.....?.....
Cedar Breaks	.....
Bat Well	.....?.....
Salado	.....?.....
San Marcos	
E. rathbuni	.....N.....L...P.....
E. nana	.....N.....I...P.....
Southeastern	
E. sosorum	.....?.....I...P.....
E. latitans	.....N.....I...P.....
E. trid. BW Pit	.....?.....I...P.....
E. trid. H.C.Cave	??.....?.....I...P.....
E. trid. Eb. Cave	.....??.....I...P.....
Boardhouse	.....N?.....I...P.....
Cibolo Creek	??.....?.....I...P.....
Cloud Hollow	??.....N.....I...P.....
Comal Springs	.....N?.....I...P.....
H.C.Cave Spring	.....N.....I...P.....
E. neotenes	??.....?.....I...P.....
Pedernales	??...??.?..??.....I...P.....
Rebecca Creek	??.....?.....I...P.F.....
Southwestern	
E. troglodytes	??.....??.....P.....
176 Spring	??.....?.....P.....
Camp Mystic	.....N.....P.....
Carson Cave	??.....N.....S.P.....
Greenwood Valley	??.....?.....P.....
Sabinal Canyon	.....N.....P.....
Smith's Spring	??.....??.....P.....
Sutherland Hollow	??.....??.....P.....
Trough Spring	??.....?.....P.....
Tucker H. Cave	??....?....?.....P.....
Outgroup	
E. bislineata	.....N.....L...P.....
E. longicauda	.....?....?.....I...P.....
E. multiplicata	.....S.....P.....V...
E. quad SC	.....S.....P.....S...
E. quad TX	.....S.....I...P.....S...
E. wilderae	.....??.....L...P?.....
Haideotriton	.....S.....I...P.F.....S...
Typhlotriton	.....S.....I...P.....S...

Appendix 4 continued

Northern	
Testudo Tube	SSVAHICRDVNYGWLVRSIHTNGASLFFICMYLHIGRGLYYGSYMFKE
Ilex Cave	.....
Kretschmarr Cave	.....I.....
Stillhouse Hollow	.....I.I.....
Horsethief Hollow	.....I.I.....
Round Rock	.....I.I.....
Cedar Breaks	.....I.....
Bat Well	.?.....I.....
Salado	.....I.....
San Marcos	
E. rathbuni	.....M.N.....I....I.....Y..
E. nana	.....I....I.....Y..
Southeastern	
E. sosorum	.....I....I.....Y..
E. latitans	.....I....I.....Y..
E. trid. BW Pit	.....I....I.....Y..
E. trid. H.C.Cave	.....I....I.....Y..
E. trid. Eb. Cave	.....I....I.....Y..
Boardhouse	.....I....I.....Y..
Cibolo Creek	.....I....I.....Y..
Cloud Hollow	.....I....I.....Y..
Comal Springs	.....I....I.....Y..
H.C.Cave Spring	.....I....I.....Y..
E. neotenes	.....I....I.....Y..
Pedernales	.....I....I.....Y..
Rebecca Creek	.....I....I.....Y..
Southwestern	
E. troglodytes	.....I....I.....Y..
176 Spring	.....?.....I....I.....Y..
Camp Mystic	...I.....I....I.....Y..
Carson Cave	.....T....I.....Y..
Greenwood Valley	.....I....I.....Y..
Sabinal Canyon	.....I....I.....Y..
Smith's Spring	.....T....I.....Y..
Sutherland Hollow	.....IS...I.....Y..
Trough Spring	...I.....N.....IS...I.....Y..
Tucker H. Cave	.....V....I.....Y..
Outgroup	
E. bislineata	.....N.....I....I.....Y..
E. longicauda	.....IS?..I.....Y..
E. multiplicata	.....I....I.....Y..
E. quad SC	.....I....I.....Y..
E. quad TX	.....N.....I....I.....Y..
E. wilderae	.....?.....N.....I....I.....?..
Haideotriton	.....N.....I....I.....Y..
Typhlotriton	.....NV.....I....I.....Y..

Appendix 4 continued

Northern	
Testudo Tube	TWNIGVILLFLVMATAFVG??
Ilex Cave	..................??
Kretschmarr Cave	.............?..?????
Stillhouse Hollow	.............?.....YV
Horsethief Hollow	.............?.....YV
Round Rock	..................??
Cedar Breaks	..............??????
Bat Well	..............??????
Salado	..............?????
San Marcos	
E. rathbuni	..................??
E. nana	..............?.???
Southeastern	
E. sosorum	..................???
E. latitans	.............?.??????
E. trid. BW Pit	.............?.??????
E. trid. H.C.Cave	..............??????
E. trid. Eb. Cave	.............?.??????
Boardhouse	..............??????
Cibolo Creek	..................????
Cloud Hollow	.............?..?????
Comal Springs	..................???
H.C.Cave Spring	..................???
E. neotenes	..............?.???
Pedernales	..................?
Rebecca Creek	..............?????
Southwestern	
E. troglodytes	.............?..?????
176 Spring	...........I.....????
Camp Mystic	..............??????
Carson Cave	..................??
Greenwood Valley	.............?..?????
Sabinal Canyon	..................???
Smith's Spring	.............?.....??
Sutherland Hollow	..................????
Trough Spring	..............??????
Tucker H. Cave	..............?????
Outgroup	
E. bislineata	..................???
E. longicauda	..............??????
E. multiplicata	..................???
E. quad SC	..............?..??
E. quad TX	..................??
E. wilderae	......?.........?????
Haideotriton	..............?...??
Typhlotriton	..................?

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VITA

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